Impaired $GABA_B$ Receptor Signaling Dramatically Up-Regulates *Kiss1* Expression Selectively in Nonhypothalamic Brain Regions of Adult but Not Prepubertal Mice

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Kisspeptin, encoded by *Kiss1*, stimulates reproduction and is synthesized in the hypothalamic anteroventral periventricular and arcuate nuclei. *Kiss1* is also expressed at lower levels in the medial amygdala (MeA) and bed nucleus of the stria terminalis (BNST), but the regulation and function of *Kiss1* there is poorly understood. γ-Aminobutyric acid (GABA) also regulates reproduction, and female $GABA_{B1}$ receptor knockout (KO) mice have compromised fertility. However, the interaction between $GABA_B$ receptors and *Kiss1* neurons is unknown. Here, using double-label in situ hybridization, we first demonstrated that a majority of hypothalamic *Kiss1* neurons coexpress $GABA_{B1}$ subunit, a finding also confirmed for most MeA *Kiss1* neurons. Yet, despite known reproductive impairments in $GABA_{B1}$KO mice, *Kiss1* expression in the anteroventral periventricular and arcuate nuclei, assessed by both in situ hybridization and real-time PCR, was identical between adult wild-type and $GABA_{B1}$KO mice. Surprisingly, however, *Kiss1* levels in the BNST and MeA, as well as the lateral septum (a region normally lacking *Kiss1* expression), were dramatically increased in both $GABA_{B1}$KO males and females. The increased *Kiss1* levels in extrahypothalamic regions were not caused by elevated sex steroids (which can increase *Kiss1* expression), because circulating estradiol and testosterone were equivalent between genotypes. Interestingly, increased *Kiss1* expression was not detected in the MeA or BNST in prepubertal KO mice of either sex, indicating that the enhancements in extrahypothalamic *Kiss1* levels initiate during/after puberty. These findings suggest that $GABA_B$ signaling may normally directly or indirectly inhibit *Kiss1* expression, particularly in the BNST and MeA, and highlight the importance of studying kisspeptin populations outside the hypothalamus. **(*Endocrinology* 155: 1033–1044, 2014)**

Reproduction is governed by an intricate interaction of many hormones, neuropeptides, and neurotransmitters. Many of these regulatory factors modulate, directly or indirectly, the secretion of GnRH, the final output of the neural network regulating fertility. Kisspeptin, a neuropeptide encoded by the *Kiss1* gene, is a key upstream stimulator of GnRH secretion (1–8). In mammals, both kisspeptin and its receptor, Kiss1r (formerly Gpr54), have been demonstrated to be necessary for puberty and reproduction (4, 9, 10). In the rodent hypothalamus, *Kiss1* is highly expressed in 2 discrete nuclei: the continuum of the anteroventral periventricular nucleus and rostral periventricular nucleus (AVPV/PeN) and the arcuate nucleus (ARC) (11, 12). Adult females have more *Kiss1* mRNA

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Abbreviations: ARC, arcuate nucleus; AVPV, anteroventral periventricular nucleus; BNST, bed nucleus of the stria terminalis; DIG, digoxigenin; E_2, estradiol; ER, estrogen receptor; GABA, γ-aminobutyric acid; $GABA_AR$, $GABA_A$ receptor; $GABA_BR$, $GABA_B$ receptor; ISH, in situ hybridization; KO, knockout; MeA, medial amygdala; PeN, periventricular nucleus; PND, postnatal day; qPCR, real-time PCR; SSC, sodium citrate, sodium chloride; WT, wild type.

and kisspeptin immunoreactivity in the AVPV/PeN than adult males, regardless of the sex steroid milieu (11, 13, 14), whereas there is no such *Kiss1* sex difference in the adult ARC, especially when sex steroids are controlled (13, 15–17). In addition to the ARC and AVPV/PeN, recent evidence indicates that *Kiss1* is also expressed, to a lesser extent, in several regions outside the hypothalamus, including the medial amygdala (MeA) (18, 19) and bed nucleus of the stria terminalis (BNST) (14, 20, 21).

Neural *Kiss1* expression is strongly regulated by sex steroids, testosterone (T) and estradiol (E_2), in a brain region-specific manner: in adult rodents, T and E_2 down-regulate *Kiss1* levels in the ARC, via either androgen receptor (AR) or estrogen receptor (ER) pathways (18, 22, 23), and up-regulate *Kiss1* levels in the AVPV/PeN, through ER pathways (2, 13, 22, 23). As in the AVPV/PeN, *Kiss1* levels in the MeA are strongly up-regulated by sex steroids (both T and E_2) in rodents of both sexes, likely via ER pathways (18). Likewise, Xu et al (14) similarly reported that E_2 stimulates the expression of *Kiss1* gene and kisspeptin protein in the rat MeA and BNST. Given their hormonal regulation and neuroanatomic location in known reproductive nuclei, the ARC and AVPV/PeN kisspeptin populations are thought to play important roles in mediating negative and positive feedback effects, respectively, of sex steroids (24, 25). In contrast, the function and regulation of *Kiss1* neurons in other brain regions is far less understood. We previously demonstrated that, in addition to their sex steroid responsiveness, *Kiss1* neurons in the MeA of adult, gondal-intact rats and mice were more prevalent in males than females (18). Another study reported no *Kiss1* signal in the MeA of postnatal rats before 3 weeks of age, suggesting that its expression in this region may arise around puberty (19). However, other than this limited information, little is known about the function, development, or possible regulation of extrahypothalamic *Kiss1* neurons by factors other than sex steroids.

GABA, the main inhibitory neurotransmitter in the brain of adult mammals, can regulate the reproductive axis acting through ionotropic $GABA_{A/C}$ and metabotropic $GABA_B$ receptors. The presence of both $GABA_A$ receptor ($GABA_AR$) and $GABA_B$ receptor ($GABA_BR$) has been described in GnRH neurons (26–28), and GABA signaling can directly regulate GnRH secretion (29). However, GABA may also modulate GnRH secretion indirectly by inhibiting upstream neuronal afferents of GnRH neurons, such as opiate or noradrenergic neurons (30, 31). Yet, at present, the possible interaction of GABA with kisspeptin neurons is not fully characterized. Whereas several groups have suggested that kisspeptin may interact with, or even directly modulate, GABA signaling to GnRH neurons (32–36), the reciprocal ability of GABA to regulate kisspeptin neurons remains far less studied. Recently, Kurian et al (37) used pharmacologic techniques to demonstrate that before puberty in monkeys, but not after, GnRH release is inhibited by tonic GABA input through kisspeptin neurons; this inhibitory action of GABA on pubertal kisspeptin signaling is exerted through $GABA_AR$, although the presence of $GABA_BR$ in kisspeptin neurons was not assessed, nor was a possible role of $GABA_BR$ signaling. Indeed, although $GABA_BR$ has been shown to modulate GnRH secretion and influence characteristics of fertility in rodents, to our knowledge, no direct link between $GABA_BR$ and kisspeptin neurons has yet been reported.

We previously demonstrated that adult $GABA_{B1}$KO female mice, which lack functional $GABA_BR$, have had decreased hypothalamic GnRH protein content and compromised cyclicity and fertility that is not due to behavioral alterations in mating (38–40). The kisspeptin system was not previously examined in these mice lacking functional $GABA_BR$. Therefore, this study aimed to evaluate whether the reproductive impairments in $GABA_{B1}$KO mice were partly due to alterations in the brain *Kiss1* system that might be brought about by nonfunctional $GABA_BR$ signaling. We evaluated: 1) $GABA_{B1}R$ colocalization in *Kiss1* neurons by double-label in situ hybridization (ISH), 2) neural *Kiss1* mRNA expression, in both hypothalamic and extrahypothalamic regions, in adult male and female $GABA_{B1}$KO mice, determined by ISH and real-time PCR (qPCR), and 3) *Kiss1* mRNA expression in prepubertal $GABA_{B1}$KO mice, to evaluate possible developmental changes in the *Kiss1* system in the absence of $GABA_BR$ signaling.

Materials and Methods

Animals

$GABA_{B1}$ heterozygous mice in the BALB/C inbred strain were bred to produce wild-type (WT) and $GABA_{B1}$KO littermates, the latter of which lack the $GABA_{B1}$ subunit and have nonfunctional $GABA_BR$ (39). All mice were genotyped by PCR analysis, as described previously (38), and housed in groups on a 12-hour light, 12-hour dark cycle (lights on at 7:00 AM), with free access to laboratory chow and water. All animal studies were performed according to protocols for animal use, approved by the Institutional Animal Care and Use Committee (IBYME-CONICET) that follows NIH guidelines.

Adult and postnatal day 14 (PND14; day of birth = PND1) WT and $GABA_{B1}$KO mice of both sexes were killed by rapid decapitation in the morning (9:00 AM to 11:00 AM). All adult females were in estrus. Brains were collected and immediately frozen on dry ice and stored at −80°C until cutting for ISH assays or micropunching for qPCR. Blood samples and gonads of adults

were collected at time of death and stored at −20°C until being assayed for serum and gonadal E_2 and T content.

Single-label and double-label ISH

For ISH assays, 5 coronal series of 20-μm brain sections were cut on a cryostat, thaw mounted onto Superfrost-plus slides, and stored at −80°C. Single-label ISH for *Kiss1* was performed as previously described (16, 41), using a validated mouse *Kiss1* riboprobe. Briefly, one set of slide-mounted brain sections was fixed in 4% paraformaldehyde, pretreated with acetic anhydride, rinsed in 2× SSC (sodium citrate, sodium chloride), delipidated in chloroform, dehydrated in ethanols, and air dried. Radiolabeled (^{33}P) *Kiss1* antisense riboprobe (0.04 pmol/mL) was combined with tRNA, heat denatured, added to hybridization buffer, and applied to each slide (100 μL/slide). Slides were coverslipped and placed in a 55°C humidity chamber overnight. The slides were then washed in 4× SSC and placed into RNase A treatment for 30 minutes at 37°C, then in RNase buffer without RNase at 37°C for 30 minutes. After washing in 2× SSC at room temperature, slides were washed in 0.1× SSC at 62°C for 1 hour, dehydrated in ethanols, and air dried. Slides were then dipped in Kodak NTB emulsion, air dried, and stored at 4°C for 4–5 days (depending on the assay) before being developed and coverslipped.

For double-label ISH of $GABA_{B1}$ subunit and *Kiss1*, slide-mounted brain sections were treated similarly to single-label ISH with the following modifications (42). Digoxigenin (DIG)-labeled antisense mouse *Kiss1* riboprobe was synthesized with DIG labeling mix (Roche). Radiolabeled (^{33}P) antisense $GABA_{B1}$ subunit (GenBank NM_001177511; 0.05 pmol/mL), designed to bind bp 385–856 of the mouse $GABA_{B1}$ subunit mRNA, and DIG-labeled *Kiss1* (1:500) riboprobes were combined with tRNA, heat denatured, and dissolved together in hybridization buffer. The probe mix was applied to slides (100 μL/slide) and hybridized at 55°C overnight. After the 62°C washes on day 2, slides were incubated in 2× SSC with 0.05% Triton X-100 containing 3% normal sheep serum for 1 hour at room temperature. Slides were then incubated overnight at 21°C with anti-DIG antibody conjugated to alkaline phosphatase (Roche; diluted 1:500 in Buffer 1 containing 1% normal sheep serum and 0.3% Triton X-100). Slides were then washed with Buffer 1 and incubated with Vector Red alkaline phosphatase substrate (Vector Laboratories) for 1 hour. Slides were then air dried, dipped in emulsion, stored at 4°C, and developed and coverslipped 9 days later. No staining was detected with sense probes.

Quantification and analysis of ISH data

Single-label ISH slides were analyzed with an automated image-processing system (Dr. Don Clifton, University of Washington) by a person blind to the treatment group. The software counts the number of silver grain clusters representing *Kiss1* cells, as well as the number of silver grains over each cell (a semiquantitative index of *Kiss1* mRNA content per cell) (15, 16, 18). Cells were considered *Kiss1* positive when the number of silver grains in a cluster exceeded that of background by 3-fold. For double-label assays, DIG-containing *Kiss1* cells were identified under red fluorescence microscopy and the grain-counting software was used to quantify silver grains (representing $GABA_{B1}$ subunit mRNA) overlying each cell. Signal-to-background ratios for individual cells were calculated, and a *Kiss1* cell was considered double labeled with $GABA_{B1}$ subunit if its ratio was greater than 3.

RNA extraction and qPCR

As in a previous report (18), total RNA from 500-μm-thick frozen micropunches of select brain regions (AVPV/PeN [~Plates 28–32 of *Paxinos and Franklin Mouse Atlas*], ARC [Plates 44–48], MeA [Plates 44–48], BNST [Plates 30–34], and lateral septum [Plates 25–29]) was extracted using the RNeasy Lipid Tissue Mini kit (QIAGEN). RNA was reverse transcribed using the Omniscript RT kit (QIAGEN), and cDNA was stored at −20°C. qPCR was performed on each cDNA sample using the Bio-Rad iCycler Detection System and Quantitect SYBR Green PCR kit (QIAGEN). To detect *Kiss1*, specific primers were used (*Kiss1* forward: 5′-CAA AAG TGA AGC CTG GAT CC-3′; and *Kiss1* reverse: 5′-GTT GTA GGT GGA CAG GTC C-3′) (41). Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) or β-actin (*Actb*) was used as housekeeping gene, depending on the brain region (41). Standard curves were generated for each product using cloned cDNAs for *Kiss1* and *GAPDH* to quantify the abundance of cDNA in each sample. For standard curves, a dilution series of cloned *Kiss1* and *GAPDH* templates ranging from 10 to 10^8 copies were used. The qPCR cycling parameters were 1 cycle of 95°C for 15 minutes, followed by 50 cycles of 94°C for 15 seconds, 60°C for 30 seconds, and 72°C for 30 seconds. Data collection was taken at the 72°C extension phase.

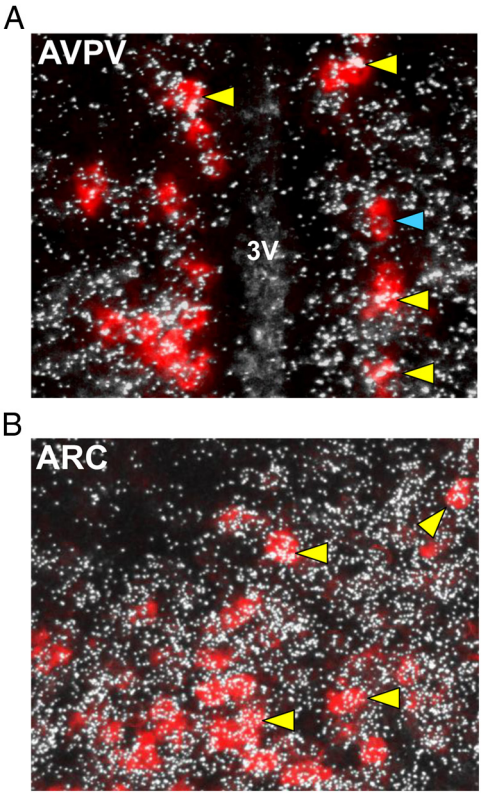

Figure 1. Representative images of $GABA_{B1}$ subunit mRNA expression (silver grains) in *Kiss1* neurons (red fluorescence) in the AVPV/PeN (panel A) and ARC (panel B) of adult mice. The majority (~75%-97%) of *Kiss1* cells coexpressed $GABA_{B1}$ subunit in each region. 3V, third ventricle. Yellow arrowheads denote examples of Kiss1-$GABA_{B1}$ coexpression. Blue arrowheads denote Kiss1 cell without $GABA_{B1}$.

To ensure the presence of a single product, a dissociation curve was performed after each run. 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* or *Actb*, the expression of which is constant and not different between groups. The size of the products was confirmed by 1% agarose gels.

Gonadal and serum hormone levels

To assess gonadal sex steroid content, gonads were obtained from adults at time of death and stored at −20°C until subsequent homogenization, ethyl-ether extraction, and determination of sex hormone levels by RIA, as previously described (38). E_2- and T-specific antisera were kindly provided by Dr. G. Niswender (Colorado State University, Fort Collins, CO). Tritiated hormones were purchased from New England Nuclear. Assay sensitivities were: E_2, 7 pg; and T, 10 pg. Intra- and interassay coefficients of variation were: E_2, 6.8% and 11.7%; and T, 7.8% and 12.3%, respectively.

For serum hormone levels, blood was collected from adult WT and $GABA_{B1}$KO mice. In females, serum was analyzed for E_2 levels by a mouse ELISA (sensitivity: 3.0 pg/mL; coefficient of variation, 4.1%) (43) and in males, serum T was assessed by RIA. Both procedures were performed by the University of Virginia Ligand Assay Core.

Statistics

Data are presented as the mean ± SEM for each group. The differences between means of 2 groups or more were analyzed by Student's *t* test or two-way ANOVA followed by the Tukey honestly significant difference test, respectively. $P < .05$ was considered statistically significant.

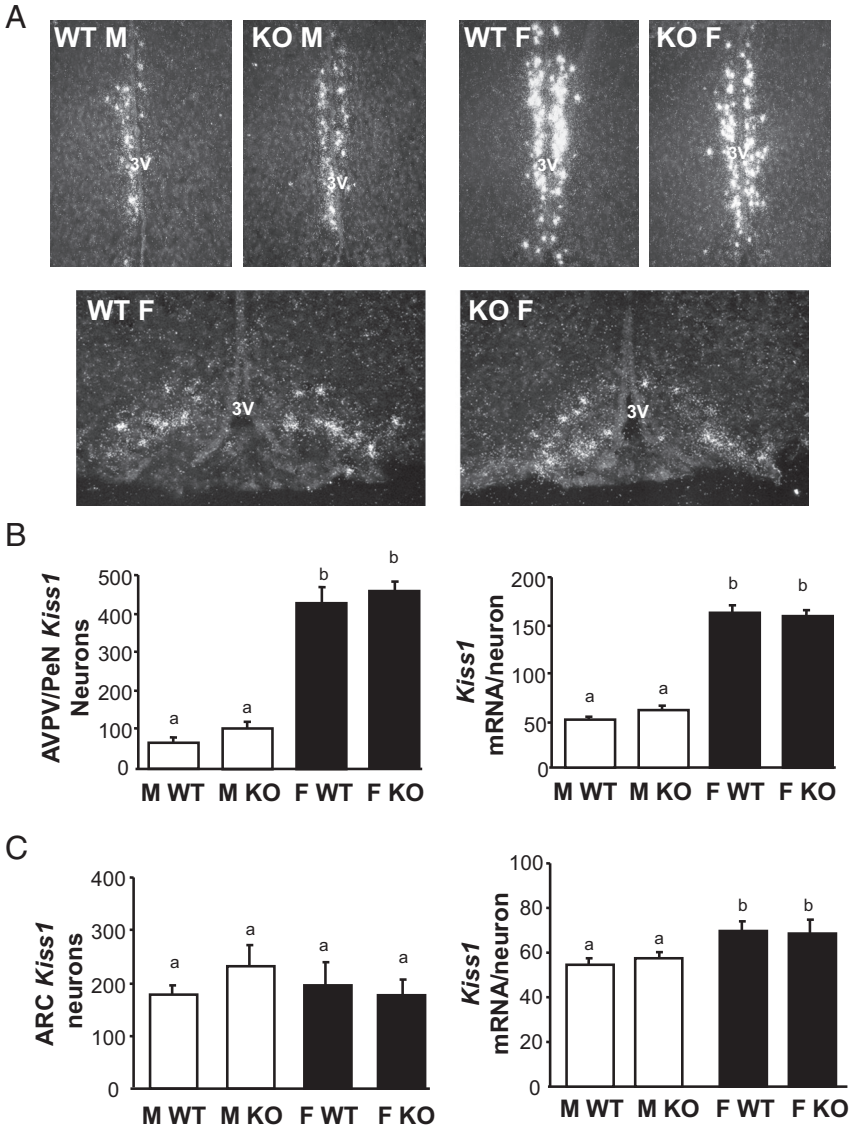

Figure 2. *Kiss1* expression in the AVPV/PeN and ARC of adult $GABA_{B1}$KO and WT mice. A, Representative images of *Kiss1* expression in the AVPV/PeN (top row) and ARC (bottom row). 3V, third ventricle. B, Mean numbers of *Kiss1* neurons in the AVPV/PeN and mean relative *Kiss1* mRNA content per neuron in the AVPV/PeN. C, Mean numbers of *Kiss1* neurons in the ARC and mean *Kiss1* levels per neuron in the ARC. Different letters denote significantly different from each other. F, female; M, male.

Results

Assessment of $GABA_{B1}$ subunit colocalization in hypothalamic Kiss1 neurons

To determine whether $GABA_B$ signaling could potentially directly influence the kisspeptin system, we first used double-label ISH to assess whether $GABA_{B1}$ subunit colocalizes in *Kiss1* neurons. Using hypothalamic tissue from adult female mice (estrus; n = 3), we found that virtually all *Kiss1* neurons in the AVPV/PeN (97.7 ± 0.89%) coexpress $GABA_{B1}$ subunit (Figure 1A). Similarly, a majority of *Kiss1* neurons in the ARC (71.3 ± 4.0%) also express $GABA_{B1}$ subunit (Figure 1B). This is the first demonstration of $GABA_{B1}$ subunit in rodent *Kiss1* neurons.

Kiss1 expression in the AVPV/PeN and ARC of mice lacking $GABA_B$R signaling

Having demonstrated the coexpression of $GABA_{B1}$ subunit in most *Kiss1* neurons, we next studied whether the absence of functional $GABA_B$Rs alters *Kiss1* mRNA levels in the ARC and AVPV/PeN, the two primary *Kiss1* populations linked to reproduction. Single-label ISH for *Kiss1* was performed in brains from

adult $GABA_{B1}$KO and WT mice of both sexes (females in estrus; n = 5–7/group). In the AVPV/PeN, *Kiss1* expression (both detectable number of *Kiss1* cells as well as *Kiss1* mRNA per cell) was higher in WT females than WT males, as expected ($P < .05$; Figure 2, A and B). However, despite known reproductive impairments in $GABA_{B1}$KO mice, AVPV/PeN *Kiss1* expression was similar between genotypes: $GABA_{B1}$KO females exhibited higher *Kiss1* levels than males of both genotypes, similar to that of WT females (Figure 2, A and B). Likewise, *Kiss1* levels in the AVPV/PeN of $GABA_{B1}$KO males were not different from that of WT males (Figure 2B). A similar outcome was detected in the ARC: the number of *Kiss1* cells in the ARC did not differ significantly between any group (Figure 2, A and C), and relative *Kiss1* mRNA per cell was slightly higher in females than males for both genotypes (Figure 2, A and C).

Increased Kiss1 expression in the MeA of $GABA_{B1}$KO mice

The previous experiment determined that *Kiss1* levels were unaltered in the ARC and AVPV/PeN of adult $GABA_{B1}$KO mice. We therefore next determined, using ISH, whether *Kiss1* gene expression in $GABA_{B1}$KO mice was altered in the MeA, an extrahypothalamic region shown to have moderate *Kiss1* expression and perhaps play a role in reproduction (18). As shown previously, adult WT male mice had a moderate number of detectable MeA *Kiss1* neurons that was significantly greater than in WT females ($P < .05$; Figure 3). Interestingly, unlike in the ARC and AVPV/PeN, there was a dramatic alteration in *Kiss1* levels in the MeA of $GABA_{B1}$KO mice. MeA *Kiss1* cell number was robustly elevated in $GABA_{B1}$KO males, being more than 6-fold higher than in WT males ($P < .05$; Figure 3). Likewise, the number of *Kiss1* cells was markedly higher in $GABA_{B1}$KO females than WT females (>5-fold higher; all females in estrus, $P < .05$; Figure 3). As with cell number, the relative *Kiss1* mRNA level per cell in the MeA was also significantly higher in $GABA_{B1}$KO mice than in WT mice ($P < .05$; Figure 3). We found the same result if we analyzed only the posterior ventral or posterior dorsal portion of the MeA (data not shown).

Enhanced Kiss1 expression in the BNST and septum of $GABA_{B1}$KO mice

Our surprising finding above of enhanced *Kiss1* levels in the MeA of $GABA_{B1}$KO mice prompted us to examine another extrahypothalamic region, the BNST, that reportedly normally expresses low *Kiss1* levels. The BNST lies dorsal-lateral to the AVPV/PeN, communicates with the medial amygdala, and has been implicated in reproductive and social behavior (44, 45). Confirming previous reports, we found low but detectable levels of *Kiss1* expression in the BNST of adult WT mice, with no significant sex difference in either *Kiss1* cell number or *Kiss1* mRNA per cell (Figure 4). Interestingly, as in the MeA, $GABA_{B1}$KO mice of both sexes had significantly more BNST *Kiss1* neurons than WT mice ($P < .05$ for each sex; Figure 4). This marked increase in BNST *Kiss1* cells in $GABA_{B1}$KO mice was 4- to 8-fold higher than in WT mice (Figure 5). BNST *Kiss1* levels were significantly higher in $GABA_{B1}$KO males than $GABA_{B1}$KO females ($P < .05$; Figure 4). Although cell number was robustly higher, the relative expression of *Kiss1* mRNA per cell was similar between genotypes (Figure 4).

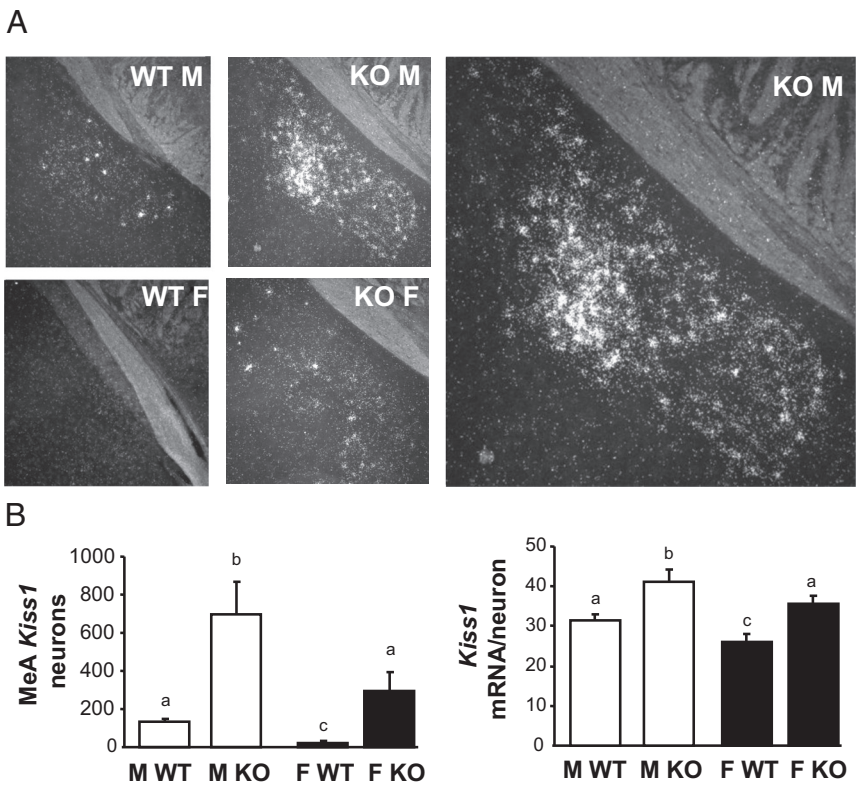

Figure 3. *Kiss1* expression in the MeA of adult $GABA_{B1}$KO and WT mice A, Representative images of *Kiss1* expression in the adult MeA. The large image on the right is a higher magnification of the KO male. B, Mean numbers of *Kiss1* neurons in the MeA and mean relative *Kiss1* mRNA content per neuron in the MeA. Different letters denote significantly different from each other. F, female; M, male.

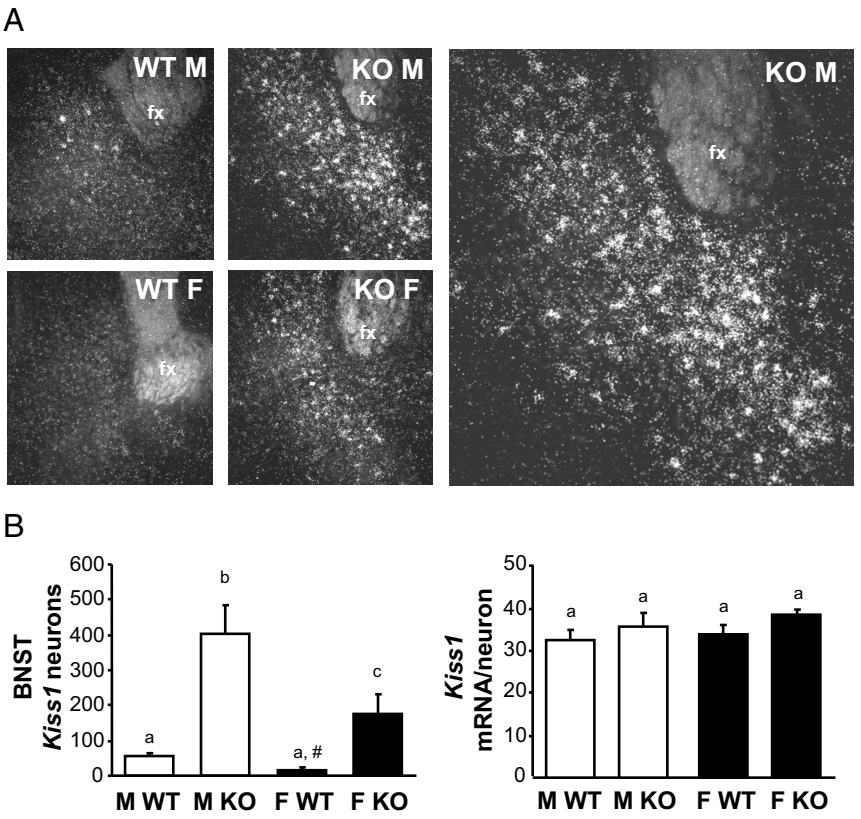

Figure 4. *Kiss1* expression in the BNST of adult GABA$_{B1}$KO and WT mice A, Representative images of *Kiss1* expression in the adult BNST. The large image on the right is a higher magnification of the KO male. B, Mean numbers of *Kiss1* neurons in the BNST and mean relative *Kiss1* mRNA content per neuron in the BNST. Different letters denote significantly different from each other. Fx, fornix; F, female; M, male. #, nonsignificant trend, $P = .10$ vs WT males.

In counting our BNST assay, we noted circumstantially notable *Kiss1* expression in some mice in the lateral septal area, just anterior and dorsal to the BNST region. We therefore analyzed *Kiss1* expression levels in all mice in this lateral septal region. In WT mice, there was virtually no detection of *Kiss1* in either sex in the septum with only a small handful of very weak cells very rarely identified (Figure 5). However, as with the MeA and BNST, adult GABA$_{B1}$KO mice of both sexes had a dramatically increased number of *Kiss1* neurons in this septal area, compared with WT mice ($P < .05$; Figure 5). In females, *Kiss1* mRNA levels per cell in the septum were also significantly higher in GABA$_{B1}$KO than WT mice ($P < .05$, Figure 5).

qPCR analysis of Kiss1 expression in hypothalamic and extrahypothalamic regions in GABA$_{B1}$KOs

Our ISH data indicated a dramatic enhancement of *Kiss1* expression in MeA, BNST, and septum, but not in the 2 main hypothalamic populations (AVPV/PeN and ARC). Because ISH is only semiquantitative, we next used sensitive qPCR to measure absolute *Kiss1* mRNA levels in the various *Kiss1* populations in adult GABA$_{B1}$KO mice. Micropunches of tissue from the AVPV/PeN, ARC, MeA, BNST, and septum were collected for male and female (estrus) GABA$_{B1}$KO and WT mice (n = 5–10/group per region). qPCR analysis of micropunch RNA mirrored the ISH outcomes. In the ARC and AVPV/PeN, *Kiss1* levels were identical between genotypes for both sexes (Figure 6). There were no sex differences in *Kiss1* levels in the ARC, but in the AVPV/PeN, *Kiss1* levels were sexually differentiated as expected (higher in females than males; $P < .05$). Confirming our ISH findings, absolute *Kiss1* levels, as determined by qPCR, were significantly higher in the extrahypothalamic regions of GABA$_{B1}$KO mice relative to WT mice (Figure 6). These elevated *Kiss1* levels were present in all 3 regions (MeA, BNST, and septum) and true for both sexes ($P < .05$ vs WT for each region). The increase in *Kiss1* levels was similar between GABA$_{B1}$KO males and females for all 3 extrahypothalamic regions.

Assessment of GABA$_{B1}$ subunit colocalization in extrahypothalamic Kiss1 neurons

To determine whether GABA$_B$ signaling might potentially occur directly in extrahypothalamic *Kiss1* cells, we used double-label ISH to assess *Kiss1*/*GABA$_{B1}$* subunit coexpression in the MeA. This could not be determined in WT mice because the level of *Kiss1* expression per cell in extrahypothalamic regions, unlike in the hypothalamus, is not high enough to detect with DIG-labeled ISH probes. However, because *Kiss1* levels in the MeA are significantly elevated in GABA$_{B1}$KOs (Figures 3 and 6), their tissue can be used to better detect *Kiss1* cells in this region (our *GABA$_{B1}$* probe targets early exons of the *GABA$_{B1}$* gene that have not been disrupted in the knockout [KO] mice). Using tissue from adult GABA$_{B1}$KO mice (n = 4 males and 2 females), we found that a majority (~66%) of detectable *Kiss1* neurons in the MeA coexpress *GABA$_{B1}$* subunit (Figure 7), similar in both sexes. Analogous analyses were not possible for *Kiss1* neurons in the BNST or septum, because the level of *Kiss1* mRNA expression per cell is not

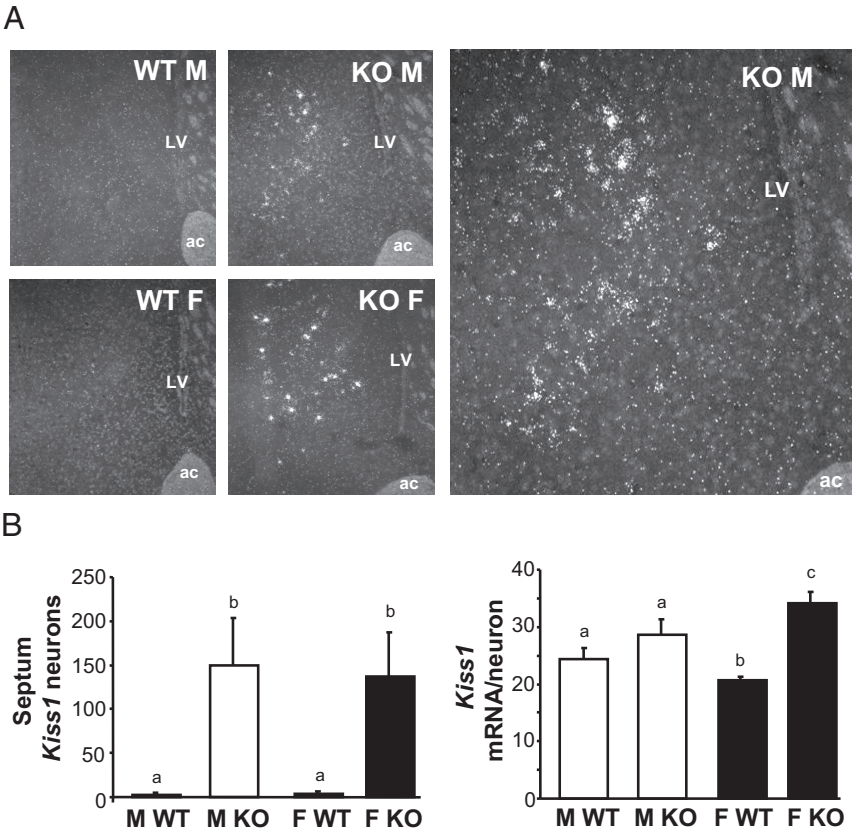

Figure 5. *Kiss1* expression in the lateral septum of adult $GABA_{B1}$KO and WT mice. A, Representative images of *Kiss1* expression in the lateral septum. The large image on the right is a higher magnification of the KO male. B, Mean numbers of *Kiss1* neurons in the lateral septum and mean relative *Kiss1* mRNA content per neuron in the septum. Different letters denote significantly different from each other. ac, anterior commissure; LV, lateral ventricle; F, female; M, male.

high enough in these regions, even in KO mice, for detection of DIG-labeled *Kiss1* cells.

Sex steroid levels in adult $GABA_{B1}$KO mice are normal

Because circulating sex steroids can strongly alter *Kiss1* expression, it is possible that the observed alterations in *Kiss1* levels in adult $GABA_{B1}$KO mice might reflect higher circulating sex steroid levels. We therefore measured gonadal content and blood serum levels of E_2 (estrous females) and T (males), as well as gonad and uterus weights, in adult $GABA_{B1}$KO and WT mice. Both gonadal and circulating sex steroid levels were similar between genotypes, even after adjusting for gonadal weight (Supplemental Table 1 published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org). In addition, gonad and uterine weights did not differ significantly between genotypes (data not shown). Thus, the high *Kiss1* expression in extrahypothalamic regions is not due to higher circulating sex steroids in the $GABA_{B1}$KO mice.

Kiss1 expression in prepubertal mice lacking $GABA_BR$ signaling

Because we found large alterations in *Kiss1* levels in extrahypothalamic areas (eg, MeA, BNST) in adult $GABA_{B1}$KO mice, we next tested whether similar increases in expression were present earlier in development, before puberty. We analyzed hypothalamic and extrahypothalamic *Kiss1* expression by ISH in PND14 male and female mice (n = 6–7/group). *Kiss1* expression at this developmental age was readily detected in the AVPV/PeN and ARC, with no genotype differences in either region, as in adulthood (Figure 8). Surprisingly, unlike in adulthood, no genotype differences in *Kiss1* levels were present between $GABA_{B1}$KO and WT mice in any of the extrahypothalamic brain areas examined in either sex (Figure 8). In the MeA, *Kiss1* was virtually undetectable in all prepubertal groups, regardless of sex or genotype. In the BNST, *Kiss1* levels in prepubertal mice were low to moderate, with all groups displaying equal *Kiss1* expression similar to adult WT levels. Thus, the elevated *Kiss1* expression observed in extrahypothalamic regions of adult $GABA_{B1}$KO mice is not yet present in prepubertal development.

Discussion

The possible interaction between $GABA_BR$ signaling and *Kiss1* neurons has not been previously addressed. Here, we evaluated whether the known reproductive alterations previously observed in $GABA_{B1}$KO mice were due to impairments in the neural *Kiss1* system. Contrary to our hypothesis, and despite the high coexpression of $GABA_{B1}$R in most *Kiss1* neurons, we found that *Kiss1* levels in the AVPV/PeN and ARC, assessed by both ISH and qPCR, were identical between genotypes for both sexes. Thus, the reproductive impairments previously observed in adult $GABA_{B1}$KO females are not due to deficits in *Kiss1* expression in the main hypothalamic nuclei that control fertility. However, surprisingly, subsequent ISH and qPCR analyses demonstrated that *Kiss1* levels outside

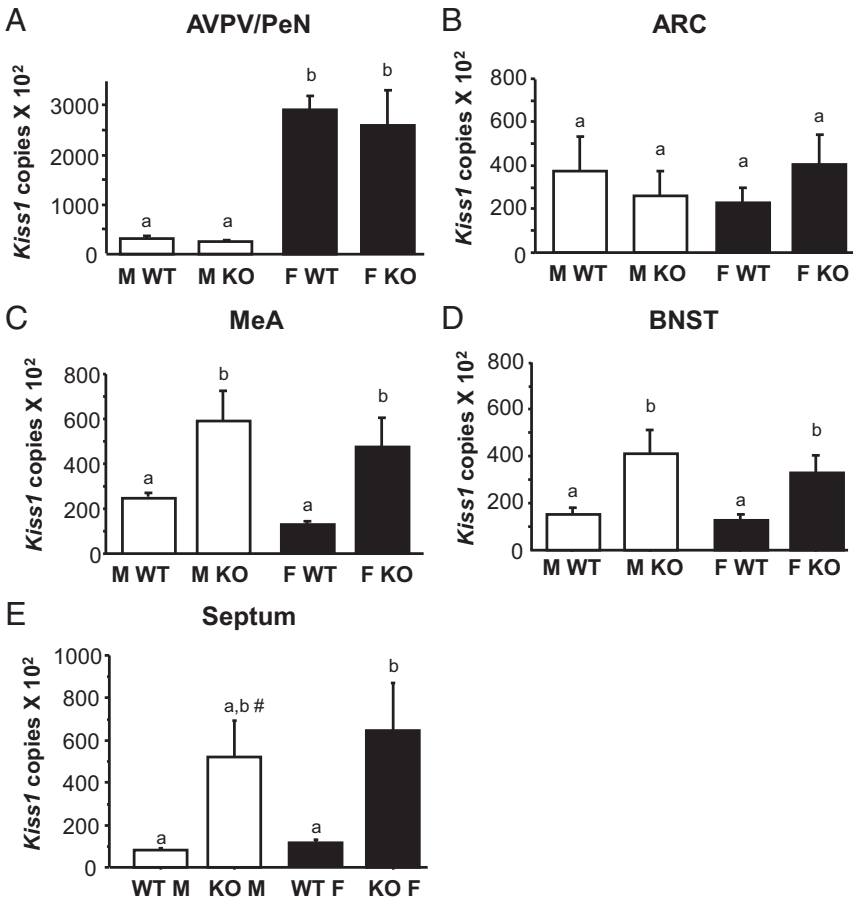

Figure 6. Mean numbers of *Kiss1* mRNA copies, determined via qPCR, in micropunches from the AVPV/PeN (panel A), the ARC (panel B), the MeA (panel C), the BNST (panel D), and the lateral septum (panel E) taken from adult GABA$_{B1}$KO and WT mice of both sexes Different letters denote significantly different from each other. #, nonsignificant trend; $P = .07$ vs WT males and WT females. F, female; M, male.

The presence of GABA receptors in *Kiss1* neurons has not been previously determined. Here we demonstrate, for the first time, that the GABA$_{B1}$ subunit is expressed in a very high percentage of *Kiss1* neurons of adult rodents. Despite this high coexpression, the lack of functional GABA$_B$Rs in GABA$_{B1}$KO mice surprisingly did not affect *Kiss1* expression in the AVPV/PeN or ARC, two important reproductive nuclei. This result was unexpected, given previous findings of subfertility and alterations in GnRH levels and pulsatility in GABA$_{B1}$KO mice (40). Based on this outcome, we propose that the reproductive impairments previously observed in adult GABA$_{B1}$KO mice are not due to underlying alterations in *Kiss1* expression in the main hypothalamic nuclei, ie, AVPV/PeN and ARC, that control fertility. Rather, other reproductive circuits or factors, like neurokinin B, dynorphin, or glutamate, might be involved, or the observed deficits in GnRH/fertility may be mediated directly by GABA$_B$R signaling in GnRH cells. We also cannot rule out that despite normal *Kiss1* levels in the ARC or AVPV/PeN, kisspeptin protein levels or secretion patterns in these regions might be altered in GABA$_{B1}$KO mice.

the hypothalamus, in the MeA and BNST, as well as the lateral septum, were dramatically increased in GABA$_{B1}$KO mice of both sexes, an effect that was not due to altered sex steroid levels. Intriguingly, this enhanced *Kiss1* expression was not detected in the MeA or BNST of prepubertal mice, indicating that the extrahypothalamic *Kiss1* alterations occur during or after puberty. Collectively, these novel findings indicate that impaired GABA$_B$ signaling can lead to dramatically altered *Kiss1* neurons in a region-specific manner, particularly in the MeA, BNST, and septum. Although it is unknown if these effects on *Kiss1* expression are due to direct or indirect (or secondary) effects of GABA signaling on *Kiss1* neurons, the presence of GABA$_{B1}$R in extrahypothalamic *Kiss1* neurons suggests the possibility for direct regulation. If so, it is possible that GABA$_B$ signaling might normally act to inhibit *Kiss1* expression in extra hypothalamic regions. These novel findings underscore the importance of studying the regulation and potential function of other kisspeptin populations outside the hypothalamus.

In addition to the ARC or AVPV/PeN, smaller *Kiss1* neuron populations resided in several extrahypothalamic brain areas, such as the MeA and BNST (14, 18–21), although kisspeptin's physiological roles in these regions are still under investigation. Interestingly, in stark contrast to the AVPV/PeN and ARC *Kiss1* systems, we found a dramatic increase of *Kiss1* expression in these extrahypothalamic regions in GABA$_{B1}$KO mice. In both the BNST and MeA of GABA$_{B1}$KO mice, *Kiss1* expression was higher in males than in females, although *Kiss1* was robustly increased in GABA$_{B1}$KO mice of both sexes compared with WT mice. The expression of *Kiss1* in the MeA and BNST is known to be up-regulated by sex steroids, primarily via ER-signaling pathways (14, 18). However, we found that E_2 and T serum levels were similar between genotypes, as was gonadal sex steroid content. Thus, the large increases in *Kiss1* expression in MeA and BNST are not due to a secondary effect of higher sex steroid secretion in

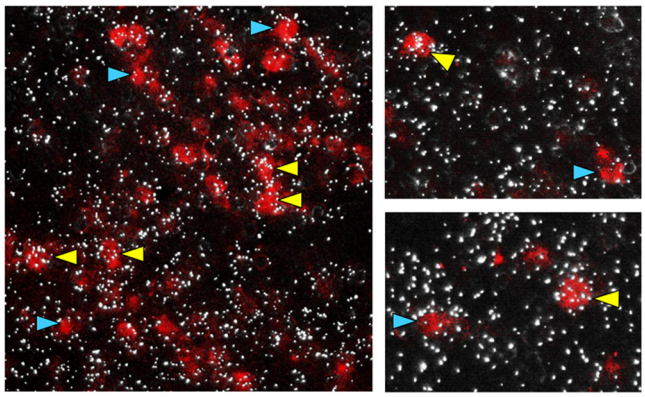

Figure 7. Representative images of *GABA$_{B1}$* subunit mRNA expression (silver grains) in *Kiss1* neurons (red fluorescence) in the MeA of adult male mice. Yellow arrowheads denote examples of *Kiss1*-GABA$_{B1}$ coexpression. Blue arrowheads denote *Kiss1* cell without GABA$_{B1}$. The majority (~66%) of MeA *Kiss1* cells coexpressed *GABA$_{B1}$* subunit.

GABA$_{B1}$KOs (although we cannot rule out higher levels of local neural steroidogenesis that could, in theory, occur in the MeA or BNST).

The functional implications of the dramatic increases in the *Kiss1* cell population in the MeA and BNST in GABA$_{B1}$KO mice remain to be elucidated, but our results lead us to speculate that under normal adulthood conditions, *Kiss1* expression in these areas is negatively regulated, directly or indirectly, by GABA$_B$R signaling. Whether this effect of GABA$_B$R signaling is direct or indirect on MeA and BNST *Kiss1* neurons is currently unknown, although our present finding of GABA$_{B1}$ coexpression in a majority of MeA *Kiss1* cells allows for the potential of a direct route of regulation by GABA, at least for this particular *Kiss1* population. This possibility of direct regulation will be important to directly test in future studies for each of the extrahypothalamic regions. In support of this possibility, prior evidence indicated that GABA can signal directly, via GABA$_B$R, to neurons in the MeA and BNST (46–49), although whether any of those neurons were *Kiss1* neurons was not determined.

The MeA and BNST, both of which express GABA$_B$R, participate in wide variety of processes and functions, including the modulation of various social and sexual behaviors through the integration of environmental and hormonal signals. Lesions of the MeA impair male sexual behavior in several rodent species (50, 51), whereas lesions of the posterior amygdala decrease sexual receptivity in female rats (52). The BNST has been shown to participate in proceptive and solicitational behaviors, sexual satiety, and maternal behavior (53–55), the latter of which was also influenced by GABA$_B$R signaling (56). Additionally, both the MeA and BNST have been implicated in anxiety behaviors (57–59). Adding complexity to the matter, each subregion of the amygdala sends and receives projections to and from other amygdala subnuclei, as well as other brain nuclei (60–63), including the BNST and hypothalamus (61, 64, 65). Moreover, both the AVPV and medial preoptic nucleus, which play important roles in ovulation and male copulatory behavior, respectively, receive regulatory input from the BNST and MeA (66). Although GABA$_{B1}$KO females do not demonstrate any deficit in their sexual receptivity, neither maternal and proceptive social behaviors nor male copulatory behaviors have yet been quantitatively studied in these mice. Kisspeptin originating from BNST or MeA could, in theory, participate in the modulation of any number of these social or behavioral processes; however, at present, only 2 studies have addressed the behavioral roles, if any, of kisspeptin. Whereas one study in mice found no direct role for kisspeptin signaling in male or female sexual behaviors (2), another study in rats determined that exogenous kisspeptin treatment increased both anxiety and locomotor behaviors (67). Interestingly, both anxiety and locomotion are similarly increased in GABA$_B$KO mice (68–70), but whether elevated endogenous kisspeptin originating from the MeA or BNST is driving these ob-

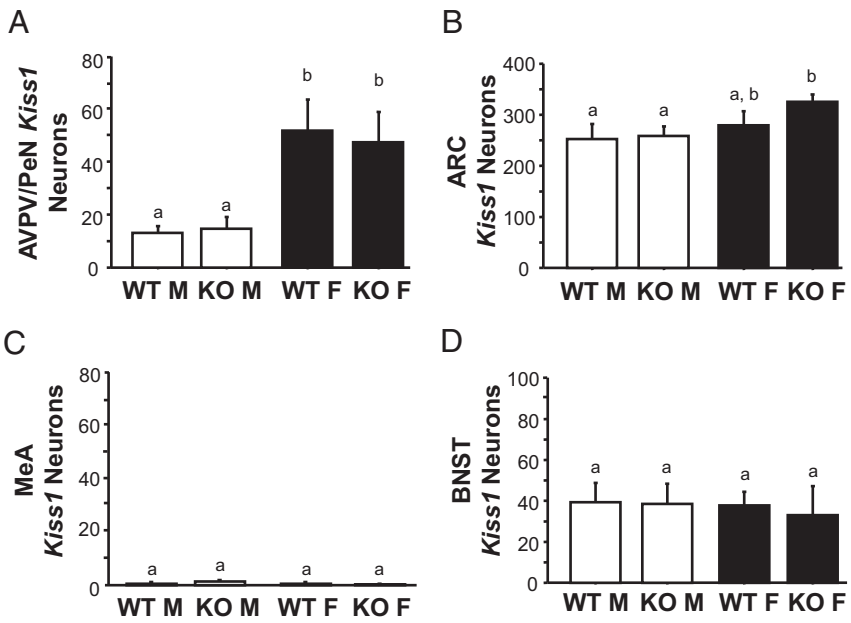

Figure 8. Mean *Kiss1* mRNA expression, determined via ISH, in brains from prepubertal (PND 14) GABA$_{B1}$KO and WT mice of both sexes A, AVPV/PeN; B, ARC; C, MeA; D, BNST. Different letters denote significantly different from each other. There were no genotype differences detected in any brain region of prepubertal mice. F, female; M, male.

served behavioral effects is unknown.

Concomitantly with the dramatic increase in *Kiss1* observed in the MeA and BNST, we also identified a remarkable increase in *Kiss1* expression in the lateral septum of adult $GABA_{B1}$KO mice, a region where *Kiss1* expression is essentially undetectable in WT mice. In this area, no sex difference in *Kiss1* expression was observed in the $GABA_{B1}$KO mice, with both KO males and females showing equally large increases in *Kiss1* expression. To our knowledge, this is the first report showing *Kiss1* neurons in the lateral septum of non-*Kiss1* transgenic mice. Previous studies of transgenic *Kiss1* mice have reported low levels of fluorescence (serving as a proxy for *Kiss1* neurons) in the septum (71). Although the authors interpreted that expression as "ectopic," because similar expression had never been reported in normal mice, our present findings argue that such *Kiss1* expression in the lateral septum may be real and may only normally be revealed when $GABA_B$ signaling is decreased, as in our $GABA_{B1}$KO mice. Indeed, $GABA_B$Rs are present in this region (72) and activation of $GABA_B$Rs can influence lateral septum dopamine release (73), indicating that GABA can act in this region via $GABA_B$R. Although the role of *Kiss1* neurons in the lateral septum remains to be determined, *Kiss1* expression in this region, like that in the MeA and BNST, seems to be under negative (direct or indirect) $GABA_B$R regulation in adult animals.

Interestingly, the high *Kiss1* expression observed in adult $GABA_{B1}$KO mice was not present in prepubertal mice of either sex (age PND 14). In the MeA, virtually no *Kiss1* neurons were found in any group, including WT mice, suggesting that MeA *Kiss1* expression is developmentally regulated to turn on during or after puberty. This matches a recent report in rats demonstrating no *Kiss1* expression in the MeA of PND 19 females (19). In the BNST, low to moderate *Kiss1* expression was detected in both genotypes, indicating that *Kiss1* is already expressed in this nucleus before puberty (even in WT mice), at low levels fairly similar to adult levels. Regardless, the absence of enhanced BNST *Kiss1* expression in prepubertal $GABA_{B1}$KO mice indicates that $GABA_B$R's effect on BNST *Kiss1*, like that in the MeA, is not present at all ages, but developmentally begins sometime after PND 14.

Collectively, our findings indicate that, despite high coexpression of *Kiss1* and $GABA_B$R, AVPV/PeN and ARC *Kiss1* gene expression is not altered in $GABA_{B1}$KO mice and therefore unlikely to contribute to the subfertility previously observed in these mice. Nevertheless, $GABA_{B1}$KO mice have dramatic changes in extrahypothalamic neural *Kiss1* expression, particularly in the MeA, BNST, and septum. Whether kisspeptin protein or secretion patterns are also altered in these regions remains to be determined. Preliminary immunohistochemical analysis did not successfully identify kisspeptin-immunoreactive cells in the MeA of $GABA_{B1}$KO mice (V. Lux-Lantos, unpublished observation), although only one pilot assay has been attempted thus far. Although it is unknown whether the observed increases in extrahypothalamic *Kiss1* gene expression are due to the loss of direct or indirect (or secondary) effects of $GABA_B$ signaling on *Kiss1* neurons, the observed presence of $GABA_{B1}$R in many MeA *Kiss1* neurons suggests the potential for direct regulation by GABA (at least in this region). If so, our data suggest that $GABA_B$ signaling may normally serve to inhibit *Kiss1* expression in extrahypothalamic regions. Because the MeA, BNST, and septal areas are involved in the regulation of reproductive and social behaviors, among other physiological processes like anxiety, the abnormally high *Kiss1* expression detected in $GABA_{B1}$KOs may cause alterations that contribute directly or indirectly to some of the phenotypes observed in these mice. Overall, our findings further emphasize the importance of both studying other kisspeptin populations outside of the hypothalamus and considering developmental vs adulthood differences in kisspeptin neurons and their regulation.

Acknowledgments

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