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Local kisspeptin excitation of rat oxytocin neurones in late pregnancy

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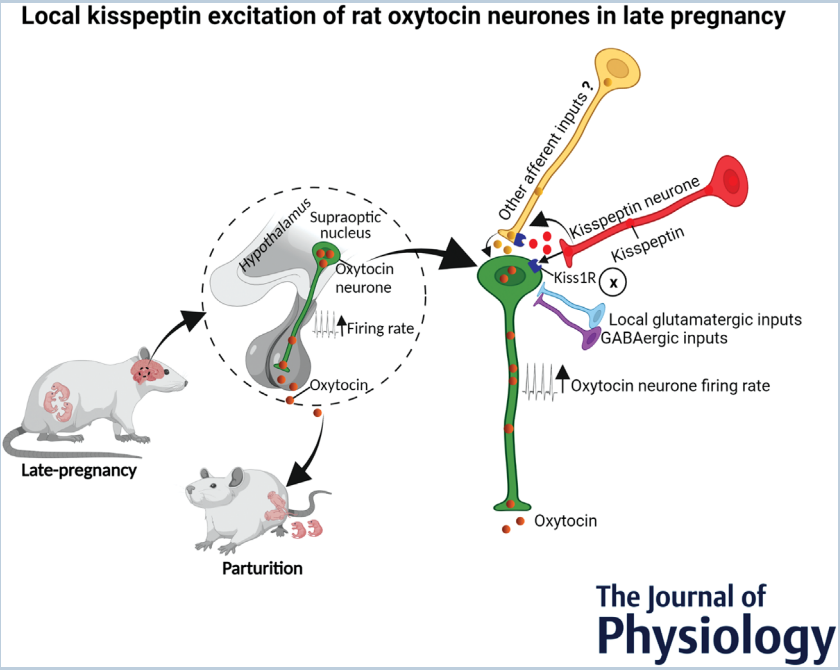

Abstract The hormone, oxytocin, is synthesised by magnocellular neurones of the supraoptic and paraventricular nuclei and is released from the posterior pituitary gland into the circulation to trigger uterine contractions during parturition. Kisspeptin fibre density increases around the supraoptic nucleus over pregnancy and intracerebroventricular kisspeptin excites oxytocin neurones only in late

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pregnancy. However, the mechanism of this excitation is unknown. Here, we found that microdialysis administration of kisspeptin into the supraoptic nucleus consistently increased the action potential (spike) firing rate of oxytocin neurones in urethane-anaesthetised late-pregnant rats (gestation day 18–21) but not in non-pregnant rats. Hazard analysis of action potential firing showed that kisspeptin specifically increased the probability of another action potential firing immediately after each action potential (post-spike excitability) in late-pregnant rats. Patch-clamp electrophysiology in hypothalamic slices showed that bath application of kisspeptin did not affect action potential frequency or baseline membrane potential in supraoptic nucleus neurones. Moreover, kisspeptin superfusion did not affect the frequency or amplitude of excitatory postsynaptic currents or inhibitory postsynaptic currents in supraoptic nucleus neurones. Taken together, these studies suggest that kisspeptin directly activates oxytocin neurones in late pregnancy, at least in part, via increased post-spike excitability.

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Abstract figure legend At parturition, oxytocin neurones produce a large amount of oxytocin that induces powerful contraction of the uterus until offspring delivery. Kisspeptin expression increases in the periventricular nucleus fibres surrounding the supraoptic nucleus in late-pregnant rats and oxytocin neurones are excited by kisspeptin. We found that intra-supraoptic nucleus kisspeptin increased the firing rate of oxytocin neurones in late-pregnant rats only. The local kisspeptin-induced excitatory effects on oxytocin neurones might be direct via Kiss1Rs or indirect via other unidentified afferent inputs but these are not mediated via local glutamatergic and GABAergic inputs. Hence, kisspeptinergic drive might be important for the activation of oxytocin neurones during parturition. Created with BioRender.com.

Key points

- Oxytocin secretion is triggered by action potential firing in magnocellular neurones of the hypothalamic supraoptic and paraventricular nuclei to induce uterine contractions during birth.
- In late pregnancy, kisspeptin expression increases in rat periventricular nucleus neurones that project to the oxytocin system.
- Here, we show that intra-supraoptic nucleus administration of kisspeptin increases the action potential firing rate of oxytocin neurones in anaesthetised late-pregnant rats, and that the increased firing rate is associated with increased oxytocin neurone excitability immediately after each action potential.
- By contrast, kisspeptin superfusion of hypothalamic slices did not affect the activity of supraoptic nucleus neurones or the strength of local synaptic inputs to supraoptic nucleus neurones.
- Hence, kisspeptin might activate oxytocin neurons in late pregnancy by transiently increasing oxytocin neuron excitability after each action potential.

Introduction

The hormone oxytocin is required for normal parturition in rats. Oxytocin receptor antagonist administration delays the onset of parturition and increases the interval between delivery of pups (Antonijevic *et al*. 1995). Oxytocin is synthesised by magnocellular neurones of the hypothalamic supraoptic and paraventricular nuclei and oxytocin neurones project to the posterior pituitary gland where action potentials trigger oxytocin release into the circulation from axon terminals (Brown *et al*. 2013). Oxytocin neurone activity increases during parturition for delivery of the offspring (Summerlee, 1981) but the mechanisms that underpin this increased activity of oxytocin neurones are still to be fully characterised.

The RF-amide, kisspeptin, is synthesised in rodents by neurones of the hypothalamic arcuate nucleus, periventricular nucleus and anteroventral periventricular nucleus, which project widely throughout the brain (Lehman *et al*. 2013), including to the supraoptic nucleus (Desroziers *et al*. 2010). The kisspeptin projection to the supraoptic nucleus arises specifically from the periventricular nucleus, and the density of this kisspeptin

projection increases at the end of pregnancy in rats, particularly in the perinuclear zone surrounding the supraoptic nucleus (Seymour *et al.* 2017). The perinuclear zone is a rich source of glutamatergic and GABAergic innervation of the supraoptic nucleus that relays afferent information to oxytocin neurones from many other brain areas (Brown *et al.* 2013). While intravenous (I.V.) kisspeptin increases circulating oxytocin levels in non-pregnant rats (Kotani *et al.* 2001) via vagal activation of oxytocin neurones (Scott & Brown, 2011), intracerebroventricular (I.C.V.) kisspeptin only excites oxytocin neurones in late-pregnant rats (Scott & Brown, 2013; Seymour *et al.* 2017; Augustine *et al.* 2018). Hence, it appears that central kisspeptin might specifically increase the activity of oxytocin neurones at the end of pregnancy, but the mechanisms that underpin this excitation are unknown.

Therefore, we used *in vivo* and patch-clamp electrophysiology to test the hypothesis that kisspeptin excites oxytocin neurones via modulation of perinuclear zone glutamatergic and GABAergic inputs. We found that the firing rate of oxytocin neurones was consistently increased *in vivo* by intra-supraoptic nucleus kisspeptin administration only in late-pregnant rats and that this excitation was associated with transiently increased excitability after each action potential. However, kisspeptin did not affect excitatory postsynaptic currents (EPSCs) or inhibitory postsynaptic currents (IPSCs) in supraoptic nucleus neurones *in vitro*. Therefore, we conclude that central kisspeptin principally increases oxytocin neurone excitability at the end of pregnancy via modulation of channels that regulate post-spike excitability.

Methods

Ethical approval

All experimental procedures were approved by the University of Otago Animals Ethics Committee (approval number: D56/17) and completed in accordance with the New Zealand Animal Welfare Act and associated guidelines.

Animals

Adult female Sprague–Dawley rats (6–12 weeks of age) were obtained from the Animal Facility of the University of Otago and housed under controlled temperature (22–24°C) and light–dark cycle (12 h light/12 h dark period scheduled) conditions, with free access to standard laboratory rodent food and water. To prevent any potential confounding effects of the oestrous cycle, non-pregnant rats were freely cycling virgin rats and were housed in groups of three to five. Primiparous pregnant rats were housed individually from gestation day 14 (G14) and used on G18−21 (G21 being the expected day of parturition).

For timed mating, oestrous cycle stage was assessed by vaginal cytology. At pro-oestrus, rats were placed overnight with a male for mating, and the next morning was considered to be G0 after confirmation of the presence of sperm in the vaginal smear.

In vivo electrophysiology

On experimental days, freely cycling non-pregnant rats (210–300 g) or late-pregnant rats (G18−21; 320–450 g) were anaesthetised by intraperitoneal injection of urethane (1.25 g kg^{-1}, ethyl carbamate, Sigma, Saint Louis, MO, USA). Deep anaesthesia was confirmed by the absence of a limb withdrawal reflex in response to a firm toe pinch. The left femoral vein was catheterised for I.V. administration of cholecystokinin-8S (CCK-8S, 20 μg kg^{-1}, 0.5 ml kg^{-1} in 0.9% saline, Sigma), and a tracheotomy was performed to maintain a patent airway throughout the experiment.

The rats were positioned supine in a stereotaxic apparatus, and the ventral surface of the brain was exposed using transpharyngeal surgery, as previously described (Brown *et al.* 2014). A U-shaped microdialysis probe (total membrane length of 2 mm; permeable to 10 kDa; in-house design) (Horn & Engelmann, 2001) was bent horizontally at an angle of 100–150° and positioned on the exposed surface of the supraoptic nucleus. Artificial cerebrospinal fluid (aCSF, in mM: 138 NaCl, 3.36 KCl, 9.52 $NaHCO_3$, 0.49 Na_2HPO_4, 2.16 urea, 1.26 $CaCl_2$, 1.18 $MgCl_2$) was dialysed at 3 μl min^{-1}.

Single-unit extracellular recordings were made using a fine-tipped glass recording micropipette (15–40 MΩ resistance) filled with 0.9% saline that was placed through the centre of the microdialysis probe. Signals were processed using a Neurolog system linked to an analog–digital converter (1401, Cambridge Electronic Design, Cambridge, UK) and recordings were captured as events and waveforms using Spike2 software (v8.09, Cambridge Electronic Design).

To confirm that recordings were from supraoptic nucleus neurones, a side-by-side bipolar stimulating electrode (SNEX-200, Science Products GmbH, Hofheim, Germany) was inserted into the pituitary stalk to evoke antidromic action potentials. Oxytocin neurones were distinguished from vasopressin neurones by transiently increasing firing rate by ≥0.5 spikes s^{-1} over 5 min in response to I.V. CCK-8S (Brown *et al.* 1996). Phasic activity in vasopressin neurones was characterised as periods of activity that lasted ≥5 s with ≥20 spikes, ≥5 s interburst interval and <1 spike s^{-1} between bursts. Vasopressin neurones for which these parameters partitioned

≥95% spikes into bursts were categorised as phasic (Scott *et al*. 2009).

To determine the effect of intra-supraoptic nucleus kisspeptin, dialysis fluid was switched to aCSF containing 100 μM kisspeptin (kisspeptin-10, Merck, Darmstadt, Germany) for 60 min. After recordings were complete, rats were euthanised by 0.5 ml 3M KCl I.V. Pup death *in utero* was confirmed after the dam was euthanised.

In vivo electrophysiological data analysis

The firing rate of all neurones were calculated in 10 min bins from 10 min before the start of kisspeptin administration. To determine whether any changes in the firing rate of supraoptic nucleus neurones were associated with post-spike excitability, hazard functions were constructed for the 10 min before, and the last 10 min during, kisspeptin administration. For this, interspike interval histograms were generated with a 0.01 s bin width for 1.5 s following each action potential and hazard functions were calculated using the formula:

$$\text{Hazard}_{(i-1,i)} = n_{(i-1,i)}/(N - n_{(0,i-1)})$$

$\text{Hazard}_{(i-1,i)}$ is the hazard value at interval i, $n_{(\text{i}-1,\text{i})}$ is the number of action potentials in interval i, $n_{(0,i-1)}$ is the total number of action potentials preceding the current interval and N is the total number of action potentials in all intervals (Brown *et al*. 2005). Hazard functions assess the inferred probability (as a decimal) that a neurone will generate an action potential in any interval following action potentials, given that no other action potential occurred before (Brown & Leng, 2000).

In vitro electrophysiology

Non-pregnant rats or late-pregnant rats were decapitated by guillotine. Pup death *in utero* was confirmed after decapitation of the dam. Coronal slices of 300 μm were cut through the supraoptic nucleus in carbogenated (95% O_2–5% CO_2) ice-cold slicing aCSF (in mM: 87 NaCl, 2.5 KCl, 25 $NaHCO_3$, 1.25 NaH_2PO_4, 0.5 $CaCl_2$, 6 $MgCl_2$, 75 D-sucrose, 25 D-glucose). Slices were kept for at least 60 min at 32°C in carbogenated recording aCSF (in mM: 120 NaCl, 3 KCl, 26 $NaHCO_3$, 1 NaH_2PO_4, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 11 D-glucose) before transfer to a recording chamber continuously superfused with recording aCSF (2 ml min^{-1}) at 20–25°C. Supraoptic nucleus neurones were viewed using differential inference contrast (DIC) optics on a light microscope (BX51WI, Olympus, Tokyo, Japan) attached to a CCD camera (RI-1000E, Dage-MTI, Michigan City, IN, USA), and relayed to a monitor through a video to PC converter (CM398, Cypress Technology, Taiwan). Recording micropipettes (2–5 MΩ) were pulled from borosilicate capillary tubes (1.5 mm outer diameter and 1.17 mm inner diameter) using a P97 puller (Sutter Instrument Co., Novato, CA, USA). Signals were filtered at 1 kHz and digitised at 10 kHz. Acquisition was performed using Clampex (v 10.1, Molecular Devices, Union City, CA, USA). All recordings were made from the dorsal, oxytocin neurone-rich region of the supraoptic nucleus (Hou-Yu *et al*. 1986).

Cell-attached recordings (10–20 MΩ seal) were obtained in voltage-clamp (0 mV holding potential) from supraoptic nucleus neurones to investigate the effect of kisspeptin superfusion on spontaneous action potential firing. Recording micropipettes (2–5 MΩ) were filled with (in mM): 10 HEPES, 120 NaCl, 3 KCl, 26 $NaHCO_3$, 1 NaH_2PO_4, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 11 D-glucose. The $GABA_A$ receptor antagonist bicuculline (10 μM, Tocris, Bristol, UK) was perfused throughout the recording period; 20 mM KCl was applied for 5 min at the end of recordings, and only recordings from neurones that were excited by KCl were included in the analysis.

Whole-cell recordings (>1 GΩ seal) were obtained in voltage clamp (−60 mV) from supraoptic nucleus neurones to investigate the effect of kisspeptin superfusion on holding current, EPSCs and IPSCs. Recording micropipettes (3–5 MΩ) were filled with (in mM): 10 HEPES, 2 MgATP, 0.2 NaGTP, 1 EGTA, 0.1 $CaCl_2$ with 124 potassium gluconate for EPSCs or 130 KCl for IPSCs (to increase IPSC amplitude); pH: 7.2; 295 mOsmol kg^{-1}.

EPSCs were measured during continuous superfusion of picrotoxin (200 μM, Sigma) to block GABAergic transmission, and IPSCs were measured in the presence of 6-cyano-7-nitroquinoxaline-2-3-dione (CNQX, 10 μM, Sigma) to block glutamatergic transmission. Miniature EPSCs and IPSCs (mEPSCs and mIPSCs) were isolated from spontaneous EPSCs and IPSCs (sEPSCs and sIPSCs) using tetrodotoxin citrate (TTX, 500 nM, Alomone Laboratories, Jerusalem, Israel).

In all experiments, after recording a stable pre-kisspeptin period for 5 min, kisspeptin (1 μM in recording aCSF) was applied for 5 min and a post-kisspeptin period (washout) was recorded for 5 min. sEPSCs/sIPSCs were recorded first with kisspeptin applied and washed out, and then TTX was washed in to isolate mEPSCs/mIPSCs before kisspeptin was re-applied.

In vitro electrophysiological data analysis

Recordings were analysed using Clampfit (v 10.1, Molecular Devices). The final 2 min of the pre-kisspeptin, kisspeptin and post-kisspeptin recording periods were used for all analyses. Action currents were identified using a threshold search in cell attached recordings. Holding current was calculated as the mean holding current during each analysis period. EPSCs and IPSCs were identified

using a template based on averaging 15–20 pre-kisspeptin EPSCs/IPSCs within each recording.

Statistical analysis

All data are presented as means (standard deviation, SD). Student's *t*-test was conducted to determine differences between two groups, and two-way repeated measures (RM) ANOVA was used to determine differences between multiple groups. All-pairwise Holm–Sidak tests were used for *post hoc* analyses when the *F*-ratio was significant. Pearson's product moment correlations were used for determining correlations. Results were considered significant when $P \leq 0.05$.

Results

Basal firing rate of supraoptic nucleus oxytocin and vasopressin neurones *in vivo*

In vivo extracellular recordings were obtained from 60 supraoptic nucleus neurones in 18 non-pregnant and 30 late-pregnant rats. There was no difference in the basal firing rate of oxytocin neurones between non-pregnant rats (3.57 (1.74) spikes s^{-1}, $n = 10$) and late-pregnant rats (3.50 (2.88) spikes s^{-1}, $n = 12$, Student's *t*-test, $P = 0.952$), and no difference in the basal firing rate of vasopressin neurones between non-pregnant rats (4.75 (2.33) spikes s^{-1}, $n = 17$) and late-pregnant rats (6.20 (3.72) spikes s^{-1}, $n = 31$, Student's *t*-test, $P = 0.153$).

Intra-supraoptic nucleus kisspeptin increases the firing rate of oxytocin neurones in late-pregnant rats

The effects of intra-supraoptic nucleus kisspeptin administration (100 μM in the dialysate) were tested on the firing rate of seven oxytocin neurones from six non-pregnant rats and seven oxytocin neurones from five late-pregnant rats. Kisspeptin consistently increased the firing rate of oxytocin neurones in late-pregnant rats but not in non-pregnant rats (reproductive status (RS) and time (T) interaction (RS × T): $F_{(1,12)} = 10.3$, $P < 0.001$, two-way RM-ANOVA; Fig. 1). There was no correlation between the response to kisspeptin and the day of gestation ($r = -0.546$, $P = 0.204$).

Kisspeptin excitation of oxytocin neurones in late-pregnant rats correlates with changes in post-spike excitability in late-pregnant rats

To determine whether excitatory effects of kisspeptin on oxytocin neurones were associated with changes in oxytocin neurone post-spike excitability, the peak early

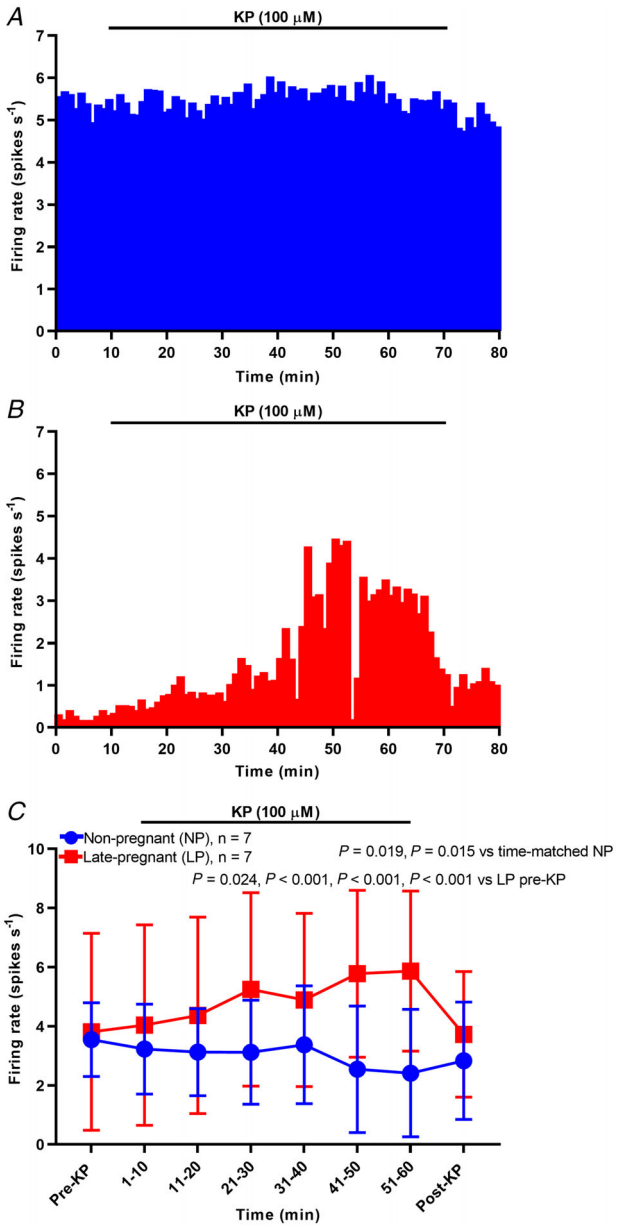

Figure 1. Intra-supraoptic nucleus kisspeptin excites oxytocin neurones in late-pregnant rats
A and *B*, example ratemeter recordings (in 1 min bins) of oxytocin neurone firing rate in a non-pregnant rat (*A*) and a late-pregnant rat (*B*) before, during (60 min) and after kisspeptin (KP) administration (100 μM in the dialysate). *C*, mean (SD) firing rate (in 10 min bins) of oxytocin neurones recorded from non-pregnant (blue circles, $n = 7$) and late-pregnant (red squares, $n = 7$) rats before, during and after kisspeptin administration. Two-way repeated measures ANOVA revealed no main effect of reproductive status ($F_{(1,6)} = 3.09$, $P = 0.104$) but a main effect of time ($F_{(1,6)} = 5.00$, $P < 0.001$) and a significant interaction between reproductive status × time ($F_{(1,12)} = 10.3$, $P < 0.001$). *Post hoc* Holm–Sidak tests revealed a significant effect of kisspeptin in late-pregnant rats compared to time-matched non-pregnant rats ($P = 0.019$ within 50 min and $P = 0.015$ within 60 min), and within-group significant increases in firing rate at 30 ($P = 0.024$), 40, 50 and 60 min (all $P < 0.001$) *versus* pre-kisspeptin. [Colour figure can be viewed at wileyonlinelibrary.com]

hazard (maximum hazard ≤0.07 s after each action potential) and mean late hazard (mean hazard from 0.4 to 0.5 s after each action potential) were calculated for the 10 min before and the last 10 min during kisspeptin administration (seven oxytocin neurones from non-pregnant rats and seven oxytocin neurones from late-pregnant rats; Fig. 2*A*–*F*). Intra-supraoptic nucleus kisspeptin had no effect on the peak early hazard of oxytocin neurones in non-pregnant or late-pregnant rats (RS: $F_{(1,1)} = 3.34$, $P = 0.092$; T: $F_{(1,1)} = 1.88$, $P = 0.195$; RS × T: $F_{(1,2)} = 1.93$, $P = 0.189$, two-way RM-ANOVA). The mean late hazard was significantly higher during kisspeptin in late-pregnant rats compared to non-pregnant rats (RS: $F_{(1,1)} = 4.04$, $P = 0.067$; T: $F_{(1,1)} = 0.194$, $P = 0.667$; RS × T: $F_{(1,2)} = 14.7$, $P = 0.002$).

There was no correlation between the change in firing rate and the maximum difference in peak early hazard ($r = 0.183$, $P = 0.694$; Fig. 2*G*) or mean late hazard of oxytocin neurones in non-pregnant rats ($r = -0.238$, $P = 0.607$; Fig. 2*H*). However, there was a positive correlation between the change in firing rate and the maximum difference in peak early hazard of oxytocin neurones in late-pregnant rats ($r = 0.924$, $P = 0.003$; Fig. 2*I*), but not between the change in firing rate and the maximum difference in mean late hazard ($r = -0.235$, $P = 0.610$; Fig. 2*J*).

Intra-supraoptic nucleus kisspeptin effects on vasopressin neurone activity

To determine whether the excitatory effects of kisspeptin were specific to oxytocin neurones in late-pregnant rats, we determined the effects of kisspeptin on the firing rate of 15 vasopressin neurones from 12 non-pregnant rats and 22 vasopressin neurones from 18 late-pregnant rats.

Vasopressin neurones displayed either continuous or phasic activity. Therefore, we analysed kisspeptin effects on the firing rate of continuously-active vasopressin neurones (10 in non-pregnant rats and 16 in late-pregnant rats) and phasic vasopressin neurones (five in non-pregnant rats and six in late-pregnant rats) separately. Kisspeptin had no effect on the firing rate of continuously-active vasopressin neurones in non-pregnant or late-pregnant rats (RS: $F_{(1,6)} = 1.38$, $P = 0.250$; T: $F_{(1,6)} = 0.245$, $P = 0.961$; RS × T: $F_{(1,12)} = 0.580$, $P = 0.746$; Fig. 3). By contrast, intra-supraoptic nucleus kisspeptin significantly reduced the firing rate of phasic vasopressin neurones in late-pregnant rats (RS × T: $F_{(1,12)} = 2.94$, $P = 0.016$; Fig. 4).

While kisspeptin decreased overall firing rate in phasic vasopressin neurones in late-pregnant rats, there was no significant effect on intraburst firing rate (RS: $F_{(1,1)} = 0.007$, $P = 0.933$; T: $F_{(1,1)} = 0.111$, $P = 0.747$; RS × T: $F_{(1,2)} = 0.921$, $P = 0.362$; Fig. 5*A*), burst duration (RS: $F_{(1,1)} = 0.005$, $P = 0.944$; T: $F_{(1,1)} = 0.022$, $P = 0.885$; RS × T: $F_{(1,2)} = 0.127$, $P = 0.729$; Fig. 5*B*) or interburst interval (RS: $F_{(1,1)} = 1.04$, $P = 0.334$; T: $F_{(1,1)} = 1.20$, $P = 0.300$; RS × T: $F_{(1,2)} = 0.009$, $P = 0.926$; Fig. 5*C*).

Correlation between kisspeptin-induced changes in the firing rate and post-spike excitability of vasopressin neurones

The peak early hazard and mean late hazard were calculated for continuously-active vasopressin neurones (nine in non-pregnant rats and 16 in late-pregnant rats; Fig. 6*A*–*F*) and phasic vasopressin neurones (five in non-pregnant rats and six in late-pregnant rats; Fig. 7*A*–*F*) in the 10 min before and the last 10 min during kisspeptin administration.

Intra-supraoptic nucleus kisspeptin had no effect on the peak early hazard (RS: $F_{(1,1)} = 0.648$, $P = 0.429$; T: $F_{(1,1)} = 0.659$, $P = 0.425$; RS × T: $F_{(1,2)} = 1.15$, $P = 0.294$, two-way RM-ANOVA) or mean late hazard (RS: $F_{(1,1)} = 0.892$, $P = 0.354$; T: $F_{(1,1)} = 0.184$, $P = 0.671$; RS × T: $F_{(1,2)} = 0.372$, $P = 0.547$) of continuously active vasopressin neurones in non-pregnant rats or late-pregnant rats.

In continuously-active vasopressin neurones of non-pregnant rats, there was a positive correlation between the change in firing rate and the maximum difference in peak early hazard ($r = 0.711$, $P = 0.031$; Fig. 6*G*) but no correlation between the change in firing rate and the maximum difference in mean late hazard ($r = 0.619$, $P = 0.075$; Fig. 6*H*). Furthermore, in late-pregnant rats, there was a positive correlation between the change in firing rate of continuously-active vasopressin neurones and the maximum difference in peak early hazard ($r = 0.959$, $P < 0.0001$; Fig. 6*I*) and mean late hazard ($r = 0.756$, $P = 0.0007$; Fig. 6*J*)

Similarly, intra-supraoptic nucleus kisspeptin had no effect on the peak early hazard (RS: $F_{(1,1)} = 0.057$, $P = 0.815$; T: $F_{(1,1)} = 0.392$, $P = 0.546$; RS × T: $F_{(1,2)} = 1.31$, $P = 0.281$, two-way RM-ANOVA) or mean late hazard (RS: $F_{(1,1)} = 0.019$, $P = 0.892$; T: $F_{(1,1)} = 0.085$, $P = 0.777$; RS X T: $F_{(1,2)} = 0.235$, $P = 0.639$) of phasic vasopressin neurones in non-pregnant rats or late-pregnant rats.

In phasic vasopressin neurones of non-pregnant rats, there was also a positive correlation between the change in firing rate and the maximum difference in peak early hazard ($r = 0.918$, $P = 0.027$; Fig. 7*G*) but no correlation between the change in firing rate and the maximum difference in mean late hazard ($r = 0.573$, $P = 0.311$; Fig. 7*H*). In late-pregnant rats, there was no correlation between the change in firing rate of phasic vasopressin

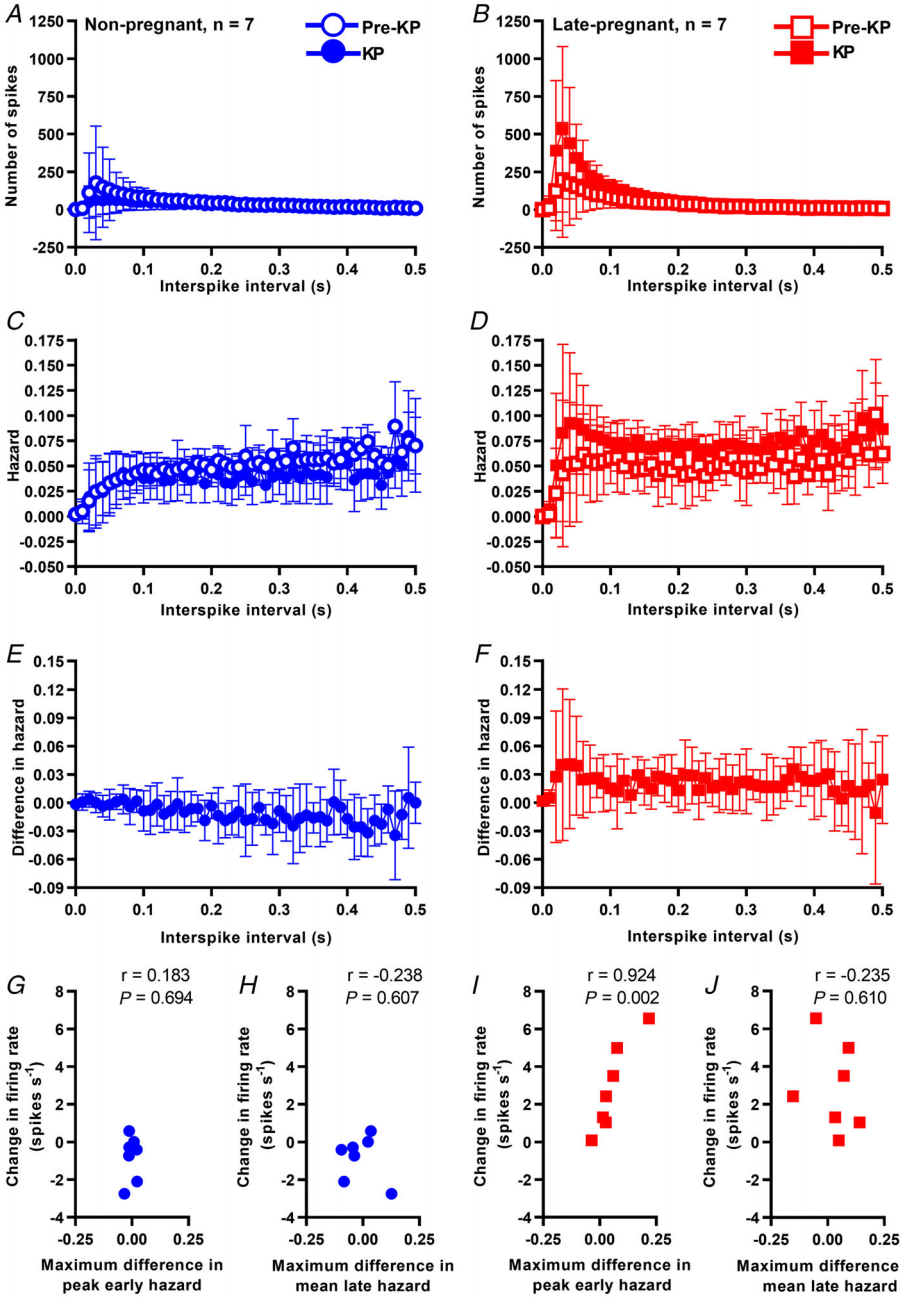

Figure 2. Intra-supraoptic nucleus kisspeptin excitation of oxytocin neurones is associated with increased post-spike excitability in late-pregnant rats
A and *B*, mean (SD) interspike intervals of oxytocin neurones in the 10 min before kisspeptin (KP) administration (open symbols) and the final 10 min during kisspeptin administration (filled symbols) from non-pregnant rats (n = 7) (*A*) and late-pregnant rats (n = 7) (*B*). *C* and *D*, mean (SD) hazard functions of the interspike intervals of the oxytocin neurones before and during kisspeptin from non-pregnant rats (*C*) and late-pregnant rats (*D*). *E* and *F*, subtraction plot of the differences between the hazard functions before and during kisspeptin from non-pregnant rats (*E*) and late-pregnant rats (*F*). *G*–*J*, scatter plots of the change in firing rate of oxytocin neurones before and during kisspeptin *versus* the maximum difference in peak early hazard (*G*) and mean late hazard (*H*) in non-pregnant rats, and peak early hazard (*I*) and mean late hazard (*J*) in late-pregnant rats. There was no correlation between the change in firing rate and the maximum difference in peak early hazard (r = 0.183, P = 0.694, Pearson's product moment correlation) or mean late hazard (r = −0.238, P = 0.607) of oxytocin neurones in non-pregnant rats. However, there was a positive correlation between the change in firing rate and the maximum difference in peak early hazard of oxytocin neurones in late-pregnant rats (r = 0.924, P = 0.003) but no correlation between the change in firing rate and maximum difference in mean late hazard of oxytocin neurones in late-pregnant rats (r = −0.235, P = 0.610). [Colour figure can be viewed at wileyonlinelibrary.com]

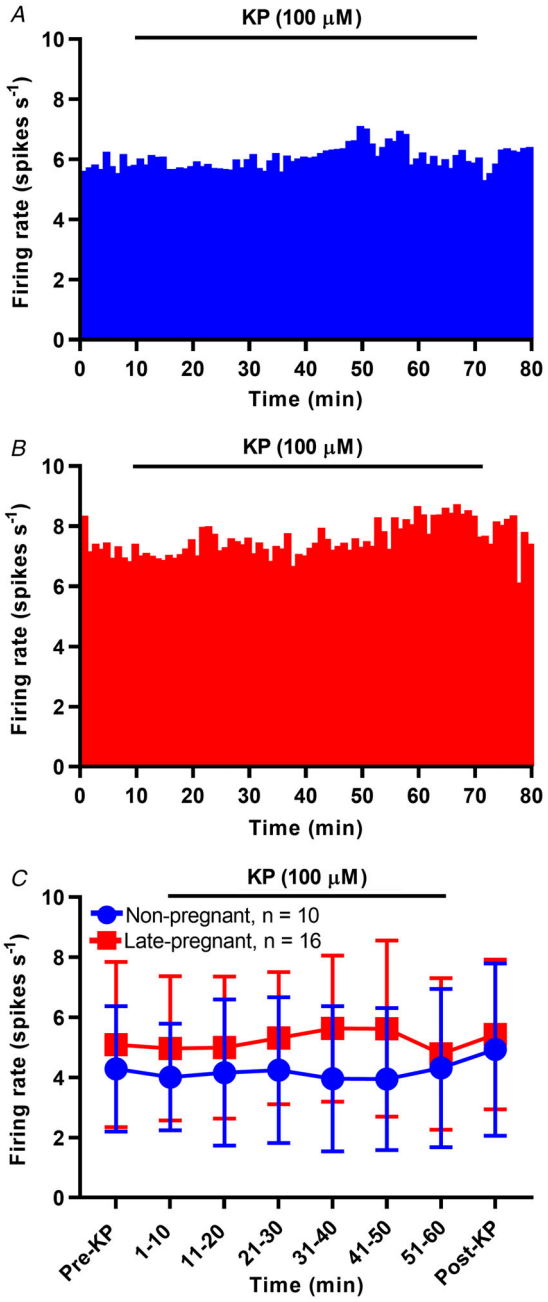

Figure 3. Intra-supraoptic nucleus kisspeptin does not affect the firing rate of continuously active vasopressin neurones in non-pregnant or late-pregnant rats
A and *B*, example ratemeter recordings (in 1 min bins) of continuously-active vasopressin neurone firing rate in a non-pregnant rat (*A*) and a late-pregnant rat (*B*) before, during (60 min) and after kisspeptin (KP) administration (100 μM in the dialysate). *C*, mean (SD) firing rate (in 10 min bins) of continuously-active vasopressin neurones recorded from non-pregnant (blue circles, n = 10) and late-pregnant (red squares, n = 16) rats before, during and after kisspeptin administration. Two-way repeated measures ANOVA revealed no main effect of reproductive status ($F_{(1,6)}$ = 1.38, P = 0.250) or time ($F_{(1,6)}$ = 0.245, P = 0.961) and no interaction between reproductive status × time ($F_{(1,12)}$ = 0.580, P = 0.746). [Colour figure can be viewed at wileyonlinelibrary.com]

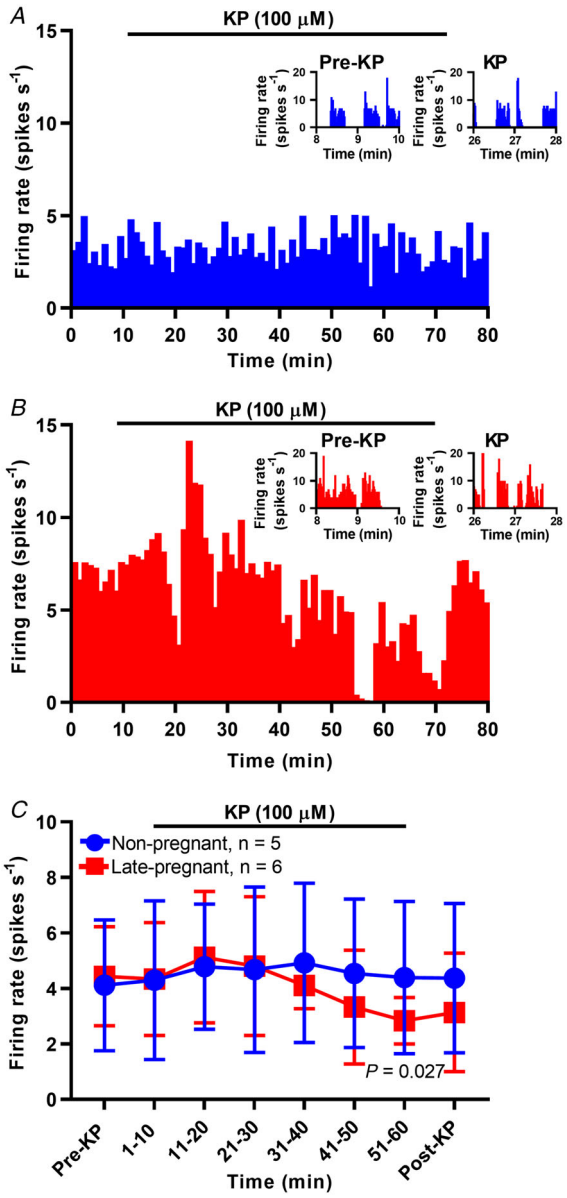

Figure 4. Intra-supraoptic nucleus kisspeptin inhibits phasic vasopressin neurones in late-pregnant rats
A and *B*, example ratemeter recordings (in 1 min bins) of phasic vasopressin neurone firing rate in a non-pregnant rat (*A*) and a late-pregnant rat (*B*) before, during (60 min) and after kisspeptin (KP) administration (100 μM in the dialysate). The insets show 2 min segments of recording (in 1 s bins) from each graph before (Pre-KP) and during (KP) kisspeptin administration to illustrate phasic bursts. *C*, mean (SD) firing rate (in 10 min bins) of phasic vasopressin neurones recorded from non-pregnant (blue circles, n = 5) and late-pregnant (red squares, n = 6) rats before, during and after kisspeptin administration. Two-way repeated measures ANOVA revealed no main effect of reproductive status ($F_{(1,6)}$ = 0.328, P = 0.581) but a main effect of time ($F_{(1,6)}$ = 3.18, P = 0.010) and a significant interaction between reproductive status × time ($F_{(1,12)}$ = 2.94, P = 0.016). *Post hoc* Holm–Sidak tests revealed a significant decrease in the firing rate within late-pregnant group at 60 min *versus* pre-kisspeptin but the firing rate of the non-pregnant group remain unaffected by kisspeptin (P = 0.027). [Colour figure can be viewed at wileyonlinelibrary.com]

neurones and the maximum difference in peak early hazard ($r = -0.461$, $P = 0.356$; Fig. 7*I*) and mean late hazard ($r = 0.565$, $P = 0.242$; Fig. 7*J*).

Kisspeptin does not affect spontaneous action potential firing or whole-cell holding current in supraoptic nucleus neurones *in vitro*

To determine whether kisspeptin activates supraoptic oxytocin neurones *in vitro*, we analysed the effect of kisspeptin on action potential frequency in cell-attached recordings from 11 supraoptic nucleus neurones from seven non-pregnant rats and 12 neurones from seven late-pregnant rats. Kisspeptin had no effect on the action potential frequency of supraoptic nucleus neurones in non-pregnant or late-pregnant rats (RS: $F_{(1,1)} = 3.68$, $P = 0.069$; T: $F_{(1,2)} = 1.05$, $P = 0.358$; RS × T: $F_{(1,3)} = 0.829$, $P = 0.444$, two-way RM-ANOVA; Fig. 8).

To test whether kisspeptin depolarises supraoptic nucleus neurones, we determined the effect of kisspeptin on baseline holding currents in whole-cell voltage clamp recordings in nine neurones from six non-pregnant rats and six neurones from five late-pregnant rats. Kisspeptin had no effect on the mean holding currents in supraoptic nucleus neurones from non-pregnant or late-pregnant rats (RS: $F_{(1,1)} = 0.294$, $P = 0.597$; T: $F_{(1,2)} = 0.202$, $P = 0.818$; RS × T: $F_{(1,3)} = 0.094$, $P = 0.910$).

Kisspeptin does not affect glutamatergic transmission in supraoptic nucleus neurones

To test whether kisspeptin excites supraoptic nucleus neurones via glutamatergic inputs, we measured the frequency and amplitude of sEPSCs and mEPSCs in whole-cell recordings in 10 supraoptic nucleus neurones from five non-pregnant rats and nine neurones (for sEPSCs) and eight neurones (for mEPSCs) from seven late-pregnant rats.

While sEPSC frequency was lower in supraoptic nucleus neurones from late-pregnant rats (RS: $F_{(1,1)} = 6.66$, $P = 0.019$; two-way RM-ANOVA), kisspeptin had no effect on sEPSC frequency (T: $F_{(1,2)} = 2.57$, $P = 0.090$; RS × T: $F_{(1,3)} = 0.731$, $P = 0.730$, Fig. 9*A*, *B* and *E*). While there was no main effect of reproductive status on sEPSC amplitude ($F_{(1,1)} = 0.025$, $P = 0.876$), sEPSC amplitude reduced during kisspeptin administration but this effect was independent of reproductive status (T: $F_{(1,2)} = 5.48$, $P = 0.008$; RS × T: $F_{(1,3)} = 0.375$, $P = 0.690$) and did not recover post-kisspeptin ($P = 0.015$ compared to pre-kisspeptin and $P = 0.778$ compared to kisspeptin; Fig. 9*C*, *D* and *F*), suggesting that it was not a response to kisspeptin.

Similarly, mEPSC frequency was lower in supraoptic nucleus neurones from late-pregnant rats (RS: $F_{(1,1)} = 5.76$, $P = 0.028$) but kisspeptin had no effect on mEPSC frequency (T: $F_{(1,2)} = 0.366$, $P = 0.696$; RS × T:

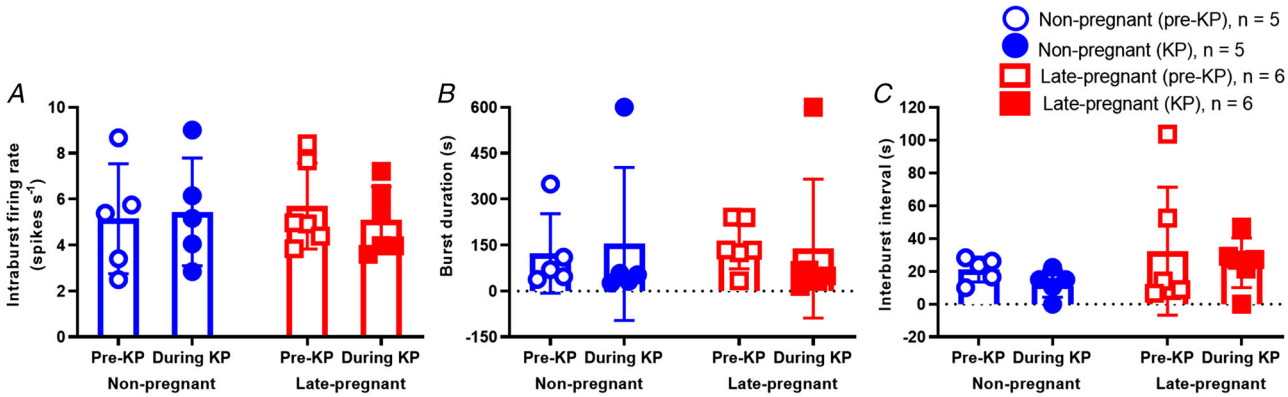

Figure 5. Intra-supraoptic nucleus kisspeptin does not affect burst parameters in phasic vasopressin neurones
A–C, burst parameters of phasic vasopressin neurones in the 10 min before kisspeptin (KP) administration (open symbols) and the 10 min during kisspeptin administration (filled symbols) from non-pregnant rats ($n = 5$) and late-pregnant ($n = 6$) rats. *A*, mean (SD) intraburst firing rate before and during kisspeptin administration (100 μM in the dialysate). Two-way repeated measures ANOVA revealed no main effect of reproductive status ($F_{(1,1)} = 0.007$, $P = 0.933$) or time ($F_{(1,1)} = 0.111$, $P = 0.747$) and no interaction between reproductive status × time ($F_{(1,2)} = 0.921$, $P = 0.362$). *B*, mean (SD) burst duration before and during kisspeptin administration (100 μM in the dialysate). Two-way repeated measures ANOVA revealed no main effect of reproductive status ($F_{(1,1)} = 0.005$, $P = 0.944$) or time ($F_{(1,1)} = 0.022$, $P = 0.885$) and no interaction between reproductive status × time ($F_{(1,2)} = 0.127$, $P = 0.729$). *C*, mean (SD) interburst interval before and during kisspeptin administration (100 μM in the dialysate). Two-way repeated measures ANOVA revealed no main effect of reproductive status ($F_{(1,1)} = 1.04$, $P = 0.334$) or time ($F_{(1,1)} = 1.20$, $P = 0.300$) and no interaction between reproductive status × time ($F_{(1,2)} = 0.009$, $P = 0.926$). [Colour figure can be viewed at wileyonlinelibrary.com]

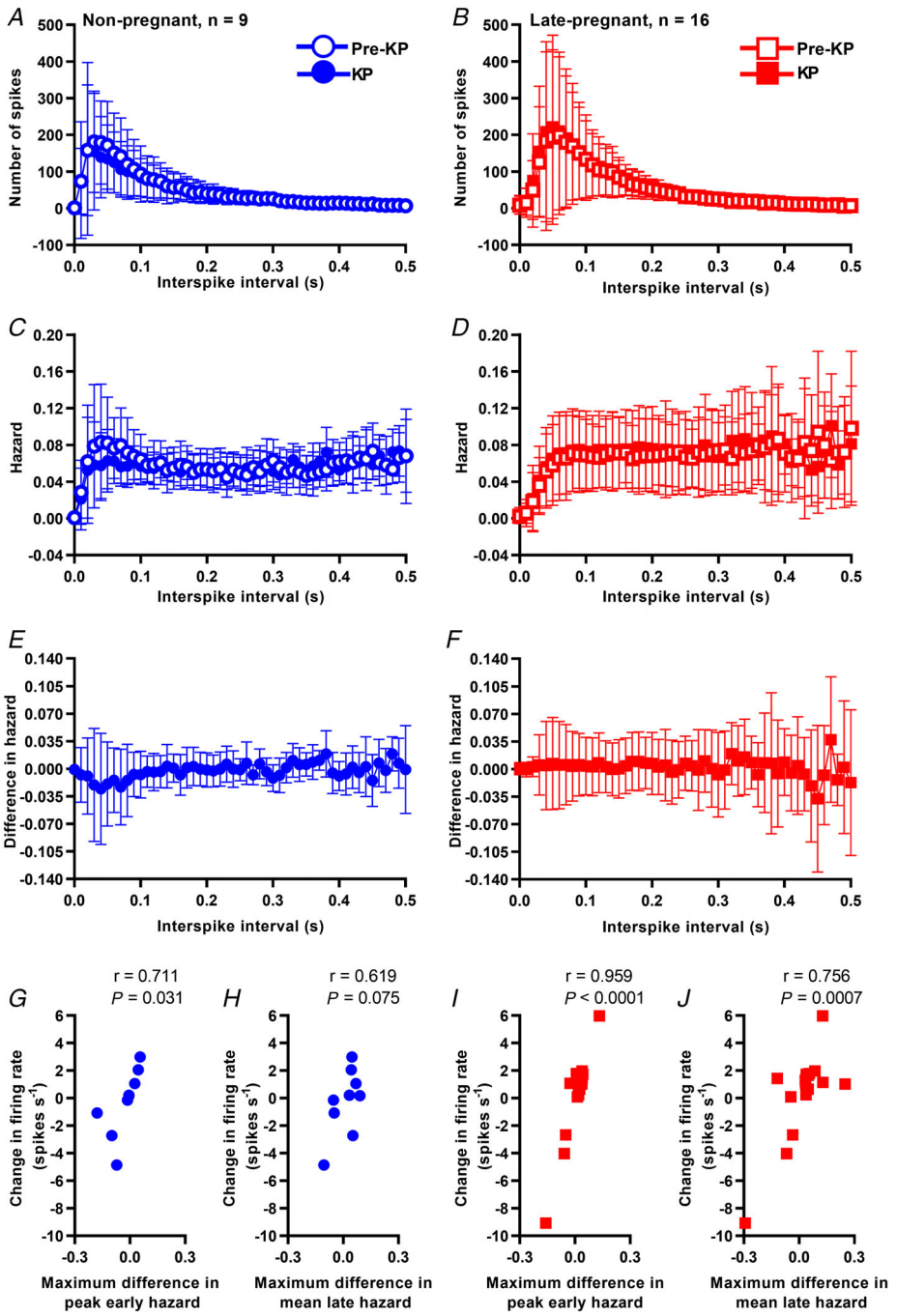

Figure 6. Intra-supraoptic nucleus kisspeptin effects on the post-spike excitability of continuously active vasopressin neurones
A and *B*, mean (SD) interspike intervals of continuously-active vasopressin neurones in the 10 min before kisspeptin (KP) administration (open symbols) and the final 10 min during kisspeptin (filled symbols) from non-pregnant rats (n = 9) (*A*) and late-pregnant rats (n = 16) (*B*). *C* and *D*, mean (SD) hazard functions (hazard per 0.01 s) of the inter-spike intervals of the continuously-active vasopressin neurones before and during kisspeptin from non-pregnant rats (*C*) and late-pregnant rats (*D*). *E* and *F*, subtraction plot of the differences between the hazard functions of continuously active vasopressin neurones before and during kisspeptin from non-pregnant (*E*) and late-pregnant (*F*) rats. *G–J*, scatter plots of the change in firing rate of continuously active vasopressin neurones before and during kisspeptin *versus* the maximum difference in peak early hazard (*G*) and mean late hazard (*H*) in non-pregnant rats, and peak early hazard (*I*) and mean late hazard (*J*) in late-pregnant rats. There was a positive correlation between the change in firing rate and the maximum difference in peak early hazard of continuously-active vasopressin neurones in non-pregnant rats (r = 0.711, P = 0.031, Pearson's product moment correlation) but no correlation

between the change in firing rate and the maximum difference in mean late hazard of continuously-active vasopressin neurones in non-pregnant rats ($r = 0.619$, $P = 0.075$). Furthermore, there was a positive correlation between the change in firing rate and the maximum difference in peak early hazard ($r = 0.959$, $P < 0.0001$) and mean late hazard ($r = 0.756$, $P = 0.0007$) of continuously-active vasopressin neurones in late-pregnant rats. [Colour figure can be viewed at wileyonlinelibrary.com]

$F_{(1,3)} = 0.492$, $P = 0.616$; Fig. 9*G*, *H* and *K*) or amplitude (RS: $F_{(1,1)} = 0.530$, $P = 0.477$; T: $F_{(1,2)} = 0.263$, $P = 0.770$; RS × T: $F_{(1,3)} = 0.379$, $P = 0.688$; Fig. 9*I*, *J* and *L*).

Kisspeptin does not affect GABAergic synaptic transmission in supraoptic nucleus neurones

To test whether kisspeptin excites oxytocin neurones via GABAergic inputs, we measured the frequency and amplitude of sIPSCs and mIPSCs in whole-cell recordings from six supraoptic nucleus neurones from four non-pregnant rats and nine supraoptic nucleus neurones from five late-pregnant rats.

While sIPSC frequency was higher in supraoptic nucleus neurones from late-pregnant rats (RS: $F_{(1,1)} = 5.89$, $P = 0.030$, two-way RM-ANOVA), kisspeptin had no effect on sIPSC frequency (T: $F_{(1,2)} = 2.51$, $P = 0.100$; RS × T: $F_{(1,3)} = 0.076$, $P = 0.927$; Fig. 10*A*, *B* and *E*) or amplitude of sIPSCs (RS: $F_{(1,1)} = 3.95$, $P = 0.068$; T: $F_{(1,2)} = 0.104$, $P = 0.902$; RS × T: $F_{(1,3)} = 0.648$, $P = 0.531$; Fig. 10*C*, *D* and *F*).

Similarly, mIPSC frequency was higher in supraoptic nucleus neurones from late-pregnant rats (RS: $F_{(1,1)} = 5.49$, $P = 0.035$) but kisspeptin had no effect on mIPSC frequency (T: $F_{(1,2)} = 0.668$, $P = 0.521$; RS × T: $F_{(1,3)} = 0.246$, $P = 0.783$; Fig. 10*G*, *H* and *K*) or amplitude of mIPSCs (RS: $F_{(1,1)} = 3.20$, $P = 0.096$; T: $F_{(1,2)} = 1.41$, $P = 0.262$; RS × T: $F_{(1,3)} = 0.715$, $P = 0.498$; Fig. 10*I*, *J* and *L*) in non-pregnant or late-pregnant rats.

Discussion

Here, we found that central kisspeptin excitation of oxytocin neurones in late pregnancy occurs via a local action within the supraoptic nucleus and that this excitation is associated with a transient increase in post-spike excitability. Moreover, kisspeptin did not affect baseline holding current or local glutamatergic and GABAergic transmission in supraoptic nucleus neurones, suggesting that kisspeptin might excite oxytocin neurons via activation of the channels that underpin post-spike excitability.

Mechanism of intra-supraoptic nucleus kisspeptin excitation of oxytocin neurones in late pregnancy

Kisspeptin fibre density increases around the supraoptic nucleus over the course of pregnancy but this increase is more prominent in the perinuclear zone surrounding the supraoptic nucleus than in the supraoptic nucleus itself, and there are no obvious close appositions between those kisspeptin fibres that penetrate the supraoptic nucleus and oxytocin neurones (Seymour *et al.* 2017). Therefore, we tested whether local microdialysis administration of kisspeptin directly into the supraoptic nucleus excites oxytocin neurons; the resulting kisspeptin-induced excitation was robust and consistent, suggesting that the excitation is mediated locally. The longer time-course of the excitation by dialysed kisspeptin compared to that elicited by I.C.V. kisspeptin likely reflects the time required for dialysed drugs to reach sufficient concentration within the tissue to have an effect (Ludwig & Leng, 1997).

Because glutamatergic and GABAergic neurones of the perinuclear zone densely innervate oxytocin neurones (Brown *et al.* 2013) and kisspeptin fibre density increases in the perinuclear zone at the end of pregnancy (Seymour *et al.* 2017), we expected that kisspeptin would excite oxytocin neurones via modulation of perinuclear zone glutamatergic and GABAergic inputs. However, we found that the frequency and amplitude of EPSCs and IPSCs were not affected by kisspeptin superfusion in supraoptic nucleus brain slices from non-pregnant and late-pregnant rats. Therefore, it appears that local kisspeptin likely does not excite oxytocin neurones via perinuclear glutamate or GABA interneurons.

The frequency of sEPSCs was similar to the frequency of mEPSCs in each group, suggesting that few EPSCs were driven by action potentials in the brain slices from non-pregnant rats and late-pregnant rats, and the same was true for sIPSCs and mIPSCs. The lack of effect of kisspeptin on the frequency of EPSCs or IPSCs is unlikely to reflect a failure to deliver sufficient kisspeptin to have an effect because 10–100 nM excites gonadotrophin releasing hormone neurones in 1–3 min (Han *et al.* 2005). Rather, it likely reflects a failure of kisspeptin to affect release probability at glutamatergic or GABAergic synapses on supraoptic nucleus neurones, or induce action potential firing in glutamatergic or GABAergic afferents within the brain slice. Furthermore, the lack of effect of kisspeptin on EPSC or IPSC amplitude suggests that the kisspeptin-induced increase in oxytocin neurone firing rate evident *in vivo* was not underpinned by altered responsiveness of supraoptic nucleus neurones to glutamate or GABA.

While there was no effect of kisspeptin on EPSCs or IPSCs, the frequency of sEPSCs and mEPSCs was lower in supraoptic nucleus neurones from late-pregnant

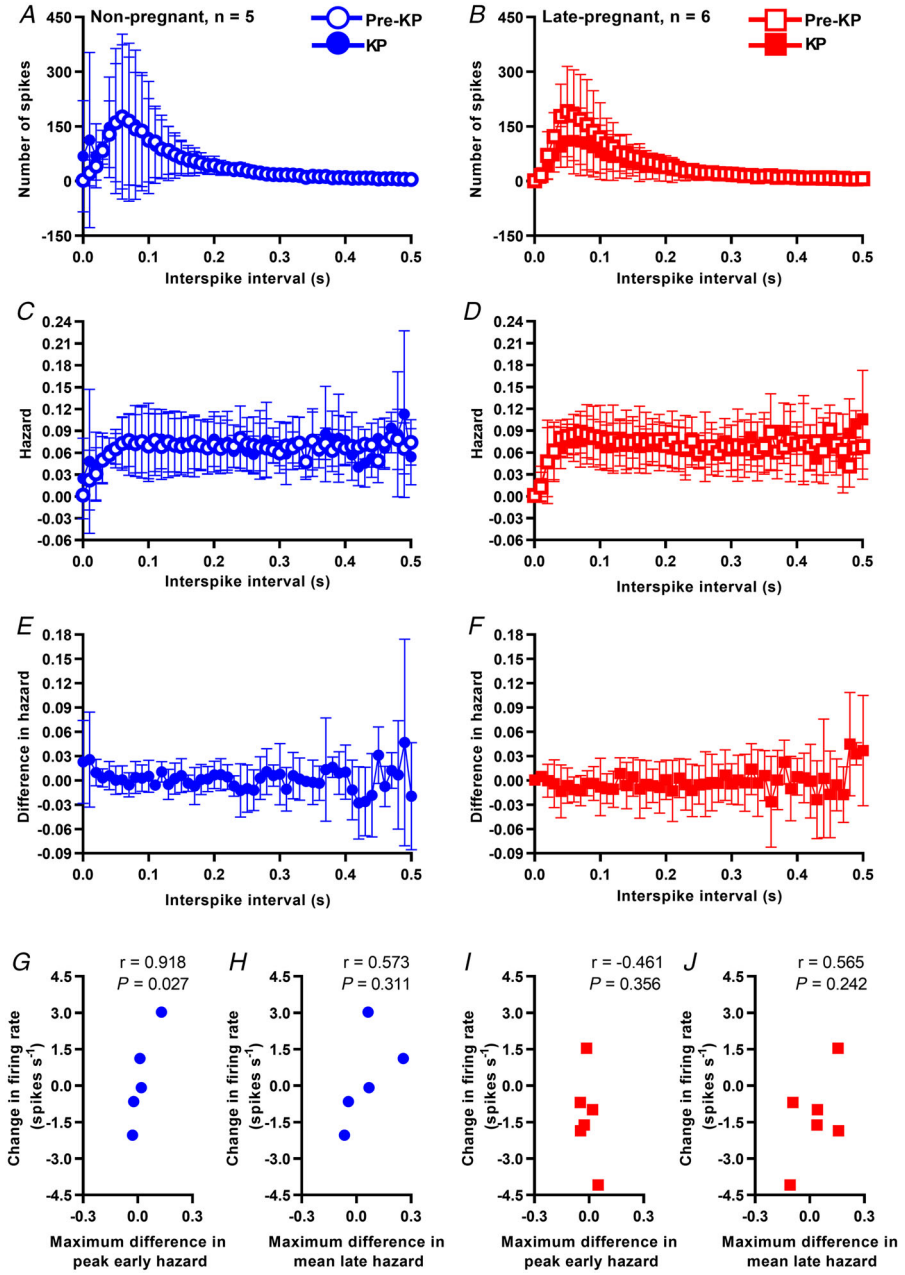

Figure 7. Intra-supraoptic nucleus kisspeptin effects on the post-spike excitability of phasic vasopressin neurones

A and *B*, mean (SD) interspike intervals of phasic vasopressin neurones in the 10 min before kisspeptin (KP) administration (open symbols) and the final 10 min during kisspeptin administration (filled symbols) from non-pregnant rats (n = 5) (*A*) and late-pregnant rats (n = 6) (*B*). *C* and *D*, mean (SD) hazard functions (hazard per 0.01 s) of the interspike intervals of the phasic vasopressin neurones before and during kisspeptin from non-pregnant rats (*C*) and late-pregnant rats (*D*). *E* and *F*, subtraction plot of the differences between the hazard functions of phasic vasopressin neurones before and during kisspeptin from non-pregnant rats (*E*) and late-pregnant rats (*F*). *G–J*, scatter plots of the change in firing rate of phasic vasopressin neurones before and during kisspeptin *versus* the maximum difference in peak early hazard (*G*) and mean late hazard (*H*) of non-pregnant rats, and peak early hazard (*I*) and mean late hazard (*J*) of late-pregnant rats. There was a positive correlation between the change in firing rate and the maximum difference in peak early hazard of phasic vasopressin neurones in non-pregnant rats (r = 0.918, P = 0.027, Pearson's product moment of correlation) but no correlation between the change in firing rate and the maximum difference in mean late hazard of phasic vasopressin neurones in non-pregnant rats (r = 0.573, P = 0.311). Also, there was no correlation between the change in firing rate and the maximum difference in peak early hazard (r = −0.461, P = 0.356) or mean late hazard (r = 0.565, P = 0.242) of phasic vasopressin neurones in late-pregnant rats. [Colour figure can be viewed at wileyonlinelibrary.com]

rats, while the frequency of sIPSCs and mIPSCs was generally higher in supraoptic nucleus neurones from late-pregnant rats. The lower excitatory synaptic drive and higher inhibitory synaptic drive on oxytocin neurones in late pregnancy appear counterintuitive but might help reduce the risk of preterm delivery by restraining oxytocin neurone activity prior to the onset of parturition.

It should be noted that we did not differentiate between oxytocin and vasopressin neurones in our patch-clamp recordings. However, all recordings were made from the dorsal, oxytocin neurone-rich region of the supraoptic nucleus (Hou-Yu *et al.* 1986) so the majority of recordings would be expected to be from oxytocin neurones. Furthermore, kisspeptin increases mEPSC frequency in identified supraoptic nucleus vasopressin neurones in male rats (Yokoyama *et al.* 2014) and we found no effect of kisspeptin on EPSCs or IPSCs in neurones from non-pregnant rats and late-pregnant rats. While we cannot rule out the possibility that the different result arises from sex differences, the most likely explanation is that our recordings are predominantly from oxytocin neurones.

We also tested whether local kisspeptin release might directly excite oxytocin neurones via volume transmission, as it does for gonadotrophin-releasing hormone neurones (Liu *et al.* 2021), but baseline holding current (and by inference, membrane potential) was not affected by kisspeptin superfusion. However, neuronal excitability is modulated by post-spike potentials as well as baseline membrane potential, and the proportion of oxytocin neurones that express an afterdepolarisation is higher in late-pregnant rats than non-pregnant rats (Teruyama & Armstrong, 2002). Post-spike potentials transiently modulate neuronal excitability after each action potential, and this is also more prominent in late pregnancy (Perkinson *et al.* 2021).

The influence of post-spike potentials on excitability is revealed by hazard analysis of action potential firing (Sabatier *et al.* 2004). The hazard function illustrates the probability of the next action potential firing at any time following each action potential, with the transient changes immediately following each action potential (peak early hazard) largely dictated by the contribution of post-spike potentials to membrane potential, while the steady-state hazard (mean late hazard) reflects the combined influence of baseline membrane potential and ongoing synaptic inputs (Brown *et al.* 2007).

The mean late hazard was increased by kisspeptin in oxytocin neurones of late-pregnant rats but neither baseline membrane potential nor EPSCs or IPSCs were affected by kisspeptin in supraoptic nucleus neurones in brain slices. Therefore, the kisspeptin-induced increase in steady-state excitability might be mediated by effects on afferent inputs that are severed in the brain slice. Brainstem noradrenergic inputs to the supraoptic nucleus are activated at parturition (Meddle *et al.* 2000), releasing noradrenaline (Herbison *et al.* 1997) to activate oxytocin neurones via α_1-adrenoreceptors (Douglas *et al.* 2001). Hence, noradrenergic afferents might mediate the kisspeptin-induced increase in steady-state excitability of oxytocin neurones in late-pregnant rats, but further work will be required to determine whether this is the case.

While mean late hazard was increased by kisspeptin in oxytocin neurones of late-pregnant rats, there was no correlation between the change in mean late hazard and the change in firing rate during kisspeptin. By contrast, there was no kisspeptin-induced change in peak early hazard in oxytocin neurones of late-pregnant rats. However, the change in peak early hazard was highly variable across oxytocin neurones during kisspeptin administration and there was a robust correlation between the change in peak early hazard and the change in firing rate during kisspeptin, suggesting that the kisspeptin-induced increase in firing rate might be driven more by a transient increase in post-spike excitability than by an increase in steady-state excitability. In addition

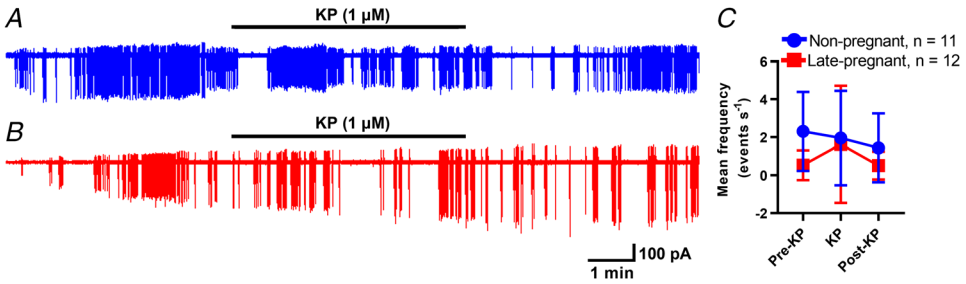

Figure 8. Kisspeptin does not affect the action current frequency of supraoptic nucleus neurones
A and *B*, example cell-attached recordings of supraoptic nucleus neurones (15 min) from a non-pregnant rat (*A*) and a late-pregnant rat (*B*) before, during (5 min) and after kisspeptin (KP) administration (1 μM). *C*, mean (SD) frequency of action currents in spontaneously active supraoptic nucleus neurones from non-pregnant (blue circles, n = 11) and late-pregnant (red squares, n = 12) rats before, during and after kisspeptin administration. Two-way repeated measures ANOVA revealed no main effect of reproductive status ($F_{(1,1)}$ = 3.68, P = 0.069) or time ($F_{(1,2)}$ = 1.05, P = 0.358) and no interaction between reproductive status × time ($F_{(1,3)}$ = 0.829, P = 0.444). [Colour figure can be viewed at wileyonlinelibrary.com]

to the afterdepolarisation, the peak early hazard of oxytocin neurones is also influenced by the medium afterhyperpolarisation and transient outward rectification (Brown *et al.* 2005) and it remains to be determined which of these kisspeptin modulates to increase post-spike excitability of oxytocin neurones in late pregnancy.

The failure of kisspeptin to increase action potential firing in the slice preparation likely results from the slice being a reduced preparation in which long-range inputs are severed. As we report here, spontaneous activity is typically much lower in the slice than *in vivo*. The influence of post-spike potentials is only evident when action potentials occur close enough together for summation of their associated post-spike potentials to bring membrane potential closer to threshold (Brown, 2016) and the firing rate of supraoptic nucleus neurones in our experiments did not enter this range, even in the presence of bicuculline to try to increase excitability.

While kisspeptin modulation of post-spike excitability of oxytocin neurones in late pregnancy is presumably a direct effect on the oxytocin neurones themselves, the receptor involved remains to be determined. While the Kiss1R mRNA is expressed in oxytocin neurones (Higo *et al.* 2016; Seymour *et al.* 2017), supraoptic nucleus

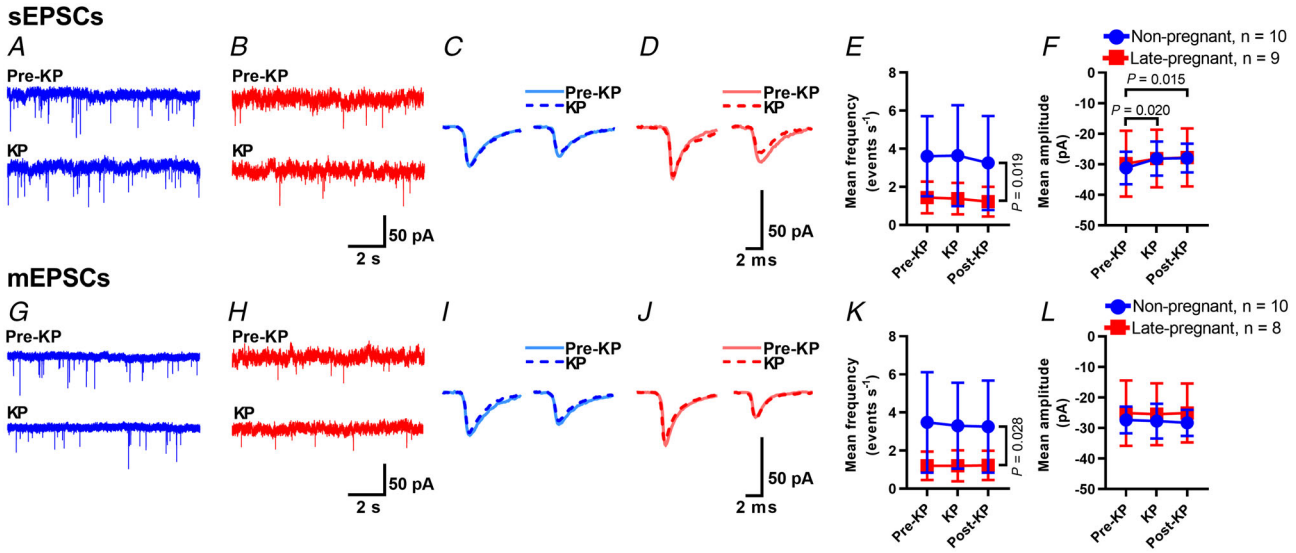

Figure 9. Kisspeptin does not affect the frequency or amplitude of sEPSCs or mEPSCs in supraoptic nucleus neurones
A and *B*, example whole-cell recordings (10 s) of supraoptic nucleus neurones showing sEPSCs from a non-pregnant rat (*A*) and a late-pregnant rat (*B*) before and during kisspeptin (KP) administration (1 μM). Each downward deflection represents one sEPSC. *C* and *D*, examples of averaged sEPSCs events of supraoptic nucleus neurones from non-pregnant rats (*C*) and late-pregnant rats (*D*) before (continuous lines) and during (dashed lines) kisspeptin administration (1 μM). *E*, mean (SD) frequency of sEPSCs before, during and after kisspeptin administration (1 μM) in supraoptic nucleus neurones from non-pregnant (blue circles, n = 10) and late-pregnant (red squares, n = 9) rats. Two-way repeated measures ANOVA revealed a main effect of reproductive status ($F_{(1,1)}$ = 6.66, P = 0.019) but no effect of time ($F_{(1,2)}$ = 2.57, P = 0.090) and no interaction between reproductive status × time ($F_{(1,3)}$ = 0.731, P = 0.730). *F*, mean (SD) amplitude of sEPSCs before and during kisspeptin administration (1 μM) in supraoptic nucleus neurones from non-pregnant (blue circles, n = 10) and late-pregnant (red squares, n = 9) rats. Two-way repeated measures ANOVA revealed no main effect of reproductive status ($F_{(1,1)}$ = 0.025, P = 0.876), a main effect of time ($F_{(1,2)}$ = 5.48, P = 0.008) but no interaction between reproductive status × time ($F_{(1,3)}$ = 0.375, P = 0.690). *Post hoc* Holm–Sidak tests revealed a significant increase in amplitude during kisspeptin *versus* pre-kisspeptin (P = 0.020) and post-kisspeptin *versus* pre-kisspeptin (P = 0.015) in non-pregnant and late-pregnant rats. *G* and *H*, example whole-cell recordings (10 s) of supraoptic nucleus neurones showing mEPSCs from a non-pregnant rat (*G*) and a late-pregnant rat (*H*) before and during kisspeptin administration (1 μM). Each downward deflection represents one mEPSC. *I* and *J*, examples of averaged mEPSCs events in supraoptic nucleus neurones from non-pregnant rats (*I*) and late-pregnant rats (*J*) before (continuous lines) and during (dashed lines) kisspeptin administration (1 μM). *K*, mean (SD) frequency of mEPSCs before, during and after kisspeptin administration (1 μM) in supraoptic nucleus neurones from non-pregnant (blue circles, n = 10) and late-pregnant (red squares, n = 8) rats. Two-way repeated measures ANOVA revealed main effect of reproductive status ($F_{(1,1)}$ = 5.76, P = 0.028) but no main effect of time ($F_{(1,2)}$ = 0.366, P = 0.696) and no interaction between reproductive status × time ($F_{(1,3)}$ = 0.492, P = 0.616). *L*, mean (SD) amplitude of mEPSCs before, during and after kisspeptin administration (1 μM) in supraoptic nucleus neurones from non-pregnant (blue circles, n = 10) and late-pregnant (red squares, n = 8) rats. Two-way repeated measures ANOVA revealed no main effect of reproductive status ($F_{(1,1)}$ = 0.530, P = 0.477) or time ($F_{(1,2)}$ = 0.263, P = 0.770) and no interaction between reproductive status × time ($F_{(1,3)}$ = 0.379, P = 0.688). [Colour figure can be viewed at wileyonlinelibrary.com]

Kiss1R mRNA expression does not change over pregnancy (Seymour *et al.* 2017). While the emergence of excitation by kisspeptin might reflect a change in surface expression, sensitivity and/or intracellular coupling of Kiss1R, it is possible that the effects are mediated by other RF-amide receptors, such as neuropeptide FF receptors, which appear to be expressed on oxytocin neurones (Kim *et al.* 2016) and for which kisspeptin also has high affinity (Oishi *et al.* 2011).

Kisspeptin effects on vasopressin neurones *in vivo*

While vasopressin neurones were not a specific focus of the current study, we measured the effects of intra-supraoptic nucleus kisspeptin on vasopressin neurone firing rate to determine whether excitation was specific to oxytocin neurones. Consistent with our previous observations of a lack of effect of I.C.V. kisspeptin on vasopressin neurones (Scott & Brown, 2011; Seymour *et al.* 2017), intra-supraoptic nucleus kisspeptin did not affect the firing rate of continuously active vasopressin neurones. However, there was a clear kisspeptin-induced inhibition of phasic vasopressin neurones in late-pregnant rats. Phasic activity is characterised by bursts of activity that last tens of seconds that are separated by silent periods that also last tens of seconds (Brown *et al.* 2006). The kisspeptin inhibition of phasic neurone firing rate was not underpinned by a change in any specific parameter of phasic bursts (intra-burst firing rate, burst duration or inter-burst interval). Furthermore, hazard analyses of

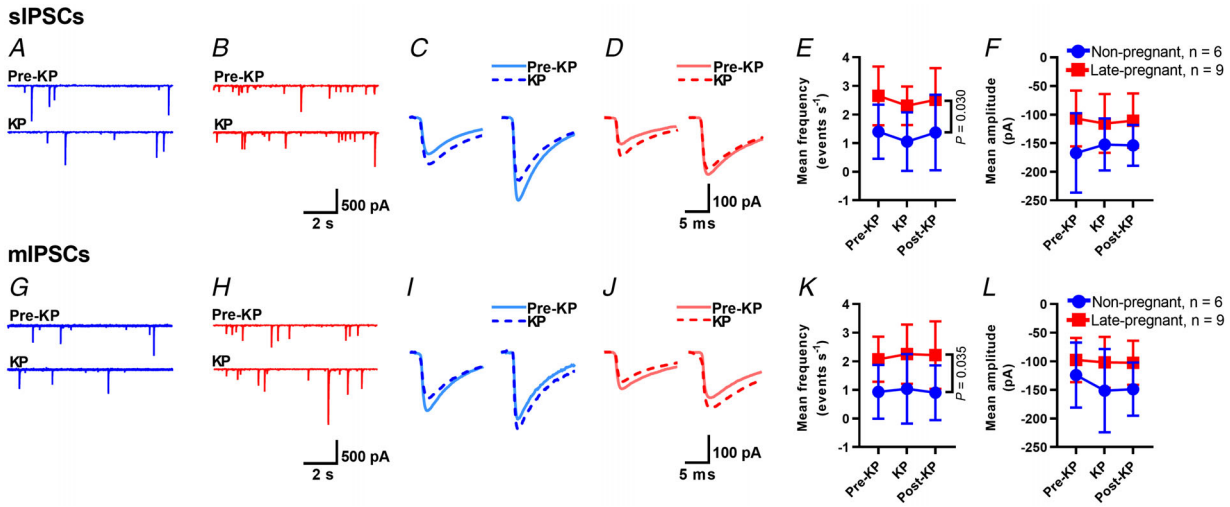

Figure 10. Kisspeptin does not affect the frequency or amplitude of sIPSCs or mIPSCs in supraoptic nucleus neurones
A and *B*, example whole-cell recordings (10 s) of supraoptic nucleus neurones showing sIPSCs from a non-pregnant rat (*A*) and a late-pregnant rat (*B*) before and during kisspeptin (KP) administration (1 μM). Each downward deflection represents one sIPSC. *C* and *D*, examples of averaged sIPSCs events of supraoptic nucleus neurones from non-pregnant rats (*C*) and late-pregnant rats (*D*) pre-kisspeptin (continuous lines) and during (dashed lines) kisspeptin administration (1 μM). *E*, mean (SD) frequency of sIPSCs before, during and after kisspeptin administration (1 μM) in supraoptic nucleus neurones from non-pregnant (blue circles, $n = 6$) and late-pregnant (red squares, $n = 9$) rats. Two-way repeated measures ANOVA revealed main effect of reproductive status ($F_{(1,1)} = 5.89$, $P = 0.030$) but no effect of time ($F_{(1,2)} = 2.51$, $P = 0.100$) and no interaction between reproductive status × time ($F_{(1,3)} = 0.076$, $P = 0.927$). *F*, mean (SD) amplitude of sIPSCs before and during kisspeptin administration (1 μM) in supraoptic nucleus neurones from non-pregnant (blue circles, $n = 6$) and late-pregnant (red squares, $n = 9$) rats. Two-way repeated measures ANOVA revealed no main effect of reproductive status ($F_{(1,1)} = 3.95$, $P = 0.068$) or time ($F_{(1,2)} = 0.104$, $P = 0.902$) and no interaction between reproductive status × time ($F_{(1,3)} = 0.648$, $P = 0.531$). *G* and *H*, example whole-cell recordings (10 s) of supraoptic nucleus neurones showing mIPSCs from a non-pregnant rat (*G*) and a late-pregnant rat (*H*) before and during kisspeptin administration (1 μM). Each downward deflection represents one IPSC. *I* and *J*, examples of averaged mIPSCs events in supraoptic nucleus neurones from non-pregnant rats (*I*) and late-pregnant rats (*J*) before (continuous lines) and during (dashed lines) kisspeptin administration (1 μM). *K*, mean (SD) frequency of mIPSCs before, during and after kisspeptin administration (1 μM) in supraoptic nucleus neurones from non-pregnant (blue circles, $n = 6$) and late-pregnant (red squares, $n = 9$) rats. Two-way repeated measures ANOVA revealed main effect of reproductive status ($F_{(1,1)} = 5.49$, $P = 0.035$) but no effect of time ($F_{(1,2)} = 0.668$, $P = 0.521$) and no interaction between reproductive status × time ($F_{(1,3)} = 0.246$, $P = 0.783$). *L*, mean (SD) amplitude of mIPSCs before, during and after kisspeptin administration (1 μM) in supraoptic nucleus neurones from non-pregnant (blue circles, $n = 6$) and late-pregnant (red squares, $n = 9$) rats. Two-way repeated measures ANOVA revealed no main effect of reproductive status ($F_{(1,1)} = 3.20$, $P = 0.096$) or time ($F_{(1,2)} = 1.41$, $P = 0.262$) and no interaction between reproductive status × time ($F_{(1,3)} = 0.715$, $P = 0.498$). [Colour figure can be viewed at wileyonlinelibrary.com]

vasopressin neurone activity revealed an overall shift in post-spike excitability, with kisspeptin-induced changes in both peak early and mean late hazard generally correlating with changes in firing rate. Taken together, these observations suggest that kisspeptin affects vasopressin neurones through a different mechanism to oxytocin neurones and further work will be required to determine this mechanism.

Vasopressin controls body fluid osmolality and the osmotic threshold for vasopressin release is reduced in pregnancy (Durr *et al*. 1981; Suzuki *et al*. 2000), which increases vasopressin release to allow blood volume expansion to cope with the cardiovascular demands of the developing offspring. Phasic activity enhances vasopressin release into the circulation (Dutton & Dyball, 1979). Hence, the emergence of kisspeptin inhibition of phasic vasopressin neurones in pregnancy appears counter-intuitive, but might be a compensatory mechanism to prevent over-secretion of vasopressin in the face of the reduced osmotic threshold for vasopressin release in late pregnancy to allow the secretory load to be spread across the population of vasopressin neurones (Leng *et al*. 2008).

Concluding remarks

Central kisspeptin appears to excite oxytocin neurones in late pregnancy by a direct action that increases post-spike excitability. Increased post-spike excitability is believed to support burst firing of oxytocin neurones that is essential for normal parturition (Brown *et al*. 2013). Hence, increased kisspeptin excitation of oxytocin neurones in late pregnancy might specifically facilitate burst firing of oxytocin neurones for successful delivery of the offspring.

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Additional information

Data availability statement

Data are available upon reasonable request to the corresponding author.

Competing interests

The authors have no competing financial interests.

Author contributions

All authors contributed to the design and interpretation of the experiments. M.A., M.R.P., A.J.S. and C.H.B. performed the experiments. M.A., A.J.S. and C.H.B. analysed data. M.A. prepared the figures and prepared the first draft of the manuscript. All authors reviewed the manuscript for intellectual content. All authors have read and approved the final version of this manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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kisspeptin, oxytocin, pregnancy, supraoptic nucleus, vasopressin

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Translational perspective

Preterm birth is a major cause of infant mortality and lifelong morbidity. Appropriate activation of oxytocin neurones is critical for normal birth and early activation of oxytocin neurones can lead to preterm delivery. Our research shows that central kisspeptin directly excites oxytocin neurones in late pregnancy. Hence, antagonising kisspeptin activation of oxytocin neurones might provide a novel therapeutic target to reduce the risk of preterm birth in at-risk pregnancies.