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RESEARCH ARTICLE

GABAergic input through $GABA_B$ receptors is necessary during a perinatal window to shape gene expression of factors critical to reproduction such as *Kiss1*

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Bizzozzero-Hiriart M, Di Giorgio NP, Libertun C, Lux-Lantos V. GABAergic input through $GABA_B$ receptors is necessary during a perinatal window to shape gene expression of factors critical to reproduction such as *Kiss1*. *Am J Physiol Endocrinol Metab* 318: E901–E919, 2020. First published April 14, 2020; doi:10.1152/ajpendo.00547.2019.—Lack of $GABA_B$ receptors in $GABA_{B1}$ knockout mice decreases neonatal ARC kisspeptin 1 (*Kiss1*) expression in the arcuate nucleus of the hypothalamus (ARC) in females, which show impaired reproduction as adults. Our aim was to selectively impair $GABA_B$ signaling during a short postnatal period to evaluate its impact on the reproductive system. Neonatal male and female mice were injected with the $GABA_B$ antagonist CGP 55845 (CGP, 1 mg/kg body wt sc) or saline from postnatal *day 2* (*PND2*) to *PND6*, three times per day (8 AM, 1 PM, and 6 PM). One group was killed on *PND6* for collection of blood samples (hormones by radioimmunoassay), brains for gene expression in the anteroventral periventricular nucleus-periventricular nucleus continuum (AVPV/PeN), and ARC micropunches [quantitative PCR (qPCR)] and gonads for qPCR, hormone contents, and histology. A second group of mice was injected with CGP (1 mg/kg body wt sc) or saline from *PND2* to *PND6*, three times per day (8 AM, 1 PM, and 6 PM), and left to grow to adulthood. We measured body weight during development and parameters of sexual differentiation, puberty onset, and estrous cycles. Adult mice were killed, and trunk blood (hormones), brains for qPCR, and gonads for qPCR and hormone contents were obtained. Our most important findings on *PND6* include the CGP-induced decrease in ARC *Kiss1* and increase in neurokinin B (*Tac2*) in both sexes; the decrease in AVPV/PeN tyrosine hydroxylase (*Th*) only in females; the increase in gonad estradiol content in both sexes; and the increase in primordial follicles and decrease in primary and secondary follicles. Neonatally CGP-treated adults showed decreased ARC *Kiss1* and ARC gonadotropin-releasing hormone (*Gnrh1*) and increased ARC glutamic acid decarboxylase 67 (*Gad1*) only in males; increased ARC $GABA_B$ receptor subunit 1 (*Gabbr1*) in both sexes; and decreased AVPV/PeN *Th* only in females. We demonstrate that ARC *Kiss1* expression is chronically downregulated in males and that the normal sex difference in AVPV/PeN *Th* expression is abolished. In conclusion, neonatal GABAergic input through $GABA_B$ receptors shapes gene expression of factors critical to reproduction.

GABA; $GABA_B$ receptors; gonadotropins; Kiss1; sex steroids

INTRODUCTION

The gonadotropin-releasing hormone (GnRH) neurons represent the final output pathway of the neuronal network controlling reproduction in all mammalian species. GnRH neuron physiology is regulated by external (e.g., light and temperature), as well as internal (e.g., neurotransmitters, neuropeptides, and sex steroids), inputs. Kisspeptins (22, 85) and GABA (2, 27, 49, 56, 79) are two of the most significant factors regulating GnRH neurons in all stages of life.

The circuitry that will control reproduction in adults is sex-specific, and its establishment starts during embryonic life and ends around puberty. Perinatal sex steroids are the main factor contributing to this sexual dimorphism: they induce organizational effects on critical traits, such as the onset of the gonadotropin surge in females, but not in males (4, 34, 60, 64).

GABA, the main inhibitory neurotransmitter in the brain, can function as an important stimulatory developmental signal early in life. GABA, acting on both $GABA_A$ and $GABA_B$ receptors ($GABA_AR$ and $GABA_BR$), regulates migration, neurite growth, synapse formation, and plasticity (30–32, 52, 92). Regarding reproduction, GABA participates in the migration of GnRH neurons from the olfactory placode into the hypothalamus. During this developmental stage, GnRH neurons express $GABA_AR$ and $GABA_BR$ (93). Furthermore, GABA contributes to puberty onset and regulates the preovulatory luteinizing hormone (LH) surge in adult females (17, 41, 61, 68, 91, 93, 95). Interestingly, some of the sex-differentiating actions of testosterone/estradiol (E_2) are brought about by various mediators, including GABA (5, 62), particularly in the arcuate nucleus of the hypothalamus (ARC) (63).

Two main reproduction-related neuron populations are located in the hypothalamus, in the anteroventral periventricular nucleus-periventricular nucleus continuum (AVPV/PeN) and the ARC. AVPV/PeN kisspeptin 1 (*Kiss1*) neurons project to and stimulate GnRH cell bodies, whereas ARC *Kiss1* neurons also project to GnRH cell bodies but, mainly, contact GnRH fiber terminals in the median eminence controlling GnRH secretion (45, 55, 78). Expression of *Kiss1* in the AVPV/PeN has been reported to appear around postnatal *day 10* (*PND10*) in rodents, although recent work demonstrated a transient male-specific increase during late embryogenesis, peaking on the day of birth and disappearing soon thereafter (20). In adults, AVPV/PeN *Kiss1* expression is sexually dimorphic

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(females >> males) (18, 43), and circulating E_2 is essential for the prepubertal developmental increase in kisspeptin within this area in females (16). ARC *Kiss1* is expressed during embryonic life in both sexes. Perinatally, its expression is transiently higher in females than in males; it then increases to attain adult levels around puberty, without marked sex differences (82, 84). However, some recent studies show increased ARC kisspeptin immunoreactivity in adult gonadally intact and gonadectomized female mice compared with males (12). Interestingly, ARC *Kiss1* neurons already contact GnRH neurons during embryonic development (20, 54). The sex differences in the adult *Kiss1* neuron populations reflect the organizational effects of the sex steroids mentioned above.

The phenotype and function of *Kiss1* neurons differ between the ARC and the AVPV/PeN. Rodent ARC *Kiss1* neurons coexpress dynorphin and neurokinin B (known as KNDy neurons), whereas AVPV/PeN neurons do not; AVPV/PeN *Kiss1* neurons coexpress tyrosine hydroxylase (*Th*), while ARC *Kiss1* neurons do not (43, 44, 71). While AVPV/PeN *Kiss1* neurons are essential for the trigger of the preovulatory GnRH/LH surge in females, ARC *Kiss1* neurons provide tonic stimulatory input to GnRH neurons and the sex steroid-mediated negative feedback to gonadotropin secretion in both sexes (45, 73). In addition, ARC KNDy neurons are now postulated to form a critical component of the GnRH pulse generator (65, 69, 70).

In adult rodents, kisspeptin neurons express both $GABA_AR$ and $GABA_BR$, and effects of each of these receptors have been described (3, 23, 26, 28, 35). Whether GABA also modulates kisspeptin neurons early in development, similar to its effects on GnRH neurons, is not well established. A recent study demonstrated activation of *Kiss1* gene expression by muscimol, a $GABA_A$ agonist, in kisspeptin-producing rHypoE8 cells, as well as in primary cultures of the neuronal cells from fetal rat brains (42). These results suggest that GABA receptors might be active at this developmental age. We previously showed that female *PND4* mice lacking $GABA_B$ signaling ($GABA_{B1}KO$) show decreased ARC *Kiss1* expression, thus eliminating the normal sex difference in *Kiss1* expression observed in this area at this stage of development (25). Remarkably, adult $GABA_{B1}KO$ females have compromised reproduction (14), without alterations in AVPV/PeN or ARC *Kiss1* expression (26).

Our aim was to selectively impair $GABA_B$ signaling during a short postnatal period (*PND2–PND6*) critical in the organization of neuronal connections to evaluate its impact on the reproductive system at different end points (neonatal and adult mice), with particular interest in the ARC kisspeptin neuron population, which is active during this postnatal stage.

MATERIALS AND METHODS

Animals

BALB/c mice from the Instituto de Biología y Medicina Experimental (IBYME) colony were housed in groups in ventilated racks (22°C), with lights on from 7 AM to 7 PM, 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-National Scientific and Technical Research Council), in accordance with the Division of Animal Welfare, Office for Protection from Research Risks, National Institutes of Health, Animal Welfare Assurance for the Institute of Biology and Experimental Medicine (protocol no. 016/2017). Breeding pairs were kept to provide successive litters. Litters were weaned at 21 days of age.

For the following experiments we used CGP 55845 (CGP), a potent selective $GABA_BR$ antagonist with an IC_{50} of 5 nM. Its potency is >1,000-fold higher than that reported previously for other $GABA_BR$ antagonists (9). Neonatal male and female mice were subcutaneously injected with CGP [1 mg/kg body weight (BW); F CGP and M CGP groups] or saline (F control and M control groups) from *PND2* to *PND6* ± 1, three times per day (8 AM, 1 PM, and 6 PM). Mice were killed at different end points. In *protocol 1*, mice were euthanized at 3 PM on the day of the last injections (after 2 injections on this day at 8 AM and 1 PM) under minimal stress for collection of blood samples for hormone measurements [radioimmunoassay (RIA)], brains for quantitative PCR (qPCR) assays, and gonads for qPCR assays, hormone contents (RIA), and histology. In *protocol 2*, mice were treated as described for *protocol 1* but were left to grow to adulthood. BW was measured during postnatal development (*PND7* and *PND21*), and parameters of sexual differentiation and puberty onset were determined. In adulthood, estrous cycles were determined in females, and mice were killed (females in diestrus). Trunk blood was collected for hormone measurements (RIA), brains for qPCR assays, and gonads for qPCR assays and hormone contents (RIA).

In addition, nontreated adult male BALB/c mice were injected with CGP (1 mg/kg sc) or saline as control for 5 days, three times per day (8 AM, 1 PM, and 6 PM). Mice were killed at 3 PM (after 2 injections on the last day) under minimal stress for collection of brains for qPCR assays.

Assessment of Sexual Differentiation and Puberty Onset

Sexual differentiation was assessed by evaluating the anogenital index [AGI = anogenital distance (AGD)/BW] on *PND21* (38). It has been shown that AGD covaries with body size, so normalization to BW is warranted (36).

Puberty onset was determined in females by the age of vaginal opening (VO) (89) and males by the age of preputial separation (PS) (53). Females were examined daily starting on *PND21* and males starting on *PND24*. In both cases, these measurements were also calculated relative to BW.

RNA Isolation and Reverse Transcription

Brains obtained from *protocols 1* and *2* were frozen on dry ice and kept at −70°C. ARC and AVPV/PeN micropunches from CGP-treated and control mice in *protocols 1* and *2* were obtained from 400- and 500-μm-thick frozen brain slices, respectively, as previously described (25, 76). Medial amygdala (MeA) and ARC micropunches from adult male CGP-treated and control mice were obtained from 400-μm-thick frozen brain slices (26). In addition, from another group of mice in *protocol 1*, preoptic area-anterior hypothalamic fragments (POA-AH) were obtained for *Gnrh1* expression, as previously described (26).

Total RNA from brain micropunches (ARC, AVPV/PeN, and MeA), POA-AH fragments, and gonads was isolated using TRI Reagent (Molecular Research Center, Inc.) according to the manufacturer's protocol and stored at −70°C. A 20-μL reaction using Moloney murine leukemia virus retrotranscriptase (RT) (Invitrogen) and $oligo(dT)_{15}$ primers (Biodynamics, Buenos Aires, Argentina) was used for reverse transcription of 0.4–1 μg of total RNA. The RT 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 it was used for qPCR.

Gene Expression Assays

Quantitative PCR. Primer sets were designed for the specific amplifications of murine genes encoding for *Kiss1*, *Kiss1* receptor

(*Kiss1r*), neurokinin B (*Tac2*), prodynorphin (*Pdyn*), *Th, Gnrh1*, estrogen receptor-α (*Esr1*), estrogen receptor-β (*Esr2*), progesterone receptor (*Pgr*), aromatase (*Cyp19A1*), glutamic acid decarboxylase 67 (*Gad1*, the rate-limiting enzyme in GABA synthesis), $GABA_BR$ subunits 1 and 2 (*Gabbr1* and *Gabbr2*), brain-derived neurotropic factor (*Bdnf*), and the housekeeping control gene cyclophilin B (*Ppib2*) (Table 1). For qPCR, the amplification efficiency of each primer set was calculated from the slope of a standard amplification curve of log(ng cDNA) per reaction vs. C_t value [$E = 10^{-(1/\text{slope})}$]. Efficiencies of 2 ± 0.1 were considered optimal. Quantitative measurements of *Kiss1r, Tac2, Pdyn, Th, Esr1, Esr2, Pgr, Gnrh1, Cyp19A1, Gad1, Gabbr1, Gabbr2*, and *Ppib2* cDNA were performed by qPCR in a total volume of 10 μL, as previously described (24), whereas *Kiss1* qPCR was performed in a total volume of 30 μL. Amplification was carried out in StepOne real-time PCR systems (Applied Biosystems). Each sample was analyzed in duplicate along with nontemplate controls to monitor possible contamination. Quantitative differences in the cDNA target between samples were determined as previously described using the mathematical model of Pfaffl (77), which compares expression with that in a single randomly selected calibrator male.

To determine quantitative *Kiss1, Kiss1r, Tac2, Pdyn, Pgr, Gnrh1*, and *Bdnf* expression in micropunches, qPCR was performed using FastStart Universal SYBR green master mix (ROX) (Sigma-Aldrich). Quantitative *Th, Esr1, Esr2, CypA19, Gad1, Gabbr1, Gabbr2*, and *Ppib2* gene expression was measured with 5× HOT FIREpol EvaGreen qPCR Mix Plus (ROX) (Solis Biodyne).

Hormone determinations by RIA. Trunk blood was collected from mice of the different experimental groups, and sera were obtained and frozen for hormone determinations. Serum LH and follicle-stimulating hormone (FSH) levels were determined by RIA with kits from the National Institute of Diabetes and Digestive and Kidney Diseases National Hormone and Pituitary Program and Dr. A. F. Parlow, as previously described (25). Results are expressed in terms of RP3 rat LH and FSH standards, as these systems recognize mouse samples. Assay sensitivities were 0.15 ng/ml for LH and 1.56 ng/ml for FSH. Intra- and interassay coefficients of variation were 7.2 and 11.4% for LH and 8.0 and 13.2% for FSH, respectively.

Ovarian and testicular E_2 and testosterone contents were determined by RIA using specific antisera (kindly provided by Dr. G. D. Niswender, Colorado State University, Fort Collins, CO) after ethyl ether extraction (25). Tritiated hormones were purchased from New England Nuclear. Assay sensitivities were 13.5 pg for E_2 and 125 pg for testosterone. Intra- and interassay coefficients of variation were 6.8 and 11.7% for E_2 and 7.8 and 12.3% for testosterone, respectively.

Gonad morphological analysis. Ovaries and testes from *PND6* male and female CGP-treated and control mice were fixed in 4% buffered formalin for 12 h at room temperature and embedded in paraffin for morphological studies. Ovaries and testes were cut in a microtome and mounted at 50-μm intervals onto microscope slides to prevent counting the same structures twice. Slides were stained with hematoxylin-eosin. For ovaries, the numbers of primordial, primary, and secondary follicles were determined under a light microscope, as described elsewhere (11). Results are expressed as the number of specific ovarian structures relative to total structures (%). For testes, morphological structures were analyzed and compared between treatments. There were four animals in each group.

Statistics

Data are presented as means ± SE. The differences between means of two groups were analyzed by two-tailed *t* test. More than two groups were analyzed by two-way ANOVA, with sex and treatment as main factors, followed by post hoc tests. Different measurements in the same animal were evaluated by repeated-measures ANOVA. Differences in percentages were analyzed with the χ^2 test, or data were arcsine-transformed for conversion from a binomial to a normal distribution and analyzed by ANOVA or Student's *t* test. $P < 0.05$ was considered statistically significant. Analyses were performed with Statistica (data analysis software system) version 8.0 (StatSoft, Inc., 2007).

RESULTS

Effect of CGP Administration on Hypothalamic Gene Expression on PND6

Previous work in neonatal $GABA_{B1}$KO mice lacking functional $GABA_BR$ showed that the absence of $GABA_B$ signaling in female mice decreased *Kiss1* mRNA expression in the ARC, but not the AVPV/PeN, which has very low expression at this age (25). Adult $GABA_{B1}$KO females have compromised reproduction (14).

To evaluate whether blocking the activation of $GABA_BR$ by a pharmacological approach had a similar effect, male and

Table 1. *Primer sequences and annealing temperature for gene expression assays by quantitative PCR*

Gene	GenBank Accession No.	Primer		Annealing Temp, °C
		Forward (5′–3′)	Reverse (5′–3′)	
Kiss1	NM 178260	CAAAAGTGAAGCCTGGATCC	GTTGTAGGTGGACAGGTCC	60
Kiss1r	NM 053244	TGCAA ATTCGTCAAC TACATC	GGAACACAGTCACATACCAG	55
Tac2	NM 009312	CGTGACATGCACGACTTC	CCAACAGGAGGACCTTAC	60
Pdyn	NM 018863	AGCTTGCCTCCTCGTGATG	GGCACTCCAGGGAGCAAAT	60
Pgr	NM 008829	GGTCCTTGGAGGTCGTAAGT	CTGGCTCTCGTTAGGAAGGC	60
Th	NM 009377	CCAGAGAGGACAAGGTTCCC	ATACGCCTGGTCAGAGAAGC	60
Cyp19a1	NM 007810	CGGGCTACGTGGATGTGTT	GAGCTTGCCAGGCGTTAAAG	55
Esr1	NM 007956	ATGAAAGGCGGCATACGGAAAG	CACCCATTTCATTTCGGCCTTC	60
Esr2	NM 207707	CCAGACTGCAAGCCCAAATGT	AGAAGCGATGATTGGCAGTGG	55
Gnrh1	NM 008145	GAACCCCAGCACTTCGAATGT	TGGCTTCCTCTTCAATCAGACTTT	58
Gabbr1	NM 019439	AGATGACTGAGGCGGTGGA	TTCAGCCGCTTGGTTAGTTTC	59
Gabbr2	NM 001081141	GGCTACATCGGAGTGG	TGTATTCTCTTTCGTATTGCT	54
Gad1	NM 008077	GCGGGAGCGGATCCTAATA	TGGTGCATCCATGGGCTAC	58
Bdnf	NM 007540	GGTATCCAAAGGCCAACTGA	CTTATGAATCGCCAGCCAAT	56
Ppib2	NM 011149	GACCCTCCGTGGCCAACGAT	ACGACTCGTCCTACAGATTCATCTC	60

Bdnf, brain-derived neurotropic factor; *Cyp19a1*, aromatase; *Esr1* and *Esr2*, estrogen receptors-α and -β; *Gabbr1* and *Gabbr2*, $GABA_B$ receptor subunits 1 and 2; *Gad1*, glutamic acid decarboxylase 67; *Gnrh*, gonadotropin-releasing hormone; *Kiss1*, kisspeptin 1; *Kiss1r*, *Kiss1* receptor; *Pdyn*, prodynorphin; *Pgr*, progesterone receptor; *Ppib2*, cyclophilin B (housekeeping gene); *Tac2*, neurokinin B; *Th*, tyrosine hydroxylase.

female mice were injected with CGP or saline (control) three times per day from *PND2* to *PND6*.

We observed the typical sex difference (females > males) in ARC *Kiss1* expression at early stages of postnatal life. In addition, *Kiss1* expression was significantly reduced in the ARC of CGP-treated males and females, while the sex difference remained [2-way ANOVA: interaction, not significant (NS); main factor sex, $P < 0.001$; main factor treatment, $P < 0.001$; Fig. 1*A*].

As ARC KNDy neurons also express *Tac2* and *Pdyn*, these genes were also evaluated. Interestingly, *Tac2* showed the same sex differences (females > males) as *Kiss1*, but, in contrast to *Kiss1, Tac2* expression was significantly increased in both sexes by CGP treatment (2-way ANOVA: interaction, NS; main factor sex, $P < 0.001$; main factor treatment, $P < 0.001$; Fig. 1*B*), suggesting that these two genes are oppositely regulated by $GABA_BR$ in this area at *PND6*. *Pdyn* expression showed a clear sex difference (females > males) without treatment differences (2-way ANOVA: interaction, NS; main factor sex, $P < 0.001$; main factor treatment, NS; Fig. 1*C*).

To evaluate whether CGP indirectly affects the expression of *Kiss1* and *Tac2* by modulating the expression of other genes in the hypothalamus, we measured ARC mRNA levels of aromatase (*Cyp19a1*), estrogen receptors-α and -β (*Esr1* and *Esr2*), progesterone receptor (*Pgr*), kisspeptin receptor (*Kiss1r*), glutamic acid decarboxylase 67 (the rate-limiting enzyme of GABA synthesis, *Gad1*), *Gabbr1*, and *Gabbr2*. CGP did not modify the expression of any of these genes (Fig. 1, *D–K*).

In AVPV/PeN punches, CGP treatment did not modify *Kiss1*, *Kiss1r*, *Cyp19a1*, *Esr1*, *Esr2*, *Pgr*, *Gabbr1*, *Gabbr2*, or *Gad1* expression (Fig. 2, *A–I*). Interestingly, *Th* expression showed a clear sex difference (females > males) in controls in this area at *PND6*. This sex difference was completely abolished in CGP-treated mice, as *Th* expression in CGP-treated females decreased to male levels (Fig. 2*J*; 2-way ANOVA: interaction, $P < 0.001$; F control significantly different from F CGP, M control, and M CGP, $P < 0.02$ or less).

Additionally, *Gnrh1* expression in POA-AH fragments was also evaluated in control and CGP-treated mice but did not show sex or treatment differences (data not shown).

Effect of CGP Administration to Adult Male Mice on ARC and MeA Kiss1 Expression and on MeA Bdnf Expression

In adult $GABA_{B1}$KO mice, which lacked $GABA_B$ signaling from conception onward, *Kiss1* expression was normal in the ARC and AVPV/PeN but dramatically increased in extrahypothalamic areas such as the MeA, bed nucleus of the stria terminalis, and lateral septum (26). Here we evaluated whether the same CGP administration scheme and dose that decreased *Kiss1* expression in the neonate ARC affected *Kiss1* expression in the MeA in adult males. We examined the ARC as a control area. No changes in *Kiss1* expression were observed (Fig. 3, *A* and *B*). As a positive control, *Bdnf* expression, which has been shown to be modified by $GABA_B$ analogs (51), was assessed in the MeA punches. CGP significantly decreased *Bdnf* mRNA expression in the MeA to one-third of that in controls (Fig. 3*C*; $P < 0.001$).

Thus, whereas CGP treatment is able to modify *Kiss1* expression in neonates, it no longer does so when administered in adulthood, suggesting organizational effects of $GABA_B$ input in kisspeptin neuron development.

Overall, these results suggest that the effect of $GABA_BR$ antagonism on gene expression is gene-, site-, and age-specific.

Effect of CGP Administration on Pituitary and Gonad Parameters on PND6

$GABA_BR$ are also present in the pituitary and the gonads. Increased gonad E_2 content and increased LH serum levels have been reported in neonate $GABA_{B1}$KO mice (25).

We therefore evaluated the effect of CGP on serum gonadotropin levels. FSH, which shows sexually dimorphic levels (females > males) at this age (7), was increased in CGP-treated males compared with control males and was similar to levels in females. Female titers were not affected by CGP treatment (Fig. 4*A*; 2-way ANOVA: interaction, $P < 0.05$; M control significantly different from M CGP, F control, and F CGP, $P < 0.05$ or less). Thus the sexual difference in FSH levels was lost in CGP-treated mice. A different pattern was observed for LH. Serum LH was higher in control females than control males, as expected. GCP-treated females showed significantly lower LH titers than control females, similar to levels in males, which did not differ due to CGP treatment (Fig. 4*B*; 2-way ANOVA: interaction, $P < 0.03$; F control significantly different from F CGP, M control, and M CGP, $P < 0.01$ or less). Interestingly, LH levels paralleled ARC Kiss1 expression in females. Again, the normal sex difference in LH levels was lost in CGP-treated mice.

As 4-day-old $GABA_{B1}$KO mice had increased E_2 content in ovaries and testes, gonad E_2 content was determined in *PND6* CGP-treated mice and controls. Similar to $GABA_{B1}$KO mice, E_2 was significantly increased in both males and females by CGP treatment (Fig. 4*C*; 2-way ANOVA: interaction, NS; main factor sex, $P < 0.02$; main factor treatment, $P < 0.001$). This did not seem to depend on circulating gonadotropins, at least in females. Testosterone content in testis was similar between groups (Fig. 4*D*).

To evaluate whether this increased ovarian E_2 content had an impact on the ovaries of neonatal mice, ovaries were weighed at euthanasia and the ovarian morphology was analyzed in hematoxylin-eosin-stained sections. Ovary weight was reduced by 58% in the CGP-treated group (Fig. 4*E*; $P < 0.001$). CGP induced a significant increase in the percentage of primordial follicles ($P < 0.01$) and a significant decrease in the percentage of primary ($P < 0.01$) and secondary ($P < 0.03$) follicles Fig. 4, *G* and *H*), clearly altering follicle development.

Likewise, to evaluate whether increased E_2 content had an impact on the testes of neonatal males, testis weight was evaluated but did not differ between groups (Fig. 4*F*). Morphological analysis of hematoxylin-eosin-stained sections did not show differences due to treatment (data not shown).

To investigate how CGP induces this local increase in E_2, gene expression of *Cyp19a1*, *Esr1*, and *Esr2* was measured by qPCR in ovaries of control and CGP-treated mice. Interestingly, *Cyp19a1* ($P < 0.05$), *Esr1* ($P < 0.001$), and *Esr2* ($P < 0.05$) were markedly increased by the $GABA_B$ antagonist (Fig. 5, *A–C*). These increases in the E_2-synthesizing enzyme and both E_2 receptors may explain the high E_2 content and the alterations in ovary weight and follicle distribution.

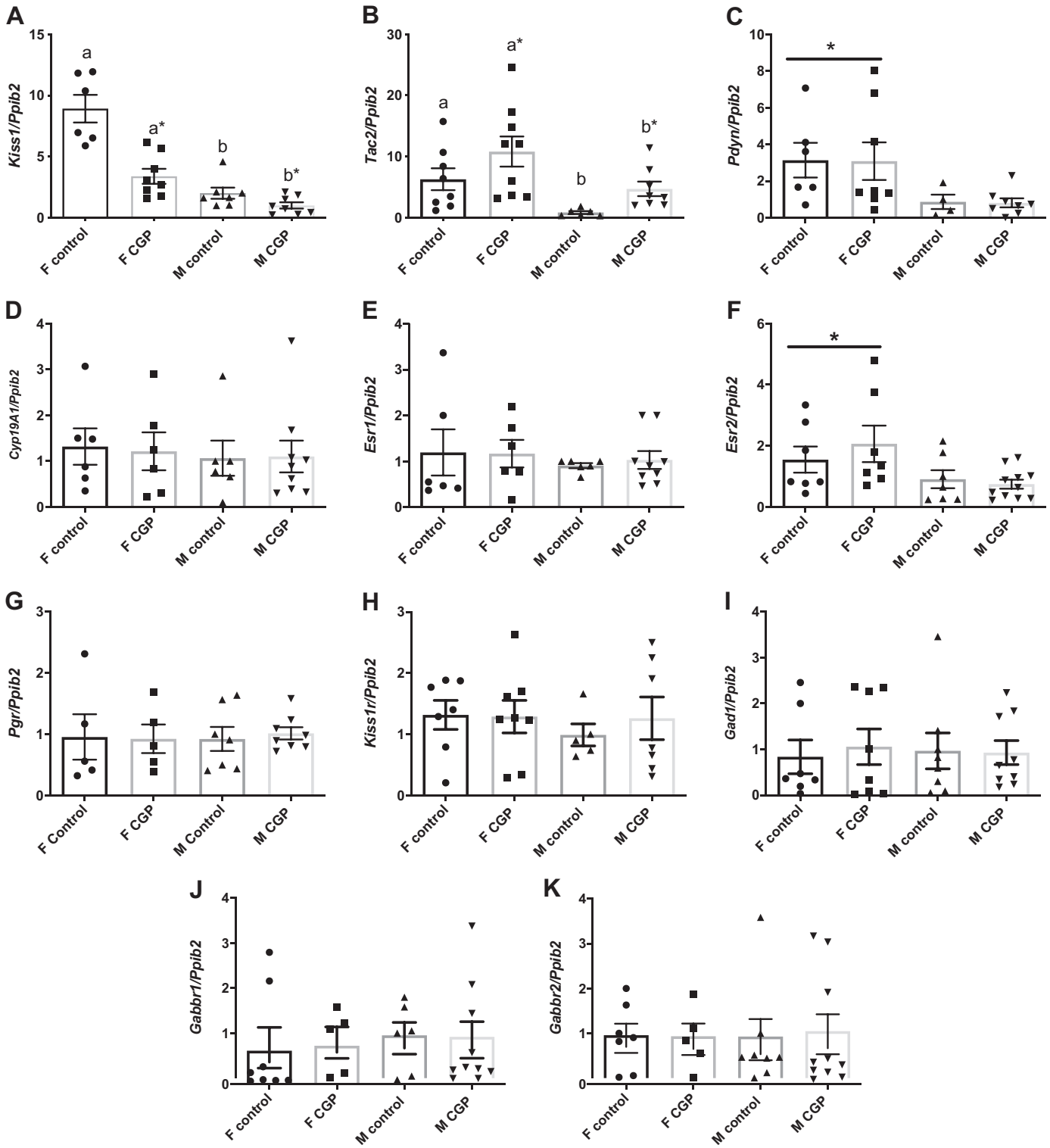

Fig. 1. Effect of CGP 55845 (CGP) administration from postnatal *day 2* (*PND2*) to *PND6* on gene expression in the arcuate nucleus (ARC) on *PND6*. Expression of kisspeptin 1 (*Kiss1*), neurokinin B (*Tac2*), prodynorphin (*Pdyn*), aromatase (*Cyp19a*), estrogen receptors-α and -β (*Esr1* and *Esr2*), progesterone receptor (*Pgr*), *Kiss1* receptor (*Kiss1r*), glutamic acid decarboxylase 67 (*Gad1*), and $GABA_B$ receptor subunits 1 and 2 (*Gabbr1* and *Gabbr2*) were determined in brain ARC micropunches from *PND6* control and CGP-treated mice (3 daily CGP doses of 1 mg/kg body wt sc from *PND2* to *PND6* or saline as control). Data were normalized to cyclophilin B (*Ppib2*) expression. *A*: *Kiss1* (n = 6–8). Two-way ANOVA: interaction: not significant (NS); main factor sex, $P < 0.001$ [females (a) ≠ males (b)]; main factor treatment, $P < 0.001$ (*CGP ≠ control). *B*: *Tac2* (n = 7–9). Two-way ANOVA: interaction, NS; main factor sex, $P < 0.001$ [females (a) ≠ males (b)]; main factor treatment, $P < 0.001$ (*CGP ≠ control). *C*: *Pdyn* (n = 5–8). Two-way ANOVA: interaction, NS; main factor sex, $P < 0.001$; main factor treatment, NS; *females > males. *D*: *Cyp19a1* (n = 6–8). Two-way ANOVA: NS. *E*: *Esr1* (n = 6–8). Two-way ANOVA: NS. *F*: *Esr2* (n = 7–8). Two-way ANOVA: interaction, NS; main factor sex, $P < 0.001$; main factor treatment, NS; *females > males. *G–K*: *Pgr, Kiss1r*, *Gad1*, *Gabbr1*, and *Gabbr2* (n = 5–10). Two-way ANOVA: NS. F control; female control; F CGP, CGP-treated female; M control, male control; M CGP, CGP-treated male.

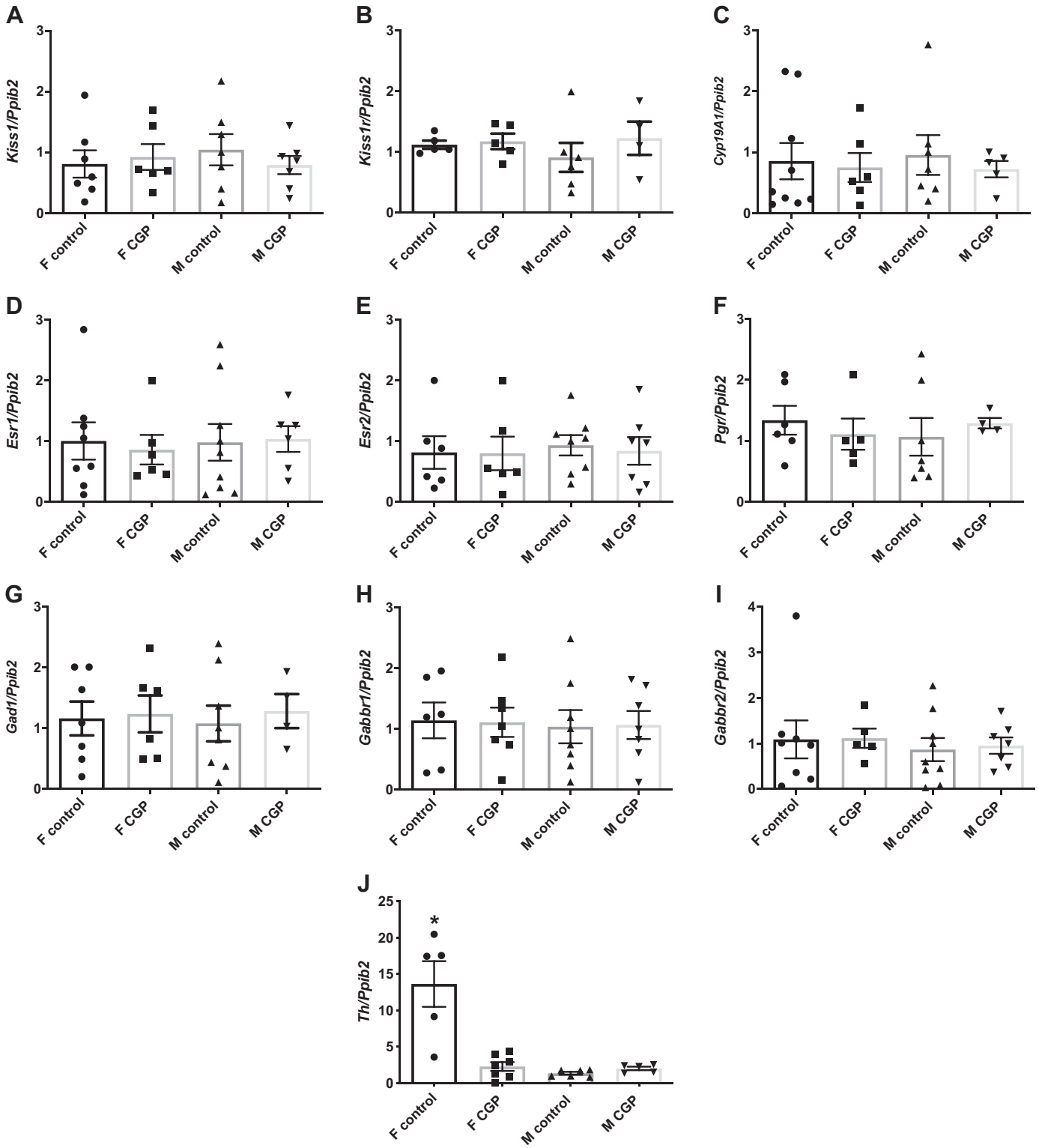

Fig. 2. Effect of CGP 55845 (CGP) administration from postnatal *day 2* (*PND2*) to *PND6* on gene expression in the anteroventral periventricular nucleus-periventricular nucleus continuum (AVPV/PeN) on *PND6*. Expressions of kisspeptin 1 (*Kiss1*), *Kiss1* receptor (*Kiss1r*), aromatase (*Cyp19a*), estrogen receptors-α and -β (*Esr1* and *Esr2*), progesterone receptor (*Pgr*), glutamic acid decarboxylase 67 (*Gad1*), $GABA_B$ receptor subunits 1 and 2 (*Gabbr1* and *Gabbr2*), and tyrosine hydroxylase (*Th*) were determined in brain AVPV/PeN micropunches from *PND6* control and CGP-treated mice. Data were normalized to cyclophilin B (*Ppib2*) expression. *A–I*: *Kiss1*, *Kiss1r*, *Cyp19a*, *Esr1*, *Esr2*, *Pgr*, *Gad1*, *Gabbr1*, and *Gabbr2* ($n = 6–8$). Two-way ANOVA: not significant. *J*: *Th* ($n = 4–8$). Two-way ANOVA: interaction, $P < 0.001$; *F control significantly different from F CGP, M control, and M CGP ($P < 0.02$ or less). F control; female control; F CGP, CGP-treated female; M control, male control; M CGP, CGP-treated male.

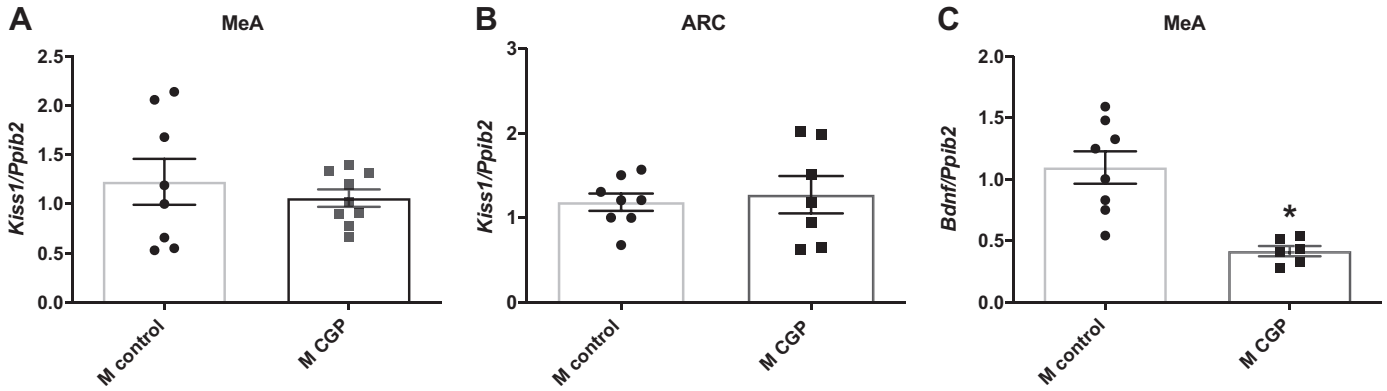

Fig. 3. Effect of CGP 55845 (CGP) administration to adult male mice on arcuate nucleus (ARC) and medial amygdala (MeA) *Kiss1* expression and on MeA brain-derived neurotropic factor (*Bdnf*) expression. Adult males were injected for 5 days with 3 daily CGP doses (1 mg/kg body wt sc; M CGP) or saline (M control). Data were normalized to cyclophilin B (*Ppib2*) expression. *A* and *B*: *Kiss1* expression in Arc (*A*) and MeA (*B*) micropunches (n = 7–9). Student's t test: not significant. *C*: *Bdnf* expression in MeA micropunches (n = 6–8). Student's t test: $^*P < 0.001$.

Since CGP had modified *Kiss1* expression in the ARC and as kisspeptin and its receptor are also expressed in the gonads, we evaluated whether the $GABA_B$ antagonist also altered their expression in this tissue. In the ovaries, neither *Kiss1* nor *Kiss1r* was changed by CGP treatment (Fig. 5, *D* and *E*), indicating that the alterations in aromatase and E_2 receptors were not dependent on local kisspeptin.

None of the above-mentioned genes (*Cyp19a1, Esr1, Esr2, Kiss1*, and *Kiss1r*) could be detected in 6-day-old testes.

Effect of Neonatal CGP Treatment on Developmental Parameters: Anogenital Index and Puberty Index

To elucidate whether the CGP-induced decrease in ARC *Kiss1* and increase in ARC *Tac2* expression, along with the increase in gonad E_2 on *PND6*, had any effects on developmental parameters, body weight (BW) was measured on *PND7* and *PND21*. In females, BW was significantly decreased by CGP treatment on *PND7* (6.05 ± 0.18 and 4.99 ± 0.17 g in control and CGP-treated females, respectively; Student's t test: $P < 0.05$). On *PND21*, this decrease in BW was still evident in CGP-treated females (Student's t test: $P < 0.05$; Fig. 6*A*). In addition, the AGI (AGD-to-BW ratio) was determined on *PND21*. CGP treatment did not result in significant differences in AGD between groups on *PND21* (Fig. 6*B*). Nevertheless, the AGI was significantly increased by CGP in females on *PND21* (Fig. 6*C*; Student's t test: $P < 0.01$), indicating some degree of masculinization.

BW was significantly lower in CGP-injected than control males on *PND7* (5.75 ± 0.21 and 5.08 ± 0.18 g in control and CGP-treated males, respectively; Student's t test: $P < 0.05$), and this persisted on *PND21* (Student's t test: $P < 0.05$; Fig. 6*D*). CGP did not induce significant changes in AGD on *PND21* (Fig. 6*E*). As in females, the AGI was significantly increased by the $GABA_B$ antagonist (Fig. 6*F*; Student's t test: $P < 0.01$).

These results suggest that neonatal impairment of $GABA_B$ signaling has effects on BW and may have an effect on sexual differentiation in both sexes.

In addition, puberty onset was evaluated in females by vaginal opening (VO) from weaning (*PND21*) onward and in males by preputial separation (PS) from *PND24* onward.

Neither average BW on the day of VO nor average day of VO was significantly different between groups (Fig. 7, *A* and *B*). However, the puberty index (VO-to-BW ratio) was significantly increased in CGP-treated females (Fig. 7*C*; $P < 0.02$). This result was also evident from analysis of the percent distribution of females showing VO during development: 100% of control females attained puberty onset (VO) on *PND33*, while VO was delayed to *PND38* in CGP-treated mice (Fig. 7*D*; χ^2 for percentage of mice showing VO on *PND33*: CGP < CTRL, $P < 0.05$). In males, CGP treatment did not alter BW, PS, or PS-to-BW ratio (Fig. 7, *E–H*).

Together, these results indicate that neonatal $GABA_BR$ antagonism delays puberty onset in females, in line with increased AGI, but has no such effect in males.

Effect of Neonatal CGP 55845 Administration on Adult Hypothalamic Gene Expression

To evaluate whether transient, neonatal administration of a $GABA_BR$ antagonist from *PND2* to *PND6* could induce permanent alterations in gene expression in adulthood, the same group of genes that had been evaluated on *PND6* was evaluated in adults that had received the same neonatal CGP treatment.

In the ARC, *Kiss1* expression levels were similar between control males and females, as expected (Fig. 8*A*). Control and CGP-treated females also showed similar levels of *Kiss1* expression. Interestingly, *Kiss1* expression was significantly lower in CGP-treated males than control males, control females, and CGP-treated females (2-way ANOVA: interaction, $P < 0.03$; M CGP significantly different from M control, F control, and F CGP, $P < 0.03$ or less). This indicates that neonatal CGP treatment induces a sex difference in adult ARC *Kiss1* expression, showing the typical pattern of expression (females > males) in neonates.

Tac2 expression was higher in females than males, as previously demonstrated (94), without changes due to CGP (Fig. 8*B*; 2-way ANOVA: interaction, NS; main factor sex, $P < 0.01$; main factor treatment, NS). No differences were observed in *Pdyn* or *Kiss1r* expression among groups (Fig. 8, *C* and *D*). Since CGP treatment decreased ARC *Kiss1* expression in males, we evaluated whether *Gnrh1* was affected in this area, where GnRH dendrons reach the median eminence and

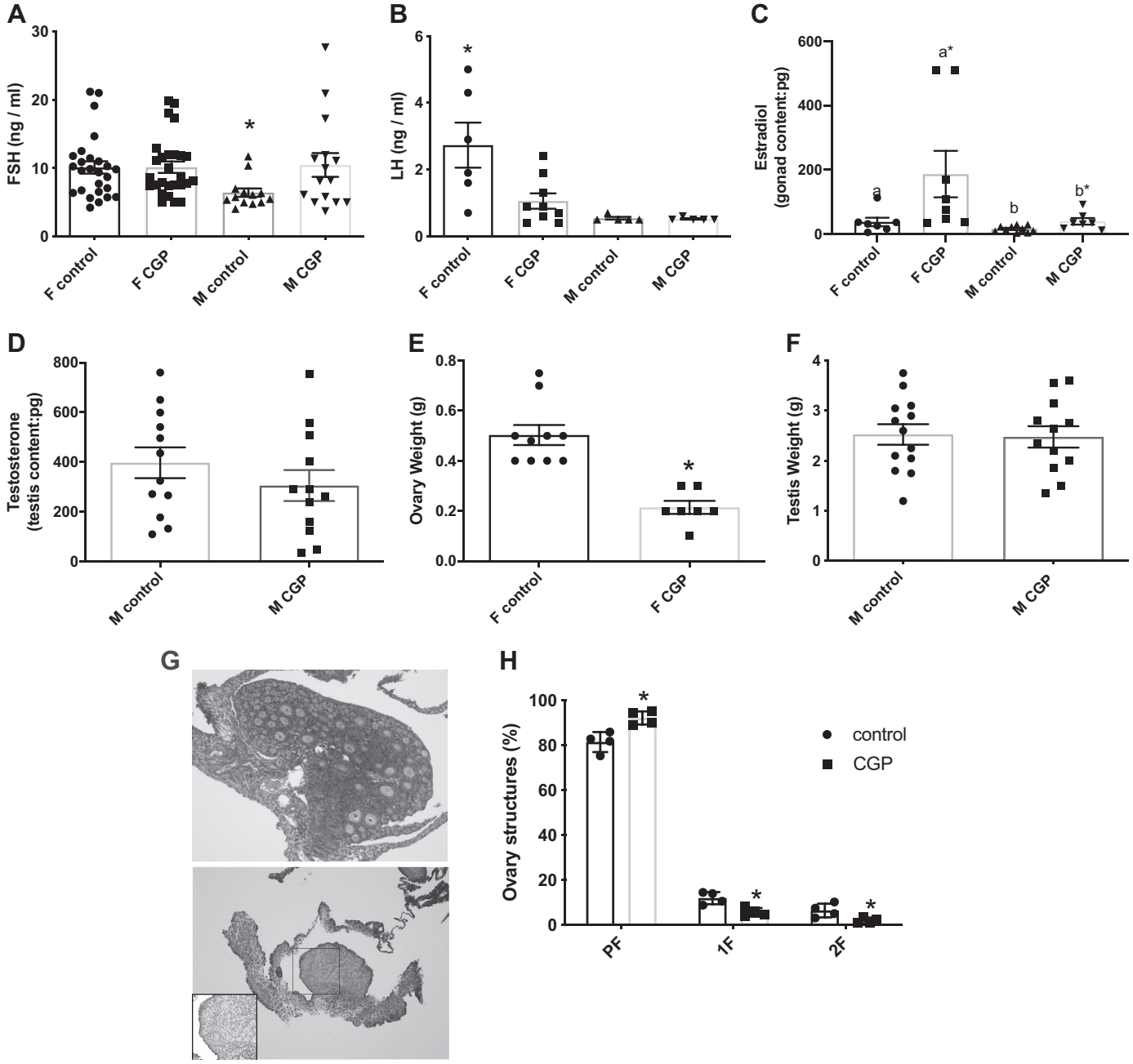

Fig. 4. Effect of CGP 55845 (CGP) administration from postnatal *day 2* (*PND2*) to *PND6* on gonadotropin serum levels and gonad parameters on *PND6*. *A*: follicle-stimulating hormone (FSH; $n = 13$–18). Two-way ANOVA: interaction, $P < 0.05$; *M control significantly different from M CGP, F control, and F CGP ($P < 0.05$ or less). *B*: luteinizing hormone (LH; $n = 5$–9). Two-way ANOVA: interaction, $P < 0.03$; *F control significantly different from F CGP, M control, and M CGP ($P < 0.01$ or less). *C*: gonad estradiol content ($n = 7$–10). Two-way ANOVA: interaction, not significant (NS); main factor sex, $P < 0.02$ [females (a) ≠ males (b)]; main factor treatment, $P < 0.001$ (*CGP ≠ control). *D*: testes testosterone content ($n = 12$). Student's *t* test: NS. *E*: ovary weight ($n = 7$–10). Student's *t* test: *$P < 0.001$. *F*: testes weight ($n = 12$–13). Student's *t* test: NS. *G*: ovarian morphology on *PND6*. Images of hematoxylin-eosin-stained 4-μm-thick ovarian slices are shown at ×10 (*insets* at ×40) magnification. *Top*: control ovary. Note the presence of primordial, primary, and secondary follicles. *Bottom*: ovary from a CGP-treated female. Note the presence of almost exclusively primordial follicles and the small size of the ovary. *H*: distribution of ovarian structures [primordial follicles (PF), primary follicles (1F), and secondary follicles (2F)] relative to total structures ($n = 4$). Student's *t* test after arcsine transformation for each structure: PF (*$P < 0.01$), 1F (*$P < 0.01$), and 2F (*$P < 0.03$). F control; female control; F CGP, CGP-treated female; M control, male control; M CGP, CGP-treated male.

can be modulated by ARC kisspeptin. ARC *Gnrh1* expression was lower in CGP males than control males, in agreement with *Kiss1* results. In addition, the sex difference (males > females) in *Gnrh1* expression in control mice in this area was abolished in CGP-treated animals (Fig. 8*E*; 2-way ANOVA: interaction, $P < 0.05$; M control significantly different from F control, M CGP, and F CGP, $P < 0.01$ or less). Interestingly, ARC *Gad1* expression was substantially increased in CGP-treated males, inducing a sex difference that was not observed in controls (Fig. 8*F*; 2-way ANOVA: interaction, $P < 0.01$; M CGP

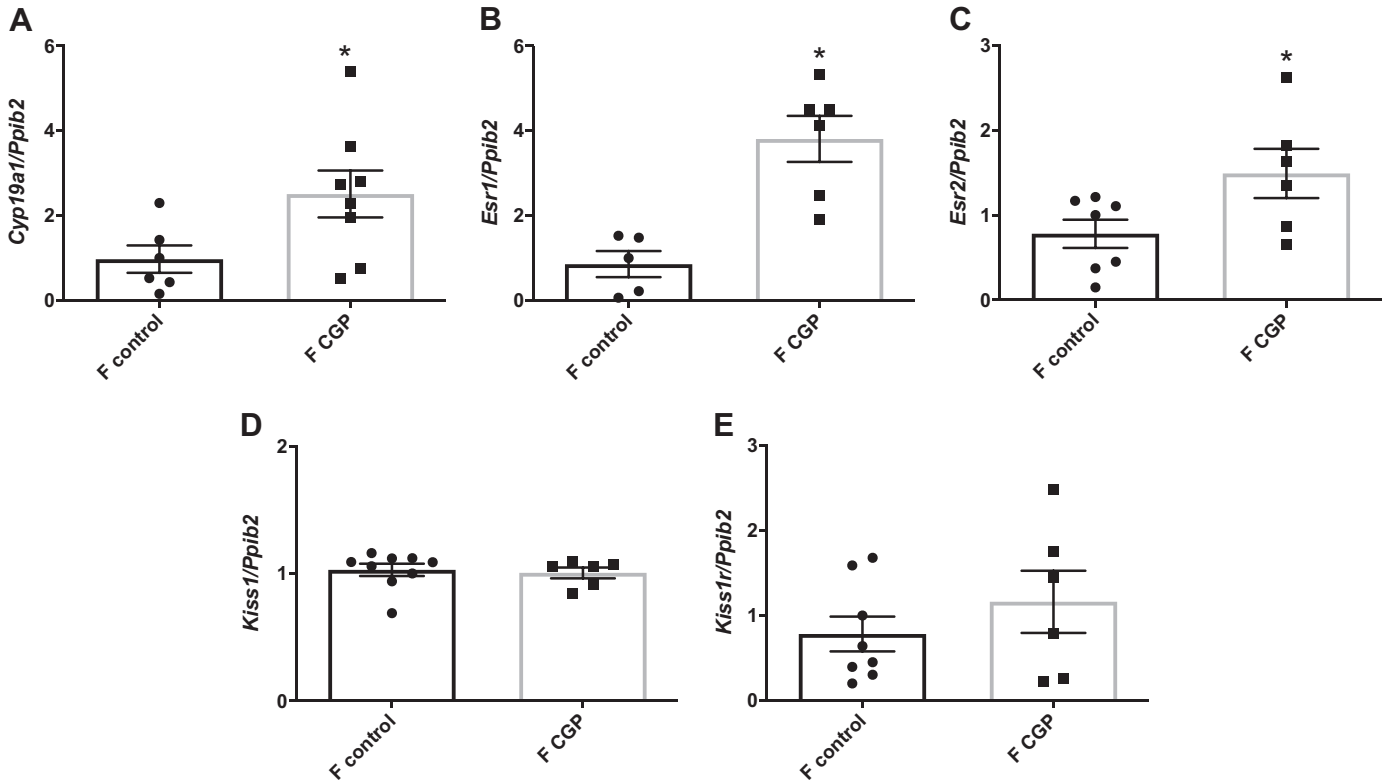

Fig. 5. Effect of CGP 55845 (CGP) administration from postnatal *day 2* (*PND2*) to *PND6* on ovarian aromatase (*Cyp19a1*), estrogen receptors-α and -β (*Esr1* and *Esr2*), kisspeptin 1 (*Kiss1*), and *Kiss1* receptor (*Kiss1r*) expression on *PND6*. Data were normalized to cyclophilin B (*Ppib2*) expression. *A–C*: *Cyp19a1*, *Esr1*, and *Esr2* ($n = 5–8$). *Student's *t* test: $P < 0.05$ (*A* and *C*), $P < 0.001$ (*B*). *D* and *E*: *Kiss1* and *Kiss1r* ($n = 6–9$). Student's *t* test: not significant. F control; female control; F CGP, CGP-treated female; M control, male control; M CGP, CGP-treated male.

significantly different from M control, F control, and F CGP, $P < 0.01$ or less). *Gabbr1* expression was significantly increased in both males and females of the CGP groups (2-way ANOVA: interaction, NS; main factor sex, NS; main factor treatment, $P < 0.01$), while *Gabbr2* was not affected (Fig. 8, *G* and *H*), suggesting long-lasting upregulation of the former by the neonatal presence of the antagonist.

In the AVPV, *Kiss1* expression showed the typical sex difference of adulthood but no effect of neonatal CGP treatment (2-way ANOVA: interaction, NS; main effect sex, $P < 0.001$; main effect treatment, NS; Fig. 9*A*). Moreover, *Kiss1r*, *Gnhr1*, *Gad1*, *Gabbr1*, and *Gabbr2* were not altered by neonatal CGP treatment (Fig. 9, *B–F*). Interestingly, *Th* expression showed the typical sex difference (females > males) in controls, but *Th* expression was significantly downregulated in neonatally CGP-treated females (Fig. 9*G*), losing this sex difference (2-way ANOVA: interaction, $P < 0.05$, F control significantly different from M control, F CGP, and M CGP, $P < 0.02$ or less), consistent with our findings on *PND6*.

These results show that neonatal CGP treatment has long-lasting effects on gene expression in adults, largely abolishing (*Gnrh1* and *Th*) and inducing (*Kiss1* and *Gad1*) sex differences in the ARC and AVPV/PeN.

Effect of Neonatal CGP Administration on Pituitary and Gonad Parameters in Adult Mice

Neonatal treatment with a $GABA_BR$ antagonist had a long-lasting impact on gonadotropin levels. Serum LH showed the expected sex differences (males > females) in both groups, but a significant increase in both sexes due to GCP administration was observed (Fig. 10*A*; 2-way ANOVA: interaction, NS; main factor sex, $P < 0.01$; main factor treatment, $P < 0.01$). FSH was lower in CGP-treated females ($P < 0.05$), while it was increased in CGP-treated males compared with same-sex controls ($P < 0.04$; Fig. 10*B*; 2-way ANOVA: interaction, $P < 0.01$).

These differences did not seem to be due to alterations in gonadal steroids, as E_2 (2-way ANOVA: NS) and testosterone (2-way ANOVA: interaction, NS; main effect sex, $P < 0.0001$; main effect treatment, NS) contents were similar between treatments in each sex (Fig. 10, *C* and *D*). Consistent with these findings, adult gonad weight was similar between treatments in each sex (Fig. 10, *E* and *F*). While E_2 gonad content was similar between sexes, ovaries weigh ~60 times less than testes. Progesterone was measured only in females and did not show differences between treatments (data not shown). *Cyp19a*, *Esr1*, and *Esr2* expression in ovaries was also similar between control and CGP-treated females at this age (data not shown). In addition, estrous cycles were similar between female groups (Fig. 10*G*).

DISCUSSION

GABA and kisspeptins are major regulators of reproduction that act on GnRH neurons. It has been well described that GABA is one of the neurotransmitters first active during central nervous system development (96) and that it regulates

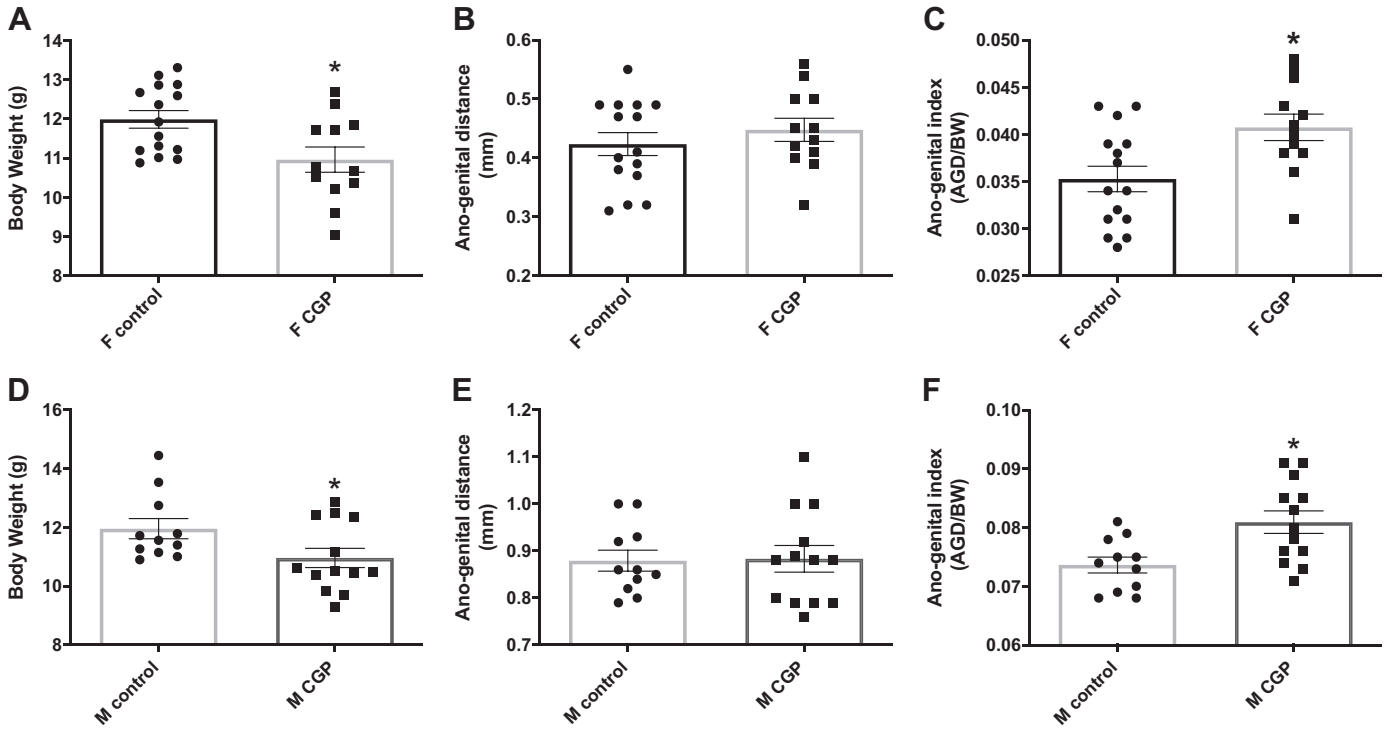

Fig. 6. Body weight (BW), anogenital distance (AGD), and anogenital index (AGI = AGD/BW) in males (n = 11–13) and females (n = 12–15) at postnatal *day 21*. *A*: BW in females. *Student's *t* test: $P < 0.05$. *B*: AGD in females. Student's *t* test: not significant (NS). *C*: AGI in females. *Student's *t* test: $P < 0.01$. *D*: BW in males. *Student's *t* test: $P < 0.05$. *E*: AGD in males. Student's *t* test: NS. *F*: AGI in males. *Student's *t* test: $P < 0.01$. F control; female control; F CGP, CGP-treated female; M control, male control; M CGP, CGP-treated male.

GnRH neurons from the very early stages of development (17, 61, 91, 93, 95). However, little is known about how GABA may regulate kisspeptin neuron function at early stages of development and whether this regulation is relevant for the development of the gonadotropic axis. Interestingly, some of the sex-differentiating actions of testosterone/E_2 are mediated by GABA (5, 62), particularly in the ARC (63). We previously showed that lack of functional $GABA_BR$ ($GABA_{B1}KO$) at neonatal stages (*PND4*) decreased *Kiss1* expression in the ARC in female mice (25), abolishing the normal sex difference in *Kiss1* expression observed at this age (84). These $GABA_{B1}KO$ females, which have no $GABA_BR$ signaling from conception onward, show compromised reproduction as adults (14, 15). In the present work our aim was to evaluate whether short-term neonatal pharmacological antagonism of $GABA_BR$ had an impact on the gonadotropic axis at this early stage of development and/or in adulthood, long after the cessation of drug administration (organizational effects).

Effects of Neonatal CGP Treatment on the Hypothalamic-Hypophyseal-Gonadal Axis on PND6

Neonatal treatment with the $GABA_B$ antagonist CGP decreased ARC *Kiss1* expression in both sexes at *PND6*, without altering the sex difference between males and females typical of this age (82, 84). In contrast, CGP did not modify *Kiss1* expression in the ARC or MeA when administered to adult males. These results indicate that the effect of CGP on *Kiss1* expression is age-specific, is operative during a critical stage of life, and cannot be replicated in adults. These findings were somewhat different from those observed in *PND4* $GABA_{B1}KO$ mice, in which only female ARC *Kiss1* expression was reduced. While *Pdyn* showed the same sex difference (females > males) as *Kiss1*, no CGP treatment effect was observed. Early postnatal expression of dynorphin in the ARC of rats has been described (59), but, to our knowledge, this is the first description of neonatal sexual dimorphic expression of ARC *Pdyn*. ARC *Tac2* showed the same sex difference as *Kiss1*, as previously described by Walker et al. (94), but, in contrast, CGP significantly increased *Tac2* expression levels in both sexes, without altering the difference in expression between sexes. These results show that the three genes that characterize KNDy neurons show the same sex differences in their expression levels at this age but suggest that the $GABA_BR$ differentially regulates each of them. Whether this is the consequence of different ways in which the $GABA_BR$ modulates each of these genes or whether the modulation of one impinges on the others remains to be discerned. Moreover, the effects on *Kiss1* and *Tac2* were specific to the lack of $GABA_B$ signaling, because several other genes (*Kiss1r, Gabbr1, Gabbr2*, and *Gad1*) did not show differences between groups.

PND6 CGP-treated mice had significantly increased gonad E_2 content (males and females), while testosterone content was not affected in males, similar to our previous observations in *PND4* $GABA_{B1}KO$ mice (25). Although normally at this age α-fetoprotein is present in plasma, sequestering E_2 and avoiding its impact on the central nervous system (6, 66), we cannot discount the possibility that, in this case, the binding capacity of this protein may have been exceeded, so that E_2 was able to lower ARC *Kiss1* levels. We believe this was not the case for several reasons. E_2 has been shown to decrease *Kiss1*, *Tac2*,

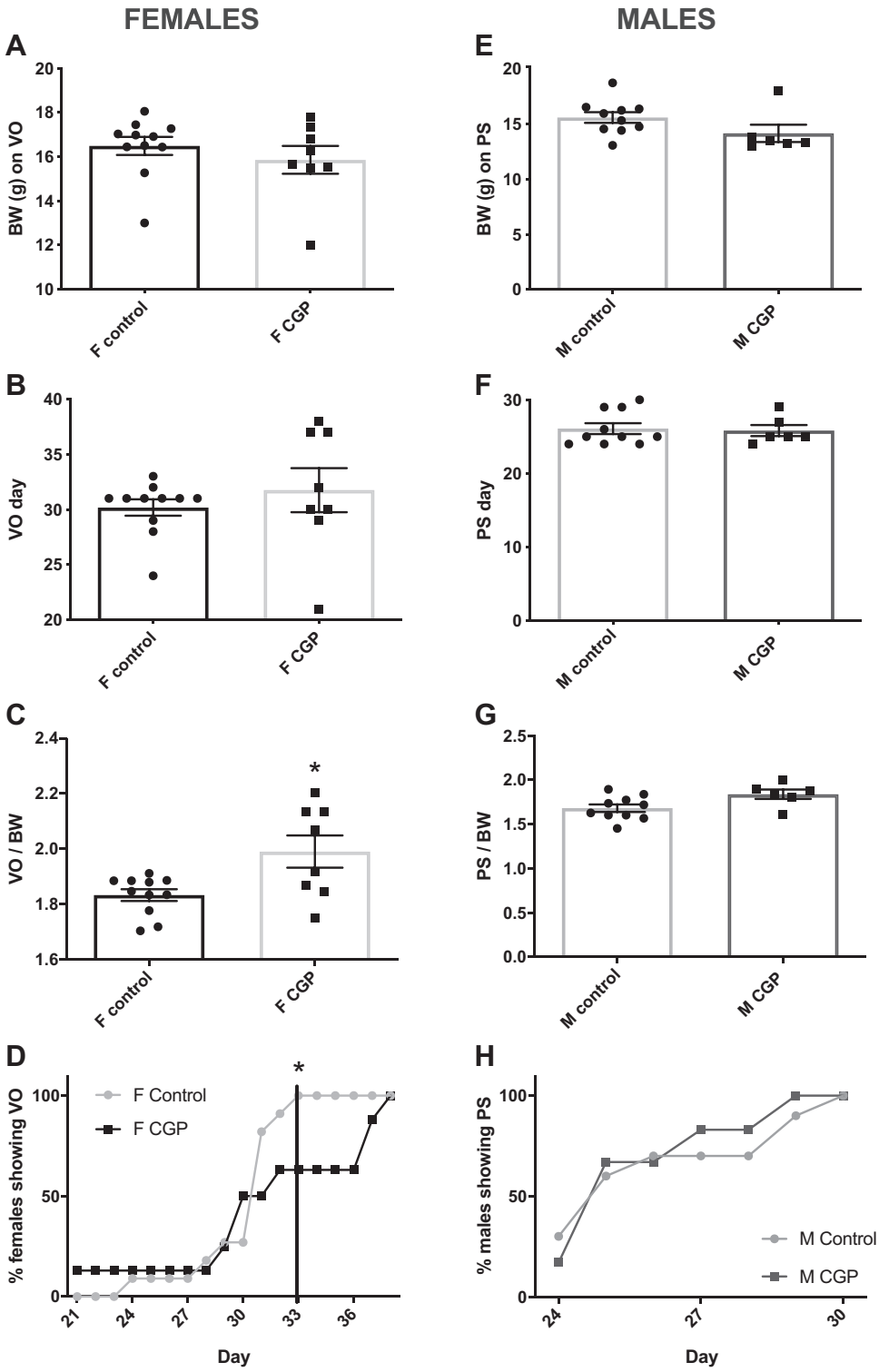

Fig. 7. Effect of CGP 55845 (CGP) administration from postnatal *day 2* (*PND2*) to *PND6* on puberty onset in males and females. Puberty onset was determined by the day of vaginal opening (VO) in females and the day of preputial separation (PS) in males. Puberty index is determined as the day of VO normalized to body weight (VO/BW) on that day in females and the day of PS normalized to BW (PS/BW) on that day in males. *A*: BW on the day of VO (n = 8–11). Student's t test: not significant (NS). *B*: mean day of VO (n = 8–11). Student's t test: NS. *C*: VO/BW (n = 8–11). *Student's t test: $P < 0.02$. *D*: distribution of females showing VO along development (n = 8–11). Note that 100% of controls had achieved VO by *PND33* (vertical line), while VO was achieved in CGP-treated females on *PND38*. *χ^2 test on *PND33*: $P < 0.05$. *E*: BW on the day of PS (n = 6–10). *F*: mean day of PS (n = 6–10). *G*: PS/BW (n = 6–10). Student's t test: NS. *H*: distribution of males showing PS along development (n = 6–10). χ^2 test on *PND27*: NS. F control; female control; F CGP, CGP-treated female; M control, male control; M CGP, CGP-treated male.

and *Pdyn* (44, 80), while we observed a decrease in *Kiss1* concurrent with an increase in *Tac2* and no change in *Pdyn*. Furthermore, high E_2 has been shown to increase *Pgr* expression (1, 40), and *Pgr* was not altered by CGP treatment. Thus the effects on gene regulation seem to be due to $GABA_BR$ antagonism on KNDy neurons, in agreement with previous results showing that 70% of adult ARC *Kiss1* neurons coexpress *Gabbr1* (26).

Increased ovarian E_2 content in CGP-treated mice could result from increased aromatase expression in this tissue; additionally, the expression of estrogen receptors-α and -β was also significantly induced in the ovaries of these mice. In contrast, the $GABA_BR$ antagonist altered none of these genes in the ARC and AVPV/PeN, where local E_2 synthesis and action have also been postulated to participate in sexual differentiation of the brain (63). This suggests a tissue-specific

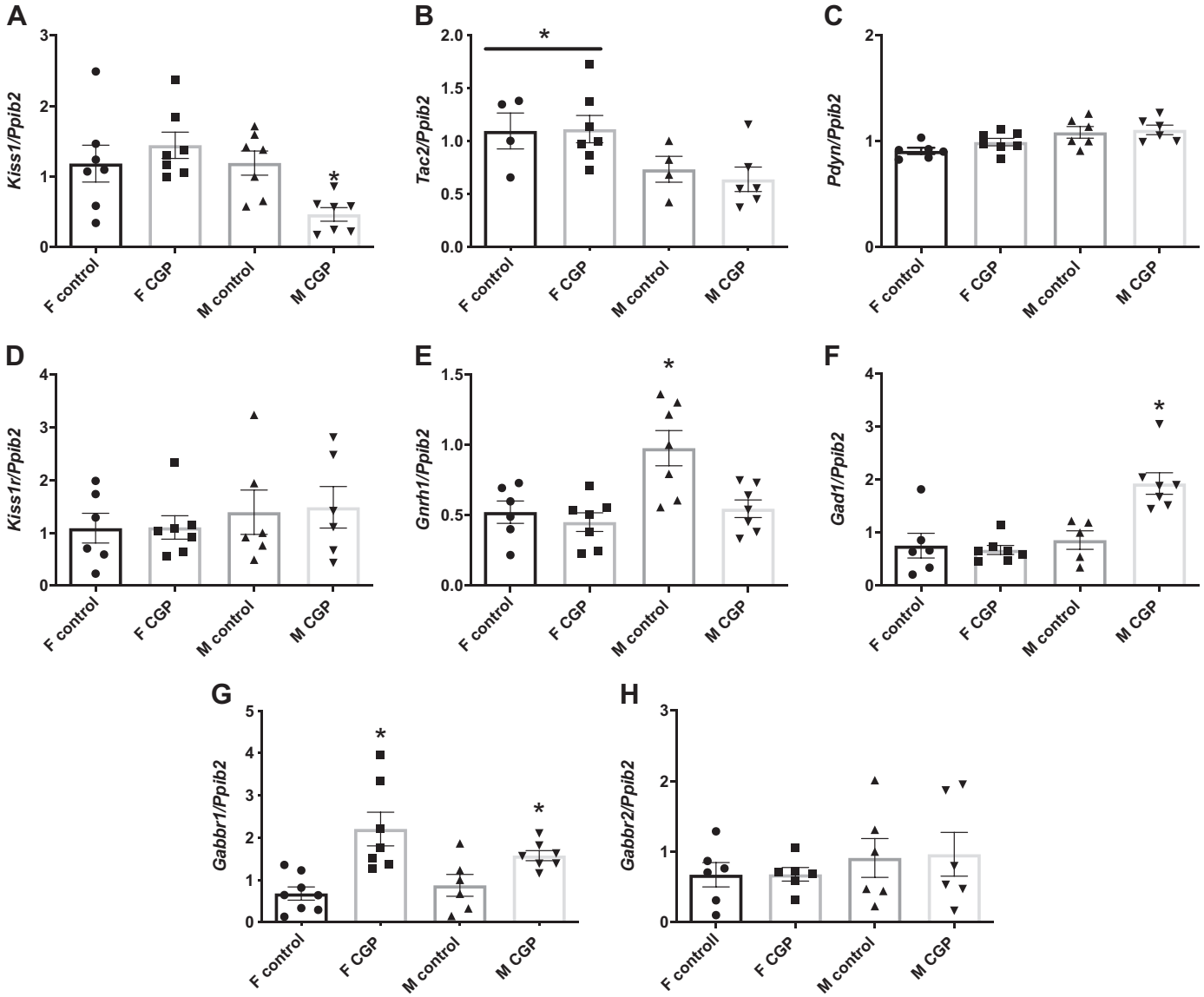

Fig. 8. Effect of neonatal CGP 55845 (CGP) administration on adult arcuate nucleus (ARC) gene expression. Expression of kisspeptin 1 (*Kiss1*), neurokinin B (*Tac2*), prodynorphin (*Pdyn*), *Kiss1* receptor (*Kiss1r*), gonadotropin-releasing hormone 1 (*Gnrh1*), glutamic acid decarboxylase 67 (*Gad1*), and $GABA_B$ receptor subunits 1 and 2 (*Gabbr1* and *Gabbr2*) were determined in adult brain ARC micropunches from control and neonatally CGP-treated mice. Data were normalized to cyclophilin B (*Ppib2*) expression. *A*: *Kiss1* ($n = 7$). Two-way ANOVA: interaction, $P < 0.03$; *M-CGP significantly different from M control, F control, and F CGP ($P < 0.03$ or less). *B*: *Tac2* ($n = 4$–7). Two-way ANOVA: interaction, not significant (NS); main factor sex, $P < 0.01$ (*females > males); main factor treatment, NS. *C* and *D*: *Pdyn* and *Kiss1r* ($n = 6$–7). Two-way ANOVA: NS. *E*: *Gnrh1* ($n = 6$–7). Two-way ANOVA: interaction, $P < 0.05$; *M control significantly different from F control, M CGP, and F CGP ($P < 0.01$ or less). *F*: *Gad1* ($n = 6$–7). Two-way ANOVA: interaction, $P < 0.01$; *M CGP significantly different from M control, F control, and F CGP ($P < 0.01$ or less). *G*: *Gabbr1* ($n = 6$–8). Two-way ANOVA: interaction, NS; main factor sex, NS; main factor treatment, *$P < 0.01$. *H*: *Gabbr2* ($n = 6$). Two-way ANOVA: NS. F Control; female control; F CGP, CGP-treated female; M Control, male control; M CGP, CGP-treated male.

effect, in agreement with the ovaries and testes expressing $GABA_BR$ (46–48). The kisspeptin/kisspeptin receptor system in the ovary has been proposed to control several functions, including steroidogenesis (39), but neither *Kiss1* nor *Kiss1r* expression was altered in the ovaries by CGP treatment, again suggesting a direct effect of CGP on local $GABA_BR$ in the regulation of E_2 synthesis and action. None of the genes measured in the ovary could be detected in the testes at *PND6*. Regarding *Kiss1*, our results are in agreement with those from Salehi et al., who could not detect *Kiss1* expression in testis on *PND7* (81).

In the AVPV/PeN, the only gene that showed a dramatic effect on its expression due to the $GABA_BR$ antagonist was tyrosine hydroxylase (*Th*), the limiting synthesizing enzyme for dopamine (19, 89). CGP induced a sharp decrease in *Th* expression in females, thus promoting loss of the typical sexual dimorphism (female > male). This sex difference in AVPV/PeN *Th* expression is well characterized in adults and depends on perinatal androgen/estrogen programming (75, 83, 86–88). To our knowledge, this is the first time this sex difference has been described at this early neonatal age, although it was previously described at *PND19* and thereafter (75). Although

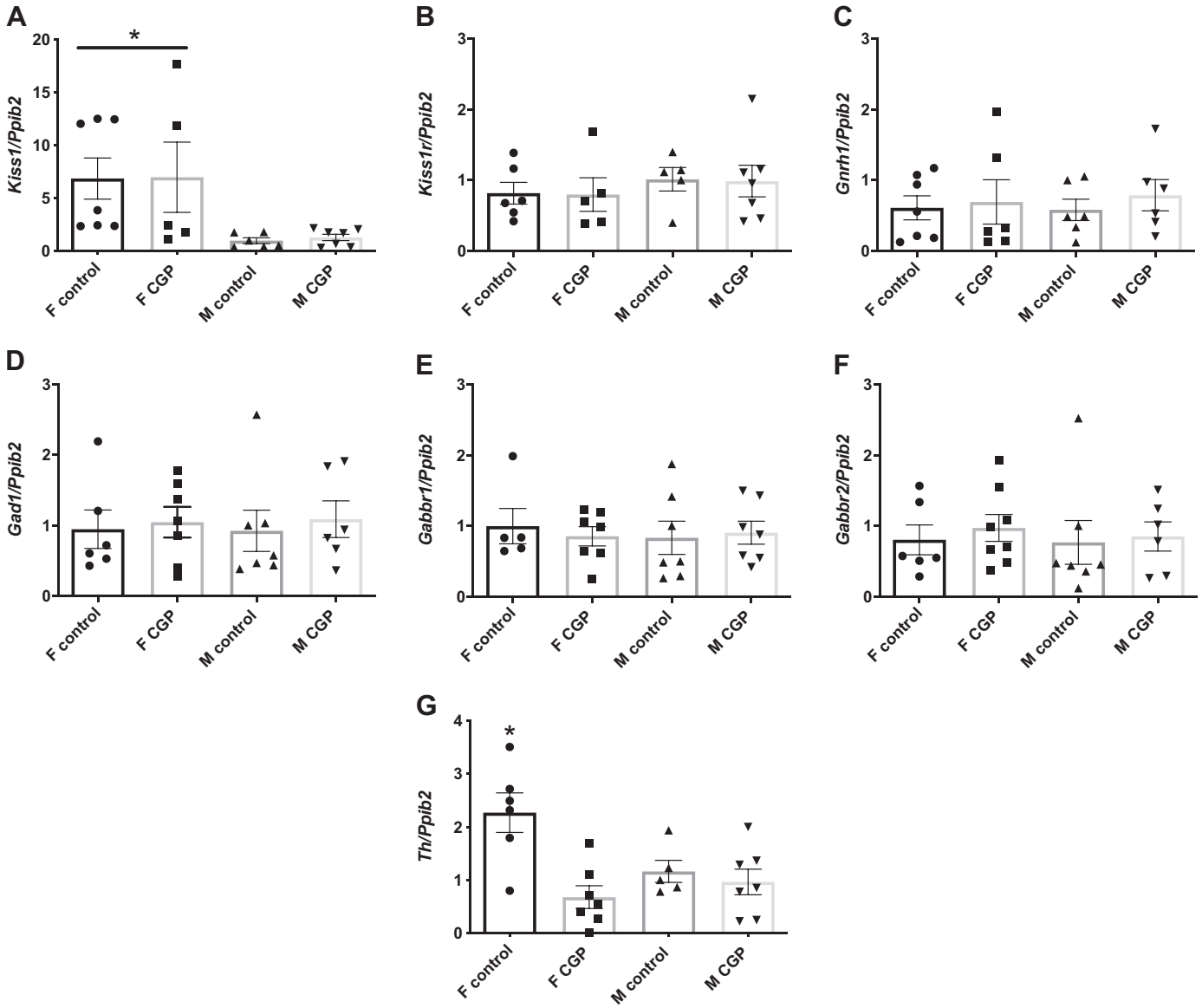

Fig. 9. Effect of neonatal CGP 55845 (CGP) administration on adult anteroventral periventricular nucleus-periventricular nucleus continuum (AVPV/PeN) gene expression. Expression of kisspeptin 1 (*Kiss1*), *Kiss1* receptor (*Kiss1r*), gonadotropin-releasing hormone 1 (*Gnrh1*), glutamic acid decarboxylase 67 (*Gad1*), $GABA_B$ receptor subunits 1 and 2 (*Gabbr1* and *Gabbr2*), and tyrosine hydroxylase (*Th*) was determined in adult brain AVPV/PeN micropunches from control and neonatally CGP-treated mice. Data were normalized to cyclophilin B (*Ppib2*) expression. *A*: *Kiss1* ($n = 6–7$). Two-way ANOVA: interaction, not significant (NS); main effect sex, $*P < 0.001$; main effect treatment, NS. *B–F*: *Kiss1r*, *Gnrh1*, *Gad1*, *Gabbr1*, and *Gabbr2* ($n = 6–8$). Two-way ANOVA: NS. *G*: *Th* ($n = 6–7$). Two-way ANOVA: interaction, $P < 0.05$; *F control significantly different from M control, F CGP, and M-CGP ($P < 0.02$ or less). F Control; female control; F CGP, CGP-treated female; M Control, male control; M CGP, CGP-treated male.

gonad E_2 content was high in CGP-treated mice at *PND6*, the results mentioned above suggest that increased E_2 did not reach the brain. Increased E_2 reaching the brain would also have masculinized *Kiss1* expression in neonate and adult females, which did not occur. The CGP-induced decrease in *Th* expression in females could be a direct effect of lack of $GABA_BR$ stimulation in these neurons, since it has been shown that $GABA_BR$ regulate dopaminergic neurons (58).

A high percentage of AVPV/PeN *Kiss1* neurons coexpress *Th* (19, 89). Nevertheless, no effect on AVPV/PeN *Kiss1* expression due to CGP administration was observed, even though 97% of these neurons express $GABA_BR$, at least in adulthood (26). This could be due to the very low level of *Kiss1* expression in this area at *PND6*, preventing us from detecting a decrease in expression. Alternatively, as is the case in KNDy neurons, different genes within the same neuron could be differentially regulated. Moreover, other dopaminergic neurons exist in the AVPV/PeN that do not coexpress *Kiss1* (19) and may be affected by the $GABA_BR$ antagonist. Exactly which population is affected and whether *Th* is also altered in other areas of the brain by neonatal $GABA_BR$ antagonism remain to be determined.

Interestingly, neonatal CGP treatment affected both gonadotropins in different ways. In control mice, serum FSH and LH showed the typical sex difference (females > males) for this age (7, 80), which was lost in CGP-treated mice due to a sharp

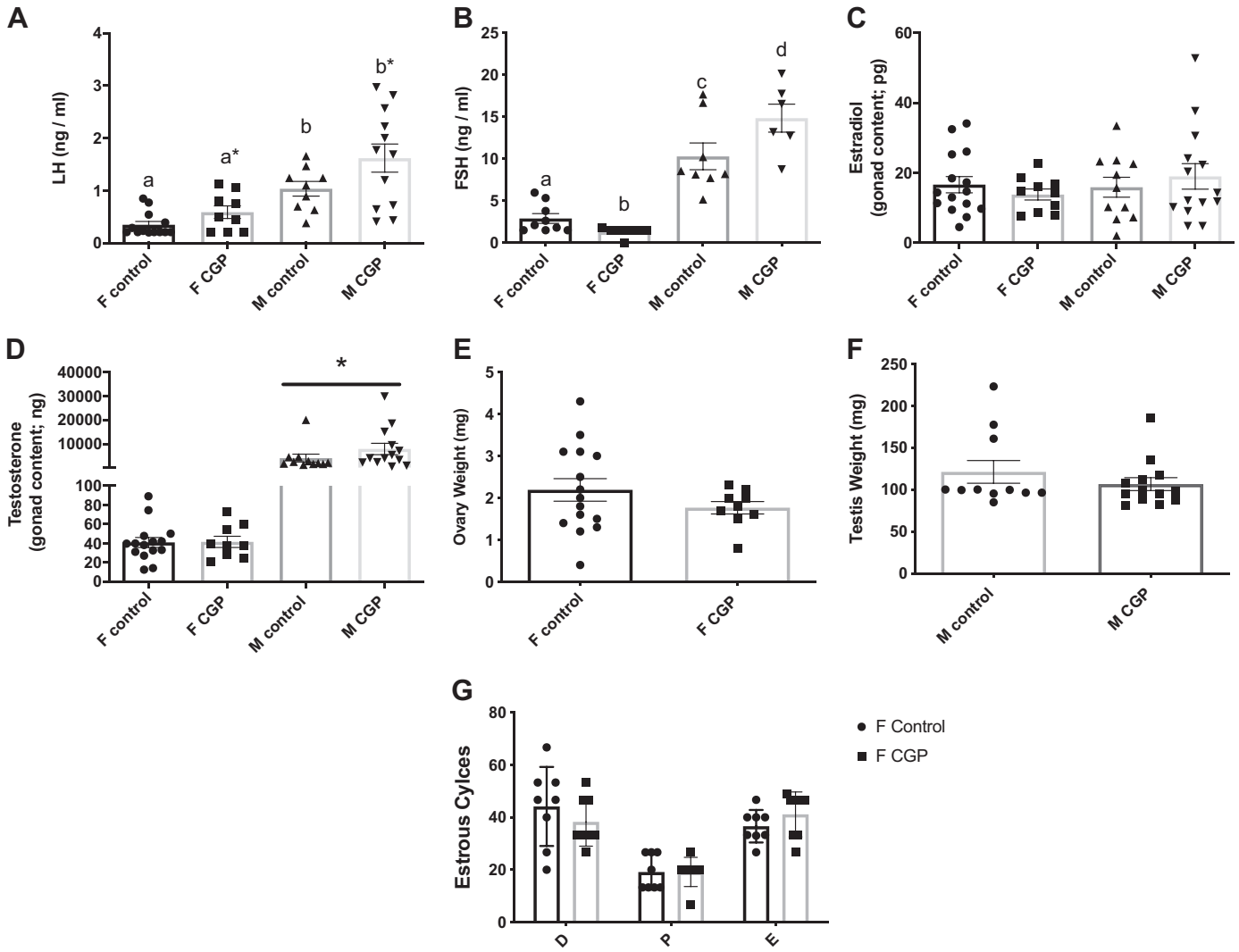

Fig. 10. Effect of neonatal CGP55845 (CGP) administration on serum gonadotropins, gonad weight, estradiol and testosterone gonad content, and estrous cycles in adult mice. *A*: luteinizing hormone (LH; $n = 9$–12). Two-way ANOVA: interaction, not significant (NS); main factor sex, $P < 0.01$ [females (a) ≠ from males (b)]; main factor treatment, $P < 0.01$ (*CGP ≠ control). *B*: follicle-stimulating hormone (FSH; $n = 6$–9). Two-way ANOVA: interaction, $P < 0.01$; different letters (a–d) indicate significant differences. *C*: estradiol gonad content ($n = 6$–9). Two-way ANOVA: NS. *D*: testosterone gonad content ($n = 10$–15). Two-way ANOVA: interaction, NS; main effect sex, $*P < 0.0001$; main effect treatment, NS. *E* and *F*: ovary and testes weight ($n = 10$–15). Student's *t* test: NS. *G*: days in each phase [diestrus (D), proestrus (P), and estrus (E)] of the estrous cycle (%; $n = 8$–11). χ^2 for each stage of the cycle: NS. F control; female control; F CGP, CGP-treated female; M control, male control; M CGP, CGP-treated male.

increase of FSH in males and a sharp decrease of LH in females. In both cases, this may imply a decrease in GnRH pulsatile frequency, as the LH-to-FSH ratio decreased in CGP-treated males and females (F control: 0.27, F CGP: 0.10, M control: 0.11, M CGP: 0.05). Neurokinin B (NKB) may trigger GnRH pulse onset by stimulating kisspeptin release from KNDy neurons, and kisspeptin serves as the output signal from KNDy neurons driving GnRH secretion. Additionally, kisspeptin and NKB receptors are anatomically segregated in GnRH and KNDy neurons, respectively (72). In CGP-treated mice, *Tac2* expression is augmented but *Kiss1* expression is decreased, possibly impairing the normal NKB-induced kisseptin secretion. Reduced kisspeptin secretion may then decrease GnRH pulsatility, which could account for the alterations in gonadotropin secretion. In males, increased *Tac2* could stimulate FSH secretion, as observed by Ruiz-Pino et al. when *PND10* males were treated with senktide, a NKB agonist (80). Unlike LH, FSH did not change in CGP-treated females, suggesting that other stimuli that preserve FSH secretion, such as activin (13) and anti-Müllerian hormone (33), are present in females at *PND6*. In any case, we cannot discount the possibility of direct effects of CGP on the pituitary gland, which expresses $GABA_BR$.

The increased FSH secretion in CGP-treated males did not alter testes weight or histology. In contrast, in CGP-treated females with similar FSH levels but significantly lower LH than controls, a marked decrease in ovary weight and altered histology, with an increase of primordial follicles and a corresponding decrease of primary and secondary follicles, were observed. Estrogen-induced suppression of primordial follicle development has been proposed (50), suggesting that ovarian estrogen due to local CGP action, rather than the decrease in LH, may account for the changes. Abnormalities in primordial follicle assembly, arrest, and development (i.e., primordial-to-

primary follicle transition) can cause pathological conditions such as premature ovarian failure and premature onset of menopause (50), perhaps explaining the reproductive impairment in adult $GABA_{B1}$KO females (14).

Therefore, neonatal short-term treatment with a $GABA_BR$ antagonist has clear effects at different levels of the gonadotropic axis. Some of these effects may also have been organizational in nature and will manifest later in life.

Effects of Neonatal CGP Treatment on Developmental Parameters

To evaluate whether this short treatment with CGP could alter developmental parameters, a second group of identically treated mice was allowed to mature. BW was ~10–20% lower in males and females on *PND7* and at weaning. The AGI (AGD-to-BW ratio), a commonly used end point for hormonally regulated sex differentiation in rodents (36, 38), is normally used to evaluate intrauterine exposure to androgens/estrogens and is also useful in adults, as it remains sensitive to gonadal steroids (67). AGD correlates with BW (36) and, thus, would have been expected to be lower in CGP-treated mice (with lower BW) if no effect on sex differentiation was present (similar AGIs). Nevertheless, AGD was similar between CGP-treated and control mice in each sex. As a consequence, AGI was increased in males and females on *PND21*, indicating some degree of masculinization. Puberty onset was delayed in CGP-treated females, in agreement with the AGI result, while it was not affected in males.

Effects of Neonatal CGP Treatment on the Hypothalamic-Hypophyseal-Gonadal Axis in Adulthood

Next we evaluated whether neonatal CGP treatment had organizational effects that became evident in adult mice. Interestingly, ARC *Kiss1* expression was significantly lower in CGP-treated males than controls, demonstrating an organizational effect of neonatal $GABA_BR$ antagonism only in males. As neither ARC *Tac2* nor *Pdyn* was affected, each gene may be differentially regulated, as previously suggested (74). In the adult ARC, GnRH is present mainly in dendrons that contact the median eminence (37). ARC *Gnrh1* expression was significantly decreased in CGP-treated males, consistent with the *Kiss1* decrease in this area. Interestingly, an upregulation of ARC *Gad1* expression was observed in CGP-treated males, but not in neonates, suggesting that it is an organizational effect that appears later in life. Moreover, an increase in *Gabbr1* expression was observed in this area in both CGP-treated males and females, probably a compensatory effect only observed in adults. These effects on gene expression demonstrate that neonatal silencing of $GABA_BR$ signaling causes permanent alterations in factors that are critical for normal reproductive function, such as kisspeptin, GnRH, and GABA, mainly in the ARC. It is interesting to note that some of these changes were the same as those observed neonatally (the decrease in *Kiss1* in males), and others emerged in adulthood (the decrease in *Gnrh1* and the increase in *Gad1* and *Gabbr1*), suggesting that different mechanisms/pathways may have been involved.

In the adult AVPV/PeN, the only organizational effect due to neonatal CGP treatment was the downregulation of *Th* expression in CGP-treated females, thus abolishing the expected sex difference (75, 83, 86–88). The CGP-induced decrease in *Th* suggests a clear organizational effect that was established early in life and persisted thereafter. In contrast, *Kiss1* expression was not altered in females in this region. As only *Th* expression was affected, it was not brought about by E_2. GABA has been described as one of the mediators of E_2-induced sex differences (63). Although $GABA_AR$ have been mainly implicated, our results demonstrate the $GABA_BR$ input is also critical to this process.

Neither E_2 nor testosterone was altered in CGP-treated adult mice, indicating that the increase in neonatal E_2 content in ovaries and testes was a transient effect and that the effects on adult gene expression were not sex steroid-dependent. Nevertheless, a long-lasting impact on gonadotropin levels was detected in CGP-treated mice. Neonatally CGP-treated adult females showed increased LH concomitant with decreased FSH. This type of shift in the levels of gonadotropin secretion is compatible with an increase in GnRH pulsatility that favors the LH-to-FSH ratio (21). Liu and Herbison demonstrated that dopamine is one of the most potent tonic inhibitors of GnRH neuron excitability (57). Our CGP-injected females showed significantly diminished AVPV/PeN *Th* expression, indicative of decreased dopamine, which could increase GnRH neuron

CGP 1-6 (neonates)			CGP 1-6 (adults)		
	F CGP	M CGP		F CGP	M CGP
ARC *Kiss1*	↓	⊡↓	ARC *Kiss1*	=	⊡↓
ARC *Tac2*	↑	↑	ARC *Tac2*	=	=
ARC *Gnrh1*	ND	ND	ARC *Gnrh1*	=	◯↓
ARC *Gad1*	=	=	ARC *Gad1*	=	◯↑
ARC *Gabbr1*	=	=	ARC *Gabbr1*	◯↑	◯↑
AVPV *Th*	⊡↓	=	AVPV *Th*	⊡↓	=
Gonad E2	↑	↑	Gonad E2	=	=

Fig. 11. Model of the main observations after treatment of neonatal male and female BALB/c mice with the $GABA_B$ antagonist CGP555845 (CGP). Findings are classified as those observed in the presence of the drug at postnatal *day 6* [*PND6* (neonates)] and those observed in adulthood (adults), long after termination of drug administration. Arrows indicate whether expression of a gene or levels of a hormone are increased (↑), decreased (↓), or not changed (=) in the treated animal compared with same-sex controls. □, Genes that were altered neonatally, with alteration persisting in adulthood (organizational changes); ○, genes that became altered in adulthood. ARC, arcuate nucleus; AVPV, anteroventral periventricular nucleus; E_2, estradiol; *Gabbr1*, $GABA_B$ receptor subunit 1; *Gad1*, glutamic acid decarboxylase 67; *Gnrh*, gonadotropin-releasing hormone; *Kiss1*, kisspeptin 1; *Tac2*, neurokinin B; *Th*, tyrosine hydroxylase.

excitability. This increased GnRH pulse frequency was observed in $GABA_{B1}KO$ mice (15). In these reproductively impaired $GABA_{B1}KO$ females (14), a decrease in AVPV *Th* expression was also detected (unpublished results), coinciding with the observation in CGP-treated females. Young CGP-treated females showed normal ovary weight and estrous cycles. These findings are in agreement with those of Stephens et al., who showed that deletion of *Th* expression, specifically from AVPV/PeN neurons, did not impair reproduction (89). Nevertheless, an increased LH-to-FSH ratio is pathognomonic of polycystic ovary syndrome (90). Whether reproductive alterations may arise later in life in these females remains to be investigated.

In adult males neonatally treated with the $GABA_BR$ antagonist, LH and FSH levels were increased compared with controls. Interestingly, ARC *Gad1* is highly expressed only in CGP-treated males. As GABA has been shown to exert a depolarizing influence on GnRH dendrons in adult mice (37), it is possible that high local GABA levels may stimulate increased GnRH secretion from terminals at the median eminence, thus stimulating gonadotropin secretion from the pituitary in CGP-treated males. This increase in gonadotropins did not affect gonad weight or testosterone content.

Together, our results clearly show that GABAergic input through $GABA_BR$ during a critical perinatal window is necessary to shape gene expression of factors critical to reproductive function, such as the neuropeptides kisspeptin, NKB, and GnRH, and the neurotransmitter-synthesizing enzymes glutamate decarboxylase and tyrosine hydroxylase. Changes in their normal expression patterns due to lack of $GABA_BR$ signaling have effects on the downstream reproductive axis. In many cases, sex differences in gene expression were abolished or induced by CGP treatment. In this regard, it has been proposed that $GABA_BR$ participate in fundamental steps of network development by regulating migration, neurite growth, synapse formation, and plasticity (31). Moreover, Bolton et al. showed that neonatal treatment with $GABA_BR$ agonists/antagonists produced sex differences in sensorimotor gating in adulthood (10), in consonance with our results and also with the notion that some of the sex-differentiating actions of testosterone/E_2 are mediated by GABA (5, 62), particularly in the ARC (63). In addition, we previously showed that $GABA_BR$ are maximally expressed in the early postnatal hypothalamus and decrease thereafter (8).

In sum, we demonstrate that neonatal $GABA_BR$ signaling modulates gene expression in several ways. *1*) It helps maintain neonatal expression at the appropriate levels in both males and females, such as with ARC *Tac2*, but this effect is transient. *2*) It helps maintain appropriate neonatal expression levels in both sexes, such as with ARC *Kiss1*; this effect is transient in females but permanent in males. *3*) It is critical for shaping the permanent sex difference (female > male) in AVPV/PeN *Th* expression that is established in early life and persists thereafter. *4*) It participates in shaping the sex difference (males > females) in adult ARC *Gnrh1* expression levels. *5*) It is critical for maintaining similar levels of expression of ARC *Gad1* in adults. A visual model of these findings is described in Fig. 11.

In view of our results, it is interesting that $GABA_BR$ agonists, antagonists, and allosteric modulators are increasingly being used or evaluated for their use in various pathologies, including spasticity, alcohol and drug addiction, anxiety, chronic pain, epilepsy, and autism (29). Our results raise the concern that these treatments, especially when administered in early stages of development, may interfere with the gonadotropic axis.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

V.L.-L. conceived and designed research; M.B.-H. performed experiments; M.B.-H. and V.L.-L. analyzed data; N.P.D.G. and V.L.-L. interpreted results of experiments; M.B.-H. prepared figures; M.B.-H. drafted manuscript; N.P.D.G. and V.L.-L. edited and revised manuscript; N.P.D.G., C.L., and V.L.-L. approved final version of manuscript.

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