FEATURED ARTICLE

Alpha-Synuclein in Colonic Submucosa in Early Untreated Parkinson's Disease

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ABSTRACT: The diagnosis of Parkinson's disease rests on motor signs of advanced central dopamine deficiency. There is an urgent need for disease biomarkers. Clinicopathological evidence suggests that α-synuclein aggregation, the pathological signature of Parkinson's disease, can be detected in gastrointestinal tract neurons in Parkinson's disease. We studied whether we could demonstrate α-synuclein pathology in specimens from unprepped flexible sigmoidoscopy of the distal sigmoid colon in early subjects with Parkinson's disease. We also looked for 3-nitrotyrosine, a marker of oxidative stress. Ten subjects with early Parkinson's disease not treated with dopaminergic agents (7 men; median age, 58.5 years; median disease duration, 1.5 years) underwent unprepped flexible sigmoidoscopy with biopsy of the distal sigmoid colon. Immunohistochemistry studies for α-synuclein and 3-nitrotyrosine were performed on biopsy specimens and control specimens from a tissue repository (23 healthy subjects and 23 subjects with inflammatory bowel disease). Nine of 10 Parkinson's disease samples were adequate for study. All showed staining for α-synuclein in nerve fibers in colonic submucosa. No control sample showed this pattern. A few showed light α-synuclein staining in round cells. 3-Nitrotyrosine staining was seen in 87% of Parkinson's disease cases but was not specific for Parkinson's disease. This study suggests a pattern of α-synuclein staining in Parkinson's disease that was distinct from healthy subjects and those with inflammatory bowel disease. The absence of this pattern in subjects with inflammatory bowel disease suggests it is not a sequel of inflammation or oxidative stress. 3-Nitrotyrosine immunostaining was common in all groups studied, suggesting oxidative stress in the colonic submucosa.

Key Words: Parkinson's disease; α-synuclein; colon; biopsy

Parkinson's disease (PD) is a common neurodegenerative disease of aging, affecting as many as 4.6 million persons in the most populated countries of the world. It is projected that nearly 10 million citizens worldwide will be affected in 2030.[1] A diagnosis of PD rests on the clinical recognition of cardinal motor signs including tremor at rest, bradykinesia, rigidity, and postural instability. These motor signs reflect decreased dopaminergic activity in the striatum, occasioned by the loss of dopamine synthetic capacity in and ultimately death of neurons of the midbrain substantia nigra pars compacta.[2] Characteristic pathological changes in affected regions include eosinophilic cytoplasmic neuronal inclusions (Lewy bodies) that stain for α-synuclein (αSYN), as well as αSYN staining in neuronal processes (Lewy neurites).[3–5] The clinical diagnosis can be difficult early in the disease, and as many as 10%–20% of PD patients may be misdiagnosed.[6,7] Moreover, the diagnostic opportunity occurs relatively late in the evolution of PD pathology, when there is already significant cell loss. Estimates are that the characteristic motor signs do not appear until the

Additional Supporting Information may be found in the online version of this article.

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midbrain degenerative process is well advanced, with loss of as many as 40%–60% of dopaminergic neurons at the onset of motor symptoms.[8,9] Structural imaging is not useful in the diagnosis, and there is currently no accepted way to objectively demonstrate the αSYN pathology characteristic of early PD.

Recent evidence suggests that αSYN pathology in PD may begin in the peripheral nervous system, possibly in neurons of the gastrointestinal submucosa.[10] From there, PD has been proposed to spread through contiguous neuronal systems, initially to the medulla oblongata. From the medulla, the disease then spreads rostrally, causing cumulative clinical signs as more numerous regions of the nervous system become affected.[10–12] The ability to reliably demonstrate characteristic αSYN pathology in peripheral tissues could lead to significant advancements in PD research, including improved diagnostic accuracy in patients with motor symptoms, as well as identifying persons with subtle motor or even "premotor" PD when there is a minimal neuronal cell loss, thus the opportunity to prevent life-altering neurological symptoms. αSYN staining has been reported in biopsies of the submucosa of the ascending colon taken during routine screening colonoscopy of subjects with mild to moderate PD, but this procedure requires preparatory colonic cleansing and sedation, and the timing of biopsy is dictated by screening guidelines.[13,14] Furthermore, many of these cases were treated with antiparkinsonian medication, and the effects of these medications on peripherally expressed αSYN are unknown. The objective of this study was to see whether αSYN staining of colonic submucosal neurons can be used as a potential biomarker for early PD. To this end, we determined whether αSYN staining is present in early, untreated PD subjects and whether simple unprepped flexible sigmoidoscopy with biopsy is well tolerated and yields tissue suitable for demonstrating submucosal αSYN. Furthermore, the association between inflammatory processes, oxidative stress, and the expression of αSYN was explored to see if αSYN staining is a simple consequence of chronic inflammation or tissue oxidative stress or is more specific for PD.

Patients and Methods

Subjects

The study was approved by the Institutional Review Board (IRB) of Rush University Medical Center (RUMC) and registered at Clinicaltrials.gov (NCT01155492), and all subjects provided written informed consent. Subjects with PD diagnosed by United Kingdom Parkinson Disease Research Society Brain Bank criteria[6] were recruited from the movement disorders clinic at RUMC. They had mild disability not sufficient to warrant symptomatic therapy with dopamine agonists or levodopa. They were excluded if they had atypical or secondary parkinsonism, had been taking antiplatelet agents or anticoagulants, or had primary gastrointestinal pathology or unstable medical, neurological, or psychiatric illness. Limited unprepped flexible sigmoidoscopy to the distal sigmoid at around 20 cm from the anal verge was performed by 1 of the authors (A.K.). No sedation was required for the procedure, which lasted less than 10 minutes. Nine cold biopsies were obtained from normal-appearing sigmoid colon using biopsy forceps. One sample from each subject was fixed in 10% formalin for 30 days, then cut in paraffin blocks. One sample from each subject was put in optimal cutting temperature mounting media, Tissue Tek, Torrance, CA. The remaining samples were snap-frozen at the time of collection in liquid nitrogen for future use. The formalin-fixed sample was studied with immunohistochemistry for alpha-synuclein and 3-nitrotyrosine and counterstained for enteric neurons with cuprolinic blue dye.

Control Samples

Control samples were taken from the IRB-approved Tissue Repository, Department of Gastroenterology and Nutrition, RUMC. Tissues came from healthy control subjects without a history of gastrointestinal or neurological disease and for the inflammation/oxidative stress control group, from subjects with inflammatory bowel disease (IBD; Crohn's disease and ulcerative colitis). The same exclusion criteria were applied to both control groups. Control samples were fixed in 10% formalin for 30 days, then cut in paraffin blocks. As positive controls for the presence of Lewy bodies, substantia nigra cases were collected from the RUMC brain bank.

Tissue Preparation and Immunostaining

Deparaffinization

Eight-micron-thick sections of colon as well as Lewy-body-positive control sections were deparaffinized using standard procedures. Paraffin was melted at 60°C for 30 minutes and removed from these tissues by rinsing the sections in xylene. Rehydration steps were then performed using different grades of alcohol and then distilled water.

Immunohistochemistry

Sections were processed using an antigen-retrieval procedure. Briefly, the sections were treated with 88% formic acid for 20 minutes, followed by treatment with citric acid (pH, 6.0 at boiling temperature) for 20 minutes, then kept outside at 25°C and processed through 3 washes of distilled water and 3 washes of phosphate-buffered solution (PBS). Sections were incubated in 3% normal horse serum and 2% bovine serum albumin for an hour, then incubated in the primary αSYN (mouse anti-alpha-synuclein, 1:500, 18-0215; Invitrogen, Camarillo, CA) or 3-

TABLE 1. Study subject demographics

	Parkinson's disease (n = 10)	Controls (n = 23)	Crohn's disease (n = 10)	Ulcerative colitis (n = 13)
Age, median (range)	59 (46–79)	54 (36–71)	47 (27–72)	43 (21–62)
Sex (male), n (%)	7 (70%)	12 (52%)	5 (50%)	4 (31%)

nitrotyrosine (3-NT; rabbit polyclonal anti-3-NT, 1:500, 06-284; Millipore, Inc., Temecula, CA) antibody for 48 hours at 4°C. The sections were washed, then sequentially incubated in the secondary antibody (biotinylated horse antimouse, 1:200; Vector Laboratories, Inc., Burlingame, CA), phosphate-buffered saline washes, and ABC solution (ABC kit, Vectastatin Elite, Vector Laboratories, Inc., Burlingame, CA). The stain was completed in a chromogen solution containing 0.05% 3′3-diaminobenzidine and 0.005% hydrogen peroxide. After stain development, the sections were dried overnight, then coverslipped with cytoseal (23244257; Fisher, Fairlawn, NJ). The same procedure was followed for positive control PD substantia nigra sections. Negative control sections followed almost the same procedure except that an irrelevant IgG was substituted for the primary antibody.

Counterstaining of enteric neurons was performed using cuprolinic blue dye (Quinolinic pthalocyanine, 17052; Polysciences Inc., Warrington, PA), as published elsewhere.[15] Briefly, cuprolinic blue solution was prepared by dissolving 0.5% cuprolinic blue in 0.05M sodium acetate buffer (pH, 5.6) to which 1.0M $MgCl_2$ was added. After washing the sample slides in distilled water, the cuprolinic acid solution was filtered through a 0.22-μm Millipore filter onto tissue-mounted slides. The counterstain was performed for 15 minutes, then differentiated with 0.05M sodium acetate buffer (pH, 5.6) to which 1.0M $MgCl_2$ had been added. Finally, the sections were rinsed with PBS and coverslipped.

Immunostained slides were reviewed and rated for the presence and intensity of αSYN and 3-NT staining by a blinded rater (H.D.) according to a 5-point scale, from 0 = no immunostaining to 4 = very intense immunostaining.

Results

Ten subjects (7 men) with early PD participated in the study. They ranged in age from 46 to 79 years (median, 59 years; see Table 1), and duration of disease from symptom onset ranged from 6 months to 8 years (median, 1.5 years) and from diagnosis ranged from 1 to 36 months (median, 7.5 months; see Table 2). No subject had ever been treated with levodopa or direct-acting dopamine agonists, although 1 had been treated with amantadine for 2 weeks at the time of sigmoidoscopy. Control sigmoid tissues included samples from 24 healthy subjects and 23 subjects with IBD (10 Crohn's disease and 13 ulcerative colitis; see Table 1.

The biopsy procedure was brief (<5 minutes), painless, and well tolerated, and there were no reported complications 7 or 30 days after the biopsy.

The tissue sample was too small for immunostaining in 1 PD subject. The 9 remaining PD subjects showed staining for αSYN in the lamina propria of the colonic submucosa in a pattern suggesting nerve fibers (Table 2, Fig. 1A,B; also see Supplemental Data). No sample from healthy or IBD subjects showed this pattern of αSYN staining. Two of 24 normal control subjects (8%) and 3 of 13 ulcerative colitis IBD controls (23%) showed light αSYN staining in round cells of unknown origin. This pattern was quite distinct from that seen in the PD subjects (Fig. 1C,D). Twenty-two of 24 healthy controls, 10 of 13 subjects with ulcerative colitis, and 10 subjects with Crohn's disease showed no αSYN staining in the colonic submucosa (Fig. 1E,F). Counterstaining 2 of the αSYN-positive PD samples with cuprolinic blue confirmed that the visualized αSYN was within neuronal tissues in the colonic submucosa (Fig. 2). There was substantial variability in the intensity of immunostaining among

TABLE 2. α-Synuclein and 3-nitrotyrosine immunostaining in subjects with PD

No.	Age	Sex	Time since onset (y)	Time since diagnosis (mo)	Total UPDRS	HY stage	αSYN	3-NT
1	55	M	4	3	28	2	1+	2+
2	66	M	1	5	27	2	1+	2+
3	56	F	1	9	15	1.5	2+	2+
4	79	M	1	6	20	2	4+	4+
5	75	M	4	1	28	2	Inadequate sample	
6	68	F	0.5	4	24	2	4+	Inadequate sample
7	46	F	2	12	16	1	4+	4+
8	47	M	8	12	18	2	4+	4+
9	61	M	1	6	18	2	4+	4+
10	57	M	2	18	28	2	3+	4+
Median (range)	59 (46–79)		1.5 (0.5–8)	7.5 (1–36)	22 (15–28)	2 (1–2)		

αSYN, alpha-synuclein; HY, Hoehn & Yahr; 3-NT, 3-nitrotyrosine; UPDRS, Unified Parkinson's Disease Rating Scale.

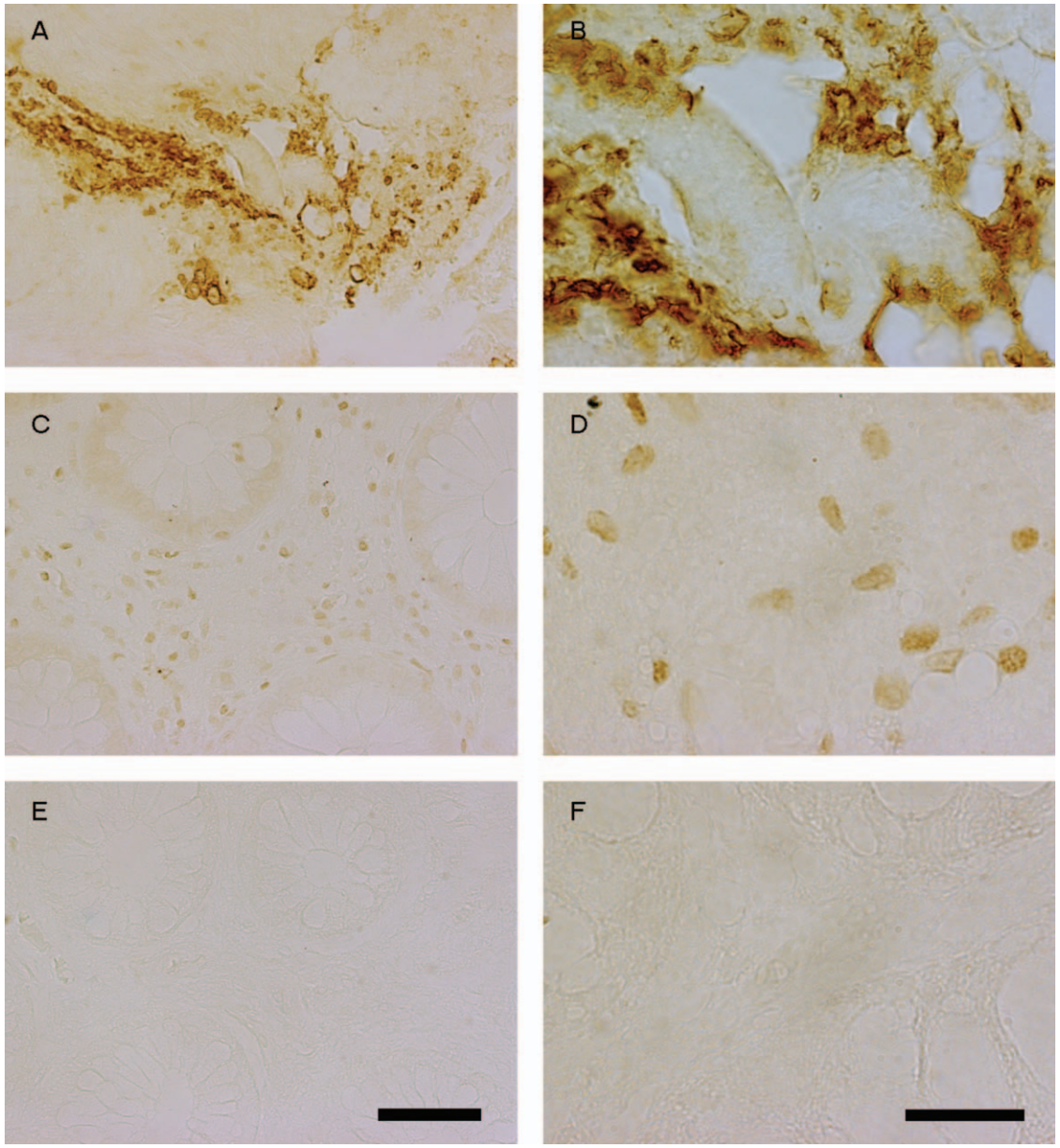

FIG. 1. αSYN immunohistochemistry of colon at 40× magnification (**A, C, E**) and 120× magnification (**B, D, F**). Nerve fiber staining was seen in PD subjects (**A, B**). Some subjects with inflammatory bowel disease showed a pattern of immunostaining in round cells of unknown origin (**C, D**). Controls and most subjects with inflammatory bowel disease showed no α-synuclein immunostaining (**E, F**). Scale bar in **E** and **F** represents 50 and 20 μm and applies to **A, C, E** and **B, D, F**, respectively.

different sections from the same biopsy and among the different PD subjects (see Supplemental Fig. 1).

To determine whether αSYN-positive neuritic staining is associated with oxidative stress, sigmoid samples were also stained for 3-NT. In 2 of 10 PD subjects, the tissue sample was inadequate for 3-NT staining. Seven of 8 PD subjects with adequate samples showed positive staining for 3-NT (Fig. 3B,D). 3-NT immunostaining was also present in 14 of 24 normal controls, 7 of 10 Crohn's disease colons, and 9 of 13 ulcerative colitis colons.

Discussion

The ultimate goal of translational research in PD is to find a neuroprotective therapy that will slow, stop, or reverse progressive neurodegeneration. Attempts to demonstrate such a therapy have been stymied by the inability to diagnose PD accurately and at a time early in the course when there are a sizable number of surviving dopaminergic neurons.

Clinical and clinicopathological data support the concept that the gastrointestinal tract might be a portal of pathogen entry in at least some patients with PD.[10,16] PD patients often report long-standing constipation, which begins before motor signs in nearly half of all PD subjects[17] and predates motor signs by 12–18 years.[17,18] Long-term epidemiological cohort studies reinforce this notion. In the Honolulu-Asia Aging study of more than 8000 men of Japanese ancestry

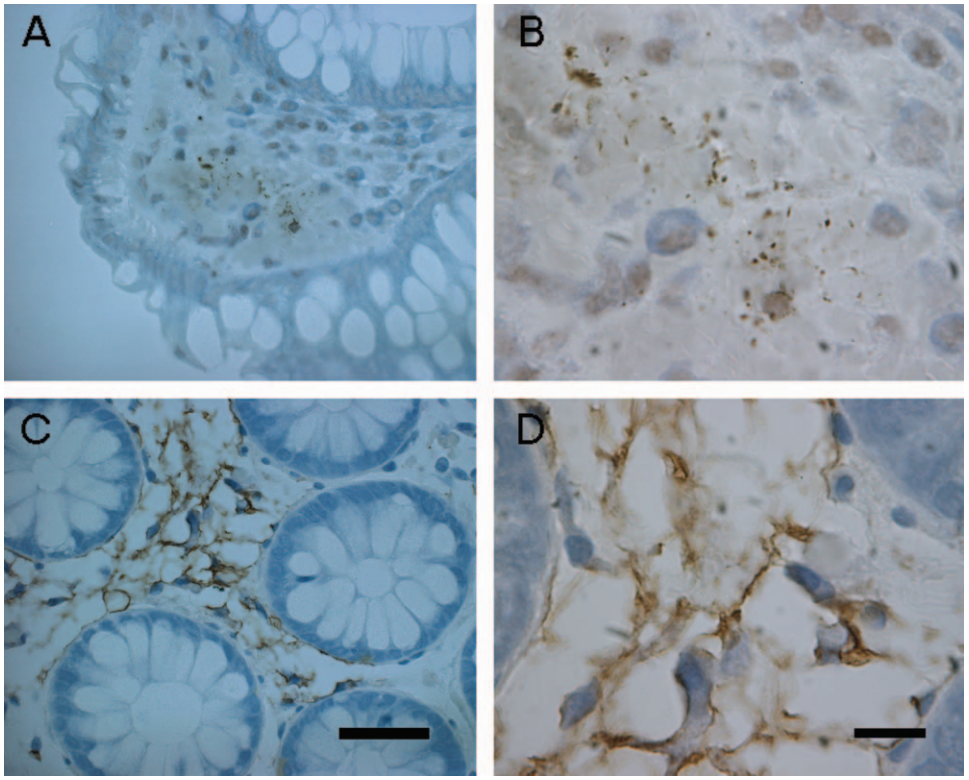

FIG. 2. αSYN and cuprolinic blue double label of colon at 60× magnification (**A, C**) and 120× magnification (**B, D**). αSYN staining was seen in nerve fibers as well as cuprolinic blue–stained neurons in PD subjects. Scale bar in **C** represents 30 μm and in **D** represents 10 μm and applies to **A, C** and **B, D,** respectively.

living in Hawaii, men with fewer bowel movements had a higher risk of PD compared with men with more normal bowel function (OR, 2.7; 95% CI, 1.3–5.5; $P = .007$), with an average time until diagnosis of 12 years.[11] αSYN-related pathology has been demonstrated in the submucosa of the gastric cardia in autopsy cases with incidental Lewy bodies.[16,19] In a series of 5 subjects with moderate PD of greater than 5 years' duration undergoing screening colonoscopy, 4 showed deposits of αSYN in the ascending colon.[13] Submucosal αSYN immunostaining was demonstrated in 21 of 29 subjects with PD in various stages undergoing routine screening colonoscopy using an antibody against phosphorylated αSYN.[14]

We have shown that it is possible to demonstrate the presence of αSYN in submucosal neuronal structures of the distal sigmoid colon in early, untreated PD subjects using a simple procedure. Tissue is obtained from the distal colon approximately 20 cm from the anal verge using a flexible sigmoidoscope and cold biopsy forceps. No colon cleansing is required, and the procedure can be performed in 5–10 minutes without the need for sedation. Although none of our subjects had any complications, published series suggest a complication rate of 0.15%.[20] We used an antibody that was not specific for phosphorylated αSYN, which may have increased the sensitivity in our study because αSYN was detected in all usable specimens compared with 70% of specimens stained with the more specific antibody.[14] Although it could be argued that use of the less specific antibody would adversely affect specificity, we found the characteristic pattern of staining was 100% specific for PD because no control showed this pattern. Our technique of unprepped distal sigmoidoscopy may be more desirable than obtaining tissue incidental to screening colonoscopy. Because there is no need for colon preparation or anesthesia and the procedure is well tolerated, it lends itself to testing at times determined by the appearance or progression of the neurological disease or to repeated assessments dictated by a study protocol, rather than at those times dictated by scheduled preventive screening.[20] Unprepped sigmoidoscopy yielded useful tissue in 90% of subjects in this small cohort. Every PD subject with adequate tissue showed a pattern of αSYN deposition in submucosal neurites. Healthy controls and subjects with inflammatory bowel disease of the small or large intestine did not show this pattern, although some showed evidence of light αSYN in round cells of unknown origin. There was considerable variability in the intensity of αSYN immunostaining between different sections taken from the same biopsy specimen and among different PD subjects. Thus, it may be difficult to standardize this analysis if performed in different laboratories, and

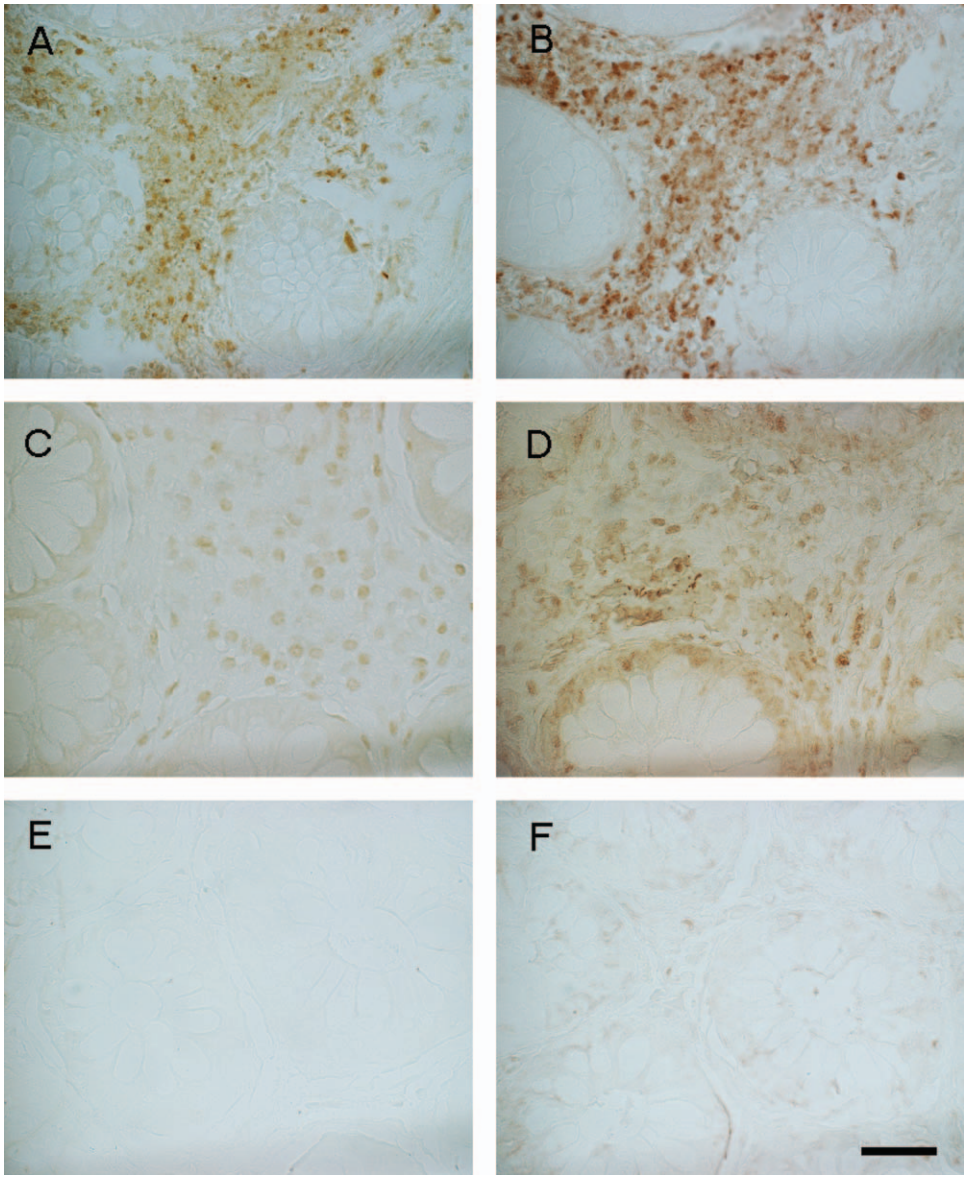

FIG. 3. Sixty times magnification of αSYN (**A, C, E**) and 3-NT (**B, D, F**) immunohistochemistry of colon in PD (**A, B**), inflammatory bowel disease (**C, D**), and control (**E, F**) subjects. Scale bar in **F** represents 30 μm and applies to **A–F**.

additional study will be required to replicate these study findings. The mechanism by which αSYN deposits within submucosal neurites remains to be established. Our initial hypothesis was that inflammatory processes might induce αSYN expression. However, our data argue against this hypothesis because general inflammatory processes in our inflammatory bowel disease control group did not elicit αSYN in submucosal neuritis. Were αSYN expression simply a result of inflammatory processes, its expression would be expected in cases with IBD, which did not occur. Another possible mechanism is oxidative stress. Oxidative injury has been proposed as a mechanism of neuronal cell death in several neurological disorders including PD.[21] Our finding of positive staining for 3-NT in the submucosal neurons supports an important role of oxidative stress in inducing αSYN expression in neuronal cell bodies and processes. However, lack of αSYN expression in neurites in patients with IBD despite the presence of the marker of oxidative stress indicates that a simple oxidative milieu is not sufficient to induce αSYN expression in neurites. Thus, alternative mechanisms must be at play and need further study.

Further study will be required to determine if this technique can be replicated in other laboratories and if this pattern is specific to PD or can be seen in atypical parkinsonism or other neurological disorders. If it can be shown that this pattern is specific to PD, it may be a useful tool to confirm the diagnosis of early PD or to study subjects who have clinical abnormalities now thought to predict the subsequent

development of PD (premotor PD). These premotor clinical abnormalities may include excessive daytime sleepiness, laxative-resistant constipation, anosmia, and rapid eye movement sleep behavior disorder.[11,16,22–24] In view of a multi-billion-dollar translational research effort that aims to identify agents that slow or stop the progression of PD, the need for accurate and timely diagnostic biomarkers, including the potential for premotor diagnosis, is particularly acute. ■

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