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Pituitary Adenylate Cyclase–Activating Polypeptide Does Not Colocalize with Vasoactive Intestinal Polypeptide in the Hypothalamic Magnocellular Nuclei and Posterior Pituitary of Cats and Rats

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Pituitary adenylate cyclase–activating polypeptide (PACAP) and vasoactive intestinal polypeptide (VIP) immunoreactive cells were demonstrated in the hypothalamic magnocellular nuclei in cats and rats. In cats these immunoreactive cells were stained without any treatment or intervention; however, in rats we had to use the pituitary stalk section to enhance the amount of PACAP and VIP for successful immunostaining. In both species the regions occupied by PACAP and VIP immunoreactive cells partially overlap each other in the paraventricular and supraoptic nuclei. Nevertheless, in either cats or rats PACAP and VIP immunoreactivities do not colocalize in the same cells studied by double labeling immunohistochemistry (IHC) or by the combination of immunohistochemistry and *in situ* hybridization. As was expected, PACAP and VIP immunoreactive materials were stored in different fibers of the posterior pituitary where the distribution of PACAP and VIP fibers also showed different patterns: PACAP fibers form a dense plexus at the periphery of the posterior lobe, in the vicinity of the intermediate lobe; however, the VIP fibers were evenly distributed mainly in the center of the posterior lobe. In spite of the high sequence homology of PACAP and VIP, the two peptides are synthesized in different subpopulations of hypothalamic neurons. This different distribution correlates well with the different role of the hypothalamic PACAP and VIP in the biologic clock and in the functions of the anterior and posterior pituitary.

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Introduction

Pituitary adenylate cyclase–activating polypeptide (PACAP) and vasoactive intestinal polypeptide (VIP) are two members of the secretin family. The N-terminal portion of PACAP shows 68% sequence homology with VIP *(1)*. Both peptides are widely distributed in the central nervous system (CNS) *(2–7)*.

In rat forebrain the highest concentration of PACAP was measured in the hypothalamus using radioimmunoassay (RIA) *(8)* and sandwich enzyme immunoassay *(9)*. With the use of RIA, VIP was demonstrated in the highest concentration in the anterior hypothalamus *(10)*. With the use of immunohistochemistry (IHC) in intact rats PACAP cells cannot be seen; however, in colchicine-treated rats PACAP immunoreactive cells mainly occupy the magnocellular regions including the supraoptic (SO), paraventricular (PV), anterior commissural, and perifornical nuclei *(4–7)*. In intact rats VIP immunoreactive cells are always present in the suprachiasmatic (SCh) nucleus *(2,11–14)*, although in special models, such as after colchicine treatment, hypophysectomy *(5,15)*, or pituitary stalk section *(16)*, VIP immunoreactive cells were also observed in SO and PV nuclei.

In intact rats PACAP immunoreactive fibers are always present in the internal zone (IZ) of the median eminence (ME), and in the posterior pituitary, VIP fibers cannot be seen *(5)*. Under special circumstances, such as after pituitary stalk section *(16)* or after the removal of the eyes (enucleation) *(17)*, both PACAP and VIP immunoreactive fibers were observed in both layers of the ME.

In intact cat hypothalamus, VIP immunoreactive cells are commonly present in SO and PV nuclei besides their

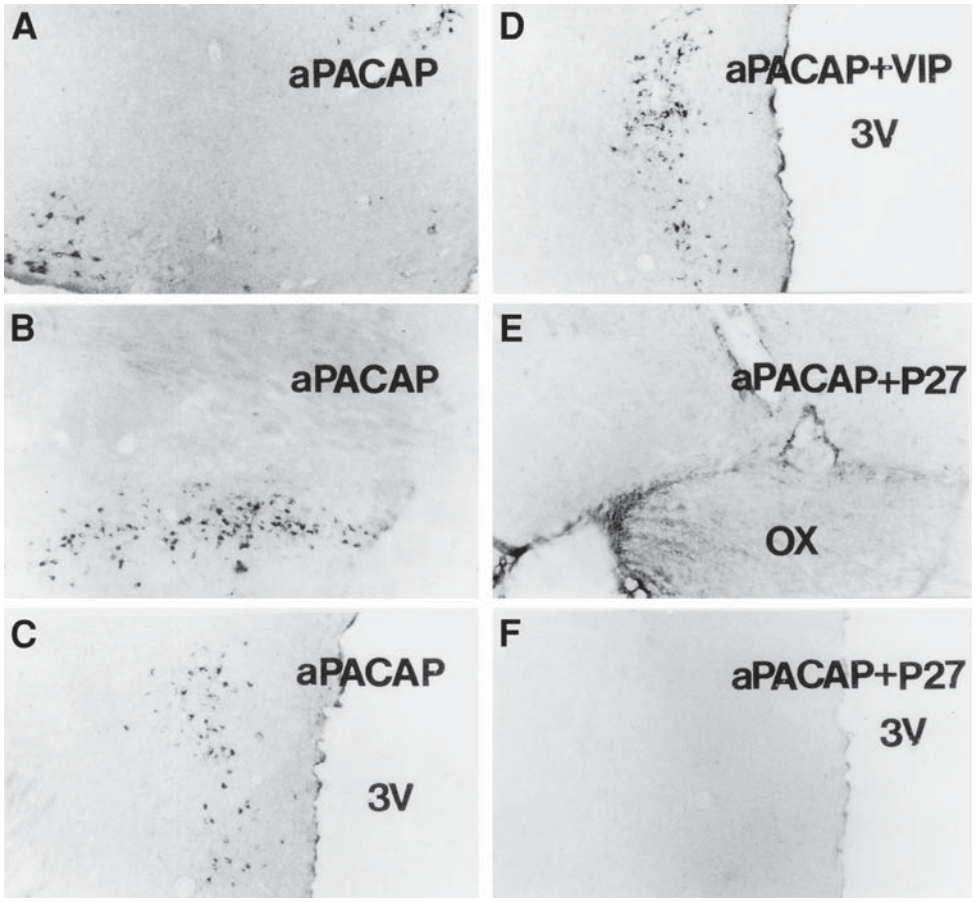

Fig. 1. Microphotographs demonstrating PACAP immunoreactivity in frontal sections of a cat hypothalamus. PACAP immunoreactive cells are present in both SO (**A,B**) and PV (**A,C,D**) nuclei. The preabsorption of PACAP antiserum with PACAP27 abolished the immunostaining in both SO (**E**) and PV (**F**) nuclei; however, the preabsorption of PACAP antiserum with VIP did not influence it. aPACAP, PACAP antiserum; OX, optic chiasm; PV, paraventricular nucleus; P27, PACAP27; SO, supraoptic nucleus; 3V, third ventricle. Scale = 200 μm.

characteristic localization in the SCh nucleus *(18)*. In spite of the fact that Obata-Tsuto et al. *(18)* did not see any immunoreactive fibers in the ME *(18)*, in our laboratory we observed VIP immunoreactive fibers in both layers of ME and in the posterior pituitary as well as cells in SO and PV nuclei.

It was demonstrated previously that the distribution of PACAP and VIP immunoreactive elements shows similarity and differences as well not only in the hypothalamus, but also in other regions of the CNS of rats *(5)*. Their localization in the same structures of the hypothalamus suggests the possibility of colocalization in the same cells because in the peripheral nervous system partial colocalization between the two immunoreactivities was observed: in the gastrointestinal tract of different species *(19,20)*, in the respiratory apparatus *(21)*, and in the wall of cerebral vessels *(22)*.

In this article, we first describe the precise distribution of PACAP and its comparison with VIP immunoreactivity in the magnocellular nuclei of intact cats and rats bearing pituitary stalk section. Pituitary stalk section was used to induce the appearance of both PACAP and VIP immunoreactive neurons in the rat magnocellular system. This intervention interrupted the axons, causing accumulation of immunoreactive PACAP and VIP in the cell bodies. The enhanced amount of PACAP and VIP was enough for successful immunostaining. We also present findings of our study of the possibility of colocalization of the two peptides in the hypothalamohypophyseal magnocellular system using double labeling IHC in intact cats and rats bearing pituitary stalk section as well as the combination of IHC and *in situ* hybridization (ISH) technique in pituitary stalk–sectioned rats.

Results

Distribution of PACAP Immunoreactivity in Cat and Rat Hypothalamus, ME, and Posterior Pituitary

The number of PACAP immunoreactive cell bodies was considerably high in the magnocellular nuclei of intact cats (Figs. 1 and 2) and rats bearing pituitary stalk section (Fig. 4). In both species, the immunoreactive cell bodies were observed in SO (Figs. 1A,B and 4A) and PV (Figs. 1C and

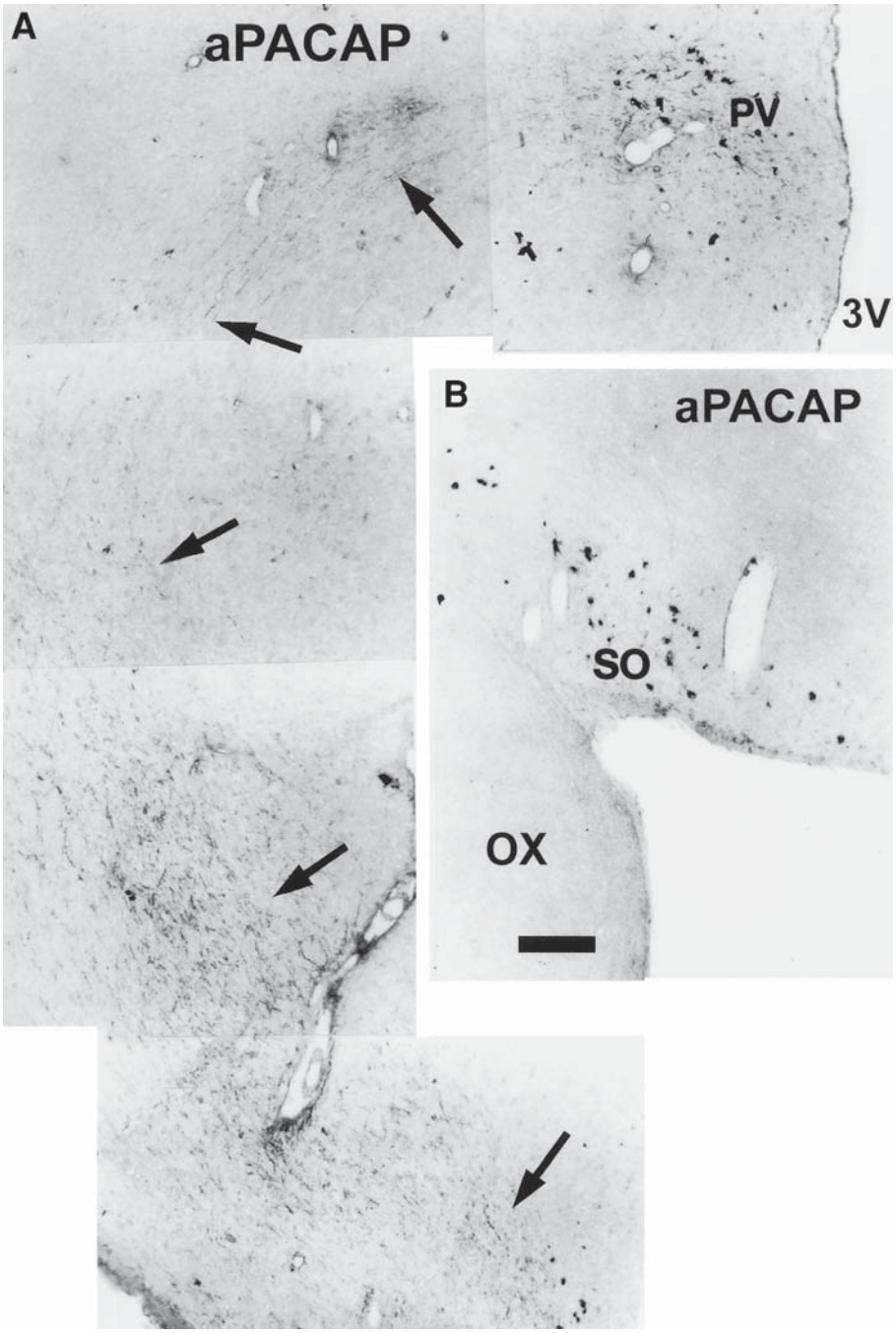

Fig. 2. Microphotographs demonstrating course of PACAP immunoreactive fibers leaving PV nucleus (**A**) and PACAP immunoreactivity in SO nucleus (**B**) in cat hypothalamus. The fibers leaving the PV nucleus descend toward the ME. Arrows outline the course of immunoreactive fibers. OX, optic chiasm; PV, paraventricular nucleus; SO, supraoptic nucleus. 3V, third ventricle. Scale = 100 μm.

4B) nuclei. Staining was specific because the preabsorption of PACAP antiserum with PACAP27 abolished the immunostaining (Fig. 1E,F); however, the preabsorption of PACAP antiserum with VIP did not influence the staining (Fig. 1D).

PACAP immunoreactive fibers leaving the PV nuclei took the same route as the oxytocin or vasopressin fibers. The fibers left the PV nucleus laterally; then turned downward; near the base of the hypothalamus turned medialward (Fig. 2A); and with fibers coming from the SO nucleus entered the ME, where in cats the immunoreactive fibers were seen in both layers (Fig. 3A); however, in intact rats they were only seen in the IZ (Fig. 4C). After pituitary stalk section in rats, PACAP fibers were also observed in both layers of ME similarly to those in cats (Fig. 4D). In the posterior pituitary, PACAP fibers exhibited special distribution.

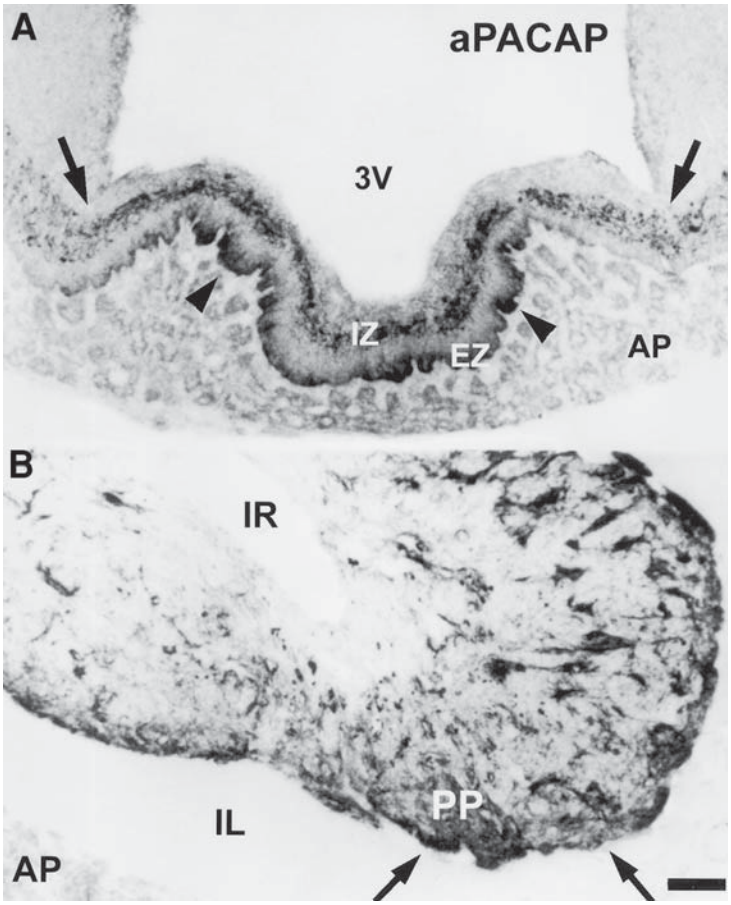

Fig. 3. PACAP immunoreactive fibers in ME and in posterior pituitary of cat. PACAP immunoreactive fibers are present in both layers of the ME (**A**). Arrowheads indicate fibers in the external zone (EZ). Arrows indicate fibers in the IZ. In the posterior pituitary (**B**) the PACAP immunoreactive fibers form a plexus at the periphery of the posterior pituitary (indicated by arrows) in the vicinity of the intermediate lobe. Scattered fibers were also present in the center of the posterior lobe. AP, anterior pituitary; IL, intermediate lobe; IR, infundibular recess; PP, posterior pituitary; 3V, third ventricle. Scale = 75 μm.

In both species the fibers formed a well-defined plexus at the periphery of the posterior lobe, in the vicinity of the intermediate lobe, and in the center of the posterior lobe they were scattered in the cats and evenly distributed in rats (Figs. 3B and 4E).

Distribution of VIP Immunoreactivity in Cat and Rat Hypothalamus, ME, and Posterior Pituitary

VIP immunoreactive cells were present in SCh nucleus of both species (Figs. 5A and 7A). In intact cats VIP immunoreactive cells were also observed in SO and PV nuclei (Fig. 5B,D). Their number was very limited in PV nucleus; however, in SO nucleus their number was several times higher. Staining was specific because the preabsorption of VIP antiserum with VIP abolished the immunostaining (Fig. 5C,E). In intact rats VIP immunoreactive cells were not observed in the magnocellular nuclei; however, VIP immunoreactive cells appeared after pituitary stalk section (Fig. 7B,C). Similarly to cats, the number of VIP immunoreactive cells was much lower in these nuclei than the number of PACAP immunoreactive cells.

VIP immunoreactive fibers were seen in both layers of ME (Fig. 6A), in the pituitary stalk (Fig. 6B), and in the posterior pituitary (Fig. 6C,D) of intact cats. In the latter structures, the fibers were evenly distributed. In intact rats VIP immunoreactive fibers could not be seen in the layers of ME, just in the wall of the vessels of the pia mater (Fig. 7D); however, after pituitary stalk section, a considerable number of fibers were seen in both layers of ME proximal to the intervention (Fig. 7F). In the posterior pituitary of intact rats, a few VIP fibers were seen along vessels (Fig. 7E).

Comparison of Distribution of PACAP and VIP Immunoreactive Elements in Cats and Rats

Figure 8 schematically illustrates the distribution of PACAP and VIP immunoreactive elements in frontal sections of the hypothalamus in intact cats (Fig. 8A) and rats bearing pituitary stalk sections (Fig. 8B) according to the stereo-

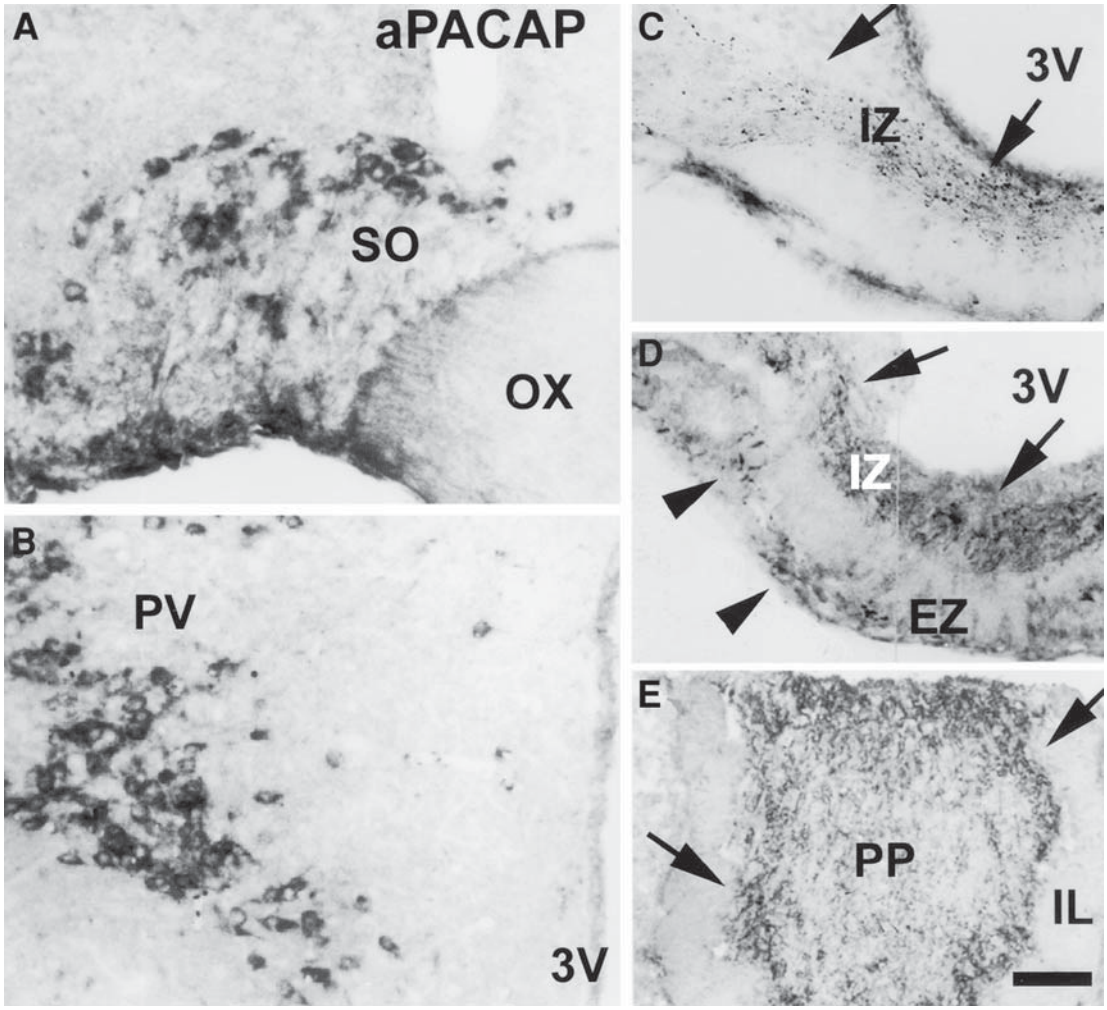

Fig. 4. Microphotographs demonstrating PACAP immunoreactivity in frontal sections of hypothalamus and ME of rat bearing pituitary stalk section (**A,B,D**) and PACAP fibers in ME and posterior pituitary of intact rat (**C,E**). Pituitary stalk section induced the appearance of PACAP immunoreactivity in the magnocellular SO (A) and PV nuclei (B) and in the EZ of the ME (D). In intact rats, the PACAP fibers were only seen in the IZ of ME (C) and in the posterior pituitary (E). At the periphery of the posterior lobe, the fibers were more densely packed than in the center of the lobe. Arrows indicate fibers in IZ and posterior pituitary, and arrowheads indicate fibers in EZ. EZ, external zone; IL, intermediate lobe; IZ, internal zone; OX, optic chiasm; SO, supraoptic nucleus; PV, paraventricular nucleus; PP, posterior pituitary; 3V, third ventricle. Scale = 50 μm.

taxic texts by Jasper and Ajmone-Marsan *(23)* and Paxinos and Watson *(24)*, respectively. The number of PACAP immunoreactive cells in the magnocellular nuclei was high; however, the number of VIP immunoreactive cells was low. When we compared the number of VIP immunoreactive cells in SO and PV nuclei in serial sections, it became evident that altogether more VIP cells were present in the SO than in the PV nucleus. These areas, which are occupied by VIP and PACAP immunoreactive cells, partially overlapped each other in both species.

PACAP and VIP Immunoreactivities Are Present in Distinct Population of Hypothalamic Magnocellular Nuclei

Double labeling IHC using the ABC technique for demonstrating VIP and immunofluorescence technique for demonstrating PACAP was not able to reveal double-labeled nerve elements in either cats or rats bearing pituitary stalk section. Figure 9 shows a portion of both SO (Fig. 9A,B) and PV nuclei (Fig. 9C,D) of intact cats. Figure 10A,B shows a portion of SO of a rat bearing pituitary stalk section. We have never found PACAP immunoreactive cells showing VIP immunoreactivity and vice versa. In the posterior pituitary of intact cats, as was expected, there was no colocalization between PACAP and VIP immunoreactivities in the fibers. PACAP fibers, as already described, formed a dense plexus at the periphery of the posterior lobe in the vicinity of the intermediate lobe; however, the VIP fibers were evenly distributed in the center of the posterior lobe (Fig. 9E,F).

With the combination of ISH for demonstrating PACAP and IHC for demonstrating VIP, we were not able to find double-labeled nerve cells in the magnocellular nuclei of

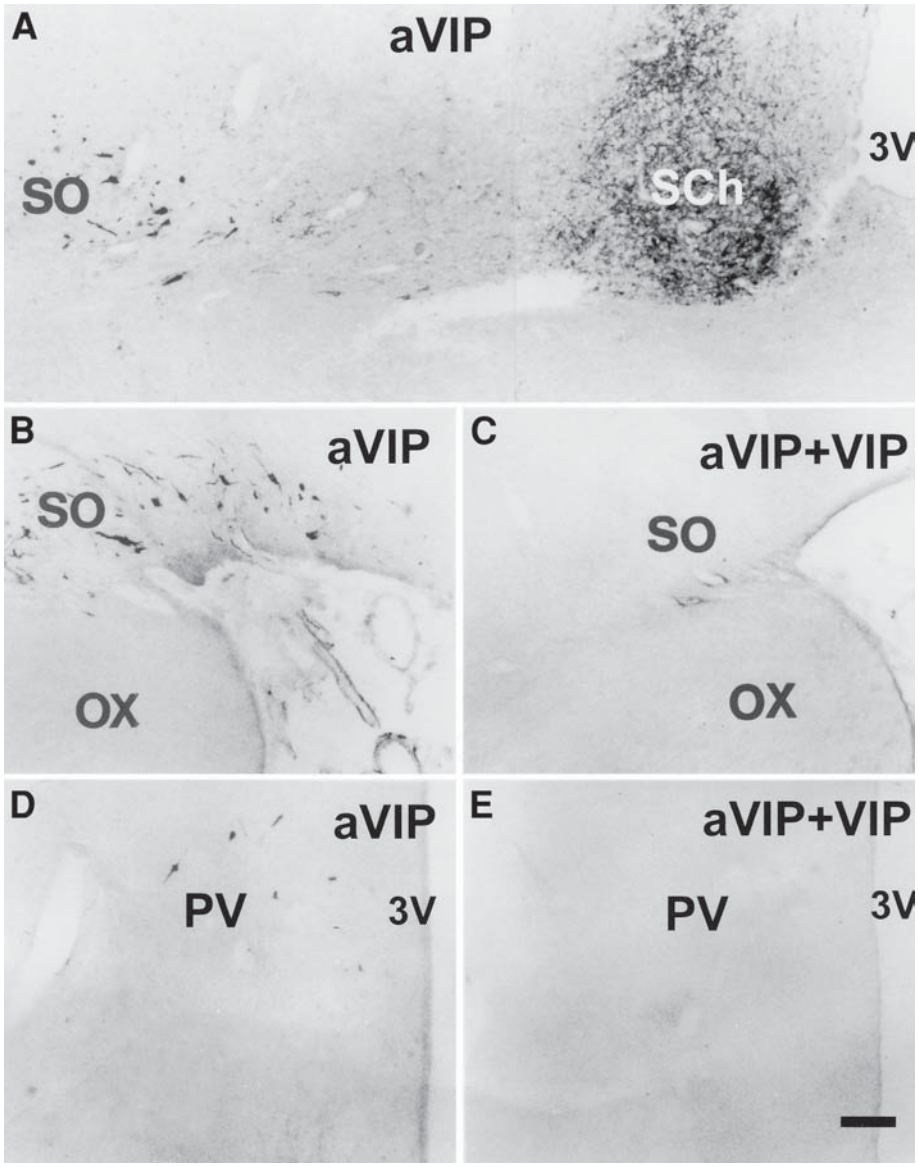

Fig. 5. Microphotographs demonstrating VIP immunoreactive cells in SCh (**A**), SO (**A,B**), and PV nuclei (**D**) in frontal sections of cat hypothalamus. Preabsorption of VIP antiserum with VIP abolished the immunostaining (**C,E**). OX, optic chiasm; PV, paraventricular nucleus; SCh, suprachiasmatic nucleus; SO, supraoptic nucleus; 3V, third ventricle. Scale = 100 μm.

rats bearing pituitary stalk section. Figure 10C shows a portion of PV nucleus of a rat bearing pituitary stalk section.

Discussion

The presence of regulatory peptides in the magnocellular oxytocin- and vasopressin-producing neurons is well established. The major part of these neurons sends the neurons' axons to the posterior pituitary. However, a subpopulation of the paraventricular magnocellular neurons is hypophysiotropic; their axons or axon collaterals terminate in the EZ of the ME *(25)*.

There is much evidence that the regulatory peptides colocalize with oxytocin and vasopressin immunoreactivities in the same cells *(26–28)*; however, there is no colocalization between the oxytocin and vasopressin immunoreactivities *(29)*.

It was previously demonstrated that VIP and PACAP, which are both members of the secretin superfamily, partially colocalize with vasopressin immunoreactivities *(30)*. In addition, PACAP partially colocalizes with oxytocin as well *(16)*. It seems that there are separate subpopulations of vasopressin neurons in the magnocellular nuclei that synthesize PACAP or VIP but not both. Oxytocin-synthesizing neurons do not produce VIP *(16)*. It seems that those

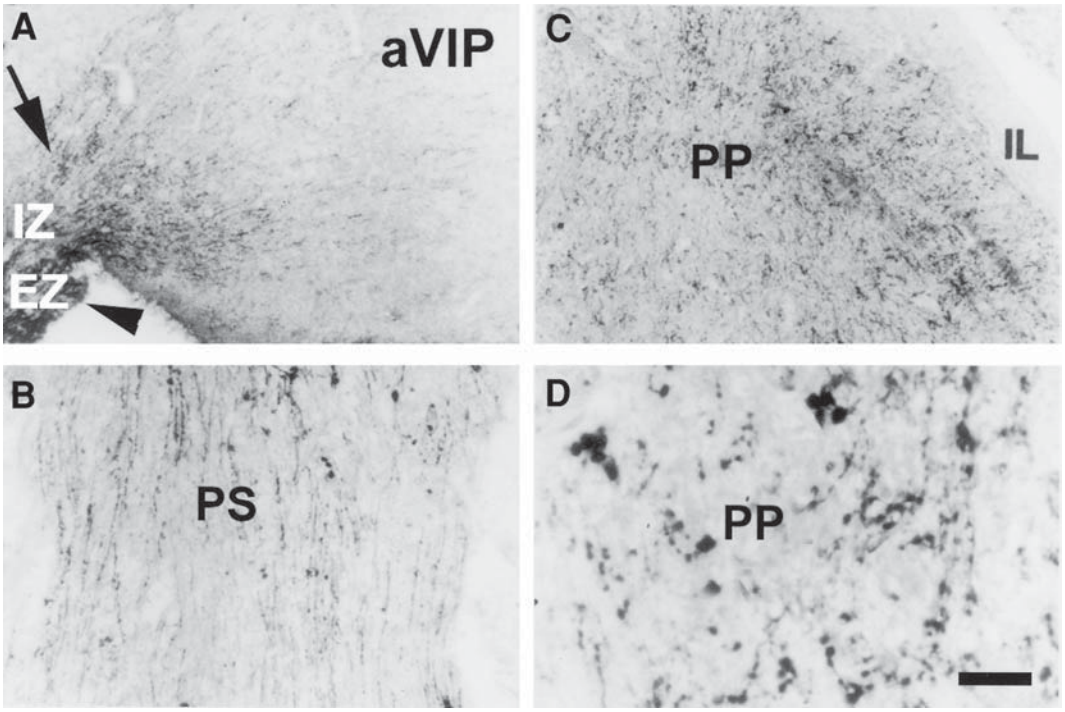

Fig. 6. Microphotographs demonstrating VIP fibers in ME, in pituitary stalk, and in posterior pituitary of cat. VIP fibers entered in both layers of the ME (**A**) and the fibers ran parallel in the pituitary stalk (**B**) to their destination in the posterior pituitary (**C**). (**D**) High-power detail of (C). The VIP fibers were evenly distributed in the posterior pituitary. IL, intermediate lobe; IZ, internal zone; EZ, external zone; PP, posterior pituitary; PS, pituitary stalk. Scale = 100 μm in (A) and (C), 50 μm in (B), and 25 μm in (D).

PACAP cells that synthesize oxytocin do not synthesize vasopressin and vice versa.

In either cats or rats it seems that PACAP and VIP are present in the same cell groups, but not in the same cells, because we were not able to demonstrate colocalization of the two peptides. In cats the localization of VIP cells inside the PV nucleus is not characteristic; sometimes these cells were observed more medially or more laterally than PACAP ones. In rats the major part of VIP cells were seen more medially than PACAP ones. The lack of colocalization is also supported by the different distribution of PACAP and VIP fibers in the posterior pituitary. It is surprising that there is no colocalization between PACAP and VIP immunoreactivities in the hypothalamus, although there is much evidence that in the autonomic nervous system PACAP and VIP occur in the same neurons *(19–21)*. The lack of colocalization in the magnocellular neurons explains the different role of the two peptides in the posterior pituitary functions: VIP stimulates the release of both oxytocin and vasopressin *(31)*; however, PACAP is able to stimulate only the release of vasopressin *(32)*.

Both peptides are involved in the function of the photoneuroendocrine system. PACAP was demonstrated in the retinohypothalamic tract *(33–35)* and VIP in a reverse connection between the hypothalamus and the eye *(25,36)*. No colocalization, but interaction, was demonstrated between PACAP containing glutamatergic retinal fibers and VIP immunoreactive cells in SCh nucleus *(37)*. The role of these two peptides in the function of the biologic clock is well established *(33)*. The removal of the eyes—i.e., the lack of the light impulses—enhances both VIP *(36)* and PACAP *(17)* immunoreactivity in the magnocellular nuclei.

The major conclusion of our results is that inspite of the fact that PACAP and VIP show a high sequence homology in the hypothalamus, they are produced by different populations of the neurons. This different distribution correlates well with the different role of the hypothalamic PACAP and VIP in the biologic clock *(33,35)* and in the functions of the anterior and posterior pituitary. For example, PACAP administered intracerebroventricularly before the critical period of the proestrous stage inhibits ovulation, diminishing the preovulatoty luteinizing hormone (LH) surge *(38)*. This effect is mediated through the corticotropic hormone–releasing hormone and endogenous opioids *(39)*; however, VIP in the same model enhances the rate of the preovulatory LH surge *(40)*. Both PACAP and VIP are stimulatory for prolactin (PRL); however, in a special model during lactation, when the litters are separated from the mother for 4 h, during this refracter period PACAP (not VIP) is the only factor that stimulates the release of PRL, probably

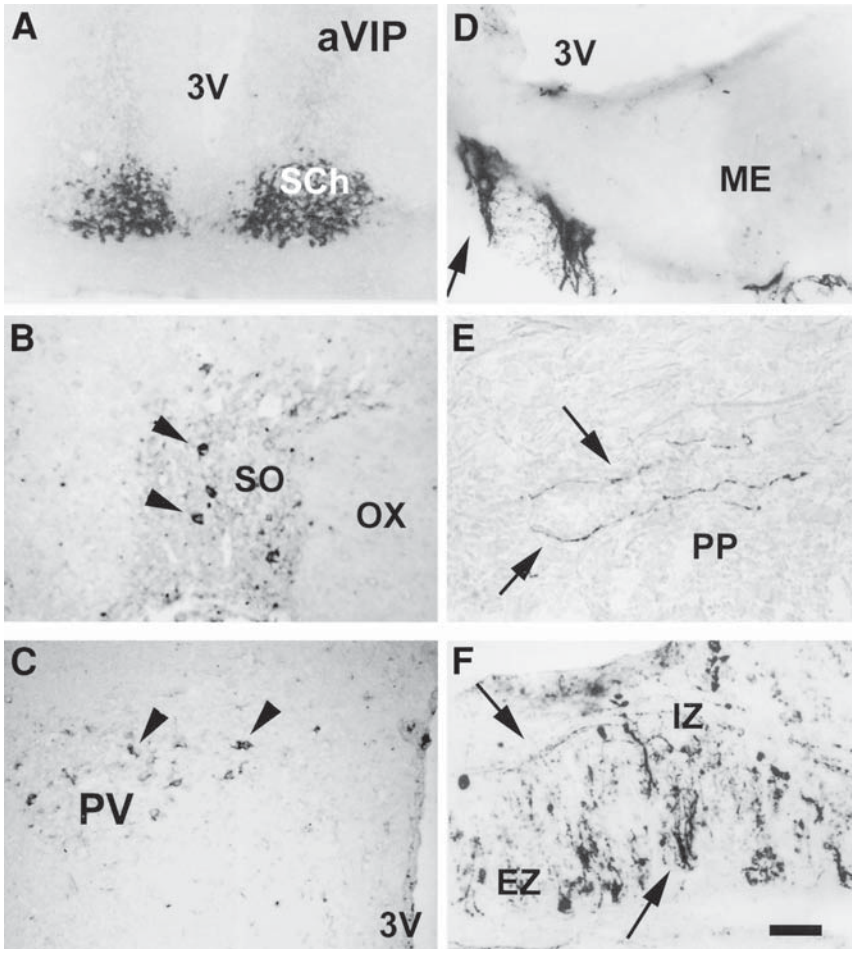

Fig. 7. Microphotographs demonstrating VIP immunoreactive elements in frontal sections of intact rat hypothalamus (**A**), ME (**D**), and posterior pituitary (**E**) and in that bearing pituitary stalk section (**B,C,F**). In an intact rat VIP immunoreactive cells were only present in the SCh nucleus (A). No immunoreactive fibers were present in the ME, but they heavily innervated the vessels in the pia mater (D). In the posterior pituitary of intact rat, a few fibers were observed accompanying vessels (E). In a rat bearing pituitary stalk section, a moderate number of VIP immunoreactive cells appeared in both SO (B) and PV nuclei (C). VIP immunoreactive fibers appeared in both layers of the ME (F), shown in a sagittal section proximal to the pituitary stalk section. Arrowheads in (B) and (C) indicate immunoreactive cells. Arrows in (D–F) indicate immunoreactive fibers. EZ, external zone; IZ, internal zone; OX, optic chiasm; SO, supraoptic nucleus; PP, posterior pituitary; PV, paraventricular nucleus; 3V, third ventricle. Scale = 100 μm in (A), 50 μm in (B) and (C), and 25 μm in (D–F).

mediated by the posterior pitutary through the short portal veins *(41)*. As mentioned, VIP stimulates the release of both oxytocin and vasopressin *(31)*; however, PACAP is able to stimulate only the release of vasopressin *(32)*.

To date, experiments with VIP and PACAP and their receptor knockout (KO) animals cannot give any explanation about the role of the magnocellular PACAP and VIP. However, it has been published that PACAP KO mice display a disturbance in lipid and carbohydrate metabolism and in the sympathetic response to insulin. PACAP null mice are temperature sensitive. Their mortality is high before puberty and it increases at low temperature *(42)*. PAC1 receptor KO female animals show a reduced fertility. The number of their litters is lower than that of intact animals *(43)*. VPAC2 receptor KO animals exhibited retarded growth and reduced insulin-like growth factor levels *(44)*.

Materials and Methods

Animals

Short-hair domestic intact male cats (6–8 mo old) and Sprague-Dawley male rats (2 to 3 mo old) were kept in a light- and temperature-controlled vivarium (lights on at 5:00 AM and off at 7:00 PM; temperature: 24 ± 2°C). The rats were divided into two groups: The first group consisted of rats bearing pituitary stalk section to enhance immunostaining ($n = 6$). The animals were anesthetized and placed in a stereotaxic instrument. The pituitary stalk was interrupted

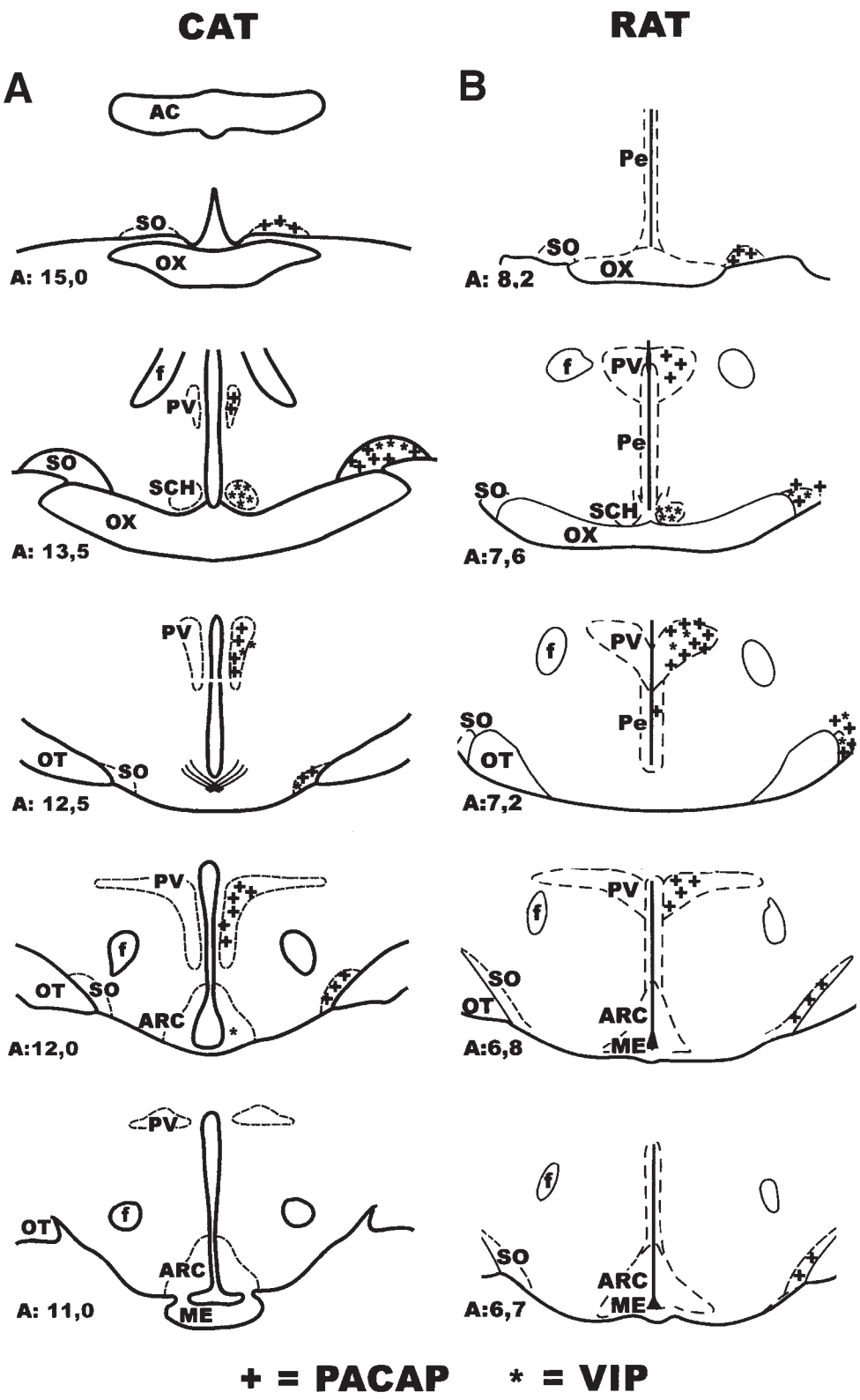

Fig. 8. Schematic illustration of comparative distribution of PACAP and VIP immunoreactive cells in both cat (**A**) and rat (**B**) hypothalami (according to the texts of Jasper and Ajmonr-Marsan *[23]* and Paxinos and Watson *[24]*, respectively). Ear bar was used as a reference line. The distance of each frontal section from the ear bar is indicated by numbers at the left side of the bottom of each section. AC, anterior commissure; ARC, arcuate nucleus; f, fornix; ME, median eminence; OT, optic chiasm; Pe, periventricular nucleus; PV, paraventricular nucleus; SCH, supraoptic nucleus; SO, supraoptic nucleus. Symbols indicate PACAP and VIP immunoreactive cells in the magnocellular nuclei. The number of the symbols does not show the correct number of the cells; just their proportion is indicated.

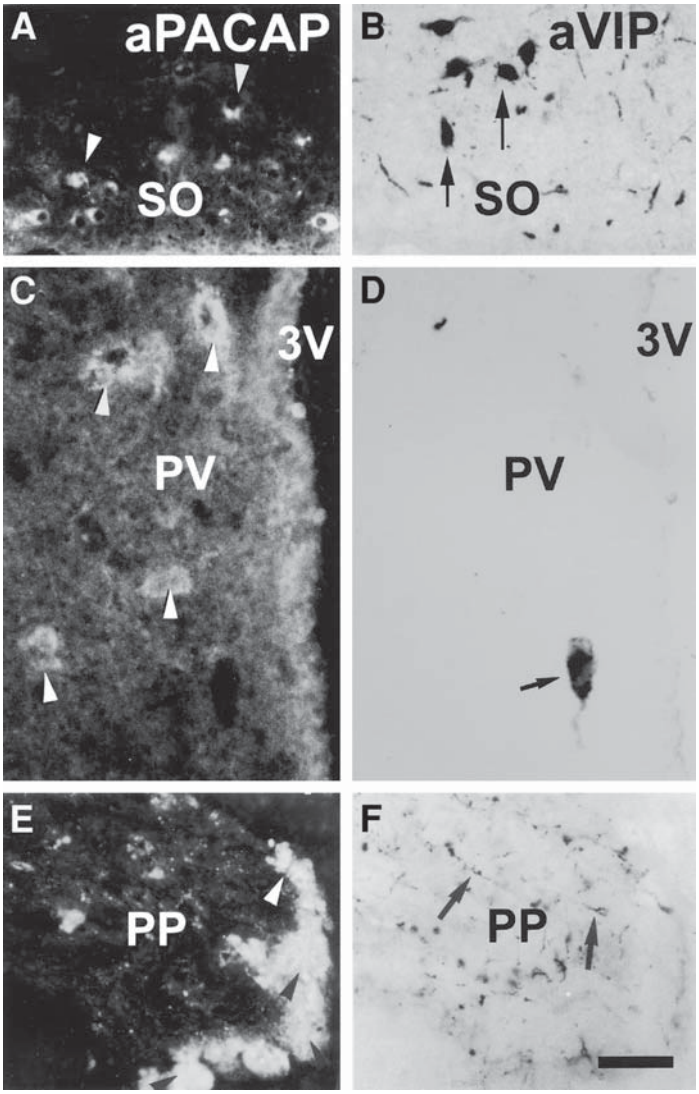

Fig. 9. Microphotographs demonstrating examples of double-labeled frontal sections of hypothalamus and posterior pituitary of cat (combination of ABC-DAB and fluorescence staining for demonstrating VIP and fluorescence staining for demonstrating PACAP). No double-labeled cells for PACAP and VIP were observed in SO (**A,B**) and PV (**C,D**) nuclei and fibers in the posterior pituitary (**E,F**). Arrowheads indicate PACAP immunoreactive cells and fibers, and arrows indicate VIP immunoreactive cells and fibers. aPACAP, PACAP antiserum; aVIP, VIP antiserum; PP, posterior pituitary; PV, paraventricular nucleus; SO, supraoptic nucleus; 3V, third ventricle. Scale = 100 μm in (A) and (B), 50 μm in (E) and (F), and 25 μm in (C) and (D).

using a piece of a razor blade. Through a hole in the calvaria, the razor blade was lowered 4.5 mm in front of the ear bar to the base of the skull according to Paxinos and Watson *(24)*. Figure 11 schematically illustrates the localization of the intervention. The second group consisted of intact rats ($n = 3$). Age-matched controls were included in this group. Half of the pituitary stalk–sectioned animals and the controls were processed for IHC, and the other half of the pituitary stalk–sectioned animals for ISH.

Preparation of Tissues for IHC

Under general hexobarbital anesthesia, 11 cats, 3 intact rats, and 6 rats bearing the pituitary stalk section 4 d after the intervention were perfused by 4% paraformaldehyde in phosphate buffer (PB) (0.1 *M*, pH 7.4) through the ascending aorta. The brains and the pituitaries were removed, and postfixed overnight at room temperature. After fixation, the tissue samples were cut on Cryotome (Shandon). The brains were sectioned in the frontal plane, and the pituitaries were sectioned in the horizontal plane at 20-μm thickness in two parallel series.

Immunohistochemistry

Endogenous peroxidase activity was blocked by application of PB containing 0.05% H_2O_2 and 1% normal goat serum (NGS). Then the sections were placed in PB containing 1% Triton X-100 to enhance penetration of the antibodies. The sections were preincubated in PB containing 10%

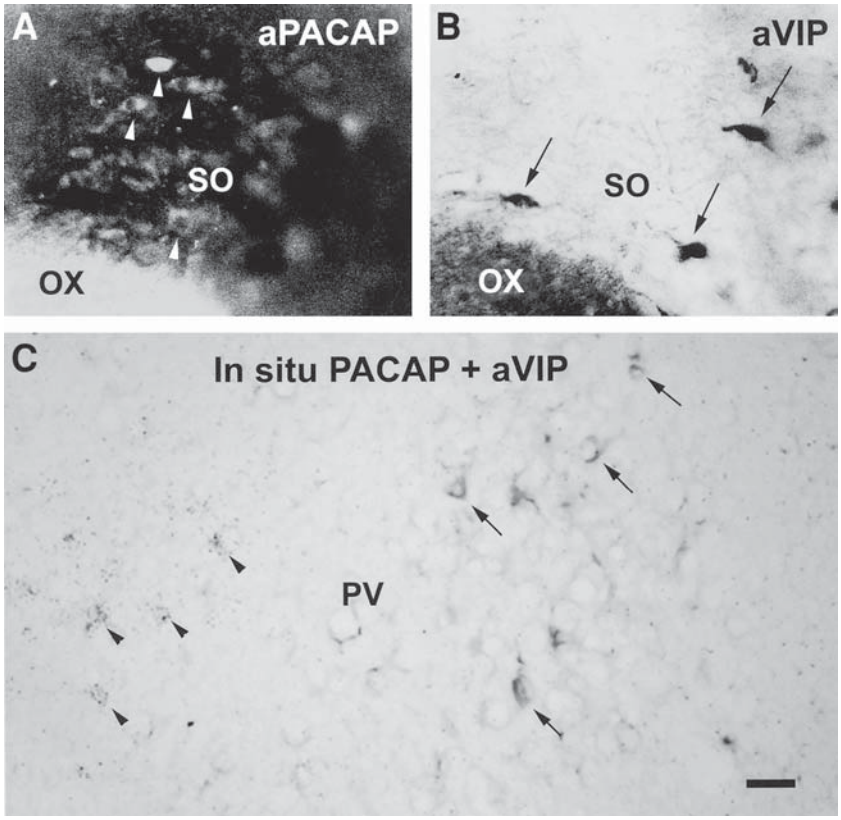

Fig. 10. Microphotographs demonstrating examples of double-labeled frontal sections of hypothalamus of rat. (**A,B**) The combination of ABC-DAB technique (for demonstrating VIP) and fluorescence staining (for demonstrating PACAP) was used; (**C**) the combination of ISH (for demonstrating PACAP) and ABC-DAB technique (for demonstrating VIP) was used. No double-labeled cells for PACAP and VIP were observed in SO (**A,B**) and PV (**C**) nuclei. Arrowheads indicate PACAP cells and arrows indicate VIP cells. aPACAP, PACAP antiserum; aVIP, VIP antiserum; OX, optic chiasm; PV, paraventricular nucleus; SO, supraoptic nucleus. Scale = 50 μm.

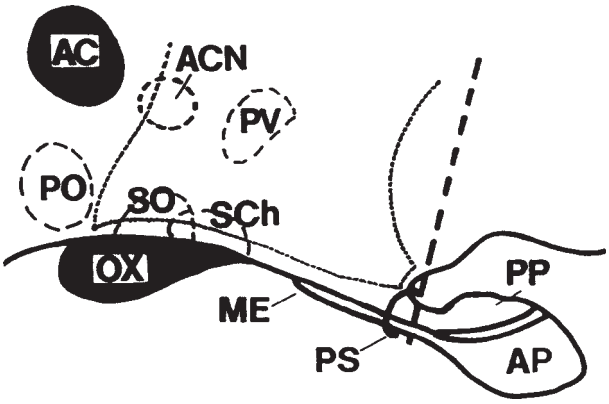

Fig. 11. Schematic illustration of pituitary stalk section in sagittal section of rat hypothalamus. The broken line indicates where the cut interrupted the pituitary stalk. AC, anterior commissure; ANC, anterior commissural nucleus; AP, anterior pituitary; ME, median eminence; OX, optic chiasm; PO, preoptic area; PP, posterior pituitary; PS, pituitary stalk; PV, paraventricular nucleus; SCH, suprachiasmatic nucleus; SO, supraoptic nucleus.

NGS. Then a series of sections was incubated in a primary antiserum raised in rabbit against VIP by Görcs and characterized by Gulyás et al. *(45)* (dilution of antiserum was 1:10,000). Another series was incubated in a primary antiserum raised in rabbit against PACAP27 by Arimura and characterized by Köves et al. *(4,5)* (dilution of antiserum was 1:6000). After incubation the tissue sections were processed according to the directions of a Vectastain ABC Kit (Vector, Burlingame, CA). The final complex was visualized using H_2O_2 and 3,3'-diaminobenzidine tetrahydrochloride (DAB) in the presence of nickel-ammonium sulfate (Reanal, Budapest, Hungary). Selected sections containing VIP immunoreactive elements in those places where PACAP immunoreactive elements were also expected were further processed for PACAP immunostaining using indirect fluorescence technique. The sections were first incubated with PACAP antiserum in a 1:500 dilution and then with fluorescein isothiocyanate in a 1:200 dilution. The double-stained sections were photographed first using a light microscope, then the same view fields with a fluorescence microscope.

Preparation of Tissues for ISH

Under general hexobarbital anesthesia, the animals, were perfused with 4% paraformaldehyde (PFA) in phosphate buffer (0.1 *M*, pH 7.4). The brains were removed and blocks containing the hypothalamus were cut off. For cryoprotec-

tion the blocks were placed in 20% sucrose solution (diluted in PB) for 24 h and then sectioned at 10 µm on a cryostat and mounted on silanized slides. The slides were dried on a 37°C plate and stored at −70°C until use.

In Situ *Hybridization*

Before hybridization the sections were treated with Triton X-100 to enhance the penetration of antibodies for subsequent VIP immunostaining. The sections were washed and then treated with 0.25% acetic anhydride in 0.1 mol of triethanolamide-HCl (pH 8.0) for 10 min. The sections were then rinsed in 0.3 mol of NaCl–0.03 mol of sodium citrate solution (2X saline sodium citrate [SSC]); dehydrated and delipidated in a subsequent series of 70, 80, 96, 100, and 96% ethanol; and finally air-dried. Hybridization was performed overnight at 55°C with 10^6 cpm/slide of radioactively labeled PACAP riboprobe in a humid chamber. The following day the sections were washed four times in 4X SSC buffer (pH 7.0) for 5 min each at room temperature; then they were treated in RNase A (20 µg/mL; Sigma, Budapest, Hungary) in buffer (pH 8.0) containing 500 m*M* NaCl, 10 m*M* Tris-HCl, and 0.25 m*M* EDTA for 30 min at 37°C; then for 5 min in each of the following buffer solutions: 2X SSC, 1X SSC, and 0.5X SSC at room temperature and finally two times in 0.1X SSC at 65°C for 30 min each. The slides were allowed to cool, and the sections were washed in 1X phosphate-buffered saline (pH 7.4) and processed for VIP immunostaining (see above). When the immunostaining was completed, the slides were dipped in NTB3 nuclear track emulsion (Eastman Kodak, Rochester NY). After 10 d of exposition time at 4°C in the dark, the reaction was developed using Kodak Dektol developer and fixer at 18°C. The sections were air-dried again and cover-slipped using Cytoseal 60 mounting medium (Richard-Allan Scientific, Kalamazoo, MI).

Preparation of PACAP Riboprobe

Isolated plasmids provided by Hashimoto et al. *(46)* (Laboratory of Molecular Neuropharmacology, Osaka University, Japan) were used to prepare riboprobes for PACAP ISH. The insert was amplified by a standard polymerase chain reaction (PCR) using T3 and T7 primers, catalyzed by *Taq* polymerase (Roche, Budapest, Hungary). The PCR product was used for in vitro transcription of the radioactively (using S35UTP; Amersham, Budapest, Hungary) labeled riboprobe. Transcription of the labeled riboprobes was performed according to a Maxi Script Kit (Ambion, Austin, TX) using T7RNA polymerase in sense and T3 polymerase in antisense direction.

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