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$GABA_{B1}$ Knockout Mice Reveal Alterations in Prolactin Levels, Gonadotropic Axis, and Reproductive Function

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Key Words

γ-Aminobutyric acid · γ-Aminobutyric acid receptors · Gonadotropins · Prolactin · Sex behavior · Puberty · Ovariectomy

Abstract

γ-Aminobutyric acid (GABA) has been implicated in the control of hypophyseal functions. We evaluated whether the constitutive loss of functional $GABA_B$ receptors in $GABA_{B1}$ knockout ($GABA_{B1}^{-/-}$) mice alters hormonal levels, under basal and stimulated conditions, and reproductive function. The serum hormone levels were measured by radioimmunoassay, the estrous cyclicity was evaluated by vaginal lavages, and the mating behavior was determined by the presence of vaginal plugs. A moderate hyperprolactinemic condition was observed, in which prolactin increase and thyroid-stimulating hormone decrease were similar between genotypes. Basal luteinizing hormone (LH), follicle-stimulating hormone, thyroid-stimulating hormone, and growth hormone levels were similar between genotypes in each sex. Analysis of the gonadotropin axis revealed no differences in puberty onset between female genotypes. In contrast, the estrous cyclicity was significantly disrupted in $GABA_{B1}^{-/-}$ female mice, showing significantly extended periods in estrus and shortened periods in proestrus. Reproduction was significantly compromised in $GABA_{B1}^{-/-}$ females, with a significantly lower proportion of mice (37.5%) getting pregnant during the first 30 days of mating as compared with wild-type controls (87.5%). Moreover, only 14% of vaginal plug positive $GABA_{B1}^{-/-}$ females had successful pregnancies as compared with 75% in the controls. In addition, the postovariectomy LH rise was significantly advanced in $GABA_{B1}^{-/-}$ mice, while the response to estradiol feedback was similar in both genotypes. In conclusion, our endocrine analysis of $GABA_{B1}^{-/-}$ mice reveals that $GABA_B$ receptors are involved in the regulation of basal prolactin titers. Moreover, the hypothalamic-hypophyseal-ovarian axis is seriously disturbed, with alterations in cyclicity, postcastration LH increase, and fertility indexes. The molecular mechanism underlying these hormonal disturbances remains to be addressed.

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Introduction

γ-Aminobutyric acid (GABA) is the main inhibitory neurotransmitter in the central nervous system (CNS). GABA participates in the control of hypophyseal secretion, by influencing hypothalamic neural circuits and by a direct action on the pituitary [1, 2]. Pituitary GABA

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derives from tuberoinfundibular GABA, neurointermediate pituitary lobe GABAergic axons, other hypothalamic GABAergic systems, and local synthesis, since the expression of glutamic acid decarboxylase (GAD), its biosynthetic enzyme, was demonstrated in the pituitary [3, 4]. GABA acts on ionotropic $GABA_{A/C}$ receptors and metabotropic $GABA_B$ receptors ($GABA_BRs$). The latter were originally described by Bowery et al. [5] over two decades ago. We and others demonstrated the participation of $GABA_BRs$ in the regulation of prolactin (PRL) and gonadotropin secretion in developing and adult rats [6–15]. The presence of $GABA_BRs$ in somatotropes has also been described [4].

It has been demonstrated [16, 17] that $GABA_BRs$ are G-protein-coupled receptors, in which heterodimeric assembly of a $GABA_{B1}$ with a $GABA_{B2}$ subunit confers functionality. Results from our laboratory [18, 19] established that $GABA_BR$ subunits have particular ontogenic expression patterns in hypothalamus and pituitary, decreasing throughout postnatal development in both sexes and being more abundant in female than in male infantile rats. This dimorphic sexual expression pattern in the pituitary is the consequence of early testosterone imprinting in males [20]. In addition, we have demonstrated [21] that pituitary $GABA_BRs$ are coupled Gi/0-type G-proteins and that their activation initiates the same intracellular signaling pathways as in the CNS.

Recently, two strains of mice deficient in either the $GABA_{B1}$ subunit [22] or the $GABA_{B2}$ subunit [23] were developed, both of which suffer from spontaneous seizures, hyperalgesia, hyperlocomotor activity, and severe memory impairment. This demonstrates that most $GABA_B$ functions depend on heterodimerization between the $GABA_{B1}$ and $GABA_{B2}$ subunits. However, $GABA_{B2}^{-/-}$ mice, but not $GABA_{B1}^{-/-}$ animals, still exhibit atypical electrophysiological $GABA_B$ responses. This indicates that in vivo $GABA_{B1}$, but not $GABA_{B2}$, can be functional in the absence of the partner subunit [23].

In the past, studies addressing the physiological roles of $GABA_BRs$ in hypophyseal regulation relied on the use of selective agonists and antagonists. The aim of the present work was to analyze the endocrine consequences of a constitutive loss of $GABA_B$ responses in $GABA_{B1}^{-/-}$ mice, with emphasis on the hypothalamic-pituitary-gonadal unit.

Materials and Methods

Animals

$GABA_{B1}^{-/-}$ mice [22] generated in the BALB/C inbred strain were obtained by intercrossing heterozygous animals. DNA isolated from fingertips biopsies (performed for identification purposes) was genotyped by polymerase chain reaction analysis, as described [22]. The 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 NIH.

Female and male mice from both genotypes were weighed from birth to adulthood every 5 days until 60 days of age and every 10 days thereafter (90 days); 6–15 animals per group were used.

Anterior Pituitary Function Evaluation

Several groups of adult mice from both sexes and genotypes were sacrificed by decapitation between 09.00 and 11.00 h. Pituitaries were dissected and weighed; trunk blood samples were collected to determine serum PRL, luteinizing hormone (LH), follicle-stimulating hormone (FSH), thyroid-stimulating hormone (TSH), and growth hormone (GH) levels by radioimmunoassay (RIA). Number of animals per group: 6–12. Ovaries and testes were also dissected and collected for other determinations (see below).

PRL and TSH Responses to Immobilization Stress

Adult male mice from both genotypes were sacrificed by decapitation under basal conditions (control group) or submitted to 15-min immobilization stress and sacrificed thereafter by decapitation. Trunk blood samples were collected to determine serum PRL and TSH levels by RIA. Number of animals per group: 7–8.

Hypothalamic-Pituitary-Gonadal Axis Evaluation in Female Wild-Type and $GABA_{B1}^{-/-}$ Mice

Puberty Onset and Cyclicity. Female knockout and wild-type mice were checked for vaginal opening, as an index of puberty onset, from day 16 of life onwards, and they were weighed on the day of vaginal opening. Starting at 60 days of age, vaginal lavages were obtained daily for assessment of the estrous cyclicity for a 50-day period. Number of animals per group: 18–22.

Reproductive Function. Female, virgin adult mice of both genotypes were mated with males of known fertility. One wild-type and 1 knockout female were put into a cage with a heterozygous male. The percentage of female mice pregnant after 30 days, the days to first delivery, and the number of pups/litter were recorded. Number of animals per group: 8. In a second set of wild-type and knockout virgin females, estrous cycles were recorded, and the mice were mated on the day of proestrus with a male of known fertility. The next morning, the animals were checked for the presence of a vaginal plug. If present, this was designated day 1 of pregnancy. The percentage of successful pregnancies reaching parturition was determined. Number of animals per group: 7–8.

LH and FSH Responses to Withdrawal and Reinstatement of the Estradiol Feedback Action. Female knockout and wild-type adult mice were anesthetized between 09.00 and 11.00 h with ketamine/xylazine, jugular blood samples for basal serum LH and FSH determinations were taken, and they were then ovariectomized (OVX). Thereafter, 1-cm Silastic capsules (0.04 in inner diameter, 0.085 in outer diameter; Dow Corning, Midland, Mich., USA) containing 2.5 μg 17β-estradiol (Sigma-Aldrich, St. Louis, Mo., USA)

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mixed into silicone type A medical adhesive were placed subcutaneously. Controls were implanted with capsules containing no estradiol [24]. One ovary from each mouse was dissected and weighed (n = 12–16). At 19.00 h of the 7th day after OVX, the mice were sacrificed by decapitation, and trunk blood samples were collected for determination of serum LH and FSH levels by RIA. Number of animals per group: 6–8.

Participation of $GABA_BRs$ in the Postcastration Rise in Gonadotropins in Female and Male Wild-Type and Knockout Mice

Female and male mice from both genotypes were castrated between 09.00 and 11.00 h under ketamine/xylazine anesthesia. One ovary or testicle from each mouse was dissected and weighed. Jugular blood samples were collected on the day prior to castration (day 0) and on days 1, 3, 4, 5, and 7 after castration under anesthesia. On day 9 after castration, the mice were sacrificed by decapitation, trunk blood was collected, and serum LH and FSH levels were determined. Number of animals per group: 8–15 females and 6–9 males.

Gonadal Parameters in Wild-Type and $GABA_{B1}^{-/-}$ Mice

Testes and ovaries from mice from both genotypes were weighed and homogenized to determine testosterone, progesterone, and estradiol contents by RIA after ethyl ether extraction. Steroid hormone levels were expressed relative to protein (μg) measured by Lowry's method in aliquots of tissue homogenates. Number of animals per group: 13–20.

Radioimmunoassays

Mouse serum LH, FSH, PRL, TSH, and GH were analyzed by RIA using kits obtained through the National Hormone and Peptide Program (Dr. A.F. Parlow, Harbor-UCLA Medical Center, Torrance, Calif., USA). LH and FSH were determined with the rat kits, and the results were expressed in terms of RP-3 and RP-2 rat LH and FSH standards, respectively. PRL, TSH, and GH were determined using the mouse kits, and results were expressed in terms of mPRL-AFP-6476C, mTSH/LH-AFP51718MP, and mGH-AFP-10783B standards, respectively. Assay sensitivities, calculated as the dilution factor of samples multiplied by the lowest dose of the standard curve, were 0.18 ng/ml for LH, 2.14 ng/ml for FSH, 0.95 ng/ml for PRL, 9.8 ng/ml for TSH, and 0.66 ng/ml for GH. Intra- and interassay coefficients of variation were for LH 7.2 and 11.4%, for FSH 8.0 and 13.2%, for PRL 7.8 and 12.4%, for TSH 8.3 and 13.5%, and for GH 8.4 and 13.3%, respectively.

Testosterone, estradiol, and progesterone testicular contents were determined by RIA using specific antisera kindly provided by Dr. G.D. Niswender (Colorado State University, Fort Collins, Colo., USA) after ethyl ether extraction [20]. Labeled hormones were purchased from PerkinElmer (Wellesley, Mass., USA). Assay sensitivities were for testosterone 125 pg, for estradiol 11.3 pg, and for progesterone 500 pg. Intra- and interassay coefficients of variation were 7.8 and 12.3% for testosterone, 6.8 and 11.7% for estradiol, and 7.1 and 12.15% for progesterone, respectively.

Statistics

The differences between mean values of two groups were analyzed by Student's t test. Differences between mean values of more than two groups were analyzed by one-way, two-way, or three-way ANOVA, followed by Tukey's honestly significant difference test for unequal numbers. Percentages were subjected to arcsine transformation before statistical analysis to convert them from a binomial to a normal distribution. Frequency distributions were analyzed using the χ^2 test. Data are presented as mean ± SEM. $p < 0.05$ was considered statistically significant.

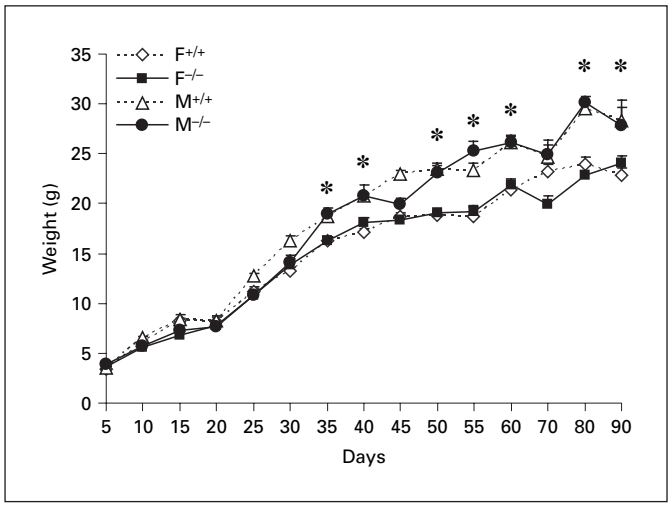

Fig. 1. Body weight from birth to adulthood in female and male wild-type and $GABA_{B1}^{-/-}$ mice. The animals were weighed every 5 days until 60 days of age and every 10 days thereafter. Three-way ANOVA. Main factors genotype, sex, and age. Triple interaction: NS; double interaction genotype and sex: NS; double interaction genotype and age: NS; double interaction sex and age: $p < 0.001$. * Significantly different from females at that age.

Results

Somatic Growth from Birth to Adulthood in Female and Male Wild-Type and $GABA_{B1}^{-/-}$ Mice

The absence of $GABA_{B1}R$ expression did not compromise somatic growth, as body weights did not vary between wild-type and knockout mice from birth to adulthood in either sex (fig. 1). Body weights were significantly higher in males than in females from 35 days onwards.

Anterior Pituitary Function Evaluation

First, we evaluated the pituitary function in adulthood under basal conditions. The pituitary weights were significantly higher in females than in males, without differences between genotypes (table 1). The serum PRL levels were significantly elevated in knockout males with regard to wild-type controls (fig. 2a); females did not show significant differences between genotypes (fig. 2b). Serum

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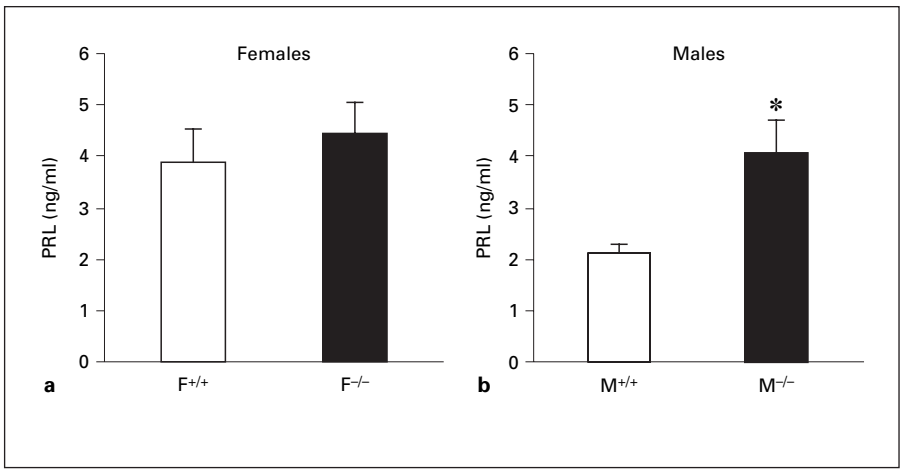

Fig. 2. Basal prolactinemia in female (**a**) and male (**b**) wild-type and $GABA_{B1}^{-/-}$ mice. * $p < 0.01$ as compared with wild-type males.

Table 1. Pituitary weights and serum basal pituitary hormone levels in adult wild-type and $GABA_{B1}^{-/-}$ female and male mice

	Females		Males		p
	$GABA_{B1}^{+/+}$	$GABA_{B1}^{-/-}$	$GABA_{B1}^{+/+}$	$GABA_{B1}^{-/-}$	
Pituitary weight, mg	2.15 ± 0.24 (6)	1.71 ± 0.34 (7)	1.04 ± 0.05 (12)	1.06 ± 0.10 (11)	<0.01
LH, ng/ml	0.21 ± 0.02 (16)	0.21 ± 0.02 (15)	0.75 ± 0.17 (8)	0.81 ± 0.11 (8)	<0.001
FSH, ng/ml	3.69 ± 0.59 (14)	3.34 ± 0.68 (13)	13.30 ± 2.44 (6)	14.57 ± 1.95 (9)	<0.001
TSH, ng/ml	16.96 ± 3.61 (14)	11.65 ± 1.81 (12)	25.12 ± 4.39 (23)	28.14 ± 4.19 (25)	<0.01
GH, ng/ml	6.96 ± 1.59 (14)	6.28 ± 1.91 (12)	11.43 ± 2.70 (29)	11.27 ± 2.76 (32)	NS

Data were analyzed by two-way ANOVA. No differences between genotypes were observed; differences between sexes are shown. Number of animals in parentheses.

LH, FSH, and TSH levels were higher in males than in females, without differences between genotypes (table 1). GH did not show significant differences between sexes or genotypes (table 1).

PRL and TSH Responses to Immobilization Stress

As the basal PRL level was increased in $GABA_{B1}^{-/-}$ males as compared with their wild-type controls, we next evaluated whether the PRL secretion was also altered under stress-stimulated conditions. Both wild-type and knockout mice responded to immobilization stress with an increase in PRL (fig. 3a). The overall PRL levels were higher in $GABA_{B1}^{-/-}$ males when compared to wild-type controls (two-way ANOVA: interaction NS; main effects genotype $p < 0.01$). However, the percent increase above basal levels was similar between genotypes (% increase: wild-type 333.1 ± 107.4 vs. $GABA_{B1}^{-/-}$ 331.1 ± 94.2; n = 3 independent experiments, NS).

TSH was also evaluated under basal and stress conditions in adult males, as immobilization stress has been reported to reduce the TSH levels in rodents [25, 26]. Both genotypes responded to immobilization stress with a similar decrease in TSH levels (fig. 3b).

Hypothalamic-Pituitary-Gonadal Axis Evaluation in Wild-Type and $GABA_{B1}^{-/-}$ Female Mice

Next we evaluated the hypothalamic-pituitary-gonadal axis in female mice by recording parameters of puberty onset, ovarian weight, and steroid contents at adulthood. Neither the day of vaginal opening nor the body weight at the time of vaginal opening differed between wild-type and knockout mice [vaginal opening (days): wild-type 21.9 ± 0.7 (n = 18) vs. knockout 20.8 ± 0.7 (n = 11), NS; body weight at vaginal opening (g): wild-type 11.2 ± 0.4 (n = 18) vs. knockout 10.0 ± 0.8 (n = 11)]. No differences were observed in ovary weight or es-

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tradiol, progesterone, or testosterone contents between genotypes at adulthood (table 2).

In contrast to this lack of $GABA_BR$ participation at onset of puberty or in ovarian parameters, the estrous cyclicity was significantly disrupted in knockout mice. Female $GABA_{B1}^{-/-}$ mice exhibited an extended estrus ($p < 0.001$) and a reduced proestrus period ($p < 0.001$) (fig. 4) as compared with wild-type controls; a decrease in time spent in diestrus did not attain statistical significance ($p < 0.110$). Representative estrous cycle profiles demonstrating these differences are depicted in figure 4. In addition to the alterations in cyclicity, the reproductive function of female $GABA_{B1}^{-/-}$ mice was significantly compromised (fig. 5). The number of mice becoming pregnant within the first 30 days of mating was significantly reduced in knockout animals (% of pregnancies in the first 30 days: wild-type 87.5, n = 8, vs. $GABA_{B1}^{-/-}$ 37.5, n = 8; χ^2 test $p < 0.05$; fig. 5a), while the interval between exposing female mice to a male and delivery of the first litter was lengthened, although this difference in comparison with wild-type mice did not attain statistical significance ($p < 0.11$; fig. 5b). No difference in the number of pups per litter between genotypes was detected (fig. 5c).

In the previous set of experiments, the difference in the pregnancy index could not discriminate between a possible lack of mating behavior, length of time in getting into proestrus, or pregnancy interruption in knockout with regard to wild-type females. Therefore, a new group of females from both genotypes was cycled and, on the day of proestrus, they were mated with a male of known fertility; the next day, the presence of a vaginal plug (index of positive mating behavior) was recorded. No differences between genotypes were observed, as 100% of the mice which mated on proestrus showed the vaginal plug (fig. 6). Nevertheless, a significant difference was observed when successful pregnancies were evaluated, as 6

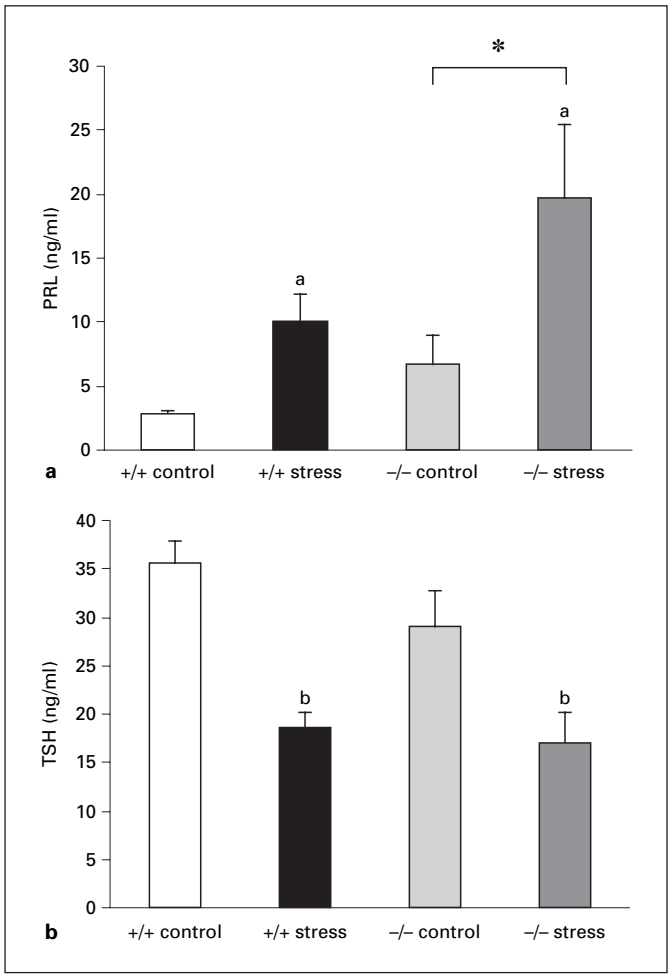

Fig. 3. PRL (**a**) and TSH (**b**) responses to immobilization stress in male wild-type and $GABA_{B1}^{-/-}$ mice. PRL: two-way ANOVA. Main factors genotype and treatment. Double interaction: NS; main effect genotype: $p < 00.1$, * significantly different from wild-type mice; main effect treatment: $p < 0.001$, [a] significantly different from basal levels. TSH: two-way ANOVA. Main factors genotype and treatment. Double interaction: NS; main effect genotype: NS; main effect treatment: $p < 0.001$, [b] significantly different from basal levels.

Table 2. Ovary weights and estradiol, progesterone, and testosterone contents in adult wild-type and $GABA_{B1}^{-/-}$ females

Ovaries	Ovary weight mg	Estradiol pg/μg protein	Progesterone pg/μg protein	Testosterone pg/μg protein
$GABA_B^{+/+}$	5.2 ± 0.4 (20)	0.019 ± 0.003 (16)	36.9 ± 6.6 (16)	0.106 ± 0.019 (16)
$GABA_B^{-/-}$	4.6 ± 0.4 (21)	0.018 ± 0.004 (17)	42.5 ± 5.0 (20)	0.132 ± 0.020 (16)

Data were analyzed by Student's t test. No differences between genotypes were observed. Number of animals in parentheses.

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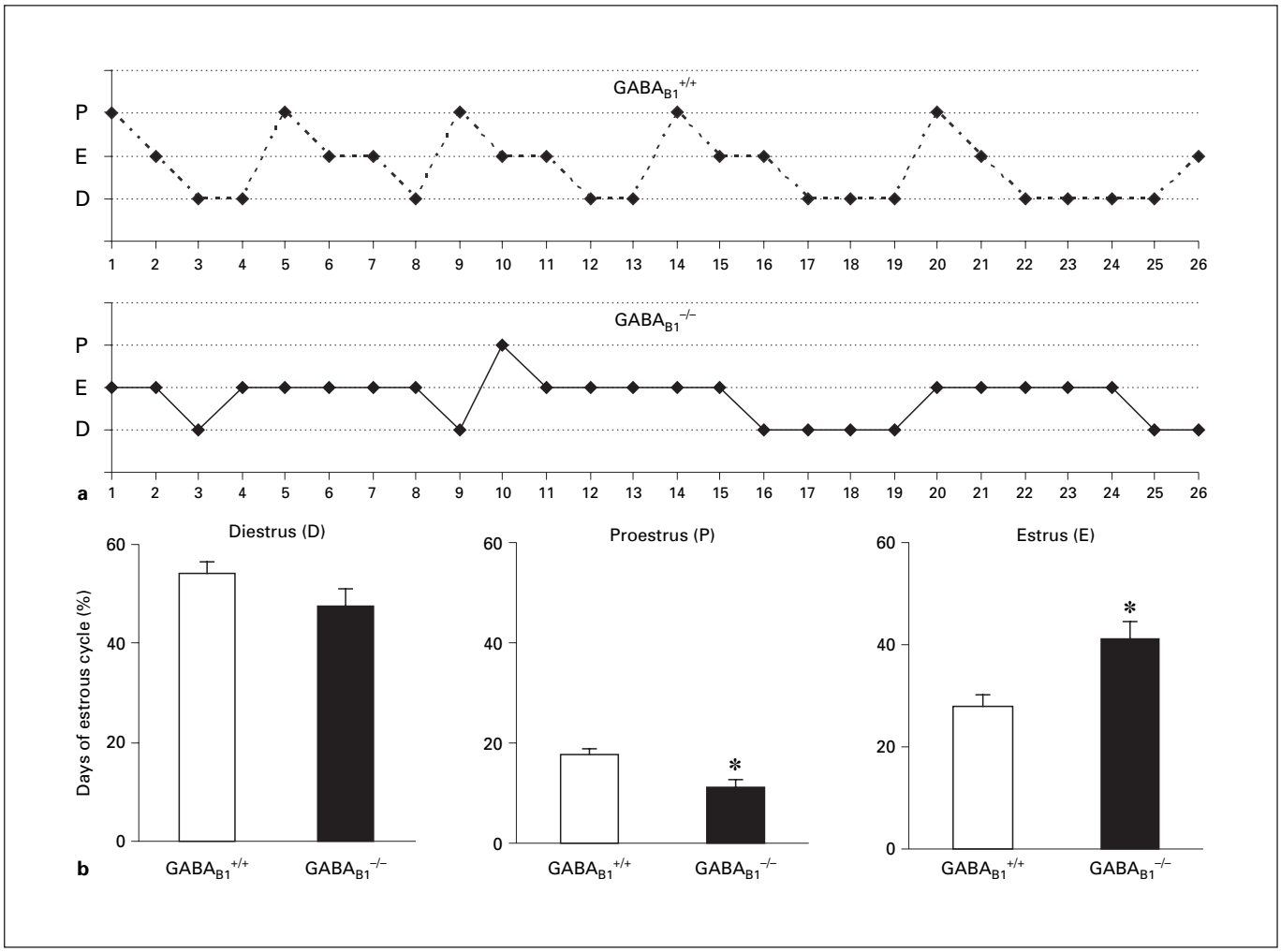

Fig. 4. a Representative profiles illustrating the disruption of estrous cyclicity in $GABA_{B1}^{-/-}$ mice as compared with wild-type controls. **b** Days in each phase of the estrous cycle in wild-type and $GABA_{B1}^{-/-}$ mice. Days of estrous cycle: Student's t test. * $p < 0.001$: significantly different from wild-type mice.

of 8 plug-positive wild-type mice reached parturition, while only 1 of 7 did so in knockout females (χ^2 test: $p < 0.03$).

As cyclicity and reproductive function were altered in female $GABA_{B1}^{-/-}$ mice, we next evaluated the functionality of the gonadotropin axis in females. We determined LH and FSH levels in female mice under basal conditions, 7 days after OVX, and after reinstatement of the estradiol-negative feedback action by estradiol administration (Silastic capsule containing 2.5 μg 17β-estradiol; OVX-E_2). No differences were observed between genotypes [LH (ng/ml): wild-type basal 0.31 ± 0.06, OVX 4.54 ± 1.06, and OVX-E_2 0.87 ± 0.21 (n = 6) vs. knockout basal 0.22 ± 0.03, OVX 4.02 ± 0.59, and OVX-E_2 0.55 ± 0.13 (n = 5); two-way ANOVA: interaction NS, main effect genotype NS, main effect treatment $p < 0.001$]. FSH showed a similar response (not shown).

Participation of $GABA_B$Rs in the Postcastration Rise in Gonadotropins in Wild-Type and Knockout Mice of Both Sexes

GABA, through activation of $GABA_A$ and $GABA_B$ receptors, has been proposed to participate in the postcastration delay in the rise of LH in female rodents as opposed to males [27]. We evaluated the gonadotropin level increase in mice from both sexes and both genotypes the first 9 days after castration. The LH rise was significantly advanced in female $GABA_{B1}^{-/-}$ mice when com-

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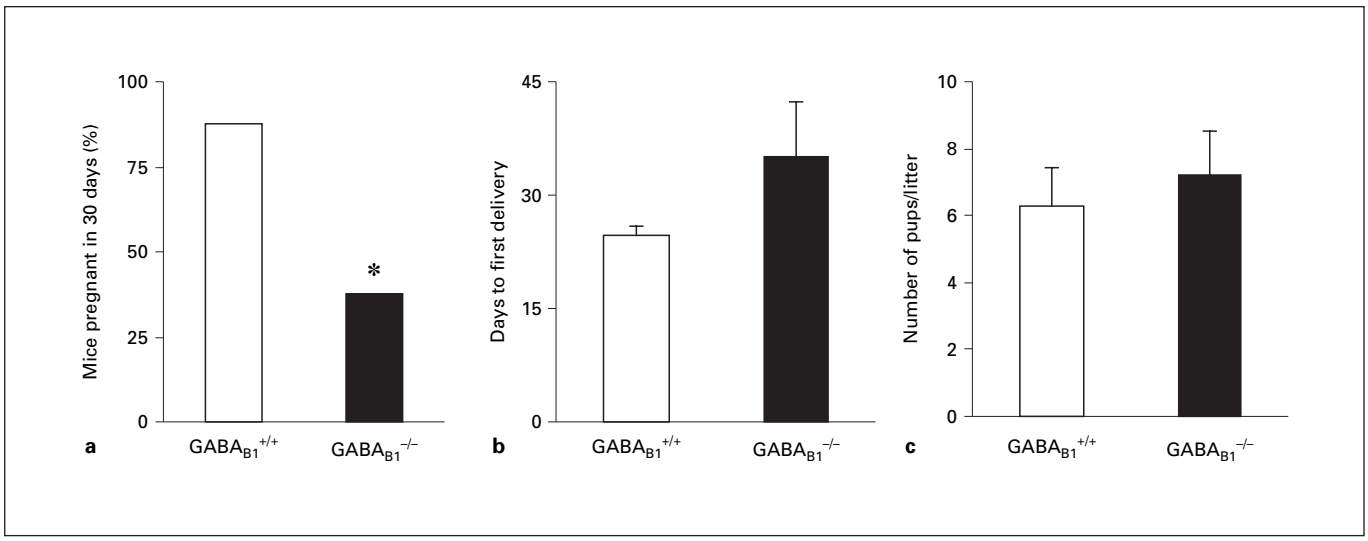

Fig. 5. Reproductive function parameters in wild-type and $GABA_{B1}^{-/-}$ female mice. **a** Mice pregnant in each genotype after 30 days of mating. Data were analyzed by the χ^2 test. * Significantly different from wild-type mice ($p < 0.05$). **b** Days from mating to first delivery. Student t test: NS. **c** Number of pups per litter in each genotype. Student t test: NS.

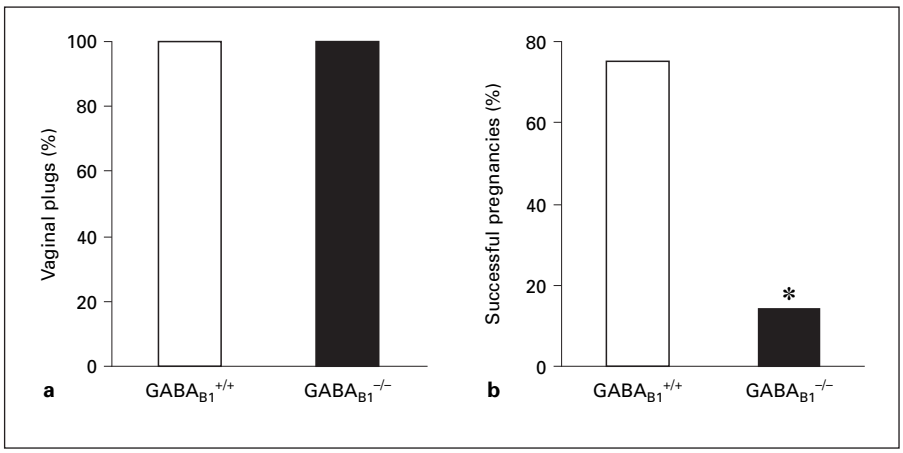

Fig. 6. Percentages of vaginal plug presence (**a**) and successful pregnancies (**b**) in wild-type and $GABA_{B1}^{-/-}$ female mice mated on proestrus. Data were analyzed by the χ^2 test. * Significantly different from wild-type mice ($p < 0.03$).

pared to wild-type controls, without significant differences between male genotypes (fig. 7a, b). No differences in the postcastration rise in FSH were observed between genotypes of either sex (fig. 7c, d).

Testicular Parameters in Wild-Type and $GABA_{B1}^{-/-}$ Mice

Since the testis expresses the GABA biosynthetic enzyme GAD, as well as $GABA_A$ and $GABA_B$ receptors, and since a physiological role for GABA in Leydig cell function has been proposed [28], testis weight and testis steroid hormone contents were determined in adult wild-type and knockout mice. No significant differences in these parameters were observed between genotypes [testis weight (mg): wild-type 0.136 ± 0.006 (n = 23) vs. knockout 0.126 ± 0.004 (n = 21), NS; testosterone (pg/μg protein): wild-type 0.40 ± 0.06 (n = 14) vs. knockout 0.68 ± 0.15 (n = 14), NS; estradiol (pg/μg protein): wild-type 0.005 ± 0.001 (n = 12) vs. knockout 0.004 ± 0.001 (n = 13), NS; progesterone (pg/μg protein): wild-type 0.99 ± 0.17 (n = 16) vs. knockout 0.78 ± 0.12 (n = 17), NS].

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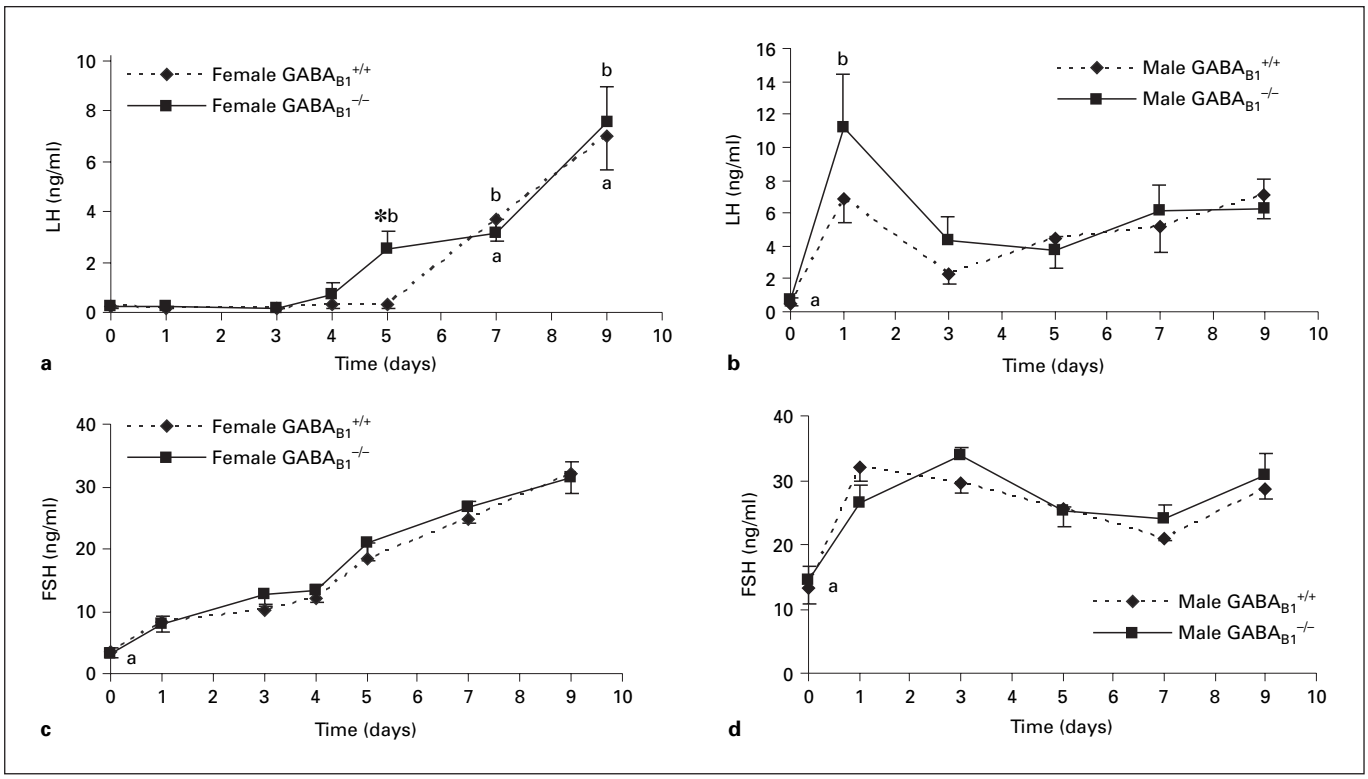

Fig. 7. Postcastration serum LH (**a**, **b**) and FSH (**c**, **d**) levels in adult female and male wild-type and $GABA_{B1}^{-/-}$ mice, evaluated over 9 days after gonadectomy. Data were analyzed by two-way ANOVA. Main effects: genotype and days. LH in females, interaction: $p < 0.001$, * significantly different from wild-type mice at that date, [a] significantly different from initial levels in $GABA_{B1}^{+/+}$ mice, [b] significantly different from initial levels in $GABA_{B1}^{-/-}$ mice. FSH in females, interaction: NS, main effect genotype: NS, main effect days: $p < 0.01$, [a] significantly different from all other dates. LH in males, interaction: NS, main effect genotype: NS, main effect days: $p < 0.001$, [a] days 1, 3, 5, 7, and 9 significantly different from day 0 ($p < 0.001$ or less), [b] day 1 levels significantly different from day 3 ($p < 0.004$). FSH in males, interaction: NS, main effect genotype: NS, main effect days: $p < 0.001$, [a] significantly different from all other dates.

Discussion

Constitutive gene ablation has become a key technique to study role and regulation of genes in the context of the intact animal, without the need of pharmacological agents which, in addition to their proposed actions, may present lack of cell specificity, toxicity, and undesirable nonspecific effects.

This is the first study addressing the role of $GABA_BRs$ in neuroendocrine functions in constitutive $GABA_{B1}^{-/-}$ mice which completely lack functional $GABA_BRs$. The results demonstrate altered PRL levels in males and compromise of the gonadotropin axis and reproduction impairment in females and thus support previous reports that GABA also regulates the hypothalamic-pituitary unit through $GABA_BRs$.

During the CNS development, early activation of $GABA_A$ and $GABA_B$ receptors appears to account for the regulation of proliferation, migration, and differentiation of cells, prior to their roles in neurotransmission [29, 30]. $GABA_BRs$ are already expressed in the pituitary early in development [18]; nevertheless, their absence does not seem to seriously influence pituitary development, as the gland weight is normal in adulthood. The sex difference observed in pituitary weight is in agreement with previous data [31].

We have previously shown that $GABA_BRs$ are implicated in the control of PRL secretion, acting both at the CNS level and directly in the pituitary. In vivo, $GABA_BR$ stimulation resulted in inhibition of PRL secretion under stimulated conditions, such as stress or suckling, in rats [6, 32, 33], with no significant effect on basal PRL titers. In vitro stimulation of $GABA_BRs$ in anterior pituitary

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cultures inhibited PRL secretion, both under basal and stimulated conditions, and this was reverted by specific antagonists [7]. On the other hand, the stimulatory effects of baclofen, a $GABA_B$ agonist [5], on PRL release were demonstrated in prepubertal rats, acting probably at the CNS [9, 10]. When evaluating basal PRL levels in $GABA_B^{-/-}$ mice, we observed a moderate hyperprolactinemic condition only in males. The more robust dopaminergic PRL-inhibitory tone characteristic of females [31, 34] may mask this response in PRL titers in knockout females. Although the PRL levels were higher in $GABA_B^{-/-}$ males than in controls, absence of $GABA_B$Rs did not modify the hyperprolactinemic response to immobilization stress, as the percent increase was similar for both genotypes. Results in $GABA_B^{-/-}$ mice confirm that $GABA_B$Rs are involved in the regulation of PRL secretion and point to a contribution in the control of basal secretion. As compared with the previous results obtained by pharmacological manipulation, our new findings are best explained by assuming that baclofen administration does not increase an already tonic inhibition of basal PRL by GABA through $GABA_B$Rs. On the other hand, the inhibitory effect of baclofen on stress-induced PRL secretion observed previously may have been a pharmacologic effect, as $GABA_B$Rs do not appear to be involved in the regulation of PRL release under stress in $GABA_B^{-/-}$ mice.

GABA has also been involved in the regulation of TSH secretion in mice and rats, mainly through activating $GABA_{A/C}$Rs [35–37]. We have previously reported that a suckling-induced TSH increase is abolished by baclofen administration in the rat [6], suggesting a participation of $GABA_B$Rs in TSH regulation. In addition, stress was shown to influence the TSH secretion, increasing or decreasing its levels, depending on the stressor used [25, 26]. We, therefore, evaluated basal TSH levels and their response to immobilization stress in null mutant and wild-type mice. Basal titers were similar in knockout and wild-type animals of both sexes, being higher in males than in females. Immobilization induced a similar decrease in TSH in both male genotypes. Taken together, our results suggest that $GABA_B$Rs are not involved in the control of either basal or the stress-modified TSH levels, in agreement with previous reports [8, 9].

Although stimulation of the GH secretion by baclofen has been well characterized in healthy humans and in psychiatric patients [38], little is known about $GABA_B$R regulation of the GH secretion in rodents. The presence of $GABA_B$Rs has been demonstrated in rat somatotropes and GH3 cells [4]; in addition, in GH3 cells, baclofen stimulated the GH secretion [4]. Others [39] failed to observe an effect of baclofen on the GH secretion in superfused anterior pituitaries. Here, we observed no differences in basal GH levels between adult wild-type and knockout mice, suggesting that $GABA_B$Rs are not involved in the regulation of the basal somatotropin secretion. In agreement with these results, similar growth curves for both genotypes in each sex were recorded. However, a compensation of possible alterations due to absence of $GABA_B$Rs may take place, as the GH secretion is under a very complex multifactor regulation [40].

Testes express the GABA biosynthetic enzyme GAD [28, 41], $GABA_A$ and $GABA_B$ receptors [28, 42, 43], as well as GABA transporters [28, 44]. Furthermore, a physiological role for GABA in the control of Leydig cell proliferation and steroid synthesis has been proposed [28, 45]. In this context, we studied testis weight and steroid hormone contents in testes of adult knockout and wild-type mice. None of these parameters differed between wild-type and $GABA_{B1}^{-/-}$ males, suggesting that these receptors are not critically involved in growth of testes or their endocrine function.

GABA has been shown to influence the hypothalamic-hypophyseal-ovarian axis through $GABA_A$ and $GABA_B$ receptor activity [11–14, 46–48]. Furthermore, $GABA_B$Rs are expressed in gonadotropin-releasing hormone (GnRH) neurons [49, 50], in the pituitary [7, 18, 20], and in the ovary [16, 51, 52]. When evaluating the hypothalamic-pituitary-ovarian axis in female mice, the parameters of puberty onset did not show significant differences between genotypes. In contrast, the estrous cyclicity was significantly disrupted in knockout mice, presenting longer periods in estrus and shorter periods in proestrus than wild-type controls. In addition, the fertility parameters were also compromised, as the number of $GABA_B^{-/-}$ females getting pregnant within the first 30 days of mating was significantly reduced with regard to wild-type controls. At this point, we could not discriminate between a possible lack of mating behavior, length of time in getting into proestrus, or pregnancy interruption in knockout females with regard to wild-type animals. The second breeding experiment demonstrated that knockout females show a normal mating behavior once they get into proestrus, as 100% showed vaginal plugs the next morning. Nevertheless, in addition to less frequent proestrus occurrence, a difficulty in ovulation, fertilization, and/or pregnancy progression was also evidenced, as only 1 of 7 plug-positive knockout mice delivered pups. Regarding the participation of $GABA_B$Rs in these processes, $GABA_B$Rs are present in the ovary [53, 54], Kaupmann et al. [16]

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described their presence in the rat placenta, and their expression in human, rabbit, and rat oviduct and myometrium has also been demonstrated, participating in the modulation of the contractility in both tissues [55–59]. Therefore, a thorough investigation will be undertaken to determine in which process or processes the absence of $GABA_BRs$ interferes with pregnancy outcome.

As cyclicity and reproductive function were compromised in $GABA_B^{-/-}$ mice, we evaluated the functionality of the gonadotropin axis. It has been proposed that baclofen blocks the estrogen- and progesterone-induced gonadotropin surge in OVX rats [11] while increasing GnRH mRNA in the preoptic/anterior hypothalamic area [47]. Baclofen was also shown to inhibit naloxone-, glutamic-acid-, or noradrenaline-induced LH increases in rats [12, 14, 46]. Moreover, Akema and Kimura [13] have proposed that $GABA_BRs$ are involved in the estrogen feedback response in castrated female rats [13], while Wagner et al. [48] suggested that attenuation of presynaptic $GABA_B$ autoreceptor function occurs as a consequence of an estrogen negative-feedback action. In addition, sex differences in gonadotropin increases after gonadectomy in rats [60, 61] have been attributed to GABA [62] acting at both $GABA_A$ and $GABA_B$ receptors [27]. As a consequence, we first evaluated the gonadotropin response to estrogen withdrawal (castration plus empty Silastic capsules) and reinstatement of the estradiol negative feedback (castration plus estradiol-filled Silastic capsules) in wild-type and knockout females. High gonadotropin levels on day 7 after castration were similar between genotypes (see below). The fact that we observed no differences in the negative-feedback action of estradiol on castration-induced gonadotropin levels between female genotypes is in agreement with the hypothesis that $GABA_BRs$ are inactivated during estradiol feedback action, as suggested by others [48]; in this situation the inhibitory action of GABA is probably exerted through $GABA_ARs$ and, therefore, should be similar between $GABA_B$ knockout and wild-type females. In agreement with the above hypothesis, we have also shown that $GABA_BRs$ are downregulated and/or desensitized by estradiol in the hypothalamus and in the pituitary in the rat [unpubl. results]. We next evaluated the early gonadotropin postcastration rise in male and female wild-type and knockout mice. In males, both gonadotropins increased markedly already 24 h after castration, without significant differences between genotypes; in contrast, in wild-type females, LH rose significantly only on day 7 after castration, similar to what was described in the rat [27]. A striking difference in this delay in the LH rise was observed between knockout females and wild-type controls. In $GABA_{B1}^{-/-}$ females, the LH titers increased markedly on day 5 after OVX, reaching fourfold higher levels than on day 4 after OVX and eightfold higher titers than wild-type controls on the same day. LH reached similar levels in both female genotypes on day 7 after OVX. These observations were specific for LH, as FSH increased in females from the 1st day after castration in both genotypes in a similar way, though more gradually than in males. Therefore, in the early maintenance of low LH levels in OVX females, $GABA_BRs$ must be playing a significant role. In the rat, the hypothalamic GABA content had been described to increase after castration in females, while decreasing in males [63], justifying the ongoing GnRH and LH inhibition in females and the immediate increase of these parameters in males. This effect of GABA was reversed by $GABA_A$ and $GABA_B$ antagonists in females, suggesting the participation of both kinds of receptors [27]. The early increase in LH in OVX knockout females corroborates the participation of $GABA_BRs$ in this response in the mouse and demonstrates that in the absence of estradiol $GABA_BRs$ are active in the control of LH secretion in the OVX wild-type female, as was suggested previously [15] in the rat. A further effect in the initial suppression of LH increase in females is probably exerted through $GABA_ARs$, since in female $GABA_{B1}^{-/-}$ mice LH did not increase as early as in males, although other factors may also be playing a role. The lack of differences in the FSH levels between female genotypes shows that this hormone is less dependent on GnRH stimulation and probably more dependent on the reduction of circulating inhibin after OVX.

Taken together, certain features in the $GABA_{B1}^{-/-}$ female, such as the lack of change in puberty onset, the alterations in the estrous cycles and in the postcastration gonadotropin increase, and a reduced reproductive function, are similar to the findings by the groups of Ojeda and coworkers [64, 65] in female rodents, with an overexpression of GAD-67 in median eminence [64] or in GnRH neurons [65]. In the above-mentioned studies, the endocrine functions are disturbed by alterations in the GABA homeostasis, while in our study, the $GABA_BR$ signaling was impaired. Future experiments will have to address whether in $GABA_{B1}$ knockout mice the output of GABA or GnRH is altered during development or in adulthood. In addition, one should take into consideration that in the knockout mice some developmental compensatory phenomena may have occurred which may obscure the function of the $GABA_BRs$ in the adult. Further work using adult animals with conditional $GABA_{B1}$

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knockout, antisense techniques, etc. will be required to rule out this possibility.

In conclusion, our study of $GABA_{B1}$ knockout mice suggests that $GABA_BRs$ are involved in regulating basal PRL release. Furthermore, the hypothalamic-hypophyseal-ovarian axis is compromised in these mice, with significant alterations in cyclicity, postcastration LH increase, and reproduction indexes. Future studies will address the molecular events underlying these alterations.

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