The roles of kisspeptin and neurokinin B in GnRH pulse generation in humans, and their potential clinical application

Richard A Anderson[1] and Robert P Millar[2]

[1]MRC Centre for Reproductive Health, Queens Medical Research Institute, University of Edinburgh, Edinburgh, UK, EH16 4 TJ

[2] Centre for Neuroendocrinology, Department of Immunology, Faculty of Health Sciences, University of Pretoria, Gezina 0084, and Department of Integrative Biomedical Sciences and Institute of Infectious Disease and Molecular Medicine, Faculty of Health Sciences, Observatory 7925 South Africa

Please address correspondence to:

Professor Richard A Anderson

MRC Centre for Reproductive Health,

Queens Medical Research Institute, University of Edinburgh, Edinburgh, UK, EH16 4 TJ

richard.anderson@ed.ac.uk

Abstract

The delivery of GnRH in a pulsatile mode to the gonadotropes has long been known to be essential for normal reproductive function. There have been numerous studies aimed at dissecting out the mechanisms underlying GnRH pulse generation. The discovery of kisspeptin as an upstream regulator of GnRH attracted the possibility that pulsatile kisspeptin governed the pulsatile secretion of GnRH. Subsequent studies have shown the importance of the neurokinin B (NKB) system in modulating kisspeptin secretion and this GnRH. A number of studies in laboratory rodents have supported this notion. In contrast we present here data from clinical studies in men and women, in a range of contexts, that show that continuous infusion of kisspeptin 10 at receptor-saturating levels gives rise to an increase in LH(GnRH) pulse frequency. This has been demonstrated in normal healthy and hypogonadal men, in normal women during the mid-cycle LH surge as well as in men and women with mutations in the genes encoding NKB or its receptor NK3R, women with polycystic ovary syndrome (PCOS) treated with NK3R antagonist and in women treated with NK3R antagonist during the LH surge. These finds indicate that pulsatile secretion and action of kisspeptin on GnRH neurons is not required for the generation of LH (GnRH) pulses in humans. We also report that there is an absence of desensitisation in humans exposed to continuous infusion of kisspeptin-10 at receptor-saturating concentrations over 22 hours and briefly review GnRH, kisspeptin and NKB analogs and their clinical application.

Keywords : GnRH, kisspeptin, LH, neurokinins

1. Introduction

The secretion of GnRH into the portal vessels in a pulsatile manner is essential for its physiological action on the gonadotropes, particularly in regulating LH secretion. This has been recognised since the pioneering studies administering GnRH; in these, continuous infusion resulted in suppression of LH secretion, whereas administration in discrete pulses stimulated LH, and consequently gonadal function with ovulation in females (1, 2). These observations have become a mainstay of clinical practice using GnRH analogues, with chronic administration of GnRH agonists used in a range of treatments and conditions where suppression of gonadotropin secretion and thus gonadal steroidogenesis is required, including assisted reproduction, medical management of uterine fibroids and endometriosis, and in prostate and breast cancer (3). The mechanisms underlying the pulsatile activity of GnRH neurones have, however, remained cryptic, and data from animal studies are reviewed in detail by other articles in this Special Edition. Here we present and discuss clinical studies of relevance to this, which have explored the stimulatory effect of kisspeptin on pulsatile GnRH to investigate whether this regulatory pathway, and that of the KNDy system more generally, is a key component of the pulse generation mechanism or not.

The kisspeptin prohormone is proteolytically processed to peptides with increasing truncation of the amino terminus to generate kisspeptin 54, 14,13 and 10, all of which have the same 10 amino acid carboxyl terminal sequence and have full intrinsic biological activity. However, Kp-54 has a longer half life (4).

2. Clinical studies with kisspeptins

Initial clinical studies on kisspeptin effects in humans involved administration of the full length Kp-54 to men and subsequently to women. These demonstrated that kisspeptin infusion (4pmol/kg for 90 min) in men resulted in a rapid stimulation of LH secretion (as a surrogate for GnRH secretion) (4). Subsequent studies in women showed that LH secretion was poorly stimulated by bolus injections of Kp-54 (up to 6.4nmol/kg) other than in the preovulatory phase (5).

Our group investigated effects on LH pulsatile secretion using the shorter 10 amino acid form of kisspeptin (Kp-10) (6), in part due to the prohibitive cost of manufacturing the necessary GMP-quality 54 amino acid peptide for clinical studies. Dose-finding studies demonstrated a rapid LH response to bolus injections of Kp-10 at doses of 0.01 to 3 mcg/kg (7.7 pmol/kg to 2.3 nmol/kg), peaking about 20 minutes after administration with a maximal response to 1mcg/kg (0.77nmol/kg) (6). Using a frequent blood sampling protocol, Seminara and colleagues showed that bolus injection of Kp-10 induced an immediate LH pulse (figure 1A and B) of longer duration than induced by either bolus injection or short infusions of GnRH, suggesting that kisspeptin induces sustained GnRH secretion (7). Kisspeptin-induced LH pulses were followed by a delay before the subsequent endogenous pulse, which was interpreted as a resetting of the GnRH pulse generator (7). This supported our more direct studies showing an increased pulse frequency during continuous infusion. When 1.5mcg/kg/hr (1.1 nmol/kg/hr) was infused, 10 minute blood sampling facilitated the analysis of the pulsatile characteristics of LH secretion, particularly pulse frequency, over a prolonged period. This demonstrated that continuous Kp-10 infusion resulted in increasing LH secretion over the subsequent 9 hours of infusion and sampling, with a robust increase in LH pulse frequency and LH mass per pulse (figure 1C and D) (6). These studies clearly showed that constant kisspeptin stimulation of GnRH neurones can drive increased frequency of pulsatile GnRH secretion in humans, and thus that other mechanisms and/or pathways are required to translate this into pulsatile GnRH activity, rather than the kisspeptin system being the pulse generator itself. Since we were using Kp-10 infusion concentrations which maximally stimulated LH (GnRH), the increase in pulse frequency appears to result from Kp-10

stimulating or enhancing activity of an upstream and/or downstream pulse generator and do not support kisspeptin being the pulse generator. These conclusions from clinical studies appear to be at odds with findings from experiments in rodents which interpret that it is pulsatile activity of kisspeptin neurons that translates into pulsatile secretion of GnRH (reviewed in (8)), and experiments in other species provide evidence for both arguments (9-11). As summarised by Teresawa (12) “Herbison and colleagues have clearly shown that kisspeptin neurons are a major cell group modifying GnRH pulse generation, However, there are several phenomena where it might be premature to call the kisspeptin neuron the pulse generator”. Certainly our clinical findings indicate that kisspeptin cannot be regarded as “the pulse generator” in humans. Reconciling these differences and conducting incisive dissection of the system in humans and animals is an exciting backdrop for future research, bearing in mind that synchronicity does not confirm causality.

We also explored the possibility of kisspeptin tachyphylaxis which has been proposed in animal studies including in non-human primate (13). We infused higher doses of Kp-10 to determine if this could result in desensitisation and thus suppression of LH secretion (6). Prolonged infusion of a maximally stimulatory dose of 4mcg/kg/hr for 22 hours resulted in continuing stimulation of LH secretion (figure 1 E), with no evidence of desensitisation, with high sustained LH concentrations of around 30 IU/L achieved in some men. Similar experiments in mildly hypogonadal men with diabetes also showed prolonged stimulation of LH secretion with increase pulse frequency (figure 1 F and G), with testosterone levels rising into the normal range by the end of 11 hours of Kp-10 infusion (14). The inability to induce desensitisation of LH secretion with Kp-10 provides further support to the concept that physiologically, kisspeptin provides a constant drive to GnRH neurones, and that kisspeptin secretion is necessary for pulsatile secretion of GnRH. It is possible that more prolonged administration might induce desensitisation, noting that the physiological LH surge lasts longer than 22 hours. Studies using potent kisspeptin peptide agonists, notably Tak448 (now termed MVT-602), do show marked suppression of LH (and testosterone secretion) when administered to men, after a period of stimulated LH secretion over the initial 2 days of administration (15). Data in women also show this initial stimulation, but longer-term responses were not reported (16). Thus the GnRH neurone is susceptible to desensitisation by a kisspeptin agonist of sufficient potency, even if this is not demonstrable using the natural ligand. This is clearly a pharmacological phenomenon (as for GnRH desensitisation) as there is no evidence that desensitisation occurs as a physiological mechanism.

In women, the effect of kisspeptin on GnRH/LH secretion varies across the menstrual cycle, being modest in the early follicular phase but more marked in the pre-ovulatory period (5, 17, 18) and is further limited during administration of exogenous sex steroids in the form of the contraceptive pill (19). This raises the possibility that kisspeptin contributes to the mid-cycle LH surge that drives ovulation. This has been directly investigated using Kp-10, and a model of the mid-cycle surge in which transdermal estradiol is administered to women in the mid-follicular phase. This initially suppresses LH secretion, through negative feedback, but this then changes to positive feedback with surge-like stimulation of LH at 48 hours (20). Importantly, it must be remembered that while there is a physiologic increase in GnRH secretion driving the midcycle surge in LH, the changing pituitary response is also a major contributor. This is most clearly demonstrated by the finding that GnRH deficient hypogonadotrophic women treated with pulsatile administration of GnRH generate an ovulatory LH surge without any change in frequency or dose of the administered GnRH, as originally demonstrated in non-human primates (2). In our studies infusion of Kp-10 from 24 hours after the start of estrogen administration (i.e. during the negative feedback period) led to a switch to positive feedback with a dramatic stimulation of LH secretion 8 hours later (21). This was confirmed with a direct positive relationship between estradiol concentrations and the LH response to Kp-10. LH pulse frequency was stimulated by Kp-10 in this model, contrasting with no effect on that parameter in women in the early follicular phase (22). Table

1 provides a summary of the effects of Kp-10 administration on LH pulse frequency in both men and women, in different models.

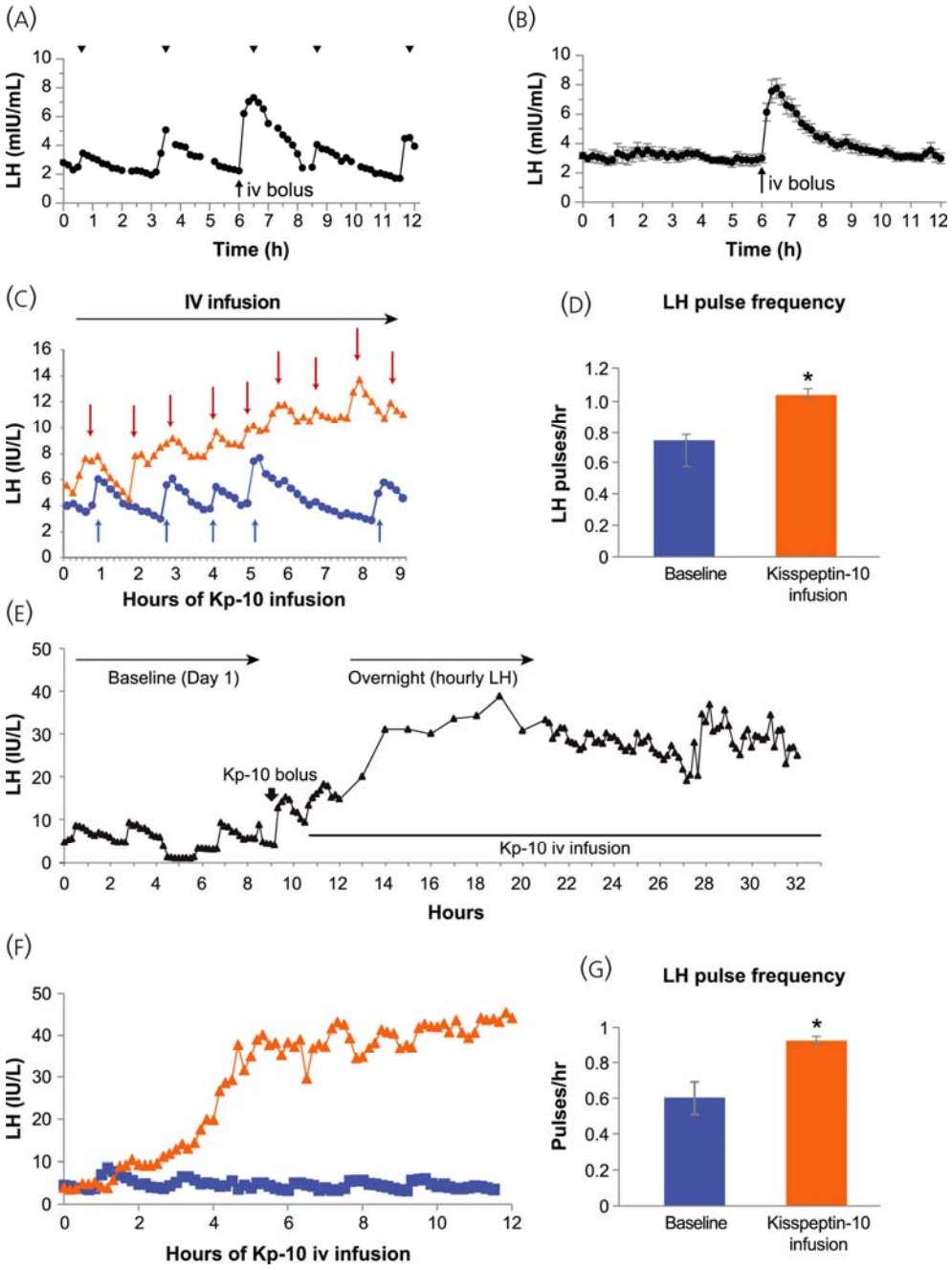

Figure 1. Kp stimulates GnRH/LH pulsatile secretion but does not readily result in desensitisation (all data in men). A and B, Kp-10 induces an immediate pulse of LH (7). A shows data from a single man with arrowheads indicating LH pulses, B shows mean±SEM, n=13. C, Kp-10 continuous infusion stimulates LH pulse frequency (blue: no treatment; orange, during Kp-10 infusion in the same man). Arrows indicated LH pulses. D, Mean LH pulse frequencies in the 6 men in the study (6). E and F, High dose (4 mcg/kg/hr) Kp-10 infusion does not result in desensitization (6, 14). E shows data from a single healthy man. In F, blue is baseline LH secretion, orange shows LH secretion in the same man with mild hypogonadism during continuous Kp-10 infusion.G, mean LH pulse frequency in the group of men represented in F (n=5). * $p<0.05$ vs baseline.

Table 1. Summary of clinical studies investigating the impact of administration of kisspeptin and neurokinin B antagonists separately and in combination on LH pulse frequency

Hormone/drug administered	Clinical setting	Effect on LH pulse frequency	Reference
Kp-10	Healthy men	Increase	George et al 2011 (6)
Kp-10	Men-mild hypogonadism	Increase	George et al 2013 (14)
Kp-10	Men and women with *TAC3* and *TAC3R* mutations	Increase	Young et al 2013 (24)
Kp-10	Women-LH surge model	Increase	Skorupskaite et al 2016 (21)
Kp-10	Women with PCOS	No change	Skorupskaite et al 2020 (29)
Kp-10 in presence of NK3R antagonist	Women-LH surge model	Increase	Skorupskaite et al 2016 (21)
Kp-10 in presence of NK3R antagonist	Women with PCOS	Increase	Skorupskaite et al 2020 (29)
NK3R antagonist	Healthy men	No change	Skorupskaite et al 2017 (27)
NK3R antagonist	Women-early follicular phase	No change	Skorupskaite et al 2018 (22)
NK3R antagonist	Women-LH surge model	Decrease	Skorupskaite et al 2016 (21)
NK3R antagonist	Postmenopausal women with hot flushes	Decrease (no change in women without hot flushes)	Skorupskaite et al 2018 (28)

Kp-10 increases LH pulse frequency, also seen in absence of NK3R signalling. NK3R antagonism can slow LH pulse frequency, most clearly when it is elevated (eg in PCOS, midcycle LH surge, menopause), but this is not clearly demonstrable when it is slower (eg in men, women in the follicular phase).

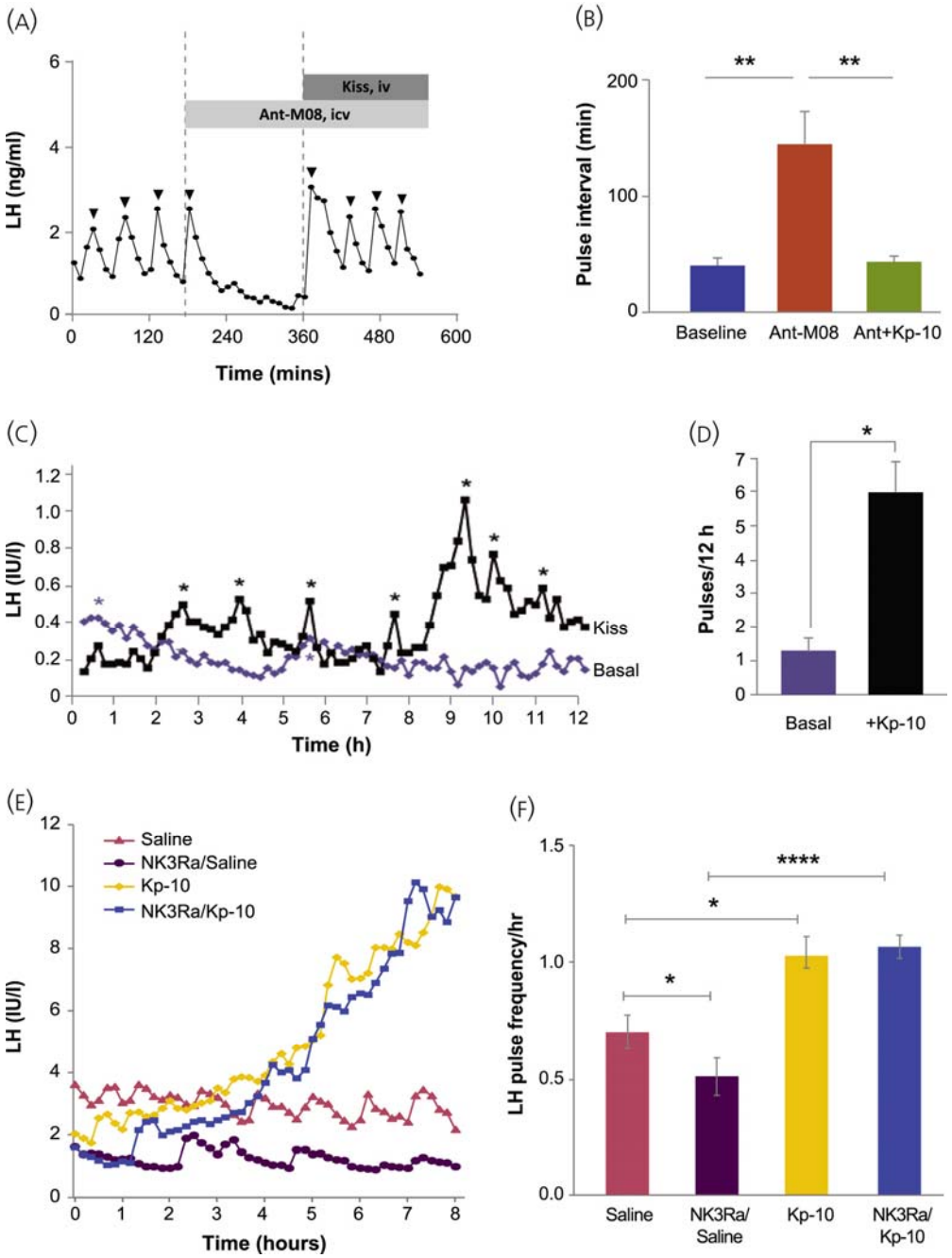

Figure 2. Inhibition of Neurokinin B signalling (antagonists or inactivating mutation in NK3R) suppresses GnRH/LH pulsatile secretion which is restored by continuous Kp-10 infusion. This shows that NKB action is upstream of KP-10 and that GnRH/LH pulsatility does not require kisspeptin pulsatility. A and B: icv administration of the NK3 antagonist MRK-08 in ovariectoimised sheep inhibits GnRH/LH pulsatility which is restored by continuous infusion of Kp-10 (23); arrowheads in A indicate LH pulses, ** $p<0.01$ between indicated groups. C and D: human male with NK3R mutation, showing baseline LH secretion (blue) and during continuous Kp-10 infusion (black), asterisks in C indicate LH pulses (24). D shows mean LH pulse frequency in this group (n=4), * $P<0.05$ vs basal. E and F: human female treated with NK3R antagonist ± iv Kp-10 infusion, E shows data from a single woman receiving the 4 different treatment regimens (21), F shows mean LH pulse frequency (n=10). NK3R antagonist inhibits GnRH/LH pulsatility which is restored by continuous exposure to Kp-10. * $p<0.05$, **** $p<0.0001$ between indicated groups.

3. Clinical studies with neurokinin B agonists and antagonists

Following the demonstration of co-expression of kisspeptin, neurokinin B and dynorphin in some hypothalamic neurons, dubbed KNDy neurons, there have been only a few clinical studies investigating the interactions between kisspeptin and neurokinin B in the regulation of GnRH pulsatility. It is clear that neurokinin B is, at least predominantly, functionally upstream of kisspeptin in the regulation of GnRH secretion, in keeping with the anatomical studies showing kisspeptin-containing neuronal projections in apposition to GnRH neurones and terminals. In a study designed to demonstrate directly these relationships we inhibited LH pulsatility in ewes by infusion of the NKB antagonist MRK-08 and then infused Kp-10 continuously, which restored LH pulsatility (figure 2 A and B) (23). This functional relationship was demonstrated in humans in our study involving administration of kisspeptin to individuals with mutations in *TAC3* or *TAC3R*, encoding neurokinin B and its cognate receptor, the neurokinin 3 receptor, respectively (24). Such individuals show a phenotype of hypogonadotrophic hypogonadism: this contrasts with rodents with genetic ablation of *Tacr3* which are fertile with no GnRH deficiency (25). This discrepancy highlights the importance of human experimental research in interpreting normal and pathological regulation and dysregulation in the HPG axis. While detailed analysis of pulsatile LH secretion shows some variability between individuals with these mutations, LH pulses are clearly reduced in both frequency and amplitude. Continuous infusion of Kp-10 increased LH secretion, with near-normalisation of the pulsatile pattern and a marked increase in LH pulse frequency (figure 2C and D), clearly showing that the response to kisspeptin is not dependant on neurokinin B signalling and neurokinin B action is upstream of kisspeptin (24). It is noteworthy that the dose of continuous infusion of Kp-10 was at a receptor saturating level (maximal dose) as in our other studies and yet again this restored pulsatile LH. These findings suggest that although kisspeptin is required for LH (GnRH) secretion, kisspeptin pulsatility is not required and that the origin of pulse generation is upstream of KNDy neurones or within GnRH neurons themselves.

The role of neurokinin B has also been explored using pharmacological antagonists at the neurokinin 3 receptor. AZ4901 (subsequently renamed MLE4901 following acquisition by Millendo Therapeutics) and similar drugs were developed for potential use in the treatment of schizophrenia but were ineffective and unexpectedly found to suppress the reproductive axis; now recognised to reflect the role of neurokinin B as a key component of the regulation of GnRH secretion (26). In men (27), in women in both the early follicular phase (28) and in the above-described model of the midcycle surge (21), and in women with polycystic ovary syndrome (PCOS) (29), the neurokinin 3 receptor antagonist reduced LH secretion but did not prevent the stimulatory effect of Kp-10 (figure 2E and F, table 1). Intriguingly however the positive relationship between estradiol concentrations and the LH response to Kp-10 was lost during neurokinin 3 receptor antagonism treatment in both the midcycle LH surge model and in women with PCOS (21, 29). While further studies are required to clarify these relationships, the current findings suggest involvement of the neurokinin B signalling pathway in sensing and mediating estrogen feedback via kisspeptin onto GnRH secretion.

4. GnRH and KNDy neurones in hot flushes and their treatment

Links between KNDy neuropeptides now known to be critical to the regulation of GnRH secretion and the common and debilitating vasomotor disturbances known as hot flushes associated with the menopause date back several decades, when Casper documented the synchrony of hot flushes and LH pulses in some women (30). These regulatory pathways are currently the subject of intense interest due to the potential for the therapeutic intervention in the treatment of hot flushes, and clinical trials of neurokinin B antagonists are in advanced stages. Tachykinins have long been associated with hot flushes, in patients with carcinoid tumours where there are elevated circulating concentrations of substance P and neurokinin A

(31, 32), and from direct administration of substance P (33). Neurokinin B (and substance P) mRNA expression is increased in some neurons in the infundibular nucleus in postmenopausal women, with those neurons also shown to express estrogen receptors (34). Subsequently kisspeptin neurons were also shown to be hypertrophied in the postmenopausal hypothalamus, with a similar distribution to the neurokinin B-expressing neurons (35). As mentioned above, a degree of synchrony between hot flushes and LH pulses had been recognised (30) and more recently confirmed (36), leading to the hypothesis that increased KNDy neuron activity in the post-menopausal hypothalamus resulting from estrogen deficiency was a key driver of hot flushes (37). These neurons project to the thermoregulatory centre of the median preoptic nucleus. A schematic representation of these projections is shown in figure 3. Elegant optico-genetic experiments have shown that activation of kisspeptin-expressing neurons of the arcuate nucleus (in rodents) induces vasomotor responses comparable to hot flushes in both male and females, that this is sensitised by ovariectomy, and that the vasomotor response is prevented by neurokinin receptor antagonists (38). Thus KNDy neurones project both to the median eminence controlling GnRH secretion through kisspeptin signalling, and to the median preoptic area controlling the vasomotor outputs, through neurokinin B signalling. Whether the latter also involves Kisspeptin and Dynorphin is uncertain.

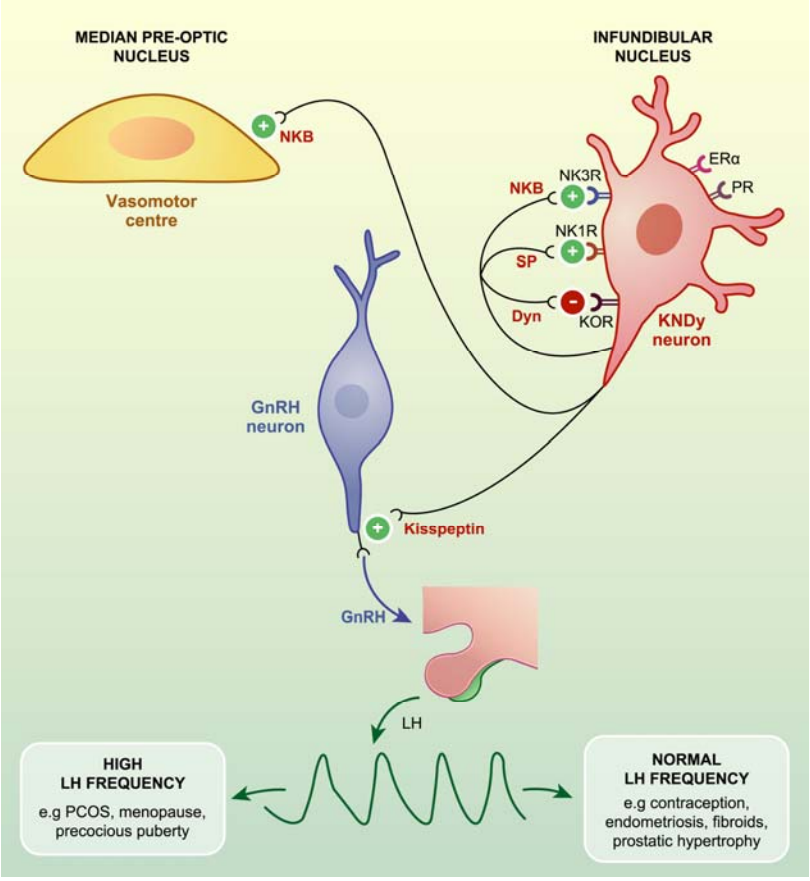

Figure 3. Schematic pathway by which KNDy neurons regulate both GnRH secretion and vasomotor activity. KNDy neurons in the infundibular nucleus project to both GnRH neurons (releasing kisspeptin acting on the KiSS1 receptor) and to the vasomotor centre (releasing neurokinin B (NKB) in the median pre-optic nucleus. Also shown are feedback projections on KNDy neurons, stimulatory in the case of NKB via its receptor NK3R and substance P (SP) via the NK1R receptor, and inhibitory via dynorphin acting on the kappa opioid receptor.

Although none of the above-described studies involving administration of kisspeptin to men or women, including post-menopausal women (19), reported induction of hot flushes, infusion of neurokinin B to premenopausal women resulted in rapid onset of hot flush-like symptoms in most subjects (39). This, with the aforementioned data, led to the development of clinical trials of neurokinin 3 receptor antagonists for this indication and the findings on several such drugs have now been reported. The first to report involved MLE4901 (40), now discontinued due to liver toxicity, but key findings from that and other early studies (28) have been substantiated in subsequent larger trials with other drugs. Most striking is the very rapid onset of the reduction of hot flush frequency, both by day and at night, generally by the 2nd day of drug administration, contrasting with the prolonged period needed for estrogen-based menopausal hormone therapy to have an effect. The degree of reduction of symptoms achieved is also dramatic, again greater than with estrogen-based treatment. The most advanced studies are with fezolinetant (previously ESN364) (41, 42), now in phase 3 trials. Another promising compound is elinzanetant (previously NT-814), which also shows good efficacy during 2 week treatment (43) and in a 12 week study (44). This drug differs in that it is an antagonist at both the neurokinin 1 as well as neurokinin 3 receptors, thus will antagonise effects of substance P in addition to NKB. Substance P infusion results in both vasodilation/flushing (33) and stimulation of LH secretion (45, 46). Thus antagonism at the neurokinin 1 receptor may be of relevance, though that is not yet clear from the clinical data. Initial pharmacodynamic data showing approximately 70% reductions in LH and testosterone with a single dose in men were reported for a further compound, SJX-653 (47), prior to anticipated testing in menopausal women, but further development has now been discontinued.

The rapid and substantial reduction in vasomotor symptoms with the above agents indicates a potential role in clinical practice, and especially where estrogen-based hormone therapy is contra-indicated (eg in women with breast cancer), but may also have potential in dual therapy with conventional hormone replacement to maximise the therapeutic benefit while minimising the necessary estrogen dose. Part of the reason for these striking characteristics of the response to these drugs may be the multiple sites of action within the one neuronal pathway, in that they inhibit KNDy neuron activity both at the level of the cell body in the arcuate/infundibular nucleus, and at the site of neurokinin B transmission at the terminals in the median preoptic nucleus (figure 3). This dual activity 'in series' (to take an electrical circuitry analogy) may therefore underpin the very effective suppression of activity at the vasomotor centre. It is noteworthy however that despite this, postmenopausal women taking these drugs show little suppression of GnRH secretion. In a small study investigating this, only limited suppression of LH secretion was seen during treatment with MLE4091, with no changes in LH pulse frequency. However in those women in the study who reported most troublesome hot flushes, which were markedly suppressed, there were small but clearer effects on LH secretion with a decrease in pulse frequency than was seen in the group as a whole (28). Effects of neurokinin 3 receptor antagonism that determine KNDy neuron drive to control GnRH in menopausal women may therefore be limited in contrast to the marked effects on what are thought to be projections of the same neurons to the vasomotor centre of the median preoptic area.

5. Mechanisms of modulation of gonadotrophin pulsatility in the KNDy-GnRH axis

The above review of the origin and modulation of gonadotropin pulsatility highlights existing and potential future clinical applications. Through our clinical studies we have gained insight into the mechanisms controlling GnRH pulsatility in humans, the involvement of the KNDy system of the arcuate nucleus, and how analogs in the system may be utilized to alter pulse frequency and applied to various reproductive pathologies. Importantly, we point out differences in pulsatility control in humans and laboratory rodents. We will now consider the mechanisms whereby these analogs can alter pulse frequency for the purpose of treating these pathologies in the hypothalamic-pituitary-gonadal axis. These discoveries have

spawned the development of new analogs and the 'repurposing' of existing CNS-directed analogs for utilization in the modulation of gonadotropin pulsatility and secretion. These molecules and their effects on gonadotropin secretion have been described above and their structures and properties reviewed in detail (48). We will, therefore, only briefly describe them as an *aide memoire* in the context of their mechanism of action.

6. GnRH

As mentioned earlier, the pioneering observation of GnRH pulsatility effects on reproduction were those of Knobil and colleagues who noted that pulsatile delivery of GnRH in monkeys gave rise to robust and continuing stimulation of gonadotropin. When GnRH was administered continuously there was a decline in gonadotropin secretion. A considerable body of research examined the mechanism underlying this desensitization of the gonadotrope response to GnRH. These included receptor downregulation through ligand-induced internalisation of the GnRH receptor, desensitisation of the intracellular signaling mechanisms including uncoupling from Gq, diminished generation of inositol tris phosphate, Ca^{2+}, and protein kinase C, and various other mechanisms. it has however become clear that receptor down regulation could not be the major mechanism as an absence of receptor would mean that GnRH could no longer have this desensitizing effect. Indeed, review of all the postulated mechanisms for continuous GnRH causing a desensitisation of the gonadotropes indicated that this was due to the inability to synthesize the beta subunit of LH. This was revealed in long term studies on gonadotropin production in patients receiving chronic GnRH agonists for prostatic cancer treatment in which it was observed that there continued to be high circulating levels of the alpha subunit after years of GnRH agonist treatment but an absence of the complete alpha/beta molecule heterodimerwhich is the biologically active form (49). This establishes that the major mechanism of desensitisation is not through receptor downregulation and post receptor inhibition of signaling mechanisms but through the decrease in biosynthesis of the beta subunit after continuous exposure to GnRH. The precise mechanisms involved have yet to be elucidated in spite of the wide use of GnRH agonists for clinical purposes over many decades and the mantra "if it works do we need to understand how?".

Although GnRH agonists are very effective pharmaceutical agents, they have a distinct limitation in that initially these analogs induce stimulation of gonadotropin and downstream steroid hormones for one to two weeks before the desensitisation phenomenon sets in. Thus in treatment of hormone-dependent disease such as endometriosis and prostatic cancer there is initially a flare of the disease state due to elevated sex steroid hormones. To overcome this disadvantage, GnRH antagonists were developed to have an immediate inhibition of pulsatile gonadotropin and sex steroid hormones. Examples of the application of GnRH antagonists having immediate inhibition are Degarelix for prostatic cancer and Cetrorelix and Ganirelix for inhibition of the LH surge for oocyte retrieval in IVF. For comprehensive reviews of peptide GnRH analogs and their application see reviews (50-52).

A significant limitation of peptide GnRH analogs is their 'all or none' effect giving rise to an inability to modify dose and partially suppress gonadotropin and sex steroids. This would be an advantage in pathologies such as endometriosis where partial suppression of estrogen production is capable of improving the condition without causing significant bone loss and other side effects such as hot flushes. A considerable effort was therefore directed at developing orally-active GnRH antagonists in which dosage could be modulated to achieve this end. Neurocrine developed an orally bioavailable non-peptide GnRH receptor antagonist, Elagolix (ORILISSA™), which has been approved by the FDA for the management of pain induced by endometriosis. Elagolix, with or without low-dose estrogen add-back therapy, has advanced through phase III for heavy menstrual bleeding associated with uterine fibroids (53) and other small molecule antagonists are similarly in development (54). However, in spite of the clear dosing advantage and convenience of an orally-active GnRH antagonist, long acting injectable peptide GnRH analog formulations, some of which last for up to a year, have advantages in compliance and convenience.

7. Kisspeptin

As reviewed above kisspeptin, as the 54 and 10 amino forms, has been used to stimulate the reproductive axis in a number of settings. Extended administration has been reported in humans to elicit what may be desensitization with a resulting lack of continuing stimulation of gonadotropin secretion in the setting of administration to women with hypothalamic amenorrhoea (55) where GnRH secretion is suppressed. However in a range of studies in men and women given continuous Kp-10 at a receptor saturating dose (ie dose eliciting maximal LH response) we observed no indication of tachyphylaxis (see above and figs 1 and 2). There is therefore some uncertainty that at high doses and prolonged exposure the natural kisspeptin peptides are robustly able to inhibit gonadotropin secretion. However highly active kisspeptin agonists developed by Takeda (eg TAK 448) were most effective in inhibiting gonadotropin in both rodent models and in men with prostatic cancer (15, 56). This may be a significant advantage as administration kisspeptin itself can be utilized to stimulate reproduction without concern for desensitization and super-active analogs can be employed in conditions requiring suppression.

The potential value of a kisspeptin antagonist for interrogating the mechanisms of pulse generation and for reproductive pathologies encouraged us to develop peptide kisspeptin antagonists. In the course of designing and testing over 100 analogs we developed a series of potent antagonists including one with a penetratin sequence at the amino terminus to facilitate penetration of the blood brain barrier. These analogs had high binding affinity and potently inhibited kisspeptin stimulation of GnRH neuron firing and GnRH secretion in rats and monkeys, LH in male and female rats and mice, and LH pulsatility (57), and ovulation in sheep (58, 59), and rats . These studies indicate that kisspeptin is required for GnRH/LH pulse generation but not necessarily that pulses of kisspeptin are needed for pulse generation. Indeed our clinical studies using continuous infusion argue the opposite. A parsimonious interpretation is that continuous kisspeptin (exogenous) or pulsatile (endogenous) drives the pulse generator.

Although there have been attempts to develop small molecule kisspeptin agonists and antagonists, to date this has led to little success. The only small molecule antagonist described (60) has not advanced into humans. There is a need to develop such molecules as they would find potential application in a number of conditions such as endometriosis, polycystic ovary syndrome, precocious puberty and induction of ovulation in IVF.

8. Neurokinin B analogs

Numerous neurokinin B antagonists have been developed as modulators in the central nervous system. These orally-active small molecules therefore provided a rich source of antagonists when the role of neurokinin B and Substance P in reproduction was discovered. As articulated above these have been developed for hot flashes and polycystic ovary syndrome. The majority of these analogs are orally-active small molecule antagonists: the only agonists developed are all peptides such as senktide.

9. Conclusion

There is a substantial body of research which shows that there is synchronisation of hypothalamic multiunit electrophysiological activity, and secretion of kisspeptin, GnRH and LH. This, together with experiments on the activation and inactivation of kisspeptin neurones and effects on GnRH and LH secretion, have led to the conclusion of causality in the relationship. However our extensive studies in humans have shown that continuous administration of kisspeptin is able to elicit or restore LH pulsatility, indicating that kisspeptin is required for LH pulsatility, but that kisspeptin pulsatility is not. From these human studies, kisspeptin therefore appears to facilitate and/or enhance upstream and/or downstream

generation of LH pulses, and that this is modulated by neurokinin B signalling, and likely by other neurotransmitters.

We summarise with these questions for further clinical research:

- What is the precise role of Dynorphin A in the KNDY system?
- To what extent does Substance P play a role along with Neurokinin B in the regulation of kisspeptin and thus GnRH secretion?
- Would tachykinin small molecule agonists be useful therapeutics?
- Kisspeptin antagonists with brain-penetrating and non-penetrating properties need to be developed for clinical investigation and as a therapeutic
- After 50 years of GnRH research, orally-active antagonists have been produced. What is the potential for new applications of these molecules?
- The GnRH pulse generator was identified almost 50 years ago. An ambition should be a definitive and complete identification and elucidation of its functioning.

Acknowledgements
The authors studies referred to herein were supported by MRC grant G0701682 and the Wellcome Trust Scottish Translational Medicine and Therapeutics Initiative (STMTI)

Conflicts of Interest
RAA has undertaken consultancy work for KaNDy Therapeutics and Sojournix Inc. RPM has no conflicts to report.

Data availability
There are no original data associated with this review

References

1. Belchetz PE, Plant TM, Nakai Y, Keogh EJ, Knobil E. Hypophysial responses to continuous and intermittent delivery of hypopthalamic gonadotropin-releasing hormone. *Science*. 1978; **202**(4368): 631-3.
2. Knobil E, Plant TM, Wildt L, Belchetz PE, Marshall G. Control of the rhesus monkey menstrual cycle: permissive role of hypothalamic gonadotropin-releasing hormone. *Science*. 1980; **207**(4437): 1371-3.
3. Anderson RC, Newton CL, Anderson RA, Millar RP. Gonadotropins and Their Analogs: Current and Potential Clinical Applications. *Endocr Rev*. 2018; **39**(6): 911-37.
4. Dhillo WS, Chaudhri OB, Patterson M, Thompson EL, Murphy KG, Badman MK, McGowan BM, Amber V, Patel S, Ghatei MA, Bloom SR. Kisspeptin-54 stimulates the hypothalamic-pituitary gonadal axis in human males. *J Clin Endocrinol Metab*. 2005; **90**(12): 6609-15.
5. Dhillo WS, Chaudhri OB, Thompson EL, Murphy KG, Patterson M, Ramachandran R, Nijher GK, Amber V, Kokkinos A, Donaldson M, Ghatei MA, Bloom SR. Kisspeptin-54 stimulates gonadotropin release most potently during the preovulatory phase of the menstrual cycle in women. *J Clin Endocrinol Metab*. 2007; **92**(10): 3958-66.
6. George JT, Veldhuis JD, Roseweir AK, Newton CL, Faccenda E, Millar RP, Anderson RA. Kisspeptin-10 is a potent stimulator of LH and increases pulse frequency in men. *J Clin Endocrinol Metab*. 2011; **96**(8): E1228-36.
7. Chan YM, Butler JP, Pinnell NE, Pralong FP, Crowley WF, Jr., Ren C, Chan KK, Seminara SB. Kisspeptin resets the hypothalamic GnRH clock in men. *J Clin Endocrinol Metab*. 2011; **96**(6): E908-15.
8. Herbison AE. The Gonadotropin-Releasing Hormone Pulse Generator. *Endocrinology*. 2018; **159**(11): 3723-36.

9. Keen KL, Wegner FH, Bloom SR, Ghatei MA, Terasawa E. An increase in kisspeptin-54 release occurs with the pubertal increase in luteinizing hormone-releasing hormone-1 release in the stalk-median eminence of female rhesus monkeys in vivo. *Endocrinology*. 2008; **149**(8): 4151-7.
10. Caraty A, Lomet D, Sebert ME, Guillaume D, Beltramo M, Evans NP. Gonadotrophin-releasing hormone release into the hypophyseal portal blood of the ewe mirrors both pulsatile and continuous intravenous infusion of kisspeptin: an insight into kisspeptin's mechanism of action. *J Neuroendocrinol*. 2013; **25**(6): 537-46.
11. Ezzat A, Pereira A, Clarke IJ. Kisspeptin is a component of the pulse generator for GnRH secretion in female sheep but not the pulse generator. *Endocrinology*. 2015; **156**(5): 1828-37.
12. Terasawa E. Mechanism of pulsatile GnRH release in primates: Unresolved questions. *Mol Cell Endocrinol*. 2019; **498**110578.
13. Seminara SB, Dipietro MJ, Ramaswamy S, Crowley WF, Jr., Plant TM. Continuous human metastin 45-54 infusion desensitizes G protein-coupled receptor 54-induced gonadotropin-releasing hormone release monitored indirectly in the juvenile male Rhesus monkey (Macaca mulatta): a finding with therapeutic implications. *Endocrinology*. 2006; **147**(5): 2122-6.
14. George JT, Veldhuis JD, Tena-Sempere M, Millar RP, Anderson RA. Exploring the pathophysiology of hypogonadism in men with type 2 diabetes: Kisspeptin-10 stimulates serum testosterone and LH secretion in men with type 2 diabetes and mild biochemical hypogonadism. *Clin Endocrinol (Oxf)*. 2013; **79**100-4.
15. MacLean DB, Matsui H, Suri A, Neuwirth R, Colombel M. Sustained exposure to the investigational Kisspeptin analog, TAK-448, down-regulates testosterone into the castration range in healthy males and in patients with prostate cancer: results from two phase 1 studies. *J Clin Endocrinol Metab*. 2014; **99**(8): E1445-53.
16. Abbara A, Eng PC, Phylactou M, Clarke SA, Richardson R, Sykes CM, Phumsatitpong C, Mills E, Modi M, Izzi-Engbeaya C, Papadopoulou D, Purugganan K, Jayasena CN, Webber L, Salim R, Owen B, Bech P, Comninos AN, McArdle CA, Voliotis M, Tsaneva-Atanasova K, Moenter S, Hanyaloglu A, Dhillo WS. Kisspeptin receptor agonist has therapeutic potential for female reproductive disorders. *J Clin Invest*. 2020; **130**(12): 6739-53.
17. Jayasena CN, Nijher GM, Comninos AN, Abbara A, Januszewki A, Vaal ML, Sriskandarajah L, Murphy KG, Farzad Z, Ghatei MA, Bloom SR, Dhillo WS. The effects of kisspeptin-10 on reproductive hormone release show sexual dimorphism in humans. *J Clin Endocrinol Metab*. 2011; **96**(12): E1963-72.
18. Chan YM, Butler JP, Sidhoum VF, Pinnell NE, Seminara SB. Kisspeptin administration to women: a window into endogenous kisspeptin secretion and GnRH responsiveness across the menstrual cycle. *J Clin Endocrinol Metab*. 2012; **97**(8): E1458-67.
19. George JT, Anderson RA, Millar RP. Kisspeptin-10 stimulation of gonadotrophin secretion in women is modulated by sex steroid feedback. *Human Reprod*. 2012; **in press**.
20. Baird DT, Thong KJ, Hall C, Cameron ST. Failure of oestrogen induced luteinizing hormone surge in women treated with mifepristone (RU 486) every day for 30 days. *Hum Reprod*. 1995; **10**(9): 2270-6.
21. Skorupskaite K, George JT, Veldhuis JD, Millar RP, Anderson RA. Interactions Between Neurokinin B and Kisspeptin in Mediating Estrogen Feedback in Healthy Women. *J Clin Endocrinol Metab*. 2016; **101**(12): 4628-36.
22. Skorupskaite K, George JT, Veldhuis JD, Anderson RA. Neurokinin B Regulates Gonadotropin Secretion, Ovarian Follicle Growth, and the Timing of Ovulation in Healthy Women. *J Clin Endocrinol Metab*. 2018; **103**(1): 95-104.
23. Clarke IJ, Li Q, Henry BA, Millar RP. Continuous Kisspeptin Restores Luteinizing Hormone Pulsatility Following Cessation by a Neurokinin B Antagonist in Female Sheep. *Endocrinology*. 2018; **159**(2): 639-46.
24. Young J, George JT, Tello JA, Francou B, Bouligand J, Guiochon-Mantel A, Brailly-Tabard S, Anderson RA, Millar RP. Kisspeptin Restores Pulsatile LH Secretion in Patients

with Neurokinin B Signaling Deficiencies: Physiological, Pathophysiological and Therapeutic Implications. *Neuroendocrinology*. 2013; **97**(2): 193-202.
25. Kung TT, Crawley Y, Jones H, Luo B, Gilchrest H, Greenfeder S, Anthes JC, Lira S, Wiekowski M, Cook DN, Hey JA, Egan RW, Chapman RW. Tachykinin NK3-receptor deficiency does not inhibit pulmonary eosinophilia in allergic mice. *Pharmacological research*. 2004; **50**(6): 611-5.
26. Topaloglu AK, Reimann F, Guclu M, Yalin AS, Kotan LD, Porter KM, Serin A, Mungan NO, Cook JR, Imamoglu S, Akalin NS, Yuksel B, O'Rahilly S, Semple RK. TAC3 and TACR3 mutations in familial hypogonadotropic hypogonadism reveal a key role for Neurokinin B in the central control of reproduction. *Nat Genet*. 2009; **41**(3): 354-8.
27. Skorupskaite K, George JT, Veldhuis JD, Millar RP, Anderson RA. Neurokinin 3 receptor antagonism decreases gonadotropin and testosterone secretion in healthy men. *Clin Endocrinol (Oxf)*. 2017; **87**(6): 748-56.
28. Skorupskaite K, George JT, Veldhuis JD, Millar RP, Anderson RA. Neurokinin 3 Receptor Antagonism Reveals Roles for Neurokinin B in the Regulation of Gonadotropin Secretion and Hot Flashes in Postmenopausal Women. *Neuroendocrinology*. 2018; **106**(2): 148-57.
29. Skorupskaite K, George JT, Veldhuis JD, Millar RP, Anderson RA. Kisspeptin and neurokinin B interactions in modulating gonadotropin secretion in women with polycystic ovary syndrome. *Hum Reprod*. 2020; **35**(6): 1421-31.
30. Casper RF, Yen SS, Wilkes MM. Menopausal flushes: a neuroendocrine link with pulsatile luteinizing hormone secreation. *Science*. 1979; **205**(4408): 823-5.
31. Conlon JM, Deacon CF, Richter G, Stockmann F, Creutzfeldt W. Circulating tachykinins (substance P, neurokinin A, neuropeptide K) and the carcinoid flush. *Scand J Gastroenterol*. 1987; **22**(1): 97-105.
32. Norheim I, Theodorsson-Norheim E, Brodin E, Oberg K. Tachykinins in carcinoid tumors: their use as a tumor marker and possible role in the carcinoid flush. *J Clin Endocrinol Metab*. 1986; **63**(3): 605-12.
33. Schaffalitzky De Muckadell OB, Aggestrup S, Stentoft P. Flushing and plasma substance P concentration during infusion of synthetic substance P in normal man. *Scand J Gastroenterol*. 1986; **21**(4): 498-502.
34. Rance NE, Young WS, 3rd. Hypertrophy and increased gene expression of neurons containing neurokinin-B and substance-P messenger ribonucleic acids in the hypothalami of postmenopausal women. *Endocrinology*. 1991; **128**(5): 2239-47.
35. Rometo AM, Krajewski SJ, Voytko ML, Rance NE. Hypertrophy and increased kisspeptin gene expression in the hypothalamic infundibular nucleus of postmenopausal women and ovariectomized monkeys. *J Clin Endocrinol Metab*. 2007; **92**(7): 2744-50.
36. Oakley AE, Steiner RA, Chavkin C, Clifton DK, Ferrara LK, Reed SD. kappa Agonists as a novel therapy for menopausal hot flashes. *Menopause*. 2015; **22**(12): 1328-34.
37. Rance NE, Dacks PA, Mittelman-Smith MA, Romanovsky AA, Krajewski-Hall SJ. Modulation of body temperature and LH secretion by hypothalamic KNDy (kisspeptin, neurokinin B and dynorphin) neurons: a novel hypothesis on the mechanism of hot flushes. *Front Neuroendocrinol*. 2013; **34**(3): 211-27.
38. Padilla SL, Johnson CW, Barker FD, Patterson MA, Palmiter RD. A Neural Circuit Underlying the Generation of Hot Flushes. *Cell Rep*. 2018; **24**(2): 271-7.
39. Jayasena CN, Comninos AN, De Silva A, Abbara A, Veldhuis JD, Nijher GM, Ganiyu-Dada Z, Vaal M, Stamp G, Ghatei MA, Bloom SR, Dhillo WS. Effects of neurokinin B administration on reproductive hormone secretion in healthy men and women. *J Clin Endocrinol Metab*. 2014; **99**(1): E19-27.
40. Prague JK, Roberts RE, Comninos AN, Clarke S, Jayasena CN, Nash Z, Doyle C, Papadopoulou DA, Bloom SR, Mohideen P, Panay N, Hunter MS, Veldhuis JD, Webber LC, Huson L, Dhillo WS. Neurokinin 3 receptor antagonism as a novel treatment for menopausal hot flushes: a phase 2, randomised, double-blind, placebo-controlled trial. *Lancet*. 2017; **389**(10081): 1809-20.

41. Depypere H, Timmerman D, Donders G, Sieprath P, Ramael S, Combalbert J, Hoveyda HR, Fraser GL. Treatment of Menopausal Vasomotor Symptoms With Fezolinetant, a Neurokinin 3 Receptor Antagonist: A Phase 2a Trial. *J Clin Endocrinol Metab*. 2019; **104**(12): 5893-905.
42. Santoro N, Waldbaum A, Lederman S, Kroll R, Fraser GL, Lademacher C, Skillern L, Young J, Ramael S. Effect of the neurokinin 3 receptor antagonist fezolinetant on patient-reported outcomes in postmenopausal women with vasomotor symptoms: results of a randomized, placebo-controlled, double-blind, dose-ranging study (VESTA). *Menopause*. 2020; **27**(12): 1350-6.
43. Trower M, Anderson RA, Ballantyne E, Joffe H, Kerr M, Pawsey S. Effects of NT-814, a dual neurokinin 1 and 3 receptor antagonist, on vasomotor symptoms in postmenopausal women: a placebo-controlled, randomized trial. *Menopause*. 2020; **27**(5): 498-505.
44. Simon J, Anderson R, Ballantyne E, Joffe H, Kerr M, Seymore S, Trower M, Pawsey S. NT-814, a Non-Hormonal Dual Neurokinin 1,3 Receptor Antagonist Markedly Improves Vasomotor Symptoms in Post-Menopausal Women; Results of a Randomised, Double-Blind, Placebo-Controlled, Dose-Finding Study (SWITCH-1). *ENDO 2020 (abstract)*. 2020.
45. Coiro V, Volpi R, Capretti L, Caiazza A, Marcato A, Bocchi R, Colla R, Rossi G, Chiodera P. Luteinizing hormone response to an intravenous infusion of substance P in normal men. *Metabolism*. 1992; **41**(7): 689-91.
46. Navarro VM, Bosch MA, Leon S, Simavli S, True C, Pinilla L, Carroll RS, Seminara SB, Tena-Sempere M, Ronnekleiv OK, Kaiser UB. The integrated hypothalamic tachykinin-kisspeptin system as a central coordinator for reproduction. *Endocrinology*. 2015; **156**(2): 627-37.
47. Anderson RA, Cormier J, Thieroff-Ekerdt R, Boyce M, van den Berg F, Grau D, Turnquist D, Corzo D, Graham P. Pharmacodynamic activity of the novel neurokinin-3 receptor antagonist SJX-653 in healthy men. *J Clin Endocrinol Metab*. 2020; **105**(12): e4857-e65.
48. Millar RP, Newton CL. Current and future applications of GnRH, kisspeptin and neurokinin B analogues. *Nat Rev Endocrinol*. 2013; **9**(8): 451-66.
49. Lahlou N, Roger M, Chaussain JL, Feinstein MC, Sultan C, Toublanc JE, Schally AV, Scholler R. Gonadotropin and alpha-subunit secretion during long term pituitary suppression by D-Trp6-luteinizing hormone-releasing hormone microcapsules as treatment of precocious puberty. *J Clin Endocrinol Metab*. 1987; **65**(5): 946-53.
50. Sealfon SC, Weinstein H, Millar RP. Molecular mechanisms of ligand interaction with the gonadotropin-releasing hormone receptor. *Endocr Rev*. 1997; **18**(2): 180-205.
51. Millar RP, Lu ZL, Pawson AJ, Flanagan CA, Morgan K, Maudsley SR. Gonadotropin-releasing hormone receptors. *Endocr Rev*. 2004; **25**(2): 235-75.
52. Newton CL, Riekert C, Millar RP. Gonadotropin-releasing hormone analog therapeutics. *Minerva Ginecol*. 2018; **70**(5): 497-515.
53. Schlaff WD, Ackerman RT, Al-Hendy A, Archer DF, Barnhart KT, Bradley LD, Carr BR, Feinberg EC, Hurtado SM, Kim J, Liu R, Mabey RG, Jr., Owens CD, Poindexter A, Puscheck EE, Rodriguez-Ginorio H, Simon JA, Soliman AM, Stewart EA, Watts NB, Muneyyirci-Delale O. Elagolix for Heavy Menstrual Bleeding in Women with Uterine Fibroids. *N Engl J Med*. 2020; **382**(4): 328-40.
54. Al-Hendy A, Lukes AS, Poindexter AN, 3rd, Venturella R, Villarroel C, Critchley HOD, Li Y, McKain L, Arjona Ferreira JC, Langenberg AGM, Wagman RB, Stewart EA. Treatment of Uterine Fibroid Symptoms with Relugolix Combination Therapy. *N Engl J Med*. 2021; **384**(7): 630-42.
55. Jayasena CN, Nijher GM, Chaudhri OB, Murphy KG, Ranger A, Lim A, Patel D, Mehta A, Todd C, Ramachandran R, Salem V, Stamp GW, Donaldson M, Ghatei MA, Bloom SR, Dhillo WS. Subcutaneous injection of kisspeptin-54 acutely stimulates gonadotropin secretion in women with hypothalamic amenorrhea, but chronic administration causes tachyphylaxis. *J Clin Endocrinol Metab*. 2009; **94**(11): 4315-23.
56. Tanaka A, Nakata D, Masaki T, Kusaka M, Watanabe T, Matsui H. Evaluation of pharmacokinetics/pharmacodynamics and efficacy of one-month depots of TAK-448 and

TAK-683, investigational kisspeptin analogs, in male rats and an androgen-dependent prostate cancer model. *Eur J Pharmacol*. 2018; **822**138-46.
57. Roseweir AK, Kauffman AS, Smith JT, Guerriero KA, Morgan K, Pielecka-Fortuna J, Pineda R, Gottsch ML, Tena-Sempere M, Moenter SM, Terasawa E, Clarke IJ, Steiner RA, Millar RP. Discovery of potent kisspeptin antagonists delineate physiological mechanisms of gonadotropin regulation. *J Neurosci*. 2009; **29**(12): 3920-9.
58. De Bond JA, Li Q, Millar RP, Clarke IJ, Smith JT. Kisspeptin signaling is required for the luteinizing hormone response in anestrous ewes following the introduction of males. *PLoS One*. 2013; **8**(2): e57972.
59. Smith JT, Li Q, Yap KS, Shahab M, Roseweir AK, Millar RP, Clarke IJ. Kisspeptin is essential for the full preovulatory LH surge and stimulates GnRH release from the isolated ovine median eminence. *Endocrinology*. 2011; **152**(3): 1001-12.
60. Kobayashi T, Sasaki S, Tomita N, Fukui S, Nakayama M, Kiba A, Kusaka M, Matsumoto S, Yamaguchi M, Itoh F, Baba A. 2-acylamino-4,6-diphenylpyridine derivatives as novel GPR54 antagonists with good brain exposure and in vivo efficacy for plasma LH level in male rats. *Bioorg Med Chem*. 2010; **18**(14): 5157-71.