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• BASIC RESEARCH •

Localization of neurokinin B receptor in mouse gastrointestinal tract

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Abstract

AIM: **To observe the location of neurokinin receptor (NK3r) in the mouse gastrointestinal tract.**

METHODS: **The abdomens of 8 male Kunming mice were opened under anaesthesia with sodium pentobarbital. The exposed gut organs were kept moisture and temperature at the same time. Then the esophagus, jejulum, ileum, colon, etc were respectively cut and the segments from the stomach to the distal colon were opened along the mesenteric border. A circular 4mm~6mm enteric part(pieces of 1 cm^2 were to be prepared) and mucosa and submucosa were removed, then the longitudinal muscle layer was pulled off from the circular muscle layer under microphotograph. They were rinsed in 50n $mol \cdot L^{-1}$ potassium phosphate-buffered saline(PBS). Immunohistochemistry and immunoreactive fluorescence were used in the staining procedures.**

RESULTS: **There was not NK3r-Like(-Li) positive material on the smooth muscle cells of the esophagus, stomach, and intestines and other regions. The nerve cell bodies with immunoreactivity for NK3r were mainly distributed in the submucousal nerve plexus or myenteric nerve plexus of the gastrointestinal tract except for the esophagus, stomach and rectum. The reaction product was located on the surface of the nerve cell plasma. It was occasionally observed in the cell plasma endosomes, but was very weakly stained. Among the NK3-like positive neurons in the plexus,the morphological type in many neurons appeared like Dogiel II type cells. Some neuron cell bodies were big, having many profiles, some were long ones or having grading structure. Cell body diameter was about 10μm-46μm and 8μm-42μm in myenteric plexus and submucous plexus.**

CONCLUSION: **This study not only described the distribution of neurokinin B receptor in the mouse gut in detail, but also provided a morphological basis for deducing the functional identity of the NK3r-LI immunoreactivity neurons, suggesting the possibility that these neurons were closely related to gastrointestinal tract contraction and relaxing activity.**

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INTRODUCTION

The tachykinin family of neuropeptides mainly includes neurokinin A(NKA) neurokinin B(NKB),and the neurotransmitters in the peripheral and central nerve systems[1]. Tachykinin and their receptors (NK1\NK2\NK3) play an important role in the nerve system. Various studies have been undertaken in NK3 receptor's distribution in rat CNS[2,3].Our previous experiments suggest that NK3 receptor in the paraventricular and supraoptic nuclei of the rat hypothalamus may be involved in the modulation of the release of vasopressin from the hypothalamus when the internal environment was disturbed[3]. Some research results[4] implicated that tachykinin located in the intestine intrawall neurons is attributable to the activation of gut and its modification. Pharmacological research work has proved that NK3r was located in neurons rather than in muscle cells[5,6]. Having characterized the antisera, researchers localized the neurokinin receptor in the rat/guinea gut[6,7]. NK2r was also detected in the muscularis mucosa of the small intestine and the colon. NK2r was localized in the plasma membrane of smooth muscle cells and was present on numberous nerve terminals in both the submucosal and the myenteric plexus of the small intestine[4,7,8], but NK1r-Li reactive product existed only in the neurons that resemble the interstitial cells of Cajal[9-11]. Mann *et al*[12] and Grady *et al*[7] reported that in rat myenteric plexus there were neurons that could express NK1r. These neurons had colocalization with NK3r-Li positive neurons. Although all these results implicated that tachykinin receptors existed in the mouse gastrointestinal tract, and that they might be related to intestinal muscle contractile activity[13] in addition to its influence in blood pressure and increase of vascular penetration. Up to now, we have not seen reports about this receptor's distribution in mouse gastrointestinal tract.Our aim was to further investigate this receptor distribution in mouse gut.

MATERIALS AND METHODS

Animals and material preparation

Ten male Kunming mice weighing 20g-30g were chosen and the abdomens of 8 mice were opened under anaesthesia with sodium pentobarbital. To keep moisture and temperature we cleaned the exposed gut organs with 0.01 $mol \cdot L^{-1}$PBS (phosphate-buffered saline, pH 7.0) continously. Then the esophagus, jejulum, ileum, colon, etc were respectively cut and the segments from the stomach to the distal colon were opened and cleaned by 0.01$mol \cdot L^{-1}$ PBS containing smooth muscle relaxing medicine. And they were filled with 40$g \cdot L^{-1}$ polyformaldehyde and the two ends were closed tightly and fixed for 6-8 h. The stomachs were fixed for 8 h following Peng Xi's suggestion(1999)[14] and incubated in 300$g \cdot L^{-1}$ sucrose for 24 h (overnight) together with other organs after cut and cleaned with 0.01 $mol \cdot L^{-1}$ PBS. Gastrointestinal tract was processed from stomach to the distal colon and was opened along the mesenteric border. A circular 4-6mm enteric part(pieces of 1 cm^2 were to be prepared)

was obtained, the longitudinal muscle layer was pulled off from the circular muscle layer with fine forceps and processed for immunohistochemical examination along the mesenteric border rinsed in phosphate-buffered saline and prepared for layer separation. Gastrointestinal tracts were cut into small pieces of 1 cm^2 and mucosa and submucosa were removed with forceps. Then they were rinsed in 50 $nmol \cdot L^{-1}$ potassium phosphate-buffered saline(PBS) and prepared for layer separation. These procedures can be done under anatomy microphotography.

Staining procedures

Staining procedures: whole-mount free-floating tissues and slide-mounted sections were washed in 50 $mmol \cdot L^{-1}$ KPBS, incubated with 0.3%H_2O_2 (30min), rinsed again in 50m $mol \cdot L^{-1}$ KPBS, and incubated in diluent containing of 50mM KPBS, 4$g \cdot L^{-1}$ Triton X-100, 10 $g \cdot L^{-1}$ bovine serum albumin, and 10 $ml \cdot L^{-1}$ normal goat serum for 30min at 22℃, then transferred to NK3r affinity-purified antibodies diluted to 1:50 in the same diluent for 48-96 h at 4℃. Tissues were washed in 50m $mol \cdot L^{-1}$ KPBS with 0.2$g \cdot L^{-1}$ TritonX-100 and incubated with biotinylated goat anti-rabbit IgG(Vector) diluted to 1:200 for 3 h at 22℃(Vector),followed by avidin-biotin complex coupled with horseradish peroxidase 1:200 for 2 h at 22. C(Vector). The horseradish peroxidase reaction product was virsualized with 0.4 $g \cdot L^{-1}$ diaminobenzidine tetrahydrochloride and 0.1 $mL \cdot L^{-1}$ H_2O_2 dissolved in 0.1 $mol \cdot L^{-1}$ sodium acetate. The reaction was terminated by two consecutive 9 $g \cdot L^{-1}$ NaCl washes.

The gastrointestinal tracts of the remaining mice were pulled off and cut on a cryostate at -20℃, rinsed by 0.01 $mol \cdot L^{-1}$ PBS and incubated in 40 $g \cdot L^{-1}$ formaldehyde fixative overnight at 4℃. They were incubated again in 300 $g \cdot L^{-1}$ sucrose and embedded by OCT on the next day. Their cryostat sections were 10μm in thickness, and immunohistochemically stainied after the slides were cooled according to the earlier procedure.

RESULTS

Immunohistochemical results showed that NK3-Li neurons and fibers existed in enteric plexus(submucous plexus and myenteric plexus) in the duodenum, jejunum, ileum and colon (Figures 1-8). Staining intensity in myenteric plexus was a little stronger. And the esophagus and the stomach were immunoreactively negative. No positive immunoreactive product was found in esophagus smooth muscle cells.

NK3-Li positive substance existed mostly in the membrane, some or a little in the plasma, but none in the nuclei. The surface of the positive neurons was well stained and was more apparent than those in the cell body(Figures 1,2,4,8). The neurons stained in myenteric plexus had high intensity, the NK3-Li neurons and fibers were web-like(Figure 3). And the fibers between the positive neurons were often of a beaded or granular appearance(Figure 7). Between fibers just like necklace among longitudinal muscle-myenteric plexus neuronal fibers there were striated fibers, that connect with them. From the cryostat sections, NK3r-Li immunoreactive product was found in intestinal myenteric plexus.

Among the NK3-like positive neurons in the plexus, the morphological type of many neurons were like Dogiel Ⅱ type cells: round or ellipse shape or presenting several dividing beaded profiles. Some neuron cell bodies were bigger, having many profiles, some of which were long or having grading structures. Results by microscopic counting are shown in Figures 1 and 2.

Table 1 The number of NK3r immunoreactivity neuron cell bodies in mouse gastrointestinal Tract*

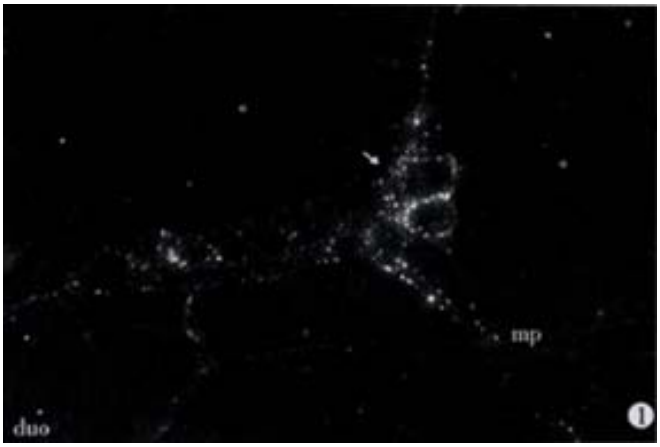
Region	Neuronal density /(cells·cm^{-2}) ($\bar{x}$±s)	
	Myenteric plexus	Submucous plexus
Esophagus	Almost no	Almost no.
Stomach	Almost no.	Almost no.
Duodenum	82±14	95±12
Jejunum	249±37	237±31
Ileum	143±24	140±19
Colon	118±17	107±15

*neurons in areas of 0.05 cm^2 were counted.

Table 2 Morphological characteristics of NK3r stained neurons in the mouse gastrointestinal tract

plexus	Region	Shape		d (cell soma)/μm	
		Unipolaror bipolar	Multipolar	Major	Minor
Myenteric	Oesophagus	(+/-)	(+)		
	Stomach	(+/-)	(+)		
	Duodenum	++	+++	17-38	10-26
	Jejunum	++	+++	25-46	13-20
	Ileum	++	+++	21-37	12-18
	Colon	+	++	15-36	14-16
	Rectum	+/-	+	13-34	8-19
Submucous	Stomach	(+/-)	(+)		
	Duodenum	++	+++	16-35	8-11
	Jejunum	++	+++	19-42	10-13
	Ileum	++	+++	14-35	9-17
	Colon	+	++	13-27	8-15

(+/-) almost no; (+) very rare; + infrequent; ++ common; +++ abundant



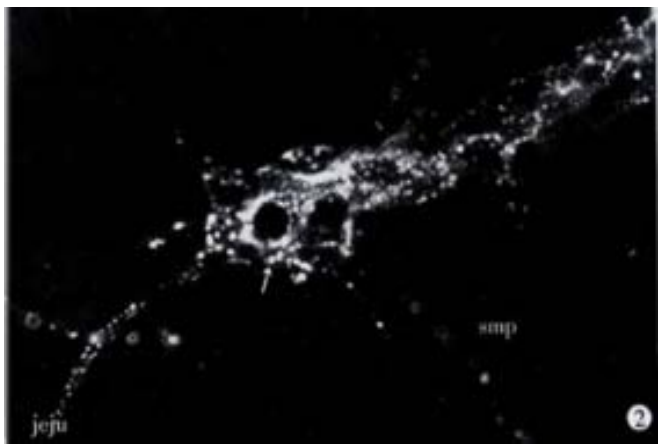


Figure 1 NK3r-like(Li) neurons in myenteric nerve plexus of duodenum, mainly located on cell membrane. Arrow: positive neuron; Duo: duodenum,mp: myenteric plexus.

Figure 2 NK3r-Li neurons in submucosa neural plexus of jejunum. Arrow: positive neuron cell bodies; Smp: submucosa nerve plexus; Jeju: jejunum.

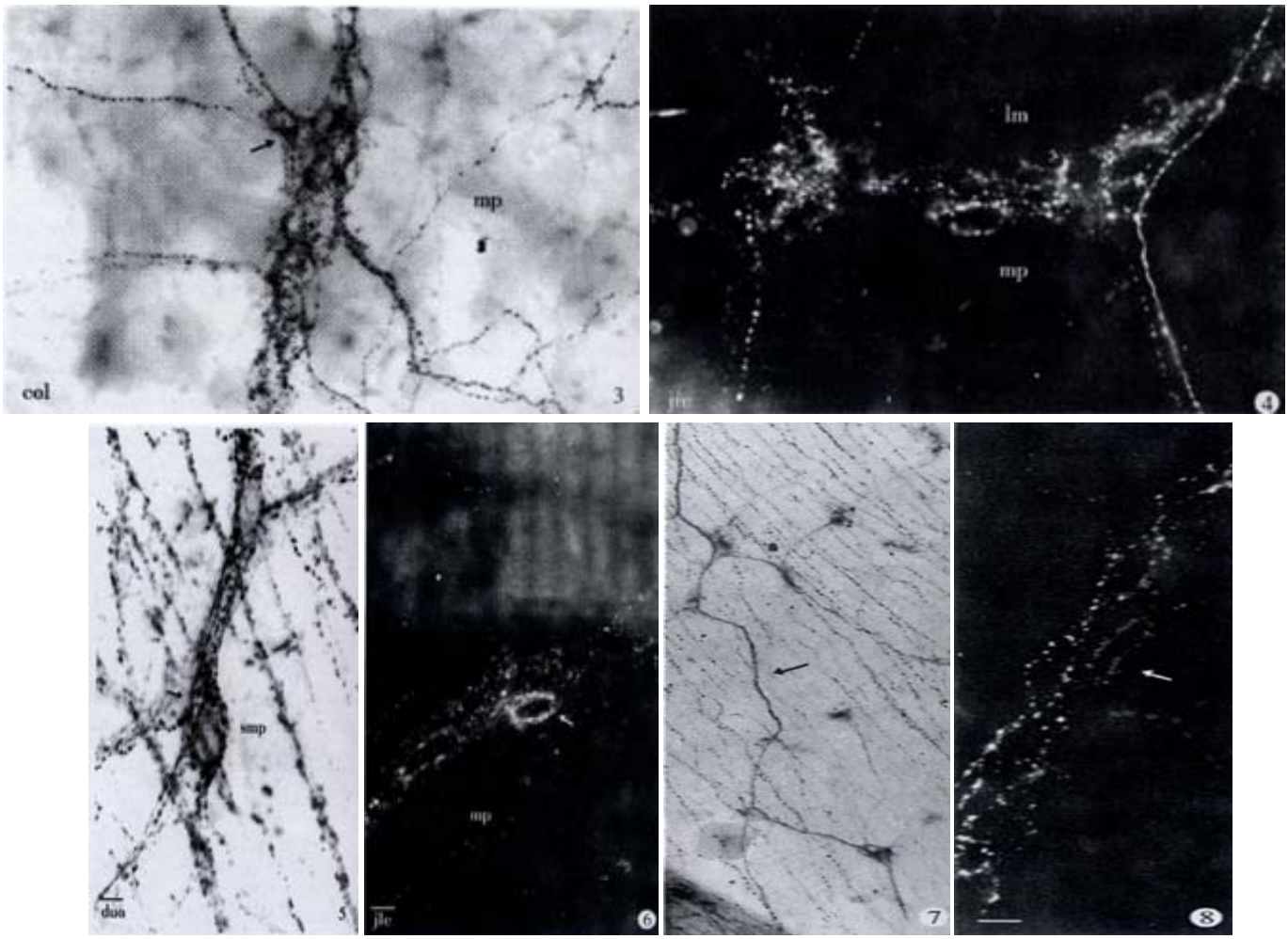


Figure 3 NK3r-Li product mainly found on the cell membrane in the colon's myenteric plexus. Dark arrow: positive neuron; Col: colon; mp: myenteric plexus.
Figure 4 The NK3r-Li neurons in the myenteric nerve plexus of the ileum. Mp: myenteric plexus; Ile: ileum; Lm: longitudinal muscles; Cm: circular muscles.
Figure 5 The NK3r-Li neurons in the submucosa nerve plexus of the duodenum. Dark arrow: positive neuron; Smp: submucosa nerve plexus. Bar=20ìm in Figures 1-5
Figure 6 NK3r-Li neurons in the neuron ganglion of the ileum myenteric nerve plexus; mp: myenteric plexus. Bar=40μm
Figure 7 The NK3r-Li nerve fibers like a necklace.
Figure 8 The higher magnification of Dogiel II type NK3r-Li neurons. Arrow: positive products on the cell membrane. Bar=6μm in Figures 7,8

DISCUSSION

There were some reports about tachykinin B receptor(NK3r) distribution in mammal gastrointestinal tract[7,12]. It was proved that NK3r-Li neurons were localized in enteric nervous system of rats and guinea pig[15]. We observed the NK3r distribution in mouse gastrointestinal tract. Our results were consistent with the previous results, and support the research work of Mann *et al*[12], and Grady *et al*[7], but we must claim several valuable differences: ① In our experiments we found that immunoreactivity was mainly located in the enteric nervous system neurons, rather than in mouse esophagus or other parts of the gut smooth muscles, which was different from the results of Maggi *et al*[5] and Guard *et al*[16]. ② We also found that there were different morphological type of NK3r-Li neurons in mouse jejulum and ileum nervous plexus: the first part gut plexus had more neurons than the second part gut plexus In ileum plexus NK3r-Li neurons were mostly of Dogiel Ⅱ type neurons[17], which was different from the results by other authors that NK3r-Li neurons were only located in the ileum. ③Although the immunoreactive products existed mainly in the enteric system neurons membrane, they were also detected in some positive neurons plasma. On the whole, cell morphology(size and shape) was similar to the cells that were observed and described by Mann *et al*. and Furness[18]. We also found that there were some small cell body neurons, which did not have clear characteristic positive shapes, so we might deduce that they had possibly different functions or belonged to dividing disparity. The location of the NK3r-Li immunoreactivity observed through the subnuclei of the NTS (solitary tract nucleus) suggested that NK3r-Li immunoreactive neurons might be involved in the medullar integration of the information conveyed by the afferent vagus nerve from the lower digestive tract[19]. The distribution of the NK3r-containing neurons coincided with the afferent fibers arising from the lower digestive tract. Vagal afferent fibers from the stomach and intestine terminated preferentially in the subnuclei medialis and commissuralis and in the substantia gelatinosa of the NTS (solitary tract nucleus)[20] as well. In the NTS, the subnucleus centralis represented the preferential termination sites for afferent fibers arising from the esophagus(vagus nerve). No or very few NK3r-Li neurons have been found in this subnucleus. We also found the supporting example for these referrences of NK3 receptor in the central nervous system and added proof to NK3 receptor in the peripheral nervous system. In the rat

brain, by situ hybridization method for NK3r mRNA, the results agreed well with the immunohistochemical results, however low levels of NK3r mRNA were found in rat stomach and intestines[21] which suggests that our detection of a large population of enteric neurons with NK3r immunoreactivity contradicts with Tsuchida *et al*'s results[22]. Binding studies also failed to detect NK3r in the gastrointestinal tract[22]. Our experiments showed that morphology of NK3r-Like neurons was in agreement with Dogiel II type neurons in size, shape and localization.The number of neurons was not very large and was less than the proportion of NK1r-Li neurons in the enteric nervous system.Considering NK3r-Li neurons' size in diameter, and the morphologic and pharmacological results, we speculate that NK3r-Li neurons are possibly a part of afferent internal neurons related to the enteric construction, relaxing the activity of mouse enteric nervous system, which was closely relatied to with some vice sympathetic nerve fiber functions. This is determined according to Furness *et al*'s[23] dividing method in guinea enteric nervous system neurons that is regarded as right, but at the same time it needs further verifications by functional test. Functional experiments supported the neuronal localization of the NK3r: the NK3 agonists NKB senktide stimulated contraction of rat duodenum and guinea pig ileum[24,25]. The response of the guinea pig ileum were abolished by tetrodotoxin and reduced by atropine which indicated the presence of NK3r in the cholinergic neurons of the myenteric plexus[26]. Indeed, senktide stimulated acetyl choline to release from the myenteric neurons[16]. Barthó *et al* (1999)[24,25] proved that NK3r existed in rodent gastrointestinal (interwall) neurons and regulated tachykinin inducing the excitement of the enteric nervous system thus influencing construction[27].Our findings that neurons in the myenteric plexus could express NK3r is of particular interest and NK3r reacting production existed mainly on the plasma membrane of gastrointestinal tract intrawall neurons, implicating that tachykinin B transmitter possibly influence membrane receptor thus influencing the contraction and relaxing smooth muscle and even regulating the exocrine of gastrointestinal tract as well as the contents that some articles have described[28-33].

In summary, our research not only observed the distribution of NK3r-like neurons and fibers in mouse gastrointestinal tract, but also provided new insight into the cellular colocalization of receptor proteins in the mouse gut tract and a base for investigating NK3r-like neuron functions in the gut tract. These results might also give implications about muscle activity of contracting or relaxing action related to the neurokinin receptor.

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