Differential modulation of gonadotropin responses to kisspeptin by aminoacidergic, peptidergic, and nitric oxide neurotransmission

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Kisspeptins (Kp), the peptide products of the Kiss1 gene, have emerged as essential elements in the control of GnRH neurons and gonadotropin secretion. However, despite considerable progress in the field, to date limited attention has been paid to elucidate the potential interactions of Kp with other neurotransmitters known to centrally regulate the gonadotropic axis. We characterize herein the impact of manipulations of key aminoacidergic (glutamate and GABA), peptidergic (NKB, Dyn, and MCH), and gaseous [nitric oxide (NO)] neurotransmission on gonadotropin responses to Kp-10 in male rats. Blockade of Kp signaling in the regulation of GnRH release (20, 21). The latter, together with the presence of projections/appositions of Kiss1 neurons onto GnRH neurons (4, 41), strongly suggests a direct mode of action of Kp for the regulation of GnRH secretion (44). This contention is reinforced by the preliminary observation that preserved Gpr54 signaling selectively in GnRH neurons seems to be sufficient to maintain puberty and fertility in the mouse (23).

Notwithstanding the above data, it is highly possible that, under physiological conditions, Kp signaling may cooperate with other neurotransmitters, neuropeptides, and peripheral hormones that are known to modulate GnRH function. In fact, the recognition of the fundamental roles of Kp in the control of GnRH secretion has forced the reassessment of the effects and mechanisms of action of other GnRH regulatory factors (44). These analyses aim to define whether the effects of such regulators are dependent or interconnected with those of Kp. Admittedly, our understanding of such interactions is still very limited.

Among the potential interactive partners of Kp in the central control of the gonadotropic axis, their interplay with aminoacidergic neurotransmission has been investigated (7, 10, 35, 38, 39). Besides their well-known functions as major regulators of GnRH secretion, excitatory and inhibitory amino acids, such as glutamate and GABA, have been proposed to interact with Kp signaling in the regulation of GnRH release (20, 21). The excitatory actions of glutamate on GnRH neurons may be mediated via metabotropic and ionotropic (NMDA and non-NMDA) receptors, which are expressed directly on GnRH neurons or in intermediate afferents (21). In addition, GABA, acting via GABAA and/or GABAB receptors, has been shown to induce either inhibitory or excitatory responses in GnRH neurons, depending on the developmental stage and the physiological status of the gonadotropic axis, with important discrepancies being found between in vivo and in vitro studies (20). In this context, an as-yet limited number of pharmacological and electrophysiological studies have suggested the...
existence of interactions between Kp and aminoacidergic neurotransmission in the central control of GnRH secretion (7, 10, 12, 38, 39). Yet the hierarchy and physiological relevance of such interactions in the regulation of gonadotropin secretion remains largely unknown.

In the same vein, the interplay of Kp with other neuropeptide transmitters in the control of GnRH neurons has been proposed on the basis of genetic, neuroanatomic, and pharmacological data, but the characteristics of such interactions need further elucidation (33, 34). As an illustrative example, neurokinin B (NKB), which is coexpressed in Kiss1 neurons in the arcuate (ARC) nucleus (25), has been shown to stimulate LH secretion in a Gpr54-dependent manner in rodents and primates (12, 26, 30, 32, 42, 43), an observation that is in keeping with the reported state of hypongondotropic hypogonadism in humans with inactivating mutations in the genes encoding NKB (TAC3), its receptor NK3R (TACR3), or GPR54 (33, 34). Collectively, these and related findings have led to the hypothesis that NKB (auto)regulates Kp output from ARC Kiss1 neurons onto GnRH neurons (33, 34, 53). However, stimulatory, inhibitory, or null effects of the agonist of NKB, senktide, on LH secretion have been reported, depending on the sex steroid milieu and the functional state of the gonadotropic axis (5, 22, 30, 32, 42, 43, 48, 51), a phenomenon whose basis remains unknown. Moreover, the ability of NKB to modulate GnRH/gonadotropin responses to Kp remains unexplored. Similarly, although Kiss1/NKB neurons in the ARC also express dynorphin (Dyn; hence, these are globally termed KNDy neurons) and Dyn inhibits GnRH secretion (31), the capacity of Dyn to regulate GnRH/gonadotropin responses to Kp has so far not been explored. Similarly, the interplay between Kp and other central regulators of GnRH, such as the metabolic neuropeptide melanin-concentrating hormone (MCH), has been proposed on the basis of electrophysiological data (52), and yet its relevance in terms of gonadotropic control in vivo has not been experimentally evaluated to date. Finally, nitric oxide (NO) has been suggested very recently to convey part of the gonadotrophic actions of Kp in male rodents (19), but its role as mediator of Kp effects in male rodents remains unexplored.

In this context, we provide herein a series of pharmacological analyses of the impact of the manipulation of key aminoacidergic, peptideergic, and gaseous neurotransmission on gonadotropin responses to central Kp-10 administration. Whereas our approach does not allow for discrimination of specific sites of action (e.g., pre- vs. postsynaptic), by the use of specific agonists and/or antagonists of glutamate, GABA, NKB, Dyn, and MCH signaling, as well as an inhibitor of NO synthesis, we intend to shed further light into the pathways whereby GnRH responsiveness to Kp is modulated in vivo and the potential interplay between kisspeptinergic, aminoacidergic, neuropetidergic, and NO transmission in the central control of the gonadotropic axis.

MATERIALS AND METHODS

Animals and Drugs

Young adult (postnatal days 45–50) Wistar male rats bred in the vivarium of the University of Cordoba were used. Animals were maintained under constant conditions of light (14:10-h light-dark) and temperature (22°C). Rats were weaned at 21 days postpartum and, until the conduction of the hormonal tests, were housed in groups of four rats per cage with free access to standard rat chow and tap water. Experimental procedures were approved by the Cordoba University Ethics Committee for animal experimentation and conducted in accordance with the European Union normative for care and use of experimental animals. All the tests were carried out in the morning between 0900 and 1000.

Since the hormonal assays involved intracerebroventricular (icv) cannulation, the animals were caged individually from the day before cannula implantation until termination of experiments. As a general procedure to target central neuroendocrine pathways, a protocol of icv injection of the different compounds tested was implemented, in keeping with previous studies (12, 27, 30). To allow delivery of drugs into the lateral cerebral ventricle, the cannulae were lowered to a depth of 3 mm beneath the surface of the skull, with an insert point at 1 mm posterior and 1.2 mm lateral to bregma. Correct positioning of the cannulae was confirmed by postmortem inspection.

Kisspeptin-10 (Kp-10; the minimal active fragment of kisspeptins) and MCH were obtained from Phoenix Pharmaceuticals (Belmont, CA). N-methyl-d-aspartate (NMDA; agonist of NMDA receptors), MK-801 (agonist of NMDA receptors); NBQX [preferential antagonist of the 2-amino-3-hydroxy-5-methyl-4-isoxazol propionic acid (AMPA) receptors], muscimol (GABA_A receptor agonist), bicuculline (GABA_A receptor antagonist), baclofen (GABA_B receptor agonist), phaclofen (GABA_B receptor antagonist), N^4-nitro-L-arginine-methyl ester (L-NAME; inhibitor of NO synthase), and senktide [sucucinyl-Asp-6-N-Me-Phe-8] substance P; agonist of NK3R) were purchased from Sigma (St. Louis, MO). (S)-3,5-dihydroxyphenylglycol (DHPG; agonist of metabotropic type-I glutamate receptors), (±)-U-50488 hydrochloride [U-50; agonist of κ-opioid receptors (κ-OR)], and norbinaltorphimine dihydrochloride (nor-BNI; antagonist of κ-OR) were supplied by Tocris Bioscience (Bristol, UK). For further details, see Table 1.

All drugs were reconstituted with saline, except for bicuculline and NBQX, which were reconstituted in 5% DMSO, and U-50, which was dissolved in 0.1% DMSO. The compounds were diluted to their working concentrations immediately before the experiment and injected icv in a final volume of 10 μl/rat. As a general procedure, blood samples (250 μl) were obtained by jugular venipuncture before and at different time points after administrations of the various drugs. Animals injected with vehicle (0.9% NaCl or 0.1–5% DMSO) served as controls. The dose regimen for the different agonists and antagonists was selected on the basis of previous references; details on dosages can be found in Table 1.

Experimental Design

In the first set of experiments, we explored the impact of the manipulation of glutamate neurotransmission on the pattern of Kp-stimulated gonadotropin response. In experiment 1, the effects of activation of ionotropic (iGlu) or metabotropic glutamate (mGlu) receptors on gonadotropin responses to Kp-10 were evaluated. Male rats (n = 10–12/group) were preinjected icv with the agonist of NMDA receptor NMDA (10 nmol/rat), the group 1 mGlu receptor agonist DHPG (500 nmol/rat), or the corresponding control. The animals were further injected icv with Kp-10 (50 nmol/rat) or saline. Blood samples were obtained at basal conditions (before iGlu agonist injection; basal) and 15 min following agonist administration (time 0 min). At this time point, Kp-10 was icv injected, and trunk blood samples were taken 15 and 60 min later for specific hormonal determinations.

Conversely, in experiment 2, we assessed the impact of central or peripheral administration of selected blockers of iGlu receptors on the profiles of gonadotropin responses to Kp-10. To this end, male rats (n = 10–12/group) were pretreated icv (central) or intraperitoneally (ip; systemic) with effective doses of the NMDA receptor antagonist MK-801 (6 nmol/rat icv or 1 mg/kg ip) or the non-NMDA AMPA receptor antagonist NBQX (3 nmol/rat icv or 0.5 mg/kg ip); animals...
injected with the corresponding vehicle served as controls. The animals were further injected ivc with an effective but submaximal dose of Kp-10 (50 pmol/rat) or saline. Blood samples (250 μL) were obtained at basal conditions (before iGlu antagonist administration; basal) and 30 (central) or 45 min (systemic) following antagonist injection (time 0 min). At this time point, Kp-10 was ivc injected, and trunk blood samples were taken 15 and 60 min later for specific hormonal determinations.

In the second set of experiments, we explored the effects of the pharmacological manipulation (either activation or blockade) of the GABAergic system in vivo on gonadotropin secretory responses to Kp-10. Of note, our preliminary tests involving systemic administration of the agonists and antagonists did not identify changes in hormone levels; hence, we focused on ivc experiments. Thus, in experiment 3, male rats (n = 10–12/group) were preinjected with effective doses of the GABA<sub>A</sub> receptor agonist muscimol (9 nmol/rat) or the GABA<sub>B</sub> receptor agonist baclofen (5 nmol/rat); animals injected with corresponding vehicle served as controls. The rats were further injected ivc with Kp-10 (50 pmol/rat) or saline. Blood samples were obtained at basal conditions (before GABA agonist injection; basal) and 15 min following agonist administration (time 0 min). At this time point, Kp-10 was ivc injected, and trunk blood samples were taken 15 and 60 min later for specific hormonal determinations.

Conversely, in experiment 4, the impact of selective blockade of the GABAergic system on Kp-stimulated gonadotropin secretion was explored. Male rats (n = 10–12/group) were preinjected ivc with the GABA<sub>A</sub> receptor antagonist bicuculline (5 nmol/rat), the GABA<sub>B</sub> receptor antagonist phaclofen (40 nmol/rat), or a combination of both. The animals were further injected ivc with Kp-10 (50 pmol/rat) or saline. Time course analyses of gonadotropin responses were conducted by blood sampling at the time points indicated in experiment 3.

In the third set of experiments, gonadotropin responses to central administration of Kp were explored following pharmacological manipulation of Dyn or NKB signaling. To this end, in experiment 5, male rats (n = 10–12/group) were preinjected ivc with the kOR agonist U-50 (1 nmol/rat), the kOR antagonist nor-BNI (2 nmol/rat), or corresponding vehicle. The animals were further treated ivc with Kp-10 (50 pmol/rat) or saline, and blood sampling was undertaken thereafter. This involved sampling at basal conditions (before Dyn compound injection; basal) and 15 min (U-50) or 90 min (nor-BNI) following agonist or antagonist administration, respectively (time 0 min). At this time point, Kp-10 was ivc injected, and trunk blood samples were taken 15 and 60 min later for specific hormonal determinations.

In turn, in experiment 6, the effects of NKB signaling on Kp-10-induced gonadotropin secretion were explored. Thus, groups of male rats (n = 10–12) were ivc injected with the agonist of NK3R, senktide (600 pmol/rat), and were further treated ivc with Kp-10 (50 pmol/rat). Blood samples were obtained at basal conditions (before senktide injection; basal) and 30 min following agonist administration (time 0 min). At this time point, Kp-10 was ivc injected, and trunk blood samples were taken 15 and 60 min later for specific hormonal determinations.

In addition, in experiment 7, the impact of selective activation of the MCH receptor on stimulated Kp-10 gonadotropin secretion was evaluated. To this end, male rats (n = 10–12/group) were preinjected ivc with MCH (40 nmol/rat) and further treated ivc with Kp-10 (50 pmol/rat). Blood samples were obtained before MCH injection (basal) and 15 min after agonist administration. At this time point, Kp-10 was ivc injected, and trunk blood samples were taken 15 and 60 min later for specific hormonal determinations.

Finally, in experiment 8, the impact of selective inhibition of NO synthesis on the ability of Kp-10 to elicit gonadotropin secretion was evaluated. For this purpose, male rats (n = 10–12/group) were preinjected ivc or ip with an effective dose of NO synthase, l-NAME (ivc: 95 nmol/rat; ip: 40 mg/kg rat), and were further treated ivc with Kp-10 (50 pmol/rat). Blood samples were obtained before l-NAME injection (basal) and 30 or 45 min after ivc or ip agonist administration (respectively). At this time point, Kp-10 was ivc injected, and trunk blood samples were taken 15 and 60 min later for specific hormonal determinations.

**Hormone Measurements**

Serum LH and FSH levels were measured using radioimmunoassay kits supplied by the National Institutes of Health (Dr. A. F. Parlow, National Hormone and Peptide Program, Torrance, CA). Rat LH-I-10 and FSH-I-9 were labeled with 125I using Iodo-gen tubes, following the instructions of the manufacturer (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 <8 and 10% for LH and 6 and 9% for FSH, respectively. The sensitivity of the assay was 5 pg/tube for LH and 20 pg/tube for FSH. Accuracy of hormone determinations was confirmed by assessment of rat serum samples of known concentrations (external controls).
Hormonal determinations were conducted in duplicate, with a minimal total number of 10 samples/group. When appropriate, integrated gonadotropin secretory responses were also calculated as the area under the curve (AUC), following the trapezoidal rule. Data are presented as means ± SE. Results were analyzed for statistically significant differences using unpaired Student’s t-test or ANOVA, followed by Student-Newman-Keuls multiple range tests (SigmaStat 3.5; Jandel, San Rafael, CA). P ≤ 0.05 was considered significant.

RESULTS
Kisspeptin-Aminoacidergic Interactions in the Control of Gonadotropin Secretion

Kisspeptin-glutamate interactions in the control of gonadotropin secretion. To explore the influence of glutamatergic neurotransmission on gonadotropin responsiveness to central injection of Kp-10, a double, partially complementary approach was implemented. In one hand, we evaluated the impact of preactivation of iGlu and mGlu pathways on the magnitude of LH and FSH responses to a submaximal dose of Kp-10. As shown in Fig. 1, central administration of effective doses of the agonists of iGlu and (group I) mGlu receptors NMDA and DHPG, respectively, evoked unambiguous LH (and to a lesser extent, FSH) responses in young adult male rats. Subsequent icv injection of a Kp-10 did not enhance further gonadotropin levels over those elicited by Kp-10 alone. In the case of mGlu neurotransmission, preactivation of group I metabotropic receptors significantly attenuated LH and FSH responses at 15 min after central injection of Kp-10 (Fig. 1).

As a complementary approach, we conducted in vivo administration of selective blockers of specific glutamate receptors, followed by central injection of a submaximal dose of Kp-10. These experiments involved both central (icv) and systemic (ip) injection of the antagonists. Central preinjection of the blocker of non-NMDA (AMPA) iGlu receptors, NBQX, resulted in significantly reduced LH responses to Kp-10, as revealed by acute hormonal determinations at 15 min and AUC values over the 60 min following Kp-10 injection. In contrast, central administration of the NMDA iGlu receptor blocker MK-801 failed to overly modify LH secretory profiles after icv Kp-10 administration (Fig. 2A). In addition, peripheral administration of either MK-801 or NBQX significantly attenuated LH responses to icv injection of Kp-10; the magnitude of suppression of LH responses tended to be greater after NBQX treatment (Fig. 2B). Regarding FSH responses, its basal levels markedly fluctuated within and between groups in this particular experiment, a phenomenon that obscured detection of any consistent changes in FSH responses to Kp-10 after central or peripheral blockade of NMDA and AMPA receptors (data not shown).

Kisspeptin-GABA interactions in the control of gonadotropin secretion. Using a similar approach, the influence of GABA receptor activation or inactivation on gonadotropin responses to Kp-10 was explored in vivo. Central administration of the agonists of GABAA and GABAB receptors muscimol and baclofen, respectively, caused a significant inhibition of basal LH levels (reduction >50% of basal values in vehicle-injected animals; see Fig. 3A, inset). However, the impact of in vivo activation of either GABAA or GABAB receptors on gonadotropin responses to Kp-10 was markedly different. Thus, whereas pretreatment with muscimol resulted in a significant decrease in the magnitude of LH and FSH secretory bursts in response to icv Kp-10 administration, administration of the GABAB receptor blocker baclofen was without effect on Kp-induced gonadotropin release (Fig. 3).

In good agreement with the agonist data, preblockade of GABAA or GABAB receptors by the administration of the antagonists bicuculline and phaclofen, respectively, had a differential impact on basal and Kp-stimulated LH levels. Thus, administration of bicuculline evoked an extraordinarily potent (~20-fold) increase in basal LH concentrations, whereas the antagonist of GABAB receptors, phaclofen, failed to do so and even induced a marginal albeit significant reduction in LH levels. Similarly, bicuculline administration enhanced basal FSH levels, whereas phaclofen did not (Fig. 4). In turn, peak LH and FSH responses to Kp-10 were not affected significantly by pretreatment with any of the antagonists. However, coadministration of GABAA and GABAB receptor blockers resulted in the magnification of the effects of bicuculline alone both on basal levels and the duration of LH and FSH responses to icv Kp-10 administration (Fig. 4).

Fig. 1. Effects of central administration of ionotropic (iGlu) or metabotropic glutamate (mGlu) receptor agonists on gonadotropin responses to kisspeptin-10 (Kp-10). Profiles of LH (A) and FSH levels (B) in young adult male rats preinjected intracerebroventricularly (icv) with effective doses of the agonist of iGlu receptor N-methyl-D-aspartate (NMDA; 10 nmol) or mGlu receptor dihydropyridinylglycol (DHPG; 500 nmol) and further treated icv with Kp-10 (50 pmol; gray arrow). Hormonal determinations were conducted before icv agonist administration (B), 15 min after agonist injections (0 min), and 15 and 60 min after icv Kp-10 injection. *P ≤ 0.05; **P ≤ 0.01 vs. control (vehicle) group (ANOVA followed by Student-Newman-Keuls multiple-range test).
Kisspeptin-Neuropeptidergic Interactions in the Control of Gonadotropin Secretion

Kisspeptin-Dyn interactions in the control of gonadotropin secretion. To evaluate the influence of Dyn signaling upon gonadotropin responsiveness to Kp-10, a similar agonist/antagonist approach was used. Activation of Dyn receptors (κ-OR) by the selective agonist U-50 did not overtly change basal LH and FSH levels, whereas it modestly but significantly reduced the integral (AUC over 60 min) responses of both gonadotropins to Kp-10 in male rats. Conversely, blockade of κ-OR by the antagonist nor-BNI increased basal LH and FSH levels significantly and enhanced acute (15 min) and AUC LH responses to Kp-10 (Fig. 5).

Kisspeptin-NKB interactions in the control of gonadotropin secretion. Analysis of the impact of NKB signaling on gonadotropin responses to Kp-10 was also explored. Central administration of senktide, the agonist of NK3R, did not change basal levels of LH or FSH in young adult male rats. In clear contrast, pretreatment with senktide did induce a significant decrease in the acute (15 min) and integral (AUC over 60 min) responses of both LH and FSH to a submaximal dose of Kp-10 (Fig. 6).

Kisspeptin-MCH interactions in the control of gonadotropin secretion. The interplay between MCH and Kp-10 in the control of gonadotropin secretion was assessed in vivo. As shown in Fig. 7, icv administration of an effective dose of MCH induced a significant reduction of the basal circulating levels of LH without affecting FSH concentration. In addition, MCH preadministration tended to inhibit gonadotropin responses to Kp-10. This inhibitory trend was more evident for FSH, as estimated by comparison of integral hormonal responses over the 60-min period (AUC) after injection of Kp-10. Moreover, FSH levels at 15 min after central Kp-10
administration were significantly lower in MCH-pretreated animals (Fig. 7).

**Kisspeptin-NO interactions in the control of gonadotropin secretion.** Finally, the eventual interplay between NO transmission and gonadotropin responsiveness to Kp-10 was explored using a pharmacological approach involving central or systemic administration of effective doses of the inhibitor of NO synthase, l-NAME. Pretreatment with l-NAME, either via icv or ip routes, did not decrease basal gonadotropin levels significantly in young male rats. Likewise, peak LH secretion after icv injection of Kp-10 was not affected by blockade of NO synthase. In contrast, Kp-induced LH secretory responses were protracted by blockade of NO synthesis, as evidenced by the fact that LH levels at 60 min after icv injection of Kp-10 were significantly higher in the l-NAME-treated groups, regardless of the central or systemic administration of the inhibitor (Fig. 8). Conversely, FSH responses to Kp-10 were not significantly modified after blockade of NO synthase, following either central or peripheral injection of l-NAME, at any time point studied. Yet, FSH levels in response to Kp-10 tended to be higher after central administration of the NO synthase blocker (data not shown).

**DISCUSSION**

Our knowledge of the signals governing GnRH secretion has enlarged recently with the identification of Kp, whose essential role in the central control of the gonadotropic axis is now universally recognized (34, 36, 44). Although experimental data strongly suggest a (predominant) direct mode of action of Kp in the regulation of GnRH neurons, evidence has also been presented for an interplay between Kp and other key modulators in the fine control of GnRH secretion. These interactions might take place either pre- or postsynaptically of GnRH.
neurons, the former through the ability of Kp to eventually regulate the input of other transmitters on GnRH neurons or vice versa and the latter via specific interactions at the level of GnRH neurons, which may affect their responsiveness to different regulators. However, the supporting experimental data remain scarce and come mostly from electrophysiological studies (10, 38, 39, 52, 55). Although the latter are an elegant approach to dissect out the actions of specific afferents directly on GnRH neurons, they may fall short in providing an integral view of how different key transmitters, including Kp, cooperate to finely modulate GnRH and hence, gonadotropin secretion. The present study complements and extends our recent work addressing the gonadotropin-releasing effects of various aminoacidergic and neuropeptidergic ligands in a Gpr54-null

Fig. 6. Effects of central administration of the NK3R agonist senktide on gonadotropin responses to Kp-10. Shown are gonadotropin profiles from young adult male rats preinjected icv with an effective dose (600 pmol/rat) of the agonist of the canonical receptor of neurokinin B (NKB) senktide and further treated icv with Kp-10 (50 pmol; gray arrow). Hormonal determinations were conducted before initiation of the treatments (B), 30 min after administration of senktide (0 min), and 15 and 60 min after icv Kp-10 injection. In addition to time course data, integral hormonal responses during the 60-min period following Kp-10 injection are depicted as AUC. *P ≤ 0.05; **P ≤ 0.01 vs. control (vehicle) group (ANOVA followed by Student-Newman-Keuls multiple-range test).

Fig. 7. Effects of central administration of melanin-concentrating hormone (MCH) on gonadotropin responses to Kp-10. Shown are gonadotropin profiles from young adult male rats preinjected icv with an effective dose of MCH and further treated icv with Kp-10 (50 pmol; gray arrow). Hormonal determinations were conducted before initiation of the treatments (B), 15 min after administration of MCH (0 min), and 15 and 60 min after icv Kp-10 injection. In addition to time course data, integral hormonal responses during the 60-min period following Kp-10 injection are depicted as AUC. *P ≤ 0.05.
Kisspeptin-Neurotransmitter Interactions in the Control of Gonadotropin Secretion

Glutamate neurotransmission is known to play a prominent excitatory role in the control of GnRH secretion (1, 21). The actions of glutamate are mediated via different iGlu (NMDA, kainate, and AMPA) and, to a lesser extent, mGlu receptors (21). Among the iGlu receptors, NMDA is a key component in the glutamatergic regulation of GnRH neurons, although its actions appear to be, to a large extent, conducted indirectly (21). In contrast, non-NMDA receptors, such as those of AMPA, are expressed abundantly in GnRH neurons (21). Our pharmacological tests demonstrated that central activation of NMDA receptors in vivo induces clear LH and FSH secretory responses per se but fails to alter the patterns of gonadotropin responses to Kp, in terms of peak secretion and duration, in young male rats. In turn, blockade of NMDA and especially AMPA receptors by specific antagonists decreased LH responses to Kp-10. Such inhibition was observed consistently after both central and systemic administration of the AMPA antagonist, whereas it was evident for the NMDA antagonist only after its peripheral (ip) administration. The later may suggest region-specific interactions between NMDA and Kp pathways in the control of GnRH neurons, as suggested recently by work in Gpr54-null mice (7), which might be more efficiently targeted after systemic delivery of NMDA blockers. The fact that inhibition of iGlu neurotransmission induced a decrease in the responses to Kp-10 is in keeping with recent electrophysiological studies showing that blockade of fast synaptic transmission, which eliminates glutamatergic inputs, reduced GnRH neuronal responses to Kp in vitro (38). Taken as a whole, these observations are compatible with the hypothesis that, at least partially, Kp effects on GnRH neurons are indirectly mediated via activation of glutamate afferents to GnRH neurons. In good agreement, it has been shown recently that Kp-10 is able to enhance glutamatergic transmission to these neurons, acting in a presynaptic manner (39).

Admittedly, initial pharmacological studies from our group documented roughly preserved LH and FSH responses to high doses of Kp-10 after effective blockade of NMDA and non-NMDA receptors in vivo (28, 29). However, those initial pharmacological tests involved the use of supramaximal doses of Kp-10: 1 nmol/icv, i.e., 20-fold higher than those in the present study. Such doses might have obscured more subtle regulatory phenomena, because supramaximal Kp stimulation directly at the GnRH neurons might compensate for the lack of indirect Kp stimulation via ionotrophic afferents. Yet it is also plausible that the reduction in GnRH/gonadotropin responsiveness to Kp-10 after NMDA and AMPA blockade may derive from the perturbation of the intrinsic secretory capacity of GnRH neurons after inhibition of such key regulatory components, thus supporting a postsynaptic interplay between Kp and ionotropic glutamate signaling within GnRH neurons. Finally, recent evidence suggests that NMDA can induce bursting activity in Kiss1 neurons, as revealed in Kiss1-CreGFP knockin mice (16). This finding is compatible with our observation that coadministration of NMDA and Kp-10 failed to enhance LH responses over those detected after icv injection of Kp-10 alone, which suggests some degree of convergence of NMDA and Kp signaling onto GnRH neurons.

In contrast to iGlu receptors, the physiological relevance of metabotropic signaling in the control of the gonadotropin axis remains ill defined (1). A previous study in the mouse identified a subset of GnRH neurons in the septal area that was insensitive to the excitatory effects of Kp-10 but highly responsive to the type I mGlu agonist DHPG (10). However, that study did not provide evidence for the hypophysiotropic relevance of such a subpopulation because it was based solely on electrophysiological recordings of GnRH neurons (10). More recently, we have documented using a Gpr54-null model that DHPG can evoke unambiguous LH responses in the mouse that were fully preserved despite elimination of Kp signaling (12). We demonstrate here the ability of DHPG to also enhance LH secretion in the rat and show for the first time that coactivation of mGlu and Kp signaling partially blunts LH responsiveness to Kp. Although the mechanism for such an inhibitory cross-talk is yet to be defined, the comparison of hormonal responses to Kp-10 after preadministration of DHPG or NMDA demonstrates important divergences between ionotopic and metabotropic pathways in the control Kp/GnRH function in rats, in keeping with our recent observations of differential responses to NMDA or DHPG in Gpr54-null mice (12).
As is the case for glutamate, solid evidence accumulated over the last decades has demonstrated an important role of GABA neurotransmission in the control of GnRH/gonadotropin secretion (20). The effects of GABA are mediated via GABA_A or GABA_B receptors that may act pre- and/or postsynaptically to modulate GnRH neuronal secretory activity (20). However, intriguing discrepancies have become apparent between in vivo and in vitro studies addressing the effects of GABAergic pathways in terms of GnRH neuronal activity and gonadotropin secretion. In fact, although administration of GABA agonists in vivo has been shown consistently to suppress both tonic and surge modes of LH secretion in a variety of species, electrophysiological recordings have documented both hyperpolarizing and depolarizing responses in GnRH neurons (20). In the context of the depolarizing effects of GABA, Kp has been shown to enhance the magnitude of GABA postsynaptic currents in GnRH neurons, whereas blockade of fast synaptic transmission, which eliminates not only glutamatergic but also GABAergic inputs, reduced GnRH neuronal responses to Kp in vitro (38). In clear contrast, our in vivo studies demonstrate that central activation of GABA_A receptors decreased basal LH levels and blunted the LH and FSH responses to Kp-10 significantly, whereas blockade of this receptor subtype enhanced serum LH and FSH levels dramatically. These observations are compatible with a preferential postsynaptic mode of action of GABA_A signaling on GnRH neurons (20) and confirms its predominant inhibitory effects on GnRH/gonadotropin secretion in vivo, in keeping with very recent findings in prepubertal female monkeys (24). Of note, our data are the first to disclose the ability of GABA_A receptors to partially suppress responsiveness to Kp. In addition, although our results do not refute recent observations on the capacity of GABA to induce depolarizing responses in GnRH neurons (20), they bring a call of caution when extrapolating electrical recordings into neurohormonal responses. Admittedly, however, in vivo studies such as those implemented here lack site specificity and hence, may obscure complex regulatory phenomena such as those that are likely to underlie GABA regulation of GnRH neurons.

Our results also suggest a less prominent role of GABA_B signaling in the inhibitory control of the gonadotropic axis, since blockade of this receptor subtype failed to enhance basal gonadotropin concentrations, whereas its activation, despite reduction of basal LH levels, did not decrease responses to Kp-10; the latter is compatible with a prominent presynaptic mode of action of GABA_B on GnRH neurons (20). This is in agreement with recent electrophysiological data showing that Kp, acting postsynaptically, can overcome the effects of GABA_B signaling on GnRH neurons (55). Yet simultaneous inactivation of GABA_A and GABA_B receptors enhanced gonadotropin responses to GABA_A receptor blockade alone and protracted LH and FSH responses to Kp-10, thus suggesting some contribution of GABA_B to restrain gonadotropin secretion in rats.

Kisspeptin-Neuropeptidergic Interactions in the Control of Gonadotropin Secretion

Recent neuroanatomic, genetic, and pharmacological data strongly suggest the tight interactions between Kp, NKB, and Dyn in the fine control of GnRH neurosecretory activity (25, 34). Regarding Dyn actions, compelling evidence has documented predominant inhibitory effects on the gonadotropic axis, which appear to be at least partially conducted upstream of GnRH neurons due to the capacity of Dyn to decrease Kp output onto GnRH neurons (31). Our present data are fully compatible with such an inhibitory effect of Dyn, as evidenced by the fact that blockade of the canonical Dyn receptor κ-OR enhanced basal LH and FSH levels and increased LH responses to Kp-10 significantly. In turn, integral (AUC) responses to Kp-10 in terms of LH and FSH secretion were modestly but significantly decreased after selective activation of κ-OR. However, our data suggest that, at least partially, the inhibitory effects of Dyn might be conducted downstream of Kp neurons eventually in GnRH neurons themselves. Nonetheless, it remains possible that the expected decrease in Kp output after Dyn activation might affect (inhibit) the capacity of GnRH neurons to respond to subsequent Kp pulses.

Regarding NKB signaling, recent evidence has demonstrated the ability of senktide, an agonist of NKB signaling, to stimulate LH secretion in a variety of species (e.g., rat, mouse, goat, sheep, and monkey) and physiological states (e.g., mainly in female animals) (2, 12, 30, 32, 42, 51). Surprisingly, in our experiments, senktide did not modify basal LH or FSH levels in young adult male rats, an observation that is in contrast to the potent stimulatory effects of this agonist on gonadotropin secretion in adult male mice (12, 32). However, the possibility of an experimental artifact can be discarded since parallel studies, using strictly similar peptide preparations and experimental conditions, revealed potent stimulatory effects of senktide on LH secretion in female rats at various developmental stages (30), as well as in male rats, during the infantile and prepubertal period (48). Moreover, despite the inability to modify basal gonadotropin levels, pretreatment of NKB signaling significantly attenuated LH and FSH responses to Kp-10. To our knowledge, this is the first evidence for an inhibitory interaction between NKB and Kp signaling. Such interplay may help to explain the predominant inhibitory action of NKB/senktide on LH secretion in conditions of stimulated gonadotropin levels, such as gonadectomy (22, 30), where endogenous Kp tone is expectedly heightened (44). In addition, our data document that, besides its ability to (presumably) increase Kp output by KNDy neurons, NKB may also conduct inhibitory actions on GnRH responsiveness to Kp under certain physiological conditions that are likely to take place downstream of KNDy neurons. Such diversity of regulatory effects and sites of action of NKB in the control of GnRH neurons may help to explain the spectrum of gonadotropin responses, from stimulatory to null or even inhibitory, that has been reported in recent literature (33, 34).

Different metabolic neuropeptides, such as neuropeptide Y, melanocortins, galanin-like peptide, and ghrelin, have been proposed to interplay with Kp pathways for ensuring a proper coupling between energy homeostasis and reproductive function (3). Among them, electrophysiological evidence has been presented for the ability of the orexigenic neuropeptide MCH to inhibit a subset of septal Kp-sensitive GnRH neurons in which MCH was capable of blocking the potent excitatory effect of Kp (52). Based on the metabolic profile of MCH, this inhibitory pathway has been proposed as critical for coupling energy balance and reproduction (52), and yet its physiological
a. Male rats

Aminoacidergic: glutamate

Ionotropic

Activation of ionotropic NMDA receptors stimulates basal LH secretion, although it does not enhance peak LH responses to Kp-10. Blockade of NMDA or AMPA receptors blunts Kp-induced LH secretion; the effects are detectable after peripheral administration of NMDA or AMPA receptor inhibitors and after central injection of AMPA but not NMDA antagonists. This suggests region-specific interactions between NMDA and Kp pathways in the control of GnRH neurons.

Metabotropic

Activation of metabotropic type I glutamate receptors stimulates basal LH secretion but partially inhibits peak LH responses to Kp-10. Comparison of the effects of ionotropic vs. metabotropic activation suggests important differences in the regulatory actions of these two glutamate pathways in the control of GnRH/gonadotropin responsiveness to Kp-10.

Aminoacidergic: GABA

GABA_A

Activation of GABA_A receptors inhibits basal LH levels and peak LH responses to Kp-10; the latter is compatible with a major postsynaptic action of GABA_A on GnRH neurons. Blockade of GABA_A enhances basal LH concentrations but not peak LH levels after Kp-10 administration.

GABA_B

Activation of GABA_B receptors inhibits LH levels but does not alter LH responses to Kp-10; this is compatible with a major presynaptic action of GABA_B on GnRH neurons. Blockade of GABA_B alone does not alter basal or peak LH levels after Kp-10, but coadministration of GABA_A and GABA_B antagonists enhances and protracts the effects of the GABA_A antagonist alone.

Peptidergic: KNDy

Dyn

Central activation of the Dyn receptor κ-OR does not change basal LH or FSH levels, but it reduces the integral responses of both gonadotropins to Kp-10, whereas κ-OR antagonism increases basal LH and FSH levels and enhances peak and integral LH responses to Kp-10. These findings are compatible with an action of Dyn as inhibitor of GnRH responsiveness to Kp.

NKB

Activation of NKB signaling by the agonist senktide does not stimulate LH or FSH secretion in young adult male rats but moderately inhibits LH responses to Kp-10. These data are in contrast with previous results showing stimulatory effects of senktide on LH secretion in female rats and other species (mouse, sheep, monkey) as well as in younger (prepubertal) male rats, thus providing evidence for the complexity of NKB actions on GnRH/gonadotropin secretion with stimulatory, null, or inhibitory responses, depending on the developmental stage, sex, species, and prevailing gonadotropin levels.

Peptidergic: MCH

Central administration of MCH decreases basal LH levels and moderately but significantly suppresses FSH responses to Kp-10. These data support an inhibitory action of MCH on the GnRH/gonadotropin axis, whose magnitude and physiological relevance appear to be modest.

Gaseous: nitric oxide

By the use of the NOS inhibitor L-NAME, we demonstrate here that NO restrains the duration of LH responses to Kp-10, thus suggesting a potential inhibitory component for the effect of NO in the control of gonadotropin secretion in vivo.

GnRH, gonadotropin-releasing hormone; Dyn, dynorphin; L-NAME, Nω-nitro-L-arginine-methyl ester. For further details, see RESULTS and DISCUSSION.

Kisspeptin-NO Interactions in the Control of Gonadotropin Secretion

A very recent study documented that Kp signaling in NO-producing neurons in the preoptic area likely plays a relevant role in the control of the gonadotropic axis and ovulation in female mice, as evidenced by the ability of Kp to enhance expression of neuronal NO synthase in this hypothalamic site and the blocking effects of inhibitors of NO synthase on the preovulatory surge of gonadotropins (19). These observations would suggest the existence of a stimulatory Kp/NO/GnRH pathway for the control of gonadotropin secretion in the female. Contrary to that model, our present studies in young male rats demonstrate that blockade of NO synthase, after either central or systemic administration of L-NAME, protracted rather than reduced the duration of LH responses to Kp-10. Although this may represent sex (male vs. female) or species (rat vs. mouse) differences in the dominant role of NO as mediator of the GnRH/gonadotropin-releasing effects of Kp, it is also worth noting that the previous study did show that NO release can restrain gonadotropin secretion at certain stages of the mouse ovarian cycle other than the preovulatory phase.
The most salient results of our study is provided in Table 2. Our approach, combining pretreatments with various agonists and antagonists and serial blood sampling before and at different time points after central injection of Kp-10, complements previous electrophysiological and in vivo studies and provides novel evidence for the ability of glutamate, GABA, NKB, Dyn, MCH, and NO signaling to variably modulate the gonadotropin releasing activity of Kp-10, using a physiologically relevant (whole body) experimental setting. These observations will help to elucidate the repertoire of interactions between Kp and other central transmitters, as well as their functional relevance, in the fine control of the gonadotropic axis.

DISCUSSIONS

In summary, we have presented herein a comprehensive series of pharmacological tests addressing the ability of different aminoacidergic, neuropeptidergic, and gaseous pathways, with proven roles in the central control of the gonadotropic axis, to modulate gonadotropin (as a surrogate marker of GnRH) responsiveness to Kp-10 in vivo. A synoptic view of the most salient results of our study is provided in Table 2. Our approach, combining pretreatments with various agonists and antagonists and serial blood sampling before and at different time points after central injection of Kp-10, complements previous electrophysiological and in vivo studies and provides novel evidence for the ability of glutamate, GABA, NKB, Dyn, MCH, and NO signaling to variably modulate the gonadotropin-releasing activity of Kp-10, using a physiologically relevant (whole body) experimental setting. These observations will help to elucidate the repertoire of interactions between Kp and other central transmitters, as well as their functional relevance, in the fine control of the gonadotropic axis.

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