New studies to unravel the connection between GABA$_B$ receptors and *Kiss1* expression and function. Evaluation in recently developed *Kiss1*-GABA$_{B1}$KO mice and global GABA$_{B1}$KO mice.

Noelia Paula Di Giorgio, PhD.

BACKROUND

The gonadotropin releasing hormone (GnRH) neurons represent the final output pathway of the neuronal network controlling reproduction in all mammalian species. The regulation of GnRH secretion depends on numerous inputs into GnRH neurons, which have been the aim of intense investigations for many years. Among these many inputs, the salient ones include kisspeptin, GABA and glutamate [1].

The neuropeptide kisspeptin, encoded by the *Kiss1* gene, is crucial for puberty and adult reproductive function. Kisspeptin is synthesized in two main hypothalamic regions (AVPV/PeN and ARC) and stimulates reproductive hormones secretion [2-4]. Additionally, *Kiss1* is also expressed outside the hypothalamus in the amygdala, bed nucleus of the *stria terminalis* (BNST), and lateral *septum* (LS) [5].

Although kisspeptin is well known to be expressed in discrete brain regions, it is also present in some peripheral tissues, such as adipose tissue and pancreas [6;7]. Likewise, its receptor (*Kiss1r)* is also expressed in multiple non-GnRH brain areas and in several peripheral tissues, including metabolic tissues like fat, liver, and pancreas [6;7]. This suggests that kisspeptin has additional roles beside reproduction. Changes in energy status or metabolic signals affect both reproduction and hypothalamic kisspeptin levels [8;9], which suggests that kisspeptin neurons mediate metabolic effects on reproductive axis. Tolson et al demonstrated in adult *Kiss1r* KO mice, which lack functional kisspeptin signaling, that in addition to reproductive alterations, the kisspeptin system is also an important player in body weight, energy balance, locomotion, and glucose metabolism regulation [10]. These effects are possibly the result of peripheral, rather than central, pathways. The role for kisspeptin on insulin secretion is controversial. Wolfe et al. demonstrated that kisspeptin at nM concentrations suppressed glucose-stimulated insulin secretion (GSIS) from control islets but not from islets lacking the *Kiss1r*, so the *Kiss1r* mediates this suppression. However, at supraphysiological levels (µM), kisspeptin stimulates GSIS through a non *Kiss1r*-mediated pathway [7].

GABA is the main inhibitory neurotransmitter in the mammalian central nervous system. It acts through two kinds of receptors, ionotropic GABA$_{A/C}$ receptors involving a chloride channel and associated with fast inhibitory/stimulatory conductance events, and metabotropic GABAB receptors (GABA$_B$R) that are Gi/0 protein linked inducing late, slow hyperpolarization, diminution in membrane Ca2+ conductance by voltage-sensitive calcium channels (VSCC), increase in membrane K+ conductance (Kir-3) and inhibition of adenylyl cyclase. GABA is also a key regulator of reproductive neural circuits, and its action through GABA$_A$ receptors has been widely studied, including its effects on both GnRH neurons and kisspeptidergic neurons [1;11]. GABA, acting through GABA$_B$R, also regulates reproduction [12], and has many important effects that are slowly coming to light. For example In adult rodents, the AVPV/PeN neuron population that co-expresses kisspeptin and GABA provides an important double excitatory input to GnRH neurons at the time of ovulation and also the hyperpolarizing stimulus needed for preovulatory rebound burst firing of kisspeptin neurons is mediated by GABA$_B$R (and also µ and κ opioid receptors); therefore, absence of GABA$_B$ signaling at this critical time may compromise the preovulatory surge [1;13].

Our previous studies demonstrated that mice globally lacking GABA$_B$R (GABA$_{B1}$KO) have a compromised reproductive axis [14;15]. More recently, we determined that hypothalamic *Kiss1* neurons co-express GABA$_B$R in the two hypothalamic regions in mice: AVPV/PeN (97%) and ARC (71%). Surprisingly, however, *Kiss1* expression was normal in both the AVPV/PeN and ARC of GABA$_{B1}$KO mice, but was dramatically increased in extra-hypothalamic regions, such as the medial amygdala (MeA), BNST, and LS [16]; co-expression of GABA$_B$R and *Kiss1* in the MeA was also demonstrated in approximately 70% of neurons, suggesting a possible direct regulation of *Kiss1* neurons by GABA$_B$ signaling. We have

recently shown that in the amygdala, BNST, and LS, *Kiss1* expression is subjected to a double regulation: stimulatory by estrogens and inhibitory by GABA$_B$R. This is different from AVPV/PeN and ARC *Kiss1* neurons, whose expression is only subjected to estrogen regulation [17].

On the other hand, GABA systems are also found in other non-neuronal peripheral tissues, such as the endocrine pancreas [18]. The GABA synthesis and degrading enzymes, glutamate decarboxylase (GAD) and GABA transaminase (GABA-T) respectively have been localized in islet β-cells [19]. In general, GABA$_B$R have been detected in insulin-producing β-cells in mice, whereas GABA$_A$Rs are located mainly in glucagon-producing α-cells [20;21]. Various in vitro studies have postulated an autocrine/paracrine role for GABA in the regulation of insulin, glucagon, and somatostatin secretion. Studies examining the role of GABA$_B$R in modulating insulin secretion have shown variable results. It was demonstrated that GABA inhibits insulin secretion specifically through GABABRs in the presence of high glucose in MIN6 cells and in rat islets [20;22], whereas in the presence of lower glucose levels it has no effect [20]. In vivo studies suggest an important role for GABA$_B$R in regulating β-cell function. For instance, the treatment of nonobese diabetic (NOD) mice with a GABA$_B$R agonist delayed onset of type 1 diabetes [23] and other studies showed GABABR-dependent improvement of β-cell survival and proliferation [24-26]. In apparent contradiction, previous results in our laboratory in GABA$_{B1}$KO mice displayed improved glucose tolerance, increased pancreatic insulin content, and elevated glucose-stimulated insulin secretion, associated with enlarged islets and insulin resistance [26] [27]. The GABA$_B$R agonist baclofen inhibited glucose-stimulated insulin secretion in wild-type but not GABA$_{B1}$KO islets [27]. Glucose metabolism and energy balance disruptions were more pronounced in GABA$_{B1}$KO males, which develop peripheral insulin resistance probably due to augmented insulin secretion. Metabolic alterations in females were milder and possibly due to previously described reproductive disorders, such as persistent estrus [28]. At this point, it appears that this receptor can have inhibitory or stimulatory effects on β-cell functions under various circumstances, and this merits further investigation. Whether peripheral kisspeptin expressing cells co-express GABAB receptors, as is the case in the CNS, is actually unknown.

The present project aims to continue investigating the participation and mechanism of action of GABA$_B$R in the regulation of neural and peripheral kisspeptin physiology, a greatly unexplored topic to date. Previously, we studied this connection in global GABA$_{B1}$KO mice. Here, we propose to advance our insight working with a new strain of mice that we have recently developed lacking GABA$_B$R exclusively in kisspeptin-expressing cells, *Kiss1*-GABA$_{B1}$KO mice.

Our hypothesis is that GABA$_B$R in *Kiss1* neurons in hypothalamic and/or extra-hypothalamic areas have an important role in the control of reproduction and/or metabolism.

To study this aim, we have crossed GABA$_{B1}$ floxed mice (GABA$_{B1}$$^{lox511/lox511}$ in a BALB/C background), kindly provided by Dr. Bernhard Bettler (Universtity of Basel, Switzerland), with *Kiss1*-Cre mice (in a C57BL/6 background) provided by The Jackson laboratory. We first crossed hemizygous *Kiss1*-Cre +/- females with GABA$_{B1}$$^{lox511/lox511}$ males, obtaining the F1. Thereafter, we backcrossed the *Kiss1*-Cre (+/-)/GABA$_{B1}$$^{lox511/wt}$ females with GABA$_{B1}$$^{lox511/lox511}$ males to obtain the F2. From the F2 we used the *Kiss1*-Cre (+/-)/GABA$_{B1}$$^{lox511/lox511}$, from now on named as *Kiss1*-GABA$_{B1}$KO mice, as kisspeptin cell specific knockouts, and the *Kiss1*-Cre (-/-)/GABA$_{B1}$$^{lox511/lox511}$ as controls. All the preliminary experiments were performed in these animals (F2). In these mice, it is very important to check for unwanted germline recombination (by a specific PCR), as *Kiss1* is also expressed in testis and ovaries. Any occasionally obtained mice with germline recombination were excluded from the study.
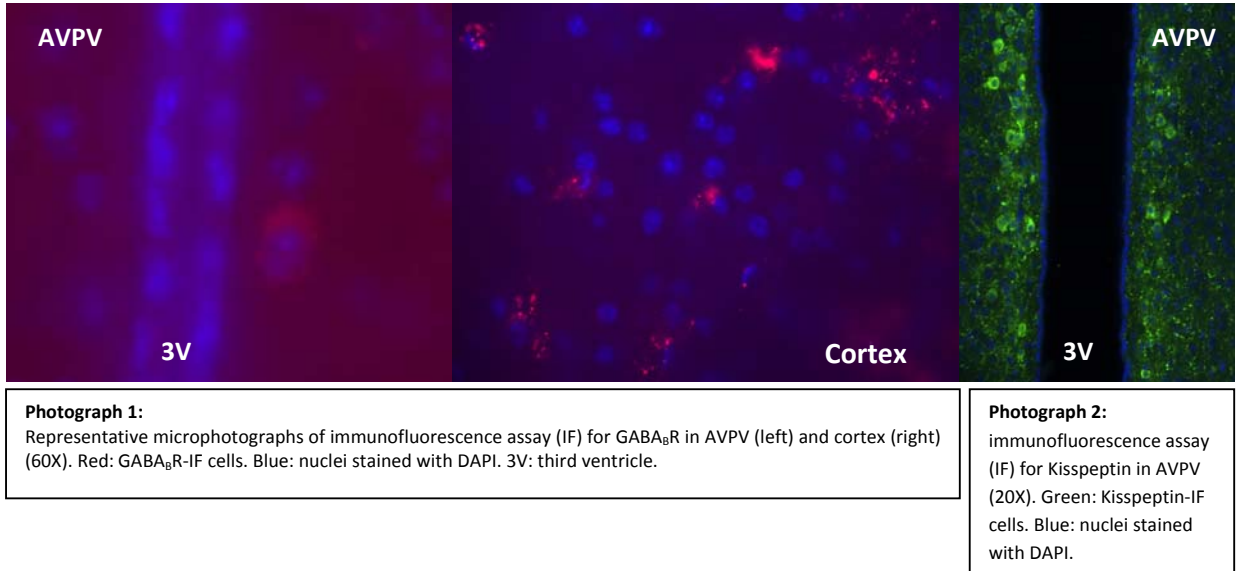
RESULTS

VERIFICATION OF THE SPECIFICITY OF THE GABA$_{B1}$ DELETION ONLY IN KISSPEPTIN-EXPRESSING CELLS.

For these experiments, adult males *Kiss1*-GABA$_{B1}$KO and control mice were anesthetized, jugular vein blood obtained (for hormone determinations by RIA) and transcardially perfused first with PBS and then with 4% paraformaldehyde in PBS. Animals were sacrificed, brains obtained and kept in 4% paraformaldehyde for 2h at 4°C and then in 20% sucrose in PBS over night at 4°C. Finally the brains were frozen in dry ice and kept at -70°C until cutting slices in a cryostat. 30µm slices were used to evaluate either kisspeptin or GABA$_{B1}$ receptor subunit expression by immunofluorescence assay (IF) with rabbit anti-Kisspeptin 10 (kp10) polyclonal antibody (AB9754, Millipore) and mouse Anti-GABA B

Receptor 1 antibody (AB55051, Abcam) and the corresponding fluorescent second antibodies. Slides were analyzed in a confocal microscope (Spinning Disk - TIRF Olympus DSU IX83).

Our specific aims were to verify that the normal development and number of kisspeptin neurons is conserved in the novel $Kiss1$-GABA$_{B1}$KO mouse and that kisspeptin neurons do not express GABA$_B$Rs but that GABA$_B$Rs are still expressed in non-kisspeptin cells in these mice. However, up to the moment we were only able to obtain the separate specific IFs for kisspeptin and GABA$_B$R. Photographs 1 and 2 are representative microphotographs of these IF, where we are able to appreciate the GABA$_B$R-IF cells in red, the kisspeptin-IF cells in green and nuclei were stained with DAPI. Adult male brains were cut, so our next aim will be to finish our verification of the specificity of the GABA$_{B1}$ deletion by double staining and the development of kisspeptin cells in both genotypes.

Photograph 1:
Representative microphotographs of immunofluorescence assay (IF) for GABA$_B$R in AVPV (left) and cortex (right) (60X). Red: GABA$_B$R-IF cells. Blue: nuclei stained with DAPI. 3V: third ventricle.

Photograph 2:
immunofluorescence assay (IF) for Kisspeptin in AVPV (20X). Green: Kisspeptin-IF cells. Blue: nuclei stained with DAPI.

TISSUE RECOMBINATION OF GABABR IN F2 $KISS1$-GABA$_{B1}$KO MICE

First, the recombination of GABABR in different brain nuclei (400μm micropunches) and tissues were analyzed in F2 WT and $Kiss1$-GABA$_{B1}$KO females by PCR and the visualization of the expected bands was performed in 1.5% agarose gels (Figure 1). The expected band for recombination is 0.36kb and we used positive and negative recombination controls obtained from ear clips. We found specific tissue recombination in AVPV/PeN, ARC, MeA and pituitary in $Kiss1$-GABA$_{B1}$KO but not in WT mice. Thus, we conclude that all this tissues express $Kiss1$ and that GABABR is deleted from them. In some tissues the recombination band is very very weak (ARC, MeA).

Figure1: Recombination of GABABR in different brain nuclei and tissues were analyzed in F2 WT and Kiss1-GABAB1KO females by PCR and visualization of the expected bands was done in a 1.5% agarose gel. AVPV: anteroventral periventricular nucleus; ARC: arcuate nuleus; MeA: medial amygdala; HIP: hypophisis. Expected band for recombination: 0.36kb.

SEXUAL AND GENOTYPE DISTRIBUTION OF $KISS1$-GABA$_{B1}$KO MICE

To our knoledge, this is the first time someone generated these mice. We did not obseved alterations in viability in the F1 or F2 pups. We then evaluated the genetic and sexual distribution of the F2 mice and we did not find differences between the real data and the expected one (Table 1 and 2).

GENETIC DISTRIBUTION		N	%	%E
CRE+/- lox511/lox511	KO	58	30,7	25
CRE-/- lox511/lox51	WT	60	31,7	25

Table 1. Genetic distribution of the F2, analyzing two of the four possible genotypes. Total N: 189. We compare real (%) and expected data (%E). χ^2 test: Non significant (NS).

TOTAL	189

SEXUAL DISTRIBUTION	N	%	%E
MALES	80	42,3	50
FEMALES	109	57,7	50
TOTAL	189		

Table 2. Sexual distribution of the F2. Total N: 189. We compare real (%) and expected data (%E). χ^2 test: Non significant (NS).

FIRST SOMATIC GROWTH AND SEXUAL DIFFERENTIATION PARAMETERS

F2 mice, both sexes and genotypes, were characterized through measurements of body weight (BW) and ano-genital index (AGI, calculated by dividing ano-genital distance (AGD) by BW). In the case of BW, pups were evaluated from postnatal day (PND) 7 to PND84. AGD (the length from the caudal base of the genital tubercle to the anterior aspect of the anus) was evaluated on PND7, PND14 and PND21 to calculate AGIs and evaluate sexual differentiation.

We found that males have similar BW curves (Figure 2A). However, $Kiss1$-GABA$_{B1}$KO females have higher BW compared to WTs and this difference is well established on PND84 (Figure 2B).
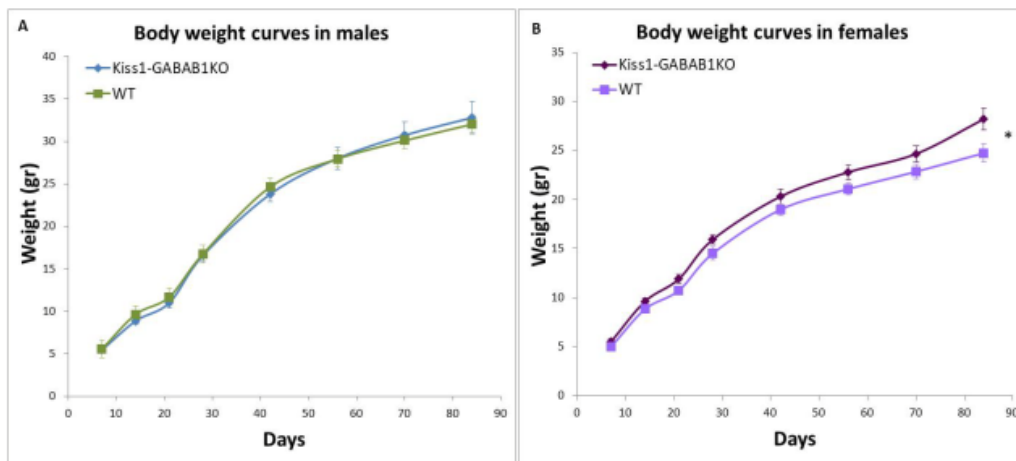
Figure 2: Body weight (BW) curves (g) in male (A) and female (B), WT and Kiss1-GABAB1KO mice. **A:** Repeated Measures ANOVA; Interaction: non significant (NS), factor days: p<0.001, factor genotype: NS. N=11WT-7KO. **B:** Repeated Measures ANOVA: Interaction: NS; factors days: p<0.001 and genotype: p<0.05. *: WT BW curve different from Kiss1-GABAB1KO BW curve. Student t test: PND84: p<0.05, WT BW lower than KO BW. N= 11WT-8KO.

To determine whether this increase in $Kiss1$-GABA$_{B1}$KO females BW was due to increase in body length, we studied this parameter on 3-month-old mice, both sexes and genotypes. However, body length was similar between genotypes in each sex (Figure 3). Then, the higher BW in $Kiss1$-GABA$_{B1}$KO females is not due to a difference in body length.
This is the first result that could indicate that KO females may have metabolic alterations in adulthood due to the lack of GABABR in $Kiss1$ cells.
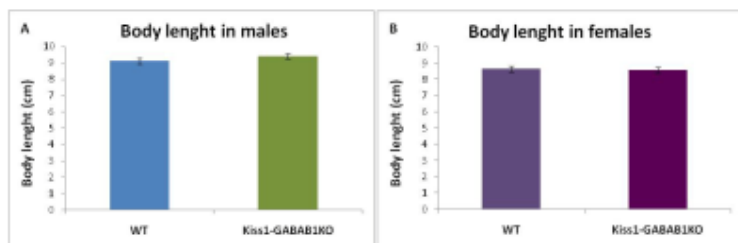
Figure 3: Body length (cm) in male (A) and female (B), WT and $Kiss1$-GABAB1KO mice (3 months of age). Student´s t-test for each determination: NS. N= 10WT-9KO.

Next, we analyzed AGDs and AGIs to determine sexual differentiation parameters in the F2. We did not find differences between genotypes in AGDs (Figure 4A-B). However, when we evaluated AGI we found that it was increased in $Kiss1$-GABA$_{B1}$KO males compared to WTs from neonatal to prepubertal ages (Figura 4C), compatible with intra-uterine masculization. On the other hand, $Kiss1$-GABA$_{B1}$KO females showed decreased AGI compared to WTs only

at PND21 (Figure 4D), compatible with feminization. Further evaluation of AGI will detemine the impact of this result in adult reproduction.
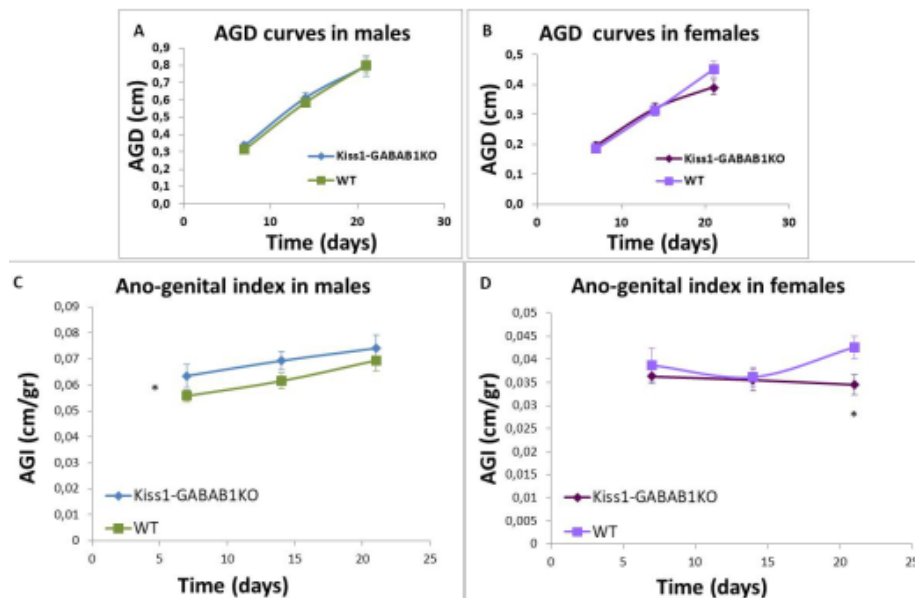

Figure 4: AGD curves in male (A) and female (B), WT and *Kiss1*-GABAB1KO pups. Repeated Measures ANOVA for each curve; Interaction: NS, Factor time: $p<0.001$, Factor genotype: NS. AGD PND21 in females: Student t test p= 0,08. AGI (AGD, cm/ BW, g) curves in male (C) and female (D), WT and *Kiss1*-GABAB1KO pups. Males: Repeated Measures ANOVA; Interaction: NS, Factor time: $p<0.05$, Factor genotype: $p<0.05$. *: AGI is higher in *Kiss1*-GABAB1KO than in WTs. N= 12WT-7KO. Females: Repeated Measures ANOVA; Interaction: NS, Factor time and genotype: NS. Student´s t test PND 21 $p<0.05$. *: AGI is lower in *Kiss1*-GABAB1KO than in WTs. N females: 11WT-9KO.

CHARACTERIZATION OF NEUROENDOCRINE-REPRODUCTIVE STATUS OF *KISS1*-GABA$_{B1}$KO F2 MICE.

ESTROUS CYCLICITY IN VIRGIN ADULT FEMALES

Previous results in our laboratory showed that adult GABA$_{B1}$KO females had alterations in estrous cyclicity (higher percentage in estrus and lower in proestrus) and reproduction. Due to these results, we decided to evaluate cyclicity in adult virgin F2 WT and *Kiss1*-GABA$_{B1}$KO females. However, our preliminary results in these females did not show differences between genotypes (Figure 5).
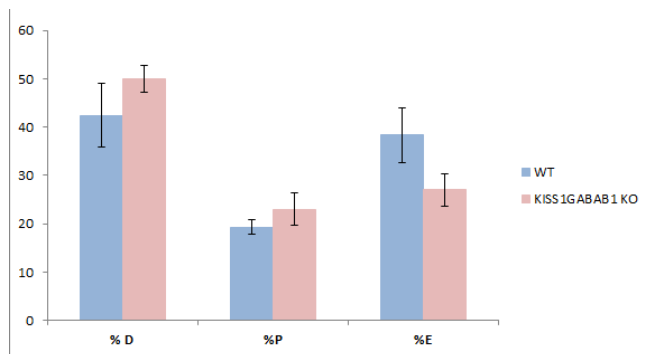
Figure 5: Cyclicity in adult virgin F2 WT and *Kiss1*-GABAB1KO females. Reapeted measures ANOVA: interaction NS; factor cycle $p<0.001$ (% diestrus different from % proestrus $p<0.001$ and % estrus $p<0.05$; factor genotype NS. N=5KO-4WT.

FERTILITY PARAMETERS IN ADULT FEMALES

We then performed a fertility assay in these virgin F2 females to evaluate reproductive indexes. They were paired with males of known fertility (Stock GABA$_{B1}$R lox511/lox511) and the percentage of pregnant females after 30 days of male exposure, days until first litter, number of pups per litter and PND2 and PND21 pup BW were determined (Table 3). In agreement with estrous cyclicity, none of the parameters evaluated showed genotype differences. Determination of hormone levels in adult females is ongoing.

FEMALES							
Genotype	Percentage of pregnancy	Days until first delivery	Number of pups	BW female pups (gr) PND2	BW male pups (gr) PND2	BW female pups (gr) PND21	BW male pups (gr) PND21
WT	79%	22,3±1,0	7,9±1,1	1,56±0,10	1,56±0,11	11,15±0,93	10,03±0,49
KO	100%	22,0±0,9	8,5±1,0	1,5±0,02	1,69±0,07	10,33±0,36	11,14±0,40

Table 3. Fertility assay in three months-old WT and *Kiss1*-GABAB1KO females. They were paired with males of known fertility (Stock ABAB1Rlox511/lox511) and percentage of pregnancy, days until first litter, number of pups per litter and PND2 and PND21 pup BW were determined. N females= 14WT-13KO. Percentage of pregnancy: X2 test NS. Student´s t-test for each determination: NS.

RETURN TO CYCLICITY AFTER WEANING

Next, we evaluated return to cyclicity in the WT and *Kiss1*-GABA$_{B1}$KO females from the fertility assay, after weaning of their first litter (Figure 6). Similar to the results obtained for virgin females, we did not find differences between genotypes in these preliminary results.
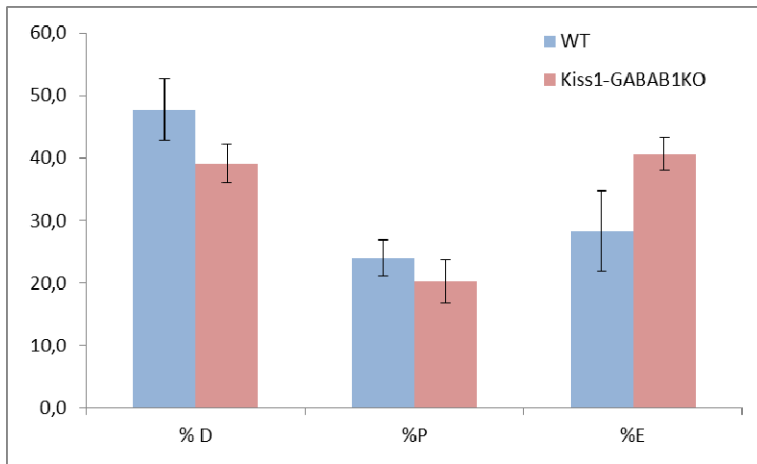

Figure 6. Return to cyclicity in adult F2 WT and *Kiss1*-GABAB1KO females after weaning of their first litter. Reapeted measures ANOVA: interaction NS; factor cycle p<0.001 (% diestrus different from % proestrus p<0.001 and % estrus p<0.05; factor genotype NS. N=4WT-6KO.

BASAL LH AND TESTOSTERONE LEVELS IN ADULT MALES

Then, we decided to evaluate basal LH serum levels (Figure 7), testosterone (T) gonad content and serum T levels by RIA in F2 WT and *Kiss1*-GABA$_{B1}$KO males (Figure 8). However, LH and testosterone levels were similar between genotypes. Although fertility was not evaluated in males, to date we did not find evidence to predict reproductive alterations in these mice.
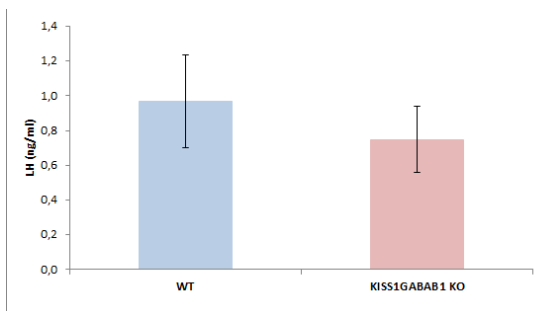
Figure 7. Serum LH levels (ng/ml, RIA) in adult F2 WT and *Kiss1*-GABAB1KO males. Student´s t-test: NS. N= 13WT-15KO.
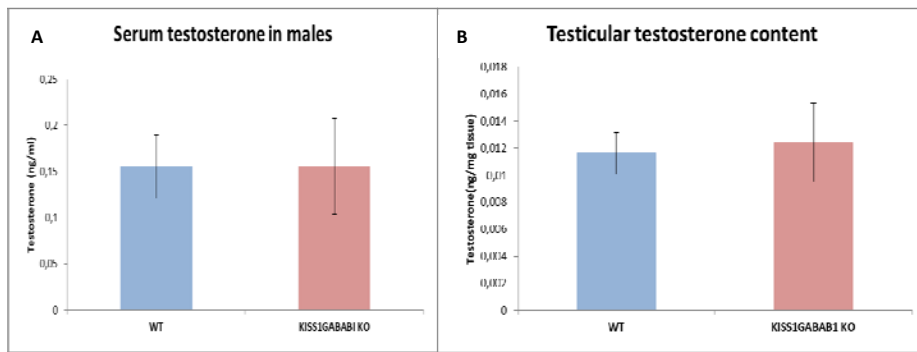
Figure 8. Basal serum (ng/ml, A) and testicular content (ng/mg tissue, B) testosterone in adult F2 WT and *Kiss1*-GABAB1KO males (RIA). Student´s t-test: NS. N (A)=9WT-13KO ; N (B): 6WT-9KO.

CHARACTERIZATION OF METABOLIC STATUS OF *KISS1*-GABA$_{B1}$KO F2 MICE.

METABOLIC PARAMETERS IN ADULT FEMALES

Due to the fact that 3-month-old *Kiss1*-GABA$_{B1}$KO females had higher BW compared to WT, we decided to evaluate non-fasting gycemia and insulin content in pancreas (Figure 9) and food intake (Table 4) in these females. For food intake evaluation, females were previously housed individually for a week (to avoid stress-induced responses) and then BW and food intake were measured during seven days.

We found that non-fasting glycemia in *Kiss1*-GABA$_{B1}$KO females was higher than in WTs (p<0,05, Figure 9A). However, insulin content was not different between genotypes with this preliminary result (Figure 9B).
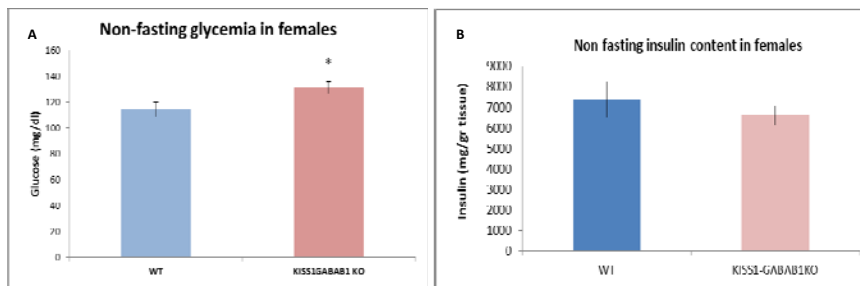
Figure 9: non-fasting glycemia (A, mg/dl) and insulin content in pancreas by RIA (B, ng/gr tissue) in WT and *Kiss1*-GABAB1KO females (3 months of age). Student´s t-test for each determination. Non-fasting glycemia in females p<0,05 (N=10WT-9KO). *: *Kiss1*-GABAB1KO glucose levels higher than WT levels. Insulin content (N=4WT-5KO): NS.

On the other hand, when we evaluated food intake we did not find differences between genotypes. However, the ratio between food intake and BW was lower in *Kiss1*-GABA$_{B1}$KO females compared to WTs (p<0.05, Table 4). This result could indicate that *Kiss1*-GABA$_{B1}$KO females eat less than WTs per grame of BW, although they have higher BW, peRhaps suggesting higher assimilation of food intake.

FEMALES			
Genotype	Body weight (gr)	Food intake (gr)	Food Intake (gr)/BW (gr)
WT	24,43±1,08	32,3±3,16	1,34±0,16 (a)
KO	27,08±0,99	27,45±2,13	1,01±0,07 (b)

Table 4. Body weight and food intake in three-month old WT and *Kiss1*-GABAB1KO females. They were housed individually and BW (g) and food intake (FI, g) were measured after seven days. Student´s t-test for each determination. BW (p=0.09) and FI: NS. FI/BW p<0.05: a≠b. N=8WT-8KO.
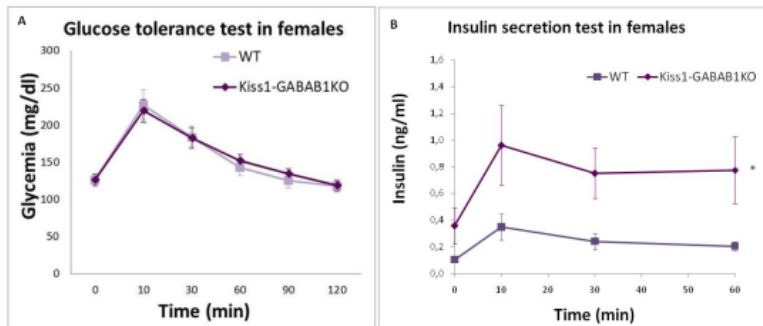
Due to the higher BW and non-fasted glycemia in *Kiss1*-GABA$_{B1}$KO females, after the food intake assay, females were fasted for 4h, fasting blood glucose was measured and glucose injected (2g/kg ip) to determine the blood glucose excursion at 10, 30, 60, 90, 120 min post injection (glucose tolerance test, GTT). In the same samples we evaluated serum insulin (ELISA) at 0, 10, 30, 60 minutes post injection (Insulin secretion test, IST) and HOMA indexes were calculated as detailed here:

HOMA-beta cell= 20 x (Fasting insulin (mU/ml) / (Fasting glucose (mmol/L) - 3.5)
**HOMA-IR**= Fasting insulin (mU/ml) x Fasting glucose (mmol/L) / 22.5

Females of both genotypes had similar fasting glucose at the beginning of the assay (Student´s t-test at 0min: NS, data not shown) and glucose curves (Figure 10A). However, IST revealed that, although basal fasting insulin levels were not significantly different (Student´s t-test at 0min: NS, data not shown), *Kiss1*-GABA$_{B1}$KO females had significantly higher insulin secretion after the glucose overload (Figure 10B, Repeated Measures ANOVA; Interaction: NS, Factor time: p<0.001, Factor genotype: p<0.05). This result showed that *Kiss1*-GABA$_{B1}$KO females secrete more insulin to achieve the same glucose levels as WTs, in agreement with insulin resistence shown below.

Figure 10. A: GTT in three-month old WT and *Kiss1*-GABAB1KO females. After the food intake assay, females were fasted for 4h, fasted blood glucose was measured (Time=0) and glucose injected (2g/kg ip), to determine blood glucose at 10, 30, 60, 90, 120 min post injection. Repeated Measures ANOVA; Interaction: NS, Factor time: p<0.001 (0min different from 10 and 30min, p<0.001), Factor genotype: NS. N= 12WT-10KO. **B:** IST (A, ng/ml) in three months-old WT and *Kiss1*-GABAB1KO females. Repeated Measures ANOVA; Interaction: NS, Factor time: p<0.001 (0min different from 10, 30 and 60min, p<0.001), Factor genotype: p<0.05. *: Insulin secretion in *Kiss1*-GABAB1KO females is higher than in WTs. N= 7WT-7KO.

HOMA indexes were calculated with basal fasting glucose and insulin levels to evaluate pancreatic and peripheric functionality (Figure 11). HOMA-beta cell index was higher in *Kiss1*-GABA$_{B1}$KO females (Student t test p<0.05, Figure 11A). On the other hand, HOMA-IR had the same pattern, higher in *Kiss1*-GABA$_{B1}$KO females, although it did not attain statistical significance (Figure 11B). Again, these results could indicate that *Kiss1*-GABA$_{B1}$KO females have insuline resistence. Whether increased insulin secretion is the cause or the consequence of insulin resistance remains to be determined.
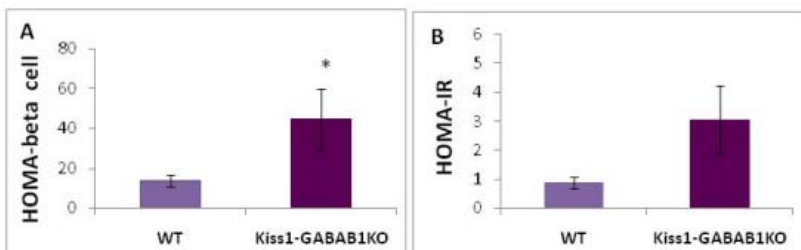
Figure 11. A: HOMA-beta cell. Student t test p<0.05. * *Kiss1*-GABAB1KO higher than in WTs. **B:** HOMA-IR index: Mann-Whitney U test NS. N= 7WT-7KO

Finally, we performed an insulin tolerance test (ITT) in these adult F2 females to determine whether pancreatic response was altered in the presence of high acute blood insulin concentration. Females were fasted for 2h, fasting blood glucose was measured and insulin injected (1unit/kg ip) to determine blood glucose at 10, 20, 30, 60, 90, 120 min post injection.

We found that *Kiss1*-GABA$_{B1}$KO females had higher glucose levels at the end of the assay compared to WTs (Figure 12, Repeated Measures ANOVA; Interaction: p<0.02). So, *Kiss1*-GABA$_{B1}$KO females are less sensitive to the same insulin concentration because they present higher glucose levels than WT females, especially at 120min.
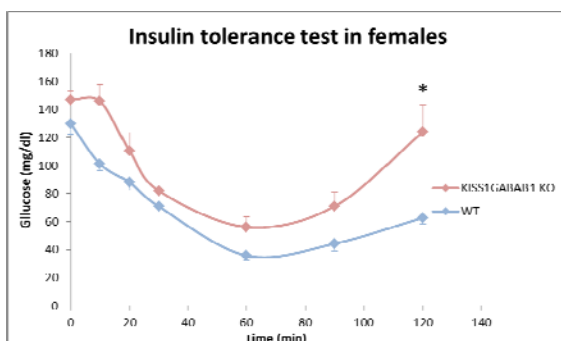

Figure 12. Insulin tolerance test (ITT) in adult F2 WT and *Kiss1*-GABAB1KO females. Repeated Measures ANOVA; Interaction: p<0.02. *: glucose levels (mg/dl) higher in *Kiss1*-GABAB1KO compared to WT females at 120min. WT: 0min different from 120min, p<0.001; KO: 0min similar to 120min. N=5WT-5KO.

In sum, *Kiss1*-GABA$_{B1}$KO females showed increased BW, non-fasting glycemia, insulin secretion and HOMA indexes, decreased insulin sensitivity and ratio food intake/BW. All these results together are compatible with kisspeptin´s metabolic effects on glucose homeostasis due to the lack of GABAB receptors in *Kiss1* cells. However, we did not find reproductive disorders up to now.

METABOLIC PARAMETERS IN ADULT MALES

First, we evaluated non-fasting glycemia and pancreatic insulin content in F2 WT and *Kiss1*-GABA$_{B1}$KO males (Figure 13). However, we did not find differences between genotypes in these parameters.
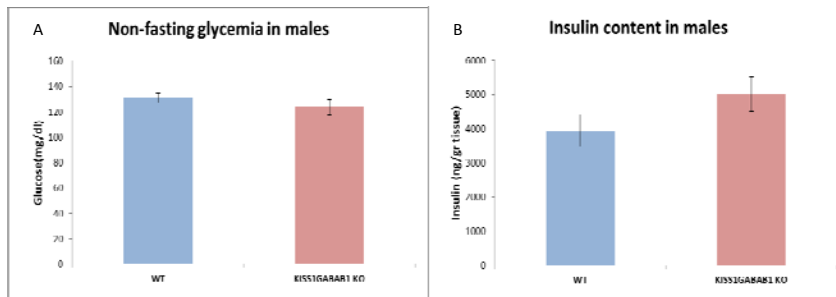

Figure 13. Non-fasting glycemia (A, mg/dl) and insulin content in pancreas by RIA (B, ng/gr tissue) in WT and *Kiss1*-GABAB1KO adult males (3 months of age). Student´s t-test for each determination: NS. N (A) = 7KO-7WT; N (B): 14KO-11WT

Then, we evaluated the glucose tolerance test in males and we did not find differences between genotypes, similar to what we have seen in females (Figure 14). In this opportunity, we were not able to determine the insulin secretion (IST) due to lack of samples.
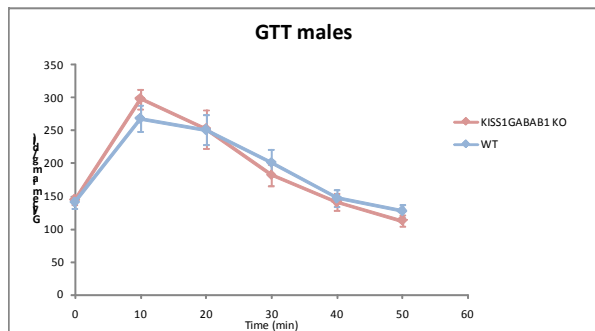
Figure 14. Glucose tolerance test (GTT) in three-month old WT and *Kiss1*-GABAB1KO males. Males were fasted for 4h, fasted blood glucose was measured (Time=0) and glucose injected (2g/kg ip), to determine blood glucose at 10, 30, 60, 90, 120 min post injection. Repeated Measures ANOVA; Interaction: NS, Factor time: p<0.001 (0min different from 10, 30 and 60min, p<0.001), Factor genotype: NS. N= 6WT-7KO.

Finally, we performed the insulin tolerance test (ITT) in F2 males (Figure 15). Again, we did not find differences between genotypes in our preliminary results, in contrast to what we had observed in females.
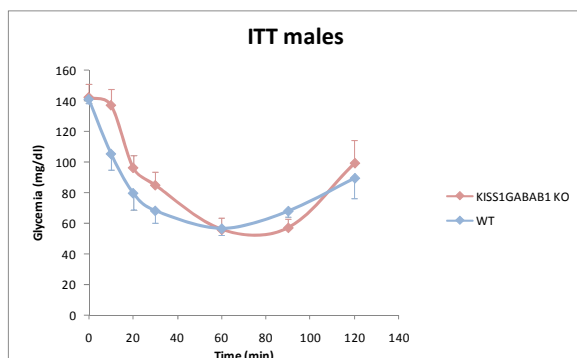
Figure 15. Insulin tolerance test (ITT) in adult F2 WT and *Kiss1*-GABAB1KO males. Repeated Measures ANOVA; Interaction: NS. Factor genotype: NS, Factor time: p<0.001 (0min different from 20, 30, 60, 90 and 120min N=4WT-5KO.

In sum, *Kiss1*-GABA$_{B1}$KO males did not present metabolic alterations. However, we need to add more cases in some of the experiments to achieve a statistically significant number of samples.

Taking into consideration that kisspeptin is an important factor in the link between reproduction and metabolism and due to our previous results in GABA$_{B1}$KO mice where we found dramatic alterations in this neuropeptide; we next decided to evaluate its mRNA expression in hypothalamic and extra-hypothalamic areas in these mice.

For this purpose, another set of adult male and female *Kiss1*-GABA$_{B1}$KO and control mice were sacrificed by decapitation, their brains removed and frozen on dry ice and kept at -80C for qPCR as previously described [16] Then, micropunches of AVPV/PeN, ARC, MeA and BNST (400µm) were obtained in a cryostat to analyze *Kiss1* expression by qPCR. Trunk blood was collected for hormonal determinations by RIA. This specific objective is under investigation at the moment, to finally confirm whether these *Kiss1*-GABA$_{B1}$KO mice showed increased *Kiss1* expression in extra-hypothalamic areas, as shown in global GABA$_{B1}$KO mice.

DISCUSION AND CONCLUSIONS

In this project we investigated the participation and mechanism of action of GABA$_B$R in the regulation of neural and peripheral kisspeptin physiology in the new developed strain of mice lacking GABA$_B$R exclusively in kisspeptin-expressing cells, *Kiss1*-GABA$_{B1}$KO mice. Here, we discuss the most important results obtained to date.

Sexual differentiation parameters during postnatal development

Anogenital distance (AGD) provides the simplest method to evaluate the correct *in utero* sex differentiation [29] and it is dependent upon prenatal exposure to androgens in mammals [30;31]
We found that AGI was increased in *Kiss1*-GABA$_{B1}$KO males compared to WTs from neonatal to prepubertal ages, compatible with intra-uterine hypermasculinization. On the other hand, *Kiss1*-GABA$_{B1}$KO females showed decreased AGI compared to WTs only at PND21, compatible with hyperfeminization.
It is well known that the neuronal components of the reproductive axis that regulate GnRH secretion are sexually differentiated by endogenous gonadal hormones, mainly estradiol, through a series of pre- and perinatal critical periods [32]. An altered AGD in male or female offspring at the time of birth indicates that the endocrine signaling, which is necessary for normal reproductive development, has been altered [33;34]. Such altered programming could have adverse consequences on reproductive functions later in life [29;34;35], even if AGD is no longer affected.
Bourguignon et al. have reported that dams exposed during pregnancy to sodium arsenite, an endocrine disruptor, had increased serum estradiol and testosterone on gestational day 18; their female offspring showed decreased AGI at birth, suggesting an hyperfeminizing effect [36]. Interestingly, Christiansen et al. described that pregnancy exposure to very low doses of the estrogenic compound Bisphenol A induced a decrease of AGD in female rats [37], similar to our observation. Moreover, Honma et al. demonstrated that male offspring from pregnant mice exposed to BPA had increased AGD in at PND60 [38]. We have not studied the AGI later in life; however, it would be really interesting to know whether these alterations persist in adulthood and also to determine gestational estradiol and testosterone serum levels in *Kiss1*-GABA$_{B1}$KO females that influence *in utero* sexual differentiation.

As stated above, sexual differentiation depends on pre- and perinatal gonadal steroid levels. Interestingly, we have previously demonstrated that postnatal day 4 global GABA$_{B1}$KO pups showed an increase in gonadal E2 content in both males (5-fold) and females (2-fold) compared to WTs. However, T was similar between genotypes [39]. Gonadal weight was not different between genotypes in each gender at this age. Moreover, Eisenberg et al. have demonstrated that AGD correlates with testosterone levels in males and can be used as a marker for testicular function [40]. For that reason it would be of our interest to evaluate E2 and T gonadal content and serum levels at this age in the new developed strain *Kiss1*-GABA$_{B1}$KO, to determine the impact of postnatal sex steroids in gonadal development and sexual differentiation.

Moreover, lack of GABA$_B$R in the new strain could be affecting AGI through the central nervous system or peripherally in the gonads. Previously, we have shown that *Kiss1* expression in the ARC was higher in PND4 WT females than in WT males, but this sex difference was lost in PND4 global GABA $_{B1}$ KO mice, notably due to a dramatic decrease in ARC *Kiss1* expression in GABA $_{B1}$ KO females [39]. So, at this early stage of life *Kiss1* expression was altered in these mice and

could lead to the reproductive alterations seen later in life. Nevertheless, GABA$_B$ receptors are also expressed in the gonads and could locally affect steroid hormones production. Interestingly, knockout mice for both *Kiss1* and *Kiss1r* show the effects of neonatal deficiency of sex steroids with decreased anogenital distance at P21. During development, the anogential distance in knockout males remains significantly smaller than in their wild-type littermates [41;42]. Evaluation of hypothalamic and gonadal *Kiss1* expression in *Kiss1*-GABA$_{B1}$KO mice will give us a better understanding of the functionality of GABA$_B$R in *Kiss1* cells.

Metabolic parameters in adult females

Interestingly, we found that *Kiss1*-GABA$_{B1}$KO females have higher BW compare to WTs on PND84. However, this difference is not due to a difference in body length. Furthermore we found that the ratio between food intake and BW was lower in *Kiss1*-GABA$_{B1}$KO females compared to WTs.

Regarding GABABR and food intake, there are some authors that showed that baclofen (GABABR agonist) induced food intake in satiated pigs and rats, acting on central GABABR [43;44]. Controversy results with chronic baclofen administration, as it was shown not alter food intake or BW in rats [45] but reduced ingestion and BW in diabetic db/db mice and obese mice on a high-fat diet [46]. Moreover, GABA$_{B1}$KO mice, lacking global GABABR, showed increase food intake and hypothalamic NPY mRNA expression [28]. NPY and POMC neurons are located in the arcuate nucleus (ARC). While NPY is a potent orexigenic stimulus, POMC neurons are suggested to decrease food intake [47]. Evidences suggest that both population respond to insulin [47] and the anorexigenic effects of insulin are exerted by inhibition of NPY in the ARC [48]. Furthermore, kisspeptin has stimulatory effects on POMC and inhibitory effects on NPY neurons in the ARC (cita 50 review dudek 2018). Thus, kisspeptin exerts a good link between metabolism and reproduction. Taking into consideration these studies, we need to continue our investigation to evaluate the mechanism behind the increase in BW in *Kiss1*-GABA$_{B1}$KO females, including the study of ARC NPY and *Kiss1* mRNA expression, in addition to other hypothalamic orexigenic and anorexigenic factors. In addition, altering kisspeptin signaling in *Kiss1r* KO females displayed markedly reduced locomotor activity, respiratory rate, and energy expenditure, which in part explained the increase in BW in these mice [10]. Therefore, we also intend to evaluate these parameters in the novel *Kiss1*-GABA$_{B1}$KO mice.

On the other hand, we have mentioned in the introduction that GABA systems are also found in other non-neuronal peripheral tissues, such as the endocrine pancreas [18]. GABA$_B$R have been detected in insulin-producing β-cells in mice, whereas GABA$_A$Rs are located mainly in glucagon-producing α-cells [20;21]. For that reason GABA has been postulated as a regulator of the glucose homeostasis.

Taking this into consideration, we evaluated different metabolic parameters in our mice and we found that non-fasting glycemia in *Kiss1*-GABA$_{B1}$KO females was higher than in WTs. Although GTT curves were similar, we found that *Kiss1*-GABA$_{B1}$KO females had higher insulin secretion in the IST. This result is in agreement with previous ones where GABA inhibits insulin secretion specifically through GABABRs in the presence of high glucose in MIN6 cells and in rat islets [20;22]. So, in the absence of GABA$_B$R in *Kiss1* cells we observed an increase in insulin secretion in the IST. Besides, previous results in our lab showed that the GABA$_B$R agonist baclofen inhibited glucose-stimulated insulin secretion in Balb/C mice *in vivo* (GTT) and in wild-type but not in GABA$_{B1}$KO islets (GSIS), [27]. Moreover, the GABABR antagonist 2-hydoxysaclofen improved the GTT and reversed the baclofen effect, also with a slight increase in insulin secretion [49].

In addition, we found that HOMA indexes were higher in *Kiss1*-GABA$_{B1}$KO females and ITT revealed that *Kiss1*-GABA$_{B1}$KO females had reduced glucose clearing. The increase in HOMA-IR index and the impaired ITT may be consequence of a sustained increase in insulin secretion that leads to insulin resistance in peripheral tissues, as has been proposed in functional insulinomas [50-52]. However, different from our results where we only observe metabolic disorders in females, global GABA$_{B1}$KO males develop peripheral insulin resistance probably due to augmented insulin secretion but metabolic alterations in GABA$_{B1}$KO females were milder, possibly due to previously described reproductive disorders, such as persistent estrus Bonaventura, 2013 3802 /id}.

Taken together these results suggest that young adult *Kiss1*-GABA$_{B1}$KO females show alterations similar to a pre-diabetic stage, with mild non-fasting hyperglycemia, normal fasting glucose levels, increased insulin secretion and peripheral insulin resistance. These results are coincident with previous results in pre-diabetic NOD mice [53;54]. Moreover, *Kiss1r* KO females had a greater BW, hyperinsulinemia, increased adiposity, elevated fasting basal glucose levels as well as impaired glucose tolerance [10]. However, the observed obesity was not due to hyperphagia, but rather reduced metabolism. *Kiss1r* KO females had dramatically decreased energy expenditure. By contrast, *Kiss1r* KO males were characterized by normal BW and glucose tolerance. Adiposity, hyperinsulinemia and decreased metabolism were

already seen at a younger age in *Kiss1r* KO females, with impaired glucose tolerance and feeding developing later in adulthood, after BW was significantly increased. Thus, an early life decrease in metabolism and energy expenditure may underlie the later emergency of the obese phenotype of adult *Kiss1r* KO females [55]. However, this phenotype of obesity may reflect peripheral rather than central metabolic impairments [56]. We do not know yet whether *Kiss1*-GABA$_{B1}$KO females develop diabetes at older ages, and it will be of a great interest to analyze the evolution of these disorders to characterize them.

Moreover, insulin release is modulated by *Kiss1r* [57]. Expression of the *Kiss1* and *Kiss1r* genes and proteins was described in both alpha and beta cells [58;59]. Kisspeptin-54 (Kp-54) increased glucose-induced insulin secretion from human and murine islets, without any effect on basal secretion [59]. Studies on rhesus monkeys [60] and rats [61] using kisspeptin-10 (Kp-10) confirmed in vitro observations. However, there are contradictory results indicating that Kp could inhibit or have no effect on insulin secretion, which may be related to the doses used and differences in protocols. Diabetic type 1 and type 2 male rats had increased pancreatic *Kiss1* mRNA levels and the *Kiss1/ Kiss1r* system may not function properly, thus being unable to control insulin levels [62]. So, it would be really interesting to analyze *Kiss1/Kiss1r* expression in the pancreas of our *Kiss1*-GABA$_{B1}$KO females to confirm whether the lack of GABABR is having an impact in this peripheral population of *Kiss1* cells, which have an important role in the control of glucose homeostasis.

In sum, the lack of GABABR in *Kiss1* cells has a clear impact on glucose homeostasis, possibly through a role in the autocrine/paracrine regulation of the pancreatic islet, in females. However, it seems not to have the same role in males, in agreement with sex differences observed in other transgenic mice [10;28;55].

This relationship between GABA, acting through GABAB receptors, and kisspeptin in the pancreas has not been studied yet. However, more studies are needed to complete our analysis in these animals. We need to evaluate other hormones involved in the regulation of glucose homeostasis, such as glucagon, somastatin, leptin, prolactin and growth hormone and the expression of *Kiss1* in the ARC of females, because neuropeptide expression of KNDy neurons (co-express kisspeptin, neurokinin B and dynorphin B) in this area depends on the metabolic status of the animal [47].

Regarding reproduction, our present results do not show a reproductive phenotype but this may be occluded by the fact that we only studied animals from the F2 generation, and it is known that strain mix generally improves reproduction. Further experiments in this regard will be performed in F7, when the background is 99% BALB/C.

POSTER PRESENTATION DURING THE FUNDING PERIOD

NOELIA P. DI GIORGIO, FLORENCIA N. TABARES, MARIANNE BIZZOZZERO, NADIA S. BOURGUIGNON, BERNHARD BETTLER, CARLOS LIBERTUN AND VICTORIA LUX-LANTOS. ***GABAB receptor deletion in Kiss1 neurons/cells alters body weight and anogenital index.*** 9th International Congress of Neuroendocrinology 2018 Toronto, Canada. July 15 - 18, 2018. Abstract book PS2.00106, page # 65.

FINANTIAL REPORT

EXPENDITURES	USD
Mouse Housing	2000
Molecular biology supplies:	1600
PCR kits and primers for genotyping	
MMLV retrotranscriptase and qPCR kits	
RNAse free tubes and tips	
Immunohistochemistry supplies	635
Antibodies	
Mounting medium with DAPI	
Other laboratory supplies	40
Technical services	225
Total amount:	**4500**

REFERENCES

1. N. P. Di Giorgio, M. Bizzozzero, C. Libertun, V. Lux-Lantos, *Reproduction.* (2019).
2. S. J. Semaan, K. P. Tolson, A. S. Kauffman, *Adv.Exp.Med.Biol.* 784, 221-252 (2013).
3. S. B. Stephens et al., *Endocrinology* 156, 3091-3097 (2015).
4. S. J. Semaan and A. S. Kauffman, *Mol.Cell Endocrinol* 401, 84-97 (2015).
5. J. Kim et al., *Endocrinology* 152, 2020-2030 (2011).
6. C. J. L. Harter, G. S. Kavanagh, J. T. Smith, *J Endocrinol* 238, R173-R183 (2018).
7. A. Wolfe and M. A. Hussain, *Front Endocrinol (Lausanne)* 9, 184 (2018).
8. R. Pasquali and A. Gambineri, *Reprod.Biomed.Online.* 12, 542-551 (2006).
9. J. M. Castellano and M. Tena-Sempere, *Adv Exp Med Biol.* 784, 363-383 (2013).
10. K. P. Tolson et al., *J Clin.Invest* 124, 3075-3079 (2014).
11. R. A. DeFazio, C. F. Elias, S. M. Moenter, *J Neurosci.* 34, 16296-16308 (2014).
12. V. A. Lux-Lantos, M. S. Bianchi, P. N. Catalano, C. Libertun, *Cell Mol.Neurobiol.* 28, 803-817 (2008).
13. C. Zhang et al., *Am J Physiol Endocrinol Metab* 305, E1384-E1397 (2013).
14. P. N. Catalano et al., *Neuroendocrinology* 82, 294-305 (2005).
15. P. N. Catalano et al., *Am.J.Physiol Endocrinol.Metab* 298, E683-E696 (2010).
16. N. P. Di Giorgio et al., *Endocrinology* 155, 1033-1044 (2014).
17. S. B. Z. Stephens et al., *Endocrinology* 159, 3389-3402 (2018).
18. Y. Wan, Q. Wang, G. J. Prud'homme, *Diabetes Metab Syndr.Obes.* 8, 79-87 (2015).
19. R. L. Sorenson, D. G. Garry, T. C. Brelje, *Diabetes* 40, 1365-1374 (1991).
20. N. L. Brice, A. Varadi, S. J. H. Aschcroft, E. Molnar, *Diabetologia* 45, 242-252 (2002).
21. Y. Shi et al., *Am.J.Physiol.Endocrinol.Metab.* 279, E684-E694 (2000).
22. M. Braun et al., *J Physiol* 559, 397-409 (2004).
23. P. E. Beales et al., *Acta Diabetol.* 32, 53-56 (1995).
24. I. Purwana et al., *Diabetes* 63, 4197-4205 (2014).
25. J. Tian et al., *Diabetes* 62, 3760-3765 (2013).
26. M. Crivello et al., *Am J Physiol Endocrinol Metab* (2013).
27. M. M. Bonaventura et al., *Am.J.Physiol Endocrinol.Metab* 294, E157-E167 (2008).
28. M. M. Bonaventura et al., *Life Sci* 92, 175-182 (2013).
29. M. Welsh et al., *J Clin.Invest* 118, 1479-1490 (2008).
30. C. Wolf, Jr. et al., *Toxicol.Ind.Health* 15, 94-118 (1999).
31. A. Thankamony, K. K. Ong, D. B. Dunger, C. L. Acerini, I. A. Hughes, *Environ.Health Perspect.* 117, 1786-1790 (2009).
32. B. Cooke, C. D. Hegstrom, L. S. Villeneuve, S. M. Breedlove, *Front.Neuroendocrinol.* 19, 323-362 (1998).
33. C. J. Bowman, N. J. Barlow, K. J. Turner, D. G. Wallace, P. M. Foster, *Toxicol.Sci.* 74, 393-406 (2003).
34. A. K. Hotchkiss et al., *Biol.Reprod.* 71, 1852-1861 (2004).
35. S. Christiansen et al., *Int.J Androl* 31, 241-248 (2008).
36. N. S. Bourguignon et al., *Reprod.Toxicol.* 69, 1-12 (2017).
37. S. Christiansen et al., *Reproduction.* (2013).
38. S. Honma et al., *Reprod.Toxicol.* 16, 117-122 (2002).
39. N. P. Di Giorgio et al., *Neuroendocrinology* 98, 212-223 (2013).
40. M. L. Eisenberg, T. K. Jensen, R. C. Walters, N. E. Skakkebaek, L. I. Lipshultz, *J Urol.* 187, 594-598 (2012).
41. S. B. Seminara et al., *N.Engl.J.Med.* 349, 1614-1627 (2003).
42. R. Lapatto et al., *Endocrinology* 148, 4927-4936 (2007).
43. I. S. Ebenezer, *Neuroreport* 1, 73-76 (1990).
44. I. S. Ebenezer and B. A. Baldwin, *Br.J.Pharmacol.* 101, 559-562 (1990).
45. S. M. Patel and I. S. Ebenezer, *Eur.J.Pharmacol.* 593, 68-72 (2008).
46. I. Sato et al., *FEBS Lett.* 581, 4857-4864 (2007).
47. M. Dudek, K. Ziarniak, J. H. Sliwowska, *Front Endocrinol (Lausanne)* 9, 145 (2018).
48. I. Sato et al., *J.Neurosci.* 25, 8657-8664 (2005).
49. M. M. Bonaventura et al., *Eur J Pharmacol* 677, 188-196 (2012).
50. A. Nankervis, J. Proietto, P. Aitken, F. Alford, *Diabetologia* 28, 427-431 (1985).
51. P. Sbraccia et al., *Diabetologia* 39, 220-225 (1996).
52. J. Skrha, G. Sindelka, T. Haas, J. Hilgertova, V. Justova, *Horm.Metab Res* 28, 595-598 (1996).
53. A. Amrani et al., *Endocrinology* 139, 1115-1124 (1998).
54. A. Jansen, J. G. Rosmalen, F. Homo-Delarche, M. Dardenne, H. A. Drexhage, *J.Autoimmun.* 9, 341-348 (1996).
55. K. P. Tolson, C. Garcia, I. Delgado, N. Marooki, A. S. Kauffman, *Endocrinology* 157, 4192-4199 (2016).
56. J. P. De Bond, K. P. Tolson, C. Nasamran, A. S. Kauffman, J. T. Smith, *J Neuroendocrinol.* 28, (2016).
57. T. A. Schwetz, C. A. Reissaus, D. W. Piston, *PLoS.One.* 9, e113020 (2014).
58. J. H. Lee et al., *J Natl.Cancer Inst.* 88, 1731-1737 (1996).
59. A. C. Hauge-Evans et al., *Diabetologia* 49, 2131-2135 (2006).
60. F. Wahab, R. Bano, S. Jabeen, S. Irfan, M. Shahab, *Horm.Metab Res* 42, 570-574 (2010).
61. J. E. Bowe et al., *Diabetologia* 52, 855-862 (2009).
62. M. Dudek et al., *Neuropeptides* 56, 41-49 (2016).