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Research report

GABAergic neuronal activity and mRNA levels for both forms of glutamic acid decarboxylase (GAD_{65} and GAD_{67}) are reduced in the diagonal band of Broca during the afternoon of proestrus

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Abstract

There is considerable evidence that GABAergic neurons play an important role in the regulation of gonadotropin-releasing hormone (GnRH) secretion, and that these neurons may mediate the feedback actions of gonadal steroids on GnRH neurons. The aim of the present study was to investigate whether endogenous changes in ovarian steroid secretion during the estrous cycle influenced GABAergic neuronal activity in the preoptic region of the hypothalamus, and in other steroid-sensitive brain regions. Intact, adult female rats were sacrificed at various times during the days of metestrus or proestrus. GABAergic neuronal activity was estimated by measuring the rate of accumulation of GABA in microdissected brain regions after pharmacological inhibition of GABA degradation. Concentrations of mRNA for both forms of glutamic acid decarboxylase (GAD_{65} and GAD_{67}) were quantified in microdissected brain regions by a microlysate ribonuclease protection assay. In the diagonal band of Broca at the level of the organum vasculosum of the lamina terminalis (DBB(ovlt)), GABAergic neuronal activity was significantly reduced during the afternoon of proestrus compared with the morning of either proestrus or metestrus. In the lateral septal nucleus, GABAergic neuronal activity was significantly increased in the afternoon of proestrus compared with the morning. There were no significant effects of time of day or day of estrous cycle in the medial preoptic nucleus, median eminence, ventromedial nucleus, suprachiasmatic nucleus, medial septal nucleus, hippocampus (CA1 region), or cingulate cortex. In the DBB(ovlt), mRNA levels for both GAD_{65} and GAD_{67} were significantly reduced in the afternoon of proestrus compared with the afternoon of metestrus. By contrast, there was no change in GAD_{65} and GAD_{67} mRNA levels in the cingulate cortex at any of the times examined. These results demonstrate that GABAergic neuronal activity, and mRNA levels for both GAD_{65} and GAD_{67}, are reduced in the DBB(ovlt) during the afternoon of proestrus. These results support the hypothesis that decreased GABAergic neuronal activity in this region plays a major permissive role in the generation and maintenance of the estrogen-induced LH surge.

Keywords: γ-Aminobutyric acid turnover; Gonadal steroid; Gonadotropin-releasing hormone; Hypothalamus; Medial preoptic nucleus; Rat

1. Introduction

There is increasing evidence that the inhibitory amino acid neurotransmitter γ-aminobutyric acid (GABA) plays an important role in the central neuroendocrine regulation of gonadotropin-releasing hormone (GnRH) secretion. Neuroanatomical studies have demonstrated direct synaptic contacts between GABAergic and GnRH neurons in the preoptic area [35], and GnRH neurons express the mRNA for the $GABA_A$ β_3 subunit [44]. Administration of exogenous GABA or GABA agonists into the medial preoptic nucleus (MPN) inhibits luteinizing hormone (LH) release [2,13,23,26], reduces GnRH gene expression [4,29], and blocks the estrogen-induced LH surge [1,18,40,48]. While GnRH neurons are not thought to contain gonadal steroid receptors in male or female rats [19,24,49], many GABAergic neurons in the MPN concentrate estrogen [12]. Exogenous estrogen acutely increases GABA turnover in the MPN [36], and increases GABA concentrations in MPN microdialysates [9,20]. In male rats, castration specif-

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ically reduces GABAergic neuronal activity in the diagonal band of Broca at the level of the organum vasculosum of the lamina terminalis (DBB(ovlt)), the MPN and the median eminence (ME) [14–16], suggesting that GABAergic neurons terminating in these areas are tonically stimulated by testosterone. Hence, it is likely that hypothalamic GABAergic neurons are involved in mediating the gonadal steroid feedback regulation of GnRH neurons.

In contrast to the stimulatory actions of gonadal steroids on GABAergic neurons described above, studies suggest that GABA activity in the medial preoptic area (MPOA) may be reduced during the estrogen-induced GnRH surge in ovariectomized rats [9,25,27,48]. Thus, removal of tonic GABAergic inhibition may be a critical factor in the generation of the estrogen and progesterone-induced LH surge. The aim of the present study was to investigate whether endogenous changes in ovarian steroid secretion during the estrous cycle in the intact adult female rat influenced GABAergic neuronal activity in the preoptic region of the hypothalamus, and also in other steroid-sensitive brain regions.

Two separate isoforms of glutamic acid decarboxylase (GAD), the rate-limiting enzyme responsible for GABA synthesis, are present in the rat CNS (GAD_{65} and GAD_{67}) [11], and GAD mRNA levels often correlate with GAD activity in nerve terminals (for review, see [37]). Levels of GAD mRNA expression appear to be regulated by gonadal steroids in both male [47] and female [21,33,54] rats, and estrogen has differential effects on GAD_{65} and GAD_{67} in different brain regions [38]. At least one of these forms, GAD_{67}, has been reported to be reduced in the MPN during the afternoon of proestrus [21]. Thus, to confirm and extend observations using a neurochemical estimate of GABAergic neuronal activity, mRNA levels for both GAD isoforms were quantified in micropunches from the rostral hypothalamus during the estrous cycle.

2. Materials and methods

2.1. Animals

Adult female Sprague-Dawley-derived rats, weighing 200–250 g, were obtained from Zivic-Miller Laboratories (Allison Park, PA) and maintained under a 14:10 h light/dark cycle (lights on at 04.00 h) with free access to food and water. Stages of the estrous cycle were monitored by daily vaginal lavage, and only animals which had exhibited at least two consecutive 4-day estrous cycles were used for this study. For determination of GABAergic neuronal activity during the estrous cycle (Experiment 1), groups of 20 animals were sacrificed after neurochemical manipulations (described below) at 07.00–09.00 h and 15.00–17.00 h on metestrus, and at 07.00–09.00 h, 11.00–13.00 h and 15.00–17.00 h on proestrus. For quantitation of GAD mRNA levels (Experiment 2), further groups of 6 animals each were sacrificed at 09.00–10.00 h and 15.00–16.00 h on metestrus and at 09.00–10.00 h and 15.00–16.00 h on proestrus. Trunk blood was collected from all rats, allowed to clot at 4°C, and the serum was stored at −20°C for radioimmunoassay of LH. The brains were rapidly removed and frozen on dry ice. Serial 300-μm coronal sections of the brain were cut in a cryostat at −9°C, thaw mounted onto glass microscope slides, and then immediately refrozen. Using the micropunch dissection technique [42], samples of brain were removed with sharpened 21-gauge tubing (approximately 500 μm internal diameter) from 10 brain regions (illustrated in Fig. 1). For quantitation of tissue GABA content, the tissue was sonicated in 50–100 μl of ice-cold 0.1 N $HClO_4$ and then centrifuged. The acid extract was stored at −20°C for measurement of GABA by HPLC, and the tissue precipitate redissolved in 50 μl of 1.0 M NaOH and stored at 4°C for assay of tissue protein content [5]. For measurement of GAD mRNA levels, tissue punches from two brain regions (DBB(ovlt) and cingulate cortex (CC)) were placed into 20 μl of 6 M guanidine thiocyanate (GuSCN, Sigma Chemical Company, St. Louis, MO), 0.13 m ETDA at room temperature, as described elsewhere [51]. GAD_{65} and GAD_{67} mRNA content were then determined by a microlysate ribonuclease (RNase) protection assay. All procedures adhered to the Guidelines for the Humane Care and Use of Research Animals of the American Physiological Society, and animal protocols were approved by the University of Maryland School of Medicine IACUC.

2.2. Experiment 1: measurement of GABAergic neuronal activity

2.2.1. Neurochemical manipulations

In vivo GABAergic neuronal activity was estimated in microdissected brain regions by measuring the accumulation of GABA in the tissue after aminooxyacetic acid (AOAA, Sigma) induced inhibition of the GABA degrading enzyme, GABA transaminase (GABA-T). We have previously shown that GABA concentrations in microdissected brain regions increase in a linear manner for approximately 90 min after a single i.p. injection of AOAA [14]. Rats were decapitated either without treatment ($n = 8$ rats per time point), or 60 min after injection of AOAA (100 mg/kg b. wt., i.p.) ($n = 12$ rats per time point). To prevent non-specific postmortem synthesis of GABA, all animals were injected with 3-mercaptopropionic acid (3-MPA; 1.2 mmol/kg b. wt., i.p.; Sigma) exactly 2.5 min before decapitation [53] to block GAD activity. The relative increase in GABA concentrations in the AOAA-treated rats compared with the untreated rats was taken as an estimate of GABAergic neuronal activity.

2.2.2. Determination of GABA

GABA concentrations in the microdissected tissue were determined by isocratic reverse phase HPLC with electro-

chemical detection, as described previously [14]. The mobile phase consisted of 0.1 M Na_2HPO_4 in 35% methanol, with the pH adjusted to 6.75 with 85% phosphoric acid. Pre-column derivatization of the amino acids in the sample was achieved by adding 25 μl of a solution containing 0.9 mg/ml o-phthalaldehyde (Eastman-Kodak, Rochester, NY) and 0.125 μl/ml β-mercaptoethanol (Fisher Scientific, Pittsburgh, PA) in 0.1 M sodium tetraborate buffer (pH 9.3) to 10 μl of tissue extract or standard GABA solution. To correct for minor variance in derivatization efficiency, 10 μl of 0.1 N $HClO_4$ containing 20 ng of homoserine (Sigma) was added to each sample as an internal standard. Exactly 2 min after mixing the derivatization reagent with the sample, 20 μl of the reaction mixture was injected onto the column. GABA content was determined by comparing the ratio of the integrated peak areas of GABA and the internal standard homoserine in sample chromatograms with the same ratio of peak areas in chromatograms from external GABA standards.

2.3. Experiment 2: Microlysate RNase protection assay for GAD_{65} and GAD_{67}

2.3.1. PCR amplification of GAD template

Templates for the GAD riboprobes were generated by the polymerase chain reaction (PCR) from plasmids containing the full rat cDNA sequence for each gene. The plasmids were a gift from Dr. Allan Tobin, Department of Biology, UCLA. Oligonucleotide primers were synthesized by the Biopolymer Laboratory of the Department of Microbiology and Immunology, University of Maryland, School of Medicine. To provide a substrate for later riboprobe synthesis, specific RNA polymerase promoters were incorporated onto the 5′ end of each primer [50]. Thus, the upper GAD_{65} primer (5′-^{87}GGCTCTGGCTTTTGGTCCTTC^{107}-3′) was synthesized to contain the T3 promoter (5′-AATTAACCCTCACTAAAGGGAAG-3′) at its 5′ end (44 bases, total). The lower GAD_{65} primer (5′-^{683}GTTCGTGTTTGCTGTTGATGT^{663}-3′) was synthesized to contain the T7 promoter (5′-TAATACGACTCACTATAGGGAGA-3′) at its 5′ end. This primer pair was designed to generate a cDNA template of 597 bp, with phage promoters for synthesis of sense (T3) or antisense (T7) mRNA probes for GAD_{65} (see Fig. 2). The upper GAD_{67} primer (5′-^{227}GAGCGGATCCTAATACTACCA^{247}-3′) was synthesized to contain the T7 promoter at its 5′ end. The lower GAD_{67} primer (5′-^{1006}TGCCTTTTGTTTTCACTTCTG^{986}-3′) was synthesized to contain the SP6 promoter (5′-AATTAGGTGACACTATAGAATAG-3′) at its 5′ end. This primer pair was designed to generate a cDNA template of 780 bp, with phage promoters for synthesis of sense (T7) or antisense (SP6) mRNA probes for GAD_{67} (see Fig. 2).

PCR was carried out in a 100 μl volume using the

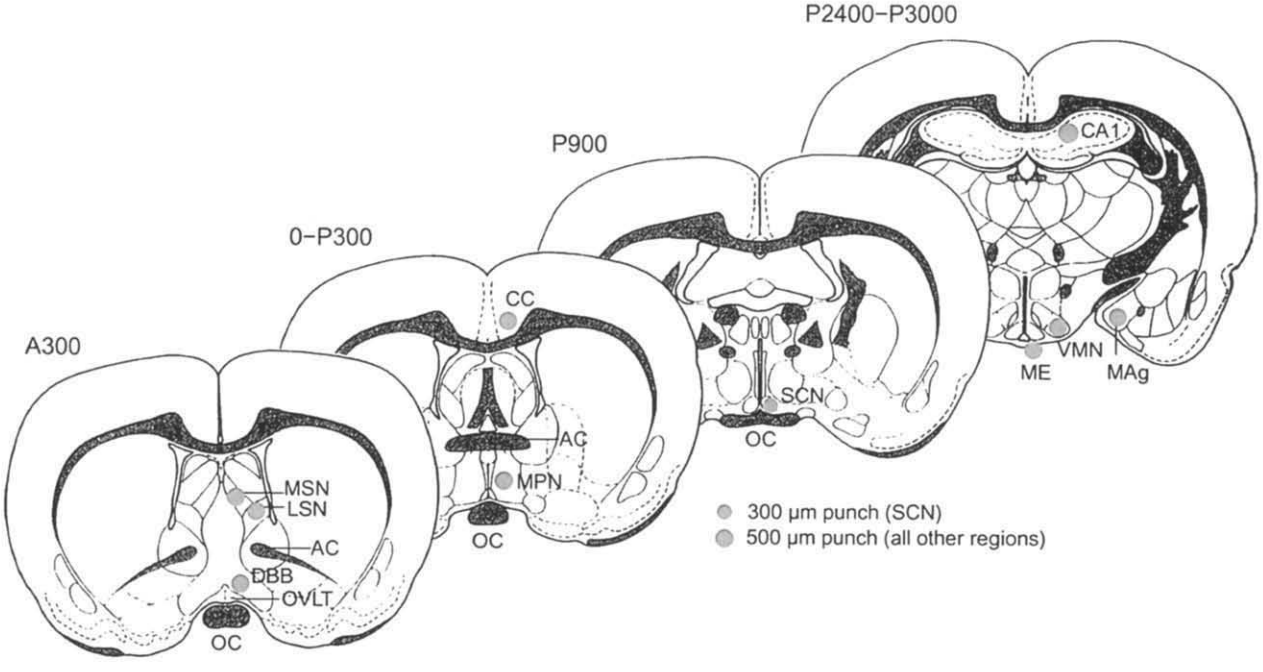

Fig. 1. Coronal sections of the rat brain representing the position of the micropunches (gray circles) taken from discrete brain regions. Drawings are modified from the microdissection atlas of Palkovits and Brownstein [42]. The coordinate at the top left of each section represents the approximate position of the rostral face of each section in microns, relative to Bregma (0). A = anterior to Bregma, P = posterior. Where a range is given, two or three consecutive 300-μm-thick sections have been used to sample the full extent of some brain nuclei. Abbreviations: AC, anterior commissure; CA1, hippocampus CA1 region; CC, cingulate cortex; DBB, diagonal band of Broca; LSN, lateral septal nucleus; MAg, medial amygdala; ME, median eminence; MPN, medial preoptic nucleus; MSN, medial septal nucleus; OC, optic chiasm; OVLT, organum vasculosum of the lamina terminalis; SCN, suprachiasmatic nucleus; VMN, ventromedial nucleus.

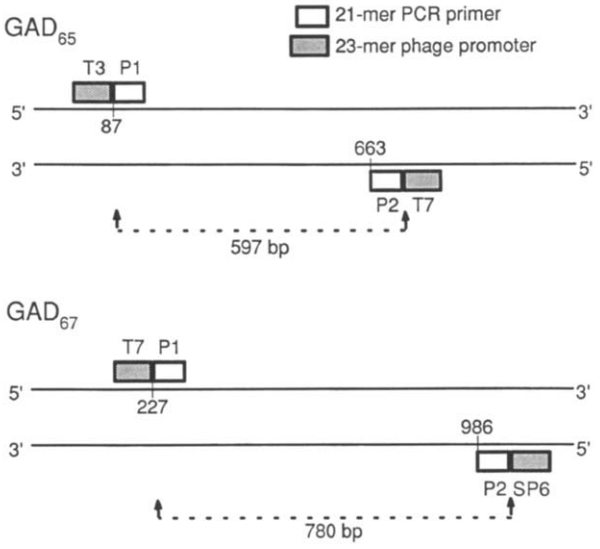

Fig. 2. Diagrammatic representation of PCR approach used to generate cDNA templates for the GAD_{65} and GAD_{67} riboprobes. Specific phage promoters were incorporated onto the 5′ end of the PCR primers, such that the PCR product could be used as a template to synthesize either sense or antisense riboprobes.

following reagents: (i) 10 × reaction buffer, containing 10 mM MgCl (10 μl; Perkin Elmer, Branchburg, NJ), (ii) DEPC-treated water (80.5 μl), (iii) dNTP mixture, containing 10 mm each of dATP, dCTP, dGTP and dTTP (2 μl; Promega, Madison, WI), (iv) 1.0% BSA (1 μl; Promega), (v) 100 mm DTT (1μl; Promega), (vi) 1 μl of the plasmid containing the cDNA template, (vii) 2 μl of each primer (0.02 μm), and (viii) AmpliTaq DNA polymerase (0.5 μl; Perkin-Elmer). The mixture was overlaid with a drop of mineral oil, and then incubated through 30 cycles in a programmable thermal cycler. Cycling parameters were 92°C for 60 s, annealing at 55°C for 45 s, then extension at 72°C for 90 s. At the completion of the 30 cycles the samples underwent a further 10-min extension period at 75°C, and then were held at 4°C. PCR products were analyzed by electrophoresis on a 2% agarose gel, and each PCR (using primers for GAD_{65} or GAD_{67}) yielded a single band of DNA corresponding to the predicted lengths described above. The PCR product was purified using the Qiaquick-spin purification kit (Quiagen, Chatsworth, CA).

2.3.2. Synthesis of riboprobes

Radiolabelled antisense riboprobes for GAD_{65} and GAD_{67} were transcribed using T7 and SP6 RNA polymerases (Promega) respectively, and [^{32}P]UTP (New England Nuclear, 800 Ci/mmol, 10 mCi/ml). For a control mRNA, an antisense riboprobe specific for rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was also transcribed, using T3 RNA polymerase (375 bp, template from Ambion). Probes were transcribed to a specific activity of approximately 10^8 cpm/μg. The reaction was carried out in a total volume of 30 μl, containing 5 × transcription buffer (6 μl), 100 mm DTT (1.5 μl), RNasin (1 μl), 10 mm rATP, rCTP and rGTP (1.5 μl each), 0.2 mm UTP (1.9 μl), [^{32}P]UTP (8 μl), purified PCR-amplified DNA template (1 μl), RNA polymerase (1.5 μl) and 4.5 μl of DEPC-treated water. The reaction was incubated for 1 h at room temperature, followed by template degradation with RNase-free DNase for 30 min at 37°C. The transcripts were then purified by elution through a Sephadex G-50 column (Quick-Spin Columns, Boehringer Mannheim, Indianapolis, IN).

2.3.3. Microlysate ribonuclease protection assay

The RNase protection method was carried out directly in the tissue microlysate, after the method of Strauss and Jacobowitz [51]. A mixture of the three riboprobes (GAD_{65}, GAD_{67} and GAPDH; 4–10 ng each in 5 μl total volume) was added to the tissue punches contained in 20 μl of the GuSCN solution (as described above), such that the total radioactivity added to each tube was between 1.2–3 × 10^6 cpm. The probes were allowed to hybridize overnight (12–24 h) at 37°C. The next morning, RNase solution containing 20 μg/ml RNase A (Boehringer Mannheim) and 0.35 mg/ml degraded herring sperm DNA in 450 μl 0.4 m NaCl was added to the lysate/riboprobe mixture, and incubated for 1 h at 37°C. Samples were then treated with 10 μl of proteinase K (20 mg/ml, Boehringer Mannheim) and 20 μl of 10% SDS for 1 h at 37°C to inactivate the RNase. Protein and lipid components were then removed by serial organic extractions with Tris-equilibrated phenol (500 μl) followed by chloroform/isoamyl alcohol (24:1; 500 μl). Ice-cold absolute ethanol (1.1 ml) was added to the final aqueous phase to precipitate the hybrids, and the samples were centrifuged at 15 800 × *g* for 30 min at 0°C. Pellets were washed in cold 90% ethanol, and dried at room temperature. The protected hybrids were then resuspended in 15 μl of loading buffer, and electrophoresed on a 6% polyacrylamide gel until the bromophenol blue exited the gel. The gels were dryed on an EasyBreeze drier (Hoefer Scientific), and labelled with phosphorescent autoradiography markers (Glogos, Stratagene) to facilitate subsequent alignment of the gel and autoradiograph. Gels were exposed to X-ray film for 3 h, then using the autoradiograph, full-length protected fragments (597 nt for GAD_{65}, 780 nt for GAD_{67}, and 353 nt for GAPDH) were excised from the gel for scintillation counting. All 48 samples in this experiment were processed in a single assay run.

2.4. Radioimmunoassay of serum LH

Plasma concentrations of LH were measured in all trunk blood samples by double-antibody radioimmunoassay, using reagents provided by the NIDDK National Hormone and Pituitary Program. Details of our assay method have been published elsewhere [43]. Reference preparation rLH-RP-3 was used, and samples were assayed in 20–100 μl aliquots. Calculating the least detectable dose as 15%

displacement (i.e. 85% of total binding), the assay sensitivity ranged from 20–25 pg LH/tube. Inter- and intra-assay coefficients of variation in the linear portion of the standard curve (15–85% binding) were less than 10%.

2.5. Statistical analysis

All data are presented as mean ± S.E.M. GABA concentrations in discrete brain regions are expressed as ng/μg protein. Differences between means were compared by one-way analysis of variance, followed by the Newman-Keuls multiple range test [55]. The rate of GABA accumulation after injection of AOAA was determined by linear regression, and is expressed as ng GABA/μg protein/h. Statistical comparison between the regression coefficients was made by one-tailed t-test with $(n_1 + n_2 - 4)$ degrees of freedom [10]. GAD mRNA levels in the different experimental groups are presented as the ratio of the cpm in the GAD protected fragment to the cpm in the GAPDH band. Comparison between ratios was made by the Kruskal-Wallis analysis of variance for non-parametric data, followed by individual comparisons between means using the Mann-Whitney U-test [55]. All statements of statistical significance refer to a probability level of less than 5% (i.e. $P < 0.05$).

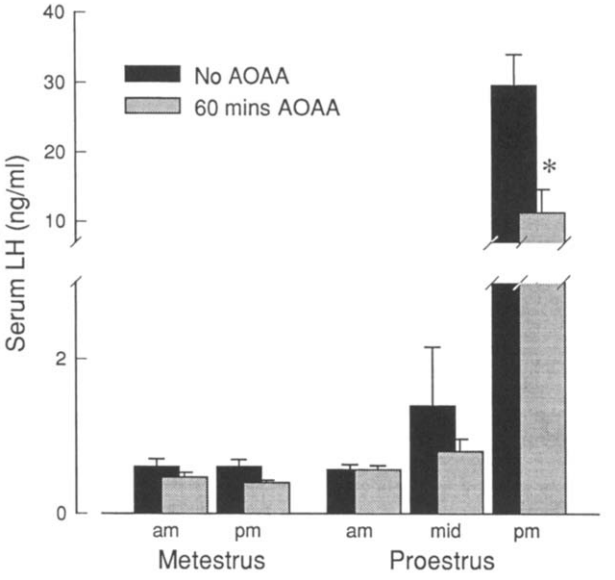

Fig. 3. Serum LH concentrations at different times on metestrus and proestrus (am = 07.00–09.00 h; mid = 11.00–13.00 h; pm = 15.00–17.00 h). Animals were sacrificed for GABA turnover measurments, either without treatment with AOAA ($n = 8$ per time point), or 60 min after i.p. injection of AOAA ($n = 12$ per time point). All animals were injected with 3-MPA exactly 2.5 min before decapitation. * Significantly reduced compared with animals that did not receive AOAA ($P < 0.05$).

3. Results

3.1. Serum LH concentrations

Serum LH concentrations for the five groups in Expt. 1 are depicted in Fig. 3. Serum LH was typically low in metestrous animals and in animals sacrificed in the morning of proestrus. On the afternoon of proestrus, all animals had greatly elevated serum levels of LH characteristic of the preovulatory LH surge. Interestingly, mean LH levels were significantly lower during the afternoon of proestrus in the animals treated with AOAA compared with those that did not receive this drug. Thus, increased GABA concentrations throughout the brain following inhibition of GABA-T results in a significant suppression of LH release, consistent with the hypothesis that GABA maintains an inhibitory tone over GnRH neuronal activity.

Serum LH in animals from Expt. 2 followed a very similar pattern, with low levels during the morning (0.99 ± 0.26 ng/ml) and afternoon of metestrus (0.50 ± 0.16 ng/ml), and the morning of proestrus (0.38 ± 0.05 ng/ml), and very high levels in animals sacrificed during the afternoon LH surge (14.19 ± 4.84 ng/ml). Peak values are somewhat lower than in Expt. 1, as animals were sacrificed slightly earlier during proestrous afternoon in this second experiment.

3.2. GABAergic neuronal activity

Steady state concentrations of GABA were not significantly affected by time of day, or by day of the estrous

Table 1
Steady-state GABA concentrations during the estrous cycle (ng/μg protein)

Brain region	Metestrus		Proestrus		
	am	pm	am	mid	pm
Medial septal nucleus	6.0 ± 1.0	6.7 ± 0.8	7.03 ± 0.9	7.4 ± 1.3	6.7 ± 2.2
Lateral septal nucleus	7.0 ± 0.9	6.3 ± 1.1	6.7 ±2.4	6.0 ± 1.0	7.0 ± 1.6
Diagonal band of Broca	13.7 ± 1.3	13.9 ± 1.9	12.9 ±1.1	15.6 ± 1.9	13.6 ± 0.7
Medial preoptic nucleus	13.7 ± 1.1	12.7 ± 0.9	11.8 ±0.9	13.5 ± 1.2	12.3 ± 1.9
Suprachiasmatic nucleus	21.5 ± 2.2	22.2 ± 2.1	19.9 ±2.4	22.7 ± 2.1	18.0 ± 2. 5
Hippocampus CA1	13.7 ± 0.8	12.0 ± 1.0	11.9 ±1.3	14.1 ± 2.0	11.5 ± 1.6
Cingulate cortex	3.1 ± 0.6	3.2 ± 0.6	3.0 ±0.4	3.1 ± 0.6	3.6 ± 0.6
Ventromedial nucleus	11.1 ± 1.6	11.5 ± 3.0	12.8 ±1.7	14.3 ± 2.9	10.0 ± 2.0
Median eminence	7.0 ± 1.1	7.9 ± 1.9	6.4 ±1.0	7.3 ± 1.1	6.1 ± 1.0

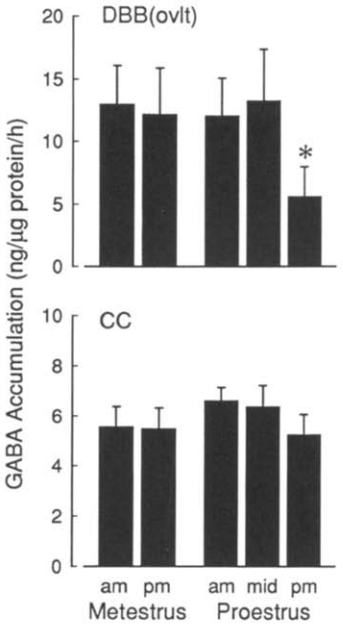

Fig. 4. GABAergic neuronal activity during the estrous cycle in the DBB(ovlt), and in a control brain region, the cingulate cortex (CC). Data represent the rate of accumulation of GABA in the tissue after administration of AOAA at different times on metestrus and proestrus generated from 20 animals at each time point, 8 sacrificed without treatment with AOAA and 12 sacrificed 60 min after i.p. injection of AOAA (am = 07.00–09.00 h; mid = 11.00–13.00 h; pm = 15.00–17.00 h). Note the differences in scale on the ordinate axes. * Significantly reduced compared with the metestrous morning and proestrous morning ($P < 0.05$).

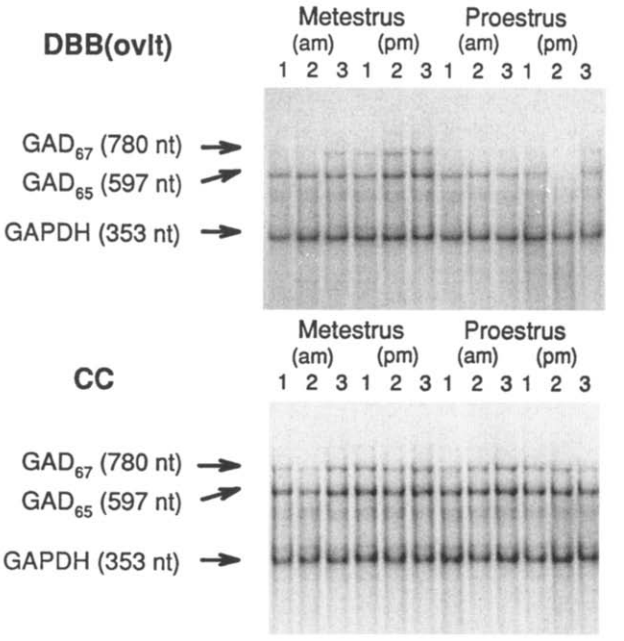

Fig. 5. Representative autoradiograms from the RNase protection assay for GAD_{65} and GAD_{67} in the DBB(ovlt) (upper panel) and in the CC (lower panel). Each lane contains protected mRNA fragments from tissue punches taken from a single animal. Three individual animals are presented from each experimental group (am = 09.00–10.00 h; pm = 15.00–16.00 h). Protected fragments appear as dark bands at the appropriate size for GAD_{67}, GAD_{65} and GAPDH.

cycle in any of the brain regions examined (Table 1). GABAergic neuronal activity during the estrous cycle, as estimated by AOAA-induced accumulation of GABA in the microdissected brain regions, are presented in Fig. 4 and Table 2. In the DBB(ovlt), there was a significant decrease in GABA accumulation during the afternoon of proestrus compared with proestrous morning or metestrous morning (Fig. 4). A similar trend also occurred in the ME, but this decrease did not attain statistical significance (Table 2). By contrast, in the LSN, GABA accumulation was significantly higher at midday and during the afternoon of proestrus compared with proestrous morning or metestrous morning (Table 2). There were similarly elevated levels of GABA accumulation on the afternoon of metestrus in the MSN, suggestive of a possible circadian rhythm in GABA activity in the septal region. In all other brain regions, including the MPN, no significant changes were observed at the time points examined (see Table 2).

3.3. GAD_{65} and GAD_{67} mRNA levels

The ribonuclease protection assay for GAD_{65} and GAD_{67} was carried out in the DBB(ovlt) to determine whether the above described decrease in GABAergic neuronal activity was associated with a change in either or both GAD isoforms. As a control region in which we did not expect to see any changes, we also examined GAD mRNA levels in the CC. Fig. 5 shows autoradiograms of a representative gel from each region. In the DBB(ovlt) the protected band

Table 2
GABA turnover in other brain regions during the estrous cycle (ng/µg protein/h)

Brain region	Metestrus		Proestrus		
	am	pm	am	mid	pm
Medial septal nucleus	7.0 ± 1.4	9.4 ± 1.2	6.9 ± 1.2	6.3 ± 1.9	11.4 ± 3.1
Lateral septal nucleus	5.5 ± 1.3	7.9 ± 1.6	4.1 ± 0.8	8.8 ± 1.4 *	8.1 ± 2.1 *
Medial preoptic nucleus	9.2 ± 2.1	8.4 ± 2.5	12.4 ± 1.6	13.1 ± 1.8	13.1 ± 1.9
Suprachiasmatic nucleus	14.3 ± 4.0	16.8 ± 4.4	21.8 ± 5.7	18.1 ± 3.8	15.2 ± 4.3
Hippocampus CA1	14.9 ± 6.1	22.0 ± 4.3	21.9 ± 3.3	17.9 ± 4.6	18.8 ± 5.0
Ventromedial nucleus	7.6 ± 2.7	8.6 ± 4.4	7.2 ± 2.5	9.9 ± 4.2	6.4 ± 2.7
Median eminence	7.9 ± 1.6	7.2 ± 2.6	7.2 ± 1.3	8.6 ± 1.6	5.2 ± 1.3

* Significantly increased compared with proestrous morning.

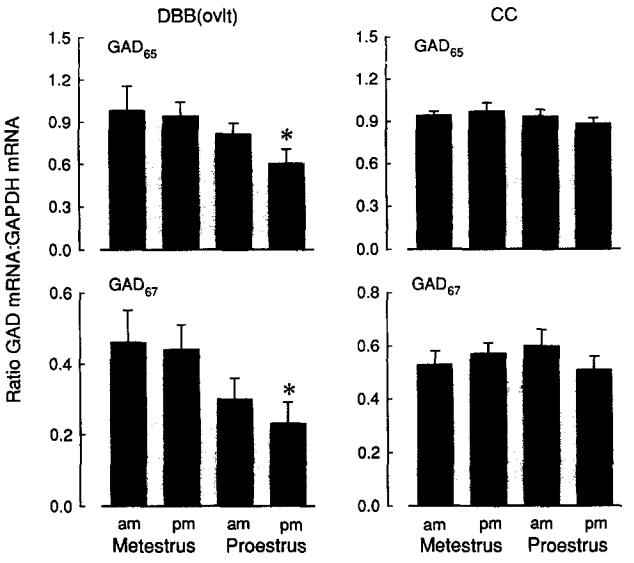

Fig. 6. GAD_{65} (upper panel) and GAD_{67} (lower panel) mRNA levels during the estrous cycle in the DBB(ovlt) (left) and CC (right). Data are presented as the ratio of radioactivity in the GAD band to that in the GAPDH band ($n = 6$ rats per time point, am = 09.00–10.00 h; pm = 15.00–16.00 h). * Significantly reduced compared with metestrous afternoon ($P < 0.05$).

for GAD_{67}, and to a lesser degree the band for GAD_{65}, are much reduced in samples collected from proestrous animals compared with metestrous animals. Levels of GAPDH are relatively constant throughout all experimental groups. In contrast, in the CC the levels of the protected bands for GAD_{65} and GAD_{67} appear similar in all experimental groups. The protected bands were excised from the gels, and radioactivity quantified by liquid scintillation spectroscopy (Fig. 6). Based on the assumption that GAPDH mRNA is not regulated during the estrous cycle, data are presented as the ratio of radioactivity in the GAD band to that in the GAPDH band to normalize for any small variations in tissue content in each dissected brain region. In the DBB(ovlt), both GAD_{65} and GAD_{67} tended to be lower on proestrus than metestrus, and this difference was significant when comparing proestrous afternoon with metestrous afternoon. By contrast, in the CC, there was no significant effect of time during the estrous cycle on mRNA levels of either GAD isoform.

4. Discussion

This study employed two independent experimental approaches to examine GABAergic neuronal activity in specific brain regions during the estrous cycle, both based on the relatively precise neuroanatomical localization provided by micropunch microdissection. First, a neurochemical estimate of GABA turnover was obtained by measuring the rate of increase of GABA content in the tissue after inhibition of GABA degradation with AOAA. AOAA-induced accumulation of GABA was significantly decreased in the DBB(ovlt) during the afternoon of proestrus compared with the morning. Secondly, using a new microlysate RNase protection assay which enabled measurement of mRNA concentrations directly in microdissected brain nuclei without requiring prior isolation of RNA, mRNA levels for both GAD_{65} and GAD_{67} have been examined in discrete brain regions during the estrous cycle for the first time. Expression of both forms of GAD mRNA was significantly reduced in the DBB(ovlt) during the afternoon of proestrus compared with the afternoon of metestrus. Taken together, these findings indicate that GABAergic neuronal activity within this brain region is significantly reduced during the time of the preovulatory LH surge. Furthermore, the data suggest that the reduction in neuronal activity may be mediated by suppression of expression of the two separate genes encoding different forms of GAD.

The dissection of the DBB(ovlt) included tissue ranging from the posterior-ventral region of the vertical limb of the DBB to the anterior part of the MPN, also known as the anteroventral periventricular nucleus (see Fig. 1). This region of the rostral hypothalamus contains the highest concentration of GnRH neuronal cell bodies, and GnRH neurons in this area are activated during the proestrous LH surge, as evidenced by expression of the immediate early genes (IEG) c-*fos* [30,31,52] and c-*jun* [32]. GnRH neurons are directly innervated by GABAergic nerve terminals [34], and GABA appears to exert a direct inhibitory tone on GnRH secretion. GABAergic agonists can inhibit pulsatile LH secretion in OVX rats [2,13,23,26], and block both norepinephrine- and *N*-methyl-d-aspartic acid-induced LH release [3], while GABA antagonists markedly increase norepinephrine-induced LH secretion [17]. Thus, it seems likely that the observed reduction in GABAergic neuronal activity during the afternoon of proestrus would result in a disinhibition of the GnRH neurons. Indeed, the timing of the expression of IEGs in GnRH neurons during the proestrous LH surge [30–32,52] and the decrease in GABAergic neuronal activity in this region are very similar.

The present studies, showing decreased GABAergic neuronal activity during proestrous afternoon in the intact rat, are supported by previously reported observations that in ovariectomized rats, extracellular GABA concentrations [9,25,27] and GABA turnover [48] in the MPN are reduced during the estrogen-induced LH surge. Interestingly, premature reduction of GABAergic tone induced by administration of a $GABA_A$ receptor antagonist during proestrous morning, advanced the onset of the LH surge [28]. Thus, it appears that the precisely timed removal of tonic GABAergic inhibition may be a major factor mediating the positive-feedback actions of estradiol to stimulate the preovulatory LH surge. Removal of GABAergic inhibition is apparently critical for the activation of the GnRH neurons, because preventing the decrease in GABA by administration of GABAergic agonists has been shown to block the

LH surge [1,18,40,48]. Indeed, even the acute increase in GABA concentration in the rostral hypothalamus induced by AOAA in the present study, significantly reduced the peak of the LH surge.

Previous studies in male and female rats have reported a correlation of GABAergic neuronal activity with the plasma levels of gonadal steroids. Thus, castration reduces GABA turnover in the male [14–16], an effect which can be prevented by testosterone treatment [15,16]. In ovariectomized rats, estradiol increases GABA turnover [36] and extracellular GABA concentrations [20] in the MPN, associated with an inhibition of LH secretion. These findings are consistent with the hypothesis that gonadal steroids stimulate GABAergic neuronal activity, and that GABAergic neurons mediate the negative-feedback actions of gonadal steroids on GnRH release. Estrogen modulation of GABAergic neurons may also be importantly involved in the regulation of female sexual behavior. Using quantitative in situ hybridization histochemistry, an estrogen-induced increase in GAD_{67} mRNA has been observed in the dorsomedial nucleus of the hypothalamus and this may be responsible for the GABAergic facilitation of lordosis in this brain region [38]. With respect to the positive-feedback action of estradiol, it is well established that rising levels of estrogen are responsible for the neuroendocrine changes which mediate the LH surge. The present findings, of reduced GABAergic neuronal activity in the face of these high levels of estradiol during the afternoon of proestrus, reflect an apparent change in the response of the hypothalamic GABAergic neurons to estradiol. This change may be the major mechanism resulting in the positive-feedback action of estradiol. The exact nature of this mechanism, however, remains enigmatic. Estrogen may act directly on GABAergic neurons in the rostral hypothalamus, as these neurons are known to express the estrogen receptor [12]. Alternatively, or perhaps in addition, one or more of the estrogen-sensitive pathways involved in stimulating the LH surge may exert its action by reducing the inhibitory GABAergic tone. For example, ultrastructural evidence suggests that catecholamines may regulate GnRH neurons indirectly, through presynaptic modulation of GABAergic interneurons [35].

Consistent with the neurochemical studies, GAD mRNA levels in the DBB(ovlt) were significantly reduced during the afternoon of proestrus. This result is supported by a previous study, in which GAD_{67} mRNA concentrations, as quantified by in situ hybridization histochemistry, decreased during the LH surge in the MPN, but not the DBB [21]. The region of the DBB examined in that study was considerably more rostral and dorsal than the level of the OVLT, and measurements from the MPN were more comparable to the region we have designated DBB(ovlt) (Allan Herbison, personal communication). Interestingly, in the present study, both GAD_{65} and GAD_{67} mRNA levels were reduced during proestrous afternoon. Expression of the two forms of GAD is regulated by different mechanisms (for review, see [37]). GAD_{67} is thought to be involved in constitutive synthesis of GABA, providing for metabolic requirements and tonic synaptic release. Changes in neuronal activity are accompanied by long-term regulation of GAD_{67} expression to maintain GABA synthesis levels. GAD_{65} protein is located primarily at nerve terminals, and is present largely as the inactive apo-enzyme. Thus, it has been suggested that GAD_{65} may be important for acute regulation of the transmitter pool of GABA, responding to increased neuronal activity by increasing the conversion of the inactive apo-GAD to the active holo-enzyme. Such acute changes would not necessarily result in changes to GAD_{65} gene transcription, and, indeed, there have been few reports of experimentally-induced changes in GAD_{65} mRNA levels [37]. Thus the present observation of decreased GAD_{65} mRNA in the DBB(ovlt) is quite unusual, and may reflect a marked decrease in synaptic release of GABA, with a corresponding lack of demand for synthesis of new GAD enzyme. While the changes in GAD mRNA concentrations suggest that gonadal steroids may act by a genomic mechanism to reduce expression of the rate-limiting enzyme responsible for GABA synthesis, leading to a reduction in GABA release, this study cannot rule out the possibility that GAD mRNA levels may be modified as a consequence of steroid-induced changes in GABAergic neuronal activity.

The changes in GABAergic neuronal activity discussed above were specifically restricted to the DBB(ovlt), and therefore, are likely to be involved primarily with the regulation of the GnRH neurons in this region. Surprisingly, there was no effect on GABA activity observed in the MPN, another region containing a relatively high number of GnRH neuronal cell bodies. Thus, it would appear that different subpopulations of GABAergic neurons terminating within the rostral-caudal extent of the distribution of GnRH cell bodies might be differentially regulated during the afternoon of proestrus. It is possible that such regional changes in GABAergic neuronal activity might induce regional differences in activation of GnRH neurons, implying that the GnRH neurons at the level of the OVLT might be primarily involved in stimulating the high levels of LH secretion during the proestrous surge. In support of this hypothesis is evidence that subpopulations of GnRH neurons within the DBB(ovlt) region might be regulated differently from those in the caudal MPN during the afternoon of proestrus [22,46].

GABAergic neuronal activity in the ME was not significantly reduced during the afternoon of proestrous, suggesting that removal of putative GABAergic inhibition of GnRH release at the level of the GnRH nerve terminals [15,16] is not a major factor in stimulating the LH surge. Similarly, in the ventromedial nucleus and hippocampus (CA1), regions containing significant GABAergic innervation [41] and estradiol-sensitive neurons [45], no estrous cycle-dependent or time-of-day-dependent changes in GABAergic neuronal activity were observed. In the septal

nuclei, particularly in the lateral septum, GABAergic neuronal activity was increased during the afternoon of both metestrus and proestrus compared with the morning, indicative of a possible circadian rhythm of GABAergic neuronal activity in this region. A similar result has been reported in ovariectomized rats treated with estradiol [48]. Circadian fluctuations in GABA content have been reported previously in the hypothalamus [8] and in tuberoinfundibular GABAergic neurons [7], and the principle circadian pacemaker in the mammalian brain, the suprachiasmatic nucleus, contains extensive GABAergic innervation and GAD-positive neuronal cell bodies [6,39]. Thus, it has been suggested that GABAergic neurons may participate in the regulation of pacemaker activity. In the present study there was no clear pattern of changes in GABA content or turnover in the suprachiasmatic nucleus. It is possible, however, that the times of sample collection, chosen to be optimal relative to the LH surge rather than the light/dark cycle, may have missed circadian changes in this nucleus.

The present study has demonstrated that GABAergic neuronal activity, and mRNA levels for both GAD_{65} and GAD_{67}, are reduced in the DBB(ovlt) during proestrous afternoon. Thus it appears that a major mechanism of the positive-feedback action of estradiol is to reduce GABAergic neuronal activity in the rostral hypothalamus, probably by decreasing expression of both forms of mRNA for GAD. The region of the DBB(ovlt) contains a large proportion of GnRH neuronal cell bodies, and the timing of the decrease in inhibitory GABAergic tone correlates very well with the previously observed activation of GnRH neurons during the preovulatory LH surge [30–32,52]. It is likely that steroid-induced disinhibition resulting from decreased GABAergic neuronal activity in this region plays a significant permissive role in the generation and maintenance of the LH surge.

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References

[1] Akema, T. and Kimura, F., The mode of $GABA_B$ receptor-mediated inhibition of the preovulatory luteinizing hormone surge in female rats, *Brain Res.*, 562 (1991) 169–172.

[2] Akema, T. and Kimura, F., Modulation of pulsatile LH secretion by baclophen, a selective $GABA_B$ receptor agonist, in ovariectomized rats, *Neuroendocrinology*, 56 (1992) 141–147.

[3] Akema, T. and Kimura, F., Differential effects of $GABA_A$ and $GABA_B$ receptor antagonists on NMDA-induced and noradrenaline-induced luteinizing-hormone release in the ovariectomized estrogen-primed rat, *Neuroendocrinology*, 57 (1993) 28–33.

[4] Bergen, H.T., Hejtmancik, J.F. and Pfaff, D.W., Effects of gamma-aminobutyric acid receptor agonists and antagonist on LHRH-synthesizing neurons as detected by immunocytochemistry and in situ hybridization, *Exp. Brain Res.*, 87 (1991) 46–56.

[5] Bradford, M.M., A rapid and sensitive method for the quantitation of microgram quantities of protein using the principle of protein-dye binding, *Anal. Biochem.*, 72 (1976) 248–254.

[6] Buijs, R.M., Hou, Y.-X., Shinn, S. and Renaud, L.P., Ultrastructural evidence for intra and extranuclear projections of GABAergic neurons of the suprachiasmatic nucleus, *J. Comp. Neurol.*, 340 (1994) 381–391.

[7] Casanueva, F., Apud, J.A., Masotto, C., Cocchi, D., Locatelli, V., Racagni, G. and Muller, E.E., Daily fluctuations in the activity of the tuberoinfundibular GABAergic system and prolactin levels, *Neuroendocrinology*, 39 (1984) 367–370.

[8] Cattabeni, F., Maggi, A., Monduzzi, M., De Angelis, L. and Racagni, N., GABA: Circadian fluctuations in rat hypothalamus, *J. Neurochem.*, 31 (1978) 565–567.

[9] Demling, J., Fuchs, E., Baumert, M. and Wuttke, W., Preoptic catecholamine, GABA, and glutamate release in ovariectomized and ovariectomized estrogen-primed rats utilizing a push-pull cannula technique, *Neuroendocrinology*, 41 (1985) 212–218.

[10] Edwards, A.L. 1954 *Statistical Methods for the Behavioural Sciences*, Rinehart, New York, 1954, pp. 300–314.

[11] Erlander, M.G. and Tobin, A.J., The structural and functional heterogeneity of glutamic acid decarboxylase: A review, *Neurochem. Res.*, 16 (1991) 215–226.

[12] Flügge, G., Oertel, W.H. and Wuttke, W., Evidence for estrogen receptive GABAergic neurons in the preoptic/anterior hypothalamic area of the rat brain, *Neuroendocrinology*, 43 (1986) 1–5.

[13] Fuchs, E., Mansky, T., Stock, K.-W., Vijayan, E. and Wuttke, W., Involvement of catecholamines and glutamate in GABAergic mechanism regulatory to luteinizing hormone and prolactin secretion, *Neuroendocrinology*, 38 (1984) 484–489.

[14] Grattan, D.R. and Selmanoff, M., Regional variation in γ-aminobutyric acid turnover: Effect of castration on γ-aminobutyric acid turnover in microdissected brain regions of the male rat, *J. Neurochem.*, 60 (1993) 2254–2264.

[15] Grattan, D.R. and Selmanoff, M., Castration-induced decrease in the activity of medial preoptic and tuberoinfundibular GABAergic neurons is prevented by testosterone, *Neuroendocrinology*, 60 (1994) 141–149.

[16] Grattan, D.R. and Selmanoff, M., Prolactin- and testosterone-induced inhibition of luteinizing hormone secretion after orchidectomy: Role of preoptic and tuberoinfundibular γ-aminobutyric acidergic neurons, *J. Endocrinol.*, 143 (1994) 165–174.

[17] Hartman, R.D., He, J.-R. and Barraclough, C.A., γ-Aminobutyric acid-A and -B receptor antagonists increase luteinizing hormone-releasing hormone neuronal responsiveness to intracerebroventricular norepinephrine in ovariectomized estrogen-treated rats, *Endocrinology*, 127 (1990) 1336–1345.

[18] Herbison, A.E. and Dyer, R.G., Effect on luteinizing hormone secretion of GABA receptor modulation in the medial preoptic area at the time of proestrous luteinizing hormone surge, *Neuroendocrinology*, 53 (1991) 317–320.

[19] Herbison, A.E. and Theodosis, D.T., Localization of oestrogen receptors in preoptic neurons containing neurotensin but not tyrosine hydroxylase, cholecystokinin or luteinizing hormone-releasing hormone in the male and female rat, *Neuroscience*, 50 (1992) 283–298.

[20] Herbison, A.E., Heavens, R.P., Dye, S. and Dyer, R.G., Acute action of oestrogen on medial preoptic gamma-aminobutyric acid neurons: Correlation with estrogen negative feedback on luteinizing hormone secretion, *J. Neuroendocrinol.*, 3 (1991) 101–106.

[21] Herbison, A.E., Augood, S.J. and McGowen, E.M., Expression of glutamic acid decarboxylase messenger RNA in rat medial preoptic area neurones during the oestrus cycle and after ovariectomy, *Mol. Brain Res.*, 14 (1992) 310–316.

[22] Hiatt, E.S., Brunetta, P.G., Seiler, G.R., Barney, S.A., Selles, W.D., Wooledge, K.H. and King, J.C., Subgroups of luteinizing hormone-releasing hormone perikarya defined by computer analyses in the basal forebrain of intact female rats, *Endocrinology*, 130 (1992) 1030–1043.

[23] Hiruma, H., Sano, A. and Kimura, F., Injection of bicuculline elicits firing of luteinizing hormone-releasing hormone pulse generator in muscimol-treated ovariectomized rats, *Brain Res.*, 641 (1994) 191–197.

[24] Huang, X. and Harlan, R.E., Absence of androgen receptors in LHRH immunoreactive neurons, *Brain Res.*, 624 (1993) 309–311.

[25] Jarry, H., Perschl, A. and Wuttke, W., Further evidence that preoptic anterior hypothalamic GABAergic neurons are part of the GnRH pulse and surge generator, *Acta Endocrinol. (Copenh.)*, 118 (1988) 573–579.

[26] Jarry, H., Leonhardt, S. and Wuttke, W., Gamma-aminobutyric acid neurons in the preoptic/anterior hypothalamic area synchronize the phasic activity of the gonadotropin-releasing hormone pulse gererator in ovariectomized rats, *Neuroendocrinology*, 53 (1991) 261–267.

[27] Jarry, H., Hirsch, B., Leonhardt, S. and Wuttke, W., Amino acid neurotransmitter release in the preoptic area of rats during the positive feedback action of estradiol on LH release, *Neuroendocrinology*, 56 (1992) 133–140.

[28] Kimura, F. and Jinnai, K., Bicuculline infusions advance the timing of luteinizing hormone surge in proestrous rats: Comparisons with naloxone effects, *Horm. Behav.*, 28 (1996) 424–430.

[29] Lee, S. and Pelletier, G., Chronic administration of muscimol and pentobarbital decreases gonadotropin-releasing hormone mRNA levels in the male rat hypothalamus determined by quantitative in situ hybridization, *Neuroendocrinology*, 58 (1993) 136–139.

[30] Lee, W.-S., Smith, M.S. and Hoffman, G.E., Luteinizing hormone-releasing hormone neurons express Fos protein during the proestrous surge of luteinizing hormone, *Proc. Natl. Acad. Sci. USA*, 87 (1990) 5163–5167.

[31] Lee, W.-S., Smith, M.S. and Hoffman, G.E., cFos activity identifies recruitment of luteinizing hormone-releasing hormone neurons during the ascending phase of the proestrous luteinizing hormone surge, *J. Neuroendocrinol.*, 4 (1992) 161–166.

[32] Lee, W.S., Abbud, R., Smith, M.S. and Hoffman, G.E., LHRH neurons express cJun protein during the proestrous surge of luteinizing hormone, *Endocrinology*, 130 (1992) 3101–3103.

[33] Leigh, A.J., Carter, N.D., Horten, R., Silverlight, J.J. and Wilson, C.A., Ovarian steroid regulation of glutamic acid decarboxylase gene expression in individual hypothalamic nuclei, *J. Neuroendocrinol.*, 2 (1990) 433–438.

[34] Léránth, C., MacLusky, N.J., Sakamoto, H., Shanabrough, M. and Naftolin, F., Glutamic acid decarboxylase-containing axons synapse on LHRH neurons in the rat medial preoptic area, *Neuroendocrinology*, 40 (1985) 536–539.

[35] Léránth, C., MacLusky, N.J., Shanabrough, M. and Naftolin, F., Catecholaminergic innervation of luteinizing hormone-releasing hormone and glutamic acid decarboxylase immunopositive neurons in the rat medial preoptic area, *Neuroendocrinology*, 48 (1988) 591–602.

[36] Mansky, T., Mestres-Ventura, P. and Wuttke, W., Involvement of GABA in the feedback action of estradiol on gonadotropin and prolactin release: Hypothalamic GABA and catecholamine turnover rates, *Brain Res.*, 231 (1982) 353–364.

[37] Martin, D.L. and Rimvall, K., Regulation of γ-aminobutyric acid synthesis in the brain, *J. Neurochem.*, 60 (1993) 395–407.

[38] McCarthy, M.M., Kaufman, L.C., Brooks, P.J. and Pfaff, D.W., Estrogen modulation of mRNA for two forms of glutamic acid decarboxylase (GAD) in female rat brain, *J. Comp. Neurol.*, 360 (1995) 685–697.

[39] Moore, R.Y. and Speh, J.C., GABA is the principal neurotransmitter of the circadian system, *Neurosci. Lett.*, 150 (1993) 112–116.

[40] Morello, H., Caligaris, L., Haymal, B. and Taleisnik, S., Daily variations in the sensitivity of proestrous LH surge in the inhibitory effect of intraventricular injection of 5-HT or GABA in rats, *Can. J. Physiol. Pharmacol.*, 70 (1992) 447–451.

[41] Mugnaini, E. and Oertel, W.H., An atlas of the distribution of GABAergic neurons and terminals in the rat CNS as revealed by GAD immunocytochemistry. In A. Björklund and T. Hökfelt (Eds.), *Handbook of Chemical Neuroanatomy, Vol. 4: GABA and Neuropeptides in the CNS, Part 1*, Elsevier, Amsterdam, 1985, pp. 436–608.

[42] Palkovits, M. and Brownstein, M.J., *Maps and Guide to Microdissection of the Rat Brain*, Elsevier, New York, 1988.

[43] Park, S.-K. and Selmanoff, M., Dose-dependent suppression of postcastration luteinizing hormone secretion exerted by exogenous prolactin administration in male rats: A model for studying hyperprolactinemic hypogonadism, *Neuroendocrinology*, 53 (1991) 404–410.

[44] Peterson, S.L., McCrone, S., Coy, D., Adelman, J.P. and Mahan, L.C., $GABA_A$ receptor subunit mRNAs in cells of the preoptic area: Colocalization with LHRH mRNA using dual label in situ hybridization histochemistry, *Endocr. J.*, 1 (1993) 29–34.

[45] Pfaff, D.W. and Keiner, M., Atlas of estradiol-concentrating cells in the central nervous system of the female rat, *J. Comp. Neurol.*, 151 (1973) 121–158.

[46] Porkka-Heiskanen, T., Urban, J.H., Turek, F.W. and Levine, J.E., Gene expression in a sub-population of luteinizing hormone-releasing hormone (LHRH) neurons prior to the preovulatory gonadotropin surge, *J. Neurosci.*, 14 (1994) 5548–5558.

[47] Sagrillo, C.A. and Selmanoff, M., Castration decreases single cell levels of mRNA encoding glutamic acid decarboxylase in the diagonal band of Broca and the sexually dimorphic nucleus of the preoptic area, *J. Neuroendocrinol.*, (1996) in press.

[48] Seltzer, A.M. and Donoso, A.O., Restraining action of GABA on estradiol-induced LH surge in the rat: GABA activity in brain nuclei and effects of GABA mimetics in the medial preoptic nucleus, *Neuroendocrinology*, 55 (1992) 28–34.

[49] Shivers, B.D., Harlan, R.E., Morrell, J.I. and Pfaff, D.W., Absence of estradiol concentration in cell nuclei of LHRH-immunoreactive neurons, *Nature*, 304 (1983) 345–347.

[50] Stoflet, E.S., Koeberl, D.D., Sarkar, G. and Sommer, S.S. Genomic amplification with transcript sequencing, *Science*, 239 (1988) 491–494.

[51] Strauss, K.I. and Jakobowitz, D.M., Quantitative measurement of calretinin and β-actin mRNA in rat brain micropunches without prior isolation of RNA, *Mol. Brain Res.*, 20 (1993) 229–239.

[52] Van der Beek, E.M., Van Oudheusden, H.J., Buijs, R.M., Van der Donk, H.A., Van der Hurk, R. and Wiegant, V.M., Preferential induction of c-fos immunoreactivity in vasoactive intestinal polypeptide-innervated gonadotropin-releasing hormone neurons during a steroid-induced luteinizing hormone surge in the female rat, *Endocrinology*, 134 (1994) 2636–2644.

[53] Van der Hayden, J.A.M. and Korf, J., Regional levels of GABA in the brain: Rapid semiautomated assay and prevention of post mortem increase by 3-mercaptopropionic acid, *J. Neurochem.*, 31 (1978) 197–203.

[54] Weiland, N.G., Glutamic acid decarboxylase messenger ribonucleic acid is regulated by estradiol and progesterone in the hippocampus, *Endocrinology*, 131 (1993) 2697–2702.

[55] Winer, B.J., *Statistical Principles in Experimental Design*, McGraw-Hill, New York, 2nd Edn., 1971.