

LOCALIZATION OF SUBSTANCE P-IMMUNOREACTIVE NERVE STRUCTURES IN THE RAT COLON

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Abstract

The tachykinins, including their major representative substance P (SP), are an essential component of its non-adrenergic, non-cholinergic transmission of the myenteric plexus of the enteric nervous system. Hence, the understanding of the role that SP plays in the conduction of a wide variety of motility and secretory reflexes lies in the evidence-based understanding of the sites of its expression. In the present study, the presence, distribution and staining intensity of the SP-positive nerve structures in the myenteric plexus of the proximal and distal colon of the adult rat have been investigated by means of immunohistochemistry at the light microscopic level. We observed an abundance of intensely stained beaded SP-immunopositive fibres, surrounding the SP-immunonegative myenteric neuronal cell bodies in a basket-like manner. Fine bundles of SP-immunostained varicose fibres were also registered in the adjacent smooth muscle layers. The statistical analysis revealed discernible prevalence of the staining intensity in the proximal colon, compared to the distal one. In summary, our findings provide immunohistochemical evidence for the occurrence of abundant SP-immunopositive nerve structures in the colonic region of the rat. Taken together with previous morphological and pharmacological research the results of this study suggest that the SP-mediated neurotransmission is of physiological and likely of pathological significance for the examined region.

Key words: myenteric plexus, substance P, tachykinins, colon, rat

Introduction. The enteric nervous system (ENS), with its two constituent parts – the submucosal and myenteric plexuses, is the major executor of a wide variety of colonic reflexes and, thus, includes a neuronal network with neurochemical coding of astonishing complexity. Each of these plexuses possesses, therefore, the entire array of neuronal functional types, including a well-formed population of excitatory motor neurons and intrinsic primary afferent neurons. As opposed to the classical paradigm that the major transmitters of these neurons are the acetylcholine (ACh) and noradrenaline, over time the non-adrenergic, non-cholinergic (NANC) transmission system was proven to be much more diverse and widespread.

The tachykinin neurotransmitter family has been shown to be a significant participant in the NANC transmission and, therefore, profound understanding of its participation in the enteric circuits is mandatory. Tachykinins are a group of neurotransmitter substances with similar structure, which were very difficult to differentiate between even by means of specific imaging techniques, like immunohistochemistry. Proving that the major tachykinin involved in the enteric circuits is in fact substance P (SP) was a long and doubtful process, finally resulting in the modern concept [1,2], according to which, SP is a co-transmitter not of a single neuronal system, but rather a few.

Since most of this knowledge is acquired by physiological and pharmacological experiments, the purpose of the present study is to examine the presence, exact localization, and regional differences of the SP-positive structures in the myenteric plexus of the rat colon by means of immunohistochemical technique at the light microscopic level.

Materials and methods. In our study we used nine mature (3-month-old) Wistar rats of both sexes with average weight (200–300 g). The experiments were carried out at the Institute of Neurobiology, Bulgarian Academy of Sciences in accordance with ethical principles for the care and use of laboratory animals. Following a routine transcardial perfusion with 4% paraformaldehyde, we collected two segments, each measuring approximately 10 mm, from proximal and distal colonic segments, which were fixed additionally in the same fixative for 24 h. Tissue sections (30 µm thick) were cut on a freezing microtome (Reichert Jung), collected in a free-floating state, washed overnight in several changes of PBS and then processed for immunohistochemistry, according to the avidin-biotin-horseradish peroxidase method.

Briefly, the sections were permeabilized with PBS containing 0.3% Triton X 100 (Merck) to increase the penetration of the antibody and then treated with 1.2% H₂O₂ in absolute methanol (Merck) to neutralize the endogenous peroxidase. In order to reduce the non-specific peroxidase staining, the sections were preincubated in 5% normal goat serum (Sigma) for 30 min at room temperature.

The sections were then incubated with the primary rabbit SP-antiserum (Abcam, Cambridge, UK, Cat. No ab43826; dilution 1:6000) for 24 h at 4 °C. The secondary incubation was performed with biotinylated anti-rabbit goat IgG-Biotin

(Jackson ImmunoResearch Lab. Inc., USA, Cat. No 75749, diluted 1:250) for 2 h at room temperature. Afterwards, the sections were incubated for 90 min in ABC complex diluted 1:100 (Vectastain ABC kit, Vector Laboratories Inc, Burlingame, USA) for 2 h in a moist chamber at room temperature. The reaction was visualized with 3,3-DAB/H₂O₂ (Sigma Chemicals, St Louis, MO, USA). The intensity of the reaction was increased with 0.05% nickel ammonium sulfate. Thereafter, the sections were washed with Tris-HCl buffer, mounted on chrome-gelatin coated slides, air dried for 24 h, dehydrated in absolute alcohol, cleared in xylene and coverslipped with Entellan (Merck). Control sections were incubated in the absence of the primary antibody and the results were negative.

The slides were examined and photographed with Nikon Eclipse 80i (Japan), equipped with image analysis software NIS-Elements Advanced Research (ver. 2.30), and the images processed with Adobe Photoshop CC software. The SP staining intensity quantification was performed by means of the program ImageJ 1.48v (NIH, Bethesda, MD, USA). The statistical analyses between groups were performed using the SigmaStat[®] 11.0 (Systat Software Inc., San Jose, CA, USA) software package. We implemented Mann-Whitney *U*-test and the results were considered statistically significant with $p < 0.05$.

Results. At a low magnification, we found considerable staining which contrasted with the surrounding muscle layers, the latter being seemingly unstained (Fig. 1A, C). The observation at a higher magnification (Fig. 1B, D) revealed abundance of immunoreactive fibres in the myenteric ganglia and a distinctive presence of perisomatic varicosities associated with them. We did not register staining in the neuronal perikarya whatsoever. However, the fibres surrounded the spaces which inevitably contained the neuronal bodies in a basket-like manner and had such a contrasting staining, that made it possible to define the outlines of the bodies. Also, since the largest and most intensely stained varicosities were located exactly on the borders of these reaction-negative spaces, it is only logical to assume that those were the microscopic equivalent of axo-somatic/dendritic synapses. Internodal strands, showing the same staining reaction and varicosities, were also present in the plane between the muscle layers (not shown).

In the two muscular layers, surrounding the plexus, the immunoreactive fibres we noticed were barely noticeable and had a course parallel to the orientation of the muscle cells. However, they showed much less intense staining reaction than the one in the plane of the myenteric plexus, and only scattered varicosities.

The differences in the staining intensity were evaluated by means of the Mann-Whitney *U* test. To present the distribution of the data we have displayed them as box-and-whisker plots (Fig. 2). Compared to the distal colon, the SP staining density of the nerve structures in the proximal colon was significantly higher, with median values 81.189% for the proximal, versus 70.344% for the distal colon ($p < 0.05$).

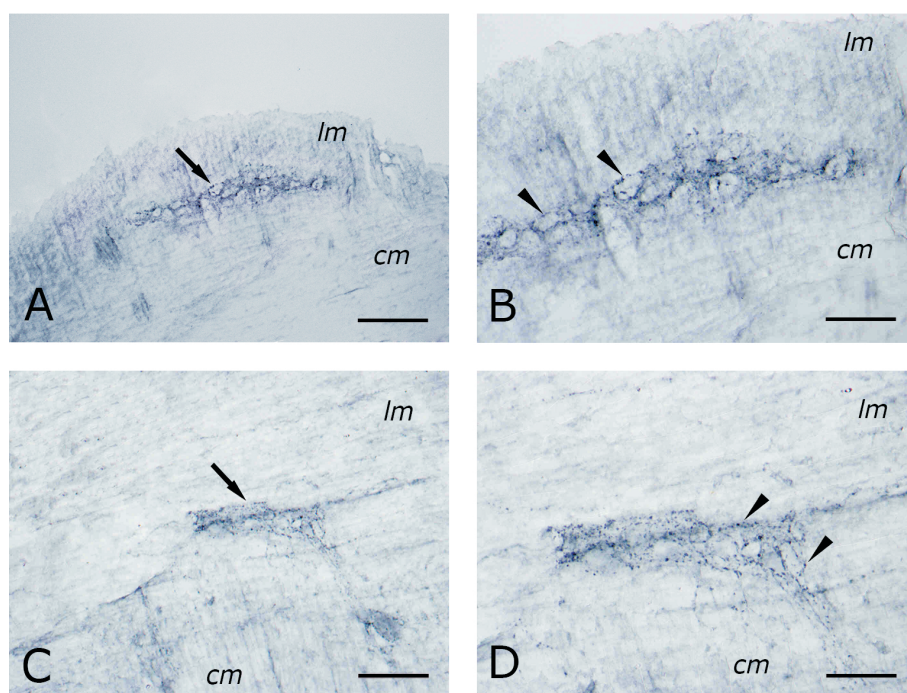


Fig. 1. SP-immunoreactive nerve structures in the myenteric plexus of the rat proximal (A, B) and distal (C, D) colon. Significant staining was observed in the myenteric ganglia (arrows). Note the immunopositive beaded fibres which surround the immunonegative ganglionic perikarya in a basket-like manner (arrowheads). *lm* – longitudinal muscle layer; *cm* – circular muscle layer. Scale bar: A, C – 100 μ m; B, D – 50 μ m

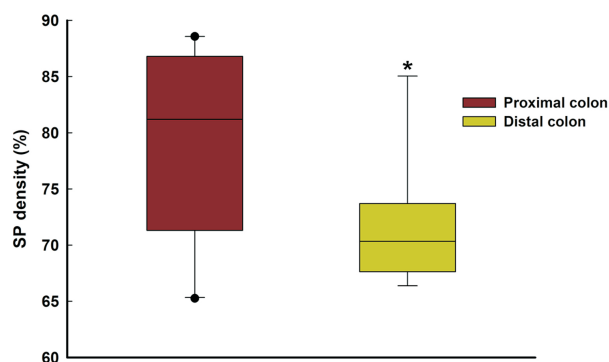


Fig. 2. Statistical comparison of the staining intensity of SP-positive nerve structures in the myenteric plexus of the proximal and distal rat colon. Data are compared using Mann–Whitney’s *U*–test and presented as box-and-whisker plots. In each box plot the central line represents the median, whilst the upper and lower borders of the box are the 75th and the 25th percentiles, respectively. The whiskers on each box are the 95th and the 5th percentiles for the group. The results were considered significant, where $p < 0.05$

Discussion. The significance of substance P for maintenance of bowel contents has been investigated in a variety of animal models and digestive tract segments. In the rat intestines, for instance, the SP-positive myenteric neurons have been suggested to mediate intrinsic motility and secretory reflexes induced both by physiological [3] and pathophysiological [4] stimuli.

Several lines of evidence suggest that SP-mediated transmission is likely to participate in three regulatory neuronal systems. Firstly, SP is recognized as a co-transmitter of the excitatory myenteric motor neurons, together with ACh [5,6]. However, their functional significance, measured by their ability to induce contraction, is uneven. Blockage of the cholinergic transmission leads to much higher dysfunction than that of the tachykininergic one, showing the secondary role of SP in the excitatory motor system. Secondly, SP has been registered as a co-transmitter in ascending (orally projecting) interneurons, together with ACh and calretinin. Naturally, these neurons are a conduit for ascending pathways that are components of propulsive colonic reflexes. Lastly, SP is proven to be a co-transmitter (together with ACh and calbindin) of the myenteric intrinsic primary afferent neurons, which essentially are the primary sensory neurons of the ENS [7-9]. In this regard, our present findings strongly reinforce the perception that SP-positive structures are an important component of enteric circuitry.

The absence of significant staining in the neuronal bodies, as in our study, has been previously reported [10,11]. On the other hand, other investigations have resulted in more intense staining of neuronal bodies [12,13]. A possible explanation for this phenomenon is the presumption that the perikaryal staining requires preincubation with colchicine [14].

It is speculative to draw conclusions about the nature of the perikarya, which were surrounded by the beaded fibres in our study. However, it must be highlighted that these findings are not in any contradiction to recent reports that myenteric SP-positive neuronal bodies express both Dogiel type I (motor neurons and interneurons) and type II (intrinsic primary afferent neurons) morphology [15]. The same is valid for canine research results, showing that similar beaded SP-positive fibres synapse on the surface of NK1 receptor-positive, but SP-negative myenteric neurons [16].

In general, without specific procedures with intracellular tracers, it is impossible to surely determine the origin of these fibres. However, our findings in the rat colon almost completely mimic the ones in the much more examined guinea-pig colon. Therefore, it is probable that the SP-immunoreactive fibres that we observed in the myenteric plexus were mostly of intrinsic origin, as is the case in the guinea-pig [17].

The presence of SP-immunoreactive fibres in the circular muscle layer is in accordance with previous experiments, conducted on a wide variety of animal models [15,18]. The findings in the longitudinal muscle layer, however, are inconsistent. For instance, the porcine longitudinal muscle layer is reported to be

completely devoid of SP-positive structures [19], whereas in the guinea-pigs and rats they are not only a common finding, but are even proved to have a quite significant excitatory motor influence [20].

An interesting finding of the present study is the site-specific differences in the staining intensity of SP-positive structures in the rat colonic region. As the median values of our plot imply, there is a significant predominance in the density of SP-positive neuronal structures in the proximal colonic segment, compared to the distal colon.

In conclusion, our findings provide immunohistochemical evidence for the presence and distribution of SP-positive structures in the myenteric plexus of the colonic region of the rat, with specific emphasis on the regional differences in their staining density. Additionally, they confirm previous findings related to the distribution of immunoreactive fibres in the adjacent muscle layers. However, further investigations and understanding of the role and mechanisms of action of these structures are the key for development of modern therapies for colonic motility disorders.

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