Immunocytochemical Detection of Orexin A in Endocrine Cells of the Developing Mouse Gut

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SUMMARY Orexins are novel neuropeptides that were originally localized in neurons of the hypothalamus and neuronal fibers of the brain. Recently orexin A and its receptor have also been reported in neurons and endocrine cells of the gastrointestinal tract. Because no studies have been done at the embryonic period, we studied the appearance and distribution of orexin A during the development of mouse gastrointestinal tract using immunocytochemical methods. Immunoreactivity to orexin A was detected in neuroendocrine cells of the pyloric region of the stomach at gestational Day 14 and 1 day after in the small intestine. The numbers of immunoreactive cells progressively increased through development until the adult pattern was reached. Staining of reverse-face sections demonstrated that orexin A and serotonin co-localized in some endocrine cells of the mouse stomach and small intestine. These findings suggest that orexin A may be relevant in the growth and maturation of the gastrointestinal tract during intrauterine life.

KEY WORDS
orexin
serotonin
gastrin
endocrine cells
gastrointestinal tract
ontogeny
development
immunocytochemistry

Orexins are novel amidated neuropeptides that were first localized in neurons within the lateral hypothalamus and are involved in different aspects of feeding behavior (Nambu et al. 1999; Takahashi et al. 1999), stimulating appetite and food consumption (Wolf 1998). Two types of orexins, A and B, both derived from the same precursor by proteolytic processing, have been identified (Sakurai et al. 1998).

Previous reports indicated that orexins were restricted to neuronal cell bodies of the dorsal and lateral hypothalamic areas and that immunoreactivity was associated with large granular vesicles at synapses (de Lecea et al. 1998). However, later studies showed that a subset of neurons and endocrine cells of the gastrointestinal tract exhibited orexin A-like immunoreactivity and express functional receptors (Kirchgessner and Liu 1999). In the rat stomach, orexin A immunoreactivity was found in endocrine cells of the pyloric glands and a subset of these cells co-stored gastrin (Kirchgessner and Liu 1999). The same authors, using double labeling with antibodies to serotonin (5HT) and orexin A, established that a large subset of the orexin A-immunoreactive cells of the intestine contained this amine (Kirchgessner and Liu 1999).

There are few studies on orexins at embryonic and postnatal ages, all of them performed in rat hypothalamus. De Lecea et al. (1998) showed that orexin A mRNA was not detected in the hypothalamus from the embryonic period to postnatal Day 5. Yamamoto et al. (2000) observed that the first immunoreactivity for orexin A appeared at postnatal Day 15, although the same authors demonstrated mRNA expression for prepro-orexin A and orexin receptors before this date. Because no histological studies have been done concerning the presence of orexins in fetal mouse gastrointestinal (GI) tract, we decided to study the appearance and distribution of orexin A during development and the first days of postnatal life in an attempt to better understand the possible role of orexins in ontogenic development.

Materials and Methods
Swiss mice bred at the Harlan-INTERFAUNA-IBÉRICA, S.A (Barcelona, Spain) were used in this study. The mean...
length of gestation in this species is approximately 19 days and Day 1 is considered to be the day after fertilization. Pregnant females were sacrificed by cervical dislocation. The abdominal cavity was opened and the fetuses were removed and placed on ice (Danneman and Mandrell 1997). We studied seven embryos from each stage of embryonic (E) development (E14 to E19) and 10 adult mice. They were dissected out and immersed in Bouin’s fluid for 24 hr. After fixation the tissues were embedded in paraffin and sectioned.

### Antisera

We used a polyclonal antiserum specific for orexin A (cat. OXA11A) from Alpha Diagnostics (San Antonio, TX). The optimal dilution was 1:200. Gastrin and 5HT endocrine cells were identified with a monoclonal antibody against gastrin (#93), a kind gift from Dr. Walsh (CURE, UCLA) and a polyclonal antiserum anti-5HT (code 20080) from Incstar (Stillwater, MN) (Table 1).

### Antigen Retrieval Microwave (MW) Heating Technique

Before the immunohistochemical procedure for orexins, tissue sections were deparaffinized and rehydrated to water, and endogenous peroxidase was blocked with 3% H₂O₂ for 10 min. Slides were washed with distilled water for 5 min, placed in citrate buffer 0.01 M (pH 6), and heated in the MW (Balay W-2112, 1150–700 W; Madrid, Spain) for 15 min at maximal power and 15 min at medium power. After

<table>
<thead>
<tr>
<th>Specificity</th>
<th>Origin</th>
<th>Code</th>
<th>Dilution</th>
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*aAlpha Diagnostics, San Antonio, TX.
*bDr. J.H. Walsh, CURE, UCLA, Los Angeles, CA.
*cIncstar, Stillwater, MN.

### Table 1 Antisera used in this study

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### Table 2 Frequency and distribution of immunoreactivity for orexin A, gastrin, and serotonin in mouse gastrointestinal tract

<table>
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<tr>
<th></th>
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<th>E-15</th>
<th>E-16/E-19</th>
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<td>+ + +</td>
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<tr>
<td>Serotonin</td>
<td>− −</td>
<td>− −</td>
<td>+ + +</td>
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*Frequency of immunoreactive endocrine cells was classified according to the following scale: − − − −, absent; − − −, few cells; − −, moderate number of cells; + + + +, many cells.

### Antisera

- **Orexin A**: ADI (Alpha Diagnostics, San Antonio, TX), code OXA11A, dilution 1:200, results +.
- **Gastrin**: Dr. Walsh (CURE, UCLA), code #93, dilution 1:60,000.
- **Serotonin**: Incstar (Stillwater, MN), code 20080, dilution 1:80,000.

**Table 1** Antisera used in this study

- **Table 2** Frequency and distribution of immunoreactivity for orexin A, gastrin, and serotonin in mouse gastrointestinal tract

**Figure 1** Absorption control for the orexin A antiserum. The immunoreactivity for orexin A in a section of small intestine (A) disappeared in the contiguous section when the antiserum was preabsorbed with the corresponding antigen (B). Adult mouse; Envision method. Bars = 40 μm.

**Figure 2** Co-localization of orexin A (A) and 5HT (B) in endocrine cells of the adult stomach. A, Envision method; B, ABC method. Bars = 20 μm.

**Figure 3** Pair of reverse-face sections of the adult mouse stomach, demonstrating that orexin A (A) and gastrin (B) are not detected in the same cells. Note the cytoplasmic processes that orexin A-positive cells display towards neighboring cells (inset). A, Envision method; B, ABC method. Bars = 20 μm.

**Figure 4** Co-localization of orexin A (A) and 5HT (B) in endocrine cells of adult mouse small intestine. A, Envision method; B, ABC method. Bars = 40 μm.
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rinsing in tapwater, the immunohistochemical procedure was performed as usual.

**Immunocytochemistry**

Paraffin sections (3 μm thick) were mounted on slides coated with Vectabond (Vector Laboratories, Burlingame, CA; code SP-1800). Immunohistochemical staining for orexin was performed using the Envision+ System, HRP consisting of a secondary antibody, goat anti-rabbit Ig, coupled to a peroxidase-labeled dextran polymer (DAKO, Carpinteria, CA; code K4011, polyclonal). To prevent nonspecific background, the sections were treated with goat normal serum, 1:20 in TBS, for 30 min and then incubated overnight at 4°C with the primary antisera. Slides were incubated for 30 min with Envision+ System, HRP. Then the peroxidase activity was revealed in 0.03% 3,3'-diaminobenzidine (DAB) in 0.1 M sodium acetate/acetate acid buffer, pH 6.0, containing 2.5% nickel ammonium sulfate, 0.2% β-d-glucoside, 0.04% ammonium chloride, and 0.001% glucose oxidase (Shu et al. 1988). Sections were lightly counterstained with hematoxylin.

Immunohistochemical staining for gastrin or 5HT was performed by the avidin–biotin–peroxidase method (ABC) (Hsu et al. 1981). The detailed protocol used was previously described by García–Vitoria et al. (2000). To test the specificity of the reagents, the following controls were done on each specimen: (a) omission of the primary antibodies; and (b) application of primary antibodies, previously absorbed by incubation overnight at 4°C with 0.1–1 nmol of the synthetic peptide antigens per ml of optimally diluted antiserum.

**Results**

The distribution of orexin A immunoreactivity in the mouse GI tract through development and adult life is shown in Table 2. The absorption test confirmed the specificity of the orexin A antiserum (Figure 1).

**Adult Mouse**

Orexin A but not orexin B immunoreactivity was found in endocrine cells of the mouse GI tract (Figures 1–4), the number of positive cells being higher in the small intestine than in the stomach.

In the stomach, orexin-A immunoreactive cells were found scattered in the pyloric glands, with a tendency to be located at the bottom of the glands (Figures 2A and 3A), whereas the region of the body remained unlabeled. Some of the positive cells were equipped with long, slender basal processes reaching neighboring cells, indicating that the peptide might be released directly onto the target (paracrine secretion) (Figure 3A). To determine which particular endocrine cell types expressed orexin, we performed immunocytochemical techniques on serial reverse-face sections. Different types of gastric endocrine cells were identified with antibodies for gastrin, somatostatin, histamine, calcitonin gene-related peptide (CGRP), neuropeptide Y (NPY) substance P, glucagon-like peptide-1 (GLP1), and calcitonin. By using reverse-face sections, we found that a set of orexin A-immunoreactive cells were also immunoreactive for serotonin (Figure 2) but for none of the other markers tested, including gastrin (Figure 3). In the small intestine, many orexin A-immunoreactive cells were scattered in the villi and the crypts of the epithelium (Figures 1A and 4A), being in general more intensely stained than those of the stomach. The immunolabeled cells showed the typical morphology of intestinal endocrine cells. A subset of the orexin A-immunoreactive cells was also positive for 5HT (Figure 4), the two immunoreactivities showing differences in their intracellular distribution. In general, the immunostaining with 5HT occupied a wider cytoplasmic area, also reaching the luminal process, whereas the immunostain with orexin A was more restricted to the perinuclear and basal regions of the cell (Figure 4).

**Mouse Embryonic Development**

In the developing mouse, the immunoreactivity for orexin A was first detected at E14 in the antropyloric region (Figure 5A). At this stage, the antropyloric epithelium consists of several cell layers with slight foldings from which will arise the antral glands of the stomach at the end of development. Early orexin A-immunoreactive cells, normally basal and round, progressively increased in number with age parallel to the growth of the gut (Figures 5A–5D). At the end of development, orexin A-immunoreactive cells were scattered throughout the antal epithelium with a tendency to be located at the bottom of the gastric glands and exhibiting the typical traits of adult endocrine cells. In further studies of serial reverse-face sections, we did not observe co-localization of orexin A and gastrin as occurs in adults. In the intestine, the first immunoreactive cells for orexin A were detected at E15, 1 day later than in the stomach. During ontogeny the number of immunoreactive cells increased more

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**Figure 5** Evolution of the immunoreactivity for orexin A in developing mouse gut: E14 (A), E15 (B), E18 (C), E19 (D). (A–D) Immunoreactivity for orexin A is detected for the first time at E14 (A) in cells of the immature gastric glands of the pyloric region. (C) In the small intestine (I), the numbers of immunostained cells progressively increase through development and, at the end of the development, are much more numerous than in the stomach. Envision method. S, stomach; I, intestine. Bars = 40 μm.

**Figure 6** Orexin A (A) and 5HT (B) detected in reverse-face sections of E18 mouse intestine. Some cells are positive for both orexin A and 5HT (arrowheads). A, Envision method; B, ABC method. Bars = 40 μm.
rapidly in the intestine, being at the end of development much more numerous than in the stomach (Figure 5C). Using pairs of reverse-face sections of the small intestine, we detected a population of cells immunoreactive for both orexin A and 5HT antisera (Figure 6) but we also observed cells showing only one of the two immunoreactivities.

Discussion
This study shows that orexin A is present in endocrine cells of the GI tract from E14 of mouse development and that the number of orexin-immunoreactive cells progressively increases until adult life. Only one previous study had detected the presence of orexins in the GI tract of mammals, both in neurons and endocrine cells (Kirchgessner and Liu 1999). To our knowledge, no studies of the ontogeny of orexin-producing cells have been performed in the digestive system during embryonic development. There is only one report of orexin in embryonic tissues, in which the authors did not detect orexin mRNA in rat brains using Northern blotting analysis (de Lecea et al. 1998). During postnatal development, orexins A and B have been detected by immunocytochemical methods only from Day 15 in the rat hypothalamus (Yamamoto et al. 2000).

We have examined the fetal and neonatal development of orexin A immunoreactive cells in the mouse gastrointestinal tract. The early presence at E14 of endocrine cells producing orexin A in the gut indicates that this hormone may play an important role at the embryonic stage. In the adult, orexins are involved in several aspects of feeding behavior (Nambu et al. 1999; Takahashi et al. 1999), stimulating appetite and food consumption. During the postnatal period, orexins have been suggested to be associated with developmental changes such as weaning, feeding, and sleep/wakefulness states (Yamamoto et al. 2000). In particular, the dramatic increase of orexin during the weaning period has been related to the development of adipocytes, which produce leptin (Yamamoto et al. 2000). However, during embryogenesis the adult influences on feeding behavior would make no sense, and the possible roles of orexins must therefore be different at this stage. A similar situation has been described for gastrin, another amidated hormone whose main functions in the adult stomach are stimulation of acid secretion and mucosal growth of the GI tract but which in the fetus exerts a trophic role (Wolfe and Soll 1988; Walsh 1990). Another coincidence is that orexins and gastrin are amidated peptides and, interestingly, some observations suggest that amidated peptides may play essential roles early in development (Zhang et al. 1997). Orexin A-immunoreactive cells were first localized in the antropyloric region. However, at the end of development the immunostained endocrine cells became more numerous in the small intestine as occurs in the adult. In the gut, development progresses in a proximal to distal direction so that the different segments of the gut are at different stages of maturation (Montuenga et al. 1997). This could explain the different appearance times for the same hormone in different organs and may imply a specific regulation according to each state of maturation. Furthermore, in the intestine orexin A cells were more intensely stained than in the stomach, probably due to the higher concentration of the peptide.

To determine which endocrine cell types of the mouse stomach and intestine expressed orexin A, we applied immunocytochemical techniques on consecutive serial sections with antisera against different regulatory substances known to be present in these endocrine cells. We detected orexin A immunoreactivity in 5HT producing cells in both stomach and intestine. Our results are in only partial agreement with the previous report of Kirchgessner and Liu (1999). These authors have found co-localization of orexin A with gastrin in endocrine cells of the pyloric glands and with 5HT in intestinal endocrine cells of several mammalian species, including the mouse.

The formation of organs during embryogenesis is highly dependent on the sequential and time-regulated expression of a number of regulatory substances. Many endocrine cell types must be carefully co-ordinated in time and space during fetal development (Snow and Tam 1980). The results of the present study indicate that orexin A is already expressed in endocrine cells of the mouse gastrointestinal tract from E14 onwards. The importance of its earliest appearance must be clarified but, like other amidated hormones that are present during gestation, orexin A may play an important role even at the embryonic stage.

Acknowledgments
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We thank the donors of the antisera used in this study (Table 1). Monoclonal antibodies for gastrin were generated at the DDC Antibody RIA Core, CURE, UCLA with an NIH grant (41301). We also thank Ms Isabel Ordoqui and Ms Ainhoa Urbiola for technical assistance.

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