Differential regulation of *Kiss1* gene expression by oestradiol in the hypothalamus of the female Damaraland mole-rat, an induced ovulator

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Highlights

- Differential regulation of kisspeptin neurons in the hypothalamus by oestradiol.
- Oestradiol downregulates *Kiss1* gene expression in the arcuate nucleus.
- Oestradiol upregulates *Kiss1* gene expression in the preoptic region.

ABSTRACT

Kisspeptin, a product of the *Kiss1* gene is considered a potent stimulator of gonadotropin release, by interacting with its receptor, the G protein-coupled receptor 54. *Kiss1* neurons are known to mediate the positive and negative feedback effects of oestradiol on GnRH neurons that control the pulsatile and surge secretion of GnRH. While in spontaneously ovulating mammals the GnRH/LH surge is initiated by a rise in ovarian oestradiol secreted from maturing follicles, in induced ovulators, the primary trigger is the mating stimulus. Damaraland mole rats (*Fukomys damarensis*) are cooperatively breeding, subterranean rodents that exhibit induced ovulation. We have previously described in this species the distribution and differential expression pattern of *Kiss1*-expressing neurons in the hypothalamus of males and females. Here we examine whether oestradiol (E2) regulates the hypothalamic *Kiss1* expression in a similar way as described for spontaneously ovulating rodent species. By means of *in situ* hybridisation, we measured *Kiss1* mRNA among groups of ovary-intact, ovariectomized (OVX) and OVX females treated with E2 (OVX+E2). In the arcuate nucleus (ARC), *Kiss1* expression increased after ovariectomy and decreased with E2 treatment. In the preoptic region, *Kiss1* expression after gonadectomy was similar to the level of wild-caught gonad-intact controls, but was dramatically upregulated with E2 treatment. The data suggest that, similar to other species, *Kiss1* neurons in the ARC, which are inhibited by E2, play a role in the negative feedback control on GnRH release. The exact role of the *Kiss1* neuron population in the preoptic region, which is stimulated by E2, remains to be determined.

KEYWORDS: *Kiss1*, *in situ* hybridization, *Fukomys damarensis*, oestradiol, ovariectomy

1. INTRODUCTION

The secretion of gonadotropin-releasing hormone (GnRH) from the hypothalamus into the portal vasculature, which in turn stimulates the release of the gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the anterior pituitary is essential for the regulation of mammalian reproductive function (Pohl and Knobil 1982). The predominant mode of GnRH secretion is the pulsatile release, which is critical for gonadal development and normal regulation of the ovarian cycle. The surge mode, on the other hand, generates a massive release of GnRH and subsequently LH to induce ovulation. These two modes underlie different feedback effects of gonadal steroid hormones (Goodman 2015; Herbison 2015). The GnRH surge in spontaneously ovulating mammals is brought about by the positive feedback action of rising ovarian oestradiol levels at the end of the follicular phase of the female cycle. In induced ovulators, it is the sensory stimulus associated with mating that leads to the activation of the GnRH neurons and to the GnRH surge, which results in the pre-ovulatory LH surge (Bakker and Baum 2000; Kauffman and Rissman 2006). As GnRH neurons do not express steroid hormone receptors, the positive and negative feedback action of oestradiol is mediated through other, steroid hormone-sensitive neurons that project to GnRH neurons (Wintermantel et al. 2006; Herbison 2008).

Kisspeptin (*Kiss1*), a member of the RF-amide peptide family, has been identified as a strong activator of GnRH neurons (Gottsch et al. 2004; Navarro et al. 2004; Messager et al. 2005) and a key regulator of GnRH and LH secretion (for review, see Kauffman 2022). *Kiss1*-expressing neuron populations have been found mainly in two regions of the hypothalamus, in the preoptic area and in the arcuate nucleus (ARC). In rodents such as mice, rats and hamsters, the rostral population is located in the anteroventral periventricular nucleus (AVPV) and adjacent periventricular nucleus (PeN). In female rodents, the kisspeptin neurons in the AVPV/PeN are thought to mediate the oestradiol (E2)-induced GnRH/LH surge, while those in the ARC are thought to control GnRH pulses through negative feedback action of E2 (for

review, see Smith 2013). This positive and negative feedback regulation is mediated by oestrogen receptor α (ERα), which is coexpressed in both populations of *Kiss1* neurons (Smith et al. 2005; Poling et al. 2017). Indeed, in rodents and sheep it was shown that *Kiss1* gene expression in both regions is differentially regulated by steroid hormones. In ovariectomized females, *Kiss1* expression in the AVPV/PeN is greatly reduced while oestradiol treatment leads to an upregulation of *Kiss1* mRNA. The opposite pattern is seen in the ARC. In the ARC, ovariectomy stimulates *Kiss1* expression and oestradiol treatment reverses this effect (Smith et al. 2005, 2007; Gottsch et al. 2009).

African mole-rats (family Bathyergidae) are subterranean rodents, which show a wide variety of social organization, from strictly solitary to highly social such as naked mole-rats (*Heterocephalus glaber*) and Damaraland mole-rats (*Fukomys damarensis*, Bennett and Faulkes 2000). Damaraland mole-rats are cooperative breeders, which live in groups with an average group size of 11 individuals. Reproduction is partitioned unequally among group members with dominant individuals monopolizing breeding opportunities (Bennett and Jarvis 1988). In cooperative breeders, the high reproductive skew is maintained by reproductive suppression of subordinate group members (Keller and Reeve 1994). In the Damaraland mole-rat, reproduction is monopolized by a single female (queen) in each group and subordinates, which are the offspring of the breeding pair, constitute the work force and help with colony maintenance, foraging and raising the offspring (Bennett and Jarvis 1988). Subordinate females in the presence of the queen are anovulatory (Molteno and Bennett 2000). Damaraland mole-rats are induced ovulators and anovulation in subordinate females occurs through a lack of copulation (Voigt et al. 2021). It is likely that such females remain in a prepubertal stage until virgin mating occurs. In musk shrews (*Suncus murinus*), the latter has been shown to activate ovarian steroidogenesis, thereby priming the hypothalamic-pituitary-gonadal (HPG) axis and inducing the onset of puberty (Rissman 1992). In relation, non-reproductive female Damaraland mole-rats possess significantly less *Kiss1*-expressing cells and a reduced *Kiss1* mRNA content

per cell in the ARC compared to reproductively active females (Voigt and Bennett 2018). In the preoptic hypothalamus, however, *Kiss1*-expressing cells are scarce and restricted to a few cells scattered around the AVPV, with no differences between reproductive and non-reproductive females (Voigt and Bennett 2018). This contrasts with findings in other rodents such as rats and mice, where females show moderate levels of *Kiss1* expression in this region (Smith et al. 2005; Kauffman et al. 2007). Furthermore, a study in another mole-rat species, the naked mole-rat, using immunocytochemistry reported substantial kisspeptin immunoreactivity in the AVPV/PeN, with breeding females having significantly more kisspeptin-ir cells than non-breeding females (Zhou et al. 2013). Moreover, these authors reported that gonadectomy had no effect on kisspeptin immunoreactivity in either the ARC or AVPV/PeN. This result is somewhat surprising because in a wide variety of other animals such as other rodents, sheep, pigs and primates, gonadectomy significantly affects the hypothalamic *Kiss1* expression (for review, see Goodman 2022). In most of these species, coexpression of *Kiss1* and *ERα* has been confirmed, which supports the view that *Kiss1* neurons mediate the feedback actions of ovarian oestradiol on GnRH neurons through ERα signaling (Franceschini et al. 2006; Adachi et al. 2007; Clarkson et al 2008; Poling et al 2017). A study using quantitative PCR confirmed hypothalamic *ERα* expression in naked mole-rats and showed that breeding females have increased levels of *ERα* compared to subordinate females (Swift-Gallant et al. 2015), similar findings, using *in situ* hybridisation have been obtained by us in Damaraland mole-rats (Voigt et al. 2014). Based on these conflicting data, the objective of the present study was to determine whether the hypothalamic *Kiss1* mRNA expression in female Damaraland mole-rats is regulated by oestradiol. Therefore, we used *in situ* hybridisation to compare the *Kiss1* mRNA expression in groups of intact wild-caught, ovariectomized (OVX) and OVX females receiving oestradiol replacement.

2. MATERIALS AND METHODS

2.1. Animals

Adult female Damaraland mole-rats (*Fukomys damarensis*) were used. The study comprises four groups of animals: 1) non-reproductive females subjected to ovariectomy (OVX; N=7); 2) non-reproductive females subjected to ovariectomy and oestradiol replacement (OVX+E2; N=7); 3) wild-caught reproductive females (breeder, syn. queen, N=7); 4) wild-caught non-reproductive females (non-breeder, N=7). The animals subjected to ovariectomy originated from the breeding stock of the University of Pretoria. Mole-rat colonies were kept under 12L:12D cycle at 25° in plastic containers (1.0m x 0.5m x 0.5m) containing wood shavings and were provided with nesting material and perspex tubes for environmental enrichment. They were fed on sweet potato, gem squash and apples. In each colony, the reproductive status was determined for all adult females. Females were considered breeders if they had produced a litter previously in captivity, or were identified based on the presence of a perforate vagina and prominent teats. Non-breeders were discerned by their non-perforate vagina. (Bennett & Jarvis, 1988). Each colony only contained one queen. After ovariectomy, females were kept singly until sacrifice. Wild-caught animals were obtained from colonies of mole rats that were captured in July and August 2020 near the village of Black Rock, Northern Cape, South Africa (27°7′S, 22°50′E) with Hickman live-traps under permission from Northern Cape Nature Conservation authorities. All animals were kept in their original colonies until sacrifice. Queens could be readily distinguished from non-reproductive females by the presence of a perforate vagina and prominent teats. None of the females was pregnant at the time of sacrifice. At the time of brain collection, body weight of all animals was recorded to the nearest gram. All experimental procedures were approved by the University of Pretoria Animal Ethics Committee (EC043-17, EC013-18).

2.2. Ovariectomy and E2 replacement

Ovaries were removed from female Damaraland mole-rats under isoflurane anaesthesia by a registered veterinarian. Immediately afterwards, E2-filled silastic capsules were implanted subcutaneously via a small incision at the base of the neck. The implants were made from Silastic tubing (Helix Medical, Carpinteria, CA, USA; inner diameter: 1.47mm, outer diameter: 1.96 mm), packed with crystalline 17ß-estradiol (Sigma E8875) and sealed with silicone. The dose of E2 had been determined in a preliminary study. Implants had a length of 8mm. The tubing length refers to the length of the tubing actually filled with hormone. Before implantation, silastic capsules were washed with 100% ethanol and immersed in 0.9% saline overnight. All females in the OVX group received empty implants.

2.3. Experimental design

The purpose of the study was to examine the effects of OVX and E2 replacement on the *Kiss1* mRNA expression within the hypothalamus. Two experimental groups of females were used. These were females subjected to ovariectomy (OVX group) and females subjected to ovariectomy and E2 replacement (OVX+E2). Two groups of females served as controls, reproductively-active females (breeder) and non-reproductive females (non-breeder). We have previously shown that reproductive status affects the hypothalamic *Kiss1* expression (Voigt and Bennett 2018). In the morning of day 8 after ovariectomy, females were killed by decapitation. Trunk blood was collected for the oestradiol 17β assay. The wild-caught females that served as controls were killed within one day of capture.

2.4. Brain histology

Mole-rats were killed by decapitation, brains were dissected out of the skull, immediately frozen on dry ice and stored at -80°C until used. Before sectioning, brain weight was recorded to the nearest milligram. Frozen brains were cut on a cryostat into 20 µm coronal sections. The

plane of the sections was adjusted to match as closely as possible the plane of the rat brain atlas[30] Sections were mounted onto Superfrost Plus slides (Menzel-Gläser, Braunschweig, Germany) in six different series, so that one series of slides contained a section every 120 µm. *In situ* hybridization was carried out for the localization of *Kiss1* mRNA.

2.5. Cloning of cDNA probes

The cloning of the *Kiss1* sequence has been described in detail previously (Voigt and Bennett 2018). The cloned *Kiss1* sequence [GenBank: KY081957] is 277 bp in length and encompasses 72 bp of the coding region and 205 bp of the 5' untranslated region.

2.6. In situ hybridization

The expression of *Kiss1* in brain sections was detected with antisense RNA probes labeled with ^{35}S-CTP. Labelling of the probes with ^{35}S-CTP (1250 Ci/mmol; Perkin Elmer, Rodgau, Germany) was performed using the Riboprobe System (Promega). Our *in situ* hybridization procedure followed a previously published protocol (Whitfield et al. 1990) with modifications as described elsewhere (Gahr and Metzdorf 1997). Signal detection was done via emulsion autoradiography, which allows the study of gene expression at the cellular level. With this technique, the number of silver grains overlying single cells is related to the radioactivity concentration and therefore represents an estimate of the target mRNA content per cell. To localize autoradiographic silver grains, sections were processed with NTB photographic emulsion (Carestream Health, Stuttgart, Germany). Exposure time was 7 days. Sections were developed with Kodak Dektol Developer and Kodak Fixer and counterstained with thionin. Brain sections from both groups of females were run through the entire procedure at the same time to avoid any possible effect of small differences in procedures on the observed group differences. We have previously demonstrated that there is no signal with the radiolabeled sense probe (Voigt and Bennett 2018, 2021).

2.7. Blood sampling and hormone assays

Upon decapitation of the animal, trunk blood was collected into heparin-coated collection tubes. Blood samples were centrifuged, the plasma collected and frozen at –40°C until the hormone assay was conducted. The oestradiol 17β assay was performed as described (Ngalameno et al. 2017) using the Coat-a-Count MG 12101 oestradiol kit (IBL International GmbH Hamburg, Germany). The assay involved non-extraction and measured native hormone. The cross-reactivity of the antibody to all naturallyoccurring steroids was <0.2% except for oestrone (1%), oestriol (0.6%) and ethinylestradiol (0.2%). The intra-assay coefficient of variation for repeated measures of a quality control was 6.1% and sensitivity of the assay was 2.7 pg/ml. The oestradiol 17β assay has previously been validated for *F. damarensis* (Voigt and Bennett 2018).

2.8. Data analysis

Slides were analysed under darkfield illumination with a Leitz Aristoplan microscope (Leitz Wetzlar,Germany) and images were video digitized using a PC equipped with an image analysis system (SPOT, vs. 5.2, Visitron Systems, Germany). Before analysis, sections of all animals were anatomically matched according to the anterior commissure (AC) at the level where it reaches its largest extension. The complete signal for *Kiss1* was analysed in adjacent sections along the rostro-caudal axis throughout the hypothalamus. This comprised for the preoptic hypothalamus covering the AVPV and the PeN on average between 6 to 16 sections depending on treatment group. For the tuberal hypothalamus covering the ARC it comprised on average between 12 to 16 sections. *Kiss1*-expressing cells were identified by isolated clusters of silver grains overlying cells as described previously (Voigt and Bennett 2021; Fig. 1A, B). As semi-quantitative estimate of mRNA content, the number of silver grains over individual cells was determined. On each section, the number of *Kiss1*-positive cells were counted and the silver grain density overlying each cell was measured (Chowen et al. 1991; Burton et al. 1992). Grain counting on clusters of silver grains was performed manually with a 40x objective under

darkfield illumination. On the digitized images, the centre of each grain cluster was located and silver grains were assigned to the cell if they were found within a 30-µm radius from the centre of the grain cluster otherwise they were counted as background. The number of silver grains was counted in the 60-µm diameter circular measurement area that was drawn on the digitized images using the built-in function of the image analysis software. To obtain an estimate of the background grain density, similar measurements were carried out on the same section in an area considered not to express the gene of interest. Cells were considered labelled when the number of silver grains in a cluster exceeded that of background by 3-fold. The number of silver grains assigned to the cell was adjusted by subtracting the number of silver grains assigned to the background. For each animal, the number of *Kiss1*-positive cells and the average number of silver grains/cell was determined and these values were combined to give group means.

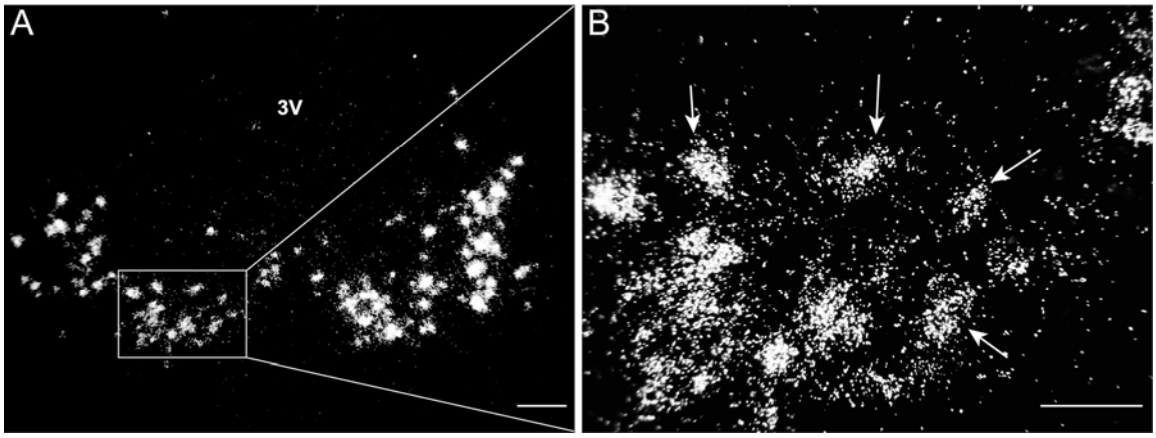

Figure 1. Darkfield photomicrographs of an ovariectomized female (OVX) showing the hybridisation signal for *Kiss1* in the ARC. The boxed area in panel A is shown under higher magnification in panel B to illustrate the clusters of silver grains (marked by arrows). Abbreviations: ARC, arcuate nucleus; 3V, third ventricle. Scale bars: A = 100 µm; B = 50 µm.

2.9. Statistical analysis

Statistical analyses were carried out using JMP software. Data are presented as means ± SEM. Morphological differences between groups were analyzed with One-Way-ANOVA. Body weight changes in the experimental animals during the 7-day treatment period were analysed

with paired t-tests. Because plasma E2 levels were not normally distributed and transformation was not feasible, nonparametric statistics were used (Siegel and Castellan 1988). Hormone levels were compared among groups with Kruskal–Wallis test and planned post hoc comparisons using Dunn's test. For analysis of *Kiss1* gene expression, a REML-model was employed with treatment (OVX, OVX+E2, breeder, non-breeder) and brain region (AVPV/PeN vs. ARC) as fixed factors and animal ID as random factor. Post hoc analyses were performed with the Tukey HSD test. All tests were two-tailed and the significance level was fixed at $p<0.05$. Data are presented as means ± SEM.

3. RESULTS

3.1. Morphological measurements and plasma E2

The four groups of females differed in body weight ($F_{3,24}=6.97$, $P=0.002$). Wild-caught non-reproductive females were significantly lighter than wild-caught reproductive females (Tukey's post hoc test, $P<0.05$) and females treated with E2 (OVX+E2; Tukey's post hoc test, $P<0.01$, Fig. 2). During the 7-day treatment period no significant changes in body weight occurred in the experimental animals (OVX: $t=0.97$, $df=6$, $P=0.37$; E2: $t=0.13$, $df=6$, $P=0.90$). No significant group differences were found in brain mass ($F_{3,24}=1.40$, $P=0.266$). Plasma E2 levels were, as expected, significantly different between groups (Kruskal–Wallis $H = 17.53$; $P = 0.0005$). OVX+E2 females had significantly higher E2 levels than OVX females (3.17±0.17 ng/ml vs. 0.03±0.02 ng/ml; $P<0.05$). Both wild-caught reproductive and non-reproductive females had E2 levels that were close to the detection limit of the assay (0.009±0.005 ng/ml vs. 0.008±0.003 ng/ml) and were not significantly different from OVX females ($P=0.82$).

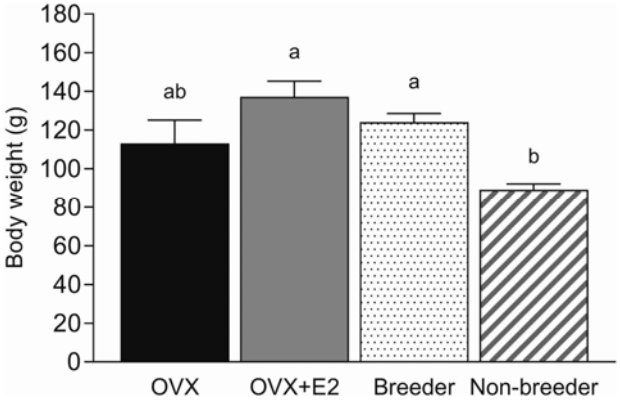

Figure 2. Body weight (g) of ovariectomized females (OVX), ovariectomized females treated with E2 (OVX+E2) and wild-caught breeding and non-breeding females. For each region, values without common letters differ significantly.

3.2. Distribution of Kiss1 mRNA

In all groups of females, *Kiss1*-expressing cells were found in two hypothalamic regions, the preoptic area and the ARC (Fig. 3). Within the preoptic area, wild-caught control females, irrespective of reproductive status, had very low *Kiss1* expression, which was restricted to few cells scattered throughout the AVPV (Fig. 3 G, H). However, in E2-treated females, *Kiss1*-expression in this region was dramatically increased and extended to the adjacent periventricular nucleus (PeN, Fig. 3 F, J). The data presented for the preoptic area therefore comprise both regions the AVPV and the PeN. Within the ARC, nearly all *Kiss1*-expressing cells were found in the caudal part of the nucleus. Labelling was not found in other brain regions.

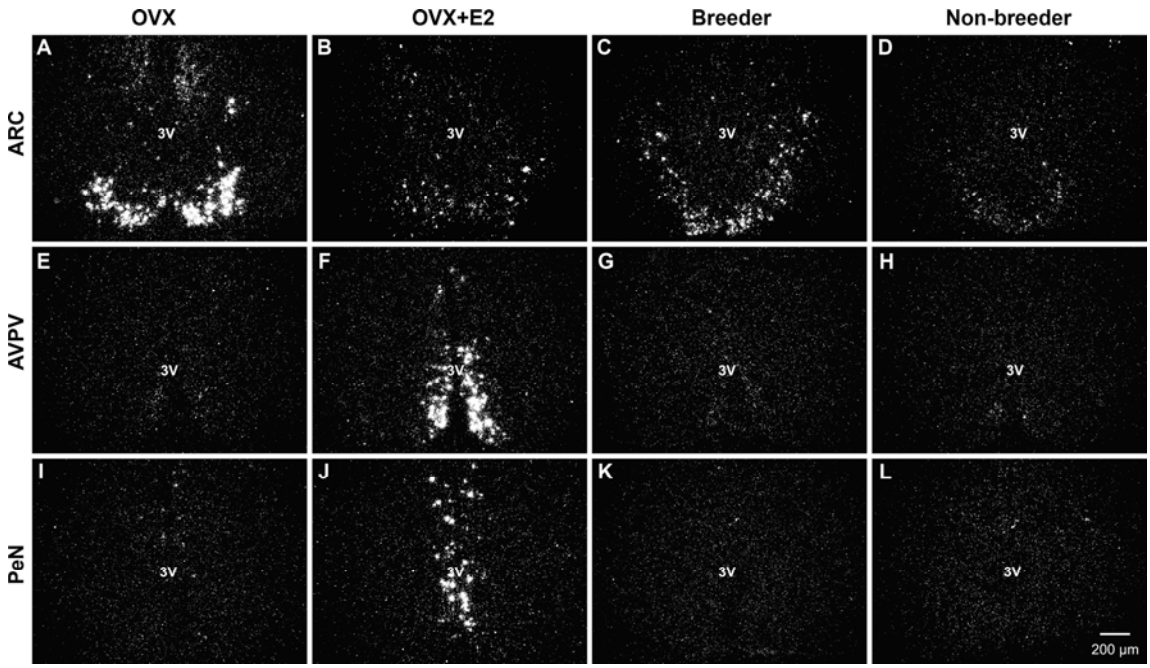

Figure 3. Darkfield photomicrographs illustrating the expression of *Kiss1* in the caudal ARC and in the preoptic hypothalamus covering the AVPV/PeN of an ovariectomized female (OVX; A, E, I), an ovariectomized female treated with E2 (OVX+E2; B, F, J), a wild-caught breeding female (breeder; C, G, K) and a wild-caught non-breeding female (non-breeder; D, H, L). In E-H, the anterior commissure is to the top. Abbreviations: ARC, arcuate nucleus; AVPV, anteroventral periventricular nucleus; PeN, periventricular nucleus; 3V, third ventricle.

3.3. Effect of OVX and E2 treatment on Kiss1 expression

The total number of *Kiss1*-expressing cells in the AVPV/PeN and in the ARC differed significantly between the four groups of females (treatment: $F_{3,22.4}$=19.17, P<0.0001; region: $F_{1,22.2}$=133.14, P<0.0001; interaction: $F_{3,22.2}$=45.39, P<0.0001, Fig. 4). In the ARC, wild-caught reproductive females had nearly three times more *Kiss1*-expressing cells compared to wild-caught non-reproductive females (P<0.01). OVX increased the number of *Kiss1*-expressing cells by 1.3-fold compared to reproductive controls (P>0.05) and by 3.6-fold compared to non-reproductive controls (P<0.01). Treatment with E2 reversed this effect (OVX vs. OVX+E2, P<0.01; Fig. 4A). In the AVPV/PeN, opposite results were obtained. In both groups of wild-caught controls, very few *Kiss1*-expressing cells were found. OVX females remained at the

same level as the control females. Treatment with E2 lead to a 30-fold increase of *Kiss1* cell number (OVX+E2 vs. all other groups, P<0.001, Fig. 4A).

A similar pattern as for the total number of *Kiss1*-expressing cells was seen for the *Kiss1* mRNA content per cell (treatment: $F_{3,24.0}$=12.43, P<0.0001; region: $F_{1,23.6}$=17.14, P=0.0004; interaction: $F_{3,23.6}$=50.00, P<0.0001, Fig. 4). In the ARC, both groups of wild-caught females had similar levels of *Kiss1* mRNA expression per cell (P>0.05). OVX increased the cellular content of *Kiss1* mRNA by 2-fold compared to the wild-caught controls (P<0.01). Treatment with E2 reversed this effect (OVX+E2 vs. all other groups, P<0.001, Fig. 4B). In the AVPV/PeN, OVX females had similarly low *Kiss1* mRNA content per cell as both wild-caught controls. Treatment with E2 lead to a 3-fold increase (OVX+E2 vs. all other groups, P<0.001, Fig. 4B).

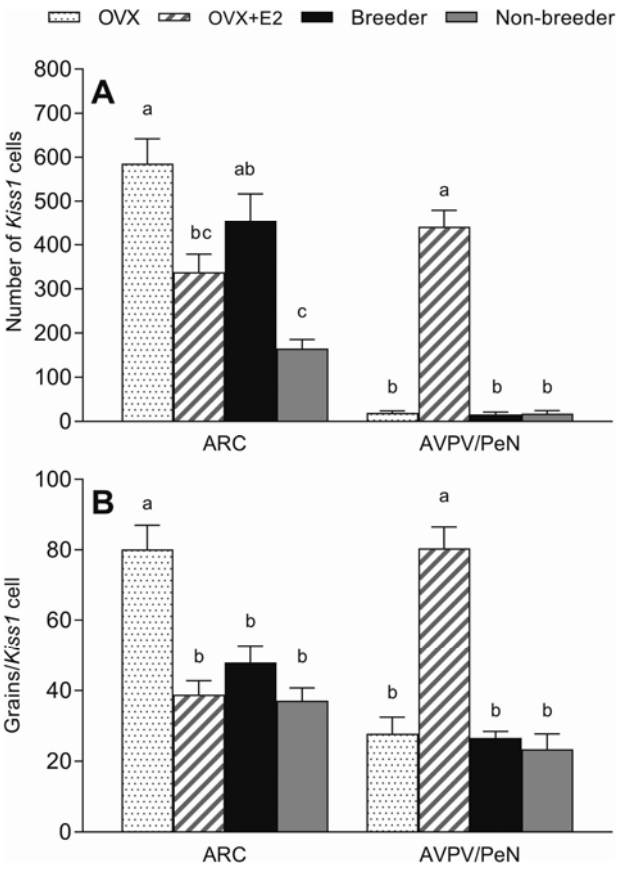

Figure 4. Total number of *Kiss1*-expressing cells (A) and the average number of silver grains per cell (an estimate for the *Kiss1* mRNA content, B) in the ARC and the AVPV/PeN of ovariectomized females (OVX),

ovariectomized females treated with E2 (OVX+E2) and wild-caught breeding and non-breeding females. For each region, values without common letters differ significantly.

4. DISCUSSION

We have demonstrated in an induced ovulator, the Damaraland mole-rat, that kisspeptin neurons in the ARC and in the AVPV/PeN of females are differentially regulated by E2. In the ARC, E2 inhibits *Kiss1* expression while in the AVPV/PeN it stimulates *Kiss1* expression. Conversely, when E2 levels are very low such as after gonadectomy, *Kiss1* expression is increased in the ARC and decreased in the AVPV/PeN. These results are in agreement with previous findings in other species. The regulation of kisspeptin neurons by steroid hormones has been well-studied in spontaneous ovulators such as mice (Gottsch et al. 2004; Smith et al. 2005), rats (Smith et al. 2006; Kauffman et al. 2007), sheep (Smith et al. 2007) and primates (Smith et al. 2010). There, kisspeptin neurons are involved in mediating the positive and negative feedback effects of steroid hormones on GnRH neurons, controling the pulsatile and surge modes of GnRH release. The regulation of kisspeptin neurons has so far only been studied in one other induced ovulator besides Damaraland mole-rats, in the musk shrew (*Suncus murinus*, Inoue et al. 2011). Similar to the data of the present study, kisspeptin neurons were found to be target of positive and negative feedback effects of E2. Moreover, mating was shown to upregulate *Kiss1* neurons in the preoptic area but not in the ARC and treatment with exogenous kisspeptin induced ovulation in female musk shrews by mimiking the mating stimulus (Inoue et al. 2011).

4.1. Plasma oestradiol levels

The physiological range of plasma oestradiol in female Damaraland mole-rats was previously found to be 0.02-0.06 ng/ml in non-reproductive (N=9) and 0.02-0.48 ng/ml in reproductively active females (N=9, Voigt and Bennett 2018). Similar to other induced ovulators such as musk

shrews, minks (*Mustela vison*) and rabbits (*Oryctolagus cuniculus*), circulating oestradiol levels in female Damaraland mole-rats are generally very low and may only increase in response to mating (Hillard and Eaton 1971; Fortune et al. 1992; Lagerkvist et al. 1992). However, so far, no such data exist for this species. Therefore, we do not know the upper limit of the physiological range. The silastic implants in our study produced oestradiol levels that were about 6 times higher than the highest previously measured plasma level in a wild-caught unmanipulated breeding female (0.48 ng/ml, Voigt and Bennett 2018). It is likely that this is above the physiological range of this species. However, we think the use of these implants was justified for our research aim because the E2-responsiveness of the preoptic kisspeptin neurons could not be detected in females with circulating oestradiol levels (Voigt and Bennett 2018, this study). The functional significance of the activation of this kisspeptin neuron population remains currently elusive.

4.2. Regulation of Kiss1 neurons in the ARC

It is now established across several species that oestradiol inhibits kisspeptin neurons in the ARC (for review, see Smith 2013). The ARC kisspeptin neurons colocalize two additional neuropeptides, neurokinin B (NKB; encoded by the *Tac3* gene) and the endogenous opioid peptide dynorphin (encoded by the *Pdyn* gene). This neuron population is termed the "KNDy" (kisspeptin/neurokinin B/dynorphin) neurons and is indeed considered the mammalian GnRH pulse generator (Herbison 2018, Nagae et al. 2021). The model proposes that the release of NKB leads through a positive feedback loop to increased KNDy neuronal activity, kisspeptin release and subsequent GnRH secretion, and with a time delay, to upregulation of dynorphin, which terminates the kisspeptin release and the GnRH pulse (Herbison 2018). The KNDy neurons are activated synchronously before each pulse and in a temporal order, suggesting that subsets of neurons play different roles in the initiation, maintenance and termination of the GnRH pulse (Moore et al. 2022). However, recent data obtained from mice suggest a departure

from the proposed model (Han et al. 2023). The authors demonstrate that glutamate signalling through AMPA receptors is critical for ARC kisspeptin neurons to synchronize their activity in order to drive the pulsatile GnRH release, with NKB and dynorphin only playing modulatory roles (Han et al. 2023). These results were obtained from males and whether the same mechanisms apply to females needs to be seen.

The activity of the KNDy neuron population within the ARC is regulated by circulating levels of gonadal steroids (Goodman et al. 2022). Consequently, when circulating E2 levels in females decrease, GnRH pulse frequency will increase, leading to increased pulsatile release of LH from the pituitary and subsequently, increased steroid synthesis in the ovaries. In line with this, OVX females in our study had upregulated *Kiss1* expression, whereas OVX females treated with E2 showed *Kiss1* expression levels similar to untreated females. It is possible that this negative feedback effect of E2 on kisspeptin neurons is mediated by ERα, as has been shown in several other species (Franceschini et al. 2006; Adachi et al. 2007; Poling et al. 2017), because Damaraland mole-rats express abundandly ERα in the ARC (Voigt et al. 2014). Although E2 levels were similarly low in OVX females and in reproductively active control females, the former were found to have higher *Kiss1* expression. Such an observation has also been made in female musk shrews (Inoue et al. 2011). It is possible that in reproductively active control females, chronically low E2 levels lead to a reduced activation of *Kiss1* neurons and thereby exerting a negative feedback effect on GnRH neuron activity.

Damaraland mole-rats are cooperative breeders and therefore, groups possess a social hierarchy and reproductive skew. There is usually only one reproductively active female per group (Bennett and Jarvis 1988; Jarvis and Bennett 1993). We have previously reported that the mRNA expression patterns of several hypothalamic genes implicated in the control of reproduction differ between such breeding females and subordinate non-reproductive females. For example, female reproductively active Damaraland mole-rats have significantly more *Kiss1*-expressing cells, increased NKB and decreased dynorphin gene expression in the ARC

compared to non-reproductive females (Voigt and Bennett, 2018, 2019, this study). These reproductive status-dependent gene expression patterns of KNDy neurons are in line with the observation that in all mammals the GnRH pulse generator reactivates at puberty after juvenile quiescence (for review, see Herbison, 2016). Therefore, it is likely that the gene expression patterns found in non-reproductive female Damaraland mole-rats actually reflect their pre-pubertal stage and that these will become upregulated upon the onset of puberty (Terasawa et al. 2013). For example, reproductively quiescent female naked mole-rats show increased expression of RFamide-related peptide -3 (RFRP-3) in the dorsomedial hypothalamus when compared to reproductively active females (Peragine et al. 2017). RFRP-3 is a potent regulator of GnRH neurons, which has an inhibitory effect on GnRH neuronal activity and subsequent gonadotropin release (Kriegsfeld et al. 2006; Ducret et al. 2009). Moreover, administration of exogenous RFRP-3 prevents puberty onset in subordinate female naked mole-rats (Peragine et al. 2017). We found similar reproductive status-related differences concerning RFRP-3 gene expression in Damaraland mole-rats (Voigt and Bennett 2018).

4.3. Regulation of Kiss1 in the AVPV/PeN

Rodents such as rats and mice, show robust *Kiss1* expression in the ARC and in the AVPV/PeN. The kisspeptin neuron population in the AVPV/PeN projects to GnRH neurons in the preoptic area and has been shown to be directly involved in mediating the E2-positive feedback leading to the generation of the preovulatory GnRH/LH surge in females (Clarkson et al. 2006; Adachi et al. 2007). In spontaneous ovulators, the signal initiating this process is the rise in circulating E2 produced by maturing follicles. Additionally, in rodents the GnRH/LH surge is coupled to a circadian signal and takes place in the late afternoon of proestrus when E2 levels are elevated. At this time, *Kiss1* expression in the AVPV/PeN is increased (Robertson et al. 2009; Williams et al. 2011; Smarr et al. 2012). In contrast, in induced ovulators such as rabbits, voles and ferrets, the preovulatory GnRH/LH surge cannot be induced by increased E2 levels, which suggests

that the E2-positive feedback on GnRH release is absent (for review, see Bakker and Baum 2000). Instead, coitus is the primary stimulus to induce the GnRH/LH surge and subsequently ovulation. The genital-somatosensory stimulus, which is received during mating is thought to be mediated by noradrenergic neurons in the brainstem to stimulate kisspeptin and/or GnRH neurons in the preoptic area. (for review, see Goodman et al. 2022). In female musk shrews, the number of *Kiss1*-expressing cells in the preoptic area is significantly upregulated in response to mating (Inoue et al. 2011). However, there is so far no direct evidence that this activation results from the noradrenergic input. Further experimental work is necessary to identify the role of the kisspeptin neurons in the generation of the mating-induced preovulatory surge. In Damaraland mole-rats, it is possible that *Kiss1* expression in intact reproductively active females is only upregulated during a narrow time window after the mating stimulus has occurred. This could be confirmed by sacrificing females after mating and analysing the coexpression of *Kiss1* and *cfos* by means of double-label ISH.

Inoue et al. (2011) also showed that the *Kiss1* neuron population in the preoptic area is positively regulated by E2. *Kiss1* mRNA expression is significantly higher in OVX females treated with E2 compared to OVX females. The results of the present study in Damaraland mole-rats are consistent with these findings. OVX females showed very low *Kiss1* expression in the AVPV/PeN, similar to wild-caught control females, whereas E2 treatment of OVX females dramatically increased *Kiss1* expression in this region. This steroid hormone sensitivity of kisspeptin neurons suggests that ovarian oestradiol could act stimulatory on the neural pathway that controls the GnRH/LH surge. Female Damaraland mole-rats have abundant ERα expression in the AVPV/PeN, which is elevated in reproductively active females (Voigt et al. 2014). In a recent study in female rats, Inoue et al. (2023) demonstrate that ATP, a purinergic receptor ligand, originating from brainstem purinergic neurons, stimulates AVPV kisspeptin neurons and consequently, triggers ovulation. These purinergic neurons, located in hindbrain noradrenergic regions such as A1, A2 and A6, project directly to the AVPV and are activated

by high levels of circulating E2. Therefore, the ATP-purinergic receptor signalling could be responsible for mediating the E2-induced increase in AVPV/PeN kisspeptin neurons leading to the preovulatory GnRH/LH surge in females of spontanously ovulating species (Inoue et al. 2023). In induced ovulators, where the mating stimulus activates brainstem noradrenergic regions, ATP-receptor signalling could also play a critical role in activating AVPV kisspeptin neurons and E2 could facilitate this pathway. However, it is currently unknown whether the noradrenergic neurons of A1, A2 and A6 in such species are also purinergic and coexpress ERα. Similarly, ATP receptor expression needs to be confirmed in AVPV kisspeptin neurons.

In conclusion, we have shown that in Damaraland mole-rats the hypothalamic *Kiss1* mRNA expression is differentially regulated by E2, with E2 downregulating *Kiss1* in the ARC and upregulating *Kiss1* in the AVPV/PeN. Our data suggest that the *Kiss1* neuron population in the ARC is involved in the E2-mediated negative feedback effect on GnRH secretion. Further experiments are needed to clarify the role of the AVPV/PeN kisspeptin neuron population and and the functional significance of their steroid hormone sensitivity.

CRediT authorship contribution statement

Cornelia Voigt: Conceptualization, Methodology, Data curation, Investigation, Writing – original draft, Writing – review & editing. Manfred Gahr: Conceptualization, Writing – review & editing, Funding acquisition. Nigel C. Bennett: Conceptualization, Methodology, Data curation, Writing – review & editing, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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REFERENCES

Adachi S, Yamada S, Takatsu Y, Matsui H, Kinoshita M, Takase K, Sugiura H, Ohtaki T, Matsumoto H, Uenoyama Y, Tsukamura H, Inoue K, Maeda K. Involvement of anteroventral periventricular metastin/kisspeptin neurons in estrogen positive feedback action on luteinizing hormone release in female rats. *J Reprod Dev.* 2007; **53**: 367-378.

Bakker J, Baum MJ. Neuroendocrine regulation of GnRH release in induced ovulators. *Front Neuroendocrinol*. 2000; **21**(3): 220-262.

Bennett NC, Jarvis JUM. The social structure and reproductive biology of colonies of the mole-rat *Cryptomys damarensis* (Rodentia: Bathyergidae). *J Mammal.* 1988; **69**: 293–302.

Bennett, N.C., Faulkes, C.G., 2000. African mole-rats. Ecology and eusociality. Cambridge: Cambridge Univ Press.

Burton, K.A., Kabigting, E.B., Clifton, D.K., Steiner, R.A. 1992. Growth hormone receptor messenger ribonucleic acid distribution in the adult male rat brain and its colocalization in hypothalamic somatostatin neurons. Endocrinology 131, 958-963.

Chowen, J.A., Steiner, R.A., Clifton, D.K. 1991. Semiquantitative analysis of cellular somatostatin mRNA levels by in situ hybridization histochemistry. Methods Neurosci 5, 137–158.

Clarkson J, Herbison AE. Postnatal development of kisspeptin neurons in mouse hypothalamus; sexual dimorphism and projections to gonadotropin-releasing hormone neurons. *Endocrinology* 2006; **147**: 5817-5825.

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.

Ducret E, Anderson GM, Herbison AE. RFamide-related peptide-3, a mammalian gonadotropin-inhibitory hormone ortholog, regulates gonadotropin-releasing hormone neuron firing in the mouse. Endocrinology 2009;150:2799-2804.

Fortune JE, Eppig JJ, Rissman EF. Mating stimulates estradiol production by ovaries of the musk shrew (*Suncus murinus*). *Biol Reprod.* 1992; **46**: 885-891.

Franceschini I, Lomet D, Cateau M, Delsol G, Tillet Y, Caraty A. Kisspeptin immunoreactive cells of the ovine preoptic area and arcuate nucleus co-express estrogen receptor alpha. *Neurosci Lett.* 2006; **401**: 225-230.

Gahr M, Metzdorf R. Distribution and dynamics in the expression of androgen and estrogen receptors in vocal control systems of songbirds. *Brain Res Bull.* 1997; **44**: 509–517.

Goodman RL. Neuroendocrine control of gonadotropin secretion: comparative aspects, in: Plant, T.M., Zeleznik, A.J. (Eds.), Knobil and Neill's Physiology of Reproduction (Fourth Edition). 2015. San Diego: Academic Press, pp. 1537-1574.

Goodman RL, Herbison AE, Lehman MN, Navarro VM. Neuroendocrine control of gonadotropin-releasing hormone: Pulsatile and surge modes of secretion. *J Neuroendocrinol.* 2022; **34**(5): e13094.

Gottsch ML, Cunningham MJ, Smith JT, Popa SM, Acohido BV, Crowley WF, Seminara S, Clifton DK, Steiner RA. A role for kisspeptins in the regulation of gonadotropin secretion in the mouse. *Endocrinology* 2004; **145**: 4073-4077.

Gottsch ML, Navarro VM, Zhao Z, Glidewell-Kenney C, Weiss J, Jameson JL, Clifton DK, Levine JE, Steiner RA. Regulation of Kiss1 and dynorphin gene expression in the murine brain by classical and nonclassical estrogen receptor pathways. *J Neurosci.* 2009; **29**(29): 9390-9395.

Han SY, Morris PG, Kim JC, Guru S, Pardo-Navarro M, Yeo SH, McQuillan HJ, Herbison AE. Mechanism of kisspeptin neuron synchronization for pulsatile hormone secretion in male mice. *Cell Reports* 2023; **42**(1): 111914.

Herbison AE. Estrogen positive feedback to gonadotropin-releasing hormone (GnRH) neurons in the rodent: the case for the rostral periventricular area of the third ventricle (RP3V). *Brain Res Rev*. 2008; **57**(2): 277-287.

Herbison AE. Physiology of the adult gonadotropin-releasing hormone neuronl network, in: Plant, T.M., Zeleznik, A.J. (Eds.), Knobil and Neill's Physiology of Reproduction (Fourth Edition). 2015. San Diego: Academic Press, pp. 399-466.

Herbison AE. Control of puberty onset and fertility by gonadotropin-releasing hormone neurons. *Nat Rev Endocrinol.* 2016; **12**(8): 452-66.

Herbison AE. The Gonadotropin-Releasing Hormone Pulse Generator. *Endocrinology* 2018; **159**: 3723-3736.

Hillard, J., Eaton, LW. Estradiol-17β, Progesterone and 20α-Hydroxypregn-4-en-3-one in Rabbit Ovarian Venous Plasma. II. From Mating Through Implantation, *Endocrinology* 1971; **89**: 522–527

Inoue, N., Sasagawa, K., Ikai, K., Sasaki, Y., Tomikawa, J., Oishi, S., Fujii, N., Uenoyama, Y., Ohmori, Y., Yamamoto, N., Hondo, E., Maeda, K., Tsukamura, H. Kisspeptin neurons mediate reflex ovulation in the musk shrew (*Suncus murinus*). *Proc. Natl. Acad. Sci. U S A.* 2011; **108**: 17527-17532.

Inoue N, Hazim S, Tsuchida H, Dohi Y, Ishigaki R, Takahashi A, Otsuka Y, Yamada K, Uenoyama Y, Tsukamura H. Hindbrain Adenosine 5-Triphosphate (ATP)-Purinergic

Signaling Triggers LH Surge and Ovulation via Activation of AVPV Kisspeptin Neurons in Rats. *J Neurosci.* 2023; **43** (12): 2140-2152.

Jarvis JUM, Bennett NC. Eusociality has evolved independently in two genera of bathyergid mole-rats - but occurs in no other subterranean mammal. *Behav Ecol Sociobiol*. 1993; **33**: 353-360.

Kauffman AS. Neuroendocrine mechanisms underlying estrogen positive feedback and the LH surge. *Front Neurosci.* 2022; 16: 953252.

Kauffman AS, Rissman, EF. Neuroendocrine Control of Mating-Induced Ovulation. In: Neill JD, ed. *Knobil and Neill's Physiology of Reproduction*, 3rd ed. Elsevier; 2006: 2283-2326.

Kauffman AS, Gottsch ML, Roa J, Byquist AC, Crown A, Clifton DK, Hoffman GE, Steiner RA, Tena-Sempere M. Sexual differentiation of Kiss1 gene expression in the brain of the rat. *Endocrinology* 2007; **148**: 1774-1783.

Keller L, Reeve KH. Partitioning of reproduction in animal societies. *Trends Ecol Evol.* 1994; **9**: 98-102.

Kriegsfeld LJ, Mei DF, Bentley GE, Ubuka T, Mason AO, Inoue K, Ukena K, Tsutsui K, Silver R. Identification and characterization of a gonadotropin-inhibitory system in the brains of mammals. *Proc Natl Acad Sci U S A.* 2006; **103**: 2410-2415.

Lagerkvist G, Einarsson EJ, Forsberg M, Gustafsson H. Profiles of oestradiol-17 beta and progesterone and follicular development during the reproductive season in mink (*Mustela vison*). *J Reprod Fertil.* 1992; 94: 11-21.

Messager, S., Chatzidaki, E.E., Ma, D., Hendrick, A.G., Zahn, D., Dixon, J., Thresher, R.R., Malinge, I., Lomet, D., Carlton, M.B., Colledge, W.H., Caraty, A., Aparicio, S.A. Kisspeptin directly stimulates gonadotropin-releasing hormone release via G protein-coupled receptor 54. *Proc Natl Acad Sci USA* 2005; **102**: 1761-1766.

Molteno AJ, Bennett NC. Anovulation in non-reproductive female Damaraland mole-rats (*Cryptomys damarensis*). *J Reprod and Fertil.* 2000; **119**: 35-41.

Moore AM, Coolen LM, Lehman MN. In vivo imaging of the GnRH pulse generator reveals a temporal order of neuronal activation and synchronization during each pulse. *Proc Natl Acad Sci U S A.* 2022; **119**(6): e2117767119.

Nagae M, Uenoyama Y, Okamoto S, Tsuchida H, Ikegami K, Goto T, Majarune S, Nakamura S, Sanbo M, Hirabayashi M, Kobayashi K, Inoue N, Tsukamura H. Direct evidence that KNDy neurons maintain gonadotropin pulses and folliculogenesis as the GnRH pulse generator. *Proc Natl Acad Sci U S A.* 2021; **118**(5): e2009156118.

Navarro VM. Interactions between kisspeptins and neurokinin B. *Adv Exp Med Biol.* 2013; 784: 325-347.

Navarro VM, Castellano JM, Fernández-Fernández R, Barreiro ML, Roa J, Sanchez-Criado JE, Aguilar E, Dieguez C, Pinilla L, Tena-Sempere M. Developmental and hormonally regulated messenger ribonucleic acid expression of KiSS-1 and its putative receptor, GPR54, in rat hypothalamus and potent luteinizing hormone-releasing activity of KiSS-1 peptide. *Endocrinology* 2004; **145**: 4565-4574.

Ngalameno MK, Bastos ADS, Mgode G and Bennett NC. The pattern of reproduction in the mole-rat Heliophobius from Tanzania: do not refrain during the long rains! *Canad J Zool.* 2017; **95**: 107-114.

Peragine DE, Pokarowski M, Mendoza-Viveros L, Swift-Gallant A, Cheng HM, Bentley GE, Holmes MM. RFamide-related peptide-3 (RFRP-3) suppresses sexual maturation in a eusocial mammal. *Proc Natl Acad Sci USA* 2017; **114**: 1207-1212.

Pohl CR, Knobil E. The role of the central nervous system in the control of ovarian function in higher primates. *Annu Rev Physiol.* 1982; **44**: 583-593.

Poling MC, Luo EY, Kauffman AS. Sex Differences in Steroid Receptor Coexpression and Circadian-Timed Activation of Kisspeptin and RFRP-3 Neurons May Contribute to the Sexually Dimorphic Basis of the LH Surge. *Endocrinology* 2017; **158**(10): 3565-3578.

Rissman, E.F. Mating induces puberty in the female musk shrew. *Biol Reprod*. 1992; **47**: 473-477.

Robertson JL, Clifton DK, de la Iglesia HO, Steiner RA, Kauffman AS. Circadian regulation of Kiss1 neurons: implications for timing the preovulatory gonadotropin-releasing hormone/luteinizing hormone surge. *Endocrinology* 2009; **150**(8): 3664-3671.

Smarr BL, Morris E, de la Iglesia HO. The dorsomedial suprachiasmatic nucleus times circadian expression of Kiss1 and the luteinizing hormone surge. *Endocrinology* 2012; 153(6): 2839-2850.

Smith JT. Sex steroid regulation of kisspeptin circuits. *Adv Exp Med Biol* 2013; **784**: 275-295.

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**: 3686-3692.

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**: 6687-6694.

Smith JT, Clay CM, Caraty A, Clarke IJ. KiSS-1 messenger ribonucleic acid expression in the hypothalamus of the ewe is regulated by sex steroids and season. *Endocrinology* 2007; **148**: 1150-1157.

Smith JT, Shahab M, Pereira A, Pau KY, Clarke IJ. Hypothalamic expression of KISS1 and gonadotropin inhibitory hormone genes during the menstrual cycle of a non-human primate. *Biol Reprod*. 2010; 83(4): 568-577.

Swift-Gallant A, Mo K, Peragine DE, Monks DA, Holmes MM. Removal of reproductive suppression reveals latent sex differences in brain steroid hormone receptors in naked mole-rats, Heterocephalus glaber. *Biol Sex Differ.* 2015; **6**: 31.

Terasawa E, Guerriero KA, Plant TM. Kisspeptin and puberty in mammals. *Adv Exp Med Biol.* 2013; **784**: 253-273.

Voigt C, Bennett NC. GnRH mRNA expression in the brain of cooperatively-breeding female Damaraland mole-rats. *Reproduction* 2017; **153**: 453–460.

Voigt C, Bennett NC. Reproductive status-dependent Kisspeptin and RFamide-related peptide (Rfrp) gene expression in female Damaraland mole-rats *J Neuroendocrinol*. 2018, **30**: e12571

Voigt C, Bennett NC. Reproductive status-dependent dynorphin and neurokinin B gene expression in female Damaraland mole-rats. *J Chem Neuroanat.* 2019; **102**: 101705

Voigt C, Bennett NC. Gene expression pattern of Kisspeptin and RFamide-related peptide (Rfrp) in the male Damaraland mole-rat hypothalamus. *J Chem Neuroanat.* 2021; **118**: 102039.

Voigt C, Gahr M, Leitner S, Lutermann H, Bennett NC. Breeding status and social environment differentially affect the expression of sex steroid receptor and aromatase mRNA in the brain of female Damaraland mole-rats. *Front Zool.* 2014; **11**: 38.

Voigt C, Medger K, Bennett NC. The oestrous cycle of the Damaraland mole-rat revisited: evidence for induced ovulation. *J Zoology Lond.* 2021; **314**: 85-95

Whitfield HJ Jr, Brady LS, Smith MA, Mamalaki E, Fox RJ, Herkenham M. Optimization of cRNA probe in situ hybridization methodology for localization of glucocorticoid receptor mRNA in rat brain: a detailed protocol. *Cell Mol Neurobiol*. 1990; **10**: 145-157.

Williams WP 3rd, Jarjisian SG, Mikkelsen JD, Kriegsfeld LJ. Circadian control of kisspeptin and a gated GnRH response mediate the preovulatory luteinizing hormone surge. *Endocrinology* 2011; **152**(2): 595-606

Wintermantel TM, Campbell RE, Porteous R, Bock D, Gröne HJ, Todman MG, Korach KS, Greiner E, Pérez CA, Schütz G, Herbison AE: Definition of estrogen receptor pathway critical for estrogen positive feedback to gonadotropin-releasing hormone neurons and fertility. *Neuron* 2006; **52**: 271-280.

Zhou S, Holmes MM, Forger NG, Goldman BD, Lovern MB, Caraty A, Kalló I, Faulkes CG, Coen CW. Socially regulated reproductive development: analysis of GnRH-1 and kisspeptin neuronal systems in cooperatively breeding naked mole-rats (*Heterocephalus glaber*). *J Comp Neurol.* 2013; **521**: 3003-3029.