

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_BRs 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.

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Introduction

Fetuses and newborns are exposed to gonadal steroids derived from their mother, their own gonads, and synthesized locally in their brains. Estradiol (E₂), 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-

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 E₂ 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- μ m 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% H₂O₂ 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 μ m 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 on 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 μ l 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- μ m, 25 cm \times 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 μ g of total RNA was reverse-transcribed in a 20- μ l 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- μ m-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 ($E = 10^{-(1/\text{slope})}$). 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 μ l 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
<i>Gnrh1</i>	qPCR	NM 008145	GAACCCAGCACTTCGAATGT	TGGCTTCCTCTTCAATCAGACTTT	94	58
<i>Gad1</i>	qPCR	NM 008077	GCGGGAGCGGATCCTAATA	TGGTGCATCCATGGGCTAC	79	58
<i>Kiss1</i>	qPCR	NM 178260	CAAAAGTGAAAGCCTGGATCC	GTTGTAGGTGGACAGGTCC	254	60
<i>Cyclophilin</i>	qPCR	NM 011149	GTGGCAAGATCGAAGTGGAGAAAC	TAAAAATCAGGCCTGTGGAATGTG	210	62
<i>Gapdh</i>	qPCR	NM 008084	TGCACCACCAACTGCTTAG	GGATGCAGGGATGATGTTTC	177	60
<i>Kiss1</i>	RT-PCR	NM 178260	AGCTGCTGCTTCTCCTCTGT	AGGCTTGCTCTCTGCATAACC	140	60
<i>Gapdh</i>	RT-PCR	NM 008084	CCAGAACATCATCCCTGCAT	GTTTCAGCTCTGGGATGACCTT	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 10⁸ 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 μ l 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 E₂ 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: E₂ 11.3 pg and T 125 pg. Intra- and interassay coefficients of variation were: E₂ 6.8 and 11.7% and T 7.8 and 12.3%, respectively. n = 4–6.

Statistics

Data are presented as the mean \pm 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 correla-

tion 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 brain 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 OVLTPOA, 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-

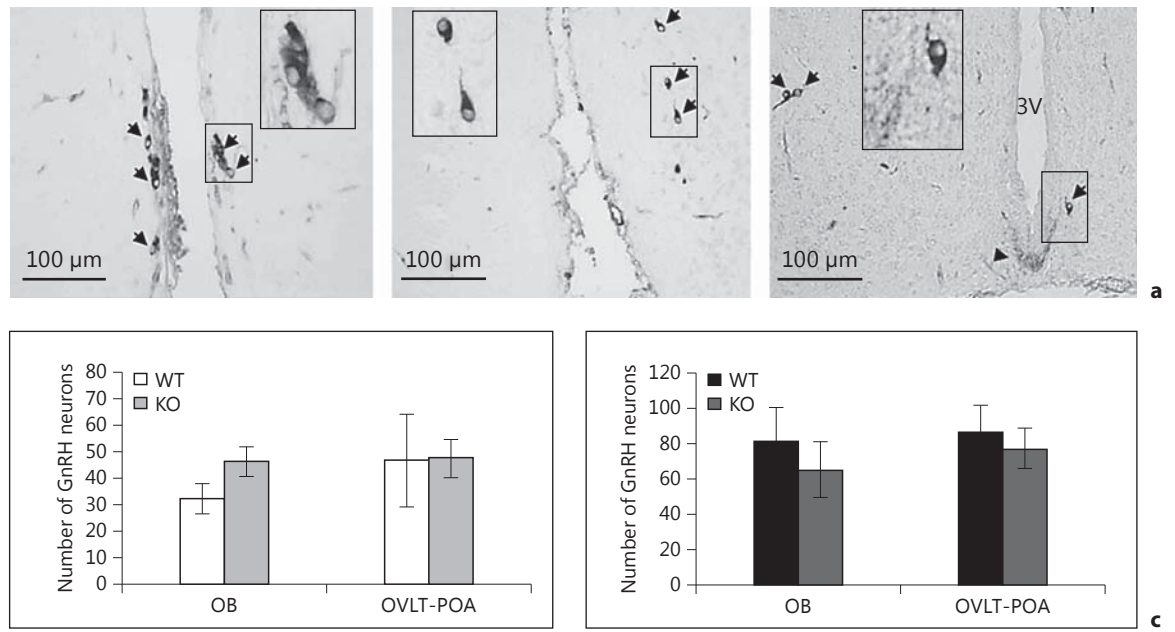


Fig. 1. Localization of ir-GnRH neurons (IHC) in coronal sections at PND4 mice. **a** Representative micrographs (20 \times) from OB (left panel), medial septum (medium panel) and OVLT-POA (right panel). Arrows show ir-GnRH cell bodies and arrowheads ir-fibers running caudally parallel to the brain floor. 3V = Third ventricle. **Inset** Detailed amplification of lined square. Number of animals =

4. Scale bar = 100 μ m. **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.

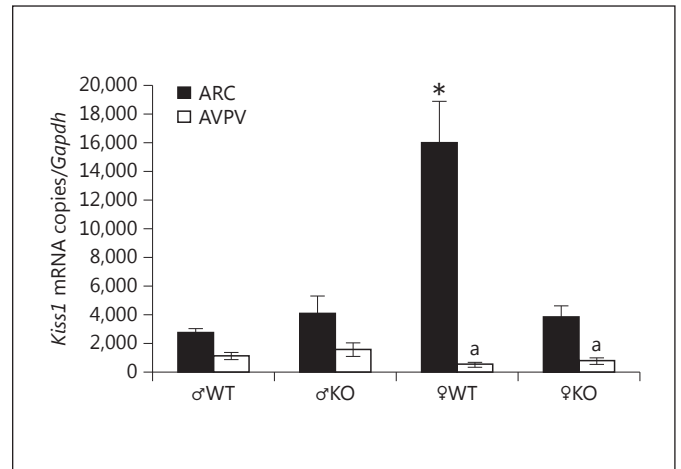
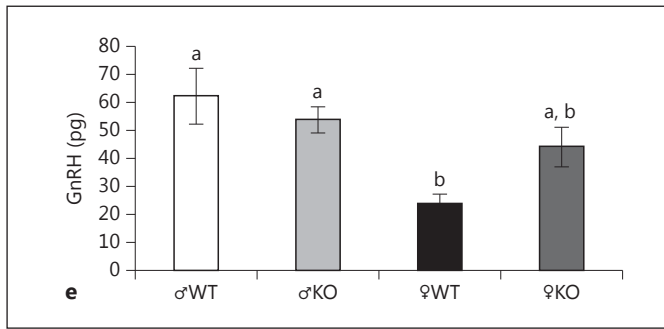
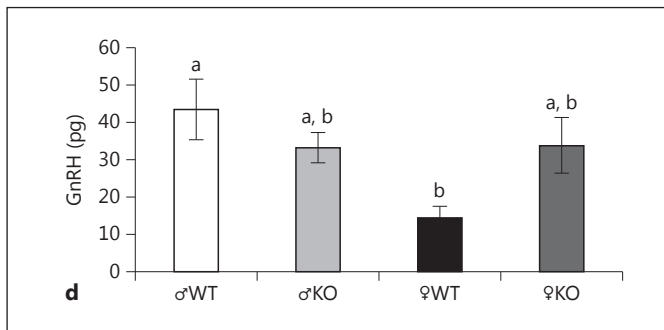
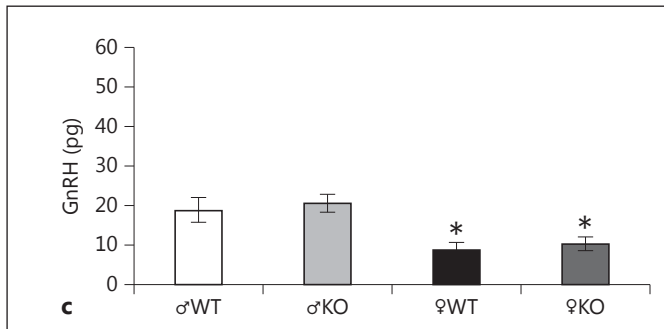
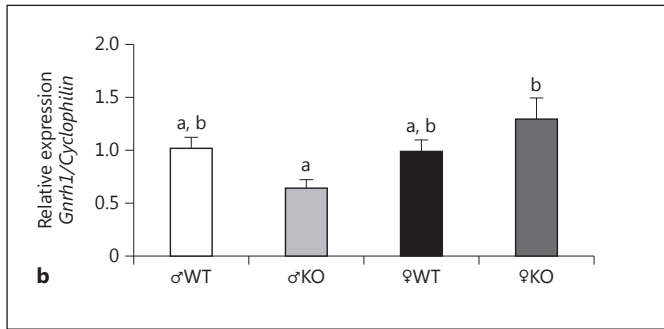
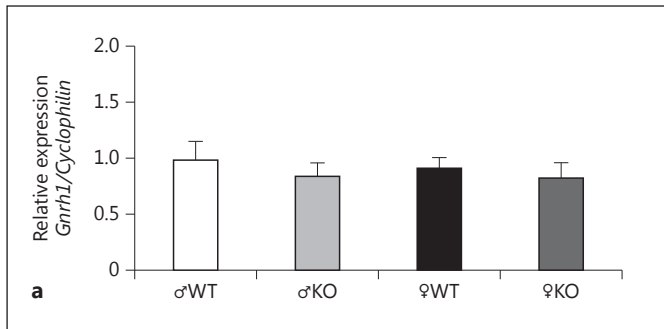


Fig. 3. *Kiss1* expression in discrete nuclei of the hypothalamus. *Kiss1* mRNA expression in micropunches of ARC and AVPV nuclei (qPCR). Three-way ANOVA, factors sex, genotype and nucleus: triple interaction: $p < 0.05$. * ARC *Kiss1* expression in WT females significantly different from ARC *Kiss1* 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.

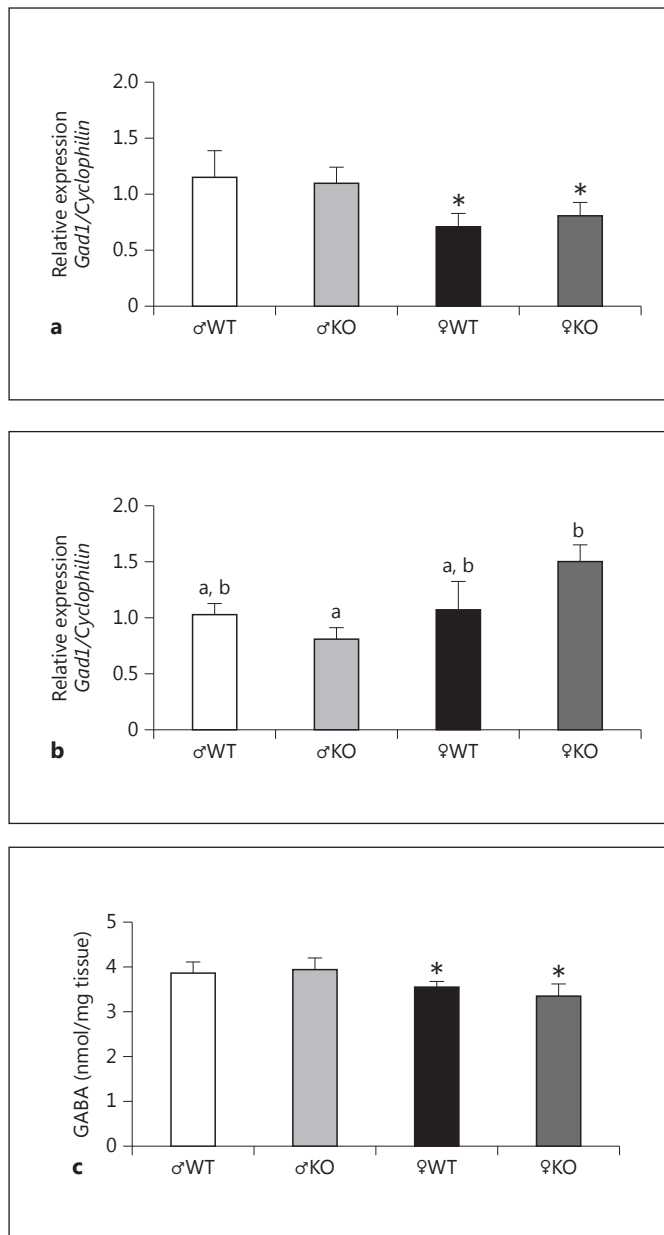


Fig. 4. *Gad1* expression and GABA amino acid hypothalamic content. *Gad1* mRNA expression (qPCR) in POA-AH (**a**) and MBH (**b**). GABA content (HPLC) in total HT (**c**). **a** Two-way ANOVA, factors sex and genotype: interaction: NS; main effect sex: $p < 0.05$; main effect genotype: NS. * *Gad1* 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 micro-punches 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

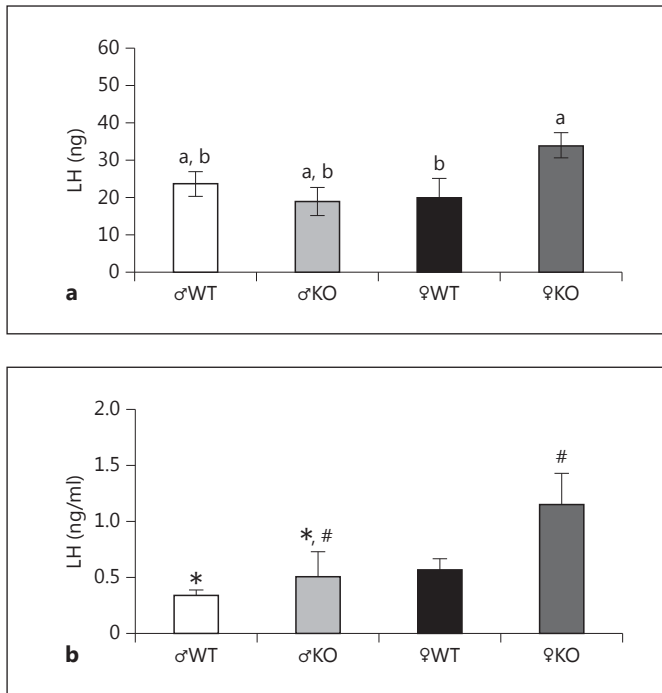


Fig. 5. Pituitary content and serum LH. Pituitary content (**a**, ng/pituitary, RIA) and serum LH (**b**, ng/ml, RIA). **a** Two-way ANOVA, factors sex and genotype: interaction: $p < 0.05$; different letters mean statistically significant differences, $p < 0.05$ or less: LH content in $GABA_{B1}KO$ 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 WT. 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).

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).

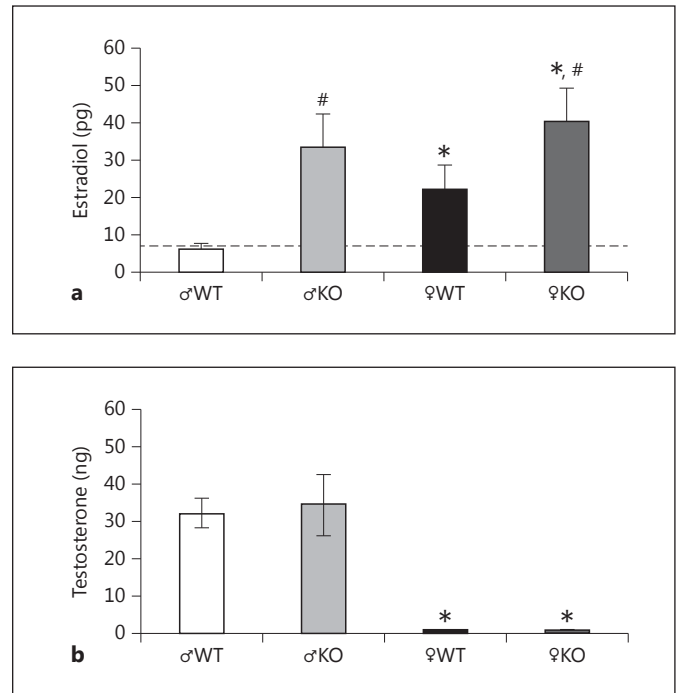


Fig. 6. Gonadal steroid hormones content. Gonadal E₂ (pg/ovary or testis, **a**) and T (ng/ovary or testis, **b**) contents (RIA). **a** Two-way ANOVA, factors sex and genotype: interaction: NS; main effect sex: $p < 0.05$; main effect genotype: $p < 0.01$. * E₂ content in females significantly different from males. # E₂ content in $GABA_{B1}KO$ mice significantly different from WT. 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.

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 E₂ in ovaries and testes. E₂ content was higher in females than in males ($p < 0.05$), as expected. Interestingly, GABA_{B1}KO mice showed an increase in E₂ 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_BRs causes alterations in the adult reproductive axis, mainly in females [5, 6]. Here, we were interested in addressing whether absence of GABA_BRs 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_BRs, 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_BRs 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_BRs. *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_BRs. It has been established that direct GABA action through GABA_ARs 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_BRs 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 E₂-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*-positive 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_BRs affects both genes in the same way. This observation also suggests that GABA_BRs 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_BRs affects *Gad1* expression in a different way in each gender. In adults, the lack of functional GABA_BRs 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_BRs 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 adenohipophyseal GABA_BR 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 ad-

dition, pituitary GABA_BRs are maximally expressed neonatally, and GABA_BR 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 E₂ 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 E₂ content in gonads of GABA_{B1}KO mice may be the consequence of the absence of local GABA_BRs, which are normally expressed in ovaries [67, 68] and testes [69], suggesting in this case that GABA_BRs may be negatively modulating aromatase synthesis and/or activity. Although ovarian E₂ 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, E₂ capability to surpass α -feto-protein binding capacity is difficult to establish. Nevertheless, E₂ 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 E₂ 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 E₂ to induce the sex differentiation of the ARC, and GABA_ARs have been postulated as the pathway through which GABA exerts its developmental actions [1]. Our results demonstrate that GABA_BRs 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.

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References

- Schwarz JM, McCarthy MM: Steroid-induced sexual differentiation of the developing brain: multiple pathways, one goal. *J Neurochem* 2008;105:1561–1572.
- Sheikh SN, Martin SB, Martin DL: Regional distribution and relative amounts of glutamate decarboxylase isoforms in rat and mouse brain. *Neurochem Int* 1999;35:73–80.
- McCarthy MM, Auger AP, Perrot-Sinal TS: Getting excited about GABA and sex differences in the brain. *Trends Neurosci* 2002;25:307–312.
- Maffucci JA, Gore AC: Hypothalamic neural systems controlling the female reproductive life cycle gonadotropin-releasing hormone, glutamate, and GABA; in Kwang WJ (ed): *International Review of Cell and Molecular Biology*. Burlington, Academic Press, 2009, pp 69–127.
- Catalano PN, Bonaventura MM, Silveyra P, Bettler B, Libertun C, Lux-Lantos VA: GABA_{B1} knockout mice reveal alterations in prolactin levels, gonadotropic axis, and reproductive function. *Neuroendocrinology* 2005;82:294–305.
- Catalano PN, Di Giorgio N, Bonaventura MM, Bettler B, Libertun C, Lux-Lantos VA: Lack of functional GABA_B receptors alters GnRH physiology and sexual dimorphic expression of GnRH and GAD-67 in the brain. *Am J Physiol Endocrinol Metab* 2010;298:E683–E696.
- Herbison AE, Moenter SM: Depolarising and hyperpolarising actions of GABA_A receptor activation on gonadotropin-releasing hormone neurones: towards an emerging consensus. *J Neuroendocrinol* 2011;23:557–569.
- Garcia-Galiano D, Pineda R, Roa J, Ruiz-Pino F, Sanchez-Garrido MA, Castellano JM, Aguilar E, Navarro VM, Pinilla L, Tena-Sempere M: Differential modulation of gonadotropin responses to kisspeptin by aminoacidergic, peptidergic, and nitric oxide neurotransmission. *Am J Physiol Endocrinol Metab* 2012;303:E1252–E1263.
- Fueshko SM, Key S, Wray S: Luteinizing hormone releasing hormone (LHRH) neurons maintained in nasal explants decrease LHRH messenger ribonucleic acid levels after activation of GABA_A receptors. *Endocrinology* 1998;139:2734–2740.
- Fujioka H, Yamanouchi K, Akema T, Nishihara M: The effects of GABA on embryonic gonadotropin-releasing hormone neurons in rat hypothalamic primary culture. *J Reprod Dev* 2007;53:323–331.
- Lee K, Porteous R, Campbell RE, Luscher B, Herbison AE: Knockdown of GABA_A receptor signaling in GnRH neurons has minimal effects upon fertility. *Endocrinology* 2010;151:4428–4436.
- Akema T, Kimura F: Modulation of pulsatile LH secretion by baclofen, a selective GABA_B receptor agonist, in ovariectomized rats. *Neuroendocrinology* 1992;56:141–147.
- Martínez de la Escalera G, Choi AL, Weiner RI: Biphasic GABAergic regulation of GnRH secretion in GT1 cell lines. *Neuroendocrinology* 1994;59:420–425.
- Mogulevsky JA, Wuttke W: Changes in the control of gonadotrophin secretion by neurotransmitters during sexual development in rats. *Exp Clin Endocrinol Diabetes* 2001;109:188–195.
- Constantin S, Piet R, Iremonger K, Hwa YS, Clarkson J, Porteous R, Herbison AE: GnRH neuron firing and response to GABA in vitro depend on acute brain slice thickness and orientation. *Endocrinology* 2012;153:3758–3769.
- Bergen HT, Hejtmanck JF, Pfaff DW: Effects of γ -aminobutyric acid receptor agonists and antagonist on LHRH-synthesizing neurons as detected by immunocytochemistry and in situ hybridization. *Exp Brain Res* 1991;87:46–56.
- Kang SH, Seong JY, Cho S, Cho H, Kim K: Acute increase of GABAergic neurotransmission exerts a stimulatory effect on GnRH gene expression in the preoptic/anterior hypothalamic area of ovariectomized, estrogen- and progesterone-treated adult female rats. *Neuroendocrinology* 1995;61:486–492.
- Cho BN, Kim K: Differential effect of baclofen on hypothalamic GnRH and pituitary LH β gene expression in steroid-treated rats. *Mol Cells* 1997;7:605–609.
- Heger S, Seney M, Bless E, Schwarting GA, Bilger M, Mungenast A, Ojeda SR, Tobet SA: Overexpression of glutamic acid decarboxylase-67 (GAD-67) in gonadotropin-releasing hormone neurons disrupts migratory fate and female reproductive function in mice. *Endocrinology* 2003;144:2566–2579.
- Lee JM, Tiong J, Maddox DM, Condie BG, Wray S: Temporal migration of gonadotropin-releasing hormone-1 neurones is modified in GAD67 knockout mice. *J Neuroendocrinol* 2008;20:93–103.
- Wierman ME, Kiseljak-Vassiliades K, Tobet S: Gonadotropin-releasing hormone neuron migration: initiation, maintenance and cessation as critical steps to ensure normal reproductive function. *Front Neuroendocrinol* 2011;32:43–52.
- Tobet SA, Bless EP, Schwarting GA: Developmental aspect of the gonadotropin-releasing hormone system. *Mol Cell Endocrinol* 2001;185:173–184.
- McClellan KM, Calver AR, Tobet SA: GABA_B receptors role in cell migration and positioning within the ventromedial nucleus of the hypothalamus. *Neuroscience* 2008;151:1119–1131.
- Bony G, Szczurkowska J, Tamagno I, Shelly M, Contestabile A, Cancedda L: Non-hyperpolarizing GABA_B receptor activation regulates neuronal migration and neurite growth and specification by cAMP/LKB1. *Nat Commun* 2013;4:1800.
- Fukui M, Nakamichi N, Yoneyama M, Ozawa S, Fujimori S, Takahata Y, Nakamura N, Taniura H, Yoneda Y: Modulation of cellular proliferation and differentiation through GABA_B receptors expressed by undifferentiated neural progenitor cells isolated from fetal mouse brain. *J Cell Physiol* 2008;216:507–519.
- Schwirtlich M, Emri Z, Antal K, Mate Z, Katarova Z, Szabo G: GABA_A and GABA_B receptors of distinct properties affect oppositely the proliferation of mouse embryonic stem cells through synergistic elevation of intracellular Ca²⁺. *FASEB J* 2010;24:1218–1228.
- Tu H, Rondard P, Xu C, Bertaso F, Cao F, Zhang X, Pin JP, Liu J: Dominant role of GABA_{B2} and G β for GABA_B receptor-mediated-ERK1/2/CREB pathway in cerebellar neurons. *Cell Signal* 2007;19:1996–2002.
- Fiorentino H, Kuczewski N, Diabira D, Ferland N, Pangalos MN, Porcher C, Gaiarsa JL: GABA_B receptor activation triggers BDNF release and promotes the maturation of GABAergic synapses. *J Neurosci* 2009;29:11650–11661.
- Kauffman AS: Coming of age in the kisspeptin era: sex differences, development, and puberty. *Mol Cell Endocrinol* 2010;324:51–63.
- Pinilla L, Aguilar E, Dieguez C, Millar RP, Tena-Sempere M: Kisspeptins and reproduction: physiological roles and regulatory mechanisms. *Physiol Rev* 2012;92:1235–1316.
- Zhang C, Bosch MA, Ronnekleiv OK, Kelly MJ: Gamma-aminobutyric acid B receptor-mediated inhibition of gonadotropin-releasing hormone neurons is suppressed by kisspeptin-G protein-coupled receptor 54 signaling. *Endocrinology* 2009;150:2388–2394.
- Fiorini Z, Jasoni CL: A novel developmental role for kisspeptin in the growth of gonadotropin-releasing hormone neurites to the median eminence in the mouse. *J Neuroendocrinol* 2010;22:1113–1125.
- Pielecka-Fortuna J, Chu Z, Moenter SM: Kisspeptin acts directly and indirectly to increase gonadotropin-releasing hormone neuron activity and its effects are modulated by estradiol. *Endocrinology* 2008;149:1979–1986.
- Schuler V, Luscher C, Blanchet C, Klis N, Sansig G, Klebs K, Schmutz M, Heid J, Gentry C, Urban L, Fox A, Spooren W, Jatou AL, Vigouret J, Pozza M, Kelly PH, Mosbacher J, Froestl W, Kaslin E, Korn R, Bischoff S, Kaupmann K, van der Putten H, Bettler B: Epilepsy, hyperalgesia, impaired memory, and loss of pre- and postsynaptic GABA_B responses in mice lacking GABA_{B1}. *Neuron* 2001;31:47–58.
- Bonthuis PJ, Cox KH, Searcy BT, Kumar P, Tobet S, Rissman EF: Of mice and rats: key species variations in the sexual differentiation of brain and behavior. *Front Neuroendocrinol* 2010;31:341–358.

- 36 Raynaud JP: Influence of rat estradiol binding plasma protein on uterotrophic activity. *Steroids* 1973;21:249–258.
- 37 Belayew A, Tilghman SM: Genetic analysis of α -fetoprotein synthesis in mice. *Mol Cell Biol* 1982;2:1427–1435.
- 38 Park MK, Wakabayashi K: Preparation of a monoclonal antibody to common amino acid sequence of LHRH and its application. *Endocrinol Jpn* 1986;33:257–272.
- 39 Paxinos G, Halliday G, Watson C, Koutchev Y, Wang HQ: Atlas of the developing mouse brain at E17.5, P0, and P6. Amsterdam, Academic Press, 2007.
- 40 Soriano EM, Fernandez MO, Catalano PN, Mongiat LA, Somoza GM, Libertun C, Lux-Lantos VA: Differential gonadotropin releasing hormone (GnRH) expression, autoregulation and effects in two models of rat luteinized ovarian cells. *Life Sci* 2005;77:2141–2155.
- 41 Bianchi MS, Catalano PN, Bonaventura MM, Silveyra P, Bettler B, Libertun C, Lux-Lantos VA: Effect of androgens on sexual differentiation of pituitary γ -aminobutyric acid receptor subunit GABA_B expression. *Neuroendocrinology* 2004;80:129–142.
- 42 Kim J, Semaan SJ, Clifton DK, Steiner RA, Dhamija S, Kauffman AS: Regulation of Kiss1 expression by sex steroids in the amygdala of the rat and mouse. *Endocrinology* 2011;152:2020–2030.
- 43 Gonzalez B, Ratner LD, Di Giorgio NP, Poutanen M, Huhtaniemi IT, Calandra RS, Lux-Lantos VA, Rulli SB: Endogenously elevated androgens alter the developmental programming of the hypothalamic-pituitary axis in male mice. *Mol Cell Endocrinol* 2011;332:78–87.
- 44 Pfaffl MW: A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* 2001;29:e45.
- 45 Semaan SJ, Dhamija S, Kim J, Ku EC, Kauffman AS: Assessment of epigenetic contributions to sexually-dimorphic Kiss1 expression in the anteroventral periventricular nucleus of mice. *Endocrinology* 2012;153:1875–1886.
- 46 Semaan SJ, Murray EK, Poling MC, Dhamija S, Forger NG, Kauffman AS: BAX-dependent and BAX-independent regulation of Kiss1 neuron development in mice. *Endocrinology* 2010;151:5807–5817.
- 47 Lowry OH, Rosebrough NH, Farr AL, Randall RJ: Protein measurement with Folin phenol reagent. *J Biol Chem* 1951;193:265–275.
- 48 Livne I, Gibson MJ, Silverman AJ: Biochemical differentiation and intercellular interactions of migratory gonadotropin-releasing hormone cells in the mouse. *Dev Biol* 1993;159:643–656.
- 49 Kimura F, Funabashi T: Two subgroups of gonadotropin releasing hormone neurons control gonadotropin secretion in rats. *News Physiol Sci* 1998;13:225–231.
- 50 Herman AP, Tomaszewska-Zaremba D: Effect of endotoxin on the expression of GnRH and GnRHR genes in the hypothalamus and anterior pituitary gland of anestrus ewes. *Anim Reprod Sci* 2010;120:105–111.
- 51 Wierman ME, Pawlowski JE, Allen MP, Xu M, Linseman DA, Nielsen-Preiss S: Molecular mechanisms of gonadotropin-releasing hormone neuronal migration. *Trends Endocrinol Metab* 2004;15:96–102.
- 52 Livne I, Gibson MJ, Silverman AJ: Biochemical differentiation and intercellular interactions of migratory gonadotropin-releasing hormone cells in the mouse. *Dev Biol* 1993;159:643–656.
- 53 Gore AC, Roberts JL, Gibson MJ: Mechanisms for the regulation of gonadotropin-releasing hormone gene expression in the developing mouse. *Endocrinology* 1999;140:2280–2287.
- 54 Gore AC, Roberts JL: Regulation of gonadotropin-releasing hormone gene expression in vivo and in vitro. *Front Neuroendocrinol* 1997;18:209–245.
- 55 Cao J, Patisaul HB: Sexually dimorphic expression of hypothalamic estrogen receptors α and β and Kiss1 in neonatal male and female rats. *J Comp Neurol* 2011;519:2954–2977.
- 56 Takumi K, Iijima N, Ozawa H: Developmental changes in the expression of kisspeptin mRNA in rat hypothalamus. *J Mol Neurosci* 2011;43:138–145.
- 57 Davis AM, Grattan DR, Selmanoff M, McCarthy MM: Sex differences in glutamic acid decarboxylase mRNA in neonatal rat brain: implications for sexual differentiation. *Horm Behav* 1996;30:538–552.
- 58 Flügge G, Wuttke W, Fuchs E: Postnatal development of transmitter systems: sexual differentiation of the GABAergic system and effects of muscimol. *Int J Dev Neurosci* 1986;4:319–326.
- 59 Takumi K, Iijima N, Iwata K, Higo S, Ozawa H: The effects of gonadal steroid manipulation on the expression of Kiss1 mRNA in rat arcuate nucleus during postnatal development. *J Physiol Sci* 2012;62:453–460.
- 60 Poling MC, Kauffman AS: Sexually dimorphic testosterone secretion in prenatal and neonatal mice is independent of kisspeptin-Kiss1r and GnRH signaling. *Endocrinology* 2012;153:782–793.
- 61 Tobet SA, Schwarting GA: Minireview: recent progress in gonadotropin-releasing hormone neuronal migration. *Endocrinology* 2006;147:1159–1165.
- 62 Wu TJ, Gibson MJ, Rogers MC, Silverman AJ: New observations on the development of the gonadotropin-releasing hormone system in the mouse. *J Neurobiol* 1997;33:983–998.
- 63 Silverman AJ, Jhamandas J, Renaud LP: Localization of luteinizing hormone-releasing hormone neurons that project to the median eminence. *J Neurosci* 1987;7:2312–2319.
- 64 Lux-Lantos V, Becu-Villalobos D, Bianchi M, Rey-Roldán EB, Chamson-Reig A, Pignataro O, Libertun C: GABA_B receptors in anterior pituitary cells. Mechanism of action coupled to endocrine effects. *Neuroendocrinology* 2001;73:334–343.
- 65 Bianchi MS, Rey-Roldán EB, Bettler B, Ristig D, Malitschek B, Libertun C, Lux-Lantos V: Ontogenic expression of anterior pituitary GABA_B receptor subunits. *Neuropharmacology* 2001;40:185–192.
- 66 Andric N, Ascoli M: Mutations of the lutropin/choriogonadotropin receptor that do not activate the phosphoinositide cascade allow hCG to induce aromatase expression in immature rat granulosa cells. *Mol Cell Endocrinol* 2008;285:62–72.
- 67 Kaupmann K, Huggel K, Heid J, Flor P, Bischoff S, Mickel S, McMaster G, Angst C, Bittiger H, Froestl W, Bettler B: Expression cloning of GABA_B receptors uncovers similarity to metabotropic glutamate receptors. *Nature* 1997;386:239–246.
- 68 Kaupmann K, Malitschek B, Schuler B, Heid J, Froestl W, Beck P, Mosbacher J, Bischoff S, Kulik A, Shigemoto R, Karschin A, Bettler B: GABA_B receptor subtypes assemble into functional heteromeric complexes. *Nature* 1998;396:683–687.
- 69 Vidal RL, Ramirez A, Castro M, Concha II, Couve A: Marlin-1 is expressed in testis and associates to the cytoskeleton and GABA_B receptors. *J Cell Biochem* 2008;103:886–895.
- 70 Haisenleder DJ, Schoenfelder AH, Marcinko ES, Geddis LM, Marshall JC: Estimation of estradiol in mouse serum samples: evaluation of commercial estradiol immunoassays. *Endocrinology* 2011;152:4443–4447.