Reproductive status-dependent Kisspeptin and RFamide-related peptide (*Rfrp*) gene expression in female Damaraland mole-rats

Cornelia Voigt* **and Nigel C Bennett**

Department of Zoology and Entomology, University of Pretoria, 0028 Pretoria, South Africa

* Correspondence to:

Cornelia Voigt, Department of Zoology and Entomology, University of Pretoria, 0028 Pretoria, South Africa

Email: cornelia.voigt@gmail.comα

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ABSTRACT

Damaraland mole rats (*Fukomys damarensis*) are cooperatively breeding, subterranean mammals, which exhibit high reproductive skew. Reproduction is monopolized by the dominant female of the group while subordinates are physiologically suppressed to the extent that they are anovulatory. It is thought that in these latter animals normal GnRH secretion from the hypothalamus is disrupted. The RFamide peptides kisspeptin (Kiss1) and RFamid-related peptide-3 (RFRP-3) are considered potent regulators of gonadotropin release. To assess whether these neuropeptides are involved in the mechanism of reproductive suppression we investigated the distribution and gene expression of *Kiss1* and *Rfrp* by means of *in situ* hybridisation in wild-caught female Damaraland mole-rats with different reproductive status. In both reproductive phenotypes, substantial *Kiss1* expression was found in the arcuate nucleus and only few *Kiss1*-expressing cells were detected in the AVPV, potentially due to low circulating estradiol concentrations in breeding and non-breeding females. *Rfrp* gene expression occurred in the dorsomedial nucleus, the paraventricular nucleus and the periventricular nucleus. While in female breeders and non-breeders plasma oestradiol levels were low and not significantly different, quantification of the hybridisation signal for both genes revealed significant differences in relation to reproductive status. Reproductively active females had more *Kiss1*-expressing cells and a higher number of silver grains per cell in the arcuate nucleus when compared to non-reproductive females. This difference was most pronounced in the caudal part of the nucleus. No such differences were found in the AVPV. Furthermore, breeding status was associated with a reduced number of *Rfrp*-expressing cells in the anterior hypothalamus. This reproductive status-dependent expression pattern of *Kiss1* and *Rfrp* suggests that both neuropeptides play a role in the regulation of reproduction in Damaraland mole-rats. Enhanced long-term negative feedback effects of oestradiol could be responsible for the lower *Kiss1* expression in the arcuate nucleus of reproductively suppressed females.

INTRODUCTION

In animal societies with high reproductive skew, subordinate group members are often inhibited from breeding opportunities, such as in honey bees (*Apis mellifera*), naked mole-rats (*Heterocephalus glaber*) or meerkats (*Suricata suricatta*).[1-3] In naked mole-rats, for example, the dominant female monopolizes reproduction while the subordinate females of the group are physiologically suppressed by her to such an extent that gonadal development, ovarian cyclicity and ovulation are completely blocked.[4]

In spontaneously ovulating mammals the positive feedback action of ovarian oestradiol leads to release of GnRH, which in turn stimulates the preovulatory LH surge from the anterior pituitary. The structurally related neuropeptides, kisspeptin and RFamide-related peptide -3 (RFRP-3) have been identified as potent regulators, though with opposing effects, of gonadotropin release.[5-8] Kisspeptin, a product of the *Kiss1* gene, is a strong activator of GnRH neurons and is considered essential for reproduction and the timing of puberty onset in mammals (for review, see [9,10]). *Kiss1*-expressing neuron populations have been found mainly in two regions of the hypothalamus, the anteroventral periventricular nucleus (AVPV) and in the arcuate nucleus (ARC). In female rodents, oestrogen (E2) stimulates *Kiss1* neurons in the AVPV while it inhibits those in the ARC. This positive and negative feedback regulation is mediated by oestrogen receptor α (ER α), which is coexpressed in both populations of *Kiss1* neurons.[11] *Kiss1* neurons in the AVPV project to GnRH neurons and have been shown to be directly involved in the generation of the preovulatory GnRH/LH surge.[12,13] Interestingly, in another rodent, the guinea pig, very few *Kiss1* neurons are found in the preoptic area while the majority is located in the ARC and this population is implicated in the positive feedback effect of oestrogen on GnRH neurons.[14] In contrast, RFRP-3, encoded by the *Rfrp* gene and considered the mammalian homolog of the gonadotropin-inhibiting hormone (GnIH) in birds,

has been shown to have inhibitory effects on GnRH neuron activity[15,16] and on gonadotropin release.[7,8] However, regarding the inhibitory effect on LH secretion, data are inconsistent. Several studies using intracerebroventricular administration of RFRP-3 failed to find an effect on LH secretion in female rats and sheep[17,18] and opposite, stimulatory effects were reported in males from two species of hamsters[19,20] suggesting sex- and species-specific differences in RFRP-3 action.

African mole-rats (Bathyergidae) exhibit a wide degree of reproductive skew; from species being solitary to those being highly social such as naked mole-rats or Damaraland mole-rats (*Fukomys damarensis*).[21] In these species, subordinate females show no signs of sexual behaviour, have low urinary oestradiol and progesterone levels, exhibit interrupted follicular development and show a reduced LH response to exogenous GnRH indicating that the normal GnRH secretion from the hypothalamus is disrupted (for review, see [22]). We have recently shown in Damaraland mole-rats that reproductive status affects the neuroendocrine phenotype of females with subordinates having reduced gene expression of androgen receptor (AR) and/or ERα in several preoptic-hypothalamic brain regions, including the AVPV and ARC. Furthermore, reduced GnRH gene expression in the anterior preoptic area of subordinates was found in these females compared to breeders.[23,24] Because in rodents GnRH neurons coexpress receptors for both kisspeptin and RFRP-3, these neuropeptides could act directly on the GnRH neuronal network.[25,26] Moreover, the presence of the RFRP-3 receptor (GPR147) in arcuate *Kiss1* neurons suggests that kisspeptin neurons also communicate with RFRP-3-expressing neurons.[27] Lastly, subpopulations of *Kiss1*- and *Rfrp*-expressing neurons are steroid hormone-sensitive, allowing to be targeted directly by circulating estradiol.[11,28]

In view of these findings, we hypothesize that both neuropeptides play a significant role in regulating the GnRH pathway that leads to physiological suppression of reproductive function in female Damaraland mole-rats. In particular, we predict that reproductively active females show increased *Kiss1*- and decreased *Rfrp* gene expression in the hypothalamus

compared to suppressed females. In the present study, we used *in situ* hybridization to determine the distribution and gene expression of *Kiss1* and *Rfrp* in the forebrain of reproductively active and reproductively suppressed females.

MATERIALS AND METHODS

Animals

Adult female Damaraland mole-rats (*Fukomys damarensis*) were used. Data were obtained from nine reproductive females (breeder, syn. queen) and nine non-reproductive females (non-breeder). Colonies of mole rats were captured between April and July 2015 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. Colony size ranged from 3 to 14 animals with an average size of 7.5 ± 0.8 individuals. Prior to sacrifice, animals were housed for a maximum period of 12 weeks in captivity 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. Queens could be readily distinguished from non-reproductive females by the presence of a perforate vagina and prominent teats[29]. None of the females was pregnant at the time of sacrifice. All animals were kept in their original colonies until sacrifice. At the time of brain collection, body mass of all animals was recorded to the nearest gram. All experimental procedures were approved by the University of Pretoria Animal Ethics Committee (EC103-13).

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 mass 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* and *Rfrp* mRNA.

Cloning of cDNA probes

Based on sequence information available from the Damaraland mole-rat, PCR was used to amplify fragments of the *Kiss1 and Rfrp* gene. For *Kiss1* 5' RACE was carried out using the SMARTer RACE 5'/3' kit (Clontech Laboratories). The preparation of 5'RACE cDNA and PCR was done according to the manufacturer's instructions. The gene-specific antisense primer was -5'CCTTGGGAGAACCCCTGGCAAAA-3'. 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. Additionally, a synthetic construct was obtained (501 bp, [GenBank: KY081958], MWG Biotech, Germany) containing the entire coding region (402 bp). Both sequences gave identical expression patterns in the *in situ* hybridization, therefore, the 5'RACE fragment was used for all analyses. The coding region shows 82% homology with guinea pig *Kiss1* [GenBank: HM030726.2]. For *Rfrp*, total RNA was extracted from mole-rat hypothalamus by using the RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany). The synthesis of first-strand cDNA was done with SUPERSCRIPT III Reverse Transcriptase (Invitrogen, Karlsruhe, Germany) and random primers. The resulting RNA-DNA hybrids were subsequently used in PCR to generate pieces of the appropriate gene. The forward primer was 5'-CAATGGGACATCTTCACAGCA-3' and the reverse primer was 5'-AGAAATCTGGAGCCTGTCCCTAA-3'. PCR was carried out for 40 cycles by using the following parameters: 94°C for 1 minute, 55°C for 45 seconds, 72°C for 1 minute. The amplified fragment was purified and cloned into the pCRII TOPO vector using the TOPO-TA cloning kit (Invitrogen, Karlsruhe, Germany). Resultant clones were sequenced to verify the

authenticity and fidelity of the amplification. The cloned *Rfrp* sequence [GenBank: MF101869] is 548 bp in length and shows 79% homology with human *RFRP* [GenBank: NM_013476].

***In situ* hybridization**

The expression of GnRH 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[31] with modifications as described elsewhere.[32] For signal detection, 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 cresyl violet. 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. Control sections processed with the sense probes showed no signal.

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[33] using the Coat-a-Count MG 12101 oestradiol kit (IBL International GmbH Hamburg, Germany). The cross reactivity of the antibody to all naturally occurring steroids was 10% with oestrone, <5% with oestriol, oestrone-b-D- glucoronide, oestrone-3-sulphate, D-eequilenin, 17β oestradiol-3-monosulphate, testosterone and androsterone. The intra-assay coefficient of variation for repeated measures of a quality control was 8.3% and sensitivity of the assay was 10 pg/ml. The assay was validated for plasma of *F. damarensis* using serial dilutions of unextracted mole-rat plasma obtained from a female with high oestradiol concentrations and serial dilutions paralleled the standard curve.

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. Anatomical landmarks were obtained from parallel sections from one individual stained with the Nissl-stain thionin. The number of silver grain clusters representing cells was counted in adjacent sections along the rostro-caudal axis throughout the hypothalamus. The complete signal for *Kiss1* and *Rfrp* was analysed. For *Kiss1*, this comprised on average 3 sections within the preoptic hypothalamus and 14 sections covering the arcuate nucleus. *Rfrp* expressing cells were found in the anterior and tuberal hypothalamus and the signal comprised on average 14 sections per individual. As semi-quantitative estimate of mRNA content the number of silver grains over individual cells was counted using a 40 x objective. Silver grains were assigned to the cell if they were within a 30 µm radius from the centre of the grain cluster. Cells were considered labelled when the number of silver grains in a cluster exceeded that of background by 3-fold. For each gene, results are presented as total number of cells, as total number of silver grains (an estimate of total mRNA) and the average number of silver grains per cell. Rostro-caudal analysis of expression was carried out for *Kiss1* from 2.28-3.60 mm posterior of the anterior commissure and for *Rfrp* from 1.20-2.52 mm posterior of the anterior commissure.

Statistical analysis

Statistical analyses were carried out using JMP software (SAS Institute, Cary, NC, USA). Data are presented as the mean ± SEM. Morphological differences between groups were analysed with *t* tests. Because plasma E_2 levels were not normally distributed, they were analysed by means of nonparametric statistics. For analysis of *Kiss1* gene expression, a restricted maximum likelihood (REML)-model was employed with reproductive status

(breeder vs nonbreeder) and brain region (AVPV vs ARC) as fixed factors and animal ID as random factor. Post-hoc analyses were performed with the 'test slices' comparison in JMP. The expression of *Rfrp* was analysed between groups by *t* tests. Analysis of the total number of *Kiss1*- and *Rfrp*-expressing cells along the rostro-caudal level was conducted using a REML-model with reproductive status and brain region as fixed factors and ID as random factor. $P < .05$ (two-tailed) was considered statistically significant.

RESULTS

Morphological measurements and plasma E2

The two groups of females differed significantly in body mass, with breeders (115.8 ± 3.1 g) being heavier than nonbreeders (83.3 ± 4.0 g, $t = 6.32$, $df = 16$, $P < .0001$). No significant group differences were found in brain mass ($t = 1.24$, $df = 16$, $P = .23$). Plasma E_2 levels were very low in both breeders (0.09 ± 0.05 ng mL^{-1}, range: 0.02-0.481 ng mL^{-1}) and nonbreeders (0.032 ± 0.004 ng mL^{-1}, range: 0.016-0.059 ng mL^{-1}) and not significantly different between groups (Mann-Whitney $U = 20.0$, $P = .073$). Only one breeding female had an elevated E_2 level (0.481 ng mL^{-1}).

Distribution of *Kiss1* and *Rfrp* mRNA

Kiss1-expressing cells were found in the preoptic and tuberal hypothalamus. Within the preoptic hypothalamus expression was scarce with few cells scattered throughout the AVPV and the periventricular preoptic nucleus (PVpo). Within the tuberal hypothalamus expression was found in the ARC, with nearly all *Kiss1*-expressing cells residing in the caudal part of the nucleus (Fig. 1). Labelling was not found in other brain regions.

Rfrp mRNA expression was detected in the anterior and tuberal hypothalamus comprising the region of the periventricular nucleus (PeN), the paraventricular nucleus (PVH) and the dorsomedial nucleus (DMH). Occasionally, single *Rfrp*-expressing cells were also found in the lateral hypothalamic area, the lateral retrochiasmatic area, the retrochiasmatic part of the supraoptic nucleus and within the ARC.

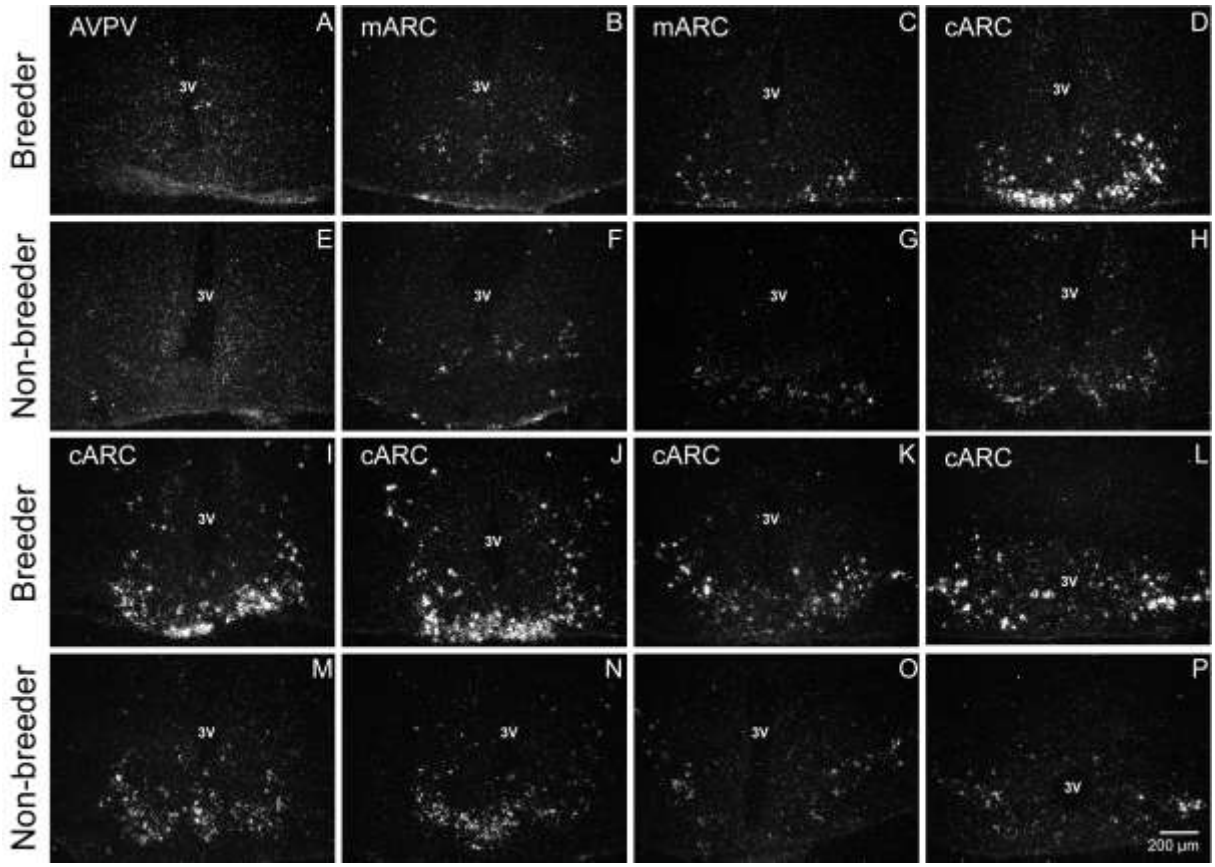

Figure 1. Darkfield photomicrographs illustrating the expression of *Kiss1* in rostral to caudal direction in the hypothalamus of a breeding female (A-D, I-L) and a reproductively suppressed female (E-H, M-P). A, E: AVPV; the anterior commissure is to the top. B, C, F, G: middle ARC; D, I-P, caudal ARC. 3V, third ventricle. The region of the ARC comprised by the sections corresponds to the distance of -2.40 to -3.60 posterior of the anterior commissure.

Kiss1 and *Rfrp* gene expression related to reproductive status

We quantified *Kiss1* mRNA expression in the AVPV and in the ARC by counting the number of *Kiss1*-expressing cells and the number of silver grains over each cell. Reproductive females had significantly more *Kiss1*-expressing cells in ARC than non-reproductive females. Further, the total number of silver grains (as an estimate of total mRNA) and the average number of silver grains per cell was higher in ARC of reproductive than non-reproductive females. No significant group differences were found in the AVPV (Table 1; Fig. 1, 2). *Rfrp* mRNA expression was quantified by counting the number of *Rfrp*-expressing cells and the number of silver grains over each cell of the entire signal visible. Reproductive females had significantly

less *Rfrp*-expressing cells (t=2.34, df=15, P=0.034) and a lower total number of silver grains (t=2.34, df=15, P=0.033) within the hypothalamus compared to non-reproductive females. The average number of silver grains per cell was similar between both groups (t=0.24, df=15, P=0.81; Fig. 3, 4).

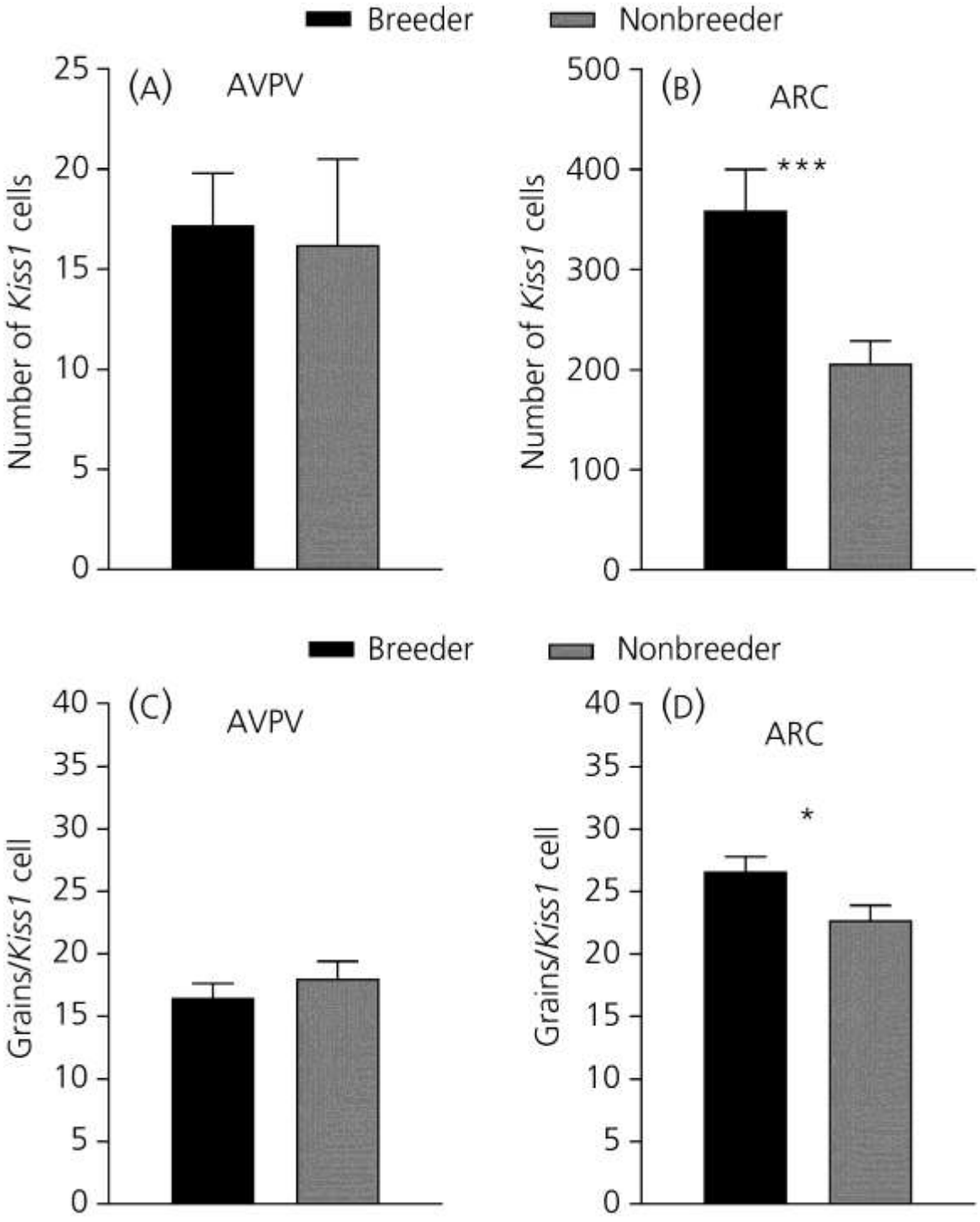

Figure 2. Number of *Kiss1*-expressing cells (A, B) and number of silver grains per *Kiss1* cell (C, D) in AVPV and ARC of female breeders and non-breeders (*** p< 0.001; * p < 0.05).

Table 1: Linear mixed model results for the effect of reproductive status (reproductive vs. non-breeder) and brain region (AVPV vs. ARC) on gene expression of *Kiss1* in female Damaraland mole-rats.

	F	**df**	***P***
Kiss1 total no. of cells			
Status	10.29	1, 16	**0.006**
Region	131.17	1, 16	**0.0001**
Status x Region	10.64	1, 16	**0.005**
Kiss1 total no. of silver grains			
Status	9.80	1, 16	**0.007**
Region	78.74	1, 16	**0.0001**
Status x Region	9.78	1, 16	**0.007**
Kiss1 average no. of silver grains/cell			
Status	1.04	1, 16	0.324
Region	41.28	1, 16	**0.0001**
Status x Region	5.03	1, 16	**0.039**

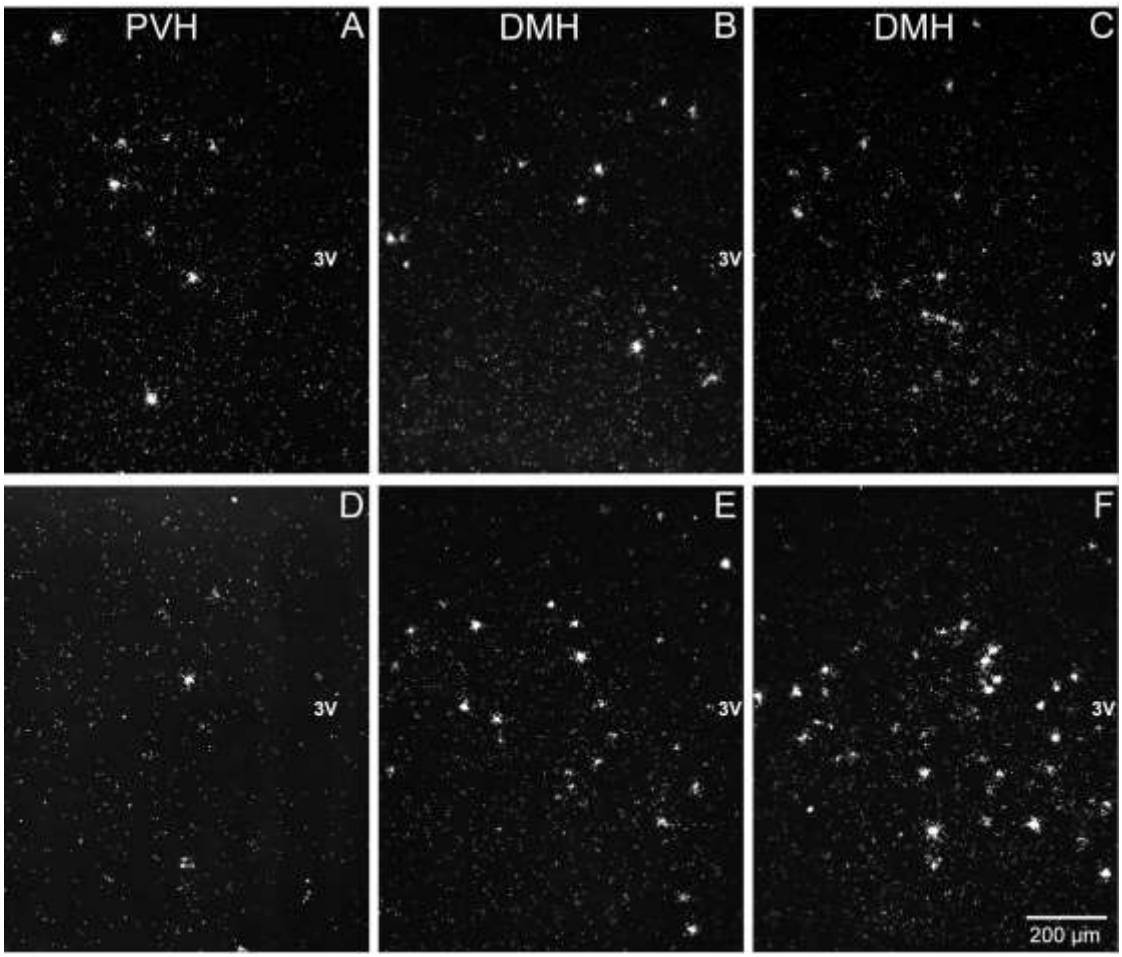

Figure 3. Darkfield photomicrographs illustrating the expression of *Rfrp* in rostral to caudal direction at the level of the PVH (A, D) and the DMH (B, C, E, F) from a breeding female (A-C) and a reproductively suppressed female (D-F). 3V, third ventricle.

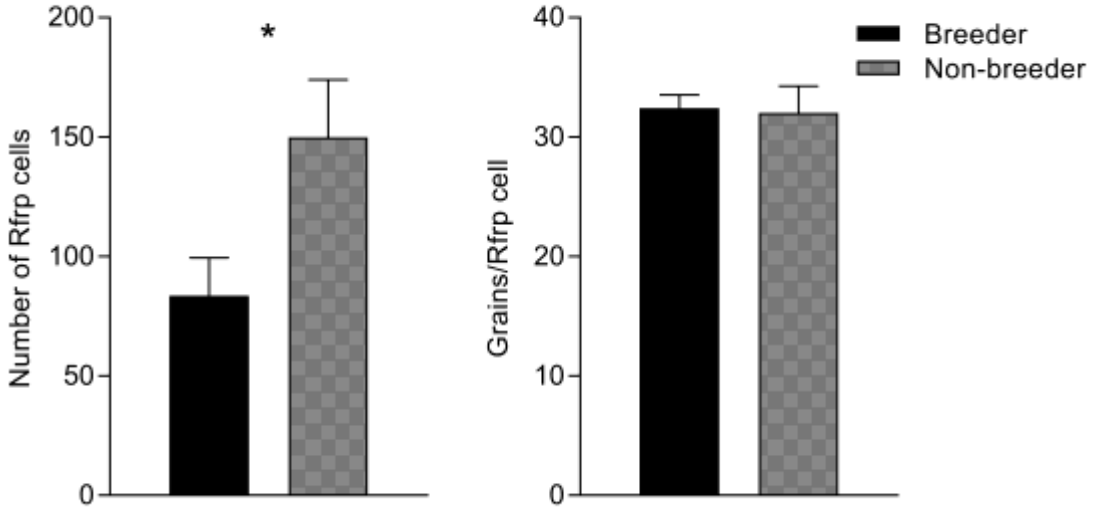

Figure 4. Number of *Rfrp*-expressing cells and number of silver grains per *Rfrp* cell in the hypothalamus of female breeders and non-breeders (*** p< 0.001; * p < 0.05).

We further analysed the differences between the two groups of females in the number of *Kiss1*-expressing cells in ARC and of *Rfrp*-expressing cells in PVH/DMH along the rostro-caudal level. Reproductive females had significantly more *Kiss1*-expressing cells in the caudal part of ARC than non-reproductive females (status: $F_{1,15.97}$=6.28, P=0.023; region: $F_{11,155.6}$=7.93, P=0.0001; interaction: $F_{11,155.6}$=2.51, P=0.006). The latter had more *Rfrp*-expressing cells compared to reproductive females in the anterior hypothalamus at the level of the caudal limits of PVH (status: $F_{1,14.22}$=4.63, P=0.049; region: $F_{11,137.3}$=7.76, P=0.0001; interaction: $F_{11,137.3}$=1.48, P=0.147, Fig. 5).

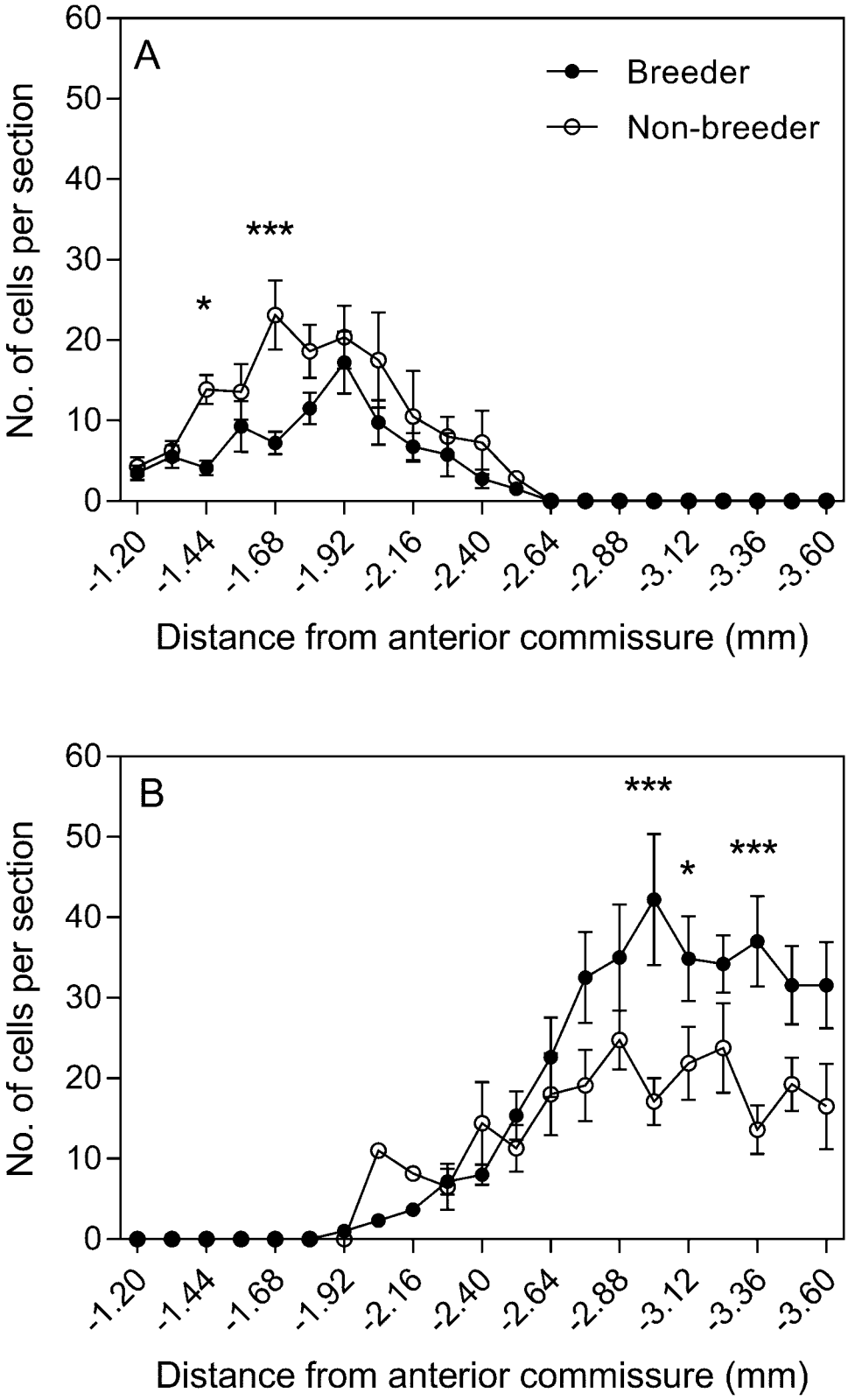

Figure 5. Number of *Rfrp*-expressing cells (A) and *Kiss1*-expressing cells (B) along the rostro-caudal axis throughout the anterior and tuberal hypothalamus in female breeders and non-breeders (* $p < 0.05$, *** $p < 0.001$).

DISCUSSION

Female subordinate Damaraland mole-rats within their natal colony possess an anovulatory phenotype.[34] The mechanisms mediating this reproductive suppression are still not well understood. Here we report the distribution and differential gene expression of the two RFamide neuropeptides kisspeptin and RFRP that are implicated in the control of female reproduction.

Distribution of *Kiss1* and *Rfrp*-expressing cells

In rodents such as mice, rats, hamsters and guinea pigs, Kiss1-expressing cells have been detected previously by ISH in mainly two locations, the preoptic hypothalamus comprising the region of the AVPV/PVpo and in the tuberal hypothalamus comprising the ARC.[5,14,25,35] Our study is in agreement with this distribution. However, unlike in mice, rats and hamsters, labelling in the mole-rat preoptic hypothalamus is scarce, which matches reports from the guinea pig. In the latter, a small percentage of GnRH neurons within the ARC innervate the median eminence and those are being essential for gonadotropin release.[36] In relation, the kisspeptin neurons of the caudal ARC rather than the AVPV have been implicated in the positive feedback regulation of oestrogen on GnRH neurons in this species.[14] There is also evidence from a non-rodent model, the sheep, that ARC kisspeptin neurons are involved in the GnRH/LH surge of females[37]. Whether this is the case in mole-rats, too, needs to be determined. Using ICC and ISH it was shown previously that in Damaraland mole-rats the majority of GnRH cell bodies are found in the preoptic hypothalamus with only few cells being present in the tuberal hypothalamus suggesting that mainly the preoptic GnRH neurons constitute the median eminence innervation[24,38] The distribution of Kiss1-expressing cells detected by ICC studies does not completely match those using ISH. In mice and sheep, Kiss1-immunoreactive cells have been described in the DMH where no *Kiss1* mRNA is found.[12,39] In naked mole-rats, in addition to the DMH, Kiss1-ir cell bodies have been found in the anterior part of the periventricular nucleus (PVa).[40] The present study on a closely

related species did not detect mRNA expression in the PVa and the DMH. This discrepancy is likely due to the non-specificity of the kisspeptin antisera used.

The distribution of *Rfrp* neurons matched previous reports from ISH and ICC studies in other animals.[7,41,42] However, in addition to neurons in the DMH, we found mRNA expression in the PVH (Fig. 3A, D), which has been described in ewes but not in rodents.[43] A recent ICC study in naked mole-rats reports substantial Rfrp-3-ir cell bodies in the DMH and in the ARC, but not in the PVH.[44] *Rfrp* gene expression in the ARC could not be confirmed with the present ISH study and is not known from previous studies in any other animal model. Interestingly, in Damaraland mole-rats, single, often high-expressing *Rfrp* cells were found in the lateral retrochiasmatic area, in close proximity to the supraoptic nucleus and in the retrochiasmatic part of the supraoptic nucleus. In these areas we also detected GnRH neurons.[24] Double-label ISH could confirm which GnRH neuron populations coexpress the RFRP-3 receptor GPR147.

Influence of reproductive status on *Kiss1* and *Rfrp* expression

While we have previously reported that GnRH and ERα gene expression within the preoptic hypothalamus differs according to female reproductive status, the present study does not detect such a difference concerning *Kiss1* gene expression in that region. Moreover, in both groups of females very few *Kiss1*-expressing cells were found in AVPV compared to other rodents.[11,45] It is a limitation to our study that we were not able to assess the oestrous cycle stages using vaginal cytology and thus could not sample all breeders at the preovulatory stage. Therefore, it must be assumed that the reproductive females in our study were in different stages of their ovarian cycle. Only one out of nine females had elevated E2 level indicative of follicular development and possibly proestrus. If, similar to rats, *Kiss1* expression fluctuates throughout the stages of the oestrous cycle, this could explain the observed absence of an elevation in the group of breeding females. In rats, *Kiss1* mRNA is significantly increased during the afternoon of proestrus compared to estrus and diestrus[13,46]. Because of this narrow

time window it is likely that we missed this upregulation in our animals. Alternatively, it is possible that the preoptic kisspeptin neurons are not important for the regulation of ovulation in mole-rats. However, reproductively active females possess significantly elevated ERα gene expression in the AVPV compared to suppressed females, which shows that E2 in this brain region plays a role in female reproduction in this species.[23] Investigating *Kiss1* gene expression in OVX and E2-treated females could reveal if this neuron population is regulated by circulating oestradiol.

In the ARC, we found that the number of *Kiss1*-expressing cells and the total amount of *Kiss1* mRNA (represented by the number of silver grains) was significantly higher in reproductively active compared to suppressed females. In mice, rats, hamsters and guinea pigs, kisspeptin neurons in this region are involved in the negative feedback action of E2 on GnRH neurons. Ovariectomy increases and E2 treatment decreases *Kiss1* expression.[45] According to this, one would have expected a higher level of *Kiss1* expression in the ARC of suppressed female Damaraland mole-rats, considering their anovulatory phenotype. However, E2 levels in our animals were similarly low in both groups of females. Therefore, other factors must contribute to the regulation of *Kiss1* expression in this brain region. Studies in seasonal breeders reveal a much more complex scenario, with additional positive and negative feedback actions of E2 within the ARC.[47] In seasonally anestrous ewes, the chronically very low E2 levels during the non-breeding season constitute a negative feedback effect on GnRH neuron activity and are associated with reduced *Kiss1* expression in the ARC.[43,48] Similar results were obtained from Syrian hamsters, which are reproductively inactive during short-day conditions.[49] Such long-term negative feedback effect of E2 could be acting on ARC neurons of reproductively suppressed Damaraland mole-rats, leading to decreased levels of *Kiss1* gene expression. Our rostro-caudal analysis showed that the group differences in the number of *Kiss1* cells was most pronounced in the caudal ARC. It has been suggested previously that *Kiss1* neuron subpopulations within ARC may show differential

responsiveness to E2 levels[50] and our data support a role for *Kiss1* neurons in caudal ARC in mediating the long-term negative feedback action of E2.

As we had expected, reproductively suppressed female Damaraland mole-rats possessed more *Rfrp*-expressing cells within the hypothalamus than breeding females. Such results were recently also reported in an ICC study from naked mole-rats, but sample size was low and the antibody used produced staining in a brain region (ARC), not known to possess RFRP-3 expressing cells.[44] Our results agree with the seasonal studies performed in sheep, where in anestrous ewes (during the non-breeding season), when no ovulation occurs, *Rfrp* gene expression and RFRP-3-ir cell number are higher than when reproductively active. Also, fewer GnRH cells are contacted by RFRP-3 fibers during this stage.[43,51] However, besides this direct action of RFRP-3 on GnRH neurons, *Kiss1*-expressing neurons, mainly in the ARC, also receive RFRP-3 fibre contacts.[27] Thus, the exact mechanism by which *Rfrp*-expressing neurons exert their inhibiting effect in Damaraland mole-rats needs to be determined.

In conclusion, our data suggest that kisspeptin and RFRP gene expression are involved in mediating reproductive suppression in Damaraland mole-rats. It seems likely that an enhanced long-term negative feedback effect of E2 plays a role in suppressing fertility similar as described for seasonally breeding species. Studying the steroid hormone sensitivity of both neuropeptides will provide further inside into this mechanism.

DECLARATION OF INTEREST

The authors of the manuscript have no conflicts of interest to declare.

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REFERENCES

1. Keller L, Reeve KH. Partitioning of reproduction in animal societies. Trends Ecol Evol 1994;9:98-102.
2. Clutton-Brock TH, Brotherton PN, Russell AF, O'Riain MJ, Gaynor D, Kansky R, Griffin A, Manser M, Sharpe L, McIlrath GM, Small T, Moss A, Monfort S. Cooperation, control, and concession in meerkat groups. Science 2001;291:478-481.
3. Lacey EA, Sherman PW. Cooperative breeding in naked mole-rats: Implications for vertebrate and invertebrate sociality. In: Solomon NG, French JA. Cooperative Breeding in Mammals. Cambridge: Cambridge University Press; 1997. pp. 268-301.
4. Faulkes CG, Abbott DH. The physiology of a reproductive dictatorship: Regulation of male and female reproduction by a single breeding female in colonies of naked mole rats. In: Solomon NG, French JA. Cooperative Breeding in Mammals. Cambridge: Cambridge University Press; 1997. pp. 302-334.
5. 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.
6. 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.

7. 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.
8. Clarke IJ, Sari IP, Qi Y, Smith JT, Parkington HC, Ubuka T, Iqbal J, Li Q, Tilbrook A, Morgan K, Pawson AJ, Tsutsui K, Millar RP, Bentley GE. Potent action of RFamide-related peptide-3 on pituitary gonadotropes indicative of a hypophysiotropic role in the negative regulation of gonadotropin secretion. Endocrinology 2008;149:5811-5821.
9. Pinilla L, Aguilar E, Dieguez C, Millar RP, Tena-Sempere M. Kisspeptins and reproduction: physiological roles and regulatory mechanisms. Physiol Rev 2012;92:1235-1316.
10. Putteeraj M, Soga T, Ubuka T, Parhar IS. A "timed" kiss is essential for reproduction: lessons from mammalian studies. Front Endocrinol 2016;7:121.
11. 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.
12. 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.
13. 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.
14. Bosch MA, Xue C, Rønnekleiv OK. Kisspeptin expression in guinea pig hypothalamus: effects of 17β-estradiol. J Comp Neurol 2012;520:2143-2162.

15. Anderson GM, Relf HL, Rizwan MZ, Evans JJ. Central and peripheral effects of RFamide-related peptide-3 on luteinizing hormone and prolactin secretion in rats. Endocrinology 2009;150:1834-1840.
16. 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.
17. Decourt C, Anger K, Robert V, Lomet D, Bartzen-Sprauer J, Caraty A, Dufourny L, Anderson G, Beltramo M. No Evidence That RFamide-Related Peptide 3 Directly Modulates LH Secretion in the Ewe. Endocrinology 2016;157:1566-1575.
18. Rizwan MZ, Porteous R, Herbison AE, Anderson GM. Cells expressing RFamide-related peptide-1/3, the mammalian gonadotropin-inhibitory hormone orthologs, are not hypophysiotropic neuroendocrine neurons in the rat. Endocrinology 2009;150:1413-1420.
19. Ancel C, Bentsen AH, Sébert ME, Tena-Sempere M, Mikkelsen JD, Simonneaux V. Stimulatory effect of RFRP-3 on the gonadotrophic axis in the male Syrian hamster: the exception proves the rule. Endocrinology 2012;153:1352-1363.
20. Ubuka T, Inoue K, Fukuda Y, Mizuno T, Ukena K, Kriegsfeld LJ, Tsutsui K. Identification, expression, and physiological functions of Siberian hamster gonadotropin-inhibitory hormone. Endocrinology 2012;153:373-385.
21. Faulkes CG, Bennett NC. Family values: group dynamics and social control of reproduction in African mole-rats. Trends Ecol Evol 2001;16:184-190.
22. Bennett NC, Faulkes CG, Jarvis JUM. Socially-induced infertility incest avoidance and the monopoly of reproduction in cooperatively breeding African mole-rats. Advances in the study of Behaviour 1999,28:75-113.

23. 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. Frontiers Zool 2014;11:38.
24. Voigt C, Bennett NC. GnRH mRNA expression in the brain of cooperatively-breeding female Damaraland mole-rats. Reproduction 2017;153: 453–460.
25. Irwig MS, Fraley GS, Smith JT, Acohido BV, Popa SM, Cunningham MJ, Gottsch ML, Clifton DK, Steiner RA. Kisspeptin activation of gonadotropin releasing hormone neurons and regulation of KiSS-1 mRNA in the male rat. Neuroendocrinology 2004;80:264-272.
26. Rizwan MZ, Poling MC, Corr M, Cornes PA, Augustine RA, Quennell JH, Kauffman AS, Anderson GM. RFamide-related peptide-3 receptor gene expression in GnRH and kisspeptin neurons and GnRH-dependent mechanism of action. Endocrinology 2012;153:3770-3779.
27. Poling MC, Quennell JH, Anderson GM, Kauffman AS. Kisspeptin neurones do not directly signal to RFRP-3 neurones but RFRP-3 may directly modulate a subset of hypothalamic kisspeptin cells in mice. J Neuroendocrinol 2013;25:876-886.
28. Poling MC, Kim J, Dhamija S, Kauffman AS. Development, sex steroid regulation, and phenotypic characterization of RFamide-related peptide (Rfrp) gene expression and RFamide receptors in the mouse hypothalamus. Endocrinology 2012;153:1827-1840.
29. 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.
30. Paxinos G, Watson C. The Rat Brain in Stereotaxic Coordinates. Amsterdam: Academic Press; 2007.
31. 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.

32. 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.
33. 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! Canadian Journal of Zoology 2017;95:107-114.
34. Molteno AJ, Bennett NC. Anovulation in non-reproductive female Damaraland mole-rats (Cryptomys damarensis). J Reproduction and Fertility 2000;119:35-41.
35. Revel FG, Ansel L, Klosen P, Saboureau M, Pévet P, Mikkelsen JD, Simonneaux V. Kisspeptin: a key link to seasonal breeding. Rev Endocr Metab Disord 2007;8:57-65.
36. Silverman AJ., Livne I, and Witkin JW. The Gonadtropin-Releasing Hormone, Neuronal Systems: Immunocytochemistry and In Situ Hybridization. In: Knobil E, Neill JD. The Physiology of Reproduction. New York: Raven Press; 1994. pp 1683-1709.
37. Merkley CM, Porter KL, Coolen LM, Hileman SM, Billings HJ, Drews S, Goodman RL, Lehman MN. KNDy (kisspeptin/neurokinin B/dynorphin) neurons are activated during both pulsatile and surge secretion of LH in the ewe. Endocrinology 2012;153:5406-5414.
38. Molteno AJ, Kallo I, Bennett NC, King JA, Coen CW. A neuroanatomical and neuroendocrinological study into the relationship between social status and GnRH system in cooperatively breeding female Damaraland mole-rats, Cryptomys damarensis. Reproduction 2004;127:13-21.
39. 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.

40. 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.
41. Hinuma S, Shintani Y, Fukusumi S, Iijima N, Matsumoto Y, Hosoya M, Fujii R, Watanabe T, Kikuchi K, Terao Y, Yano T, Yamamoto T, Kawamata Y, Habata Y, Asada M, Kitada C, Kurokawa T, Onda H, Nishimura O, Tanaka M, Ibata Y, Fujino M. New neuropeptides containing carboxy-terminal RFamide and their receptor in mammals. Nat Cell Biol 2000;2:703-708.
42. Legagneux K, Bernard-Franchi G, Poncet F, La Roche A, Colard C, Fellmann D, Pralong F, Risold PY. Distribution and genesis of the RFRP-producing neurons in the rat brain: comparison with melanin-concentrating hormone- and hypocretin-containing neurons. Neuropeptides 2009;43:13-19.
43. Smith JT, Coolen LM, Kriegsfeld LJ, Sari IP, Jaafarzadehshirazi MR, Maltby M, Bateman K, Goodman RL, Tilbrook AJ, Ubuka T, Bentley GE, Clarke IJ, Lehman MN. Variation in kisspeptin and RFamide-related peptide (RFRP) expression and terminal connections to gonadotropin-releasing hormone neurons in the brain: a novel medium for seasonal breeding in the sheep. Endocrinology 2008;149:5770-5782.
44. 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.
45. 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.

46. 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.

47. Clarke IJ, Caraty A. Kisspeptin and seasonality of reproduction. Adv Exp Med Biol 2013;784:411-430.

48. 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.

49. Ansel L, Bolborea M, Bentsen AH, Klosen P, Mikkelsen JD, Simonneaux V. Differential regulation of kiss1 expression by melatonin and gonadal hormones in male and female Syrian hamsters. J Biol Rhythms 2010;25:81-91.

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

51. Dardente H, Birnie M, Lincoln GA, Hazlerigg DG. RFamide-related peptide and its cognate receptor in the sheep: cDNA cloning, mRNA distribution in the hypothalamus and the effect of photoperiod. J Neuroendocrinol 2008;20:1252-1259.