

Chronic rotenone exposure reproduces Parkinson's disease gastrointestinal neuropathology

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ABSTRACT

Gastrointestinal disorders, particularly severe constipation and delayed gastric emptying, are core symptoms of Parkinson's disease that affect most patients. However, the neuropathological substrate and physiological basis for this dysfunction are poorly defined. To begin to explore these phenomena in laboratory models of PD, rats were treated with either vehicle or rotenone (2.0 mg/kg, i.p.; 5 days/week) for 6-weeks. Myenteric plexus α -synuclein aggregate pathology and neuron loss were assessed 3-days and 6-months after the last rotenone injection. Gastrointestinal motility was assessed at 3-days, 1-month and 6-months after the last rotenone injection. Rotenone treatment caused an acute reduction in α -synuclein-immunoreactivity, but this was followed 6 months later by a robust increase in aggregate pathology and cytoplasmic inclusions that were similar in appearance to enteric Lewy-bodies in idiopathic PD. Rotenone-treated rats also had a moderate but permanent loss of small intestine myenteric neurons and an associated modest slowing of gastrointestinal motility 6-months after treatment. Our results suggest that a circumscribed exposure to an environmental toxicant can cause the delayed appearance of parkinsonian α -synuclein pathology in the enteric nervous system and an associated functional deficit in gastrointestinal motility. The rotenone model may therefore, provide a means to investigate pathogenic mechanisms and to test new therapeutic interventions into gastrointestinal dysfunction in PD.

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Introduction

Idiopathic Parkinson's disease (PD) is a multi-system, complex disorder with an uncertain etiology, that affects selected neuronal populations throughout the central and peripheral nervous systems (Bonifati et al., 2004; Hirsch, 1999; Schapira, 1998; Sherer et al., 2002a). Although PD is a prototypical movement disorder, many non-motor symptoms occur in the disease, including cognitive dysfunction, sleep disorders, psychiatric symptoms and, most commonly, gastrointestinal (GI) dysfunction (Poewe, 2008). Symptoms such as dysphagia, nausea and distension as a result of impaired gastric emptying, and bowel dysfunction, including both reduced bowel movement frequency and difficulty defecating, are among the most common non-motor symptoms of PD (Cersosimo and Benarroch, 2008; Natale et al., 2008; Pfeiffer, 1998; Poewe, 2008). As such, GI dysfunction is now often considered a cardinal PD symptom that can dominate the clinical picture for some patients.

Little is known about the GI dysfunction of PD. Interestingly, GI problems may precede the onset of classical motor symptoms by many years, and their occurrence in otherwise healthy people is

associated with an increased risk of developing PD (Abbott et al., 2001). A pathological hallmark of PD in the brain is the presence of Lewy-bodies and Lewy-neurites, which are cytoplasmic inclusions of insoluble, aggregated proteins, including α -synuclein (Spillantini et al., 1997). Lewy-bodies and Lewy-neurites are also found in neurons of the myenteric and submucosal plexuses (Braak et al., 2006; Leboviev et al., 2008; Wakabayashi et al., 1988). Lewy-body pathology in the enteric nervous system (ENS) represents an early event in the disease, as they have been observed in both clinically diagnosed cases with advanced pathology, and in non-symptomatic subjects with PD-related brain lesions limited to the lower brainstem (Braak et al., 2006). In fact, according to the hypothesis proposed by Braak, the presence of Lewy-bodies in the GI tract may represent one of the earliest manifestations of the disease. It is still unclear if there is overt enteric neuron loss in PD. Attempts at quantifying enteric neuron loss in PD have been limited. In one report, a majority of PD patients showed reduced dopamine neurons in the colon myenteric and submucosal plexuses with little difference in other neuronal subtypes (Singaram et al., 1995). Similarly, another group failed to find overt neuronal loss in the colon submucosal plexus in colonic biopsies taken from a few patients (Leboviev et al., 2008). It is clear that detailed post-mortem studies of ENS innervation throughout the GI tract are lacking.

Few studies have explored GI dysfunction in laboratory models of PD. Systemic administration of the selective dopaminergic neurotoxin

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MPTP causes a loss of enteric dopamine neurons in mice and non-human primates (Anderson et al., 2007; Chaumette et al., 2009; Tian et al., 2008), and interestingly, has been associated with an increased number of neurons per ganglia in the MPTP-treated monkey (Chaumette et al., 2009). Functionally, in mice, MPTP causes an increase in stool frequency and colonic relaxation defects in mice which disappeared within one week after MPTP cessation (Anderson et al., 2007; Tian et al., 2008). These deficits are not consistent with the permanent slowing of GI motility that occurs in PD. Further, although inhibitory enteric dopamine neurons may be affected in PD, it is unlikely that they are the primary or exclusive neuropathological target of the disease. Enteric DA neurons represent a small proportion of total neurons and their inhibitory role in GI muscle contraction suggests they do not play a role in PD-related gastroparesis or slowing of motility. That is, loss of inhibition of contraction would be expected to speed rather than slow motility – and this is what was found with MPTP treatment. Further, neuropathology is observed in non-dopaminergic neurons (Wakabayashi et al., 1990). Thus, it is likely that other, non-dopaminergic neuronal populations are primarily affected in the disease. An accurate disease model should reflect this.

GI function has also been explored in transgenic mice over-expressing wild-type α -synuclein driven by the Thy-1 promoter (Wang et al., 2008). These mice had delayed colonic transit and impaired stress-induced motility. Histopathological data from the ENS have yet to be reported from these mice, but the initial findings suggest that genetic models may also provide important information regarding GI dysfunction in PD. Together, these studies highlight a pressing need to continue to develop and characterize models that reproduce PD-related GI dysfunction.

To date, there has not been a good experimental model that reproduces both the GI pathology and dysfunction of PD. Chronic systemic exposure to rotenone recapitulates key pathological and clinical hallmarks of PD in the central nervous system and is a common *in vivo* and *in vitro* laboratory model of the disease (Betarbet et al., 2000; Sherer et al., 2002b, 2003). Rotenone is a lipophilic compound that easily crosses biological membranes. Following systemic administration, rotenone evenly distributes throughout the body and gains access to all cells, including those within the GI tract (Greenamyre et al., 1992). It is a potent mitochondrial complex I inhibitor that hinders ATP production and promotes reactive oxygen species formation. Oxidative stress and ROS play roles in PD pathogenesis (Hald and Lotharius, 2005; Jenner, 2003; Onyango, 2008; Schapira, 1995; Zhang et al., 2000), and may also contribute to ENS pathology and GI symptoms (Braak et al., 2003b).

Recently, Greene et al. (2009) showed that chronic rotenone treatment (22–28 days) impaired gastric emptying in a subset of rats, and transiently decreased stool frequency and impaired longitudinal muscle contraction in response to electrical stimulation, indicative of ENS defects. No loss of ENS neurons was observed in this study and α -synuclein pathology was not examined. These initial findings suggest that rotenone administration may more accurately recapitulate parkinsonian gastroparesis and slowing of motility than does MPTP. Therefore, identifying the pathological and functional defects in the rotenone model may enhance our understanding of the nature of GI disorders in PD.

The present studies investigate the immediate and long-term effects of chronic rotenone on gastrointestinal function and PD-related ENS pathology. Here we show that chronic rotenone exposure recapitulates the primary pathological hallmark of PD in the ENS, an increase in α -synuclein-positive protein aggregates that are reminiscent of enteric Lewy-bodies described by Braak and Del Tredici (2008). Rotenone also caused a moderate loss of small intestine myenteric neurons. These pathological changes were associated with a modest slowing of GI motility.

Materials and methods

Animals

Male Lewis rats ($n=23$) age 3–4 months were obtained from Hilltop Laboratories (Scottsdale, PA). Animals were individually housed and maintained in a temperature ($22 \pm 1^\circ\text{C}$) and light controlled (12L:12D) room. Food and water were provided *ad libitum*. The University of Pittsburgh Institutional Animal Care and Use Committee approved all experiments described herein.

Rotenone administration

Rats were injected with either rotenone (2.0 mg/kg, 1.0 ml/kg, i.p.) dissolved in dimethyl sulfoxide (DMSO) and diluted in Miglyol 812N (Sasol North America), a medium chain fatty acid (2% final DMSO concentration, 98% final Miglyol concentration) or vehicle alone ($n=12$). Rats received five injections per week (Monday–Friday) for 6 weeks. At a higher dose of 3 mg/kg, this rotenone administration regimen produces relatively uniform nigrostriatal lesions (Cannon et al., 2009); however, animals become severely akinetic and thus are not well-suited for identifying physiological defects in GI function. In contrast, at a dose of 2 mg/kg, animals do not develop any motor/behavioral defects, and there is no lesion of nigrostriatal dopamine neurons.

Animal weight and food consumption were monitored throughout the experiment (Fig. 1). Rats were injected at the same time every day to ensure an equal interval between injections. Pathology was assessed 3-days ($n=12$: 5 control/7 rotenone) and 6-months ($n=11$: 6 control/5 rotenone) following the last injection as described

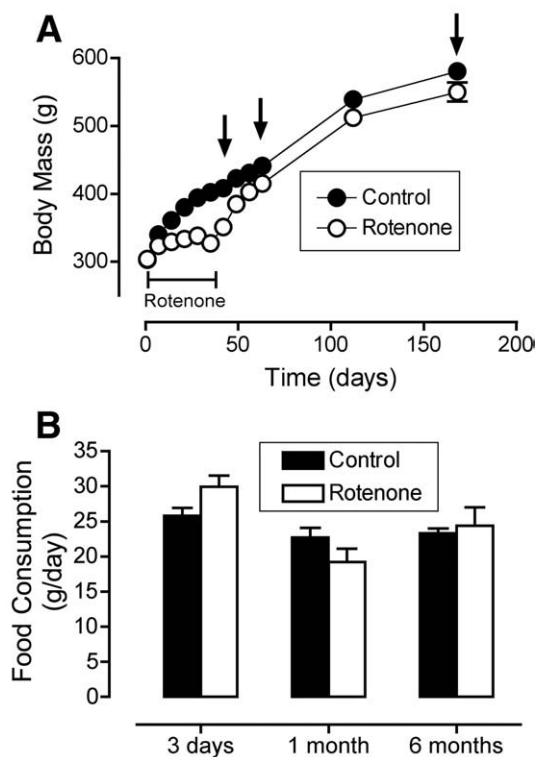


Fig. 1. Body mass and food intake over the course of the study. (A) Rats were weighed daily during the injection period and once a week after treatment ended. Weights of rotenone-treated rats lagged behind controls until treatment stopped and then quickly returned to control values. Bar indicates rotenone treatment period. (B) Daily food consumption in control and rotenone-treated rats at the time of the 1st (3-days), 2nd (1-month) and 3rd (6-months) motility assays, as denoted by arrows in panel A. Food consumption is shown as grams of dry food consumed per day. There was no statistically significant difference in food intake between groups at any time point.

below. Reduced food consumption and weight loss are common in laboratory models of PD. It is therefore difficult to attribute differences in GI function to a physiological deficit rather than acute systemic toxicity or fasting-induced motility changes. To avoid this, in the present study, GI motility was assessed at multiple time points (3-days, 1-month and 6-months after the final injection, $n = 11$: 6 control/5 rotenone) after rotenone administration ended. At these time points food intake and weight gain were equivalent between experimental groups (Fig. 1).

Gastrointestinal transit time assay

Gastrointestinal motility was determined using a modification of the *Bacillus stearothermophilus* motility assay described by Li et al. (2006). Rats were orally gavaged with 0.2 ml of CW800 Carboxylate (5 nM) a non-absorbable infrared dye (Li-Cor, Lincoln NE) dissolved in PBS. Following oral gavage, fecal pellets were collected at 6, 18, 22, 26, 30 and 42 h. Fecal pellet CW800 dye content was determined using an Odyssey infrared imager (Li-Cor). All pellets for each animal were assayed at the same time. The IR800 signal was normalized to IR700 signal, number of pellets, or pellet weight. Mean transit time for each animal was determined as the sum of the normalized IR800 signal \times time (h) at each time point collected over total normalized IR800 signal (Li et al., 2006).

Tissue processing

Three days or 6-months after the final rotenone injection rats were deeply anesthetized and perfused transcardially with saline followed by 4% paraformaldehyde. During saline perfusion, the small intestine was excised and placed into ice-cold 0.1 M PBS (pH 7.4). Segments (5 cm) of duodenum, jejunum and ileum were isolated, bisected along the longitudinal axis and pinned flat in a sylgard-coated Petri dish with mucosal side facing down. Care was taken to isolate and compare similar segments from each animal. Small intestine segments were then post-fixed overnight in 4% paraformaldehyde. A dissecting microscope was used to prepare whole mounts of small intestine myenteric plexus with longitudinal muscle (LMMP). LMMP whole mounts were then stored in ice-cold PBS with 0.05% sodium azide until immunohistochemical analysis.

Immunohistochemistry

Small intestine LMMP

All immunohistochemistry were performed on free-floating LMMP sections. For detection of α -synuclein aggregates: sections were washed 3 times in PBS and then incubated in either 100% formic acid or proteinase K (1.0 mg/ml, 25 °C) for 10 min. Following wash and serum block, sections were incubated for 48 h in primary mouse anti- α -synuclein (Syn1 BD Transduction Laboratories, 1:2000) followed by biotin-conjugated anti-mouse secondary antibody (Jackson ImmunoResearch, West Grove, PA). Bound peroxidase was visualized with 0.05% 3-3'-diaminobenzidine tetrahydrochloride with 0.01% hydrogen peroxide using an ABC Elite kit (Vector Laboratories, Burlingame, CA).

For enteric neuron detection and quantification, sections were blocked then incubated in mouse anti-HuC/HuD (1:1000, Invitrogen) a pan-neuronal primary antibody and rabbit anti-nNOS (1:1000, Upstate). The HuC/HuD primary antibody labels ~97% of all enteric neurons (Murphy et al., 2007). Primary antibodies were detected with Cy-3 and Alexa-488 conjugated secondary antibodies.

Rating α -synuclein aggregate immunoreactivity

Similar to the method for rating Lewy-body pathology in human PD cases (Beach et al., 2008), a rater blinded to the status of the

animals examined and scored α -synuclein pathology on a 0–5 scale where 0 was mild/light and 5 was intense immunoreactivity. Sections were evaluated based on: the extent, intensity, and the pattern of α -synuclein-immunoreactivity in LMMP sections. For each animal, 3 or 4 LMMP sections (~2.5 cm each) were evaluated.

Quantification of myenteric plexus neurons

HU-immunoreactive myenteric neurons were visualized with a laser scanning confocal microscope (Olympus BX61, Fluoview FV1000) at 20 \times . This magnification is ideal for visualizing and counting neurons, however many ganglia exceed the field of view. In these cases, the field of view was focused on the middle region of the ganglion. Because myenteric ganglia are uneven and do not always lie in a single plane of view, the confocal microscope acquired several (5 cm, z-stack) images through the entirety of the ganglion to ensure all neurons were counted. As depicted in Fig. 3, region of interest (ROI) lines were traced around the perimeter of each ganglion and the area within each ROI was measured. The number of HU-immunoreactive neurons within each ROI was counted and expressed as HU+ neurons/mm². At least 20 ganglia were counted for each animal and the total area sampled for each rat was kept consistent. Since ganglia density measurements may theoretically be susceptible to error introduced from tissue stretching, an additional measurement of neuron density was made to assess whether rotenone caused a loss of neurons. Similar to previously described methods (de Souza et al., 1993; Phillips and Powley, 2001), neuronal density measurements were made by counting total HU+ neurons in a large sampling frame (confocal z-stack images) and by sampling from many frames. Total HU+ neurons were then normalized to the counting area and expressed as HU+ neurons per mm².

Statistical analysis

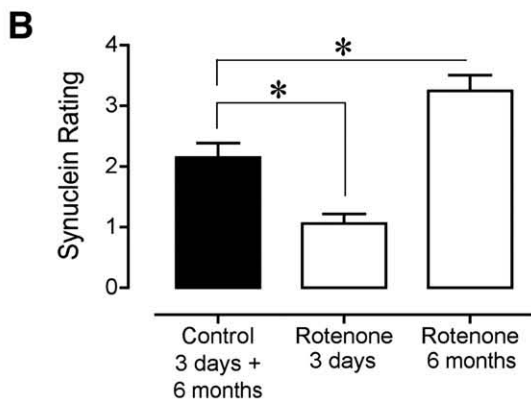
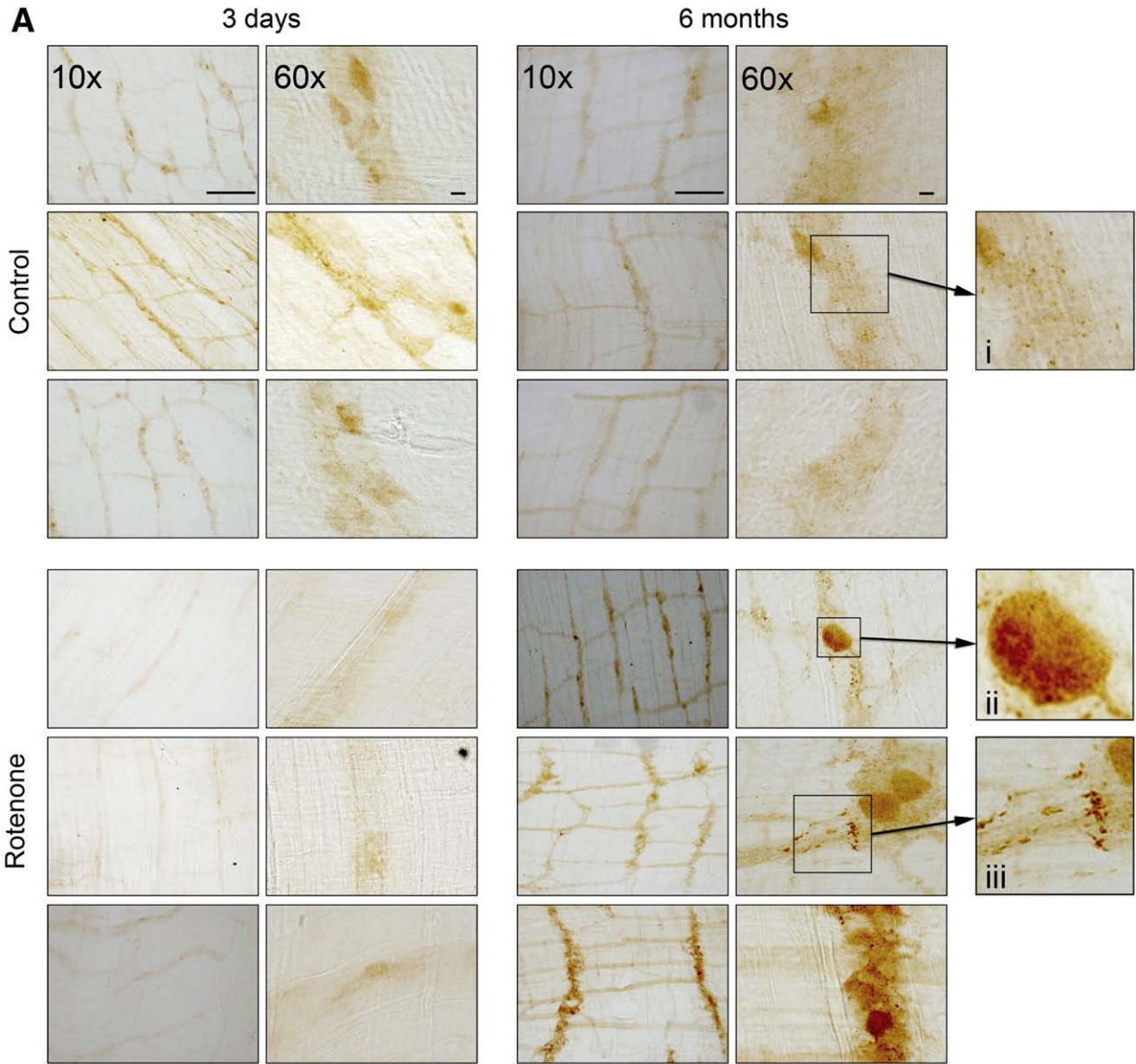
Graph Pad Prism 5 software was used to make statistical comparisons among groups using one or two-way analysis of variance. If a significant interaction was detected, post-hoc analysis was followed by between-group comparisons using Tukey's test. For α -synuclein ranking ordinal data, Graph Pad software was used to determine median and range and a Mann–Whitney *U* test was used to determine statistical significance between medians of groups. Differences with a probability of error of less than 5% were considered statistically significant.

Results

Progressive increase in formic acid-resistant α -synuclein-immunoreactivity

Lewy-bodies and Lewy-neurites are pathological hallmarks of Parkinson's disease and, similar to the brain, they are found in enteric neurons in the gut. To determine whether rotenone-induced oxidative stress causes α -synuclein pathology, the small intestine myenteric plexus (duodenum, jejunum and ileum) of control and rotenone-treated rats was examined for α -synuclein-positive protein inclusions with immunohistochemical methods used to identify Lewy-bodies in human tissue (formic acid or proteinase K digestion) (Beach et al., 2008; Braak et al., 2003a). Small intestine, as opposed to gastric or colonic, myenteric plexus was chosen because many whole mount preparations can be made throughout the extent of the small intestine allowing α -synuclein pathology and enteric neuron loss to be assessed in many adjacent sections in multiple experiments. As such, a more comprehensive view of GI pathology can be compiled in control and rotenone-treated rats.

In vehicle-treated rats, relatively faint formic acid-resistant α -synuclein-immunoreactivity was easily identifiable in myenteric ganglia (Fig. 2A). Higher magnification images show α -synuclein-



C

	3 Days		6 Months	
	Control	Rotenone	Control	Rotenone
Median	2.5	1.0*	2.0	3.5*
Range	0 – 4.5	0 – 2	1.5 – 3.5	1.5 – 4.5

Fig. 2. Formic acid-resistant α -synuclein aggregates in the small intestine myenteric plexus of vehicle and rotenone-treated rats. (A) α -Synuclein-immunoreactivity in control rats (upper panels) and rotenone-treated rats (lower panels) shown at 10 \times and 60 \times magnification. Each pair of 10 \times and 60 \times images represents a different animal. Insets show higher magnification images depicting α -synuclein aggregates in control (inset i) and rotenone-treated animals in cell bodies (inset ii) and axons (inset iii). Scale bars: 200 μ m at 10 \times ; 10 μ m at 60 \times . Note the relative decrease in α -synuclein-immunoreactivity at 3 days in rotenone-treated rats and the marked increase in α -synuclein aggregates at 6 months after rotenone treatment. (B) α -Synuclein-immunoreactivity was quantified on a 0–5 rating scale (0=Mild; light–no staining; 5=heavy; dense–severe staining) by a blinded rater. There was no difference between control rats at 3 days and 6-months post-treatment, so data were collapsed for ease of interpretation. Bars represent means \pm SEM. * p <0.05 compared to control rats at the same time point. (C) Group medians and range. * p \leq 0.05.

immunoreactivity was found in both cell bodies and axons. The majority of staining appeared diffuse within cell bodies, with some punctate staining found in axon bundles of enteric nerves. α -Synuclein-immunoreactivity was not in all enteric neurons, nor was it restricted to neurons of a particular morphology as it was observed in both large and small neurons. In control rats, there was no change in the intensity or pattern of α -synuclein-immunoreactivity between 3-days and 6-months post-treatment.

In rotenone-treated rats, 3 days after treatment ended, α -synuclein-immunoreactivity was markedly reduced relative to vehicle-treated rats (Fig. 2A). However, 6-months after the last rotenone injection there was a dramatic increase in α -synuclein aggregates compared to controls (Fig. 2A). Most striking was the increase in punctate α -synuclein aggregates that were easily visible at higher magnification. Such punctate aggregates were observed in both neuronal cell bodies and enteric axons, but were most pronounced in axons. The aggregates were virtually identical in appearance to the Lewy-bodies and Lewy-neurites found in the myenteric plexus in human PD cases (Braak et al., 2006; Braak and Del Tredici, 2008; Hawkes et al., 2007). Similar to the description by Braak et al. (2006), within cell bodies, α -synuclein aggregates were often either globose, filling large portions of the cell soma (Fig. 1A inset ii) or punctate in appearance. Punctate α -synuclein aggregates also marked select neuronal fibers of various size and shape that coursed within and between myenteric ganglia (Fig. 1A, inset iii). These processes were often swollen and dystrophic in appearance. Proteinase-K pre-treatment of tissue sections produced similar results to that of formic acid (not shown). Consistent with the lipophilic nature of rotenone and its ability to gain access to all cells, the α -synuclein aggregates were seen in neurons of all sizes and morphologies and were not restricted to a particular segment of the small intestine (duodenum, jejunum, or ileum). Thus, chronic rotenone administration reproduces the major GI pathological hallmark in PD – formic acid- or proteinase-K-resistant α -synuclein-positive protein aggregates.

Loss of enteric neurons following rotenone

It is unclear whether enteric neuron loss contributes to GI motility problems in PD; two studies failed to find evidence in support of this (Lebouvier et al., 2008; Singaram et al., 1995). However, the amount of GI tissue examined in these studies was minimal as it was restricted to small colonic biopsies from a total of 16 patients. Further investigation is therefore necessary to determine if enteric neurodegeneration occurs in PD. To determine if rotenone causes a loss of enteric neurons, the small intestine myenteric plexus from control and rotenone-treated rats were stained immunohistochemically for the pan-neuronal marker HuC/HuD. The area of each ganglion and the number of neurons per ganglion were determined using laser scanning confocal microscopy and used as an index of myenteric ganglion neuronal density. In control rats, the average neuronal densities of myenteric ganglia were 1300 ± 41 and 1383 ± 43 neurons/mm² at 3-days and 6-months after vehicle treatment, respectively. In rotenone-treated rats, however, there was a 25% decrease in neurons in the myenteric plexuses at both 3-days and 6-months post rotenone treatment (Fig. 3). Similar to α -synuclein aggregate pathology, we did not observe a difference in cell loss based on proximal or distal location within the small intestine. Further, since ganglia neuronal density may be affected by tissue stretching, we also quantified total neuronal density in samples of small intestine tissue, randomly including areas that did and did not contain ganglia. Similar to the specific reduction in myenteric ganglia neuronal density, rotenone caused $25 \pm 4\%$ and $35 \pm 14\%$ decreases of small intestine enteric neuronal density at 3-days and 6-months, respectively. Thus, using indices of ganglia and total neuronal density, the present findings suggest that enteric neurons are susceptible to oxidative stress-

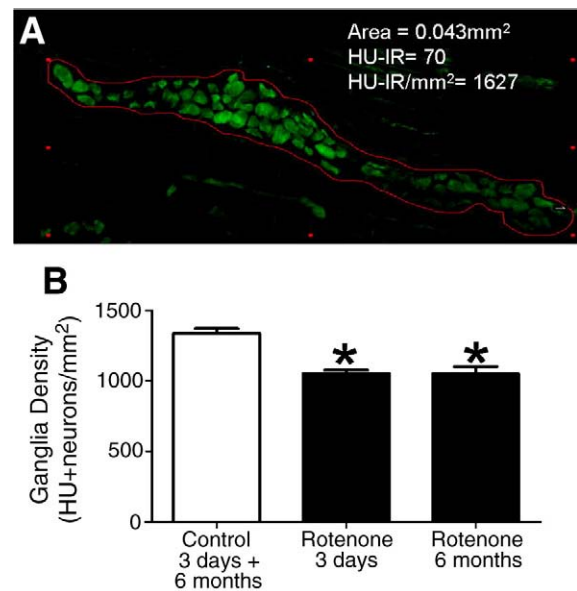


Fig. 3. (A) HU-immunoreactive neurons in the myenteric plexus of a control rat. Region of interest (ROI) lines were generated around the perimeter of the ganglion using Olympus Fluoview software and the area was measured. The number of HU+ neurons in each ROI was counted and expressed as HU+ neurons per mm². (B) Small intestine enteric neuron density in control and rotenone-treated rats. A minimum of 20 ganglia were counted and averaged for each rat. Bars represent means \pm SEM. * $p \leq 0.05$ compared to control rats at the same time point.

induced neurodegeneration and therefore, may also contribute to gastrointestinal symptoms in PD.

GI motility after rotenone administration

To determine whether rotenone causes a defect in GI function similar to PD, total GI transit time was assessed at 3-days, 1-month, and 6-months after completion of vehicle or rotenone treatment (Fig. 4). This assay was chosen in order to make repeated measurements of GI motility in the same animals over time and detect any perturbations to global GI function. In control rats, 3-days after the last vehicle injection, mean gastrointestinal transit time of CW800 carboxylate was 21.5 ± 0.25 h and did not change over subsequent time points (Fig. 4A). In rotenone-treated rats, there was more variability in GI transit time at all time points, but by 6 months post-treatment, the mean transit time of rotenone-treated rats had slowed to 23.5 ± 0.5 h ($p < 0.05$; Fig. 4A). All but one of the animals had a GI transit time that was at least 0.5 h slower than controls and, for 3 of the 5 rats, transit time was approximately 3 h slower than controls. Except for the rat with the slowest transit time at 1-month, the transit times of each of the animals slowed between 1 month and 6 months post-treatment (Fig. 4C); however, this difference between 1 and 6 months was not statistically significant. In summary, chronic rotenone treatment caused a modest slowing of GI motility.

Discussion

Rotenone, a naturally-occurring pesticide and inhibitor of mitochondrial complex I, has been used to model PD and, with chronic administration, it recapitulates key behavioral and pathological aspects of the disease, including intracellular aggregates of α -synuclein similar to Lewy-bodies. Here, we have shown that chronic low-grade exposure to rotenone (at a dose that does not lesion nigrostriatal dopamine neurons or produce motor impairments) reproduces an essential 'non-motor' aspect of the disease – GI neuropathology and dysfunction. This suggests that common pathogenic mechanisms are responsible for pathology in the central and

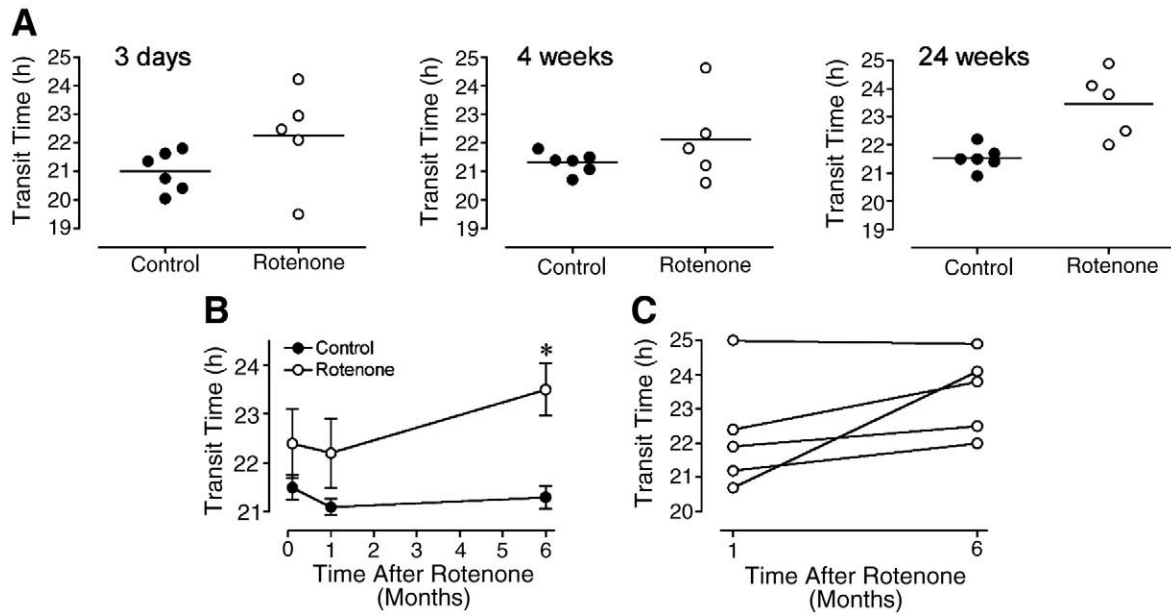


Fig. 4. Gastrointestinal transit time following vehicle and rotenone treatment. (A) Using the CW800 carboxylate transit assay, gastrointestinal transit was measured 3-days, 1-month and 6-months following completion of vehicle or rotenone administration (2.0 mg/kg, i.p.). Data points represent transit times of individual animals and horizontal bars indicate group means. (B) Time-course comparison of GI transit times of control and rotenone-treated rats after cessation of rotenone treatment. Note that the y-axis scale (Transit Time) does not include a zero value since a transit time of zero – or one that is less than control values – is not physiologically relevant for the current studies. Data points reflect means of groups \pm SEM. * $p \leq 0.05$. (C) Scatter plot of GI transit times for rotenone-treated rats 1 and 6 months after rotenone treatment. Except for the animal with the longest transit time at 1 month, all animals showed a further prolongation of GI transit time at 6 months compared to 1 month.

enteric nervous systems, and suggests the ENS may be particularly sensitive to oxidative stress and mitochondrial dysfunction.

Although GI function is commonly affected in PD, the enteric neuropathology of the disease is relatively poorly characterized. Nevertheless, it is clear that there is extensive α -synuclein accumulation and aggregation in the ENS and that it may occur early in the disease. This aspect of the disease is reproduced well by the rotenone model described here: the formic acid or proteinase-K-resistant aggregates seen in our model are reminiscent of those described in the ENS of individuals with PD (Braak et al., 2006), and, as in the human disease, they occur in both cell bodies and axons. It is notable, however, that while enteric Lewy-bodies in post-mortem human tissue are not yet well-characterized, limited ultrastructural studies from a few PD cases suggest they are very similar to those found in the substantia nigra (Wakabayashi et al., 1988). Further, there is evidence that enteric Lewy-bodies also contain serine-129 phosphorylated α -synuclein (Lebouvier et al., 2008). Additional ultrastructural and biochemical studies of both human and rat tissue will be necessary to determine the extent to which the enteric α -synuclein aggregates observed in the rotenone model are truly reminiscent of the Lewy-bodies in PD.

With regard to the human ENS neuropathology of PD, an issue that requires clarification is whether Lewy pathology affects specific subtypes of neurons selectively. Some studies have suggested that Lewy-bodies tend to localize selectively to vasoactive intestinal peptide (VIP)-containing enteric neurons, the majority of which are a subset of nitrergic neurons (Wakabayashi et al., 1990). However, this has only been shown by a single laboratory in a total of 3 PD autopsy cases, so further neuropathological characterization is needed. In the central nervous system, Lewy pathology is not restricted to a particular neurotransmitter phenotype, as it is found in cholinergic, dopaminergic, and noradrenergic neurons (Braak et al., 2003a). The present studies suggest that, similar to the brain, there is not a selective localization of α -synuclein aggregates to a particular neuronal subtype, since they were noted in both large and small neurons throughout the ENS. Further, preliminary studies suggest that rotenone-induced α -synuclein pathology is found in both nitrergic

and cholinergic neurons (not shown) and therefore is unlikely to be specific to VIP neurons.

Another issue that must be resolved is whether there is frank degeneration and loss of myenteric neurons in PD. Thus far, attempts at quantifying enteric neuron loss have been restricted to colonic biopsies taken relatively few patients (Lebouvier et al., 2008; Singaram et al., 1995). Detailed post-mortem studies of ENS innervation, particularly in the myenteric plexus and throughout the GI tract are certainly lacking. Assessing enteric neuron loss is difficult because of the variability in GI tissues and regions, the amount of tissue sampled, and collection methods. The present study attempted to minimize these confounds by (i) comparing similar GI segments, (ii) counting from many myenteric ganglia, (iii) using confocal microscopy and 'z-stack' reconstruction of cells to avoid staining and tissue variability, and (iv) using indices of ganglia density and total enteric neurons. Using these methods, we showed that rotenone caused a moderate, and apparently permanent decrease in myenteric ganglion (and total) neuronal density. It remains to be determined whether similar methodology will also reveal enteric neuron loss in human PD. In this regard, however, it should be noted that, in addition to reproducing many behavioral, biochemical and pathological features of PD, the rotenone model actually predicted what we would find in the human post-mortem parkinsonian brain with respect to mechanisms of iron accumulation (Mastroberardino et al., 2009) and may serve a similar predictive function with respect to GI neuropathology. It is also worth noting that while our study focused on small intestine, there is currently a lack of information regarding GI neuropathology in the small intestine in PD, because most studies have focused on the stomach or colon.

In addition to α -synuclein pathology, and loss of enteric neurons, we found that rotenone exposure caused a delayed slowing of GI transit time. Since there has been very little study of GI dysmotility in PD models, we chose to explore this phenomenon using a rather gross measurement – total GI transit time – to detect any perturbation in GI motility. The advantage of this approach is that changes in motility can be assessed repeatedly in the same animal at multiple time points after toxin administration. However, a limitation of this motility assay

is that compensatory changes in one GI region could mask deficits in other regions. Thus, it is conceivable that more robust deficits in GI function could exist in selected regions of the GI tract (stomach, small intestine, or colon). In fact, similar to the report from Greene et al. (2009) preliminary results from a separate cohort of animals indicate that rotenone substantially slows gastric emptying (not shown). Such gastroparesis is a common problem in PD and may lead to levodopa “dose failures” and motor fluctuations. Currently it is unclear how the α -synuclein pathology, enteric neuron loss, and impaired GI transit observed in rotenone-treated rats are related and whether a cause-effect relationship exists. Elucidating the complex relationship between these phenomena will be an important task of future studies and may aid in understanding the nature of GI dysfunction in PD.

Presently, there are no effective therapies for the GI dysmotility of PD. Cisapride and tegaserod, two “prokinetic” drugs that increase acetylcholine release in the ENS by stimulating 5-HT₄ receptors, have been recently withdrawn from the US market because of their potential cardiotoxicity (Smalley et al., 2000; Thompson, 2007; Wysowski et al., 2001). Other prokinetics widely used in clinical practice include dopamine antagonists acting on D₂-like receptors, such as metoclopramide; however, because of their dopamine blocking properties, such compounds are contraindicated in PD. There is a pressing need for an appropriate animal model with which to screen drugs to improve GI motility in PD patients. The present findings suggest that the current model may be a step in that direction.

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