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Immunohistochemical detection of prolactin-releasing peptide2 in the brain of the inshore hagfish *Eptatretus burgeri*

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ABSTRACT

Prolactin-releasing peptide2 (PrRP2) belongs to the RFamide peptide group and is a paralog of prolactin-releasing peptide (PrRP). Recent studies demonstrated that PrRP2, but not PrRP, regulates prolactin release in teleosts. The evolutionary origin of PrRP and PrRP2 dates back to at least early vertebrates because homologs of PrRP/PrRP2 were identified in lampreys, one of the earliest branch of vertebrates class Agnatha. However, PrRP/PrRP2 remains to be identified in hagfish, another representative species of class Agnatha. Here, we examined the distribution of PrRP2 in the brain and pituitary of the inshore hagfish *Eptatretus burgeri* to obtain further understanding of the neuroendocrine system of PrRP2. PrRP2-immunoreactive (ir) cell bodies were detected in the infundibular nucleus of hypothalamus (HYinf). PrRP2-ir fibers were restricted around PrRP2-ir cell bodies and were not detected in the dorsal wall of the neurohypophysis compared to the abundant PrRP2-ir fiber distribution in the brain and innervation to the pituitary in other vertebrates. To examine possible reciprocal connections of PrRP2 and other neuropeptides, we further conducted dual-label immunohistochemistry of PrRP2 and the PQRFamide (PQRFa) peptide or corticotropin-releasing hormone (CRH). Reciprocal connections are suggested between PrRP2 and PQRFa neurons as well as between PrRP2 and CRH neurons. The present study demonstrates, for the first time, that PrRP2 is expressed in the brain of inshore hagfish. The restricted distribution of PrRP2-ir fibers in the HYinf suggests that PrRP2 does not directly regulate the pituitary gland, but regulates the function of the HYinf where PQRFa and CRH are expressed.

1. Introduction

Neuropeptides with the Arg-Phe-NH_2 motif at the C-terminal have been identified in both vertebrates and invertebrates. These neuropeptides are termed RFamide peptides. The RFamide peptide family consists of five groups: gonadotropin-inhibitory hormone (GnIH), kisspeptin, PQRFamide peptide (NPFF), pyroglutamylated RFamide peptide (QRFP)/26RF amide, and prolactin-releasing peptide (PrRP) groups (for reviews, see Tsutsui, 2009; Tsutsui et al., 2010, 2012; Tsutsui and Ubuka, 2015).

In teleost fishes, PrRP is a hypothalamic neuropeptide that was initially isolated from the Japanese crucian carp *Carassius auratus langsdorfii* and thus termed *Carassius* RFamide (C-RFa) (Fujimoto et al., 1998). To date, PrRP has been isolated from several teleost fish species, such as chum salmon *Oncorhynchus keta* (Moriyama et al., 2002), Atlantic salmon *Salmo salar* (Monterfusco-Siegmund et al., 2006), Mozambique tilapia *Oreochromis mossambicus* (Seale et al., 2002), and silver sea bream *Sparus sarba* (Kwong and Woo, 2008). The amino acid sequences of these teleost PrRP peptides are identical to those of C-RFa. The function of PrRP in teleost fish is to stimulate prolactin (PRL) secretion, regulate food intake, and promote adaptation to new osmotic conditions in euryhaline teleosts (for a review, see Tachibana and Sakamoto, 2014).

It has recently been proposed that the widely used name of “PrRP”

Abbreviations: AH, adenohypophysis; CT, connective tissue; D, diencephalon; HB, habenula; Hp, periventricular hypothalamus; Hyinf, infundibular nucleus of hypothalamus; Hyp, hypothalamus; lr, lateral recess; Med, medulla oblongata; Mes, mesencephalon; NH, neurohypophysis; OB, olfactory bulb; ON, optic nerve; P1, pallial layer 1; P2, pallial layer 2; T, telencephalon; vIII, third ventricle

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in non-mammalian vertebrates should be renamed "PrRP2" because synteny analysis suggested that PrRP and C-RFa originated through gene duplication from a common ancestral gene (see Tachibana and Sakamoto, 2014). In the present study, we follow this new nomenclature.

In the sea lamprey *Petromyzon marinus* (class Agnatha), two RFamide peptides (RFa-A and RFa-B) have been identified (Moriyama et al., 2007). Although synteny analysis has not been available for the genes of these RFamide peptides, RFa-A and RFa-B have amino acid sequences similar to PrRP and PrRP2, respectively. In particular, the amino acid sequence of the C-terminal of RFa-B is identical with that of teleost PrRP2. Lampreys do not appear to possess PRL (Bently, 1998), suggesting that PrRP appeared prior to the occurrence of PRL. Therefore, the original role of PrRP/PrRP2 would not be the regulation of PRL release; rather, PrRP/PrRP2 would regulate growth hormone (GH) release and feeding (see Tachibana and Sakamoto, 2014). Indeed, lamprey RFa-A and RFa-B dose-dependently inhibited GH mRNA expression *in vitro* in the sea lamprey *Petromyzon marinus* (Moriyama et al., 2007).

Hagfish are included in the class Agnatha with lampreys. The hagfish is commonly thought to represent one of the earliest evolutionary branches among vertebrates (Forey and Janvier, 1993). Thus, it is of interest to examine the involvement of neuropeptides in the regulation of pituitary hormone secretion in hagfish from an evolutionary perspective, as described below. Four novel PQRFamide peptides have been identified in the brain of the brown hagfish *Paramyxine atami* (Osugi et al., 2011). It has been reported that PQRFa (one of the PQRFamide peptides)-immunoreactive (ir) cell bodies were detected in the infundibular nucleus of hypothalamus (HYinf) of the brown hagfish (Osugi et al., 2011).

Since PrRP2 has not been identified in the hagfish, we investigated whether PrRP2-ir peptide exists in the brain and pituitary of the inshore hagfish *Eptatretus burgeri* by immunohistochemistry to obtain further understanding of the neuroendocrine system in this species. Moreover, the distribution of PrRP2-ir cell bodies and PQRFa-ir cell bodies was compared by dual-label immunohistochemistry to examine possible reciprocal connections of PrRP2 and PQRFa neurons. The distribution of PrRP2-ir cell bodies and corticotropin-releasing hormone (CRH)-ir cell bodies was also compared since CRH-ir cell bodies were detected by immunohistochemistry in the preoptic nucleus, periventricular preoptic nucleus, HYinf, and in the nucleus "A" of Kusunoki et al. (1982) in the medulla oblongata (Amano et al., 2016).

2. Materials and methods

2.1. Experimental fish

The inshore hagfish of both sexes (body weight 180–400 g) collected in Sagami Bay were purchased commercially. Fish were fixed just after arrival at the laboratory. The experiment was performed following the guidelines of the animal care committee of Kitasato University.

2.2. Dot blot assay

A rabbit polyclonal antibody raised against salmon PrRP2 was used for dot blot assay. This antibody detects immunoreactivity in the brain of rainbow trout (Moriyama et al., 2002), the ovoviviparous fish species *Poecilia reticulata* (guppy) (Amano et al., 2007), and the sea lamprey (Moriyama et al., 2007). The specificity of the antibody against other RFamide peptides in hagfish, i.e., PQRFa, PQRFa-RP-1, PQRFa-RP-2, and LPQRFa, was examined by a dot blot assay according to Yokoyama et al. (2011), with a slight modification. Briefly, various amounts of PrRP2 (8, 40, 200, and 1000 ng) were spotted onto a 0.2 μm nitrocellulose membrane (Bio-Rad, CA, USA). The blot was incubated overnight at 4 °C with the primary antibody, which had been diluted 2000-fold with 0.1 M phosphate buffer (pH 7.4) containing 0.75% NaCl and 0.3% Triton X-100 (0.1 M PBST). A Histofine immunostaining kit (Nichirei, Tokyo, Japan) was used to detect immunoreactivity. To test the specificity of the reaction, the membrane was also incubated with the antibody that had been pre-absorbed overnight at 4 °C with an excess amount of synthetic PrRP2 (100 μg PrRP2 in 1 ml of diluted antibody). The subsequent procedure was the same as the normal antibody. Immunoreactions with other PQRFamide peptides were also examined. The subsequent procedure was the same as in the first experiment except that only 200 ng of each peptide was spotted.

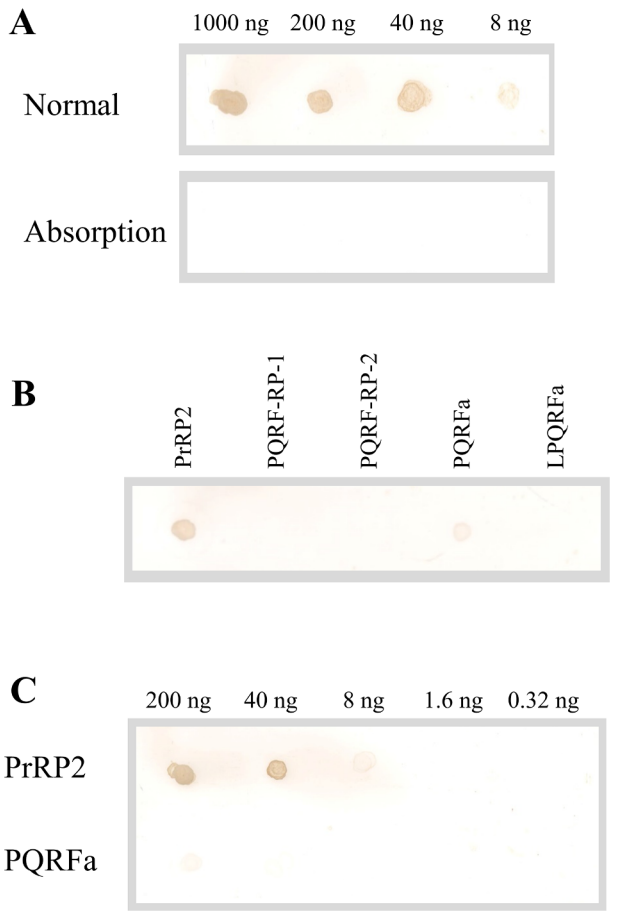

Fig. 1. Dot blot assay for determining the specificity the anti-salmon PrRP2 antibody. (A) Various amounts of PrRP2 (8, 40, 200, and 1000 ng) are spotted onto a 0.2 μm nitrocellulose membrane, which was incubated with the anti-salmon PrRP2 antibody diluted 2000-fold with 0.1 M PBST (upper) or with the anti-salmon PrRP2 antibody (2000-fold with 0.1 M PBST) that had been pre-absorbed overnight at 4 °C with an excess amount of synthetic PrRP (lower). (B) PrRP2 and RFamide peptides (PQRFa-RP-1, PQRFa-RP-2, PQRFa, and LPQRFa) are spotted onto a 0.2 μm nitrocellulose membrane (200 ng each). The membrane was incubated with the anti-salmon PrRP2 antibody diluted 2000-fold with 0.1 M PBST. (C) Various amounts of PrRP2 and PQRFa (0.32, 1.6, 8, 40, and 200 ng) are spotted onto a 0.2 μm nitrocellulose membrane. The membrane was incubated with the anti-salmon PrRP2 antibody diluted 2000-fold with 0.1 M PBST.

2.3. Immunohistochemistry for PrRP2

The fish were anesthetized with crushed ice. The brains were excised, fixed with Bouin's fluid for 24 h at 4 °C, rinsed in cold 70% ethanol, dehydrated through an ascending series of ethanol, and embedded in paraplast (Monoject, Sherwood Medical, St Louis, MO, USA). Sagittal or frontal sections were cut at 8 or 12 μm and mounted on MAS-GP coated slides (Matsunami, Osaka, Japan).

Immunohistochemistry was conducted according to Amano et al.

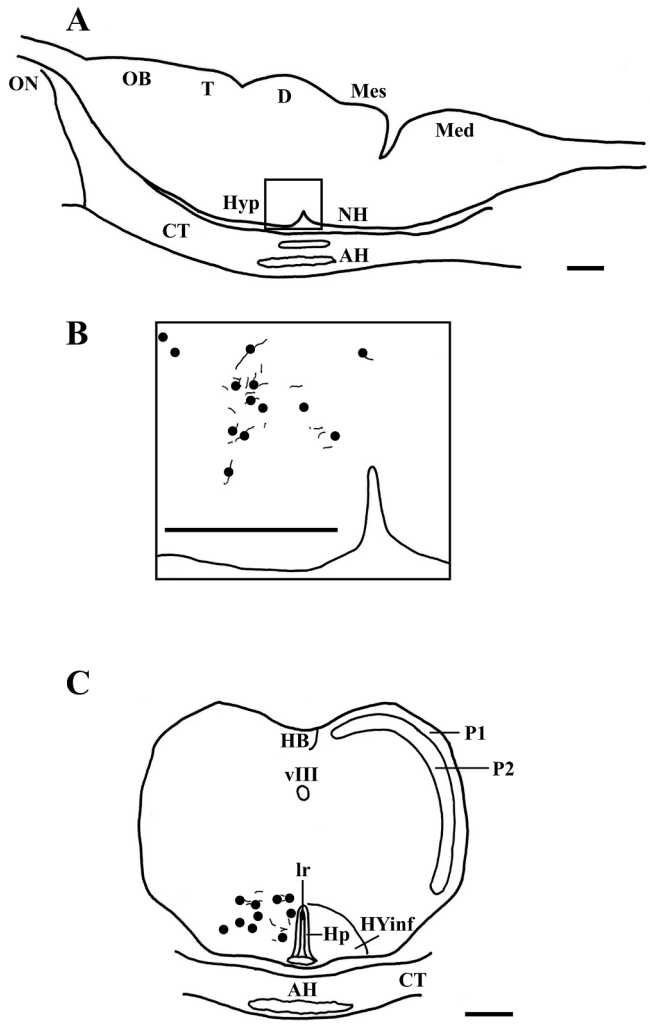

Fig. 2. Schematic illustration of the distribution of PrRP2-ir cell bodies (closed circles) and fibers (lines). (A) A midsagittal section of the hagfish brain. (B) Higher magnification of boxed area in (A). PrRP2-ir cell bodies (dots) and fibers are observed. (C) A frontal section of the hypothalamus of the hagfish brain. PrRP2-ir cell bodies (dots) and fibers are observed. Rostral is to the left for sagittal section and top is to the dorsal for frontal section. Bar indicates 1 mm. For the abbreviations of brain nuclei and regions, see the list of abbreviations.

(2016). The sections were incubated overnight at 4 °C with the antibody raised against salmon PrRP2 that had been diluted 4000-fold with 0.1 M PBST. A Histofine immunostaining kit was used for immunohistochemical reactions. After the sections were washed three times with 0.1 M PBST, biotin-labeled anti-rabbit IgG was added for 15 min at room temperature. Subsequently, the sections were washed three times with 0.1 M PBST and treated with peroxidase-labeled streptavidin for 15 min at room temperature. The sections were again washed three times with 0.1 M PBST, after which 3,3′-diaminobenzidine tetrahydrochloride (DAB) and hydrogen peroxide were added for 15 min at room temperature to visualize the peroxidase reaction. Finally, the sections were rinsed in distilled water and mounted using Permount (Fischer, Fair Lawn, NJ, USA).

To test the specificity of the immunohistochemical reactions, control sections were incubated in antisera that had been preabsorbed overnight at 4 °C with an excess amount of synthetic PrRP2 (2.5 μg PrRP2 in 1 ml of diluted antiserum). The subsequent procedure was identical to that used for the experimental sections.

For histological identification of the nuclear boundaries, adjacent sections were stained with cresyl violet. We followed the terminology of brain atlas of the Pacific hagfish *Eptatretus stouti* (Braun et al., 1995) and the Atlantic hagfish *Myxine glutinosa* (Wicht and Nieuwenhuys, 1998).

2.4. Dual-label immunohistochemistry for PrRP2 and PQRFa and for PrRP2 and CRH

Since PrRP2-ir cell bodies were detected in the HYinf, where PQRFa-ir cell bodies (Osugi et al., 2011) and CRH-ir cell bodies (Amano et al., 2016) were detected, we compared the distribution of PrRP2-ir cell bodies and PQRFa-ir cell bodies and that of PrRP2-ir cell bodies and CRH-ir cell bodies in the hypothalamus by dual-label immunohistochemistry, according to Amano et al. (2016). The rabbit polyclonal antibody raised against lamprey PQRFa was used (Osugi et al., 2006). The specificity of the antibody had been examined by enzyme linked immunosorbent assay (ELISA): the antibody shows cross-reactivity of about 1% for PrRP2 (Osugi et al., 2006). This antibody was used to detect immunoreactivities in the brain of the brown hagfish (Osugi et al., 2011). Since the anti-salmon PrRP2 antibody was found to slightly cross-react with PQRFa by dot blot assay, the anti-lamprey PQRFa antibody had been pre-absorbed overnight at 4 °C with an excess amount of synthetic PrRP2 (10 μg PrRP2 in 1 ml of 1000-fold diluted antiserum). A rabbit polyclonal antibody raised against human/mouse/rat CRH (Cat. # AB-02, Advanced Targeting Systems, San Diego, CA, USA) was also used. We have already clarified the cross-reactivity of the antibody against other CRH family peptides, such as urocortin-I, II, III, urotensin-I, and sauvagine: the cross-reactivity of anti-CRH antibody against CRH family peptides was found to be less than 0.01%, indicating the specificity of the antibody (Amano et al., 2016). The sections were reacted with the anti-salmon PrRP2 antibody diluted 4000-fold with 0.1 M PBST containing 0.02% BSA, as described above. After the DAB reaction, the sections were washed three times with distilled water and maintained first at 40 °C in 100 mM glycine-HCl buffer (pH 2.0) for 60 min and then at 40 °C in an 8 M urea solution for 60 min, in order to prevent interaction between the first and second immunostaining systems. Subsequently, the sections were washed three times with 0.1 M PBST and incubated overnight at 4 °C with the absorbed anti-lamprey PQRFa antibody or anti-CRH antibody (1000-fold). Biotin-labeled anti-rabbit IgG was added to the sections for 15 min at room temperature after rinsing them three times with 0.1 M PBST. This was followed by re-rinsing the sections three times with 0.1 M PBST and the addition of alkaline phosphatase-labeled streptavidin (PerkinElmer Life Sciences Japan, Tokyo, Japan) diluted 100-fold with 0.1 M PBST for 15 min at room temperature. Next, after three washes with 0.1 M PBST, BCIP/NBT Substrate Solution (PerkinElmer, Boston, MA, USA) containing 0.024% levamisole was added to the sections for 30 min at room temperature under dark condition to facilitate the visualization of alkaline phosphatase. Finally, the sections were rinsed in distilled water and mounted with Aqua-Poly/Mount (Polysciences, Inc, Warrington, PA, USA).

3. Results

3.1. Dot blot assay

A dose-dependent signal was observed using the anti-salmon PrRP2 antibody: strong, moderate, and weak signals were seen for 1000 ng, 200 and 40 ng, and 8 ng PrRP2, respectively. No signal was detected when using the anti-salmon PrRP2 antibody that had been pre-absorbed overnight at 4 °C with an excess amount of PrRP2 (Fig. 1A). The anti-salmon PrRP2 antibody also slightly cross-reacted with 200 ng of PQRFa, but not with PQRFa-RP-1, PQRFa-RP-2, and LPQRFa (Fig. 1B). Strong, moderate, and weak signals were seen for 200 ng, 40 ng, and 8 ng PrRP2, respectively, and no signal was detected for under 1.6 ng of PrRP2. In contrast, weak and slight signals were seen for 200 ng and 40 ng PQRFa, respectively, and no signal was detected under 8 ng of PQRFa (Fig. 1C).

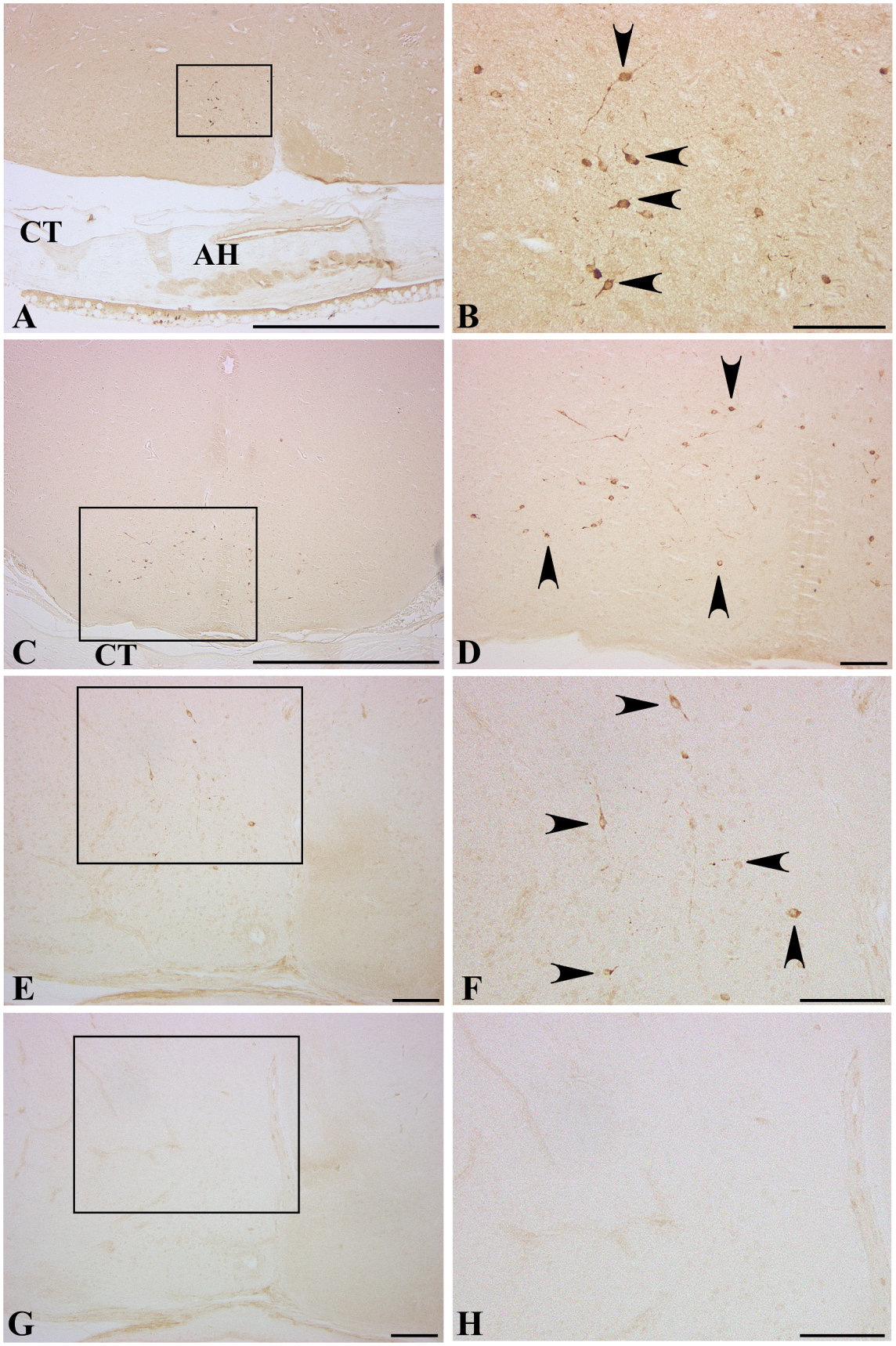

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Fig. 3. (A) Sagittal section through the preopticohypothalamic area and the NH. (B) Higher magnification of boxed area in (A). PrRP2-ir cell bodies (arrowheads) and fibers are observed. (C) Frontal section through the hypothalamus. (D) Higher magnification of boxed area in (C). PrRP2-ir cell bodies (arrowheads) and fibers are observed. (E) Sagittal section through the preopticohypothalamic area. (F) Higher magnification of boxed area in (E). PrRP2-ir cell bodies (arrowheads) and fibers are observed. (G) Adjacent section of (E). (H) Higher magnification of boxed area in (G). No PrRP2-ir cell bodies and fibers are observed when the anti-salmon PrRP2 antibody was pre-absorbed overnight at 4 °C with an excess amount of PrRP2. Rostral is to the left. Bars indicate 1 mm (A, C) and 100 μm (B, D, E, F, G, H). AH adenohypophysis, CT connective tissue.

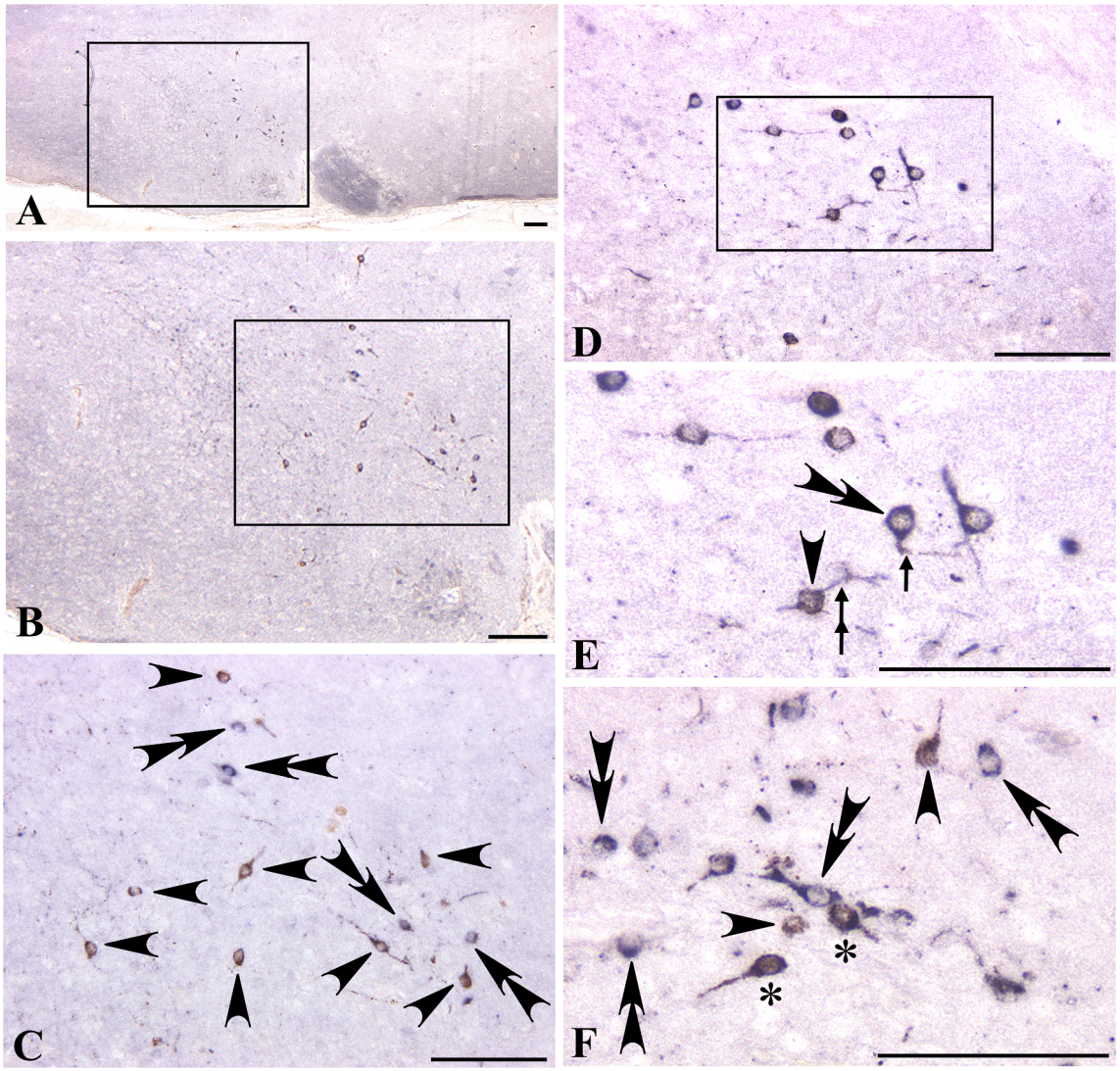

Fig. 4. (A) Photographs showing dual-label immunohistochemistry for PrRP2 and PQRFa in the hypothalamus (sagittal section). (B) Higher magnification of boxed area in (A). (C) Higher magnification of boxed area in (B). PrRP2-ir cell bodies (brown, single arrowheads) and PQRFa-ir cell bodies (blue, double arrowheads) are distinguishable. (D) Photographs showing dual-label immunohistochemistry for PrRP2 and PQRFa in the hypothalamus (sagittal section). (E) Higher magnification of boxed area in (D). PrRP2-ir fibers (brown, single arrow) are in close contact with PQRFa-ir cell body (blue, double arrowhead) and PQRFa-ir fibers (blue, double arrow) are in close contact with PrRP2-ir cell body (brown, single arrowhead). (F) Photographs showing dual-label immunohistochemistry for PrRP2 and PQRFa in the hypothalamus (sagittal section). PrRP2-ir cell bodies (brown, single arrowhead), PQRFa-ir cell bodies (blue, double arrowhead), and cell bodies that appear as a mix of brown and blue colors (asterisks) are observed. Rostral is to the left. Bars indicate 100 μm.

3.2. Immunohistochemistry for PrRP2

The distribution of PrRP2-ir cell bodies and fibers in the brain and pituitary is summarized in Fig. 2. PrRP2-ir cell bodies were detected only in the HYinf of the hypothalamus (Figs. 2B, C, 3A, B, C, D, E, F). PrRP2-ir fibers were distributed around the HYinf and did not seem to project to other brain regions or the dorsal wall of the neurohypophysis (NH) (Fig. 2B, C). Compared to the normal immunostaining in the adjacent section, no PrRP2-ir cell bodies or fibers were observed when the anti-salmon PrRP2 antibody was pre-absorbed overnight at 4 °C with an excess amount of PrRP2 (Fig. 3G, H), indicating the specificity of immunoreaction.

3.3. Dual-label immunohistochemistry for PrRP2 and PQRFa and for PrRP2 and CRH

PrRP2-ir cell bodies (brown) and PQRFa-ir cell bodies (blue) were distinguishable in the hypothalamus (Fig. 4A, B, C). PQRFa-ir cell bodies (blue) and fibers were detected in HYinf, as previously reported in the brown hagfish (Osugi et al., 2011). The number of PQRFa-ir cell bodies seemed larger than that of PrRP2-ir cell bodies. Some PrRP2-ir fibers were in close contact with PQRFa-ir cell bodies (Fig. 4E). In

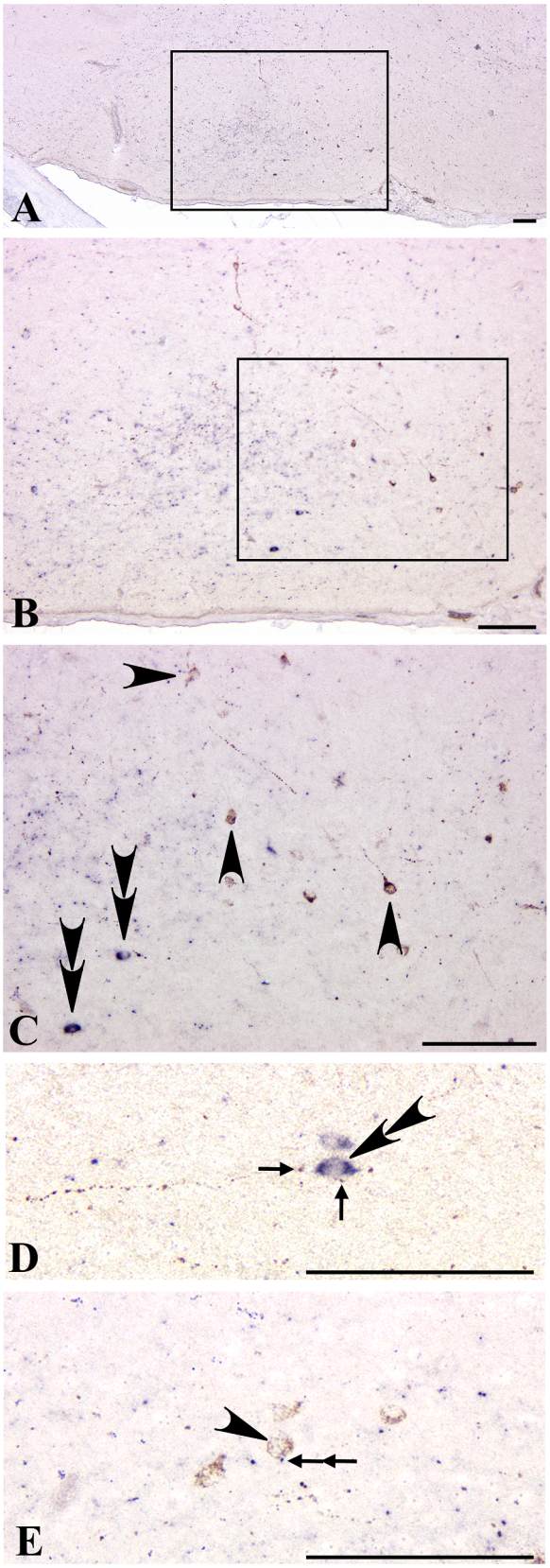

Fig. 5. (A) Photographs showing dual-label immunohistochemistry for PrRP2 and CRH in the hypothalamus (sagittal section). (B) Higher magnification of boxed area in (A). (C) Higher magnification of boxed area in (B). PrRP2-ir cell bodies (brown, single arrowheads) and CRH-ir cell bodies (blue, double arrowheads) are distinguishable. (D) PrRP2-ir fibers (brown, single arrow) are in close contact with CRH-ir cell body (blue, double arrowhead) in the hypothalamus (sagittal section). (E) CRH-ir fibers (blue, double arrow) are in close contact with PrRP2-ir cell body (brown, single arrowhead) in the hypothalamus (sagittal section). Rostral is to the left. Bars indicate 100 μm.

addition, some PQRFa-ir fibers were in close contact with PrRP2-ir cell bodies (Fig. 4E). Cell bodies that appeared as a mix of brown and blue colors were also observed (Fig. 4F).

PrRP2-ir cell bodies (brown) and CRH-ir cell bodies (blue) were also distinguishable in the hypothalamus (Fig. 5A, B, C). CRH-ir cell bodies (blue) and fibers were detected in the HYinf, as previously reported in the inshore hagfish (Amano et al., 2016). Some PrRP2-ir fibers were in close contact with CRH-ir cell bodies (Fig. 5E). In addition, some CRH-ir fibers were in close contact with PrRP2-ir cell bodies (Fig. 5F).

4. Discussion

It is well established that neuropeptides in the hypothalamus of vertebrates are involved in the regulation of pituitary hormone secretion. In the present study, the distribution of PrRP2-ir cell bodies and fibers in the brain and pituitary of the inshore hagfish *Eptatretus burgeri* was elucidated by immunohistochemistry to obtain further understanding of the neuroendocrine system of the hagfish.

Immunohistochemistry showed that PrRP2-ir cell bodies were located only in the HYinf of the hypothalamus. In the sea lamprey *Petromyzon marinus*, PrRP2-ir cell bodies were detected in the ventral part of the hypothalamus, and PrRP2-ir fibers were abundant throughout the brain and innervated the pituitary gland, as revealed by immunohistochemistry using the anti-salmon PrRP2 antibody (Moriyama et al., 2007). Distribution of PrRP2-ir cell bodies and fibers was reported in some teleost fish. In the rainbow trout, PrRP2-ir cell bodies were detected in the posterior part of the hypothalamus and PrRP2-ir fibers were abundant from the hypothalamus to the ventral telencephalon (Moriyama et al., 2002). In the ovoviviparous fish species *Poecilia reticulata* (guppy), PrRP2-ir cell bodies were detected in the nucleus lateralis tuberis pars posterioris in the hypothalamus, and PrRP2-ir fibers were detected not only in the hypothalamus but also in areas such as the optic tectum and thalamus (Amano et al., 2007). Thus, the localization of PrRP2-ir cell bodies was similar among hagfish, lamprey, and teleost species, i.e., PrRP2-ir cell bodies were detected in the infundibular nucleus in the hypothalamus. On the other hand, the distribution of PrRP2-ir fibers was different among these animals. In hagfish, PrRP2-ir fibers in the brain were extremely limited, whereas in lamprey and teleost species, PrRP2-ir fibers were widely distributed in the hypothalamus and/or in the pituitary, suggesting that the functions of PrRP2 would have diversified during fish evolution. Based on the phylogenetic position, hagfish may have conserved the ancestral features of PrRP2 in its distribution and function.

Although it remains unclear whether PRL is present in the hagfish pituitary, a slight immunoreaction of PRL was observed in the pituitary of the inshore hagfish by an anti-sturgeon PRL antibody (Nozaki et al., 2005). Even if PRL exists in the pituitary of the hagfish, PrRP2 might not directly regulate PRL secretion, since PrRP2-ir fibers did not seem to project to the dorsal wall of the NH. Thus, it is suggested that PrRP2 has functions other than stimulation of PRL secretion. This is also true for the sea lamprey. In the sea lamprey, RFamide peptides, which are teleost PrRP2 homologs, inhibit the expression of GH and melanotropin genes in the pituitary (Moriyama et al., 2007). It has been suggested that GH is the ancestral hormone of the GH family, and that the emergence of PRL and somatolactin (SL) resulted from gene duplication events during the evolution of vertebrates because GH, but neither PRL nor SL, have been detected in agnathans (Kawauchi and Sower, 2006). Indeed, GH has been detected in the pituitary of three hagfish species by immunohistochemistry, although some species-specific differences

were observed (Nozaki et al., 2005, Nozaki et al., 2007, Nozaki, 2008). Although PrRP2 may be involved in GH secretion, as in the case of the sea lamprey (Moriyama et al., 2007), it is unlikely that PrRP2 directly regulates GH secretion because PrRP2-ir fibers were not observed in dorsal wall of the NH.

In the brown hagfish, four PQRFamide peptides (hagfish PQRFa, PQRFa-RP-1, PQRFa-RP-2 and LPQRFa) were identified as endogenous ligands (Osugi et al., 2011). PQRFa-ir cell bodies were detected in the HYinf. In the inshore hagfish, CRH-ir cell bodies also were detected in the hypothalamus (Amano et al., 2016). To examine possible reciprocal connections of PrRP2 neurons and PQRFa or CRH neurons in the hypothalamus of the inshore hagfish, we compared the localization of PrRP2-ir cell bodies and those of PQRFa-ir or CRH-ir cell bodies in the brain by dual-label immunohistochemistry. The results suggested that reciprocal connections likely exist between the PrRP2 and PQRFa neurons, and between PrRP2 and CRH neurons. Furthermore, dual-label immunohistochemistry for PrRP2 and PQRFa showed that the cell bodies appeared as a mix of brown and blue colors suggesting co-localization of PrRP2 and PQRFa. As for the distribution of the immunoreactive fibers, in the brown hagfish, no PQRFa-ir fibers were observed in the NH and the adenohypophysis (AH); instead, PQRFa-ir fibers terminated at blood vessels in the HYinf, suggesting that PQRFa is released into the general circulation, thus reaching the pituitary (Osugi et al., 2011). Indeed, one of the PQRFamide peptides, LPQRFa, significantly increased the expression of gonadotropin β (GTHβ) mRNA levels in primary cultures of adult brown hagfish pituitaries *in vitro* (Osugi et al., 2011); thus GTH has been identified in the brown hagfish (Uchida et al., 2010). Bundles of CRH-ir fibers were detected in the dorsal but not in the ventral wall of the NH. Moreover, no CRH-ir fibers were present close to adrenocorticotropic hormone (ACTH) cells in the AH (Amano et al., 2016). Even if CRH also regulates ACTH secretion in the hagfish, it is likely that CRH in the NH reaches the AH via diffusion, as previously reported by Tsukahara et al. (1986). In contrast to PQRFa-ir and CRH-ir fibers, PrRP2-ir fibers were restricted around PrRP2-ir cell bodies. The limited distribution of PrRP2-ir fibers suggests that PrRP2 acts as a neuromodulator or neurotransmitter to regulate the function of the HYinf, or indirectly regulates peripheral organs, including the pituitary, via other peptide neurons.

In summary, immunohistochemistry results showed that PrRP2-ir peptide is expressed in the brain of the inshore hagfish. This is the first observation demonstrating the distribution of PrRP2-ir cell bodies and fibers in the brain of the inshore hagfish. Further studies are needed to clarify the relationship between PrRP2 and other peptides, including PQRFa and CRH, in the hagfish.

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