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Expression of brain prolactin releasing peptide (PrRP) changes in the estrous cycle of female rats

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Abstract

Prolactin releasing peptide (PrRP) is a neuropeptide with 31 or 20 amino acid residues and regarded as a potent and specific stimulator of pituitary prolactin. PrRP immunoreactive (PrRP-ir) neurons and mRNA are found in medulla oblongata and hypothalamus and the fibers containing PrRP are widely distributed in rat brains. Therefore, it is postulated that PrRP might act as a neurohormone or a neurotransmitter as well as a neuromodulator in the brain. In the present study, we probed the expression of brain PrRP in the estrous cycle of female rats and the relationship between brain PrRP and GnRH. Female rats were divided into four groups: the diestrus, the proestrus, the estrus and the metaestrus, which were identified by the vaginal cytological examination. Immunohistochemistry, reverse transcriptase-polymerase chain reaction (RT-PCR) and immunofluorescent double labeling histochemistry combining confocal laser scanning microscope (CLSM) were used. The results showed that PrRP immunoreactive neurons in nucleus of solitary tract (NTS) and ventrolateral reticular nucleus (VLRN) in the proestrus were less than those in the diestrus, the estrus and the metaestrus. Similarly, the relative optical density of PrRP-ir fibers of the bed nucleus of stria terminalis (BST) in the proestrus was decreased compared with those in other three groups. However, the brain PrRPmRNA level was higher in the proestrus and estrus than those in the metaestrus and diestrus. We also observed the co-localization of GPR10-immunoreactive (GPR10-ir) and GnRH-immunoreactive (GnRH-ir) neurons in hypothalamic medial preoptic area (MPO). The present results provide morphological evidences that PrRP in the female rat brains might participate in the regulation of the rat estrous cycle at least in a direct way.
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Keywords: Prolactin releasing peptide (PrRP); Gonadotropin-releasing hormone (GnRH); Estrous cycle

Prolactin releasing peptide (PrRP) was isolated and identified primarily from bovine hypothalamus in 1998 and found to specifically stimulate prolactin secretion, at least in vitro [5]. The distribution of PrRP neurons and mRNA signals suggested many additional functions other than a prolactin releasing factor [10,14,13,21]. In rat brains, immunohistochemical results show that PrRP neurons mainly locate in the nucleus of the solitary tract (NTS) and ventrolateral reticular nucleus (VLRN). Its immunoreactive terminals have been found to project to hypothalamic neuroendocrine nucleus [3,7,18]. Strong PrRPmRNA signals are detected in NTS and VLRN and weak signals in posterior hypothalamus [11,12]. Therefore, it is postulated that PrRP might act as a neurohormone or a neurotransmitter as well as a neuromodulator in the brain. The PrRPmRNA expression in female rats shows normal sexual gonadal cycles and administration of estrogen or progesterone after ovariectomy induces an increase of PrRPmRNA expression in the NTS [8]. It suggests that PrRP might be related to hypothalamus–pituitary–ovary axis (HPOA). Even though there was a contradictory report in 2003 [2] to indicate that no significant changes in PrRPmRNA expression were noted in any sampled region between proestrus, estrus or diestrus in hypothalamus, but because of the low level of PrRPmRNA expression in hypothalamus and micropunch brain samples used in experiment, the conclusion seems to need more evidences about the expression of PrRP protein. For providing a new insight into this field, we examined PrRP immunoreactive (PrRP-ir) neurons and PrRPmRNA level of medulla oblongata and hypothalamus to learn the expression of PrRP during rat estrous cycles and detected further the co-localization of GnRH immunoreactive (GnRH-

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ir) and GPR10 immunoreactive (GPR10-ir) substances in hypothalamus.

Forty-eight adult female Sprague–Dawley rats (200–220 g), with regular 4-day estrous cycle, were purchased from the Medical Experimental Animals Center of Fudan University (Shanghai, China). Animals were kept in group cages with ad libitum access to food and water, under the circumstance of controlled temperature at 22 ± 1 °C and a schedule of 12/12 (light/dark). The stages of the animal sexual cycle were monitored by examination of daily exfoliative epithelia in vaginal smears. All experimental procedures were in strict accordance with the guidelines of the Shanghai Medical College of Fudan University.The animals in different groups ($n = 6$ per group) were sacrificed and then perfused via the left cardiac ventricle with cold 0.9% sodium chloride 200 ml and 4% formaldehyde polymerisatum in 0.1 M phosphate buffer (PB) 300 ml (pH 7.4) to fix quickly. The rat brains were taken out and post-fixed in 0.1 M PBS containing 20% sucrose for 24 h at 4 °C and subsequently in 0.1 M PBS containing 30% sucrose at least for 48 h at 4 °C. Serially frozen frontal sections (35 μm) were cut from every brain. The brain sections were taken from the VLRN, NTS (both between Bregma −12.80 and −13.20) and the hypothalamic BST (Bregma −1.60 and −2.40), stored at −20 °C. The sections were washed in 0.01 M PBS (5 min × 3) and then incubated with 10% sheep serum for 1 h at 37 °C. Thereafter, monoclonal rabbit antibody against rat PrRP (1:500 dilution, primary antibody, Phoenix Co., USA) was added and incubated continuously at 37 °C for 2 h and at 4 °C for 70 h. It was then washed and incubated in goat anti-rabbit IgG conjugated with biotin (1:200 dilution, second antibody, SABC Ltd., China,) for 1 h at 37 °C and processed by the avidin–biotin–peroxidase conjugate kits (ABC immunocytochemistry methods, SABC Ltd., China). The controls were set up in the absence of primary antibody or in the place of primary antibody with normal rabbit serum. The number of PrRP cells in one side NTS and VLRN and the optical density of PrRP-ir fibres in one side BST were calculated under the power light microscope (Leica, Germany), and their images were treated by the Software (Leica QW in V.3).

To compare the level of PrRPmRNA expression at different estrous cycle stages, RT-PCR method was adapted to provide a semiquantitative measure of mRNA levels. The rat brains in every group ($n = 6$) were rapidly removed and immediately frozen in dry ice. The brain regions of medulla oblongata (Bregma −12.80 and −13.20) and hypothalamus (Bregma −1.60 and −4.30) were taken out. Total RNA were extracted with TRIZOL reagent (Sangon Inc., China) and stored at −80 °C. The concentration of RNA was estimated by spectrophotometry using UV absorbance at 260 nm and 280 nm. The integrity of RNA was determined by denaturing agarose gel electrophoresis and ethidium bromide (EB) staining. Primers were synthesized based on published reports [2]. The following primers for PrRP gene were used: Sense 5′-CATGGAGACAAGAACCCCTG-3′ and Antisense 5′-ACGCTGAGAGAACTTGGTGC-3′ generating fragment of 163 bp; for β-actin: Sense 5′-CCTCTATGCCAACACAGTGC-3′ and Antisense 5′-ATACTCCTGCTTGCTGATCC-3′ for 211 bp. Total RNA (5 μl) was reversely transcribed in a final volume of 20 μl, using 3.5 μM anchored oligo (dT)15 (1 μl, Promega, USA), 200 IU M-MuLV reverse transcriptase (1 μl, Sangon Inc., China), 2.5 mM deoxy-NTP mixture 4 μl and 20 IU RNasin (1 μl, Sangon Inc. China) for 60 min at 37 °C before getting heat denatured for 5 min at 95 °C. The cDNAs obtained were further amplified by PCR using PCR primers (Sangon Inc., China). Each PCR reaction was performed with RT reaction solution 2 μl, 10× PCR buffer 2.5 μl, $MgCl_2$ solution 1.5 μl, amplification primer 1 (20 μM) 1 ul, amplification primer 2 (20 μM) 1 μl, deoxy-NTP Mix 4 μl, and *Taq* DNA polymerase (3 units) and adding sterilized H_2O to total volume of 25 μl, mixed by gently flicking the bottom of each tube. We used 30 PCR amplification cycles (94 °C, 1 min; 59 °C, 1 min; and 72 °C, 1 min) for PrRP and 30 cycles (94 °C, 1 min; 57 °C, 1 min; and 72 °C, 1 min) for β-actin. Samples were all finished with the final extension at 72 °C for 5 min. To check the presence of DNA contamination, RT-PCR was performed on 5 μl of total RNA without M-MuLV reverse transcriptase (negative control). An internal control (water instead of RNA) for each RT-PCR was performed to investigate RNA contamination of the mixture. For each sample 5 μl of the PCR amplification products were analyzed on

Table 1
The number of PrRP-ir cells in NTS, VLRN and the relative optical density of PrRP-ir fibers in BST of the diestrus, proestrus, estrus and metaeastrus groups (mean ± S.D., $n = 6$)

Groups	NTS	VLRN	BST
Diestrus	189.6 ± 11.1	241.1 ± 57.6	100.0 ± 40.0
Proestrus	97.8 ± 11.9*	146.5 ± 28.7*	30.0 ± 13.3**
Estrus	192.4 ± 23.6	204.8 ± 37.9	85.0 ± 12.3
Metaestrus	205.2 ± 22.0	229.4 ± 14.2	82.5 ± 20.7

** $p < 0.01$ vs. diestrus.
* $p < 0.05$ vs. diestrus.

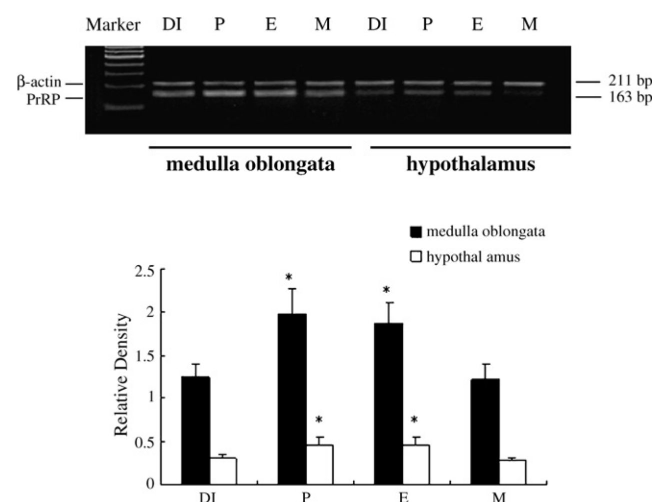

Fig. 1. RT-PCR analysis of medulla oblongata and hypothalamic PrRPmRNA of the rats ($n = 6$). The upper picture shows the gel electrophoresis of the RT-PCR products for the PrRP. Densitometry analysis of the mRNA concentration using PrRP/β-actin was expressed as the mean with S.D. in each column indicated in the lower panel ($n = 6$ per group). *$p < 0.05$ vs. DI. DI: diestrus; P: proestrus; E: estrus; M: metaestrus.

1.6% agarose gels and stained with EB. The optical intensities of the bands were evaluated using the Image Master Software (SYDR-1990, SYNGENE, USA.).

For exploration of the co-localization of GPR10-ir and GnRH-ir neurons in hypothalamus, immunofluorescent double labeling and CLSM was used. The brain sections were washed and incubated in 0.01 M PBS containing 10% sheep serum albumin for 2 h at 37 °C, then with both mouse anti-GnRH antibody (1:500, Chemicon Inc., USA) and rabbit anti-GPR10 antibody (1:400, Novus Biologicals Inc., USA) diluted in PB containing 1% BSA at 37 °C for 2 h and then at 4 °C for 70 h. After they had been rinsed in 0.01 M PBS (3 times, 5 min each), the sections were sequentially incubated in secondary antibody solutions with the same blocking sera. Consequently, the sections were incubated in goat anti-rabbit IgG conjugated to tetramethyl rhodamine isothiocyanate (TRITC) (1:200 dilution, Sigma chemical Co., USA) for 2 h at 37 °C. After that they were washed (3 times, 30 min each) and incubated in goat anti-mouse IgG conjugated to fluorescein isothiocyonate (FITC, 1:200 dilution, KANGCHEN, China) for 2 h under the same conditions. Finally, they were washed (at least 3 times, 30 min each) until the background was reduced enough and mounted on slides for coverslipping. Data were analyzed by CLSM (Leica confocal software, TCS SP2). The adjacent two slices were used to illustrate GPR10-ir and GnRH-ir cells, respectively. The antibodies and processes were the same as those described previously, except diaminobenzidine (DAB) as chromogen. DAB staining, with ABC immunochemistry kits (SABC Ltd., China), was processed according to the manufacturer's instructions for anti-rabbit and anti-mouse secondary antiserum conjugated to peroxidase. Specificity of GPR10 and GnRH staining was determined by preincubation of antiserum for 24 h at 4 °C with varying concentration of GPR10 and GnRH, with primary antibody omitted to identify non-specific staining as well.

All results were expressed as mean ± S.D. and analyzed by the Statistical Package for the Social Sciences (SPSS) statistical software (version 11.5). The raw data were analyzed by one-way analysis of variance (ANOVA) and significance of difference was determined by the Student–Newman–Keuls post hoc test. Significance was set at $p < 0.05$ and $p < 0.01$ in two-tailed testing chosen.

The number of PrRP-ir neurons in rat medulla oblongata and hypothalamus are presented in Table 1. The ABC method immunohistochemistry showed that PrRP-ir positive neurons or fibers were found in rat NTS and VLRN of medulla oblongata or hypothalamus. Further enumerative results of PrRP-ir neurons revealed that the number of PrRP-ir neurons in NTS and VLRN of the proestrus rats were less than those of the diestrus, estrus and metaestrus groups. Similarly, in BST, less intensity of PrRP-ir fibers in proestrus compared with those of other three groups ($p < 0.05$) was observed. The tissue sections processed for immunohistochemistry using antiserum after preabsorption with excessive antigens and omission of primary antibody showed the staining as expected. Densitometry analysis of the mRNA concentration using target product/β-actin was expressed as the mean with S.D. The ratios of medulla oblongata PrRP to β-actin of proestrus (1.975 ± 0.300) and estrus (1.873 ± 0.241) groups increased significantly compared with those of diestrus (1.237 ± 0.159) and metaestrus (1.214 ± 0.182) rats ($p < 0.05$). And in hypothalamus, the ratio of proestrus (0.449 ± 0.105) and estrus (0.458 ± 0.089) was higher than the other two groups (diestrus: 0.308 ± 0.042; metaestrus: 0.283 ± 0.026. $p < 0.05$) (Fig. 1).

To ascertain whether PrRP receptor (GPR10) co-localized with GnRH in hypothalamus, we applied immunochemistry (Fig. 2) and fluorescent double labeling immunocytochemistry of GPR10 and GnRH (Fig. 3). As shown in photos, some of the neurons showed GPR10-ir and GnRH-ir simultaneously in

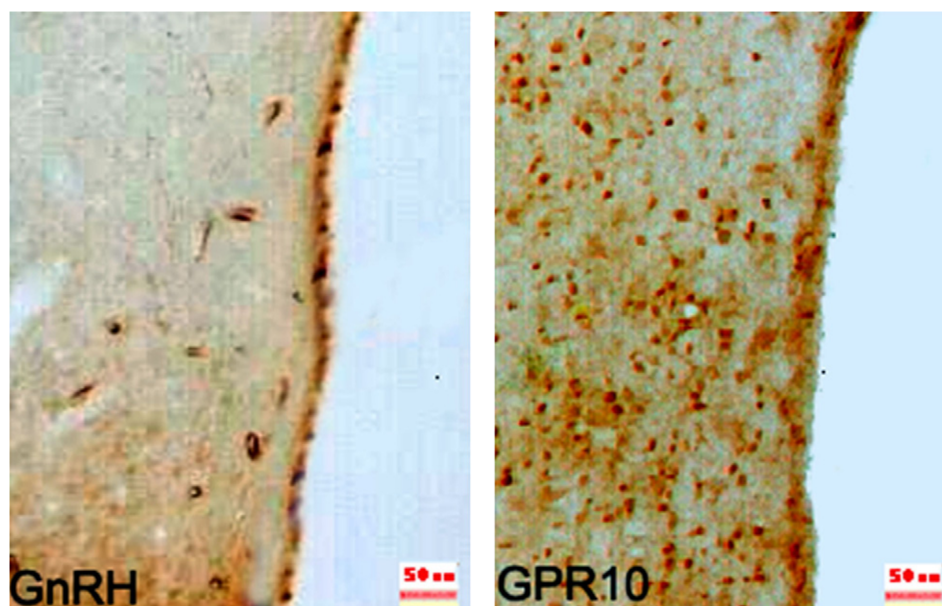

Fig. 2. Light micrography illustrating the distribution of GnRH-ir and GPR10-ir at the region of hypothalamic MPO by immunohistochemical method (ABC). The two slices were used to illustrate GnRH-ir and GPR10-ir cells in rat MPO.

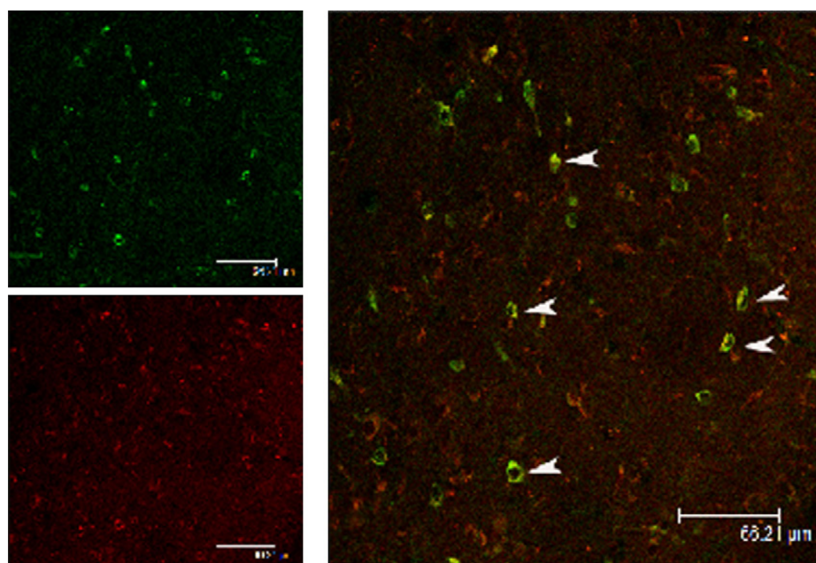

Fig. 3. The co-localization of GPR10-ir and GnRH-ir in hypothalamic MPO neurons by immunofluorescent double labeling histochemistry-combining CLSM. GnRH-ir elements are shown by green colour, GPR-10-ir elements are shown in red colour, and double-labeled cells are shown in yellow colour. The arrows indicate the co-localization of GPR10-ir and GnRH-ir. About 25% GnRH-ir cells contained GPR10-ir substance on their membranes.

hypothalamic MPO. Big green neurons were GnRH-ir whereas GPR10-ir was membrane receptors, and about 25% GnRH-ir contained GPR10-ir substance on their membranes. These findings proved the co-localization of GnRH neurons and GPR10.

It is well known that PrRP is not a classic hypothalamus neuropeptide, and researches about it still focus on the studies of morphology and physiological functions [5,16]. Some previous studies suggested that PrRP-ir neurons distributed in medulla oblongata of rats, mainly in NTS and VLRN [4,18], are in coincidence with our finding. But the expressions of PrRP in different stages of estrous cycle detected by immunohistochemistry and RT-PCR showed disparity. For example, in preoestrus, PrRPmRNA level reached the peak while the number of PrRP-ir were least. Therefore, we supposed that the release of PrRP was maximum in this stage when the LH level increased. It was interesting to note that in the medial preoptic area (MPO), the release of GnRH increased prior to the initiation of the LH surge, and PrRP even started rising earlier than GnRH [17]; the endogenous PrRP could induce LH surge, and anti-PrRP-31 serum could descend the LH and PRL peak [6], which had been confirmed. PrRP also significantly increased the release of GnRH from hypothalamic explants [15]. In our previous study, we observed that PrRP was involved in the regulation of subnormal function of HPOA in ovariectomized rats [1,20]. These results suggested that PrRP might play a role by activating GnRH neurons. So we concluded that the release of PrRP would increase in proestrus stage when GnRH and LH release increasingly to achieve the peak. And the release of PrRP increased prior to the initiation of the GnRH surge. This indicated that PrRP was an important factor to regulate GnRH peak and was favorable for GnRH pulse release.

For further understanding of the relationship between PrRP and GnRH, we wanted to know whether there is a co-localization relationship in some brain region. GnRH neurons were few in rat brain and the highest density of the cells is located in MS, DBB and MPO. Although the project area of PrRP-ir fibers were not located in MPO as reported [9,12], we did observe the few PrRP-ir fibers in MPO and searched the same result in Yano's paper [19]. Therefore, we consider that the MPO is the possible co-localized area. Luckily, PrRP receptor GPR10 immunohistochemical signals were found in MPO (Fig. 2). Furthermore, with immunofluorescent double labeling histochemistry-combining CLSM, we confirmed the co-localization of GnRH and GPR10 in hypothalamic MPO, which might provide the direct morphological evidence that PrRP might be partly responsible for the regulation of GnRH release via its receptor—GPR10 in female rat brain.

The present results suggested that PrRP in the female rat brain might participate directly in the regulation of female estrous cycle via its receptors. It will be investigated further if the estrous cycle could be affected by brain PrRP to change the release of brain neurotransmitters, such as DA, 5-HT, GABA and so on.

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