Kisspeptin Signaling Is Indispensable for Neurokinin B, but not Glutamate, Stimulation of Gonadotropin Secretion in Mice

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Kisspeptins (Kp), products of the *Kiss1* gene that act via Gpr54 to potently stimulate GnRH secretion, operate as mediators of other regulatory signals of the gonadotropic axis. Mouse models of *Gpr54* and/or *Kiss1* inactivation have been used to address the contribution of Kp in the central control of gonadotropin secretion; yet, phenotypic and hormonal differences have been detected among the transgenic lines available. We report here a series of neuroendocrine analyses in male mice of a novel Gpr54 knockout (KO) model, generated by heterozygous crossing of a loxP-*Gpr54*/Protamine-Cre double mutant line. Gpr54-null males showed severe hypogonadotropic hypogonadism but retained robust responsiveness to GnRH. Gonadotropic responses to the agonist of ionotropic glutamate receptors, *N*-methyl-D-aspartate, were attenuated, but persisted, in Gpr54-null mice. In contrast, LH secretion after activation of metabotropic glutamate receptors was totally preserved in the absence of Gpr54 signaling. Detectable, albeit reduced, LH responses were also observed in Gpr54 KO mice after intracerebroventricular administration of galanin-like peptide or RF9, putative antagonist of neuropeptide FF receptors for the mammalian ortholog of gonadotropin-inhibiting hormone. In contrast, the stimulatory effect of senktide, agonist of neurokinin B (NKB; cotransmitter of Kiss1 neurons), was totally abrogated in Gpr54 KO males. Lack of Kp signaling also eliminated feedback LH responses to testosterone withdrawal. However, residual but sustained increases of FSH were detected in gonadectomized Gpr54 KO males, in which testosterone replacement failed to fully suppress circulating FSH levels. In sum, our study provides novel evidence for the relative importance of Kp-dependent *vs*. -independent actions of several key regulators of GnRH secretion, such as glutamate, galanin-like peptide, and testosterone. In addition, our data document for the first time the indispensable role of Kp signaling in mediating the stimulatory effects of NKB on LH secretion, thus supporting the hypothesis that NKB actions on GnRH neurons are indirectly mediated via its ability to regulate Kiss1 neuronal output. (***Endocrinology*** **153: 316–328, 2012)**

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Abbreviations: ARC, Arcuate nucleus; DHPG, (*S*)-3,5-dihydroxyphenylglycol; Dyn, dynorphin; GALP, galanin-like peptide; GNX, gonadectomy; HH, hypogonadotropic hypogonadism; icv, intracerebroventricular; Kp, kisspeptin; KO, knockout; NKB, neurokinin B; NMDA, *N*-methyl-D-aspartic acid; NPFF, neuropeptide FF; nor-BNI, nor-binaltorphimine dihydrochloride; ORX, orchidectomized; RFRP, RFamide-related peptide; T, testosterone.

Kisspeptins (Kp), a family of structurally related peptides encoded by the *Kiss1* gene, act via the G protein-coupled receptor, Gpr54 (also termed "Kiss1R"), to regulate puberty onset and fertility (1, 2). The reproductive dimension of Kp was unveiled by the identification of inactivating mutations of the *GPR54* gene in patients with impuberism and hypogonadotropic hypogonadism (HH) (3, 4), observations that have been recently complemented by the discovery of activating mutations of *KISS1* and *GPR54* genes in patients with precocious puberty (5, 6). Similarly, genetically engineered mice lacking either functional *Kiss1* or *Gpr54* have been shown to be a phenocopy of humans with perturbed Kp signaling (7). Accordingly, a large body of genetic, anatomic, physiological, and pharmacological data has set the contention that Kp are pivotal elements of the networks controlling GnRH secretion and reproductive function, as extensively documented elsewhere (1, 2, 8–10).

Recognition of the fundamental roles of Kp in the control of GnRH neurons has not only allowed identification of discernible neural pathways for key regulatory phenomena of the reproductive axis, but has also prompted the reassessment of the functional roles and mechanisms of action of other neurotransmitters, neuropeptides, and peripheral hormones, known to modulate GnRH function, to define whether their effects are dependent or interconnected with those of Kp. However, our understanding of the interactions of Kp with other regulatory signals of GnRH neurons remains incomplete. Nonetheless, the important role of Kiss1 neurons as mediators of feedback actions of sex steroids has been long recognized (2, 10, 11), and characterization of the putative interplay between glutamate (mainly via ionotropic receptors), γ-amino butyric acid, and Kp signaling in the control of GnRH secretion has been recently initiated (12–16). Similarly, possible interactions between Kp and RFRP, orthologs of the avian gonadotropin-inhibiting hormone, have been suggested in rodents and sheep (17–19). Whether Kp interplay with other members of the RF-amide superfamily, with proven roles in gonadotropin regulation, such as 26RFa (20), remains unexplored.

Recent data has documented that neurokinin B (NKB) is coexpressed in Kiss1 neurons in the arcuate nucleus (ARC) in different species (21), and activation of NKB signaling stimulates LH secretion in rat, sheep, and monkey (22–24). Based on indirect evidence, NKB has been proposed to enhance Kp output onto GnRH neurons (25, 26). The relevance of such NKB-Kp interplay is indirectly supported by clinical observations of HH in humans with inactivating mutations of the genes encoding NKB (*TAC3*) or its receptor (*TACR3*) (27). Kiss1/NKB neurons in the ARC also express dynorphin (Dyn), which conducts inhibitory actions on Kiss1 neurons (26). However, most of the experimental evidence supporting the above interactions comes from indirect approaches, whereas the direct assessment of these interactions *in vivo* remains scarce. In addition, the putative relationships between Kp and other central regulators of GnRH, such as metabolic neuropeptides with discernible effects on the gonadotropic axis, have received limited attention to date.

Mouse models of genetic inactivation of *Gpr54* or *Kiss1* have been (sporadically) used to dissect out the relative contribution of Kp signaling in the control of GnRH/gonadotropin secretion (28). Whereas these studies have unambiguously documented the importance of the Kiss1 system in the central control of reproductive function, they have also underscored notable phenotypic and hormonal differences among the transgenic lines available (28). For example, disparate results on the ability of estrogen to elicit positive feedback and surge-like LH responses have been reported in two different Gpr54 KO mouse lines (29, 30). Similarly, although the absence of LH rise after gonadectomy (GNX) in Gpr54- or Kiss1-null models would suggest an essential role of Kp signaling in mediating negative feedback (30, 31), detection of marginal, albeit significant, FSH responses to GNX in male and female Kiss1 and Gpr54 KO mice underscored a Kp-independent component for this phenomenon (31), the features of which need to be fully elucidated.

To shed further light on the potential interactions and relative importance of Kp signaling in the control of the reproductive axis, we report here a series of neuroendocrine analyses monitoring LH and FSH responses to different key regulators, including central transmitters and gonadal hormones, in adult male mice of a novel Gpr54 KO model, generated by heterozygous crossing of a loxP-*Gpr54*/Protamine-Cre double mutant line. Our study not only documents the validity of this line as (additional) model of profound HH, due to congenital absence of Gpr54, but discloses also interesting differences in the relative importance of Kp-dependent *vs.* -independent actions of several key regulators of GnRH secretion, such as glutamate, GALP, testosterone (T), and NKB.

Materials and Methods

Generation and validation of Gpr54-null mice

A conditional Gpr54 KO mouse was developed at Lexicon Pharmaceuticals (The Woodlands, TX), as described in detail in the legend of Supplemental Fig. 1 published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org. The *Gpr54* locus was modified by the insertion of flanking loxP sites. Total Gpr54 KO mice were obtained by crossing the conditional Gpr54 mutant mice with the Protamine-Cre deletor

strain to remove the Gpr54 coding sequence selectively in male germ cells (32). By crossing heterozygous ($Gpr54^{+/-}$) males and females (F_1 generation), homozygous ($Gpr54^{-/-}$) KO mice were obtained. All animal experiments pertaining to validation of the novel $Gpr54^{-/-}$ mouse line were approved by the MSD-Organon Animal Ethics Committee.

Drugs

Mouse *Kp-10* and *GALP* were obtained from Phoenix Pharmaceuticals Ltd (Belmont, CA). GnRH, *N*-methyl-D-aspartic acid (NMDA), and T were purchased from Sigma Chemical Co. (St. Louis, MO). RF9, putative antagonist of neuropeptide FF (NPFF)1R and NPFF2R, was provided by MSD (Oss, The Netherlands). (S)-3,5-Dihydroxyphenylglycol (DHPG) and norbinaltorphimine dihydrochloride (nor-BNI) were supplied by Tocris Bioscience (Bristol, UK). Senktide [(Succinyl-Asp-6;N-Me-Phe-8)-Substance P], agonist of the NKB receptor, NK3R, was purchased from Enzo-Life Sciences (Lörach, Germany) and Sigma. Unless otherwise stated, drugs were dissolved in saline (0.9% NaCl). Doses and timings were selected on the basis of previous studies (22, 33–35).

Blood analyses

A series of hematological, blood clotting, and biochemical parameters were determined in wild-type (wt) and Gpr54 KO mice, as described in detail in Supplemental Tables 1 and 2.

Staining for β-galactosidase activity

As complementary analysis, the patterns of Gpr54 expression were monitored using β-galactosidase activity as marker, because in our model expression of LacZ reporter gene is driven by the *Gpr54* promoter. Methodological details of histological analyses can be found in the legend of Supplemental Fig. 1.

Histopathology

Full phenotypic analyses were performed in 9-wk-old male and female mice (wt, heterozygous, and homozygous; n = 6/group). Additional studies were performed on mice at the age of 3-wk (before sexual maturation) and 7 months. A detailed description of the histopathological analyses and the tissues evaluated can be found in the legend of Supplemental Figs. 2 and 3.

Experimental design

General procedures

All the experimental protocols were approved by the Córdoba University Ethical Committee of animal experimentation and conducted in accordance with the European Union guidelines for use of experimental animals. Neuroendocrine characterization of Gpr54 KO mice involved assessment of basal gonadotropin levels and (lack of) responses to an effective dose of Kp-10. For the latter, $Gpr54^{-/-}$ (n = 8) and $Gpr54^{+/+}$ (n = 18) male mice received a single ip bolus of 7.5 nmol Kp-10; route of administration and dose were selected as effective on the basis of previous literature (35). Blood samples (200 μl) were obtained by jugular venipuncture before (basal) and 15 min after ip administration of Kp-10. In addition, protocols of intracerebroventricular (icv) administration of different neurotransmitters and neuropeptides were implemented, as described elsewhere (35, 36). Adult (3- to 4 month-old) male mice of both genotypes ($Gpr54^{-/-}$, $Gpr54^{+/+}$) were used. To allow delivery of drugs into the lateral cerebral ventricle, the cannulae were lowered to a depth of 2 mm beneath the surface of the skull, with an insert point at 1 mm posterior and 1.2 mm lateral to bregma. To exclude the possibility that defective gonadotropin responses to the various stimuli may stem from insufficient pituitary responsiveness to GnRH due to its low endogenous tone, which is characteristic of Gpr54-null animals (2), KO mice were subjected to a protocol of GnRH priming during 2 d before each neuroendocrine test, to heighten pituitary responsiveness. The priming protocol, which was adapted from previous literature in other species (23), consisted of five successive ip boluses of a low dose of GnRH (0.15 μg/each), with the following schedule: at 1000 h, 1700 h, and 2350 h on d 1; at 0800 and 1600 on d 2. To assess the effectiveness of the priming protocol, blood samples were taken 30 min after the last bolus of GnRH (at 1630 h on d 2). $Gpr54^{+/+}$ male mice, submitted to a similar protocol of five ip injections (but of physiological saline, because no GnRH priming was considered necessary), served as controls. Neuroendocrine tests were conducted between 0900 h and 1100 h of the following day. Blood samples were taken by jugular venipuncture either before (basal levels) or 15 min after injection of the testing compounds. The former involved a large number of determinations per genotype (at preinjection conditions; >20 for wt, >10 for KO animals), as a mean to define basal reference values in $Gpr54^{-/-}$ and $Gpr54^{+/+}$ mice.

Experiment 1

LH and FSH responses to central administration of agonists of glutamate receptors were explored in $Gpr54^{+/+}$ and $Gpr54^{-/-}$ male mice (n ≥ 7 per group). Effective doses of NMDA (1 nmol/5 μl; agonist of ionotropic receptors of the NMDA subtype) and DHPG (200 nmol/5 μl; agonist of type-I metabotropic receptors) were icv injected, and blood samples were obtained by jugular venipuncture 15 min later.

Experiment 2

Analysis of LH and FSH responses to the putative antagonist of NPFF1R and NPFF2R, RF9, was conducted in Gpr54 KO male mice, following a similar experimental protocol. Male $Gpr54^{+/+}$ and $Gpr54^{-/-}$ mice (n ≥ 7 per group) were icv injected with an effective dose of RF9 (20 nmol/5 μl; for reference see Ref. 33), and blood samples were obtained 15 min later.

Experiment 3

Given the potent stimulatory effects of NMDA and RF9 on LH secretion in wt animals, and to exclude the possibility that reduced LH responses in Gpr54 KO mice may derive from their severely decreased T levels (28), rather than from the primary defect of Gpr54 signaling, LH and FSH responses to NMDA and RF9 were monitored in orchidectomized (ORX) $Gpr54^{+/+}$ and $Gpr54^{-/-}$ male mice receiving effective T replacement. The mice were subjected to bilateral ORX via abdominal route and, 3 wk later, were implanted with SILASTIC brand (Dow Corning, Midland, MI) tubing elastomers (15 mm length; inner diameter, 1.98 mm; exterior diameter, 3.18 mm) containing T, so that androgen levels would be equal in both genotypes. Seven days after T supplementation, $Gpr54^{+/+}$ and $Gpr54^{-/-}$ mice (n = 4/group) were submitted to testing of gonadotropic responses

15-min after central administration of 20 nmol RF9 or 1 nmol NMDA, as described above.

Experiment 4

LH and FSH responses to senktide, the agonist of NKB, and nor-BNI, the antagonist of κ-opioid receptor, were assayed in Gpr54 KO mice. Groups of $Gpr54^{+/+}$ and $Gpr54^{-/-}$ male mice were icv injected with effective doses of senktide (600 pmol/5 μl) ($n \geq 7$/group) or nor-BNI (2 nmol/5 μl) (n = 4/group), and blood samples were drawn by jugular venipuncture 15 min after administration.

Experiment 5

Gonadotropic responses to the neuropeptide GALP were also explored. Groups of $Gpr54^{+/+}$ and $Gpr54^{-/-}$ male mice ($n \geq 9$) were icv injected with an effective dose of GALP (1 nmol/5 μl), and LH and FSH levels were assayed in jugular blood samples 15 min later.

Experiment 6

Finally, the time course of gonadotropic responses to ORX and T replacement and/or subsequent withdrawal were explored in Gpr54 KO mice. Groups of $Gpr54^{+/+}$ and $Gpr54^{-/-}$ adult male mice ($n \geq 9$/group) were submitted to bilateral ORX as described in Experiment 3, and blood samples were obtained by jugular venipuncture 2 d and 20 d after ORX. In parallel, groups of ORX mice of both genotypes ($n \geq 7$) received T implants (SILASTIC elastomers; 15 mm length) 3 wk after surgery, and blood samples were obtained 7 d after T replacement. Finally, in the same group of animals, T implants were removed and additional blood samples taken 2 d and 20 d after removal of T capsules.

Hormone measurements

Serum LH and FSH levels were measured using RIA 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 ^{125}I using Iodo-gen tubes, following the instructions of the manufacturer (Pierce Chemical Co., Rockford, IL). Hormone concentrations were expressed using reference preparations LH-RP-3 and FSH-RP-2 as standards. Intra- and interassay coefficients of variation (CV) were less than 8 and less than 10%, respectively.

Presentation of data and statistics

Hormonal determinations after neuroendocrine tests were conducted in duplicate, with group sizes equal to or greater than seven samples per group. Data are presented as the means ± SEM. Results were analyzed for statistically significant differences, using unpaired Student's *t* test or ANOVA followed by Student-Newman-Keuls multiple-range tests (SigmaStat 8.0; Jandel, San Rafael, CA). $P \leq 0.05$ was considered significant.

Results

Generation of Gpr54 KO mice: validation and phenotypic analyses

Complete Gpr54-null mice were generated by heterozygous crossing of a floxP-*Gpr54*/Protamine-Cre double mutant line, using a strategy illustrated in Supplemental Fig. 1. Homozygous lack of *Gpr54* in the F_2 generation was confirmed by primer-specific PCR genotyping. In good agreement, RT-PCR analyses conclusively demonstrated the absence of detectable *Gpr54* mRNA in the hypothalamus, as major target tissue for Kp, in Gpr54 KO mice (Supplemental Fig. 1C). Likewise, gonadal expression of *Gpr54* mRNA that was detected in wt animals was completely absent in $Gpr54^{-/-}$ male and female mice (data not shown).

Gpr54 KO phenotype-general characterization

No behavioral differences were observed in 9-wk-old Gpr54 KO mice when compared with wt and heterozygous littermates. Blood analyses revealed significant changes in some hematological, blood clotting (Supplemental Table 1), and biochemical parameters (Supplemental Table 2), although the pathophysiological relevance of such mild alterations is considered limited. Body weight data showed a decrease in weight of the $Gpr54^{-/-}$ males compared with wt and heterozygous littermates, whereas no decrease in body weight of the $Gpr54^{-/-}$ females was detected. In both male and female Gpr54 KO mice, a decrease in kidney weight was observed. Furthermore, $Gpr54^{-/-}$ females displayed a modest decrease in adrenal gland weight, whereas the $Gpr54^{-/-}$ males showed an increase in spleen and thymus weight and a decrease in liver weight (Supplemental Table 3). Reproductive organs were macroscopically very small in 9-wk-old male and female Gpr54 KO mice, and fresh dissection for weighing was not possible. However, weights of the reproductive organs of additional groups (3 wk, 9 wk, and 7 months of age) were assessed after fixation. No difference in ovarian and uterus weights was detected between wt and $Gpr54^{-/-}$ females in 3-wk-old animals. In contrast, in males, a decrease in testis weight was observed already at this age in $Gpr54^{-/-}$ KO mice. In 9-wk-old and 7-month-old animals, there was a significant decrease in reproductive organ weights in both the male and female $Gpr54^{-/-}$mice compared their wt littermates (Supplemental Fig. 2).

Histopathological alterations in Gpr54 KO mice

Severe histological changes were observed in the reproductive organs of both the male and female Gpr54 KO mice; in all other tissues, no overt histopathological changes were detected in null mice when compared with wt and $Gpr54^{+/-}$ animals. In adult wt males, the testes showed all stages of spermatogenesis, and the epididymis displayed abundant spermatozoa in the lumen, whereas in wt females, all stages of follicular development and corpora lutea were detected in the ovaries. In marked con-

trast, in Gpr54$^{-/-}$ males, the testes and epididymis were macroscopically very small, and the seminal vesicles and prostate gland were frequently not identifiable. No spermatozoa were found in the lumina of epididymis, and the testes displayed incomplete spermatogenesis, with small seminiferous tubules that contained only spermatogonia and spermatocytes. In Gpr54$^{-/-}$ females, follicles from primordial to preantral stage were detected in the ovaries; however, atretic follicles were frequently observed, and a conspicuous lack of corpora lutea was detected (Supplemental Fig. 3).

LacZ expression under Gpr54 promoter

Because of insertion of LacZ reporter cassette into the *Gpr54* locus, tissues expressing LacZ (as marker of endogenous *Gpr54* expression) can be screened based on their β-galactosidase activity. For validation purposes, analyses were focused on brain tissues; however, more than 40 organs and tissues were studied for β-galactosidase activity (for further details, see legend of Supplemental Fig. 1). Highly specific staining was observed in the brain, with prominent β-galactosidase signal in the hypothalamus, as well as in the cerebellum, cortex, and hippocampus (Supplemental Fig. 1). Weak staining was also observed in the epithelial cell layers of the reproductive organs. LacZ staining studies were performed at three age points (3 wk, 9 wk, and 7 months), with no major difference in the expression profiles between ages (data not shown).

Hormonal phenotype and responses to GnRH priming in Gpr54 KO male mice

A series of neuroendocrine analyses were applied to our model of Gpr54 inactivation as a means to dissect out Kp-dependent *vs.* -independent mechanisms for the control of the gonadotropic axis. To avoid the potential variability of hormone responses in wt females associated with ovarian cyclicity, these analyses were selectively pursued in male mice. The neurohormonal and reproductive phenotypes of Gpr54 KO male mice were assessed by monitoring the circulating levels of LH and FSH in basal conditions in adults, as well as different indices of pubertal maturation, including testicular weight/histology and fertility. As documented in Table 1, circulating levels of both gonadotropins and testicular weights were severely reduced in adult Gpr54$^{-/-}$ male mice. In keeping with initial validation studies, Gpr54-null mice displayed also a marked atrophy of seminiferous tubules and a reduction in the number of mature Leydig cells in the testis. In addition, they failed to show the consensus external index of puberty, balano-preputial separation, even at 7 months postpartum, and were infertile. Similarly, Gpr54 KO mice had lower weights of epididymis, seminal vesicles, and prostate, as well as reduced penis length. In addition, anogenital distance was substantially decreased in Gpr54$^{-/-}$ animals (data not shown).

Lack of functional Gpr54 signaling, evidenced by genotypic and expression analyses (see Supplemental Fig. 1), was further confirmed in Gpr54$^{-/-}$ mice by testing the gonadotropin-releasing effects of an effective bolus of Kp-10 (7.5 nmol ip/rat). In contrast to the potent LH-releasing responses in wt animals, injection of Kp-10 to Gpr54 KO mice failed to evoke any significant elevation of LH levels (Table 1). Likewise, FSH responses to Kp-10 were null in Gpr54 KO males (data not shown). Nonetheless, Gpr54 KO mice retained their ability to potently respond to GnRH priming. As shown in Fig. 1, Gpr54 KO males had severely reduced basal LH and FSH levels. However, repeated GnRH administration elicited robust LH and FSH responses in these animals, with approximately 20-fold increases in circulating levels of both gonadotropins over basal concentrations 30 min after the last injection of GnRH. Of note, 18 h after the last GnRH injection, serum LH levels in Gpr54 KO mice had returned to basal, preinjection values. In contrast, FSH levels remained elevated 18 h after cessation of GnRH priming (Fig. 1). These levels (~10-fold higher than basal) were considered as reference preinjection FSH levels for the corresponding neuroendocrine tests.

Gonadotropin responses to glutamate receptor agonists and RF9 in Gpr54 KO male mice

Validation of the HH phenotype of our Gpr54-null model was followed by testing of the gonadotropin responses to

TABLE 1. A compilation of representative phenotypic and hormonal indices of HH in wt and Gpr54$^{-/-}$ male mice

Genotype	Testis weight (mg)	Basal LH (ng/ml)	Basal FSH (ng/ml)	LH response to Kp-10 (ng/ml)
Gpr54+/+	91.45 ± 1.7 (n = 11)	0.34 ± 0.04 (n = 25)	18.0 ± 1.00 (n = 22)	7.3 ± 0.5[a] (n = 10)
Gpr54−/−	6.57 ± 0.6[b] (n = 7)	0.10 ± 0.01[b] (n = 9)	0.51 ± 0.17[b] (n = 4)	0.15 ± 0.05[b] (n = 4)

[a] $P < 0.01$ vs. corresponding basal LH levels in the same genotype (unpaired Student's *t* test).

[b] $P < 0.01$ *vs.* corresponding wt Gpr54$^{+/+}$ mice (unpaired Student's *t* test). The number of individual determinations per group is indicated in *parenthesis*.

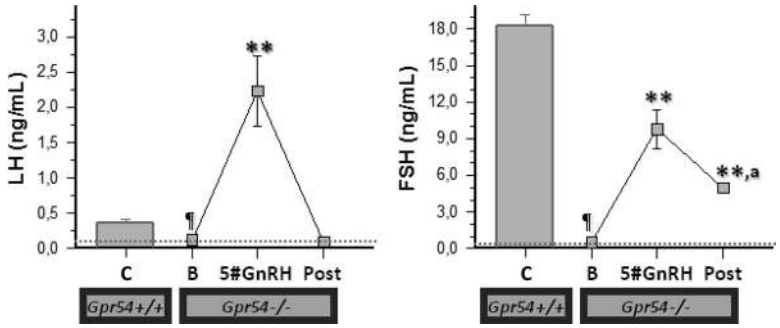

FIG. 1. Gonadotropin responses to GnRH priming in Gpr54 KO male mice. Profiles of LH and FSH responses to repeated GnRH injection (priming) in Gpr54$^{-/-}$ male mice are illustrated. B, Basal preinjection values (also represented as *dotted lines*); 5#GnRH, hormonal values 30 min after the injection of the fifth bolus of the protocol of GnRH priming; Post, hormonal values 18 h after injection of the last GnRH bolus. For reference purposes, circulating levels of both gonadotropin in untreated Gpr54$^{+/+}$ males are also presented (C). **, $P < 0.01$ *vs.* corresponding basal, preinjection values; a, $P < 0.01$ *vs.* corresponding 5#GnRH hormonal values (ANOVA followed by Student-Newman-Keuls multiple range test).

central administration of different agonist or antagonists of key signals putatively involved in the control of GnRH secretion. First, Kp-signaling dependency of the gonadotropin-releasing effects of glutamate pathways was explored by central injection of NMDA, agonist of a subset of ionotropic glutamate receptors, and DHPG, agonist of type I metabotropic receptors. Administration of NMDA evoked a significant (8-fold) increase in the circulating levels of LH in wt male mice, whereas a nonsignificant elevation was detected for FSH (Fig. 2). Likewise, LH levels were elevated in Gpr54 KO mice after icv injection of NMDA; however, the magnitude of such response (~3-fold increase) was substantially attenuated. In addition, Gpr54 KO males displayed significant increases in circulating FSH levels after central injection of NMDA, which nonetheless did not reach control values in wt animals (Fig. 2).

Activation of type I metabotropic glutamate receptors by DHPG evoked a significant 2.5-fold increase in serum LH concentrations in wt males, whereas no significant changes in circulating FSH were detected. Similarly, central injection of DHPG heightened serum LH levels in Gpr54 KO mice, to a similar extent as in wt animals (Fig. 2). Indeed, considering the lowering of basal LH values in null animals, these absolute responses equaled a relative increase of approximately 6-fold over preinjection LH concentrations in Gpr54 KO mice. As for wt mice, DHPG did not cause any significant modification of FSH levels in Gpr54-null males (Fig. 2).

RF9, a putative antagonist of NPFF receptors type 1 and 2, has been recently shown to elicit potent LH responses in rodents (33). Because these might be suggestive of a role of the endogenous RFRP tone, gonadotropin responses to RF9 were explored in Gpr54-null males. In keeping with previous reports, central injection of RF9 evoked a dramatic more than 20-fold increase in circulating LH levels in wt mice, without inducing significant modifications of serum FSH concentrations (Fig. 3). Gpr54 KO mice displayed persistent LH responses to RF9, the absolute magnitude of which was, nonetheless, about one tenth of that observed in wt animals. Yet, due to the lowering of basal LH levels in these animals, relative increases represented more than 8-fold elevation over preinjection LH values. As in wt controls, RF9 failed to change FSH levels in Gpr54-null mice (Fig. 3).

Because our Gpr54 KO mice displayed severe hypogonadism, we tested the possibility that diminished responses to NMDA and RF9 may stem

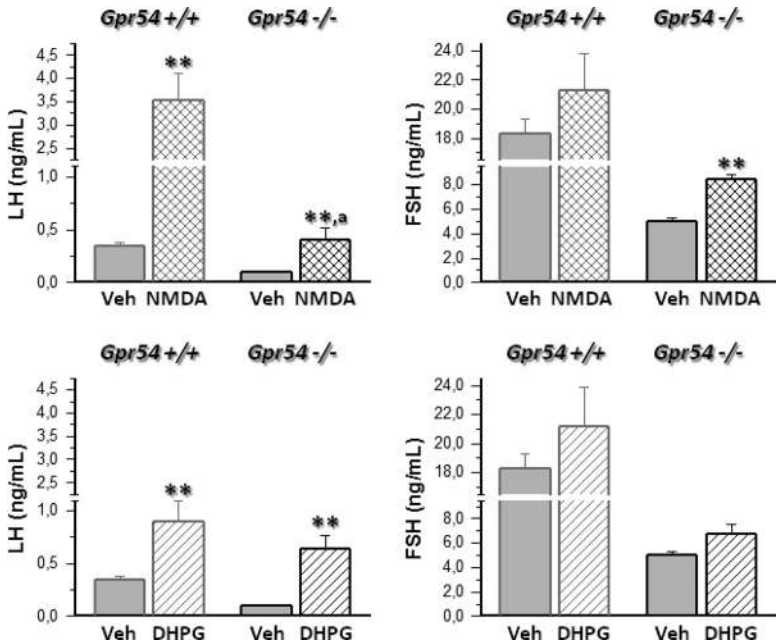

FIG. 2. Gonadotropin responses to agonists of glutamate receptors in Gpr54 KO male mice. Profiles of LH and FSH responses in Gpr54$^{+/+}$ and Gpr54$^{-/-}$ male mice after central administration of effective doses of NMDA (agonist of ionotropic glutamate receptors; *upper panels*) and DHPG (agonist of type-I metabotropic glutamate receptors; *lower panels*). Hormonal levels were assayed 15 min after icv administration of the compounds. Animals injected with vehicle (Veh) served as controls. **, $P < 0.01$ *vs.* corresponding values in vehicle-injected animals (irrespective of the phenotype); a, $P < 0.01$ *vs.* corresponding stimulated values in wt Gpr54$^{+/+}$ mice (ANOVA followed by Student-Newman-Keuls multiple-range test).

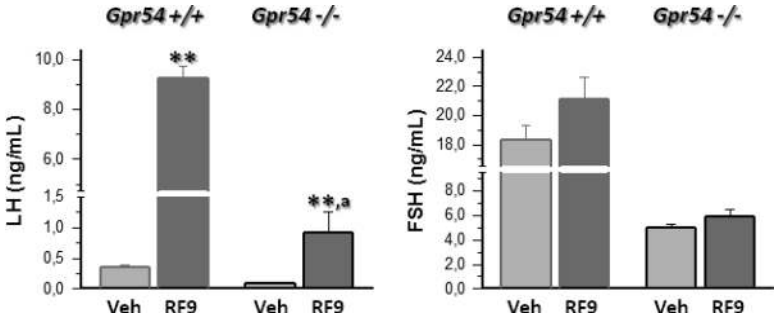

FIG. 3. Gonadotropin responses to RF9 in Gpr54 KO male mice. Profiles of LH and FSH responses in Gpr54$^{+/+}$ and Gpr54$^{-/-}$ male mice after central administration of an effective dose of RF9, putative antagonist of NPFF1R and NPFF2R. Hormonal levels were assayed 15 min after icv administration of the compounds. Animals injected with vehicle (Veh) served as controls. **, $P < 0.01$ *vs.* corresponding values in vehicle-injected animals (irrespective of the phenotype); a, $P < 0.01$ *vs.* corresponding stimulated values in wt Gpr54$^{+/+}$ mice (ANOVA followed by Student-Newman-Keuls multiple-range test).

from the absence of T input rather than primary alterations due to the lack of Gpr54. To this end, ORX+T mice were submitted to similar protocols of gonadotropin monitoring after icv injection of these compounds; the protocol of T supplementation was set on the basis of previous references as to be able to normalize post-ORX levels of LH (36). Central injection of NMDA elicited a robust elevation (>10-fold) of LH levels in wt animals, where it also evoked a modest but significant increase of FSH levels. In contrast, LH and FSH responses to icv injection of NMDA were null in ORX+T Gpr54 KO mice (Fig. 4). Central administration of RF9 induced extraordinarily potent LH responses in ORX Gpr54+/+ receiving a fixed dose of T; responses that represented approximately 100-fold increase over corresponding preinjection values and were 3 times higher than those elicited by RF9 in intact wt animals. However, LH responses to RF9 were totally suppressed in ORX Gpr54 KO mice receiving T implant. In contrast, unambiguous FSH responses to RF9 were observed in ORX+T wt and Gpr54 KO mice, responses that in the KO animals represented a 2-fold increase over corresponding preinjection levels (Fig. 4). Of note, no significant FSH responses to RF9 were detected in gonadal-intact Gpr54+/+ and −/− mice (see Fig. 3).

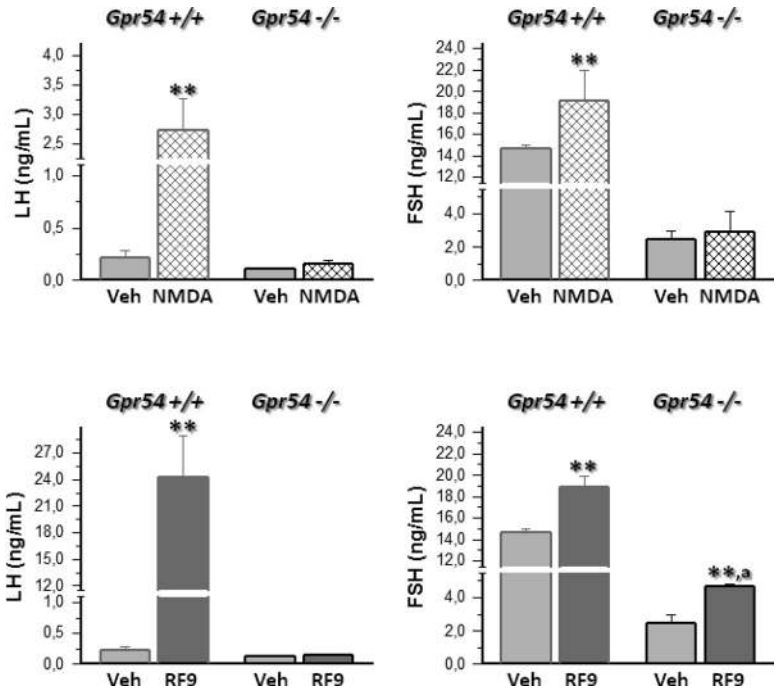

FIG. 4. Influence of T levels on gonadotropin responses to NMDA and RF9 in Gpr54 KO male mice. Profiles of LH and FSH responses in Gpr54$^{+/+}$ and Gpr54$^{-/-}$ male mice, submitted to ORX and hormonal replacement with a fixed, supraphysiological dose of T, after central administration of effective doses of NMDA (*upper panels*) and RF9 (*lower panels*). Hormonal levels were assayed 15 min after icv administration of the compounds. Animals injected with vehicle (Veh) served as controls. **, $P < 0.01$ *vs.* corresponding values in vehicle-injected animals (irrespective of the phenotype); a, $P < 0.01$ *vs.* corresponding stimulated values in wt Gpr54$^{+/+}$ mice (ANOVA followed by Student-Newman-Keuls multiple-range test).

Gonadotropin responses to senktide, nor-BNI, and GALP in Gpr54 KO male mice

The effects of senktide, as agonist of the NKB receptor, NK3R, were also monitored in wt and Gpr54-null mice. As shown in Fig. 5, central injection of senktide evoked a robust 10-fold increase in circulating LH levels, and significantly elevated FSH levels, in control wt animals. In marked contrast, LH and FSH responses to senktide were totally abrogated in Gpr54 KO males. In addition, the effects of the antagonist of κ-opioid receptor, as mediator of Dyn actions on GnRH secretion, were also explored. Central injection of nor-BNI failed to increase LH levels but induced a moderate elevation of serum FSH levels in wt animals. In contrast, in Gpr54 KO males no significant gonadotropin responses to nor-BNI were detected; however, a trend for increase in LH levels was observed in Gpr54$^{-/-}$ mice that did not reach statistical significance due to large variability within this group (Fig. 5).

The effects of the neuropeptide, GALP, on gonadotropin secretion were also explored in wt and Gpr54 KO mice. In keeping with previous litera-

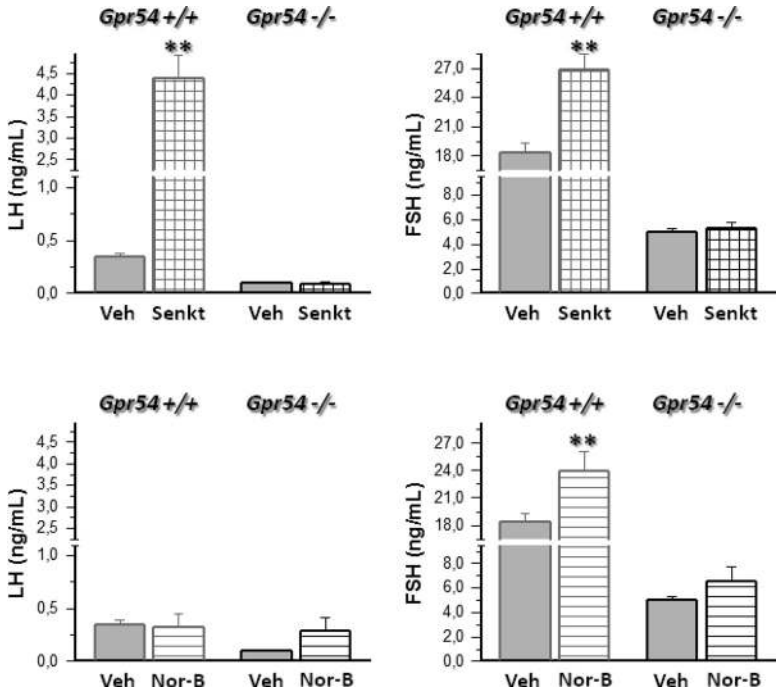

FIG. 5. Gonadotropin responses to manipulation of NKB and Dyn pathways in Gpr54 KO male mice. Profiles of LH and FSH responses in Gpr54$^{+/+}$ and Gpr54$^{-/-}$ male mice after central administration of effective doses of Senktide (agonist of the NKB receptor, NK3R; *upper panels*) and nor-BNI (Nor-B; antagonist of the Dyn receptor, κ-opioid receptor; *lower panels*). Hormonal levels were assayed 15 min after icv administration of the compounds. Animals injected with vehicle (Veh) served as controls. **, $P < 0.01$ *vs.* corresponding values in vehicle-injected animals (ANOVA followed by Student-Newman-Keuls multiple-range test).

ture, icv injection of GALP was found to induce a significant (8-fold) elevation of circulating LH levels in wt mice, where it also significantly stimulated FSH secretion (Fig. 6). Significant LH responses to GALP were also detected in Gpr54 KO males, but their absolute magnitude was severely blunted, and the relative increase represented only a 2-fold elevation over corresponding preinjection levels. No FSH responses to GALP were detected in Gpr54 KO mice.

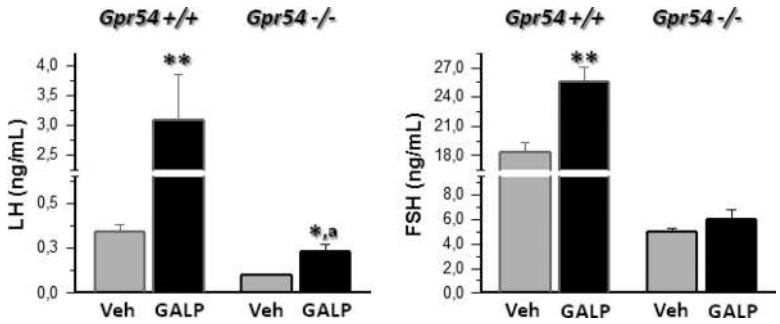

FIG. 6. Gonadotropin responses to GALP in Gpr54 KO male mice. Profiles of LH and FSH responses in Gpr54$^{+/+}$ and Gpr54$^{-/-}$ male mice after central administration of an effective dose of GALP. Hormonal levels were assayed 15 min after icv administration of the compounds. Animals injected with vehicle (Veh) served as controls. **, $P < 0.01$ *vs.* corresponding values in vehicle-injected animals; a, $P < 0.01$ *vs.* corresponding stimulated values in wt Gpr54$^{+/+}$ mice (ANOVA followed by Student-Newman-Keuls multiple-range test). *, $P < 0.05$ *vs.* corresponding values in vehicle-injected Gpr54$^{-/-}$ mice (unpaired Student's *t* test).

Gonadotropin responses to gonadectomy and/or T supplementation in Gpr54 mice

The dynamic fluctuations in circulating LH and FSH levels in response to ORX, T replacement, and T withdrawal were studied in wt and KO Gpr54 mice. As shown in Fig. 7, a significant elevation in serum LH levels took place in wt mice at 2 d after ORX, which further increased by d 20 after ORX. In contrast, LH levels remained invariant in Gpr54 KO mice at 2 and 20 d after ORX. Hormone replacement of wt ORX mice by means of T implants prevented the rise of LH concentrations after ORX and lowered LH levels below basal, pre-ORX values. In this model, removal of T implants in wt mice evoked a substantial rise of circulating LH values at 2 and 20 d, which reached levels higher than those of equivalent time points after surgical ORX. Again, although LH levels could not be further lowered by T in Gpr54 KO animals, no detectable increase of circulating LH levels was detected at 2 and 20 d after removal of T implants.

Profiles of FSH responses to ORX, T replacement, and T withdrawal were similar to those of LH in wt animals; however, the magnitude of FSH increases was similar after ORX or removal of T implants (Fig. 7). However, in clear contrast with LH profiles, FSH levels were indeed elevated at 2 and 20 d after ORX in Gpr54 KO mice. Moreover, T supplementation in ORX Gpr54-null animals failed to completely suppress FSH levels to pre-ORX values in this genotype. Finally, removal of T implants in ORX Gpr54 KO animals did not further increase circulating FSH levels, but rather resulted in the progressive decline of FSH concentrations, at 2 and 20 d after T withdrawal (Fig. 7).

Discussion

Mouse models of congenital inactivation of *Gpr54* or *Kiss1* genes have helped to define the putative roles of Kp signaling in key facets of GnRH regulation, such as negative and positive

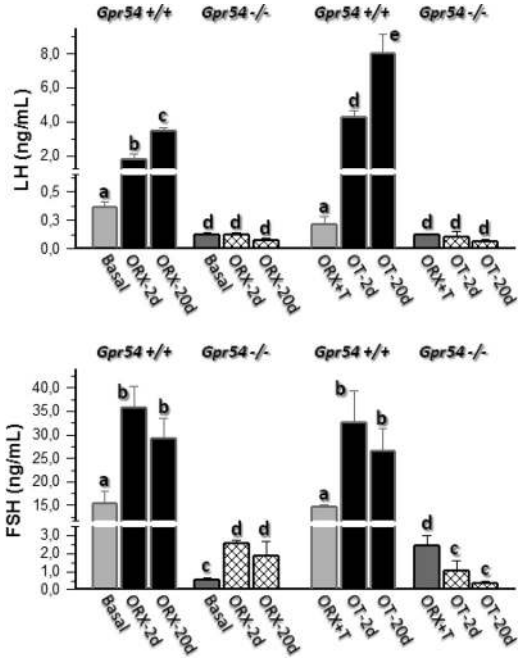

FIG. 7. Gonadotropin responses to testis and T withdrawal in Gpr54 KO male mice. Profiles of LH (*upper panel*) and FSH (*lower panel*) responses to orchidectomy (ORX; at 2 and 20 d after ORX), T replacement (ORX+T; at 1 wk after T administration to ORX animals) and T removal (OT; at 2 and 20 d after ORX) in $Gpr54^{+/+}$ and $Gpr54^{-/-}$ adult male mice. Differences between groups with *different superscript letters* are statistically significant ($P < 0.05$; ANOVA followed by Student-Newman-Keuls multiple-range test).

feedback (29–31) and, very recently, its potential interaction with NMDA pathways (37). However, notable phenotypic and endocrine differences seem to exist among the different mouse lines so far reported. Thus, whereas all Kiss1 and Gpr54 KO mouse lines generated to date are infertile, the magnitude of their predicted hypogonadotropic hypogonadism appears to vary considerably, ranging from severe impact to rather mild affectation. Indeed, some Gpr54- or Kiss1-null lines have been reported to display only modestly delayed puberty onset and grossly preserved basal LH levels (38). In clear contrast, our Gpr54 KO model is characterized by a profound hypogonadotropic state, associated with persistent absence of external signs of puberty and severe gonadal hypoplasia, despite preserved responsiveness to exogenous GnRH. In addition to potential differences in terms of KO strategy (28), the genetic background is likely a determinant factor for the above discrepancies, because milder reproductive impacts of *Gpr54* or *Kiss1* inactivation have been reported in Sv129 mice (28), whereas Gpr54-null mice of the C57BL6 background seem to display a more severe phenotype. This would nicely illustrate the importance of genetic covariables for the penetrance of reproductive impairment due to defective Gpr54 signaling; a phenomenon documented in humans (39). In any event, the features of our null mouse, as complete phenocopy of humans harboring inactivating mutations of *GPR54* (4), make it a suitable model for the neuroendocrine analyses reported herein.

In keeping with the crucial role of Kp signaling for proper function of GnRH neurons, Gpr54-null males not only showed a profound suppression of basal LH and FSH levels, but displayed also decreased gonadotropic responses to most of the elicitors tested. The fact that, despite the preserved responsiveness to exogenous GnRH, LH secretory responses were globally reduced after central administration of a diversity of secretagogues, operating via different receptors and modes of action, strongly suggests that the congenital absence of Gpr54 is deleterious for the intrinsic secretory activity of GnRH neurons and their capacity to respond to different regulators, even if they are not acting directly via Gpr54. This is in keeping with recent reports showing that Kp-10 is able to enhance the amplitude of postsynaptic currents in GnRH neurons in response to other key neurotransmitters, such as γ-amino butyric acid (12). In any event, for most of the compounds tested, Gpr54 KO mice displayed discernible LH and/or FSH responses that were clearly different from the null responses to Kp injection, thus allowing a reliable estimate of the relative importance of Kp-dependent *vs.* -independent pathways in the control of GnRH secretion.

Glutamate neurotransmission is known to play a prominent excitatory role in the control of puberty onset and gonadotropin secretion (40, 41). The actions of glutamate are mediated via different ionotropic [NMDA, kainate, alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA)] and, to a lesser extent, metabotropic receptors, the relative contribution of which has been the subject of active investigation recently (40, 41). The ionotropic glutamate receptor, NMDA, has been shown to be a key component in the glutamatergic regulation of GnRH neurons. Yet, whether it operates via direct actions and/or intermediate afferents remains partially unsolved (40). In our study, central NMDA injection did not only elicit a robust elevation of LH levels in wt animals, but evoked also unambiguous LH and FSH responses in Gpr54 KO mice. Indeed, the increase of FSH levels after central NMDA injection became significant only in Gpr54-null mice. Admittedly, absolute levels of LH after NMDA administration were about 10 times lower in Gpr54-null animals; however, due to their hypogonadotropic state, these LH responses represented an approximately 3-fold elevation over basal preinjection values. In keeping with our present results, d'Anglemont de Tassigny *et al.* (37) recently reported preserved LH responses to central NMDA administration in their Gpr54 and Kiss1 KO lines. These previous and present findings collectively support that a

component of the glutamate/NMDA neurotransmission that regulates GnRH secretion is independent of Kp signaling. However, in the former study, absolute LH responses to central NMDA injection in Gpr54 KO males were similar to those of wt animals (37). Although the reasons for such discrepancy remain open, it is stressed that our study involved injection of much lower (and presumably, more physiological) doses of NMDA (1 nmol *vs.* 7 nmol); admittedly, higher doses of NMDA might have elicited greater gonadotropic responses but may pose also the risk of unspecific excitotoxic effects (42, 43). In addition, the fact that the previous study was conducted in the 129S6 background, which seems to display a milder gonadotropic alteration after Gpr54 inactivation, may partially explain the above differences. In fact, our present results suggest that the mechanisms whereby NMDA activates GnRH secretion involve also a Kp-dependent component. Whether this would imply direct stimulation of Kp input on GnRH neurons by NMDA and/or postsynaptic modulation of GnRH responsiveness to NMDA by Kp signaling is yet to be elucidated. In favor of the first possibility, only a modest fraction of GnRH neurons have been shown to express functional NMDA glutamate receptors (40), therefore suggesting a indirect mode of action that might involve activation of Kp afferents. In contrast to NMDA, electrophysiological studies have demonstrated the abundant expression of kainate/ alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) glutamate receptors in GnRH neurons (40), thus suggesting predominant direct actions, the potential interplay with Kp signaling of which merits investigation.

Metabotropic neurotransmission has been also involved in the regulation of the GnRH system (40, 44). However, only a small proportion of GnRH neurons seem to express functional metabotropic receptors (40), and the physiological relevance of metabotropic signaling in the control of the gonadotropic axis remains ill defined (41). Interestingly, a recent report in the mouse suggested the presence of two distinct subsets of GnRH neurons in the septal area, defined on the basis of their differential responsiveness to type I metabotropic receptor agonist, DHPG, and Kp-10 (15). Thus, whereas one group of GnRH neurons was surprisingly insensitive to the excitatory effects of Kp-10 but highly responsive to DHPG, a second population of GnRH neurons was potently excited by Kp-10, but not by DHPG. That study, however, was solely based on electrophysiological recordings of GnRH neurons and did not include assessment of hormonal endpoints (15). In keeping with those findings, our present work documented clear-cut LH responses to DHPG in wt male mice, responses that were detected also in Gpr54 null animals. Notably, LH secretory responses to DHPG were similar in magnitude in wt and Gpr54 KO animals, which is in contrast to the overall suppression of gonadotropic responses to other elicitors, such as NMDA, RF9, and GALP, in Gpr54-null mice. Together with previous electrophysiological results (15), our present *in vivo* data demonstrate that type I metabotropic receptors participate in the control of a subset of GnRH neurons, which seem to be unaffected by the lack of Gpr54 signaling and play a distinct role in the excitatory control of LH secretion.

In recent years, neuropeptides other than Kp have also gained appreciation as putative modulators of the gonadotropic axis. Among those, the gonadotropin-inhibitory peptide RFRP3, as the mammalian ortholog of the avian gonadotropin-inhibiting hormone (45, 46), and GALP, as central transmitter that may participate in the functional coupling of metabolic state and GnRH secretion (34, 47), have been actively investigated. Based on our recent data (33), we tested in our Gpr54-null model the gonadotropin-releasing effects of RF9, a putative antagonist of NPFF1R, a receptor that mediates the biological effects of RFRP (46). In keeping with our previous study, central injection of RF9 evoked very potent LH-releasing actions in wt mice, LH-secretory responses that were also detected, albeit at a lower magnitude, in Gpr54-null animals. Although the presence of detectable LH responses to RF9 in Gpr54 KO might be compatible with the existence of a Kp-independent, RFRP-dependent pathway in the control of GnRH/gonadotropin secretion, it must be stressed, as general call of caution, that RF9 has been shown to inhibit NPFF2R with equal potency, namely, the receptor of neuropeptide FF (48). Moreover, the possibility that RF9 may be acting through other, as yet unknown, targets has not been conclusively ruled out to date. In any event, our pharmacological data unambiguously demonstrate that at least part of the potent LH-releasing effect of RF9, a compound with potential therapeutic interest in the management of opioid-induced tolerance and hyperalgesia (48–50), takes place in a Gpr54-independent manner. Likewise, our data are the first to document that a fraction of the LH-secretory activity of GALP, which was detected also in Gpr54-null mice, occurs via Kp-independent mechanisms.

Given the prominent roles of sex steroids in the modulation of gonadotropic responses to Kp (2), we assessed in our study whether the prevailing hypogonadal state in Gpr54 KO mice, with very low T levels, may contribute to the overall suppression of LH- and FSH-secretory responses to most of the secretagogues tested. Our analyses in ORX+T mice strongly suggest that this is not the case, because Gpr54 KO males with constant, supraphysiological levels of T replacement did not display enhanced responsiveness to NMDA or RF9; on the contrary, LH re-

sponses to NMDA and RF9 that were detectable in gonadal-intact Gpr54-null mice were abrogated in ORX+T KO, probably reflecting the summative influence of the lack of Gpr54 signaling and negative feedback of high T concentrations (*e.g.* at the pituitary level). These observations suggest that the substantial reduction of gonadotropin responses to various elicitors detected in our Gpr54 KO line is a genuine phenomenon, directly related to the lack of Kp signaling and not secondary to the marked suppression of endogenous sex steroid levels.

In the last 2 yr, NKB has been recognized as an essential central regulator of the gonadotropic axis in humans and very probably other mammalian species (21, 27, 51). The fact that NKB is coexpressed in the ARC population of Kiss1 neurons in rodents, sheep, goat, and primates (51), species in which activation of NKB receptors has been shown to evoke potent LH-secretory responses (22–24), has added further interest to the study of potential Kp-NKB interactions in the control of GnRH neurons. Indeed, based on expression and functional data, mostly from rodent and ovine studies (25, 26), a model had been proposed in which NKB may act mainly on Kiss1 neurons as (auto)regulator of the Kp output onto GnRH neurons; however, this hypothetical model is founded mostly on indirect evidence. To our knowledge, our study is the first to provide conclusive functional validation of such a hypothesis, because the very potent secretagogue effect of the agonist of NKB receptor, senktide, was totally abrogated in Gpr54-null mice. This observation suggests that preserved Kp signaling is mandatory for the stimulatory actions of NKB on GnRH neurons, and therefore is compatible with the proposal that NKB is upstream of the Kp pathways projecting to GnRH neurons. The consensus hypothesis also postulates that Dyn, which is also expressed in Kiss1 neurons, is a negative autoregulator of Kp pathways (26, 51). In our study, the antagonist of the κ-opioid (Dyn) receptor, nor-BNI, significantly enhanced FSH levels in wt animals, a response that was absent in Gpr54-null mice. This would support the hypothesis that the inhibitory effect of Dyn on GnRH/gonadotropin secretion is conducted via modulation of Kp signaling. However, the fact that blockade of Dyn receptors failed to enhance serum LH levels in wt animals is intriguing and merits further investigation; however, it remains possible that a longer time lag is needed for the effects of the Dyn antagonist, nor-BNI, on LH secretion to surface.

Finally, our Gpr54-null model was used also to interrogate the contribution of Kp signaling in mediating the negative feedback effects of testicular hormones on LH and FSH secretion. Admittedly, previous studies using other mouse models of Kiss1 or Gpr54 inactivation had set the contention that Kp pathways play an indispensable role in conveying the inhibitory effects of sex steroids on LH secretion (30, 31). However, previous analyses had also disclosed marginal elevations of FSH levels after GNX of Gpr54-null mice (31). Of note, conclusions from previous studies were based solely on single-time point determinations after GNX. Moreover, these former studies did not take into account the fact that the lack of responses to gonadal withdrawal in Gpr54 KO mice might be secondary to their severe hypogonadism, because removal of negligible sex steroid levels should not be sufficient to raise serum LH concentrations. In our study, we monitored short- (48 h) and long-term (20 d) responses not only to ORX, but also to the withdrawal of constant, supraphysiological doses of T replacement in wt and Gpr54 KO mice. Our results conclusively demonstrate that the lack of LH responses to GNX in Gpr54 KO mice is a genuine phenomenon due to the primary impairment of GnRH neurons to respond to the elimination of negative feedback inputs in the absence of Kp signaling. In addition, our work further documents the persistent elevation of FSH levels after GNX in Gpr54-null males. This observation, together with the fact that supraphysiological doses of T failed to fully suppress FSH concentrations in $Gpr54^{-/-}$ male mice, strongly suggest that other testicular factors, such as inhibins, may account for this fraction of Kp-independent negative feedback control of FSH secretion. Curiously enough, removal of T replacement in long-term ORX Gpr54-null mice did not induce further elevations of FSH levels, but rather lowered them. One possible explanation for this paradoxical phenomenon is that T replacement may produce some positive effects on FSH biosynthesis directly at the pituitary level in the context of low GnRH secretion, as previously reported in the rat (52).

In sum, we have presented herein the detailed phenotypic characterization of a novel Gpr54-null mouse line, defined by a marked and persistent state of hypogonadotropic hypogonadism. Testing of gonadotropic responses to a diversity of elicitors in this mouse model has allowed us to dissect out the differential contribution of Kp-dependent *vs.* -independent modes of action of key regulators of the reproductive axis, from glutamate neurotransmission to sex steroids. In addition to intriguing differences in terms of Gpr54 signaling dependency for the gonadotropin-releasing effects of ionotropic *vs.* metabotropic glutamate pathways, our results are the first to provide direct functional support to the hypothesis that the stimulatory effects of NKB are dependent on the integrity, and thus likely upstream, Kp signaling in GnRH neurons.

Acknowledgments

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