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Effect of gonadotropin inhibitory hormone (GnIH) on luteinizing hormone secretion in ~~man~~humans.

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Abbreviated title: Gonadotropin inhibitory hormone in the human.

Precis: Exogenous GnIH decreased LH secretion in post-menopausal women. The stimulation of LH secretion in response to kisspeptin in men was not abrogated by co-administration of GnIH.

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Abstract

Gonadotropin inhibitory hormone (GnIH, human homologue of RFRP-3) suppresses gonadotropin secretion in animal models, but its effects have not been studied in the human.

Objective

We tested the hypotheses that exogenous GnIH inhibits LH secretion a) in postmenopausal women, and b) in men concurrently administered exogenous kisspeptin.

Design

Following *in vitro* and *in vivo* pre-clinical studies to functionally characterize the GnIH peptide, a dose-finding study (human GnIH 1.5 to 150 µg/kg/h, iv for 3h) was undertaken, and 50 µg/kg/h selected for further evaluation.

Five postmenopausal women were administered 50 µg/kg/h iv infusion for 3h or vehicle on two separate days.

Four men were administered kisspeptin-10 (0.3 µg/kg iv bolus) with simultaneous infusion of GnIH (50 µg/kg/h, iv for 3h) or vehicle.

Participants.

Healthy postmenopausal women (mean age 58±2 years, LH: 30.8±2.9 IU/L, FSH: 78.7±6.4 IU/L, estradiol: <50 pmol/L) and men (39.8±2.1 years, mean total testosterone 12.1±1.8 nmol/L, LH 2.2±0.2 IU/L).

Primary Outcome

Change in area-under-curve of LH during GnIH vs. vehicle.

Results

During GnIH administration in postmenopausal women, LH secretion decreased (Δ AUC -9.9±1.8 IU/3h) vs. vehicle (Δ AUC -0.5±1.7 IU/3h) (P= 0.02). Kisspeptin-10 stimulated LH responses in men was not affected by GnIH co-administration (60-min AUC of LH 6.2±0.8 IU/h with kisspeptin-10 alone, 6.3±1.0 IU/h, kisspeptin-10 with GnIH, P = 0.72).

Exogenous GnIH was well tolerated, with no adverse events reported.

Conclusions.

GnIH decreased LH secretion in postmenopausal women in this first-in-human study. Kisspeptin-stimulated LH secretion in men was not inhibited during concomitant administration of GnIH.

Introduction

Pulsatile secretion of gonadotropin releasing hormone (GnRH) stimulates the secretion of gonadotropins (LH and FSH) and the downstream secretion of gonadal steroid hormones.[1-3] Gonadotropin inhibitory hormone (GnIH) is a hypothalamic neuropeptide initially discovered in birds as an inhibitor of LH secretion[4] but its activity has not been studied in humans. We report the first human studies on the effect of exogenous GnIH on LH secretion.

The avian GnIH peptide (SIKPSAYLPLRF-NH_2) has one human homologue which is inactive and, two orthologues which have the characteristic carboxyl terminal RF-amide. RFRP-1 (MPHSFANLPLRF-NH_2) and RFRP-3 (VPNLPQRF-NH_2,), were isolated and structurally identified in the human hypothalamus.[5] GnIH inhibits GnRH-stimulated mobilization of intracellular calcium in avian[4], ovine[6] and bovine[7] gonadotropes *in vitro* – suggesting a pituitary locus of action. These data, and other evidence from a range of species[5, 8-14], reviewed recently elsewhere[15], indicate that GnIH is a specific inhibitor of gonadotropin secretion in mammals, signaling through the GnIH receptor (GnIH-R, GPR147) via inhibition of cyclic AMP production. GnIH neurons display close apposition to GnRH neurons in sheep[16] , non-human primate[17] and in human[5] hypothalami, suggesting that GnIH may also directly regulate GnRH secretion.

Following pre-clinical studies to characterize the activity of the GnIH peptide *in vitro* and in the ovariectomized ewe, we investigated the effect of GnIH on LH secretion in two human models of increased GnRH secretion: in postmenopausal women, and in in healthy men administered exogenous kisspeptin.

Methods

GMP kisspeptin and GnIH

Kisspeptin-10 and GnIH peptides were custom synthesized to GMP standards (Bachem GmBH, Weil am Rhein, Germany). Purity was assessed by HPLC at 97% with a mass balance of 98.8%. Both peptides were made up within the hour before injection/infusion by diluting 1 mg of lyophilized peptide in 5 ml sterile normal saline. The bolus dose of

kisspeptin-10 (0.3 μg/kg) was selected for acute LH stimulation tests based on our previous dose-finding study of kisspeptin-10 [18] and other published data.[3]

Characterisation of GnIH *in vitro*

The objective of these *in vitro* studies was to compare the biological activity of the custom-synthesised GnIH peptide used in the present study with that of GnIH previously used in published data involving non-human species. Whole-cell receptor binding and CRE-luciferase assays were carried out in parallel using the custom-synthesized GMP GnIH and a custom synthesized preparation (EzBiolab, Carmel, IN, USA) used extensively by our laboratory.

Whole-cell receptor binding assay

The methods are described in detail elsewhere[19] but in brief, COS-7 cells were electroporated with 10 μg *NPFFR1 (*GPR147) and seeded in 12 well plates. Forty eight hours post transfection cells were incubated in HEPES-DMEM with 100000 cpm ^{125}I radiolabelled-RFRP3/well and increasing concentrations of non-radiolabelled peptide in the range of 0-1μM for 4 hours at 4°C. Cells were then rapidly washed twice with cold PBS (+MgCl2/CaCl2). Thereafter, 0.5mL 0.1M NaOH was added to the cells and incubated at room temperature for 15 minutes to lyse the cells. Cell lysates were transferred to plastic tubes and bound radioactivity counted with a Berthold LB2111 gamma counter for 1 minute.

CRE-Luciferase Assay

HEK 293T cells were seeded in 24 well plates coated with Matrigel (BD Biosciences). The next day, cells were chemically transfected (X-tremeGENE HP DNA Transfection Reagent (Roche)) with GPR147 and CRE-Luc reporter gene plasmid DNA in a 1:1 DNA ratio. Plates were incubated for 24 hours post transfection and then washed twice with phosphate buffered saline (PBS+). Thereafter, starving media (DMEM [Dulbecco's modified Eagle's medium], 1% pen/strep, 4mM L-glutamine, 10mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HEPES) was added to the cells overnight. Media was aspirated and replaced 0.5mL of compound dilutions prepared in starving media. Plates were incubated for 6 hours at 37 °C after which they were transferred to ice and washed with cold PBS+. Diluted passive lysis buffer (20 μL) was added to each well and plates placed on a shaker at 1050 rpm for 15

minutes at room temperature. Lysates were diluted 1:4 and 20 µL was transferred to a white, flat bottomed 96 well plate. Luciferase activity was measured for 10 seconds on a Lumat LB9501 Luminometer after 100 µL LARII was added to each well.

LH inhibition by GnIH in ewes

Animals and peptide infusion regime

Groups of 4 ovariectomised ewes were held in single pens and both jugular veins were cannulated on the day prior to experimentation to allow blood sampling and infusion. Venous blood samples were taken at 10 min intervals for 7.5 hours, in 2.5 hour blocks, before, during and after infusion of either GnIH or vehicle (saline). The animals received a loading dose of 1mg and then an infusion of 1 mg/h, using an infusion rate of 1ml/h. All ovine experiments were carried out at the University of Monash (IJC) in compliance with regulations and ethical standards.

Radioimmunoassay of ovine LH

LH in plasma was measured as previously described[20] using NIH-oLH-S18 as the standard and NIDDK-anti-oLH-I as the antiserum. Iodinated ovine LH (125 I-NIDDK-AFD-9598B) was used as tracer. Assay sensitivity was 0.1 ng/ml. Intra-assay coefficient of variation (CV) was <10% between 0.6 and 13.7 ng/ml and inter-assay CV was 7.8% at 10.4 ng/ml and 9.5% at 20.3 ng/ml.[21]

LH Pulse analysis in ovine studies

An LH pulse was defined as having occurred when the assay value of a given sample exceeded the assay value of the previous sample by at least 3 standard deviations, as well as other criteria detailed previously[22], using error estimates generated by the computer program of Salamonsen et al.[23] Comparisons of LH pulse amplitude, inter-pulse interval, and mean LH between groups infused with GnIH and vehicle were made using two-way ANOVA.

Human studies

Participants

Five healthy postmenopausal women (mean age 58±2 yrs, 12.4±2.6 yrs since last menstrual period, LH: 30.8±2.9 IU/L, FSH: 78.7±6.4 IU/L, estradiol: <50 pmol/L) and four healthy adult men (mean age 39.8±2.1 yrs, total testosterone 12.1±1.8 nmol/L, LH 2.2±0.2 IU/L and FSH 4.8±12 IU/L took part in the study. LH, FSH and testosterone were all within normal range in the men, and this was confirmed on at least one additional morning sample). Baseline physical examination, full blood count, renal function, liver function, and serum electrolytes were within normal limits. All volunteers provided written informed consent to take part in this study, which was approved by the South East Scotland Research Ethics Committee (10/S1101/53). The study was also approved by the Edinburgh Clinical Research Facility Phase 1 and First-in-Human Study Review Committee (Ref E10903).

Peptide infusion protocols for human studies

First, we set to establish a safe and effective dose that achieved nanomolar concentrations of RFRP3 in the peripheral circulation when administered as an intravenous infusion in two post-menopausal women (dose finding study). Second, the effects of the maximally effective dose (identified from the dose-finding study as approximately 50 µg/kg/h) was studied in 3 additional post-menopausal women, to give a total n=5 for this dose. Third, four healthy men were given intravenous boluses of kisspeptin in the presence or absence of GnIH 50 µg/kg/h administered concurrently as intravenous infusion (Kisspeptin-GnIH Study).

Effects of GnIH on LH in post-menopausal women

a) Dose-finding study

The median inhibitory concentration of GnIH at its cognate receptor (GPR147) is 1-2 nM[24]. The objective of this study was therefore to identify a dose at which circulating GnIH concentrations greater than 1 nM were achieved.

Two healthy post-menopausal women were admitted six times to our clinical research facility, with a minimum inter-visit interval of a week. Each of these visits involved six hours of frequent (every 10-min) venous blood sampling to assess effects on LH secretion. After

three hours of baseline sampling, an infusion of GnIH (1.5, 5, 15, 50 and 150 µg/kg/h) or vehicle (normal saline) was administered for a further three hours. At the start of this infusion, an intravenous bolus of GnIH (25% of hourly infusion dose) or vehicle was administered, simulating the studies in which GnIH was effective in ovariectomised ewes. For safety reasons, doses were administered in increasing order in an unblinded manner under medical supervision and participants were observed in the research facility for at least an hour after the infusion completed. LH sampling was opportunistically continued at 10-min interval during this additional hour of safety monitoring.

In addition to collection of serum for hormone assays, plasma samples were collected immediately before as well as at regular intervals up to four hours (at 30, 60, 120, 150, 179, 190, 200, 210, 220, 230 and 240 minutes) after commencing the infusion for pharmacokinetic assessment to quantify circulating GnIH. GnIH concentrations were estimated using liquid chromatography tandem mass spectrometry. A DPP-IV inhibitor (Diprotin A, 0.1mM final concentration; Sigma I9759) and protease inhibitor cocktail (Sigma P8340, 1x final concentration; both sourced from Sigma-Aldrich, St. Louis, MO, USA) were added to these samples to inhibit breakdown of GnIH after serum collection.

Blood pressure, pulse rate, and peripheral oxygen saturations were measured with standard automated techniques. Full blood count, serum electrolytes, liver and renal function were checked at the beginning and end of each visit.

b) Effects of 50 µg/kg/h intravenous GnIH in post-menopausal women

In addition to the two women who participated in dose-finding study, three more healthy postmenopausal women received 50 µg/kg/h intravenous infusions of GnIH (with a 12.5 µg/kg intravenous bolus at the start of the infusion) and vehicle for three hours each on two separate days, at least a week apart, using the LH sampling protocol outlined above.

LH pulsatility in all five women during the 50 µg/kg/h of GnIH was compared with the pulsatility observed during the infusion of vehicle.

Effects of GnIH on kisspeptn-10 stimulation of LH in healthy men

The objective of this study was to test the hypothesis that co-administration of GnIH would attenuate the stimulatory LH response elicited by kisspeptin.

Healthy male volunteers (n=4) were admitted twice to our clinical research facility for 7.5 hours and LH samples obtained at 10-minute intervals. Two intravenous boluses of kisspeptin-10 (0.3 µg/kg, 23 pmol/kg) were administered at 60-minutes and 270 minutes. On the first visit, an intravenous infusion of saline was started at 240 minutes. On the second visit, a 50 µg/kg/h intravenous infusion of GnIH was commenced at the identical time-point (240 minutes) to that of vehicle infusion, along with a 12.5 µg/kg intravenous bolus of GnIH administered at the start of this infusion which continued for 180 minutes. This protocol is identical to the 50 µg/kg/h infusion protocol used in the dose-finding and replication studies described above.

Human LH pulsatility analysis and statistical comparisons

Blood samples were centrifuged immediately at 4 C for 10 min at 3000 rpm and serum frozen at -20 C until analysis. LH was determined by ELISA as previously described[18]. Inter-assay coefficient of variation for all hormonal assays was less than 5% at the concentrations measured. Intra-assay coefficient of variation of LH was 2.9%. All samples from each of the study visits were analyzed together.

Area-under-curve (AUC) of LH was calculated using trapezoid integration. In studies to assess the impact of exogenous GnIH on LH secretion, 180-min AUC before and during the infusion of GnIH were calculated to derive ΔAUC LH - the arithmetic difference of AUC over three hours before and during the infusion. Paired Student's *t* tests used to compare ΔAUC LH during vehicle and GnIH infusions.

In the kisspeptin-GnIH study, 60-min AUC was calculated during three periods: Baseline (first hour of the study) and 60-min after each of the kisspeptin-10 boluses. Repeated measures ANOVA was used to assess variance in 60-min AUC and multiple comparisons

made using Fishers Least Significant Difference (LSD) test. We have previously shown the sensitivity of this approach to detect LH response to kisspeptin-10 [18, 25, 26].

LH pulses were identified and mass-per-pulse (MPP) and basal LH secretion calculated using a deconvolution algorithm using cluster analysis with 93% sensitivity and specificity on blinded data[27]. Paired Student's t test was used to assess changes in pulse frequency, mass-per-pulse and basal LH secretion.

Data are presented as mean±SEM. A two-sided $P<0.05$ was regarded as statistically significant for all analyses. The statistical software package Graphpad Prism 6 for Mac OSX (Graphpad, La Jolla, CA 92037 USA) was used.

Results

In vitro studies

GMP custom-synthesised GnIH (Bachem) and comparator peptide (EZbiolab) induced an inhibition of forskolin-induced accumulation of cAMP with similar potencies (Supplementary Fig 1). Both peptides also displayed high affinity for the receptor with no statistically significant difference in the IC50 (data not shown).

In summary, the GnIH peptide used in the present human study thus has identical binding and signalling characterstics to a peptide preparation previously used in non-human studies at the GPR147 receptor.

LH Inhibition by GnIH in Ewes

Infusion of GnIH significantly reduced the amplitude of LH pulses in ovariectomised ewes (Supplementary Fig 2). LH pulse amplitude decreased from 0.9±0.1 ng/ml before the GnIH infusion to 0.4±0 ng/ml during it ($P<0.002$) and returned to baseline (0.7±0.1 ng/ml) following discontinuation. In the control vehicle infusion group, LH pulse amplitude remained unchanged at 1±0.1 ng/ml before, 1.3±0.2 ng/ml during and 1.3±0.3 ng/ml after infusion.

There were no significant changes in LH pulse frequency or mean LH during either GnIH or vehicle infusions. GnIH infused sheep had 3.8±0.25 pulses before, and 4.0±0 pulses during the infusion, and 4.0±0.4 pulses in the post-infusion study period. Control animals infused with vehicle were observed to have 3.5±0.5, 3.75±0.25 and 3.75±0.5 pulses during corresponding sampling windows.

Individual animal-level data on LH secretion in both GnIH and vehicle treated groups are tabulated in supplementary table 1.

Effects of GnIH on LH in postmenopausal women

Dose finding study

All doses of GnIH were well tolerated, with no adverse events. Both women showed modest but dose-dependant suppression of LH secretion during GnIH infusion. One subject achieved maximal reduction in AUC of LH at 15 µg/kg/h dose (Δ AUC -9.7 IU/L/3h) and the other at 50 µg/kg/h (Δ AUC -10.9 IU/L/3h) (Fig 1A).

During infusion of 50 µg/kg/h, circulating concentrations of GnIH rose from below the limits of quantification (1ng/mL) before administration to 2.4 ±0.4 ng/ml during the infusion (range 1.2-3.8 ng/ml; 1.2-3.9 nM) and 6.5±1.8 ng/ml (range 1.9-11.9 ng/ml; 2-12.3 nM) in the two women. GnIH became undetectable 10 minutes after the end of the infusion.

The highest dose studied (150 µg/kg/h) resulted in higher plasma concentrations of GnIH (10.1±1.5 ng/mL; 10.4±1.5 nM) and (11.1±2.5 ng/mL; 11.5±2.6 nM) in the two subjects. However, this increase in circulating peptide concentration was not associated with larger reductions in LH secretion than that observed with the 50 µg/kg/h dose. Therefore, the lowest dose that achieved nanomolar concentrations in peripheral circulation, i.e. 50 µg/kg/h, was selected for further studies.

LH inhibition by GnIH in post-menopausal women

GnIH infusion (50 µg/kg/h) significantly decreased LH secretion (Δ AUC -9.9±1.8 IU/3h) when compared to the infusion of vehicle (Δ AUC -0.5±1.7 IU/3h) in the five women (*p*=

0.02, Fig 1B). Decreases in mean LH was observed in four out of the five women studied. Individual volunteer-level data on mean LH secretion before, during and after saline and GnIH treatments are tabulated in supplementary table 2.

There was no effect on pulsatile LH secretion (figure 2). Baseline pulse frequency of 0.8±0.1 pulse/h remained unchanged during GnIH treatment, at 0.8±0.1 pulse/h. Nevertheless, mass-per-pulse showed a numerical increase, from 20.2±4.5 IU/l at baseline to 27.8±6.4 IU/l during GnIH infusion (P = 0.052). Consistent with these, the total pulsatile secretion of LH also showed a numerical increase without statistical significance - at 100.6±26.6 IU/L/3h at baseline to 139.1±38.2 IU/L/3h (P = 0.124) during GnIH infusion.

Co-administration of GnIH and kisspeptin-10 in men

Kisspeptin-10 bolus (0.3 µg/kg iv) significantly increased LH secretion, with 60-min AUC of LH increasing from 2.8±0.8 IU/L/h to 6.5±0.9 after each of the two boluses in the control visit. (ANOVA P = 0.005, Fisher's LSD P = 0.003, baseline vs. kisspeptin boluses; 0.96 (comparing the two kisspeptin boluses), Fig 3A.

During concurrent administration of GnIH, both boluses of kisspeptin-10 also elicited significant increases in LH secretion, with 60-min AUC of LH increasing from 3.1±0.5 IU/L/h to 6.2±0.8 IU/L/h with kisspeptin-10 alone vs. 6.3±1.0 IU/L/h with kisspeptin-10 in the presence of GnIH respectively (Fisher's LSD P <0.005). There was no statistical difference between responses to the two kisspeptin-10 boluses administered before or during GnIH infusion (Fisher's LSD P = 0.715), Fig 3B. Individual volunteer-level 60-min AUC during baseline and after the two kisspeptin-10 boluses are tabulated in supplementary table 3. Figure 4 provides exemplar LH profiles from a participant during GnIH and vehicle infusion visits.

Safety of GnIH

GnIH infusions were well tolerated with no serious adverse events reported. Heart rate and blood pressure, which were monitored intensely during study visits, showed no significant

change. Serum electrolytes, renal function, full blood count and liver function monitored at all study visits also remained unchanged.

Discussion

The present study provides first-in-human data on the effects of exogenous GnIH effects on LH secretion in women and men, in paradigms of increased GnRH secretion. In postmenopausal women, GnIH administration resulted in a modest suppression of LH secretion, while it did not affect kisspeptin-stimulated LH secretion in men. GnIH was well tolerated by men and women who took part in this first in man study, providing reassurance about its safety for future clinical studies.

Infusions of GnIH (up to the dose of 150 µg/kg/h in two post-menopausal women, 50 µg/kg/h in all other men and women) for three hours were well tolerated. Serum concentrations in the low nanomolar range were achieved with continuous intravenous infusion of GnIH at 50 µg/kg/h and associated with significant decrease in LH secretion (AUC of LH) in post-menopausal women. We demonstrated that the circulating concentrations of 1.2-12.3 nM/L achieved are sufficient to bind to the GPR147 receptor and inhibit forskolin-stimulated cAMP generation (Fig 1).

Our results are consistent with decreased LH secretion observed in large animal models (ewes and calves) exposed to GnIH.[6, 7] In rodents, the effects of GnIH appear to show sexual dimorphism. Male rats[9, 14] and hamsters[8] respond with gonadotropin suppression, while female rats showed no change in LH or FSH when the peptide was administered intracerebroventricularly. [28]

The pattern of reduction in LH secretion observed in post menopausal women in our study during GnIH administration appears to be broadly concordant with that observed in previously published ovine[6] and bovine models[7]. Post-menopausal women in the present study showed no change in LH pulse frequency; the corresponding ovine model (ovariectomised sheep) also showed no reduction in pulse frequency in the present study as

well as in previous studies.[6] A previous study using a bovine model did show a reduction in pulse frequency[7] but in that study GnIH was administered as multiple boluses rather than by infusion. Furthermore, the bovine models used were castrated males, which does not allow direct comparison with the data in the present study.

The magnitude of reduction of LH in human volunteers in the present study appears to be lower than the marked reduction in serum LH observed in ovariectomised sheep.[6] There is considerable inter-species difference in the effects of GnIH[29], which may explain some of the differences. Therefore, the most parsimonious explanation for the differential responses in ovine models and human volunteers is potential inter-species variability. Furthermore, as our study was based only on a small number of participants, the true effect size of LH response to GnIH in a wider population cannot be reliably extrapolated. In addition, administration of exogenous peptide hormones, i.e., the experimental paradigm employed here, is not an appropriate technique to study the physiological role of the peptide. However, even when taking study limitations into consideration, the limited suppression of LH observed in the present study suggests that pharmacological application of GnIH (or its agonists) in human disorders of excess LH secretion may not be feasible.

GnIH had no effect on kisspeptin-stimulated LH secretion in men in the present study where 50 μg/kg/h was administered as continuous intravenous infusion. In addition to its main objectives, the present study also assessed the effects of repeated doses of kisspeptin-10 boluses within the same individual – four boluses on two separate study days. These data showed that there was remarkable intra-individual consistency in LH response to kisspeptin-10 (supplemental table 3). This is distinct from the tachyphylaxis observed on repeated dosing of kisspeptin-54[3] or with longer-acting kisspeptin analogues.[30] This finding could lend support for the continuing development of kisspeptin-10 as a physiologic probe to ascertain GnRH function *in* or as a potential therapeutic option to stimulate GnRH endogenously.[3]

We chose kisspeptin-10 administration as a model of LH stimulation to study the effects of GnIH on LH secretion in men. Kisspeptin acts via endogenous GnRH secretion and results in a doubling in peripheral LH concentrations, comparable to an endogenous LH pulse, and is thus a more physiologically relevant stimulus than exogenous GnRH, which elicits around a 10-fold rise in LH.[3] Furthermore, we have previously shown that the acute LH-response to kisspeptin in men is not affected by prevailing sex-steroid status.[25] LH response to kisspeptin remained unchanged with concurrent administration of GnIH.

With the limited LH suppression observed in these first-in-man studies in both men and women, it is reasonable to speculate that the role of GnIH in the regulation of LH secretion in the human may be less robust than in other species. Nevertheless these studies have limitations. As a first-in-human study with associated safety considerations, the exposure to human volunteers in the study was limited to 3 hours and only a small number of volunteers were studied. Furthermore, we did not undertake a separate dose-finding study in men, but have assumed similar pharmacokinetics in men and women.

GnRH-induced gonadotropin subunit gene transcription is suppressed by GnIH orthologues by inhibiting adenylate cyclase/cAMP/protein kinase A-dependent ERK activation.[31] This transcriptional effect suggests that a longer exposure paradigm may have achieved greater suppression of LH secretion. However, the rapid clearance of GnIH from circulation observed here makes it challenging to deliver sufficient quantities of the native peptide intravenously to maintain stable serum concentrations over long durations. This challenge has been effectively overcome for GnRH and other small peptide hormones by incorporation of D-amino acids to reduce proteolytic degradation and by formulating in biodegradable polymers which can deliver efficacious levels of peptide for up to one year. Therapeutic potential of GnIH to treat disorders characterised by increased LH secretion (e.g. PCOS, menopausal hot flushes, precocious puberty) needs to be assessed in context of emerging data on kisspeptin and neurokinin B modulation. Prima facie, GnIH appears to be a less potent tool in comparison to these approaches.[3, 32]

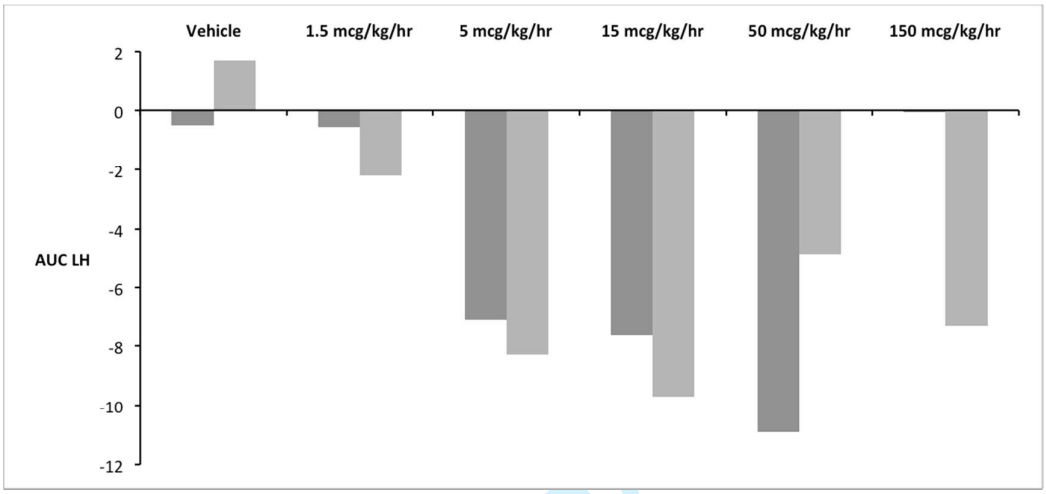
In conclusion, in this first-in-human study, we have demonstrated that exogenously administered GnIH has a suppressive effect on LH secretion in postmenopausal women and that kisspeptin-10 mediated LH secretion in men is unaffected during concomitant administration of GnIH. Further studies, are required to fully elucidate the physiological mechanisms and the therapeutic potential for GnIH.

Author Contributions

RPM conceived the study, JTG developed the study protocol and managed the study, guided by RPM, RAA and RW. JTG supervised the administration of all doses of GnIH, collated biochemical results and wrote the first draft of this manuscript. Deconvolution analysis of LH pulsatility was carried out by JDV on blinded data. IJC designed, supervised and reported the ovine studies of GnIH. MG carried out *in vitro* studies. All authors contributed to the revision of this manuscript and have approved the final version.

Figure 1: Reductions in 180-minute Area-under-curve (ΔAUC) of Luteinising Hormone in postmenopausal women administered GnIH. **A**, ΔAUC of LH in two postmenopausal women administered vehicle and GnIH infusions at 1.5, 5, 15, 50 and 150 µg/kg/h. Each of the data series represents data from an individual volunteer. Each series represents a single woman receiving all five doses and vehicle. One subject only had 0.07 unit decrease. **B**, ΔAUC of LH in five postmenopausal women administered vehicle (white column) and RFPR-3 infusion at 50 µg/kg/h (black column). The data include those of the 50 µg/kg/h from the two women receiving the various doses (**1A** above) and four additional women (* P=*0.02*)

1A



1B

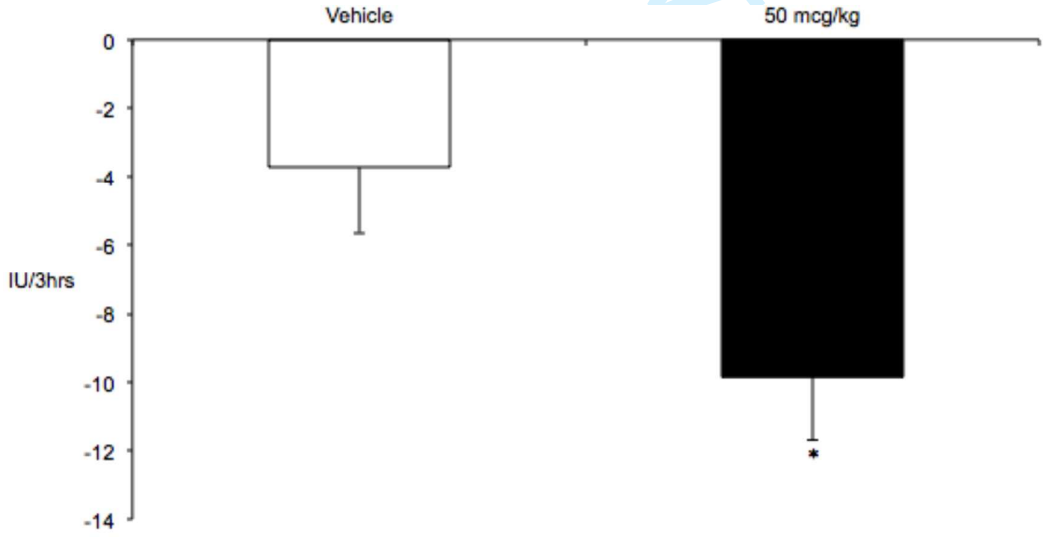


Figure 2: LH pulse profiles of five postmenopausal women administered 50 mcg/kg/hour GnIH (red squares) and saline infusions (blue triangles). Blood samples were drawn at 10-min intervals throughout the study. Intravenous infusion of GnIH was maintained for three hours, from 0 to 180 min, and an intravenous bolus of GnIH (12.5 µg/kg) was administered at time 0.

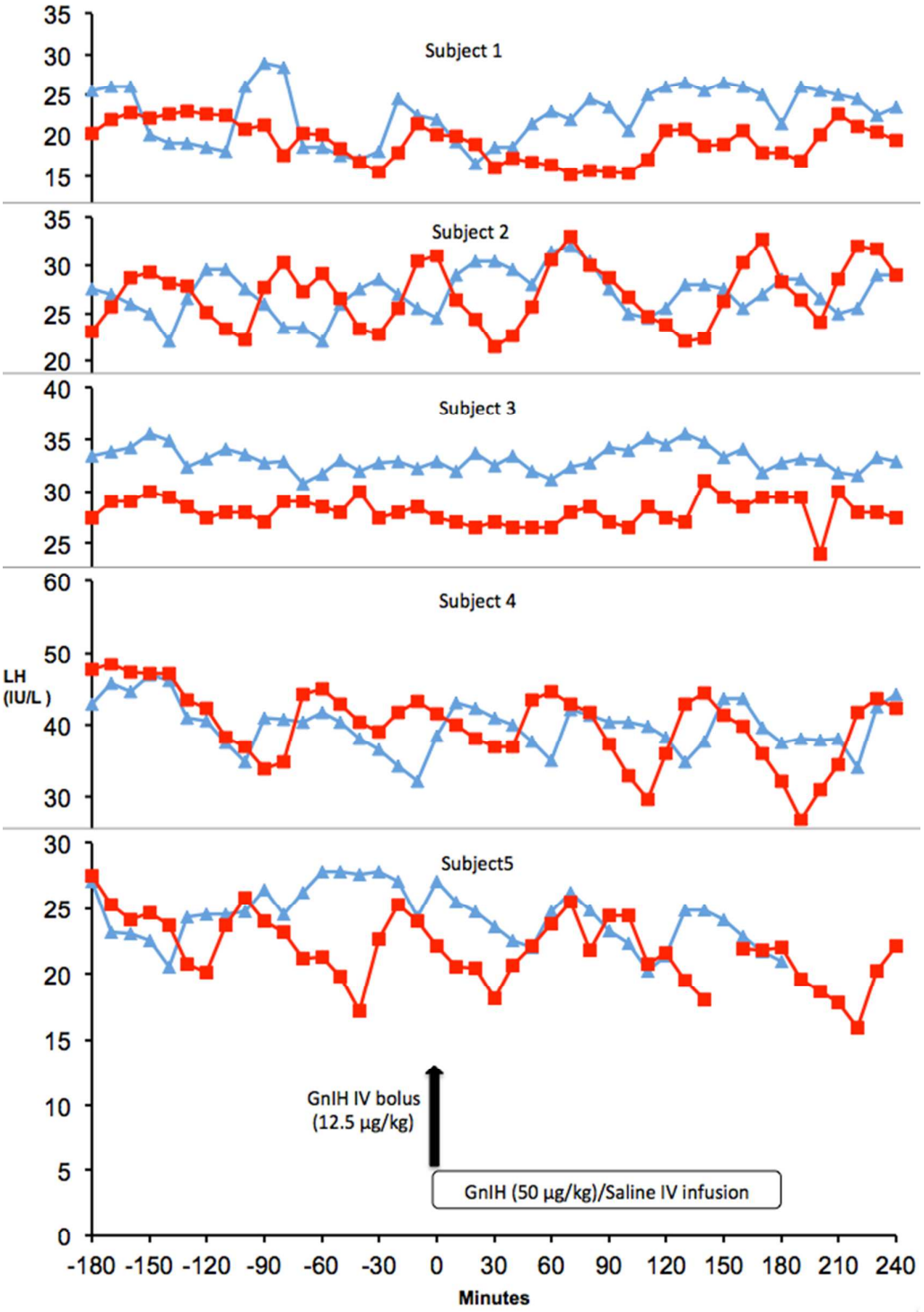
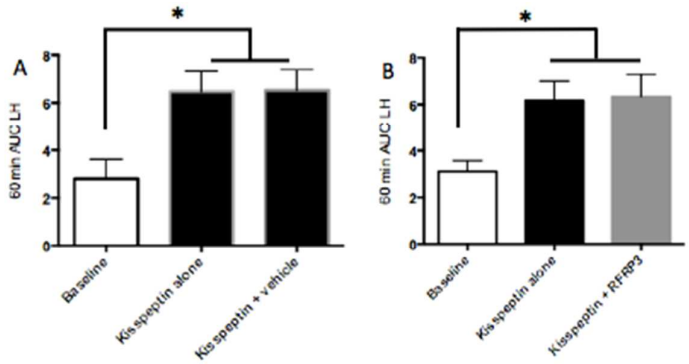


Figure 3. LH secretion (60 minute AUC, IU/h) at baseline and during the hour after healthy men were administered boluses of kisspeptin-10 (0.3 μg/kg). Co-administration of vehicle (A) or GnIH (B) did not impact on the stimulatory effect of kisspeptin on LH secretion. * $p < 0.05$.



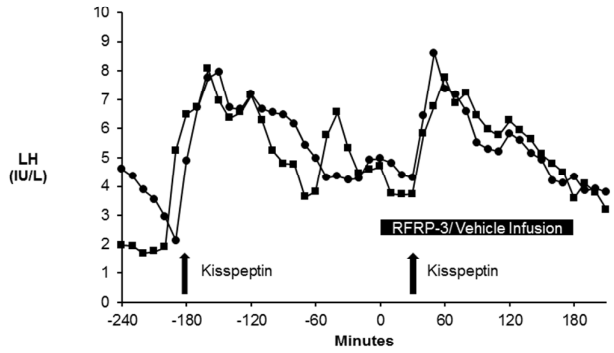


Figure 4: LH pulse profiles of one of the male volunteers on the two days when vehicle (squares) and GnIH (circles) were administered for three hours - from 0 min to 180 min. Patients had one hour of LH profiling at the beginning of each session to quantify baseline LH secretion (-240 to -180 min). Kisspeptin was administered at -180 and +30 mins during both these study visits, to quantify 60-min area-under-curve of LH following each of these.

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Supplementary data

		Mean LH (IU)	Number of Pulses	Pulse Amplitude (IU)	IPI (Min)
Sheep 1	Prior to infusion	1.85	3	1.34	36.7
	During infusion	2.6	4	1.94	43.3
	After infusion	2.74	3	2.07	50
Sheep 2	Prior to infusion	3.67	3	3.36	36.7
	During infusion	3.68	4	4.59	42.5
	After infusion	3.93	4	3.31	42.5
Sheep 3	Prior to infusion	1.17	4	0.74	35
	During infusion	1.38	4	0.53	37.5
	After infusion	1.39	5	0.43	32
Sheep 4	Prior to infusion	2.66	3	2.1	40
	During infusion	2.83	4	2.9	42.5
	After infusion	2.98	3	4.72	43.3
Sheep 5	Prior to infusion	2.71	4	2.12	35
	During infusion	2.36	4	0.89	40
	After infusion	3.33	3	6.74	43.3
Sheep 6	Prior to infusion	2.79	4	1.53	35
	During infusion	3.31	4	1.89	40
	After infusion	3.38	4	2.31	40
Sheep 7	Prior to infusion	1.74	5	1.61	30
	During infusion	1.97	4	1.96	35
	After infusion	1.86	5	1.01	34

Sheep 8	Prior to infusion	3.5	3	2.94	46.7
	During infusion	3.89	3	2.43	43.3
	After infusion	3.95	4	2.21	42.5

Supplementary table 1: LH secretory parameters (mean LH, pulse frequency, pulse amplitude and inter-pulse interval (IPI) in ovariectomised sheep administered GnIH (1mg/hr) or vehicle. Sheep 1, 4 , 7 and 8 were controls to sheep 2,3, 5 and 6 receiving GnIH.

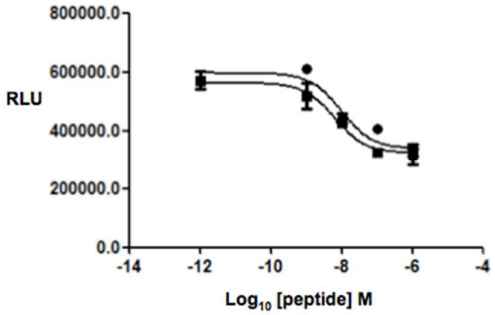
		Mean LH (IU/L) Saline administration	Mean LH (IU/L) GnIH administration
Subject 1	Prior to infusion	21.8±0.9	20.4±0.5
	During infusion	22.8±0.7	17.7±0.4
	After infusion	24.5±0.7	20.1±0.4
Subject 2	Prior to infusion	26.0±0.5	26.7±0.6
	During infusion	28.3±0.5	26.7±0.9
	After infusion	27.3±0.5	28.6±0.9
Subject 3	Prior to infusion	33.1±0.3	28.4±0.2
	During infusion	33.3±0.3	27.8±0.3
	After infusion	32.6±0.3	27.8±0.3
Subject 4	Prior to infusion	40.3±0.9	42.4±1.0
	During infusion	40.0±0.6	38.8±1.0
	After infusion	39.2±0.6	36.7±1.0
Subject 5	Prior to infusion	25.4±0.5	23.0±0.6
	During infusion	23.4±0.4	21.6±0.5
	After infusion	N/A	19.0±0.5

Supplementary table 2: Mean LH concentrations in five post menopausal women administered GnIH (50 mcg/kg) or saline infusions for 180 min. Data expressed as Mean ±SEM of peripheral LH concentrations obtained at 10-min intervals for the 180 minutes preceding and 60 minutes following infusion.

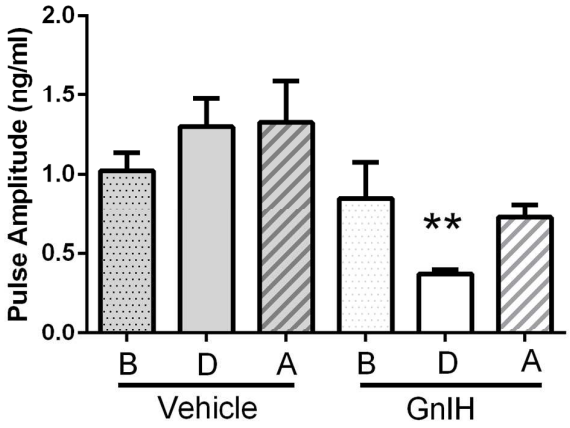
	Saline administration visit			GnIH administration visit		
	60-min AUC Baseline	60-min AUC after first kisspeptin bolus	60-min AUC after second kisspeptin bolus	60-min AUC Baseline	60-min AUC after first kisspeptin bolus	60-min AUC after second kisspeptin bolus
Subject 1	2.0	8.1	8.4	3.7	7.4	8.4
Subject 2	5.1	6.8	6.9	3.4	6.5	6.4
Subject 3	2.8	6.9	6.6	3.6	7.0	6.8
Subject 4	1.3	4.1	4.2	1.8	3.8	3.7

Supplementary table 3: Area-Under-Curve (IU/hr) in four male volunteers administered GnIH or saline infusions concurrently with kisspeptin-10 boluses. Increase in AUC after first kisspeptin-10 bolus indicates the stimulatory effect of kisspeptin-10 (0.3mcg/kg) on its own. The second bolus was administered concurrently with GnIH (50mcg/kg) or normal saline. AUCs following these second boluses of kisspeptin-10 were comparable to the first, suggesting a lack of modulatory effect of GnIH on kisspeptin-stimulated LH release.

Supplementary Figures



Supplementary Figure 1: *In vitro* comparsion of biological activity of the custom-synthesised GMP grade human GnIH used in the present study with that of GnIH previously used in published data involving non-human species. Both peptides induced an inhibition of forskolin-induced accumulation of cAMP with similar potencies. RLU= relative luciferase units. N=3.



Supplementary Figure 2: LH pulse amplitude before (B), during (D) and after (A) vehicle or GnIH (1mg/hr) infusions in ovariectomised ewes (n=4) each. Significant reduction in the amplitude of LH pulses (ANOVA $p<0.002$) was observed during GnIH infusion.

575