Reproductive status-dependent dynorphin and neurokinin B gene expression in female Damaraland mole-rats

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Short title: *Pdyn* and *Tac3* mRNA expression in Damaraland mole-rats

Highlights

• Differential distribution of dynorphin and NKB within the forebrain.
• Reproductive suppression associated with altered dynorphin and NKB gene expression in the arcuate nucleus.
• Elevated dynorphin and reduced NKB gene expression within the arcuate nucleus of suppressed females.

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. The blockade

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of reproduction results from an inhibition of ovulation, which is 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. The neuropeptides dynorphin and neurokinin B are expressed together with kisspeptin in a subpopulation of neurons in the arcuate nucleus (ARC). This neuron population is termed KNDy neurons and is considered to constitute the GnRH pulse generator. To assess whether dynorphin (encoded by the *Pdyn* gene) and neurokinin B (NKB, encoded by the *Tac3* gene) are involved in the mechanism of reproductive suppression we investigated the distribution and gene expression of *Pdyn* and *Tac3* by means of *in situ* hybridisation in wild-caught female Damaraland mole-rats with different reproductive status. In both reproductive phenotypes, substantial *Pdyn* expression was found in several brain regions of the telencephalon including the cerebral cortex, the striatum, the hippocampus, the amygdala and the olfactory tubercle. Within the hypothalamus *Pdyn* expression occurred in the paraventricular nucleus, the dorsomedial nucleus, the supraoptic nucleus, the ventromedial nucleus and the ARC. Prominent *Tac3* expression was found in the habenula, the bed nucleus of the stria terminalis, the cerebral cortex, the striatum, the hippocampus, the amygdala, the dorsomedial nucleus, the ARC and the lateral mammillary nucleus. Quantification of the gene expression levels in the ARC revealed decreased *Pdyn* and increased *Tac3* expression in breeding compared to nonbreeding females. This suggests that both neuropeptides play a role in the regulation of reproduction in Damaraland mole-rats. Their exact role in mediating the inhibition of GnRH release in nonbreeding females remains to be determined.

1. Introduction

The regulation of reproductive function in mammals is tightly linked to the secretion of gonadotropin-releasing hormone (GnRH) from the hypothalamus into the portal vasculature, which in turn stimulates the release of the gonadotropins LH and FSH from the anterior pituitary (Pohl and Knobil 1982). GnRH release is governed by two different mechanisms, the pulsatile and the surge mode. The former, the GnRH pulse generator, regulates pulsatile LH secretion, which is critical for gonadal development and normal regulation of the ovarian cycle. In contrast, the surge mode is activated by increasing amounts of ovarian oestradiol during the preovulatory period and generates a massive release of GnRH and thus LH to induce ovulation. These different modes of secretion underlie different feedback effects of gonadal steroids and are controlled by different neural networks (for review, see Goodman 2015; Herbison 2015). The RFamide peptide kisspeptin (Kiss1) has been identified as a strong activator of GnRH neurons (Gottsch et al. 2004; Navarro et al. 2004; Messager et al. 2005) and it is now considered a key regulator of GnRH and LH secretion (for review, see Simonneaux 2018). *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, the kisspeptin neurons in the AVPV are thought to mediate the oestrogen (E2)-induced GnRH/LH surge while those in the ARC control GnRH pulses through negative feedback action of E2. This positive and negative feedback regulation is mediated by oestrogen receptor α (ER α), which is coexpressed in both populations of Kiss1 neurons (Smith et al. 2005). In rats and mice, 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 (Clarkson et al. 2006; Adachi et al. 2007). 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 (Bosch et al. 2012). In relation,

ARC kisspeptin neurons in ewes are implicated in the control of both pulsatile and surge secretion of GnRH (Merkley et al. 2012).

In contrast to the rostral kisspeptin neuron population, the ARC kisspeptin neurons colocalize two additional neuropeptides, neurokinin B (NKB; encoded by the *Tac3* gene) and the endogenous opioid peptide dynorphin (encoded by the *Pdyn* gene), which are strongly implicated in the control of GnRH release. By means of multiple-label immunocytochemistry, the colocalization was originally discovered in sheep and later on shown in other species (Goodman et al. 2007; Navarro et al. 2009; Ramaswamy et al. 2010; True et al. 2011). The neuron population was termed as "KNDy" (kisspeptin/neurokinin B/dynorphin) neurons and is thought to constitute the GnRH pulse generator responsible for generating the pulsatile release of GnRH. The model proposes that NKB is the signal responsible for pulse onset. The release of NKB leads through a positive feedback loop to increased KNDy neural activity, kisspeptin release and GnRH secretion. Most likely, kisspeptin acts directly to drive GnRH neuron activity as the majority of KNDy neuron projections are to the mediobasal hypothalamus where they directly contact GnRH cell bodies. About 5 min after the onset of the GnRH pulse the release of dynorphin terminates the kisspeptin release and the GnRH pulse (for review, see Moore et al. 2018). In females, there is approximately one pulse per hour during the follicular phase and one pulse every 3-4 hours during the luteal phase of the ovarian cycle (Herbison 2018).

In group-living mammals, reproductive suppression, where dominant individuals inhibit the reproductive capabilities of subordinates via behavioural and other interactions, is commonly found (Keller and Reeve 1994). 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 (*Heterocephalus glaber*) or Damaraland mole-rats (*Fukomys damarensis*; Bennett and Faulkes 2000). 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 anterior pituitary of such females is desensitized. (for review, see Bennett et al. 1999).

Damaraland mole-rats 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. This blockade of reproduction results from an inhibition of ovulation, which appears to be caused by inadequate secretion of LH from the pituitary. Such females were found to have 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). Moreover, GnRH gene expression level in the rostral preoptic area of nonbreeders is reduced compared to breeders (Voigt and Bennett 2017).

Based on work in rats and mice it was proposed that the ARC kisspeptin neurons participate in the control of GnRH pulses and those in the rostral hypothalamus are responsible for the E2-induced GnRH surge. Across species, there is substantial support for the former, but data supporting the latter are limited. In guinea pigs, sheep and primates the rostral kisspeptin neuron population is much smaller than in rats and mice and there is evidence that in the former mentioned species the ARC kisspeptin neurons contribute to both the surge and pulsatile GnRH release (for review, see Goodman and Lehman 2012). In relation to this finding, in female Damaraland mole-rats, only few kisspeptin neurons were detected in the AVPV while a substantial kisspeptin neuron population was found in the ARC. Furthermore, breeders were found to have significantly higher *Kiss1* expression in this nucleus than nonbreeders (Voigt and Bennett 2018a). Based on the "KNDy hypothesis", where ARC neurons expressing kisspeptin, NKB and dynorphin interact to generate GnRH pulses, the neuropeptides NKB and dynorphin should show a reproductive status-dependent expression pattern similar to *Kiss1*. In particular, we predict that breeders possess increased NKB and decreased dynorphin gene expression in the ARC compared to nonbreeders. In the present study, we used *in situ* hybridization to

determine the distribution and gene expression level of *Tac3* and *Pdyn* in the forebrain of reproductively active and reproductively suppressed females.

2. Materials and Methods

2.1. Animals

Adult female Damaraland mole-rats (*Fukomys damarensis*) were used in this study. For *Tac3*, data were obtained from eight breeders and eight nonbreeders and for *Pdyn*, data were obtained from eight breeders and seven nonbreeders. The females came from colonies that were captured between April and July 2015. Mole-rats were captured 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 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 they were fed on sweet potato, gem squash and apples. In each colony, the reproductive status was determined for all adults. Breeding females (syn. queens) could be readily distinguished from non-reproductive females by the presence of a perforate vagina and prominent teats. None of the females were pregnant or lactating 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 (EC003-12).

2.2. 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 20 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 situ* hybridization was carried out on adjacent series of sections for the localization of prodynorphin (*Pdyn*) and tachykinin 3 (*Tac3*) mRNA. Brain mass did not differ in relation to social status ($t = 1.24$, *df* = 16, *P* = 0.23). The same brains were used in a previous study investigating the expression of the prolactin receptor (Voigt and Bennett 2018b).

2.3. Cloning of cDNA probes

Based on sequence information available from other mole-rat species, PCR was used to amplify a fragment of the *Pdyn* gene. 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. The forward primer was 5'-AATCCCCTGATTTGCTCTCTGG-3' and the reverse primer was 5'-CGAGTAGGCATTGGGGTCTTC-3'. PCR was carried out for 40 cycles by using the following parameters: 94°C for 1 minute, 60°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 *Pdyn* sequence [GenBank: MK910765] is 600bp in length and shows 73% homology with rat prodynorphin [GenBank: NM_019374.3] and 86% homology with guinea pig prodynorphin [GenBank: NM_001172997.1]. For *Tac3,* 3' RACE was carried out using the SMARTer RACE 5'/3' kit (Clontech Laboratories). The preparation of 3'RACE cDNA and PCR was done according to the manufacturer's instructions. The gene-specific antisense primer was 5'-GAGGAGTCACAGGAGCAGGTGGTG -3'. Subsequently, nested PCR with a second gene-specific primer was performed from the first PCR product. The primer for the nested PCR was

5'-GAGGGATTGCTGAAAGTGCTGAGCAAGG-3'. The cloned *Tac3* sequence [GenBank: MK910766] is 491bp in length and shows 83% homology with rat tachykinin 3 [GenBank: NM_019162.2].

2.4. In situ hybridization

The expression of *Pdyn* and *Tac3* 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). Our *in situ* hybridization procedure followed a previously published protocol (Whitfield et al. 1990) with modifications as described elsewhere (Gahr and Metzdorf 1997). For signal detection, sections were exposed to autoradiographic film (Kodak Biomax MR, Rochester, NY, USA) for 4 weeks. Brain sections from reproductive and non-reproductive animals 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 for each gene were obtained from two female 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. To obtain more detailed anatomical information the brain sections of two females were processed with emulsion autoradiography. 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 were counterstained with thionin.

2.5. 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 represents the average measurement from both hemispheres. Brain regions were identified using the atlas of the rat (Paxinos and Watson 2007). Before analysis, sections of all animals were anatomically matched according to the anterior commissure (AC) at the level where it reaches its largest extension. For quantification of the mRNA expression levels in the ARC, adjacent sections along the rostro-caudal axis were measured throughout the extent of the labelling that was visible. Additionally, we analysed expression levels for both genes in an adjacent region, which served as a control. We measured three consecutive sections in VMHdm for the expression of *Pdyn* and in DMH for the expression of *Tac3*.

2.6. Statistical analysis

Statistical analysis was carried out using JMP software. Data are presented as means ± SEM. *G*ene expression in the ARC was analysed by using a REML-model with reproductive status (breeder, nonbreeder) and gene (*Pdyn, Tac3*) as fixed factors and animal ID as random factor. Post hoc analysis were performed with the 'test slices' comparison in JMP. All tests were two-

tailed and the significance level was fixed at p<0.05. Gene expression in the control regions (VMHdm, DMH) between the groups was analysed separately for each gene with a t-test.

3. Results

3.1. Distribution of Pdyn and Tac3 mRNA

Pdyn expression was most prominent in several areas of the telencephalon and diencephalon (Fig. 1, 2). In the telencephalon these include the cerebral cortex, the caudate-putamen, the hippocampus, the olfactory tubercle and the amygdala. Within the diencephalon, the most prominent *Pdyn* expression was found in the paraventricular nucleus, the supraoptic nucleus, the dorsomedial part of the ventromedial nucleus and in the ARC. Less strong expression was found in the dorsomedial hypothalamic nucleus and in the premammillary nucleus. Prominent *Tac3* expression in the telencephalon was found in the cerebral cortex, the hippocampus, the bed nucleus of the stria terminalis and in the amygdala (Fig. 1, 2). Expression in the caudate-putamen was low. Within the diencephalon, the strongest hybridization signal of *Tac3* was found in the medial habenula. Strong expression was also detected in the dorsomedial hypothalamic nucleus, the ARC and the lateral mammillary nucleus.
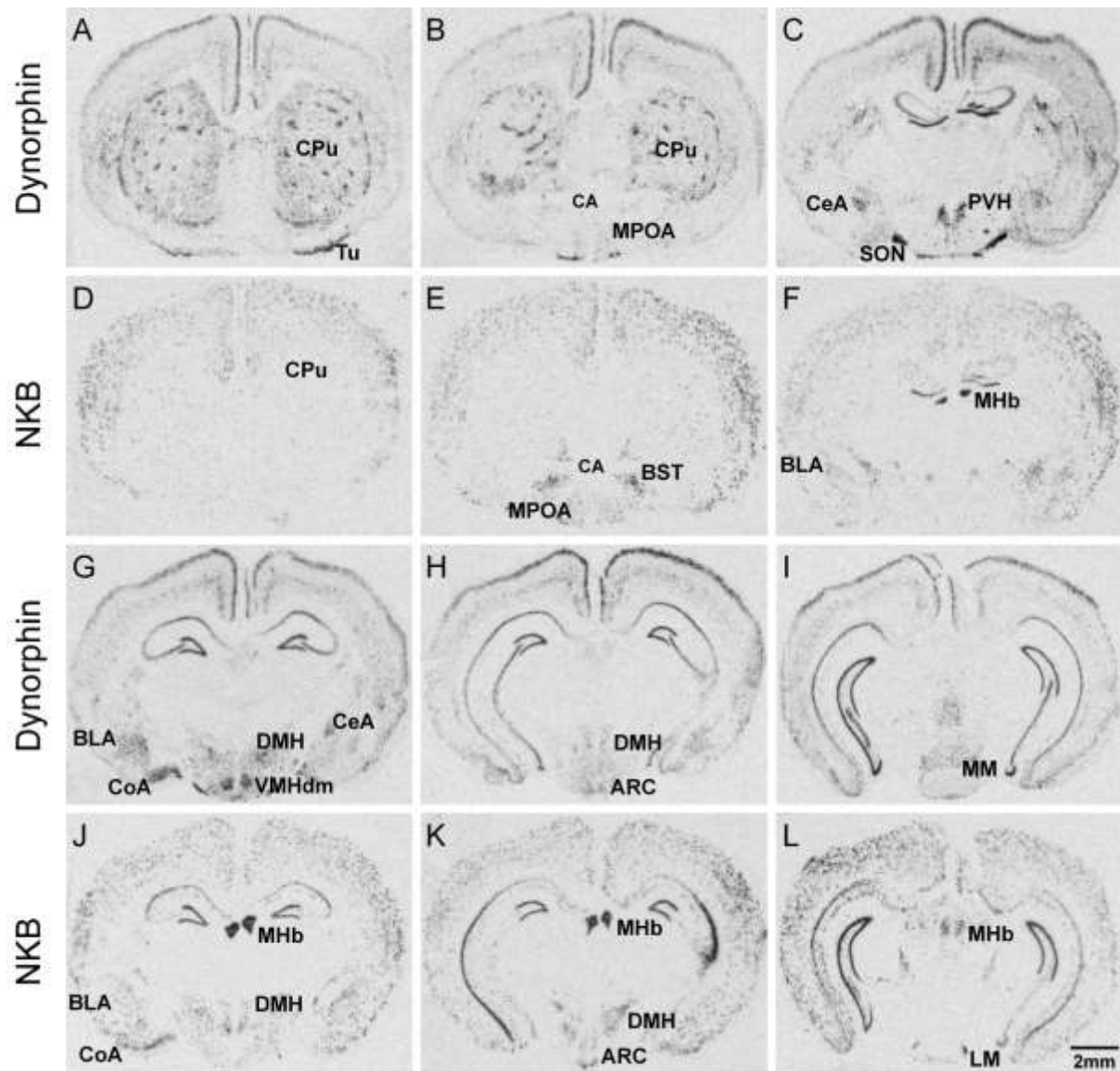
Fig. 1. Brightfield photomicrographs of autoradiograms from coronal sections through the mole-rat brain illustrating the distribution of *Pdyn* mRNA (dynorphin, Panels A–C, G–I) and *Tac3* mRNA (NKB, Panels D–F, J–L) visualized by *in situ* hybridization. Panels illustrate sections from a nonbreeding female. Abbreviations: ARC, arcuate nucleus; BLA, basolateral nucleus of the amygdala; BST, bed nucleus of the stria terminalis; CA, anterior commissure; CeA, centromedial nucleus of the amygdala; CoA, cortical nucleus of the amygdala; CPu, caudate-putamen; DMH, dorsomedial nucleus; MM, medial mammillary nucleus; LM, lateral mammillary nucleus; MHb, medial habenula; MPOA, medial preoptic area; PVH, paraventricular nucleus; SON, supraoptic nucleus; Tu, olfactory tubercle. VMHdm, ventromedial hypothalamic nucleus, dorsomedial part
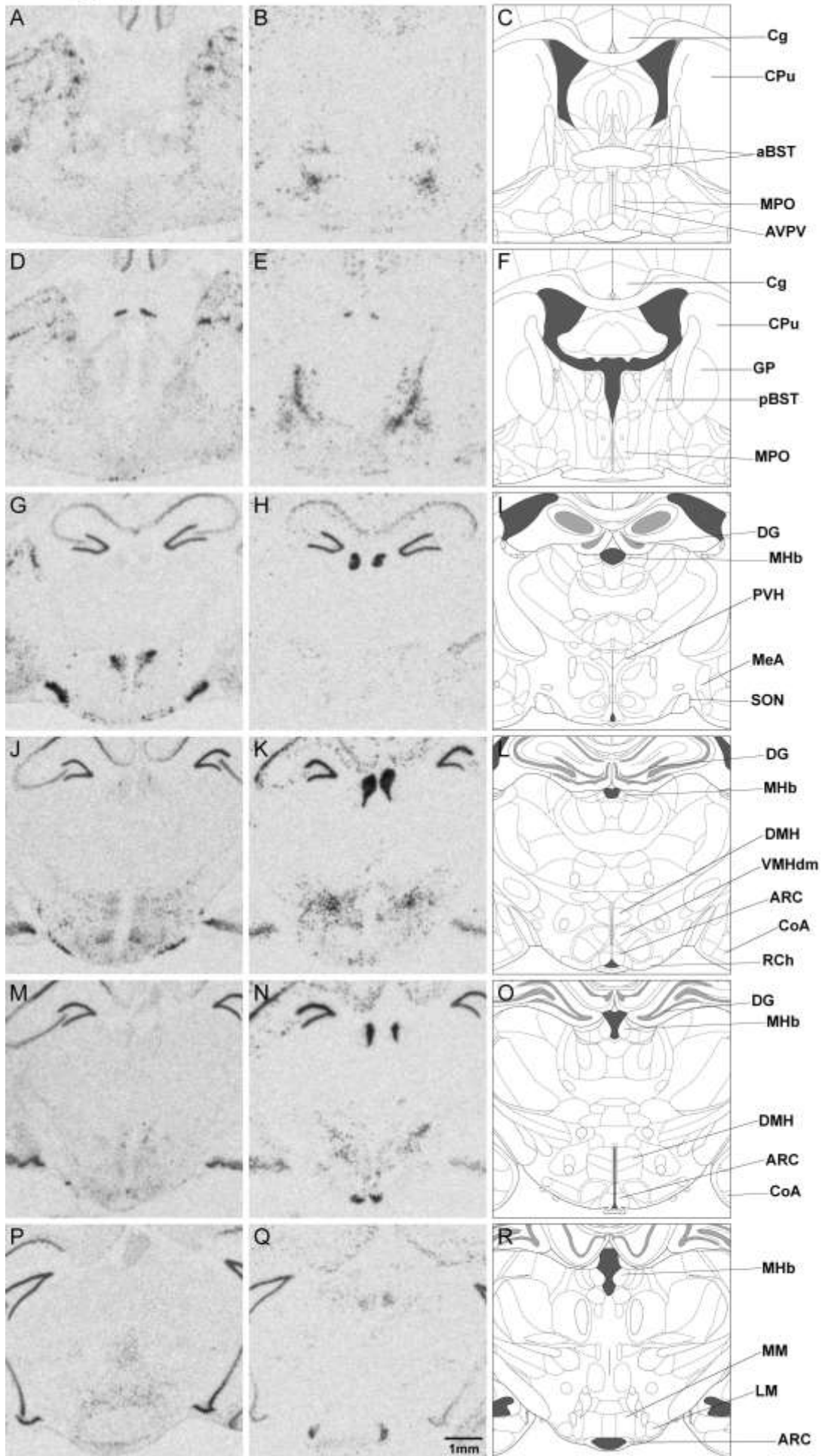
Dynorphin NKB

A B C — Cg, CPu, aBST, MPO, AVPV

D E F — Cg, CPu, GP, pBST, MPO

G H I — DG, MHb, PVH, MeA, SON

J K L — DG, MHb, DMH, VMHdm, ARC, CoA, RCh

M N O — DG, MHb, DMH, ARC, CoA

P Q R — MHb, MM, LM, ARC

1mm

Fig. 2. Brightfield photomicrographs of autoradiograms from coronal sections through the hypothalamus of a breeding female showing the expression of *Pdyn* mRNA (dynorphin; Panels A, D, G, J, M, P) and *Tac3* mRNA (NKB; Panels B, E, H, K, N, Q). Abbreviations: ARC, arcuate nucleus; AVPV, anteroventral periventricular nucleus; BLA, basolateral nucleus of the amygdala; aBST, bed nucleus of the stria terminalis, anterior part; pBST, bed nucleus of the stria terminalis, posterior part; CoA, cortical nucleus of the amygdala; Cg, cingulate cortex; CPu, caudate-putamen; DMH, dorsomedial nucleus; DG, dentate gyrus; GP, globus pallidus; MeA, medial nucleus of the amygdala; MPO, medial preoptic nucleus; MM, medial mammillary nucleus; LM, lateral mammillary nucleus; MHb, medial habenula; PVH, paraventricular nucleus; RCh, retrochiasmatic area; VMHdm, ventromedial hypothalamic nucleus, dorsomedial part.

3.3. ARC gene expression of Pdyn and Tac3 related to reproductive status

Measurement of the average optical density of the two genes in ARC revealed significant group differences (Fig. 3, 4). The analysis of the *Pdyn* and *Tac3* hybridisation signal revealed no overall effect of status ($F_{1,13.85}=0.01$, $p=0.92$), but a significant effect of gene ($F_{1,13.66}=7.54$, $p=0.016$) and a significant interaction between reproductive status and gene ($F_{1,13.66}=14.06$, $p=0.002$). Breeding females had significantly elevated *Tac3* expression and reduced *Pdyn* expression in the ARC compared to nonbreeding females ($p<0.05$). Further, while in the ARC of breeding females *Tac3* expression was significantly higher than *Pdyn* expression ($p<0.001$), no such difference was seen in nonbreeding females ($p>0.05$). Within the two control regions, VMHdm and DMH, no group differences in gene expression levels of dynorphin and NKB, respectively were found (VMHdm, breeder: 0.27 ± 0.04, nonbreeder: 0.32 ± 0.04, $t=0.86$, df=13, $p=0.40$; DMH, breeder: 0.20 ± 0.01, nonbreeder: 0.20 ± 0.02, $t=0.09$, df=14, $p=0.93$).
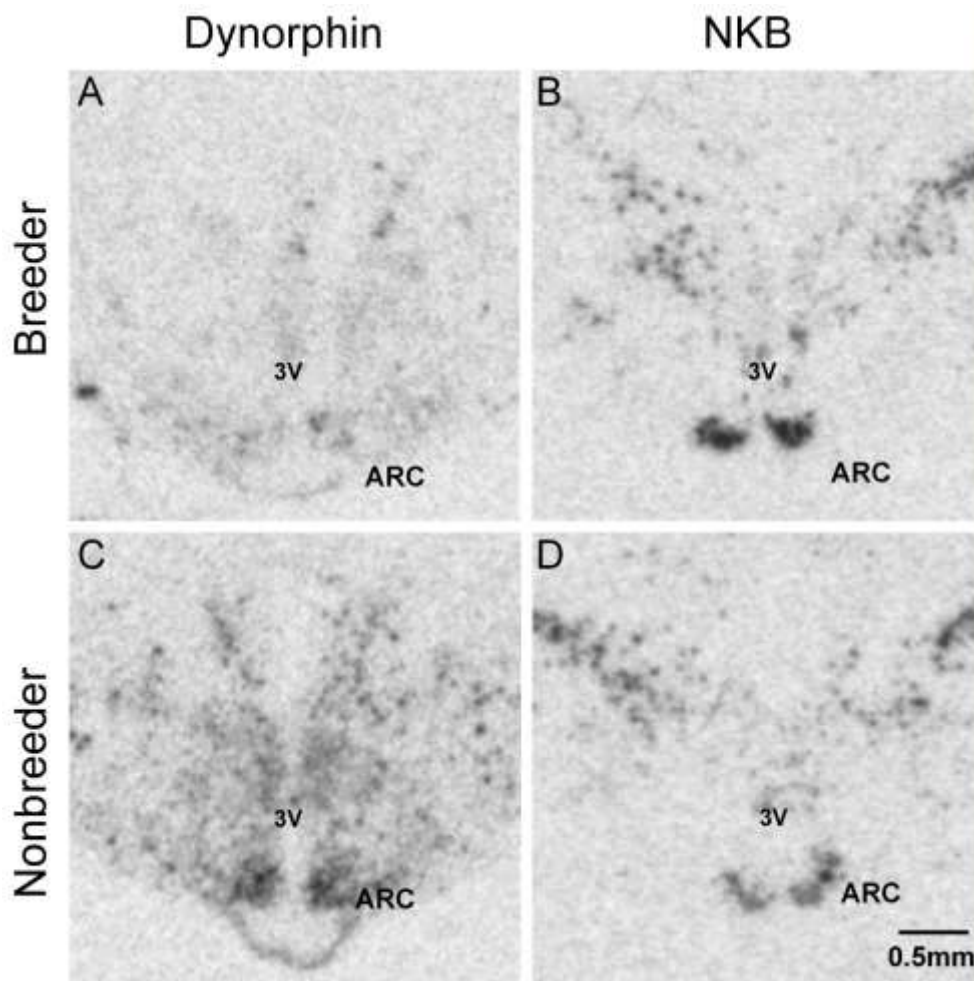
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Fig. 3. Brightfield photomicrographs of autoradiograms showing the expression of *Pdyn* mRNA (dynorphin; A, C) and *Tac3* mRNA (NKB, B, D) in the arcuate nucleus of a breeding (A, B) and a nonbreeding female (C, D).
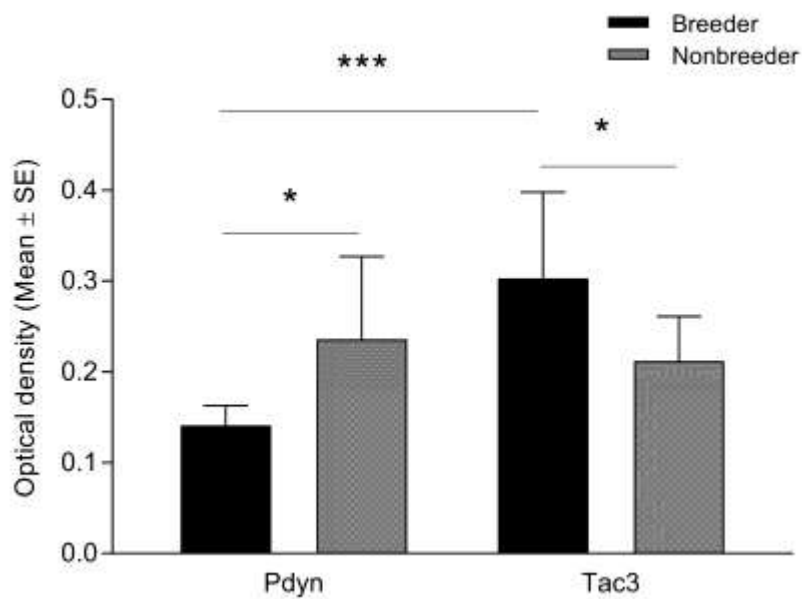

Fig. 4. Average optical density of the hybridization signal for *Pdyn* (dynorphin) *Tac3* (NKB) in the arcuate nucleus of breeding and nonbreeding females. *** $p < 0.001$; * $p < 0.05$.

4. DISCUSSION

Female subordinate Damaraland mole-rats, in the presence of the queen, experience strong physiological suppression to the extent of being anovulatory (Bennett 2011). The mechanisms mediating this reproductive suppression are still not well understood. Here we report the distribution and the differential gene expression within the ARC of the two neuropeptides dynorphin and neurokinin B that are implicated in the control of female reproduction.

4.1. Distribution of Pdyn and Tac3 expression

Overall, the brain distribution of *Pdyn* and *Tac*3 was distinctly different from each other. Dynorphin was found to be widely expressed throughout the mole-rat brain and its distribution matched previous reports of peptide and mRNA expression in rats, mice, hamsters and sheep (Neal and Newman 1989; Alvarez-Bolado et al. 1990; Foradori et al. 2005; Lin et al. 2006). There, intensively labelled telencephalic areas include the olfactory tubercle, the striatum, the amygdala, the cerebral cortex and the hippocampus. Within the hypothalamus, pronounced *Pdyn* expression is described in the preoptic area, the paraventricular nucleus, the supraoptic nucleus, the ventromedial nucleus, the dorsomedial nucleus and the ARC. Unlike in other rodents, *Pdyn* expression in the preoptic area of Damaraland mole-rats was extremely low and restricted to few scattered cells. *Pdyn* expression in the anteroventral periventricular nucleus (AVPV) is regulated by E2 and increases significantly in ovariectomized rats after E2 treatment (Simerly 1991). However, the exact mechanism through which dynorphin mediates its inhibitory effects on GnRH secretion is currently not known. Damaraland mole-rats exhibit pronounced estrogen receptor α (ERα) gene expression in the AVPV, but circulating E2 levels in both breeding and nonbreeding females are generally very low (Voigt et al. 2014; Voigt and Bennett 2018a).

NKB-expressing cells have been detected previously by ICC and ISH in rodents, sheep and humans in many brain regions including the cerebral cortex, the hippocampal formation, the amygdala, the bed nucleus of the stria terminalis, the habenula, the ARC and the lateral

mammillary nuceus (Warden and Young 1988; Merchenthaler et al. 1992; Marksteiner et al. 1992; Chawla et al. 1997; Foradori et al. 2006). Our study is in general agreement with this distribution. Especially striking was the strong labelling seen in the dorsal and ventral aspects of the medial habenula. This brain region sends projections to the interpeduncular nucleus, but their functional significance is still not fully understood. Recent studies suggest a role of the medial habenula in anxiety, stress, memory, mood disorders and addiction (Viswanath et al. 2014). Within the hypothalamus, scattered expression was found in the anterior preoptic area while more pronounced *Tac3* expression was visible in the dorsomedial hypothalamic nucleus, the ARC and the lateral mamillary nucleus. Although our study was not designed to detect colocalization of *Pdyn* and *Tac3* mRNA, it was apparent from processing of adjacent sections that some brain areas contained both transcripts, most notably the ARC. There, the gene expression of both neuropeptides was concentrated in the middle and caudal part of the nucleus. Similarly, pronounced kisspeptin gene expression was found in this region of Damaraland mole-rats (Voigt and Bennett 2018a). Therefore, these neurons presumably constitute the KNDy neuron network described for all other mammalian species studied to date (for review, see Moore et al. 2018).

4.2. Influence of reproductive status on Pdyn and Tac3 gene expression

The model of the extrinsic pulse generator proposes that the pulsatile release of GnRH is initiated by the release of NKB from the KNDy neuron network within the ARC, which in turn stimulates the release of kisspeptin followed by an increase in GnRH secretion. The release of dynorphin from the KNDy neuron network serves as a stop signal, which eventually terminates the GnRH pulse (Lehman et al. 2010). Recent work shows that dynorphin is released onto KNDy cells almost immediately after pulse onset while its release onto GnRH neurons within the mediobasal hypothalamus occurs only at pulse termination (Weems et al. 2018). Our findings of increased NKB and decreased dynorphin gene expression in breeding females generally agrees with the proposed model. Moreover, female breeding Damaraland mole-rats

were previously found to have increased *Kiss1* expression in the ARC compared to nonbreeders (Voigt and Bennett 2018a). It is now generelly accepted that the stimulatory effects of NKB are mediated via kisspeptin stimulation (Fergani and Navarro 2017). Our data suggest that in nonbreeding females the normal pulsatile GnRH release is disturbed, which could impact pulse amplitude or frequency or both and be reflected in reduced LH pulse amplitude and/or frequency. This is in line with the earlier findings of increased GnRH concentrations in the median eminence and proximal pituitary stalk indicating reduced release of GnRH, and reduced concentrations of pituitary LH in nonbreeding females (for review, see Bennett 2011). Moreover, the ovaries of such females rarely contain Graafian follicles, contain no corpora lutea of ovulation, but instead possess unruptured luteinized follicles resulting from atresia of developing follicles (Bennett et al. 1994).

Based on our previous finding of decreased numbers of *Kiss1*-expressing cells and decreased levels of *Kiss1* gene expression in the ARC of reproductively inhibited female Damaraland mole-rats we had argued that chronically very low E2 levels in such females constitute a negative feedback effect of E2 on GnRH neuron activity similar as was described for seasonally anestrous ewes (Smith et al. 2007). In the latter, not only *Kiss1* expression in the ARC is reduced but also *Tac3* expression, and i.c.v. infusion of NKB is able to activate ARC kisspeptin neurons and to elicit LH release in these animals (Sakamoto et al. 2012; Weems et al. 2017). Our present data would still be in line with this reasoning. However, studying the steroid hormone sensitivity of the KNDy neuropeptides in Damaraland mole-rats would reveal insight into the mechanism. Furthermore, studies involving the administration of kisspeptin, NKB receptor agonists and kappa opioid receptor (KOR) antagonists can demonstrate whether subordinate females can be reproductively activated. For example, in species such as rats, guinea pigs and primates, administration of a single dose of naloxone, a non-specific antagonist to endogenous opioid peptide receptors, results in a significant increase in basal LH release (Van Vugt et al. 1981; 1983; Gore and Terasawa 2001). A similar study done in female breeding

and nonbreeding Damaraland mole-rats, failed to observe such an effect (Molteno and Bennett 2002). In two further species, which exhibit socially induced infertility of subordinates, highveld mole-rats (*Cryptomys hottentotus pretoriae*) and marmoset monkeys (*Callithrix jacchus*) comparable results were obtained (Abbott 1988; du Toit et al. 2006). Moreover, recent evidence from sheep suggests that the blockade of KOR increases LH secretion in ovariectomized individuals treated with E2 at pre-pupertal age, but not at post-pubertal age. During the former period, the hypothalamus of the ewe lamb is highly sensitive to the inhibitory actions of small amounts of E2 (Lopez et al. 2016). These data are in support of our hypothesis of a long-term negative feedback effect of E2 acting on KNDy neurons of reproductively suppressed female Damaraland mole-rats leading to reduced activation of GnRH neurons. It remains presently unclear why KOR antagonist administration in such females had no effect on LH release (Molteno and Bennett 2002). Our present data reporting increased dynorphin gene expression in reproductively suppressed compared to reproductively active females, suggests that endogenous opioid peptides are indeed involved in the regulation of normal reproductive function. It is a limitation to our study that we were not able to report plasma oestradiol levels in these females. We will take this into account in future studies.

Taken together, our data show a differential gene expression pattern of NKB and dynorphin in the ARC according to reproductive phenotype. It confirms that both neuropeptides play a role in the regulation of normal reproduction in Damaraland mole-rats. Further experimental work is required to elucidate their contribution to the inhibition of GnRH release in reproductively suppressed females.

COMPETING INTERESTS

The authors declare that they have no competing interests.

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