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Age-related changes in substance P-immunoreactive nerve structures of the rat recto-anal region

Research Article

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Abstract: The recto-anal region is innervated by extrinsic and intrinsic nerves and a number of neuropeptides including substance P (SP) have been suggested to participate in the regulation of intestinal movements. We examined the age-related changes in the distribution of SP-immunoreactive nerve structures in the distal part of the rat large intestine. Using immunohistochemistry, the presence of SP was studied in fresh tissues from Wistar rats at different ages taken at three sampling sites, the distal rectum, anal canal and internal anal sphincter. In the15-day old rats the myenteric plexus of the distal rectum and anal canal was well outlined by numerous SP-immunoreactive varicose nerve fibres encircling immunonegative perikarya. In the circular muscle layer, nerve fibres and small nerve bundles ran parallel to the muscle cells, while in the longitudinal muscle layer, only occasional nerve fibres were seen. At the level of the internal anal sphincter, no myenteric ganglia were present. Here, thin varicose fibers ran parallel to the smooth muscle cells. In the 3-month old rats, a larger number of intensely staining SP-immunoreactive nerve fibres were found and in the circular muscle layer, thicker nerve strands were observed. In the 26-month old rats, the density and staining intensity of SP-immunopositive nerve fibres in the myenteric plexus was lower than in the 3-month-old rats. Similar changes in the SP-immunostained fibres in the internal anal sphincter were observed. Degenerative alterations in SP-containing fibres during aging appear to play a role in ano-rectal motility and sphincter control.

Keywords: *Aging, Anal canal • Distal rectum • Internal anal sphincter • Myentric ganglia • Substance P • Recto-anal region • Rat*

1. Introduction

The distal region of the large intestine, the rectum and the anal canal, are involved in the final processing and expulsion of bowel contents, culminating in defecation. Below the rectum, a well defined involuntary smooth muscle, the internal anal sphincter, is found at the termination of the alimentary canal. It comprises a distinct inner ring of smooth muscle surrounded by an outer ring of skeletal muscle (external anal sphincter). Although both muscles are important in the maintenance of continence, the internal anal sphincter is an involuntary muscle under a state of chronic contraction, due both to intrinsic myogenic properties and external innervation [1,4]. The recto-anal region is innervated by extrinsic nerves from the autonomic nervous system and intrinsic nerves originating from the enteric nervous system (ENS) [2,3]. However, it has been previously shown that gastrointestinal motility is independent of extrinsic autonomic nervous supply, and only projections from intramural intrinsic nerves serve to control the internal anal sphincter [4]. Nonetheless, there is good communication between the internal and

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external innervation. In fact, intrinsic nerves are involved in the local neural network to stimulate the inhibitory control of the internal anal sphincter [5]. On the other hand, extrinsic neural pathways serve as controller mechanisms in the coordination of motility in the distal regions of the gastrointestinal tract [6].

Despite recent progress in neurogastroenterology, little information is available on the neural circuits that control motor and secretory functions in the rat recto-anal region and the neurotransmitters involved. In addition, the neural mechanisms that coordinate gut function rely on a complex interplay between the established neurotransmitters and their receptors. Overall, more than 20 putative neuroactive substances have been identified within the ENS including well-known substances such as acetylcholine, norepinephrine and serotonin as well as peptide transmitters such as substance P, vasoactive intestinal polypeptide, and the gaseous messenger nitric oxide [7].

Among the enteric neuropeptides, substance P (SP) has received considerable attention because of its role as a pivotal sensory mediator of intestinal motility in animal species, including humans [8-10]. This peptide was first isolated from extracts of the brain and gut. Over time it has been shown to have powerful stimulatory effects on gastrointestinal muscle [11] and to be an excitatory gastrointestinal neurotransmitter [12]. SP is released from intestinal nerves to stimulate acetylcholine release from other intestinal nerves, and for production of rhythmic contraction in the intestinal smooth muscle [13]. A possible role has also been suggested for SP-containing nerves in the effector control of the internal anal sphincter and its dysfunction [14]. SP is a member of the tachykinin family of peptides and its effects are mediated through the stimulation of tachykinin NK1, NK2 and NK3 receptors, preferentially by the NK1 receptor [15]. In the gastrointestinal tract of rats, NK1 receptors are expressed in both neuronal and non-neuronal cells in the submucosal and myenteric plexus, in particular in intrinsic primary afferent neurons, excitatory and inhibitory motor neurons, secretomotor neurons, and interstitial cells of Cajal [16].

Our previous studies have shown that SP-immunoreactive perikarya occasionally occur in the hind gut of the rat [10]. However, changes in the distribution, dimension and frequency of appearance of the SP immunoreactive nerve structures in the recto-anal region of the rat during normal aging still remain uncertain. This may be an important issue since there is a well documented age-related increase in gastrointestinal diseases and impairment of intestinal function. Observations derived from animal studies are largely applicable to human disorders but studies are lacking investigating the relationship between age-dependant changes in function and intrinsic innervation in the recto-anal region.

The goal of this study was to determine the presence of SP immunoreactivity in the recto-anal region of the rat and examine the age-related changes in the distribution of SP-immunoreactive nerve structures in that area.

2. Material and Methods

We used nine male Wistar rats of different ages (15 days, 3 months and 26 months of age). The housing facility and procedures were approved by the Animal Care and Use Committee of the Medical University-Sofia and were in accordance with the ethical guidelines of the European Convention for the protection of vertebrate animals used for experimental and other scientific purposes (1986). The rats were maintained under standard conditions at 21ºC, with a 12 hour light–dark cycle. The animals were supplied with water ad libitum and fed a normal diet of standard rat chow, providing 50 g of food daily.

Under deep anesthesia with Thiopental (50 mg/kg, i.p.), the animals were transcardially perfused, first with warm heparinized 0.05 M phosphate buffered saline (PBS), pH 7.3, followed by freshly prepared 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.3. Circumferential segments of the recto-anal region were excised and postfixed overnight in the same fixative at 4ºC. The intestinal pieces were then transferred into 20% sucrose in 0.1 M PB at 4ºC until they sank. Samples from the distal rectum, anal canal and internal anal sphincter were prepared. The tissue blocks from the rectum and the anal canal were cut longitudinally while those from the internal anal sphincter were cut transversally to the longitudinal direction of the gut. 30 µm thick sections were cut using a Reichert Jung freezing microtome, collected in a free-floating state in two subsets, washed overnight in several changes of PBS and then processed for immunohistochemistry. Every first section of the subset was mounted on a glass slide and stained using Nissl's method to investigate the presence of ganglion cells in the myenteric plexus.

For the immunohistochemistry every second section of the subsets was stained for SP according to the avidin-biotin-horseradish peroxidase (ABC) method [17]. Briefly, the sections were permeabilized with PBS containing 0.3% Triton X 100 (Fluka, Buchs, Switzerland) to increase the penetration of the antibody and then treated with 1.2% H_2O_2 in absolute methanol (Merck, Darmstadt, Germany) to neutralize the endogenous peroxidase. In order to reduce nonspecific peroxidase staining, the sections were preincubated in 5% normal

Figure 1. A Nissl-stained section showing a cluster of neurons (thick arrow) in the rectal myenteric ganglion of a 15-day-old rat.

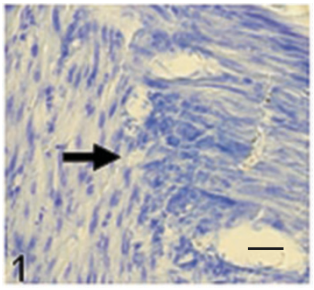


Figure 2. A group of large neurons (thick arrow) in the myenteric ganglion of the anal canal in a 3-month-old rat, visualized by the Nissl staining.

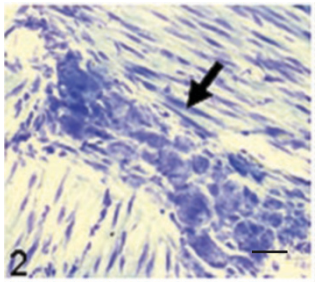
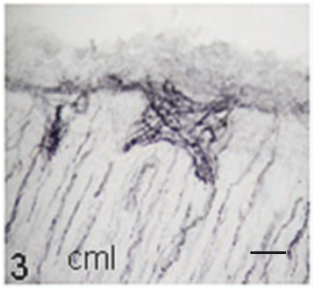


Figure 3. SP-immunoreactive nerve fibers in the myenteric ganglion of the distal rectum of a 15-day-old rat. Note that the immunostained fibers surround the cell bodies of large immunonegative myenteric neurons. Some immunopositive fibers are also visible in the circular muscle layer (cml).



goat serum (Sigma, MO, USA) for 30 minutes at room temperature. Thereafter, the tissue sections were incubated overnight in a rabbit polyclonal antibody against SP (Abcam, Cambridge, UK, diluted 1:6000) kept at room temperature, shaking. A second antibody, a biotinylated goat anti-rabbit IgG (1:500; Dianova, Hamburg, Germany), was applied for 90 minutes at room temperature. Following rinsing in a PBS/Triton solution, the sections were placed in ABC complex (Vectastain Elite Kit; Vector Laboratories, Burlingame, CA, USA) for 90 minutes. Visualization of the reaction product was done using either 3,3'-diaminobenzidine tetrahydrochloride (DAB; Sigma, St. Louis, MO, USA), dissolved in Tris/HCl buffer with 0.05% H_2O_2 (Fluka, Buchs, Switzerland) alone or by nickel intensification of the DAB reaction product with nickel ammonium sulphate. After the development procedure, the sections were washed in Tris-HCl buffer, mounted on chrome-gelatin coated slides, air dried for 24 hours, dehydrated in absolute ethanol, cleared in xylene and coverslipped with Entellan (Merck, Darmstadt, Germany). The slides were examined and photographed with a Nikon digital camera linked to a Jenaval research microscope. All digital images were saved in a TIFF format and matched for brightness and contrast using Adobe Photoshop CS3 software (Adobe Systems, Inc., San Jose, CA, USA).

For immunoreaction specificity testing, omission of the primary antibody from the incubation medium or its replacement with normal goat serum was performed. No immunoreactivity was found under these conditions.

3. Results

The light microscopic observations after the Nissl staining demonstrated that the myenteric plexus of the distal part of the large intestine in 15 day old rats is composed of a cluster of neuronal somata of small to large neurons and satellite cells situated between the longitudinal and circular muscle layers (Figure 1). Most of the myenteric neurons were medium in size and their somata exhibited a longitudinal diameter of 35-40 μm (Figure 1). In the 3-month old rats, (Figure 2) the myenteric neurons were larger than those observed in the15 day old rats and the longitudinal diameter of their perikarya ranged from 65 to 70 μm. At the level of the internal anal sphincter, in the space between the two muscle layers, no myenteric ganglia were observed. Conversely, the presence of a large number of nerve fibres was noted in this area.

The immunohistochemistry for SP revealed that the myenteric ganglia of the hind gut in 15 day old rats were outlined by a large number of fine SP-immunoreactive varicose nerve fibres. In addition, the immunostained beaded fibers encircled, in a basket-like manner, the cell bodies of the small SP-immunonegative myenteric neurons in both the rectum (Figure 3) and the anal canal (Figure 4). Within the ganglia, SP-containing perikarya were occasionally observed. The circular muscle layer was innervated by sparse thin varicose fibers (Figure 3) as well as by small nerve bundles running parallel to the muscle cells. Similarly, a few fine immunopositive varicosities were observed within the longitudinal muscle layer (Figure 5). In the internal anal sphincter, no myenteric ganglia were detected however, faintly staining varicose nerve fibers were scattered among the smooth muscle cells (Figure 6).

Figure 4. SP-immunoreactivity in the myenteric plexus of the anal canal. The immunoreactive fibers envelop the immunonegative perikarya in a basket-like manner.

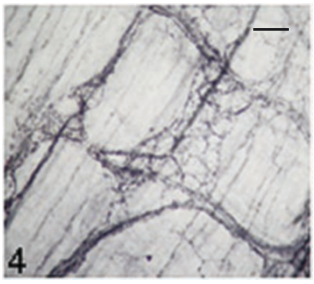


Figure 6. Tortuous, varicose, and lightly stained SP-immunoreactive nerve fibres (arrows) among smooth muscle cells of the internal anal sphincter. Scale bars = 100 μm for Fig. 1 and 50 μm for Figs. 2-6.

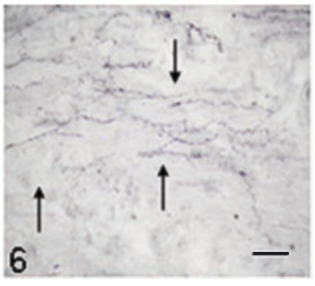


Figure 8. A large number of single varicose fibers and nerve bundles of a different caliber in the myenteric ganglion (thick arrow) of the anal canal. Lml, longitudinal muscle layer.

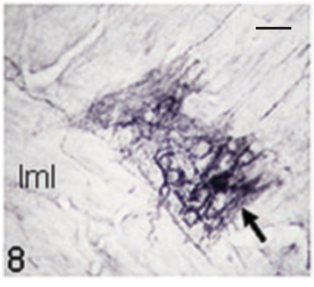


Figure 5. Immunopositive varicose single nerve fibers (arrows) in the longitudinal muscle layer (lml) of the anal canal. The border between the longitudinal and circular muscle layer (cml) is indicated by arrowheads.

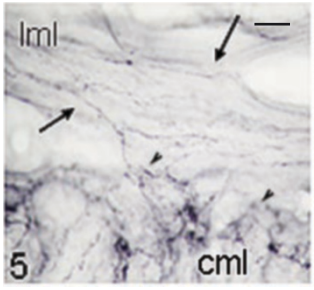


Figure 7. SP-immunoreactive nerve structures in the myenteric ganglion (thick arrow) of the rectum in a 3-month-old rats. Single immunostained fibers are also observed in the longitudinal muscle layer (lml).

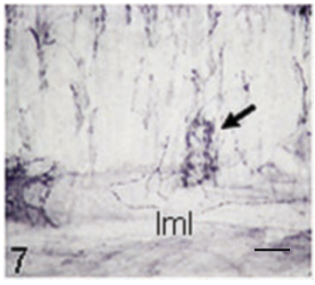
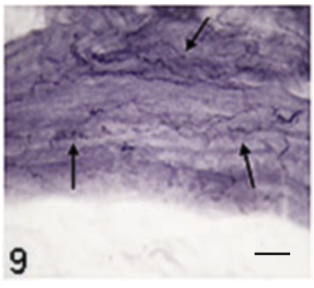


Figure 9. Intensely stained varicose and twisted nerve fibers (arrows) are seen in the internal anal sphincter.



In the myenteric ganglia in the distal rectum of 3-month old rats, fine terminal fields of SP-immunopositive nerve fibers surrounded the negatively stained perikarya (Figure 7). The immunostained nerve fibres were more numerous in number, closely packed in their arrangement and more intensely staining than the SP-immunoreactive fibres in the younger rats. The SP-immunonegative myenteric neuronal perikarya were larger in size than those seen in the 15 day old rats. The nerve strands of various sizes in the circular muscle layers in the anal canal of the 3-month old rats were tightly clustered, showed an increase in staining (Figure 8) and were more densely clustered than in 15 day old rats. Similar distribution of SP-containing nerve plexus of thin, varicose fibers in the longitudinal muscle layer was observed when compared with those in the 15 day old rats. The SP-containing nerve fibres in the internal anal sphincter were abundant, tortuous and more intensely staining than in the young rats (Figure 9).

In adult rats (aged 26-months) the SP-immunopositive nerve fibres in the distal rectum were compatible in number and density (Figure 10), while those in the anal canal were scarce and tended to be less intensely stained than those observed in the 3-month old rats (Figure 11). These fibres encircled the immunonegative perikarya in the myenteric ganglia in a basket-like manner. In the circular muscle layers, SP-immunopositive nerve fibres and/or nerve bundles were

Figure 10. SP-immunoreactive nerve fibers showing moderate staining intensity in the myenteric ganglion (arrow) of the distal rectum in a 26-month-old rat.

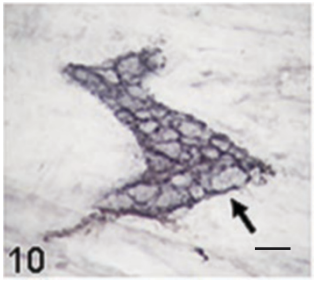


Figure 11. Sparse nerve fibers around the perikarya of myenteric neurons in the anal canal of a 26-month-old rat.

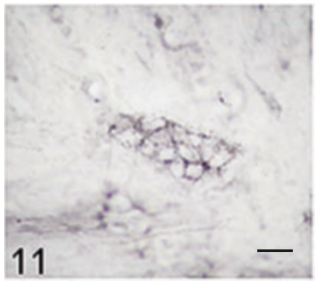
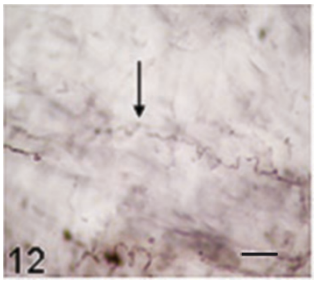


Figure 12. Scant SP-immunostained nerve fibers (arrow) in the internal anal sphincter of a 26-month-old rat. Scale bars = 50 μm.



significantly fewer and almost always less intensely stained when compared with the immunostained fibres in 3-month old rats. In this area, as well as in the internal anal sphincter, occasional immunopositive varicose fibres were visible (Figure 12).

4. Discussion

The maintenance of bowel contents and their evacuation is a complex process largely mediated by intramural pathways and triggered by a number of neurotransmitters, including SP. It is also known that SP plays an important role in the regulation of smooth muscle contraction in the internal anal sphincter utilizing intracellular calcium and calmodulin dependent pathways [18]. The results of the present study indicate that the ENS in the distal part of the digestive tract is well developed in 15 day old rats. This finding corresponds with prior results on the innervation of the smooth muscle of the human large intestine during postnatal development [19]. At this stage, the SP-containing nerves are abundant and their organization closely resembles what has been observed in other mammalian species including humans. Our data also show that SP-immunoreactive nerve fibres are present in the myenteric ganglia of the rectum and the anal canal, in the circular and longitudinal muscle layers, as well as in the internal anal sphincter of 3-month old rats. The distribution of SP in the distal part of the large intestine is consistent with earlier findings in rats [20], guinea pigs [21] and humans [22]. Therefore, the presence and distribution of the SP-positive cell population in the recto-anal region indicates more similarities rather than species differences.

An interesting finding of the current study are the age-related differences in the distribution, morphological appearance and staining intensity of the SP-immunoreactive nerve structures in the rat recto-anal region. In general, SP-immunostained fibres are more common in the young and adult animals than in the elderly rats. Our study specifically demonstrated a larger number of SP-immunoreactive nerve fibres in the distal part of the rat large intestine in 15 day old and 3-month old rats. However, in the elderly animals (26-month old) the overall number and density of SP-immunoreactive nerve fibres is decreased and most of them are in a state of degeneration. Previously, Fehér and Penzes [23] have observed by electron microscopy, degenerated neuronal profiles in the myenteric ganglia of the small intestine of ageing rats. According to the findings of Nilsson et al. [12] SP-immunopositive cell bodies are entirely absent in the mouse gut. This, as well as findings in our previous study [10], show that SP-containing perikarya in the myenteric ganglia of the rat recto-anal region are rare and thus occasionally seen. It appears that SP immunostained fibres may arise from neurons located in the myenteric plexus of the upper gut, although an extrinsic sympathetic origin cannot be entirely excluded. Thus, our observations concur with those previously noted for the human jejunum and distal ileum [13].

Concerning the effects of malnutrition on enteric neuron morphological characteristics, it has been a long standing view that the number of myenteric plexus structures is largely dependent on the diet and that protein deprivation alters their histological features [24,25]. Since the animals used in our study were fed a normal diet, we did not observe any alterations in the number and size of neuronal structures in the recto-anal region due to poor nutrition.

On the other hand, it is now well established that neurodegenaration is a key factor in the ageing gut [26,27]. Our study clearly indicates degenerative alterations in SP-containing fibres in the terminal part of the rat colon which also support this finding. Also of interest, notably from a clinical point of view, is that disturbance and disruption of SP innervation of the aganglionic colon in Hirschsprung's disease, resulting in limited occurrence of immunoreactive fibres in the internal anal sphincter, are well documented [22,28]. Both of these findings suggest that, although age-dependent SPergic neuronal loss does not cause dramatic changes in intestinal motility, it may be an underlying factor in the higher frequency of ano-rectal motility disorders encountered in the elderly [26].

In conclusion, our findings provide direct immunohistochemical evidence that there are striking differences in the distribution, density and staining intensity of SP-containing nerves in the rat recto-anal wall during ageing. Further investigations are needed to identify the presence of SP receptors in this area. Although the clinical significance of the present findings will require further study, it is likely that SPergic nerves play a regulatory role in smooth muscle contraction and internal anal sphincter control in health and disease. Lastly, knowledge of the distribution of SP-immunoreactive nerve structures and further progress in our understanding of their role and mechanisms of action in this region are the key to the development of new and effective therapies for ano-rectal motility disorders such as constipation and diarrhea.

Acknowledgments

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