# Critical Roles of Kisspeptins in Female Puberty and Preovulatory Gonadotropin Surges as Revealed by a Novel Antagonist

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Kisspeptins (Kp) have recently emerged as master regulators of the reproductive axis and among the most potent elicitors of GnRH-gonadotropin secretion. Despite their paramount importance in reproductive physiology and their potential therapeutic implications, development of Kp antagonists has remained elusive, and only recently has the first compound with the ability to block Kp actions *in vitro* and *in vivo*, namely p234, been reported. However, previous *in vivo* studies all used acute central injections, whereas characterization of the effects of the antagonist after continuous or systemic administration, which poses pharmacological challenges, is still pending. We report herein a comprehensive series of analyses on the impact of continuous intracerebroventricular infusion of p234 on puberty onset and the preovulatory surge of gonadotropins in the female rat. In addition, the effects of systemic (ip) administration of a tagged p234-penetratin, with a predicted higher permeability at the blood-brain barrier, on Kp-10 induced gonadotropin secretion were evaluated. Central infusion of p234 to pubertal females delayed vaginal opening and decreased uterine and ovarian weights at the expected time of puberty, without affecting body weight. Likewise, chronic intracerebroventricular administration of p234 for 4 d prevented the preovulatory surges of LH and FSH. In addition, systemic (ip) administration of p234-penetratin significantly attenuated acute LH and FSH responses to Kp-10, either after intracerebroventricular or ip injection of Kp. Our data document the validity of p234 for antagonizing Kp actions *in vivo* and provide direct experimental evidence for the important role of Kp signaling in the key events of female reproduction, such as puberty onset and the preovulatory surge of gonadotropins. **(*Endocrinology* 151: 722–730, 2010)**

Kisspeptins (Kp) are a family of structurally related peptides, encoded by the *KISS1* gene, that operate through the G protein-coupled receptor GPR54 (also termed Kiss1 receptor; 1, 2). Although Kp were originally defined as metastasis suppressors, most of the recent attention has been directed at their role as central gatekeepers of puberty onset and fertility (2). Such a reproductive "facet" was initially disclosed by the observation that inactivating mutations of the *GPR54* gene were linked to a failure to go through puberty and hypogonadotropic hypogonadism (3, 4), a phenotype also observed for Kiss1 inactivation in mice (5). Thereafter, Kp have been documented as extraordinarily potent elicitors of gonadotropin secretion in a variety of species, from nonmammals to

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Abbreviations: AUC, Area under the curve; GPR54, G protein-coupled receptor 54; icv, intracerebroventricular; Kp, kisspeptin(s).

rodents, sheep, and primates, including humans (1, 2, 6, 7), by acting primarily on the hypothalamus to activate GnRH neurons. In addition, neuroanatomical studies have allowed identification of discrete populations of Kiss1 neurons at different hypothalamic areas (8–10), as well as the characterization of their patterns of development (*e.g.* along puberty) and physical contacts with GnRH neurons (2, 11, 12), thus unveiling the involvement of Kp in the central networks controlling reproductive function.

Given their putative key roles in fundamental aspects of reproductive maturation and function, the physiology of the Kiss1-GPR54 system has been thoroughly scrutinized during the past 5 yr by a large number of experimental studies, ranging from gene-protein expression analyses to electrophysiological recordings and pharmacological tests (1, 2). However, despite the obvious potential of Kp as targets for therapeutic manipulation of the gonadotropic axis, the latter studies have been based mostly in protocols of single or repeated administration of agonists of GPR54, whereas development of effective Kp antagonists has remained elusive for years. Indeed, the lack of tools for blockade of Kp signaling *in vivo* has made it difficult to provide direct experimental proof for the actual physiological roles of endogenous Kp in key reproductive functions. Very recently, however, the first compound with the ability to block Kp actions *in vivo* and *in vitro*, namely p234, has been reported (13). This peptide antagonist inhibits the firing rate of GnRH neurons and GnRH secretion and suppresses LH responses to exogenous Kp (Kp-10) and gonadectomy (13). Kp antagonists will facilitate the dissection of the physiological roles of the KISS1-GPR54 system and have potential for pharmacological intervention in reproductive diseases.

Despite the considerable advancement posed by the emergence of this first generation of antagonists, the protocols of Kp blockade reported so far have been restricted to the acute, central [intracerebroventricular (icv)] administration of the compounds. From a pharmacological standpoint, however, it is mandatory to define effective protocols of peripheral (rather than central) and/or continuous (rather than acute) antagonization of Kp actions. Moreover, from a physiological perspective, the possibility of persistently blocking Kp signaling provides a unique tool for the direct assessment of the importance of this system in key reproductive phenomena. In this context, the aim of the present work was 2-fold: 1) to characterize the effects of continuous icv administration of the leading compound, p234, on the timing of puberty onset and the generation of the preovulatory surge of gonadotropins, as a trigger for ovulation, in the female rat; and 2) to evaluate the effects of systemic administration of the antagonist on the gonadotropin responses to either centrally or peripherally injected Kp-10. For the latter, a variant of p234 tagged with a penetratin extension, thereby putatively provided with higher permeability at the blood-brain barrier, was used.

## Materials and Methods

### Animals

Wistar male and female rats, bred in the vivarium of the University of Córdoba, were used. Experimental procedures were approved by the Córdoba University Ethical Committee and conducted in accordance with the European Union normative for use of experimental animals. The animals were weaned at d 21 postpartum and were maintained under constant conditions of light (14 h of light, from 0700 h) and temperature (22 C), with free access to pelleted food and tap water. For experiments involving adult female rats, adult virgin animals were monitored for estrous cyclicity by daily vaginal cytology; only rats with at least three consecutive regular 4-d estrous cycles were subsequently used, in keeping with previous work (14, 15).

### Peptides

Kp (110–119)-$NH_2$, or Kp-10, was obtained from Phoenix Pharmaceuticals Ltd. (Belmont, CA). Peptide analogs p234 and p234-penetratin were designed by A.R. and R.P.M. and synthesized by EZBiolab Inc. (Carmel, IN). Peptide sequence of p234 is ac-(D-A)NWNGFG(D-W)RF-NH2, as recently described elsewhere (13). For systemic administration experiments, p234 was modified by the addition of a penetratin tag to increase its penetration of the blood-brain barrier. Penetratin is a seven-amino acid cationic cell-penetrating peptide with the sequence RRMKWKK-NH2 (16), and this was attached via a Tyr residue; the chemical structure of p234-penetratin is RRMKWKKY(D-A)NWNGFG(D-W)RF-NH2. Of note, addition of the penetratin tag to p234 did not affect its binding affinity to GPR54 (see supplemental Fig. 1, published as supplemental data on The Endocrine Society's Journals Online web site at http://endo.endojournals.org), as assayed following previously published procedures (13).

### Experimental designs

In the first set of studies, protocols of continuous central infusion of the recently reported antagonist of Kp, p234, were implemented in female rats. In experiment 1, p234 was chronically infused to pubertal females. As general procedure for central delivery of the antagonist, prepubertal females (n = 15/group) were implanted intradermally with osmotic minipumps (1 $\mu$l/h delivery rate $\times$ 7 d; Alzet mini-osmotic pump model no. 2001, Durect, Cupertino, CA) that were connected to icv cannulae, as previously described (17). Antagonist concentration per mini-pump was adjusted to 10 nmol/24 $\mu$l. Pair-aged females infused with vehicle served as controls. The treatment spanned from postnatal d 30 to d 36. Along treatment, the animals were monitored for daily food intake, body weight gain, and vaginal opening. On d 36, the animals were killed by decapitation, trunk blood was collected, and ovarian and uterine tissues were dissected out and weighed. In addition, in experiment 2, a similar protocol of central infusion of p234 was implemented in adult,

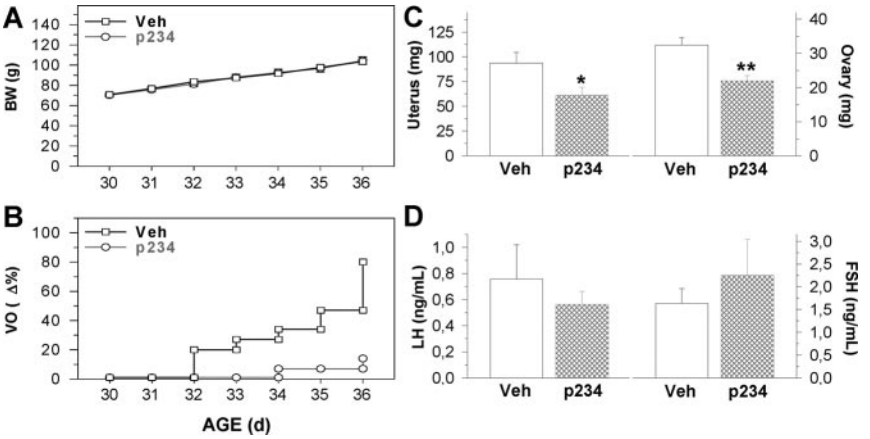


**FIG. 1.** Effects of continuous infusion of Kp antagonist on puberty onset in female rats. The impact of chronic icv infusion of the antagonist of Kp, p234, to pubertal female rats (d 30 to d 36) on different indices of puberty onset is documented. Body weights (A), vaginal opening (B), uterine and ovarian weights (C), and terminal serum LH and FSH levels (D) are presented for animals infused with vehicle or p234. Note that scales are different for LH and FSH levels. *, $P \leq 0.05$; **, $P \leq 0.01$ *vs.* control (vehicle) group (Student's *t* test). BW, Body weight; Veh, vehicle; VO, vaginal opening.

cyclic female rats. The animals (n = 9) were implanted with osmotic mini-pumps in the morning of estrus, and infusion was continued until the following estrus. On the afternoon of proestrus, blood samples (250 $\mu$l) were obtained by jugular venipuncture at 2-h intervals, from 1200 h onward, following previously published procedures (14, 15). Additional blood samples were taken from each animal between 0900 h and 1000 h of the following estrus. A group of cyclic female rats (n = 11) infused with vehicle served as controls.

In the second set of studies, the effects of systemically delivered antagonist, tagged with a penetratin extension, on gonadotropin responses to Kp-10 were evaluated in adult male rats. In experiment 3, the ability of ip injections of p234-penetratin to block the effects of centrally injected Kp-10 was studied. To this end, groups (n = 10–12) of adult male rats were implanted with icv cannulae 24–48 h before testing, as described in detail elsewhere (13). Pharmacological tests involved three 100-$\mu$l injections, via the ip route, of 5-nmol boluses of the antagonist p234-penetratin at 60-min intervals. The last injection was linked to the icv injection of an effective (but submaximal) dose of 100 pmol Kp-10. Animals ip injected with vehicle (instead of the antagonist) and icv injected with Kp-10 served as controls. Blood samples (250 $\mu$l) were taken by jugular venipuncture at 15 and 60 min after each ip injection. In addition, blood samples were taken immediately before initiation of the experiment (time: 0 min) and at 120 min after the last injection (time: 240 min). In addition, in experiment 4, the ability of ip injections of p234-penetratin to antagonize the effects of peripherally injected Kp-10 was evaluated in adult male rats. To this end, a similar experimental-sampling procedure was implemented, except for the fact that animals were not implanted with icv cannulae and received a bolus of Kp-10 (6 nmol) via the ip route coinciding with the last injection of the antagonist.

### Hormone measurements

Serum LH and FSH levels were determined in a volume of 25–50 $\mu$l using a double-antibody method and RIA kits supplied by the National Institutes of Health (Dr. A. F. Parlow, National Institute of Diabetes and Digestive and Kidney Diseases National Hormone and Peptide Program, Torrance, CA). Rats LH-I-10 and FSH-I-9 were labeled with $^{125}$I using Iodo-gen tubes, following the manufacturer's instructions (Pierce, Rockford, IL). Hormone concentrations were expressed using reference preparations LH-RP-3 and FSH-RP-2 as standards. Intra- and interassay coefficients of variation were, respectively, less than 8% and 10% for LH and less than 6% and 9% for FSH. The sensitivity of the assay was 5 pg/tube for LH and 20 pg/tube for FSH. For each hormone, all samples were measured in the same assay. Accuracy of hormone determinations was confirmed by assessment of rat serum samples of known hormone concentrations.

### Presentation of data and statistics

Hormone determinations were conducted in duplicate. Hormonal data are presented as mean ± SEM; when relevant, individual secretory profiles are shown and integrated secretory responses, calculated as area under the curve (AUC) using the trapezoidal rule, are also displayed. Results were analyzed using Student's *t* tests or two-way ANOVA followed by Student-Newman-Keuls multiple range test (SigmaStat 2.0, Jandel Corp., San Rafael, CA). $P \leq 0.05$ was considered significant.

## Results

### Central infusion of p234 inhibits puberty onset in the female rat

To assess directly the functional relevance of Kp signaling in the central networks controlling puberty onset, a protocol of icv infusion of the antagonist p234 was applied in peripubertal female rats, following previous work (17). Thus, osmotic mini-pumps were implanted to deliver a constant rate of 10 nmol/24 h p234 during 7 d, from d 30 to d 36, and phenotypic and hormonal analyses were applied to monitor the progression of puberty. Infusion of Kp antagonist failed to induce overt changes in body weight gain over the study period (Fig. 1A); neither did it cause modifications in daily food intake (data not shown). In contrast, icv administration of p234 to peripubertal female rats evoked a marked delay in the timing of puberty; thus, although 80% of animals infused with vehicle displayed complete canalization of vagina on d 36, only 13% of the females treated with the antagonist showed complete vaginal opening at that age (Fig. 1B). In good agreement, central infusion of p234 caused a significant reduction of ovarian and uterine weights at the end of treatments (Fig. 1C). Of note, however, no overt differences in mean LH and FSH levels were detected between control and p234-treated groups in blood determinations conducted on the morning of the last day (d 36) of infusion of the antagonist (Fig. 1D).

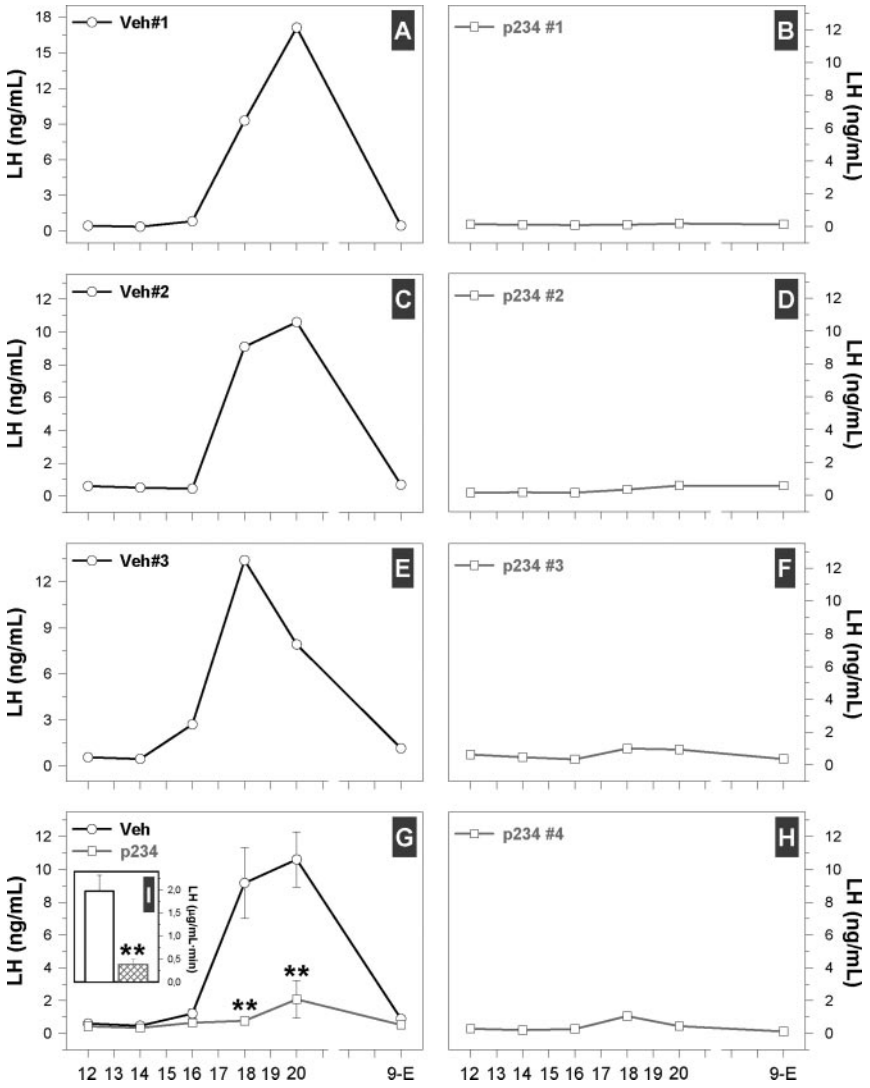


**FIG. 2.** Effects of continuous infusion of Kp antagonist on preovulatory surge of LH. Individual hormonal profiles of LH secretion along the proestrus-to-estrus transition are presented from representative female rats centrally infused with either vehicle (A, C, and E) or p234 (B, D, F, and H). In addition, mean serum LH levels in both groups (G), as well as integrated LH secretion between 14:00 h and 20:00 h of proestrus (AUC; *bar graph* in I), are also shown. Numeric values on x-axis represent daytime (hour) along the afternoon and evening of proestrus. Hormonal levels at the morning of estrus (9-E) are also shown. For further details, see text. **, $P \leq 0.01$ *vs.* corresponding control group (ANOVA followed by Student-Newman-Keuls multiple range test or Student's *t* test for bar graph data). Veh, Vehicle.

## Central infusion of p234 inhibits the preovulatory surge of gonadotropins in the female rat

A similar protocol of central infusion of p234 was applied to adult, cycling female rats to further define the importance of Kp signaling in the generation of the preovulatory surge of gonadotropins. Regularly cycling female rats, showing at least three consecutive 4-d vaginal cycles, were implanted in the morning of estrus with osmotic mini-pumps to allow icv delivery of p234 at a constant rate of 10 nmol/24 h. The infusion was continued until the afternoon of the following proestrus, when the animals were subjected to serial blood sampling along the afternoon and evening of proestrus and the morning of estrus, in keeping with our previous work (14, 15). In cyclic females infused with vehicle, 10 of 11 animals displayed the expected preovulatory surge of LH during the afternoon of proestrus, with a progressive rise of serum concentrations between 1600 h and 2000 h, followed by a drop in LH levels on the morning of estrus; the hormonal profiles of three representative individuals of this group are shown in Fig. 2, A, C, and E. In striking contrast, seven of nine females icv infused with p234 failed to display the prototypical peak of LH levels at proestrus; LH profiles of four representative individuals of this group are depicted in Fig. 2 (B, D, F, and H). However, two of the females infused with p234 did show roughly preserved proestrous peaks of LH (data not shown); in these animals, no overt failure in the delivery system (osmotic pump + icv cannulae) of the antagonist was observed at the time of sampling. For the calculation of mean representative LH levels in both groups, these outliers were selectively excluded. The results of this calculation are shown in Fig. 2G, which documents the ability of the Kp antagonist to potently block the preovulatory surge of LH in cyclic rats. Likewise, Fig. 2I (*inset*) plots the integrated LH secretion (calculated as AUC) during the afternoon or evening (1400 h to 2000 h) of proestrus, showing a 5-fold decrease in the net secretory mass of LH during the preovulatory period in animals infused with p234 antagonist.

In addition, serum FSH levels were monitored in control and p234-treated female rats, during the afternoon or evening of proestrus and the morning of estrus. For simplicity, only mean FSH concentrations, calculated as described for LH determinations, are depicted in Fig. 3. As shown, animals infused with vehicle displayed a detectable increase in serum FSH levels between 1600 h and 2000 h of proestrus (primary surge), which was followed by persistently elevated FSH concentrations at the morning of estrus (secondary surge). In clear contrast, central infusion of p234 abrogated the primary surge of FSH and nullified the elevation of FSH levels on the morning of estrus. Accordingly, the inte-

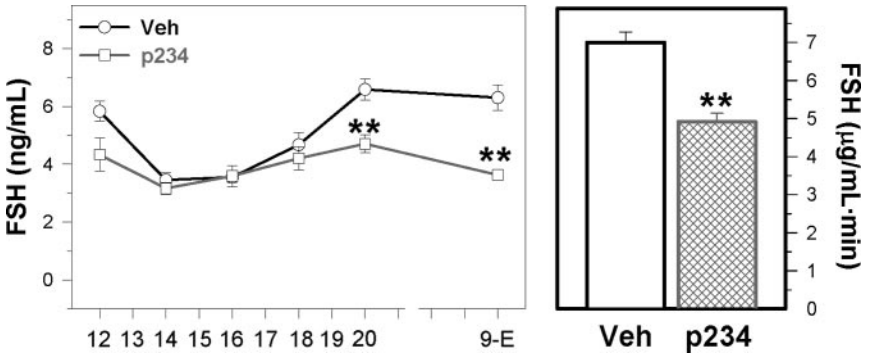


**FIG. 3.** Effects of continuous infusion of Kp antagonist on preovulatory surge of FSH. Mean serum FSH levels along the proestrus-to-estrus transition for female rats centrally infused with either vehicle or p234. Numeric values on x-axis represent daytime (hours) along the afternoon or evening of proestrus. Hormonal levels at the morning of estrus (9-E) are also shown. In addition, integrated FSH secretion between 14:00 h and 20:00 h of proestrus (AUC) is also shown as *bar graph*. For further details, see text. **, $P \leq 0.01$ *vs.* corresponding control group (ANOVA followed by Student-Newman-Keuls multiple range test or Student's *t* test for bar graph data). Veh, Vehicle.

grated FSH secretion encompassing the afternoon or evening of proestrus to the morning of estrus was significantly attenuated in females infused with p234 antagonist. These effects were not detected in the two (out of nine) females displaying conserved LH surges despite icv infusion of p234 (see above) because they also showed elevated levels of FSH at the expected times of the primary and secondary surges (data not shown).

### Systemic administration of p234-penetratin blunts gonadotropin responses to Kp-10

In addition to protocols of continuous central infusion of p234, the ability of systemic (ip) administration of the antagonist to block the gonadotropin-releasing effects of exogenous Kp-10 was tested in adult male rats. For these experiments, a variant of p234, with a penetratin N-terminal extension, as to enhance its permeability through the blood-brain barrier, was used. The blocking effects of this antagonist were assayed against central (icv) and peripheral (ip) injection of a single bolus of Kp-10; p234-penetratin was administered as three ip boluses of 5 nmol each, at −120, −60, and 0 min before administration of Kp-10.

Adult male rats receiving three ip boluses of vehicle and subsequently injected icv with 100 pmol Kp-10 displayed the expected increase in serum LH levels (7), which peaked (>6-fold increase) at 15 min and declined thereafter, with circulating LH levels becoming similar to preinjection values at 120 min after Kp-10 administration. This hormonal response was significantly blunted by pretreatment with p234-penetratin because the LH peak after icv administration of Kp-10 achieved only a 2.5-fold increase over basal levels and was no longer detected at 60 min after injection. Indeed, when represented as the AUC over the 120 min after Kp-10 administration, integrated LH secretory responses to Kp-10 were significantly decreased by pretreatment with the antagonist (Fig. 4, *upper panel*). Likewise, rats injected icv with Kp-10 showed the expected elevation in serum FSH levels, with a mean 2-fold increase over preinjection values that persisted 120 min after administration of Kp. Again, this FSH response was significantly attenuated by ip pretreatment with p234-penetratin; integrated FSH responses over the 120 min after Kp-10 administration were significantly reduced in animals receiving the Kp antagonist (Fig. 4, *lower panel*).

Similarly, adult male rats injected ip with 6 nmol Kp-10 showed a significant increase in circulating LH levels that peaked at 15 min and declined thereafter. The magnitude of this response was lower (2-fold increase) than after icv injection of Kp, probably because of its peripheral route of administration (7). In this setting, pretreatment with three ip boluses of p234-penetratin totally nullified LH responses to Kp-10, as evidenced by time-course profiles and integrated secretory mass after Kp administration (Fig. 5, *upper panel*). Similarly, the modest but detectable rise in serum FSH levels after ip injection of 6 nmol Kp-10 was abrogated by the pretreatment with p234-penetratin (Fig. 5, *lower panel*).

## Discussion

In recent years, Kp and GPR54 have taken central stage in reproductive biology because compelling experimental evidence has strongly suggested their involvement in key aspects of reproductive maturation and function, such as sexual differentiation of the brain, timing of puberty onset, negative feedback of sex steroids on gonadotropin secretion, generation of the preovulatory surge of gonadotropins, and metabolic regulation of fertility (2, 18). However, interpretation of the actual roles of Kp in these phenomena has relied almost entirely on neuroanatomical, pharmacological (*i.e.* activation of GPR54 by exogenous Kp), or functional genomic approaches, the latter involving the congenital inactivation of either Kiss1 or GPR54. The availability of Kp antagonists would facilitate the delineation of the roles of the endogenous Kiss1 system in the neuroendocrine regulation of the gonadotropic system. Our present results extend and refine recent data on the characterization of the first peptide antagonist of GPR54 (13) because they are the first to provide experimental support for protocols of continuous or periph-

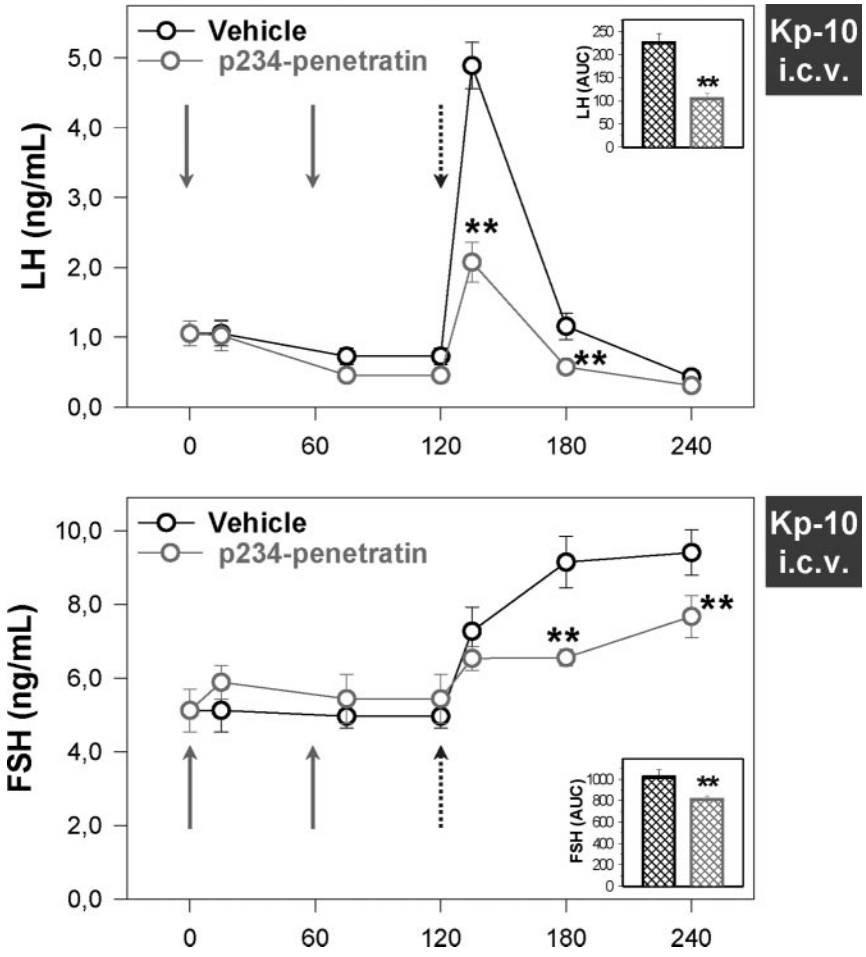


**FIG. 4.** Effects of systemic Kp antagonist on gonadotropin responses to icv Kp-10. LH and FSH secretory profiles are shown from adult male rats receiving three consecutive ip injections (5 nmol each) of the antagonist of Kp, p234-penetratin (*arrows*); the last injection was associated with an icv bolus of Kp-10 (100 pmol). Numeric values on x-axis represent time (minutes) after the first injection of the antagonist. Integrated secretory responses after Kp-10 administration, calculated as AUC, are also depicted as *bar graphs*. **, $P \leq 0.01$ *vs.* corresponding control group (ANOVA followed by Student-Newman-Keuls multiple range test or Student's *t* test for bar graph data).

eral administration of Kp antagonists as a tool for the manipulation of the hypothalamic-gonadotropic axis. In addition to help for a better dissection of Kp biological actions *in vivo*, these observations also have potential therapeutic implications.

The involvement of Kp signaling in the timing of puberty onset was originally suggested on the basis of observations of lack of puberty linked to congenital deficiency of GPR54 (3, 4) and has been further documented by a wealth of pharmacological and neuroanatomical data (11, 19–21). However, the actual role of Kiss1 neurons, either as trigger or as amplifier of the neurosecretory activity of the GnRH system at the time of puberty, has yet to be fully characterized (12). In this context, our present data demonstrate the important activational role of Kp signaling in the onset of puberty in the female rat because the classical indices, such as vaginal opening and the increase in uterine and ovarian weights, were dramatically suppressed (if not totally nullified) by central infusion of the antagonist. Of note, these phenotypic effects were not apparently associated with an overt decrease in mean circulating levels of LH and FSH at the end of treatment. This observation is in line with data from present and previous studies in adult rats (13), where administration of p234 suppressed LH elevation resulting from gonadectomy but not basal gonadotropin concentrations. Given the intracerebral delivery of the antagonist in our experiment, it is tempting to speculate, although yet to be proven, that the observed delay in puberty might be caused by central inhibition of the afternoon minisurges of LH that precede the occurrence of vaginal opening and full pubertal awakening in the rat female (22). Although the effect of peripheral administration was not explored in pubertal females, from a therapeutic perspective our current results prove the principle that occurrence of normal (and possibly precocious) puberty can be suppressed or delayed by the use of Kp antagonists, a possibility that awaits to be confirmed by protocols of continuous systemic administration, in line with our present acute data.

Hypothalamic Kp signaling has also been implicated in the generation of the preovulatory surge of gonadotropins (23). Kiss1 neurons located in the anteroventral periventricular nucleus of rodent hypothalamus are activated by the peak of estradiol preceding the LH surge (23) and make direct synaptic contacts with GnRH neurons (11). However, the actual role of anteroventral periventricular kisspeptinergic afferents in the generation of the ovulatory surge appears controversial because opposite findings on the persistence of positive feedback of estradiol have been reported in different mouse lines with null mutations of GPR54 (24, 25). Of note, however, the latter studies were based on models of congenital absence of the receptor. Our results clarify the situation because they support a mandatory role of Kp in the generation of the preovulatory surge of gonadotropins because acute, transient suppression of Kp signaling prevented the occurrence of the primary surges of LH and FSH, and the secondary peak of FSH, in the majority of cyclic females treated with the antagonist. However, in

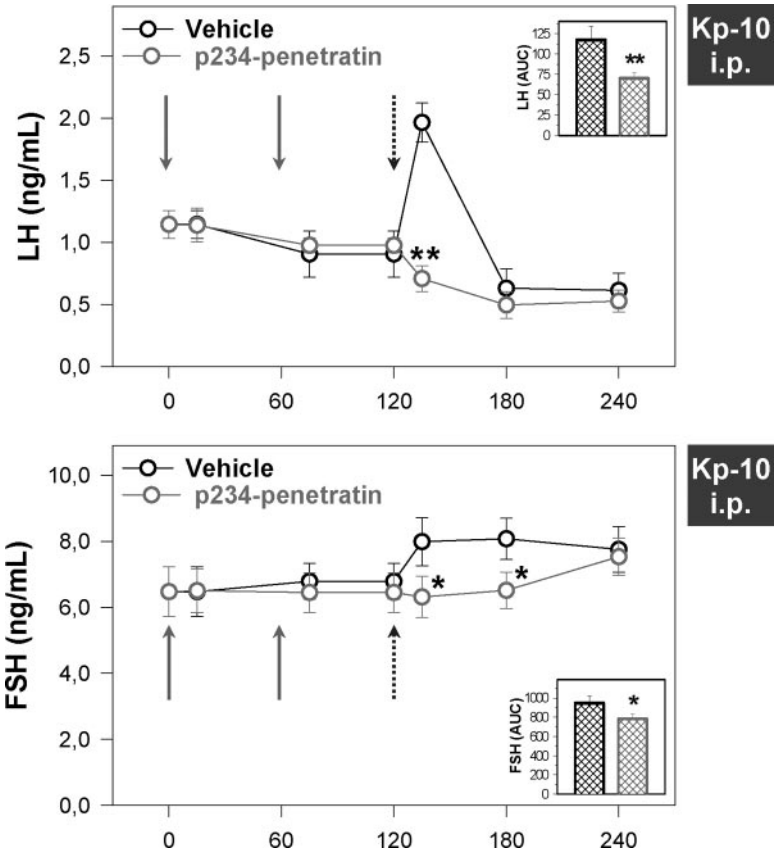


**FIG. 5.** Effects of systemic Kp antagonist on gonadotropin responses to ip Kp-10. LH and FSH secretory profiles are shown for adult male rats receiving three consecutive ip injections (5 nmol each) of the antagonist of Kp, p234-penetratin (*arrows*); the last injection was associated with an ip bolus of Kp-10 (6 nmol). Numeric values on x-axis represent time (minutes) after the first injection of the antagonist. Integrated secretory responses after Kp-10 administration, calculated as AUC, are also depicted as *bar graphs*. *, $P \leq 0.05$; **, $P \leq 0.01$ *vs.* corresponding control group (ANOVA followed by Student-Newman-Keuls multiple range test or Student's *t* test for bar graph data).

two of nine animals infused with the antagonist, the preovulatory surges of gonadotropins were not ablated. Although it is possible that there was a failure of the delivery system in these particular animals, it is also important to stress that the dose of daily infusion of the antagonist (10 nmol/24 h) was intentionally set at the low range, making it possible that interindividual variations in pharmacokinetics and/or sensitivity to the antagonist might have allowed these two rats to escape its blocking effects. In any event, the fact that the occurrence of the preovulatory surges of gonadotropins was totally abrogated in seven of nine females infused with p234 proves the principle that pending adjustments of doses and routes of administration, the hormonal trigger of ovulation can be prevented by appropriate use of Kp antagonists. This is in line with initial data from studies on central immune neutralization of endogenous Kp (26), thus paving the way for the development of tenable protocols of pharmacological blockade of ovulation based on inhibition of Kp signaling. Notably, despite the inhibition of hormonal surges, basal LH and FSH levels were not overtly decreased by administration of the antagonist in cyclic females, thereby supporting the possibility of designing therapeutic approaches selectively targeting the preovulatory surge but devoid of the potential side effects of persistent lowering of the basal gonadotropic input to the gonads. It is important that the lack of detectable decreases in basal gonadotropin levels in p234-treated animals cannot be attributed to limitations in the sensitivity of our assays because these are capable of detecting the marked suppression of circulating LH and FSH levels after treatment with a GnRH antagonist (20, 27, 28). Our demonstration of inhibition of the LH and FSH surges without affecting basal gonadotropin levels suggests the potential application of Kp antagonists in inhibiting ovulation with the maintenance of ovarian steroids in female contraception.

In addition to protocols of central infusion, we also evaluated the ability of a variant of p234, tagged with a terminal penetratin extension to potentially facilitate penetration of the blood-brain barrier, to suppress the gonadotropin-releasing effects of Kp-10. From a pharmacological perspective, this is a relevant issue because preliminary testing of the native p234 suggested that this compound may not be efficacious after systemic delivery (Roseweir, A., and R. P. Millar, unpublished data). Our present results conclusively demonstrate that peripheral injection of p234-penetratin is able to suppress the LH and FSH secretory responses to Kp. Pending elucidation of important pharmacokinetic aspects of this or related analogs, such as circulating half-life and permeability through the blood-brain barrier, these observations point to the potential of systemic administration of Kp antagonists for the pharmacological manipulation of the gonadotropic axis. It is important that ip-injected p234-penetratin was capable of inhibiting the effects of both centrally and peripherally injected Kp-10, suggesting that systemically administered antagonists access both potential sites of action of Kp stimulation of GnRH neurons—that is, at perikarya, mainly located at the preoptic area, or nerve terminals, mostly at the median eminence (29). However, the fact that the effects of peripherally injected Kp-10 were totally abrogated whereas the effects of centrally injected Kp-10 were only partially blunted by the systemic antagonist might be indicative of differential preferential sites of action of Kp in the control of gonadotropin secretion. In addition, it is also possible that these

distinct responses could be the result of differences in the effective dose and actual access of the systemically delivered antagonist to such different sites. In any event, the availability of Kp antagonists with different permeability at the blood-brain barrier, and the comparison of their effects after central or peripheral delivery, may be instrumental in a better dissection of the preferential sites of action of Kp on GnRH neurons.

In conclusion, we have presented a series of studies on the effects of continuous central infusion, and peripheral administration, of Kp antagonists on gonadotropin secretion and the occurrence of key reproductive events, such as puberty onset and the preovulatory surge of gonadotropins, in the rat. Our findings underline the potential application of peptide analogs of Kp as tools for the manipulation of the gonadotropic axis, also via peripheral routes of administration. In addition, our current validation of protocols of continuous antagonism of GPR54 will facilitate the delineation of the plethora of neuroendocrine and peripheral actions of Kp.

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