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Delayed gastric emptying and enteric nervous system dysfunction in the rotenone model of Parkinson's disease

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Abstract

Gastrointestinal (GI) dysfunction is the most common non-motor symptom of Parkinson's disease (PD). Symptoms of GI dysmotility in PD include early satiety and weight loss from delayed gastric emptying and constipation from impaired colonic transit. Understanding the pathophysiology and treatment of these symptoms in PD patients has been hampered by the lack of investigation into GI symptoms and pathology in PD animal models. We report that the parkinsonian neurotoxin and mitochondrial complex I inhibitor rotenone causes delayed gastric emptying and enteric neuronal dysfunction when administered chronically to rats in the absence of major motor dysfunction or CNS pathology. When examined 22–28 days after initiation of rotenone infusion by osmotic minipump (3 mg/kg/day), 45% of rotenone-treated rats had a profound delay in gastric emptying.

Electrophysiological recording of neurally-mediated muscle contraction in isolated colon from rotenone-treated animals confirmed an enteric inhibitory defect associated with rotenone treatment. Rotenone also induced a transient decrease in stool frequency that was associated with weight loss and decreased food and water intake. Pathologically, no alterations in enteric neuron numbers or morphology were apparent in rotenone-treated animals. These results suggest that enteric inhibitory neurons may be particularly vulnerable to the effects of mitochondrial inhibition by parkinsonian neurotoxins and provide evidence that parkinsonian gastrointestinal abnormalities can be modeled in rodents.

Keywords

Parkinson; gastrointestinal; dopamine; stomach; gastric; rotenone; constipation; enteric; gut; myenteric

Introduction

Parkinson's disease (PD) is a common neurodegenerative disorder typically associated with prominent motor symptoms thought secondary to degeneration of substantia nigra dopamine neurons in the midbrain.

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As the motor symptoms of PD have begun to be ameliorated by effective pharmacological and surgical treatments, the prominence and severity of non-motor symptoms in PD patients has been more fully recognized (Hely, et al., 2005, Temlett and Thompson, 2006, Woodford and Walker, 2005). Gastrointestinal dysfunction is the most common non-motor symptom of Parkinson's disease, and the majority of symptoms, such as constipation, bloating, and dysphagia result from abnormal motility of the GI tract (Edwards, et al., 1992, Natale, et al., 2008, Pfeiffer, 2003). These symptoms have a significant negative impact on quality of life and frequently precede the onset of motor abnormalities (Abbott, et al., 2001, Pfeiffer and Quigley, 1999, Ueki and Otsuka, 2004). Despite the important consequences that GI dysmotility has for patients and its potential relationship to incipient PD, there is a dearth of information concerning the pathophysiology of the GI tract in PD and PD model systems.

The mitochondrial complex I inhibitor rotenone has been shown to mimic certain behavioral and neuropathological aspects of PD when administered systemically to rats, including motor abnormalities and dopamine neuron loss (Betarbet, et al., 2000, Fleming, et al., 2004, Sherer, et al., 2003). The fact that rotenone is an environmental contaminant due to its use as an organic pesticide has stimulated significant interest in this animal model of PD.

In this study, we evaluated the effects of systemic rotenone administration on gastrointestinal motility in rats. In particular, we examined behavioral GI motility and electrophysiological function of the enteric nervous system (ENS), the semi-autonomous neural network that predominantly controls GI motility. Our results suggest that delayed gastric emptying occurs during rotenone intoxication in the setting of a functional inhibitory deficit in the enteric nervous system. This study is one of the first to examine GI function in a PD model system and points to the ENS as a possible site of dysfunction that may cause GI dysmotility in PD.

Materials and Methods

Animals and rotenone treatment

All experimental procedures were in accordance with the NIH Guide for the Care and Use of Experimental Animals and approved by the Emory University Institutional Animal Care and Use Committee. Male Lewis rats (250g) were subcutaneously implanted with osmotic minipumps resulting in administration of rotenone (3 mg/kg/day) or vehicle (50:50 DMSO:polyethylene glycol). Infusion was continuous for 22–28 days (Sherer, et al., 2003). During the first week of infusion, rats were given access to hydrated chow on the floor of the cage. At the end of infusion, rats were anesthetized with isoflurane and killed by decapitation. Brain, stomach, small intestine, and colon were removed from the animals for electrophysiological and histopathological analyses. Two separate experimental groups of 20 rats each (8 vehicle- and 12 rotenone-treated per group) were used for behavioral and pathological analyses. Electrophysiological experiments were performed on a separate group of 14 animals.

General health assessment and gross motor function

Rats were observed twice daily during the first week of infusion and daily thereafter. General health and gross motor function were assessed by observing in-cage behavior and during brief gentle handling to check for rigidity (hunched posture and increased tail tone), bradykinesia (slowed movement and/or absence of rearing), dystonia (clenched paws), autonomic signs (piloerection), abnormal thermoregulation, and signs of dehydration (dry mucus membranes or tenting skin).

Measurement of colon motility by one-hour stool collection

One-hour stool frequency was measured prior to pump implantation and on days 8, 11, 14, 18, and 21 after the start of rotenone infusion. Assays were performed between 10:00 and 12:00 on each day. Each animal was removed from its home cage and placed in a clean, clear plastic cage without food or water for one hour. Stools were collected immediately after expulsion and placed in sealed tubes. The total stools were weighed to provide a wet weight, then dried overnight at 65°C and weighed again to provide a dry weight. Results were normalized to body weight (Li, et al., 2006).

Solid gastric emptying

Following a 12-hour fast, rats were allowed free access to food for one hour. Two hours later, animals were killed and the stomach contents were weighed. Food was weighed before and after the feeding period to confirm equal consumption between groups (Anderson, et al., 2007, Whited, et al., 2006). Results were normalized to body weight.

Isometric muscular force recording

Segments of proximal colon were collected for isometric muscle recordings using electrical field stimulation (EFS) according to previously published methods (Anderson, et al., 2007, Anitha, et al., 2006). Samples of longitudinal muscle along with their enteric innervation were attached to two hooks placed between platinum electrodes and suspended in Krebs buffer parallel to normal flow. Each sample was tightened to a force of 3 mN and allowed to equilibrate for at least 1 hour. Amplitude and frequency of contractions were assessed following this normalization period. Magnitude of relaxation induced by EFS (24V, 4Hz, 0.1 msec pulse width, 30 sec) was assessed after pre-contraction using 10 μ M serotonin (5-HT) in the presence of atropine (1 μ M) and guanethidine (1 μ M). For quantification, the difference in force during the 30 second period of EFS was compared to the maximum force from the period immediately prior to EFS and the minimum baseline force. Results are expressed as percentage relaxation with -100% being complete relaxation of the tissue.

Preparation of tissue samples

Wholemounts of ileum and proximal colon were prepared for staining according to previously published methods (Anitha, et al., 2006; Anderson et al., 2007) (Anderson, et al., 2007, Anitha, et al., 2006). Intestinal segments were excised from the rat, and the contents were washed out with phosphate buffered saline (PBS). Pieces of intestine were cut along the mesentery, stretched flat on a silicone coated plate, and fixed in 4% formaldehyde at room temperature for two hours. After fixing, longitudinal muscle and the underlying myenteric plexus were peeled from the submucosa using a dissecting microscope.

The pyloric region was embedded in OCT embedding medium and frozen on dry ice. 20 micron sections were collected onto slides using a cryostat and stored at -80°C until use. Prior to staining, sections were fixed with 4% paraformaldehyde for 30 min at room temperature.

The brain was fixed overnight in 4% paraformaldehyde and then transferred to 30% sucrose for 3 days. Forty micron sections through the striatum and midbrain were collected using a sliding microtome and stored at -20 °C in cryoprotectant solution.

Immunostaining

Samples were stained for vasoactive intestinal peptide (1:100; rabbit anti-VIP; Millipore), HuC/D (1:100; mouse anti-HuD; Millipore), choline acetyltransferase (1:100; goat anti-ChAT; Millipore), nitric oxide synthase (1:1000; rabbit anti-NOS; Millipore), or tyrosine hydroxylase (1:100; rabbit anti-TH; Millipore). On the first day, sections were washed in tris-buffered saline

(TBS) with 1% Triton X-100, blocked with NGS or NHS for 30 minutes, and then incubated with primary antibody solution overnight at 4 °C. The following day, sections were washed with TBS and incubated with secondary antibody for 1 hour (1:500; AlexaFluor488 goat anti-rabbit (Invitrogen), Cy3 goat anti-mouse (Jackson), biotinylated donkey anti-goat (Jackson), or biotinylated goat anti-rabbit (Jackson). For histochemical detection, after treatment with 0.3% hydrogen peroxide, sections were exposed to ABC solution (Vectastain) for 1 hour, washed, and incubated with DAB in de-ionized water for ten minutes.

NADPH diaphorase staining

Pieces of intestinal wholemount were washed in PBS and incubated in diaphorase solution (NADPH, 1 mg/ml; nitroblue tetrazolium, 0.1 mg/ml; and 0.3% Triton-X 100 in PBS) for 1 hour at 37 °C. After washing, tissue was mounted on glass slides, dehydrated, and coverslipped (Anderson, et al., 2007, Anitha, et al., 2006). Previous reports have demonstrated that NADPH-diaphorase and NOS immunostaining label the same population of rat myenteric neurons (Belai, et al., 1992, Saffrey, et al., 1992).

Neuron counting

Neuron counting was performed by a reader blinded to treatment group. VIP-, HuD-, NOS-, and ChAT-positive neurons were counted using a 1 cm² grid at 20X magnification (0.25 mm²). The average number of positive neurons in wholemounts from each of five randomly chosen grids is reported per mm² (Anderson, et al., 2007, Anitha, et al., 2006). In addition, the average percentage of neurons expressing a particular neuronal phenotype relative to the total number of HuC/D neurons is reported.

Tyrosine hydroxylase immunoblotting

Tissue samples were homogenized in Tissue Protein Extraction Reagent, TPER (Pierce) supplemented with 700 U/ml DNase and 1% β-mercaptoethanol (Sigma) and centrifuged at 10,000×g for 10 min. Supernatant was collected, and protein content was assayed using BioRad protein assay (BioRad) according to manufacturer's protocol. Protein samples were run on 12% polyacrylamide gels, transferred to PVDF membranes (Immobilon, Millipore) and probed for tyrosine hydroxylase (1:1000, mouse anti-TH, Millipore). Rabbit polyclonal anti-TUJ (1:1000, Covance) was used to confirm equal loading of wells. AlexaFluor 680 donkey anti-mouse (1:5,000; Molecular Probes, Eugene, OR) and IRDye 800 goat anti-rabbit (1:5,000; Rockland, Gilbertsville, PA) secondary antibodies were used. Blots were dried, scanned, and quantified with an Odyssey Infrared Imaging System (Li-Cor Biosciences) (Betarbet, et al., 2006).

Statistics

Results are expressed as means. Except for stool frequency and body weight, which entailed repeated measures, data were compared with unpaired t-tests. For stool frequency, a 2 × 5 mixed design ANOVA was used to compare mean stool frequency per hour across either treatment or gastric emptying (between factor) and day after start of infusion (repeated factor) with Bonferroni posttests. A p-value of less than 0.05 was considered significant.

Results

Surviving rotenone-treated rats have normal brain TH neurons and gross motor behavior

The first 7–10 days of rotenone administration was associated with signs of systemic illness, such as piloerection, decreased activity, and weight loss, which is expected in this model. Food and water intake was also lower early in the infusion. We and others have observed that rats that go on to develop motor parkinsonism and central dopamine lesions in this model system

typically have a phenotype of either continued or precipitous weight loss and/or motor decline, with evidence of gross motor abnormalities or central dopamine pathology being rare in surviving animals (Sherer, et al., 2003). Other groups have reported that a significant proportion of surviving animals do show alterations in dopamine pathology associated with subtler motor findings, such as impaired rearing behavior (Fleming, et al., 2004, Zhu, et al., 2004). In this experiment, surviving rotenone-treated animals (>95% of our animals) showed grossly normal behavior and normal food and water intake after 2 weeks of treatment. To minimize any effects of acute illness on the results, electrophysiological and pathological evaluation was performed on animals after 3–4 weeks of treatment. Motor examination, including posture, tail position and tone, gait, and rearing behavior was grossly normal. Additionally, examination of brains from these animals revealed neither morphological abnormality in tyrosine hydroxylase immunohistochemistry nor decreased TH protein content in striatum (Fig 1). Midbrain TH morphology (not shown) and protein content [100 ± 26 v. 104 ± 24 ; mean $\pm$ SEM; vehicle (N = 5) v. rotenone (N = 5)] were also not altered.

Rotenone-treated rats have delayed gastric emptying

Rotenone-treated animals had higher residual stomach content two hours after a meal, indicating a significant delay in solid gastric emptying associated with rotenone intoxication (Fig 2A). Food intake between the groups during the test was the same (9.6 ± 1.5 v. 9.5 ± 0.9 mg/g body weight; mean $\pm$ SEM; N = 11 each; rotenone v. vehicle). There were two populations of rotenone animals apparent, one that retained a large amount of food in the stomach after a meal, and one that was similar to control animals (Fig 2B). There was no correlation between initial weight loss, used as a marker of acute rotenone toxicity, and subsequent gastric emptying after 22–28 days of rotenone exposure.

Rotenone-treated rats have a transient decrease in stool frequency

There was a transient decrease in colon motility as assessed by one-hour stool frequency in rotenone-treated rats. ANOVA revealed a significant main effect of treatment $F(1,144) = 13.75$, $p < 0.001$, but no main effect of treatment day $F(4,144) = 1.66$, $p > 0.05$, or treatment times day interaction $F(4,144) = 1.93$, $p > 0.05$. Post hoc analysis revealed a significant difference between rotenone- and vehicle-treated rats on day 8 after the start of infusion ($p < 0.05$).

The decline in stool frequency paralleled body weight in rotenone animals in that when stool frequency recovered, so did weight gain (Fig 3B). For body weight, ANOVA revealed a significant main effect of treatment $F(1,144) = 113.24$, $p < 0.0001$, a main effect of treatment day $F(4,144) = 80.62$, $p < 0.0001$, and a significant treatment times day interaction $F(4,144) = 48.61$, $p < 0.0001$. Post hoc analysis revealed a significant difference between rotenone- and vehicle-treated rats on each day after the start of infusion ($p < 0.05$).

There was lower stool frequency in rotenone-treated animals subsequently shown to have delayed gastric emptying (Fig 3C). ANOVA revealed a significant main effect of gastric emptying $F(1,36) = 6.95$, $p < 0.05$, but no main effect of treatment day $F(4,36) = 2.61$, $p > 0.05$, or gastric emptying times day interaction $F(4,36) = 0.16$, $p > 0.95$. Post hoc analysis revealed no significant differences at any individual day after the start of infusion.

Stool water content, another indicator of colon function, was no different between groups on day 21 of infusion ($59 \pm 8\%$ vehicle v. $60 \pm 4\%$ rotenone; mean $\pm$ SEM).

Rotenone-treated rats exhibit a physiological defect of inhibitory neurons in the ENS

Longitudinal colon muscle from rotenone-treated rats generated 41% more isometric contractile force in response to $10 \mu\text{M}$ serotonin than colon muscle from vehicle-treated rats (Fig 4A–C). Electrical field stimulation of the ENS following serotonin contraction caused a

tetrodotoxin-sensitive relaxation of colon muscle that was reduced by 30% in rotenone-treated animals compared to controls (Fig 4A,B,D). These findings together are consistent with an inhibition defect in the ENS of rotenone-treated rats.

Rotenone-treated rats have normal numbers of enteric neurons

Given the physiological impairment of ENS inhibitory function in rotenone-treated animals, major inhibitory ENS neurotransmitters were examined histologically. The number of neurons and character of innervation was not changed for NADPH-diaphorase (NOS) neurons or vasoactive intestinal peptide (VIP) neurons (Fig 5A–C). The morphological appearance of tyrosine hydroxylase (catecholaminergic) processes in the ENS was not changed (Fig 5B), nor was the level of protein assessed by western blot [100 ± 6 v. 116 ± 12 ; mean $\pm$ SEM; vehicle (N = 12) v. rotenone (N = 20)]. Numbers of cholinergic (ChAT) neurons, the major excitatory transmitter in the ENS, were the same between groups (Fig 5A, D). Not surprisingly given the above results, the total number of enteric neurons as assessed by HuD immunostaining was unaffected by rotenone (Fig 5C). The results presented in Figure 5 are from ileum, but analyses of the pyloric region and proximal colon yielded similar results. As can be seen in Table 1, the relative percentage of NOS, VIP, and TH myenteric neurons was unchanged by rotenone. Qualitatively, immunostaining for NOS, VIP, and TH was similar among vehicle- and rotenone-treated rats.

Discussion

Parenteral administration of the parkinsonian neurotoxin rotenone disrupted enteric nervous system function and caused delayed gastric emptying in rats. These findings are provocative because delayed gastric emptying is one of the characteristic GI motility disturbances seen in PD, and both behavioral and electrophysiological abnormalities occurred in the absence of severe motor signs or central dopaminergic toxicity. This suggests that enteric neuron function may be particularly susceptible to mitochondrial toxins.

Abnormal gastric emptying has been described in 43–88% of PD patients and can worsen as PD progresses (Djaldetti, et al., 1996, Goetze, et al., 2006, Goetze, et al., 2005, Hardoff, et al., 2001, Muller, et al., 2006). Gastric motility disturbance contributes to symptoms such as nausea, bloating, early satiety, and weight loss. Interestingly however, objective gastric motility abnormalities are frequently detected even in PD patients without subjective GI complaints (Chen, et al., 2005). In addition to problematic GI discomfort, erratic absorption of medications with resultant motor fluctuations can be a serious complication of abnormal gastric motility for PD patients (Djaldetti, et al., 1996, Kurlan, et al., 1988, Muller, et al., 2006).

Our behavioral data indicate that rotenone delays gastric emptying in rats, as evidenced by elevated residual stomach content after a meal. Closer inspection of the data revealed a bimodal distribution of rotenone-treated animals whereby 45% were severely delayed and others were more similar to vehicle-treated animals. Variability in toxin motor effects and central pathology has been commonly observed during rotenone intoxication of rats (Betarbet, et al., 2000, Fleming, et al., 2004, Sherer, et al., 2003, Zhu, et al., 2004). Similar variability was suggested in a recent article describing slower colon motility in another potential animal model of PD, α -synuclein transgenic mice (Wang, et al., 2008). It is also seen in PD where only a subset of patients exhibit symptoms or signs referable to gastric dysmotility. Possibilities to explain the difference include differential exposure to toxin caused by metabolic differences or differential susceptibility to complex I inhibition. The electrophysiological data suggest that ENS dysfunction is more consistent than the behavioral finding, implying that more sensitive behavioral assays may be more likely to reveal a continuum of gastric responses.

The simplicity of this assay makes it ideal for a preliminary evaluation of stomach function, but it is limited by its one-time nature and single time point (Whited, et al., 2006). Longitudinal assessment of gastric function using radiological or nuclear medicine techniques might provide a more detailed understanding of the behavioral effects of rotenone on the stomach. Likewise, use of the rotenone model as a tool to explore the pathogenesis and treatment of gastric dysmotility in PD will require validation using more sophisticated methods with the potential for repetition on live animals (Cabezos, et al., 2008, Choi, et al., 2007, Matsumoto, et al., 2008, Symonds, et al., 2002, Whited, et al., 2004).

Gastric emptying is a complex process subject to regulation at many levels (Patrick and Epstein, 2008, Quigley, 2006). Coordinated contraction of the stomach body and fundus against a closed pylorus facilitates mixing and mechanical digestion of stomach contents. Upon completion of adequate stomach digestion and mixing, the pylorus relaxes to allow chyme to enter the duodenum. This relaxation is thought to be mediated by enteric inhibitory neurons and vagus nerve projections. Hormones such as cholecystokinin, gastrin, and ghrelin also modulate coordinated emptying of stomach content. Ineffective function at any stage may delay gastric emptying (Quigley, 2006).

Our *ex vivo* physiological data suggest dysfunction in the intrinsic enteric nervous system (ENS) during rotenone treatment, and we hypothesize that delayed gastric emptying may be related to this abnormality. The ENS is a largely autonomous neural network that lines the entire GI tract and regulates GI motility, secretion, and absorption. As shown by its sensitivity to tetrodotoxin, field-induced intestinal muscle relaxation during isometric recording is an active neurally-mediated event. Impaired relaxation in rotenone-treated animals indicates dysfunction of ENS inhibitory neurons (Anderson, et al., 2007, Anitha, et al., 2006). More robust contraction associated with the application of serotonin supports this finding.

Isometric force recording is a reliable way to assess intestinal ENS function, but is not well-suited to evaluation of the stomach or pylorus (Anderson, et al., 2007, Anitha, et al., 2006). The crisscrossed orientation of stomach muscle layers does not allow for examination by unidirectional force recording, and the pylorus is difficult to isolate reliably from surrounding muscular components of the stomach and intestine. Direct assessment of the stomach and pylorus using pressure recording techniques would help confirm impaired relaxation as the substrate causing prolonged gastric emptying during rotenone exposure (Sivarao, et al., 2008).

Despite persistent neural dysfunction in the colonic ENS, there was no lasting abnormality of stool frequency detected in rotenone-treated rats evaluated for three weeks. The transient decrease in stool output associated with rotenone infusion appears to be correlated with body weight. Weight loss associated with rotenone infusion has been well-described, and it is clear that animals do not eat and drink sufficiently during early time points (Betarbet, et al., 2000, Fleming, et al., 2004, Sherer, et al., 2003). This makes it difficult to ascertain whether the early decrease in stool frequency is merely due to decreased food and water intake or that slowed colon motility is a superimposed finding. Perhaps *ex vivo* analysis of colonic propulsion would help delineate this difference.

Weight loss during rotenone infusion has been variously linked to “systemic illness” or poor motor function. In the absence of other evidence, it would seem reasonable to attribute the early decrease in weight and stool frequency observed in this study to decreased food and water intake; however, it is possible that the order of events is reversed, and that abnormal GI motility is a cause, rather than a result, of decreased intake and weight loss. In PD patients, abnormal upper and lower GI motility can affect absorptive effectiveness of the GI tract and result in food aversion due to discomfort or bloating following meals. Both can contribute to weight

loss. If this is the sequence of events during rotenone treatment, as compensatory changes occur in the GI tract, improved motility would drive a recovery of intake and body weight. Results we have previously observed in a separate rodent model of PD, acute MPTP treatment in mice, support this view (Anderson, et al., 2007). In that setting, an abnormality in enteric neuron function outlasted a transient change in stool frequency, suggesting that compensatory mechanisms were engaged to normalize motility in the presence of a continued enteric abnormality. Potential mechanisms of compensation include partial recovery of function in enteric neurons affected by rotenone, reorganizational or plastic changes in the ENS, and modulation of extrinsic sympathetic or parasympathetic input via central and peripheral pathways. Support for ENS plasticity in response to parkinsonian toxins is derived from a recent study that described enteric neurochemical alterations in response to MPTP intoxication in monkeys (Chaumette, et al., 2008). An incomplete or dysfunctional compensatory response to rotenone in the proximal GI tract may contribute to impaired gastric function in the current study, a hypothesis supported by the finding that at earlier time points of rotenone infusion, rats with delayed gastric emptying had a more profound decrease in stool frequency than those with normal gastric emptying. A similar mechanism may be at work in the aforementioned α -synuclein transgenic mice that may display deficits in colon motility (Wang, et al., 2008). Further investigation will be required to delineate the nature and extent of enteric neuroplasticity and compensatory effects in parkinsonism.

Relaxation of intestinal smooth muscle is thought to be primarily mediated by inhibitory enteric neurotransmitters, such as nitric oxide, vasoactive intestinal peptide, and catecholamines (Bornstein, et al., 2004). Our histopathological analysis showed no effect of rotenone on number or morphology of nitric oxide, VIP, or catecholaminergic neurons in the ENS, indicating the enteric neuronal deficit caused by rotenone using this paradigm is more subtle than loss of neurons or processes. It is possible that administration of higher rotenone doses would cause frank damage to enteric neurons. Further analysis of different doses or routes of administration of rotenone will be required to determine if that is the case. We suspect that at this stage and level of rotenone intoxication, the enteric deficit is primarily a functional one. It should be noted that the gastrointestinal deficit in PD is also primarily functional, in that no loss of enteric neurons has been described in gastrointestinal samples from human cases (Singaram, et al., 1995).

Despite no loss of enteric neurons, enteric α -synuclein pathology has been consistently described in PD; however, the relationship between it and GI symptoms is far from clear and requires detailed investigation. We have not observed any abnormal accumulation of α -synuclein in the GI tract of these animals (not shown) despite confirming the prominent basal expression of α -synuclein in the rat ENS that was recently reported (Phillips, et al., 2008). Examination of modified forms of α -synuclein, such as oxidized or phosphorylated forms, may be informative in PD and PD model systems.

While delayed gastric emptying and impaired inhibitory ENS function both occur during rotenone treatment, the finding of one doesn't necessarily indicate a causal relationship to the other. As stated above, regulation of gastrointestinal motility and gastric emptying in particular is a complex process with disruption possible at multiple points. Dysfunction of intrinsic enteric inhibitory neurons appears to play some role in rotenone-induced gastroparesis, but dysfunction of central neurons, particularly in the vagal nuclei, or of muscle and secretory function in the gut may also be important. Pathological examination of the DMV from rotenone-treated rats revealed no obvious cell loss or damage to TH-positive innervation (not shown), but as with the ENS, that does not rule out a functional lesion. We did not evaluate intestinal secretory or absorptive function directly, but the frequency and amplitude of spontaneous contractions in colon longitudinal smooth muscle was not affected by rotenone.

The mechanisms by which enteric neurons are susceptible to rotenone intoxication are not known. ENS neurons are consistently active, a state which results in high metabolic demand (Kann and Kovacs, 2007, Vanden Berghe, 2004, Wong-Riley, 1989). In addition, the prominent enteric transmitter nitric oxide can impose significant additional stress on neurons due to its potential to cause damage via second messenger systems, aberrant nitrosylation, and free radical production (Bolanos, et al., 2008, Moncada and Bolanos, 2006, Tsang and Chung, 2008). The early effect of rotenone on enteric neurons is interesting given that enteric neuron damage has recently been hypothesized to be a sentinel event in PD pathogenesis (Braak, et al., 2006, Braak, et al., 2003).

We did not evaluate these animals for more subtle motor signs, which several studies indicate can develop in these animals (Fleming, et al., 2005, Zhu, et al., 2004). However, those studies have also reported TH abnormalities in surviving animals, which we and others have rarely observed (Betarbet, et al., 2006, Sherer, et al., 2003). The discrepancy is potentially related to many factors and consistent with the reported variability in the model, but the relatively high survival rate in this study suggests a relatively mild rotenone insult. Given that, the high incidence of gastric dysmotility in these animals is particularly interesting. Regardless, while it appears that delayed gastric emptying is an early finding in these animals, caution should be taken not to overinterpret the time course in the absence of data from detailed motor testing.

In summary, we report enteric nervous system dysfunction and delayed gastric emptying in the rotenone-treated rat model of Parkinson's disease. These results contribute to the understanding and manifestation of non-motor symptoms in animal models of Parkinson's disease, an area that has been woefully understudied to this point. They suggest that enteric neurons may be particularly vulnerable to the effects of environmental toxicants associated with PD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Surviving rotenone-treated rats have normal tyrosine hydroxylase content and morphology in the brain

Micrographs depicting tyrosine hydroxylase immunohistochemistry in striatum of vehicle- (A) and rotenone-treated (B) rats. C. Quantification of TH protein content from striatum revealed no difference between vehicle- (N=5) and rotenone-treated (N=5) animals.

Figure 2. Rotenone infusion caused delayed gastric emptying in rats

A. Content remaining in the stomach after a meal was significantly higher in rotenone-treated animals (N=11 per group). * $p < 0.05$, unpaired, two-tailed t-test. B. Delayed gastric emptying in rotenone-treated rats did not correlate with acute rotenone toxicity as assessed by weight loss after 1 week of infusion.

Figure 3. Stool frequency was transiently decreased in rotenone-treated rats

A. Stools per one hour were diminished for the first 10 days of rotenone exposure and then recovered to control values. * $p < 0.05$, 2×5 mixed factor ANOVA with Bonferroni posttest. B. Rats lost weight for the first 10 days of rotenone exposure and then recovered a normal rate of gain. * $p < 0.05$, 2×5 mixed factor ANOVA with Bonferroni posttest. C. Rotenone-treated rats with delayed gastric emptying trended toward lower stool frequency than those with normal gastric emptying. 2×5 mixed factor ANOVA $p < 0.05$ for main effect of gastric emptying.

Figure 4. Rotenone-treated rats have impaired enteric neural inhibition in proximal colon

A. Example force recording of longitudinal colon muscle contraction from a vehicle-treated rat. Note the contractile response to 5-HT and the relaxation induced by ENS stimulation. B. Example force recording from a rotenone-treated rat. Note the augmentation of 5-HT contraction and the lesser relaxation response. C. The 5-HT contraction response was greater in rotenone-treated rats. D. Field stimulation caused a lesser relaxation in rotenone-treated rats. N=6–7 per group. * $p < 0.05$, unpaired, two-tailed t-test.

Figure 5. No change in enteric neuron number or innervation quality in rotenone-treated rats

A. No difference in neuron numbers for NADPH-diaphorase (nNOS) neurons (N=12 vehicle and 18 rotenone), vasoactive intestinal peptide (VIP) neurons (N=6 per group), or choline acetyltransferase (ChAT) neurons (N=6 vehicle and 11 rotenone) in the enteric nervous system from rotenone-treated rats. B. Quality of enteric innervation for NADPH-diaphorase (blue) and TH (brown) was not different between groups. C. Quality of VIP innervation (green) was not different between groups. Red fluorescence represents HuD staining of all enteric neurons. D. Quality of cholinergic innervation as assessed by ChAT immunostaining (brown) was not different between groups.

Table 1
No change in proportions of enteric inhibitory neurons in rotenone-treated rats

	Pyloric region		Ileum		Proximal Colon	
	VEH	ROT	VEH	ROT	VEH	ROT
NOS	29 ± 4 (7)	34 ± 3 (4)	31 ± 3 (6)	26 ± 4 (6)	20 ± 2 (4)	20 ± 2 (7)
VIP	36 ± 1 (7)	39 ± 6 (4)	10 ± 3 (6)	9 ± 1 (6)	0.5 ± 0.1 (6)	0.6 ± 0.1 (7)
TH	16 ± 2 (5)	17 ± 3(4)	rare	rare	1.3 ± 0.2 (6)	1.1 ± 0.2 (8)

Mean ± SEM (N) relative percentage of enteric inhibitory neurons compared to total enteric neurons (HuD-positive) in different segments of the GI tract.