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AN IMMUNOHISTOCHEMICAL STUDY OF THE GONADOTROPHIN-RELEASING HORMONE 1 SYSTEM IN SOLITARY CAPE MOLE-RATS, *GEORYCHUS CAPENSIS*, AND SOCIAL NATAL MOLE-RATS, *CRYPTOMYS HOTTENTOTUS NATALENSIS*

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Abstract—Mole-rat species within the family *Bathyergidae* exhibit a wide range of reproductive strategies and social systems. Various forms of reproductive suppression are displayed within this family: in the solitary species, breeding is suspended for part of the year and in the social species, reproduction is suppressed in subordinate animals. This study investigated the gonadotrophin-releasing hormone 1 (GnHR-1) systems of breeding and non-breeding solitary Cape mole-rats and social Natal mole-rats for possible inter-and/or intra-species differences. In both species, GnRH-1 cell bodies are predominantly in the medial septum region of the diagonal band or the preoptic area, with relatively few in the mediobasal hypothalamus; a dense concentration of GnRH-1-immunoreactive (ir) processes is present in the region of the organum vasculosum of the lamina terminalis. In Cape mole-rats, GnRH-1-ir processes are particularly dense within the lateral margins of the median eminence, which is enfolded by a large pars tuberalis of the pituitary gland. Natal mole-rats display GnRH-1-ir processes across the breadth of the median eminence, which is abutted by a relatively small pars tuberalis. There are more GnRH-1-ir cell bodies in Natal mole-rats than in Cape mole-rats (~720 vs. ~420). No significant differences were found in the number, distribution or size of GnRH-1-ir cell bodies according to season in Cape mole-rats or according to reproductive status or sex in Natal mole-rats. In female and male Natal mole-rats, GnRH-1-immunoreactivity in the median eminence is less dense in the reproductive animals; no such difference was found in Cape mole-rats between the breeding and non-breeding seasons. These immunohistochemical results are discussed in the light of earlier studies which identified no functional neuroendocrine impediments underlying regulated reproduction in either Cape or Natal mole-rats. The cumulative findings suggest that the principal factors determining seasonal or socially induced suppression of reproduction in these species are behavioral rather than neuroendocrine.

Key words: GnRH, LHRH, seasonal breeding, reproductive suppression, dominance, subordination.

African mole-rats of the family Bathyergidae are subterranean rodents endemic to sub-Saharan Africa. This remarkable family includes species displaying a wide range of reproductive strategies and social systems, from strictly solitary to highly cooperative (Bennett and Faulkes, 2000). All of the solitary mole-rat species investigated thus far have been found to breed seasonally (Bennett and Jarvis, 1988; Herbst et al., 2004; Hart and Bennett, 2006, and Šumbera et al., 2008). In contrast, social mole-rat species live in family groups in which reproduction usually occurs throughout the year; only two of the social mole-rat species, the common mole-rat (*Cryptomys hottentotus hottentotus*) and the Highveld mole-rat (*Cryptomys hottentotus pretoriae*), show seasonally regulated breeding (Janse van Rensburg et al., 2002; Spinks et al., 1997, 1999). Nevertheless, reproduction in all social mole-rat species is subject to regulation by non-seasonal factors. Procreation in each colony is restricted to a single female and one to three putative males. The remaining colony members perform supporting roles, such as digging tunnels, foraging and caring for offspring. Reproductive suppression in the subordinates may be effected by behavioral interventions from dominant animals, or by a neuroendocrine response to the presence of the dominants which suppresses the hypothalamo-pituitary-gonadal (HPG) axis. Additionally, subordinate animals may refrain from reproducing due to inbreeding avoidance. When an unrelated male is introduced into the colony, subordinate females become aggressive and reproductively active (Cooney and Bennett, 2000).

Social mole-rat species are widespread in sub-Saharan Africa and occur in a variety of different habitats. In the species inhabiting arid regions, suppression of reproduction in subordinates appears to involve neuroendocrine functions; in contrast, in the species living in mesic regions, with more frequent rainfall, reproductive suppression seems to depend on behavioral interactions (Bennett and Faulkes, 2000).

In neuroendocrinologically suppressed mole-rats, a higher content of gonadotrophin-releasing hormone 1 (GnRH-1) in the median eminence may be expected, reflecting GnRH-1 retention. This has been found in female Damaraland and Highveld mole-rats (Molteno et al., 2004; Du Toit et al., 2006), two species in which a neuroendo-

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Abbreviations: DB, diagonal band; GnHR-1, gonadotrophin-releasing hormone 1; HPG, hypothalamo-pituitary–gonadal; ir, immunoreactive; LH, luteinizing hormone; MBH, mediobasal hypothalamus; MS, medial septum; OVLT, organum vasculosum of the lamina terminalis; PBS, phosphate-buffered saline; PFA, paraformaldehyde; POA, preoptic area; SCN, suprachiasmatic nucleus.

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crine suppression of subordinate females is demonstrated by their reduced plasma level of luteinizing hormone (LH) before and after exogenous GnRH-1 (Bennett et al., 1993; Van der Walt et al., 2001).

Extensive evidence from many mammalian species indicates that seasonal breeding may involve seasonal changes in the reproductive neuroendocrine system; such changes have been reported for the release of GnRH-1, the sensitivity of gonadotrophs to GnRH-1 and the secretion and action of gonadal steroids (Clay et al., 1989; Caillol et al., 1990; Horan et al., 1993; Karsch et al., 1993; Rhim et al., 1993; Lehman et al., 1997; Gerlach and Aurich, 2000; Kawamoto et al., 2000; Millesi et al., 2002). In white-footed mice, photoperiod-induced gonadal regression is associated with elevated GnRH-1-immunoreactivity in the median eminence, which may reflect decreased GnRH-1 release; there is also an increase in the number of GnRH-1-immunoreactive (ir) cell bodies (Glass, 1986). In Syrian hamsters, the elevated hypothalamic GnRH-1 content during photoperiod-induced inhibition of the HPG axis (Pickard and Silverman, 1979; Jackson et al., 1984; Steger et al., 1982) is associated with an increase in the size of GnRH-1-ir cell bodies, but no change in their number (Urbanski et al., 1991) or in GnRH mRNA expression (Brown et al., 2001); such findings indicate that the regulation of GnRH release is of primary functional importance for seasonal breeding.

Recent endocrinologic studies on two species of mole-rats indicate unusual characteristics in their regulation of reproduction. Thus, neither seasonal inhibition of breeding in solitary Cape mole-rats (*Georychus capensis*) nor socially suppressed reproduction in Natal mole-rats (*Cryptomys hottentotus natalensis*) is associated with a reduced plasma level of LH either before or after exogenous GnRH-1 (Bennett and Jarvis, 1988; Oosthuizen and Bennett, 2007; Oosthuizen et al., in press). The present study initiates research into hypothalamic aspects of the HPG axis in these two species. The GnHR-1 systems of Cape and Natal mole-rats have been investigated immunohistochemically to elucidate possible inter- and/or intra-species differences.

EXPERIMENTAL PROCEDURES

Mole-rats were captured using Hickman live traps (Hickman, 1979). Female Cape mole-rats were trapped in the Darling area, Western Cape Province (33º22′S, 15º25′E), in August, during the breeding season (n=6), and in February, during the non-breeding season (n=7). Male and female Natal mole-rats, which breed aseasonally, were obtained from Glengarry, KwaZulu Natal (29°19′S, 29°43′E) at various times during the year (seven reproductive females, six non-reproductive females, eight reproductive males, seven non-reproductive males). Experiments conformed to the Animal Use and Care Committee of the University of Pretoria and the guidelines of the American Society of Mammalogists (Animal Care and Use Committee 1998) The number of animals used and their suffering were minimized.

Prior to perfusion, animals were kept at the University of Pretoria for no more than 2 weeks; Cape mole-rats were housed individually and Natal mole-rats were maintained within their colonies. Under fluorothane anesthesia (Zeneca, RSA), animals were perfused intracardially with 0.9% saline, followed by 4% paraformaldehyde (PFA; Saarchem, RSA) in 0.1 M phosphate buffer (pH 7.4) (Sigma). The brains were stored in 2% PFA until further treatment. Prior to sectioning, brains were placed in 30% sucrose until saturated for cryoprotection. Twenty-five micrometer thick coronal sections were cut on a cryostat (Bright Cryostats, UK). Every sixth section was used for GnRH-1 immunohistochemistry. The sections were pre-treated in 0.5% Triton-X100 (BDH Chemical Company, Poole, UK). Endogenous peroxidase was suppressed using 0.02% H_2O_2. The sections were washed in phosphate-buffered saline (PBS) and incubated in 2% normal donkey serum for an hour; they were then incubated in a GnRH-1 primary antibody (raised in rabbits, Incstar Corporation, Stillwater, MN, USA) for 48 h at 4 °C (dilution 1:20,000). After a PBS wash, sections were incubated in secondary biotin-SP conjugated AffiniPure Donkey anti-rabbit IgG antibody for 2 h (dilution 1:200, Jackson Immunoresearch, West Grove, PA, USA). Following a wash in PBS the tissue was incubated in an avidin–biotin peroxidase complex (1:1000, Elite Kit; Vector Laboratories, Peterborough, UK). After a wash in Tris buffer the sections were incubated in 0.05% diaminobenzidine (DAB) with 0.15% ammonium nickel sulfate and 0.005% H_2O_2 to visualize GnRH immunoreactivity. All of the sections used for a specific comparison were processed concurrently. Increasing dilutions of the primary antibody led to a commensurate attenuation of the ir signal in both species. No immunoreactivity was observed when the primary or secondary antibody was omitted. We have recently demonstrated (Kalamatianos et al., 2005) that despite some nucleotide base changes comparable to those found in the guinea-pig GnRH gene, the amino acid sequence of the mole-rat GnRH decapeptide corresponds to that of "mammalian" GnRH, as identified in all mammals thus far investigated with the exception of the guinea pig (in which there are two amino acid substitutions).

Analysis

The distribution of GnRH-1-ir processes was identified and the total number of GnRH-1-ir cell bodies was counted in every sixth section from the confluence of the two hemispheres rostrally to the posterior hypothalamus caudally. Only cell bodies with a visible nucleus were counted. The data were corrected for a sampling rate of one in six sections. Image analysis software (ImageJ version 1.30, National Institutes of Health, USA) was used to determine the size of the cell body of 20 randomly selected GnRH-1-ir cell bodies for each animal, according to the method of Robinson et al. (1997). The optical density of GnRH-1-immunoreactivity in the median eminence was quantified as the mean area occupied by GnRH-ir processes according to the method of Molteno et al. (2004). The statistical analyses for this study were undertaken using the Statistica 6.0 software package (Statsoft, Tulsa, USA). Non-parametric Mann-Whitney *U* tests were used. Data are presented as mean±S.E.M. Significance was assumed at $P<0.05$.

RESULTS

Distribution of GnRH-1 cell bodies

Cape mole-rats. GnRH-1-ir cell bodies of female Cape mole-rats were typically found to be spindle-shaped, with smooth contours, and either unipolar or bipolar (Fig. 1a, b); multipolar morphology was seen only rarely. In these animals, GnRH-1-ir cell bodies are distributed along the septo-preoptico-infundibular continuum (Fig. 2). Most of the GnRH-1-ir cell bodies (~85%) are located in the medial septum, region of the diagonal band or preoptic area (MS/DB/POA), with only a small proportion located more caudally in the mediobasal hypothalamus (MBH; Fig. 2,