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Compensatory mechanisms for GnRH production in Fgf8-deficient mice

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Abstract:

GnRH (gonadotropin-releasing hormone) is a peptide hormone produced by neurons from the brain and is indispensable for the onset and maintenance of vertebrate reproduction. The development of GnRH neurons relies greatly on fibroblast growth factor (Fgf) signaling. In fact, it has been shown that in fibroblast growth factor 8-deficient (F8 Het) mice, the number of GnRH neurons is reduced by about 50%. Interestingly, F8 Het males and females appear to compensate for this reduction by undergoing normal puberty and producing normal levels of the GnRH peptide before 60 days of age. However, the mechanisms for this compensation are unknown. We hypothesized that F8 Het mice may compensate for a defected GnRH system by increasing the production of transcripts for: (1) *GnRH*, (2) two GnRH prohormone processing enzymes, *CPE* and *PCSK2*, and (3) *KiSS1*, an upstream stimulator of GnRH release and neuronal activity. Accordingly, the goal of this project was to use quantitative polymerase chain-reaction (qPCR) to investigate if genes involved in these three levels of GnRH system control were upregulated in F8 Het mice. Our results suggest that none of mechanisms proposed were responsible for the plasticity observed. In fact, F8 Het mice harbored significant defects in both *GnRH* and *KiSS1* expression. Our results highlight the complex levels of control that drive the function of the GnRH system and suggest other compensatory mechanisms that we have not yet identified are at play.

Keywords: GnRH, Fgf signaling, puberty, transgenic mice, Fgf8, *KiSS1,* GnRH prohormone

Introduction:

The functionality of the vertebrate reproductive system relies heavily on the proper secretion of neurohormones from the brain. Specifically, pulsatile secretion of gonadotropin-releasing hormone (GnRH) by GnRH neurons located in the preoptic area (POA) is crucial for proper reproductive onset and maintenance in all vertebrates (Maffucci et al., 2009). Secreted GnRH acts on the anterior pituitary gland to signal the production and release of gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH). These gonadotropins enter the blood and stimulate gonadal growth, gametogenesis, and steroid hormone production. Collectively, these endocrine organs form the hypothalamic-pituitary-gonadal (HPG) axis which drive reproduction in vertebrates.

Many peptide hormones, including GnRH, are generated by the posttranslational processing of the larger prohormones into the final mature hormones before secretion. This processing includes the cleavage of the GnRH prohormone first by prohormone convertase 2 (PCSK2) (Wetsel et al., 1995) to remove the GnRH-associated peptide (GAP), then by carboxypeptidase E (CPE), which remove the excess amino acids in the C-terminus, and finally, modifications including pyroglutimation and amidation of the peptide to elicit proper effects (Wetsel et al., 2002). Once the active GnRH peptide has been created, GnRH secretion is influenced by a variety of neurotransmitters and neuromodulators from within the brain (Genazzani et al., 2000).

Well-known regulators of GnRH neurons are peptides encoded by the *KiSS1* gene called kisspeptins. Kisspeptins are secreted by two neuronal populations within the brain and act as upstream stimulators of GnRH release from GnRH neurons. Because of the

dominant role of *KiSS1* neurons in the activation of GnRH neurons, *KiSS1* neurons are also referred to as the "gatekeepers of puberty" (Pinilla et al., 2012). In mice, the two populations of *KiSS1* neurons are found in the anteroventral periventricular (AVPV) and arcuate (ARC) nuclei, and both *KiSS1* neuronal populations project to GnRH neurons to stimulate GnRH secretion (Yip et al., 2015).

Evidence shows that members of the fibroblast growth factor (Fgf) signaling family are required for the appropriate development of the GnRH neuronal system in mice (Tsai et al., 2011). In humans, loss-of-function mutations on *Fgf8*, an Fgf ligand, or *Fgfr1,* an Fgf receptor, have been causally linked to disorders such as hypogonadotropic hypogonadism (HH), a disorder characterized by reduced gonadotropins and absent or delayed puberty, and Kallmann syndrome (KS) (Hardelin et al., 2008; HyY et al., 2010; Topaloglu et al., 2016). KS is characterized by an impaired sense of smell comorbid with HH. Whereas HH can stem from low levels of GnRH, gonadotropins, or pituitary GnRH responsiveness, KS is caused exclusively by GnRH shortage (Tsai et al., 2001). It has been determined that Fgf8 binding to Fgfr1 is critical for successful GnRH neuron specification, genesis, and emergence from the nasal placode, the birthplace of GnRH neurons (Chung et al., 2008, Chung et al., 2010). However, some humans with *Fgf8* mutations and transgenic mice with partial *Fgf8* loss undergo normal puberty and are fertile. For example, a study of mice deficient in Fgf8 showed that genetically altered mice with one *Fgf8* hypomorphic allele (F8 Het) exhibited a 50% loss of GnRH neurons permanently after birth (Zhang et al., 2015). However, despite this reduction, F8 Het mice (males and females) were able to produce normal levels of GnRH peptide before puberty (PN20) and at the onset of puberty (PN30) (Zhang et al., 2015), suggesting an inherent

compensatory mechanism that allowed the smaller GnRH neuronal population to produce normal levels of the GnRH peptide.

In this study, we explored three potential mechanisms used by F8 Het mice to produce normal levels of the GnRH peptide despite their smaller GnRH neuronal population. These mechanisms include enhanced transcription of GnRH in the POA, increased posttranslational processing of GnRH prohormone, and increased stimulation of GnRH neurons by elevated *KiSS1* expression. All mechanisms were studied using relative quantitative reverse transcriptase polymerase chain reaction (qPCR). Our results demonstrated that GnRH neurons in F8 Het mice did not enhance *GnRH* transcription, GnRH prohormone processing, or *KiSS1* compared to the wildtype (WT) mice. Additional unknown mechanisms may be at play to compensate for the smaller GnRH neuronal population in F8 Het mice. Interestingly, these data revealed novel insight into changes in *KiSS1* mRNA expression in F8 Het mice during puberty and suggested that the *KiSS1* system in females can be negatively affected by *Fgf8* deficiency.

Methods:

Animals

F8 Het mice (129p2/OlaHsd* CD-1) originally obtained from the Mouse Regional Resource Centers (Davis, CA, USA) were inbred in the University of Colorado Gold Conventional animal facility. They were housed under a 12-hour light:dark cycle and fed rodent chow and water *ad libitum*. These animals have an allele with a neo cassette insertion upstream of the second exon in a noncoding region of the *Fgf8* gene. This forms an artificial splice site that decreases the amount of functional *Fgf8* transcript by about 55% in mice homozygous for *Fgf8* hypomorphic allele and about 27% in mice

herterozygous for *Fgf8* hypomorphic allele (Meyers et al., 1998). We included WT and F8 Het offspring generated from a WT and F8 Het cross in this study. Age of mice were determined by designating date of birth as postnatal day (PN) 0 and weaned at PN20 into same-sex housing conditions. At weaning or sacrifice, tail biopsies were taken to establish genotype using polymerase chain reaction from isolated genomic DNA. WT and F8 Het male and female mice at ages PN20, PN30 and PN60 were included for the study each with a sample size of *N*=4-5 per group. All animal procedures complied with protocols approved by the Institutional Animal Care and Use Committee at the University of Colorado Boulder.

Tissue Collection:

Animals were sacrificed by isuflorane-induced anesthesia followed by rapid decapitation in the mid-afternoon. Brains were dissected, removed and blocked to isolate the dominate GnRH neuron containing region, the POA, and one of the two hypothalamic regions that contain *KiSS1* neurons, the anteroventral periventricular nucleus (AVPV). Brain blocking was conducted by a caudal cut using a razor blade and a matrix at Bregma -0.22 and a rostral cut approximately 2 mm anterior, Bregma -0.02. Three modifying cuts were made following blocking. The first was just inferior to the corpus callosum, the second and third cuts were made lateral to the internal capsules. Tissue blocks were immediately placed on dry ice and stored at -70˚C until processed.

RNA Isolation, cDNA Synthesis, and Relative Quantification of GnRH, PCSK2, CPE and KISS1 Transcript:

RNA from POA/hypothalamic blocks were homogenized and isolated using Trizol Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The quality of RNA was verified by gel electrophoresis as previously described and performed

(Brooks et al., 2010). cDNA was synthesized from 1 µg of total RNA using the Superscript III First-Strand Synthesis SuperMix (Invitrogen) according to protocol. Initial efficiency tests for µL per reaction of cDNA were run to optimize CT values to be within a readable range. Optimal amount of cDNA template per reaction was 1µL for *GnRH*, 0.5 µL for *PCSK2* and *CPE*, and 1.3 µL for *KISS1*. Relative qPCR of *GnRH*, *PCSK2*, *CPE* and *KISS1* transcripts was performed using FastStart DNA Master SYBR Green I kit (Roche Pharmaceuticals, Indianapolis, IN). Primer sequences were 5'-TCAGGATCTGCGAGGAG-3' (forward) and 5'-GGGCCAGTGCATCTACAT-3' (reverse) for *GnRH*, 5'-GGCGTGTTTGCATTAGCTTT-3' (forward) and 5'-GCACAGTCAGATGTTGCATGT-3' (reverse) for *PCSK2*, 5'-CCGGAAGAGACTCTCAAAAGC-3' (forward) and 5'-CGGACAAACCCTTTAACACC-3' (reverse) for *CPE*, and 5'-AGCTGCTGCTTCTCCTCTGT-3' (forward) and 5'-GCATACCGCGATTCCTTTT-3' (reverse) for *KISS1*. Samples were run in duplicates for the target genes and the housekeeping gene, hypoxanthine phosphoribosyltransferase (*HPRT*) with primer sequence 5'- AGCAGTACAGCCCCAAAATGG-3' (forward) and 5'-TGCGCTCATCTTAGGCTTTGT-3' (reverse). *HPRT* gene expression is not impacted by *Fgf8* deficiency. Therefore, it was deemed as an appropriate housekeeping gene. All amplifications were performed using Mastercycler® ep realplex* for 40 cycles with an annealing temperature of 60°C for *GnRH*, *PCSK2*, and *CPE* and 56°C for *KISS1*. The 2-ΔΔCT method was used to the relative expression of all target genes as previously described (Livak et al., 2001).

Statistical Analysis

All statistical analyses were performed were done using Prism (GraphPad, La Jolla, CA, USA). The Mann-Whitney test was used to compare relative expression within age between different genotypes. The Kruskal-Wallis tests were used to compare relative expression within genotypes between different ages followed by the Dunn's Multiple Comparisons post-hoc test. Differences were significant if $P<0.05$.

Results:

GnRH mRNA

To determine the effects of F8 Het deficiency on the ability of GnRH neurons to produce GnRH, the *GnRH* mRNA levels in the POA were analyzed in F8 Het and WT mice using relative qPCR as the first mechanism of compensation. The Mann-Whitney test showed *GnRH* mRNA was significantly lower in F8 Het mice compared to WT at all ages of the study (Figure 1A). Specifically, the reduction of *GnRH* mRNA in F8 Het mice was about 33% at PN20 ($P=0.0011$), 32% at PN30 ($P=0.0037$) and 50% at PN60 ($P<0.0001$). To determine if these reductions in *GnRH* mRNA were related to the change in age, *GnRH* mRNA levels were examined within each genotype (Figure 1B). Kruskal-Wallis test followed by Dunn's post-hoc test revealed that PN60 WT mice had significantly increased *GnRH* mRNA compared to PN20 WT mice ($P<0.001$) and PN30 WT ($P<0.05$) (Figure 1B). These results suggested a significant elevation of the *GnRH* transcript after the onset of puberty. Unlike WT mice, the F8 Het mice showed no significant differences in the levels of *GnRH* mRNA at any of the ages studied, suggesting the age-dependent increase in *GnRH* mRNA was blunted by Fgf8 deficiency.

CPE and PCSK2 mRNA

Despite our current data showing that *GnRH* mRNA levels were reduced in F8 Het mice in all ages examined (Figure 1A), previous results (Zhang et al., 2015) indicated normal GnRH peptide levels in PN20 and PN30 F8 Het mice. This discrepancy raised the possibility that post-translational processing of GnRH could be enhanced in F8 Het mice to compensate for their lower *GnRH* mRNA levels. To examine this possibility, we analyzed the expression of two key enzymes involved in GnRH prohormone processing, *CPE* and *PCSK2*, in the preoptic/hypothalamic region for our second proposed mechanism. The *CPE* and *PCKS2* mRNA levels were compared within age between F8 Het and WT mice using the Mann-Whitney test. No differences in *CPE* (Figure 2A) or *PCSK2* transcript levels (Figure 2B) were found between genotypes at any of the ages studied. Therefore, the general upregulation of *CPE* and *PCSK2* in the preoptic/hypothalamic region was unlikely the mechanism used by F8 Het mice to elevate the production of the GnRH peptide.

KiSS1 mRNA

The last mechanism studied was the transcript levels of *KiSS1*, an upstream stimulator of GnRH. To do this, the AVPV was assessed for *KiSS1* mRNA using relative qPCR. Because evidence shows that the *KiSS1* neuronal population in the AVPV is sexually dimorphic within mice (Simerly 2002), the levels of *KiSS1* mRNA were analyzed separately for males and females. The Mann-Whitney test revealed that *KiSS1* mRNA levels did not differ between genotypes at any of the ages studied in females (Figure 3A) or males (Figure 3B). However, the change of GnRH expression with increasing age prompted the effort to understand how *KiSS1* mRNA levels changed with age for both

males and females. The Kruskal-Wallis test followed by Dunn's post-hoc test revealed a significant increase in *KiSS1* mRNA for female WT mice at PN30 (P<0.05), the time of pubertal onset, compared to PN20, where the level of *KiSS1* at PN30 almost tripled (Figure 4A). After puberty at PN60, *KiSS1* mRNA levels plateaued in WT females and did not differ from either PN20 or PN30 (Figure 4A). Similar to the female WT mice, there was an increase in *KiSS1* mRNA at pubertal onset (PN30) in F8 Het female mice compared to PN20 F8 Het KiSS1 mRNA (P<0.05) followed by no change in *KiSS1* transcript after the initial surge (Figure 4A). However, the difference in *KiSS1* levels between PN20 (pre-pubertal) and PN30 (pubertal onset) in WT females was more robust than that observed in F8 Het animals (Figure 4A). For the male mice, *KiSS1* mRNA was unchanged between ages studied for both WT and F8 Het mice (Figure 4B).

Discussion:

Many steps are required for the ability of GnRH neurons to properly stimulate the activity of the HPG axis. These steps include the integration of a variety of stimuli by the GnRH neuronal network, accurate transcription of the *GnRH* gene, efficient translation of the GnRH peptide, proper proteolytic cleavage of the GnRH prohormone, functional packaging of the mature GnRH peptide into secretory granules, and proper release of the peptide from the median eminence (Herbison et al., 2016). All of these also depend on the proper formation of a mature GnRH neuronal system during development. Importantly, it has been shown that *Fgf8* deficiency disrupts the fate identity of GnRH progenitor cells, leading to specification failure of GnRH neurons and a significant reduction of GnRH neuronal population at birth (Chung et al., 2008; Falardeau et al., 2008; Chung et al., 2010; Sabado et al., 2012). Despite having a permanent reduction in

the GnRH neuronal population, F8 Het mice are fertile and produce normal levels of GnRH peptide before and during puberty (Tata et al., 2012; Zhang et al., 2015). The ability of a significantly compromised GnRH neuronal population to initiate reproduction in F8 Het mice speaks to the profound plasticity of this system in order to drive sexual maturation and propagate offspring. To understand the mechanisms underlying this plasticity, we examined various levels of the GnRH system function in F8 Het mice. The current studies aimed at understanding the impacts of *Fgf8* deficiency on the expression of *KiSS1* mRNA in the AVPV, the level of *GnRH* transcript, and two enzymes involved in GnRH prohormone processing, CPE and PCSK2, at ages that correspond to before puberty (PN20), the onset of puberty (PN30), and after puberty (PN60).

We discovered that *GnRH* mRNA levels in F8 Het mice were significantly lower than WT mice at every age examined, suggesting that these mice did not compensate for the loss of GnRH neuron numbers by enhancing *GnRH* transcription (Figure 1A). Compared to PN20 and PN30, the PN60 time point was also associated with a more deleterious reduction (50%) of *GnRH* mRNA in the F8 Het mice (Figure 1A). An analysis comparing changes in *GnRH* mRNA across different ages (Figure 1B) supported this notion. The WT mice displayed a profound increase in GnRH mRNA expression after puberty (PN60) when compared to both animals before puberty (PN20) and at the onset of puberty (PN30). Interestingly, the F8 Het mice lacked this pubertal increase in *GnRH* mRNA (Figure 1B). Consistent with these findings, a previous study reported a reduction in GnRH peptide levels in F8 Het mice at PN60 (Zhang et al., 2015). Taken together with the previous results (Zhang et al., 2015), our currently data suggested that F8 Het mice exhibited a consistent defect in GnRH peptide and transcript levels after puberty (PN60),

but were able to compensate for lower GnRH transcript levels before (PN20) and at puberty (PN30) by unknown mechanisms to produce normal levels of the GnRH peptide.

In addition to *GnRH* transcript levels, we examined the relative expression of *CPE* and *PCSK2*. We hypothesized that F8 Het animals may increase the expression of prohormone processing enzymes in order to enhance the formation of the mature GnRH peptide. Our data showed that *CPE* and *PCSK2* expression in F8 Het mice did not differ from WT (Figures 2A, 2B). However, these data need to be interpreted with some caveats. For example, these enzymes are ubiquitously used throughout the brain for the prohormone processing of a variety of neurohormones. The tissue fragments used to harvest RNA samples for the qPCR analysis of these enzymes did not include only GnRH neurons, but also other heterogeneous cell types. Consequently, *CPE* and *PCSK2* expression levels in these tissue fragments would not be specific only to GnRH neurons. More specific analysis of GnRH neurons between F8 Het and WT mice could reveal more accurate levels of *CPE* and *PCSK2* and allow us to understand how these prohormone processing enzymes contribute to the production of normal GnRH peptide levels in F8 Het mice.

We next examined the relative expression of *KiSS1* mRNA in the AVPV. The AVPV population of kisspeptin-producing neurons has been determined to critically stimulate GnRH neurons to release high levels of GnRH needed for the ovulatory LH surge (Kauffmann et al., 2010) and pubertal onset in females (Clarkson et al., 2008), but contains few kisspeptin neurons in males (Clarkson et al., 2006).

For males, there were no differences in *KiSS1* expression across different ages studied Figure 4B). There also were no differences between the genotypes within the age group (Figure 3B). Our results were consistent with the published literature that, *KiSS1* expression in the AVPV should not be robustly upregulated during puberty in males. Importantly, our data revealed that for females and males at all ages studied, there were no differences in *KiSS1* mRNA levels between different genotypes within the same age group (Figures 3A, 3B). This suggested that F8 Het mice did not upregulate *KiSS1* mRNA levels to compensate for their deficient GnRH system. These results were consistent with a previous study showing comparable numbers of AVPV kisspeptin-producing neurons before puberty in F8 Het and WT mice (Tata et al., 2012). However, when we examined changes in *KiSS1* transcript levels across the ages studied, we observed subtle differences between WT and F8 Het mice before and during puberty in females (Figure 4A). According to our data, the WT females had a more robust 3-fold increase in *KiSS1* mRNA expression between PN20 and PN30 compared to a more modest 2-fold increase in F8 Het females (Figure 4A). A previous study found that F8 Het mice had significantly fewer kisspeptin neurons at PN30 compared to WT (Tata et al., 2012). Together, these data suggest that F8 Het females had less robust pubertal increases in *KiSS1* mRNA expression compared to WT females, possibly due to the decreased number of kisspeptin neurons around the onset of puberty. Overall, we found no evidence that *KiSS1* expression was upregulated in F8 Het females.

Our observations that AVPV *KiSS1* expression in F8 Het mice was not upregulated did not preclude a role of *KiSS1* in compensating for GnRH deficits in F8 Het mice. Future studies should examine the expression of *KiSS1* Receptor (*KISS1R*) in GnRH neurons in

both WT and F8 Het mice. A previous study reported that the responsiveness of GnRH neurons to kisspeptin increased with sexual maturation (Han et al., 2005). Given this finding, the amount of kisspeptins or *KiSS1* gene expression may not reveal the full impact of kisspeptins on GnRH neurons. Future studies will address if GnRH neurons in F8 Het mice express high levels of *KiSS1R* to drive the greater activation of a smaller population of GnRH neurons.

This study investigated three potential mechanisms utilized by F8 Het animals to produce normal levels of GnRH peptide despite their permanent reduction in GnRH neuron numbers. Although none of the proposed mechanisms was observed in the F8 Het animals, our data supported the deficits in GnRH and kisspeptin systems previously seen in the F8 Het mice (Tata et al., 2012; Zhang et al., 2015). Further, they shed light on the potential compensatory mechanisms to rule out or explore in the future, including enhanced *KISS1R* or translational efficiency. This study stresses the complex nature of reproductive control, and suggests the involvement of other factors that may activate GnRH neurons to ensure fertility in *Fgf8*-deficient mice and humans.

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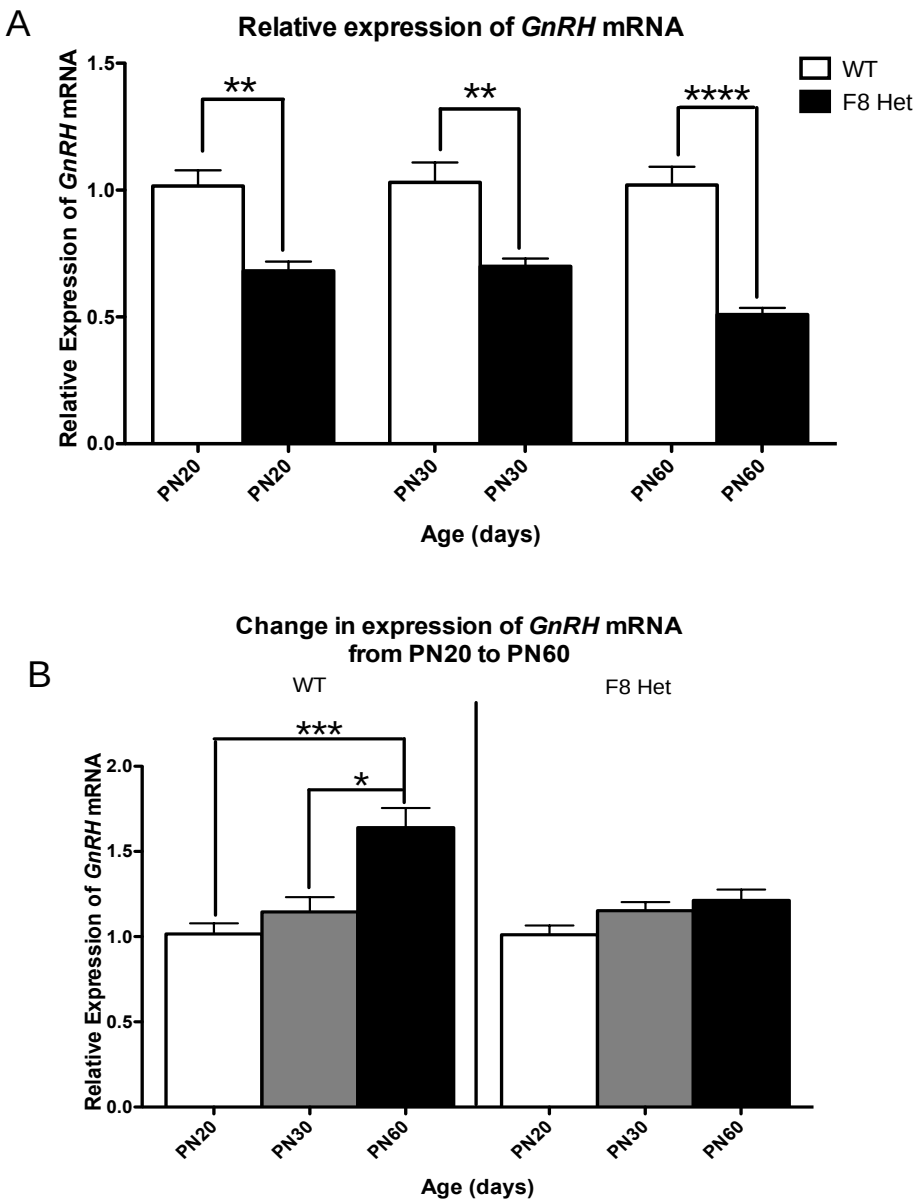

Figure 1. **Relative expression of *GnRH* mRNA** A) within age comparisons between mixed-sex F8 Het mice was and mixed-sex WT mice. B) within genotype comparisons between PN20 mixed-sex F8 Het and WT mice to F8 Het and WT at PN30 and PN60 respectively. Each bar represents mean ± SEM *N*=9-10. *denotes *P*<0.05 **denotes p<0.01 ***denotes *P*<0.001 ****denotes *P*<0.0001

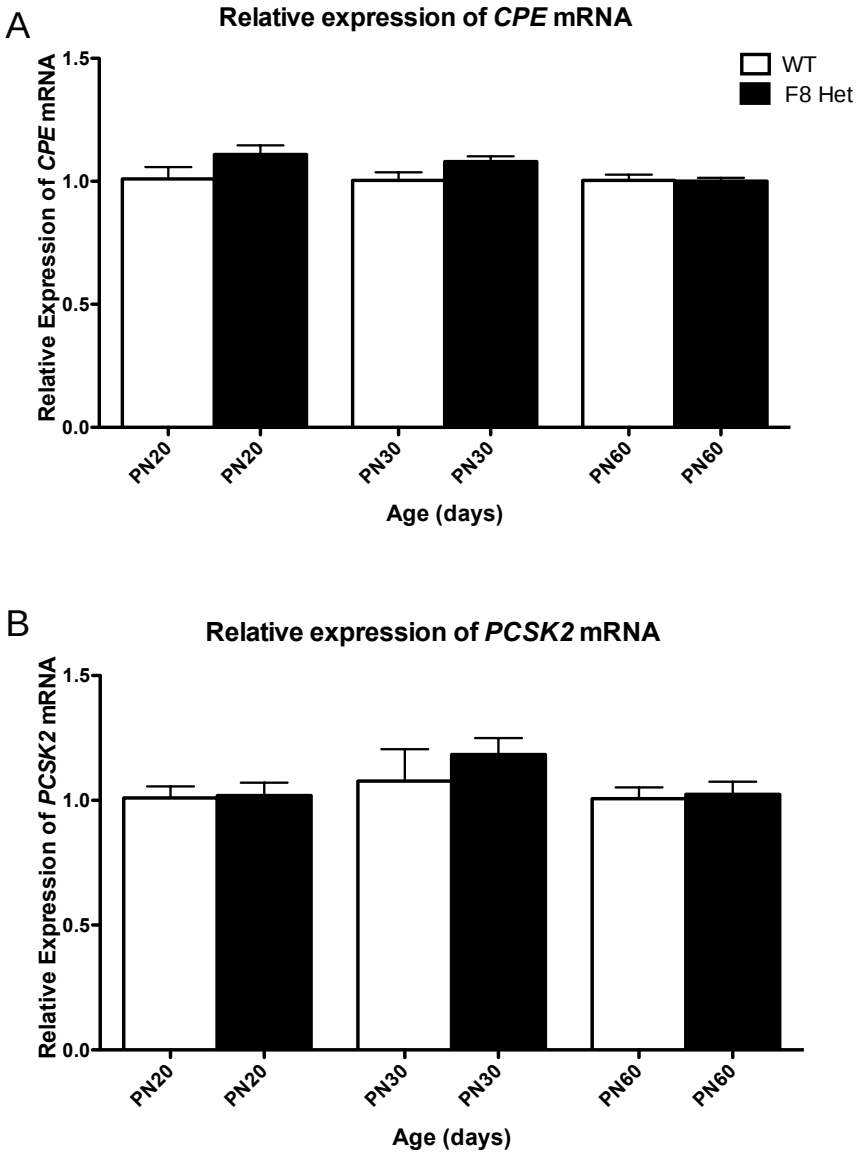

Figure 2. A) **Relative *CPE* and B) *PCSK2* mRNA expression** within age comparisons between mixed-sex F8 Het and mixed-sex WT mice. Each bar represents mean ± SEM *N*=9-10. No parameters were statistically significant from each other.

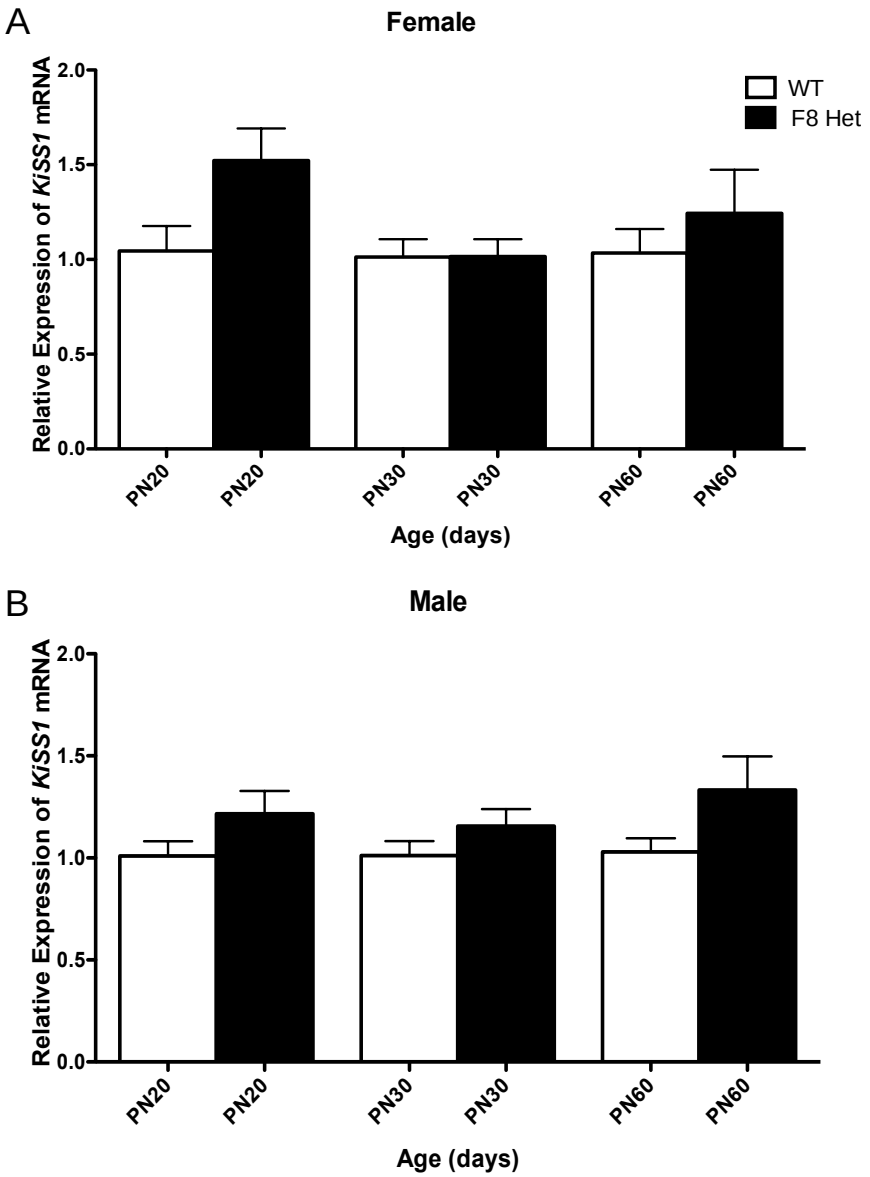

Figure 3. **Relative *KiSS1* mRNA expression** A) between female F8 Het mice were compared to female WT within the same ages. B) between male F8 Het mice were compared to male WT within the same ages. Each bar represents mean ± SEM *N*=4-5. No parameters were statistically significant from each other.

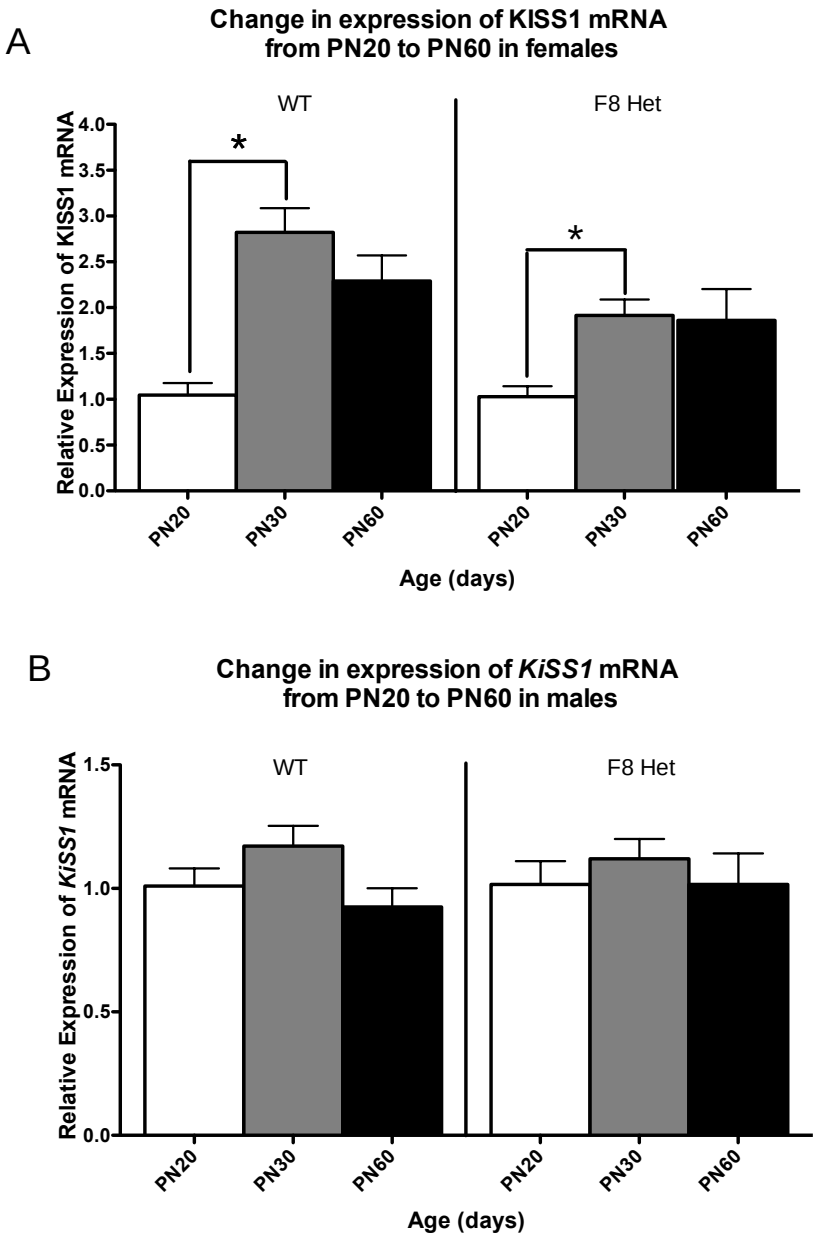

Figure 4. **Relative *KiSS1* mRNA expression** within genotype comparisons between PN20 mixed-sex F8 Het and WT mice to F8 Het and WT at PN30 and PN60 respectively for A) females and B) males were performed. Each bar represents mean ± SEM *N*=4-5. * denotes *P*<0.05