Targeting the Gut-Brain axis in Parkinson's disease

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Targeting the Gut-Brain axis in Parkinson's Disease

De darm-brein-as: als aangrijpingspunt voor de ziekte van Parkinson

(met een samenvatting in het Nederlands)

Proefschrift

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door

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CHAPTER 1

General Introduction

Parkinson's disease (PD) is the most common progressive movement disorder, with increasing age being the greatest risk factor for its development. The mean age of onset of PD is around 60 years, with an average disease duration of around 15 years.

PD is hallmarked by the progressive degeneration of dopaminergic nigrostriatal neurons, with reductions in striatal dopamine levels resulting in the characteristic motor impairments. Another characteristic of PD is alpha-synuclein containing inclusion bodies in the surviving neurons in different areas of the central and peripheral nervous system[1–3]. Although PD is generally considered as a movement disorder, it has long been recognized that the symptoms go beyond motor dysfunction since PD patients very often develop non-motor symptoms[4], including olfactory and sleep disturbances[5–7], depression[8], cognitive decline[9], and most commonly gastrointestinal dysfunctions[10–13]. Among gastrointestinal dysfunctions, constipation is the most prominent and it might precede motor symptoms by over a decade[10,11]. The occurrence of constipation before the manifestation of motor symptoms in PD patients was reported to be 87%[14].

There are two forms of PD; familial and sporadic. The familial form is caused by genetic aberrations, but the causes of sporadic PD onset remain unknown. However, some progress has been made in the search for potential causes and it is thought that it probably has multifactorial origins, with contributions from genetic predisposition, environmental factors, and aging. The early involvement of the gastrointestinal tract in PD supports the hypothesis that environmental factors could exert its influence on PD development and progression via the gut.

AIM AND OUTLINE OF THIS THESIS

This thesis aims to gain insight in the importance of gut-brain interactions in PD pathogenesis. For that purpose, two separate rotenone-induced PD models (oral and intrastriatal rotenone models) are employed.

The major aims of the thesis are as follows:

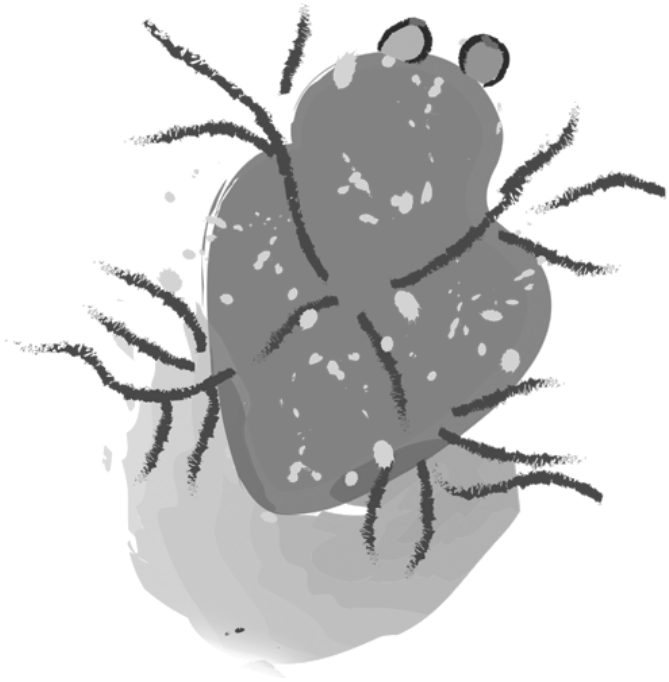
1. To investigate the involvement of bidirectional communications between the gut and the brain for the genesis of PD-like phenotype and pathology in two mouse models of PD.
2. To investigate the importance of TLR4-mediated inflammation and changes in microbiota composition in the genesis of PD.
3. To investigate the effects of nutritional interventions containing phospholipid precursors, cofactors, and prebiotic fibers in the treatment before and after disease induction of motor and non-motor symptoms in the rotenone models of PD.

In **Chapter 2,** a review of the current scientific literature on Braak's hypothesis is presented. The support and the criticism for the hypothesis are discussed and it is concluded that Braak's hypothesis is highly relevant for a large subset of PD patients.
Chapter 3 demonstrates that both oral and intrastriatal administration of rotenone induced similar PD-like motor deficits and gastrointestinal dysfunctions in mice. Moreover, it shows that the uridine and docosahexaenoic acid containing diet prevented rotenone-induced motor and gastrointestinal dysfunctions in both models. **Chapter 4** demonstrates that PD patients have intestinal barrier disruption, enhanced number of TLR4 positive cells and higher pro-inflammatory gene profiles in the colonic biopsy samples. Furthermore, using Tlr4 KO mice treated orally with rotenone, we show mitigated neuroinflammation, neurodegeneration and intestinal dysfunction when compared to WT. **Chapter 5** subsequently describes the alterations in microbiota composition and possibly associated metabolic pathways observed in oral rotenone-exposed mice. **Chapter 6** demonstrates that intrastriatal rotenone-induced motor and non-motor problems were alleviated by a therapeutic dietary intervention (given after full development of disease) providing uridine and DHA plus other nutrients that increase phospholipid synthesis as well as prebiotic fibers. In **Chapter 7,** a review giving a comprehensive overview of the evidences supporting the hypothesis that PD could initiate in the gut is presented. How food-based therapies might impact PD pathology and/or improve non-motor as well as motor symptoms in PD is also considered in this chapter. **Chapter 8** demonstrates that the combined administration of the dietary intervention used in chapter 6 together with oral levodopa, the most common used drug for PD treatment, shows additive beneficial effects on motor function in the intrastriatal rotenone PD model in mice. Finally, the main findings of this thesis are summarized and discussed with perspectives for future research in **Chapter 9**.

References

1. Djaldetti, R., Lev, N. & Melamed, E. Lesions outside the CNS in Parkinson's disease. *Mov. Disord. Off. J. Mov. Disord. Soc.* **24,** 793–800 (2009).
2. Beach, T. G. *et al.* Multi-organ distribution of phosphorylated alpha-synuclein histopathology in subjects with Lewy body disorders. *Acta Neuropathol. (Berl.)* **119,** 689–702 (2010).
3. Del Tredici, K. & Braak, H. Review: Sporadic Parkinson's disease: development and distribution of α-synuclein pathology. *Neuropathol. Appl. Neurobiol.* **42,** 33–50 (2016).
4. Lee, H. M. & Koh, S.-B. Many Faces of Parkinson's Disease: Non-Motor Symptoms of Parkinson's Disease. *J. Mov. Disord.* **8,** 92–97 (2015).
5. Haehner, A., Hummel, T. & Reichmann, H. Olfactory dysfunction as a diagnostic marker for Parkinson's disease. *Expert Rev. Neurother.* **9,** 1773–1779 (2009).
6. Ponsen, M. M., Stoffers, D., Twisk, J. W. R., Wolters, E. C. & Berendse, H. W. Hyposmia and executive dysfunction as predictors of future Parkinson's disease: a prospective study. *Mov. Disord. Off. J. Mov. Disord. Soc.* **24,** 1060–1065 (2009).
7. Ross, G. W. *et al.* Association of olfactory dysfunction with incidental Lewy bodies. *Mov. Disord. Off. J. Mov. Disord. Soc.* **21,** 2062–2067 (2006).
8. Reijnders, J. S. A. M., Ehrt, U., Weber, W. E. J., Aarsland, D. & Leentjens, A. F. G. A systematic review of prevalence studies of depression in Parkinson's disease. *Mov. Disord. Off. J. Mov. Disord. Soc.* **23,** 183–189; quiz 313 (2008).
9. Aarsland, D., Andersen, K., Larsen, J. P., Lolk, A. & Kragh-Sørensen, P. Prevalence and characteristics of dementia in Parkinson disease: an 8-year prospective study. *Arch. Neurol.* **60,** 387–392 (2003).
10. Pfeiffer, R. F. Gastrointestinal dysfunction in Parkinson's disease. *Parkinsonism Relat. Disord.* **17,** 10–15 (2011).
11. Fasano, A., Visanji, N. P., Liu, L. W. C., Lang, A. E. & Pfeiffer, R. F. Gastrointestinal dysfunction in Parkinson's disease. *Lancet Neurol.* **14,** 625–639 (2015).
12. Jost, W. H. Gastrointestinal dysfunction in Parkinson's Disease. *J. Neurol. Sci.* **289,** 69–73 (2010).
13. Savica, R. *et al.* Medical records documentation of constipation preceding Parkinson disease: A case-control study. *Neurology* **73,** 1752–1758 (2009).
14. Cersosimo, M. G. *et al.* Gastrointestinal manifestations in Parkinson's disease: prevalence and occurrence before motor symptoms. *J. Neurol.* **260,** 1332–1338 (2013).

CHAPTER 2



Exploring Braak's hypothesis of Parkinson's disease

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Abstract

Parkinson's disease (PD) is a neurodegenerative disorder for which there is no cure. Most patients suffer from sporadic PD, which is likely caused by a combination of genetic and environmental factors. Braak's hypothesis states that sporadic PD is caused by a pathogen that enters the body via the nasal cavity, and subsequently is swallowed and reaches the gut, initiating Lewy pathology (LP) in the nose and the digestive tract. A staging system describing the spread of LP from the peripheral to the central nervous system was also postulated by the same research group. There has been criticism to Braak's hypothesis, in part because not all patients follow the proposed staging system. Here, we review literature that either supports or criticizes Braak's hypothesis, focused on the enteric route, digestive problems in patients, the spread of LP on a tissue and a cellular level, and the toxicity of the protein αSynuclein (αSyn), which is the major constituent of LP. We conclude that Braak's hypothesis is supported by *in vitro, in vivo* and clinical evidence. However, we also conclude that the staging system of Braak only describes a specific subset of patients with young onset and long duration of the disease.

1. Introduction

Parkinson's disease (PD) is an incurable neurodegenerative disease hallmarked by damage to the dopaminergic neurons of the substantia nigra (SN), and αSynuclein (αSyn) containing inclusion bodies (Lewy pathology; LP) in the surviving neurons, resulting in characteristic motor impairment. The prevalence of PD in Europe ranges between 65.6 to 12,500 per 100,000, and the annual incidence rate ranges between 5 to 346 per 100,000 [1]. The variation in these prevalence and incidence rates could be due to genetic or environmental factors, differences in case ascertainment or diagnostic criteria, or different age distributions in the populations (countries) studied[1]. In the US population of 65 years and older, PD is more common in Caucasians and Hispanics, than Afro-Americans and Asians[2,3], indicating a genetic factor may be (partially) responsible for the differences found in the European study. Current treatments for PD include medicinal treatment using levodopa[4,5], and surgical treatment using deep brain stimulation[6]. Although these treatments offer relief of symptoms, they do not cure the disease. All in all it is clear that PD is an important neurodegenerative disorder to study, even with the more conservative estimations of prevalence and incidence, since currently no cure or preventative treatment exists.

There are two forms of PD; familial and sporadic. The familial form is caused by genetic aberrations, amongst others in the gene for αSyn (point mutations A30P[7], A53T[8], E46K[9], H50Q[10,11], and G51D[12], or locus duplication[13,14] or triplication[15,16]). The cause for sporadic PD is not known, but some progress has been made in the search for potential causes, implicating both genetic and environmental factors. The pesticides rotenone and paraquat[17], and the toxin MPTP[18] (1-methyl-4-fenyl-1,2,3,6-tetrahydropyridine; a toxic byproduct of the opioid analgesic desmethylprodine, MPPP, a synthetic heroin), are known to cause PD in humans, explaining some cases of sporadic PD. Additionally, two twin studies have found that sporadic PD has a significant genetic component[19,20]. As mentioned above, in the US a difference was found in the incidence and prevalence of PD between the Caucasian and Hispanic vs Afro-American and Asian population, also showing a genetic influence[2]. On the other hand, a recent review by Pan-Montojo and Heinz Reichmann suggests an important role of toxic environmental substances in the etiology of sporadic PD[21]. Although the exact influence of genetic and environmental factors in sporadic PD is not known, some elements of disease development have been identified, most importantly neuroinflammation, oxidative stress, and αSyn misfolding and aggregation[22–29]. Misfolding and aggregation of αSyn is suspected to lead to LP in surviving neurons, and thus combatting αSyn aggregation has been suggested to be of potential therapeutic value[30]. It seems likely that both environmental and genetic factors interact to cause sporadic PD. As a result, the search for potential environmental factors has been ongoing in PD research.

2. Braak's hypothesis

In 2003 Braak et al. postulated the hypothesis that an unknown pathogen (virus or bacterium) in the gut could be responsible for the initiation of sporadic PD[31], and they presented an associated staging system for PD based on a specific pattern of αSyn spreading[32]. These publications were followed by the more encompassing dual-hit hypothesis, stating that sporadic PD starts in two places; the neurons of the nasal cavity and the neurons in the gut[33,34]. This is now known as Braak's hypothesis. From these places the pathology is hypothesized to spread according to a specific pattern, via the olfactory tract and the vagal nerve respectively, towards and within the central nervous system (CNS). This process has been visualized in figure 1. Interestingly, the hypothesized spread of disease to the spinal cord only takes place after the CNS has already become involved, and so the spinal cord is not considered to be a potential route for the spread of the disease from the periphery to the brain[33,35].

Pre-clinical and clinical evidence

There is experimental and clinical evidence supporting Braak's hypothesis. Gastrointestinal problems like dysphagia, nausea, constipation and defecatory difficulty[36,37], and the olfactory problem of the loss of smell[38] have been reported in PD. Additionally, the presence of LP in the neurons of the olfactory tract[39,40] and the enteric nervous system (ENS)[41–43] has been confirmed. Severe LP in the ENS is positively correlated with constipation and motor problems in PD patients[44]. There is also clinical evidence that LP in the nasal and gastrointestinal regions potentially precedes the diagnosis of the disease[32,43,45], leading to complaints of the digestive tract[46,47] and problems with olfaction[48,49] during the earlier stages of PD, before the onset of motor symptoms (this stage is also known as incidental Lewy Body Disease[50]).

In animal models similar results have been found. Gastrointestinal problems have been described in models of advanced PD suffering from motor impairment[51–58], and in both genetic and toxin-induced models for earlier stages of PD without motor problems[59–61]. Additionally, αSyn aggregations were found in the gastrointestinal tract of animal models of early[59,60,62] and advanced[51,55] PD.

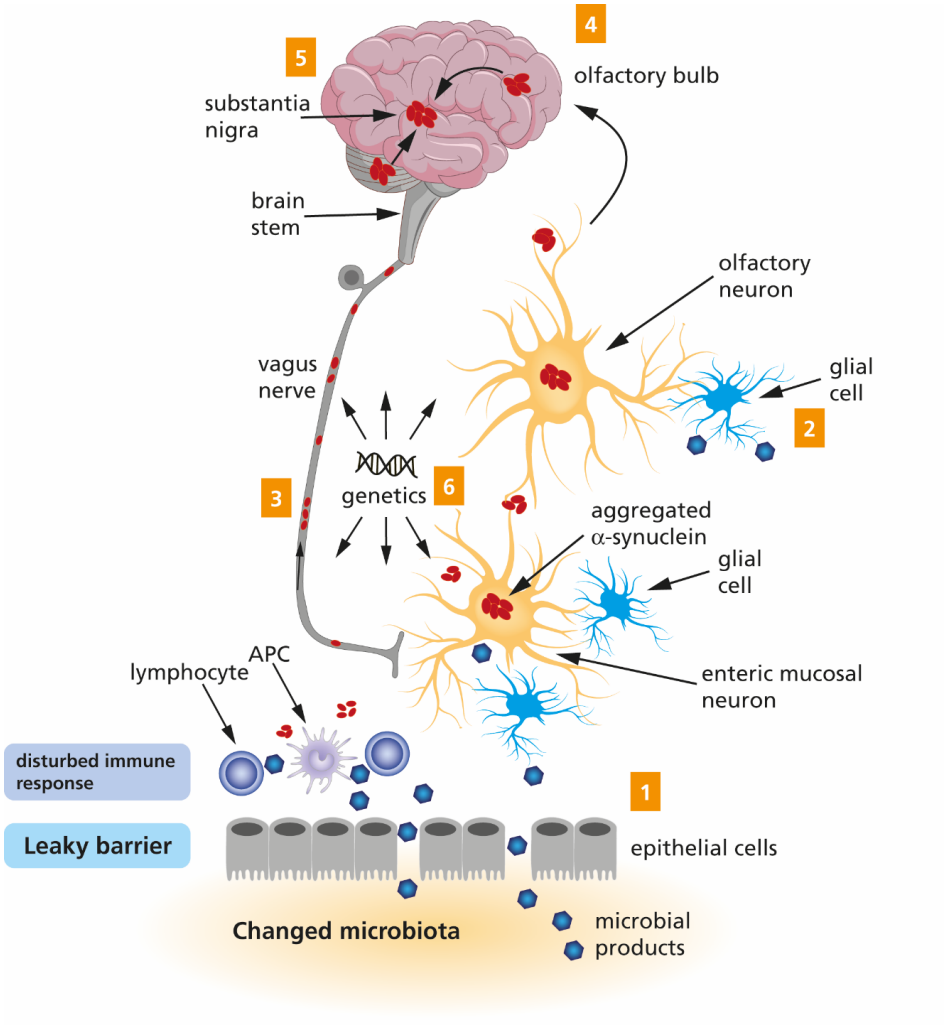


Figure 1. A schematic representation of Braak's hypothesis of Parkinson's disease. Microbial products come into contact with olfactory and/or enteric neurons, which triggers the aggregation of α-Synuclein (1&2). The aggregated α-Synuclein spreads towards the central nervous system via the olfactory bulb and the vagus nerve (3&4). Eventually the aggregated α-Synuclein arrives at the substantia nigra (5). Geneticfactors are likely to contribute to Parkinson's disease, but the exact mechanism remains to be elucidated (6).

Enteric route: clinical evidence

From here on this review will focus on the enteric route of Braak's hypothesis . The importance of the ENS for PD is emphasized by circumstantial clinical evidence. The microbiome of control subjects contains a higher relative abundance of Prevotellaceae bacteria compared to PD patients, and within PD patients a higher relative abundance of

Enterobacteriaceae is associated with more postural and gait symptoms and less tremors[63]. PD patients also suffer from increased inflammation in the colon, although colonic inflammation does not seem to be related to severity of gastrointestinal or motor problems[64]. However, in PD patients another sign of intestinal inflammation, an increased permeability of the intestinal barrier, seems to be related to increased staining in the intestinal mucosa for bacteria, oxidative stress and αSyn[65]. If changes in the microbiome predispose the (future) PD patient to a more pro-inflammatory environment in the intestines and increased barrier permeability, this could potentially lead to oxidative stress in the ENS. This oxidative stress could then trigger αSyn misfolding and aggregation, which could potentially spread from the ENS to the CNS, and eventually cause the hallmark motor problems. Therefore, changes in the microbiome and increased inflammation could directly negatively affect neurons of the ENS, and be related to PD development, which is in accordance with Braak's hypothesis.

Dietary components and dietary patterns have a considerable effect on the composition of the gut microbiome[66]. The commensal gut microbiota thrive on the substrates that escape absorption in the small intestine and are available for colonic bacterial fermentation[67]. For example, fiber-rich diets can enhance the growth of colonic bacteria that produce short chain fatty acids (SCFA). These SCFA , have systemic anti-inflammatory effects[68] and could therefore influence PD pathogenesis through this gut-mediated mechanism. Another example is Western diet (high in saturated fat and refined carbohydrates) that might result in dysbiotic microbiota (e.g., lower bifidobacteria, higher firmicutes and proteobacteria)[69–71] and that could ultimately lead to a pro-inflammatory response an promote αSyn pathology. Therefore, it is essential to continue to research specific foods and dietary patterns that can improve gut health for PD risk reduction.

Enteric route: αSyn spreading via vagal nerve

Another vital part of Braak's hypothesis is the spread of αSyn pathology from the ENS to the CNS via the vagal nerve and the dorsal motor nucleus of the vagus (DMV) in the medulla oblongata, and the spread of pathology within the CNS from lower brainstem regions, towards the SN, and eventually the neocortex. Although these specific areas of the nervous system are affected by PD, certain neighboring areas seem to be spared, such as the nucleus tractus solitarius that is located next to and connected to the DMV. This indicates a non-uniform and specific pattern of the spreading of disease, which cannot be explained by the nearest neighbor rule[72]. This specific pattern of spreading is supported by experimental and clinical evidence, although discussion about the validity of Braak's hypothesis is still ongoing. In PD patients LP has been found in the vagal nerve[73,74] and the DMV[73,75–78], and cell loss in the DMV of PD patients has also been reported[79]. Lewy pathology has been shown to occur in vagal nerves and DMV before it spreads to other parts of the CNS[32,45,76,80], like the locus coeruleus and the SN, the mesocortex, the neocortex and the prefrontal cortex[32]. Additionally, truncal vagotomy might be associated with a decreased long-term risk of

developing PD, which could be related to a hindrance of the spreading of disease via the vagal nerve, although this cannot yet be concluded from this single study[81]. The spread of αSyn from the ENS to the CNS has also been studied in animal models. When the protein αSyn was injected in the wall of the stomach and duodenum of rats it was able to spread through the vagal nerve to the DMV[82]. Additionally, intragastric rotenone treatment of mice resulted in αSyn inclusions in the ENS, DMV and SN, and cell loss in the SN[83]. This rotenone-induced αSyn spreading could be stopped by vagotomy[84]. These results show the vagus nerve is involved in and essential for the spread of αSyn pathology from the ENS to the CNS in both rats and mice.

Enteric route: spread of αSyn within CNS

Clinical evidence for the cellular transport of LP within the CNS comes from studies of PD patients whose grafts of fetal dopaminergic neurons showed LP and degeneration, indicating potential spread of pathology from host cells to graft cells[85–90]. Host-to-graft transmission of αSyn has also been shown for mouse cortical neuronal stem cells[91] and mouse embryonic dopaminergic neurons[92] implanted in transgenic mice overexpressing human αSyn, and for rat embryonic dopaminergic neurons implanted in human αSyn overexpressing rats with[93] or without[94] striatal dopamine depletion. These results show that healthy neurons in the CNS are vulnerable to spread of disease by taking up LP from surrounding LP-affected neurons, although it does not indicate any specific pattern for this spreading.

Transport of αSyn between neurons

The ability of LP to spread through the nervous system raises the question what is the exact mechanism of transport of LP between neurons, and why the spread of LP follows a specific pattern, as suggested by Braak's hypothesis. Both neuronal cell lines and primary neurons are able to excrete αSyn monomers, oligomers and fibrils through unconventional calcium dependent exocytosis from large dense core vesicles or via exosomes[84,95–97]. Once the αSyn is present in their environment, both neuronal cell lines and primary neurons seem able to take up free or exosome bound fibrils and oligomers by endocytosis after which they are degraded in lysosomes (SH-SY5Y cells), while monomers seem to diffuse across the cell membrane and are not degraded[91,97,98]. In a different study the uptake was only found in proliferating SH-SY5Y neurons, but not in differentiated SH-SY5Y neurons, which could be due to the type of αSyn which was different from the other studies (radioactively labelled cell produced αSyn, versus different forms of recombinant human or non-human αSyn)[96]. The transfer of specific αSyn molecules between by cells of neuronal cell lines was proven in a coculture study of SH-SY5Y neurons expressing the same human αSyn labelled either green or red[92]. Coculture resulted in double labelled neurons, showing the process of subsequent excretion and uptake of αSyn by neighboring cells. After uptake αSyn can be transported anterograde or retrograde through axons and passed on to other neurons[82,84,99–101], providing a potential highway for the spread of LP between connected nervous system regions in PD patients. A recent study shows that neuron-to-neuron αSyn transmission could

be initiated by binding the transmembrane protein lymphocyte activation gene 3 (LAG3). The study demonstrated that LAG3 binds αSyn preformed fibrils (PFF) with high affinity and initiates αSyn PFF endocytosis, transmission and toxicity in SH-SY5Y cells. Moreover, mice lacking LAG3 showed delayed αSyn PFF-induced pathology and reduced toxicity[102] .

It is known that the neurons in the area's affected by LP in PD have specific characteristics that cause a high metabolic burden, which seems to make these neurons especially sensitive to oxidative stress and αSyn misfolding. These neurons have high levels of endogenous αSyn, they use monoamine neurotransmitters, have long and highly branched axons with no or poor myelination, and characteristic continuous activity patterns[72,103,104]. Together this could explain why PD pathology develops in the specific pattern proposed by Braak, specifically affecting interconnected regions with vulnerable neurons like the DMV, while sparing neighboring areas like the nucleus tractus solitaries[72].

Neurotoxicity of αSyn

It has been suggested that αSyn acts prion-like in PD. In this theory pathologic, misfolded αSyn is an infectious protein spreading toxicity by forming a toxic template that seeds misfolding for nearby αSyn protein, turning the previously healthy protein into a toxic protein, causing LP. Excellent reviews on the prion-like theory of αSyn have been previously published[105,106]. The prion-like theory fits into Braak's hypothesis, since the staging system of Braak is based on the regional presence (or absence) of LP and the spreading of LP, linking LP to severity of disease[32]. The toxicity of αSyn in its different forms is still undecided and remains the topic of many experiments, with one study reporting a cytoprotective function of αSyn aggregation[107], while others suggest the oligomeric form of αSyn is the most toxic form of the protein[108–110]. Foreign αSyn induces LP-resembling inclusion bodies in recipient neurons[91], caused by fibrils acting as exogenous seeds and recruiting endogenous αSyn into the inclusion body[92,111], even in cells not overexpressing αSyn[101]. Neuronal death resulting from αSyn exposure has also been shown[91], with a higher toxicity for oligomeric compared to monomeric species[96], and a higher toxicity of exosome bound oligomers compared to free oligomers[97]. Inclusion bodies are linked to cell death, involving the loss of synaptic proteins and reduction in network connectivity[101].

In animal studies injection of aggregated αSyn (derived from symptomatic transgenic mice) or synthetic αSyn fibrils into the brain of young, asymptomatic transgenic mice accelerated the formation and spread of αSyn inclusions throughout the brain, resulted in early onset motor symptoms, and reduced the lifespan of these mice[112,113]. Synthetic αSyn fibrils injected in the striatum also induced widespread LP, cell death of dopamine neurons in the SN and motor deficits in wild type mice[114]. It has even been shown that fibril-seeded αSyn inclusions specifically increase neuronal death in αSyn transgenic mice in an experiment where neurons with or without inclusions were followed *in vivo*, providing direct evidence that αSyn inclusions were responsible for neuronal death[115]. Injection of wild type mice with

patient-derived Lewy Body (LB) αSyn just above the SN resulted in degeneration of the dopamine fibers and cell bodies in the SN, and concomitant development of inclusion bodies exclusively consisting of endogenous αSyn, and reduced motor coordination and balance[116]. Mice treated with non-LB αSyn (monomers) did not develop these lesions. Similar results were found in rhesus monkeys; injection of patient-derived LB αSyn in the striatum or SN resulted in reduced nigrostriatal dopaminergic innervation, increased αSyn immunoreactivity in connected brain regions after striatal injection (but not after SN injection), without LP or motor symptoms[116]. Taken together, these results do not definitively confirm or reject the prion-like theory in the context of Braak's hypothesis. However a picture emerges where αSyn oligomers are likely toxic to neurons, and inclusion bodies are linked to neuronal death, which might or might not lead to motor symptoms. Although the studies included here were performed in the CNS, the emerging picture of oligomer toxicity and inclusion body-induced neuronal death could also be applicable to the ENS and other parts of the peripheral nervous system.

3. Criticism to Braak's hypothesis

Criticism to the specific pattern of spreading

Despite the *in vitro*, *in vivo* and clinical support for Braak's hypothesis, there is also doubt whether it accurately describes the development of PD in all patients[117,118]. A large subset of 51%-83% of PD patients follow Braak's staging, while a smaller subset of 7%-11% do not have LP in the DMV while higher brain regions are affected[119–124]. Additionally, there is no correlation between severity of LP in the DMV, and in the limbic system or neocortex [125]. Also, LP in the ENS is not correlated to olfactory problems, and 27%-33% of PD patients did not show any LP in the ENS, which does not support the dual-hit hypothesis[64,126], although it is known LP can be restricted to the olfactory system in the early stage of the disease[124]. Additionally, people with incidental Lewy Body Disease seem to have a similar distribution but milder expression of LP compared to PD patients[50,127], and can show LP in the SN and other areas of the brain without LP or neuronal loss in the DMV[77,122,128,129] or LP in the vagus nerve[45], favoring multiple origination sites for LP instead of a spread from ENS to CNS via the vagus nerve. Additionally, Braak's hypothesis does not explain how or why cardiac sympathetic nerves are affected in early PD[129]. Therefore it seems safe to conclude that not all PD patients adhere to the specific pattern of LP spread proposed by Braak.

Criticism to the link between LP, neuronal loss, and PD symptoms

Other studies have shown that the link between LP and clinical PD symptoms should be questioned. Only 45% of people with widespread LP in the brain are diagnosed with dementia or motor symptoms[121] and only about 10% of people with LP in the SN, DMV and/or basal forebrain are diagnosed with PD[130]. Additionally, neurodegeneration in the SN might precede LP[131]. Therefore the spreading of LP, whether according to Braak's staging

system or not, might not be as tightly bound to clinical symptoms as has been suggested by Braak. The basic science underlying Braak's hypothesis has also been questioned[118,132], because in the initial studies all cases were preselected for LP in the DMV[32,76], systematically excluding any cases where LP in higher brain regions was found in the absence of LP in the DMV, which seems to have led to a selection bias and the inclusion of non-representative samples in the pre-clinical PD group in the original research[132]. The limited clinical information on the pre-clinical PD group and the absence of information on neuronal cell loss in the original Braak papers has also been criticized[117,118,132]. It has been suggested that neuronal loss and activation of glial cells should be part of future pathological analysis of PD to better describe disease progression, since the clinical significance of LP is not yet clear and might be less important than previously thought[121,130,131].

Studying neuronal loss and glial activation in future PD research

Studying neuronal loss together with LP during PD development is important because neuronal loss in the SN shows a linear relationship with motor symptoms[133], while LP in the overall brain only shows a trend for positive correlation with motor symptoms[124]. Additionally, LP is not related to dopaminergic cell loss in the striatum[124], and may [124] or may not[134] be related to dopaminergic cell loss in the SN of PD patients. Therefore it can be concluded that neuronal loss and LP are not interchangeable hallmarks for PD progression or severity of disease, but should rather be seen as complimentary to each other.

Studying the activation of glial cells is important because neuroinflammation is an important factor in PD development, and glial cells are major contributors to neuroinflammation, partially through Toll-like receptors (TLRs)[22–27]. Especially TLR2 and -4 are important in PD, since their expression is increased in the brain of PD patients, and a polymorphism resulting in lower expression of TLR2 tends to be linked to an increased risk of PD[135–138]. Pre-clinical research has confirmed the importance of TLR2 and -4 for PD, and has specifically shown their importance in the context of glial-induced inflammation and αSyn uptake by glial cells[138–149].

4. Conclusion

Reviewing the current literature it can be concluded that there is much evidence to support Braak's hypothesis. Enteric and olfactory pathology and dysfunction are well known characteristics of early and late PD. The vagus nerve and DMV form a likely route for αSyn pathology to spread from the ENS to the CNS, and αSyn is able to spread cellularly within the CNS. Neurons are able to transmit different forms of αSyn protein to each other, and to transport αSyn via their axons, which enables the spread of the potentially toxic oligomeric variety of the protein, which could be the basic mechanism underlying the specific pattern of LP spread in PD as proposed by Braak. It seems then possible that a pathogen or

environmental toxin might provoke local inflammation and oxidative stress in the gut, thereby initiating αSyn deposition that is subsequently disseminated to the CNS. Hypothetically, the toxic αSyn can lead to neuronal death. (Micro)glial cells and surviving neurons can then be activated through the release of danger associated molecular patterns and subsequent activation of Toll-like receptors. This would trigger a vicious circle of neuroinflammation.

However, it can also be concluded that a significant portion of PD patients do not follow Braak's staging system. It has been discovered that a subgroup of levodopa-responsive PD patients who develop PD at a young age and have a long duration clinical course with predominantly motor symptoms, and dementia only at the later stages, seem to follow Braak's staging, while other levodopa-responsive PD patients did not[80]. In addition to this, a Lewy Body staging system has been proposed which encompasses all patient groups, a system wherein LP staging correlates well with motor symptoms and cognitive decline[124], and allowing for patients who show a spread of LP not accounted for in Braak's hypothesis. Unfortunately the staging system is only describing the different observed patterns of LP spread, while not answering the question as to the cause of the non-Braak patterns. What is the reason or explanation for these other types of patterns to occur? This question remains to be answered.

We conclude that Braak's hypothesis and the Braak staging system are valuable and useful for the future study of PD, and these theories are likely to accurately describe disease initiation and progression in a subgroup of PD patients with young onset and long duration of disease. However, a similar theory describing the initiation and disease progression in other PD patients is still sorely lacking and deserves to be elucidated. To better understand the progression of LP and PD in different patient groups it is necessary to study people longitudinally during disease development, and especially in the earliest stages of PD. This should lead to a larger theory describing different disease processes, all leading to PD, including Braak's hypothesis. This theory could offer useful insight into specific targets for disease prevention or disease treatment, dependent on the type of LP disease the patient is likely suffering from. Either more optimal treatment with currently available drugs and technology, or the development of new treatments could be the result.

References

1. von Campenhausen, S. *et al.* Prevalence and incidence of Parkinson's disease in Europe. *Eur. Neuropsychopharmacol. J. Eur. Coll. Neuropsychopharmacol.* **15,** 473–490 (2005).
2. Wright Willis, A., Evanoff, B. A., Lian, M., Criswell, S. R. & Racette, B. A. Geographic and ethnic variation in Parkinson disease: a population-based study of US Medicare beneficiaries. *Neuroepidemiology* **34,** 143–151 (2010).
3. Van Den Eeden, S. K. *et al.* Incidence of Parkinson's disease: variation by age, gender, and race/ethnicity. *Am. J. Epidemiol.* **157,** 1015–1022 (2003).
4. Fahn, S. The medical treatment of Parkinson disease from James Parkinson to George Cotzias. *Mov. Disord.* **30,** 4–18 (2015).
5. Warren Olanow, C. *et al.* Factors predictive of the development of Levodopa-induced dyskinesia and wearing-off in Parkinson's disease. *Mov. Disord. Off. J. Mov. Disord. Soc.* **28,** 1064–1071 (2013).
6. Williams, Z. M. Good vibrations with deep brain stimulation. *Nat. Neurosci.* **18,** 618–619 (2015).
7. Kruger, R. *et al.* Ala30Pro mutation in the gene encoding alpha-synuclein in Parkinson's disease. *Nat. Genet.* **18,** 106–108 (1998).
8. Polymeropoulos, M. H. *et al.* Mutation in the alpha-synuclein gene identified in families with Parkinson's disease. *Science* **276,** 2045–2047 (1997).
9. Zarranz, J. J. *et al.* The new mutation, E46K, of alpha-synuclein causes Parkinson and Lewy body dementia. *Ann. Neurol.* **55,** 164–173 (2004).
10. Appel-Cresswell, S. *et al.* Alpha-synuclein p.H50Q, a novel pathogenic mutation for Parkinson's disease. *Mov. Disord. Off. J. Mov. Disord. Soc.* **28,** 811–813 (2013).
11. Proukakis, C. *et al.* A novel alpha-synuclein missense mutation in Parkinson disease. *Neurology* **80,** 1062–1064 (2013).
12. Lesage, S. *et al.* G51D alpha-synuclein mutation causes a novel parkinsonian-pyramidal syndrome. *Ann. Neurol.* **73,** 459–471 (2013).
13. Chartier-Harlin, M. C. *et al.* Alpha-synuclein locus duplication as a cause of familial Parkinson's disease. *Lancet* **364,** 1167–1169 (2004).
14. Nishioka, K. *et al.* Clinical heterogeneity of alpha-synuclein gene duplication in Parkinson's disease. *Ann. Neurol.* **59,** 298–309 (2006).
15. Singleton, A. B. *et al.* alpha-Synuclein locus triplication causes Parkinson's disease. *Science* **302,** 841 (2003).
16. Farrer, M. *et al.* Comparison of kindreds with parkinsonism and alpha-synuclein genomic multiplications. *Ann. Neurol.* **55,** 174–179 (2004).
17. Tanner, C. M. *et al.* Rotenone, paraquat, and Parkinson's disease. *Environ. Health Perspect.* **119,** 866–872 (2011).
18. Langston, J. W., Ballard, P., Tetrud, J. W. & Irwin, I. Chronic Parkinsonism in humans due to a product of meperidine-analog synthesis. *Science* **219,** 979–980 (1983).
19. Piccini, P., Burn, D. J., Ceravolo, R., Maraganore, D. & Brooks, D. J. The role of inheritance in sporadic Parkinson's disease: evidence from a longitudinal study of dopaminergic function in twins. *Ann. Neurol.* **45,** 577–582 (1999).
20. Wirdefeldt, K., Gatz, M., Reynolds, C. A., Prescott, C. A. & Pedersen, N. L. Heritability of Parkinson disease in Swedish twins: a longitudinal study. *Neurobiol. Aging* **32,** 1923.e1-1923.e8 (2011).
21. Pan-Montojo, F. & Reichmann, H. Considerations on the role of environmental toxins in idiopathic Parkinson's disease pathophysiology. *Transl. Neurodegener.* **3,** 10–9158–3–10. eCollection 2014 (2014).
22. Drouin-Ouellet, J. & Cicchetti, F. Inflammation and neurodegeneration: the story 'retolled'. *Trends Pharmacol. Sci.* **33,** 542–551 (2012).
23. Tomé, C. M. L. *et al.* Inflammation and α-Synuclein's Prion-like Behavior in Parkinson's Disease—Is There a Link? *Mol. Neurobiol.* **47,** 561–574 (2013).
24. Hirsch, E. C., Vyas, S. & Hunot, S. Neuroinflammation in Parkinson's disease. *Parkinsonism Relat. Disord.* **18,** S210–S212 (2012).
25. Clairembault, T., Leclair-Visonneau, L., Neunlist, M. & Derkinderen, P. Enteric glial cells: New players in Parkinson's disease? *Mov. Disord.* (2014).
26. Lee, H. J. *et al.* Direct transfer of alpha-synuclein from neuron to astroglia causes inflammatory responses in synucleinopathies. *J. Biol. Chem.* **285,** 9262–9272 (2010).
27. Rogers, J., Mastroeni, D., Leonard, B., Joyce, J. & Grover, A. Neuroinflammation in Alzheimer's disease and Parkinson's disease:

are microglia pathogenic in either disorder? *Int. Rev. Neurobiol.* **82,** 235–246 (2007).
28. Niranjan, R. The role of inflammatory and oxidative stress mechanisms in the pathogenesis of Parkinson's disease: focus on astrocytes. *Mol. Neurobiol.* **49,** 28–38 (2014).
29. Zaltieri, M. *et al.* Mitochondrial Dysfunction and-Synuclein Synaptic Pathology in Parkinson's Disease: Who's on First? *Park. Dis.* **2015,** (2015).
30. Dehay, B. *et al.* Targeting α-synuclein for treatment of Parkinson's disease: mechanistic and therapeutic considerations. *Lancet Neurol.* (2015).
31. Braak, H., Rub, U., Gai, W. P. & Del Tredici, K. Idiopathic Parkinson's disease: possible routes by which vulnerable neuronal types may be subject to neuroinvasion by an unknown pathogen. *J. Neural Transm. Vienna Austria 1996* **110,** 517–536 (2003).
32. Braak, H. *et al.* Staging of brain pathology related to sporadic Parkinson's disease. *Neurobiol. Aging* **24,** 197–211 (2003).
33. Hawkes, C. H., Del Tredici, K. & Braak, H. Parkinson's disease: a dual-hit hypothesis. *Neuropathol. Appl. Neurobiol.* **33,** 599–614 (2007).
34. Hawkes, C. H., Del Tredici, K. & Braak, H. Parkinson's disease: the dual hit theory revisited. *Ann. N. Y. Acad. Sci.* **1170,** 615–622 (2009).
35. Del Tredici, K. & Braak, H. Review: Sporadic Parkinson's disease: development and distribution of α-synuclein pathology. *Neuropathol Appl Neurobiol* **42,** 33–50 (2016).
36. Pfeiffer, R. F. Gastrointestinal dysfunction in Parkinson's disease. *Parkinsonism Relat. Disord.* **17,** 10–15 (2011).
37. Cersosimo, M. G. & Benarroch, E. E. Pathological correlates of gastrointestinal dysfunction in Parkinson's disease. *Neurobiol. Dis.* **46,** 559–564 (2012).
38. Doty, R. L. Olfaction in Parkinson's disease and related disorders. *Neurobiol. Dis.* **46,** 527–552 (2012).
39. Beach, T. G. *et al.* Olfactory bulb alpha-synucleinopathy has high specificity and sensitivity for Lewy body disorders. *Acta Neuropathol. (Berl.)* **117,** 169–174 (2009).
40. Hubbard, P. S., Esiri, M. M., Reading, M., McShane, R. & Nagy, Z. Alpha-synuclein pathology in the olfactory pathways of dementia patients. *J. Anat.* **211,** 117–124 (2007).
41. Wakabayashi, K., Takahashi, H., Takeda, S., Ohama, E. & Ikuta, F. Parkinson's disease: the presence of Lewy bodies in Auerbach's and Meissner's plexuses. *Acta Neuropathol. (Berl.)* **76,** 217–221 (1988).
42. Braak, H., de Vos, R. A., Bohl, J. & Del Tredici, K. Gastric alpha-synuclein immunoreactive inclusions in Meissner's and Auerbach's plexuses in cases staged for Parkinson's disease-related brain pathology. *Neurosci. Lett.* **396,** 67–72 (2006).
43. Shannon, K. M. *et al.* Alpha-synuclein in colonic submucosa in early untreated Parkinson's disease. *Mov. Disord.* **27,** 709–715 (2012).
44. Lebouvier, T. *et al.* Colonic biopsies to assess the neuropathology of Parkinson's disease and its relationship with symptoms. *PloS One* **5,** e12728 (2010).
45. Bloch, A., Probst, A., Bissig, H., Adams, H. & Tolnay, M. α-Synuclein pathology of the spinal and peripheral autonomic nervous system in neurologically unimpaired elderly subjects. *Neuropathol. Appl. Neurobiol.* **32,** 284–295 (2006).
46. Abbott, R. D. *et al.* Bowel movement frequency in late-life and incidental Lewy bodies. *Mov. Disord. Off. J. Mov. Disord. Soc.* **22,** 1581–1586 (2007).
47. Cersosimo, M. G. *et al.* Gastrointestinal manifestations in Parkinson's disease: prevalence and occurrence before motor symptoms. *J. Neurol.* **260,** 1332–1338 (2013).
48. Ross, G. W. *et al.* Association of olfactory dysfunction with incidental Lewy bodies. *Mov. Disord.* **21,** 2062–2067 (2006).
49. Ross, G. W. *et al.* Association of olfactory dysfunction with risk for future Parkinson's disease. *Ann. Neurol.* **63,** 167–173 (2008).
50. Dickson, D. W. *et al.* Evidence that incidental Lewy body disease is pre-symptomatic Parkinson's disease. *Acta Neuropathol. (Berl.)* **115,** 437–444 (2008).
51. Hallett, P. J., McLean, J. R., Kartunen, A., Langston, J. W. & Isacson, O. Alpha-synuclein overexpressing transgenic mice show internal organ pathology and autonomic deficits. *Neurobiol. Dis.* **47,** 258–267 (2012).
52. Kuo, Y. M. *et al.* Extensive enteric nervous system abnormalities in mice transgenic for artificial chromosomes containing Parkinson disease-associated alpha-synuclein gene mutations precede central nervous system changes. *Hum. Mol. Genet.* **19,** 1633–1650 (2010).

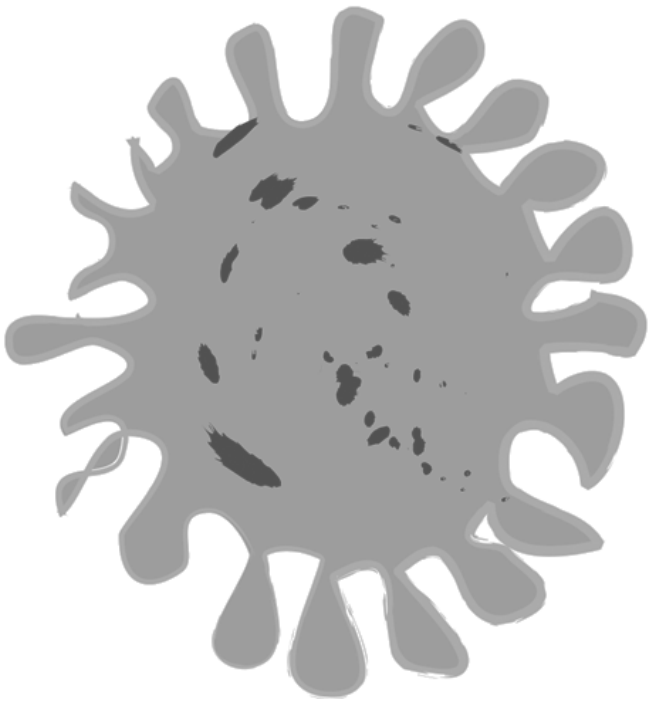
53. Anderson, G. *et al.* Loss of enteric dopaminergic neurons and associated changes in colon motility in an MPTP mouse model of Parkinson's disease. *Exp. Neurol.* **207,** 4–12 (2007).
54. Greene, J. G., Noorian, A. R. & Srinivasan, S. Delayed gastric emptying and enteric nervous system dysfunction in the rotenone model of Parkinson's disease. *Exp. Neurol.* **218,** 154–161 (2009).
55. Natale, G. *et al.* MPTP-induced parkinsonism extends to a subclass of TH-positive neurons in the gut. *Brain Res.* **1355,** 195–206 (2010).
56. Fleming, S. M. *et al.* Early and progressive sensorimotor anomalies in mice overexpressing wild-type human alpha-synuclein. *J. Neurosci. Off. J. Soc. Neurosci.* **24,** 9434–9440 (2004).
57. Hunot, S. *et al.* JNK-mediated induction of cyclooxygenase 2 is required for neurodegeneration in a mouse model of Parkinson's disease. *Proc. Natl. Acad. Sci. U. S. A.* **101,** 665–670 (2004).
58. Fleming, S. M. *et al.* Behavioral and immunohistochemical effects of chronic intravenous and subcutaneous infusions of varying doses of rotenone. *Exp. Neurol.* **187,** 418–429 (2004).
59. Wang, L. *et al.* Mice overexpressing wild-type human alpha-synuclein display alterations in colonic myenteric ganglia and defecation. *Neurogastroenterol. Motil.* **24,** e425–e436 (2012).
60. Drolet, R. E., Cannon, J. R., Montero, L. & Greenamyre, J. T. Chronic rotenone exposure reproduces Parkinson's disease gastrointestinal neuropathology. *Neurobiol. Dis.* **36,** 96–102 (2009).
61. Noorian, A. R. *et al.* Alpha-synuclein transgenic mice display age-related slowing of gastrointestinal motility associated with transgene expression in the vagal system. *Neurobiol. Dis.* **48,** 9–19 (2012).
62. Kelly, L. P. *et al.* Progression of intestinal permeability changes and alpha-synuclein expression in a mouse model of Parkinson's disease. *Mov. Disord.* **29,** 999–1009 (2014).
63. Scheperjans, F. *et al.* Gut microbiota are related to Parkinson's disease and clinical phenotype. *Mov. Disord.* (2014).
64. Devos, D. *et al.* Colonic inflammation in Parkinson's disease. *Neurobiol. Dis.* **50,** 42–48 (2013).
65. Forsyth, C. B. *et al.* Increased intestinal permeability correlates with sigmoid mucosa alpha-synuclein staining and endotoxin exposure markers in early Parkinson's disease. *PloS One* **6,** e28032 (2011).
66. Turnbaugh, P. J. *et al.* The effect of diet on the human gut microbiome: a metagenomic analysis in humanized gnotobiotic mice. *Sci. Transl. Med.* **1,** 6ra14 (2009).
67. Wong, J. M. W. *et al.* Gut microbiota, diet, and heart disease. *J. AOAC Int.* **95,** 24–30 (2012).
68. Singh, N. *et al.* Activation of Gpr109a, receptor for niacin and the commensal metabolite butyrate, suppresses colonic inflammation and carcinogenesis. *Immunity* **40,** 128–139 (2014).
69. Brinkworth, G. D., Noakes, M., Clifton, P. M. & Bird, A. R. Comparative effects of very low-carbohydrate, high-fat and high-carbohydrate, low-fat weight-loss diets on bowel habit and faecal short-chain fatty acids and bacterial populations. *Br. J. Nutr.* **101,** 1493–1502 (2009).
70. De Filippo, C. *et al.* Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa. *Proc. Natl. Acad. Sci. U. S. A.* **107,** 14691–14696 (2010).
71. Fava, F. *et al.* The type and quantity of dietary fat and carbohydrate alter faecal microbiome and short-chain fatty acid excretion in a metabolic syndrome 'at-risk' population. *Int. J. Obes. 2005* **37,** 216–223 (2013).
72. Surmeier, D. J. & Sulzer, D. The pathology roadmap in Parkinson disease. *Prion* **7,** 85–91 (2013).
73. Del Tredici, K. & Braak, H. A not entirely benign procedure: progression of Parkinson's disease. *Acta Neuropathol. (Berl.)* **115,** 379–384 (2008).
74. Del Tredici, K., Hawkes, C. H., Ghebremedhin, E. & Braak, H. Lewy pathology in the submandibular gland of individuals with incidental Lewy body disease and sporadic Parkinson's disease. *Acta Neuropathol. (Berl.)* **119,** 703–713 (2010).
75. Takeda, S., Yamazaki, K., Miyakawa, T. & Arai, H. Parkinson's disease with involvement of the parasympathetic ganglia. *Acta Neuropathol. (Berl.)* **86,** 397–398 (1993).
76. Del Tredici, K., Rub, U., De Vos, R. A., Bohl, J. R. & Braak, H. Where does parkinson disease pathology begin in the brain? *J. Neuropathol. Exp. Neurol.* **61,** 413–426 (2002).
77. Jellinger, K. A. Lewy body-related α-synucleinopathy in the aged human brain. *J. Neural Transm.* **111,** 1219–1235 (2004).

78. Hely, M. A., Reid, W. G. J., Adena, M. A., Halliday, G. M. & Morris, J. G. L. The Sydney multicenter study of Parkinson's disease: the inevitability of dementia at 20 years. *Mov. Disord.* **23,** 837–844 (2008).
79. Gai, W. P., Blumbergs, P. C., Geffen, L. B. & Blessing, W. W. Age-related loss of dorsal vagal neurons in Parkinson's disease. *Neurology* **42,** 2106–2111 (1992).
80. Halliday, G., Hely, M., Reid, W. & Morris, J. The progression of pathology in longitudinally followed patients with Parkinson's disease. *Acta Neuropathol. (Berl.)* **115,** 409–415 (2008).
81. Svensson, E. *et al.* Vagotomy and subsequent risk of Parkinson's disease. *Ann Neurol* **78,** 522–9 (2015).
82. Holmqvist, S. *et al.* Direct evidence of Parkinson pathology spread from the gastrointestinal tract to the brain in rats. *Acta Neuropathol. (Berl.)* **128,** 805–820 (2014).
83. Pan-Montojo, F. *et al.* Progression of Parkinson's disease pathology is reproduced by intragastric administration of rotenone in mice. *PloS One* **5,** e8762 (2010).
84. Pan-Montojo, F. *et al.* Environmental toxins trigger PD-like progression via increased alpha-synuclein release from enteric neurons in mice. *Sci. Rep.* **2,** (2012).
85. Kordower, J. H., Chu, Y., Hauser, R. A., Freeman, T. B. & Olanow, C. W. Lewy body–like pathology in long-term embryonic nigral transplants in Parkinson's disease. *Nat. Med.* **14,** 504–506 (2008).
86. Li, J.-Y. *et al.* Lewy bodies in grafted neurons in subjects with Parkinson's disease suggest host-to-graft disease propagation. *Nat. Med.* **14,** 501–503 (2008).
87. Kordower, J. H., Chu, Y., Hauser, R. A., Olanow, C. W. & Freeman, T. B. Transplanted dopaminergic neurons develop PD pathologic changes: a second case report. *Mov. Disord.* **23,** 2303–2306 (2008).
88. Li, J. *et al.* Characterization of Lewy body pathology in 12-and 16-year-old intrastriatal mesencephalic grafts surviving in a patient with Parkinson's disease. *Mov. Disord.* **25,** 1091–1096 (2010).
89. Chu, Y. & Kordower, J. H. Lewy body pathology in fetal grafts. *Ann. N. Y. Acad. Sci.* **1184,** 55–67 (2010).
90. Kurowska, Z. *et al.* Signs of degeneration in 12–22-year old grafts of mesencephalic dopamine neurons in patients with Parkinson's disease. *J. Park. Dis.* **1,** 83–92 (2011).
91. Desplats, P. *et al.* Inclusion formation and neuronal cell death through neuron-to-neuron transmission of alpha-synuclein. *Proc. Natl. Acad. Sci. U. S. A.* **106,** 13010–13015 (2009).
92. Hansen, C. *et al.* alpha-Synuclein propagates from mouse brain to grafted dopaminergic neurons and seeds aggregation in cultured human cells. *J. Clin. Invest.* **121,** 715–725 (2011).
93. Kordower, J. H. *et al.* Transfer of host-derived alpha synuclein to grafted dopaminergic neurons in rat. *Neurobiol. Dis.* **43,** 552–557 (2011).
94. Angot, E. *et al.* Alpha-synuclein cell-to-cell transfer and seeding in grafted dopaminergic neurons in vivo. *PLoS One* **7,** e39465 (2012).
95. Lee, H. J., Patel, S. & Lee, S. J. Intravesicular localization and exocytosis of alpha-synuclein and its aggregates. *J. Neurosci. Off. J. Soc. Neurosci.* **25,** 6016–6024 (2005).
96. Emmanouilidou, E. *et al.* Cell-produced alpha-synuclein is secreted in a calcium-dependent manner by exosomes and impacts neuronal survival. *J. Neurosci. Off. J. Soc. Neurosci.* **30,** 6838–6851 (2010).
97. Danzer, K. M. *et al.* Exosomal cell-to-cell transmission of alpha synuclein oligomers. *Mol. Neurodegener.* **7,** 42 (2012).
98. Lee, H.-J. *et al.* Assembly-dependent endocytosis and clearance of extracellular a-synuclein. *Int. J. Biochem. Cell Biol.* **40,** 1835–1849 (2008).
99. Jensen, P. H., Li, J., DahlstroÈm, A. & Dotti, C. G. Axonal transport of synucleins is mediated by all rate components. *Eur. J. Neurosci.* **11,** 3369–3376 (1999).
100. Freundt, E. C. *et al.* Neuron-to-neuron transmission of α-synuclein fibrils through axonal transport. *Ann. Neurol.* **72,** 517–524 (2012).
101. Volpicelli-Daley, L. A. *et al.* Exogenous α-synuclein fibrils induce Lewy body pathology leading to synaptic dysfunction and neuron death. *Neuron* **72,** 57–71 (2011).
102. Mao, X. *et al.* Pathological α-synuclein transmission initiated by binding lymphocyte-activation gene 3. *Science* **353,** (2016).
103. Braak, H. & Del Tredici, K. Poor and protracted myelination as a contributory factor to neurodegenerative disorders. *Neurobiol. Aging* **25,** 19–23 (2004).

104. Lebouvier, T. *et al.* The second brain and Parkinson's disease. *Eur. J. Neurosci.* **30,** 735–741 (2009).
105. Visanji, N. P., Brooks, P. L., Hazrati, L.-N. & Lang, A. E. The prion hypothesis in Parkinson's disease: Braak to the future. *Acta Neuropathol Commun* **1,** 12 (2013).
106. Jucker, M. & Walker, L. C. Self-propagation of pathogenic protein aggregates in neurodegenerative diseases. *Nature* **501,** 45–51 (2013).
107. Tanaka, M. *et al.* Aggresomes formed by alpha-synuclein and synphilin-1 are cytoprotective. *J. Biol. Chem.* **279,** 4625–4631 (2004).
108. Andreasen, M., Lorenzen, N. & Otzen, D. Interactions between misfolded protein oligomers and membranes: A central topic in neurodegenerative diseases? *Biochim. Biophys. Acta BBA-Biomembr.* (2015).
109. Lindström, V. *et al.* Immunotherapy targeting α-synuclein protofibrils reduced pathology in (Thy-1)-h [A30P] α-synuclein mice. *Neurobiol. Dis.* **69,** 134–143 (2014).
110. Roberts, H. L. & Brown, D. R. Seeking a Mechanism for the Toxicity of Oligomeric α-Synuclein. *Biomolecules* **5,** 282–305 (2015).
111. Luk, K. C. *et al.* Exogenous alpha-synuclein fibrils seed the formation of Lewy body-like intracellular inclusions in cultured cells. *Proc. Natl. Acad. Sci. U. S. A.* **106,** 20051–20056 (2009).
112. Luk, K. C. *et al.* Intracerebral inoculation of pathological alpha-synuclein initiates a rapidly progressive neurodegenerative alpha-synucleinopathy in mice. *J. Exp. Med.* **209,** 975–986 (2012).
113. Mougenot, A.-L. *et al.* Prion-like acceleration of a synucleinopathy in a transgenic mouse model. *Neurobiol. Aging* **33,** 2225–2228 (2012).
114. Luk, K. C. *et al.* Pathological alpha-synuclein transmission initiates Parkinson-like neurodegeneration in nontransgenic mice. *Science* **338,** 949–953 (2012).
115. Osterberg, V. R. *et al.* Progressive Aggregation of Alpha-Synuclein and Selective Degeneration of Lewy Inclusion-Bearing Neurons in a Mouse Model of Parkinsonism. *Cell Rep.* **10,** 1252–1260 (2015).
116. Recasens, A. *et al.* Lewy body extracts from Parkinson disease brains trigger α-synuclein pathology and neurodegeneration in mice and monkeys. *Ann. Neurol.* **75,** 351–362 (2014).
117. Jellinger, K. A. A critical reappraisal of current staging of Lewy-related pathology in human brain. *Acta Neuropathol. (Berl.)* **116,** 1–16 (2008).
118. Kalaitzakis, M. E., Graeber, M. B., Gentleman, S. M. & Pearce, R. K. B. Evidence against a reliable staging system of α-synuclein pathology in Parkinson's disease. *Neuropathol. Appl. Neurobiol.* **35,** 125–126 (2009).
119. Kalaitzakis, M. E., Graeber, M. B., Gentleman, S. M. & Pearce, R. K. B. The dorsal motor nucleus of the vagus is not an obligatory trigger site of Parkinson's disease: a critical analysis of α-synuclein staging. *Neuropathol. Appl. Neurobiol.* **34,** 284–295 (2008).
120. Attems, J. & Jellinger, K. A. The dorsal motor nucleus of the vagus is not an obligatory trigger site of Parkinson's disease. *Neuropathol. Appl. Neurobiol.* **34,** 466–467 (2008).
121. Parkkinen, L., Pirttilä, T. & Alafuzoff, I. Applicability of current staging/categorization of α-synuclein pathology and their clinical relevance. *Acta Neuropathol. (Berl.)* **115,** 399–407 (2008).
122. Jellinger, K. A. α-Synuclein pathology in Parkinson's and Alzheimer's disease brain: incidence and topographic distribution—a pilot study. *Acta Neuropathol. (Berl.)* **106,** 191–202 (2003).
123. Zaccai, J. *et al.* Patterns and stages of alpha-synucleinopathy: Relevance in a population-based cohort. *Neurology* **70,** 1042–1048 (2008).
124. Beach, T. G. *et al.* Unified staging system for Lewy body disorders: correlation with nigrostriatal degeneration, cognitive impairment and motor dysfunction. *Acta Neuropathol. (Berl.)* **117,** 613–634 (2009).
125. Kingsbury, A. E. *et al.* Brain stem pathology in Parkinson's disease: an evaluation of the Braak staging model. *Mov. Disord.* **25,** 2508–2515 (2010).
126. Lebouvier, T. *et al.* Colonic Neuropathology is Independent of Olfactory Dysfunction in Parkinson's Disease. *J. Park. Dis.* **1,** 389–394 (2011).
127. Beach, T. G. *et al.* Multi-organ distribution of phosphorylated α-synuclein histopathology in subjects with Lewy body disorders. *Acta Neuropathol. (Berl.)* **119,** 689–702 (2010).
128. Parkkinen, L., Soininen, H. & Alafuzoff, I. Regional Distribution of α-Synuclein Pathology

in Unimpaired Aging and Alzheimer Disease. *J. Neuropathol. Exp. Neurol.* **62,** 363–367 (2003).
129. Orimo, S. *et al.* Degeneration of cardiac sympathetic nerve begins in the early disease process of Parkinson's disease. *Brain Pathol.* **17,** 24–30 (2007).
130. Parkkinen, L., Kauppinen, T., Pirttilä, T., Autere, J. M. & Alafuzoff, I. α-Synuclein pathology does not predict extrapyramidal symptoms or dementia. *Ann. Neurol.* **57,** 82–91 (2005).
131. Milber, J. M. *et al.* Lewy pathology is not the first sign of degeneration in vulnerable neurons in Parkinson disease. *Neurology* **79,** 2307–2314 (2012).
132. Kalaitzakis, M. E., Graeber, M. B., Gentleman, S. M. & Pearce, R. K. B. Controversies over the staging of α-synuclein pathology in Parkinson's disease. *Acta Neuropathol. (Berl.)* **116,** 125–128 (2008).
133. Greffard, S. *et al.* Motor score of the Unified Parkinson Disease Rating Scale as a good predictor of Lewy body–associated neuronal loss in the substantia nigra. *Arch. Neurol.* **63,** 584–588 (2006).
134. Parkkinen, L. *et al.* Disentangling the relationship between Lewy bodies and nigral neuronal loss in Parkinson's disease. *J. Park. Dis.* **1,** 277–286 (2011).
135. Doorn, K. J. *et al.* Microglial phenotypes and toll-like receptor 2 in the substantia nigra and hippocampus of incidental Lewy body disease cases and Parkinson's disease patients. *Acta Neuropathol. Commun.* **2,** 90–91 (2014).
136. Kalinderi, K., Bostantjopoulou, S., Katsarou, Z. & Fidani, L. TLR9 -1237 T/C and TLR2 -194 to -174 del polymorphisms and the risk of Parkinson's disease in the Greek population: a pilot study. *Neurol. Sci. Off. J. Ital. Neurol. Soc. Ital. Soc. Clin. Neurophysiol.* **34,** 679–682 (2013).
137. Noguchi, E. *et al.* An association study of asthma and total serum immunoglobin E levels for Toll-like receptor polymorphisms in a Japanese population. *Clin. Exp. Allergy J. Br. Soc. Allergy Clin. Immunol.* **34,** 177–83 (2004).
138. Drouin-Ouellet, J. *et al.* Toll-like receptor expression in the blood and brain of patients and a mouse model of Parkinson's Disease. *Int. J. Neuropsychopharmacol.* pyu103 (2014).
139. Kim, C. *et al.* β1-integrin-dependent migration of microglia in response to neuron-released α-synuclein. *Exp Mol Med* **46,** (2014).
140. Qiao, H. *et al.* Elevated neuronal α-synuclein promotes microglia activation after spinal cord ischemic/reperfused injury. *Neuroreport* **26,** 656–61 (2015).
141. Daniele, S. *et al.* Activation of MyD88-dependent TLR1/2 signaling by misfolded α-synuclein, a protein linked to neurodegenerative disorders. *Sci Signal* **8,** ra45 (2015).
142. Beraud, D. *et al.* alpha-Synuclein Alters Toll-Like Receptor Expression. *Front. Neurosci.* **5,** 80 (2011).
143. Roodveldt, C. *et al.* Preconditioning of Microglia by α-Synuclein Strongly Affects the Response Induced by Toll-like Receptor (TLR) Stimulation. *PloS One* **8,** e79160 (2013).
144. Lehnardt, S. *et al.* Activation of innate immunity in the CNS triggers neurodegeneration through a Toll-like receptor 4-dependent pathway. *Proc. Natl. Acad. Sci. U. S. A.* **100,** 8514–8519 (2003).
145. Stefanova, N. *et al.* Toll-like receptor 4 promotes alpha-synuclein clearance and survival of nigral dopaminergic neurons. *Am. J. Pathol.* **179,** 954–963 (2011).
146. Fellner, L. *et al.* Toll-like receptor 4 is required for α-synuclein dependent activation of microglia and astroglia. *Glia* **61,** 349–60 (2013).
147. Rannikko, E. H., Weber, S. S. & Kahle, P. J. Exogenous α-synuclein induces toll-like receptor 4 dependent inflammatory responses in astrocytes. *BMC Neurosci.* **16,** 57 (2015).
148. Noelker, C. *et al.* Toll like receptor 4 mediates cell death in a mouse MPTP model of Parkinson disease. *Sci. Rep.* **3,** 1393 (2013).
149. Kim, C. *et al.* Antagonizing Neuronal Toll-like Receptor 2 Prevents Synucleinopathy by Activating Autophagy. *Cell Rep* **13,** 771–82 (2015).

CHAPTER 3



Gut-brain and brain-gut axis in Parkinson's disease models: effects of a uridine and fish oil diet.

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Abstract

Recent investigations have focused on the potential role of gastrointestinal abnormalities in the pathogenesis of Parkinson's disease (PD). The "dual-hit" hypothesis of PD speculates that a putative pathogen enters the brain via two routes: the olfactory system and the gastrointestinal system. Here, we investigated [1] whether local exposures of the neurotoxin rotenone in the gut or the brain of mice, could induce PD-like neurological and gastrointestinal phenotypes as well as a characteristic neuropathology in accordance with this "dual-hit hypothesis" and [2] the effects of a diet containing uridine and fish oil providing docosahexaenoic acid, in both models. Mice were given rotenone either orally or by an injection in the striatum. Dietary interventions were started 1 week before rotenone exposures.

We found that: [1] both oral and intrastriatal administration of rotenone induced similar PD-like motor deficits, dopaminergic cell loss, delayed intestinal transit, inflammation and alpha-synuclein accumulation in the colon; [2] the uridine and docosahexaenoic acid containing diet prevented rotenone-induced motor and gastrointestinal dysfunctions in both models.

The models suggest possible bidirectional communication between the gut and the brain for the genesis of PD-like phenotype and pathology. The dietary intervention may provide benefits in the prevention of motor and non-motor symptoms in PD.

1. Introduction

Patients suffering from Parkinson's disease (PD) often develop non-motor symptoms such as hyposmia[1,2] and gastrointestinal (GI) dysfunctions[3,4]. These symptoms may precede the classical motor symptoms by many years[5–7] and their occurrence in otherwise healthy people is associated with an increased risk of developing PD[5,8]. Therefore, a better understanding of these non-motor impairments may provide important insights into the etiology and progression of PD. In recent years, special focus has been placed upon the GI-tract and the associated enteric nervous system (ENS) in the development of PD[9–12]. The ENS is a major player in the gut-brain axis which is a bidirectional communication system between the central nervous system (CNS) and the GI-tract[13].

Animal models are invaluable tools to investigate the underlying mechanisms of the pathogenesis of PD and to test potential symptomatic, neuroprotective, and neurorestorative therapies. To date, many studies have used different compounds and routes of administration in order to reproduce a PD phenotype in animals. Most of these studies focused on the motor symptoms and only few explored the role of the gut-brain or brain-gut axis in the development of the disease[14–17].

The pesticide rotenone is a potent mitochondrial complex I inhibitor that promotes reactive oxygen species formation. Mitochondrial respiratory chain has shown to be vulnerable during PD pathophysiology. Although there are many different rotenone models of PD, all of which may differ in the resulting phenotype depending on the procedural details, many hallmarks of PD have been replicated in these models, including loss of dopaminergic cell bodies in the substantia nigra (SN)[18], alpha-synuclein aggregation[19,20], and GI dysfunction[15,16,21]. Rotenone exposure is known to be associated with an increased risk of developing PD in humans[22]. Therefore, rotenone might be a good candidate to mimic the human PD-like characteristics in animal models.

In early untreated PD patients and in subjects with PD-related brain pathology but still without motor symptoms, neurons of the ENS and the olfactory bulbs were found to contain alpha-synuclein aggregates[23,24]. Braak and co-workers proposed in their 'dual-hit hypothesis' that alpha-synuclein pathology primes in the ENS and spreads to the brain, thereby suggesting an active retrograde transport via the vagal nerve (gut to brain)[25,26]. Environmental factors might also start the pathology in the olfactory bulbs, affecting the brain more directly and then spreading to the ENS (brain to gut)[25]. This study aimed to investigate whether rotenone exposures in the gut or the brain, would either induce pathology and symptoms restricted to the gut or the brain respectively, or could induce PD-like pathology in accordance to Braak's hypothesis[25] and thereby develop a similar PD-like phenotype including both motor problems and GI dysfunction.

In addition, we investigated the effects of a specific dietary intervention combining uridine and docosahexaenoic acid (DHA) on motor and non-motor symptoms, in both rotenone mouse models. Uridine and DHA are dietary precursors for membrane phospholipid

synthesis and their administration may synergistically support synaptic membrane formation, relevant to PD[27,28]. This dietary intervention was shown to partially restore dopaminergic neurotransmission in the 6-OHDA model of PD in rats[27]. A recent study demonstrated that dietary fat intake may modify PD risk directly or by altering the response to environmental neurotoxins including pesticides; high levels of polyunsaturated fatty acids (PUFAs), like DHA decreased the association of PD with pesticide exposure[29]. Individually, both DHA and uridine have been shown to induce favorable effects with preventive intake in various animal models of PD, albeit by different modes of action. In the MPTP mouse model, both DHA[30] and uridine[31] prevented neurodegeneration. Similarly, in the 6-OHDA rat model, both DHA[32] and uridine[33] reduced drug-induced rotational behavior, possibly by enhancing dopamine turnover in remaining neurons. To date, there have been no studies exploring the beneficial effects of this active diet on the GI dysfunction associated with PD. Now we test the combined administration of DHA and uridine on non-motor and motor symptoms of PD using two different rotenone mouse models of PD.

2. Methods

Mice

Seven week-old C57BL/6J male mice (Charles River, The Netherlands) were housed at room temperature under 12h light/dark cycle. Food and water was provided *ad libitum.* Animal procedures were approved by the Ethical Committee of Animal Research of Utrecht University, Utrecht, The Netherlands. We used male mice because males have a higher relative risk for developing PD[34]. The age of the animals was based on previous studies with similar models of PD[17,35].

Diets

Mice were fed either a control or an active diet one week prior to either the start of rotenone administration (oral model) or before surgery (intrastriatal model) and continuing for the duration of the experiment. For both models, animals were divided into four groups (n=10). Iso-caloric diets were produced by Research Diet Services (Wijk bij Duurstede, The Netherlands) based on standard food for laboratory rodents AIN-93M[36]. The control diet contained 14% protein (casein), 5% lipids (soy, coconut, and corn oil), 71% carbohydrates (corn starch, maltodextrin, sucrose, and dextrose), 5% fibers (cellulose), and 5% of standard AIN-93M mineral and vitamin mixes and additives. For the active diet, part of the lipid blend of the control diet was replaced by fish oil, providing DHA (0.74 g/100g diet) and EPA (0.29 g/100g diet), and uridine-monophosphate was added as a source of uridine (0.51 g/100g diet), replacing its weight equivalent of corn starch.

Induction of mitochondrial dysfunction by rotenone: two murine models

For the oral rotenone model, mice received 10mg/kg freshly prepared rotenone solution once a day for 28 days by oral gavage (this dose has been shown to cause a reduction of TH immunoreactivity in the SN)[35]. Control animals received vehicle (4% carboxymethylcellulose and 1.25% chloroform). It has been previously shown that rotenone is not detected in the brain of mice receiving oral doses of 10mg/kg or lower[17], although the effects of circulating rotenone on the brain are unknown. On day 28, mice were sacrificed by decapitation.

For the intrastriatal rotenone model, mice underwent stereotaxic surgery under isoflurane anesthesia: a hole was drilled in the skull, a cannula inserted in the right striatum and 5.4 µg of freshly prepared rotenone (dissolved in 2µl DMSO) was infused. In a pilot experiment, this dose of rotenone was the lowest effective dose without increasing mortality. The following stereotaxic coordinates were used: AP +0.4, ML -2.0 from bregma and DV -3.3 below dura. Control animals were injected with vehicle. Forty days after surgery, mice were euthanized by decapitation.

Motor function assessment

The motor function of each mouse was assessed by the Rotarod test as described before[35]. Briefly, mice were placed on an accelerating rod with speeds starting with 2 rpm and gradually increasing to 20 rpm. The latency to fall was recorded for a maximum of 300s. Oral rotenone-treated mice were tested at baseline and every 7 days until day 28. Intrastriatal rotenone-injected animals were tested at baseline and every 5 days until day 40.

Intestinal transit and colon length

Intestinal transit was assessed in all animals. Thirty minutes before sacrificing the mice, a solution of 2.5% Evans blue in 1.5% methylcellulose (0.3mL per animal) was administered intragastrically. After euthanasia, intestinal transit was measured as the distance from the pylorus to the most distal point of migration. In addition, the length of the colon was measured.

Tissue preparation and immunohistochemistry

Coronal brain slices of 40 µm were sectioned using a cryostat (CM3050, Leica Microsystems) and incubated with 0.3% H_2O_2 for 30 min. Following serum block, brain sections were incubated overnight with rabbit anti-tyrosine hydroxylase (TH) (Santa Cruz Biotechnology) 1:1000 followed by biotinylated goat anti-rabbit IgG (Jackson ImmunoResearch) 1:200 for 2h. The avidin-biotin method was used to amplify the signal (ABC kit, Vector) and 3,3'-diaminobenzidine tetrachloride (DAB) was used as chromogen.

The ileum and the colon of the animals were embedded in paraffin. Tissue sections (15 µm) were incubated with 0.3% H_2O_2 for 30 min, rehydrated and incubated with citrate buffer. Sections of ileum and colon were then incubated overnight with the primary antibodies [rabbit anti-alpha-synuclein (1:1000, millipore), rabbit anti-ZO-1 (1:500, abcam) and rabbit anti-CD3 (1:1000, abcam)].

For alpha-synuclein and CD3 staining, sections of colon were incubated with biotinylated secondary antibodies (1:200, Dako). The avidin-biotin method was used to amplify the signal (ABC Kit; Vector) and DAB was used as chromogen. Sections were counterstained with Mayer's haematoxylin (Merck Millipore).

For ZO-1 staining, the sections of ileum and colon were incubated with a fluorescent secondary antibody donkey anti-rabbit (1:200, Alexa Fluor®) and mounted using Vectashield® mounting medium with DAPI (1:10000, Vector).

Image analysis

For immunostained sections, images were captured with an Olympus BX50 microscope equipped with a Leica DFC320 digital camera. TH-immunopositive neurons were quantified stereologically on regular spaced brain sections throughout the SN[37]. To measure alpha-synuclein expression in colon, the optical density (OD) in the area of interest was measured

and corrected for non-specific background. Alpha-synuclein OD was analyzed in at least 100 crypts (with similar orientation) per animal and is expressed per 5 crypts. Stereology was performed to quantify the number of CD3 positive cells on regular spaced sections throughout the colon[38]. Immunofluorescence images of ZO-1 staining were made using a Keyence BZ-9000 microscope.

ZO-1 integrity was measured by analyzing a minimum of 15 images with crypts and epithelial lining sites per animal. All images were taken in blind manner assigned with a code. Tight junction protein ZO-1 integrity for each case was evaluated with scoring scale 0-3 where 0= no ZO-1 expression and 3= continuous great ZO-1 integrity. Minimum of 20 individual crypt and epithelial lining sites were scored using the integrity scale and average was taken for each case.

Statistical analysis

Experimental results are expressed as mean ± SEM. Differences between groups were statistically analyzed with a two-way ANOVA [analyzing significant effects of the treatments (rotenone vs vehicle), diets (active diet vs control diet) and interactions (between diets and treatments)] followed by a Tukey's multiple comparison test. Rotarod test results were analyzed with a general linear model repeated measure ANOVA, with the within subject factor time and the between subject factors treatment and diet. Results were considered statistically significant when $p<0.05$. Analyses were performed using SPSS 22.0.

3. Results

Both oral and intrastriatal rotenone exposures caused motor deficits in mice and these motor deficits were mitigated by the active diet.

To investigate whether oral or intrastriatal rotenone administration and dietary intake affected motor coordination, the rotarod test was performed. The latency to fall from the rod was used for analysis of motor function.

In the oral rotenone experiment, rotarod data (Fig 1A) showed a significant interaction effect between time and treatment (rotenone vs vehicle) [$F(4,132)=2.715$, $p<0.05$]. Rotenone treated mice exhibited a reduction in the latency to fall from the rod starting on day 21 after first rotenone treatment [$F(1,36)=17.31$, $p <0.001$] and this deficit increased on day 28 [$F(1,36)=32.35$, $p<0.0001$]. By then the active diet had beneficial effects on motor function as evidenced by a significant difference between diets (active vs control diet) [$F(1,36)=4.24$, $p<0.05$] and an interaction effect between diets and treatment [$F(1,36)=5.52$, $p<0.05$]. More specifically, on day 28, rotenone-treated mice on the active diet showed a better motor function than rotenone-treated mice on control diet ($p<0.05$) (Fig 1A) suggesting active diet mitigated motor deficits in the oral rotenone model.

In the intrastriatal rotenone experiment, rotarod data (Fig 1B) showed significant differences between treatments (rotenone vs vehicle) [$F(1,33)=131.11$, $p<0.0001$] and diets

[F(1,33)=28.94, p<0.0001]. Repeated measures showed an effect of time [F(7,231)=9.36, p<0.0001]. Rotenone-injected mice developed motor dysfunction over time compared to sham-operated mice [interaction effect between treatment and time (F(7,231)=16.19, p<0.0001)]. Furthermore, there was also an interaction between diets and time [F(7,231)=2.65, p<0.05], between diets and treatments [F(1,33)=10.93, p<0.01], and between treatment, diets and time [F(7,231)=4.97, p<0.0001]. Rotenone-injected mice showed impairments in their ability to remain on the rod starting on day 25 after surgery compared to the sham-operated mice [F(1,36)=21.84 p<0.0001]. The beneficial effects of the active diet were significant on day 25 [F(1,36)=45.78 p<0.0001] and remained significant for the duration of the experiment (F(1,36)=227.2 p<0.0001 day 40). Post-hoc analysis showed that rotenone operated animals on the active diet performed better on the rotarod compared to animals on control diet (p<0.01 day 25, p<0.001 day 30, p<0.05 day 35, p<0.001 day 40)(Fig 1B) suggesting beneficial effects of the active diet in the intra-striatal rotenone model.

Both oral and intrastriatal rotenone exposures caused dopaminergic cell loss in the SN of mice and this cell loss was protected by the active diet.

To investigate the motor impairments associated neurodegeneration, we performed unbiased stereology for the estimated number of TH positive dopaminergic cells in the SN.

In the oral rotenone experiment, we observed a significant bilateral decrease in the number of TH positive cells of rotenone compared to vehicle-treated animals [F(1,24)=85.79, p<0.0001]. There was an overall effect of the diets [F(1,24)=12.00, p<0.01] and an interaction effect between diets and treatment [F(1,24)=12.90, p<0.01]. More specifically, rotenone-treated animals on active diet had more TH positive cells compared to rotenone treated animals on control diet (p<0.001) (Fig 1C: a-d; 1D).

Similar results were found in the intrastriatal model, where significant differences were observed in the number of TH positive cells of rotenone-injected mice compared to sham [F(1,24)=73.57, p<0.0001]. There was an overall effect of the diets [F(1,24)=5.77, p<0.05] and an interaction effect between diets and treatments [F(1,24)=8.34, p<0.01]. Post-hoc analysis revealed a smaller reduction in the number of TH positive cells in rotenone-infused mice on active diet in comparison to rotenone-operated mice on control diet (p<0.01) (Fig 1C:e-h; 1E). Rotenone injection in the striatum caused bilateral dopaminergic cell loss since no differences were found in the estimated number of TH positive cells in the SN between the two hemispheres.

Both oral and intrastriatal rotenone exposures caused gastrointestinal dysfunction in mice and this dysfunction was reduced by the active diet.

We recorded intestinal transit time (distance travelled by the Evans blue dye in the GI-tract) as a GI dysfunction parameter to assess the effects of rotenone in both models.

In the oral rotenone experiment, the intestinal transit (Fig 2A) in the rotenone-treated mice was reduced compared to vehicle-treated mice [$F(1,36)=24.41$, $p<0.0001$]. The active diet had an overall effect [$F(1,35)=7.290$, $p<0.05$] and there was a significant interaction effect between treatment and diets [$F(1,35)=12.51$, $p<0.01$]. Increased intestinal transit (increased distance covered by the dye) was observed in rotenone-treated mice on active diet compared to rotenone-treated mice on control diet ($p<0.01$) (Fig 2A).

In the intrastriatal rotenone model, Rotenone injection decreased intestinal transit (decreased distance travelled by the dye) compared to sham-operated mice [$F(1,31)=25.39$, $p<0.0001$]. The active diet increased the distance [$F(1,31)=8.348$, $p<0.01$]. More specifically, rotenone-operated mice on active diet showed an increased intestinal transit compared to rotenone-operated mice on control diet ($p<0.01$) (Fig 2B).

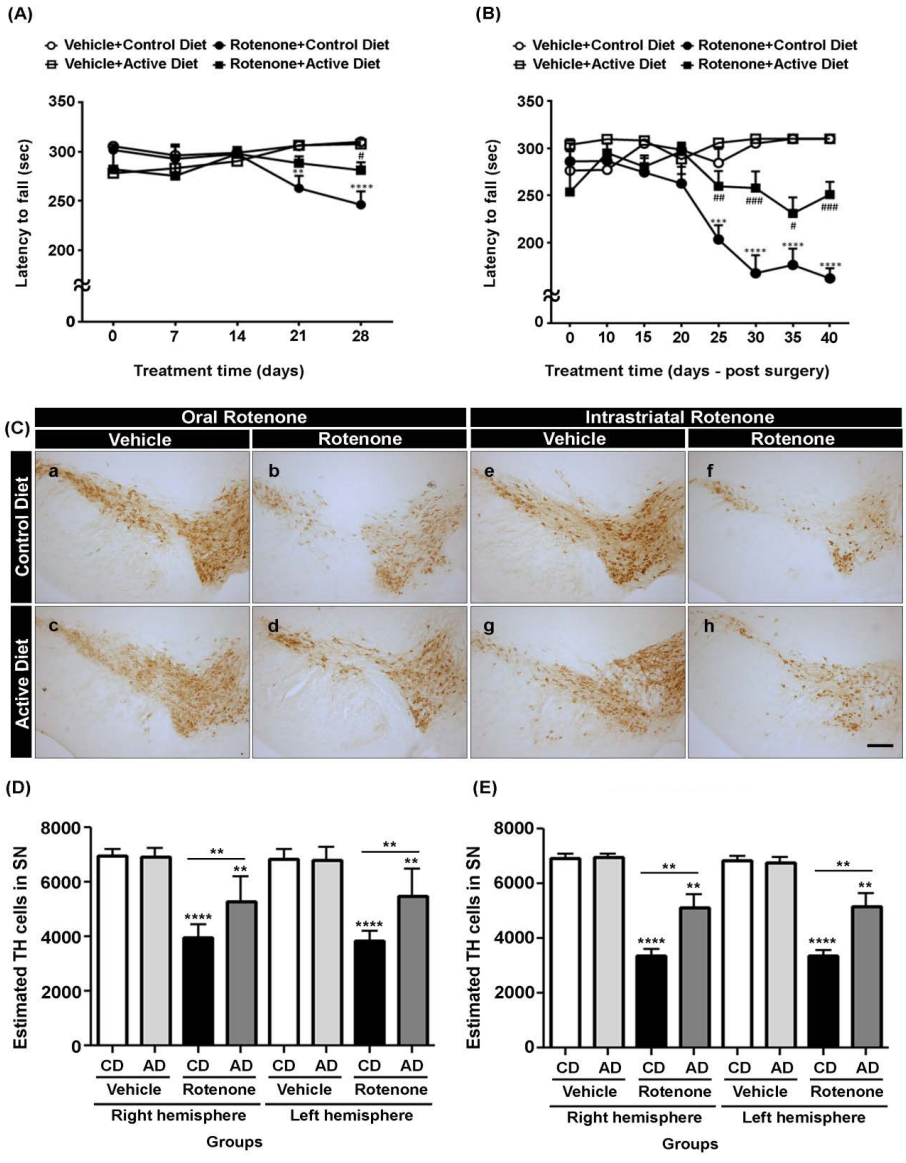

Figure 1: Effects of the active diet on the latency to fall and dopaminergic cell loss in the substantia nigra in **(A, C: a-d, D)** orally rotenone-treated mice and **(B, C: e-h, E)** intrastriatal rotenone-injected mice. Both rotenone treatments induced motor dysfunction and decreased the number of dopaminergic cells. The active diet showed beneficial effects in both parameters for both models. **p<0.01, ***p<0.001, ****p<0.0001 compared to vehicle control diet; #p<0.05 ##p<0.01, ###p<0.001 compared to rotenone control diet. (Scale bars: 100µm applies to all panels).

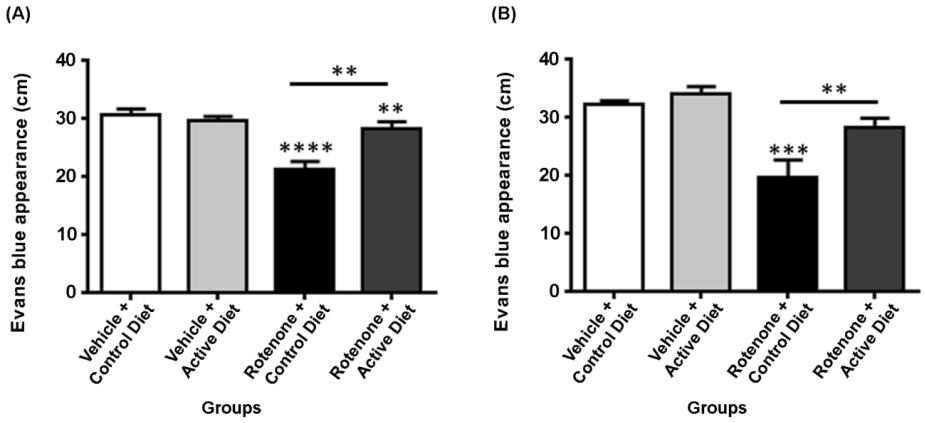

Figure 2: Effects of the active diet on intestinal transit indicated by the total distance travelled by the Evans blue dye in the GI tract 30 min after its injection by oral gavage in **(A)** orally rotenone-treated mice and **(B)** intrastriatal rotenone-injected mice. Both rotenone treatments reduced intestinal transit time and the active diet had beneficial effects on intestinal transit time for both models.**p<0.01, ***p<0.001, ****p<0.0001.

Both oral and intrastriatal rotenone exposures increased alpha-synuclein expression in the colon of mice and its levels were reduced by the active diet.

Light microscopic analysis showed alpha-synuclein expression in the myenteric plexuses of all animals (Fig 3). In both oral and intrastriatal rotenone models, rotenone administration increased alpha-synuclein expression in the plexuses of the colon compared to vehicle-exposed mice [oral experiment: $F(1,16)=59.97$, $p<0.0001$ and intrastriatal experiment: $F(1,14)=56.74$, $p<0.0001$]. Optical density analysis for alpha-synuclein intensity showed that the animals on the active diet had less alpha-synuclein accumulation in the colon [oral experiment: $F(1,16)=12.19$, $p<0.01$ and intrastriatal experiment: $F(1,14)=9.824$, respectively]. More specifically, rotenone-treated animals receiving active diet showed less alpha-synuclein accumulation compared to rotenone-treated mice on control diet ($p<0.01$ for both models) (Fig 3A, 3B, 3C).

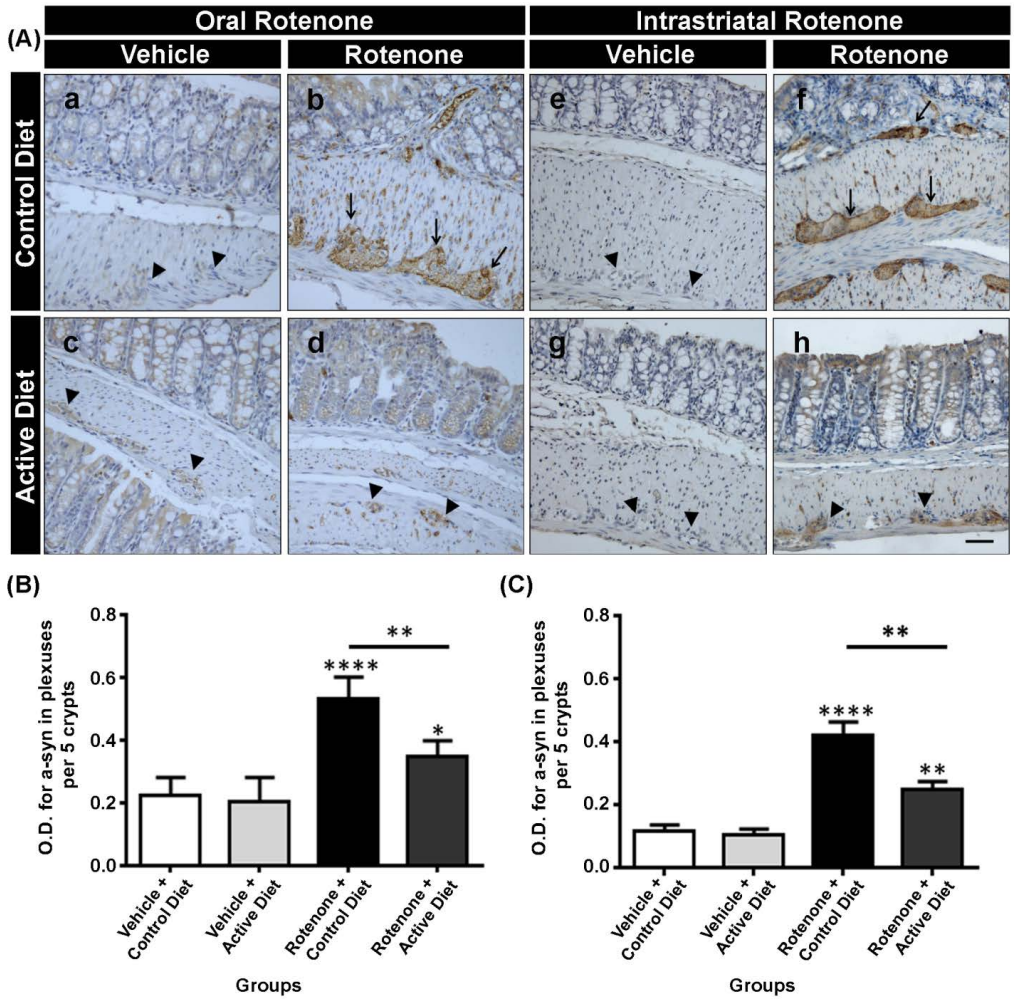

Figure 3: Effects of the active diet on alpha-synuclein expression in the colon in **(A: a-d, B)** orally rotenone-treated mice and **(A: e-h, C)** intrastriatal rotenone-injected mice. For both models, rotenone increased alpha-synuclein in the colon and the active diet was able to reduce rotenone-induced alpha-synuclein overexpression.*p<0.05, **p<0.01, ****p<0.0001. (Scale bars: 50µm applies to all panels).

Only oral rotenone exposures affected small intestinal and colonic barrier integrity deficits in mice and active diet mitigated this impairment.

One of the major intestinal tight junction proteins, Zonula Occludens 1 (ZO-1) was evaluated to assess the barrier integrity in both models using immunofluorescence method.

In the oral rotenone experiment, the vehicle-treated mice showed an intact ZO-1 expression around the mucosa in ileum and colon (Fig 4A) samples, while the rotenone-treated mice showed comparatively significant reduced levels of ZO-1 protein in the ileum [$F(1,36)=22.71$, $p<0.0001$] (Fig 4C) and colon [$F(1,36)=24.16$, $p<0.0001$] (Fig 4B) samples. Overall, oral rotenone treatment resulted in completely diminished levels of ZO-1 expression at the epithelial junctions of the intestine suggesting dysfunctional barrier integrity. On the other hand, rotenone-treated animals on active diet showed a higher tight-junction protein expression compared to the rotenone-treated animals on control diet ($p< 0.05$ for both ileum and colon).

Intrastriatal rotenone injection showed no significant changes in the levels of ZO-1 expression in both ileum and colon samples (data not shown).

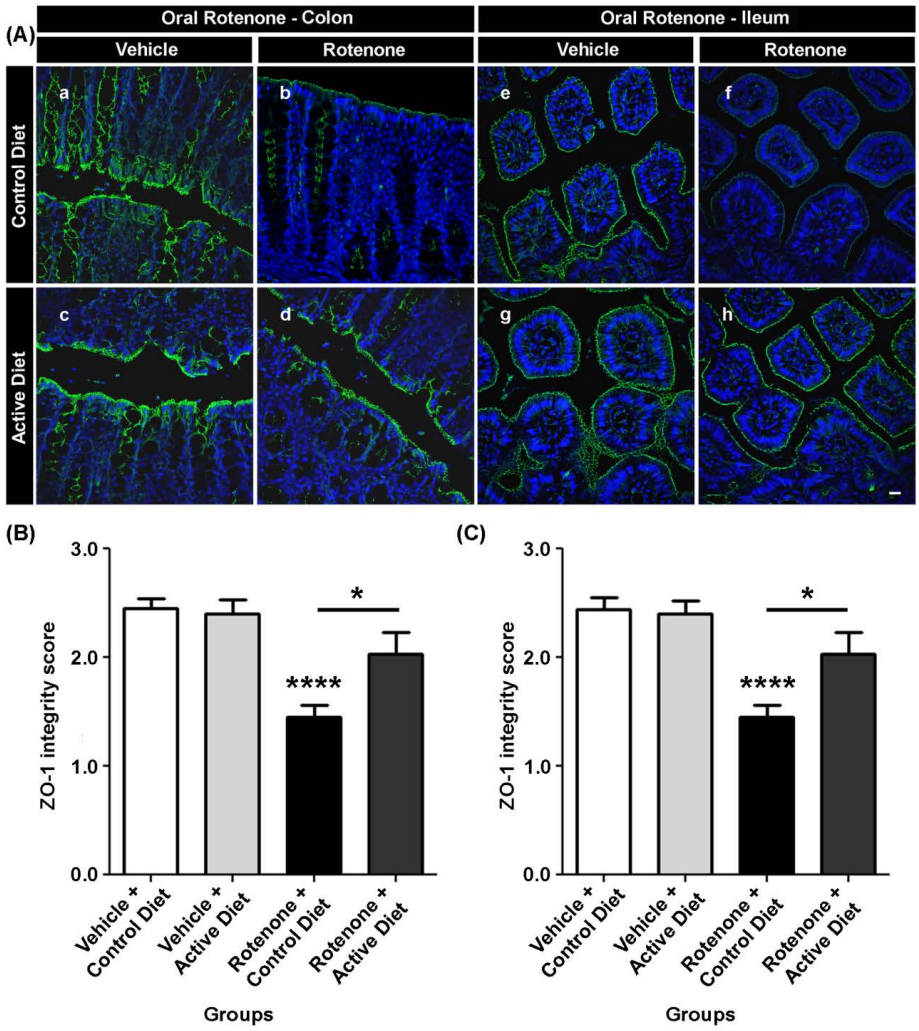

Figure 4: Effects of oral rotenone treatment and diet on epithelial integrity of the small intestine and of the colon assessed by ZO-1 tight junction protein immunoreactivity. Vehicle treated mice showed an intact barrier in small intestine and colon **(a, c, e, g)** (shown in green). The rotenone treated mice on control diet almost lacked that barrier completely **(b, f)**. The rotenone treated animals on the active diet **(d, h)** maintained the tight junction protein expression when compared to rotenone treated animals on control diet. (Scale bar: 50µm applies to all panels)**.**

Both oral and intrastriatal rotenone exposures showed evidence of colonic inflammation and immune activation in mice and it was reduced with the active diet.

To assess inflammation and immune activation, the colon length (a gross indicator of inflammation) [39], and the number of T-cells in the colonic mucosa were quantified.

In both oral and intrastriatal rotenone models, rotenone treatment resulted in significant reduced colon length [oral experiment: $F(1,36)=6.3$, $p<0.0001$ and intrastriatal experiment : $F(1,34)=108.4$, $p<0.0001$]. The active diet reduced the disruptive effect on colon length caused by rotenone [oral experiment: $F(1,36)=6.831$, $p<0.05$ and intrastriatal experiment: $F(1,34)=24.50$, $p<0.0001$] and there was an interaction effect between diets and treatment [oral experiment: $F(1,36)=6.831$, $p<0.05$ and intrastriatal experiment: $F(1,34)=28.62$, $p<0.0001$]. Post-hoc analysis revealed a smaller decrease in colon length in both oral and intrastriatal rotenone-treated mice on the active diet compared to the control diet (oral experiment: $p<0.01$ and intrastriatal experiment: $p<0.0001$) (Fig 5A, 5B).

The number of T-cells in the colon was increased after rotenone exposure in both models [oral experiment: $F(1,16)=47.46$, $p<0.001$and intrastriatal experiment: $F(1,24)=25.80$, $p<0.0001$]. There was an interaction effect between diet and rotenone treatment in both experiments [oral experiment: $F(1,16)=6.125$, $p<0.05$ and intrastriatal experiment: $F(1,24)=9.306$, $p<0.01$] (Fig 5C, 5D, 5E). The number of T-cells in the colon increased to a lesser extent for both experiments in rotenone-treated animals receiving active diet compared to animals with same treatment receiving control diet ($p<0.05$ for both models).

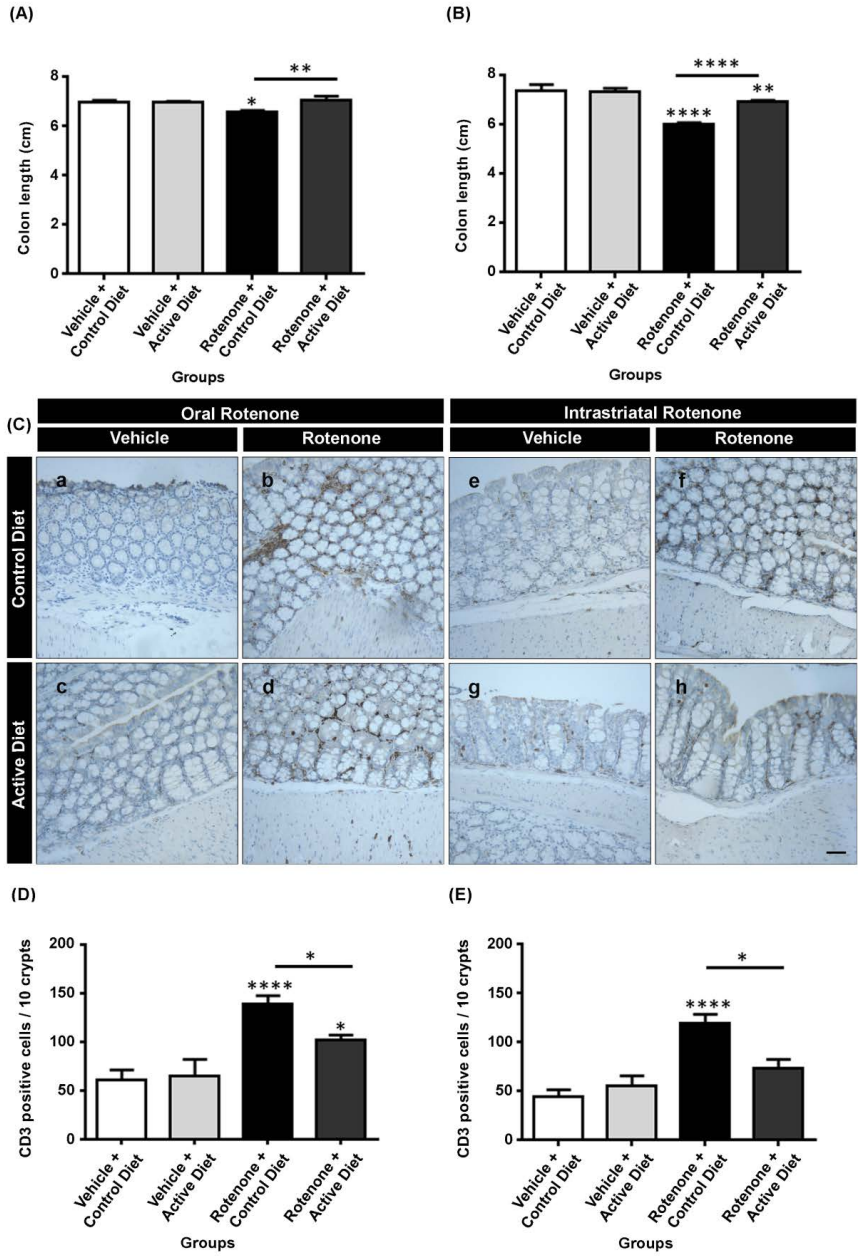

Figure 5: Effects of the active diet in colon length and in the number of T-cells in the colon for the oral model **(A, C: a-d, D)** and intrastriatal model **(B, C :e-h, E).** In both models, colon length was decreased and the number of T-cells was increased after rotenone exposures. The active diet restored rotenone-induced deficits, as evidenced by normalizations of colon length and T-cells infiltration. *p<0.05, **p<0.01, ****p<0.0001. (Scale bars: 50µm applies to all panels).

4. Discussion

The present study demonstrated a possible bidirectional communication pathway between the brain and the gut in PD pathogenesis using two separate rotenone-induced PD models (oral and intrastriatal rotenone models). In the second part of the study, we showed that motor and GI abnormalities caused by rotenone were reduced by a dietary intervention providing uridine and DHA, irrespective of the toxin exposure route.

Overall, rotenone exposures in the murine striatum led to a reduction of TH positive cells in the SN, impaired motor function, delayed intestinal transit, increased alpha-synuclein expression, colonic inflammation (such as reduced colon length) and signs of immune activation. Oral administration of rotenone led to a similar phenotype and an additional decrease of ZO-1 expression. The striking similarity in pathology and broad symptomatology induced by the two separate models suggests that initial pathological processes in the development of PD may take place in either the brain or the gut.

Braak and coworkers hypothesized that environmental factors might start a pathological process at two sites, in the olfactory bulbs and within enteric nerve cell plexus secondary to swallowing nasal secretions[40], causing inflammation and oxidative stress and thereby initiating alpha-synuclein accumulation[26]. The vagal nerve might provide a conduit for the spread of alpha-synuclein pathology from the ENS to the brain whereas the initiation of the pathological process in the olfactory bulbs can directly affect the brain[40] and could be transferred to distal organs including the ENS. In accordance with this hypothesis, it has been shown that alpha-synuclein can be retrogradely transported from the intestinal wall to the brain in rats[41]. Moreover, full truncal vagotomy has been associated with a decreased risk of PD development, supporting the idea that the vagal nerve might provide a conduit to spread PD pathology from the gut to the brain[42]. The analysis of phosphorylated alpha-synuclein in future studies could further characterize the animal models. An important component of this theory would be the disruption of the intestinal barrier integrity or microbiota changes that allows the putative factors to start pathological changes such as alpha-synuclein accumulation in the gut. The increased intestinal permeability in PD patients has been reported before[43] and our current finding showing the reduction of ZO-1 tight junction protein expression after oral rotenone treatment supports that theory. We studied intestinal and colonic ZO-1 expression and not functional permeability, but the disruption of that tight junction protein has been shown to be a reliable marker for barrier integrity[44,45]. It is well known that leaky gut due to barrier disruption could result in peripheral low grade chronic inflammation[46]. Chronic peripheral inflammation is associated with central neuroinflammation and degeneration[46]. It is then likely that oral rotenone administration induces chronic low grade inflammation in the periphery affecting pathological changes in the gut and the brain via dysfunctional gut-brain axis. Microbiota composition was not evaluated in this study but future studies are warranted to examine its role in this model.

Our current observation that rotenone injection in the brain also caused a GI phenotype supports the theory that the brain-to-gut axis could also be involved in PD progression and suggests that environmental factors may also start a detrimental process in the brain, subsequently affecting the GI-tract. Pellegrini and colleagues also reported increased GI disturbances and colonic inflammation with intra-nigral injection of the neurotoxin 6-OHDA[47]. As suggested by the authors, central dopaminergic neurodegeneration (triggered by 6-OHDA or rotenone) might lead to an inflammatory abdominal condition through an impairment of the dorsal motor nucleus of the vagus-vagus nerve anti-inflammatory pathways[47]. These inflammatory conditions might trigger alpha-synuclein accumulation in the ENS.

Further studies targeting the olfactory bulbs are warranted to further confirm the dual-hit hypothesis in PD.

Remarkably, our results from the intrastriatal rotenone experiment revealed that unilateral injection of rotenone in the mouse striatum caused a bilateral reduction of dopaminergic cells in the SN. Others have previously shown that unilateral injection of rotenone in the striatum in rats may lead to ipsilateral depletion of dopaminergic cells in the SN[48,49], although it is not uncommon that degeneration is also observed in the contralateral hemisphere including hippocampus and cortex after unilateral intrastriatal injection of rotenone in rats[50]. However, to our knowledge, this is the first study describing the effects of unilateral injection of rotenone in the striatum in mice. Several mechanisms may have contributed to the bilateral pathology after unilateral injection. For instance, the pesticide rotenone is extremely lipophilic and might diffuse more widely in the mouse brain causing a reduction in TH positive cells in both hemispheres. It is also possible that dopamine depletion in the ipsilateral striatum creates cascades of inflammatory events that ultimately causing degeneration in the contralateral SN[51].

The specific dietary intervention providing uridine and DHA has never been tested in the rotenone-induced PD rodent models. Also, previous studies did not characterize the beneficial effects of this diet on the non-motor symptoms in any PD rodent models. Lately it was recognized that non-motor symptoms have a higher impact on quality of life of PD patients than the typical motor symptoms[52,53]. Furthermore, GI complications may have important implications for the absorption of levodopa, the most common used drug in the treatment of PD, interfering with the drug's action and contributing to response fluctuations[54].

In the investigated rotenone models, dopaminergic cell loss and motor dysfunction were less pronounced in the animals on the active diet. It cannot be differentiated whether the diet had neuroprotective properties or could have interacted with rotenone toxicity and therefore increase the survival of dopaminergic cells in the SN and motor function. However, several animal studies, including aged animals and models of neurodegeneration, have shown that combined supplementation of dietary phospholipid precursors such as uridine, omega-3 fatty acids, and choline may act via the Kennedy pathway to increase brain

phospholipids, neurite outgrowth, synaptic proteins, dendritic spine formation, and neurotransmission[27,28,55–57].

All this together suggests that these dietary phospholipid precursors act together to increase the synthesis of synaptic membranes and, consequently synaptogenesis, as reviewed by Wurtman and colleagues[55]. Interestingly, the phospholipid precursors are included in a specific nutrient combination designed to target synaptic dysfunction in Alzheimer's disease[58] and shown to support brain functional connectivity in mouse models of this neurodegenerative disorder[59,60].

In addition, the diet reduced alpha-synuclein accumulation, the shortening of the colon, T-cell infiltration and delayed intestinal transit caused by rotenone. Omega-3 PUFAs, like DHA, have anti-inflammatory properties[61] that might reduce oxidative stress and therefore reduce alpha-synuclein accumulation. Alpha-synuclein accumulation and other abnormalities in the ENS might negatively affect peristalsis and gastric emptying[62] and therefore be responsible for the abnormalities in intestinal transit time. This diet could also affect microbiota, shifting pro-inflammatory microbiota profile towards anti-inflammatory microbiota profile, which could explain some of the beneficial effects on GI dysfunctions.

In conclusion, our results obtained with both rotenone-induced mouse models clearly show the possible role of brain-to-gut as well as a gut-to-brain axis in PD pathogenesis. This bidirectional processes might create a vicious cycle that could sustain the neuroinflammatory cascade leading to progression/exacerbation of PD symptoms. This study also describes the beneficial preventive effects of a dietary intervention containing fish oil and uridine in two different rotenone models for PD. The active diet was effective for both the motor symptoms and the gastrointestinal phenotype. Further research is needed to further clarify a specific role of the GI-inflammation for the diet-induced beneficial effects in the gut and the brain. Future microbiome and metabolomic studies are required to explore possible effects of the diet via microbiota-gut-brain axis. Overall, the results of this study reveal that rotenone mouse models are valuable tools for PD research and that DHA and uridine may be beneficial in the prevention of motor and GI disturbances of PD.

References

1. Ross, G. W. *et al.* Association of olfactory dysfunction with incidental Lewy bodies. *Mov. Disord. Off. J. Mov. Disord. Soc.* **21,** 2062–2067 (2006).
2. Ponsen, M. M. *et al.* Idiopathic hyposmia as a preclinical sign of Parkinson's disease. *Ann. Neurol.* **56,** 173–181 (2004).
3. Pfeiffer, R. F. Gastrointestinal dysfunction in Parkinson's disease. *Lancet Neurol.* **2,** 107–116 (2003).
4. Fasano, A., Visanji, N. P., Liu, L. W. C., Lang, A. E. & Pfeiffer, R. F. Gastrointestinal dysfunction in Parkinson's disease. *Lancet Neurol.* **14,** 625–639 (2015).
5. Abbott, R. D. *et al.* Frequency of bowel movements and the future risk of Parkinson's disease. *Neurology* **57,** 456–462 (2001).
6. Gao, X., Chen, H., Schwarzschild, M. A. & Ascherio, A. A prospective study of bowel movement frequency and risk of Parkinson's disease. *Am. J. Epidemiol.* **174,** 546–551 (2011).
7. Chen, H. *et al.* Meta-analyses on prevalence of selected Parkinson's nonmotor symptoms before and after diagnosis. *Transl. Neurodegener.* **4,** 1 (2015).
8. Ponsen, M. M., Stoffers, D., Twisk, J. W. R., Wolters, E. C. & Berendse, H. W. Hyposmia and executive dysfunction as predictors of future Parkinson's disease: a prospective study. *Mov. Disord. Off. J. Mov. Disord. Soc.* **24,** 1060–1065 (2009).
9. Pan-Montojo, F. *et al.* Environmental toxins trigger PD-like progression via increased alpha-synuclein release from enteric neurons in mice. *Sci. Rep.* **2,** 898 (2012).
10. Clairembault, T., Leclair-Visonneau, L., Neunlist, M. & Derkinderen, P. Enteric glial cells: new players in Parkinson's disease? *Mov. Disord. Off. J. Mov. Disord. Soc.* **30,** 494–498 (2015).
11. Klingelhoefer, L. & Reichmann, H. Pathogenesis of Parkinson disease-the gut-brain axis and environmental factors. *Nat. Rev. Neurol.* **11,** 625–636 (2015).
12. Felice, V. D., Quigley, E. M., Sullivan, A. M., O'Keeffe, G. W. & O'Mahony, S. M. Microbiota-gut-brain signalling in Parkinson's disease: Implications for non-motor symptoms. *Parkinsonism Relat. Disord.* **27,** 1–8 (2016).
13. Cryan, J. F. & Dinan, T. G. Mind-altering microorganisms: the impact of the gut microbiota on brain and behaviour. *Nat. Rev. Neurosci.* **13,** 701–712 (2012).
14. Miwa, H., Kubo, T., Suzuki, A. & Kondo, T. Intragastric proteasome inhibition induces alpha-synuclein-immunopositive aggregations in neurons in the dorsal motor nucleus of the vagus in rats. *Neurosci. Lett.* **401,** 146–149 (2006).
15. Drolet, R. E., Cannon, J. R., Montero, L. & Greenamyre, J. T. Chronic rotenone exposure reproduces Parkinson's disease gastrointestinal neuropathology. *Neurobiol. Dis.* **36,** 96–102 (2009).
16. Murakami, S., Miyazaki, I., Sogawa, N., Miyoshi, K. & Asanuma, M. Neuroprotective effects of metallothionein against rotenone-induced myenteric neurodegeneration in parkinsonian mice. *Neurotox. Res.* **26,** 285–298 (2014).
17. Pan-Montojo, F. *et al.* Progression of Parkinson's disease pathology is reproduced by intragastric administration of rotenone in mice. *PloS One* **5,** e8762 (2010).
18. Betarbet, R. *et al.* Intersecting pathways to neurodegeneration in Parkinson's disease: effects of the pesticide rotenone on DJ-1, alpha-synuclein, and the ubiquitin-proteasome system. *Neurobiol. Dis.* **22,** 404–420 (2006).
19. Sherer, T. B., Kim, J. H., Betarbet, R. & Greenamyre, J. T. Subcutaneous rotenone exposure causes highly selective dopaminergic degeneration and alpha-synuclein aggregation. *Exp. Neurol.* **179,** 9–16 (2003).
20. Johnson, M. E. & Bobrovskaya, L. An update on the rotenone models of Parkinson's disease: their ability to reproduce the features of clinical disease and model gene-environment interactions. *Neurotoxicology* **46,** 101–116 (2015).
21. Greene, J. G., Noorian, A. R. & Srinivasan, S. Delayed gastric emptying and enteric nervous system dysfunction in the rotenone model of Parkinson's disease. *Exp. Neurol.* **218,** 154–161 (2009).
22. Tanner, C. M. *et al.* Rotenone, paraquat, and Parkinson's disease. *Environ. Health Perspect.* **119,** 866–872 (2011).
23. Braak, H., de Vos, R. A. I., Bohl, J. & Del Tredici, K. Gastric alpha-synuclein immunoreactive inclusions in Meissner's and Auerbach's plexuses in cases staged for

Parkinson's disease-related brain pathology. *Neurosci. Lett.* **396,** 67–72 (2006).
24. Shannon, K. M. *et al.* Alpha-synuclein in colonic submucosa in early untreated Parkinson's disease. *Mov. Disord. Off. J. Mov. Disord. Soc.* **27,** 709–715 (2012).
25. Hawkes, C. H., Del Tredici, K. & Braak, H. Parkinson's disease: a dual-hit hypothesis. *Neuropathol. Appl. Neurobiol.* **33,** 599–614 (2007).
26. Hawkes, C. H., Del Tredici, K. & Braak, H. A timeline for Parkinson's disease. *Parkinsonism Relat. Disord.* **16,** 79–84 (2010).
27. Cansev, M., Ulus, I. H., Wang, L., Maher, T. J. & Wurtman, R. J. Restorative effects of uridine plus docosahexaenoic acid in a rat model of Parkinson's disease. *Neurosci. Res.* **62,** 206–209 (2008).
28. Holguin, S., Huang, Y., Liu, J. & Wurtman, R. Chronic administration of DHA and UMP improves the impaired memory of environmentally impoverished rats. *Behav. Brain Res.* **191,** 11–16 (2008).
29. Kamel, F. *et al.* Dietary fat intake, pesticide use, and Parkinson's disease. *Parkinsonism Relat. Disord.* **20,** 82–87 (2014).
30. Bousquet, M. *et al.* Beneficial effects of dietary omega-3 polyunsaturated fatty acid on toxin-induced neuronal degeneration in an animal model of Parkinson's disease. *FASEB J. Off. Publ. Fed. Am. Soc. Exp. Biol.* **22,** 1213–1225 (2008).
31. Klivenyi, P. *et al.* Neuroprotective effects of oral administration of triacetyluridine against MPTP neurotoxicity.
Neuromolecular Med. **6,** 87–92 (2004).
32. Delattre, A. M. *et al.* Evaluation of chronic omega-3 fatty acids supplementation on behavioral and neurochemical alterations in 6-hydroxydopamine-lesion model of Parkinson's disease. *Neurosci. Res.* **66,** 256–264 (2010).
33. Myers, C. S., Fisher, H. & Wagner, G. C. Uridine reduces rotation induced by L-dopa and methamphetamine in 6-OHDA-treated rats. *Pharmacol. Biochem. Behav.* **52,** 749–753 (1995).
34. Wooten, G. F., Currie, L. J., Bovbjerg, V. E., Lee, J. K. & Patrie, J. Are men at greater risk for Parkinson's disease than women? *J. Neurol. Neurosurg. Psychiatry* **75,** 637–639 (2004).
35. Inden, M. *et al.* Neurodegeneration of mouse nigrostriatal dopaminergic system induced by repeated oral administration of rotenone is prevented by 4-phenylbutyrate, a chemical chaperone. *J. Neurochem.* **101,** 1491–1504 (2007).
36. Reeves, P. G., Nielsen, F. H. & Fahey, G. C. AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. *J. Nutr.* **123,** 1939–1951 (1993).
37. West, M. J., Slomianka, L. & Gundersen, H. J. Unbiased stereological estimation of the total number of neurons in thesubdivisions of the rat hippocampus using the optical fractionator. *Anat. Rec.* **231,** 482–497 (1991).
38. Gundersen, H. J. *et al.* Some new, simple and efficient stereological methods and their use in pathological research and diagnosis. *APMIS Acta Pathol. Microbiol. Immunol. Scand.* **96,** 379–394 (1988).
39. Axelsson, L. G., Landström, E. & Bylund-Fellenius, A. C. Experimental colitis induced by dextran sulphate sodium in mice: beneficial effects of sulphasalazine and olsalazine. *Aliment. Pharmacol. Ther.* **12,** 925–934 (1998).
40. Hawkes, C. H., Del Tredici, K. & Braak, H. Parkinson's disease: the dual hit theory revisited. *Ann. N. Y. Acad. Sci.* **1170,** 615–622 (2009).
41. Holmqvist, S. *et al.* Direct evidence of Parkinson pathology spread from the gastrointestinal tract to the brain in rats. *Acta Neuropathol. (Berl.)* **128,** 805–820 (2014).
42. Svensson, E. *et al.* Vagotomy and Subsequent Risk of Parkinson's Disease. *Ann. Neurol.* (2015).
doi:10.1002/ana.24448
43. Forsyth, C. B. *et al.* Increased intestinal permeability correlates with sigmoid mucosa alpha-synuclein staining and endotoxin exposure markers in early Parkinson's disease. *PloS One* **6,** e28032 (2011).
44. Balda, M. S. & Matter, K. Tight junctions at a glance. *J. Cell Sci.* **121,** 3677–3682 (2008).
45. Poritz, L. S. *et al.* Loss of the tight junction protein ZO-1 in dextran sulfate sodium induced colitis. *J. Surg. Res.* **140,** 12–19 (2007).
46. Maes, M., Kubera, M. & Leunis, J.-C. The gut-brain barrier in major depression: intestinal mucosal dysfunction with an increased translocation of LPS from gram negative enterobacteria (leaky gut) plays a role in the inflammatory
pathophysiology of depression. *Neuro Endocrinol. Lett.* **29,** 117–124 (2008).

47. Pellegrini, C. *et al.* Alteration of colonic excitatory tachykininergic motility and enteric inflammation following dopaminergic nigrostriatal neurodegeneration. *J. Neuroinflammation* **13,** 146 (2016).
48. Mulcahy, P., Walsh, S., Paucard, A., Rea, K. & Dowd, E. Characterisation of a novel model of Parkinson's disease by intra-striatal infusion of the pesticide rotenone. *Neuroscience* **181,** 234–242 (2011).
49. Carriere, C. H., Kang, N. H. & Niles, L. P. Neuroprotection by valproic acid in an intrastriatal rotenone model of Parkinson's disease. *Neuroscience* **267,** 114–121 (2014).
50. Abdel-Salam, O. E. *et al.* Effect of a single intrastriatal rotenone injection on oxidative stress and neurodegeneration in the rat brain. *Comp. Clin. Pathol.* **23,** 1457–1467 (2014).
51. Paumier, K. L. *et al.* Intrastriatal injection of pre-formed mouse α-synuclein fibrils into rats triggers α-synuclein pathology and bilateral nigrostriatal degeneration. *Neurobiol. Dis.* **82,** 185–199 (2015).
52. Martinez-Martin, P. The importance of non-motor disturbances to quality of life in Parkinson's disease. *J. Neurol. Sci.* **310,** 12–16 (2011).
53. Martinez-Martin, P. *et al.* Prevalence of nonmotor symptoms in Parkinson's disease in an international setting; study using nonmotor symptoms questionnaire in 545 patients. *Mov. Disord. Off. J. Mov. Disord. Soc.* **22,** 1623–1629 (2007).
54. Pfeiffer, R. F. Gastrointestinal dysfunction in Parkinson's disease. *Parkinsonism Relat. Disord.* **17,** 10–15 (2011).
55. Wurtman, R. J., Cansev, M., Sakamoto, T. & Ulus, I. Nutritional modifiers of aging brain function: use of uridine and other phosphatide precursors to increase formation of brain synapses. *Nutr. Rev.* **68 Suppl 2,** S88-101 (2010).
56. Sakamoto, T., Cansev, M. & Wurtman, R. J. Oral supplementation with docosahexaenoic acid and uridine-5'-monophosphate increases dendritic spine density in adult gerbil hippocampus. *Brain Res.* **1182,** 50–59 (2007).
57. Cansev, M. *et al.* Specific multi-nutrient enriched diet enhances hippocampal cholinergic transmission in aged rats. *Neurobiol. Aging* **36,** 344–351 (2015).
58. van Wijk, N. *et al.* Targeting synaptic dysfunction in Alzheimer's disease by administering a specific nutrient combination. *J. Alzheimers Dis. JAD* **38,** 459–479 (2014).
59. Zerbi, V. *et al.* Multinutrient diets improve cerebral perfusion and neuroprotection in a murine model of Alzheimer's disease. *Neurobiol. Aging* **35,** 600–613 (2014).
60. Wiesmann, M. *et al.* A Dietary Treatment Improves Cerebral Blood Flow and Brain Connectivity in Aging apoE4 Mice. *Neural Plast.* **2016,** 6846721 (2016).
61. Miller, R. L., James-Kracke, M., Sun, G. Y. & Sun, A. Y. Oxidative and inflammatory pathways in Parkinson's disease. *Neurochem. Res.* **34,** 55–65 (2009).
62. Cersosimo, M. G. & Benarroch, E. E. Autonomic involvement in Parkinson's disease: pathology, pathophysiology, clinical features and possible peripheral biomarkers. *J. Neurol. Sci.* **313,** 57–63 (2012).

CHAPTER 4

The role of TLR4 in the gut-brain axis in Parkinson's disease: a translational study from men to mice

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Abstract

Recent evidence suggesting an important role of gut-derived inflammation in brain disorders has opened up new directions to explore the possible role of the gut-brain axis in neurodegenerative diseases. Given the prominence of colonic dysfunction in Parkinson's disease (PD) patients, we propose that intestinal dysfunction could contribute to intestinal and central inflammation in PD related neurodegeneration. The first part of this study shows that PD patients have intestinal barrier disruption, enhanced markers of microbial translocation and higher pro-inflammatory gene profiles in the colonic biopsy samples compared to healthy controls. In this regard, we found increased expression of the bacterial endotoxin specific ligand toll-like receptor 4 (TLR4), CD3 positive T cells and cytokine expression in colonic biopsies from PD subjects. To further specify the role of TLR4 in PD-induced neuroinflammation, rotenone treatment in Tlr4 knockout (KO) mice revealed less intestinal inflammation, intestinal and motor dysfunction, brain neuroinflammation and neurodegeneration relative to rotenone-treated wild type animals. Taken together, these studies suggest that TLR4-mediated inflammation plays an important role in intestinal and/or brain inflammation leading to neurodegeneration in PD.

1.Introduction

The mechanism of neurodegeneration in Parkinson's disease (PD) remains unknown but converging evidence suggests that inflammation-derived oxidative stress and cytokine toxicity may play a critical role[1–10]. The intestinal tract, especially the colon, could be a contributing factor to the neurodegenerative processes in PD. Intestinal impairments including constipation are common in PD and can begin decades before the onset of motor symptoms. In this regard, studies by our group[11,12] and others[13–16] suggest that the intestinal tract, especially the colon, could be a major source of inflammation contributing to neurodegeneration.

The intestinal tract is a large surface area in direct contact with microorganisms[17] that have the capability to provoke inflammatory responses. Our group and others report that PD patients exhibit a pro-inflammatory microbiota profile[12,15] with a reduction in beneficial products such as short chain fatty acids[16]. Reconstitution of the fecal micriobiota in germ-free alpha-synuclein (α-syn) transgenic PD mice using stool from PD patients exacerbates motor symptoms and pathology in this PD model[18]. In addition, the microbiota via mechanisms including metabolite production can impact immune and inflammatory pathways leading to the peripheral and central immune activation and inflammation[19,20]. Finally, the intestinal barrier is critical to separate the pro-inflammatory contents of the intestine from the systemic circulation. Intestinal hyper-permeability is observed in PD patients[11] and likely contributes to inflammation in PD.

Consequences of intestinal dysbiosis and/or intestinal barrier dysfunction include immune activation and inflammation. Examples include involvement of TLR pathway activations and abnormal immune activations such as CD3 T cells. Increased T-cell trafficking into the colonic mucosa is a feature associated with constipated PD patients[21]. In addition, activation of various toll-like receptors (TLR), specifically TLR4 is of interest since the gram-negative bacteria activate this receptor. If abnormalities in the microbiome (especially favoring lipopolysaccharide (LPS) producers) are an initial trigger for PD pathogenesis in the intestinal tract, TLR4 might be the first point of microbial interaction.

There are several potential mechanisms by which intestinal inflammation and immune activation secondary to intestinal dysbiosis or intestinal barrier dysfunction might lead to central nervous system pathology. Inflammation and immune activation could initiate α-syn pathology in the enteric nervous system (ENS) that then spreads in a prion-like fashion via the vagus nerve to the lower brainstem, and then the substantia (SN)[22]. Our group and others have shown α-syn accumulations in colonic tissue from PD patients prior to the onset of motor symptoms[23–25]. Alternatively, gut-derived bacterial products or the peripheral inflammatory response (e.g., cytokine production) could impact the brain through systemic mechanisms including disruption of the blood brain barrier as is observed in PD patients[26]. Thus, chronic activation of intestinal and peripheral immune cells and proinflammatory cytokines could initiate or promote neurodegeneration leading to PD.

Taken together, we hypothesize that intestinal dysbiosis and gut leakiness to bacterial endotoxins activates TLR4-mediated inflammatory cascades leading to neurodegeneration in PD. To test this hypothesis we performed a series of studies in both human tissue and a murine model of PD. Inflammation and immune activation were measured in colonic mucosal samples from PD and healthy controls (HC) subjects and rotenone or vehicle-treated mice. To further assess the role of the TLR4 receptor, we used TLR4 knock out (KO) mice in conjunction with oral rotenone administration to model PD.

2. Methods

Part I: Human study - subjects and methods

Healthy control and PD subjects

Healthy control subjects (HC, N=6) were recruited by the gastroenterology group at Rush University Medical Center (RUMC; Chicago, IL). Parkinson's disease patients (PD, N=6) were recruited by the movement disorders clinic at RUMC. All subjects signed a consent form for use of their samples and data to be part of a RUMC Institutional Review Board (IRB)-approved GI repository and the ongoing research. Control subjects had no history of GI or neurological disorders. Subjects with PD diagnosed by United Kingdom Parkinson Disease Research Society Brain Bank criteria were recruited by the movement disorders clinic as in our previously published protocol[23]. Exclusion criteria were also as per our previously published work [23].

Collection of colon biopsy samples and blood samples

Subjects underwent an unprepped, limited sigmoidoscopy by one of the authors [AK] as published previously[23]. The reason for performing the procedure without colon preparation was to avoid any potential confounding factors from use of laxative. No sedation was required for this procedure. No bleeding, fever or any other side effects were reported in any subjects. Eight mucosal biopsy samples were taken from distal sigmoid colon [20 cm from the anal verge] from each subject. Four samples were placed in either 10% formalin (89379-094, VWR) or in OCT (25608-930, VWR) to perform histology studies. Others were snap frozen in liquid nitrogen for gene array analysis.

Six milliliters of blood was collected using normal aseptic techniques and stored in ice buckets. Within 20 min of blood collection, plasma was separated by centrifugation at 1500 × g at 4°C for 15 min. Collected plasma was stored in a fresh polypropylene tube at −70°C for LBP measurements.

Intestinal permeability and endotoxin (LBP) measurements

All PD (N=6) and HC (N=6) subjects followed our established protocol to measure total gut permeability (24 hours)[11]. All subjects fasted overnight and subsequently ingested a sugar

mixture containing 2 grams mannitol (M9546, Sigma), 7.5 grams lactulose (NDC66220-729-01, Cumberland Pharmaceuticals), 40 gm sucrose (S9378, Sigma) and 1 gram sucralose (105200, Tate and Lyle). Urine samples were collected twenty-four hours after oral sugar probe administration. Urine samples were frozen in liquid nitrogen for gas chromatography analyses as established[28] to measure total excreted sugar levels and data were expressed as percent oral dose excreted in the urine.

Systemic levels of bacterial endotoxin were determined using LPS-binding protein (LBP) measurements according to our established procedure from N=5 PD and N=5 HC subjects[29]. Blood samples were not available from one PD and one HC subjects. In brief, Human LBP Elisa kit (HK315, Hycult Biotech) was used to process all samples concurrently to maintain consistency of the measurements. Samples and standards were incubated in microtiter wells (in a duplicate manner) coated with antibodies recognizing human LBP. LBP was captured using biotinylated tracer antibody. Streptavidin-peroxidase enzyme and tetramethylbenzidine substrate were used to initiate the enzyme reaction, which was stopped by the addition of oxalic acid. The absorbance was measured at 450 nm with a spectrophotometer. A standard curve was obtained by plotting the absorbance values and the corresponding concentrations of the human LBP standards (log). This standard curve was used to measure the concentration of samples.

Histology

Histology in human biopsies (N=6 PD and N=6 HC subjects) was performed as per established protocol[29]. Formalin fixed biopsy samples were processed through dehydration protocol and embedded in paraffin (22-900-700, Fisher). Paraffinized tissue block was sectioned at 6µm thickness and mounted on a glass slide for staining. On the day of staining, sections were heated at 60°C using an oven to remove paraffin. Sections were there processed through xylenes (X3P, Fisher), different grades of alcohol (111000200, Fisher) to water. Antigen retrieval was performed for 10 or 20 minutes using citrate buffer (S1699, Dako) in a pressure cooker at high pressure. Samples were then brought to the room temperature. Sodium periodate and serum blocks were performed to avoid any background staining. Primary antibody incubation was performed for overnight at room temperature (TLR4: 1:500, ab22048, Abcam; ZO-1: 1:200, 617300, Abcam and CD3: 1:200, ab5690, Abcam). Next day, a specific secondary antibody was applied for an hour at room temperature. Immunofluorescence staining was performed for ZO-1 and TLR4 markers using fluorophore tagged secondary antibody (donkey anti-rabbit 488: 1:400, A21206, Thermofisher Scientific or donkey anti-mouse 488, 1:400, A21202, Thermofisher Scientific). Immunoperoxidase staining was performed for TLR4 and CD3 markers using biotinylated secondary antibodies (horse anti-mouse: 1:200, BA2000, Vector lab or goat anti-rabbit: 1:200, BA1000, Vector lab), followed by actin biotin complex incubation (PA1000, Vector lab). Color reaction was performed using DAB chromogen (D5637, Sigma) and hydrogen peroxide (H1009, Sigma). Immunofluorescence sections were counter stained using 4',6-diamidino-2-Phenylindole

(DAPI) (1:10000, D1306, Thermofischer Scientific). Immunoperoxidase sections were counter stained using hematoxylin (SH26-500D, Fisher Scientific) according to manufacturer's protocol. Immunofluorescence slides were coverslipped using aqueous mounting media (F4680, Sigma-Aldrich) and stored in 4°C. Immunoperoxidase slides were dehydrated using alcohol and xylenes. These slides were then cover slipped using non-aqueous mounting media (8310-16, Thermofisher Scientific) and stored at room temperature.

Microscopic analyses

Fluorescence images were taken using confocal microscope (LSM700, Zeiss). Levels of laser intensity, confocal aperture, photomultiplier voltage, offset, scan speed, image size, filter and magnification were kept at constant levels to keep consistencies among all samples. These settings were maintained for analysis of each marker. Using a 40x1 magnification objective and a 488nm excitation source, in combination with 405nm (DAPI) excitation source, images were acquired at each sampling site from lamina propria (colonic epithelial lining and crypts) of all subjects. Images were overlaid using Adobe Photoshop (v.CS3, Adobe, San Jose, CA) software.

Confocal microscopic analyses were performed to evaluate ZO-1 tight junction barrier integrity.

Data for tight junction barrier ZO-1 integrity analyses was performed as established protocol[29]. One slide containing colonic biopsy was used per subject for imaging analyses. Sections were assigned a code and analysed in a blind manner. Minimum of twenty crypts and epithelial linings were analyzed per subject. Arbitrary scoring scale 0-3 was used to evaluate tight junction integrity as: 0 = no ZO-1 immunofluorescence; 1 = very light and discontinuous ZO-1 immunofluorescence; 2 = intense and discontinuous ZO-1 immunofluorescence; 3 = smooth continuous and well organized ZO-1 immunofluorescence. Mean values were collected for comparisons between groups and represented at Mean±SEM.

Unbiased stereology was performed for TLR4 and CD3+ cells as per established protocol (Engen, Dodiya et al. 2016) using light microscope attached to stereoinvestigator cell count probe (S.I.-V.1.5, MBF Bioscience). One slide containing colonic sigmoid mucosa per subject was used for stereology for each marker. Under low magnification (10x), the lamina propria was outlined in each colonic sigmoid mucosa sample. Under high magnification (60x), using grid size 100µmX100µm and counting frame 90µmX90µm all counting sites were visited and cells were marked. Total estimated number of cells and total counting area (mm^2) were collected using probe run list to compare the data between groups. These data were presented as the estimated number of cells/counting area. Light microscope (BX61, Olympus, Waltham, MA) was used to image immunoperoxidase performed sections.

mRNA expression analyses

Genes specific for microbiota-induced intestinal inflammation and gut dysfunction were evaluated as per manufacturer's protocol (QuantiGene 55 plex assay, Santa Clara) and according to published protocol[30]. The 55 genes measured are listed in Fig 3. Tissue homogenates were prepared from N=6 PD and N=4 HC using Affymetrix lysis buffer and processed according to the manufacturer's instructions. Tissue homogenates were not available form N=2 HC subjects. Levels of mRNA were determined using a Luminex-based custom multiplex bead array. Background values were subtracted from the raw intensity readings for each sample. Values that were less than or equal to 0 were set to 1 (floor effect), and all values were log 2 transformed. These values were then normalized to the housekeeping. Selection of housekeeping gene was based on a screen plot of eigenvalues with two variables being consistent with an inflection break in the scree plot; thus, *HGPRT* was selected. Genes that did not have more than 50% of the samples in the detectable range (above background) were excluded based on unreliable gene expression data. Samples were analyzed via Significance Analysis of Microarrays (SAM) software add-on for Microsoft Excel. The criteria for significance differences were selected for SAM analysis as median false discovery rate 0.03% or less and change more than 1.5-fold.

Statistical analyses

All data are expressed as mean ± SEM. Differences between HC and PD groups were statistically analysed with an Unpaired Two-tailed T Test. Correlations were performed using non-parametric analysis. Significance was selected at $P<0.05$. Analyses were performed using Graph Pad Prism V1.5.

Part II: Mice study - materials and methods

Animal housing

Seven week old C57BL/6J wild type (WT) and Tlr4 KO mice (MGI 1860755) Jackson Laboratories) were housed under a 12h light/dark cycle. Food and water were provided *ad libitum.* Animal procedures were approved by the Ethical Committee of Animal Research of Utrecht University, Utrecht, The Netherlands.

Induction of mitochondrial dysfunction by rotenone in mice

WT and Tlr4 KO mice received freshly prepared rotenone (Sigma-Aldrich, The Netherlands) solution (10mg/kg body weight; suspended freshly in 4% carboxymethylcellulose (Sigma-Aldrich, The Netherlands) and 1.25% chloroform vehicle) once a day for 28 days by oral gavage as decribed before[31]. Control animals received vehicle for similar time. Pan-Montojo and colleagues showed that rotenone is not detected in the brain of mice receiving oral doses of 10mg/kg or lower[32]. On day 28, mice were sacrificed by decapitation and the brain and the intestinal tissue were collected for further analysis.

Motor function assessment

Motor function of mice was assessed by the Rotarod test as described before[31] Briefly, mice were placed on an accelerating rod with speeds starting with 2 rpm and gradually increasing to 20 rpm. Time to fall was recorded for a maximum of 300s and reported as latency to fall. Mice were tested at baseline and every 7 days until sacrifice.

Intestinal transit and colon length

Thirty minutes before sacrificing the mice, a solution of 2.5% Evans blue (Sigma-Aldrich, The Netherlands) in 1.5% methylcellulose (0.3ml per animal) was intragastrically administered to the mice. After euthanasia, intestinal transit was measured as the distance from the pylorus to the most distal point of migration. In addition, the length of the colon was measured.

Immunohistochemistry and image analysis

Antibodies for Tlr4 (ab13867, abcam), ZO-1 (ab59720, abcam), glial fibrillary acidic protein (GFAP) (Z0334, Dako), CD3 (ab49943, abcam) and α-synuclein (04-1053, Millipore) were used to evaluate the gut pathology in the different experimental groups. The detailed protocol is explained in supplemental methods. Data collection for tight junction barrier integrity analyses (ZO-1 integrity) and stereology analyses are explained in supplemental methods. The brains were stained with tyrosine hydroxylase (TH) (sc-14007, Santa Cruz Biotechnology) and iba-1 (019-197410, Wako) antibodies to assess the amount of dopaminergic neurons and microglia morphology in the SN. TH-immunopositive neurons were assessed using stereology counting as described in supplemental methods. For microglia analysis z-stacks were imaged at 1µm step and analysed with Image-J software. The experimenter designates individual cells and the software quantified the number of branches, the number of branches endpoints, the branch length, the cell body size and the total cell size. 20 cells per region per animal were analysed.

Statistical analyses

Experimental results are expressed as mean ± SEM. Differences between groups in the animal experiment were statistically analyzed with a two-way ANOVA followed by a Tukey's multiple comparison test. Rotarod test results were analyzed with a general linear model repeated measure ANOVA, with the within subject factor time and the between subject factors treatment (vehicle vs rotenone) and genotype (WT vs Tlr4 KO). Pearson correlations were applied to associate the different symptoms developed with rotenone exposure in mice. Results were considered statistically significant when $p<0.05$. Analyses were performed using SPSS 22.0.

3. Results

Part I: Human study in PD and HC subjects

PD subjects showed intestinal barrier dysfunction (intestinal hyperpermeability)

There were no statistically significant differences in age, gender, race or BMI between PD and HC subjects (Table 1). The current study confirmed intestinal hyperpermeability in PD subjects (Mean±SEM: 2.33±0.30 %sucralose excretion) compared to HC (0.97±0.28) as we have shown previously[11]. Specifically, PD subjects had significantly higher 24 hour urinary excretion of sucralose after ingestion of the sugar cocktail (n=6, unpaired two-tailed *t*-test: $t_{(10)}$=3.36, p=0.01; Fig 1A). However, no group differences were observed in five-hour urinary lactulose, mannitol or the lactulose/mannitol ratio (data not shown) suggesting that only colonic permeability (not small bowel permeability) is impacted in PD subjects.

Intestinal barrier dysfunction permits the passage of bacteria and bacterial products such as endotoxin (i.e., LPS) into the intestinal mucosa and into the systemic circulation. LPS binds to the soluble acute-phase protein LPS binding protein (LBP) presenting LPS to cell surface pattern receptors such as CD14 and TLR4, which are responsible for consequent innate immunity. LBP has a dual role in the initiation of cellular responses to LPS. Lower levels of LBP potentiate the cell's response to LPS by facilitating transfer of LPS molecules to its receptor CD14[33] whereas higher levels of LBP inhibit cell responses to LPS by transferring the LPS to high-density lipoproteins[34] or by promoting internalization of LPS without triggering inflammatory cell stimulation[35]. As shown previously[28], reduced levels of LBP were found in plasma samples from PD (15.73±3.75µg/ml) compared to HC (33.47±6.20 µg/ml) subjects (n=5, unpaired two-tailed *t*-test: $t_{(8)}$=2.45, p=0.04; Fig 1B).

The intestinal barrier is maintained by a series of apical junctional proteins including a major tight junction protein zonula occludens 1 (ZO-1). Immunofluorescence staining in healthy controls displayed continuous expression of ZO-1 protein at the apical surface of the crypts (Fig 1C-a,b). In contrast, ZO-1 imunofluorescence in PD cases was disrupted or reduced in the colonic sigmoid mucosa (Fig 1C-c,d). Similar to the crypts, epithelial lining also showed diminished or disrupted expression of ZO-1 in PD mucosa compared to HC (Suppl Fig 1). Integrity for ZO-1 was scored in a blind manner using an arbitrary integrity scale. Average integrity scoring data per group showed significant disruption in the ZO-1 expression in PD (1.58±0.05) subjects compared to HC (2.32±0.18) for combined both apical junction of the crypts and epithelial lining (n=6, Unpaired two-tailed T test: $t_{(10)}$=3.917, p=0.0029; Fig 1D). Taken together these data demonstrate that PD patients have disrupted colonic barrier integrity.

	Age	Gender	Race	BMI	PD duration (Years)
PD1	73	F	W	27	12
PD2	63	M	W	27	2
PD3	57	F	W	18	22
PD4	55	M	W	24	6
PD5	57	M	W	24	1
PD6	58	M	W	24	2
Control1	42	M	W	25	
Control2	41	F	B	28	
Control3	72	M	W	24	
Control4	70	F	W	23	
Control5	57	F	W	22	
Control6	53	F	W	25	

Table 1: Demographic information of PD patients (N=6) and HC (N=6). Age, gender and BMI did not differ significantly between PD and HC cases.

PD subjects showed evidence of mucosal inflammation and immune activation

As previously mentioned, TLR4 receptors are important mediators of gut-derived inflammation; thus, we evaluated the localization and or staining intensity of TLR4 positive cells. Analyses of immunoperoxidase staining in HC subjects showed occasional TLR4 immunoreactivity in the lamina propria (Fig 1E-a,b). This staining profile was enhanced in PD subjects (Fig 1E-c,d). Stereological analyses of TLR4+ cells in the lamina propria supported the qualitative observations. In this regard, healthy controls displayed (41.30±9.76) cells; an estimate significantly lower that PD cases (127.9±30.47) (n=6; unpaired two-tailed *t*-test: $t_{(10)}$=2.71, p=0.02, Fig 1F). For further evaluation, immunofluorescence staining was performed. Confocal microscope showed restricted TLR4 immunoreactivity to the apical surface of the crypt in samples from HC subjects with minimal expression in the lamina propria (Suppl Fig 1). In comparison to HC subjects, biopsies from PD subjects showed TLR4 immunoreactivity at the apical surface and the basolateral surface of the crypts but importantly TLR4 immunoreactivity was also observed in the lamina propria of colonic tissue (Suppl Fig 1). These data demonstrate that PD is associated with an increase in TLR4+ cells in the intestinal mucosa.

CD3+ T cell staining was assessed in the sigmoid mucosa. Occasional CD3+ T cells were observed in the lamina propria of HC samples (Fig 1G-a,b). These cells were in close proximity to the basolateral surface of the crypts. In contrast, PD subjects showed higher CD3+ T cell numbers in the lamina propria as opposed to being in close proximity to the

basolateral surface (Fig 1G-c,d). Stereological analyses estimated a significantly greater number of CD3+ T cells in the lamina propria of PD patients (89.02±14.43) compared to HC (28.63±5.89) (n=6; unpaired two-tailed *t*-test: $t_{(10)}$=3.87, p<0.001; Fig 1H). These data demonstrate that CD3+ cells have penetrated the intestinal mucosa in PD subjects.

To further characterize the immune pathways and inflammation in PD mucosa, a microarray analysis was performed on sigmoid mucosal samples (PD n=6, HC n=4) including 50 genes relevant to microbial translocation and inflammatory pathways (Fig 2). Out of 50 genes, 15 were significantly upregulated and one gene was significantly dowregulated in biopsies obtained from PD compared to HC subjects. In agreement with the histological findings, there was significantly higher TLR4 mRNA expression in PD biopsies compared to those from HC subjects. Downstream signalling molecules associated with TLR4 activation were altered in a way that was consistent with increased TLR4 signalling. For example, increased IRAK2 expression was observed while levels of the inhibitory molecule TOLLIP was significantly lower in colonic biopsy tissue from PD subjects compared to HC subjects (Fig 2). Cytokines (IL1-β, IFN-γ, CCL5 (RANTES)) and chemokines (CCL2, CCL5, CCR5) were elevated in PD sigmoid mucosa compared to HC (Fig 2). In addition, tissue from PD subjects had significantly more mRNA expression of CD3+ T cell markers (CD3G, CD3E) and Th1/Th17 inflammatory cytokines (IFN-γ, IL1β, IFN-β, IL17A, IL8) and IL7R compared to tissue from HC subjects. These data are consistent with increased activation of TLR4 and/or CD3+ immune activation. There were no significant changes in the mRNA levels of other TLRs (1, 2, 3, 5, 7, 8, 9) in the colonic mucosa.

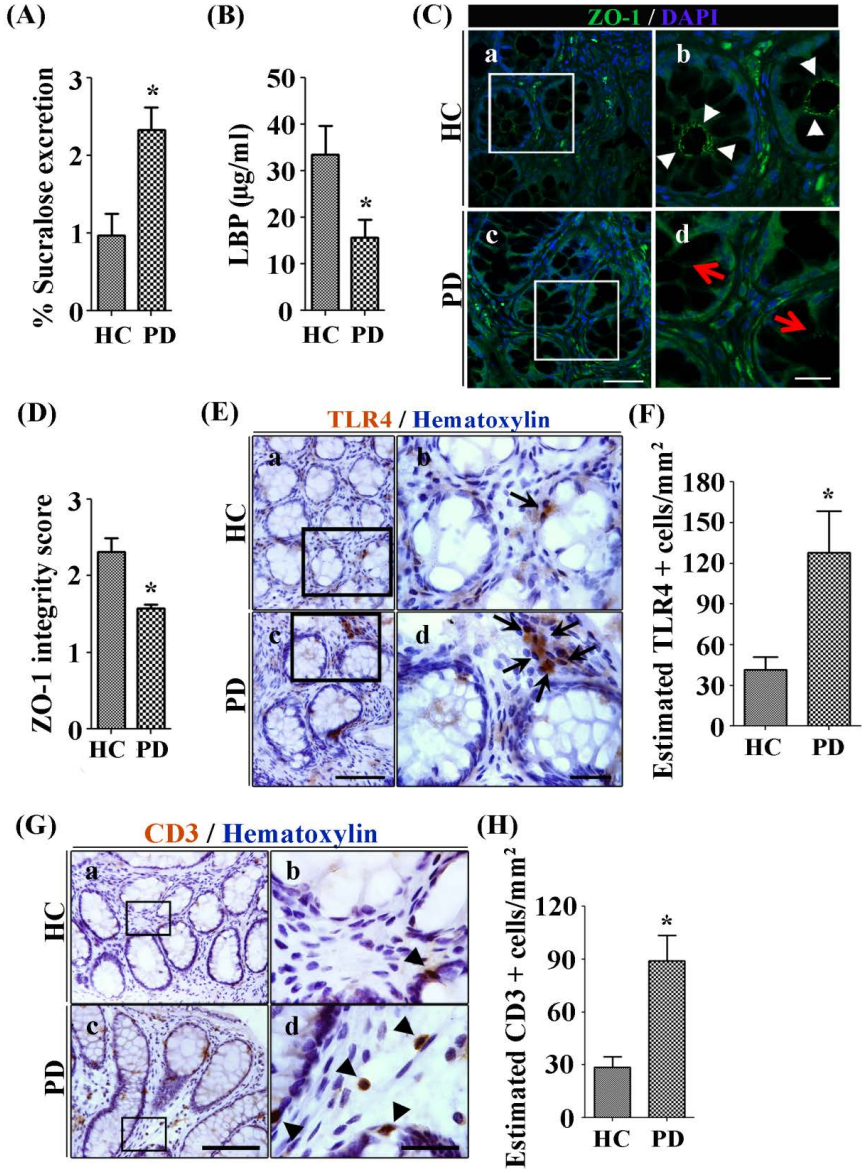


Figure 1: PD patients show increased colonic permeability and associated colonic inflammatory as well as immune markers in their biopsies compared to HC. Graph in (A) shows % excretion of sucralose in the urine samples as an intestinal permeability marker. Graph in (B) represents level of LPS-binding protein (LBP) as a marker for systemic endotoxin in the plasma samples. Photomicrographs in (C) represent immunofluorescence staining of a major tight junction protein ZO-1 expression in HC (a, b) and PD (c, d) colonic mucosa. White arrowheads indicate continuous expression of ZO-1 at the apical junction of the HC mucosa and red arrows indicate complete lack of/very minimal expression of ZO-1 at the apical surface of PD mucosa. Graph in (D) represents integrity scoring for ZO-1 tight junction protein expression in colonic samples. Scoring scale 0-3 represents: 0 = no ZO-1 expression, 1 = very poor ZO-1 expression, 2 = moderate expression of ZO-1, 3 = great

continuous expression of ZO-1. Photomicrographs in (E) represent immunoperoxidase stained TLR4+ cells in lamina propria of the HC mucosa (a, b) and PD mucosa (c, d). Black arrows indicate TLR4+ cells. Graph (F) represent stereological assessment of TLR4+ cells in lamina propria of colonic mucosa samples. Photomicrographs in (G) represent immunoperoxidase stained CD3+ T cells in colonic mucosa of HC (a, b) and PD (c, d). Black arrowheads indicate CD3+ T cells. Graph in panel (H) represent stereological assessment of CD3+ cells in lamina propria of colonic samples. Counterstaining using DAPI and haematoxylin was performed for panels C and (E, G) respectively. Scale bars in panel C(c) and C(d) represent 50µm and 20µm and applies to C(a, c) and C(b, d) respectively. Scale bars in panel E(c) and E(d) represent 75µm and 25µm and applies to E(a, c) and E(b, d) respectively. Scale bars in panel G(c) and G(d) represent 40µm and 25µm and applies to G(a, c) and G(b, d) respectively. *p<0.05,**p<0.001, ***p<0.0001 Data represent Mean $\pm$ SEM. HC = healthy controls, PD = Parkinson's disease, TLR4 = toll-like receptor 4**.**

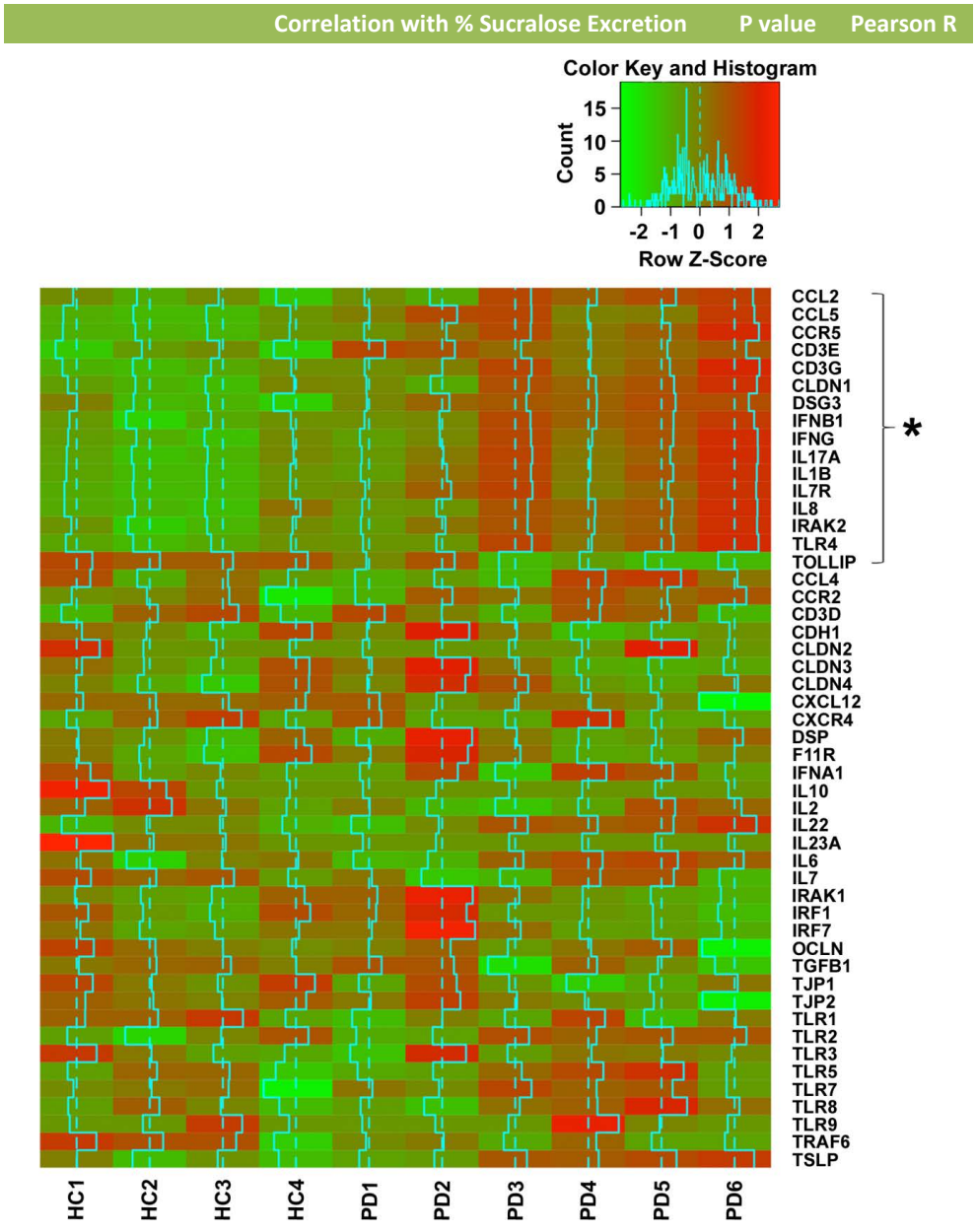


Figure 2: Higher pro-inflammatory milieu was observed in the colonic biopsy of PD compared to HC subjects. Genes specific for microbiota-induced intestinal inflammation and gut dysfunction were evaluated using QuantiGene 55 plex assay kit from HC (HC1, HC2, HC3 and HC4) and PD (PD1, PD2, PD3, PD4, PD5, PD6) subjects. * denotes significantly different gene in PD compared to HC (*p<0.03). Green color indicates lower expression and red color indicates higher expression of gene as shown in top right panel.

Demographics	Age of Subjects	0.6211	0.159
	BMI of Subjects	0.0809	-0.523
Barrier impairments	ZO-1 scoring	0.1777	-0.417
Endotoxins	LBP levels in Plasma	0.4433	-0.274
Cell counts	Number of TLR4+ve cells	0.3170	0.316
	Number of CD3+ve cells	0.1219	0.471
Gene Expressions	CLD1	0.1152	0.530
	IFNB1	0.0545	0.623
	IFNG	0.0715	0.592
	IL17A	0.0528	0.626
	IL1B	0.0299	0.682
	CCL5	0.0702	0.594
	CCR5	0.0249	0.698
	IRAK2	0.0817	0.576
	TOLLIP	0.0231	-0.704
	TLR4	0.0305	0.680
	DSG3	0.0586	0.615
	IL7R	0.0354	0.666
	IL8	0.1168	0.528

Table 2: Human study correlations between Leaky Gut (% Sucralose) and other factors

Demographics	Age of Subjects	0.740	-0.107
	BMI of Subjects	0.700	-0.124
Endotoxins	LBP levels in Plasma	0.003	0.831
Cell counts	TLR4 cell counts	0.142	-0.450
	CD3 cell counts	0.149	-0.442
Gene Expressions	CLD1	0.053	-0.626
	IFNB1	0.014	-0.741
	IFNG	0.034	-0.670
	IL17A	0.025	-0.698
	IL1B	0.016	-0.734
	CCL5	0.008	-0.777
	CCR5	0.019	-0.721
	IRAK2	0.012	-0.755
	TOLLIP	0.068	0.598
	TLR4	0.042	-0.649
	DSG3	0.0003	-0.905
	IL7R	0.009	-0.769
	IL8	0.090	-0.563

Table 3: Human study correlations between intestinal barrier dysfunction (ZO-1 scoring data) and other factors

Part II: Rotenone-induced PD mouse model

Tlr4KO mice are partially protected from rotenone-induced effects on the intestine

Rotenone serves as model of Parkinson's disease. To initially validate its use in the gut-brain axis, we evaluated whether this toxin recapitulates aspects of gastrointestinal physiology observed in human PD patients. Indeed, the rotenone model recapitulated aspects of intestinal dysfunction associated with PD. In this regatd, rotenone-treated animals showed a reduction in colon length compared to vehicle-treated mice ($p<0.0001$; Fig 3A) as well as negatively affected intestinal transit (i.e., reduced distance travelled by the Evans Blue dye in the intestinal tract compared to vehicle) ($F_{(1, 26)}=81.53$, $p<0.0001$; Fig 3B).WT-vehicle treated mice had an intact tight junction barrier with a continuous lattice pattern around the epithelial lining in colon tissue samples. In contrast, WT-rotenone mice showed reduced/disrupted levels of ZO-1 expression ($p<0.01$) (Fig 3C, D) in a manner similar to that seen in in human PD subjects (Fig 1C).

Similar to human PD biopsy tissue, rotenone-treated mice demonstrated increased numbers of Tlr4+ and CD3+ cells in the intestinal mucosa relative to vehicle-treated mice. Rotenone-treated mice had a greater number of Tlr4+ cells in the mucosa of colon tissue while control mice showed occasional Tlr4 expression in close proximity to basolateral crypts. Specifically, rotenone-treated mice had evidence of immune activation with a higher number of Tlr4+ cells in the ileum and the colon compared vehicle treated mice (Fig 3E, F) ($p<0.0001$). Stereological analyses showed increased number of CD3+ T-cells in the colon of WT rotenone-treated mice compared to vehicle treated mice (Fig 3G, H); again similar to what we saw in PD human study (Fig 3G).

To establish whether TL4 was critical for this effect, we examined the effects of rotenone in Tlr4 knockout (KO) mice with the premise that if the intestinal microbiota and gut-derived inflammation contribute to rotenone-induced PD-like pathology then Tlr4 KO mice should be protected from rotenone-induced effects. Tlr4 KO did not impact rotenone-induced decrease in colon length (Fig 3A); however, the Tlr4 KO mice were partially protected from rotenone-induced deficits in intestinal transit time ($p<0.05$) (Fig 3B). Finally, while rotenone significantly decreased ZO-1 integrity in WT mice, the Tlr4 KO mice were protected from disruptive effects of rotenone on intestinal barrier integrity (Fig 3C, D).

Rotenone-induced increase in CD3+ T cells in the mucosa of WT mice was mitigated in Tlr4 KO mice ($F_{(1, 36)}=91.98$, $p<0.0001$, Fig 3G, H). There was a significant main effect of genotype ($F_{(1, 36)}=8.97$, $p<0.01$) as well as an interaction effect between treatment (i.e., rotenone vs control) and genotype ($F_{(1, 36)}=9.30$, $p<0.01$).

Our next step was to determine if these changes in immune markers are accompanied by biological changes . Thus, we assessed GFAP as a marker of enteric glial activity and α-syn accumulation in the myenteric plexus. While WT-rotenone group showed an increased number of GFAP+ enteric glial cells in myenteric plexuses compared to WT-vehicle the Tlr4 KO, rotenone-treated mice had mitigated expression of GFAP+ enteric glial cells (Fig 4A). Optical density analyses showed increased GFAP staining in the myenteric plexuses of WT-

rotenone mice while Tlr4 KO mice were unaffected ($F_{(1, 36)}$=9.25, p<0.01; Fig. 4B). There was a significant main effect of genotype ($F_{(1, 36)}$=7.86, p<0.01) and an interaction between genotype and treatment ($F_{(1, 36)}$=9.15, p<0.01). Post-hoc analysis showed that GFAP intensity in myenteric plexuses of Tlr4 KO-rotenone group was similar to Tlr4 KO-vehicle and WT-vehicle but it was significantly lower than the WT-rotenone group (p<0.01). Analyses showed higher α-syn positive structures in the myenteric plexuses (Fig 4C) and mucosa (Fig S2) of WT-rotenone compared to other groups. There was no difference in α-syn immunoreactivity between WT-vehicle, Tlr4 KO-vehicle and Tlr4 KO-rotenone (Fig 4C). Optical density analyses for α-syn intensity (Fig 4D) in the myenteric plexuses showed an increased α-syn expression in WT-rotenone ($F_{(1, 36)}$=34.19, p<0.0001). There was an effect of the genotype on α-syn expression in the colon ($F_{(1, 36)}$=28.68, p<0.0001) and an interaction between treatment and genotype ($F_{(1, 36)}$=24.82, p<0.0001). Post-hoc analysis revealed lower levels of α-syn in Tlr4 KO-rotenone group compared to WT-rotenone (p<0.0001).

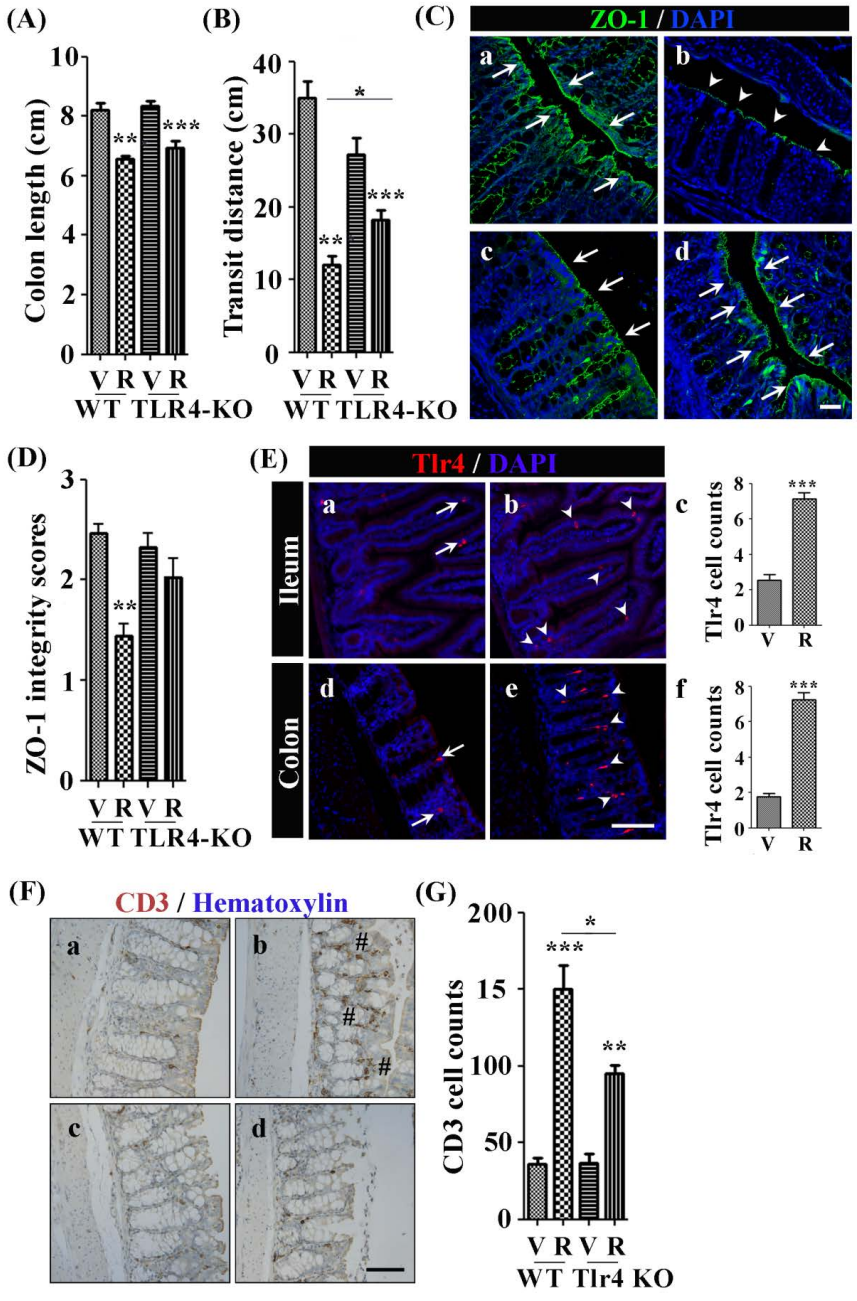


Figure 3: Rotenone PD model recapitulates GI physiology as observed in human PD patients and TLR4KO partially protects against them. Graphs represent data for (A) colon length (B) transit distance. Photomicrographs in panel C represent immunofluorescence staining for ZO-1 in (a) WT-vehicle (b) WT-rotenone (c) Tlr4 KO-vehicle and (d) Tlr4 KO-rotenone. White arrows in panels (D-a, D-c, D-d) represent continuous lattice structure of ZO-1 barrier integrity while white arrowheads in panel (D-b) represent non-

continuous (disrupted) as well as lower expression of ZO-1 barrier. Graph in panel (D) represent ZO-1 integrity scores (using scoring scale 0-3 represents: 0 = no ZO-1 expression, 1 = very poor ZO-1 expression, 2 = moderate expression of ZO-1, 3 = great continuous expression of ZO-1). Photomicrographs in (E) represent Tlr4 immunofluorescence staining for (a) ileum of WT-vehicle, (b) ileum of WT-rotenone, (d) colon of WT-vehicle and (e) colon of WT-rotenone. White arrows in panels a and d represent occasional Tlr4+ cells at the basolateral crypts while white arrowheads in panels b and e indicate higher infiltration of Tlr4+ cells into lamina propria of tissue samples. Graphs represent estimated Tlr4+ cell counts in (c) ileum and (f) colon samples. Photomicrographs IN (F) represents CD3 stained cells in colonic samples of (a) WT-vehicle, (b) WT-rotenone, (c) Tlr4 KO-vehicle and (d) Tlr4 KO-rotenone. Black number sign (#) in panel (b) represent increased CD3+ T cells infiltration into lamina propria. Graph in (G) represent quantitative analyses of cell counts for CD3+ T cells in lamina propria. Counterstaining with DAPI and hematoxyline was used in (C, E) and F panels respectively. Scale bar in panel C-d represent 100µm and applies to (C-a to C-d). Scale bar in panel E-e represent 100µm and applies to (E-a, E-b, E-d, E-e). Scale bar in F-d represent 100 µm and applies to (F-a to F-d). ***$p<0.001$. Data represent as mean + SEM. V= vehicle treatment, R = rotenone treatment, WT = wild type C57BL6, Tlr4 KO = Tlr4 knockout.

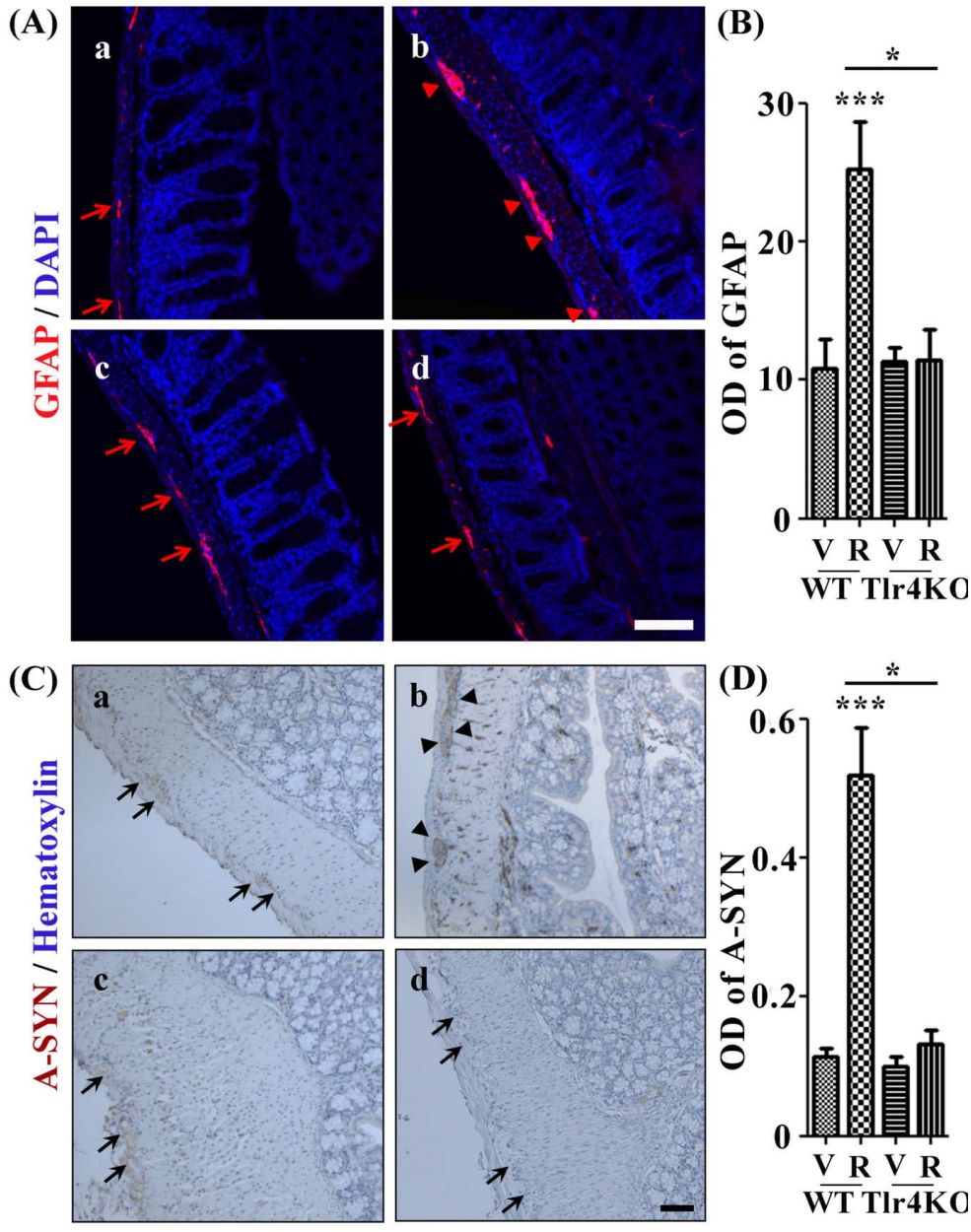


Figure 4: TLR4KO-rotenone partially protects against enteric glial inflammation and α-syn expression in the colonic tissue compared to WT-rotenone mice. Photomicrographs represent histology for (A) GFAP and (C) a-syn markers in colonic samples of (a) WT-vehicle, (b) WT-rotenone, (c) Tlr4 KO-vehicle and (d) Tlr4 KO-rotenone. Red arrows in panels (B-a, B-b, B-c) represent normal distribution of GFAP in myenteric plexuses of colon while red arrowheads in panel (B-d) represent increased GFAP in myenteric plexuses. Black arrows in

panels (C-a, C-b, C-c) represent normal distribution of a-syn into myenteric plexuses while black arrowheads in panel (C-b) represent higher expression of a-syn into myenteric plexuses of colon. Graphs represent quantitative analyses of (B) OD data for GFAP in myenteric plexuses and (D) OD data for a-syn expression in myenteric plexuses. Counterstaining using hematoxylin was performed for immunoperoxidase staining (A) and DAPI was used for immunofluorescence (C). Scale bar in panel (A-d) represents 100µm and applies to panels (A-a to A-d). Scale bar in panel (C-d) represents 100µm and applies to panels (C-a to C-d). *p<0.05,**p<0.001, ***p<0.0001. Data represented as mean + SEM. V= vehicle treatment, R = rotenone treatment, WT = wild type C57BL6, Tlr4 KO = Tlr4 knockout, GFAP = Glial fibrillary acidic protein, A-SYN = α-synuclein.

Tlr4 KO mice are partially protected from rotenone-induced effects in the brain

Microglia are morphologically and functionally dynamic CNS cells, upon activation microglia transform from thin cell bodies with highly ramified extensions into amoeboid cells with fewer branches[36] with these morphological changes indicative of a transition to pro-inflammatory/phagocytic microglia. This analysis focused on the SN a region highly relevant for PD. Compared to vehicle-treated WT mice, microglia in WT-rotenone animals displayed a decreased number of branches ($p<0.001$, Fig 5A), decreased number of branch endpoints ($p<0.01$, Fig 5B), decreased branch length ($p<0.05$, Fig 5C) and increased ratio of cell body size/total cell size ($p<0.01$, Fig 5D). A comparison between WT and Tlr4 KO rotenone-treated mice revealed that Tlr4 KO mice had significantly more branches ($p<0.05$), branch length and a decrease in the ratio of cell body size/total cell size compared to WT-rotenone mice ($p<0.01$). Rotenone treatment in WT or Tlr4 KO mice resulted in a similar reduction of number of branches endpoints.

Tyrosine hydroxylase (TH) staining was performed to assess the number of dopaminergic neurons in PD relevant brain areas. Light microscopic stereological analyses showed significantly lower number of TH+ cells in the SN after rotenone treatment in WT group compared to vehicle (Fig 5F). In WT mice, rotenone significantly reduced the number of TH+ cells in the SN; however, rotenone treatment in Tlr4 KO mice impacted the number of TH+ to a lesser extent (Fig 5F). Cell count analyses showed significantly decreased number of TH+ cells in the SN in WT-rotenone group compared to others ($F_{(1, 36)}=35.89$ $p<0.0001$) (Fig 5G). There was a significant interaction between treatment (rotenone vs vehicle) and genotype (WT vs KO) ($F_{(1, 36)}=6.48$ $p<0.05$). Post-hoc analysis showed that the WT-rotenone group had significantly lower number of TH+ cells compared to Tlr4 KO-rotenone mice ($p<0.05$) (Fig 5G).

A rotarod test was used to assess motor function and data are represented as latency to fall from the apparatus in seconds, shorter latency to fall indicates motor impairment (Fig 5H). There was an overall effect of treatments (rotenone vs vehicle) ($F(1,32)=60.11$, $p<0.0001$) and of genotype ($F_{(1,32)}=9.60$, $p<0.01$) on rotarod performance. Repeated measures showed an effect of time ($F_{(3,96)}=22.49$, $p<0.0001$). WT-rotenone group showed greater motor dysfunction over time compared to WT-vehicle (interaction effect treatment x time $F_{(3,96)}=1.33$ $p<0.0001$). WT-rotenone mice showed a decrease in rotarod performance

starting on day 21 compared to the WT-vehicle ($F_{(1,32)}$=6.20, p<0.05 on day 21 and $F_{(1,34)}$=72.14, p<0.0001 on day 28). On day 28 there was a significant effect of the genotype ($F_{(1,34)}$=8.05, p<0.001) on rotarod performance. Post-hoc analysis showed that Tlr4 KO-rotenone mice performed significantly better on the rotarod compared to WT-rotenone (p<0.05) (Fig 5H).

Evidence to support the gut-immune-brain axis involvement in rotenone-induced PD mice

In an effort to understand how multiple variables are similar or dissimilar from each other correlation analysis was performed. While correlation does not equate to causation, these relationships allow us to infer potential relationships. ZO-1 integrity score inversely associated with the number of CD3 positive T cells in colon (r=-0.64, p<0.0001; Fig 6A). The number of T-cells in colon positively correlated with α-syn in the colon (r=0.59, p<0.0001; Fig 6B). α-Syn in the colon positively correlated with a marker of microglial activation in the SN (i.e., the ratio of microglial cell body size/total cell size) (r=0.76, p<0.0001; Fig 6C). Likewise, presence of phagocytic-like microglial phenotype in the SN (increased ratio cell body size/ total cell size) inversely correlated with the number of dopaminergic cells (TH positive cells) in the SN (r=-0.63, p<0.01; Fig 6D). Finally, the number of dopaminergic cells in the SN positively correlated with motor function (latency to fall in the rotarod) (r=0.72, p<0.0001; Fig 6E).

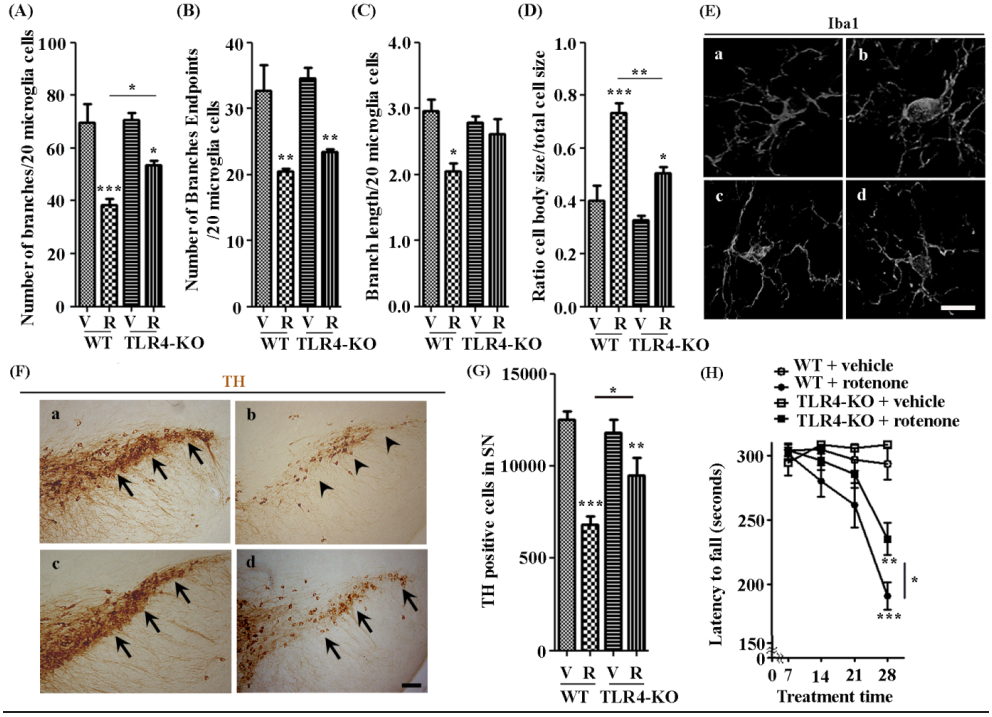


Figure 5: TLR4KO-rotenone mice showed less pro-inflammatory microglia, less dopamine cell loss and less behaviour deficits compared to WT-rotenone. Graphs in panel (A,B,C and D) shows the average number of branches, branches endpoints, branch length and the ratio cell body size/total cell size of microglia in the SN. Photomicrographs in panel (E) represent microglial cells for each condition: a = WT-Vehicle, b= WT-Rotenone, c=TLR4KO+Vehicle, d=TLR4KO+Rotenone. Tlr4 KO mice showed microglia with less pro-inflammatory phenotype in the SN compared to WT-rotenone mice. Photomicrographs in panel (F) represent TH stained dopamine cells in (a) WT-vehicle, (b) WT-rotenone, (c) Tlr4 KO-vehicle and (d) Tlr4 KO-rotenone groups. Black arrows indicate higher number of TH+ cells while black arrowheads represent lower number of TH+ cells. Graph in panel (G) shows estimated cell counts for TH stained dopamine cells into SN. WT-rotenone showed profound loss of TH cells while Tlr4 KO-rotenone showed a mitigated TH+ cell loss in the SN. Graph in panel (H) shows rotarod data to assess motor function associated behavior deficits. Tlr4 KO-rotenone group showed reduced rotenone-induced motor-dysfunction compared to WT-rotenone. Scale bar in panel (E-d) represent 10µm and it applies to panels (E-a to E-d). Scale bar in panel (F-d) represent 200µm and it applies to panels (F-a to F-d). *p<0.05,**p<0.001, ***p<0.0001. Data represented as mean + SEM. V= vehicle treatment, R = rotenone treatment, WT = wild type C57BL6, Tlr4 KO = Tlr4 knockout.

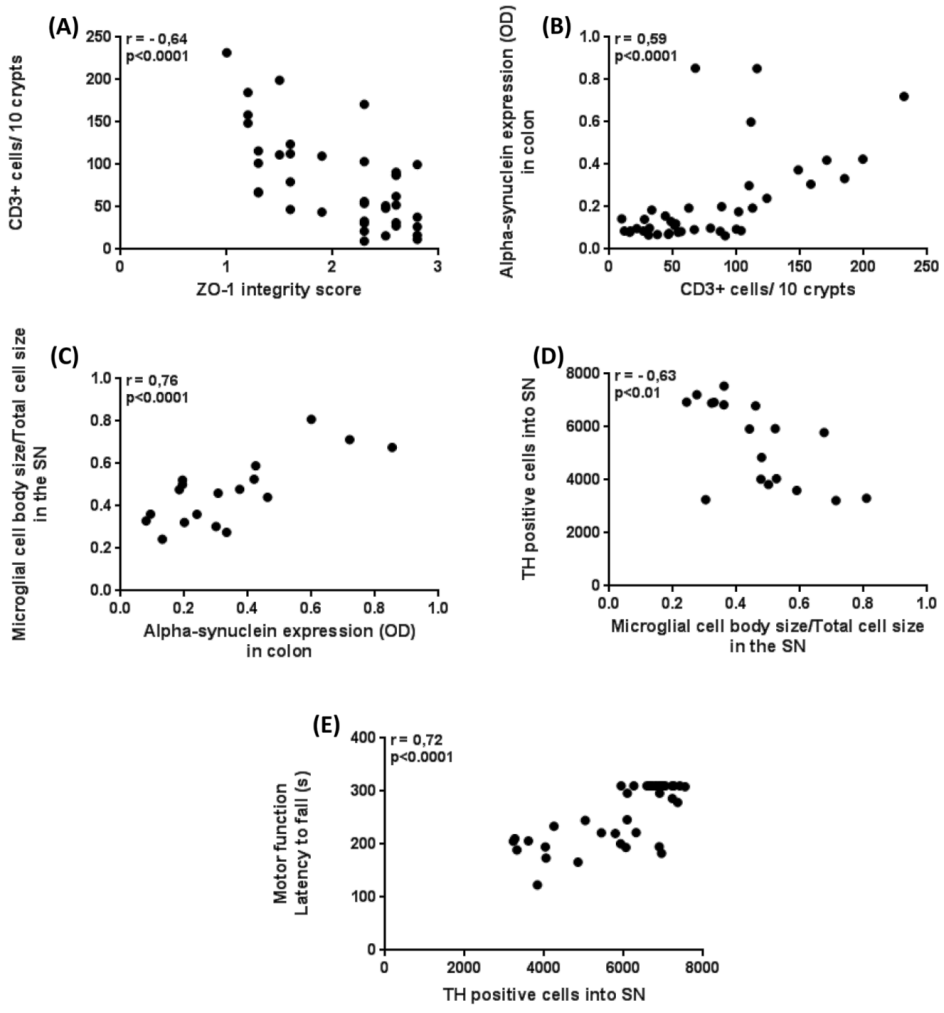


Figure 6: Correlations support the gut-immune-brain axis involvement in rotenone-induced parkinsonism in mice. Graph in panel (A) shows that ZO-1 integrity score, decreased with rotenone treatment, was inversely associated with the number of T-cells (CD3 positive cells) in colon. (B) The number of T-cells in colon, significantly increased with rotenone, was positively associated with α-syn expression in colon. (C) α-syn expression in colon was positively correlated with the ratio microglial cell body size/total cell size in the SN. Rotenone exposure changed microglial phenotype into a more pro-inflammatory state (increased ratio cell body size/ total cell size) in the SN. (D) This state inversely correlated with the number TH positive cells in the SN. (E) The number of TH positive cells in the SN, decreased after rotenone exposure, was positively associated with motor function (latency to fall in the rotarod).

4. Discussion

We propose that immune activation occurring secondary to changes in the intestinal host-microbe interaction could be a key factor responsible for ongoing chronic neuroinflammation and neurodegeneration in PD. Indeed, PD subjects demonstrate intestinal barrier dysfunction[11,28] and intestinal microbiota dysbiosis which are two factors that would be expected to have pro-inflammatory consequences in the intestinal mucosa, systemic circulation, and potentially in remote organs such as the brain. Data in this study revealed increased presence of CD3+ T cells, TLR4+ cells, and associated pro-inflammatory cytokines and chemokines in colon tissue from PD subjects. In a rodent model of PD the number of CD3+ T cells and TLR4+ cells in the colonic mucosa were also increased associated with enhanced number of GFAP+ enteric glial cells in the colonic myenteric plexuses. Taken together, these findings may suggest that intestinal inflammation leads to activation of enteric glial cells. Increased activation of enteric glial cells has also been reported in PD patients[13,37]. Others have shown similar results of α-syn pathology in colonic samples of mice orally exposed to rotenone[31,38,39].

Increased T-cell trafficking into the colonic mucosa is a feature associated with PD. A recent study reports increased T cell trafficking into colonic mucosa of constipated PD patients[21]. Likewise, analysis of biopsy tissue from PD subjects in the current study revealed higher CD3+ cell counts compared to HC subjects concurrent with an increased chemoattractant signals such as CCR5 which may account for the increased CD3+ T-cells in the PD colon. Increased CD3+ T cells were also present in the colon of rotenone-treated mice. It is possible that the intestinal barrier dysfunction[11] and/or abnormal microbiota found in PD patients[12,15,16] is promoting T cell trafficking into the intestine. However, future studies with manipulation of intestinal microbiota will be necessary to verify this effect.

To the best of our knowledge, the current study is the first to show an upregulation of TLR4 mRNA and protein in the colon of PD patients. In addition, there were changes in the levels of downstream signalling molecules of TLR4 including increased IRAK2 and lower TOLLIP as well as higher levels of TLR4-activated MyD88-dependent pathway mediated cytokines such as IL1-β, IFN-γ, IFN-β and CCL5 levels in colon tissue from PD compared to HC subjects. The idea that TLR4 mediated signalling impacts the brain is supported by research demonstrating that knocking out TLR4 mitigates neuroinflammation in the brain[40]. The importance of TLR4 signalling for PD is suggested by data in the current study whereby TLR4 KO mice were protected against many of the PD-like consequences of rotenone-induced effects including: intestinal barrier integrity, myenteric plexus GFAP expression, colonic α-syn, SN microglial activation and dopaminergic cell loss and motor function impairment. Taken together, this evidence suggests a possible role for TLR4-mediated inflammation in neuroinflammation and neurodegeneration in PD.

We hypothesize that immune activation occurring secondary to changes in the intestine could be a key factor responsible for ongoing chronic neuroinflammation and

neurodegeneration in PD. Indeed, there is a plethora of literature showing that manipulation of the intestinal microbiota influences brain function and inflammation[41–43]. It is possible that the link between the microbiota and the brain (i.e., the gut-brain axis) is the immune system. The changes in immune cell populations in the intestinal mucosa may be induced by a number of factors including dysfunction of the intestinal barrier as well as pro-inflammatory changes in the intestinal microbiota. TLR4 is particularly interesting in this regard since our group has reported high relative abundance of LPS producing bacteria [12] in PD patient stool as well as changes LBP indicating exposure to LPS[11]. In combination with the intestinal barrier dysfunction observed in PD, it is conceivable that pro-inflammatory bacteria (especially LPS producers) could initiate TLR4 and/or CD3 cell-mediated gut inflammation. Up-regulated cytokines in the intestinal tract could exacerbate inflammatory conditions by recruiting inflammatory cells, enhancing local inflammation and disturbing immune homeostasis. The local inflammation in the intestine might affect the brain through the systemic circulation or via neuronal terminals of the vagus nerve.

Future studies exploring the intestinal microbiota are warranted to investigate the contribution of the microbiota to TLR4 mediated signalling in PD. Site targeted gene silencing such as siRNA or miRNA for TLR4 pathway blocking mechanisms could also help delineate the spread of gut-derived inflammation to the brain. Pharmacological modulation of the TLR4 pathway could also represent a novel therapeutic approach in the treatment of PD.

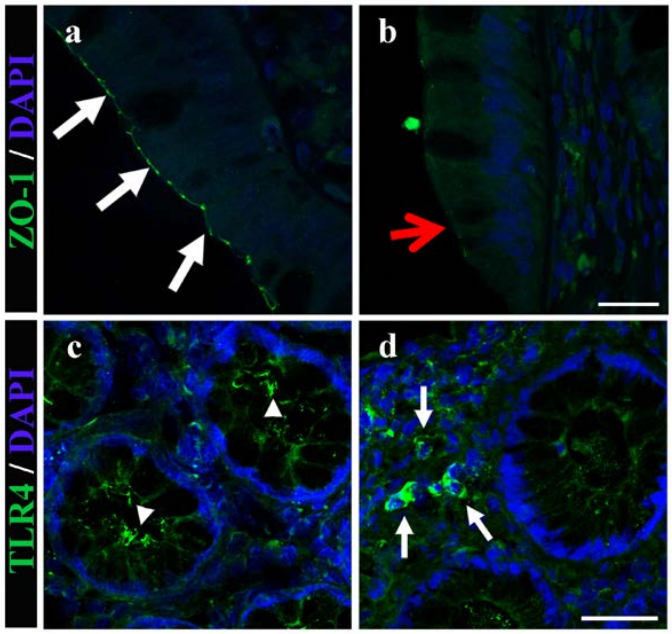
Results from our PD patients support ongoing chronic intestinal inflammation. Using Tlr4 KO+ rotenone mice model, we observed mitigated neuroinflammatory and neurodegenerative effects in the ENS and CNS suggesting a possible role of endotoxemia relevant TLR4-mediated intestinal inflammation in PD pathogenesis.

References

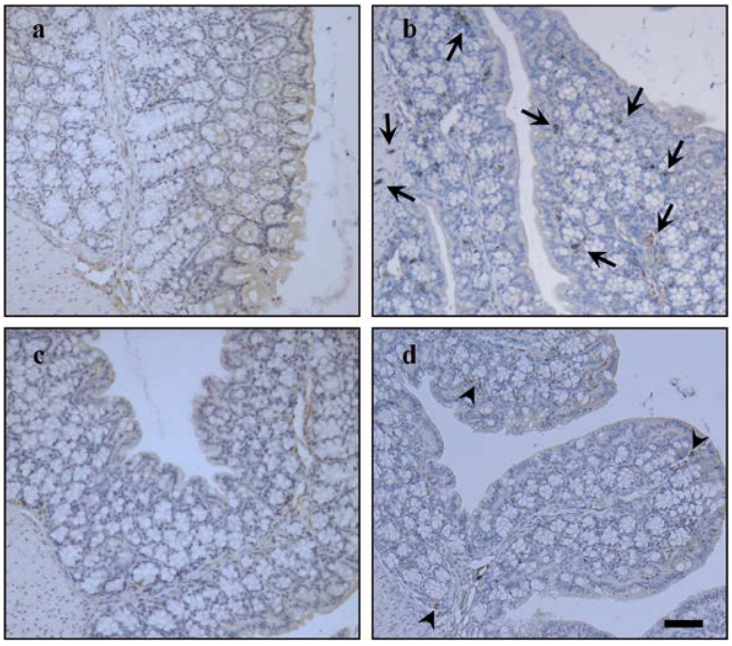
1. Hirsch, E. C. & Hunot, S. Neuroinflammation in Parkinson's disease: a target for neuroprotection? *Lancet Neurol.* **8,** 382–397 (2009).
2. Hirsch, E. C., Vyas, S. & Hunot, S. Neuroinflammation in Parkinson's disease. *Parkinsonism Relat. Disord.* **18 Suppl 1,** S210-212 (2012).
3. Xanthos, D. N. & Sandkühler, J. Neurogenic neuroinflammation: inflammatory CNS reactions in response to neuronal activity. *Nat. Rev. Neurosci.* **15,** 43–53 (2014).
4. Mogi, M. *et al.* Interleukin-1 beta, interleukin-6, epidermal growth factor and transforming growth factor-alpha are elevated in the brain from parkinsonian patients. *Neurosci. Lett.* **180,** 147–150 (1994).
5. Mogi, M. *et al.* Tumor necrosis factor-alpha (TNF-alpha) increases both in the brain and in the cerebrospinal fluid from parkinsonian patients. *Neurosci. Lett.* **165,** 208–210 (1994).
6. Garcia-Esparcia, P., Llorens, F., Carmona, M. & Ferrer, I. Complex deregulation and expression of cytokines and mediators of the immune response in Parkinson's disease brain is region dependent. *Brain Pathol. Zurich Switz.* **24,** 584–598 (2014).
7. Blum-Degen, D. *et al.* Interleukin-1 beta and interleukin-6 are elevated in the cerebrospinal fluid of Alzheimer's and de novo Parkinson's disease patients. *Neurosci. Lett.* **202,** 17–20 (1995).
8. Mogi, M. *et al.* Interleukin (IL)-1 beta, IL-2, IL-4, IL-6 and transforming growth factor-alpha levels are elevated in ventricular cerebrospinal fluid in juvenile parkinsonism and Parkinson's disease. *Neurosci. Lett.* **211,** 13–16 (1996).
9. Brodacki, B. *et al.* Serum interleukin (IL-2, IL-10, IL-6, IL-4), TNFalpha, and INFgamma concentrations are elevated in patients with atypical and idiopathic parkinsonism. *Neurosci. Lett.* **441,** 158–162 (2008).
10. Reale, M. *et al.* Peripheral cytokines profile in Parkinson's disease. *Brain. Behav. Immun.* **23,** 55–63 (2009).
11. Forsyth, C. B. *et al.* Increased intestinal permeability correlates with sigmoid mucosa alpha-synuclein staining and endotoxin exposure markers in early Parkinson's disease. *PloS One* **6,** e28032 (2011).
12. Keshavarzian, A. *et al.* Colonic bacterial composition in Parkinson's disease. *Mov. Disord. Off. J. Mov. Disord. Soc.* **30,** 1351–1360 (2015).
13. Devos, D. *et al.* Colonic inflammation in Parkinson's disease. *Neurobiol. Dis.* **50,** 42–48 (2013).
14. Tan, A. H. *et al.* Small intestinal bacterial overgrowth in Parkinson's disease. *Parkinsonism Relat. Disord.* **20,** 535–540 (2014).
15. Scheperjans, F. *et al.* Gut microbiota are related to Parkinson's disease and clinical phenotype. *Mov. Disord. Off. J. Mov. Disord. Soc.* **30,** 350–358 (2015).
16. Unger, M. M. *et al.* Short chain fatty acids and gut microbiota differ between patients with Parkinson's disease and age-matched controls. *Parkinsonism Relat. Disord.* (2016). doi:10.1016/j.parkreldis.2016.08.019
17. Helander, H. F. & Fändriks, L. Surface area of the digestive tract - revisited. *Scand. J. Gastroenterol.* **49,** 681–689 (2014).
18. Sampson, T. R. *et al.* Gut Microbiota Regulate Motor Deficits and Neuroinflammation in a Model of Parkinson's Disease. *Cell* **167,** 1469–1480.e12 (2016).
19. Cryan, J. F. & O'Mahony, S. M. The microbiome-gut-brain axis: from bowel to behavior. *Neurogastroenterol. Motil. Off. J. Eur. Gastrointest. Motil. Soc.* **23,** 187–192 (2011).
20. Lyte, M. Microbial endocrinology in the microbiome-gut-brain axis: how bacterial production and utilization of neurochemicals influence behavior. *PLoS Pathog.* **9,** e1003726 (2013).
21. Chen, Y. *et al.* Clinical characteristics and peripheral T cell subsets in Parkinson's disease patients with constipation. *Int. J. Clin. Exp. Pathol.* **8,** 2495–2504 (2015).
22. Braak, H. & Del Tredici, K. Invited Article: Nervous system pathology in sporadic Parkinson disease. *Neurology* **70,** 1916–1925 (2008).
23. Shannon, K. M., Keshavarzian, A., Dodiya, H. B., Jakate, S. & Kordower, J. H. Is alpha-synuclein in the colon a biomarker for premotor Parkinson's disease? Evidence from 3 cases. *Mov. Disord. Off. J. Mov. Disord. Soc.* **27,** 716–719 (2012).
24. Hilton, D. *et al.* Accumulation of α-synuclein in the bowel of patients in the pre-clinical phase of Parkinson's disease. *Acta Neuropathol. (Berl.)* **127,** 235–241 (2014).

25. Stokholm, M. G., Danielsen, E. H., Hamilton-Dutoit, S. J. & Borghammer, P. Pathological α-synuclein in gastrointestinal tissues from prodromal Parkinson disease patients. *Ann. Neurol.* **79,** 940–949 (2016).
26. Guan, J. *et al.* Vascular degeneration in Parkinson's disease. *Brain Pathol. Zurich Switz.* **23,** 154–164 (2013).
27. Shaikh, M., Rajan, K., Forsyth, C. B., Voigt, R. M. & Keshavarzian, A. Simultaneous gas-chromatographic urinary measurement of sugar probes to assess intestinal permeability: use of time course analysis to optimize its use to assess regional gut permeability. *Clin. Chim. Acta Int. J. Clin. Chem.* **442,** 24–32 (2015).
28. Pal, G. D. *et al.* Abnormal lipopolysaccharide binding protein as marker of gastrointestinal inflammation in Parkinson disease. *Front. Neurosci.* **9,** 306 (2015).
29. Engen, P. A. *et al.* The Potential Role of Gut-Derived Inflammation in Multiple System Atrophy. *J. Park. Dis.* (2017). doi:10.3233/JPD-160991
30. Voigt, R. M. *et al.* HIV-associated mucosal gene expression: region-specific alterations. *AIDS Lond. Engl.* **29,** 537–546 (2015).
31. Perez-Pardo, P. *et al.* Gut–brain and brain–gut axis in Parkinson's disease models: Effects of a uridine and fish oil diet. *Nutr. Neurosci.* 1–12 (2017). doi:10.1080/1028415X.2017.1294555
32. Pan-Montojo, F. *et al.* Progression of Parkinson's disease pathology is reproduced by intragastric administration of rotenone in mice. *PloS One* **5,** e8762 (2010).
33. Gutsmann, T. *et al.* Dual role of lipopolysaccharide (LPS)-binding protein in neutralization of LPS and enhancement of LPS-induced activation of mononuclear cells. *Infect. Immun.* **69,** 6942–6950 (2001).
34. Wurfel, M. M., Kunitake, S. T., Lichenstein, H., Kane, J. P. & Wright, S. D. Lipopolysaccharide (LPS)-binding protein is carried on lipoproteins and acts as a cofactor in the neutralization of LPS. *J. Exp. Med.* **180,** 1025–1035 (1994).
35. Gegner, J. A., Ulevitch, R. J. & Tobias, P. S. Lipopolysaccharide (LPS) signal transduction and clearance. Dual roles for LPS binding protein and membrane CD14. *J. Biol. Chem.* **270,** 5320–5325 (1995).
36. Erny, D. *et al.* Host microbiota constantly control maturation and function of microglia in the CNS. *Nat. Neurosci.* **18,** 965–977 (2015).
37. Clairembault, T., Leclair-Visonneau, L., Neunlist, M. & Derkinderen, P. Enteric glial cells: new players in Parkinson's disease? *Mov. Disord. Off. J. Mov. Disord. Soc.* **30,** 494–498 (2015).
38. Drolet, R. E., Cannon, J. R., Montero, L. & Greenamyre, J. T. Chronic rotenone exposure reproduces Parkinson's disease gastrointestinal neuropathology. *Neurobiol. Dis.* **36,** 96–102 (2009).
39. Pan-Montojo, F. J. & Funk, R. H. W. Oral administration of rotenone using a gavage and image analysis of alpha-synuclein inclusions in the enteric nervous system. *J. Vis. Exp. JoVE* (2010). doi:10.3791/2123
40. Maes, M., Kubera, M. & Leunis, J.-C. The gut-brain barrier in major depression: intestinal mucosal dysfunction with an increased translocation of LPS from gram negative enterobacteria (leaky gut) plays a role in the inflammatory pathophysiology of depression. *Neuro Endocrinol. Lett.* **29,** 117–124 (2008).
41. Rea, K., Dinan, T. G. & Cryan, J. F. The microbiome: A key regulator of stress and neuroinflammation. *Neurobiol. Stress* **4,** 23–33 (2016).
42. Dinan, T. G. & Cryan, J. F. Gut instincts: microbiota as a key regulator of brain development, ageing and neurodegeneration. *J. Physiol.* **595,** 489–503 (2017).
43. Dinan, T. G. & Cryan, J. F. Gut-brain axis in 2016: Brain-gut-microbiota axis - mood, metabolism and behaviour. *Nat. Rev. Gastroenterol. Hepatol.* **14,** 69–70 (2017).

Supplemetary material



Supplementary Figure 1: Immunofluorescence staining for ZO-1 and TLR4 in PD and HC subjects. Photomicrographs represent immunofluorescence staining for a major tight junction protein ZO-1 (a, b) and TLR4 (c, d). Similar to ZO-1 expression in crypts (Fig 1), continuous lattice structure of ZO-1 was present at epithelial lining in healthy control (shown by white arrows) and PD subject showed disappearance of ZO-1 at the epithelial lining (shown by red arrows). TLR4 immunofluorescence staining showed TLR4 expression restricted to the apical surface of the crypts in HC (white arrowheads) compared to PD subjects who had more TLR4+ cells in lamina propria (white arrows). Scale bars in panels (a, b) and (c, d) represent 20µm and 40µm respectively.



Supplementary Figure 2: Rotenone exposure increased a-syn structures in colonic mucosa of WT mice, which was lower in Tlr4 KO mice. Photomicrographs represent immunohistochemistry for a-syn in (a) WT-vehicle (b) WT-rotenone (c) Tlr4 KO-vehicle and (d) Tlr4 KO-rotenone. Black arrows (panel b) indicate increased a-syn positive structures in mucosa samples of WT-rotenone group. Black arrowheads (panel d) indicate occasional but very few a-syn structures in mucosa of Tlr4 KO-rotenone group. There were no definite structures for a-syn reactivity in mucosa of vehicle treated groups (a, c). Scale bar in panel d is 100µm and it applies to all panels a-d.

CHAPTER 5

Gut bacterial composition in a mouse model of Parkinson's disease

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Manuscript in preparation

Abstract

The mechanism of neurodegeneration in Parkinson's disease (PD) remains unknown but it has been hypothesized that the intestinal tract could be an initiating and contributing factor to the neurodegenerative processes. In PD patients as well as in animal models for PD alpha-synuclein-positive enteric neurons in the colon and evidence of colonic inflammation have been demonstrated. Moreover, several studies reported pro-inflammatory bacterial dysbiosis in PD patients. Here we report for the first time significant changes in the composition of the intestinal microbiota (mucosal and luminal bacteria in the cecum) and the associated metabolic pathways in a rotenone-induced mouse model for PD. The mouse model for PD induced by the pesticide rotenone is associated with enhanced abundance of (human relevant) pro-inflammatory intestinal bacteria at the expense of beneficial commensal bacteria. Bacterial dysbiosis might play an important role in the disruption of intestinal epithelial integrity, intestinal inflammation leading to alpha-synuclein aggregation and PD pathology.

1. Introduction

Parkinson's disease (PD) is the second most common age-associated neurodegenerative disease characterized by motor impairments due to loss of dopamine cells in the basal ganglia of the mid-brain[1,2]. The main hallmark of the disease is the presence of misfolded, aggregated and neurotoxic forms of the protein alpha-synuclein in the remaining dopamine neurons of the substantia-nigra[3]. Besides the brain pathology, PD patients also develop non-motor symptoms, including gastrointestinal (GI) dysfunctions[4,5]. These symptoms may precede the motor symptoms by several years and their occurrence in otherwise healthy people is associated with an increased risk of developing PD[6,7]. Moreover, GI dysfunctions are major determinants for the quality of life[8,9] and remain undertreated[10].

The mechanism of neurodegeneration in PD remains unknown but it has been hypothesized that the intestinal tract could be an initiating and contributing factor to the neurodegeneration processes[11–14]. In PD patients, as well as in animal models for PD, alpha-synuclein-positive enteric neurons in intestinal mucosal samples have been found[15–19]. In the oral rotenone-induced mouse model for PD, enteric alpha-synuclein aggregates and intestinal inflammation were associated with reduced intestinal transit[19]. In PD patients alpha-synuclein-positive neurons in the colonic mucosa were detected from 2 to more years before the onset motor symptoms and were significantly associated with abnormal intestinal permeability and endotoxemia[15,20]. Moreover, the intestinal barrier disruption observed in PD patients and in the PD rotenone model was significantly associated with higher pro-inflammatory cytokine profiles and T-cell infiltration in the colonic biopsy samples compared to healthy controls[21].

Very recently, a prominent role for TLR4 was demonstrated in the oral rotenone mouse model for PD. The intestinal PD phenotype was associated with increased expression of TLR4 in the intestinal mucosa of PD patients and mice undergoing rotenone-induced PD-like. Compared to rotenone-treated wild type animals, rotenone-treated Tlr4KO animals had less intestinal inflammation, intestinal and motor dysfunction, brain neuroinflammation and neurodegeneration[21]. These studies suggest that TLR4-mediated inflammation could play an important role in intestinal and/or brain inflammation leading to neurodegeneration and PD.

We hypothesize that the intestinal phenotype, such as the Tlr4 overexpression, disrupted intestinal barrier, inflammation, alpha-synuclein accumulation in ENS and intestinal transit deficits found in the oral rotenone murine model for PD are linked to an altered intestinal microbiota composition.

Bacteria in the intestinal tract are a primary source of proinflammatory products that in the case of dysbiosis can impact intestinal barrier function and immune homeostasis causing inflammation and oxidative stress and thereby initiating alpha-synuclein accumulation[22].

Alpha-synuclein pathology might then spread to the brain in a prion-like fashion[23,24]. Alternatively, gut-derived bacterial products or the peripheral inflammatory response might impact the brain through systemic mechanisms including disruption of the blood brain barrier as observed in PD patients[25].

Several studies linking intestinal microbiota to PD pathology have been performed in humans[13,14,26,27] and one in alpha-synuclein overexpressing mice where colonization with microbiota from PD patients was shown to exacerbate neuroinflammation and motor symptoms[28]. Here we report for the first time changes in the composition of the intestinal microbiota (cecal tissue and content) in a rotenone-induced mouse model for PD.

2. Methods

Animal housing

Seven week old C57BL/6J wild type (Jackson Laboratories) were housed under a 12h light/dark cycle. Food and water were provided ad libitum. Animal procedures were approved by the Ethical Committee of Animal Research of Utrecht University, Utrecht, The Netherlands.

Induction of mitochondrial dysfunction by rotenone in mice

Mice received freshly prepared rotenone (Sigma-Aldrich, The Netherlands) solution (10mg/kg body weight; suspended freshly in 4% carboxymethylcellulose (Sigma- Aldrich, The Netherlands) and 1.25% chloroform (vehicle) once a day for 28 days by oral gavage as described before[29]. Control animals received vehicle. On day 28, mice were sacrificed by decapitation and the brain and the intestinal tissue were collected for further analysis.

Immunohistochemistry and image analysis

Antibodies for ZO-1 (ab59720, abcam), glial fibrillary acidic protein (GFAP) (Z0334, Dako), CD3 (ab49943, abcam) and alpha-synuclein (04-1053, Millipore) were used to evaluate the gut pathology in the different experimental groups. Data collection for tight junction barrier integrity analyses (ZO-1 integrity) and stereology analyses were performed as described before[21]. The brains were stained with tyrosine hydroxylase (TH) (sc-14007, Santa Cruz Biotechnology) and iba-1 (019-197410, Wako) antibodies to assess the amount of dopaminergic neurons and microglia morphology in the SN. TH-immunopositive neurons were assessed using stereology counting as previously describe[21]. For microglia analysis z-stacks were imaged at 1µm step and analyzed with Image-J software. The experimenter designates individual cells and the software quantified the number of branches, the number of branches endpoints, the branch length, the cell body size and the total cell size. 20 cells per region per animal were analyzed.

Microbiota profiling and bioinformatics analyses.

Total genomic DNA extracted from mice cecum mucosa and cecum content samples (FastDNA bead-beating Spin Kit for Soil, MP Biomedicals, Solon, OH), was amplified using primers targeting the V4 variable region of the microbial 16S rRNA gene (Earth Microbiome Project primer set, adapted for the Illumina platform)[30], and these amplicons were sequenced on an Illumina MiSeq (2x151bp reads) at Argonne National Laboratory. Negative controls were used with each set of amplifications, which indicated no contamination. The raw sequence data (FASTQ files) were deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA), under the BioProject identifier PRJNA387564.

Forward and reverse reads were merged, quality trimmed and sequences longer than 250 bases were discarded (CLC Genomics Workbench, v7.0, CLC Bio, Qiagen, Boston, MA). Sequences were screened for chimeras (usearch61 algorithm)[31], and putative chimeric sequences were removed from the dataset (QIIME v1.8.)[32]. Each sample sequence set was rarefied to 25,000 sequences[33] and data were pooled, renamed, and clustered into operational taxonomic units (OTU) at 97% similarity (usearch61algorithm). Representative sequences from each OTU were extracted and classified using the uclust consensus taxonomy assigner (Greengenes 13_8 reference database). A biological observation matrix (BIOM)[34] was generated at each taxonomic level ("make OTU table" algorithm) and analyzed and visualized using Primer7[35].

Statistical analyses

Primer7 was used to calculate alpha diversity indices and to perform analysis of similarity (ANOSIM)calculations; ANOSIM was performed at the taxonomic level of family, using square-root transformed data. In SPSS (V.22), one and two factor analysis of variance (ANOVA), with Tukey's multiple comparison test, were used to analyze differences for parametric data satisfying test assumptions in alpha diversity indices.

Significant differences in the relative abundance of individual taxa between defined groups were detected using a Kruskal-Wallis test generating a Benjamini-Hochberg false-discovery rate (FDR) corrected p-value (FDR-P<0.05), implemented within the software package QIIME[32]. Taxa with an average abundance of <1% across the sample set were removed from the analysis. All graphs were created using GraphPad Prism (v5.00) software.

Microbial community functional predictions were performed using PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States)[36]. Differences in Kyoto Encyclopedia of Genes and Genomes (KEGG) ortholog (KO) abundances between groups were identified (Kruskal-Wallis test)[37]. KEGG pathways were analyzed using the KEGG Mapper pathway search function[38]. PICRUSt analysis significance was accepted at (FDR-P<0.05).

Pearson correlations were applied to associate the different symptoms developed with rotenone exposure in mice. Results were considered statistically significant when $p<0.05$. Analyses were performed using SPSS 22.0.

3. Results

Mucosal associated and luminal bacteria in a rotenone-induced mouse model for PD.
Oral rotenone causes cecal mucosal associated and luminal dysbiotic bacterial composition. The microbial diversity and OTU richness and evenness assessed with alpha diversity metrics on OTU data showed a significant increased richness in cecal mucosa of rotenone-treated mice compared to vehicle-treated mice (Fig 1 A). In contrast, rotenone treatment did not affect evenness, Shannon index (increases as diversity increases) and Simpson index (the higher the value the lower in diversity) (Fig 1 C, D & E). In cecal content no significant differences were found in microbial diversity and richness.

At the taxonomic level of family, the microbial community structures in cecal mucosa of rotenone-treated mice significantly differed from vehicle-treated mice (Fig 2A). Similar effects of rotenone treatment in mice were observed in cecal content microbial community structures (Fig 2B).

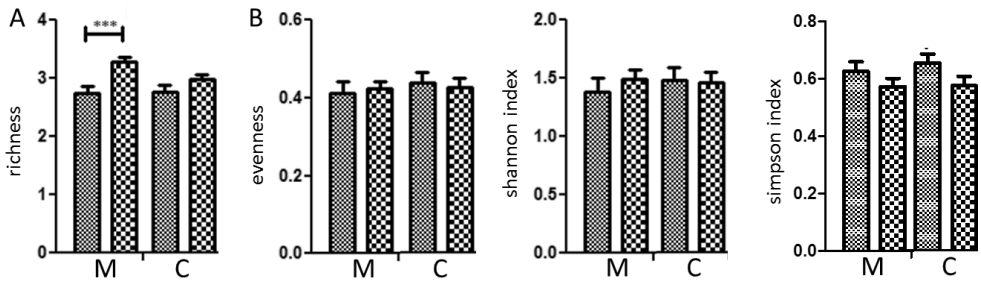


Figure 1: Alpha diversity (within samples) comparisons between vehicle and rotenone treatments in mice in cecal mucosa and content at the taxonomic level of family. A - richness; B – Evenness; C - Shannon index; D – Simpson index (dot pattern= vehicle treated mice; square pattern= rotenone treated mice). 2 Way ANOVA, Bonferroni test for n= 9-10 per group; ***P<0.001.

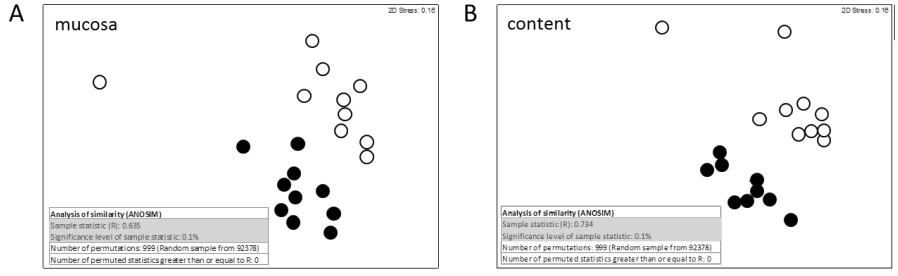


Figure 2: Ordination plots (nMDS plots) of microbial community structure at the taxonomic level of family using analysis of similarity ANOSIM comparing rotenone and vehicle treatment associated with cecal mucosa (A) and content (B), n=9-10 mice per group.

The relative abundance of 3 phyla, 7 families and 5 genera was significantly different between vehicle and rotenone treatment in cecal mucosa (Fig 3 and Table 1).

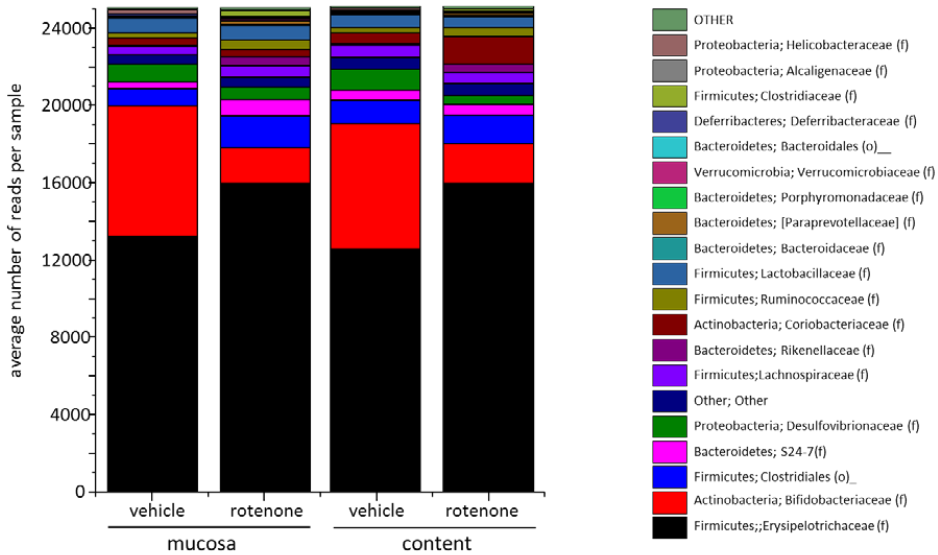


Figure 3. Stacked column plots showing average microbial community composition at the taxonomic level of family.

	cecal mucosa			cecal content		
	vehcile	rotenone	veh vs ROT FDR-*P*	vehcile	rotenone	veh vs ROT FDR-*P*
Phylum						
Actinobacteria	7109,20	2226,00	**0,00**	6987,90	3505,20	**0,00**
Bacteroidetes	540,50	1606,70	**0,01**	810,90	1221,40	0,29
Firmicutes	15572,20	19782,60	**0,00**	15334,90	19102,00	**0,00**
Proteobacteria	1181,80	629,00	0,76	1204,60	452,20	0,32
Unassigned;Other	490,60	533,40	0,76	614,60	637,40	0,76
Family						
Actinobacteria; Bifidobacteriaceae	6731,80	1835,20	**0,00**	6453,00	2069,60	**0,01**
Actinobacteria; Coriobacteriaceae	392,00	383,60	0,68	545,70	1456,20	0,82
Bacteroidetes; Rikenellaceae	63,10	476,10	**0,00**	87,40	423,60	**0,01**
Bacteroidetes; S24-7	356,90	860,70	**0,01**	533,20	578,70	0,48
Firmicutes; Clostridiales (o)	892,60	1663,40	**0,01**	1182,40	1453,90	0,18
Firmicutes; Erysipelotrichaceae	13190,20	15885,00	**0,01**	12496,90	15862,30	*0,06*
Firmicutes; Lachnospiraceae	423,20	589,10	*0,06*	617,10	536,00	0,48
Firmicutes; Lactobacillaceae	790,80	737,20	0,88	654,30	583,20	0,82
Firmicutes; Ruminococcaceae	222,70	508,30	**0,01**	296,50	435,20	0,18
Proteobacteria; Desulfovibrionaceae	922,40	612,40	0,88	1089,90	444,70	0,56
Unassigned;Other	489,40	526,40	0,88	609,40	633,00	0,82
Genus						
Actinobacteria; Bifidobacteriaceae; Bifidobacterium	6734,70	1830,30	**0,00**	6448,60	2074,60	**0,01**
Actinobacteria; Coriobacteriaceae; g__	280,90	288,60	0,97	395,60	1321,50	0,62
Bacteroidetes; Rikenellaceae;g__	59,70	469,20	**0,00**	85,20	418,30	**0,01**
Bacteroidetes; S24-7;g__	354,10	854,40	**0,02**	536,20	588,10	0,51
Firmicutes; Clostridiales (o);f__;g__	891,70	1661,90	**0,02**	1186,80	1468,70	0,24
Firmicutes; Erysipelotrichaceae; Allobaculum	13166,10	15878,10	**0,02**	12476,80	15843,60	*0,06*
Firmicutes; Lachnospiraceae; g__	290,40	312,00	0,13	397,00	286,30	0,51
Firmicutes; Lactobacillaceae; Lactobacillus	784,70	734,10	0,97	659,50	583,10	0,78
Proteobacteria; Desulfovibrionaceae; Desulfovibrio	913,10	567,70	0,97	1070,50	421,30	0,62
Unassigned;Other;Other	489,30	534,50	0,97	613,60	633,10	0,82

Table 1. Rotenone-induced changes in taxa in cecal mucosa and content associated bacteria of mice. Group-significant testing was performed using Kruskal-Wallis test plus false discovery rate correction (FDR-*P*), n=9-10 mice per group.

Rotenone treatment induced a significant higher abundance of the phylum Bacteroidetes and Firmicutes and a lower relative abundance of Actinobacteria compared to vehicle treatment in cecal mucosa (table 1). Whereas in the cecal content rotenone induced only a significant higher relative abundance of the phylum Firmicutes and a lower abundance of Actinobacteria (table 1). A significant lower Firmicutes-to-Bacteroidetes ratio was observed in cecal mucosa of rotenone-treated mice ($p<0.001$, Tukey's multiple comparison test) compared to vehicle treatment (Fig 4). For the cecal content, bacterial communities showed no significant differences of this ratio (Fig 4).

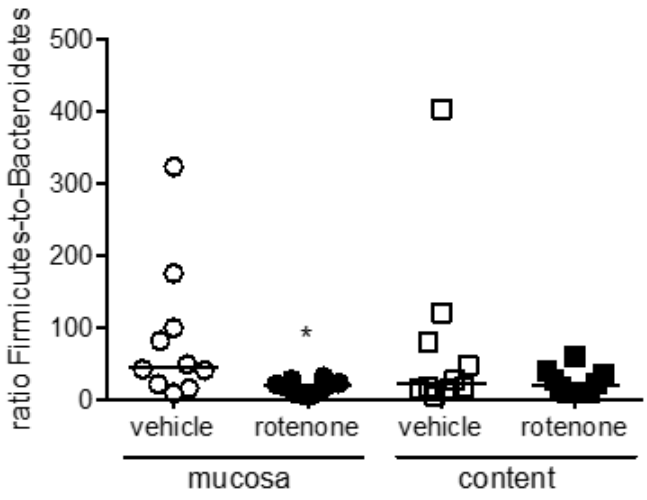


Figure 4. Firmicutes to Bacteriodetes ratio in the colonic mucosa and content associated bacteria. Data are expressed as median and analyzed with two-way ANOVA - Tukey's multiple comparison test, * P=0.05; **P=0.01, n=9-10 per group.

Rotenone treatment resulted in a significant higher relative abundance at the family or genus taxa level of mucosa-associated bacteria Rikenellaceae, and *S24-7* (Bacteroidetes; class Bacteroidia; genus unspecified), Ruminoccaceae, Lachnospiraceae (trend p=0.06) and an unassigned family (Firmicutes; class Clostridia; genus unspecified), and *Allobaculum* (Firmicutes; class Erysipelotrichi; family Erysipelotrichaceae) (table 1 & Fig 5). Furthermore, rotenone treatment resulted in lower relative abundance of the *Bifidobacterium* (family Actinobacteria; class Actinobacteria; family Bifidobacteriaceae) in cecal mucosa (Fig 5).

The effects of rotenone on cecal bacterial content at the family or genus levels in mice were trending similar, but less pronounced compared to mucosa-associated bacteria (Fig 5 and table 1).

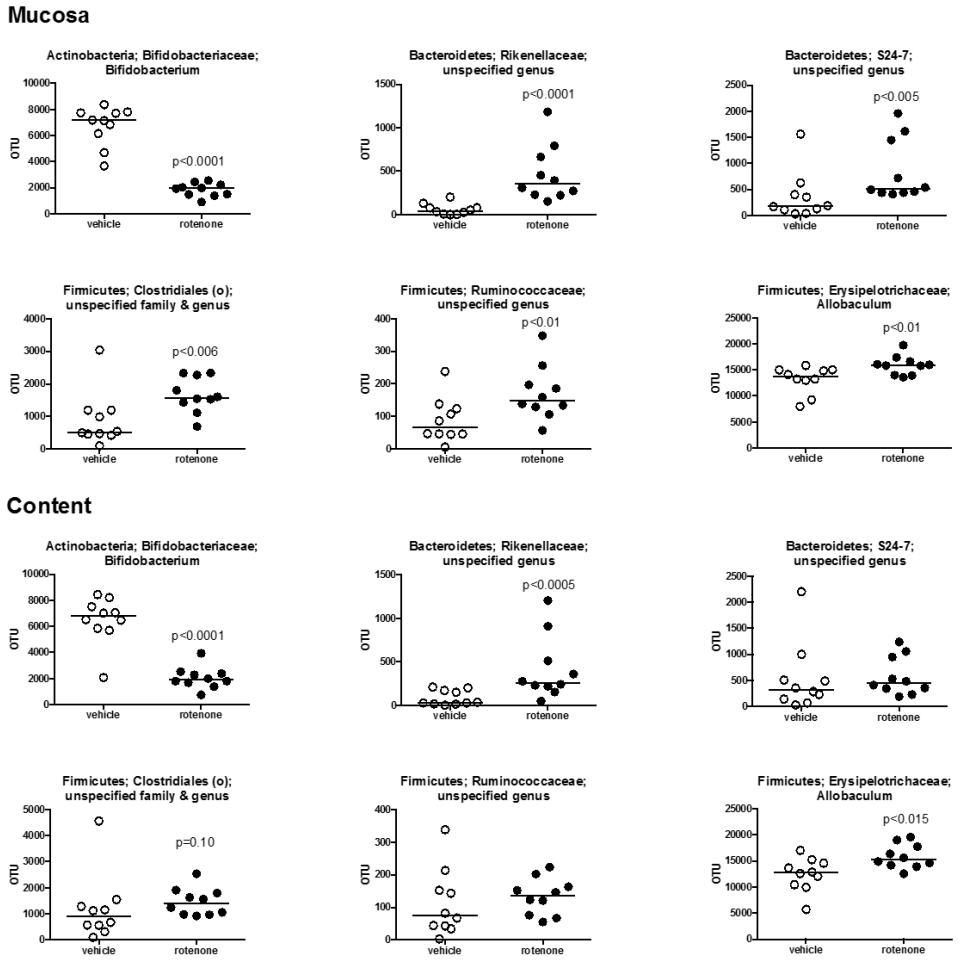


Figure 5. Effect of rotenone on OUTs of cecal mucosa-associated and content bacteria in mice. Mice were orally treated with rotenone (closed dots) or vehicle (open dots). Data are expressed as median and analyzed with Mann Whitney test for n=9-10 per group.

Evidence to support the microbiota-gut-brain axis involvement in Rotenone-induced PD mice.

In an effort to understand how multiple variables are similar or dissimilar from each correlation analysis were performed at the taxa level of family. While correlation does not equate to causation these relationships allow us to infer potential relationships. First, correlations were assessed to investigate the association between cecal bacterial content and intestinal barrier integrity, inflammation and alpha-synuclein accumulation in the ENS.

The intestinal epithelial barrier integrity score, assessed by ZO-1 expression, significantly and positively correlated with Bifidobacteriaceae (phylum Actinobacteria)*;* Rikenellaceae and S24-7 (phylum Bacteroidetes); unassigned family of the order Clostridiales and Ruminoccaceae (class Clostridia, phylum Firmicutes) and Erysipelotrichaceae (class Eripsipelotrichi, phylum Firmicutes; trend) in the cecal mucosa (table 2). Cecal content correlations were similar except for S24-7 and unassigned family of the order Clostridiales where no significant correlation with ZO-expression was found. The number of CD3+ T-cells in colon, marker of intestinal inflammation/immune activation, correlated with the same bacteria families in cecal mucosa and content as for the epithelial barrier integrity score, except for Erysipelotrichaceae in both cecal mucosa and content. In addition, Ruminoccaceae in cecal content did not correlate at all with CD3+ T-cells. The alpha-synuclein accumulation in the colonic plexi correlated with the same bacteria families in cecal mucosa and content as found for the intestinal epithelial barrier integrity score, with the exception of Ruminoccaceae in cecal content (table 2).

Next, the correlation analysis were performed to investigate the association between cecal bacteria and neuroinflammation and dopaminergic cells loss in the substantia nigra (SN). Microglial activation in the SN was only positively associated with Rikenellaceae in cecal mucosa and content (table 2). On the other hand, the dopaminergic cell number in the SN, assessed by the number of TH+ cells, significantly inversely correlated with Rikenellaceae, Erysipelotrichaceae, and Ruminoccaceae and S24-7 (only for mucosal associated bacteria), and positively correlated with Bifidobacteriaceae.

				mucosa					content				
Phylum	Class	Order	Familiy	ZO-1 in colon	CD3+ cells in colon	α-synuclein in colon	microglial activation SN	TH+ cells SN	ZO-1 in colon	CD3+ cells in colon	α-synuclein in colon	microglial activation SN	TH+ cells SN
Actinobacteria	Actinobacteria	Bifidobacteriales	Bifidobacteriaceae	r=0.79 p<0.0001	r=-0.77 p<0.0001	r=-0.77 p<0.0001	r=-0.22 ns	r=0.80 p<0.0001	r=0.72 p=0.0003	r=-0.74 p=0.0002	r=-0.73 p=0.0003	r=-0.22 ns	r=0.78 p<0.0001
Bacteriodetes	Bacteroidia	Bacteroidales	Rikenellaceae	r=-0.67 p=0.0011	r=0.77 p<0.0001	r=0.68 p=0.0010	r=0.54 p=0.0885	r=-0.68 p=0.001	r=-0.54 p=0.0142	r=0.56 p=0.0097	r=0.52 p=0.0176	r=0.57 p=0.0689	r=-0.62 p=0.0038
			S24-7	r=-0.47 p=0.0346	r=0.63 p=0.0032	r=0.38 p=0.0962	r=0.08 ns	r=-0.38 p=0.0960	r=-0.10 ns	r=0.20 ns	r=-0.01 ns	r=-0.09 ns	r=-0.06 ns
Firmicutes	Clostridia	Clostridiales	-	r=-0.51 p=0.0217	r=0.40 p=0.0782	r=0.54 p=0.0134	r=-0.06 ns	r=-0.30 ns	r=-0.15 ns	r=0.14 ns	r=0.23 ns	r=-0.17 ns	r=-0.19 ns
			Lachnospiraceae	r=-0.25 ns	r=0.19 ns	r=0.17 ns	r=-0.13 ns	r=0.01 ns	r=0.08 ns	r=-0.02 ns	r=-0.04 ns	r=-0.43 ns	r=-0.00 ns
			Ruminoccaceae	r=-0.72 p=0.0003	r=0.67 p=0.0012	r=0.64 p=0.021	r=-0.01 ns	r=-0.41 p=0.0691	r=-0.46 p=0.0430	r=0.32 ns	r=0.35 ns	r=-0.11 ns	r=-0.17 ns
	Erysipelotrichi	Erysipelotrichales	Erysipelotrichaceae	r=-0.40 p=0.0822	r=0.35 ns	r=0.42 p=0.0614	r=0.22 ns	r=-0.63 p=0.0030	r=-0.46 p=0.0395	r=0.36 ns	r=0.52 p=0.0180	r=0.47 ns	r=-0.46 p=0.0437

Table 2. Correlations between intestinal barrier dysfunction (ZO-1 scoring data); colonic T-cell infiltration (CD3+ cells in colon); accumulation of alpha-synuclein in enteric plexi of colon; microglial activation and dopaminergic cells loss (loss of TH+cells) in substantia nigra (SN) with those bacterial family associated with cecal mucosa or found in the cecal content that were changed by rotenone treatment expressed as Pearson correlation coefficients.

Functional prediction of microbiota associated metabolic pathways

Predictive assessment of the microbial community functional potential (PICRUSt) was used to infer functional differences in the microbiota of rotenone treated versus control mice. PICRUSt is a computational tool that allows, using 16S rRNA amplicon data, to predict the genes that are present, to calculate their abundance, assign them to metabolic pathways using KEGG and then test the difference between rotenone and vehicle treated mice. In cecal mucosa associated bacteria most KEGG pathways had more genes in the rotenone treated mice. Among all the tested metabolic pathways, twelve were significantly upregulated and nine showed a trend when comparing rotenone-treated mice with controls. In particular, rotenone induced a significant enhancement of the following pathways: glycan biosynthesis and metabolism; metabolism of terpenoids and polyketides; xenobiotics biodegradation and metabolism; metabolism of other amino acids; biosynthesis of other secondary metabolites amino acid metabolism lipid metabolism (table 3A). Only three metabolic pathways were found to be significantly downregulated in the cecal mucosa-associated bacteria after rotenone treatment in mice: xenobiotics biodegradation and metabolism and metabolism of cofactors and vitamins (table 3A). For the cecal content associated bacteria, KEGG pathways had less genes in the rotenone treated mice. Eight metabolic pathways were significantly downregulated and four pathways showed a trend when comparing rotenone-treated mice with controls (table 3B). In particular, rotenone induced a significant reduction of the following pathways: xenobiotics biodegradation and

metabolism; metabolism of cofactors and vitamins; carbohydrate metabolism; amino acid metabolism and fatty acid metabolism (table 3B). No significantly different KEGG pathways with greater abundance in rotenone relative to vehicle treated mice were found.

Table 3A. List of different KEGG pathways with greater abundance in rotenone relative to vehicle treated WT mice & greater abundance in vehicle relative to rotenone treated WT mice, as inferred using PICRUSt analysis of ceacal mucosal microbiomes

Pathway	Cecum Mucosa[b]	FDR-P[a]	Abundance Mean, rotenone WT	Abundance Mean, vehicle WT	rotenone/vehicle Ratio
gbm	Glycosphingolipid biosynthesis - ganglio series	0.05	4956	1516	3.27
gbm	Glycosaminoglycan degradation	0.04	7945	2477	3.21
mtp	Biosynthesis of siderophore group nonribosomal peptides	0.04	1180	483	2.44
xbm	Atrazine degradation	0.07	2976	1592	1.87
xbm	Caprolactam degradation	0.10	3436	2054	1.67
xbm	Styrene degradation	0.09	2322	1413	1.64
moa	beta-Alanine metabolism	0.05	29818	19531	1.53
	Sporulation	0.09	81254	54120	1.50
xbm	Aminobenzoate degradation	0.05	25969	17778	1.46
bsm	Butirosin and neomycin biosynthesis	0.05	13650	9429	1.45
aam	Lysine degradation	0.05	38598	26753	1.44
lm	Lipid metabolism	0.05	18865	13190	1.43
bsm	Biosynthesis and biodegradation of secondary metabolites	0.05	13581	9641	1.41
aam	Amino acid metabolism	0.05	51344	37920	1.35
aam	Tryptophan metabolism	0.08	42834	32187	1.33
mt	Phosphotransferase system (PTS)	0.08	336956	254162	1.33
xbm	Bisphenol degradation	0.05	22504	17193	1.31
mcv	Porphyrin and chlorophyll metabolism	0.08	120779	92810	1.30
moa	Cyanoamino acid metabolism	0.08	50406	38980	1.29
lm	Glycerolipid metabolism	0.05	98758	78235	1.26
xbm	Nitrotoluene degradation	0.07	15314	12352	1.24

	Pathway Cecum Mucosa[b]	FDR-P[a]	Abundance Mean, vehicle WT	Abundance Mean, rotenone WT	vehicle/rotenone Ratio
xbm	Chlorocyclohexane and chlorobenzene degradation	0.01	6763	2847	2.38
mcv	Retinol metabolism	0.01	8011	3648	2.20
xbm	Metabolism of xenobiotics by cytochrome P450	0.02	7823	3664	2.13

FDR-P[a] Value: P < 0.05, significant different; P ≤ 0.10, trend [a]FDR-P Value: P < 0.05, significant different; P ≤ 0.10, trend Metabolism:

[b]Metabolism: xbm: xenobiotics biodegradation and metabolism; mcv: metabolism of cofactors and vitamins; aam: amino acid metabolism; lm: lipid metabolism; gbm: glycanbiosynthesis and metabolism; mtp: metabolism of terpenoids and polyketides; moa: metabolism of other amino acids; bsm: Biosynthesis of other secondary metabolites; Environmental Information Processing; membrane transport (mt)

Table 3B. List of different KEGG pathways with greater abundance in vehicle relative to rotenone treated WT mice, as inferred using PICRUSt analysis of cecum content microbiomes

Pathway Cecum Content[b]		FDR-P[a]	Abundance Mean, vehicle WT	Abundance Mean, rotenone WT	vehicle/rotenone Ratio
xbm	Chlorocyclohexane and chlorobenzene degradation	0.01	8078	3191	2.53
mcv	Retinol metabolism	0.01	9561	3781	2.53
xbm	Metabolism of xenobiotics by cytochrome P450	0.01	9450	3768	2.51
xbm	Xylene degradation	0.02	15011	7748	1.94
xbm	Dioxin degradation	0.02	15092	7883	1.91
xbm	Naphthalene degradation	0.02	30669	17127	1.79
mcv	Lipoic acid metabolism	0.04	6852	4283	1.60
xbm	Toluene degradation	0.10	16909	10616	1.59
cm	C5-Branched dibasic acid metabolism	0.10	57449	36995	1.55
aam	Valine, leucine and isoleucine biosynthesis	0.08	152653	112703	1.35
xbm	Chloroalkane and chloroalkene degradation	0.04	60944	45352	1.34
fam	Fatty acid metabolism	0.06	73741	57431	1.28

[a]FDR-P Value: P < 0.05, significant different; P ≤ 0.10, trend

[b]xbm: xenobiotics biodegradation and metabolism; mcv: metabolism of cofactors and vitamins; cm: carbohydrate metabolism; aam: amino acid metabolism; fam: fatty acid metabolism

Note: no significantly different KEGG pathways with greater abundance in rotenone relative to vehicle treated mice were found.

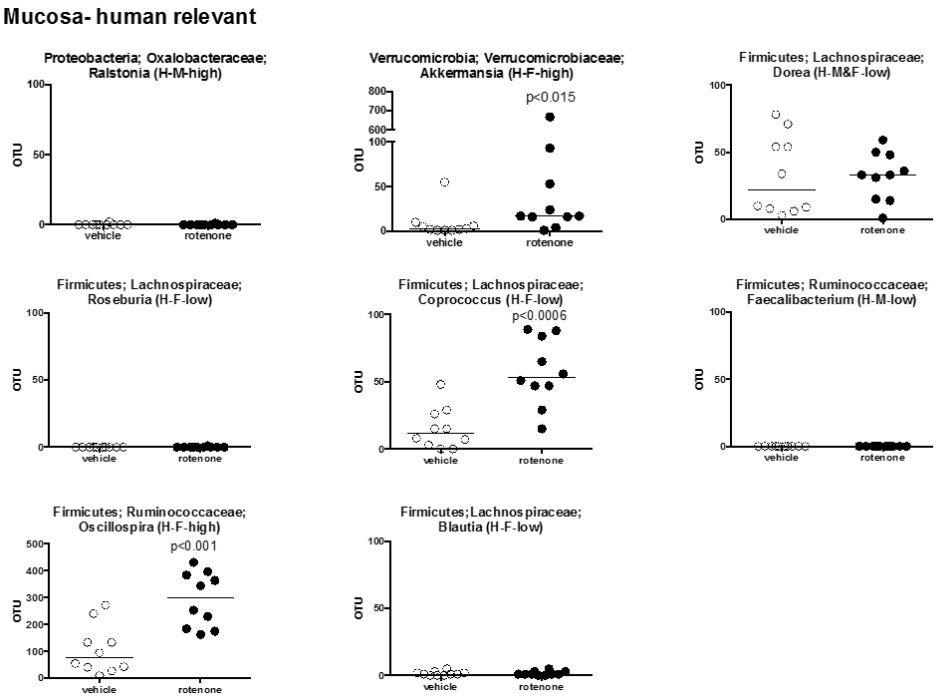


Figure 6. Effect of rotenone on OUTs of cecal mucosa-associated bacteria in mice demonstrated in PD patients to be affected. Mice were orally treated with rotenone (closed dots) or vehicle (open dots). Data are expressed as median and analyzed with Mann Whitney test for n=9-10 per group.

4. Discussion

This is the first study investigating changes in microbiota composition and possibly associated metabolic pathways in a mouse model for PD. Our group has previously shown that oral exposure to rotenone induced intestinal dysfunction and inflammation in the colon[29]. Here, we showed that oral rotenone exposure resulted in significant differences at all taxonomic levels in cecal microbiota composition. In contrast to the human colonic mucosal associated and luminal microbiota composition[26], the murine microbiota composition of cecal mucosa-associated and cecal content were more or less similar regardless of the treatment. All in all, the effects of rotenone were more pronounced on the mucosa-associated microbiota composition. This can be explained by the direct epithelium damaging effects of rotenone leading to a disruption of the microbiota-host interaction.

Rotenone-induced dysbiosis can (further) disrupt the epithelial integrity, leading to gut leakiness, innate immune activation and possibly systemic inflammation[39–41]. Enhanced exposure to more pro-inflammatory bacteria and their products, originated from the cecum and possibly the colon, might activate mucosal immune cells that in turn can modulate the local and remote (upper intestinal tract and even other organs) immune responses[42]. Pro-inflammatory bacteria and/or bacterial endotoxins, such as LPS, might induce inflammation, immune activation and the associated oxidative stress locally in the intestinal tract, but possibly also remotely in the brain. Oxidative stress could initiate alpha-synuclein pathology in the ENS[43] that could spread in a prion-like fashion through connected neurons to the SN[23,24,44,45]. Moreover, gut-derived bacterial products or the peripheral inflammatory response could impact the brain through systemic mechanisms.

Significant correlations between rotenone treatment associated microbial changes (at the family taxonomic level), intestinal inflammation and enteric alpha-synuclein pathology were found in this study supporting the importance of the microbiota in local immune responses. Neuroinflammation and dopaminergic cells loss in the SN also correlated with several bacterial families. This suggests that the CNS response in the mouse model for PD might be regulated by specificbacterial communities. Recent studies in mice overexpressing alpha-synuclein fully support the importance of the microbiota in PD symptoms development. Germ-free or antibiotic treated alpha-synuclein overexpressing mice were protected against neuroinflammation and motor dysfunction[28].

In order to translate the data obtained in the mouse model to the human situation, fecal and mucosal microbiota data of PD patients and healthy controls measured at the same institution, using identical sequencing platform, as the presented mice data were used for comparison[26]. It should be noted that in mice the differences observed when comparing cecal content and cecal mucosa associated microbiota composition found in humans was not evident. At the phylum level a significant lower Firmicutes to Bacteriodetes ratio was found in the cecal mucosa of rotenone treated mice compared to vehicle. A similar finding was

observed in fecal samples of PD patients when compared to healthy individuals[26]. In addition, both in human as well as in the mouse model for PD a significant decrease in Actinobacteria was found. At the taxa of family and genus, rotenone treated mice exhibited significant lower relative abundance of *Bifidobaterium* which was not observed in PD patients. PD patients showed lower abundances of the butyrate producers *Blautia*, *Roseburia* and *Coprococcus* in fecal samples. In the mouse PD model, *Blautia* and *Roseburia* were barely detectable and the relative abundance of *Coprococcus* was significantly enhanced.

It has been postulated that the lower abundance of butyrate-producing bacteria in PD patients feces could be harmful for the intestinal epithelial integrity and immune function[26,46,47]. Short chain fatty acids including butyrate have been shown to have beneficial effects on the host by keeping the intestinal PH low, improving epithelial barrier function and by having anti-microbial and anti-inflammatory/immunomodulatory effects[48–50]. *Bifidobacterium* has been shown to interact with butyrate-producing bacteria because of its ability to produce acetate and lactate during carbohydrate fermentation, organic acids that in turn can be converted into butyrate by other colonic bacteria through cross-feeding interactions[51,52]. Thus, our observed lower abundance of *Bifidobacterium* in rotenone-treated mice could lead to low level of butyrate in the colon. Moreover, reductions in *Bifidobacteria* abundancies are associated with intestinal inflammation in inflammatory bowel diseases and irritable bowel syndrome[53,54]. The reduced abundance of this beneficial bacterium in the presented mouse model for PD could lead to aberrant short chain fatty acid production, resulting in barrier disruption, intestinal (neuro)inflammation and the associated intestinal dysfunction as well as alpha-synuclein accumulation in ENS.

Besides the low abundance of *Bifidobacterium*, enhancement of proinflammatory intestinal bacteria may also promote inflammation and immune activation locally in the intestinal tract as well as remotely in the brain. Indeed, relative abundance of several putative pro-inflammatory bacteria were increased in rotenone-treated mice. In the rotenone-induced PD mouse model significant increased levels of the following familes/orders/genera were found: Rikenellaceae (unspecified genus); Clostridiales (unspecified family, genus); Erysipelotrichaceae (*Allobaculum*) Verrucommicrobiaceae (*Akkermansia*), and Ruminococcaceae (*Oscillospira*). Mice fed with a high fat diet developed an increased abundance of Rikenellaceae and colonic inflammation[55]. This high fat diet-induced increase of Rikenellaceae might exacerbate inflammation via the TLR4 signaling pathway . Erysipelotrichaceae (*Allobaculum*) has been shown to be positively correlated with intestinal inflammation and leaky gut, induced by high cholesterol diet in rats[61]. In addition, western type diet induced overweight, leaky gut and intestinal inflammation is also associated with high levels of *Allobaculum*[62]. Taken together, the enhanced abundance of Erysipelotrichaceae in the rotenone-induced PD model might be involved in the intestinal

inflammatory response. However, in the human PD study no differences within this family were found[26].

Rotenone treatment resulted in higher abundance of unclassified Clostridiales in cecal mucosa. Clostridiales species have been shown to suppress the growth of Bacteroides and to prevent intestinal inflammation[56,57]. Therefore, the enhanced abundance of Clostridiales in the rotenone PD mouse model can be regarded as a secondary response to the chemically induced inflammation. A comparable enhancement of Clostridiaceae was found in feces of PD patients[26]. Furthermore, both in PD[26] as well as in the rotenone PD model an increased abundance of Verrucommicrobiaceae (*Akkermansia)* and Ruminococcaceae (*Oscillospira*) were found. A recent study has shown that *Akkermansia* has a protective role in metabolic disease/obesity and inflammation[58]. Similarly, high abundance of *Oscillospira* has been associated with leanness[59] and PD patients often report weight loss[60].

The relative abundance of the family Lachnospiraceae was enhanced (trend) in the mucosa of rotenone-treated mice. Lachnospireaceae are associated with obesity and diabetes[63]. Furthermore, Lachnospiraceae was the only family whose increase in the mucosa-associated bacteria was also reflected in the lumen of villin-TLR4 mice compared with that of WT littermate mice[64]. This is of interest since increased epithelial TLR4 expression is observed in intestinal inflammation[65].

In conclusion, the mouse model for PD induced by the pesticide rotenone is associated with enhanced abundance of (human relevant) pro-inflammatory intestinal bacteria at the expense of beneficial commensal bacteria in cecal mucosa and cecal content. This study only investigated the changes in the microbiota composition in cecum of rotenone and vehicle treated mice. The intestinal phenotype in this model includes reduced transit time and disturbed stomach emptying more associated with the small intestine[29]. In addition, in PD patients small intestinal bacterial overgrowth has been described[12,66]. Therefore, future studies should characterize the microbiota in other parts of the intestinal tract in PD patients as well as in rodent models for PD.

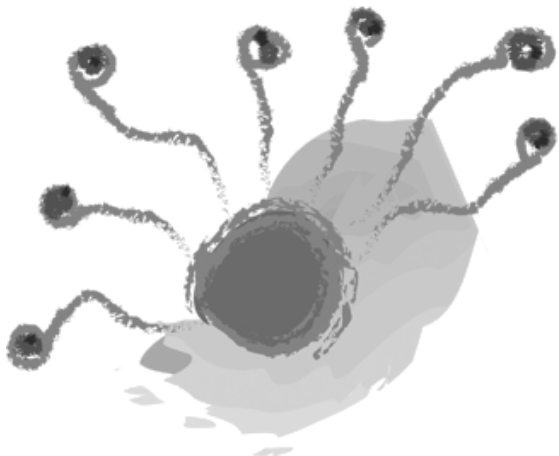
References

1. de Rijk, M. C. *et al.* Prevalence of Parkinson's disease in Europe: A collaborative study of population-based cohorts. Neurologic Diseases in the Elderly Research Group. *Neurology* **54,** S21-23 (2000).
2. Nussbaum, R. L. & Ellis, C. E. Alzheimer's disease and Parkinson's disease. *N. Engl. J. Med.* **348,** 1356–1364 (2003).
3. Crossman, A. R. Neural mechanisms in disorders of movement. *Comp. Biochem. Physiol. A* **93,** 141–149 (1989).
4. Pfeiffer, R. F. Gastrointestinal dysfunction in Parkinson's disease. *Parkinsonism Relat. Disord.* **17,** 10–15 (2011).
5. Fasano, A., Visanji, N. P., Liu, L. W. C., Lang, A. E. & Pfeiffer, R. F. Gastrointestinal dysfunction in Parkinson's disease. *Lancet Neurol.* **14,** 625–639 (2015).
6. Abbott, R. D. *et al.* Frequency of bowel movements and the future risk of Parkinson's disease. *Neurology* **57,** 456–462 (2001).
7. Adams-Carr, K. L. *et al.* Constipation preceding Parkinson's disease: a systematic review and meta-analysis. *J. Neurol. Neurosurg. Psychiatry* **87,** 710–716 (2016).
8. Martinez-Martin, P. The importance of non-motor disturbances to quality of life in Parkinson's disease. *J. Neurol. Sci.* **310,** 12–16 (2011).
9. Müller, B., Assmus, J., Herlofson, K., Larsen, J. P. & Tysnes, O.-B. Importance of motor vs. non-motor symptoms for health-related quality of life in early Parkinson's disease. *Parkinsonism Relat. Disord.* **19,** 1027–1032 (2013).
10. Chaudhuri, K. R. & Schapira, A. H. V. Non-motor symptoms of Parkinson's disease: dopaminergic pathophysiology and treatment. *Lancet Neurol.* **8,** 464–474 (2009).
11. Devos, D. *et al.* Colonic inflammation in Parkinson's disease. *Neurobiol. Dis.* **50,** 42–48 (2013).
12. Tan, A. H. *et al.* Small intestinal bacterial overgrowth in Parkinson's disease. *Parkinsonism Relat. Disord.* **20,** 535–540 (2014).
13. Scheperjans, F. *et al.* Gut microbiota are related to Parkinson's disease and clinical phenotype. *Mov. Disord. Off. J. Mov. Disord. Soc.* **30,** 350–358 (2015).
14. Unger, M. M. *et al.* Short chain fatty acids and gut microbiota differ between patients with Parkinson's disease and age-matched controls. *Parkinsonism Relat. Disord.* doi:10.1016/j.parkreldis.2016.08.019
15. Shannon, K. M., Keshavarzian, A., Dodiya, H. B., Jakate, S. & Kordower, J. H. Is alpha-synuclein in the colon a biomarker for premotor Parkinson's disease? Evidence from 3 cases. *Mov. Disord. Off. J. Mov. Disord. Soc.* **27,** 716–719 (2012).
16. Hilton, D. *et al.* Accumulation of α-synuclein in the bowel of patients in the pre-clinical phase of Parkinson's disease. *Acta Neuropathol. (Berl.)* **127,** 235–241 (2014).
17. Stokholm, M. G., Danielsen, E. H., Hamilton-Dutoit, S. J. & Borghammer, P. Pathological α-synuclein in gastrointestinal tissues from prodromal Parkinson disease patients. *Ann. Neurol.* **79,** 940–949 (2016).
18. Kelly, L. P. *et al.* Progression of intestinal permeability changes and alpha-synuclein expression in a mouse model of Parkinson's disease. *Mov. Disord. Off. J. Mov. Disord. Soc.* **29,** 999–1009 (2014).
19. Perez-Pardo, P. *et al.* Gut-brain and brain-gut axis in Parkinson's disease models: effects of a uridine and fish oil diet. *Nutr. Neurosci.* (2017). doi:10.1080/1028415X.2017.1294555
20. Forsyth, C. B. *et al.* Increased intestinal permeability correlates with sigmoid mucosa alpha-synuclein staining and endotoxin exposure markers in early Parkinson's disease. *PloS One* **6,** e28032 (2011).
21. Paula Perez-Pardo & Hemraj B. Dodiya. The role of TLR4 in the gut-brain axis in Parkinson's disease: a translational study from men to mice. *Submitted*
22. Hawkes, C. H., Del Tredici, K. & Braak, H. A timeline for Parkinson's disease. *Parkinsonism Relat. Disord.* **16,** 79–84 (2010).
23. Hawkes, C. H., Del Tredici, K. & Braak, H. Parkinson's disease: the dual hit theory revisited. *Ann. N. Y. Acad. Sci.* **1170,** 615–622 (2009).
24. Braak, H. & Del Tredici, K. Invited Article: Nervous system pathology in sporadic Parkinson disease. *Neurology* **70,** 1916–1925 (2008).
25. Guan, J. *et al.* Vascular degeneration in Parkinson's disease. *Brain Pathol. Zurich Switz.* **23,** 154–164 (2013).
26. Keshavarzian, A. *et al.* Colonic bacterial composition in Parkinson's disease. *Mov. Disord. Off. J. Mov. Disord. Soc.* **30,** 1351–1360 (2015).
27. Hasegawa, S. *et al.* Intestinal Dysbiosis and Lowered Serum Lipopolysaccharide-Binding Protein in Parkinson's Disease. *PloS One* **10,** e0142164 (2015).
28. Sampson, T. R. *et al.* Gut Microbiota Regulate Motor Deficits and Neuroinflammation in a Model of Parkinson's Disease. *Cell* **167,** 1469–1480.e12 (2016).
29. Perez-Pardo, P. *et al.* Gut–brain and brain–gut axis in Parkinson's disease models: Effects of a uridine and fish oil diet. *Nutr. Neurosci.* 1–12 (2017). doi:10.1080/1028415X.2017.1294555

30. Caporaso, J. G. *et al.* Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *ISME J.* **6,** 1621–1624 (2012).
31. Edgar, R. C. Search and clustering orders of magnitude faster than BLAST. *Bioinforma. Oxf. Engl.* **26,** 2460–2461 (2010).
32. Caporaso, J. G. *et al.* QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods* **7,** 335–336 (2010).
33. Gihring, T. M., Green, S. J. & Schadt, C. W. Massively parallel rRNA gene sequencing exacerbates the potential for biased community diversity comparisons due to variable library sizes. *Environ. Microbiol.* **14,** 285–290 (2012).
34. McDonald, D. *et al.* The Biological Observation Matrix (BIOM) format or: how I learned to stop worrying and love the ome-ome. *GigaScience* **1,** 7 (2012).
35. CLARKE, K. R. Non-parametric multivariate analyses of changes in community structure. *Aust. J. Ecol.* **18,** 117–143 (1993).
36. Langille, M. G. I. *et al.* Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. *Nat. Biotechnol.* **31,** 814–821 (2013).
37. Kanehisa, M. & Goto, S. KEGG: kyoto encyclopedia of genes and genomes. *Nucleic Acids Res.* **28,** 27–30 (2000).
38. Kanehisa, M., Goto, S., Sato, Y., Furumichi, M. & Tanabe, M. KEGG for integration and interpretation of large-scale molecular data sets. *Nucleic Acids Res.* **40,** D109-114 (2012).
39. Ivanov, I. I. & Honda, K. Intestinal commensal microbes as immune modulators. *Cell Host Microbe* **12,** 496–508 (2012).
40. Nishio, J. & Honda, K. Immunoregulation by the gut microbiota. *Cell. Mol. Life Sci. CMLS* **69,** 3635–3650 (2012).
41. Caricilli, A. M., Castoldi, A. & Câmara, N. O. S. Intestinal barrier: A gentlemen's agreement between microbiota and immunity. *World J. Gastrointest. Pathophysiol.* **5,** 18–32 (2014).
42. Lathrop, S. K. *et al.* Peripheral education of the immune system by colonic commensal microbiota. *Nature* **478,** 250–254 (2011).
43. Shults, C. W. Lewy bodies. *Proc. Natl. Acad. Sci. U. S. A.* **103,** 1661–1668 (2006).
44. Desplats, P. *et al.* Inclusion formation and neuronal cell death through neuron-to-neuron transmission of alpha-synuclein. *Proc. Natl. Acad. Sci. U. S. A.* **106,** 13010–13015 (2009).
45. Holmqvist, S. *et al.* Direct evidence of Parkinson pathology spread from the gastrointestinal tract to the brain in rats. *Acta Neuropathol. (Berl.)* **128,** 805–820 (2014).
46. Hamer, H. M. *et al.* Review article: the role of butyrate on colonic function. *Aliment. Pharmacol. Ther.* **27,** 104–119 (2008).
47. Canani, R. B. *et al.* Potential beneficial effects of butyrate in intestinal and extraintestinal diseases. *World J. Gastroenterol.* **17,** 1519–1528 (2011).
48. Ganapathy, V., Thangaraju, M., Prasad, P. D., Martin, P. M. & Singh, N. Transporters and receptors for short-chain fatty acids as the molecular link between colonic bacteria and the host. *Curr. Opin. Pharmacol.* **13,** 869–874 (2013).
49. Singh, N. *et al.* Activation of Gpr109a, receptor for niacin and the commensal metabolite butyrate, suppresses colonic inflammation and carcinogenesis. *Immunity* **40,** 128–139 (2014).
50. Maslowski, K. M. & Mackay, C. R. Diet, gut microbiota and immune responses. *Nat. Immunol.* **12,** 5–9 (2011).
51. De Vuyst, L. & Leroy, F. Cross-feeding between bifidobacteria and butyrate-producing colon bacteria explains bifdobacterial competitiveness, butyrate production, and gas production. *Int. J. Food Microbiol.* **149,** 73–80 (2011).
52. Rivière, A., Gagnon, M., Weckx, S., Roy, D. & De Vuyst, L. Mutual Cross-Feeding Interactions between Bifidobacterium longum subsp. longum NCC2705 and Eubacterium rectale ATCC 33656 Explain the Bifidogenic and Butyrogenic Effects of Arabinoxylan Oligosaccharides. *Appl. Environ. Microbiol.* **81,** 7767–7781 (2015).
53. Di Gioia, D., Aloisio, I., Mazzola, G. & Biavati, B. Bifidobacteria: their impact on gut microbiota composition and their applications as probiotics in infants. *Appl. Microbiol. Biotechnol.* **98,** 563–577 (2014).
54. Grimm, V., Westermann, C. & Riedel, C. U. Bifidobacteria-host interactions--an update on colonisation factors. *BioMed Res. Int.* **2014,** 960826 (2014).
55. Kim, K.-A., Gu, W., Lee, I.-A., Joh, E.-H. & Kim, D.-H. High fat diet-induced gut microbiota exacerbates inflammation and obesity in mice via the TLR4 signaling pathway. *PloS One* **7,** e47713 (2012).
56. Hakansson, A. & Molin, G. Gut microbiota and inflammation. *Nutrients* **3,** 637–682 (2011).
57. Harris, N. L. Intimate gut interactions: helminths and the microbiota. *Cell Res.* **26,** 861–862 (2016).
58. Schneeberger, M. *et al.* Akkermansia muciniphila inversely correlates with the onset of inflammation, altered adipose tissue metabolism and metabolic disorders during obesity in mice. *Sci. Rep.* **5,** 16643 (2015).

59. Konikoff, T. & Gophna, U. Oscillospira: a Central, Enigmatic Component of the Human Gut Microbiota. *Trends Microbiol.* **24,** 523–524 (2016).
60. Abbott, R. A., Cox, M., Markus, H. & Tomkins, A. Diet, body size and micronutrient status in Parkinson's disease. *Eur. J. Clin. Nutr.* **46,** 879–884 (1992).
61. Lee, S.-M., Han, H. W. & Yim, S. Y. Beneficial effects of soy milk and fiber on high cholesterol diet-induced alteration of gut microbiota and inflammatory gene expression in rats. *Food Funct.* **6,** 492–500 (2015).
62. Boudry, G. *et al.* Bovine milk oligosaccharides decrease gut permeability and improve inflammation and microbial dysbiosis in diet-induced obese mice. *J. Dairy Sci.* **100,** 2471–2481 (2017).
63. Kameyama, K. & Itoh, K. Intestinal colonization by a Lachnospiraceae bacterium contributes to the development of diabetes in obese mice. *Microbes Environ.* **29,** 427–430 (2014).
64. Dheer, R. *et al.* Intestinal Epithelial Toll-Like Receptor 4 Signaling Affects Epithelial Function and Colonic Microbiota and Promotes a Risk for Transmissible Colitis. *Infect. Immun.* **84,** 798–810 (2016).
65. Ye, J. *et al.* Bacteria and bacterial rRNA genes associated with the development of colitis in IL-10(-/-) mice. *Inflamm. Bowel Dis.* **14,** 1041–1050 (2008).
66. Gabrielli, M. *et al.* Prevalence of small intestinal bacterial overgrowth in Parkinson's disease. *Mov. Disord. Off. J. Mov. Disord. Soc.* **26,** 889–892 (2011).

CHAPTER 6



Promising effects of neurorestorative diets on motor, cognitive, and gastrointestinal dysfunction after symptom development in a rotenone model of Parkinson's disease.

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Abstract

Parkinson's disease (PD) is characterized by the progressive degeneration of dopaminergic nigrostriatal neurons, with reductions in the function and amount of dopaminergic synapses. Therefore, synapse loss and membrane-related pathology provide relevant targets for interventions in PD. We previously showed the beneficial preventive effects of a dietary intervention containing uridine and DHA, two precursors for membrane synthesis, in the intrastriatal rotenone model for PD. Here, we examined the therapeutic potential of the same dietary intervention on motor, cognitive and gastrointestinal symptoms. In addition, we tested the effects of an extended nutritional formula based on the same precursors plus other nutrients that increase membrane phospholipid synthesis as well as prebiotic fibers. C57BL/6J mice received a unilateral rotenone injection in the striatum. Dietary interventions started 28 days after surgery, when motor-symptoms had developed. Readout parameters included behavioral tasks measuring motor function and spatial memory as well as intestinal function and histological examination of brain and gut to assess PD-like pathology. Our results show that rotenone-induced motor and non-motor problems were partially alleviated by the therapeutic dietary interventions providing uridine and DHA. The extended nutritional intervention containing both precursors and other nutrients that increase phospholipid synthesis as well as prebiotic fibers was more effective in normalizing rotenone-induced motor and non-motor abnormalities. The latter diet also restored striatal DAT levels, indicating its neurorestorative properties. This is the first study demonstrating beneficial effects of specific dietary interventions, given after full development of symptoms, on a broad spectrum of motor and non-motor symptoms in a mouse model for PD.

1. Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disease after Alzheimer's disease (AD)[1,2]. The clinical picture is dominated by motor impairments due to a progressive degeneration of dopaminergic nigrostriatal neurons, with reductions in striatal dopamine levels, dopaminergic synapses, and the density of dendritic spines on striatal medium spiny neurons[3,4]. PD patients also develop non-motor symptoms, including cognitive impairment[5] and gastrointestinal (GI) dysfunctions[6,7]. These non-motor symptoms are major determinants of quality of life[8–10] and remain undertreated[11].
The most commonly used drug in the treatment of PD is L-Dopa that compensates for dopaminergic cell loss by enhancing dopamine synthesis in the remaining terminals. This therapy has several side effects[12], it does not prevent dopaminergic neuron degeneration, and has no effects on non-motor symptoms[13]. Considering that some of the non-responsive symptoms like GI dysfunctions may contribute to L-Dopa response fluctuations[14], there is a clear need to develop additional therapies for PD treatment.

Oral administration of two circulating phosphatide precursors, uridine and docosahexaenoic acid (DHA), increases dopaminergic neurotransmission, synaptic membrane formation, as well as the density of dendritic spines[15–18]. Indeed, preventive treatment with precursors uridine and DHA reduced rotational behavior in the unilateral 6-OHDA rat model for PD[19]. We have also shown beneficial preventive effects of a dietary intervention containing uridine and DHA in the intrastriatal rotenone mouse model for PD. Intrastriatal injection of rotenone caused several motor and non-motor symptoms associated with PD. The preventive dietary intervention was not only effective for the motor symptoms but also for the GI phenotype[20].

In the present study we examine the therapeutic potential of the same dietary intervention in the intrastriatal rotenone mouse model of PD given after the development of full motor symptoms, i.e. 4 weeks after rotenone injection, to elucidate if the diet has neurorestorative properties. We also investigate the therapeutic potential of an extended diet containing the same dietary precursors (uridine and DHA) plus additional nutrients that increase membrane phospholipid synthesis and prebiotic fibers. The additional nutrients displayed synergistic effects with the precursors in enhancing synapse formation and function, in counteracting loss of functional connectivity in neurodegeneration, and in improving behavior and cognitive functions[21]. In mouse models of Alzheimer-like pathology, the extended diet showed higher efficacy than interventions with single nutrients or incomplete formultions[22,23]. Prebiotic fibers have been shown to have beneficial effects on immune function[24–26], bowel motility and constipation[27–29] relevant for inflammation and GI-related symptoms in PD.

2. Methods

Mice

Seven week-old C57BL/6J mice (Charles River, The Netherlands) were housed at room temperature under 12h light/dark cycle. Food and water was provided *ad libitum.* All animal procedures were approved by the Ethical Committee of Animal Research of Utrecht University, The Netherlands.

Surgery

Mice underwent stereotaxic surgery under isoflurane anesthesia: a hole was drilled in the skull, a cannula inserted in the right striatum and 5.4 µg of freshly prepared rotenone (dissolved in 2µl DMSO) was infused. The following stereotaxic coordinates were used: AP +0.4, ML -2.0 (from bregma) and DV -3.3 below dura. Sham-treated animals were injected with vehicle. Ten weeks after surgery the mice were euthanized by decapitation.

Diets

Mice were fed either the Control diet, Diet1, or Diet2, starting 28 days after surgery when motor symptoms leveled out and continuing for the duration of the experiment. Animals were divided into six groups (n=10) , as specified in Table 1. Iso-caloric diets were produced by Research Diet Services (Wijk bij Duurstede, The Netherlands) and were based on the Control diet, i.e. the standard animal food for laboratory rodents AIN-93M[30] with 5% fat. For Diet1, UMP as a source of uridine (0.51 g/100g diet) was added and part of the lipid blend of control diet was replaced by fish oil, providing DHA (0.74 g/100g diet) and EPA (0.29 g/100g diet).

Diet2 provided the phospholipid precursors from Diet1, but also choline, phospholipids, selenium, folic acid, and vitamins B6, B12, C, D, and E. The fish oil in Diet2 provided DHA (0.75 g/100g diet) and EPA (0.50 g/100g diet). In addition, the cellulose fibers from Control diet and Diet1 were replaced by prebiotic fibers (1.5 g/100g diet GOS, 0.17 g/100g diet lcFOS, 1.67 g/100g diet scFOS, and 1.67 g/100g diet nutriose) for Diet2. Further specifications of the diets are listed in Table 2.

Experimental groups		Nutritional intervention		
		Control diet	**Diet1**	**Diet2**
Surgery	**Sham**	1. Sham surgery + Control diet throughout the experiment	2. Sham surgery + Diet1 starting 4 weeks after surgery	3. Sham surgery + Diet2 starting 4 weeks after surgery
	Rotenone	4. Rotenone injection + Control diet throughout the experiment	5. Rotenone injection + Diet1 starting 4 weeks after surgery	6. Rotenone injection + Diet2 starting 4 weeks after surgery

Table 1: Specification of the 6 experimental groups.

Experimental diets	Control [g/100g]	Diet1 [g/100g]	Diet2 [g/100g]
Proteins	14.0	14.0	14.0
Carbohydrates	71.1	70.6	68.7
Fats	5.0	5.0	5.0
Soy oil	1.9		
Coconut oil	0.9	0.1	0.2
Corn oil	2.2	1.7	1.2
Fish oil		3.2	3.6
providing DHA		0.74	0.75
providing EPA		0.29	0.50
Mineral mix (AIN-93M-MX)	3.5	3.5	3.5
Vitamin mix (AIN-93-VX)	1.0	1.0	1.0
Fibers	5.0	5.0	5.0
Cellulose	5.0	5.0	
GOS			1.50
lcFOS			0.17
scFOS			1.67
Nutriose			1.67
Additions			
L-cystine	0.18	0.18	0.18
Choline bitartrate	0.25	0.25	0.25
Tert-butylhydroquinone	0.0008	0.0008	0.0008
Uridine		0.51	0.51
Choline chloride			0.40
Soy lecithin			0.75
Sodium selenite			0.00023
Pyridoxine			0.0041
Folic acid			0.00067
Cyanocobalamin			0.058
Ascorbic acid			0.16
dl-α-Tocopheryl acetate (500 IU/g)			0.47
Cholecalciferol (400.000 IU/g)			0.00031

Table 2: Compositions of the experimental diets

Motor symptoms assessment

The motor function of each mouse was assessed by the Rotarod test as described before[31]. Briefly, mice were placed on an accelerating rod with speeds starting with 2rpm and gradually increasing to 20rpm. Time to first fall was recorded for a maximum of 300s. The test was performed at baseline and after every 5 days until day 70, in order to look at symptom development in time and functional recovery during dietary intervention.

Muscle strength of the four limbs was measured on day 70 after surgery using the inverted screen test as described before[32]. Briefly, the mouse was placed in the center of a wire mesh screen, which was subsequently rotated to an inverted position. Latency to fall was measured in seconds.

Muscular forelimb strength was also measured on day 70 with a grip strength tester as described before[33].

Spatial recognition test

Animals' ability to react to a spatial novelty after a 3-min delay was measured every 14 days after surgery and for the duration of the experiment, to assess model- and diet-induced changes over time. As described before[34], mice were individually submitted to seven consecutive, 6-min sessions. During session 1, mice were placed into the empty open field. During sessions 2-4, five objects were present, and mice were placed into the apparatus to habituate to the objects configuration (habituation phase). During the 3-min intersession interval, the animals were returned to a waiting cage. During the spatial test session (session 5), the objects configuration was changed by moving two objects (displaced objects, DO) and leaving the other three objects in the same position (non-displaced objects, NDO). In all sessions, the total activity of the animal was measured. From sessions 2 to 5, object exploration was evaluated on the basis of the mean time spent by the animal in contact with the different objects. The animals' ability to selectively react to the spatial change was analyzed by calculating the spatial re-exploration index (DO[S5] – DO[S4] = DO and NDO[S5] – NDO[S4] = NDO). The time the animals interact with the DO minus the time they interact with the NDO is used for analysis (DO-NDO). Data are expressed as percentage with respect to baseline (day 14).

Intestinal transit and colon length

Intestinal transit was assessed in all animals. Thirty minutes before sacrificing the mice, a 2.5% Evans blue solution in 1.5% methylcellulose (0.3mL per animal) was intragastrically administered. After euthanasia, intestinal transit was measured as the distance from the pylorus to the most distal point of migration of the Evans blue dye. In addition, the length of the colon was measured.

Tissue preparation and immunohistochemistry

Coronal slices of 40 µm were sectioned using a cryostat (CM3050, Leica Microsystems). Sections were incubated with 0.3% H_2O_2 for 30 min. Following blocking serum, sections were incubated overnight with rabbit anti-tyrosine hydroxylase (TH) (Santa-Cruz Biotechnology) 1:1000 or with rat anti-dopamine transporter (DAT) 1:1000. Next day, sections were incubated with the appropriate biotinylated secondary antibody (Jackson ImmunoResearch) 1:200 for 2h. The avidin-biotin method was used to amplify the signal (ABC Kit, Vector) and 3,3'-diaminobenzidine tetrachloride (DAB) was used as the chromogen.

The colon of the animals were embedded in paraffin. 15 µm sections were incubated with 0.3% H_2O_2 for 30 min, rehydrated and incubated with citrate buffer. Following serum block, sections were incubated overnight with the primary antibodies (rabbit anti-alpha-synuclein (1:1000, millipore), rabbit anti-GFAP (1:1000, Dako) or rabbit anti-CD3 (1:1000, abcam)).
For alpha-synuclein and GFAP, slides were incubated with a fluorescent secondary antibody: Alexa®488 donkey anti-rabbit and mounted using Vectashield® mounting medium for fluorescence with DAPI (Vectro Laboratories). For CD3 staining, a biotinylated secondary antibody (1:200, Dako) was used. The avidin-biotin method was used to amplify the signal and DAB was used as chromogen. Sections were counterstained with Mayer's haematoxylin (Merck Millipore).

Image analysis

For immunostained sections, digital images were captured with an Olympus BX50 microscope equipped with a Leica DFC 320 digital camera. To count TH-immunopositive cells, every fourth section of each mouse brain was stained. TH-immunopositive neurons were quantified stereologically on regular spaced sections. To measure DAT and alpha-synuclein expression the optical density in the area of interest was measured and corrected for non-specific background. Stereology was performed to quantify the number of CD3 positive cells in the colon on regular spaced sections. Analysis were performed by researchers that were blind to the treatment condition of the animal. Immunofluorescence images were made using a Keyence BZ-9000 microscope. The Corrected Total Fluorescence (CTF) was calculated with the formula: integrated density – (area × mean fluorescence of background reading).

Statistical analysis

Experimental results are expressed as mean ± SEM. Differences between groups were statistically analyzed with a two-way ANOVA [analyzing significant effects of the treatments (rotenone vs vehicle), diets (control diet, Diet1 and Diet2) and interactions (between diets and treatment)] followed by a Tukey's multiple comparison test. For the rotarod test and spatial memory test, data were analyzed with a general linear model repeated measure ANOVA with the within subject factor time and the between subject factors treatment and

diet. Results were considered statistically significant when $p<0.05$. Analyses were performed using SPSS 22.0.

3. Results

Dietary interventions have a restorative effect on rotenone-induced motor symptoms.
To investigate whether dietary intake affected rotenone-induced motor dysfunction, the rotarod test was performed. The time spent on the rod was used for analysis of motor function. There was an overall effect of treatment (rotenone vs vehicle) ($F(1,44)=492.62$, $p<0.0001$) and of diet ($F(2,44)=3.60$, $p<0.05$) on rotarod performance. Repeated measures showed an effect of time ($F(13,572)=50.58$, $p<0.0001$). Rotenone-injected mice developed motor dysfunction over time compared to sham (interaction effect treatment x time $F(13,572)=59.25$ $p<0.0001$). Furthermore, there was an interaction effect between diet and time ($F(26,572)=2.16$, $p<0.01$), between diet and treatment ($F(2,44)=3.55$, $p<0.05$) and between diet, treatment and time ($F(26,572)=2.15$, $p<0.01$). Rotenone-injected mice showed a decrease in rotarod performance starting on day 25 after surgery compared to the sham-operated mice ($F(1,54)=226.41$, $p<0.0001$). On day 55 after surgery there was a significant effect of the diets ($F(2,53)=3.30$, $p<0.05$) on rotarod performance that remained for the duration of the experiment ($F(2,54)=8.87$, $p<0.0001$ day 70). Post-hoc analysis showed that rotenone-treated animals on Diet1 performed better on the rotarod compared to rotenone-treated animals on control diet starting from day 65 ($p<0.05$). Rotenone-treated animals on Diet2 remained longer on the rod compared to rotenone treated animals on control diet starting from day 55 ($p<0.01$). Moreover, Diet2 was significantly more effective in normalizing rotenone-induced motor-dysfunction compared to Diet1 (Fig 1A).

Muscle strength was measured with the inverted screen test 70 days after surgery, where the latency to fall was used for analysis. Significant differences were observed in the latency to fall of rotenone-injected mice compared to sham ($F(1,53)=417.5$, $p<0.0001$). There was an overall effect of diet ($F(2,53)=17.41$, $p<0.0001$) and a diet by treatment interaction ($F(2,53)=13.10$, $p<0.0001$). Post-hoc analysis revealed an increase in the latency to fall in the test in rotenone-treated mice on Diet1 ($p<0.01$) and Diet2 ($p<0.0001$) compared to rotenone-treated mice on Control diet. The extended nutritional intervention (Diet2) was more effective than Diet1 in reducing rotenone-induced muscle strength loss ($p<0.01$) (Fig 1B).

Similar results were found in the forelimb grip strength test, rotenone-treated animals showed a decrease in their forelimb grip strength compared to sham ($F(1,53)=130.1$, $p<0.0001$) 70 days after surgery. The diet had an overall effect on grip strength ($F(2,53)=14.7$, $p<0.0001$) and there was a diet by treatment interaction ($F(2,53)=9.3$, $p<0.001$). More specifically, rotenone-injected mice fed the Diet1 and the Diet2 had more strength in the forelimbs compared to rotenone-injected mice on Control diet ($p<0.01$ and

p<0.0001 respectively). Diet2 was again more effective than Diet1 in reducing rotenone-induced forelimb grip strength loss (p<0.05) (Fig 1C).

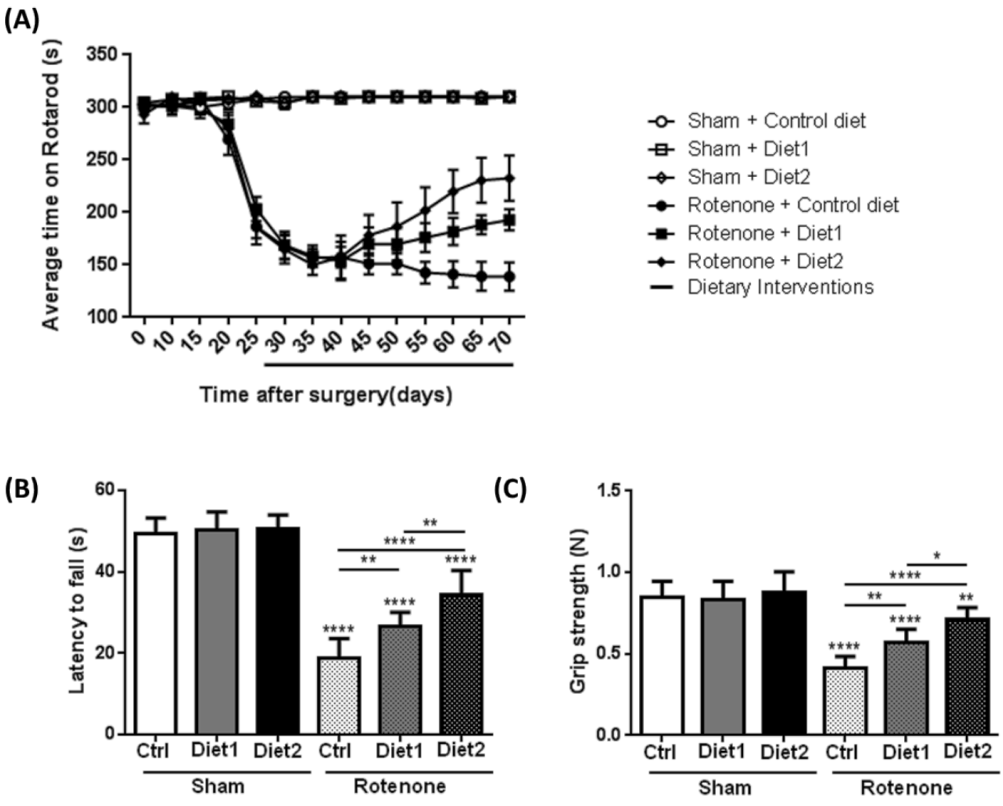


Figure 1: Effects of the therapeutic dietary interventions on: (A) rotarod performance, (B) inverted screen test, and (C) forelimb grip strength test. Unilateral rotenone injection induced motor dysfunction and grip strength loss. Dietary interventions had beneficial effects on motor function and grip strength. The extended nutritional intervention (Diet2) was more effective than Diet1 for all the tests. Data are shown as mean ± SEM. *p<0.05, **p<0.01, ****p<0.0001. (n=10 per group).

Dietary interventions have no effects on rotenone-induced dopaminergic cell loss but Diet2 has restorative effects on dopamine transporter expression.

To investigate the motor impairments associated neurodegeneration, we performed unbiased stereology to estimate the number of TH positive dopaminergic cells in the SN. 70 Days after surgery rotenone-treated mice showed a decrease in the number of TH positive cells compared to vehicle-injected mice ($F(1,30)=76.17$, $p<0.001$). No differences were found in the total number of TH positive cells in the SN between the two brain hemispheres. There was no overall effect of diet ($F(2,30)=0.57$, $p=0.57$) and no significant interaction between treatment and diet ($F(2,30)=0.90$, $p=0.42$) (Fig 2A).

Dopamine transporter (DAT) expression was measured in the striatum by measuring the optical density after DAT immunostaining. Rotenone caused a decrease in DAT expression in the striatum ($F(1,36)=26.49$, $p<0.0001$) 70 days after surgery. Unilateral-injection of rotenone caused a bilateral reduction of the DAT expression in the striatum; no differences were found in DAT immunoreactivity between the two brain hemispheres. There was an overall effect of the diets on DAT expression ($F(2,36)=3.44$, $p<0.05$) (Fig 2B). Diet2 increased DAT expression as compared to Control diet in rotenone-treated mice, in the absence of a significant treatment by diet interaction.

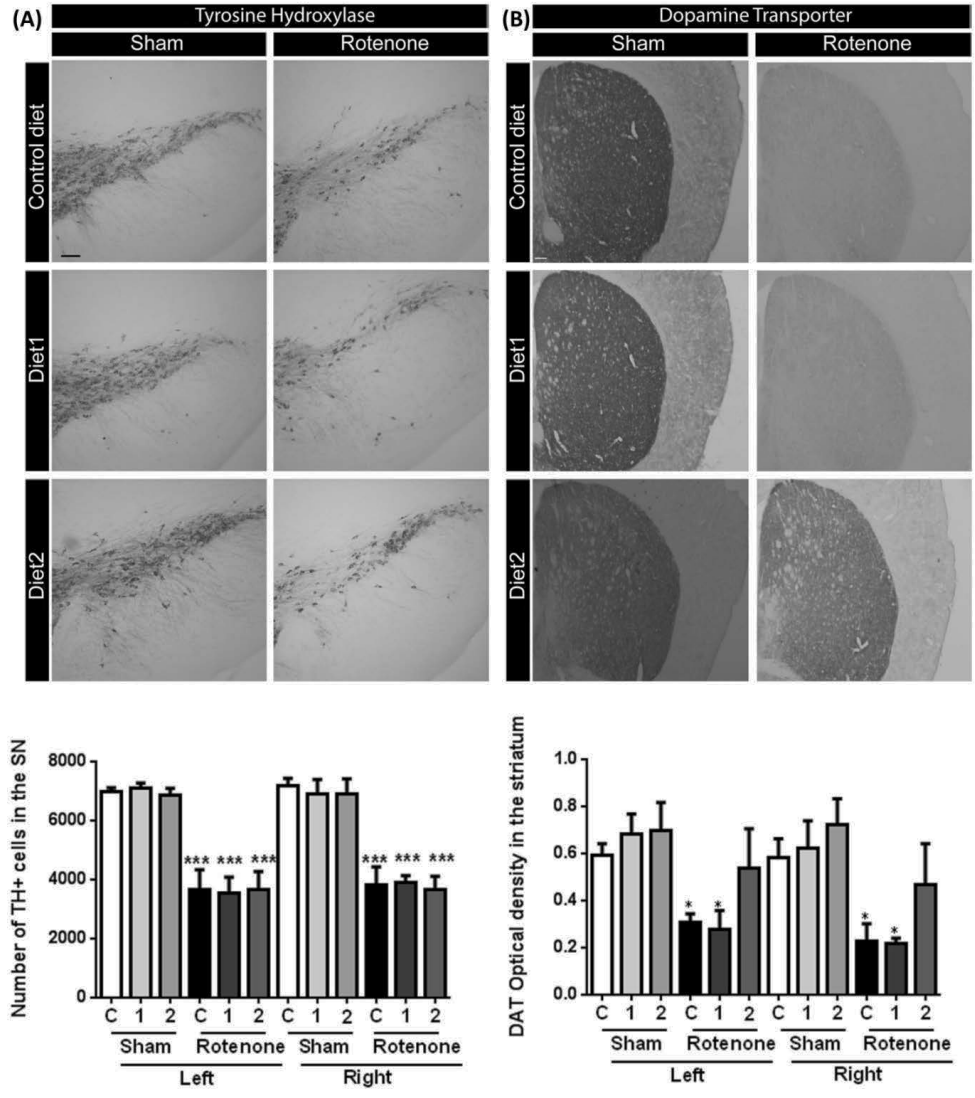


Figure 2: Effects of therapeutic dietary interventions on: (A) the number of dopaminergic cells indicated by the number of tyrosine hydroxylase (TH) immunoreactive cells in the substantia nigra, (B) dopamine transporter (DAT) expression in the striatum. Rotenone injection decreased the number of dopaminergic cells in the substantia nigra and the level of DAT expression in the striatum. Dietary interventions had no effect on the number of TH positive cells but Diet2 increased DAT expression. Data are shown as mean ± SEM. *p<0.05, ***p<0.01. (Scale bar: 200µm applies to all panels). (n=6 for TH immunostaining and n=4 for DAT immunostaining).

Diet 2 has restorative effects on rotenone-induced spatial recognition impairment.
Sham-operated animals selectively re-explored the displace object (DO) as compared to the non-displaced object (NDO) during the whole experiment, demonstrating that they were able to selectively react to the spatial change. Rotenone treatment negatively affected the rodents' ability to react to a spatial novelty ($F(1,54)=4.701$, $p<0.05$). Repeated measures showed an effect of time ($F(4,216)=11.06$, $p<0.0001$) and an effect of rotenone over time ($F(4,216)=5.82$, $p<0.0001$). Spatial recognition was affected by rotenone from day 42 after surgery onwards ($F(1,54)=7.10$, $p<0.05$) and by the diet on day 56 after surgery (day 28 after dietary interventions started) ($F(2,54)=3.52$, $p<0.05$). Post-hoc comparisons showed no differences in spatial discrimination abilities between rotenone-treated mice on Control diet and on Diet1. However, rotenone-injected mice on Diet2 had a better spatial recognition than rotenone-injected animals on Control diet ($p<0.05$) on day 70 (Fig 3). No significant differences were found in the total activity in the open field between experimental groups.

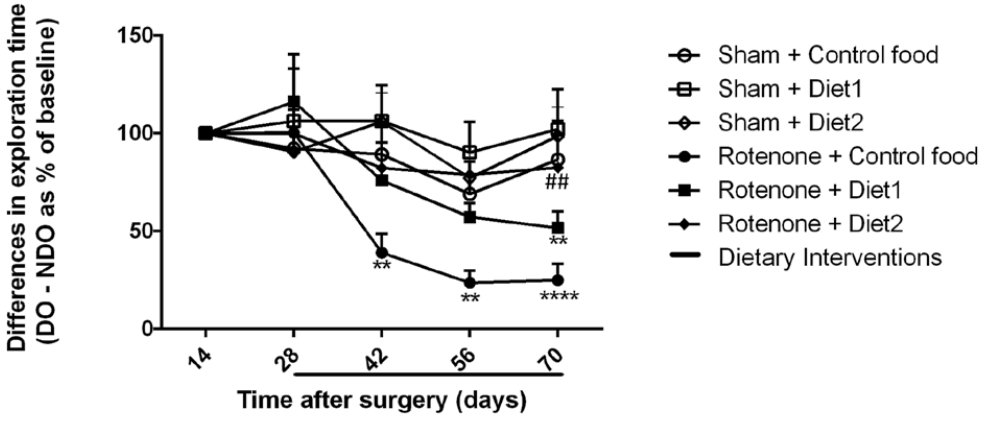


Figure 3: Effects of therapeutic dietary interventions on spatial object recognition test. Sham-operated animals selectively re-explored the displace object (DO) as compared to the non-displaced object (NDO) throughout the experiment. Rotenone decreased animals' ability to react to a spatial novelty from day 42 after surgery onwards. On day 70 after surgery, rotenone-injected animals on Diet2 showed better spatial discrimination abilities compared to rotenone-injected animals on control diet. Data are shown as mean ± SEM. **p<0.01, ****p<0.0001 compared to Sham + Control diet. ## p<0.01 compared to Rotenone + Control diet (n=10 per group).

Diet 2 has a restorative effect on rotenone-induced gastrointestinal dysfunction.
Rotenone injection in the striatum negatively affected intestinal transit, i.e. decreased the distance travelled by the Evans Blue dye in the GI-tract, as compared to vehicle-injected mice ($F(1,51)=18.81$, $p<0.0001$). There was an overall effect of diet on intestinal transit ($F(2,51)=4.19$, $p<0.05$) and an interaction effect between treatment and diet ($F(2,51)=3.33$, $p<0.05$). The beneficial effect of Diet2 on intestinal transit after rotenone exposure was more pronounced ($p<0.001$) (Fig 4A).

Dietary interventions have a restorative effect on rotenone-induced alpha-synuclein accumulation in the colon.
Rotenone administration in the striatum increased alpha-synuclein accumulation in the enteric plexus of the colon compared to vehicle exposed mice ($F(1,51)=72.14$, $p<0.0001$). There was an effect of diet on alpha-synuclein accumulation in the colon ($F(2,51)=3.56$, $p<0.05$). Post-hoc analysis revealed a reduction in alpha-synuclein levels for rotenone-injected mice on Diet1 and Diet2 compared to rotenone-injected mice on Control diet (both $p<0.05$). No differences were found in alpha-synuclein levels for rotenone-animals on Diet1 compared to rotenone-treated animals on Diet2 (Fig 4B).

Dietary interventions have a restorative effect on rotenone-induced inflammation in the gut.
To measure reactive enteric glial cells in the enteric nervous system (ENS) of the colon, GFAP (glial fibrillary acidic protein) expression was quantified after immunostaining. Rotenone exposure in the brain increased GFAP expression in the colon ($F(1,54)=23.94$, $p<0.0001$). There was also an effect of diet ($F(2,54)=6.36$, $p<0.01$) and an interaction effect between diet and treatment ($F(2,54)=7.83$, $p<0.01$). Post-hoc analysis showed that GFAP expression was normalized in rotenone-injected mice on Diet1 or Diet2 compared to rotenone-treated animals on Control diet (both $p<0.001$) (Fig 4C).

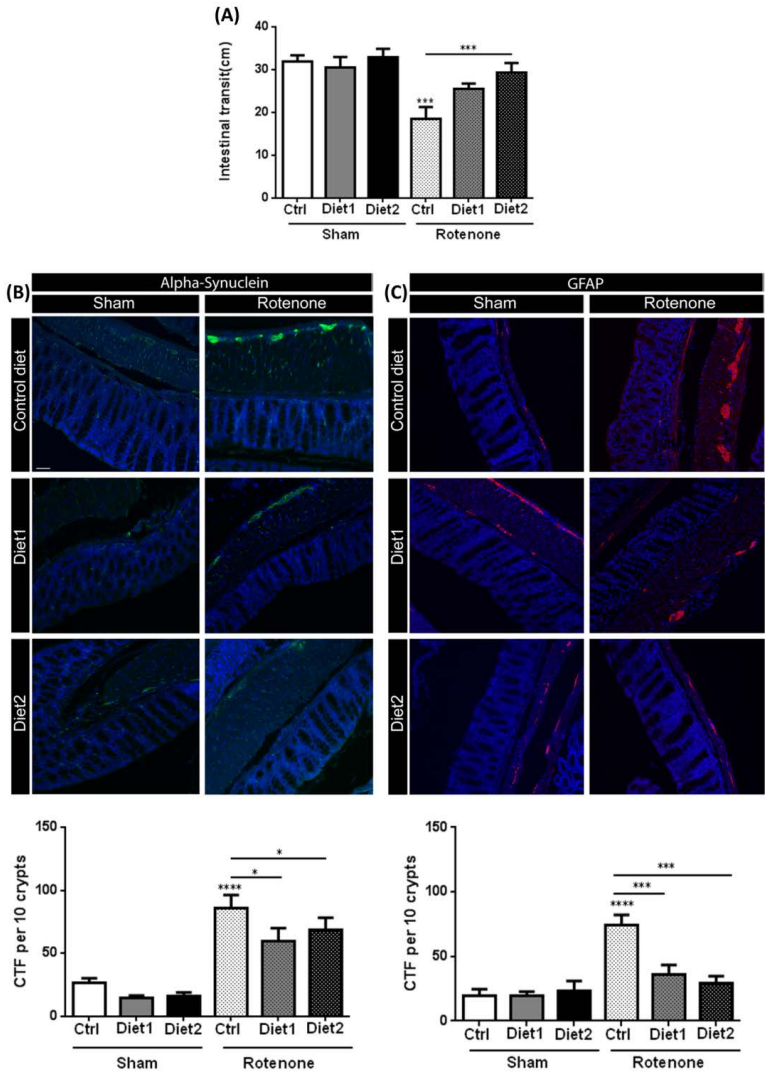


Figure 4: Effects of therapeutic dietary interventions on: (A) intestinal transit indicated by the total distance travelled by the Evans blue dye in the GI tract 30 min after its injection by oral gavage, (B) alpha-synuclein expression in the colon and (C) on enteric glial cells expression in the colon as indicated by the glial fibrillary acidic protein (GFAP). Rotenone injection reduced intestinal transit time and increased alpha-synuclein (shown in green) and GFAP (shown in red) expression in the myenteric and submucosal plexus of the colon. Dietary interventions improved rotenone-induced delayed intestinal transit and reduced rotenone-induced alpha-synuclein and GFAP overexpression in the colon. The beneficial effect of Diet2 on intestinal transit after rotenone exposure was more pronounced that the effect of Diet1. The Corrected Total Fluorescence (CTF) was calculated with the formula: integrated density – (area × mean fluorescence of background reading). Data are shown as mean ±SEM. *p<0.05, ***p<0.001, ****p<0.0001. (Scale bar: 50µm applies to all panels) (n=9 or 10 per group).

The length of the colon of all the animals was measured as a gross indicator of inflammation. There was an overall effect of the treatment (rotenone vs vehicle) ($F(1,54)=72.25$, $p<0.0001$) as well as an effect of diet ($F(2,54)=10.42$, $p<0.001$) and an interaction effect between treatment and diet ($F(2,54)=7.32$, $p<0.01$). More specifically, rotenone-treated animals on Diet1 and Diet2 revealed a smaller decrease in colon length compared to the ones on Control diet ($p<0.0001$ and $p<0.001$ respectively). No differences in colon length were found between rotenone-treated mice on Diet1 and rotenone-treated mice on Diet2 (Fig 5A).

The number of T-cells in the colon was increased after rotenone injection in the brain ($F(1,36)=51.41$, $p<0.0001$). There was an effect of diet ($F(2,36)=17.48$, $p<0.0001$) and an interaction effect between treatment and diet ($F(2,36)= 12.80$, $p<0.0001$). Post-hoc analysis revealed that rotenone-injected mice on Diet1 and Diet2 had less infiltration of T-cells in the colon compared to rotenone-injected mice on Control diet ($p<0.001$ and $p<0.0001$ respectively). Diet2 was more effective than Diet1 in reducing rotenone-induced T-cell infiltration ($p<0.05$) (Fig 5B).

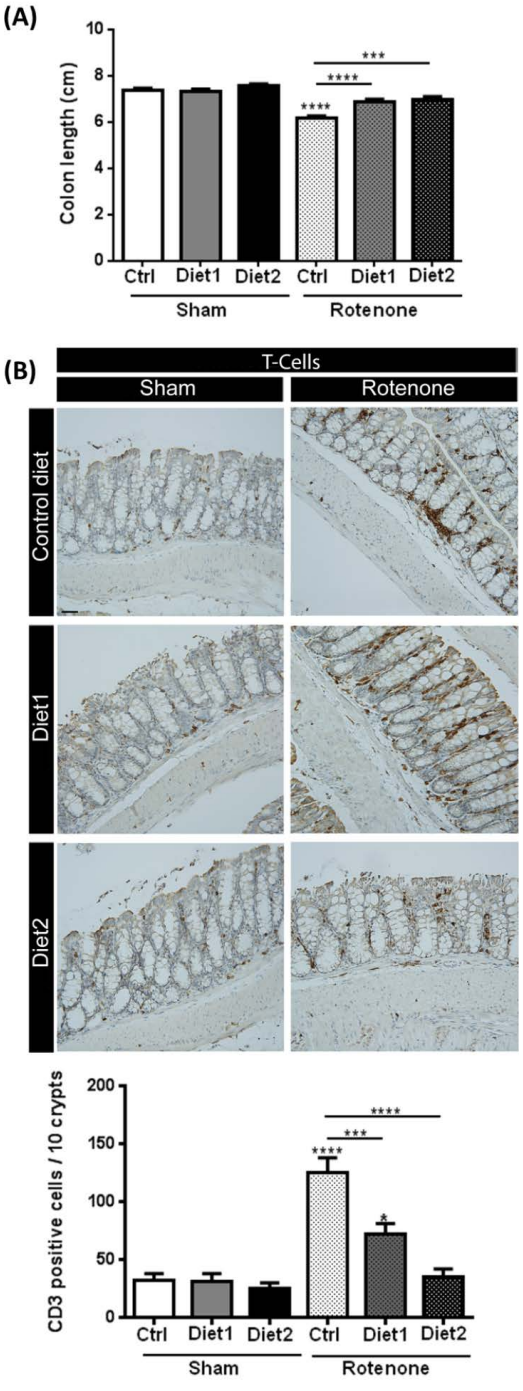


Figure 5: Effects of therapeutic dietary interventions on (A) colon length and (B) in the number of T-cells in the colon. Rotenone reduced the length of the colon and increased the number of T-cells (shown in brown). Both dietary interventions increased rotenone-induced reduction of the colon and reduced T-cell infiltration. Diet2 was more effective in normalizing rotenone induced T-cell infiltration than Diet1. Data are shown as mean ± SEM. ***p<0.001, ****p<0.0001. (Scale bar: 50µm applies to all panels). (n= 10 per group for colon length and n=7 for T-cell infiltration 10).

4. Discussion

In the present study we replicated previous finding[20] by showing that unilateral rotenone injection in the striatum caused a disturbed motor function, bilateral dopaminergic cell loss in the SN, delayed intestinal transit, alpha-synuclein accumulation in the ENS and colonic inflammation. In addition, we demonstrated that intrastriatal rotenone injection caused grip strength loss, spatial recognition deficits and an increase in the amount of reactive enteric glial cells in the colon. These new findings broaden the range of motor and non-motor symptoms observed and increase the relevance of the model for PD in patients where cognitive symptoms[35,36] and increased inflammation markers in colonic biopsies are reported[37]. Furthermore, in our mouse model the spatial recognition deficits developed later than the motor dysfunction, similar to PD patients where cognitive symptoms tend to occur later in the course of the disease. Therefore, the intrastriatal rotenone model in mice is a consistent model of PD that recapitulates key features of the pathology and symptomatology observed in humans. Whether other non-motor symptoms, in addition to cognitive and gastrointestinal dysfunction, are also induced by the model remains to be determined.

In the present study we also extended our recent observations regarding the effects of specific dietary interventions on a broad range of motor and non-motor symptoms in this mouse model of PD. It was previously shown that uridine and DHA were able to reduce circling behavior in the 6-OHDA animal model[19] and we showed that uridine and DHA prevented the induction of motor deficits as well as the development of GI dysfunctions in rotenone models of PD[20]. Here we show that the same diet (Diet1) given after the occurrence of motor problems, was able to reduce motor dysfunction, grip strength loss, cognitive deficits, delayed intestinal transit, colonic inflammation and alpha-synuclein accumulation in the ENS. This is the first study demonstrating a clear therapeutic effect of specific dietary interventions in a mouse model for PD.

Dietary interventions were started after the motor symptoms had clearly developed. As a consequence, we see no effects of the diets on the number of dopaminergic cells in the SN, indicating that the diets did not reduce the loss of dopaminergic cells, i.e. they did not interfere with rotenone toxicity. The diets helped to improve the functioning of the remaining dopaminergic neurons, demonstrating that they have neurorestorative properties and therefore may have disease-modifying potential.

There are several relevant mechanisms that may have contributed to the restoration of functions observed in the present study. A series of animal studies have shown that combined intake of phospholipid precursors including uridine and omega-3 fatty acids such as DHA, can increase brain phospholipid levels, synaptic protein levels, neurite outgrowth, dendritic spine formation, and dopaminergic neurotransmission[15,15–18]. Similarly to previous

observations in the 6-OHDA rat model of PD, where uridine plus DHA restored nigrostriatal markers and ameliorated biochemical defects[19], it is likely that a diet-induced enhancement of functional connectivity may underlie the presently observed recovery from behavioral deficits.

In addition to replicating the beneficial effects of Diet1 in the striatal rotenone model, we show that the extended nutritional intervention (Diet2) containing both precursors and other nutrients that increase phospholipid synthesis as well as prebiotic fibers was more effective in normalizing rotenone-induced motor and non-motor abnormalities. Although the present study was not designed to test the contribution of all individual nutrients, there are several factors that may have contributed to this enhanced effectiveness.

First, Diet2 contained specific prebiotic fibers such as fructo-oligosaccharides and galacto-oligosaccharides that have previously been shown to have beneficial effects on immune function[24,26] , bowel motility, and constipation[27,29], by altering the microbiota composition and their metabolic activity. Changes in microbiota composition might also alter the enteric immune and nervous system and subsequently the central nervous system[38–40]. In addition, the prebiotic fibers have microbiota-independent immunomodulatory properties[41]. Thus, adding prebiotic fibers in Diet2 may have contributed to its better effects on intestinal transit and colonic inflammation compared to Diet1, and possibly also to the beneficial effects on motor and cognitive functioning.

Second, Diet2 contained a broader combination of nutrients that has been shown to act synergistically to increase the synthesis of synaptic membranes[21]. On the one hand it contained a full set of precursors for membrane synthesis (i.e. DHA, EPA, uridine, choline, and phospholipids) that act by enhancing the substrate-saturation of the enzymes that catalyze the rate-limiting steps of phospholipid synthesis. On the other hand it also contained B-vitamins, vitamin C, vitamin E, selenium and phospholipids that act as cofactors by increasing the availability of membrane precursors or by directly affecting the neuronal membrane or membrane synthesis[21,42]. Next to synergies between individual precursors[17,18], the added value of the cofactors has for instance been reported on the activation of G-protein-coupled receptors[43]. In several in vivo experiments, the diet has repeatedly been shown to be more effective than supplementation of single nutrients or incomplete nutritional combinations[22,23,44]. Also in the present study Diet2 was more effective than the combination of uridine plus DHA (Diet1) on all functional and behavioral parameters. In addition, only intervention with this extended diet resulted in a restoration of the expression of DAT in the striatum, which might be one of the mechanisms by which the diet enhances dopaminergic transmission in the remaining nigrostriatal neurons resulting in a better functional output. Together these data suggest that especially the combined intake of these nutrients, rather than the use of single component supplements, could be beneficial to maintain brain structure and function.

The present results regarding the effectiveness of the extended dietary intervention (Diet2) in restoring functional connectivity in the rotenone model of PD are in line with previous observations in other models of either ongoing neurodegeneration [45,46] or acute neurotrauma[47,48]. In these experiments Diet2 improved both neuronal connectivity and behavioral output, suggesting a broad applicability of this nutritional technology. This, together with positive results regarding improved brain connectivity and functioning obtained in patients with AD[49,50] and preliminary findings in patients with frontotemporal dementia[51] , as well as a favorable safety profile of the intervention[52] warrant further clinical testing in other patient populations[53], including PD.

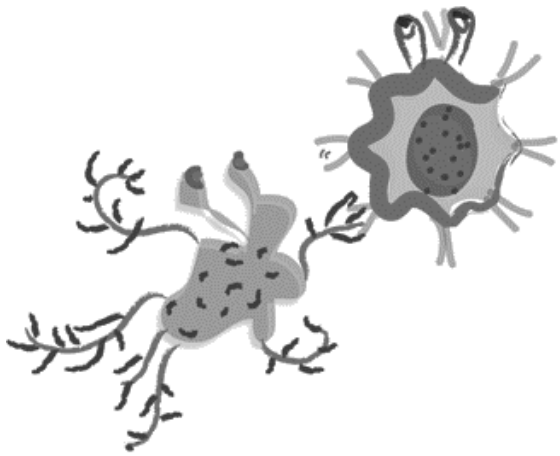
In summary, our study demonstrated clear therapeutic effects of specific dietary interventions in a mouse model of PD given after disease induction. The extended nutritional intervention (Diet2) was more effective than supplementation of only uridine and DHA (Diet1) in normalizing rotenone-induced motor and non-motor symptoms and PD-like pathologies in brain and gut. Our results suggest that this nutritional intervention might confer clinical benefits to patients suffering from PD.

References

1. de Rijk, M. C. *et al.* Prevalence of Parkinson's disease in Europe: A collaborative study of population-based cohorts. Neurologic Diseases in the Elderly Research Group. *Neurology* **54,** S21-23 (2000).
2. Nussbaum, R. L. & Ellis, C. E. Alzheimer's disease and Parkinson's disease. *N. Engl. J. Med.* **348,** 1356–1364 (2003).
3. Albin, R. L., Young, A. B. & Penney, J. B. The functional anatomy of basal ganglia disorders. *Trends Neurosci.* **12,** 366–375 (1989).
4. Crossman, A. R. Neural mechanisms in disorders of movement. *Comp. Biochem. Physiol. A* **93,** 141–149 (1989).
5. Aarsland, D., Andersen, K., Larsen, J. P., Lolk, A. & Kragh-Sørensen, P. Prevalence and characteristics of dementia in Parkinson disease: an 8-year prospective study. *Arch. Neurol.* **60,** 387–392 (2003).
6. Fasano, A., Visanji, N. P., Liu, L. W. C., Lang, A. E. & Pfeiffer, R. F. Gastrointestinal dysfunction in Parkinson's disease. *Lancet Neurol.* **14,** 625–639 (2015).
7. Pfeiffer, R. F. Gastrointestinal dysfunction in Parkinson's disease. *Parkinsonism Relat. Disord.* **17,** 10–15 (2011).
8. Martinez-Martin, P., Rodriguez-Blazquez, C., Kurtis, M. M., Chaudhuri, K. R. & NMSS Validation Group. The impact of non-motor symptoms on health-related quality of life of patients with Parkinson's disease. *Mov. Disord. Off. J. Mov. Disord. Soc.* **26,** 399–406 (2011).
9. Müller, B., Assmus, J., Herlofson, K., Larsen, J. P. & Tysnes, O.-B. Importance of motor vs. non-motor symptoms for health-related quality of life in early Parkinson's disease. *Parkinsonism Relat. Disord.* **19,** 1027–1032 (2013).
10. Schrag, A., Jahanshahi, M. & Quinn, N. How does Parkinson's disease affect quality of life? A comparison with quality of life in the general population. *Mov. Disord. Off. J. Mov. Disord. Soc.* **15,** 1112–1118 (2000).
11. Chaudhuri, K. R. & Schapira, A. H. V. Non-motor symptoms of Parkinson's disease: dopaminergic pathophysiology and treatment. *Lancet Neurol.* **8,** 464–474 (2009).
12. Schrag, A. & Quinn, N. Dyskinesias and motor fluctuations in Parkinson's disease. A community-based study. *Brain J. Neurol.* **123 (Pt 11),** 2297–2305 (2000).
13. Lee, H. M. & Koh, S.-B. Many Faces of Parkinson's Disease: Non-Motor Symptoms of Parkinson's Disease. *J. Mov. Disord.* **8,** 92–97 (2015).
14. Poewe, W., Antonini, A., Zijlmans, J. C., Burkhard, P. R. & Vingerhoets, F. Levodopa in the treatment of Parkinson's disease: an old drug still going strong. *Clin. Interv. Aging* **5,** 229–238 (2010).
15. Sakamoto, T., Cansev, M. & Wurtman, R. J. Oral supplementation with docosahexaenoic acid and uridine-5'-monophosphate increases dendritic spine density in adult gerbil hippocampus. *Brain Res.* **1182,** 50–59 (2007).
16. Wang, L., Pooler, A. M., Albrecht, M. A. & Wurtman, R. J. Dietary uridine-5'-monophosphate supplementation increases potassium-evoked dopamine release and promotes neurite outgrowth in aged rats. *J. Mol. Neurosci. MN* **27,** 137–145 (2005).
17. Wurtman, R. J., Cansev, M., Sakamoto, T. & Ulus, I. Nutritional modifiers of aging brain function: use of uridine and other phosphatide precursors to increase formation of brain synapses. *Nutr. Rev.* **68 Suppl 2,** S88-101 (2010).
18. Wurtman, R. J., Cansev, M., Sakamoto, T. & Ulus, I. H. Use of phosphatide precursors to promote synaptogenesis. *Annu. Rev. Nutr.* **29,** 59–87 (2009).
19. Cansev, M., Ulus, I. H., Wang, L., Maher, T. J. & Wurtman, R. J. Restorative effects of uridine plus docosahexaenoic acid in a rat model of Parkinson's disease. *Neurosci. Res.* **62,** 206–209 (2008).
20. Paula Perez-Pardo *et al.* Gut-brain and brain-gut axis in Parkinson's disease models: effects of a uridine and fish oil diet.
21. van Wijk, N. *et al.* Targeting synaptic dysfunction in Alzheimer's disease by administering a specific nutrient combination. *J. Alzheimers Dis. JAD* **38,** 459–479 (2014).
22. Broersen, L. M. *et al.* A specific multi-nutrient diet reduces Alzheimer-like pathology in young adult AβPPswe/PS1dE9 mice. *J. Alzheimers Dis. JAD* **33,** 177–190 (2013).
23. Zerbi, V. *et al.* Multinutrient diets improve cerebral perfusion and neuroprotection in a murine model of Alzheimer's disease. *Neurobiol. Aging* **35,** 600–613 (2014).
24. de Kivit, S., Kraneveld, A. D., Garssen, J. & Willemsen, L. E. M. Glycan recognition at the interface of the intestinal immune system: target for immune modulation via dietary components. *Eur. J. Pharmacol.* **668 Suppl 1,** S124-132 (2011).
25. Jeurink, P. V., van Esch, B. C. A. M., Rijnierse, A., Garssen, J. & Knippels, L. M. J. Mechanisms underlying immune effects of dietary oligosaccharides. *Am. J. Clin. Nutr.* **98,** 572S–7S (2013).
26. van Hoffen, E. *et al.* A specific mixture of short-chain galacto-oligosaccharides and long-chain

fructo-oligosaccharides induces a beneficial immunoglobulin profile in infants at high risk for allergy. *Allergy* **64,** 484–487 (2009).
27. Meksawan, K. *et al.* Effects of fructo-oligosaccharide supplementation on constipation in elderly continuous ambulatory peritoneal dialysis patients. *Perit. Dial. Int. J. Int. Soc. Perit. Dial.* (2014). doi:10.3747/pdi.2014.00015
28. Rasmussen, H., Piazza, B., Forsyth, C. & Keshavarzian, A. in *Pharma-Nutrition* (eds. Folkerts, G. & Garssen, J.) **12,** 213–242 (Springer International Publishing, 2014).
29. Scholtens, P. A. M. J., Goossens, D. A. M. & Staiano, A. Stool characteristics of infants receiving short-chain galacto-oligosaccharides and long-chain fructo-oligosaccharides: a review. *World J. Gastroenterol.* **20,** 13446–13452 (2014).
30. Reeves, P. G., Nielsen, F. H. & Fahey, G. C. AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. *J. Nutr.* **123,** 1939–1951 (1993).
31. Inden, M. *et al.* Neurodegeneration of mouse nigrostriatal dopaminergic system induced by repeated oral administration of rotenone is prevented by 4-phenylbutyrate, a chemical chaperone. *J. Neurochem.* **101,** 1491–1504 (2007).
32. Deacon, R. M. J. Measuring the strength of mice. *J. Vis. Exp. JoVE* (2013). doi:10.3791/2610
33. Leiter, J. R. S., Peeler, J. & Anderson, J. E. Exercise-induced muscle growth is muscle-specific and age-dependent. *Muscle Nerve* **43,** 828–838 (2011).
34. De Leonibus, E. *et al.* Spatial deficits in a mouse model of Parkinson disease. *Psychopharmacology (Berl.)* **194,** 517–525 (2007).
35. Boller, F. *et al.* Visuospatial impairment in Parkinson's disease. Role of perceptual and motor factors. *Arch. Neurol.* **41,** 485–490 (1984).
36. Giraudo, M. D., Gayraud, D. & Habib, M. Visuospatial ability of parkinsonians and elderly adults in location memory tasks. *Brain Cogn.* **34,** 259–273 (1997).
37. Clairembault, T. *et al.* Enteric GFAP expression and phosphorylation in Parkinson's disease. *J. Neurochem.* **130,** 805–815 (2014).
38. Clemente, J. C., Ursell, L. K., Parfrey, L. W. & Knight, R. The impact of the gut microbiota on human health: an integrative view. *Cell* **148,** 1258–1270 (2012).
39. Cryan, J. F. & Dinan, T. G. More than a gut feeling: the microbiota regulates neurodevelopment and behavior. *Neuropsychopharmacol. Off. Publ. Am. Coll. Neuropsychopharmacol.* **40,** 241–242 (2015).
40. Maslowski, K. M. & Mackay, C. R. Diet, gut microbiota and immune responses. *Nat Immunol* **12,** 5–9 (2011).
41. de Kivit, S. *et al.* Intestinal epithelium-derived galectin-9 is involved in the immunomodulating effects of nondigestible oligosaccharides. *J. Innate Immun.* **5,** 625–638 (2013).
42. van Wijk, N. *et al.* Dietary Crude Lecithin Increases Systemic Availability of Dietary Docosahexaenoic Acid with Combined Intake in Rats. *Lipids* **51,** 833–846 (2016).
43. Savelkoul, P. J. M. *et al.* A specific multi-nutrient formulation enhances M1 muscarinic acetylcholine receptor responses in vitro. *J. Neurochem.* **120,** 631–640 (2012).
44. Janickova, H. *et al.* Lipid-Based Diets Improve Muscarinic Neurotransmission in the Hippocampus of Transgenic APPswe/PS1dE9 Mice. *Curr. Alzheimer Res.* **12,** 923–931 (2015).
45. Jansen, D. *et al.* Effects of specific multi-nutrient enriched diets on cerebral metabolism, cognition and neuropathology in AβPPswe-PS1dE9 mice. *PloS One* **8,** e75393 (2013).
46. Wiesmann, M. *et al.* A Dietary Treatment Improves Cerebral Blood Flow and Brain Connectivity in Aging apoE4 Mice. *Neural Plast.* **2016,** 6846721 (2016).
47. Pallier, P. N. *et al.* A nutrient combination designed to enhance synapse formation and function improves outcome in experimental spinal cord injury. *Neurobiol. Dis.* **82,** 504–515 (2015).
48. Wiesmann, M. *et al.* A specific dietary intervention to restore brain structure and function after ischemic stroke.
49. de Waal, H. *et al.* The effect of souvenaid on functional brain network organisation in patients with mild Alzheimer's disease: a randomised controlled study. *PloS One* **9,** e86558 (2014).
50. Scheltens, P. *et al.* Efficacy of Souvenaid in mild Alzheimer's disease: results from a randomized, controlled trial. *J. Alzheimers Dis. JAD* **31,** 225–236 (2012).
51. Pardini, M. *et al.* Souvenaid reduces behavioral deficits and improves social cognition skills in frontotemporal dementia: a proof-of-concept study. *Neurodegener. Dis.* **15,** 58–62 (2015).
52. Cummings, J. *et al.* Effect Size Analyses of Souvenaid in Patients with Alzheimer's Disease. *J. Alzheimers Dis. JAD* **55,** 1131–1139 (2017).
53. Cummings, J. L. Food for thought: Souvenaid in mild Alzheimer's disease. *J. Alzheimers Dis. JAD* **31,** 237–238 (2012).

CHAPTER 7



The gut-brain axis in Parkinson's disease: possibilities for food-based therapies

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Abstract

Parkinson's disease (PD) is usually characterized by cardinal motor impairments. However, a range of non-motor symptoms precede the motor-phase and are major determinants for the quality of life. To date, no disease modifying treatment is available for PD patients. The gold standard therapy of levodopa is based on restoring dopaminergic neurotransmission, thereby alleviating motor symptoms, whereas non-motor symptoms remain undertreated. One of the most common non-motor symptoms is gastrointestinal dysfunction usually associated with alpha-synuclein accumulations and low-grade mucosal inflammation in the enteric nervous system. Accumulating evidence suggest that the enteric nervous system is involved in PD pathological progression towards the central nervous system. Moreover, different components of the gut could provide a central role in the gut-brain axis, which is as a bidirectional communicational system between the gastrointestinal tract and central nervous system. Dietary components might influence the gut-brain axis by altering microbiota composition or by affecting neuronal functioning in both the ENS and the CNS. This review gives a comprehensive overview of the evidences supporting the hypothesis that PD could initiate in the gut. We also consider how food-based therapies might then have an impact on PD pathology and/or improve non-motor as well as motor symptoms in PD.

1. Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disease after Alzheimer's disease and is hallmarked by damage to the dopaminergic neurons of the substantia nigra (SN) and by alpha-synuclein containing inclusion bodies (Lewy pathology; LP) in the surviving neurons, resulting in the characteristic motor impairment. It has a prevalence of 0.3% in the general population and 1-3% in the population over the age of 65[1,2]. Although PD is generally considered as a movement disorder, it has long been recognized that the symptoms go beyond motor dysfunction since PD patients very often develop non-motor symptoms, including cognitive impairment[3], hyposmia[4–6], pain[7], depression[8], tiredness, orthostatic hypotension[9] and most commonly, gastrointestinal (GI) dysfunction[10–13]. Some of these symptoms may precede the classical motor symptoms by several years[14–16] and their occurrence in otherwise healthy people has been associated with an increased risk of developing PD[5,14].

In recent years, special focus has been placed upon the GI tract and the associated enteric nervous system (ENS) in the development of PD[17–20]. The ENS is an integrative network of neurons in the GI wall and a major player in the gut-brain axis which is a bidirectional communication system between the central nervous system (CNS) and the GI tract[21]. It has been also lately recognized that the gut-brain interactions might be essentially influenced by the gut microbiota[22–24].

During the first stages of PD, neurons of the ENS and the olfactory bulbs (OB) were found to contain aggregated and phosphorylated alpha-synuclein[25,26]. The ENS and OB are gateways to the external environment and new evidence suggests that alpha-synuclein deposition in neurons might begin in the ENS and/or in the OB, where a toxin or a pathogen and associated immune/inflammatory responses might start the detrimental process and spread according to a specific pattern, via the vagal nerve and olfactory tract respectively, to the SN and further areas of the CNS[19,27–29]. It is also possible that these inflammatory responses in the gut might signal to specific parts of the brain systemically and through dysfunctional blood brain barrier structures as seen in PD patients[30].

Levodopa is the most commonly used drug in the treatment of PD. It suppresses some of the motor symptoms and compensates for dopaminergic cell loss by enhancing dopamine synthesis in the remaining terminals. This therapy has several side effects[31], it does not prevent dopaminergic neuron degeneration, and has no effects on non-motor symptoms[32]. Moreover, PD-associated GI dysfunction contributes to levodopa response fluctuations[33]. Thus, there is an urgent need to better understand gut-brain interactions in PD and to develop new therapeutic strategies targeting the gut-brain axis in order to impact PD pathogenesis.

2. Gastrointestinal dysfunction in Parkinson's disease

Non-motor symptoms in PD were already highlighted by James Parkinson in 1817. Nowadays they are well defined but they remain undertreated. An international study showed that 62% of non-motor symptoms are not reported by PD patients due to embarrassment or because patients are unaware that these symptoms are related to PD[34,35]. One of the most common non-motor symptoms in PD are GI dysfunction, with a prevalence of 70-80%[36]. GI symptoms are identified as bloating, drooling, constipation, nausea, delayed gastric emptying and prolonged intestinal transit time[10,11,14,35,37–41] and they are major determinants of quality of life[36,42]. The occurrence and prevalence of different GI dysfunctions vary among patients and have been extensively reviewed[11]. Among them, constipation is the most prominent and it might precede motor symptoms by over a decade[10,11]. The occurrence of constipation before the manifestation of motor symptoms in PD patients was reported to be 87%[38]. In addition, constipation is assumed to be a harbinger and is associated with an increased risk of developing PD[39,40]

The factors responsible for the initiation of the pathophysiological cascade in PD remain unknown. However, it is likely that environmental factors play a key role[43,44]. The early involvement of the GI tract in PD supports the hypothesis that environmental factors could exert its influence on PD development and progression via the gut.

3.Gut pathology

Alpha-synuclein accumulation in the ENS

Alpha-synuclein is a protein abundantly expressed in the CNS, mainly in the presynaptic terminals. It is thought to be involved in the regulation of neurotransmission and synaptic homeostasis[45,46]. A pathological characteristic for PD is the presence of cytoplasmatic eosinophilic alpha-synuclein inclusions in the form of Lewy bodies in cell somata and Lewy neurites in axons and dendrites[47,48]. It has been suggested that alpha-synuclein could act like a prion protein during PD pathogenesis. In this theory pathologic, misfolded alpha-synuclein is an 'infectious' protein spreading pathology by forming a template that seeds misfolding for nearby alpha-synuclein protein, turning the previously healthy protein into a pathogenic protein[49,50].

Several clinical studies revealed that PD patients expressed alpha-synuclein accumulation in the ENS[25,51–55]. Alpha-synuclein accumulations are associated with damage in the enteric neurons and possibly underlie GI dysfunction[53,54]. They affect both the myenteric and submucosal plexuses of the gut in PD patients and are distributed in the GI tract from the esophagus to its most distal point, the rectum[56].

Braak and colleagues hypothesized that alpha-synuclein pathology might start in either the OB and/or in the ENS possibly by an unknown pathogen and/or environmental toxin and

then progresses towards the SN and further areas in the CNS. The vagal nerve might provide a path for the spread of alpha-synuclein pathology from the ENS to the brain through the brainstem, midbrain, basal forebrain and finally the cortical areas[27,57], whereas the initiation of the pathological process in the OB can more directly affect the brain via the olfactory tract [19,28,29]. Our recent studies[51,58] suggest that gut-initiated pathological processes in PD do not necessarily require a pathogen and/or an environmental toxin since they can be triggered by the intestinal microbiota.

Alpha-synuclein spreading from the enteric nervous system towards the brain
Environmental factors such as microorganisms, including nasal/gut microbiota, and toxins like pesticides might start a pathological process at two sites, in the OB and within enteric nerve cell plexus[59], causing mucosal inflammation and oxidative stress and thereby initiating alpha-synuclein accumulation[29].

In accordance with this hypothesis it has been shown that alpha-synuclein can be retrogradely transported from the intestinal wall to the brain in rats[60]. Others have shown *in vitro* and *in vivo* that alpha-synuclein is transmitted via endocytosis to neighboring neurons[61,62]. In a transgenic mouse model for PD, alpha-synuclein was shown to be transmitted to engrafted neuronal precursor cells, where it created inclusions[61,63]. Similarly, autopsies of PD patients who had received fetal mesencephalic transplants, showed alpha-synuclein accumulation in the grafted neurons[64,65].

Moreover, in a recent study full truncal vagotomy was associated with a decreased risk of developing PD compared to highly selective vagotomy (affecting only acid producing portion of gastric body) or no vagotomy supporting the idea that the vagal nerve might provide a conduit to spread PD pathology from the gut to the brain[66]. Another study showed that PD-like neuropathology was mimicked by gastric administration of pesticide rotenone in mice and occurred in the absence of detectable levels of rotenone in the brain and blood[67]. The local effect of pesticides on the ENS might be sufficient to induce PD-like progression and to reproduce the neuroanatomical and neurochemical features of PD staging, from the ENS to the CNS. Two years later, the same research group showed that the progression of pathologically expressed alpha-synuclein towards the brain could be halted by the resection of sympathetic and parasympathetic nerves prior to oral rotenone treatment[17]. Since the mucosal sides in relation to OB and the ENS are exposed to substances from the environment through inhalation or ingestion, it seems plausible that environmental factors such as diets, toxins, intestinal microorganisms and other environmental pathogens might have an important role in triggering and propagating PD pathology, probably against a background of genetic vulnerability (Figure 1).

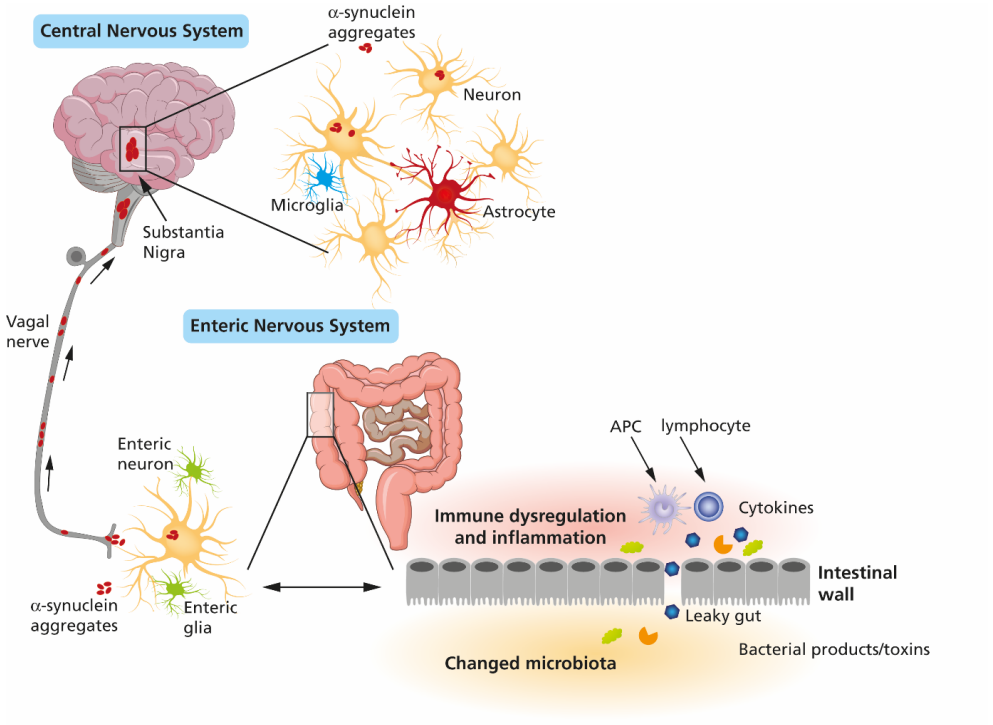

Figure 1: A schematic representation of alpha-synuclein accumulation and spreading from the ENS towards the brain. Environmental factors such as microorganisms, including the gut microbiota, and toxins like pesticides might start a pathological process within enteric nerve cell plexus, causing mucosal inflammation and oxidative stress and thereby initiating alpha-synuclein accumulation. The vagal nerve might provide a path for the spread of alpha-synuclein pathology from the ENS to the brain through the brainstem, midbrain, basal forebrain and finally the cortical areas.

Changes in gut bacterial composition

PD patients show an increased intestinal permeability, also known as leaky gut, that correlated with intestinal alpha-synuclein accumulation[51]. The increased intestinal permeability and the translocation of bacteria and inflammatory bacterial products (e.g., lipopolysaccharide, LPS) might lead to inflammation and oxidative stress in the GI tract and thereby initiating alpha-synuclein accumulation in the ENS[51,68,69]. In addition, gut-derived LPS can promote the disruption of the blood brain barrier[70,71] and thus facilitate neuroinflammation and injury in the SN that is triggered by the above stated environmental factors.

In support of this hypothesis, biopsies of colonic tissue retrieved from PD patients revealed an increased expression in the levels of pro-inflammatory cytokines, such as TNF-alpha, IFN-gamma, IL-6 and IL-1 beta as well as an increased activation of enteric glial cells[72].

There is now mounting evidence that the microbiota is altered in people suffering from PD. The first study demonstrating this was published in 2015[73] where a reduction of *Prevotellaceae* (77.6%) in fecal samples of PD patients was found. They further detected a relative abundance of *Enterobacteriaceae* that positively correlated with the severity of postural instability and gait difficulty[73]. The under-representation of *Prevotellaceae* diminishes the levels of health-promoting neuroactive short chain fatty acids (SCFA) and the capacity for biosynthesis of thiamine and folate[74], which is in line with decreased levels of these vitamins in PD patients[75,76]. The authors further indicate that a decrease in *Prevotella* might be related with a reduction in mucin synthesis which is associated with increased gut permeability[51,77] intensifying the translocation of bacterial antigens. In addition, a decreased abundance of *Prevotella* and an increased abundance of *Lactobacilliceae* have been associated with lower concentrations of ghrelin. Ghrelin is a gut hormone that may be involved in the maintenance and protection of normal nigrostriatal dopamine function[78] and impaired ghrelin secretion has been reported in PD patients[79].
Another study from our group revealed differences in mucosal and fecal microbial community of PD patients in comparison to healthy subjects[58]. The study showed a lower abundance of bacteria associated with anti-inflammatory properties such as of SCFA butyrate-producing bacteria from the genera *Blautia*, *Coprococcus*, and *Roseburia* in PD fecal samples, thereby concluding that a reduction in SCFA might contribute to gut leakiness. In addition, genes involved in lipopolysaccharide biosynthesis and type III bacterial secretion systems were higher in stool samples of PD patients compared to controls. Type III secretion systems are generally involved in pathogenicity and translocation of proteins that could facilitate bacterial product-induced inflammation[80,81]. We concluded that PD pathogenesis may be caused or exacerbated by dysbiotic microbiota-induced inflammatory responses that could promote alpha-synuclein pathology in the intestine and the brain or by rostral to caudal cell-to-cell transfer of alpha-synuclein pathology caused by increased oxidative stress (due to an increase in pro-inflammatory bacteria).

A more recent study showed that not only gut microbiota but also fecal SCFA concentrations are reduced in PD patients compared to age-matched controls[82]. They found a significant reduction of acetate, propionate and butyrate in PD fecal samples. The reduction in SCFA might induce alterations in the ENS and contribute to GI dysmotility in PD.
Moreover, SCFA butyrate has anti-inflammatory properties thought to be owing to an epigenetic mechanism or to the activation of SCFA receptors leading to anti-inflammatory effects, anti-microbial effects, and to a decreased intestinal barrier leakiness[83–86].

Small intestinal bacterial overgrowth (SIBO) is a malabsorption syndrome associated with increased bacterial density and/or the presence of colonic-type species in the small intestine[87]. Abnormalities of GI motility might increase the occurrence of SIBO which is highly prevalent in PD patients[54,77], even in recently diagnosed PD patients[88]. PD patients

suffering from SIBO were not reported to develop worse GI dysfunction. However, they were independently predisposed to worse motor-dysfunction[88]. SIBO might cause changes in intestinal permeability and contribute to an increase in bacterial translocation and therefore induce an inflammatory response[89].

It is not possible to determine if changes in the gut microbiota are a cause or a consequence of PD pathogenesis. However, it might still play a role in neuronal loss by perpetuating inflammatory cascades and oxidative injury in the brain through LPS-mediated mechanism.

4. Current treatments for PD: interactions with GI dysfunction

Current anti-parkinsonian medication is based on compensating for dopaminergic cell loss and primarily targeted towards alleviation of motor symptoms by enhancing dopaminergic neurotransmission. The most commonly used symptomatic anti-parkinsonian agents are dopamine receptor agonists and the dopamine precursor L-3,4-dihydroxyphenylalanine (levodopa). The oral substitution of levodopa is, so far, the most regulative and efficient drug in the treatment of PD. Between 2001 and 2012, levodopa was used by 85% of patients suffering from PD, whereas dopamine agonists were used by 28%[90]. Unfortunately, levodopa treatment does not stop the disease progression and has major shortcomings. Many of the non-motor symptoms are unresponsive to dopaminergic treatments[31,32]. The prolonged use of levodopa induces severe side effects such as dyskinesia and motor fluctuation[31] and as the disease progresses patients might eventually develop levodopa-resistance[32,91]. Moreover, it has been shown that levodopa-unresponsive features and constipation were positively associated with the amount of Lewy neurites in the ENS[91].
Since levodopa treatment is taken orally, a good functioning of the GI tract is required in order to absorb the drug at a beneficial rate. Many efforts have been made to improve levodopa bioavailability by developing more effective oral formulations including combining levodopa with carbidopa to inhibit peripheral metabolism of levodopa. In addition, immediate and extended release levodopa-carbidopa oral formulations are under development[92].
Several clinical studies revealed that single or multiple dosages of levodopa induced delayed gastric emptying in healthy volunteers[93–96] and that it might therefore exacerbate the GI symptoms already developed in PD patients. Delayed gastric emptying in PD patients dampens the proper absorption of levodopa or dopamine agonists, causing lower peak plasma concentrations and on-off fluctuations of the drug[97–99]. It would be interesting to test new therapies in combination with levodopa that could impact PD pathology and/or improve GI dysfunction and therefore improving levodopa uptake and availability. The dose of levodopa given to patients could then be lowered in the treatment of PD, reducing the negative secondary effects and possibly contributing to a longer beneficial use of the drug.

5. Targeting the Gut-Brain axis in Parkinson's disease with food-based therapies

No current therapeutic strategies have a favorable influence on PD progression. Moreover, none of them directly targets the gut-brain axis to prevent the spread of PD pathology or to alleviate non-motor as well as motor symptoms. Nutrition based interventions including phospholipid membrane precursors and/or microbiota-directed therapy like prebiotics and probiotics might provide opportunities to complement the traditional PD therapies and overcome some of their shortcoming including lack of efficacy for GI symptoms/dysfunction. Dietary interventions might influence the gut-brain axis by altering microbiota composition (and therefore altering PD pathogenesis)[21,86,100] or by affecting neuronal functioning in both the ENS and the CNS (Figure 2).

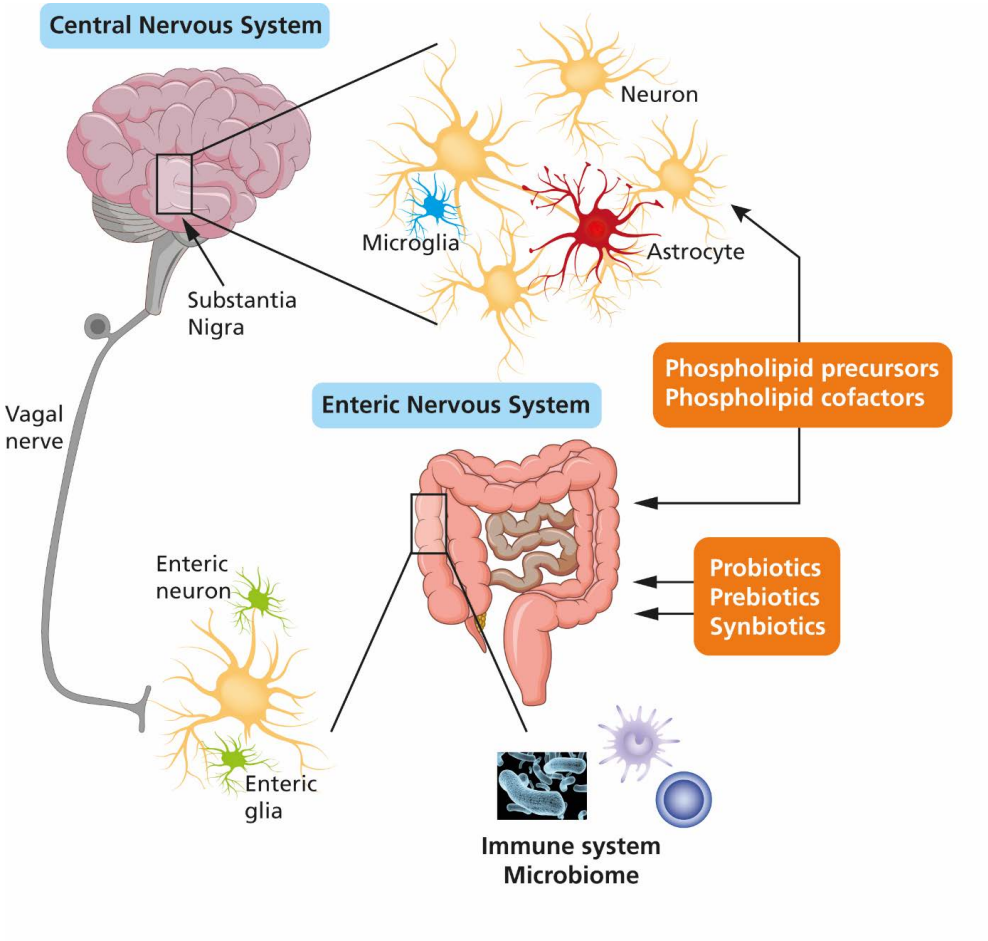

Figure 2: Dietary phospholipid precursors and cofactors can increase neuronal membrane formation and function, and reduce inflammation, affecting both the ENS and CNS and reducing motor and non-motor abnormalities in PD. Probiotics, prebiotics and/or synbiotics might impact the gut microbiota composition, enhance intestinal epithelial integrity and reduce the pro-inflammatory response, impacting initiation or progression of the neurodegenerative process.

Nutritional membrane precursors and cofactors

Specific nutrient combination containing neuronal precursors and cofactors may counteract synaptic loss and reduce membrane-related pathology in the CNS and the ENS of PD patients. It might confer clinical benefits to patients suffering from PD since they are able to reduce motor and non-motor abnormalities in preclinical studies. Its combination with prebiotic fibers might have an added therapeutic value[101].

Synapse loss and membrane-related pathology provide compelling targets for interventions in PD. Uridine (as uridine monophosphate, UMP), the omega-3 fatty acid docosahexaenoic acid (DHA) and choline are phospholipid precursors needed for the formation and maintenance of neuronal membranes[102,103]. They enhance the substrate-saturation of the enzymes responsible for the catalysis of the rate-limiting steps of phospholipids synthesis required for membrane formation[104]. Since phospholipids precursors are obtained basically from the circulation, increasing blood levels of these precursors with nutritional interventions might have a huge impact on the overall rate of phospholipid synthesis[105]. In addition, cofactors in the synthesis of phospholipids, such as B-vitamins, vitamin C, vitamin E and selenium can increase the availability of the above mentioned membrane precursors by enhancing precursors uptake and metabolism[104].
Several studies in rodents showed that co-administration of uridine, DHA, and choline can increase the amount of phospholipids, synaptic proteins, dendritic spine density and neurite outgrowth[106–108]. The combination of these phospholipid precursors has also shown to partially restore dopaminergic neurotransmission in the 6-OHDA model of PD in rats [109]. A recent study demonstrated that dietary fat intake may modify the risk of developing PD directly or by altering the response to environmental neurotoxins; high levels of polyunsaturated fatty acids (PUFAs), like DHA decreased the association of PD with pesticides[110]. Individually, both uridine and DHA have been shown to induce favorable effects with preventive intake in animal models of PD[111,112]. In the 6-OHDA rat model, both DHA[112] and uridine[113] reduced drug-induced rotational behavior. We have also shown the beneficial preventive effects of a dietary intervention containing uridine, DHA and choline in the unilateral rotenone model for PD. Intrastriatal injection of rotenone caused several motor and non-motor symptoms associated with PD. The preventive dietary intervention was not only effective for the mitochondrial dysfunction-induced motor symptoms but also reduced alpha-synuclein accumulation and inflammation in the colon[114]. PUFAs, like DHA, have anti-inflammatory effects[115] and can improve mitochondrial dysfunction[116,117] leading to a reduction in oxidative stress and alpha-synuclein accumulation. In another study we showed that the same diet given in a therapeutic setting (i.e. after the occurrence of full motor problems) in the intrastriatal rotenone model was also able to reduce motor dysfunction, colonic inflammation and alpha-synuclein accumulation in the ENS, demonstrating that the diet is not interacting with rotenone toxicity but rather has neurorestorative properties[101]. The same study showed that an extended therapeutic

nutritional intervention containing the same phospholipid precursors plus cofactors for phospholipid synthesis as well as prebiotic fibers (GOS and FOS) was more effective in normalizing motor and GI abnormalities. Adding cofactors to the diet might increase neuronal membrane formation by increasing the availability of membrane precursors or by directly affecting the neuronal membrane or membrane synthesis [118] that might explain its better effects in motor-symptoms. In other in vivo studies, similar nutritional combinations have shown to be more effective than supplementation of single nutrients or incomplete formulations in animal models for Alzheimer's disease[119–121]. Prebiotic fibres added to the diet might explain its better effects on GI-function since GOS and FOS have shown to have beneficial effects on immune function[122], bowel motility[123,124], but they might also contribute to its better efficacy on motor-function since the prebiotic fibres might positively affect microbiota composition that in turn can alter the enteric immune and nervous system and subsequently the CNS[86,100,125].

Probiotics

Probiotics are specific microorganisms that when administered in adequate amounts can exert a health benefit on the host by restoring microbiota and maintaining immune homeostasis[126]. The most common probiotic bacteria currently used are representatives of *Lactobacilli*, *Enterococci, Bifidobacteria*, yeasts and mixtures of different beneficial bacteria [127]. Various studies have reported the beneficial effects of probiotics by enhancing intestinal epithelial integrity, protecting from barrier disruption, stimulating a healthy homeostasis of the mucosal immune system and suppressing pathogenic bacterial growth[128–132]. Moreover, different strains of probiotic bacteria have been shown to be effective in stimulating intestinal motility and reducing GI dysfunction. For example, in elderly orthopedic patients, probiotics showed to have positive effects on bowel movements by lowering the incidence of diarrhea and constipation severity[133]. In a double blind placebo controlled trial, *Lactobacillus reuteri* supplementation improved bowel movement frequency in adults with chronic functional constipations, but did not show to have an effect on stool consistency[134]. Furthermore, studies have shown that is possible to modulate brain function by improving anxiety and depression using probiotics. In a mouse model of autism spectrum disorder (ASD), Hsiao and colleagues showed that the administration of *Bacteroides fragilis* reversed the abnormalities in gut permeability and ASD related behaviors[135]. Ingestion of selected probiotics also exhibited beneficial effects on brain function in humans. The administration of *Lactobacillus casei* strain Shirota in chronic fatigue syndrome patients significantly decreased anxiety symptoms[136]. Studies regarding the use of probiotics for the treatment of PD are very limited. One study showed that PD patients suffering from chronic constipation receiving fermented milk containing *Lactobacillus casei* Shirota for five weeks improved stool consistency and reduced bloating and abdominal pain[137].

Probiotics might be a powerful tool in order to alter PD-associated microbiota composition and improve GI function and therefore reduce gut leakiness, bacterial translocation and the associated neuro-inflammation in the ENS. Improving GI function by supplementation with probiotics might not solely lead to a better functionality and/or protection of the intestine, but might also improve levodopa absorption and reduce behavioral and cognitive deficits such as anxiety, depression and memory problems[138,139], which are common in PD patients.

Prebiotics

Prebiotics are non-digestible oligosaccharides, that beneficially affect the host by selectively stimulating the growth and/or activity of a limited number of bacteria in the gut[140]. Two well-known non-digestible carbohydrates are the galacto-oligosaccharides (GOS), based on lactose and fructo-oligosaccharides (FOS), synthesized from fructose. GOS and FOS reach the colon more or less unchanged where they are metabolized by most of the *Bifidobacteria*, but only a few representatives of other strains. SCFA, lactose, hydrogen, methane, and carbon dioxide are metabolic products that lead to an acidic milieu in the colon, which antagonizes the survival and the proliferation of pathogenic bacteria[141]**.** SCFA are essential for the maintenance of intestinal epithelial integrity, and the homeostasis and regulation of mucosal immunological responses[142]**.**

Prebiotic fibers have been shown to have beneficial effects on immune function[143,122,144], bowel motility and constipation[123,124,145] that might be very relevant for inflammation and GI-related symptoms in PD. Moreover, GOS and FOS have been shown to increase the levels of brain-derived neurotrophic factor (BDNF) in the dentate gyrus of the hippocampus in rats[146]. As BDNF signaling is critical for neuronal protection, survival and plasticity[147], GOS and FOS supplementation might have implications on brain neuroprotection.

Despite the evidence supporting the use of prebiotics for GI dysfunction, immune function, and neuroprotection, their use has never been investigated in patients with PD. Moreover, as mentioned above, the fecal microbial community of PD showed a lower abundance of SCFA butyrate-producing bacteria [58,82] that could be corrected by the use of prebiotic fibers.

Synbiotics

The term *synbiotic* is used when a product contains both probiotics and prebiotics. The term is reserved for products in which the prebiotic compound selectively favors the probiotic compound[148,149]. Synbiotics have shown to have beneficial effects on immune function, dysbiosis and bowel function, very relevant for PD patients.

A clinical study showed that the probiotic *Lactobacillus salivarius* decreased inflammatory markers in healthy subjects, and its effect when *combined* with FOS was more pronounced [150]. Furthermore, another trial demonstrated that females with functional constipation and receiving *Bifidobacterium animalis combined with FOS showed an increase in bowel movement, stool quantity and quality* compared to controls[151]. As mentioned before, SIBO is highly prevalent in PD[77,78] and PD patients that tested positive for SIBO are predisposed to

worse motor-dysfunction[88]. Interestingly, patients positively tested for SIBO treated with antibiotics followed by synbiotic supplementation, containing *Bacillus coagulans* and *FOS, showed a better response than* patients on the same regimen without synbiotic supplementation. 93% of the subjects treated with the synbiotic tested negative for SIBO compared to 67% of the controls. It also significantly decreased abdominal pain, flatulence and diarrhea[152].

5. Conclusion

To date, there is no treatment designed to cure PD. The most common used anti-parkinsonian medication is levodopa which does not prevent neurodegeneration, and has no effects on non-motor symptoms. Moreover, GI dysfunction in PD patients contributes to levodopa response fluctuations.

Current evidences indicate that alpha-synuclein deposition in PD might start in the ENS initiated by a toxin or pathogen and propagates to the CNS by transsynaptic cell-to-cell transmission. Bacterial translocation could also induce a pro-inflammatory environment that could signal to specific parts of the brain systemically and through dysfunctional blood brain barrier structures.

Therefore, a better understanding of the gut-brain interactions might bring new insight in PD pathological progression as well as lead to new therapeutic approaches. Pharmacological or dietary interventions should be aimed at alleviating both motor and non-motor symptoms.

Dietary membrane precursors and cofactors can increase neuronal membrane formation and function, and reduce inflammation, affecting both the ENS and CNS and reducing motor and non-motor abnormalities in PD.

Probiotics, prebiotics and/or synbiotics might impact the gut microbiota composition and possibly enhance intestinal epithelial integrity and reduce the pro-inflammatory response, impacting initiation or progression of the neurodegenerative process.

The above mentioned food-based therapies might therefore have an influence in PD pathological progression. Moreover, these therapies have shown to have beneficial effects on GI dysfunction and when combined with levodopa treatment they might increase levodopa uptake and availability, allowing a reduction in the doses of levodopa given to patients and reducing the negative secondary effects produced by the drug.

These compelling pre-clinical data have provided strong scientific rationale to conduct high quality randomized placebo controlled trial to assess the effectiveness of dietary supplementation (such as phospholipid membrane precursors, microbiota-directed therapy or a combination of them) in PD patients.

References:

1. de Rijk, M. C. *et al.* Prevalence of Parkinson's disease in Europe: A collaborative study of population-based cohorts. Neurologic Diseases in the Elderly Research Group. *Neurology* **54,** S21-23 (2000).
2. Nussbaum, R. L. & Ellis, C. E. Alzheimer's disease and Parkinson's disease. *N. Engl. J. Med.* **348,** 1356–1364 (2003).
3. Aarsland, D. *et al.* Cognitive decline in Parkinson disease. *Nat. Rev. Neurol.* **13,** 217–231 (2017).
4. Haehner, A., Hummel, T. & Reichmann, H. Olfactory dysfunction as a diagnostic marker for Parkinson's disease. *Expert Rev. Neurother.* **9,** 1773–1779 (2009).
5. Ponsen, M. M., Stoffers, D., Twisk, J. W. R., Wolters, E. C. & Berendse, H. W. Hyposmia and executive dysfunction as predictors of future Parkinson's disease: a prospective study. *Mov. Disord. Off. J. Mov. Disord. Soc.* **24,** 1060–1065 (2009).
6. Ross, G. W. *et al.* Association of olfactory dysfunction with incidental Lewy bodies. *Mov. Disord. Off. J. Mov. Disord. Soc.* **21,** 2062–2067 (2006).
7. Waseem, S. & Gwinn-Hardy, K. Pain in Parkinson's disease. Common yet seldom recognized symptom is treatable. *Postgrad. Med.* **110,** 33–34, 39–40, 46 (2001).
8. Remy, P., Doder, M., Lees, A., Turjanski, N. & Brooks, D. Depression in Parkinson's disease: loss of dopamine and noradrenaline innervation in the limbic system. *Brain J. Neurol.* **128,** 1314–1322 (2005).
9. Lim, S.-Y. & Lang, A. E. The nonmotor symptoms of Parkinson's disease--an overview. *Mov. Disord. Off. J. Mov. Disord. Soc.* **25 Suppl 1,** S123-130 (2010).
10. Pfeiffer, R. F. Gastrointestinal dysfunction in Parkinson's disease. *Parkinsonism Relat. Disord.* **17,** 10–15 (2011).
11. Fasano, A., Visanji, N. P., Liu, L. W. C., Lang, A. E. & Pfeiffer, R. F. Gastrointestinal dysfunction in Parkinson's disease. *Lancet Neurol.* **14,** 625–639 (2015).
12. Jost, W. H. Gastrointestinal dysfunction in Parkinson's Disease. *J. Neurol. Sci.* **289,** 69–73 (2010).
13. Savica, R. *et al.* Medical records documentation of constipation preceding Parkinson disease: A case-control study. *Neurology* **73,** 1752–1758 (2009).
14. Abbott, R. D. *et al.* Frequency of bowel movements and the future risk of Parkinson's disease. *Neurology* **57,** 456–462 (2001).
15. Gao, X., Chen, H., Schwarzschild, M. A. & Ascherio, A. A prospective study of bowel movement frequency and risk of Parkinson's disease. *Am. J. Epidemiol.* **174,** 546–551 (2011).
16. Chen, H. *et al.* Meta-analyses on prevalence of selected Parkinson's nonmotor symptoms before and after diagnosis. *Transl. Neurodegener.* **4,** 1 (2015).
17. Pan-Montojo, F. *et al.* Environmental toxins trigger PD-like progression via increased alpha-synuclein release from enteric neurons in mice. *Sci. Rep.* **2,** 898 (2012).
18. Clairembault, T., Leclair-Visonneau, L., Neunlist, M. & Derkinderen, P. Enteric glial cells: new players in Parkinson's disease? *Mov. Disord. Off. J. Mov. Disord. Soc.* **30,** 494–498 (2015).
19. Klingelhoefer, L. & Reichmann, H. Pathogenesis of Parkinson disease-the gut-brain axis and environmental factors. *Nat. Rev. Neurol.* **11,** 625–636 (2015).
20. Mulak, A. & Bonaz, B. Brain-gut-microbiota axis in Parkinson's disease. *World J. Gastroenterol. WJG* **21,** 10609–10620 (2015).
21. Cryan, J. F. & Dinan, T. G. Mind-altering microorganisms: the impact of the gut microbiota on brain and behaviour. *Nat. Rev. Neurosci.* **13,** 701–712 (2012).
22. Rhee, S. H., Pothoulakis, C. & Mayer, E. A. Principles and clinical implications of the brain-gut-enteric microbiota axis. *Nat. Rev. Gastroenterol. Hepatol.* **6,** 306–314 (2009).
23. Grenham, S., Clarke, G., Cryan, J. F. & Dinan, T. G. Brain-gut-microbe communication in health and disease. *Front. Physiol.* **2,** 94 (2011).
24. Borre, Y. E., Moloney, R. D., Clarke, G., Dinan, T. G. & Cryan, J. F. The impact of microbiota on brain and behavior: mechanisms & therapeutic potential. *Adv. Exp. Med. Biol.* **817,** 373–403 (2014).
25. Braak, H., De Vos, R. a I., Bohl, J. & Del Tredici, K. Gastric α-synuclein immunoreactive inclusions in Meissner's and Auerbach's plexuses in cases staged for Parkinson's disease-related brain pathology. *Neurosci. Lett.* **396,** 67–72 (2006).
26. Shannon, K. M. *et al.* Alpha-synuclein in colonic submucosa in early untreated Parkinson's disease. *Mov. Disord. Off. J. Mov. Disord. Soc.* **27,** 709–715 (2012).
27. Braak, H., Rüb, U., Gai, W. P. & Del Tredici, K. Idiopathic Parkinson's disease: Possible routes by which vulnerable neuronal types may be subject to neuroinvasion by an unknown pathogen. *J. Neural Transm.* **110,** 517–536 (2003).
28. Hawkes, C. H., Del Tredici, K. & Braak, H. Parkinson's disease: the dual hit theory revisited. *Ann. N. Y. Acad. Sci.* **1170,** 615–622 (2009).

29. Hawkes, C. H., Del Tredici, K. & Braak, H. A timeline for Parkinson's disease. *Parkinsonism Relat. Disord.* **16,** 79–84 (2010).
30. Guan, J. *et al.* Vascular degeneration in Parkinson's disease. *Brain Pathol. Zurich Switz.* **23,** 154–164 (2013).
31. Schrag, A. & Quinn, N. Dyskinesias and motor fluctuations in Parkinson's disease. A community-based study. *Brain J. Neurol.* **123 (Pt 11),** 2297–2305 (2000).
32. Lee, H. M. & Koh, S.-B. Many Faces of Parkinson's Disease: Non-Motor Symptoms of Parkinson's Disease. *J. Mov. Disord.* **8,** 92–97 (2015).
33. Poewe, W., Antonini, A., Zijlmans, J. C., Burkhard, P. R. & Vingerhoets, F. Levodopa in the treatment of Parkinson's disease: an old drug still going strong. *Clin. Interv. Aging* **5,** 229–238 (2010).
34. Chaudhuri, K. R. *et al.* The nondeclaration of nonmotor symptoms of Parkinson's disease to health care professionals: an international study using the nonmotor symptoms questionnaire. *Mov. Disord. Off. J. Mov. Disord. Soc.* **25,** 704–709 (2010).
35. Chaudhuri, K. R. & Schapira, A. H. Non-motor symptoms of Parkinson's disease: dopaminergic pathophysiology and treatment. *Lancet Neurol.* **8,** 464–474 (2009).
36. Martinez-Martin, P. The importance of non-motor disturbances to quality of life in Parkinson's disease. *J. Neurol. Sci.* **310,** 12–16 (2011).
37. Abbott, R. D. *et al.* Bowel movement frequency in late-life and incidental Lewy bodies. *Mov. Disord. Off. J. Mov. Disord. Soc.* **22,** 1581–1586 (2007).
38. Cersosimo, M. G. *et al.* Gastrointestinal manifestations in Parkinson's disease: prevalence and occurrence before motor symptoms. *J. Neurol.* **260,** 1332–1338 (2013).
39. Kaye, J., Gage, H., Kimber, A., Storey, L. & Trend, P. Excess burden of constipation in Parkinson's disease: A pilot study. *Mov. Disord.* **21,** 1270–1273 (2006).
40. Sakakibara, R. *et al.* Colonic transit time and rectoanal videomanometry in Parkinson's disease. *J. Neurol. Neurosurg. Psychiatry* **74,** 268–272 (2003).
41. Verbaan, D. *et al.* Patient-reported autonomic symptoms in Parkinson disease. *Neurology* **69,** 333–341 (2007).
42. Gallagher, D. A., Lees, A. J. & Schrag, A. What are the most important nonmotor symptoms in patients with Parkinson's disease and are we missing them? *Mov. Disord. Off. J. Mov. Disord. Soc.* **25,** 2493–2500 (2010).
43. Kieburtz, K. & Wunderle, K. B. Parkinson's disease: evidence for environmental risk factors. *Mov. Disord. Off. J. Mov. Disord. Soc.* **28,** 8–13 (2013).
44. Wirdefeldt, K., Adami, H.-O., Cole, P., Trichopoulos, D. & Mandel, J. Epidemiology and etiology of Parkinson's disease: a review of the evidence. *Eur. J. Epidemiol.* **26 Suppl 1,** S1-58 (2011).
45. Burré, J. *et al.* Alpha-synuclein promotes SNARE-complex assembly in vivo and in vitro. *Science* **329,** 1663–1667 (2010).
46. Dikiy, I. & Eliezer, D. Folding and misfolding of alpha-synuclein on membranes. *Biochim. Biophys. Acta* **1818,** 1013–1018 (2012).
47. Gibb, W. R. & Lees, A. J. The significance of the Lewy body in the diagnosis of idiopathic Parkinson's disease. *Neuropathol. Appl. Neurobiol.* **15,** 27–44 (1989).
48. Braak, H., Sandmann-Keil, D., Gai, W. & Braak, E. Extensive axonal Lewy neurites in Parkinson's disease: a novel pathological feature revealed by alpha-synuclein immunocytochemistry. *Neurosci. Lett.* **265,** 67–69 (1999).
49. Visanji, N. P., Brooks, P. L., Hazrati, L.-N. & Lang, A. E. The prion hypothesis in Parkinson's disease: Braak to the future. *Acta Neuropathol. Commun.* **1,** 2 (2013).
50. Jucker, M. & Walker, L. C. Self-propagation of pathogenic protein aggregates in neurodegenerative diseases. *Nature* **501,** 45–51 (2013).
51. Forsyth, C. B. *et al.* Increased intestinal permeability correlates with sigmoid mucosa alpha-synuclein staining and endotoxin exposure markers in early Parkinson's disease. *PLoS ONE* **6,** (2011).
52. Gelpi, E. *et al.* Multiple organ involvement by alpha-synuclein pathology in lewy body disorders. *Mov. Disord.* **29,** 1010–1018 (2014).
53. Gold, A., Turkalp, Z. T. & Munoz, D. G. Enteric alpha-synuclein expression is increased in Parkinson's disease but not Alzheimer's disease. *Mov. Disord. Off. J. Mov. Disord. Soc.* **28,** 237–240 (2013).
54. Sánchez-Ferro, Á. *et al.* In vivo gastric detection of α-synuclein inclusions in Parkinson's disease. *Mov. Disord. Off. J. Mov. Disord. Soc.* **30,** 517–524 (2015).
55. Shannon, K. M., Keshavarzian, A., Dodiya, H. B., Jakate, S. & Kordower, J. H. Is alpha-synuclein in the colon a biomarker for premotor Parkinson's Disease? Evidence from 3 cases. *Mov. Disord.* **27,** 716–719 (2012).
56. Beach, T. G. *et al.* Multi-organ distribution of phosphorylated alpha-synuclein histopathology in

subjects with Lewy body disorders. *Acta Neuropathol. (Berl.)* **119,** 689–702 (2010).
57. Hawkes, C. H., Del Tredici, K. & Braak, H. Parkinson's disease: a dual-hit hypothesis. *Neuropathol. Appl. Neurobiol.* **33,** 599–614 (2007).
58. Keshavarzian, A. *et al.* Colonic bacterial composition in Parkinson's disease. *Mov. Disord. Off. J. Mov. Disord. Soc.* **30,** 1351–1360 (2015).
59. Hawkes, C. H., Del Tredici, K. & Braak, H. Parkinson's disease: the dual hit theory revisited. *Ann. N. Y. Acad. Sci.* **1170,** 615–622 (2009).
60. Holmqvist, S. *et al.* Direct evidence of Parkinson pathology spread from the gastrointestinal tract to the brain in rats. *Acta Neuropathol. (Berl.)* **128,** 805–820 (2014).
61. Desplats, P. *et al.* Inclusion formation and neuronal cell death through neuron-to-neuron transmission of alpha-synuclein. *Proc. Natl. Acad. Sci. U. S. A.* **106,** 13010–13015 (2009).
62. Angot, E. *et al.* Alpha-synuclein cell-to-cell transfer and seeding in grafted dopaminergic neurons in vivo. *PloS One* **7,** e39465 (2012).
63. Brundin, P., Li, J.-Y., Holton, J. L., Lindvall, O. & Revesz, T. Research in motion: the enigma of Parkinson's disease pathology spread. *Nat. Rev. Neurosci.* **9,** 741–745 (2008).
64. Li, J.-Y. *et al.* Lewy bodies in grafted neurons in subjects with Parkinson's disease suggest host-to-graft disease propagation. *Nat. Med.* **14,** 501–503 (2008).
65. Kordower, J. H. & Brundin, P. Lewy body pathology in long-term fetal nigral transplants: is Parkinson's disease transmitted from one neural system to another? *Neuropsychopharmacol. Off. Publ. Am. Coll. Neuropsychopharmacol.* **34,** 254 (2009).
66. Svensson, E. *et al.* Vagotomy and Subsequent Risk of Parkinson's Disease. *Ann. Neurol.* (2015). doi:10.1002/ana.24448
67. Pan-Montojo, F. *et al.* Progression of Parkinson's disease pathology is reproduced by intragastric administration of rotenone in mice. *PLoS ONE* **5,** (2010).
68. Quigley, E. M. M. & Quera, R. Small intestinal bacterial overgrowth: roles of antibiotics, prebiotics, and probiotics. *Gastroenterology* **130,** S78-90 (2006).
69. Glass, C. K., Saijo, K., Winner, B., Marchetto, M. C. & Gage, F. H. Mechanisms underlying inflammation in neurodegeneration. *Cell* **140,** 918–934 (2010).
70. Banks, W. A. *et al.* Nitric oxide isoenzymes regulate lipopolysaccharide-enhanced insulin transport across the blood-brain barrier. *Endocrinology* **149,** 1514–1523 (2008).
71. Banks, W. A. & Erickson, M. A. The blood–brain barrier and immune function and dysfunction. *Spec. Issue Blood Brain Barrier* **37,** 26–32 (2010).
72. Devos, D. *et al.* Colonic inflammation in Parkinson's disease. *Neurobiol. Dis.* **50,** 42–48 (2013).
73. Scheperjans, F. *et al.* Gut microbiota are related to Parkinson's disease and clinical phenotype. *Mov. Disord. Off. J. Mov. Disord. Soc.* **30,** 350–358 (2015).
74. Arumugam, M. *et al.* Enterotypes of the human gut microbiome. *Nature* **473,** 174–180 (2011).
75. dos Santos, E. F. *et al.* Evidence that folic acid deficiency is a major determinant of hyperhomocysteinemia in Parkinson's disease. *Metab. Brain Dis.* **24,** 257–269 (2009).
76. Luong, K. V. Q. & Nguyễn, L. T. H. The beneficial role of thiamine in Parkinson disease. *CNS Neurosci. Ther.* **19,** 461–468 (2013).
77. Brown, C. T. *et al.* Gut microbiome metagenomics analysis suggests a functional model for the development of autoimmunity for type 1 diabetes. *PloS One* **6,** e25792 (2011).
78. Andrews, Z. B. *et al.* Ghrelin promotes and protects nigrostriatal dopamine function via a UCP2-dependent mitochondrial mechanism. *J. Neurosci. Off. J. Soc. Neurosci.* **29,** 14057–14065 (2009).
79. Unger, M. M. *et al.* Postprandial ghrelin response is reduced in patients with Parkinson's disease and idiopathic REM sleep behaviour disorder: a peripheral biomarker for early Parkinson's disease? *J. Neurol.* **258,** 982–990 (2011).
80. Hueck, C. J. Type III protein secretion systems in bacterial pathogens of animals and plants. *Microbiol. Mol. Biol. Rev. MMBR* **62,** 379–433 (1998).
81. Galán, J. E. & Collmer, A. Type III secretion machines: bacterial devices for protein delivery into host cells. *Science* **284,** 1322–1328 (1999).
82. Unger, M. M. *et al.* Short chain fatty acids and gut microbiota differ between patients with Parkinson's disease and age-matched controls. *Parkinsonism Relat. Disord.* (2016). doi:10.1016/j.parkreldis.2016.08.019
83. Forsythe, P. & Kunze, W. A. Voices from within: gut microbes and the CNS. *Cell. Mol. Life Sci. CMLS* **70,** 55–69 (2013).
84. Ganapathy, V., Thangaraju, M., Prasad, P. D., Martin, P. M. & Singh, N. Transporters and receptors for short-chain fatty acids as the molecular link between colonic bacteria and the host. *Curr. Opin. Pharmacol.* **13,** 869–874 (2013).
85. Singh, N. *et al.* Activation of Gpr109a, receptor for niacin and the commensal metabolite butyrate, suppresses colonic inflammation and carcinogenesis. *Immunity* **40,** 128–139 (2014).

86. Maslowski, K. M. & Mackay, C. R. Diet, gut microbiota and immune responses. *Nat. Immunol.* **12,** 5–9 (2011).
87. Gasbarrini, A. *et al.* Small intestinal bacterial overgrowth: diagnosis and treatment. *Dig. Dis. Basel Switz.* **25,** 237–240 (2007).
88. Tan, A. H. *et al.* Small intestinal bacterial overgrowth in Parkinson's disease. *Parkinsonism Relat. Disord.* **20,** 535–540 (2014).
89. Chen, W. C. & Quigley, E. M. M. Probiotics, prebiotics & synbiotics in small intestinal bacterial overgrowth: opening up a new therapeutic horizon! *Indian J. Med. Res.* **140,** 582–584 (2014).
90. Crispo, J. a. G. *et al.* Trends in inpatient antiparkinson drug use in the USA, 2001–2012. *Eur. J. Clin. Pharmacol.* (2015). doi:10.1007/s00228-015-1881-4
91. Lebouvier, T. *et al.* Colonic biopsies to assess the neuropathology of Parkinson's disease and its relationship with symptoms. *PloS One* **5,** e12728 (2010).
92. Freitas, M. E., Ruiz-Lopez, M. & Fox, S. H. Novel Levodopa Formulations for Parkinson's Disease. *CNS Drugs* **30,** 1079–1095 (2016).
93. Epprecht, L. *et al.* Unchanged gastric emptying and visceral perception in early Parkinson's disease after a high caloric test meal. *J. Neurol.* **262,** 1946–1953 (2015).
94. Robertson, D. R. *et al.* The influence of levodopa on gastric emptying in man. *Br. J. Clin. Pharmacol.* **29,** 47–53 (1990).
95. Robertson, D. R. *et al.* The influence of levodopa on gastric emptying in healthy elderly volunteers. *Eur. J. Clin. Pharmacol.* **42,** 409–412 (1992).
96. Waller, D. G., Roseveare, C., Renwick, A. G., Macklin, B. & George, C. F. Gastric emptying in healthy volunteers after multiple doses of levodopa. *Br. J. Clin. Pharmacol.* **32,** 691–695 (1991).
97. Doi, H. *et al.* Plasma levodopa peak delay and impaired gastric emptying in Parkinson's disease. *J. Neurol. Sci.* **319,** 86–88 (2012).
98. Hardoff, R. *et al.* Gastric emptying time and gastric motility in patients with Parkinson's disease. *Mov. Disord. Off. J. Mov. Disord. Soc.* **16,** 1041–1047 (2001).
99. Marrinan, S., Emmanuel, A. V. & Burn, D. J. Delayed gastric emptying in Parkinson's disease. *Mov. Disord. Off. J. Mov. Disord. Soc.* **00,** 1–10 (2013).
100. Clemente, J. C., Ursell, L. K., Parfrey, L. W. & Knight, R. The impact of the gut microbiota on human health: An integrative view. *Cell* **148,** 1258–1270 (2012).
101. Perez-Pardo, P. *et al.* Promising Effects of Neurorestorative Diets on Motor, Cognitive, and Gastrointestinal Dysfunction after Symptom Development in a Mouse Model of Parkinson's Disease. *Front. Aging Neurosci.* **9,** 57 (2017).
102. Araki, W. & Wurtman, R. J. How is membrane phospholipid biosynthesis controlled in neural tissues? *J. Neurosci. Res.* **51,** 667–674 (1998).
103. Wurtman, R. J. A nutrient combination that can affect synapse formation. *Nutrients* **6,** 1701–1710 (2014).
104. Van Wijk, N. *et al.* Targeting synaptic dysfunction in Alzheimer's disease by administering a specific nutrient combination. *J. Alzheimers Dis.* **38,** 459–479 (2014).
105. Wurtman, R. J., Cansev, M., Sakamoto, T. & Ulus, I. H. Use of phosphatide precursors to promote synaptogenesis. *Annu. Rev. Nutr.* **29,** 59–87 (2009).
106. Holguin, S., Huang, Y., Liu, J. & Wurtman, R. Chronic administration of DHA and UMP improves the impaired memory of environmentally impoverished rats. *Behav. Brain Res.* **191,** 11–16 (2008).
107. Sakamoto, T., Cansev, M. & Wurtman, R. J. Oral supplementation with docosahexaenoic acid and uridine-5'-monophosphate increases dendritic spine density in adult gerbil hippocampus. *Brain Res.* **1182,** 50–59 (2007).
108. Wurtman, R. J. *et al.* Synaptic proteins and phospholipids are increased in gerbil brain by administering uridine plus docosahexaenoic acid orally. *Brain Res.* **1088,** 83–92 (2006).
109. Cansev, M., Ulus, I. H., Wang, L., Maher, T. J. & Wurtman, R. J. Restorative effects of uridine plus docosahexaenoic acid in a rat model of Parkinson's disease. *Neurosci. Res.* **62,** 206–209 (2008).
110. Kamel, F. *et al.* Dietary fat intake, pesticide use, and Parkinson's disease. *Parkinsonism Relat. Disord.* **20,** 82–87 (2014).
111. Bousquet, M. *et al.* Beneficial effects of dietary omega-3 polyunsaturated fatty acid on toxin-induced neuronal degeneration in an animal model of Parkinson's disease. *FASEB J. Off. Publ. Fed. Am. Soc. Exp. Biol.* **22,** 1213–1225 (2008).
112. Delattre, A. M. *et al.* Evaluation of chronic omega-3 fatty acids supplementation on behavioral and neurochemical alterations in 6-hydroxydopamine-lesion model of Parkinson's disease. *Neurosci. Res.* **66,** 256–264 (2010).
113. Myers, C. S., Fisher, H. & Wagner, G. C. Uridine reduces rotation induced by L-dopa and methamphetamine in 6-OHDA-treated rats. *Pharmacol. Biochem. Behav.* **52,** 749–753 (1995).
114. Perez-Pardo, P. *et al.* Gut–brain and brain–gut axis in Parkinson's disease models: Effects of a uridine and fish oil diet. *Nutr. Neurosci.* 1–12 (2017). doi:10.1080/1028415X.2017.1294555

115. Miller, R. L., James-Kracke, M., Sun, G. Y. & Sun, A. Y. Oxidative and inflammatory pathways in Parkinson's disease. *Neurochem. Res.* **34,** 55–65 (2009).
116. Afshordel, S. *et al.* Omega-3 polyunsaturated fatty acids improve mitochondrial dysfunction in brain aging - Impact of Bcl-2 and NPD-1 like metabolites. *Prostaglandins Leukot. Essent. Fatty Acids* **92,** 23–31 (2014).
117. Bazan, N. G., Molina, M. F. & Gordon, W. C. NIH Public Access. 321–351 (2012). doi:10.1146/annurev.nutr.012809.104635.Docosahexaenoic
118. van Wijk, N. *et al.* Targeting synaptic dysfunction in Alzheimer's disease by administering a specific nutrient combination. *J. Alzheimers Dis. JAD* **38,** 459–479 (2014).
119. Broersen, L. M. *et al.* A specific multi-nutrient diet reduces Alzheimer-like pathology in young adult AβPPswe/PS1dE9 mice. *J. Alzheimers Dis. JAD* **33,** 177–190 (2013).
120. Zerbi, V. *et al.* Multinutrient diets improve cerebral perfusion and neuroprotection in a murine model of Alzheimer's disease. *Neurobiol. Aging* **35,** 600–613 (2014).
121. Janickova, H. *et al.* Lipid-Based Diets Improve Muscarinic Neurotransmission in the Hippocampus of Transgenic APPswe/PS1dE9 Mice. *Curr. Alzheimer Res.* **12,** 923–931 (2015).
122. de Kivit, S., Kraneveld, A. D., Garssen, J. & Willemsen, L. E. M. Glycan recognition at the interface of the intestinal immune system: target for immune modulation via dietary components. *Eur. J. Pharmacol.* **668 Suppl 1,** S124-132 (2011).
123. Meksawan, K. *et al.* EFFECTS OF FRUCTO-OLIGOSACCHARIDE SUPPLEMENTATION ON CONSTIPATION IN ELDERLY CONTINUOUS AMBULATORY PERITONEAL DIALYSIS PATIENTS. *Perit. Dial. Int. J. Int. Soc. Perit. Dial.* (2014). doi:10.3747/pdi.2014.00015
124. Scholtens, P. A. M. J., Goossens, D. A. M. & Staiano, A. Stool characteristics of infants receiving short-chain galacto-oligosaccharides and long-chain fructo-oligosaccharides: a review. *World J. Gastroenterol.* **20,** 13446–13452 (2014).
125. Cryan, J. F. & Dinan, T. G. More than a gut feeling: the microbiota regulates neurodevelopment and behavior. *Neuropsychopharmacol. Off. Publ. Am. Coll. Neuropsychopharmacol.* **40,** 241–242 (2015).
126. Reid, G. *et al.* Microbiota restoration: natural and supplemented recovery of human microbial communities. *Nat. Rev. Microbiol.* **9,** 27–38 (2011).
127. Varankovich, N. V., Nickerson, M. T. & Korber, D. R. Probiotic-based strategies for therapeutic and prophylactic use against multiple gastrointestinal diseases. *Front. Microbiol.* **6,** 685 (2015).
128. Ait-Belgnaoui, A. *et al.* Prevention of gut leakiness by a probiotic treatment leads to attenuated HPA response to an acute psychological stress in rats. *Psychoneuroendocrinology* **37,** 1885–1895 (2012).
129. Corridoni, D. *et al.* Probiotic bacteria regulate intestinal epithelial permeability in experimental ileitis by a TNF-dependent mechanism. *PloS One* **7,** e42067 (2012).
130. Patel, R. M. *et al.* Probiotic bacteria induce maturation of intestinal claudin 3 expression and barrier function. *Am. J. Pathol.* **180,** 626–635 (2012).
131. Sartor, R. B. Probiotic therapy of intestinal inflammation and infections. *Curr. Opin. Gastroenterol.* **21,** 44–50 (2005).
132. Zareie, M. *et al.* Probiotics prevent bacterial translocation and improve intestinal barrier function in rats following chronic psychological stress. *Gut* **55,** 1553–1560 (2006).
133. Zaharoni, H. *et al.* Probiotics improve bowel movements in hospitalized elderly patients--the PROAGE study. *J. Nutr. Health Aging* **15,** 215–220 (2011).
134. Ojetti, V. *et al.* The effect of Lactobacillus reuteri supplementation in adults with chronic functional constipation: a randomized, double-blind, placebo-controlled trial. *J. Gastrointest. Liver Dis. JGLD* **23,** 387–391 (2014).
135. Hsiao, E. Y. *et al.* Microbiota modulate behavioral and physiological abnormalities associated with neurodevelopmental disorders. *Cell* **155,** 1451–1463 (2013).
136. Rao, A. V. *et al.* A randomized, double-blind, placebo-controlled pilot study of a probiotic in emotional symptoms of chronic fatigue syndrome. *Gut Pathog.* **1,** 6 (2009).
137. Cassani, E. *et al.* Use of probiotics for the treatment of constipation in Parkinson's disease patients. *Minerva Gastroenterol. Dietol.* **57,** 117–121 (2011).
138. Liang, S. *et al.* Administration of Lactobacillus helveticus NS8 improves behavioral, cognitive, and biochemical aberrations caused by chronic restraint stress. *Neuroscience* (2015). doi:10.1016/j.neuroscience.2015.09.033
139. Maes, M., Kubera, M. & Leunis, J.-C. The gut-brain barrier in major depression: intestinal mucosal dysfunction with an increased translocation of LPS from gram negative enterobacteria (leaky gut) plays a role in the inflammatory pathophysiology of depression. *Neuro Endocrinol. Lett.* **29,** 117–124 (2008).

140. Gibson, G. R. & Roberfroid, M. B. Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. *J. Nutr.* **125,** 1401–1412 (1995).
141. Kovács, Z. *et al.* Recent developments in manufacturing oligosaccharides with prebiotic functions. *Adv. Biochem. Eng. Biotechnol.* **143,** 257–295 (2014).
142. Delzenne, N. M. Oligosaccharides: state of the art. *Proc. Nutr. Soc.* **62,** 177–182 (2003).
143. Jeurink, P. V., van Esch, B. C. A. M., Rijnierse, A., Garssen, J. & Knippels, L. M. J. Mechanisms underlying immune effects of dietary oligosaccharides. *Am. J. Clin. Nutr.* **98,** 572S–7S (2013).
144. van Hoffen, E. *et al.* A specific mixture of short-chain galacto-oligosaccharides and long-chain fructo-oligosaccharides induces a beneficial immunoglobulin profile in infants at high risk for allergy. *Allergy* **64,** 484–487 (2009).
145. Rasmussen, H., Piazza, B., Forsyth, C. & Keshavarzian, A. in *Pharma-Nutrition* (eds. Folkerts, G. & Garssen, J.) **12,** 213–242 (Springer International Publishing, 2014).
146. Savignac, H. M. *et al.* Prebiotic feeding elevates central brain derived neurotrophic factor, N-methyl-D-aspartate receptor subunits and D-serine. *Neurochem. Int.* **63,** 756–764 (2013).
147. Numakawa, T. *et al.* BDNF function and intracellular signaling in neurons. *Histol. Histopathol.* **25,** 237–258 (2010).
148. Frei, R., Akdis, M. & O'Mahony, L. Prebiotics, probiotics, synbiotics, and the immune system. *Curr. Opin. Gastroenterol.* **31,** 153–158 (2015).
149. Schrezenmeir, J. & de Vrese, M. Probiotics, prebiotics, and synbiotics--approaching a definition. *Am. J. Clin. Nutr.* **73,** 361S–364S (2001).
150. Rajkumar, H. *et al.* Effect of Probiotic Lactobacillus salivarius UBL S22 and Prebiotic Fructo-oligosaccharide on Serum Lipids, Inflammatory Markers, Insulin Sensitivity, and Gut Bacteria in Healthy Young Volunteers: A Randomized Controlled Single-Blind Pilot Study. *J. Cardiovasc. Pharmacol. Ther.* **20,** 289–298 (2015).
151. De Paula, J. A., Carmuega, E. & Weill, R. Effect of the ingestion of a symbiotic yogurt on the bowel habits of women with functional constipation. *Acta Gastroenterol. Latinoam.* **38,** 16–25 (2008).
152. Khalighi, A. R. *et al.* Evaluating the efficacy of probiotic on treatment in patients with small intestinal bacterial overgrowth (SIBO)--a pilot study. *Indian J. Med. Res.* **140,** 604–608 (2014).

CHAPTER 8

Additive effects of levodopa and a neurorestorative diet in a mouse model of Parkinson's disease.

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Abstract

Parkinson's disease (PD) clinical picture is usually dominated by motor impairment. However non-motor symptoms, such as cognitive decline and gastrointestinal dysfunctions, may already develop before the motor symptoms and are major determinants of quality of life. The dopamine precursor levodopa is the most commonly used drug in the treatment of motor symptoms but has serious side-effects and does not stop the degeneration process. Moreover, gastrointestinal dysfunctions of PD patients interfere with the absorption of levodopa and modify the effectiveness of the drug. There is a great need for additional therapies that reduce/modulate both motor and non-motor symptoms. In previous experiments we have shown that a diet containing nutritional precursors and cofactors required for membrane phospholipid synthesis, as well as prebiotic fibres, had therapeutic effects in a mouse model for PD. The purpose of the present study was to investigate the effects of combined administration of the same dietary intervention together with levodopa treatment in this model. C57BL/6J mice were injected with rotenone or vehicle in the striatum. The diet intervention started four weeks after surgery when PD-like symptoms were developed. The effects of oral treatment with different doses of levodopa were assessed weekly. Motor and cognitive functions were tested, intestinal transit and colon length were analysed and histological examination of the brain and the colon was assessed. Our results show that rotenone-induced motor and non-motor problems were alleviated by the therapeutic dietary intervention. Levodopa showed an additive beneficial effect on rotarod performance in rotenone-treated animals fed with the diet. No negative interaction effects were found between the diet and levodopa treatment. Our results suggest that the dietary intervention might confer clinical benefits on patients receiving levodopa treatment.

1. Introduction

Parkinson's disease (PD) is the second most frequent age-related neurodegenerative disorder for which there is no cure. It is hallmarked by the progressive degeneration of dopaminergic nigrostriatal neurons, with reductions in striatal dopamine levels resulting in the characteristic motor impairment. Another characteristic of PD is alpha-synuclein containing inclusion bodies (Lewy pathology; LP) in the surviving neurons in different areas of the central and peripheral nervous system[1–3]. Although PD is generally considered as a movement disorder, many patients suffer from non-motor symptoms[4], including olfactory and sleep disturbances[5–7], depression[8], cognitive decline with regard to visuospatial perception and working memory[9], and most commonly gastrointestinal dysfunctions[10–13]. Some of these symptoms may precede the motor phase by several years[14–16], are major determinants of quality of life[17–19], and remain undertreated[20].

L-3,4-di-hydroxy-phenylalanine (levodopa) is a precursor of the neurotransmitter dopamine that suppresses some of the PD motor symptoms since it compensates for dopaminergic cell loss by enhancing dopamine synthesis in the remaining terminals. The oral supplementation with levodopa is, so far, the most efficient drug in the treatment of PD. Between 2001 and 2012, levodopa was used by 85% of patients suffering from PD[21]. However, this therapy has several side effects[22], it does not prevent dopaminergic neuron degeneration, and many of the non-motor symptoms are unresponsive to levodopa[4]. Moreover, PD-associated gastrointestinal dysfunction contributes to levodopa response fluctuations[23]and on-off oscillations[24–27].

We have previously demonstrated beneficial effects of specific dietary interventions in a mouse model of PD when given therapeutically, i.e. after the full induction of motor symptoms[28]. Our previous results showed that rotenone-induced motor, cognitive, and gastrointestinal dysfunctions were significantly alleviated by the therapeutic dietary intervention containing both precursors and cofactors for phospholipid synthesis as well as prebiotic fibers[28].

The purpose of the present study is to see whether levodopa is effective in the rotenone model applied and to examine whether there are interactions and/or additive effects between the diet and levodopa.

2. Methods

Mice

Forty-eight seven week-old C57BL/6J male mice (Charles River, The Netherlands) were housed at room temperature under a 12h light/dark cycle. Food and water were provided *ad libitum.* All animal procedures were approved by the Ethical Committee of Animal Research of Utrecht University, The Netherlands (DEC number 2014.I.12.106).

Surgery

Mice underwent stereotaxic surgery under isoflurane anesthesia: a hole was drilled in the skull and a cannula inserted in the right striatum at the following stereotaxic coordinates: AP +0.4, ML -2.0 from bregma, and DV -3.3 below dura. Rotenone-treated animals were injected with 5.4 µg of freshly prepared rotenone solution (dissolved in 2µl DMSO). Sham-treated animals were injected with the vehicle (2µl DMSO). 93 days after surgery the mice were euthanized by decapitation.

Levodopa effectiveness in the rotenone mouse model

In order to investigate whether levodopa treatment could effectively improve motor function in the intrastriatal rotenone mouse model for PD, all animals were tested on the rotarod and in the grip strength test (as described below) 1h after oral administration of levodopa (20 mg/kg) or vehicle. 30 min before the oral levodopa administration, all animals received a subcutaneous injection of the decarboxylase inhibitor benserazide (6.25 mg/kg). The rotarod tests were performed on days 26 and 27 after surgery, i.e. when rotenone-induced motor dysfunctions were fully developed[28] and before the dietary intervention was started. The order of treatments (levodopa and saline) was balanced according to a Latin square design (Table 1).

allocate	Day 26	Day 27
Sham n=12	saline	levodopa
Sham n=12	levodopa	saline
Rotenone n=12	saline	levodopa
Rotenone n=12	levodopa	saline

Table 1: Specification of the order of treatments received by the animals according to a Latin square design.

Diets

Mice were fed either the Control diet (CD) or the Active diet (AD), starting 28 days after surgery, i.e. when motor symptoms plateaued, and continuing for the duration of the experiment. Animals were divided into four groups of 12 animals (Sham+CD, Sham+AD, Rotenone+CD, and Rotenone+AD). Iso-caloric diets were produced by Research Diet Services (Wijk bij Duurstede, The Netherlands) and were based on the CD, i.e. the standard animal food for laboratory rodents AIN-93M[30] with 5% fat. For the active diet, uridine (0.51 g/100g diet) was added and part of the lipid blend of control diet was replaced by fish oil, providing DHA (0.75 g/100g diet) and EPA (0.50 g/100g diet). The AD also contained supplementary amounts of choline, phospholipids, selenium, folic acid, and vitamins B6, B12, C, D, and E, above standard levels in the control diet. In addition, the cellulose fibers from the CD were replaced by prebiotic fibers (1.5 g/100g diet GOS, 0.17 g/100g diet lcFOS, 1.67 g/100g diet scFOS, and 1.67 g/100g diet nutriose) in the AD.

Experimental design

From day 65 after surgery onward, i.e. when the AD was shown to have beneficial effects on motor performance in a previous study[29], until day 93 (4 weeks in total), animals from the 4 different groups underwent motor function and spatial recognition testing once a week after oral administration of saline or one of 3 doses of levodopa (5, 10, and 20 mg/kg), the order of which was balanced according to a Latin square design (Table 2). All animals received a subcutaneous injection of the decarboxylase inhibitor benserazide (6.25 mg/kg) or vehicle 30min prior to the oral administration of levodopa. The rotarod test was followed by spatial recognition test and grip strength test were started 1.5h after benserazide administration and performed as described below.

Allocate for each experimental group	WEEK 1	WEEK 2	WEEK 3	WEEK 4
n=3	saline	levodopa 20	levodopa 10	levodopa 5
n=3	levodopa 5	saline	levodopa 20	levodopa 10
n=3	levodopa 10	levodopa 5	saline	levodopa 20
n=3	levodopa 20	levodopa 10	levodopa 5	saline

Table 2: Specification of the order of treatments received by the different experimental groups according to a Latin square design.

Motor function tests

The motor performance of each mouse was assessed in the Rotarod test as described before[31]. Briefly, mice were placed on an accelerating rod with speeds starting with 2rpm and gradually increasing to 20rpm. Time to first fall was recorded for a maximum of 300s. The test was performed in order to look at symptom development in time, functional recovery during dietary intervention, and the effects of oral levodopa treatment. Muscular forelimb strength was measured using a grip strength tester as described before[32].

Spatial recognition test

Animals' ability to react to a spatial novelty after a 3-min delay was measured as described before[33]. Mice were individually submitted to seven consecutive, 6-min sessions. During session 1, mice were placed into the empty open field. During sessions 2-4, five objects were present, and mice were placed into the apparatus to habituate to the object configuration (habituation phase). During the 3-min intersession interval, animals were returned to a waiting cage. During the spatial test session (session 5), the object configuration was changed by moving two objects (displaced objects, DO) and leaving the other three objects in the same position (non-displaced objects, NDO). In all sessions, the total activity of the animal was measured. From sessions 2 to 5, object exploration was evaluated on the basis of the mean time spent by the animal in contact with the different objects. The animals' ability to selectively react to the spatial change was analyzed by calculating the spatial re-exploration index (DO[S5] – DO[S4] = DO and NDO[S5] – NDO[S4] = NDO). The time the

animals interacted with the DO minus the time they interact with the NDO was used for analysis (DO-NDO).

Intestinal transit and colon length

Intestinal transit was assessed in all animals. Thirty minutes before sacrificing the mice, a 2.5% Evans blue solution in 1.5% methylcellulose (0.3mL per animal) was intragastrically administered. After euthanasia, intestinal transit was measured as the distance from the pylorus to the most distal point of migration of the Evans blue dye. In addition, the length of the colon was measured.

Tissue preparation and immunohistochemistry

Coronal slices of 40 µm were sectioned using a cryostat (CM3050, Leica Microsystems). Sections were incubated with 0.3% H_2O_2 for 30min. Following blocking serum, sections were incubated overnight with rabbit anti-tyrosine hydroxylase (TH) (Santa-Cruz Biotechnology, 1:1000). Next day, sections were incubated with a biotinylated secondary antibody (Jackson ImmunoResearch, 1:200) for 2h. The avidin-biotin method was used to amplify the signal (ABC Kit, Vector) and 3,3'-diaminobenzidine tetrachloride (DAB) was used as the chromogen.

Image analysis

For immunostained sections, digital images were captured with an Olympus BX50 microscope equipped with a Leica DFC 320 digital camera. To count TH-immunopositive cells, every fourth section of each mouse brain was stained. TH-immunopositive neurons were quantified stereologically on regular spaced sections. Analyses were performed by researchers that were blind to the treatment condition of the animal.

Statistical analysis

Experimental results are expressed as mean ± SEM. Differences between groups were statistically analyzed with a three-way ANOVA, analyzing the effects of the between subject factors surgery (rotenone vs vehicle) and diet (CD vs AD), the within subject factor treatment (levodopa doses 0, 5, 10, and 20 mg/kg), and their interactions. ANOVAs were followed by a Tukey's multiple comparison test when appropriate. For the rotarod test performance over time, data were analyzed with a general linear model repeated measure ANOVA with the within subject factor time and the between subject factors surgery and diet. Results were considered statistically significant when $p<0.05$. Analyses were performed using SPSS 22.0.

3.Results

Oral administration of levodopa is effectively reducing motor dysfunction in the rotenone model for PD.

In order to investigate whether levodopa treatment was effective in the intrastriatal rotenone mouse model for PD and could thus improve motor function, we measured performance on the rotarod (time on rotarod) and the forelimb grip strength 60 minutes after administration of 20 mg/kg oral levodopa on days 26 and 27 after surgery. Rotenone injection in the striatum negatively affected animals' ability to remain on the rod ($F(1,92)=130.5$, $p<0.0001$) and their forelimb grip strength ($F(1,92)=383.4$, $p<0.0001$), as compared to sham-treated mice. There was an overall effect of levodopa on the animals' motor performances ($F(1,92)=24.77$, $p<0.0001$ for the rotarod test and $F(1,92)=4.6$, $p<0.05$ for the grip strength test) and an interaction effect between surgery and treatment in both the rotarod test ($F(1,92)=27.66$, $p<0.0001$) and the grip strength test ($F(1,92)=4.12$, $p<0.05$). Post-hoc analyses revealed a significant increase in motor function for rotenone-injected mice treated with levodopa (20mg/kg) as compared to rotenone-injected mice treated with saline ($p<0.0001$ for the rotarod test, $p<0.05$ for the grip strength test), while levodopa administration had no effect on motor performance in sham-treated mice (Fig 1).

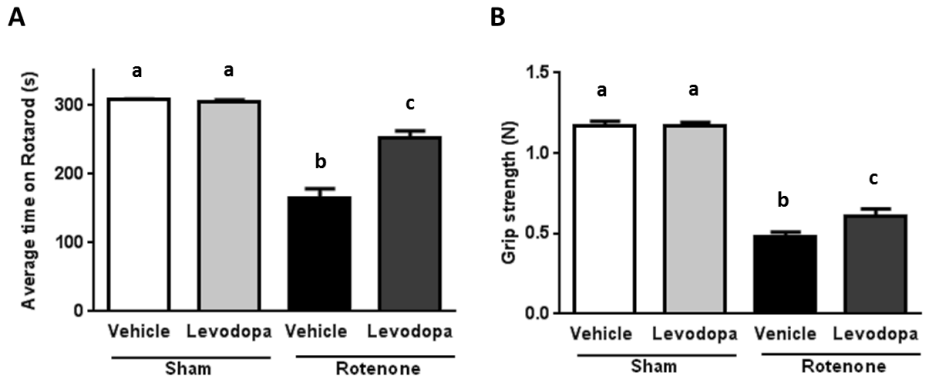


Figure 1: Effects of oral levodopa treatment (20mg/kg) on (A) rotarod performance and (B) grip strength test in sham and rotenone treated groups of mice. Intrastriatal rotenone injection induced motor dysfunction and grip strength loss. Oral Levodopa treatment had beneficial effects on motor function and grip strength in rotenone treated animals. Data are shown as mean ± SEM. Different letters indicate mean values were significantly different ($p<0.05$).

The dietary intervention has a restorative effect on rotenone-induced motor dysfunction.
Rotarod performance was measured every 7 days to analyze the effects of rotenone injection and the dietary intervention over time. These measurements were conducted at least one day before animals received levodopa to solely analyze the effectiveness of the dietary intervention.

The time spent on the rod was used for analysis of motor function. There was an overall effect of surgery ($F(1,44)=132.08$, $p<0.0001$) and of diet ($F(1,44)=6.97$, $p<0.05$) on rotarod performance. Repeated measures showed an effect of time ($F(10,440)=36.71$, $p<0.0001$). Rotenone-injected mice developed motor dysfunction over time compared to sham (interaction effect surgery x time $F(10,440)=39.55$ $p<0.0001$). Furthermore, there was an interaction effect between diet and time ($F(10,440)=4.32$, $p<0.0001$), between diet and surgery ($F(1,44)=5.48$, $p<0.05$) and between diet, surgery and time ($F(10,440)=5.08$, $p<0.0001$). Rotenone-injected mice showed a decrease in rotarod performance starting from day 28 after surgery onwards compared to the sham-operated mice ($F(1,44)=48.63$, $p<0.0001$). On day 56 after surgery there was a significant effect of the diets ($F(1,44)=10.71$, $p<0.01$) on rotarod performance that remained for the duration of the experiment ($F(1,44)=31.47$, $p<0.0001$ day 91) (Fig2).

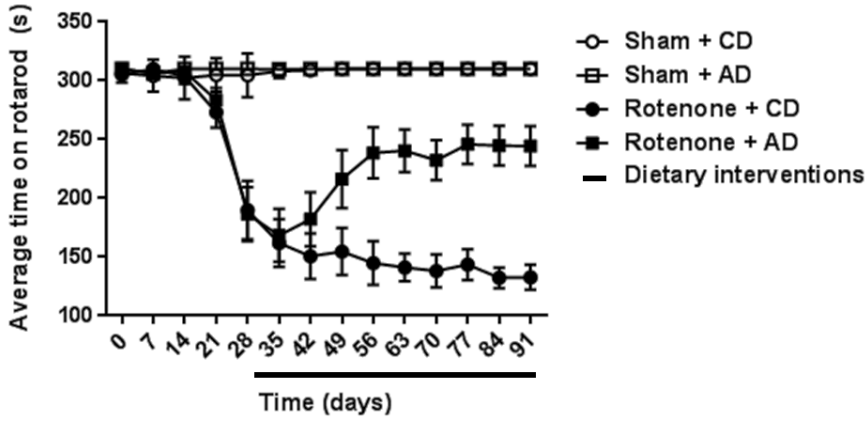


Figure 2: Effects of surgery and dietary intervention on rotarod performance over the course of the experiment. Intrastriatal rotenone injection induced a clear motor dysfunction. Dietary intervention started at day 28 after surgery, i.e. after the full development of rotenone-induced motor dysfunction. As compared to Control diet (CD), the Active diet (AD) showed beneficial effects on motor function from day 56 onward, until the end of the experiment. Data are shown as mean ± SEM.

The dietary intervention has no effects on rotenone-induced dopaminergic cell loss.

To investigate the rotenone-induced neurodegeneration, we performed unbiased stereology to estimate the number of TH positive dopaminergic cells in the SN at the end of the experiment (day 93). Rotenone-treated mice showed a decrease in the number of TH positive cells compared to sham-injected mice ($F(1,24)=318$, $p<0.001$). No differences were found in the total number of TH positive cells in the SN between the two brain hemispheres. There was no main effect of diet and no significant interaction between surgery and diet (Fig3).

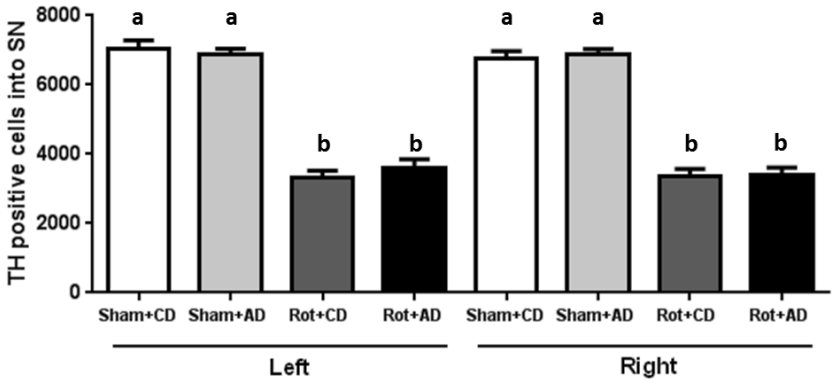


Figure 3: Effects of dietary intervention started after development of full motor dysfunction on the number of dopaminergic cells indicated by the number of tyrosine hydroxylase (TH) immunoreactive cells in the substantia nigra. The dietary intervention had no effect on the number of TH positive cells. Data are shown as mean ± SEM. Different letters indicate mean values were significantly different ($p<0.05$).

The dietary intervention has a restorative effect on rotenone-induced delayed intestinal transit and reduced colon length.

Rotenone injection in the striatum negatively affected intestinal transit, i.e. decreased the distance travelled by the Evans Blue dye in the intestinal tract, as compared to sham-injected mice ($F(1,40)=58.94$, $p<0.0001$). There was an overall effect of diet on intestinal transit ($F(1,40)=4.24$, $p<0.05$) and an interaction effect between surgery and diet ($F(1,40)=25.23$, $p<0.0001$). Improved intestinal transit (increased distance covered by the dye) was observed in rotenone-injected mice on AD compared to rotenone-injected mice on control diet ($p<0.0001$).

The length of the colon of all the animals was measured as a gross indicator of inflammation. There was a main effect of surgery ($F(1,44)=54.20$, $p<0.0001$) and of diet ($F(1,44)=26.78$, $p<0.001$), as well as an interaction effect between surgery and diet ($F(1,44)=13.40$, $p<0.001$). Post-hoc analyses revealed a smaller decrease in colon length in rotenone-injected mice on the AD compared to the CD ($p<0.0001$) (Fig4).

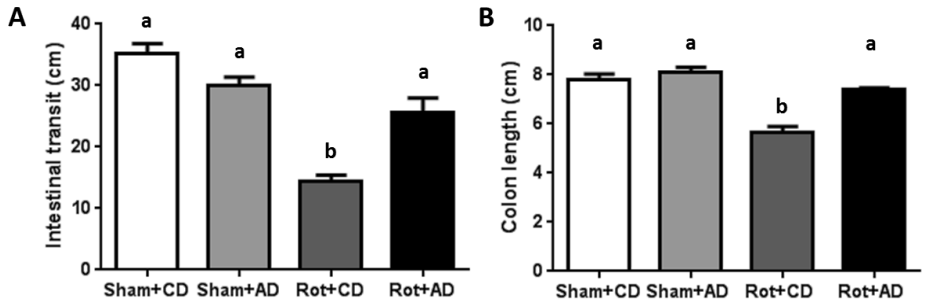


Figure 4: Effects of the dietary intervention started after development of full motor dysfunction on: (A) intestinal transit indicated by the total distance travelled by the Evans blue dye in the GI tract 30 min after its administration by oral gavage, and on (B) colon length. Rotenone injection reduced intestinal transit and colon length. The Active dietary intervention improved rotenone-induced delayed intestinal transit and improved rotenone-induced shortening of the colon. Data are shown as mean ± SEM. Different letters indicate mean values were significantly different ($p<0.05$).

The dietary intervention combined with oral levodopa showed to have additive effects on rotarod performance.

Between day 65 and day 93, dose-response relationships for levodopa on behavioral performances were assessed in rotenone- and sham-treated animals.

Rotenone treated animals exhibited a deterioration of rotarod performance compared to sham-treated animals ($F(1,176)=425.95$, $p<0.0001$). Rotarod data showed that there was an effect of the diet ($F(1,176)=48.28$, $p<0.0001$) and of levodopa treatment ($F(3,176)=10.40$, $p<0.0001$). Furthermore, there were interaction effects between surgery and diet ($F(1,176)=44.89$, $p<0.0001$), and between surgery and levodopa treatment ($F(3,176)=8.94$, $p<0.0001$).

Post-hoc analyses showed that rotenone-treated animals on CD performed significantly better on the rotarod when treated with the highest dose of levodopa (20mg/kg) compared to animals treated with the lowest dose (5mg/kg) or saline ($p<0.01$). Within the rotenone-injected mice on AD, animals treated with levodopa 10 or 20 mg/kg had a better ability to remain on the rod than animals on saline or 5 mg/kg ($p<0.001$), showing an additive beneficial effect of levodopa.

Rotenone treated mice on AD performed significantly better than the animals on CD diet at all doses (0, 5, 10, and 20 mg/kg) (all $p < 0.05$) (Fig5A).

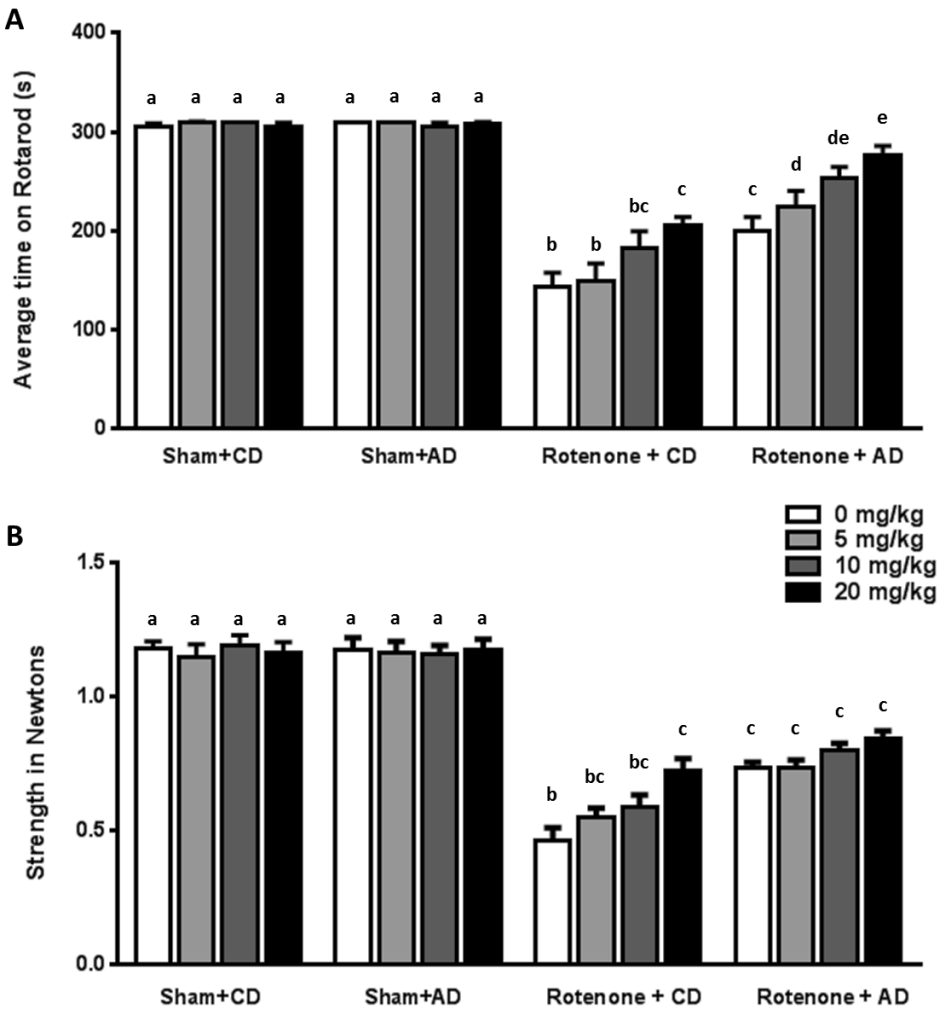


Figure 5: Effects of the dietary intervention and different levodopa doses on (A) rotarod performance and (B) forelimb grip strength. Both levodopa and dietary treatments alleviated rotenone-induced motor dysfunction. The combined administration of the diet and levodopa showed additive beneficial effects on rotarod performance. Data are shown as mean ± SEM. Different letters indicate mean values were significantly different ($p<0.05$).

The dietary intervention combined with oral-levodopa showed to have additive effects on grip strength

Rotenone-treated mice showed a decrease in grip strength when compared to sham-treated mice ($F(1,176)=697.81$, $p<0.0001$). Grip strength was affected by diet ($F(1,176)=26.04$, $p<0.0001$) and by levodopa treatment ($F(3,176)=3.68$, $p<0.05$). Moreover, there were significant interactions between surgery and diet ($F(1,176)=28.38$, $p<0.0001$), and between surgery and levodopa treatment ($F(3,176)=5.51$, $p<0.001$).

More specifically, rotenone-treated animals on CD showed a significant increase in grip strength when treated with the highest dose of levodopa (20 mg/kg) compared to animals treated with saline (0 mg/kg)($p<0.001$). No differences were found on grip strength between animals on AD and treated with different doses of levodopa or saline. Rotenone-treated animals on AD performed significantly better than rotenone- treated animals on CD at all levodopa doses (0, 5, 10, and 20 mg/kg, $p<0.05$) (Fig5B).

The dietary intervention is effective in reducing rotenone-induced spatial memory impairments whereas levodopa has no effects.

Sham-operated animals selectively re-explored the displace object (DO) as compared to the non-displaced object (NDO) during the experiment, showing that they were able to selectively react to the spatial change. However, rotenone treatment negatively affected the rodents' ability to react to a spatial novelty ($F(1,175)=33.44$, $p<0.0001$). Moreover, there was an effect of the diet ($F(1,175)=19.68$, $p<0.0001$) and an interaction effect between surgery and diet ($F(1,175)=24.40$, $p<0.0001$). No effects of levodopa treatment on spatial memory were found ($F(3,175)=0.091$, $p=0.96$). Post-hoc analyses showed that rotenone-injected mice on AD had a better spatial recognition than rotenone-injected animals on CD independently of the levodopa dose received ($p<0.01$) (Fig6). No significant differences were found in the total activity in the open field apparatus between the different experimental groups.

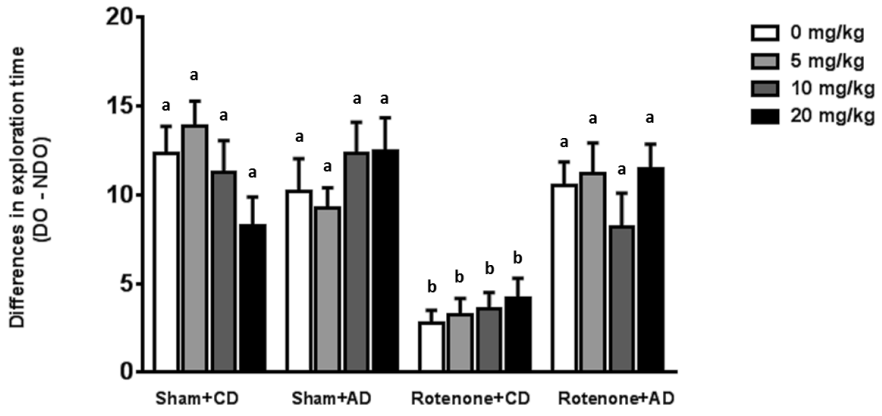


Figure 6: Effects of the dietary intervention and different levodopa doses on spatial object recognition test. Sham-operated animals selectively re-explored the displace object (DO) as compared to the non-displaced object (NDO). Rotenone decreased animals' ability to react to a spatial novelty. Rotenone-injected animals on the active diet showed better spatial discrimination abilities compared to rotenone-injected animals on control diet. Levodopa treatments did not affect spatial memory. Different letters indicate mean values were significantly different ($p<0.05$).

4. Discussion

In the present study we replicated previous finding[28] by showing that rotenone injection in the striatum caused a disturbed motor function, dopaminergic cell loss in the SN, grip strength loss, spatial recognition deficits, and intestinal dysfunctions. Furthermore, we also demonstrated that oral administration of levodopa positively affects motor symptoms in the intrastriatal rotenone model for PD since the rotenone-injected animals orally treated with levodopa showed a better performance on the rotarod test and a better forelimb grip strength. Oral levodopa administration did not show any beneficial effect on spatial memory, which is in line with observations in human PD patients where levodopa mainly affects motor symptoms.

Our study also shows therapeutic effects of a specific dietary intervention containing phosphatide precursors uridine and DHA plus additional nutrients that increase membrane phospholipid synthesis[34] and prebiotic fibers, in a mouse model of PD given only after full induction of motor symptoms in line with previous findings[29]. Our results are complementary to previous observations in other models of ongoing neurodegeneration[35,36] or acute neurotrauma[37,38] where the dietary intervention prevented both the neuronal connectivity disturbances and the behavioral output deficits.

The AD was effective in normalizing rotenone-induced disturbances in motor, gastrointestinal, and spatial memory functioning, but showed no effects on the number of

dopaminergic cells in the SN as previously demonstrated[28]. The improvement in motor performance could be explained by an increased function of the remaining neurons resulting in a better functional output. Increased levels of dopamine transporter (DAT) observed after the current dietary intervention support this idea[28].

The present study demonstrates that the AD has no negative effects on levodopa effectiveness on motor function. Moreover, the AD combined with oral levodopa treatment is more effective in reducing motor dysfunction (rotarod performance) than the diet or levodopa administered separately. The combined intake of phospholipid precursors including uridine and omega-3 fatty acids such as DHA, can increase brain phospholipid levels, synaptic protein levels, neurite outgrowth, dendritic spine formation, and dopaminergic neurotransmission[47–51]. Therefore it is likely that the AD in the present study enhanced synaptic functioning and neurotransmission in the dopaminergic terminals leading to an improvement of motor performance and an added effect of oral levodopa intake on motor-functioning.

The AD was also shown to have beneficial effects on gastrointestinal functioning in the rotenone model for PD, and could be hypothesized to improve levodopa uptake and bioavailability with long-term treatment. Since most of PD patients take levodopa orally, a good functioning of the intestinal tract is required in order to absorb the drug at a beneficial rate. Several clinical studies revealed that single or multiple doses of levodopa induced delayed gastric emptying in healthy volunteers[39–42]and that it might therefore exacerbate the gastrointestinal dysfunction already present in PD patients[43]. Levodopa is absorbed through the duodenum and proximal jejunum by a large neutral amino acid transporter system. Chronic use of oral levodopa is associated with response fluctuations partially due to slow rate of gastric emptying. Delayed gastric emptying in PD patients delays the proper absorption of levodopa, leading to a lower blood plasma concentrations of the drug and the occurrence of on-off oscillations[24,25,27]. These motor fluctuations, together with dyskinesias are considered as the major side effects of long-term treatment with levodopa[44–46]. Further research is warranted to clarify the effects of the diet on long-term levodopa administration.

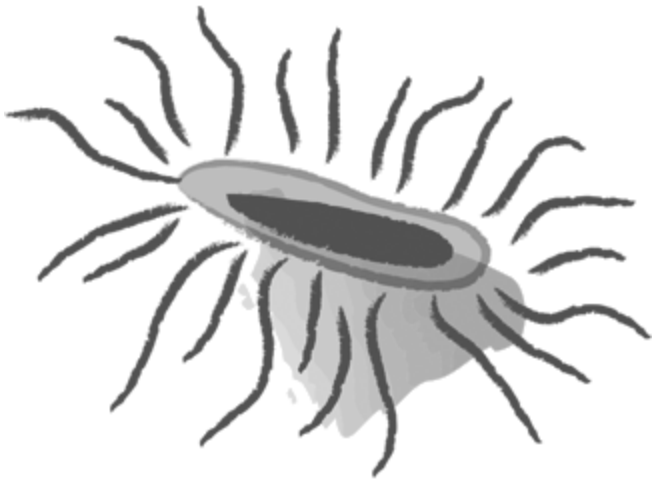
In summary, the current AD was shown to diminish a broad range of PD-like symptoms in a rotenone mouse model. The AD reduced gastrointestinal dysfunction and did not negatively influence the biological effect of levodopa treatment. In fact, the AD had an additive effect to levodopa on motor performance. Our results suggest that this dietary intervention might confer clinical benefits on patients receiving levodopa treatment. Due to the described additive effects, the combination of the diet with the drug might allow a reduction in the doses given to patients, reducing the negative secondary effects and contributing to a longer beneficial use of the drug.

References

1. Djaldetti, R., Lev, N. & Melamed, E. Lesions outside the CNS in Parkinson's disease. *Mov. Disord. Off. J. Mov. Disord. Soc.* **24,** 793–800 (2009).
2. Beach, T. G. *et al.* Multi-organ distribution of phosphorylated alpha-synuclein histopathology in subjects with Lewy body disorders. *Acta Neuropathol. (Berl.)* **119,** 689–702 (2010).
3. Del Tredici, K. & Braak, H. Review: Sporadic Parkinson's disease: development and distribution of α-synuclein pathology. *Neuropathol. Appl. Neurobiol.* **42,** 33–50 (2016).
4. Lee, H. M. & Koh, S.-B. Many Faces of Parkinson's Disease: Non-Motor Symptoms of Parkinson's Disease. *J. Mov. Disord.* **8,** 92–97 (2015).
5. Haehner, A., Hummel, T. & Reichmann, H. Olfactory dysfunction as a diagnostic marker for Parkinson's disease. *Expert Rev. Neurother.* **9,** 1773–1779 (2009).
6. Ponsen, M. M., Stoffers, D., Twisk, J. W. R., Wolters, E. C. & Berendse, H. W. Hyposmia and executive dysfunction as predictors of future Parkinson's disease: a prospective study. *Mov. Disord. Off. J. Mov. Disord. Soc.* **24,** 1060–1065 (2009).
7. Ross, G. W. *et al.* Association of olfactory dysfunction with incidental Lewy bodies. *Mov. Disord. Off. J. Mov. Disord. Soc.* **21,** 2062–2067 (2006).
8. Reijnders, J. S. A. M., Ehrt, U., Weber, W. E. J., Aarsland, D. & Leentjens, A. F. G. A systematic review of prevalence studies of depression in Parkinson's disease. *Mov. Disord. Off. J. Mov. Disord. Soc.* **23,** 183–189; quiz 313 (2008).
9. Aarsland, D., Andersen, K., Larsen, J. P., Lolk, A. & Kragh-Sørensen, P. Prevalence and characteristics of dementia in Parkinson disease: an 8-year prospective study. *Arch. Neurol.* **60,** 387–392 (2003).
10. Pfeiffer, R. F. Gastrointestinal dysfunction in Parkinson's disease. *Parkinsonism Relat. Disord.* **17,** 10–15 (2011).
11. Fasano, A., Visanji, N. P., Liu, L. W. C., Lang, A. E. & Pfeiffer, R. F. Gastrointestinal dysfunction in Parkinson's disease. *Lancet Neurol.* **14,** 625–639 (2015).
12. Jost, W. H. Gastrointestinal dysfunction in Parkinson's Disease. *J. Neurol. Sci.* **289,** 69–73 (2010).
13. Savica, R. *et al.* Medical records documentation of constipation preceding Parkinson disease: A case-control study. *Neurology* **73,** 1752–1758 (2009).
14. Abbott, R. D. *et al.* Frequency of bowel movements and the future risk of Parkinson's disease. *Neurology* **57,** 456–462 (2001).
15. Chen, H. *et al.* Meta-analyses on prevalence of selected Parkinson's nonmotor symptoms before and after diagnosis. *Transl. Neurodegener.* **4,** 1 (2015).
16. Gao, X., Chen, H., Schwarzschild, M. A. & Ascherio, A. A prospective study of bowel movement frequency and risk of Parkinson's disease. *Am. J. Epidemiol.* **174,** 546–551 (2011).
17. Martinez-Martin, P., Rodriguez-Blazquez, C., Kurtis, M. M., Chaudhuri, K. R. & NMSS Validation Group. The impact of non-motor symptoms on health-related quality of life of patients with Parkinson's disease. *Mov. Disord. Off. J. Mov. Disord. Soc.* **26,** 399–406 (2011).
18. Müller, B., Assmus, J., Herlofson, K., Larsen, J. P. & Tysnes, O.-B. Importance of motor vs. non-motor symptoms for health-related quality of life in early Parkinson's disease. *Parkinsonism Relat. Disord.* **19,** 1027–1032 (2013).
19. Schrag, A., Jahanshahi, M. & Quinn, N. How does Parkinson's disease affect quality of life? A comparison with quality of life in the general population. *Mov. Disord. Off. J. Mov. Disord. Soc.* **15,** 1112–1118 (2000).
20. Chaudhuri, K. R. & Schapira, A. H. V. Non-motor symptoms of Parkinson's disease: dopaminergic pathophysiology and treatment. *Lancet Neurol.* **8,** 464–474 (2009).
21. Crispo, J. a. G. *et al.* Trends in inpatient antiparkinson drug use in the USA, 2001–2012. *Eur. J. Clin. Pharmacol.* (2015). doi:10.1007/s00228-015-1881-4
22. Schrag, A. & Quinn, N. Dyskinesias and motor fluctuations in Parkinson's disease. A community-based study. *Brain J. Neurol.* **123 (Pt 11),** 2297–2305 (2000).
23. Poewe, W., Antonini, A., Zijlmans, J. C., Burkhard, P. R. & Vingerhoets, F. Levodopa in the treatment of Parkinson's disease: an old drug still going strong. *Clin. Interv. Aging* **5,** 229–238 (2010).
24. Doi, H. *et al.* Plasma levodopa peak delay and impaired gastric emptying in Parkinson's disease. *J. Neurol. Sci.* **319,** 86–88 (2012).
25. Hardoff, R. *et al.* Gastric emptying time and gastric motility in patients with Parkinson's disease. *Mov. Disord. Off. J. Mov. Disord. Soc.* **16,** 1041–1047 (2001).
26. Müller, T. *et al.* Impact of gastric emptying on levodopa pharmacokinetics in Parkinson disease patients. *Clin. Neuropharmacol.* **29,** 61–67 (2006).

27. Marrinan, S., Emmanuel, A. V. & Burn, D. J. Delayed gastric emptying in Parkinson's disease. *Mov. Disord. Off. J. Mov. Disord. Soc.* **00,** 1–10 (2013).
28. Perez-Pardo, P. *et al.* Promising Effects of Neurorestorative Diets on Motor, Cognitive, and Gastrointestinal Dysfunction after Symptom Development in a Mouse Model of Parkinson's Disease. *Front. Aging Neurosci.* **9,** 57 (2017).
29. Perez-Pardo, P. *et al.* Promising effects of neurorestorative diets on motor, cognitive, and gastrointestinal dysfunction after symptom development in a mouse model of parkinson's disease. *Front. Aging Neurosci.* **9,** 57 (2017).
30. Reeves, P. G., Nielsen, F. H. & Fahey, G. C. AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. *J. Nutr.* **123,** 1939–1951 (1993).
31. Inden, M. *et al.* Neurodegeneration of mouse nigrostriatal dopaminergic system induced by repeated oral administration of rotenone is prevented by 4-phenylbutyrate, a chemical chaperone. *J. Neurochem.* **101,** 1491–1504 (2007).
32. Leiter, J. R. S., Peeler, J. & Anderson, J. E. Exercise-induced muscle growth is muscle-specific and age-dependent. *Muscle Nerve* **43,** 828–838 (2011).
33. De Leonibus, E. *et al.* Spatial deficits in a mouse model of Parkinson disease. *Psychopharmacology (Berl.)* **194,** 517–525 (2007).
34. van Wijk, N. *et al.* Targeting synaptic dysfunction in Alzheimer's disease by administering a specific nutrient combination. *J. Alzheimers Dis. JAD* **38,** 459–479 (2014).
35. Jansen, D. *et al.* Effects of specific multi-nutrient enriched diets on cerebral metabolism, cognition and neuropathology in AβPPswe-PS1dE9 mice. *PloS One* **8,** e75393 (2013).
36. Wiesmann, M. *et al.* A Dietary Treatment Improves Cerebral Blood Flow and Brain Connectivity in Aging apoE4 Mice. *Neural Plast.* **2016,** 6846721 (2016).
37. Pallier, P. N. *et al.* A nutrient combination designed to enhance synapse formation and function improves outcome in experimental spinal cord injury. *Neurobiol. Dis.* **82,** 504–515 (2015).
38. Wiesmann, M. *et al.* A specific dietary intervention to restore brain structure and function after ischemic stroke. *Theranostics* **7(2),** 493–512 (2017).
39. Epprecht, L. *et al.* Unchanged gastric emptying and visceral perception in early Parkinson's disease after a high caloric test meal. *J. Neurol.* **262,** 1946–1953 (2015).
40. Robertson, D. R. *et al.* The influence of levodopa on gastric emptying in man. *Br. J. Clin. Pharmacol.* **29,** 47–53 (1990).
41. Robertson, D. R. *et al.* The influence of levodopa on gastric emptying in healthy elderly volunteers. *Eur. J. Clin. Pharmacol.* **42,** 409–412 (1992).
42. Waller, D. G., Roseveare, C., Renwick, A. G., Macklin, B. & George, C. F. Gastric emptying in healthy volunteers after multiple doses of levodopa. *Br. J. Clin. Pharmacol.* **32,** 691–695 (1991).
43. Bestetti, A., Capozza, A., Lacerenza, M., Manfredi, L. & Mancini, F. Delayed Gastric Emptying in Advanced Parkinson Disease: Correlation With Therapeutic Doses. *Clin. Nucl. Med.* **42,** 83–87 (2017).
44. Müller, T. & Russ, H. Levodopa, motor fluctuations and dyskinesia in Parkinson's disease. *Expert Opin. Pharmacother.* **7,** 1715–1730 (2006).
45. Poewe, W., Antonini, A., Zijlmans, J. C., Burkhard, P. R. & Vingerhoets, F. Levodopa in the treatment of Parkinson's disease: an old drug still going strong. *Clin. Interv. Aging* **5,** 229–38 (2010).
46. Jankovic, J. Motor fluctuations and dyskinesias in Parkinson's disease: clinical manifestations. *Mov. Disord. Off. J. Mov. Disord. Soc.* **20 Suppl 11,** S11-16 (2005).
47. Sakamoto, T., Cansev, M. & Wurtman, R. J. Oral supplementation with docosahexaenoic acid and uridine-5'-monophosphate increases dendritic spine density in adult gerbil hippocampus. *Brain Res.* **1182,** 50–59 (2007).
48. Wang, L., Pooler, A. M., Albrecht, M. A. & Wurtman, R. J. Dietary uridine-5'-monophosphate supplementation increases potassium-evoked dopamine release and promotes neurite outgrowth in aged rats. *J. Mol. Neurosci. MN* **27,** 137–145 (2005).
49. Wurtman, R. J., Cansev, M., Sakamoto, T. & Ulus, I. Nutritional modifiers of aging brain function: use of uridine and other phosphatide precursors to increase formation of brain synapses. *Nutr. Rev.* **68 Suppl 2,** S88-101 (2010).
50. Wurtman, R. J., Cansev, M., Sakamoto, T. & Ulus, I. H. Use of phosphatide precursors to promote synaptogenesis. *Annu. Rev. Nutr.* **29,** 59–87 (2009).
51. Cansev, M., Ulus, I. H., Wang, L., Maher, T. J. & Wurtman, R. J. Restorative effects of uridine plus docosahexaenoic acid in a rat model of Parkinson's disease. *Neurosci. Res.* **62,** 206–209 (2008).

CHAPTER 9



Summarizing discussion

Patients suffering from Parkinson's disease (PD) are known to experience both motor and non-motor symptoms. However, these patients are not diagnosed with PD until the motor-symptoms such as bradykinesia, tremor, rigidity and postural instability are clinically evident[1]. Yet, some of the non-motor symptoms such as olfactory dysfunction[2–4], cognitive impairment[5] and most commonly, gastrointestinal (GI) dysfunction[6–9] might precede the onset of motor-symptoms by several years[10–12] and have a major impact on health-related quality of life[13–15]. Moreover, current treatments for PD include levodopa[16,17] and deep brain stimulation[18] which offer relief from motor-symptoms but do not provide a cure and show little effects on non-motor dysfunction.

This thesis describes preclinical findings on gut-brain interactions in mice models of PD and their possible role in PD pathogenesis. Moreover, opportunities for dietary interventions in the prevention but also the treatment after disease induction of GI, cognitive and motor dysfunction in these models were investigated. This chapter summarizes the major findings of this thesis and describes the potential translational impact for the prevention and treatment of PD.

THE GUT-BRAIN AXIS IN MICE MODELS OF PD

One of the most common non-motor symptom in PD is GI dysfunction, with a prevalence of 70-80%[14]. GI symptoms are identified as bloating, constipation, nausea, delayed gastric emptying and prolonged intestinal transit time[7,19–22] (reviewed in **Chapter 5**). These symptoms have been attributed to alpha-synuclein aggregation and abnormalities in the dorsal motor nucleus of the vagus nerve (DMV) and the enteric nervous system (ENS) but also to changes in the gut microbiota composition. Moreover, accumulating evidence suggests that the ENS is involved in PD pathological progression towards the central nervous system (CNS). During the early stages of PD, neurons of the ENS and the olfactory bulbs (OB) have been found to contain alpha-synuclein aggregates[23,24]. Braak and colleagues proposed in their 'dual-hit hypothesis' (reviewed **Chapter 2**) that alpha-synuclein pathology primes in the ENS and/or the OB and spreads from the periphery to the CNS according to a specific pattern[25,26].

Chapter 3 reveals that the GI phenotype is present in two separate rotenone-induced PD models (oral and intrastriatal rotenone models). Rotenone exposures in both the striatum and the gut induced delayed intestinal transit, increased colonic alpha-synuclein expression, colonic inflammation and signs of immune activation but also led to a reduction of TH positive cells in the substantia nigra (SN) and impaired motor function. The similarity in the pathological development induced by the two different rotenone models suggests that initial pathological processes in the development of PD may take place in the brain, the gut or in both.

The models also suggest a possible bidirectional communication between the gut and the brain for the derivation of PD-like phenotype. These bidirectional processes might create a vicious cycle which may possibly sustain the neuroinflammatory cascade leading to progression and/or exacerbation of PD associated symptoms.
Further research is needed to clarify the mechanism underlying the development of a similar phenotype after rotenone administration in mice in the gut and or the brain. Studying the effects of the resection of autonomic nerves in both models would provide answers regarding the involvement of sympathetic and/or parasympathetic nerves in PD pathological progression.
A better understanding of the gut-brain communication and interaction might lead to new insights in PD pathological progression and perhaps allow earlier diagnosis by focusing on peripheral biomarkers within the GI tract.

Role of TLR4

Intestinal barrier disruption, enhanced markers of microbial translocation and higher pro-inflammatory gene profiles were observed in the PD colonic biopsies samples compared to the healthy controls (**Chapter 4**). Furthermore, PD subjects showed an increased expression of the bacterial endotoxin specific ligand toll-like receptor 4 (TLR4), CD3 positive T-cells and cytokines. To better understand the role of TLR4 in PD-induced neuroinflammation, oral rotenone treatments in Tlr4 KO mice were performed. Tlr4 KO mice treated with rotenone revealed less intestinal disrupted epithelial integrity and inflammation, reduced intestinal and motor dysfunction, and decreased brain neuroinflammation and neurodegeneration relative to rotenone-treated wild type animals.

These results, together with the fact that high relative abundance of LPS producing bacteria have been found in PD patient stool [27] suggest that pro-inflammatory bacteria (especially LPS producers) could initiate TLR4-mediated gut inflammation. An up-regulation of cytokine release in the GI tract could exacerbate inflammatory conditions enhancing local inflammation and disturbing immune homeostasis. The local inflammation in the intestine might affect the brain through the systemic circulation or via neuronal terminals of the vagus nerve. TLR4 might be an important target for future treatments of PD. If pro-inflammatory bacteria are indeed the initial trigger for PD in the gut, TLR4 might be the first point of microbial interaction, offering a potential target for PD. Therefore, pharmacological modulation of the TLR4 pathway could represent a novel therapeutic approach in the treatment of PD.

Further investigations focusing on the effects of specific blocking or knock-out of the TLR4 pathway only in the GI tract on rotenone-induced symptoms would provide answers on the involvement of TLR4 in PD development through the gut-brain axis. Moreover, the effects of

TLR4 stimulation in the gut should also be study for the effects on motor and non-motor symptoms development after rotenone exposure in mice. Other studies have shown the possible implication of other TLRs in PD pathogenesis, such as TLR2 and TLR9 [28–30] and therefore there is a high relevance for future experiments to further study their role in PD.

Changes in microbiota composition

In addition to GI dysfunction and inflammation in the colon, changes in cecal microbiota composition were observed in mice after oral rotenone exposure (**Chapter 5**). Rotenone exposure enhanced the abundance of (human relevant) pro-inflammatory bacteria at the expense of beneficial commensal bacteria. This in line with clinical studies, in which a compositional dysbiosis was found in PD patients [27,31–33].

It is not possible to determine whether changes in the gut microbiota are a cause or a consequence of PD pathogenesis. However, these changes are very likely involved in PD pathogenesis by perpetuating inflammatory cascades and oxidative injury through LPS-mediated mechanism. In addition, it is possible that the pesticide rotenone itself directly affects microbiota composition leading to an inflammatory response. Alternatively, rotenone might be responsible for the inflammation that will impact the microbiota composition, creating a vicious cycle.

The importance of the intestinal microbiome in PD was recently demonstrated in a study showing that under germ-free conditions, or when bacteria are depleted with antibiotics, mice overexpressing human alpha-synuclein displayed reduced microglia activation, alpha-synuclein inclusions, and motor deficits compared to animals with a complex microbiota. These results suggest that gut microbiota is required for PD phenotype development. Moreover, reconstitution of the fecal microbiota in germ-free alpha-synuclein transgenic PD mice using stool from PD patients exacerbates motor symptoms and pathology in this model[30].

Taken together, these findings imply that changes in microbiota composition influences the outcome of the disease. Therefore, the altered microbiota composition in our study might also have contributed to symptom development. Investigating the effects of microbiota modulation on motor and non-motor symptoms using pre-, pro-, antibiotics or fecal transplantation in the rotenone model for PD could provide further insights.

In addition, bacteria are abundant members of the gut microbiome, but not the only residents. Hence, studying changes in viral, fungal and archaeal composition in PD patients and animal models will provide a broader picture on the mechanism by which the gut microbiota influences PD outcome.

DIETARY INTERVENTIONS

No current therapeutic strategies have a favorable influence on PD progression. Moreover, none of them directly targets both motor and non-motor symptoms. Nutrition-based interventions including phospholipid membrane precursors and/or microbiota-directed therapies are of scientific interest since they bring opportunities to complement the traditional PD therapies and overcome some of their shortcomings including lack of efficacy for non-motor symptoms (reviewed in **Chapter 7**).

In **chapter 3** the preventive effects of a specific dietary intervention on motor and non-motor symptoms are investigated in both the oral and the intrastriatal mouse models of PD. The diet contained the precursors for membrane phospholipid synthesis uridine, DHA, and choline that have been shown to increase phospholipids in the brain, synaptic proteins, neurite outgrowth, dopaminergic neurotransmission and dendritic spine formation[35–37]. Moreover, omega-3 PUFAs, like DHA, have anti-inflammatory properties[38] that might reduce oxidative stress.
The preventive dietary intervention was effective for motor symptoms and dopaminergic cell loss but also improved intestinal transit and reduced alpha-synuclein accumulation and inflammation in the colon.

In **Chapter 6** the effects of the same diet given in a therapeutic setting (i.e. after the occurrence of full motor problems) are investigated in the intrastriatal rotenone mouse model for PD. Here, the diet was able to reduce motor dysfunction, cognitive impairment, colonic inflammation and alpha-synuclein accumulation in the ENS, demonstrating that the diet is not interacting with rotenone toxicity but rather has neurorestorative properties and therefore might have disease-modifying potential.

Results from the same study indicated that an extended therapeutic dietary intervention containing the same phospholipid precursors plus cofactors for phospholipid synthesis as well as prebiotic fibers (galacto- and fructooligosaccharides, GOS and FOS) was more beneficial for motor and non-motor abnormalities. The addition of cofactors to the diet might beneficially affect neuronal membrane formation by increasing the availability of membrane precursors or by directly affecting the neuronal membrane or membrane synthesis[39], which would explain its enhanced effects on motor symptoms. Prebiotic fibres have previously been shown to have beneficial effects on immune function[40] and bowel motility[41,42], possibly describing the positive effects on GI-function when added to the diet. Furthermore, the prebiotic fibres GOS and FOS might also contribute to the increased efficacy of the extended dietary intervention on motor function. Additionally GOS/FOS might positively affect microbiota composition and its metabolic activity which in turn can alter the intestinal immune and nervous system and subsequently the CNS[43–45].

In line with our observations, in previous studies in other models of neurodegeneration[46,47] and acute neurotrauma[48,49] the same diet improved neuronal connectivity and behavioral output, suggesting an extensive applicability of this dietary intervention.

The dopamine precursor levodopa is the most effective and commonly used drug in the treatment of PD. However, it has serious side-effects and does not stop the neurodegeneration process. Moreover, GI dysfunctions in PD patients interfere with the absorption of levodopa and modify the effectiveness of the drug.

In **Chapter 8** the effects of the combined administration of the same extended dietary intervention (containing the phospholipid precursors uridine, choline, and DHA plus cofactors for phospholipid synthesis and prebiotic fibers) together with levodopa treatment in the intrastriatal rotenone model were investigated. The diet had no negative influences on the effectiveness of levodopa on motor function in the model. Moreover, the diet combined with oral levodopa treatment was more effective in reducing motor dysfunction than the diet or levodopa administered separately.

The therapeutic dietary intervention on PD might enhance synaptic functioning and neurotransmission in the dopaminergic terminals leading to an improvement of motor performance and an additional effect of oral levodopa intake on motor-functioning. Additionally, the diet was also shown to have beneficial effects on GI functioning in the rotenone model for PD, and therefore might improve levodopa uptake and bioavailability.

In summary, the results of these studies imply that the extended dietary intervention containing the phospholipid precursors uridine, choline, and DHA plus cofactors and prebiotic fibers is more effective in reducing motor and non-motor symptoms in a rotenone mouse model for PD than the diet containing only the phospholipid precursors uridine and DHA. Both diets, but especially the extended formula showed to have therapeutic effects in the model. In addition, the extended diet showed to have an additive effect to levodopa on motor performance. These findings, suggest that this dietary intervention might confer clinical benefits on PD patients that are already under levodopa treatment. Furthermore, since additive effects of the diet to levodopa have been proven, the combination of the diet with levodopa might allow a reduction in the doses given to patients, reducing the secondary effects and contributing to a longer beneficial use of the drug. Future studies measuring membranes phospholipid contents, neuronal integrity and dendritic spine density in the dopaminergic neurons of the SN but also in the ENS and DMV in the rotenone mouse model for PD after the treatment with the specific dietary intervention might provide further insights on the mechanism by which this diet beneficially affects PD symptoms. Moreover, future microbiota and metabolomics studies are required to further explore the possible effects of the diet via microbiota-gut-brain axis.

OVERALL CONCLUSION

This thesis provides evidence for the occurrence of motor symptoms and GI dysfunction in two separate rotenone-induced PD mouse models. The models suggest a bidirectional gut-brain communication involved in the genesis of PD-like phenotype where TLR4-mediated gut inflammation and changes in microbiota composition may play an important role.

A nutritional intervention containing phospholipid precursors, cofactors, and prebiotic fibers has been proven effective in the treatment after disease induction of motor and non-motor symptoms in the rotenone model. Moreover, the diet shows an additive effect to levodopa, the most commonly used drug in the treatment of PD. Hence, this specific diet may result in a beneficial treatment for PD when combined with the oral levodopa treatment.

Combined, the findings in this thesis create more insight into the pathophysiological relevance of gut-brain interactions in PD and demonstrate that the nutritional intervention may be beneficial in the prevention but also the management of the disease.

References

1. Hughes, A. J., Daniel, S. E., Kilford, L. & Lees, A. J. Accuracy of clinical diagnosis of idiopathic Parkinson's disease: a clinico-pathological study of 100 cases. *J. Neurol. Neurosurg. Psychiatry* **55,** 181–184 (1992).
2. Haehner, A., Hummel, T. & Reichmann, H. Olfactory dysfunction as a diagnostic marker for Parkinson's disease. *Expert Rev. Neurother.* **9,** 1773–1779 (2009).
3. Ponsen, M. M., Stoffers, D., Twisk, J. W. R., Wolters, E. C. & Berendse, H. W. Hyposmia and executive dysfunction as predictors of future Parkinson's disease: a prospective study. *Mov. Disord. Off. J. Mov. Disord. Soc.* **24,** 1060–1065 (2009).
4. Ross, G. W. *et al.* Association of olfactory dysfunction with incidental Lewy bodies. *Mov. Disord. Off. J. Mov. Disord. Soc.* **21,** 2062–2067 (2006).
5. Aarsland, D. *et al.* Cognitive decline in Parkinson disease. *Nat. Rev. Neurol.* **13,** 217–231 (2017).
6. Pfeiffer, R. F. Gastrointestinal dysfunction in Parkinson's disease. *Parkinsonism Relat. Disord.* **17,** 10–15 (2011).
7. Fasano, A., Visanji, N. P., Liu, L. W. C., Lang, A. E. & Pfeiffer, R. F. Gastrointestinal dysfunction in Parkinson's disease. *Lancet Neurol.* **14,** 625–639 (2015).
8. Jost, W. H. Gastrointestinal dysfunction in Parkinson's Disease. *J. Neurol. Sci.* **289,** 69–73 (2010).
9. Savica, R. *et al.* Medical records documentation of constipation preceding Parkinson disease: A case-control study. *Neurology* **73,** 1752–1758 (2009).
10. Abbott, R. D. *et al.* Frequency of bowel movements and the future risk of Parkinson's disease. *Neurology* **57,** 456–462 (2001).
11. Gao, X., Chen, H., Schwarzschild, M. A. & Ascherio, A. A prospective study of bowel movement frequency and risk of Parkinson's disease. *Am. J. Epidemiol.* **174,** 546–551 (2011).
12. Chen, H. *et al.* Meta-analyses on prevalence of selected Parkinson's nonmotor symptoms before and after diagnosis. *Transl. Neurodegener.* **4,** 1 (2015).
13. Gallagher, D. A., Lees, A. J. & Schrag, A. What are the most important nonmotor symptoms in patients with Parkinson's disease and are we missing them? *Mov. Disord. Off. J. Mov. Disord. Soc.* **25,** 2493–2500 (2010).
14. Martinez-Martin, P. The importance of non-motor disturbances to quality of life in Parkinson's disease. *J. Neurol. Sci.* **310,** 12–16 (2011).
15. Müller, B., Assmus, J., Herlofson, K., Larsen, J. P. & Tysnes, O.-B. Importance of motor vs. non-motor symptoms for health-related quality of life in early Parkinson's disease. *Parkinsonism Relat. Disord.* **19,** 1027–1032 (2013).
16. Fahn, S. The medical treatment of Parkinson disease from James Parkinson to George Cotzias. *Mov. Disord.* **30,** 4–18 (2015).
17. Warren Olanow, C. *et al.* Factors predictive of the development of Levodopa-induced dyskinesia and wearing-off in Parkinson's disease. *Mov. Disord. Off. J. Mov. Disord. Soc.* **28,** 1064–1071 (2013).
18. Williams, Z. M. Good vibrations with deep brain stimulation. *Nat. Neurosci.* **18,** 618–619 (2015).
19. Abbott, R. D. *et al.* Bowel movement frequency in late-life and incidental Lewy bodies. *Mov. Disord. Off. J. Mov. Disord. Soc.* **22,** 1581–1586 (2007).
20. Cersosimo, M. G. *et al.* Gastrointestinal manifestations in Parkinson's disease: prevalence and occurrence before motor symptoms. *J. Neurol.* **260,** 1332–1338 (2013).
21. Chaudhuri, K. R. & Schapira, A. H. Non-motor symptoms of Parkinson's disease: dopaminergic pathophysiology and treatment. *Lancet Neurol.* **8,** 464–474 (2009).
22. Verbaan, D. *et al.* Patient-reported autonomic symptoms in Parkinson disease. *Neurology* **69,** 333–341 (2007).
23. Braak, H., de Vos, R. A. I., Bohl, J. & Del Tredici, K. Gastric alpha-synuclein immunoreactive inclusions in Meissner's and Auerbach's plexuses in cases staged for Parkinson's disease-related brain pathology. *Neurosci. Lett.* **396,** 67–72 (2006).
24. Shannon, K. M. *et al.* Alpha-synuclein in colonic submucosa in early untreated Parkinson's disease. *Mov. Disord. Off. J. Mov. Disord. Soc.* **27,** 709–715 (2012).
25. Hawkes, C. H., Del Tredici, K. & Braak, H. Parkinson's disease: a dual-hit hypothesis. *Neuropathol. Appl. Neurobiol.* **33,** 599–614 (2007).
26. Hawkes, C. H., Del Tredici, K. & Braak, H. A timeline for Parkinson's disease. *Parkinsonism Relat. Disord.* **16,** 79–84 (2010).

27. Keshavarzian, A. *et al.* Colonic bacterial composition in Parkinson's disease. *Mov. Disord. Off. J. Mov. Disord. Soc.* **30,** 1351–1360 (2015).
28. Ros-Bernal, F. *et al.* Microglial glucocorticoid receptors play a pivotal role in regulating dopaminergic neurodegeneration in parkinsonism. *Proc. Natl. Acad. Sci. U. S. A.* **108,** 6632–6637 (2011).
29. Zhu, K., Teng, J., Zhao, J., Liu, H. & Xie, A. Association of TLR9 polymorphisms with sporadic Parkinson's disease in Chinese Han population. *Int. J. Neurosci.* **126,** 612–616 (2016).
30. Kalinderi, K., Bostantjopoulou, S., Katsarou, Z. & Fidani, L. TLR9 -1237 T/C and TLR2 -194 to -174 del polymorphisms and the risk of Parkinson's disease in the Greek population: a pilot study. *Neurol. Sci. Off. J. Ital. Neurol. Soc. Ital. Soc. Clin. Neurophysiol.* **34,** 679–682 (2013).
31. Scheperjans, F. *et al.* Gut microbiota are related to Parkinson's disease and clinical phenotype. *Mov. Disord. Off. J. Mov. Disord. Soc.* **30,** 350–358 (2015).
32. Unger, M. M. *et al.* Short chain fatty acids and gut microbiota differ between patients with Parkinson's disease and age-matched controls. *Parkinsonism Relat. Disord.* (2016). doi:10.1016/j.parkreldis.2016.08.019
33. Hasegawa, S. *et al.* Intestinal Dysbiosis and Lowered Serum Lipopolysaccharide-Binding Protein in Parkinson's Disease. *PloS One* **10,** e0142164 (2015).
34. Sampson, T. R. *et al.* Gut Microbiota Regulate Motor Deficits and Neuroinflammation in a Model of Parkinson's Disease. *Cell* **167,** 1469–1480.e12 (2016).
35. Wang, L., Pooler, A. M., Albrecht, M. A. & Wurtman, R. J. Dietary uridine-5'-monophosphate supplementation increases potassium-evoked dopamine release and promotes neurite outgrowth in aged rats. *J. Mol. Neurosci. MN* **27,** 137–145 (2005).
36. Wurtman, R. J., Cansev, M., Sakamoto, T. & Ulus, I. Nutritional modifiers of aging brain function: use of uridine and other phosphatide precursors to increase formation of brain synapses. *Nutr. Rev.* **68 Suppl 2,** S88-101 (2010).
37. Wurtman, R. J., Cansev, M., Sakamoto, T. & Ulus, I. H. Use of phosphatide precursors to promote synaptogenesis. *Annu. Rev. Nutr.* **29,** 59–87 (2009).
38. Miller, R. L., James-Kracke, M., Sun, G. Y. & Sun, A. Y. Oxidative and inflammatory pathways in Parkinson's disease. *Neurochem. Res.* **34,** 55–65 (2009).
39. van Wijk, N. *et al.* Targeting synaptic dysfunction in Alzheimer's disease by administering a specific nutrient combination. *J. Alzheimers Dis. JAD* **38,** 459–479 (2014).
40. de Kivit, S., Kraneveld, A. D., Garssen, J. & Willemsen, L. E. M. Glycan recognition at the interface of the intestinal immune system: target for immune modulation via dietary components. *Eur. J. Pharmacol.* **668 Suppl 1,** S124-132 (2011).
41. Meksawan, K. *et al.* Effects of fructo-oligosaccharide supplementation on constipation in elderly continuous ambulatory peritoneal dialysis patients. *Perit. Dial. Int. J. Int. Soc. Perit. Dial.* (2014). doi:10.3747/pdi.2014.00015
42. Scholtens, P. A. M. J., Goossens, D. A. M. & Staiano, A. Stool characteristics of infants receiving short-chain galacto-oligosaccharides and long-chain fructo-oligosaccharides: a review. *World J. Gastroenterol.* **20,** 13446–13452 (2014).
43. Clemente, J. C., Ursell, L. K., Parfrey, L. W. & Knight, R. The impact of the gut microbiota on human health: An integrative view. *Cell* **148,** 1258–1270 (2012).
44. Cryan, J. F. & Dinan, T. G. More than a gut feeling: the microbiota regulates neurodevelopment and behavior. *Neuropsychopharmacol. Off. Publ. Am. Coll. Neuropsychopharmacol.* **40,** 241–242 (2015).
45. Maslowski, K. M. & Mackay, C. R. Diet, gut microbiota and immune responses. *Nat. Immunol.* **12,** 5–9 (2011).

46. Jansen, D. *et al.* Effects of specific multi-nutrient enriched diets on cerebral metabolism, cognition and neuropathology in AβPPswe-PS1dE9 mice. *PloS One* **8,** e75393 (2013).
47. Wiesmann, M. *et al.* A Dietary Treatment Improves Cerebral Blood Flow and Brain Connectivity in Aging apoE4 Mice. *Neural Plast.* **2016,** 6846721 (2016).
48. Pallier, P. N. *et al.* A nutrient combination designed to enhance synapse formation and function improves outcome in experimental spinal cord injury. *Neurobiol. Dis.* **82,** 504–515 (2015).
49. Wiesmann, M. *et al.* A specific dietary intervention to restore brain structure and function after ischemic stroke. *Theranostics* **7(2),** 493–512 (2017

Appendices

Nederlandse samenvatting

Patiënten die aan de ziekte van Parkinson lijden, kennen zowel motorische als niet-motorische symptomen. De diagnose wordt echter niet gesteld voordat de motorische symptomen zoals bradykinesie (trager worden van bewegingen), tremor (trillen), rigiditeit (stijfheid) en een verstoorde lichaamshouding klinisch vastgesteld kunnen worden. Toch kunnen sommige van de niet-motorische symptomen, zoals verminderde reuk, cognitieve stoornissen en de veel voorkomende maag-darmproblemen, meerdere jaren voorafgaand aan het ontstaan van motorische symptomen aanwezig zijn. Deze symptomen hebben een grote impact op de kwaliteit van leven. De huidige behandelingsmethoden voor de ziekte van Parkinson omvatten het geneesmiddel levodopa en diepe hersenstimulatie, die motorische symptomen verzachten, maar geen genezing brengen en weinig effect hebben op niet-motorische symptomen.
Het doel van dit proefschrift was om meer inzicht te krijgen in de interacties tussen de darmen en het brein in muizenmodellen voor de ziekte van Parkinson. Bovendien werden mogelijkheden voor voedingsinterventies in de preventie, maar ook de behandeling van maag-darm, cognitieve en motorische verstoringen na ziekte-inductie in deze modellen onderzocht. In dit hoofdstuk worden de belangrijkste bevindingen van dit proefschrift samengevat en wordt de mogelijke betekenis van de bevindingen voor de preventie en behandeling van de ziekte van Parkinson beschreven.

DE DARM-BREIN AS IN MUIZENMODELLEN VOOR DE ZIEKTE VAN PARKINSON
Problemen in het maagdarmkanaal behoren met een prevalentie van 70-80% tot de meest voorkomende niet-motorische symptomen van de ziekte van Parkinson. De intestinale symptomen bestaan uit een opgeblazen gevoel, constipatie, misselijkheid, vertraagde maag-lediging en een verlengde passagetijd door de darmen (beschreven in **Hoofdstuk 5**). Deze symptomen worden toegeschreven aan de ophoping van het eiwit alfa-synucleïne en abnormaliteiten in de dorsale motorkern van de nervus vagus en het enterisch zenuwstelsel, maar ook aan veranderingen in de samenstelling van de micro-organismen in de darm. Er wordt steeds meer bewijs geleverd dat het enterisch zenuwstelsel betrokken is bij de ontwikkeling van de ziekte van Parkinson. In de vroege stadia van de ziekte van Parkinson worden alfa-synucleïne ophopingen aangetroffen in neuronen van het enterisch zenuwstelsel en in de olfactorische lobben van het brein. Braak en collega's stelden in hun 'dual-hit' hypothese (beschreven in **Hoofdstuk 2**) voor, dat alfa-synucleïne pathologie in het enterisch zenuwstelsel en/of de olfactorische lobben zich verspreidt van de perifere zenuwen naar het centrale zenuwstelsel volgens een specifiek patroon.
Hoofdstuk 3 beschrijft dat het maagdarmkanaal-fenotype aanwezig is in twee verschillende rotenon-geïnduceerde modellen voor de ziekte van Parkinson in de muis (het orale en het intra-striatale rotenon-model). Blootstelling aan rotenon van zowel het striatum als in de darmen leidde tot een vertraagde darmpassage, ophoping van alfa-synucleïne in de

neuronale plexi van de dikke darm, ontsteking van de dikke darm en activiteit van het mucosale immuunsysteem, maar leidden ook tot een vermindering van dopamine neuronen (tyrosine hydroxylase-positieve cellen) in de substantia nigra en een verminderd motorisch functioneren. De gelijkenis in de ontwikkeling van pathologie veroorzaakt door de twee verschillende manieren van toediening van rotenon suggereert dat initiële pathologische processen bij de ontwikkeling van de ziekte van Parkinson in de hersenen, in de darm, of in beide kunnen plaatsvinden.
De modellen suggereren ook een mogelijke bidirectionele communicatie tussen de darmen en de hersenen bij de ziekte van Parkinson. Deze bidirectionele processen kunnen een vicieuze cyclus creëren die mogelijk de neuro-inflammatoire cascade kan opleveren die leidt tot ontwikkeling en/of verergering van met de ziekte van Parkinson geassocieerde symptomen.
Verder onderzoek is nodig om het mechanisme te duiden dat betrokken is bij de ontwikkeling van Parkinson pathologie na de toediening van rotenon in de darm en/of de hersenen van muizen. Het bestuderen van de effecten van de resectie van autonome zenuwen in beide modellen zou antwoorden kunnen geven op de betrokkenheid van sympathische en/of parasympathische zenuwen bij de ontwikkeling van Parkinson pathologie. Een beter inzicht in de communicatie en interactie tussen de darmen en de hersenen kan leiden tot nieuwe inzichten in de pathologie van de ziekte van Parkinson en mogelijk tot een vroegere diagnose met behulp van perifere biomarkers uit het maagdarmkanaal.

De rol van Toll-like receptor 4

In darmbiopten van de ziekte van Parkinson patiënten werden verstoring van de intestinale barrière –de lekkende darm–, verhoogde markers van microbiële translocatie (met name lipopolysaccharide (LPS) producerende bacteriën) en hogere pro-inflammatoire gen profielen waargenomen in vergelijking met die van gezonde individuen (**Hoofdstuk 4**). Daarnaast werd in de darmbiopten van Parkinson patiënten meer Toll-like receptoren (specifiek TLR4), een toename in het aantal CD3-positieve T-cellen en cytokines aangetoond. Om de rol van TLR4 bij Parkinson-geïnduceerde ontsteking beter te begrijpen, werden orale rotenon behandelingen in zogenaamde Tlr4 KO-muizen uitgevoerd. Deze Tlr4 KO muizen hebben geen TLR4. Tlr4 KO muizen behandeld met rotenon vertoonden minder lekkende darm en ontsteking, minder darm- en motorstoornissen, en verminderde neuroinflammatie en neurodegeneratie in het brein ten opzichte van de met rotenon behandelde wild type dieren.
Deze resultaten, samen met de bevinding dat er meer LPS-producerende bacteriën aanwezig is in de ontlasting van Parkinson-patiënten, suggereert dat pro-inflammatoire bacteriën (vooral LPS-producenten) TLR4-gemedieerde darmontsteking kunnen initiëren. Een verhoogde cytokine-afgifte in het maagdarmkanaal kan de ontstekingsomstandigheden

verergeren hetgeen de lokale ontsteking versterkt en de immuun-homeostase verstoort. De lokale ontsteking in de darm kan de hersenen beïnvloeden via de systemische circulatie of via zenuwuiteinden van de nervus vagus. TLR4 kan een belangrijk aangrijpingspunt zijn voor toekomstige behandelingen van de ziekte van Parkinson. Als pro-inflammatoire bacteriën inderdaad de initiële trigger voor de ziekte van Parkinson in de darm zijn, kan de darmflora ook een nieuw aangrijpingspunt voor de behandeling van de ziekte van Parkinson zijn.

Veranderingen in samenstelling van darmbacteriën

Naast een verstoorde maagdarmkanaal functie en een ontsteking van de dikke darm, werden veranderingen in de samenstelling van de darmbacteriën in het ceacum waargenomen bij muizen na orale blootstelling aan rotenon (**Hoofdstuk 5**). De behandeling met rotenon verhoogde het aantal van (voor de mens relevante) pro-inflammatoire bacteriën ten koste van gunstige commensale bacteriën. Dit is in overeenstemming met klinische studies, waarin een dysbiose bij Parkinson patiënten werd gevonden.

Het is niet mogelijk om te bepalen of veranderingen in de darmbacteriën een oorzaak of gevolg zijn van de ziekte van Parkinson. Echter, deze veranderingen zijn zeer waarschijnlijk betrokken bij de pathogenese van de ziekte van Parkinson door het voort laten duren van inflammatoire cascades en oxidatieve schade door middel van LPS/TLR4-gemedieerde mechanismes. Daarnaast is het mogelijk dat het pesticide rotenon zelf direct de darmbacterie-samenstelling beïnvloedt, wat leidt tot een ontstekingsreactie. Het kan ook zo zijn dat rotenon verantwoordelijk is voor de ontsteking die vervolgens de darmbacterie-samenstelling beïnvloedt, waardoor een vicieuze cirkel ontstaat.

Het belang van de darmbacteriën in de ziekte van Parkinson is onlangs aangetoond in een studie die laat zien dat onder kiemvrije condities of wanneer bacteriën bestreden zijn met antibiotica, muizen met een over-expressie van alfa-synucleïne een verminderde activatie van microglia, ophoping van alfa-synucleïne en motorfunctie verstoringen lieten zien in vergelijking met dieren met complexe samenstelling van darmbacteriën. Deze resultaten suggereren dat darmbacteriën nodig zijn voor het ontwikkelen van de ziekte van Parkinson. Bovendien verergerde de transplantatie van ontlasting van patiënten met de ziekte van Parkinson in kiemvrije alfa-synucleïne transgene muizen de pathologie en de motorische symptomen.

Samenvattend impliceren deze bevindingen dat veranderingen in samenstelling van de darm micro-organismen de uitkomst van de ziekte beïnvloeden. Daarom kan de gewijzigde darmbacterie samenstelling in onze studie ook bijgedragen hebben aan de ontwikkeling van symptomen. Het onderzoeken van de effecten van het moduleren van de darmbacteriën met behulp van pre-, pro-, en antibiotica of fecale transplantatie op motorische en niet-motorische symptomen in het rotenon model voor de ziekte van Parkinson kan verdere inzichten verschaffen.

Bacteriën zijn niet de enige elementen in de darmflora. Daarom zal het bestuderen van veranderingen in de samenstelling van virussen, schimmels en archeae in Parkinson-

patiënten en diermodellen een breder beeld opleveren van het mechanisme waarmee darmmicro-organismen het verloop van de ziekte van Parkinson beïnvloeden.

VOEDINGSINTERVENTIONS

Geen van de huidige therapeutische strategieën heeft een gunstige invloed op het voortschrijden van de ziekte van Parkinson. Bovendien grijpt geen van hen direct aan op zowel motorische als niet-motorische symptomen. Voedings-gerelateerde interventies, waaronder die gericht op membraanfosfolipide voorloperstoffen en/of darmbacteriën, zijn van wetenschappelijk belang omdat ze mogelijkheden bieden om de traditionele ziekte van Parkinson-therapieën aan te vullen en sommige van hun tekortkomingen op te heffen, waaronder het gebrek aan effect op niet-motorische symptomen (beschreven in **Hoofdstuk 7**).

In **Hoofdstuk 3** worden de preventieve effecten van een specifieke voedingsinterventie op motor- en niet-motorische symptomen onderzocht in zowel het orale als het intra-striatale muismodellen voor de ziekte van Parkinson. Het dieet bevatte de voorloperstoffen voor membraan fosfolipide synthese, uridine, docosahexaeenzuur (DHA) en choline, waarvan is aangetoond dat zij fosfolipiden in de hersenen, synaptische eiwitten, neuriet-uitgroei, dopaminerge neurotransmissie en de vorming van dendritische uitlopers verhogen. Bovendien hebben omega-3 meervoudig onverzadigde vetzuren, zoals DHA, anti-inflammatoire eigenschappen die oxidatieve stress kunnen verminderen.

De preventieve dieetinterventie was effectief voor motorische symptomen en het verlies van dopaminerge cellen, maar verbeterde ook de darmpassage en verminderde alfa-synucleïne ophoping en ontsteking van de dikke darm.

In **Hoofdstuk 6** worden de effecten van hetzelfde dieet gegeven in een therapeutische opzet (d.w.z. na het ontwikkelen van de motorische problemen) onderzocht in het intra-striatale rotenon muizenmodel voor de ziekte van Parkinson. In deze situatie was het dieet in staat om motorische dysfunctie, cognitieve stoornissen, ontsteking van de dikke darm en alfa-synucleïne ophoping in het enterische zenuwstelsel te verminderen. Deze resultaten tonen aan dat het dieet niet interacteert met de toxiciteit van rotenon, maar eerder neuro-restoratieve en ontstekingsremmende eigenschappen bezit en daarom ziekte-modificerende capaciteiten kan hebben. Uit resultaten van dezelfde studie bleek dat een uitgebreidere therapeutische dieetinterventie met dezelfde fosfolipide voorloperstoffen plus cofactoren voor fosfolipidesynthese alsook prebiotische vezels (galacto- en fructo-oligosacchariden, GOS en FOS) significant meer gunstige effecten had op motorische en niet-motorische afwijkingen. De toevoeging van de cofactoren aan het dieet kan de vorming van neuronale membranen gunstig beïnvloeden door de beschikbaarheid van voorloperstoffen te verhogen of door de neuronale membraan of membraan synthese direct te beïnvloeden, die de verbeterde effecten op motorische symptomen zou verklaren. Van prebiotische vezels is

eerder aangetoond dat zij gunstige effecten hebben op de intestinale barrière, het functioneren van het immuunsysteem en op darmmotiliteit, die mogelijk de positieve effecten op de maag-darm functie duiden wanneer ze aan het dieet worden toegevoegd. Bovendien kunnen de prebiotische vezels GOS en FOS ook bijdragen tot de verhoogde werkzaamheid van de uitgebreidere dieetinterventie op het motorisch functioneren. Tot slot kan GOS/FOS de samenstelling van de darmbacteriën en de metabolische activiteit van de bacteriën positief beïnvloeden, wat het immuun- en zenuwstelsel van de darm en vervolgens het centrale zenuwstelsel positief kan beïnvloeden.
In lijn met onze waarnemingen, verbeterde hetzelfde uitgebreide dieet in de voorafgaande studies in andere modellen van neurodegeneratie en acute neurotrauma de neuronale connectiviteit en gedrag, hetgeen een brede toepasbaarheid van deze voedingsinterventie suggereert.

De dopamine voorloperstof levodopa is het meest effectieve en meest gebruikte geneesmiddel bij de behandeling van de ziekte van Parkinson. Het heeft echter ernstige bijwerkingen en stopt het neurodegeneratieve proces niet. Bovendien verstoren maag-darmproblemen bij Parkinson-patiënten de absorptie van levodopa en veranderen zo de effectiviteit van het geneesmiddel. In **Hoofdstuk 8** werden de effecten van de gecombineerde toediening van de uitgebreide voedingsinterventie (die de fosfolipide voorloperstoffen uridine, choline en DHA alsmede de cofactoren voor fosfolipidesynthese en de prebiotische vezels bevatten) samen met levodopabehandeling in het intra-striatale rotenon-model onderzocht. Het dieet had geen negatieve invloeden op de effectiviteit van levodopa op het motorisch functioneren in het model. Bovendien was het dieet gecombineerd met orale levodopa-behandeling effectiever bij het verminderen van motorstoornissen dan het dieet of levodopa afzonderlijk. De therapeutische dieetinterventie zou de werking van synapsen en neurotransmissie in de dopaminerge terminals kunnen verbeteren, wat leidt tot een verbetering van de motorische prestatie en een extra effect bovenop dat van levodopa op het motorisch functioneren. Daarnaast bleek dat het dieet ook gunstige effecten heeft op de werking van het maagdarmsysteem in het rotenon-model van de ziekte van Parkinson, waardoor de opname en de biologische beschikbaarheid van levodopa mogelijk zou zijn verbeterd.

Samengevat impliceren de resultaten van deze studies dat de uitgebreide dieetinterventie die de fosfolipide voorloperstoffen uridine, choline en DHA plus de cofactoren en de prebiotische vezels bevat, effectiever is bij het verminderen van motorische en niet-motorische symptomen in een rotenon muizenmodel voor de ziekte van Parkinson dan het dieet dat alleen de fosfolipide voorloperstoffen uridine en DHA bevat. Beide diëten, maar vooral de uitgebreidere formule bleken therapeutische effecten in het model te hebben. Daarnaast bleek dat het uitgebreidere dieet een additief effect heeft op levodopa op motorische prestaties. Deze bevindingen suggereren dat deze dieetinterventie klinische

voordelen kan opleveren voor Parkinson-patiënten die al levodopa gebruiken. Bovendien, aangezien we additieve effecten van het dieet op levodopa lieten zien, zou de combinatie van het dieet met levodopa een vermindering van de dosering van het geneesmiddel kunnen opleveren voor patiënten, hetgeen ook de bijwerkingen zou verminderen en zou bijdragen aan een langere gunstig gebruik van levodopa.

ALGEMENE CONCLUSIE

Dit proefschrift levert bewijs voor het optreden van motorische symptomen en maag-darmproblemen in twee afzonderlijke rotenon-geïnduceerde muismodellen voor de ziekte van Parkinson. De modellen suggereren een bidirectionele darm-brein communicatie betrokken bij de ontstaan en verloop van de ziekte van Parkinson waar TLR4-gemedieerde darmontsteking en veranderingen in de samenstelling van de darmbacteriën een belangrijke rol kunnen spelen. Een voedingsinterventie die fosfolipide voorloperstoffen, cofactoren en prebiotische vezels bevat, is bewezen effectief in de behandeling na inductie van ziekte-gerelateerde motorische en niet-motorische symptomen in het rotenon-model. Bovendien laat het dieet een additief effect zien op levodopa, het meest gebruikte geneesmiddel bij de behandeling van de ziekte van Parkinson. Vandaar dat dit specifieke dieet kan leiden tot een gunstige behandeling van de ziekte van Parkinson in combinatie met de orale levodopa behandeling.

De bevindingen beschreven in dit proefschrift geven meer inzicht in de pathofysiologische relevantie van darm-brein interacties in de ziekte van Parkinson en tonen aan dat voedingsinterventies mogelijk zowel voor de preventie alsook voor de behandeling van de ziekte van Parkinson ingezet kunnen worden.

Resumen en Español

Los pacientes con la enfermedad de Parkinson (EP) experimentan problemas motores y no motores. Sin embargo, muchos enfermos no son diagnosticados con la EP hasta que problemas motores como bradicinesia, temblor y rigidez son clínicamente evidentes. Además, algunos de los síntomas no motores como la disfunción olfativa , el deterioro cognitivo y más comúnmente la disfunción intestinal, pueden desarrollarse antes que los problemas motores y tienen un fuerte impacto en la calidad de vida. Los tratamientos actuales para la EP incluyen levodopa y estimulación cerebral profunda, los cuales ofrecen alivio para los problemas motores pero no representan una cura para la enfermedad y no son efectivos en el tratamiento de los síntomas no motores.
Esta tesis describe los hallazgos preclínicos de las interacciones en el eje cerebro-intestino en modelos de ratón para la EP y su posible papel en la patogénesis de la enfermedad. Además, se han investigado las posibilidades para el uso de intervenciones dietéticas en la prevención y el tratamiento de las disfunciones gastrointestinales, cognitivas y motoras en los modelos de ratón. Este capítulo resume los mayores hallazgos de esta investigación y describe las potenciales aplicaciones para la prevención y el tratamiento de la EP.

EL EJE INTESTINO-CEREBRO EN MODELOS DE RATÓN PARA LA ENFERMEDAD DE PARKINSON

Uno de los síntomas no motores más comunes de la EP es la disfunción gastrointestinal |(GI) con una prevalencia del 70-80%. Los síntomas GI se han definido como hinchazón abdominal ,diarrea, náuseas, estreñimiento y un tránsito intestinal prolongado (capítulo **5**). Estos síntomas se han atribuido a la agregación de alfa-sinucleína y a anormalidades en el núcleo dorsal motor del nervio vago y el sistema nerviosos entérico, pero también a cambios en la composición de la microbiota intestinal. La evidencias sugieren que el sistema nervioso entérico está relacionado con la progresión patológica de la EP hacia el sistema nerviosos central . Durante las fases iniciales de la EP se encontraron agregados de alfa-sinucleína en neuronas del sistema nervioso entérico y de los bulbos olfativos. Braak y su equipo han propuesto en su 'dual-hit hypothesis' (revisada en el **capítulo 2**) que la patología de la alfa-sinucleína prima en el sistema nervioso entérico y/o en los bulbos olfativos y se extiende desde la periferia al sistema nervioso central siguiendo un patrón específico.
El **capítulo 3** revela que el fenotipo GI está presente en los dos modelos diferentes de ratón para la EP inducidos por rotenona. La exposición a la rotenona, tanto en el cuerpo estriado del cerebro como en el intestino, ralentiza el tránsito intestinal, incrementa la expresión de alfa-sinucleína en el colon, así como los signos de activación inmune y reduce el número de células TH positivas en la *substantia nigra* y la actividad motora. La similitud en el desarrollo de la patología inducida en los dos modelos de rotenona sugiere que el inicio de los procesos patológicos en el desarrollo de la EP pueden tener lugar en el cerebro, el intestino o en ambos. Los modelos también sugieren la existencia de una comunicación bidireccional

entre el intestino y el cerebro involucrada en la derivación de un fenotipo similar. Estos procesos bidireccionales podrían crear un círculo vicioso que sustentaría una cascada neuroinflamatoria dando lugar al avance y/o agravamiento de los síntomas asociados a la EP. Es necesaria más investigación para clarificar el mecanismo subyacente al desarrollo de un fenotipo similar después de la administración de rotenona en ratones en el cerebro o el intestino. El estudio de la escisión de los nervios autónomos en ambos modelos podría proporcionar respuestas acerca de la participación de los nervios simpáticos y/o parasimpáticos en la progresión patológica de la enfermedad. Una mejor compresión de la comunicación intestino-cerebro y de sus interacciones podría llevar a nuevas perspectivas en el entendimiento de la progresión patológica de la EP y quizá permitir un diagnóstico más temprano al concentrarse en biomarcadores periféricos del tracto intestinal.

El Papel de TLR4

En biopsias de colon de pacientes aquejados de la EP se observó una disrupción de la barrera intestinal, un incremento en los marcadores de translocación microbiana y un perfil más elevado de genes pro-inflamatorios en comparación con los individuos sanos (**Capítulo 4**).
Además, los sujetos con EP demostraron un incremento en la expresión de la endotoxina bacteriana ligada a receptores de tipo Toll 4 (TLR4) y un aumento en el número de células T positivas y de citoquinas. Para comprender mejor el papel de TLR4 en la neuroinflamación inducida por EP, se realizaron tratamientos orales con rotenona en ratones Tlr4 KO. Estos ratones KO tratados con rotenona revelaron una menor inflamación y disrupción del epitelio intestinal, una menor disfunción motora e intestinal y una menor inflamación y degeneración neuronal en relación con los animales *wild-type* igualmente tratados con rotenona.
Estos resultados, sumados al aumento en la abundancia relativa de bacterias productoras de lipopolisacáridos (LPS) hallado en muestra de heces de pacientes con EP, sugiere que las bacterias pro-inflamatorias (especialmente productoras de LPS) pueden iniciar , mediante TLR4 , inflamación intestinal. Un incremento en la liberación de citoquinas en el tracto digestivo puede agravar las condiciones inflamatorias, incrementando la inflamación local y provocando una desregulación de la homeostasis inmune. Esta inflamación local del intestino puede afectar al cerebro a través la circulación sistémica o a través la terminales neuronales del nervio vago.
TLR4 puede ser una diana importante en futuros tratamientos de la EP. Si las bacterias pro-inflamatorias son en realidad el desencadenante de la EP en el intestino, TLR4 podría ser el primer punto de interacción microbiana, ofreciendo una posible diana para el tratamiento de la EP.

Cambios en la composición de la microbiota.

En ratones expuestos rotenona por vía oral, además de disfunción GI e inflamación en el colón, se observaron cambios en la composición de la microbiota fecal (**capítulo 5**). La

exposición a rotenona incrementó la abundancia relativa de bacteria pro-inflamatorias (relevantes en el caso de pacientes con la EP) a expensas de las bacterias comensales beneficiosas. Este hallazgo concuerda con estudios clínicos donde también se ha descrito la presencia de disbiosis en pacientes con la EP.

Resulta imposible determinar si los cambios en la composición de la microbiota intestinal son la causa o la consecuencia de la patogénesis en la EP. En cualquier caso, estos cambios están probablemente involucrados en el desarrollo de la EP, mediante la perpetuación de cascadas inflamatorias y estrés oxidativo a través de mecanismos inducidos por LPS. Además, es posible que la misma rotenone afecte directamente a la composición de la microbiota desencadenando un respuesta inflamatoria. Alternativamente , la rotenona puede causar directamente inflamación causando cambios en la composición microbiana.

La importancia de la microbiota intestinal en la EP ha sido demostrada recientemente en un estudio. En condiciones libres de gérmenes, o tras la exposición a antibióticos, los ratones que sobreexpresan alfa-sinucleina humana mostraron una reducción en la activación de la microglia , una disminución en las inclusiones de alfa-sinucleina y un descenso en los déficits motores en comparación con animales con una microbiota compleja. Estos resultados sugieren que la microbiota intestinal es fundamental en el desarrollo del fenotipo de la EP. Además, la reconstitución de la microbiota fecal en ratones libres de gérmenes que sobre-expresan alfa sinucleina humana, usando una muestra de pacientes con EP , empeora los síntomas motores y la patología en este modelo.

Tomados en conjunto, estos hallazgos implican que los cambios en la composición de la microbiota influencian el desarrollo de la enfermedad. Por tanto, la alteración de la composición microbiótica en nuestro estudio puede haber contribuido al desarrollo de los síntomas. Investigar los efectos de la modulación de la microbiota (usando antibióticos, prebióticos, probióticos o transplantes fecales) en el desarrollo de síntomas motores y no motores en el modelo de rotenona para EP puede proporcionar nuevas perspectivas.

Por otra parte, las bacterias son organismos abundantes en la microbiota intestinal, pero no son los únicos residentes. Por tanto el estudio de los cambios en las poblaciones virales, fúngicas o de arqueas en pacientes con la EP y en modelos animales puede proporcionar una imagen más amplia del mecanismo mediante el cual la microbiota intestinal influencia el desarrollo de la EP.

INTERVENCIONES DIETÉTICAS

En la actualidad, no existen estrategias terapéuticas que tengan una influencia favorable en el desarrollo de la EP. Además, ninguna de ellas se centra en tratar conjuntamente síntomas motores y no motores. Las intervenciones basadas en la nutrición, incluyendo precursores de membranas de fosfolípidos y/o terapias dirigidas a influenciar la microbiota son métodos de gran interés científico ya que podrían complementar los métodos tradicionales en el tratamiento de la EP o superar algunas de sus deficiencias como su falta de eficiencia en los síntomas no motores. (revisados en el **capítulo 7**)

En el **capítulo 3** , se investigaron los efectos preventivos de una intervención dietética en síntoma motores y no motores en dos modelos de ratón para la EP.
La dieta contenía uridina, DHA y colina, precursores para la síntesis de fosfolípidos. Estos precursores han demostrado que pueden incrementar la cantidad de fosfolípidos en el cerebro, el número de proteínas sinápticas, el crecimiento de las neuritas, la neurotransmisión dopaminérgica y la formación de espinas dendríticas. Además, los ácidos grasos poliinsaturados omega-3, como DHA, han demostrado tener propiedades antiinflamatorias que pueden reducir el stress oxidativo. La intervención dietética fue efectiva para los síntomas motores y para la prevenir la pérdida de células dopaminérgicas pero también demostró mejorar el tránsito intestinal y redujo la acumulacion de alfa-sinucleina y la inflamación en el colon.

En el **capítulo 6** se investigan los efectos terapéuticos de la misma dieta (administrada tras el desarrollo de los problemas motores) en el modelo de ratón donde se inyectó rotenona en el cuerpo estriado. En este caso la dieta redujo la disfunción motora, el deterioro cognitivo , la inflamación del colon y la acumulacion de alfa sinucleina en el sistema nervioso entérico, demostrando que la dieta no interacciona con la toxicidad de la rotenona si no que posee propiedades neurorestaurativas y por tanto tiene potencial para actuar sobre la enfermedad.
Los resultados del mismo estudio indican que una intervención dietética más extensa, que contiene los mismos precursores de fosfolípidos además de cofactores para la síntesis de fosfolípidos así como fibras prebióticas (galacto y fructooligosacáridos , GOS, y FOS) fue más beneficiosa en el tratamiento de disfunciones motoras y no motoras. La adición de de cofactores en la dieta puede afectar beneficiosamente a la formación de las membranas neuronales, incrementando la disponibilidad de precursores o beneficiando directamente a la síntesis de la membrana, lo cual explicaría sus efectos aumentados en los síntomas motores.
Se ha demostrado previamente que las fibras pre-bioticas tienen efectos beneficiosos en la función inmune y la movilidad intestinal, lo cual explicaría sus efectos positivos en la función GI cuando son añadidos a la dieta. Adicionalmente, las fibras prebióticas FOS y GOS pueden contribuir a incrementar la eficacia de la intervención dietética sobre la función motora. GOS/FOS pueden afectar positivamente a la composición de la microbiota y a su actividad metabólica, lo cual a su vez puede puede alterar los sistemas inmunes nervioso e intestinal y subsecuentemente al sistema nervioso central.
En línea con nuestras observaciones, estudios previos en otros modelos de neurodegeneración y neurotrauma agudo, la misma dieta mejoró la conectividad neuronal y el comportamiento, sugiriendo una gran variedad de aplicaciones del tratamiento dietético.

El precursor de dopamina levodopa es la sustancia más comúnmente usada en el tratamiento de la EP. No obstante tiene importantes efectos secundarios y no detiene el

proceso neurodegenerativo. Además, las disfunciones GIs en pacientes con la EP interfieren con la absorción de levodopa y por tanto con la efectividad de esta sustancia.

En el **capítulo 8** se investigan los efectos de la administración combinada de la misma intervención dietética junto con tratamientos de levodopa en el modelo de ratón donde se inyectó rotenone en el cuerpo estriado. La dieta no demostró tener influencias negativas en la efectividad de la levodopa en la función motora. No solo eso sino que la dieta combinada con levodopa fue más efectiva en la reducción de la disfunción motora que la levodopa o la dieta administradas de forma independiente.

La intervención dietética terapéutica en la EP puede incrementar la función sináptica y la neurotransmission en la terminales sinápticas, mejorando la función motora. La dieta también demostró tener efectos positivos en el funcionamiento GI en el modelo de rotenona para la EP y por tanto podría mejorar la absorción de levodopa y su biodisponibilidad.

Para resumir, los resultados de estos estudios implican que una intervención dietética conteniendo los precursores de fosfolípidos uridina, colina, y DHA, además de cofactores y fibras prebióticas, es más efectivo en la reducción de los síntomas motores y no motores en un modelo de rotenona para la EP, que la dieta que contiene solo los precursores de fosfolípidos. Ambas dietas pero especialmente la fórmula extendida han demostrado tener efectos terapéuticos en el modelo. Además la dieta extendida demuestra un efecto añadido a la levodopa en la función motora. Estos hallazgos sugieren que la intervención dietética puede proporcionar beneficios clínicos en pacientes con EP que ya están en tratamiento con levodopa. Asimismo, como se ha demostrado que la dieta tiene efectos aditivos sobre el tratamiento de levodopa, ambos tratamientos tomados conjuntamente podrían permitir reducir las dosis de levodopa administrada a los pacientes y reducir así los efectos secundarios de esta y posiblemente contribuir al uso beneficios de la misma durante periodos más largos.

CONCLUSION GENERAL

Esta tesis provee evidencias sobre la manifestación de síntomas motores y GI en dos modelos separados de ratón para la EP. Estos modelos sugieren una comunicación bi-direccional intestino-cerebro involucrada en la génesis del fenotipo de la EP donde la inflamación intestinal a través de TLR4 y cambios en la composición microbiótica juegan un papel importante.

Una intervención nutricional que contiene precursores de fosfolípidos, cofactores y fibras prebióticas ha demostrado ser más efectiva en el tratamiento de síntomas motores y no motores en el modelo de rotenona. Además, la dieta muestra un efecto aditivo cuando se combina con un tratamiento de levodopa, la sustancia más común en el tratamiento de la EP. Por tanto una dieta específica puede resultar beneficiosa en el tratamiento de EP cuando se combina con levodopa administrada por vía oral.

Combinados, los hallazgos descritos en esta tesis, crean un mayor conocimiento sobre la relevancia de las interacciones entre el intestino en la EP y demuestran que las intervenciones nutricionales pueden ser beneficiosos para el tratamiento de la enfermedad.

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About the author

Paula Perez Pardo was born in Madrid, Spain on August 21, 1985. After finishing high school at the "*Lycée Français* de *Madrid"* in 2003 she studied Biology at the "Universidad Autónoma de Madrid" and she spend an academic year at the Freie Universität in Berlin following an Erasmus Program. She pursued a master degree in Molecular Biology at the same University in Madrid. Thereafter, she spend 3 years as a junior scientist at the Center of Molecular Biology in Madrid prior to joining Drug Innovation PhD program of Utrecht University in The Netherlands under the supervision of Prof. dr. Aletta Kraneveld and Prof. dr. Johan Garssen from the division of Pharmacology and dr. Laus Broersen from Nutricia Research. Paula's project focused on gut-brain interactions in Parkinson's disease. The results from this research are presented in this thesis.

List of Publications

Paula Perez-Pardo, Hemraj B Dodiya, Phillip A Engen, Ankur Naqib; Christopher B Forsyth, Stefan Green , Johan Garssen, Ali Keshavarzian, Aletta D Kraneveld
Gut bacterial composition in a mouse model of Parkinson's disease
Manuscript in preparation

Paula Perez-Pardo[¶], Hemraj B Dodiya[¶], Christopher B Forsyth, Andrea M Huschens, Maliha Shaikh, Robin M Voigt, Jeffrey H Kordower, Kathleen M Shannon, Johan Garssen, Aletta D Kraneveld†, Ali Keshavarzian†
The role of TLR4 in the gut-brain axis in Parkinson's disease: a translational study from men to mice
Submitted for publication
¶ These two authors contributed equally to this work as First Authors
† These two authors contributed equally to this work as the senior Authors

Paula Perez-Pardo, Laus M Broersen, Tessa Kliest, Nick van Wijk, Amos Attali, Johan Garssen, Aletta D Kraneveld
Additive effects of levodopa and a neurorestorative diet in a mouse model of Parkinson's disease
Submitted for publication

Paula Perez-Pardo, Tessa Kliest, Hemraj B Dodiya , Laus M Broersen , Johan Garssen , Ali Keshavarzian , Aletta D Kraneveld
The gut-brain axis in Parkinson's disease: possibilities for food-based therapies
Eur J Pharmacol. 2017 May 23. pii: S0014-2999(17)30373-4.

Paula Perez-Pardo, Esther M de Jong, Laus M Broersen, Nick van Wijk, Amos Attali, Johan Garssen, Aletta D Kraneveld
Promising effects of neurorestorative diets on motor, cognitive, and gastrointestinal dysfunction after symptom development in a rotenone model of Parkinson's disease
Front Aging Neurosci. 2017 Mar 20;9:57

Paula Perez-Pardo, Hemraj B Dodiya, Laus M Broersen, Hidde Douna, Nick van Wijk, Sofia Lopes da Silva, Johan Garssen, Ali Keshavarzian, Aletta D Kraneveld
Gut–brain and brain–gut axis in Parkinson's disease models: Effects of a uridine and fish oil diet
Nutr Neurosci. 2017 Mar 9:1-12

Carmen D Rietdijk, **Paula Perez-Pardo**, Johan Garssen, Richard A. van Wezel, Aletta D Kraneveld
Exploring Braak's hypothesis of Parkinson's disease
Front Neurol.2017 Feb 13;8:37