Available online at www.sciencedirect.com
ScienceDirect
Progress in Natural Science 18 (2008) 245–249
Progress in Natural Science

Short communication

Distribution of ghrelin-like immunoreactive cells in amphioxus, *Branchiostoma belcheri* – A study of immunohistochemistry

You-Zhu Weng, Hai-Xia Song, Yong-Qiang Fang *

The Third Institute of Oceanography of the State Oceanic Administration, Xiamen 361005, China

Received 16 July 2007; received in revised form 14 August 2007; accepted 14 August 2007

Abstract

The distribution of ghrelin-like immunoreactive cells in amphioxus (*Branchiostoma belcheri*) was investigated by using immunohistochemical staining with rabbit antiserum against synthetical mammalian ghrelin. The results showed that ghrelin-like immunoreactive cells were distributed widely in the nervous system, Hatschek's pit, wheel organ, digestive tract and gonads (ovary and testis). In nervous system, ghrelin-like immunoreactive neurons and their protrusions were distributed specifically on the dorsal side, ventral side and funnel part of brain vesicle, with a few dispersive immunoreactive nerve cells and their fibers in nerve tube. Ghrelin-like immunoreactivities were also detected in Hatschek's pit epithelial cells and wheel organ cells, with positive substance located along cell membrane. In digestive tract, ghrelin-like immunoreactive cells existed in hepatic diverticulum, anterior and posterior region of midgut, and could be classified into two types, closed- and opened-type endocrine cells. The number of positive cells was most in hepatic diverticulum, secondary in posterior region of midgut and least in anterior region of midgut. In gonads, ghrelin-like immunoreactive substance was detected in oogonia, oocytes and follicle cells in ovary at the small and large growth stages and in early spermatogenic cells and Sertoli cells in testis. The extensive distribution of ghrelin-like cells in amphioxus suggested that these kinds of cells are conservative in evolution and diversified in function. At the same time, we found for the first time that ghrelin-like immunoreactive cells existed in brain vesicle and Hatschek's pit, which provided new morphological evidence for the existence of an activation pathway between brain vesicle and Hatschek's pit for the regulation of growth hormone excretion.

Keywords: Ghrelin; Amphioxus; Nervous system; Hatschek's pit; Digestive tract; Gonad

1. Introduction

Ghrelin, a 28-amino acid peptide, was originally isolated and purified from rat stomach by Kojima et al. and was identified as a natural endogenous ligand for the growth hormone secretagogue receptor (GHS-R) that functions in the stimulation of growth hormone (GH) release [1,2]. Thus, ghrelin is thought to be the stimulating system for hypothalamic regulation of pituitary GH excretion and to be a third regulator for GH release. Ghrelin had been demonstrated to be distributed in various tissues in vertebrates, mammalian (including human) [3–5], avian [6], amphibian [7] and fish [8–10]. It exerts many physiological functions. However, data about ghrelin in reptile and cyclostomata are not available now, and we do not know whether ghrelin exists in cephalochordate amphioxus, which is a transitional animal evolving from invertebrate to vertebrate. In this study, we carried out an immunohistochemical localization of ghrelin in nervous system (brain vesicle and nerve tube), Hatschek's pit, wheel organ, digestive tract and gonads (ovary and testis) of amphioxus using the antiserum against synthetical mammalian ghrelin,

* Corresponding author. Tel.: +86 592 2195277; fax: +86 592 2086646.
E-mail address: fant98@public.xm.fj.cn (Y.-Q. Fang).

1002-0071/$ - see front matter
doi:10.1016/j.pnsc.2007.08.006

which helped us to understand the distribution and function of ghrelin in amphioxus.

2. Materials and methods

2.1. Animals

Amphioxus (*Branchiostoma belcheri*) were collected during a period from May 2005 to April 2006 along the Qiongtou coast, Tongan' County, Xiamen, South China. Animals were reared in laboratory for a week and fed on cultured algae twice a day. A total of 20 amphioxus of both sexes, with a length of 43.8–56.6 mm, were used in this study.

2.2. Preparation of samples

After being anaesthetized at low temperature, the animals were fixed for 24 h in freshly prepared Bouin's solution without acetic acid. Specimens were cut into head, middle and caudal part in the process of dehydration through a graded ethanol series, and the head and middle part were subsequently embedded in paraplast. Paraplast blocks were sectioned at 6–7 μm thickness and mounted on poly-L-lysine-coated slides. Sections with the structure of brain vesicle, nerve tube, Hatschek's pit, different parts of digestive tract and gonads (ovary and testis) at different developmental stages were picked out under a microscope and selected for immunohistochemical staining.

2.3. Immunohistochemical staining

Sections were stained by the streptavidin–biotin–peroxidase (SABC) method. Deparaffinized sections were incubated in 3% H_2O_2 for 10 min to remove endogenous peroxidase. After heat-induced antigen retrieval and rinsing with distilled water, the sections were immersed in 0.01 mol/L PBS (pH 7.2–7.4) for 5 min, and then incubated with normal goat serum (1:10 dilution) for 15 min at room temperature. The sections were reacted with the polyclonal antibody against synthetical mammalian ghrelin (1:150 dilution, purchased from Wuhan Biological Technology Company) at 4 °C for 36 h. After being rinsed with PBS, sections were incubated for 20 min at room temperature with goat anti-rabbit IgG, which was followed by incubation for 20 min again with a streptavidin–biotin–peroxidase complex. Visualization of antigens was achieved with diaminobenzidine (DAB)/H_2O_2 solution. Negative controls included replacing the primary antibody with normal rabbit serum or PBS or omitting the primary antibody in the SABC reaction.

3. Results

The results of immunohistochemical staining showed that ghrelin-like immunoreactive cells were distributed in nervous system, Hatschek's pit, wheel organ, digestive tract, testis and ovary of amphioxus.

3.1. Ghrelin-like immunoreactive neurons in nervous system

The nervous system of amphioxus has a structure of solid tube traversing the whole body and it can be divided into brain vesicle and nervous tube (spinal cord). The results of immunohistochemical staining showed that ghrelin-like immunoreactive neurons were located on the dorsal side and ventral side of brain vesicle and on the funnel part near Hatschek's pit extending from the right side of brain vesicle. In these neurons deep brown immunoreactive substance was located along cell membrane but the nucleus showed negative reaction (Fig. 1(a) and (b)). On the longitudinal sections of nerve tube, positively stained neurons and their protrusions and nerve fibers were dispersedly distributed on the dorsal and ventral side and center of the nerve tube (Fig. 1(c)).

3.2. Ghrelin-like immunoreactivity in Hatschek's pit

The Hatschek's pit of amphioxus is the primitive pituitary of vertebrate and consists of three kinds of cells: (1) epithelial cells at the basal part with a prismatic or irregular shape. They appear in an irregular crisscross arrangement of 2–3 layers. Result of immunohistochemical staining showed that strong ghrelin-like immunoreactivity was detected on each layer of epithelial cells and wheel organ cells (Fig. 1(d)). At the same time, on the longitudinal section of brain vesicle and Hatschek's pit different sizes of neurons in the funnel part of brain vesicle were positive for ghrelin staining, which closely contacted the epithelial cells in Hatschek's pit (Fig. 1(e)). The control section with the primary antibody replaced by normal rabbit serum was immunonegative (Fig. 1(f)).

3.3. Ghrelin-like immunoreactive cells in digestive tract

The amphioxus digestive tract is a straight tract which consists of pharynx, short oesophagus, hepatic diverticulum, anterior and posterior region of midgut, hindgut and anus. Ghrelin-like immunopositive cells were found to be distributed in the hepatic diverticulum, anterior and posterior region of midgut, with the number most in hepatic diverticulum, secondary in the posterior region of midgut and least in the anterior region of midgut. The distribution patterns of these positive cells were different in different positions of digestive tract. Most cells in hepatic diverticulum were spindle-shaped with slightly bulgy apex and base parts, and a very fine middle part. Cytoplast of some cells extended to the base membrane (Fig. 2(a)). These cells were typical openedle-type gastrointestinal endocrine cells and could also be seen in the anterior region of midgut (Fig. 2(b)). There were two kinds of immunopositive cells in the posterior region of

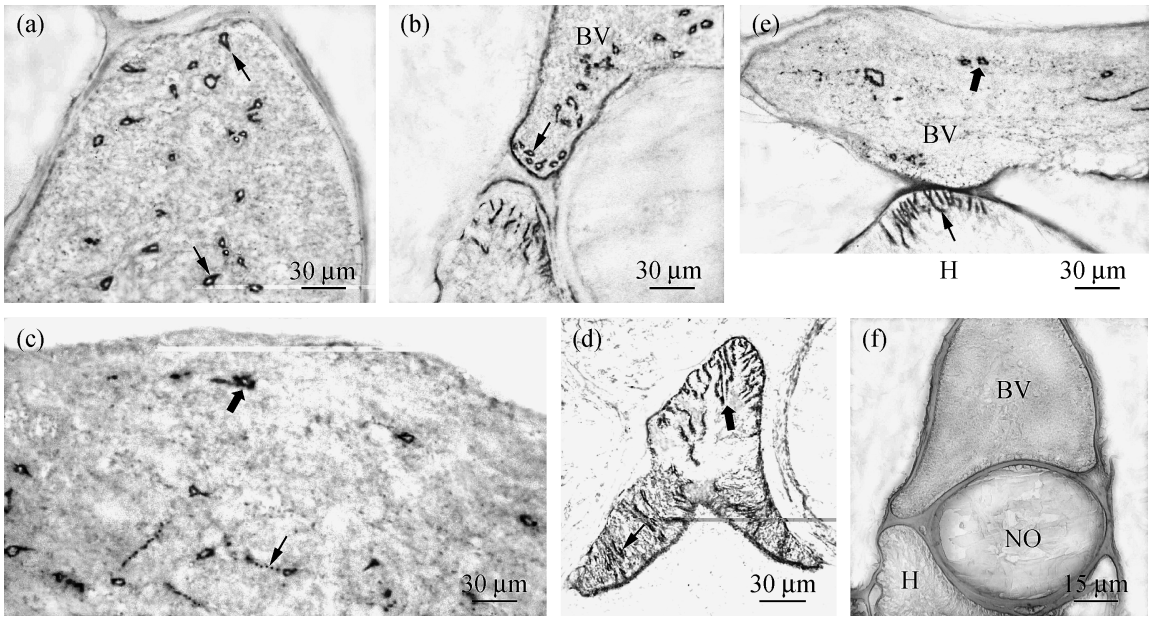

Fig. 1. Distribution of ghrelin in nerve system and Hatschek's pit of amphioxus. (a) Cross-section of head in male amphioxus. Ghrelin-like immunoreactive neurons and their protrusions were distributed on the dorsal and ventral side of brain vesicle (arrow); (b) ghrelin-like immunoreactive neurons were located on the funnel part on the right side of brain vesicle (arrow); (c) longitudinal section of nerve tube in female amphioxus. Ghrelin-like immunoreactive neurons and their protrusions (thick arrow) as well as nerve fibers (thin arrow) were dispersed on the dorsal and ventral side of nerve tube; (d) cross-section of head in female amphioxus. Ghrelin-like immunoreactive substance was distributed in epithelial cells (thick arrow) of 1–3 layers at the basal part of Hatschek's pit and in wheel organ cells (thin arrow); (e) longitudinal section of head in male amphioxus. Ghrelin-like immunoreactive neurons (thick arrow) in brain vesicle and immunoreactive cells (thin arrow) in Hatschek's pit were observed; (f) control section of head in male amphioxus was immunonegative by replacing the primary antibody with normal rabbit serum. BV, brain vesicle; H, Hatschek's pit; NO, notochord.

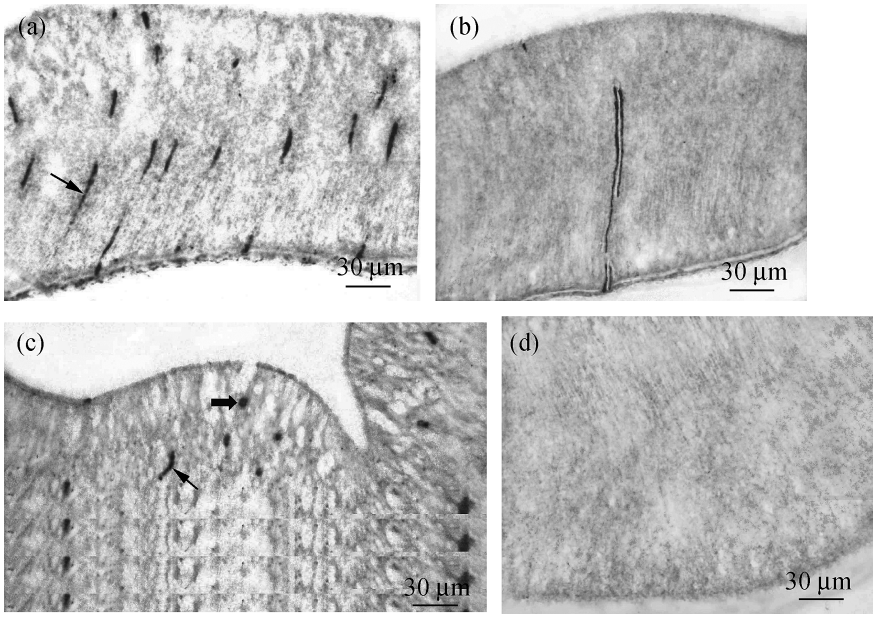

Fig. 2. Distribution of ghrelin in the digestive tract of amphioxus. (a) Cross-section of middle part in male amphioxus. Ghrelin-like immunoreactive cells were distributed in hepatic diverticulum and belonged to opened-type endocrine cells (arrow); (b) ghrelin-like immunoreactive cells in the anterior region of midgut; (c) ghrelin-like immunoreactive cells in the posterior region of midgut, which belonged to opened-type (thin arrow) and closed-type (thick arrow) cells, respectively; (d) the control section of anterior region of midgut was negative.

midgut, one was in circular or elliptic shape, located sporadically near enterocele, the other was spindle-shaped or in coniform. The former was a kind of typical closed-type gastrointestinal endocrine cells and the latter was opened-type cells (Fig. 2(c)). Other parts of digestive tract in amphioxus were all immunonegative for ghrelin. The control section omitting the primary antibody in the SABC reaction was negative (Fig. 2(d)).

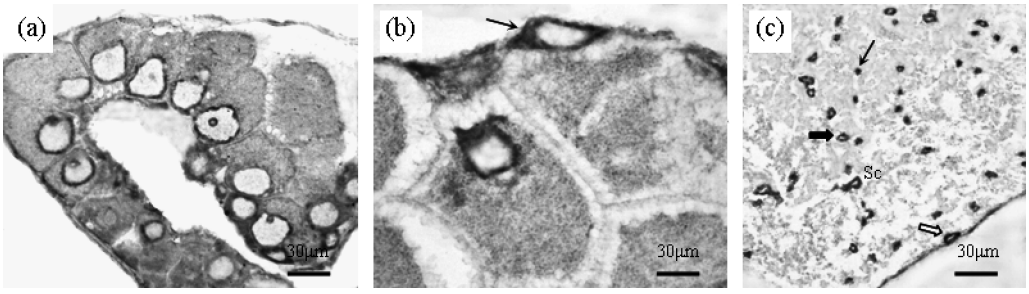

Fig. 3. Distribution of ghrelin in the gonads of amphioxus. (a) Ghrelin-like immunoreactive substance was located in karyotheca, nucleolus membrane, and cytoplast of oogonium and oocyte in ovary at stage I–II; (b) ghrelin-like immunoreactive substance in cytoplast of oocyte and follicle cells in ovary at stage III–IV; (c) ghrelin-like immunoreactive substance was detected in spermatogonia (hollow arrow), primary spermatocytes (thick arrow), second spermatocytes (thin arrow) and Sertoli cells (Sc) in testis at stage II–III.

3.4. Ghrelin-like immunoreactivity in gonads

In ovary, ghrelin-like immunoreactive substance was located in the karyotheca, nucleolus membrane, and cytoplast of oogonium and oocyte at small growth stage and large growth stage, and also in follicle cells, while the karyoplasm was negative for ghrelin (Fig. 3(a) and (b)). In testis, ghrelin-like substance was located in early spermatogenic cells (spermatogonia and primary spermatocytes) and Sertoli cells showed a deep brown color, while spermatid and spermatozoa were negative (Fig. 3(c)).

4. Discussion

Since Kojima et al. found ghrelin firstly in 1999, studies about its tissue localization, molecular structure, physiological function and regulation have become one of study hotspots of endocrinology. Ghrelin-like immunoreactive cells are widely distributed in many tissues of animals including stomach, intestine, brain, pituitary, gonad, pancreas, kidney, liver, spleen, gill and head kidney of most vertebrates. In the present study, we found for the first time that ghrelin-like immunoreactive cells were distributed widely in the brain vesicle, nerve tube, Hatschek's pit, wheel organ, digestive tract, testis and ovary of cephalochordate amphioxus, indicating that ghrelin-like cells are highly conservative in the progress of vertebrates' evolution.

Existing data indicated that ghrelin is a kind of multifunction hormone and its primary function is to stimulate appetite and GH release from pituitary. A series of physiological experiments *in vitro* and *in vivo* revealed that administration of ghrelin could strongly stimulate GH release from pituitary in a dose-dependent manner which had been demonstrated in human [11], rat [1], chicken [12], frog [13] and fish [14] with a potency equal or excess to that induced by hypothalamic growth hormone releasing hormone (GHRH) and that the action mechanism of stimulation of GH release from pituitary by ghrelin was different from that by GHRH [15]. We have reported that a regulatory system for inhibition of GH release from Hatschek's pit might have been developed between brain and Hatschek's pit in amphioxus as that in vertebrates [16]. However, we did not know whether a stimulating system (such as GHRH and ghrelin) for regulating GH release from Hatschek's pit also existed in the brain vesicle of amphioxus.

In the present study, we found that ghrelin-like immunoreactive neurons existed in the brain vesicle of amphioxus, which provided morphological evidence for the existence of a stimulating system between brain vesicle and Hatschek's pit in amphioxus. In addition, we think that as the ghrelin cells in the pituitary of vertebrates [17] these ghrelin-like immunoreactive cells located in Hatschek's pit of amphioxus might directly influence the secretion activity of GH cells in Hatschek's pit in an autocrine and/or paracrine manner.

Studies in mammals (including human) and fish indicated that ghrelin exerted strong stimulation effect on food in take [18] via a kind of appetite-stimulating peptide in brain, namely neuropeptide (NPY) [19]. We also found in our experiment that NPY immunoreactive neurons existed in the brain of amphioxus [20]. Thus, combining with the observation in this study, we assume that two kinds of neurons in the brain of amphioxus constitute the appetite regulation center in this species. However, the function of ghrelin- and NPY-positive neurons in appetite regulation in amphioxus needs further studies.

The structure and function of hepatic diverticulum in amphioxus are similar to those of stomach in vertebrates. We observed that most of ghrelin-like immunoreactive cells existed in hepatic diverticulum as that in mammal, chicken and frog [1,7,8], indicating that hepatic diverticulum is the main localization for ghrelin in the digestive tract of amphioxus. In addition, two kinds of ghrelin-like immunoreactive cells in the gastrointestinal tissue of amphioxus were detected, similar to that in rat [21]. In amphioxus, opened-type ghrelin-like cells are characterized by their cytoplasm protrusion of cells extending to the intestinal cavity, a character similar to that in mullet [22], and by their long cytoplasm protrusion extending to the base membrane. These opened-type ghrelin-like cells might influence the secretion activity of intestinal epithelial cells in a paracrine manner.

Data about effects of ghrelin on the reproduction function of animal almost came from mammals. Ghrelin had been found to be expressed in the luteal cells and hilus interstitial cells in ovary [23,24] of primates (including human) and rodent, but not in non-mammals. The present study demonstrated that oogonia, oocyte and follicle cells in ovary and Sertoli cells and spermatogenic cells in testis were all immunoreactive to ghrelin antibody, which will provide an important clue for further tracing the function of ghrelin during the gonad development in non-mammals.

In conclusion, the present study demonstrated that ghrelin-like immunoreactive cells and substance existed in the brain vesicle, nerve tube, Hatschek's pit, wheel organ, digestive tract, ovary and testis of cephalochordate amphioxus, and suggested that amphioxus might have built a stimulating mechanism for regulating GH secretion from Hatschek's pit as that in vertebrates. Existence of ghrelin in various tissues of amphioxus indicated that ghrelin exerts different physiological functions in amphioxus. To reveal ghrelin function will have scientific significance in elucidating the evolution of endocrine system in vertebrates and non-vertebrates.

Acknowledgement

This work was supported by National Natural Science Foundation of China (Grant Nos. 30470224, 40576075).

References

[1] Kojima M, Hosoda H, Date Y, et al. Ghrelin is a growth-hormone-releasing acylated peptide from stomach. Nature 1999;402:656–60.
[2] Kojima M, Kangawa K. Ghrelin: structure and function. Physiol Rev 2005;85:495–522.
[3] Kamegai J, Tamura H, Shimizu T, et al. Regulation of the ghrelin gene: growth hormone-releasing hormone upregulates mRNA in the pituitary. Endocrinology 2001;142:4154–7.
[4] Date Y, Nakazato M, Murakami N, et al. Ghrelin acts in the central nervous system to stimulate gastric acid secretion. Biochem Biophys Res Commun 2001;280:904–7.
[5] Mondal MS, Date Y, Yamaguchi H, et al. Identification of ghrelin and its receptor in neurons of the rat arcuate nucleus. Regul Pept 2005;126:55–9.
[6] Neglia S, Arcamone N, Esposito V, et al. Presence and distribution of ghrelin-immunopositive in the chicken gastrointestinal tract. Acta Histochem 2005;107:3–9.
[7] Galas L, Chartrel N, Kojima M, et al. Immunohistochemical localization and biochemical characterization of ghrelin in the brain and stomach of the frog *Rana esculenta*. J Comp Neurol 2002;450:34–44.
[8] Parhar IS, Sato H, Sakuma Y. Ghrelin gene in cichlid fish is modulated by sex and development. Biochem Biophys Res Commun 2003;305:169–75.
[9] Kaiya H, Kojima M, Hosoda H, et al. Amidated fish ghrelin: purification, cDNA cloning in the Japanese eel and its biological activity. J Endocrinol 2003;176:415–23.
[10] Kaiya H, Small BC, Bilodeau AL, et al. Purification, cDNA cloning, and characterization of ghrelin in channel catfish, *Ictalurus punctatus*. Gen Comp Endocrinol 2005;143:201–10.
[11] Arvat E, Di-Vito L, Broglio F, et al. Preliminary evidence that Ghrelin, the natural GH secretagogue (GHS)-receptor ligand, strongly stimulates GH secretion in humans. J Endocrinol Invest 2000;23:493–5.
[12] Baudet ML, Harvey S. Ghrelin-induced GH secretion in domestic fowl *in vivo* and *in vitro*. J Endocrinol 2003;179(1):97–105.
[13] Kaiya H, Kojima M, Hosoda H, et al. Bullfrog ghrelin is modified by *n*-octanoic acid at its third threonine residue. J Biol Chem 2001;276:40441–8.
[14] Kaiya H, Kojima M, Hosoda H, et al. Peptide purification, complementary deoxyribonucleic acid (DNA) and genomic DNA cloning, and functional characterization of ghrelin in rainbow trout. Endocrinology 2003;144:5215–26.
[15] Yamazaki M, Kobayashi H, Tanaka H, et al. Ghrelin-induced growth hormone release from isolated rat anterior pituitary cells depends on intracellular and extracellular Ca^{2+} sources. J Neuroendocrinol 2004;16(10):825–31.
[16] Fang YQ, Weng YZ. Distribution of growth hormone-like immunoreactive cells and somatostatin receptors in the nervous system and Hatschek's pit of amphioxus, *Branchiostoma belcheri*. Prog Nat Sci 2006;16:359–65.
[17] Korbonits M, Bustin SA, Kojima M, et al. The expression of the growth hormone secretagogue receptor ligand ghrelin in normal and abnormal human pituitary and other neuroendocrine tumors. J Clin Endocrinol Metab 2001;86:881–7.
[18] Unniappan S, Canosa LF, Peter RE. Orexigenic actions of ghrelin in goldfish: feeding-induced changes in brain and gut mRNA expression and serum levels, and responses to central and peripheral injections. Neuroendocrinology 2004;79:100–8.
[19] Cowley MA, Smith RG, Diano S, et al. The distribution and mechanism of action of ghrelin in the CNS demonstrates a novel hypothalamic circuit regulating energy homeostasis. Neuron 2003;37(4):649–61.
[20] Weng YZ, Fang YQ. Distribution of neuropeptide Y-like and β-endorphin-like immunoreactivity in the nervous system and Hatschek's pit of amphioxus, *Branchiostoma belcheri*. Acta Biol Exp Sin 2002;35(3):205–10.
[21] Sakata I, Nakamura K, Yamazaki M, et al. Ghrelin-producing cells exist as two types of cells, closed- and opened-type cells, in the rat gastrointestinal tract. Peptides 2002;23:531–6.
[22] Fang YQ, Weng YZ, Hu XX, et al. Immunohistochemical localization of endocrine cells in gastrointestinal tract of *Mugil cephalus*. J Fish China 2002;26(6):481–5.
[23] Gaytan F, Morales C, Barreiro ML, et al. Expression of growth hormone secretagogue receptor type 1a, the functional ghrelin receptor, in human ovarian surface epithelium, mullerian duct derivatives, and ovarian tumors. J Clin Endocrinol Metab 2005;90:1798–804.
[24] Barreiro ML, Suominen JS, Gaytan F, et al. Developmental, stage-specific, and hormonally regulated expression of growth hormone secretagogue receptor messenger RNA in rat testis. Biol Reprod 2003;68:1631–40.