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The 3rd World Conference on Kisspeptin, "Kisspeptin 2017: Brain and Beyond": Unresolved questions, challenges and future directions for the field

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In Memoria:

We dedicate this article to the memory of our dear friend and colleague, Dr. Kei-Ichiro Maeda, who contributed so much to our field.

Abstract

The 3rd World Conference on Kisspeptin, "Kisspeptin 2017: Brain and Beyond" was held March 30-31 at the Rosen Centre Hotel in Orlando, Florida, providing an international forum for multidisciplinary scientists to meet and share cutting-edge research on kisspeptin biology and its relevance to human health and disease. The meeting built upon previous world conferences focused on the role of kisspeptin and associated peptides in the control of gonadotropin-releasing hormone (GnRH) secretion and reproduction. Based on recent discoveries, the scope of this meeting was expanded to include functions of kisspeptin and related peptides in other physiological systems including energy homeostasis, pregnancy, ovarian and uterine function,

and thermoregulation. In addition, discussions addressed the translation of basic knowledge of kisspeptin biology to the treatment of disease, with the goal of seeking consensus about the best approaches to improve human health. The two-day meeting featured a non-traditional structure, with each day starting with poster sessions followed by lunch discussions and facilitated large-group sessions with short presentations to maximize the exchange of new, unpublished data. Topics were identified by a survey prior to the meeting, and focused on major unresolved questions, important controversies, and future directions in the field. Finally, career development activities provided mentoring for trainees and junior investigators, and networking opportunities for those individuals with established researchers in the field. Overall, the meeting was rated as a success by attendees and covered a wide range of lively and provocative discussion topics on the changing nature of the field of "kisspeptinology" and its future.

I. Introduction and scope of the conference

Since the discoveries that loss-of-function mutations in kisspeptin signaling genes cause reproductive deficits in humans and mice (1-4), the field of kisspeptin (KISS1) research has exploded. Two previous international meetings were held on kisspeptin biology, in 2008 and 2012, as a forum for capturing that progress, sharing new data, and facilitating collaboration. Both earlier meetings focused predominantly on understanding the basic roles of kisspeptin and related peptides, such as neurokinin B (NKB), in the central control of reproduction via GnRH neurons. The 3rd World Conference on Kisspeptin, built upon this foundation and expanded our perspective to explore the wider universe of kisspeptin biology extending to other homeostatic functions of the brain and beyond, including peripheral tissues such as the gonads, liver, and placenta.

The avalanche of research on kisspeptin over the past decade and a half has demonstrated the quintessential role of kisspeptin neurons in awakening pulsatile GnRH secretion at puberty and supporting reproductive function in adulthood in all mammals. Kisspeptin neurons are now viewed as essential central processors of reproduction, regulating both pulsatile and surge modes of GnRH secretion. However, there is clearly much more to discover. Indeed, with sophisticated new technologies and tools, including optogenetics, virally-introduced constructs for "designer receptors exclusively activated by designer drugs" or DREADDs, *in vivo* voltage-sensing and calcium-imaging technologies, we stand at the threshold of more discoveries and opportunities to improve human health and treat disorders of reproduction and beyond. At the level of kisspeptin control of GnRH, current research is aimed at expanding our understanding of i) the molecular, cellular, and biophysical components of the kisspeptin-GnRH pulse generating mechanisms (5-6), and the inter- and intracellular signaling pathways that mediate physiological and developmental control of reproduction (94); ii) the mechanisms by which peripheral and metabolic cues gate pubertal activation of kisspeptin and GnRH neurons; and iii) the role of kisspeptin neurons in mediating the influence of other internal and environmental cues (7-9) on the development and adult functioning of the reproductive axis.

At the same time, previously unimagined new chapters on kisspeptin signaling have begun to emerge involving its role in cells, tissues and physiological systems beyond GnRH. For example, recent data suggests that kisspeptin signaling plays a critical role in oocyte development and sperm motility (10-14), but we have only begun to scratch the surface of this new realm of KISS1 biology. Other recent studies have implicated hepatocyte-derived KISS1 in the regulation of glucose homeostasis and insulin secretion (15), which dovetails with new ideas about the role of kisspeptin signaling in the regulation of metabolism and body weight regulation (16). Kisspeptin may play a role in trophoblast invasion (17), and recent evidence supports a role for kisspeptin in implantation and maintenance of early pregnancy (18-20). The interplay between kisspeptin and NKB present in the brain may even extend to the placenta— yet, there is little known about this placental dialogue and its possible implications for placental physiology and disorders, such as preeclampsia (21).

As mentioned above, a pressing frontier of knowledge in this field is an understanding of the translation of basic knowledge of kisspeptin and related peptides to the treatment of disease. As such, it is important to consider both reproductive and non-reproductive aspects of KISS1 biology, including with respect to pharmacotherapeutics related to kisspeptin, NKB, and dynorphin signaling for treatment of infertility, PCOS and other disorders (22-25). Indeed, the discovery of the role of NKB as a key component of the GnRH pulse generator (5,6) has opened a completely new frontier in translational research. For example, NKB antagonists are now being investigated as treatments for PCOS (25) as well as for postmenopausal hot flushes (also commonly referred to as "hot flashes") (26-27, 84). Finally, given growing evidence for the role of kisspeptin in the central and peripheral control of body weight and metabolism (15,16,87), the possibility exists for future therapeutic interventions in humans that act at the intersection of reproduction and metabolism.

The 3rd World Conference was convened as a forum to share information on all of these new roles for kisspeptin and related peptides in human health and disease, and to frame the conversation within the context of major unresolved questions and controversies in the field. As noted above, we also considered the present and future of the translational pipeline that leads from drug discovery to clinical application, and what challenges lay ahead in development of drugs that mimic or antagonize kisspeptin and related peptides. In addition to expanding the scope of the meeting, early discussions among the members of the Organizing and Program Committees (Fig. S1) also led to the decision to take a non-traditional approach to the planning and format of the conference (see Appendix I for details). In brief, topics for the meeting were solicited from a large, international group of prospective attendees, who were asked to identify the "big questions" that remain in our field. Based on this survey, issues and areas were selected as topics for large and small group sessions. In addition, the meeting format was purposely designed to promote informal interaction and discussion; facilitated, large group sessions were centered around short, informal presentations of unpublished data and unresolved issues, and small group lunch table discussions focused on additional questions and

controversies. Finally, poster sessions, career development activities for trainees and junior faculty, and social events, served as vehicles for further interactions, both formal and informal. Our hope was that by providing this type of interactive forum for sharing new ideas, and promoting networking among established scientists, new investigators and trainees, we would lay a path for future research in the field. We hope this report will provide a useful summary for those who attended the meeting, as well as others who were unable to attend, and serve as a useful template for future scientific gatherings in this expanding area of research.

II. Major unresolved questions, controversies, and research directions for the future

As noted in the introduction, a major aim of the conference was to stimulate open and speculative discussions of unresolved major issues related to the emerging functional roles of kisspeptin, including current obstacles and challenges to future progress and eventual clinical/translational impact.

The following are synopses of the discussions held in each of the facilitated large group sessions, reflecting those areas identified by the pre-meeting interest survey. Other topics concerning unresolved questions and future directions were the subject of the informal lunch table discussions held each day of the conference. These smaller, table discussions were just as fruitful as the large group sessions. The individual topics and questions addressed during the luncheons are listed in the scientific program (Fig. S2); because of space limitations they are not summarized here.

1. **How do kisspeptin neurons participate in the gating of puberty?**

Over a decade ago, *Science* magazine identified 'what triggers puberty?' as one of 125 questions needing an answer (96). This was two years after the discovery of kisspeptin as a major excitatory neuromodulator of reproductive neuroendocrine function. Over the intervening time, arcuate kisspeptin neurons have been postulated to mediate steroid negative

feedback and potentially rhythm generation for GnRH release, both of which are critical aspects of the pubertal transition. In this session, five leaders in the field briefly presented their latest research relevant to the questions of whether or not sex differences in the tempo of puberty and pubertal disorders are due to kisspeptin sex differences, and what turns on kisspeptin at puberty. The session leaders then held a group discussion on these and other topics.

Consensus emerged on a number of major issues. One point was that sex differences in puberty of both primate and rodent species may involve later development of arcuate kisspeptin (KNDy) neurons in males (88). Precisely which attributes of KNDy neurons are critical is still a point of debate, with signs pointing to NKB in some species and kisspeptin in others. This suggests that NKB and kisspeptin may act as independent signals at different stages of reproductive neuroendocrine development. Some of these differences may arise from the mechanisms of estradiol feedback, which in itself is still controversial as a basis for prepubertal restraint of GnRH neuron output. Does estradiol merely regulate expression of neuromodulators and if so how (e.g., epigenetic changes)? Does centrally-synthesized estradiol play a role and is this species-specific? Another area of lively debate was the definition of puberty. This arose from a disconnect between typical external measures of puberty (e.g., vaginal opening and estrous cycles in females, preputial separation in males) vs. a desire to directly assess maturation of underlying neural circuit function, which may be happening at a different developmental time. For example, estrogens are needed for the external manifestation of puberty in females and a common presumption has been that this is subsequent to activation/reactivation of neuroendocrine systems. In control animals, this likely means neuroendocrine puberty occurs before external signs. In some infertile models, there may be early development of external pubertal signs (e.g., early vaginal opening in kisspeptin-targeted knockout of estrogen receptor alpha) but a failure to complete the pubertal process. Should that animal be considered postpubertal because of vaginal opening, or still in the process because estrous cyclicity and fertility are not established? Another area of discussion was whether or not there are neuronal circuits upstream of kisspeptin neurons (e.g., glutamatergic, GABAergic, Tac1, substance P) that participate in the pubertal process. Both new unpublished data on additional tachykinins

(substance P and neurokinin A) that arise outside KNDy neurons and a plethora of historical data from the 'prekisspeptin" era suggest the functional reproductive neuroendocrine system extends upstream from hypothalamic kisspeptin populations.

Although the field has made progress, the question posed in *Science* twelve years ago ("what forces childhood to end?") remains unanswered. The focus of the field has been on what is measurable: peptide and mRNA levels, cycles and genital development. This can be used as a starting point for further mechanistic studies. For example, how does neuronal connectivity and activity change over development? What is the role of glia, neural immune cells, androgens and the microbiome? Can puberty or reproduction happen (under the right circumstances) without kisspeptin? When critical functional elements are identified, what are the main controlling factors (e.g., epigenetics, microRNAs?). How different and how similar are the sexes and species? Ultimately, can responsiveness to kisspeptin and GnRH be used to make clean diagnoses of pubertal disorders at younger ages in children in order to optimize therapeutic interventions?

2. **What is the mechanistic basis of pulse generation?**

This session began with a summary of the development of the KNDy model for GnRH pulse generation, with an emphasis on similarities and differences of it in rodents and ruminants (28). Two speakers presented evidence in mice that optogenetic inhibition of KNDy neurons in vivo inhibited episodic LH secretion and that optogenetic stimulation of KNDy neurons in a slice preparation stimulated this population in the contralateral arcuate and induced a circuit-wide excitation via an NKB-driven slow EPSP. The third presentation demonstrated that endogenous dynorphin was released during the entire period of an NKB-induced LH pulse in sheep and the fourth speaker moved beyond the hypothalamus with data that kisspeptin acts in the medial amygdala of rats to increase LH release and that optogenetic stimulation of kisspeptin neurons in this area induces an LH pulse in mice.

The attendees discussed differences in the colocalization of the three KNDy peptides among rodent, ovine, human and non-human primate species. First, in the rodent (29,30) and ovine brain (31), kisspeptin is highly colocalized with neurokinin B and dynorphin in the arcuate nucleus of the hypothalamus. However, dual-immunolabelling studies within the infundibular nucleus (arcuate nucleus) and stalk of the human (32) revealed either few or no dynorphin cell bodies, despite previous reports of dynorphin mRNA within the area (33). The inability to detect dynorphin colocalization with kisspeptin or NKB cells was considered a challenge to the translational value of the KNDy neuron hypothesis generated in sheep and rodents. However, it was debated if the lack of labeling reflects a paucity in the dynorphin protein, or, a lack in the sensitivity of currently available immunolabelling techniques. Second, despite strong data obtained from the ewe (34) and goat (35), pharmacological and electrophysiological studies in rodents have not supported the proposed role of endogenous dynorphin in terminating the GnRH/LH pulse (36-39), although recent *in vitro* data on control of slow EPSPs are consistent with this hypothesis (40). Thus, one major unresolved issue is whether dynorphin or an alternate signal terminates the GnRH/LH pulse in rodents.

Additional future directions discussed in this forum included investigation of non-KNDy neurons that could contribute to the control of GnRH/LH pulse generation, such as kisspeptin neurons of the medial amygdala. The continued investigation of the mechanistic basis of GnRH/LH pulse generation promises to be explored using exciting new tools in neuroscience that can correlate the activity of isolated neuronal circuits with GnRH and LH release, such as optogenetics, fast-scan cyclic voltammetry and calcium imaging (e.g., ref. 85).

3. **Negative feedback and hormone regulation of ARC kisspeptin cells**

Based on observations of brain region-specific regulation of kisspeptin mRNA and peptide in response to gonadal steroids (41), one of the first functional roles proposed for kisspeptin was that of the arcuate (ARC) subpopulation of kisspeptin cells in mediating the negative feedback control of pulsatile GnRH secretion. Though considerable progress has been made since, there

are still a number of important unresolved questions concerning this central role of kisspeptin, and multiple issues were considered during this session. These included the enduring major question of whether ARC kisspeptin cells are necessary for estrogen negative feedback to occur, independent of the questions of where and on what neurons is the direct action of estrogens required? Related questions discussed included the possible role of neuronal-derived estrogens in negative feedback, as well as the interactions between estrogens and progesterone required for feedback control under normal physiological conditions (89,90).

Several major obstacles were identified as challenges to answering questions related to the role of ARC kisspeptin neurons in negative feedback. Among these was the challenge of determining estrogen receptor alpha expression in the membrane of ARC kisspeptin neurons, in conjunction with understanding the functional roles of non-classical estrogen receptor in mediating estrogen negative feedback. Current controversies discussed include the issue of possible species difference in the role of dynorphin in estrogen negative feedback, as well as differences between ruminants and rodents in neuronal activation in ARC kisspeptin cells following removal of estrogen negative feedback.

A number of exciting new pieces of data and directions for future research were discussed in this session. This included recent electrophysiological findings (42) showing that estradiol decreases glutamatergic inputs to ARC kisspeptin neurons, and that glutamatergic transmission to the same cells is increased in female mice bearing kisspeptin-specific knock out of estrogen receptor alpha. Perhaps most exciting was evidence that serial LH pulse bleeds in kisspeptin-ER knockout mice showed significant impairment in estrogen negative feedback, in contrast to the lack of changes seen in previous studies involving single measurements of LH. Finally, as an unresolved issue for the future, the question of whether estrogen negative feedback is really necessary for regulation of neuronal firing or electrical activity of ARC kisspeptin neurons was raised.

4. **Positive feedback and hormone control of preoptic kisspeptin neurons.**

Ovulation in female mammals is gated at the neuroendocrine level by an estrogen-mediated positive feedback induction of LH secretion. This "surge" release of LH is itself controlled by a preceding surge in GnRH secretion from the brain. The secretion of GnRH is itself tightly regulated by a collection of hormones and upstream neurotransmitters and neuropeptides. The neural and molecular mechanisms that underlie the neuroendocrine GnRH and LH surges still remain incompletely characterized, but mounting evidence in rodents supports kisspeptin neurons in the anteroventral periventricular and rostral periventricular (AVPV/PeN) region (also called the rostral periventricular area of the third ventricle, R3PV) as key participants in this pre-ovulatory event. Presentations in this session reviewed a number of pieces of evidence regarding this role as well as unanswered questions. For example, estradiol positive feedback and the LH surge are also dependent on proper progesterone signaling, but how this works has not been entirely clear. It was recently demonstrated that progesterone signaling directly in KISS1 neurons is required to mount an LH surge using an estradiol-positive feedback model (43,44), suggesting these neurons integrate both estradiol and progesterone steroid signaling to generate the surge.

In rodents and other species, the GnRH/LH surge is precisely timed in a circadian fashion, occurring just before or at the onset of nightly activity. Circadian gating of the LH surge depends on temporal input from the brain's master circadian clock, the suprachiasmatic nucleus (SCN) (45) and current evidence points to AVPV/PeN kisspeptin neurons as targets of the SCN. For example, data from multiple rodent species demonstrate a circadian pattern of AVPV/PeN *KISS1* gene expression and neuronal activation (46,47). Moreover, recent evidence indicates that the SCN-derived neuropeptide, vasopressin, induces AVPV/PeN KISS1 neuronal activity and may serve as the circadian integrating signal for the LH surge (48). Along with other data, this supports the notion that AVPV/PeN KISS1 neurons can incorporate both ovarian positive feedback (estrogen) and circadian cues (vasopressin) to drive GnRH activity and mount a bolus surge of LH that ultimately triggers ovulation. How the circadian and steroid hormone cues are

integrated within the KISS1 neuron, and what intra-cellular signaling pathways and processes are activated (or inhibited), still remain to be determined.

Based on receptor expression and hormone treatment studies, AVPV/PeN kisspeptin neurons are sensitive to both ovarian sex steroids (estradiol and progesterone) and also prolactin (PRL) (49-51), though the functional relevance of the latter remains unknown. AVPV/PeN kisspeptin neurons project to multiple downstream brain regions, including areas containing GnRH neurons, but also to other areas, such as the PVN and ARC. Likewise, the AVPV/PeN neurons receive synaptic input from neurons in several regions, including the SCN and ARC (40,52). The projections from the ARC may include glutamate or dynorphin, but their role in the surge process remains to be fully explored. Indeed, aside from the SCN's temporal input onto the kisspeptin cells, it is unclear if and how these other inputs and downstream targets are involved in AVPV/PeN kisspeptin-induced GnRH/LH surge or if they are involved in other processes unrelated to the surge. It is also unknown if there are "sub-populations" of kisspeptin neurons within the larger AVPV/PeN population, and if so, do these sub-populations serve different physiological processes? Continued *in vivo* work using advanced genetic and viral technology will help to uncover the mechanism(s) by which AVPV/PeN kisspeptin neurons function to trigger the LH surge and ovulation, as well as contribute to other non-surge processes.

5. **How do kisspeptin neurons participate in regulation of body temperature, stress and other non-reproductive functions?**

This session addressed the issue of how kisspeptin neurons participate in regulation of body temperature and stress and, more broadly, how kisspeptin neurons participate in the control of autonomic functions. Since reproduction is a fundamental homeostatic function it is possible that a set of central "command" neurons coordinates all of these other functions with reproduction. Therefore, this session covered diverse functions, such as temperature, circadian rhythms, parturition, lactation, energy homeostasis, stress, and even aspects of social behavior. Emphasized throughout this session was the role of the co-localized neurotransmitters in the

kisspeptin neurons of ARC and those in the AVPV/PeN. One of the highlights of this session was a review and update on the role of neurokinin B, originating from ARC kisspeptin (KNDy) neurons in the steroidal regulation of body temperature. Since all three KNDy peptides are increased in the postmenopausal state, it has been postulated that NKB transmission may play a role in hot flushes. Indeed, basic findings on the critical role of KNDy neurons and their NKB projections to the medial preoptic area in the control of body temperature (91) set the stage for exciting clinical trials for treating hot flushes in postmenopausal women with NKB receptor (NK3R) antagonists (84). A point of discussion was where exactly at the cellular level are the NK3R antagonists acting to block KNDy neuronal transmission—presynaptically or postsynaptically?

Other talks in this session highlighted the role of arcuate KISS1 neurons in regulating circadian rhythms and non-REM sleep. Studies were presented in which synaptic transmission of KNDy neurons was blocked by using a viral vector containing tetanus toxin targeted to arcuate *Kiss1-cre* neurons. However, questions arose as to what transmitter or transmitters released from KNDy mediate these actions and whether they have direct or indirect actions on suprachiasmatic neurons. Evidence was also presented for a role of AVPV/PeN kisspeptin neurons in parturition via synapses on oxytocin neurons. This communication (via fiber projections) is enhanced in late gestation and may provide excitatory input to oxytocin neurons for parturition. Although *icv* kisspeptin increases oxytocin cell neural firing, the exact cellular site of action is still not known. Additional evidence presented suggested that kisspeptin may be involved in lactation and prolactin secretion through its actions on dopamine neurons, and these actions are via the neuropeptide FF receptor 1 (NPFFR1). A general point of discussion was the fact that we are lacking the pharmacological tools—selective KISS1R and NPFFR1 antagonists—to appropriately and rigorously parse out this pathway. Finally, interesting findings were presented on the role of kisspeptin in mediating social behavior in the *medaka* fish. A kisspeptin homolog activates KISS1R in neuropeptide B neurons that synapse on and excite vasotocin/isotocin neurons in this species, regulating social behaviors associated with mating.

The overarching theme throughout this session was the observation that kisspeptin neurons are very complex with respect to the neurotransmitters that they express, how they are regulated during different physiological states, and the projections/synapses that they establish. One aspect that has been largely ignored is the fact that they also express fast amino acid neurotransmitters, predominantly GABA in the AVPV/PeN kisspeptin neurons and glutamate in ARC kisspeptin neurons, that may be conveying some of these functions. Also, are the postsynaptic actions of substances released by kisspeptin cells determined by the selectivity of the postsynaptic receptors and/or are the peptide and amino acid neurotransmitters differentially released at different firing frequencies as earlier findings (40) would suggest? Key to addressing these and other challenges is the need for better pharmacological tools (peptide agonists and antagonists) to parse out specific functions. Cellular physiological studies will help to establish the efficacy and selectivity of agonists and antagonists such that they can be moved forward into whole animal experiments and ultimately into the clinic. Certainly, the current ongoing development of NKB antagonists for treatment of postmenopausal hot flushes is a strong proof of concept for this approach (84).

6. **What are the reproductive functions of kisspeptin/KISS1R systems in the periphery?**

While KISS1 and KISS1R are expressed widely outside of the brain, the roles of KISS1R signalling in the periphery remain relatively poorly understood. KISS1 and/or KISS1R are expressed in reproductive tissues such as the ovary, testes, endometrium, and the placenta (53) leading to the hypotheses that KISS1R signalling is involved in the local regulation of reproduction within these tissues. This session featured presentations and discussions that focused on the roles of the kisspeptin signalling system in these tissues.

The use of genetically-modified mice has revealed potential roles for KISS1/KISS1R signalling in ovarian and endometrial function, with loss of KISS1R signalling causing defects in ovarian reserve, implantation and pregnancy. Whether or not these findings are relevant to human

disorders of reproduction is an important, outstanding question. It was reported that a woman bearing a mutation in the KISS1R (54) who had a failed pregnancy, was later able to become pregnant following IVF treatment and carry the pregnancy to term. Whether the mutation resulted in a complete loss-of-function (55) or may be associated with spontaneous reversal of infertility (56) was not resolved. A published study (57) further highlighted species differences by revealing that kisspeptin, while detectable in very high levels in the blood during pregnancy in humans and some primates, are barely detectable in other species. The role of circulating kisspeptin in humans and the reason for its potential absence in other species remains to be elucidated.

The ability to study the role of KISS1/KISS1R signalling in the periphery remains difficult and further research, particularly in humans, is required. A recently developed mouse model lacking peripheral signalling but retaining intact central signalling (86) has served as a powerful tool to investigate the peripheral roles for the ovarian and endometrial KISS1/KISS1R in regulating fertility (20). However, many questions remain, including whether circulating kisspeptin originates from the placenta, the liver (15), or elsewhere, does it regulate placental function and/or the timing of parturition, and can its levels be used as a biomarker for pre-eclampsia risk? Further, if circulating kisspeptin is important for pregnancy, is there a compensatory mechanism in the absence of KISS1R signalling in humans? These important questions must be addressed as progress is made to better understand the reproductive functions of kisspeptin/KISS1R systems in the periphery.

7. **Metabolic functions of kisspeptin in the periphery**

There is an obvious connection between gonadal function and metabolism, via multiple convergent regulatory pathways, acting in the brain and peripheral tissues (58). While evidence indicates that metabolic signals can influence, directly or indirectly, KISS1 neurons to modulate reproductive function, recent, as yet fragmentary evidence suggests a *reverse* interplay in

which KISS1 signaling may operate as an important regulator of metabolism, glucose homeostasis and, eventually, adipose function.

However, the nature and physiological relevance of such direct metabolic actions of kisspeptins are yet to be fully defined and still under considerable debate. While initial results failed to document any detectable effects of kisspeptin on food intake (59), more recent data showed that congenital, whole body absence of kisspeptin signaling increased body weight and adiposity, as well as glucose intolerance, in female mice, irrespective of gonadal status (16). This would suggest a primary role of kisspeptin signaling in body weight/glucose homeostasis, whose underpinnings remain unclear. Intriguingly, KISS1R null mice eat less, pointing out potential alterations in the thermogenic program, which need to be investigated, in order to explain the conundrum of lower food intake but yet higher body weight. In addition, liver-derived kisspeptin has been suggested also to participate in glucose homeostasis, via a bidirectional pancreatic-liver loop, whereby glucagon stimulates hepatic kisspeptin output to suppress β-cell insulin production (15). Dysregulation of such a circuit has been suggested to be causative for hyperglycaemia and type-2 diabetes. Yet, no conclusive evidence for dysregulated glycaemia has emerged so far from pharmacological studies addressing the impact of kisspeptin administration to laboratory animals and humans, and data for stimulatory rather than inhibitory effects of kisspeptin on insulin secretion (60), depending on prevailing glucose concentrations, have been also reported. Finally, fragmentary evidence suggested that the adipose tissue may expresses KISS1 itself (61); yet, the magnitude and functionality of such adipose expression remain obscure, and warrant further investigation on the actual roles, if any, of kisspeptin, its receptor and eventual co-transmitters, in key aspects of adipocyte biology, such as differentiation, thermogenic programing and potential secretory activity.

Given the gaps in existing evidence, additional efforts are needed to fully clarify the metabolic roles of kisspeptin, teasing apart its genuine direct effects from the potential secondary actions due to its capacity to modulate gonadal steroid secretion. Similarly, the actual impact and pathophysiological relevance of kisspeptin, not only from the liver, but also from the placenta

(62) in the control of glucose homeostasis need to be defined, as prerequisite for safety validation and/or eventual new indications of kisspeptin-based therapies. Likewise, the as yet undefined roles of kisspeptin signaling in the control of thermogenesis or adipocyte biology warrant further investigation. All these efforts will allow us to elucidate the putative metabolic dimension of kisspeptin, its eventual physiological relevance and possible therapeutic implications.

8. **What are the challenges and likely outcomes of drug development in the field?**

Basic and translational research has identified kisspeptin and the neurokinins as key regulators of various aspects of reproductive physiology and pathology (63-65). In parallel to this, several agonists and antagonists for both kisspeptin and NKB receptors are under development (66). Combining these developments seems like a perfect route to clinical applications, however several challenges remain.

First, there is still a need to clearly delineate the roles of these peptides in both physiologic and pathophysiologic states. These include both common disorders such as polycystic ovary syndrome (PCOS) (67) as well as more rare conditions such as idiopathic hypogonadotropic hypogonadism (68). Human studies often generate greater impact when they are able to draw upon observations made in animal models (69-71). These complex and time-consuming mechanistic studies are critical to ensure appropriate and evidence-based clinical applications.

Second, to target central reproductive and non-reproductive pathways, agonists and antagonists for kisspeptin and NKB receptors may need to access the brain and cross the blood-brain-barrier (BBB). For example, brain penetration for neurokinin 3 antagonists may prove to be fundamental to their efficacy for treating hot flushes and PCOS (72-74). In addition, recent data suggests that different formulations of kisspeptin have different capabilities with respect to crossing the BBB when administered peripherally (75). Probing these considerations is not

only important in understanding the biology of these neuropeptides but will be relevant when considering which form of kisspeptin to use in a clinical setting.

Third, clinical trial protocols should be developed in reflection of important unmet clinical needs while at the same time balancing the unique requirements of vulnerable populations such as adolescents (76) and pregnant women (77). Clear, well-defined end-points, better assay tools, and a thorough understanding of off-target effects will be essential. In addition, appropriate doses and routes of administration need to be determined for the current and future arsenal of agonists, antagonists as well as mixed agonists-antagonists (78).

Finally, advancing fundamental basic and translational research to produce meaningful clinical impact requires the development of fruitful partnerships between academia and the pharmaceutical industry. Academic organizations and pharmaceutical companies often have different expectations for drug development, with difficulties encountered in technology transfer and carrying out trials for 'off the shelf' compounds. Furthermore, these difficulties vary from country to country. However, with potentially exciting new diagnostic and treatment options for a variety of disorders, including idiopathic hypogonadotropic hypogonadism (68,79), hypothalamic amenorrhoea (80), hyperprolactinaemia (81), infertility (22), menopausal hot flushes (74), psychosexual disorders (82,83) and PCOS (73) already on the horizon, overcoming the challenges in drug development for the kisspeptin field is an important and worthy goal.

9. **What are the biggest technical and conceptual impediments or concerns to progress in the field?**

The role of kisspeptin-KISS1R signaling in regulating and maintaining reproductive function is well established. However, many obstacles exist regarding the optimal approach to study the mechanisms and physiological impact of kisspeptin signaling at the level of the brain and in the periphery.

Important questions regarding kisspeptin neurocircuitry remain to be answered in order to move the field forward. Although hypothalamic kisspeptin neurons are well established to be intricately involved with the regulation of GnRH neurons and gonadotropin release, pinpointing the exact nuclei (AVPV, PeN and ARC) responsible for different modes of GnRH release is very difficult. The field needs to adopt and develop methodologies that enable specific kisspeptin populations (and sub-populations) to be regulated in freely behaving mice. In part, this can be achieved through the viral transduction of specific regional kisspeptin populations with optogenetic and chemogenetic tools. The investigation of neurochemically-defined sub-groups within the AVPV/PeN and ARC kisspeptin populations may be achieved by intersectional transgenic strategies. To this end the development of mice in which specific kisspeptin neural populations express Cre in an inducible manner would be invaluable in deciphering adult physiology.

It is also necessary to better understand the mechanistic processes by which kisspeptin targets are triggered and regulated. The further development of selective KISS1R antagonists and the generation of antisera specific for KISS1R would be extremely useful for identifying and understanding the cells targeted by kisspeptin and their responses. Moreover, the generation of mice in which KISS1R is epitope-tagged (e.g., HA or FLAG) could enable the localization, tracking, and interactions of KISS1R within cells to be examined in a variety of tissues.

Improved assays for measuring kisspeptin output and its functional effects on various organ systems are also needed. Several kisspeptin-secreting cell lines have recently become available and will enable assessment of mechanistic interactions between receptors and second messenger cascades within the kisspeptin cell. The eventual validation of these cell lines as being specific models of AVPV/PeN or ARC (or other) kisspeptin-secreting cells will be critical. Although it has been possible to use conventional approaches to measure kisspeptin concentrations in serum of some species and, indeed, even in the monkey mediobasal hypothalamus, it would be invaluable to develop more advanced kisspeptin biosensing or bioassay methodologies. This would facilitate greatly the dynamic measurement of kisspeptin

concentrations even in small tissues or brain regions of the rodent. From the perspective of measuring the output of the kisspeptin neurons themselves, the substantial advances in voltage sensing and calcium imaging technologies will make it possible to record the activity of specific kisspeptin cell types in freely behaving rodents (85).

Recently, the emergence of single-cell RNA sequencing (scRNA-seq) provides the most direct and unbiased method to define a cell type based on its transcriptional profile, which can provide additional insights into connectivity and function of the cell. This method can facilitate the discovery of gene expression profile of Kiss1 neurons, identifying the repertoire of surface receptors that may mediate physiological responses (93,94). This approach can help us to gain tremendous insights into upstream signals that converge on Kiss1 neurons to modulate the reproductive axis. Furthermore, an advanced approach for translational profiling of neurons based on connectivity using viral translating ribosome affinity purification (vTRAP) has been reported recently (95). In this approach, CRE-dependent AAV or other retrograde viruses (rabies or canine adenovirus) is engineered to express an EGFP-tagged ribosome protein enabling rapid access to translating mRNAs from a discrete CRE-expressing neural population. Projection-specific translational profiling is achieved by selectively precipitating neuronal ribosomes based on connectivity. Quantitative PCR is then used for selected target genes or high-throughput RNA sequencing to determine the neuronal identity without the need for detailed anatomical or electrophysiological investigation (95).

Much has been achieved in the kisspeptin field. The ongoing revolutions in techniques will enable the detailed investigation of defined populations of cells and their interactions at both network and whole organism levels to continue to produce exciting insights. The application of these methodologies will provide unprecedented opportunities to advance kisspeptin research and generate a comprehensive understanding of physiology of kisspeptin signaling throughout the body.

III. Summary and conclusions

The 3rd World Conference on Kisspeptin was attended by a total of 166 registrants, of which over 40% were trainees. Registrants represented 17 countries from Africa, Asia, Australia/Oceania, Europe, North and South America. Seventy-three abstracts were presented in the two morning poster sessions. Over 120 of the attendees participated in the small group, lunch table discussions on each of the two days.

To assess the effectiveness of the meeting and its format (Fig. S2), attendees were asked during the session to complete a brief survey (Fig. S3) before leaving the conference hall for the concluding social event. Attendees were very positive about the meeting experience, with a large majority either satisfied or very satisfied with the major elements of the conference. For example, 92% of the survey respondents were either satisfied or very satisfied with the overall structure of the meeting and its emphasis on informal discussions, 89% satisfied or very satisfied with the opportunities offered for networking, and 82% satisfied or very satisfied with the facilitated, large group discussions. There were a number of comments and specific suggestions for improvements: a number of participants suggested increasing the length of short presentations in the facilitated group sessions to 5 min. each, blending in longer presentations as needed while maintaining time for questions and discussion. In general, attendees were enthusiastic about the different type of meeting structure, and comments included “amazing format”, “format very informative and productive” and “I really liked this meeting format that encourages engaging discussions”.

Overall, the 3rd World Conference on Kisspeptin, “Kisspeptin 2017: Brain and Beyond”, provided a comprehensive and forward-looking view of a scientific field that is continuing to evolve in both scientific scope and range of impact. The interactive, non-traditional meeting format was well received and facilitated the informal exchange of new data and ideas, interactions between researchers at varying career stages, and open discussion of the challenges and obstacles to be overcome in order for the field to move forward. The participation of researchers from a number of pharmaceutical companies added value to the discussions of the

clinical translation and potential impact of basic discoveries in kisspeptin biology on human health. While the field of "kisspeptinology" is clearly at a crossroads, transitioning beyond its initial focus on one peptide and physiological system, opportunities for new discoveries, based on the array of tools and technologies recently available, have never been greater and the coming years are certain to be exciting ones.

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References

APPENDIX I: Kisspeptin 2017: a different kind of scientific meeting

Planning for this conference grew out of informal discussions among several reproductive neuroendocrinologists attending the annual meeting of the Endocrine Society in 2016. There was general agreement that the two previous Kisspeptin World Conferences (Cordoba, Spain in 2008 and Tokyo, Japan in 2012) had been extremely useful and that enough new information had been developed since 2012 to warrant a third meeting. It was thought that the only closely related meeting (International Congress of Neuroendocrinology that meets every 4 years) was too large and broadly focused to facilitate the informal, focused discussions planned for this meeting. Based on these discussions, Dr. Allan Herbison emailed kisspeptin biologists from around the world to determine if there was sufficient interest in another meeting. After email exchanges, the general consensus was to hold a two-day meeting as a satellite conference with the 2017 meeting of the Endocrine Society (ENDO2017) in Orlando, Florida, to minimize travel costs and maximize participation. Organizing and Program Committees were quickly formed (see Fig. S1 for membership list), with Dr. Michael Lehman serving as the meeting organizer and chair of the two committees.

There was also general agreement that the scope and topic of the meeting should build and expand on those of the previous conferences. The 1st World Conference occurred within five years of the first reports that the kisspeptin receptor, GPR54 (KISS1R), was essential for puberty and fertility in humans and focused on the role of kisspeptin signaling in puberty onset and control of GnRH. The 2nd World Conference extended this focus to include the roles of NKB and dynorphin co-localized with kisspeptin in a subpopulation of kisspeptin neurons (KNDy neurons), the electrophysiology of these neurons, the control of kisspeptin release by internal (e.g., estradiol) and external (e.g., nutrition and photoperiod) input, and potential clinical applications. As noted above, the 3rd World Conference expanded beyond these GnRH-centric topics to consider the functional roles of kisspeptin in other areas of the brain as well as extending beyond the brain and the control of reproduction into other important physiological systems. The intent was to take the opportunity to ripen and harvest the fruits of this new

research — morphing the field from a focus on one neuropeptide and reproduction to another that comprises a larger scope, to include the gonads, placenta, liver and beyond. In addition, the goal was to place this knowledge in the context of larger issues in physiology (*e.g.,* puberty, pregnancy, obesity, prenatal origins of disease) rather than to solely focus on the role of KISS1 by itself.

In addition, there was strong consensus for a meeting format that facilitated discussion centered around short, informal presentations of unpublished data and unresolved issues, rather than the more traditional plenary sessions with formal talks that largely present data that has been already published. Specifically, it was recognized that most scientific meetings invite presenters based on their general area of inquiry; these presenters then share with the audience their particular question(s) and how they answered them. For this meeting, we flipped this approach – we asked a large, international group of prospective attendees to name the big questions (see below), and then invited presenters in these selected areas to share their ideas, either with their own data or provocative thinking. In addition, we reversed the usual placement of poster sessions at the end of the day, and instead started each day with a poster session/breakfast grouped around specific topics related to the scientific questions to be featured later at lunch discussions and general sessions. Our goal was to use poster sessions to encourage people to share with others their most up-to-date research activity— then use the afternoon sessions to delve further into these areas, tackle unresolved matters and debate controversies.

To identify major themes and topics for group discussion, we sent an email to a large group of investigators in the field, including most attendees of the previous World Conferences, asking the following questions:

- What are the most important contemporary questions in the realm of kisspeptin biology?
- What are the biggest impediments (technically or otherwise) to answering these questions?
- What are the biggest controversies/debates/disagreements related to kisspeptin biology?

Based on the responses received, the Organizing and Program Committees identified major areas of interest for lunch table and large group discussions and began to shape the overall structure of the scientific program (Fig. S2).

On the first day of the meeting, there was a short welcome session that included a general orientation to the meeting format and process. Then, as mentioned, the morning of each day was taken up by posters followed by Lunch Table Discussions on a variety of topics selected from responses to the pre-meeting questionnaire and not used for the large group, facilitated sessions. At each table, a member of the Organizing and Program Committees served as discussion leader. Attendees submitted their preferences for table topics before the meeting and were assigned tables (8-10 per table) for each day. We purposely assigned at least one senior investigator and several trainees to each table in order to ensure a mix of backgrounds and career stages, and thereby used this format as an additional opportunity for informal networking for trainees attending the meeting (also see Career Development activities below).

Afternoon sessions consisted of large group facilitated discussions for all attendees that were focused on the major unresolved questions, issues and controversies identified in the pre-meeting questionnaire (Fig. S2). Sessions ran in series, rather than in parallel, so the entire group could attend each topic. Sessions were 45 minutes long and consisted of short presentations from a facilitator (5 min), 3-5 invited lead speakers (3 min/each), and any additional attendees who wished to share unpublished data (1 min/each). The facilitator provided a general background/setting for each session, identifying the major questions and issues to be discussed. The facilitator also served as moderator, helping to move presentations and discussions along, and ensure that multiple points of view were expressed freely and openly. A large group session at the end of the meeting reviewed the highlights, consensus items, and discussed open questions, opportunities, and next steps. Social events were held on each evening, as way to further encourage informal networking and interactions among the meeting attendees.

In addition to the poster sessions, lunch table discussions, and facilitated large group sessions, a number of activities were organized during the meeting to encourage and support the professional career development of early stage investigators and trainees. With the support of a scientific conference grant from the National Institutes of Health, USA (NIH R13HD092038 to M.N.L.), travel awards were given on a competitive basis for early stage investigators and trainees. Awards to help defray the cost of child care during the meeting were also made available with all attendees being eligible. Before the meeting, each trainee attending the meeting (including but not limited to travel award recipients) was appointed a mentor with career track experience that matched the individual trainee's career goals, with the aim of providing trainees with networking opportunities. A breakfast event for mentor-mentee meetings was held on the first morning of the conference. On the second day of the conference, a career workshop was held featuring multiple speakers from academia and pharmaceutical companies attending the conference, who addressed research-related job opportunities in multiple career settings. These activities were held before the poster sessions so there was no conflict with the remainder of the scientific program. Activities were mandatory for travel awardees but all other trainees attending the meeting were welcomed and encouraged to participate. Participation in these events was very high, reflecting the high value with which they were viewed by trainees and early stage investigators.