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CHAPTER 6

The Suprachiasmatic nucleus is part of a Kisspeptin feedback network involving the anterior ventral part of the third ventricle and Arcuate nucleus.

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To be submitted

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Abstract

The suprachiasmatic nucleus (SCN) plays an essential role in the timing of reproduction. This is based on absent reproductive cycles in female rats after lesioning the SCN, on the strict circadian pattern of ovulation in rodents, and on the connections of the SCN with gonadotropin releasing hormone (GnRH) neurons as well as with Kisspeptin (Kiss) neurons, both receiving input from SCN vasoactive intestinal peptide neurons (VIP) or vasopressin (AVP) neurons, respectively. These SCN projections are thought to be important for timing the LH peak. On the basis of previous findings that the RF-amide related peptide-3 (RFRP-3) neurons of the dorsomedial nucleus of the hypothalamus (DMH), known to inhibit the luteinizing hormone (LH) surge, were found to project to the SCN, we investigated the presence of Kiss innervation in the rat SCN. We observed small caliber Kiss fibers forming a dense network in the ventral SCN, targeting the same VIP neuronal area as where RFRP-3 fibers terminate. Surprisingly, in female as well as in male animals the intensity of this SCN-Kiss innervation varied with a higher fiber density in the morning as compared to the end of day. Interestingly, in spite of major opposite changes in immunoreactivity of Kiss neurons in the anterior ventral part of the third ventricle (AVPV) and arcuate nucleus (ARC) following ovariectomy, SCN-Kiss fiber density was not influenced by ovariectomy or estrogen treatment. Retrograde tracing experiments demonstrated that Kiss neurons innervating the SCN were present both in the AVPV and ARC. The present observations put the SCN at the center of a reciprocal network involved in timing of the LH surge.

Introduction

Female reproduction in mammals requires careful synchronization of a series of physiological and behavioral events in order to assure that conception takes place at the right moment in time. Therefore, it is not surprising that more and more evidence is building up showing that the biological clock has a pivotal role in the timing of reproduction, including that of humans (Kerdelhué et al. 2002). For example, in order to induce ovulation in rat, elevated levels of circulating estradiol (Norman, Blake, and Sawyer 1973; Legan and Karsch 1975) have to coincide with a suprachiasmatic nucleus (SCN) driven luteinizing hormone (LH) peak at the end of the day (Brown-Grant and Raisman 1977; Samson and McCann 1979). Numerous SCN projections are candidate to be responsible for associating and synchronizing reproductive behavior with reproductive physiology. For instance, SCN neurons containing vasoactive intestinal peptide (VIP) terminate on Gonadotrophin releasing hormone (GnRH) neurons in the rat preoptic area (POA) (Van Der Beek et al. 1997), thus providing a temporal niche for the LH surge (Christian and Moenter 2008). Moreover, infusion of arginine vasopressin (AVP) in the anterior ventral part of the third ventricle (AVPV) in SCN lesioned animals was able to induce an LH surge (Palm et al. 1999) proving the importance of AVP in regulating the LH peak. Later, it was shown that the presence of Kisspeptin (Kiss) neurons in this area receiving AVP input from the SCN was associated with this effect (Williams et al. 2011).

Kiss is a neuropeptide essential for reproductive function as Kiss inactivating mutations cause abnormal sexual maturation and infertility in human due to hypogonadism (de Roux et al. 2003; Seminara et al. 2003) which results in the impairment of pubertal maturation and of reproductive function. In the absence of pituitary or hypothalamic anatomical lesions and of anosmia (Kallmann syndrome. Kiss expressing neurons are predominantly found in the AVPV, the preoptic periventricular nucleus (PeN), the arcuate nucleus (ARC) (Gottsch et al. 2004; Desrozier et al. 2010) which bind to a G protein-coupled receptor known as GPR54. Mutations or targeted disruptions in the GPR54 gene cause hypogonadotropic hypogonadism in humans and mice, suggesting that kisspeptin signaling may be important for the regulation of gonadotropin secretion. To examine the effects of kisspeptin-54 (metastin and sparsely in extra-hypothalamic areas (Cravo et al. 2011; Kim et al. 2011). In the ARC Kiss neurons are also known as KNDy neurons because they co-express kisspeptin, neurokinin B, dynorphin, and glutamate amongst others (Lehman, Coolen, and Goodman 2010). Within the central nervous system, Kiss has a pivotal role in the regulation of reproductive functions through its projections to GnRH neurons in the POA inducing the LH surge and ovulation (Gu and Simerly 1997; Simonian, Spratt, and Herbison 1999; de Roux et al. 2003; Seminara et al. 2003; Kauffman, Clifton, and Steiner 2007) mediating hormonal feedback on gonadotropin secretion. The results of anterograde transport experiments indicate that the AVPV sends ascending projections to the ventral part of the lateral septal nucleus, the parastrial nucleus, and

the region adjacent to the vascular organ of the lamina terminalis (OVLt). The majority of GnRH neurons express kisspeptin-1 receptors (Kiss1r), suggesting that Kiss is able to directly modify the activity of these cells (Irwig et al. 2004). Indeed injections of Kiss in the POA results in activation of GnRH neurons followed by an LH peak (Matsui et al. 2004; Patterson et al. 2006). Moreover, Kiss1r knockout animals lack GnRH release and Kiss injections do not produce an LH peak in these animals (Messenger et al. 2005) we investigate the possible central mode of action of GPR54 and kisspeptin ligand. First, we show that GPR54 transcripts are colocalized with gonadotropin-releasing hormone (GnRH) confirming the importance of Kiss for the LH surge.

The vast majority of Kiss neurons in the AVPV and ARC express sex steroid receptors, namely estrogen receptor α (ER α). Kiss expression in both nuclei is estrogen-dependent with estrogen being stimulatory in the AVPV and inhibitory in the ARC (Smith et al. 2005; Smith 2008; Clarkson et al. 2009). Since electrolytic lesions restricted to the AVPV result in an impaired estrous cycle and are able to block an exogenous estrogen induced LH peak (Wiegand, Terasawa, and Bridson 1978; Wiegand et al. 1980) here designated as the medial preoptic nucleus (MPN), the AVPV-GnRH Kiss pathway is thought to be essential for positive feedback on the reproductive axis. On the other hand, studies also support a role for Kiss neurons in the ARC in regulating the reproductive cycle; e.g. a negative energy balance suppresses Kiss expression in the ARC and is associated with an inhibition of the reproductive cycle (Roa 2013).

Considering the timing of the LH surge, the SCN is involved in the positive arm of the reproductive cycle with AVP neurons projecting to Kiss neurons in the AVPV indirectly stimulating GnRH release and the LH peak (Vida et al. 2010; Williams et al. 2011). Furthermore, VIP neurons show direct projections to GnRH neurons and exert an excitatory effect dependent on estrogen levels and time of day (Van der Beek et al. 1997; Christian and Moenter 2008). Interestingly, the SCN is also involved in adapting negative feedback from the reproductive cycle. SCN VIP neurons project to RFRP-3 neurons in the dorsomedial hypothalamus (DMH) (Gibson et al. 2008), which are associated with a tonic inhibition of LH secretion (Henningsen et al. 2017). Moreover, melatonin, which is regulated by SCN activity, feeds back onto RFRP-3 neurons enabling a seasonal organization of fertility and breeding (Ubuka et al. 2012; Henningsen, Gauer, and Simonneaux 2016) and mammals including humans. The identified avian and mammalian GnRH peptides universally possess an LPXRFamide (X = L or Q). The SCN not only provides input to the reproductive network, it also receives input from it. For instance, DMH RFRP-3 neurons receive SCN innervation but also have projections to the ventral part of the SCN (Acosta-Galvan et al. 2011) the biological clock. Consequently, a food-entrained oscillator has been proposed to be responsible for meal time estimation. Recent studies suggested the dorsomedial hypothalamus (DMH) indicating the negative arm of a feedback circuit between the LH surge and the SCN.

In the present study we hypothesized that similar to DMH RFRP-3 neurons, Kiss

neurons form a feedback circuit with the SCN. Analyzing Kiss immunoreactivity in the anterior hypothalamus with an antibody with a high capacity to stain Kiss fibers (Oakley, Clifton, and Steiner 2009), we observed a dense Kiss immuno-positive innervation of the ventrolateral SCN showing diurnal variation with a higher staining intensity of Kiss fibers in the SCN in the morning, whereas late in the day Kiss fiber density was strongly diminished. We show that both Kiss neurons in the AVPV and ARC contribute to this SCN projection, with both nuclei showing an estrogen dependent temporal organization of staining intensity and mRNA synthesis. These observations provide evidence for the incorporation of the SCN in a neuronal feedback circuit within the reproductive system.

Materials and Methods

Animals and ethical approval. Experiments were performed on male and female Wistar rats (~250g) individually housed in a temperature and humidity controlled environment under a 12:12 h LD cycle (lights on, 0700 h). Rats were given food and water ad libitum. All animal experiments were performed following approval from the Committee for Ethical Evaluation at the Institute for Biomedical Research, Universidad Nacional Autónoma de México, in accordance with Mexican (Norma Oficial Mexicana, NOM-062-ZOO-1999) and ASPA guidelines for animal handling.

Surgery. All animals undergoing surgery were anesthetized with ketamine (50 mg/kg) and xylazine (2 mg/kg) (Pisa-Agropecuaria S.A. de C.V.; Atitalaquia Hgo., Mexico).

SCN CtB injections. In testing our hypothesis that the SCN receives direct innervation from the AVPV and ARC, we performed unilateral Cholera toxin B (CtB) injections in the SCN. Cholera toxin B (CtB) iontophoretic injections (Molecular Probes, Eugene, OR, USA) were made using alternate current 10 sec on 10 sec off, 7uA 30-50V via 15 microns tip diameter glass micropipette, placed unilaterally into the SCN. Thirteen female rats received anesthesia, and were mounted in a stereotact (David Kopf Instruments; Tujunga, USA) using coordinates for SCN injections as described before (Buijs et al., 2014). In short, the animal was placed in the stereotact with tooth bar at -3.4mm and the point of the micropipette placed 0.05mm anterior; 0.09mm lateral from Bregma and 8.6mm ventral from the dura, under an angle of 4°. Animals were allowed to recover for 7-10 days to allow sufficient time for retrograde transport to all cell bodies with terminals inside the injection site. Five of these animals were used for further analysis as injections showed minimal leakage along the injection tract and injections were positioned completely inside the SCN as determined by CtB immunohistochemistry.

OVX. To avoid interference from fluctuating estradiol blood levels over the estrous cycle during experiments, ovariectomy was performed on forty-two female rats. A 2 cm long peritoneal incision was made; the two ovaries were reached and removed by cutting the

oviduct. Surgical silk (Atramat MR) was used for stitches. For SHAM, 20 animals received a silastic tube (Dow Corning SILASTIC Brand 0.24" ID) of 20mm long was cut, filled with sesame oil, sealed with medical grade adhesive and placed subcutaneously in the dorsal thoracic area.

OVXE. Following ovariectomy, twenty-two animals received a silastic tube (Dow Corning SILASTIC Brand 0.24" ID) of 20mm filled with a solution of 180 µg E2-estradiol/mL sesame oil (E2; SIGMA E1024-1G) and sealed with medical grade adhesive, which was implanted subcutaneously in the dorsal thoracic area. This regimen has been shown to produce hormone serum levels that are within the physiological range for at least four weeks (Ström, Theodorsson, and Theodorsson 2008). All animals recovered from the surgery for at least 2 weeks in LD. All following experiments were performed on ZT2 or ZT11. At time of sacrifice just prior to perfusion, blood (200µl) was taken and centrifuged for 5 min at 5000 rpm after which supernatant was frozen at 20°C. Estradiol was measured by ELISA (SE120084; Sigma-Aldrich, MO, USA) according to the manufacturer's protocol.

Immunohistochemistry. Under an overdose of sodium pentobarbital (Sedal-Vet 65 mg/mL) male (n=3) and female rats (n=8-10 per time point, per group) were perfused transcardially with 0.9% saline followed by 4% paraformaldehyde in 0.1M Phosphate buffered saline (PBS; pH 7.5) (Buijs et al. 1993). Following sacrifice, brains were removed, post fixed for 24h, cryoprotected in 30% sucrose for 48-72h, frozen and cut in coronal sections of 30µm at -20°C. Free floating sections were processed for Kisspeptin (Rabbit AB9754, 1:500; EMD Millipore, Billerica, MA, USA), VIP (Rabbit, 1:2000; (R. Buijs et al. 1989), AVP (Rabbit, 1:1000 made in house), CtB (Goat, 1:2000; Sigma-Aldrich Corp., MO, USA) immunohistochemistry using the avidin-biotin-peroxidase procedure followed by 3,3'-diaminobenzidine (DAB) staining (Acosta-Galvan et al. 2011)the biological clock. Consequently, a food-entrained oscillator has been proposed to be responsible for meal time estimation. Recent studies suggested the dorsomedial hypothalamus (DMH). The Kisspeptin anti-body has proven efficacy for Kiss specific staining not showing cross-reactions with other peptides (True et al. 2013; Beale et al. 2014; Hu et al. 2015)raising the possibility that CART plays a role in reproductive inhibition during negative metabolic conditions. The current study characterized CART's regulatory influence on GnRH and kisspeptin (Kiss1).

Analysis SCN CtB injections. In order to visualize projections from the SCN, brain sections were incubated with goat anti-CtB (Sigma-Aldrich) at 4°C overnight. After rinsing, sections were incubated in biotinylated donkey-secondary antibody (Jackson ImmunoResearch, West Grove, PO, USA; 1:400) for 1.5h and then put in an avidin-biotin complex (Vector, Burlingame, CA, USA, 1:500) solution. Staining was performed in a solution of 0.025% diaminobenzidine (DAB), 10% NiNH4SO4 and 0.01% H2O2 (Sigma-Aldrich) in Tris-buffered saline (TBS, 0.01M, pH7.6), for 10 minutes. Sections were mounted on gelatinized

slides, dried, dehydrated with graded solutions of ethanol, soaked in xylene, and finally coverslipped with an Entellan embedding agent (Merck).

In situ hybridization. In situ hybridization (ISH) was carried out as described earlier (Buijs et al. 2017). In short.

Primers. The primers were synthesized by Sigma Aldrich: Forward CAGCTGCTAGCACAGCG and Reverse GGGCAGTGTGTTTCATCCTGA. The reverse primers were labeled in 5' with T7 polymerase promoter. An antisense DIG-RNA label was obtained using 200ng purified PCR fragment for Kiss, 2µL of DIG-RNA labeling mix (Roche; Basel Switzerland), 2 µL T7 RNA and polymerase (Roche), 2 µL 10X concentrated transcription buffer (Roche) and 40U RNaseOUT (Invitrogen; CA, USA), then supplemented with MilliQ diethyl pyrocarbonate (DEPC) water until 20µL. Following 2h 37°C incubation, 1µl RNA polymerase was added for enhanced labeling and incubated for 1h at 37°C. We added 2µl of 0.2M EDTA pH 8.0, 2.5µl 4M LiCl, 75µl pre-chilled (-20°C) 100% ethanol and incubated overnight at -20 °C. Tubes were centrifuged 30 minutes (14.000rpm, 4 °C), supernatant removed and washed with 50µl pre-chilled 70% ethanol then centrifuged 10 minutes (14.000rpm, 4 °C). Supernatant was removed, the tube put on ice to dry for 10 minutes and resuspended in 100µl autoclaved milliQ DEPC water. The labeling efficiency of the DIG-labeling reaction was determined in a spot assay. The RNA dilutions were spotted on Nylon Membrane (Zeta probe, BioRad; CA, USA), incubated with anti-DIG POD (Roche) and developed with DAB. The RNA was compared to labelled control RNA (Roche).

In situ hybridization. Following a short rinse in PBS, sections were fixed in 4% paraformaldehyde in PBS for 5 min and treated with H₂O₂ 3% in PBS-DEPC for 30 min. Sections were washed three times for 5 min in PBS and incubated in PBS-DEPC 0.1% for 30 min at room temperature followed by 10 min post-fixation in 4% paraformaldehyde in PBS. Sections were washed 3 times for 5 min in PBS, rinsed with MilliQ DEPC water and incubated 10 min at room temperature with 0.25% acetic anhydride in 0.1M triethanolamine/ Milli Q DEPC water. After washed 3 times, free floating sections were incubated in hybridization solution (4X SSC, 50% deionized formamide, 1X Denhardtts solution) for 2h at 63°C. The RNA probe (400 ng/ml) in hybridization solution was denatured for 5 min at 85°C and put on ice for 5 min. Sections were submerged in hybridization mix and incubated overnight at 63°C in a moist hybridization oven. They were subsequently rinsed in 5X SSC at room temperature and washed in 5X SSC (50% formamide) for 30 min, 2X SSC (50% formamide) for 20 min, 0.2X SSC (50% formamide) for 20 min at 63°C, 0.2X SSC for 5 min at room temperature, 5 min in buffer 1 (100 ml Tris HCl 1M pH 7.4, 30 ml NaCl 5M and 870 ml milliQ water with DEPC) and incubated with blocking solution 1% (Roche) in buffer 1 for 30 min in order to block nonspecific protein binding. Sections were washed for 5 min in buffer 1 and incubated with anti-DIG-POD (Roche) 1:1000 in buffer 1 for 2h, then washed 3 times in buffer 1 for 5 min and the signal was amplified with TSA plus biotin kit (Perkin Elmer) according to instructions. Finally, sections were incubated

with Streptavidin HRP (Jackson Labs) 1:500 in buffer 1 for 30 min. Following 3 washes in buffer 1 the reaction was revealed with DAB-Nickel and sections were mounted with Entelan as described above.

Quantification. *Kiss*. Pictures of the stained sections were taken using an Axioplan microscope (Zeiss, Jena, Germany) equipped with an Infinity2-2 digital color camera (Lumenera; Ottawa ON, Canada). From each brain, three sections ~90 μ m apart (between bregma -0.90 to -1.20) were taken and the ventrolateral and dorsomedial part of the SCN were bilaterally outlined and analyzed. The mean optic density of *Kiss* fibers in the SCN for both areas were quantified using ImageJ software (NIH; Bethesda, MA, USA), with the background subtracted from the positive staining. For the AVPV, three sections ~60 μ m apart (between bregma 0.12 to -0.12) were taken and bilaterally outlined manually. For the ARC three sections ~150 μ m apart (between bregma -2.20 to -2.70) were taken and bilaterally outlined manually. For both nuclei the mean optic density of *Kiss* fibers were quantified using ImageJ software, with the background subtracted from the positive staining.

Kiss mRNA. The AVPV and ARC were manually outlined as described above; the *Kiss* mRNA-positive cells were automatically detected by means of size and staining threshold detection, using ImageJ software. Further processing was done as stated above. The total number *Kiss* positive cells were counted using ImageJ software.

Statistics. All data were normally distributed and are expressed in mean \pm SE. Statistical comparisons were performed using Prism 6 (GraphPad software, San Diego, CA, U.S.). Data were analyzed using two-way ANOVA with Bonferroni post-hoc test when appropriate. $P < 0.05$ was considered significant. Analyzing mRNA and IHC staining a two-way ANOVA was used for factor group, i.e., OVX vs. OVXE (two levels) and factor time, i.e., ZT2 and ZT11 (2 levels). This was followed by a Bonferroni multi-comparison post-hoc test. $P < 0.05$ was considered significant.

Results

The ventrolateral SCN receives elaborate Kiss innervation. We observed the presence of an elaborate network of very thin Kiss fibers in the SCN of male rats (Fig. 1A) and a similar presence of Kiss fibers in female rats (Fig. 1B). Both males and females showed the same distribution with nearly all Kiss innervation located in the ventrolateral area of the SCN, suggesting possible interaction with VIP neurons. Kiss fibers inside the SCN were of very small caliber with the presence of only a few larger caliber fibers.

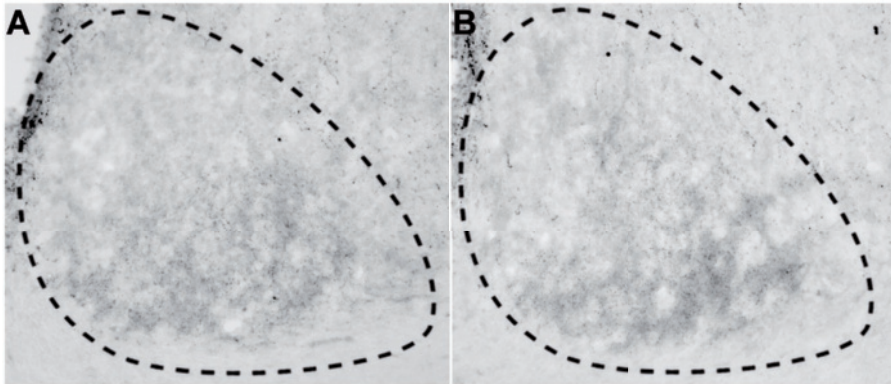


Figure 1. Kisspeptin projections show a specific distribution in the SCN with near to all innervation located in the ventrolateral area of the SCN in both males and females. (A) Representative photomicrograph of the left SCN of a male Wistar rat showing immunohistochemical staining for Kisspeptin. (B) Shows a representative photomicrograph of the left SCN of a female Wistar rat demonstrating a similar distribution of the Kisspeptin staining as with males.

SCN Kiss fibers contact VIP neurons but not AVP neurons. The ventrolateral area of the SCN is known to be involved in receiving both retinal and non-photoc input (Herzog and Schwartz 2002; Buijs et al. 2016) temperature-compensated circadian clocks have been localized to discrete sites within the nervous systems of a number of organisms. In mammals, the master circadian pacemaker is the bilaterally paired suprachiasmatic nucleus (SCN and it contains the majority of VIP expressing neurons (Shinohara et al. 1993; Nakagawa and Okumura 2010). Hence we investigated whether Kisspeptin fibers located in the ventrolateral SCN were contacting VIP neurons. Double labeling of Kiss and VIP immunoreactivity demonstrated Kiss fibers in close apposition to VIP neurons in the ventrolateral part of the SCN (Fig. 2). We did not find any clear appositions to AVP neurons. This is in agreement with the observation that the dorsomedial part of the SCN shows only sparse Kiss innervation.

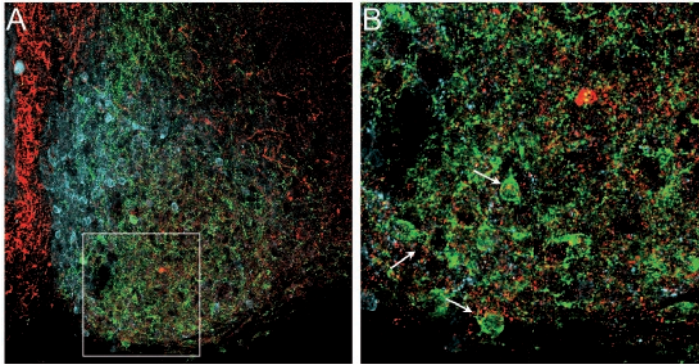


Figure 2. Kisspeptin projections oppose VIP neurons in the ventrolateral part of the SCN but do not oppose AVP neurons. Immunofluorescence staining of SCN sections show Kisspeptin (Red), VIP (Green) and AVP (Turquoise) with Kiss fibers forming direct apposition with VIP but not AVP neurons in the ventrolateral part of the SCN (vISCN). **(A)** A photomicrograph of the SCN with VIP neurons visible in the ventrolateral part of the SCN in green and AVP neurons in the dorsomedial SCN in turquoise. The square outlined in A represents the magnification shown in B. **(B)** Magnification of the vISCN with white arrows illustrating Kiss fibers (Red) apposing VIP neurons (Green).

Kisspeptin neurons of the AVPV and ARC project to the SCN. To assess the origin of the Kiss neuronal input to the SCN, the anterograde and retrograde tracer Cholera toxin B (CtB) was injected into the SCN of 13 female Wistar rats by iontophoresis. In animals where CtB injections were successfully placed within the anatomical borders of the SCN (n=5), we observed retrogradely traced cell bodies in hypothalamic areas known to contain Kiss neurons, i.e., the PeN, AVPV and ARC. Interestingly, especially with injections placed in the ventrolateral part of the SCN we observed co-localization of CtB with Kiss cell bodies in both the AVPV and ARC (Fig. 3). In contrast, injections placed in the dorsal medial part of the SCN hardly demonstrated any co-localization of CtB with Kiss neurons in AVPV or ARC in agreement with the lack of Kiss innervation in the dorsal SCN. Unilateral SCN CtB injections predominantly resulted in ipsilateral presence of cell bodies in the AVPV and ARC (Fig. 3). Besides CtB stained cell bodies, also CtB stained fibers were detected in the AVPV and ARC, confirming previously demonstrated SCN projections to both nuclei (Saeb-Parsy et al. 2000; Gerhold, Horvath, and Freeman 2001; Vida et al. 2010; Williams et al. 2011; Buijs et al. 2017).

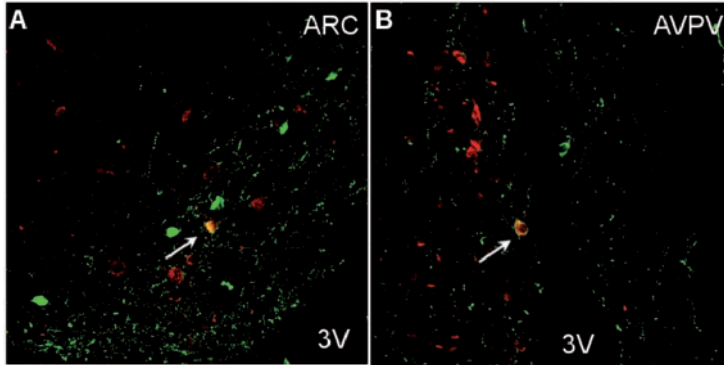


Figure 3. Kisspeptin neurons of the ARC and anterior ventral part of the AVPV project to the SCN. Retrograde tracing following SCN CtB injections (Red), demonstrating ipsilateral colocalisation with Kisspeptin neurons (Green) shown in sections of both the ARC and the AVPV. **(A)** Representative photomicrographs of the ARC and **(B)** AVPV, demonstrating numerous Kiss neurons co-localizing with CtB (Yellow). This was especially notable when the injection was placed in the ventral part of the SCN. 3V, third ventricle.

The SCN demonstrates a diurnal variation in Kiss fiber density. In rodents, the expression of Kiss mRNA in the AVPV under the influence of estrogen, peaks around ZT11, coinciding with the LH surge, and shows a trough around ZT2. Ovariectomized (OVX) animals not receiving estrogen do not show such rhythmicity. However, not all studies were able to see a significant rhythm in AVPV Kiss mRNA expression under high estrogen conditions (Smith et al. 2006; Maeda et al. 2007; Robertson, Clifton, de la Iglesia, et al. 2009; B. L. Smarr, Morris, and De La Iglesia 2012). Based on these past observations we investigated whether the observed Kiss innervation in the SCN could show a diurnal variation at these two time points. Indeed Kiss innervation of the SCN shows differences through time with a denser Kiss innervation at ZT2 as compared to ZT11 (Fig. 4). OVX animals show low Kiss mRNA expression in the AVPV and high expression in the ARC while OVX+estrogen (OVXE) animals show a reverse pattern (Smith et al. 2006). This shift in pattern is thought to be associated with the differential role of the AVPV and ARC conveying the positive and negative feedback effects of sex steroids, although it might not be as straightforward (Helena et al. 2015). We investigated whether the variation in Estrogen dependent Kiss immunoreactivity would be reflected in Kiss innervation density of the SCN, thus providing an indication where this diurnal variation in Kiss innervation might come from. Surprisingly no change in Kiss innervation density could be observed regardless of the animal being either OVX or OVXE. Two-way ANOVA analyses demonstrated no significant interaction ($F_{1, 29}=0.6068$, $P=0.4423$) or effect of group ($F_{1, 29}=0.2618$, $P=0.6128$), showing that SCN Kiss fiber density did not vary due to the estrogen treatment protocol. Yet, notwithstanding the Estrogen treatment, statistical

analysis did show a significant effect of time ($F_{1, 29}=12.39$, $P=0.0014$) with Bonferroni post-hoc analysis indicating a significantly higher fiber density at ZT2 as compared to ZT11 in OVX animals ($P=0.0086$) with OVXE animals missing significance ($P=0.1139$; Fig. 4).

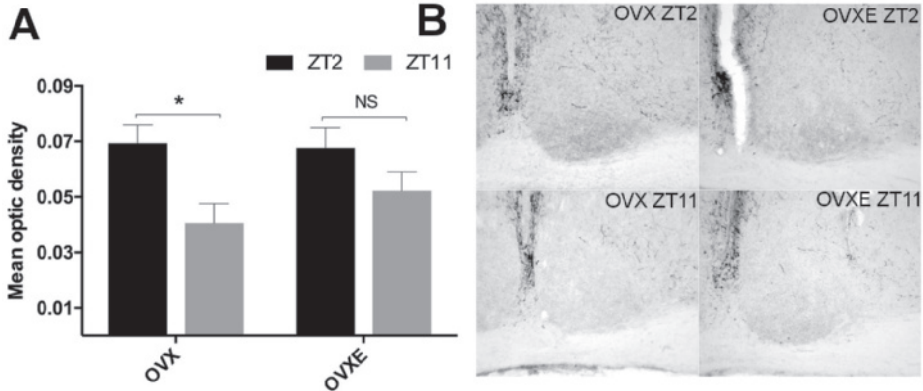


Figure 4. The SCN demonstrates a diurnal variation in Kiss fiber density. (A) Analysis of SCN sections showing Kisspeptin immunoreactivity demonstrated that the ventrolateral part of the SCN has higher fiber density at ZT2 as compared to ZT11 in OVX animals ($P=0.0086$) with a trend for higher ZT2 fiber density in OVXE animals but missing significance ($P=0.1139$) according to 2-way ANOVA Bonferroni post-hoc analysis. Data shown is mean \pm SEM. (B) Representative photomicrographs of SCN sections showing Kisspeptin immunoreactivity in OVX and OVXE animals at ZT2 and ZT11.

The AVPV and ARC demonstrate a diurnal variation in Kiss-ir but not in Kiss-mRNA expression. We next examined whether this diurnal variation in SCN-Kiss fiber density could be related to fluctuations between synthesis and storage/release of Kiss in the AVPV and ARC. Hereto we compared Kiss mRNA and IHC staining of AVPV and ARC sections in OVX and OVXE animals.

For the AVPV, analysis of the number of kiss mRNA positive cells showed a significant effect of group (ANOVA, $F_{1, 24}=85.83$, $P<0.0001$) with a lower Kiss expression in OVX as compared to OVXE animals. We found no significant interaction ($F_{1, 24}=2.541$, $P=0.124$) nor a significant effect of time ($F_{1, 24}=0.9054$, $P=0.3508$), indicating no significant difference in OVX or OVXE animals between the ZT2 or ZT11 time points (Fig. 5A,B). This was confirmed by Bonferroni post-hoc analysis in OVX ($P=0.99$) and OVXE ($P=0.1478$). However, previously there have been reports of significant higher Kiss synthesis at ZT11 (Smith et al. 2006; Robertson, Clifton, de la Iglesia, et al. 2009) suggesting that in OVXE animals indeed the AVPV could have a peak in Kiss mRNA synthesis at ZT11.

Looking at Kiss-ir staining intensity in the AVPV and analyzing the optic density of Kiss-ir in OVX/OVXE animals at ZT2 and ZT11 showed a significant interaction (ANOVA, $F_{1, 21}=6.173$; $P=0.0215$), as well as a significant effect of group ($F_{1, 21}=27.43$;

$P < 0.0001$) and time ($F_{1, 21} = 18.20$; $P = 0.0003$). Bonferroni post hoc analysis did not show a difference in Kiss-ir staining between ZT2 and ZT11 in OVX animals ($P = 0.5255$) but did show a significantly higher optic density at ZT2 as compared to ZT11 in OVXE animals ($P < 0.001$; Fig. 6A,B). When comparing the high ZT2 Kiss-ir staining with low ZT2 mRNA synthesis, as shown in previous reports (Smith et al. 2006; Robertson, Clifton, de la Iglesia, et al. 2009), this could indicate a higher peptide storage/release at ZT2 in the AVPV only in OVXE animals. This diurnal variation in Kiss signaling is only apparent under high estrogen levels in OVXE animals suggesting it to be estrogen dependent.

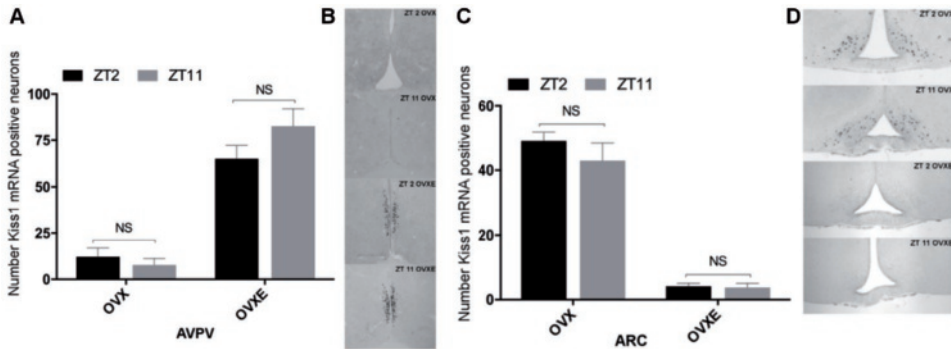


Figure 5. The AVPV and ARC demonstrate a diurnal rhythm in mRNA expression. (A) Bar graphs showing relative levels of Kisspeptin mRNA in the AVPV. A trend towards a peak at ZT11 can be seen in the AVPV of OVXE animals ($P = 0.1478$; 2-way ANOVA Bonferroni post-hoc analysis). Data shown is mean \pm SEM. (B) Representative photomicrographs of AVPV sections showing Kisspeptin mRNA expression in OVX and OVXE animals at ZT2 and ZT11. (C) Bar graphs showing relative levels of Kisspeptin mRNA in the ARC. In the ARC, mRNA expression showed no variation between ZT2 and ZT11 in OVX ($P = 0.2776$) or OVXE ($P = 0.99$) animals according to 2-way ANOVA Bonferroni post-hoc analysis. (D) Representative photomicrographs of ARC sections showing Kisspeptin immunoreactivity in OVX and OVXE animals at ZT2 and ZT11. Data shown is mean \pm SEM.

For the ARC, analysis of the number of Kiss mRNA positive cells showed a significant effect of group (ANOVA, $F_{1, 30} = 231.3$, $P < 0.0001$) demonstrating a higher Kiss expression in OVX as compared to OVXE animals for both time points. There was no significant interaction ($F_{1, 30} = 0.1639$, $P = 0.2103$), nor a significant effect of time ($F_{1, 30} = 0.2020$, $P = 0.1656$; Fig. 5C,D) indicating no significant difference between time-points as was confirmed by Bonferroni post-hoc analysis (OVX, $P = 0.2776$; OVXE, $P = 0.99$). The lower mRNA levels in OVXE animals implies an overall reduced Kiss synthesis in the ARC during high estrogen conditions as compared to low estrogen levels in OVX animals.

Looking at the optic density of Kiss-ir in the ARC, we saw a significant effect of group (ANOVA, $F_1, 29=61.59$; $P<0.0001$), interaction ($F_1, 29=14.23$; $P=0.0007$) and of time ($F_1, 29=6.228$; $P=0.0185$). Bonferroni post-hoc analysis showed a significant peak in Kiss-ir at ZT2 in OVX animals ($P=0.0008$), but not in OVXE animals ($P=0.6334$; Fig. 6C,D). Again, this could indicate a higher peptide storage at ZT2 in the ARC only in OVX animals. Thus, only in OVX animals the ARC demonstrates high ZT2 Kiss-ir staining and in OVXE animals only the AVPV shows high Kiss-ir staining at ZT2 in the AVPV in the ARC. Together with the demonstrated Kiss projections from both the AVPV and ARC to the SCN and the diurnal variation in Kiss-ir intensity in the SCN with higher levels at ZT2 in both OVX and OVXE animals suggests an ARC dominant Kiss activity in the SCN at ZT2 in OVX animals and an AVPV dominant Kiss activity in the SCN in OVXE animals at the same time point.

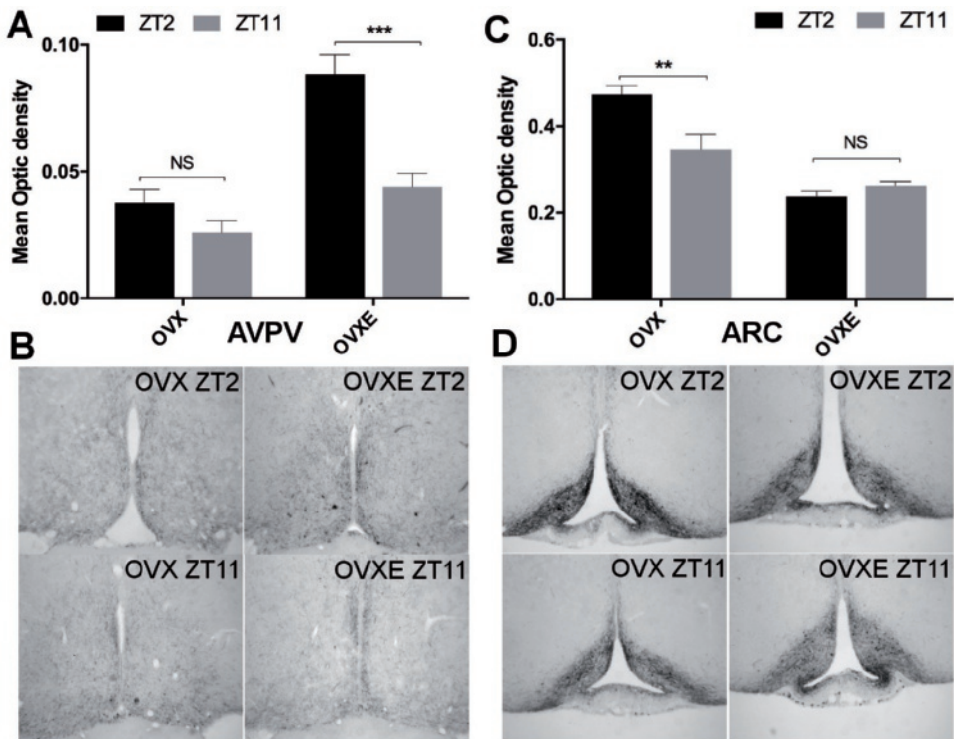


Figure 6. The AVPV and ARC demonstrate a diurnal rhythm in Kiss immunoreactivity. (A) Bar graphs showing optic density analysis of Kiss immunoreactivity demonstrate a peak in staining intensity at ZT2 in the AVPV of OVXE animals ($P<0.0001$), but not in OVX animals ($P>0.05$), according to 2-way ANOVA Bonferroni post-hoc analysis. Data shown is mean \pm SEM. (B) Representative photomicrographs of the AVPV at ZT2 and ZT11 in OVX and OVXE animals showing Kiss staining intensity. (C) Bar graphs showing optic density analysis of Kiss immunoreactivity in sections of the ARC. The ARC demonstrated a peak at ZT2 in OVX animals ($P=0.0008$) not seen in OVXE ($P=0.6334$) according to 2-way ANOVA Bonferroni post-hoc analysis. Data shown is mean \pm SEM. (D) Representative photomicrographs of the ARC at ZT2 and ZT11 in OVX and OVXE animals showing Kiss staining intensity.

Discussion

For long it has been known that the SCN is essential for the generation and timing of the LH surge in rodents (Brown-Grant and Raisman 1977; Samson and McCann 1979). More recently it was demonstrated that SCN vasopressin neurons project to AVPV Kiss neurons relaying a circadian signal enabling a timed estrogen dependent LH surge (Williams et al. 2011). The present findings show that not only does the SCN mediate circadian signals to different hypothalamic nuclei timing the reproductive cycle, but the SCN also receives signals from AVPV and ARC Kiss efferents.

For the first time, we provide evidence of the existence of temporal feedback circuits between Kiss expressing neurons in the AVPV and ARC and the SCN.

We demonstrate that, in both male and female Wistar rats, the SCN receives elaborate innervation from Kiss fibers targeting predominantly the ventrolateral SCN. Through retrograde CtB-tracer injections into the SCN we find that Kiss cell bodies in both the AVPV and ARC of female rats project to the SCN. Our observations show that in spite of major opposing changes in Kiss-ir intensity in the ARC and AVPV after OVX or OVXE, the diurnal pattern of SCN innervation seems to remain balanced towards a higher staining density at ZT2 as compared to ZT11, though missing significance in OVXE animals. This could indicate that the majority of the Kiss feedback comes from the ARC since it also lacks a significant difference between ZT2 and ZT11 values in OVXE animals. The importance of these observations is that in addition to the confirmed circadian input of SCN vasopressin neurons to Kiss neurons in Wistar rats, these Kiss neurons appear to return a diurnal signal to SCN VIP neurons. The temporal constancy of this feedback seems to be vital, as it is independent of estrogen levels in the animal.

The observation that male and female rats show a similar distribution of Kiss innervation suggests the presence of a general, non-sex-dependent function of Kisspeptin in the SCN. Neurons in the vlSCN are best known for receiving light input and to convey this synchronizing photic cue to the rest of the SCN, with VIP being critical in maintaining an autonomous rhythm and thus critical for the circadian drive of physiological functions (Shinohara et al. 1993; Harmar 2003; An et al. 2013; Hughes, Guilding, and Piggins 2011) by enzyme immunoassay, daily and circadian patterns of the concentrations of three peptides, which are located in the ventrolateral subdivision of the suprachiasmatic nucleus (SCN. VIP neurons are also target of peripheral cues adjusting SCN rhythmicity to environmental cycles (Aton et al. 2005; Vosko et al. 2007; Nakagawa and Okumura 2010; An et al. 2011)synchrony between neurons, or both. We found that *Vip*^{-/-}. The presently found Kiss projections to the vlSCN could provide a novel feedback pathway for the circadian regulation of reproduction and functions that are associated with metabolic control (Hussain et al 2015).

Kisspeptin shows diurnal oscillations in the AVPV, ARC and SCN. In rodents, the expression of Kiss mRNA in the AVPV peaks at approximately ZT11 together with the end of the LH surge, though this peak in Kiss mRNA is subtle at best (Smith et al. 2006; Maeda et al. 2007; Robertson, Clifton, de la Iglesia, et al. 2009; Smarr, Morris, and De La Iglesia 2012). In the AVPV, the daily peak of clock gene *Per1* expression runs in tandem with Kiss mRNA expression (Smarr, Gile, and de la Iglesia 2013). This circadian rhythm is likely driven by the SCN since AVP efferents directly appose Kiss neurons in the AVPV in hamsters and mice (Vida et al. 2010; Williams et al. 2011). This could clarify the circuit through which SCN AVP release peaks together with *c-fos* activity in the AVPV just before the LH surge and subsequent ovulation (Kalsbeek et al. 1995; Robertson, Clifton, De La Iglesia, et al. 2009). Together with our observation that SCN projecting Kiss efferents appose VIP but not AVP neurons, dismisses the existence of a “simple” feedback circuit.

Through immunohistochemistry and *in situ* hybridization we find diurnal variation in the storage/release and synthesis of Kiss in the AVPV and ARC. This variance with high Kiss-ir staining at ZT2 in the AVPV and ARC, of OVXE and OVX animals respectively, coincides with diurnal variation in Kiss-ir we observed in the SCN of OVX and OVXE animals. These findings, together with data found in literature on the temporal synthesis of Kiss with a peak at ZT11 (Smith et al. 2006; Maeda et al. 2007; Robertson, Clifton, de la Iglesia, et al. 2009; B. L. Smarr, Morris, and De La Iglesia 2012) suggest that Kiss accumulates in cell bodies and fibers at ZT2 while it is released at around ZT11 in the target areas of the Kiss neurons (Williams et al. 2011). However, there are multiple mechanisms regulating Kiss-ir intensity in the ARC (Lehman, Hileman, and Goodman 2013) and two time-points are arguably insufficient for interpreting changes based on immunohistochemistry thus further investigation is needed.

It is surprising that in spite of the enormous changes in Kiss synthesis in AVPV and ARC under the influence of estrogen, the diurnal variation of innervation density in the SCN remains stable and independent of estrogen levels. Considering the fact that the SCN only sparsely expresses estrogen receptors with specifically VIP neurons expressing none (Vida et al. 2008), a possible mechanism through which the SCN can receive positive and negative estrogen feedback is via its Kiss innervation shown in this present study. This would imply that the SCN receives distinct Kiss input from the AVPV and ARC whereby differences in co-transmitters released from these Kiss expressing terminals either from AVPV or ARC might transmit a differential signal.

For example, it is known that the majority of ARC Kiss neurons uniquely co-localize Dynorphin and Neurokinin B (Lehman, Coolen, and Goodman 2010; True et al. 2011; Weems et al. 2016) KNDy neurons are capable of transmitting both stimulatory (Kisspeptin and Neurokinin B) and inhibitory (Dynorphin) signals (Lehman, Coolen, and Goodman 2010; Helena et al. 2015), distinguishing KissARC signaling from KissAVPV stimuli. KNDy neurons are also likely involved in relaying nutritional status and stress on the reproductive system (Lehman, Coolen, and Goodman 2010). We show that the SCN

is an important feedback target for both KissAVPV and KNDy neurons. Furthermore, Kiss mediated sex steroid feedback on SCN neuronal activity could explain why estrogen has an effect on the circadian rhythm of activity in free running animals (Morin, Fitzgerald, and Zucker 1977; Albers 1981). On the day of estrous, hamsters and rats show a phase advance in locomotor activity (Moline and Albers 1988) and running-wheel activity is increased on the day of estrous by the effects of estrogen on the AVPV (Ogawa et al. 2003) little is known about the separate roles of two types of estrogen receptors, ERalpha and ERbeta, both of which are expressed in mPOA neurons. In the present study the effects of continuous estrogen treatment on running wheel activity were examined in male and female mice specifically lacking either the ERalpha (alphaERKO). Similarly, it is well established that the absence of estrogen importantly alters rhythmic behavior and physiology (Thomas and Armstrong 1989). Ovariectomy increases and, by extension, peripheral sex steroids decrease the coupling between the control of body temperature rhythms and sleep (Li and Satinoff 1996). The different responses of the AVPV and ARC to estrogen and their diurnal varying input to the SCN might allow the SCN to adjust its output accordingly, without having to receive direct sex steroid input.

A possible role for KissAVPV feedback to the SCN could be to synchronize ovulation with the temporal cycle of reproductive behavior. Kiss projections could serve to stimulate VIP-containing neurons that form appositions with GnRH neurons in the POA (van der Beek et al. 1997). Hereby, VIP can stimulate GnRH neurons, which are dependent on both estrogen, time of day and Kisspeptin input (Christian and Moenter 2008). VIP neurons have reciprocal connections with RFRP-3 neurons in the DMH (Gibson et al. 2008; Acosta-Galvan et al. 2011) the biological clock. Consequently, a food-entrained oscillator has been proposed to be responsible for meal time estimation. Recent studies suggested the dorsomedial hypothalamus (DMH, we demonstrate that similar to RFRP-3 neurons Kiss shows projections to the ventral part of the SCN where they appose VIP neurons innervation. This indicates a complex role for the SCN and VIP in regulating the circadian-timed preovulatory LH surge under the influence of inhibitory and excitatory sex-steroid feedback.

The ARC is the hypothalamic metabolic integration center and fulfills an important role in the metabolic control of reproduction (Hill, Elmquist, and Elias 2008; Popa, Clifton, and Steiner 2008). As such, fasting has been shown to induce a pronounced decrease in estrogen (Otukonyoung 2000) and thus a stronger Kiss signal from the ARC. Hence we suggest that the ARC, while under the influence of fluctuating estrogen levels, could relay an integrated metabolic and estrogen signal to the SCN. We find KissARC neurons project to the SCN but it has also been demonstrated that KissARC neurons project to KissAVPV neurons where they have direct synaptic apposition to GnRH terminals (True et al. 2011; Goodman et al. 2013; Yip et al. 2015) such as kisspeptin (Kiss1). We show that next to the AVPV, the SCN is a possible target through which the ARC is able to affect, and possibly reduce the LH surge thus suppressing ovulation in negative

energy balance conditions (Matsuzaki et al. 2011) which is the product of the *kiss1* gene and its receptor *kiss1r*, have emerged as the essential gatekeepers of reproduction. The present study used gonadally intact female rats to evaluate fasting-induced suppression of the KiSS-1 system of anteroventral periventricular nucleus (AVPV).

Similarly, we previously demonstrated the importance of a synchronized interaction between the SCN and multiple hypothalamic nuclei. For temperature rhythmicity, we demonstrated in rats that for an adequate temperature decrease at dawn, SCN AVP output needs to coincide with temporal α -MSH ARC output in the MnPO (Guzmán-Ruiz et al. 2015) daily changes in body temperature (T_b). In addition we have shown that the ARC-SCN interaction is essential for the organization of multiple physiological rhythms (Buijs et al 2017). The demonstrated KissARC and KissAVPV input to the SCN offers an interesting illustration of how the circadian system is dependent on different neuronal inputs to time physiological functions associated with reproduction.

Conclusion

It has long been known that the SCN is essential for the temporal organization of reproduction but more recently a pivotal role for Kisspeptin in the regulation of reproduction has emerged (Williams and Kriegsfeld 2012; Simonneaux and Bahougne 2015) estradiol-sensitive neural circuits that converge to optimally drive hypothalamo-pituitary-gonadal (HPG). Still much remains to be uncovered about the exact role of the SCN and that of Kisspeptin in the timing of the reproductive cycle. Here we demonstrate the existence of a temporally organized Kiss feedback pathway from both the AVPV and ARC to the SCN. This feedback circuit possibly mediates reproductive and metabolic feedback whereby both peak and trough estrogen levels are incorporated. This adds to the growing evidence that the SCN is an integral part of a hypothalamic multi-oscillatory network synchronized through different feedback pathways. We show the AVPV-SCN-ARC Kisspeptin systems are additional feedback circuits to be considered, adding to the complexity of circadian regulation of physiology and reproductive function.

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