A Conditional Tetracycline-Regulated Increase in Gamma Amino Butyric Acid Production near Luteinizing Hormone-Releasing Hormone Nerve Terminals Disrupts Estrous Cyclicity in the Rat*

MARIE BILGER†, SABINE HEGER‡, DARRELL W. BRANN, ALFONSO PAREDES§, AND SERGIO R. OJEDA

Division of Neuroscience, Oregon Regional Primate Research Center/Oregon Health Sciences University (M.B., S.H., A.P., S.R.O.), Beaverton, Oregon 97006; and Department of Physiology, Medical College of Georgia (D.W.B.), Augusta, Georgia 30912

ABSTRACT

Gamma amino butyric acid (GABA) is the main inhibitory neurotransmitter controlling LH-releasing hormone (LHRH) secretion in the mammalian hypothalamus. Whether alterations in GABA homeostasis within discrete regions of the neuroendocrine brain known to be targets of GABA action, such as the median eminence, can disrupt the ability of the LHRH releasing system to maintain reproductive cyclicity is not known but amenable to experimental scrutiny. The present experiments were undertaken to examine this issue. Immortalized BAS-8.1 astroglial cells were genetically modified by infection with a regulatable retroviral vector to express the gene encoding the GABA synthesizing enzyme glutamic acid decarboxylase-67 (GAD-67) under the control of a tetracycline (tet) controlled gene expression system. In this system, expression of the gene of interest is repressed by tet and activated in the absence of the antibiotic. BAS-8.1 cells carrying this regulatory cassette, and cultured in the absence of tet ("GAD on"), expressed abundant levels of GAD-67 messenger RNA and GAD enzymatic activity, and released GABA when challenged with glutamate. All of these responses were inhibited within 24 h of exposure to tet ("GAD off"). Grafting "GAD on" cells into the median eminence of late juvenile female rats, near LHRH nerve terminals, did not affect the age at vaginal opening, but greatly disrupted subsequent estrous cyclicity. These animals exhibiting long periods of persistent estrus, interrupted by occasional days in proestrus and diestrus, suggesting the occurrence of irregular ovulatory episodes. Administration of the tetracycline analog doxycycline (DOXY) in the drinking water inhibited GAD-67synthesis and restored estrous cyclicity to a pattern indistinguishable from that of control rats grafted with native BAS-8.1 cells. Animals carrying "GAD on" cells showed a small increase in serum LH and estradiol levels, and a marked elevation in serum androstenedione, all of which were obliterated by turning GAD-67 synthesis off in the grafted cells. Morphometric analysis of the ovaries revealed that both groups grafted with GABA-producing cells had an increased incidence of large antral follicles (>500 μm) compared with animals grafted with native BAS-8.1 cells, but that within this category the incidence of steroidogenically more active follicles (*i.e.* larger than 600 μm) was greater in "GAD on" than in "GAD off" rats. These results indicate that a regionally discrete, temporally controlled increase in GABA availability to LHRH nerve terminals in the median eminence of the hypothalamus suffices to disrupt estrous cyclicity in the rat, and raise the possibility that similar local alterations in GABA homeostasis may contribute to the pathology of hypothalamic amenorrhea/oligomenorrhea in humans. (*Endocrinology* **142:** 2102–2114, 2001)

IT IS WELL established that the neuronal input to the LHRH neuronal network is provided by both stimulatory and inhibitory neurotransmitters (1–3). Among these transsynaptic regulatory systems, amino acid neurotransmitters appear to have a major role in the control of LHRH secretion (4–6). Although glutamate stimulates LHRH release via ionotropic (6) receptors, γ aminobutyric acid (GABA)— the dominant neurotransmitter in inhibitory synapses of the hypothalamus (7)—affects LHRH secretion via two different classes of membrane anchored recognition molecules: $GABA_A$ receptors, which are ligand-gated anion channels (8–10), and $GABA_B$ receptors, which are seven-transmembrane-domain receptors negatively coupled to adenylate cyclase via GTP- binding proteins (11–14).

Experiments in nonhuman primates have shown that GABA acting via $GABA_A$ receptors restrain LHRH release during sexual development (15) and that removal of this restraining influence results in increased LHRH secretion (16) and advances the onset of female puberty (17). Removal of an inhibitory GABA tone also appears to play a role in the activation of the preovulatory surge of gonadotropins in the adult rat. Although GABA release in the preoptic area decreases before the LH discharge (18), intrahypothalamic infusion of the amino acid in this region of the brain during the

Address all correspondence and requests for reprints to: Sergio R. Ojeda, Division of Neuroscience, Oregon Regional Primate Research Center, 505 NW 185th Avenue, Beaverton, Oregon 97006. E-mail: ojedas@ohsu.edu.

* This work was supported by a grant from NICHD/NIH through cooperative U54 HD18185–16 as part of the Specialized Cooperative Centers Program in Reproduction Research (to S.R.O.), NIH Grants RR00163 for the operation of the Oregon Regional Primate Center, HD-28964 (to D.W.B.), and Eli Lilly & Co. Pediatric Endocrine Fellowship grant (to M.B.).

† Pediatric Endocrinology Research Fellow supported by a grant from the Department of Pediatric Endocrinology, Oregon Health Sciences University, 3181 SW Sam Jackson Park Road, Portland, Oregon 97201. Present address: Valley Children's Clinic, 2401 River Road, Eugene, Oregon 97404.

‡ Pediatric Endocrinology Research Fellow supported by a grant from the European Society for Pediatric Endocrinology (ESPE).

§ Graduate student, Biochemistry Graduate Program, Faculty of Chemistry and Pharmaceutical Sciences, University of Chile. Supported by a fellowship from NICHD TW/HD00668 Fogarty International Training and Research in Population and Health grant.

afternoon of proestrus abolishes the surge (19). Conversely, blockade of $GABA_A$ receptors at this time advances the timing of the surge (20), suggesting that—as in the monkey—the inhibitory effect that GABA exerts on LHRH secretion is mediated by $GABA_A$ receptors. Studies in female rats have shown that this inhibitory capacity only develops around the time of puberty (21–23).

It appears that, at least in rodents, GABA also acts via $GABA_B$ receptors to reduce LHRH secretory activity, as pharmacological activation of these receptors inhibits both the preovulatory LH surge (24), and neurotransmitter-mediated activation of LHRH release (25, 26). In addition, both receptor systems are involved in mediating the inhibitory actions of GABA on pulsatile LH release (27–30). However, not always GABA inhibits LHRH secretion, as stimulatory $GABA_A$ receptor-mediated effects of GABA on LHRH release are well-documented (28, 31–33). Although part of this stimulatory capacity appears to be developmentally regulated (28, 31), it also appears to be related to a $GABA_A$ receptor-mediated increase in excitatory amino acid release (34), and—perhaps more importantly—to a direct excitatory GABA action on LHRH neurons, as shown in a LHRH neuronal cell line (35, 36). That such a direct excitatory effect may be of physiological importance is suggested by the recent findings that normal LHRH neurons *in situ* contain the complement of $GABA_A$ receptor subunits (37, 38) required for the formation of a functional chloride channel responsive to GABA binding and, as transformed LHRH neurons, respond to $GABA_A$ receptor stimulation with depolarization in the presence of appropriate chloride concentrations (39, 40). Of added significance is the observation that, in female rats, the inhibitory and excitatory effects of GABA on LHRH secretion appear to be anatomically segregated, with the inhibitory effects most prominently demonstrated in the preoptic area (18, 28, 30), and the excitatory ones in the medial basal hypothalamus/median eminence (33, 41–43).

It is, therefore, clear that substantial information exists concerning the involvement of GABA in both the control of puberty and the acute regulation of the preovulatory surge of gonadotropins. In contrast, little—if anything—is known about the overall contribution of the amino acid to the maintenance of adult reproductive cyclicity, and the potential importance that specific hypothalamic sites of action may have for the manifestation of such GABA regulatory effect(s). Such knowledge is important as it may offer new insights into the cell-cell mechanisms underlying human reproductive diseases of central origin, but of unknown etiology, such as hypothalamic amenorrhea/oligomenorrhea. To address this issue, we have used a gene transfer-cell grafting system and a conditional gene expression system to produce, in a temporally controlled fashion, regionally circumscribed increases in GABA release in response to the availability of endogenous precursors within the microenvironment surrounding LHRH neurons. Because much less is known about the consequences that GABA actions on LHRH nerve terminals may have on reproductive function, as compared with those on the preoptic region, we targeted the median eminence for grafting GABA-producing cells. The results show that these genetically modified cells disrupt estrous cyclicity via a mechanism that, initiated by an alteration in LHRH output, ultimately compromises ovarian secretory activity. A partial report of these findings has appeared (44).

Materials and Methods

Animals

Female rats of the Sprague Dawley strain (B & K Universal, Fremont, CA) were used in these studies. They were housed in a room with a controlled photoperiod (14-h light, 10-h dark; lights on from 0500–1900 h) and temperature (23−25 C), and were given free access to tap water and pelleted rat chow. The animals were used in accordance with the NIH guide for the Care and Use of Laboratory Animals and the experimental protocols were approved by the Institutional Research Animal Committee.

Cloning of a glutamic acid decarboxylase (GAD)-67 complementary DNA (cDNA) into a tet-regulatable retroviral vector

To generate cells able to release GABA in a regulatable fashion, we engineered immortalized astrocytes to express GAD-67, one of the two enzymes involved in GABA synthesis (45), under the control of a tetracycline (tet)-controlled gene expression system (46, 47). We selected the GAD-67 gene for transgenic expression, as opposed to GAD-65, because GAD-67 is the isoenzyme that may preferentially respond to tonic transsynaptic regulation (48) and, importantly, is the most critical enzyme in GABA synthesis (49). The tet regulatable system employed uses microbial proteins and microbial DNA response elements to drive expression of mammalian genes in heterologous cells (46). In *Escherichia coli*, transcription of genes conferring tet resistance is inhibited by a tet repressor. In the presence of tet, the repressor (tetR) does not bind to its operators contained in the promoter of the tet operon, so that transcription is allowed to proceed. Gossen and Bujard fused the active domain of VP-16, a potent transcriptional activator, to tetR, creating a hybrid (tTA, tet-controlled transactivator) that stimulates, instead of inhibiting, minimal promoters containing tet operator (tetO) sequences (46). The promoter $P_{hCMV^{*}-1}$, which in the present case drives GAD-67 expression, is almost silent in the presence of tet, because the antibiotic prevents binding of tTA to the tetO sequences. Thus, in the presence of tet GAD-67 synthesis is repressed and GABA is not produced even in the presence of glutamate precursor. Conversely, in the absence of tet, GAD-67 synthesis is activated, but GABA can only be produced if glutamate is made available to the cell.

To obtain tet-regulated GAD-67 expression we used the retroviral vector LINX (kindly provided by F. Gage, The Salk Institute, La Jolla, CA). LINX is a Moloney murine leukemia virus-based vector that contains all of the components required for tet regulation in a "tet-off" manner (50), as well as a neomycin resistance selection marker (Fig. 1). A 1,972-bp rat GAD-67 cDNA encoding the entire open reading frame of GAD-67 messenger RNA (mRNA) (45, 51) [nucleotides (nt) −42 to 1930; a generous gift from A. Tobin (Department of Biology, UCLA, Los Angeles, CA)] was excised from pBS-SK II by digestion with *Sac*II and *Hin*dIII, blunted and cloned into the unique (blunted) *Cla*I site of LINX located directly downstream of the heptamerized tet operator (TetO) sequence fused to the human cytomegalovirus (CMV) immediate early minimal promoter ($P_{hCMV^{*}-1}$, Fig. 1A). The same vector was recently used by Behrstock *et al.* (52) to express GAD-65, the isoform of GAD-67, under tetracycline control in the conditionally immortalized astrocytic cell line BAS-8.1 (see below).

Cell culture

Two cell lines were used: mouse BAS-8.1 immortalized astrocytic cells (kindly provided by A. Campagnoni, Mental Retardation Research Center, UCLA, Los Angeles, CA) for expression of the LINX-GAD-67 retroviral construct, and the ecotropic packaging cell line Phoenix-E (a generous gift from G. Nolan, Department of Molecular Pharmacology Stanford University, Palo Alto, CA) for the production of replication-defective transmissible viruses (53). The BAS-8.1 cell line is derived from mouse cortical astrocytes immortalized by stable incorporation of a temperature-sensitive mutant of the SV40 large T antigen oncogene into their genome (54). To select the cells that incorporated the oncogene, the

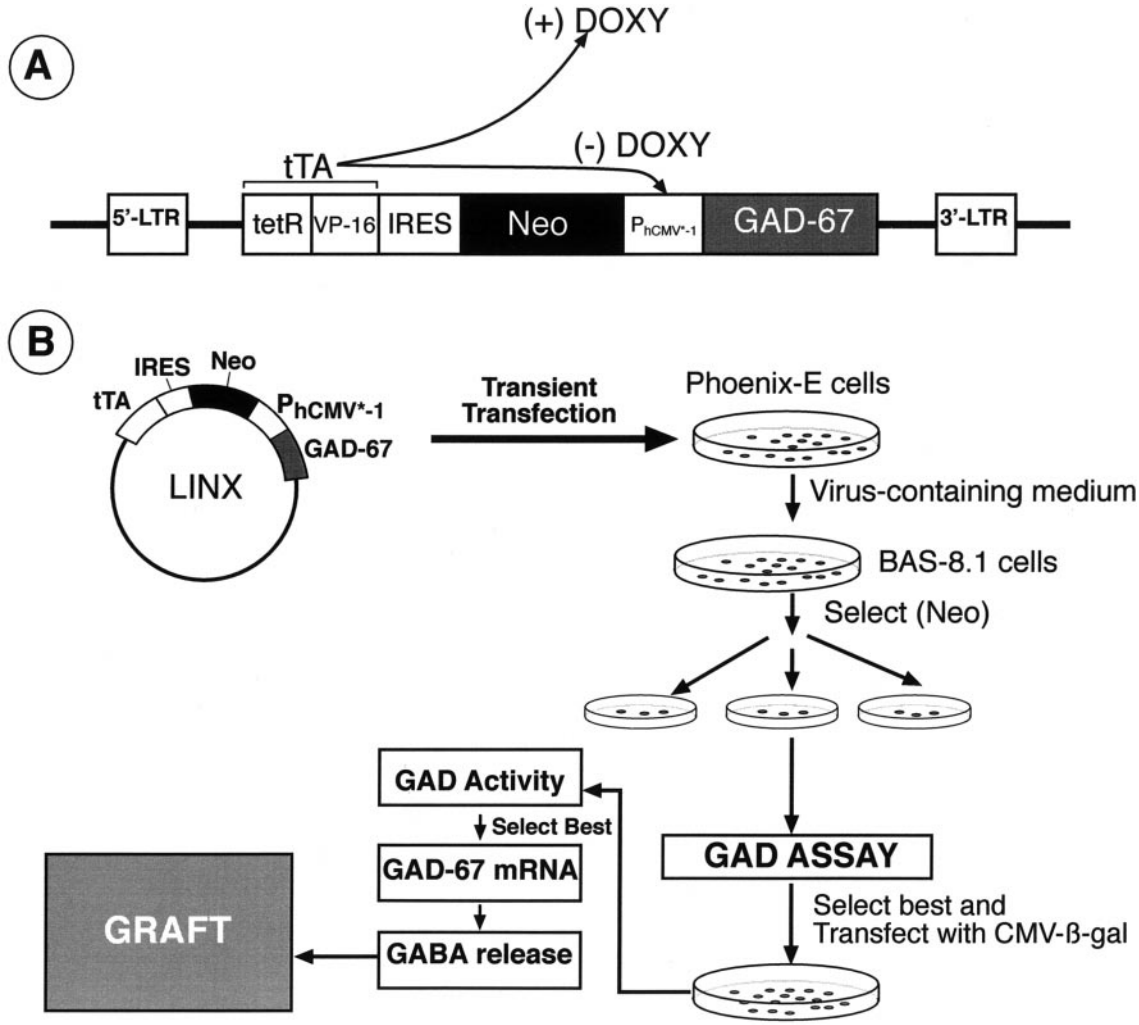

FIG. 1. A, Diagram of the LINX tetracycline (tet)-regulatable retroviral vector used to generate cells overexpressing the GAD-67 gene. The viral LTR transcribes a bicistronic mRNA that in the presence of an IRES (internal ribosomal entry site) element is translated into two protein: tTA (tet repressor-VP16 fusion gene) and neomycin phosphotransferase (neo). In the absence of tetracycline (-tet), tTA binds to, and activates an heptamerized tetracycline operator sequence (tetO) fused to the human cytomegalovirus (CMV) early immediate minimal promoter (CMV TATA) termed P_{hCMV^*-1}, resulting in expression of GAD-67. In the presence of tet (+tet), tTA cannot bind to the tetO sequences and expression levels of the GAD-67 mRNA decrease to those determined by the activity of the basal promoter. B, Outline of the gene transfer and selection procedure employed to generate GABA-producing BAS-8.1 cells. For details see *Materials and Methods*.

immortalizing tsA58 vector was engineered to contain a gene encoding for puromycin resistance, instead of the neomycin resistance gene. Because the thermolabile T antigen protein is active at the permissive temperature of 33−34 C, the host cells can proliferate at this temperature. Upon switching the cells to 37 C, the mutant protein is inactivated, resulting in cessation of cell proliferation and establishment of their differentiated phenotype. Both the packaging cell line and the BAS −8.1 cells were grown in DMEM (Sigma, St. Louis, MO) containing 10% FCS (HyClone Laboratories, Inc., Logan, UT), penicillin G (100U/ml; Sigma), and streptomycin sulfate (100 μg/ml; Sigma). The BAS-8.1 cells had, in addition, puromycin (3 μg/ml; CLONTECH Laboratories, Inc., Palo Alto, CA) added to maintain expression of the T antigen oncogene under selective pressure. A recent report demonstrated the ability of these cells to express GAD-65 and release GABA under the control of the tetracycline-regulated gene expression system (52).

Transfections and infections

The Phoenix-E packaging cell line was transiently transfected with the LINX-GAD-67 retroviral vector using Lipofectamine (Life Technologies, Inc., Grand Island, NY). The cells were seeded at 300,000 cells per well in a 6-well plate. After 24 h, they were washed with PBS solution (PBS) and 1 ml of a transfection cocktail containing 2 μg/ml LINX-GAD67 retroviral vector and 5 μl/ml Lipofectamine in Optimem medium (Life Technologies, Inc.) was added to each well. After 5 h, the transfection medium was replaced with 2 ml of DMEM-10% FCS. Forty-eight hours after transfecting the Phoenix-E cells, the host BAS-8.1 cells were seeded at 350,000 cells per well in a six-well plate, and fresh media were added to the Phoenix cells. Virus containing media was collected from the Phoenix cells 48–72 h after transfection and was passed through a 45 μm filter. Hexadimethrine bromide (Polybrene, Sigma) was added to the virus-containing medium to a final concentration of 4 μg/ml and the medium was applied to the BAS-8.1 cells at 1 ml per well. After 24 h, fresh cultured medium was added to the cells. Selection with neomycin (CLONTECH Laboratories, Inc.) at 500 μg/ml was started 48 h after infection (Fig. 1B).

Cell colonies growing under neomycin selection were isolated, replated, and tested for GAD activity (Fig. 1B, see below). The colony showing the highest activity was expanded and transfected with a plasmid constitutively expressing the β-galactosidase (β-gal) gene under the control of the CMV promoter (pCMV-SPORT-β-gal, Life Technologies, Inc.). For selection of β-gal expressing colonies, the cells were cotransfected with a plasmid encoding the hygromycin gene under the control

of the thymidine kinase promoter (pTK-Hygro, CLONTECH Laboratories, Inc.). The plasmid was cotransfected with pCMV-SPORT-β-gal at a 1/10th ratio and selection was initiated 48 h later by adding hygromycin B at 100 μg/ml to the cultures. Colonies growing under triple selection (puromycin, neomycin and hygromycin) were seeded in duplicate 24-well plates and one of the duplicate wells was stained for β-gal using the procedure described by Sanes *et al.* (55).

After selecting several β-gal expressing colonies, they were isolated and again assayed for GAD activity. The colonies showing the highest GAD activity were then evaluated for GAD-67 mRNA content by RNase protection assay following a 24 h treatment with two different doses of doxycycline (DOXY, 1 and 10 μg/ml). The colony containing the highest level of GAD activity and GAD-67 mRNA content in response to DOXY withdrawal was selected for assessment of GABA release in response to a glutamate challenge, and then used for grafting (Fig. 1B).

GAD assay

Native and GAD-67-producing BAS-8.1 cells were trypsinized and transferred to a 1.6 ml microcentrifuge tube in PBS. Following centrifugation at low speed, the cell pellets were collected and frozen at −85 C until assay for GAD activity (45, 56). For the assay, the cell pellets were resuspended in a homogenization buffer containing 60 mM potassium phosphate, 0.5% Triton X-100, and the protease inhibitors, 2-aminoethylisothiouronium bromide and phenylmethylsulfonyl fluoride at 1 mM each. Twenty microliters of the cell lysate mixture were then incubated in airtight tubes with 0.1 μCi of ^{14}C-L-Glutamate (NEN Life Science Products, Boston, MA), pH 7.0, in 0.1 mM EDTA, 0.5% Triton X-100, 0.1 mM dithiothreitol, 9.0 mM L-glutamate, 30 mM potassium phosphate, and 0.05 mM pyridoxal-5′-phosphate for 1 h at 37 C in a total volume of 60 μl. The $^{14}CO_2$ released by the GAD-mediated decarboxylation of ^{14}C-labeled glutamic acid was trapped on filters that had been saturated with hyamine hydroxide and allowed to dry before being placed in the tubes above the reaction ingredients. The reaction was stopped with 10 ml of 10% trichloroacetic acid, and the mixture was incubated for another hour at 37 C. After the final incubation the filters were removed, placed in scintillation vials with ScintiVerse BD solution (Fisher Scientific, Pittsburgh, PA) and counted on a Packard 1500 Tri-Carb liquid scintillation counter. The lysate mixture was then assayed for protein content (Protein Assay, Bio-Rad Laboratories, Inc., Hercules, CA), and the amounts of $^{14}CO_2$ released were calculated as pmol/mg protein·h.

GABA assay

Cells were seeded at 200,000 per well in a 6-well plate with and without DOXY at 1 μg/ml. Twenty-four hours later, the medium was changed to serum-free, glutamate-free medium (DMEM supplemented with 5 μg/ml insulin and 100 μM putrescine) with and without DOXY at 1 μg/ml. Three days later, the cells were washed with PBS and equilibrated in PBS for 30 min before 200 μM of glutamate was added (57) still in the presence or absence of DOXY. The medium was collected 60 min later and was frozen at −85 C until processed for GABA detection by HPLC. All samples (50 μl, run in duplicate) were derivatized with 5 μl of fluoraldehyde (Orthophthaldehyde, Pierce Chemical Co., Rockford, IL) for exactly 10 min. Then, 10 μl from each sample were injected onto a reverse-phase column (Econosphere C-18, 5 μM, 250 × 4.6 mM) and separated using gradient elution. Standards of known concentrations were treated in exactly the same way to circumvent the problem of adduction during the elution process.

The HPLC system (Beckman Coulter, Inc., Fullerton, CA) consisted of a Model 421 microprocessor, Model 110A pumps, an injector with a 20 μl sample loop and a fluorescence detector (Gilson Specra, Gilson Medical Electronics, Inc., Middleton, WI), coupled to a recording integrator (Beckman Coulter, Inc., Altex Scientific Operation, Berkeley, CA). The fluorescence detector used a standard flow cell, a 7–51 excitation filter and a 3–72 M emission filter. The mobile phase in pump A consisted of 0.1 M NaH_2PO_4 buffer, pH 6.0 containing 10% acetonitrile; that in pump B contained only acetonitrile. The mobile phase was begun at a flow-rate of 1.0 ml/min at 10%B and increased to 40%B over 30 min at which time an isocratic hold lasting 4.5 min was instituted. At the end of the isocratic period, B was increased to 100% over 1 min and maintained at 100% for an additional 10 min. The column was the re-equilibrated with A-B (90:10) for 10 min. Quantification of sample peaks was accomplished by comparing peak areas with those of known concentrations of standards. The data were expressed as pmol GABA/μl sample.

RNA extraction

Total RNA was extracted using the acid phenol method (58, 59) for the extraction of total RNA from cultured cells, as reported (60).

RNase protection assay

The changes in GAD-67 mRNA levels resulting from exposing BAS-8.1 cells carrying the GAD-67 gene to doxycycline were analyzed by RNase protection assay, using 5 μg of total RNA and a procedure described in detail elsewhere (61). Each RNA sample was simultaneously hybridized to 500,000 cpm of a gel-purified ^{32}P-labeled GAD-67 complementary RNA (cRNA) probe and 5,000 cpm of a ^{32}P-labeled cyclophilin antisense RNA probe to correct for procedural variabilities (61). The GAD-67 cRNA probe was generated by *in vitro* transcription of a 220-bp cDNA template complementary to nt 303–523 in the GAD-67 mRNA coding region (51). The cyclophilin probe was transcribed from a 158-bp PCR-generated cDNA fragment corresponding to nt 265 to 422 in rat cyclophilin mRNA (62).

Grafting

Native BAS-8.1 cells or cells carrying the GAD-67 gene under the control of the tetracycline-inducible system were grafted into median eminence-medial basal hypothalamus of 28- to 30-day-old female rats using an stereotaxic approach described in detail elsewhere (63). To maintain GAD-67 production inhibited before implantation, some cells were exposed *in vitro* to DOXY (1 μg/ml) for 3- 5 days before grafting. Cells intended to synthesize GAD-67 were left untreated. On the day of grafting, the cells were harvested and processed for implantation following a procedure previously described (63). Each animal received two injections, each containing 40,000 cells in 1 μl of complete phosphosaline buffer, on both sides of the median eminence, 0.4 mm from the midline, as reported (63).

Evaluation of estrous cyclicity and reproductive competence

Starting on the day of grafting, and throughout rest of the experiment, the rats were provided with drinking water containing 0.5% sucrose or 0.5% sucrose plus DOXY at concentrations of 0.1, 1, or 2 mg/ml. The animals were inspected every afternoon for vaginal opening, starting 2 days after grafting; once the vagina became patent, vaginal lavages were obtained daily for assessment of estrous cyclicity. Seven to 42 days after grafting, the animals were anesthetized with tribromoethanol (2.5 mg/100 g BW) and their brains were perfusion-fixed for histochemical evaluation of the grafted cells (see below). Before perfusion, the uterus and ovaries were dissected out, cleaned of adipose tissue and weighed, and the ovaries were inspected for the presence of corpora lutea. The ovaries were fixed in Kahle's solution (64), embedded in paraffin, sectioned at 8 μm, and stained with methylene blue for histological examination.

Because cyclicity was disrupted in animals carrying GAD-67 producing cells, the fertility of some of these rats was evaluated by placing them with a fertile male for 5 days at a time on two separate occasions. Following introduction of the male into the cage containing the experimental animals, several parameters were evaluated, including the ability of the animals to carry a pregnancy to term, the interval between the introduction of the male and delivery of pups, and the number and body weight of the pups at birth.

In situ localization of the grafted cells and assessment of their functional status

The brains were fixed for either immunohistofluorescence or *in situ* hybridization, using procedures described in detail elsewhere (65, 66).

a) Immunohistofluorescence-confocal microscopy. This procedure was employed to determine the localization of the grafted cells in relation to the LHRH nerve terminals of the median eminence. LHRH nerve terminals were identified with polyclonal antibodies HFU60 (67) diluted 1:1,000

and the reaction was developed with a fluorescein (FITC)-labeled goat antirabbit γ globulin (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA; 1:200), as outlined (38). The BAS-8.1 cells carrying the GAD-67 gene were identified with a monoclonal antibody to β-galactosidase (Roche Molecular Biochemicals, Indianapolis, IN; 500 ng/ml). The reaction was developed with a Texas Red-labeled goat antimouse γ globulin (Jackson ImmunoResearch Laboratories, Inc.; 1:200). Cell nuclei were detected by staining the sections with Hoechst 33258 (bisbenzimide) (Molecular Probes, Inc., Eugene, OR) at 0.1 μg/ml potassium PBS for 1 min after completion of the immunohistochemical reactions. The immunofluorescence images were acquired with a Leica Corp. TCS-SP laser scanning confocal system (Heidelberg, Germany) and a Leica Corp. IRBE microscope, as previously described (68, 69).

*b) Immunohistochemistry/*in situ *hybridization.* This procedure was employed to determine whether the grafted cells were indeed responding to the DOXY treatment with the expected changes in GAD-67 expression. BAS-8.1-GAD-67 cells were identified by their content of immunoreactive β-gal (using the same monoclonal antibody described above) and their content of GAD-67 mRNA was determined by hybridization histochemistry using the same GAD-67 cRNA probe used for RNase protection assays, but labeled with ^{35}S-UTP.

Ovarian morphology

Paraffin-embedded 8 μm serial sections stained with methylene blue (64) were used to determine the number and size distribution of antral follicles. Every fifth section was imaged on a Carl Zeiss Axioplan (Carl Zeiss, Jena, Germany), using a CoolSnap camera (Roper Scientific, Stillwater, MN). Follicle size was estimated only in follicles containing a visible oocyte nucleus (70, 71) by averaging the minimum and maximum diameters of the follicle. Both these measurements and counting of the follicles were carried out using MetaMorph 4.0 (Universal Imaging Co., West Chester, PA). The presence of follicular cysts and precystic structures was also determined using criteria previously reported (72).

RIAs

Circulating LH and FSH levels were measured by RIA as reported (73). Serum levels of ovarian steroids were measured as described (74).

Statistics

The differences between groups were analyzed by ANOVA followed by the Student-Newman-Keuls' multiple comparison test for unequal replications. Frequency distributions were analyzed using the x^2 test. Percentages were subjected to arc-sine transformation before statistical analysis to convert them from a binomial to a normal distribution (75).

Results

BAS-8.1 cells carrying a LINX-GAD-67 retroviral construct express GAD enzymatic activity in a tetracycline-dependent fashion

BAS-8.1 cells stably transfected with LINX-GAD-67 expressed very low GAD activity in the presence of DOXY (1 μg/ml). Three days after removing the antibiotic from the culture medium, GAD activity (measured by the release of $^{14}CO_2$ from 1-^{14}C-labeled glutamate) increased severalfold. Figure 2 depicts the GAD activity in several colonies of genetically modified BAS-8.1 cells cultured in the presence and absence of DOXY. Basal levels of GAD activity were similar in native and DOXY-treated LINX-GAD-67 cells. Removal of the antibiotic led to a striking increase in GAD activity in several colonies. Colonies 6 and 8 were the most active and thus were selected for tagging with a reported gene. We chose the β-gal gene because its protein product can be readily identified by immunohistochemistry to localize the grafted cells in brain tissue (54). Stable incorporation of

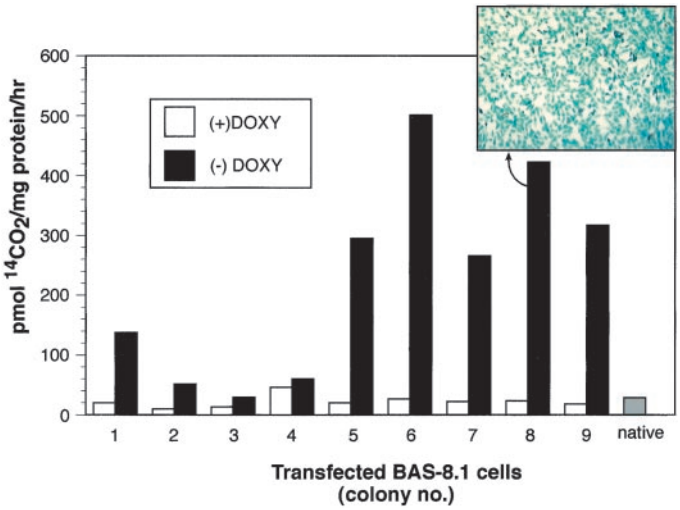

FIG. 2. Detection of GAD activity in BAS-8.1 cells selected in neomycin-containing medium for stable expression of the GAD-67 gene under tet regulatory control. GAD activity was determined by the ability of cell extracts to induce the release of ^{14}C from 1-^{14}C-labeled glutamic acid. Several individual neomycin-resistant colonies were analyzed. Numbers under each pair of columns identify the colony assayed. Colony 8 (*arrow*) was selected for transfection with a CMV-β-gal expression plasmid that, once incorporated into the cell genome, results in constitutive expression of the *Escherichia coli* β-galactosidase (*lac*Z) gene. *Inset,* Histochemical detection of β-gal in BAS-8.1 cells stably transfected with CMV-β-gal.

a CMV driven β-gal transgene into their genome resulted in abundant β-gal expression, as assessed by histochemical detection of the protein (Fig. 2, *inset*).

LINX-GAD-67 cells express GAD-67 mRNA and release GABA in a tet-dependent fashion

Native BAS-8.1 cells had undetectable levels of GAD-67 mRNA (Fig. 3, *upper panel*) and did not release GABA (Fig. 3, *lower panel*) in the presence of 200 μM glutamate. In contrast, when the LINX-GAD-67 cells selected above were cultured in the absence of DOXY they showed markedly elevated levels of GAD-67 mRNA (Fig. 3, *upper panel*) and responded to glutamate with a substantial release of GABA into the culture medium (Fig. 3, *lower panel*). Addition of DOXY markedly reduced, but did not abolish, GAD-67 mRNA content and GABA release from the modified cells (Fig. 3).

LINX-GAD-67 cells tagged with the β-gal gene and grafted into the median eminence can be identified by their immunoreactive β-gal content

Figure 4 depicts confocal microscope images of LINX-GAD-67 cells grafted into the median eminence of the hypothalamus and stained with monoclonal antibodies to β-gal 6 weeks after grafting. Panel A shows the presence of β-gal-positive cells (*red*) in the vicinity of LHRH nerve terminals (*green*) in the mid-to-lateral ventral portion of the median eminence. Panel B demonstrates at a higher magnification, and in a more lateral plane, that some of the β-gal-positive cells establish direct contact with LHRH nerve terminals (*arrows*). Panel C shows a similar view of this relationship,

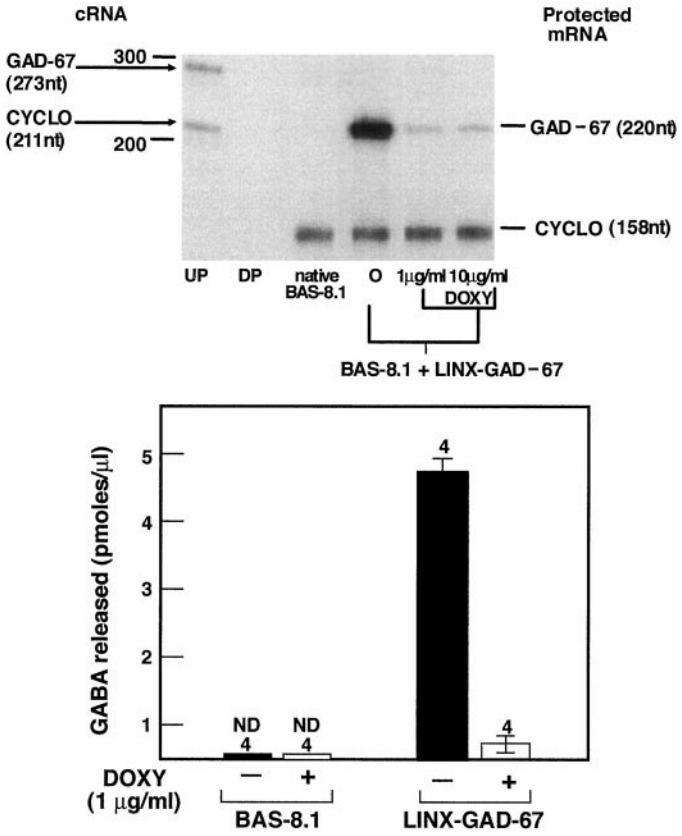

FIG. 3. *Upper panel*, Tetracycline-dependent expression of GAD-67 mRNA in BAS-8.1 cells carrying a LINX retrovirus in which transcription of the GAD-67 gene is under the control of the tetracycline-regulatable gene expression system. The cells were grown in the absence or presence of doxycycline (DOXY, 1 or 10 μg/ml) for 24 h, at the end of which total RNA was extracted and analyzed for GAD-67 mRNA by RNase protection assay. UP, Undigested probes; DP, digested probes; CYCLO, cyclophilin. *Lower panel*, GABA release from BAS-8.1 cells carrying the GAD-67 gene under the control of the tetracycline regulated gene expression system. The cells were cultured in glutamate-free medium for 24 h before adding L-glutamate; 200 μM) to the culture wells. The media were collected 1 h later and their GABA content was determined by HPLC. *Numbers above bars* are number of wells per group. *Vertical bars* are SEM. ND, Not detectable.

but in the lateral median eminence, where the bulk of LHRH nerve terminals converge to release their secretory products into the portal system. Panel D shows the presence of β-gal-containing cells in the tract of the needle used for grafting and that runs parallel to the wall of the third ventricle.

In vivo treatment with DOXY turns off GAD-67 mRNA expression in LINX-GAD-67 cells grafted into the median eminence of the hypothalamus

LINX-GAD-67 cells grafted into the median eminence of rats not receiving DOXY treatment expressed high levels of GAD-67 mRNA, which were reduced to undetectable values in animals treated with DOXY. Figure 5A depicts a darkfield image of the median eminence of an animal not treated with DOXY demonstrating the presence of a focus of GAD-67 mRNA expression in the latero-ventral aspect of the median eminence (*white frame*). That this hybridizing hot spot corresponds to GAD-67 mRNA expressed in the grafted cells is shown in Fig. 5C which demonstrates, at a higher magnification and in a brightfield image, that the hybridization signal (*white grains*) is present in β-gal immunopositive cells (*brown staining*). Figure 5B demonstrates the absence of focal hybridization in the median eminence of a rat grafted with GAD-67-producing cells and treated with DOXY. This absence of focal expression was due to undetectable levels of GAD-67 mRNA (*white grains*) in the grafted cells, identified by their β-gal immunoreactivity (*brown color*; Fig. 5D).

GABA-producing cells grafted into the median eminence disrupt estrous cyclicity in a tet-dependent manner

The age at vaginal opening was similar in rats grafted with "GAD on" BAS-8.1 cells compared with animals grafted with native BAS-8.1 cells (37.4 $\pm$ 0.5 *vs.* 37.0 $\pm$ 0.7 days, n = 8 for both groups). Surprisingly, rats grafted with "GAD off" cells, *i.e.* given DOXY in the drinking water showed a significant delay in vaginal opening (41.2 $\pm$ 1.2 days, n = 8). This delay, however, did not appear to be caused by leakage of the delivery system, *i.e.* the release of small amounts of GABA by the grafted cells (see Fig. 3), but instead was related to the DOXY treatment. Administration of the antibiotic to rats grafted with native BAS-8.1 cells or intact animals resulted in ages at vaginal opening (40.2 $\pm$ 2.3 and 39.7 $\pm$ 0.6 days, n = 5 for both groups) that were similar to that of rats grafted with "GAD off" cells.

In contrast to the lack of effect of the grafts on the onset of puberty, estrous cyclicity was markedly disrupted in animals receiving a graft of GABA-producing cells. As illustrated in Fig. 6, these animals exhibited estrous cycles characterized by prolonged periods of estrus sporadically interrupted by occasional days in proestrus and diestrus. An initial dose of 100 μg DOXY/ml water was not sufficient to reverse this profile toward normal cyclicity. Increasing the dose to 2,000 μg/ml was, however, effective. Statistical analysis of the estrous cycle of each animal demonstrated that animals carrying "GAD on" cells spent a greater ($P < 0.01$) percentage of time in estrus, and much less time ($P < 0.01$) in proestrus and diestrus than controls rats grafted with native cells (Fig. 7). Turning off GABA release by administration of DOXY in the drinking water fully restored the incidence of each phase of the estrous cycle to the values seen in control animals grafted with native cells (Fig. 7).

Rats grafted with either native BAS-8.1 cells or "GAD off" cells became pregnant within the first 5 days of exposure to a fertile male (n = 5). In contrast, of three rats receiving "GAD on" cells, two become pregnant only after a second 5-day exposure to a male, and the third one failed to become pregnant even after three consecutive 5-day exposure periods. No significant differences in the number of pups/litter were found among the three groups.

GABA-producing cells grafted into the median eminence of the hypothalamus disrupt pituitary and ovarian hormone output in a tet-dependent manner

Circulating gonadotropin and sex steroid levels were measured in sera collected 4–6 weeks after the intrahypothalamic grafting of GABA-producing cells. Although FSH lev-

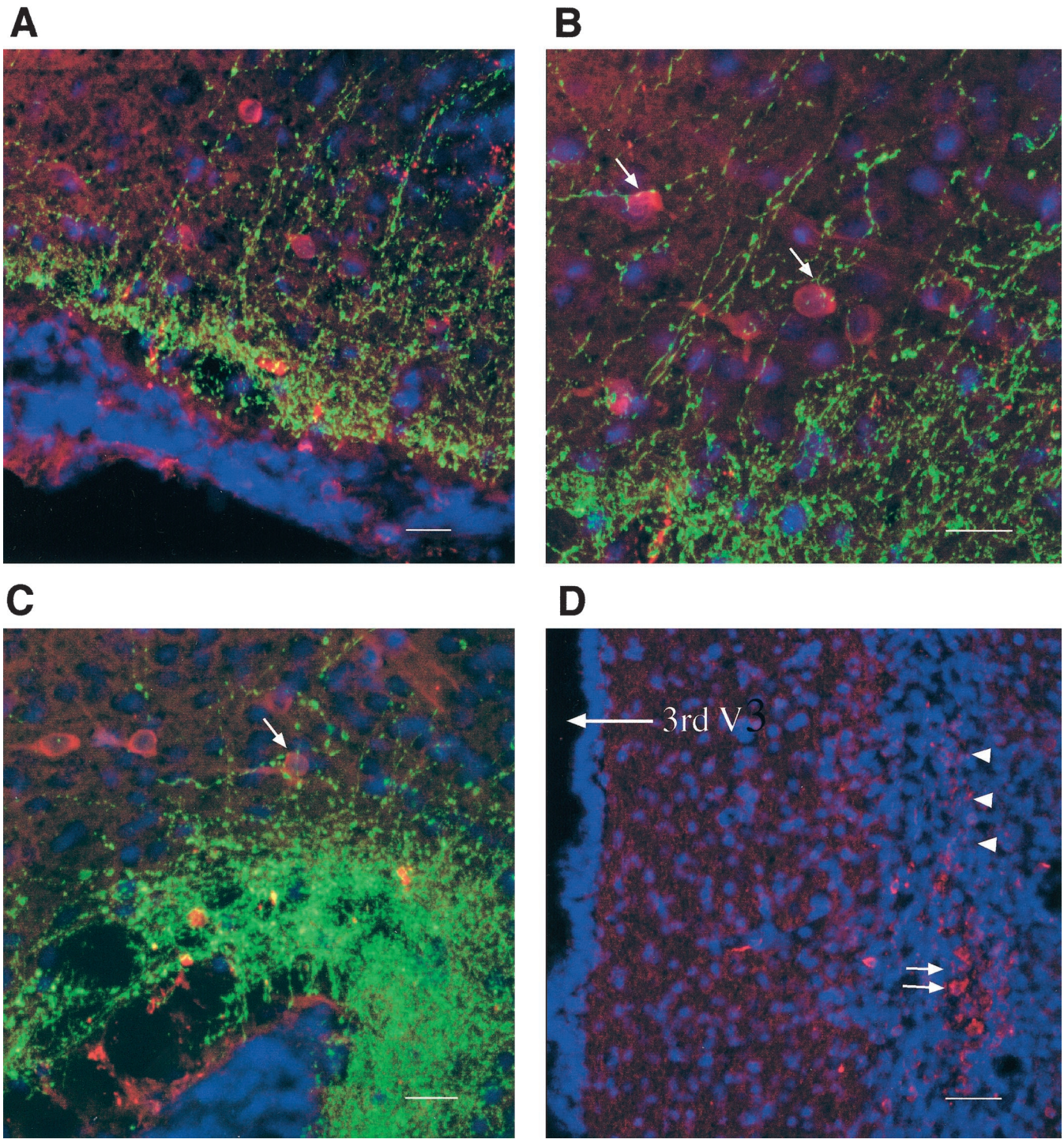

FIG. 4. Detection of BAS-8.1 cells grafted near LHRH nerve terminals in the median eminence of the hypothalamus by double immunofluorescence-confocal microscopy. LHRH nerve terminals (*green color*) were visualized with a rabbit polyclonal antiserum to the LHRH decapeptide and an FITC-conjugated second antibody. The grafted cells (*red color*) were identified with a monoclonal antibody to β-galactosidase and a Texas Red-conjugated second antibody. Cell nuclei (*blue color*) were stained with Hoerscht dye. A, β-gal-positive cells in the vicinity of LHRH nerve endings in the medio-lateral aspect of the median eminence. B, Higher magnification view of a similar field from another animal. C, β-gal-positive cells near LHRH nerve endings in the lateral aspect of the median eminence. Notice in B and C the close apposition of some LHRH nerve terminals to β-gal-positive cells (*arrows*). D, β-gal-positive cells (examples denoted by *double arrows*) along the tract of the needle used for grafting (denoted by *arrowheads*). 3rd V, Third ventricle. *Bars* in A–C, 5 μm; *bar* in D, 10 μm.

els were not affected (Fig. 8, *middle upper panel*), animals grafted with "GAD on" cells showed a 50% increase in LH levels, which was obliterated ($P < 0.05$) by DOXY treatment (Fig. 8, *left upper panel*). As a consequence of these changes, the LH:FSH ratio tended to increase in animals carrying "GAD on" cells with respect to the ratio in rats grafted with

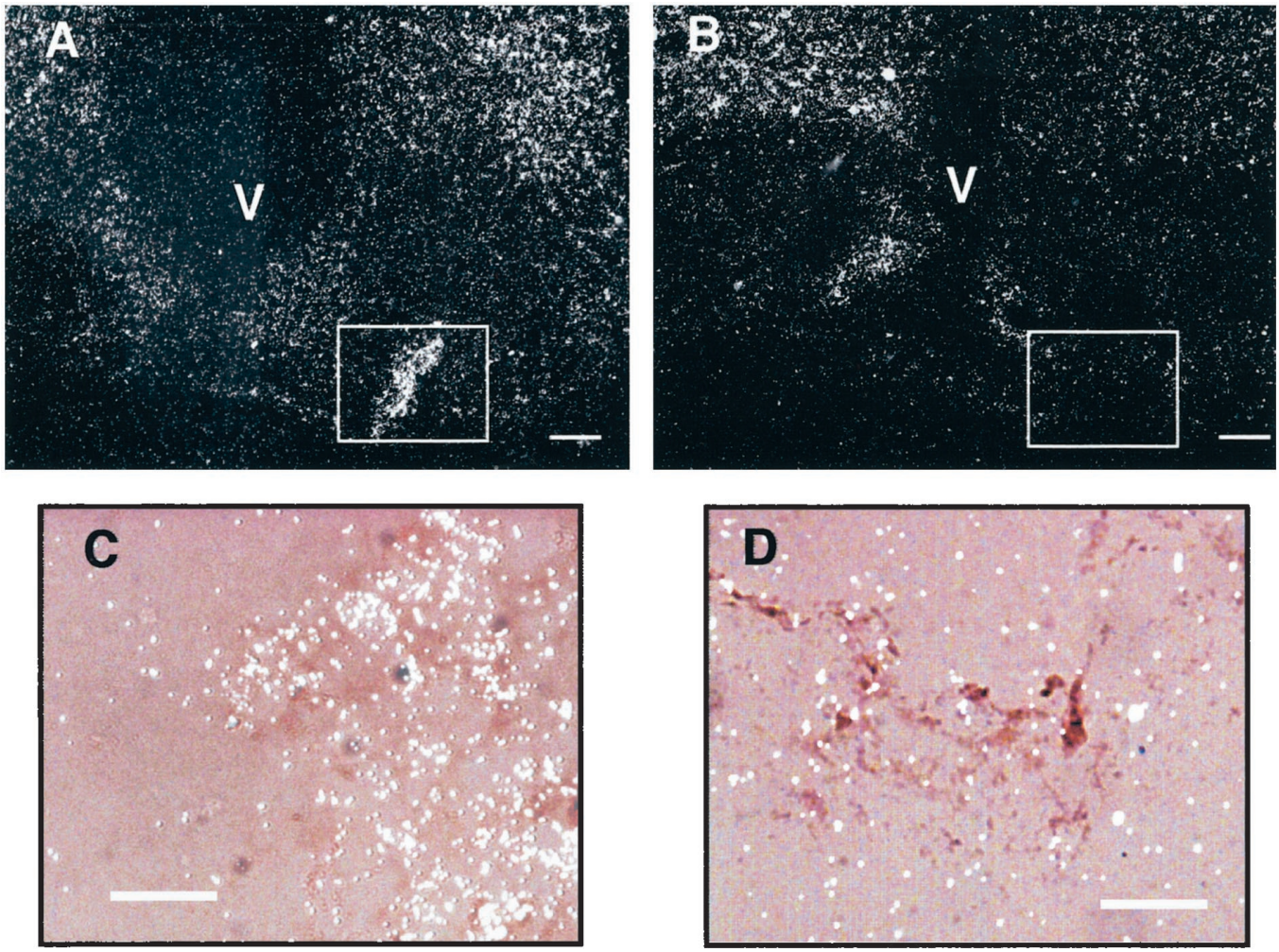

FIG. 5. *In vivo* regulation of GAD-67 mRNA expression in cells carrying the GAD-67 gene under the control of the "tet off" tetracycline-regulated gene expression system, and grafted near the median eminence of the hypothalamus. In this system, GAD-67 synthesis and GABA release are activated in the absence of the tetracycline analog DOXY ("GAD on"), and repressed in its presence ("GAD off"). A, Darkfield image showing a hot spot of ^{35}S-UTP-GAD-67 cRNA hybridization in the latero-ventral aspect of the median eminence from a female rat grafted with "GAD on" cells. B, Absence of focal ^{35}S-UTP-GAD-67 cRNA hybridization in the median eminence of a rat grafted with BAS-8.1 GAD-67 cells and treated with DOXY (2 mg/ml) in the drinking water to inhibit GAD-67 mRNA expression. C, Brightfield image showing that the hybridization signal shown in A (seen here as *white grains*) is on grafted cells identified by their content of immunoreactive β-gal (*brown color*). D, Brightfield image showing the lack of detectable GAD-67 cRNA hybridization to the β-gal positive cells (*brown color*) grafted in the *boxed area* depicted in C. *Bars* for A and B, 200 μm; *bars* for C and D, 50 μm.

BAS-8.1 or "GAD off" cells (Fig. 8, *right upper panel*). Whereas a similar tendency was observed in serum estradiol levels (Fig. 8, *left lower panel*), serum P levels were similar in all three groups of animals (Fig. 8, *middle lower panel*). In contrast to the subtle alterations in LH and estradiol levels, serum androstenedione values were noticeably elevated in rats carrying "GAD on" cells, an increase that was absent in rats grafted with "GAD off" cells (Fig. 8, *right lower panel*).

Ovarian follicular dynamics is altered in animals carrying GABA-producing cells into the median eminence of the hypothalamus

The mean size of ovarian follicles in the 300–499 μm and >500 μm range was similar in the three groups studied, *i.e.* rats grafted with native BAS-8.1 cells and rats grafted with "GAD on" or "GAD off" cells (Table 1). However, the relative distribution of these two populations was different as both "GAD on" and "GAD off" groups had a significantly greater incidence of >500 μm follicles than control rats grafted with native BAS-8.1 cells (Table 1). A more detailed examination of this follicle population in the two groups grafted with GAD-67 synthesizing cells (Table 2) revealed that in "GAD on" rats there were more large (>600 μm) than intermediate (500–599 μm) size follicles (37/26) and that in the "GAD off" rats this proportion was reversed, *i.e.* there were more intermediate than large-size follicles (28/37, $P < 0.025$ by x^2 test). In all three groups a low incidence of follicular cysts and precystic structures (follicle type III) (72) was detected in the population of follicles larger than 500 μm. This incidence was similar in all three groups (6 of 36 follicles in the group receiving BAS-8.1 cells; 6 of 63 in the "GAD on" group; and 4 of 65 in the "GAD off" group).

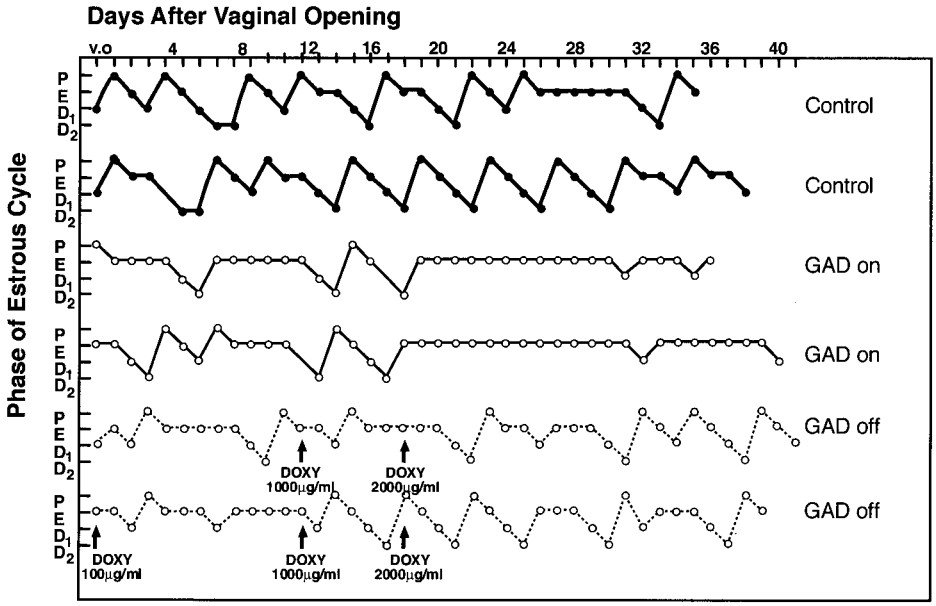

FIG. 6. Disruption of estrous cyclicity in postpubertal rats by tetracycline-dependent activation of GABA release from BAS-8.1 cells carrying the GAD-67 gene under the control of the "tet off" tetracycline regulated gene expression system and grafted into the median eminence of the hypothalamus. Two animals from each group are represented. Control animals were grafted with native BAS-8.1 cells. Notice that estrous cyclicity is restored by the 1,000 and 2,000 μg/ml doses of DOXY by not by the lower dose of 100 μg/ml. P, Proestrus; E, estrus; D_1, diestrous day 1; D_2, diestrous day 2.

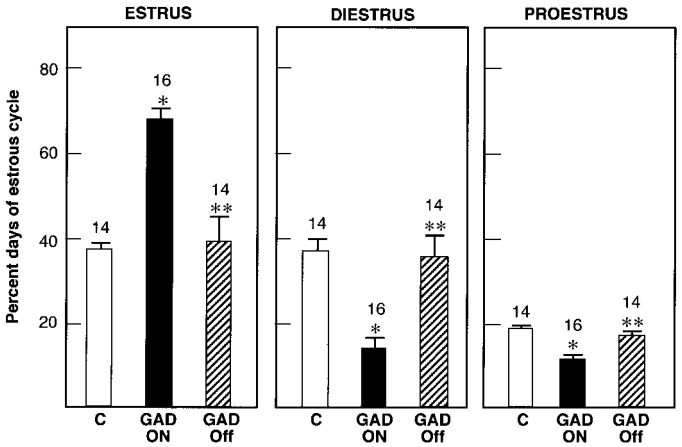

FIG. 7. Analysis of the alterations in estrous cyclicity caused by the tetracycline-dependent activation of GABA release from BAS-8.1 cells carrying the GAD-67 gene under the control of the "tet off" tetracycline regulated gene expression system and grafted into the median eminence of the hypothalamus. The tetracycline-controlled transgene is designed to activate GAD-67 synthesis (and, therefore, GABA release) in the absence of tetracycline (*i.e.* no DOXY in the drinking water, "GAD on") and to repress GAD-67 synthesis in the presence of DOXY (2,000 μg/ml, "GAD off"). Animals carrying "GAD on" cells exhibited almost twice as many days in estrus than control rats grafted with native BAS-8.1 cells and 3 to 4 times fewer days in proestrus and diestrus, respectively. Treatment with DOXY to turn off GAD-67 expression restored the incidence of all three stages of the estrous cycle to control values. *, $P < 0.1$ *vs.* control and "GAD off" groups; **, $P < 0.1$ *vs.* "GAD on" group.

Discussion

The present results demonstrate that conditional activation of GABA release near LHRH nerve terminals in the median eminence of the hypothalamus disrupts estrous cyclicity and reduces fertility in female rats. A regionally restricted increase in GABA release was attained by grafting immortalized astrocytes genetically modified to express GAD-67, one of the GABA synthesizing enzymes, under the control of the tetracycline-dependent gene expression system. Although in this system activation of GAD-67 synthesis is regulated by exogenous manipulation of tetracycline levels, the formation of GABA itself can only occur if endogenous glutamate levels are sufficiently elevated within the microenvironment where the cells were grafted. Thus, the brain is provided with cells able to synthesize GABA, but actual production of the neurotransmitter requires the availability of endogenous precursors. Because of these characteristics, GABA-synthesizing immortalized astrocytes are currently being tested as vehicles for gene therapy of the central nervous system (52, 57, 76).

The neurotransmitter GABA is synthesized from glutamate via a reaction catalyzed by the enzyme GAD. GAD exists in two forms, encoded by two separate genes, GAD-65 and GAD-67 (45). Deletion of the GAD-65 and GAD-67 genes has, however, demonstrated that GAD-67 is the key enzyme for GABA synthesis, as loss of GAD-67 results in more than 90% depletion of GABA levels in the brain, as compared with no reduction in mice deficient in GAD-65 (49). Because most of neuronal GAD-67 is present as an active holoenzyme (45), GABA synthesis would be expected to increase rapidly in the presence of adequate levels of glutamate. Such a change does, in fact, occur in the intact brain (77) and, as shown in the present study, is also observed in GAD-67-expressing cells challenged with glutamate. Previous studies showed that GAD-67-expressing astrocytes preferentially convert glutamate into GABA (57), and rapidly release GABA in response to glutamate, presumably via activation of reverse GABA transporters (57).

Our results show that when GABA availability to LHRH nerve terminals is increased at the end of the juvenile period, the onset of female puberty is not delayed. A delay would have been predicted by earlier studies demonstrating that a) GABA acts on the median eminence of prepubertal rhesus monkeys to inhibit LHRH release (15, 16), and b) that the ability of GABA to delay the pubertal process in rats becomes established during the juvenile period of development (21–23). Although several more mundane explanations come to

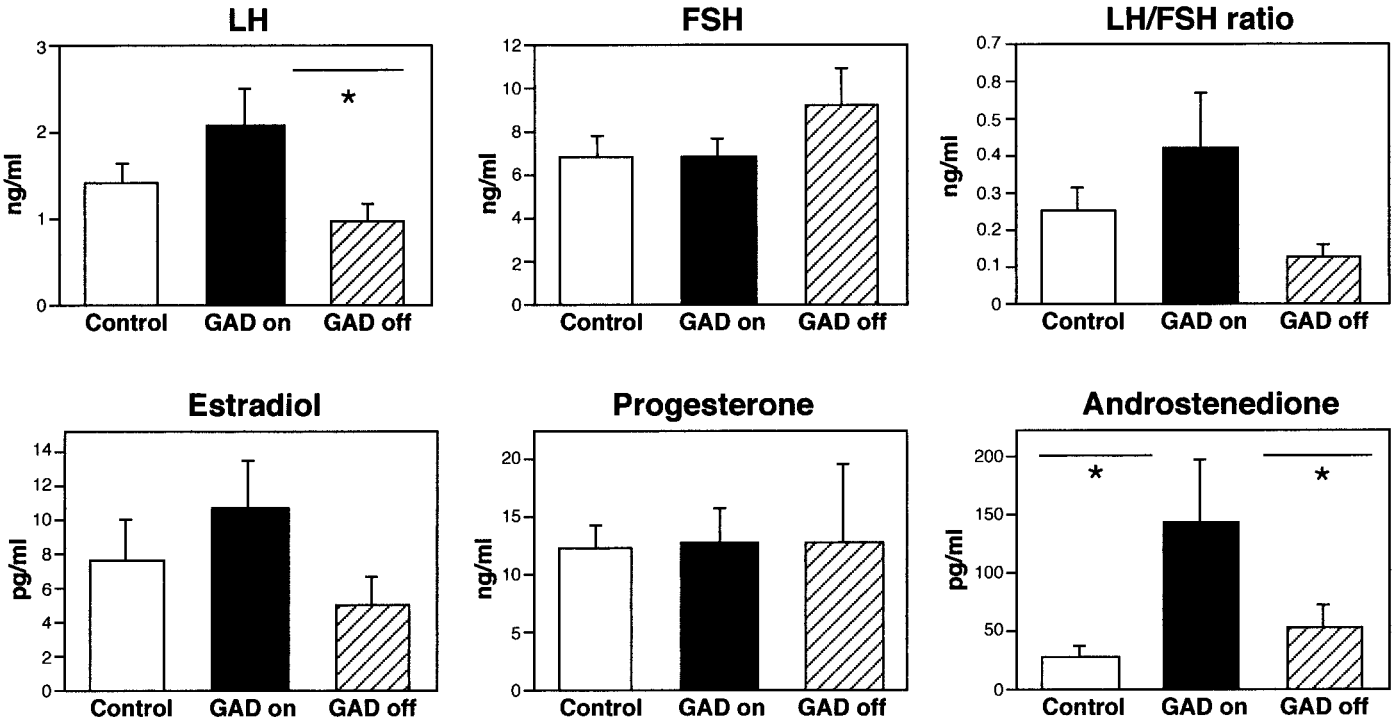

FIG. 8. Serum hormone levels in postpubertal rats grafted in the median eminence of the hypothalamus with BAS-8.1 cells carrying the GAD-67 gene under the control of the "tet off" tetracycline regulated gene expression system. The cells were grafted at 28–30 days of age and the serum was collected 4–6 weeks later for hormone measurements. Control rats (grafted with native BAS-8.1 cells) and "GAD on" rats were provided with 0.5% glucose in the drinking water. "GAD off" rats received DOXY (2 mg/ml) in 0.5% glucose in their drinking water. *, $P < 0.05$ *vs.* control and/or "GAD off" groups.

TABLE 1. Incidence of medium and large size antral follicles in rats carrying intrahypothalamic grafts of GABA-producing cells under the control of the tetracycline-regulated gene expression system

Class of antral follicles	Groups receiving grafts[a]		
	Native BAS-8.1 cells[b]	GAD on cells[c]	GAD off cells[d]
Total No. of > 500 μm follicles/Total No. of 300–499 μm follicles[e]	36/84	63/73[g]	65/56[g]
Actual size of > 500 μm class	644.7 ± 19[f] (36)	670 ± 18 (63)	640.1 ± 16 (65)
Actual size of 300–499 μm class	387.8 ± 6 (84)	386.6 ± 7 (73)	396.6 ± 8 (56)

[a] BAS-8.1 cells were grafted into the median eminence of the hypothalamus on postnatal day 28–30. Ovaries were collected 4–6 weeks later for histological analysis.
[b] Conditionally immortalized BAS-8.1 cells not carrying a LINX-GAD-67 construct.
[c] BAS-8.1 cells carrying a LINX-GAD-67 construct; recipient rats given 0.5% sucrose in the drinking water.
[d] BAS-8.1 cells carrying a LINX-GAD-67 construct; recipient rats given DOXY (2 mg/ml) in 0.5% sucrose drinking water to suppress GAD-67 expression.
[e] Calculated from a total of 5 rats/group.
[f] Mean ± SEM (number of follicles in *parentheses*).
[g] $P < 0.001$ (x^2 test) *vs.* group carrying native BAS-8.1 cells.

mind (for instance, the cells were grafted too late in development, and/or the amount of GABA produced may have been insufficient to suppress LHRH release), we believe that a more tenable explanation for our findings is that—within the rodent hypothalamus—the inhibitory effects of GABA on LHRH secretion are preferentially exerted in the preoptic region, where the LHRH cell bodies are located (18, 28, 30), instead of the median eminence, which was the area selected for grafting.

Although specific experiments may have to be designed to resolve this issue, the intention of the present study was not to reexamine the effect of GABA on the onset of puberty, but instead, to determine whether a discrete change in GABA availability to LHRH nerve terminals, endogenously driven by the availability of glutamate, the GABA precursor, would disrupt adult reproductive cyclicity. The tet-controlled system we employed may not be useful to study developmental aspects of reproductive neuroendocrine control because of the unexpected side effects observed in animals given tet in the drinking water. These animals exhibited a significant delay in vaginal opening as compared with rats drinking only glucose-containing water. The delay was independent of both the surgical procedure used to graft the cells and the presence of foreign cells in the median eminence, as it was also observed in intact rats receiving DOXY. The delay is more likely related to the animals drinking less water due to

TABLE 2. Changes in the size distribution of large antral follicles in the ovaries of rats carrying intrahypothalamic grafts of GABA-producing cells under the control of the tetracycline-regulated gene expression system

Class of large antral follicles	Groups receiving grafts[a]	
	GAD on cells[b]	GAD off cells[c]
Total No. of > 600 μm follicles/Total No. of 500–599 μm follicles[d]	37/26[e]	28/37
Actual size of > 600 μm class	759.6 ± 19 (37)	759.5 ± 21 (28)
Actual size of 500–599 μm class	539.7 ± 8 (26)	549.8 ± 4 (37)

[a] Cells were grafted into the median eminence of the hypothalamus on postnatal day 28–30 and the ovaries were collected 4–6 weeks later for histological analysis.
[b] BAS-8.1 cells carrying a LINX-GAD-67 construct; recipient rats receiving 0.5% sucrose drinking water.
[c] BAS-8.1 cells carrying a LINX-GAD-67 construct; recipient rats given DOXY (2 mg/ml drinking water containing 0.5% sucrose) to suppress GAD-67 expression.
[d] Calculated from a total of 5 rats/group.
[e] $P < 0.025$ *vs.* GAD off cells (by x^2 test).

the bitter taste of DOXY, a reaction observed despite the presence of glucose in the water. Future studies using the tet-controlled system in developing animals will have to search for alternative means of providing the antibiotic orally. Incorporating DOXY to the pelleted food seems to represent a viable alternative for drug administration (78). However, adult animals may be much less sensitive to the treatment than developing rats, because DOXY-treated postpubertal rats cycle and become pregnant as normally as untreated controls receiving grafts of native BAS-8.1 cells.

The tet off-dependent activation of GABA release from GAD-67 producing cells grafted near the median eminence was strikingly effective in disrupting reproductive cyclicity. The GABA dependency of this disruption was indicated by the ability of DOXY treatment—which turns off GABA release—to normalize the estrous cycle toward a pattern indistinguishable from that observed in rats grafted with native BAS-8.1 cells (which are incapable of producing GABA). Two alternative explanations can be offered for these findings. The first assumes that the tet-dependent, focal increase in GABA production near LHRH nerve terminals inhibits LHRH release from those terminals near the grafted cells. This mode of action would be consistent with the well-established inhibitory effects that GABA exerts on LHRH release from the monkey median eminence (15) (reviewed in Ref. 79). However, because in our experiments GABA-producing cells led to an apparent stimulation of the hypothalamic-pituitary-ovarian axis (see below), one would have to assume that, if GABA inhibited LHRH release, there was also a compensatory increase in LHRH release from terminals not accessed by GABA. The second explanation, which we favor, is that GABA actually stimulates LHRH release by a direct action on LHRH nerve terminals (42, 43). Such a direct stimulatory effect is in keeping with the demonstration that the direct actions of GABA on LHRH neurons are of excitatory nature (35, 36, 40, 80), and that exposure of isolated medial basal hypothalamic fragments (which contain LHRH terminals without their perikarya) or the median eminence itself to GABA receptor agonists increases, instead of inhibits LHRH release (42, 43).

Although we did not determine the *in vivo* LHRH response to GABA-producing cells, measurement of serum pituitary gonadotropins and sex steroids levels suggested that, indeed, the hypothalamic-pituitary-ovarian system in rats carrying "GAD on" cells was tonically activated, instead of depressed, by the focal increase in GABA availability. This activation was evidenced by several parameters including the mildly elevated serum LH and estradiol levels, the clearly increased serum androstenedione values, and the higher incidence of large, steroidogenically active (81) ovarian follicles detected in these animals. It thus appears that the presence of GABA-producing cells near a subpopulation of LHRH nerve terminals in the median eminence alters the pattern of LHRH secretion sufficiently to disrupt reproductive cyclicity and cause: 1) an LH-dependent increase in ovarian steroidogenic output; 2) a relative inability of these higher circulating steroid levels to suppress gonadotropin (and presumably LHRH) secretion (because LH levels are not reduced by the high androstenedione and mildly elevated estradiol levels; and c) a disruption of the central mechanism underlying the preovulatory surge of gonadotropins (because "GAD on" rats ovulate sporadically despite the presence of ovulatory competent (82) antral follicles in their ovaries).

Taken altogether, the present results are consistent with the concept that chronic disruption of GABA homeostasis within the confines of the median eminence of the hypothalamus leads to reproductive dysfunction because of an inappropriate increase in LHRH secretion. The disruption in estrous cyclicity, without complete loss of ovulatory capacity, observed in these animals raises the possibility that similar alterations in GABA homeostasis in the same or other relevant regions of the human hypothalamus may contribute to the genesis of at least a fraction of hypothalamic oligomenorrhea/amenorrhea syndromes.

Acknowledgments

We are grateful to Soshana Behrstock and Allan Tobin (Department of Biology, UCLA, Los Angeles, CA) for providing us with the rat GAD-67 cDNA and for their generous help and advice in the preparation and functional analysis of the GAD-67 retroviral construct), and to Les Dees (Texas A & M University, College Station, TX) for measuring serum gonadotropins. We also thank Diane Hill, Veronica Tapia, and Maria E. Costa for their expert technical assistance, and Pablo Ojeda for performing the morphometric analysis of the ovaries.

References

1. **Ojeda SR, Urbanski HF** 1994 Puberty in the rat. In: Knobil E, Neill JD (eds) The Physiology of Reproduction, ed 2. Raven Press, New York, vol 2:363–409
2. **Plant TM** 1994 Puberty in primates. In: Knobil E, Neill J (eds) The Physiology of Reproduction, ed 2. Raven Press, New York, vol 2:453–485
3. **Terasawa E** 1995 Mechanisms controlling the onset of puberty in primates: the

role of GABAergic neurons. In: Plant TM, Lee PA (eds) The Neurobiology of Puberty. Journal of Endocrinology Ltd., Bristol, UK, pp 139–151
4. **Gay VL, Plant TM** 1987 *N*-methyl-D,L-aspartate (NMDA) elicits hypothalamic GnRH release in prepubertal male rhesus monkeys. Endocrinology 120:2289–2296
5. **Urbanski HF, Ojeda SR** 1990 A role for *N*-methyl-D-aspartate (NMDA) receptors in the control of LH secretion and initiation of female puberty. Endocrinology 126:1774–1776
6. **Brann DW, Mahesh VB** 1994 Excitatory amino acids: function and significance in reproduction and neuroendocrine regulation. Front Neuroendocrinol 15:3–49
7. **Decavel C, van den Pol AN** 1990 GABA: a dominant neurotransmitter in the hypothalamus. J Comp Neurol 302:1019–1037
8. **Wisden W, Seeburg PH** 1992 $GABA_A$ receptor channels: from subunits to functional entities. Curr Opin Neurobiol 2:263–269
9. **Sieghart W** 1995 Structure and pharmacology of gamma-aminobutryic $acid_A$ receptor subtypes. Pharmacol Rev 47:181–234
10. **Rabow LE, Russek SJ, Farb DH** 1995 From ion currents to genomic analysis: recent advances in $GABA_A$ receptor research. Synapse 21:189–274
11. **Bowery N** 1989 $GABA_B$ receptors and their significance in mammalian pharmacology. Trends Pharmacol Sci 10:401–411
12. **Bormann J** 1988 Electrophysiology of $GABA_A$ and $GABA_B$ receptor subtypes. Trends Neurosci 11:112–116
13. **Mott DD, Lewis DV** 1994 The pharmacology and function of central $GABA_B$ receptors. Int Rev Neurobiol 36:97–223
14. **Kaupmann K, Huggel K, Heid J, Flor PJ, Bischoff S, Mickel SJ, McMaster G, Angst C, Bittiger H, Froestl W, Bettler B** 1997 Expression cloning of $GABA_B$ receptors uncovers similarity to metabotropic glutamate receptors. Nature 386:239–246
15. **Mitsushima D, Hei DL, Terasawa E** 1994 Gamma-aminobutyric acid is an inhibitory neurotransmitter restricting the release of luteinizing hormone-releasing hormone before the onset of puberty. Proc Natl Acad Sci USA 91:395–399
16. **Mitsushima D, Marzban F, Luchansky LL, Bruich AJ, Keen KL, Durning M, Golos TG, Terasawa E** 1996 Role of glutamic acid decarboxylase in the prepubertal inhibition of the luteinizing hormone releasing hormone release in female rhesus monkeys. J Neurosci 16:2563–2573
17. **Keen KL, Burich AJ, Mitsushima D, Kasuya E, Terasawa E** 1999 Effects of pulsatile infusion of the $GABA_A$ receptor blocker bicuculline on the onset of puberty in female rhesus monkeys. Endocrinology 140:5257–5266
18. **Jarry H, Hirsch B, Leonhardt S, Wuttke W** 1992 Amino acid neurotransmitter release in the preoptic area of rats during the positive feedback actions of estradiol on LH release. Neuroendocrinology 56:133–140
19. **Herbison AE, Dyer RG** 1991 Effect on luteinizing hormone secretion of GABA receptor modulation in the medial preoptic area at the time of proestrous luteinizing hormone surge. Neuroendocrinology 53:317–320
20. **Mitsushima D, Jinnai K, Kimura F** 1997 Possible role of the γ-aminobutyric acid-A receptor system in the timing of the proestrous luteinizing hormone surge in rats. Endocrinology 138:1944–1948
21. **Moguilevsky JA, Carbone S, Szwarcfarb B, Rondina D** 1991 Sexual maturation modifies the GABAergic control of gonadotrophin secretion in female rats. Brain Res 563:12–16
22. **Szwarcfarb B, Carbone S, Stein ML, Medina J, Moguilevsky JA** 1994 Sexual differences in the effect of the GABAergic system on LH secretion and in the hypothalamic ontogenesis of $GABA_A$ receptors in prepubertal rats. Brain Res 646:351–355
23. **Mitsushima D, Kimura F** 1997 The maturation of $GABA_A$ receptor-mediated control of luteinizing hormone secretion in immature male rats. Brain Res 748:258–262
24. **Adler BA, Crowley WR** 1986 Evidence for gamma-aminobutyric acid modulation of ovarian hormonal effects on luteinizing hormone secretion and hypothalamic catecholamine activity in the female rat. Endocrinology 118:91–97
25. **Masotto C, Negro-Vilar A** 1987 Activation of gamma-amino butyric acid B-receptors abolishes naloxone-stimulated luteinizing hormone release. Endocrinology 121:2251–2255
26. **Akema T, Kimura F** 1993 Differential effects of $GABA_A$ and $GABA_B$ receptor agonists on NMDA-induced and noradrenaline-induced luteinizing-hormone release in the ovariectomized estrogen-primed rat. Neuroendocrinology 57:28–33
27. **Akema T, Kimura F** 1992 Modulation of pulsatile LH secretion by baclofen, a selective $GABA_B$ receptor agonist, in ovariectomized rats. Neuroendocrinology 56:141–147
28. **Jarry H, Leonhardt S, Wuttke W** 1991 Gamma-aminobutyric acid neurons in the preoptic/anterior hypothalamic area synchronize the phasic activity of the gonadotropin-releasing hormone pulse generator in ovariectomized rats. Neuroendocrinology 53:261–267
29. **Akema T, Chiba A, Kimura F** 1990 On the relationship between noradrenergic stimulatory and GABAergic inhibitory systems in the control of luteinizing hormone secretion in female rats. Neuroendocrinology 52:566–572
30. **Jarry H, Perschl A, Wuttke W** 1988 Further evidence that preoptic anterior hypothalamic GABAergic neurons are part of the GnRH pulse and surge generator. Acta Endocrinol 118:573–579
31. **Feleder C, Jarry H, Leonhardt S, Wuttke W, Moguilevsky JA** 1996 The GABAergic control of gonadotropin-releasing hormone secretion in male rats during sexual maturation involves effects on hypothalamic excitatory and inhibitory amino acid systems. Neuroendocrinology 64:305–312
32. **Nikolarakis KE, Loeffler J-PH, Almeida OFX, Herz A** 1988 Pre- and postsynaptic actions of GABA on the release of hypothalamic gonadotropin-releasing hormone (GnRH). Brain Res Bull 21:677–683
33. **Ondo JG** 1974 Gamma-aminobutyric acid effects on pituitary gonadotropin secretion. Science 186:738–739
34. **Fleischmann A, Makman MH, Etgen AM** 1995 $GABA_A$ receptor activation induces GABA and glutamate release from preoptic area. Life Sci 56:1665–1678
35. **Hales TG, Sanderson MJ, Charles AC** 1994 GABA has excitatory actions on GnRH-secreting immortalized hypothalamic (GT1–7) neurons. Neuroendocrinology 59:297–308
36. **Martínez de la Escalera G, Choi ALH, Weiner RI** 1994 Biphasic gabaergic regulation of GnRH secretion in GT_1 cell lines. Neuroendocrinology 59:420–425
37. **Petersen SL, McCrone S, Coy D, Adelman JP, Mahan LC** 1993 $GABA_A$ receptor subunit mRNAs in cells of the preoptic area: colocalization with LHRH mRNA using dual-label *in situ* hybridization histochemistry. Endocr J 1:29–34
38. **Jung H, Shannon EM, Fritschy J-M, Ojeda SR** 1997 Several $GABA_A$ receptor subunits are expressed in LHRH neurons of juvenile female rats. Brain Res 780:218–229
39. **Sim JA, Skynner MJ, Pape JR, Herbison AE** 2000 Late postnatal reorganization of GABAA receptor signalling in native GnRH neurons. Eur J Neurosci 12:3497–3504
40. **Spergel DJ, Krüth U, Hanley DF, Sprengel R, Seeburg PH** 1999 GABA- and glutamate-activated channels in green fluorescent protein-tagged gonadotropin-releasing hormone neurons in transgenic mice. J Neurosci 19:2037–2050
41. **Vijayan E, McCann SM** 1978 The effects of intraventricular injection of γ-aminobutyric acid (GABA) on prolactin and gonadotropin release in conscious female rats. Brain Res 155:35–43
42. **Donoso AO, López FJ, Negro-Vilar A** 1992 Cross-talk between excitatory and inhibitory amino acids in the regulation of luteinizing hormone-releasing hormone secretion. Endocrinology 131:1559–1561
43. **Masotto C, Wisniewski G, Negro-Vilar A** 1989 Different γ-aminobutyric acid receptor subtypes are involved in the regulation of opiate-dependent and independent luteinizing hormone-releasing hormone secretion. Endocrinology 125:548–553
44. **Heger S, Bilger M, Brann DW, Ojeda SR** 2000 Tetracycline-regulated increase in GABA production near LHRH nerve terminals disrupt estrous cyclicity in the rat. Horm Res 53:19
45. **Erlander MG, Tillakaratne NJK, Feldblum S, Patle N, Tobin AJ** 1991 Two genes encode distinct glutamate decarboxylases. Neuron 7:91–100
46. **Gossen M, Bujard H** 1992 Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. Proc Natl Acad Sci USA 89:5547–5551
47. **Gossen M, Bonin AL, Bujard H** 1993 Control of gene activity in higher eukaryotic cells by prokaryotic regulatory elements. Trends Biochem Sci 18:471–475
48. **Martin DL, Rimvall K** 1993 Regulation of γ-aminobutyric acid synthesis in the brain. J Neurochem 60:395–407
49. **Asada H, Kawamura Y, Maruyama K, Kume H, Ding R-G, Kanbara N, Kuzume H, Sanbo M, Yagi T, Obata K** 1997 Cleft palate and decreased brain γ-aminobutyric acid in mice lacking the 67-kDa isoform of glutamic acid decarboxylase. Proc Natl Acad Sci USA 94:6496–6499
50. **Hoshimuru M, Ray J, Sah DWY, Gage FH** 1996 Differentiation of the immortalized adult neuronal progenitor cell line HC2S2 into neurons by regulatable suppressino of the v-*myo* oncogene. Proc Natl Acad Sci USA 93:1518–1523
51. **Michelsen BK, Petersen JS, Boel E, Moldrup A, Dyrberg T, Madsen OD** 1991 Cloning, characterization and autoimmune recognition of rat islet glutamic acid decarboxylase in insulin-dependent diabetes mellitus. Proc Natl Acad Sci USA 88:8754–8758
52. **Behrstock SP, Anantharam V, Thompson KW, Schweitzer ES, Tobin AJ** 2000 Conditionally-immortalized astrocytic cell line expresses GAD and secretes GABA under tetracycline regulation. J Neurosci Res 60:302–310
53. **Pear WS, Scott ML, Nolan GP** 1998 Production of retroviruses by transient transfection. http://www-leland.stanford.edu/group/nolan/
54. **Bongarzone ER, Foster LM, Byravan S, Verity AN, Landry CF, Schonmann V, Amur-Umarjee S, Campagnoni AT** 1996 Conditionally immortalized neural cell lines: potential models for the study of neural cell function. Methods: A Companion to Methods in Enzymology 10:489–500
55. **Sanes JR, Rubenstein JLR, Nicolas JF** 1986 Use of a recombinant retrovirus to study post-implantation cell lineage in mouse embryos. EMBO J 5:3133–3142
56. **Krieger NR, Heller JS** 1979 Localization of glutamic acid decarboxylase within laminae of the rat olfactory tubercle. J Neurochem 33:299–302
57. **Sacchettoni SA, Benchaibi M, Sindou M, Belin MF, Jacquemont B** 1998 Glutamate-modulated production of GABA in immortalized astrocytes transduced by a glutamic acid decarboxylase-expressing retrovirus. Glia 22:86–93

58. **Peppel K, Baglioni C** 1990 A simple and fast method to extract RNA from tissue culture cells. Biotechniques 9:711–713
59. **Salvatori R, Bockman RS, Guidon Jr PT** 1992 A simple modification of the Peppel/Baglioni method for RNA isolation from cell culture. Biotechniques 13:510–512
60. **Loeb DM, Maragos J, Martin-Zanca D, Chao MV, Parada LF, Greene LA** 1991 The *trk* proto-oncogene rescues NGF responsiveness in mutant NGF-nonresponsive PC12 cell lines. Cell 66:961–966
61. **Ma YJ, Dissen GA, Rage F, Ojeda SR** 1996 RNase protection assay. Methods: A Companion to Methods in Enzymology 10:273–278
62. **Danielson PE, Forss-Petter S, Brow MA, Calavetta L, Douglass J, Milner RJ, Sutcliffe JG** 1988 p1B15: a cDNA clone of the rat mRNA encoding cyclophilin. DNA 7:261–267
63. **Rage F, Hill DF, Sena-Esteves M, Breakefield XO, Coffey RJ, Costa ME, McCann SM, Ojeda SR** 1997 Targeting transforming growth factor α expression to discrete loci of the neuroendocrine brain induces female sexual precocity. Proc Natl Acad Sci USA 94:2735–2740
64. **Hirshfield AN, DeSanti AM** 1995 Patterns of ovarian cell proliferation in rats during the embryonic period and the first three weeks postpartum. Biol Reprod 53:1208–1221
65. **Berg-von der Emde K, Dees WL, Hiney JK, Hill DF, Dissen GA, Costa ME, Moholt-Siebert M, Ojeda SR** 1995 Neurotrophins and the neuroendocrine brain: different neurotrophins sustain anatomically and functionally segregated subsets of hypothalamic dopaminergic neurons. J Neurosci 15:4223–4237
66. **Rage F, Lee BJ, Ma YJ, Ojeda SR** 1997 Estradiol enhances prostaglandin E_2 receptor gene expression in luteinizing hormone-releasing hormone (LHRH) neurons and facilitates the LHRH response to PGE_2 by activating a glia-to-neuron signaling pathway. J Neurosci 17:9145–9156
67. **Urbanski HF** 1990 A role for *N*-methyl-D-aspartate receptors in the control of seasonal breeding. Endocrinology 127:2223–2228
68. **Ma YJ, Hill DF, Creswick KE, Costa ME, Ojeda SR** 1999 Neuregulins signaling via a glial erbB2/erbB4 receptor complex contribute to the neuroendocrine control of mammalian sexual development. J Neurosci 19:9913–9927
69. **Ojeda SR, Hill J, Hill DF, Costa ME, Tapia V, Cornea A, Ma YJ** 1999 The oct-2 POU-domain gene in the neuroendocrine brain: a transcriptional regulator of mammalian puberty. Endocrinology 140:3774–3789
70. **Mayerhofer A, Dissen GA, Costa ME, Ojeda SR** 1997 A role for neurotransmitters in early follicular development: induction of functional follicle-stimulating hormone receptors in newly formed follicles of the rat ovary. Endocrinology 138:3320–3329
71. **Mayerhofer A, Weis J, Bartke A, Yun JS, Wagner TE** 1990 Effects of transgenes for human and bovine growth hormones on age-related changes in ovarian morphology in mice. Anat Rec 227:175–186
72. **Lara HE, Dissen GA, Leyton V, Paredes A, Fuenzalida H, Fiedler JL, Ojeda SR** 2000 An increased intraovarian synthesis of nerve growth factor and its low affinity receptor is a principal component of steroid-induced polycystic ovary in the rat. Endocrinology 141:1059–1072
73. **Hiney JK, Srivastava V, Nyberg CL, Ojeda SR, Dees WL** 1996 Insulin-like growth factor I of peripheral origin acts centrally to accelerate the initiation of female puberty. Endocrinology 137:3717–3728
74. **Dissen GA, Lara HE, Leyton V, Paredes A, Hill DF, Costa ME, Martínez-Serrano A, Ojeda SR** 2000 Intraovarian excess of nerve growth factor increases androgen secretion and disrupts estrous cyclicity in the rat. Endocrinology 141:1073–1082
75. **Zar JH** 1984 Biostatistical Analysis, ed. 2. Prentice Hall, Englewood Cliffys, NJ
76. **Anantharam V, Behrstock S, Thompson K, Weatherwax R, Bongarzone ER, Campagnoni AT, Tobin AJ** 1997 Conditionally immortalized astrocytic cell line (BAS8.1) engineered to express glutamate decarboxylase (GAD_{65}) synthesize and release GABA. Soc Neurosci Abstr 23:118
77. **Sloviter RS, Dichter MA, Rachinsky TL, Dean E, Goodman HJ, Sollas AL, Martin DL** 1996 Basal expression and induction of glutamate decarboxylase and GABA in excitatory granule cells of the rat and monkey hippocampal dentate gyrus. J Comp Neurol 373:593–618
78. **Mansuy IM, Winder DG, Moallem TM, Osman M, Mayford M, Hawkins RD, Kandel ER** 1998 Inducible and reversible gene expression with the rtTA system for the study of memory. Neuron 21:257–265
79. **Terasawa E** 1999 Hypothalamic control of the onset of puberty. Curr Opin Endocrinol Diabetes 6:44–49
80. **Spergel DJ, Krsmanovic LZ, Stojilkovic SS, Catt KJ** 1995 L-type Ca^{2+} channels mediate joint modulation by gamma-amino-butyric acid and glutamate of $[Ca^{2+}]_i$ and neuropeptide secretion in immortalized gonadotropin-releasing hormone neurons. Neuroendocrinology 61:499–508
81. **Meijs-Roelofs HMA, Osman P, Kramer P** 1982 Ovarian follicular development leading to first ovulation and accompanying gonadotrophin levels as studied in the unilaterally ovariectomized rat. J Endocrinol 92:341–349
82. **Osman P** 1975 Preovulatory changes in the ovaries during the first spontaneous pro-oestrus in the rat. J Endocrinol 67:259–265