Effects of constant intravenous kisspeptin administration on luteinizing hormone release in cyclic mares

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Acute Injection and Chronic Perfusion of Kisspeptin Elicit Gonadotropins Release but Fail to Trigger Ovulation in the Mare[1]

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ABSTRACT

Kisspeptin has emerged as the most potent gonadotropin-releasing hormone (GnRH) secretagogue and appears to represent the penultimate step in the central control of reproduction. In the sheep, we showed that kisspeptin could be used to manipulate gonadotropin secretion and control ovulation. Prompted by these results, we decided to investigate whether kisspeptin could be used as an ovulation-inducing agent in another photoperiodic domestic mammal, the horse. Equine kisspeptin-10 (eKp10) was administered intravenously as bolus injections or short- to long-term perfusions to Welsh pony mares, either during the anestrus season or at various stages of the cycle during the breeding season. In all the experimental conditions, eKp10 reliably increased peripheral concentrations of both luteinizing hormone and follicle-stimulating hormone. The nature of the response to eKp10 was consistent across experimental conditions and physiological states: the increase in gonadotropins was always rapid and essentially transient even when eKp10 was perfused for prolonged periods. Furthermore, eKp10 consistently failed to induce ovulation in the mare. To gain insights into the underlying mechanisms, we used acute injections or perfusions of GnRH. We also cloned the equine orthologues of the kisspeptin precursor and Kiss1r; this was justified by the facts that the current equine genome assembly predicted an amino acid difference between eKp10 and Kp10 in other species while an equine orthologue for Kiss1r was missing altogether. In light of these findings, potential reasons for the divergence in the response to kisspeptin between ewe and mare are discussed. Our data highlight that kisspeptin is not a universal ovulation-inducing agent.

GPR54, GnRH, horse, gonadotropins, Kiss1R, kisspeptin, ovulation, reproduction, seasonality

INTRODUCTION

Kisspeptins (Kp) are a family of peptides derived from a precursor encoded by the gene *Kiss1*. The last 10 amino acid C-terminal sequence (Kp10), shared by all Kp isoforms, is well conserved across mammals and sufficient to obtain maximal activity upon binding to its cognate receptor Kiss1r (a member of the GPCR family). Loss-of-function mutations of the *Kiss1* gene or *Kiss1r* gene lead to hypogonadotropic hypogonadism [1, 2]. Indeed, it is now obvious that the Kp system is a gatekeeper of virtually every aspect of reproduction in mammals, including sexual differentiation of the brain, initiation of puberty, and regulation of gonadotropin secretion by sex steroids and has a role in the control of fertility by metabolic cues [1, 2]. Kisspeptins also play a prominent role in the control of seasonal reproduction in several species, including golden hamster [3, 4] and sheep and goats [5–8].

In mammals, neurons expressing Kp are mainly located within the arcuate nucleus (Arc), and the anteroventral periventricular nucleus (AVPV) or the preoptic area (POA), depending on the species. There is general agreement that the direct effect of Kp on gonadotropin-releasing hormone (GnRH) neurons, which express Kiss1r, constitutes the major route through which Kp impinge on the gonadotropic axis. Notwithstanding sex and species differences, a refined model is emerging in which Kp neurons of the AVPV/POA and Arc make preferential contact with either GnRH cell bodies or GnRH nerve terminals in the median eminence [1, 2, 9–12]. Nevertheless, there is evidence that Kp might also act upon the gonadotropic cells of the adenohypophysis and at the level of the gonads, albeit any relevant role of Kp at these sites remains controversial [1, 2, 13].

Mares are long-day polyestrous seasonal breeders with a natural breeding season that usually extends from April to October in the Northern hemisphere. Most breed registries consider January first as the official birth date for all foals born in a given year. Therefore, having yearlings born as close as possible to this date is an important goal for breeders as it provides an efficient way of maximizing future racing performance. A classical photoperiodic treatment, which consists of 35 long days given from December onward, has been used for decades to reliably advance the next breeding season in mares [14–17] as well as other seasonal breeders, including sheep [18]. In mares, the induction of ovulation per se can be obtained by the use of GnRH and its analogs or human chorionic gonadotropin [19–21]. Although such treatments do indeed trigger ovulation, its exact timing differs across breeds and individuals [22, 23]. The ability to timely induce ovulation represents a means of enhancing fertility by precisely programming breeding/insemination, which in turn minimizes the cost of hiring the stallion or using valuable

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semen. Considerable financial interests associated with the control of ovulation are therefore at stake for the equine industry. The difficulty to establish a reliable easy-to-use and cost-effective method for timely ovulation in mare may, in part, be explained by some specific endocrine features of her estrous cycle compared to that of other domestic mammals. These include a lengthy follicular phase, the ability for the follicles to grow without progesterone priming, and the requirement for a sustained luteinizing hormone (LH) increase over several days to induce ovulation [20].

As the most potent endogenous GnRH secretagogue identified to date, Kp is a prime candidate to manage reproduction in domestic animals [8, 24]. Indeed, we successfully employed it to induce ovulation in anestrus ewes and to advance and synchronize ovulation in a flock during the breeding season [7, 25]. We wished to extend these findings and investigate if Kp could overcome the problems encountered when managing ovulation in mares. Therefore, we designed a series of in vivo experiments to thoroughly investigate the potential usefulness of Kp as a novel ovulation-inducing agent in the Welsh pony mare. Additionally, we noticed that an orthologue for the *Kiss1r* gene was missing from the Thoroughbred equine genome assembly and therefore wished to attempt to clone full-length cDNAs for Kiss1r and the Kiss1 precursor from the Welsh pony mare to appraise a possible breed difference.

MATERIALS AND METHODS

Animals

All animal experiments were conducted in accordance with the French national law (authorizations 37–118 and C37-175-2 of the French Ministry of Agriculture) implementing the European Communities Council Directive 86/609/EEC and validated by the local Animal Ethics Committee. All the experiments were conducted in adult (7–9 yr) Welsh pony mares from the INRA herd of Nouzilly, France (latitude 47° North). Animals were kept indoors in individual stalls but were exposed to the natural photoperiod. They were fed wheat straw and given water ad libitum. A hay supplement was also provided twice daily.

Intravenous Perfusion and Blood Collection

Mares were sedated with 0.25 ml/100 kg Romifidin (Sedivet; Boehringer Ingelheim France S.A.S.) and two intravenous silicone catheters were introduced into the jugular vein. The first (inner diameter 1.2 mm, outer diameter 2.0 mm; Nutricath S; Vygon) allowed perfusion of the solutions while the second (inner diameter 2.2 mm, length 80 mm; Intraflon 2; Vygon) was used to sample jugular blood. Perfusion was performed with a peristaltic pump (RP-Q1; Sercom). Sterile vehicle solution (0.9% NaCl) was perfused at a flow rate of 24 ml/h; equine kisspeptin-10 (eKp10) and GnRH were perfused at various flow rates according to the experiment (see below). Serial blood samples were collected with heparin vacutainers and plasma was stored at −20°C.

Peptides

The peptide YRWNSFGLRY-NH_2 corresponding to the equine Kp C-terminal decapeptide sequence was synthesized by GeneCust and diluted into sterile water. The GnRH analog (buserelin [Suprefact]; Aventis Pharma) was diluted into sterile water and was used at 1 mg/ml. GnRH (Fertagyl; Intervet) was diluted into sterile water and used at 6.25 μg/ml.

Validation of the Perfusion Procedure

Prior to starting the perfusion procedures, a thorough validation had been performed to ensure that eKp10 was being delivered at the expected dose: eKp10 was diluted at 0.5 ng/ml, placed in the perfusion pocket at room temperature, and assayed by radioimmunoassay (RIA) as described previously [9] either immediately, in samples collected every hour for 6 h at the end of the tubing, after 24 h in the pocket, or in a sample that had been frozen/thawed after 24 h at room temperature. Albeit the values were lower than expected, the amount of eKp10 was virtually identical in all the experimental conditions (between 0.32 and 0.33 ng/ml); we therefore concluded that eKp10 is stable in our perfusion system over time.

Experimental Design

Experiments 1 and 2 were performed during the nonbreeding (anestrus) season. A mare was considered acyclic when the size of all the follicles was <17 mm, and if serum concentrations of progesterone had been <1 ng/ml for at least four consecutive weeks. Experiments 3–6 were performed during the breeding season. Luteolysis was induced by an intramuscular injection of 250 mg prostaglandin F2 alpha (PGF2α) (cloprostenol [Estrumate]; Schering-Plough), which was administered 5–6 days after ovulation. For experiments 4–6, treatments were started on the day following PGF2α treatment. Ovaries were scanned twice daily by ultrasonography (equipped with a 5 MHz linear probe; Aloka) to track follicular growth and to assign mares into groups.

Experiment 1 (nonbreeding season): effect of acute successive intravenous injections of eKp10 and GnRH analogue. Four anestrus acyclic mares received successively at 2 h intervals a single intravenous (i.v.) dose of eKp10 (1 mg then 6 mg) followed by the GnRH analog (4 mg). Jugular blood samples were taken 20, 10, and 0 min before injection and 5, 10, 20, 30, 60, 90, and 120 min after each injection.

Experiment 2 (nonbreeding season): effect of a short/midterm perfusion of eKp10. Sixteen anestrus acyclic mares were first perfused with vehicle for 6 h then randomly assigned to one of four groups (n = 4/group). Each group was perfused for 72 h with either vehicle, eKp10 at 3 mg/h, eKp10 at 6 mg/h, or GnRH at 20 μg/h. Jugular blood was sampled every 2 h before the beginning of treatment and during the first 10 h of treatment, then blood was sampled four times a day until the end of the treatment, and finally thrice daily for the next 72 h.

Experiment 3 (breeding season): is there a stage-specific effect of eKp10 during the follicular phase? Twelve mares were randomly assigned to one of three groups (n = 4/group): early follicular phase (diameter of the follicles <17 mm), midfollicular phase (diameter of the follicles = 20–25 mm), and late-follicular phase (diameter of the follicles = 32–35 mm). These phases were obtained on average 1, 2, and 5 days after PGF2α injection, respectively. Mares were first perfused with vehicle for 3 h followed by eKp10 at 6 mg/h for 6 h. This dose was chosen based on results from a preliminary experiment that had revealed high interindividual variability in the response with a lower dose (2.5 mg/h for 6 h; data not shown). Jugular blood was sampled every 15 min during infusion of the vehicle, then at 5, 10, 20, and 30 min and every 30 min during eKp10 infusion.

Experiment 4 (breeding season): dose-response to eKp10 during the early follicular phase. This experiment was performed during the breeding season at the beginning of a follicular phase (diameter of the follicles <17 mm, see experiment 3) in 16 mares randomly assigned to one of four groups (n = 4/group): 0.5, 1, 3, and 6 mg/h. Mares were first perfused with vehicle for 1 h followed by eKp10 for 3 h. Jugular blood was sampled every 10 min throughout the experiment.

Experiments 5a–5c (breeding season): could eKp10 be used during the early follicular phase to advance and synchronize ovulation? This experiment (5a) was performed during the early follicular phase (diameter of the follicles <17 mm) in 12 mares randomly assigned to one of three groups (n = 4/group): vehicle, eKp10 at 3 mg/h, and eKp10 at 6 mg/h. Mares were first perfused with vehicle for 4 h followed by either vehicle or eKp10 for 72 h. Jugular blood was sampled every 2 h before perfusion and during the first 10 h of perfusion, then every 6 h until the end of treatment, and finally once daily until about 5 days after ovulation, as detected using ultrasonography.

Another experiment (5b) was performed to test the effect of a perfusion of eKp10 started at the beginning of the early follicular phase and prolonged for the entire follicular phase. To this end, eight mares were perfused from the early follicular phase with either vehicle (n = 4) or the largest dose of eKp10 (6 mg/h, n = 4) and for the entire duration of the follicular phase (8–9 days) until ovulation occurred. Jugular blood was sampled every 2 h before perfusion and during the first 10 h of perfusion, then every 8 h until ovulation (detected by ultrasonography), and finally once daily for another 5 days postovulation.

A third experiment (5c) was performed on 16 mares to test the effect of a perfusion of eKp10 started at the late follicular phase. Mares were perfused either with eKp10 at 3 mg/h (n = 8) or with vehicle (n = 8) for 72 h. Jugular blood was sampled every 15 min for 1 h before perfusion, then every 2 h for the first 10 h of perfusion and every 6 h until the end of perfusion, and finally once daily until 5 days after ovulation (detected by ultrasonography).

Experiment 6 (breeding season): an attempt to investigate the mechanism(s) underlying the short-lasting response to eKp10 in cyclic mares. This experiment was performed in eight mares in the early follicular phase. Mares were first perfused with vehicle for 4 h, then perfused with eKp10 at 6 mg/h (n = 4) or vehicle (n = 4) for 30 h. Mares received a single i.v. injection of 1 mg eKp10 after 20 h of perfusion followed 5 h later by a single

i.v. injection of 25 μg GnRH. Jugular blood was sampled every 2 h before the beginning of treatment and every hour during the first 10 h of treatment. Samples were also taken every 15 min for 1 h before and after both injections, then every 30 min for 4 h.

Hormone assays. Plasma LH concentration was assayed by RIA following a procedure adapted from Guillaume et al. [26]. All the samples were run in duplicate. Standards were prepared using plasma from a mare vaccinated against GnRH with an equine reference hormone (eLH, NHPP AFP 5130A; Dr. A.F. Parlow). The eLH was labeled with ^{125}I (PerkinElmer NEN Radiochemicals) using Iodogen (Pierce, Interchim). The antibody (anti-LH AFP-240580; Dr. A.F. Parlow) was used at a final dilution of 1:440 000. The detection limit of the assay was 125 pg/ml. The mean intra- and interassay coefficients of variation (CV) for plasma containing 3.5 ng/ml of LH were 5% and 14%, respectively.

Plasma follicle-stimulating hormone (FSH) concentration was assayed by RIA using a previously validated procedure [27]. All the samples were run in duplicate. Standards were prepared using plasma from a hypophysectomized mare with equine reference hormones (eFSH 1368; Dr. Y. Combarnous). The eFSH was labeled as described above for LH. The antibody was used at a final dilution of 1:160 000 (anti-FSH 803; Dr. Y. Combarnous). The detection limit of the assay was 780 pg/ml. The mean intra- and interassay CV for plasma containing 3 ng/ml of FSH were 12% and 18%, respectively.

Plasma estradiol-17β concentration was determined using a commercially available kit (KIP0629; DIAsource ImmunoAssay S.A.). Briefly, plasma was extracted with ethyl acetate-cyclohexane, dried, and recovered in PBS before being assayed by RIA; the recovery varied between 49%–53%. A slight cross-reactivity is observed with estrone (1.8%), estriol (1.2%), and other steroids (<1%). The detection limit of the assay was 3.12 pg/ml. The intraassay CV for PBS containing 15 pg/ml of estradiol-17β was 6%.

Plasma progesterone concentration was determined using a previously validated procedure [26]. The detection limit was 125 pg/ml. The intraassay CV for plasma containing 1.2 ng/ml of progesterone was 9.3%.

Molecular cloning. The complete open reading frames (ORF) for Kiss1 and Kiss1r were obtained using hypothalamic mRNA from a single Welsh pony mare. The cloning procedures have been described in detail elsewhere [28]. Kiss1 was cloned using a standard homology procedure whereby multiple mammalian Kiss1 sequences were aligned to define regions of homology in the untranslated regions; once defined, PCR primers were designed. This approach could not be applied to Kiss1r. A pair of Kiss1r universal primers was therefore used to screen an equine BAC (bacterial artificial chromosome) library (see the *Results* section). A clone was identified and used to obtain the 5′ and 3′ ends of Kiss1r ORF. This information served to design primers for PCR. Kiss1 and Kiss1r cDNA were generated by RT-PCR using a reverse transcription kit (Qiagen) and Accuprime GC-rich DNA polymerase (Invitrogen) or Phusion GC master mix (NEB). PCR fragments were cloned in pGEMT (Promega) and four to six independent clones were sequenced (Eurofins MWG Operon).

Data presentation and statistical analyses. Data are presented as the mean ± SEM, unless otherwise stated. Integrated LH and FSH secretory responses were estimated by calculation of the area under the curve (AUC) of the concentration versus time plots above the baseline (mean level preceding the peptide injection/perfusion). AUC was calculated with the trapezoidal rule over the period after/during administration of the peptide.

Because of the limited number of animals, statistical analyses were performed with nonparametric exact tests (StatXact 5 software; Cytel Software Corporation). The level of statistical significance was set at 5%. When groups were paired (experiment 1), the values for the AUC were compared using the Friedman test. When the difference was significant, pairwise comparisons were performed with a permutation test for paired samples with Bonferroni correction and one-tailed *P* value. When groups were independent (experiments 2, 3, and 5a) the values for the AUC were compared between groups using the Kruskal-Wallis test. When the difference was significant, groups were compared only to the vehicle group (experiment 2) or two-by-two (experiments 3 and 5a) using a permutation test for independent samples with Bonferroni correction and one-tailed *P* value. To evaluate the effect of a treatment within groups over time, the Friedman test for paired samples was used (experiments 3 and 4). When the difference was significant, the LH or FSH rise between the 1 h or 3 h period preperfusion and the perfusion period 3–6 h (experiment 3) or 2–3 h (experiment 4) was assessed with a Page test for paired samples. For experiment 6, the mean values of AUC were compared with a permutation test for independent samples with a two-tailed *P* value.

RESULTS

Cloning of the Equine Kiss1 *Precursor*

The GenBank database comprises an equine *Kiss1* mRNA sequence (GenBank accession number XM_001489036) predicted from genomic data (genome assembly EquCab2, Thoroughbred breed). Importantly, the deduced Thoroughbred sequence for eKp10 differs from Kp10 sequences in all other mammals in that the second amino acid on the N-terminal is an Arg instead of an Asn. We therefore cloned a Kiss1 cDNA (GenBank accession number KF418371) to rule out any annotation issue or breed difference between sequences in the Welsh pony mare and the Thoroughbred; the two sequences are 100% identical at the amino acid level. Akin to other mammals, the equine *Kiss1* ORF exhibits a very high GC content of 75% (mouse 67%, sheep 74%, human 67%).

In Vivo Experiments

Experiment 1. The effect of a single acute injection of eKp10 (1 or 6 mg) was tested in anestrus mares. A rapid increase of both LH and FSH was observed within 5 min after successive i.v. injections of eKp10 or the GnRH analog (Fig. 1A). The AUC is different between each injection for FSH ($P < 0.05$; Fig. 1C) but not for LH ($P = 0.43$; Fig. 1B) reflecting a more pronounced effect of the GnRH analog compared to eKp10 toward FSH. Both eKp10 doses had similar effects on gonadotropin levels.

Experiment 2. Results from experiment 1 showed that acute eKp10 injection elicited a transient increase of gonadotropin levels. We therefore wished to test whether the response could be sustained using short/midterm chronic perfusion of eKp10. Because a preliminary experiment showed that a dose of 0.5 mg/h for 6 h was inefficient (data not shown), we tried larger doses delivered for a longer duration (72 h). We decided to analyze separately acute and chronic effects of the perfusion and therefore arbitrarily chose to consider effects during either the first 10 h or the entire duration of 72 h. Perfusion of eKp10 at 6 mg/h (but not 3 mg/h; $P = 0.17$) or GnRH led to a first significant increase in plasma LH concentration during the first 10 h compared to vehicle ($P < 0.05$; Fig. 2A). Peak LH levels were reached within 2 h of perfusion with either GnRH at 20 μg/h, eKp10 at 3 mg/h, or eKp10 at 6 mg/h. Plasma concentrations of LH declined rapidly as the perfusion continued. FSH significantly increased during perfusion of eKp10 at 6 mg/h (but not at 3 mg/h; $P = 0.17$) or GnRH at 20 μg/h compared to vehicle ($P < 0.05$; Fig. 2B). Peak FSH levels were reached within 2 h of perfusion with GnRH at 20 μg/h, eKp10 at 3 mg/h, and eKp10 at 6 mg/h. Similar to LH, plasma concentrations of FSH steadily declined as the perfusion proceeded.

Considering the entire duration (72 h) of the perfusion and following the initial rise in LH, concentrations appeared to stay at intermediate levels in the groups given eKp10 at 3 or 6 mg/h, while they steadily increased in the group perfused with GnRH at 20 μg/h. At the end of the perfusion, LH concentrations declined and reached basal level within 24 h in the three groups. The AUC during the 72 h of perfusion was significantly higher in all the treated groups versus vehicle group ($P < 0.05$; Fig. 2C). For FSH, after the initial rise, the concentrations rapidly returned and stayed at basal level throughout the remainder of the perfusion. The AUC during the 72 h perfusion was similar between groups ($P = 0.095$; Fig. 2D). The treatments had no effect upon estradiol plasma concentrations ($P = 0.36$; data not shown).

Experiment 3. We then wished to test whether eKp10 could be used to trigger and/or synchonize ovulations during the breeding season. We first attempted to determine if eKp10 would be more efficient during a specific part of the follicular phase. To this end, the effect of a short-term perfusion (6 mg/h for 6 h) provided at different stages of the follicular phase of

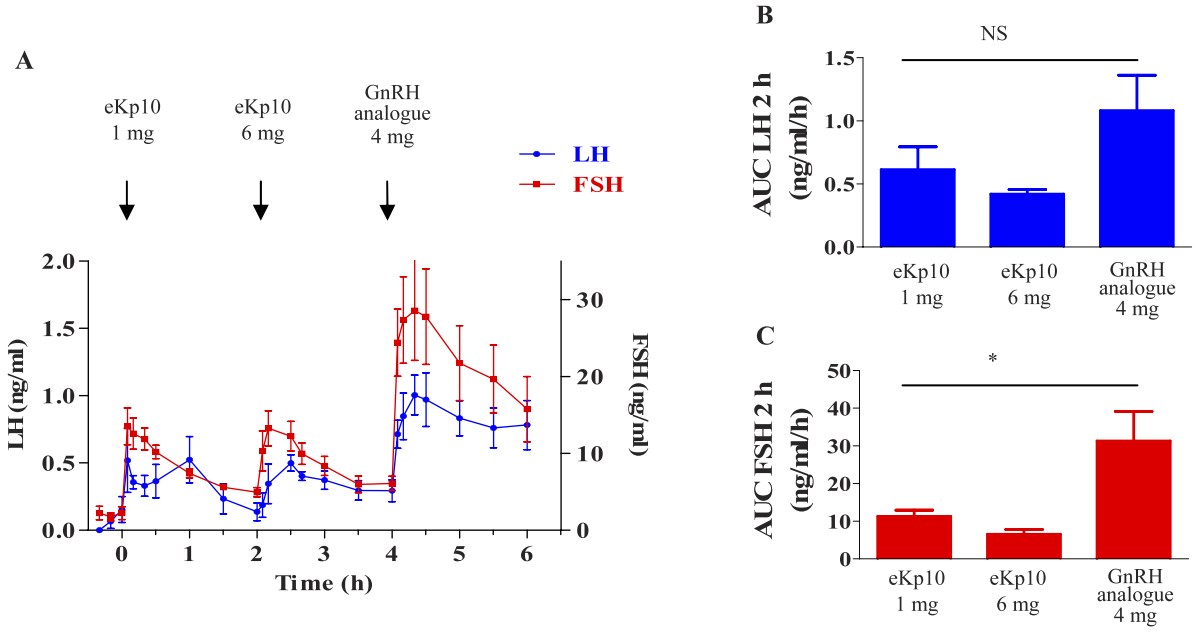

FIG. 1. Experiment 1: effects of an acute injection of eKp10 in the anestrus acyclic mare. **A**) Effects of successive acute injection of eKp10 (1 mg then 6 mg) and GnRH analog (buserelin, 4 mg) on LH (blue symbols) and FSH (red symbols). **B**, **C**) AUC for LH and FSH during the 2 h postinjection. *$P < 0.05$ (Friedman test), $P > 0.05$, permutation test for paired samples with Bonferroni correction and one-tailed P value. NS, not significant.

cyclic mares (early, middle, and late follicular phases) was tested (Fig. 3, A and B). This treatment had an effect on plasma LH concentrations (Fig. 3A, right panels) whatever the stage of the follicular phase ($P < 0.05$) and LH concentrations progressively increased between the periods −3 to 0 h and 3 to 6 h in the three groups ($P < 0.05$). However, the AUC was greater for the early follicular phase compared to mid- and late follicular phases ($P < 0.05$; Fig. 3A, lower panel). An effect was also observed for plasma FSH concentrations (Fig. 3B, right panels) when the perfusion was given during either the early or midfollicular phase ($P < 0.05$), but not during the late follicular phase ($P = 0.2$). A very modest increase in FSH levels between the periods −3 to 0 h and 3 to 6 h was observed only for the midfollicular phase ($P < 0.05$) and, similar to LH, the AUC was greater for the early follicular phase compared to mid- and late follicular phases ($P < 0.05$, Fig. 3B, lower panel). There was some degree of interindividual variability in the response to eKp10, which is shown in Supplemental Figure S1 (all the Supplemental Data are available online at www.biolreprod.org). The stage of the follicular phase at which eKp10 was perfused had no effect on the timing of ovulation, which occurred on average (mean ± SD) 11 ± 2.16, 10 ± 1.41, and 9.25 ± 0.83 days after PGF2α injection (early, middle, and late follicular phases, respectively).

Experiment 4. Experiment 3 showed that the increase in gonadotropin secretion is greatest when eKp10 is perfused during the early follicular phase. We then tried to define the smallest efficacious dose of eKp10 required to elicit gonadotropin release. Increasing doses of eKp10 (0.5, 1, 3, or 6 mg/h) were therefore perfused at this particular stage for 3 h. Although a progressive increase of plasma LH concentrations was observed for all the treatments, the effect was larger with the 1, 3, and 6 mg/h doses than with the 0.5 mg/h dose (Fig. 4A). An increase of plasma FSH concentrations was only observed with the 1, 3, and 6 mg/h doses (Fig. 4B). The interindividual variability in the response appeared to be slightly lower with 3 and 6 mg/h compared to 0.5 and 1 mg/h (data not shown).

Experiment 5a. Results of experiments 3 and 4 demonstrated that the largest effects of eKp10 are observed when doses of either 3 or 6 mg/h are perfused at the beginning of the follicular phase. Taking this into account, an attempt was made to influence the time of ovulation: eKp10 was perfused for 72 h at either 3 or 6 mg/h during the early follicular phase.

Effects on LH and FSH. Similar to previous results, an initial increase in plasma LH concentrations was observed: maximal LH values of 4.14 ± 0.94 ng/ml and 3.70 ± 0.89 ng/ml were reached after 6 and 4 h of perfusion with eKp10 at 3 and 6 mg/h, respectively. This increase was not sustained, and LH was back to basal levels by the end of the 72 h of perfusion (Fig. 5A, black bar indicates perfusion). The LH response (AUC) over the 72 h of perfusion differed between the three groups, and pairwise comparisons showed that the AUC is higher in the 3 mg/h group than in the vehicle-treated group ($P < 0.05$; Fig. 5B), and tended to be higher in the 6 mg/h group than in the vehicle-treated group ($P = 0.08$). Contrasting with this initial response, there were no differences in the AUC (Fig. 5C) or amplitude (data not shown) of the periovulatory LH surge. Compared to the vehicle-treated group, the onset of the periovulatory LH surge (determined as an increase greater than twice the basal level) tended to occur earlier in animals treated with eKp10 at the dose of 6 mg/h ($P = 0.07$) but not in animals treated with eKp10 at the dose of 3 mg/h (mean ± SD; control, 10.09 ± 1.08; 3 mg/h, 9.29 ± 0.5; 6 mg/h, 7.04 ± 1.79 days after PGF2α injection).

The immediate response to eKp10 perfusion showed a similar trend for plasma FSH concentrations: maximal values of 11.51 ± 1.10 ng/ml and 8.87 ± 1.16 ng/ml were reached after 2 and 4 h of perfusion with eKp10 at 3 and 6 mg/h, respectively (Fig. 5D). Plasma FSH was back to basal levels after approximately 36 h. The AUC for FSH over the 72 h of perfusion were different between the three groups, and pairwise comparisons showed that the AUC was higher in the 3 mg/h group than in the vehicle group ($P < 0.05$; Fig. 5E) and tended to be higher in the 6 mg/h group than in the vehicle group ($P = 0.08$). Contrasting with this initial response, there were no

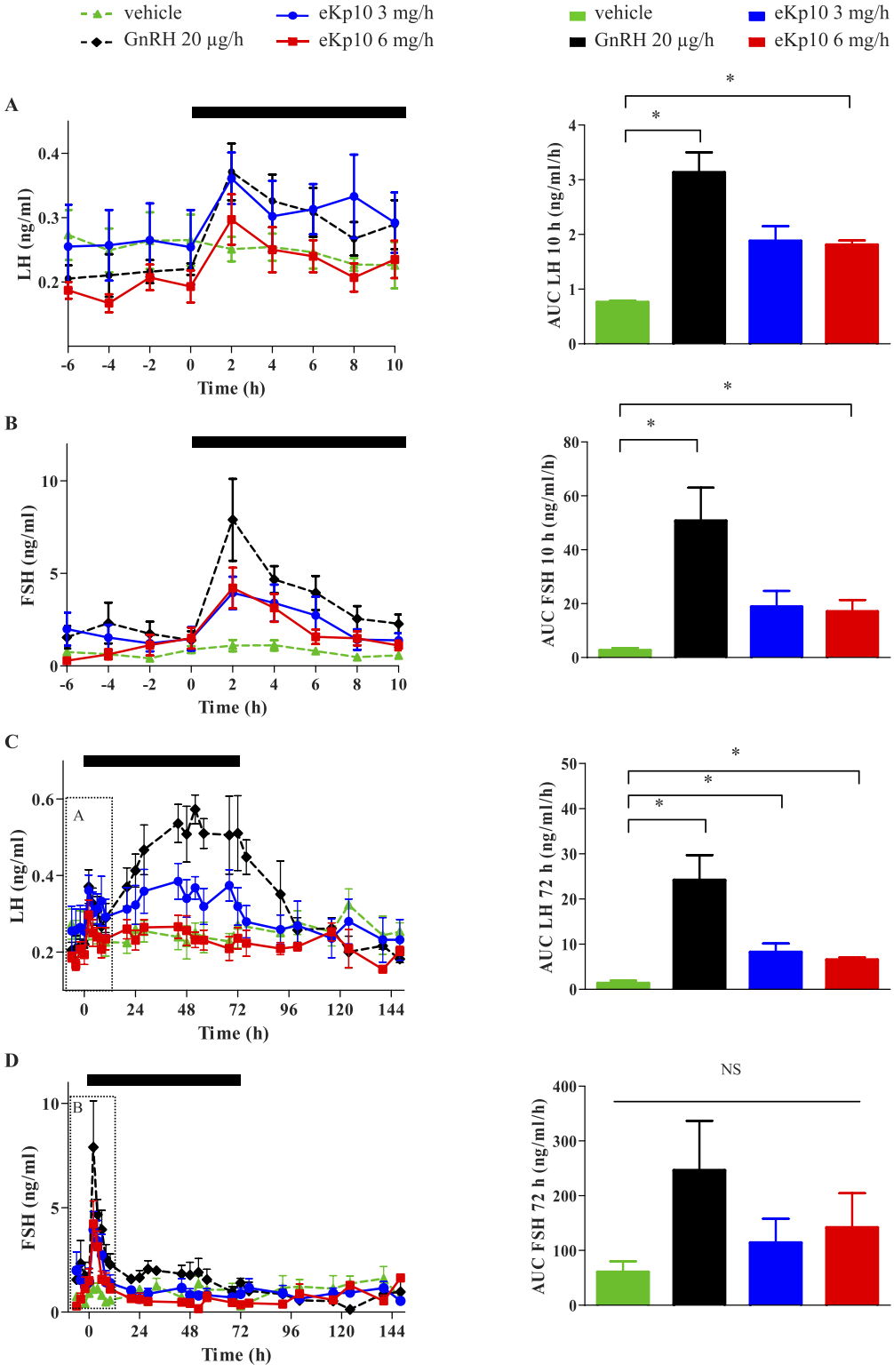

FIG. 2. Experiment 2: effect of a short/midterm perfusion of eKp10 in the anestrus acyclic mare. **A**, **B**) Effects of GnRH (black symbols, 20 µg/h), eKp10 at 3 mg/h (blue symbols), eKp10 at 6 mg/h (red symbols), or vehicle (green symbols) on LH and FSH levels, respectively, during the first 10 h of perfusion (corresponding to dashed boxes in **C** and **D**). Left panels show mean levels (± SEM) during the 6 h period before perfusion and during the first 10 h of perfusion. The black bar indicates the time of perfusion. Right panels show the integrated hormone response (AUC) over the 10 h period of perfusion. **C**, **D**) Effects of GnRH (20 µg/h), eKp10 at 3 mg/h, eKp10 at 6 mg/h, or vehicle on LH and FSH, respectively, over the entire duration (150 h) of the experiment (same color code for symbols as above). Left panels show mean levels (± SEM) during the 6 h period before perfusion, during the 72 h of perfusion, and during the next 72 h. The black bar indicates the time of perfusion. The dashed boxes correspond to the first 10 h, detailed in **A** and **B**. Right panels show the integrated hormone response (AUC) over the 72 h period of perfusion. $P < 0.001$ (Kruskal-Wallis), $^*P < 0.05$ permutation test for independent samples with Bonferroni correction and one-tailed P value. NS, not significant.

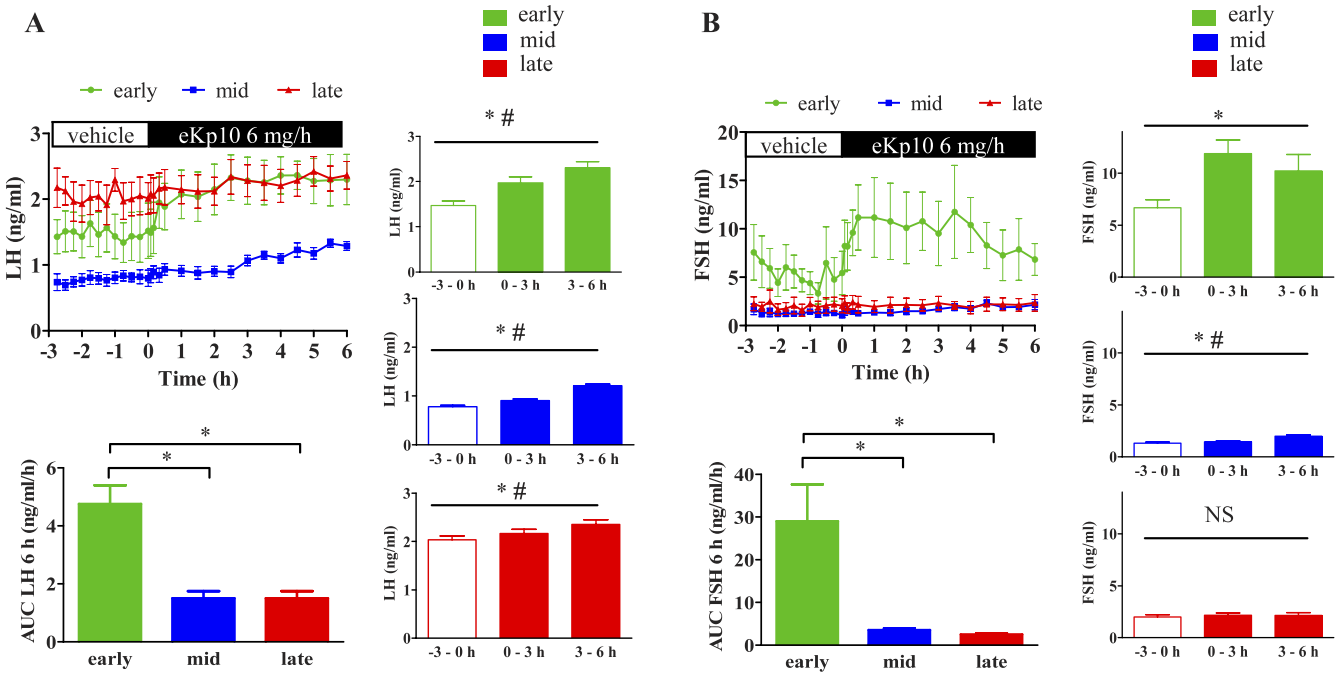

FIG. 3. Experiment 3: effects of a short-term perfusion of eKp10 in the cyclic mare. Is there a phase-specific effect of eKp10? **A**, **B**) Effect of eKp10 perfusion (at 6 mg/h for 6 h) on LH and FSH profiles at early (green symbols), middle (blue symbols), and late (red symbols) follicular phases. Upper left panels show mean levels (± SEM) during the 9 h period of perfusion. Lower left panels show the integrated hormone response (AUC) over the 6 h period of eKp10 perfusion. Right panels show pooled data (mean ± SEM) for three consecutive 3 h periods: −3 to 0 h (vehicle perfusion, white bars), 0 to 3 h, and 3 to 6 h (eKp10 perfusion). Comparison of the AUC values: $P < 0.001$ (Kruskal-Wallis), $^*P < 0.05$ permutation test for independent samples with Bonferroni correction and one-tailed P value. Effect of treatment within groups and over time: $^*P < 0.05$ (Friedman test), $\#P < 0.05$ (Page test). NS, not significant.

differences in the AUC (Fig. 5F) of FSH levels considering the same time period used to define the periovulatory LH surge.

Effects on estradiol, progesterone, and timing of ovulation. The mean plasma estradiol concentrations increased progressively during the 72 h of eKp10 perfusion (Fig. 5G). Analysis of the AUC during the 72 h of perfusion showed that plasma concentrations of estradiol were different between the three groups, with higher levels in the 3 and 6 mg/h groups compared to the vehicle-treated group ($P < 0.05$; Fig. 5H). There were no differences in the AUC (Fig. 5I) or amplitude (data not shown) of the periovulatory estradiol surge. Although not statistically significant, progesterone levels (Fig. 5J) tended to increase earlier in both eKp10-treated groups compared to the vehicle-treated group. The timing of ovulation itself, as defined twice daily by ultrasonography, was not different between the three groups ($P = 0.15$). Considering the day of PGF2α injection as a reference (day −1 with reference to the treatments), ovulation occurred on average (mean ± SD) after 11.75 ± 0.83, 10.75 ± 0.43, and 10.25 ± 0.83 days in vehicle-treated, eKp10 at 3 mg/h, and eKp10 at 6 mg/h groups, respectively.

Experiments 5b and 5c. Gonadotropin profiles reminiscent of those observed for experiment 5a were obtained when the duration of perfusion was extended to the entire follicular phase (8–9 days) and prolonged until ovulation occurred (data not shown). Ovulation occurred at (mean ± SD) 9.5 ± 0.4 and 9.5 ± 0.6 days after PGF2α injection in eKp10 at 6 mg/h and vehicle-treated groups, respectively ($P = 0.61$). Finally, when perfusion of eKp10 at 3 mg/h was administered for 72 h at the end of the follicular phase, the timing of ovulation again remained similar to that of vehicle-treated animals (data not shown).

Experiment 6. Similar to our findings during the anestrus season, it appeared that perfusion of eKp10 could not sustain gonadotropin output. To gain insights into the mechanism(s) underlying this transient gonadotropin secretion, we tested whether acute injection of either eKp10 or eKp10 followed by GnRH could elicit an increase in serum gonadotropins 20 h after the beginning of an eKp10 perfusion. In line with results from the preceding experiments, an initial rise of plasma concentrations of LH and FSH was observed at the onset of the perfusion in the group receiving eKp10 at 6 mg/h (Fig. 6, A and B). Again, this increase was not sustained over time, and LH/FSH levels declined as perfusion continued. Nevertheless, AUC for both LH (Fig. 6A, right panel) and FSH (Fig. 6B, right panel) over the first 10 h of perfusion were larger in the treated group than in the control group ($P < 0.05$). After 20 h of perfusion of either vehicle solution or eKp10 at 6 mg/h, a single i.v. injection of 1 mg of eKp10 was given. No statistically significant difference was observed between the two groups in the AUC for LH for the 5 h following injection of eKp10, although there appeared to be a slight trend for higher levels in animals pretreated with vehicle. The acute injection of eKp10 led to an increase in the AUC for FSH in the group that had been first perfused with vehicle but not in the group pretreated with eKp10 at 6 mg/h ($P < 0.05$). At the end of the 5 h period, gonadotropins were back to basal levels in the two groups. After 25 h of perfusion, and 5 h after the eKp10 injection, a single i.v. injection of 25 μg of GnRH elicited similarly robust discharges of LH and FSH in the two groups. Values of the AUC for LH and FSH during the 5 h post-GnRH injection were not different between the two groups ($P = 0.39$ and 0.48, respectively).

Cloning of the Equine Kiss1r

We had noticed that the *Kiss1r* gene was missing from the equine genome assembly, EquCab2, and therefore wished to

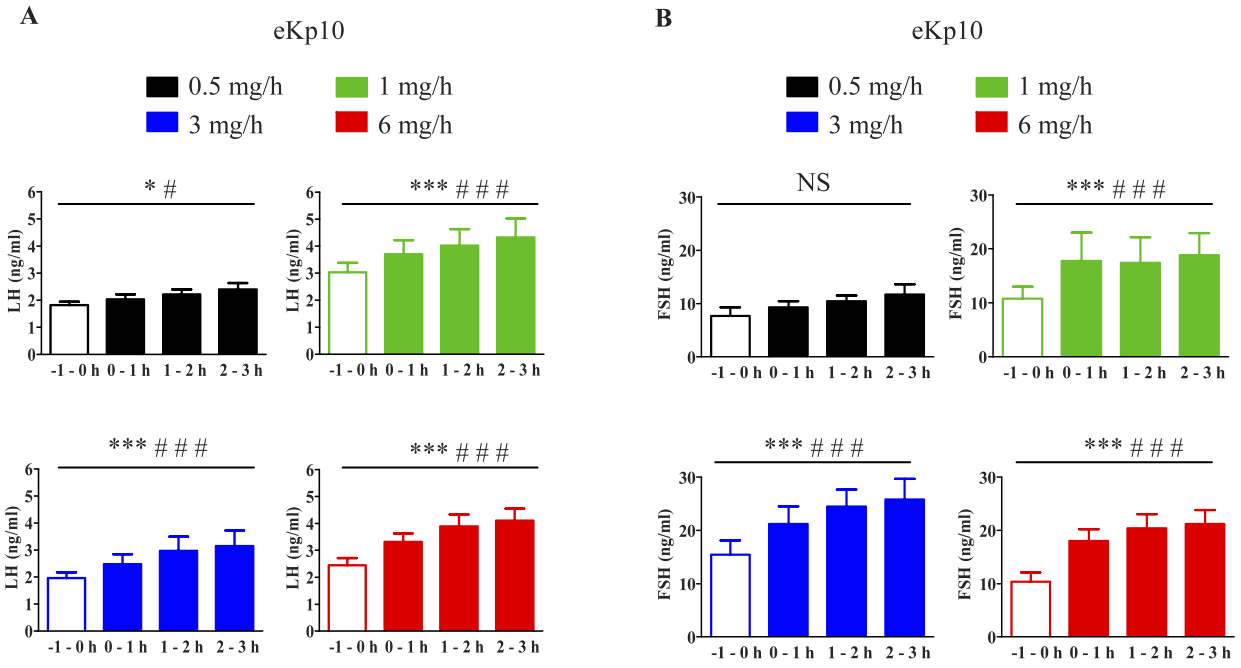

FIG. 4. Experiment 4: dose-response to eKp10 during the early follicular phase. **A**, **B**) Effects of a 3 h perfusion of increasing doses of eKp10 (0.5, 1, 3, or 6 mg/h) on levels of LH and FSH during the early follicular phase. Data are presented as mean (± SEM) concentrations for four successive 3 h periods: −1 to 0 h (vehicle perfusion, white bars), 0 to 1 h, 1 to 2 h, and 2 to 3 h (eKp-10 perfusion of 0.5 mg/h, black bars; 1 mg/h, green bars; 3 mg/h, blue bars; or 6 mg/h, red bars). Effect of treatment within groups and over time: $*P < 0.05$, $***P < 0.001$ (Friedman test), $\#P < 0.05$, $\#\#\#P < 0.001$ (Page test). NS, not significant.

identify it. The human *Kiss1r* locus is located on chromosome 19, in a region which maps to part of the equine chromosome 7. Synteny revealed that an interval of ~260 kilobases, containing the *Kiss1r* locus and found between the loci for *Ptbp1* and *Hmha1*, was absent from EquCab2 and compared to the human genome assembly GRCh37. Taking the latter as a reference revealed that several genes within this interval (*Tmem259, WDR18, Arid3A, R3hdm4, Med16,* and *Prtn3*) do exist as unplaced scaffolds in EquCab2, while others (*Kiss1r, ABCA7, CNN2, Grin3B, Cfd, Elane, Azu1,* and *LPPR3*) are notably absent. This most likely reflects incomplete sequencing of the equine genome. The use of RT-PCR on genomic DNA revealed the existence of a putative *Kiss1r* gene (GenBank accession number KF361519) whose expression was confirmed using hypothalamic cDNA from a Welsh pony mare. An equine BAC library (from a Thoroughbred mare) was screened by PCR, which yielded a single hit (clone EBAB 6H11); the 5′ and 3′ ends of the locus were sequenced (GenBank accession numbers KF361516 and KF361517, respectively). Based on these data, a full-length clone was generated from hypothalamic cDNA of a Welsh pony mare (GenBank accession number KF361518). The deduced *Kiss1r* sequence has an ORF of 1140 bp, yielding a protein of 380 amino acids, which bears 88%–94% identity compared to KISS1R of other mammals. Akin to other mammals, the equine *Kiss1r* ORF exhibits a very high GC content of 73% (mouse 65%, sheep 70%, human 72%). Sequencing the ends of the BAC insert revealed that the equine *Kiss1* gene locus is located within a contig flanked by loci for *Hmha1* and *LmnB2*.

DISCUSSION

Collectively, our data show that acute, short-term or long-term chronic peripheral delivery of eKp10 in the mare during either the nonbreeding or the breeding season consistently results in a short-term increase of the plasma levels of gonadotropins. Most importantly, the increase in LH/FSH levels elicited by eKp10 is consistently only transient, even in the face of a long-term chronic eKp10 application. Finally, we showed that the perfusion of eKp10 during the early follicular phase, the stage of the follicular phase when eKp10 appeared to be most efficient in inducing the release of LH/FSH, is not able to trigger, or even advance, the time of ovulation of the dominant follicle. These conclusions concur with those of Magee et al. [29] who investigated the effect of repeated injections of eKp10 in light-horse mares but contrast with our previous findings in sheep where Kp10 perfusion reliably synchronized ovulation during the breeding season and even induced it when applied during the anestrus [7]. There are therefore marked species differences in the response to Kp10 for the control of ovulation, and the usefulness of eKp10 for this purpose in the mare is not readily obvious. The inability to induce ovulation with eKp10 in the mare may be due to a combination of specifics of the equine model, which are considered and discussed below. These specifics pertain to 1) the general endocrinology governing ovulation, 2) the desensitization of the GnRH receptor (GnRHR), 3) the sequence of eKp10 itself, 4) the desensitization of the Kiss1r, 5) the neuroanatomy and functioning of the GnRH system, and 6) the estradiol positive feedback governing the GnRH/LH surge.

The endocrine requirements for the development of ovarian follicles in the mare differs considerably from that in other mammals [20, 23]. The mare estrous cycle presents an extended periovulatory LH surge that lasts 4–6 days compared to a few hours in other species, including the ewe [30–33]. Indeed, peak levels of LH in the mare do not precede ovulation but instead follow it by about 24 h, and there is evidence that only a fraction of the total amount of LH is sufficient to induce ovulation [34]. The general pattern of estradiol levels is similar, albeit asynchronous, to that of LH: the levels rise over several days in the mare and reach peak values about 2–4 days before

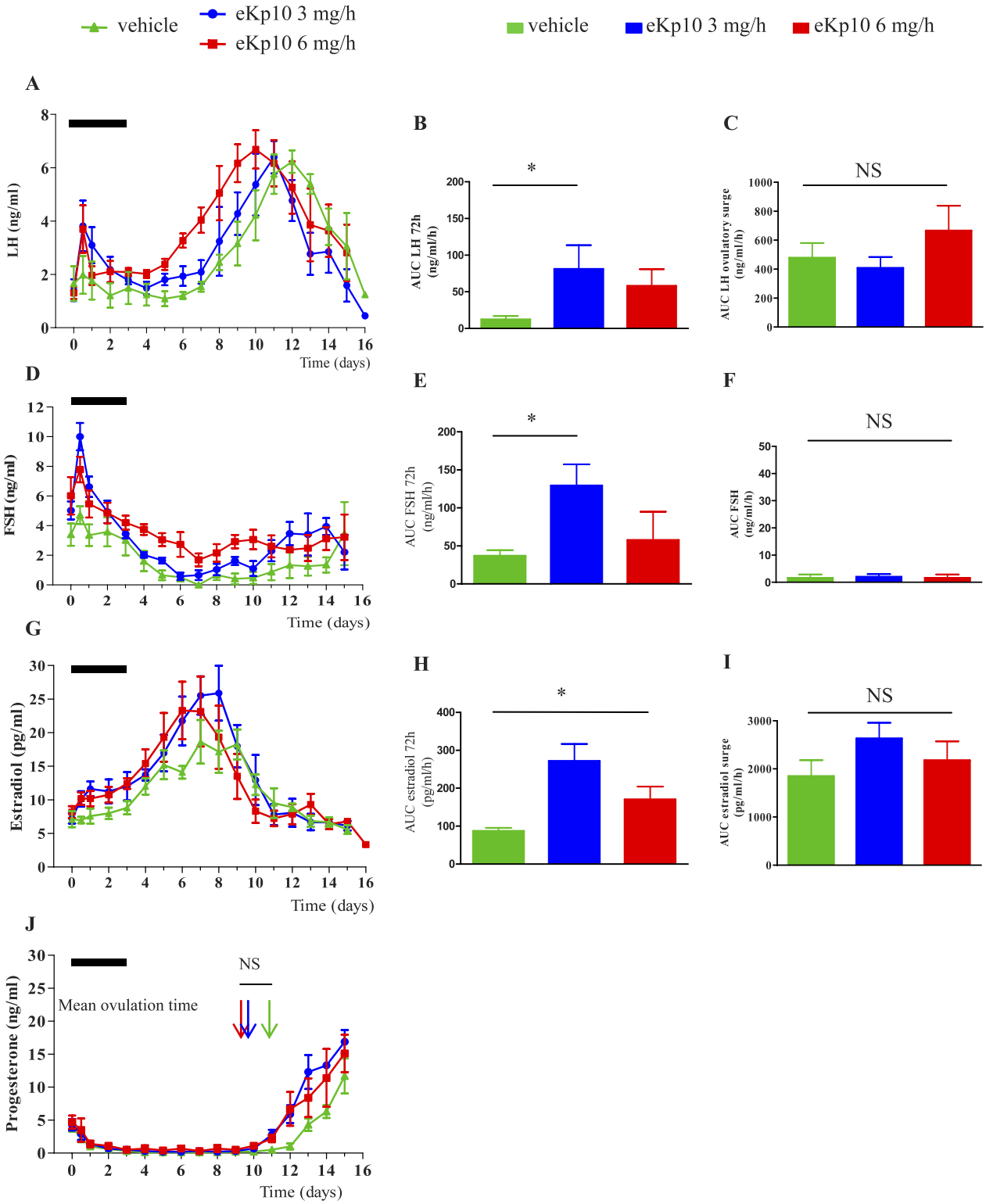

FIG. 5. Experiment 5a: effects of midterm perfusion of eKp10 during the early follicular phase on plasma concentrations (mean ± SEM) of LH (**A**, **B**, and **C**), FSH (**D**, **E**, and **F**), estradiol (**G**, **H**, and **I**), and progesterone (**J**) throughout the cycle. Mares were perfused for 72 h with either vehicle (green symbols), eKp10 at 3 mg/h (blue symbols), or eKp10 at 6 mg/h (red symbols), and hormonal profiles were followed until 5 days after ovulation (as detected by ultrasonography). The black bar shows the 72 h period of perfusion. **B**, **E**, **H**) Integrated hormone response (AUC) for the 72 h of perfusion for LH, FSH, and estradiol, respectively. **C**, **F**, **I**) Integrated hormone response (AUC) for the periovulatory surge for LH, FSH, and estradiol, respectively. Arrows in **J** indicate the mean ovulation time in the three groups (color coded as above) as determined by ultrasonography. $P < 0.05$ (Kruskal-Wallis), $^*P < 0.05$, permutation test for independent samples with Bonferroni correction and one-tailed P value. NS, not significant.

peak LH levels are attained (that is 2 days before ovulation, [35] and Fig. 5), consistent with the proposed role for estradiol in the positive feedback leading to ovulation [25, 30, 33]. Nevertheless, in contrast to ovine or cattle, for instance, the very existence of a positive feedback effect of estradiol at any point of the periovulatory LH surge in mare remains controversial (Refs. [35–37], and see below). The long-lasting LH increase in mare appears mandatory to induce maturation of dominant-size follicles leading to ovulatory competence [38]. As a consequence, when used as an ovulation-inducing agent, exogenous GnRH needs to be provided at larger doses and for much longer in the anestrus mare compared to the anestrus ewe [38, 39].

The fact that gonadotropic cells of the mare pituitary manage to sustain their LH output for several days in the face of persistent GnRH stimulation may be explained in part by the relative resistance to desensitization and slow rate of endocytosis of the equine GnRHR compared to other species, including sheep [40, 41]. In line with the reduced down-regulation of the receptor by its ligand, GnRHR content within the hypothalamus does not show any conspicuous seasonal variations in spite of the fact that both total GnRH in the hypothalamus and LH within the pituitary are drastically reduced during deep anestrus compared to the breeding season [42]. Such a difference in pituitary LH levels most likely underlies the large difference in basal LH levels we observed between seasonally acyclic and cyclic mares. In line with previous findings [43], we did not find any overt seasonal difference in basal FSH levels. The relative paucity of releasable pituitary LH during anestrus might explain, at least in part, the small response to eKp10. However, exhaustion of pituitary gonadotropins as a cause for the weak and short-lived response to eKp10 can be ruled out because injection of a GnRH analog following injection of eKp10 readily elicited LH/FSH release during anestrus. Furthermore, when GnRH is perfused for 3 days, the LH response (but not the FSH response) is sustained for the full duration of the perfusion. The same applies for the breeding season: acute GnRH injection triggers increases in LH/FSH during a chronic perfusion of eKp10. Actually, notwithstanding the different basal levels, the relative magnitude of the LH and FSH response to the same dose of eKp10 (6 mg/h) is not overtly larger in cyclic mares; indeed, the response is actually comparatively weaker during the mid- to late stages of the follicular phase than during anestrus. The reason for this difference in the ability of eKp10 to raise gonadotropin levels between mid- to late and early follicular phase is unclear but may reflect the increased negative feedback of estradiol toward the pituitary that develops throughout the follicular phase.

Compared to the sheep, the LH response to eKp10 in the mare is small. This may indicate that eKp10 is not as potent a GnRH secretagogue as mouse Kp10 (identical to ovine Kp10) or human Kp10. The cloning of Kiss1 in the Welsh pony mare reveals that eKp10—as predicted from genomic data in Thoroughbred horse—differs from Kp10 in other species in that the second amino acid on the N-terminal is an Arg instead of an Asn. Whether such a difference in sequence translates into a difference in biological activity is unknown. However, we deem it unlikely because the characteristics of the response obtained by others with either rat Kp10 [44] or eKp10 [29] in light-horse mares appear similar and are also in good agreement with those we report here. Furthermore, the amplitude of the gonadotropin response to eKp10 is not overtly smaller than that to GnRH, albeit comparatively larger doses of eKp10 have to be used. This seems to imply that stimulation triggered by eKp10 at the Kiss1r is able to elicit a significant GnRH release and would seem to rule out a grossly deficient signaling through the equine Kiss1r as a potential explanation. Finally, in line with other species and notwithstanding the difference in basal levels mentioned above, production and release of gonadotropins from the pituitary can be pharmacologically elicited throughout the year. Therefore a reduced GnRH output, most likely due to a reduced stimulatory input to GnRH neurons, appears to be the primary reason for the low LH level, which appears as the primary factor for the anovulatory follicular waves typical of the anestrus state in the mare [43].

If one combines these considerations with the fact that GnRH application can sustain high levels of gonadotropins (in part due to the low desensitization of the GnRHR already mentioned) and induce ovulation in the mare, it would then appear that the blockade resides in the ability of eKp10 target tissues to provide a sustained response to the ligand. Whether sustained eKp10 stimulation leads to desensitization and/or internalization of the mare Kiss1r was not directly addressed in vitro, but such a mechanism is plausible and has indeed been reported in other species [9, 45, 46]. Actually, the characteristics of the in vivo LH/FSH response to eKp10 reported here—short-lived effect whatever the reproductive status and inability of an acute eKp10 injection to elicit gonadotropin release during a chronic eKp10 perfusion—appear fully compatible with the hypothesis of a rapid desensitization. To start to address this possibility, we identified and cloned the equine orthologue of Kiss1r/GPR54 in the horse, which is missing from the genome assembly EquCab2 and subsequent genomic [47] or transcriptomic sequencing datasets [48]. This gene is expressed in the Welsh mare hypothalamus, the length of the mRNA consists of an ORF similar to that of other species, and the deduced eKiss1r protein sequence possesses several of the key features of GPCRs, albeit some amino acid positions differ with respect to other mammals. Whether eKiss1r is subject to a more rapid desensitization than Kiss1r of other mammals remains unknown, and addressing this question will require the development of comparative in vitro studies.

Besides a potential role for Kiss1r desensitization, another reason for the inability of eKp10 to sustain LH/FSH levels may emerge if one considers the sites of action for Kp. When a large bolus of Kp10 was injected peripherally in the ewe, Kp10 remained undetectable in the cerebrospinal fluid, which indicates that the peptide does not cross the blood-brain barrier [12]. Therefore, any effect of the peptide injected through this route necessarily reflects action in structures outside the blood-brain barrier, including circumventricular organs such as the median eminence. If the full effect of eKp10 in mare requires combined action at the level of both GnRH cell bodies in the hypothalamus and nerve terminals in the median eminence, then peripheral injection may be insufficient to sustain high GnRH output. However, because Kp10 is a very efficient ovulation-inducing agent in the ewe, such a line of reasoning would be valid only if one concedes significant differences—anatomical and/or otherwise—between the two species, while admitting that the inability of Kp10 to cross the blood-brain barrier also applies to the mare. The hypothesis that Kp10 injected peripherally does not exert its full effect may also be relevant when considered in the context of the comparative neuroanatomy of GnRH systems. In mammals, GnRH cell bodies are found as a rather continuous band of scattered cells that runs from the medial septum/diagonal band of Broca (dbB) to the tubero-infundibular region of the medio-basal hypothalamus (MBH), including the Arc. There are, however, marked species differences in the relative numbers of GnRH neurons in any given region. For instance, in the ovine, the populations

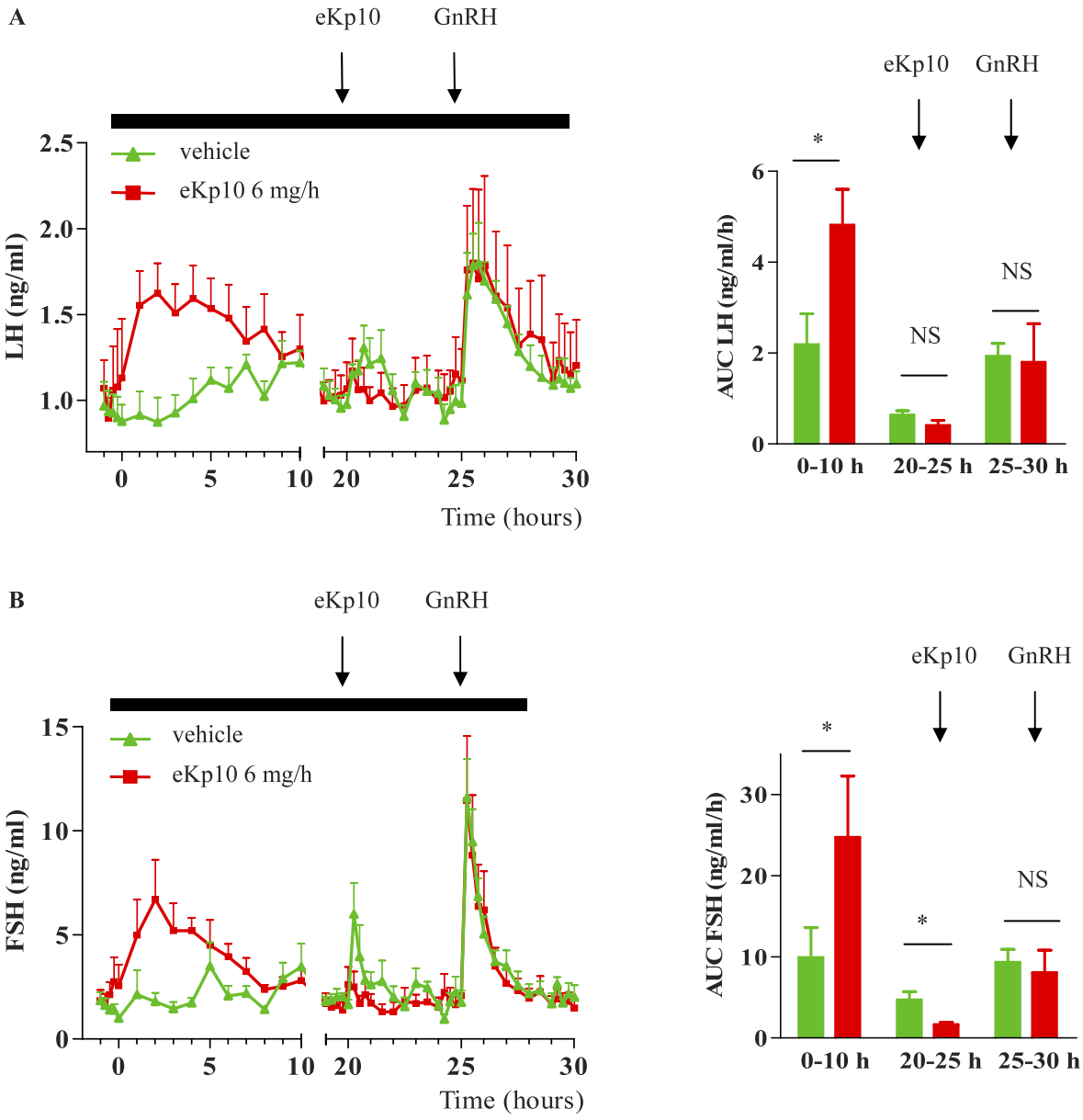

FIG. 6. Experiment 6: effects of acute eKp10 and GnRH injections during midterm perfusion of eKp10 in cyclic mares. **A**, **B**) Left panels are plasma concentrations (mean ± SEM) of LH and FSH during the 30 h of eKp10 at 6 mg/h (red symbols) or saline (green symbols) perfusion at early follicular phase. Mares received a single i.v. injection of eKp10 after 20 h (1 mg, first black arrow) followed by a single i.v. injection of GnRH (25 μg) 5 h later (second black arrow). The period of perfusion (30 h) is represented by a black bar. Right panels are AUC for LH and FSH during the 0–10 h period of perfusion of eKp10 at 6 mg/h or vehicle, and after eKp10 and GnRH bolus injections at, respectively, 20 and 25 h (same color code as for **A**). *$P < 0.05$, permutation test for independent samples with two-tailed P value. NS, not significant.

located within the POA/dbB and the MBH represent 40%–50% and 15%–20% of GnRH neurons, respectively [49, 50]. This is virtually opposite to the equine, where up to 60% of GnRH neurons are found within the MBH while the POA/dbB population comprises only about 10% of the total [44, 51]. This anatomical difference may be functionally meaningful because Kp neurons located within the anterior hypothalamus appear to preferentially project onto GnRH cell bodies while Kp neurons of the Arc may act mostly on GnRH nerve terminals within the median eminence [1, 2]. In other words, the proportion of GnRH neurons located rostrally—hence sheltered from Kp injected peripherally—is larger in ewe than in mare. Although speculative, such a functional arrangement may endow the ewe with the ability to maintain a higher basal level of GnRH production than the mare during Kp stimulation. In the mare, peripheral Kp injection would elicit an immediate release of GnRH through its action at the median eminence—similar to the ewe—but GnRH stocks may thereafter be replenished only at a much lower pace.

This organizational difference might also be relevant to the apparent functional duality of Kp neurons in the Arc and the POA. In rodents, Kp neurons of the AVPV are responsible for the positive feedback effect of estradiol leading to the GnRH/LH surge while Kp neurons of the Arc mediate the negative feedback effect [1, 2]. In the ovine, the role of the MBH in the negative feedback effect has been clearly established, but the site(s) and mechanisms responsible for the GnRH/LH surge remain controversial. Some data appear consistent with a major role for the POA, hence a rodent-like mechanism [52], but most data suggest a combined contribution from both POA and

MBH [53–55]. Indeed, because peripherally injected Kp in the ewe does not cross the blood-brain barrier but nevertheless triggers the LH surge, a direct effect of Kp toward GnRH cell bodies within the rostral hypothalamus does not appear to be the primary mechanism by which the surge is initiated. To the best of our knowledge, there are no data regarding these issues in mare, and the very existence of a positive feedback remains questionable [37]. Classical experiments in sheep have shown that pulsatility of GnRH governs pulsatile release of LH [56, 57], a feature that appears critical for the function of the hypothalamo-pituitary-gonadal axis [8]. In the mare, these aspects of pulsatility remain largely unexplored, and whether LH pulses are indeed triggered by GnRH pulses is yet to be determined [21]. Interestingly, compared to continuous perfusion, pulsatile GnRH application appears more efficient in eliciting LH release in anestrus mares [39]. Whether eKp10 needs to be released in a pulsatile manner to sustain GnRH/LH output leading to the surge remains unknown but is a distinct possibility [12]. Such a mode of release would also presumably limit considerably any potential desensitization of the eKiss1r.

As discussed previously, levels of estradiol in the cycling mare display particular patterns, which seem crucial for the occurrence of the LH surge. In contrast to the situation in the ewe, where photoperiod and sex steroids interact to yield the seasonal pattern of reproduction [58], seasonal variations of LH persist in ovariectomized mares [43, 59, 60]. Varying degrees of steroid-independence in the seasonal control over gonadotropins are observed in other species such as the snowshoe hare [61], the golden hamster [62], and the quail [63]. In all these species, the effect of ovarian steroids is secondary to that of photoperiod and responsible for the cyclical LH/FSH secretion during the breeding season [35, 59, 61]. There is unambiguous evidence that Kp neurons within the Arc are responsible for this dual response to photoperiod and sex steroids and indeed constitute a crucial center of integration and relay toward GnRH neurons in hamsters [3] and sheep [5, 6]. The photoperiodic control over *Kiss1* expression may be dependent on differential photoperiodic triiodothyronine production within the MBH, which is itself dependent on the effect of melatonin within the adjacent pars tuberalis [4, 18]. Similar mechanisms for triiodothyronine production and action might be at play also in horses because the median eminence/pars tuberalis region of this species, akin to what has been consistently observed in all mammals studied to date, is by far the central site displaying the most important density of melatonin receptors [64]. The scarce neuroanatomical evidence in mare suggests that the general organization of the Kp system is roughly similar to that in sheep [44, 65]. However, there are no data regarding photoperiodic expression or regulation of *Kiss1* by sex steroids within the Arc of the horse.

In conclusion, whatever the procedure of administration, eKp10 stimulation of the gondadotropic axis in the mare is always transient and appears insufficient to elicit a prolonged LH surge, a mandatory prerequisite for ovulation. Several not mutually exclusive reasons might account for this failure, amongst which is desensitization of the Kiss1r. The cloning of the equine Kiss1r now provides us with the molecular tool required to undertake in vitro experiments to further our understanding of this potentially relevant mechanism. If one considers the apparent importance of GnRH/LH pulsatility and the potential desensitization of Kiss1r in face of continuous eKp10 application, pulsatile release of eKp10 instead of long-term perfusion or repeated injections might be the way forward. Arguably this mode of administration, even if it proved efficient, would be of little value for practical application in the field but may be helpful to elucidate potential species differences. Investigating further the existence and potential mechanisms mediating estradiol positive feedback as well as the impact of photoperiod and sex steroids on Kp expression within the Arc and the POA will be crucial for our understanding of the mechanisms underlying the control of reproduction in the mare and may assist us in developing more efficient strategies to induce ovulation.

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