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Downregulation of neuronal vasoactive intestinal polypeptide in Parkinson's disease and chronic constipation

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Abstract

Background—Chronic constipation (CC) is a common and severe gastrointestinal complaint in Parkinson's disease (PD), but its pathogenesis remains poorly understood. This study evaluated functionally distinct submucosal neurons in relation to colonic motility and anorectal function in PD patients with constipation (PD/CC) vs. both CC and controls.

Methods—Twenty-nine PD/CC and 10 Rome III-defined CC patients were enrolled. Twenty asymptomatic age-sex matched subjects served as controls. Colonic transit time measurement and conventional anorectal manometry were evaluated in PD/CC and CC patients. Colonoscopy was performed in all three groups. Colonic submucosal whole-mounts from PD/CC, CC and controls were processed for immunohistochemistry with antibodies for vasoactive intestinal polypeptide

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DISCLOSURES: The authors have no competing interests.

AUTHOR CONTRIBUTION: Study concept and design: RDeG, FG, FT, UV, MG, AT and CS; acquisition of data: FG, FT, RR, FB, RL, AI, MM, MG; analysis and interpretation of data: FG, FT, AI, UV, AT, CS and RDeG; drafting of the manuscript: FG, FT, UV, AT, CS and RDeG; critical revision of the manuscript for important intellectual content: RC, PC, AT, CS, statistical analysis: FG; obtained funding: RDeG; study supervision and guarantor of the article: RDeG. All authors approved the final version of the manuscript. All authors approved the final version of the article, including the authorship list.

SUPPORTING INFORMATION

Supporting materials and methods

Supporting results

(VIP) and peripheral choline acetyl-transferase, markers for functionally distinct submucosal neurons. The mRNA expression of VIP and its receptors was also assessed.

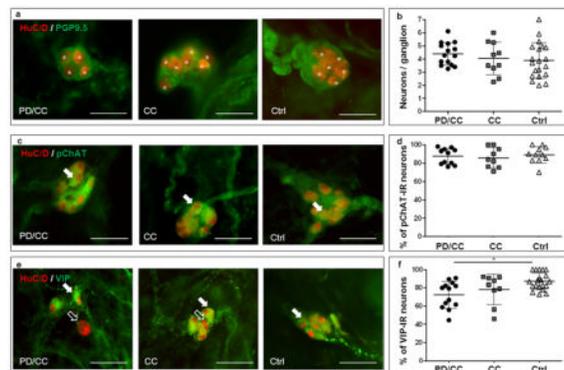
Key Results—Four subgroups of PD/CC patients were identified: delayed colonic transit plus altered anorectal manometry (65%); delayed colonic transit (13%); altered manometric pattern (13%); no transit and manometric impairment (9%). There were no differences in the number of neurons/ganglion between PD/CC vs. CC or vs. controls. A reduced number of submucosal neurons containing VIP immunoreactivity was found in PD/CC vs. controls ($P<.05$). *VIP*, *VIPR1* and *VIPR2* mRNA expression was significantly reduced in PD/CC vs. CC and controls ($P<.05$).

Conclusions & Inferences—Colonic motor and rectal sensory functions are impaired in most PD/CC patients. These abnormalities are associated with a decreased VIP expression in submucosal neurons. Both sensory-motor abnormalities and neurally-mediated motor and secretory mechanisms are likely to contribute to PD/CC pathophysiology.

Abbreviated abstract

Chronic constipation is usually a severe gastrointestinal dysfunction in patients with Parkinson's disease, but its pathogenesis remains poorly understood. This study evaluated functionally distinct submucosal neurons in relation to colonic motility and anorectal function in PD patients with constipation (PD/CC) vs. both CC and controls.

Colonic motor and rectal sensory functions resulted impaired in most parkinsonian constipated patients. Compared to controls, they display a decreased number of submucosal secretomotor neurons containing VIP immunoreactivity accompanied by a reduced mRNA expression of VIP and VIP-receptors.



Keywords

anorectal manometry; secretomotor neurons; slow transit constipation; cholinergic neurons

1. INTRODUCTION

Parkinson's disease (PD) is a chronic neurodegenerative multi-system condition affecting about 1% of the elderly population, though a subset (~ 10%) of patients develops symptoms before 50 years of age.¹ The pathological hallmarks of PD are a progressive degeneration of the dopamine-containing neurons in the *substantia nigra pars compacta* along with

intraneuronal aggregates of eosinophilic inclusions (mainly phosphorylated α -synuclein), i.e. Lewy bodies (LBs) and Lewy neurites (LNs).² Although PD is regarded as a prototypical movement disorder, non-motor manifestations, such as autonomic dysfunctions, particularly those involving the gastrointestinal (GI) tract, are increasingly recognised as being a major aspect of the PD clinical picture.³

Virtually all parkinsonian patients experience GI dysfunctions such as dysphagia, delayed gastric emptying (up to gastroparesis), and severe chronic constipation (CC).^{4–5} Specifically, CC is a dominant manifestation in up to 80% of PD patients⁴ and it occurs 2–4 times more frequently in PD compared to age-sex matched non-PD subjects.⁶ Furthermore, the administration of L-Dopa, the most used drug to manage motor symptoms of PD patients, induces a prominent inhibitory action on GI motility, thus worsening the severity of CC.⁷ However, a delayed colonic transit is observed in PD patients also independently of drug treatment.⁸ The severe CC in parkinsonian patients is often unresponsive to first-line treatment (e.g. osmotic laxatives) and it may evolve to severe complications such as megacolon and intestinal pseudo-obstruction.^{9–11}

The enteric nervous system (ENS) is a prime target of the pathological process of PD as indicated by autopsies of parkinsonian patients revealing the widespread presence of LBs and/or LNs in myenteric and submucosal neurons throughout the GI tract.^{12–13} Furthermore, full-thickness biopsies of patients with idiopathic CC undergoing colectomy for treatment-resistant, severe slow transit CC show changes in myenteric and submucosal neurons.¹⁴ Taken together, these features support strongly the role of altered ENS function in patients with PD and CC. The recent demonstration that colonic mucosal biopsies including submucosal tissue with its ganglionated plexus can be obtained during colonoscopy provided an important tool to investigate changes in the ENS at the cellular and molecular level occurring in patients with PD and GI dysfunction.¹⁵

Thus, the present study was designed to investigate and correlate functional and neuronal features in a cohort of PD/CC patients in comparison to non-parkinsonian CC and healthy controls. Our morphological analysis focused on enteric neurons of submucosal plexuses, which play a critical role in controlling secretomotor functions but also participate to motility regulation in the GI tract. Understanding the mechanisms underlying bowel dysfunction in PD patients would ultimately allow for a better knowledge of the management and treatment of CC in these patients.

2. MATERIALS AND METHODS

2.1 Patient recruitment

The study design included three groups of patients: n= 29 PD/CC (9F; age range: 48–83 years); n= 10 patients with chronic constipation CC (6F; age range: 36–87 years); n= 20 control subjects (7F; age range: 33–89 years). PD/CC patients were consecutively enrolled at the Movement Disorder Center of the Neurology Unit of St. Orsola-Malpighi Hospital in Bologna, Italy. The diagnosis of PD was defined according to well established guidelines of the United Kingdom Parkinson's Disease Society Brain Bank clinical diagnostic criteria, United Kingdom Parkinson's Disease Survey Brain society.¹⁶ Patients with a significant

cognitive impairment Mini Mental State Examination (MMSE) score < 19 were excluded. Data concerning duration of PD, Hoehn & Yahr (HY) stage, MMSE score, parkinsonian features evaluated by Unified PD rating scale (UPDRS),¹⁷ along with daily medication dosage of L-Dopa, were collected for each PD patient (Supplementary Table 1). CC patients with or without PD were diagnosed according to Rome III criteria¹⁸ at the Gastroenterology outpatient clinic of St. Orsola-Malpighi Hospital in Bologna, Italy. Each PD/CC and CC patient reported an average Bristol stool scale of 1–2.¹⁹ Asymptomatic, otherwise healthy subjects undergoing screening colonoscopy for polyps served as control group.

Each patient / subject signed an informed consent form before entering the study. The study protocol was approved by the Ethical Committee of St. Orsola-Malpighi Hospital, Bologna, Italy (N° 66/2011/U/Tess).

2.2 GI functional assessment

Both colonic transit time (TT) (expressed as the number of radiologically detected intracolonic radiopaque markers after five days) and anorectal manometry (AM) were performed in any CC patients with or without PD based on standardised methods^{20–21} (see Supporting information for methodological details). The asymptomatic controls underwent colonoscopy (neither TT nor AM were assessed).

2.3 Tissue collection

During colonoscopy, n= 4 mucosal biopsies (including the submucosa) were taken from the descending colon in each PD/CC, CC and control subject. Biopsies were processed according to standard protocols (see Supporting information).

2.4 Immunohistochemistry and neuronal cell bodies quantification

Submucosal whole mounts were analyzed using previously validated immunohistochemical protocols (see Supporting information). The following primary antibodies were used: two general pan-neuronal markers, i.e. protein gene product9.5 (PGP9.5) recognizing perikarya and nerve fibers, and HuC/D detecting only neuronal cell bodies, for quantitative analysis. Neuronal counts were expressed as number of cell bodies/ganglion (mean ± SD). At least 20–30 neurons from 5–6 ganglia/whole-mount were quantified in each patient or control subject. Submucosal secretomotor neurons were identified by using the anti-peripheral choline acetyl-transferase (pChAT) antibody and vasoactive intestinal polypeptide (VIP). Double-labeling immunohistochemistry included HuC/D and pChAT or VIP. The percentages of HuC/D positive neurons colocalising with either pChAT or VIP were counted and data were expressed as mean ± SD.

2.5 RT-qPCR analysis

RT-qPCR was performed for each patient / subject on the total RNA extracted from one frozen biopsy (see Supporting information). The relative gene expression analysis evaluated the following genes: *VIP*, *VIP receptor 1 (VIPR1)* and *VIP receptor 2 (VIPR2)*. Data were calculated with C_T method using *18S* as a reference gene.

2.6 Statistical analysis

Statistical analysis was performed with the commercial software SPSS (for Windows, version 13.0; SPSS Inc, Chicago, Illinois) according to the appropriate tests for each considered variable. A Kolmogorov-Smirnov non-parametric test was applied to verify the normality of the distributions. Continuous data were reported as mean \pm SD, and categorical data were described as frequencies. One-way analysis of variance, Fisher exact test, and χ^2 test were applied. Correlation analyses were performed by Pearson χ^2 and Spearman's rank test. Two-tailed P values less than .05 were considered significant. Graphical representations of data were obtained using GraphPad software (GraphPad Prism version 5.00 for Windows, GraphPad Software Inc., La Jolla, CA, USA).

3. RESULTS

Among PD/CC patients, n= 17 (58%) completed the study; n= 7 (24%) refused colonoscopy (although they underwent TT and AM testing), n= 1 (3.5%) refused both TT and AM assessment (but that patient underwent colonoscopy), n= 3 (10%) refused TT (they had colonoscopy and AM) and n= 1 (3.5%) refused AM (but had colonoscopy and TT). In the group of CC patients, n= 9 (90%) underwent TT and AM, while n= 1 performed only AM.

3.1 Functional constipation assessment in PC/CC and CC

Evaluation of colonic TT and AM was performed in 24 and 27 PD/CC patients, respectively (Table 1). In the PD/CC cohort that underwent transit study, 75% (18 / 24) showed a delayed colonic TT, while 85% (23 / 27) of the PD/CC patients who underwent AM had one or more AM abnormalities: increased basal anal sphincter pressure (2 / 27), decreased basal anal sphincter pressure (5 / 27), ultra-slow waves (2 / 27), and defective squeezing pressure, i.e. inability to contract the anus properly, (7 / 27) (specifically, 3 patients lacking anal contractions, while 4 showing short-lived anal contractions). In 8 / 27 PD/CC patients AM showed anismus, i.e. paradoxical sphincter contraction during straining attempts. Also, PD/CC patients showed a rectal sensory dysfunction characterised by a reduced ampullary threshold (7 cases), although not associated with hyposensitivity (Table 1). The recto-anal inhibitory reflex was detected in all patients. Both colonic TT and AM were performed in 23 / 29 PD/CC patients and the resultant features allowed for the identification of four subgroups of patients: 1) delayed TT and altered AM (65%); 2) delayed TT only (13%); 3) altered AM pattern only (13%); 4) no functional impairment (9%) (Fig. 1A).

Conversely, non-PD CC patients were subdivided in two groups only: 1) delayed TT and altered AM (33%); and 2) delayed TT only (67%) (Fig. 1B). However, there were no differences in the mean number of pellets found in PD/CC vs. CC patients (12.4 ± 7.78 vs. 11.9 ± 4.65 ; $P=.28$) (Fig. 1C).

3.2 Submucosal neuron count

In order to define possible differences existing in the number of neurons among PD/CC, CC and controls, submucosal whole mount preparations were analyzed by labeling neuronal cell bodies with HuC/D. Virtually all perikarya were labeled by HuC/D and quantitative assessment revealed no significant differences in terms of the mean number of HuC/D

immunoreactive (IR) ganglion cell bodies/ganglia in the three groups (4.4 ± 0.86 vs. 4.0 ± 1.23 vs. 4.0 ± 1.35 in PD/CC, CC and controls, respectively; ANOVA test, $P = .357$) (Fig. 2A–B).

3.3 Submucosal cholinergic and VIP containing neurons

Both cholinergic and VIP containing neurons are widely represented in the human colonic submucosal plexus.²² pChAT and VIP-IRs were readily detectable in the cell bodies as well as nerve processes branching off the ganglia. The proportion of pChAT-IR neurons was calculated for each patient and control on the total number of HuC/D-IR neuronal cell bodies. There were no changes in the number of HuC/D/pChAT-IR neurons among the three groups ($87 \pm 8.7\%$ vs. $86 \pm 10.9\%$ vs. $89 \pm 8.7\%$ in PD/CC, CC and controls, respectively; $P = .770$) (Fig. 2C–D). In contrast, the percentage of HuC/D/VIP-IR neurons was significantly reduced in PD/CC ($72 \pm 14.6\%$) vs. controls ($87 \pm 9.2\%$) ($P = .007$), while no differences were observed between PD/CC vs. CC ($78 \pm 16.4\%$; $P = .292$) and between CC vs. controls ($P = .321$) (Fig. 2E–F).

3.4 VIP and VIP receptor mRNA expression

The reduced number of VIP containing neurons in the absence of changes in the total number of HuC/D and cholinergic neurons, suggests VIP downregulation. Therefore, we tested whether *VIP* and its receptors, *VIPR1* and *VIPR2*, gene expression was altered in PD/CC, CC and controls (Fig. 3A–C). Figure 3A demonstrated a significant reduction of VIP mRNA expression in PD/CC vs. controls (0.012 ± 0.045 vs. 1.059 ± 0.177 ; $P < .0001$) and vs. CC (0.012 ± 0.045 vs. 0.189 ± 0.39 ; $P = .036$) as well as in CC vs. controls (1.059 ± 0.177 vs. 0.189 ± 0.39 ; $P = .001$).

Compared to controls, *VIPR1* expression (Fig. 3B) was significantly reduced in both groups of constipated patients, PD/CC vs. controls (0.00008 ± 0.000085 vs. 1.04 ± 0.226 ; $P < .0001$) and CC vs. controls (0.003 ± 0.0055 vs. 1.04 ± 0.226 ; $P < .0001$). Notably, PD/CC *VIPR1* mRNA expression was significantly lower than that detected in CC (0.00008 ± 0.000085 vs. 0.003 ± 0.0055 ; $P < .0001$). Similar results were obtained for *VIPR2* expression (Fig. 3C). Our data showed reduced *VIPR2* mRNA expression levels in PD/CC vs. controls (0.002 ± 0.0063 vs. 0.93 ± 0.379 ; $P < .0001$) and vs. CC (0.002 ± 0.0063 vs. 0.05 ± 0.126 ; $P = .001$) as well as in CC vs. controls (0.05 ± 0.126 vs. 0.93 ± 0.379 ; $P < .0001$).

In patients with PD/CC and CC with slow transit, there was a reduction (although not significant, $P = .07$) in *VIP* mRNA levels in PD/CC vs. CC. However, PD/CC with slow transit showed a significant decrease of *VIPR1* and *VIPR2* mRNA expression ($P = .014$ and $P = .002$ respectively) vs. CC with slow transit (Fig. 4A–C).

Other results concerning clinical-pathological correlations have been reported in the Supporting information (with Supplementary Fig. S1).

4. DISCUSSION

The present study demonstrated: a) a downregulation of VIP submucosal neurons and of VIP mRNA as well as of both VIPRs, *VIPR1* and *VIPR2*, in PD/CC and CC patients compared to controls, with the downregulation being more pronounced in PD/CC patients

vs. CC patients; and b) an impairment of colonic motor and rectal sensory function in both PD/CC and CC patients. Because of the demanding nature of the study protocol that required many visits and several tests, several patients, particularly in the PD/CC group, did not complete both functional GI assessment and colonoscopy with related histopathological analysis resulting in a smaller number of subjects than the initial enrolled number. The vast majority of PD/CC patients in our study (91%) showed abnormalities of either TT or AM, indicative of sensory-motor abnormalities; 65% had a delayed TT combined with anorectal sensory-motor impairment. Taken together our data indicate a severe functional motor impairment in the colon and rectum of PD/CC patients. In contrast, in the non-parkinsonian CC group, only 33% of patients showed combined slow TT and altered AM patterns, but a high proportion (67%) displayed slow transit. Overall, the two groups, PD/CC and CC, were comparable in terms of slow transit assessed by objective measurements. Our functional data on PD/CC extend previous studies evaluating constipated parkinsonian patients.^{23–24} In fact, Sakakibara et al. demonstrated that slow transit constipation was prevalent in PD/CC patients, while anorectal abnormalities were identified only in a small proportion of patients.²⁴ In contrast, Edwards et al. found anorectal manometric abnormalities in 77% of the PD/CC cases and slow transit only in 31%. In that study, the acute administration of apomorphine has been shown to improve dyssynergic defecation symptoms in a subset of patients with PD/CC, thus implying that a dopaminergic dysfunction is also responsible for dyssynergia.²³ The differences between our findings and those reported by Sakakibara et al. and Edwards et al. may be explained by the severity of PD per se and sample size, i.e. the number of patients enrolled in the two mentioned studies (approximately 50% less than the current study). In addition, the heterogeneity of pathogenetic factors contributing to CC in PD (e.g., delayed transit vs. sensory-motor abnormalities) also needs to be taken into consideration, thus different groups of patients might show differences in their GI abnormalities and symptoms. Taken together, both this current study as well as previous data indicate that both a delayed TT and abnormal anorectal manometric patterns contribute to CC in PD patients. The origin of these abnormalities is quite complex and, to date, two mechanisms have been proposed: *i)* ENS changes mainly in the myenteric plexus associated with LBs and LNs with or without neuronal loss;^{11, 25} and *ii)* an altered extrinsic nerve input to the lower gut, thereby affecting the colonic and anorectal sensory-motor function.²⁶ In the human colonic submucosa, using specific antibodies to phosphorylated α -synuclein, Lebouvier et al. showed LNs (but not LBs) in about 72% of PD/CC cases. The number of LNs positively correlated with the severity of Rome III defined PD/CC patients, although such correlation was lost when data were adjusted by age.¹⁵ The impact of LNs on altered gut physiology (transit time and sensory-motor function) remains plausible, but not yet proven. Thus, Lewy pathology may be a marker of PD involvement in the ENS, although its actual role in terms of gut dysfunction remains to be assessed. Finally, it should be stressed that the identification of Lewy pathology does not represent a reliable bio-marker of PD as strong evidence points to its demonstration in the nervous system (either central or peripheral) in patients with a variety of neurological disorders,²⁷ as well as ageing people⁴ and even in healthy subjects.²⁸

In our study, we used a recently established methodology of obtaining deep biopsies including the mucosa and submucosa during colonoscopy to obtain a submucosal

preparation with the submucosal ganglia.¹⁵ The submucosa contains secretomotor / vasomotor neurons innervating mucosa and vasculature.^{29–30} We have found that both PD/CC and CC patients have a significantly reduced expression of VIP, as well its receptors, at both the protein and mRNA level, whereas there were no significant changes in the expression of ChAT, a marker of cholinergic neurons. Both VIP and acetylcholine are critical signaling molecules for the regulation of colonic secretion and fluid movements^{30–31} and are largely co-stored in enteric, specifically myenteric, neurons of the human GI tract. In the submucosal plexus of the human colon, however, a considerable number of VIP containing neurons are distinct from those containing acetylcholine.³² Upon release from submucosal neurons, VIP activates the specific constitutive receptor, VIPR1, expressed by the enteric epithelial lining. This results in a cAMP-related HCO₃⁻ excretion and movement of Na⁺ and H₂O into the gut lumen, thereby enhancing fluid secretion.³³ The proportion of neurons immunoreactive for VIP was decreased, although not significantly in PD/CC patients *vs.* CC patients; however, the decrease in VIP mRNA and related receptors, VIPR1 and VIPR2, was significantly higher in PD/CC than in CC patients and controls. The decrease of VIP immunoreactive neurons in the absence of changes in other neuronal populations such as ChAT and in the total number of enteric neurons suggests a downregulation of VIP rather than neuronal loss. This is further evidenced by the concomitant decrease in VIP mRNA levels. The difference in the expression of VIP protein and mRNA may be explained by the difficulty in detecting peptides at the level of cell bodies, due to axonal transport and peptide release.³⁴ The reason(s) for the VIP downregulation remain(s) unknown. Given the crucial regulatory role exerted by VIP in gut physiology, it is reasonable to speculate that this peptide can be selectively targeted by PD.

When comparing the PD/CC and CC patients with slow transit, without taking into consideration the anorectal alteration, our data indicated a trend toward reduced VIP mRNA expression and a significant downregulation of VIPR1 and VIPR2 in slow transit PD/CC *vs.* CC. Overall, these findings suggest that downregulation of VIP and VIPR expression are more prominent in slow transit PD/CC than slow transit CC. The observation that human submucosal neurons project to the muscle layers in addition to the mucosa³⁵ raises the possibility that a downregulation of VIP, which inhibits GI smooth muscle, alters the normal reflex motility pattern, thus contributing to transit and anorectal abnormalities. Also, VIP containing neurons bear some important neurobiological implications. For example, whether VIP containing enteric neurons are a selective target of α -synuclein aggregation (and thereby degeneration) in PD patients remains to be determined. The identification of LNs in VIPergic neurons of the gut of parkinsonian patients supports this possibility.^{36–37}

The downregulation of VIP and its related receptors does not appear to be a unique feature underlying PD/CC. In fact, CC patients had a reduced expression of VIP, VIPR1 and VIPR2 compared to controls, albeit to a lower extent than PD/CC patients. It is likely that the VIP reduction shown in the investigated PD/CC patients reflects the long-standing nature of their condition which, in turn, contributes to their VIPR1 and VIPR2 downregulation. In addition, the evidence that both VIP receptors are downregulated suggests a generalized impairment of the VIP signaling. Furthermore, inflammatory changes, which are known to occur in the colonic mucosa of both PD/CC³⁸ and CC patients,³⁹ can affect the expression of VIPRs, in particular the inducible VIPR2 isoform. The involvement of other factors in addition to

inflammatory stimuli, in the reduction of VIP, VIPR1 and VIPR2 mRNA expression cannot, however, be excluded. Taken together, our data suggest that a downregulation of VIP and VIPRs may contribute to the pathophysiology of CC in both parkinsonian and non-parkinsonian patients by impairing secretion in addition to inducing colonic and anorectal sensory-motor dysfunction. Secretory abnormalities can affect the composition of the fecal water content thereby leading to hard stools and resultant delayed colonic TT.⁴⁰

In conclusion, our study provides evidence for a downregulation of the VIP system, in addition to sensory-motor colonic and rectal abnormalities in patients with PD/CC. VIP containing neurons represent a major subset of submucosal, mainly secretomotor, neurons thus a decrease in VIP neurons, as well as VIPR1 and VIPR2, might lead to dysfunctional secretory processes. VIP is a major stimulant of fluid secretion, thus a reduced production of VIP is likely to result in reduced fluid secretion, which might account for the development or aggravation of CC in PD patients. Sensory-motor and secretory abnormalities are likely to represent two prominent mechanisms contributing to the pathogenesis of CC particularly in PD patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

AM	anorectal manometry
CC	chronic constipation
ENS	enteric nervous system
GI	gastrointestinal
HY	Hoehn & Yahr
IR	immunoreactive
LBs	Lewy bodies
LN s	Lewy neurites
pChAT	peripheral choline acetyl-transferase
PD	Parkinson's disease

PD/CC	Parkinson's disease with chronic constipation
PGP9.5	protein gene product9.5
TT	colonic transit time
UPDRS	unified Parkinson's disease rating scale
VIP	vasoactive intestinal polypeptide
VIPR1	VIP receptor 1
VIPR2	VIP receptor 2

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Key Points

- Chronic constipation is usually a severe gastrointestinal dysfunction in patients with Parkinson's disease, but its pathogenesis remains poorly understood.
- Colonic motor and rectal sensory functions are impaired in most parkinsonian constipated patients. Compared to controls, they display a decreased number of submucosal secretomotor neurons containing VIP immunoreactivity accompanied by a reduced mRNA expression of VIP and VIP-receptors.
- The identification of molecular targets underlying constipation in parkinsonian patients may pave the way to new therapeutic strategies.

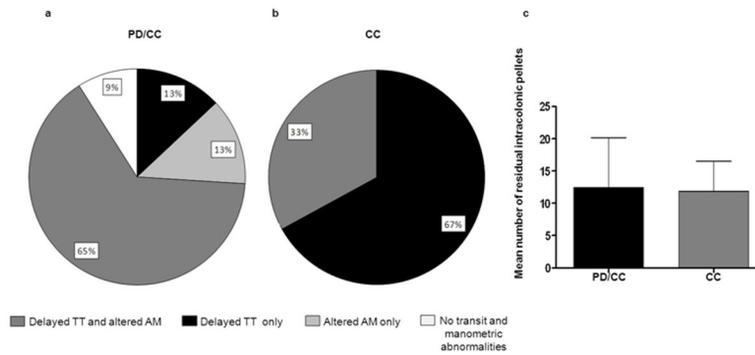


Figure 1. Functional assessment of total colonic transit time (TT) and anorectal manometry (AM) in PD/CC and CC patients
(A) Percentages of PD/CC (n= 23) showing delayed TT and altered AM (65%) (dark gray); delayed TT only (13%) (black); altered AM only (13%) (light gray); and no transit and manometric abnormalities (9%) (white). **(B)** Percentages of CC (n= 9) patients showing delayed TT and altered AM (33%) (dark gray); and delayed TT (67%) (black). **(C)** No differences were detected between PD/CC (black) vs. CC (dark gray) patients in delayed transit as indicated by the mean number of residual intracolonic pellets.

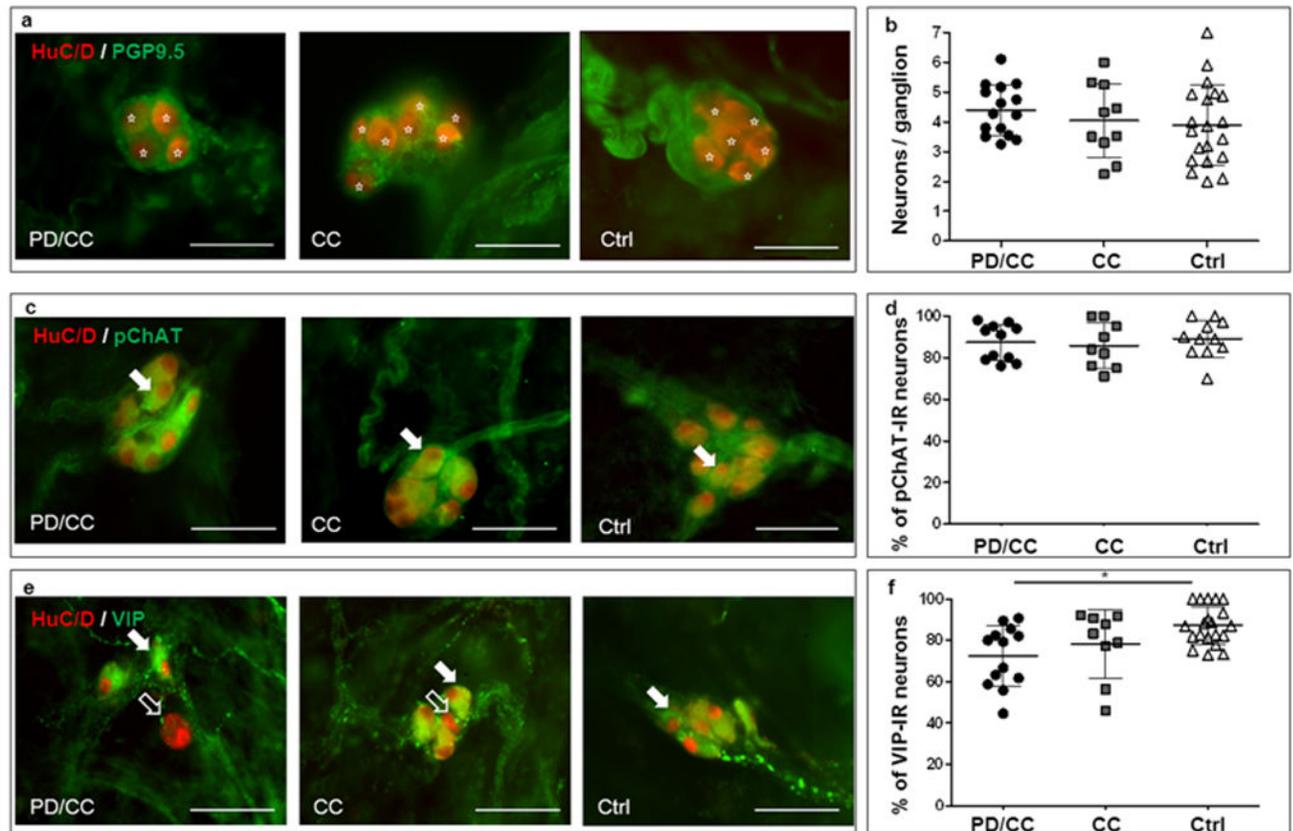


Figure 2. Submucosal neuronal distribution and quantification in PD/CC, CC and control (Ctrl) groups

(A) Photomicrographs showing double-labeling of HuC/D (red) and PGP9.5 (green) in neuronal cell bodies and ganglia. Stars indicate neuronal cell bodies. Scale bar: 50 μ m. (B) Plots represent the mean \pm SD number of neurons/ganglion. (C) Photomicrographs showing HuC/D (red) and pChAT (green) double-labeling identifying cholinergic (pChAT positive) neurons indicated by white filled arrows. Scale bar: 50 μ m. (D) Plots represent the mean \pm SD percentages of HuC/D/pChAT-IR neurons. (E) Photomicrographs showing HuC/D (red) and VIP (green) double-labeling identifying VIP containing neurons indicated by white filled arrows. White empty arrows point to ganglion cell bodies not immunoreactive for VIP. Scale bar: 50 μ m. (F) Plots represent the mean \pm SD percentages of HuC/D/VIP immunoreactive neurons. Note that compared to controls (Ctrl), PD/CC patients have a significantly lower number of VIP containing neurons (* $P < .05$).

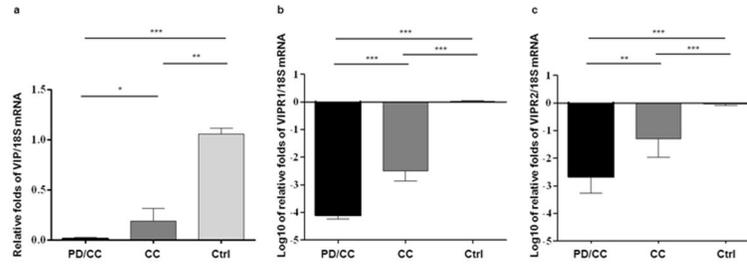


Figure 3. VIP and its receptors, VIPR1 and VIPR2 mRNA expression in PD/CC, CC and control (Ctrl) groups

(A) VIP mRNA levels. Data are expressed as relative folds compared to the mean value of the control (Ctrl) group (mean \pm SD; *** P < .0001; ** P < .001; * P < .05). (B) and (C) VIPR1 and VIPR2 mRNA levels. Data are expressed in logarithmic scale as relative folds compared to the mean value of the Ctrl group (mean \pm SD; ** P < .001; *** P < .0001).

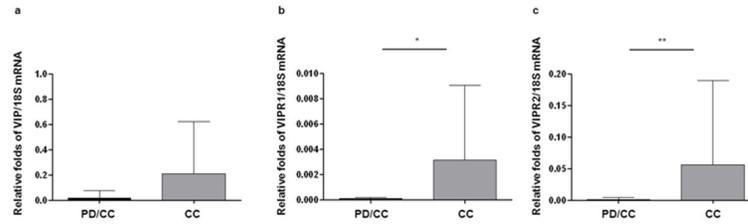


Figure 4. VIP, VIPR1 and VIPR2 mRNA expression in PD/CC vs. CC patients with delayed transit time

(A) VIP mRNA levels were not significantly different in slow transit PD/CC vs. CC patients (mean \pm SD; ns). (B) VIPR1 mRNA levels resulted significantly decreased in PD/CC vs. CC patients ($* P < .05$). (C) VIPR2 mRNA levels resulted significant decreased in PD/CC vs. CC patients ($**P < .001$). Data are expressed as relative folds compared to the mean value of the control (Ctrl) group.

Table 1

Colonic transit time and anorectal manometric features of PD/CC patients.

PD/CC	TT	AM	RP	SQ	SP	S
P1	0	Normal	Normal	Normal	Normal	Normal
P2	-	Altered	Decreased	Normal	Normal	Hypersensitive
P3	15	-	-	-	-	-
P4	0	Normal	Normal	Normal	Normal	Normal
P5	-	Altered	Normal	Normal	Paradoxical increase	Normal
P6	0	Normal	Normal	Normal	Normal	Normal
P7	14	Altered	Normal	Normal	Paradoxical increase	Hypersensitive
P8	20	Altered	Normal	Normal	Paradoxical increase	Normal
P9	19	Altered	Decreased	Defective	Normal	Normal
P10	15	Altered	Normal	Defective	Normal	Normal
P11	0	Altered	Decreased	Normal	Normal	Normal
P12	17	Normal	Normal	Normal	Normal	Normal
P13	19	Altered	Normal	Normal	Paradoxical increase	Normal
P14	20	Altered	Normal	Defective	Normal	Normal
P15	0	Altered	Normal	Normal	Normal	Hypersensitive
P16	17	Altered	Normal	Normal	Paradoxical increase	Normal
P17	17	Altered	Normal	Normal	Normal	Hypersensitive
P18	18	Altered	Normal	Defective	Normal	Normal
P19	-	Altered	Normal	Normal	Paradoxical increase	Hypersensitive
P20	8	Altered	Increased	Normal	Normal	Normal
P21	-	-	-	-	-	-
P22	20	Altered	Normal	Normal	Normal	Hypersensitive
P23	14	Altered	Decreased	Defective	Normal	Normal
P24	15	Altered	Normal	Defective	Normal	Normal
P25	0	Altered	Increased	Normal	Normal	Normal
P26	15	Altered	Normal	Normal	Normal	Hypersensitive
P27	20	Altered	Normal	Normal	Paradoxical increase	Normal
P28	15	Altered	Normal	Normal	Normal	Normal

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PD/CC	TT	AM	RP	SQ	SP	S
P29	-	Altered	Decreased	Normal	Paradoxical increase	Normal

Notes: AM, Anorectal manometry; PD/CC, Parkinson's disease with chronic constipation; RP, resting pressure; S, sensitivity; SQ, squeezing pressure; SP, strain pattern; TT, total colonic transit time (number of pellets).