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Development, validation, and utilization of a novel antibody specific to the type III chicken gonadotropin-releasing hormone receptor

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Abstract

Two gonadotropin-releasing hormone receptors (GnRH-Rs) have been characterized in chickens to date: cGnRH-R-I and cGnRH-R-III, with cGnRH-R-III being the predominant pituitary form. The purpose of the present study was to first validate a novel antibody for the specific detection of cGnRH-R-III and second, using this antibody, detect changes in cGnRH-R-III protein levels in the pituitary gland of male and female chickens during a reproductive cycle. The localization of cGnRH-R-III within the anterior pituitary gland was also determined. Western blotting of pituitary extracts and transiently transfected COS-7 cell lysates revealed that our antibody is highly specific to cGnRH-R-III protein. Similarly, when used in immunocytochemistry, this antibody specifically detects cells expressing cGnRH-R-III and not cGnRH-R-I. Western blot analyses of chicken pituitary gland homogenates show that cGnRH-R-III protein levels are significantly greater in sexually mature birds than in immature birds or birds at the end of a reproductive cycle ($P < 0.0001$). A similar pattern was observed for both males and females. Additionally, the antibody was able to detect cGnRH-R-III in cells along the periphery of the cephalic and caudal lobes of the anterior pituitary where the cells containing the gonadotropins are located. In summary, we successfully validated a novel antibody to cGnRH-R-III and showed levels of cGnRH-R-III protein in the pituitary fluctuate with respect to the reproductive status in both male and female chickens.

Keywords: GnRH; Receptor; Chicken; Reproduction; Pituitary

1. Introduction

In avian species, reproduction is under the control of the hypothalamopituitary-gonadal axis. Light perceived by photoreceptors within the hypothalamus is transduced into nervous impulses that initiate the synthesis and release of gonadotropin-releasing hormone (GnRH). GnRH is then delivered to the anterior pituitary gland, where it stimulates the synthesis and release of the gonadotropins: luteinizing hormone (LH) and follicle-stimulating hormone (FSH) [1,2]. In turn, LH and FSH stimulate the growth and development of the gonads as well as the production of androgens, progesterone, and estrogen [3–6].

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Two variants of chicken GnRH (cGnRH) have been characterized and classified to date. cGnRH-I is a hypothalamus-specific variant [7,8] found in the preoptic and septal areas and in the periventricular nucleus [9]. Gonadotropin-releasing hormone II, a highly conserved variant across vertebrate species [10], is found not only in the hypothalamus but also in the mesencephalon [9]. Because cGnRH-I fibers project to the median eminence [9] and immunization of laying hens against cGnRH-I and not GnRH-II results in a decrease in plasma LH and regression of the reproductive system [1], cGnRH-I has been proposed as the peptide controlling gonadotrope function in chickens, whereas GnRH-II may primarily be involved in the control of behavior [11].

Similarly, two GnRH receptor isoforms (cGnRH-Rs) have been identified in chickens. The first receptor isoform characterized (cGnRH-R-I) was expressed in reproductive (testes) as well as non-reproductive tissues (spleen and heart) and throughout the brain and the pituitary gland [12]. More recently, we cloned a second receptor, cGNRH-R2, which is predominantly expressed in the pituitary [13,14]. Using bioinformatic and phylogenic analyses, we further classified this cGNRH-R2 as a type III receptor homolog [14], and we will thus refer to it as cGnRH-R-III from now on.

Expression of this novel receptor in the anterior pituitary was much more abundant than that of cGnRH-R-I [14]. Furthermore, levels of cGnRH-R-III messenger ribonucleic acid (mRNA) change with respect to reproductive status [13,14]. In laying hens (white Leghorn), mRNA levels significantly increase from immature to peak of lay and then decrease toward the end of lay [13]. Similarly, in broiler breeder hens, cGnRH-R-III mRNA was higher in sexually mature birds compared with juveniles [14]. In white Leghorn males, cGnRH-R-III mRNA levels also significantly increase following photostimulation and decrease afterward [13]. Taken together, our studies suggest that in chickens, type III GnRH-R is the physiologically relevant form controlling the reproductive axis. However, to date no antibody specific to cGnRH-Rs exists and most data available rely on qualitative and quantitative analyses of mRNA levels rather than protein [13,14]. To overcome this limitation, the present study describes the generation, validation, and use of a specific polyclonal antibody for the detection of cGnRH-R-III protein by western blot and immunocytochemistry.

2. Materials and methods

2.1. Generation of the antibody specific to cGnRH-R-III

Antibodies against cGnRH-R-III were developed by Affinity BioReagents at their facility using the BioPerformancePlus protocol (Affinity BioReagents, Golden, CO, USA). In brief, rabbits were co-injected with two peptides corresponding to regions of the cGnRH-R-III N-terminal extracellular domain sequence deduced from the complementary deoxyribonucleic acid (cDNA) sequence described by Shimizu and Bédécarrats [13] (peptide 1, position 1 to 11: Met-Ala-Arg-Leu-Gly-Gly-Gly-Thr-Gly-Gln-Asp; peptide 2, position 47 to 58: Ser-Pro-Arg-Leu-Glu-Ser-Ala-Glu-Glu-Pro-Leu-Leu). These peptides were chosen because they show little homology with cGnRH-R-I and other GnRHR types (Fig. 1). Immunization was performed according to the BioPerformancePlus protocol and after serum collection specific immunoglobulins to each peptide were purified separately by affinity chromatography with the peptide used as an antigen.

2.2. Validation of the antibody specific to cGnRH-R-III

2.2.1. Dot-blot analysis

Dot-blot analysis was performed to determine the affinity of immunoglobulins against each peptide used for immunization. Increasing concentrations (0 ng, 0.2 ng, 2 ng, 20 ng, 200 ng, and 2 μg) of the 2 peptides were dotted onto nitrocellulose membranes, which were then incubated with specific immunoglobulins to either peptide at a concentration of 0.2, 2, or 20 μg/mL in blocking buffer (1% bovine serum albumin (BSA),

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                Peptide 1                                          Peptide 2
Chicken III : MARLGGGTGQDAAAAGGGWLDPGPTVGNVSTEPSSSTSHPKRGCAWSPRLESAEEPLLLPTFS
Bullfrog I  : MNISKEVSIKGCNNAQ--WLSSSCDLD---VNMTSTNG--------------THTHFQLPTFS
Chicken I   : MCVPAALIEAEPPHHP---------TTEGDTNTSATH---------------CLEHWVEPRFT
Bullfrog II : MAMQLAIVNQSHLVVP---------DNA----VSSLG---------------FPGPWSEPTFT
Human I     : MANSASPEQNQNHCSA--------------INNSIP----------------LMQGNLPTLT
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Fig. 1. Sequence alignment of the selected immunogen segments with other gonadotropin-releasing hormone receptors (GnRH-Rs). The N-terminal extracellular domain sequence of various GnRH-R subtypes (type III, chicken III and bullfrog I; type I, chicken I, bullfrog II, and human I) were aligned according to Joseph et al [14]. The sequences of peptide 1 and peptide 2 used as immunogens are shown as underlined in the chicken GnRH-R-III sequence.

1% nonfat dry milk, and 0.1% Tween 20 in Tris-buffered saline (TBS; 0.01 M Tris-HCl and 0.15 M NaCl solution)). Membranes were washed 3 times with 0.1% Tween 20 in TBS (0.1% TBS-T) and then incubated in horseradish peroxidise–conjugated goat anti-rabbit secondary antibody (Jackson ImmunoResearch Laboratories, Inc, West Grove, PA, USA) at a dilution of 1:20,000. Membranes were washed 3 times in 0.1% TBS-T and once in TBS, and immunocomplexes were then detected and visualized using the Pierce enhanced chemiluminescence western blotting substrate according to the manufacturer's instructions (Pierce, Thermo Fisher Scientific, Ottawa, ON, Canada), followed by exposure to Pierce CL-XPosure radiograph films.

2.2.2. Peptide-competition assay

To identify the location of the bands specific to cGnRH-R-III on western blots and to discriminate between specific and nonspecific signals, a peptide competition assay was performed 7 times. A pituitary (positive control) and heart (negative control) were harvested from a 34-wk-old Plymouth Barred Rock rooster and homogenized by sonication using an ultrasonic probe and stored in RIPA buffer (1% Nonidet P-40 (NP-40), 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), and 0.02% sodium azide in phosphate-buffered saline (PBS)) containing protease inhibitors (phenylmethysulfonyl fluoride, aprotinin, and sodium orthovanadate). Protein concentration in homogenates was determined by Coomassie (Bradford) protein assay kit (Pierce Biotechnology, Thermo Fisher Scientific) and 2.5 μg proteins of pituitary and 10 μg proteins of heart lysates were diluted in 4 times concentrated Laemmli loading buffer containing β-mercaptoethanol and denatured at 100 °C for 5 min. Samples were then immediately loaded into a 10% SDS-polyacrylamide gel (SDS-PAGE). After electrophoresis, proteins were transferred onto an immobilon polyvinylidene difluoride membrane (Millipore Corporation, Billerica, MA, USA). The membrane was then incubated overnight at 4 °C in blocking buffer (1% BSA, 1% nonfat dry milk, 0.2% Tween 20 in TBS). Simultaneously, anti-cGnRH-R-III immunoglobulins against peptide 1 (2 μg/mL) were preincubated in blocking solution at 4 °C overnight with serial amounts of peptide 1 (0, 5, or 10 μg). Membranes were then incubated for 4 h at room temperature with the cGnRH-R-III immunoglobulin/peptide 1 solution, washed 3 times with 0.2% TBS-T (0.2% Tween 20 in TBS), and incubated with the secondary antibody (horseradish peroxidise–conjugated goat anti-rabbit; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) for 60 min at a 1:40,000 dilution. Finally, membranes were washed 3 times with 0.2% TBS-T and 2 times with TBS and bands were visualized using the Pierce Supersignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific) according to the manufacturer's instructions, followed by exposure to Pierce CL-XPosure radiograph films (Thermo Fisher Scientific). Images were scanned and bands were analyzed using ImageQuant TL Software (Amersham Biosciences, GE Healthcare, Baie d'Urfe, QC, Canada).

2.2.3. Detection of cGnRH-R-III in cell lysates

Chicken GnRH-R subtypes (type I and type III) were transiently transfected in COS-7 cells as outlined previously [14]. After a 24-h incubation period, the medium was removed and replaced with a serum-free medium for 16 h prior to cell lysate preparation. The incubation was terminated by placing the cells on ice, aspirating off the medium, and washing the cells once with 4 °C PBS + Ca^{2+} + Mg^{2+}. Cell lysis was performed with 200 μl per culture dish of a NP-40-based solubilization buffer (250 mM NaCl, 5 mM HEPES, 10% glycerol, 0.5% NP-40, 2 mM ethylenediaminetetraacetic acid (EDTA; pH 8) supplemented with 1 mM sodium orthovanadate, 1 mM phenylmethysulfonyl fluoride, and 1 mg/mL leupeptin) at 4 °C. After dishes were scraped, cell lysates of each transfected receptor construct were pooled and centrifuged at 14,000 rpm for 10 min at 4 °C. Lysates were then sonicated and subjected to another centrifugation at 14,000 rpm for 10 min at 4 °C. One hundred microliters of the supernatant was recovered and mixed with an equal volume of 2× Laemmli sample buffer containing β-mercaptoethanol. A 20-μl aliquot of each sample was then denatured at 100 °C for 3 min and loaded onto a 4% to 20% gradient SDS Tris-glycine PAGE (Invitrogen, Carlsbad, CA, USA). After electrophoresis, proteins were transferred onto an Immobilon-FL polyvinylidene difluoride membrane (Millipore Corporation) and blocked for 60 min in blocking buffer (50% Odyssey blocking buffer, 50% PBS). Membranes were then incubated with anti-cGnRH-R-III immunoglobulins against peptide 1 (2 μg/mL diluted in blocking buffer, 0.01% sodium azide, and 0.05% Tween 20) for 60 min, washed 3 times in PBS-T, and incubated for 60 min with secondary antibody diluted at 1:10,000 (in blocking solution + 0.05% Tween 20 and 0.01% SDS). Finally, the membranes were washed 3 times in PBS-T and once in PBS prior

to immunocomplexes being visualized on a Licor gel scanner. This experiment was repeated 3 times.

2.2.4. Immunocytochemistry

To determine whether the antibody is able to specifically recognize the cGnRH-R-III in cells, immunocytochemistry was performed on COS-7 cells transiently transfected with cDNA encoding either the cGnRH-R-I or the cGnRH-R-III previously cloned into pcDNA3.1 expression vector [13]. The immunocytochemistry procedure was performed 3 times, each in duplicate. Cells plated at 40% confluency in Lab-Tek 8-well chamber slides (0.8 cm^2/well culture area; Nunc, Thermo Fisher Scientific) were transfected with 1 μg/well of cGnRH-R-III, cGnRH-R-I, or empty pcDNA3.1 expression vectors. In addition, 1 chamber of COS-7 cells was left untransfected. The transfection efficiency of COS-7 cells was consistently about 50%. Twenty-nine h post-transfection, cells were fixed with 4% paraformaldehyde in HyClone Dulbecco's PBS (DPBS, Thermo Fisher Scientific) for 60 min, washed twice in DPBS, and blocked at 4 °C overnight in 5% BSA, 0.1% Tween 20 in DPBS (blocking solution). Cells were then incubated with 16.8 μg of immunoglobulins against peptide 1 in 200 μl blocking solution per well for 2 h at room temperature and washed twice in blocking solution. FITC-conjugated anti-rabbit immunoglobulin G (IgG; Sigma, Saint Louis, MO, USA) was applied as a secondary antibody at a concentration of 3.1 μg/mL for 60 min at room temperature in the dark. Cells were washed twice with blocking solution and then treated with 0.5 μg/mL Hoechst bisbenzimide 33258 fluorochrome stain. After 2 additional washes with DPBS, slides were mounted with a fluorescent mounting medium and observed under a Leica DMR fluorescent upright microscope (Leica Microsystems, Bannockburn, IL, USA).

2.3. Analyses of cGnRH-R-III protein levels in pituitaries from chickens at different reproductive stages

2.3.1. Animals and tissue collection

Plymouth Barred Rock chickens were raised at the University of Guelph poultry research station (Arkell, ON, Canada) on an 8-h photoperiod until 17 (males) and 18 (females) wk of age prior to photostimulation by an abrupt transfer to a 14-h photoperiod. For both males and females, pituitary glands were collected at 2 immature stages: 6 wk and 16 wk of age (5 females and 5 males from each age group), 4 wk post-photostimulation (21 wk, 5 males; 22 wk, 5 females), at peak sexual maturity (35 wk of age, 5 females and 5 males), and at the end of a reproductive period (84 wk of age, 5 females and 5 males). After chickens were euthanized by cervical dislocation, pituitaries were harvested, flash frozen in liquid nitrogen, and stored at −80 °C until use. All birds were euthanized in the morning, between 2 and 5 h after lights were turned on. All animal procedures were conducted under the guidelines of the Canadian Council for Animal Care.

2.3.2. Western blotting

Pituitary glands (section 2.3.1) were homogenized individually as described in section 2.2.2 and protein concentration in lysates was determined by Bradford assay (Pierce Biotechnology, Thermo Fisher Scientific). To allow for quantification between blots, a general pool serving as an interblot calibrator was generated by mixing equal amounts of every pituitary homogenate. One microgram of each pituitary, interblot calibrator, or negative tissue (skeletal muscle) was loaded per well. The western blot procedure was performed as described in section 2.2.2 with the exception that membranes were incubated with 2 μg/mL of anti-cGnRH-R-III immunoglobulin solution not precleared with peptide 1. Following detection of cGnRH-R-III, all blots were stripped by incubation in Pierce stripping buffer (Pierce Biotechnology, Thermo Fisher Scientific) for 15 min and reprobed with a glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mouse monoclonal antibody (Invitrogen) at a concentration of 1 μg/mL for normalization within blots. As described in section 2.2.2, images were scanned and bands were analyzed using ImageQuant TL Software (Amersham Biosciences, GE Healthcare). To quantify the relative amount of the immunoreactive bands within a blot, the densitometric values for cGnRH-R-III protein in individual samples were divided by corresponding GAPDH protein values. To compare samples across multiple blots, individual cGnRH-R-III values corrected for GAPDH protein were normalized by the pooled pituitary sample value from that blot.

The effects of age or sex on the level of cGnRH-R-III protein were analyzed by analysis of variance (ANOVA) using the general linear model procedure of Statistical Analysis Software (SAS 9.2, SAS Institute, Inc., Cary, NC, USA). Tukey's Student range test was used as a post hoc test to analyze differences in cGnRH-R-III between each age group. $P < 0.05$ was considered statistically significant.

2.3.3. In situ detection of cGnRH-R-III by immunohistochemistry

Chicken GnRH-R-III-immnoreactive (ir) cells in the anterior pituitary gland were detected by immunohis-

tochemistry as described previously with some modifications [15]. Anterior pituitary glands collected from sexually mature female Leghorn chickens (n = 4) were fixed in Bouin's solution, dehydrated, cleared, and embedded in paraffin using an automated tissue processor. Four-micrometer pituitary sections (3 tissue sections/slide; 2 slides/animal) in the sagittal plane were deparaffinized and hydrated in descending concentrations of ethyl alcohol in water and rinsed in TBS (pH 7.4). Endogenous peroxidase activity was quenched in the tissue section by treatment with 3% hydrogen peroxide. Following several washes in TBS containing 0.5% Triton X-100 (Sigma-Aldrich, Saint Louis, MO, USA), slides were treated in a blocking solution (2.5% normal goat serum in TBS containing 0.5% Triton X-100) for 60 min. Tissue sections were then incubated with anti-cGnRH-R-III IgG (1 μg/ml) at 4 °C for approximately 18 h. Following washes in TBS, sections were incubated for 60 min with biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA, USA). Tissue sections were washed in TBS and then treated for 60 min with an avidin peroxidase complex (Vector ABC Elite kit, Vector Laboratories, Burlingame, CA, USA). A brown-colored immunoreaction product was developed using 3,3′-diaminobenzidine tetrahydrochloride and hydrogen peroxide (Vector Laboratories). After several washes in TBS, tissue sections were dehydrated, cleared in Histoclear, and coverslipped. As a negative control, the primary antibody was substituted with rabbit IgG during immunohistochemical staining. Immunoreactive cells in the pituitary gland were examined and photomicrographs taken using an Olympus BX61 microscope fitted with an Olympus DP71 digital camera (Olympus America, Melville, NY, USA).

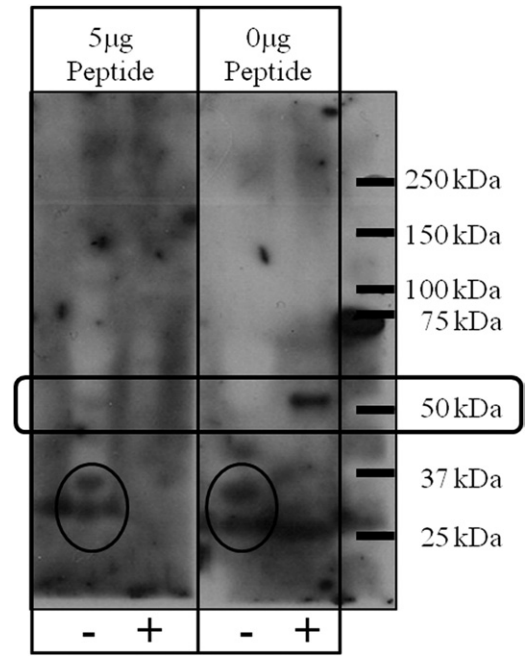

Fig. 2. Western blot of the peptide-competition analysis. Samples from a pituitary gland (positive, +) and heart (negative, −) were loaded on a 10% acrylamide gel and transferred to a polyvinylidene difluoride membrane for detection by western blot. Competition was performed by preincubating chicken GnRH-R-III immunoglobulins with increasing amounts (0, 5, or 10 μg (not shown)) of peptide 1 prior to use in western blot. A strong band at around 50 kDa present only in the positive tissue was competed out with increasing concentrations of peptide 1 (rectangular box). Additional bands present in both negative and positive tissues (circles) were not completely competed out. Representative picture of 7 separate experiments.

3. Results and discussion

3.1. Validation of the antibody specific to cGnRH-R-III

Nitrocellulose membranes were dotted with increasing concentrations of either peptide 1 or peptide 2. Membranes were then incubated with specific immunoglobulins to either peptide at various concentrations and visualized using enhanced chemiluminescence. Immunoglobulins against peptide 1 appeared to have a higher affinity than immunoglobulins against peptide 2 and were able to detect as little as 20 ng of peptide at a dilution of 2 μg/mL. Thus, only immunoglobulins against peptide 1 were selected for further validation.

Although GnRH-Rs have been shown to be expressed in numerous tissues in vertebrates, in chickens cGnRH-R-III is predominantly expressed in the pituitary gland [13,14], and contrary to cGnRH-R-I, it is not expressed in the heart [12,13]. Thus, these tissues were used as positive and negative controls for western blot analyses, respectively. A strong band located at approximately 50 kDa was detected in the pituitary sample, whereas it was absent from the heart sample (Fig. 2). Furthermore, this band was specifically competed out with pre-incubation of the antibody with increasing concentrations of peptide 1 (Fig. 2). In the negative tissue (heart sample), 2 bands could be visualized below the 50-kDa marker; however, these bands could not be completely competed out, suggesting that they correspond to nonspecific immunocomplexes. Based on amino acid composition, the calculated molecular weight of cGnRH-R-III protein is 46 kDa. With several potential glycosylation sites, cGnRH-R-III is most likely modified posttranslation and thus might migrate at a slightly higher molecular weight. Furthermore, we used prestained molecular weight markers for western blot, which only give an approximate relative

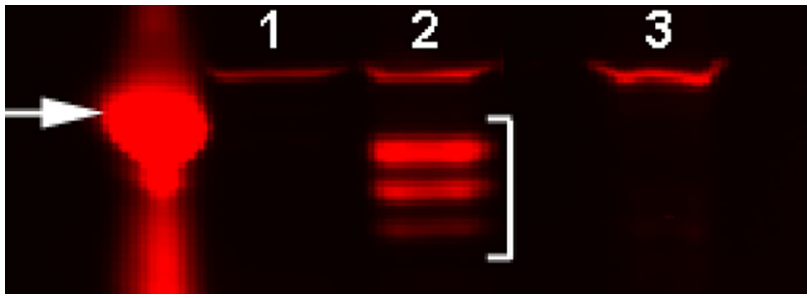

Fig. 3. Immunoblot of cell lysates from transiently transfected COS-7 cells expressing gonadotropin-releasing hormone receptors (GnRH-Rs). The presence of the receptor proteins in cell lysates was analyzed using immunoblotting with anti-chicken GnRH-R-III (cGnRH-R-III) antibody (red). 1 = chicken GnRH-R-I, 2 = cGnRH-R-III, and 3 = nontransfected control. The bracket indicates the detection of cGnRH-R-III protein. The arrow indicates prestained broad-range SDS-PAGE standards (Bio-Rad) of approximately 56 kDa. Representative picture of 3 separate experiments.

determination. Thus, the specific band detected at approximately 50 kDa is most likely our band of interest. Several additional bands with lower molecular weight were also detected in both negative and positive tissues. However, these bands were not completely displaced by the preincubation with peptide 1 (Fig. 2), suggesting they most likely also correspond to nonspecific immunocomplexes. Occasionally, a 40-kDa band that could be displaced was detected in the heart sample. However, although we cannot exclude the possibility of a spliced variant of cGnRH-R-III in this tissue, the lack of mRNA [13] and the inconsistent pattern suggest it may correspond to a nonspecific signal.

In an attempt to validate the specificity of the anti cGnRH-R-III antibody (against peptide 1), western blots were performed on lysates from cells transiently transfected with cGnRH-R-I, cGnRH-R-III, or a nontransfected control (Fig. 3). The results presented show detection of cGnRH-R-III in lysates of transiently transfected COS-7 cells expressing cGnRH-R-III and not cGnRH-R-I or the nontransfected control. Immunoblotting with the anti-cGnRH-R-III antibody yielded 3 bands for cGnRH-R-III at approximately 50 kDa (Fig. 3), which further validates the striking specificity of the antibody for cGnRH-R-III and not cGnRH-R-I despite the 53% sequence identity at the protein coding level of the 2 chicken receptor subtypes [14]. Because several possible glycosylation sites have previously been identified in the protein sequence of the cGnRH-R-III [13,14], it is possible that the additional bands observed in lysates from transfected COS-7 cells may correspond to a differential glycosylation pattern in that cell model. Alternatively, because western blot on cell lysates was performed on a 4% to 20% gradient PAGE, whereas pituitary homogenates were separated on a 10% gel, it is possible that glycosylated variants in chicken pituitary samples comigrated and could not be visualized separately.

In further support of the validation of the specificity of the cGnRH-R-III antibody, immunohistochemical studies were performed. Using immunoglobulins against peptide 1, immunocytochemistry performed on COS-7 cells transiently transfected with cGnRH-R-I, cGnRH-R-III, or empty pcDNA3.1 expression vectors

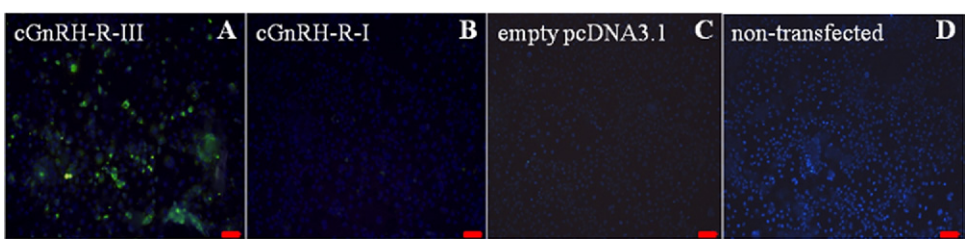

Fig. 4. Immunocytochemical detection of chicken gonadotropin-releasing hormone receptor (cGnRH-R)-III in transfected COS-7 cells. COS-7 cells were transfected with (A) cGnRH-R-III, (B) cGnRH-R-I, or (C) empty pcDNA3.1 expression vectors or (D) left as nontransfected COS-7 cells. After incubation with anti-cGnRH-R-III immunoglobulins, immunoreactive cells were detected with fluorescein isothiocyanate–conjugated anti-rabbit immunoglobulin G (green fluorescence). Individual cells were visualized by staining with Hoechst bisbenzimide 33258 fluorochrome (blue fluorescence). Pictures correspond to an overlay of green and blue fluorescence images. The red bar in the lower right-hand corner represents 100 pixels.

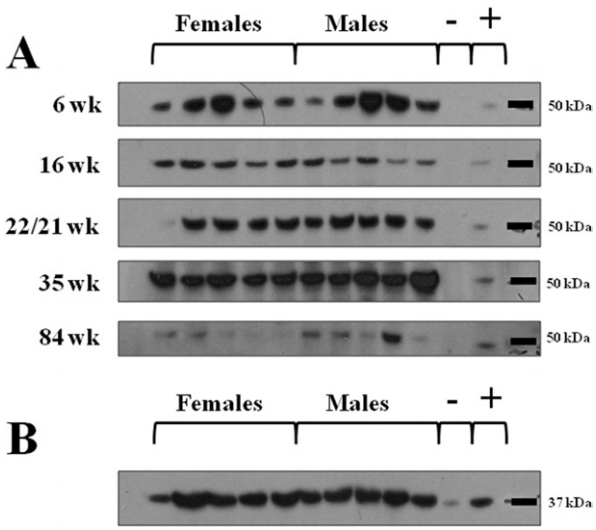

Fig. 5. Representative blots of individual pituitary samples. The positive control (+) corresponds to a pooled pituitary sample that was used as an interblot calibrator, whereas a skeletal muscle sample was used as negative control (−). (A) Chicken gonadotropin-releasing hormone receptor III protein immunoreactive band used for quantification. (B) Representative incubation with glyceraldehyde 3-phosphate dehydrogenase antiserum (21-wk age group).

revealed that on intact cells, the anti-cGnRH-R-III antibody is specific for the type III receptor and does not cross-react with cGnRH-R-I (Fig. 4).

As highlighted in a recent editorial [16], it is a common problem for antibodies against G protein–coupled receptors to lack selectivity. Thus, as suggested by the authors [16], we felt it necessary to fully validate the specificity of our developed cGnRH-R-III antibody by 4 techniques: western blotting using negative and positive tissues, competition in the presence of blocking peptide, western blotting on transfected cells, and immunocytochemistry. Although Michel et al [16] recommend the use of knockout animals for the specific receptor, this is not a feasible approach in chickens. Nonetheless, based on our validation results we are confident that our antibody is specific to cGnRH-R-III and can be successfully used in both immunocytochemistry and western blotting techniques.

3.2. Utilization of the antibody specific to cGnRH-R-III

Following successful validation of our cGnRH-R-III antibody, we investigated whether receptor protein levels in the pituitary gland of male and female chickens change with respect to reproductive status.

To cover the entirety of a reproductive lifespan, tissues were collected at critical ages. From hatch, chickens must be exposed to short day lengths for 8 to 12 wk to be receptive to an increase in photoperiod [17]. The immature age groups were chosen to reflect this, with birds at 6 and 16 wk of age being nonphotoreceptive and photosensitive, respectively. Although photostimulation can trigger early sexual maturation once birds are photosensitive, a spontaneous start in egg laying naturally occurs around 24 wk even if birds are left under nonstimulatory photoperiods [17]. To prevent spontaneous maturation and focus on the effect of stimulatory inputs, we decided to photostimulate birds at 17 (males) and 18 (females) wk of age; this ensured that by 24 wk all hens were in lay and roosters were producing spermatozoa; by 35 wk they were at peak production. In avian species, active reproduction is followed by a photorefractory period during which the rate of lay declines and the pituitary no longer responds to GnRH [18]. At 84 wk, our animals were at the end of a reproductive period and most birds were photorefractory.

As shown in Fig. 5, when compared with the general pool calibrator sample, cGnRH-R-III protein levels appeared much higher in the 35-wk-old birds than in all other age groups for both males and females. This was further confirmed after densitometric analysis (Fig. 6). Receptor levels were low in immature birds (6 and 16 wk) and birds postphotostimulation (21 and 22 wk) and significantly increased ($P < 0.0001$) in birds at peak sexual maturation (35 wk). Receptor protein levels then

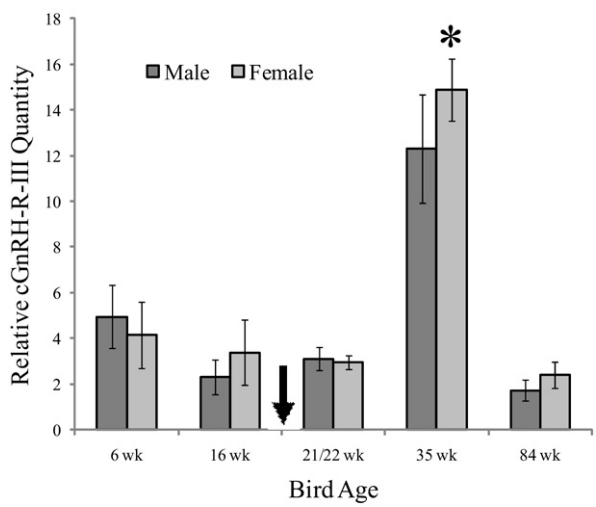

Fig. 6. Changes in chicken gonadotropin-releasing hormone receptor III protein levels in the chicken pituitary. Values for individual samples were normalized for glyceraldehyde 3-phosphate dehydrogenase protein and corrected with the interblot calibrator (pooled sample). The arrow indicates time of photostimulation (17 wk, males; 18 wk, females). The asterisk represents the significant difference ($P < 0.0001$) between the 35-wk-old groups and all other groups.

significantly decreased ($P < 0.0001$) as both males and females reached the end of reproduction (84 wk). Overall, levels of cGnRH-R-III were significantly higher in pituitary glands from birds at 35 wk of age when compared with all other collection dates. Interestingly, no significant difference was observed between males and females ($P > 0.1$).

Previous research conducted in our laboratory showed that cGnRH-R-III mRNA levels in layer-type chickens (white Leghorn) were low in immature males and females, significantly increased in mature animals, and decreased thereafter [13]. Similarly, in female broiler-type chickens, levels of mRNA were also shown to increase upon sexual maturation [14]. However, when looking at our dataset, cGnRH-R-III did not significantly increase until birds reach peak maturation (35 wk), suggesting that the increase in mRNA levels observed following photostimulation [13,14] precedes the increase in protein. We showed that in layer-type males, cGnRH-R-III mRNA levels follow a similar profile to females with an increase observed 4 wk postphotostimulation [13]. Conversely, in broiler-type chickens, mRNA levels were higher in immature than in mature males [14]. As shown in Fig. 6, the increase in cGnRH-R-III protein appears to be transient; thus, the age at photostimulation and tissue collection time points may be critical factors that could explain discrepancies between our previous studies [13,14].

We have previously proposed a model to describe the interactions among cGnRH-I, chicken gonadotropin-inhibitory hormone (cGnIH), and their respective receptors (cGnRH-R-III and cGnIH-R) during a reproductive cycle in chickens [19]. According to this model, in immature birds under a short day photoperiod, the cGnRH-R-III/cGnIH-R ratio in the pituitary gland is low, resulting in low levels of gonadotropin secretion. Upon photostimulation, the pituitary becomes more responsive to cGnRH-I and the cGnRH-R-III/cGnIH-R ratio increases, leading to the full activation of the hypothalamopituitary-gonadal axis. Our results, combined with our previous mRNA data [13,14], further support this model. However, although cGnIH-R mRNA levels were down-regulated in pituitary glands from immature hens treated with estradiol [20], the molecular mechanisms controlling cGnRH-R-III gene expression in gonadotropes are still not known and whether receptor levels fluctuate throughout diurnal or ovulatory cycles is currently under investigation.

Photomicrographs of chicken anterior pituitary gland tissue sections in the midsagittal plane showing distribution of the cGnRH-R-III-ir cells are provided in Fig. 7A–C. Replacing anti-cGnRH-R-III IgG with nonimmunized rabbit IgG did not produce staining (Fig. 7D), indicating the specificity of anti-cGnRH-R-III antibody. The cGnRH-R-III immunoreactive product was found in the pituicyte cytoplasm and particularly around the nucleus. cGnRH-R-III-ir cells were scattered throughout the cephalic and caudal lobes of the anterior pituitary gland but were more predominantly found concentrated in the peripheral edges of the cephalic lobe (Fig. 7A–C). LH-containing gonadotrophs were previously found to be distributed throughout the anterior pituitary, including the peripheral margins of both cephalic and caudal lobes where FSH-containing gonadotrophs are absent [15]. Similar to the distribu-

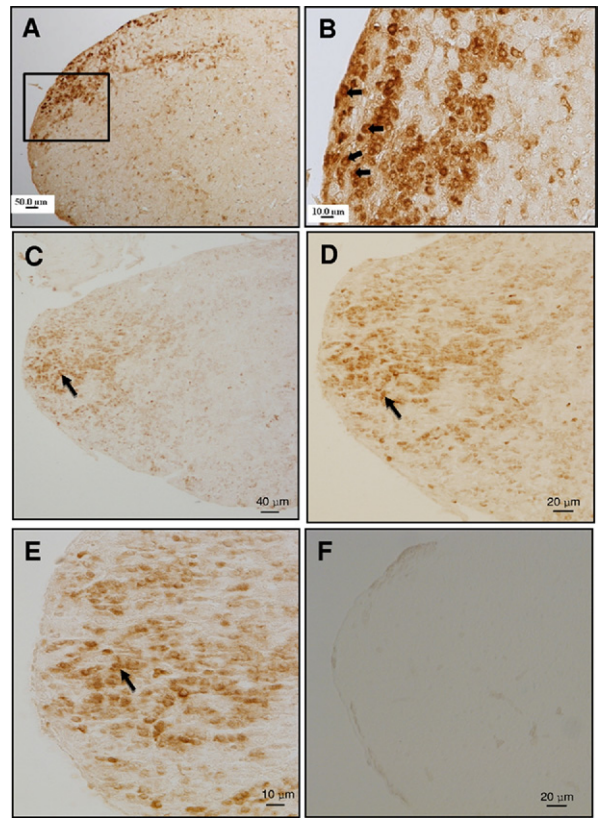

Fig. 7. Representative photomicrographs of chicken anterior pituitary gland tissue sections showing chicken gonadotropin-releasing hormone receptor (GnRH-R)-III-immunoreactive (ir) cells. Anterior pituitary gland tissue sections from sexually mature female chickens (n = 4) were immunostained using anti-cGnRH-R-III IgG. A representative photomicrograph is shown in (A), with (B) corresponding to a magnification of the boxed area in (A). The distribution of cGnRH-R-III-ir cells in the cephalic lobe is shown at various magnifications in (C–E). As a negative control, replacing cGnRH-R-III IgG with nonimmunized rabbit immunoglobulin G did not produce any staining (F). Arrows indicate representative pituicytes positive for cGnRH-R-III.

tion of cGnRH-R-III-ir cells, the gonadotropin-inhibitory hormone receptor (cGnIH-R)-ir cells were found distributed throughout the anterior pituitary gland and more predominantly in the peripheral regions [20]. Based on the anatomical distribution of cells expressing LH and cGnRH-R-III, it is likely that cGnRH-R-III mediate the effect of cGnRH on LH secretion.

In summary, using our antibody, we were able to detect cGnRH-R-III protein by immunocytochemistry in chicken pituitary sections as well as in transfected COS-7 cells. Similarly, using this antibody we were also able to detect the cGnRH-R-III protein by western blot in both chicken pituitary glands and crude lysates of COS-7 cells transiently transfected. Furthermore, we were able to quantify changes in cGnRH-R-III protein levels in the pituitary gland of chickens at various reproductive stages and showed that levels remain low until peak sexual activity is reached.

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References

[1] Sharp PJ, Talbot RT, Main GM, Dunn IC, Fraser HM, Huskisson NS. Physiological roles of chicken LHRH-I and -II in the control of gonadotrophin release in the domestic chicken. J Endocrinol 1990;124:291–9.

[2] Chaiseha Y, El Halawani ME. Neuroendocrinology of the female turkey reproductive cycle. J Poult Sci 2005;42:87–100.

[3] Burns JM. The influence of chicken and mammalian luteinizing hormones on ovarian estrogen synthesis in the pullet (*Gallus domesticus*). Comp Biochem Physiol 1972;42:1011–7.

[4] Brown NL, Bayle JD, Scanes CG, Follett BK. Chicken gonadotrophins: Their effects on the testes of immature and hypophysectomized Japanese quail. Cell Tissue Res 1975;156:499–520.

[5] Maung SL, Follett BK. The endocrine control by luteinizing hormone of testosterone secretion from the testis of the Japanese quail. Gen Comp Endocrinol 1978;36:79–89.

[6] Phillips A, Scanes CG, Hahn DW. Effect of androgens and gonadotropins on progesterone secretion of chicken granulose cells. Comp Biochem Physiol 1985;81:847–52.

[7] King JA, Millar, RP. Structure of chicken hypothalamic luteinizing hormone-releasing hormone. J Biol Chem 1982; 257:10722–8.

[8] Miyamoto K, Hasegawa Y, Minegishi T, Nomura M, Takahashi Y, Igarashi M. Isolation and characterization of chicken hypothalamic luteinizing hormone-releasing hormone. Biochem Biophys Res Commun 1982;107:820–7.

[9] Mikami S, Yamada S, Hasegawa Y, Miyamoto K. Localization of avian LHRH-immunoreactive neurons in the hypothalamus of the domestic fowl, *Gallus domesticus*, and the Japanese quail, *Coturnix coturnix*. Cell Tissue Res 1988;251:51–8.

[10] Miyamoto K, Hasegawa Y, Nomura M, Igarashi M, Kangawa K, Matsuo H. Identification of the second gonadotropin-releasing hormone in chicken hypothalamus: Evidence that gonadotropin secretion is probably controlled by two distinct gonadotropin-releasing hormones in avian species. Proc Natl Acad Sci U S A 1984;81:3874–8.

[11] Maney DL, Richardson RD, Wingfield JC. Central administration of chicken gonadotropin-releasing hormone-II enhances courtship behaviour in a female sparrow. Horm Behav 1997; 32:11–8.

[12] Sun YM, Dunn IC, Baines E, Talbot RT, Illing N, Millar RP, Sharp PJ. Distribution and regulation by oestrogen of fully processed and variant transcripts of gonadotropin releasing hormone I and gonadotropin releasing hormone receptor mRNAs in the male chicken. J Neuroendocrinol 2001;13:37–49.

[13] Shimizu M, Bedecarrats GY. Identification of a novel pituitary-specific chicken gonadotropin-releasing hormone receptor and its splice variants. Biol Reprod 2006;75:800–8.

[14] Joseph NT, Morgan K, Sellar R, McBride D, Millar RP, Dunn IC. The chicken type III GnRH receptor homologue is predominantly expressed in the pituitary, and exhibits similar ligand selectivity to the type I receptor. J Endocrinol 2009; 202:179–90.

[15] Proudman JA, Vandesande F, Berghman LR. Immunohistochemical evidence that follicle-stimulating hormone and luteinizing hormone reside in separate cells in the chicken pituitary. Biol Reprod 1999;60:1324–8.

[16] Michel MC, Wieland T, Tsujimoto G. How reliable are G-protein-coupled receptor antibodies? Naunyn-Schmied Arch Parmacol 2009;379:385–8.

[17] Sharp PJ. Photoperiodic control of reproduction in the domestic hen. Poult Sci 1993;72:897–905.

[18] Nicholls TJ, Goldsmith AR, Dawson A. Photorefractoriness in birds and comparison with mammals. Physiol Rev 1988;68: 133–76.

[19] Bedecarrats GY, McFarlane H, Maddineni SR, Ramachandran R. Gonadotropin-inhibitory hormone receptor signalling and its impact on reproduction in chickens. Gen Comp Endocrinol 2009;163:7–11.

[20] Maddineni S, Ocon-Grove OM, Krzysik-Walker SM, Hendricks GL, III Proudman JA, Ramachandran R. Gonadotrophin-inhibitory hormone receptor expression in the chicken pituitary gland: Potential influence of sexual maturation and ovarian steroids. J Neuroendocrinol 2008;20:1078–88.