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Revised

Sex- and sub region-dependent modulation of arcuate kisspeptin neurons by vasopressin and vasoactive intestinal peptide

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Short title: Neuropeptide modulation of arcuate kisspeptin neurons

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Abstract

A population of kisspeptin neurons located in the hypothalamic arcuate nucleus (ARN) very likely represent the gonadotrophin-releasing hormone pulse generator responsible for driving pulsatile luteinizing hormone secretion in mammals. As such, it has become important to understand the neural inputs that modulate the activity of ARN kisspeptin (ARN^{KISS}) neurons. Using a transgenic GCaMP6 mouse model allowing the intracellular calcium levels ($i[Ca^{2+}]$) of individual ARN^{KISS} neurons to be assessed simultaneously, we examined whether the circadian neuropeptides vasoactive intestinal peptide (VIP) and arginine vasopressin (AVP) modulated the activity of ARN^{KISS} neurons directly. To validate this methodology, we initially evaluated the effects of neurokinin B (NKB) on $i[Ca^{2+}]$ in kisspeptin neurons residing within the rostral, middle and caudal ARN subregions of adult male and female mice. All experiments were undertaken in the presence of tetrodotoxin and ionotropic amino acid antagonists. NKB was found to evoke an abrupt increase in $i[Ca^{2+}]$ in 95-100% of kisspeptin neurons throughout the ARN of both sexes. In marked contrast, both VIP and AVP were found to primarily activate kisspeptin neurons located in the caudal ARN of female mice. Whereas 58 and 59% of caudal ARN kisspeptin neurons responded to AVP and VIP, respectively, in female mice, only 0-8% of kisspeptin neurons located in other ARN subregions responded in females and 0-12% of cells in any subregion in males ($p<0.05$). These observations demonstrate unexpected sex differences and marked heterogeneity in functional neuropeptide receptor expression amongst ARN^{KISS} neurons organized on a rostro-caudal basis. The functional significance of this unexpected influence of VIP and AVP on ARN^{KISS} neurons remains to be established.

Introduction

The pulsatile release of luteinizing hormone (LH) is critical for fertility. Adult males exhibit an LH pulse approximately every 3h while females show variable LH pulse rates ranging from a pulse every hour in the follicular phase to one every 3h in the luteal phase of the cycle (1). However, a variety of internal and external factors can drive LH pulse frequency outside this normal range to suppress fertility. For example, the high LH pulse frequency of females with polycystic ovary syndrome and slow pulsatility observed in hypothalamic amenorrhea are often associated with infertility (2-4).

Studies examining a range of mammalian species over recent years have indicated that kisspeptin neurons co-expressing neurokinin B and dynorphin (KNDy neurons) located in the hypothalamic arcuate nucleus are responsible for generating the pulsatile pattern of gonadotropin-releasing hormone (GnRH) release that drives pulsatile LH secretion (1, 5). As such, the arcuate nucleus KNDy or kisspeptin (ARN^{KISS}) neurons have become a focal point for investigators wanting to understand how different physiological and pathophysiological factors influence LH pulse frequency (1, 6, 7).

Although the definition and role of circadian inputs to the preoptic population of kisspeptin neurons has received much attention (8, 9), no information exists regarding the potential circadian regulation of the ARN^{KISS} neurons. Studies in humans have identified sleep-wake variations in LH pulse frequency (10-12) raising the possibility that some form of circadian input may also be directed at the ARN^{KISS} neurons. To begin to investigate this possibility, we have examined whether ARN^{KISS} neurons in the mouse express functional receptors for arginine vasopressin (AVP) and vasoactive intestinal peptide (VIP), the two major neuropeptidergic outputs from the suprachiasmatic nucleus (SCN). Studies in rats and mice have identified receptors for both of these neuropeptides in the arcuate nucleus (13-15) and SCN neurons are known to project to and modulate the activity of ARN neurons in the rat (16, 17). However, we note that many neural populations expressing VIP and AVP are also found outside the SCN and may conceivably have a role in regulating LH secretion.

To examine the potential effects of AVP and VIP, we have established a transgenic GCaMP6 calcium imaging approach that enables the effects of neurotransmitters on adult ARN^{KISS} neurons to be assessed in the acute brain slice. To ensure that we measure direct effects of these neuropeptides on kisspeptin neurons, intracellular calcium concentrations ($[Ca^{2+}]i$) were assessed in the presence of amino acid receptor antagonists and tetrodotoxin (TTX).

Materials and Methods

Experimental animals

Mice were generated by crossing *Kiss1*-Cre$^{+/-}$ (18) and homozygous floxed GCaMP6f (Ai95(RCL-GCaMP6f)-D)(19) lines to generate mixed background *129S6Sv/Ev C57BL6 Kiss1-Cre::GCaMP6f-lox-STOP-lox* (*Kiss1::GCaMP6*) mice. Mice were group-housed under conditions of controlled temperature (22±2^{0}C) and lighting (12-hour light/12-hour dark cycle (lights on at 6:00h and off at 18:00h) with *ad libitum* access to food and water. The University of Otago Animal Ethics Committee approved all animal experimental protocols.

Immunohistochemistry

Four adult female *Kiss1::GCaMP6f* mice were ovariectomized under Halothane anesthesia and 3-weeks later anesthetized and perfused through the heart with 4% paraformaldehyde, phosphate-buffered saline for free-floating dual immunofluorescence histochemistry as reported previously (20, 21). Mice were ovariectomized so as to increase the level of kisspeptin peptide in ARN neurons to improve immunohistochemical detection. Primary antisera raised against GFP (rabbit 1:5,000, Invitrogen; RRID:AB_221570) and kisspeptin (sheep 1:1,000, AC053 gift of Alain Caraty, Nouzilly, France)(22) were used to increase the GCaMP6 signal and detect kisspeptin, respectively. Secondary antisera were biotinylated donkey anti-sheep immunoglobulins (1:200, Jackson ImmunoResearch Labs, PA) followed by streptavidin-568 (1:200, Molecular Probes), and donkey anti-rabbit-488 (1:200, Jackson), respectively. Dual-fluorescence images were captured on a NikonA1+

inverted confocal microscope. Two sections at each of the rostral, middle and caudal levels of the ARN were analyzed in each mouse by counting the total number of cells that expressed GFP (GCaMP6) and/or kisspeptin.

Calcium imaging

The i[Ca^{2+}] of multiple ARN^{KISS} neurons was monitored simultaneously in acute brain slices using a methodology previously established for the preoptic kisspeptin neurons (23). Coronal brain slices (250μm-thick) containing the rostral, middle and caudal regions of the ARN were prepared from adult male and diestrous-stage female *Kiss1::GCaMP6f* mice between 10:00-11:00h (N=4 for each sex, region and neuropeptide) and constantly perfused (1mL/min) with 30^0C, 95%O_2/5%CO_2 equilibrated, artificial cerebrospinal fluid (aCSF) comprised of (mM) NaCl 120, KCl 3, $NaHCO_3$ 26, NaH_2PO4 1, $CaCl_2$ 2.5, $MgCl_2$ 1.2 and glucose 10. To ensure that only direct neuropeptide responses were recorded from ARN^{KISS} neurons, the aCSF contained TTX (0.5 μM) and the ionotropic $GABA_A$ and glutamate receptor antagonists picrotoxin (100 μM), CNQX (10 μM), and AP5 (40 μM) at all times (all sourced from Tocris Biosciences). Slices were placed under an upright Olympus BX51W1 microscope and multiple individual cells in a plane of focus visualized through a 40x immersion objective using a xenon arc light source (300 W, filtered by a GFP filter cube (excitation 470-490 nm, Chroma)) and a DG-4 shutter (Sutter Instruments) providing 100ms duration light at 2Hz. Epifluorescence (495 nm long pass and emission 500-520 nm) was collected using a Hamamatsu ORCA-ER digital CCD camera.

The effects of neuropeptides on ARN^{KISS} neuron GCaMP6f fluorescence were assessed by measuring basal fluorescence over a 4 min period and then adding the test neuropeptide to the aCSF for a two-min period before switching back to aCSF only. Regions of interest over individual, non-overlapping, and in-focus fluorescent somata were selected and analyzed using ImageJ software and custom R scripts. Individual cells were considered to have responded if they exhibited an increase in fluorescence during the 2-min test period that was greater than their mean baseline level + 2 standard deviations (SD) derived from basal recordings. To accommodate for the gradual decline in fluorescence levels that occurs over the test period, the fluorescence levels over the first 4-min basal period were divided into the first (b1) and second (b2) two-minute basal recording periods and an adjusted baseline (*F*) for the 2-min stimulation period set at = b2-(b1-b2). For visualizing data, values are presented as relative fluorescence changes using $\frac{\Delta F}{F} = \frac{Ft - F}{F} * 100$ where F is the adjusted baseline and F_t is the fluorescence measured.

To assess effects of neuropeptides on ARN^{KISS} neuron located at different rostro-caudal levels of the ARN, four rostral, four middle and four caudal slices from four separate mice were tested with each neuropeptide. The different rostrocaudal levels were determined by the distinctive topography of kisspeptin neurons in each area and the shape of the median eminence (Fig.1). Each slice received only one test. Results are reported as number of cells examined (n) and numbers of slices or mice (N). The effects of neurokinin B (NKB, 50nM), AVP (300nM) and VIP (1µM) (Tocris Bioscience) were examined. Prior studies in the laboratory have shown that these concentrations are effective in activating the firing of kisspeptin, GnRH and other neurons in acute brain slices (23-25). Statistical analysis comparing between sexes and ARN subregions was undertaken with two-way ANOVA and *post-hoc* Tukey tests.

Results

Expression of GCaMP6 in arcuate nucleus kisspeptin neurons

The distribution of GCaMP6-expressing cells throughout the rostro-caudal extent of the ARN in *Kiss1::GCaMP6f* mice (Fig.1A) was identical to that reported for kisspeptin neurons (21) with the largest numbers of cells detected in the caudal ARN (Table). Dual-label immunofluorescence demonstrated that 88-95% of GCaMP6 cells expressed kisspeptin and virtually all (99%) kisspeptin neurons contained GCaMP6 (Fig.1B-D, Table).

GCaMP6 calcium imaging

The GCaMP6 imaging enabled the fluorescence levels of 8-25 ARN^{KISS} neurons to be evaluated simultaneously in each brain slice. Initial control experiments in slices from four diestrous female mice showed that 53 of the 54 recorded cells (N=4 slices) exhibited a stable baseline level of fluorescence that gradually declined over time (Fig.2). Only one of the 54 cells was found to show spontaneous fluctuations (Fig.2).

NKB activates all kisspeptin neurons in the ARN of males and females

To test the validity of this preparation, we first examined the effects of NKB as this neuropeptide has been reported to have direct stimulatory effects on the firing of nearly all ARN^{KISS} neurons in male mice (24, 26, 27). Exposure to 50nM NKB was found to evoke an increase in fluorescence levels in essentially all GCaMP6-expressing cells located throughout the ARN (Fig.3A-C). Responses could be abrupt or take over 1 min to occur with baseline levels typically restored to normal within 4-5 min of the response (Fig.3C). In four diestrous *Kiss1::GCaMP6f* female mice, 37 rostral, 100 middle, and 65 caudal cells were tested in four brain slices from each region with 100% of cells exhibiting a change in fluorescence signal that was > basal + 2SD. The same result was found for male mice (N=4) with 28/28 rostral, 75/79 (95%) middle and 84/87 (97%) caudal cells responding to NKB (Fig.3D). Basal fluorescence signals in males were the same as those observed for females with only occasional evidence of spontaneous calcium transients. No significant differences were detected between any regions or sexes (p>0.05, Two-way ANOVA).

VIP activates caudal arcuate kisspeptin neurons in a sexually dimorphic manner.

The administration of 1μM VIP was found to have no effects upon GCaMP6 fluorescence in 35 kisspeptin neurons (N=4) located in the rostral-aspect of the ARN in female *Kiss1::GCaMP6f* mice (Fig.4A). While the middle ARN (N=4) was similar with only 2/58 (3%) cells responding, 46/80 (58%) kisspeptin neurons in the caudal ARN (N=4) were activated by VIP (Fig.4A,B; p<0.05 compared with other subregions, Two-way ANOVA, post-hoc Tukey's tests). These responses could take up to 2 min to initiate and typically exhibited a fluctuating profile before returning to baseline up to

10 min later (Fig.4B). Although a relationship between the basal level of fluorescence and the magnitude of any response existed, responding cells could not be predicted by their basal level of fluorescence. In male *Kiss1::GCaMP6f* mice, no cells in the rostral (n=32, N=4) or middle aspects of the ARN (n=71, N=4) responded to VIP with only 3/57 (6%, N=4) kisspeptin neurons in the caudal stimulated ($p<0.05$ compared to females, Two-way ANOVA, post-hoc Tukey's tests; Fig. 4C).

AVP activates caudal arcuate kisspeptin neurons in a sexually dimorphic manner.

Exposure to 300nM AVP had minimal effects on fluorescence levels in kisspeptin neurons located in the rostral (2/34 cell responded, 6%, N=4) and middle (5/66 cells, 8%, N=4) aspects of the ARN but increased intracellular calcium levels in 46 of 78 (59%, N=4) cells located in the caudal ARN (Fig.5A; $p<0.05$ compared with other subregions, Two-way ANOVA, post-hoc Tukey's tests). Responses evoked by AVP were typically immediate upon entry of AVP into the bath but short-lived, sometimes terminating during the application period (Fig.5B). In male *Kiss1::GCaMP6f* mice, 4-12% of kisspeptin neurons responded throughout the rostro-caudal extent of the ARN; 1/24 rostral (4%), 5/67 middle (8%) and 10/81 caudal (12%)(N=4 each, Fig.5C). The numbers of kisspeptin neurons responding to AVP were significantly different in the caudal ARN of females compared to other subregions and males ($p<0.05$ compared to females, Two-way ANOVA, post-hoc Tukey's tests; Fig. 5C).

Discussion

We report here the unexpected observation that kisspeptin neurons located at different rostro-caudal locations within the ARN can express different functional neuropeptide receptors and, further, that this is sexually dimorphic. Whereas all ARN^{KISS} neurons in both males and females express tachykinin receptors activated by NKB, the kisspeptin neurons activated by AVP and VIP are located primarily within the caudal ARN and exhibit a marked female-dominant sex difference. These observations highlight the functional heterogeneity and striking sexually dimorphic nature of the ARN^{KISS} neuron population and, further, indicate that this may be organized in a rostro-caudal topographic manner.

The *Kiss1::GCaMP6f* mouse line was found to provide high fidelity targeting of GCaMP6 to the ARN^{KISS} neuron population with essentially 100% of kisspeptin neurons expressing GCAMP6 and these cells representing 88-95% of all GCaMP6 neurons located in the ARN. As such, this mouse line provides a good model preparation for examining the direct responses of *ex-vivo* ARN^{KISS} neurons to putative neurotransmitters and neuropepitdes. Nevertheless, the approach has caveats, principally being that inhibitory effects of transmitters, or receptor activation that does not directly or indirectly modulate i[Ca^{2+}], will not be revealed. Thus, false negatives may occur but positive responses will be indicative of the presence of cognate receptors for the transmitter examined.

Prior electrophysiological brain slice studies have shown that 90-100% of ARN^{KISS} neurons are activated by NKB or tachykinin receptor agonists in male mice (24, 26, 27). In good agreement, we find that essentially 100% of ARN^{KISS} neurons in intact male mice respond directly to NKB with elevated i[Ca^{2+}] and now extend this to demonstrate that this is also the case in diestrous female mice. Prior studies using dual-label *in situ* hybridization have reported that 75-100% of ARN^{KISS} neurons located in the middle aspects of the ARN express *Tacr3* transcripts in female mice (27, 28). By targeting recordings to the rostral, middle and caudal aspects of the ARN we are able to demonstrate remarkable consistency in the functional expression of NKB-activated receptors by ARN^{KISS} neurons in both males and females. As a sub-population of ARN^{KISS} neurons will be the GnRH pulse generator, these observations are in good agreement with *in vivo* data showing that intracerebroventricular administration of senktide, an NK3R agonist, activates LH secretion in both intact male and diestrous-stage female mice (29).

In striking contrast to the effects of NKB, both AVP and VIP exerted subregion- and sex-dependent effects on ARN^{KISS} neuron i[Ca^{2+}]. Whereas 58% of caudal ARN^{KISS}

neurons responded to VIP in females only 6% were activated in male, and essentially no cells were stimulated in the rostral or middle regions of the ARN in either sex. Similarly, 58% of caudal ARN^{KISS} neurons were activated by AVP compared with only 4 -12% of neurons in other regions or in males. This indicates that female caudal ARN^{KISS} neurons preferentially express functional receptors for VIP and AVP. Although not addressed in this study, it is possible that changes in ARN^{KISS} neuron sensitivity to AVP and VIP may occur with postnatal development or across the estrous cycle in females.

Similar numbers of caudal ARN^{KISS} neurons are activated by AVP and VIP in female but they evoke very different profiles of changing $i[Ca^{2+}]$. The molecular identities of the receptors activated are not known although VIP at 1μM concentrations would be expected to activate both type 1 (VPAC1) and type 2 (VPAC2) VIP receptors in addition to the pituitary adenylate cyclase-activating peptide (PACAP) receptor (30). Recent studies have shown that PACAP neurons located in the premammillary nucleus project to ARN^{KISS} neurons and can directly activate a sub-population of caudal ARN neurons in female mice (31). Hence, it is possible that PACAP is the endogenous ligand for VIP receptors expressed by ARN^{KISS} neurons. Recent transcriptome-based cell sorting strategies failed to identify any AVP or VIP/PACAP receptor transcripts in pools of ARN^{KISS} neurons from young female or mixed male/female mice (32, 33). Given the sex-dependent and highly regionalised nature of AVP and VIP receptor expression revealed here, future transcriptomic studies of ARN^{KISS} neuron will need to take potential subregion and sex differences into consideration.

The sex- and region-specific nature of AVP and VIP effects identified here would not support the concept of a generalized direct circadian regulation of ARN^{KISS} neurons by these neuropeptides. Studies have found day-night differences in LH pulse frequency in both human males and females (10-12) although their dependence on circadian cues, as opposed to environmental influences such as sleep and stress, has been challenged (34). Further, while studies in rodents have identified circadian-like changes in LH secretion in peripubertal females, it has remained unclear whether this was related to pulse or surge modes of LH secretion (35). Hence, it is possible that there is no direct or substantial circadian modulation of the different ARN^{KISS} neuron sub-populations. Instead, VIP/PACAP and AVP inputs to ARN^{KISS} neurons may originate from neural populations located outside the suprachiasmatic nucleus, as has recently been demonstrated for PACAP neurons of the premammilary nucleus (31). Interestingly, preliminary viral retrograde tracing data indicate that vasopressin neurons in the supraoptic nucleus project to ARN^{KISS} neurons (Yeo & Colledge, unpublished).

The function of neuropeptidergic inputs directed at caudal ARN^{KISS} neurons in the female can only be speculated upon at present. Sex differences in ARN^{KISS} morphology and function have been documented (20, 36) but the extent to which this depends on sexually differentiated inputs is unknown. Further, the functions of AVP/VIP-sensitive ARN^{KISS} neurons are unknown and may even be unrelated to the regulation of GnRH neurons. Indeed, tract tracing studies have shown that caudal ARN^{KISS} neurons project to multiple limbic brain region in the mouse (37). On the other hand, recent optogenetic studies have indicated that the ARN^{KISS} neuron pulse generator may be located in the middle-caudal aspects of the nucleus (21) although no further features or markers of this population have been identified as yet. One intriguing speculation is that SCN inputs to ARN^{KISS} neurons may modulate the LH surge. Although there is little evidence for the ARN to be involved in the timing of the LH surge (38), recent studies have suggested that the ARN^{KISS} neurons may be involved in regulating the amplitude of the LH surge (39, 40), potentially through direct projections to preoptic area kisspeptin neurons (41).

In summary, these studies reveal marked sex- and subregion-specific effects of two neuropeptides on ARN^{KISS} neurons. These observations reinforce the concept of functional heterogeneity amongst the ARN^{KISS} neuron population. Alongside other evidence (21, 37), it seems that this heterogeneity may, in part, be organized on a rostro-caudal basis within the ARN of the mouse. In contrast, we find that the response of ARN^{KISS} neurons to NKB is extremely uniform impacting upon essentially all kisspeptin neurons throughout the nucleus in both males and females. The functional relevance of this heterogeneity with respect to pulse generation as well as other functions of the ARN^{KISS} neuron population awaits elucidation. More specifically, the surprising observation of sexually dimorphic and region-specific AVP and VIP signaling within the ARN^{KISS} neuron population raises intriguing questions as to the roles of these neuropeptides within the ARN.

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References

1. Herbison AE. The gonadotropin-releasing hormone pulse generator. Endocrinology. 2018: *Endocrinology*, en.2018-00653.
2. Jayasena CN, Franks S. The management of patients with polycystic ovary syndrome. Nature reviews Endocrinology. 2014; 10: 624-636.
3. Burt Solorzano CM, Beller JP, Abshire MY, Collins JS, McCartney CR, Marshall JC. Neuroendocrine dysfunction in polycystic ovary syndrome. Steroids. 2012; 77: 332-337.
4. Fourman LT, Fazeli PK. Neuroendocrine causes of amenorrhea--an update. J Clin Endocrinol Metab. 2015; 100: 812-824.
5. Goodman RL, Okhura S, Okamura H, Coolen LM, Lehman MN. KNDy hypothesis for generation of GnRH pulses; evidence from sheep and goats. In: Herbison AE, Plant TM, eds. *The GnRH neuron and its control*. Hoboken, NJ, USA: John Wiley and Sons Ltd 2018: 289-324.
6. Herbison AE. Control of puberty onset and fertility by gonadotropin-releasing hormone neurons. Nature reviews Endocrinology. 2016; 12: 452-466.
7. Yeo SH, Colledge WH. The Role of Kiss1 Neurons As Integrators of Endocrine, Metabolic, and Environmental Factors in the Hypothalamic-Pituitary-Gonadal Axis. Frontiers in endocrinology. 2018; 9: 188.
8. Kriegsfeld LJ. Circadian regulation of kisspeptin in female reproductive functioning. Adv Exp Med Biol. 2013; 784: 385-410.
9. Simonneaux V, Bahougne T. A Multi-Oscillatory Circadian System Times Female Reproduction. Frontiers in endocrinology. 2015; 6: 157.
10. Spratt DI, O'Dea LS, Schoenfeld D, Butler J, Rao PN, Crowley WF, Jr. Neuroendocrine-gonadal axis in men: frequent sampling of LH, FSH, and testosterone. Am J Physiol. 1988; 254: E658-666.
11. Boyar R, Finkelstein J, Roffwarg H, Kapen S, Weitzman E, Hellman L. Synchronization of augmented luteinizing hormone secretion with sleep during puberty. N Engl J Med. 1972; 287: 582-586.
12. Collins JS, Beller JP, Burt Solorzano C, Patrie JT, Chang RJ, Marshall JC, McCartney CR. Blunted day-night changes in luteinizing hormone pulse frequency in girls with obesity: the potential role of hyperandrogenemia. J Clin Endocrinol Metab. 2014; 99: 2887-2896.
13. Lukas M, Bredewold R, Neumann ID, Veenema AH. Maternal separation interferes with developmental changes in brain vasopressin and oxytocin receptor binding in male rats. Neuropharmacology. 2010; 58: 78-87.
14. Mounien L, Bizet P, Boutelet I, Gourcerol G, Fournier A, Vaudry H, Jegou S. Pituitary adenylate cyclase-activating polypeptide directly modulates the activity of proopiomelanocortin neurons in the rat arcuate nucleus. Neuroscience. 2006; 143: 155-163.
15. Ronnekleiv OK, Fang Y, Zhang C, Nestor CC, Mao P, Kelly MJ. Research resource: Gene profiling of G protein-coupled receptors in the arcuate nucleus of the female. Mol Endocrinol. 2014; 28: 1362-1380.

16. Yi CX, van der Vliet J, Dai J, Yin G, Ru L, Buijs RM. Ventromedial arcuate nucleus communicates peripheral metabolic information to the suprachiasmatic nucleus. Endocrinology. 2006; 147: 283-294.
17. Guzman-Ruiz M, Saderi N, Cazarez-Marquez F, Guerrero-Vargas NN, Basualdo MC, Acosta-Galvan G, Buijs RM. The suprachiasmatic nucleus changes the daily activity of the arcuate nucleus alpha-MSH neurons in male rats. Endocrinology. 2014; 155: 525-535.
18. Yeo SH, Kyle V, Morris PG, Jackman S, Sinnett-Smith LC, Schacker M, Chen C, Colledge WH. Visualisation of Kiss1 neurone distribution using a Kiss1-CRE transgenic mouse. J Neuroendocrinol. 2016; 28.
19. Madisen L, Garner AR, Shimaoka D, Chuong AS, Klapoetke NC, Li L, van der Bourg A, Niino Y, Egolf L, Monetti C, Gu H, Mills M, Cheng A, Tasic B, Nguyen TN, Sunkin SM, Benucci A, Nagy A, Miyawaki A, Helmchen F, Empson RM, Knopfel T, Boyden ES, Reid RC, Carandini M, Zeng H. Transgenic mice for intersectional targeting of neural sensors and effectors with high specificity and performance. Neuron. 2015; 85: 942-958.
20. Han SY, McLennan T, Czieselsky K, Herbison AE. Selective optogenetic activation of arcuate kisspeptin neurons generates pulsatile luteinizing hormone secretion. Proc Natl Acad Sci U S A. 2015; 112: 13109-13114.
21. Clarkson J, Han SY, Piet R, McLennan T, Kane GM, Ng J, Porteous RW, Kim JS, Colledge WH, Iremonger KJ, Herbison AE. Definition of the hypothalamic GnRH pulse generator in mice. Proc Natl Acad Sci U S A. 2017; 114: E10216-E10223.
22. Franceschini I, Yeo SH, Beltramo M, Desroziers E, Okamura H, Herbison AE, Caraty A. Immunohistochemical evidence for the presence of various kisspeptin isoforms in the Mammalian brain. J Neuroendocrinol. 2013; 25: 839-851.
23. Piet R, Fraissenon A, Boehm U, Herbison AE. Estrogen permits vasopressin signaling in preoptic kisspeptin neurons in the female mouse. J Neurosci. 2015; 35: 6881-6892.
24. de Croft S, Boehm U, Herbison AE. Neurokinin B activates arcuate kisspeptin neurons through multiple tachykinin receptors in the male mouse. Endocrinology. 2013; 154: 2750-2760.
25. Piet R, Dunckley H, Lee K, Herbison AE. Vasoactive intestinal peptide excites GnRH neurons in male and female mice. Endocrinology. 2016: en20161399.
26. Ruka KA, Burger LL, Moenter SM. Regulation of arcuate neurons coexpressing kisspeptin, neurokinin B, and dynorphin by modulators of neurokinin 3 and kappa-opioid receptors in adult male mice. Endocrinology. 2013; 154: 2761-2771.
27. Navarro VM, Gottsch ML, Wu M, Garcia-Galiano D, Hobbs SJ, Bosch MA, Pinilla L, Clifton DK, Dearth A, Ronnekleiv OK, Braun RE, Palmiter RD, Tena-Sempere M, Alreja M, Steiner RA. Regulation of NKB pathways and their roles in the control of Kiss1 neurons in the arcuate nucleus of the male mouse. Endocrinology. 2011; 152: 4265-4275.
28. Navarro VM, Gottsch ML, Chavkin C, Okamura H, Clifton DK, Steiner RA. Regulation of gonadotropin-releasing hormone secretion by kisspeptin/dynorphin/neurokinin B neurons in the arcuate nucleus of the mouse. J Neurosci. 2009; 29: 11859-11866.
29. Navarro VM, Bosch MA, Leon S, Simavli S, True C, Pinilla L, Carroll RS, Seminara SB, Tena-Sempere M, Ronnekleiv OK, Kaiser UB. The integrated hypothalamic

tachykinin-kisspeptin system as a central coordinator for reproduction. Endocrinology. 2015; 156: 627-637.

30. Harmar AJ, Fahrenkrug J, Gozes I, Laburthe M, May V, Pisegna JR, Vaudry D, Vaudry H, Waschek JA, Said SI. Pharmacology and functions of receptors for vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide: IUPHAR review 1. Br J Pharmacol. 2012; 166: 4-17.
31. Ross RA, Leon S, Madara JC, Schafer D, Fergani C, Maguire CA, Verstegen AM, Brengle E, Kong D, Herbison AE, Kaiser UB, Lowell BB, Navarro VM. PACAP neurons in the ventral premammillary nucleus regulate reproductive function in the female mouse. eLife. 2018; 7.
32. Chen R, Wu X, Jiang L, Zhang Y. Single-Cell RNA-Seq Reveals Hypothalamic Cell Diversity. Cell reports. 2017; 18: 3227-3241.
33. Campbell JN, Macosko EZ, Fenselau H, Pers TH, Lyubetskaya A, Tenen D, Goldman M, Verstegen AM, Resch JM, McCarroll SA, Rosen ED, Lowell BB, Tsai LT. A molecular census of arcuate hypothalamus and median eminence cell types. Nat Neurosci. 2017; 20: 484-496.
34. Klingman KM, Marsh EE, Klerman EB, Anderson EJ, Hall JE. Absence of circadian rhythms of gonadotropin secretion in women. J Clin Endocrinol Metab. 2011; 96: 1456-1461.
35. Urbanski HF, Ojeda SR. The juvenile-peripubertal transition period in the female rat: establishment of a diurnal pattern of pulsatile luteinizing hormone secretion. Endocrinology. 1985; 117: 644-649.
36. Desroziers E, Mikkelsen JD, Duittoz A, Franceschini I. Kisspeptin-immunoreactivity changes in a sex- and hypothalamic-region-specific manner across rat postnatal development. J Neuroendocrinol. 2012; 24: 1154-1165.
37. Yeo SH, Herbison AE. Projections of arcuate nucleus and rostral periventricular kisspeptin neurons in the adult female mouse brain. Endocrinology. 2011; 152: 2387-2399.
38. Herbison AE. Physiology of the adult GnRH neuronal network. In: Plant TM, Zeleznik AJ, eds. *Knobil and Neill's Physiology of Reproduction*. 4th ed: Academic Press 2015: 399-467.
39. Mittelman-Smith MA, Krajewski-Hall SJ, McMullen NT, Rance NE. Ablation of KNDy Neurons Results in Hypogonadotropic Hypogonadism and Amplifies the Steroid-Induced LH Surge in Female Rats. Endocrinology. 2016; 157: 2015-2027.
40. Helena CV, Toporikova N, Kalil B, Stathopoulos AM, Pogrebna VV, Carolino RO, Anselmo-Franci JA, Bertram R. KNDy Neurons Modulate the Magnitude of the Steroid-Induced Luteinizing Hormone Surges in Ovariectomized Rats. Endocrinology. 2015; 156: 4200-4213.
41. Qiu J, Nestor CC, Zhang C, Padilla SL, Palmiter RD, Kelly MJ, Ronnekleiv OK. High-frequency stimulation-induced peptide release synchronizes arcuate kisspeptin neurons and excites GnRH neurons. eLife. 2016; 5: e16246.

Figure legends

Figure 1. Distribution of GCaMP6 in arcuate nucleus (ARN). **A**. Images from the same adult female mouse showing the GFP-immunofluorescence (GCaMP6) in the rostral, middle and caudal levels of the ARN. **B-D**, confocal images showing dual fluorescence for GFP- (B) and kisspeptin- (C) immunofluorescence, and with the overlapping signals represented in D.

Figure 2. *Baseline GCaMP6 fluorescence levels in arcuate kisspeptin neurons*. **A**. Raw fluorescence levels of eight ARN kisspeptin neurons located in a middle arcuate nucleus brain slice over 12 min. **B.** Higher resolution change in fluorescence levels of 5 of these cells showing the one cell (cell 3) that exhibited spontaneous fluctuations.

Figure 3. *NKB activates essentially all kisspeptin neurons in both male and female mice.* **A.** Single frame photograph of GCaMP6 fluorescence in the middle ARN of a slice being imaged. The five cells shown in C are labelled. **B.** Raw fluorescence recordings from 21 kisspeptin neurons in that brain slice showing their response to a 2-min application of 50nM NKB (grey bar) in the presence of TTX and amino acid receptor antagonists. **C.** Higher resolution change in fluorescence levels from the five cells depicted in A. Colors are the same as in B. **D**. Histogram showing the percentage of kisspeptin neurons in the rostral, middle and caudal aspects of the ARN that responded to NKB in male and female mice (N=4, each).

Figure 4. *VIP preferentially activates caudal ARN kisspeptin neurons in female mice.* **A.** Raw fluorescence traces from the rostral, middle and caudal aspects of the ARN from the same diestrous female *Kiss1::GCaMP6f* mouse. The two-min 1μM VIP exposure period is indicated by the grey bar and each colored line represents a different cell. **B.** Higher resolution changes in fluorescence showing the response profiles of six caudal kisspeptin neurons to VIP. **C.** Histogram showing the percentage of kisspeptin neurons in the rostral, middle and caudal aspects of the ARN responding to VIP in

509 male and female mice (N=4 each) * $p<0.05$ compared to all other groups, Two-way
510 ANOVA with *post-hoc* Tukey tests.
511
512 **Figure 5.** *AVP preferentially activates caudal ARN kisspeptin neurons in female mice.*
513 **A.** Raw fluorescence traces from the rostral, middle and caudal aspects of the ARN
514 from diestrous female *Kiss1::GCaMP6f* mouse. The two-min 300nM AVP exposure
515 period is indicated by the grey bar and each colored line represents a different cell.
516 Note the orange cell in the caudal traces that was discarded from analysis as it
517 exhibited a spontaneous fluctuation in fluorescence prior to the test with AVP. **B.**
518 Higher resolution change in fluorescence images showing the AVP response profiles
519 of five of the caudal kisspeptin neurons shown in A. **C**. Histogram showing the
520 percentage of kisspeptin neurons in the rostral, middle and caudal aspects of the ARN
521 responding to AVP in male and female mice (N=4 each). * $p<0.05$ compared to all
522 other groups, Two-way ANOVA with *post-hoc* Tukey tests.
523

524 Table

	nos. Kiss neurons / hemi-section	% GCaMP6 cells +ve for kisspeptin	% kisspeptin cells +ve for GCaMP6
rostral ARN	37±14	88±7%	99±1%
middle ARN	60±7	94±1%	99±1%
caudal ARN	68±8	95±2%	99±1%

525

526 Table showing the numbers of kisspeptin-immunoreactive neurons detected in the
527 three subregions of the ARN and their levels of co-expression with GCaMP6. N = 4
528 ovariectomized female mice.

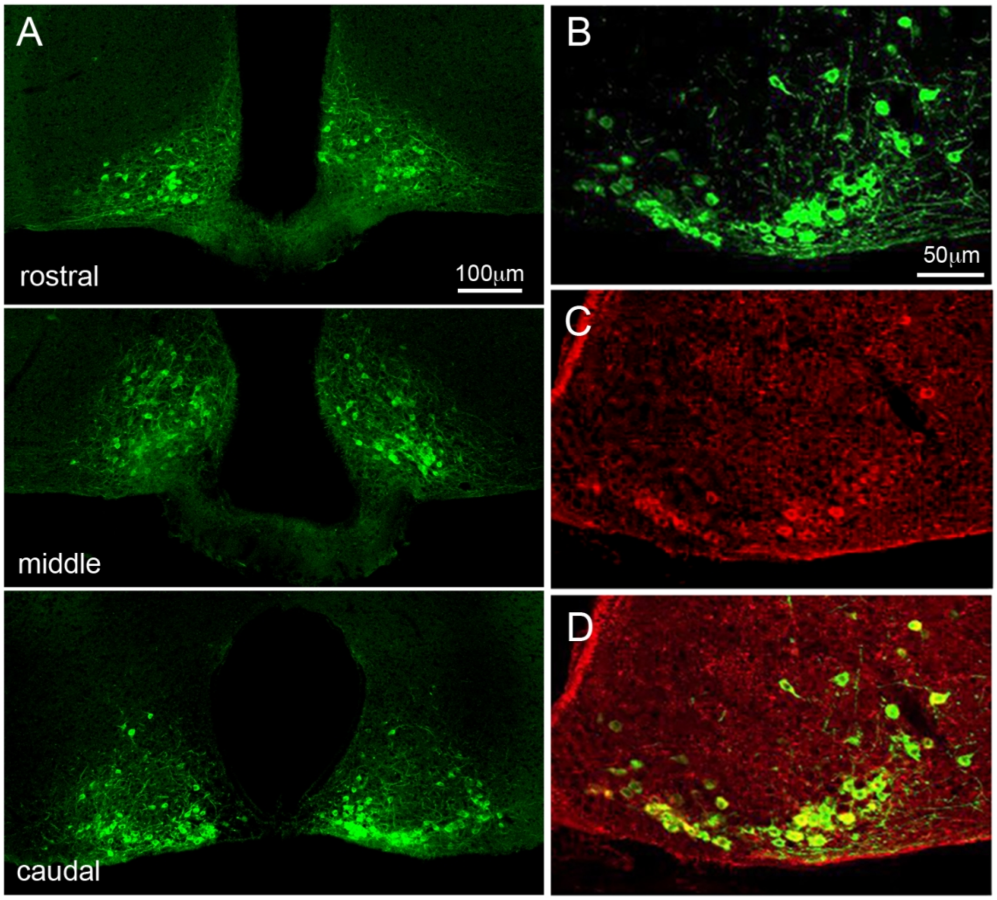

Figure 1.

Figure 1. Distribution of GCaMP6 in arcuate nucleus (ARN). A. Images from the same adult female mouse showing the GFP-immunofluorescence (GCaMP6) in the rostral, middle and caudal levels of the ARN. B-D, confocal images showing dual fluorescence for GFP- (B) and kisspeptin- (C) immunofluorescence, and with the overlapping signals represented in D.

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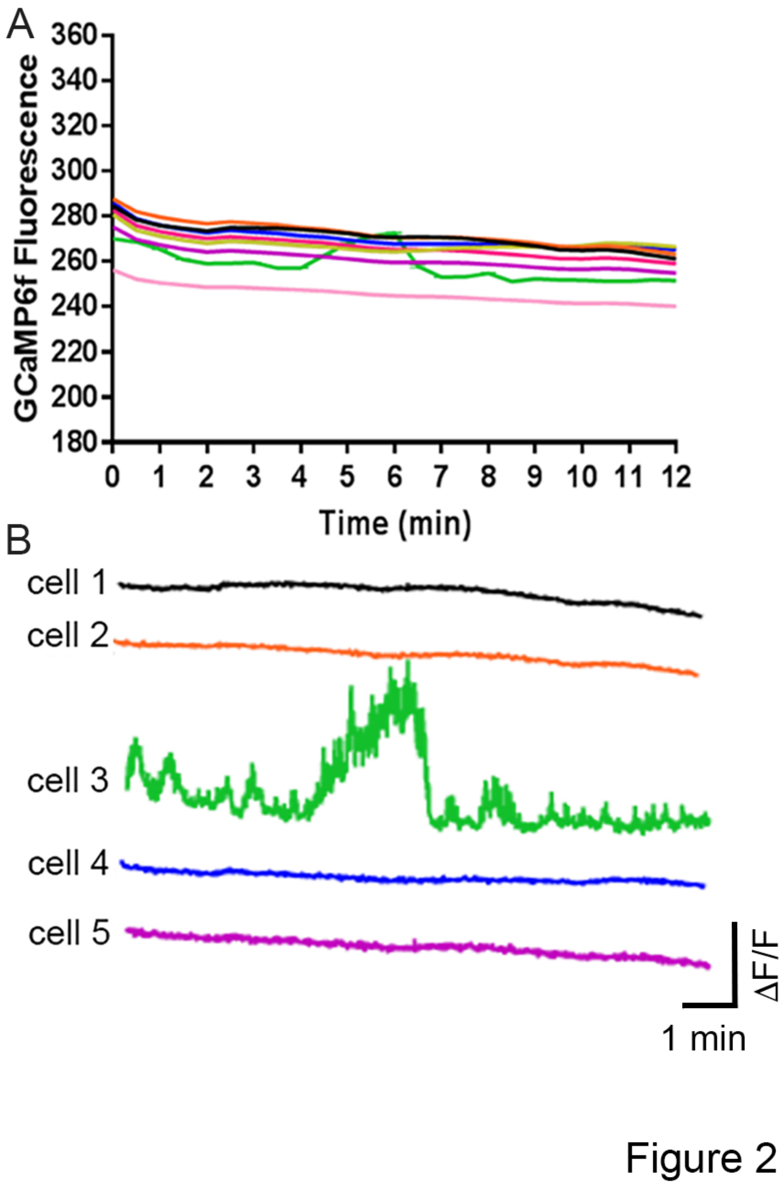

Figure 2. Baseline GCaMP6 fluorescence levels in arcuate kisspeptin neurons. A. Raw fluorescence levels of eight ARN kisspeptin neurons located in a middle arcuate nucleus brain slice over 12 min. B. Higher resolution change in fluorescence levels of 5 of these cells showing the one cell (cell 3) that exhibited spontaneous fluctuations.

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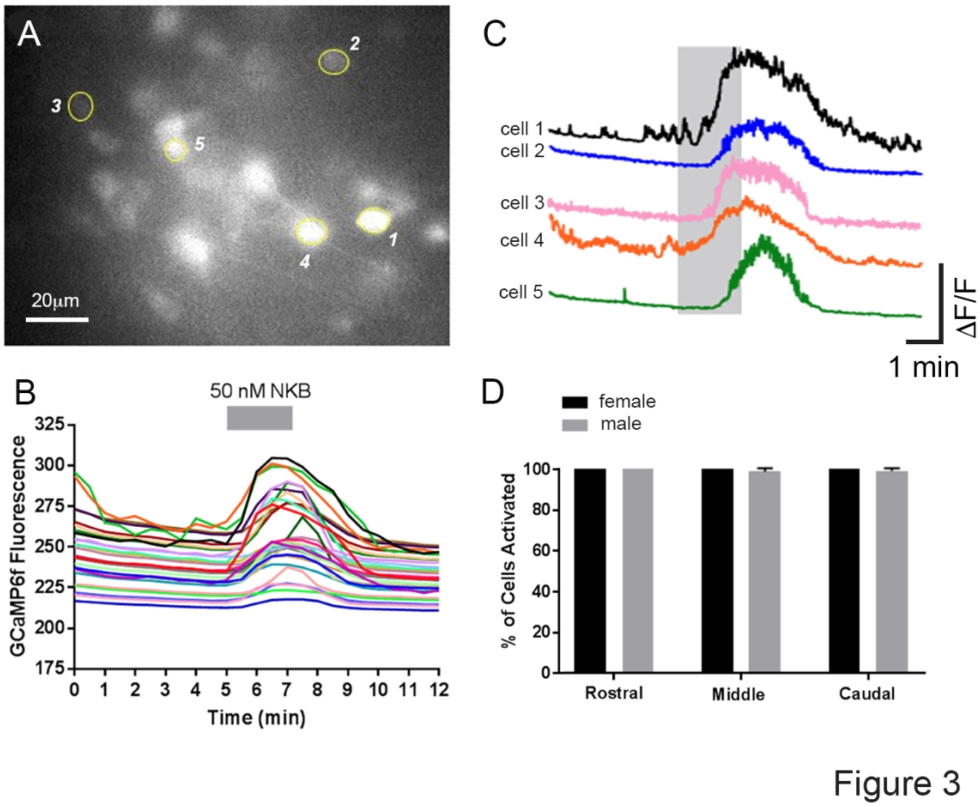

Figure 3. NKB activates essentially all kisspeptin neurons in both male and female mice. A. Single frame photograph of GCaMP6 fluorescence in the middle ARN of a slice being imaged. The five cells shown in C are labelled. B. Raw fluorescence recordings from 21 kisspeptin neurons in that brain slice showing their response to a 2-min application of 50nM NKB (grey bar) in the presence of TTX and amino acid receptor antagonists. C. Higher resolution change in fluorescence levels from the five cells depicted in A. Colors are the same as in B. D. Histogram showing the percentage of kisspeptin neurons in the rostral, middle and caudal aspects of the ARN that responded to NKB in male and female mice (N=4, each).

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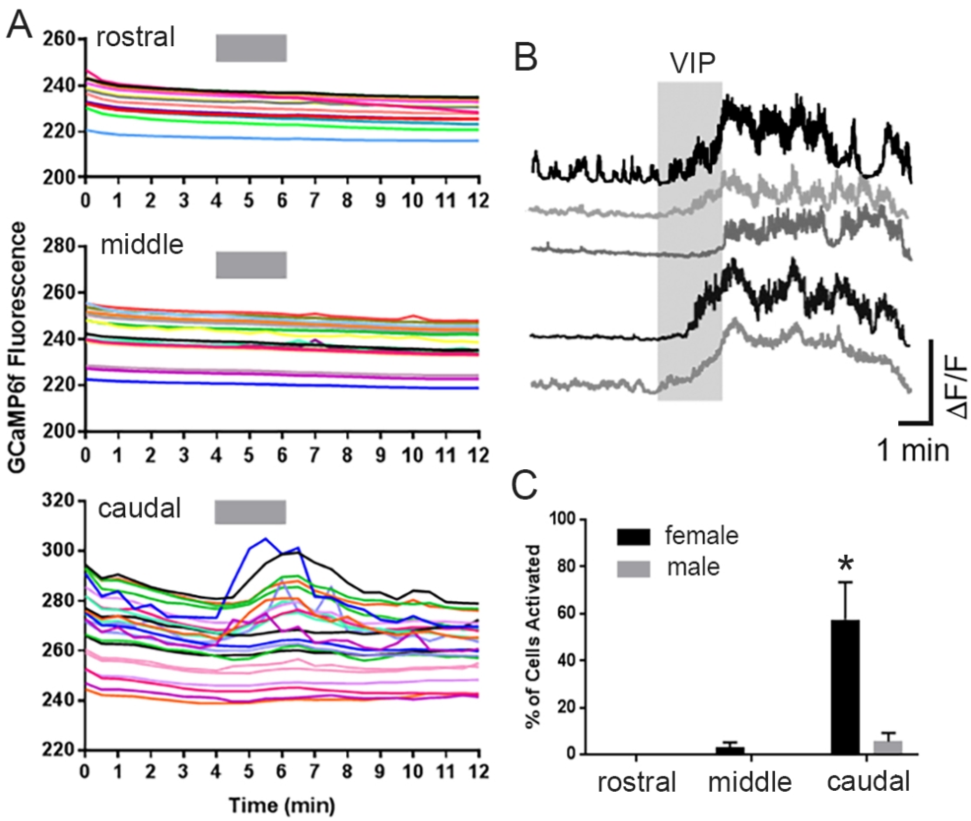

Figure 4

Figure 4. VIP preferentially activates caudal ARN kisspeptin neurons in female mice. A. Raw fluorescence traces from the rostral, middle and caudal aspects of the ARN from the same diestrous female Kiss1::GCaMP6f mouse. The two-min 1□M VIP exposure period is indicated by the grey bar and each colored line represents a different cell. B. Higher resolution changes in fluorescence showing the response profiles of six caudal kisspeptin neurons to VIP. C. Histogram showing the percentage of kisspeptin neurons in the rostral, middle and caudal aspects of the ARN responding to VIP in male and female mice (N=4 each) * $p<0.05$ compared to all other groups, Two-way ANOVA with post-hoc Tukey tests.

98x90mm (300 x 300 DPI)

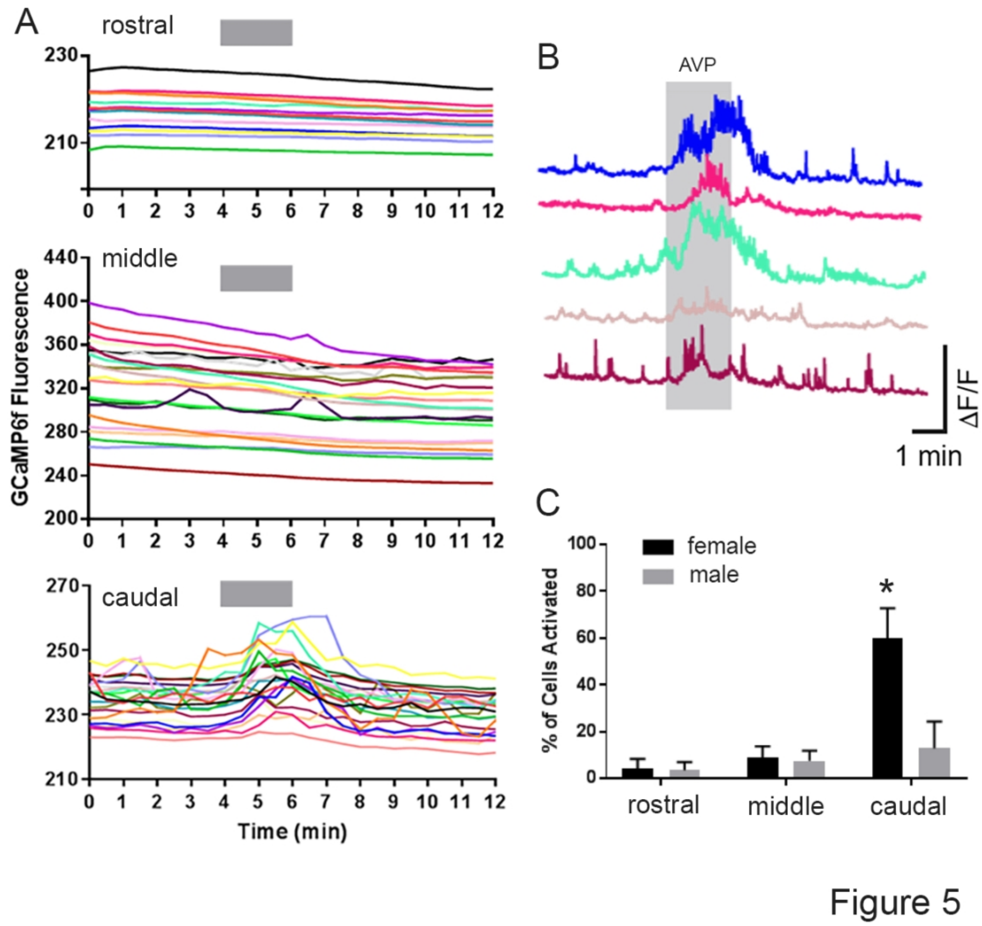

Figure 5. AVP preferentially activates caudal ARN kisspeptin neurons in female mice. A. Raw fluorescence traces from the rostral, middle and caudal aspects of the ARN from diestrous female Kiss1::GCaMP6f mouse. The two-min 300nM AVP exposure period is indicated by the grey bar and each colored line represents a different cell. Note the orange cell in the caudal traces that was discarded from analysis as it exhibited a spontaneous fluctuation in fluorescence prior to the test with AVP. B. Higher resolution change in fluorescence images showing the AVP response profiles of five of the caudal kisspeptin neurons shown in A. C. Histogram showing the percentage of kisspeptin neurons in the rostral, middle and caudal aspects of the ARN responding to AVP in male and female mice (N=4 each). * $p<0.05$ compared to all other groups, Two-way ANOVA with post-hoc Tukey tests.

107x102mm (300 x 300 DPI)