Phosphodiesterase expression targeted to gonadotropin-releasing hormone neurons inhibits luteinizing hormone pulses in transgenic rats

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Experiments in the GT1 gonadotropin-releasing hormone (GnRH) cell line have shown that the cAMP signaling pathway plays a central role in regulating the excitability of the cells. Lowering cAMP levels by expressing the constitutively active cAMP-specific phosphodiesterase PDE4D1 in GT1 cells inhibited spontaneous Ca^{2+} oscillations and intrinsic pulsatile GnRH secretion. To address the role of cAMP levels in endogenous GnRH neurons, we genetically targeted expression of PDE4D1 (P) to GnRH neurons in transgenic rats (R) by using the GnRH gene promoter/enhancer regions (G). Three lines of transgenic rats, GPR-2, -4, and -5, were established. *In situ* hybridization and RT-PCR studies demonstrated that transgene expression was specifically targeted to GnRH neurons. Decreased fertility was observed in female but not in male rats from all three lines. The mean luteinizing hormone (LH) levels in ovariectomized rats were significantly reduced in the GPR-4 and -5 lines but not in the GPR-2 line. In castrated male and female GPR-4 rats, the LH pulse frequency was dramatically reduced. Six of twelve GPR-4 females studied did not ovulate and had polycystic ovaries. The remaining six females ovulated, but the magnitude of the preovulatory LH surge was inhibited by 63%. These findings support the hypothesis that cAMP signaling may play a central role in regulating excitability of GnRH neurons *in vivo*. The GPR-4 line of transgenic rats provides a genetic model for the understanding of the role of pulsatile gonadotropin release in follicular development.

The pulsatile release of gonadotropin-releasing hormone (GnRH) underlies reproductive function in male and female mammals. Understanding the molecular mechanisms regulating pulsatile GnRH secretion has been hampered by the low number of GnRH neurons and their scattered localization throughout the preoptic area in rodents. Further complicating the understanding of these questions is the complexity of the neural connectivity of GnRH neurons. The development of the highly differentiated GT1 GnRH neuronal cell lines (1) and the demonstration that pulsatile release was an intrinsic property of the cells provided a model to study molecular events underlying pulsatile GnRH release (2–4). However, one is always faced with the problem of translating findings from an immortalized cell line to the physiological setting. We have attempted to bridge observations made in GT1 cells regarding signaling events regulating GnRH pulsatility to the physiological setting by developing a transgenic rat model. We used an established genetic approach to alter the activity of a signaling pathway that could be used in parallel experiments in GT1 cells and transgenic rats. Transgenic rats, unlike transgenic mice, permit the reliable analysis of pulsatile luteinizing hormone (LH) release that closely mimics pulsatile GnRH release in castrated animals (5).

The secretion of GnRH in GT1 neurons is stimulated by elevations in cAMP levels (6–8). The stimulation of GnRH secretion by elevated cAMP levels appears to be regulated via cAMP-gated cation channels expressed by the cells. We showed that GT1 neurons express the three subunits that comprise the olfactory cyclic nucleotide-gated (CNG) channels (CNG 2, 4.3, and 5), and that the subunits formed functional channels with similar electrophysiological properties to those observed in olfactory neurons (9, 10). CNG channels play an important role in regulating the excitability of GT1 neurons (10). Increases in cAMP increase CNG channel activity, permitting the entry of cations, e.g., Na^+ or Ca^{2+}, into the neurons, and resulting in increased excitability of the neurons leading to depolarization, the opening of voltage-gated Ca^{2+} channels, and the exocytosis of GnRH.

GT1 cells show spontaneous changes in cell excitability (11). In GT1–1 cells loaded with Fura2, spontaneous Ca^{2+} oscillations were observed. Calcium oscillations were preceded by the occurrence of an action potential. Spatially propagated intracellular Ca^{2+} waves were also observed. We recently determined whether cAMP levels in GT1 cells played a role in regulating spontaneous changes in cell excitability, i.e., Ca^{2+} oscillations and pulsatile GnRH secretion. We used a genetic approach to lower cAMP levels in GT1 cells by overexpression of the cAMP-specific constitutively active phosphodiesterase PDE4D1 (12). This approach was successfully used to inhibit human chorionic gonadotropin-stimulated increases in cAMP levels in MA-10 Leydig tumor cells. Expression of PDE4D1 in GT1 cells blocked the ability of forskolin to increase cAMP levels and to stimulate GnRH secretion (unpublished data). Furthermore, expression of PDE4D1 decreased the frequency of spontaneous Ca^{2+} oscillations and pulsatile GnRH release from the cells. From these data, we hypothesized that cAMP levels are a major regulator of changes in the excitability of GT1 cells associated with pulsatile GnRH release. We reasoned that this genetic approach to lowering intracellular cAMP levels could be extended to the *in vivo* setting by targeted expression of PDE4D1 to GnRH neurons in transgenic rats.

Data in the literature were consistent with the idea that cAMP levels were also important in the regulation of GnRH release in rat hypothalamus. Elevations in cAMP levels stimulated GnRH release from rat median eminence, the region containing GnRH nerve terminals (13). We demonstrated by *in situ* hybridization and double immunofluorescence that endogenous GnRH neurons also contain the mRNAs and proteins for all three subunits for the cAMP-gated CNG channels (14). In addition to the localization of the channel proteins in GnRH perikarya, subunit proteins were observed in the median eminence in regions overlapping with the GnRH terminals. To test the hypothesis that cAMP levels regulated the excitability of GnRH neurons in a physiological setting, we targeted expression of PDE4D1 to GnRH neurons in transgenic rats and analyzed reproductive function and LH secretion. Transgene expression was targeted

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Abbreviations: GnRH, gonadotropin-releasing hormone; LH, luteinizing hormone; CNG, cyclic nucleotide-gated; PDE4D1, constitutively active cAMP-specific phosphodiesterase; GPR, GnRH promoter (G) PDE4D1 cDNA (P) transgenic rat line (R).

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by using the promoter/enhancer regions of the rat GnRH gene. Consistent with the hypothesis in the GPR-4 line of transgenic rats, targeting of PDE4D1 to GnRH neurons dramatically inhibited the preovulatory LH surge in cycling females and the pulsatile release of LH in castrated males and females. Interestingly, inhibition of gonadotropin secretion is associated with polycystic ovaries in half of the GPR-4 females.

Materials and Methods

Transgene Construction. The promoter/enhancer region of the rat GnRH gene (G) (−3,002 to +88) (15) was inserted upstream of the B intron of rabbit β globin gene. The PDE4D1 cDNA (P) was inserted downstream of the B intron followed by the human growth hormone poly(A) sequence. Inclusion of a heterologous intron 5′ of cDNAs increases transgene expression (16). Unique restriction sites were engineered at the 5′ and 3′ ends for isolation of the transgene for injection. We have successfully made numerous lines of transgenic mice with these intronic and poly(A) sequences, which give robust levels of transgene expression (17).

Production and Analysis of Transgenic Rats. Transgenic rats (R) were produced by using procedures modified from standard techniques for making transgenic mice (18). Thirty 45-day-old female Sprague–Dawley rats (Charles River Breeding Laboratories) were superovulated by priming with 15 units of pregnant mare serum (PMS) followed 2 days later with 10 or 15 units of human chorionic gonadotropin, placed with fertile males, and euthanized the next morning for embryo harvest. The transgene (4–8 ng/μl) was microinjected into the male pronucleus by using an Eppendorf Transjector. Injected embryos were transferred to the oviduct of pseudopregnant adult female Sprague–Dawley rats. At weaning, transgenic animals were identified by tail blot analysis. Three lines of transgenic rats were established, GPR-2, -4, and -5. Because of problems with fertility, all experiments were performed with animals heterozygous for the transgene.

Evaluation of Transgene Expression by RT-PCR. Total RNA from the preoptic area, posterior hypothalamus, and control tissues was prepared in TRIZOL reagent (GIBCO). First-strand cDNA synthesis was performed by using oligo(dT) primers (Life Technologies, Gaithersburg, MD) and Moloney–murine leukemia virus (H-) reverse transcriptase (Promega), as recommended by the manufacturer. A sense primer against a sequence in the 3′ region of the PDE4D1 cDNA (5′-CTTAGAGGAAGATGGCGAGTC-3′) and an antisense primer against an expressed 5′ sequence in the human growth hormone poly(A) (5′-CTGGTGGGCACTGGAGT-3′) were used. PCR conditions were 95°C/45 sec, 64°C/60 sec, and 72°C/60 sec, 25 cycles.

Evaluation of Transgene Expression by *in Situ* Hybridization. The level and specificity of targeting of the transgene expression were analyzed by *in situ* hybridization on adjacent 6–8 μm frozen serial sections of the preoptic area, hippocampus, neocortex, cerebellum, caudate nucleus, and thalamus. Sections were acetylated, dehydrated progressively, and hybridized overnight at 55°C with the different probes following the protocol suggested by the manufacturer (Boehringer Mannheim). To localize the transgene mRNA, a 73-bp fragment from the 5′ end of the human growth hormone poly(A) fragment was used to generate a digoxigenin-labeled antisense riboprobe. This region was transcribed and was contained within the transgene mRNA. GnRH neurons were identified with a 345-bp digoxigenin-labeled antisense riboprobe to the rat GnRH cDNA (19). The sense strand for both probes was used as a control. For each pair of serial sections, one section was hybridized with the GnRH riboprobe, whereas the adjacent section was hybridized with the transgene riboprobe. After hybridization, the slides were washed under high stringency and processed directly for detection of the digoxigenin signal. The sections were examined with a Leica DMR photomicroscope under bright field optics.

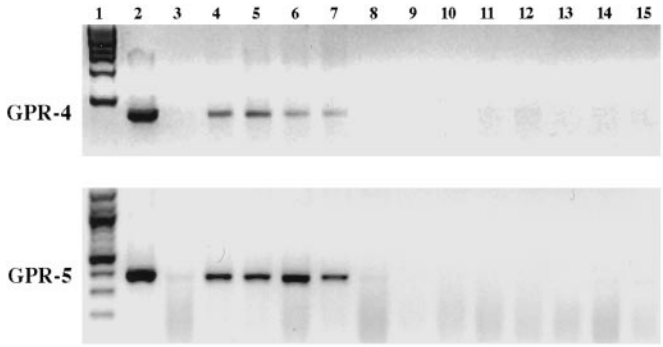

Fig. 1. RT-PCR fragments (expected size, 383 bp) amplified from total mRNA extracted from tissues from transgenic lines GPR-4 and GPR-5. Lanes contain data from: 1, DNA ladder; 2, plasmid control; 3, blank control; 4 and 5, preoptic area; 6 and 7, posterior hypothalamus; 8, cerebral cortex; 9, thalamus; 10, septum; 11, caudate nucleus; 12, anterior pituitary; 13, GPR-4 testes and GPR-5 ovary; 14, liver; and 15, kidney. Analysis was performed on two rats from each line; however, the bands for both animals are shown only for the preoptic and posterior hypothalamus.

Evaluation of Reproductive Phenotype. Breeding records were maintained for male and female transgenic animals. Occurrence of estrous cycles was evaluated by vaginal cytology. Ovaries obtained at the time of castration or euthanasia were fixed in 4% paraformaldehyde, and 6-μm paraffin sections were stained with hematoxylin/eosin.

To assess the preovulatory LH surge in cycling rats, a right atrial catheter was inserted (20) on the morning of proestrous (before 1200 h) under tribromoethanol anesthesia (21). Pulsatile LH release was assessed in female and male rats castrated for 4 weeks. Plasma samples obtained from a right atrial catheter were inserted the day before sampling under tribromoethanol anesthesia. Plasma LH levels were measured in duplicate by RIA (National Hormone and Peptide Program, National Institute of Diabetes and Digestive and Kidney Diseases) and the data analyzed for LH pulse amplitude and pulse frequency by using "cluster analysis," a statistically based peak-detection algorithm (22). Cluster sizes (nadir/peak) were defined by 2 × 2 points and detection of peaks defined by t values (up and down) >3.

At the time of euthanasia, the preoptic area and hypothalamus were dissected, homogenized in 0.1 M acetic acid and radioimmunoassayed for GnRH (23). The landmarks for the hypothalamic dissection were: anterior to the optic chiasma; posterior to the mammalary bodies; lateral at the hypothalamic sulcus; and superior to the anterior commissure. Data were expressed as the mean ± SD and statistically analyzed by the nonparametric Mann–Whitney U test.

Results

Transgene Specifically Targeted to GnRH Neurons. We established three lines of transgenic rats termed GPR-2, -4, and -5. The numbering is based on the number of the founder. We determined by RT-PCR and *in situ* hybridization studies whether transgene expression was specifically targeted to GnRH neurons. In RT-PCR experiments, a band of the predicted size (383 bp) and sequence was obtained from total RNA extracted from the preoptic area of all three lines (Fig. 1; data from the GPR-2 line not shown). Bands of the same size were observed from total

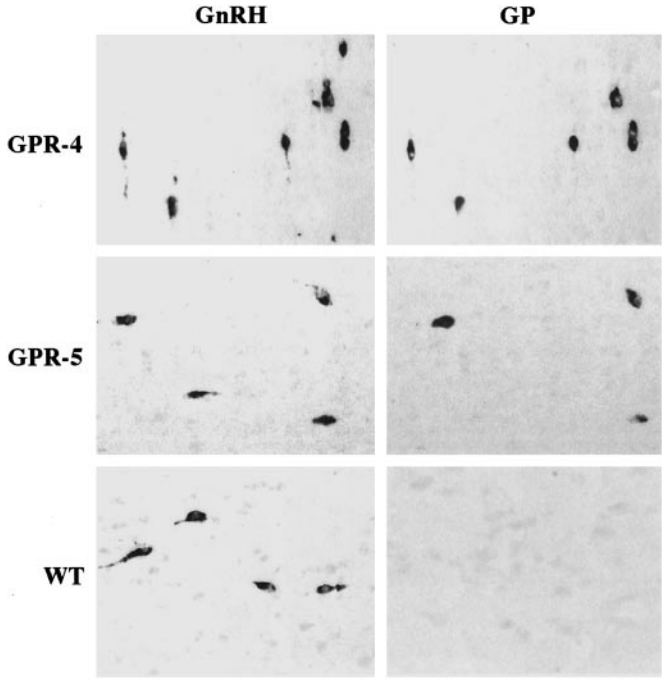

Fig. 2. Localization by *in situ* hybridization of GnRH and the GP transgene mRNA-containing neurons preoptic area of control (WT) and GPR-4 and -5 transgenic rats. Serial sections were hybridized with digoxigenin-labeled GnRH or growth hormone poly(A) riboprobes. In line GPR-4, six of seven GnRH neurons were colabeled for the transgene and in line GPR-5, three of four. No expression of the transgene is observed in the control. In the sections from transgenics, no staining was observed outside of GnRH neurons (×8).

RNA extracted from posterior hypothalamic fragments. In general, the intensity of the bands from posterior hypothalamic extracts was less intense, consistent with the observation that the preoptic area contains most of the GnRH cell bodies (24). No expression was seen in the septum, thalamus, caudate nucleus, hippocampus, cerebral cortex, pituitary, ovary, testes, kidney, or liver.

By *in situ* hybridization with specific riboprobes against GnRH and the transgene mRNA, we showed that the transgene was expressed in ≈75% of GnRH neurons in the GPR-4 (28 of a total of 38) and GPR-5 (25 of 33) lines (Fig. 2). The distribution of stained GnRH neurons in preoptic sections did not appear to be different between control and transgenic sections. The GPR-2 line was not analyzed by *in situ* hybridization, because the line had been lost before establishment of the technique. There was no ectopic expression of the transgene in non-GnRH neurons in the preoptic region or in other brain regions not containing GnRH neuron cell bodies, including the caudate nucleus, cerebellum, cerebral cortex, hippocampus, and thalamus. The *in situ* and RT-PCR data together convincingly show that the transgene expression was specifically targeted to the majority of endogenous GnRH neurons. No detectable ectopic expression of the transgene was observed in the brain regions or tissues studied.

Decreased Fertility in Transgenic Lines. Fertility was compromised to varying degrees in all three lines. Over time, the GPR-2 and -5 lines were lost. Only detailed data were obtained on the fertility of the GPR-4 line. The majority of males of the GPR-4 line were fertile. For example, 75% of 6-mo-old GPR-4 males ($n = 8$), when placed with wild-type females, produced litters. However, the GPR-4 female rats showed a rapid decrease in fertility with age. Eighty percent of 2.5-mo-old GPR-4 females ($n = 10$) placed with wild-type males produced normal-sized litters, whereas none of the six 6-mo-old GPR-4 females ($n = 6$) became pregnant.

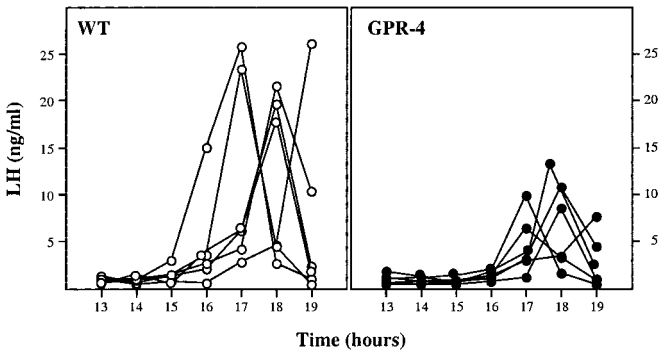

Fig. 3. A right atrial catheter was placed on the morning of proestrus in six nontransgenic littermate controls (WT, open circles) and six GPR-4 rats (filled circles). Plasma samples obtained every hour from 1300 to 1900 hours were assayed for LH by RIA. The large characteristic preovulatory surge in WT females was significantly diminished in GPR-4 females.

To try to understand the basis for the infertility in the GPR-4 females, we followed vaginal smears in 3-mo-old GPR-4 females ($n = 12$) and nontransgenic littermate controls ($n = 12$) for 6 weeks. In 83% of the nontransgenic controls, normal 4 or 5 day estrous cycles were observed. In half of the transgenic females clear 4- or 5-day cycles were also observed. However, in the remaining six animals, no clear cyclicity was observed. To understand the hormonal basis of the differences observed in vaginal cyclicity, several parameters were studied. The preovulatory LH surge was evaluated in cycling controls ($n = 6$) and cycling transgenics ($n = 6$) on the afternoon of proestrus. The remainder of the controls ($n = 6$) and the noncycling GPR-4 females ($n = 6$) were castrated for 4 weeks, and pulsatile LH secretion evaluated. The hypothalamus and ovaries were obtained from these animals for measurement of GnRH and histological evaluation, respectively.

Inhibition of Preovulatory LH Surge. We studied the preovulatory surge of LH in the cycling GPR-4 females ($n = 6$) and cycling littermate controls ($n = 6$). In the rat, on the afternoon of proestrus, a large increase in LH secretion is initiated by the release of GnRH into the portal blood (25). Nontransgenic littermate controls had large characteristic LH surges starting between 1500 and 1600 h (Fig. 3). In the cycling GPR-4 transgenic females, a small LH surge was observed beginning at a similar time. The average mass of the LH surge, as measured by the area under the peak (36.7 ± 7.3 vs. 13.6 ± 3.4 ng/ml/surge), was significantly reduced by 63%. On the next morning, animals were euthanized and the number of ovulated ova counted in the oviduct. A similar number of ova were retrieved from controls and transgenics (7.0 ± 1.5 vs. 6.5 ± 2.0). That significantly reduced levels of LH are sufficient to induce ovulation was previously observed after partial blockade of LH surge with pentobarbital anesthesia (26).

Inhibition of Pulsatile LH Release. We hypothesized that if the cAMP levels were decreased by expressing PDE4D1, pulsatile GnRH release would be inhibited. Supporting this idea in GT1 cells, expression of PDE4D1 decreased spontaneous pulsatile GnRH release (unpublished data). To eliminate the effects of the negative feedback of gonadal steroids that were likely altered in the transgenic rats, control females and noncycling GPR-4 females were castrated for 4 weeks. Nontransgenic control males and GPR-4 males from the same litters were also castrated.

Table 1. LH pulse parameters (mean ± SD) in castrated control (WT) and GPR-4 transgenic female and male rats

	LH, ng/ml	Pulse frequency (pulses/2 h)	Pulse amplitude, ng/ml
Female WT ($n = 6$)	7.0 ± 1.2	5.3 ± 0.5	4.7 ± 0.8
Female GPR-4 ($n = 6$)	1.8 ± 0.7*	1.5 ± 0.6*	4.5 ± 1.4
Male WT ($n = 6$)	5.8 ± 0.9	4.8 ± 0.5	3.1 ± 0.5
Male GPR-4 ($n = 6$)	2.1 ± 0.7*	2.5 ± 0.6*	2.7 ± 0.4

*Significantly lower than control at $P < 0.01$ with Mann–Whitney U test.

In castrated GPR-4 females the mean plasma LH levels were decreased by greater than 3-fold compared with six castrated littermate nontransgenic controls (Table 1). In the ovariectomized GPR-4 rats, the LH pulse frequency was also dramatically reduced (Fig. 4). However, no change was seen in the pulse amplitude. The pulse frequency in the nontransgenic controls was in close agreement with published data (27). The decrease in pulsatile LH secretion in the GPR-4 females did not appear to depend on the fact that the animals were not cycling at the time of castration. In additional experiments in two GPR-4 females that were cycling before castration, a decrease in mean and pulsatile LH release was observed (mean LH = 2.7 ± 0.5 ng/ml; pulse frequency = 3.0 ± 0.0 pulses/2 h).

As in the castrated GPR-4 females, the mean LH levels in the castrated GPR-4 males were significantly decreased relative to castrated littermate controls. Furthermore, the LH pulse frequency was dramatically reduced (Fig. 4). Again the pulse amplitude was not different.

In earlier studies with the GPR-2 and -5 lines, before they were lost, it was impossible to reliably analyze pulsatile LH release, because few pulses were observed in nontransgenic controls. However, in the GPR-5 line a significant ($P < 0.05$) 31% decrease in plasma LH levels was observed in five castrated females compared with six castrated nontransgenic controls (4.3 ± 1.4 vs. 6.2 ± 1.7 ng/ml). However, no change in the mean LH levels was observed in six castrated GPR-2 females compared with six castrated nontransgenic controls (5.3 ± 2.1 vs. 6.2 ± 1.7 ng/ml).

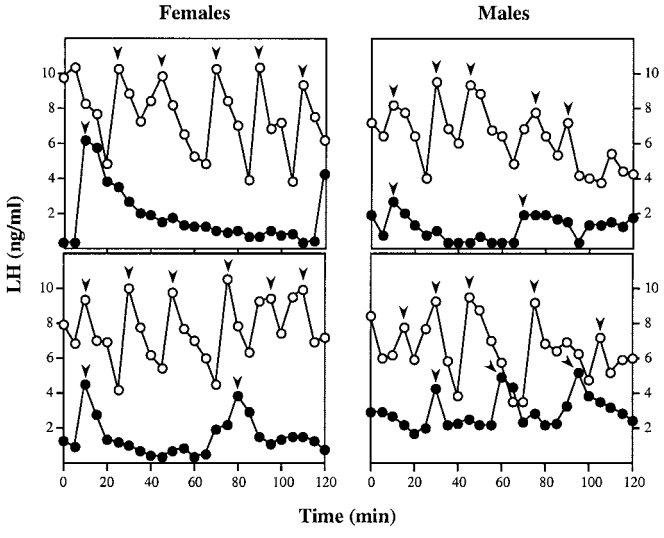

Fig. 4. Plasma LH levels measured in catheterized castrated females: controls ($n = 2$, open circles) and GPR-4 transgenic ($n = 2$, filled circles); and castrated male: controls ($n = 2$, open circles) and two transgenics ($n = 2$, filled circles). Arrowheads denote pulses, as determined by a statistically based peak-detection algorithm ''cluster analysis.'' Data shown are representative of findings from six experiments in each group.

Hypothalamic GnRH Levels. We measured the hypothalamic content of GnRH to roughly assess whether expression of the transgene affected the availability of GnRH. The hypothalamic GnRH content on the morning of estrous in the cycling controls ($n = 6$) and cycling GPR-4 females ($n = 6$) was unchanged (313 ± 35 vs. 346 ± 44 pg/mg protein, respectively). The hypothalamic GnRH content in the castrated controls ($n = 6$) and castrated GPR-4 females ($n = 6$) was also not significantly different (124 ± 19 vs. 170 ± 40 pg/mg protein, respectively). The well characterized large decrease in GnRH content associated with castration (28) was observed in both groups. Similarly, no change in hypothalamic GnRH content was observed in castrated controls ($n = 6$) and in six castrated GPR-4 males (123 ± 42 vs. 164 ± 41 pg/mg protein).

Development of Polycystic Ovaries. The ovarian weights at 5 mo of age of the control ($n = 12$) and cycling GPR-4 transgenic females ($n = 6$) were not different; however, the ovarian weight of the noncycling females ($n = 6$) was significantly decreased (31.7 ± 4, 31.4 ± 6, 20.7 ± 6 mg/100 g body weight, respectively). By histological evaluation of hematoxylin/eosin-stained sections, the ovaries from controls contained large numbers of corpora lutea and developing follicles, as seen in normal rats (29) (Fig. 5). No dramatic changes in ovarian histology were observed in the six cycling GPR-4 rats. The presence of large numbers of corpora lutea is consistent with the recovery of a normal number of ova from the oviduct of these animals on the morning of estrous. However, the ovaries of the noncycling GPR-4 females had no corpora lutea, demonstrating the complete inhibition of ovulation. In addition, the ovaries of the noncycling transgenics contained numerous large cystic follicles and type 3 precystic follicles. Because we observed an ovarian phenotype, we performed additional RT-PCR studies to confirm that the transgene was not expressed in the ovaries of three GPR-4 and three control females. Although we confirmed expression of the transgene in the anterior hypothalamus of the three transgenic animals, no transgene expression was observed in the ovary (data not shown).

The testes weights of 5-mo-old male GPR-4 rats ($n = 6$) were significantly lower than in the six nontransgenic controls (64.2 ± 6.6 vs. 76.0 ± 9.4 mg/100 g body weight). The histology of the testes appeared normal. No experiments were performed in males from lines GPR-2 and -5.

Discussion

The finding that the frequency of pulsatile LH secretion is decreased in the GPR-4 rats supports the hypothesis that the level of cAMP in GnRH neurons plays a central role in regulating the excitability of the neurons. The assumed decrease in cAMP levels in GnRH neurons by targeted expression of PDE4D1 appears to decrease neuron excitability, making it less likely that a GnRH pulse was initiated. In GT1 cells, Ca^{2+} oscillations result from bursts of Na^{+}-dependent action potentials, resulting in the influx of Ca^{2+} through L-type voltage-gated channels (11). The increase in the frequency of Ca^{2+} oscillations in response to elevated cAMP levels is consistent with an increase in excitability. Expression of PDE4D1 in GT1 cells decreases spontaneous Ca^{2+} oscillations, consistent with the idea that spontaneous alterations in cAMP levels participate in spontaneous changes in the excitability of GT1 cells (unpublished data). It appears that activation of CNG channels by cAMP modulates the excitability of GT1 cells by altering the depolarization drive that underlies spontaneous oscillations in membrane potentials (10).

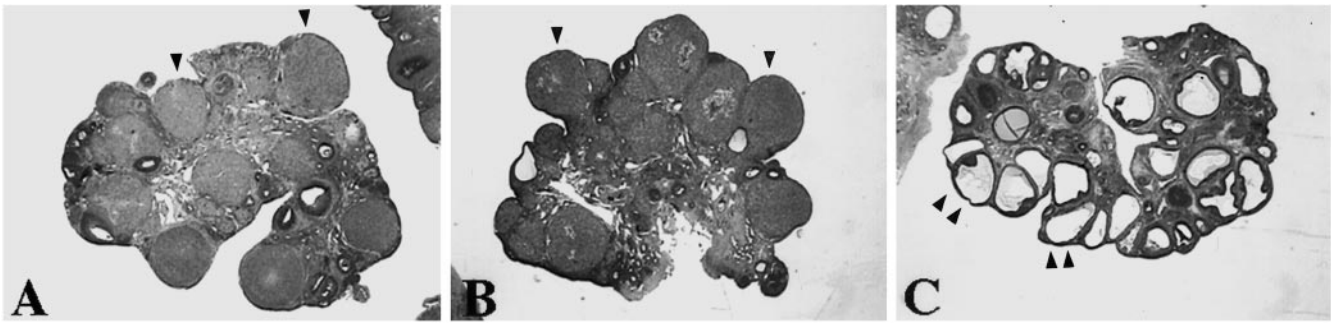

Fig. 5. Photomicrographs of sections of ovaries stained with hematoxylin/eosin. The ovaries of a nontransgenic control (*A*) and a cycling GPR-4 transgenic (*B*) are mainly composed of corpora lutea (▼), whereas the ovary from a noncycling GPR-4 transgenic (*C*) contains numerous large cystic follicles (▲▲) (×10).

Pulsatility appears to be an intrinsic property of GnRH neurons or networks of GnRH neurons. Intrinsic pulsatile GnRH secretion, first seen in the GT1 GnRH cell lines (2–4), has now been observed in GnRH neurons isolated from the olfactory placode of the monkey (30), rat (31), and sheep (32). Pulsatile GnRH secretion appears to be a property of individual GT1 cells (33). A model for the pulsatile behavior of networks of GnRH neurons has been described that predicts that interconnected cells with periodic alterations in cell excitability would show pulsatile behavior (34). No pacemaker neurons are necessary for this model. The waxing and waning of cAMP levels could constitute a timing mechanism for change in cell excitability. Consistent with this idea, we have recently shown that expression of PDE4D1 in GT1–1 cells inhibits the frequency of pulsatile GnRH secretion (unpublished data). Cyclical changes in cAMP are known to function as biological clocks. In the slime mold, Dictyostelium, rhythmic changes in cell aggregation are timed by cyclical changes in cAMP (35).

The decrease in the magnitude of the ovulatory surge in LH seen in half of the GPR-4 females could also result from a decrease in the excitability of the GnRH neurons. This would make the GnRH neurons less responsive to neurotransmitters released by synapses of afferent inputs. A second interpretation could be that the neurotransmitters released from afferent inputs signal by increasing intracellular cAMP levels in GnRH neurons. For example, vasoactive intestinal peptide $(VIP)_2$ receptors that are positively coupled to adenylate cyclase are expressed by rat GnRH neurons (36). Central administration of an antiserum to VIP delays and attenuates the LH surge (37). Overexpression of PDE4D1 could inhibit the VIP-induced increase in cAMP. In GT1 cells, expression of PDE4D1 inhibits the dopamine-induced increases in cAMP levels and GnRH secretion (unpublished observation).

Several observations in the literature support the hypothesis that cAMP levels regulate the excitability of endogenous GnRH neurons. Forskolin-induced increases in cAMP levels stimulated GnRH release from fragments of the rat median eminence (13) and from GT1 cells (9). We have shown by *in situ* hybridization and double immunostaining that endogenous GnRH neurons express the same three CNG channel subunits expressed in GT1 cells (14). Interestingly, the subunit proteins are present in GnRH nerve terminals in the median eminence. Some mechanism must exist for GnRH neurons to coordinate secretion from the subset of neurons constituting a pulse. The median eminence is the only region in which large numbers of GnRH neurons can directly communicate through physical approximation.

The use of transgenic rats rather than transgenic mice was clearly advantageous for the assessment of hormone levels necessary for these studies. The success of the current study in transgenic rats depended on the ability to genetically target GnRH neurons. The data obtained by RT-PCR and *in situ* hybridization studies strongly indicate that 3 kb of the rat GnRH gene 5′ flanking regions specifically target expression to GnRH neurons. Although no previous studies had been performed in transgenic rats, this same promoter specifically directed expression of green fluorescent protein to GnRH neurons in transgenic mice (38). The infertility of the transgenic rats supports the use of overexpression of a phosphodiesterase (PDE) as a strategy to manipulate cAMP responses *in vivo*. Given the limited pharmacological strategies available to decrease cAMP in discrete sets of cells *in vivo*, the transgenic overexpression of a PDE will be very useful to probe the effect of a decreased cAMP signaling in selected loci of the brain and in other organs.

One question that arises is why only half of the female GPR-4 rats were acyclic. One explanation is that the level of transgene expression varies from animal to animal. If large differences in transgene expression occurred, one would expect that the ovulatory surges observed in cycling GPR-4 females would vary from animal to animal. However, the magnitude of the LH surge showed little variation in the six animals studied. Another potential explanation is that in the cycling GPR-4 animals, just barely enough GnRH is released to cause ovulation. In females that were ovulating, the preovulatory LH surge was dramatically decreased. All of the current experiments were performed in animals that were heterozygous for the transgene. Potentially, the increased expression of the transgene in homozygous animals may lead to the earlier loss of cyclicity and the blockade of follicular development. Another variable that may be important is the age of the animals. With time, more and more of the females become acyclic. The studies reported here were with relatively young rats of 3–4 mo of age. At 6 mo of age, all of the animals are acyclic. Possibly age-related changes in energy metabolism and obesity could be involved in the inhibition of cyclicity, and the development of polycystic ovaries. Obesity is well recognized as playing some pathological role in polycystic ovarian syndrome in women (39).

These data show that changes in GnRH release in transgenic animals dramatically alter follicular development. The absence of an ovulatory LH surge could result in the arrest of preovulatory follicles. However, the possibility also exists that changes in the pulsatile release of LH and potentially follicle-stimulating hormone (FSH) contribute to alterations in follicular development. We assume that changes in pulsatile LH secretion are the result of changes in the pulsatile release of GnRH. Because GnRH regulates both LH and FSH secretion, we conclude that it is likely the pulsatile FSH secretion is also altered. We are currently performing experiments measuring pulsatile FSH secretion in castrated GPR-4 rats. The large number of cystic follicles should provide a useful model for studying abnormal follicular development. In other rat and mouse polycystic ovarian models, only an occasional cystic follicle was observed (40, 41). The GPR-4 rat could provide a model

for understanding the role of pulsatile LH and possibly FSH release on follicular development.

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