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Expression Of Pituitary Adenylate Cyclase Activating Peptide In The Uterine Cervix, Lumbosacral Dorsal Root Ganglia And Spinal Cord Of Rats During Pregnancy

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Abstract

The uterine cervix is highly innervated by the sensory nerves containing neuropeptides which change during pregnancy and are regulated, in part, by estrogen. These neuropep- tides act as transmitters both in the spinal cord and cervix. The present study was under- taken to determine the expression pattern of the neuropeptide pituitary adenylate cyclase activating peptide (PACAP) in the cervix and its nerves during pregnancy and the influence of estrogen on this expression using immunohistochemistry, radioimmunoassay and RT- PCR. PACAP immunoreactivity was detected in nerves in the cervix, lumbosacral (L6-S1) dorsal root ganglia (DRG) and spinal cord. PACAP immunoreactivity was highest at day 15 of pregnancy in the cervix and dorsal spinal cord, but then decreased over the last trimester of pregnancy. However, levels of PACAP mRNA increased in the L6-S1 DRG at late pregnancy relative to early pregnancy. DRG of ovariectomized rats treated with estrogen showed increased PACAP mRNA synthesis in a dose-related manner, an effect partially blocked by the estrogen receptor (ER) antagonist ICI 182 780. We postulate that synthesis of PACAP in L6-S1 DRG and utilization in the cervix and spinal cord increase over pregnancy and this synthesis is the under influence of the estrogen-ER system. Since PACAP is expressed by sensory nerves and may have roles in nociception and vascular function, collectively, these data are consistent with the hypothesis that sensory nerve-derived neuronal factors innervate the cervix and play a role in cervical ripening.

1. Introduction

The uterine cervix is highly innervated with sensory and autonomic nerves [4,5,32,37–39,46]. When the cervical sensory nerves are bilaterally transected (bilateral pelvic neurectomy, BPLN) dystocia results [3,15]. Although the exact mechanism(s) of this effect are unclear, we have shown that systemic injection of substance P (SP), a neuropeptide synthesized by uterine cervix-related sensory nerves, induces inflammatory-

like responses and vascular changes resembling those that occur during cervical ripening [5]. Moreover, key sensory neuropeptides SP and calcitonin gene-related peptide (CGRP) co-exist in uterine sensory neurons [43] and their receptors are expressed in the cervix [5,41]. We have shown that the expression patterns of SP and CGRP have temporal relationships with cervical ripening [27,28]. In addition, BLPN alters the expression of factors involved in vascular and inflammatory responses, such as vascular endothelial growth factor (VEGF),

tissue inhibiting metalloproteinase 1 (TIMP-1) and LIX; and VEGF, with its associated basic signaling factors, has a temporal relationship with SP, CGRP and cervical ripening during pregnancy [29,30]. Collectively, these data indicate that sensory nerves and their neuropeptides play a role in remodeling the cervix in late pregnancy, in part, by influencing factors that regulate the vasculature and inflammatory responses.

The present study builds on our previous observations of sensory SP- and CGRP-containing nerves in the cervix and was designed to determine if the neuropeptide pituitary adenylate cyclase-activating polypeptide (PACAP) could also play a role in cervical function, such as cervical ripening. PACAP has been identifi in nerves in the female genital tract [7,8,9], autonomic neurons of the paracervical ganglia [8] and sensory neurons of the dorsal root ganglia [6,11,25] and dorsal horn of the spinal cord [6,13,18,25,40]. PACAP, first identified by Miyata and co-workers [23,24], was isolated from an extract of ovine hypothalamus and was named based on its ability to stimulate formation of cAMP in rat pituitary cells [23,24]. PACAP is a member of the VIP-glucagon-GRF-secretin superfamily of structurally related peptides and is costored with VIP in parasympathetic nerves in reproductive organs [8], but is also costored with SP/CGRP in a subpopulation of sensory fibers [25] and is considered a sensory neuropeptide. PACAP, and its three cloned receptors (PAC_1, $VPAC_1$ and $VPAC_2$), have widespread distributions in the CNS, PNS and peripheral organs, consistent with its pleiotropic biological effects that include vasodilation, bronchodilation, and regulation of neurotransmitter release ([13] and review by [47]).

The objective of the present study was to determine if PACAP is present in uterine cervix-related dorsal root ganglia (DRG) sensory neurons, changed over the course of pregnancy, and responded to estrogen treatment. Here, we show that PACAP synthesis increased in L6-S1 DRG, but levels of PACAP decreased in the spinal cord and cervix in late pregnancy. PACAP mRNA levels in the L6-S1 DRG increased with estrogen treatment and the estrogen-induced increase was blocked by ER blocker, ICI 182 780.

2. Materials and methods

2.1. Animals

Non-pregnant and timed-pregnant (gestational days 10, 15, 20, parturient (day 22) and 2-day postpartum) Sprague-Dawley rats (SASCO strain from Charles Rivers) were used. Pregnant animals included $n = 6$ for radioimmunoassay, $n = 5$ for immunohistochemistry, and $n = 3$ for RT-PCR at five representative time points and $n = 3$ for bilateral pelvic neurectomy. Nonpregnant animals included $n = 9$ for treatment with 17b-estradiol, $n = 3$ for treatment with ER blocker ICI 182780, and $n = 3$ for vehicle controls. All experiments were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals (NIH Publications No. 86–23) revised 1985 and efforts were made to minimize both animal suffering and numbers of animals used.

2.2. Immunohistochemistry

Immunohistochemical studies of the expression of PACAP in the cervix, dorsal root ganglia (DRG), and spinal cord used intact rats. For additional immunohistochemical studies of PACAP in DRG neurons, pregnant rats were subjected to bilateral pelvic neurectomy (BLPN). For BLPN, rats on day 8 of pregnancy were anesthetized with sodium pentobarbital (45 mg/kg, i.p.), a laparatomy performed, the pelvic nerve identified, and a 5 mm segment removed from the nerves. The muscle layers were sutured, skin closed with wound clips, and rats allowed to recover until day 22 (time that parturition should occur). For tissue harvesting, the rats were deeply anesthetized (100 mg/kg sodium pentobarbital, i.p.) and exsanguinated by cardiac perfusion with saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.3. The cervix, L6-S1 spinal cord segments, and DRG were removed, cryoprotected in 30% sucrose, frozen, and sectioned (14 mm thick) on a cryostat. Sections were processed on slide for immunohistochemistry to localize PACAP by application of antibodies for immunostaining according to protocols routinely used in our lab [34,35]. Briefly, tissue sections were incubated for 16–24 h at room temperature with primary antibodies: rabbit generated antibody against PACAP (dilution 1:1000; Peninsula Laboratories Inc., San Carlos, CA) or mouse monoclonal antibody (JHH1, [14]). Sections were washed in phosphate buffered saline (PBS), incubated in appropriate secondary antibodies: goat anti-rabbit IgG Alexa Fluor 594 (1:600 dilution) (Molecular Probes), goat anti-rabbit Alexa 488, or goat anti-mouse Alexa 488 (1: 100 dilution) (Molecular Probes) for 1 h and mounted in Vectashield (Vector Labs). Some immunostained sections were developed with the Vectastain ABC Kit Elite Standard using DAB and nickel sulfate to produce a blue-purple color signal in transmitted light.

Some sections of the L6-S1 spinal cord were double immunostained using a cocktail of antibodies generated in different species for antigens located in different cells and cellular compartments; i.e., for PACAP (using mouse mono-clonal anti-PACAP JHH1) and estrogen receptor-a (ER-a) (using a rabbit generated antibody against ER-a; 1:10,000 dilution; Upstate Biotechnology, Charlottesville, VA).

The mouse PACAP antibody is well characterized [14]. The ER-a antiserum coded C1355 was raised against the last 14 amino acids of the ER and its characterization is established [10,35]. Controls included omission of the primary antiserum or omission of the secondary antibody.

Tissue sections were viewed with an Olympus Provis Microscope equipped for epifluorescence and brightfield microscopy. Images were captured with a SPOT™ Digital Camera (Diagnostics Instruments, Sterling Heights, Michigan), imported into PhotoShop™ V 6.0 (Adobe Systems Inc., San Jose, CA), contrast and brightness adjusted if necessary and then labeled.

2.3. Radioimmunoassay

Pregnant rats were euthanized with sodium pentobarbital (100 mg/kg body weight, i.p.), and perfused intracardially with 0.9% sodium chloride. The cervix, L6-S1 DRG and spinal cord

segments (dorsal one-half only) were carefully removed and stored frozen at -80 8C until processing.

Before PACAP analysis the frozen tissue specimens were weighed and extracted in boiling water/acetic acid [14]. The extracted samples were reconstituted in assay buffer and analyzed by a radioimmunoassay specific for PACAP 38. The PACAP antiserum (code No. 733C) was raised in rabbit and does not cross-react with PACAP 27 or structurally related peptides [14]. The detection limit of the assay was 5 pmol/l and the working range 5–50 pmol/l. The within and between assay coefficients of variation were <10%. All tissue extracts were assayed in duplicate in at least two different dilutions.

2.4. Estrogen treatment

Nonpregnant rats were ovariectomized, after anesthesia with sodium pentobarbital (45 mg/kg, i.p.), via two small dorsal incisions. Incisions were closed with a single stitch to the muscle layer and a single wound clip to the skin. Two weeks following surgery, some of the rats were treated with 17b-estradiol or 17b-estradiol + an estrogen receptor (ER) blocker in order to determine whether estrogen regulates PACAP synthesis via the classical ER pathway. Rats were treated either with increasing doses of 17b-estradiol (Sigma Chemicals Co., St. Louis, MO, USA) (0.03, 0.3, 3.0, 30.0 mg per rat daily for 4 days, s.c., sacrificed 12 h after last injection) or ER antagonist ICI 182 780 (AstraZeneca, Cheshire, UK) (s.c., 4 mg/kg body weight plus 0.3 mg 17b-estradiol daily for 3 days—initial injection of ER blocker was given 12 h prior to 0.3 mg 17b-estradiol) dissolved in 100 ml of sesame oil. Control animals were ovariectomized and treated with vehicle (100 ml).

2.5. Total RNA isolation

Pregnant (early pregnant day 10 and late pregnant day 20 were compared) and nonpregnant ovariectomized estrogen or estrogen blocker treated rats were euthanized with sodium pentobarbital (100 mg/kg body weight; i.p.) and perfused only with 0.9% sodium chloride. The L6-S1 DRG were removed and stored at -80 8C until processing. Total RNA was isolated from L6-S1 DRG of individual animals using RNeasy Mini Kit (Qiagen, Valencia, CA). The amount and purity of total RNA for each sample were estimated by spectrophotometric analysis at A260 and A280. The quality of RNA was determined by agarose gel electrophoresis following ethidium bromide staining. Aliquots of total RNA were diluted in diethylpyrocarbonated (DEPC)-treated water and stored at -80 8C.

2.6. Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA from DRG was reverse transcribed and amplified in an Eppendorf Master Cycler, using reagents from Gene AMP Gold RNA PCR Kit (P.E. Biosystems, Foster City, CA), according to manufacturer's instructions. Briefly, 0.5–1 mg total RNA was reverse transcribed and amplified in a 50 ml reaction mixture containing the following: 5X RT-PCR buffer, 1.75 mM $MgCl_2$, 1.2 mM dNTP's, 10 U RNase inhibitor, 5.0 mM DTT, 1 mM random hexamer, 200 nM PACAP (forward), 200 nM PACAP (reverse), 5.0 U AmpliTaq Gold DNA polymerase, 30 U MultiScribe reverse

transcriptase and DEPC-treated water. The RT-PCR protocol used for PACAP (modification of [20]) was as follows: pre-heated for 10 min at 94 8C followed by 1 min at 94 8C, 1 min at 60 8C, 2 min at 72 8C for 29 cycles and finally 10 min at 72 8C. The sequences used for PACAP were as follows: (i) PACAP: forward: 5^0-CAC-CAA-TGT-GGG-CTC-TGA-AG-3^0, and (ii) reverse: 5^0-CCG-CTT-GAG-GTT-TAG-CAG-AG-3^0 (370-bp fragment).

The RT-PCR reactions were normalized across runs using 18S ribosomal RNA as a standard. A Quantum RNA kit with 18S primers and competimers for quantitative RT-PCR was used (Ambion, Austin, Texas). The ribosomal primers for this kit yielded a 488-bp PCR fragment. The PCR product was separated using agarose gel electrophoresis, visualized after staining with SYBR Green 1 and scanned using the Kodak 1D Image Station (Rochester, NY).

2.7. Statistical analysis

For RIA, comparisons among groups were made by ANOVA and data are shown as S.E.M. (Fig. 4). RT-PCR data are shown as means and S.E.M. (Fig. 5); comparisons among groups were made by t-test (Fig. 5A and C) and ANOVA (Fig. 5B). P values < 0.05 were considered significant.

3. Results

3.1. Immunohistochemistry

3.1.1. Cervix
PACAP-immunoreactive nerve fibers were evident in the fibromuscular stroma of the cervix (Fig. 1A), in nerve trunks at the periphery of the cervix (Fig. 1B), and coursing in the endocervix (Fig. 1C). Within the endocervix, PACAP-immunoreactive fibers were closely adjacent to the walls of the vasculature, especially venules (Fig. 1D). In addition, PACAP-positive fibers coursed near the epithelium lining the lumen of the cervix (Fig. 1E).

3.1.2. DRG
PACAP-immunoreactive neurons were evident as small clusters among nonimmunoreactive neurons in the L6-S1 DRG (Fig. 2A) as well as scattered throughout the DRG sections as solitary neurons (Fig. 2B). Such neurons were in the small (<20 mm diameter) to medium-sized (21–40 mm diameter) categories. In addition, PACAP-immunoreactive nerve fibers were evident throughout the DRG (Fig. 2B and C).

3.1.3. Spinal cord
Sections of the L6-S1 spinal cord immunostained for PACAP showed positive fibers in a dense band in the superficial areas of the dorsal horn (DH) (Fig. 3A), along the medial border of the DH and coursing to the dorsal intermediate gray (DIG) of the central spinal cord (Fig. 3B), and along the lateral border of the DH in the lateral collateral pathway (Fig. 3A) coursing to the sacral parasympathetic nucleus (SPN) (Fig. 3A and C). Some PACAP-immunoreactive fibers were closely adjacent to ER-a-immunoreactive neurons in the LCP and SPN (Fig. 3D and E).

In general, changes in immunostaining or density of neurons or fibers in the cervix, DRG or spinal cord over the
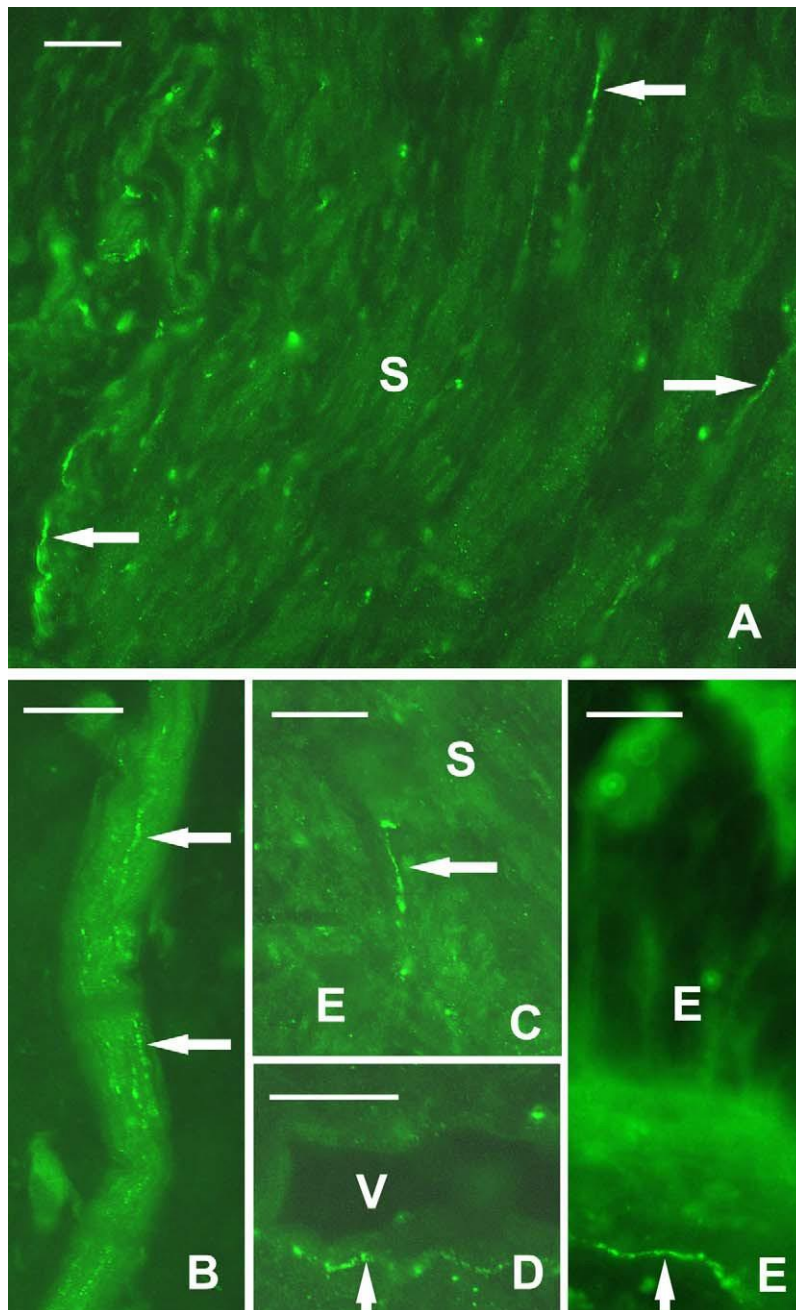
Fig. 1 – Sections of the uterine cervix immunostained for PACAP-immunoreactive nerves. (A) Nerve fibers (arrows) are evident coursing through the fibromuscular stroma (S) of the cervix at day 10 of pregnancy. (B) A nerve trunk at the edge of the cervix from a parturient rat showing a subset of nerve fibers immunoreactive for PACAP (arrows). (C) A PACAP-immunoreactive nerve fiber (arrow) courses from the fibromuscular stroma (S) to the endocervix (E) of a day 10 pregnant rat. (D) PACAP-immunoreactive nerve (arrow) in a day 10 pregnant rat closely adjacent to a venule (V) in the endocervix. (E) Immunoreactive nerve fiber (arrow) subjacent to the epithelium (E) lining the lumen of the cervix of a parturient rat. Scale bar = 25 μm.

course of pregnancy were not readily evident with immunohistochemistry.

3.2.   Radioimmunoassay (RIA)

Levels of immunoreactive PACAP change in the cervix and spinal cord during pregnancy (Fig. 4); levels of PACAP immunoreactivity in individual DRG were not readily detectable in our assay procedure. Levels of PACAP in the cervix and L6-S1 spinal cord were highest over the early to middle stages of pregnancy (days 10 and 15) and then levels began to decrease during the third trimester of pregnancy, reach a nadir at parturition (day 22), and remain low at 2 days postpartum (Fig. 4A and B). There were notable and significant differences
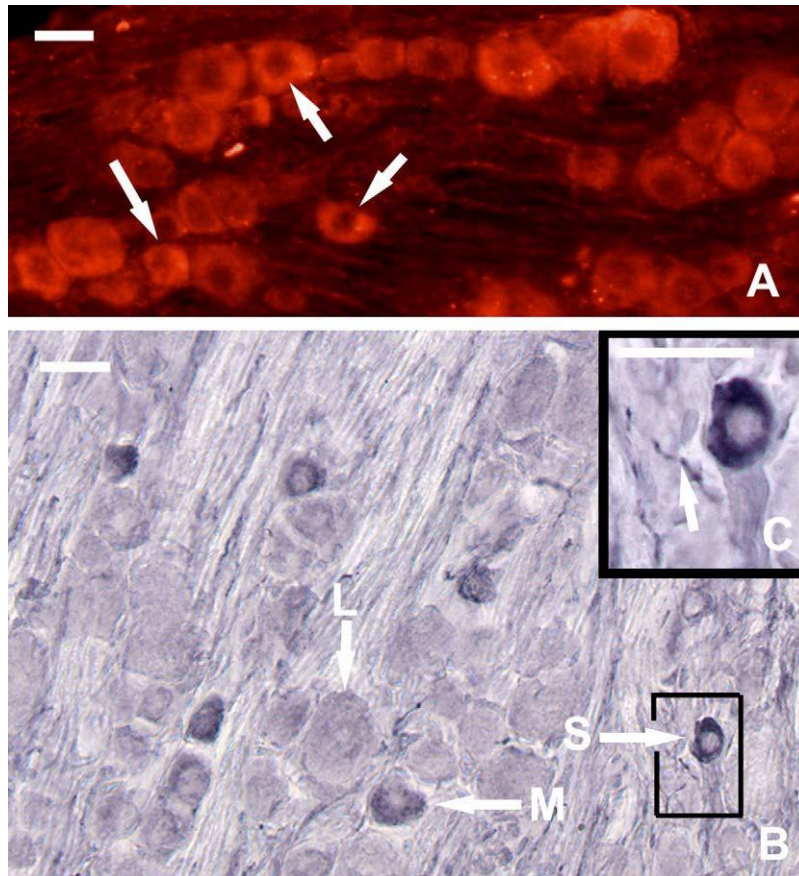
Fig. 2 – Cryostat sections of the L6-S1 dorsal root ganglia (DRG) immunostained for PACAP. (A) DRG from a day 15 pregnant rat. In some instances small clusters of PACAP-positive neurons (arrows) were evident in the DRG. These neurons were small to medium in size. (B) Low magnification view of the DRG (from a bilaterally pelvic neurectomized rat) at day 22 of pregnancy showing PACAP-immunoreactive neurons scattered throughout the section. Both small (S) and medium (M) sized neurons show staining. Large neurons (L) are unstained for PACAP. (C) Higher magnification view of the area of the DRG shown in the box of (B) illustrating PACAP-immunoreactive nerve fibers (arrow) within the DRG. Scale bar = 25 mm.

in tissue concentrations of PACAP between the second and third trimesters of pregnancy (days 10–15 versus days 20–22) (Fig. 4A and B) ($p < 0.05$).

3.3. RT-PCR

PACAP mRNA in L6-S1 DRG is up-regulated in late pregnancy, increased by exogenous 17b-estradiol treatment, and this increase is blocked by an ER antagonist. PACAP mRNA bands showed an apparent increase in density in late pregnancy (day 20) compared to early pregnancy (day 10) (Fig. 5A). When the bands were scanned using the Kodak 1D Image Station to produce a histogram of the net intensity of the bands the changes were more apparent (Fig. 5A). The difference in net intensity of bands in late (day 20) versus early pregnancy (day 10) was statistically different ($p < 0.05$).

Increasing doses of 17b-estradiol (0.03, 0.3, 3 and 30 mg per rat per day for 4 days) in ovariectomized rats produced dose-related increases of PACAP mRNA in L6-S1 DRG between the ranges of 0.03–3.0 mg (no further response was observed beyond 3.0 mg) (Fig. 5B). Differences between vehicle-treated and 0.03 mg were statistically significant ($p < 0.001$).

Exogenous estrogen up-regulates PACAP mRNA expression, and ERs are expressed in neurons of DRG [34,45]. Thus, we examined whether estrogen effects on PACAP mRNA levels were ER-mediated. Ovariectomized rats treated with the ER antagonist ICI 182 780, 12 h prior to 17b-estradiol, exhibited reduced effects of estrogen (at 0.3 mg) on PACAP mRNA expression (Fig. 5C). The partial blockage of PACAP mRNA expression by ICI 182 780 was statistically significant compared to the 17b-estradiol-treated animals ($p < 0.0014$). This suggests that the effects of estrogen on PACAP mRNA expression are mediated, in part, via ER signaling pathway.

4. Discussion

The important findings of this study are that (1) in the female rat, neurons in the L6-S1 DRG express PACAP and apparently transport this sensory neuropeptide to nerve terminals in the uterine cervix and in the corresponding levels of the spinal cord, (2) PACAP mRNA increases in the DRG during pregnancy, whereas levels of immunoreactive PACAP in the cervix and spinal cord decline toward the end of pregnancy, and (3)
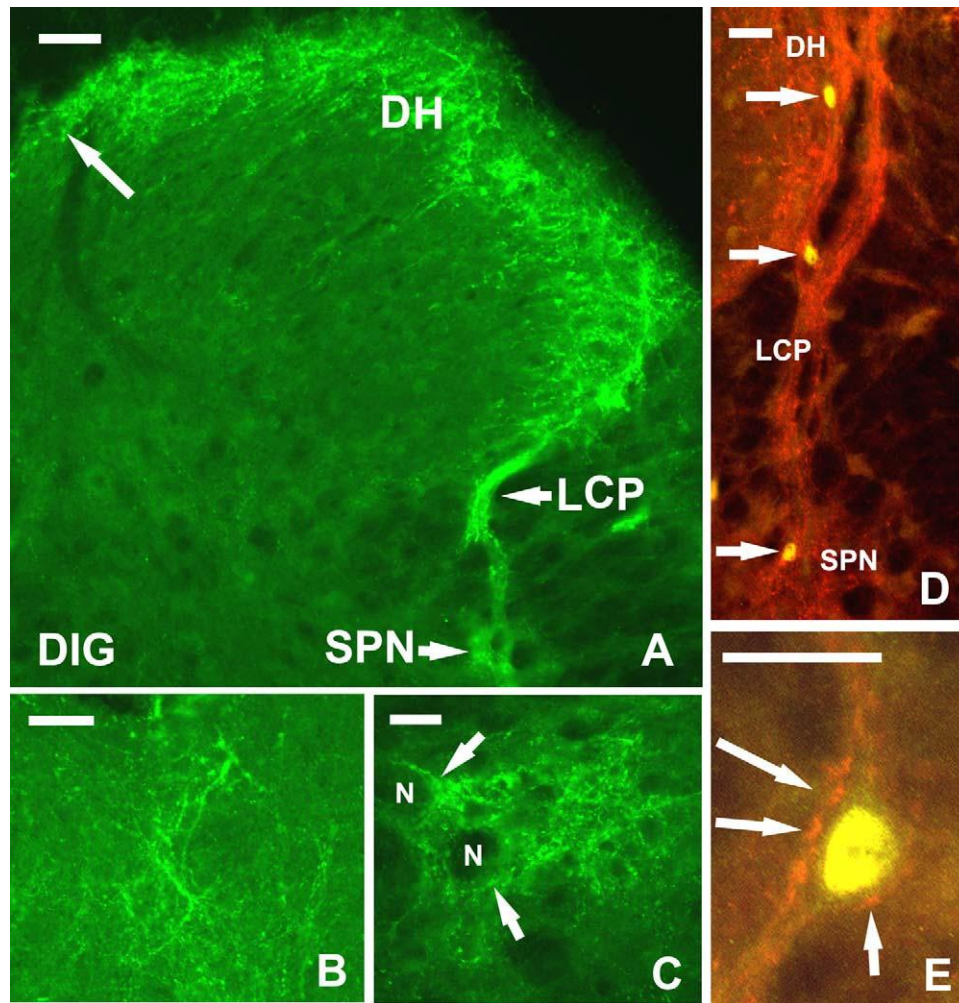
Fig. 3 – Cryostat sections of the L6/S1 spinal cord from a parturient rat (A–C) and a day 10 pregnant rat (D and E). (A) Shows a plexus of PACAP-immunoreactive nerve fibers in the superficial dorsal horn (DH) of the spinal cord. Also evident are bundles of fibers coursing along the lateral collateral pathway (LCP) toward the area of the sacral parasympathetic nucleus (SPN). Fibers from the medial aspect of the DH (arrow) course to the dorsal intermediate gray (DIG) (dorsal to the central canal). (B) Plexus of PACAP-immunoreactive fibers in the DIG of the spinal cord. (C) Higher magnification view of PACAP-immunoreactive fibers (arrows) surrounding neurons (N) in the SPN. (D) Spinal cord section immunostained for PACAP (red) and ER-*a* (in a nuclear location and appears yellow when photographed with the filter for Alexa Fluor 594 used for PACAP). ER-*a*-immunoreactive neurons (arrows) are evident among the PACAP-positive fibers of the lateral DH, LCP and SPN. (E) High magnification view of PACAP immunoreactive varicosities (arrows) closely adjacent to an ER-*a*-positive neuron (yellow nucleus) in the SPN. Scale bar = 25 *m*m.

synthesis of PACAP is influenced by the estrogen-ER system. These data, taken together with the current literature, imply that PACAP-expressing sensory neurons represent a subtype of neuron innervating the cervix that could be influenced by estrogen and ultimately play a role in cervical ripening in preparation for birthing.

It is important to recognize that PACAP in the genital organs may, in addition to sensory neurons, also be derived from parasympathetic neurons in the pelvic paracervical ganglion [8]. That most PACAP-immunoreactive fibers in the genital organs, as well as other tissues, were sensitive to capsaicin treatment and costore ''sensory neuropeptides'' substance P (SP) and calcitonin gene-related peptide (CGRP) [8,25] suggests that they are small unmyelinated sensory

fibers. A small number of capsaicin-resistant PACAP-immunoreactive fibers are likely derived from autonomic neurons and costore vasoactive intestinal peptide (VIP) [8].

The presence and distribution of PACAP immunoreactivity in the L6-S1 spinal cord of female rats shown in the present study is consistent with that shown by other groups in various levels of the spinal cord [6,13,18,40]. PACAP-immunoreactive fibers were prevalent in laminae I and II of the dorsal horn which are the sites for termination of many central sensory axons of nociceptive neurons in the DRG. In addition, PACAP-positive fibers were noted to course (1) medially along the dorsal horn to an area dorsal to (dorsal intermediate gray, DIG) and around the central canal and (2) laterally along the gray matter of the dorsal horn as the lateral collateral pathway

(A) Cervix

PACAP RIA in the Cervix

(B) Spinal cord.
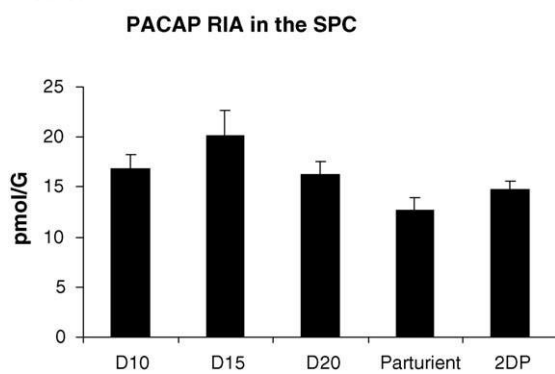
PACAP RIA in the SPC
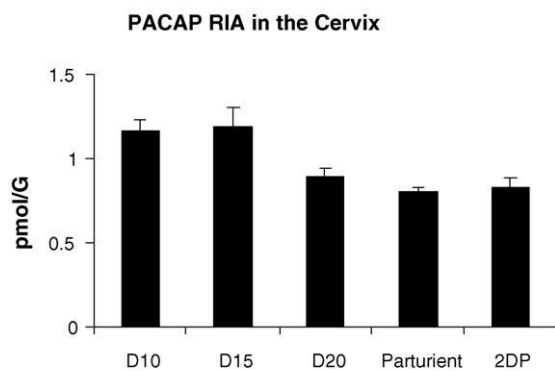
Fig. 4 – Concentration of immunoreactive PACAP in extracts of the cervix (A) and dorsal half of the L6-S1 spinal cord (SPC) (B). Levels of PACAP in the cervix and spinal cord peaked at about the end of the second trimester of pregnancy and then progressively declined until delivery.

(LCP) to the area of the sacral parasympathetic nucleus. The DIG and SPN are target areas of many visceral sensory fibers; indeed sensory SP- and CGRP-immunoreactive fibers terminate and are closely adjacent to estrogen receptor (ER) expressing neurons in the DIG and SPN [33,36]. As shown in the present study, PACAP-immunoreactive varicosities, presumably central terminals of primary sensory neurons from viscera, also are closely adjacent to ER-a expressing neurons. Given the DRG-spinal cord localization of PACAP, it is possible that PACAP functions in nociceptive processing and output of autonomic neurons, some of which is influenced by estrogen.

Small primary afferent neurons serve a dual role: as sensory neurons they transmit information about stimuli from peripheral sites to the spinal cord; as efferent effectors they can release neuropeptides at peripheral sites to mediate such responses as neurogenic inflammation (which consists of vasodilatation, plasma extravasation, and recruitment of immune cells). We previously reported the expression patterns of SP and CGRP, two neuropeptides derived from L6-S1 DRG, over pregnancy and demonstrated their potential functional significance in neurogenic inflammation and influencing cervical ripening as well as conveying information to the spinal cord. In the present study, we examined the

expression pattern of another sensory nerve-derived neuropeptide, PACAP. Fahrenkrug and Hannibal [8] and Fahrenkrug et al. [9] previously reported PACAP-immunoreactive nerves in the cervix; the present study detailed their distribution and corroborated the results of the earlier report. The pattern of PACAP-immunoreactive fibers in the cervix paralleled that of other sensory nerves containing such neuropeptides as SP and CGRP [32,46], though they were less dense. A subpopulation of PACAP-immunoreactive fibers is capsaicin-sensitive, costores SP and CGRP, and likely originates from small sensory neurons of DRG [8,25]. PACAP-positive fibers were frequently evident subjacent to venules, a location from which their neuropeptide could influence vascular activities as do other sensory neuropeptides such as SP and CGRP [5,16,17,22,41]. In addition, fibers were associated with the epithelium lining the lumen of the cervix, a location where sensory fibers could respond to activity that impinges on the cervical lining such as noxious or mechanical stimuli. PACAP-immunoreactive nerves were evident among the nonvascular smooth muscle of the cervix. PACAP released from such fibers could have an important function in the cervix during late pregnancy and parturition as PACAP has an inhibitory effect on nonvascular (and vascular) smooth muscle in the female reproductive system [9,44]. Thus, PACAP may be a factor to inhibit motility and promote relaxation of the sphincteric function of the cervix for completion of birthing. Our immunohistochemical studies did not reveal any obvious changes in density or distribution of PACAP-positive nerves in the cervix during pregnancy. However, RIA indicated that PACAP concentrations tended to be high until the end of the second trimester/beginning of the third trimester when PACAP immunoreactivity decreased and remained lower through pregnancy. This could indicate a release and utilization of PACAP in the cervix. Along this line, RT-PCR data showed an increase in PACAP mRNA in L6-S1 DRG at this same time. It is likely that the increased mRNA levels for PACAP reflect increased synthesis. It is, however, possible that other mechanisms are involved, e.g., increased mRNA stability. Thus, it appears that as pregnancy advances there could be an increase in synthesis in the DRG with an increased utilization at terminal sites. Consequently, PACAP, like SP and CGRP, influences the function of nonvascular and vascular smooth muscle [7,9,44], inflammatory actions [52], nociceptive signaling [49,50,54,55] and cervical ripening.

Our study localized PACAP immunoreactivity in small-medium size sensory neurons of the L6-S1 DRG of intact pregnant rats and the visualization of PACAP was enhanced by bilateral pelvic neurectomy (BLPN—which entailed transecting the primary afferent nerves to the urogenital organs). These observations are consistent with previous studies showing enhanced PACAP immunoreactivity and/or PACAP mRNA after colchicine treatment [25], or perturbations to sensory nerves such as axotomy of the sciatic nerve [51,53], spinal cord injury or chronic cystitis [48,56] thus illustrating plasticity in this system. PACAP, under normal physiological conditions, was localized in small sensory neurons [6,18,25], which are responsible for mediating nociception, but also in medium-sized sensory neurons after inflammation-inducing processes, such as injection of adjuvant in the rat paw [52], suggesting a role for PACAP in inflammation. Such an effect is thought to be mediated by the PACAP preferring receptor,
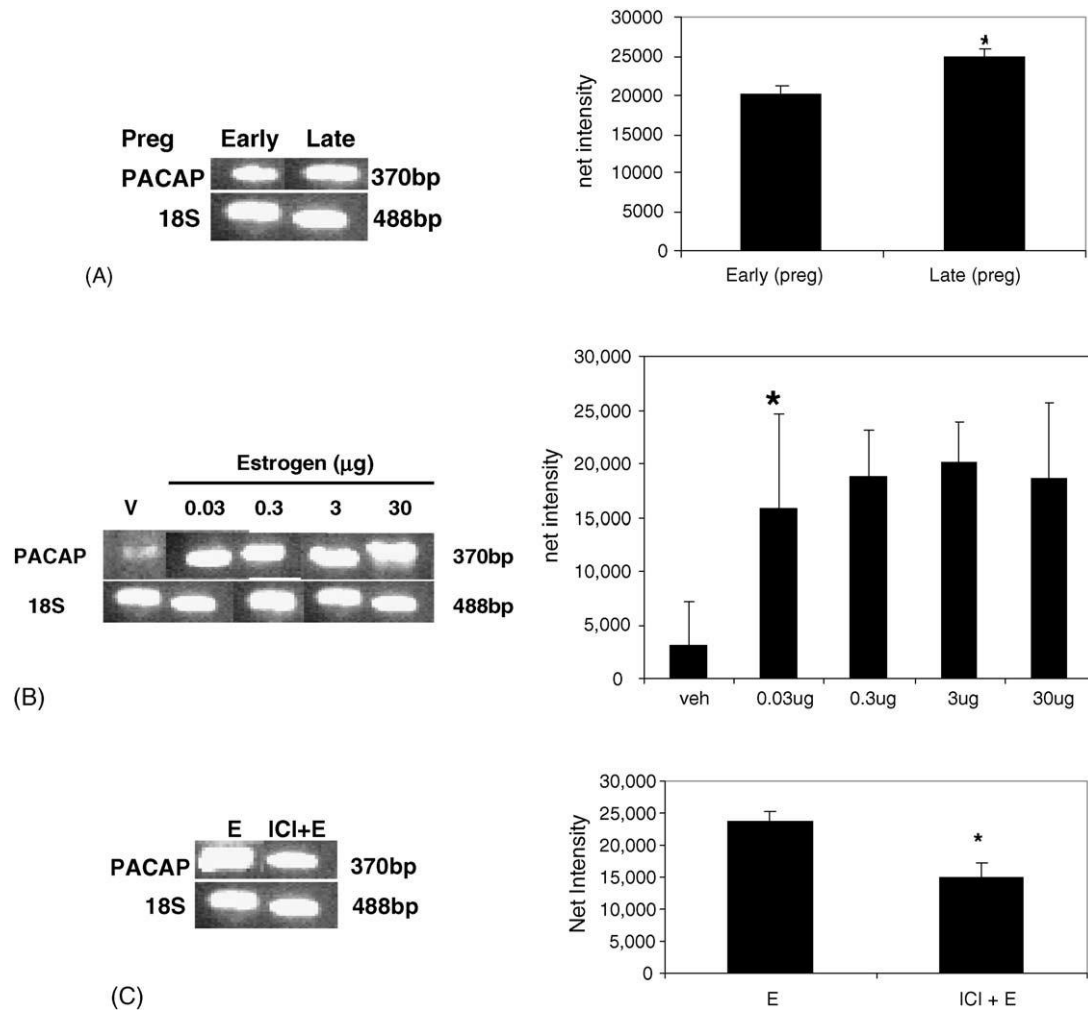
Fig. 5 – PACAP mRNA levels in L6-S1 DRG (A–C). Levels of PACAP mRNA increased in late (day 20) pregnancy compared to early pregnancy (day 10) (A). In ovariectomized rats estrogen increased PACAP mRNA synthesis in a dose-related manner (B), an effect partially blocked by the ER antagonist ICI 182 780 (C). Asterisk indicates a significant difference between: in (A) early and late pregnancy; in (B) treatment and vehicle control; and in (C) treatment with estrogen plus blocker and estrogen alone.

PAC$_1$, since in the formalin test; mice lacking PAC$_1$ had a profoundly decreased nociceptive response in the late, inflammatory phase [19]. Cervical ripening is a normal physiological process in which inflammatory-like changes occur, in part, through neuropeptides, such as SP and CGRP [4]. Since levels of PACAP increase during inflammatory conditions [52], it is plausible that PACAP mRNA (synthesis) should increase in DRG, and PACAP decrease in the spinal cord and cervix, during the inflammation-like conditions associated with cervical remodeling in the last trimester of pregnancy. This could help explain the rise in PACAP mRNA in L6-S1 DRG and decrease of PACAP in the cord and cervix at late pregnancy in the present study. Moreover, PACAP-immunoreactive nerves innervate mast cells and release histamine in humans and rodents [26,31].

Other factors, in addition to inflammation, likely to be involved in regulating synthesis of PACAP in uterine cervix-related sensory neurons during pregnancy are neurotrophins (such as NGF [19]) and sex steroids (such as estrogen). Because

sex steroid hormones have a significant modulating effect during pregnancy, including the synthesis of neuropeptides SP and CGRP [27,28], it is plausible that they influence PACAP synthesis observed in the present study. The synthesis of hypothalamic PACAP is regulated by sex steroid hormones, including estrogen and progesterone [1,12]. Moreover, the temporal correlation in the rat (at about day 20) in the rise of serum estrogen (and decline of progesterone) [2,21,42], up-regulation of ER-a mRNA [27], and PACAP mRNA (present study) in L6-S1 DRG argues in favor of the notion that estrogen, working through ERs, positively influences PACAP expression.

Although PACAP has been demonstrated in the neurons of the DRG, the present study is the first to show a relationship between pregnancy and PACAP synthesis and the dose-dependent effect of exogenous estrogen on PACAP mRNA. Because prior administration of an estrogen (receptor) blocker, ICI 182 780, to ovariectomized rats treated with estrogen attenuates the effects of estrogen, we suggest that the effects of estrogen on PACAP synthesis, as with SP and CGRP [27,28],

are mediated by ERs. The specific ER subtype (*a* or *b*) in the DRG neurons mediating up-regulation of PACAP was not determined in the present study. However, our previous studies using PCR have shown that only ER-*a* mRNA levels, but not ER-*b*, increase during pregnancy [27,28].

The exact role of PACAP in the remodeling cervix has not been elucidated. However, our working hypothesis is that, like other neuropeptides, PACAP is axonally transported (shown by [51]) to the cervix and impacts cervical ripening by influencing the local microvasculature. The present data, showing proximity of nerve terminals immunoreactive to PACAP to the microvasculature of the cervix, is consistent with this hypothesis.

Acknowledgements

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