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Kisspeptin Can Stimulate Gonadotropin-Releasing Hormone (GnRH) Release by a Direct Action at GnRH Nerve Terminals

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The G protein-coupled receptor GPR54, and its peptide ligand kisspeptin (Kp), are crucial for the induction and maintenance of mammalian reproductive function. GPR54 is expressed by GnRH neurons and is directly activated by Kp to stimulate GnRH release. We hypothesized that Kp may be able to act at the GnRH nerve terminals located in the mediobasal hypothalamus (MBH) region. To test this hypothesis, we used organotypic culture of MBH explants challenged with Kp, followed by RIA to detect GnRH released into the cultured medium. Kp stimulation for 1 h induced GnRH release from wild-type male MBH in a dose-dependent manner, whereas this did not occur in MBH explants isolated from *Gpr54* null mice. Continuous Kp stimulation caused a sustained GnRH release for 4 h, followed by a decrease of GnRH release, suggesting a desensitization of GPR54 activity. Tetrodotoxin did not alter the Kp-induced GnRH release, indicating that Kp can act directly at the GnRH nerve terminals. To localize *Gpr54* expression within the MBH, we used transgenic mice, in which *Gpr54* expression is tagged with an IRES-LacZ reporter gene and can be visualized by β-galactosidase staining. *Gpr54* expression was detected outside of the median eminence, in the *pars tuberalis*. In conclusion, our results provide evidence for a potent stimulating effect of Kp at GnRH nerve terminals in the MBH of the mouse. This study suggests a new point at which Kp can act on GnRH neurons. (*Endocrinology* 149: 3926–3932, 2008)

THE ACTIVATION AND maintenance of fertility are centrally controlled by GnRH neurons (1). In the mouse, GnRH cell bodies are located in the preoptic region of the hypothalamus and project caudally to the median eminence (ME). GnRH is released at the ME into the fenestrated capillaries of the pituitary-portal plexus, and transported to gonadotropes of the anterior pituitary to stimulate production and secretion of LHs and FSHs. GnRH release is regulated by several molecules, including neurotransmitters, glial cell factors, sex steroids (1, 2), and the recently identified kisspeptin (Kp) neuropeptide (3).

Kp signaling in the brain is vital for reproduction. Disruption of either the *Kiss1* gene that encodes Kp (4, 5), or the *Gpr54* gene that encodes the receptor for Kp (4, 6), causes hypogonadotropic hypogonadism in the mouse. Kp has a potent effect on the release of the gonadotropins LH and FSH (7, 8). Central injection of very small amounts of Kp (<1 nmol) is sufficient to evoke maximal LH responses in the rat (8–10), sheep (11), and monkey (12, 13). To a lesser degree, plasma FSH levels are also increased after Kp stimulation (7, 9). Systemic administration of Kp also elicits LH release in humans (14), rodents (8, 9, 11), and monkeys (13). Kp acts upstream of GnRH release from the hypothalamus to activate the pituitary-gonadal axis. The use of potent GnRH antagonists prevents Kp-induced LH release (8, 15). Moreover, Kp induces c-*fos* expression in rat GnRH neurons (16, 17), which are known to express *Gpr54* (11, 16), and causes depolarization of 90% of GnRH neurons in the adult mouse (18). Anatomically, Kp immunoreactive fibers surround the GnRH cell bodies in the preoptic area (POA) to make synaptic appositions in the mouse (19). The consensus view is that Kp stimulates GnRH neurons by binding to GPR54 receptors on the cell bodies localized in the rostral POA. Several *in vitro* and *in vivo* studies have investigated the effect of the GPR54/Kp pathway on GnRH release from the intact whole hypothalamus (20), but none has examined the possible effect of Kp at GnRH nerve terminals.

In this context the present experimental work was undertaken to dissect further the site(s) of action of Kp on GnRH release and to test the hypothesis that Kp can act directly at the ME. To this end, we explanted mediobasal hypothalamic (MBH) fragments into culture and challenged them with Kp10 at increasing concentrations. As continuous Kp infusion desensitizes GPR54-induced GnRH release in the rhesus monkey (13), we also examined the effect of continuous Kp10 stimulation on MBH explants. *Gpr54* null mice (6) were used as controls for these studies to verify that the responses were mediated by GPR54. We used tetrodotoxin (TTX) to block the possible action potentials from the few GnRH cell bodies left in the MBH fragment. Finally, we used the property that *Gpr54* mutant mice express β-galactosidase under control of the *Gpr54* promoter to identify the possible cell type express-

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Abbreviations: ARH, Arcuate nucleus; AVPV, anteroventral periventricular nucleus; GPR, G protein-coupled receptor; KCl, potassium chloride; Kp, kisspeptin; MBH, mediobasal hypothalamus; ME, median eminence; MECA, Mouse pan ECA-32; PB, phosphate buffer; POA, preoptic area; RT, room temperature; TTX, tetrodotoxin.

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ing *Gpr54* that could be involved in these responses at the ME.

Materials and Methods

Animals and reagents

Adult (2–4 months old) male wild-type and transgenic (*Gpr54*^{tm1PAR}) (6) mice were used for the following experiments. These animals were maintained as an inbred stock on a 129S6/SvEv genetic background. The mice were housed in cages on a 1200-h day and 1200-h night cycle (light on at 0700 h) at controlled temperature (22 C), and were provided with food and water *ad libitum*. All experiments were performed under the authority of a United Kingdom Home Office Project License and were approved by a local ethics panel.

Kp10 [human Metastin (45–54) amide, M-2816] was purchased from Sigma-Aldrich Co. Ltd. (Dorset, UK). Kp10 consists of the 10 C-terminal amino acids of Kp54 and has produced comparable biological effects to full-length metastin (15, 21, 22). TTX (Calbiochem, Nottingham, UK) was kindly provided by Dr. David Parker (Department of Physiology Development and Neuroscience, Cambridge University, Cambridge, UK). The rabbit anti-GnRH antibody was a gift from Dr. Vincent Prévot (Institut National de la Santé et de la Recherche Médicale U837, University of Lille 2, Lille, France) initially obtained and validated by Professor Gerard Tramu (23) (University of Bordeaux 1, Talence, France). The rat monoclonal antimouse pan ECA-32 (MECA-32) antibody was a generous gift from Professor Britta Engelhardt (Theodor Kocher Institute, University of Bern, Bern, Switzerland). Anti-MECA-32 specifically recognizes endothelial cells outside of the blood-brain barrier in the adult mouse (24). The rabbit anti-LH β-antibody was validated on rat pituitary tissue (25) and was obtained from Dr. Albert F. Parlow and the National Hormone and Peptide program (Torrance, CA). Secondary goat anti-rabbit (BA-1000) and goat anti-rat (BA-9400) biotin-conjugated antibodies were purchased from Vector Laboratories (Peterborough, UK). Primary antibodies were tested at different concentrations, and for each of the antibodies, we obtained immunostaining results in accordance with the expected pattern.

Experimental design

Mice used in our experiments were killed between 0900 and 1000 h by CO_2 exposure, and the brain was rapidly removed. The MBH was dissected under a binocular microscope following the limits: posterior border of the optic chiasm and the anterior border of the mamillary bodies. The MBH explants comprise the ME, the arcuate nucleus (ARH), as well as the ventromedial nuclei.

MBH fragments were incubated individually in wells of a 96-well plate filled with 250 μl culture medium made of DMEM (21063; Life Technologies, Inc., Gaithersburg, MD) with 20 μM bacitracin (B0125; Sigma-Aldrich) saturated with 95% O_2-5% CO_2, and placed at 37 C in a 5% CO_2 atmosphere cell culture incubator. The medium was replaced every hour with freshly prepared medium. Fragments were moved to fresh medium, and the old medium was harvested, stabilized with 12.5 μl 0.2 M EDTA, and stored at −20 C until assayed for GnRH. Experiments were performed by incubating each tissue fragment alone for 1 h in a 250-μl well (acclimatization) before any treatment. Two hundred fifty microliters of medium with no MBH were simultaneously incubated under the same conditions to serve as a control for background. The RIA value corresponding to the background was subtracted from RIA values obtained for cultured MBH. Treatments with Kp10 or vehicle began after a 1-h acclimatization period and went on for 1 h (in experiment 1), 2 h (in experiment 3), or 6 h (in experiment 2). To determine the viability of the tissue fragments at the end of the experiment, MBH fragments were challenged for 1 h with 60 mM KCl to elicit GnRH release. Any MBH fragment displaying no significant GnRH release after KCl stimulation was removed from the study.

Experiment 1: GnRH response to acute (1 h) Kp10 stimulation in cultured MBH fragments

To determine the dose response of Kp on GnRH release in cultured MBH fragments, the tissue explants were challenged with increasing Kp10 concentrations. MBH from wild-type mice were dissected and placed in culture. After 1-h acclimatization to the culture medium, this was replaced by fresh medium containing different concentrations of Kp10 (0, 0.5, 5, 50, or 500 nM) or untreated. MBH from GPR54−/− mice were treated with Kp10 (500 nM) and served as a negative control. After 1-h Kp10 treatment, MBH fragments were incubated with 60 mM KCl for viability control. Total incubation time was 3 h.

Experiment 2: GnRH response to continuous (6 h) Kp10 stimulation in cultured MBH

To investigate the effects of continuous Kp stimulation on GnRH release, MBH fragments were incubated with 50 nM Kp10 for 6 h. After 1-h acclimatization, the culture medium was replaced every hour with freshly oxygenated medium containing newly prepared 50 nM Kp10. The final incubation hour was performed with culture medium containing 60 mM KCl to elicit GnRH release to assess the tissue viability. Total incubation time was 8 h.

Experiment 3: effect of TTX on Kp10-induced GnRH release from cultured MBH

After 1-h acclimatization to the culture medium, wild-type MBH fragments were incubated for 1 h in medium containing 1 μM TTX or no TTX. MBH fragments were then placed in a medium containing TTX 1 μM plus 500 nM Kp10 or 500 nM Kp10 only. Finally, MBH fragments were incubated with 60 mM KCl for viability control. As for previous experiments, the medium was collected every hour, EDTA added to give a final concentration of 10 μM, and stored until RIA. Total incubation time was 4 h.

Experiment 4: assessment of Gpr54 expression in the MBH

To determine whether *Gpr54* is expressed within the ME, we used transgenic *Gpr54* null mice in which the *LacZ* gene is under the control of the *Gpr54* promoter (6). Adult male mice were deeply anesthetized by pentobarbital ip and intracardially perfused with ice-cold PBS 0.1 M containing 0.5 mM EDTA, followed by 1% paraformaldehyde in PBS. Brains were collected and placed in the same fixative for 2 h at 4 C and subsequently incubated in 25% sucrose-containing PBS overnight at 4 C. The next day, brains were washed twice with PBS and incubated overnight at 37 C in a β-galactosidase staining solution [PBS 0.1 M containing 1 mM $MgCl_2$, 0.4 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal, MB1001; Melford Laboratories, Chelsworth, Ipswich, UK), 5 mM potassium ferrocyanide, and 5 mM potassium ferricyanide]. Brains were incubated overnight at 4 C in PBS with 25% sucrose for cryoprotection, and then embedded in Cryo-M-Bed (Bright, Huntingdon, UK) and frozen on dry ice. Coronal 15-μm thick sections at the level of the ME were collected on Polysine microscope slides (VWR International Ltd., Leicestershire, UK) and air-dried overnight under vacuum. For immunohistochemistry, sections were blocked in phosphate buffer (PB) (4 μM KH_2PO_4 and 16 μM K_2HPO_4) with 5% goat serum (G-9023; Sigma-Aldrich) and 0.3% Triton X-100 (X100; Sigma-Aldrich) for 1 h at room temperature (RT). Incubation with either anti-MECA-32 (1:25), anti-GnRH (1:3000), or anti-LH β (1:1000) antibodies was performed in the same blocking solution overnight at 4 C. After washing, sections were incubated with either anti-rat or anti-rabbit IgG biotin-conjugated secondary antibodies (1:500) diluted in PB with 5% goat serum and 0.3% Triton X-100 for 1 h at RT, then in 1% hydrogen peroxide in PB for 10 min at RT to quench the residual endogenous peroxidase activity. Detections were done using the avidin-biotin complex (ABC PK-4000; Vector Laboratories), with 3,3′-diaminobenzidine (SK-4100; Vector Laboratories) as chromogen. Sections were rinsed in distilled water, dehydrated, and mounted with DPX (Sigma-Aldrich). A minimum of four animals was used for each staining.

GnRH RIA

The incubation medium was assayed for GnRH content by RIA using a kit provided by Phoenix Pharmaceuticals (RK-040-02; Karlsruhe, Germany) (sensitivity 4 pg/tube, intraassay variation 4.7%, interassay variation, 8.3%). Two replicates per sample were performed for each test.

The RIA protocol was performed as indicated in the guidelines provided by the manufacturer.

Statistical analysis

In experiment 1, the differences among several groups were analyzed by one-way ANOVA, followed by the Student-Newman-Keuls multiple comparison test for unequal replication. In experiment 2, the significance of differences between mean GnRH concentrations after Kp10 treatment was determined by one-way ANOVA with repeated measures. In experiment 3, samples exposed to TTX and samples without TTX were separated for comparison. One-way ANOVA with repeated measures followed by the Student-Newman-Keuls multiple comparison test was used to compare mean GnRH concentrations between acclimatization, with or without TTX, and Kp10 stimulation samples. The unpaired *t* test was used to compare the Kp10-induced GnRH concentration between samples exposed to TTX and samples with no TTX. The level of significance was set at $P < 0.05$.

Results

Kp10 stimulates GnRH release from mouse MBH explants

To test the hypothesis that Kp can stimulate GnRH release in the absence of GnRH neuronal cell bodies, we challenged MBH fragments with Kp10 at different concentrations; the number of MBH explants used per condition is indicated in Fig. 1. We determined the lowest effective dose of Kp10 that stimulated GnRH secretion in our experimental conditions by testing a range of Kp10 concentrations from 0.5–500 nM for 1-h incubation on cultured MBH explants. Kp10 produced a significant increase in the secretion of GnRH from the cultured MBH fragments. GnRH released into the culture medium was significantly increased after 1-h exposure to 500 nM Kp10 compared with other Kp10 concentrations in wild type ($P < 0.001$; Fig. 1). To ascertain if this effect was mediated by GPR54, MBH fragments from GPR54−/− age-matched mice were incubated in the absence or presence of 500 nM Kp10. This concentration of Kp10 did not significantly increase GnRH secretion from GPR54−/− MBH compared with no Kp10 treatment, whereas the same Kp10 concentration significantly increased GnRH release from wild-type MBH fragments ($P < 0.001$). Both wild-type and GPR54−/− MBH explants responded to 60 mM KCl by releasing GnRH, indicating viable tissue at the end of the procedure (Fig. 1, *inset*).

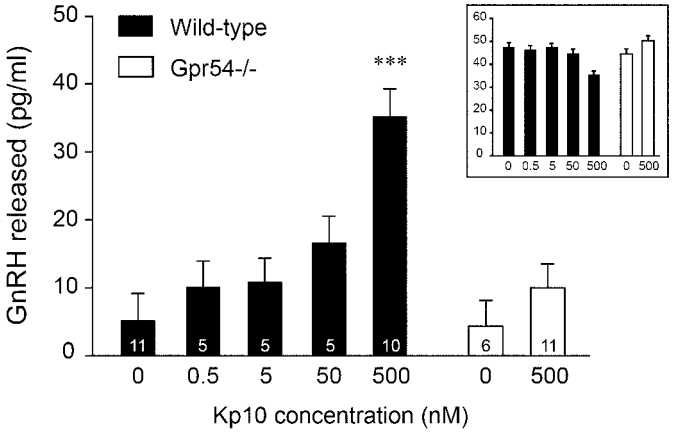

FIG. 1. Direct stimulatory effect of Kp on GnRH release from MBH explants from male mice. After 1-h acclimatization, MBH explants were exposed to different concentrations of Kp10 (0, 0.5, 5, 50, and 500 nM for wild type, and 0 and 500 nM for GPR54−/−) for 1 h and then challenged with 60 mM KCl for 1 h. *Columns* represent the mean (± SEM) of the GnRH released into the culture medium after Kp10 stimulation. *Inset* shows responses to 60 mM KCl. ***, $P < 0.001$ compared with all other treatments on both wild type and GPR54−/−. One-way ANOVA followed by the Student-Newman-Keuls multiple range test. The number of animals used per conditions is indicated at the *bottom of each column*.

Continuous Kp10 stimulation of MBH explants leads to desensitization of GPR54 response to Kp10

To test whether desensitization occurred in our MBH explants, we investigated the effect of 6-h continuous Kp10 stimulation on GnRH release. In wild-type MBH explants (n = 11), GnRH release was significantly increased for 4 h using 50 nM Kp10 compared with no Kp10 addition ($P < 0.01$; Fig. 2A). GnRH release decreased after 5 and 6-h continuous Kp10 stimulation to levels significantly lower than the first 4-h exposure to Kp10 ($P < 0.05$) and not statistically different from the initial level of GnRH release in the absence of Kp10. Final incubation with 60 mM KCl produced a large increase of GnRH release into the culture medium (Fig. 2A), suggesting that the lack of Kp10 stimulation in the fifth and sixth hour of continuous stimulation was not caused by depletion of the pool of GnRH in axon terminals but rather a desensitization of GPR54 signaling. Wild-type MBH fragments cultured in the absence of Kp10 (n = 3; Fig. 2B) did not release GnRH. There was a significant decrease in GnRH release from vehicle-treated wild-type MBH fragments after the first hour, which corresponded to an acclimatization period ($P < 0.01$; Fig. 2B). Continuous Kp10 stimulation for 6 h did not elicit GnRH release from GPR54−/− MBH (n = 5; Fig. 2C). All tissues responded to KCl stimulation at the end of the experiment, with the GPR54−/− MBH explants releasing more GnRH than wild type, although this was not significant (Fig. 2, C and D).

Kp10 still stimulates GnRH release in the presence of TTX

To verify that these responses were independent of action potential propagation from the very low number of GnRH cell bodies still present in the explant, wild-type MBH explants were incubated with or without 1 μM TTX before and during stimulation with 500 nM Kp10. TTX is a potent voltage-gated Na+ channel-blocking agent and, thus, stops action potentials from any Kp10-activated GnRH cell bodies in the MBH fragment that may project to the ME (26). Kp10 significantly induced GnRH release from MBH fragments in both treatment groups compared with samples collected prior to Kp10 stimulation (n = 6 per condition; $P < 0.001$; Fig. 3). Comparison of Kp10-induced GnRH release between TTX-treated or vehicle-treated MBH showed no significant difference ($P = 0.321$, unpaired *t* test). The response to KCl exposure was in the same range as 500 nM Kp10 stimulation (data not shown).

GPR54 expression revealed by β-galactosidase activity within the MBH region of GPR54−/− mice

Expression of *Gpr54* gene can be visualized in *Gpr54* mutant mice by staining for β-galactosidase activity (6, 11). Our observations are based on brain stainings performed in 5

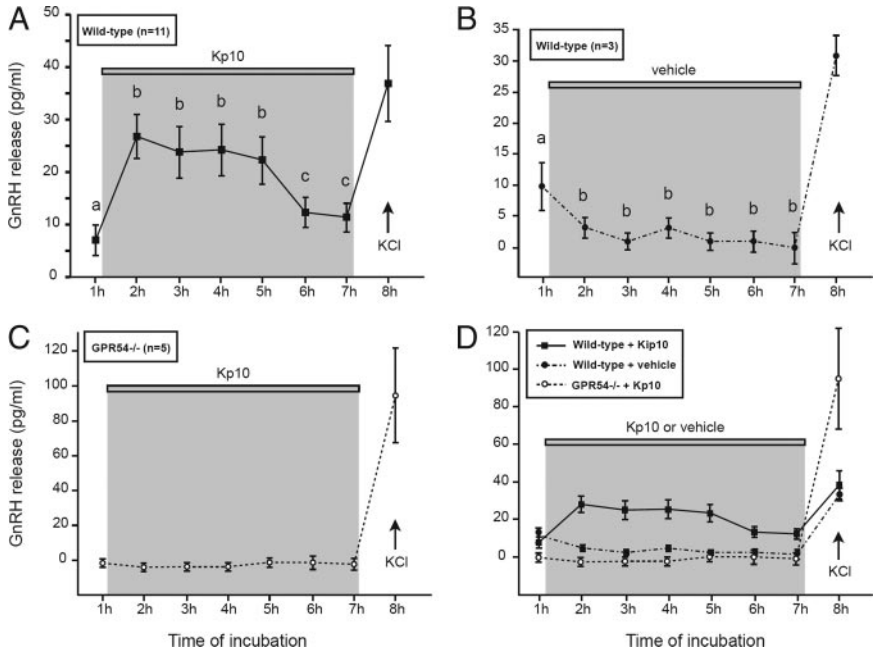

FIG. 2. Effect of continuous Kp stimulation on GnRH release from MBH explants from male mice. After 1-h acclimatization, MBH explants from wild-type mice were challenged with vehicle or 50 nM Kp10, whereas MBH explants from GPR54−/− mice were treated with 50 nM Kp10 only. All tissues were treated with 60 mM KCl at the end of the experiment. Culture medium was collected and replaced every hour with freshly prepared medium containing either Kp10 or vehicle. A, Effect of continuous Kp10 exposure on wild-type MBH explants (n = 11) (a *vs.* b, $P < 0.01$; b *vs.* c, $P < 0.05$; a *vs.* c, not statistically different). B, Effect of vehicle exposure on wild-type MBH explants (n = 3) (a *vs.* b, $P < 0.01$). C, Effect of continuous Kp10 exposure on GR54−/− MBH explants (n = 5). D, Merge of the curves in A–C. Statistics were performed by using one-way ANOVA repeated measures, followed by the Student-Newman-Keuls method.

Gpr54 null and heterozygous mice. A macroscopic observation of the ventral part of the brain after β-galactosidase staining showed blue staining at the level of the MBH (Fig. 4, A and B). After sectioning and counting, one to five scattered blue-stained cells were found within the MBH region corresponding to the explant (data not shown). GnRH nerve terminals would not be expected to stain for β-galactosidase activity because the enzyme cannot migrate down the axon from the cell body region. We observed that the majority of β-galactosidase positive cells were located in the very external layer of the ME. These *GPR54*-expressing cells are distinct and distant from the GnRH-positive nerve terminals (Fig. 4, C and D). We used an anti-MECA-32 antibody that recognizes endothelial cells that do not participate in the blood-brain barrier (24) to show that the *GPR54*-expressing cells do not colocalize with endothelial cells (Fig. 4, E and F). Coupling LH β-immunostaining to β-galactosidase activity staining allowed us to establish that the *GPR54*-expressing cells are localized in the *pars tuberalis*, a subdivision of the adenohypophysis (27) (Fig. 4, G and H).

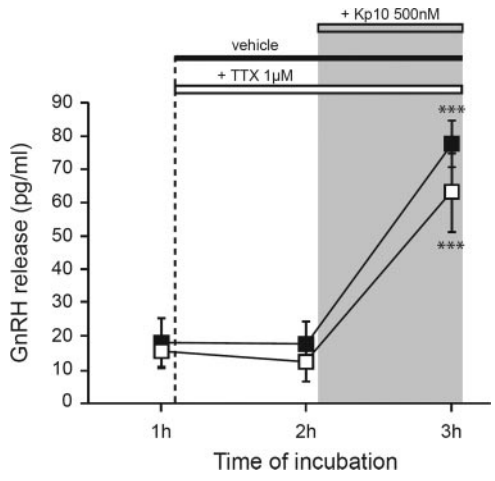

FIG. 3. Effect of TTX on Kp-induced GnRH release from MBH from wild-type male mice. After 1-h acclimatization, MBH explants were exposed for 1 h to either 1 μM TTX or vehicle before challenge with 500 nM Kp10 for 1 h. MBH explants treated with TTX (□), MBH explants treated with vehicle (■). ***, $P < 0.001$ *vs.* acclimatization (1 h) and vehicle or TTX-treated samples (2 h), one-way ANOVA repeated, measures followed by the Student-Newman-Keuls method (n = 6 per condition). Comparison of the two points at 3-h incubation, *i.e.* Kp10 treated samples with or without TTX, showed no statistical difference ($P = 0.321$, unpaired *t* test).

Discussion

Considerable evidence indicates that GnRH neurons that express GPR54 are a direct target for Kp. In the sheep, a single dose of Kp54 into the third ventricle of the brain results in an increase in GnRH secretion into the cerebrospinal fluid (11), and most of the adult mouse GnRH neurons respond electrophysiologically to Kp14 (18). *In vitro* studies using rat hypothalamic explants with the POA region demonstrated that Kp10 stimulates GnRH secretion (9, 28). Although Kp neurons innervate GnRH neuronal cell bodies and proximal dendrites within the rostral region of the POA (19), any action of Kp on GnRH nerve terminals has not been determined. This study shows that Kp10 can stimulate GnRH release from nerve terminals in the absence of GnRH neuronal cell bodies. This was demonstrated using adult mouse MBH explants containing the ME region, which contains the GnRH nerve terminals, but lacking the POA, which contains the GnRH cell bodies. The MBH explants were sectioned after the experiment, and it was verified that they only contained a few GnRH neuronal cell bodies (data not shown). Moreover, GnRH secretion was insensitive to TTX, providing additional proof that this response was independent of GnRH neuronal cell bodies and action potential transmission.

GnRH secretion from the MBH explants was dependent upon GPR54 because no release was found in explants

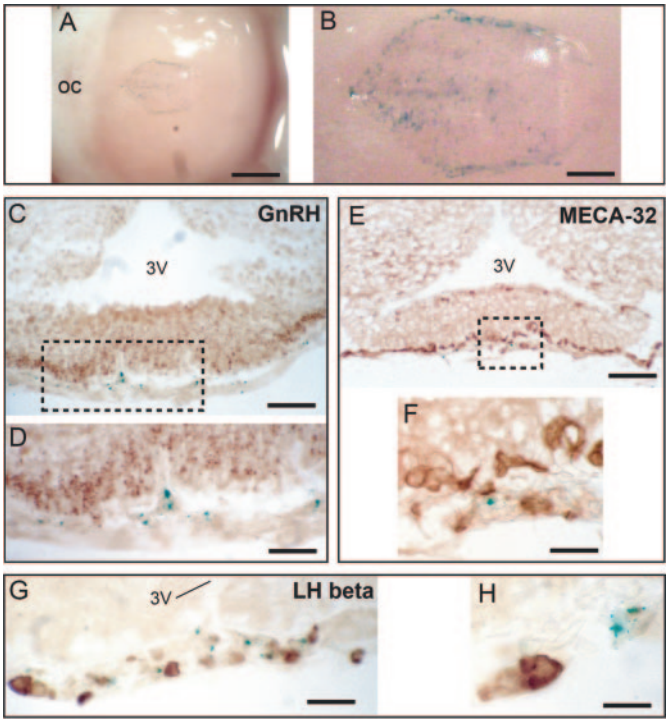

FIG. 4. Gpr54 expression revealed by β-galactosidase activity in the ME region of Gpr54−/− mice. A and B, Ventral view of Gpr54−/− female mouse brain after β-galactosidase activity staining. *Scale bars*, 0.5 mm (*left*), 0.2 mm (*right*); C–H, Fifteen-micrometer thick coronal section performed at the level of the ME from Gpr54−/− female brain. C, GnRH immunostaining showing GnRH nerve terminals (*brown*) within the ME at low-power view. *Scale bar*, 100 μm. D, Magnified image from C (*dashed box*) showing that the GnRH positively stained nerve terminals (*brown*) are distinct from GPR54-expressing cells. *Scale bar*, 50 μm. E, MECA-32 immunostaining showing endothelial cells of the ME (*brown*) at low-power view. *Scale bar*, 100 μm. F, Magnified image from E (*dashed box*) showing GPR54 expression (*blue*) outside of the endothelial cell layer (*brown*). *Scale bar*, 50 μm. G, LH β immunostaining (*brown*) showing gonadotropes and GPR54-expressing cells (*blue*) in the same area. Note that gonadotropes do not coexpress GPR54. *Scale bar*, 30 μm. H, High-powered image showing a gonadotrope and a GPR54-expressing cell in the *pars tuberalis*. *Scale bar*, 15 μm. oc, Optic chiasm; 3V, third ventricle.

from *Gpr54* null mice. This indicates that *Gpr54* must be expressed in the region around the GnRH nerve terminals. It is known that GnRH neurons express *Gpr54*, but whether the receptor is localized to the nerve terminals has not yet been determined because there are currently no suitable antibodies for this study. *Gpr54* expression is tagged with a *LacZ* transgene in our mice (6), but the β-galactosidase protein does not provide information about the subcellular distribution of GPR54. It is likely that Kp acts directly on GnRH nerve terminals via GPR54, but we have also found a minor population of cells within the *pars tuberalis* that expresses *Gpr54*, and a role for these cells in the responses we observe cannot be discounted. Although it has been shown that *Gpr54* mRNA expression occurs within the anterior pituitary (22), *Gpr54* expression within the *pars tuberalis* has not been previously reported. The *pars tuberalis* is composed of different cellular types, including some gonadotropes, but most of the cells are "*pars tuberalis*-specific" and contain melatonin receptors (29).

The minimum Kp10 concentration that elicited significant GnRH release in our experiments (50 nM) is higher than the concentrations normally used *in vivo* (9, 11, 28) but is consistent with other *in vitro* studies in which a minimum of 10 nM Kp10 was required to trigger GnRH secretion from rat whole hypothalamic explants (9, 28). Tissue explants probably require higher concentrations of Kp than *in vivo* due to the more limited delivery of peptide to the middle of the explant. However, the failure of the highest concentration of Kp10 (500 nM) to stimulate GnRH secretion from MBH explants from *Gpr54* null mice demonstrates that the effects observed are not due to nonspecific secretion.

Our data also support the suggestion of Plant and colleagues (13, 30) that desensitization of the responses to continuous Kp exposure mainly occurs at the level of the hypothalamus. Continuous Kp infusion in male rhesus monkeys abolished LH secretion after an initial acute stimulatory effect (13, 30). The ability to stimulate LH release by GnRH during this desensitization period suggested that the desensitization was via a direct action on GPR54 rather than an effect on the pituitary (13). Our results are consistent with this because we show a desensitization of GnRH release directly on hypothalamic tissue after 5-h continuous Kp10 stimulation. This was not simply due to GnRH depletion because potassium depolarization after the desensitization period still induced GnRH release. It is known that GnRH inhibits its own release in cultured hypothalamic explants (31), but we eliminated this effect in our experiments by changing the medium every hour. Our desensitization data are also consistent with those of Han *et al.* (18), who found sustained electrophysiological activation of GnRH neurons in brain slices after Kp treatment for periods up to 60 min.

GnRH is released in a pulsatile manner in rodents (2), and continuous exposure of the pituitary to GnRH leads to desensitization of the GnRH receptor (32). GPR54 desensitization after continuous Kp10 stimulation of MBH explants suggests that pulsatile release of Kp will also be required to keep the GPR54 pathway active at the ME. It has been shown that peripheral infusions of Kp10 every hour in the rhesus monkey result in a robust and pulsatile LH response, without decrease throughout the 48-h treatment regime (33). Kp could be an important factor that modulates pulsatile GnRH release from the ME, leading to pulsatile LH secretion. It is perhaps significant that pulsatile GnRH release from the hypothalamus does not require GnRH neuronal cell bodies (34), consistent with a direct action at the ME.

Whether Kp is endogenously produced within the ME remains to be established. Two brain regions containing Kp neurons are suggested to regulate GnRH secretion: the anteroventral periventricular nucleus (AVPV) and the ARH. A large neuronal population in the ARH (35) and a smaller population in the AVPV (36) send axons to the ME. Kp neurons from the AVPV project toward the rostral POA and make synaptic apposition with GnRH neurons in the mouse (19), and are proposed to directly activate GnRH neuron activity. Kp-positive fibers have been found in the external zone of the ovine ME (37). Investigation of the Kp

neuronal connections to the ME by viral tracing experiments should help to understand better the function of Kp at the GnRH nerve terminals.

It is clear that peripheral injections of Kp lead to a rapid increase in LH release (7, 12, 17), suggesting that one site of action of Kp stimulation of GnRH release could be circumventricular organs, such as the ME, with weak or no blood-brain barrier. However, peripheral Kp also induced c-*fos* immunoreactivity in GnRH cell bodies in the rat (17), suggesting that peripheral Kp does not only act at the nerve terminals but can also act directly on the GnRH cell bodies. The relative contribution of Kp in GnRH release at GnRH nerve terminals *vs*. GnRH cell bodies will require further investigation.

In summary, we have demonstrated that Kp stimulates GnRH release at nerve terminals, in a GPR54-dependant manner. GPR54 is desensitized after continuous stimulation in the adult male mouse. Furthermore, we have shown that *Gpr54* is only expressed in a few cells belonging to the *pars tuberalis*, an adenohypophysis structure outside of the brain. These observations indicate that Kp can also regulate GnRH release via GPR54 stimulation within the ME.

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