Gonadotrope Plasticity at Cellular and Population Levels

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Hormone-secreting cells within the anterior pituitary gland may form organized and interdigitated networks that adapt to changing endocrine conditions in different physiological contexts. For gonadotropes, this might reflect a strategy to cope with acute changes throughout different female reproductive stages. The current study examined gonadotropes in female mice at characteristically different hormonal stages: prepubertal, postpubertal, and lactating. Gonadotrope plasticity was examined at the level of the whole population and single cells at different stages by imaging both fixed and live pituitary slices. The use of a model animal providing for the identification of selectively fluorescent gonadotropes allowed the particular advantage of defining cellular plasticity specifically for gonadotropes. *In vivo* analyses of gonadotropes relative to vasculature showed significantly different gonadotrope distributions across physiological states. Video microscopy studies using live slices *ex vivo* demonstrated pituitary cell plasticity in the form of movements and protrusions in response to GnRH. As positive feedback from rising estradiol levels is important for priming the anterior pituitary gland for the LH surge, experiments provide evidence of estradiol effects on GnRH signaling in gonadotropes. The experiments presented herein provide new insight into potential plasticity of gonadotropes within the anterior pituitary glands of female mice. **(*Endocrinology* 153: 4729–4739, 2012)**

The reproductive axis is dependent on communication among the hypothalamus, pituitary gland, and gonads (the hypothalamic-pituitary-gonadal axis). Some anterior pituitary cells are organized in complex networks that adapt to changing endocrine conditions in different physiological contexts (1–5). For gonadotropes, this might reflect a strategy to cope with acute changes throughout different female reproductive stages. For example, plasticity may be essential for rapid changes that are important for exquisitely timed hormonal surges such as that of LH needed to drive ovulation (6). The adult anterior pituitary gland adapts and responds to the cyclic fluctuations of hormones throughout the different hormonal stages a female experiences as well as across other major epochs of the life span including from prepubertal to postpubertal, pregnancy, and lactation. Although lactotropes are well documented as a population of cells that demonstrate plasticity in the anterior pituitary gland (7, 8), there is less evidence for plasticity in other populations of cells.

GnRH released from the median eminence of the hypothalamus in a pulsatile manner acts upon gonadotropes in the anterior pituitary gland to result in the release of LH and FSH into the pituitary vasculature. Although inhibitory or negative feedback of gonadal hormones control the hypothalamic-pituitary-gonadal axis, in females, preovulatory estradiol (E_2) changes from providing negative feedback to positive feedback. For successful ovulation, a large increase in circulating E_2 provides positive feedback at

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Abbreviations: ACSF, Artificial cerebrospinal fluid; AP, anterior pituitary; CAGS, chicken β-actin promoter and cytomegalovirus enhancer; DMSO, dimethylsulfoxide; E_2, estradiol; eR26-τGFP, ROSA26-CAGS-τGFP; GFP, green fluorescent protein; τGFP, fusion of the microtubule-associated protein τ with GFP; GnRHR, GnRH receptor; GRIC, GnRHR-IRES-Cre; PP, posterior pituitary; R26-YFP, ROSA26-YFP; YFP, yellow fluorescent protein.

both the hypothalamic and pituitary levels to promote the LH surge. The cellular and molecular events underlying estradiol's action(s) upon the anterior pituitary gland, specifically gonadotropes, remain elusive.

Studies suggest both a genomic and nongenomic site of E_2 action for priming the anterior pituitary gland in response to rising levels of circulating E_2. E_2 has been demonstrated in several species to increase GnRH receptor (GnRHR) number in primary culture including sheep (9), rat (10, 11), and mouse (12). These results have been replicated *in vitro* in dissociated gonadotrope preparations (13), and in transgenic mice with a fragment of the ovine *GnRHR* promoter driving luciferase expression (14). In ovariectomized ewes, E_2 treatment also produces acute and latent effects on LH levels, suggesting a nongenomic and genomic site of action (15). Other studies have suggested a membrane-initiated site of E_2 action in gonadotropes by activating second-messenger signaling cascades and intracellular calcium levels. Iqbal *et al.* (16) reported an increase in intracellular levels of phosphorylated ERK-1/2 immunoreactivity in gonadotropes within 15 min of E_2 treatment.

One purpose of GnRH-induced changes in cell mobilization may be to increase appositions to vascular endothelium and gain more immediate access to the bloodstream for hormone release from basement membranes (17). This hypothesis had reasonable precedent in dynamic experiments using dissociated gonadotropes showing process extension in response to GnRH within 10 min (18, 19), and static experiments showing that GnRH-stimulated gonadotropes developed processes that extended to blood vessels during peak LH secretory episodes (20). Work with other pituitary cell types including corticotropes (1, 21) and somatotrophs (22) all further indicate the potential for pituitary cell plasticity relative to vasculature.

The current study examined gonadotropes in female mice at characteristically different hormonal stages: prepubertal, postpubertal, and lactating. Gonadotrope plasticity was explored at both whole population and single cell levels by imaging both fixed pituitaries and live organotypic pituitary slices. In an initial study, video microscopy used with live slices *ex vivo* demonstrated pituitary cell plasticity in the form of movements and protrusions in response to GnRH (17). In that initial study, the cells were presumed gonadotropes based on responsiveness to GnRH, but they were fluorescent based on nonspecific methods. The subsequent development of a model animal providing for the selective fluorescent identification of gonadotropes provided an advantage for defining cellular plasticity specifically for gonadotropes (23). As positive feedback from rising E_2 levels is important for priming the anterior pituitary gland for the LH surge, experiments within sought to gain a greater understanding of E_2 effects on GnRH signaling in gonadotropes. The experiments presented herein provide new insight into potential plasticity of gonadotropes within the anterior pituitary glands of female mice.

Materials and Methods

Mice

Mice were maintained on a 12-h light, 12-h dark cycle with access to rodent chow (Harlan 2918) and water *ad libitum*. For all experiments, a knock-in mouse model was used in which gonadotropes selectively express yellow fluorescent protein (YFP) (23) or τ-green fluorescent protein (GFP) (24). This model is based on using a cross between two mouse strains: homozygous mice with the *GnRHR* promoter driving Cre recombinase expression [GnRHR-IRES-Cre (GRIC) mice] (23) and homozygous mice with the *ROSA26* promoter with a floxed transcriptional stop signal driving fluorescent protein expression. Two different ROSA26 reporter lines were used in this study. ROSA26-YFP (R26-YFP) reporter mice (25) and ROSA26-CAGS-τGFP (eR26-τGFP) mice (24), which express a fusion of the microtubule-associated protein τ with GFP (τGFP) (26) under control of the chicken β-actin promoter and cytomegalovirus (CMV) enhancer (CAGS) (27) in the *ROSA26* locus after Cre-mediated excision of a stop sequence. When crossed to GRIC mice, Cre recombinase excises the transcriptional stop signal resulting in constitutive expression of YFP or τGFP in the GnRHR and ROSA26-expressing cells (23, 24) (Supplemental Fig. 1, published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org). All experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Animal Welfare Committee of the Universities of Hamburg and the Colorado State University Animal Care and Use Committee. GRIC, eR26-τGFP, and R26-YFP mice were kept in a mixed (129/SvJ and C57BL/6J) background.

Perfusion with rhodamine-coupled gelatin and confocal imaging of whole-mount pituitary gland preparations

Gelatin was coupled to rhodamine following a protocol by Hashimoto *et al.* (28). Briefly, 20 g bovine gelatin (G9391; Sigma-Aldrich, Irvine, UK) were dissolved in 75 ml distilled water in a boiling water bath for 2 h. The pH of the solution was adjusted to 11.0 with 1 N NaOH, and the final concentration of gelatin was adjusted to 20%. One hundred milligrams of rhodamine B isothiocyanate (R1755; Sigma-Aldrich) were dissolved in 5 ml dimethylsulfoxide (DMSO; Sigma-Aldrich), added to the gelatin solution and allowed to react under mild agitation in the dark at 37 C overnight. The rhodamine-conjugated gelatin was then dialyzed (dialyzing tube D9527; Sigma-Aldrich) in the dark at 37 C against 0.01 M sodium PBS containing 0.01% NaN_3 (pH 7.4). The dialyzing buffer was changed every 12 h. Dialysis continued for 7 d until no free rhodamine B isothiocyanate was found in the dialyzing buffer. The rhodamine-conjugated gelatin solution was subsequently solidified and stored in the dark at 4

C. Pre- (3 wk old, n = 6) and postpubertal [12 wk old; proestrous stage, n = 6 (animals killed during LH surge); diestrous stage, n = 6; as assessed by vaginal cytology] as well as lactating (12–15 wk old, n = 6; 6–9 suckling pubs per litter) females were anesthetized with ketamine/xylazine (Bayer, Leverkusen, Germany) and perfused intracardially with 4 ml of PBS containing 20 IU heparin, followed by 6 ml of 10% rhodamine-coupled gelatin (warmed to 37 C) in PBS. After perfusion animals were kept for 15 min on ice. Pituitary glands were removed and postfixed for 2 h in Roti-Histofix 4% (Roth, Karlsruhe, Germany). Whole pituitaries were mounted on glass slides (Roth), and the ventral side was analyzed using a confocal laser-scanning microscope (Fluoview 1000; Olympus, Tokyo, Japan). Z-stack images extending 68 μm deep into the tissue (z-axis) were taken every 4 μm. Photographic images were processed using Imaris (Bitplane, Zürich, Switzerland) and Adobe's Photoshop and Illustrator CS5 software (Adobe Systems, San Jose, CA).

Pituitary gland dissociation and quantification of gonadotropes

To determine the total number of gonadotropes per animal, pituitary glands of pre- (3 wk old, n = 8) and postpubertal (8–15 wk old, n = 9) GRIC/eR26-τGFP females were harvested and acutely dissociated as previously described (29). τGFP+ cells were counted using a Neubauer improved hemocytometer. Three independent counts were performed per pituitary gland. Numbers were analyzed by two-tailed Student's *t* test. Differences were taken as significant when $P < 0.05$.

To determine the relative number of gonadotropes in the caudal, lateral, and rostral (area of the pars tuberalis) parts of the anterior pituitary, respectively, we analyzed confocal z-stacks of entire pituitary glands from pre- (n = 3) and postpubertal females (n = 3) imaged from the ventral side as described above. The 1.4 mm^3 volumes [317 μm (x-axis) × 317 μm (y-axis) × 14 μm (z-axis)] were randomly chosen within the respective areas (n = 2–8) and τGFP+ cells within these volumes were counted using Imaris software (Bitplane). Numbers were analyzed by one-way ANOVA followed by Student Newman-Keuls multiple comparison test. Differences were taken as significant at $P < 0.05$.

Confocal time-lapse video microscopy of pituitary gland slices

Adult GRIC/R26-YFP females were deeply anesthetized with isofluorane and decapitated. The pituitary was rapidly removed from the skull and submerged in ice-cold sucrose-modified artificial cerebrospinal fluid (ACSF) containing (in millimoles) 125 NaCl, 25 $NaHCO_3$, 1.25 NaH_2PO_4, 2.5 KCl, 0.05 $CaCl_2$, 6 $MgCl_2$, 2.5 glucose, 20 sucrose, and 3 kynurenic acid. This solution was continuously oxygenated with Carbogen (95% O_2 and 5% CO_2) to maintain a pH of 7.2–7.3. The pituitary was then embedded in 2% agarose made up with ACSF containing (in millimoles) 125 NaCl, 25 $NaHCO_3$, 1.25 NaH_2PO_4, 2.5 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 2.5 glucose and 22.5 sucrose. Coronal slices of 200 μm thickness were prepared on a vibrating microtome (Leica VT1200S) in ice-cold oxygenated sucrose-modified ACSF and incubated in a holding chamber for a least 30 min at room temperature in oxygenated ACSF. Slices were transferred to an experimental chamber filled with HEPES-buffered ACSF (pH 7.4) containing (in millimoles) 10 HEPES, 135 NaCl, 5 KCl, 2 $CaCl_2$, 2 $MgCl_2$, and 15 glucose and secured in place with a weighted net. Images were captured on a confocal laser-scanning microscope (Fluoview 1000; Olympus) at ×20 magnification at intervals of 5 min over periods of up to 3 h. After 30 min of baseline video, GnRH was added to a final concentration of 20 nM.

Time-lapse video microscopy of organotypic pituitary gland slices

Adult female mice 2–8 months of age were killed in the diestrus 1 phase of the estrus cycle as assessed by vaginal cytology. Murine pituitaries were dissected in cold Krebs' solution (126 mM NaCl; 2.5 mM KCl; 2.5 mM $CaCl_2$; 1.2 mM $MgCl_2$; 1.2 mM NaH_2PO_4; 11 mM glucose; 25 mM $NaHCO_2$) embedded in 8% agarose (type VII-A; Sigma-Aldrich; maintained as liquid at 39 C) for sectioning at 200 μm in the sagittal plane using a vibrating microtome (Leica VT1000S; Leica Microsystems, Heidelberg, Germany). Slices were then placed in a postcutting sterile filtered Kreb's cutting buffer containing 0.01 M HEPES, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 0.1 mg/ml gentamicin for a minimum of 15 min but no longer than 2 h. Slices chosen for video were then plated on glass-bottom, 35-mm culture dishes (Mat Tek, Ashland, MA) that were coated with poly-D-lysine and collagen (Advanced BioMatrix PureCol, San Diego, CA; purified bovine collagen solution no. 5005-B). Slices were then covered with 40 μl collagen solution containing 1 ml collagen, 125 μl 10× MEM, 23 μl pen-strep (10,000 U penicillin and 10 mg/ml streptomycin), and 33 μl of 1 M sodium carbonate. This was allowed to polymerize for up to 1 h in a high humidity incubator. One milliliter of serum-free media was added to the slices [adult neurobasal medium (GIBCO BRL Laboratories, Gaithersburg, MD) with B27 supplement and supplemented further with 25 μM glutamate, 1.34 mM glutamine, 0.5% glucose, 134 U/ml penicillin, and 0.13 mg/ml streptomycin]. Slices were kept at 36 C in an incubator with 5% CO_2. For estradiol treatment studies, slices were treated with one of three hormone treatment durations: long-term 10 nM E_2 (>14 h before time of video acquisition), short-term 10 nM E_2 (treated at time of video acquisition, therefore before GnRH treatment exposure for 1.5 h), or vehicle treated (1 μl per 1ml of 100% ethanol.)

Time-lapse video microscopy was conducted 1–2 d after pituitaries were harvested to allow for estradiol treatment paradigms. There were no differences in results between slices examined at 1 *vs.* 2 d after harvesting. Slices were maintained at 36–37 C for the duration of video recording. Images were captured using a Hamamatsu C10600 ORCA camera interfaced with a Dell Precision T3500 computer using Metamorph software (version 7.0; Universal Imaging Corp., Downingtown, PA). Images were acquired every 5 min for a total video time of 3 h with each frame exposed for 30 msec using a YFP filter set (23). The first 21 frames (1 h 30 min) were vehicle, long-term 10 nM E_2, or short-term 10 nM E_2. The last 21 frames (1 h 30 min) were in the presence of 100 nM GnRH.

Images were analyzed for movement during baseline hormone treatment (1.5 h) and during GnRH treatment (1.5 h). The percentage of cells with process extensions was quantified throughout the video duration (3 h) along with the time dependence for the first, second, and third 30-min segments after GnRH treatment (note that some cells were responding in each segment of the video or possibly two of three time periods.) Five cells were analyzed from each video with prominent process ex-

tensions after GnRH treatment to quantify average process length from edge of cell to end of process extension using National Institutes of Health ImageJ software (version 1.37v; Bethesda, MD) and the data are reported in micrometers.

Results

Experimental strategy

The distribution of gonadotropes in the anterior pituitary at different female reproductive stages was analyzed using GRIC mice to express fluorescent markers specifically in these cells (23). GRIC mice carry a mutant *GnRHR* allele, which is transcribed into a bicistronic mRNA, from which the GnRHR and Cre recombinase are independently translated (23). Previous experiments had shown that greater than 99% of gonadotropes coexpress Cre recombinase with the GnRHR in these animals (23). GRIC mice were bred to eR26-τGFP mice (24), which express a fusion of the microtubule-associated protein τGFP (26) under control of the chicken β-actin promoter and cytomegalovirus (CMV) enhancer (CAGS) (27) in the *ROSA26* locus after Cre-mediated excision of a stop sequence (Supplemental Fig. 1). The CAGS fragment boosts expression levels in the *ROSA26* locus (30) and thus provides a robust fluorescent signal during extensive confocal imaging (24). GRIC/eR26-τGFP mice were born with Mendelian frequencies and were fertile.

Plasticity of the gonadotrope population in the anterior pituitary gland

Gonadotropes are homogeneously distributed in the anterior pituitary before puberty

The distribution of the gonadotrope population was analyzed in prepubertal (3 wk old) GRIC/eR26-τGFP females with blood vessels labeled with rhodamine-coupled gelatin (Fig. 1A). The whole-mount preparations of entire pituitary glands were imaged from their ventral side using confocal microscopy (Fig. 1, A–C). Gonadotropes were homogeneously distributed throughout the lateral, caudal, and rostral (pars tuberalis) areas of the anterior pituitary (AP) (Fig. 1, B, D–F) but absent in the posterior pituitary (PP) (Fig. 1B). Consistent with this, gonadotrope numbers in the lateral, ventral, and rostral areas of the AP were not significantly different in the prepubertal animals [205.6 ± 21.7 gonadotropes per 1.4 mm^3 volume in the rostral area compared with 194 ± 12.3 gonadotropes in the lateral and 171.2 ± 13.5 gonadotropes in the caudal area ($P > 0.05$, Fig. 1G)]. Irrespective of their relative position within the AP, the gonadotropes were always located close to blood vessels (Fig. 1, D–F).

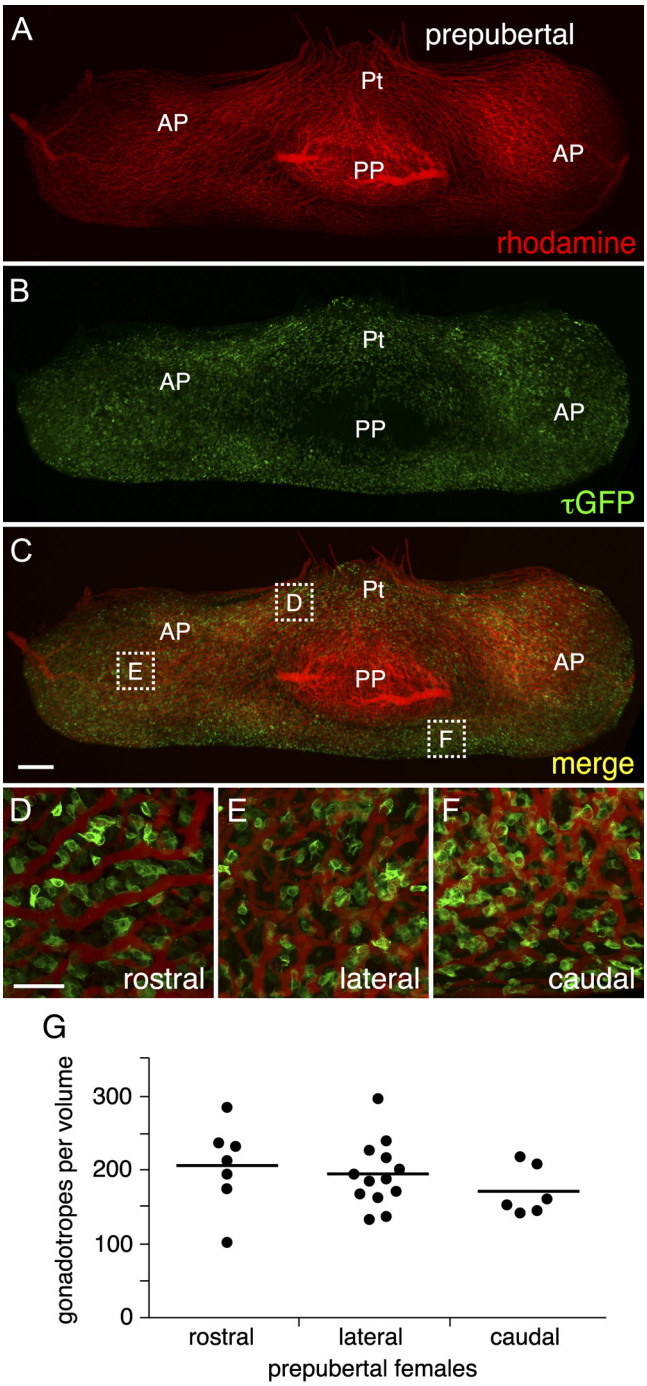

FIG. 1. Gonadotropes were homogeneously distributed in the anterior pituitary before puberty. Ventral view of a three-dimensional reconstruction (68 μm z-stack) of a whole-mount preparation of an entire pituitary gland prepared from a female 3-wk-old GRIC/eR26-τGFP mouse is shown. A, Vascular architecture was visualized with rhodamine-coupled gelatin (*red*). Note the hypophyseal portal vessels located in the pars tuberalis (Pt) and the capillary bed of the AP and PP. B, Gonadotropes (*green*) were visualized homogeneously distributed throughout the AP but were absent from the PP. C, Merge. Gonadotropes were homogeneously distributed throughout the rostral (D), lateral (E), and caudal (F) areas of the AP. G, Quantification of gonadotrope cells. *Scale bars*, 200 μm (A–C), 50 μm (D–F).

Reorganization of the gonadotrope population during reproductive maturation

The prepubertal gonadotrope distribution was strikingly different from that in postpubertal females (Fig. 2). Clear changes were noted in the relative distribution of gonadotropes within the gland. The number and density of gonadotropes was greatly increased in the rostral (area of the pars tuberalis), compared with the lateral and ventral areas of the AP in postpubertal animals (Fig. 2, C, G–I). There were 251.2 ± 12.5 gonadotropes per 1.4 mm^3 volume in the rostral area compared with 156 ± 8 gonadotropes in the lateral and 167.7 ± 11.6 gonadotropes in the caudal area (Fig. 2J, ***, $P < 0.001$). The total number of gonadotropes was increased (*, $P < 0.05$) from 15826 ± 1527 cells in the prepubertal animals (n = 8) to 26617 ± 3326 cells in postpubertal animals (n = 9) (Fig. 2K). Taken together, these data suggest a major reorganization of the gonadotrope population during reproductive maturation. To address whether gonadotropes are redistributed in the AP at different stages of the estrous cycle, we examined the distribution of the gonadotrope population in cycling GRIC/eR26-τGFP mice at diestrus (Fig. 2, A, C, and E) and proestrus (Fig. 2, B, D, and F). There were, however, no gross differences in the distribution of the gonadotrope population between pro- and diestrus.

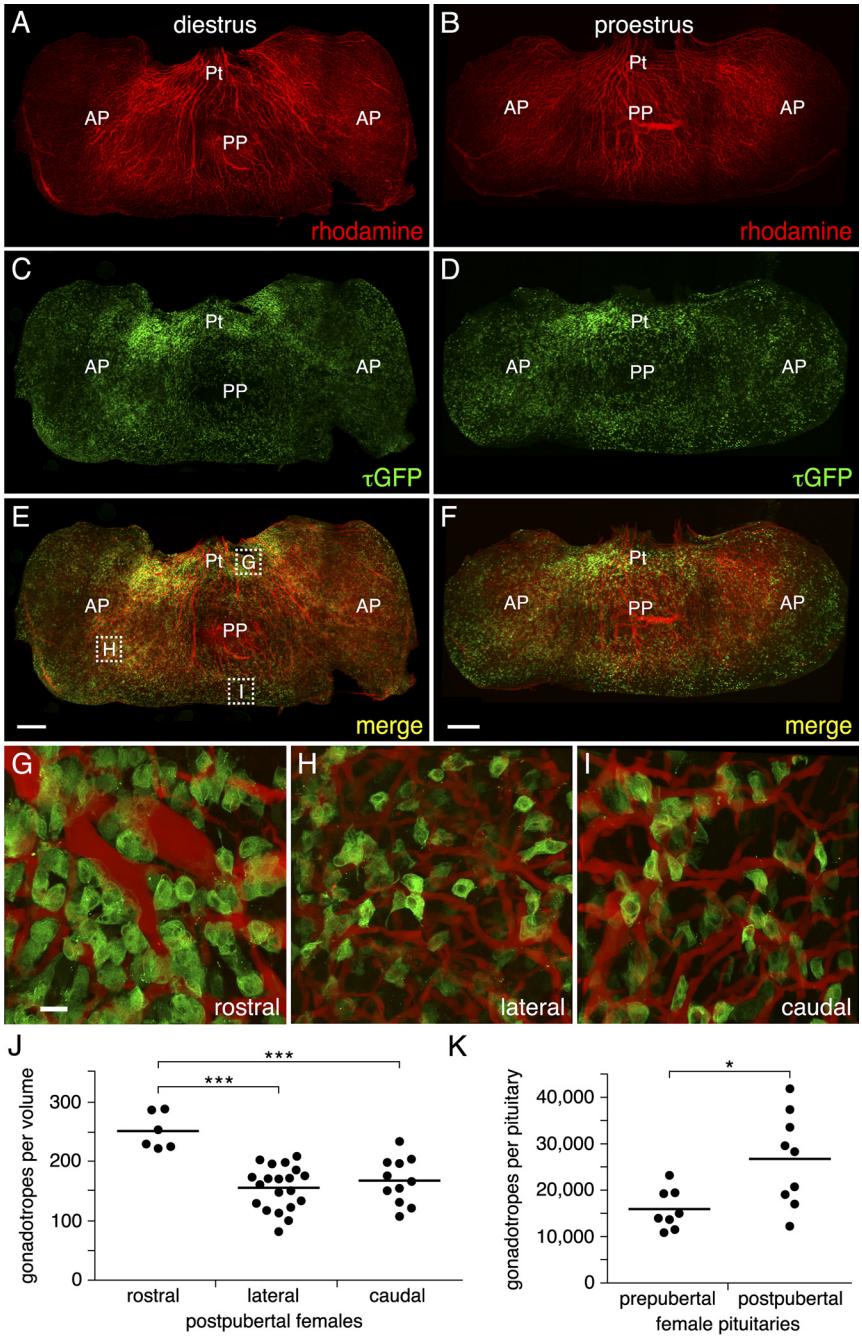

FIG. 2. Gonadotropes were concentrated at the pars tuberalis in the anterior pituitary after puberty. Ventral view of a three-dimensional reconstruction (68 μm z-stack) of a postpubertal female pituitary gland prepared from a 14-wk-old GRIC/eR26-τGFP mouse at diestrus (A, C, E, G, H, and I) and proestrus (B, D, and F) is shown. Vascular architecture was visualized with rhodamine-coupled gelatin (*red*; A and B). Gonadotropes (*green*) were concentrated at the pars tuberalis (Pt) (C and D). E, and F, Merge. The overall distribution of gonadotropes within the AP did not change during the different stages of estrus. J, Increased number of gonadotropes in the Pt in postpubertal females. K, The total number of gonadotropes per animal increased during reproductive maturation. *Scale bars*, 300 μm (A–C), 50 μm (D–F).

Redistribution of gonadotropes during lactation

The distribution of the gonadotrope population in lactating GRIC/eR26-τGFP mice was strikingly different from both prepubertal and postpuberal cycling mice. Pituitary glands of animals (n = 6) 1 wk into lactation (six to nine suckling pubs per litter) were found to contain discrete clusters of gonadotropes in the lateral and ventral areas of the AP (Fig. 3B). Such clusters contained 17.4 ± 1.5 cells (19 clusters analyzed from three animals; Fig. 3D). Taken together, these data demonstrate plasticity suggesting that major reorganizations of gonadotropes occur in the female pituitary during the life span.

Plasticity of the Pituitary Gland at the Single Gonadotrope level

Gonadotropes form protrusions to contact blood vessels

Previous studies raised the possibility that gonadotropes may display plas-

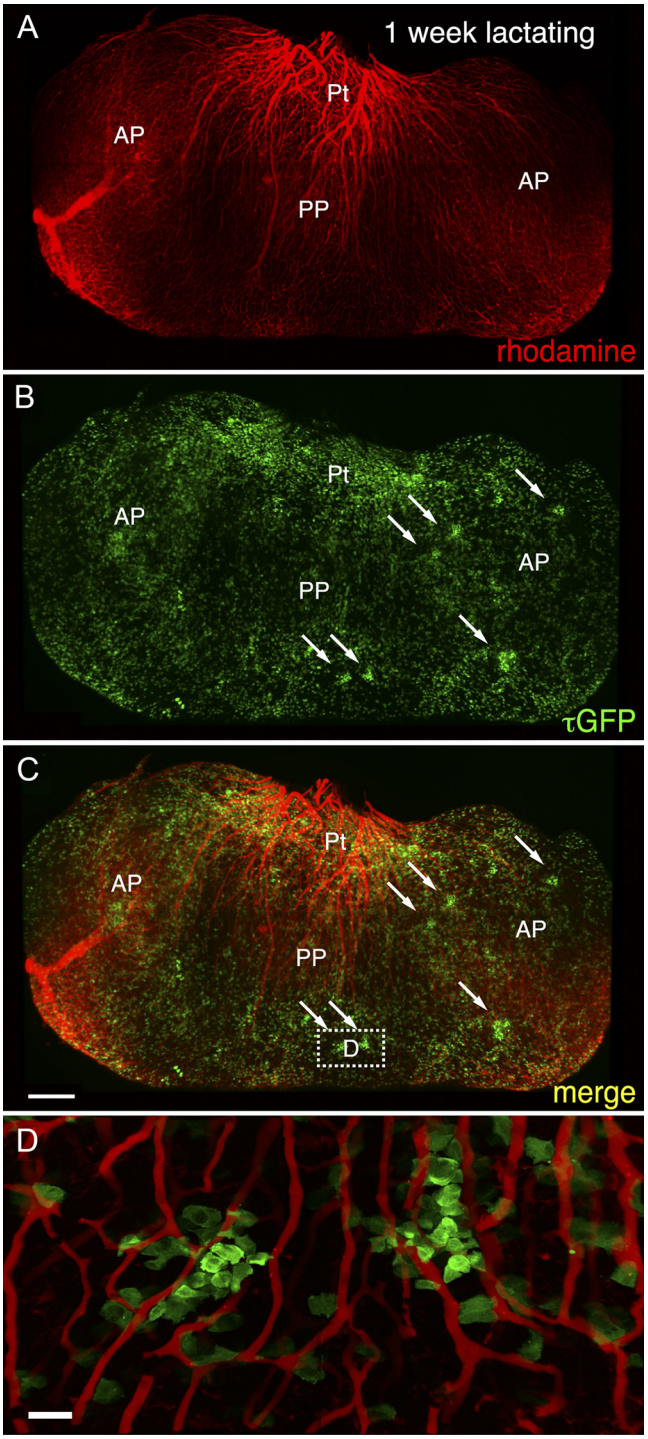

FIG. 3. Redistribution of gonadotropes during lactation. Ventral view of a three-dimensional reconstruction of an adult GRIC/eR26-τGFP pituitary after 1 wk of lactation and after perfusion with rhodamine-coupled gelatin (*red*; A, C, and D) is shown. The *arrows* highlight clusters of gonadotropes (*green*; B, C, and D) that have formed in the AP of a lactating GRIC/eR26-τGFP mouse. *Scale bar*, 300 μm (A, B, and C). D, The box in panel C at higher magnification. The *scale bar* (D), 30 μm.

ticity at the cellular level and form protrusions to contact blood vessels (17). Consistent with this, individual frames from a 60-min confocal time-lapse movie of an acute pituitary slice prepared from an adult GRIC/R26-YFP mouse illustrate the formation of a new protrusion in the direction of a blood vessel (Fig. 4). Gonadotropes forming protrusions were found with a frequency of 19.6 ± 3.7% (487 gonadotropes analyzed from five animals) and suggest gonadotrope plasticity at the single-cell level. Interestingly, we found that many gonadotropes came close to more than one blood vessel through multiple protrusions (Fig. 5). Quantitative analysis (178 gonadotropes in three different pituitary areas from two animals analyzed) of three-dimensional reconstructions of postpubertal female pituitary glands showed that only 4 ± 0.2% of gonadotropes were not in apparent apposition to any blood vessel. In contrast, 23.9 ± 2.7% of gonadotropes were found to come close to one, 50 ± 1.7% were found to come close to two, 15.2 ± 3.3% were found to come close to three, and 6.8 ± 0.3% were in apparent apposition to four or more blood vessels.

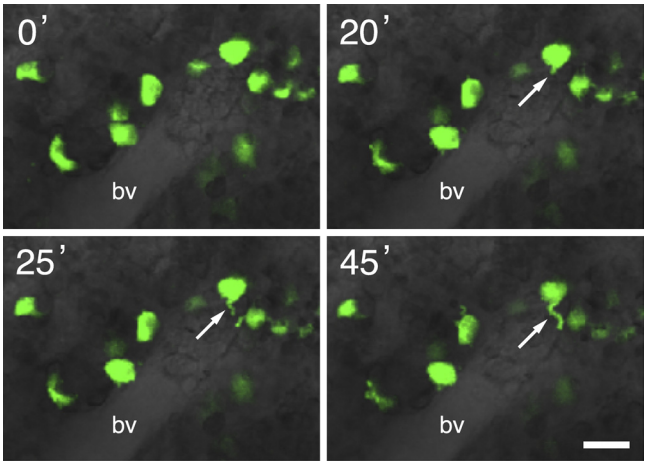

FIG. 4. Confocal time-lapse imaging. Individual image frames from a 60-min confocal time-lapse video of an acute 200-μm pituitary slice prepared from a GRIC/R26-YFP mouse. *Arrows* mark a new protrusion forming in the direction of a blood vessel (bv).

Long-term exposure to estradiol increases responsiveness of gonadotropes to GnRH

Time-lapse video microscopy of live murine pituitary slices was further used to analyze cellular plasticity induced by E_2 treatment of different durations in combination with GnRH treatment. In Fig. 6, before and after GnRH exposure, gonadotrope morphology is portrayed with slices treated long-term with E_2 (>14 h). Process extensions formed only after GnRH stimulation (*e.g.* Fig. 6). There was no impact on the percentage of gonadotropes with process extensions with 10 nM estradiol treatment, short 1.5 h or long greater than 14 h, compared with vehicle at baseline video in the absence of GnRH treatment (Fig. 7A). However, after GnRH treatment, gonadotropes in slices exposed longer term to 10 nM E_2 (n = 11 pituitary

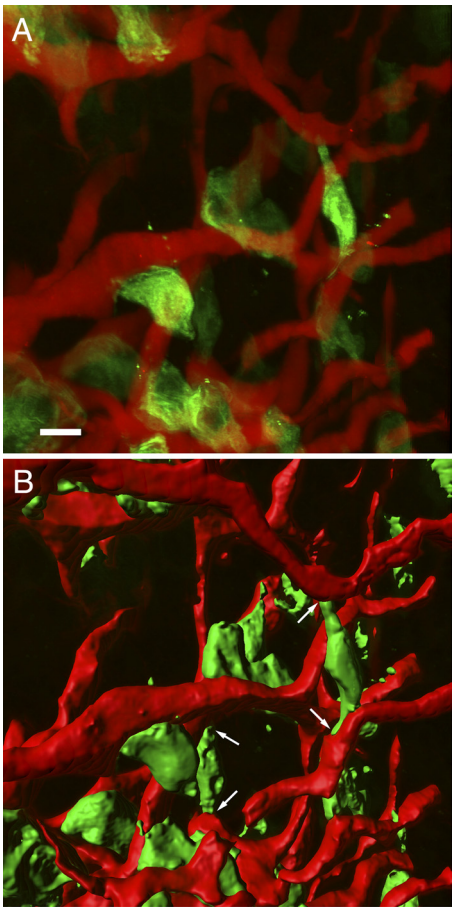

FIG. 5. Gonadotropes contact multiple blood vessels. Three-dimensional reconstruction (20 μm z-stack) of the caudal area of postpubertal female pituitary gland prepared from a 14-wk-old GRIC/eR26-τGFP mouse at diestrus after perfusion with rhodamine-coupled gelatin is shown. The *arrows* mark gonadotrope protrusions extending in the direction of blood vessels (*red*). B, A three-dimensional surface rendering (Imaris) of A for better visualization of blood vessels. *Scale bars*, 15 μm.

slices) had a significantly greater percentage of gonadotropes with process extensions with an average of 41.8% gonadotropes compared with short-term estradiol treatment (n = 9 pituitary slices) with 21.1% and vehicle (n = 8 pituitary slices) with 26.1% gonadotropes in a given video field (Fig. 7B) [$F(2, 25) = 5.96$; $P < 0.01$].

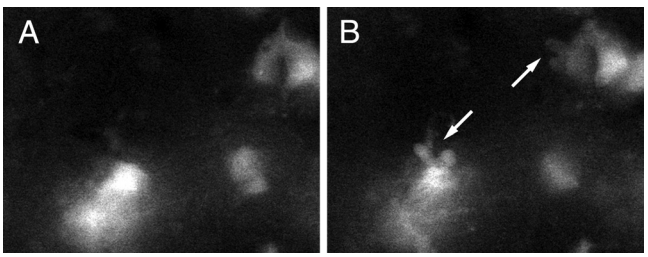

FIG. 6. Gonadotrope process extension with long-term E_2 and GnRH exposure. A, Organotypic pituitary gland slices were exposed to E_2 for 14 h. After 100 nM GnRH, processes extended from several of the YFP+ gonadotropes (B; *white arrows*).

No change in time-dependent process extension activation

Gonadotropes responding with a protrusion were often visualized within 5 min of GnRH treatment, consistent with prior results (17). However, there was no time-dependence observed in gonadotropes that responded to GnRH between treatment groups divided into three 30-min periods (Fig. 7B). Some cells were seen responding in one, two, or three of the three 30-min segments and were included in the count in each segment if responding. Although there appears to be a difference between 30 and 60 min of video acquisition in short-term and long-term estradiol treatment (Fig. 7B) [$F(4, 50) = 2.33$; $P < 0.07$], the difference did not reach statistical significance. It appeared that more gonadotropes responded within the first 30 min than those responding in the last 30 min; however, it was also not statistically significant.

No difference observed in process extension length with estradiol treatment

Time-lapse video microscopy was analyzed for process extension length quantified by measuring from the edge of a cell to end of process extension using ImageJ software (National Institutes of Health). Reported in micrometers, the average length between all three treatment groups (vehicle, 1.5 h E_2, > 14 h E_2) was similar (Fig. 7C), suggesting an all-or-none response of protrusions. If a gonadotrope responded to GnRH, a process extension was approximately 10 μm in length and did not vary significantly with estradiol treatment.

Discussion

The anterior pituitary gland adapts to influence the function of the reproductive axis as one of the major endocrine organs in the body. The experiments presented provide new insight into potential plasticity of the anterior pituitary gland. Using a model animal with cell-restricted fluorescent protein expression, gonadotropes could be examined selectively at both whole-population and individual-cell levels. Gonadotropes within the anterior pituitary gland are a heterogeneous population of cells (23, 31). In a prior study using the same line of mice, only 50% of gonadotropes expressing YFP responded to GnRH by releasing LH quantified by reverse hemolytic plaque assay (23). The current studies add to our understanding of heterogeneity among gonadotropes because only a subpopulation changed after puberty or during lactation relative to pituitary vasculature *in vivo* and only a subpopulation responded with process extensions to GnRH and E_2 at the cellular level *ex vivo*.

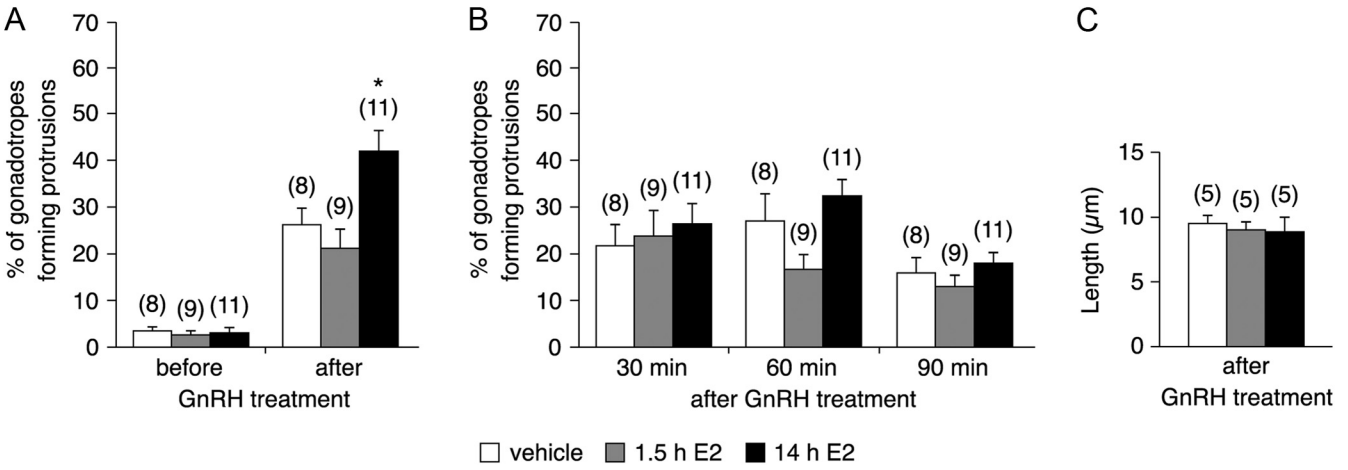

FIG. 7. Long-term estradiol treatment increased GnRH-induced plasticity in live pituitary slices. Adult female GRIC/R26-YFP mice in diestrus 1 with YFP expression in gonadotropes were used for live time-lapse video microscopy of organotypic pituitary gland slices. A, The graph depicts estradiol or vehicle exposure before and after GnRH treatment (100 nM). There was no effect of estradiol treatment alone on the percentage of gonadotropes with process extensions in a given region of interest. Long-term estradiol treatment (14 h+) significantly increased GnRH-induced cell process extension compared with vehicle or 1.5 h E_2 (10 nM). There were no discernible effects of short-term estradiol treatment (1.5 h) on GnRH-treated gonadotropes. Veh: Vehicle (n = 8 pituitary slices); 1.5 h: 1.5 h of E_2 treatment exposure started at start of video acquisition (n = 9 pituitary slices); 14 h: 14 h+ of estradiol treatment before start of video acquisition (n = 11 pituitary slices); *, $P < 0.01$. B, There were no differential effects of estradiol on the process extensions over time. Time-lapse video images were captured from live murine pituitary slices treated with GnRH for 90 min. Video acquisition began at the start of GnRH treatment and was analyzed in 30-min segments to quantify time-dependent cell process extension (note: some cells had process extension for more than one 30 min segment and were counted in each time period they had a process extension.) Estradiol treatment (10 nM) for the short term (1.5 h) and long term (14 h+) was analyzed compared with vehicle (1 μl per 1 ml of 100% ethanol). C, There was no effect of estradiol pretreatment on the length of cell process extensions after GnRH treatment. The lengths of the prominent process extensions from five gonadotropes per slice after GnRH treatment were analyzed to quantify the average length of the process extension. There was no difference between estradiol treatment groups and vehicle. Length is reported in micrometers from the edge of the cell to the edge of the process extension.

Female reproduction is characterized by many unique physiological states corresponding to puberty, gestation, and lactation. Many changes must take place for each of these physiological states to occur, yet few studies have looked at the changes at the level of the anterior pituitary gland on the gonadotrope cell population that secretes FSH and LH. The current study examined the anterior pituitary glands of female mice before and after puberty and during lactation. There was a significant difference between pre- and postpubertal anterior pituitary gland gonadotrope populations in the number of gonadotropes in the whole pituitaries and in the distribution of gonadotropes, particularly in the pars tuberalis. Furthermore, there was a strikingly altered distribution of gonadotropes in the anterior pituitaries of 1-wk lactating females that formed discrete clusters of approximately 17 cells per cluster always in association with blood vessels. Similarly sized clusters were not observed in the other reproductive stages analyzed and suggest a unique plasticity occurring in conjunction with lactation. Although striking plasticity of lactotropes has been reported during lactation in rats (32), a recent study in mice suggests species-specific caution (33), and in neither study was a relationship to vasculature noted.

The pituitary gland is highly vascularized, and reports have indicated that hormone-secreting endocrine cells in rats show a close spatial relationship with blood vessels (1, 5), consistent with the observations in the current study. Many gonadotropes came close to blood vessels and protrusions extended in the direction of pituitary vasculature. Gonadotrope protrusions may extend in the direction of blood vessels to increase the secretory impact of gonadotropes.

As reviewed in the introductory text, numerous studies indicate the importance of E_2 in regulating pituitary sensitivity to GnRH. The current study examined the potential impact of E_2 on gonadotropes in two different ways. First, the distribution of gonadotropes was examined in the pituitary at different stages of the estrous cycle and in relationship to the vasculature. Although we did not observe major gonadotrope population differences between the proestrous and diestrous stages of the estrus cycle, future more detailed studies will be needed to address the question of whether gonadotrope contacts to the vasculature change directly as a function of cyclicity. This might not be unusual, considering the estrous cycle-dependent movement of GnRH neuronal terminals relative to capillary loops in the median eminence (34). The second way in which the influence of E_2 on gonadotropes was assessed was more directly *ex vivo*. Pretreatment with 10 nM E_2 was capable of significantly enhancing gonadotrope respon-

siveness to GnRH with respect to process extension. As in the first case, additional experiments will be necessary to determine whether this motion would be specifically directed toward vasculature *in vivo*.

An increasing number of studies are demonstrating that estradiol can have rapid effects on cell physiology by working through membrane receptors (35–37). Results from the *ex vivo* pituitary slice experiments suggested that estradiol impacts gonadotrope sensitivity to GnRH at the cellular level. Given sufficient time, experiments in the current study showed a stimulatory effect of long-term (14 h) but not short-term (1.5 h) estradiol on the ability of GnRH to stimulate process extensions from identified gonadotropes. Only 25% of gonadotropes responded with process extension(s) to GnRH in the absence of exogenous estradiol. Long-term E_2 treatment, however, increased the percentage of cells responding to GnRH with a process extension by 64%. The time dependence may indicate an effect for which time is essential, perhaps requiring protein synthesis.

It is interesting to note that the percentage of gonadotropes responding to GnRH, even in the presence of long-term estradiol, did not rise above 50%. The experiments were conducted using pituitaries harvested from females in diestrus I, which is characterized by low estradiol levels. The exposure duration to GnRH did not have an observable effect on the percentage of cells recruited to show process extensions over time or the length of process extensions. This suggests that GnRH-induced formation of process extensions may be an all-or-none response. The percentage of cells responding to GnRH to secrete LH- and GnRH-induced plasticity of gonadotropes brings to question the heterogeneity of this population of cells and what the potential function(s) might be mediated by such heterogeneity. After Cre-mediated removal of the transcriptional stop signal in the *ROSA26* locus, genetic labeling of gonadotropes is independent of the activity of the *GnRHR* promoter (23). Future studies will need to address whether the nonresponding gonadotropes actually express GnRHR at the time of analysis.

The choice of animals in the diestrus I stage may have impacted the percentage of gonadotropes with process extensions because Funabashi *et al.* correlated levels of *GnRHR* mRNA levels with the stages of the estrous cycle (38). They concluded that the highest levels of *GnRHR* mRNA was observed during the end of diestrus II and the beginning of proestrus. These findings are congruent with studies that have demonstrated estradiol levels impact gonadotropes and that estradiol increases GnRHR synthesis (13, 31, 39). Because the E_2 effect presented in these studies is an increase in responsiveness to GnRH signaling, long-term E_2 may mediate these effects by increasing the levels of the GnRHR gene expression. Because short-term E_2 exposure had no effect on the percentage of cells responding to GnRH-induced plasticity, it suggests there is not a short-term, membrane-initiated site of action.

In previous experiments, cell movements were observed in response to GnRH when cells were visualized in *ex vivo* live murine pituitary slices infected with an adenovirus-containing Rous sarcoma virus driving GFP (17). However, cells that changed positions may not have been gonadotropes. The cell movements were GnRH sensitive, but GnRHR binding has been localized to somatotropes and gonadotropes (40). Therefore, cells moving may have been a different population of pituitary cells than gonadotropes. In the current study, cells were confirmed gonadotropes based on a high-fidelity YFP reporter. In this case, gonadotropes responded to GnRH treatment with process extensions, but not cell body movements.

In summary, the current study further establishes plasticity for gonadotropes in the anterior pituitary of prepubertal and postpubertal female mice. This plasticity was evident at two different levels. First, gonadotrope locations within the anterior pituitary depended on the physiological state and varied relative to the vasculature. Second, in postpubertal females when pituitary gonadatropes were selectively examined *ex vivo*, 14 h of E_2 priming significantly increased the ability of GnRH to activate process extension. The effect of E2 was likely indirect, if not genomic, because short treatment (1.5 h) did not influence process extensions with or without GnRH treatment. It is clear that the pituitary is a site of significant adult cellular plasticity (7, 22), and the current study provides new views of gonadotropes as strong participants.

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