Continuous Kisspeptin restores Luteinizing Hormone pulsatility following cessation by a Neurokinin B antagonist in female sheep

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Abstract

Pulsatile secretion of gonadotropin releasing hormone (GnRH) drives pulsatile secretion of luteinizing hormone (LH), with evidence that this depends upon kisspeptin (Kiss) input to GnRH neurons. Kiss administration causes acute GnRH/LH secretion and electrophysiological data suggest that Kiss neurons may act in a phasic manner to drive GnRH secretion, but there is not definitive evidence for this. We have shown previously that continuous delivery of Kiss-10 to anestrous ewes can cause a surge in GnRH secretion and ovulation, and increases LH pulse frequency in humans. Here we tested the hypothesis that continuous Kiss-10 delivery can support pulsatile GnRH/LH secretion in the sheep. Neurokinin B (NKB) provides positive drive to Kiss neurons, so we therefore infused an NKB antagonist (ANT-08) intracerebroventricularly to induce cessation of pulsatile GnRH/LH secretion, with or without concomitant continuous Kiss-10 infusion. ANT-08 suppressed GnRH/LH pulsatility, which was immediately restored with continuous Kiss-10 infusion. These data support the notion that Kiss action is downstream of NKB signaling and that continuous Kiss-10 stimulation of GnRH neurons is sufficient to support a pulsatile pattern of GnRH/LH secretion. This offers further support to the theory that GnRH pulse generation is intrinsic to GnRH neurons and pulsatile GnRH release can be effected with continuous stimulation by Kiss.

Introduction

Following reports indicating that Kisspeptin (Kiss) signaling is mandatory for puberty in mice and humans [1-3] it became apparent that Kiss is a major regulator of GnRH secretion. Kiss-10, which is the amidated 10-decapeptide C-terminal sequence of the Kiss protein, stimulates secretion of gonadotropin releasing hormone (GnRH) into the hypophysial portal blood which, in turn, elicits the pulsatile secretion of luteinizing hormone (LH) from the gonadotropes of the pituitary gland [4]. This is effected through the Kiss receptor (GPR54), which is expressed by GnRH cells [5-7]. GnRH pulse generation is thought to be driven by the neurons of the arcuate nucleus that co-produce Kiss, neurokinin B (NKB) and dynorphin A, known as KNDY cells [6, 7].

The projections of the GnRH neurons to the median eminence possess unique properties, with spiney processes that suggest regulation can occur on these so-called 'dendrons' [8]. In addition, the KNDY neurons project into the neurosecretory zone of the median eminence, allowing Kiss regulation of GnRH secretion at this level. Indeed, we [6] recently showed that Kiss-10 can elicit a pulse secretion of GnRH, when micro-injected into the median eminence. This is consistent with other data from studies in mice and non-human primates, suggesting that the control of pulsatile GnRH is at this level [7, 9]. When endogenous pulses occur, however, both the KNDY and the glutamate cells of the arcuate nucleus display increased activity [6], suggesting that the so-called 'pulse generator' is not a single entity. Evidence that pulsatile GnRH secretion is driven by Kiss action on the neurosecretory terminals in the median eminence is also provided by the data of Caraty and colleagues, who

showed that intravenous (i.v.) administration of Kiss-10 caused immediate release of GnRH, even though the peptide did not cross the blood-brain barrier [10]. Further support for the notion that KNDY neurons are an essential component of the 'pulse generator' for GnRH secretion was indicated by multi-unit activity (MUA) recordings in the arcuate nucleus of the goat, which aligns with pulsatile LH secretion [11]. This proposition does, however, rely on the unequivocal demonstration that this MUA originates exclusively from the KNDY neurons, which has not been forthcoming. As to whether Kiss neurons represent the 'pulse generator' for GnRH neurons, and whether *pulsatile* input of Kiss to GnRH neurons is unequivocally mandatory for pulsatile secretion of GnRH remains an open question.

NKB stimulates the KNDY cells [12] in a manner that could be 'autocrine' in part, although there are other cells in this region of the brain that produce NKB, but not Kiss [13]. NKB stimulates LH secretion in sheep [14], which taken together with the foregoing, is consistent with a model in which this peptide activates KNDY cells, which in turn, stimulate GnRH secretion. The importance of NKB in this model is exemplified by the loss of GnRH/LH secretion in response to infusion of an NKB antagonist [15]. This antagonist effect is most likely on the KNDY neurons, since only a small number of NKB receptors are found on the soma of GnRH neurons, at least in rats and sheep [16, 17]. Further evidence that NKB supports the function of Kiss neurons in the brain comes from studies in humans in which inactivating mutations in the NKB gene or its receptor gene cause profound lowering of LH pulse frequency and amplitude. In such patients, LH pulse frequency is restored to normal by continuous infusion of Kiss-10 [18]. We have also demonstrated that continuous infusion of Kiss-10 in normal men [19] and in diabetic men [20] induces a robust

increase in LH pulse frequency. A role for Kiss in GnRH/LH pulse frequency was further suggested by the demonstration that a Kiss antagonist reduced GnRH pulse frequency in ovariectomised rats [21], in female rhesus monkeys and in ovariectomised (OVX) sheep [22, 23].

Whilst natural GnRH secretion may be due to phasic input from KNDY neurons to GnRH neurons, at the level of the median eminence, continuous i.v. infusion of Kiss-10 is able to elevate GnRH/LH secretion in sheep and cause ovulation in anestrous ewes [24]. Other data from anestrous ewes provide some suggestion that continuous i.v. infusion of 10 or 20 µg/h Kiss-10 may elicit pulsatile GnRH secretion in some animals, although the interpretation of the data sets was that no pulses of GnRH or LH were evident during infusion [10]. These data and those from men cited above question whether phasic Kiss input to GnRH neurons is mandatory for pulsatile GnRH secretion. We have addressed this question by blocking GnRH/LH secretion with an NKB antagonist and then superimposing a constant infusion of Kiss-10. We show that, in this model, pulsatile LH secretion (reflecting GnRH secretion) clearly occurs with non-pulsatile, continuous delivery of Kiss-10.

Materials and Methods

Ethics

The experiments described herein were performed in accordance with the Prevention of Cruelty to Animals Act, Victorian Government, 1986 and the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes and were approved by the Animal Ethics Committee of Monash University.

Animals

We used adult OVX Corriedale ewes which were kept in natural lighting in the field and housed for one week in natural lighting environment (identical to that in the field) in single pens to enable infusion and blood sampling.

Experimental Procedure

All animals received a cannula into the lateral cerebral ventricles, the tip of which was located above the Foramen of Munro [25] for intracerebroventricular (icv) infusion of the NKB antagonist. Bilateral cannulae were inserted into both jugular veins for blood sampling and infusion respectively. Jugular venous blood samples (5 ml) were taken from groups of OVX ewes (n=3-4) at 10 min intervals for 9 h, with treatment periods (A, B and C) as shown in Table 1. Plasma was harvested by centrifugation at 4 C and stored at -20 C for LH assay. An NKB antagonist (ANT-08), which is equivalent to MRK-08 (Merck Australia, Bayswater, VIC, Australia), was synthesized in house and was infused i.c.v. at a dose of 80 nmol/h in a vehicle of 5 % dimethylsuphoxide in artificial cerebrospinal fluid (aCSF). ANT-08/MRK-08 has high selectivity for TACR3 (NKB3R) with a relative affinity for TACR2 which is 17 fold lower and relative affinity for TACR1 >300 fold lower. It has a high receptor affinity (4.0 nM), good stability in human microsome studies (<5% turnover after 15 min) and a low metabolic clearance - t1/2 of 6.5h. It was a potent inhibitor of inositol phosphate stimulation by 3 nM senktide with an IC_{50} of 3.0 nM [26]. We have shown previously that this dose of ANT-08 suppresses LH pulsatility in OVX ewes [13].

Murine Kiss-10 (NeoMPS, Strasbourg, France) was infused i.v. at a dose of 25 μg/h (19 nmole/h) in normal saline. Infusion rates were 200 μl/h for icv infusion and 1

Table 1. Experimental protocol for i.c.v. infusion of 80 nmol/h NKB antagonist (ANT-08) and/or i.v. infusion of 19 nmol/h Kisspeptin and the respective vehicles. For i.c.v. infusion of ANT-08, the vehicle was 5 % dimethylsuphoxide in artificial cerebrospinal fluid (aCSF) and for i.v. infusion of Kiss-10, the vehicle was normal saline (0.9% NaCl).

Treatment	n	Period A: 0-3h	Period B: 3-6h	Period C: 6-9h
Vehicle i.c.v. & vehicle i.v.	3	No treatment	i.c.v. vehicle	i.c.v. vehicle & i.v. vehicle
Kiss & i.c.v. & vehicle i.v.	4	No treatment	i.c.v. vehicle	i.c.v. vehicle & i.v. Kiss
ANT-08 i.c.v. & vehicle i.v.	3	No treatment	ANT-08	ANT-08 & i.v. vehicle
ANT-08 i.c.v. & Kiss i.v.	4	No treatment	ANT-08	ANT-08 & Kiss

ml/h for iv infusion, using Graseby pumps (Graseby MS16A; Graseby Medical Ltd., Gold Coast, Australia). This dose of kiss-10 is essentially equivalent to that (20 μg/h) used by Caraty et al [10] to elicit robust LH responses in anestrous ewes.

The details of the experimental groups and the periods of infusion of the vehicles, ANT-08 and/or Kiss-10 are shown in Table 1. The experiment was conducted with a repeated measures design, over 4 experimental series, such that every animal received each treatment; thus, each group consisted of 3-4 animals (two blocked cannulae eliminated 2 sheep in the vehicle/vehicle group and the ANT-08/vehicle group). At least 2 days separated each of the 4 experimental days.

LH Assay

LH concentration in plasma were assayed using ovine LH (NIH-S26) as standard as described previously [27]. Computation with a program [28] that provides error estimates for each sample, allowed analysis of pulse amplitude and frequency [29, 30]. The assay sensitivity was 0.06 ng/ml, within assay maximum precision was at 2.8 ng/ml and between assay coefficient of variation (%) was 5.7 at 3.5 ng/ml and 1.0 at 6.3 ng/ml respectively.

Statistics

Identification of LH pulses was according to the criteria described previously [31]. Average values for mean LH levels, LH pulse amplitude and LH inter-pulse interval were calculated for periods A, B and C (Table 1). In the case of ANT-08 treatment, the occurrence of an LH pulse shortly after the commencement of infusion was not taken into account when analyzing pulse amplitude because no further pulses were

recorded. This delay in the effect of the antagonist seems most likely due to the fact that it was infused into a lateral ventricle and it would have taken some time for effective levels to be reached in CSF. In addition, the mechanism of suppressive action would require antagonist binding to the KNDY cells and the time required for sub-cellular signaling to be negated. For the analysis of LH interpulse interval, this was taken as the time of the entire period if no pulses were recorded. Data were analysed by repeated measures analysis of variance, with treatment (group) and period (A, B, C as shown in Table 1) as factors. All data was checked for homogeneity of variance and equal distribution. Data for the LH pulse amplitude and inter-pulse interval were subjected to log (x + 1) transformation to account for zero values and to achieve homogeneity of variance. Post-hoc analyses were performed using the least significant difference test. P values of <0.05 were considered significant.

Results

Examples of the plasma LH profiles for each treatment are shown in Fig 1. There were no effects of either iv vehicle or icv vehicle on plasma LH values (Fig 1; Fig 2).

Infusion of Kiss-10 to OVX animals with vehicle treatment caused a slight but non-significant increase in LH (Fig 1B). The NKB receptor antagonist, ANT-08 led to virtual elimination of pulsatile LH secretion, albeit with a slight delay (Fig 1C); mean LH levels and LH pulse amplitude were significantly reduced and LH inter-pulse interval was significantly increased (Fig 2). Notable features in some animals (exemplified in Fig 1, Panels C and D) were that the suppression of LH pulsatility was delayed after the start of ANT-08 infusion and a number of small excursions in

Figure 1. Examples of plasma LH profiles from four separate representative OVX ewes from each of the four experimental groups: (A) ICV vehicle and IV vehicle; (B) ICV vehicle and IV Kiss; (C) ICV ANT-08 andIV vehicle; and (D) ICV ANT-08 and IV Kiss-10. Each panel shows plasma LH levels during (A columns) baseline, (B columns) infusion of either vehicle or ANT-08 alone, or (C columns) either vehicle with either ANT-08 or Kiss-10 infusion or during combined ANT-08/Kiss-10 infusion. Arrowheads indicate important LH pulses. Note that centrally infused ANT-08 suppresses pulsatile LH secretion, but this is restored to normal with IV Kiss-10 infusion.

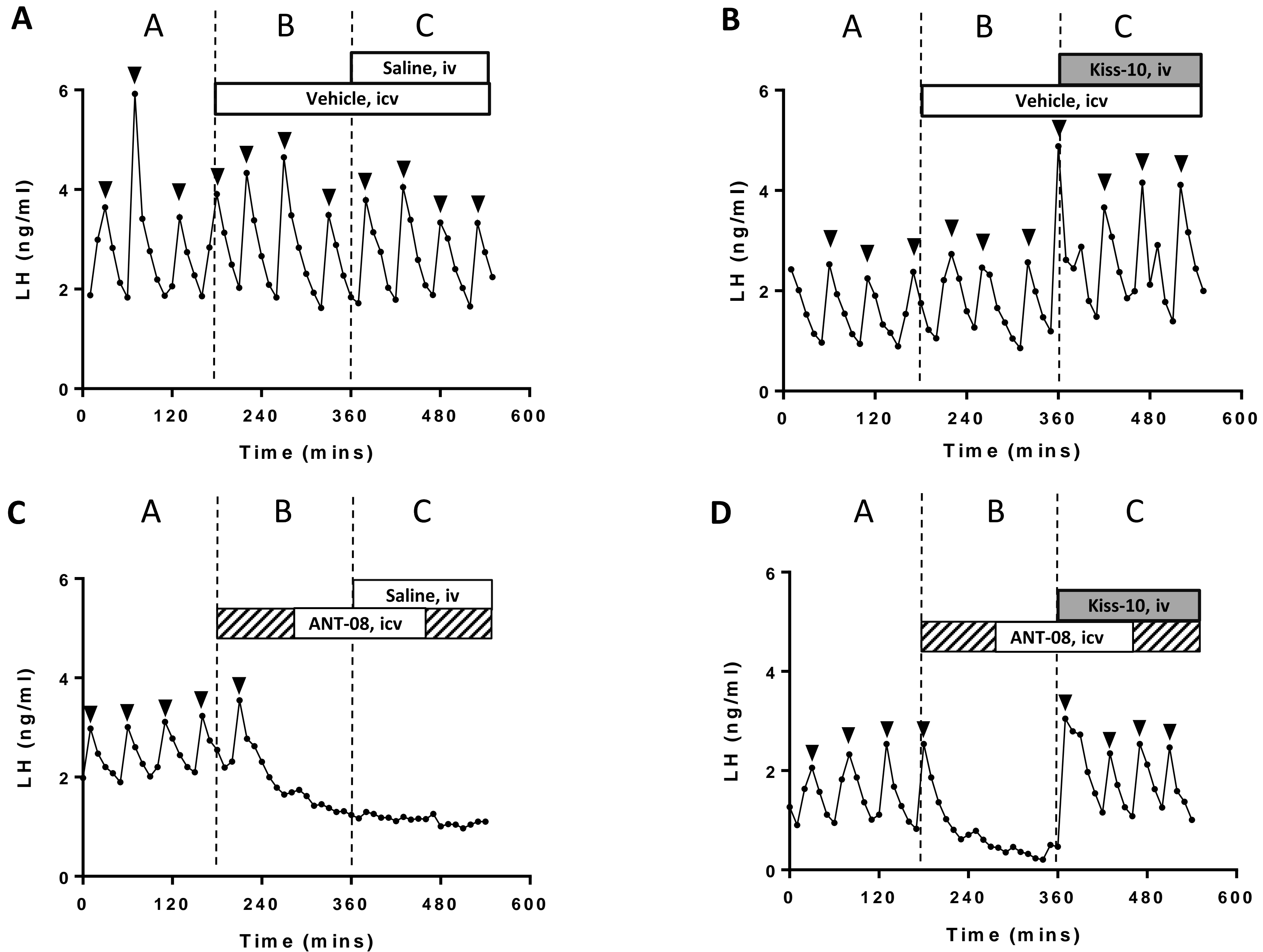
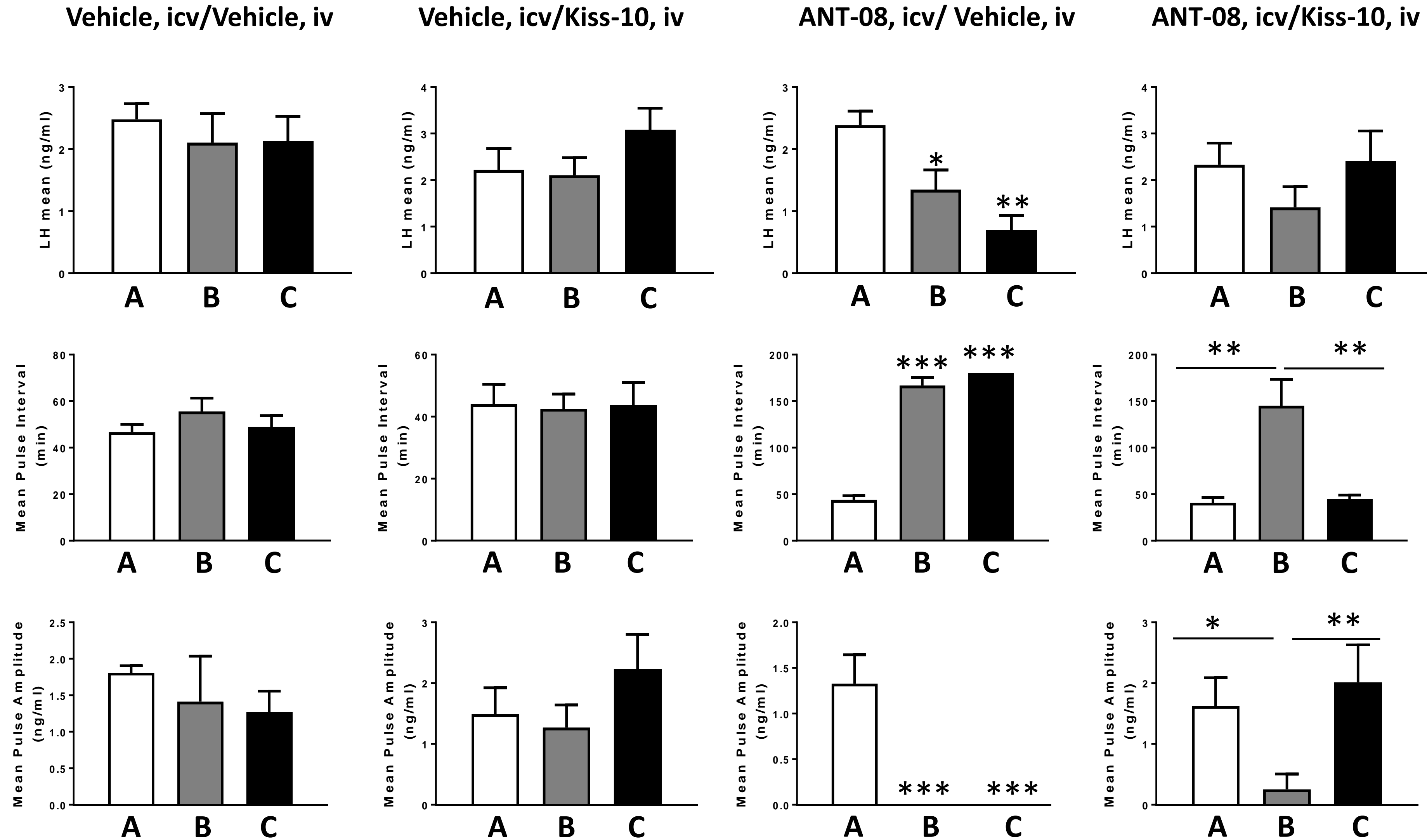


Figure 2. Means (±standard error of the mean) of average plasma LH levels, plasma LH pulse amplitude, and plasma LH interpulse interval in periods A, B, and C for OVX ewes infused with ANT-08, Kiss-10, or respective vehicles, as indicated in Table 1. Note the differences in scale of the y-axis for the interpulse interval. *P < 0.05; **P < 0.01; ***P < 0.001.



LH values were seen during ANT-08 infusion, which might be interpreted as small LH pulses (reflecting small GnRH pulses). None of these apparent small LH pulses were statistically significant as determined by our pulse criteria. Infusion of Kiss-10 in combination with MRK-08 caused an immediate and robust response, with pulsatile LH secretion returning to pre-infusion levels (Fig 1D and Fig 2).

Discussion

Our study demonstrates in a robust manner that central delivery of an NKB antagonist in OVX ewes substantially eliminates the pulsatile secretion of LH, indicative of the suppression of pulsatile GnRH secretion. In addition, we demonstrated that continuous infusion of Kiss-10 is able to restore pulsatile secretion of GnRH in ewes treated with ANT-08, returning LH pulse frequency and amplitude to normal values with immediate effect. The finding confirms earlier studies suggesting that NKB signalling is upstream of Kiss function and positively regulates Kiss neurons, but also indicates that this is an *acute* response. The data are consistent with our earlier study in humans [18], showing that iv continuous infusion of Kiss-10 restores LH pulse frequency to normal in subjects with inactivating mutations in either NKB or its cognate receptor.

NKB co-localises with Kiss in KNDY cells of the arcuate nucleus in this sheep [32] and other species. KNDY cells also express the NKB receptor [16], strongly suggesting an autoregulatory mechanism within these cells, whereby NKB positively regulates Kiss production/signalling. Indeed, there is good evidence that the KNDY cells of the arcuate nucleus are activated by NKB, substantiating the notion that NKB

signalling is fundamental to KNDY cell activation [12] and the downstream function of Kiss to stimulate GnRH secretion [33]. Importantly, the NKB receptor is not expressed by GnRH neurons of the ovine brain [16], reinforcing this fact. The present data are consistent with this theory, showing that blockade of NKB receptor suppresses pulsatile LH secretion, a surrogate of GnRH pulsatility. There are some issues particular to the present data, one being that the response to ANT-08 was somewhat delayed, such that a pulse of LH was seen after the start of infusion. This could be explained as follows. Firstly, the half-life of LH is 20 min and if a pulse began shortly after the initiation of the infusion, this would take 20 min to dissipate to 50% of peak value in peripheral blood. Secondly, the infusion was into a lateral ventricle, so it would take some time for levels to increase in the entire CSF space. Thirdly, for the antagonist to take effect, it would need to bind to receptors on KNDY cells and then allow for the 'inactivation' of pathways within the cell that drive kisspeptin secretion. 'Delayed' responses to centrally administered antagonists, in terms of pulsatile LH secretion, have also been seen in the ewe in other studies [33].

Pulsatile secretion of LH from the pituitary gonadotropes [34] is dependent upon the pulsatile release of GnRH from the brain [35]. When endogenous GnRH secretion is eliminated, intermittent (pulsatile) delivery of GnRH can restore both LH and FSH secretion, but continuous infusion of GnRH does not do so [36]. More recently, it has become accepted that Kiss provides a positive stimulus to GnRH neurons [4, 37], with evidence that there is phasic input of the former to the latter [6], which drives pulsatile GnRH secretion. Inactivating mutations of the Kiss gene or its cognate receptor, GPR54 cause loss of pulsatile gonadotropin secretion in humans and laboratory rodents, reflecting loss of pulsatile GnRH secretion [1-3, 38]. Similarly icv

administration of Kiss antagonists eliminates pulsatile LH secretion in various species, as indicated in the Introduction. It is generally held that Kiss release from KNDY neurons is phasic, giving rise to pulses of secretion of GnRH. Accordingly, a concordance of 70% between 'pulses' of Kiss and GnRH has been reported in hypothalamic microdialysis samples taken from rhesus monkeys [7] and KNDY cells of the arcuate nucleus are activated (cFos expression) at the time of a natural LH pulse in normally cycling ewes [6]. Paradoxically, continuous Kiss administration to rhesus monkeys caused loss of pulsatile LH secretion [7], suggestive of desensitisation of the GnRH neurones or the gonadotropes. Such a phenomenon has been seen in other species (reviewed in [39]). Continuous infusion of Kiss-10 elevates basal LH secretion in acyclic anestrous ewes, with some evidence of small pulses of GnRH and LH being generated over a 6h period, although these were not identified as pulses in the report [10]. The occurrence of pulses in these animals is a matter of conjecture and the difference between that study and the present one is that we used OVX ewes, in which there is a free running generation of GnRH/LH pulses, unencumbered by the negative feedback effects of gonadal steroids. In Syrian hamsters a dose of 6 nmol/day stimulated reproductive activity in animals on short day (inhibitory) photoperiod, whereas a dose of 10 nmol/day did not, perhaps suggestive of desensitization [40]. These data implicate dose as a factor as to whether desensitization occurs with constant infusion. Indeed, recent studies in women with hypothalamic amenorrhea demonstrated that pulsatile LH secretion could occur with continuous Kiss infusion, the number of pulses being greatest at 0.03-0.1 nmol/kg/h, but reducing at higher doses [41]. The data of the present paper are consistent with these data obtained in humans, although we did not test responses across such a wide range of Kiss doses.

Inactivating mutations of the NKB ligand or its cognate receptor (TAC3R) result in low LH pulse frequency and amplitude in humans [42, 43]. Continuous administration of Kiss-10 to such patients restores pulsatility of LH secretion to normal [18], further indicating that, while Kiss is mandatory for pulsatile GnRH secretion, it need not be delivered to the GnRH neurons in 'pulses'. The clear indication in the present paper that Kiss-10 may fully restore pulsatile LH secretion to normal in OVX ewes in combination with an infusion of an NKB antagonist supports the notion that it is Kiss arising from the KNDY cells, and not NKB, that drives GnRH secretion.

GnRH may be secreted in a pulsatile manner by GnRH cells cultured from the nasal placode, demonstrating inherent phasic function of the cells [44]. Thus, it is possible that constant Kiss exposure amplifies this endogenous activity or one generated in GnRH neurons through afferent inputs. Further studies at the cellular level will be required to answer these questions, but it is clear that NKB signaling is essential for the function of KNDY cells which are, in turn, important drivers of GnRH secretion. The most plausible explanation for the data obtained in the present paper and in preceding papers reporting response to continuous Kiss-10 infusion is that elevation of Kiss 'tone' allows pulsatile secretion from GnRH neurons at least within a short timeframe; 3h in this study and 8h in recent human studies [41]. Because the action of Kiss to facilitate pulsatile secretion of GnRH is directed to the secretory bed within the external zone of the median eminence, this opens the possibility that long acting enteral or parenteral preparations may be used to stimulate reproduction.

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