

# Distribution and biological activity of obestatin in the rat

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## Abstract

Obestatin, a 23 amino acid peptide recently isolated from the rat stomach, is encoded by the same gene that encodes ghrelin. With the use of an antiserum directed against the mouse/rat obestatin, obestatin immunoreactivity (irOBS) was detected in cells of the gastric mucosa, myenteric plexus, and in Leydig cells of the testis in Sprague–Dawley rats. Double labeling the myenteric plexus with obestatin antiserum and choline acetyltransferase (ChAT) antiserum revealed that nearly all irOBS neurons were ChAT positive and vice versa. For comparative purposes, myenteric ganglion cells, cells in the gastric mucosa, and Leydig cells of the testis were shown

to be immunoreactive to preproghrelin. The biological activity of obestatin on rat central neurons was assessed by the calcium microfluorimetric Fura-2 method. Obestatin (100 nM) administered to dissociated and cultured rat cerebral cortical neurons elevated cytosolic calcium concentrations  $[Ca^{2+}]_i$  in a population of cortical neurons. The result provides the first immunohistochemical evidence that obestatin is expressed in cells of the gastric mucosa and myenteric ganglion cells, and also in Leydig cells of the testis; the peptide is biologically active on central neurons.

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## Introduction

Obestatin is a newly discovered peptide of 23 amino acids derived from the same precursor protein as ghrelin (Zhang *et al.* 2005, Broglio *et al.* 2006, Gualillo *et al.* 2006). Notwithstanding the same precursor, the biological activity of obestatin is reportedly opposite to that of ghrelin. For example, obestatin by i.p. administration resulted in a suppression of food intake and body weight gain in mice and rats, which is opposite to that produced by ghrelin (Zhang *et al.* 2005). Central administration of obestatin promoted sleep in rats (Szentirmai & Krueger 2006), whereas ghrelin induced wakefulness (Szentirmai *et al.* 2006). In isolated mouse jejunum muscle strips, obestatin decreased isometric force contraction and opposed the stimulatory effect of ghrelin (Zhang *et al.* 2005). Moreover, ghrelin and obestatin interact with two distinct subtypes of G-protein-coupled receptor. Ghrelin is identified as the endogenous ligand for the growth hormone secretagog (GHS) receptor (McKee *et al.* 1997, Kojima *et al.* 1999); whereas, obestatin is proposed to be the cogent ligand for GPR39, which is expressed in a wide variety of tissues, with the highest level being found in the gut, liver, and brain (Zhang *et al.* 2005).

Obestatin was isolated and purified from the rat stomach (Zhang *et al.* 2005). The type(s) of cells in the gastrointestinal tract expressing obestatin has not yet been identified. Although obestatin is thought to be peripheral in origin,

the peptide appears to interact with receptors distributed to the peripheral and central sites and mediate a specific behavioral response. For example, inhibition of contraction of isolated jejunum is probably mediated by a local action of obestatin; whereas, suppression of food intake and reduction of body weight gain by intracerebroventricular or i.p. injection of obestatin are proposed to be mediated through a central action (Zhang *et al.* 2005). For these reasons, the present study was undertaken to identify obestatin-containing cells in the rat peripheral tissues, but investigate the biological activity of obestatin on dissociated and cultured rat cortical neurons using the Fura-2 fluorescence as a functional assay.

## Materials and Methods

Adult male Sprague–Dawley rats, weighing 200–250 g and neonatal rats (1–3 days old; Ace Animals, Inc., Boyertown, PA, USA), were used in this study. Animal protocols were reviewed and approved by the University Animal Care and Use Committee.

### Immunohistochemical procedures

Animals were anesthetized with urethane (1.2 g/kg, i.p.) and intracardially perfused with 0.1 M PBS followed by freshly prepared 4% paraformaldehyde/0.2% picric acid in PBS.

A segment of stomach, small and large intestine were removed, postfixed for 2 h, and stored in 30% sucrose/PBS solution overnight. In preparing the myenteric plexus, layers of smooth muscles were scraped as much as possible by cotton swabs and the plexus was processed as a whole mount (Dun *et al.* 1997). In single staining, tissues were processed for obestatin immunoreactivity (irOBS) by the avidin–biotin complex procedure (Brailoiu *et al.* 2005b). Tissues were first treated with 3% H<sub>2</sub>O<sub>2</sub> to quench endogenous peroxidase, washed several times and blocked with 10% normal goat serum. Tissues were then incubated in obestatin or preproghrelin antiserum for 48 h at 4 °C with gentle agitation. Obestatin antiserum, a rabbit polyclonal directed against the rat/mouse obestatin (FNAPFDVGIKLSGA-QYQQHGRAL-NH<sub>2</sub>; Phoenix Pharmaceuticals, Inc., Belmont, CA, USA), was used at a dilution of 1:1000 with 0.4% Triton X-100 and 1% BSA in PBS. Rabbits were immunized with obestatin conjugated to thyroglobulin by the *N*-ethyl-*N'*-(3-dimethylaminopropyl) carbodiimide method. Preproghrelin antiserum, a rabbit polyclonal directed against the human preproghrelin (86–117; LSGVQYQQHSQALGKFLQDILWEEAKEAPADK; Phoenix Pharmaceuticals, Inc.), was used at a dilution of 1:750–1000. After thorough rinsing, sections were incubated in biotinylated anti-rabbit IgG (1:150 dilution, Vector Laboratories, Burlingame, CA, USA) for 2 h. Sections were rinsed with PBS and incubated in avidin–biotin complex solution for 1 h (1:100 dilution, Vector Laboratories). Following several washes in Tris-buffered saline, sections were incubated in 0.05% diaminobenzidine/0.001% H<sub>2</sub>O<sub>2</sub> solution and washed for at least 2 h with Tris-buffered saline. Sections were mounted on slides with 0.25% gel alcohol, air-dried, dehydrated with absolute alcohol followed by xylene, and coverslipped with Permount. To establish the specificity of obestatin or preproghrelin antiserum, myenteric sections were processed with obestatin- or preproghrelin antiserum pre-absorbed with the rat/mouse obestatin peptide or preproghrelin (86–117; 1 µg/ml) overnight.

In the case of double-labeling experiments, tissue sections were first blocked with normal goat serum (1:10 in PBS, 0.5% BSA, 0.4% Triton X-100) and then incubated in obestatin antiserum (1:750 dilution) for 48 h in a cold room, with gentle agitation. Following several washes with PBS, sections were incubated with biotinylated anti-rabbit IgG (1:50, Vector Laboratories) for 2 h. After several washes with PBS, tissues were incubated with Avidin Texas Red for 3 h. After rinsing with PBS for 1 h, tissues were blocked with normal donkey serum, and incubated with choline acetyltransferase (ChAT) antiserum (1:1000; a guinea pig polyclonal from Bachem Bio Sciences, Inc., King of Prussia, PA, USA) for 48 h in a cold room. After washing with PBS for 30 min, tissues were incubated in fluorescein isothiocyanate (FITC)-conjugated Affini Pure donkey anti-guinea pig IgG (1:50, Jackson Laboratories, West Grove, PA, USA) for 3 h. Sections were washed for 1 h with PBS, mounted in Citifluor and

coverslipped. Sections were examined under a confocal scanning laser microscope (Leica TCS SL) with excitation/emission wavelengths set to 488 nm/520 nm for FITC and 543 nm/620 nm for Texas Red in the sequential mode.

#### *Specificity of obestatin and preproghrelin antisera*

Obestatin antiserum reacts 100% with rat/mouse obestatin and rat/mouse Des (1–10) obestatin, and does not cross-react with human/monkey obestatin, mouse/rat or human ghrelin, human preproghrelin (86–117), preproghrelin (52–75), or preproghrelin (101–117). Preproghrelin antiserum reacts 100% with human preproghrelin (86–117), and does not cross-react with rat/mouse obestatin or rat/mouse ghrelin (Phoenix Pharmaceuticals, Inc.).

#### *Neuronal cell culture*

Cells were isolated from the cerebral cortex of postnatal 1–3-day-old rats by enzymatic digestion with 0.5 mg papain/100 mg tissue (Brailoiu *et al.* 2005a). Cells were plated at a density of 10<sup>3</sup>/mm<sup>2</sup> in a Neurobasal-A medium, supplemented with 10% fetal calf serum, 2 mM glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin (all from Invitrogen), and maintained at 37 °C in a humidified atmosphere with 5% CO<sub>2</sub>. Glial cell growth was inhibited by the mitotic inhibitor cytosine β-arabino furanoside (1 µM; Sigma). Neurons were cultured for 5 days. Cells were transferred to a medium without fetal serum 12 h prior to Ca<sup>2+</sup> measurements.

#### *Cytosolic Ca<sup>2+</sup> concentrations*

Cytosolic Ca<sup>2+</sup> concentrations, [Ca<sup>2+</sup>]<sub>i</sub>, were measured by the microfluorimetric technique, as previously described (Brailoiu *et al.* 2005a). Dissociated and cultured neurons on coverslips were loaded with the fluorescent Ca<sup>2+</sup> indicator Fura-2 AM (3 µM) by incubation of the cells in Hank's balanced salt solution (HBSS) plus Fura-2 AM for 45 min, and HBSS alone for an additional 15–60 min to allow de-esterification of the dye. Coverslips were placed in a custom-designed bath and transferred to the stage of an inverted Nikon epifluorescence microscope equipped with a C&L Instruments Fluorimeter System (Brailoiu *et al.* 2005a). Cells were perfused with HBSS at a flow rate of 2.5 ml/min. Fura-2 fluorescence (excitation wavelength = 340 and 380 nm, emission wavelength = 520 nm) of single cells was acquired at a frequency of 1 Hz. The ratio of the fluorescence signals (340 nm/380 nm) was converted to Ca<sup>2+</sup> concentrations (Grynkiewicz *et al.* 1985).

#### *Statistical analysis*

In calcium measurement experiments, statistical significance between groups was tested using one-way ANOVA followed by Bonferroni test, *P* < 0.05 being considered significantly different.

## Results

Longitudinal sections of stomach, small and large intestine, myenteric plexus, and testis from seven rats were labeled with obestatin antiserum. irOBS was present in the myenteric plexus, gastrointestinal mucosa, and the testis.

### Myenteric plexus

Ganglion cells in the myenteric plexus expressed irOBS of varying intensity (Fig. 1A). Immunoreactivity appeared in granule-like vesicles and was distributed throughout the cytoplasm, excluding the nucleus (Fig. 1A). Strands of irOBS cell processes emanating from myenteric ganglion cells formed a ladder-like network (Fig. 1A). The myenteric plexus processed with obestatin antiserum pre-absorbed with obestatin (1 µg/ml) showed no immunoreactivity (Fig. 1B).

Myenteric ganglion cells were immunoreactive to preproghrelin (irGRL; Fig. 1C and D). The pattern of distribution of irGRL cells in the myenteric plexus was similar to that of irOBS cells. Pre-absorption of the antiserum with the peptide resulted in no irGRL in the myenteric plexus (not shown).

In the guinea pig myenteric plexus, ghrelin immunoreactivity is expressed in ChAT-containing neurons (Xu *et al.* 2005). The possibility that irOBS myenteric neurons contain ChAT was explored here. Double labeling the sections with obestatin- and ChAT antisera revealed that nearly all irOBS myenteric ganglion cells were ChAT positive and vice versa (Fig. 2).

### Gastrointestinal mucosa

In addition to the myenteric plexus, irOBS cells were noted in the mucosa throughout the gastrointestinal tract (Fig. 3). Labeled cells were more abundant in the stomach and fewer in the small intestine (not shown). Further, irOBS cells occurred more often in the glandular base, next to the muscularis mucosae, and less frequently in the glandular neck (Fig. 3A and B).

Cells immunoreactive to preproghrelin were noted in the gastrointestinal mucosa, with a pattern of distribution similar to that of irOBS cells (Fig. 3C and D).

### Testis

Ghrelin is expressed in rat testicular interstitial Leydig cells (Barreiro *et al.* 2002). Here, irOBS and irGRL were detected in Leydig cells of the rat testis (Fig. 4).

### Ca<sup>2+</sup> responses to obestatin in dissociated and cultured cortical neurons

The neuronal activity of obestatin was evaluated by monitoring changes of intracellular Ca<sup>2+</sup> concentration, [Ca<sup>2+</sup>]<sub>i</sub>, in dissociated and cultured cortical neurons.

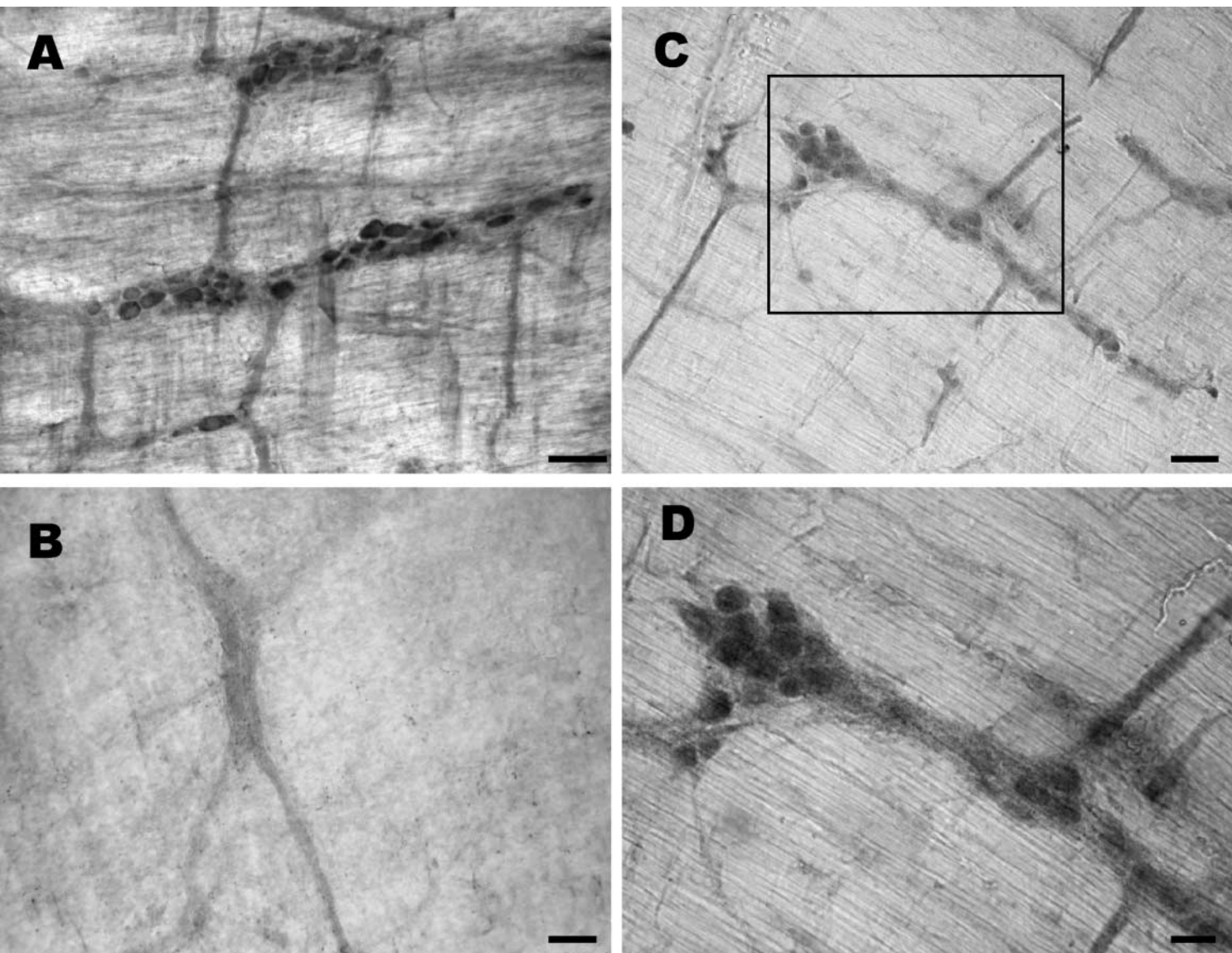
The basal [Ca<sup>2+</sup>]<sub>i</sub> value of rat dissociated cortical neurons varied from 70 to 180 nM, which is in agreement with the concentration (30–200 nM) reported in mammalian central neurons (Connor 1986). In Ca<sup>2+</sup>-containing saline, application of obestatin (100 nM) induced a fast rise in [Ca<sup>2+</sup>]<sub>i</sub> of 658 ± 8.3 nM, which gradually returned to the basal level in 26 of 80 cells tested; a representative trace is shown in Fig. 5A. In Ca<sup>2+</sup>-free saline, obestatin produced a transitory elevation in [Ca<sup>2+</sup>]<sub>i</sub> of 184 ± 4.6 nM (*n* = 10; Fig. 5B). Response elicited in a Ca<sup>2+</sup>-free medium was significantly smaller (*P* < 0.01) as compared with that induced in a Ca<sup>2+</sup>-containing saline. The mean increase in [Ca<sup>2+</sup>]<sub>i</sub> induced by obestatin (100 nM) in a medium with or without calcium is shown in Fig. 5C.

## Discussion

Obestatin and ghrelin, two peptides of 23 and 28 amino acids, are derived from the same precursor protein. The predicted sequence of obestatin as well as ghrelin is 100% identical in the rat and mouse (Zhang *et al.* 2005). Obestatin antiserum used in this study is raised in rabbits and directed against the rat/mouse obestatin peptide. As pre-absorption of the antiserum with the peptide obestatin results in no labeling in any of the tissues investigated, the antiserum appears to be specific. It is worth noting that a similar pattern of distribution of irOBS cells is observed in the myenteric plexus, mucosa membrane, and testis of ICR mice (S.L. Dun, G.C. Brailoiu, J. Yang, J.K. Chang and N.J. Dun, unpublished results). These results, collectively, provide the first evidence supporting the hypothesis that obestatin is expressed in specific groups of cells in the periphery.

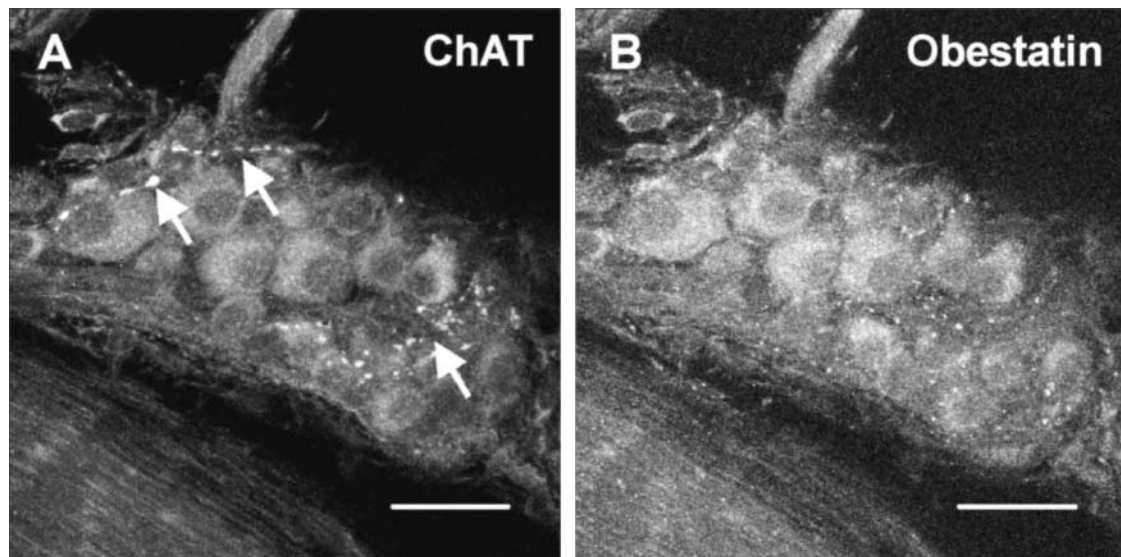
In the rat, irOBS is expressed in gastrointestinal tract and testis. In the gastrointestinal tract, irOBS is detected in cells of gastric mucosa and in cells and fibers of the myenteric plexus. In the testis, irOBS is noted in Leydig cells, which form clusters in the interstitium. In a separate series of studies, immunoreactivity to the precursor protein preproghrelin is noted in cells of the gastric mucosa, myenteric plexus and in interstitial Leydig cells. Thus, the distribution pattern of irGRL cells parallels that of irOBS cells in the rats. Since these two antisera were raised in rabbits, double-labeling experiments that would address the issue of co-localization of obestatin and preproghrelin could not be reliably performed. Notwithstanding the same precursor protein, the similarity in tissue distribution between these two peptides supports the hypothesis that they are distributed in the same cells.

Insofar as ghrelin immunoreactivity is concerned, several earlier studies show that it is detected in cells of the gastrointestinal mucosa from stomach to colon (Date *et al.* 2000, Dornonville de la Cour *et al.* 2001, Sakata *et al.* 2002), myenteric plexus (Xu *et al.* 2005), and testis (Barreiro *et al.* 2002, Tena-Sempere *et al.* 2002). A parallel expression pattern between ghrelin-containing cells reported by others and obestatin-expressing cells observed in this study is



**Figure 1** Obestatin- and preproghrelin-immunoreactive cells in the rat myenteric plexus. (A) Several myenteric ganglia contain clusters of obestatin-immunoreactive cells. (B) Immunoreactivity is not detected in myenteric plexus processed with obestatin antiserum pre-absorbed with the peptide (1  $\mu\text{g/ml}$ ) overnight. (C) Several myenteric ganglia contain preproghrelin-immunoreactive cells of varying intensities. (D) A higher magnification of the area shown in C, where several preproghrelin-immunoreactive ganglion cells are clearly seen. Scale bar: A–C, 50  $\mu\text{m}$  and D, 25  $\mu\text{m}$ .





**Figure 2** Confocal images of a rat myenteric ganglion double labeled with choline acetyltransferase and obestatin antisera. (A) A group of myenteric ganglion cells exhibiting varying intensities of ChAT immunoreactivity. (B) The same group of myenteric neurons labeled with obestatin antisera. All the ganglion cells shown here contain both ChAT- and obestatin immunoreactivity; several strands of ChAT immunoreactive fibers (arrows) seem to lack obestatin immunoreactivity. Scale bar: 30  $\mu$ m.

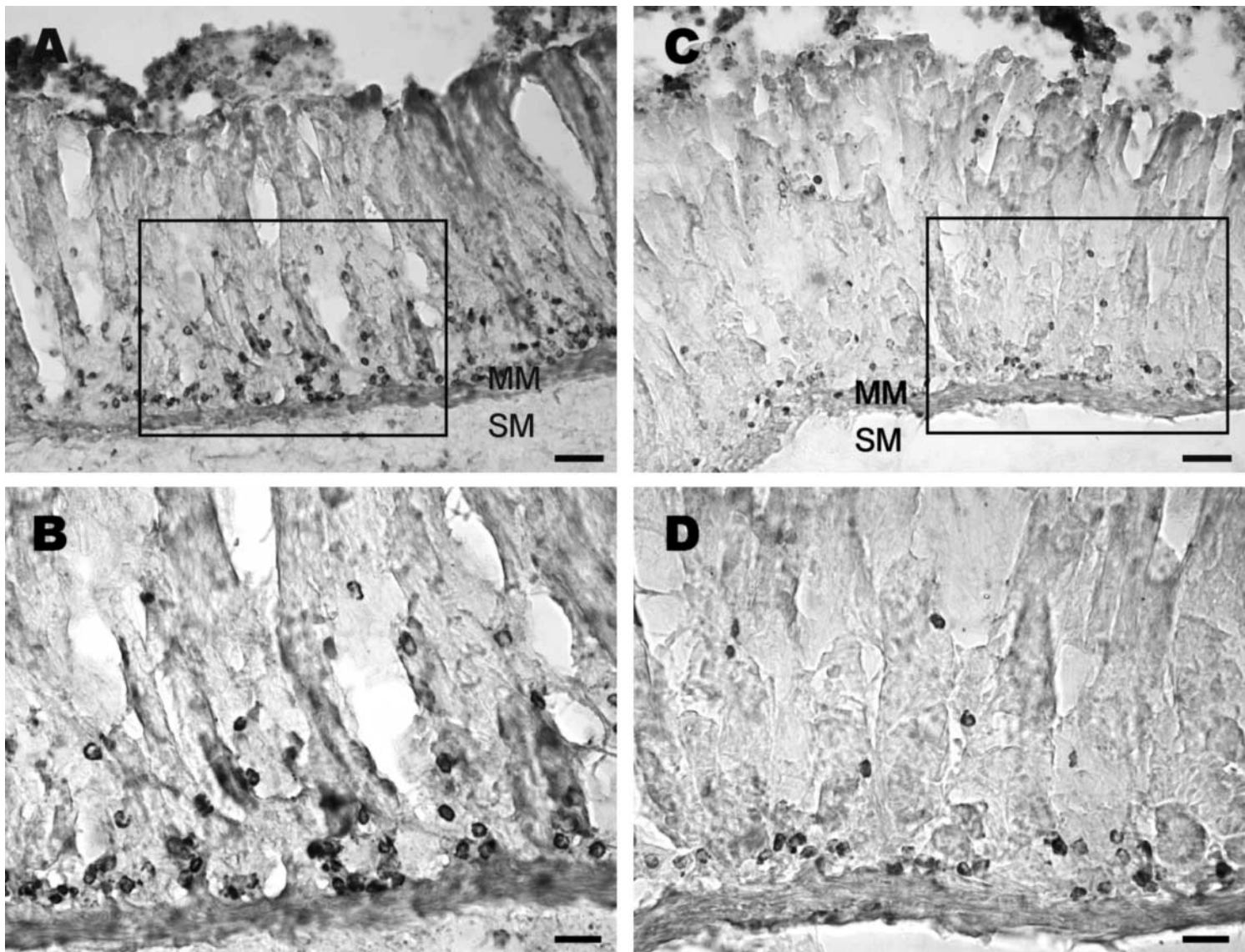
consistent with the fact that they arise from the same precursor protein. However, distribution of ghrelin immunoreactivity in myenteric plexus and mucosa membrane appears to be species dependent. For example, ghrelin immunoreactivity has been detected in cells of the gastrointestinal mucosa but absent in the myenteric plexus of the rat (Sakata *et al.* 2002). A more recent study shows the presence of ghrelin immunoreactivity in the guinea pig myenteric plexus (Xu *et al.* 2005). Our finding that irOBS and irGRL are detectable in myenteric neurons of the rat supports the contention that ghrelin is expressed in myenteric neurons of the rat. Further, the number of ghrelin-immunoreactive cells and their contents has been reported to be lower in the hamster gastric mucosa as compared with that in the mouse or rat (Yabuki *et al.* 2004).

In the guinea pig myenteric plexus, a majority (>70%) of ghrelin-immunoreactive cells are reported to be ChAT positive (Xu *et al.* 2005). Our result shows that nearly all irOBS cells in the myenteric plexus of the rat were ChAT positive and vice versa, supporting the contention that irOBS is distributed in cholinergic neurons of the myenteric plexus. Our observation coupled with the earlier finding of co-expression of ghrelin immunoreactivity and ChAT (Xu *et al.* 2005) raise the possibility that a majority of cholinergic neurons of the myenteric plexus contain both obestatin and ghrelin. A large percentage of ChAT-containing neurons in the myenteric plexus are secretomotor neurons, though some are thought to be intrinsic primary afferent neurons (Pfannkuche *et al.* 1998, Sang & Young 1998, Mann *et al.* 1999). It remains to be determined whether or not obestatin

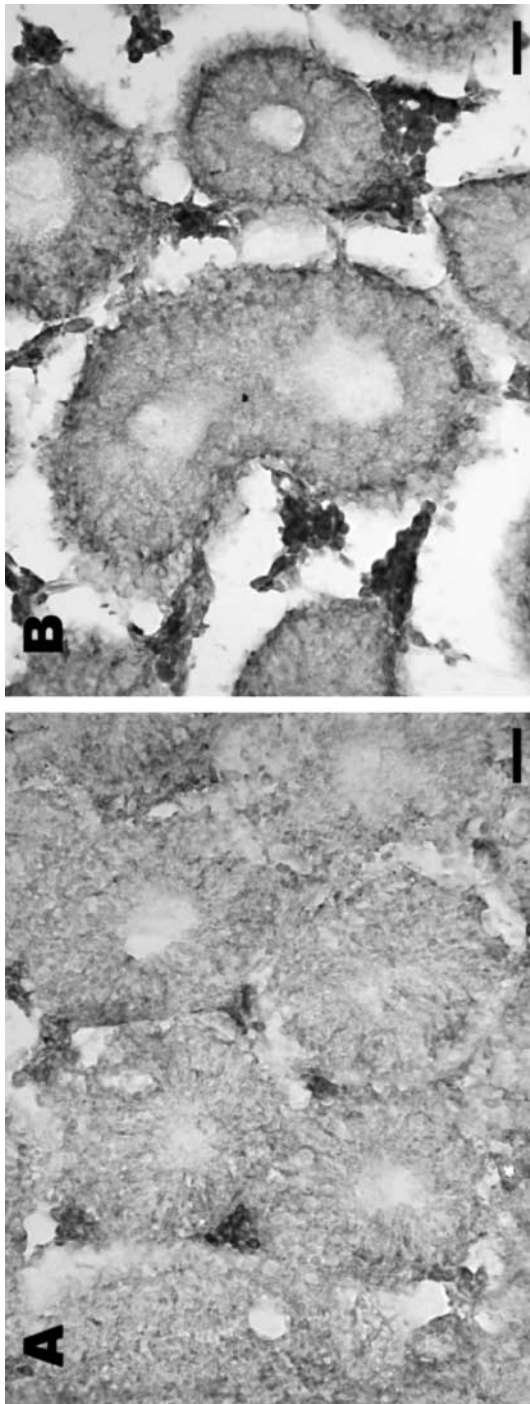
is distributed in ChAT-positive secretomotor or primary afferent neurons or both. Exogenous obestatin, when applied to isolated mouse jejunum, inhibits isometric contractions and opposes the stimulatory action of ghrelin (Zhang *et al.* 2005), suggesting that obestatin is inhibitory to gastrointestinal tract motility. On the other hand, ghrelin is stimulatory to gastric motility (Peeters 2005). The concept that two peptides derived from the same precursor protein may differentially modulate the activity of smooth muscle cells and other target cells is intriguing.

In addition to the myenteric plexus, irOBS or irGRL is detected in cells of the mucosa membrane and in interstitial Leydig cells of the testis. Neither the type(s) of cells expressing irOBS in the mucosa membrane is known nor is the physiological role of obestatin in relation to gastric function. As in the case of ghrelin-containing cells (Sakata *et al.* 2002), irOBS cells are more numerous in the glandular base and fewer in the glandular neck of the gastric glands; also the number of irOBS cells is smaller in the small intestine as compared with that of the stomach. On the basis of their morphology, two types of ghrelin-containing cells are distinguishable: an open and closed type (Sakata *et al.* 2002). In an ultrastructural study, ghrelin immunoreactivity is identified in a distinct type of endocrine cells, with round, electron dense granules, referred to as X/A cells, from the mucosal layer of the stomach (Date *et al.* 2000).

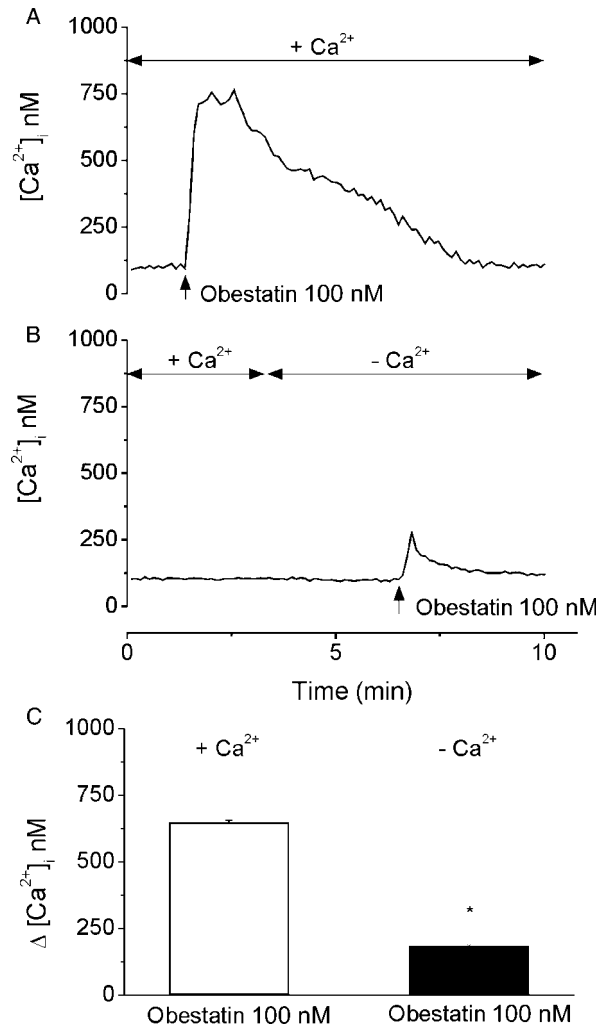
Interstitial Leydig cells of the rat testis contain ghrelin throughout postnatal development and adulthood (Barreiro *et al.* 2002, Tena-Sempere *et al.* 2002). Our result shows the detection of irOBS as well as irGRL in Leydig cells, which



**Figure 3** Longitudinal strips of rat stomach labeled with obestatin- or preproghrelin antiserum. (A) Obestatin-immunoreactive cells occur mostly in the glands of gastric mucosa. (B) A higher magnification of the area in A where strongly labeled cells are seen in the glandular base. (C) Preproghrelin-immunoreactive cells are localized near the base of the glands. (D) A higher magnification of the area in C showing immunoreactive cells localized mainly to the glandular base. MM, muscularis mucosae and SM, submucosa. Scale bar: A and C, 100  $\mu$ m; B and D, 25  $\mu$ m.



**Figure 4** Transverse sections of rat testis labeled with obestatin or preproghrelin antisera. (A and B) Interstitial Leydig cells express obestatin and preproghrelin immunoreactivity. Scale bar: 25  $\mu\text{m}$ .



**Figure 5** Effects of obestatin on cytosolic calcium concentrations in dissociated and cultured rat cortical neurons. (A) Obestatin (100 nM) causes a large and long-lasting increase of  $[\text{Ca}^{2+}]_i$  in a  $\text{Ca}^{2+}$ -containing medium. (B) Obestatin (100 nM) causes a small and transitory increase of  $[\text{Ca}^{2+}]_i$  in a  $\text{Ca}^{2+}$ -free medium. (C) Mean increases of  $[\text{Ca}^{2+}]_i$  elicited by obestatin (100 nM;  $n=10$ ) in a medium with or without calcium. \*Statistically significant difference,  $P<0.01$ .

again seems to support the hypothesis that ghrelin and obestatin derived from the same precursor. Recently, insulin-like peptide 6 has been identified in the Leydig cells of the mouse testis (Brailoiu *et al.* 2005b). Information regarding the physiological function of ghrelin or insulin-like peptide 6 in relation to reproductive biology is limited. Ghrelin signaling has been suggested to be involved in the direct control of gonadal function in male rat (Tena-Sempere *et al.* 2002). In addition, ghrelin has central effects on hypothalamo-pituitary-gonadal axis, for example, inhibiting luteinizing hormone secretion (Fernandez-Fernandez *et al.* 2004). A physiological role of obestatin in reproduction remains to be identified. The



possibility that ghrelin and obestatin may cause opposing effects in the reproductive system is intriguing.

We show for the first time that activation of obestatin receptors elevated intracellular  $\text{Ca}^{2+}$  in a population of cultured rat cortical neurons. An increase in neuronal  $[\text{Ca}^{2+}]_i$  is produced by an influx of calcium through plasmalemmal calcium channels and/or release from internal stores (Berridge 1998). In our model, obestatin was able to elevate  $[\text{Ca}^{2+}]_i$  in  $\text{Ca}^{2+}$ -free saline, indicating the participation of calcium released from internal calcium stores. The response in  $\text{Ca}^{2+}$ -free saline was significantly smaller than that in  $\text{Ca}^{2+}$ -containing saline, indicating that obestatin causes a large calcium influx through plasmalemmal channels. Collectively, our result suggests that obestatin increases intracellular calcium by facilitating calcium influx and by releasing calcium from internal sources. Calcium mobilization subsequent to receptor activation is associated with an array of neuronal activities; i.e., synaptic transmission, neurite outgrowth or retraction, and cell growth or apoptosis (Berridge 1998, Augustine *et al.* 2003). The type(s) of neuronal responses associated with activation of obestatin receptors remains to be investigated.

GPR39, which is present in many regions of the brain including the cerebral cortex, is proposed to be the cogent receptor for obestatin (Zhang *et al.* 2005). Results from more recent studies seem to be in variant with the concept that obestatin is an anti-orexigenic agent released from peripheral cells and exerts its effect by interacting with GPR39 distributed to central neurons, as proposed by Zhang *et al.* (2005). For example, GPR39 mRNA, while detectable in many areas of the brain including the mouse hypothalamus by reverse transcriptase-PCR method (Zhang *et al.* 2005), is not detected in the mouse hypothalamus by *in situ* hybridization (Jackson *et al.* 2006). In addition, the serum level of obestatin is high in the mouse; however, the peptide is apparently absent from the brain because it is not transported across the blood-brain barrier due to a high degradation rate in the circulation (Pan *et al.* 2006). The reported absence of circulating obestatin in the brain (Pan *et al.* 2006) and GPR39 in the hypothalamus (Jackson *et al.* 2006) coupled with the lack of information regarding central neurons immunoreactive to obestatin render the hypothesis put forth by Zhang *et al.* (2005) less compelling. Our finding that obestatin mobilizes calcium in cortical neurons neither supports nor refutes necessarily the hypothesis that obestatin is the endogenous ligand for GPR39 (Zhang *et al.* 2005). Receptor characterization awaits the development of pharmacological tools specific for analyzing GPR39.

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