Lack of functional GABA<sub>B</sub> receptors alters GnRH physiology and sexual dimorphic expression of GnRH and GAD-67 in the brain

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Submitted 27 August 2009; accepted in final form 10 December 2009

Catalano PN, Di Giorgio N, Bonaventura MM, Bettler B, Libertun C, Lux-Lantos VA. Lack of functional GABA<sub>B</sub> receptors alters GnRH physiology and sexual dimorphic expression of GnRH and GAD-67 in the brain. Am J Physiol Endocrinol Metab 298: E683–E696, 2010. First published December 15, 2009; doi:10.1152/ajpendo.00532.2009.—GABA, the main inhibitory neurotransmitter, acts through GABA<sub>A/C</sub> and GABA<sub>B</sub> receptors (GABA<sub>B</sub>Rs); it is critical for gonadotropin regulation. We studied whether the lack of functional GABA<sub>B</sub>Rs in GABA<sub>B1</sub> knockout (GABA<sub>B1</sub>KO) mice affected the gonadotropin axis physiology. Adult male and female GABA<sub>B1</sub>KO and wild-type (WT) mice were killed to release, except during the preovulatory period in females when bursts or pulses, with essentially no secretion between bursts of reproductive function. The secretion of GnRH occurs in discrete peptidergic hormone produced by specific neurons in the hypothalamus, forms the final common pathway regulating reproductive function. The secretion of GnRH occurs in discrete bursts or pulses, with essentially no secretion between bursts of release, except during the preovulatory period in females when a GnRH surge occurs, inducing the pituitary gonadotropin preovulatory peaks (67, 70). Regulation of the gonadotropin axis is provided by both stimulatory and inhibitory inputs conveyed by neurotransmitters, neurohormones, and peripheral peptide and steroid hormones. Among the neurotransmitters involved in this regulation, GABA has been proposed to be of prime importance (16, 26, 43, 65). GABA can regulate the hypothalamic-pituitary-gonadal axis by acting at ionotropic GABA<sub>A/C</sub> and/or metabotropic GABA<sub>B</sub> receptors (GABA<sub>B</sub>Rs). In the brain, GABA can directly act at GnRH neurons, since glutamic acid decarboxylase (GAD)-containing axons synapse on GnRH neurons in the rat medial preoptic area (53), and the presence of both GABA<sub>A</sub> and GABA<sub>B</sub>Rs has been described in these GnRH neurons (30, 51, 86). GABA also can act indirectly by inhibiting interneurons involved in the regulation of GnRH secretion, such as opiate or noradrenergic neurons (21, 45). Interestingly, GABA also has been proposed to have a role during embryogenesis (92) and to guide the migration of GnRH neurons across the nasal septum and cribriform plate into the brain to their final destination, mainly in the preoptic area-anterior hypothalamus (POA-AH) and medial basal hypothalamus (80, 100). In addition, a dual effect of GABA on GnRH release has been postulated, with GABA stimulating at early stages of development and inhibitory in later stages of development and during adulthood, both effects being mediated by GABA<sub>A</sub> receptors (40); nevertheless, recent studies propose that its direct effect on GnRH neurons also could be stimulatory in adulthood (19, 43). The actions of GABA on GnRH secretion through different GABA receptors from embryos to adults was recently reviewed by Maffucci and Gore (57). As stated in their report, the effects of GABA on GnRH cell migration have been described to occur through GABA<sub>A</sub> receptors, although GABA<sub>B</sub> receptors are also expressed in GnRH neurons at these early developmental stages. Interestingly, both receptor types show developmental changes in protein subunit composition in hypothalamic areas, which include GnRH neurons. The period of the pubertal transition is one in which pharmacological properties of the GABARs undergo interesting changes; whereas baclofen, the GABAB agonist, is inhibitory on GnRH and gonadotropin release at both postnatal days 16 and 30, muscimol, the GABA<sub>A</sub> agonist, was stimulatory on these end points on day 16 and inhibitory on day 30. In adulthood, both GABA<sub>A</sub>Rs and GABA<sub>B</sub>Rs are involved in a complex regulation of GnRH/LH pulsatility and probably interact to attain the very precise regulation of this system. Moreover, the participation of GABA in delaying the postcastration LH increase in adult females involves both GABA<sub>A</sub> and GABA<sub>B</sub> receptors. A decrease in GABA levels in...
the POA-AH just before the GnRH surge has been described, suggesting a disinhibition of GnRH neurons allowing the GnRH release to increase; both receptors have been shown to be involved in GABA inhibition of the preovulatory LH surge in rats. In addition, in anestrous ewes, GABA_A receptors attenuate GnRH release, whereas GABA_B receptors decrease or increase GnRH secretion, depending on the brain area. Regarding GABA actions on GnRH gene expression, various results have been obtained and gonadal steroid levels influence these results; in general, in ovariectomized rats, both GABA_A and GABA_B antagonists tend to increase GnRH expression, whereas the agonists tend to decrease it.

The presence of GABA_A, GABA_B, and GABA_C receptors in the pituitary is well documented (3, 24, 29, 56). Their ligand, GABA, reaches the pituitary by different hypothalamic pathways but is also synthesized locally (28, 94). Ovaries and testes also have been shown to express intrinsic GABAergic components (13, 32, 42, 44, 48).

Results from our laboratory established the participation of GABA_A receptors in the control of pituitary hormone secretion (for review, see Ref. 55) and GABA_B intracellular signaling pathways in the adenohypophysis (54). Functional GABA_A receptors are obligate heterodimers composed of a GABA_A1 and a GABA_A2 subunit (12, 48). In this regard, we showed that GABA_A receptors have particular ontogenic expression patterns in the rat hypothalamus and the pituitary (9, 10) and that their expressions show sexual dimorphisms at early stages of development (8, 9). In GABA_A knockout (GABA_A KO) mice, which lack functional GABA_A receptors (79), we demonstrated that GABA_A receptors are involved in regulating basal prolactin (PRL) release in males and that the hypothalamic-hypophyseal-ovarian axis is compromised in females, with significant alterations in cyclicity, postcastration LH increase, and reproduction indexes (14). Because GABA_A receptors have been demonstrated to participate in the regulation of the gonadotrophic axis, and taking into consideration our previous results in GABA_A KO mice, we studied different parameters involved in the regulation of the hypothalamic-pituitary gonadotrophic axis in adult male and female GABA_A KO mice to establish the impact of the loss of functional GABA_A receptors. Specifically, we determined hypothalamic GnRH expression, content, and pulsatility; hypothalamic GABA, glutamate, and taurine contents and hypothalamic GAD-67 expression; and gonadotropin serum levels, pituitary contents, and, in vitro secretion.

**MATERIALS AND METHODS**

**Animals**

GABA_A KO (79) generated in the BALB/C inbred mouse strain were obtained by intercrossing heterozygous animals, and the day of birth was recorded. DNA isolated from toe clips (performed for identification purposes) was genotyped by PCR analysis, as described previously (14). Animals were given free access to laboratory chow and tap water. All studies were performed according to protocols for animal use, approved by the Institutional Animal Care and Use Committee (IBYME-CONICET) and by the National Institutes of Health. Wild-type (WT) and GABA_A KO female and male adult mice were killed by decapitation in the morning (9:00–11:00 AM); females were euthanized in estrus, since GABA_A KO females showed marked estrus persistence (14).

**Western Blot Analysis of the GABA_B1 Subunit in Cortex Membranes**

Western blot analysis for the GABA_B1 subunit was performed in WT and GABA_B1 KO male mice frontoparietal cerebral cortex membranes. Briefly, 20 mg of proteins of cortex membrane preparations, obtained as previously described (9), were subjected to 8% SDS-PAGE. Proteins were transferred onto nitrocellulose membranes. Blots were blocked for 2 h in PBS-0.2% Tween 20–5% nonfat milk and incubated overnight at 4°C with a guinea pig anti-GABA_B1 antibody (1:4,000 in PBS-0.2% Tween 20–5% nonfat milk), which specifically binds the GABA_B1 (130 kDa) and GABA_B1 (100 kDa) isoforms of the GABA_B1 subunit of the GABA_B receptor (antibody GP311, a gift from Marta Margeta, Jan Lab, University of California, San Francisco (59)). Secondary antibody was horseradish peroxidase coupled (1:1,000 in PBS-0.1% Tween 20, for 60 min at room temperature; Sigma, St. Louis, MO). Detection was performed using an enhanced chemiluminescence Western blot analysis system (Western blotting chemiluminescence LuminoL reagent; Santa Cruz Biotechnology). The absence of the protein of the GABA_B1 subunit of the GABA_B1 receptor in cortex in GABA_B1 KO is shown in Fig. 1.

**GnRH Content Determination**

For GnRH hypothalamic content, whole hypothalami including the POA were excised and rapidly frozen. The anterior limit of the fragments was a plane at the height of the anterior commissure, lateral limits were the hypothalamic fissures, the posterior limit was behind the mammillary bodies, and the in-depth limit was the subthalamic sulcus, as previously described (8). The olfactory bulbs (OB) and frontoparietal cortex (CT) were also rapidly dissected and frozen. Tissues were homogenized in 100 μl of ice-cold 0.1 N HCl as described by Heger et al. (43). The homogenate was centrifuged for 30 min at 15,000 g at 4°C and the supernatant was recovered; the procedure was repeated. Samples were stored at −20°C until assayed for GnRH by RIA, as previously described (87). GnRH assay sensitivity was 1.5 pg, and intra- and interassay coefficients of variation were 7.1 and 11.6%, respectively. The number of animals per group was 5–12.

**Gonadotropins, Pituitary Content, and Serum Levels**

Trunk blood was collected, and sera were obtained and frozen for hormone determinations. Pituitary gland was rapidly dissected and frozen. To determine LH and FSH pituitary content, each pituitary was homogenized in 100 μl of PBS with protease inhibitors. Protein
content was measured using Lowry, and the result determined the aliquot of the homogenate that was measured by RIA. The number of animals per group was 6 – 12.

LH and FSH were determined by RIA with kits from the National Hormone and Pituitary Program, National Institute of Diabetes and Digestive and Kidney Diseases, and Dr. A. F. Parlow. Results are expressed in terms of RP3 rat LH and FSH standards. Assay sensitivities were as follows: LH, 0.015 ng/ml; and FSH, 0.1175 ng/ml. Intra- and interassay coefficients of variation were as follows: LH, 7.2 and 11.4%; and FSH, 8.0 and 13.2%, respectively.

Amino Acidic Neurotransmitter Determination by HPLC

Whole hypothalami with the POA and CT (control tissue) were rapidly dissected and frozen. GABA, glutamate, and taurine contents were determined by HPLC as previously described (8). Briefly, aminooacidic dansyl derivates were measured using a HPLC system, which consisted of a model 125 Programmable Solvent Module (Beckman Instruments, Fullerton, CA), a 5-μm 25 cm × 4.6 mm Ultrasphere ODS reversed-phase column (Beckman), a model 166 programmable UV detector module (254 nm; Beckman), and System Gold software (Beckman). The mobile phase consisted of a water-acetonitrile mixture (82.18; vol/vol) containing 0.15% (vol/vol) phosphoric acid. The number of animals per group was 6 – 12.

GnRH Pulsatility Studies

Pulsatility studies were performed in vitro as described by Heger et al. (43). Briefly, whole hypothalami were incubated in 1.5-ml microfuge tubes containing 250 μl of Krebs-Ringer bicarbonate buffer with 4.5 mg/ml glucose and 16 mM HEPES at 37°C for 6 h. After 30 min of preincubation, the medium from each tube was collected at 8.5-min intervals and replaced with fresh medium. GnRh in the incubation media was measured by RIA as previously described (87). The number of animals per group was 6 – 7.

GnRH pulses were identified and their parameters determined using the computer algorithm Cluster8 analysis developed by Veldhuis and Johnson (93). A 2 × 2 cluster configuration and a t statistic of 2 for the upstroke and downstroke were used to maintain false-positive and false-negative error rates below 10%, as suggested by Martinez de la Escalera et al. (62). The statistical significance of the pulse parameters calculated by the program was tested using Student’s t-test or two-way ANOVA. For analysis, percentages were subjected to arc-sine transformation to convert them from a binomial to a normal distribution.

Anterior Pituitary Cell Cultures

Monolayer adenohypophyseal cell cultures from adult male and estrous female wild-type and GABA1KO mice were obtained as previously described (69). Briefly, 50,000 cells were seeded per well in P96 multiwell dishes and maintained in the incubator for 4 days until hormonal studies were performed. Thereafter, cells were incubated in DMEM-F12 (Invitrogen, Carlsbad, CA) with CO3HNa (2.2 g/l) and BSA (0.1%). Cells were stimulated for 1 h with GnRH (1.10-6 M) or medium as control. LH and FSH were measured in media samples by RIA. In each experiment, four to six mice of each sex and genotype were used; experiments were repeated four times, and each treatment was performed in quadruplicate.

Cell Viability Assay

After the GnRH stimulation studies, to detect possible variation in cell numbers between groups, cell viability was colorimetrically determined using the MTS method (Promega, Madison, WI), as previously described (87). Gonadotropin secretion was normalized to cell number to compare between groups.

GnRH and GAD-67 mRNA Expression

To determine GnRH and GAD-67 expression by qRT-PCR, total RNA was isolated from hypothalamic fragments, OB, and CT. The hypothalami were divided into POA-AH and medial basal-posterior hypothalami (MBH-PH) to determine whether there were differences in the expression of these genes in the area involved in surge GnRH secretion (POA-AH) and/or in the area involved in pulsatile GnRH secretion (MBH-PH) due to differences in genotype and/or sex.

POA-AH. This area is a triangle limited anteriorly by a plane at the height of the anterior commissure, laterally by the hypothalamic fissures, posteriorly by a plane passing immediately behind the optic chiasm, and in depth by the subthalamic sulcus.

MBH-PH. The anterior limit is the plane passing immediately behind the optic chiasm, lateral limits are hypothalamic fissures, posterior limits are the mammillary bodies, and the in-depth limit is the subthalamic sulcus.

RNAs were obtained using Trizol reagent (Invitrogen) according to the manufacturer’s protocol and kept at −70°C until used. The RNA concentration of all final preparations was calculated using the Qubit Quantitation Platform (Invitrogen) according to the manufacturer’s protocol. The number of animals per group was 6 – 8.

cDNA Synthesis

First-strand cDNA was synthesized from 1 μg of total RNA in the presence of 10 mM MgCl2, 50 mM Tris · HCl (pH 8.6), 75 mM KCl, 1 mM deoxy-NTPs, 0.1 mM DTT, 2 U/μl RNaseOUT (Invitrogen), 0.2 μg of oligo(dT)17 primer (Biodyomics, Buenos Aires, Argentina), and 20 units of MMLV reverse transcriptase (Epicentre, Madison, WI). The reverse transcriptase was omitted in control reactions, where the absence of PCR-amplified DNA indicated the isolation of RNA free of genomic DNA.

qRT-PCR

Sense and antisense oligonucleotide primers for GnRH and cyclophilin were designed based on the published cDNA sequences using the PrimerExpress software (Applied Biosystems, Foster City, CA). Oligonucleotides were obtained from Invitrogen. The sequences of these primers were as follows: GnRH: sense, GAACCCGAGCAGCTTC- GAATGT, antisense, TGGCTTCCTCTCTCAATCACTTCTT; cyclophilin: sense, GTGGCAAGATCGAAGTG, antisense, TAAAAATCAGG- GCCGTGG (85); and GAD-67: sense, CCGGGACCGGATCCTAATA, antisense, TGGTGCACTCATGGGCTAC (75).

Quantitative measurements of GnRH and GAD-67 cDNA were performed by kinetic PCR using SYBR green PCR master mix (Applied Biosystems) according to the manufacturer’s protocol with an additional annealing step of 58°C for 15 s. GnRH and GAD-67 primers concentration were 0.4 and 0.2 μM, respectively.

Quantitative measurements of cyclophilin cDNA were performed by kinetic PCR using SYBR green PCR master mix (Applied Biosystems) according to the manufacturer’s protocol with an additional annealing step of 58°C for 15 s. GnRH and GAD-67 primers concentration were 0.4 and 0.2 μM, respectively.

Quantitative measurements of cyclophilin cDNA were performed by kinetic PCR using SYBR green I as fluorescent dye (Invitrogen) and ROX as passive reference dye (Epicentre Biotechnologies). PCR reaction consisted of 0.4 μM primers, 10 mM Tris · HCl, 50 mM KCl, 3.5 mM MgCl2, 0.15 mM deoxy-NTPs, 0.63 units of Taq polymerase (Invitrogen), and an appropriate dilution of cDNA in a final volume of 13 μl. After denaturing at 95°C for 1 min, the cDNA products were amplified with 40 cycles, each cycle consisting of denaturing at 95°C for 20 s, annealing at 62°C for 40 s, and extension at 72°C for 40 s.

The accumulating DNA products were monitored using the ABI7500 sequence detection system (Applied Biosystems), and data were stored continuously during the reaction. The results were validated based on the quality of dissociation curves, generated at the end of the PCR runs by ramping the temperature of the samples from 60°C to 95°C, meanwhile continuously collecting fluorescence data. Product purity was confirmed by 2.3% agarose gel electrophoresis. Each sample was analyzed in triplicate along with nontemplate controls to
monitor contaminating DNA. For each target gene, the relative gene expression was normalized to that of cyclophilin housekeeping gene.

Quantitative differences in the cDNA target between samples were determined using the mathematical model of Pfaffl (18, 74) in which an expression ratio was determined for each sample by calculating $\frac{(E_{\text{target}})^{\Delta C_{\text{target}}}}{H_{\text{cyclophilin}}^{\Delta C_{\text{cyclophilin}}}}$, where $E$ is the efficiency of the primer set and $\Delta C = C_{\text{reference}} - C_{\text{experimental}}$. The amplification efficiency of each primer set was calculated from the slope of a standard amplification curve of log concentration of cDNA (in ng/μl) per reaction vs. $C_t$ value over a wide-range serial dilution of cDNA that covered the amount of target mRNA expected in the experimental samples. The amplification efficiency of GnRH primers was $E = 2.09$ (MBH-PH cDNA), $E = 2.13$ (OB cDNA), and $E = 2.09$ (CT cDNA); in GAD-67 primers, $E = 1.83$ (MBH-PH cDNA), $E = 1.93$ (PA-AH cDNA), and $E = 2.06$ (CT cDNA); and in cyclophilin primers, $E = 2.01$ (MBH-PH cDNA), $E = 1.90$ (OB cDNA), and $E = 1.99$ (CT cDNA). Results are expressed as arbitrary units (AU) for comparison among samples. AU is defined as the expression level relative to the reference sample (a WT male chosen at random).

Statistics

Data are means ± SE. The differences between means of two groups were analyzed using Student’s $t$-test. The differences between means of more than two groups were analyzed using one- or two-way ANOVA, followed by Tukey’s honestly significant difference test for unequal $n$. $P < 0.05$ was considered significant.

RESULTS

GnRH Contents in Hypothalamus, OB, and CT in Male and Female WT and GABA B1KO Mice

When analyzing GnRH contents in whole hypothalamic fragments including the POA, important differences were observed between genotypes and sexes (Fig. 2A). Male levels were significantly higher than in females, and knockout contents were significantly lower than in WTs [2-way ANOVA, interaction: not significant (NS), main effect sex: $P < 0.02$, main effect genotype: $P < 0.01$].

In OB, as expected, GnRH contents were significantly lower than in hypothalamus (Fig. 2B). No significant sex differences were observed in OB; nevertheless, like in the hypothalamus, knockout females showed significantly lower GnRH titers than WT controls (2-way ANOVA, factors: sex and genotype, interaction: $P < 0.02$, WT females vs. GABA B1KO females, $P < 0.01$).

In contrast to hypothalamus and OB, GnRH content in cortex showed no genotype differences (Fig. 2C). Females showed significantly lower cortex GnRH titers than males, as also observed in hypothalamus (2-Way ANOVA, interaction: NS; factor sex: $P < 0.005$). Very low levels of GnRH content were detected in this tissue.
GnRH mRNA Expression in Hypothalamus, OB, and CT in Male and Female WT and GABA B1KO Mice

When GnRH mRNA expression were analyzed in MBH-PH, sex and genotype differences were observed: male levels were significantly lower than in females, and knockout mice GnRH expression was significantly higher than in WT controls (2-way ANOVA, interaction: NS, main effect sex: P < 0.005, main effect genotype: P < 0.01) (Fig. 3A). POA-AH showed a different pattern of GnRH expression: GnRH mRNA was significantly higher in WT males than in WT females or in GABA B1KO males (Fig. 3B). In addition, GABA B1KO females showed increased expression with regard to their WT controls and to GABA B1KO males (2-way ANOVA, interaction: P < 0.001). These results suggest an inversion of sexual dimorphism in GnRH expression in this area. Interestingly, in both hypothalamic fragments, GnRH expression was increased in GABA B1KO females. In contrast, in GABA B1KO males, GnRH expression was lower in POA-AH and higher in MBH-PH compared with WT controls. In OB and CT, GnRH mRNA expression was similar in all groups (2-way ANOVA: NS) (Fig. 3, C and D).

GnRH Pulsatility in Hypothalamic Fragments from Male and Female WT and GABA B1KO Mice

Because hypothalamic GnRH mRNA expression and peptide content were altered in hypothalami of GABA B1KO mice, we measured GnRH release from whole hypothalamic explants. GnRH analyzed in media samples every 8.5 min revealed a pulsatile pattern of secretion with a similar frequency in control females and males [GnRH (pulses/h): WT males: 0.80 ± 0.09 (n = 6) vs. WT females: 0.74 ± 0.03 (n = 7), Student's t-test: NS].

Representative pulsatility profiles of WT and GABA B1KO females are depicted in Fig. 4A. GABA B1KO females showed a significant increase in the frequency of GnRH pulses with regard to WT controls (Fig. 4B). Other parameters such as peak amplitude, peak duration, mean interpulse interval, or mean secretion pulse mass were not different between female genotypes (Table 1). To determine whether there were differences in the distribution of the interpulse intervals (43), we grouped the intervals (a parameter provided by Cluster8 algorithm) into two categories: those lasting 10 min or less and those lasting more than 10 min. Analysis of the data showed that whereas in WT females, the large amount of pulses occurred at longer intervals (>10 min: 76%), in GABA B1KO females, the number of pulses occurring at short and long intervals did not differ (Fig. 4C), in agreement with the increase in frequency observed. In males, no significant differences in GnRH pulse frequency were detected, but mean secretion pulse mass decreased to one-third in GABA B1KO males (Table 1).

GABA, Glutamate, and Taurine Contents in Hypothalamus and CT in Male and Female WT and GABA B1KO Mice

To evaluate whether the differences in hypothalamic GnRH content, GnRH mRNA expression, and GnRH pulsatility in GABA B1KO mice could be due to alterations in stimulatory and/or inhibitory neurotransmitters related to the GABAergic system, we determined GABA, glutamate, and taurine contents by HPLC in whole hypothalami and cortex (as a control area) in adult mice. Hypothalamic GABA contents varied depending on sex and genotype (Fig. 5A) (2-way ANOVA, interaction: P < 0.02). A clear sex difference in GABA content was observed between male and female WT mice, with males showing significantly higher levels than females (P < 0.003). GABA B1KO males showed hypothalamic GABA contents similar to those of WT controls. In contrast, GABA B1KO females showed significantly higher levels than WT females.
similar to those observed in males and thus failing to show the sex differences observed in WTs.

Glutamate hypothalamic contents showed a pattern similar to that of GABA (Fig. 5B) (2-way ANOVA, interaction: \( P < 0.05 \)). The same sex difference in glutamate contents was observed in WTs (\( P < 0.01 \)) and was lost in GABAB1KO mice. Interestingly, in GABAB1KO females, glutamate was elevated, significantly different from WT females (\( P < 0.04 \)), as also observed for GABA.

Hypothalamic taurine showed a similar pattern of content, but statistical analysis determined that only sexual differences were significant, with male levels higher than female levels in both genotypes (2-way ANOVA, factors: genotype and sex, interaction: NS, main effect genotype: NS, main effect sex: \( P < 0.001 \)) (Fig. 5C).

Amino acid neurotransmitter contents in cortex are shown in Fig. 6. Sex and genotype differences in GABA content were observed in cortex; male levels were higher than in females, and GABAB1KO contents were higher than in WT mice (Fig. 6A) (2-way ANOVA, factors: genotype and sex, interaction: NS, genotype: \( P < 0.03 \), sex: \( P < 0.001 \)). Glutamate did not show any differences between sexes or genotypes (Fig. 6B). Similar to GABA, taurine cortex contents were significantly increased in GABAB1KO mice compared with controls, although titer levels were similar between sexes (Fig. 6C) (2-way ANOVA, factors genotype and sex, interaction: NS, genotype \( P < 0.01 \), sex: NS).

GAD-67 mRNA Expression in Hypothalamus and CT in Male and Female WT and GABAB1KO Mice

To evaluate whether alterations in GABA contents were influenced by differential GAD-67 expression, we determined the expression of its mRNA in POA-AH, MBH-PH, and cortex of adult male and female mice of both genotypes (Fig. 7).

GABAB1KO animals showed a threefold increase in GAD-67 mRNA expression in MBH-PH; no differences between sexes were observed (Fig. 7A) (2-way ANOVA, factors: genotype and sex, interaction: NS, main effect sex: NS, main effect genotype: \( P < 0.001 \)). POA-AH showed a different pattern of GAD-67 mRNA expression with respect to MBH-PH, with a clear sexual dimorphism between WT males and females, with males expressing significantly higher levels; this sexually specific expression pattern was inversed in GABAB1KO mice (2-way ANOVA: interaction: \( P < 0.001 \)) (Fig. 7B). Interestingly, the pattern observed for GAD-67 mRNA expression in POA-AH is very similar to the one observed for GnRH mRNA expression in this area.

Table 1. Parameters of secreted GnRH pulses identified by cluster analysis in WT and GABAB1KO female and male mice

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<thead>
<tr>
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<th>Females</th>
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<tr>
<td></td>
<td>WT</td>
<td>KO</td>
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<td>KO</td>
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<tr>
<td>Number of peaks in 6 h</td>
<td>4.46 ± 0.20</td>
<td>5.17 ± 0.16*</td>
<td>4.78 ± 0.55</td>
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<td>Peak amplitude, pg/mg HT</td>
<td>4.49 ± 1.22</td>
<td>3.77 ± 1.46</td>
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<td>Peak duration, min</td>
<td>41.64 ± 2.21</td>
<td>41.10 ± 2.64</td>
<td>47.31 ± 4.47</td>
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<td>Mean interpeak interval, min</td>
<td>62.82 ± 4.20</td>
<td>57.41 ± 3.64</td>
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<td>Mean secretion pulse mass</td>
<td>2.06 ± 0.59</td>
<td>1.71 ± 0.40</td>
<td>2.67 ± 0.71</td>
<td>0.94 ± 0.16</td>
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<tr>
<td>Number of cases</td>
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<td>6</td>
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Values are means ± SE. WT, wild-type control; KO, GABAB1 knockout; HT, hypothalamus. *\( P < 0.05 \), significantly different from WT.
GAD-67 mRNA expression in CT, selected as a control area, was increased in WT females, significantly different from WT males (Fig. 7C) (2-way ANOVA: interaction: \( P < 0.02 \)). This sex difference was lost in GABA\(_{B1}\)KO mice.

**Pituitary LH and FSH Contents and Serum Levels in Male and Female WT and GABA\(_{B1}\)KO Mice**

*Pituitary gonadotropin contents.* No genotype differences were observed in either LH or FSH pituitary contents. In females, LH and FSH contents were significantly lower in males (2-way ANOVA: interaction: NS, main effect sex: \( P < 0.001 \), main effect genotype: NS) (Fig. 8, A and B).

*Serum gonadotropins.* Results similar to those for pituitary gonadotropin contents were observed. LH and FSH were significantly lower in females than in males, without differences due to genotype (2-way ANOVA: interaction: NS, main effect: genotype: NS, main effect age: \( P < 0.001 \)) (Fig. 8, C and D).

**Basal and GnRH-Induced LH and FSH Secretion in Adenohypophyseal Cell Cultures From Male and Female WT and GABA\(_{B1}\)KO Mice**

To evaluate whether there were differences at the gonadotropin levels between genotypes in the absence of central and peripheral feedback regulation, gonadotropin secretion was evaluated in anterior pituitary cell cultures of both sexes and genotypes (Fig. 9). Significant sex and genotype differences were observed in basal LH secretion from primary adenohypophyseal cell cultures [LH (ng/50,000 cells): WT males, 13.8 ± 1.7; WT females, 4.4 ± 0.3; KO males, 11.7 ± 1.5; KO females, 6.1 ± 0.5 (n = 4); 2-way ANOVA, factors: sex and genotype, interaction: \( P < 0.01 \)]. WT male pituitary cells secreted significantly more LH to the culture medium than WT female cells (\( P < 0.01 \)) in agreement with the more abundant pituitary LH content observed in males; pituitary cells from GABA\(_{B1}\)KO males secreted basal LH levels similar to those in control male cells; knockout female cells showed significantly increased basal LH secretion compared with WT female cells (\( P < 0.02 \)) but still maintained the sex difference with regard to GABA\(_{B1}\)KO males (\( P < 0.01 \)).

Regarding basal FSH secretion, again, sex and genotype differences were observed [FSH (ng/50,000 cells): WT males, 2.05 ± 0.09; WT females, 2.71 ± 0.05; KO males, 2.31 ± 0.1; KO females, 3.24 ± 0.17; 2-way ANOVA, factors: sex and genotype, interaction: \( P < 0.05 \)]. Male pituitary cells of both genotypes secreted similar FSH levels that were significantly lower than those of female cells (\( P < 0.01 \), even though the FSH pituitary content was increased in males. Similar to LH, cells from GABA\(_{B1}\)KO females secreted increased basal FSH titers compared with WT females (\( P < 0.03 \)).

GnRH stimulated an increase in LH (Fig. 9A) and FSH (Fig. 9B) secretion in males. The GnRH-induced FSH increase was lower in cells from GABA\(_{B1}\)KO males (Fig. 9B, inset). In females, GnRH also stimulated LH increases (Fig. 9C) in cells from both genotypes, but this increase was less pronounced in pituitary cells from GABA\(_{B1}\)KO mice (Fig. 9C, inset); in contrast to female WT cells, the GnRH-induced FSH increase did not reach statistical significance in cells from GABA\(_{B1}\)KO mice (Fig. 9D).

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**Fig. 5.** Hypothalamic GABA (A), glutamate (B), and taurine contents (C) contents in male and female WT and GABA\(_{B1}\)KO mice. Number of animals: 12 WT males, 12 KO males, 6 WT females, and 6 KO females. A: GABA. 2-way ANOVA: factors genotype and sex, interaction: \( P < 0.02 \). *WT females were significantly different from WT males (\( P < 0.003 \)), KO males (\( P < 0.001 \)), and KO females (\( P < 0.01 \)). B: glutamate. 2-way ANOVA: factors genotype and sex, interaction: \( P < 0.05 \). *WT females were significantly different from WT males (\( P < 0.01 \)), KO males (\( P < 0.003 \)), and KO females (\( P < 0.04 \)). C: taurine. 2-way ANOVA, factors genotype and sex, interaction: NS, main effect genotype: NS, main effect sex: \( P < 0.001 \). *Females significantly different from males.
In a previous work we demonstrated significant alterations in estrous cyclicity, postcastration LH increase, and reproduction indexes in female GABA$_{B1}$KO mice (14). This suggested that GABA$_B$Rs participate in the regulation of these functions, as was also proposed on the basis of pharmacological experiments in rats (1, 2, 41, 55). In the present study we have demonstrated that the absence of functional GABA$_B$ receptors from conception alters the sexual dimorphic expression of critical genes involved in reproduction such as GnRH and GAD-67 in the POA-AH, as well as modifying GnRH physiology, mainly in female mice.

**GnRH Content and mRNA Expression**

We analyzed GnRH expression and content in hypothalamus, OB, and CT. GnRH neurons have been shown to originate in the nasal compartment in mammals and migrate along olfactory nerves to the cribriform plate, where most of them migrate caudally to the forebrain to reach their final destination in the hypothalamus, while a second population remains in the olfactory region (80, 81, 92, 95). GnRH receptors and some isolated GnRH neurons were also observed in cerebral cortex in humans and rats (31, 76, 97). Interestingly, increases of GnRH levels were detected in the cortex when migration of GnRH neurons toward their natural location was impaired, as in mice overexpressing GAD-67 in GnRH neurons (43) or in netrin 1 knockout mice (81). GABA$_B$Rs are expressed in GnRH neurons early in development; they were demonstrated in several immortalized GnRH neuron cell lines (30, 63, 91), in green fluorescence protein-expressing embryonic GnRH neurons (26), and in situ hypothalamic neurons (86). Intriguingly, GABA$_B$Rs were recently shown to control cell migration and cell positioning within the ventromedial nucleus of the hypothalamus (64) and in the POA-AH (99) in mice. In addition, GABA$_B$Rs may also modulate cellular proliferation and differentiation of neural progenitor cells in mice (27).

The lack of functional GABA$_B$Rs expression may influence proliferation, positioning, and or migration of GnRH neurons, as already proposed for GABA$_A$ receptors (11). In this study, patterns of GnRH expression and content were observed in the different brain areas analyzed in mice lacking a functional GABA$_B$ receptor.

In whole hypothalami GnRH, peptide content was higher in males than in females, as previously described in rats (15), and this sex difference is maintained in KO mice. GnRH levels were reduced in GABA$_{B1}$KO mice of both sexes but especially pronounced in females, where a 60% decrease was observed. Regarding GnRH mRNA expression, in the MBH-PH, sex differences were observed, with increased expression in females. Moreover, expression was augmented in GABA$_{B1}$KO MBH-PH of both sexes. In contrast, POA-AH GnRH expression was fourfold higher in WT males than in WT females, and this sex difference was reversed in GABA$_{B1}$KO mice, indicating that the absence of functional GABA$_B$ receptors critically alters the sexual differentiation of this typically dimorphic area, which determines cyclical activity in females (25). These results are in line with observations from Wolfe et al. (99) showing the participation of GABA$_B$ receptors in inducing the sexually dimorphic localization of cell populations in the POA-AH. In addition, hyperprolactinemia is known to inhibit...
GnRH synthesis and secretion (37); we have previously observed hyperprolactinemia in GABA_B1KO male mice (14). This characteristic also may have contributed to the lower GnRH mRNA expression observed in the POA-AH of GABA_B1KO males. Our results in adult POA-AH GnRH expression differ from those of Gore et al. (36), because they report higher levels in female than in male mice; nuclei were included in the area analyzed, so the mouse strains and detection methods employed may account for this discrepancy. In general, it seems that GnRH mRNA expression in POA-AH, where most GnRH cell bodies are located (36), bears more weight on GnRH peptide content in whole hypothalami, because the sex difference in WTs is observed in both, but not in MBH-PH GnRH mRNA expression. On the other hand, it is interesting to note that GnRH mRNA expression is increased in GABA_B1KO females in both hypothalamic areas, although GABA_B1KO females show the lowest GnRH peptide content. In this case, the low content may be explained by increased GnRH secretion, because GABA_B1KO females show an increase in GnRH pulse frequency (discussed below). Alternatively, it has been demonstrated that GnRH synthesis can be controlled at multiple levels, including transcriptional, posttranscriptional, translational, and posttranslation sites of regulation (35, 78), and any of these may be altered in GABA_B1KO females. Future studies on GnRH cell migration in both sexes and genotypes are needed to help determine whether differences in GnRH content and expression are due to GABABR regulation of the migration process, affecting cell number and localization.

GnRH neurons in the OB have been shown to participate in olfaction in land vertebrates and mammals such as the prairie vole (72, 98), as well as in lordosis behavior in mice and rats (34, 49). In addition, the presence of GABA_ARs in neurons in the OB also has been demonstrated in rats (47). Interestingly, similar to the hypothalamus, GnRH content in OB was markedly decreased in adult female GABA_B1KOs but not in males. In contrast to hypothalamus, no significant sex differences in OB GnRH contents were observed. In addition, GnRH mRNA expression was similar in both sexes and genotypes in OB, again pointing to the importance of multiple sites of regulation in GnRH synthesis and stability of the mRNA. Although GnRH neurons in the OB share their embryological origin with hypothalamic GnRH neurons, they do not show the sexually dimorphic GnRH expression typical of the POA-AH; in addition, the lack of functional GABA_ARs only affects GnRH content in females in this area.

In cortex, GnRH mRNA levels did not vary with sex or genotype. Very low levels of GnRH peptide were detected, as expected, without differences due to genotype.

These results show that the lack of functional GABA_ARs impact GnRH expression with area specificity; the most important alteration is the loss and inversion of the sexual dimorphic expression in the POA-AH, which could be causally

Fig. 7. Glutamic acid decarboxylase-67 (GAD-67) mRNA expression determined by qRT-PCR in MBH-PH (A), POA-AH (B), and CT (C) from male and female WT and GABA_B1KO mice. A: 2-way ANOVA: factors genotype and sex, interaction: NS, main effect sex: NS, main effect genotype: P < 0.001. *KOs significantly different from WTs. Number of animals: 6 WT males, 6 KO males, 7 WT females, and 5 KO females. B: 2-way ANOVA: factors genotype and sex, interaction: P < 0.001. *WT females significantly different from WT males (P < 0.01) and KO females (P < 0.05). Number of animals: 6 WT males, 6 KO males, 8 WT females, and 5 KO females. C: 2-way ANOVA: factors genotype and sex, interaction: P < 0.02. *WT females significantly different from WT males (P < 0.05). Number of animals: 8 WT males, 6 KO males, 6 WT females, and 5 KO females.
linked to the impaired reproductive parameters observed in females.

**GnRH Pulsatility**

To further analyze GnRH physiology in GABA<sub>B1</sub> KO mice, we measured pulsatile GnRH release from whole hypothalami. GABA<sub>B1</sub> KO females exhibited increased GnRH pulse frequency, with an altered distribution of interpeak intervals, as also described in mice bearing GAD overexpression in GnRH neurons (43). This increased pulsatility may account for the decrease in GnRH content observed in hypothalamus of GABA<sub>B1</sub> KO females, as stated above. Furthermore, the increase in GnRH pulsatility also may be related to the reproductive disorders observed in GABA<sub>B1</sub> KO females (14). Increased GnRH pulsatility has been described in the polycystic ovarian syndrome (60) or experimental rat models of this pathology (23), in which estrus persistence is also a characteristic. In GABA<sub>B1</sub> KO males, pulsatile secretion was not altered but the mean secretion pulse mass was significantly decreased, correlating with the lower hypothalamic GnRH content and the lower GnRH mRNA expression in the POA-AH area determined in GABA<sub>B1</sub> KO males, even though the arcuate nucleus in the MBH is the main area involved in controlling pulsatility (50).

**Neurotransmitter Content and GAD-67 Expression**

GnRH pulsatility is partly intrinsic to GnRH neurons (20, 89) but is also influenced by various neurotransmitters (88, 89). We investigated amino acidic neurotransmitters relevant in GnRH regulation, such as GABA (16, 17, 39, 61, 63) and glutamate, the precursor of GABA biosynthesis (17, 58). In adult WT mice, GABA, glutamate, and taurine titers show levels higher than those of females, as previously shown for GABA in rats (38, 84). Importantly, this sex difference is lost in GABA<sub>B1</sub> KO mice for both GABA and glutamate. In addition, GABA<sub>B1</sub> KO females showed a significant increase in GABA and glutamate levels with regard to their WT controls. Moreover, GAD-67 mRNA also shows a clear sexually dimorphic expression in the POA-AH of WT mice, as previously described in other brain areas (84). This sex difference is also lost in GABA<sub>B1</sub> KO animals; in fact, a sexual inversion in GAD-67 mRNA expression is observed in this genotype, identical to the one observed for GnRH mRNA in the POA-AH. Sexually dimorphic GAD expression, and therefore GABA levels, in the neonatal period is crucial for the development of sexually dimorphic reproductive characteristics (82); recently, it was demonstrated that neonatal gluatamate is decisive for the defeminization process in the male (83). Whether the alteration in GAD expression and neurotransmitter levels we observed in the present work are also found at neonatal stages of development is still unknown. In addition, whether it is the increase in GAD-67 expression and GABA and glutamate contents in GABA<sub>B1</sub> KO females that in turn alters GnRH expression, as previously suggested (46, 52), or whether these are parallel phenomena remains to be determined. Also, it has to be taken into account that an increase in GABA and glutamate contents does not necessarily mean an increase in neurotransmitter release. However, these results demonstrate that the lack of functional GABA<sub>B1</sub>Rs profoundly affects sexual differentiation of the POA-AH, an effect previ-
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...ously mainly attributed to GABA_A receptors. Our results suggesting the participation of GABAB1Rs in determining sexual dimorphic gene expression of specific areas of the brain are in agreement with recent work demonstrating that GABAB1Rs control cell migration and cell positioning within the POA-AH (99) and the ventromedial nucleus of the hypothalamus (64), also a sexually dimorphic nucleus. This pattern of GABA, glutamate, and GAD-67 expression varying with sex and genotype is specific of the POA-AH, because it is not observed in the MBH-PH or in cortex. Both GABA and glutamate are intrinsically related to GnRH secretion (17, 71, 77). GnRH output is thought to be, to a great extent, the balance between glutamate and GABA inputs (73). Although GABA has been shown to inhibit GnRH and gonadotropins secretion, lately it has been proposed that the direct effect of GABA on GnRH neurons is stimulatory even in adulthood in mice (43, 68). Therefore, the increase in GnRH pulse frequency observed in GABAB1KO adult females may reflect the increase in GABA and glutamate in the hypothalamus. Interestingly, Heger et al. (43) have shown that overexpression of GAD-67 in GnRH neurons, which increased GABA levels in the hypothalamus, also increased GnRH pulsatility in young female mice, as well as altering cyclicity and fertility, as we also have described in GABAB1KO females (14).

Other critical players in sexual dimorphism and regulation of the gonadotropic axis are gonadal steroids. Testosterone, through its aromatization to estradiol, is fundamental in determining sex differences in the developing brain, and one of the molecules considered to be a major effector of hormone action is GnRH, acting through both GABA_A and GABA_B receptors (90), as commented above. In addition, one of the key mediators of the estradiol positive and negative feedback regulation of GnRH neurons in females is GABA (66). Although serum steroid (estradiol, testosterone, and progesterone) levels were not determined in the present work, we have previously shown that gonad steroid contents are not modified due to genotype in females (14). This does not preclude the possibility that either serum or central nervous system estrogen levels may be altered in GABAB1KO mice and participate in establishing the differences in GnRH expression and content, GABA and glutamate levels, and GAD expression. Pituitary Gonadotropin Content and LH and FSH Serum Levels

As shown above, GnRH pulsatility was increased in GABAB1KO females, and GnRH pulse frequency is critical for pituitary physiology (5, 96). We therefore analyzed gonadotropin levels in both sexes and genotypes. Neither LH and FSH pituitary contents nor basal serum levels showed genotype differences; titers were higher in males than in females. Nevertheless, a possible alteration in LH pulsatile secretion in GABAB1KO females cannot be discarded by the present results. To further evaluate the participation of the pituitary in the alterations of...
the gonadotropic axis determined in GABA_B1KO mice, LH and FSH secretion were assessed in primary cultures of adeno-hypophyseal cells, a situation in which cells are deprived from the regulatory inputs from the gonads and the brain. The most relevant result from this experimental design was the demonstration that anterior pituitary cells from female GABA_B1KO mice have an increased basal secretion of gonadotropins and an impaired response to GnRH. We have previously demonstrated that GABA_BR activation inhibits gonadotropins secretion acting directly on pituitary cells by blocking voltage-gated calcium channels (54); therefore, the increased basal gonadotropin secretion demonstrated in the present study would be in agreement with our previous results. The increase in basal secretion in GABA_B1KO female cells may be the cause of the decreased response to GnRH, because fewer gonadotropin-containing granules may be stored and ready to respond to a stimulatory input in these cells, as suggested in other similar situations (33). Whether this alteration of in vitro gonadotrop physiology is solely a consequence of lack of local GABA_BR expression or also a consequence of increased in vivo GnRH pulsatility remains to be determined. In any case, it may also contribute, to some extent, to the dysfunction of the gonadotropic axis observed in GABA_B1KO female mice.

Together, our results demonstrate that the absence of functional GABA_BRs alters the sexually dimorphic expression pattern of critical genes, such as GnRH or GAD-67, in key areas involved in specific reproductive functions of the adult brain, such as the POA-AH. In addition, lack of GABA_BRs profoundly affects GnRH physiology, especially in the female. GABA_B1KO females show increased GnRH pulsatile secretion and increased GABA and glutamate contents, as well as modified gonadotrope physiology. All these features contribute to the estrous cyclicity disruption and fertility impairments previously observed in GABA_B1KO female mice and also reveal the importance of GABA_B receptors in the development and regulation of the gonadotropic axis.

GRANTS

This work was supported by Consejo Nacional de Investigaciones Científicas y Técnicas, Argentina Grant PIP 5540, Agencia Nacional de Promoción Científica y Tecnológica, Argentina Grants ME 048 and 038, and Swiss Science Foundation Grant 3100A0-117816 (to B. Bettler).

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