FINAL REPORT ISN-CAEN FELLOWSHIP

Category 1: Research A. Visit by the applicant to another laboratory
Fellow awarded: Noelia Paula Di Giorgio, MD.
Host Laboratory: Alexander Kauffman, PhD (UCSD, CA, USA)

Background to project

My project is focused on studying the relationship between the neurotransmitter GABA, acting through its $GABA_B$ receptors, and the neuropeptide kisspeptin in the regulation of the hypothalamic-pituitary-gonadal endocrine axis in mammals. My project utilizes a knockout mouse model, the $GABA_{B1}$ receptor subunit knockout mouse ($GABA_{B1}$KO), which has yet to be used to study regulation of GnRH and kisspeptin and therefore provides a novel and powerful approach.

Previous results in our laboratory revealed the interesting finding that adult female $GABA_{B1}$KO mice have compromised fertility and that these reproductive impairments were not due to behavioural modifications that had been described in these animals (1;2). Moreover, $GABA_{B1}$KO mice have alterations in hypothalamic GnRH, the key brain hormone that is essential for activating the rest of the reproductive system. Specifically, $GABA_{B1}$KO females had altered GnRH cellular content, mRNA expression, and pulsatile secretion, as well as changes in the expression of glutamate decarboxilase-67 (GAD-67), the enzyme that converts L-glutamate into GABA (1;2). Recently, we have been studying developmental aspects of the regulation of the reproductive axis at early postnatal stages of development, in particular at postnatal day 4 (PND4). We discovered that $GABA_{B1}$KO mice have significant alterations in hypothalamic GnRH content and mRNA expression, as well as GAD-67 mRNA expression, at this early developmental age. However, these PND4 alterations differ from those observed in adulthood, suggesting that additional levels of regulation occur in later stages of development. In view of these results, we decided to evaluate whether reproductive disturbances observed in $GABA_{B1}$KO females are due to alterations in the expression and/or secretion of kisspeptin (an important reproductive neuropeptide encoded by the *Kiss1* gene) and its receptor (*Kiss1R*) in the brain. Kisspeptin is a recently-identified stimulator of GnRH neurons in all mammals studied, and has been shown to be both necessary and sufficient for activation of GnRH secretion (and hence, activation of the reproductive axis). Our preliminary results in PND4 mice showed that *Kiss1* mRNA expression is decreased in the medial basal hypothalamus (MBH) of $GABA_{B1}$KO females.

Hypothesis to be tested

I am testing the hypothesis that impairments in the reproductive axis in $GABA_{B1}$KO mice are partly due to alterations in the brain's *Kiss1* system, brought about by the absence of functional $GABA_B$ receptors.

To test this hypothesis, I chose Dr. Kauffman's lab at U.C. San Diego as host institution because they have been studying sexual differentiation and development of reproductive circuits in the brain (and the physiological processes they control) during critical developmental periods, including the early postnatal and pubertal periods. Given the critical role of *Kiss1* in regulating the reproductive axis, Dr. Kauffman's lab is currently studying sexual differentiation and development of the *Kiss1* and *Kiss1R* systems, and are also investigating the regulatory mechanisms that govern and time the development of these important reproductive brain circuits (3).

Specific aims

The purpose of this research fellowship was to be able to detect and analyze *Kiss1* mRNA levels by *in situ* hybridization (ISH) in the brains of adult mice that lack $GABA_{B1}$ signalling. This powerful technique (which Dr. Kauffman's lab has expertise in) enabled us to evaluate simultaneously both the gene expression levels and specific localization of *Kiss1* neurons in the brains of these $GABA_{B1}$KO mice, identifying the precise nuclei or area where *Kiss1* expression is altered, thus widening our previous approaches to the study of this system. Taking in consideration *Kiss1* results and our previous data, we decided to analyze GnRH mRNA by ISH too.

Furthermore, we determined the expression of *Kiss1* and progesterone receptor (Pgr), as a marker for estradiol levels, in WT and $GABA_B$ KO mice by Real Time PCR (qPCR), a technique the Kauffman lab also routinely performs. In addition, we determined the estradiol levels in females and testosterone levels in males by RIA.

On the other hand, we determined the expression of tyrosine hydroxylase (TH) by qPCR since it will also help us to establish whether the alterations observed in *Kiss1* expression in the absence of functional $GABA_B$ receptors are unique to this gene or are a more generalized phenomenon, affecting other sexually dimorphic brain systems (like TH).

Materials and Methods

Animals

$GABA_{B1}$KO generated in the BALB/C inbred strain (4) were obtained by intercrossing heterozygous animals and the day of birth was recorded. Mice were genotyped by PCR analysis, as described previously (1). All animals were housed in groups in air-conditioned rooms (22 C), with lights on from 7AM to 7PM, and given free access to laboratory chow and tap water. All studies were performed according to protocols for animal use, approved by the Institutional Animal Care and Use Committee (IBYME-CONICET) that follows the NIH guidelines.

PND4 and adult, male and female, WT and $GABA_{B1}$KO mice were killed by decapitation in the morning (9-11AM). Their brains were immediately frozen on dry ice and stored at -80 C. Blood samples were collected to determine serum hormone levels. Pituitaries were collected and stored at -80 C until RNA obtention.

In situ hybridization

Five coronal series of 20µm brain sections were cut on a cryostat thaw mounted onto Superfrost-plus slides, and stored at -80 C until ISH.

Kiss1 cRNA probe was cloned from adult mouse hypothalamic cDNA into pBluscript II SK(-) transcription plasmid (Stratagene, La Jolla, CA) between *Hin*dIII and *Kpn*I restriction sites, and antisense probes transcribed with T7 polymerase, as previously described (5;6) . Gnrh cRNA probe was cloned as previously described (7).

Single-label ISH was performed as previously described (5;6). Briefly, slide-mounted sections were fixed in 4% paraformaldehyde, pretreated with acetic anhydride, rinsed in 2X SSC (sodium citrate, sodium chloride), delipidated in chloroform, dehydrated in ethanols, and air dried. Radiolabeled (33P) *Kiss1* or GnRH antisense riboprobe (0.04 pmol/ml) was combined with tRNA, heat denatured, added to hybridization buffer, and applied to each slide (100µl/slide). Slides were coverslipped and placed in a 55 C humidity chamber overnight. The slides were then washed in 4X SSC and placed into RNase A treatment for 30 min at 37 C, then in RNase buffer without RNase at 37 C for 30 min. After

washing in 2X SSC at room temperature, slides were washed in 0.1X SSC at 62Cfor 1 h, dehydrated in ethanols, and air dried. Slides were then dipped in Kodak (Eastman Kodak, Rochester, NY) NTB emulsion, air dried, and stored at 4 C for 3–4 d (depending on the assay) before being developed and coverslipped. No staining was detected with sense probes.

ISH slides were analyzed with an automated image processing system (Dr. Don Clifton, University of Washington) by a person unaware of the treatment group of each slide. For single-label experiments, the software counted the number of silver grain clusters representing cells, as well as the number of silver grains over each cell (a semiquantitative index of mRNA content per cell) (5;8;9). Cells were considered *Kiss1* or GnRH positive when the number of silver grains in a cluster exceeded that of background by 3-fold.

Quantitative real-time PCR

Total RNA from 400-µm-thick frozen micropunches of the AVPV/PeN and 500-µm-thick frozen micropunches of the ARC (8)were extracted using the RNeasy Lipid Tissue Mini kit (QIAGEN, Valencia, CA) according to the manufacturer's protocol. RNA was reverse transcribed using the Omniscript RT kit (QIAGEN), and cDNAwas stored at -20 C until use in PCR. Quantitative real-time PCR (qPCR) was performed on each cDNA sample using the Bio-Rad iCycler Detection System and Quantitect SYBR Green PCR kit (QIAGEN). To detect *Kiss1*, Pgr and TH 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´(6); Pgr forward: 5´-CTC CTG GAT GAG CCT GAT G-3´and Pgr reverse: 5´-CGC GGA TAT AGC TTG ATC TC-3´; TH forward: 5´-GTA CTT TGT GCG CTT CGA GG-3´ and TH reverse:5´- CAG GTG GTG ACA CTT ATC C-3´) were used. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as housekeeping gene (6) Standard curves were generated for each product using cloned cDNAs for *Kiss1*, TH and GAPDH to quantify the abundance of cDNA in each sample. For standard curves, a dilution series of cloned *Kiss1*, TH and GAPDH templates ranging from 10 to 10^8 copies were used. For Pgr we checked the efficiency of the primers with serial dilutions of pooled samples and due to the lack of cloned product we only measured the relative expression to GAPDH. The quantitative PCR cycling parameters were one cycle of 95 C for 15 min, followed by 50 cycles of 94 C for 15 sec, 60 C for 30 sec, and 72 C for 30 sec. Data collection was taken at the 72 C extension phase. 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*, Pgr and TH values were normalized to GAPDH, whose expression is constant. The size of the products was confirmed by 1% agarose gels.

Serum hormone levels

Blood was collected and serum obtained from adult WT and $GABA_{B1}$KO mice to detect estradiol in females and testosterone in males. Blood serum was analyzed for estradiol levels via a mouse ELISA assay and serum testosterone was assessed by RIA. Both procedures were performed by the University of Virginia Ligand Assay Core.

Statistics

Data were presented as the mean ± SEM for each group. The differences between means of more than two groups were analyzed by one- or two-way ANOVA, followed by the Tukey HSD test. $p<0.05$ was considered statistically significant. All analysis were performed in STATISTICA (data analysis software system), version 8.0, StatSoft, Inc. (2007).

Results

Kiss1 expression in PND4 and adult mice.

Kiss1 expression in the AVPV/PeN of PND4 mice was sexually dimorphic in WTs, higher in males than females (Figure 1). However, *Kiss1* expression in the ARC was higher in WT females than in WT males and this sex difference was lost in $GABA_{B1}$KO mice (Figure 2).

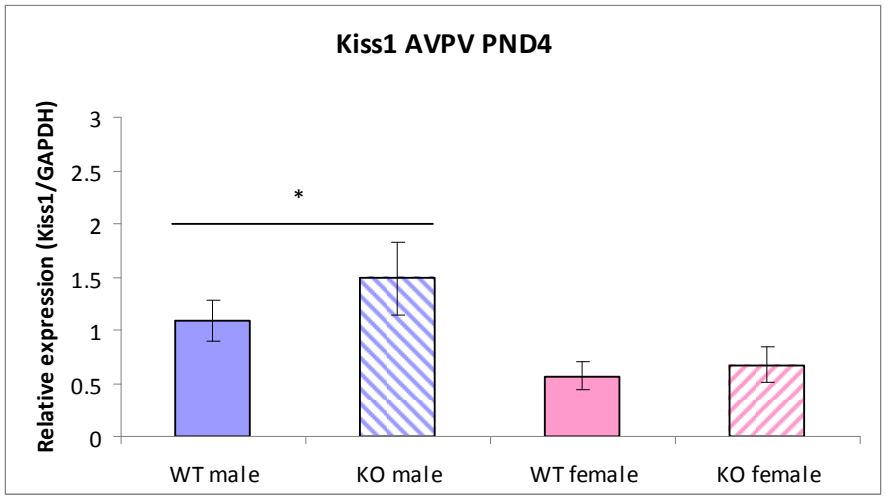


FIGURE 1: Relative expression of *Kiss1* in the AVPV/PeN of PND4 mice (qPCR). N=5-8. Two-Way ANOVA: interaction NS; main effect sex: p<0,01. *: males higher than females.

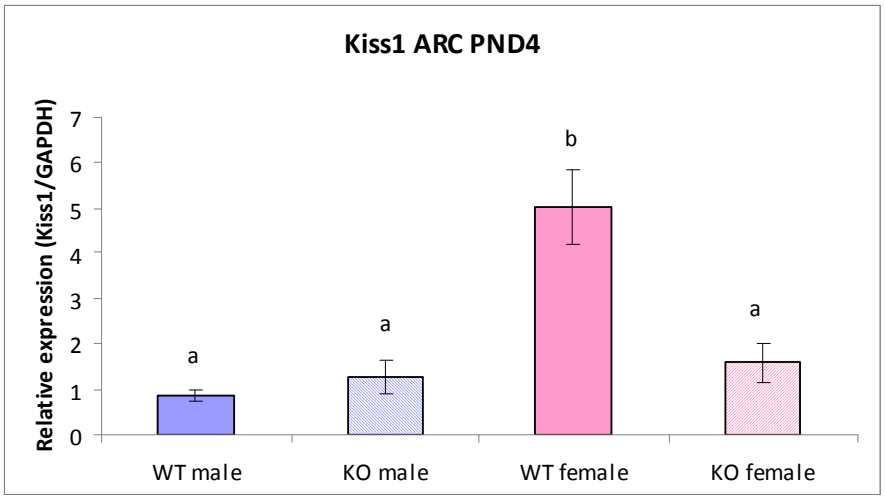


FIGURE 2: Relative expression of *Kiss1* in the ARC of PND4 mice (qPCR). N=4-8. Two-Way ANOVA: interaction: p<0,02 ; different letters indicate statistical significant differences between groups, p<0,01.

On the other hand, when we analyzed the relative expression of *Kiss1* in the AVPV/PeN of adult mice, we found that Kiss1 was higher in WT females than in WT males and this sex difference was also observed in $GABA_{B1}$KO mice. Furthermore, we found that $GABA_{B1}$KO females had much higher expression than WT females (Figure 3).*Kiss1* expression in the ARC of adult mice was not statistically different between groups (Figure 4).

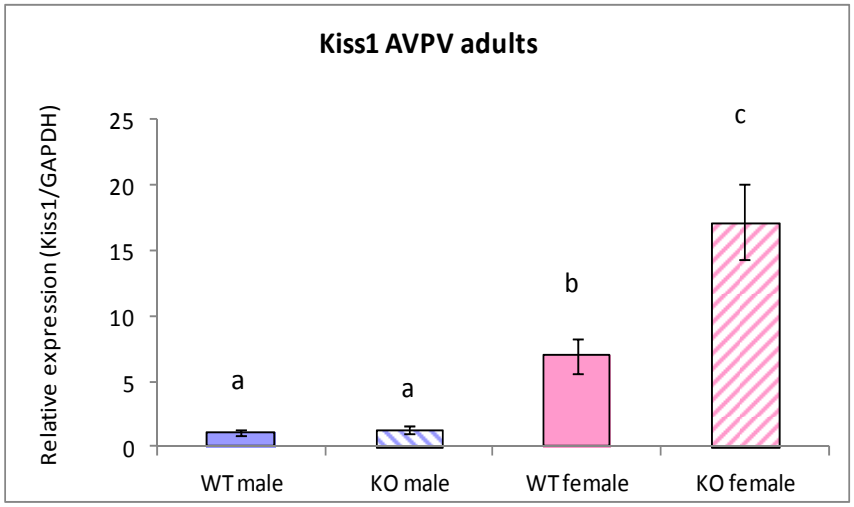


FIGURE 3: Relative expression of *Kiss1* in the AVPV/PeN of adult mice (qPCR). N=4.
Two-Way ANOVA: interaction: p<0,03 ; different letters indicate statistical significant differences between groups, p<0,03.

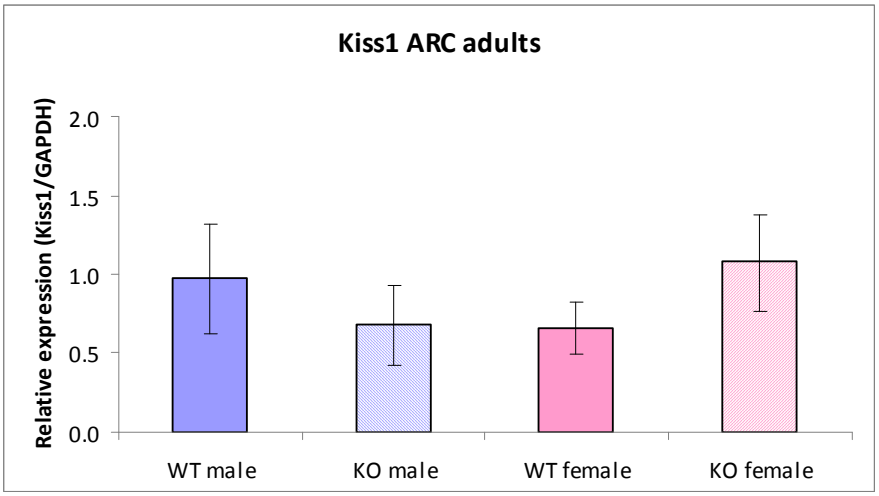


FIGURE 4: Relative expression of *Kiss1* in the ARC of adult mice (qPCR). N=5-7.
Two-Way ANOVA: interaction and main effects: NS.

Moreover, when we compared the number of copies of *Kiss1* between areas at the same age we found that all groups had higher expression of *Kiss1* in the ARC compared to AVPV/PeN at PND4 (Figure 5), especially in females. However, the AVPV/PeN expressed more *Kiss1* in adult females, while males expressed similar Kiss1 levels between areas in adulthood (Figure 6).

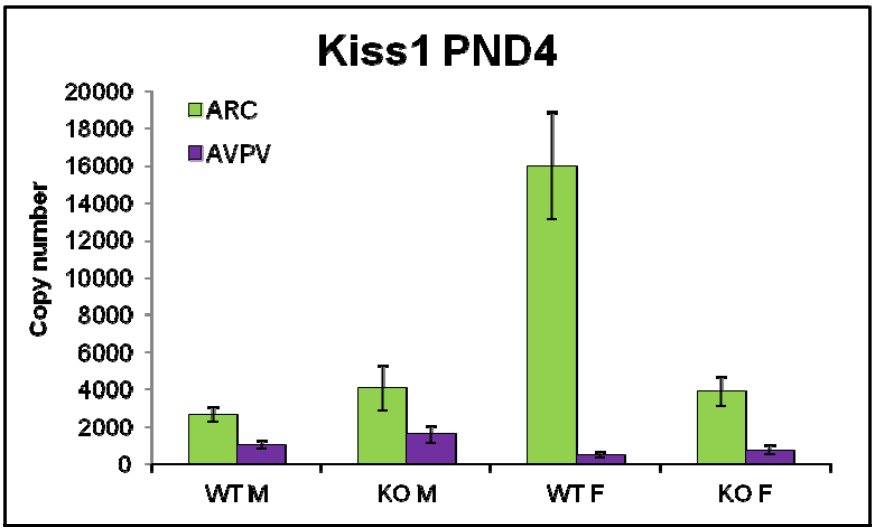


FIGURE 5: Relationship between areas in the copy number of *Kiss1* at PND4 mice (qPCR).

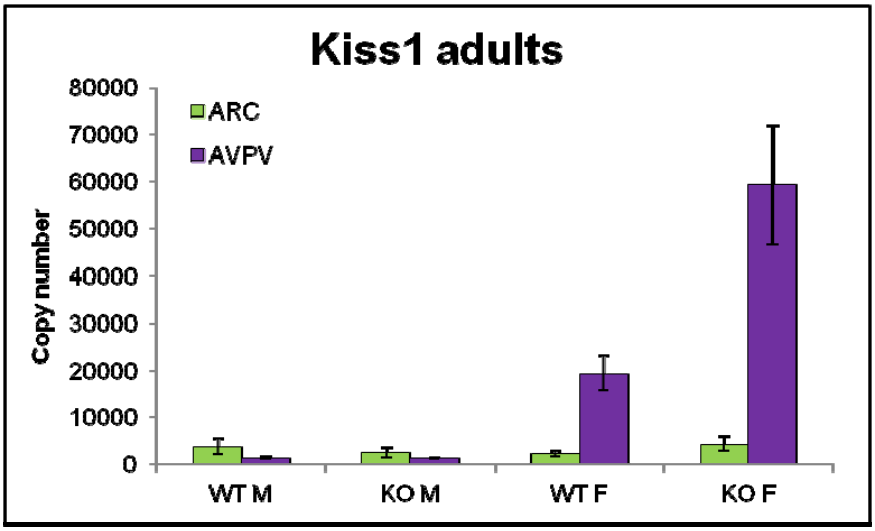


FIGURE 6: Relationship between areas in the copy number of *Kiss1* in adult mice (qPCR).

Furthermore, we corroborate that *Kiss1* expression in the AVPV/PeN was increased in adult females compared to PND4 females, while males had similar expression in the AVPV/PeN in the two ages evaluated (Figure 7). Interestingly, *Kiss1* expression in the ARC was similar between ages in all groups except for the WT females, who had higher *Kiss1* expression in the ARC at PND4 (Figure 8).

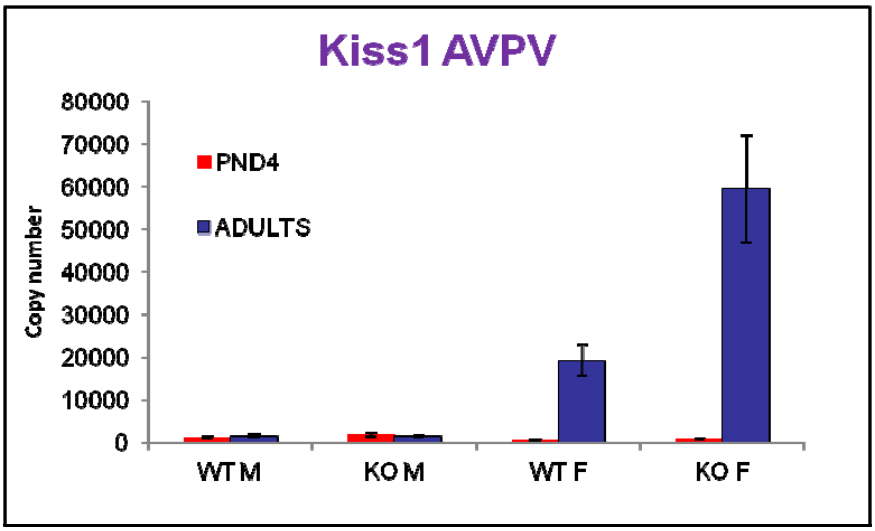


FIGURE 7: Relationship between ages in the copy number of *Kiss1* in the AVPV/PeN (qPCR).

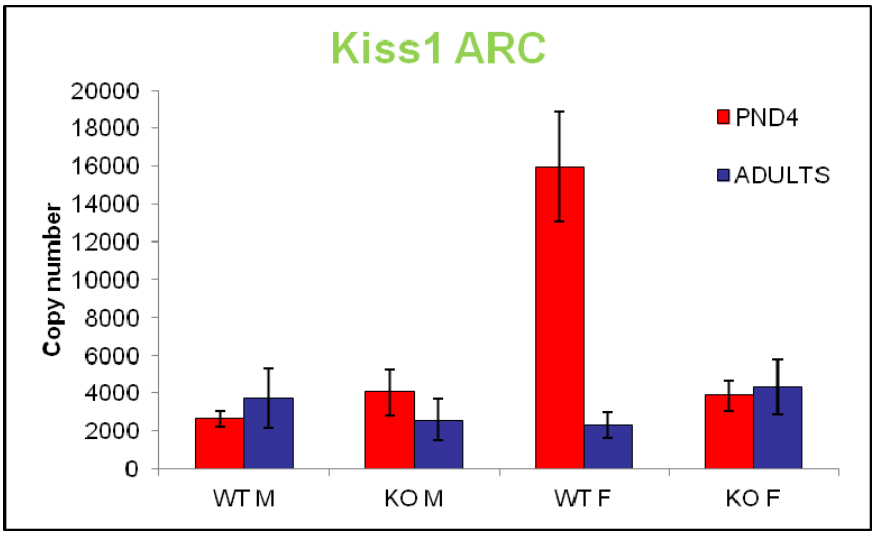


FIGURE 8: Relationship between ages in the copy number of *Kiss1* in the ARC (qPCR).

To have a more complete scenario of the expression and localization of the *Kiss1* neurons, we performed an ISH in adult mice. We evaluated the number of *Kiss1* cells and its expression per cell in the AVPV/PeN and ARC of the different groups.

Regarding the AVPV/PeN area we found that females had more *Kiss1* neurons and *Kiss1* mRNA per cell than males (Figure 9-10), without genotype differences.

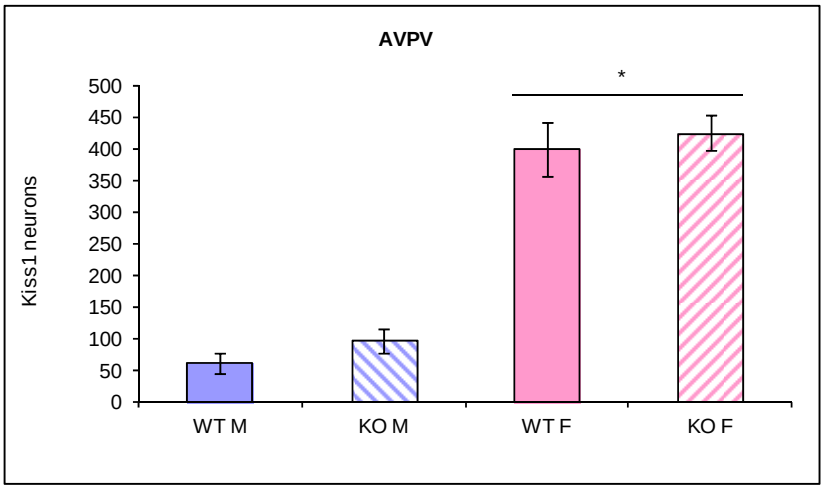


FIGURE 9: *Kiss1* neurons in the AVPV/PeN of adult mice (ISH). N=7.
Two-Way ANOVA: interaction: NS; main effects sex: p<0,001. *: females higher than males.

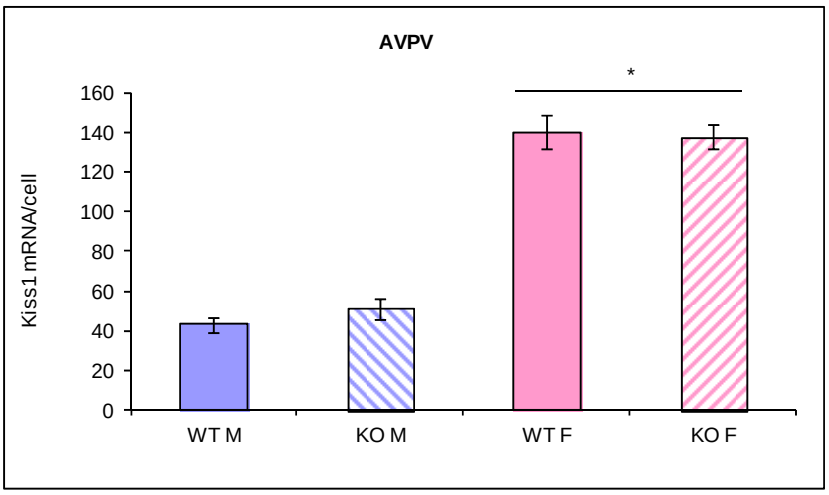


FIGURE 10: *Kiss1* mRNA/cell in the AVPV/PeN of adult mice (ISH). N=7.
Two-Way ANOVA: interaction: NS; main effect sex: p<0,001. *: females higher than males.

When we analyzed the ARC, we found that the number of *Kiss1* neurons was similar between groups, although the *Kiss1* mRNA per cell was slightly but significantly higher in females compared with males, without genotype differences (Figure 11-12).

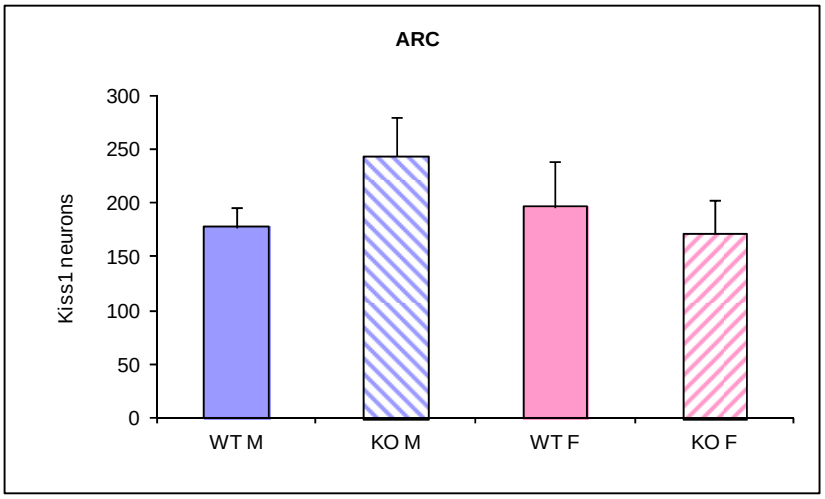


FIGURE 11: *Kiss1* neurons in the ARC of adult mice (ISH). N=7.
Two-Way ANOVA: interaction and main effects: NS.

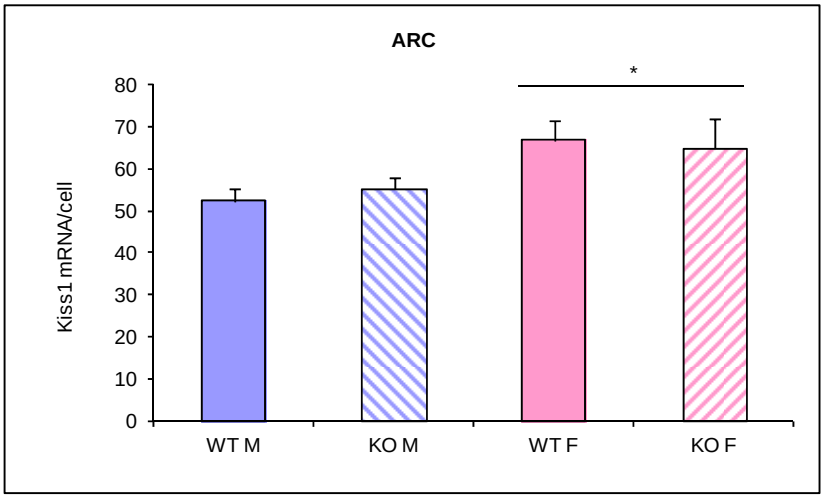
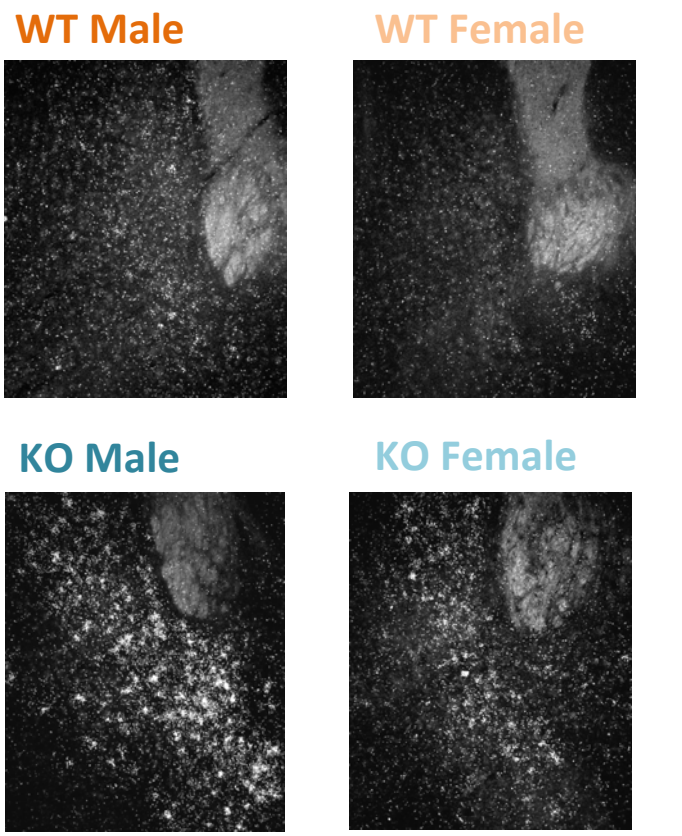


FIGURE 12: Kiss1 mRNA/cell in the ARC of adult mice (ISH). N=7.
Two-Way ANOVA: interaction: NS; main effect sex: p<0,02. *: females higher than males.

Although the results for AVPV/PeN showed the same sex difference by qPCR and ISH, by ISH we did not detect higher expression of *Kiss1* in $GABA_{B1}$KO females than in WTs, as we were able to detect by qPCR. Due to these results, and taking into consideration that when we micropunch the AVPV area we can take some portion of the bed nucleus of the stria terminalis (BNST), which is near the dorsal part of the AVPV, we decided to evaluate *Kiss1* neurons in that area.

Surprisingly, we found that the BNST in $GABA_{B1}$KO mice had more *Kiss1* neurons than in WTs (Figure 13), maintaining the same gender difference (male>female). The expression of *Kiss1* per cell was similar between groups (data not shown).



Note: white matter in each of the micrographs is the

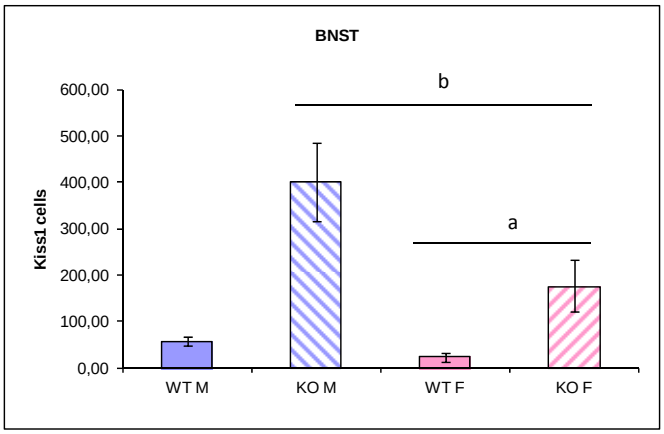


FIGURE 13: micrographs and quantification of *Kiss1* neurons in the BNST of adult mice (ISH). N=7. Two-Way ANOVA: interaction: NS; main effect sex (a): p<0,002 (males > females); main effect genotype (b): p<0,0001 ($GABA_{B1}$KO > WT)

In addition to those areas, we evaluated the number of *Kiss1* neurons in the posterior dorsal medial amygdala (MePD), which has been shown to have *Kiss1* cells and may have a role in reproduction (8). Our results showed that the same sex difference in *Kiss1* , as reported previously (males>females). Moreover, $GABA_{B1}$KO male and female mice had much higher *Kiss1* neurons and expression in the MePD than WTs (Figure 14). We found the same results if we analyzed the MePValone or the total amigdala (MeA: MePD and MePV) (data not shown).

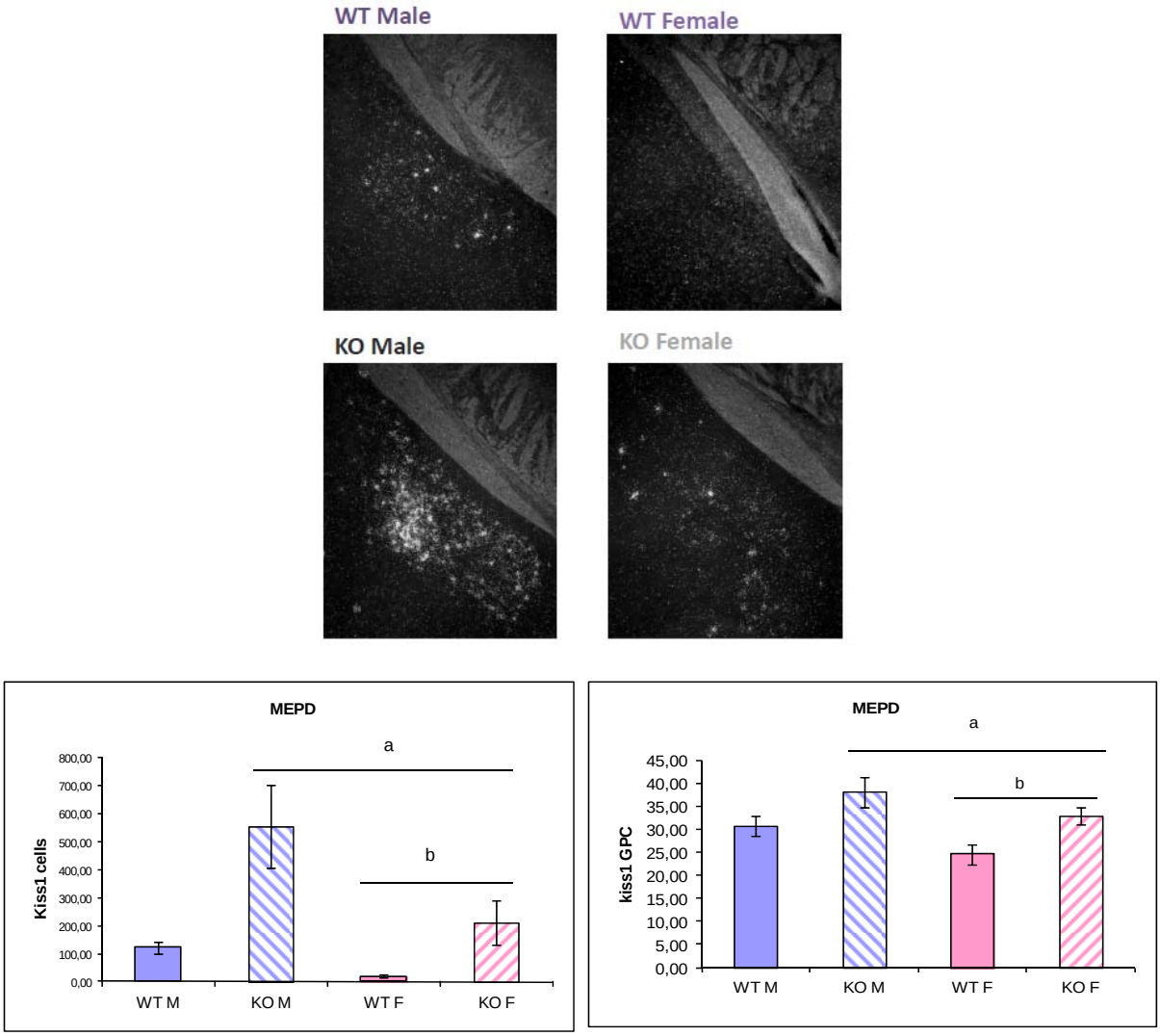
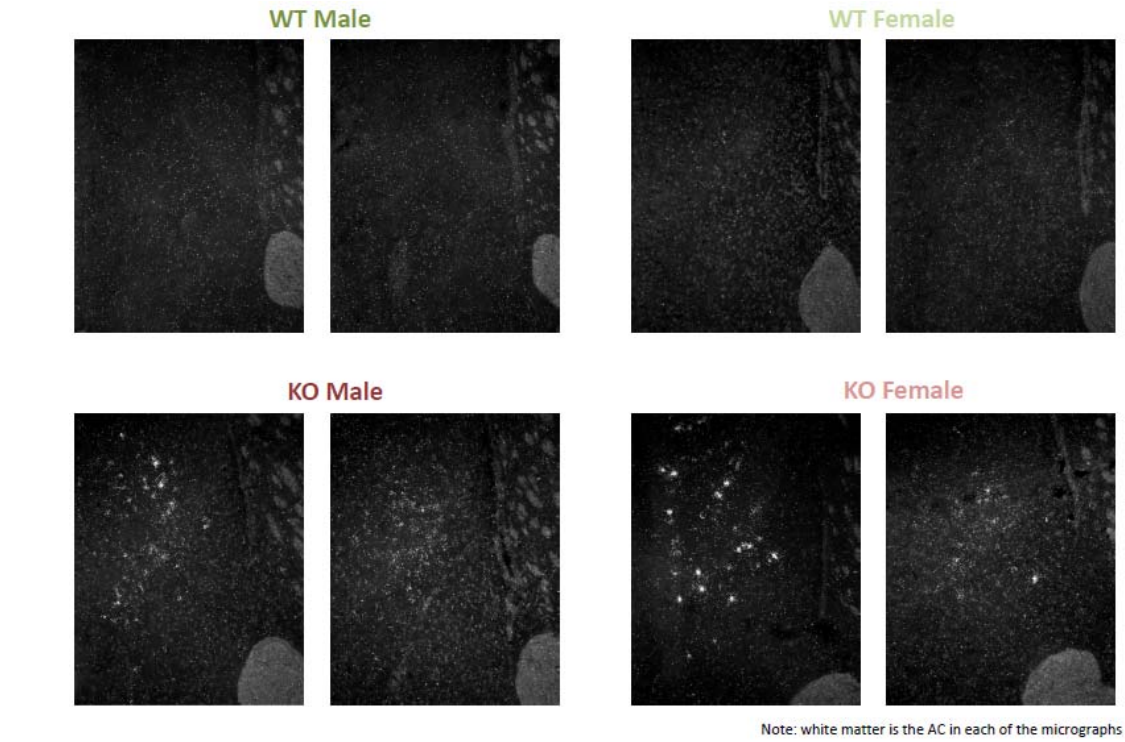


FIGURE 14: micrographs and quantification of *Kiss1* neurons and grains per cell (GPC) in the posterior dorsal medial amygdala (MePD region) of adult mice (ISH). N=7. Two-Way ANOVA *Kiss1* cells (left): interaction: NS; main effect sex (b): p<0,0001 (males > females); main effect genotype (a): p<0,0001 ($GABA_{B1}$KO > WT). Two-Way ANOVA *Kiss1* GPC (right): interaction: NS; main effect sex (b): p<0,04 (males > females); main effect genotype (a): p<0,004 ($GABA_{B1}$KO > WT)

The septal area, which lies frontally to the AVPV, has basically no *Kiss1* neurons in WT mice. However, we found that male and female $GABA_{B1}$KO mice had a significantly increased number of *Kiss1* neurons and its expression per cell in this area when compared to WTs (Figure 15).



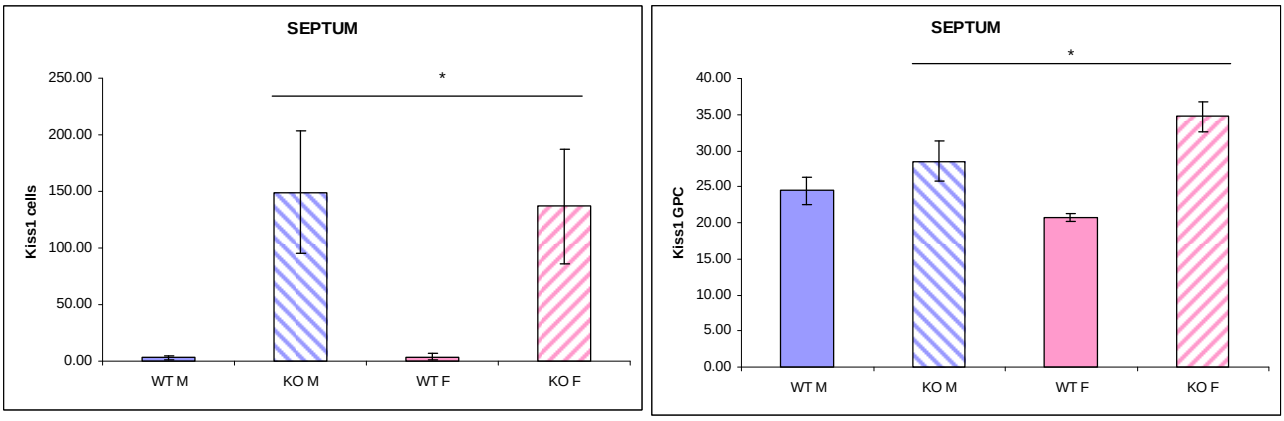


FIGURE 15: micrographs and quantification of *Kiss1* neurons and grains per cell (GPC) in the septal area of adult mice (ISH). N=7. Two-Way ANOVA: interaction: NS; main effect sex: NS; main effect genotype: p<0,01 (*: $GABA_{B1}$KO > WT).

Pgr expression and estradiol

Due to the fact that we found some important genotype differences in *Kiss1* expression by qPCR and ISH in different areas of the brain and that it is well known that estradiol is the major regulator of *Kiss1* neurons, we decided to evaluate Pgr expression, which is used as a marker of estradiol levels and signaling, in the same micropunches by qPCR.

We did not find differences between groups at PND4 (Figure 16-17). However, we found interesting results in adulthood, where Pgr expression was sexually dimorphic in the AVPV/PeN of WT mice (males higher than females) and this was lost in $GABA_{B1}$KO mice. Moreover, $GABA_{B1}$KO females had higher expression than WTs (Figure 18). Regarding the ARC, we found that $GABA_{B1}$KO mice had lower Pgr expression than WTs, without sex differences (Figure 19).

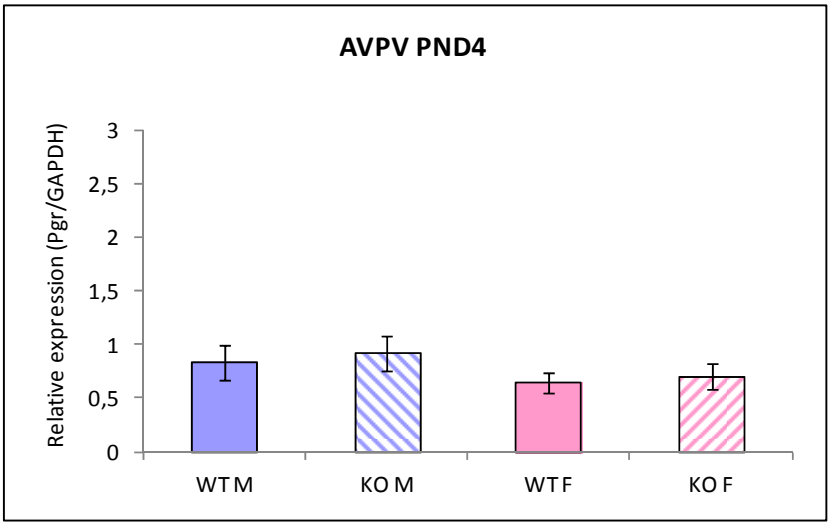


FIGURE 16: Relative expression of Pgr in the AVPV/PeN of PND4 mice (qPCR). N=5-9.
Two-Way ANOVA: interaction NS and main effects: NS

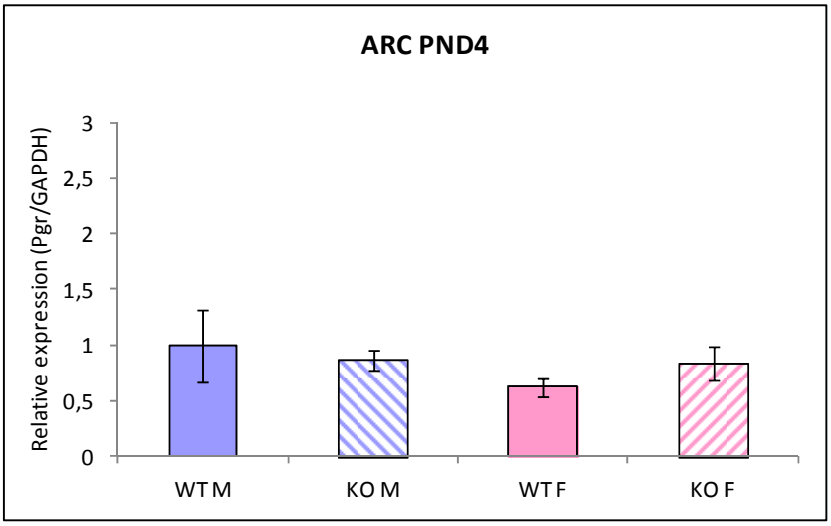


FIGURE 17: Relative expression of Pgr in the ARC of PND4 mice (qPCR). N=5-10.
Two-Way ANOVA: interaction NS and main effects: NS

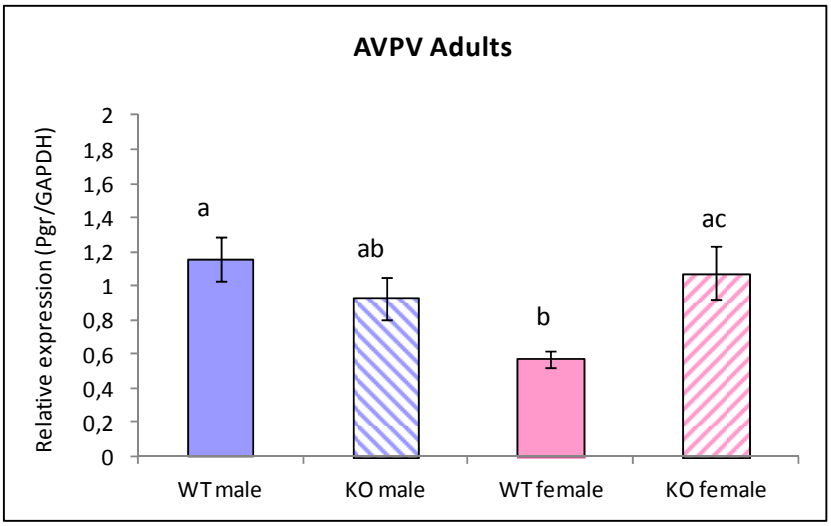


FIGURE 18: Relative expression of Pgr in the AVPV/PeN of adult mice (qPCR). N=5-7. Two-Way ANOVA: interaction: p<0,01; different letters indicate statistical significant differences between groups, p<0,04.

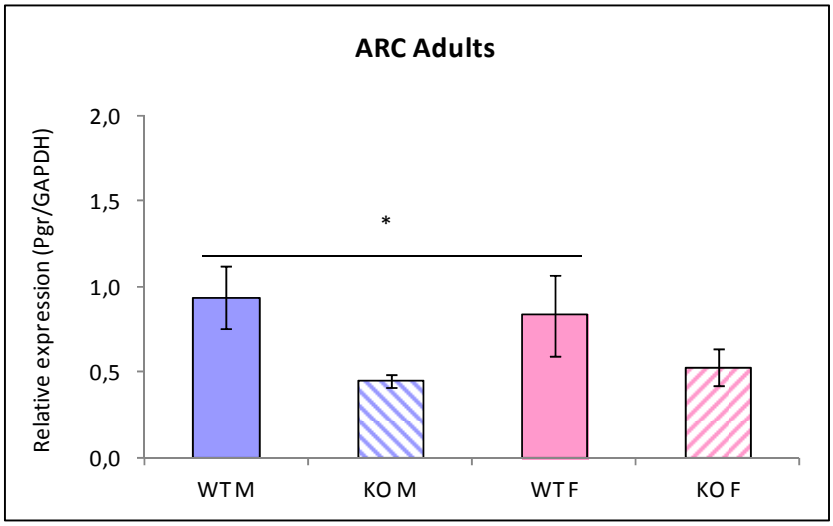


FIGURE 19: Relative expression of Pgr in the ARC of adult mice (qPCR). N=5-7. Two-Way ANOVA: interaction: NS; main effect genotype: p<0,03. *: WT mice higher than $GABA_{B1}$KO mice.

Since we found that the pattern of *Kiss1* and Pgr expression was similar between WT and $GABA_{B1}$KO females in the AVPV (increased in $GABA_{B1}$KOs) and as it is well known that Pgr expression is stimulated by estradiol in that area, we decided to evaluate if serum estradiol was altered in adult $GABA_{B1}$KO females. Testosterone serum levels were also determined in $GABA_{B1}$KO and WT males. Interestingly, we did not find significant differences in circulating sex steroids between genotypes (Figure 20-21), suggesting that other mechanisms participate in inducing genotype differences in $GABA_{B1}$KO mice.

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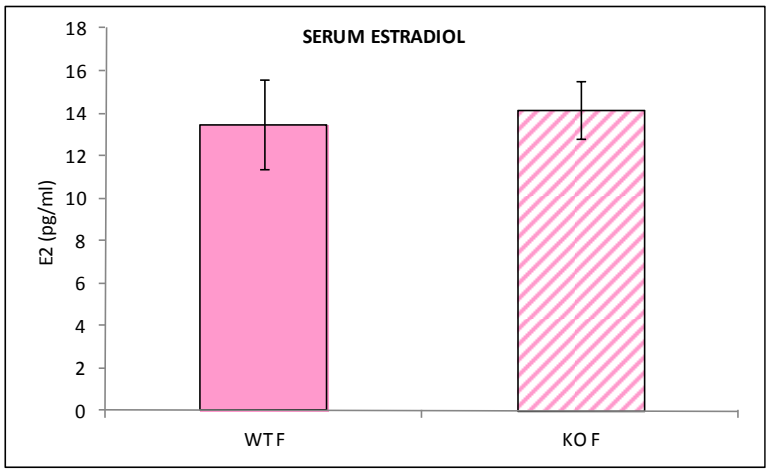

Figure 20: Serum estradiol (pg/ml) in adult, WT and $GABA_{B1}$KO females (ELISA). N=7-8
One-way ANOVA: NS.

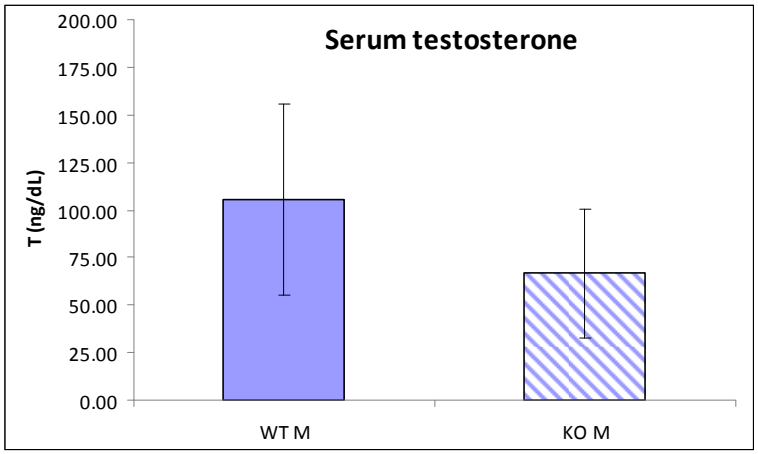

Figure 21: Serum testosterone (ng/dl) in adult, WT and $GABA_{B1}$KO males (RIA). N=5-6
One-way ANOVA: NS.

GnRH expression in adult mice

Kiss1 neurons in the AVPV and ARC regulate the gonadotrophic axis acting directly to GnRH neurons through *Kiss1r*. So, it is very important to evaluate whether the alterations observed in *Kiss1* expression in AVPV, BNST, amygdala and septum of adult mice could be impacting GnRH expression. For this reason, we decided to do ISHs and to look at GnRH neurons in the POA. We found that the number of GnRH neurons and the GnRH mRNA expression per cell did not vary between groups (Figure 22-23).

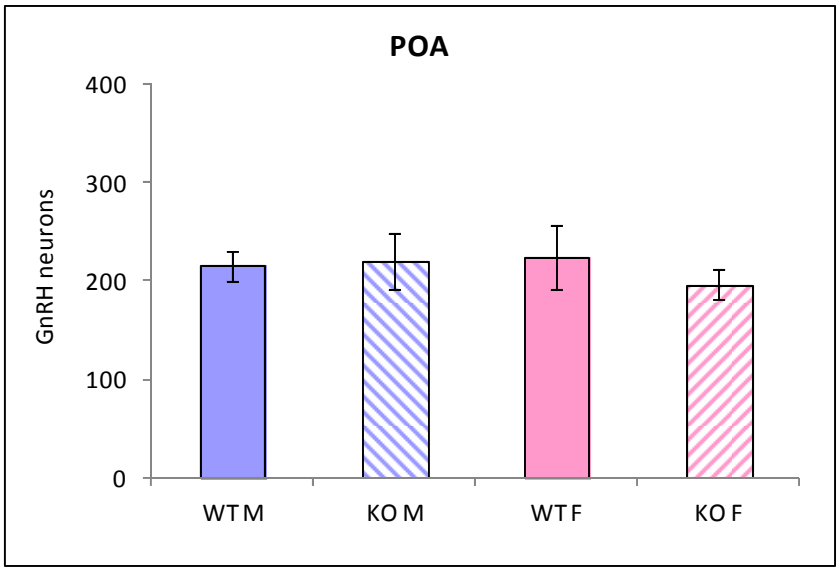

FIGURE 22: GnRH neurons in the POA of adult mice (ISH). N=7.
Two-Way ANOVA: interaction and main effects: NS.

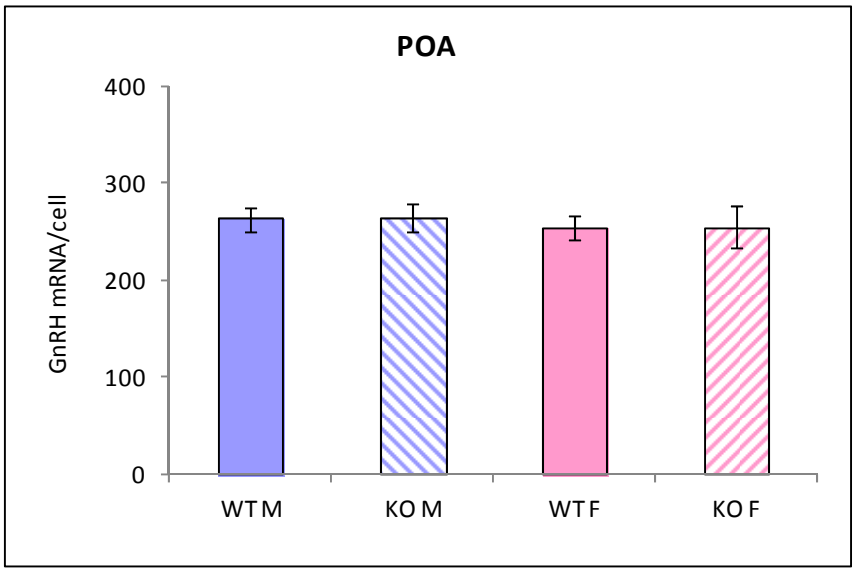

FIGURE 23: GnRH mRNA/cell in the POA of adult mice (ISH). N=7.
Two-Way ANOVA: interaction and main effects: NS.

TH expression in PND4 and adult mice

We then decided to evaluate whether the alterations observed in *Kiss1* expression in the absence of functional $GABA_B$ receptors are unique to this gene or are a more generalized phenomenon, affecting other sexually dimorphic brain systems. So, we measured TH mRNA expression, known to be sexually dimorphic, by qPCR in the same areas at PND4 and adult mice.

We did not find any differences between groups at PND4, either in the AVPV or in the ARC (Figure 24-25). However, in the AVPV of adult mice, we found that TH expression was sexually dimorphic in WT mice (females higher than males), as expected (5), and this sex difference was lost in the $GABA_{B1}$KO mice. On the other hand, we found the inverse sexual dimorphism in the ARC, without genotype differences (Figure 26-27).

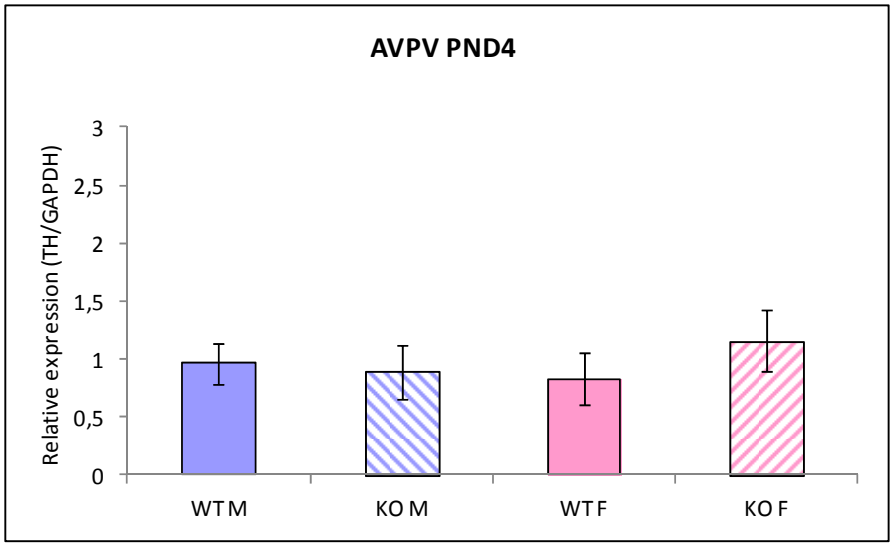

FIGURE 24: Relative expression of TH in the AVPV/PeN of PND4 mice (qPCR). N=7-9. Two-Way ANOVA: interaction and main effects: NS.

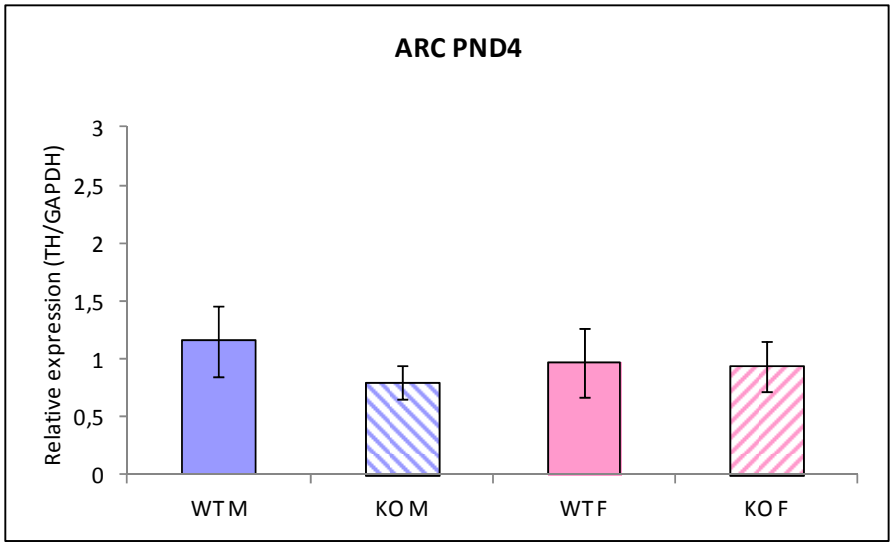

FIGURE 25: Relative expression of TH in the ARC of PND4 mice (qPCR). N=5-10. Two-Way ANOVA: interaction and main effects: NS.

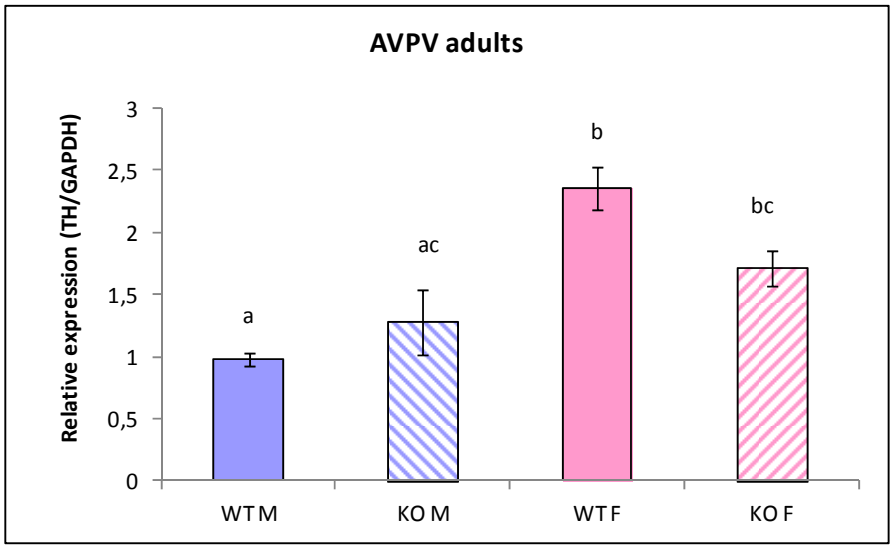

FIGURE 26: Relative expression of TH in the AVPV/PeN of adult mice (qPCR). N=4.
Two-Way ANOVA: interaction: p<0,03; different letters indicate statistical significant differences between groups, p<0,05.

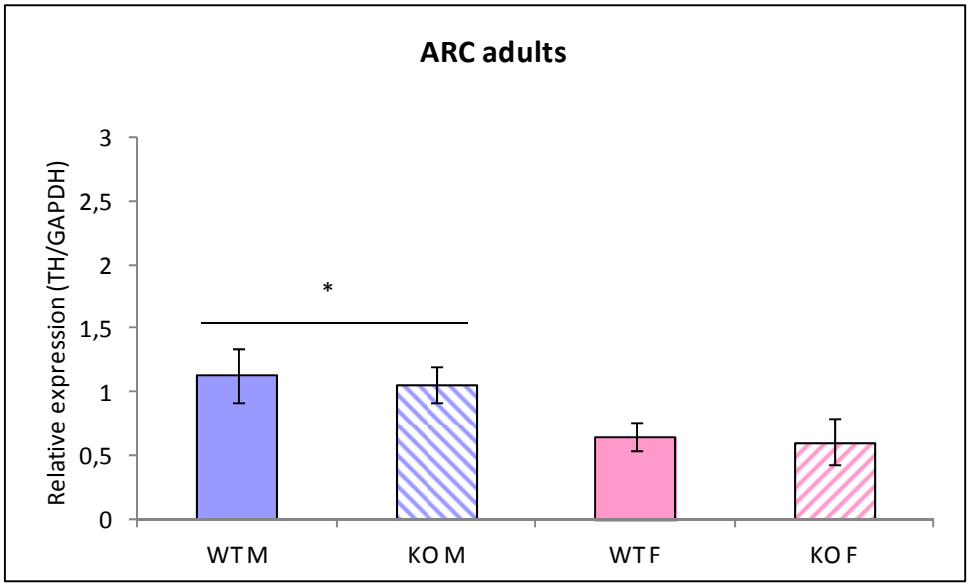

FIGURE 27: Relative expression of TH in the ARC of adult mice (qPCR). N=5-7.
Two-Way ANOVA: interaction: NS; main effects sex: p<0,02. *: males higher than females.

When we analyzed the copy number of TH in the different areas at the same age, we found that TH is expressed in higher amounts in the ARC compared to the AVPV in all groups at PND4 (Figure 28). However, in adulthood, TH is higher in the ARC of males compared to the AVPV but similar between areas in females (Figure 29). Moreover, TH expression in the AVPV increased in adults compared to PND4 but in the ARC it increased only in males (Figure 30-31).

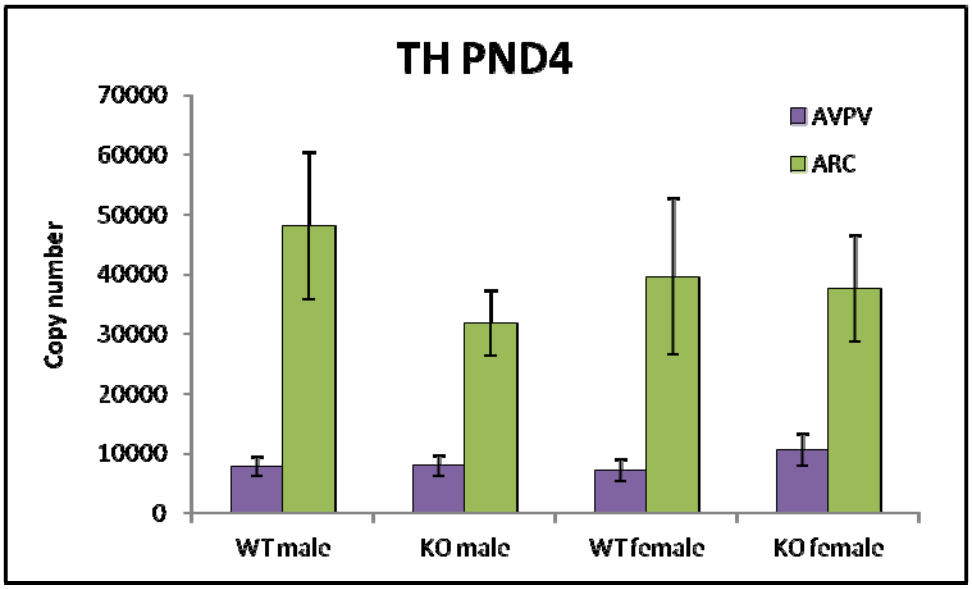

FIGURE 28: Relationship between areas in the copy number of TH at PND4 mice (qPCR).

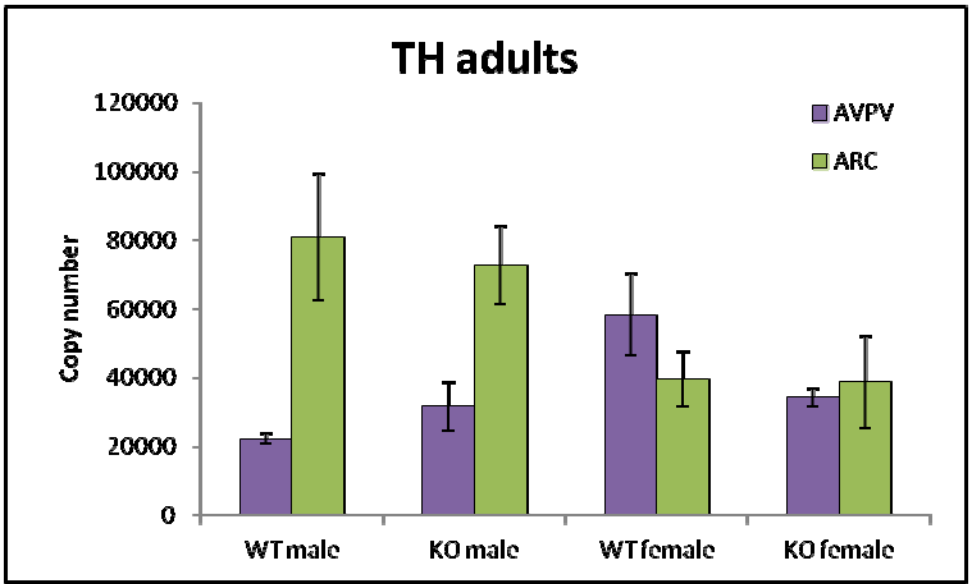

FIGURE 29: Relationship between areas in the copy number of TH in adult mice (qPCR).

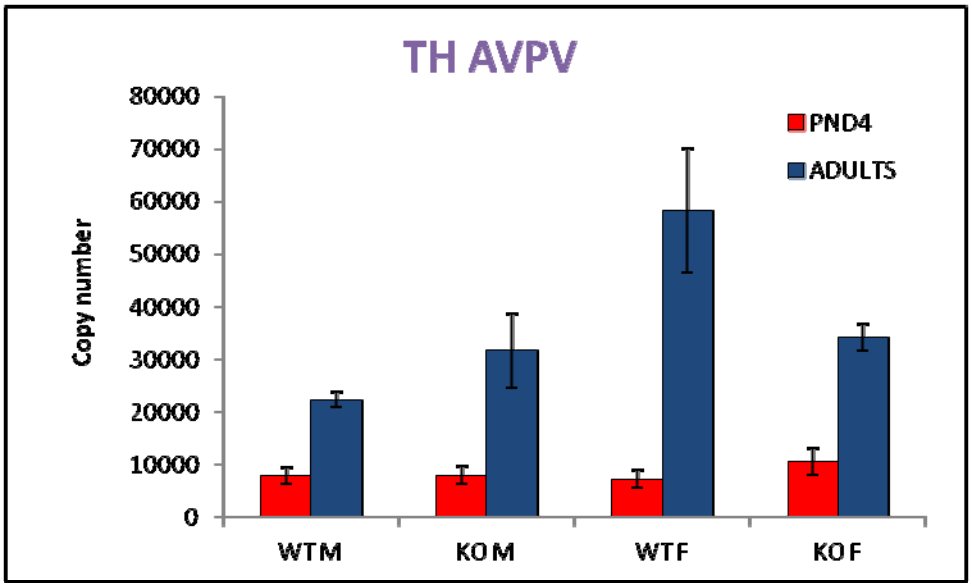

FIGURE 30: Relationship between ages in the copy number of TH in the AVPV/PeN (qPCR).

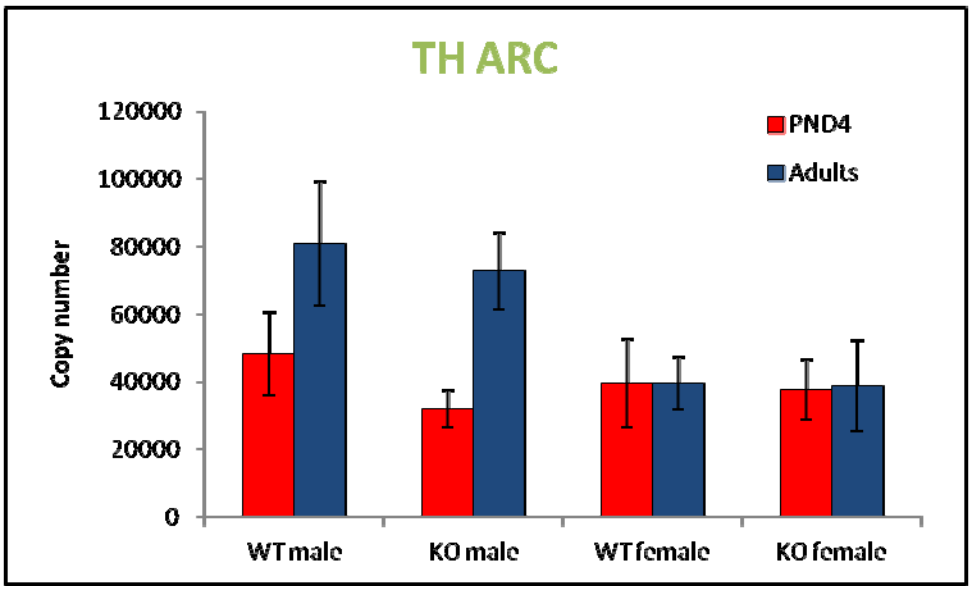

FIGURE 31: Relationship between ages in the copy number of TH in the ARC (qPCR).

Co-localization of *Kiss1* and $GABA_{B1}$ receptor

As we found very interesting results due to the lack of a functional $GABA_B$ receptor, we decided to evaluate whether the $GABA_B$ receptor mRNA is synthesized in *Kiss1* neurons by using double label ISH, a question never studied before that will help us to understand the relationship between GABA and kisspeptin system in the reproductive axis.

These are our preliminary results in two females for each genotype. . We found that $GABA_B$ receptor is expressed in *Kiss1* neurons in WT and $GABA_{B1}$KO females. Although the outcome was very obvious, we are going to analyze a great number of females and males soon to confirm the results.

Animal	Group	# of AVPV Kiss1 Cells analyzed	# of double labeled Cells (SBR >3)	% Co-labelled
3496	KO F	128	122	95.31%
3510	WT F	125	124	99.20%
3514	KO F	232	226	97.41%
3535	WT F	185	183	98.92%

Discussion

Kisspeptin system

It is well documented that adult females have more *Kiss1* mRNA and kisspeptin immunoreactivity in the AVPV/PeN than males (10-12)and that there is not a sex difference in the ARC at that time (5;9;10). Moreover, the expression of *Kiss1* mRNA in the ARC has been studied since PND1 (13), although it was not detected until PND 10 in the AVPV of mice (5) . In adults *Kiss1* neurons in the ARC mediate the negative feedback of sex steroids on the gonadotropic axis in both genders, while in females *Kiss1* neurons in the AVPV mediate the positive feedback elicited by estradiol which triggers the preovulatory LH surge (14).

Although the regulation and function of *Kiss1* neurons in the AVPV and ARC areas has been well studied, the presence and importance of *Kiss1* neurons in other brain regions is less understood. Recently, Dr. Kauffman´s group have shown that male rats and mice have higher *Kiss1* neurons and mRNA per cell than females in the MeA (8). Furthermore, they demonstrated that *Kiss1* is expressed and regulated by sex steroids in the MeA of both sexes.

In addition, Xu et al. have demonstrated in rats that estrogen stimulates the expression of Kiss1 gene and peptide in the rostral periventricular area of the third ventricle (RV3P), the ventromedial hypothalamus nucleus (VMH), the MeA, the bed nucleus of the stria terminalis (BNST) and paraventricular hypothalamic nucleus (PaAP) (12). Furthermore, they showed that the sensitivity to estrogen in RV3P, VMH, BNST and PaAP is dimorphic, with a greater sensitivity in females rats, whereas ARC and MeA did not show a sex differences.

Interestingly, the AVPV, BNST, VMH and MeA have been demonstrated to be involved in the regulation of sexual behavior (15-17). And it has been reported that these regions express ERα, which is necessary for sexual behavior, although no one has examined the existence of this receptor in *Kiss1* neurons, except in the AVPV. Thus, it is possible that kisspeptin participates in the modulation of dimorphic sexual behavior such as mounting and lordosis. Additionally, the amygdala sends neuronal projections to target sites known to be involved in reproduction, including the preoptic area (where GnRH neurons reside), the AVPV, and ARC (8).

GABA and $GABA_B$ receptors are widely expressed in the brain, and little is known of their interaction with kisspeptidegic neurons. Therefore, in this work we analyzed *Kiss1* expression in the AVPV and

ARC of $GABA_{B1}$KO and control WT mice by qPCR and ISH. In addition, we also analyzed *Kiss1* mRNA expression in BNST, MeA and septum by ISH in adult mice of both genders and genotypes.

Kiss1 cell number (ISH) and expression (ISH and qPCR) in the **AVPV/PeN** of **adult** WT mice were sexually dimorphic, females had more Kiss1 neurons and expressed more than males, in agreement with previous results (10-12). However, although we did not find a genotype difference by ISH, we found a clear genotype difference in the AVPV of females by qPCR, where the expression of *Kiss1* was increased in $GABA_{B1}$KO compared to WTs, holding the same sex difference. The difference in the results obtained by each technique may be due to their specific sensitivity (qPCR>ISH).

Kiss expression in the AVPV of PND4 mice by qPCR, showed low expression, with males expressing significantly more than females, without genotype differences. At this age *Kiss1* can not be detected by ISH in this area, questioning the physiological importance of the sex differences found by qPCR, but suggesting again differences in assay sensitivities.

Interestingly we found that *Kiss1* expression, sexually dimorphic in both genotypes (males>females), was significantly increased in the **BNST** of adult $GABA_{B1}$KO compared to WT mice. This finding posed the question whether the increased *Kiss1* detection by qPCR in $GABA_{B1}$KO females could be due to contamination of the sample with part of the BNST when obtaining the micropunches. Nevertheless, this does not seem to be the case, since no increase in *Kiss1* expression in the AVPV of $GABA_{B1}$KO males was observed, even though they have the highest *Kiss1* expression in the BNST. Therefore, we maintain that differences in method sensitivity may account for this discrepancy.

Regarding ***Kiss1*** expression in the **ARC of adult mice**, we did not find differences in the number of *Kiss1* neurons (ISH) or the relative expression of *Kiss1* (qPCR), although we found a small but statistically significant sex difference in *Kiss1* expression/cell by ISH. As we mentioned before, it is well documented that adult mice have similar number of *Kiss1* neurons in the ARC during the adulthood (14). Although no difference was observed by qPCR in adult mice, ***Kiss1* expression in the ARC of PND4** was sexually dimorphic in WTs (females>males) and its expression decreased significantly in $GABA_{B1}$KO females with regard to WT controls, without sex differences in this genotype. Males showed no genotype differences.

These observations are in agreement with previous results in our laboratory, where we found that the main alterations in adult $GABA_{B1}$KO females were at the anterior hypothalamus (2), where the AVPV is located, while PND4 $GABA_{B1}$KO mice had important alterations at the medial basal hypothalamus which contains the ARC (18).

Another interesting result was that when we evaluated the number of copies **of *Kiss1* between areas** (qPCR) we found that *Kiss1* expression was predominant in the ARC of PND4 mice. However, *Kiss1* expression in the AVPV is higher compared to its expression in the ARC in adult females. Thus, it is possible that kisspeptin in the ARC is already functional at this early stage of life.

We also analyzed other areas of the brain, such as the amygdala and the septum. We found a clear sex difference in WT mice, males higher than females, in the MeA, as described by Kim J et al. (8). Furthermore, *Kiss1* cell number and *Kiss1* mRNA per cell were significantly increased $GABA_{B1}$KO compared to WT mice of both genders. Interestingly, in the septum, in which *Kiss1* expression is normally, practically non-existent, we observed a very high expression in $GABA_{B1}$KO mice, without

sex differences. These results need to be investigated ti find which is the role of Kiss1 in those areas and why it is altered in $GABA_{B1}$KO mice.

Finally, all the alterations due to genotype observed in the expression of *Kiss1* in PND4 and adult mice, did not affect the number of cells and mRNA per cell of GnRH in the POA of adult $GABA_{B1}$KO mice (ISH), postulating that Kiss1 regulation at this site may be exerted at the posttranscriptional level.

Role of steroid hormones in kisspeptin system

In adult rodents, ARC *Kiss1* neurons are inhibited by sex steroids and may mediate negative feedback effects of gonadal hormones on pulsatile GnRH secretion (3). In contrast, AVPV/PeN *Kiss1* neurons are stimulated by sex steroids, primarily estradiol (E2), and may mediate E2's positive feedback induction of the preovulatory LH surge, a sexually-dimorphic event occurring only in females (3;19). Recently, Xu et al. (12) have demonstrated in rats that estrogen stimulates the expression of *Kiss1* gene and peptide in the rRV3P, VMH, MeA, (BNST and PaAP. However, its expression is regulated in a negative way by estrogen in ARC. Furthermore, they showed that the sensitivity to estrogen in RV3P, VMH, BNST and PaAP is dimorphic, with a greater sensitivity in female rats, whereas ARC and MeA did not show a sex difference.

As we found higher *Kiss1* expression in the AVPV of $GABA_{B1}$KO adult females and in BNST, MeA and septum of both sexes $GABA_{B1}$KO mice, we decided to evaluate whether the serum steroids were elevated in adult $GABA_{B1}$KOs. We analyzed the expression of **Pgr in the AVPV and ARC,** a well established biomarker for circulating estradiol levels, which induces higher expression of hypothalamic Pgr (20-23). We found that $GABA_{B1}$KO adult females had higher expression of Pgr in the AVPV but lower in the ARC compared to WT adult females, thus theoretically correlating with higher estradiol in $GABA_{B1}$KO females. However, the expression of Pgr did not vary between genotypes in the AVPV of males but was lower in the ARC of $GABA_{B1}$KO males.

To clarify this situation, we decided to evaluate the sex hormones **serum levels.** Although the measure of estradiol in mice is still controversial, we sent our samples to a lab using the reliable method (24). Surprisingly, serum estradiol and testosterone levels were similar between genotypes. It may still be possible that estradiol is produced in greater amounts locally in the brain of adult $GABA_{B1}$KO mice, we are now working on this hypothesis.

On the other hand, we did not find differences in the expression **of Pgr at PND4** in the areas studied, although we found that *Kiss1* expression was decreased in $GABA_{B1}$KO females at that time. Our previous results in our lab showed that estradiol content/mg tissue is increased in the gonads of $GABA_{B1}$KO mice at PND4. Moreover, Takumi et al. (25) postulated that *Kiss1* neurons in the ARC are already sensitive to estradiol at PND3, although the existence of estrogen receptor alpha in *Kiss1* neurons at this age has not been established. So, we will need to determine whether this increase leads to increased serum levels that could affect *Kiss1* expression in the ARC at this stage of development, taking into account the presence of serum alpha fetoprotein, that binds estrogen, at this age. A possible increase in brain synthesized estrogen must also be determined.

Tyrosine hydroxylase system and role of steroid hormones

Finally, we decided to evaluate whether the alterations observed in *Kiss1* system in $GABA_{B1}$KO mice were a particular phenomenon or a more generalized one that included other sexually dimorphic systems, such as TH.

Our TH expression results by qPCR showed that only the AVPV in adults was compromised in $GABA_{B1}$KO mice. We found a clear sex difference in WT mice as was previously reported (26) (females higher than males), but it was lost in $GABA_{B1}$KOs. It is known that estradiol downregulates TH expression in the adult AVPV in both sexes (27). Moreover, whereas adult steroids levels can affect overall TH cell number, the sex difference in the AVPV is determined by neonatal concentration of steroids (27;28). Taking into account these observations, we postulate that in $GABA_{B1}$KO mice neonatal circulating levels of testosterone/estradiol may be influencing TH expression observed in the adulthood. As no differences in serum sex steroids were observed.

Nevertheless, the alterations observed in *Kiss1* expression in the different nuclei, seems to be specific for this gene in each region.

Conclusions

In summary, we found that adult $GABA_{B1}$KO mice have impairments in the brain's *Kiss1* system, more specifically in the AVPV, BNST, MeA and septum, brought about by the absence of functional $GABA_B$ receptors. This abnormal expression may cause alterations in the reproductive axis that lead to the phenotype observed in adulthood. We propose to continue our studies to evaluate the importance of these populations of *Kiss1* neurons highly expressed in $GABA_{B1}$KO mice and their relation with GABA system in reproduction.

Regarding PND4 mice, the results obtained from *Kiss1* expression in $GABA_{B1}$KO mice further hint to the important role of the ARC in modulating the gonadotropic axis at this age.

References

1. **Catalano PN, Bonaventura MM, Silveyra P, Bettler B, Libertun C, Lux-Lantos VA** 2005 GABA(B1) Knockout Mice Reveal Alterations in Prolactin Levels, Gonadotropic Axis, and Reproductive Function. Neuroendocrinology 82:294-305
2. **Catalano PN, Di Giorgio N, Bonaventura MM, Bettler B, Libertun C, Lux-Lantos VA** 2010 Lack of functional GABA(B) receptors alters GnRH physiology and sexual dimorphic expression of GnRH and GAD-67 in the brain. Am J Physiol Endocrinol Metab 298:E683-E696
3. **Kauffman AS** 2010 Coming of age in the kisspeptin era: sex differences, development, and puberty. Mol Cell Endocrinol 324:51-63
4. **Schuler V, Luscher C, Blanchet C, Klix N, Sansig G, Klebs K, Schmutz M, Heid J, Gentry C, Urban L, Fox A, Spooren W, Jaton AL, Vigouret J, Pozza M, Kelly PH, Mosbacher J, Froestl W, Kaslin E, Korn R, Bischoff S, Kaupmann K, van der Putten H, Bettler B** 2001 Epilepsy, hyperalgesia, impaired memory, and loss of pre- and postsynaptic GABA(B) responses in mice lacking GABA(B(1)). Neuron 31:47-58
5. **Semaan SJ, Murray EK, Poling MC, Dhamija S, Forger NG, Kauffman AS** 2010 BAX-dependent and BAX-independent regulation of Kiss1 neuron development in mice. Endocrinology 151:5807-5817

6. **Semaan SJ, Dhamija S, Kim J, Ku EC, Kauffman AS** 2012 Assessment of epigenetic contributions to sexually-dimorphic kiss1 expression in the anteroventral periventricular nucleus of mice. Endocrinology 153:1875-1886
7. **Finn PD, Steiner RA, Clifton DK** 1998 Temporal patterns of gonadotropin-releasing hormone (GnRH), c-fos, and galanin gene expression in GnRH neurons relative to the luteinizing hormone surge in the rat. J Neurosci 18:713-719
8. **Kim J, Semaan SJ, Clifton DK, Steiner RA, Dhamija S, Kauffman AS** 2011 Regulation of kiss1 expression by sex steroids in the amygdala of the rat and mouse. Endocrinology 152:2020-2030
9. **Kauffman AS, Navarro VM, Kim J, Clifton DK, Steiner RA** 2009 Sex differences in the regulation of Kiss1/NKB neurons in juvenile mice: implications for the timing of puberty. Am J Physiol Endocrinol Metab 297:E1212-E1221
10. **Kauffman AS, Gottsch ML, Roa J, Byquist AC, Crown A, Clifton DK, Hoffman GE, Steiner RA, Tena-Sempere M** 2007 Sexual differentiation of Kiss1 gene expression in the brain of the rat. Endocrinology 148:1774-1783
11. **Clarkson J, Herbison AE** 2006 Postnatal development of kisspeptin neurons in mouse hypothalamus; sexual dimorphism and projections to gonadotropin-releasing hormone neurons. Endocrinology 147:5817-5825
12. **Xu Z, Kaga S, Mochiduki A, Tsubomizu J, Adachi S, Sakai T, Inoue K, Adachi AA** 2012 Immunocytochemical localization of kisspeptin neurons in the rat forebrain with special reference to sexual dimorphism and interaction with GnRH neurons. Endocr J 59:161-171
13. **Poling MC, Kauffman AS** 2012 Sexually dimorphic testosterone secretion in prenatal and neonatal mice is independent of kisspeptin-Kiss1r and GnRH signaling. Endocrinology 153:782-793
14. **Poling MC, Kauffman AS** 2012 Organizational and activational effects of sex steroids on kisspeptin neuron development. Front Neuroendocrinol
15. **Crews D** 2005 Evolution of neuroendocrine mechanisms that regulate sexual behavior. Trends Endocrinol Metab 16:354-361
16. **Paredes RG, Baum MJ** 1997 Role of the medial preoptic area/anterior hypothalamus in the control of masculine sexual behavior. Annu Rev Sex Res 8:68-101
17. **Simerly RB** 1998 Organization and regulation of sexually dimorphic neuroendocrine pathways. Behav Brain Res 92:195-203
18. **Di Giorgio N, Catalano PN, Bettler B, Libertun C, Lux-Lantos V,** Alterations in the Regulation of the Gonadotrophic Axis In Neonatal GABAB1KO Mice. (Abstract)
19. **Herbison AE** 2008 Estrogen positive feedback to gonadotropin-releasing hormone (GnRH) neurons in the rodent: the case for the rostral periventricular area of the third ventricle (RP3V). Brain Res Rev 57:277-287
20. **Quadros PS, Pfau JL, Goldstein AY, De Vries GJ, Wagner CK** 2002 Sex differences in progesterone receptor expression: a potential mechanism for estradiol-mediated sexual differentiation. Endocrinology 143:3727-3739
21. **Quadros PS, Goldstein AY, De Vries GJ, Wagner CK** 2002 Regulation of sex differences in progesterone receptor expression in the medial preoptic nucleus of postnatal rats. J Neuroendocrinol 14:761-767
22. **Wagner CK, Pfau JL, De Vries GJ, Merchenthaler IJ** 2001 Sex differences in progesterone receptor immunoreactivity in neonatal mouse brain depend on estrogen receptor alpha expression. J Neurobiol 47:176-182
23. **Wagner CK, Nakayama AY, De Vries GJ** 1998 Potential role of maternal progesterone in the sexual differentiation of the brain. Endocrinology 139:3658-3661
24. **Haisenleder DJ, Schoenfelder AH, Marcinko ES, Geddis LM, Marshall JC** 2011 Estimation of estradiol in mouse serum samples: evaluation of commercial estradiol immunoassays. Endocrinology 152:4443-4447

25. **Takumi K, Iijima N, Ozawa H** 2011 Developmental changes in the expression of kisspeptin mRNA in rat hypothalamus. J Mol Neurosci 43:138-145
26. **Simerly RB, Swanson LW, Gorski RA** 1985 The distribution of monoaminergic cells and fibers in a periventricular preoptic nucleus involved in the control of gonadotropin release: immunohistochemical evidence for a dopaminergic sexual dimorphism. Brain Research 330:55-64
27. **Simerly RB** 1989 Hormonal control of the development and regulation of tyrosine hydroxylase expression within a sexually dimorphic population of dopaminergic cells in the hypothalamus. Brain Res Mol Brain Res 6:297-310
28. **Bodo C, Kudwa AE, Rissman EF** 2006 Both estrogen receptor-alpha and -beta are required for sexual differentiation of the anteroventral periventricular area in mice. Endocrinology 147:415-420