

**Original citation:**

Skorupskaite, Karolina, George, Jyothis T., Veldhuis, Johannes D, Millar, Robert P and Anderson, Richard A. (2017) Neurokinin 3 receptor antagonism decreases gonadotropin and testosterone secretion in healthy men. *Clinical Endocrinology*.

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Article type : 1 Original Article - UK, Europe

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## Neurokinin 3 receptor antagonism decreases gonadotropin and testosterone secretion in healthy men

Running title: NKB regulates GnRH/LH secretion in men

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**Acknowledgements:** We thank the men who volunteered to take part in the studies and the staff at the Royal Infirmary of Edinburgh Clinical Research Facility. We are grateful to Cat Graham for

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/cen.13445

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statistical advice, and to Forbes Howie and Kirsten Wilson for hormone measurements. We are grateful to AstraZeneca for the supply of AZD4901 (now known as MLE4901) used in this study.

**Conflict of interest:** This study was funded by the Wellcome Trust through the Scottish Translational Medicine and Therapeutics Initiative 102419/Z/13/A, and Medical Research Council grant G0701682. JTG has undertaken consultancy work for AstraZeneca and Takeda Pharmaceuticals and is an employee of Boehringer Ingelheim. RAA has undertaken consultancy work for AstraZeneca, Takeda Pharmaceuticals and Ferring Pharmaceuticals. RPM consults for Ogeda and is CEO of Peptocrone. JDV and KS have nothing to disclose.

## Summary

**Objective:** Patients with mutations of NKB and its receptor show hypogonadotrophic hypogonadism, but there is little evidence for the importance of this pathway in reproductive function in normal men, or its functional hierarchy with kisspeptin.

**Design:** An open label study wherein men (n=6) were administered the NK3R antagonist MLE4901 40mg orally twice a day for 7 days. Kisspeptin-10 (0.3 µg/kg iv bolus) was given before and on day 7 of NK3R antagonist treatment.

**Patients:** Subjects were healthy men.

**Measurements:** Reproductive hormones were measured before and during the NK3R antagonist administration, including frequent sampling on two occasions for analysis of pulsatile LH secretion.

**Results:** LH, FSH and testosterone secretion were decreased during NK3R antagonist administration. LH showed a biphasic response, being reduced after 24 hours of treatment ( $4.5 \pm 0.6$  IU/l pre-treatment to  $1.7 \pm 0.2$  IU/l,  $p < 0.05$ ), with partial recovery thereafter; but it was again decreased on day 7 ( $2.5 \pm 0.6$  IU/l,  $p < 0.05$  vs pre-treatment). FSH secretion was also suppressed, with a similar temporal pattern to that of LH. Testosterone secretion was decreased from 24 hours ( $18.4 \pm 1.6$  pre-treatment vs  $5.6 \pm 1.5$  nmol/l,  $p < 0.01$ ) and remained suppressed throughout the treatment period. Analysis of LH pulsatility showed that both basal and pulsatile LH secretion were markedly suppressed but there was no detected change in LH pulse frequency. Kisspeptin-10 stimulated LH secretion, with similar responses before and during NK3R antagonist administration.

**Conclusions:** These data demonstrate a central role for NKB/NK3R in the physiological regulation of reproductive function in men, and that this is functionally upstream of kisspeptin-mediated GnRH secretion.

**Key words (7 words):** Neurokinin B, Kisspeptin, GnRH pulsatility, Gonadotropins, Testosterone

## Introduction

Kisspeptin and neurokinin B (NKB) are hypothalamic neuropeptides now recognised to be key regulators of GnRH secretion and thus central to the control of the human hypothalamo-pituitary-gonadal (HPG) axis (Skorupskaite et al. 2014). Men and women with loss-of-function mutations in kisspeptin, neurokinin B or their cognate receptors (KISS1R and NK3R) show reduced GnRH secretion and hypogonadotropic pubertal delay (de Roux et al. 2003; Seminara et al. 2003; Topaloglu et al. 2009; Topaloglu et al. 2012), whilst activating mutations in the kisspeptin receptor are associated with precocious puberty (Teles et al. 2008).

Whilst kisspeptin potently stimulates gonadotropin secretion (George et al. 2011; Dhillo et al. 2005; Jayasena et al. 2015; Jayasena et al. 2011; Chan et al. 2011) and increases LH pulse frequency in men (George et al. 2011), the effects of exogenous NKB administration are unclear. In animal studies, both stimulatory and inhibitory actions of NKB on LH secretion has been observed (Billings et al. 2010; Navarro, Castellano, et al. 2011; Ramaswamy et al. 2011), whilst it had no effect on reproductive hormone concentrations or LH pulsatility when infused for 8 hours to healthy men and women (Jayasena et al. 2014). Administration of the NK3R antagonist ESN364 to normal men suppressed LH and testosterone but not FSH secretion (Fraser et al. 2016). However, the effect on LH lasted only few hours with recovery to baseline within 24 hours (Fraser et al. 2016). Antagonism of NK3R has been shown to decrease LH secretion and reduce the frequency of LH pulses in women with PCOS (George et al. 2016) and in gonadectomised animals (Li et al. 2015; Fraser et al. 2015), indicating a role for NKB-NK3R signalling in the regulation of pulsatile GnRH and thus LH secretion.

Kisspeptin and NKB are co-expressed by some neurones within the hypothalamus, in humans and other species. These neurones can also co-express the opioid dynorphin and thus have been termed KNDy neurones (Cheng et al. 2010). The interaction and functional hierarchy between kisspeptin and neurokinin B are inferred from observations from preclinical and clinical studies. Thus in animal models, *Kissr1* knockout mice were unable to show a stimulatory effect of the NK3R agonist senktide on LH secretion (Garcia-Galiano et al. 2012), and in juvenile male monkeys, kisspeptin-10 stimulated LH secretion after desensitization with senktide infusion (Ramaswamy et al. 2011). These results suggest the action of NKB is upstream of kisspeptin. This is supported by studies in patients with inactivating genetic defects in NKB (*TAC3*) and its NK3 receptor (*TAC3R*), showing slow GnRH secretion as inferred from low LH secretion, in whom kisspeptin-10 increased LH pulse frequency (Young et al. 2013). Women treated with an NK3R antagonist in a model of the mid-cycle LH surge showed largely preserved LH response to kisspeptin administration, although there were effects on timing and duration and the relationship to circulating estradiol concentrations was lost (Skorupskaite et al. 2016), providing evidence that neurokinin B is functionally upstream of kisspeptin in the regulation of GnRH secretion in healthy women.

We therefore sought to determine whether NKB has a physiological role in the regulation of the male reproductive axis by the administration of the specific NK3R antagonist MLE4901, with detailed analysis of potential effects on GnRH/LH pulsatility, and to explore the hierarchy between NKB and kisspeptin in the control of this pathway.

# Accepted Article

## **Materials and Methods**

### **Study subjects**

Six healthy men, aged 23-39 years were recruited to this study; all volunteers provided informed written consent and the study received Ethics Committee approval. Subjects had normal physical examination and serum LH, FSH and testosterone concentrations, and full blood count, renal function, electrolytes, liver function and electrocardiogram were within normal limits.

### **Study drugs**

The NK3R antagonist MLE4901 (previously known as AZD4901, kindly provided by AstraZeneca) was administered orally at 40 mg twice daily. It has been shown to completely block neurokinin B agonist senktide induced calcium influx in human NK3R expressing Chinese hamster ovary cells with high selectivity (Malherbe et al. 2011). This dose of MLE4901 reduced LH secretion and pulse frequency in normal women (Skorupskaite et al. 2016) and in women with Polycystic Ovary Syndrome (George et al. 2016). Kisspeptin-10 was administered by an intravenous bolus at 0.3 µg/kg as previously described (George et al. 2011), with the dose chosen as the lowest that caused maximal stimulation in LH secretion (George et al. 2011).

### **Protocol**

*Investigation of the effect of NK3R antagonism on gonadotropin and testosterone secretion.*

A schematic representation of the protocol is shown in Figure 1. Volunteers were administered the NK3R antagonist for 7 days. Single time point daily peripheral venous blood was sampled for LH, FSH and testosterone measurement between 08.00 and 10.00 hours at 24 hours before treatment started (day -1), and on days 2, 4, 6 and 7 of NK3R antagonist administration. During treatment, the daily blood sampling was performed immediately prior to the next dose of NK3R antagonist, i.e. 12 hours after the previous dose.

*Investigation of the effect of NK3R antagonist on LH pulsatility.*

On day -1 and on the last day of NK3R antagonist administration (day 7) volunteers attended our clinical research facility for 8 hours. On day 7, volunteers took the NK3R antagonist within an hour before starting the pulsatility assessment blood sampling. All visits commenced between 08.00 and 09.00 hours. Blood samples were collected via an indwelling intravenous cannula at 10 min intervals for 6 hours for assessment of LH pulsatility.

*Establishment of the hierarchy between kisspeptin and neurokinin B in the regulation of LH secretion.*

Kisspeptin-10 was administered at 6 hours of the pulsatility study described above, with blood sampling every 10 minutes for 2 hours after administration.

Safety blood tests including full blood count, renal function, electrolytes, and liver function were checked before commencing MLE4901, at the end of 7-day treatment and 2-3 weeks later. Post-treatment testosterone concentrations were measured 2 weeks after treatment.

## Hormone assays

Blood samples were centrifuged and serum frozen at  $-20^{\circ}\text{C}$  or below until analysis. LH and FSH were measured by ELISA as previously described (George et al. 2011). Testosterone was measured by liquid chromatography-tandem mass spectrometry. LH pulsatility was assessed by deconvolution analysis on blinded data with ApEn quantified as a measure of secretory regularity (Liu et al. 2009).

## Statistical analysis

Mean LH, FSH and testosterone concentrations over time were compared using one-way analysis of variance (ANOVA) with repeated-measures followed by Bonferroni's post hoc correction for multiple comparisons. Area under the curve (AUC) of LH during frequent sampling was determined by trapezoid integration on day -1 (control) and on day 7 of NK3R antagonist administration, and the change in AUC LH during one hour pre kisspeptin-10 injection and for the 2 consecutive hours thereafter was calculated. Comparisons across both time and between treatments were performed using repeated-measures two-way ANOVA with Bonferroni's multiple comparisons post hoc analysis. Paired Student's t-test (for parametric data) or Wilcoxon matched-pairs signed rank test (for non-parametric data) were used to assess changes in measures of LH pulsatility. Data are presented as mean  $\pm$  SEM.

## Ethical approval

The study protocol was approved by South East Scotland Research Ethics Committee (Ref: 09/S1101/67).

## Results

### NK3R antagonist decreases gonadotropin and testosterone secretion

LH secretion based on daily LH values decreased during NK3R antagonist administration ( $p < 0.03$ ), with a biphasic response: LH concentrations were significantly reduced after 24 hours of treatment ( $4.5 \pm 0.6$  IU/l on day -1 to  $1.7 \pm 0.2$  IU/l on day 2,  $p < 0.05$ ), then recovered ( $4.2 \pm 0.7$  IU/l on day 4 and  $3.7 \pm 0.7$  IU/l on day 6) but were again decreased on day 7 ( $2.5 \pm 0.6$  IU/l vs day -1,  $p < 0.05$ ) (Fig. 2A). To detect subtle changes in hormone secretion over time potentially overlooked by performing single time blood sampling, a more detailed analysis of LH secretion every 10 minutes for 6 hours post NK3R antagonist dose on day 7 of treatment showed a decrease in LH AUC throughout the 6 hours post-treatment compared to day -1 ( $16.8 \pm 2.7$  vs  $8.7 \pm 1.5$  IU/l over 6hr with NK3Ra,  $p = 0.0007$ , Fig. 2B).

FSH secretion was also suppressed during NK3R antagonist administration ( $p = 0.001$ ), being significantly lower after 24 hours of treatment ( $3.6 \pm 0.3$  vs  $2.4 \pm 0.4$  IU/l,  $p < 0.001$ ) (Fig. 2C). FSH showed a comparable pattern to changes in LH over the following days, being significantly lower than day -1 on days 2, 4 and 7 (each  $p < 0.05$  vs day -1) but not on day 6 of NK3R antagonist administration.

Testosterone secretion declined rapidly in response to NK3R antagonist at 24 hours ( $18.4 \pm 1.6$  day<sup>-1</sup> vs  $5.6 \pm 1.5$  nmol/l,  $p < 0.01$ ) but in contrast to LH, was consistently suppressed for the remainder of the treatment period ( $p < 0.05$  on days 4, 6 and 7 vs day -1) (Fig. 2D). Serum testosterone recovered to pre-treatment concentrations in all subjects 2 weeks later ( $19.8 \pm 2.0$  nmol/l).

### **Effect of NK3R antagonist on pulsatile LH secretion in men**

The characteristics of the pulsatile secretion of LH were determined before and after 7 days of NK3R antagonist treatment. The LH pulse profile from all individual subjects is shown in figure 3. LH pulse frequency was unchanged by NK3R antagonist ( $0.50 \pm 0.09$  vs  $0.47 \pm 0.07$  pulses/hr, ns) (Fig. 4A). However, consistent with the overall suppression of LH secretion seen in the daily time point analysis, other parameters of LH secretion were reduced. Both basal (nonpulsatile) and pulsatile mass of LH secretion were lower with NK3R antagonist treatment compared with day -1 (Fig. 4B-C, both  $p = 0.02$ ). The secretory mass of LH per pulse (Fig. 4D) was not significantly changed ( $p < 0.09$ ). NK3R antagonist increased the orderliness of the LH secretion pattern as assessed by approximate entropy (ApEn) ( $p = 0.02$ ; Fig. 4E).

### **NK3R antagonist does not reduce kisspeptin-induced LH secretion**

To assess the hierarchical interaction between kisspeptin and neurokinin B in the regulation of LH secretion in men, an intravenous bolus injection of kisspeptin-10 was administered after 6 hours of blood sampling on day-1 and on day 7 of NK3R antagonist treatment (Fig. 5). Kisspeptin-10 elicited a rapid increase in LH concentration ( $3.0 \pm 0.6$  at 6 hours vs  $4.8 \pm 0.5$  IU/L at 7 hours,  $p < 0.05$  and AUC LH  $2.7 \pm 0.4$  at 6 hours vs  $5.2 \pm 0.5$  IU/l hr at 7 hours,  $p < 0.01$ ; Fig. 5A-B). The stimulation of LH secretion by kisspeptin-10 was maintained in the presence of NK3R antagonist (mean LH and AUC LH for kisspeptin-10+NKB antagonist: at 6 hours vs at 7 and 8 hours, both  $p < 0.01$ ; Fig. 5A-B) and stimulated LH concentrations were similar to those after kisspeptin-10 alone (AUC LH at 7 and 8 hours kisspeptin-10:  $5.2 \pm 0.5$  and  $3.9 \pm 0.4$  vs  $5.2 \pm 0.7$  and  $4.0 \pm 0.6$  IU/l hr with NK3R antagonist, ns). However, calculation of the LH response to kisspeptin-10 as  $\Delta$ AUC over the 2 hours following administration suggested an enhanced response during NK3R antagonist treatment (kisspeptin-10 alone  $\Delta$ AUC LH 7 and 8 hours:  $2.5 \pm 0.2$  and  $1.2 \pm 0.2$ , vs kisspeptin-10 with NK3R antagonist  $3.8 \pm 0.5$  and  $2.5 \pm 0.4$ , both  $p < 0.05$ ; Fig. 5C) reflecting the lower LH concentrations with the NK3R antagonist at the time of kisspeptin administration with similar LH concentrations after administration of kisspeptin with and without the NK3R antagonist.

### **Tolerability and safety**

MLE4901 treatment was well tolerated. One man reported reduced libido whilst on NK3R antagonist and this recovered after the completion of the study. Haematology and biochemistry safety parameters remained stable in all subjects throughout the study period.

### **Discussion**

The present study investigated the role of neurokinin B in the control of the reproductive axis in healthy men and its interaction with kisspeptin in the regulation of GnRH secretion, using LH as a surrogate. Pharmacological blockade of NKB-NK3R signalling caused a rapid and marked decrease in



LH, FSH and testosterone secretion. However, the stimulatory LH response to kisspeptin after 7 days of NK3R antagonist was maintained. NK3R antagonism did not affect the frequency of LH pulses in men, but other aspects of the pulsatile nature of LH secretion were markedly reduced. These data provide novel evidence that NKB is a major regulator of the physiological regulation of GnRH/LH and testosterone secretion in men. They also support a predominantly hierarchical relationship whereby central action of neurokinin B is proximal to kisspeptin in the modulation of GnRH/LH secretion, as previously indicated in non human primates (Ramaswamy et al. 2011), in men with NKB/NK3R mutations (Young et al. 2013), and in healthy women (Skorupskaite et al. 2016).

These data demonstrate that in men, NK3R antagonist administration decreased LH secretion in a biphasic pattern, with a marked reduction after 24 hours administration (on day 2), and on day 7. Consistent with this being by suppression of GnRH secretion, NK3R antagonist also lowered FSH concentrations on days 2, 4 and 7 of treatment, albeit to a lesser extent than LH secretion. NK3R antagonists have been reported to suppress LH secretion in ovariectomised ewes (MRK-08) and castrate monkeys (ESN364) (Li et al. 2015; Fraser et al. 2015) but with only a transient effect in normal men (ESN364) (Fraser et al. 2016) without suppression of FSH secretion in either men (Fraser et al. 2016) or castrate monkeys (Fraser et al. 2015). This may reflect the greater effect on LH (and by inference GnRH) observed with MLE4901 at 40 mg BD than with the other antagonists investigated.

Sustained suppression of testosterone secretion was observed throughout the 7 days of treatment. Previously administration of this NK3R antagonist, MLE4901, reduced both LH and testosterone secretion in women with PCOS after 7 days (George et al. 2016). The simultaneous decrease in serum LH and testosterone concentrations within 24 hours of treatment suggests a central role of neurokinin B in the regulation of GnRH/LH secretion, which is further supported by changes in the pattern of LH pulsatility. It is possible that recovery of suppressed LH secretion while testosterone levels remain low on days 4 and 6 may indicate a direct gonadal effect of the NK3R antagonist, as *NKB-NK3R* mRNA has been detected in human granulosa cells (Garcia-Ortega et al. 2014; Cejudo Roman et al. 2012). However, *NKB-NK3R* mRNA has not been reported in human testis. It is however plausible that the initial fall in testosterone results in reduced negative feedback with a secondary recovery in LH secretion, although caution must be taken when interpreting those findings given that once only daily blood sampling may miss subtle changes in hormone secretion especially when there are relatively rapid changes in steroid feedback and the pathways that mediate that feedback are likely to be impacted by this drug. Nevertheless, analysis over 6 hours after dosing on day 7 showed consistent suppression of LH suggesting that suppression would persist with chronic antagonism of NKB-NK3R signalling. FSH secretion is less sensitive to rapid changes in GnRH secretion, which may account for the slightly different profile of the FSH response to NK3R antagonism over the 7 days of treatment.

The present study has shown that in normal men NK3R antagonist administration markedly reduced both basal (i.e. nonpulsatile) LH secretion and the total amount of LH secreted in pulses, contributing to overall lower serum LH concentrations. The regularity of the LH secretory pattern and by inference GnRH secretion, showed greater orderliness, as indicated by reduced ApEn. NK3R antagonist did not alter the frequency of LH pulses, although the ability to detect a change in LH pulse frequency may have been limited by the small number of men studied here, and the limited duration of frequent sampling. This contrasts with data from administration of the same NK3R



antagonist to women with PCOS (George et al. 2016) and to normal women in a model of the LH surge (Skorupskaite et al. 2016) and to administration of different NK3R antagonists (MRK-08 and ESN364) to ovariectomised ewes (Li et al. 2015; Fraser et al. 2015), where reductions in LH pulse frequency were demonstrated. This may however reflect sexual dimorphism in that changes in the frequency of LH pulses are not seen in male reproduction (Veldhuis et al. 2012), unlike the changes in LH pulse frequency that characterise (but are not essential for) the normal menstrual cycle (Filicori et al. 1986; Hurley et al. 1984). Sexual dimorphism is well recognised in the kisspeptin-induced LH response, with a marked increase in LH in men but a variable response in women dependent on the sex-steroid milieu (Dhillon et al. 2007; George et al. 2012). The female hypothalamus has significantly more kisspeptin cell bodies and fibres compared to men (Hrabovszky et al. 2010), and there is a greater abundance of neurokinin B-expressing neurones in ewes than rams (Goubillon et al. 2000), indicating gender differences in the NKB-kisspeptin pathway. Testosterone is also a regulator of LH pulse frequency in men (Veldhuis et al. 2008), thus the fall in serum testosterone is likely to have counteracted any suppressive effect of NK3R antagonism on LH pulse frequency. Nevertheless, the changes in LH secretion induced by the NK3R antagonist in this study, in concordance with a stimulatory action of neurokinin B and an inhibitory action of NK3R antagonist on LH pulses in animal studies (Li et al. 2015; Fraser et al. 2015), supports the notion that neurokinin B acts centrally to modulate pulsatile GnRH secretion in men and that there are complex neurokinin B/kisspeptin interactions that result in the characteristics of the pulsatile secretion of GnRH and thus LH.

Kisspeptin-10 stimulated LH secretion in the presence of NK3R antagonist in normal men. This supports findings from animal and human studies, in particular the demonstration that kisspeptin-10 infusion restored pulsatile LH secretion in men and women with inactivating mutations in neurokinin B pathway (Young et al. 2013) and in normal women in a model of the mid-cycle LH surge (Skorupskaite et al. 2016), that neurokinin B signalling is functionally upstream of kisspeptin. There was a greater change in LH secretion in response to kisspeptin-10 in the presence of the NK3R antagonist, but this is likely to reflect the lower basal LH concentrations following 7 days of NK3R antagonist administration. There was no difference in mean LH levels after kisspeptin-10 administration alone or following NK3R antagonist treatment. NKB may modulate kisspeptin action by altering the sensitivity of GnRH neurones to the latter and/or by acting on GnRH terminals enhancing, either synergistically or additively, kisspeptin effects. Although NKB/Kp immunoreactive terminals are in close apposition to GnRH terminals in the median eminence (Burke et al. 2006), no NK3R expression on GnRH neurones has been shown in mice and ewes (Amstalden et al. 2010; Navarro, Gottsch, et al. 2011). Direct action of NKB on GnRH neurones therefore seems unlikely.

Conversely, co-infusion of neurokinin B itself and kisspeptin-54 in healthy men stimulated gonadotropin and testosterone secretion significantly less than with kisspeptin-54 alone and had no effect on LH pulsatility (Narayanaswamy et al. 2016). As reductions in *GnRH* and *Kiss1r* mRNA were observed after administration of the NKB agonist senktide in rats (Grachev et al. 2012), it was proposed that NKB can act to inhibit GnRH and kisspeptin transcription to reduce the stimulatory action of kisspeptin on gonadotropins (Narayanaswamy et al. 2016). Gonadotropin inhibitory hormone (GnIH) also appears to be upstream of kisspeptin, having no suppressive effect on kisspeptin-induced LH secretion in a recent study in men (George et al. 2017), although its interaction with neurokinin B on modulating GnRH secretion remains unknown. While it is possible that NKB exerts variable stimulatory and/or inhibitory downstream actions on kisspeptin signalling,

perhaps in response to the sex steroid feedback environment, the present data clearly support a stimulatory role of NKB signalling in the normal male HPG axis.

The present study has strengths in that a specific neurokinin-3 receptor antagonist was used, detailed LH pulse profiling was performed with blinded pulse analysis, and paired data were obtained for the control period and during administration of NK3R antagonist. The lack of suppression in the frequency of LH pulses by the NK3R antagonist in men is in contrast to the same dose of MLE4901 reducing LH pulse frequency in women with PCOS (George et al. 2016) and in normal women (Skorupskaite et al. 2016), which may reflect sexual dimorphism. However, the sample size is small and a placebo control was not employed in this mechanistic exploratory study.

In summary, we have shown that NK3R antagonism resulted in suppression of both LH and FSH concentrations, and a sustained decrease in testosterone secretion in healthy men over a 7 day period, indicating an important role for neurokinin B in the physiological regulation of the male HPG axis. We showed that the NK3R antagonist reduced some aspects of pulsatile LH secretion in men. Assessment of the interaction between neurokinin B and kisspeptin showed that LH response to kisspeptin was maintained in the presence of NK3R antagonist, supporting a functionally hierarchical relationship of neurokinin B being predominantly proximal to kisspeptin. These data thus indicate that neurokinin B modulates GnRH/LH secretion in men, with potential for therapeutic development as well as exploration of its contribution to disorders of reproductive function.

**Authors' roles:** KS, JTG, RPM and RAA contributed to conception and design of the study. KS was responsible for acquisition of data. KS, RAA and JDV analysed the data. All authors were involved in data interpretation. KS drafted the manuscript, which was edited by JTG, RPM, JDV and RAA. All authors have approved the final manuscript.

**Funding:** This study was funded by the Wellcome Trust Scottish Translational Medicine and Therapeutics Initiative (STMTI) and MRC grant G0701682 to RAA and RPM.

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## Figures:

**Figure 1. Study protocol.** 6 healthy men were administered the NK3R antagonist MLE4901 orally for 7 days. LH, FSH and testosterone were measured on days -1, 2, 4, 6 and 7 during the study. LH pulsatility was assessed for 6 hours on day -1 and on day 7, the last day of NK3R antagonist treatment. Kisspeptin (KP)-10 was administered by intravenous bolus at 6 hours with further frequent blood sampling for 2 hours on day -1 and day 7.

**Figure 2. LH, FSH and testosterone response to administration of NK3R antagonist in healthy men (n=6).** (A) Mean of daily LH concentrations during 7 days of NK3R antagonist treatment and (B) LH AUC during 6 hours of 10 minute LH sampling on day-1 and on day 7 of NK3R antagonist administration. Mean FSH (C) and testosterone (D) response to NK3R antagonist administration. Data are presented as mean  $\pm$  SEM. \*,  $p<0.05$ ; \*\*,  $p<0.01$ ; \*\*\*,  $p<0.001$ ; \*\*\*\*,  $p<0.0001$  vs day -1.

**Figure 3.** LH profiles sampled at 10 min intervals from 6 individual subjects on control day -1 (*black circles*) and on day 7 of NK3Ra administration (open circles). Arrows indicate LH pulses detected by blinded deconvolution with (open arrows) and without (black arrows) NK3R antagonist treatment.

**Figure 4. Analysis of 6 hour LH secretory pattern on day 7 of NK3R antagonist treatment compared to control day -1 in healthy men.** Mean LH pulse frequency (A), basal (nonpulsatile) LH secretion (B), pulsatile LH secretion (C), LH secretory mass per pulse (D) and the relative orderliness/regularity of LH secretory pattern (E) on day 7 of NK3R antagonist treatment compared to day -1. Data are presented as mean  $\pm$  SEM. \*,  $p=0.02$ .

**Figure 5. LH response to kisspeptin-10 injection with and without NK3R antagonist pre-treatment in healthy men.** (A) Mean LH concentrations from 5 to 8 hours of frequent sampling following kisspeptin-10 administration at hour 6; (B) AUC LH and (C)  $\Delta$ AUC LH were compared over one hour before kisspeptin-10 administration (hour 6), with 2 hours post kisspeptin-10 administration (hours 7 and 8), with (day 7) and without (day -1) NK3R antagonist pre-treatment during frequent blood sampling for LH every 10 minutes. Data are presented as mean  $\pm$  SEM. \*,  $p<0.05$ ; \*\*,  $p<0.01$ ; \*\*\*\*,  $p<0.0001$ .



Days of NK3Ra

-1

1

2

3

4

5

6

7

Intervention

**Neurokinin 3 receptor  
antagonist**  
40mg BD oral 7 days

Pulse study

**10 min LH sampling**  
for 6 hours

Hierarchy study

**KP-10 (0.3µg/kg)**  
iv bolus at 6 hours

**10 min LH sampling**  
for 2 hours

**Repeated on  
day 7** of  
neurokinin 3  
receptor  
antagonist  
treatment

Spot LH, FSH, T









