Gut–brain and brain–gut axis in Parkinson's disease models: Effects of a uridine and fish oil diet

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Recent investigations have focused on the potential role of gastrointestinal (GI) 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 GI 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 GI 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 (DHA), 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 DHA containing diet prevented rotenone-induced motor and GI 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.

Keywords: Rotenone Parkinson's model, Gut–brain and brain–gut axis, GI dysfunction, Uridine, Docosahexaenoic acid

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

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DOI 10.1080/1028415X.2017.1294555

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 coworkers 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 with 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 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (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.

Methods

Mice

Seven- week-old C57BL/6J male mice (Charles River, The Netherlands) were housed at room temperature under 12 h 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. 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 1 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/100 g diet) and EPA (0.29 g/100 g diet), and uridine–monophosphate was added as a source of uridine (0.51 g/100 g diet), replacing its weight equivalent of corn starch.

Induction of mitochondrial dysfunction by rotenone: two murine models

For the oral rotenone model, mice received 10 mg/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% carboxymethyl cellulose and 1.25% chloroform). It has been previously shown that rotenone is not detected in the brain of mice receiving oral doses of 10 mg/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 2 μl 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 300 s. 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 (0.3 mL 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, Rijswijk, The Netherlands) 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, Heidelberg, Germany) 1 : 1000 followed by biotinylated goat anti-rabbit IgG (Jackson ImmunoResearch, Amsterdam, The Netherlands) 1 : 200 for 2 h. 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 hematoxylin (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 4',6-diamidino-2-phenylindole (DAPI) (1 : 10 000; 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. A 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 factor treatment and diet. Results were considered statistically significant when $P < 0.05$. Analyses were performed using SPSS 22.0.

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 the 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

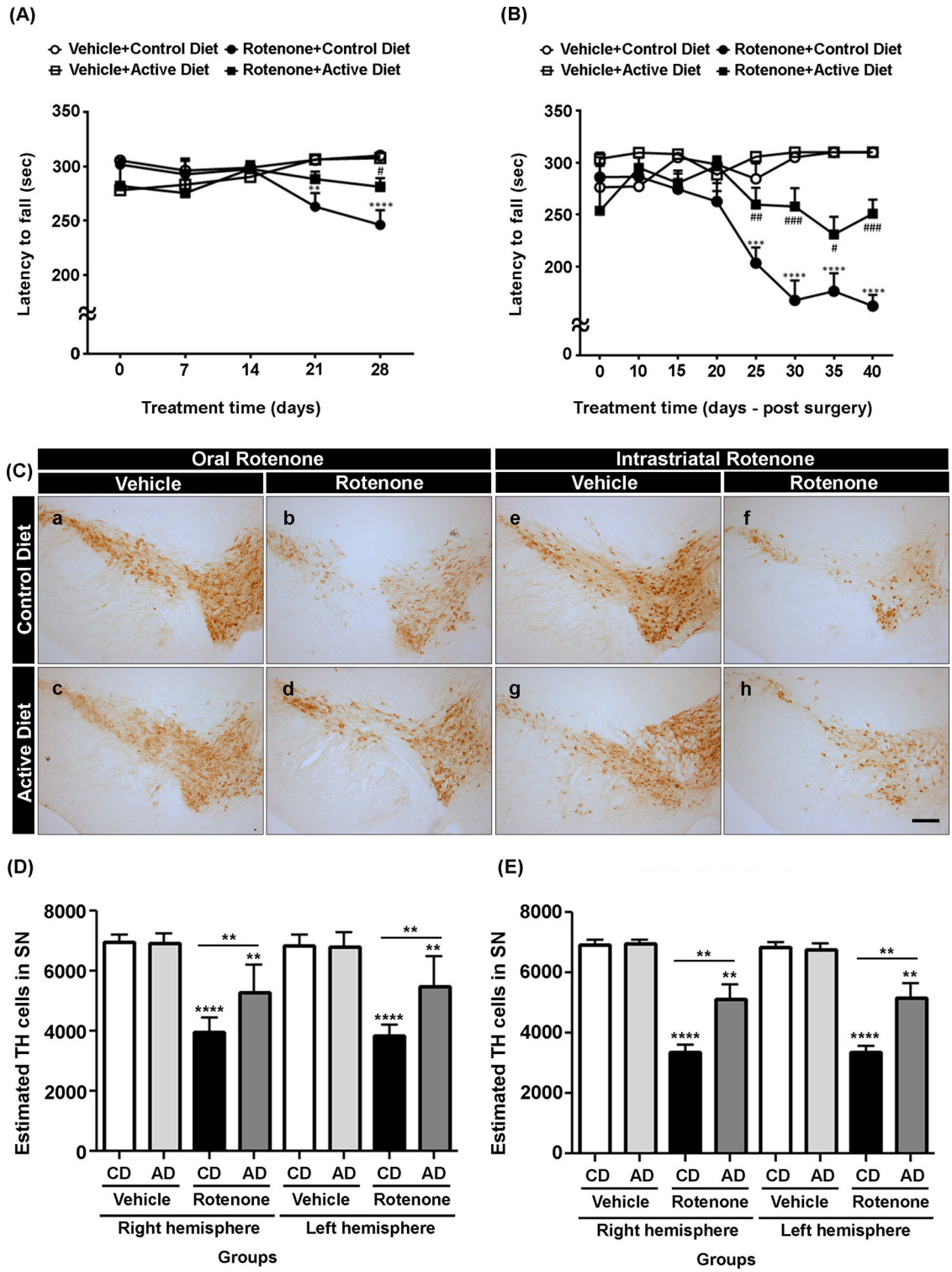

Figure 1 **Effects of the active diet on the latency to fall and dopaminergic cell loss in the SN in (A, C: a–d and D) orally rotenone-treated mice and (B, C: e–h and E) intrastriatal rotenone-injected mice. Both rotenone treatments induced motor dysfunction and decreased the number of dopaminergic cells. 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: 100 μm applies to all panels).**

($F(1,36) = 17.31$, $P < 0.001$), 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-treated animals compared to those of 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 and D).

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-operated mice ($F(1,24) = 73.57$, $P < 0.0001$). 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 and E). Rotenone injection in the striatum caused bilateral dopaminergic cell loss because 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 GI dysfunction in mice, and this dysfunction was reduced by the active diet

We recorded intestinal transit time (distance traveled 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 (Fig. 2A), rotenone-treated mice showed reduced intestinal transit 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 ($F(1,35) = 12.51$, $P < 0.01$). Rotenone-treated mice showed increased intestinal transit (increased distance covered by the dye) on active diet compared to rotenone-treated mice on control diet ($P < 0.01$) (Fig. 2A).

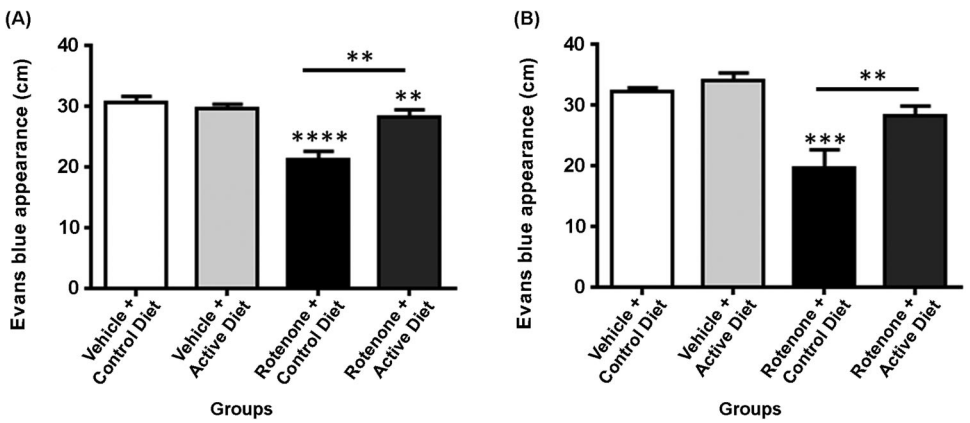

Figure 2. Effects of the active diet on intestinal transit indicated by the total distance traveled by the Evans blue dye in the GI tract 30 min after its injection by oral gavage in (A) orally rotenone-treated mice and (B) intrastriatal rotenone-injected mice. Both rotenone treatments reduced intestinal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

In the intrastriatal rotenone model, rotenone-injected mice showed decreased intestinal transit (decreased distance traveled 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).

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: $F(1,14) = 56.74$, $P < 0.0001$). OD 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: $F(1,14) = 9.824$). 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–C).

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 the immunofluorescence method.

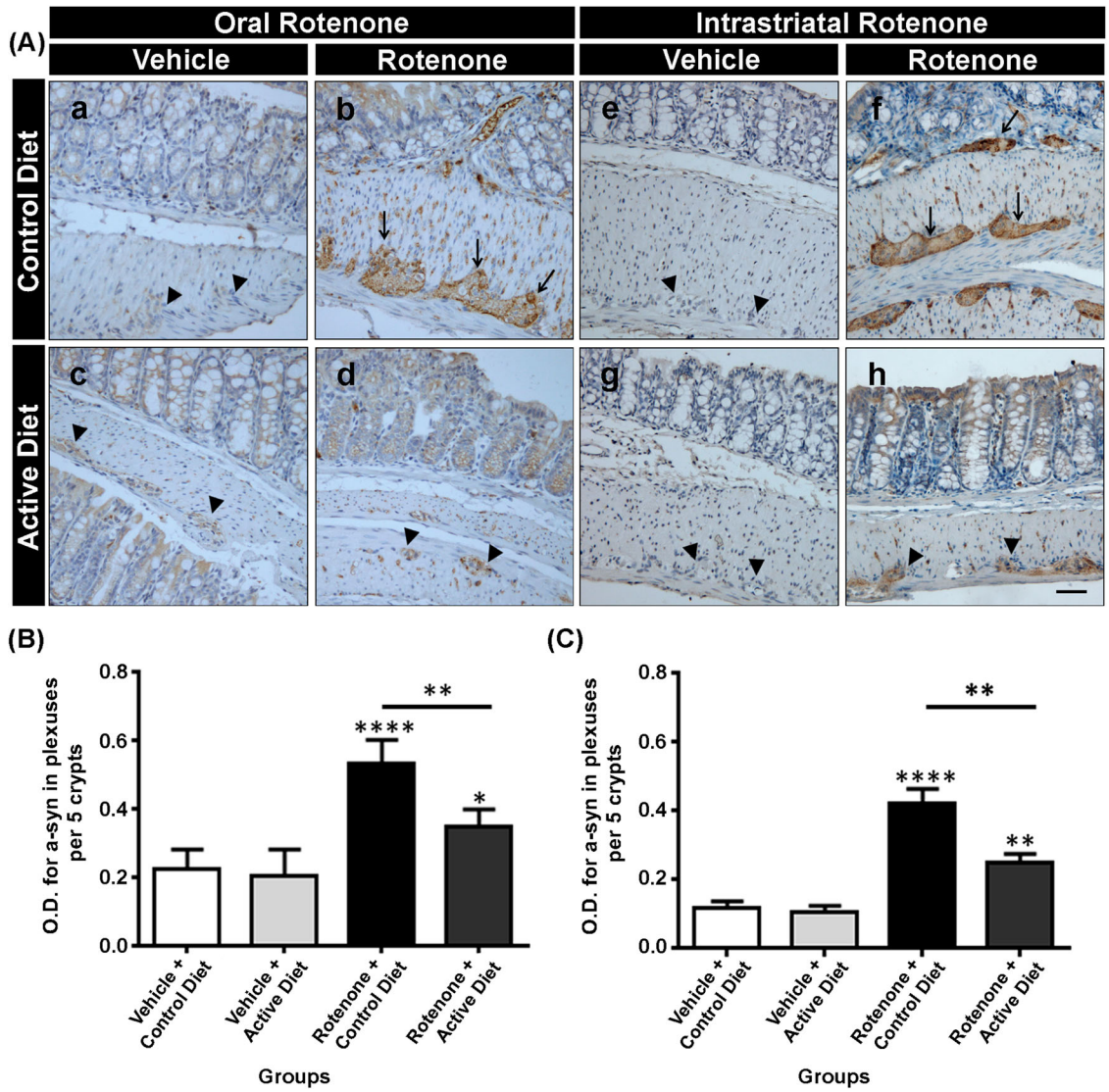

Figure 3. Effects of the active diet on alpha-synuclein expression in the colon in (A: a–d and B) orally rotenone-treated mice and (A: e–h and C) intrastriatal rotenone-injected mice. For both models, rotenone increased alpha-synuclein in the colon and the active diet was able to reduce rotenone-induced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

In the oral rotenone experiment, the vehicle-treated mice showed an intact ZO-1 expression around the mucosa in the 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 the ileum and colon). Intrastriatal rotenone injection showed no significant changes in the levels of ZO-1 expression in both the ileum and colon samples (data not shown).

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]

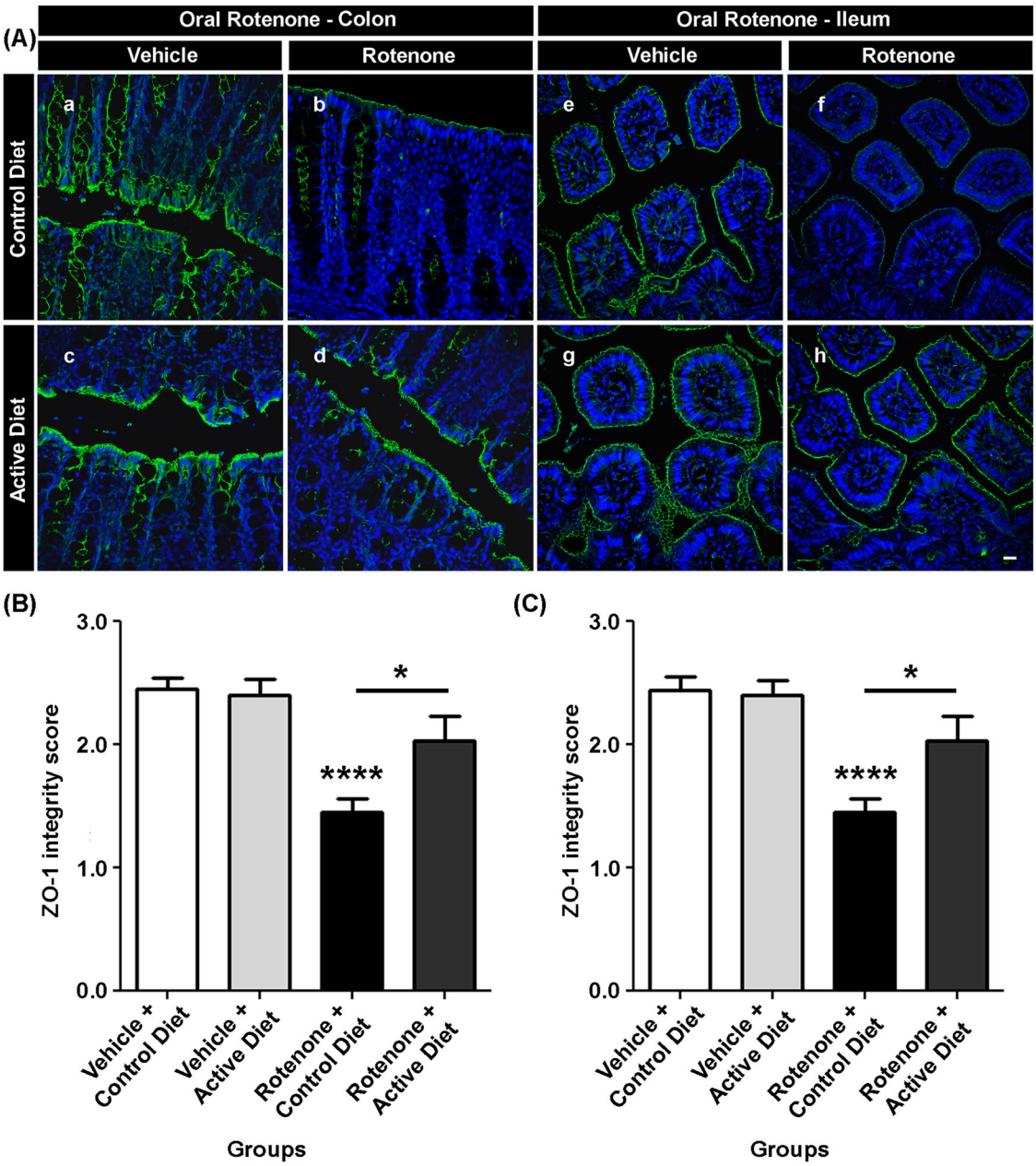

Figure 4. Effects of oral rotenone treatment and diet on epithelial integrity of the colon (A: a–d and B) and small intestine (A: e–h and C) assessed by ZO-1 tight junction protein immunoreactivity. Vehicle-treated mice showed an intact barrier in colon and small intestine (a, c, e, g). The rotenone-treated 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 the control diet (scale bar: 50 μm applies to all panels).

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 and B).

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–E). 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).

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

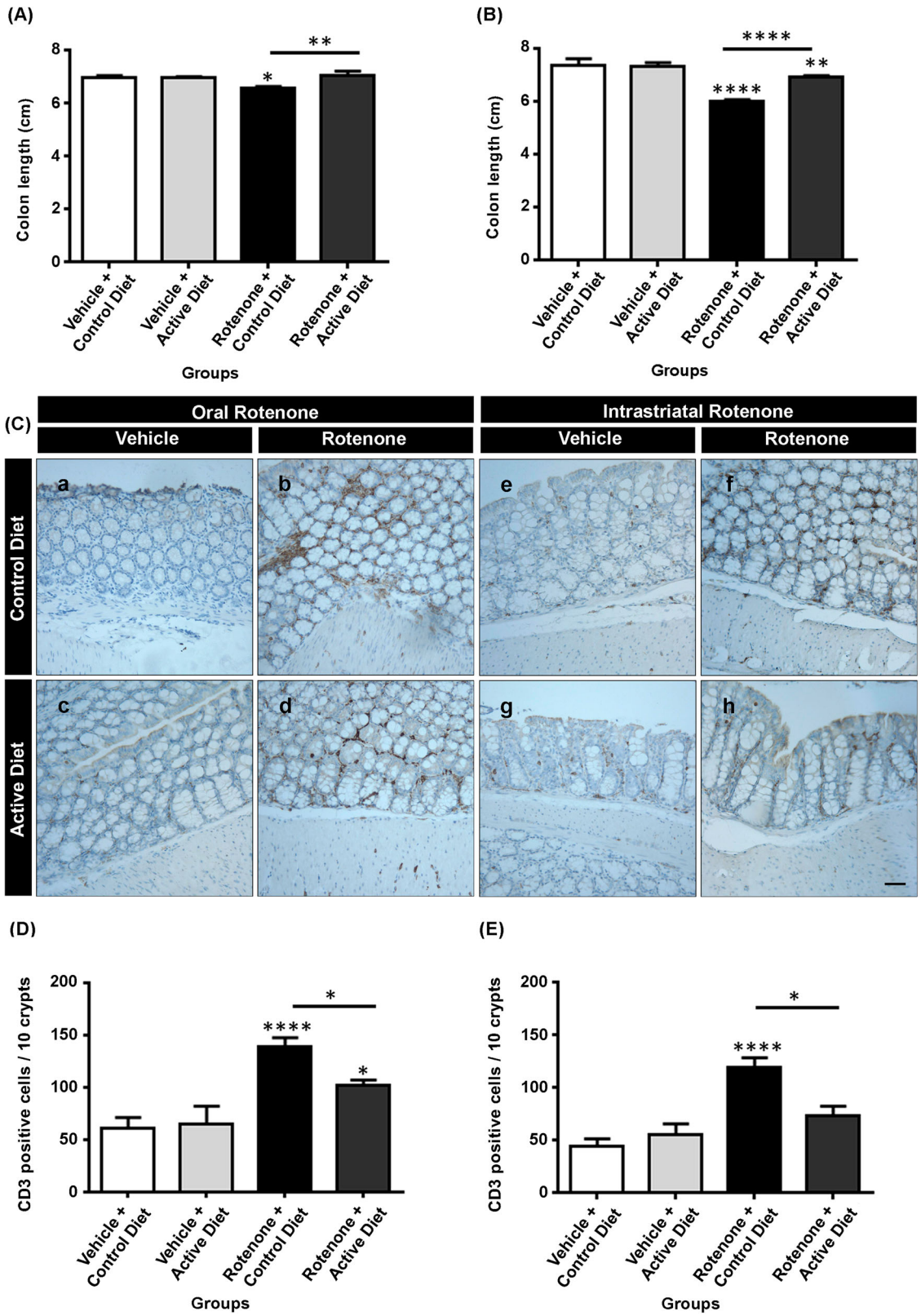

Figure 5. Effects of the active diet in colon length and in the number of T cells in the colon for the oral model (A, C: a–d and D) and intrastriatal model (B, C :e–h and E). In both models, colon length was decreased and the number of T cells was increased after rotenone exposures. The active diet restored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

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 cause 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 the 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 these together suggest 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 proinflammatory microbiota profile toward 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. These 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 GI 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.

Disclaimer statements

Contributors None.

Funding This work is part of the Utrecht University 'Focus en Massa program' and financially supported by Nutricia Research.

Conflict of interest Prof. Dr Johan Garssen, Dr Laus M Broersen, and Nick Van Wijk are employees of Nutricia Research, Utrecht, The Netherlands. Dr Sofia Lopes da Silva was an employee of Nutricia Research, Utrecht, The Netherlands, at the time of the study. All other authors report no potential conflicts of interest.

Ethics approval None.

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