Diurnal regulation of hypothalamic kisspeptin is disrupted during mouse pregnancy

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Abstract

Kisspeptin, the neuropeptide product of the *Kiss1* gene, is critical in driving the hypothalamic–pituitary–gonadal (HPG) axis. Kisspeptin neurons in the anteroventral periventricular nucleus (AVPV) and arcuate nucleus (Arc) of the hypothalamus mediate differential effects, with the Arc regulating negative feedback of sex steroids and the AVPV regulating positive feedback, vital for the preovulatory surge and gated under circadian control. We aimed to characterize hypothalamic *Kiss1* and *Kiss1r* mRNA expression in nonpregnant and pregnant mice, and investigate potential circadian regulation. Anterior and posterior hypothalami were collected from C57BL/6J mice at diestrus, proestrus, and days 6, 10, 14, and 18 of pregnancy, at six time points across 24 h, for real-time PCR analysis of gene expression. Analysis confirmed that *Kiss1* mRNA expression in the AVPV increased at ZT13 during proestrus, with a luteinizing hormone surge observed thereafter. No diurnal regulation was seen at diestrus or at any stage of pregnancy. Anterior hypothalamic *Avp* mRNA expression exhibited no diurnal variation, but *Avpr1a* peaked at 12:00 h during proestrus, possibly reflecting the circadian input from the suprachiasmatic nucleus to AVPV *Kiss1* neurons. *Rfrp* (*Npvf*) expression in the posterior hypothalamus did not demonstrate diurnal variation at any stage. Clock genes *Bmal1* and *Rev-erba* were strongly diurnal, but there was little change between diestrus/proestrus and pregnancy. Our data indicate the absence of the circadian input to *Kiss1* in pregnancy, despite high gestational estradiol levels and normal clock gene expression, and may suggest a disruption of a kisspeptin-specific diurnal rhythm that operates in the nonpregnant state.

Key Words

- ▶ kisspeptin
- ▶ hypothalamus
- ▶ circadian
- ▶ pregnancy
- ▶ mouse

Journal of Endocrinology
(2016) **229**, 307–318

Introduction

Kisspeptin neurons are the afferent population that is critical for stimulation of gonadotropin-releasing hormone (GnRH) neurons, to drive the hypothalamic–pituitary–gonadal (HPG) axis. Gonadal sex steroids exert feedback actions onto GnRH neurons via kisspeptin regulation,

with kisspeptin populations in the anteroventral periventricular (AVPV) nucleus and arcuate (Arc) nucleus being involved in positive and negative feedback control of estradiol, respectively (Gottsch *et al.* 2004, Smith *et al.* 2005a,b). Neurons expressing RFamide-related peptides

(RFRPs (NPVF)) such as RFRP-3, produced from a precursor peptide encoded by the neuropeptide VF precursor (*Npvf*) gene and the actions of which are conducted through the neuropeptide FF receptor (NPFFR) (Clarke *et al.* 2009), are found in the dorsomedial nucleus (DMN). RFRP-3 neurons are proposed inhibitors of GnRH secretion, which are thought to modulate the negative feedback effects of estrogen across most of the ovulatory cycle (Kriegsfeld *et al.* 2006, Ducret *et al.* 2009, Williams & Kriegsfeld 2012). For a short period just before ovulation, estrogen feedback to the reproductive axis switches from negative to positive, and this causes a surge of GnRH and thus LH secretion from the pituitary (Karsch *et al.* 1997, Levine 1997). This switch is thought to be governed, in part, by the interplay between kisspeptin and RFRP3.

The pre-ovulatory LH surge that occurs in female rodents is a product of interactions between circadian inputs and estrogenic signals. Specifically, high concentrations of estradiol and a circadian signal predicting the onset of darkness are both essential for triggering the increase in kisspeptin levels and the subsequent LH surge (Levine 1997, Christian & Moenter 2010, Williams *et al.* 2011). In female rodents, the LH surge is precisely timed to occur in the late afternoon of proestrus, being tightly controlled by the master circadian oscillator located in the suprachiasmatic nucleus (SCN) of the hypothalamus. Kisspeptin also appears to be under the circadian control, as evidenced by studies showing that increases in kisspeptin expression in the AVPV of the hypothalamus are synchronous with the LH surge during proestrus in female rats (Smith *et al.* 2006), mice (Robertson *et al.* 2009), and hamsters (Williams *et al.* 2011). Furthermore, studies in hamsters have shown that RFRP-3 cells are indirectly modulated via SCN vasoactive intestinal peptide (VIP)-ergic neurons (Russo *et al.* 2015) and act directly on GnRH neurons to inhibit their activity (Gibson *et al.* 2008). Data also suggest that RFRP-3 exhibits reduced expression during proestrus, and thus lowers GnRH inhibition and positively drives the GnRH/LH surge and ovulation (Gibson *et al.* 2008). Other studies showing that estrogen lowers *RFRP3* mRNA levels also suggest that RFRP-3 may also play a role in estrogen positive feedback (Molnár *et al.* 2011, Poling *et al.* 2012).

Arginine vasopressin (AVP) neurons are responsible for the circadian input to the AVPV and originate from the dorsomedial SCN to contact kisspeptin neurons expressing the AVP receptor subtype V1a (Leak & Moore 2001, Williams *et al.* 2011). As the master pacemaker, the SCN exerts tight circadian control over many biological processes through

endogenous rhythms generated by positive and negative feedback gene transcription and translation loops of clock genes, including *Clock*, *Bmal1*, *Per1-3*, *Cry 1-2*, and *Rev-erba* (Reppert & Weaver 2001). These clock genes are also expressed in numerous peripheral tissues (Boden *et al.* 2010). We have recently shown that the expression of core clock genes in the SCN changes significantly across gestation in the mouse (Wharfe *et al.* 2016), and Wharfe and coworkers have also previously demonstrated that clock gene rhythms in the rat liver are altered by pregnancy (Wharfe *et al.* 2011). Additionally, despite the high estradiol levels during pregnancy, the HPG axis is dormant and ovulation does not occur, suggesting the shutdown of kisspeptin signaling, reduced GnRH neuron or gonadotrope sensitivity, or a combination of each. We therefore speculate that disruption of normal hypothalamic circadian rhythms, including those of kisspeptin in the AVPV, occurs in the pregnant state and suppresses brain mechanisms controlling ovulation. In this study, we sought to characterize the hypothalamic expression of the kisspeptin signaling system in the mouse during pregnancy, and to investigate whether this expression exhibits a circadian pattern, as is observed in proestrus females.

Materials and methods

Animals

Nulliparous C57Bl/6J mice (6 weeks old) were supplied by the Animal Resources Centre (Murdoch, Australia). Mice were maintained in an environmentally controlled room under a 12h light:12h darkness cycle (lights on from 07:00 to 19:00h) with access to food and water *ad libitum*. By convention in chronobiology, lights off at 19:00h was defined as Zeitgeber time (ZT) 12, with sampling times described as relative to ZT12. Female mice were subjected to a daily vaginal smear to determine the estrous cycle stage and monitored for three full cycles. A subgroup of female mice was mated overnight, and pregnancy confirmed by observation of a vaginal plug the following morning, which was designated day 1 of pregnancy. Whole-brain and blood sample collections were made at 4h intervals commencing at 08:00h on diestrus II/proestrus of the cycle or day 6, 10, 14, or 18 of pregnancy (term = 19 days). Diestrus and proestrus were chosen as days of the cycle as hormone levels and gene expression typically show the most variation between those two days. The days of pregnancy chosen represent developmental milestones: day 6 is postimplantation, day 10 is the embryonic

period, day 14 represents early fetal life, and day 18 late fetal life. We have previously shown that six time points (4h: intervals) are adequate for the determination of circadian regulation of genes (Wharfe *et al.* 2016). All procedures involving animals were conducted with the approval of the Animal Ethics Committee of the University of Western Australia, Perth, WA, Australia.

Tissue collection

Whole brains were collected from mice under isoflurane anaesthesia at 08:00h (ZT1), 12:00h (ZT5), 16:00h (ZT9), 20:00h (ZT13), 24:00h (ZT17), or 04:00h (ZT21) in order to obtain a reasonable circadian profile of gene expression and plasma hormone levels. A red light (>600nm wavelength) was used to facilitate collection of tissues in the dark phase. Whole brains were frozen by being placed on dry ice immediately following removal. A blood sample was obtained from each mouse under anesthesia through a cardiac puncture and collected in a tube containing EDTA (100 µL/mL of blood). Plasma was obtained following centrifugation of the blood sample and stored at –20°C until required.

Hypothalamic gene expression

RNA extraction Hypothalami were dissected from whole-brain samples and bisected into anterior (containing the AVPV and SCN) and posterior (containing the ARC) portions as described by Quennell and coworkers (Quennell *et al.* 2011). Total RNA was extracted from anterior and posterior hypothalami using Qiazol (Qiagen) according to the manufacturer's instructions.

The RNA pellet was dissolved in 50 µL of RNase-free water, placed on ice for 5 min and thoroughly vortexed. RNA was quantitated using the Nanodrop ND-1000 spectrophotometer (Thermo Scientific) at 260nm and stored at –80°C until required.

Reverse transcription RNA samples were treated with RQ1 RNase-free DNase (Promega, cat# M6101) to remove any genomic DNA present in the sample. Reactions were made up to a total volume of 10 µL with 1 µg of RNA, 1 µL of RQ1 RNase-Free DNase 10× Buffer, 2 µL of RQ1 RNase-Free DNase and nuclease-free water, and incubated at 37°C for 30 min. About 1 µL of RQ1 DNase Stop Solution was added to each sample to terminate the reaction, and samples were incubated at 65°C for 10 min to deactivate the DNase.

Total RNA (1 µg) was reverse transcribed to cDNA with random primers (Promega, cat# C1181) using Moloney Murine Leukemia Virus Reverse Transcriptase, RNase H Minus, Point Mutant (M-MLV RT (H–)) (Promega, cat# M3683). The resultant cDNAs were purified using the UltraClean PCR Clean-Up Kit (MoBio Laboratories, Carlsbad, CA, USA; cat# 12500-250), according to the manufacturer's instructions.

Real-time PCR Analyses of mRNA levels for total *Kiss1*, *Kiss1* receptor (*Kiss1r*), AVP (*Avp*), AVP receptor 1a (*Avpr1a*), neuropeptide VF precursor (*Npvf*), and NPFPR (*Npffr*) transcripts were carried out by quantitative RT-PCR on the Rotorgene 6000 (Corbett Life Science, Concorde, New South Wales, Australia) using iQ SYBR Green Supermix (Bio-Rad, cat# 170-8880). Primers (Table 1) for total *Kiss1*,

Table 1 Primer sequences and conditions for quantitative PCR.

Gene	Sequence	Size (bp)	Annealing temp (°C)
<i>Kiss1</i>	F: 5'-CTCTGTGTCGCCACCTATGG-3' R: 5'-AGGCTTGCTCTCTGCATACC-3'	126	60
<i>Kiss1r</i>	F: 5'-TGCTGGGAGACTTCATGTGC-3' R: 5'-CATACCAGCGGTCCACACTC-3'	102	60
<i>Bmal1</i>	F: 5'-CGTGCTAAGGATGGCTGTTC-3' R: 5'-CTTCCCTCGGTACATCCTA-3'	166	60
<i>Rev-erba</i>	F: 5'-ATTGCCCAGGGGCGAGAGA-3' R: 5'-GCCAAAAGAGCGGGCAGGGT-3'	292	60
<i>Hprt</i>	F: 5'-GCAGTACAGCCCCAAATGG-3' R: 5'-AGTCTGGCCTGTATCCAACAC-3'	80	60
<i>Sdha</i>	F: 5'-ATGGAAAATGGGGAGTGCCG-3' R: 5'-ACAGCTGAAGTAGGTTCCGGC-3'	123	60
<i>Actb</i>	F: 5'-TCCACACCCGCCACCAG-3' R: 5'-GGCCTCGTCACCCACATAG-3'	197	62

Kiss1r, *Bmal1*, and *Rev-erba*, and the reference genes hypoxanthine-guanine phosphoribosyltransferase (*Hprt*), succinate dehydrogenase subunit A (*Sdha*), and beta actin (*Actb*) were designed using Primer-BLAST (<http://www.ncbi.nlm.nih.gov>). Each of the selected primer pairs was positioned to span an intron to ensure that no product was amplified from genomic DNA. The resulting amplicons were sequenced to confirm specificity. Standard curves for each product were generated from gel-extracted PCR products (QIAquick Gel Extraction Kit, Qiagen) using ten-fold serial dilutions and Rotorgene 6000 software. The PCR cycling conditions were as follows: initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 1 s, annealing at 60°C for 15 s, and extension at 72°C for 5 s. Melt-curve analysis was carried out to confirm amplification specificity for each gene.

Primers for *Avp*, *Avpr1a*, *Npvf*, and *Npffr* were purchased as QuantiTect Primer Assays (Qiagen, cat# QT00249389, QT00113169, QT00278551, QT001169196). The PCR conditions were as follows: initial activation 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 5 s, and combined annealing/extension at 60°C for 10 s. Melt-curve analysis was carried out to confirm amplification specificity.

All target genes were standardized against housekeeping genes *Hprt*, *Sdha* and *Actb* using the GeNorm algorithm (Vandesompele *et al.* 2002). No differences were seen in these genes across days or time points (data not shown).

Plasma hormone analyses

Pituitary hormones Plasma levels of follicle-stimulating hormone (FSH), luteinizing hormone (LH), prolactin, and adrenocorticotrophic hormone (ACTH) were measured in a 10 µL sample using the Milliplex Map Mouse Pituitary Magnetic Bead kit (Merck Millipore; cat# MPTMAG-49K) according to the manufacturer's instructions. The plate was run on a MAGPIX system (Luminex Corporation, Austin, TX, USA) with xPONENT software (Luminex) to analyze the median fluorescent intensity data and obtain analyte concentrations. The lower limits of detection for this assay are as follows: FSH, 9.5 pg/mL; LH, 1.9 pg/mL; and prolactin, 46.2 pg/mL.

Steroid hormones Before assay, plasma samples (50 µL) were extracted in 75 µL of acetonitrile, vortexed for 5 s, and incubated for 10 min at room temperature. Samples were centrifuged at 17,000g for 5 min, and the

supernatant was transferred into separate tubes. The samples were dried by using SpeedVac (Thermo Scientific) at the highest setting and reconstituted with 40 µL of assay buffer for subsequent assay.

Plasma levels of estradiol and progesterone were measured in 25 µL of extracted sample using the Milliplex Map multi species Steroid/Thyroid Hormone panel kit (Merck Millipore; cat # STTHMAG-21K) according to the manufacturer's instructions. The plate was run on a MAGPIX system (Luminex Corporation) with xPONENT software to analyse the median fluorescent intensity data and obtain analyte concentrations. The lower limits of detection for this assay are as follows: estradiol, 16 pg/mL; and progesterone, 90 pg/mL.

Statistical analysis

Statistical analyses were conducted using GraphPad Prism 6 (GraphPad Software). Data are expressed as the mean $\pm$ S.E.M., with an *n* of 5–7 per ZT on each day measured. Differences were considered to be significant when $P < 0.05$. One-way ANOVAs were used to analyze plasma estradiol, progesterone, prolactin, ACTH, and FSH levels. Two-way ANOVAs were used to analyze gene expression data and plasma LH levels, with day and time as factors, followed by Tukey's *post hoc* tests where appropriate. One-way ANOVAs with time as a factor were conducted within each day, where significant interaction effects were observed in two-way ANOVA analyses.

Results

Hormone levels

There was a significant effect of day on plasma LH concentrations ($P < 0.001$), which was highest at proestrus. Within proestrus, LH increased around two-fold between ZT5 and ZT17 ($P < 0.05$), and no diurnal variation was observed at diestrus or day 18 of pregnancy (Fig. 1A). Plasma FSH levels showed no difference between any of the days measured (Fig. 1B).

Plasma estradiol levels increased significantly during pregnancy, almost doubling from diestrus to day 18 of pregnancy ($P < 0.05$) (Fig. 1C). Similarly, plasma progesterone levels rose dramatically during pregnancy, increasing 45-fold by day 6 ($P < 0.01$) and 170-fold by day 18 ($P < 0.0001$; Fig. 1D). Plasma prolactin concentrations peaked at day 6 of pregnancy, increasing nearly twofold compared with proestrus ($P < 0.0001$) and falling to pre-pregnancy levels by day 18 ($P < 0.0001$; Fig. 1E).

Figure 1

Plasma hormone concentrations in nonpregnant animals and during pregnancy. (A) LH levels peaked at ZT17 on the day of proestrus. (B) FSH levels were unchanged in nonpregnant animals and pregnant mice. (C) Estradiol levels were significantly increased at day 18 of pregnancy compared with day 6 of proestrus (Pro) and diestrus (Die). (D) Progesterone (P4) levels were significantly increased at day 6 of pregnancy and further increased at day 18 of pregnancy. (E) Prolactin levels peaked at day 6 of pregnancy. Data are mean $\pm$ S.E.M., $n=4-7$ per group. Two-way ANOVA; * $P<0.05$ within-day effect; # $P<0.001$ between-day effect. For C and D, values without common notations differ; $P<0.05$.

Gene expression

Kiss1 expression in the anterior hypothalamus, representing the AVPV population, differed significantly between days and peaked at day 10 of pregnancy, increasing twofold compared with diestrus ($P<0.001$). As for within-day effects, on the day of proestrus, *Kiss1* increased more than two-fold from ZT9 to ZT13 ($P<0.05$) and returned to baseline by ZT17 (Fig. 2A). No time-of-day variation was observed during diestrus or any other day of pregnancy (Fig. 2A). In the posterior hypothalamus, representing the ARC population of KISS1 neurons, no change was noted between days. Within days, *Kiss1* levels increased over fourfold from ZT5 to ZT9 ($P<0.05$) and returned to baseline by ZT13 on day 18 of pregnancy, whereas diurnal variation was not observed on any other day of pregnancy, diestrus, or proestrus (Fig. 2B).

Kiss1r expression in the anterior hypothalamus was different among days ($P<0.0001$; Fig. 3), with elevated mRNA levels at day 14 of pregnancy, having nearly doubled compared with diestrus ($P<0.0001$), but showed no diurnal variation on any day of the cycle or pregnancy.

Avp expression in the anterior hypothalamus changed between days as pregnancy progressed ($P<0.0001$); levels peaked in the late stages, with an increase of 2.4-fold from day 10 to day 14 of pregnancy ($P<0.0001$), which was sustained at day 18 ($P<0.01$). However, no diurnal variation was observed at any stage (Fig. 4A).

There was an effect of day on *Avpr1a* mRNA levels in the anterior hypothalamus ($P<0.0001$); *Avpr1a* was high throughout diestrus/proestrus and the early stages of pregnancy, then decreased by 55% from day 10 to days 14 and 18 of pregnancy ($P<0.0001$; Fig. 4B). As for within-day effects, *Avpr1a* levels in the anterior hypothalamus increased nearly two-fold from ZT1 to ZT5 during proestrus ($P<0.05$) and 1.6-fold between ZT5 and ZT13 on day 10 of pregnancy ($P<0.05$; Fig. 4B).

Npvf expression in the posterior hypothalamus, representing the dorsomedial hypothalamus (DMH) population of RFRP-3 neurons, was different among days ($P<0.05$), with mRNA levels declining after day 10 of pregnancy to 65% by day 18 ($P<0.05$). However, diurnal variation was not observed at any stage (Fig. 5A). *Npffr* mRNA levels in the anterior hypothalamus varied across

Figure 2

Kiss1 diurnal gene expression in nonpregnant animals and during pregnancy. (A) *Kiss1* levels in the AVPV were significantly increased at ZT13 on the day of proestrus. This effect was not seen at diestrus or during pregnancy. (B) Diurnal variation in *Kiss1* seen at day 18 of pregnancy in the ARC. Data are mean $\pm$ s.e.m., $n=5-7$ per group. Two-way ANOVA; * $P<0.05$ within-day effect; # $P<0.001$ between-day effect compared with diestrus.

pregnancy ($P<0.01$); *Npffr* was high throughout diestrus/proestrus and the early stages of pregnancy, then fell by 55–65% at day 14 through to day 18 of pregnancy ($P<0.01$). As for time-of-day effects, *Npffr* levels increased 3.5-fold from ZT1 to ZT13 ($P<0.01$) during proestrus, but there was no diurnal variation evident at diestrus or during pregnancy (Fig. 5B).

Bmal1 and *Rev-erba* exhibited strong time-of-day effects on both diestrus and proestrus ($P<0.05$ for both genes on both days) (Fig. 6). They also demonstrated an antiphase relationship, with *Rev-erba* peaking in the afternoon between

ZT5 and ZT9, and *Bmal1* peaking in the early hours of the morning between ZT21 and ZT1 (Fig. 7). *Bmal1* and *Rev-erba* and other central circadian clock genes also exhibited robust diurnal rhythms during pregnancy, which appeared unchanged despite an apparent upward shift in overall expression from mid-to-late gestation (Wharfe *et al.* 2016).

Discussion

The present findings demonstrate an alteration in the diurnal regulation of AVPV *Kiss1* and *Avpr1a* expression

Figure 3

Anterior hypothalamus *Kiss1r* diurnal gene expression in nonpregnant animals and during pregnancy. No diurnal variation was observed at any stage. Data are mean $\pm$ s.e.m., $n=5-7$ per group. Two-way ANOVA; # $P<0.0001$ between-day effect compared with diestrus and proestrus.

Figure 4

Avp and *Avpr1a* diurnal gene expression in the anterior hypothalamus in nonpregnant animals and during pregnancy. (A) *Avp* mRNA levels did not exhibit diurnal variation at any stage. (B) *Avpr1a* expression increased at ZT5 on the day of proestrus and showed some diurnal variation at day 10 of pregnancy. Data are mean $\pm$ S.E.M., $n=5-7$ per group. Two-way ANOVA; * $P<0.05$ within-day effect compared with ZT1 and days 9, 13, and 21; # $P<0.01$, ## $P<0.0001$ between-day effect compared with diestrus, proestrus, and days 6 and 10.

during pregnancy in the mouse, which is associated with the cessation of the preovulatory LH surge, in spite of elevated estradiol concentrations and a functional master circadian clock. *Kiss1* gene expression in the AVPV peaked at ZT13 during proestrus, 4h before the LH peak at ZT17, which is consistent with that reported in the literature, although the peak in *Kiss1* mRNA levels has previously been found to be roughly coincident with the LH surge (Smith *et al.* 2006, Robertson *et al.* 2009).

We also found that AVP receptor *Avpr1a* increased in expression in the AVPV at ZT5 during proestrus, before the rise in *Kiss1* expression and the LH surge. This is consistent with work by de la Iglesia and colleagues on the rat model, showing a peak in *Avpr1a* gene expression 4h before that of *Kiss1* (Smarr *et al.* 2013). It is possible that this temporal arrangement of events may reflect the transmission of circadian information from the SCN to kisspeptin neurons in the AVPV, although the 8h gap observed in this study decreases the likelihood of a direct connection between the two events. Although its receptor showed an increase in expression at proestrus, *Avp* itself did not mirror this increase at any time on the day of proestrus. However, previous data have shown that *Avp*

gene expression in the mice SCN follows a circadian pattern, being highest at the end of the day and lowest at the end of the night, although the sex of the mice was not specified (Dardente *et al.* 2004). This pattern of *Avp* expression would be consistent with the sequence of events observed in this study, which comprise the increase in *Avpr1a* and *Kiss1* expression and subsequent LH surge. It is possible that in this study, the diurnal rhythm of *Avp* mRNA specifically within the SCN has been masked; numerous parvocellular and magnocellular neurosecretory cells of the paraventricular and supraoptic nuclei located in the anterior hypothalamus express AVP as well (Alves *et al.* 1998, Nomura *et al.* 2002). *In situ* hybridization is needed to determine *Avp* mRNA levels specifically in the SCN.

The transcription factor albumin D-site binding protein (*Dbp*) in the AVPV has been hypothesised to be the conduit through which AVP drives the circadian rhythm of *Kiss1*; *Dbp* is capable of triggering *Kiss1* transcription and exhibits elevated levels in the afternoon of proestrus, similar to *Kiss1* expression (Xu *et al.* 2011). Data from Kriegsfeld and coworkers suggest that the initiation of the LH surge by AVP is gated

Figure 5

Npvf (*Rfrp*) and *Npffr* (*Rfrpr*) diurnal gene expression in nonpregnant animals and during pregnancy. (A) *Npvf* expression in posterior hypothalamus showed no diurnal variation at any stage. (B) *Npffr* expression in the anterior hypothalamus increased from ZT1 to ZT13 on the day of proestrus. Data are mean $\pm$ S.E.M., $n=5-7$ per group. Two-way ANOVA; * $P<0.01$ within-day effect; # $P<0.05$ between-day effect compared with days 6, 14, and 18; ## $P<0.01$ between-day effect compared with diestrus, proestrus, and days 6 and 10.

by GnRH neurons, whereby kisspeptin neurons are indiscriminately activated by AVP but GnRH neurons are selectively responsive to kisspeptin stimulation, depending on the time of day (Williams *et al.* 2011). This mechanism of control ensures that the LH surge is limited to the late afternoon of proestrus. In a further layer of circadian control, AVPV kisspeptin neurons

appear to possess a circadian oscillator independent of the master pacemaker in the SCN. Recent work by Chassard and coworkers demonstrates a daily period circadian clock 1 (PER1) rhythm within AVPV kisspeptin cells that is E_2 -sensitive and phase delayed compared with that in the SCN (Chassard *et al.* 2015), suggesting that there may exist yet another level of circadian gating of the LH surge.

In this study, both *Kiss1* and *Avpr1a* mRNAs in the anterior hypothalamus exhibited a peak in expression during proestrus that was absent at diestrus. These estrous cycle differences in expression profiles do not appear to be driven by clock genes as the rhythms of *Bmal1* and *Rev-erba* were unchanged between diestrus and proestrus, suggesting that the likely major stimulus for the increase in *Kiss1* and *Avpr1a* expression at proestrus is rising estradiol levels (Robertson *et al.* 2009, Williams *et al.* 2011, Smarr *et al.* 2013). Unlike *Kiss1* expression in the AVPV, *Kiss1* expression in the ARC was not different between proestrus and diestrus, exhibiting no diurnal variation on either day. The different expression patterns reflect the distinct roles of the two neuronal populations, with the kisspeptin neurons in the ARC responsible for the

Figure 6

Anterior hypothalamus diurnal clock gene expression in nonpregnant animals. (A) *Bmal1* expression showed a robust diurnal rhythm on diestrus and proestrus. (B) *Rev-erba* showed a robust diurnal rhythm on diestrus and proestrus. Expected antiphase relationship between *Bmal1* and *Rev-erba* was observed. Data are mean $\pm$ S.E.M., $n=5-7$ per group. Two-way ANOVA; * $P<0.05$ within-day effect.

Proestrus PM

Late Pregnancy

Figure 7

Proposed model indicating the change between proestrus and late pregnancy with the role of AVPV kisspeptin neurons in generating the GnRH/LH surge. At proestrus, kisspeptin neurons in the AVPV receive circadian information from the SCN via vasopressin receptors (V1a), and sense circulating estradiol via estrogen receptors (ER). Kisspeptin neurons are activated in the event that estrogenic and circadian signals are both high, activating GnRH neurons. In late pregnancy, despite high estrogen and an operant clock, there is a disruption of kisspeptin output possibly due to reduction in V1a and high progesterone and/or prolactin. Figure adapted under the terms of a Creative Commons Attribution-Noncommercial-Share Alike 3.0 Unported License from (Smith *et al.* 2006).

tonic control of gonadotropin secretion, as opposed to generating the preovulatory LH surge (Smith *et al.* 2005b, Beale *et al.* 2014).

Diurnal rhythmicity in the SCN (as part of the anterior hypothalamus) during pregnancy remains intact, even though the expression of core clock genes (including *Bmal1* and *Rev-erba*) is altered during pregnancy compared with diestrus (Wharfe *et al.* 2016). The persistence of these rhythms throughout pregnancy suggests that the master pacemaker in the SCN is functioning normally, and thus any changes in the circadian input to the AVPV are likely to occur downstream of the SCN. Consequently, the pathway that involves AVP neurons transmitting circadian information from the SCN to kisspeptin neurons in the AVPV is potentially disrupted in pregnancy (Leak & Moore 2001, Williams *et al.* 2011).

Although the suppression of diurnal regulation of *Kiss1* and *Avpr1a* expression in the AVPV during pregnancy compared with proestrus is consistent with our hypothesis, the mechanisms underlying this suppression are unclear and require further investigation. Despite the rise in estradiol levels throughout pregnancy, established in the literature and confirmed by our data, the surge in kisspeptin and LH does not occur at any stage of pregnancy. As both the LH surge and AVPV *Kiss1* levels require the combination of elevated estradiol and a circadian signal (Robertson *et al.* 2009), the lack of an LH surge in the face of peak estradiol concentrations would indicate a disruption of the circadian signal.

Because diurnal rhythmicity of all the core clock genes in the SCN appears to be intact during pregnancy (Wharfe *et al.* 2016), one possibility is that there is an alteration in the AVP neuronal connections between the SCN and the AVPV, leading to a loss in circadian information. Alternatively, it could be that other hormone(s) that experience an upsurge during pregnancy are overriding the estrogenic and circadian signals that give rise to the increase in kisspeptin in the AVPV.

Potential candidate hormones are progesterone and prolactin, both of which are elevated in the plasma during pregnancy, as observed in this study, and exhibit expression patterns that are in agreement with previously reported data from both the rat and the mouse. Plasma prolactin concentrations are high in early pregnancy with a biphasic pattern of secretion, characterized by one nocturnal and one diurnal surge, which cease after day 8 of pregnancy (Smith & Neill 1976). This coincides with the beginning of an increase in placental lactogen secretion (which peaks at day 11) (Smith & Neill 1976), and there is evidence to suggest that the latter causes the former (Tonkiewicz & Voogt 1983). Prolactin levels fall and plateau in mid- and late pregnancy, then surge again immediately before parturition (Morishige *et al.* 1973, Murr *et al.* 1974a). Plasma progesterone exhibits almost the opposite pattern to prolactin, rising continually until very late in pregnancy, when levels fall precipitously to signal the onset of parturition (Murr *et al.* 1974b, Barkley

et al. 1979, Waddell *et al.* 1989). Our data failed to capture the sudden decrease in progesterone levels before parturition; however, it is likely that this would have occurred at a later time had the pregnancies been allowed to progress further. In addition, recent work has shown that AVPV kisspeptin neurons require progesterone receptor signaling to display normal *c-Fos* induction and to mount an LH surge in response to elevated estradiol (Stephens *et al.* 2015). However, it does not appear to be gonadal progesterone but rather estradiol-induced local synthesis of neuroprogesterone in the hypothalamus that is critical for the LH surge (Micevych & Sinchak 2011). Furthermore, kisspeptin expression in the ARC and AVPV is inhibited by high prolactin levels, resulting in the suppression of LH secretion (Araujo-Lopes *et al.* 2014, Brown *et al.* 2014). The substantial effects of progesterone and prolactin on kisspeptin, as well as the important role that these hormones play during gestation, likely implicate them in the disruption of circadian signaling, which results in the suppression of kisspeptin activation in pregnancy.

Although no diurnal regulation of AVPA *Kiss1* mRNA expression during pregnancy was noted in our study, we did reveal a number of changes in gene expression during pregnancy. AVPV *Kiss1* expression was elevated at day 10 of pregnancy compared with diestrus, in line with the findings of Roa and coworkers showing elevated *Kiss1* mRNA in the whole hypothalamus during pregnancy (Roa *et al.* 2006), although the authors suggest that this is not likely attributable to high levels of estradiol and progesterone. Moreover, at day 18, we saw a within-day increase in ARC *Kiss1*, despite relatively high estradiol levels and no observable increase in plasma LH levels. We also saw an increase in *Kiss1r* at day 18. *Avp* mRNA expression increased at day 14 and 18 of pregnancy, and within-day effects in *Avpr1a* were seen at day 10. We can only speculate what these changes reflect, but an attractive idea may be that changes relate to parturition or the phenomenon of postpartum ovulation in the mouse. Clearly, this requires further study.

Although this study demonstrated a 4 h time lag between the peak in *Kiss1* mRNA levels and that of plasma LH levels, Robertson and coworkers have previously shown that these two events occur in synchrony (Robertson *et al.* 2009). This discrepancy in timing may be due in part to the shorter time intervals between collections in the Robertson *et al.* (2009) study; 1–2 h intervals around the time of the LH surge, compared with 4 h intervals in this study, which may have failed to capture the true

peak of LH concentrations as well as *Kiss1* mRNA levels. In this study, another contributing factor could be the large variation in plasma LH concentrations at each time point. Samples were collected across a 1 h period, within 30 min of the designated time; thus, variations in short-lived peaks of LH levels would contribute to variation in the four-hourly measures.

Finally, we observed no difference in *Npvf* expression between diestrus, proestrus, or any stage of pregnancy. This is in contrast to some of the known literature, which indicates that RFRP expression is lowest at proestrus and maximal at diestrus (Gibson *et al.* 2008), consistent with its role as a GnRH inhibitor and in the negative feedback effects of estradiol present for the majority of the female mice ovulatory cycle (Kriegsfeld *et al.* 2006, Gibson *et al.* 2008, Ducret *et al.* 2009). The disparity between our data and previous research may be explained by the differing methods used to measure RFRP levels; Gibson and colleagues used immunohistochemical procedures to determine the number of RFRP-expressing cells (Gibson *et al.* 2008). In this case, it is likely that *Npvf* message levels are not an accurate reflection of RFRP protein levels; therefore, further studies using either western blots or immunohistochemistry are needed to confirm our results. Moreover, our anterior hypothalamus *Nppfr* data would seem to show the inverse of the expected trend, with expression at proestrus increased around the time of the LH surge, suggesting that RFRP may even follow a similar pattern to kisspeptin activation. However, it is also important to note that there is currently no consensus in the literature on the regulation of RFRP neurons by estradiol. Estradiol treatment has been shown to have markedly different effects on RFRP levels in female rodents: in some instances decreasing (Molnár *et al.* 2011, Poling *et al.* 2012), increasing (Iwasa *et al.* 2012), or having no effect (Quennell *et al.* 2010).

The current findings reveal a spike in kisspeptin expression during proestrus that is concurrent with the preovulatory LH surge and preceded by an increase in AVP receptor expression, which may indicate the transmission of a circadian signal from the SCN. Pregnancy onset abolishes these temporal patterns and the subsequent LH surge. Although RFRP-3 may inhibit GnRH and/or gonadotropin secretion, we find that neither the peptide nor its receptor exhibits a lower level of expression during proestrus around the time of the LH surge; furthermore, there was no evidence of diurnal variation in RFRP-3 before or during pregnancy. Our data are the first to show that the diurnal variation in hypothalamic kisspeptin expression seen at proestrus is

abolished during pregnancy, in spite of high estradiol levels. We speculate that this is due to a disruption of the circadian signal, possibly along the SCN–AVPV pathway or at a hormonal level.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This work was supported by the Western Australian Department of Health (grant number 1062158 2014).

Acknowledgment

The authors thank Dijana Tesic and Celeste Wale for their technical assistance.

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Received in final form 24 March 2016

Accepted 11 April 2016

Accepted Preprint published online 11 April 2016