Mechanism of arcuate kisspeptin neuron synchronization in acute brain slices from female mice

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Abstract

The mechanism by which arcuate kisspeptin (ARN^{KISS}) neurons co-expressing glutamate, neurokinin B, and dynorphin intermittently synchronize their activity to drive pulsatile hormone secretion remains unclear in females. In order to study spontaneous synchronization within the ARN^{KISS} neuron network, acute brain slices were prepared from adult female Kiss1-GCaMP6 mice. Analysis of both spontaneous synchronizations and those driven by high frequency stimulation of individual ARN^{KISS} neurons revealed that the network exhibits semi-random emergent excitation dependent upon glutamate signaling through AMPA receptors. No role for NMDA receptors was identified. In contrast to male mice, ongoing tachykinin receptor tone within the slice operated to promote spontaneous synchronizations in females. As previously observed in males, we found that ongoing dynorphin transmission in the slice did not contribute to synchronization events. These observations indicate that a very similar AMPA receptor-dependent mechanism underlies ARN^{KISS} neuron synchronizations in the female mouse supporting the "glutamate two-transition" model for kisspeptin neuron synchronization. However, a potentially important sex difference appears to exist with a more prominent facilitatory role for tachykinin transmission in the female.

Introduction

Sexual development and fertility in mammals are enabled by a hypothalamic central pattern generator that drives pulsatile gonadotropin-releasing hormone (GnRH) release. A group of kisspeptin-expressing cells located in the arcuate nucleus (ARN), defined as the 'GnRH pulse generator', release kisspeptin in an episodic manner on distal GnRH neuron processes to evoke the episodic GnRH secretion underlying pulsatile gonadotropin release critical for fertility (1,2).

In order to drive this ultradian rhythm of pulsatile GnRH and LH secretion, ARN kisspeptin (ARN^{KISS}) neurons must synchronize their activity. Recent *in vivo* fiber photometry experiments have shown a perfect correlation between ARN^{KISS} neuron synchronization events (SEs) and their corresponding LH pulses (3-5). Remarkably, the great majority of ARN^{KISS} neurons contribute to each SE in both male and female mice (6,7). It is now important to dissect the mechanisms underlying ARN^{KISS} neuron synchronization activity to understand how fertility is maintained and to provide potential new targets for the development of therapies in the clinic.

Alongside glutamate, ARN^{KISS} neurons co-express neurokinin B (NKB) and dynorphin in almost all mammals (8,9), and experiments in mice have shown that essentially all ARN^{KISS} neurons synthesize functional tachykinin and kappa opioid receptors that activate and suppress their firing, respectively (10,11). On this basis, the KNDy hypothesis posits that reciprocal interconnections between ARN^{KISS} neurons release tachykinin and dynorphin sequentially to activate and then terminate synchronization in the network (12-14). We recently tested this model using *in vivo* fiber photometry and a new *ex-vivo* acute brain slice preparation that maintains spontaneous synchronization within the ARN^{KISS} neuron network (7). We observed that ARN^{KISS} neuron synchronizations were driven by glutamate transmission via AMPA receptors (AMPARs) with NKB operating sequentially to facilitate the glutamatergic activation. Dynorphin was not found to have any role in synchronization termination that, instead, appears to be facilitated by intrinsic mechanisms within the ARN^{KISS} neurons themselves. Dynorphin was, however, identified to operate as a state-dependent gate for synchronization activation. As such, we proposed a new "glutamate two-transition" model for understanding ARN^{KISS} neuron synchronization (7).

An important caveat of the glutamate two-transition model is that the supporting data were obtained solely from male mice. We have begun to address potential sex differences in the pulse generator synchronization mechanism by undertaking the same *ex-vivo* acute brain slice studies in female mice as performed previously in males (7).

Materials and Methods

Animals

Mice were generated and housed as detailed previously (7). This involved crossing the 129S6Sv/Ev C57BL/6 *Kiss1*$^{Cre/+}$ and C57BL/6 Ai162 (TIT2L-GC6s-ICL-tTA2)-D Cre-dependent GCaMP6s lines (JAX stock #031562)(15) with mice group-housed in cages with environmental enrichment under conditions of controlled temperature (22±2^{0}C) and lighting (12-hour light/12-hour dark cycle; lights on at 7:00h and off at 19:00h) with *ad libitum* access to food (RM1-P, SDS, UK) and water. All animal experimental protocols were approved by the University of Cambridge, UK (P174441DE). Mice were 10-22 weeks old at the time of experimentation.

Brain slice preparation

Brain slices were prepared exactly as reported previously (7). Kiss1-Cre,Ai126D mice were anaesthetized using isoflurane, decapitated, and the brain removed into oxygenated, ice-cold slicing solution composed of (mM): NaCl 52.5; sucrose 100; glucose 25; $NaHCO_3$ 25; KCl 2.5; $CaCl_2$ 1; $MgCl_2$ 5; NaH_2PO_4 1.25; kynurenic acid 0.1 (95% O_2 / 5% CO_2). Coronal slices containing the ARN were prepared at 320 µm thickness using a VT1200S tissue slicer (Leica Biosystems UK) before being transferred to a submersion chamber containing an oxygenated (95% O_2 / 5% CO_2) aCSF recording solution composed of (mM): NaCl 124; glucose 30; $NaHCO_3$ 25; KCl 3.5; $CaCl_2$ 1.5; $MgCl_2$ 1; NaH_2PO_4 0.5 and incubated at 30°C for 1-5 hours prior to use.

Brain slice calcium imaging and analysis

Brain slices containing the ARN were transferred to the stage of an Olympus BX51WI upright microscope with differential interference contrast optics, and constantly perfused with oxygenated aCSF at 30 ± 1 °C. The variation in intracellular calcium concentration of ARN^{KISS} neurons was estimated by recording their GCaMP6s fluorescence using a Prime BSI Express sCMOS camera (Teledyne Photometrics UK) and CoolLED *p*E-300 ultra light source via an Olympus 40x immersion objective and GFP filter cube (Chroma). The excitation waveband was 470-490 nm, applied at 2 Hz for 100 ms, and emission collected at 500-520 nm with a 495 nm long-pass filter. For analysis, ImageJ (v1.53c) was used to obtain mean fluorescence intensities over the image time series: active cell soma were selected manually as regions of interest (ROIs), and for each, the mean fluorescence values of a nearby background ROI was subtracted. Fluorescence intensity data and all metrics described below were analysed using custom Python scripts. The change in fluorescence (ΔF/F) was calculated and individual calcium events in cells and population miniature synchronization events (mSEs) were registered as previously described (7). Briefly, events are recorded where the ΔF/F trace exceeded 2 SD above the trace mean. An mSE exists when the peak of calcium events from at least two neurons occurs within 10 s of each other. Event and mSE rates are presented as 'per cell, per hour', thereby controlling for the variation in the number of neurons being recorded in each brain slice.

Within each experiment, a pre-drug baseline was obtained, followed by a drug application period, and a wash period. All of these measurement periods, from which event/mSE rates were calculated, were 12 minutes in duration and followed/preceded by a two-minute gap to allow wash-in/out of compounds.

Shape properties of calcium events were obtained as described previously (7): briefly, a trace baseline value was calculated as the median value of all data points below a threshold of 0.5 SD above the trace mean. Each datapoint shown is a mean of means, representing one slice/experiment, and a minimum of three neurons meeting these criteria was required to form a datapoint for that experiment. Half width is defined as the width of the calcium event at 50% amplitude from the calculated baseline to the peak. Rise time is the length of time taken to rise from 20% to 80% of maximum event amplitude, and decay time the reverse.

For the temporal correlation analysis, experiments were performed over 40 minutes and brain slices with a minimum of three mSEs involving ≥5 cells were analyzed in order to calculate the correlation coefficient. Cell activation order within mSEs was calculated using the event peak time of each cell. In generating heatmaps and scatter plots, neurons were sorted in order of the mean event order over all mSEs occurring, making it possible to visualise good correlations should they occur. Spearman correlation coefficients were calculated for scatterplots, representing the consistency of the order in

which ARN^{KISS} neurons reach peak amplitude. High correlation coefficients indicate a consistent temporal order of firing whilst low correlation implies stochastic recruitment of neurons.

Brain slice electrophysiology and calcium imaging experiments

Patch pipettes were pulled from standard borosilicate glass (GC150F, Warner Instruments) to a resistance of 2.5–4 MΩ and filled with an intracellular solution composed of (mM): potassium gluconate 130; sodium gluconate 5, HEPES 10; $CaCl_2$ 1.5; sodium phosphocreatine 4; Mg-ATP 4; Na-GTP 0.3; pH 7.3; filtered at 0.2 µm. Experiments were performed as per other calcium imaging experiments, but a single GCaMP-expressing kisspeptin neuron was patched and stimulated with alternating ~5-10 and ~20-40 pA current injections for 10-15 s in order to achieve low (~2.5 Hz) and high (maximal) frequency firing. To mimic spontaneous depolarising behaviour observed in individual ARN^{KISS} neurons, we applied inward current in a ramping manner with a 2 s time constant. Co-incident activation of the patched neuron and other cells in the slice were evaluated as above.

Receptor and channel antagonists

NorBNI, CNQX, and DAP5 were obtained from Sigma-Aldrich UK, and SDZ-NKT 343, GR 94800, and SB 222200 obtained from Tocris (Bio-Techne) UK. Standard laboratory salts were purchased from Fisher Scientific UK.

Quantification and statistical analyses

Statistical analyses were performed in Prism 9 (GraphPad software Inc.). All values given in the text and within figures are mean ± SEM, and significance is defined as $p<0.05$*, $p<0.01$**, $p<0.001$***, or $p<0.0001$****. The Shapiro-Wilk normality test was used to assess the distribution of unpaired datasets, and residuals for paired datasets: nonparametric tests were applied where data did not pass the normality test. All analyses were two-tailed, and all experiments replicated in a minimum of five animals per group, with n representing the number of brain slices unless otherwise stated.

Results

Characterization of spontaneous GCaMP calcium events and synchronizations

Acute brain slices were prepared from diestrous-stage female *Kiss1*-Cre,Ai162D mice. Coronal brain slices from the mid to caudal ARN were used for experiments, with 8-23 kisspeptin neurons visible within the focal plane for simultaneous GCaMP fluorescence imaging. In slices prepared from 55 female mice, individual calcium events occurred at a rate of 14.7 ± 0.9 events/cell/h and mSEs at 10.3 ± 0.8 mSEs/cell/h. The mean half-width of calcium events was 14.9 ± 0.9 s with a rise time of 4.2 ± 0.5 s and decay of 6.8 ± 0.3 s. All visible ARN^{KISS} neurons exhibited episodes of activity that were synchronized with at least one other ARN^{KISS} neuron.

Synchronizations varied in their scale with many consisting of 2 or 3 neurons, but large synchronizations involving 5-12 neurons (to a maximum of 66 % of all recorded cells) also occurred regularly. Individual ARN^{KISS} neurons took part in such large synchronizations (≥5 cells) at a rate of 0.66 ± 0.1 /cell/hr (n = 9 slices; mean 16.4 cells/slice), which represents an overall rate of 9.4 + 1.5 per hour

within the experimental field of view. The spatiotemporal participation of individual ARN^{KISS} neurons in such synchronizations was analyzed (Fig.1A,B; n = 9 slices with 3–14 mSEs). The participation of neurons in mSEs was inconsistent, with none involved in every mSE and others only active during a single mSE: on average, a single neuron participated in 46.9% ± 2.2% of mSEs. We found a low to moderate degree of consistency in the order of firing, with neurons which peaked first during one mSE slightly more likely to peak early during other mSEs. The mean Spearman correlation coefficient was 0.43 (range 0.21–0.74; Table 1). The anatomical relationship between neurons was also assessed, with their sequence of activation having no apparent topographical relationship with their proximity to each other within the experimental field of view (Fig.1A,B). These results highlight the generally unpredictable nature of synchronizations within the ARN^{KISS} neuron network *ex-vivo*.

Both AMPA- and tachykinin mediated transmission contribute to ARN^{KISS} neuron synchronization

To explore the contribution of glutamate, NKB, and dynorphin in mediating spontaneous synchronizations, brain slices were prepared from intact diestrous female mice and antagonists included in the aCSF for 15 minutes. Control experiments switching to aCSF alone resulted in no change in individual event frequency (15.8 ± 2.0 events/cell/h pre-vehicle, 16.1 ± 1.4 events/cell/h during vehicle; n = 11 slices; mean 13.5 cells/slice) or mSE frequency (Fig.2A,C; 10.4 ± 1.8 mSE/cell/h pre-vehicle, 11.2 ± 1.5 mSE/cell/h during vehicle).

Application of ionotropic glutamate receptor antagonists CNQX (20 µM) and DAP5 (20 µM) together resulted in significant reductions in the rates of both calcium events and mSEs. Events were reduced by 54% (19.7 ± 2.2 events/cell/h pre-drug and 9.1 ± 1.4 in CNQX + DAP5; p = 0.004; Wilcoxon; n = 9 slices; mean 9.8 cells/slice) and mSEs by 65% (Fig.2D; 13.1 ± 2.5 mSE/cell/h pre-drug and 4.6 ± 1.1 in CNQX + DAP5; p = 0.004; Wilcoxon).

Inhibition of AMPA receptors alone using CNQX caused a similar 55% reduction in events (14.9 ± 2.1 events/cell/h pre-drug and 6.7 ± 1.3 in CNQX; p = 0.016; Wilcoxon; n = 7 slices; mean 15.4 cells/slice) and a 63% reduction in mSEs (Fig.2B,E; 10.8 ± 1.8 mSE/cell/h pre-drug and 4.0 ± 1.3 in CNQX; p = 0.016; Wilcoxon). Washing out CNQX often resulted in rebound activity, illustrated in Fig.2B.

Application of the NMDA receptor antagonist DAP5 alone had no effect on the rate of events (13.0 ± 1.8 events/cell/h pre-drug and 11.2 ± 1.6 in DAP5; p = 0.64; Wilcoxon; n = 8 slices; mean 11.8 cells/slice) or mSEs (Fig.2F; 7.1 ± 1.4 mSE/cell/h pre-drug and 5.8 ± 1.0 in DAP5; p = 0.56; Wilcoxon).

Application of a cocktail of tachykinin receptor antagonists targeting NK1 (SDZ-NKT 343; 1 µM), NK2 (GR 94800; 1 µM), and NK3 (SB 222200; 3 µM) receptors led to a 57% reduction in events (16.0 ± 2.6 events/cell/h pre-drug and 6.8 ± 0.8 under inhibition; p = 0.023; Wilcoxon; n = 8 slices; mean 10.6 cells/slice) and a 65% reduction in mSEs (Fig.3A,B; 8.9 ± 1.6 mSE/cell/h pre-drug and 3.1 ± 0.7 under inhibition; p = 0.039; Wilcoxon).

Application of the kappa opioid receptor antagonist NorBNI (10 µM) had no effect on the rate of events (12.7 ± 2.1 events/cell/h pre-drug and 12.5 ± 2.2 in NorBNI; p = 0.74; Wilcoxon; n = 8 slices; mean 10.9 cells/slice) or mSEs (Fig.3C; 8.7 ± 1.8 mSE/cell/h pre-drug and 9.2 ± 1.8 in NorBNI; p = 0.94; Wilcoxon). No significant changes in half-width, rise time, or decay time were found in response to NorBNI application or any other antagonist (Table 2).

ARN^{KISS} neuron activation promotes primarily glutamatergic responses in nearby neurons

The ability of individual ARN^{KISS} neurons to directly promote synchronization in nearby cells was examined by direct electrical stimulation (Fig.4). Cells exhibiting spontaneous synchronized activity were identified and one cell in the group selected for whole-cell patch clamp. These patched ARN^{KISS} neurons (n=30) had a mean capacitance of 15.8 ± 0.8 pF, input resistance of 743 ± 47 MΩ, and resting membrane potential of −58.7 ± 0.8 mV (Table 3).

The patched cell in each experiment was driven to fire at high and low frequencies ("HF" and "LF" respectively) for 15 s by gradually increasing current injection and the effects on GCaMP fluorescence observed in all other ARN^{KISS} neurons in the field of view (Figs.4 & 5). Current injections were calibrated on a per neuron basis, with the aim of achieving the highest possible firing rate over 15 s for HF stimulation and slow firing (~5 Hz) for LF (Fig.4A). Actual firing rates were measured, with a peak firing frequency of 26.9 ± 1.6 Hz under HF stimulation and 5.9 ± 0.5 Hz under LF stimulation. Over the course of each 15 s stimulation there was a consistent and substantial decrease in firing rate typically found with spike frequency adaptation (Fig.4A). As such, over the entire 15 s period, HF stimulation produced a mean firing frequency of 12.3 ± 1.2 Hz, and LF stimulation generated a mean of 2.5 ± 0.3 Hz. Driven stimulation produced transient GCAMP fluorescence increases in the patched neuron, but due to micropipette dialysis this generally faded over the course of each experiment.

Events in nearby neurons were considered to be coincident if their onset occurred before the end of the stimulation period in the patched cell and peaked either during stimulation or within 10 s of its termination (Fig.5). In unstimulated control periods of the same length prior to HF and LF stimulations, a coincident event was found to occur in at least one other neuron within the group in 40.5% of 79 trial periods (Fig.4B). The LF stimulation did not alter this with coincident events occurring in 41.0% of 39 trials (Fig.4B). Following HF stimulation, a significant increase occurred with a coincident event occurring in at least one other neuron in 61.9% of 42 trials ($p = 0.007$, two-tailed binomial test; 15 brain slices; mean 8.8 cells/slice) (Fig.4B). Coincident calcium events did not occur consistently between the stimulated cell and other neurons (Fig.5). It is possible that repeated stimulations may gradually result in a change in success rate over time. To test this, we recorded the rate of coincident events in the 1^{st} and 2^{nd} pre-drug HF stimulations across all experiments. No reduction was found, with coincident events in 58% of 26 trials at stimulation 1 and 73% of 26 trials at stimulation 2 (n=26 slices). Whilst an analysis of success over the first two recorded HF stimulations is limited, the robustness of spontaneous synchronisation over 40-min duration experiments indicates little is likely to change over shorter periods.

Following a set of repeated stimulations to establish baseline values, CNQX was then applied, and the stimulations continued (Fig.5A). Under these conditions, CNQX substantially and significantly reduced the occurrence of coincident events in all groups compared to pre-CNQX (Fig.4B & 5A); 10.0% of 50 trials in controls with no electrical stimulation ($p < 0.0001$, two-tailed binomial test), 14.3% of 21 trials following LF ($p = 0.013$, two-tailed binomial test) and 13.8% of 29 trials after HF ($p < 0.0001$, two-tailed binomial test) (Fig.4B). These observations showed that CNQX almost completely blocked spontaneous mSEs, as expected, as well as the effects of electrically activating one kisspeptin neuron on other nearby kisspeptin neurons.

In order to evaluate the contribution of NKB to synchronizations, we repeated these experiments using the tachykinin receptor antagonist cocktail (Fig.5B). As NKB release from kisspeptin neurons is only evoked by high stimulation frequencies (14), the effects of the antagonist cocktail were only examined using HF stimulation. In this series of experiments, at least one other neuron was activated by HF stimulation in 73.9% of 23 trials (Figs.4B & 5B; 11 slices; mean 9.5 cells/slice). This was significantly above unstimulated controls (34.8% of 23 trials; $p = 0.0002$, two-tailed binomial test) taken from periods of the same length but with no stimulation applied. Following application of the NKR

antagonist cocktail, a coincident event occurred under HF stimulation in 56.3% of 32 trials, significantly lower than HF stimulation under control conditions (Fig.4B, p = 0.041, two-tailed binomial test). During periods of the same length in NKR antagonists in which stimulation was not applied, an event occurred in 40.6% of 32 trials, which was not significantly different from the pre-drug control value. These experiments demonstrate that tachykinin receptor antagonists are able to block approximately 25% of coincident activity between an HF-stimulated kisspeptin neuron and other neurons.

Comparisons between females and males

For reference, we provide a comparison in Table 3 between each variable measured in the present study and that observed previously for males in the same slice preparation (7). Both events and synchronizations in females occurred at a significantly higher rate in comparison to males. The ARN^{KISS} neurons from female mice had a lower mean capacitance, greater input resistance, and a slightly more depolarized resting membrane voltage in comparison to those from males (Table 3).

Discussion

We report here that ARN^{KISS} neurons from female mice intermittently synchronize with each other in an acute brain slice preparation, and that these spontaneous synchronizations are dependent on glutamate transmission via AMPA receptors as well as NKB. Rates of synchronization were markedly lowered by inhibiting AMPA but not NMDA receptors underscoring the importance of glutamate neurotransmission in mediating synchronization within the ARN^{KISS} neuron network in females. This study follows our characterisation of the pulse generator in male mice, in which we observed an identical dependence on glutamate signalling via AMPA receptors (7). However, in contrast to that study, we find a contribution of NKB to spontaneous synchronized activity in female brain slices, possibly representing a sex difference in the mechanism of ARN^{KISS} neuron synchronization.

The fundamental mechanism of synchronization is the same in male and female mice

We previously demonstrated that ARN^{KISS} neurons in males synchronize their activity through the stochastic-like emergence of coordinated activity mediated by glutamate-AMPA receptor transmission (7). The present observations indicate that the same fundamental process is involved in ARN^{KISS} neuron synchronization in female mice. Individual ARN^{KISS} neurons were observed to couple together in an unpredictable manner and while the electrical stimulation of a single neuron increases overall population synchrony it does not reliably activate other individual neurons. This loose coupling likely underlies the very modest degree of consistency in their order of neuron activation within each synchronization. As before in male brain slices, we find no robust evidence for the concept of “leader cells” in these synchronizations. Our prior GRIN lens study examining the activity of up to 50 individual ARN^{KISS} neurons *in vivo* similarly found no evidence for leader cells in gonadectomized males (16) although another study looking at ~13 cells in ovariectomized females did (17).

Temporally aligned excitatory input from at least two other kisspeptin neurons, and perhaps more, may be required to reliably elicit an event in a typical ARN^{KISS} neuron. Modelling studies from other pattern generator networks suggest that each population burst, similar to a full-scale SE, can be generated better via an assembly of loosely coupled neurons with diverse intrinsic properties than an orderly recruitment beginning with pacemakers (18,19). Variations in the firing patterns of individual ARN^{KISS} neurons suggest that there is heterogeneity in their ion channel and/or receptor densities, although this has not been explored fully (20).

There is substantial variation in the scale of synchronizations in brain slices with most comprising two or three neurons activating together within the limited field of view. Larger mSEs also occur which are able to recruit up to two-thirds of recorded neurons. These larger mSEs may represent activity that would be more likely to transition to a full-scale SE *in vivo* (16). In the absence of pacemaker cells, the ability of a loosely coupled network to generate a large-scale synchronization may well depend on the gradual removal of multiple inhibitory influences created during the *previous* large-scale synchronization; this may include the depletion of neurotransmitters and peptides and receptor desensitization or internalization. If so, there would be a very high chance of a full-scale SE occurring immediately following a sudden removal of inhibition. Indeed, we occasionally observed large synchronizations following the sudden removal of artificial inhibition (CNQX) in the slice preparation (Fig.2B).

As found previously in male slices (7), we observe a very substantial reduction in calcium events and mSEs in the presence of CNQX and DAP5 or CNQX alone. We also note that CNQX effectively abolishes the ability of electrical stimulation of a single ARN^{KISS} neuron to activate other kisspeptin neurons. Together this demonstrates that AMPAR-mediated glutamate transmission is central to synchronization within the network in both males and females. It is worth noting that spontaneous synchronization is not entirely abolished by application of CNQX, possibly due to the origin of such activity sometimes being too deep within our relatively thick slices for effective drug penetration. In contrast, electrical driven stimulation originates at a patched cell near the surface. We also directly examined the potential role of NMDA receptors by undertaking experiments using DAP5 alone but found no effects on calcium event dynamics or mSEs. While not excluding a role for NMDA receptors in synaptic plasticity (21), this indicates that glutamate transmission through NMDA receptors is not essential for the synchronized burst firing of ARN^{KISS} neurons in the brain slice. Interestingly, the tightly synchronized calcium oscillations observed between ARN^{KISS} neurons in neonatal organotypic cultures are paused by application of DAP5 (22).

A role for NKB in spontaneous synchronizations in female mice

We find here that the inhibition of tachykinin signalling in the female brain slice results in a substantial reduction in the frequency of calcium events and mSEs indicating the presence of tonic stimulatory tachykinin transmission involving ARN^{KISS} neurons. This was not observed in male brain slices although the *in vivo* suppression of male SE amplitude by the same receptor cocktail led us to conclude that tachykinins operate as a "second transition" to facilitate glutamate-dependent emergent synchronization (7). To try and establish the relative importance of tachykinin signalling in female brain slice synchronization we used a high frequency electrical stimulation paradigm and tested with CNQX or the tachykinin receptor cocktail. Whereas CNQX reduced the ability of HF activation to couple a single ARN^{KISS} neuron with others by 78%, the tachykinin receptor cocktail was less effective resulting in only a 24% reduction. Thus, it appears that glutamate remains the key driver of synchronizations with likely contemporal facilitation by NKB in female slices.

Curiously, we found that the tachykinin receptor antagonist cocktail was much less effective in reducing spontaneous kisspeptin neuron couplings in control periods within the electrical stimulation experiments. This may be due to prior high frequency stimulations promoting on-going glutamatergic synchronizations in the slice; for example the activation of ARN^{KISS} neurons by NKB generates an initial tachykinin stimulation followed by robust on-going glutamate-driven synchronizations in male slices (7).

These results suggest that a sex difference exists in NKB transmission within the ARN^{KISS} neuronal network. The precise nature of any sex differences and how it may impact on activity remains unclear.

On one hand, it seems unlikely that there are substantial sex differences in tachykinin receptor expression by ARN^{KISS} neurons. When compared side-by-side, the effects of NKB on ARN^{KISS} neuron electrical or GCaMP calcium activity are not different in intact males and females (23,24). Furthermore, all ARN^{KISS} neurons in female mice express *Tacr3*, half have *Tacr1*, and none contain *Tacr2* transcripts (25) and this correlates well with evidence that all ARN^{KISS} neurons in males respond to the NK3R agonist senktide, half are activated by an NK1R agonist, and none respond to an NK2R agonist (26).

It may instead be the case that functional sex differences exist in NKB neuropeptide expression or release mechanisms in kisspeptin neurons. For example, there is evidence that more ARN^{KISS} neurons express NKB in females compared with males and that overall *Tac2* gene expression in the ARN is higher in females (27,28). Thus, it is possible that enhanced NKB release or tone within the ARN^{KISS} neuronal network of females is responsible for the more prominent facilitatory role of tachykinins in synchronizations observed here. While issues regarding developmental compensation cannot be overlooked, sex differences in tonic NKB transmission may also underly the multiple observations of disordered adult female fertility but normal adult male fertility in a range of tachykinin ligand or receptor knockout mouse models (27,29,30).

Sex differences in ARN^{KISS} neuron mSEs and membrane properties

When comparing the data presented here with that obtained using the same *ex-vivo* brain slice preparation in males, we find a 30% higher rate of calcium events and 58% increase in mSEs frequency within the female ARN^{KISS} neuron network. These calcium events represent short episodes of burst firing in ARN^{KISS} neurons (7) and, alongside their higher input resistance (Table 3), indicate that females have heightened excitability compared with males. Very few studies have made direct electrophysiological comparisons between male and female ARN^{KISS} neurons (31) so it is unclear whether this may represent variation in their intrinsic properties (20) or different levels of neurotransmitter input. As mSEs recorded in the brain slice may represent the foundations of eventual full SEs *in vivo* (7), it is interesting to observe that the higher frequency of mSEs in females mirrors their more frequent synchronizations *in vivo* (4,5).

We also observed that ARN^{KISS} neuron capacitance, a proxy for total membrane surface area, was lower in females. Previous work has found that ARN^{KISS} neuron size is increased in post-menopausal women (32) and chronic estradiol treatment of OVX female mice decreased ARN^{KISS} neuron capacitance (33,34). To our knowledge the only previous direct sex comparison of ARN^{KISS} neuron capacitance found a similar, albeit non-significant, trend for females to have lower values than males (35).

Dynorphin has no role in determining the dynamics of synchronization episodes in female mice

We found that application of NorBNI to inhibit kappa opioid receptors did not alter the rate of mSEs or the dynamics of calcium events. Thus, as in male brain slices (7), there is little or no impact of any tonic dynorphin transmission on the ability of ARN^{KISS} neurons to synchronize or, in particular, terminate their synchronized burst firing. As observed with males, we find evidence of spike frequency adaptation in female kisspeptin neurons and this is very likely to contribute to the mechanism terminating the 1-2 min burst of firing during a synchronization (7). Nevertheless, based on our recent *in vivo* studies in males, there is likely to an important role for dynorphin transmission within the kisspeptin network in gating the ease with which emergent glutamate-driven network activity transcends to achieve a full-scale SE. Understanding the likely multi-faceted role of dynorphin in controlling the generation of SEs in females will require detailed *in vivo* analyses.

Conclusion

The "glutamate two-transition" model for ARN^{KISS} neuron synchronization involves the initiation of synchronization by emergent glutamatergic network activity that needs to pass through a dynorphin gate as a first transition before being facilitated by NKB as a second transition (7). Taken together with our previous study, the current results support this model by demonstrating that emergent glutamate-AMPA transmission underlies ARN^{KISS} neuron synchronization in both male and female mice. However, we identify a more prominent role for tachykinins in contributing to spontaneous mSEs in female brain slices although this appears to be modulatory. Thus, in males, synchronizations occur through glutamate-dependent emergent activity that is later amplified by NKB release, whereas, in females, NKB may have a more continual facilitatory role during the generation and amplification of synchronous behavior. Alongside sex differences in their intrinsic properties, this variation in NKB signalling may underly the faster rate of full-scale ARN^{KISS} neuron SEs observed in females *in vivo (4)*. It will now be essential to undertake *in vivo* investigations to examine the functional significance of the current brain slice studies and, more generally the applicability of the "glutamate two-transition" model to females.

Data availability statement

Some or all datasets generated during and/or analyzed during the current study are not publicly available but are available from the corresponding author on reasonable request.

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Figure Legends

Figure 1. *Spatiotemporal analysis of ARN^{KISS} neuron synchronization*. **A & B**, Two representative examples showing the temporal relationships between individual ARN^{KISS} neurons (14 and 12 cells) across five and six miniature synchronization events (SE1-6). The heatmap codes for order with early neurons in dark blue and late neurons in light blue. Empty squares indicate that the cell did not participate in the SE. A correlation scatterplot of the same information is provided below. Alongside is given the spatial location of individual ARN^{KISS} neurons in the coronal imaging plane showing their sequence of activity for the third (**A**) and second (**B**) mSE.

Figure 2. *Glutamatergic signaling is essential for ARN^{KISS} neuron synchronization in vitro*. **A**, Example brain slice control experiment showing GCaMP fluorescence recorded from 14 neurons with a 15-min vehicle application. mSEs are indicated by blue bars, with dark blue indicating mSEs consisting of

more neurons and light blue indicating fewer. The key indicates exact number of cells taking part. **B**, Example brain slice showing the effect of 15-min exposure to CNQX (20 µM) on GCaMP fluorescence recorded from 14 neurons. **C–F**, Histograms showing the individual data points and mean (+SEM) mSE rate for the pre-drug, drug-applied and wash periods in response to vehicle, CNQX + DAP5, CNQX alone, and DAP5 alone in slices from diestrous female mice (∗p < 0.05, ∗∗p < 0.01, Wilcoxon).

Figure 3. *NKB signaling also contributes to ARN^{KISS} neuron synchronization in female mice.* **A**, Example brain slice showing the effect of 15-min exposure to the tachykinin receptor antagonist cocktail (SDZ-NKT 343 1 µM, GR94800 1 µM, SB 222200 3 µM on GCaMP fluorescence recorded from 9 neurons. mSEs are indicated by blue bars, with dark blue indicating mSEs consisting of more neurons and light blue indicating fewer. The key indicates exact number of cells taking part. **B & C**, Histograms showing the individual data points and mean (±SEM) mSE rate for the pre-drug, drug-applied and wash periods in response to the tachykinin receptor antagonist cocktail and NorBNI (10 µM) in slices from diestrous female mice (∗p < 0.05, Wilcoxon).

Figure 4. *Effects of high and low frequency activation of single ARN^{KISS} neurons on other cells.* **A**, Representative examples of low- and high-frequency firing evoked in a patched ARN^{KISS} neuron by current injection over 10-15 s. Note the marked spike frequency adaptation during high frequency stimulation. **B,** Histograms showing the percentage of trials in which coincident calcium events occurred between ARN^{KISS} neurons in the brain slice under unstimulated conditions (white) and following low frequency (green) and high frequency (red) stimulation of a single neuron in the absence and presence of CNQX or neurokinin receptor (NKR) antagonists. ∗p< 0.05, ∗∗p<0.01, ∗∗∗p<0.001, Binomial test versus Control or as indicated; number in base gives the number of stimulation trials for each group (CNQX, 15 brain slices; NKR 11 brain slices).

Figure 5. *Direct electrical excitation of one ARN^{KISS} increases GCaMP activity in nearby ARN^{KISS} neurons in an AMPA receptor dependent manner.* **A**, Example experiments showing GCaMP traces from 9 ARN^{KISS} neurons in the same slice as Neuron #10 (bottom trace) that was patched. Artificial activation of Neuron #10 at high (HF; maximal) and low (LF; mean 2.5 Hz) frequencies are highlighted in pink and green respectively, which are continued through CNQX (20 µM) application. Above-threshold GCAMP calcium transients are highlighted in blue. **B**, Example experiments showing GCaMP traces from 9 ARN^{KISS} neurons in the same slice as Neuron #10 (bottom trace) that was patched. Artificial activation of Neuron #10 at high (HF; maximal) frequency are highlighted in pink, which are continued through the application of a cocktail of neurokinin receptor (NK1-3R) antagonists. Above-threshold GCAMP calcium transients are highlighted in blue.

Table 1. *Spearman test statistics for temporal order analysis (Fig.1).*

Brain slice #	Correlation Coefficient	df	t-score	p
1	0.381	42	2.67	0.0108*
2	0.231	38	1.46	0.152
3	0.209	84	1.96	0.0536
4	0.740	15	4.26	0.00068***
5	0.581	27	3.71	0.00096***

6	0.414	30	2.49	0.0186*
7	0.477	17	2.24	0.0390*
8	0.361	26	1.98	0.0589
9	0.484	77	4.85	<0.00001****
	Mean = 0.431			

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560 **Table 2.** *No changes occur in the dynamics of GCaMP calcium events in the presence of antagonists for*
561 *AMPA receptors (CNQX), NMDA receptors (DAP5), tachykinin receptors (NKR antagonists) or kappa*
562 *opioid receptors (NorBNI) in diestrous female mice.*

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	Half width (s)	**Rise time (s)**	**Decay time (s)**	
CNQX				n=7
Pre-drug	14.6 ± 2.08	4.62 ± 1.13	6.57 ± 0.984	
Drug	11.6 ± 0.819	3.84 ± 0.488	6.55 ± 0.521	
DAP5				n=8
Pre-drug	15.5 ± 0.962	5.71 ± 0.665	6.74 ± 0.684	
Drug	14.4 ± 1.58	4.67 ± 0.678	7.01 ± 0.449	
NKR antagonists				n=8
Pre-drug	13.9 ± 0.649	3.55 ± 0.573	6.43 ± 0.517	
Drug	11.6 ± 0.951	2.98 ± 0.373	6.42 ± 0.689	
NorBNI				n=7
Pre-drug	13.2 ± 1.38	4.71 ± 4.71	6.35 ± 0.297	
Drug	12.9 ± 1.88	4.25 ± 0.871	7.43 ± 0.799	

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566 **Table 3.** *Comparative summary of ARN^{KISS} neuron and network properties between diestrous female*
567 *and male mice. Male data originate from (7).*

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	Female	**n**	**Male**	**n**	**p**
SEs/cell/hr	10.3 ± 0.8	55	6.5 ± 0.9	36	0.00048***
Events/cell/hr	14.7 ± 0.9	55	11.3 ± 1.0	36	0.025*
Event half width (s)	14.9 ± 0.9	22	14.5 ± 1.2	35	n.s.
Event rise time (s)	4.2 ± 0.5	22	4.3 ± 0.4	35	n.s.
Event decay time (s)	6.8 ± 0.3	22	6.5 ± 0.2	35	n.s.
Capacitance (pF)	15.8 ± 0.8	30	21.4 ± 1.3	14	0.0004***
Input resistance (MΩ)	743 ± 47	30	597 ± 21.1	14	0.042*
Resting Vmem (mV)	-58.7 ± 0.8	30	−61.4 ± 1.7	14	n.s.

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