Original Paper

Neuro
-endocrinology

Neuroendocrinology 2013;98:212–223
DOI: 10.1159/000355631

Received: March 26, 2013
Accepted after revision: September 10, 2013
Published online: November 6, 2013

Lack of Functional $GABA_B$ Receptors Alters *Kiss1*, *Gnrh1* and *Gad1* mRNA Expression in the Medial Basal Hypothalamus at Postnatal Day 4

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Key Words
$GABA_B$ receptors · GnRH · GABA · *Kiss1* · Gonadotropins · Sex steroids

Abstract
Background/Aims: Adult mice lacking functional $GABA_B$ receptors ($GABA_{B1}$KO) show altered *Gnrh1* and *Gad1* expressions in the preoptic area-anterior hypothalamus (POA-AH) and females display disruption of cyclicity and fertility. Here we addressed whether sexual differentiation of the brain and the proper wiring of the GnRH and kisspeptin systems were already disturbed in postnatal day 4 (PND4) $GABA_{B1}$KO mice. ***Methods:*** PND4 wild-type (WT) and $GABA_{B1}$KO mice of both sexes were sacrificed; tissues were collected to determine mRNA expression (qPCR), amino acids (HPLC), and hormones (RIA and/or IHC). ***Results:*** GnRH neuron number (IHC) did not differ among groups in olfactory bulbs or OVLT-POA. *Gnrh1* mRNA (qPCR) in POA-AH was similar among groups. *Gnrh1* mRNA in medial basal hypothalamus (MBH) was similar in WTs but was increased in $GABA_{B1}$KO females compared to $GABA_{B1}$KO males. Hypothalamic GnRH (RIA) was sexually different in WTs (males > females), but this sex difference was lost in $GABA_{B1}$KOs; the same pattern was observed when analyzing only the MBH, but not in the POA-AH. Arcuate nucleus *Kiss1* mRNA (micropunch-qPCR) was higher in WT females than in WT males and $GABA_{B1}$KO females. *Gad1* mRNA in MBH was increased in $GABA_{B1}$KO females compared to $GABA_{B1}$KO males. Serum LH and gonadal estradiol content were also increased in $GABA_{B1}$KOs. ***Conclusion:*** We demonstrate that $GABA_B$Rs participate in the sexual differentiation of the ARC/MBH, because sex differences in several reproductive genes, such as *Gad1*, *Kiss1* and *Gnrh1*, are critically disturbed in $GABA_{B1}$KO mice at PND4, probably altering the organization and development of neural circuits governing the reproductive axis.

Introduction

Fetuses and newborns are exposed to gonadal steroids derived from their mother, their own gonads, and synthesized locally in their brains. Estradiol (E_2), from aromatization of testosterone (T), exerts permanent organizational effects on the developing brain and thereby establishes adult hormonal responsiveness that commands sex-specific physiology and behavior. The neu-

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rotransmitter GABA is one of the key signaling molecules selected by E_2 to govern the development of brain circuits and neuronal populations [1]. Synthesized mainly by glutamic acid decarboxylase-67 *(Gad1)* in mice [2], GABA acts by binding two classes of receptors: the ionotropic $GABA_{A/C}$ and the metabotropic $GABA_B$ receptors ($GABA_BRs$). $GABA_ARs$ have been demonstrated to participate in the establishment of sexual differences in the hypothalamus (HT) [1, 3, 4]. Here our aim was to analyze the contribution of $GABA_BRs$ to the developmental organization of the neuroendocrine reproductive axis.

GABA regulates the reproductive axis at multiple sites and during all developmental stages, from GnRH neuron migration to the preovulatory luteinizing hormone (LH) surge [4]. We previously showed that adult, female $GABA_{B1}KO$ mice, with absence of functional $GABA_BRs$, display increased GnRH pulsatility, disruption of estrous cycles, and infertility [5]. These reproductive changes are associated with changes in sexual dimorphic expression of key genes in the preoptic-anterior hypothalamic area, such as *Gnrh1* and *Gad1* [6].

Concerning the effects of GABA on GnRH secretion, $GABA_AR$ activation has been postulated to stimulate GnRH release early in development but to mainly inhibit it thereafter [4, 7, 8]. $GABA_AR$ stimulation also modulates *Gnrh1* mRNA expression in a species-, age-, and model-specific manner [9, 10]. Despite this, knockdown of $GABA_ARs$ in GnRH neurons has minimal effects on fertility [11]. Regarding $GABA_BRs$, an inhibitory effect on GnRH and LH release has been demonstrated [12–15]. However, much less is known about the regulation of *Gnrh1* expression by $GABA_BR$, especially in development. Baclofen, a $GABA_BR$ agonist, decreased *Gnrh1* in the preoptic area (POA) of ovariectomized adult rats [16] but stimulated *Gnrh1* expression in steroid-treated adult rats [17, 18], suggesting $GABA_BR$ signaling may be influenced by the gonadal steroid milieu. Regarding the migration of GnRH neurons from the nasal compartment, GABA inhibits this process through $GABA_ARs$ [19–21]. In contrast, pharmacological reports suggested that $GABA_BRs$ do not participate in this event [4, 22]. However, because $GABA_BRs$ (a) are present in migrating GnRH neurons [10, 22], (b) participate in neuron migration and differentiation [23, 24], (c) are present in neural progenitors and in embryonic stem cells [25, 26], and (d) participate in E_2-induced sexual differentiation of several hypothalamic nuclei [27, 28], their participation on GnRH neuron migration and subsequent *Gnrh1* expression warrants further investigation.

The interaction of GABA and the kisspeptidergic system, a key regulator of GnRH and reproduction [29, 30], has been poorly studied. *Kiss1* is expressed in two critical areas of the HT involved in GnRH regulation: the anteroventral periventricular nucleus-periventricular nucleus continuum (AVPV-PeN) in the anterior hypothalamus (AH), a sexually dimorphic area where E_2 exerts its positive feedback effects in females, and the arcuate nucleus (ARC) in the mediobasal hypothalamus (MBH), where E_2 and T exert their negative feedback effects [29, 31]. In addition, kisspeptin from the ARC was recently identified as a novel stimulator of GnRH neurite growth at embryonic day 13.5, possibly to facilitate GnRH fiber innervation of the median eminence (ME) [32]. Moreover, in adult rodents, kisspeptin activation of GnRH neurons in the presence of E_2 can be either direct or mediated indirectly by GABAergic and glutamatergic neurons [33]. In addition, Zhang et al. [31] showed that while $GABA_BR$ agonists hyperpolarized adult GnRH neurons, this response was abrogated by addition of kisspeptin-10, suggesting an interaction between kisspeptin and $GABA_BR$ signaling in the regulation of GnRH.

Taking into account the above considerations, we were interested in addressing whether sexual differentiation of the brain and the proper developmental wiring of the GnRH and kisspeptin systems were already disturbed in early postnatal development in $GABA_{B1}KO$ mice. We selected postnatal day 4 (PND4) for this research because this is an age when the pre- and postnatal T surges that initiate sexual differentiation have already occurred yet major steps towards completing brain sexual differentiation are still ongoing and activational effects of estrogens are not yet present [1]. Therefore, we analyzed the contribution of $GABA_BRs$ to the developmental organization of the neuroendocrine reproductive axis at PND4 by studying hypothalamic GnRH, GABA and *Kiss1* systems, as well as pituitary and gonadal hormones in male and female $GABA_{B1}KO$ and wild-type (WT) mice.

Materials and Methods

Animals

$GABA_{B1}KO$ mice, generated in the BALB/C inbred strain [34], were obtained by intercrossing heterozygous animals and the day of birth was recorded. Mice were genotyped by PCR analysis, as described previously [5]. All animals were housed in groups in mouse ventilated racks (22°C), with lights on from 7:00 to 19:00 h, and 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) that follows the NIH guidelines.

PND4 female and male WT and $GABA_{B1}$KO mice were killed in the morning (9:00–11:00 h) under minimal stress to collect tissues and blood samples. We have previously demonstrated that pup weights do not differ between sex or genotype at this age [5].

PND4 is a critical stage of development just after both T androgenizing peaks (embryonic day 18 and PND1) have taken place and during which time major developmental changes occur towards permanently inducing brain sex differences and wiring regulatory neural circuits [1, 35]. Furthermore, at this neonatal stage of life we are able to discard the activational effects of E_2 in females, due to the presence of α-fetoprotein [36, 37], which could potentially mask or confound existing neural sex differences.

Localization of ir-GnRH Neurons

PND4 mice were killed and brains were fixed overnight in Bouin's fixative at 4°C, stored in 70% ethanol, and later processed by routine methods to perform immunohistochemistry (IHC). Serial 6-μm coronal sections from each brain were obtained (Leica RM 2125RT) and kept at 4°C until used. Male and female brains were processed in two different assays. First, brain sections were blocked with 0.3% H_2O_2 for 35 min and then with 5% skimmed milk 0.3% Triton X-100 in PBS for 30 min. Thereafter, they were incubated at 22°C overnight with a monoclonal antibody against GnRH (LRH13 1:1,000, generously donated by the Hormone Assay Centre, Institute of Endocrinology, University of Gumma, Japan [38]). Then, sections were rinsed twice with PBS, and incubated with a secondary biotinylated antibody (LSAB2 System-HRP; Dako) at 22°C for 30 min. After rinsing with PBS, sections were incubated with streptavidin (LSAB2 System-HRP; Dako) at 22°C for 35 min and revealed with diaminobenzidine (DAB, 3 min). The slides were then analyzed with a light Nikon Eclipse E600 microscope and photographed. One slide every 40 μm was analyzed to count only once every ir-neuron. We considered a neuron if it had a clear nucleus and was cut approximately along the middle of it. The number of immunoreactive GnRH (ir-GnRH) neurons in the olfactory bulbs (OB), organum vasculosum of the lamina terminalis (OVLT) and POA were counted approximating the coordinates from an atlas for PND6 mice [39]: OB: from P6 #1 0.99 mm to P6 #6 2.07 mm; OVLT: from P6 #17 3.39 mm to P6#18 3.51 mm, and POA: from P6 #22 3.99 mm to P6 #24 4.23 mm. Number of animals per group (n) = 4.

GnRH Protein Content Determination

For hypothalamic GnRH protein content, the AH (including the POA, where many GnRH cell bodies reside: POA-AH), and the MBH (including the ME, where most GnRH axonal fibers target) were excised and rapidly frozen, as previously described [6]. To determine the total hypothalamic (HT) content, we considered the sum of the content (pg of GnRH) from both POA-AH and MBH fragments. The OB and frontoparietal cortex (CT, taken as control tissue) were also rapidly dissected and frozen. Tissues were processed as previously described [6]. Briefly, tissues were homogenized in 200 μl ice-cold 0.1 N HCl, the homogenate was centrifuged at 13,000 *g* at 4°C for 30 min, and the supernatant was recovered. Samples were stored at –20°C until assayed for GnRH by RIA [40]. GnRH assay sensitivity was 1.5 pg. Intra- and interassay coefficients of variation: 7.1 and 11.6%, respectively. Number of samples: OB 4–6, CT 5–8, and POA-AH and MBH 6–13.

Hypothalamic GABA Amino Acid Content

HT and CT were rapidly dissected and frozen. GABA content was determined by HPLC as previously described [41]. Briefly, amino acidic dansyl derivatives were measured using a HPLC system, which consisted of a Model 125 Programmable Solvent Module (Beckman Instruments, Fullerton, Calif., USA), a 5-μm, 25 cm × 4.6 mm Ultrasphere® ODS reversed-phased 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, v/v) containing 0.15% (v/v) phosphoric acid. n = 5–8.

RNA Isolation and Reverse Transcription

Hypothalamic fragments (POA-AH and MBH), OB and CT, were obtained as above. The tissues were kept in RNA Later Solution (Ambion, Austin, Tex., USA) at 4°C for 24 h to preserve the RNA, then removed and kept at –70°C until RNA isolation. Total RNA was isolated using TRIzol Reagent (Invitrogen, Carlsbad, Calif., USA) according to the manufacturer's protocol and kept at –70°C until used. 1 μg of total RNA was reverse-transcribed in a 20-μl reaction using MMLV reverse transcriptase (Epicentre, Madison, Wisc., USA) and oligo$(dT)_{15}$ primers (Biodynamics, Buenos Aires, Argentina).

ARC and AVPV micropunches (2 mm diameter) of PND4 mice were obtained from 500- and 400-μm-thick frozen brain slices respectively, as previously described [42]. Total RNA was extracted using the RNeasy Lipid Tissue Mini kit (Qiagen, Valencia, Calif., USA) according to the manufacturer's protocol. RNA (500 ng) was reverse transcribed using the Omniscript RT kit (Qiagen).

The reverse transcriptase was omitted in control reactions, where the absence of an amplification product indicated the isolation of RNA free of genomic DNA. cDNA was stored at –20°C until use in RT-PCR or qPCR.

Gene Expression Assays

Primer sets were designed for the specific amplifications of murine *Gnrh1, Gad1* and *Kiss1,* and the housekeeping control genes *Cyclophilin b* and *Gapdh* (table 1). Semiquantitative determination of *Kiss1* expression by RT-PCR in MBH was performed as previously described [43]. n = 5.

For quantitative determinations (qPCR) the amplification efficiency of each primer set was calculated from the slope of a standard amplification curve of log (ng cDNA) per reaction versus Ct value ($E = 10^{-(1/slope)}$). Efficiencies of 2 ± 0.1 were considered optimal. Quantitative measurements of *Gnrh1*, *Gad1* and *Cyclophilin b* cDNA from POA-AH, MBH, OB and CT were performed by qPCR in a total volume of 13 μl as previously described [6] (see table 1 for details). Amplification was carried out in an ABI7500 Sequence Detection System (Applied Biosystems). Results were validated based on the quality of dissociation curves and the size of the product was confirmed by 2.3% agarose gel electrophoresis. Each sample was analyzed in duplicate along with non-template controls to monitor contaminating DNA. Quantitative differences in the cDNA target between samples were determined as previously described [6] using the mathematical model of Pfaffl [44] which refers expression to a single randomly selected WT male. n = 9–10.

To determine quantitative *Kiss1* and *glyceraldehyde 3-phosphate dehydrogenase (Gapdh)* expression in micropunches, qPCR was performed using the Bio-Rad iCycler Detection System and

Table 1. Primer sequences and details used for gene expression assays by qPCR or RT-PCR

Gene	Assay	GenBank accession No.	Primer forward 5′–3′	Primer reverse 5′–3′	Product size, bp	Annealing temp., °C
Gnrh1	qPCR	NM 008145	GAACCCCAGCACTTCGAATGT	TGGCTTCCTCTTCAATCAGACTTT	94	58
Gad1	qPCR	NM 008077	GCGGGAGCGGATCCTAATA	TGGTGCATCCATGGGCTAC	79	58
Kiss1	qPCR	NM 178260	CAAAAGTGAAGCCTGGATCC	GTTGTAGGTGGACAGGTCC	254	60
Cyclophilin	qPCR	NM 011149	GTGGCAAGATCGAAGTGGAGAAAC	TAAAAATCAGGCCTGTGGAATGTG	210	62
Gapdh	qPCR	NM 008084	TGCACCACCAACTGCTTAG	GGATGCAGGGATGATGTTC	177	60
Kiss1	RT-PCR	NM 178260	AGCTGCTGCTTCTCCTCTGT	AGGCTTGCTCTCTGCATACC	140	60
Gapdh	RT-PCR	NM 008084	CCAGAACATCATCCCTGCAT	GTTCAGCTCTGGGATGACCTT	67	60

Quantitect SYBR Green PCR kit (Qiagen) as previously described [45, 46]. Standard curves were generated for each product using cloned cDNAs for *Kiss1* and *Gapdh* to quantify the abundance of cDNA in each sample. For standard curves, a dilution series of cloned *Kiss1* and *Gapdh* templates ranging from 10 to 10^8 copies were used. Data were collected from threshold values using the automatic function of the Bio-Rad MyIQ software. All samples were run in duplicate and *Kiss1* was normalized to *Gapdh*, whose expression is constant. The size of the products was confirmed by 1% agarose gels. n = 4–8.

Pituitary Gonadotropin Content and Serum Hormone Concentrations

Trunk blood of PND4 mice was collected and sera were obtained and frozen for hormone determinations. In each treatment, some samples were pooled to obtain the necessary volume (between 2 and 4 animals). The number of pooled samples per group was 5–9. Pituitary glands were rapidly dissected and frozen. To determine LH and FSH pituitary contents, each pituitary (individually) was homogenized in 100 µl PBS with protease inhibitors. Protein content was measured by Lowry et al. [47] and the result determined the aliquot of the homogenate which was measured by RIA. The number of samples per group was 5–8. For both blood serum and pituitary homogenates, LH and FSH protein levels were determined by RIA with kits from NHPP, NIDDK & Dr. Parlow. Results were expressed in terms of RP3 rat LH and FSH standards, as these systems recognize mouse samples. Assay sensitivities: LH 0.015 ng/ml, FSH 0.1175 ng/ml. Intra- and interassay coefficients of variation: LH 7.2 and 11.4% and FSH 8.0 and 13.2%, respectively.

Gonadal Steroids Contents

Ovarian and testicular E_2 and T contents from PND4 mice 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 [41]. Tritiated hormones were purchased from New England Nuclear. Assay sensitivities were: E_2 11.3 pg and T 125 pg. Intra- and interassay coefficients of variation were: E_2 6.8 and 11.7% and T 7.8 and 12.3%, respectively. n = 4–6.

Statistics

Data are presented as the mean ± SEM. The differences between means of: (a) two groups were analyzed by Student's t test; (b) more than two groups were analyzed by two- or three-way ANOVA, followed by the Tukey HSD test. Pearson's correlation test was used to analyze the possible lineal association or correlation between two variables measured in the same samples (r = Pearson's correlation coefficient). $p < 0.05$ was considered statistically significant. All analyses were performed in Statistica (data analysis software system), version 8.0, StatSoft, Inc. (2007).

Results

Localization of ir-GnRH Neurons

To evaluate whether the previously reported alterations in the reproductive axis in adult $GABA_{B1}$KO mice could be due to abnormal migration or alterations in the number of GnRH neurons, we determined the localization and the number of ir-GnRH neurons by IHC in PND4 mice of both sexes and genotypes. Coronal brains sections of the PND4 mice, from the OB to the POA, were analyzed; a representative series is shown in figure 1a. We did not observe statistical differences in distribution or cell number due to genotype along the OB or OVLT-POA, either in males (fig. 1b) or in females (fig. 1c). Very few cell bodies were detected with this technique in more caudal sites, such as the caudal POA, but this is not surprising as the vast majority of ir-GnRH cell bodies are located in more rostral regions. Moreover, we did observe ir-GnRH fibers running caudally parallel to the brain floor, which finally target the ME (fig. 1a).

Gnrh1 mRNA Expression and Protein Content

Although localization of GnRH neurons was not altered in $GABA_{B1}$KO PND4 mice, we next evaluated *Gnrh1* mRNA expression in POA-AH and MBH, as well as in the OB and CT (as negative control), in both sexes and genotypes, since we had previously found alterations in *Gnrh1* mRNA expression in the HT of adult mice [6]. In the POA-AH of PND4 mice, *Gnrh1* mRNA expression did not vary between groups (fig. 2a). Although it has been described that few GnRH cell bodies reside in the MBH [48, 49], axonal transport of *Gnrh1* mRNA and regulation of its expression has been proposed [50]. There-

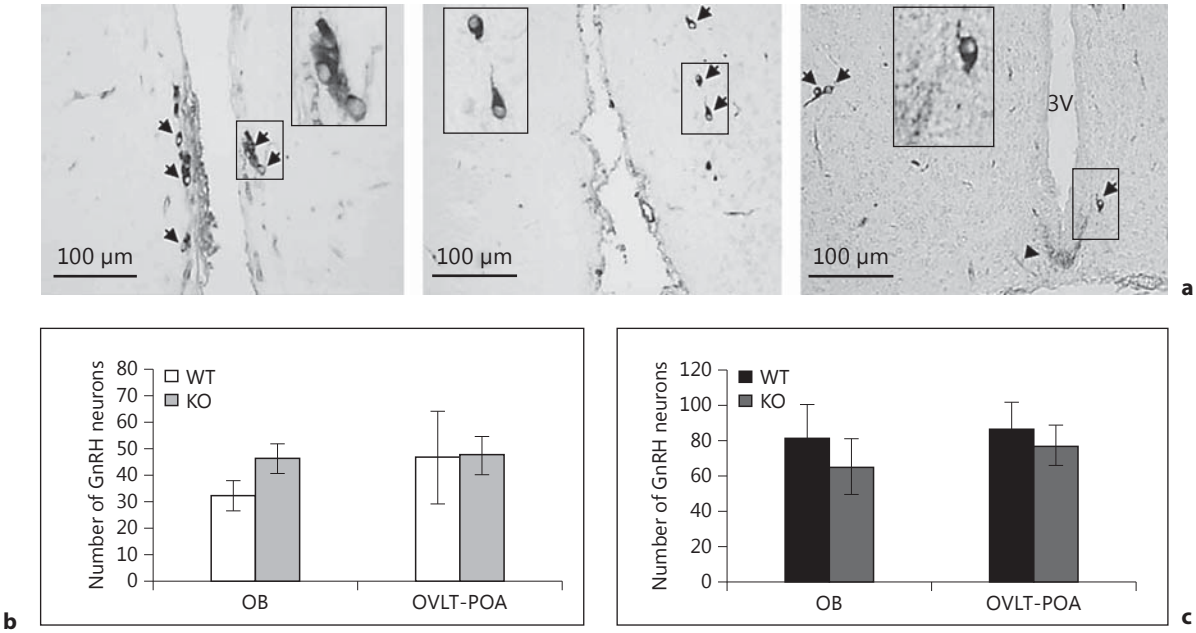


Fig. 1. Localization of ir-GnRH neurons (IHC) in coronal sections at PND4 mice. **a** Representative micrographs (20×) from OB (left panel), medial septum (medium panel) and OVLT-POA (right panel). Arrows show ir-GnRH cell bodies and arrowheads ir-fibers running caudally parallel to the brain floor. 3V = Third ventricle. **Inset** Detailed amplification of lined square. Number of animals = 4. Scale bar = 100 µm. **b** Number of ir-GnRH neurons in OB and OVLT-POA in WT and $GABA_{B1}$KO males. One-way ANOVA in OB or OVLT: main effect genotype: NS. **c** Number of ir-GnRH neurons in OB and OVLT-POA in WT and $GABA_{B1}$KO females. One-way ANOVA in OB or OVLT: main effect genotype: NS.

fore, we also examined *Gnrh1* mRNA expression in the MBH. Similar to the POA-AH, WT mice did not show sex differences in MBH (fig. 2b). In contrast, *Gnrh1* mRNA expression in MBH from $GABA_{B1}$KO mice was sexually different, with higher expression in females than in males ($p < 0.01$).

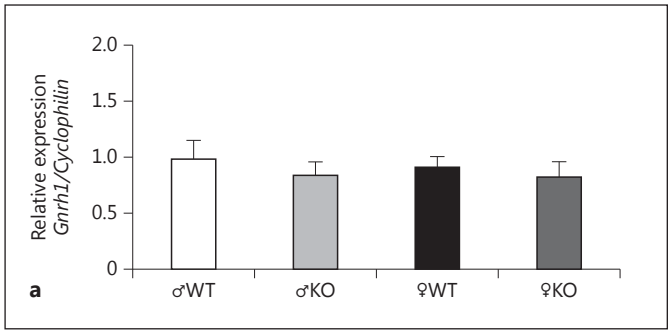
Gnrh1 mRNA expression in OB was not different among groups (WT males = 0.9 ± 0.29, KO males = 0.9 ± 0.16, WT females = 0.72 ± 0.12, KO females = 0.74 ± 0.13; two-way ANOVA: NS). Similar levels were observed in all groups in CT (data not shown).

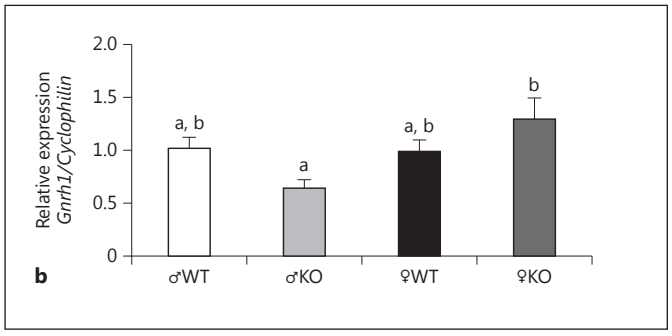
We also analyzed GnRH peptide content in POA-AH and MBH fragments from PND4 mice of both sexes and genotypes. In both genotypes, GnRH peptide content was sexually different in the POA-AH, with males having higher GnRH levels than females ($p < 0.001$), without genotype differences (fig. 2c). In the MBH (the target site of GnRH axons) GnRH content was higher than in the POA at PND4. In addition, MBH GnRH content depends on sex and genotype (two-way ANOVA, interaction: $p < 0.05$) (fig. 2d). In WT mice, the same gender difference as in the POA-AH was observed ($p < 0.05$), while it was lost in $GABA_{B1}$KO mice. We also analyzed GnRH content in whole HT from both sexes and genotypes (fig. 2e). HT GnRH content was found to be very similar to the MBH pattern, suggesting a greater contribution of this area to the total GnRH protein content. In addition, in HT as in MBH, GnRH tended to increase in $GABA_{B1}$KO females compared with WT females, although it did not attain statistical significance ($p = 0.11$).

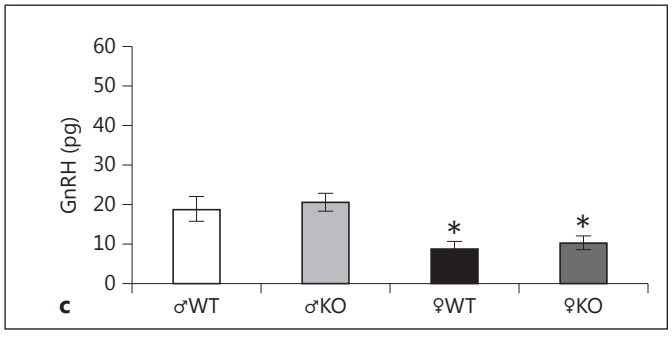
GnRH content was sexually different in OB, similar to the POA-AH, males higher than females, without genotype differences [GnRH content (pg/OB): WT males = 19.5 ± 3.2, KO males = 20.6 ± 5.0, WT females = 9.4 ± 1.7, KO females = 9.8 ± 2.0; two-way ANOVA, interaction: NS; main effect sex: $p < 0.01$; main effect genotype: NS]. We did not find differences between groups in CT, where decapeptide levels were scarce (data not shown).

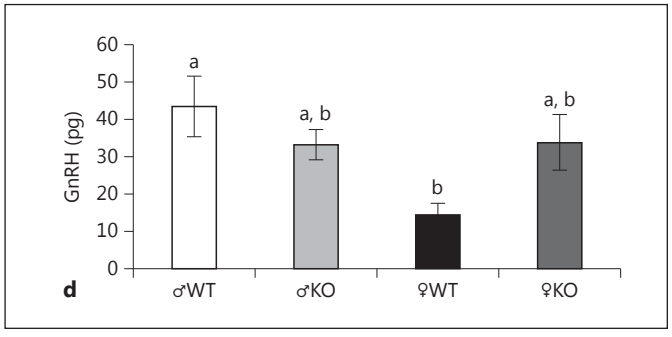
Kiss1 mRNA Expression in the Hypothalamus

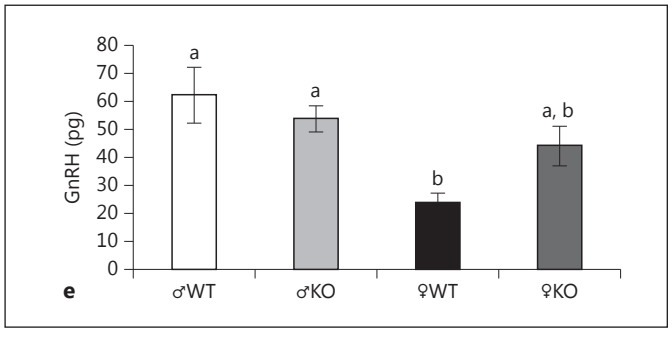
Because we found alterations in the mRNA expression and peptide levels of GnRH in the MBH and because kisspeptin is a major regulator of GnRH neurons and has also been described to play a developmental role, we evaluated whether the expression of *Kiss1* mRNA was also altered in PND4 $GABA_{B1}$KO mice.











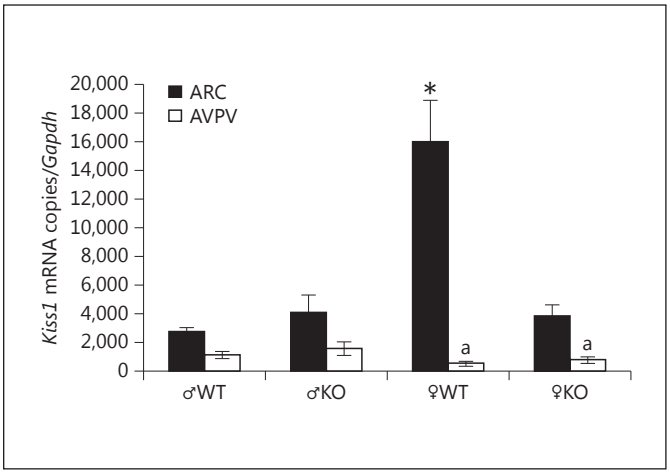
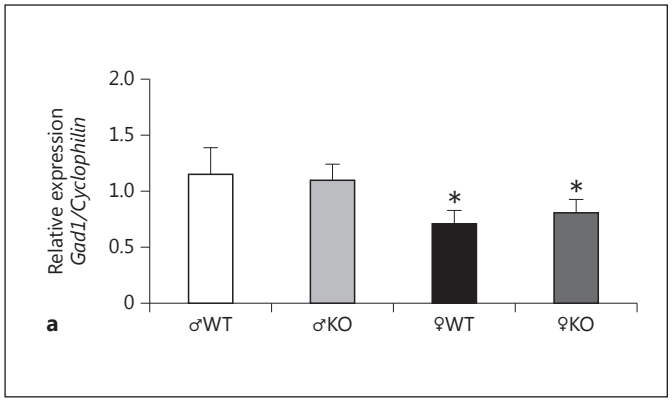
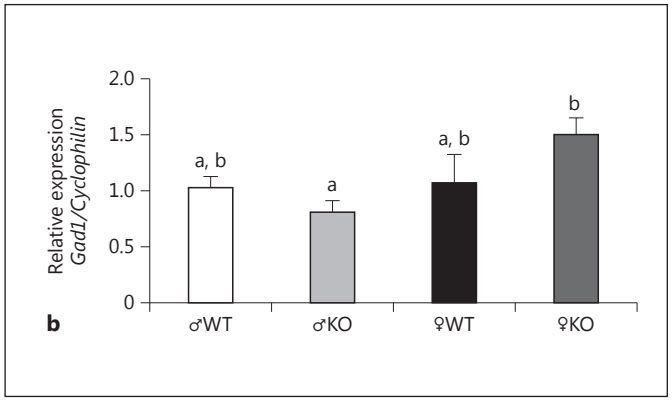


Fig. 3. *Kiss1* expression in discrete nuclei of the hypothalamus. *Kiss1* mRNA expression in micropunches of ARC and AVPV nuclei (qPCR). Three-way ANOVA, factors sex, genotype and nucleus: triple interaction: $p < 0.05$. * ARC *Kiss1* expression in WT females significantly different from ARC Kiss1 expression in WT males ($p < 0.05$) and in $GABA_{B1}$KO females ($p < 0.01$). [a] *Kiss1* expression in AVPV significantly different from ARC in females of both genotypes. Number of animals: ♂WT = 4–7, ♂KO = 5–6, ♀WT = 7–8, ♀KO = 8.

Fig. 2. *Gnrh1* hypothalamic expression and peptide content. *Gnrh1* mRNA expression (qRT-PCR) and content (RIA) in POA-AH (**a**, **c**) and MBH (**b**, **d**). Total hypothalamic GnRH content (RIA) (**e**). ♂ = Male; ♀ = female. **a** Two-way ANOVA, factors sex and genotype: NS. Number of animals: ♂WT = 10, ♂KO = 10, ♀WT = 10, ♀KO = 10. **b** Two-way ANOVA, factors sex and genotype: interaction: $p < 0.02$; different letters mean statistically significant differences, $p < 0.05$ or less: *Gnrh1* mRNA expression in $GABA_{B1}$KO males significantly different from $GABA_{B1}$KO females ($p < 0.01$). Number of animals: ♂WT = 9, ♂KO = 10, ♀WT = 9, ♀KO = 10. **c** Two-way ANOVA, factors sex and genotype: interaction: NS; main effect genotype: NS; main effect sex: $p < 0.001$. * GnRH content in females significantly different from males. Number of animals: ♂WT = 9, ♂KO = 9, ♀WT = 6, ♀KO = 13. **d** Two-way ANOVA, factors sex and genotype: interaction: $p < 0.05$; different letters mean statistically significant differences, $p < 0.05$ or less: GnRH content in WT males significantly different from WT females ($p < 0.05$). Number of animals: ♂WT = 9, ♂KO = 9, ♀WT = 6, ♀KO = 13. **e** Two-way ANOVA, factors sex and genotype: interaction: $p < 0.05$; different letters mean statistically significant differences, $p < 0.05$ or less: GnRH content in WT males significantly different from WT females ($p < 0.01$). Number of animals: ♂WT = 9, ♂KO = 9, ♀WT = 6, ♀KO = 13.





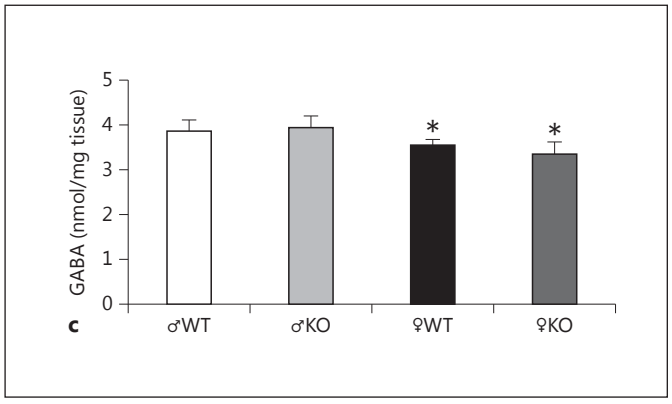


Fig. 4. *Gad1* expression and GABA amino acid hypothalamic content. *Gad1* mRNA expression (qPCR) in POA-AH (**a**) and MBH (**b**). GABA content (HPLC) in total HT (**c**). **a** Two-way ANOVA, factors sex and genotype: interaction: NS; main effect sex: $p < 0.05$; main effect genotype: NS. * *Gad1* mRNA expression in females significantly different from males. Number of animals: ♂WT = 9, ♂KO = 8, ♀WT = 10, ♀KO = 9. **b** Two-way ANOVA, factors sex and genotype: interaction: $p < 0.05$. Different letters mean statistically significant differences, $p < 0.05$ or less: *Gad1* mRNA expression in $GABA_{B1}$KO males significantly different from $GABA_{B1}$KO females ($p < 0.05$). Number of animals: ♂WT = 8, ♂KO = 8, ♀WT = 7, ♀KO = 8. **c** Two-way ANOVA, factors sex and genotype: interaction: NS; main effect sex: $p < 0.05$; main effect genotype: NS. * GABA content in males significantly different from females. Number of animals: ♂WT = 6, ♂KO = 5, ♀WT = 8, ♀KO = 5.

First, we evaluated *Kiss1* expression by semiquantitative RT-PCR in the MBH (which contains the ARC, a major *Kiss1* population). MBH *Kiss1* expression showed a significant sex difference in WT mice, with females having higher *Kiss1* levels than males ($p < 0.01$). However, this sex difference was lost in $GABA_{B1}$KO mice, particularly because MBH *Kiss1* mRNA was significantly lower in $GABA_{B1}$KO females than in WT females ($p < 0.001$) (data not shown).

As *Kiss1* is expressed in discrete nuclei in the HT, we decided to evaluate its expression by qPCR in micropunches of the ARC nucleus, which is located in the MBH and also in the AVPV nucleus, located in the AH (fig. 3). As with the semiquantitative RT-PCR results, *Kiss1* expression in the ARC was higher in PND4 WT females than in WT males ($p < 0.05$), but this sex difference was lost in PND4 $GABA_{B1}$KO mice, notably due to a dramatic decrease in ARC *Kiss1* expression in $GABA_{B1}$KO females ($p < 0.01$). We did not find differences between groups in *Kiss1* expression in the AVPV. Although males have similar *Kiss1* expression between nuclei, females express significantly more *Kiss1* mRNA in the ARC compared to AVPV nucleus at this stage of life ($p < 0.001$).

Gad1 mRNA Expression and GABA Amino Acid Content

Due to the fact that GAD1 is the main enzyme to convert glutamate into GABA, and that this neurotransmitter is also in charge of controlling GnRH neurons, we analyzed *Gad1* mRNA expression in the POA-AH and MBH and also in CT (as control area) at PND4. *Gad1* expression was sexually different in the POA-AH, males greater than females ($p < 0.05$), without genotype differences (fig. 4a). In contrast, *Gad1* expression in the MBH (fig. 4b) in WT mice was similar between sexes. However, in the MBH of $GABA_{B1}$KO mice, a sexually different *Gad1* expression emerged, with $GABA_{B1}$KO females showing higher expression levels than $GABA_{B1}$KO males ($p < 0.05$). Interestingly, in males *Gad1* expression was 3-fold higher in the POA-AH than in the MBH, while *Gad1* expression was similar in both areas in females (data not shown).

In HT, GABA amino acid content was higher in males than in females ($p < 0.05$), without genotype differences (fig. 4c). We did not find differences in GABA content in CT (data not shown).

Because we found that the pattern of expression of *Gnrh1* and *Gad1* was very similar in the MBH, we decided to evaluate a possible correlation between the mRNA

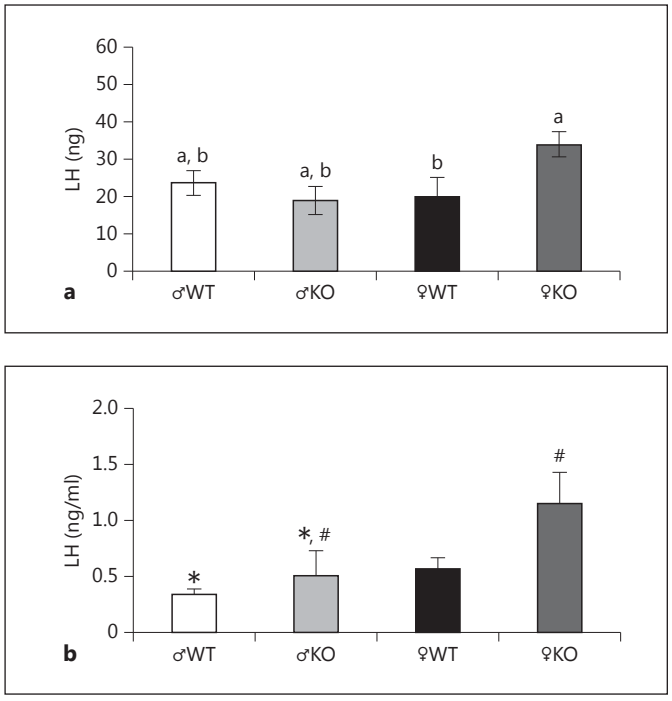


Fig. 5. Pituitary content and serum LH. Pituitary content (**a**, ng/pituitary, RIA) and serum LH (**b**, ng/ml, RIA). **a** Two-way ANOVA, factors sex and genotype: interaction: $p < 0.05$; different letters mean statistically significant differences, $p < 0.05$ or less: LH content in $GABA_{B1}KO$ females significantly different from WT females ($p < 0.05$). Number of animals: ♂WT = 6, ♂KO = 5, ♀WT = 8, ♀KO = 5. **b** Two-way ANOVA, factors sex and genotype: interaction: NS; main effect sex: $p < 0.05$; main effect genotype: $p < 0.05$. * Serum LH in females significantly different from males. # Serum LH in $GABA_{B1}KO$ mice significantly different from WTs. Number of animals: ♂WT = 8, ♂KO = 9, ♀WT = 6, ♀KO = 5. Nevertheless, the genotype difference should be mainly attributed to females, as when each sex was analyzed separately by Student's t test, the difference was significant between females ($p < 0.02$) but not between males (NS).

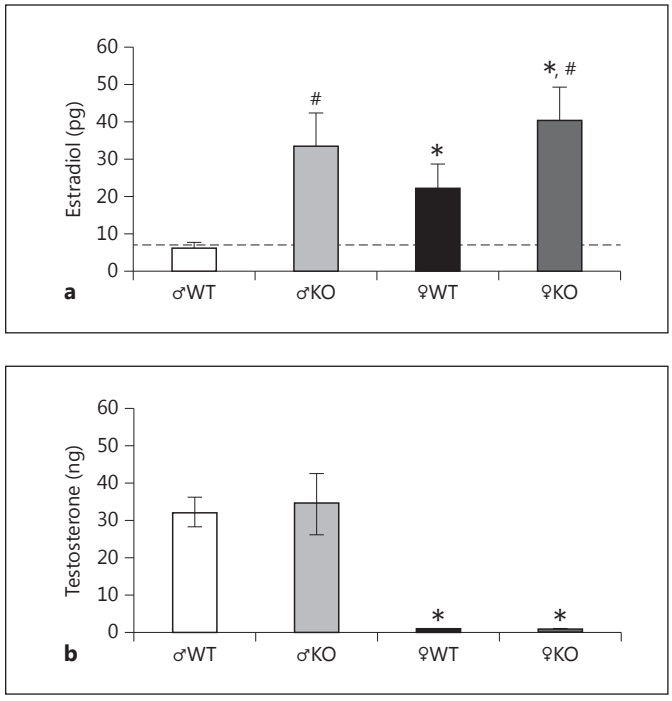


Fig. 6. Gonadal steroid hormones content. Gonadal E_2 (pg/ovary or testis, **a**) and T (ng/ovary or testis, **b**) contents (RIA). **a** Two-way ANOVA, factors sex and genotype: interaction: NS; main effect sex: $p < 0.05$; main effect genotype: $p < 0.01$. * E_2 content in females significantly different from males. # E_2 content in $GABA_{B1}KO$ mice significantly different from WTs. Dotted line: assay detection limit. Number of animals: ♂WT = 5, ♂KO = 5, ♀WT = 4, ♀KO = 5. **b** Two-way ANOVA, factors sex and genotype: interaction: NS; main effect sex: $p < 0.001$; main effect genotype: NS. * T content in females significantly different from males. Number of animals: ♂WT = 5, ♂KO = 6, ♀WT = 4, ♀KO = 5.

expressions of these genes in the two different areas evaluated. Effectively, *Gnrh1* and *Gad1* present a positive correlation in MBH ($r = 0.53$; $p < 0.01$) while there is no such correlation in POA-AH.

Gonadotropin Contents and Serum Concentrations

Pituitary LH content did not show sex differences at this early stage of development in WT mice (fig. 5a). Interestingly, LH content was significantly increased in $GABA_{B1}KO$ females compared to WT females ($p < 0.05$) and nearly significant compared to $GABA_{B1}KO$ males ($p = 0.06$). FSH content did not differ among groups (data not shown).

Regarding serum LH (fig. 5b), two-way ANOVA showed a non-significant interaction, females showed higher levels than males ($p < 0.05$) and a genotype difference was also observed, $GABA_{B1}KO$ > WT ($p < 0.05$). Nevertheless, the genotype difference should be mainly attributed to females, as when each sex was analyzed separately by Student's t test, the difference was significant between females ($p < 0.02$) but not between males (NS). Serum FSH was higher in females than in males, as expected at this age, without differences due to genotype (ng/ml: WT males = 6.1 ± 0.7, KO males = 6.0 ± 0.9, WT females = 8.7 ± 0.3, KO females = 8.7 ± 0.8; two-way ANOVA, interaction: NS; main effect sex: $p < 0.001$; main effect genotype: NS).

Gonadal Steroids Contents

We also measured T and E_2 in ovaries and testes. E_2 content was higher in females than in males ($p < 0.05$), as expected. Interestingly, $GABA_{B1}$KO mice showed an increase in E_2 content ($p < 0.01$) in both males (5-fold) and females (2-fold) (fig. 6a). T was higher in males than in females ($p < 0.001$), without genotype differences (fig. 6b). Gonadal weight was not different between genotypes in each gender at this age [weight (mg): WT males = 2.70 ± 0.36 vs. KO males = 2.52 ± 0.31, NS; WT females = 0.88 ± 0.18 vs. KO females = 0.90 ± 0.14, NS].

Discussion

We have previously shown that the absence of functional $GABA_B$Rs causes alterations in the adult reproductive axis, mainly in females [5, 6]. Here, we were interested in addressing whether absence of $GABA_B$Rs affected sexual differentiation and development of the brain and the proper wiring of the GnRH and kisspeptin systems early in development at PND4, an age when major steps towards brain sexual differentiation and development are occurring [1].

Because GABA has previously been implicated in the regulation of GnRH neuron migration [19–21], a process which is completed by birth in mice [51], we first evaluated GnRH neurons in brains from WT and $GABA_{B1}$KO mice. We did not find differences due to genotype in either the number or localization of GnRH-ir neurons in the OB or in the OVLT-POA at PND4 in either gender. Thus, we did not observe any evidence of abnormal GnRH cell migration in the absence of $GABA_B$Rs, in agreement with previous pharmacological studies [22]. Although we did not find alterations in GnRH neuron migration, GnRH pulsatility has been found to be increased in adult $GABA_{B1}$KO females [5, 6]. For GnRH neurons to play their normal physiological role in adulthood, their axons must extend from the somata in the rostral hypothalamus to the ME in the MBH, a process which occurs early in development [52]. Recently, a role of $GABA_B$Rs in axon/dendrite morphology maturation was reported [24] which could justify the alterations in physiology we observed previously in adults.

In the POA-AH, GnRH at either the mRNA or peptide levels was not altered by lack of functional $GABA_B$Rs. *Gnrh1* mRNA expression was similar in both genders, in agreement with previous results from Gore et al. [53]. Regarding GnRH peptide content in the POA-AH, we observed higher peptide levels in males than females that did not correlate with *Gnrh1* mRNA expression, suggesting posttranscriptional regulation of GnRH [54], apparently in a sex-specific manner. Although *Kiss1* expression was not consistently detected in the AVPV-PeN by ISH before PND10 [29, 46, 55, 56], we evaluated its expression by sensitive qPCR at this age. We determined very low levels of *Kiss1* mRNA in this area that did not show either sex or genotype differences.

We then evaluated *Gad1* mRNA expression in the POA-AH and established that it was higher in males than in females, as previously proposed [57] with concomitant higher GABA content in whole hypothalami of PND4 males, in agreement with Flügge et al. [58]. Neither POA-AH *Gad1* mRNA nor HT GABA content was altered by the absence of functional $GABA_B$Rs. It has been established that direct GABA action through $GABA_A$Rs is mainly stimulatory in GnRH neurons from embryonic to prepubertal stages [4]. We therefore propose that high GABA in the POA-AH of PND4 males may stimulate GnRH peptide synthesis. Taken together, these results demonstrate a sex difference in GnRH peptide and GABA at the POA-AH at this early stage of development and that the lack of functional $GABA_B$Rs does not influence these particular sexually dimorphic parameters. This finding is not surprising, as GABA does not participate in establishing sex differences in this area [1].

A different scenario seems to be occurring at the ARC/MBH where GABA has been postulated as a main effector of E_2-induced sex differentiation [1]. Although few GnRH cell bodies are present in this area [48, 49], *Gnrh1* mRNA has also been reported to be axonally transported and regulated at this level [50]. A particular pattern of *Gnrh1* expression was observed in the MBH without differences between WT mice, as in the POA-AH, while a sex difference became apparent in $GABA_{B1}$KO mice (females higher than males). Moreover, as in the POA-AH, GnRH peptide content in the MBH was sexually different in WT mice (males higher than females) and this sex difference was lost in $GABA_{B1}$KO mice. Furthermore, MBH GnRH content tended to increase in $GABA_{B1}$KO compared to WT females, similar to *Gnrh1* mRNA expression.

The observed genotypic and sexually different patterns of GnRH mRNA and peptide expression could not be attributed to kisspeptin input, since *Kiss1* expression in the ARC of WT PND4 mice showed the previously-described higher postnatal expression in females with regard to males [55, 59, 60]. Interestingly, this gender difference in ARC *Kiss1* expression was lost in $GABA_{B1}$KO mice. In addition, ARC *Kiss1* expression was markedly inhibited in $GABA_{B1}$KO compared to WT females, clearly suggesting

that the enhanced GnRH synthesis in PND4 $GABA_{B1}$KO females is not being driven by kisspeptin, in keeping with other reports [60]. Recently, Fiorini and Jasoni [32] discovered that *Kiss1*, secreted by the *Kiss1*-possitive cell bodies in the developing ARC, stimulates the growth of GnRH neurites only once they are in or very near the ME, and thereby acts to ensure target innervations. Moreover, *Kiss1* expression is present as early as E13.5 in the MBH, which is coincident with the arrival of the first GnRH neurites to that area [61–63]. So, the decrease in *Kiss1* expression observed at PND4 in $GABA_{B1}$KO females, which could already be present at earlier ages, may affect normal GnRH neurite development and cause the alterations in GnRH physiology observed in adulthood [6].

Interestingly, *Gad1* mRNA expression in MBH showed the same pattern as *Gnrh1* mRNA expression in this area, including a sex difference present in $GABA_{B1}$KO mice which is absent in WT mice. A significant, positive correlation between these two genes further points to GABA regulating *Gnrh1* expression in this area and/or that absence of functional $GABA_B$Rs affects both genes in the same way. This observation also suggests that $GABA_B$Rs are normally modulating *Gad1* expression in the MBH at this stage of development, in contrast to the POA-AH, and that the absence of functional $GABA_B$Rs affects *Gad1* expression in a different way in each gender. In adults, the lack of functional $GABA_B$Rs altered GnRH and *Gad1* expression in both the POA-AH and the MBH [6], whereas here we show that only the MBH is disrupted at PND4. The MBH controls tonic pulsatile GnRH secretion, active neonatally, as opposed to surge secretion, which is controlled by the POA-AH and which develops later at puberty [49]. These results suggest that the POA-AH may become affected in $GABA_{B1}$KO mice once this region starts to become active in GnRH regulation later in pubertal and adult life.

Physiologically, alterations in the GnRH system may have consequences on the pituitary. We detected an increase in LH content in the pituitaries of $GABA_{B1}$KO females compared to WTs which could be due to altered GnRH secretion/pulsatility, as we previously described in adult $GABA_{B1}$KO mice [6], and/or to the absence of functional $GABA_B$Rs in the pituitary. Regarding serum LH, a sex difference was observed, females greater than males, as also demonstrated on PND1 by Poling and Kauffman [60]. In addition, serum LH was elevated in both male and female $GABA_{B1}$KO mice. We have previously shown that adenohypophyseal $GABA_B$R stimulation inhibits LH secretion [64] and that absence of functional receptors induces an increase in basal LH secretion in adult mouse pituitary cell cultures from $GABA_{B1}$KO mice [6]. In addition, pituitary $GABA_B$Rs are maximally expressed neonatally, and $GABA_B$R protein levels are higher in females than in males [65]. Whatever the origin of the LH increase in our PND4 mice, it appears to stimulate estrogen synthesis in the gonads, as E_2 content was significantly elevated in testes and ovaries from $GABA_{B1}$KO mice, with no alterations in T content. Although the natural stimulus for aromatase is FSH, it has been postulated that LH can induce aromatase synthesis and activity in various tissues, especially when LH receptors are present at low levels [66]. Alternatively, increased E_2 content in gonads of $GABA_{B1}$KO mice may be the consequence of the absence of local $GABA_B$Rs, which are normally expressed in ovaries [67, 68] and testes [69], suggesting in this case that $GABA_B$Rs may be negatively modulating aromatase synthesis and/or activity. Although ovarian E_2 was increased in PND4 $GABA_{B1}$KO females, its serum levels are not readily detectable at this age [A.S. Kauffman, pers. observation; 70]. Furthermore, E_2 capability to surpass α-fetoprotein binding capacity is difficult to establish. Nevertheless, E_2 may play a role in decreasing ARC *Kiss1* expression in $GABA_{B1}$KO females, as this negative feedback loop is already functional at this early age [56]. It is also possible that the increase in ovarian E_2 content at this early age in $GABA_{B1}$KO females could disrupt normal ovary development and may contribute to the impaired reproduction previously observed in adult mice.

Collectively, these results contribute to the hypothesis that GABA is critical for sex differentiation and development of the ARC/MBH. GABA has been shown to be a key factor co-opted by perinatal E_2 to induce the sex differentiation of the ARC, and $GABA_A$Rs have been postulated as the pathway through which GABA exerts its developmental actions [1]. Our results demonstrate that $GABA_B$Rs also participate in this process, because in their absence the sex differences in key genes such as *Gad1*, *Kiss1* and *Gnrh1* are critically disturbed at PND4 in the ARC/MBH, probably altering the subsequent organization and development of the reproductive axis.

Acknowledgements

This work was supported by Consejo Nacional de Investigaciones Científicas y Técnicas, Argentina (CONICET; PIP 00363), Agencia Nacional de Promoción Científica y Tecnológica, Argentina (ANPCyT: PICT 2007 Nº 01050 to C.L., PICT 2006 Nº 00200 to V.L.-L. and PICT 2008 Nº 1383 to G.M.S.), Universidad de Buenos Aires, Argentina (UBA, ME 043 and 038) and the Swiss National Science Foundation (3100A0-117816 to B.B.). Additional support provided by NIH grant R01 HD065856 (A.S.K.). N.P.D.G. is supported by a CONICET PhD and by an ISN-CAEN fellowship.

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