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Immunohistochemical Localization of Some Neurotransmitters in Mole-rats (*Spalax leucodon*) Intestinal Plexus

The aim of this study was to investigate the presence of calcitonin gene-related peptide (CGRP), substance P (SP), neuropeptide Y (NPY), vasoactive intestinal peptide (VIP) and serotonin immunoreactivity in the intestinal plexus of mole-rats (*Spalax leucodon*). Immunohistochemical methods were conducted on the ileum and colon of the small and large intestine. CGRP immunoreactive neuronal structures were detected in the form of enteric ganglia within the myenteric plexus of ileum and colon. SP immunoreactivity in both of muscle layer of these intestine regions were identified in the form of running nerve fibers and myenteric ganglia. Also, NPY immunoreactive nerve cell bodies and fibers were present in the lamina propria and myenteric plexus of ileum and colon. However, VIP and serotonin immunoreactive neuronal structures were not detected in any other region of the small and large intestines. In conclusion, this study demonstrated the presence of different neuronal populations in the mucosal and ganglionated plexuses in the small and large intestine of the mole rats.

Key Words: Enteric nervous system, immunohistochemistry, intestinal plexus, mole-rats.

Kör farenin (*Spalax leucodon*) İntestinal Pleksüslerinde Bazı Nörotransmitterlerin İmmunohistokimyasal Lokalizasyonu

Bu çalışmanın amacı kör fare (*Spalax leucodon*) intestinal pleksüslerinde kalsitonin gene-related peptid (CGRP), substance P (SP), nöropeptid Y (NPY), vasoaktif intestinal peptid (VIP) ve serotonin immunoreaktivitesinin varlığını araştırmaktır. İmmunohistokimyasal incelemeler ince ve kalın bağırsağın ileum ve kolon bölümlerinde yapıldı. CGRP immunoreaktif nöronal yapılar ileum ve kolonun miyenterik pleksüsleri içinde enterik ganglionlar şeklinde belirlendi. SP immunoreaktivitesi bu bağırsak bölümlerinin her iki kas tabakası içinde uzayan sinir telleri ve miyenterik ganglionlar şeklinde ayırtıldı. Ayrıca NPY sinir hücre cisimcikleri ve iplikleri ileum ve kolonun miyenterik pleksüs ve lamina propriyasında mevcuttu. Buna karşın VIP ve serotonin immunoreaktif yapılar ince ve kalın bağırsağın herhangi bir bölümünde tespit edilemedi. Sonuç olarak, bu çalışma kör farenin ince ve kalın bağırsaklarının mukozal ve gangliyoner pleksüslerinde farklı nöronal popülasyonların varlığını ortaya koydu.

Anahtar Kelimeler: Enterik sinir sistemi, immunohistokimya, intestinal pleksüs, kör fare.

Introduction

The enteric nervous system (ENS) is the intrinsic nervous system of the gastrointestinal tract. It contains complete reflex circuits that detect the physiological condition of the gastrointestinal tract, integrate information about the state of the gastrointestinal tract, and provide outputs to control gut movement, fluid exchange between the gut and its lumen, and local blood flow (1, 2). There are several distinct features of enteric neurons which contribute to their ability to regulate gastrointestinal functions (3). The function of the ENS is to coordinate the complex interactions of the enteric networks, which consist of sensory, inter, motor and secretomotor neurons (4).

The ENS is usually formed from two major plexus: the myenteric plexus mainly regulating muscle activity and the submucous plexus mainly regulating mucosal functions. The myenteric plexus is an endogenous source for motor innervation to the muscular layers and secretomotor innervation to the mucosa. Similar to other nervous systems, the functions of the myenteric plexus are mediated by various neurochemical substances (2, 5, 6).

The extrinsic and intrinsic components of the enteric nervous system appear to be the primary mechanisms involved in motility regulation. It is well known that besides the noradrenergic and cholinergic regulations, serotonergic and peptidergic ones are present, too (7), some of them having a facilitatory action, others having inhibitory effects upon classical neurotransmitters (8-11).

Calcitonin gene-related peptide (CGRP) which was originally identified as a splicing product of the alternative RNA processing of the calcitonin gene in the rat brain, affects a variety of biological activities in the ENS, such as release of gastrointestinal hormone, co-ordination of gastrointestinal motility, excitation of myenteric neurons and vasodilatation (12).

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Substance P (SP) is a member of a family of peptides called the tachykinins (13) and a neurotransmitter that plays an important role in regulating gastrointestinal motility (14). SP was isolated from the gut and can be detected in a dense nerve fiber network within the myenteric plexus. SP-containing nerve fibers are an intrinsic contractor of the longitudinal muscle layer (5, 14, 15).

NPY is widely distributed in the central and peripheral nervous system and represents one of the most abundant neuropeptides (16). It has been functionally related to regulation of blood pressure, circadian rhythms, feeding behavior, anxiety, memory processing, and cognition in the central nervous system, and to vasoconstriction and gastrointestinal tract motility in the peripheral nervous system (17, 18).

Although the immunoreactivity of the above-mentioned neurotransmitters has been studied in ENS of several laboratory animals (14, 19-22), these neurotransmitters have not been examined in small and large intestine of mole-rats (*Spalax leucodon*). Thus, we have investigated the location of CGRP, SP, NPY, VIP and serotonin immunoreactivity in the extrinsic and intrinsic innervation of the mole-rats (*Spalax leucodon*) intestines.

Materials and Methods

Animal and tissue samples: In the present study, six adult mole-rats, trapped by the farmers, were used. After the mole-rats (*Spalax leucodon*) were anaesthetized with 2.5% pentathol (6 mL/kg), the left carotid artery was cannulated at the base of the neck and allowed to exsanguinate. Tissue samples were taken from the small and large intestine (ileum and colon) and fixed in 10% neutral-buffered formalin for 24 hr. Then tissues were dehydrated through graded ethanol and embedded in paraffin. Five μ m thick sections, mounted on poly-L-lysine coated glass slides, were obtained and processed for immunohistochemical staining.

Immunohistochemistry: Immunohistochemical staining was performed by using avidin-biotin-peroxidase complex (ABC) methods. According to the ABC methods, tissues were incubated in citrate buffer (10 mM citric acid, pH 6.0) in a microwave oven at 750 W for 20 minutes to retrieve antigenicity. Quenching of the endogenous peroxidase activity was done with 3% hydrogen peroxide (H_2O_2) in methanol for 10 minutes. In order to block unspecific binding incubation with normal goat serum (1:10) in 0.1 M phosphate buffered saline (PBS), pH 7.2 was performed. Sections were incubated with primary antibodies for 16–20 hours at 4°C. The working dilutions and the sources of the primer antibodies were listed in Table 1. The primary antibodies were diluted in PBS containing 0.25% sodium azide and 2.5% bovine serum albumin (BSA). Negative control sections were performed by replacing the primary antibodies with PBS. The sections were then incubated with biotinylated goat anti-rabbit immunoglobulin G (Sigma) followed by avidin-biotin-peroxidase complex (Dako) both at dilution of 1.50 in PBS for 1 hour at room

temperature. Sections were washed in PBS for 30 minutes after each incubation step and finally immersed in glucose oxidase–DAB (diaminobenzidine)–nickel ammonium sulphate substrate (23) for 10 minutes. After washing in distilled water and counterstaining with hematoxylin, sections were dehydrated and coverslips mounted with mounting medium. Primary antibodies were omitted in negative controls; no immunoreactivity was detected in these preparations.

The specificity of each immunohistochemical reaction was determined as recommended by Sternberger (24) including the replacement of specific antiserum by the same antiserum which had been preincubated with its corresponding antigen. Sections were examined with microscope (Olympus BX–51, New York) and the photographs were taken.

Table 1. Details of antibodies used in this study

Primer Antibodies	Dilution	Trade Name	Cat. No.
CGRP	1:500	Chemicon (Millipore), Canada	AB5920
SP	1:500	Chemicon (Millipore), Canada	AB1566
NPY	1:200	Chemicon (Millipore), Canada	AB1915
VIP	1:100	Chemicon (Millipore), Canada	AB982
Serotonin	1:500	Zymed (Invitrogen), UK	18-0077

All antisera were raised in rabbit (polyclonal). CGRP, Calcitonin Gene-Related Peptide; SP, Substance P; NPY, Neuropeptide Y; VIP, Vasoactive Intestinal Peptide.

Results

In the present study CGRP, SP and NPY immunoreactivities were found in the nerve cell bodies and fibers of the intestinal myenteric plexus and submucous plexus of the mole-rats (*Spalax leucodon*). However, VIP and serotonin immunoreactivity were not observed in any intestinal region.

CGRP immunoreactivity was very numerous in the small and large intestine. These immunoreactive neuronal structures were detected within the myenteric plexus. The myenteric plexus was located between the inner and outer musculature. In addition, structures belonging to the myenteric plexus were often embedded in the circular musculature of the ileum. This plexus was arranged in an almost continuous layer which contained both nerve cell bodies and fibers in the ileum (Figure 1A). Also, the neuronal cell bodies exhibiting strong CGRP immunoreactivity was in the form of enteric ganglia in the colon myenteric plexus (Figure 1B).

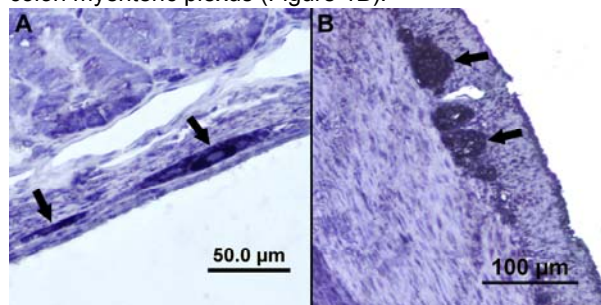


Figure 1. CGRP-immunoreactive neuronal cell bodies (arrows) are observed in the myenteric plexus of ileum (A) and colon (B)

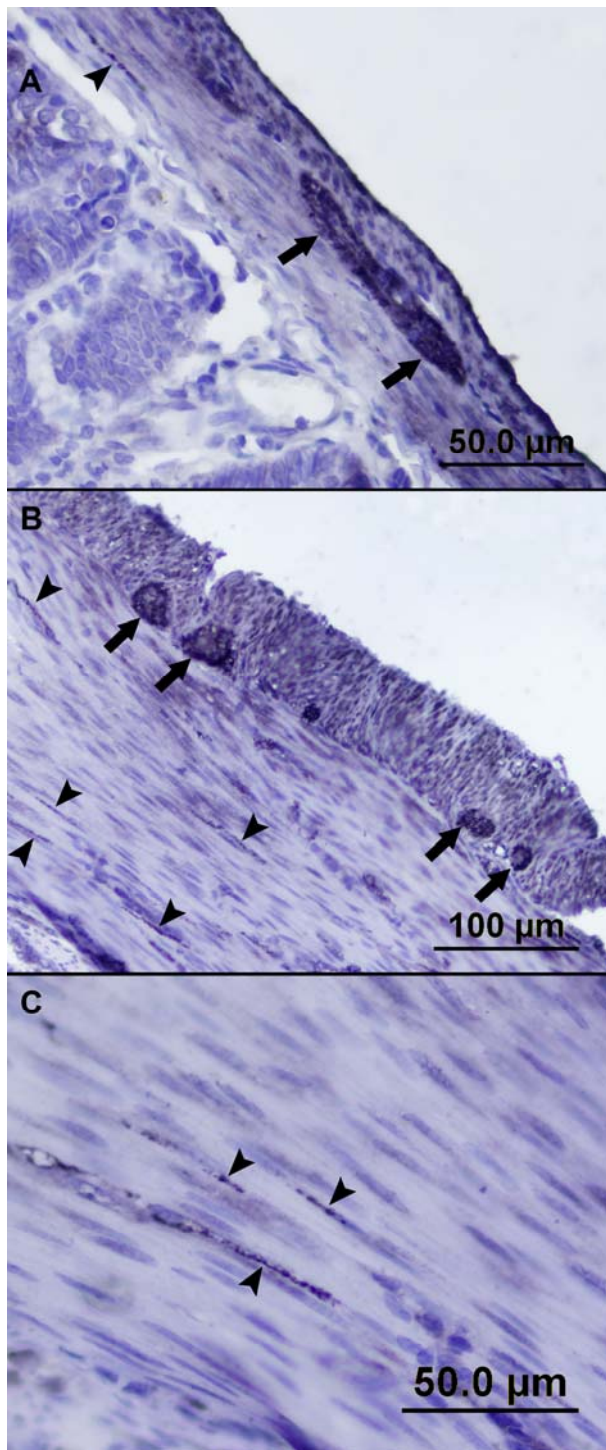


Figure 2. SP-immunoreactive neuronal cell bodies (arrows) are shown in the myenteric plexus of ileum. Note that nerve fiber varicosities (arrowheads) lie in the circular layer of the tunica muscularis (A). SP-immunoreactive neuronal cell bodies are seen in the myenteric plexus of colon (B). SP immunoreactive nerve fiber varicosities (arrowheads) lie in the circular layer of the tunica muscularis colon (B and C)

SP immunoreactivity was detected in the tunica muscularis of the small and large intestine. The immunoreactivity was discernible with particular evidence within nerve fibers running in both the musculature and myenteric ganglia of ileum (Figure 2A). SP-immunoreactive cell bodies of myenteric neurons were localized in the small groups within longitudinal musculature of colon (Figure 2B). In addition, these immunoreactive nerve fibers were found in the circular musculature of this intestinal region (Figure 2C).

NPY immunoreactive nerve cell bodies and fibers were present in the lamina propria, myenteric and sub-serosal plexus of small and large intestine. The immunoreactive nerve cell bodies were seen between the inner and outer musculature layers of ileum, sometimes opposed to negative nerve cell bodies. Also, it was observed in the sub-serosal plexuses of this intestinal region (Figure 3A). In the colon, NPY immunoreactivity was detected in subtle nerve fibers of the lamina propria. Additionally, IR nerve cell bodies were mostly grouped in small numbers, forming tiny ganglia in the myenteric plexus of the colon (Figure 3B).

VIP and serotonin immunoreactive neuronal structures were not detected any other region of the small and large intestine.

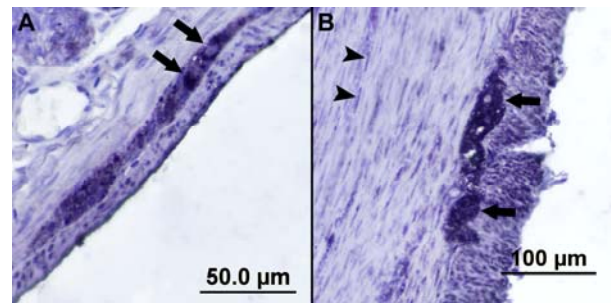


Figure 3. NPY-immunoreactive neuronal cell bodies (arrows) are shown in the myenteric plexus of ileum (A). NPY-immunoreactive neuronal cell bodies (arrows) are seen in the myenteric plexus as well as nerve fiber varicosities in the circular layer of colon tunica muscularis (B).

Discussion

The basic organisation and function of the ENS in all species is much the same (25). However, there are some differences between large and small mammals (26). CGRP (27, 28) and various other peptides such as SP, NPY and VIP are expressed in the myenteric neurons of the variety mammals (19, 29-33). No information concerning the occurrence of the neuronal structures in the gastrointestinal tract in subspecies of mole-rats (*Spalax leucodon*) is available apart from the regional distribution and relative frequency of the endocrine cells in the gastrointestinal tract (34). The present study was identified the existence and distribution of neuronal structures in the small and large intestine of the mole-rats. This is the first report showing that the CGRP, SP

and NPY innervation in the mole-rat (*Spalax leucodon*) intestines.

Neurons expressing the CGRP were present in the myenteric ganglia of the intestine in many mammalian species (22, 35, 36). CGRP immunoreactivity was reported in the myenteric plexus and submucosal plexus in the human small intestine (37). CGRP-IR was also localized to neurons of the myenteric plexus and to the fibers in the mucosal folds of the mice small intestine (32). Chiocchetti et al. (33) reported that the existence of CGRP-IR in fibers and nerve cell bodies located in the myenteric and submucosal plexuses of lamb ileum. In addition, CGRP-containing neurons were observed in the myenteric plexus of the rat colon (19, 22, 30). CGRP-containing neurons in many species, including the rat, exhibit morphological similarities. However, in this study, CGRP-IR was observed in wide ganglioner form within myenteric plexus in both the small and large intestine. In the submucosal plexus, the neuronal elements were not present. The neuronal density was too high particularly in the circular musculature. The results of this study were in agreement with the immunohistochemical studies reported by some authors (19, 22, 30, 32).

SP immunoreactive neurons that innervate the longitudinal muscle of the guinea pig ileum were identified in the longitudinal muscle plexus (31). A study in the canine ileum demonstrated SP-IR varicose nerve fibers in the myenteric plexus and both external smooth muscle layers (29). Sang et al. (38) reported that SP immunoreactive nerve fibers were located in the circular musculature of the mouse large intestine. In addition, some studies reported that the presence of SP-IR nerve fibers in the myenteric plexus and longitudinal

musculature of the guinea pig colon (39). Similar to the findings reported for canine, SP-IR nerve fibers showed the same localisation in ileum in the present study (29). Furthermore, as in the guinea pig colon (39), SP-IR was found in the myenteric plexus and the longitudinal musculature of the mole rats.

Autoradiographic studies had shown the presence of NPY receptor in the submucosal and myenteric ganglia in the intestine (40, 41). In the rat intestinal tract, Y1 receptor immunoreactivity was detected in the submucosal and myenteric nerve cell bodies (20). In addition, NPY-IR nerve cell bodies were only observed in the myenteric plexus of the rat small intestine and canine ileum (21, 29). The other study was reported that NPY-IR were present immunoreactive fibers forming a dense network and immunoreactive nerve cell bodies in the lamina propria and submucosal plexus of the rat small intestine; they were few in this location in the colon (42). Also, another study was demonstrated the presence of NPY receptor immunoreactivity in the myenteric and submucosal nerve plexuses of the human colon (43). As in the some studies (20, 21, 42, 43), NPY immunoreactivity was detected in the lamina propria, myenteric and sub-serosal plexuses of small and large intestine in this study. Our findings showed the presence of dense immunoreactive localization in the colon myenteric plexus by contrast with Sundler (42).

In conclusion, this study demonstrated the presence of different neuronal populations in the mucosal, submucosal and ganglionated plexuses in the small and large intestine of the mole rats. This is the first report of neuronal localization of CGRP, SP and NPY in the mole rat intestines.

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