

Distribution of α -synuclein in normal human jejunum and its relations with the chemosensory and neuroendocrine system

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ABSTRACT

Alpha-synuclein (α -syn) is a presynaptic neuronal protein and its structural alterations play an important role in the pathogenesis of neurodegenerative diseases, such as Parkinson's disease (PD). It has been originally described in the brain and aggregated α -syn has also been found in the peripheral nerves including the enteric nervous system (ENS) of PD patients. ENS is a network of neurons and glia found in the gut wall which controls gastrointestinal function independently from the central nervous system. Moreover, two types of epithelial cells are crucial in the creation of an interface between the lumen and the ENS: they are the tuft cells and the enteroendocrine cells (EECs). In addition, the abundant enteric glial cells (EGCs) in the intestinal mucosa play a key role in controlling the intestinal epithelial barrier. Our aim was to localize and characterize the presence of α -syn in the normal human jejunal wall. Surgical specimens of proximal jejunum were collected from patients submitted to pancreaticoduodenectomy and intestinal sections underwent immunohistochemical procedure. Alpha-syn has been found both at the level of the ENS and the epithelial cells. To characterize α -syn immunoreactive epithelial cells, we used markers such as choline acetyltransferase (ChAT), useful for the identification of tuft cells. Then we evaluated the co-presence of α -syn with serotonin (5-HT), expressed in EECs. Finally, we used the low-affinity nerve growth factor receptor (p75NTR), to detect peripheral EGCs. The presence of α -syn has been demonstrated in EECs, but not in the tuft cells. Additionally, p75NTR has been highlighted in EECs of the mucosal layer and co-localized with α -syn in EECs but not with ChAT-positive cells. These findings suggest that α -syn could play a possible role in synaptic transmission of the ENS and may contribute to maintain the integrity of the epithelial barrier of the small intestine through EECs.

Key words: Small intestine; jejunum; tuft cells; enteroendocrine cells (EECs); α -synuclein (α -syn); enteric

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Contributions: AC, worked on the study design, performed morphological and immunohistochemical experiments and wrote the paper; RM, captured pictures, interpreted the results and wrote the paper, formatting the manuscript as an article; CLM, worked in the preliminary research reports about α -synuclein, helped in the experimental and revisional aspects; LP, PO, monitored the experiments and performed a critical revision of the manuscript; PC, collected the several samples; RV, coordinated the work, interpreted the results and performed a critical revision of the manuscript.

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Introduction

Alpha-synuclein (α -syn) is a 140 amino acid protein, belonging to the synuclein family, expressed in mammalian neurons. Alpha-syn has been first cloned and isolated from the electric organ of *Torpedo californica*¹ and localized at the level of the presynaptic nerve terminals and in the nuclear envelope.² The normal functions of α -syn are not entirely known, but its high concentration in presynaptic terminals suggests a role in the maintenance of synaptic vesicles pools and in neurotransmitter release.³⁻⁵ Structural alterations of α -syn as well as its overexpression have been related to the onset and the progression of several human neurodegenerative diseases, known as synucleinopathies.⁶⁻¹¹ In fact, α -syn aggregates are the main component of the Lewy bodies (Lbs), considered as pathological hallmarks of synucleinopathies.^{6,12} Abnormal processing of α -syn leads to pathological changes in its binding properties with overexpression and accumulation of α -syn resulting in compromised synaptic function and axonal transport.^{13,14}

The jejunum is composed of repeating crypts and villi with an important niche of stem cells to sustain the rapid turnover of its epithelium.¹⁵ The intestinal stem cells (ISCs) are located at the base of the crypts and produce five different cell types: enterocytes, which are the real absorptive cells, the goblet cells, which secrete mucus into the lumen, the tuft cells, which are considered luminal sensory cells, the enteroendocrine cells (EECs), which are able to produce several hormones, and Paneth cells with antimicrobial activity.¹⁶ Some of these cell types are crucial in the interactions between the intestinal epithelium and the enteric nervous system (ENS), forming an effective interface between the lumen and nerve fibers.^{17,18} These are the tuft cells and EECs. In particular, the tuft cells or brush cells, characterized by a fusiform shape with apical and long microvilli, can transfer sensory signals from the lumen to other epithelial cells or neurons.¹⁹ Tuft cells express choline acetyltransferase (ChAT), responsible for acetylcholine (ACh) production, and secrete cytokines and cyclooxygenase to regulate inflammatory responses.^{20,21} They are part of the diffuse chemosensory system (DCS), first described by Sbarbati *et al.*^{22,23} DCS might be thought to be involved in a wide range of diseases of both the respiratory and digestive apparatuses, as well as in systemic diseases.²⁴ A renewed interest in these tuft cells is linked to the involvement of the olfactory and digestive system by COVID-19.²⁵ Furthermore, also EECs have been linked not only to the hormone production but also to the chemosensory aspects. In fact, EECs in the mucosal layer have the apical surface open into the intestinal lumen and they can sense the luminal contents.²⁶ EECs contain different peptides and hormones such as 5-HT and express nutrient-sensing GPCRs (G-protein-coupled receptors) regulating the local innervation.^{27,28} Recently, it has been shown that EECs express α -synuclein in mouse and human intestine, discovering a direct transmission from the intestine to the brain through the vagus nerve to the dorsal motor nucleus of the vagus in the brain stem, in a retrograde manner.^{18,29} Remarkably, recent reports have shown that the lesions in the ENS occurred at a very early stage of the PD disease, even before the involvement of the central nervous system. For these reasons, the ENS could be critical in the pathophysiology of Parkinson's disease (PD).^{30,31} In such a scenario, the enteric glial reaction observed in PD may play a key role by modulating intestinal permeability as greater gut permeability has been observed in experimental parkinsonism.³² Although Lbs, as well as α -syn pathological aggregates, have been observed throughout the autonomic nervous system projecting to the gut of patients affected by PD^{30,33-36} or other neurodegenerative diseases.^{31,37} For that reason, knowledge about the normal distribution of α -syn could lead to a better understanding of its biological activity and its precise role in neurodegeneration, so the aim of the

present study was to analyze the distribution pattern of α -syn in the proximal tract of human normal small intestine.

Materials and Methods

All reagents were obtained from Dako-Agilent (Santa Clara, CA, USA) unless otherwise stated. The antibodies for ChAT, 5-HT and p75NT receptor were obtained from Merk Millipore (Temecula, CA). The antibodies for α -syn, together with Ultracruz aqueous mounting medium with DAPI were produced by Santa Cruz Biotechnology (Santa Cruz, CA, USA).

All the specific secondary antibodies AlexaFluor were obtained from Invitrogen, Life Technologies Ltd. (Paisley, UK).

Patients and tissues

Specimens of intestinal wall were collected from the 2nd jejunal loop (30 cm from the duodenojejunal flexure). Histological samples were obtained from surgical specimens from ten patients (seven males, three females) within the age range of 45 to 84 years, submitted to a pancreaticoduodenectomy for pancreatic cancer. All patients gave informed consent to the study as part of the research investigation for gastrointestinal tract disorders.

Tissue specimens were fixed with cold 4% paraformaldehyde solution in 0.01 M saline phosphate buffer (PBS) for 24-48 h at +4°C, washed, dehydrated, paraffin embedded and cut into 5-7 μ m thick serial sections which were mounted on albumin coated slides.

The study has previously received ethical approval by the Institutional Review Board of the "Sapienza" University of Rome in accordance with the Declaration of Helsinki.

Hematoxylin-eosin (H&E) staining and immunohistochemistry

Morphological analysis was performed using H&E staining. Firstly, sections of human jejunum were colored with Hematoxylin solution for 10 min and then washed in tap water for other 10 min. After washing, we performed the passage in Eosin for 1 min and deoxygenated water. Before mounting the slides with a specific mounting medium, the samples were dehydrated with ethanol of different concentration gradients and xylene.

For immunohistochemistry after deparaffinization, rehydration and wash in 1x phosphate buffered saline (PBS), sections were pre-treated for 20 minutes at room temperature with H₂O₂, to inactivate the endogenous peroxidase activity. Then, sections were incubated with antibodies for: i) mouse anti- α -syn (sc-58480 Santa Cruz Biotechnology, Santa Cruz, CA) 1: 250; ii) goat anti-ChAT (AB144P Merk Millipore, Temecula, CA) 1:100; or iii) rabbit anti-5-HT (AB938 Merk Millipore, Temecula, CA, USA) 1: 500 overnight at 4°C. The day after, samples were rinsed twice with 1x PBS for 5 min, incubated for 20 min at room temperature with secondary biotinylated antibody (LSAB+ System-HRP, code K0690; Dako-Agilent, Glostrup, Denmark) and then with Streptavidin-HRP (LSAB+ System-HRP, code K0690, Dako-Agilent). Diaminobenzidine (DAB, Dako-Agilent) was used as substrate, and some sections were counterstained with hematoxylin. In some sections the localization of peroxidase activity was visualized by reacting the sections for 3 min at room temperature with a solution containing 0.04% 3-3'-diaminobenzidine tetrahydrochloride (DAB; Fluka, Buchs, Switzerland), 0.4% nickel ammonium sulfate, and 0.003% H₂O₂ in 0.05 M Tris-HCl buffer, pH 7.6 giving a dark blue granular precipitate. Then sections were counterstained with the Nuclear Fast Red (Kernechtrot) solution (Cat# 368458-500G, Sigma-Aldrich, St. Louis, MO, USA) after IHC procedure. Intestinal sections were visualized and examined using the light microscope Leica Microsystems DM 4500.

Immunofluorescence

The expression of α -syn, ChAT, 5-HT and p75NTR receptor was evaluated by immunofluorescence (IF) in human jejunum samples from normal patients. For IF, non-specific protein binding was blocked by 5% bovine serum albumin (BSA). Specimens were incubated with the previous primary antibodies and rabbit anti-p75NTR (AB1554, Merk Millipore, Temecula, CA). Then, specimens were washed and incubated for 1 h at room temperature with labeled isotype-specific secondary antibodies (AlexaFluor-488 or 594 respectively anti-mouse, anti-goat and anti-rabbit, Invitrogen, Life Technologies Ltd.) and counterstained with 4,6-diamidino-2-phenylindole (DAPI) (Ultracruz aqueous mounting medium with DAPI, sc.24941, Santa Cruz Biotechnology) for visualization of cell nuclei.

Image acquisition and processing

Images were visualized using Leica Microsystems DM 4500 B Fluorescence Microscopy (Wetzlar, Germany) equipped with a JenoptikProg Res C10 Plus Videocam (Jena, Germany) or using an AX70 Provis microscope (Olympus Optical, Tokyo, Japan) with a cooled CCD digital camera (Spot, Diagnostic Instruments, Sterling Heights, MI, USA). Images were captured using the IAS 2000 software (Delta Sistemi, Rome, Italy) and saved as .tiff files. Images were digitally processed in Adobe Photoshop CS5 (San Jose, CA, USA). Only general contrast adaptations were made, and figures were not otherwise manipulated. The final figure composition was done using Microsoft Office 2020 Powerpoint software (Redmond, WA, USA).

Results

Morphological analysis of the jejunal wall

From the inner to the outer side, the wall of normal human jejunum is characteristically made up of the mucosa, submucosa, muscularis externa and serosa (Figure 1A). The mucosa contains crypts and villi, and it is formed by a single layer of epithelial columnar cells. But we can find at least other two subpopulations: i) cells with a secretory capacity, such as the goblet cells (Figure 1A, blue arrows); and ii) specialized signaling cells, such as the EECs (Figure 1B, green cells) and the tuft cells (Figure 1B, red cells).¹⁶ Since the intestinal epithelium has a rapid turnover, it also contains a niche of intestinal stem cells (ISC) that are responsible for ongoing epithelial regeneration and are located at the base of the crypts of Lieberkuhn (Figure 1A yellow arrows).³⁸ We also took into consideration the tunica muscularis externa with the typical inner circular (CM) and outer longitudinal (LM) layers of smooth muscle, controlled by ganglion cells and nerve fibers of the submucosal (Meissner's) plexus and the myenteric (Auerbach's) plexus (Figure 1A, green arrows).

α -syn distribution in the jejunal wall

We found a pattern of α -syn expression consistent with previous and recent studies. It is widely expressed in the intestinal wall, in particular at the level of the enteric innervation.¹⁸ In fact, we found α -syn immunoreactivity between the glands at the base of the jejunal epithelium (Figure 2A, black arrows). In addition, the presence of α -syn is much evident in the ganglia of the myenteric plexus, where axons project and travel to the mucosal villi within the normal gut wall (Figure 2B, white arrows). Frequently, α -syn not only was present in the ganglion with dendritic branches of the

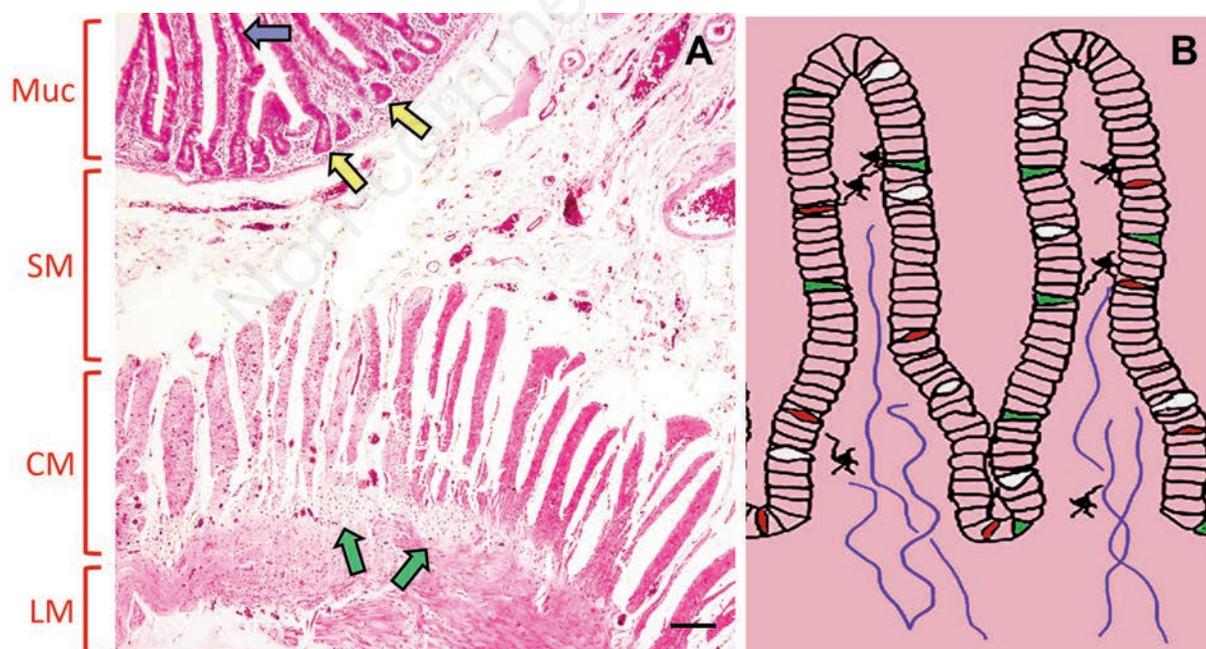


Figure 1. A) Morphological aspects of a normal human jejunum through H&E routinely staining; in this representative picture is shown the typical aspect of the jejunal wall with the mucosa (Muc), submucosa (SM), muscularis with the inner circular layer (CM) and the outer longitudinal layer (LM); in the mucosa are visible plentiful enterocytes and goblet cells (blue arrows); at the base of the crypts of Lieberkuhn (yellow arrows) there are intestinal stem cells niche; in the muscularis, between the double layer are present characteristic ganglia of Auerbach's plexus (green arrows); original magnification: 10x; scale bar :100 μ m. B) Schematic drawings of jejunal mucosa; both nerve fibers and EGCs are in close contact with epithelial cells; green, EECs; red, tuft cells; white, goblet cells; black, EGCs; blue: nerve fibers.

intrinsic neurons, but also encircled it in a perfect way, as shown in the IF of Figure 2B. α -Syn is not only present in the enteric nerves, but also in the epithelial mucosal cells. In addition, we found that α -syn is not expressed by the enterocytes.

α -syn and ChAT expression in the jejunal epithelium

To determine if α -syn is present in other mucosal subpopula-

tions, we have used specific markers. The first one tested, was ChAT, the enzyme choline acetyltransferase important to produce ACh and broadly utilized as a marker for the cholinergic tuft cells.³⁹ Through IHC, we have analyzed the expression of ChAT in normal jejunal epithelium, and we have localized the presence of the enzyme at the level of tuft cells (Figure 3A, red arrows). Then, using a double IF, we have studied the expression of ChAT together with α -syn to find

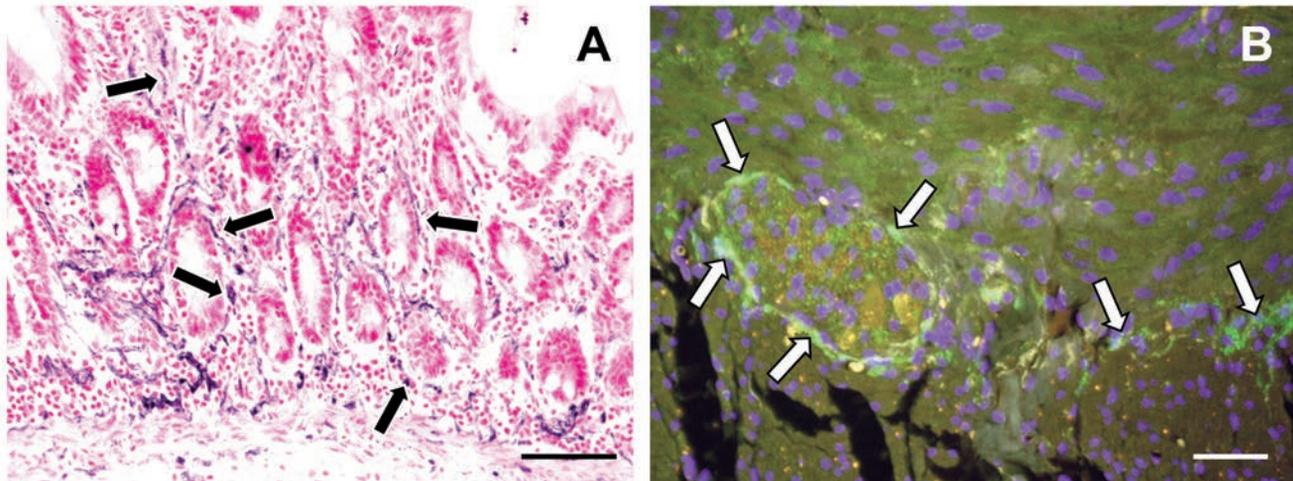


Figure 2. Immunohistochemistry (A) and immunofluorescence (B) in paraffin sections of normal human jejunum showing the typical expression of α -syn. A) α -syn varicose nerve fibers are evident in dark blue, and it are widespread in the glands at the base of the villi (black arrows); original magnification: 10x; scale bar: 100 μ m. B) α -syn is stained with a green fluorochrome at the level of a myenteric ganglion, and it continues in the chain of connection between ganglia (white arrows); original magnification: 10x; scale bar: 50 μ m.

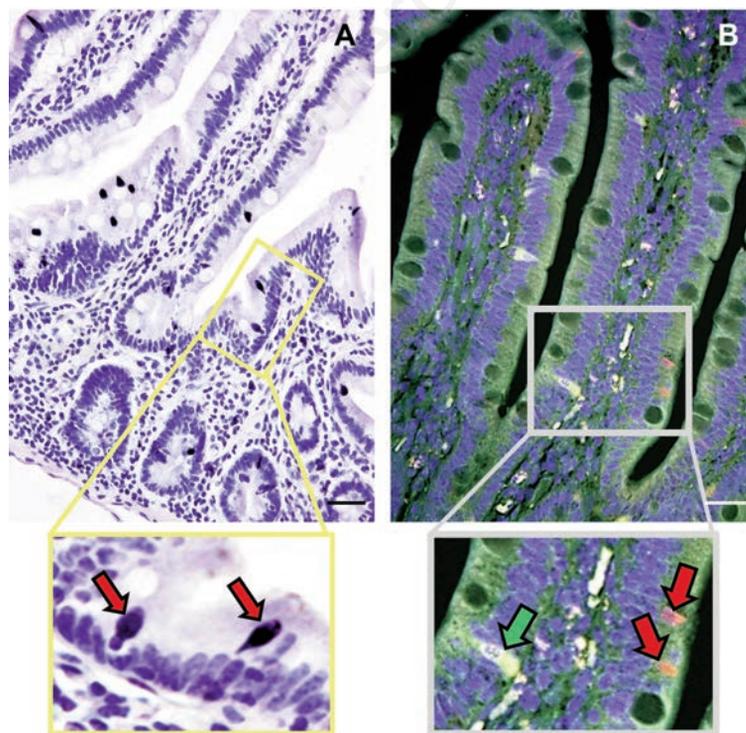


Figure 3. A) Representative immunoreaction of normal jejunal tissue for the presence of ChAT (dark blue); the expression of ChAT has been widely studied on tuft cells, that are considered part of the non-neuronal cholinergic system; for that reason, the rare cells stained in dark blue in mucosal epithelium are tuft cells; in the high-magnification field, the positive cells are more evident (red arrows); original magnification: 10x; scale bar: 50 μ m. B) Double immunofluorescence to identify the expressions of α -syn and ChAT; green arrows indicate scattered α -syn-positive cells, whereas red arrows point ChAT-positive cells; they do not co-localize in the same epithelial cell, meaning that different mucosal cells express differentially α -syn and ChAT; original magnification: 10x; scale bar: 50 μ m.

that the two antibodies did not co-localize in the same epithelial cells (Figure 3B). In detail, we have confirmed the immunoreaction of ChAT at the level of tuft cells in normal human jejunum (Figure 3B, red color), while the expression of α -syn was at the level of different occasional mucosal cells (Figure 3B, green color). For that reason, the next step has been to discover which type of cells express α -syn.

Presence of α -syn in EECs and co-localization with p75NTR

Having established the different presence of α -syn compared to ChAT in the mucosal tunica, we have used 5-HT, one of the typical markers of EECs (28). First, we have tested the antibody through IHC, and we have localized 5-HT-containing enterochromaffin cells, which represent the enteroendocrine subpopulation of the mucosal layer (Figure 4A, red arrows). Similarly, to the previous experiments, we have performed a double IF to co-localize α -syn and 5-HT. As shown in human duodenum,¹⁸ also in normal human jejunum α -syn is expressed in EECs (Figure 4B, yellow arrows). In fact, the IF has shown a co-localization with a yellow color in the cytoplasm of some specific cells. Moreover, we have identified a higher expression of α -syn at the base of the EECs, where the terminals of the α -syn-positive enteric nerve are present and could communicate and transmit information in the mucosal layer. To confirm that a relationship between the ENS and the epithelial layer through the EECs exists, we have continued to evaluate another nervous marker: the neurotrophin receptor p75 (p75NTR). In particular, since nerve growth factor (NGF) plays an important role in the intestinal pathophysiology, such as in barrier function

and mobility,⁴⁰ we have investigated a possible cross talk between α -syn and NGF at the level of EECs. Through another double IF, we have displayed a co-staining of α -syn and p75NTR at the level of the EECs (Figure 5, yellow arrows). Also in these experiments, we have observed the main expression of α -syn at the base of the cells, closer to the internal part of the villus, where enteric neuronal branches α -syn-positive are present (small green arrows). In fact, with this double IF we found several portions of the EECs positive independently for α -syn (Figure 5, small green arrows) and p75NTR (Figure 5, small red arrows) inside the villi. In the present figure, we can appreciate a long branch α -syn-positive that from the inner of the villus reaches an EEC (red oval), to confirm the important role of α -syn in the communications between ENS with the EECs.

Presence of ChAT in tuft cells without a co-localization with p75NTR

To complete the study and to exclude or include even a probable participation of tuft cells in the crosstalk with NGF, we co-localized ChAT with p75NTR. But, in normal human jejunum, we found that the tuft cells did not express the NGF receptor (Figure 6). The presence of ChAT was confirmed in the tuft cells (Figure 6, red arrows), but in these cells we did not find a co-localization with p75NTR, excluding the possible communication of the enteric innervation with tuft cells both through α -syn and NGF. Whereas we found other cells that stained in green and were positive for p75NTR (Figure 6, green arrows).

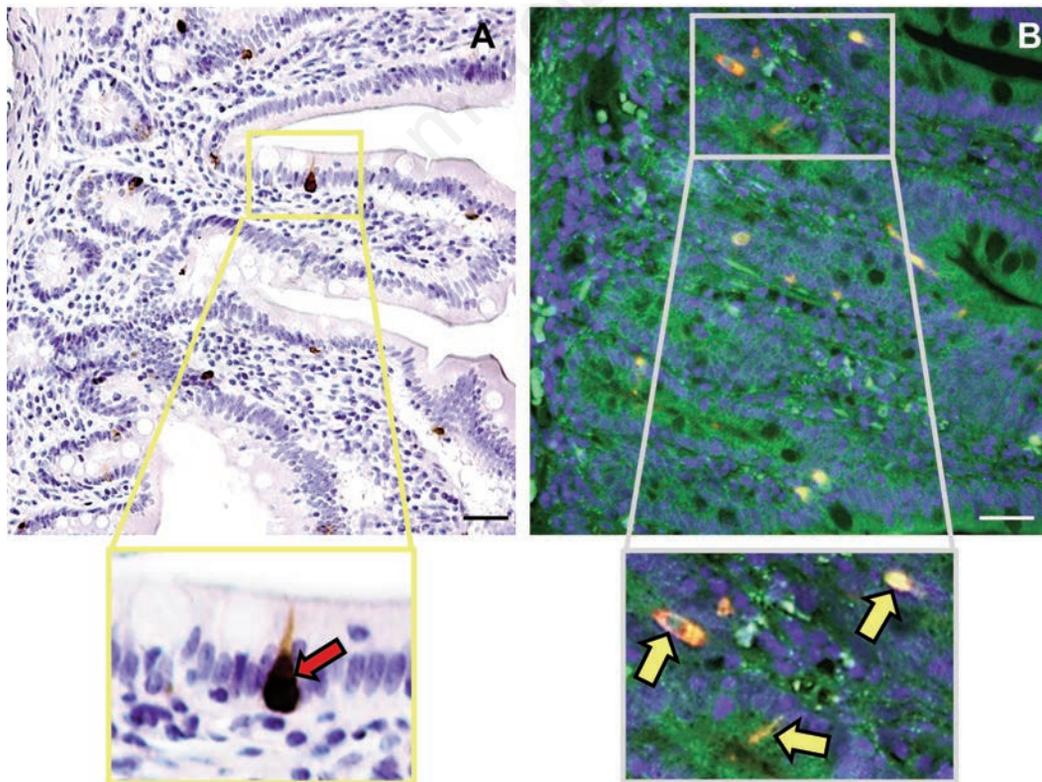


Figure 4. A) IHC to evaluate the presence of 5-HT and its distribution in secreting EECs; paraffin section of human jejunum shows immunoreactive EEC in brown along the mucosal layer (red arrows) with different aspects depending on the typical tridimensional shape of EEC cells; original magnification: 10x; scale bar: 50 μ m. B) Representative double IF for α -syn (in green) and 5-HT (in red); the staining displays co-localization different cells; these yellow cells may represent the enteroendocrine compartment of the small intestine in relation with the nervous control of the jejunum; original magnification: 10x; scale bar: 50 μ m.

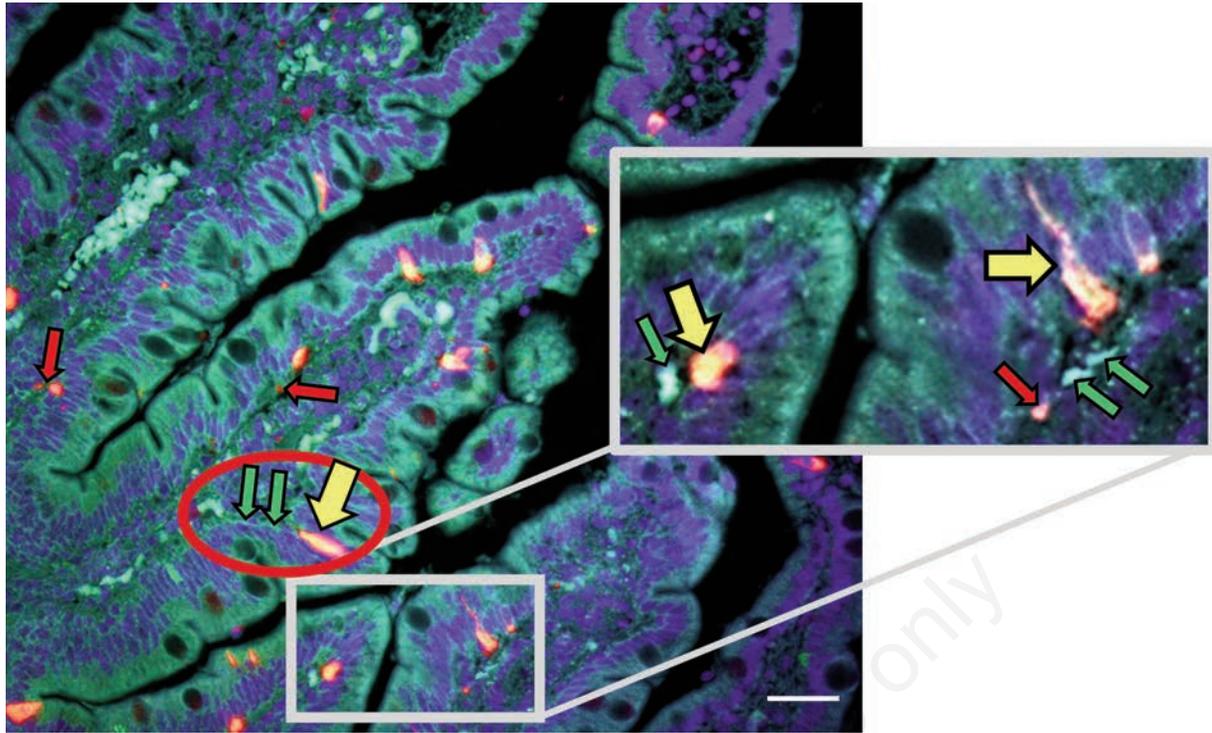


Figure 5. A cross section of human jejunum in which α -syn (in green) and p75 NTR (in red) are co-localized at the level of EEC cells (yellow arrows). The expression of α -syn (green arrows) is also widespread at the base of mucosal layer. In the grey box, at the base of the co-stained cells is evident the green α -syn to support the regulatory function of EEC cells in a normal jejunum between the lumen and the ENS. In the red oval, it is clearly represented a fiber to connect the internal part of the villus with the epithelial cells. A green α -syn-positive branch (small green arrows) runs from the inner side of the villus to the base of a yellow ECC, positive both for α -syn and p75NTR, like an electric wire to its lamp; original magnification: 20x; scale bar: 50 μ m.

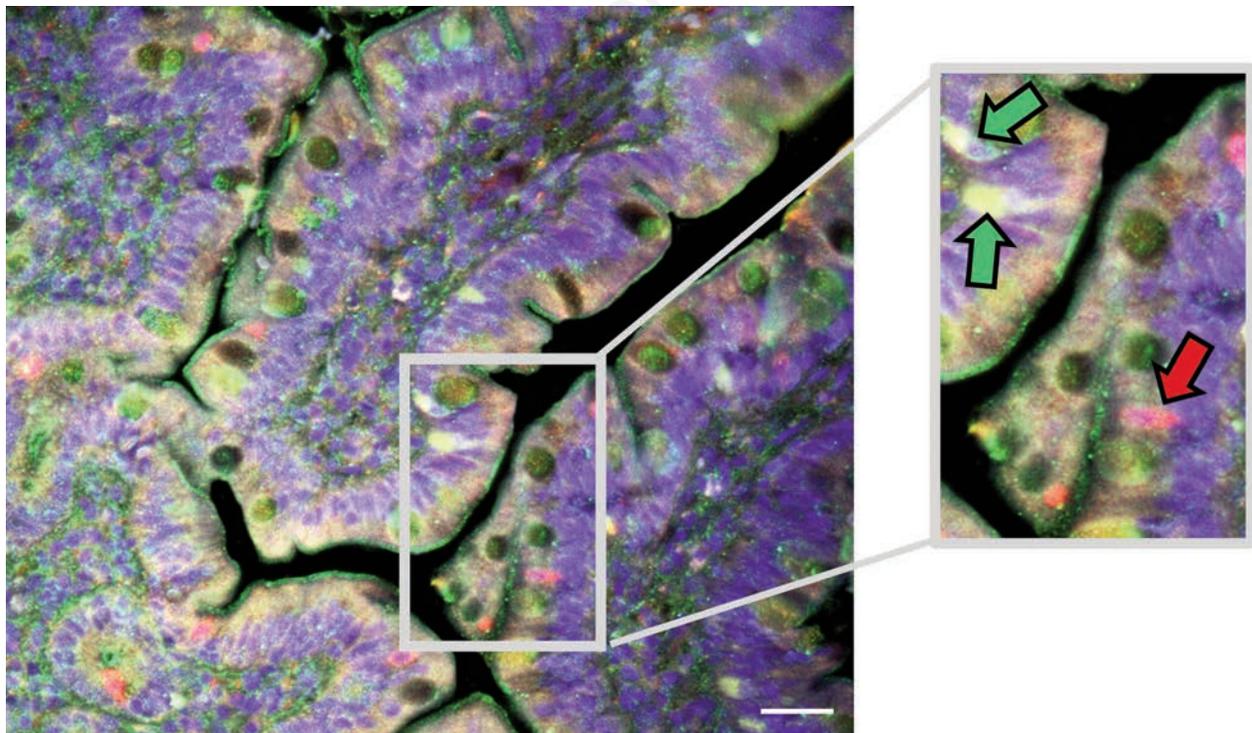


Figure 6. A double IF for p75NTR (in green) and ChAT (in red) to show their different localization. The expression of ChAT is always at the level of the tuft cells, evident in the red cells. Whereas the low-affinity NGF receptor is present in other cells and at the base of the epithelium (green arrows). This picture endorses the previous data showing that the possible communications between the intestinal lumen and the ENS is managed by the α -syn-positive EECs. Tuft cells could act and regulate the communications between ENS and intestinal lumen through another pathway; original magnification: 20x; scale bar: 50 μ m.

Discussion

In the present study, we have investigated the distribution of α -syn in the human normal jejunal wall focusing on the relations between some specific epithelial cells, such as EECs and tuft cells, with enteric nerve fibers and EGCs. Our first results have confirmed the typical localization of α -syn between the glands and in the ganglia of the submucosal plexus.

Additionally, we have shown the presence of scattered EECs expressing α -syn which colocalize with 5-HT in normal human jejunum. But we did not find a similar situation in ChAT-positive tuft cells. Then, we have co-localized p75NTR in α -syn-positive EECs but not in ChAT-positive tuft cells. The gastrointestinal epithelium contains sensory cells, such as tuft and endocrine cells, which can transport luminal signals to the neighboring cells or to the nerve terminals of the ENS. In fact, enteric nerves cannot reach the intestinal lumen and, they do not have a direct contact with the intestinal content but through these sensory cells.^{18,41} In detail, 5-HT containing EECs are the most abundant type of EEC in the intestine.⁴²⁻⁴⁵ They were discovered to possess many neuron-like features including neurofilament-containing axon-like processes called neuropods and neurotrophin receptors, pre- and post-synaptic proteins.⁴⁶ Expression of synaptic proteins raised the possibility that EECs meet nerves, connecting the gut lumen with the nervous system.²⁶ In addition, production and distribution of EECs are strictly linked to the inflammatory process, mediating the connections between the immune, endocrine and nervous systems in the gastrointestinal tract. In fact, colonic inflammation is crucially correlated to changes in the expression of α -syn.⁴⁷ Our data are consistent with other articles, where the presence of α -syn has been discovered in the basal surface of the EECs suggesting that these cells diffused throughout the mucosal lining can sense luminal contents and also connect to enteric nerves.¹⁸ By virtue of their location at the interface of the gut lumen and enteric nerves, EECs may be subject to pathogen or toxin exposure that could affect α -syn.¹⁸ α -syn should misfold in EECs, its transmission to α -syn-containing enteric neurons could be the first step in a prion-like cascade leading to PD. The other intestinal sensory population is represented by the cholinergic tuft cells.^{39,48} Current evidence demonstrated that tuft cells derived ACh contribute to maintain epithelial homeostasis, modulating airway remodeling, regulating reflexes, promoting muscle constriction, inducing neurogenic inflammation, initiating carcinogenesis, and producing ATP, but their mechanism remain still not completely elucidated.³⁹ In fact, ChAT-immunoreactive tuft cells are separate from α -syn-positive EECs, as our results have shown. Both cellular populations seem to be involved in the same functions and activities, but apparently in different ways, and our interest has been to investigate a possible connection between these different type of cells with the ENS.

The intestinal epithelial barrier (IEB) serves as the first boundary of defense between the blood circulation and the luminal environment, blocking the passage of noxious substances.^{49,50} It consists of a continuous monolayer of proliferating and differentiating intestinal epithelial cells, maintained together by intercellular junctional complexes.⁵¹⁻⁵³ The abundant EGCs in the intestinal mucosa play a key role in controlling IEB.⁵⁴ In fact, at the base of the intestinal epithelial cells we have a population of astrocyte-like cells that corresponds to the enteric glia. They can be considered as astrocytes of the central nervous system both for morphological aspects and for the expression of specific markers.⁵⁵ In our study, we found sparse EGCs in the mucosal layer, positive for p75NTR, these cells are in close contact with the α -syn positive and 5-HT-positive cells. In particular, we have displayed the co-localization of α -syn and p75NTR at the level of the EECs, while cholinergic tuft cells did not express the NGF receptor. EGCs serve a function

of structural support to enteric neurons and to constitute large communication networks to integrate neurons and other cells in the gut.⁵⁶ EGCs may play a crucial role in the regulation of epithelial cell proliferation and gut motility. In fact, mucosal EGCs are suggested to be involved in the maintenance of intestinal epithelial barrier, and alterations of their activity occur in different disorders, including PD.⁵⁷ Even if it is so, the underlying mechanisms of how EGCs may influence epithelial cells and the maintenance of intestinal epithelial barrier function remain undefined.⁵⁸ Through our results, we can speculate that the α -syn positive EECs may act as a possible chemosensory link between mucosal EGCs and the intestinal lumen, inducing changes in the intestinal homeostasis at the base of the gut disorders. In the end, our findings show three protagonists of the intestinal changes, anatomically localized in strategic positions: EECs, EGCs and ENS. They play a fundamental role in the pathological staging of gastrointestinal diseases, where the environmental factors can cross the intestinal barrier through the EECs, start the process of enteric neurodegeneration through the EGCs and can spread from the gut to the brain through the ENS. All these activities may be in part regulated through α -syn, as confirmed in some pathological studies with postmortem tissues of the brain and peripheral nervous structures, where it has been found that pathological α -syn nucleation and aggregation may occur in the enteric neurons of the gastrointestinal tract and can propagate from the intestine to the central nervous system.^{30,59-61}

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