MPTP-induced parkinsonism extends to a subclass of TH-positive neurons in the gut

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ABSTRACT

Gastrointestinal (GI) dysfunction occurs frequently in early Parkinson’s disease (PD) and it is supposed to anticipate motor symptoms. About 80% of PD patients suffer from constipation before the onset of movement disorders. Despite such a high prevalence of gut impairment in PD, the molecular mechanisms remain poorly investigated. This is also due to the scarcity of experimental studies. In the present work, we tried to reproduce digestive abnormalities observed in PD patients by administering the parkinsonism-inducing neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) to C57BL mice. We show that in these mice, MPTP (20 mg/kg×3) while producing the classic striatal dopamine (DA) denervation, persistently delays colonic motility, produces constipation, and reduces the number of enteric TH-positive neurons. The loss of TH-positive cells in the gut is selectively due to the disappearance of DA neurons within both myenteric and mostly submucosal plexus in the intestine, while no change is detected in the esophagus and stomach. In contrast, norepinephrine (NE) neurons are not affected. These data were confirmed by immunohistochemistry and by HPLC showing the significant loss of DA levels while NE and 5-HT content was not affected. Dopamine cell loss was associated with increased α-synuclein levels. These functional, biochemical, and morphological findings extend the PD-mimicking effects of MPTP to GI dysfunctions and provide a useful experimental model to understand gut dysfunction in PD and to find effective treatments for digestive symptoms.

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1. Introduction

Parkinson’s disease (PD) is a chronic, progressive neurodegenerative disease characterized by nigrostriatal dopamine (DA) and locus coeruleus (LC) norepinephrine (NE) neuronal degeneration which is considered to underlie the most overt motor symptoms such as tremor, bradykinesia, stiffness, and postural instability (Hornykiewicz, 1975; Zarow et al., 2003;
Besides the classic motor impairment, the onset and progression of the disease is also associated with nonmotor features, including altered behavior, abnormal sleep-waking cycle, and pain, as well as autonomic dysfunctions (Chaudhuri et al., 2005). While experimental modeling of PD pathology in the central nervous system (CNS) is extensively carried out, peripheral autonomic dysfunction has been overlooked. Only recent studies focused on setting up experimental models which address peripheral alterations such as cardiac denervation consisting in the loss of NE axons directed to the heart (Fukumitsu et al., 2006; which led to the definition of “Parkinsonian heart,” see Fornai et al., 2007 for a review) or reduced testosterone levels (Ruffoli et al., 2008). The gastrointestinal (GI) dysfunction represents a common syndrome, which often precedes the onset of motor disabilities. This already appeared in the former description by James Parkinson (Parkinson, 1817), and now, it is considered an early and critical feature of PD. In fact, a recently developed questionnaire (SCOPA-AUT) focusing on the evaluation of autonomic dysfunction in PD revealed a high frequency of GI disturbances (Verbaan et al., 2007). In contrast, the pathophysiology of GI dysfunction still remains uncertain and experimental models are still in progress while an effective cure is lacking (see Natale et al., 2008 for a review).

The entire GI tract may be affected in PD, with hypersalivation, oropharyngeal and esophageal dysphagia, gastroparesis, small intestine and colonic dysmotility, and anorectal dysfunction. However, the most common symptom consists of constipation (Edwards et al., 1991; Jost, 1997; Pfeiffer, 2003, Natale et al., 2008).

GI dysfunction was hypothesized to depend on a degenerative process involving the enteric nervous system (ENS), similarly to what occurs in the CNS (Singaram et al., 1995; Wakabayashi et al., 1988). The heterogeneous pathological findings leading to a variety of GI alterations, as well as the difficulty in reconciling the bowel physiology with what occurs in PD, call for an appropriate animal model to investigate in depth GI impairment in PD. This is also justified by the lack of an in depth knowledge on the physiological role of NE/DA in the gut. At present, despite the plethora of research efforts aimed at elucidating the etiology of central nigrostriatal damage, only a few studies approached the GI dysfunctions in PD. This paucity of data is quite surprising considering how critical GI dysfunction is to the patient’s quality of life and management of the disease.

A few papers aimed at studying the effects of PD-inducing neurotoxins in the gut were produced in the last two decades. Recently, the effects of MPTP in the mouse colon have been evaluated in vivo by Anderson et al. (2007). These authors found that 10 days after MPTP administration, there is a 40% reduction of TH-positive neurons. Furthermore, in an elegant study, the loss of myenteric neurons was replicated in the rat following rotenone exposure (Drolet et al., 2009). It remains unclear whether damaged TH-positive neurons correspond to DA, NE,
or, although unlikely, adrenaline-containing neurons. In keeping with the mouse MPTP model, important issues need to be addressed. (i) Which is the specific site in the gut wall where neurons undergo MPTP-induced degeneration? (ii) Are these cells submucosal or localized in the myenteric plexus (MP), or are they scattered along the whole enteric wall? (iii) Is there still evidence for a morphological damage under a plain haematoxylin and eosin (H&E) staining? The latter point is critical to conclude whether a real cell loss occurs, since a loss of the TH protein could occur specifically, in the absence of any real cell loss. We believe that a concomitant staining for TH and the DA transporter (DAT) or NE transporter (NET), joined with haematoxylin counterstaining, would provide important information for the specificity of a potential catecholamine cell loss. Moreover, it is also critical to perform a quantitative measurement of neurotransmitter levels DA, NE, adrenaline, and even 5-HT in the gut homogenate to understand which cell phenotype is affected by MPTP treatment. Finally, both during parkinsonism in monkeys (Purissai et al., 2005; McCormack et al., 2008) and following MPTP administration in mice (Kühn et al., 2003), an increased expression of alpha-synuclein (α-syn) in the nigrostriatal pathway is demonstrated. This was recently replicated in the gut of rotenone-administered rats (Drolet et al., 2009).

In the present study, we investigated all these open issues in the gut of MPTP-treated mice.

2. Results

2.1. Behavioural and functional tests

Colonic transit assay showed a 33.60% delay in MPTP-treated mice compared with controls (4.56 ± 0.713 sec vs 6.87 ± 0.677 sec, respectively; Fig. 1a).

MPTP-treated mice exhibited a 30.97% reduction of daily stool weight (p < 0.0001; Fig. 1b) in comparison with their control littermates, whereas food and water intake did not change significantly between the two groups (p = 0.16) (Figs. 1c and d). Thus, MPTP administration produced a delayed transit and constipation, which was lasting at least 1 week following the exposure to the neurotoxin.
2.2. Immunohistochemistry

Immunohistochemical analysis carried out in striatum and substantia nigra from MPTP-treated mice confirmed the classic reduction of TH immunopositivity in both brain areas examined compared to saline-treated animals (data not shown).

In small intestine and colon from control mice, TH immunopositivity was well evident in both myenteric and submucosal plexuses, appearing as continuous markedly stained rings (Figs. 2a and c). From the submucosal plexus, nervous fibers and neurons extended to the mucosa up to the axes of the villi. In MPTP-treated mice, TH-immunopositive neurons were severely lost in both plexuses and the continuous ring-like staining pattern was broken, especially in small intestine (Figs. 2b and d). The quantitative analysis revealed that such a reduction was significant in the small intestine but not in the colon (Fig. 3).

In contrast, TH immunohistochemistry in mouse esophagus (Figs. 4a and b) and stomach (Figs. 4c and d) showed no significant difference compared with controls.

Sections of small intestine from control animals (Figs. 5a and c) exposed to DAT antibodies developed a dark brown immune reaction in both myenteric and submucosal plexuses. In MPTP-treated mice, DAT immunoreactivity was noticeable in the myenteric plexus, whereas it appeared markedly reduced in the submucosal plexus (Figs. 5b and d).

NET immunohistochemistry in duodenum from salinetreated (Figs. 6a and c) and MPTP-treated mice (Figs. 5b and d) did not show any evident difference either in myenteric or submucosal plexus.

The counting of DAT- and NET-immunopositive cells (Figs. 7a and b) at the level of both myenteric and submucosal plexuses indicates that the decrease in TH-immunopositive cells in the small intestine (Fig. 2b) is due to a specific depletion of DA (Fig. 7a) rather than NE neurons (Fig. 7b).

In the duodenum, but not in the colon, MPTP produced a marked increase in the immunofluorescence for α-syn (Fig. 8).

2.3. Western blotting

This was confirmed in homogenates from the duodenum, where the band of the α-syn protein was significantly increased following MPTP, while no difference was observed between saline or MPTP at the level of the colon (see Fig. 8 for optical density and representative gels).

2.4. HPLC analysis of dopamine, norepinephrine, and serotonin content

To document quantitatively the selective loss of DA neurons, we measured DA, NE, and 5-HT levels from intestinal tissue of saline- and MPTP-treated mice. MPTP treatment induced a significant (p=0.03) decrease in DA (from 0.67±0.06 ng/mg of protein to 0.50±0.03 ng/mg of protein; Fig. 9a), but did not induce a significant change in the tissue levels either of NE (2.94±0.30 ng/mg of protein in comparison with controls: 2.77±0.31 ng/mg of protein; Fig. 9b) or 5-HT (11.88±2.23 ng/mg of protein in comparison with controls: 12.47±2.68 ng/mg of protein; Fig. 9c).

3. Discussion

The present data indicate that, in the mouse MPTP administration produces an impairment in GI function which is reminiscent of what described in PD patients. In fact, these mice develop a persistent constipation analogous to what described in humans (for a review, see Chaudhuri et al., 2005; Natale et al., 2008; Lebouvier et al., 2009). Such an effect is accompanied at morphological level by a massive depletion of TH immunostaining, which appears to be due to the selective loss of enteric DAergic neurons, as confirmed by the selective damage to DAT-containing cells and the specific loss of DA in the homogenates from the gut. Conversely, loss of TH was not related to alterations of NE-containing neurons. In fact, we could detected neither a reduction of NET-containing neurons nor a loss of enteric NE content. These findings further extend the validity of the MPTP model to replicate the GI disorder that occurs in PD patients. In fact, postmortem and biopitic studies in humans (parkinsonian patients compared with controls, Edwards et al., 1992; Singaram et al., 1995; Lebouvier et al., 2008) suggest that the loss of TH immunostaining observed in the gut PD patients is due to a selective DA cell death. These results prove the reliability of the mouse MPTP model as an
experimental setting to investigate GI alterations occurring in PD. Remarkably, the degeneration of enteric DA neurons was observed for the very same doses of MPTP, which produce damage to the nigrostriatal pathway.

Despite the high prevalence of GI dysfunction in PD, the lack of an experimental model, which is able to mimic such a dysfunction, greatly limited the investigations on PD in the gut, leaving open the question whether a real cell loss occurs and whether this happens specifically for a subclass of autonomic neurons. Interestingly, a very recent paper in the rat by Drolet et al. (2009) found the occurrence of GI dysfunction following systemic administration of rotenone, which analogous to MPTP, is a parkinsonism-inducing neurotoxin which inhibit mitochondrial complex I.

Only a few experimental studies using animal models addressed GI dysfunctions in PD, thus limiting the understanding of the pathophysiology and treatment of these symptoms. Since PD does not naturally occur in animal species, experimental models need to be artificially developed. It is now widely established that among rodents only the mouse is valuable to reproduce parkinsonism leading to avoid rat studies. This restricts the number of work on MPTP to very few evidence.

In this respect, an acute inhibition of GI motility, measured by the transit of charcoal, was reported in mice by Haskel and Hanani (1994) who attributed this effect to both epinephrine and DA changes. Analogously, in an in vitro study, Hanani (1990) observed a muscle relaxation in the mouse colon. However, despite their significant contribution, all these effects were measured immediately after MPTP exposure, when the cell degeneration is not yet produced. Therefore, the effects very likely depend on acute NE release and depletion from peripheral nerve endings. In fact, the neurotoxin produces acutely a nonspecific peripheral and central (Pileblad and Carlsson, 1988) catecholamine release, which is followed by stable damage only after a few days. The MPTP-induced neurotransmitter release, which occurs peripherally immediately after injection is nonrelated to the subsequent damage (Giovanni et al., 1994a,b; Fornai et al., 1997). This point is crucial since as recently shown by Blandini et al. (2009), it is likely that even the mere damage to the nigrostriatal pathway, in the absence of peripheral toxicity may contribute to produce parkinsonian constipation. In a very interesting study, Anderson et al. (2007) found alterations in enteric motility at ten days after the administration of a total dose of 60 mg/kg of MPTP in the mouse.
The main criticism posed to the MPTP model is based on the fact that MPTP is unable to replicate the time course and the anatomical topography of PD whose clinical manifestations are also due to non-DAergic lesions with associated non motor symptoms (Braak et al., 2003). The present data, joined with previous findings obtained in other peripheral organs extend the significance of the MPTP model to reproduce reliably peripheral alterations occurring in PD. Our findings are in line with a recent commentary by Goetze and Woitalla (2008) recommending the use of MPTP in experimental studies aimed at analyzing GI activity in PD. These data joined with the recent report of Drolet et al. (2009) indicate that mitochondrial complex 1 inhibitors are suitable neurotoxins to study the GI dysfunction in rats (rotenone) and mice (MPTP).

In fact, in this study, MPTP reproduced most alterations occurring in the digestive system of PD patients ranging from the functional impairment to the morphological and biochemical correlates. In detail, the morphology shows that MPTP administration causes neurodegeneration in the ENS, in both myenteric and submucosal plexus, which was evidenced by haematoxylin histochemistry of enteric neurons joined with TH immunostaining. This occurs more severely in the small intestine. At this level, the reduced DAT, but not NET, immunoreactivity from MPTP-treated animals suggests a specific involvement of DAergic neurons, which is confirmed by the assay of DA and NE levels. Again, we found that MPTP increased α-syn both at immunoblotting and at immunofluorescence in the small intestine. These latter findings confirm what it was found by Drolet et al. (2009) who explored mainly the myenteric plexus. In the present report, the DA lesion was reported both in the myenteric and submucosal plexus.

As a side observation, it is noticeable that these DA neurons are abundant in control mice suggesting that DA plays a role in modulating GI function. Previous studies described DAergic neurons in the ENS, mostly in the myenteric plexus forming an intrinsic nervous network (Anlauf et al., 2003; Cersosimo and Benarroch, 2008). Similar to mice, in humans ENS, some neurons are DAergic, and they have been evidenced in human biopsies (Lebouvier et al., 2008).

However, the role of DA in modulating bowel motility remains controversial. In fact, mice lacking DAT (high extracellular DA levels stimulating DA receptors) possess a reduced bowel motility (Walker et al., 2000), which is in line with an increased propulsive motility observed in mice knocked out for D2 receptors. In keeping with this, the use of peripheral D2 antagonists (in various species including humans) promotes enteric motility. These data suggest that, in the presence of an intact enteric DA innervation, the
activation of DA receptors in the gut inhibits motility, probably via presynaptic blockade of cholinergic myenteric nerve terminals (Li et al., 2006). This apparently contrasts with the occurrence of constipation following enteric DA denervation. In fact, here we show a reduced intestinal transit and a moderate slowing of gastrointestinal motility following DA denervation, which is in line with what was recently reported using rotenone in the rat (Drolet et al., 2009). It is likely that the constipation we observed in these mice depends on several morphofunctional alterations. In detail, the reduced amount of stool associated with constipation is likely to depend on reduced DA activity in the long small intestine. The concomitant reduction in the expulsion test is likely to rely on terminal alterations in the anorectal motility, which adds on constipation, and it is likely to depend on central motor mechanisms, which, as shown by Blandini et al. (2009), might interfere with the expulsion phase.

The possibility that PD might rearrange the neuronal pattern in ENS has been proposed by Chaumette et al. (2009): in the bowel of MPTP-treated monkeys, the neurochemical plasticity leads to an increase in nitric oxide synthase immunoreactivity is induced by the loss of TH-positive neurons.

As recently suggested, it is likely that, similar to the postsynaptic changes described in the CNS, the physiology of DA receptor stimulation in the gut is completely altered following the loss of enteric DA neurons (Gerfen, 2003; Natale et al., 2008). Despite the scarce knowledge on the role of D₂ and D₁ receptors, it is also unclear which receptor activity prevails in the presence of different DA concentrations in the gut (which varies from the healthy state to the disease) and it is uncertain what happens to the pattern of expression of DA receptor subtype following DA denervation. In particular, are both D₁-like and D₂-like DA receptors equally upregulated? Or is there a selective prevalence of one subtype? Is the ability of NE on the gut to bind to D₁ and D₂ receptors comparable? In the absence of DA, is it possible that NE becomes the main endogenous agonist for DA receptors? Since the DA replacement therapy in PD is largely based on the use of D₂-like preferring agonists, what is the impact of the ongoing DA substitution therapy on the prevalence of constipation?

Further studies using reliable models of enteric DA denervation are critical to address these issues, which might solve the enteric distress of PD patients.

Although these points need to be fully addressed by a variety of investigations, it is worth mentioning that in a

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**Fig. 6** – NET immunohistochemistry in the duodenum from controls (a, c) and mice treated with MPTP (20 mg/kg × 3, 2 h apart) (b, d). There is no difference in the immunohistochemical staining, either in myenteric or in submucosal plexus. Scale bars: 70 μm (a, b), and 35 μm (c, d).
recent study, colonic biopsies in the submucosal plexus in PD patients complaining of functional constipation showed higher α-syn-immunopositivity (Lebouvier et al., 2008). In line with these findings, in the present study, we observed pronounced α-syn immunostaining in the duodenum of MPTP-treated mice. Interestingly, reduced enteric propulsive motor activity was observed in mice overexpressing α-syn (Wang et al., 2008). In line with this, mice expressing mutant α-syn develop abnormalities in ENS function, recapitulating the early gastrointestinal deficits observed in human PD (Kuo et al., 2010).

4. Experimental procedures

4.1. Animals

Male 9-week-old C57 black mice (Charles River, Lecco, Italy) were used throughout the study. They were fed standard laboratory chow and tap water ad libitum and were not used for at least 1 week after their delivery to the laboratory. The animals were housed, six in a cage, in temperature-controlled rooms on a 12-hour light cycle at 22–24 °C and 50–60% humidity. The experiments were approved by the local ethical committee at the University of Pisa and were conducted in accordance with the Guidelines of the National Institutes of Health for the Conduct of experiments in rodents. After MPTP treatment, the animals were housed individually in single cages to be able to measure for each mouse the amount of food consumption and stool weight daily.

4.2. Experimental protocol

Animals were randomly assigned to the following experimental groups:

Control group (n=20): mice were injected intraperitoneally (i.p.) with saline;
MPTP group (n=20): mice were injected i.p. with MPTP (20 mg/kg×3, 2 h apart).

During the first week after treatment, food intake, daily stool weight, and colonic motility were monitored to document an impairment in the gut motility. One week after the treatment, all animals were sacrificed by decapitation. The brain and the samples of the digestive tract (esophagus, stomach, small intestine, and colon) were rapidly removed and immersed in fixative solution (buffered paraformaldehyde 4%) for 24 h at room temperature to be then processed following standard procedures for morphology (H&E and immunohistochemistry for TH, DAT, NET, and α-syn). Furthermore, samples of the gut were frozen for the following measurement of monoamines (DA, NE and 5-HT) by high-performance liquid chromatography with electrochemical detection (HPLC-ED).

4.3. Behavioral and functional tests

4.3.1. Food and water consumption

Food and water consumption was studied over the course of a 144-h observation period. Food and water consumption was calculated from the difference in weights of the food or in milliliters of water, respectively, at the beginning and the end of the observation period.

4.3.2. Twenty-four hours of stool collection

The fecal production from each mouse was individually collected and weighed daily over the first week after treatment. Each measurement was repeated daily for each mouse starting at 48 h after MPTP administration. In the same way, we measured the food intake following MPTP administration.

4.3.3. Colonic transit assay

Colonic propulsion was measured according to the method described by Raffa et al. (1987): distal colonic transit was determined by measuring the time required for the expulsion of the glass 3-mm bead inserted 2 cm into the distal colon of each mouse. Bead insertion was accomplished with a glass rod, one end of which was fire-polished so as to be rendered nontraumatic. After bead insertion, mice were placed in individual plastic cages lined with white paper to aid visualization of bead expulsion. Each measurement was repeated daily for each mouse starting at 48 h after MPTP administration.
4.4. Immunohistochemistry

After dissection, all samples were dehydrated, embedded in paraffin, and cut at microtome. Deparaffinized 5-μm-thick transverse sections were dehydrated and incubated with 3% methanolic H₂O₂ for 10 min to inhibit the intrinsic peroxidase. After treatment with blocking serum for 2 h, the tissue sections were incubated with primary antibodies overnight at 4 °C with following primary antibodies: anti-TH (dilution 1:200; Sigma, St. Louis, MO, USA), rat anti-DAT (dilution 1:350; Chemicon, Temecula, CA, USA), rabbit anti-NET (dilution 1:500; Chemicon, Temecula, CA, USA), and mouse anti-α-syn (dilution 1:50; BD Transduction Laboratories, San Jose, CA, USA). After primary antibody incubation, sections were rinsed in phosphate-buffered saline (PBS, 1×, pH 7.4) and incubated for 1 h at room temperature in the following secondary antibodies: for TH, DAT and NET, biotinylated goat anti-mouse, anti-rat, or anti-rabbit IgG, respectively (dilution 1:200; Vector Laboratories, Burlingame, CA, USA); for α-syn, fluorescein-conjugated goat anti-mouse IgM (dilution 1:100; Vector Laboratories, Burlingame, CA, USA). The avidin–biotin complex (ABC Elite Kit; Vector Laboratories, Burlingame, CA, USA) and 3,3-diaminobenzidine tetrachloride (DAB; Sigma) were used to visualize bound biotinylated antibody. Some sections were lightly counterstained with haematoxylin.

With respect to brain samples, eight 10-μm-thick serial slices from striatum and substantia nigra were alternatively stained with H&E and immunocytochemistry for TH (dilution of 1:1000), according to the previously described procedure. The sections were observed using a Nikon Eclipse 80i light microscope equipped with software for image analysis.

To perform a quantitative analysis, the number of TH-, DAT-, and NET-immunopositive cells in both the submucosal and myenteric plexuses was counted in small intestine and colon where the qualitative difference with respect to control was more evident. Ten slices were examined for each mouse (N=20) for a total of 200 slides per group. The neuronal loss in the gut wall (proximal duodenum and distal colon) was estimated by counting the number of neurons at 20× magnification in 7-μm-thick slices. This procedure provided a useful tool to virtually rebuild the neuronal three-dimensional size, avoiding to count the same neuron twice (sections spaced 40 μm). The counting was made in blind conditions by two independent observers (G.N. and O.K.).
4.5. Western blotting

Samples of the whole colonic or duodenal wall from mice treated with saline ($N=20$) or MPTP ($N=20$) to be immunoblotted were homogenized in a buffer containing 50 mM Tris, 2 mM EDTA, 150 mM NaCl, 1% NP-40 at pH 7.6, containing 0.1% of phenylmethylsulfonyl fluoride (PMSF), and 10 $\mu$g/mL of protease inhibitors and centrifuged at 10,000 $\times g$ for 20 min at 4 °C. An aliquot of supernatant from each sample was used to determine the protein concentration by a protein assay kit (Sigma). Sample containing 40 $\mu$g of total protein were separated by SDS–PAGE (15% acrylamide). Following electrophoresis, the proteins were transferred to PVDF membrane (Millipore, Bedford, MA, USA). The membrane was immersed in blocking solution (5% nonfat dried milk in 20 mM Tris, 137 mM NaCl at pH 7.6 containing 0.05% Tween-20) for 3 h at 4 °C. Subsequently, the membrane was probed using a commercial anti- $\alpha$-syn (1:400; BD Biosciences, Pharmingen, San Diego, CA, USA) antibody. The immunoreactive bands were visualized by enhanced chemoluminescence using horseradish peroxidase-linked secondary antibodies (Amersham, Pharmacia Biotech, Buckinghamshire, UK). Films were scanned, and the optical density (OD) of the bands was measured by using NIH IMAGE 1.61. The relative values for each band were calculated by subtracting the background of the OD value from the OD measured for the bands. Groups used for statistical analyses were always determined within the same Western blot.

4.6. HPLC measurement of dopamine, norepinephrine, and serotonin content in the gut

The contents of NE, DA, 5-HT, and metabolites were measured using HPLC and electrochemical detection. Briefly, after sacrifice, tissue samples were taken and rapidly frozen in 600 $\mu$L of cold perchloric acid (0.1 mol/L) containing 10 ng/mL 3,4-dihydroxybenzylamine (Sigma Chemicals Co., St. Louis, MO, USA) as the internal standard, and stored immediately at 80 °C until use. When ready for the assay, the samples were sonicated, and an aliquot of homogenate (50 $\mu$L) was extracted for protein measurement (Lowry et al., 1951). The homogenate was centrifuged for 10 min at 10,000 $\times g$ at 0 °C. Following centrifugation, 400 $\mu$L of supernatant was recovered. Twenty microliters was immediately injected into the HPLC system, while a 380-$\mu$L volume was stored at 80 °C for duplicates and triplicates.

Quantitative monoamine concentrations were obtained by regression analysis of curves obtained specifically for a range of standard concentrations of each monoamine; the content of DA, NE, and 5-HT was expressed as nanograms per milligram of protein.

4.7. Drugs

The following drug was used: MPTP hydrochloride (Sigma Chemicals Co., St. Louis, MO, USA). Other reagents were of analytical grade.

4.8. Statistics

Data concerning colonic motility, stool weight and, food consumption were collected from each mouse daily. The mean±SEM of each daily measurement starting at 24 h following MPTP was reported and compared with saline-injected mice, and data were evaluated with $t$ test (when two groups were compared: Western blotting) or analysis of variance (ANOVA) and Scheffè’s post hoc test followed by Fisher post hoc test, when appropriate. The statistical tests were performed by the software package Statview (Abacus Concepts Inc., Berkeley, CA, USA). $p<0.05$ was chosen to reject the $H_0$ hypothesis. Results were expressed as the means ± SD.

Cell count data were analyzed by expressing the mean±SEM and comparing the groups using Student’s $t$ test for unpaired data.

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