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Distribution of beacon immunoreactivity in the rat brain

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ARTICLE INFO

Article history:
Received 24 April 2005
Received in revised form
13 July 2005
Accepted 13 July 2005
Published on line 12 September 2005

Keywords:
Beacon
Corticotrophin-releasing hormone
Rat brain
Feeding behavior

ABSTRACT

Beacon is a novel peptide isolated from the hypothalamus of Israeli sand rat. In the present study, we determined the distribution of beacon in the rat brain using immunohistochemical approach with a polyclonal antiserum directed against the synthetic C-terminal peptide fragment (47–73). The hypothalamus represented the major site of beacon-immunoreactive (IR) cell bodies that were concentrated in the paraventricular nucleus (PVN) and the supraoptic nucleus (SON). Additional immunostained cells were found in the septum, bed nucleus of the stria terminalis, subfornical organ and subcommissural organ. Beacon-IR fibers were seen with high density in the internal layer of the median eminence and low to moderate density in the external layer. Significant beacon-IR fibers were also seen in the nucleus of the solitary tract and lateral reticular formation. The beacon neurons found in the PVN were further characterized by double label immunohistochemistry. Several beacon-IR neurons that resided in the medial PVN were shown to coexpress corticotrophin-releasing hormone (CRH) and most labeled beacon fibers in the external layer of median eminence coexist with CRH. The topographical distribution of beacon-IR in the brain suggests multiple biological activities for beacon in addition to its proposed roles in modulating feeding behaviors and pituitary hormone release.

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1. Introduction

Beacon is a 73-amino acid peptide encoded by a hypothalamic gene first reported in the Israeli sand rat [4]. Intracerebroventricular (i.c.v.) administration of beacon increased food intake, body weight and neuropeptide Y (NPY) gene expression in the hypothalamus [4,21]. Immunohistochemical studies revealed that beacon was widely expressed in the hypothalamus of rodents [1,2,4,22]. Within the paraventricular nucleus (PVN), beacon-immunoreactive (IR) perikarya were present in both the magnocellular and parvocellular divisions [1,2]. Double-labeling the hypothalamic sections revealed that beacon joins a list of peptides that co-located with oxytocin (OT) or vasopressin (VP) in a population of hypothalamic neurons [2], but its distribution character in the parvocellular divisions of PVN has not been reported. More over, other neuropeptides identified in the hypothalamus, such as corticotrophin-releasing hormone (CRH), VP and OT were also widely expressed in extrahypothalamic systems [17–19], but it is not known for beacon.

In order to obtain more information upon the possible role played by beacon in the central nerve system, it seems

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doi:10.1016/j.peptides.2005.07.011

worthwhile to characterize the detailed brain distribution of this neuropeptide. In the present study we performed histochemical analysis to determine the distribution of beacon neurons throughout the rat brain using immunostaining techniques. Moreover, beacon reactive neurons were also examined with its possible coexistence with CRH.

2. Materials and methods

2.1. Animals

Six adult male and three female Sprague–Dawley rats (250–300 g) were obtained from Vital Company (Beijing) and kept under artificial light/dark cycle (12 h/12 h) with standard laboratory chow and water given ad libitum. All of the steps of the experimental procedures were conducted in agreement with the guiding principles for the care and use of experimental animals approved by the Society for Neuroscience.

Three male rats were anaesthetized with sodium pentobarbital (50 mg/kg, i.p.) and placed on a stereotaxic apparatus (Kopf, CA). The rats were stereotaxically injected into the lateral ventricle (0.8 mm caudal and 1.5 mm lateral to bregma, depth 3.6 mm) with 100 μg of colchicine (Sigma) dissolved in sterile 0.9% saline, and kept in the animal room for 24 h before sacrifice for double label immunohistochemistry. The purpose of using colchicine is to block the axonal transportation of neuropeptides, to enrich their content in the cell body and facilitate its detection through immunocytochemical techniques [5].

All of the rats were then anaesthetized and intracardially perfused with 150 ml of isotonic saline followed by 250 ml of 4% paraformaldehyde. Brains were removed, postfixed in the same fixative overnight, and immersed in 10, 20, 30% sucrose/PBS solution overnight. Serial coronal sections of 30 μm thickness were prepared with Cryostat (Leica, Nussloch, Germany). A consistent angle of cut was maintained by examining the shape of the third ventricle and anterior commissure.

2.2. Single labeling immunohistochemistry

Free-floating sections were pre-incubated in a solution containing 4% normal goat serum, 1% bovine serum albumin and 0.3% Triton X-100 at 37 °C for 30 min and subsequently incubated with the primary antibody (rabbit-anti-rat antiserum against beacon peptide at a dilution of 1:4000, Phoenix, CA) for 48 h at 4 °C, anti-rabbit biotinylated IgG (1:200, Vector Labs, Burlingame, CA) for 4 h at room temperature, and finally streptavidin-biotin-peroxidase complex (1:200, Vector Labs) for 2 h at room temperature. The sections were then developed in 100 mmol/L acetate buffer containing 0.02 % 3,3′-diaminobenzidine (DAB) (Sigma), 4% nickel ammonium sulphate and 0.03% H_2O_2 for 10 min at room temperature. Negative controls were carried out by similarly treated adjacent sections omitting the primary antibody, as well as using primary antibody preabsorbed with excess antigen. In both cases, no positive immunostaining was detected.

2.3. Double labeling immunohistochemistry

After preincubation in 10% normal donkey serum for 30 min, sections were incubated in CRH-antiserum (1:1000; Santa Cruz, CA) for 48 h at 4 °C. Following several washes with PBS, sections were incubated with TRITC conjugated donkey anti-goat IgG (1:100; Santa Cruz) for 2 h. After thorough washing with PBS for 1 h, tissues were blocked with normal bovine serum, and incubated in rabbit polyclonal beacon antiserum (1:1000; Phoenix) for 48 h at 4 °C. After washing with PBS for several times, tissues were incubated in FITC conjugated bovine anti-rabbit IgG (1:100, Santa Cruz) for 2 h. They were then washed for 1 h with PBS, transferred to slides, air-dried, and mounted with 90% glycerin in PBS. Sections were examined on a laser scanning confocal microscope (Leica TCS NT) with excitation/emission wavelengths 488/530 nm for FITC and 568/620 nm for TRITC, respectively.

3. Results

3.1. Beacon expression in the brain

Representative micrographs of beacon-IR for coronal sections through the brain were presented in Figs. 1–4, showing brain regions containing significant beacon-IR. There were no qualitative differences in cell body or fiber distribution

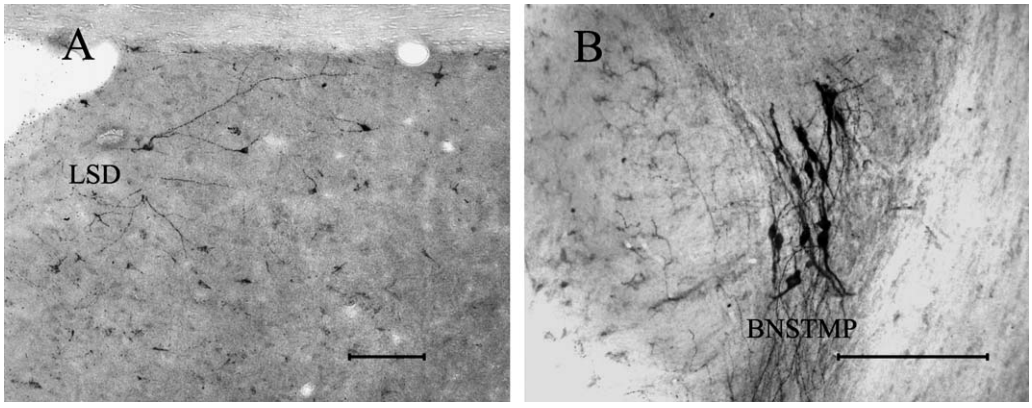

Fig. 1 – Coronal sections illustrating the distribution of beacon-containing cell bodies and nerve fibers in the dorsal lateral septal nucleus (LSD) (A) and the medial posterior region of the bed nucleus of the stria terminalis (BNSTMP) (B). Scale bar = 200 μm.

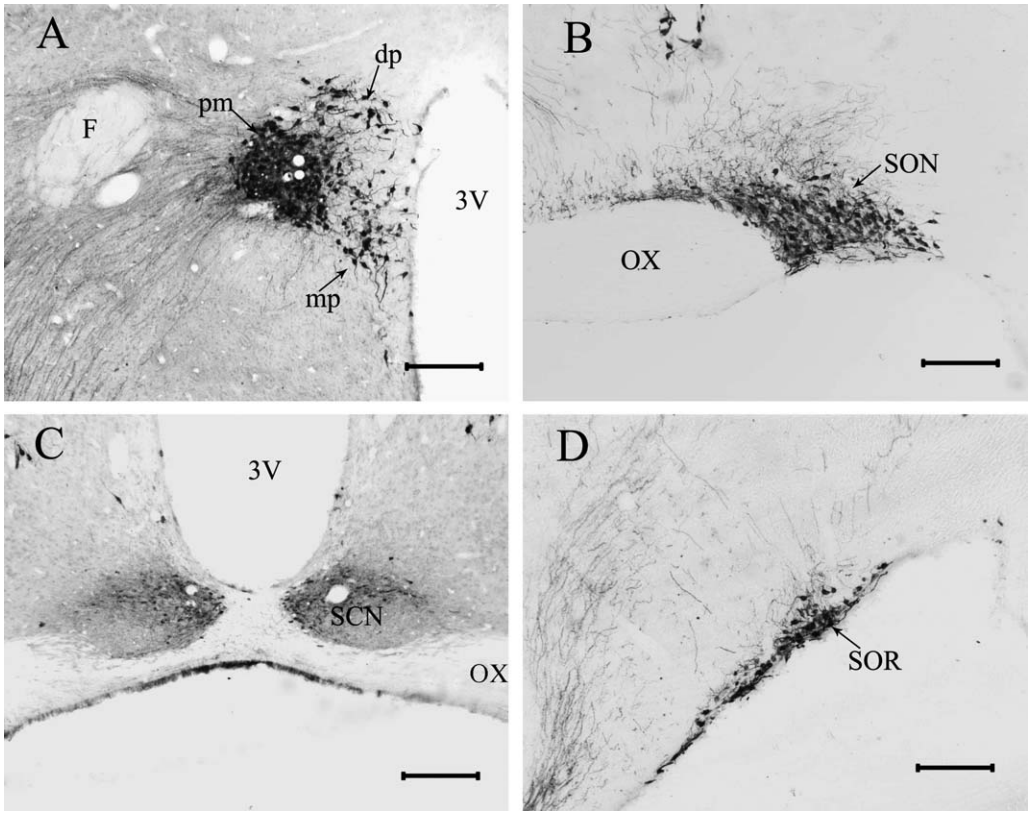

Fig. 2 – Coronal sections showing neurons containing beacon-IR in the hypothalamus. The main population of beacon-IR cells were noted in the PVN (A) and SON (B). In the PVN, beacon-positive cell bodies and nerve fibers were found both in the magnocellular and parvocellular part of the nucleus (A). Quite a number of beacon-IR neurons and nerve fibers were detected in the SCN (C) and SOR (D). Abbreviation: dp, dorsal parvocellular; mp, medial parvocellular; F, fornix; OX, optic chiasm; 3V, third ventricle. Scale bar = 200 μm.

between male and female rats, and labeled regions of the brain were consistent from animal to animal.

3.1.1. *Telencephalon*

No beacon-IR fibers were found in the cortical area. Small amounts of beacon-IR cells and fibers were detected in the septal nucleus, with a relatively high concentration in the dorsal lateral corner (Fig. 1A). A group of intensely labeled beacon-IR cells occupy the medial posterior region of the bed nucleus of the stria terminalis (Fig. 1B). Only occasional scattered fibers were detected in the hippocampus, and little to none in the central amygdaloid nucleus.

3.1.2. *Diencephalon*

We failed to find beacon-IR in the thalamus. In the hypothalamus, the distribution of beacon-IR was similar to

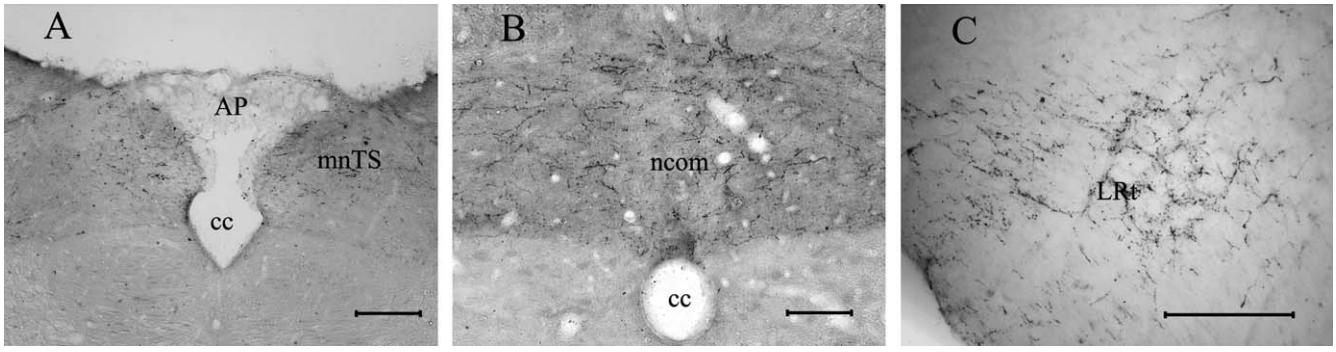

Fig. 3 – Coronal sections illustrating beacon-stained nerve fibers in the nucleus of the solitary tract (NTS) (A and B) and the lateral reticular nucleus (LRt) (C). Abbreviation: AP, area postrema; cc, central canal; ncom, commissural nucleus of NTS; mNTS, medial subnucleus of NTS. Scale bar = 200 μm.

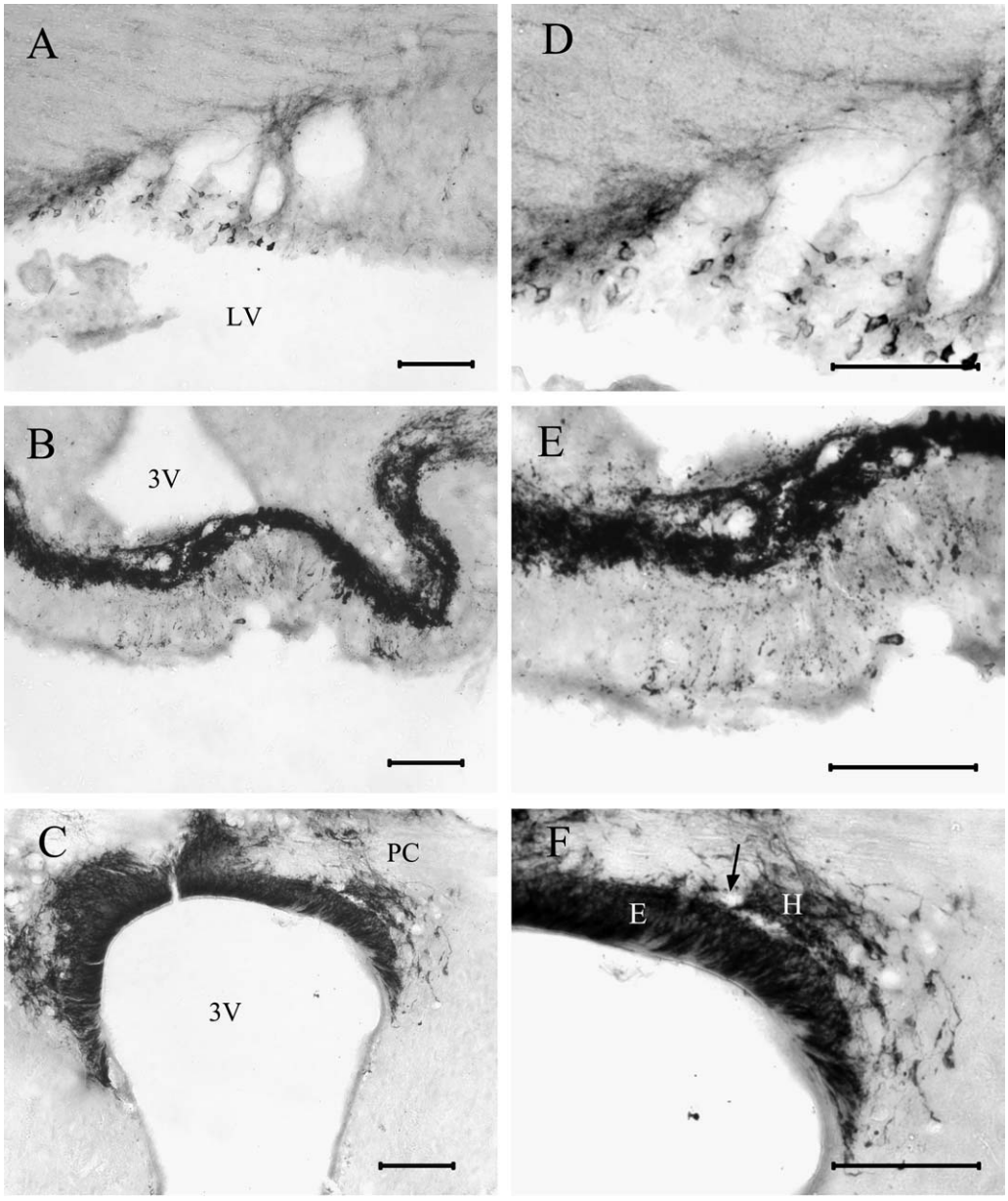

Fig. 4 – Coronal sections illustrating the localization of beacon-IR in circumventricular organs including SFO (A and C), ME (B and D) and SCO (C and F). A group of intensely stained beacon-positive cell bodies was detected in the lateral margin of the SFO (A and C). In the ME, most Beacon-IR fibers were seen in the internal layer (B and D). In the SCO, both the ependymal (E) and hypendymal (H) cells are beacon-IR. The arrow shows the blood vessel between the hypendymal cells and basal processes of the ependymal cells. Abbreviation: LV, lateral ventricle; 3V, third ventricle; PC, posterior commissure. Scale bar = 100 μm.

that reported recently in rats and mice [1,2]. The main populations of beacon-IR cells were found in the PVN, supraoptic nucleus (SON), and accessory neurosecretory nuclei (Fig. 2A and B). Within the PVN, intensely labeled cells were revealed in both the magnocellular and parvocellular divisions (Fig. 2A). Fibers from the magnocelluar beacon-IR cells projected ventrolaterally to form the paraventriculo-supraoptico-neurohypophyseal tract. Beacon-IR fibers were seen with high density in the internal layer of the median eminence (ME) and low to moderate density in the external layer (Fig. 4B and E). Additionally, quite a number of beacon-IR cell bodies and nerve fibers were detected in the suprachiasmatic nucleus (SCN) and the supraoptic retrochiasmatic nucleus (SOR) (Fig. 2C and D). A few cell bodies were scattered in the periventricular nucleus, the medial and the lateral preoptic area, as well as anterior and lateral hypothalamic areas.

3.1.3. *Brainstem*

Only sparse or light beacon-IR fibers were found throughout the brainstem, with notable exception of the nucleus of the solitary tract (NTS) and lateral reticular nucleus (LRt). Very few

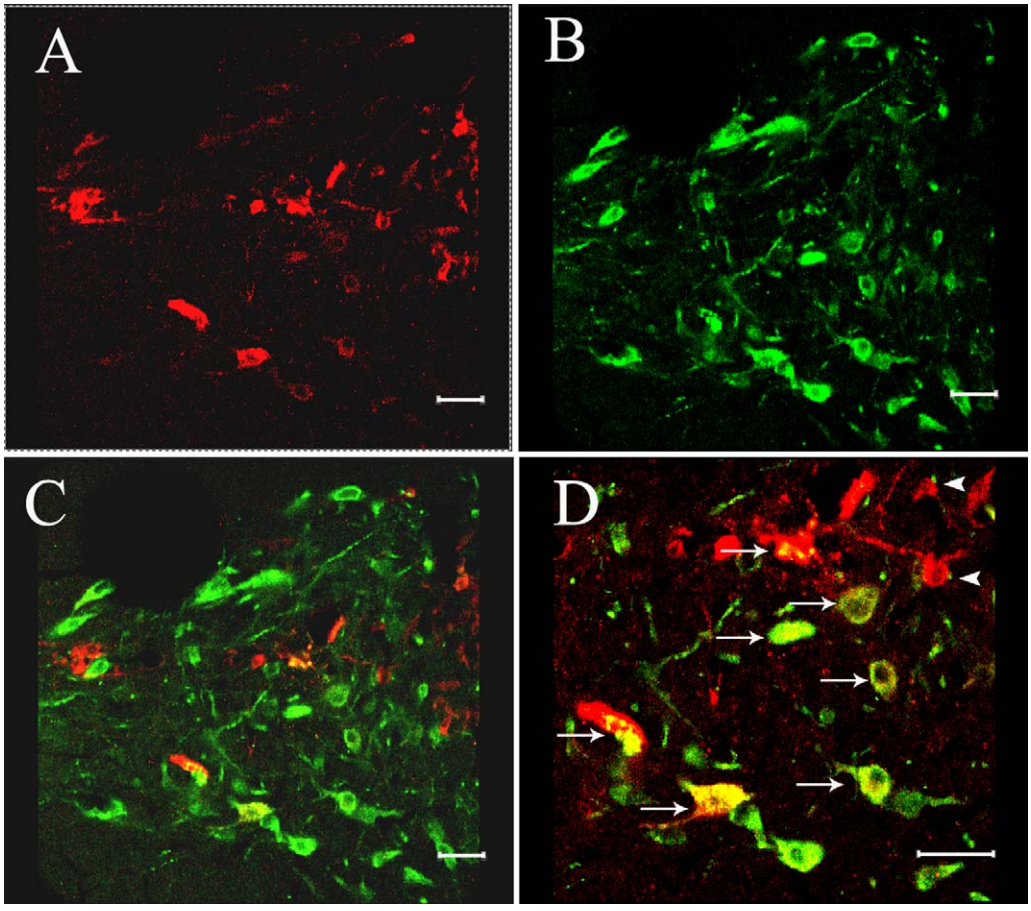

Fig. 5 – Confocal images of rat paraventricular hypothalamic nucleus double-labeled with beacon-antiserum and CRH-antiserum. Section labeled with beacon- (A) and CRH-antiserum (B); and an overlay of the images (A) and (B) (C); (D) is a high magnification of (C). Several beacon-containing neurons were CRH-IR positive (arrows) and some beacon-containing fibers were in close contact with CRH-IR neurons (arrowheads). Scale bar = 40 μm.

signals appeared in the rostral NTS. Beacon-IR positive nerve fibers were visualized from the posterior area postrema (AP) to the caudal medulla oblongata, i.e., restricted to the caudal NTS. Most signals were concentrated in the medial and commissural parts, and a few were scattered in other subnuclei (Fig. 3A and B). In the caudal medulla oblongata, another site with positive signal was LRt where a few positive fibers were visualized (Fig. 3C).

3.1.4. *Circumventricular organs*

A group of intensely stained beacon-IR cell bodies was detected in the lateral margin of the subfornical organ, close

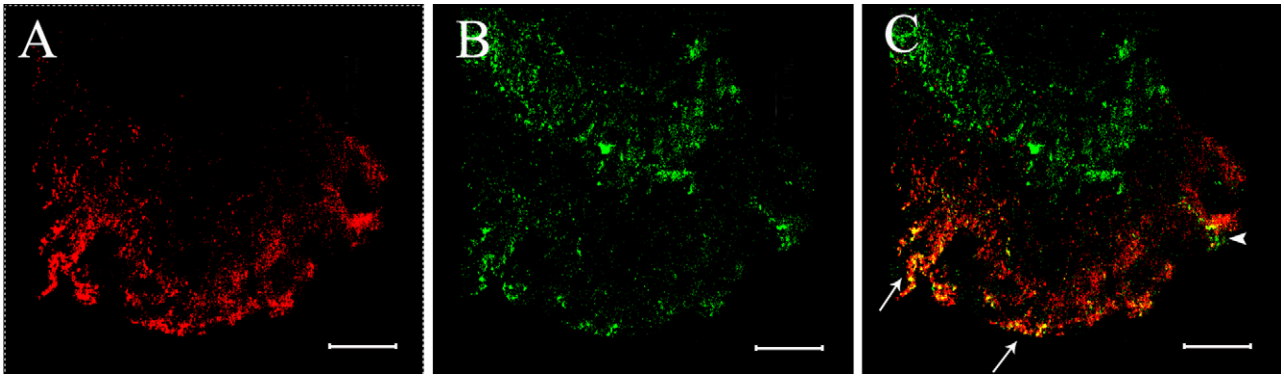

Fig. 6 – Confocal images of rat ME double-labeled with beacon-antiserum and CRH-antiserum. Most beacon-containing fibers in the external layer are CRH-immunoreactive (arrows), but several beacon-IR fibers (arrowhead) are not CRH-positive. Scale bar = 40 μm.

to the large blood vessels and lateral ventricle (Fig. 4A and D). Very few scattered cell bodies were seen in its central region and lateral region around blood vessels. In the subcommissural organ (SCO), the cells in ependyma showed a significant beacon-IR with their basal processes contacting with blood vessels. Some hypendymal cells grouped in clusters were also positive for beacon-IR, and intensely labeled beacon-IR fibers extended into the junction of the posterior commissure (Fig. 4C and F). The distribution of beacon-IR in the ME has already been described in the previous paragraph. No significant beacon-IR was detected in the vascular organ of the lamina terminalis and AP.

3.2. *CRH/beacon-immunoreactivity*

Hypothalamic sections from three male rats were double-labeled with beacon and CRH antiserum. Several beacon-IR neurons that resided in the medial parvocellular nucleus were shown to coexpress CRH, and some beacon-containing fibers were in close contact with CRH-IR neurons (Fig. 5). CRH-IR fibers were present mainly in the external layer of median eminence, and most beacon labeled fibers in the external layer of ME were found to coexist with CRH (Fig. 6).

4. Discussion

Using a specific polyclonal antiserum directed to the beacon fragment 47–73 for immunohistochemistry, we have shown for the first time a detailed distribution of beacon-IR through out the adult rat brain. Analysis of the distribution of beacon-containing cell bodies and fibers revealed that the hypothalamus was the region containing by far the highest beacon immunoreactivity. In addition to the hypothalamus, beacon-positive signals were also detected in the septum, bed nucleus of the stria terminalis, NTS, LRt, and several circumventricular organs.

Similar to the previous studies, we also detected beacon-IR cells both in the hypothalamic magnoceullar and paricellular system. It has been reported that beacon was found to express in VP- and OT-containing neurons, which are two major cell types in the magnocellular hypothalamic nuclei [10]. In the present study, double-labeling the sections with beacon- and CRH-antiserum revealed that several beacon-IR neurons resided in the medial parvocellular nucleus were shown to coexist with CRH, and most labeled beacon fibers in the external layer of ME also coexisted with CRH under normal physiological conditions. A recent study suggests that beacon may exert a negative modulatory action on the pituitary-adrenal axis, which is functionary opposite to CRH [13,19,22]. The histochemical interconnection between beacon and CRH reported in the present study provides a morphological evidence supporting a possible role of beacon in regulating hypothalamic–pituitary–adrenal axis.

Beacon-IR was also found to exist in extrahypothalamic regions as was reported for OT and/or VP [17,18], except for a few differences. In the central amygdaloid nucleus where abundance of VP-positive signals were reported [18], we found little to no beacon-IR fibers. In the brainstem, the distribution of beacon-IR was more restricted than that of OT and/or VP.

It was notable that we detected a high level of beacon-IR in the SCO, where no OT or VP positive cells were observed [18]. The SCO is a highly specialized circumventricular organ, composed of a well-modified ependymal cell layer and an additional hypendyma. SCO cells located in both layers exhibit structural characteristics of secretory cells with the possibility of releasing their secretory products into the ventricle to form a thread-like structure – Reissner's fiber (RF), or into the local blood vessels [15]. In the present study, we found that the two layers of the SCO cross-react with the anti-beacon antibody, suggesting that beacon may have been the synthetic and secretory product of the SCO that can be released into the CSF, local blood vessels or stored in the RF. Consistent with a recent study [20], we provided another piece of evidence that the SCO-RF complex synthesizes and discharges not only glycoproteinous materials but also neuropeptides. By now, the significance of the SCO function is still obscure. It may play a role in the pathophysiology of hydrocephalus [3,14], the activity on neurite outgrowth [7,12] and the reproductive physiology and salt/water balance [11,16]. The abundant amount of beacon in SCO cells and in the RF of the third ventricle suggests that beacon may take part in some of these activities.

Recently, a widespread expression of beacon in endocrine glands of the rat was detected by RT-PCR and Immunocytochemistry [22]. Furthermore, beacon has also been described as ubiquitin-like protein-5, or homologous to ubiquitin peptide-1 [4,6]. Since beacon appears to be closely related to ubiquitin, its widespread expression in numerous cells and organs is not surprising. However, the absence of a diglycine motif in the C-terminus precludes any typical ubiquitin like function for beacon [8]. Recently, beacon has been identified as a protein interacting with cdc2/cdc28-like kinases CLK1, 2 and 4, and it has been suggested that by way of these kinases it can regulate the expression of specific genes involved in central regulation of energy homeostasis [9]. Data obtained in the present study imply that beacon may function as a neurotransmitter, a neuromodulator or a neuroendocrine regulator depending on its localization. Up to now, no specific receptor was characterized for beacon, which hinders the development of specific and selective non-peptide agonists and/or antagonists thus delaying the investigation on the physiological roles of brain beacon. In this regards, in situ genetic manipulations as well as local antibody sequestration can be used as powerful approaches for the elucidation of its functions.

Acknowledgments

This research was funded by the National Natural Science Foundation of China (30270624) to D.R. TIAN, and a grant from the Ministry of Science and Technology (973 project for basic research 2003CB515407) to J.S. HAN.

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