The Role of Kisspeptin in Sexual Behavior

Vincent Hellier, PhD[1,2] Olivier Brock, PhD[1,3] Julie Bakker, PhD[1]

[1] GIGA Neurosciences, Liège University, Liège, Belgium
[2] Group of Neurocircuit Wiring and Function (S. Steculorum group), Max Planck Institute for Metabolism Research, Koln, Germany
[3] Department of Basic and Clinical Neuroscience, Headache Group, James Black Centre, King's College London, London, United Kingdom

Address for correspondence Julie Bakker, PhD, GIGA Neurosciences, Liège University, Avenue Hippocrate 15, B36, 4000 Liège, Belgium (e-mail: jbakker@uliege.be).

Semin Reprod Med 2019;37:84–92

Abstract

Sexual behavior is essential for the perpetuation of a species. In female rodents, mate preference and lordosis behavior depend heavily on the integration of olfactory cues into the neuroendocrine brain, yet its underlying neural circuits are not well understood. We previously revealed that kisspeptin neurons in the anteroventral periventricular nucleus/periventricular nucleus continuum (AVPv/PeN) are activated by male olfactory cues in female mice. Here, we further reveal that male-directed mate preferences and lordosis are impaired in kisspeptin knockout mice but are rescued by a single injection with kisspeptin. Acute ablation of AVPV/PeN kisspeptin neurons in adult females impaired mate preference and lordosis behavior. Conversely, optogenetic activation of these neurons triggered lordosis behavior. Kisspeptin neurons act through classical GPR54/GnRH signaling in stimulating mate preferences, but unexpectedly, GPR54/GnRH neuronal ablation did not affect lordosis behavior. Therefore, to identify the downstream components of the neural circuit involved in lordosis behavior, we employed genetic transsynaptic tracing in combination with viral tract tracing from AVPV/PeN kisspeptin neurons. We observed that kisspeptin neurons are communicating with neurons expressing the neuronal form of nitric oxide synthase. These results suggest that hypothalamic nitric oxide signaling is an important mechanism downstream of kisspeptin neurons in the neural circuit governing lordosis behavior in female mice.

Keywords
- sexual behavior
- hypothalamus
- olfaction
- GnRH
- nitric oxide

Reproductive behavior (i.e., any activity directed toward the perpetuation of a species) is a major behavioral trait in all animals. Sexual behavior enables mammals to copulate with the opposite sex and ensures fertilization and consequently reproductive success. In rodent species, males typically show mounting behavior, whereas females respond by displaying lordosis behavior which is characterized by an arched back and an immobile posture to facilitate intromission and fertilization. The display of sexual behavior is under tight control of gonadal sex steroids, such as testicular testosterone in males and ovarian estradiol and progesterone in females. In contrast to males, females show lordosis behavior only when they are in estrus coinciding with ovulation.[1]

The ability to show sex-specific reproductive behaviors is organized during perinatal development under the influence of sex steroids. In male rodents, testosterone which can be converted into estradiol by the enzyme aromatase in the brain masculinizes and defeminizes the brain; that is, males will show high levels of mounting behavior and will lose the ability to show lordosis behavior in adulthood. For instance, male rats treated with the aromatase inhibitor 1,4,6-androstatriene-3,17-dione (ATD) from day 1 after birth until postnatal day 10 show only low levels of mounting behavior when paired with an estrous female, but high levels of lordosis behavior when paired with a sexually active male.[2] Accordingly, perinatal exposure to estradiol abolishes the ability of female mice to show lordosis behavior and increases mounting behavior in adulthood.[3,4] The finding that lordosis behavior is easily observed in male rats which were either treated with ATD or castrated directly after birth suggested that the ability

Issue Theme Kisspeptin, Neurokinin B and New Players in Reproduction: Part 1; Guest Editor, Waljit S. Dhillo, MD

DOI https://doi.org/10.1055/s-0039-3400992.
ISSN 1526-8004.

to display female sexual behavior develops by default (i.e., in the absence of any hormonal secretions). Additional evidence for this supposition came from the observation that the ovaries are quiescent during perinatal development and do not start to secrete any estrogens before the second week after birth. However, recent studies using aromatase knockout (ArKO) mice have shown that estradiol is actually required over a specific prepubertal period to feminize the brain.[4,5] Female ArKO mice show very low levels of lordosis behavior in adulthood following ovariectomy and sequential treatment with estradiol and progesterone. However, when treated with estradiol benzoate between postnatal days 15 and 25, lordosis behavior was almost completely restored up to wild-type levels.[4] These findings did not only challenge the default theory of female brain organization but also showed that there are most likely different developmental time windows for male- versus female-typical sexual differentiation: male-typical neural and behavioral characteristics develop predominantly pre- and early postnatally under the influence of testosterone and/or estradiol, whereas female-typical neural and behavioral characteristics develop postnatally, perhaps extending into puberty.

At present, the neural network underlying lordosis behavior is relatively well described (reviewed in Pfaff).[6] Briefly, three brain regions have been identified as playing a key role in lordosis behavior: the ventromedial hypothalamus (VMH), the medial preoptic area (MPOA), and the midbrain periaqueductal gray (PAG). The VMH has been shown to be critical for the facilitatory effects of estradiol and progesterone on lordosis behavior since (1) lesions of the VMH abolish lordosis in female rats and (2) local implants of estradiol and progesterone into the VMH of ovariectomized females can stimulate lordosis. By contrast, the MPOA seems to play primarily an inhibitory role in lordosis behavior. For example, electrical stimulation of the MPOA inhibits lordosis, whereas lesions stimulate the behavior.[7,8] More recently, a specific lordosis-inhibitory circuit with a central role for the MPOA and the arcuate nucleus (ARC) has been proposed in female rats.[9] Briefly, estradiol provokes the release of neuropeptide Y in the ARC which then activates NPY-1 receptors expressed on ARC β-endorphin (β-END) neurons projecting to the MPOA. As a result, μ-opioid receptors (MORs) in MPOA neurons are internalized and by consequence exert a tonic inhibitory action on target lordosis control neurons in the VMH. Progesterone reverses the estradiol-induced MOR internalization, thereby reducing the inhibition of VMH neurons, which in turn facilitates the expression of lordosis.

Pharmacological and lesion studies have shown that the PAG, which receives direct projections from the VMH and the MPOA, plays primarily a stimulatory role in lordosis behavior.[10] Using a combination of anterograde tracing with biotinylated dextrin amine and retrograde viral tracing using pseudorabies virus, it has been shown that these three brain regions are interconnected and that they are connected to the paraventricular nucleus (PVN), the lateral hypothalamus, and the nucleus paragigantocellularis.[11] Furthermore, olfactory structures such as the vomeronasal organ (VNO) and the accessory olfactory bulb as well as brain regions receiving direct projections from the olfactory systems, such as the medial amygdala (MeA) and the bed nucleus of the stria terminalis (BNST), are also part of this neural network as sexual behavior highly depends on the detection and processing of olfactory cues in rodents. Removal of the VNO or chemical ablation of the main olfactory epithelium (MOE) by infusion of zinc sulfate significantly reduces the expression of lordosis behavior in female mice.[12,13] Most of these brain regions express estradiol receptors and are thus potential neural targets of the organizing actions of estradiol in the prepubertal female mouse brain.

Gonadotropin-releasing hormone (GnRH) neurons are at the core of hypothalamic control of reproduction (i.e., the hypothalamic–pituitary–gonadal (HPG) axis). GnRH secreted in the median eminence triggers the release of the gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the pituitary, which in turn stimulates the production of sex hormones (i.e., testosterone and estradiol), which are crucial for the display of sexual behavior. In addition, GnRH might have direct action in the brain itself as it has been shown that a peripheral[14,15] or an intracerebroventricular[16,17] injection with GnRH facilitates lordosis behavior in female rodents. Accordingly, it was shown using a mouse model in which the transneuronal tracer barley lectin (BL) was specifically expressed by GnRH neurons (GnRH-BL) that GnRH neurons make synaptic connections with subsets of neurons in several brain areas implicated in sexual behavior.[18] These include the MeA and the posteromedial part of the BNST, both known to relay pheromonal signals, as well as the MPOA, the ventrolateral part of the VMH, and the ventral part of the premamillary nucleus. Accordingly, GnRH-R expressing neurons have been detected in these same brain areas, including the PAG.[19] Interestingly, using the GnRH-BL mouse model, it was also shown that GnRH neurons receive signals from both main and accessory olfactory relay areas in the brain.[18] Bidirectional contacts were evident suggesting that GnRH neurons can also modulate odor- and pheromone-signal processing. None of these inputs and outputs were found to differ between the sexes.[18] This is surprising because it has been shown that pheromones modulate GnRH activity in a sex-dependent manner. For example, exposure to female pheromones activates GnRH neurons in male mice, consequently leading to a LH/testosterone surge, and vice versa, male pheromones induce LH release in female mice. This suggests that additional neuronal populations must be involved in transferring chemosensory information to GnRH neurons in a sexually dimorphic manner. Kisspeptin neurons are a potential candidate. Kisspeptin, the processed peptide product of the *Kiss1* gene and the endogenous agonist for the GPR54 receptor (also known as KISS1R), has been identified as a potent stimulator of GnRH neurons and has been implicated as a major regulator of the HPG axis (for review, see Castellano et al.).[20–22] Kisspeptin-expressing neurons are mainly localized in two hypothalamic brain nuclei (i.e., the anteroventral periventricular nucleus/periventricular nucleus continuum [AVPv/PeN] and the ARC) and are modulated by sex steroids.[23–27] Some scattered kisspeptin neurons are also

observed in the amygdala.[28–30] Over the last 15 years, the different roles of these two kisspeptin neuronal populations in reproductive maturation and function were further refined by including their participation in the sexual differentiation of the brain, puberty onset, feedback regulation of gonadotropin secretion, neuroendocrine control of ovulation, metabolic modulation of fertility, environmental (photoperiod) control of reproduction in seasonal species, as well as in metabolism modulation and obesity.[25,26,31–36] Interestingly, the AVPV/PeN population shows important sex differences with females having greater numbers of kisspeptin neurons than males, suggesting a particular function of this population in female reproductive physiology. Furthermore, this particular kisspeptin population seems to develop during the prepubertal period under the influence of estradiol. In a first study by Clarkson and Herbison, it was shown that AVPV/PeN kisspeptin expression begins around postnatal day 15 (P15) and rapidly increases to achieve adult-like levels by P30, the time of puberty onset.[37] Ovariectomy of female pups at P15 resulted in a 70 to 90% reduction in the number of neurons expressing kisspeptin of mice killed either at P30 or P60. Administration of estradiol to P15-ovariectomized mice until P30 completely restored the number of kisspeptin-expressing neurons.[37] Likewise, in adult female ArKO mice, we observed a strong reduction in the number of AVPV/PeN kisspeptin neurons, whereas postnatal treatment with estradiol between P15 and P25 significantly augmented the number of kisspeptin neurons, although not to wild-type levels.[27,38] Finally, exposure to male (but not female) olfactory cues induced the expression of the immediate early-gene *c-fos* in AVPV/PeN kisspeptin neurons in female mice, suggesting a specific role for this neuronal population in olfactory-driven behaviors.[38,39] Therefore, we asked whether kisspeptin neurons in the AVPV/PeN might present the missing link in how olfactory cues can elicit sexually differentiated behavioral and neuroendocrine responses. To dissect the role of kisspeptin in female sexual behavior, we used a variety of different transgenic mouse models in combination with cell-specific viral ablation techniques.

Kisspeptin Neurons Are Part of a Motivational Circuit Triggered by Male Olfactory Cues

Female rodents typically control the initiation and timing of copulatory contacts with a male. This is achieved by a succession of precopulatory behaviors to bring the female in contact with the male and is driven by sexual motivation and mate preferences. Mice are nocturnal species and thus relay heavily on olfactory cues to find potential mates. By consequence, the male mouse secretes a wide variety of pheromonal cues through urine and feces but also through other glands. For example, exocrine gland-secreting peptide 1 (ESP1) that is released in male tear fluids and binds to the vomeronasal receptor V2Rp5 has been shown to stimulate lordosis behavior in female mice.[40]

We previously showed that exposure to male (but not female) olfactory cues contained in either urine or soiled bedding specifically activated AVPV/PeN kisspeptin neurons, as measured by Fos/kisspeptin double-labeling immunohistochemistry, in female wild-type mice.[38,39] Interestingly, we observed significantly less Fos/kisspeptin double labeling in female ArKO mice and no double labeling in female α-fetoprotein knockout (AFP-KO) mice, which have been overexposed to estrogens prenatally as they lack the protective actions of AFP against maternal estrogens.[38,39] These results suggest a potential link between odor-induced activation of kisspeptin neurons and the ability to show female sexual behavior as female ArKO mice show very low levels of lordosis behavior, whereas AFP-KO female mice show no lordosis behavior at all.[3,5] Therefore, we asked whether kisspeptin neurons are part of the neural circuit mediating female sexual behaviors. We used several different genetic strategies to address this question. First, we focused on a potential role of kisspeptin in olfactory mate preferences. Female mice lacking a functional *Kiss1* gene ($Kiss^{-/-}$) failed to show any male-directed preference in adulthood following ovariectomy and subsequent treatment with estradiol and progesterone. Interestingly, a single injection with kisspeptin-10 (KP-10 at the dose of 0.13 mg/kg) induced a strong male-directed preference in $Kiss^{-/-}$ females, suggesting that kisspeptin indeed stimulates male-directed preferences.[41] However, it should be noted that $Kiss^{-/-}$ mice lack kisspeptin since ontogeny; so, possible developmental effects could not be ruled out. In addition, it could not be determined which kisspeptin population (AVPV/PeN or Arc or amygdala) is important in mediating mate preferences. Therefore, in a second experiment, we used Kiss-Cre mice and injected an adeno-associated virus (AAV) encoding a Cre-recombinase-dependent caspase 3, bilaterally into the AVPV/PeN to specifically ablate the AVPV/PeN kisspeptin population. Female Cre^{+} mice failed to show any male-directed preference upon viral injection, but mate preference could be restored by a single injection with KP-10. Successful ablation of kisspeptin neurons by this viral strategy was confirmed at the histological level; that is, a strong decrease (~71%) in the number of kisspeptin-immunoreactive neurons was observed in the AVPV/PeN of Kiss-Cre^{+} females compared with Kiss-Cre^{-} females. No effect was observed on kisspeptin expression in the ARC, confirming that only the AVPV/PeN population was affected.[41] Taken together, these two experiments strongly suggest that AVPV/PeN kisspeptin neurons are part of a neural circuit mediating mate preferences in female mice.

Kisspeptin Neurons Are Essential for Lordosis Behavior

Once the female is in direct contact with the male, the female will display specific receptive behaviors, such as the lordosis posture. So, the next question was to determine whether kisspeptin neurons are also important for lordosis behavior. In a first experiment, ovary-intact females which were monitored for their estrous cycle were paired with a sexually active male on the day of estrus to determine whether mating stimulation would activate kisspeptin neurons by means of significant Fos/Kisspeptin double labeling. Interestingly, we observed that approximately 30% of AVPV/PeN kisspeptin

neurons expressed Fos protein upon receiving intromissive stimulation, whereas no such double labeling was observed in estrous females not exposed to the male.[41] As a next step, we investigated the effects of a single injection with KP-10 on lordosis behavior. Interestingly, stimulatory effects of KP-10 were already visible 15 minutes after the injection and remained present 2 hours later. It could be argued that KP-10 injected peripherally might not pass the blood–brain barrier and might thus not exert its effects in the brain itself but through the pituitary gonadotrophs which also express GPR54. However, Comninos et al convincingly showed that radiolabeled kisspeptin injected peripherally in male mice could be detected in numerous brain regions 30 to 60 minutes later.[42] Furthermore, GnRH neurons are most likely the site of action and peripheral kisspeptin can access GnRH neurons via their dendritic terminals in the organum vasculosum of the lamina terminalis (OVLT) which lies outside the blood–brain barrier.[43] Nevertheless, we performed an additional experiment, in which KP-10 was injected directly into the lateral ventricle and tested females for lordosis behavior. We found that an intracerebroventricular injection of KP-10 strongly stimulated lordosis behavior up to levels which were very comparable to what was observed with a peripheral injection. In subsequent experiments, the important role of AVPV/PeN kisspeptin neurons in lordosis behavior was further confirmed. As expected, Kiss$^{-/-}$ females showed very low levels of lordosis behavior in comparison with WT females, and a single injection with KP-10 was sufficient to restore lordosis behavior in this mouse model. Likewise, kisspeptin neuronal ablation by bilateral injection of an AAV-Caspase 3 into the AVPV/PeN of Kiss-Cre^{+} mice led to a significant decrease in lordosis behavior, which was again restored by a subcutaneous KP-10 injection. Conversely, blue light photostimulation of kisspeptin neurons facilitated lordosis behavior in female Kiss-Cre^{+} mice which were bilaterally injected into the AVPV/PeN with an AAV encoding a Cre-dependent channel rhodopsin (ChR2).[41] Taken together, these experiments showed that AVPV/PeN kisspeptin neurons are an essential part of the neural network involved in both mate preference and lordosis behavior.

Kisspeptin Acts through GnRH Neurons in Stimulating Mate Preferences, but Not Lordosis Behavior

It has been clearly shown that kisspeptin neurons directly innervate GnRH neurons. Since previous studies from our laboratory and others have shown that a single peripheral injection with GnRH reliably stimulates lordosis behavior in female rodents, it was reasonable to assume that kisspeptin's effects on sexual behavior are mediated through GPR54 expressed by GnRH neurons.[12,14–17] To determine whether kisspeptin is acting through GnRH neurons, we first tested mate preferences and lordosis behavior in female GPIC/R26-iDTR mice following an acute peripheral injection with diphtheria toxin (DT) to ablate all GPR54-expressing neurons (which includes GnRH neurons).[44] In line with our hypothesis, mate preferences were clearly affected in these mice following DT injection, but rather surprisingly, they showed normal levels of lordosis behavior when paired with a male. Since 5% of GnRH neurons do not express GPR54 and were thus not ablated (confirmed by histological examinations), we used an additional transgenic mouse model, that is, GnRH::Cre;Dicer$^{loxP/loxP}$ mice in which GnRH immunoreactivity is completely absent in adulthood.[45] Female GnRH::Cre;Dicer$^{loxP/loxP}$ mice showed no preference for the male and even a small preference for the female. Furthermore, a male-directed preference was induced by a peripheral injection with GnRH but not with kisspeptin, suggesting that kisspeptin induces male-directed preferences through GnRH neurons.[41] However, as was observed in GPIC/R26-iDTR females, lordosis behavior was not affected in GnRH::Cre;Dicer$^{loxP/loxP}$ females, suggesting that kisspeptin does not stimulate lordosis behavior through GnRH neurons. In addition, it questions whether kisspeptin acts through its canonical receptor GPR54 as ablation of GPR54-expressing neurons in the GPIC/R26-iDTR mouse model did not affect lordosis behavior as well. The latter results are in accordance with Kauffmann and colleagues who showed that female GPR54KO mice also expressed normal levels of lordosis behavior.[26] Several studies have demonstrated that kisspeptin can also activate neuropeptide FF receptors (NPFFR1 and NPFFR2).[44–48] Kisspeptin can, for example, modulate arcuate neuron excitability at least partially via NPFF receptors independently of GPR54.[49] As both NPFFR1 and NPFFR2 have been detected in brain areas important in female sexual behavior, such as the POA, the PVN, and the VMH, it is thus tempting to speculate that these receptors may play a key role in the facilitation of lordosis behavior.[50] Consistent with this, we observed that an intracerebroventricular injection with 5 ng of the NPFFR1 antagonist BIBP3226 into the third ventricle decreased lordosis behavior in female mice (►**Fig. 1a**). To further confirm a potential role for NPFFR, we analyzed lordosis behavior in NPFFR1$^{-/-}$ mice. We did not observe any deficits in their lordosis behavior, whereas a subcutaneous injection with KP-10 was successful in inducing lordosis behavior in these mice (►**Fig. 1b**). Furthermore, a subcutaneous injection with RFRP3, the most important ligand of NPFFR, failed to stimulate lordosis behavior in estradiol-treated wild-type female mice (►**Fig. 1c**). These data thus did not confirm a role for NPFFR1 as alternative receptor to GPR54 to induce lordosis behavior. However, NPFFR2 could have compensated the lack of NPFFR1 in the NPFFR1$^{-/-}$ mouse model. Therefore, more research is clearly warranted, for instance by analyzing lordosis behavior in a double KO model for NPFFR1 and -2.

Alternative Pathway Involving NO-Synthesizing Neurons in Lordosis Behavior

To identify potential candidate neurons downstream of AVPV/PeN kisspeptin neurons, we used KissIC/R26-BlZ mice which express the transsynaptic tracer BL exclusively in kisspeptin neurons to label synaptically connected cells. In a first experiment, we observed BL+ neurons in the PVN, which is consistent with previous studies demonstrating

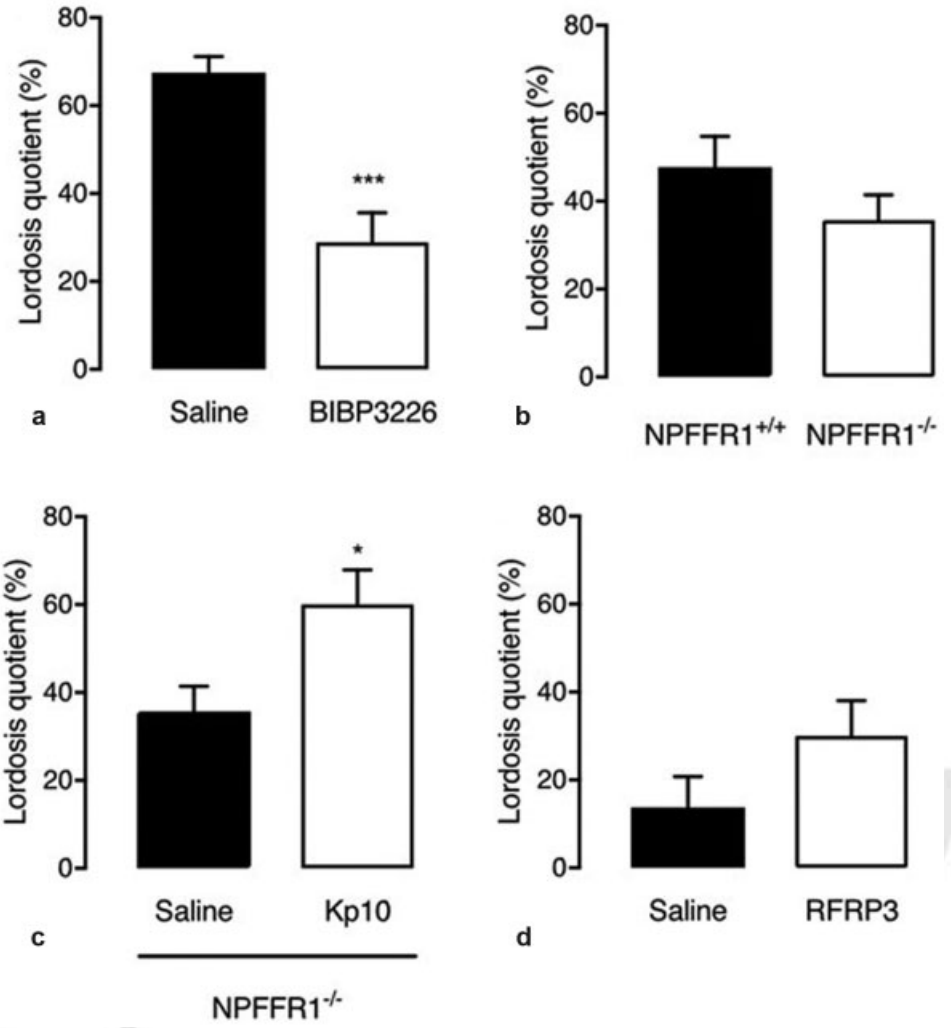

Fig. 1 The role of NPFFRs in lordosis behavior. (**a**) An intracerebroventricular injection of the NPFFR1 antagonist BIBP3226 significantly decreased lordosis behavior in ovariectomized female wild-type mice treated with estradiol and progesterone (saline: $n = 8$; BIBP3226: $n = 9$). (**b**) Lordosis behavior is not affected in mice carrying a mutation in the NPFFR1 gene ($NPFFR1^{+/+}$: $n = 9$; $NPFFR1^{-/-}$: $n = 7$). (**c**) A subcutaneous injection with kisspeptin (KP-10) significantly increased lordosis behavior in female $NPFFR1^{-/-}$ mice. (**d**) An intracerebroventricular injection with RFRP3, which is an agonist of NPFFRs, did not significantly increase lordosis behavior in ovariectomized female wild-type mice treated with estradiol, but not with progesterone before the lordosis test (saline: $n = 8$; RFRP3: $n = 9$).

kisspeptidergic innervation of this nucleus.[28] Subsequent immunohistochemical analyses of these cells showed that the BL+ neurons in the PVN predominantly expressed neuronal nitric oxide synthase (nNOS; ►**Fig. 2a**), which has previously been implicated in reproductive behaviors.[51,52] These data thus demonstrated that kisspeptin neurons communicate with subsets of nNOS neurons in the PVN. To further define the role of NO signaling in this neural circuit, we next analyzed nNOS phosphorylation in the PVN following lordosis. We found that nNOS was robustly activated in the PVN upon mating (►**Fig. 2b**). We then analyzed mice deficient in nNOS and found that nNOS knockout ($nNOS^{-/-}$) females showed a strong decrease in lordosis behavior compared with control littermates (►**Fig. 2c**), but showed lordosis behavior comparable to WT females when treated with the NO donor SNAP (►**Fig. 2c**). $Kiss^{-/-}$ females injected subcutaneous with SNAP also showed wild-type–like levels of lordosis behavior (►**Fig. 2d**). Importantly, subcutaneous injection of either kisspeptin or GnRH failed to stimulate lordosis behavior in nNOS-KO females, further confirming that nNOS neurons are downstream of kisspeptin in this neural circuit (►**Fig. 2c**).

Since it has been shown that approximately 35% of the nNOS neurons in the PVN also coexpress oxytocin (OT), we wanted to determine the role of this particular neuropeptide in lordosis behavior. OT has been implicated in maternal attachment, social recognition, and uterine contractions, but also in female sexual behavior, since female $OT^{-/-}$ mice showed strong deficits in lordosis behavior.[53,54] Therefore, we determined whether OT injected into the lateral ventricle would stimulate lordosis behavior in wild-type female mice (which were ovariectomized and treated with estradiol, but not with progesterone). We found a significant effect of OT on lordosis behavior: 1 and 5 ng of OT, but not the lowest dose of 0.3 ng, significantly stimulated lordosis behavior (►**Fig. 3**). Furthermore, although OT expression in the PVN seems to be stimulated by estradiol and progesterone with the highest numbers when females are sexually receptive, it did not

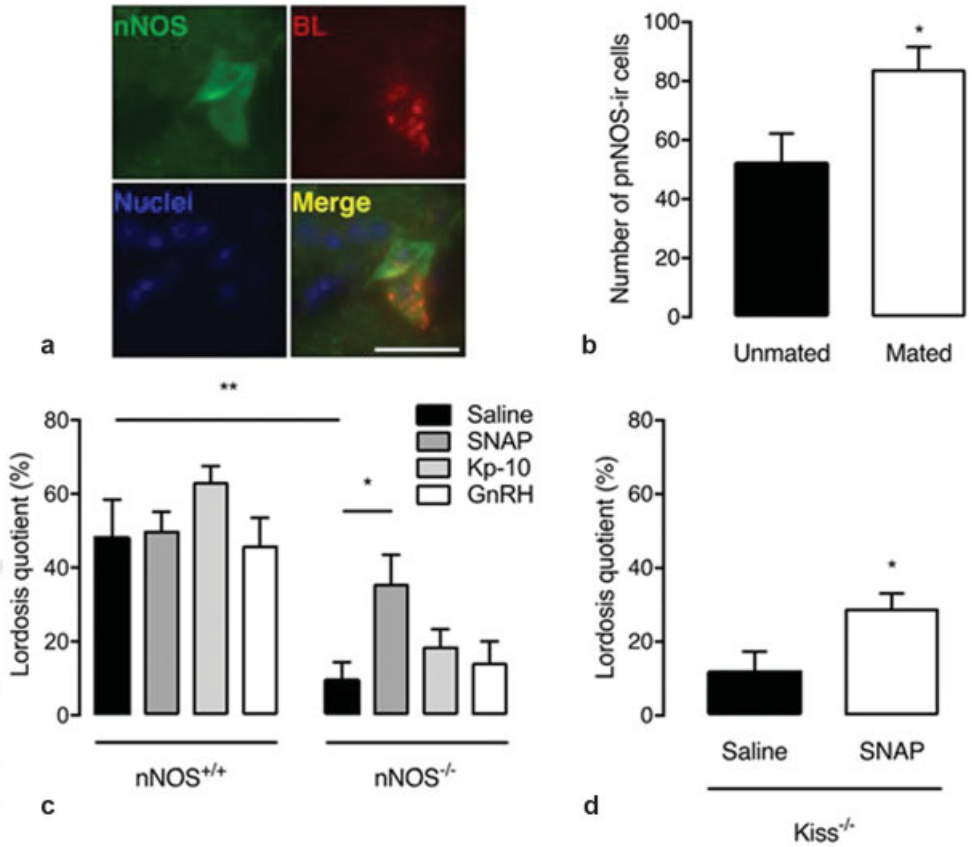

Fig. 2 nNOS neurons are downstream of kisspeptin neurons in the lordosis circuit. (**a**) Transfer of the transsynaptic tracer barley lectin from kisspeptin neurons reveals that kisspeptin neurons communicate with nNOS neurons in the PVN. (**b**) Mating triggered phosphorylation (and thus activation) of nNOS in the PVN (unmated: $n = 5$; mated: $n = 5$). (**c**) Lordosis behavior is disrupted in nNOS$^{-/-}$ mice, but restored by a peripheral injection of the NO donor SNAP. By contrast, a peripheral injection of either kisspeptin or GnRH failed to restore lordosis in nNOS$^{-/-}$ mice (nNOS$^{+/+}$: $n = 6$; nNOS$^{-/-}$: $n = 7$). (**d**) A peripheral injection with SNAP restored lordosis in Kiss$^{-/-}$ mice ($n = 10$). KP-10, kisspeptin; nNOS, neuronal form of nitric oxide synthase; PVN, paraventricular nucleus; SNAP, S-nitroso-N-acetylpenicillamine. Bars represent the mean ± SEM. $^{*}p \leq 0.05$, $^{**}p \leq 0.01$. Scale bar represents 20 µm.

reach statistical significance (data not shown). So, the role of OT neurons in the PVN in lordosis behavior needs to be further confirmed.

The KissIC/R26-BlZ mouse model has an important limitation that we could not distinguish between projections coming from either the AVPV/PeN or the ARC kisspeptin population. Therefore, in an additional experiment, we injected a Cre-dependent mCherry AAV-virus bilaterally into the AVPV/PeN of KissIC/R26-BlZ mice to delineate only the projections coming from AVPV/PeN kisspeptin neurons. We observed a cluster of BL+ cells in the ventrolateral part of the VMH (VMHvl), which has shown to be critical in lordosis behavior, but no BL+ neurons in the PVN. The latter suggests that the PVN most likely receives projections from the ARC kisspeptin population, but this needs to be confirmed in future experiments. Subsequent immunohistochemical analyses showed that the BL+ neurons in the VMHvl expressed nNOS, suggesting that this particular nNOS population might be a potential important downstream relay of AVPV/PeN kisspeptin neurons in governing lordosis behavior. It is interesting to note that close to 100% of neurons expressing estradiol receptors in the VMHvl are actually nNOS expressing neurons.[55] However, it cannot be ruled out that other nNOS populations

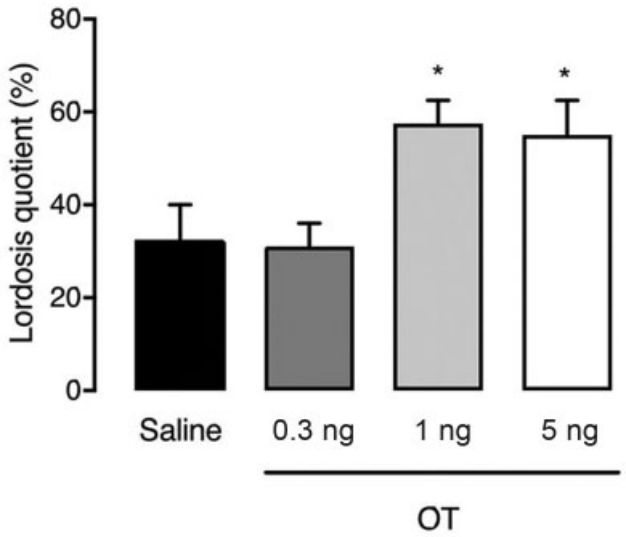

Fig. 3 Role of oxytocin in lordosis behavior. An injection with 1 and 5 ng significantly stimulated lordosis behavior in female wild-type mice ($n = 9$ for each group) which were ovariectomized in adulthood and treated with estradiol through a Silastic implant. OT, oxytocin.

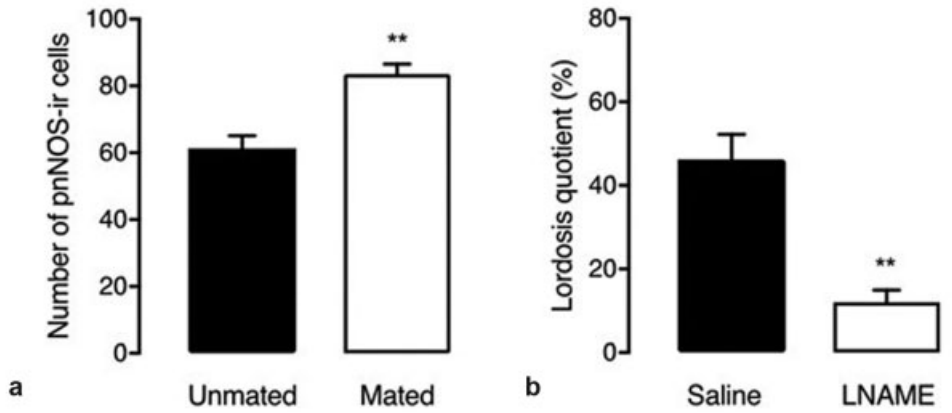

Fig. 4 Role of nNOS neurons in the OVLT in lordosis behavior. (**a**) Mating triggered phosphorylation (and thus activation) of nNOS in the OVLT (unmated: $n = 5$; mated: $n = 5$). (**b**). Continuous infusion via a mini osmotic pump (Alzet) with L-NAME, a nNOS inhibitor, into the OVLT significantly decreased lordosis behavior in female wild-type mice which were ovariectomized and treated with estradiol and progesterone. OVLT, organum vasculosum of the lamina terminalis. Saline: $n = 8$; L-NAME: $n = 5$.

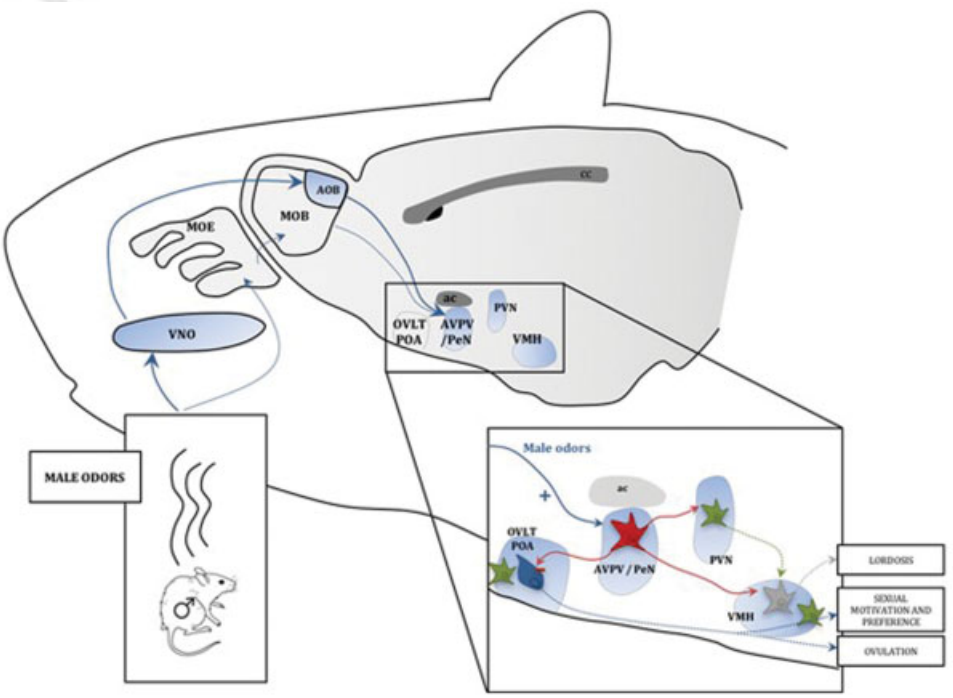

Fig. 5 Schematic overview of the neural network important in female sexual behavior in the female mouse. ac, anterior commissure; AOB, accessory olfactory bulb; AVPV, anteroventral periventricular area; cc, corpus callosum; MOB, main olfactory bulb; MOE, main olfactory epithelium; OVLT, organum vasculosum of the lamina terminalis; POA, preoptic area; PVN: paraventricular nucleus; VMH, ventromedial hypothalamus; VNO: vomeronasal organ.

are also involved, for instance, in the OVLT, where nNOS neurons are part of a neural circuit regulating ovarian cyclicity and ovulation.[56] It is interesting to note that mating stimulation induced an activation of nNOS neurons in the OVLT as measured by the number of phosphorylated nNOS (pnNOS) neurons (►**Fig. 4a**) as well as that a continuous infusion with L-NAME, an inhibitor of nNOS, significantly decreased lordosis behavior (►**Fig. 4b**). Taken all available data together, there is strong evidence that kisspeptin acts through nNOS neurons in stimulating lordosis behavior. This is based on the different neuronal tracing experiments using the KissIC/R26-BlZ mouse model, as well as on the phenotype of nNOS-KO mice showing very little lordosis behavior which cannot be restored by either GnRH or kisspeptin administration. However, which nNOS neuronal population (OVLT, PVN, or VMHvl) is particularly important needs to be further confirmed in future experiments.

Conclusion

To summarize, we have revealed a novel neural pathway controlling sexual behavior in female mice in which AVPV/PeN kisspeptin neurons play a central role (►**Fig. 5**). AVPV/PeN kisspeptin neurons are activated by male olfactory cues

detected and processed through the accessory olfactory pathway.[38,39,41] Then AVPV/PeN kisspeptin neurons presumably act through GnRH neurons and its canonical receptor GPR54 in stimulating male-directed preferences, but they might act through an alternative pathway involving nNOS neurons in the VMHvl to stimulate lordosis behavior. Some questions remain, however, on this neural circuit and some refinement is clearly needed. For instance, it has to be confirmed whether kisspeptin can act through a different receptor than GPR54 in stimulating lordosis behavior such as NPFFR. Furthermore, it has to be confirmed which nNOS neuronal population is critical (i.e., the VMHvl, PVN, or OVLT) for lordosis behavior. Finally, it needs to be determined what is the precise role of GnRH neurons in this neural circuit as it has been shown that a single injection with GnRH can stimulate lordosis behavior in female mice and rats?

Acknowledgments

We would like to thank Dr. Manuel Tena-Sempere for generously providing us with the NPFFR1-KO mouse model. This research was supported by grants from the Fonds National de la Recherche Scientifique (FNRS T.0207.13 and J.0004.19). Julie Bakker is a research director of the FNRS.

References

1 Boling JL, Blandau RJ. The estrogen-progesterone induction of mating responses in the spayed female rat. Endocrinology 1939; 25:359–364
2 Bakker J, Brand T, van Ophemert J, Slob AK. Hormonal regulation of adult partner preference behavior in neonatally ATD-treated male rats. Behav Neurosci 1993;107(03):480–487
3 Bakker J, De Mees C, Douhard Q, et al. Alpha-fetoprotein protects the developing female mouse brain from masculinization and defeminization by estrogens. Nat Neurosci 2006;9(02):220–226
4 Brock O, Baum MJ, Bakker J. The development of female sexual behavior requires prepubertal estradiol. J Neurosci 2011;31(15): 5574–5578
5 Bakker J, Honda S, Harada N, Balthazart J. The aromatase knockout mouse provides new evidence that estradiol is required during development in the female for the expression of sociosexual behaviors in adulthood. J Neurosci 2002;22(20):9104–9112
6 Pfaff DW. Estrogens and Brain Function: Neural Analysis for a Hormone - Controlled Mammalian Reproductive Behavior. New York: Springer-Verlag; 1980
7 McCarthy MM, Malik KF, Feder HH. Increased GABAergic transmission in medial hypothalamus facilitates lordosis but has the opposite effect in preoptic area. Brain Res 1990;507(01):40–44
8 Olster DH. Ibotenic acid-induced lesions of the medial preoptic area/anterior hypothalamus enhance the display of progesterone-facilitated lordosis in male rats. Brain Res 1993;626(1-2): 99–105
9 Mills RH, Sohn RK, Micevych PE. Estrogen-induced mu-opioid receptor internalization in the medial preoptic nucleus is mediated via neuropeptide Y-Y1 receptor activation in the arcuate nucleus of female rats. J Neurosci 2004;24(04):947–955
10 Sakuma Y, Pfaff DW. Modulation of the lordosis reflex of female rats by LHRH, its antiserum and analogs in the mesencephalic central gray. Neuroendocrinology 1983;36(03):218–224
11 Marson L, Murphy AZ. Identification of neural circuits involved in female genital responses in the rat: a dual virus and anterograde tracing study. Am J Physiol Regul Integr Comp Physiol 2006;291 (02):R419–R428
12 Keller M, Pierman S, Douhard Q, Baum MJ, Bakker J. The vomeronasal organ is required for the expression of lordosis behaviour, but not sex discrimination in female mice. Eur J Neurosci 2006;23 (02):521–530
13 Keller M, Douhard Q, Baum MJ, Bakker J. Destruction of the main olfactory epithelium reduces female sexual behavior and olfactory investigation in female mice. Chem Senses 2006;31(04):315–323
14 Moss RL, McCann SM. Induction of mating behavior in rats by luteinizing hormone-releasing factor. Science 1973;181(4095): 177–179
15 Pfaff DW. Luteinizing hormone-releasing factor potentiates lordosis behavior in hypophysectomized ovariectomized female rats. Science 1973;182(4117):1148–1149
16 Moss RL, Foreman MM. Potentiation of lordosis behavior by intrahypothalamic infusion of synthetic luteinizing hormone-releasing hormone. Neuroendocrinology 1976;20(02):176–181
17 Sirinathsinghji DJ. GnRH in the spinal subarachnoid space potentiates lordosis behavior in the female rat. Physiol Behav 1983;31 (05):717–723
18 Boehm U, Zou Z, Buck LB. Feedback loops link odor and pheromone signaling with reproduction. Cell 2005;123(04):683–695
19 Wen S, Götze IN, Mai O, Schauer C, Leinders-Zufall T, Boehm U. Genetic identification of GnRH receptor neurons: a new model for studying neural circuits underlying reproductive physiology in the mouse brain. Endocrinology 2011;152(04):1515–1526
20 de Roux N, Genin E, Carel JC, Matsuda F, Chaussain JL, Milgrom E. Hypogonadotropic hypogonadism due to loss of function of the KiSS1-derived peptide receptor GPR54. Proc Natl Acad Sci U S A 2003;100(19):10972–10976
21 Seminara SB, Messager S, Chatzidaki EE, et al. The GPR54 gene as a regulator of puberty. N Engl J Med 2003;349(17):1614–1627
22 Castellano JM, Roa J, Luque RM, et al. KiSS-1/kisspeptins and the metabolic control of reproduction: physiologic roles and putative physiopathological implications. Peptides 2009;30(01):139–145
23 Smith JT, Dungan HM, Stoll EA, et al. Differential regulation of KiSS-1 mRNA expression by sex steroids in the brain of the male mouse. Endocrinology 2005;146(07):2976–2984
24 Smith JT, Cunningham MJ, Rissman EF, Clifton DK, Steiner RA. Regulation of Kiss1 gene expression in the brain of the female mouse. Endocrinology 2005;146(09):3686–3692
25 Clarkson J, Herbison AE. Postnatal development of kisspeptin neurons in mouse hypothalamus; sexual dimorphism and projections to gonadotropin-releasing hormone neurons. Endocrinology 2006;147(12):5817–5825
26 Kauffman AS, Park JH, McPhie-Lalmansingh AA, et al. The kisspeptin receptor GPR54 is required for sexual differentiation of the brain and behavior. J Neurosci 2007;27(33):8826–8835
27 Brock O, Bakker J. The two kisspeptin neuronal populations are differentially organized and activated by estradiol in mice. Endocrinology 2013;154(08):2739–2749
28 Clarkson J, d'Anglemont de Tassigny X, Colledge WH, Caraty A, Herbison AE. Distribution of kisspeptin neurones in the adult female mouse brain. J Neuroendocrinol 2009;21(08):673–682
29 Kim J, Semaan SJ, Clifton DK, Steiner RA, Dhamija S, Kauffman AS. Regulation of Kiss1 expression by sex steroids in the amygdala of the rat and mouse. Endocrinology 2011;152(05):2020–2030
30 Comninos AN, Anastasovska J, Sahuri-Arisoylu M, et al. Kisspeptin signaling in the amygdala modulates reproductive hormone secretion. Brain Struct Funct 2016;221(04):2035–2047
31 Revel FG, Saboureau M, Masson-Pévet M, Pévet P, Mikkelsen JD, Simonneaux V. Kisspeptin mediates the photoperiodic control of reproduction in hamsters. Curr Biol 2006;16(17): 1730–1735
32 Smith JT, Popa SM, Clifton DK, Hoffman GE, Steiner RA. Kiss1 neurons in the forebrain as central processors for generating the preovulatory luteinizing hormone surge. J Neurosci 2006;26(25): 6687–6694

33 Tena-Sempere M. Hypothalamic KiSS-1: the missing link in gonadotropin feedback control? Endocrinology 2005;146(09): 3683–3685
34 Clarkson J, d'Anglemont de Tassigny X, Moreno AS, Colledge WH, Herbison AE. Kisspeptin-GPR54 signaling is essential for preovulatory gonadotropin-releasing hormone neuron activation and the luteinizing hormone surge. J Neurosci 2008;28 (35):8691–8697
35 Simonneaux V, Ansel L, Revel FG, Klosen P, Pévet P, Mikkelsen JD. Kisspeptin and the seasonal control of reproduction in hamsters. Peptides 2009;30(01):146–153
36 Tolson KP, Garcia C, Yen S, et al. Impaired kisspeptin signaling decreases metabolism and promotes glucose intolerance and obesity. J Clin Invest 2014;124(07):3075–3079
37 Clakson J, d'Anglemont de Tassigny X, Colledge WH, Caraty A, Herbison AE. Distribution of kisspeptin neurones in the adult female mouse brain. J Neuroendocrinol 2009;21(08):673–682
38 Bakker J, Pierman S, González-Martínez D. Effects of aromatase mutation (ArKO) on the sexual differentiation of kisspeptin neuronal numbers and their activation by same versus opposite sex urinary pheromones. Horm Behav 2010;57(4-5):390–395
39 Taziaux M, Bakker J. Absence of female-typical pheromone-induced hypothalamic neural responses and kisspeptin neuronal activity in α-fetoprotein knockout female mice. Endocrinology 2015;156(07):2595–2607
40 Haga S, Hattori T, Sato T, et al. The male mouse pheromone ESP1 enhances female sexual receptive behaviour through a specific vomeronasal receptor. Nature 2010;466(7302):118–122
41 Hellier V, Brock O, Candlish M, et al. Female sexual behavior in mice is controlled by kisspeptin neurons. Nat Commun 2018;9 (01):400
42 Comninos AN, Wall MB, Demetriou L, et al. Kisspeptin modulates sexual and emotional brain processing in humans. J Clin Invest 2017;127(02):709–719
43 d'Anglemont de Tassigny X, Fagg LA, Carlton MBL, Colledge WH. Kisspeptin can stimulate gonadotropin-releasing hormone (GnRH) release by a direct action at GnRH nerve terminals. Endocrinology 2008;149(08):3926–3932
44 Mayer C, Boehm U. Female reproductive maturation in the absence of kisspeptin/GPR54 signaling. Nature Neuroscience 2011;14:704–710
45 Messina A, Langlet F, Chachlaki K, et al. A microRNA switch regulates the rise in hypothalamic GnRH production before puberty. Nat Neurosci 2016;19(06):835–844
46 Lyubimov Y, Engstrom M, Wurster S, Savola JM, Korpi ER, Panula P. Human kisspeptins activate neuropeptide FF2 receptor. Neuroscience 2010;170(01):117–122
47 Oishi S, Misu R, Tomita K, et al. Activation of neuropeptide FF receptors by kisspeptin receptor ligands. ACS Med Chem Lett 2010;2(01):53–57
48 Elhabazi K, Humbert JP, Bertin I, et al. Endogenous mammalian RF-amide peptides, including PrRP, kisspeptin and 26RFa, modulate nociception and morphine analgesia via NPFF receptors. Neuropharmacology 2013;75:164–171
49 Liu X, Herbison A. Kisspeptin regulation of arcuate neuron excitability in kisspeptin receptor knockout mice. Endocrinology 2015;156(05):1815–1827
50 Gouardères C, Faura CC, Zajac JM. Rodent strain differences in the NPFF1 and NPFF2 receptor distribution and density in the central nervous system. Brain Res 2004;1014(1-2):61–70
51 Mani SK, Allen JM, Rettori V, McCann SM, O'Malley BW, Clark JH. Nitric oxide mediates sexual behavior in female rats. Proc Natl Acad Sci U S A 1994;91(14):6468–6472
52 González-Flores O, Etgen AM. The nitric oxide pathway participates in estrous behavior induced by progesterone and some of its ring A-reduced metabolites. Horm Behav 2004;45 (01):50–57
53 Argiolas A, Melis MR. Neuropeptides and central control of sexual behaviour from the past to the present: a review. Prog Neurobiol 2013;108:80–107
54 Becker RO, Lazzari VM, Menezes IC, et al. Sexual behavior and dendritic spine density of posterodorsal medial amygdala neurons in oxytocin knockout female mice. Behav Brain Res 2013; 256:95–100
55 Chachlaki K, Nalone SA, Qualls-Creekmore E, Hrabovsky E, Munzberg H, Giacobini P, Ango F, Prevot V. Phenotyping of nNOS neurons in the postnatal and adult female mouse hypothalamus. J Comp Neurol 2017;525(15):3177–3189
56 Hanchate NK, Parkash J, Bellefontaine N, Mazur D, Colledge WH, d'Anglemont de Tassigny X, Prevot V. Kisspeptin-GPR54 signaling in mouse NO-synthesizing neurons participates in the hypothalamic control of ovulation. J Neurosci 2012;32:932–945