Breeding status affects the expression of androgen and progesterone receptor mRNA in the brain of male Damaraland mole-rats

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ABSTRACT

The eusocial Damaraland mole-rat (*Fukomys damarensis*) represents an extreme example of reproductive skew, in that reproduction is completely blocked in female subordinate group members. Similarly, male subordinates within the colony show no sexual behaviour. In contrast to females, however, non-reproductive males have functional gonads and do not differ in circulating levels of pituitary hormones and testosterone from reproductive males. Nevertheless, they have reduced numbers of follicle-stimulating hormone receptors in their testes and they produce fewer spermatozoa with a large proportion of immature spermatozoa and precursors. To understand the mechanism of reproductive suppression operational in subordinate males we studied the expression of androgen and progesterone receptor genes in forebrain regions involved in the control of reproductive behaviour in male breeders and non-breeders from intact colonies. While it is well documented that testosterone activates male-typical behaviour, the role of progesterone in this process is less clear as previous studies have produced contradictory results. We found expression of androgen receptor (AR) and progesterone receptor (PGR) genes in several forebrain regions of male Damaraland mole-rats. The distribution of AR in males matches our previous findings in females. This is the first report showing the distribution of PGR in mole-rats. We found PGR in all areas which were also sensitive to androgens and estrogens. Analysis of the optical densities of the AR and PGR hybridisation signal revealed that breeding males had increased expression of AR and PGR compared to non-breeders in most brain regions examined, which include the medial preoptic area, the bed nucleus of the stria terminalis, the ventromedial nucleus of the hypothalamus, the arcuate nucleus and the medial amygdala. These status-related differences were more pronounced for PGR than for AR. This study shows that breeding position affects the neuroendocrine phenotype of male Damaraland mole-rats. Further, it suggests that androgens and progesterone might act synergistically in activating sexual behaviour in males.

KEYWORDS: androgen receptor, progesterone receptor, breeding, *in situ* hybridization, *Fukomys damarensis*, social status

INTRODUCTION

Reproductive suppression is common in group-living mammals, where dominant individuals inhibit the reproductive capabilities of subordinates via behavioural and other interactions (Keller and Reeve 1994). Extremes of high reproductive skew are represented by societies with a single breeding pair and helpers, such as in callitrichid primates (Abbott 1984), mongooses and meerkats (Rasa 1973, Doolan and Macdonald 1997) and mole-rats (Bennett and Faulkes 2000). The mechanism by which reproduction is suppressed in female subordinates varies between species and in extreme instances reproduction is completely blocked by physiological means (Abbott 1984, Bennett 1994). In contrast, among males of most mammalian cooperative breeders the control of reproduction seems to be predominantly behavioural rather than physiological. For example, subordinate male marmosets in a family group show very little sexual behaviour, but will mate when introduced to unfamiliar females (Abbott 1984). Similarly, in mongooses, male subordinates are primarily suppressed from reproduction by behavioural interactions with the dominant male and not by endocrine deficiencies (Creel et al. 1992).

The mole-rats of the family Bathyergidae exhibit a wide range of social behaviour and restricted reproduction has been found in several species (Bennett and Faulkes 2000). Eusocial Damaraland mole-rats (*Fukomys damarensis*), with an average group size of 11 individuals, represent an extreme example of reproductive skew, in that reproduction is completely blocked in female subordinate group members (Bennett 1994, Bennett et al. 1994). This blockade results from an inhibition of ovulation, which is caused by a disruption in the normal GnRH secretion from the hypothalamus (Molteno et al. 2004). Furthermore,

reproductive status and social suppression affects the neuroendocrine phenotype of females with breeders having elevated expression of steroid hormone receptor genes in the preoptic-hypothalamic brain regions compared to non-breeders (Voigt et al. 2014). Among male Damaraland mole-rats within the colony there is no sexual behaviour of subordinates, but when introduced to unfamiliar females, copulatory behaviour occurs rapidly (Bennett et al. 1996). In contrast to females, non-reproductive males have functional gonads. Further, males of different status are similar in endocrine parameters such as plasma concentrations of luteinizing hormone (LH), follicle-stimulating hormone (FSH) and testosterone as well as in the response of the pituitary to an exogenous GnRH challenge (for review, see Bennett 2011). However, non-reproductive males have reduced numbers of FSH receptors in their testes (Nice et al. 2010). Furthermore, they produce fewer spermatozoa with a large proportion of immature spermatozoa and precursors (Maswanganye et al. 1999).

Our recent findings in female Damaraland mole-rats (Voigt et al. 2014), led us to investigate to what extent the difference in reproductive behaviour affects the neuroendocrine phenotype of males in this species. Previous work identified social status-related gross-morphological differences concerning two brain areas involved in reproductive behaviour, the principal nucleus of the bed nucleus of the stria terminalis (BSTp) and the paraventricular nucleus (PVN) being larger in reproductive males than in non-reproductive males (Anyan et al. 2011). However, the properties of steroid-hormone sensitive neuron populations in hypothalamic and limbic regions of males are unknown. Here we focus on the mRNA expression of androgen receptors (AR) and progesterone receptors (PGR). It has long been established that androgens are essential in the control of male sexual behaviour (Beach 1942, Baum and Vreeburg 1973) and spermatogenesis (Steinberger 1971). Less clearly understood is the role of progesterone in males, as previous studies have produced contradictory results. While pharmacological doses of progesterone have been found to have anti-androgenic effects in male mice and guinea pigs (Diamond 1966, Erpino 1973, Connolly et al. 1988) low

dosages of progesterone act facilitatory on male sexual behaviour (Witt et al. 1995, Phelps et al. 1998). For example, exogenous progesterone, producing plasma levels in the physiological range, is able to fully restore sexual behaviour in castrated rats even in the absence of other gonadal hormones and treatment with a progesterone antagonist abolishes these effects (Witt et al. 1995). Furthermore, testosterone treatment only completely restores sexual behaviour when administered together with progesterone indicating a synergizing action of both hormones (Witt et al. 1995). Finally, male progesterone receptor knockout (PRKO) mice exhibit a reduction in mounting frequency and are less responsive to exogenous testosterone (Phelps et al. 1998). Progesterone receptors (PGR) have been found in several rodent forebrain regions that are also sensitive to androgens and estrogens and exhibit high aromatase activity (Simerly et al. 1990, Lauber et al. 1991, Roselli et al 1985).

These data suggest that the difference in mating behaviour between reproductive and non-reproductive male Damaraland mole-rats could potentially result from a differential activation of PGR-mediated mechanisms. We therefore analysed, by using *in situ* hybridization, the mRNA expression levels of both AR and PGR in hypothalamic and limbic brain regions involved in sexual behaviour of breeder and non-breeder males kept in their natal colonies.

MATERIALS AND METHODS

Animals

In the current study, adult male Damaraland mole-rats (*Fukomys damarensis*) were used. Data were obtained from nine reproductive males (breeder) and nine non-reproductive males (non-breeder). Complete colonies of mole rats were captured between April and June 2012 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. Average colony size was 9.3±1.2 individuals. Prior to sacrifice, animals were housed for a maximum period of

10 weeks at the University of Pretoria under 12L:12D cycle at 25°C in plastic containers (1.0x0.5x0.5m) containing wood shavings and they were fed on sweet potato, gem squash and apples. In each colony, the reproductive status of all individuals was determined. Queens or breeders could be readily distinguished from non-reproductive females by the presence of a perforate vagina and prominent teats. Reproductive males were identified by their bulging abdominal testes, heavily stained cheeks around the mouth and by observing copulations with the reproductive female. Non-reproductive males are not involved in copulations, do not have bulging testes or stained cheeks (Bennett and Jarvis 1988). Animals for the breeder and non-breeder groups were kept in their original colonies until sacrifice. Body mass of all animals was recorded to the nearest gram. At the time of brain collection, testes mass and brain mass was recorded to the nearest milligram. All experimental procedures were approved by the University of Pretoria Animal Ethics Committee (EC0003-12).

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 hormone assays were conducted. Plasma samples were assayed for testosterone using the commercially available coated tube assay kit (Coat-a-Count, Diagnostic Products Corporation, Los Angeles, CA) as described before (Bennett et al. 1994, Voigt et al. 2014). All samples were assayed in duplicate and the intra-assay coefficient of variation was 4.3% for testosterone. Blood samples to analyze circulating progesterone levels were not available.

Brain histology

Mole-rats were killed by decapitation using a small mammal commercially available guillotine, 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 the plane of the rat brain atlas (Paxinos and Watson 2007). Sections were mounted onto Superfrost Plus slides (Menzel-Gläser, Braunschweig, Germany) in five different series, so that one series of slides contained a section every 150 µm. *In situ* hybridization was carried out on adjacent series of sections for the localization of androgen receptor mRNA (AR) and progesterone receptor mRNA (PGR).

Cloning of cDNA probes

The cloning of a partial Damaraland mole-rat AR cDNA [GenBank: KF574039] was done in our laboratory and has been described previously (Voigt et al 2014). Based on sequence information available from other species, PCR was used to amplify a fragment of the PGR gene from Damaraland mole-rat. Total RNA was extracted from the 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. Degenerate primers were designed and the forward primer was 5'-CTGGSATGGTCCTTGGAGGT-3' and the reverse primer was 5'-CAAACTCCTGYGGKATYTGC-3' (degenerate bases were S= C and G, Y=C and T and K= G and T). The cloned PGR sequence [GenBank: KJ634483] is 525bp in length and shows 83% homology with mouse progesterone receptor [GenBank: NM_008829.2]. PCR was carried out for 40 cycles by using the following parameters: 94°C for 1 minute, 58°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. Two isoforms of PGR are described, a larger PGR-B form and a shorter PGR-A form. Both isoforms, differing only at the N-terminal region, are encoded by a single

gene, and are under the control of two distinct promotors (Kastner et al. 1990). Our PGR sequence comprises the hormone-binding domain, therefore, the riboprobe prepared from our PGR sequence detects both isoforms.

In situ hybridization

The expression of AR and PGR in brain sections was detected with antisense RNA probes labeled with ^{35}S-CTP as described before (Voigt et al. 2014). Labeling of the probes with ^{35}S-CTP (1250 Ci/mmol; Perkin Elmer, Rodgau, Germany) was performed using the Riboprobe System (Promega). For signal detection, sections were exposed to autoradiographic film (Kodak Biomax MR, Rochester, NY, USA) for 12 (PGR) and 14 weeks (AR) respectively. Brain sections from both groups of males were run through the entire procedure at the same time and placed on each autoradiographic film to avoid any possible effect of small differences in procedures on the observed group differences. Control sections processed with the sense probes were obtained from two male mole-rats and were labeled by the same procedure as described above. Autoradiograms from these sections showed no signal. These control data will therefore not be discussed below.

Data analysis

Images from autoradiograms were scanned with an Epson Perfection V750 Pro scanner connected to a PC running the image analysis software Image J 1.43u (NIH, USA; see http://rsb.info.nih.gov/ij/). Before acquisition the system was calibrated by 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 Image J [y=d+(a-d)/(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 never reached saturation. Regions of interest in each section (defined by the presence of a denser signal

density than surrounding areas) were delineated on screen with the computer mouse and their average optical density (OD) was calculated by built-in functions of the software. Background optical density of the film was measured in a rectangular area ($1mm^2$) in the same image immediately ventral to the brain section of interest. Final OD measurements were obtained by subtracting the film background OD value from the OD value of the region of interest and represent the average measurement from both hemispheres. Brain regions were identified using the atlases of the rat (Paxinos and Watson 2007) and the naked mole-rat (Xiao, Levitt, Buffenstein 2006). The region of interest was measured in parallel sections hybridized with AR and PGR. For quantification of the mRNA expression levels, three adjacent sections along the rostro-caudal axis were measured in each selected brain region.

Statistical analysis

Statistical analyses were carried out using Systat 13.0 (Systat Software, Point Richmond, CA, USA). Data are presented as means ± SEM. Hormone data were analyzed by non-parametric statistics. Morphological differences between groups were analysed with t-tests. Repeated measures ANOVAs were used to analyse the AR and PGR mRNA expression in the brain, with status as independent factor and brain regions as repeated factor. They were followed, when appropriate, by *post-hoc* t-tests with sequential Bonferroni correction. All tests were two-tailed, and the significance level was set at $p<0.05$.

RESULTS

Body mass, gonad mass and circulating testosterone levels

Reproductive males were significantly heavier than non-reproductive males (148.5±7.8g vs. 124.7±5.6g; t=2.49, df=16, p=0.024). To control for the effects of body size, testes mass was corrected for body mass (mg/g body mass). Both groups of males did not differ in the corrected testicular mass (t=0.10, df=16, p=0.918). Further, no significant group differences

were found in brain mass (t=1.46, df=11.4, p=0.171). Plasma testosterone levels were similar in breeders (3.1±1.6 nmol/l, range: 0.0-6.2 nmol/l) and non-breeders (2.9±1.2 nmol/l, range: 0.0-11.4 nmol/l; Mann Whitney U=31.5, p=0.453; Figure 1).

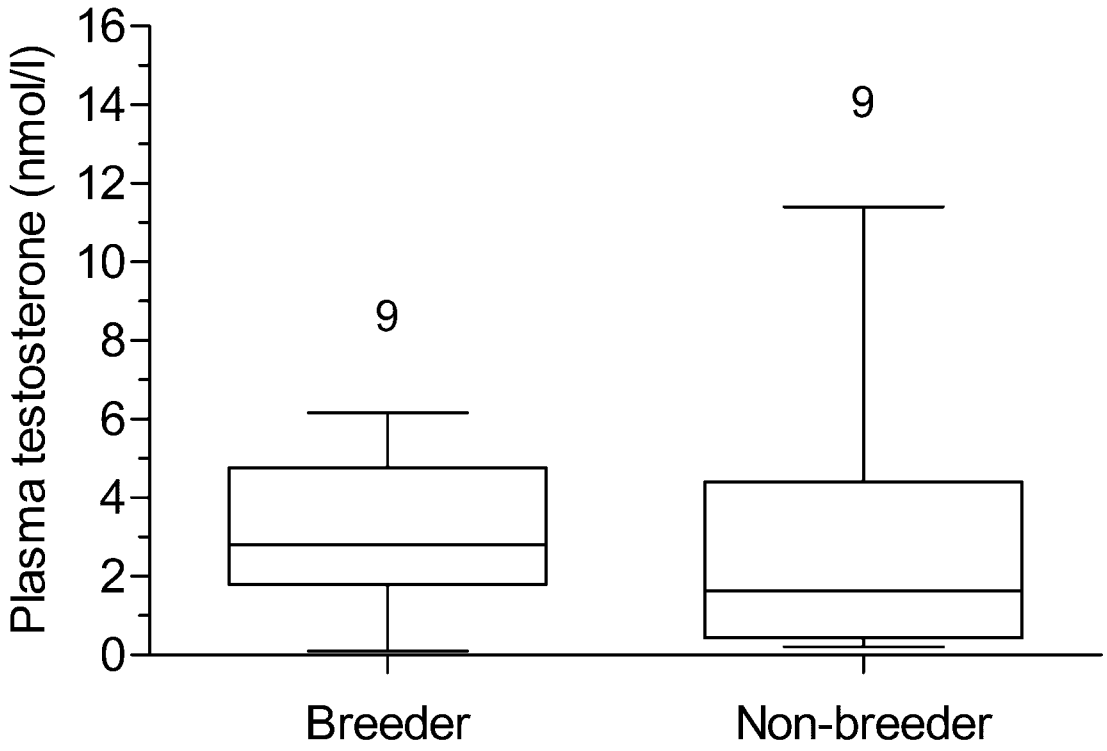

Figure 1. **Plasma testosterone levels do not differ between reproductive and non-reproductive males.** Median, lower and upper quartiles and range of plasma testosterone levels of breeding and non-breeding male Damaraland mole-rats.

Gene expression related to reproductive status

We quantified the mRNA expression levels of AR and PGR in the AVPV, MPOA, ARC, BSTp, MeA and the ventrolateral part of the VMH (VMHvl; Figure 2). Measurement of the average optical density in the six different cell groups revealed significant group differences (Figure 3). The analysis of the AR hybridisation signal in all nuclei revealed an overall effect of status ($F_{1,16}$=9.78, p=0.006), no significant effect of the brain region ($F_{5,80}$=1.72, p=0.14) and no significant interaction between these two factors ($F_{5,80}$=0.51, p=0.77). *Post-hoc*

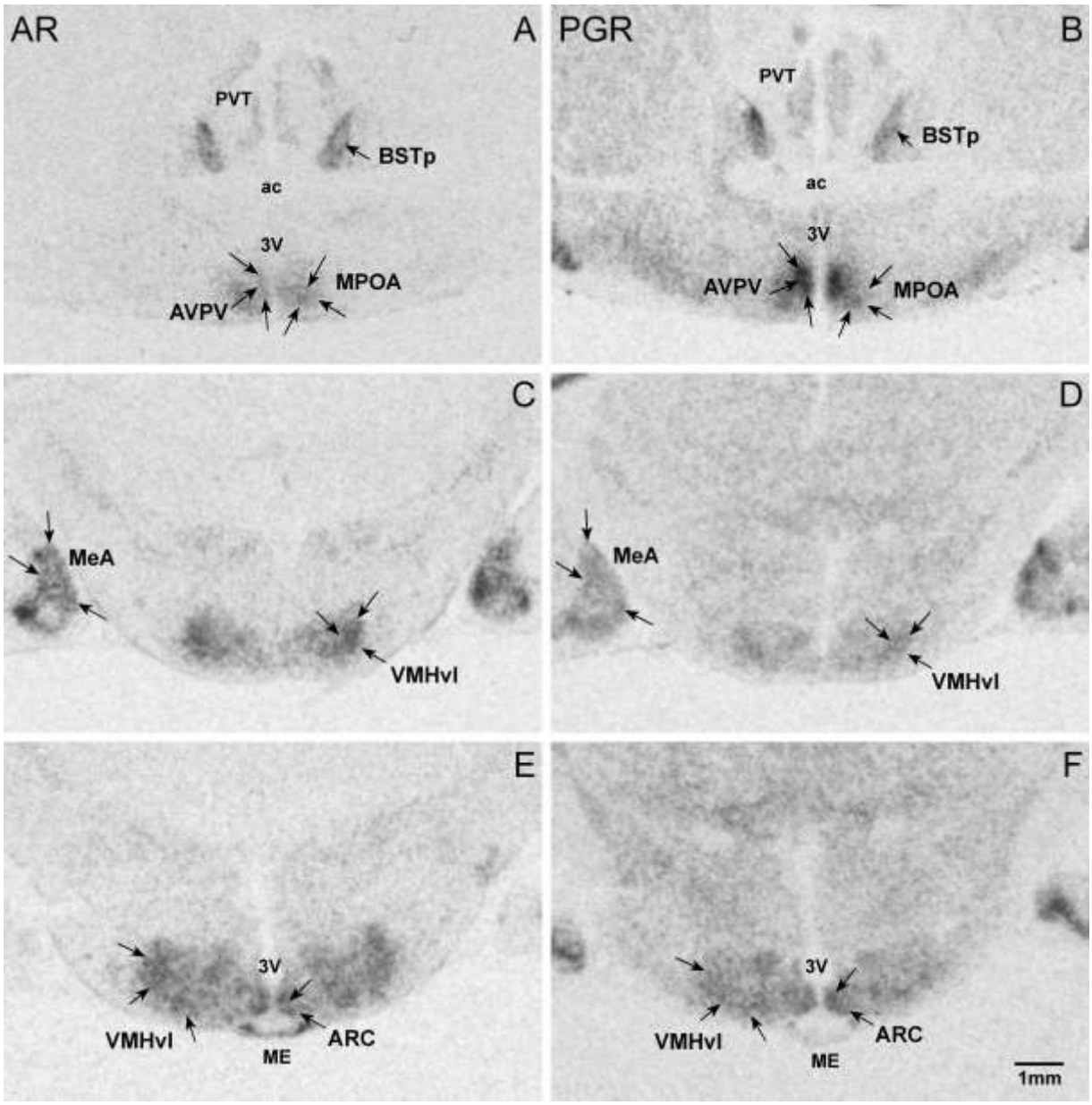

Figure 2. **Expression pattern of AR and PGR mRNA in forebrain regions of breeding male Damaraland mole-rats.**

Brightfield photomicrographs of autoradiograms from coronal sections through the mole-rat brain illustrating the distribution of AR and PGR mRNA visualized by *in situ* hybridization. Panels A, C and E illustrate sections from three male breeders at the level of the preoptic area and caudal hypothalamus processed for AR mRNA. Panels B, D and F show adjacent sections from the same males, respectively, processed for PGR mRNA. Abbreviations: ac, anterior commissure; ARC, arcuate nucleus; AVPV, anteroventral periventricular nucleus; BSTp, bed nucleus of the stria terminalis, principal nucleus; ME, median eminence; MeA, medial amygdaloid nucleus; MPOA, medial preoptic area; PVT, paraventricular nucleus of the thalamus; VMHvl, ventromedial hypothalamic nucleus, ventrolateral part; 3V, third ventricle.

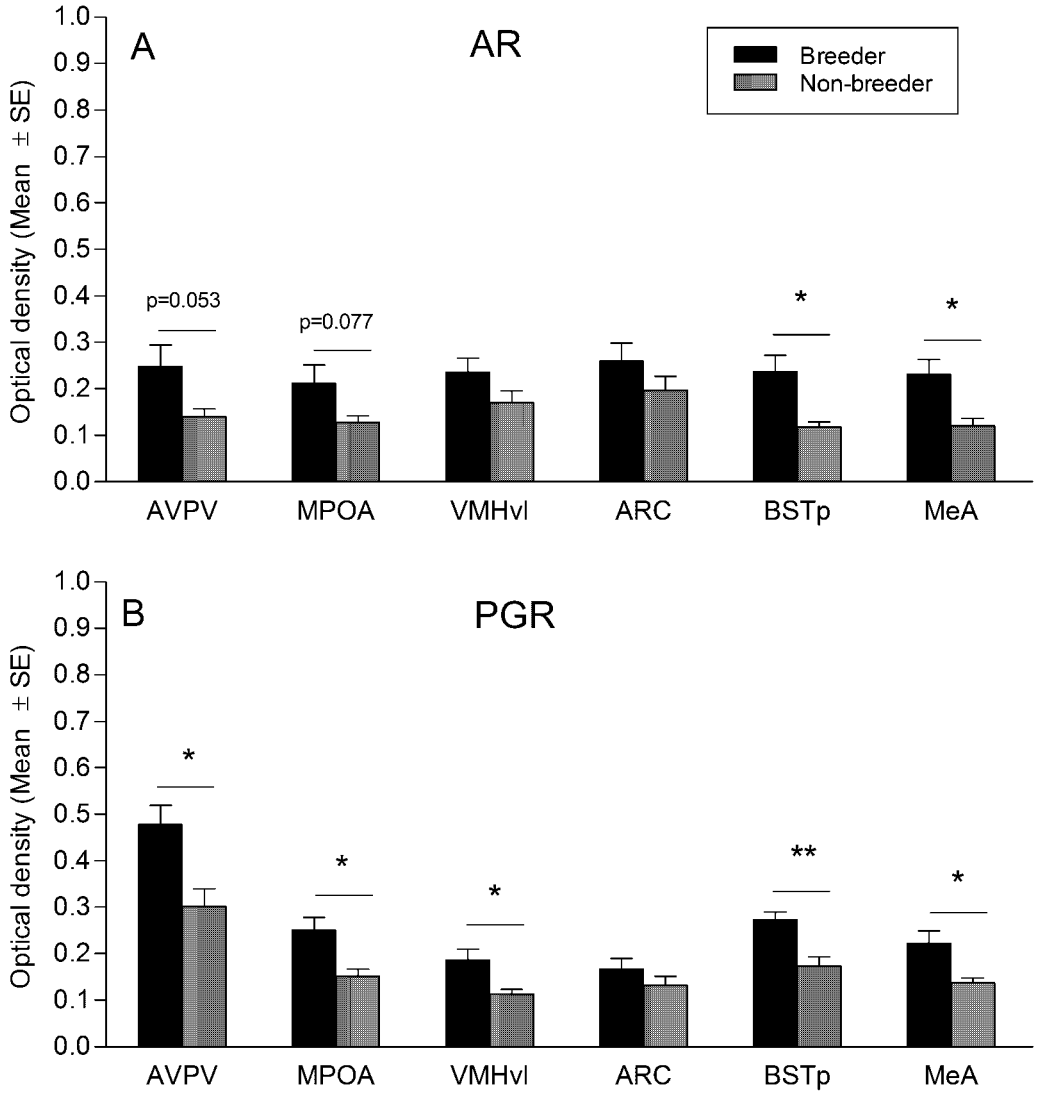

Figure 3. **Breeding status affects the mRNA expression levels of AR and PGR.**

Average optical density of the hybridization signal for AR (A) and PGR (B) for different forebrain regions of male breeders and non-breeders. Symbols above the bars indicate the results of the *post hoc* t-tests with sequential Bonferroni correction comparing both groups of males for different brain regions.** $p < 0.01$; * $p < 0.05$.

Bonferroni tests identified a significant difference between both groups of males in the MeA and BSTp, with breeders having a higher AR expression than non-breeders. The AR expression was also elevated in AVPV and POM of breeders compared to non-breeders but

just failed to be significant (AVPV: p=0.053, POM: p=0.077; Figure 3A). The analysis of the PGR hybridisation signal in all nuclei revealed an overall effect of status ($F_{1,16}$=14.67, p=0.001), a significant effect of the brain region ($F_{5,80}$=45.85, p=0.0001) and a significant interaction between these two factors ($F_{5,80}$=3.05, p=0.048; Figure 2B). *Post-hoc* Bonferroni tests identified a significantly higher PGR expression in all brain regions except for ARC of breeders than non-breeders (Figure 3B).

DISCUSSION

Here we report for the first time the mRNA expression patterns of AR and PGR in the brain of male Damaraland mole-rats. While female subordinates, in the presence of the queen, experience strong physiological suppression to the extent of being anovulatory, reproductive suppression in males seems to be less pronounced (Bennett 2011). By using *in situ* hybridization, we identify status-related neural differences in brain areas that are implicated in the regulation of reproductive behaviour.

Testosterone levels

Males of different breeding status in our study did not differ in circulating plasma testosterone levels, which agrees with previous findings in this species Bennett 1994, Maswanganye et al. 1999). Furthermore, earlier work showed that reproductive and non-reproductive males have similar plasma LH and FSH levels and a similar pituitary response to exogenous GnRH challenge (Bennett 2011). In stark contrast is the pattern found in eusocial naked mole-rats. Gonadal function of non-reproductive males is inhibited by a reduced pituitary secretion of LH (possibly by disruption of GnRH secretion), which results in lower circulating testosterone levels as well as inhibited spermatogenesis (Faulkes et al. 1991, Faulkes et al. 1994). Although the endocrine parameter obtained from Damaraland mole-rats suggests that the pathway of the hypothalamic-pituitary-gondadal axis (HPG axis) is not disrupted in non-

reproductive males, Nice et al. (2010) reported a significant reduction of FSH receptors (FSH-R) within the testicular tissue of non-reproductive males. Furthermore, the release from social suppression caused a rapid increase in FSH-R immuno-positive cells. Therefore, the oligospermic and azoospermic conditions found in non-reproductive males (Maswanganye et al. 1999) could result from an inhibition of the FSH signaling pathway. FSH-R mutant mice were found to be fertile, but to display spermatogenic failures (Krishnamurthy et al. 2000, Abel et al. 2008).

Breeding status affects mRNA expression of AR and PGR

The present study in male Damaraland mole-rats identifies AR in the same brain regions as previously described for females of this species (Voigt et al. 2014) and agrees with the distribution reported from *in situ* hybridization and immunocytochemical studies in other species (Simerly et al. 1990, Handa et al. 1996, Sar et al. 1990). Similar to females, AR mRNA expression in males was affected by the animal's reproductive status. Breeders had increased AR levels in several of the regions examined compared to non-breeders, however, the extent was less pronounced than in females. We found a significant increase in AR levels of breeders in BSTp and MeA and those in AVPV and MPOA just failed to be significant while VMHvl and ARC showed no status-related differences. As breeders and non-breeders did not differ in circulating testosterone levels, the elevated AR mRNA expression of breeders does not seem to result from autologous feedback mechanisms. One possibility is that AR in breeders is upregulated by estrogens as has been shown for the MPOA of the male rat (Handa et al. 1996). The regulation of male sexual behaviour is controlled by the synergistic actions of androgens and estrogens. Moreover, AR- and estrogen receptor (ER)-containing neuron populations in hypothalamic and limbic regions of the rodent brain have overlapping distributions (Greco et al. 1998). Lesion studies confirmed that the MPOA and the mediobasal hypothalamus (MBH) are crucial for mediating male mating behaviour (Phoenix 1961,

Heimer and Larsson 1967). However, in our study we observe the largest increase in AR in the BSTp and MeA, which are part of the pathway that relays olfactory information related to reproduction (Simerly et al. 1989). Similar to our findings in female Damaraland mole-rats, our present data from males do not match previous reports in naked mole-rats showing that AR immunoreactivity is elevated in the brain of non-breeders compared to breeders regardless of sex (Holmes et al. 2008). However, the studies differ in regard to the social environment of the breeding individuals. While we sampled socially dominant individuals from intact colonies in the wild, Holmes et al generated breeders by pairing subordinate animals and therefore removed the influence of social dominance on these individuals.

Besides elevated AR levels, breeding male Damaraland mole-rats were found to have elevated PGR mRNA expression compared to non-breeders in nearly all regions analyzed. This status-related difference of PGR was more pronounced than that of AR. The observed upregulation of PGR in breeder males suggest that the activation of this pathway is important for reproductive behaviour and thus, PGRs are functionally linked to the neural areas controlling male sexual behaviour. The distribution of PGR in this study generally matches the distribution of the gene and its corresponding protein observed in other species (Lauber et al. 1991, Blaustein et al. 1988, Williams et al. 2013) and in Damaraland mole-rats, PGR is present in all areas, which also express AR, ERα and aromatase (Voigt et al. 2014).

First evidence for a stimulatory effect of progesterone on male sexual behaviour came from studies in whiptail lizards. Exogenous progesterone induces male-like pseudosexual behaviour in the parthenogenetic species *Cnemidophorus uniparens* and it reinstates courtship and copulatory behaviour in castrated males of the sexually reproducing species *C. inornatus* (Grassman and Crews 1986, Lindzey and Crews 1986). Further, testosterone and progesterone synergize in the activation of male sexual behaviour (Lindzey and Crews 1992). Moreover, androgens upregulate progesterone receptors in gonadectomized whiptail lizards (Crews et al. 1996). In male rodents, the activation of copulatory behaviour by testosterone involves the

modulation of dopamine release in the preoptic area, which in turn is regulated by nitric oxide. (for review, see Hull and Dominguez 2006). In females, nitric oxide is involved in progesterone-mediated lordosis behaviour (Mani et al. 1994). Based on evidence from male whiptail lizards, where nitric oxide is upregulated by both androgens and progesterone (O'Connell et al. 2011), it seems possible that similar mechanisms are present in male rodents to facilitate copulatory behaviour since progesterone stimulates male sexual behaviour in rats and mice (Witt et al. 1995, Phelps et al. 1998). Therefore, while in female rodents estrogens and progesterone both upregulate nitric oxide, in males androgens and progesterone might act in synergism to activate sexual behaviour. The reduced PGR levels in non-breeder males in our study suggest that this pathway is inhibited either by reduced circulating progesterone levels or by other mechanisms which affect ligand-independent activation of the progesterone receptor.

The highest PGR levels were detected in the AVPV. The PGR levels in the MPOA, BSTp and MeA were still higher than those in VMH and ARC. The pronounced elevation of PGR in the AVPV relates to its importance within the circuit controlling mating behaviour. AVPV is part of a pathway that processes pheromonal stimuli. This nucleus receives a strong projection from the BSTp, which in turn relays olfactory information received from the medial amygdala to hypothalamic nuclei. This projection from BSTp to AVPV is much denser in males than in females (Hutton et al. 1998). Moreover, AVPV neurons directly innervate GnRH neurons.

In conclusion, our data provide evidence that breeding status affects the neuroendocrine phenotype of male Damaraland mole-rats. In particular, they suggest that AR- and PGR-mediated pathways are operative in order to activate male sexual behaviour. Moreover, the AVPV seems to play a key role in this process.

COMPETING INTERESTS

The authors declare that they have no competing interests.

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