Developmental Changes in GnRH Release in Response to Kisspeptin Agonist and Antagonist in Female Rhesus Monkeys (*Macaca mulatta*): Implication for the Mechanism of Puberty

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Kisspeptin (KP) and KP-1 receptor (KISS1R) have emerged as important upstream regulators in the control of puberty. However, how developmental changes in KP-KISS1R contribute to the pubertal increase in GnRH release still remains elusive. In this study, we examined the effects of the KP agonist, human KP-10 (hKP-10), and the KP antagonist, peptide 234, on *in vivo* GnRH release in prepubertal and pubertal ovarian-intact female rhesus monkeys using a microdialysis method. We found that direct infusion of hKP-10 into the medial basal hypothalamus and stalk-median eminence region stimulated GnRH release in a dose-responsive manner, whereas infusion of peptide 234 suppressed GnRH release in both developmental stages. Because ovarian steroid feedback on GnRH release becomes prominent after the initiation of puberty in primates, we further examined whether ovarian steroids modify the GnRH response to hKP-10. Results demonstrate that the hKP-10-induced stimulation of GnRH release was eliminated by ovariectomy in pubertal, but not prepubertal, monkeys. Furthermore, replacement of estradiol into ovariectomized pubertal monkeys resulted in a partial recovery of the hKP-10-induced GnRH release. Collectively, these results suggest that a KISS1R-mediated mechanism, in addition to the pubertal increase in KP-54 release we previously reported, contributes to the pubertal increase in GnRH release and that there is a switch from an ovarian steroid-independent to -dependent mechanism in the response of GnRH to KP. **(*Endocrinology* 153: 825–836, 2012)**

Despite the well-established concept that puberty onset is triggered by an ovarian steroid-independent increase in GnRH release in primates (1–4), the neurobiological mechanism triggering the pubertal increase in GnRH release remains elusive. The hope for a breakthrough arrived in 2003, when groundbreaking studies proposed that kisspeptin (KP) and the KP-1 receptor (KISS1R) play a role in the mechanism of puberty. These studies identified mutations in *KISS1R* that result in a delay in or absence of puberty in humans (5, 6), and evidence from transgenic *KISS1R*-deficient mice, which exhibit hypogonadotropic hypogonadism (5), supports this concept.

Previous studies in our laboratory in primates demonstrate that 1) KP increases along with the pubertal increase in GnRH release and 2) direct infusion of the KP agonist, human KP-10 (hKP-10), into the medial basal hypothalamus (MBH)/stalk-median eminence (S-ME) region stimulates *in vivo* GnRH release, whereas a similar infusion of the KP antagonist, peptide 234, suppresses GnRH release in ovarian-intact pubertal female rhesus monkeys (7, 8). However, it is unclear whether the pubertal increase in KP release solely contributes to the pubertal increase in GnRH release or whether developmental changes in a KISS1R-mediated mechanism also contribute to the pubertal increase in GnRH release.

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Abbreviations: CNS, Central nervous system; E_2, 17β-estradiol; hKP-10, human KP-10; KISS1R, KP-1 receptor; KP, kisspeptin; MBH, medial basal hypothalamus; OVX, ovariectomy; S-ME, stalk-median eminence.

In both male and female mice, the number of KP-expressing neurons increases before puberty onset (9, 10), although there is a clear sex difference in the timing of the pubertal increase in KP expression pattern (9), which is likely due to the difference in the age when circulating gonadal steroids increase (10, 11). In primates, KP signaling also increases at the time of puberty, as indicated by an increase in KP mRNA in ovarian-intact female and agonadal male monkeys (12) and KP release in ovarian-intact female monkeys (7). However, because a direct comparison between the two sexes has not been made, it is unclear whether there are any sex differences in the timing of the pubertal increase in KP signaling or whether this timing is influenced by gonadal steroids in primates.

The number of KISS1R-expressing neurons, including the number of GnRH neurons that express KISS1R, also increases during development (13). Interestingly, however, it appears that no sex difference in the developmental pattern of KISS1R expression exists. In both male and female mice, the number of GnRH neurons expressing KISS1R reaches adult levels by postnatal d 20 (13). Moreover, the number of GnRH neurons responding to exogenous KP increase before puberty (14), and the LH response (and presumably response of GnRH neurons) in juvenile mice to a higher (0.1 nmol) dose of KP-10 is larger than that in adult (14) male mice, indicating that the GnRH response to KP-10 increases well before puberty onset and that developmental changes in a KISS1R-mediated mechanism are independent from the influence of gonadal steroid feedback. In primates, developmental changes in KISS1R also appear to be independent of the pubertal increase in gonadal steroids, because KISS1R mRNA levels do not change at puberty in agonadal males (12). However, this is not as clear in female primates, because data on a developmental change in KISS1R mRNA has been reported only from ovarian-intact, but not ovariectomized (OVX), female monkeys (12).

Clearly, there is a gap in our knowledge as to the contribution of developmental changes in KP input to GnRH neurons *vs.* the KISS1R-mediated mechanism in GnRH neurons. In the present study, we therefore conducted a series of studies addressing the question of whether any changes in the GnRH response to a KP agonist and antagonist occur during pubertal development in female rhesus monkeys and whether these changes are dependent upon the pubertal increase in ovarian steroids.

Materials and Methods

Animals

Thirteen prepubertal (13.0–20.2 months of age) and 15 pubertal (28.0–43.7 months of age) female rhesus monkeys (*Macaca mulatta*) were used in this study. All animals were born and raised at the Wisconsin National Primate Research Center and housed in pairs (cages 172 × 86 × 86 cm) with 12 h light, 12 h dark, at a controlled temperature (22 C). They were fed a standard diet of Teklad Primate Chow (Harlan, Madison, WI) twice per day, and water was available *ad libitum*. Fresh fruit or other enrichment was provided on a daily basis. Pubertal stages of female rhesus monkeys were defined as previously reported (15). During the prepubertal stage, circulating levels of 17β-estradiol (E_2) and LH are low, no physical signs of puberty are apparent, and monkeys are typically younger that 21 months of age. During the pubertal stage, circulating levels of E_2 and LH are elevated with clear nocturnal increases in LH, and physical signs of puberty (development of perineal sex-skin, larger nipple size, and vaginal bleeding) are apparent. All experiments were conducted under the National Institutes of Health and U.S. Department of Agriculture guidelines, and the protocol was approved by the Animal Care and Use Committee, University of Wisconsin-Madison.

Experimental design

Four series of experiments using an *in vivo* microdialysis method were conducted to compare developmental changes in female rhesus monkeys at the prepubertal and pubertal stages. In experiment 1, to determine developmental changes in KISS1R in ovarian-intact prepubertal and pubertal female monkeys, the effects of the KP agonist human KP-10 (hKP-10) [hKP10 (112–121)-amide; Phoenix, Belmont, CA] or vehicle [perfusion fluid for central nervous system (CNS); CMA, Stockholm, Sweden] on GnRH release were examined. After 60 min of control sampling, 10 nM (low dose, experiment 1a) or 100 nM (high dose, experiment 1b) hKP-10 or vehicle was infused through the microdialysis probe into the MBH/S-ME for 20 min while dialysates were continuously collected at 10-min intervals. In experiment 1a, a set of four prepubertal (13.4 ± 0.4 months) and four pubertal (31.8 ± 0.2 months) females received 10 nM hKP-10. In experiment 1b, a second set of four prepubertal (15.2 ± 0.5 months) and four pubertal (30.2 ± 1.4 months) females received 100 nM hKP-10. Control data (vehicle effects) were obtained from two prepubertal and two pubertal females from each set of animals in experiments 1a and 1b.

In experiment 2, to determine whether there are developmental changes in the response of GnRH release to the blockade of endogenous KP signal, the KP antagonist peptide 234 (8) at 10 nM or vehicle (CNS perfusion fluid) was infused through the microdialysis probe for 30 min while dialysates were continuously collected at 10-min intervals. Five prepubertal (14.4 ± 0.5 months) and four pubertal (32.3 ± 0.4 months) monkeys were used for this experiment. Vehicle data were obtained from all monkeys. Because experiments 1 and 2 were concurrently conducted, three prepubertal and all of the pubertal monkeys examined in experiment 2 were also examined in experiment 1.

In experiment 3, to determine whether the developmental changes in the GnRH response to hKP-10 observed in experiment 1 are due to the pubertal increase in circulating ovarian steroids, the effects of 10 nM hKP-10 were initially examined in six prepubertal (19.2 ± 0.7 months) and four pubertal (33.6 ± 1.9 months) OVX monkeys: three of the prepubertal and two of the pubertal females used in experiments 1 and 2 and an additional three prepubertal and two pubertal females were OVX at least 1 month before experimentation. Vehicle data were ob-

tained from three of the monkeys at each developmental stage. Subsequently, the effects of a higher dose (100 nM) of hKP-10 or vehicle were examined in three additional OVX pubertal monkeys (29.6 ± 0.6 months). The protocol for this experiment was similar to that described in experiment 1.

In experiment 4, to determine the role of E_2 on the GnRH response to hKP-10, three OVX pubertal females (one from experiment 3 and two additional monkeys, 37.0 ± 4.6 months) received a 4-cm silastic capsule containing E_2 12–14 d before the microdialysis experiments, as described previously (16). The protocol for this experiment was similar to that described in experiment 1.

Cranial pedestal implantation and guide cannula insertion

All animals were well adapted to the primate chair, experimental conditions, and the researchers before experimentation. At least 1 month before microdialysis experiments, all animals were surgically implanted with a cranial pedestal under isoflurane anesthesia as previously described (17). On the day of the microdialysis experiment, the monkey was anesthetized with ketamine (10–15 mg/kg body weight) and dexmedetomidine (up to 3.0 μg/kg body weight) and placed in a stereotaxic apparatus. A microdrive unit, which allows for three-dimensional adjustment, was secured to the cranial pedestal, and a guide cannula with an inner stylet was inserted 5 mm above the S-ME with the microdrive unit. The custom-made guide cannula (CMA) consisted of a stainless steel shaft (76.0 mm in length, 0.91 mm diameter) with a removable stainless steel stylet (96.0 mm in length, 0.6 mm in diameter). Venticulographs were used to visualize and verify the x-, y-, z-coordinates of the guide cannula above the S-ME.

Microdialysis experiment

Immediately after guide cannula placement, the monkey was removed from the stereotaxic apparatus and placed in a primate chair. The inner stylet was removed, and a microdialysis probe (stainless steel shaft 96.0 mm in length, 0.6 mm in diameter) with a polyarylethersulfone membrane (20-kDa molecular mass cutoff, 5.0 mm in length, 0.5 mm in diameter) was inserted into the guide cannula so that the tip of the probe was located in the MBH/S-ME as previously described (18). CMA CNS perfusion fluid (147 mM NaCl, 2.7 mM KCl, 1.2 mM $CaCl_2$, 0.85 mM $MgCl_2$) containing bacitracin (4 U/ml) was infused through the influx tubing at 2 μl/min with a CMA/102 microdialysis pump (CMA). Perfusates were collected into borosilicate tubes at 10-min intervals with a fraction collector (FC203B; Gilson, Middleton, WI) and 130 μl buffer (0.1% gelatin, 0.01 M PO_4, 0.15 M NaCl, and 0.1% NaN_3 at pH 7.4) was added after sample collection. Samples were immediately frozen on dry ice and stored at −80 C until assayed. Experiments were conducted for up to 12 h, during which the animal was provided monkey chow, fresh fruit, other snacks, and water and seated close to a partner monkey for visualization and vocalization. After experiments, monkeys were returned to their home cage for at least 1 month before participating in a subsequent microdialysis experiment.

Radioimmunoassay

RIA for GnRH measurement was conducted using the R42 antiserum provided by Dr. Terry Nett (Colorado State University, Fort Collins, CO) with a protocol previously described (19). Assay sensitivity was 0.02 pg/tube. Intra- and interassay coefficients of variation were 8.1 and 11.3%, respectively. Circulating levels of E_2 before the first experiment from each monkey were measured as described previously (20).

Statistical analysis

As described in the experimental design, three to six animals were used for each experiment. However, in some experiments, data were obtained twice in an animal at the same developmental stage. In this case, we counted the results of the two trials as an independent entry, because the data of each trial were obtained at least 2–4 months apart, and the placement of the microdialysis probe was different for each infusion. In experiment 1a, two of the four prepubertal and all four pubertal monkeys were examined twice (n = 6, prepubertal stage; n = 8, pubertal stage). In experiment 1b, two of the four prepubertal and three of the four pubertal monkeys were examined twice (n = 6, prepubertal stage; n = 7, pubertal stage). In experiment 2, two of the five prepubertal and two of the four pubertal monkeys were examined twice (n = 7, prepubertal stage; n = 6, pubertal stage). In experiment 3, one of the six prepubertal and all four pubertal monkeys were examined twice (n = 7, prepubertal stage; n = 8 pubertal stage) with 10 nM hKP-10, and one of the three pubertal monkeys was examined twice (n = 4) with 100 nM hKP-10. In experiment 4, 1 of the 3 pubertal monkeys was examined twice (n = 4.) In experiment 1, the mean of 30-min time blocks (three successive 10-min samples) from 60 min before hKP-10 or vehicle infusion (baseline GnRH levels) to 90 min after infusion (GnRH responses) was calculated in individual trials. Subsequently, the effects of 10 nM hKP-10, 100 nM hKP-10, or vehicle on the 30-min time blocks within an experimental group were examined using two-way ANOVA with repeated measure followed by Bonferroni *post hoc* analysis (Prism version 5.00; GraphPad Software, San Diego, CA). Next, treatment effects (vehicle *vs.* 10 nM hKP-10; vehicle *vs.* 100 nM hKP-10; 10 nM hKP-10 *vs.* 100 nM hKP-10) were similarly examined using two-way ANOVA with unrepeated measure followed by Bonferroni *post hoc* analysis. Finally, treatment between developmental stages (prepubertal *vs.* pubertal) was tested with two-way ANOVA and Bonferroni *post hoc* analysis. Overall significance is described in the text (see *Results* and figure legends), but only *post hoc* analysis is depicted on the figures. In experiment 2, analysis for 1) treatment effects over time within a developmental stage, 2) the effects of treatments (vehicle *vs.* 10 nM peptide 234) also within a developmental stage, and 3) treatment between developmental stages (prepubertal *vs.* pubertal) was conducted as described in experiment 1. In experiment 3, analysis was conducted as described in experiment 1. Additionally, the effects of gonadal status (OVX *vs.* ovarian-intact) on GnRH response to hKP-10 within a developmental stage were similarly tested. In experiment 4, analysis was conducted as described in experiment 1. Additionally, the effects of gonadal status ($OVX+E_2$ *vs.* OVX or $OVX+E_2$ *vs.* ovarian-intact) were similarly tested. Circulating E_2 levels between groups were examined with Student's *t* test. Differences were considered significant at $P < 0.05$.

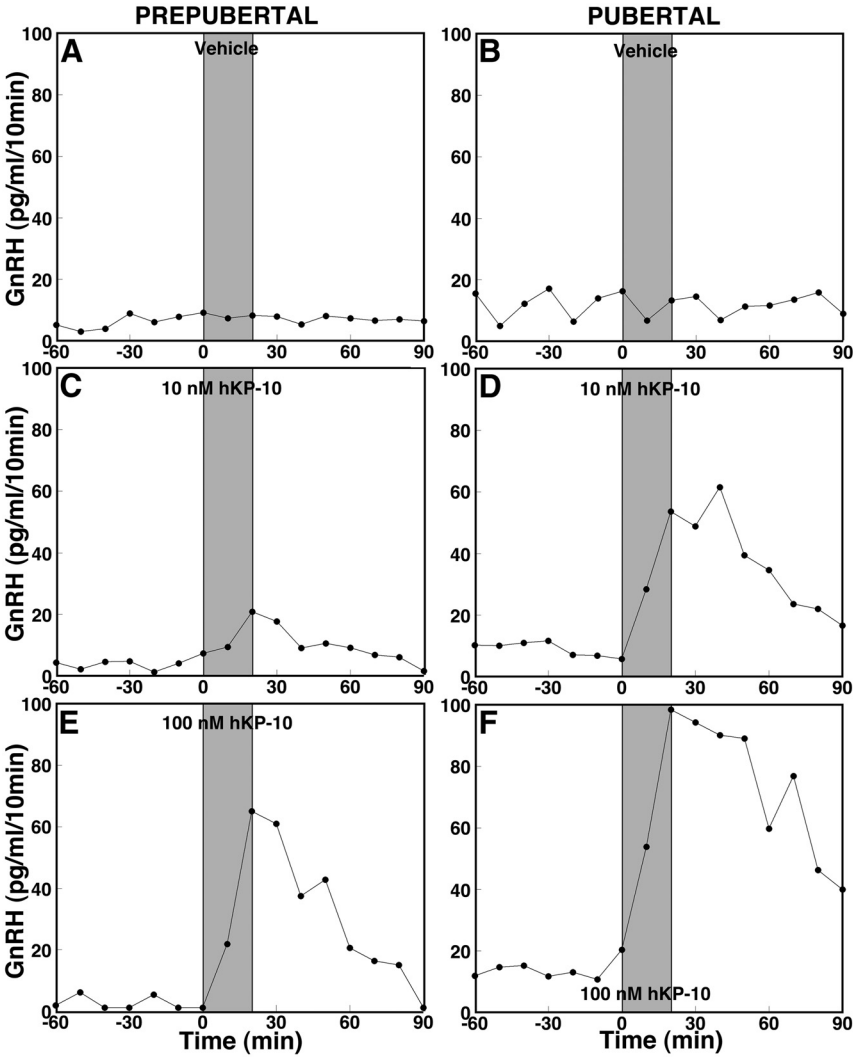

FIG. 1. Effects of hKP-10 on GnRH release in ovarian-intact prepubertal (*left panels*) and pubertal (*right panels*) female monkeys. After 60 min control sampling with microdialysis, vehicle (*top panels*), 10 nM hKP-10 (*middle panels*), or 100 nM hKP-10 (*bottom panels*) was directly infused into the S-ME for 20 min as shown by *shaded bars*, and dialysates were continuously collected for an additional 90 min. Representative cases with each treatment are shown. At both 10 nM (C) and 100 nM (E), hKP-10 clearly stimulated GnRH release *in vivo* in prepubertal monkeys, whereas vehicle (A) did not. Similarly, hKP-10 infusion at both doses (10 nM in D and 100 nM in F) stimulated GnRH release in pubertal monkeys, whereas vehicle (B) failed to induce any changes. Note that baseline levels of GnRH release are higher in the pubertal monkeys than in the prepubertal monkeys.

Results

Developmental changes in GnRH response to hKP-10 (experiment 1)

Consistent with our previous observation (1), baseline GnRH levels before hKP-10 challenges in prepubertal monkeys were lower than those in pubertal monkeys (Figs. 1 and 2). Infusion of 10 and 100 nM hKP-10 for 20 min in prepubertal monkeys stimulated GnRH release in a dose-responsive manner (Fig. 1, C and E). Statistical analysis indicated that the increases in GnRH release at both doses in prepubertal monkeys (Fig. 2, C and E) were significant (overall significance: $P < 0.05$ for low dose; $P < 0.01$ for high dose) compared with vehicle infusion (Fig. 2A). *Post hoc* analysis indicated that hKP-10 significantly ($P < 0.05$ for low dose; $P < 0.001$to $P < 0.05$ for high dose) stimulated GnRH release for up to 60 (10 nM) or 90 (100 nM) min over baseline GnRH levels as well as GnRH values with vehicle treatment at corresponding time periods (Fig. 2A). Moreover, the increase in GnRH release induced by 100 nM hKP-10 (Fig. 2E) was significantly (overall significance: $P < 0.01$) greater than that by 10 nM hKP-10 (Fig. 2C) and *post hoc* analysis indicated that all 30-min blocks of GnRH release during and up to 90 min after 100 nM hKP-10 (Fig. 2E) infusion were significantly ($P < 0.05$ to $P < 0.01$) higher than corresponding 30-min blocks with 10 nM hKP-10 (Fig. 2C).

In pubertal monkeys, 10 and 100 nM hKP-10 infusion also induced a robust increase in GnRH release (Fig. 1, D and F). Increases in GnRH release induced by hKP-10 at both doses (Fig. 2, D and F) were also significant (overall significance: $P < 0.01$ for both doses) compared with vehicle infusion. *Post hoc* analysis indicated that both 10 and 100 nM hKP-10 significantly ($P < 0.01$ for low dose; $P < 0.05$ for high dose) stimulated GnRH release for up to 60 min after the start of the infusion compared with GnRH baseline levels as well as corresponding values with vehicle treatment (Fig. 2B). Interestingly, although the GnRH response to the 100 nM hKP-10 dose was significantly (overall significance: $P < 0.05$) larger than that to the 10 nM hKP-10 dose in pubertal monkeys, *post hoc* analysis indicated only a trend toward an increase. Importantly, the GnRH response to 10 nM hKP-10 during the first 60 min was significantly greater in pubertal monkeys compared with prepubertal monkeys ($P < 0.05$ for both). Vehicle infusion had no effect on GnRH release in monkeys at either developmental stage (Figs. 1, A and B, and 2, A and B).

Developmental changes in GnRH response to peptide 234 (experiment 2)

To assess the contribution of endogenous KP signaling to GnRH release across pubertal onset, we examined the

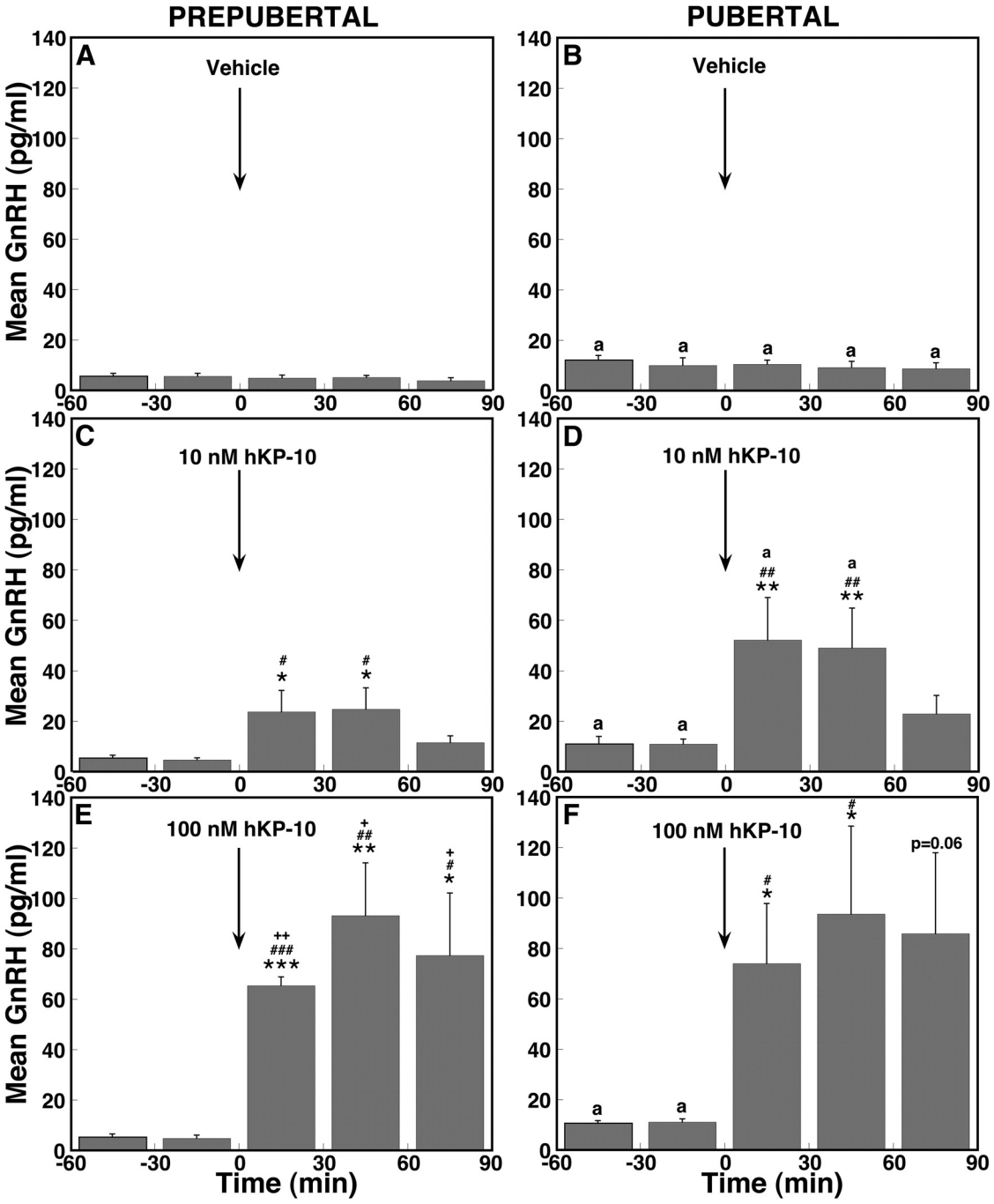

FIG. 2. Direct hKP-10 infusion stimulates mean GnRH release *in vivo* in ovarian-intact prepubertal and pubertal monkeys. Group data (mean ± SEM) from prepubertal monkeys treated with vehicle (A, n = 4), 10 nM hKP-10 (C, n = 6), and 100 nM hKP-10 (E, n = 6) and from pubertal monkeys treated with vehicle (B, n = 4), 10 nM hKP-10 (D, n = 8), and 100 nM hKP-10 (F, n = 7) are shown. In prepubertal monkeys, both 10 and 100 nM hKP-10 significantly increased GnRH release over the baseline levels ($P < 0.05$ for 10 nM and $P < 0.01$ for 100 nM hKP-10), the effect of hKP-10 at both doses was significantly higher than those in vehicle ($P < 0.05$ for 10 nM and $P < 0.01$ for 100 nM hKP-10), and the effect of 100 nM hKP-10 was significantly larger than the effect of 10 nM hKP-10 ($P < 0.01$). Similarly, in pubertal monkeys, both 10 and 100 nM hKP-10 significantly stimulated GnRH release over the baseline levels ($P < 0.01$ for 10 and 100 nM hKP-10), the effects of hKP-10 at both doses were significantly higher than those in vehicle ($P < 0.01$ for 10 and 100 nM hKP-10), and the effect of 100 nM hKP-10 was significantly larger than the effect of 10 nM hKP-10 ($P < 0.05$). Moreover, the GnRH response to 10 nM hKP-10 during the first 60 min was significantly larger than that in prepubertal monkeys. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ *vs.* before 10 or 100 nM hKP-10 infusion; #, $P < 0.05$; ##, $P < 0.01$; ###, $P < 0.001$ *vs.* vehicle infusion; +, $P < 0.05$; ++, $P < 0.01$ *vs.* 10 nM hKP-10; a, $P < 0.05$ *vs.* corresponding time block in prepubertal monkeys.

effects of the novel KP antagonist, peptide 234 (10 nM), on GnRH release. Results indicate that peptide 234 suppressed GnRH release in prepubertal monkeys (Fig. 3C). The peptide 234-induced suppression of GnRH release (Fig. 4C) in prepubertal monkeys was significant (overall significance: $P < 0.01$) compared with vehicle infusion

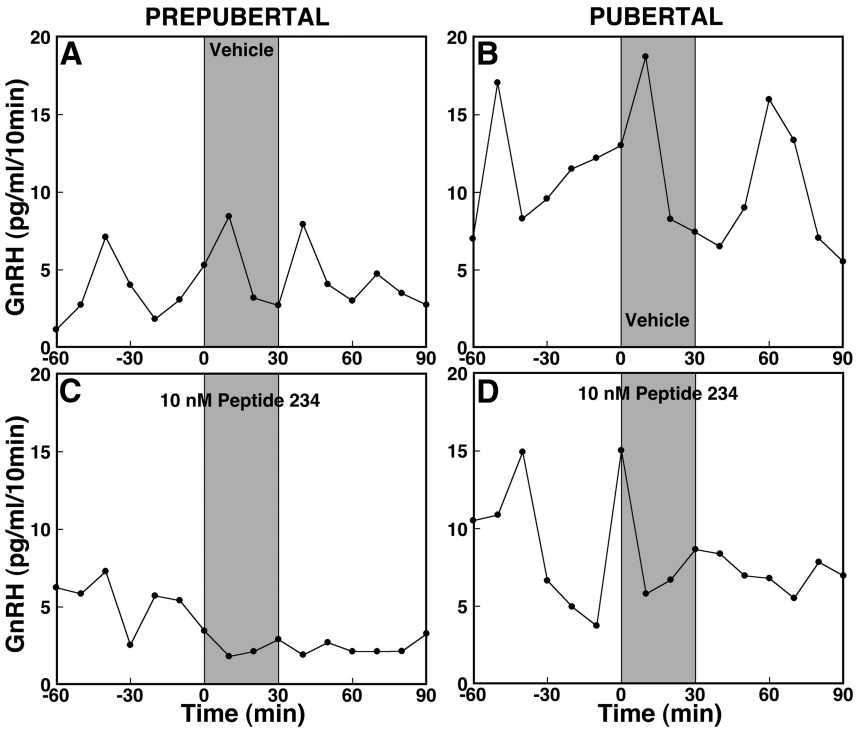

FIG. 3. Effects of peptide 234 on GnRH release in ovarian-intact prepubertal (*left panels*) and pubertal (*right panels*) female monkeys. After 60 min control sampling with microdialysis, vehicle (*top panels*) or 10 nM peptide 234 (*bottom panels*) was directly infused into the S-ME for 30 min as shown by *shaded bars*, and dialysates were continuously collected for an additional 90 min. Representative cases with each treatment are shown. Peptide 234 at 10 nM (C) clearly suppressed GnRH release *in vivo* in a prepubertal monkey, whereas vehicle (A) did not. Similarly, 10 nM peptide 234 (D) suppressed GnRH release in a pubertal monkey, whereas vehicle (B) failed to induce any changes. Note that baseline levels of GnRH release are higher in the pubertal monkeys than in the prepubertal monkeys.

(Fig. 4A). *Post hoc* analysis indicated that peptide 234 significantly ($P < 0.01$) suppressed GnRH release during and up to 90 min after the start of the infusion compared with baseline GnRH levels as well as respective mean values for vehicle treatment (Fig. 4A).

In pubertal monkeys, peptide 234 similarly suppressed GnRH release (Figs. 3D and 4D), and this suppression was significant (overall significance: $P < 0.05$) compared with vehicle infusion (Fig. 4B). Interestingly, *post hoc* analysis indicated that suppression of GnRH release by peptide 234 was significant ($P < 0.05$) during, but not after, infusion compared with baseline GnRH levels and respective vehicle treatment means. Vehicle infusion had no effect on GnRH release in monkeys at either developmental stage (Figs. 3, A and B, and 4, A and B).

Effects of OVX on GnRH response to hKP-10 (experiment 3)

Because circulating levels of ovarian steroid hormones differ before and after pubertal onset, we next examined whether ovarian steroid feedback plays a role in the developmental difference in GnRH response to hKP-10. Infusion of 10 nM hKP-10 resulted in a small increase in GnRH release in OVX prepubertal monkeys (Fig. 5D), and this increase was significant (overall significance: $P < 0.05$) compared with vehicle infusion. *Post hoc* analysis indicated that hKP-10 significantly ($P < 0.05$) stimulated GnRH release (Fig. 6D) for up to 60 min after the start of infusion over GnRH baseline levels as well compared with corresponding vehicle treatment means (Fig. 6A). Comparison between OVX and ovarian-intact prepubertal monkeys indicated that there was no overall difference in the GnRH response to hKP-10 (Fig. 2C *vs.* 6D).

In contrast, both the 10 and 100 nM hKP-10 infusion in OVX pubertal monkeys failed to induce any increase in GnRH release (Figs. 5, E and F, and 6, E and F). In fact, there was no significant difference during or after either hKP-10 infusion (10 or 100 nM) compared with before infusion as well compared with vehicle infusion. Furthermore, no difference in the GnRH response was found between the low and high dose of hKP-10. Comparison between OVX and ovarian-intact pubertal monkeys in experiment 1 indicated that OVX clearly (overall significance: $P < 0.05$) abolished the 10 nM hKP-10-induced stimulation of GnRH release (Fig. 2D *vs.* 6E). *Post hoc* analysis further indicated that GnRH levels for up to 60 min after the start of the hKP-10 infusion were significantly different ($P < 0.05$) between OVX and ovarian-intact pubertal monkeys. Similarly, OVX (overall significance: $P < 0.05$) abolished the 100 nM hKP-10-induced stimulation of GnRH observed in ovarian-intact pubertal monkeys, and *post hoc* analysis indicated that GnRH levels were significantly different ($P < 0.05$) for up to 60 min after the start of the hKP-10 infusion between OVX and ovarian-intact pubertal monkeys (Fig. 2F *vs.* 6F). Vehicle infusion had no effect on GnRH release in either developmental stage (Figs. 5, A–C, and 6, A–C).

Effects of E2 replacement on GnRH response to hKP-10 (experiment 4)

To determine whether replacement of E_2 restores the absence of the hKP-10-induced GnRH release in OVX pubertal monkeys in experiment 3, OVX pubertal monkeys were implanted with a silastic capsule containing E_2

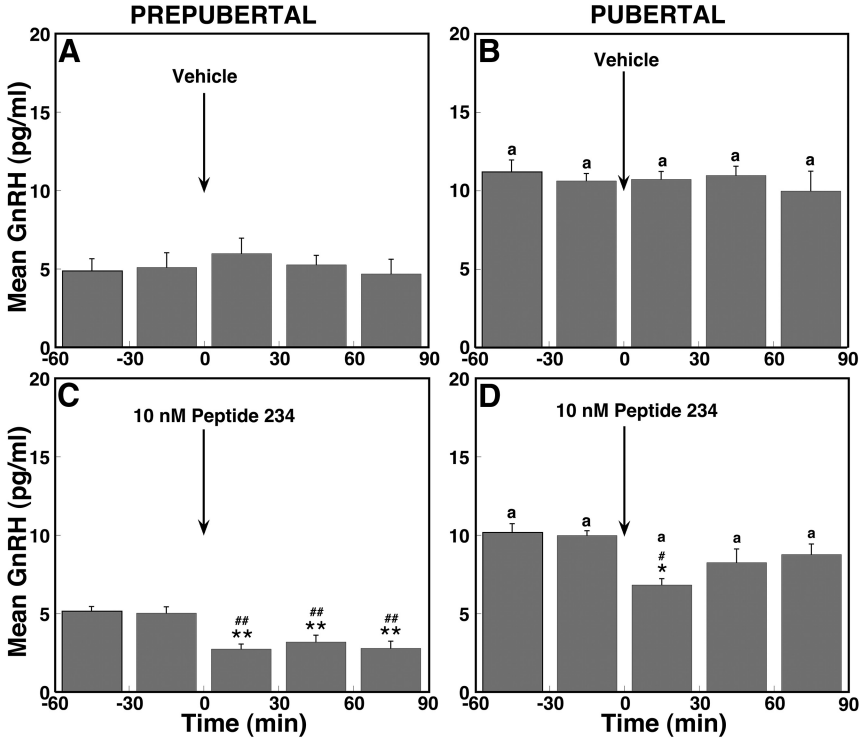

FIG. 4. Direct peptide 234 infusion suppresses mean GnRH release *in vivo* in ovarian-intact prepubertal and pubertal monkeys. Group data (mean ± SEM) from prepubertal monkeys treated with vehicle (A, n = 5) or 10 nM peptide 234 (C, n = 7) and from pubertal monkeys treated with vehicle (B, n = 4) or 10 nM peptide 234 (D, n = 6) are shown. In prepubertal monkeys, peptide 234 at 10 nM significantly decreased GnRH release compared with baseline levels ($P < 0.01$), and the effect of peptide 234 was significantly lower than that in vehicle ($P < 0.01$). Similarly, in pubertal monkeys, 10 nM peptide 234 significantly suppressed GnRH release compared with baseline levels ($P < 0.05$), and the effect of peptide 234 was significantly lower than that in vehicle. *, $P < 0.05$; **, $P < 0.01$ *vs.* before peptide 234 infusion; #, $P < 0.05$; ##, $P < 0.01$ *vs.* vehicle infusion; a, $P < 0.05$ *vs.* corresponding time block in prepubertal monkeys.

benzoate (OVX+E_2). Infusion of hKP-10, but not vehicle, induced an increase in GnRH release (Fig. 7, A and B) in OVX+E_2 pubertal monkeys. The hKP-10-induced GnRH increase was significant (overall significance: $P < 0.05$) over GnRH baseline levels (Fig. 7D). *Post hoc* analysis indicated that the effects of hKP-10 were significant during ($P < 0.05$), but not after, infusion (Fig. 7D). Comparison between OVX+E_2 and OVX monkeys indicated that hKP-10 significantly (overall significance: $P < 0.05$) stimulated GnRH release in OVX+E_2, but not OVX, pubertal monkeys. *Post hoc* analysis indicated that only GnRH levels during, but not after, the hKP-10 infusion in OVX+E_2 pubertal monkeys were significantly ($P < 0.05$) greater than those in OVX pubertal monkeys. Further comparison between OVX+E_2 and intact monkeys indicated that the GnRH response to hKP-10 was similar (no overall significant difference) during the infusion (Fig. 7D *vs.* 2D). Interestingly, the duration of the GnRH stimulation in OVX+E_2 monkeys was shorter than in ovarian-intact pubertal monkeys.

Circulating estradiol levels in females in this study

Measurement of circulating E_2 levels in monkeys from all experiments, shown in Table 1, indicated that E_2 levels in ovarian-intact prepubertal monkeys were low and similar to their OVX counterparts. Although E_2 levels in ovarian-intact pubertal monkeys were significantly ($P < 0.001$) higher than those in ovarian-intact prepubertal monkeys, OVX eliminated the elevated E_2 in pubertal monkeys, resulting in levels as low as those seen in OVX prepubertal monkeys ($P < 0.001$). Finally, implantation of the E_2 capsule significantly ($P < 0.001$) increased E_2 levels in OVX pubertal monkeys, although these restored E_2 levels were slightly higher than those in ovarian-intact pubertal monkeys ($P < 0.05$).

Discussion

In the present study, we found that 1) GnRH release was stimulated by hKP-10 in both prepubertal and pubertal monkeys in a dose-responsive manner; 2) release of GnRH in both prepubertal and pubertal monkeys was suppressed by peptide 234; 3) OVX eliminated the hKP-10-induced GnRH release in pubertal, but not prepubertal, monkeys; and 4) replacement of E_2 in OVX pubertal monkeys only partially restored the hKP-10-induced GnRH release that was absent in OVX pubertal monkeys. These results indicate that there are developmental changes in the GnRH responsiveness through a KISS1R-mediated mechanism.

Nearly a decade ago, the role of KP and its receptor, KISS1R, in the mechanism of puberty was proposed (5, 6, 21). Since then, new avenues of KP-KISS1R signaling to GnRH neurons have been revealed. GnRH neurons express KISS1R (22), KP mRNA expression in the MBH increases at puberty (9, 12, 14, 23), and administration of KP-10 advances puberty onset in rodents (23, 24), whereas administration of peptide 234 delays the onset of puberty in rodents (25). Moreover, we have previously shown parallel pubertal increases of KP-54 release and GnRH release (7). These findings, together with the observations from the present study that hKP-10 stimulates GnRH release in a dose-responsive manner in

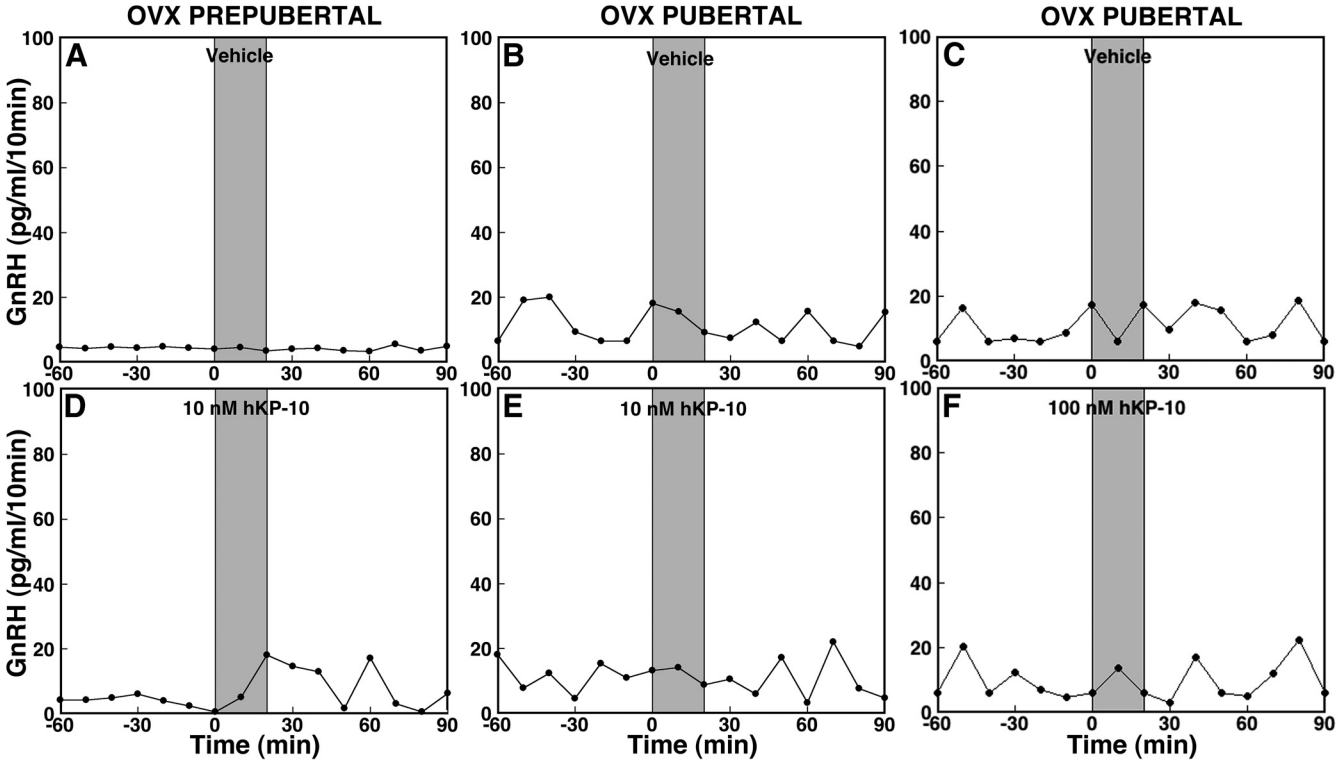

FIG. 5. Effects of 10 nM hKP-10 (D and E), 100 nM hKP-10 (F), or vehicle (A–C) on GnRH release *in vivo* in prepubertal (*left panels*) and pubertal (*middle* and *right panels*) OVX female monkeys. Representative cases are shown. Infusion of 10 nM hKP-10 clearly stimulated GnRH release in an OVX prepubertal monkey (D), whereas infusion of either 10 (E) or 100 (F) nM hKP-10 failed to stimulate GnRH release in OVX pubertal monkeys. Vehicle infusion had no effect on GnRH release in monkeys at either developmental stage.

both prepubertal and pubertal monkeys, allow us to conclude that the pubertal increase in GnRH release is, at least in part, a consequence of the pubertal increase in KP release.

The pubertal increase in GnRH release could also be a consequence of developmental changes in KISS1R. The results of the present study show that hKP-10 induced dose-dependent responses in both age groups. The absolute magnitude of the GnRH responses to hKP-10 at the 10 nM dose in pubertal monkeys was significantly larger than in prepubertal monkeys, whereas GnRH responses to hKP-10 at the 100 nM dose in prepubertal monkeys were similar to that in pubertal monkeys. Perhaps, with the high (100 nM) hKP-10 dose, GnRH neurons reach their limit of secretory capacity, because it has been reported that stimulation of LH release by KP-10 reaches a plateau with higher KP doses in humans (26) and in sheep (27). Although we did not assess the effect of hKP-10 at doses lower than 10 nM in this study, our results with the 10 nM dose are consistent with those described by Han *et al.* (14) showing that the LH response to intracerebroventricular infusion of lower (10 fmol and 0.1 pmol), not higher (0.1 nmol), KP-10 doses in male mice increases across puberty. Therefore, functional changes in KISS1R occur at the time of puberty. This view is further supported by reports that 1) there is a pubertal increase in KISS1R mRNA expression in the MBH in ovarian-intact female monkeys (12) and 2) KISS1R mRNA expression in the anteroventricular periventricular nucleus increases shortly before puberty in ovarian-intact female rats (28).

One can argue that there is no difference in the relative GnRH response to hKP-10 between prepubertal and pubertal monkeys in this study, because the baseline GnRH levels before the hKP-10 challenge differ. Initially, we had a similar viewpoint. However, the absence of a GnRH response to hKP-10 in OVX pubertal monkeys and the difference in circulating E_2 levels between two developmental stages cannot be ignored. Additionally, the relationship between baseline GnRH levels *vs.* the GnRH response to KP-10 is currently unknown. Thus, the absolute GnRH response to hKP-10 should reflect developmental changes in KISS1R signaling in the hypothalamus. Hypothetically, studies of the response to hKP-10 in single primate GnRH neurons (if the procedure becomes available) may be helpful to tease out the two possible interpretations.

In contrast to changes with the KP agonist, peptide 234, the KP antagonist, suppressed GnRH release in both prepubertal and pubertal monkeys. However, the magnitude of the suppression (maximal suppression) by peptide 234

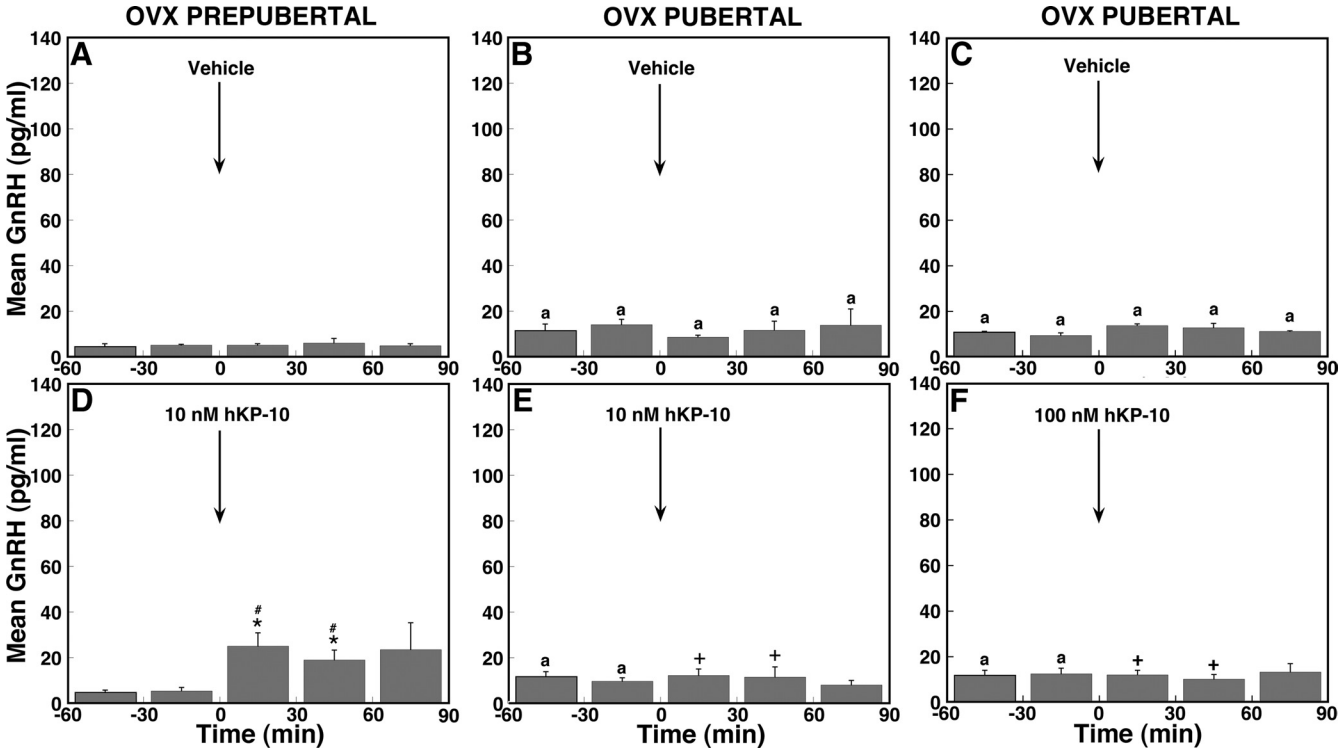

FIG. 6. Effects of OVX on mean GnRH release in response to hKP-10 infusion in prepubertal and pubertal monkeys. Group data (mean ± SEM) from prepubertal monkeys treated with vehicle (A, n = 4) or 10 nM hKP-10 (D, n = 7) or from pubertal monkeys treated with vehicle (B, n = 4; C, n = 3), 10 nM hKP-10 (E, n = 8), or 100 nM hKP-10 (F, n = 4) are shown. In prepubertal monkeys, hKP-10 at 10 nM significantly increased GnRH release compared with baseline levels ($P < 0.05$), and the effect of hKP-10 was significantly higher than that in vehicle ($P < 0.05$). In contrast, in pubertal monkeys, both 10 and 100 nM hKP-10 failed to induce any changes in GnRH release compared with baseline levels and vehicle infusion. Moreover, the GnRH responses to 10 or 100 nM hKP-10 were significantly different between OVX and ovarian-intact pubertal monkeys ($P < 0.05$ for both). *, $P < 0.05$ *vs.* before hKP-10 infusion; #, $P < 0.05$ *vs.* vehicle infusion; +, $P < 0.05$ *vs.* hKP-10 infusion in ovarian-intact pubertal monkeys; a, $P < 0.05$ *vs.* corresponding time block in prepubertal monkeys.

at the dose examined did not differ between the two age groups. Interestingly, the suppression of GnRH release by peptide 234 continued after peptide 234 infusion in prepubertal, but not pubertal, monkeys. This can be interpreted to mean that because there is more endogenous KP available in pubertal monkeys (7, 29) for competitive binding to KISS1R against peptide 234 than in prepubertal monkeys, peptide 234 is not able to continue suppressing GnRH release after infusion in pubertal monkeys. We cannot, however, exclude the possibility that there are pubertal changes in the pharmacokinetics of peptide 234 binding to KISS1R, because both age and steroid milieu can influence receptor pharmacokinetics (30, 31). Although in the present study we were not able to examine a higher concentration of peptide 234, because it occluded the microdialysis membrane, additional experiments with peptide 234 at lower doses and different steroid milieu may clarify this possibility.

In the present study, we found that in ovarian-intact female monkeys, the low dose of hKP-10 induced larger GnRH responses in pubertal monkeys compared with prepubertal monkeys. This difference is, in part, attributable to an elevated circulating E_2 concentration at puberty (15), because OVX in pubertal monkeys eliminated the GnRH response to both low and high doses of hKP-10, and results of our preliminary studies show that there is no GnRH response to peptide 234 in OVX pubertal monkeys (Guerriero, K. A., and E. Terasawa, unpublished findings). Moreover, the finding in prepubertal monkeys that hKP-10 with the low dose induced a small, but significant, increase in GnRH release, regardless of the presence or absence of the ovary indicates that feedback action of E_2 on GnRH release is not operative in prepubertal monkeys. This view is consistent with our previous findings that OVX increases pulsatile GnRH release in pubertal, but not prepubertal, monkeys (3) and that sc injection of E_2 suppresses GnRH release in OVX pubertal monkeys, but E_2 is ineffective in OVX prepubertal monkeys (4).

In this study, baseline GnRH levels in ovarian intact pubertal monkeys were not different from those in OVX counterparts. These data differ from those reported in the previous study (3). We believe that the higher variability of GnRH baseline levels in this study is due to the differences in the methods from the previous study (3). That study used a push-pull perfusion method, by which 200-μl samples were collected exclusively from the S-ME

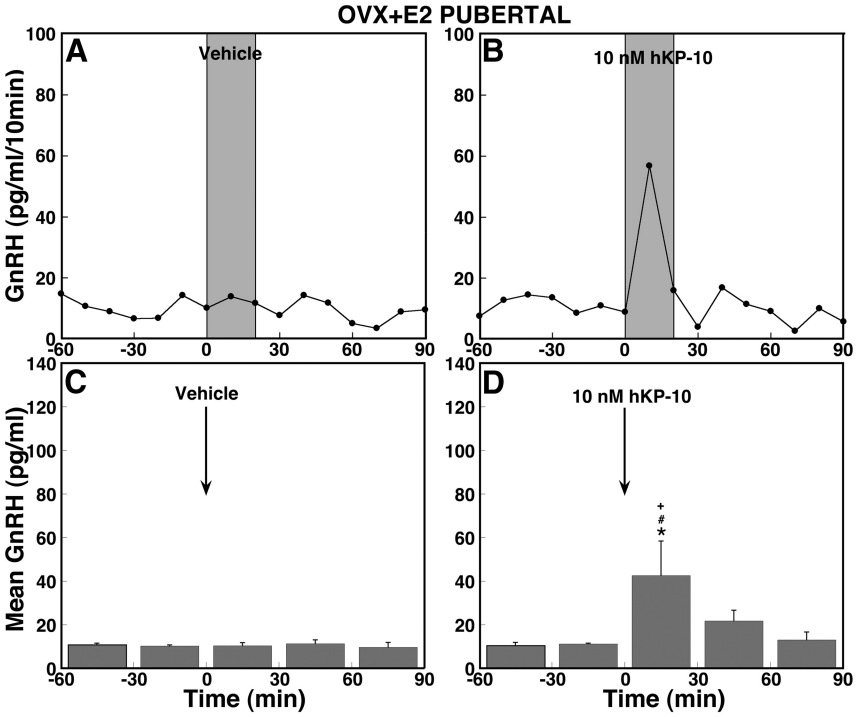

FIG. 7. Replacement with E_2 only partially restores the hKP-10-induced stimulation in the OVX pubertal monkey. Representative cases and group data (mean ± SEM) with vehicle infusion (A and C, n = 4) and 10 nM hKP-10 (B and D, n = 4) are shown. During the infusion, hKP-10 at 10 nM significantly stimulated mean GnRH release over baseline levels ($P < 0.05$), whereas vehicle infusion had no effect on GnRH release. Comparison between OVX+E_2 and OVX pubertal monkeys shows that the GnRH response to hKP-10 is significantly different during the infusion ($P < 0.05$). *, $P < 0.05$ *vs.* before hKP-10 infusion; #, $P < 0.05$ *vs.* vehicle infusion; +, $P < 0.05$ *vs.* OVX pubertal monkeys.

where GnRH neuroterminals are concentrated, whereas the current study used a microdialysis method, by which 20-μl samples were collected not only from the S-ME but also MBH. Therefore, the amount of GnRH in samples with microdialysis is likely to yield higher variability among individuals.

In contrast to our present findings, absence of the ovary does not eliminate the LH response to peptide 234 in sheep and rodents (8), nor does the LH response to KP-10 in rats (32). The difference in the results of the present study and those in sheep and rodents could be due to differences in species, sex, the age of gonadectomy, and/or developmental stages. Because in this study we examined the effects of hKP-10 only in OVX pubertal, but not adult, monkeys, it is unknown whether a significant change in a KISS1R-mediated mechanism observed in pubertal monkeys continues into adulthood or whether it is specific to puberty. Nonetheless, the loss of the GnRH response in OVX pubertal, but not OVX prepubertal, monkeys suggests that the GnRH responsiveness through KISS1R in primates appears to undergo a significant change after exposure to the pubertal rise in circulating E_2, such that this KISS1R-mediated mechanism becomes dependent upon ovarian steroids after puberty onset. The underlying mechanism of developmental changes in responsiveness of KISS1R in the absence of the ovary remains to be elucidated.

Synaptic input onto GnRH neurons increases along with puberty. The contour of GnRH neurons increases across puberty (33), presumably due to increased synaptic innervation. In fact, an increase in the number of synapses onto GnRH neurons has been reported (34). Because KP stimulates GnRH neuronal activity directly or transsynaptically through interneurons and E_2 further enhances KP-induced GnRH neuronal activity through transsynaptic networks in mice (35), it is likely that synaptic plasticity induced by E_2 after puberty onset greatly amplifies KP action to GnRH neurons. Once this feed-forward mechanism is initiated at puberty, increased E_2 in circulation further accelerates KP-induced GnRH release during pubertal progression.

Unexpectedly, replacement of E_2 in OVX pubertal monkeys only partly restored the hKP-10-induced GnRH response. The reason for this partial recovery remains speculative. First, this is not due to the amount of circulating E_2. Although E_2 capsule implantation in OVX pubertal females results in E_2 levels slightly higher than those in their ovarian-intact counterparts, this level of E_2 is within the normal range for ovarian-intact midpubertal monkeys (15). Second, other factors from the ovary besides E_2 may be involved. For example, a maximal LH response to KP-10, as observed in an estrous rat, requires replacement of both E_2 and progesterone in adult OVX

TABLE 1. Circulating E_2 levels in ovarian-intact (INT) and OVX animals with or without capsules containing E_2 in this study

Developmental stage	Gonadal status	n	E_2 (pg/ml)
Prepubertal	INT	9	42.8 ± 3.1
Pubertal	INT	8	82.6 ± 8.7[a]
Prepubertal	OVX	7	38.6 ± 1.4
Pubertal	OVX	8	38.1 ± 2.7[b]
Pubertal	OVX+E_2	3	113.6 ± 7.2[c]

Values are the mean ± SEM.
[a] $P < 0.001$ *vs.* prepubertal intact.
[b] $P < 0.001$ *vs.* pubertal intact.
[c] $P < 0.001$ *vs.* pubertal OVX.

rats (32). However, this is an unlikely explanation, because progesterone release from the ovary remains low until first ovulation (15), and the prepubertal and pubertal monkeys examined in this study are not yet capable of ovulating, as previously characterized (15). Third, it is possible that properties of estrogen receptors are altered, because we did not replace E_2 immediately after OVX. Expression of estrogen receptor-α differs depending on the time period between OVX and E_2 replacement (36). Fourth, longer exposure to E_2 may be necessary in pubertal monkeys. Although physiological levels of E_2 (15) are achieved with similar capsule implantation (37), it is possible that short-term (~2 wk) E_2 replacement is not sufficient to fully revert the GnRH responsiveness through KISS1R back to the ovarian-intact state. In adult female OVX rabbits, a short-term E_2 replacement with a physiologically equivalent E_2 level, only partially restored the GnRH-induced LH release, and implantation of two times higher the level of E_2 fully restored the effect of OVX on LH release (38). Possible effects of a longer exposure to E_2 remain to be investigated.

In the present study, we have shown that the pubertal increase in GnRH release appears to be attributable to an increase in a KISS1R-mediated mechanism on GnRH neurons. To our knowledge, this is the first study examining developmental changes in KISS1R function by direct measurement of GnRH release. Our finding adds to a previous observation (7) indicating that an increase in KP-54 release occurs with the pubertal increase in GnRH release. Importantly, the GnRH responsiveness through KISS1R appears to undergo developmental changes, switching from an ovarian steroid-independent to -dependent state. This can be interpreted to mean that in primates, an important neurocircuitry change is required before puberty onset, and this change occurs before KP-KISS1R signaling becomes a prominent factor in the control of GnRH release. Previously, we and others have shown (39–41) that in primates the enervation of predominant central inhibition is a prerequisite for the onset of puberty. Perhaps the inability of E_2 to modify stimulatory KP action on GnRH release before puberty is due to this central inhibition. After puberty onset, reduction in this central inhibition allows KP action on GnRH neurons, enhanced by E_2, to be fully functional (42). The relationship between the pubertal increase in KP release and diminution of central inhibition, however, remains to be further investigated.

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