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The Study of Pituitary Adenylate Cyclase Activating Polypeptide (PACAP)-like Immunoreactivity in the Brain of a Teleost, Stargazer, *Uranoscopus japonicus*

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ABSTRACT—Pituitary adenylate cyclase activating polypeptide (PACAP), a member of the secretin/glucagon/vasoactive intestinal polypeptide family of peptides, consists of a 38-residue (PACAP 38) and a truncated 27-residue (PACAP 27) form that play several roles in mammals. Recently, we isolated a PACAP-like peptide with a sequence highly homologous with that of tetrapod PACAP from the brain of a teleost, the blue spotted-stargazer (*Gnathagnus elongatus*). In this study, a PACAP-like peptide obtained from the brain of the stargazer, *Uranoscopus japonicus*, was examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting analysis using an anti-PACAP 27 serum. The stargazer PACAP-like peptide and synthetic human PACAPs were reacted with this antiserum. The distribution of PACAP-like immunoreactivity in the stargazer brain was also studied immunohistochemically using the peroxidase-antiperoxidase method. PACAP-like immunoreactive (LI) cells were found in the nucleus preopticus, pars parvicellularis of the hypothalamus. PACAP-LI nerve fibers and terminals were observed in the anterior neurohypophysis and in the rostral pars distalis, and many PACAP-LI nerve fibers were seen in the medulla oblongata. These results suggest that PACAP-like peptide may be involved in pituitary regulation and/or other neural functions in the stargazer brain.

INTRODUCTION

Pituitary adenylate cyclase activating polypeptide (PACAP) is a neuropeptide that was first isolated from the ovine hypothalamus (Miyata *et al.*, 1989) and two molecular forms of PACAP have been identified: a 38-residue peptide and a shorter 27-residue form (Miyata *et al.*, 1990). Structurally, PACAP is a member of the secretin/glucagon/vasoactive intestinal polypeptide family of peptides and ovine, rat and human PACAPs are identical. In addition to stimulating adenylate cyclase activity in the pituitary, PACAP has been reported to have various actions in mammals (see Arimura, 1992; Arimura and Shioda, 1995; Arimura *et al.*, 1994). In non-mammalian species, PACAP comprising 38 amino acid residues with only one substitution compared with mammalian PACAP 38 that stimulated adenylate cyclase activity in the pituitary gland was isolated from the brain of the frog, *Rana ridibunda* (Chartrel *et al.*, 1991, 1995). Nerve perikarya and fibers showing PACAP-like immunoreactivity were found to be distributed widely in the hypothalamo-pituitary complex as well as the central nervous system of *R. ridibunda* (Yon *et al.*, 1992, 1993).

Recently, we isolated a PACAP-like peptide with a highly homologous sequence with those of mammalian and frog PACAPs from the brain of a teleost, the blue spotted-stargazer (*Gnathagnus elongatus*) (Matsuda *et al.*, 1996). In this study, a PACAP-like peptide obtained from the brain of another teleost, the stargazer (*Uranoscopus japonicus*) was examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting analysis using an anti-PACAP 27 serum and the distribution of PACAP-like immunoreactivity in the stargazer brain was investigated immunohistochemically.

MATERIALS AND METHODS

Animals

Adult stargazers, *Uranoscopus japonicus* (Order Perciformes), were obtained from the fishermen in Yokata fishery harbor of Toyama City, Japan. All animal experiments were conducted in accordance with Toyama University's guidelines for the care and use of animals.

Antiserum

The primary anti-PACAP 27 serum (No. 92112-4) was raised in a rabbit, and the details of its production and characterization are

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described elsewhere (Köves *et al.*, 1990).

Electrophoresis and Western blotting analysis

An extract was obtained from 50 stargazer brains with 0.5 M acetic acid. A purified stargazer PACAP-like peptide was prepared from this extract by chromatography on a Sep-Pak C18 cartridge (Waters Associates, Milford, MA, USA) and successive high-performance liquid chromatography (Model 510, Waters Associates, Milford, MA, USA) on gel-filtration (Superose-12 HR 10/30, Pharmacia Biotech, Uppsala, Sweden), cation-exchange (CM-TOYOPEARL 650S, TOSOH, Tokyo, Japan) and two reverse-phase (Puresil C18, Waters Associates, Milford, MA, USA; Inertsil Phenyl, Gas-Liquid Science, Tokyo, Japan) columns. The details of the purification procedure are described elsewhere (Matsuda *et al.*, 1997).

Synthetic peptides: human PACAPs 27 and 38, porcine vasoactive intestinal polypeptide, porcine secretin and human glucagon were purchased from Peptide Institute Inc., Osaka, Japan. Aliquots of the extract (10 μg protein), purified peptide (60 ng) and synthetic peptides (1.5–2.5 μg) were subjected to SDS-PAGE with 15% polyacrylamide gels containing 8 M urea and 0.1% SDS at pH 8.9 on a pair of gels at 50 V, one of which was stained with 0.25% coomassie brilliant blue R-250 (MERCK, Darmstadt, Germany) and the other was used for blotting analysis. The proteins and peptides were transferred electrophoretically to a nitrocellulose membrane (Hybond-C Super, Amersham Int. plc, Buckinghamshire, UK). After the membrane was incubated with 3% bovine serum albumin (BSA; Sigma Chemical Co., St. Louis, MO, USA) in 0.01 M PBS for 1 hr, it was immunostained by the peroxidase-antiperoxidase (PAP) method using an anti-PACAP 27 serum (diluted 1:2,000) for 24 hr. Then, the membrane was treated with swine anti-rabbit immunoglobulin (diluted 1:100, DAKO A/S, Denmark) for 1 hr, incubated with the rabbit peroxidase-antiperoxidase (PAP) complex (diluted 1:100, DAKO A/S, Denmark) for 1.5 hr and treated with 20 mg 3, 3'-diaminobenzidine-4 HCl (Dojin, Tokyo, Japan) and 0.005% H_2O_2 in 100 ml 0.1 M Tris-HCl buffer (pH 7.6). The specificity of the immunostaining was checked by incubating a blotted membrane with normal rabbit serum (diluted 1:50, DAKO A/S, Denmark) instead of primary antiserum and with 1 ml primary antiserum (diluted 1:2,000) preincubated with excess (60 μg, 20 μM) synthetic human PACAP 27. Marker proteins (Rainbow™ colored protein molecular weight markers, Amersham Int. plc, Buckinghamshire, UK) were used to estimate the molecular weight of each band.

Immunohistochemical staining

Six fishes of both sexes were anesthetized with MS-222 (Sankyo Co., Tokyo, Japan), blood was flushed from the blood vessels by infusing 80 ml physiological saline for teleosts containing heparin through the aorta and then the animals were perfused with 80 ml 4% paraformaldehyde and 0.3% picric acid in 0.1 M PBS for 1 hr. The whole brains were removed immediately after perfusion, postfixed with the latter solution at 4°C for 24 hr, dehydrated, embedded in paraffin and serial frontal and sagittal sections (8 μm thick) were cut. Several sections were selected for normal staining with 0.1% cresyl violet acetate (EM Science, Gibbstown, NJ, USA). Neighboring sections were treated with the normal swine serum (diluted 1:50, DAKO A/S, Denmark), then stained with the anti-PACAP 27 serum (diluted 1:4,000) at room temperature for 24 hr, treated with swine anti-rabbit immunoglobulin (diluted 1:100, DAKO A/S, Denmark) for 1 hr, incubated with the rabbit PAP complex (diluted 1:100, DAKO A/S, Denmark) for 1.5 hr, and finally treated with 20 mg 3, 3'-diaminobenzidine-4 HCl (Dojin, Tokyo, Japan) and 0.005% H_2O_2 in 100 ml 0.05 M Tris-HCl buffer (pH 7.6). After immunostaining, all the sections were counterstained with Mayer's hematoxylin. The specificity of the immunostaining was checked by incubating sections with normal rabbit serum (diluted 1:50, DAKO A/S, Denmark) instead of the primary antiserum and with 1 ml primary antiserum (diluted 1:4,000) preincubated with excess (30 μg, 10 μM) synthetic human PACAP 27. The nomenclature of stargazer brain nuclei was based on the papers of Tuge *et al.* (1968) on the blue spotted-stargazer and Anglade *et al.* (1993) on the goldfish.

RESULTS

Figure 1 shows the SDS-PAGE (A) and Western blotting (B) patterns of each material. The acid extract of the whole brains, purified PACAP-like peptide and synthetic human PACAPs were reacted with an anti-PACAP 27 serum. The lanes containing the acid extract and purified peptide each showed a single band with a mobility similar to that of synthetic human PACAP 38 that was stained by the antiserum, whereas the other peptides demonstrated no immunoreactivity (Fig. 1B). The normal rabbit serum and the antiserum preadsorbed with PACAP 27 stained no bands (data not

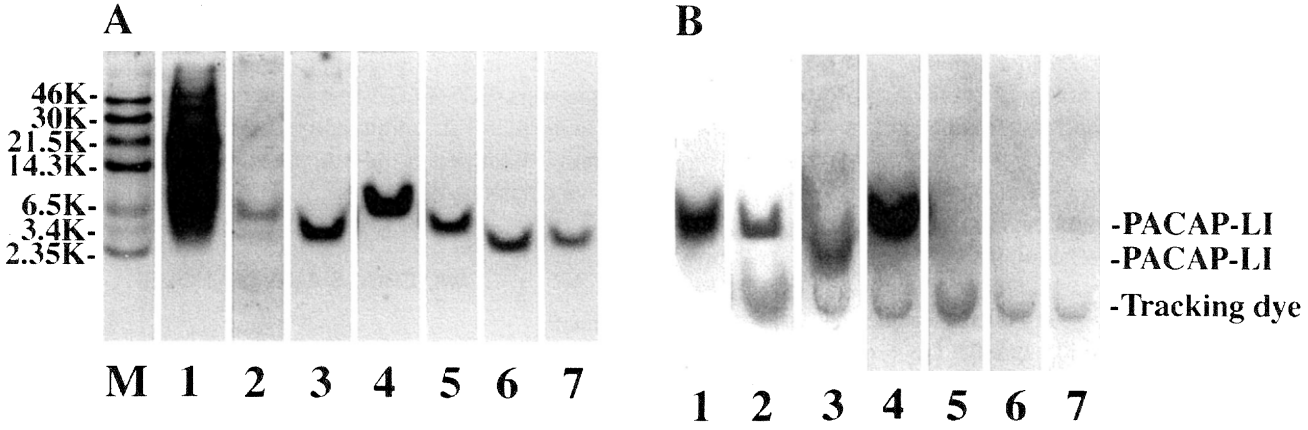

Fig. 1. Detection of PACAP-like immunoreactivities (PACAP-LI) in each material by Western blotting analysis. (**A**) SDS-PAGE patterns stained with coomassie brilliant blue. (**B**) Western blotting patterns stained with an anti-PACAP 27 serum. M, molecular weight markers. Lanes 1, Acid extract of stargazer brain; 2, purified stargazer PACAP-like peptide; 3, synthetic PACAP 27; 4, synthetic PACAP 38; 5, synthetic vasoactive intestinal polypeptide; 6, synthetic secretin; 7, synthetic glucagon.

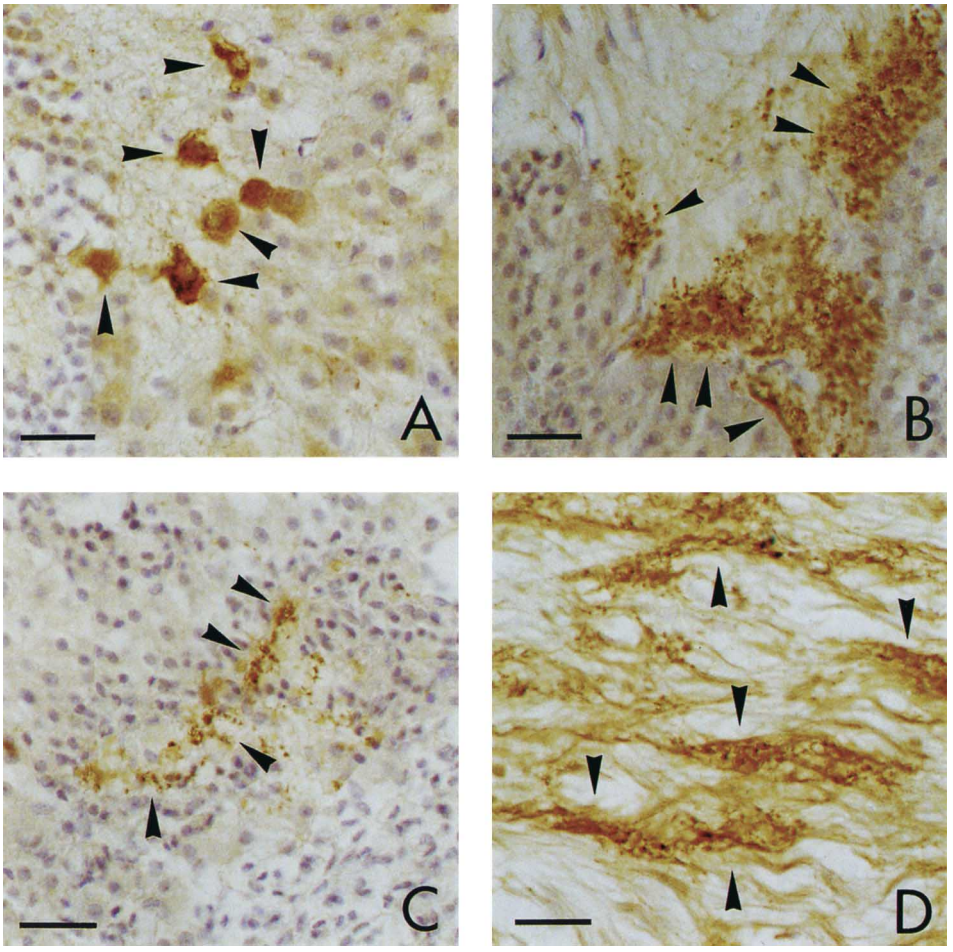

Fig. 2. (**A**) PACAP-LI neuronal cell bodies in the nucleus preopticus, pars parvicellularis. (**B**) PACAP-LI nerve fibers and terminals on the anterior neurohypophysis. (**C**) PACAP-LI nerve terminals in the rostral pars distalis. (**D**) Dense bundle of PACAP-LI nerve fibers on the dorsolateral zone of the medulla oblongata. The sections were counterstained with Mayer's hematoxylin. PACAP-LI neurons and nerve fibers are shown by arrowheads. Scale bar: 20 μm.

shown). Figure 2 shows the immunohistochemical results for the stargazer brain. A population of PACAP-like immunoreactive (LI) neuronal cell bodies was found in the nucleus preopticus, pars parvicellularis (Fig. 2A), PACAP-LI nerve fibers and terminals were located on the anterior neurohypophysis (Fig. 2B), and scattered PACAP-LI nerve terminals were seen in the rostral pars distalis (Fig. 2C). The dorsolateral zone of the medulla oblongata also exhibited a dense plexus of intensely immunoreactive fibers (Fig. 2D). No immunoreactivity was detected when the sections were treated with normal rabbit serum and antiserum preadsorbed with synthetic PACAP 27 (data not shown). Diagrams of sections showing the distributions of PACAP-LI cells and fibers throughout the brain of the stargazer are shown in Fig. 3.

DISCUSSION

Immunohistochemical examinations have revealed PACAP-like immunoreactivity in some peripheral organs, such as the chromaffin tissues (Reid *et al.*, 1995) and gastrointestinal tracts (Olsson and Holmgren, 1994) of fishes, but the present study is the first to show the distribution and localization of PACAP-like immunoreactivity in the brain of a teleost.

We have described the purification and characterization of a PACAP-like peptide with an amino acid sequence that shows high homology with those of mammalian and frog PACAPs, but contains several amino acid residue substitutions, from the blue spotted-stargazer brain on the basis of its immunoreactivity with an anti-PACAP 27 serum (Matsuda *et al.*, 1996). In this study, the results of SDS-PAGE and Western blotting analysis of the stargazer brain indicated that this anti-PACAP 27 serum recognizes the substance with a molecular weight of approximately 5,000. This substance is a PACAP-like peptide that is distinct from secretin-, glucagon- and VIP-like peptides. Recently, we actually characterized and sequenced this stargazer PACAP-like peptide, which has a molecular mass of 4623, comprises 38 amino acid residues and its amino acid sequence shows 89 and 87% homology with those of mammalian and frog PACAPs, respectively (Matsuda *et al.*, 1997).

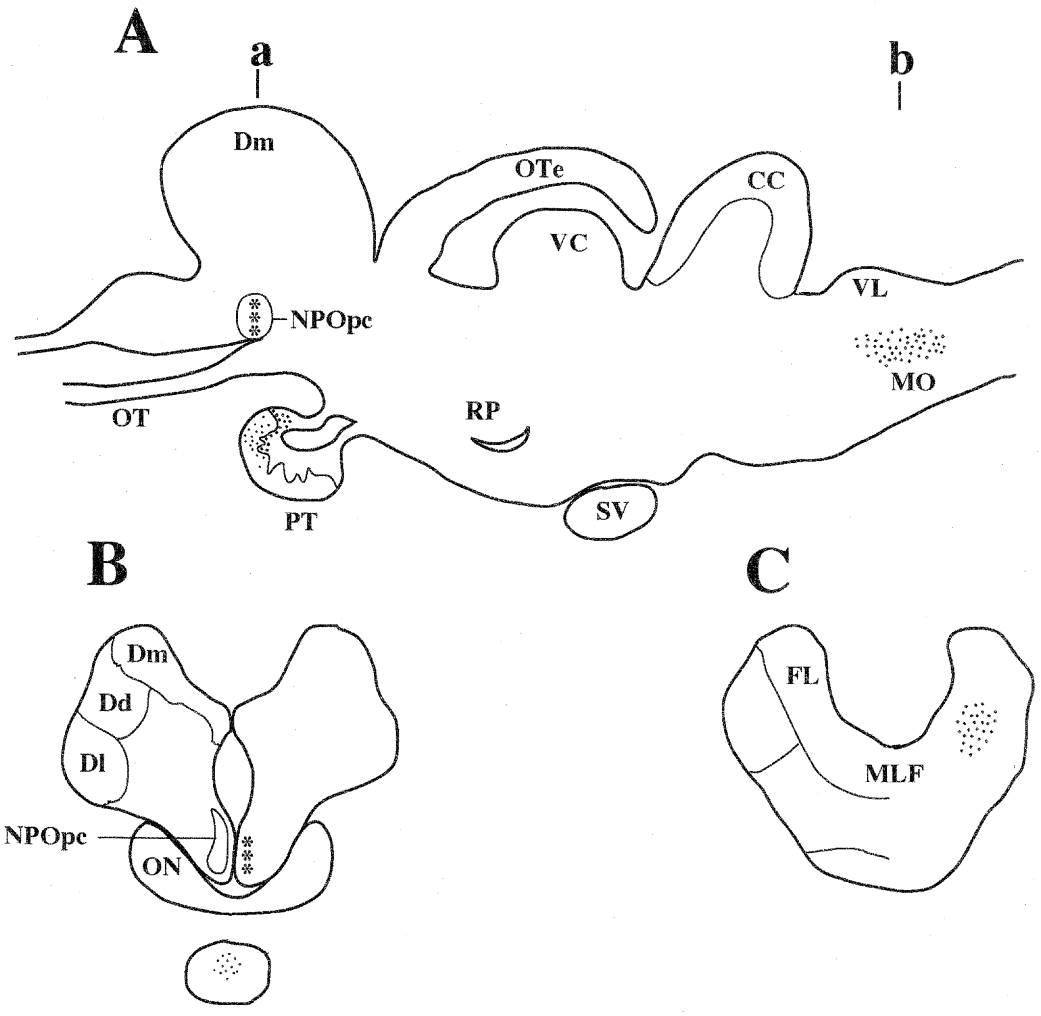

Fig. 3. Diagrams showing the distribution of PACAP-LI neuronal cell bodies (asterisks) and nerve fibers and terminals (dots) throughout the brain. Abbreviations: CC, corpus cerebelli; Dd, dorsal zone of area dorsalis telencephali; Dl, lateral zone of area dorsalis telencephali; Dm, medial zone of area of dorsalis telencephali; FL, facial lobe; MLF, medial longitudinal fascicle; MO, medulla oblongata; NPOpc, nucleus preopticus, pars parvicellularis; ON, optic nerve; OTe, optic tectum; PT, pituitary gland; RP, recessus posterior; SV, saccus vasculosus; VC, valvula cerebelli; VL, vagal lobe. (**A**) Schematic parasagittal section. (**B**) and (**C**) Schematic frontal sections at levels **a** and **b** shown in (**A**).

We showed that PACAP-LI neuronal cell bodies and fibers were located in the diencephalon, hypophysis and medulla oblongata. Neuronal cell bodies with PACAP-like immunoreactivity were observed in the nucleus preopticus, pars parvicellularis. PACAP-LI nerve fibers were observed in the anterior neurohypophysis. In teleosts, the neurohypophysis penetrates deeply into adenohypophysis and the neuronal cell bodies in the preoptic area which contain neuropeptides, such as galanin (Batten *et al.*, 1990; Holmqvist and Ekström, 1991), corticotropin releasing factor (Olivereau and Olivereau, 1988) and growth hormone releasing hormone (Olivereau *et al.*, 1990) may project their fibers to the neurohypophysis. Indeed, using a retrograde transport technique, Anglade *et al.* (1993) demonstrated that hypophysiotrophic areas are essentially restricted to the preoptic area of the goldfish brain. The distribution of PACAP-LI neurons is similar to those of neurons as mentioned above. Therefore, the preoptic area appears to be the major source of the PACAP-LI nerve fibers in the anterior neurohypophysis of the stargazer. We also observed scattered PACAP-LI nerve terminals in the rostral pars distalis suggesting that some of PACAP-LI fibers through the anterior neurohypophysis terminate in it. The dorsolateral zone of the medulla oblongata contained a dense bundle of PACAP-LI fibers. Any PACAP-LI cell bodies were not observed in the midbrain and the hindbrain.

PACAP-containing neurons in the central nervous systems of tetrapods have been well demonstrated in detail. In the mammalian hypothalami, PACAP-LI cells were located in the supraoptic and paraventricular nuclei and a dense network of PACAP-LI fibers was distributed throughout the hypothalami (Kivipelto *et al.*, 1992; Köves *et al.*, 1990, 1991; Vigh *et al.*, 1991). PACAP has been considered to be a pleiotropic neuropeptide with several functions, such as regulation of anterior and posterior pituitary hormone and neurotrans-

mitter release, and to act as a neurotrophic factor (see Arimura, 1992; Arimura and Shioda, 1995; Arimura *et al.*, 1994; Murase *et al.*, 1993; Shioda *et al.*, 1996; Uchida *et al.*, 1996). PACAP-LI cells and fibers were also found to be distributed widely throughout the frog brain and PACAP induced concentration-dependent stimulation of cAMP production in frog isolated pituitary fragments (Yon *et al.*, 1992, 1993) and stimulated calcium mobilization in frog dispersed distal pituitary cells (Gracia-Navarro *et al.*, 1992). These findings indicated that frog PACAP may also act as a hypophysiotropic hormone.

In our preliminary experiments, synthetic human PACAP 27, which has a one-amino acid residue substitution relative to the comparative portion of stargazer PACAP-like peptide, at higher concentration, stimulated cAMP production in the stargazer pituitary *in vitro* (Matsuda *et al.*, unpublished data). Our results suggest that human PACAP 27 activates adenylate cyclase in the stargazer pituitary, but the homologous peptide may be essential for full expression of this function. Although further studies are necessary to elucidate the physiological functions of stargazer PACAP-like peptide, it seems likely that it may be involved in the regulation of the pituitary hormone secretion and synthesis and in other functions as a neurotransmitter, neuromodulator or neurotrophic factor in the brain of stargazer.

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REFERENCES

Anglade I, Zandbergen T, Kah O (1993) Origin of the pituitary innervation in the goldfish. Cell Tissue Res 273: 345–355

Arimura A (1992) Pituitary adenylate cyclase activating polypeptide (PACAP): discovery and current status of research. Regul Pept 37: 287–303

Arimura A, Shioda S (1995) Pituitary adenylate cyclase activating polypeptide (PACAP) and its receptors: neuroendocrine and endocrine interaction. Frontiers in Neuroendocrinology 16: 53–88

Arimura A, Somogyvari-Vigh A, Weill C, Fiore RC, Tatsuno I, Bay V, Brenneman DE (1994) PACAP functions as a neurotrophic factor. Ann NY Acad Sci 739: 228–243

Batten TFC, Moons L, Cambre M, Vandesande F (1990) Anatomical distribution of galanin-like immunoreactivity in the brain and pituitary of teleost fishes. Neurosci Lett 111: 12–17

Chartrel N, Tonon MC, Vaudry H, Conlon JM (1991) Primary structure of frog pituitary adenylate cyclase activating polypeptide (PACAP) and effects of ovine PACAP on frog pituitary. Endocrinology 129: 3367–3371

Chartrel N, Wang Y, Fournier A, Vaudry H, Conlon JM (1995) Frog vasoactive intestinal polypeptide and galanin: primary structures and effects on pituitary adenylate cyclase. Endocrinology 136: 3079–3086

Gracia-Navarro F, Lamacz M, Tonon MC, Vaudry H (1992) Pituitary adenylate cyclase-activating polypeptide stimulates calcium mobilization in amphibian pituitary cells. Endocrinology 131: 1069–1074

Holmqvist BI, Ekström P (1991) Galanin-like immunoreactivity in the brain of teleosts: distribution and relation to substance P, vasotocin, and isotocin in the Atlantic salmon (*Salmo salar*). J Comp Neurol 306: 361–381

Kivipelto L, Absood A, Arimura A, Sundler F, Håkanson R, Panula P (1992) The distribution of pituitary adenylate cyclase-activating polypeptide-like immunoreactivity is distinct from helodermin- and helospectin-like immunoreactivities in the rat brain. J Chem Neuronal 5: 85–94

Köves K, Arimura A, Görcs TG, Somogyvari-Vigh A (1991) Comparative distribution of immunoreactive pituitary adenylate cyclase activating polypeptide (PACAP) and vasoactive intestinal polypeptide (VIP) in rat forebrain. Neuroendocrinology 54: 159–169

Köves K, Arimura A, Somogyvari-Vigh A, Vigh S, Millar J (1990) Immunohistochemical demonstration of a novel hypothalamic peptide, pituitary adenylate cyclase activating polypeptide, in the ovine hypothalamus. Endocrinology 127: 264–271

Matsuda K, Katoh J, Uchiyama M, Shioda S, Arimura A (1996) Distribution of PACAP-like immunoreactivity and isolation of this substance in the brain of teleost, *Gnathagnus elongatus*. Proc Japan Soc Comp Endocrinol 11 [Abstract]

Matsuda K, Takei Y, Katoh J, Shioda S, Arimura A, Uchiyama M (1997) Isolation and structural characterization of pituitary adenylate cyclase activating polypeptide (PACAP)-like peptide from the brain of a teleost, stargazer, *Uranoscopus japonicus*. Peptides (in press)

Miyata A, Arimura A, Dahl RR, Minamino N, Uehara A, Jiang L, Culler MD, Coy DH (1989) Isolation of a novel 38 residue-hypothalamic polypeptide which stimulates adenylate cyclase in pituitary cells. Biochem Biophys Res Commun 164: 567–574

Miyata A, Jiang L, Dahl RD, Kitada C, Kubo K, Fujino M, Minamino N, Arimura A (1990) Isolation of a neuropeptide corresponding to the N-terminal 27 residues of the pituitary adenylate cyclase activating polypeptide with 38 residues (PACAP 38). Biochem Biophys Res Commun 170: 643–648

Murase T, Kondo K, Otake K, Oiso Y (1993) Pituitary adenylate cyclase-activating polypeptide stimulates arginine vasopressin release in conscious rats. Neuroendocrinology 57: 1092–1096

Olivereau M, Olivereau J (1988) Localization of CRF-like immunoreactivity in the brain and pituitary of teleost fish. Peptides 9: 13–21

Olivereau M, Olivereau J, Vandesande F (1990) Localization of growth hormone-releasing factor-like immunoreactivity in the hypothalamo-hypophysial system of some teleost species. Cell Tissue Res 259: 73–80

Olsson C, Holmgren S (1994) Distribution of PACAP (pituitary adenylate cyclase-activating polypeptide)-like and helospectin-like peptides in the teleost gut. Cell Tissue Res 277: 539–547

Reid S, Fritsche R, Jönsson AC (1995) Immunohistochemical localization of bioactive peptides and amines associated with the chromaffin tissue of five species of fish. Cell Tissue Res 280: 499–512

Shioda S, Yada T, Nakajo S, Nakaya K, Nakai Y, Arimura A (1996) Cellular localization and functional significance of PACAP and its receptors in rat hypothalamus. Abstracts from the 11th International Symposium on Regulatory Peptides

Tuge H, Uchihashi K, Shimamura H (1968) An Atlas of the Brains of Fishes of Japan. Tsukiji Shokan Pub, Tokyo, in Japanese

Uchida D, Arimura A, Somogyvari-Vigh A, Shioda S, Banks WA (1996) Prevention of ischmia-induced death of hippocampal neurons by pituitary adenylate cyclase activating polypeptide. Brain Res 763: 280–286

Vigh S, Arimura A, Köves K, Somogyvari-Vigh A, Sitton J, Fermin CD (1991) Immunohistochemical localization of the neuropeptide, pituitary adenylate cyclase activating polypeptide (PACAP), in human and primate hypothalamus. Peptides 12: 313–318

Yon L, Feuilloley M, Chartrel N, Arimura A, Conlon JM, Fournier A,

Vaudry H (1992) Immunohistochemical distribution and biological activity of pituitary adenylate cyclase-activating polypeptide (PACAP) in the central nervous system of the frog *Rana ridibunda*. J Comp Neurol 324: 485–499

Yon L, Jeandel L, Chartrel N, Feuilloley M, Conlon JM, Arimura A, Fournier A, Vaudry H (1993) Neuroanatomical and physiological evidence for the involvement of pituitary adenylate cyclase-activating polypeptide in the regulation of the distal lobe of the frog pituitary. J Neuroendocrinol 5: 289–296

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