Neuroendocrinology

Research Article

Neuroendocrinology 2021;111:1176–1186
DOI: 10.1159/000513248

Received: June 11, 2020
Accepted: November 9, 2020
Published online: November 23, 2020

Kisspeptin-54 Accurately Identifies Hypothalamic Gonadotropin-Releasing Hormone Neuronal Dysfunction in Men with Congenital Hypogonadotropic Hypogonadism

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Keywords
Kisspeptin · Congenital hypogonadotropic hypogonadism · Gonadotropin-releasing hormone · Kallmann

Abstract
Background: Hypogonadotropic hypogonadism (HH) is hypogonadism due to either hypothalamic or pituitary dysfunction. While gonadotropin-releasing hormone (GnRH) can directly test pituitary function, no specific test of hypothalamic function exists. Kisspeptin-54 (KP54) is a neuropeptide that directly stimulates hypothalamic GnRH release and thus could be used to specifically interrogate hypothalamic function. Congenital HH (CHH) is typically due to variants in genes that control hypothalamic GnRH neuronal migration or function. Thus, we investigated whether KP54 could accurately identify hypothalamic dysfunction in men with CHH. ***Methods:*** Men with CHH (n = 21) and healthy eugonadal men (n = 21) received an intravenous bolus of either GnRH (100 μg) or KP54 (6.4 nmol/kg), on 2 occasions, and were monitored for 6 h after administration of each neuropeptide. ***Results:*** Maximal luteinizing hormone (LH) rise after KP54 was significantly greater in healthy men (12.5 iU/L) than in men with CHH (0.4 iU/L; $p < 0.0001$). KP54 more accurately differentiated CHH men from healthy men than GnRH (area under receiver operating characteristic curve KP54: 1.0, 95% CI 1.0–1.0; GnRH: 0.88, 95% CI 0.76–0.99). Indeed, all CHH men had an LH rise <2.0 iU/L following KP54, whereas all healthy men had an LH rise >4.0 iU/L. Anosmic men with CHH (i.e., Kallmann syndrome) had even lower LH rises after KP54 than did normosmic men with CHH (p = 0.017). Likewise, men identified to have pathogenic/likely pathogenic variants in CHH genes had even lower LH rises after KP54 than other men with CHH (p = 0.035). ***Conclusion:*** KP54 fully discriminated men with CHH from healthy men. Thus, KP54 could be used to specifically interrogate hypothalamic GnRH neuronal function in patients with CHH.

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Introduction

Hypogonadotropic hypogonadism (HH) is characterized by hypogonadism in the context of low or inappropriately normal gonadotropin levels, due to either pituitary or hypothalamic dysfunction. When assessing patients with HH, a specific test of pituitary gonadotropin function can be conducted, that is, a gonadotropin-releasing hormone (GnRH) test [1]. However, a direct test of hypothalamic GnRH neuronal function, which would be expected to have greater diagnostic specificity in patients with GnRH neuronal dysfunction, is yet to be clinically available [2]. Being able to specifically interrogate hypothalamic GnRH neuronal function would offer the unique ability to delineate the precise defect in patients with HH and facilitate greater precision in their diagnosis.

In 2003, 2 seminal reports described congenital HH (CHH), with a failure to proceed through puberty, due to mutations affecting the kisspeptin (KP) receptor (previously known as GPR54) [3, 4]. Subsequently, a wealth of animal data has confirmed that the neuropeptide KP is a specific stimulator of hypothalamic GnRH secretion [5]. Consistent with this, human studies have demonstrated that KP administration robustly stimulates gonadotropin secretion in healthy men and women [6–9].

The ability of KP to directly stimulate hypothalamic GnRH release avails the opportunity to specifically interrogate hypothalamic GnRH neuronal function when assessing patients with HH. A subset of patients with HH are those with CHH. Typically, such patients have variants in genes that encode for hypothalamic GnRH neuronal migration or GnRH secretion/function. Hence, one may expect that patients with CHH would not have a gonadotropin response to KP administration, especially if neurons are geographically dislocated from their biological site of action [2, 10]. In half of CHH cases, these variants are also associated with anosmia, which is termed "Kallmann syndrome" [2]. As pituitary function is typically preserved in patients with CHH, a GnRH test of pituitary function is of "poor diagnostic value" [2]. Thus, patients with CHH represent a suitable model to investigate the potential of KP to determine hypothalamic GnRH neuronal function in a cohort of patients with a known specific abnormality in GnRH neuronal functionality. Most previous studies evaluating the response to KP in CHH have used bolus administration of KP10 [11, 12]; however, the use of KP54 could be preferable based on its more stable pharmacokinetic properties ($t_{1/2}$ 28 vs. 3 min) and greater potency on luteinizing hormone (LH) secretion [13]. Furthermore, KP54 is reported to cross the blood-brain barrier, whereas KP10 does not [13]. Thus, it is possible that KP54 can act at GnRH neuronal cell bodies, rather than only at GnRH neuronal terminals, and could thus provide novel insights into the response to KP in patients with CHH. However, the response to KP54 in men with CHH has not previously been studied, and the specific threshold for LH response to differentiate healthy men from men with CHH is not known. As such, in this study, we investigated the endocrine response to KP54 in men with CHH and in healthy eugonadal men.

Materials and Method

Ethical Approval

Ethical approval for this study was granted by the West London Research Ethics Committee, London, UK (reference: 12/LO/0507), and all participants provided written informed consent. The study was conducted in accordance with the Declaration of Helsinki.

Participants

Healthy eugonadal men (n = 21) and men with CHH (n = 21) were recruited through newspaper advertisements, endocrine clinics, or the CHH online community. All participants underwent a detailed medical assessment including full medical history and clinical physical examination. Eugonadal healthy men fulfilled the following criteria: age 18–35 years, BMI 18–30 kg/m^2, absence of significant systemic disease or comorbidity, absence of recreational or therapeutic drug use, and normal clinical and biochemical reproductive function. Men with CHH were defined as having HH with a history of incomplete progression through puberty by the age of 18 years. Further examination in CHH men included measurement of testicular volume using a Prader orchidometer, evaluation for signs of hypopituitarism, and nonreproductive clinical features, for example, cleft palate or synkinesis. Olfactory function in CHH men was quantified subjectively and objectively using the 40-item University of Pennsylvania Smell Identification Test (UPSIT). All participants had measurement of basal serum levels of LH, follicle-stimulating hormone (FSH), testosterone, inhibin B (INB), anti-Müllerian hormone (AMH), prolactin, and sex hormone-binding globulin. CHH men also had genetic testing to identify genes implicated in the etiology of CHH (detailed below).

Study Protocol

Recruited participants attended the Clinical Research Unit at Imperial College Healthcare NHS Trust for 2 study-visits (KP54 administered on 1 study visit and GnRH at the other study-visit, in random order). Study visits were commenced at 9 am, and participants were asked to refrain from strenuous exercise and sexual activity and to abstain from alcohol, caffeine, and tobacco for 24 h prior to each study visit. On arrival, an intravenous cannula was inserted into the antecubital fossa. After a 30-min period of baseline blood sampling, a single intravenous bolus of either KP54 (6.4 nmol/kg) or GnRH (100 μg) was administered at each visit in random order. Serial blood-sampling was conducted every 15 min for 6 h. A summary of the study protocol is presented in Figure 1a. The

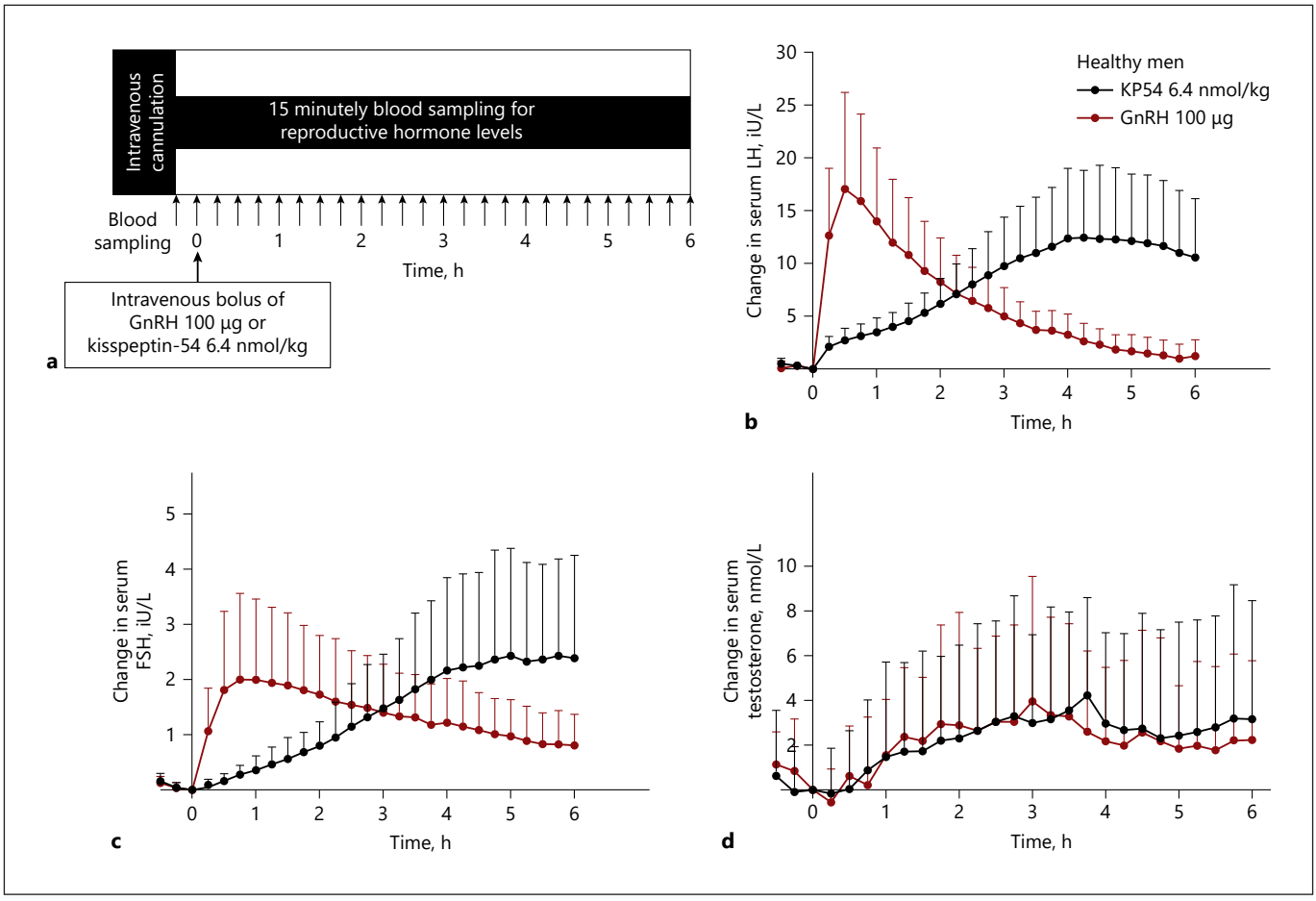

Fig. 1. a Protocol diagram for study visits for healthy men ($n = 21$) and men with CHH ($n = 21$). Reproductive hormone levels were taken every 15 min for 6 h after an intravenous bolus of KP54 6.4 nmol/kg, or GnRH 100 µg, at time 0 h, on 2 separate occasions separated by at least 1-week washout period. Reproductive hormone levels taken include LH, FSH, and testosterone levels. Mean ± SD of change from baseline levels of serum LH (iU/L) (**b**), FSH (iU/L) (**c**), and testosterone (nmol/L) (**d**) in healthy men ($n = 21$) over 6 h following a single intravenous bolus of KP54 at time 0 at a dose of 6.4 nmol/kg (black) and GnRH 100 µg (maroon). Groups were compared by two-way ANOVA. Changes in serum LH ($p = 0.054$), FSH ($p = 0.69$), and testosterone ($p = 0.92$) did not significantly differ between the 2 peptides. GnRH, gonadotropin-releasing hormone; KP54, kisspeptin-54; CHH, congenital hypogonadotropic hypogonadism; LH, luteinizing hormone; FSH, follicle-stimulating hormone; SD, standard deviation.

second study-visit was conducted following a washout period of at least 1 week.

The dose of KP54 used was selected based on doses of KP54 that had been shown to increase gonadotropin levels in previous studies [14–16] and confirmed in a preliminary dose-finding study in 5 healthy men who received a single intravenous bolus of 6.4, 12.8, and 25.6 nmol/kg on subsequent visits at least 1 week apart (see online suppl. Fig. 1a; for all online suppl. material, see www.karger.com/doi/10.1159/000513248). All 3 doses elicited robust rises in serum LH with no significant effect of dose ($p = 0.57$ by two-way ANOVA). Thus, we chose 6.4 nmol/kg to represent the lowest dose that induces a near-maximal response to KP54. Testosterone levels have been shown to not influence the response to KP in men [17]; however, to avoid suppressive levels, CHH men on testosterone gel were asked to discontinue it for at least 1 week prior to participation in the study. To fully wash out longer acting intramuscular preparations of testosterone undecanoate would have left patients hypogonadal for many months, and therefore, study-visits were conducted when patient's testosterone levels were at trough levels prior to the next injection to minimize disruption to their treatment. Men with CHH did not receive gonadotropin therapy for at least 6 weeks prior to participation in the study.

Peptides

Human KP54 was synthesized by Bachem AG (Liverpool, UK) and further purified and tested as previously described consistent with the standards for physiological research studies [9]. Vials of freeze-dried KP54 were stored at −20°C and then reconstituted with 0.9% saline. Gonadorelin 100 µg (GnRH) was purchased from Intrapharm Laboratories Ltd., (Maidenhead, Berks, UK) and was reconstituted with 1 mL of sterile water for injection prior to administration.

Table 1. Clinical characteristics at first study visit in men with CHH and healthy men

Clinical characteristic	Men with CHH (n = 21)	Healthy men (n = 21)	p value
Age, years	39.1±14.4	23.9±4.6	<0.0001
Weight, kg	78.7 (65.8, 92.9)	71.9 (63.8, 79.4)	0.19
BMI, kg/m^2	26.1±5.3	22.9±2.2	0.016
Mean testicular volume, mL	6.0±3.7	na	na
Serum LH, iU/L	0.46±0.6	2.9±1.0	<0.0001
Serum FSH, iU/L	0.4 (0.1, 1.2)	1.9 (1.6, 3.0)	<0.0001
Serum AMH, pmol/L	26.8 (12.9, 106.6)	46.6 (32.7, 79.1)	0.20
Serum INB, ng/L	35 (8, 79)	136 (111, 176)	<0.0001

Mean ± SD is presented for parametrically distributed values (age, BMI, testicular volume, LH, and AMH) and median (25th centile, 75th centile) is presented for nonparametrically distributed data (weight, FSH, INB). Parametrically distributed data were compared by the unpaired *t* test and nonparametrically distributed data by the Mann-Whitney U test. Hormonal values presented were measured at the baseline of the first study-visit. Reference ranges in men are for serum LH 2.0–12.0 iU/L, FSH 1.7–8.0 iU/L, AMH 10.2–82.8 pmol/L, and INB 25–325 ng/L. CHH, congenital hypogonadotropic hypogonadism; LH, luteinizing hormone; FSH, follicle-stimulating hormone; INB, inhibin B; AMH, anti-Müllerian hormone; SD, standard deviation.

Hormone Assays

Samples were collected in plain serum vacutainer tubes and were allowed to clot for 1 h prior to centrifugation at 1,210 *g* for 10 min. Serum was then separated and frozen at −20°C until analysis. Frozen samples were defrosted and analyzed for measurement of serum LH, FSH, and testosterone at all time points using automated chemiluminescent immunoassays (Abbott Diagnostics, Maidenhead, UK). The reference ranges in healthy men were as follows: LH 2.0–12.0 iU/L, FSH 1.7–8.0 iU/L, total testosterone 10.0–30.0 nmol/L, AMH 10.2–82.8 pmol/L, and INB 25–325 ng/L; respective intra-assay and inter-assay coefficients of variation: LH 2.7 and 4.1%, FSH 3.0 and 4.1%, total testosterone 2.8 and 4.2%, AMH 1.8 and 4.4%, and INB 6.6 and 5.6%. Analytical sensitivities were LH 0.03 iU/L, FSH 0.05 iU/L, total testosterone 0.05 nmol/L, AMH 0.07 pmol/L, and INB 2.91 pg/mL.

Statistical Methods

Statistical analyses were conducted using GraphPad Prism version 8.0. Parametrically distributed continuous variables were reported as mean ± standard deviation (SD) and compared using unpaired Student's *t* test (2 groups) or one-way ANOVA (multiple groups). Nonparametric variables were reported as median (interquartile range) and compared using the Mann-Whitney U test or Kruskal-Wallis test, as appropriate. Changes in gonadotropin levels over time were analyzed by two-way ANOVA.

Genetic Testing

Exome sequencing in CHH patients was performed by Health 2030 Genomic Center in Geneva and analyzed using previously described methods [18]. Forty-five CHH genes were included in this study: *ANOS1, AMH, AMHR2, CCDC141, CHD7, DCC, DMXL2, FEZF1, FGF17, FGF8, FGFR1, FSHB, GNRH1, GNRHR, HS6ST1, IL17RD, KISS1, KISS1R, KLB, LEP, LEPR, LHB, NDNF, NR0B1, NSMF, NTN1, OUTD4, PCSK1, PLXNA1, PNPLA6, POLR3A, POLR3B, PROK2, PROKR2, RNF216, SEMA3A, SEMA3E, SMCHD1, SOX10, SOX2, STUB1, TAC3, TACR3, TUBB3,* and *WDR11*. Nonsynonymous rare sequencing variants and splicing variants (+/−2bp) with minor allele frequency <1% in from the Genome Aggregation Database (http://gnomAD.broadinstitute.org/) were selected for further analysis. Variants were interpreted using the American College of Medical Genetics and Genomics (ACMG) criteria [19], and only variants predicted to be "pathogenic," "likely pathogenic," or "of uncertain significance" are reported in this article.

Results

Baseline Characteristics

The clinical characteristics of healthy men and men with CHH are presented in Table 1. CHH men were older, had higher BMI, but lower serum LH, FSH, and INB than healthy men. The individual clinical characteristics of each of the 21 men with CHH are presented in Table 2.

Gonadotropin Rises after KP54 and GnRH in Healthy Men

The mean maximal rise in LH in healthy men occurred at 30 min following GnRH (18.2 iU/L), but later at an average time of 4.4 h after KP54 (13.2 iU/L) (Fig. 1b). The median time point of maximal LH response was 4.25 h (interquartile range 4.0–4.63 h); a single LH measure between 4 and 4.5 h after KP54 will provide an estimate within 0.9 iU/L of the maximal LH at any time point. FSH rises are presented in Fig. 1c and testosterone levels in Fig. 1d.

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Table 2. Clinical characteristics and genetic analysis of men with congenital hypogonadotropic hypogonadism

Patient study ID	Age at screening, years	Age at diagnosis, years	Sense of smell (UPSIT-40)	TV at screening, mL	Mean TV, mL	Serum LH, iU/L	Serum FSH, iU/L	Serum INB, pg/mL	Serum AMH, pmol/L	Duration of gonadotropin therapy, years	Time since stopping gonadotropins, years	Current treatment	Genetic mutation	ACMG criteria
1	43	27	N	R-5, L-4	4.5	0.97	1.3	52.0	13.6	5	1	TU	*PLXNA1* p.D429E	VUS
2	44	37	M	R-12, L-15	13.5	<0.03	0.1	106.0	44.6	2	3	TU	*KLB* p.G34E	VUS
3	58	17	M	R-1, L-1	1.0	0.04	0.1	5.0	12.8	Nil	na	Nil	–	Nil
4	60	16	N	R-4, L-4	4.0	1.44	2.0	83.0	40.5	1	19	TU	–	Nil
5	50	16	A	R-2, L-5	3.5	0.03	0.1	26.0	46.2	1	0.5	hCG	*SEMA3A* p.R734Q	VUS
6	53	16	A	R-4, L-0	2.0	<0.03	0.1	5.0	10.4	1	35	TU	–	Nil
7	48	16	N	R-12, L-12	4.0	0.22	0.8	35.0	16.6	1	1	TU	–	Nil
8	28	18	N	R-4, L-5	4.5	0.91	1.1	157.0	754.0	Nil	na	TU	*CHD7* p.S1604T	VUS
9	65	23	A	R-8, L-5	6.5	1.01	1.9	68.0	12.8	2	34	Nil	–	Nil
10	23	17	N	R-4, L-4	4.0	0.36	0.8	5.0	47.0	Nil	na	TG	*WDR11* p.A768V	VUS
11	19	16	A	R-3, L-4	3.5	0.04	0.1	11.0	70.2	Nil	na	TU	*ANOS1* deletion of first 9 exons	P
12	17	16	N	R-7, L-7	7.0	1.42	1.1	79.0	396.3	Nil	na	TU	–	Nil
13	27	17	A	R-8, L-6	7.0	0.05	0.1	146.0	280.2	0.5	9	TU	–	Nil
14	31	17	N	R-12, L-10	11.0	1.47	3.0	38.0	26.8	2	1	TU	–	Nil
15	26	16	N	R-5, L-3	4.0	0.05	2.5	136.0	12.6	Nil	na	TU	*FGFR1* p.N724K, *PLXNA1* p.A14797T	LP VUS
16	31	18	N	R-6, L-10	8.0	0.04	0.4	5.0	24.2	Nil	na	SU	–	Nil
17	31	16	A	R-2, L-3	2.5	0.04	0.1	136.0	12.6	Nil	na	TU	*PROKR2* p.H20Lfs*24	P
18	46	18	N	R-8, L-8	8.0	1.44	0.3	58.0	22.1	Nil	na	TU		NT
19	43	19	N	R-15. L-8	11.5	0.10	2.5	29.0	556	Nil	na	SU	–	Nil
20	37	16	A	R-12, L-15	13.5	0.13	0.5	23.0	12.9	Nil	na	TU		NT
21	39	16	A	R-3, L-5	4.0	<0.03	0.6	5.0	4.9	1	6	TU	*SEMA3A* p.R531*	LP

Serum LH, FSH, INB, and AMH presented were measured at baseline of the first study visit. One patient had orchidectomy of the left testis, which is recorded as 0 mL in the table. UPSIT-40, 40-item University of Pennsylvania Smelling Identification Test (N, normosmia; M, microsmia; A, anosmia); TV, testicular volume at screening; LH, luteinizing hormone; FSH, follicle-stimulating hormone; INB, inhibin B; AMH, anti-Müllerian hormone; TU, testosterone undecanoate (Nebido); hCG, human chorionic gonadotropin; SU, sustanon; TG, testosterone gel; American College of Medical Genetics (ACMG) criteria for interpretation of sequencing variants (VUS, variants of uncertain significance; Nil, no mutation identified; P, pathogenic variant; LP, likely pathogenic variant; NT, not tested); na, not applicable.

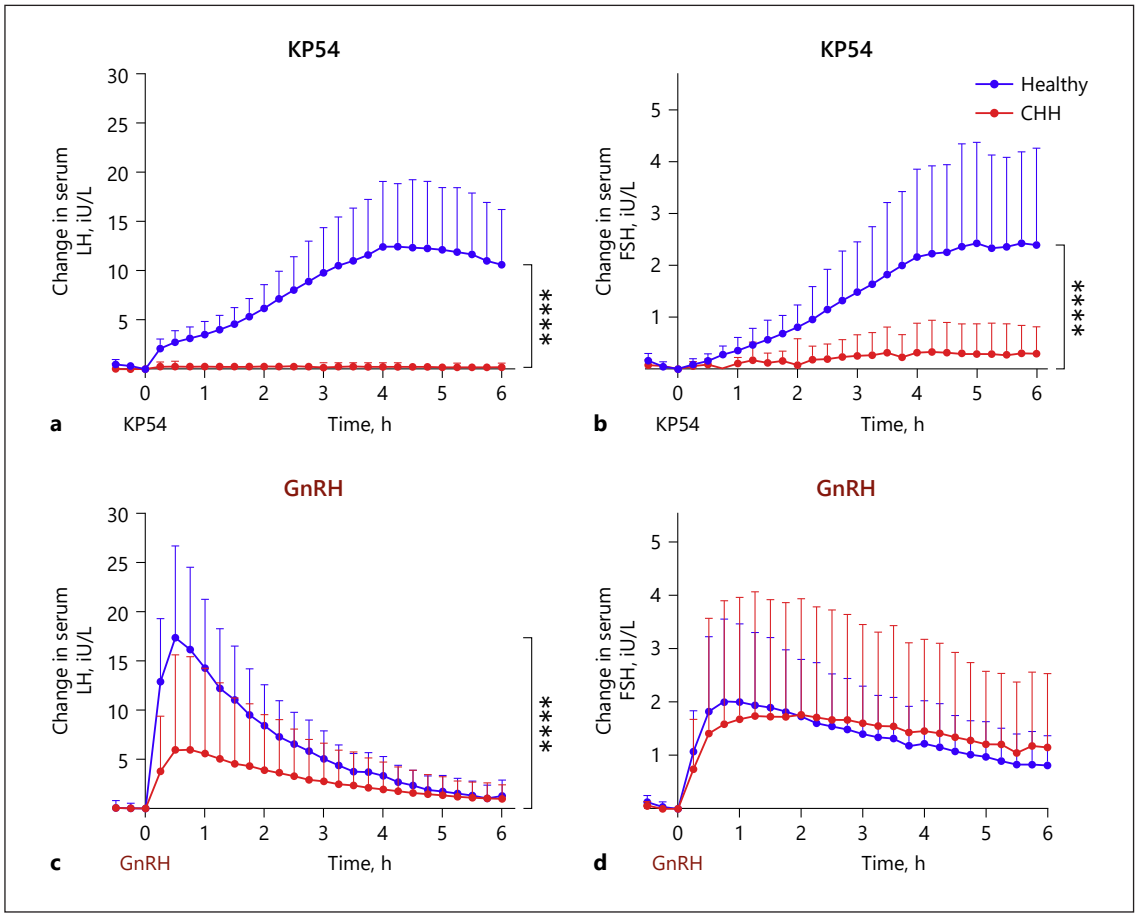

Fig. 2. Mean ± SD of change from baseline of serum LH (iU/L) (**a**) and FSH (iU/L) (**b**) in healthy men ($n = 21$) in blue and CHH men ($n = 21$) in red after 6.4 nmol/kg of KP54 over 6 h. Groups were compared by two-way ANOVA (****p value <0.0001). Mean ± SD of change from baseline of serum LH (iU/L) (**c**) and FSH (iU/L) (**d**) in healthy men ($n = 21$) in blue and CHH men ($n = 21$) in red after GnRH (100 μg) over 6 h. Groups were compared by two-way ANOVA (****p value <0.0001). GnRH, gonadotropin-releasing hormone; KP54, kisspeptin-54; CHH, congenital hypogonadotropic hypogonadism; LH, luteinizing hormone; FSH, follicle-stimulating hormone; SD, standard deviation.

Gonadotropin Rises after KP54 and GnRH in Healthy Men versus Men with CHH

LH levels remained almost unchanged following KP54 in men with CHH in comparison to healthy men ($p < 0.0001$) (Fig. 2a), whereas LH rises after GnRH were ~3-fold lower in CHH men than in healthy men ($p < 0.0001$) (Fig. 2c). Likewise, FSH rises were also markedly attenuated in CHH men after KP54 ($p < 0.0001$) (Fig. 2b), whereas FSH rises after GnRH did not significantly differ between healthy men and CHH men ($p = 0.43$) (Fig. 2d).

Ability of KP54 and GnRH to Distinguish Healthy Men from Men with CHH

The median maximal LH rise following KP54 administration was 12.5 iU/L in healthy men and 0.4 iU/L in men with CHH ($p < 0.0001$) (Fig. 3a). The lowest LH rise in healthy men was 4.1 iU/L, whereas the greatest LH rise in CHH men was 2.0 iU/L. Therefore, the LH rise following KP54 could accurately differentiate all healthy men from those with CHH (area under receiver operating characteristic curve 1.0, 95% CI 1.0–1.0) (Fig. 3b). The median maximal LH rise after GnRH administration was 18.2 iU/L for healthy men and 2.0 iU/L for CHH men ($p < 0.0001$) (Fig. 3d). However, there was overlap between the groups, with maximal LH rises as high as 40 iU/L in both CHH men and in healthy men after GnRH. Therefore, a GnRH test less accurately differentiated healthy men from those with CHH (area under ROC curve 0.88, 95% CI 0.76–0.99) (Fig. 3e). The median maximal FSH rise after KP54 administration was also signifi-

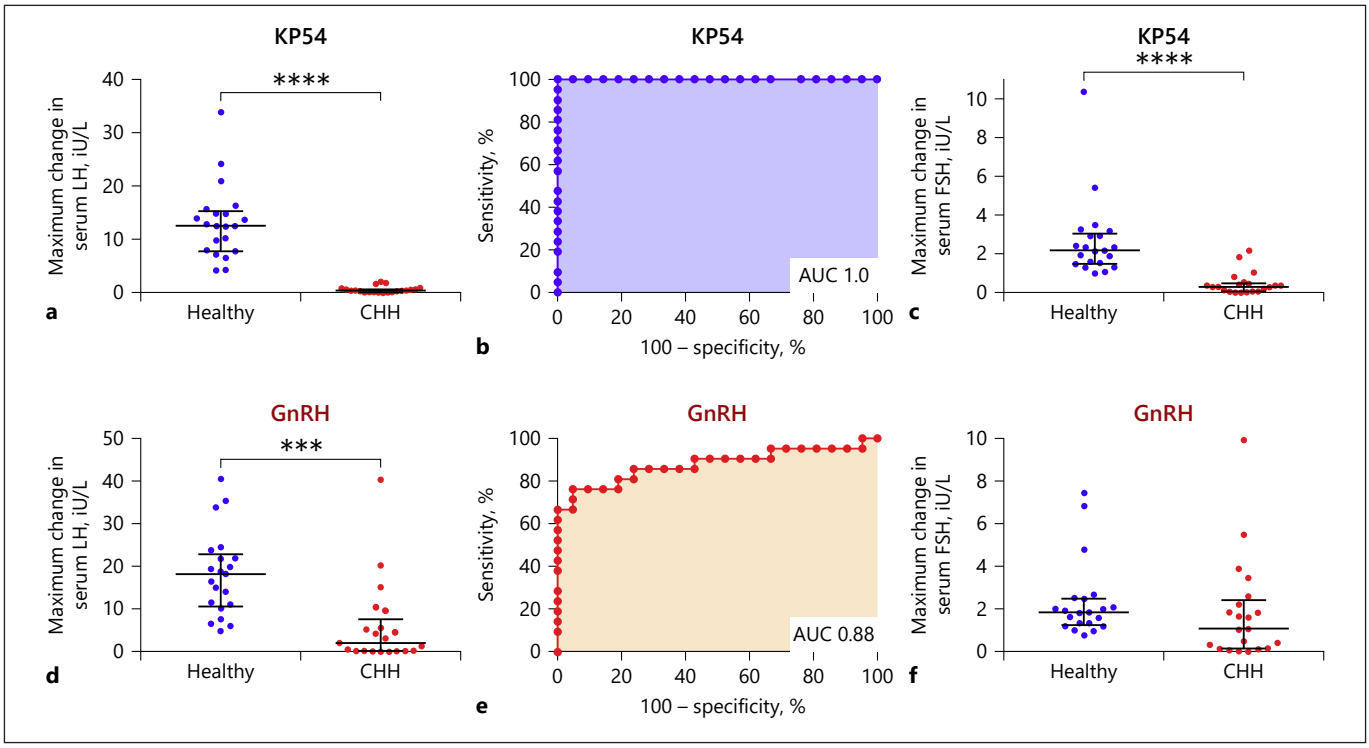

Fig. 3. Scattergram (median ± IQR) of maximum change from baseline in serum LH (iU/L) (**a**) and in FSH (iU/L) (**c**) in healthy men in blue and CHH men in red following an intravenous bolus of KP54 (6.4 nmol/kg). Groups were compared by the Mann-Whitney U test (****p value <0.0001). **b** AuROC curve for maximum change in serum LH (iU/L) to differentiate diagnosis of CHH from healthy men following an intravenous bolus of KP54. Scattergram (median ± IQR) of maximum change from baseline in serum LH (iU/L) (**d**) and in FSH (iU/L) (**f**) in healthy men in blue and CHH men in red following an intravenous bolus of GnRH (100 µg). Groups were compared by the Mann-Whitney U test (***p value <0.001). **e** AuROC curve for maximum change in serum LH (iU/L) to differentiate diagnosis of CHH from healthy men following an intravenous bolus of GnRH. GnRH, gonadotropin-releasing hormone; KP54, kisspeptin-54; CHH, congenital hypogonadotropic hypogonadism; LH, luteinizing hormone; AuROC, area under receiver operating characteristic; FSH, follicle-stimulating hormone; IQR, interquartile range.

cantly lower in CHH men (0.3 iU/L) than in healthy men (2.2 iU/L) ($p < 0.0001$) (Fig. 3c). However, the FSH rise after GnRH administration did not significantly differ between CHH men and healthy men ($p = 0.079$) (Fig. 3f).

Determinants of the Gonadotropin Rise after KP54 and GnRH Administration

The LH rise following KP54 correlated with the LH rise following GnRH in healthy men ($r^2 = 0.48$, $p = 0.0005$) (Fig. 4a). By comparison, the LH rise after KP54 was attenuated in men with CHH, even if the LH response to GnRH was preserved (Fig. 4a). Indeed, the ratio of LH rise after GnRH to that after KP54 was increased in CHH men (10.8) when compared to that in healthy men (1.5; $p = 0.0005$). The LH level at baseline predicted the subsequent maximal LH rise after KP54 in healthy men; however, in men with CHH, the rise in LH after KP54 was attenuated even when accounting for pretreatment LH levels (Fig. 4b). Baseline testosterone levels did not affect the maximum LH rise following KP54 ($p > 0.86$) (online suppl. Fig. 1b).

Genetic Analysis in Men with CHH

Among the CHH patients, we identified a partial deletion in *ANOS1*, 2 protein truncating variants in *PROKR2* (p.H20Lfs*24) and in *SEMA3A* (p.R531*), and a missense mutation in *FGFR1* (p.N724K), which was previously demonstrated to impair *FGFR1* signaling in vitro [20]. All these 4 variants were classified as pathogenic or likely pathogenic by the ACMG criteria, and thus are considered as loss-of-function mutations (Table 2). Variants of uncertain significance were identified in 5 men, while no genetic defects in known CHH genes were found in ten men (Table 2). The 4 men with pathogenic/likely pathogenic variants identified had even lower LH rises after KP54 administration than other CHH men without an identifiable

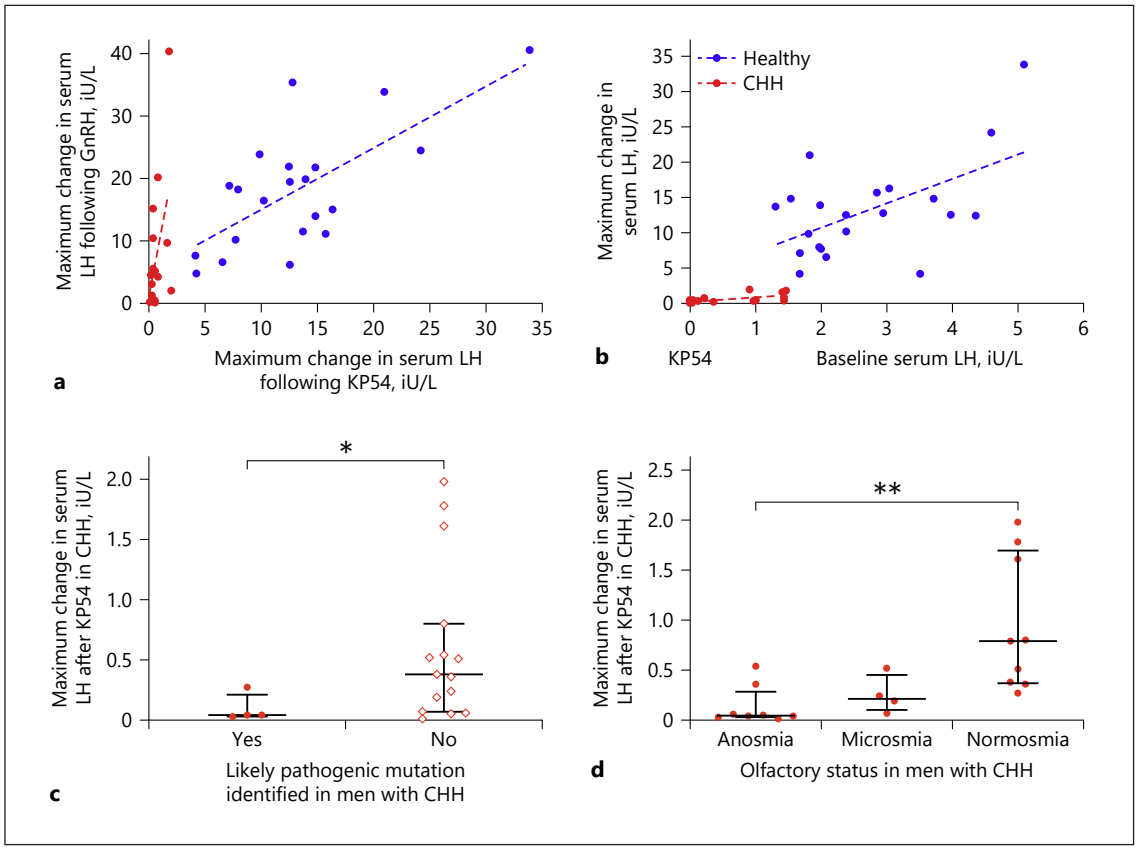

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Fig. 4. **a** Simple linear regression between the maximum change from baseline in serum LH (iU/L) following an intravenous bolus of 100 μg of GnRH, or of KP54 6.4 nmol/kg, in healthy men (n = 21) in blue (p = 0.0005, r^2 = 0.49) and CHH men (n = 21) in red (p = 0.007, r^2 = 0.33). **b** Simple linear regression between maximum change from baseline in serum LH (iU/L) following an intravenous bolus of KP54 and baseline serum LH just prior to KP54 (iU/L) in healthy men (n = 21) in blue (p = 0.009, r^2 = 0.31) and CHH men (n = 21) in red (p = 0.0007, r^2 = 0.46). **c** Scattergram median (±IQR) change in serum LH after KP54 in CHH men grouped by those with pathogenic/likely pathogenic mutations (n = 4) in closed circles, and those with VUS or no abnormality detected on genetic testing (open circles) (*p < 0.05). **d** Scattergram (median ± IQR) of maximum change from baseline of serum LH (iU/L) in CHH men (n = 21) with anosmia, microsmia, and normosmia determined by the 40-item UPSIT. Groups were compared by the Kruskal-Wallis test with post hoc Dunn's multiple comparison test (**p value <0.01). GnRH, gonadotropin-releasing hormone; KP54, kisspeptin-54; CHH, congenital hypogonadotropic hypogonadism; LH, luteinizing hormone; UPSIT, University of Pennsylvania Smell Identification Test; IQR, interquartile range; VUS, variants of uncertain significance.

genetic cause (Fig. 4c; p = 0.035). Notably, the maximal LH rise after KP54 administration in men with CHH also differed by olfactory status (normosmia 0.79 iU/L, microsmia 0.22 iU/L, and anosmia 0.05 iU/L) (Fig. 4d). Anosmic men with CHH (Kallmann syndrome) had significantly lower LH rises than normosmic men with CHH (p = 0.003).

Discussion

We report data evaluating the use of an intravenous bolus of KP54 to specifically interrogate hypothalamic GnRH neuronal function in the assessment of a large cohort of men with CHH. All men with CHH, irrespective of their underlying genetic cause, had an attenuated response to KP54 consistent with impaired hypothalamic GnRH neuronal function, when compared to healthy men. This was the case even if pituitary responsiveness to GnRH was preserved in men with CHH, highlighting the added value provided by KP54 in comparison to GnRH. The response to KP54 completely distinguished CHH men from healthy men (unlike GnRH).

Our findings in 21 men with CHH and 21 healthy men receiving KP54 build on previous data investigating the effects of KP10 in patients with CHH [11]. Chan and colleagues administered an intravenous bolus of KP10 (0.24

nmol/kg) to 9 men and 2 women with CHH [11]. LH did not rise after KP10 in patients with CHH, regardless of genotype, doses of up to 2.4 nmol/kg, repeated dosing, or GnRH priming [11].

Notably, KP54 is reported to cross the blood-brain barrier, whereas KP10 does not [13, 21], suggesting that KP54 could provide more specific information on the functionality of GnRH neuronal cell bodies (as opposed to nerve terminals at the median eminence) than KP10 [13]. To date, no previous study has investigated the performance of KP54 in the examination of hypothalamic GnRH function in men with CHH. Bolus administration of KP54 is known to induce a greater and more persistent rise in LH than equimolar doses of KP10 [13]. Thus, due to its longer half-life of KP54 ($t_{1/2}$ KP54: 28 min and KP10: 3 min) [13, 22], KP54 could possess preferable pharmacokinetic properties for intravenous bolus administration as a "KP test" of hypothalamic GnRH neuronal function. Indeed, the mean LH rise was 13.2 iU/L after KP54 in the present study, whereas the highest reported LH rise following intravenous bolus administration of KP10 is 8.3 iU/L after a dose of 0.77 nmol/kg [23]. Thus, KP54 could facilitate greater granularity in the endocrine response to differentiate healthy men from those CHH, which could be of particular value in patients with partial CHH phenotypes. However, it is not known whether the purported ability of KP54 to cross the blood-brain barrier and directly activate GnRH neuronal cell bodies impacts the resultant LH response in men with CHH as compared to KP10. The LH response curves in the present study were clearly distinct between KP54 and GnRH in healthy individuals. The exact effect of KP54 bolus on GnRH neurons and the amount of GnRH released remain uncertain in humans. The LH response observed after KP54 indicates that the mechanism is not obvious and may not be restricted to a single event of GnRH release. Chan et al. [7] estimated that a single bolus of KP10 results in GnRH release that lasts 17 min in men. Exposure of murine GnRH neurons to KP10 (10–100 nM) for 1–3 min results in GnRH neuronal electrophysiological firing lasting for at least 20 min [24] and for 55 min after 3-min exposure to 10 nM of KP54 [25]. Thus, there remain many shadow zones for KP physiology in humans.

Indeed, certain genetic variants causing CHH can be incomplete or induce a milder clinical phenotype. Whereas some variants impair GnRH neurogenesis or migration, others, such as those in genes affecting neurokinin B signaling, impair GnRH secretion [2]. Young and colleagues have shown that a 12-h infusion of KP10 in 2 patients with Tac3 mutations and 2 patients with Tac3R mutations (encoding neurokinin B and its receptor, respectively) increased mean LH (saline 0.4 iU/L and KP10 1.0 iU/L) but to a lesser extent than in healthy men (saline 5.2 iU/L and KP10 14.1 iU/L) [23]. Thus, although patients with deficits in neurokinin B signaling can respond to KP10, the response was attenuated in comparison to healthy individuals. Consequently, the greater magnitude of gonadotropin response following KP54 administration could be advantageous to more precisely differentiate patients with CHH from healthy individuals. Furthermore, the response to KP could be used to differentiate men with CHH (reduced response to KP) from those with functional cause of HH such as diabetes-related hypogonadism (in which responsiveness is preserved) [26].

In keeping with this, there was no overlap in LH responses between men with CHH and healthy men, irrespective of the specific causative mutation in the current study. However, pathogenic/likely pathogenic mutations were identified in 4 CHH men, and these men had even lower LH rises following KP54 administration than CHH men with either variants of uncertain significance or no abnormality identified on genetic testing. The identification of loss-of-function mutations in genes critical for GnRH neuronal migration (*SEMA3A, PROKR2, FGFR1*, and *ANOS1*) [27, 28] is consistent with the lower responses to KP54. Similarly, anosmic CHH men, that is, Kallmann syndrome, had even more attenuated responses after KP54 administration than normosmic CHH men. Conceivably, this is due to that patients with anosmia being more likely to have failure of GnRH neuronal migration (rather than secretion), and thus, GnRH neurons are not in the appropriate location to be able to respond to KP54. Indeed, anosmic CHH men are also reported to have a more severe phenotype than normosmic CHH men, also reflected by lower baseline LH levels (Table 2) [29, 30].

The response to KP54 is predicated on a responsive pituitary gland, and thus, a lack of GnRH priming could impair the response to GnRH and in turn to KP54 administration. Although GnRH priming was not available during the present study, recent evidence suggests that GnRH priming does not significantly alter the gonadotropin response to KP in unprimed individuals (mean LH rise 2.0 iU/L before vs. 1.2 iU/L after GnRH priming) [31]. Moreover, the response to GnRH in the same patient can be taken into account when interpreting the response to KP [31]. Accordingly, the ratio of LH rise after GnRH administration to that after KP54 administration was increased in CHH men as compared to that in healthy men (10.8 vs. 1.5; $p = 0.0005$), highlighting the differential response to

GnRH and KP54 in CHH men. In summary, we demonstrate that KP54 offers the unique opportunity to specifically interrogate hypothalamic GnRH neuronal function and provides added value in comparison to currently available investigations.

Acknowledgements

The study was designed, conducted, analyzed, and reported entirely by the authors. This article presents independent research funded by grants from the NIHR and supported by the NIHR/Wellcome Trust Imperial Clinical Research Facility and Imperial Biomedical Research Centre. The section of Metabolism, Digestion, and Reproduction was funded by grants from the MRC, BBSRC, and NIHR and was supported by the NIHR Biomedical Research Centre Funding Scheme. The views expressed are those of the author(s) and not necessarily those of the MRC, BBSRC, the NHS, the NIHR, or the Department of Health. A.A. was supported by National Institute of Health Research (NIHR) Clinician Scientist Award CS-2018-18-ST2-002. S.C. was supported by funding from an NIHR Academic Clinical Lectureship. W.S.D. was supported by an NIHR Research Professorship NIHR-RP-2014-05-001.

Statement of Ethics

Ethical approval for this study was granted by the West London Research Ethics Committee, London, UK (reference: 12/LO/0507), and all participants provided written informed consent. The study was conducted in accordance with the Declaration of Helsinki.

Conflict of Interest Statement

A.A. and W.S.D. have undertaken consultancy work for Myovant Sciences Ltd.

Funding Sources

NIHR.

Author Contributions

A.A., P.C.E., M.P., S.A.C., E.M., G.C., L.Y., C.I-E., N.S., C.N.J., A.N.C., R.A.I., J.R., C.X., R.Q., N.P., and W.S.D. designed the study, analyzed the data, prepared the manuscript, and designed the figures and tables. A.A., P.C.E., M.P., S.A.C., E.M., G.C., L.Y., and C.I.E. conducted data collection. A.A., P.C.E., M.P., and S.A.C. performed the statistical analysis. W.S.D. was the project supervisor, who reviewed and edited the manuscript, and is the guarantor of this research project. All authors have made a substantial, direct, and intellectual contribution to the work and approved the manuscript prior to its submission.

Data Availability Statement

All data generated or analyzed during this study are included in this published article.

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