

# Estrogen receptor $\alpha$ -induced cholecystokinin type A receptor expression in the female mouse pituitary

Hyun Joon Kim<sup>1,2</sup>, Mary C Gieske<sup>1,3</sup>, Susan Hudgins<sup>1</sup>, Beob Gyun Kim<sup>4</sup>, Andree Krust<sup>5</sup>, Pierre Chambon<sup>5</sup> and CheMyong Ko<sup>1,3</sup>

<sup>1</sup>Division of Clinical and Reproductive Sciences, Department of Clinical Sciences, University of Kentucky, Lexington, Kentucky 40536, USA

<sup>2</sup>Department of Anatomy and Neurobiology, School of Medicine, Institute of Health Sciences, Gyeongsang National University, Jinju, South Korea

Departments of <sup>3</sup>Biology and <sup>4</sup>Animal Sciences, University of Kentucky, Lexington, Kentucky 40536, USA

<sup>5</sup>Institut de Genetique et de Biologie Moleculaire et Cellulaire (CNRS, INSERM, ULP, College de France) and Institut Clinique de la Souris, BP10142, 67404 Illkirch-Strasbourg, France

(Correspondence should be addressed to C Ko; Email: cko2@uky.edu)

## Abstract

Estrogen plays a critical role in inducing LH surge. In the pituitary, estrogen receptor  $\alpha$  (ER $\alpha$ ) mediates the action of estrogen, while the downstream pathway of ER $\alpha$  activation is yet to be elucidated. Here, we report the finding that cholecystokinin type A receptor (CCK-AR) is an ER $\alpha$  downstream gene in the mouse anterior pituitary. In the cycling mouse pituitary, the expression of CCK-AR mRNA is markedly higher in the afternoon of proestrus compared with metestrus. Both ovariectomy (OVX) and null mutation of the ER $\alpha$  gene completely abolish CCK-AR mRNA expression. Injection of 17 $\beta$ -estradiol to OVX wild-type mice induces recovery of CCK-AR mRNA expression to levels observed at proestrus, but no such recovery is induced in OVX ER $\alpha$  knockout mice. The same pattern of estrogen dependency in inducing CCK-AR mRNA expression was

seen in cultured primary anterior pituitary cells, indicating that estrogen directly acts on pituitary cells to induce CCK-AR expression. Immunohistological analysis revealed that more than 80% of gonadotrophs express CCK-AR in the afternoon of proestrus. To test whether CCK-AR mediated the sensitizing effect of estrogen in GnRH-induced LH secretion, primary pituitary cells were primed with estrogen followed by treatment with GnRH in the presence or absence of lorglumide, a CCK-AR antagonist. While both groups secreted LH upon GnRH treatment, lorglumide treatment significantly decreased LH secretion. Taken together, this study finds CCK-AR to be an ER $\alpha$  downstream gene in the pituitary and suggests that CCK-AR may play a role in the estrogen sensitization of the pituitary response to GnRH.

*Journal of Endocrinology* (2007) **195**, 393–405

## Introduction

It is well established that, among multiple factors that contribute to the induction of luteinizing hormone (LH) secretion, the ovarian steroid estrogen plays a pivotal role by exerting positive feedback to the pituitary (Clarke 2002, Christian *et al.* 2005); however, the process by which estrogen controls these events has not been fully understood. Estrogen plays its role by modulating the activity of  $\alpha$  and/or  $\beta$  subtypes of estrogen receptors (ERs) in a tissue type-dependent manner. While both ER $\alpha$  and ER $\beta$  are present in the pituitary, ER $\alpha$  has been shown to be the effector of estrogen action in the pituitary (Sanchez-Criado *et al.* 2004, 2005). In support of this finding, ER $\alpha$  knockout (ER $\alpha$ KO) female mice are completely infertile and do not ovulate, while ER $\beta$ KO mice are fertile (Dupont *et al.* 2000, Hewitt & Korach 2003). However, the downstream pathway of ER $\alpha$  activation in the pituitary gonadotroph is not yet known.

As a nuclear receptor transcription factor, ER $\alpha$  has been speculated to regulate the expression of molecules involved in hormone secretion in the gonadotrophs (Naik *et al.* 1985, Powers 1986, Sapino *et al.* 1986, Bauer-Dantoin *et al.* 1993, Thomas & Clarke 1997, Kirkpatrick *et al.* 1998, DePasquale 1999, Clarke 2002, Rispoli & Nett 2005). In this regard, it is interesting that the cholecystokinin (CCK)/CCK type A receptor (CCK-AR) system, a well-known regulatory machinery of protein secretion, has been detected in the pituitary and shown to be involved in the LH secretion (Vijayan *et al.* 1979, Vijayan & McCann 1986, Peuranen *et al.* 1995). Furthermore, recently, it has been shown that estrogen via ER $\alpha$  influences the function and expression of the CCK/CCK-AR system in regulating satiety (Geary *et al.* 1994, 1996, 2001). These findings have led us to hypothesize that as a way of regulating LH secretion, estrogen via ER $\alpha$  may modulate CCK/CCK-AR expression in the pituitary.

CCK is a multifunctional peptide, whose action is mediated by two forms of G-protein-coupled receptors, CCK-AR and

type B (CCK-BR; Wank 1995, Williams *et al.* 2002). CCK stimulates the secretion of a variety of proteins including digestive enzymes (Sankaran *et al.* 1980, Rossetti *et al.* 1987), neuropeptides (Wank 1995, Tirassa *et al.* 1998), and hormones (Rossetti *et al.* 1987, Karlsson & Ahren 1992, Peuranen *et al.* 1995, Andren-Sandberg *et al.* 1999). Since the first cloning of CCK-AR in the pancreatic acinar cells (Sankaran *et al.* 1980), expression of CCK-AR has been reported in multiple cell types including gastric chief cells (Qian *et al.* 1993), smooth muscle cells of gastrointestinal tract (Bitar & Makhoulf 1982, Meyer *et al.* 1989), neurons (Skirboll *et al.* 1986), and endocrine cells (Kamilaris *et al.* 1992). In particular, the CCK/CCK-AR system has been implicated in the secretion of pituitary hormones including adrenocorticotrophic hormone,  $\beta$ -endorphin, growth hormone (GH), thyroid-stimulating hormone (TSH), prolactin (PRL), and LH (Vijayan *et al.* 1979, Vijayan & McCann 1986, Bondy *et al.* 1989, Kamilaris *et al.* 1992, Mannisto *et al.* 1992, Peuranen *et al.* 1995).

Here, we report evidence that estrogen induces CCK-AR expression via ER $\alpha$  in the pituitary and CCK-AR activation enhances sensitivity of estrogen-primed gonadotrophs to gonadotropin-releasing hormone (GnRH)-stimuli.

## Materials and Methods

### Reagents

Antibodies for CCK-AR were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Polyclonal antisera for mouse pituitary hormones (adrenocorticotropin hormone (ACTH), GH, PRL, follicle-stimulating hormone (FSH), LH, and TSH) were purchased from the National Hormone and Pituitary Program (Harbor-UCLA Medical Center, Torrance, CA, USA). GnRH, 17 $\beta$ -estradiol (E<sub>2</sub>), CCK-8s, and lorglumide were purchased from Sigma. Molecular reagents were purchased from Invitrogen. Cell culture reagents including Dulbecco's Modified Eagle's Medium (DMEM), gentamicin, BSA, HEPES, trypsin, trypsin inhibitor, and DNase I were purchased from Sigma. Other reagents including ITS (insulin 10  $\mu$ g/ml, transferin 5.5  $\mu$ g/ml, sodium selenite 6.7 ng/ml), fungizone, and fetal bovine serum were purchased from Gibco-BRL.

### Animals and treatments

Animal handling procedures were carried out in accordance with the University of Kentucky Animal Care and Use Committee. All mice used in this study have a C57BL/6 genetic background. For ovariectomy (OVX) and estrogen/vehicle treatment, mice were ovariectomized at 45 days of age. Three weeks later, each mouse was injected (s.c.) with 10  $\mu$ g E<sub>2</sub> or 100  $\mu$ l sesame oil at 0900 h for two consecutive days. On the second day, the mice were killed at 1500 h by carbon dioxide inhalation, and the pituitary was harvested and frozen on dry ice for later RNA extraction. For the histological analyses, cardiac

perfusion was performed using 4% neutralized buffered paraformaldehyde. After postfixation with the same fixative, tissues were stored in 20% sucrose and later frozen in OCT compound (Tissue-Tek, Sakura Finetek, Torrance, CA, USA). For determination of stages of the estrous cycle, a standard vaginal lavage technique (Becker *et al.* 2005) was applied. After daily vaginal lavages for 2 weeks, mice were killed on proestrus or metestrus at 1500 h to collect estrus cycle-specific pituitary tissues. For primary pituitary culture, 10-week-old female mice (C57BL/6) were purchased from Harlan Animal Breeding Center (Harlan, Indianapolis, IN, USA).

### Generation of ER $\alpha$ KO mice

The generation of ER $\alpha$ KO (ER $\alpha^{-/-}$ ) mice resulted from a cross of male ER $\alpha^{\text{flox/flox}}$  with female Zp3<sup>cre</sup>, a line expressing Cre recombinase specifically in the oocyte. ER $\alpha^{\text{flox/flox}}$  mice possess two loxP sites flanking exon 3 of the ER $\alpha$  gene (Dupont *et al.* 2000). The resulting F1 heterozygote ER $\alpha^{\text{flox/+}}$  Zp3<sup>cre</sup> was then bred with ER $\alpha^{\text{flox/flox}}$  to produce ER $\alpha^{\text{flox/flox}}$  Zp3<sup>cre</sup>. Female ER $\alpha^{\text{flox/flox}}$  Zp3<sup>cre</sup> mice produce ER $\alpha^{-}$  oocytes due to the deletion of floxed exon in the oocyte. Thus, oocytes fertilized by sperm from ER $\alpha^{\text{flox/flox}}$  males result in progeny that are ER $\alpha^{\text{flox/-}}$ . The breeding of female ER $\alpha^{\text{flox/flox}}$  Zp3<sup>cre</sup> with male ER $\alpha^{\text{flox/-}}$  mice produces half of progeny that are ER $\alpha^{-/-}$ . Genotyping was performed by PCR using ear biopsy DNA. Genomic DNA was isolated from ear using the Easy-DNA Kit (Invitrogen). A primer set of ER $\alpha$ P1 (5'-ttg ccc gat aac aat aac at-3') and ER $\alpha$ P3 (5'-ggc att acc act tct cct ggg agt ct-3') was used to determine whether or not exon 3 had been deleted (ER $\alpha^{-}$ ). The presence of Zp3 Cre recombinase was determined using primers Cre-P1 (5'-gga cat gtt cag gga tgc cca ggc g-3') and Cre-P85 (5'-gtg aaa cag cat tgc tgt cac tt-3').

### Primary pituitary cell culture

Anterior pituitary lobes were dissected from 10-week-old female C57BL/6 mice pituitaries after carbon dioxide inhalation. Pituitary cells were isolated as described previously (Kim *et al.* 2000) with minor modification. Briefly, anterior pituitary lobes were minced into small pieces in serum-free media, digested with trypsin for 20 min at room temperature, and dispersed in solution containing trypsin inhibitor by repeated sucking and pushing using an 18 G needle and syringe. After washing, cells were counted and plated onto poly-L-lysine-coated culture dish that contained medium (20 mM HEPES and 0.3% BSA in DMEM) supplemented with 10% fetal bovine serum or charcoal-treated fetal bovine serum. Cells were incubated in a humidified incubator at 37 °C with 5% CO<sub>2</sub>. Tissue culture medium was changed every other day.

### Cell treatment and LH assay

For assessment of the effect of CCK-AR on LH secretion, cells were counted and plated (1  $\times$  10<sup>5</sup> cells/well) in 96-well

plates coated with poly-L-lysine. After 2 days of culture, incubation media were changed with medium supplemented with 10% charcoal-treated fetal bovine serum and cultured for an additional 2 days. The cells were then treated with charcoal-treated serum containing either 0.00001% ethanol, 1 nM E<sub>2</sub> in 0.00001% ethanol, or 10 nM E<sub>2</sub> in 0.00001% ethanol for 2 days. Then, the expression of CCK-AR was examined using RT-PCR and immunocytochemistry. Experiment 1: Two days after estrogen (1 nM) treatment, GnRH (10 nM; Lindzey *et al.* 2006), CCK-8s (agonist for CCK-AR, 100 nM; Baptista *et al.* 2005), lorglumide (antagonist for CCK-AR, 10 µM; Gonzalez-Puga *et al.* 2005), and vehicle (0.00001% ethanol) were added to serum-free media for 2 h. Then, media were collected for LH concentration measurement. Experiment 2: The cells were cultured for 2 days in the presence of E<sub>2</sub> (1 nM) prior to GnRH (10 nM) administration. Lorglumide (10 µM) and vehicle were treated 15 min before GnRH treatment. Cells were washed and retreated to the same reagent with GnRH for 15 min. This treatment was repeated six times for a total span of 3 h. Upon completion of each treatment, media were collected, snap frozen, and stored at -80 °C until analysis. RIA of LH concentration was performed using a mouse LH sandwich assay provided by the University of Virginia Center for Research in Reproduction Ligand Assay and Analysis Core (NICHD (SCCPRR) Grant U54-HD28934, University of Virginia, Virginia).

#### Western blot

Total protein extracts were prepared in tri-detergent lysis buffer (1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.02% sodium azide, 100 µg/ml phenylmethylsulfonyl fluoride, 1 µg/ml aprotinin, and 0.5 µg/ml leupeptin) by grinding with disposable polypropylene grinder followed by ultrasonication. Lysates were centrifuged for 30 min at 13 000 g, and the supernatants were collected. Protein levels in the supernatants were determined using the BCA protein assay kit (Pierce, Rockford, IL, USA). Samples (30 µg each pituitary and 5 µg for pancreas) were separated by SDS-PAGE using a discontinuous buffer system. Electrophoretically separated polypeptides were transferred to a polyvinylidene fluoride (PVDF) membrane at 15 V for 20 min using a semidry transfer apparatus (Bio-Rad) submerged in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol, pH 8.3). The membrane was blocked with 1% BSA, 5% skim milk, and 0.1% Tween 20 in Tris-buffered saline (TBS, pH 7.4; 0.05 M Tris-HCl, 0.9% NaCl). After incubation for 2 h with anti-CCK-AR (goat polyclonal, Santa Cruz, sc-16172, 1/300) and β-actin (1:10,000; Sigma) antibodies, bound antibodies were detected with an enhanced chemiluminescence detection kit (Amersham Biosciences) according to the supplied protocol. The membranes were exposed to X-ray film.

#### Immunohistological analysis

For immunohistological analysis, tissues were fixed and processed as described previously (Kim *et al.* 2005). Tissue sections were incubated with 5% normal serum for 30 min at room temperature to block nonspecific binding. For CCK-AR detection, sections were incubated with anti-CCK-AR (goat polyclonal, Santa Cruz, sc-16172, 1/100) for 2 h, then incubated with a biotinylated anti-goat IgG antibody followed by incubation with streptavidin-conjugated Alexa Fluor 594 (Molecular Probes, Eugene, OR, USA; 1/1000). For double immunostaining of CCK-AR and pituitary hormones, pituitary sections were incubated with anti-CCK-AR and either LH, FSH, GH, PRL, FSH, or ACTH antibody (rabbit polyclonal antibody, 1/500) for 2 h. Biotinylated secondary antibodies were used followed by streptavidin-conjugated Alexa Fluor 594 and Alexa Fluor 488-conjugated anti-rabbit IgG antibodies to detect CCK-AR and each pituitary hormone respectively. After washing with distilled water, sections were mounted with ProLong Gold antifade reagent with DAPI (Molecular Probes). Photographs were taken using a fluorescent microscope (Olympus, Tokyo, Japan) and a digital camera (DP70, Olympus). At least three different pituitaries were used for each protein detection. To count double-labeled cells, four merged images (0.1376 mm<sup>2</sup>) were made from each group. Single-positive cells for pituitary hormone and double-positive cells for pituitary hormone and CCK-AR were separately counted using each image, and then the total number and percentages of double-labeled cells were calculated.

#### RT-PCR and DNA microarray analysis

The gene expression pattern of CCK-AR mRNA from OVX WT mice treated with E<sub>2</sub> or vehicle was analyzed by semiquantitative RT-PCR. The total RNA (1 µg/group) was used for cDNA synthesis followed by PCR. Primers used were as follows: CCK-AR forward (5'-gtg ctg att cga aac aag agg-3'), CCK-AR reverse (5'-aga tgg cta cca ggt tga agg-3'), L19 ribosomal protein forward (5'-cct gaa ggt caa agg gaa tgt g-3'), and L19 ribosomal protein reverse (5'-gtc tgc ctt cag ctt gtg gat-3'). To examine the expression patterns of CCK-AR and other CCK-AR-related genes at different conditions, DNA microarray was performed with total RNA (5 µg/group) at the University of Kentucky DNA Microarray Core Facility using the Affymetrix Mouse 430 2.0 oligonucleotide array set (Affymetrix, Santa Clara, CA, USA). Briefly, the total RNA was extracted from the pituitaries of 1) metestrus and proestrus wild-type (WT) mice, 2) OVX WT mice treated with E<sub>2</sub> or vehicle, and 3) OVX ERαKO mice. Mice were ovariectomized at 45 days of age. Three weeks after OVX, groups 2 and 3 were injected (s.c.) with 10 µg E<sub>2</sub> or 100 µl sesame oil at 0900 h on days 1 and 2. At 1500 h on day 2, mice were killed and the pituitaries harvested, snap frozen on dry ice, and stored at -80 °C for

later RNA isolation. Total RNA was extracted using Trizol reagent (Invitrogen Life Technologies Inc.) and purified using an RNeasy kit (Qiagen Inc). The integrity of RNA was checked by visualizing 28S rRNA and 18S rRNA bands on a 1.5% agarose gel. For each group, total RNA extracted from at least three different mice ( $n=5$  for groups 1 and 2,  $n=3$  for group 3) were pooled together. The microarray analysis was performed twice with different RNA samples. The data presented were the expression values provided by Affymetrix array system.

#### Quantitation of RT-PCR and western blot results and statistics

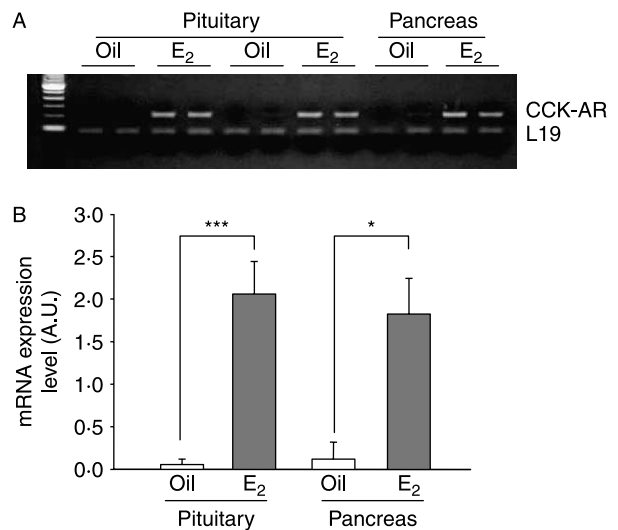
Optical density data of PCR and western blot bands were obtained using Kodak 1D software (ver. 3.63, Kodak) and analyzed using SigmaStat (ver. 3.5, Jandel Scientific Co. Ltd, Erkrath, Germany). Significance of data between two groups was evaluated using *t*-test. For RIA data analysis, data from all GnRH-treated groups were analyzed using one-way ANOVA and the Student–Newman–Keuls method or *t*-test. Statistical significance was set at  $P<0.05$ .

## Results

#### CCK-AR expression is induced by E<sub>2</sub> via ER $\alpha$ in the pituitary

To determine whether estrogen induced CCK-AR expression in the pituitary, the effect of E<sub>2</sub> treatment on the expression of CCK-AR mRNA and protein in ovariectomized mice was measured. Forty-five day old mice were ovariectomized, kept for 3 weeks, and then injected (s.c.) with either E<sub>2</sub> or sesame oil at 0900 h for two consecutive days. On the second day, the mice were killed at 1500 h, the pituitary and pancreas were collected, and the CCK-AR mRNA expression level was measured by semiquantitative RT-PCR (Fig. 1). Pancreas was used as a positive control because of its known expression of CCK-AR (Sankaran *et al.* 1980). The CCK-AR mRNA expression level in the E<sub>2</sub>-treated pituitary was 16-fold higher than the vehicle-treated group. Interestingly, CCK-AR mRNA expression in the pancreas was also dramatically increased by E<sub>2</sub> injection. Similar to the mRNA expression pattern, western blot and immunofluorescent analyses showed an increase in CCK-AR protein expression in the E<sub>2</sub>-treated pituitary (Fig. 2).

These findings led us to investigate whether the level of expression of CCK-AR changed during the estrous cycle and whether ER $\alpha$  was involved in regulating CCK-AR expression. For this purpose, the pituitaries of naturally cycling mice, OVX WT mice treated either with E<sub>2</sub> or sesame oil, and ER $\alpha$ KO mice treated either with E<sub>2</sub> or sesame oil were used to measure mRNA expression levels. We employed an extensive DNA microarray not only to measure CCK-AR mRNA expression but also to generate a genome-wide pituitary gene expression profile to determine estrogen/ER $\alpha$  effect on other genes that might be involved in the CCK-AR signaling. As expected, the CCK-AR mRNA expression appeared higher in the E<sub>2</sub>-treated OVX

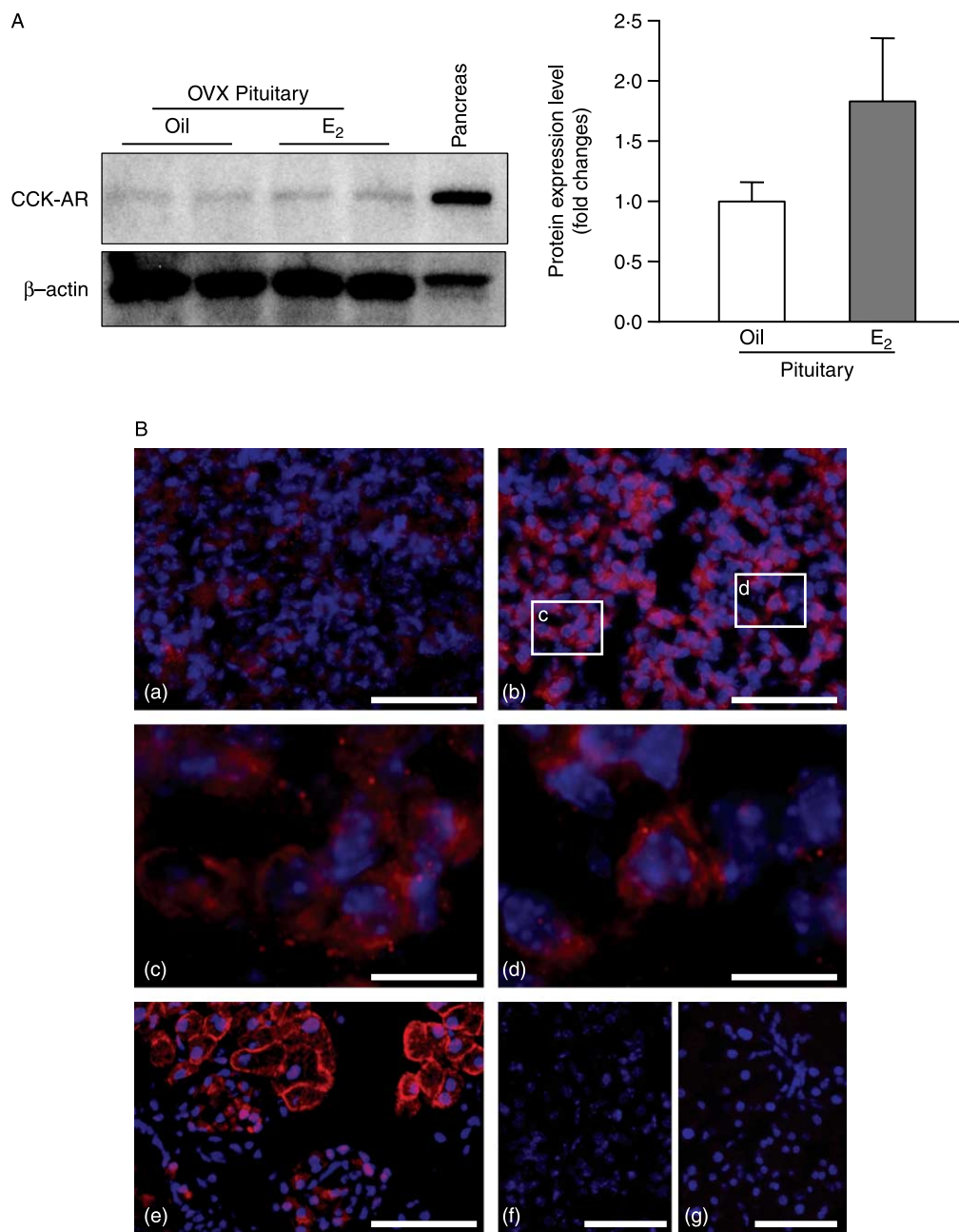


**Figure 1** The expression of CCK-AR mRNA in the pituitary and pancreas from OVX wild-type (WT) and vehicle (oil)- or E<sub>2</sub>-treated female mice. (A) Representative ethidium bromide-stained gel image of a semiquantitative RT-PCR result. (B) Quantitation of the RT-PCR results. CCK-AR mRNA expression was increased dramatically both in the pituitary and pancreas of OVX E<sub>2</sub>-treated mice. The mice were ovariectomized 3 weeks prior to E<sub>2</sub> injection (10  $\mu$ g s.c., at 0900 h for 2 days). Tissues were collected at 1500 h on the second day of E<sub>2</sub> treatment. Pituitary ( $n=4$ ,  $P<0.001$ ), pancreas ( $n=2$ ,  $P=0.035$ ).

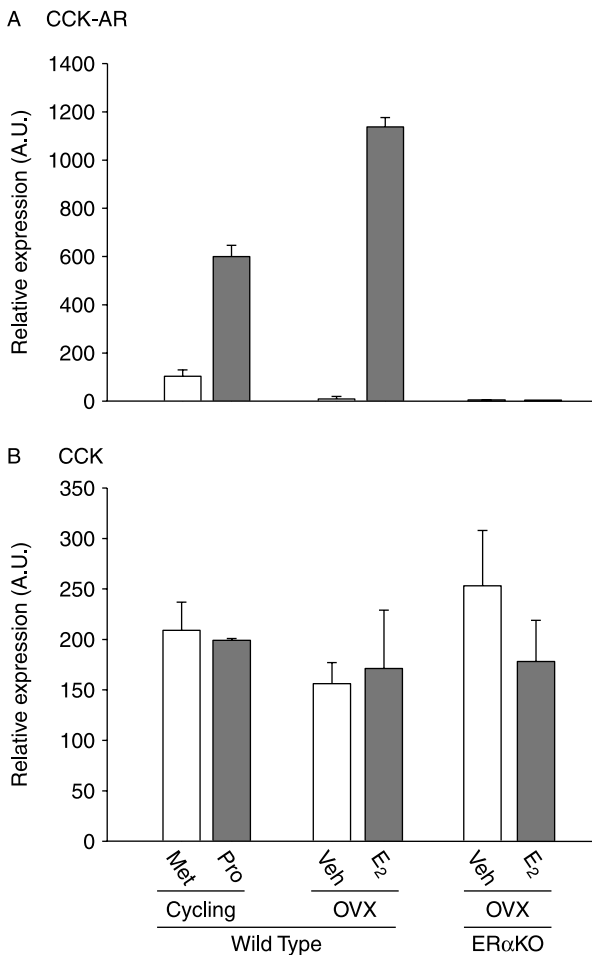
WT mouse pituitary compared with the oil-treated control. Furthermore, a sixfold induction of CCK-AR mRNA expression was observed in the pituitary at proestrus (high serum E<sub>2</sub> concentration) compared with metestrus (basal serum E<sub>2</sub> concentration; Fig. 3A). However, no CCK-AR mRNA expression was detectable in the ER $\alpha$ KO mouse pituitary regardless of E<sub>2</sub> treatment, suggesting ER $\alpha$ -dependent CCK-AR expression in the pituitary. Interestingly, no CCK-BR mRNA expression was detectable in any group (data not shown), while a low but constitutive level of mRNA expression for CCK was observed in all groups (Fig. 3B). Reflecting the mRNA expression pattern, immunohistological analyses revealed a markedly higher expression of CCK-AR protein in the pituitary of proestrus than metestrous pituitary (Fig. 4A and B). Furthermore, consistent with the E<sub>2</sub>- and ER $\alpha$ -dependent mRNA expression, neither ER $\alpha$ KO female nor WT male pituitaries expressed CCK-AR protein (Fig. 4C and D).

#### The numbers of CCK-AR-expressing gonadotrophs increase at proestrus

To determine the cell types that expressed CCK-AR, double immunofluorescent staining was employed. Combinations of antibodies against CCK-AR and each of the six pituitary hormones – LH, FSH, ACTH, TSH, GH, and PRL were used to stain adjacent pituitary sections of metestrous and proestrous mice. The CCK-AR expression was detected in a percentage of



**Figure 2** Western blot and immunofluorescent analyses of CCK-AR protein expression. (A) Western blot analysis of CCK-AR using proteins from OVX pituitaries treated with oil or E<sub>2</sub>. Pancreas was used to determine the specificity of the CCK-AR antibody. Note the increased CCK-AR protein in the E<sub>2</sub>-treated pituitary compared with oil-treated pituitary. Data are presented as mean ± s.e.m. (B) CCK-AR immunopositive signal (IS) was detected both in OVX + oil (a) and OVX + E<sub>2</sub>-treated (b–d) pituitaries. Both cell numbers and relative intensity of CCK-AR ISs were increased in the OVX E<sub>2</sub>-treated mice compared with oil-treated mice. High power images of panel b are shown (c–d). Intense CCK-AR ISs were seen in the pancreas (e). No IS were detected without primary antibody either in pituitary (f) or in pancreas (g). At least three animals were used for each group. These results were the representatives of at least four repeats that gave similar results. The mice were ovariectomized 3 weeks before E<sub>2</sub> injection (10 µg s.c., at 0900 h for 2 days). Tissues were collected at 1500 h on the second day. Red color represents Alexa Fluor-594 of CCK-AR staining and blue represents DAPI for nucleus staining. Scale bars, 50 µm (a and b), 10 µm, (c and d), 60 µm, (e–g).



**Figure 3** E<sub>2</sub>- and ER $\alpha$ -dependent expression of CCK-AR. CCK-AR (A) and CCK (B) mRNA expression levels were measured by DNA microarray as described in the Materials and Methods section. Note that while CCK-AR expression is dependent on cycling stages, E<sub>2</sub> treatment, and ER $\alpha$ , no such change was seen in the CCK expression. Data presented are mean  $\pm$  S.E.M. from two independent microarray analyses.

all five cell types, gonadotroph (LH, FSH), corticotroph (ACTH), thyrotroph (TSH), somatotroph (GH), and lactotroph (PRL), while the rest of the cells were stained only with their own cell markers (Fig. 5A). The relative CCK-AR signal intensities in all of those cell types examined were higher in the proestrous pituitary than the metestrous pituitary. Interestingly, the ratio of CCK-AR-positive cells was significantly higher in the gonadotrophs (FSH, LH) on proestrus compared with metestrus. No such change in ratios was seen in other pituitary cell types (Fig. 5B).

#### *CCK-AR enhances the sensitivity of E<sub>2</sub>-primed gonadotrophs to continued GnRH stimuli*

When treated with E<sub>2</sub>, the expression of both CCK-AR mRNA and protein increased in the primary pituitary cells

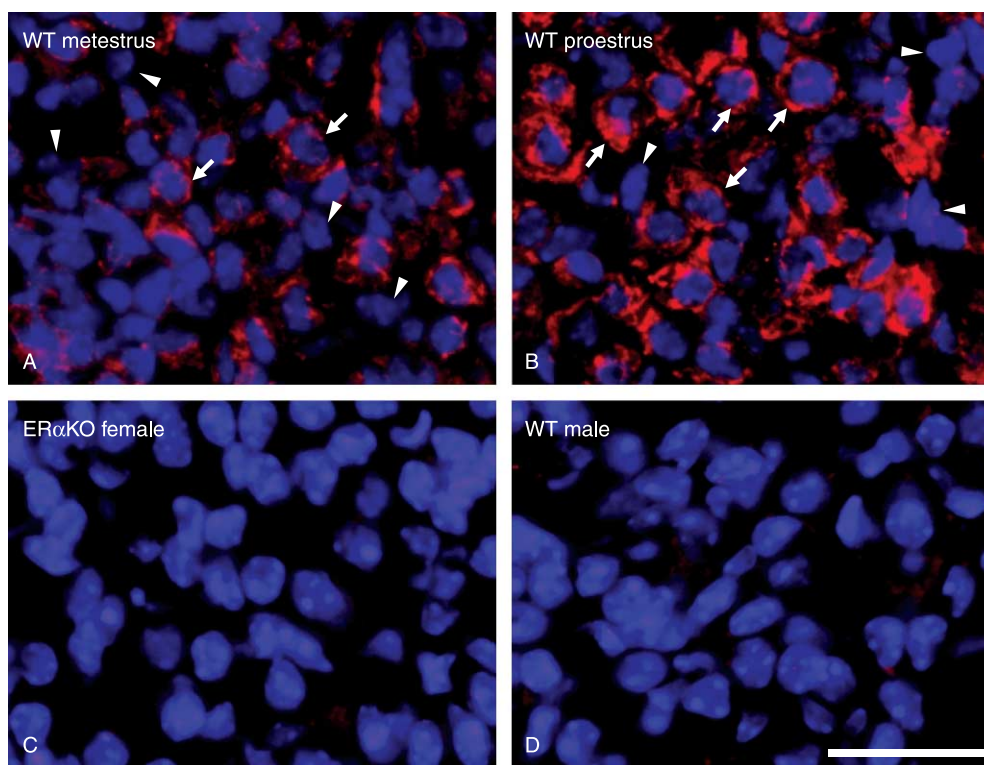
isolated from 2-month-old female mice (Fig. 6), indicating E<sub>2</sub>-induced expression of CCK-AR was regulated at the pituitary cell level. Using this culture system and a previously reported protocol (Lindzey *et al.* 2006), we tested whether CCK-AR was involved in estrogen-induced sensitization of pituitary in regulating LH secretion. The primary pituitary cells were cultured in the presence or absence of 1 nM E<sub>2</sub> for 2 days, and subjected to GnRH (10 nM) treatment in the presence or absence of a CCK-AR agonist (CCK-8s, 100 nM) or CCK-AR-specific antagonist (lorglumide, 10  $\mu$ M). Concentrations of LH in the culture media were then determined (Fig. 7A). E<sub>2</sub>-treated cells secreted a significantly higher amount of LH upon GnRH stimulation compared with control, which was consistent with the previous report (Lindzey *et al.* 2006). Meanwhile, the LH secretion from E<sub>2</sub>-treated cells was significantly decreased by co-treatment of lorglumide (Fig. 7A).

To determine whether the negative effect of lorglumide on LH secretion was repeatable upon repetitive GnRH treatment (to mimic natural GnRH pulsatile secretion), the E<sub>2</sub>-primed primary cells were cultured in the presence or absence of lorglumide (10  $\mu$ M) for 15 min, followed by GnRH (10 nM) challenge. Fifteen minutes post-GnRH treatment, the culture media were collected for LH measurement. This procedure was repeated five more times (30 min each, 3 h in total). While both vehicle- and lorglumide-treated groups showed decline in LH release upon consecutive GnRH challenges (Fig. 7B), lorglumide-treated cells secreted less amount of LH upon each GnRH challenge, and the difference in LH secretion eventually became significant upon the fifth challenge (Fig. 7B). Taken together, these results show that the CCK/CCK-AR system mediates at least in part E<sub>2</sub>-induced sensitization of pituitary in GnRH-induced LH secretion.

## Discussion

This study revealed that estrogen regulates CCK-AR expression in the pituitary via ER $\alpha$  and suggests a role for CCK-AR as a mediator of estrogen action for the LH surge. Since its identification (Sankaran *et al.* 1980), CCK-AR has been implicated in the regulation of the secretion of diverse kinds of proteins (Wank 1995, Williams *et al.* 2002). In the pancreas, CCK-AR activates exocrine secretion from acinar cells (Sankaran *et al.* 1980, Rossetti *et al.* 1987) and endocrine secretion of insulin from islet cells (Rossetti *et al.* 1987, Karlsson & Ahren 1992). In the stomach, CCK-AR mediates secretion of pepsin from gastric chief cells (Qian *et al.* 1993) and release of somatostatin from D cells of gastric mucosa (Lloyd *et al.* 1992). Not surprisingly, it has been shown that the pituitary, a major endocrine organ, has binding sites for CCK and that involvement of CCK-AR has been suggested in pituitary hormone secretion (Bondy *et al.* 1989, Kamilaris *et al.* 1992, Mannisto *et al.* 1992).

In the present study, three lines of experimental data indicate that estrogen is a key regulator of CCK-AR



**Figure 4** Immunofluorescent analyses of the CCK-AR protein expression. Pituitaries of WT metestrus (A), WT proestrus (B), ER $\alpha$ KO female (C), and WT male mouse (D) were stained with anti-CCK-AR antibody. Note that both CCK-AR immunopositive (arrow) and immunonegative (arrow head) cells are present regardless of the stages of estrus cycle, while no detectable CCK-AR signal was seen in pituitary of either ER $\alpha$ KO female or WT male mice. Three mice were used for each group, and the images are the representatives of at least four repeats that gave similar results. Red color represents Alexa Fluor-594 of CCK-AR staining and blue represents DAPI for nucleus staining. Scale bar, 50  $\mu$ m.

expression in the anterior pituitary. First, E<sub>2</sub> treatment of the OVX WT mice induced a dramatic increase in the expression of CCK-AR in the pituitary (Figs 1–3). Secondly, immunofluorescent staining showed that CCK-AR expression level was markedly higher in the afternoon of proestrus when the serum estrogen level was high than at metestrus when the estrogen level was low (Fig. 4A and B). Thirdly, CCK-AR was not expressed in the male pituitary (Fig. 4D). Interestingly, E<sub>2</sub> also induced CCK-AR expression in the pancreas of OVX WT mice (Figs 1 and 2), raising the possibility of estrogen involvement in regulating CCK-AR expression in non-reproductive tissues as well. In fact, it has been reported that E<sub>2</sub> benzoate increased CCK-AR in the OVX rat pancreas (Geary *et al.* 1996) and that E<sub>2</sub> affected satiety, a well-known physiological target where CCK-AR plays a critical role (Geary *et al.* 1994, 2001, Asarian & Geary 1999, 2006). In addition, a variety of peripheral feedback controls for eating have been shown to be E<sub>2</sub> sensitive (Asarian & Geary 2006). Thus, it will be interesting to see whether E<sub>2</sub> regulates CCK-AR expression in those tissues that are involved in satiety/eating control.

It is well known that ER $\alpha$  mediates the action of estrogen in regulating hormone secretion in the pituitary (Curtis

Hewitt *et al.* 2000). Interestingly, two lines of evidence described herein indicate that ER $\alpha$  mediates E<sub>2</sub> action in inducing CCK-AR expression in the pituitary. First, no CCK-AR expression was detectable in the ER $\alpha$ KO mouse pituitary regardless of E<sub>2</sub> treatment (Figs 3A and 4C). Secondly, CCK-AR expression was readily detectable in the major pituitary hormone secreting cell types that express ER $\alpha$  including corticotroph, somatotroph, lactotroph, thyrotroph, and gonadotroph (Mitchner *et al.* 1998; Fig. 5). While it is not known whether ER $\alpha$  directly interacts with the CCK-AR promoter, *in silico* analysis of the full-length mouse CCK-AR genomic DNA sequence (12 kb, NCBI accession no. D85605; Blesson *et al.* 2006) using transcription element search software; (TESS; <http://www.cbil.upenn.edu/tess>) revealed 15 estrogen-responsive elements in the 3 kb long promoter region (data not shown), indicating a potential direct interaction between ER $\alpha$  and the CCK-AR promoter, which needs further investigation.

CCK-AR functions as a regulator of protein secretion (Wank 1995). It regulates the secretion of digestive enzymes and endocrine hormones in the gastrointestinal tract (Sankaran *et al.* 1980, Rossetti *et al.* 1987, Lloyd *et al.* 1992, Qian *et al.* 1993), increases neurotransmitter release in the

nervous system (Crawley 1991), and is involved in the regulation of insulin secretion in the pancreas. Therefore, it is not surprising that the pituitary, a major endocrine organ secreting a variety of peptide hormones, has been speculated to be a potential target tissue of CCK action (Vijayan *et al.* 1979, Vijayan & McCann 1986, 1987). Interestingly,

however, no expression of CCK-AR has yet been reported in the pituitary. With the knowledge from our current study that pituitary CCK-AR expression would be high only when the serum estrogen level is high as at proestrus, it would be reasonable to speculate that detecting CCK-AR expression would have been challenging unless proestrous pituitary was

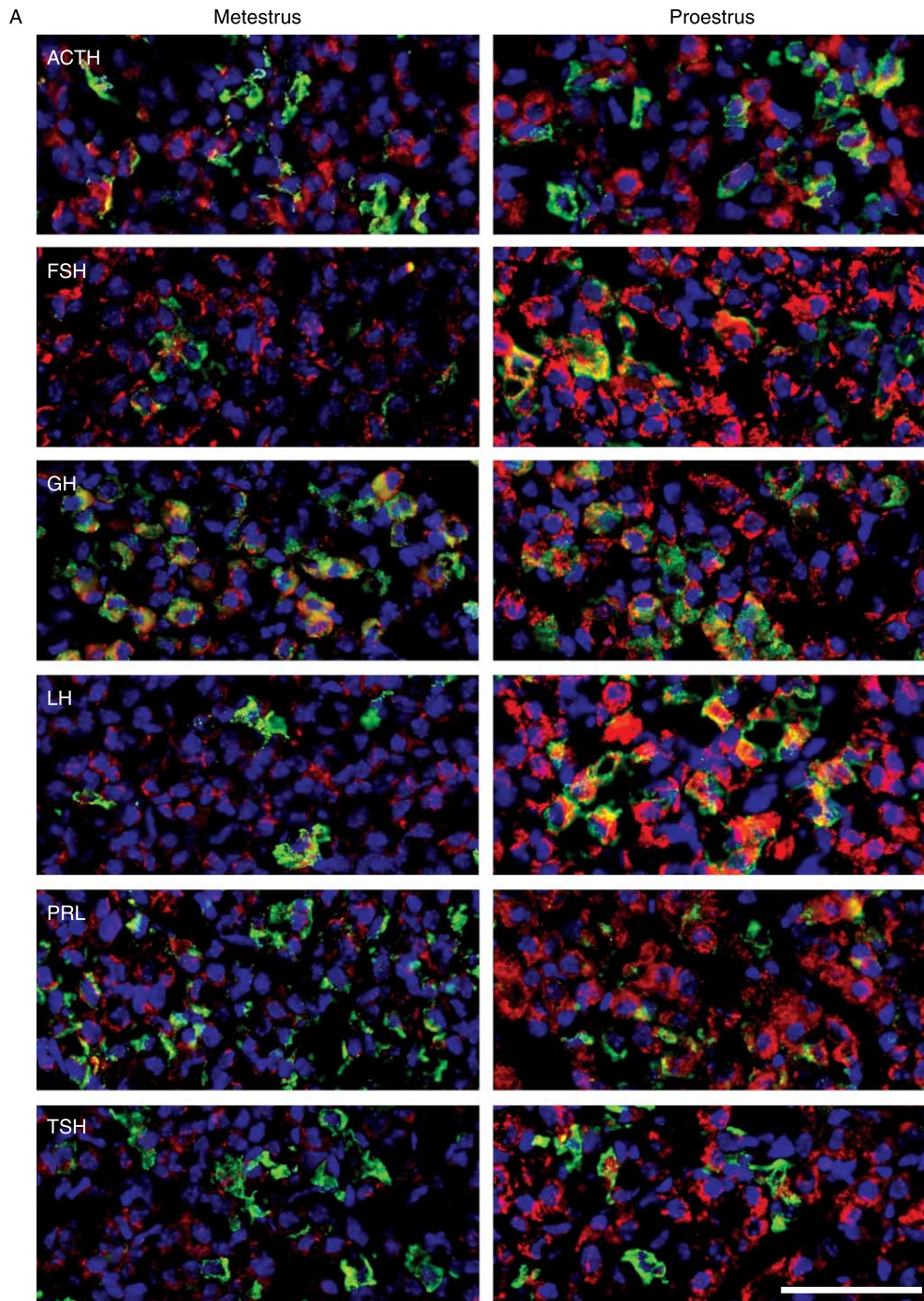
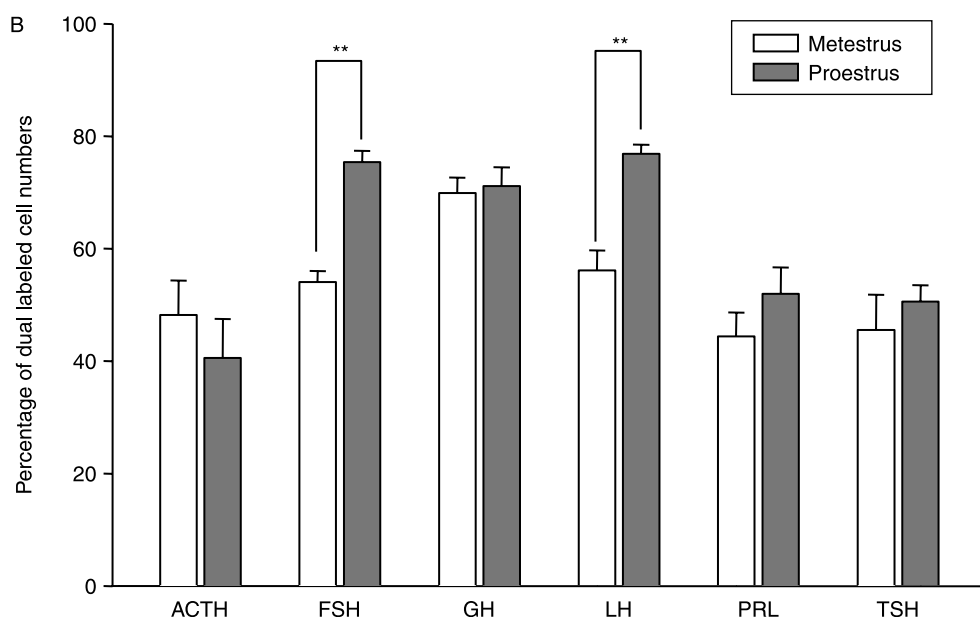


Figure 5 (continued)





**Figure 5** Colocalization of CCK-AR and pituitary hormones. (A) Representative images of double immunofluorescent staining using antibodies for CCK-AR and pituitary peptide hormones (ACTH, FSH, GH, LH, PRL, and TSH). Left panels were from metestrus, and right panels were from proestrous pituitaries. Red color represents Alexa Fluor-594 of CCK-AR, green represents Alexa Fluor-488 of each hormone, and blue represents DAPI for nucleus. Yellow color represents colocalization of CCK-AR and pituitary hormone. Scale bar, 50  $\mu$ m. (B) Quantitation of the numbers of the cells that were immunopositive for both CCK-AR and respective pituitary hormone. See the Materials and Methods section for detailed description of the quantitation. The statistical significance was evaluated by *t*-test between proestrus and metestrus. Note that only FSH- and LH-producing cells showed significantly increased double-labeled cell numbers at proestrus than metestrus. Data are presented as mean + s.e.m. ( $n=4$ ); \*\* $P<0.001$ .

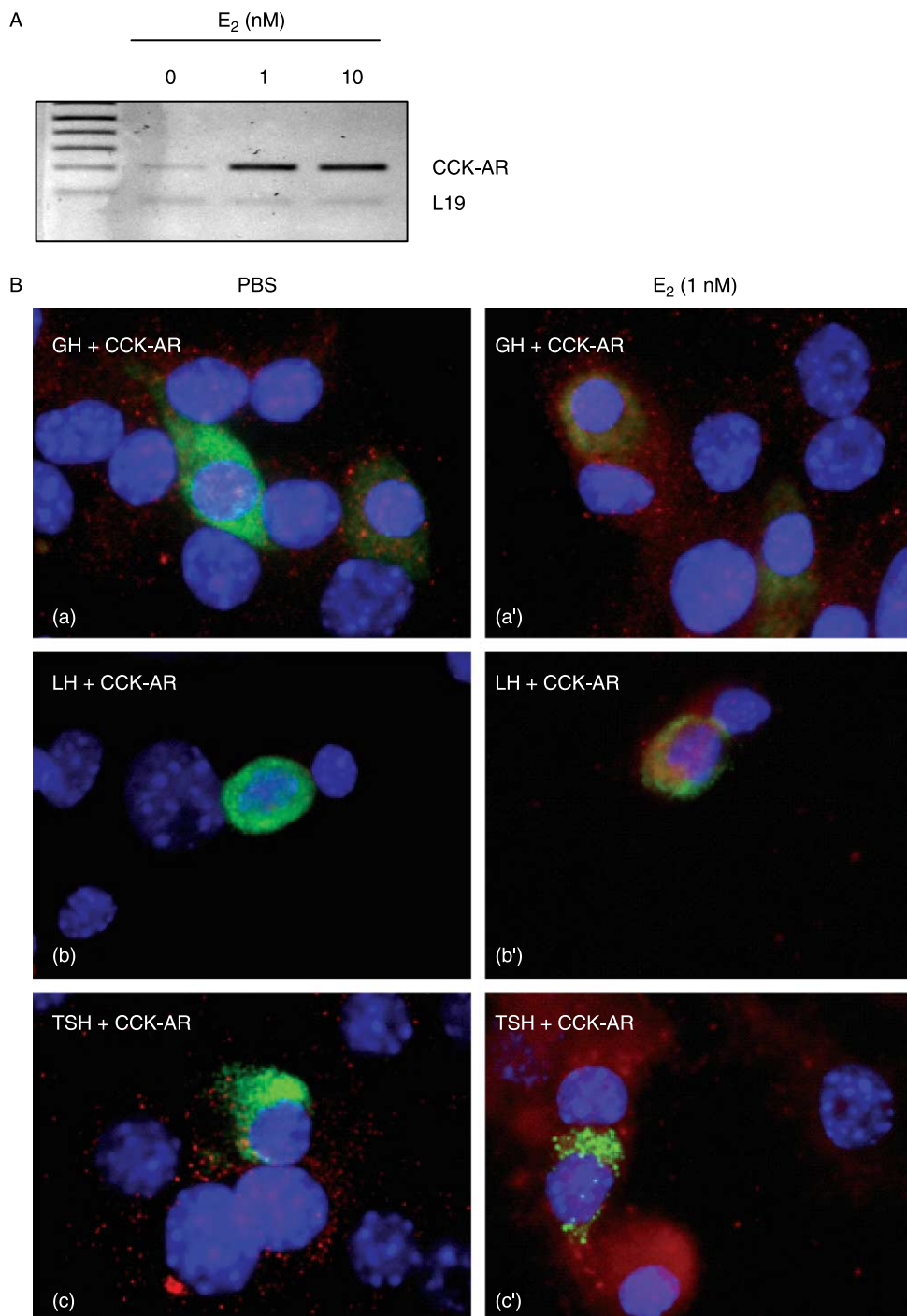
used for examination. In this study, we show that CCK-AR is expressed in at least five major endocrine cell types (Fig. 5A). In particular, the number of gonadotrophs expressing CCK-AR increased in the afternoon of proestrus (Fig. 5B), indicating an important role of CCK-AR in regulating LH secretion at this stage of the estrous cycle when the LH surge occurs (Smith *et al.* 1975, Gallo 1981).

A unique feature of LH release during the surge period is that the gonadotroph maintains its capacity to release comparable amounts of LH upon each GnRH stimulus for an extended period (Gallo 1981, van Dielen & de Koning 1995, Hoeger *et al.* 1999). In this regard, it is speculated that, at least in part, estrogen via a cohort of ER $\alpha$  downstream genes plays an important role in LH secretion, through a so-called 'estrogen-induced sensitization' (Hoeger *et al.* 1999, Turgeon & Waring 2001). Having evidence that CCK-AR is an estrogen/ER $\alpha$ -regulated gene and that its expression is dramatically induced in the proestrous gonadotrophs, it was imperative for us to determine whether estrogen-induced CCK-AR expression was a contributing factor to the enhanced sensitivity of estrogen-primed gonadotrophs upon GnRH stimuli.

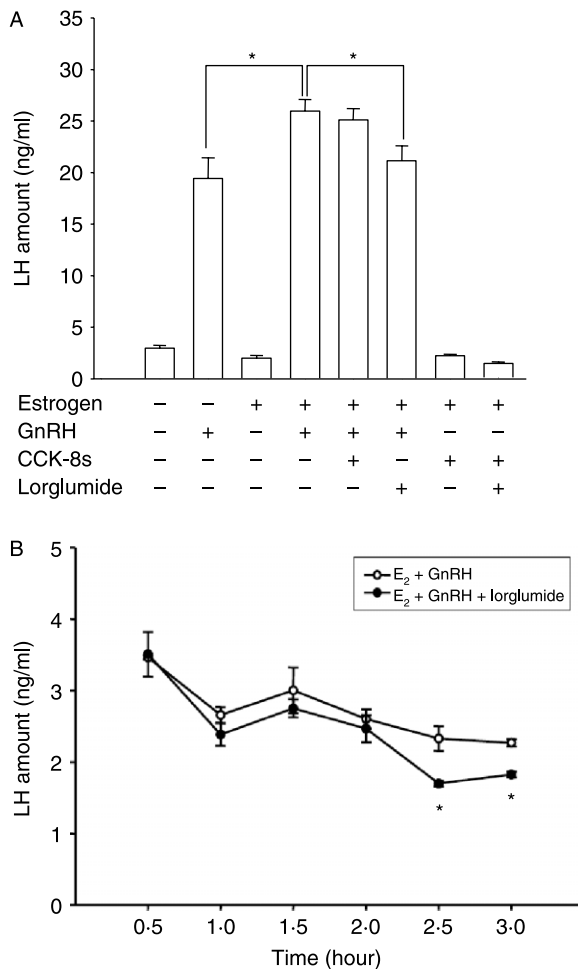
To address this question, primary anterior pituitary cells were used as an experimental system. Upon GnRH challenge, the pituitary cells secreted a large amount of LH regardless of the treatment with estrogen or lorglumide, the

CCK-AR antagonist. Pretreatment of the pituitary cells with E<sub>2</sub>, however, significantly increased the amount of LH secretion by 30% over non-treated cells (Fig. 7A). This result is consistent with the previous findings that estrogen potentiates gonadotrophs to release LH with greater pulse amplitude (Fox & Smith 1985, Hoeger *et al.* 1999) and that estrogen priming is essential for inducing the LH surge (Clarke 2002, Sanchez-Criado *et al.* 2004, 2005, Christian *et al.* 2005). In contrast, lorglumide treatment significantly decreased LH secretion in the E<sub>2</sub>-pretreated cells down to the level of non-primed cells (Fig. 7A). Furthermore, when the cells were repetitively challenged by GnRH for an extended period, lorglumide-treated cells secreted a lower amount of LH upon each GnRH challenge (Fig. 7B). It is noteworthy that while this difference looks seemingly minor, considering the fact that the surge level of serum LH is attained by the repetitive discharges of LH, the cumulative effect of the difference may eventually result in a substantial difference. Taken together, these results indicate that CCK-AR may mediate at least some portion of the priming effect of estrogen in the pituitary cells as an ER $\alpha$  downstream gene.

Upon binding to CCK, CCK-AR increases the intracellular Ca<sup>2+</sup> concentration (Yule & Williams 1994, Williams 2001), which is similar to the downstream events of GnRH receptor (GnRH-R) activation (Ghosh *et al.* 1996, Shacham *et al.* 2001). However, the consequences of



**Figure 6** The CCK-AR mRNA and protein expression in primary anterior pituitary cell culture. (A) Primary pituitary cells were treated with vehicle or E<sub>2</sub> (1 or 10 nM) for 2 days, and the CCK-AR mRNA expression level was measured by semiquantitative RT-PCR. (B) Colocalization of CCK-AR and pituitary hormones (GH, a and a'; LH, b and b'; TSH, c and c'). Left panels were from vehicle-treated cells, and right panels were from E<sub>2</sub> (1 nM)-treated cells. Red color represents Alexa Fluor-594 of CCK-AR, green represents Alexa Fluor-488 of each hormone, and blue represents DAPI of nucleus stain.



**Figure 7** The effect of CCK-AR-specific agonist (CCK-8s) and antagonist (lorglumide) on LH secretion in primary pituitary cells. (A) The primary cells isolated from anterior pituitary were treated with either vehicle (0.00001% ethanol) or E<sub>2</sub> (1 nM) for 2 days before GnRH (10 nM) administration. CCK-8s (100 nM) and/or lorglumide (10 μM) were then co-treated with GnRH (10 nM). Culture media were collected for LH concentration measurement 2 h after GnRH treatment. Statistical significance was calculated using one-way ANOVA and the Student–Newman–Keuls method. Data are presented as mean ± s.e.m. (n=4); \*P<0.05. (B) The cells were cultured in the presence of 1 nM E<sub>2</sub> for 2 days. Either vehicle or lorglumide (10 μM) were then treated for 15 min. Cells were washed and retreated to the same reagents with GnRH (10 nM) for 15 min. At the end of GnRH treatment, culture media were collected for LH concentration measurement by RIA. This procedure was repeated six times for a total span of 3 h. Data are presented as mean ± s.e.m., \*P<0.05 between E<sub>2</sub> and E<sub>2</sub>+CCK-8s+lorglumide-treated groups by *t*-test.

GnRH-R and CCK-AR activations are far different. Our data show that while GnRH alone can induce massive release of LH, CCK could not (Fig. 7A), consistent with the previous reports that when hemipituitaries were incubated with CCK, no LH release was induced (Vijayan *et al.* 1979). These

findings indicate that CCK-AR uses a unique subset of downstream signaling pathways that are different from the GnRH-R signaling pathway. In fact, CCK-AR signaling activates components of exocytosis machinery such as SNARE proteins, small G proteins, and actin filaments (Schafer *et al.* 1999, Williams 2001, Chen *et al.* 2002). Therefore, it is suggested that activation of CCK-AR may lead to the ‘tuning’ of exocytosis machinery, which eventually increase the responsiveness or sensitivity of the estrogen-primed gonadotrophs to repetitive GnRH stimuli.

Although CCK-AR may have such an important role as a mediator of estrogen action, it is not surprising that CCK-AR knockout mice are fertile (Kopin *et al.* 1999). The fertility would not mean that these mice displayed a normal LH surge; female mice with defect in LH secretion are often fertile (Xu *et al.* 2000, Thorsell & Heilig 2002). These mice may either have a redundant gene or a compensatory pathway that could be activated upon the deletion of CCK-AR in the pituitary as was shown in body weight control mechanisms in CCK-AR knockout mice (Kopin *et al.* 1999). In fact, a growing body of evidence indicates that genes involved in vesicle transportation (Thomas & Clarke 1997), cytoskeleton rearrangement (Powers 1986, Sapino *et al.* 1986, DePasquale 1999), regulation of ion channels (Clarke 2002), and energy metabolism (Simpson *et al.* 2005, Jones *et al.* 2006), as well as the receptor of GnRH itself (Naik *et al.* 1985, Bauer–Dantoin *et al.* 1993, Kirkpatrick *et al.* 1998, Rispoli & Nett 2005), are also under the regulation of estrogen. Therefore, it is suggested that the collective actions of these gene products may not only contribute to the increased responsiveness to GnRH stimuli during the period of LH surge, but also provide redundancy to this important reproductive event.

In summary, our data indicate that pituitary expression of CCK-AR is E<sub>2</sub> inducible, ERα mediated, and estrous stage dependent, and suggests that CCK-AR might be at least in part a contributing factor in maintaining the responsiveness or sensitivity of the estrogen-primed gonadotrophs to continued GnRH stimuli during the LH surge at the pituitary level.

## Acknowledgements

The authors thank Dr Phillip Bridges for his critical reading of the manuscript. This work was supported by grants P20 RR15592 and 1R01HD052694 from the National Institutes of Health, the University of Kentucky Microarray Facility, and the start-up fund provided by the University of Kentucky to C K. This work was also partially supported by the Korea Research Foundation Grant (KRF-2004-005-E00061) to H J K. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

## References

- Andren-Sandberg A, Hoem D & Backman PL 1999 Other risk factors for pancreatic cancer: hormonal aspects. *Annals of Oncology* **10** (Suppl 1) 131–135.
- Asarian L & Geary N 1999 Cyclic estradiol treatment physically potentiates endogenous cholecystokinin's satiating action in ovariectomized rats. *Peptides* **20** 445–450.
- Asarian L & Geary N 2006 Modulation of appetite by gonadal steroid hormones. *Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences* **361** 1251–1263.
- Baptista V, Zheng ZL, Coleman FH, Rogers RC & Travagli RA 2005 Cholecystokinin octapeptide increases spontaneous glutamatergic synaptic transmission to neurons of the nucleus tractus solitarius centralis. *Journal of Neurophysiology* **94** 2763–2771.
- Bauer-Dantoin AC, Hollenberg AN & Jameson JL 1993 Dynamic regulation of gonadotropin-releasing hormone receptor mRNA levels in the anterior pituitary gland during the rat estrous cycle. *Endocrinology* **133** 1911–1914.
- Becker JB, Arnold AP, Berkley KJ, Blaustein JD, Eckel LA, Hampson E, Herman JP, Marts S, Sadee W, Steiner M *et al.* 2005 Strategies and methods for research on sex differences in brain and behavior. *Endocrinology* **146** 1650–1673.
- Bitar KN & Makhlof GM 1982 Receptors on smooth muscle cells: characterization by contraction and specific antagonists. *American Journal of Physiology* **242** G400–G407.
- Blessin CS, Awasthi S, Kharkwal G, Daverey A & Dwivedi A 2006 Modulation of estrogen receptor transactivation and estrogen-induced gene expression by ormeloxifene – a triphenylethylene derivative. *Steroids* **71** 993–1000.
- Bondy CA, Jensen RT, Brady LS & Gainer H 1989 Cholecystokinin evokes secretion of oxytocin and vasopressin from rat neural lobe independent of external calcium. *PNAS* **86** 5198–5201.
- Chen X, Edwards JA, Logsdon CD, Ernst SA & Williams JA 2002 Dominant negative Rab3D inhibits amylase release from mouse pancreatic acini. *Journal of Biological Chemistry* **277** 18002–18009.
- Christian CA, Mobley JL & Moenter SM 2005 Diurnal and estradiol-dependent changes in gonadotropin-releasing hormone neuron firing activity. *PNAS* **102** 15682–15687.
- Clarke IJ 2002 Multifarious effects of estrogen on the pituitary gonadotrope with special emphasis on studies in the ovine species. *Archives of Physiology and Biochemistry* **110** 62–73.
- Crawley JN 1991 Cholecystokinin–dopamine interactions. *Trends in Pharmacological Sciences* **12** 232–236.
- Curtis Hewitt S, Couse JF & Korach KS 2000 Estrogen receptor transcription and transactivation: estrogen receptor knockout mice: what their phenotypes reveal about mechanisms of estrogen action. *Breast Cancer Research* **2** 345–352.
- DePasquale JA 1999 Rearrangement of the F-actin cytoskeleton in estradiol-treated MCF-7 breast carcinoma cells. *Histochemistry and Cell Biology* **112** 341–350.
- van Dielen JA & de Koning J 1995 From basal luteinizing hormone (LH) concentrations to the pre-ovulatory LH surge: titration of the physiological effect of gonadotrophin surge-inhibiting/attenuating factor. *Human Reproduction* **10** 3110–3116.
- Dupont S, Krust A, Gansmuller A, Dierich A, Chambon P & Mark M 2000 Effect of single and compound knockouts of estrogen receptors alpha (ERalpha) and beta (ERbeta) on mouse reproductive phenotypes. *Development* **127** 4277–4291.
- Fox SR & Smith MS 1985 Changes in the pulsatile pattern of luteinizing hormone secretion during the rat estrous cycle. *Endocrinology* **116** 1485–1492.
- Gallo RV 1981 Pulsatile LH release during the ovulatory LH surge on proestrus in the rat. *Biology of Reproduction* **24** 100–104.
- Geary N, Trace D, McEwen B & Smith GP 1994 Cyclic estradiol replacement increases the satiety effect of CCK-8 in ovariectomized rats. *Physiology and Behavior* **56** 281–289.
- Geary N, Smith GP & Corp ES 1996 The increased satiating potency of CCK-8 by estradiol is not mediated by upregulation of NTS CCK receptors. *Brain Research* **719** 179–186.
- Geary N, Asarian L, Korach KS, Pfaff DW & Ogawa S 2001 Deficits in E<sub>2</sub>-dependent control of feeding, weight gain, and cholecystokinin satiation in ER-alpha null mice. *Endocrinology* **142** 4751–4757.
- Ghosh BR, Wu JC & Miller WL 1996 Gonadotropin-releasing hormone-stimulated calcium mobilization is altered in pituitary cultures from anestrus ewes. *Biology of Reproduction* **54** 753–760.
- Gonzalez-Puga C, Garcia-Navarro A, Escames G, Leon J, Lopez-Cantarero M, Ros E & Acuna-Castroviejo D 2005 Selective CCK-A but not CCK-B receptor antagonists inhibit HT-29 cell proliferation: synergism with pharmacological levels of melatonin. *Journal of Pineal Research* **39** 243–250.
- Hewitt SC & Korach KS 2003 Oestrogen receptor knockout mice: roles for oestrogen receptors alpha and beta in reproductive tissues. *Reproduction* **125** 143–149.
- Hoeger KM, Kolp LA, Strobl FJ & Veldhuis JD 1999 Evaluation of LH secretory dynamics during the rat proestrous LH surge. *American Journal of Physiology* **276** R219–R225.
- Jones ME, Boon WC, Proietto J & Simpson ER 2006 Of mice and men: the evolving phenotype of aromatase deficiency. *Trends in Endocrinology and Metabolism* **17** 55–64.
- Kamilaris TC, Johnson EO, Calogero AE, Kalogeras KT, Bernardini R, Chrousos GP & Gold PW 1992 Cholecystokinin-octapeptide stimulates hypothalamic–pituitary–adrenal function in rats: role of corticotropin-releasing hormone. *Endocrinology* **130** 1764–1774.
- Karlsson S & Ahren B 1992 CCK-8-stimulated insulin secretion *in vivo* is mediated by CCKA receptors. *European Journal of Pharmacology* **213** 145–146.
- Kim HJ, Hwang IT, Lee HK, Yoo YB, Lee SK, Hwang DH & Lee BL 2000 Reconstituted basement membrane induces glandular-like morphogenesis but no difference in ACTH synthesis of anterior pituitary cells. *Endocrine Journal* **47** 771–776.
- Kim HJ, Sohn HJ, Ha M, Han JY, Kang SS, Choi WS & Cho GJ 2005 Prepubertal chronic ethanol administration alters TTF-1 and Oct-2 expression in the hypothalamus of female rats. *Brain Research. Molecular Brain Research* **136** 262–266.
- Kirkpatrick BL, Esquivel E, Gentry PC, Moss GE, Wise ME & Hamernik DL 1998 Regulation of amounts of mRNA for GnRH receptors by estradiol and progesterone in sheep. *Endocrine* **8** 93–99.
- Kopin AS, Mathes WF, McBride EW, Nguyen M, Al-Haider W, Schmitz F, Bonner-Weir S, Kanarek R & Beinborn M 1999 The cholecystokinin-A receptor mediates inhibition of food intake yet is not essential for the maintenance of body weight. *Journal of Clinical Investigation* **103** 383–391.
- Lindzey J, Jayes FL, Yates MM, Couse JF & Korach KS 2006 The bi-modal effects of estradiol on gonadotropin synthesis and secretion in female mice are dependent on estrogen receptor-alpha. *Journal of Endocrinology* **191** 309–317.
- Lloyd KC, Raybould HE & Walsh JH 1992 Cholecystokinin inhibits gastric acid secretion through type 'A' cholecystokinin receptors and somatostatin in rats. *American Journal of Physiology* **263** G287–G292.
- Mannisto PT, Peuranen E, Harro J & Vasar E 1992 Possible role of cholecystokinin-A receptors in regulation of thyrotropin (TSH) secretion in male rats. *Neuropeptides* **23** 251–258.
- Meyer BM, Werth BA, Beglinger C, Hildebrand P, Jansen JB, Zach D, Rovati LC & Stalder GA 1989 Role of cholecystokinin in regulation of gastrointestinal motor functions. *Lancet* **2** 12–15.
- Mitchner NA, Garlick C & Ben-Jonathan N 1998 Cellular distribution and gene regulation of estrogen receptors alpha and beta in the rat pituitary gland. *Endocrinology* **139** 3976–3983.
- Naik SI, Young LS, Charlton HM & Clayton RN 1985 Evidence for a pituitary site of gonadal steroid stimulation of GnRH receptors in female mice. *Journal of Reproduction and Fertility* **74** 615–624.
- Peuranen E, Vasar E, Koks S, Volke V, Lang A, Rauhala P & Mannisto PT 1995 Further studies on the role of cholecystokinin-A and B receptors in secretion of anterior pituitary hormones in male rats. *Neuropeptides* **28** 1–11.
- Powers CA 1986 Anterior pituitary glandular kallikrein: trypsin activation and estrogen regulation. *Molecular and Cellular Endocrinology* **46** 163–174.

- Qian JM, Rowley WH & Jensen RT 1993 Gastrin and CCK activate phospholipase C and stimulate pepsinogen release by interacting with two distinct receptors. *American Journal of Physiology* **264** G718–G727.
- Rispoli LA & Nett TM 2005 Pituitary gonadotropin-releasing hormone (GnRH) receptor: structure, distribution and regulation of expression. *Animal Reproduction Science* **88** 57–74.
- Rossetti L, Shulman GI & Zawalich WS 1987 Physiological role of cholecystokinin in meal-induced insulin secretion in conscious rats. Studies with L 364718, a specific inhibitor of CCK-receptor binding. *Diabetes* **36** 1212–1215.
- Sanchez-Criado JE, Martin De Las Mulas J, Bellido C, Tena-Sempere M, Aguilar R & Blanco A 2004 Biological role of pituitary estrogen receptors ERalpha and ERbeta on progesterone receptor expression and action and on gonadotropin and prolactin secretion in the rat. *Neuroendocrinology* **79** 247–258.
- Sanchez-Criado JE, de Las Mulas JM, Bellido C, Aguilar R & Garrido-Gracia JC 2005 Gonadotrope oestrogen receptor-alpha and -beta and progesterone receptor immunoreactivity after ovariectomy and exposure to oestradiol benzoate, tamoxifen or raloxifene in the rat: correlation with LH secretion. *Journal of Endocrinology* **184** 59–68.
- Sankaran H, Goldfine ID, Deveney CW, Wong KY & Williams JA 1980 Binding of cholecystokinin to high affinity receptors on isolated rat pancreatic acini. *Journal of Biological Chemistry* **255** 1849–1853.
- Sapino A, Pietribiasi F, Bussolati G & Marchisio PC 1986 Estrogen- and tamoxifen-induced rearrangement of cytoskeletal and adhesion structures in breast cancer MCF-7 cells. *Cancer Research* **46** 2526–2531.
- Schafer C, Clapp P, Welsh MJ, Benndorf R & Williams JA 1999 HSP27 expression regulates CCK-induced changes of the actin cytoskeleton in CHO-CCK-A cells. *American Journal of Physiology* **277** C1032–C1043.
- Shacham S, Harris D, Ben-Shlomo H, Cohen I, Bonfil D, Przeddecki F, Levy H, Ashkenazi IE, Seger R & Naor Z 2001 Mechanism of GnRH receptor signaling on gonadotropin release and gene expression in pituitary gonadotrophs. *Vitamins and Hormones* **63** 63–90.
- Simpson E, Jones M, Misso M, Hewitt K, Hill R, Maffei L, Carani C & Boon WC 2005 Estrogen, a fundamental player in energy homeostasis. *Journal of Steroid Biochemistry and Molecular Biology* **95** 3–8.
- Skirboll LR, Crawley JN & Hommer DW 1986 Functional studies of cholecystokinin-dopamine co-existence: electrophysiology and behavior. *Progress in Brain Research* **68** 357–370.
- Smith MS, Freeman ME & Neill JD 1975 The control of progesterone secretion during the estrous cycle and early pseudopregnancy in the rat: prolactin, gonadotropin and steroid levels associated with rescue of the corpus luteum of pseudopregnancy. *Endocrinology* **96** 219–226.
- Thomas SG & Clarke IJ 1997 The positive feedback action of estrogen mobilizes LH-containing, but not FSH-containing secretory granules in ovine gonadotropes. *Endocrinology* **138** 1347–1350.
- Thorsell A & Heilig M 2002 Diverse functions of neuropeptide Y revealed using genetically modified animals. *Neuropeptides* **36** 182–193.
- Tirassa P, Stenfors C, Lundeborg T & Aloe L 1998 Cholecystokinin-8 regulation of NGF concentrations in adult mouse brain through a mechanism involving CCK(A) and CCK(B) receptors. *British Journal of Pharmacology* **123** 1230–1236.
- Turgeon JL & Waring DW 2001 Luteinizing hormone secretion from wild-type and progesterone receptor knockout mouse anterior pituitary cells. *Endocrinology* **142** 3108–3115.
- Vijayan E & McCann SM 1986 The effects of the cholecystokinin antagonist, proglumide, on gonadotropin release in the rat. *Brain Research Bulletin* **16** 533–536.
- Vijayan E & McCann SM 1987 The effects of the cholecystokinin antagonist, proglumide, on prolactin secretion in the rat. *Life Sciences* **40** 629–634.
- Vijayan E, Samson WK & McCann SM 1979 *In vivo* and *in vitro* effects of cholecystokinin on gonadotropin, prolactin, growth hormone and thyrotropin release in the rat. *Brain Research* **172** 295–302.
- Wank SA 1995 Cholecystokinin receptors. *American Journal of Physiology* **269** G628–G646.
- Williams JA 2001 Intracellular signaling mechanisms activated by cholecystokinin-regulating synthesis and secretion of digestive enzymes in pancreatic acinar cells. *Annual Review of Physiology* **63** 77–97.
- Williams JA, Sans MD, Tashiro M, Schafer C, Bragado MJ & Dabrowski A 2002 Cholecystokinin activates a variety of intracellular signal transduction mechanisms in rodent pancreatic acinar cells. *Pharmacology and Toxicology* **91** 297–303.
- Xu M, Hill JW & Levine JE 2000 Attenuation of luteinizing hormone surges in neuropeptide Y knockout mice. *Neuroendocrinology* **72** 263–271.
- Yule DI & Williams JA 1994 CCK antagonists reveal that CCK-8 and JMV-180 interact with different sites on the rat pancreatic acinar cell CCKA receptor. *Peptides* **15** 1045–1051.

Received in final form 5 September 2007

Accepted 20 September 2007

Made available online as an Accepted Preprint

20 September 2007