Estrogen receptor α-induced cholecystokinin type A receptor expression in the female mouse pituitary

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Abstract

Estrogen plays a critical role in inducing LH surge. In the pituitary, estrogen receptor α (ERα) mediates the action of estrogen, while the downstream pathway of ERα activation is yet to be elucidated. Here, we report the finding that cholecystokinin type A receptor (CCK-AR) is an ERα 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 ERα gene completely abolish CCK-AR mRNA expression. Injection of 17β-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 ERα 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 ERα downstream gene in the pituitary and suggests that CCK-AR may play a role in the estrogen sensitization of the pituitary response to GnRH.

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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 α and/or β subtypes of estrogen receptors (ERs) in a tissue type-dependent manner. While both ERα and ERβ are present in the pituitary, ERα has been shown to be the effector of estrogen action in the pituitary (Sanchez-Criado *et al.* 2004, 2005). In support of this finding, ERα knockout (ERαKO) female mice are completely infertile and do not ovulate, while ERβKO mice are fertile (Dupont *et al.* 2000, Hewitt & Korach 2003). However, the downstream pathway of ERα activation in the pituitary gonadotroph is not yet known.

As a nuclear receptor transcription factor, ERα 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 ERα 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 ERα 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

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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 & Makhlouf 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 adrenocorticotropic hormone, β-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 ERα 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, 17β-estradiol (E_2), 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 μg/ml, transferin 5·5 μg/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 μg E_2 or 100 μl 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 ERαKO mice

The generation of ERαKO ($ER\alpha^{-/-}$) mice resulted from a cross of male $ER\alpha^{flox/flox}$ with female $Zp3^{cre}$, a line expressing Cre recombinase specifically in the oocyte. $ER\alpha^{flox/flox}$ mice possess two loxP sites flanking exon 3 of the ERα gene (Dupont *et al.* 2000). The resulting F1 heterozygote $ER\alpha^{flox/+}$ $Zp3^{cre}$ was then bred with $ER\alpha^{flox/flox}$ to produce $ER\alpha^{flox/flox}$ $Zp3^{cre}$. Female $ER\alpha^{flox/flox}$ $Zp3^{cre}$ mice produce $ER\alpha^{-}$ oocytes due to the deletion of floxed exon in the oocyte. Thus, oocytes fertilized by sperm from $ER\alpha^{flox/flox}$ males result in progeny that are $ER\alpha^{flox/-}$. The breeding of female $ER\alpha^{flox/flox}$ $Zp3^{cre}$ with male $ER\alpha^{flox/-}$ 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 ERαP1 (5′-ttg ccc gat aac aat aac at-3′) and ERαP3 (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 tcg 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_2. 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 (1×10^5 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_2 in 0·00001% ethanol, or 10 nM E_2 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_2 (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^2) 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_2 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 tcg 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_2 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_2 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_2 via ERα in the pituitary

To determine whether estrogen induced CCK-AR expression in the pituitary, the effect of E_2 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_2 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_2-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_2 injection. Similar to the mRNA expression pattern, western blot and immunofluorescent analyses showed an increase in CCK-AR protein expression in the E_2-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 ERα was involved in regulating CCK-AR expression. For this purpose, the pituitaries of naturally cycling mice, OVX WT mice treated either with E_2 or sesame oil, and ERαKO mice treated either with E_2 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/ERα 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_2-treated OVX

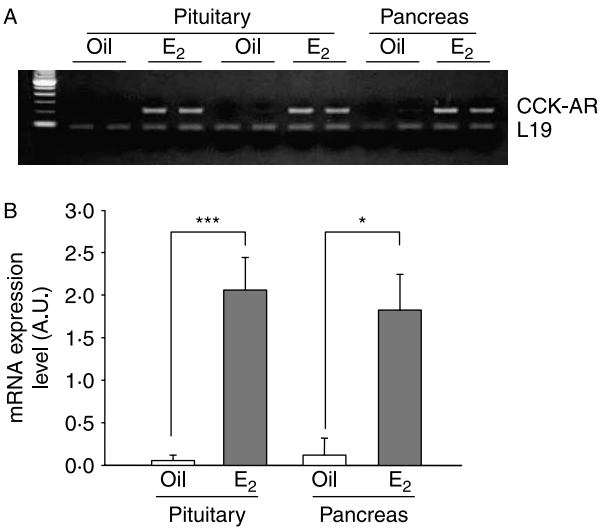

Figure 1 The expression of CCK-AR mRNA in the pituitary and pancreas from OVX wild-type (WT) and vehicle (oil)- or E_2-treated female mice. (A) Representative ethidium bromide-stained gel image of a semiquantitative RT-PCR result. (B) Quantitation of the RT-PCR results. CCK-AR mRNA expression was increased dramatically both in the pituitary and pancreas of OVX E_2-treated mice. The mice were ovariectomized 3 weeks prior to E_2 injection (10 μg s.c., at 0900 h for 2 days). Tissues were collected at 1500 h on the second day of E_2 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_2 concentration) compared with metestrus (basal serum E_2 concentration; Fig. 3A). However, no CCK-AR mRNA expression was detectable in the ERαKO mouse pituitary regardless of E_2 treatment, suggesting ERα-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 proestrous than metestrous pituitary (Fig. 4A and B). Furthermore, consistent with the E_2- and ERα-dependent mRNA expression, neither ERαKO 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

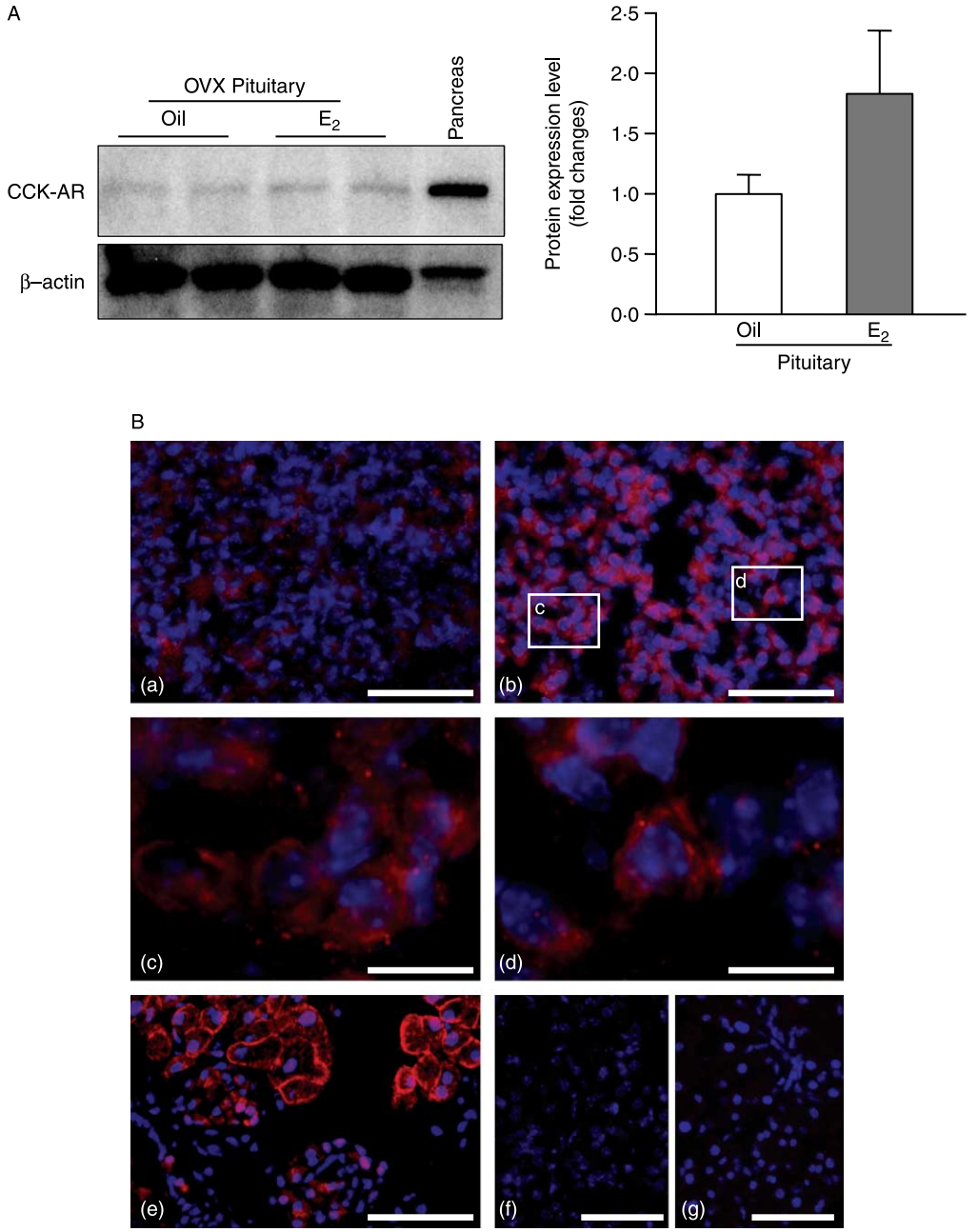

Figure 2 Western blot and immunofluorescent analyses of CCK-AR protein expression. (A) Western blot analysis of CCK-AR using proteins from OVX pituitaries treated with oil or E_2. Pancreas was used to determine the specificity of the CCK-AR antibody. Note the increased CCK-AR protein in the E_2-treated 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_2-treated (b–d) pituitaries. Both cell numbers and relative intensity of CCK-AR ISs were increased in the OVX E_2-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 was 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_2 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).

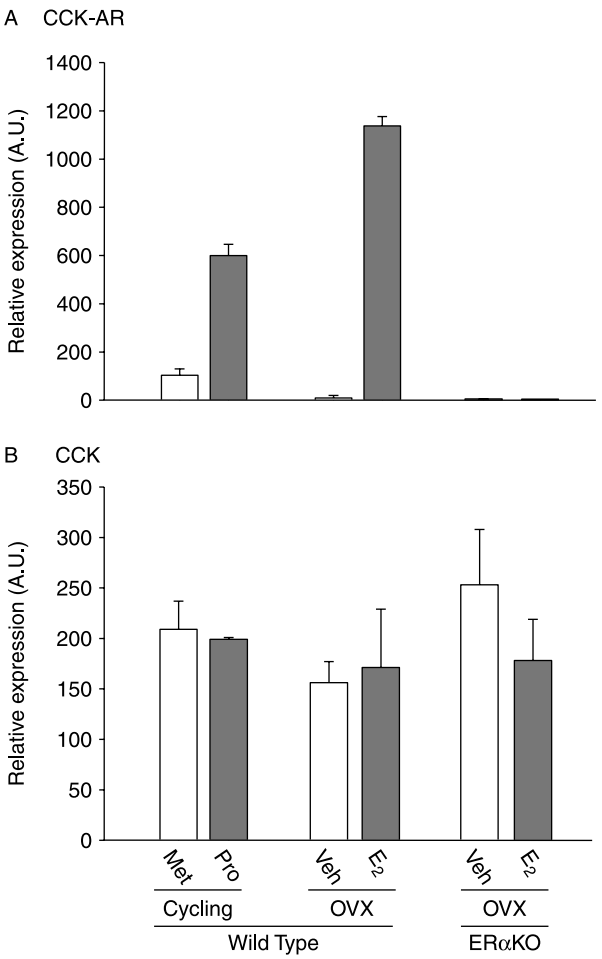

Figure 3 E_2- and ERα-dependent expression of CCK-AR. CCK-AR (A) and CCK (B) mRNA expression levels were measured by DNA microarray as described in the Materials and Methods section. Note that while CCK-AR expression is dependent on cycling stages, E_2 treatment, and ERα, no such change was seen in the CCK expression. Data presented are mean + 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_2-primed gonadotrophs to continued GnRH stimuli

When treated with E_2, 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_2-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_2 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 μM). Concentrations of LH in the culture media were then determined (Fig. 7A). E_2-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_2-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_2-primed primary cells were cultured in the presence or absence of lorglumide (10 μM) 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_2-induced sensitization of pituitary in GnRH-induced LH secretion.

Discussion

This study revealed that estrogen regulates CCK-AR expression in the pituitary via ERα 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

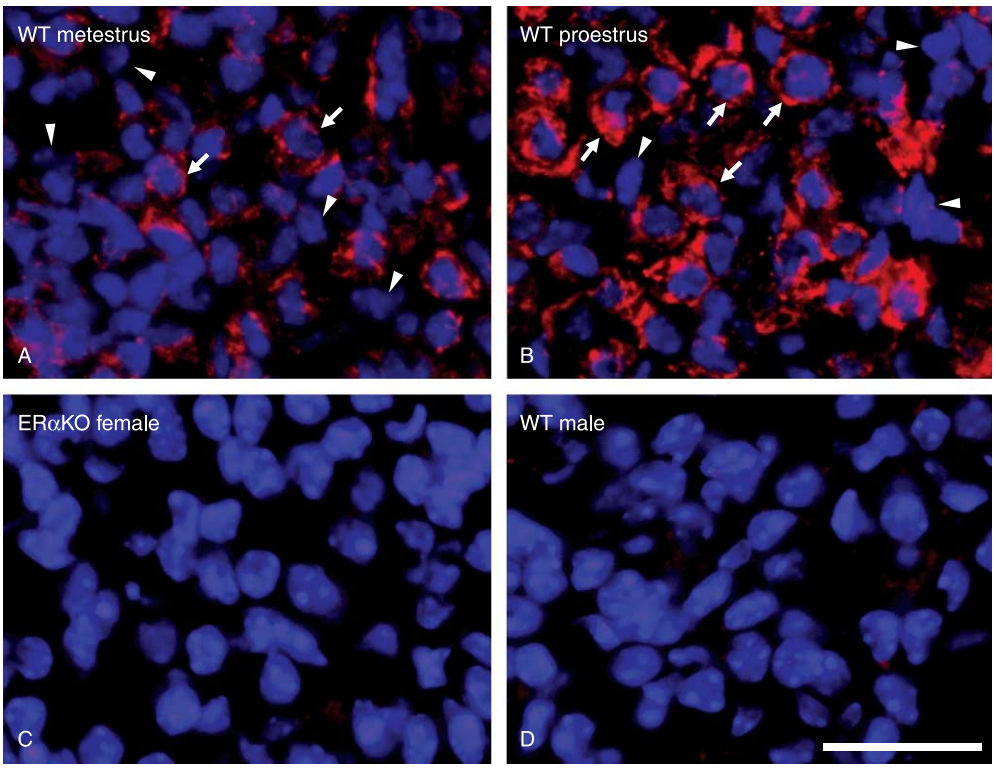

Figure 4 Immunofluorescent analyses of the CCK-AR protein expression. Pituitaries of WT metestrus (A), WT proestrus (B), ERαKO female (C), and WT male mouse (D) were stained with anti-CCK-AR antibody. Note that both CCK-AR immunopositive (arrow) and immunonegative (arrow head) cells are present regardless of the stages of estrus cycle, while no detectable CCK-AR signal was seen in pituitary of either ERαKO 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 μm.

expression in the anterior pituitary. First, E_2 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_2 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_2 benzoate increased CCK-AR in the OVX rat pancreas (Geary *et al*. 1996) and that E_2 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_2 sensitive (Asarian & Geary 2006). Thus, it will be interesting to see whether E_2 regulates CCK-AR expression in those tissues that are involved in satiety/eating control.

It is well known that ERα 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 ERα mediates E_2 action in inducing CCK-AR expression in the pituitary. First, no CCK-AR expression was detectable in the ERαKO mouse pituitary regardless of E_2 treatment (Figs 3A and 4C). Secondly, CCK-AR expression was readily detectable in the major pituitary hormone secreting cell types that express ERα including corticotroph, somatotroph, lactotroph, thyrotroph, and gonadotroph (Mitchner *et al*. 1998; Fig. 5). While it is not known whether ERα 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 ERα 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

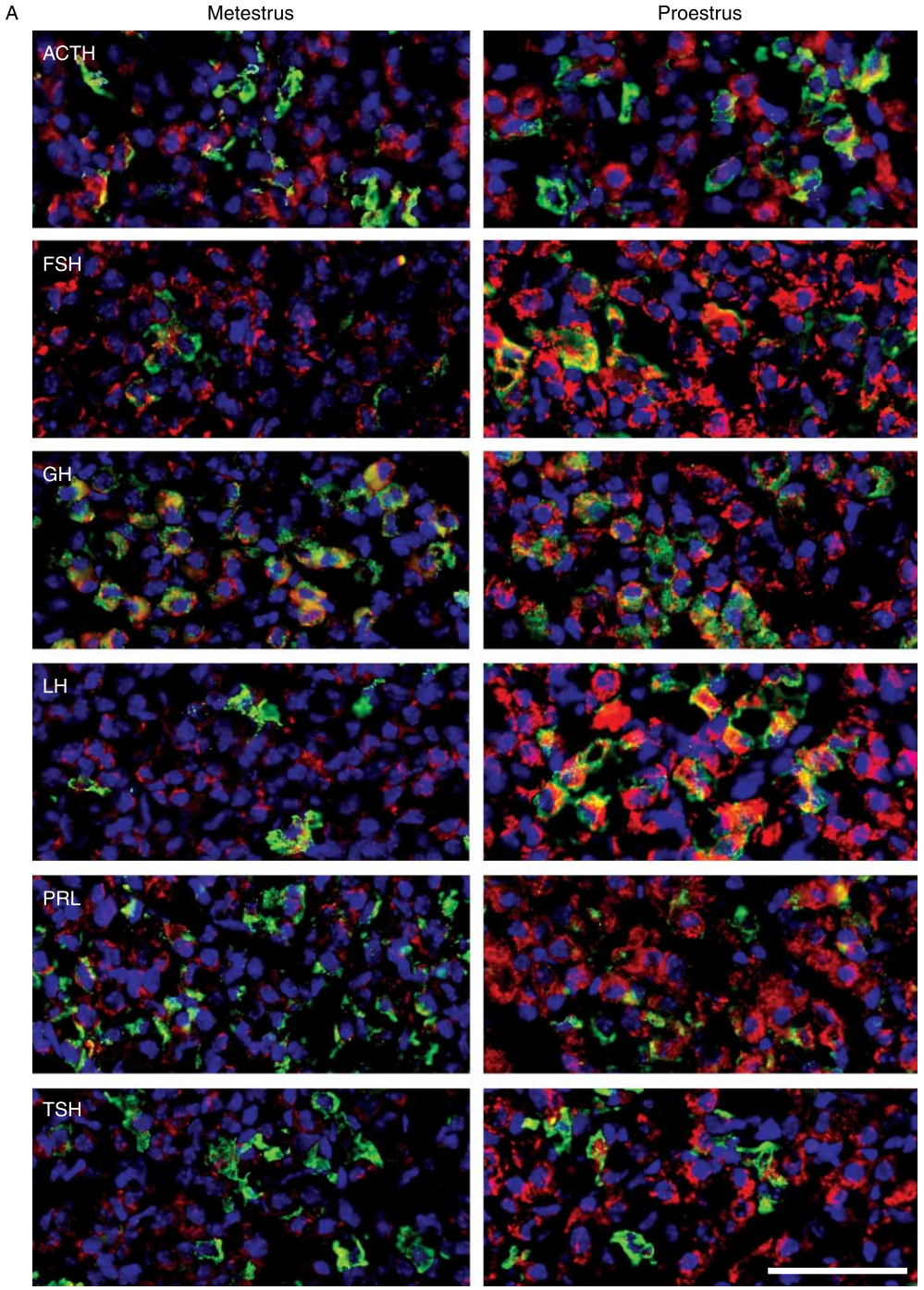

Figure 5 (*continued*)

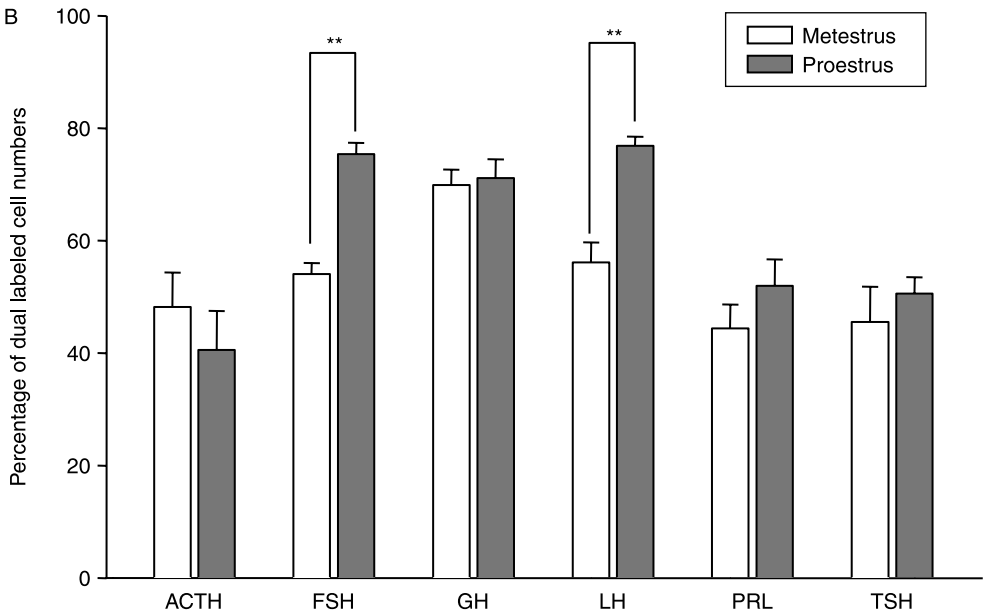

Figure 5 Colocalization of CCK-AR and pituitary hormones. (A) Representative images of double immunofluorescent staining using antibodies for CCK-AR and pituitary peptide hormones (ACTH, FSH, GH, LH, PRL, and TSH). Left panels were from metestrous, and right panels were from proestrous pituitaries. Red 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 μm. (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. ($n=4$); $^{**}P<0{\cdot}001$.

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 Dieten & de Koning 1995, Hoeger *et al*. 1999). In this regard, it is speculated that, at least in part, estrogen via a cohort of ERα 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/ERα-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_2, 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_2-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 ERα downstream gene.

Upon binding to CCK, CCK-AR increases the intracellular Ca^{2+} 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

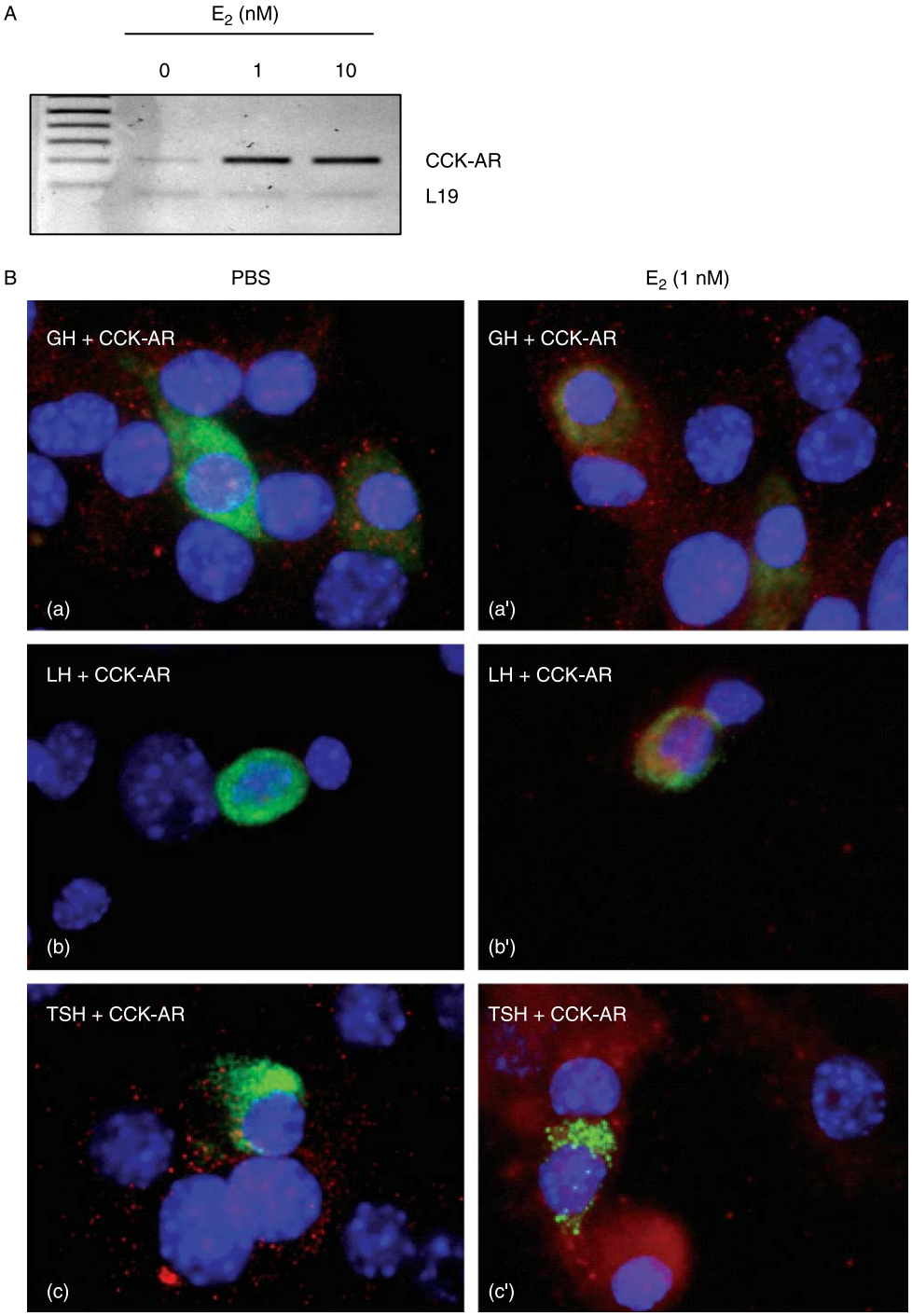

Figure 6 The CCK-AR mRNA and protein expression in primary anterior pituitary cell culture. (A) Primary pituitary cells were treated with vehicle or E_2 (1 or 10 nM) for 2 days, and the CCK-AR mRNA expression level was measured by semiquantitative RT-PCR. (B) Colocalization of CCK-AR and pituitary hormones (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_2 (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.

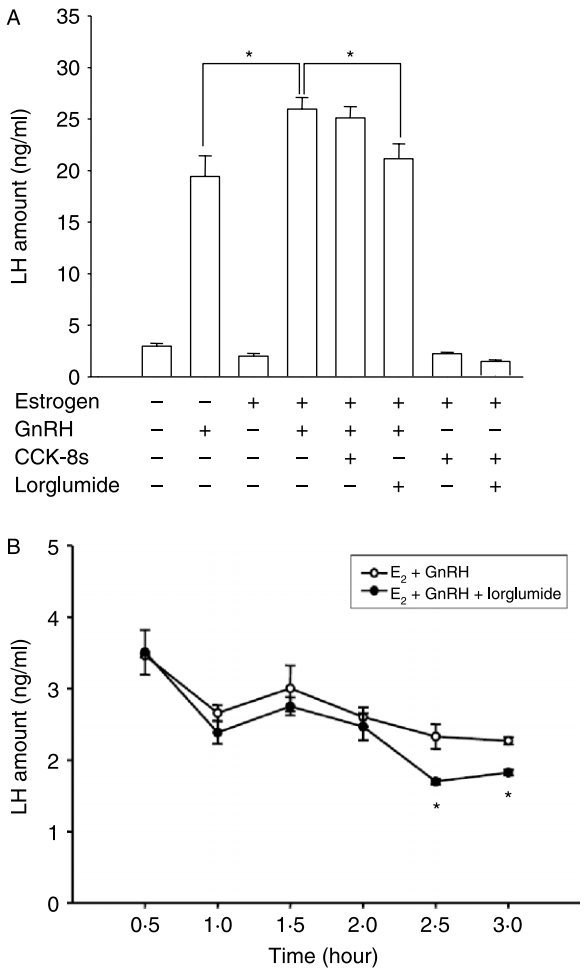

Figure 7 The effect of CCK-AR-specific agonist (CCK-8s) and antagonist (lorglumide) on LH secretion in primary pituitary cells. (A) The primary cells isolated from anterior pituitary were treated with either vehicle (0·00001% ethanol) or E_2 (1 nM) for 2 days before GnRH (10 nM) administration. CCK-8s (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_2 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_2 and EE+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_2 inducible, ERα mediated, and estrous stage dependent, and suggests that CCK-AR might be at least n 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.

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