

***In Situ* Hybridization Method Reveals (Pro)renin Receptor Expressing Cells in the Pituitary Gland of Rats: Correlation with Anterior Pituitary Hormones**

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Received August 29, 2012; accepted January 7, 2013; published online February 13, 2013

Expression of (pro)renin receptor ((P)RR), a specific receptor for renin and prorenin, was studied in rat pituitary gland. *In situ* hybridization showed that cells expressing (P)RR mRNA were widely distributed in the anterior lobe and intermediate lobe of the pituitary gland. Double-staining using *in situ* hybridization for (P)RR mRNA and immunohistochemistry for the pituitary hormones showed that (P)RR mRNA was expressed in most of the GH cells and ACTH cells in the anterior lobe. (P)RR mRNA was also expressed in a few prolactin cells and TSH cells, but not in LH cells. The present study has shown for the first time the distribution of (P)RR mRNA expressing cells in the rat pituitary gland. These findings suggest that (P)RR plays physiological roles in the pituitary gland, such as the modulation of the pituitary hormone secretion.

Key words: *in situ* hybridization, (pro)renin receptor, pituitary, immunohistochemistry, rat

I. Introduction

(Pro)renin receptor ((P)RR) is a specific receptor for renin and prorenin [15, 16]. (P)RR is a 350 amino-acid protein with a single transmembrane domain. When bound to prorenin, (P)RR activates the enzymatic activity of prorenin non-proteolytically. Moreover, it directly activates the intracellular signaling including mitogen-activated protein kinase (MAPK) ERK1/2 independently of the renin-angiotensin system (RAS) [16]. (P)RR is widely expressed in various tissues including heart, kidney, brain, and pituitary [10, 11, 15, 20, 21]. The RAS components, such as angiotensinogen, renin, angiotensin-converting enzyme, and angiotensin receptors, are all expressed in pituitary gland [9, 14, 18]. Immunocytochemistry showed that (P)RR was expressed in the paraventricular and supraoptic nuclei

of human hypothalamus and the anterior pituitary lobe [20]. However, the types of (P)RR-expressing cells have not been determined in the anterior pituitary gland. The aim of the present study is therefore to clarify the expression of (P)RR mRNA in rat pituitary gland by using *in situ* hybridization and characterization of (P)RR-expressing cells by immunohistochemistry for the pituitary hormones.

II. Materials and Methods

Animals

Male Wistar rats (aged 8–10 weeks) were purchased from Japan SLC, Inc. (Shizuoka, Japan). All animal experiments were performed after receiving approval from the Institutional Animal Experiment Committee of Jichi Medical University and were conducted in accordance with the Institutional Regulation of Animal Experiment and Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions under the jurisdiction of the Japanese Ministry of Education, Culture, Sports, Science and Technology.

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In situ hybridization and immunohistochemistry

In situ hybridization was performed with digoxigenin (DIG)-labeled cRNA probes, as described in a previous report [6]. Pituitary glands were removed from rats under anesthesia. Tissues were embedded in Tissue-Tek OCT compound (Sakura Finetechnical, Tokyo, Japan), and frozen rapidly. Frozen sections (8 μ m thick) were obtained using cryostat (CM3000; Leica Microsystems, Wetzlar, Germany). Sections were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 10 min at room temperature. Rat (P)RR cDNA fragments (Atp6ap2; NM_001007091.1; 53-713) were amplified from rat pituitary cDNA by polymerase chain reaction with forward (5'-GTG GCT CAT CTC CGC TTT AG-3') and reverse (5'-GAG AAT GAT CCT TGG CGA GA-3') primers. Amplified cDNA fragments were ligated into the pGEM-T vector (Promega, Madison, WI, USA) and cloned. Gene-specific antisense or sense DIG-labeled cRNA probes were made using the Roche DIG RNA labeling kit (Roche Diagnostics GmbH, Penzberg, Germany). DIG-labeled cRNA probe hybridization was performed at 55°C for 24 hr. Visualization of mRNA was performed with alkaline phosphatase-conjugated anti-DIG antibody (Roche Diagnostics GmbH) using 4-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate (Roche Diagnostics GmbH).

The specificity of the *in situ* hybridization method was confirmed in the following ways: No specific signal was detected in section processed with DIG-labeled sense probe; the hybridization signal was abolished after competition with 10-fold excess amounts of sense or unlabeled antisense probes; a similar positive hybridization signal was detected by means of another antisense probe to rat (P)RR, Atp6ap2 (642-1137).

For double staining, the section was immunostained for the anterior pituitary hormones after (P)RR mRNA was detected by *in situ* hybridization, as described in a previous report [5]. Sections were incubated overnight at room temperature in PBS with primary antibodies. Primary antibodies against the following proteins were used for immunostaining: adrenocorticotrophic hormone (ACTH), growth hormone (GH), prolactin, thyroid stimulating hormone (TSH) β -subunit, luteinizing hormone (LH) β -subunit as reported earlier [5, 12]. The antibody against LH β -subunit was used to identify the gonadotropes (LH/FSH cells). The ABC method (Vector Laboratories, Burlingame, CA., USA) was utilized with 3,3'-diaminobenzidine (Dojindo Laboratories, Kumamoto, Japan) as substrate. Absence of specific reaction was confirmed using the normal animal serum instead of the primary antibodies.

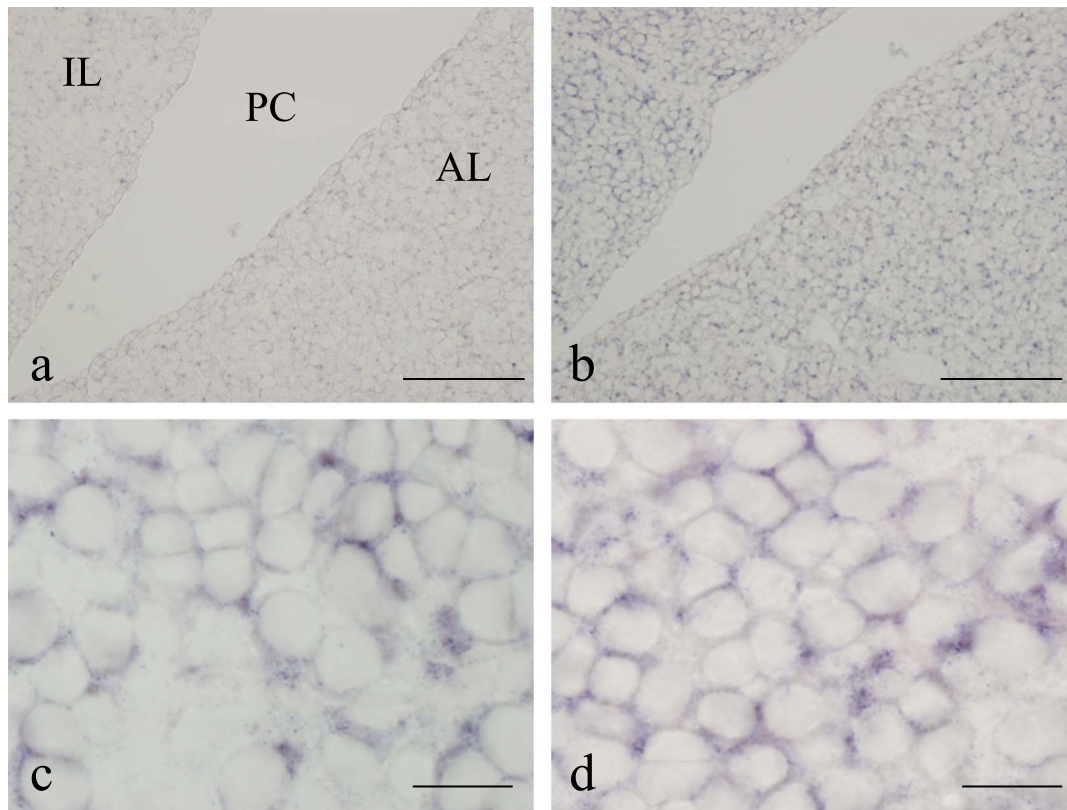


Fig. 1. *In situ* hybridization for (pro)renin receptor mRNA in the rat pituitary gland. (Pro)renin receptor mRNA was detected in the anterior lobe (AL) and intermediate lobe (IL) of the pituitary gland (**b**). (**c**) Higher magnification of the anterior lobe. (**d**) Higher magnification of the intermediate lobe. No specific signal was observed when the section was processed with a sense probe (**a**). PC, pituitary cleft. Bars=100 μ m (**a**, **b**), 10 μ m (**c**, **d**).

III. Results and Discussion

(P)RR mRNA was detected in the adult pituitary gland by *in situ* hybridization with a DIG-labeled antisense cRNA probe (Fig. 1b). (P)RR-expressing cells were widely observed in the anterior lobe (Fig. 1c) and intermediate lobe of the pituitary gland (Fig. 1d). In general, transmembrane receptor-expressing cells are difficult to identify by immunohistochemistry. Some of the reasons might be that transmembrane receptors are localized in a limited range on the cell membrane at low intracellular levels, and their immunoreactivity often depends on the fixative conditions. In this study, we therefore performed *in situ* hybridization method for detecting (P)RR-expressing cells. The present study succeeded in detecting the expression of (P)RR mRNA in the rat pituitary.

To identify cells positive for (P)RR mRNA, we performed double-staining using *in situ* hybridization for detecting (P)RR mRNA and immunohistochemistry for detecting the pituitary hormones. (P)RR mRNA was expressed in most of the GH cells (Fig. 2a) and ACTH cells in the anterior lobe (Fig. 2b). (P)RR was also expressed in a few prolactin cells (Fig. 2c) and TSH cells (Fig. 2d), but not in LH cells (Fig. 2e). (P)RR enhances the catalytic activity of prorenin on the cell surface [15, 16]. (P)RR which is expressed in the anterior pituitary lobe may promote the generation of angiotensin I. The presence of angiotensin I-converting enzyme in the anterior pituitary gland suggested production of angiotensin II [18]. Angiotensin II was reported to stimulate the secretion of GH, ACTH and prolactin from the anterior pituitary gland [1, 4, 7]. It is noteworthy that (P)RR mRNA was expressed

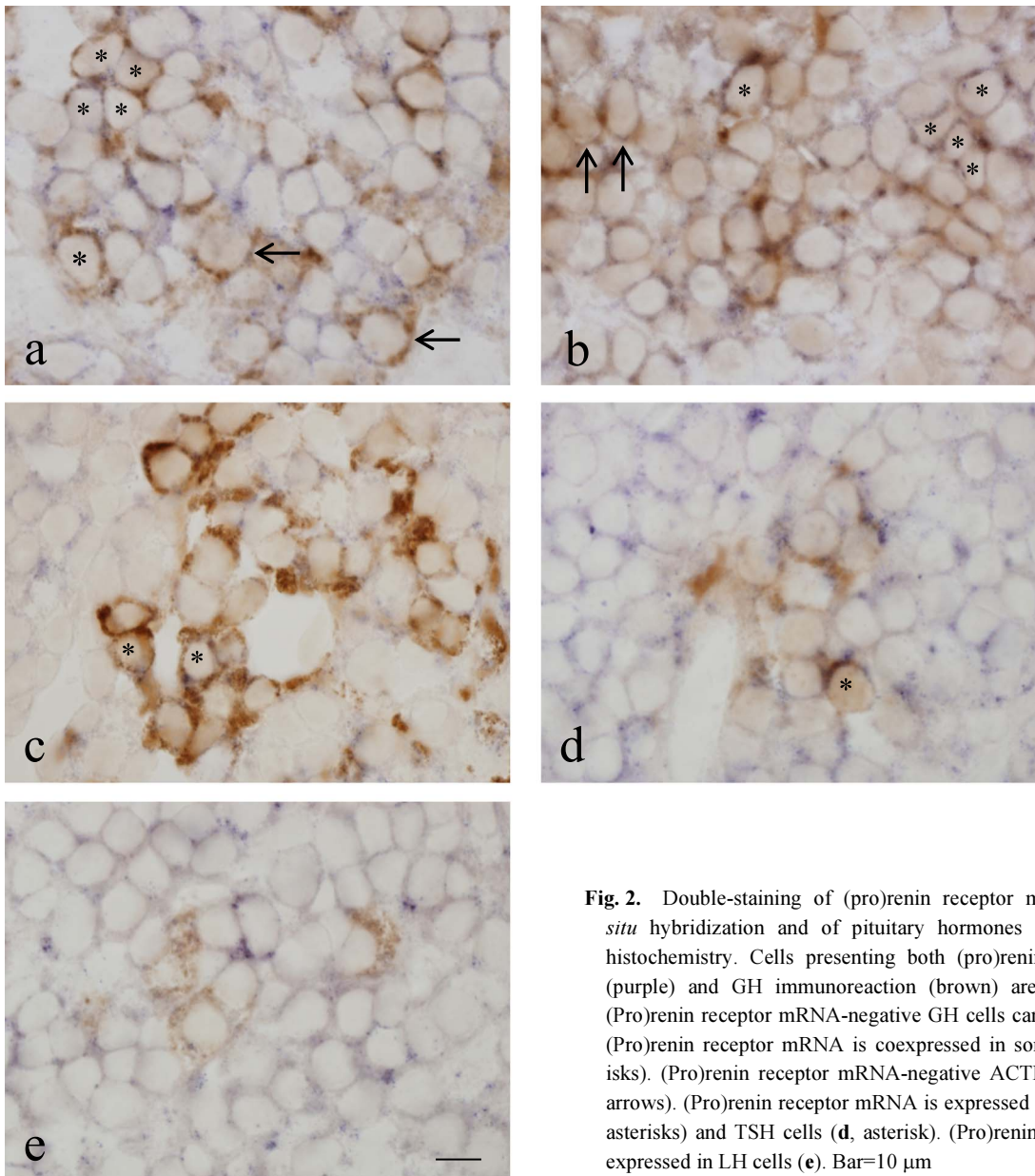


Fig. 2. Double-staining of (pro)renin receptor mRNA as detected by *in situ* hybridization and of pituitary hormones as detected by immunohistochemistry. Cells presenting both (pro)renin receptor mRNA signal (purple) and GH immunoreaction (brown) are indicated (**a**, asterisks). (Pro)renin receptor mRNA-negative GH cells can also be seen (**a**, arrows). (Pro)renin receptor mRNA is coexpressed in some ACTH cells (**b**, asterisks). (Pro)renin receptor mRNA-negative ACTH cells could be seen (**b**, arrows). (Pro)renin receptor mRNA is expressed in a few prolactin cells (**c**, asterisks) and TSH cells (**d**, asterisk). (Pro)renin receptor mRNA was not expressed in LH cells (**e**). Bar=10 μ m

in most of the GH cells and ACTH cells in the present study. (P)RR in the anterior pituitary may therefore enhance the local generation of angiotensin II, which may modify the secretion of these anterior pituitary hormones in the autocrine or paracrine manner. Because (P)RR mRNA was also expressed in the intermediate lobe of the pituitary gland, we could not deny the possibility that (P)RR may affect the secretion of intermediate pituitary hormones, such as α -melanocyte stimulating hormone. Furthermore, the intracellular signaling via (P)RR, such as MAPK, may stimulate the proliferation of the pituitary cells.

The gene encoding for PRR is named ATP6ap2 (ATPase 6 accessory protein 2), and (P)RR is the full-length form of a smaller protein described previously associated with the vacuolar H^+ -ATPase (V-ATPase) [2, 15]. This association has been shown to be functional and essential for the survival of certain cells, such as cardiomyocytes and podocytes [13, 17]. V-ATPase-mediated granular acidification also plays critical physiological roles in the pituitary gland, such as prohormone processing and hormone secretion [19]. (P)RR expressed in the anterior pituitary may be related to the survival of anterior pituitary cells, and the processing and secretion of anterior pituitary hormones, via the association with V-ATPase. Furthermore, (P)RR is involved in the Wnt/ β -catenin pathway, which is essential for adult and embryonic stem cell biology, embryonic development and tumorigenesis, including pituitary adenomas [3, 8, 15].

The present study revealed for the first time the distribution of (P)RR mRNA expressing cells in the rat pituitary gland. The findings have raised the possibility that (P)RR plays various physiological roles in the pituitary gland, such as the processing and secretion of pituitary hormones, and the differentiation and proliferation of pituitary cells.

IV. References

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