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Ghrelin prevents levodopa-induced inhibition of gastric emptying and increases circulating levodopa in fasted rats

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Abstract

Background—Levodopa (L-dopa) is the most commonly used treatment for alleviating symptoms of Parkinson's disease. However, L-dopa delays gastric emptying, which dampens its absorption. We investigated whether ghrelin prevents L-dopa action on gastric emptying and enhances circulating L-dopa in rats.

Methods—Gastric emptying of non-nutrient methylcellulose/phenol red viscous solution was determined in fasted rats treated with orogastric or intraperitoneal (ip) L-dopa, or intravenous (iv) ghrelin 10 min before orogastric L-dopa. Plasma L-dopa and dopamine levels were determined by high pressure liquid chromatography. Plasma acyl ghrelin levels were assessed by radioimmunoassay. Fos expression in the brain was immunostained after iv ghrelin (30 $\mu\text{g kg}^{-1}$) 10 min before ip L-dopa.

Key Results—L-dopa (5 and 15 mg kg^{-1}) decreased significantly gastric emptying by 32% and 62% respectively when administered orally, and by 91% and 83% when injected ip. Ghrelin (30 or 100 $\mu\text{g kg}^{-1}$, iv) completely prevented L-dopa's (15 mg kg^{-1} , orogastrically) inhibitory action on gastric emptying and enhanced plasma L-dopa and dopamine levels compared with vehicle 15 min after orogastric L-dopa. L-dopa (5 mg kg^{-1}) did not modify plasma acyl ghrelin levels at 30 min, 1 and 2 h after iv injection. L-dopa (15 mg kg^{-1} , ip) induced Fos in brain autonomic centers, which was not modified by iv ghrelin.

Conclusions & Inferences—Ghrelin counteracts L-dopa-induced delayed gastric emptying but not Fos induction in the brain and enhances circulating L-dopa levels. Potential therapeutic benefits of ghrelin agonists in Parkinson's disease patients treated with L-dopa remain to be investigated.

Keywords

dopamine; gastric emptying; ghrelin; levodopa; rats

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Contributions of authors:

LW designed and performed the research, analyzed data and wrote the manuscript; NPM performed research and analyzed data; AS and MG-S performed research; DSP contributed to synthesis of ghrelin; NTM contributed HPLC expertise and resources; YT designed the research and wrote the manuscript.

Introduction

The current therapy for Parkinson's disease (PD) is largely based on a dopamine replacement strategy, primarily using oral administration of the dopamine precursor, L-3,4-dihydroxyphenylalanine (L-dopa, levodopa) (1). This provides the largest improvement in motor functions and reduction of overall mortality in PD compared to other medical or surgical strategies currently available (1). However, long-term use of L-dopa is associated with motor fluctuations and dyskinesia (2). Moreover, oral administration of L-dopa *per se* inhibits gastric emptying in healthy human volunteers (3, 4). Likewise, in one preclinical study, L-dopa infused intravenously (iv) inhibited postprandial gastric motility in dogs measured by telemetry recording from the fundus, antrum and pylorus (5). There is also evidence in PD patients who have already developed delayed gastric emptying that L-dopa treatment worsened the gastroparesis compared to those without L-dopa (6). Therefore, the stomach is one of the hurdles on the route of oral L-dopa to the brain (1) as L-dopa is absorbed in the small intestine; thus irregular gastric emptying leads to variable absorption of the drug (1, 7, 8), resulting in response fluctuations (7-9) and greatly reducing therapeutic effectiveness.

Only a few prokinetic agents have been examined for their ability to curtail L-dopa-induced delayed GE, namely the serotonin receptor 4 (5-HT₄) agonist cisapride, tested in PD patients (10) and KDR-5169, a partial agonist at 5-HT₄ receptor and an antagonist at the dopamine receptor 2 (D₂) tested in healthy dogs (5). However, side effects of cisapride are associated with cardiac arrhythmias leading to its withdrawal from the market and limited clinical use (11).

Ghrelin (acylated, "active" form) is a gut hormone originally identified as the endogenous growth hormone (GH) secretagogue (GHS) that interacts with the GHS receptor 1a (ghrelin receptor) to stimulate GH release and food intake (12, 13). Additionally, ghrelin exerts potent prokinetic effects on gastric motility in rodents, canines and healthy human subjects (14-19). Ghrelin also improves gastroparesis in various diseases such as diabetes (20), or experimental post-operative ileus (21-23), burn injuries (24) and inflammation associated with colitis acute injection of lipopolysaccharide (25, 26) while not influencing the inhibition of gastric motor function induced by peripheral injection of urocortin 2 (27). Whether ghrelin interacts with L-dopa has not been investigated.

Here, first we compared the inhibitory effects of orogastric vs intraperitoneal (ip) L-dopa administration on gastric emptying. Then, we investigated whether iv ghrelin injection exerts a prokinetic action on the delayed gastric emptying induced by orogastric L-dopa in naïve rats. To assess whether the normalization of gastric emptying by ghrelin has pharmacokinetic consequences, we monitored the time-course of changes in plasma L-dopa and dopamine concentrations after iv ghrelin plus orogastric administration of L-dopa. We also examine whether the elevation of circulating L-dopa and dopamine is associated with altered circulating levels of endogenous ghrelin as we previously described under other conditions of inhibited gastric emptying in rats (28-30). Last, to get insight to potential brain mechanisms of interactions, we investigated whether iv ghrelin treatment modifies Fos expression in brain autonomic centers induced by ip L-dopa in rats. Peripheral administration of L-dopa is known to activate neuronal circuits in the brain assessed by Fos expression including the striatal system (31) as well as in autonomic centers, such as the paraventricular nucleus (PVN) and supraoptic nucleus (SON) of the hypothalamus, and nucleus of solitary tract (NTS) in PD animal models (32).

Materials and Methods

Animals

Adult male Sprague-Dawley rats (Harlan, San Diego, CA, USA, body weight: 280–320 g) were housed 4 animals/cage under controlled illumination (12:12 h light/dark cycle, lights on/off: 6.00 h/18.00 h) and temperature (22 ± 2 °C). Animals were fed standard rodent diet (Prolab RMH 2500; LabDiet, PMI Nutrition, Brentwood, MO, USA) and tap water *ad libitum*. Animal care and experimental procedures followed institutional ethic guidelines and conformed to the requirements of the federal authority for animal research conduct. All procedures were approved by the Animal Research Committee at Veterans Affairs Greater Los Angeles Healthcare System (animal protocol #06015-08). Experiments started between 9.00 and 10.00 h during the light phase in overnight fasted rats unless otherwise stated.

Procedures

Intravenous catheter implantation—Rats fasted overnight were anesthetized with ketamine (75 mg kg⁻¹, ip; Fort Dodge Laboratories, Fort Dodge, IA) and xylazine (5 mg kg⁻¹, ip; Mobay Corporation, Shawnee, KS). A polyethylene-50 tubing (BD Intramedic™ 23-gauge, Fisher Scientific, Pittsburgh, PA, USA) was inserted into the right jugular vein. The catheter was closed and exteriorized to the back of the neck *via* subcutaneous tunneling and secured to the skin as in our previous studies (26, 28). Rats were single housed after surgery and had three days recovery period during which time they were habituated to handling and the blood withdrawal procedure. Body weight was monitored to assess recovery and assure an anabolic state at the start of experiments.

Blood sampling and processing—Blood (0.5 ml) was withdrawn *via* the catheter while the rat was lightly hand restrained. In all experiments, blood sampling started between 9.00–9.30 h. For L-dopa measurement, blood was collected in chilled Eppendorf tubes containing ethylenediaminetetraacetic acid (EDTA 7.5%, 10 µl/0.5 ml blood; Sigma-Aldrich Corp, St. Louis, MO, USA). Plasma was separated from blood cells by centrifugation at 3,000 rpm for 15 min, aliquoted and kept at –80°C until high performance liquid chromatography (HPLC) analysis. For ghrelin (acylated form) measurement, blood was processed immediately with the RAPID method, which results in 80% greater recovery of acyl ghrelin compared to standard processing, as detailed previously (28). Briefly, blood was diluted 1:10 in ice-cold buffer (pH 3.6) containing 0.1 M ammonium acetate, 0.5 M NaCl, and enzyme inhibitors (diprotin A, E-64-d, antipain, leupeptin, chymostatin, 1 µg ml⁻¹; Peptides International, Louisville, KY, USA), and centrifuged at 3,000 rpm for 10 min at 4 °C. The supernatant was subjected to Sep-Pak chromatography (C18 cartridges, 360 mg, 55–105 µm, #WAT051910, Waters Corporation, Milford, MA, USA) and the eluted samples were dried by vacuum centrifugation and stored at –80°C until radioimmunoassay.

Reagents

Levodopa (L-dopa: 3,4-dihydroxy-L-phenylalanine) was dissolved in vehicle (pH 6.0) composed of 0.2N HCl and 7% NaHCO₃ at a ratio of 4:1.5 plus 2% ascorbic acid (all from Sigma-Aldrich) unless otherwise stated. Ghrelin (human octanoylated and C-terminal amidated) was synthesized by the solid-phase method using Rink amide resin (C-terminal amidated peptides) as solid supports with an Fmoc protection protocol (Peptide Technologies Ltd., Montréal, Québec, Canada). The peptide was cleaved from the resin with trifluoroacetic acid (TFA) in the presence of scavengers (m-cresol, triisopropylsilane, anisole) and purified by reversed-phase HPLC using 0.1% TFA:acetonitrile gradients. Peptide purity (>95%) and identity was confirmed by analytical reverse-phase HPLC on C18 3 micron columns and by LC–Mass Spectrometry. Peptide content was greater than 85%. The peptide was dissolved in saline at 1 µg µl⁻¹ and stored at –80 °C in aliquots.

Measurements

Gastric emptying—Gastric emptying of a non-nutrient viscous solution of 1.5% methylcellulose and 0.05% phenol red was determined as described in our previous studies (33). In brief, rats were fasted overnight for 18 to 20 h with access to water. Animals received an orogastric gavage 1.2 or 1.5 ml of the viscous solution depending on the protocols and were euthanized 20 min later by CO₂ inhalation followed by thoracotomy. The stomach was removed and homogenized in 100 ml of 0.1 N NaOH using a Polytron (Brinkman Instruments, Westbury, NY). Five ml of the supernatant were added to 0.5 ml 20% trichloroacetic acid, centrifuged at 3,000 rpm at 4 °C for 20 min and 3 ml of the supernatant added to 4 ml of 0.5 N NaOH. The absorbance of the samples was read at 560 nm (Shimadzu 260 Spectrophotometer, Scientific Instrument, Columbia, MD, U.S.A.) and gastric emptying was calculated as percent emptying = (1 – absorbance of test sample/absorbance of standard) × 100. Phenol red recovered from stomachs of rats euthanized immediately after gavage of the same volume of solution served as standard.

Plasma L-dopa and dopamine determinations—Plasma samples were spiked with isoproterenol internal standard, gently shaken for 10 min with aluminum oxide, 1 ml of 1.5 M Tris buffer (pH 8.6), 100 µl 0.1 M EDTA-2Na and centrifuged. Supernatants were discarded and the residue washed three times with double distilled water. Monoamines (DOPA, 3,4-dihydroxyphenylacetic acid, and dopamine) were eluted with 2% CH₃COOH/100 µM EDTA and analyzed by HPLC coupled to electrochemical detection (Antec Leyden, Palm Bay, FL, oxidation potential 0.75 V, glassy carbon electrode against Ag/AgCl reference) using a reverse phase column (SC-5ODS, 150 mm × 3.0 mm with a 4 × 5 mm AC-ODS pre-column maintained at 25°C, Eicom, Kyoto Japan) pumped at 0.5 ml min⁻¹ with mobile phase consisting of 15% methanol / 85% 0.1 M citrate-acetate buffer (pH 2.6), 110 mg/l sodium octansulfonate, 5 mg l⁻¹ EDTA-2Na. Data was collected using EzChrom software (Agilent, Santa Clara, CA). The average recovery of the isoproterenol internal standard was 81%. All samples were processed in one batch. The system was calibrated daily and monoamine concentrations corrected to the internal standard.

Radioimmunoassay for plasma acyl ghrelin—Lyophilized plasma samples were re-suspended in double distilled H₂O immediately before radioimmunoassay according to the original plasma volume of 260 µl and duplicates were used to determine acyl ghrelin levels using a specific radioimmunoassay (GHRA-88HK 100% cross-reactivity with rat acyl ghrelin, Millipore, Billerica, MA) as in our previous studies (27-28). The detection limit was 7.8 pg ml⁻¹. All samples were measured in one batch and the intra-assay variation was 3.6%.

Immunohistochemistry for Fos—The procedure was as in our previous report (35) Free-floating brain sections (30 µm thickness, every 3rd section) were incubated overnight at 4°C with rabbit polyclonal anti-Fos (1:10,000; Catalog No. P38, Millipore, Billerica, MA). Sections were incubated with biotinylated secondary goat anti-rabbit IgG (1:1000, Jackson) followed by the incubation with avidin-biotin-peroxidase complex (1:200; Vector, Burlingame, CA, USA). Incubation lasted 1 h at room temperature. The chromogen used was diaminobenzidine tetrachloride (0.025%) with hydrogen peroxide (0.01%). After staining, sections were mounted, air-dried, dehydrated in ethanol, cleared in xylene and coverslipped.

Fos-immunoreactivity (For-ir) was examined in light microscope (Axioscop II, Carl Zeiss, Germany). For quantitative assessment, the number of immunoreactive cells was counted according to a rat brain atlas (34) at anterior-posterior levels (mm relative to Bregma) -1.92 to -3.12 for the CeA, -1.56 mm to -1.92 mm for the PVN, -3.00 to -3.48 for the Arc, -9.00 to -9.36 for the LPBe, and -13.30 to -14.28 for the NTS and AP. A 10 × 10 grid of squares was

placed in one of the eyepieces of the microscope to facilitate counting cells. The average number of Fos-ir cells/section for each animal was calculated unilaterally as described previously (35). Since no consecutive sections were used for the detection of the same neuronal marker, no corrections for double counting were applied. The investigator was blinded to the treatment. Images were acquired with a digital camera (Hamamatsu, Bridgewater, NJ) using the image acquisition system SimplePCI (Hamamatsu Corporation, Sewickley, PA, USA).

Experimental Protocols

Effect of orogastric or intraperitoneal administration of L-dopa on gastric emptying—Rats fasted overnight received orogastric gavage (0.3 ml) with L-dopa (5 or 15 mg kg⁻¹) or vehicle co-administered with 1.2 ml of methylcellulose/phenol red solution. The latter was drawn in the gavage syringe first and then L-dopa dissolved in its vehicle or vehicle allowing us to gavage methylcellulose/phenol red solution and L-dopa at the same time. For ip administration, fasted rats were injected ip (0.3 ml/rat) with L-dopa (1, 5 or 15 mg kg⁻¹) or vehicle and 10 min later, rats received an orogastric gavage of 1.5 ml of methylcellulose/phenol red solution. Twenty min after gavage, rats were euthanized and gastric emptying rate was determined. L-dopa doses were selected based on previous pharmacokinetic studies in rats (36).

Effect of intravenous ghrelin alone or combined with orogastric L-dopa on gastric emptying—Rats with a chronic iv catheter were fasted overnight and injected iv (0.2 ml) with ghrelin (30 or 100 µg kg⁻¹) or vehicle (saline) 10 min before orogastric gavage of 0.3 ml of L-dopa (15 mg kg⁻¹) or vehicle co-administered with 1.2 ml of methylcellulose/phenol red. Twenty min later, rats were euthanized and gastric emptying rate was determined. The iv doses of ghrelin were based on our previous studies showing full reversal of gastric emptying inhibited by lipopolysaccharide (26).

Effect of intravenous ghrelin on orogastric L-dopa-induced plasma occurrence of L-dopa and dopamine—Rats with a chronic iv catheter were fasted overnight and injected iv with ghrelin (30 µg kg⁻¹, 0.2 ml/rat) or vehicle (saline) 10 min before the orogastric gavage of 0.3 ml of L-dopa (5 mg kg⁻¹) or vehicle. In this experiment, L-dopa was suspended in the following vehicle: 0.25% carbomethanocellulose, sodium salt (Sigma-Aldrich) plus 2% ascorbic acid as described previously (37). The use of carbomethanocellulose as a vehicle for L-dopa in this experiment is in keeping with gastric emptying studies involving oral administration of L-dopa with methylcellulose. Dissolving L-dopa in methylcellulose was precluded as the solution was unstable. Blood was withdrawn before iv ghrelin injection and at 15, 30 and 60 min after the orogastric gavage. Plasma concentrations of L-dopa and dopamine were determined by HPLC.

Effect of intravenous L-dopa on plasma ghrelin levels—L-dopa (5 mg kg⁻¹) or vehicle (0.3 ml) was injected *via* a chronic iv catheter in overnight fasted rats. Blood was withdrawn before and 30 min, 1 h and 2 h after the iv injection. Plasma acylated ghrelin levels were determined by RIA.

Effect of intravenous ghrelin and ip L-dopa on Fos expression in the brain—Non-fasted naïve rats were used in this study to avoid fasting-related increase in basal Fos expression. Rats with a chronic iv catheter were injected iv with ghrelin (30 µg kg⁻¹, 0.2 ml/rat) or vehicle (saline) 10 min before ip injection of L-dopa (15 mg kg⁻¹) or vehicle. Ninety min after the ip injection, rats were euthanized by iv injection of sodium pentobarbital (50 mg kg⁻¹, Nembutal, Abbott Laboratories, Chicago, IL, USA) and perfused transcardially with isotonic saline (0.9% NaCl) followed by 400 to 500 ml of 4% paraformaldehyde and

14% saturated picric acid in 0.1 M phosphate buffer (PB, pH 7.4). Brains were removed and processed for Fos immunohistochemistry.

Statistical Analysis

Data are expressed as mean $\pm$ SEM and analyzed by one-way ANOVA by Tukey *post hoc* test, or two-way ANOVA repeated measurement (RM) followed by Bonferroni *post hoc* test. A *p* value <0.05 was considered statistically significant.

Results

Orogastric or intraperitoneal administration of L-dopa delays gastric emptying in overnight fasted rats

L-dopa (5 and 15 mg kg⁻¹) administered by oral gavage into the stomach dose-dependently decreased gastric emptying of a non-nutrient viscous solution compared with vehicle (38.9 $\pm$ 6.5% and 23.0 $\pm$ 4.8% respectively vs 58.3 $\pm$ 3.4%; $P < 0.05$; Fig. 1A) when measured 20 min later. L-dopa (5 or 15 mg kg⁻¹) injected ip 10 min before orogastric gavage of the semi-liquid meal potently inhibited gastric emptying compared with vehicle (4.3 $\pm$ 1.7% and 8.0 $\pm$ 2.5% respectively vs 45.9 $\pm$ 4.8%; $P < 0.05$), whereas 1 mg kg⁻¹ had no effect (43.6 $\pm$ 6.4%, $P > 0.05$; Fig. 1B). There was no statistical difference between gastric emptying values in orogastric and ip vehicle groups (58.3 $\pm$ 3.4% vs 45.9 $\pm$ 4.8%). Two way ANOVA showed a significantly higher inhibitory effect of L-dopa when administered ip than orogastrically at 5 mg kg⁻¹ ($F_{1,29} = 35.6$, $P < 0.001$) and 15 mg kg⁻¹ ($F_{1,29} = 10.6$, $P < 0.01$).

Intravenous injection of ghrelin prevents orogastric L-dopa-induced delayed gastric emptying in overnight fasted rats

In rats receiving iv saline through the chronic intra-jugular catheter and orogastric L-dopa (15 mg kg⁻¹), gastric emptying was significantly reduced compared with iv saline plus orogastric vehicle (30.0 $\pm$ 4.4% vs 60.0 $\pm$ 5.2%; $P < 0.05$; Fig. 2). Ghrelin (30 or 100 μ g kg⁻¹ iv) completely prevented the L-dopa-induced delayed gastric emptying (53.2 $\pm$ 3.0% and 59.2 $\pm$ 2.3%, respectively; $P < 0.05$ vs iv saline plus orogastric vehicle). Ghrelin (30 μ g kg⁻¹, iv) significantly increased basal gastric emptying compared to iv saline in rats receiving orogastric vehicle (73.3 $\pm$ 2.3% vs 60.0 $\pm$ 5.2%, $P < 0.05$; Fig. 2).

Intravenous injection of ghrelin increases plasma L-dopa and dopamine levels after orogastric gavage of L-dopa in overnight fasted rats

Orogastric administration of L-dopa (5 mg kg⁻¹) induced the occurrence of L-dopa in the plasma 15 and 30 min after orogastric gavage in rats injected iv with saline compared with pre-injection (141.4 $\pm$ 53.6 and 105.1 $\pm$ 29.7 respectively vs 6.0 $\pm$ 2.6 nM; $P < 0.05$), and thereafter values declined to 37.9 $\pm$ 8.4 nM at 60 min although remained elevated above basal levels ($P < 0.05$; Fig. 4A). Ghrelin (30 μ g kg⁻¹, iv) pretreatment resulted in significantly higher plasma L-dopa levels 15 min after orogastric gavage of L-dopa (293.6 $\pm$ 49.4 nM) than that in rats pretreated iv with saline at the same time point. Thereafter at 30 and 60 min, plasma L-dopa values (106.9 $\pm$ 13.6 and 34.9 $\pm$ 3.5 nM respectively) were similar to those observed in the saline pretreated rats (Fig. 3A). Two-way RM ANOVA indicated a significant influence of time ($F_{3,21} = 25.4$, $P < 0.001$), and treatment $\times$ time ($F_{3,21} = 4.3$, $P < 0.05$) on plasma L-dopa concentrations after iv ghrelin.

Orogastric gavage of L-dopa (5 mg kg⁻¹) also resulted in elevated plasma dopamine concentrations 15 min after administration compared to basal levels in rats pretreated with iv saline (11.0 $\pm$ 3.5 vs 1.5 $\pm$ 0.3 nM, $P < 0.05$; Fig. 3B), that was followed by a time-related decline at 30 and 60 min (6.1 $\pm$ 2.0 and 3.1 $\pm$ 0.8 nM, $P > 0.05$ vs basal). Ghrelin (30 μ g kg⁻¹,

iv) administered before orogastric L-dopa, induced higher plasma dopamine levels (25.0 ± 3.2 nM) than in iv saline pretreated rats at 15 min ($P < 0.05$). Thereafter, dopamine values declined to 6.7 ± 1.6 and 1.9 ± 0.3 nM at 30 and 60 min respectively and were not significantly different from those observed in the vehicle pre-treated group (Fig. 3B). Two-way RM ANOVA indicated a significant influence of treatment ($F_{1,21} = 7.6$, $P < 0.05$), time ($F_{3,21} = 28.4$, $P < 0.001$), and treatment $\times$ time ($F_{3,21} = 6.3$, $P < 0.01$) on plasma dopamine concentrations after iv ghrelin.

Intravenous injection of L-dopa does not alter plasma acyl ghrelin levels in fasted rats

Plasma acyl ghrelin levels were stable at 30 min, 1 and 2 h after iv injection with vehicle ($n=7$, $P > 0.05$, Table 1). L-dopa (15 mg kg^{-1} , iv) did not modify the fasted plasma levels of acyl ghrelin compared either with vehicle at the same time points or basal within the same group ($n=7$, $P > 0.05$, Table 1). In this study, to avoid variability linked with orogastric gavage, L-dopa was administered iv (ip and iv injections showed similar pharmacokinetic at the similar doses in rats except iv injection peaked faster) (36).

Fos induction in brain autonomic centers after intravenous injection of ghrelin and/or ip L-dopa

In iv saline-pretreated rats, ip injection of L-dopa (15 mg/kg) induced Fos expression in nigrostriatal system and iv ghrelin ($30 \mu\text{g kg}^{-1}$) did not influence basal and L-dopa-stimulated Fos in that system (data not shown). With regard to the autonomic centers involved in the regulation of gastric function, ip L-dopa in iv saline-pretreated rats, increased Fos-ir cells significantly in the central nucleus of the amygdala (CeA), external subnucleus of the lateral parabrachial nucleus (LPBe), NTS and area postrema (AP) by 1.7, 2.6, 2.2 and 3.9 folds respectively compared with iv saline plus ip vehicle-treated rats that showed a low numbers of Fos-ir cells. In contrast, iv ghrelin plus ip vehicle resulted in 2.2, 2.3 and 6.7 folds increases in the number of Fos ir cells respectively in the PVN and arcuate nucleus (Arc) of the hypothalamus and AP (Table 2). The combined treatment (iv ghrelin + ip L-dopa) did not show statistically significant change in the number of Fos positive neurons in the CeA, LPBe, AP and NTS compared to iv saline + ip L-dopa, and not in the PVN, Arc and AP compared to iv ghrelin + ip vehicle (Table 2).

In the NTS of rats treated with iv saline plus ip vehicle, a few Fos-ir cells were observed in both medial and lateral areas, and ghrelin plus vehicle did not induce significant changes, while increased Fos-ir was induced by ip L-dopa in the medial NTS (Fig. 4A-D). In the Arc, iv ghrelin increased Fos expression in the ventromedial area, and ip L-dopa induced a few Fos-ir cells located in the dorsolateral areas similar to saline-vehicle controls (Fig. 4E-G). Ghrelin plus L-dopa treatment did not alter Fos expression in the Arc compared with rats treated with ghrelin plus vehicle (Fig. 4G, H).

Discussion

The present study showed that peripheral treatment with L-dopa reduced gastric emptying of a non-nutrient viscous solution in fasted rats. This inhibitory effect was more potently induced by the ip than the orogastric route of administration. Ghrelin injected iv prevented oral L-dopa-induced delayed gastric emptying and further enhanced plasma L-dopa levels compared with iv vehicle. Additionally, iv L-dopa did not affect fasted plasma ghrelin levels and ghrelin did not influence Fos expression-induced by ip L-dopa in relevant brain nuclei.

To our knowledge, this is the first report in rats showing that L-dopa (5 or 15 mg kg^{-1}) inhibits gastric emptying when administered orally (33% and 61%, respectively) or by the intraperitoneal route (91% and 83%, respectively). Previous pharmacokinetic studies in rats

showed that plasma L-dopa levels display more individual variability and lower absorption after oral administration compared to ip injection (36) which may account for the enhanced efficiency of L-dopa given ip than intragastrically in our experiments. These data provide a rodent model to study L-dopa-associated gastroparesis consistent with clinical reports showing that oral L-dopa delays gastric emptying in healthy young or older human subjects (3, 4). In addition, there is clinical evidence that L-dopa can alter gastric propulsive motility *per se* in addition to the already impaired gastric transit in the Parkinson's disease patients (6, 38).

The present study showed that plasma dopamine levels were increased along with those of L-dopa with a peak elevation 15 min after orogastric L-dopa corresponding to the same time frame in which delayed gastric emptying was observed. Such a kinetic study is also consistent with other reports showing that L-dopa levels in the circulation peak around 15 to 20 min after orogastric treatment at doses of 5 and 15 mg kg⁻¹ in rats (36) and 20 mg kg⁻¹ in rabbits (39). Elevation of circulating dopamine may play a role in the delayed gastric emptying. Dopamine reduces spontaneous and stimulated gastric contractions when applied *in vitro* to a rat stomach organ bath (40) and inhibits gastric emptying and motility when injected ip or subcutaneously in fasted rats (40, 41). Dopamine inhibitory action is mimicked by dopamine receptors D2/D3 agonists and may involve peripheral D2/D3 located on post-ganglionic cholinergic neurons (5, 42), although central mechanisms recruited by L-dopa entering into the brain cannot be excluded particularly at the level of brainstem nuclei involved in retching (43).

We showed here that the delayed gastric emptying induced by L-dopa was not associated with changes in circulating acyl ghrelin levels. Although plasma concentrations of L-dopa and dopamine were increased, plasma acyl ghrelin levels remain unchanged from 30 min to 2 h after iv injection of L-dopa in fasted rats. Our results are consistent with reports in conscious rats showing no alteration of ghrelin levels in dialysates from microdialysis probes implanted into the gastric submucosa after dopamine infusion (7.4 nmol h⁻¹) while under the same conditions, somatostatin suppressed ghrelin release (44). However, a recent *in vitro* study shows that dopamine at 10⁻⁴ M stimulates ghrelin release from a mouse ghrelinoma (MGN) 3-1 cells through interaction with the dopamine receptor subtype 1 (D1) (45). By contrast, D2/D3 receptors are involved in the inhibitory effect of dopamine on gastric motility in rats (40, 41, 46). Collectively these data demonstrate that L-dopa/dopamine-induced delayed gastric transit is not secondary to change in endogenous ghrelin release.

Importantly, we also demonstrated here that ghrelin injected iv at supraphysiological doses completely prevents the 50% inhibition of gastric emptying induced by orogastric L-dopa. Ghrelin injected iv at 30 µg kg⁻¹ increased basal gastric emptying by 22% in fasted rats within 20 min consistent with previous studies using similar dose ranges (20 and 50 µg kg⁻¹, iv) (47, 48). Ghrelin's prokinetic effect has been linked to the rapid onset increase of phase III-like contractions in the rat antrum (47, 49). In addition, we showed that the normalization of gastric emptying by iv ghrelin in orogastric L-dopa-treated rats was associated with a further enhancement of L-dopa and dopamine occurrence in the circulation 15 min after L-dopa treatment. The short duration of elevated plasma L-dopa and dopamine levels by ghrelin could be related to the short half-life and action of the peptide (50, 51). Clinical studies indicate that gastric emptying impacts L-dopa pharmacokinetics (7, 8, 39) and plasma L-dopa levels are significantly correlated with gastric motility in Parkinson's disease (8). It may be speculated based on these preclinical data that ghrelin agonists may have potential therapeutic benefits as gastrokinetic agents in Parkinson's disease patients treated with L-dopa by improving gastric emptying and delivery of L-dopa into the blood. Clinical data already support the potential use of stable ghrelin agonists to improve delayed gastric

emptying occurring under conditions of diabetes, chronic gastritis and after surgery (52, 53). However, the influence of ghrelin or stable agonists in experimental PD models will need to be investigated to ascertain the translational application of the present observations.

It is well known that L-dopa acts in the brain, and ghrelin receptors are abundantly distributed in the substantia nigra, vagal pathways and myenteric plexus (54-57). Ghrelin gastroprokinetic action is centrally mediated *via* vagal-dependent capsaicin sensitive mechanisms and peripherally via activating on myenteric neurons to activate cholinergic excitatory pathways by direct stimulation of myenteric neurons (14, 17, 58). Our study shows that Fos induction after ip injection of L-dopa occurs in the AP, medial NTS besides the CeA and LPB. The NTS, the major relay center of visceral afferents (59) is directly connected to the LPB, PVN and CeA (60-62), and plays a role in regulation of gastric motility (63, 64), which can be involved in inhibition of gastric emptying by ip L-dopa. The AP receives chemical signals outside the blood-brain-barrier and is reciprocally connected to the NTS (65). L-dopa injected ip may send signal to the NTS via the circulation. However, ghrelin does not modify Fos expression in those brain areas activated by L-dopa, while inducing increased Fos expression in the hypothalamic nuclei such as the PVN, the Arc, an appetite-stimulating center, as well as in the AP consistent with previous studies in rats treated with iv or ip ghrelin (66-68). The fact that ghrelin-induced Fos expression is persistent in rats treated with L-dopa indicates that ghrelin activated pathways are maintained to exert stimulation of gastric motility over L-dopa. It is unlikely that heterodimerization between ghrelin and dopamine as shown in the brain can account for the modulation of L-dopa action by ghrelin. The formation of ghrelin/dopamine receptor heterodimers involves D1 receptors and leads to amplification of brain dopamine rewarding processing influencing food intake (69, 70). By contrast in the present study ghrelin counteracts a dopamine inhibitory action that could be mainly mediated by D2/D3 receptor subtypes in the periphery (40, 41). The present data point to ghrelin acting through the brain stimulation of gastric cholinergic excitatory pathways (58, 71) which are maintained in presence of L-dopa thereby preventing the inhibitory effect of dopamine on postsynaptic cholinergic release (72) which needs to be assessed in further experiments.

In conclusion, orogastric and more potently intraperitoneal, L-dopa induces a rapid inhibition of gastric emptying of a non-nutrient viscous solution in rats without altering plasma levels of ghrelin. We also showed that systemic ghrelin treatment normalizes delayed gastric emptying induced by L-dopa in rats and enhanced circulating levels of L-dopa and dopamine within 20 min after orogastric administration in fasted rats. Such a restoration of basal gastric emptying in ghrelin plus L-dopa treated rats occurs without alterations in the activity of known brain neuronal circuitry activated by either ghrelin or L-dopa alone, pointing to a peripheral site of interaction under conditions of maintained brain neuronal activation by ghrelin. These observations suggest that ghrelin and its agonists which are already in clinical trials for other conditions of gastroparesis (52), may potentially also be used in Parkinson's disease patients treated with L-dopa. Such a proposition needs to be ascertained by additional experiments in experimental PD models and under chronic treatment regimens.

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Fig. 1.

L-dopa administered orogastrically (A) and intraperitoneally (B) inhibits gastric emptying of a non-nutrient semi-liquid meal in overnight fasted rats. Data are expressed as mean $\pm$ SEM. The number of animals in each group is indicated at the bottom of each column. *: $P < 0.05$ vs vehicle and #: $P < 0.05$ vs 5 mg kg⁻¹.

Fig. 2.

Ghrelin injected intravenously prevents orogastric (og) L-dopa-delayed gastric emptying of a non-nutrient semi-liquid meal in overnight fasted rats. Data are expressed as mean $\pm$ SEM. The number of animals in each group is indicated at the bottom of each column. *: $P < 0.05$ vs iv vehicle + orogastric vehicle and #: $P < 0.05$ vs iv vehicle + orogastric L-dopa.

Fig. 3. Ghrelin injected intravenously enhances plasma L-dopa (A) and dopamine (B) concentrations after orogastric L-dopa administration in fasted rats. Data are expressed as mean $\pm$ SEM, $n=4-5$ /group; *: $P < 0.05$ vs before injection (time 0) and #: $P < 0.05$ vs saline vehicle-treated rats at the same time point.

Fig. 4. Fos expression in the NTS (A-D) and Arc (E-H) in rats injected with iv saline (0.2 ml/rat) plus ip vehicle (0.3 ml/rat) (A, E), iv ghrelin ($30 \mu\text{g kg}^{-1}$) plus ip vehicle (B, F), iv saline plus ip L-dopa (15 mg kg^{-1}) (C, G), and iv ghrelin plus ip L-dopa (D, H). $n = 4/\text{group}$. Scale bar in A = $100 \mu\text{m}$ for all panels. Arc: Arcuate nucleus of the hypothalamus; AP: area postrema; DMN: dorsal motor nucleus of the vagus; NTS: nucleus tractus solitarius.

Table 1

Plasma acyl ghrelin levels before and after iv L-dopa in fasted rats.

Treatment ^a	Acyl ghrelin (ng ml ⁻¹) ^b			
	Before	30 min	1 h	2 h
Vehicle	1.29 ± 0.09	1.18 ± 0.11	1.41 ± 0.25	1.29 ± 0.11
L-dopa	1.21 ± 0.16	1.35 ± 0.40	1.33 ± 0.17	1.24 ± 0.21

^aOvernight fasted rats with chronic intra-jugular vein catheter were injected intravenously with L-dopa (5 mg kg⁻¹) or vehicle (0.3 ml). Blood was withdrawn before and 30 min, 1 h and 2 h after iv injection and plasma acyl ghrelin levels determined by RIA.

^bEach value is the mean ± SEM of 7 rats/group, $P < 0.05$ vs vehicle.

Table 2

Fos-ir cells in brain autonomic nuclei induced by iv ghrelin and ip L-dopa given alone or in combination.

Treatment ^a	Fos-ir cells/nuclei ^b			
	Saline-Vehicle	Saline-L-dopa	Ghrelin-Vehicle	Ghrelin- L-dopa
CeA	36.3 ± 9.3	99.6 ± 9.6 *	70.9 ± 18.9	89.9 ± 17.7
PVN	31.4 ± 10.4	53.4 ± 17.3	101.9 ± 22.6 *	132.8 ± 28.5 *
Arc	17.2 ± 4.9	22.2 ± 4.1	57.2 ± 10.0 *	56.7 ± 9.8 *
LPBe	13.7 ± 2.3	49.9 ± 3.4 *	26.5 ± 2.8	53.2 ± 6.0 *
AP	6.6 ± 2.5	32.4 ± 8.7 *	51.9 ± 1.9 *	54.2 ± 7.4 *
NTS	19.3 ± 3.3	62.2 ± 11.7 *	36.3 ± 6.7	73.3 ± 12.0 *

^a Non fasted rats with a chronic intra-jugular vein catheter were injected intravenously with ghrelin (30 µg kg⁻¹) or saline and 10 min later received an intraperitoneal injection of L-dopa (15 mg/kg) or vehicle and 90 min thereafter euthanasia was performed to assess Fos expression in the brain.

^b Data are means ± SEM, n = 4/group.

* $P < 0.05$ vs saline plus vehicle.