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LH-Independent Testosterone Secretion Is Mediated by the Interaction Between GNRH2 and Its Receptor Within Porcine Testes[1]

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ABSTRACT

Unlike classic gonadotropin-releasing hormone 1 (GNRH1), the second mammalian isoform (GNRH2) is an ineffective stimulant of gonadotropin release. Species that produce GNRH2 may not maintain a functional GNRH2 receptor (GNRHR2) due to coding errors. A full-length *GNRHR2* gene has been identified in swine, but its role in reproduction requires further elucidation. Our objective was to examine the role of GNRH2 and GNRHR2 in testicular function of boars. We discovered that GNRH2 levels were higher in the testis than in the anterior pituitary gland or hypothalamus, corresponding to greater GNRHR2 abundance in the testis versus the anterior pituitary gland. Moreover, GNRH2 immunostaining was most prevalent within seminiferous tubules, whereas GNRHR2 was detected in high abundance on Leydig cells. GNRH2 pretreatment of testis explant cultures elicited testosterone secretion similar to that of human chorionic gonadotropin stimulation. Treatment of mature boars with GNRH2 elevated testosterone levels similar to those of GNRH1-treated males, despite minimal GNRH2-induced release of luteinizing hormone (LH). When pretreated with a GNRHR1 antagonist (SB-75), subsequent GNRH2 treatment stimulated low levels of testosterone secretion despite a pattern of LH release similar to that in the previous trial, suggesting that SB-75 inhibited testicular GNRHR2s. Given that pigs lack testicular GNRHR1, these data may indicate that GNRH2 and its receptor are involved in autocrine or paracrine regulation of testosterone secretion. Notably, our data are the first to suggest a biological function of a novel GNRH2-GNRHR2 system in the testes of swine.

GNRH2, GNRHR2, porcine, testis, testosterone

INTRODUCTION

The classic form of gonadotropin-releasing hormone 1 (GNRH1) is regarded as the master modulator of reproduction, stimulating synthesis and secretion of the gonadotropins, luteinizing hormone (LH), and follicle stimulating hormone (FSH). Although GNRH1 conventionally regulates gonadotropin secretion [1], it has also been ascribed extrapituitary functions [2]. Both GNRH1 and its receptor (GNRHR1) have been detected in mammalian testes [3–8], and their interaction has been linked to localized testosterone secretion, independent of LH release [9–12], bypassing the central dogma of testosterone regulation.

A second form of GnRH, GNRH2, has also been identified [13], and its structure remains completely conserved throughout 500 million years of evolution [14], suggesting high selection pressure and, therefore, a critical function [15]. Like GNRH1, GNRH2 is a decapeptide but contains 3 amino acid substitutions (His[5], Trp[7], Tyr[8]) compared to GNRH1 [13]. Uniquely, GNRH2 is expressed most highly in tissues outside of the brain [16], suggesting a divergent role from GNRH1 [17]. Logically, GNRH2 has been examined for a role in gonadotropin secretion. Indeed, GNRH2 stimulates LH release, but with approximately 10% of the efficacy of GNRH1 [18], which is consistent with a report showing that GNRH2 can bind GNRHR1 with 10-fold less affinity than GNRH1 [19]. Primary cultures of porcine anterior pituitary cells release LH and FSH in response to GNRH2 [20], but this effect was attenuated with a GNRHR1 antagonist [21]. Thus, it is now postulated that only high doses (e.g., 10 μg) of GNRH2 can stimulate low levels of gonadotropin secretion through GNRHR1 in vivo and in vitro [22–25].

A receptor specific to GNRH2 (GNRHR2) has been identified in mammals [26, 27]. Similar to its ligand, *GNRHR2* mRNA is present in the brain, as well as in peripheral tissues such as the testis [26, 27], suggesting an autocrine or paracrine action [26]. Unlike most species [17], swine maintain the gene sequence for a functional GNRHR2 [28]. Using cDNA from pig pituitaries, the porcine *GNRHR2* has been cloned and sequenced, showing 90% homology with African green

monkey *GNRHR2* [21, 29]. This receptor is considered functional, as GNRH2 stimulation of cells overexpressing porcine GNRHR2 resulted in IP_3 production [20].

In mammals that produce both the ligand and the full-length receptor (e.g., musk shrews and old world monkeys [17]), GNRH2 and its receptor have been implicated in the interaction between nutritional plane and reproductive behavior [30–32], as well as feed intake [31, 33, 34]. Additionally, indirect evidence suggests that GNRH2 and its receptor may regulate porcine testicular function. For example, males immunized against GNRH2 displayed reduced levels of testosterone compared to controls, whereas LH levels remained unchanged [35]. Moreover, secretion of testosterone was suppressed in boars treated with a GNRHR1 antagonist (SB-75) despite normal concentrations of LH [36]. Additionally, SB-75 attenuated human chorionic gonadotropin (hCG)-stimulated testosterone secretion in porcine testicular explant cultures [36]. Given that GNRHR1 was not detected in swine testis [36], it was postulated that a different mechanism must govern localized testosterone release in swine [36]. Because others have reported that high concentrations of SB-75 can bind to the GNRHR2 in vitro [37], we hypothesized that the results of the latter study could be ascribed to the interaction between SB-75 and GNRHR2 in porcine testes. Therefore, we sought to examine the biological function of GNRH2 and its receptor in the boar testis.

MATERIALS AND METHODS

Ethics

All animal procedures were conducted using standard production and experimental practices in accordance with the Guide for the Care and Use of Agricultural Animals in Research and Teaching [38] and were approved by University of Nebraska-Lincoln (UNL) Institutional Animal Care and Use Committee and by the US Meat Animal Research Center (USMARC). All animals were individually housed with water available ad libitum and fed approximately 2.5 kg daily.

GNRH2 and GNRHR2 Localization

Animals. Mature Chinese Meishan boars (n = 7) from USMARC (Clay Center, NE) were used. After exsanguination, the hypothalamus, anterior pituitary gland, and testis were collected. Tissue samples (100 mg) were either snap frozen and stored at −80°C or fixed in 15 ml of 4% paraformaldehyde (testis samples only).

Antisera. A goat polyclonal antibody directed against GNRHR2 (sc-162889; lot B0810; Santa Cruz Biotechnology, Santa Cruz, CA) was used. This antibody was prepared against a synthetic human peptide derived between amino acids 60 and 75 of GNRHR2, representing the first N-terminal, intracellular domain. It was determined by NCBI protein Basic Local Alignment Search Tool (BLAST) that the epitope maintained 85% homology for porcine GNRHR2, specifically the 7-transmembrane isoform (NCBI accession number AAS68622.1). Moreover, the specificity of this antibody was confirmed in our laboratory (data not shown).

A rabbit polyclonal antibody directed against prepro-GNRH2 (sc-20942; lot C2403; Santa Cruz Biotechnology) was used. This antibody was prepared against a synthetic human peptide derived between amino acids 1 and 120 of prepro-GNRH2. It was determined by using protein BLAST that the epitope maintained 45% homology for porcine prepro-GNRH2 and 100% homology for the mature form of porcine GNRH2 (NCBI accession number XP_005672842). Use of this antibody has been previously reported [39] to detect GNRH2 in normal and human ovarian cancer cells. Furthermore, we performed several antibody validation procedures in our laboratory (data not shown).

Immunoblotting. Protein was extracted from samples of testis and anterior pituitary gland (n = 5) by homogenization in 1 ml of radioimmunoprecipitation assay buffer (20 mM Tris, 137 mM NaCl, 10% glycerol, 1% NP-40, 0.1% SDS, 0.5% deoxycholic acid, 2 mM EDTA, 1 mM PMSF, 1% protease inhibitor cocktail, and 1% phosphatase inhibitor cocktail) per 100 mg of tissue with a Tissue-Tearor (Biospec, Bartlesville, OK). Protein concentrations of all lysates were quantified using a bicinchoninic acid

(BCA) assay (Pierce Biotechnology, Rockford, IL) according to the manufacturer's instructions. Extracted protein was mixed with 4× loading dye (2% Tris [pH 6.8], 28% glycerol, 20% SDS, and Orange G) containing 100 mM dithiothreitol. Then, 20 μg of testis protein was separated using SDS-PAGE (10%) and transferred electrophoretically to a polyvinylidene difluoride membrane (Immobilon-FL; Millipore, Billerica, MA). After transfer, nonspecific binding was blocked by incubation (Odyssey blocking buffer; LI-COR Biosciences, Lincoln, NE) for 1 h at room temperature (RT). Membranes were then incubated with goat polyclonal primary antibody directed against GNRHR2 (1:1000 dilution; sc-162889; Santa Cruz Biotechnology), diluted in blocking buffer (Odyssey) with 0.05% Tween-20 and shaking at 4°C, overnight. Membranes were next incubated with a secondary donkey anti-goat antibody (1:16 000 dilution; IRDye 680; LI-COR Biosciences) in blocking buffer (Odyssey) plus 0.05% Tween-20 and 0.025% SDS for 1 h at RT. Between each step, membranes were washed in Tris-buffered saline with 0.05% Tween-20 (TBS-T). Immunoblots were visualized with a scanner (Odyssey) and image software (LI-COR Biosciences). Blots were stripped in 1× stripping buffer (NewBlot; LI-COR Biosciences) for 30 min at 37°C and reprobed with β-actin (1:2000 dilution; sc-1516; Santa Cruz Biotechnology) to serve as a loading control.

Immunohistochemistry. After overnight fixation at 4°C, testis samples were dehydrated with 100% ethanol, cleared with CitriSolv (Fisher Scientific, Pittsburgh, PA), embedded in paraffin, sectioned (7 μm), and mounted on slides (UltraStick; Gold Seal Products, Portsmouth, NH). Epitope retrieval was achieved by boiling slides in 0.01 M sodium citrate (pH 6.0) for 15 min. Nonspecific binding was blocked with 10% rabbit serum for 20 min, and slides were incubated for 30 min at RT with anti-GNRHR2 diluted 1:100 in 1× TBS (25 mM Tris, 150 mM NaCl, 2 mM KCl). Alkaline phosphatase detection was performed using a commercial kit (Vectastain; ABC-AP; Vector Blue; Vector Laboratories, Burlingame, CA) according to the manufacturer's instructions. Slides were mounted with Tris-buffered 80% glycerol and imaged using an Olympus microscope (model BX51; Center Valley, PA) and camera (model DP71).

Immunofluorescence. Slide preparation was performed as described above. Nonspecific binding was blocked with 10% goat serum (in TBS) for 1 h, and slides were incubated with an antibody directed against prepro-GNRH2, diluted 1:50 in 10% normal goat serum at 4°C overnight. Primary antibody was centrifuged (10 000 × g) for 10 min at 4°C to avoid antibody aggregates prior to use. Slides were rinsed 3 times in TBS-T (0.05% Tween-20) for 5 min. Slides were washed in TBS-T and incubated with Alexa Fluor 555-labeled goat anti-rabbit secondary antibody (1:400 dilution; 4413; Cell Signaling, Danvers, MA) for 1 h. Slides were then washed 3 times in TBS-T and incubated with 0.3% Sudan Black B (Fisher Scientific) in 70% ethanol for 10 min at RT to reduce autofluorescence [40]. Slides were washed 3 times in TBS-T and mounted with Tris-buffered 80% glycerol before sealing. Slides were imaged using an inverted confocal microscope (model IX 81; Olympus) with a TRITC filter set at the UNL Microscopy Core Facility (Lincoln, NE).

Enzyme-linked immunosorbent assay. Hypothalamus, anterior pituitary gland, and testicular tissue samples were homogenized and assayed according to the manufacturer's instructions for a precursor form of GNRH2 with a commercially available ELISA specific to the pig (CSB-EL009636PI; Cusabio, Hubei, China). The primary antibody also has a high homology with the mature decapeptide (Cusabio, personal communication). Optical density was determined using a Wallac model 1420 Victor^2 microplate reader (Perkin-Elmer, Waltham, MA) at 450 nm. The minimum sensitivity of the assay was 20 pg/ml, and the intra-assay coefficient of variation (CV) was 9.2%.

Ex Vivo Testicular Cultures

Animals. Mature Chinese Meishan boars (n = 7) were euthanized, and whole testes were collected. Testicular explant cultures were performed as described by Zanella et al. [36]. Approximately 400 mg of finely minced parenchymal tissue was incubated in 5 ml of TC199 (with Earle salts and NaHCO$_3$; Sigma-Aldrich, St. Louis, MO) buffered with a final concentration of 25 mM Hepes. Tissue was incubated in an atmosphere of 5% CO_2 in air at 37°C for 2 h. Preincubation was necessary because testosterone is released from the tissue during dissection and can mask hormone-induced testosterone secretion [41]. Media were decanted and replaced with fresh TC199, and explants were randomly assigned to treatment.

Treatments. Samples of media (250 μl) were collected to assess basal hormone levels (time 0). After media collection, explant cultures were exposed to either 30 μl of vehicle (water) or 1 μM GNRH2 (Bachem Inc., Torrance, CA) as a pretreatment. These doses were determined based on the work of Zanella et al. [36] and Darby et al. [42]. Media (250 μl) samples were collected 1 h after pretreatment from all cultures, and explants were treated with either 30 μl of vehicle (water) or 1 IU of hCG (Sigma-Aldrich). Therefore, four combinations

(pretreatment + treatment) were tested, as follows: control (vehicle + vehicle), hCG alone (vehicle + hCG), GNRH2 pretreatment without hCG (GNRH2 + vehicle), and GNRH2 pretreatment with hCG (GNRH2 + hCG). Media (250 μl) were collected at 120, 180, and 240 min after treatment, frozen immediately, and stored at −20°C until quantification of testosterone with radioimmunoassay (RIA).

In Vivo Boar Trials

Catheterization. Trial 1 was conducted at USMARC, using mature Chinese Meishan boars, whereas Trials 2 and 3 were performed at UNL Animal Science Building with mature white crossbred boars obtained from the UNL Agricultural Research and Development Center (ARDC) swine unit (Mead, NE). Catheterization of boars was conducted according to either Ford and Maurer [43] or Barb et al. [44] approximately 4 days prior to initiation of experimentation. All in vivo experiments were conducted in a crossover design, where each boar received the opposite treatment 1 week later.

Peptides. Both D-ala^6 GnRH-I (Bachem Inc.) and D-ala^6 GnRH-II (Anygen, Gwangju, Korea) were dissolved in 0.9% sterile saline. SB-75 was synthesized by the UNL Protein Core Facility (Lincoln, NE) and dissolved in 0.9% sterile saline. Trptorelix-1 (Trp-1; Anygen) was dissolved according to the manufacturer's instructions. Briefly, Trp-1 was first dissolved in 5% dimethylacetamide, mixed with 40% propylene glycol, and finally, diluted in 55% water.

Trial 1. Prior to treatment, blood samples were collected in plasma blood tubes (KMO-EDTA; S-Monovette; Sarstedt, Nümbrecht, Germany) every 20 min for 2 h to obtain a baseline hormone profile (−120 to 0 min). Boars were then given a bolus infusion (i.v.) of either a GNRHR1 (D-ala^6 GnRH-I; 150 ng/kg body weight [BW], n = 8) or GNRHR2 (D-ala^6 GnRH-II; 150 ng/kg BW, n = 6) agonist in approximately 2 ml of saline (0 min). Blood samples were drawn every 10 min for 30 min and every 20 min for 270 min thereafter. Blood samples remained at 4°C until centrifugation (2000 × g), and plasma was collected and stored at −20°C until RIA.

Trial 2. Blood was collected in sera tubes (KMO-SER; Sarstedt) at 20 and 10 min and immediately prior to injection to quantify basal hormone levels. Boars then received a dose (1 ml of saline, i.m.) of the GNRHR1 antagonist SB-75 (5 μg of biologically active compound/kg BW). Based on preliminary trials, blood was collected 7.5 h later, and then boars were given a bolus infusion (i.v.) of either GNRHR1 (D-ala^6 GnRH-I; 150 ng/kg BW, n = 5) or GNRHR2 (D-ala^6 GnRH-II; 150 ng/kg BW, n = 5) agonist in approximately 2 ml of saline. Blood samples were drawn every 10 min for 30 min and then every 20 min for 270 min after agonist treatment. Blood samples remained at 4°C until centrifugation (2000 × g), and serum was collected and stored at −20°C until RIA.

Trial 3. Prior to treatment, blood was drawn at 30 min and immediately prior to analog administration. Treatment with either GNRHR1 (SB-75; 5 μg of biologically active compound/kg BW, n = 9) or GNRHR2 (Trp-1; 5 μg/kg BW; n = 10) antagonist were given i.m. Each animal also received the vehicle of the opposite treatment (i.m.). Blood sampling occurred every 30 min for 3 h (30, 60, 90, 120, 150, and 180 min) and at 6, 12, 24, 36, and 48 h after treatment. Blood samples remained at 4°C until centrifugation (2000 × g), and serum was collected and stored at −20°C until RIA.

Radioimmunoassay. Total testosterone concentrations from explant medium samples were determined using a total testosterone Coat-a-Count RIA kit (Siemens Healthcare Diagnostics, Los Angeles, CA) in accordance with the manufacturer's instructions. This kit has been previously validated in the pig [45, 46]. The minimum sensitivity of the assay was 0.16 ng/ml. Inter- and intra-assay coefficients of variation (CVs) were 4.3 and 4.6%, respectively. Testicular explant medium samples were diluted 1:5 with a zero calibrator solution (0 ng/ml testosterone; Siemens Healthcare Diagnostics) in order to fit the standard curve, according to the manufacturer's instructions.

Plasma samples from Trial 1 were assayed for testosterone in duplicate according to Ford et al. [47]. The minimum sensitivity of the assay was 50 pg/ml, and the intra- and inter-assay CVs were 7% and 12.9%, respectively. In Trials 2 and 3, total testosterone concentrations in boar sera were measured in duplicate using a total testosterone Coat-a-Count RIA kit (Siemens Healthcare Diagnostics) according to the manufacturer's instructions. However, 2 additional standards were added to increase the sensitivity of the curve (0.04 and 0.08 ng/ml; per manufacturer's instruction), therefore the minimum sensitivity of the assay was 0.04 ng/ml. The average intra- and inter-assay CVs were 6.2 and 8.5%, respectively. LH concentrations for all trials were determined at USMARC in duplicate according to Kesner et al. [48]. The minimum sensitivity of the assay was 0.1 ng/ml. Pools of porcine serum with LH concentrations that ranged from 0.23 to 6.0 ng/ml were included in each assay (n = 9). Intra- and inter-assay CVs were 9.9 and 12%, respectively.
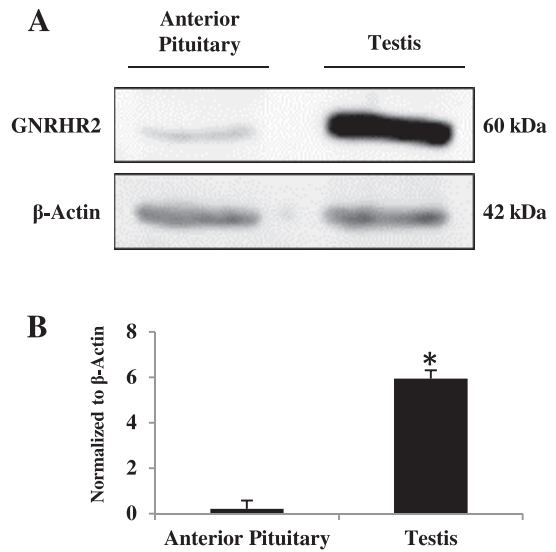

FIG. 1. GNRHR2 protein levels are significantly higher in the testis than in anterior pituitary gland. Representative Western blot of porcine anterior pituitary gland and testicular tissue (n = 5), using an antibody directed against GNRHR2 (**A**). Quantification of immunoblots revealed differences in GNRHR2 protein levels between tissue types (*$P < 0.0001$) (**B**).

Statistical Analysis

Statistical analyses were performed using the Statistical Analysis System (SAS, Cary, NC). All data were examined for normality using the Shapiro-Wilk test prior to analysis. Data that did not meet normality assumptions were log transformed, analyzed, and back-transformed to the original scale for interpretation. Western blots and ELISA data were analyzed using the general linear model procedures of SAS with animal as the experimental unit and tissue type as the fixed effect. Explant RIA data were analyzed using the MIXED procedure of SAS with time included in the model as a repeated measure and assay as a random effect. Data were first normalized to pretreatment hormone levels and then expressed as fold-change over values for the control (vehicle + vehicle) within animal.

Results from in vivo Trials 1 to 3 were analyzed with the MIXED procedure of SAS, using a statistical model that included day, treatment, time, and the interaction of treatment with time as fixed effects. Time was used as the repeated measure with boar × treatment × day as the subject. Preinjection concentrations of hormone were used as the covariate. Degrees of freedom for the pooled error term were calculated using the Satterthwaite approximation. Based on Akaike information criterion, a first-order autoregressive or heterogeneous autoregressive function with lag equal to 1 was used to model the covariance structure for the repeated measures. When a significant ($P \le 0.05$) main effect was observed, pair-wise comparisons were made using the Tukey-Kramer test adjusted for the degrees of freedom in the covariance matrix. Results are presented as means ± standard errors of the mean (SEM).

RESULTS

GNRH2 and Its Receptor Are Abundantly Expressed in the Testis

Presence of GNRHR2 in the testis and anterior pituitary gland was confirmed via Western blot analysis using an anti-GNRHR2 antibody (Fig. 1A). Quantification of relative band density indicated that the testis contained 6-fold higher amounts of GNRHR2 protein than the anterior pituitary gland ($P < 0.0001$) (Fig. 1B). Given this discovery, we examined the levels of GNRH2 in homogenates from the hypothalamus, anterior pituitary gland, and testis via ELISA. The concentration of GNRH2 protein was significantly elevated in the testis ($P < 0.0001$) compared to that in the anterior pituitary
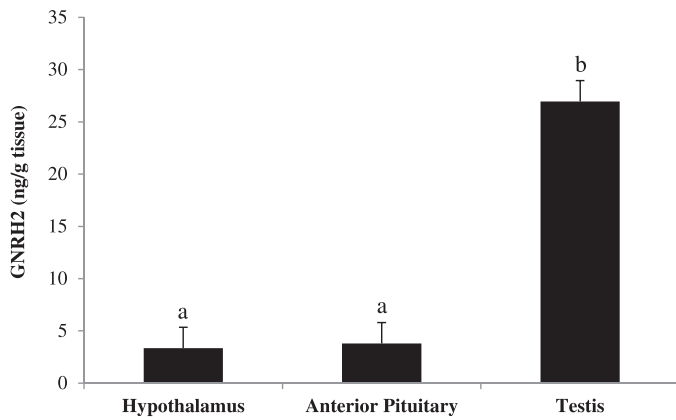
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FIG. 2. GNRH2 levels are higher in tissue homogenates from the testis than in the hypothalamus or anterior pituitary gland. GNRH2 levels were determined by ELISA in homogenates from the hypothalamus, anterior pituitary gland, and testis of boars (n = 6). [a,b] Bars with alternate letters differ ($P < 0.0001$).

gland or hypothalamus, which had similar values ($P > 0.05$) (Fig. 2).

GNRH2 Localizes Primarily to the Tubular Compartment, Whereas GNRHR2 Is Expressed in Both the Interstitial and Tubular Compartments

Immunohistochemistry of testicular tissue using a GNRHR2 antibody revealed immunoreactivity within the interstitial compartment, as well as within the seminiferous tubules (Fig. 3, B–D). However, GNRHR2 signal appeared most prominent in the interstitial compartment. Within the interstitium, staining appeared to localize to Leydig cells, based upon location and morphology, with both intracellular and peripheral signal (Fig. 3D). Immunostaining was also

visible within the seminiferous tubules and appeared localized to germ cells and Sertoli cells (Fig. 3D). In contrast, there was no signal detected in control sections incubated without the primary antibody (Fig. 3A). Based on these results, we next localized GNRH2 within testicular sections using immuno-fluorescence. GNRH2 immunostaining appeared primarily within the tubular compartment (Fig. 4, A–C), localizing to germ cells (Fig. 4, D–F). In contrast, no fluorescence signal was detected in sections processed without the primary antibody (Fig. 4, A–C insets).

GNRH2 Alone Stimulates Testosterone Secretion in Testicular Explant Cultures

Based on our immunohistochemistry results, we examined the effects of GNRH2 on steroidogenesis. There was no effect of time or treatment × time interaction for fold-change in testosterone concentration, so these effects were removed from the statistical model. We observed an effect of treatment, as treatment with either hCG or GNRH2 increased testosterone secretion compared to treatment with vehicle ($P < 0.001$) (Fig. 5). Release of testosterone from testicular explants receiving GNRH2 pretreatment was not different than explants receiving vehicle pretreatment followed by hCG ($P > 0.05$) (Fig. 5). Moreover, pretreatment with GNRH2 did not further enhance hCG-induced testosterone secretion compared with that of explant cultures receiving pretreatment with saline before hCG treatment ($P > 0.05$) (Fig. 5).

GNRH2 and GNRH1 Stimulate Testosterone Secretion Similarly Despite Differences in LH Release

To further evaluate the effect of GNRH2 on testosterone secretion, we treated boars with either D-ala[6] GnRH-I or D-ala[6] GnRH-II. We observed a treatment × time effect ($P < 0.003$) for change in LH concentrations (Fig. 6A). Within 10 min after administration of GNRH1, LH concentrations increased over
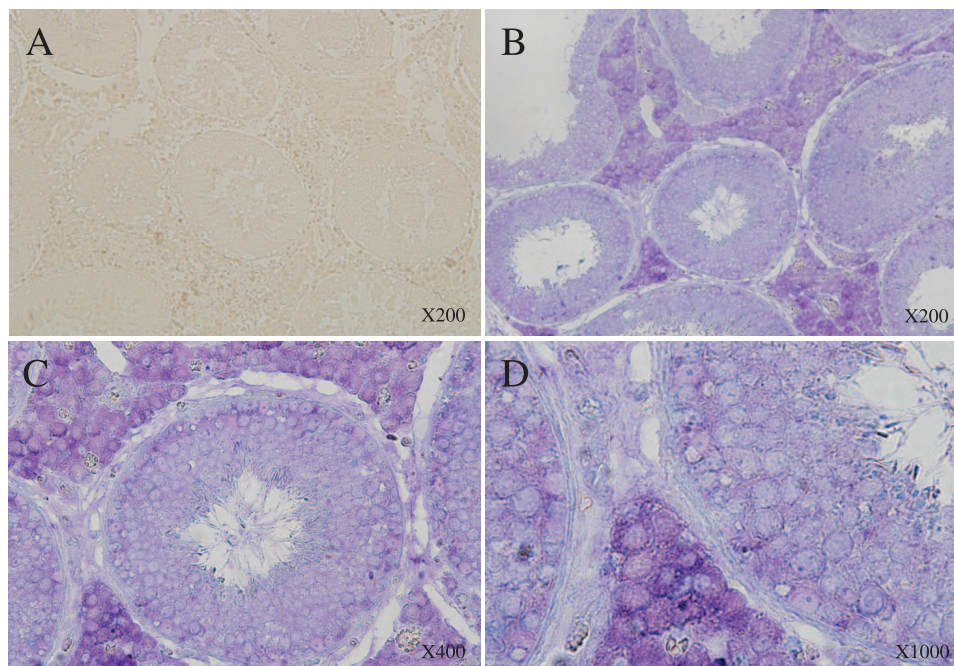
FIG. 3. GNRHR2 localizes to both the tubular and the interstitial compartments of the testis. Representative immunohistochemistry image of porcine testes (n = 7), using an antibody directed against GNRHR2 (**B–D**) compared to the negative control (**A**). Original magnification ×200 control (**A**); ×200 (**B**), ×400 (**C**), ×1000 under oil immersion (**D**).
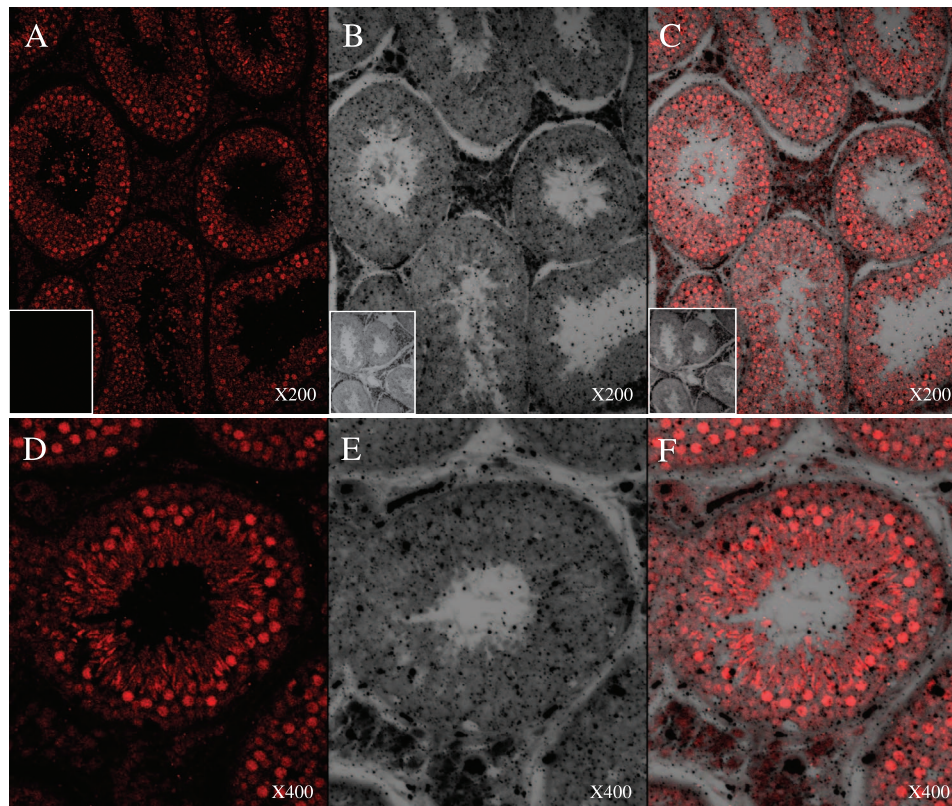
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FIG. 4. GNRH2 is localized primarily to the tubular compartment of the testis. Representative immunofluorescence image of porcine testes (n = 5), using an antibody directed against GNRH2; (**A**, **D**) fluorescence; (**B**, **E**) transmitted light; (**C**, **F**) merge of fluorescence and transmitted light. **A–C**) Insets are sections incubated without primary antibody: (**A**) fluorescence, (**B**) transmitted light; (**C**) merge of fluorescence and transmitted light. Original magnification ×200 (**A–C**); ×400 (**D–F**).

pretreatment amounts, reached maximal levels by 130 min, and remained elevated for the duration of sampling (Fig. 6A, 270 min). In contrast, LH levels increased within 10 min following GNRH2 treatment but steadily declined to pretreatment values by 50 min (Fig. 6A).

Unlike LH secretion, we did not observe a treatment × time interaction or an effect of treatment for change in testosterone levels after GNRH1 or GNRH2 treatment ($P > 0.05$) (Fig. 6B). We also observed a significant effect of time ($P < 0.0001$) (Fig. 6B), indicating that treatment with GNRH1 or GNRH2 altered testosterone secretion over time. Thus, both treatments stimulated a similar increase in testosterone secretion ($P > 0.05$) during the sampling period.

Pretreatment with SB-75 Inhibits GNRH2-Mediated Testosterone Secretion

Next, we evaluated whether the recognized GNRHR1 antagonist, SB-75, interfered with testicular GNRHR2 function. We observed a treatment × time effect for the LH response ($P < 0.001$) (Fig. 7A). After administration of the GNRHR1 antagonist, SB-75, concentrations of LH tended to be reduced in both the GNRH1 and the GNRH2 treatment groups compared with pretreatment concentrations of LH ($P < 0.10$) (Fig. 7A). After treatment with GNRH1, LH levels rose significantly above pretreatment levels within 10 min and remained elevated for 50 min before returning to basal levels for the remainder of the sampling period (270 min). In contrast, after treatment with GNRH2, serum LH concentrations were not significantly stimulated over time compared to basal hormone levels ($P > 0.05$) (Fig. 7A). Compared to post-SB-75

hormone concentrations, GNRH1 treatment stimulated LH secretion within 10 min, remaining elevated through 190 min. In contrast, GNRH2 treatment stimulated LH secretion over post-SB-75 levels at 10, 20, and 30 min before returning to basal concentrations (Fig. 7A).

For the testosterone response, we observed a tendency for a treatment × time interaction ($P < 0.10$). After treatment with SB-75, testosterone levels dropped below pretreatment concentrations at time 0 and remained suppressed ($P < 0.05$) after treatment with GNRH1 through 20 min post treatment before returning to baseline. Similarly, testosterone was suppressed below basal levels 7.5 h after SB-75 treatment (time 0) and remained reduced 10, 20, and 30 min post treatment in animals that received GNRH2. At 90 and 110 min, testosterone levels were elevated over baseline values in GNRH1-treated animals ($P < 0.05$), whereas GNRH2 treatment was unable to induce testosterone concentrations above basal levels ($P > 0.05$) at any time point. Compared to post-SB75 concentrations (time 0), testosterone was greater from 50 to 210 min following GNRH1 administration ($P < 0.05$) and from 50 to 150 min in GNRH2-treated animals ($P < 0.05$), with 170 min tending to be greater than time 0 ($P < 0.10$).

LH and Testosterone Secretion Are Reduced after Treatment with GNRHR1 Antagonist SB-75 but Not with GNRHR2 Antagonist Trp-1

To further examine the effect of receptor antagonism, we compared a GNRHR2-specific antagonist, Trp-1 [49, 50], with the GNRHR1 antagonist, SB-75. For change in LH levels,
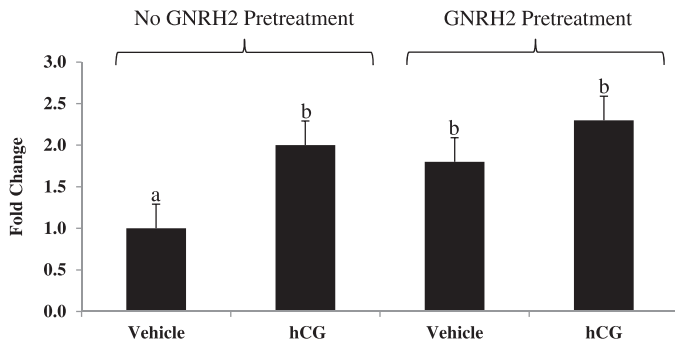
FIG. 5. GNRH2 directly stimulates testosterone production from ex vivo testicular cultures. Relative testosterone levels secreted by testicular explant cultures (n = 7) pretreated with or without GNRH2, followed by treatment with vehicle or hCG. [a,b] Bars with alternate superscripts differ ($P < 0.001$).

there was no treatment $\times$ time interaction ($P > 0.05$) or an effect of treatment ($P > 0.05$) (Fig. 8A). However, there was an effect of time ($P < 0.0001$) (Fig. 8A). Specifically, LH concentrations were suppressed compared to basal levels at 2.5, 3, 6, and 12 h after SB-75 treatment ($P < 0.05$). In contrast, Trp-1 treatment did not reduce LH secretion at any time point compared to basal levels ($P > 0.05$).
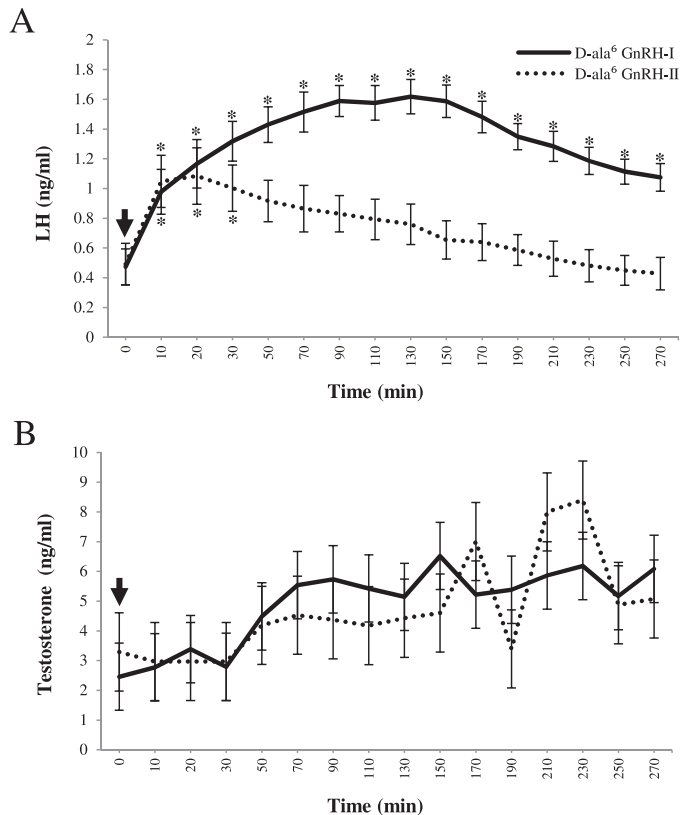

FIG. 6. GNRH2 and GNRH1 stimulate testosterone production similarly despite reduced GNRH2-induced LH secretion. Least squares means ($\pm$SEM) plasma concentrations of LH (**A**) and testosterone (**B**) after intravenous administration of D-ala^6 GnRH-I (solid line) or D-ala^6 GnRH-II (dotted line). After pretreatment sampling (-120 to 0 min), treatments were administered (0 min [arrow]). For LH, treatment, $P < 0.001$. Time, $P < 0.0001$. Treatment $\times$ time, $P < 0.03$. For testosterone, treatment, $P = 0.1929$. Time, $P < 0.002$. Treatment $\times$ time, $P = 0.7190$. *Within treatment, concentrations are different from preinjection concentrations; $P < 0.05$. GNRH1 differences are above error bars, and GNRH2 differences are below error bars.
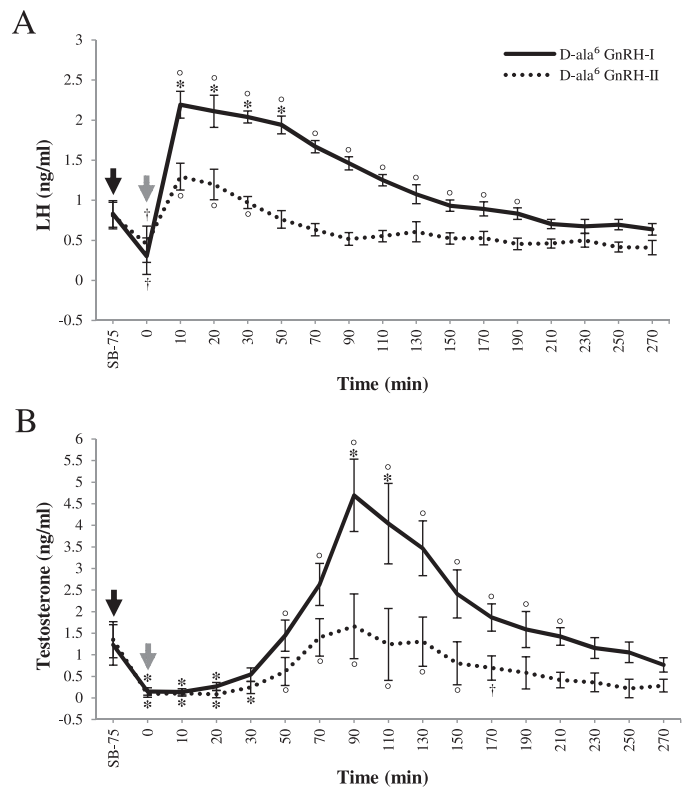

FIG. 7. GNRHR1 antagonist pretreatment mitigates GNRH2-mediated testosterone production. Least squares means ($\pm$SEM) serum concentrations of LH (**A**) and testosterone (**B**) after intramuscular administration of the GNRHR1 antagonist SB-75 at 7.5 h prior to intravenous treatment with D-ala^6 GnRH-I (solid line) or D-ala^6 GnRH-II (dotted line). After pretreatment sampling, SB-75 was administered (black arrow). Approximately 7.5 h later, blood was collected, and treatments were administered (0 min [gray arrow]). For LH, treatment, $P < 0.0001$. Time, $P < 0.0001$. Treatment $\times$ time, $P < 0.005$. For testosterone, treatment, $P = 0.1100$. Time, $P < 0.0001$. Treatment $\times$ time, $P < 0.10$. *Within treatment, concentrations are different from preinjection concentrations; $P < 0.05$. °Within treatment, concentrations are different from post-SB-75 concentrations (0 min); $P < 0.05$. †Within treatment, concentrations tend to differ from preinjection concentrations; $P < 0.10$. GNRH1 differences are above error bars and GNRH2 differences are below error bars.

We observed a treatment $\times$ time interaction ($P < 0.04$) for change in testosterone concentrations (Fig. 8B). After treatment with SB-75, serum testosterone levels were significantly reduced at 2.5, 3, 6, and 12 h compared with pretreatment values ($P < 0.05$) (Fig. 8B). After administration of Trp-1, serum testosterone concentrations were unaffected over time ($P > 0.05$).

DISCUSSION

Despite identification of mammalian GNRHR2 in 2001 [26, 27], the role of this novel receptor has been elusive [25]. However, complete conservation of the ligand sequence and presence of a functional receptor gene in just a few mammalian species [51] indicates an important biological function [17]. Thus far, most mammalian studies have examined the interaction between GNRH2 and its receptor as it relates to nutrition and reproductive behavior of old world monkeys and musk shrews [30–34, 52–54]. In fact, only a handful of studies have examined the porcine *GNRHR2* gene [21, 28, 29] or functionality (IP$_3$ and LH production) of the receptor in vitro [20]. Although these reports indicated that the porcine GNRHR2 was functional, they did not divulge a biological
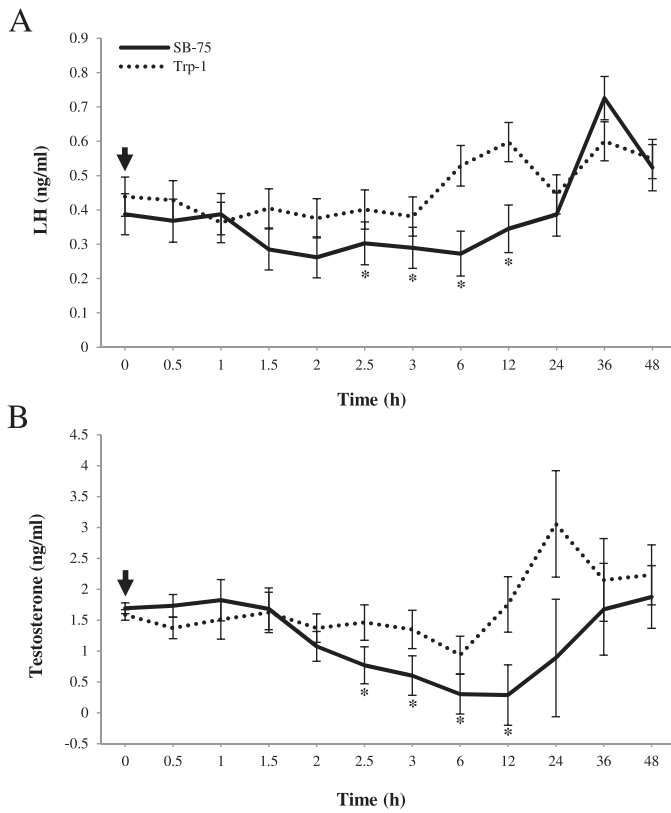
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A

B

FIG. 8. The GNRHR1 antagonist SB-75 but not the GNRHR2 antagonist Trp-1 reduces serum LH and testosterone concentrations. Least squares means (±SEM) serum concentrations of LH (**A**) and testosterone (**B**) after intramuscular administration of a GNRHR1 (SB-75 [solid line]) or GNRHR2 (Trp-1 [dotted line]) antagonist. After pretreatment (−30 to 0 min) sampling, treatments were administered (0 min [arrow]). For LH, treatment, $P = 0.2644$. Time, $P < 0.0001$. Treatment × time, $P = 0.2220$. For testosterone, treatment, $P = 0.2923$. Time, $P < 0.0001$. Treatment × time, $P < 0.04$. *Within the SB-75 treatment, concentrations are different from preinjection levels; $P < 0.05$ (below error bars).

role for GNRHR2 in pigs. As swine are one of the few mammalian species to maintain both the ligand and the functional receptor [28], we sought to examine the role of GNRH2 and its receptor in the reproductive physiology of pigs.

Evidence for *GNRHR2* mRNA in the anterior pituitary gland and testis has been reported previously in humans [26] and marmosets [27], but this is the first report of GNRHR2 protein in porcine tissues. Given the ubiquitous expression of GNRHR2 [26, 27], the presence of this receptor in the anterior pituitary gland and testis of the boar may not be surprising; however, quantitation of Western blots revealed GNRHR2 levels were greater in the testis than the anterior pituitary gland. Similarly, Millar et al. [27] demonstrated that the marmoset testis expressed the greatest amount of *GNRHR2* mRNA compared to any other tissue, including the anterior pituitary gland. Additionally, we detected prepro-GNRH2 in porcine tissue homogenates of the hypothalamus, anterior pituitary gland, and testis. Indeed, GnRH2 (mRNA or protein) has been detected in the hypothalamus [13, 16, 55], pituitary [16, 56], and testis [16, 57] of other species; however, this is the first report of GNRH2 production in pig tissues. Moreover, relative concentrations of prepro-GNRH2 in tissue homogenates have not been quantitated in other species. Concentrations of GNRH2 were greater in the testis than in the hypothalamus or anterior pituitary gland, suggesting a functional significance in porcine testis biology.

Here, we report immunoreactive staining for GNRH2 and its receptor in the swine testis. Interestingly, GNRH2 and GNRHR2 immunostaining was most intense within the tubular and interstitial compartments, respectively. Although little GNRH2 staining was detected in the interstitium, this may be a consequence of the ligand being bound to its receptor or loss of the secreted decapeptide during tissue processing. Nevertheless, it appears GNRH2 and its receptor may interact in a paracrine manner within the porcine testis. Notably, we detected intense GNRHR2 immunostaining within the interstitial compartment, localizing to Leydig cells. Logically, the identification of GNRHR2 on Leydig cells may indicate a potential role in steroidogenesis. This discovery, coupled with previous literature [35, 36], prompted us to directly test if GNRH2 alone could elicit testosterone secretion in explant cultures of testicular tissue from boars.

We demonstrated that ex vivo testicular explants secreted testosterone after stimulation with hCG, a finding consistent with those of previous studies [36, 58]. Notably, we observed that short-term treatment with GNRH2 stimulated testosterone release similar to treatment with hCG, indicating that GNRH2 is a direct stimulator of testosterone secretion within the testis. However, pretreatment with GNRH2 before hCG treatment did not elicit a synergistic release of testosterone, suggesting that GNRH2 is not priming LH receptor activity. Therefore, it appears that GNRH2 alone is sufficient to stimulate testosterone secretion in vitro. These results have precedent in species which maintain a testicular GNRHR1. Recall that GNRH1 and its receptor have been implicated in localized testosterone release [11, 12, 59] as the GNRHR1 has been identified on Leydig cells in other species [60–65]. Given that the GNRHR1 could not be isolated in porcine testis [36], the testicular GNRH2-GNRHR2 system present in pigs might function in lieu of the GNRH1-GNRHR1 system.

Based on these data, we next examined the effect of GNRH2 treatment in vivo. In the present study, males challenged with GNRH1 and GNRH2 demonstrated differential LH secretion over time. Of course, it is well known that GNRH1 stimulates LH production in the boar [66]. Additionally, high doses of GNRH2 stimulate low levels of LH secretion in other species [67]. However, this effect is likely mediated through the interaction between GNRH2 and GNRHR1. In the current study, LH was only transiently elevated in GNRH2 compared to GNRH1 treated boars, indicating that GNRH2 activated GNRHR1 within the anterior pituitary gland. Despite lower LH levels, testosterone concentrations in males treated with GNRH2 mirrored levels seen in GNRH1-treated animals. These results suggest that GNRH2 elicited testosterone secretion directly at the level of the testis, likely via interaction with its cognate receptor on Leydig cells.

In the next trial, we wanted to determine whether SB-75, an established GNRHR1 antagonist, impacted testicular GNRHR2 function, by pretreating boars with SB-75 prior to infusion with GNRH1 or GNRH2. The secretory patterns of LH following GNRH1 and GNRH2 treatment (Fig. 7A) were similar to the results from Trial 1 (Fig. 6A), confirming that GNRH2 is an ineffective stimulator of LH release in the boar. Furthermore, GNRH2 treatment restored testosterone levels from suppression after administration of SB-75 but to a lesser extent than GNRH1. These results for GNRH1- and GNRH2-induced testosterone secretion differed from testosterone profiles observed in Trial 1 (Fig. 6B). This discrepancy between GNRH1- and GNRH2-stimulated testosterone secretion in Trial 2 (Fig. 7B) compared to that in Trial 1 (Fig. 6B) could be due to the reduced levels of LH secretion stimulated by GNRH2 (Fig. 7A). However, the secretory patterns of LH from

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Trials 1 (Fig. 6A) and 2 (Fig. 7A) appeared quite similar; suggesting that the GNRH2-induced LH secretion observed in Trial 2 (Fig. 7A) should be sufficient to stimulate testosterone secretion similar to GNRH1. Thus, we focused on the major differences between Trials 1 and 2, which was the administration of SB-75. SB-75 has been reported to bind GNRHR2 in cell culture experiments [49]. If SB-75 bound to GNRHR2 within the testis, the GNRHR2 agonist would have been ineffective at eliciting testosterone secretion. This is consistent with data from Zanella et al. [36] who demonstrated that SB-75 can effectively inhibit porcine testicular GNRHRs. Moreover, the affinity of SB-75 for the GNRHR2 is not yet defined in the literature. Therefore, our data suggest that SB-75 can interact with the sole GNRHR present within the boar testis, the GNRHR2, to inhibit GNRH2-stimulated testosterone secretion.

In our last experiment, treatment with SB-75 suppressed LH and testosterone concentrations, consistent with other reports [68, 69]. Compared to pretreatment levels, however, neither LH nor testosterone concentrations were significantly reduced by Trp-1 treatment at any time points examined. The limited inhibition of testosterone following Trp-1 treatment contradicts the potent stimulatory effect of GNRH2 on testosterone release observed in Trial 1 (Fig. 6B). To our knowledge, this is the first experiment examining the in vivo effects of Trp-1 on endocrine function, as previous reports have examined the actions of Trp-1 as a prostate cancer treatment in immortalized cell lines and tumors in nude mice [37, 50, 70]. We used a dose of SB-75 (5 μg/kg BW) previously reported to be effective at suppressing LH and testosterone release in boars [71] and chose to use the same amount of Trp-1. Perhaps the level of Trp-1 used was insufficient to fully antagonize the GNRHR2 or the vehicle used (5% dimethylacetamide, 40% propylene glycol, 55% water) released Trp-1 in a different manner than the saline vehicle used for SB-75. Therefore, further investigation into the biological action of Trp-1 on endocrine function is merited.

To conclude, these data confirm that the pig transcribes the GNRHR2 gene into a functional protein. Moreover, we discovered that boars maintain a unique testicular GNRH2-GNRHR2 system. Here, we provide evidence for functionality of the GNRHR2 in vitro and in vivo. Our data suggest that GNRH2 is produced locally in the testis for interaction with its receptor. Given the identification of the GNRHR2 on Leydig cells in this study, we propose that GNRH2 and its receptor may function together to mediate localized testosterone secretion in the porcine testis.

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