ORIGINAL PAPERS

Adv Clin Exp Med 2009, **18**, 3, 243–248
ISSN 1230-025X

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A Population of Zinc Transporter 3-Like Immunoreactive Neurons is Present in the Ganglia of Human Descending Colon

Populacja neuronów immunoreaktywnych wobec trzeciego transportera cynku w śródściennych zwojach nerwowych okrężnicy zstępującej człowieka

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Abstract

Background. Zinc transporter 3 (ZnT3), a member of the SLC30 family of divalent cation transporters, has recently been reported to be present in the terminals of so-called "zinc-enriched neurons" (ZEN) located in both the central and sympathetic peripheral nervous system.
Objectives. The present study was aimed at disclosing whether ZnT3 may also be present in the enteric nervous system (ENS) of human descending colon.
Material and Methods. Tissues were collected from patients undergoing resection of the descending colon due to intestinal obstruction (n = 5) and subjected to double-label immunofluorescence using antisera directed towards protein gene product 9.5 (PGP 9.5, used here as a pan-neuronal marker) in combination with an antibody against ZnT3.
Results. ZnT3-like immunoreactive (ZnT3-LI) neurons were observed in each of the three studied plexuses. ZnT3-positive neurons constituted 31.75 ± 2.01% of all perikarya observed in the myenteric, 26.77 ± 1.09% in the outer submucous, and 24.36 ± 1.59% in the inner submucous plexus.
Conclusions. These data demonstrate for the first time ZnT3-like immunoreactivity in the ENS and suggest that ZnT3 and, consequently, ZEN terminals may participate in the neural control of human colonic activities (**Adv Clin Exp Med 2009, 18, 3, 243–248**).

Key words: enteric nervous system (ENS), zinc transporter 3 (ZnT3), descending colon, immunohistochemistry.

Streszczenie

Wprowadzenie. Trzeci transporter cynku (ZnT3) jest przedstawicielem kationowych transporterów SLC 39, które biorą udział w przenoszeniu cynku z przestrzeni pozakomórkowej do cytoplazmy. ZnT3 był obserwowany w tzw. neuronach ZEN w centralnym i obwodowym układzie nerwowym.
Cel pracy. Stwierdzenie, czy ZnT3 jest obecny w neuronach jelitowego układu nerwowego okrężnicy zstępującej człowieka.
Materiał i metody. Materiał do badań uzyskano od pacjentów (n = 5) poddanych resekcji okrężnicy zstępującej z powodu niedrożności jelit. Wycinki okrężnicy wybarwiono z użyciem techniki immunofluorescencji podwójnej, stosując surowice skierowane przeciwko PGP 9.5 (użytemu tu jako marker panneuronalny) i ZnT3.
Wyniki. Neurony ZnT3-immunoreaktywne były obserwowane w trzech zwojach śródściennych jelitowego układu nerwowego okrężnicy. W zwoju mięśniówkowym (MP) stanowiły one 31,75 ± 2,01% wszystkich komórek nerwowych, w zwoju podśluzówkowym zewnętrznym (OSP) – 26,77 ± 1,09%, a w zwoju podśluzówkowym wewnętrznym (ISP) – 24,36 ± 1,59%.

Wnioski. W niniejszych badaniach po raz pierwszy wykazano obecność ZnT3-immunoreaktywnych neuronów jelitowym układzie nerwowym człowieka, co sugeruje udział trzeciego transportera cynku i neuronów ZEN w nerwowej regulacji czynności jelit (**Adv Clin Exp Med 2009, 18, 3, 243–248**).

Słowa kluczowe: jelitowy układ nerwowy (ENS), trzeci transporter cynku (ZnT3), okrężnica zstępująca, immunohistochemia.

Zinc is the second most abundant trace element in mammalian tissues, after iron. It is necessary for vital processes and is involved in many biological functions. Zinc is known to be a cofactor of more than 300 enzymes in the mammalian organism [1]. A large amount of zinc (ca. 200 ng/mg protein) has been identified in the central nervous system (CNS), where zinc is essential for neural cell function and normal brain development [2]. Although approximately 90% of zinc ions in the CNS are bound by peptides and proteins, the remainder forms a histochemically reactive "free pool" of ionic zinc [3], which may act as a neurotransmitter/neuromodulatory factor, especially in the hippocampal circuits [4]. Furthermore, zinc ions have been found in synaptic vesicles in neuronal endings, termed zinc-enriched neuron (ZEN) terminals [3, 5]. Such nerve terminals have been traced both in the CNS, within the hippocampus [6], amygdala [7], striatum [8], cerebellum [9], spinal cord [10], and in the peripheral nervous system [11, 12]. However, knowledge concerning the physiological relevance and metabolism of ionic zinc in the nervous system is still rather fragmentary.

As zinc cation is a small hydrophilic ion that cannot freely cross biological membranes by passive diffusion, it requires proteins facilitating its active transport [12]. Zinc homeostasis thus results from a coordinated regulation by different proteins, such as metallothioneins (MTs) and transmembrane transporters of the Zrt/Irt-like protein (ZIP) and cation diffusion facilitator (CDF) families (for a review, see [13]). The mammalian members of the ZIP family have been given the systematic designation SLC39 and they were thought to play an important role in zinc transport from outside the cell into the cytoplasm. Several members of the ZIP family have been described in mouse and human tissues [14, 15]. The CDF family of transporters, which in mammals have been named "ZnT" and given the systematic name SLC30 [16], transport zinc in the direction opposite to that of the ZIP proteins, promoting zinc efflux or compartmentalization by pumping zinc from the cytoplasm out of the cell or into the lumen of an organelle [17]. In mammalian cells, 10 homologous SLC30 proteins have been described so far [13].

Zinc transporter 3 (ZnT3), the subject of the present study, is a member of the SLC30 family of zinc transporters. It has been studied in ZEN terminals in the hippocampus, amygdala, neocortex, spinal cord, and superior cervical ganglion neurons [10, 12, 18]. The presence of ZnT3 in different regions of the central and peripheral nervous system [12, 18, 19] suggests that this protein, responsible for transporting zinc ions into synaptic vesicles, can thus be used as a marker for tracing ZEN neuronal pathways. As the presence of ZnT3 in the enteric nervous system (ENS) is still unknown, the aim of the present study was to investigate the distribution pattern of ZnT3-LI structures in the ENS and evaluate the number of ZnT3-LI neurons in particular intramural ganglia of human descending colon. Thus the present study constitutes an introduction to further investigations that should focus on establishing ZnT3 and ZEN nerve functions in the gastrointestinal tract.

Material and Methods

Fragments of descending colon were collected from patients (2 women and 3 men, mean age: 61 ± ± 5 years) hospitalized at the Clinical Hospital of the Medical University in Gdańsk, Poland, just after colectomy performed due to an incurable intestinal obstruction. The samples were taken from the peripheral parts of excised colon that did not show any macroscopic or microscopic pathological changes. The tissue collection for immunohistochemical study did not affect the surgeons' decision concerning the dimension of the resection. All procedures conducted during this study were in accordance with ethical standards defined by the 1975 Helsinki Declaration (revised 1983).

The tissues were immediately fixed by immersion in a solution of freshly prepared 4% buffered paraformaldehyde (pH 7.4) for 30 minutes, rinsed for 72 h in phosphate buffer (0.1 M, pH 7.4, 4ºC), and transferred to 18% phosphate-buffered sucrose, where they were kept at 4ºC until sectioning. Finally, colon fragments were cut on a cryostat (–22ºC) into 10-µm-thick sections.

The cryostat sections were processed for routine double-label immunofluorescence. Briefly, after air-drying at room temperature (RT) for 45 min, sections were incubated with a blocking solution containing 10% normal goat serum, 0.1% bovine serum albumin, 0.01% NaN_3, Triton X-100, and thimerozal in PBS for 1 h (RT). Then they were in-

cubated (overnight, RT, in a humid chamber) with a mixture of antisera raised in different species and directed towards protein gene product 9.5 (PGP9.5, mouse monoclonal; Biogenesis, UK, working dilution: 1:2000, code: 7863-2004) and ZnT3 (rabbit polyclonal, gift from Prof. Richard D. Palmiter, Department of Biochemistry, University of Washington, USA; working dilution 1:800, for a characterization of the antibody's specificity, see [20]). Complexes of primary antisera bound to the respective antigens were visualized by incubation (1 h, RT) with species-specific secondary antisera conjugated to FITC or biotin (all from Jackson ImmunoResearch, USA; 1:800). The latter antibodies were then visualized by a streptavidin-CY3 complex (Jackson; 1:8000, 1 h, RT). Each step of the immunolabeling was followed by rinsing the sections with PBS (3 x 10 min, pH 7.4). The double-immunostained perikarya were evaluated under an Olympus BX51 microscope equipped with epi-fluorescence and appropriate filter sets, counted in each ganglionated plexus (i.e. the myenteric, outer submucous, and inner submucous plexus) found in the section studied (10 sections per patient; only neurons with a clearly visible nucleus were included), and presented as the mean ± *SEM*. Omitting the primary antibody or replacing the antibodies with normal serum eliminated the specific stainings.

Microphotographs were obtained with a digital camera connected to a PC, analyzed with AnalySIS software (version 3.02, Soft Imaging System, FRG), and printed on a wax printer (Phaser 8200, Xerox, USA).

Results

ZnT3-LI neurons were observed in all the patients in all the enteric ganglia (Fig. 1), i.e. the myenteric plexus (MP, located between the longitudinal and circular muscle layers), outer submucous plexus (OSP, located in the submucosa), and inner submucous plexus (ISP, located between the muscularis mucosa and the lamina propria). ZnT3-LI neurons were found on virtually all cross-sections of the ganglia studied. The most frequent pattern observed was the presence of two to eight ZnT3-LI cells in each studied ganglion, but ganglions with numerous ZnT3-LI neurons as well as those devoid of ZnT3-LI perikarya were observed sporadically. Generally, the distribution patterns of ZnT3-LI cells in individual plexuses were similar in all of the MP, OSP, and ISP (Fig. 1), but the average numbers of these cells differed and amounted to 31.75 ± 2.01% in the MP, 26.77 ± 1.09% in the OSP, and 24.36 ± 1.5% in the ISP. Nerve fibers containing ZnT3-LI were observed neither in the ganglia nor in the muscle layers or mucosa.

Discussion

The present study demonstrates for the first time the existence of ZnT3-LI neurons in the ENS of human descending colon. The considerable quantity of these cells, over 20% of all perikarya in each plexus, may suggest important, albeit currently unknown, function(s) of ZEN terminals within the ENS of human large intestine. Indeed, the functional relevance of ZnT3-LI ganglionic cells found not only in human intramural enteric ganglia (this study), but also in other parts of the murine peripheral nervous system, where ZnT3 was found in both adrenergic and cholinergic sympathetic neurons showing fast anterograde and retrograde axonal transport [11, 12], remains completely unknown. However, one could expect that ionic zinc in the human colon may, at least in part, be implicated as a neurotransmiter/modulatory agent, as within the CNS. Thus, although some suggestions based on the known role(s) of ionic zinc in the CNS will be discussed below, one should be aware that further studies are definitely needed to prove their validity.

It is generally accepted that ZnT3 plays an important role in the regulation of zinc level and is the key protein in synaptic vesicle zinc transport [12, 18, 19]. Previous investigations on the CNS also suggested that ZnT3-LI neural structures may be involved in either sensory transmission or the efferent control of other neuronal circuits [19, 21]. Furthermore, some ZnT3-containing cells may exhibit secretory activities [19]. It was also shown that many of the ZEN terminals in the CNS are glutamatergic and showed typical characteristics of inhibitory neurons [21, 22]. The "vesicular" zinc in glutamatergic neurons is considered to take part in multiple neuronal events in health and during pathological processes, such as cerebral ischemia, epilepsy, and brain injury, by serving as an endogenous neuromodulator of pre- or post-synaptic receptors [23] and by contributing to neuronal injury by its neurotoxic effects [24, 25, for a review, see 26].

Although glutamatergic neurons have been shown to be present in the enteric ganglia of many mammalian species, including humans [27, 28, for a review, see: 29], there are no direct data showing either the coexistence or lack of it of both ZnT3 (as an indicator of the presence of ZEN) and glutamate markers in enteric neurons.

Besides the possible functions of ZEN terminals within the CNS mentioned above, previous

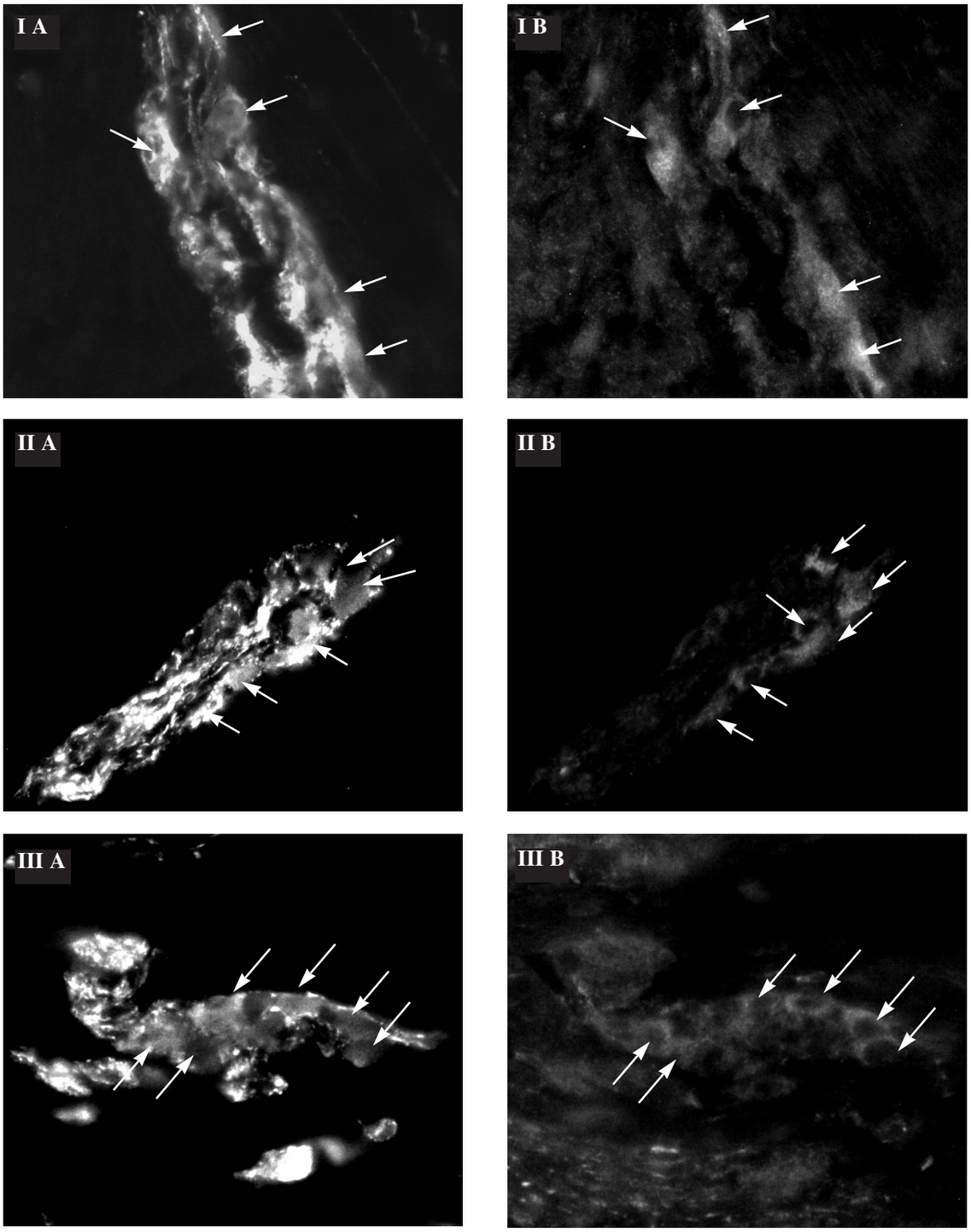

Fig. 1. Enteric ganglia in human descending colon immunostained for PGP 9.5 (A) and ZnT3 (B). I – myenteric plexus (MP), II – outer submucosus plexus (OSP), III – inner submucosus plexus (ISP). Co-localization in neurons indicated by arrows magnification ×400

Ryc. 1. Neurony zwojów jelitowego układu nerwowego okrężnicy zstępującej człowieka immunoreaktywne wobec PGP 9,5 (A) i ZNT_3 (B). I – zwój mięśniówkowy (MP), II – zwój podśluzówkowy zewnętrzny (ISP). Strzałki wskazują neurony, w których występują obydwie badane substancje, powiększenie 400×

studies have shown the presence of this nerve type in the spinal cord, where ZEN terminals both appeared in the dorsal horns and made contact with motor neurons. This may suggest that these nerves may be involved in both sensory and motor processing at the spinal cord level [10, 19, 30]. In contrast to investigations on the CNS, the functions of ZEN terminals in peripheral nervous structures are completely unknown [11, 12] and it is only possible to assume that the roles of ZEN terminals are similar in the central and peripheral nervous systems. On the other hand, it has been shown that ZnT3 is located in the neuronal somata and neuronal processes of rodent superior cervical ganglion [12], while ZnT3-LI has not be detected in neuronal somata in the CNS [19]. This may again indicate that the formation, packing, and function(s) of ZnT3 and ZEN fibers differ in the central and peripheral nervous systems.

In conclusion, the results of the present study suggest that ZnT3 may be a marker for a yet unknown population of enteric neurons.

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Conflict of interest: None declared

Received: 13.03.2009
Revised: 24.04.2009
Accepted: 19.06.2009