Gnrh mRNA expression in the brain of cooperatively breeding female Damaraland mole-rats

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Abstract

The Damaraland mole-rat (Fukomys damarensis) is a eusocial, subterranean rodent, in which breeding is limited to a single reproductive pair within each colony. Non-reproductive females, while in the confines of the colony, exhibit socially induced infertility. Anovulation is thought to be caused by a disruption in the normal gonadotropin-releasing hormone (GNRH) secretion from the hypothalamus. To assess whether social suppression is associated with altered Gnrh mRNA expression in the brain, we investigated the distribution and gene expression levels by means of in situ hybridization in female breeders and non-breeders from field captured colonies of the Damaraland mole-rat. We found expression of Gnrh mRNA as a loose network in several forebrain areas of female Damaraland mole-rats with the majority of labelling in the preoptic and anterior hypothalamus. The distribution matched previous findings using immunocytochemistry in this and other social mole-rat species. Quantification of the hybridization signal revealed no difference between breeding and non-breeding females in the average optical density of the hybridization signal and the size of the total area covered by Gnrh mRNA. However, analysis along the rostro-caudal axis revealed significantly elevated Gnrh mRNA expression in the rostral preoptic region of breeders compared to non-breeders, whereas the latter had increased Gnrh mRNA expression at the caudal level of the anterior hypothalamus. This study indicates that social suppression affects the expression of Gnrh mRNA in female Damaraland mole-rats. Furthermore, differential regulation occurs within different neuron subpopulations.

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Introduction

In cooperatively breeding birds and mammals, reproductive skew is maintained by reproductive suppression of subordinate group members (Keller & Reeve 1994). Socially induced suppression of ovulation represents one such mechanism in female mammals and is best known from callitrichid primates such as the common marmoset (Callithrix jacchus, Abbott 1984) and eusocial mole-rats (Faulkes et al. 1990, Molteno & Bennett 2000). In these species, anovulation of subordinates appears to occur due to an insufficient release of luteinizing hormone (LH) from the anterior pituitary.

African mole-rats (Bathyergidae) exhibit a wide range of social organisation, from strictly solitary to highly social species, and thus, differ in the degree of reproductive skew (Faulkes & Bennett 2001). Previous studies investigating the GNRH system by means of immunocytochemistry (ICC) have revealed inconsistent results. Among the social species, where breeding is restricted to a single female (queen) within the group, reproductive status had either no effect on the morphology and immunoreactivity of GNRH neurons (common mole-rat, Du Toit et al. 2006; naked mole-rat, Zhou et al. 2013) or non-breeders differed from breeders by showing elevated immunoreactivity in the median eminence and/or higher brain GNRH content (Damaraland mole-rat, Molteno et al. 2004; Highveld mole-rat, Du Toit et al. 2006; Natal mole-rat, Oosthuizen et al. 2008). The latter would indicate a reduced rate of GNRH release into the hypophyseal portal blood system, resulting in inadequate activation of the anterior pituitary of such suppressed females.

Damaraland mole-rats (Fukomys damarensis) with an average group size of 11 individuals represent an extreme example of socially induced infertility, in that reproduction is completely blocked in subordinate female group members. Such females show no signs of sexual behaviour, have significantly lower urinary oestradiol and progesterone levels and exhibit interrupted follicular development compared to the dominant breeding female of the group (Bennett 1994, Bennett et al. 1994). Therefore, the blockade of reproduction results from an inhibition of ovulation, which appears to be caused by inadequate secretion of luteinizing hormone (LH) from the pituitary, which in
turn might be brought about by a disruption of the normal GNRH secretion from the hypothalamus (Molteno et al. 2004). However, females removed from the presence of the queen start to ovulate spontaneously (Molteno & Bennett 2000, Snyman et al. 2006). Furthermore, we have shown recently that reproductive status and social suppression affect the neuroendocrine phenotype of female Damaraland mole-rats with subordinates having reduced expression of androgen and estrogen receptor α genes in several preoptic–hypothalamic brain regions when compared to breeders (Voigt et al. 2014).

The question arises as to whether the block to ovulation lies at the level of the hypothalamus, i.e. the GNRH neurons or at the level of the anterior pituitary, i.e. the GNRH receptors or both. Using ICC and radioimmunoassay (RIA), Molteno and coworkers (2004) investigated the distribution and morphology of GNRH-immunoreactive structures and the GNRH content in the brain of female breeders and non-breeders and of females removed from suppression. Although reproductive status did not affect the size and the total number of GNRH cells, suppressed females had a larger amount of GNRH in the median eminence and in the proximal pituitary stalk, suggesting that their release from the nerve terminals is inhibited (Molteno et al. 2004). These data would be indicative of suppression within the hypothalamus. On the other hand, subordinate females secrete significantly less LH in response to a single dose of exogenous GNRH than breeders (Bennett et al. 1993), which suggests that the anterior pituitary of such females is desensitized. GNRH receptors are positively autoregulated by physiological concentrations of GNRH (Clayton 1982); thus, a lack of GNRH may lead to their downregulation. There is evidence from several studies in rats that GNRH biosynthesis is linked to GNRH and LH release (Wise et al. 1981, Zoeller & Young 1988, Petersen et al. 1996, Spratt & Herbison 1997, Jimenez-Linan & Rubin 2001). For example, hypothalamic *Gnrh* mRNA levels were found to increase at proestrus in female rats prior to the preovulatory LH surge (Gore & Roberts 1995, Petersen et al. 1996, Jimenez-Linan & Rubin 2001). Therefore, the present study aims at investigating whether levels of *Gnrh* mRNA are altered between reproductively active and suppressed females, which may provide an indication for differential transcriptional regulation of the GNRH gene. Using *in situ* hybridization, we analysed the distribution, optical density and area covered by *Gnrh* mRNA in the forebrain of breeding females and of non-breeding females.

**Materials and methods**

**Animals**

In the current study, adult female Damaraland mole-rats (*Fukomys damarensis*) were used. Data were obtained from eight reproductive females (breeder, syn. queen) and eight non-reproductive females (non-breeder). The non-breeders derived from colonies captured between April and June 2013 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. Prior to sacrifice, animals were housed for a maximum period of 10 weeks at the University of Pretoria under 12 L:12 D cycle at 25°C in plastic containers (1.0 m × 0.5 m × 0.5 m) containing wood shavings, and they were fed on sweet potato, gem squash and apples. The breeders were captured in December 2012 as subordinates and kept in the laboratory paired with a male, respectively, for 7 months before sacrifice. Reproductive activity was confirmed for all pairs by the observation of copulations. Four females were pregnant at the time of sacrifice. The reproductive status of the females was confirmed post mortem by examination of the reproductive tract. 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 (EC003-12).

**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 30 µm coronal sections. The plane of the sections was adjusted to match as closely as possible to the plane of the rat brain atlas (Paxinos & Watson 2007). Sections were mounted onto Superfrost Plus slides (Menzel-Gläser, Braunschweig, Germany) in four different series, so that one series of slides contained a section every 120 µm. *In situ* hybridization was carried out on every fourth section for the localization of *Gnrh* mRNA.

**Cloning of cDNA probe**

Based on sequence information available from other species, PCR was used to amplify a fragment of the *Gnrh1* gene from the Damaraland mole-rat. Total RNA was extracted from mole-rat hypothalamus using the RNeasy Mini Kit (Qiagen GmbH). The synthesis of first-strand cDNA was done with SUPERSCRIPT III Reverse Transcriptase (Invitrogen) 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′-AACATCTCAAACAAGCTTGA-3′ and the reverse primer was 5′-ATCTAATAACTGTCGGCCTCTCA-3′. PCR was carried out for 40 cycles using the following parameters: 94°C for 1 min, 58°C for 45 s, 72°C for 1 min. The amplified fragment was purified and cloned into the pCRII TOPO vector using the TOPO-TA cloning kit (Invitrogen). Resultant clones were sequenced to verify the authenticity and fidelity of the amplification. The cloned *Gnrh1* sequence (GenBank: KX555603) is 421 bp in length and encompasses the entire coding region (279bp) plus 51 bp of the 5′ untranslated region and 91 bp of the 3′ untranslated region. The coding region shows

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97% homology with a previously cloned sequence (227 bp) from Highveld mole rats (*Cryptomys hottentotus pretoriae*, GenBank: AJ966357.1) (Kalaminigos et al. 2005), 77% homology with rat *Gnrh1* (GenBank: NM_012767) and 86% homology with guinea pig *Gnrh1* (GenBank: NM_001172956). The GNRH peptide of the latter species is unique in that it differs from the form found in all other mammals by two amino acid substitutions. Moreover, two polyadenylation sites are present in the precursor mRNA, resulting in two transcripts (Jimenez-Linan et al. 1997). Despite the high homology with guinea pig *Gnrh1*, the sequences isolated from the two mole-rat species represent the ‘mammalian’ GNRH.

**In situ hybridization**

The expression of GNRH in brain sections was detected with antisense RNA probes labelled with 35S-CTP. Labelling of the probes with 35S-CTP (1250 Ci/mmole; Perkin Elmer) was performed using the Riboprobe System (Promega). Our *in situ* hybridization procedure followed a previously published protocol for detecting GNRH mRNA expression in Damaraland mole-rats.
Data analysis

Images from autoradiograms were scanned with an Epson Perfection V750 Pro scanner connected to a PC running the image analysis software ImageJ 1.50u (NIH; see http://rsb.info.nih.gov/ij/). To obtain the output of the measurements in optical density (OD) the system was calibrated using a calibrated optical density step tablet (T2115CC; Stouffer Industries, Inc., Mishawaka, IN, USA) and a calibration curve was fitted with the Rodbard function of ImageJ \( y = \frac{d + (a - d)(1 + (x/c)^b)}{1 + (x/c)^b} \). This calibration was applied to all images, and it extended beyond the darkest spot to be measured in the autoradiograms so that the signals that were measured did never reach saturation. Anatomical landmarks were obtained by light microscopic examination of the counterstained sections that were used for the autoradiograms and additionally, of Nissl-stained similar sections from other individuals that had not been processed with in situ hybridization. Sections of all animals were realigned according to the anterior commissure (AC) at the level where it reaches its largest extension.

To quantify the level of Gnrh mRNA expression, the image was converted to an 8-bit grayscale image. A thresholding approach was used to measure the optical density and the area of the hybridization signal. For each section, to obtain a threshold value that separates the background from the specific labelling, I chose a rectangular area (1 mm²) immediately ventral to the brain section of interest. It must be noted that outside the areas expressing GNRH the rest of the brain was always at an optical density close to the film background. Using the threshold function in ImageJ, a threshold value was set manually, which resulted in the thresholded region being displayed in colour (Fig. 1A). The selection tool was used to mark the region of interest (ROI). The mean OD and area size of the colour-marked region within the ROI was obtained using the measurement function of the program applying the option ‘limit to threshold’. The film background OD was obtained from a 1 mm² rectangular area ventral to the brain section. The final OD measurement was the difference between the film background OD value and the OD value of the ROI. Quantification of the mRNA expression level was done in adjacent sections along the rostro-caudal axis within the preoptic and the anterior hypothalamus. The measurements comprised a region, which ranged from four sections rostral to the anterior commissure (AC+4) up to twelve sections caudal of the anterior commissure (AC−12).

Statistical analysis

Statistical analyses were carried out using JMP software. Data are presented as means ± S.E.M. Morphological differences and differences in the average optical density and total area covered by Gnrh mRNA between groups were analysed with t-tests. For analysis of GNRH gene expression along the rostro-caudal axis, a REML-model was employed with reproductive status and brain region as fixed factors and animal ID as random factor. Post hoc analysis was performed using the ‘test slices’ comparison in JMP. All tests were two-tailed, and the significance level was fixed at \( P < 0.05 \).

Results

Morphological measurements

The two groups of females differed significantly in body mass with breeders (98.3 ± 3.7 g) being heavier than non-breeders (83.9 ± 3.9 g, \( t = 2.66, df = 14, P = 0.018 \)). No significant group differences were found in brain mass (\( t = 0.95, df = 14, P = 0.36 \)).

Distribution of Gnrh mRNA

The neuroanatomical distribution of Gnrh mRNA matched largely the distribution of GNRH-ir cell
GnRH mRNA expression in Damaraland mole-rats

Distribution of GnRH mRNA

The distribution of GnRH mRNA is in overall agreement with the previous description of the distribution of GNRH cell bodies detected by immunocytochemistry in the same species (Molteno et al. 2004). Similar to their findings, in the present study, the majority of labelling was found in rostro-caudal direction from the level of the medial septum up to the level of the supraoptic nucleus (SCH). In the tuberal hypothalamus, labelling was scarce and occurred close to the retrochiasmatic part of the supraoptic nucleus. No differences were seen in the distribution of the Gnrh mRNA between breeder and non-breeder females.

Gnrh gene expression related to reproductive status

We quantified Gnrh mRNA expression in consecutive sections within the preoptic and anterior hypothalamus (AC+4–AC−12). The total area covered by Gnrh mRNA that was analysed did not differ between reproductively active and suppressed females ($t = 0.74$, $df = 14$, $P = 0.47$). Also, the average optical density was not different between the two groups ($t = 0.52$, $df = 14$, $P = 0.61$). We further analysed the optical density and the area covered by the hybridization signal at different rostro-caudal levels. Measurement of the average optical density revealed no significant effect of status and brain region, but a significant interaction between both factors on Gnrh mRNA expression (status: $F_{1,14} = 0.269$, $P = 0.61$; region: $F_{16,224} = 0.625$, $P = 0.86$; interaction: $F_{16,224} = 2.21$, $P = 0.006$). Post hoc tests showed that breeding females had increased GNRH expression compared to non-breeding females in the anterior preoptic region (AC+3; Figs 1A, G and 3), whereas the latter had elevated expression compared to breeders in the anterior hypothalamus at the level corresponding to the magnocellular division of the paraventricular nucleus (PALM) and the central portion of the supraoptic nucleus (SO, AC−10, AC−11, Figs 1C, I and 3). Measurement of the area size revealed no significant effect of status, but a significant effect of brain region and no significant interaction between both factors (status: $F_{1,14} = 0.543$, $P = 0.47$; region: $F_{16,224} = 7.70$, $P < 0.0001$; interaction: $F_{16,224} = 1.50$, $P = 0.099$).

Discussion

Here, we report for the first time the mRNA distribution and expression level of the GNRH gene in the mole-rat brain. In Damaraland mole-rats, female subordinates, in the presence of the queen, are physiologically suppressed to the extent of being anovulatory (Molteno & Bennett 2000). We provide evidence for differential regulation of Gnrh mRNA in female breeders and non-breeders. Furthermore, the data suggest GNRH biosynthesis differs between neuron subpopulations.
perikarya are localized in the MBH (Cape mole-rats, Natal mole-rats, Oosthuizen et al. 2008, Highveld mole-rats, Du Toit et al. 2006, naked mole-rats, Zhou et al. 2013, Damaraland mole-rats, Molteno et al. 2004). In contrast, in two species, the common mole-rat and Cape Dune mole-rat, about equal proportions of cell bodies are found in the MS/NDB/POA and in the MBH (Du Toit et al. 2006, Hart et al. 2008). This suggests that in the former species, the median eminence innervation is received mainly from neurons in the MS/NDB/MPOA, whereas in the latter species, GNRH neurons located within the MBH could substantially contribute to the input into the median eminence. In the guinea pig, the small percentage of GNRH neurons within the arcuate nucleus that innervate the median eminence are those being essential for gonadotropin release (Silverman et al. 1994). The functional significance of the GNRH neuron subpopulations in the different mole-rat species remains to be determined.

Influence of reproductive status on Gnrh mRNA levels

In the present study, reproductive activity had no effect on the total area covered by Gnrh mRNA in the brain of female Damaraland mole-rats. This finding supports data from ICC studies in this species and other social mole-rats, which report similar total numbers of GNRH cell bodies and similar cell body size in breeder and non-breeder females (Molteno et al. 2004, DuToit et al. 2006, Oosthuizen et al. 2008, Zhou et al. 2013). However, our results show that reproductive status influences the regulation of the GNRH gene within specific neuron subpopulations. Our detailed analysis at different rostro-caudal levels of the brain identified significantly elevated Gnrh mRNA levels in breeders vs non-breeders in the anterior preoptic area, at one level of the MS/NDB, AC+3. GNRH neurons of the MS/NDB/POA are implicated in the LH surge in female rats and hamsters as shown by double-label in situ hybridization for c-fos activation within GNRH neurons (Berriman et al. 1992, Wang et al. 1995). Furthermore, experiments involving electrical stimulation or deafferentation of the preoptic and anterior hypothalamic region strongly suggest that these neurons contribute to gonadotropin release and ovulation (Eskay et al. 1977, Palkovits et al. 1984, Köves & Molnár 1986). However, only 50–70% of the GNRH neurons actually innervate the median eminence, the remaining neurons project to other brain regions, and both types of neurons are intermingled (Silverman et al. 1987, Merchenthaler et al. 1989). This shows that the neurons regulating gonadotropin release are heterogeneously distributed, which makes it difficult to determine in the present study the functional significance of the observed mRNA expression at particular rostro-caudal levels. Four out of eight breeding Damaraland mole-rats were found to be in the stage of early to mid-pregnancy and for the others the exact stage of their estrous cycle at the time of sacrifice was unknown. The significantly elevated mRNA expression at the rostro-caudal level AC+3 of these females despite their physiological differences suggests that the activity of the GNRH neurons in the anterior preoptic region is necessary for normal gonadotropin release. More detailed information could be obtained by analysing the Gnrh mRNA expression in breeder females according to their reproductive state. In rats, caudal to the suprachiasmatic nucleus few GNRH neurons are found that project to the median eminence and deafferentations in this region revealed that only about 10% of the GNRH content of the median eminence originates from these neurons (Köves & Molnár 1986, Merchenthaler et al. 1989). This may also apply to Damaraland mole-rats as the GNRH neuron distribution is largely similar to that in rats. Therefore, the increased Gnrh mRNA expression in this region (AC+10, AC+11) of subordinate females is likely to concern GNRH neurons that have other functions in the brain than regulation of gonadotropin release.

With our method of in situ hybridization, we cannot distinguish whether the increase in Gnrh mRNA that we observed at different rostro-caudal levels in the brain of breeding and non-breeding females was due to transcriptional or post-transcriptional regulation of the gene, the latter occurring through increased stability of the primary transcript (Gore & Roberts 1995). However, in female rats in proestrus before the LH surge, the GNRH gene is transcriptionally activated as the rise in cytoplasmatic mRNA levels is preceded by a rise in primary transcript (Gore & Roberts 1995). Further, oestradiol treatment of female rats increases the GNRH gene transcription rates in the region of the organum vasculosum of the lamina terminalis (OVLT)/rostral preoptic area before the LH surge, suggesting that transcriptional regulation of the GNRH gene is involved in ovulation (Petersen et al. 1996). ERα-expressing neurons residing within the anteroventral periventricular nucleus (AVPV) are responsible for the oestrogen-positive feedback on GNRH neurons, which is essential for the LH surge (Winternantel et al. 2006). These neurons co-express kisspeptin (Kiss1), which is a potent activator of GNRH release and oestradiol upregulates Kiss1 mRNA expression in this area (for review, see Smith 2013). We have recently shown that breeding female Damaraland mole-rats exhibit significantly higher ERα-expression within the anteroventral periventricular nucleus than non-reproductives (Voigt et al. 2014). Therefore, reduced availability of oestradiol in reproductively suppressed females in this region could lead, due to insufficient Kiss1 expression, to reduced levels of GNRH gene transcription and ultimately to the observed anovulatory phenotype. Further experimental work is required to elucidate the kisspeptin-GNRH pathway and to determine its role in regulating reproductive activity in a mole-rat species with socially induced infertility.
Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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