Distribution of Vasopressin in the Forebrain of Spotted Hyenas

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ABSTRACT

The extreme virilization of the female spotted hyena raises interesting questions with respect to sexual differentiation of the brain and behavior. Females are larger and more aggressive than adult, non-natal males and dominate them in social encounters; their external genitalia also are highly masculinized. In many vertebrates, the arginine vasopressin (VP) innervation of the forebrain, particularly that of the lateral septum, is associated with social behaviors such as aggression and dominance. Here, we used immunohistochemistry to examine the distribution of VP cells and fibers in the forebrains of adult spotted hyenas. We find the expected densely staining VP immunoreactive (VP-ir) neurons in the paraventricular and supraoptic nuclei, as well as an unusually extensive distribution of magnocellular VP-ir neurons in accessory regions. A small number of VP-ir cell bodies are present in the suprachiasmatic nucleus and bed nucleus of the stria terminalis; however, there are extensive VP-ir fiber networks in presumed projection areas of these nuclei, for example, the subparaventricular zone and lateral septum, respectively. No significant sex differences were detected in the density of VP-ir fibers in any area examined. In the lateral septum, however, marked variability was observed. Intact females exhibited a dense fiber network, as did two of the four males examined; the two other males had almost no VP-ir septal fibers. This contrasts with findings in many other vertebrate species, in which VP innervation of the lateral septum is consistently greater in males than in females. J. Comp. Neurol. 498:80–92, 2006.

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Indexing terms: sex differences; lateral septum; Crocuta crocuta; androgen; masculinization

Spotted hyenas (Crocuta crocuta) do not adhere to the conventional rules of sexual differentiation. Females have a grossly elongated, fully erectile clitoris that is virtually indistinguishable from the male’s penis, and their labia are fused to form a pseudoscrotum (Watson, 1877; Matthews, 1939). In contrast to the pattern observed for most mammals, females are also more aggressive than males (Matthews, 1939; Hamilton et al., 1986). These behavioral traits correlate with the female-dominated hierarchical social system of the spotted hyena, in which all females are dominant over adult breeding males (Kruuk, 1972; Frank, 1986).

The extreme virilization of the female spotted hyena is thought to be due, at least in part, to androgen exposure during development. In contrast to other mammals, spotted hyena fetuses of both sexes are exposed to high levels of androgens resulting primarily from the conversion of maternal androstenedione to testosterone at the placenta (Licht et al., 1992; Yalcinkaya et al., 1993). Circulating androgens are also high in adults of both sexes, though the predominant androgen in each sex does differ: androstenedione in females and testosterone in males (Racey and Skinner, 1992; Yalcinkaya et al., 1993).
1979; Lindeque et al., 1986; Glickman et al., 1987; van Jaarsveld and Skinner, 1991; Goyman et al., 2001). Both hormones increase the density of fibers immunoreactive (ir) for arginine vasopressin (VP) in the lateral septum (Villalba et al., 1999), and have potent effects on sexual behavior and aggression in rats (Paup et al., 1975; Christie and Barfield, 1979).

Little is known about the hyena brain. We previously reported that spotted hyenas have a sexually dimorphic nucleus (SDN) in the preoptic area. As in rats and other mammals, the SDN in hyenas is larger in males than in females (Fenstemaker et al., 1999). Similarly, the number of motoneurons innervating the perineal muscles associated with the phallus is sexually dimorphic in the conventional manner (males > females; Forger et al., 1996). To date no neurotransmitter system has been examined in the hyena brain, nor has a descriptive neuroanatomy of the forebrain been presented. In the present study we examined the VP innervation of the forebrain in adult spotted hyenas.

The neural VP system is of particular relevance for a number of reasons. In all vertebrate classes VP, or its evolutionary precursor vasotocin (VT) in non-mammalian vertebrates, is associated with social behaviors including aggression and social dominance (Ferris et al., 1986; Ferris and Potegal, 1988; Koelhaas et al., 1990; Winslow et al., 1993; Marler et al., 1995; Delville et al., 1996; Chu et al., 1998; Goodson, 1998a,b; Semsar et al., 1998, 2001; Goodson and Adkins-Regan, 1999; Tito et al., 1999; Thompson et al., 2004; Goodson et al., 2004b; Lema and Nevitt, 2004). In addition, VP (or VT) innervation of the lateral septum exhibits what is arguably the most consistent sex difference in the vertebrate brain. In many species spanning several vertebrate classes, males have a higher density of VP/VT projections from the bed nucleus of the stria terminalis (BST) and medial amygdaloid nucleus (MeA) to the lateral septum than do females, and in many cases these sex differences are due to developmental and/or adult exposure to gonadal steroid hormones (Moore and Lowry, 1998; Goodson and Bass, 2001; De Vries and Panzica, 2005).

Lateral septal VP innervation has not been examined in species in which females are socially dominant to males, with one possible exception. In golden hamsters, females are at least as aggressive as males, and tend to dominate males of similar body weight (Payne and Swanson, 1970; Marques and Valenstein 1977; Huhman et al., 2003). Interestingly, VP innervation of the forebrain is anomalous, in that hamsters lack the dense VP innervation of the lateral septum typically found in other rodent species (Albers et al., 1991; Ferris et al., 1995; Miller et al., 1999).

The purpose of the present study was twofold: to describe the immunohistochemical distribution of VP in the spotted hyena forebrain, and to determine whether sex differences occur in this system. We hypothesized that the sex difference in VP innervation of the lateral septum observed in other vertebrates would be minimized, or possibly reversed, in spotted hyenas in accordance with their unusual social structure and developmental androgen exposure.

### MATERIALS AND METHODS

#### Animals

In a pilot study, immunohistochemistry was used to visualize the VP system in male and female spotted hyenas killed in the wild in Northern Kenya as part of a population control program by the Kenyan wildlife service. Visual observation suggested that the sex difference in lateral septal VP innervation was reversed, that is, denser in females than in males (C.V. and G.J.D., unpublished observations). However, that tissue had been in storage for several years, and nothing was known about the age, reproductive history, or social rank of the animals. To follow up, we optimized staining and systematically examined the brains of gonadally intact females (n = 3) and males (n = 4) from the captive hyena colony at the University of California, Berkeley. A fourth female whose uterus and ovaries had been removed 4 years prior to death was also included in the study. Spotted hyenas typically reach sexual maturity at 2 or 3 years of age (Kruuk, 1972), and their lifespan in captivity often exceeds 30 years. Animals in the current study were all adults between 4 and 16 years of age. The age, body weight, reproductive history, social grouping, and social rank of each subject at the time of sacrifice is given in Table 1.

Animals were lightly anesthetized with ketamine and xylazine administered by blow dart. A catheter was then inserted into the femoral artery of each animal. The catheter was connected to a pressure/flow transducer that was used to control the anesthetic. Animals were lightly anesthetized with ketamine and xylazine administered by blow dart. A catheter was then

<table>
<thead>
<tr>
<th>Abbreviations</th>
<th>Definition</th>
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<tbody>
<tr>
<td>ac</td>
<td>anterior commissure</td>
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<tr>
<td>BST</td>
<td>bed nucleus of the stria terminalis</td>
</tr>
<tr>
<td>Ca</td>
<td>caudate</td>
</tr>
<tr>
<td>cc</td>
<td>corpus callosum</td>
</tr>
<tr>
<td>Cg</td>
<td>cingulated gyrus</td>
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<tr>
<td>cing</td>
<td>cingulum bundle</td>
</tr>
<tr>
<td>En</td>
<td>entorhinal cortex</td>
</tr>
<tr>
<td>exME</td>
<td>external lamina of median eminence</td>
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<tr>
<td>fx</td>
<td>fornix</td>
</tr>
<tr>
<td>GP</td>
<td>globus pallidus</td>
</tr>
<tr>
<td>inME</td>
<td>internal lamina of median eminence</td>
</tr>
<tr>
<td>int</td>
<td>internal capsule</td>
</tr>
<tr>
<td>IVF</td>
<td>interventricular foramen</td>
</tr>
<tr>
<td>LHA</td>
<td>lateral hypothalamic area</td>
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<tr>
<td>LS</td>
<td>lateral septum</td>
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<tr>
<td>LV</td>
<td>lateral ventricle</td>
</tr>
<tr>
<td>ME</td>
<td>median eminence</td>
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<tr>
<td>MeA</td>
<td>medial nucleus of the amygdala</td>
</tr>
<tr>
<td>MEPO</td>
<td>median preoptic nucleus</td>
</tr>
<tr>
<td>MS</td>
<td>medial septum</td>
</tr>
<tr>
<td>NDB</td>
<td>nucleus of the diagonal band</td>
</tr>
<tr>
<td>och</td>
<td>optic chiasm</td>
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<tr>
<td>opt</td>
<td>optic tract</td>
</tr>
<tr>
<td>OT</td>
<td>olfactory tubercle</td>
</tr>
<tr>
<td>PNV</td>
<td>paraventricular nucleus of the hypothalamus</td>
</tr>
<tr>
<td>PVT</td>
<td>paraventricular nucleus of the thalamus</td>
</tr>
<tr>
<td>PVpo</td>
<td>preoptic periventricular nucleus</td>
</tr>
<tr>
<td>RE</td>
<td>reuniens nucleus</td>
</tr>
<tr>
<td>st</td>
<td>stria terminalis</td>
</tr>
<tr>
<td>SI</td>
<td>substantia innominata</td>
</tr>
<tr>
<td>SCN</td>
<td>suprachiasmatic nucleus</td>
</tr>
<tr>
<td>sm</td>
<td>stria medularis</td>
</tr>
<tr>
<td>SFO</td>
<td>subfornical organ</td>
</tr>
<tr>
<td>SON</td>
<td>suprachiasmatic nuclei</td>
</tr>
<tr>
<td>SoR</td>
<td>suprachiasmatic nuclei, retrochiasmatic</td>
</tr>
<tr>
<td>st</td>
<td>stria terminalis</td>
</tr>
<tr>
<td>VPa</td>
<td>ventral pallidum</td>
</tr>
<tr>
<td>3V</td>
<td>third ventricle</td>
</tr>
</tbody>
</table>
therefore isolated 5 months prior to sacrifice. Successful pairings indicates that the animal was paired, but did not copulate. 3 males were separated from the females 9 months before 75 was sacrificed. Male 67 was previously housed in a group of 3 males and 2 females. He was repeatedly attacked and therefore isolated 3 months prior to sacrifice.

Claustrum commonly used in the rat (Swanson, 1996). The names used here are based on similarities of location and structure, and generally follow the nomenclature. Anatomical structures we observed, however, were distinctive and readily recognizable from work in other species (e.g., De Vries et al., 1985; Caverson et al., 1987; Caffé et al., 1989). The names used here are based on similarities of location and structure, and generally follow the nomenclature commonly used in the rat (Swanson, 1996).

Nomenclature

There has been no previous survey of the hyena brain and no tracing or gene expression studies. Many of the anatomical structures we observed, however, were distinctive and readily recognizable from work in other species (e.g., De Vries et al., 1985; Caverson et al., 1987; Caffé et al., 1989). The names used here are based on similarities of location and structure, and generally follow the nomenclature commonly used in the rat (Swanson, 1996).

Immunohistochemistry

The right or left forebrain (approximately from the rostral pole of the lateral ventricles to the caudal pole of the third ventricle) of each animal was sectioned at 40 μm on a freezing sliding microtome and separated into four series of adjacent sections, which were stored in cryoprotectant until use. For immunohistochemical labeling, sections were washed in 0.05M Tris-buffered saline (TBS, pH 7.6), treated with 3.0% hydrogen peroxide in TBS containing 3% normal goat serum, rinsed in TBS, and treated with 0.01% sodium borohydride. Sections were then incubated in 20% normal goat serum in TBS containing 0.03% Triton X-100 for 10 minutes, followed by anti-VP antibody [1:16,000, diluted in TBS with 0.03% Triton X-100 and 2% normal goat serum (tritrigro)] for 90 minutes at 37°C. The primary antibody was a rabbit polyclonal anti-VP (ICN Biochemicals, Costa Mesa, CA; lot 1700F) raised against synthetic arginine vasopressin linked to the carrier protein, thyroglobulin. After rinsing in tritrigro at 37°C, the sections were incubated in biotinylated goat anti-rabbit secondary antibody (1.5 μg/ml; Vector Laboratories, Burlington, CA) for 45 minutes, rinsed in tritrigro, followed by TBS, and then placed in ABC complex (Vector Elite Kit, Vector Laboratories) in TBS for 45 minutes. The bound antibody complex was visualized with a 0.05% 3'-3'-diaminobenzidine solution that consisted of 0.04% ammonium chloride, 0.03% nickel ammonium sulfate, 0.15% β-d-glucose, and 0.0005% glucose oxidase in TBS. The sections were rinsed in TBS, mounted, air-dried, and covered with a coverslip.

Preabsorption of the primary VP antibody with 50 μM of VP peptide eliminated positive staining in most regions and dramatically reduced staining of the magnocellular VP immunoreactive (VP-ir) neurons in the PVN and SON. Western blots with this antibody (not shown) confirm that the antibody recognizes a peptide of the expected size of vasopressin (~6 kDa) in hyena pituitary; larger bands, presumably corresponding to VP associated with portions of the precursor protein (e.g., neurophysin II and carboxy-terminal glycopeptide) were also labeled (cf. Land et al., 1982). In addition, the antibody does not cross-react with oxytocin, as lanes with purified vasopressin, but not oxytocin contained a labeled ~6 kDa band. We conclude that the antibody specifically labels vasopressin and vasopressin precursors in the hyena.

In two males, VP-ir fibers were virtually absent in the septum and other areas that in rats receive VP innervation from the BST and MeA (see below). To determine whether this was an artifact of poor fixation or due to other limitations of the tissue, representative sections of the two males with low septal VP staining and two other animals (1 male, 1 female) were immunostained for galanin and VP in the same run, using the procedure described above. The galanin antiserum was a rabbit polyclonal (Peninsula Labs, San Carlos, CA; lot #030790-1; immunizing antigen, galanin linked to keyhole limpet hemocyanin), used at 1:2,000 dilution.

Quantification of fiber staining

The density of fiber staining was quantified in the lateral septum, subparaventricular region of the hypothalamus, anterior hypothalamic region, and anterior suprapoistic region [areas with projections of the BST/MeA, the suprachiasmatic nucleus (SCN), paraventricular nucleus (PVN), and supraoptic nucleus (SON), respectively, in rats (De Vries et al., 1985)]. Gray-level thresholding using NIH Image (ver. 1.47) was performed on one series of sections from each animal, all immunostained in the same run (cf.
Bamshad et al., 1993). Slides were coded to conceal animal identity, and light intensity and camera settings were held constant for all sections.

Five representative sections through the lateral septum were analyzed from each animal, and the number of pixels covered by fibers was determined in 8 to 10 $325 \times 325 \mu m^2$ non-overlapping areas in each section. The subparaventricular, anterior hypothalamic, and anterior supraoptic projections (Fig. 1A) were quantified in $650 \times 650 \mu m^2$ areas from three to five representative sections. Measurements of each region in a single animal were then averaged and expressed as relative area covered by VP-ir fibers.

**Radioimmunoassays**

Blood was sampled at sacrifice as described above. Plasma was collected following centrifugation and stored at $-80^\circ C$ until assayed. Radioimmunoassays for testosterone and androstenedione were performed using procedures described in Licht and colleagues (1992) and Place and coworkers (2002). Cross-reaction of the testosterone antibody (T3-125; Endocrine Sciences, Calabasas Hills, CA) with 5α-dihydrotestosterone was 44%, and cross-reaction of the androstenedione antibody (AN6-22; Endocrine Sciences) with testosterone and dihydrotestosterone was less than 2%. After extracting steroids with diethyl ether, drying under nitrogen gas, and reconstituting in PBS with gelatin, samples were incubated with $^{3}$H-labeled steroids (New England Nuclear, Boston, MA) and appropriate antiserum for 1 hour at 37°C. The intra- and interassay coefficients of variation were 5.4% and 16.5% for testosterone and 6.2% and 15.7% for androstenedione.

**Statistics**

VP fiber densities in males and females were compared using mixed-design ANOVA, with brain area as the within
factor and sex as the between factor. Data for the single ovariectomized female were not included in any of the statistical analyses, but are shown in Tables 2 and 3 for comparison. Androgen levels measured by RIA were log-transformed and the difference between sexes analyzed by Student’s t-test. Regression analysis was used to test the relationship between plasma androgens and septal VP-ir fiber density in the 7 intact animals.

### TABLE 3. Plasma Levels (ng/ml) of Androstenedione and Testosterone

<table>
<thead>
<tr>
<th>Animal</th>
<th>Androstenedione</th>
<th>Testosterone</th>
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<tbody>
<tr>
<td>Males</td>
<td></td>
<td></td>
</tr>
<tr>
<td>75</td>
<td>0.4</td>
<td>0.16</td>
</tr>
<tr>
<td>51</td>
<td>1.27</td>
<td>0.23</td>
</tr>
<tr>
<td>67</td>
<td>0.99</td>
<td>0.73</td>
</tr>
<tr>
<td>50</td>
<td>6.34</td>
<td>7.5</td>
</tr>
<tr>
<td>Mean ± SEM</td>
<td>2.25 ± 1.38</td>
<td>2.16 ± 1.78</td>
</tr>
<tr>
<td>Females</td>
<td></td>
<td></td>
</tr>
<tr>
<td>66</td>
<td>4.11</td>
<td>0.26</td>
</tr>
<tr>
<td>47</td>
<td>4.22</td>
<td>0.37</td>
</tr>
<tr>
<td>48</td>
<td>3.82</td>
<td>0.23</td>
</tr>
<tr>
<td>Mean ± SEM</td>
<td>4.05 ± 0.12</td>
<td>0.29 ± 0.04</td>
</tr>
<tr>
<td>Gdx female</td>
<td>1.32</td>
<td>n.d.²</td>
</tr>
</tbody>
</table>

1Minimum detectable limits are 0.29 ng/ml for androstenedione and 0.07 ng/ml for testosterone.
2Not detectable.

### RESULTS

#### Distribution of VP-ir cells and fibers

The distribution of VP immunoreactivity was similar in all animals, except where noted. VP-ir cells and fibers were found in the major VP pathways as established for the rat (De Vries et al., 1985). Magnocellular somata (approximately 400 μm²) were found in the PVN and SON (Figs. 1, 2B–F). In addition, we found numerous accessory magnocellular VP-ir cell groups, some of which were quite large. For example, a substantial group of VP-ir cells stretched rostrally from the SON through the area ventrally to the diagonal band of hoeca (NDB) to the olfactory tubercle (OT; Fig. 2A); a second group extended dorsally from the SON into the lateral hypothalamic area (LHA; Fig. 2D). This latter group joined a population of cells that extended laterally from the PVN (Fig. 2C,D) to form a large cluster of VP-ir cells lateral and parallel to the fornix (fx; Figs. 1C, 2E), and spilling over into the ventral region of the BST (Fig. 2D). Numerous magnocellular VP-ir cells were also found in the retrochiasmatic supraoptic nucleus (SO; Figs. 2E,F).

As expected, many large caliber VP-ir fibers (~0.45 μm diameter), presumably derived from magnocellular somata, coursed in the hypothalamo-neurohypophysial tract toward, and through, the internal zone of the median eminence (inME; Fig. 2E,F). A smaller number of VP-ir fibers were also present in the external zone of the median eminence (exME; Fig. 2F insert), presumably originating from VP-ir neurons in the PVN (Vandesande et al., 1977; Sawchenko et al., 1984). Large caliber VP-ir fibers also were found in areas other than the hypothalamo-neurohypophysial tract. For example, a bundle of fibers coursed laterally from the SON to arch dorsally through the substantia innominata (SI) toward the internal capsule (int), where they then turned sharply to run caudally and dorsomedially towards the stria terminalis (st; Fig. 2C–F). These were joined by large caliber VP-ir fibers exiting the PVN and running dorsolaterally (Fig. 2E). Large caliber fibers were also found in the organum vasculosum laminae terminalis (OVLT), the median preoptic nucleus (MEPO; Fig. 2B) and the subfornical organ (Fig. 1B). The VP-ir fibers in the OVLT did not ramify extensively, and it was not clear whether they were axon terminals or fibers-of-passage. However, in the subfornical organ, fibers occasionally could be seen forming a perineuronal structure around an unlabeled cell (Fig. 1B insert).

Parvocellular VP-ir somata (~100 μm²) were observed in the SCN (Figs. 2C insert, 3A), and small caliber fibers (~0.25 μm diameter) were found in areas that receive VP-ir innervation from the SCN in rats (Hoorneman and Buijs, 1982), that is, the OVLT and its immediate surround, the preoptic periventricular nucleus (PVT; Fig. 2C), and the paraventricular nucleus of the thalamus (PVT; Fig. 2E).

As in rats, scattered magnocellular VP-ir neurons were found in ventral posterior regions of the BST (Fig. 2D). We found very few parvocellular VP-ir cells in the BST or MeA of spotted hyenas. However, dense net-
works of small caliber VP-ir fibers were observed in possible projection sites of BST and MeA neurons, based on lesion and tracing studies in the rat (De Vries and Buijs, 1983; Caffé et al., 1987). Specifically, there was an extensive network of VP-ir fibers in the ventral pallidum (VPa; Fig. 2A) and in an area along the perimeter of the diagonal band of Broca (NDB; Figs. 2A and 4), which spilled over into the lateral and medial septum (LS, MS; Fig. 2A–D). Small caliber VP-ir fibers also were present in the MeA (Fig. 2D) and entorhinal cortex (En; Fig. 2B) of two males (#51 and #75) and two females (#48 and #66). Finally, small caliber fibers were observed in the cingulate gyrus (Cg; Fig. 2D) and nucleus reuniens (RE; Fig. 2F), and a single male showed parvocellular VP-ir cells in the medial septum at the level shown in Figure 2A.
Quantification of fiber density

We quantified VP labeling to test for a sex difference in the lateral septum and to compare immunolabeling in the lateral septum with that in three control regions. ANOVA indicated a significant main effect of brain region ($F_{3,27} = 85.19$, $P < 0.01$), with the densest label due to magnocel-
lular fibers in the anterior supraoptic region and the lightest label resulting from fine caliber fibers in the lateral septum (Table 2). We observed no main effect of sex ($F_{1,27} = 0.09$, $P = 0.77$), and no sex-by-region interaction ($F_{3,27} = 0.73$, $P = 0.55$) on fiber density. However, VP-ir staining in the lateral septum varied markedly among individuals and appeared bimodally distributed in males: two males had fiber densities similar to the highest in females, whereas the others virtually lacked VP-ir fibers in the lateral septum (approximately one-tenth the fiber density of the lowest intact female; Fig. 5 and Table 2). The single gonadectomized female also had low label in the septum (Table 2). The pattern among males was replicated in a second series of sections from the same animals. Although not quantified here, males with low VP-ir fiber density in the septum also lacked staining in other areas that receive innervation from the BST (e.g., ventral pallidum and nucleus reuniens), and VP immunoreactivity in the subparaventricular region was reduced. Staining in the anterior hypothalamic and anterior supraoptic regions was normal in males lacking septal VP (Table 2).
Galanin-ir cells and fibers

We used immunostaining for galanin to evaluate the absence of VP staining in the septa of the two males described above. Galanin is a neuropeptide that is co-expressed with VP in cells of the rat BST (Miller et al., 1993); galanin and VP fibers show overlapping distribution in the septa of several species (Goodson et al., 2004a). In accord, we find parvocellular galanin-ir cell bodies in the corresponding region of the BST of spotted hyenas (Fig. 5E). In addition, the distributions of galanin and VP-ir cells and fibers generally overlap in hyenas, except that galanin-ir fiber distribution is more extensive in ventral regions of the septum. Importantly, galanin-ir fibers could readily be demonstrated in the septa of all animals examined, including the two males lacking VP-ir septal fibers (Fig. 5).

Circulating androgens

Although plasma levels of testosterone and androstenedione were in the predicted direction (testosterone higher in males, androstenedione higher in females), mean values were not significantly different (Table 3). Both androgens were very low or undetectable in the gonadectomized female (Table 3). There was a marginally significant negative correlation between testosterone levels and relative density of VP-ir fibers in the septum ($r = .74, P = 0.06$); the correlation for androstenedione was not significant.

DISCUSSION

Several features of VP immunoreactivity in the spotted hyena forebrain are similar to what has been described in other mammals. For example, we observed magnocellular VP-ir somata in the PVN and SON, and large caliber VP-ir fibers presumably derived from those cells in the medial septum, subfornical organ, OVLT, and the hypothalamo-neurohypophyseal tract. Parvocellular VP-ir cells were found in the SCN, as were fine caliber VP-ir fibers in presumed projection areas of the SCN, such as the OVLT perimeter, the subparaventricular region, and the paraventricular nucleus of the thalamus. These features are in accord with descriptions of VP innervation of the forebrain in rats, cats, and monkeys (De Vries et al., 1985; Caverson et al., 1987; Caffé et al., 1989).

One somewhat unusual observation is the wide distribution of accessory magnocellular VP neurons outside of the PVN and SON. These cells presumably contribute to peripheral VP (Swanson and Sawchenko, 1983), also known as antidiuretic hormone, which acts to enhance water retention at the kidney. The wild relatives of our study animals inhabit a region of Africa characterized by an extended dry season, with little or no surface water available during significant portions of the year (Kruuk, 1972). Circulating VP may be especially important in maintaining water balance in these animals. Similarly, in a cross-species study of closely related rodents, those living in arid conditions had a more extensive magnocellular
system than did species inhabiting mesic environments (Hatton et al., 1972).

We observed only occasional immunolabeled cell bodies in the BST and MeA. The absence of robust cell staining in the BST or MeA is not surprising as visualization of VP-ir somata in these regions typically requires specialized fixation and staining procedures (De Vries et al., 1985), or pretreatment with an axonal transport blocker (Caffé and Van Leeuwen, 1983), neither of which were options here. We did find VP-ir fibers in the lateral septum, ventral pallidum, and nucleus reuniens, and comparative data favors the BST and MeA as the source of these fibers. Studies on the origin of VP innervation of the mammalian forebrain has been limited to rats (De Vries and Buijs, 1983; Caffé et al., 1987). This work identified the BST and MeA as the main contributors of septal and ventral pallidal VP and eliminated the SCN, PVN, and magnocellular cell groups surrounding the paraventricular nucleus as significant sources. VP-ir fibers in the lateral septum and VP-ir cells in the BST, or vasotocin cells and fibers in homologous areas in non-mammalian vertebrates, have been found in a wide variety of vertebrate species (Goodson and Bass, 2001; De Vries and Panzica, 2005). Interestingly, in the few cases where VP-ir fibers in the lateral septum were scarce or absent, such as in golden hamsters, VP-ir bodies in the BST were typically absent as well (Albers et al., 1991; Ferris et al., 1995; Miller et al., 1999). These observations suggest that the BST/MeA VP system seen in other mammals is also present in hyenas. However, we cannot exclude the possibility that the extensive clusters of magnocellular VP-ir cells seen in hyenas contribute to the septal and ventral pallidal VP innervation. It might be possible in future studies to confirm or refute the presence of VP cells in the BST and MeA using an alternative method such as in situ hybridization to locate VP mRNA.

Septal VP-ir fiber density in the forebrain is sexually differentiated by perinatal and adult exposure to gonadal steroid hormones in rats (De Vries et al., 1984; Wang et al., 1993) and other vertebrate species (Moore and Lowry, 1998; Goodson and Bass, 2001; De Vries and Panzica, 2005). Adult gonadectomy leads to the virtual elimination of VP-ir fibers in the septa of both male and female rats, and staining can be reinstated with androgenic or estrogenic metabolites of testosterone, although estrogens are more potent in this regard (De Vries et al., 1986). Consis-

Fig. 4. (A) Photomicrograph illustrating a network of fine caliber VP-ir fibers in the ventral pallidum. Magnocellular neurons of the suprachiasmatic preoptic nucleus can be seen at the bottom of the photo. The arrows in the main viewpoint to region shown at higher magnification in inset (A). Inset (B) shows the ventral pallidum in a control section in which the antibody was pre-adsorbed with purified vasopressin. Medial is left for all views. Scale bar = 200 μm in main view, 50 μm in A, and 200 μm in B.
tent with the effects of castration in rodents, the gonadectomized female in the present study had much lower androgen levels and VP staining in the lateral septum than did intact females. Paradoxically, however, testosterone levels appeared to be negatively correlated with septal staining among male hyenas. Indeed, the two males with high levels of septal VP immunoreactivity had testosterone levels in the female range or lower (Tables 2, 3). In addition, contrary to what has been reported in a wide range of vertebrate species (De Vries and Panzica, 2005), we did not find a male advantage in the density of VP innervation of the lateral septum. Although the number of animals examined was small, the near absence of septal VP fibers in 2 of the 4 males was striking. It is unlikely this lack of staining was due to poor fixation or some other tissue artifact, because the septa of these animals readily immunostained for galanin. In addition, other projections consisting of fine caliber fibers, such as those to the hypothalamic subparaventricular region and the paraventricular nucleus of the thalamus, which are derived from the SCN (Hoorneman and Buijs, 1982; Watts and Swanson, 1987), were labeled in these and all other animals.

It is not possible to know from immunohistochemistry alone whether reduced staining results from decreased production or increased release of VP. In general, however, low VP immunoreactivity is thought to reflect reduced production of peptide (De Vries et al., 1984; Miller et al., 1992; De Vries et al., 1994; but see Wang et al., 1994). In an impressive array of species, VP (or VT in non-mammals) is associated with social behaviors such as aggression and territoriality. For example, central and peripheral administration of VP facilitates aggression and aggressive communication in mammals (Ferris and Potegal, 1988; Koelhaas et al., 1990; Winslow et al., 1993; Delville et al., 1996), including humans (Thompson et al., 2004), and VT has similar effects in fish (Semsar et al., 1998, 2001; Lema and Nevitt, 2004), frogs (Marler et al., 1995; Chu et al., 1998; Tito et al., 1999), and birds (Goodson, 1998a,b; Goodson and Adkins-Regan, 1999; Goodson et al., 2004b; but see Nephew et al., 2005). In addition, a VP antagonist can reverse dominant–subordinate status in hamsters (Ferris et al., 1986), and the distribution of VP receptors correlates with social behavior in mice and voles (Insel and Young, 2001). Interestingly, some of the most consistent differences in VP receptor distribution between species are found in the lateral septum and the ventral pallidum (Bester-Meredith et al., 1999; Young et al., 2001), two regions that in the current study displayed large individual variation in VP immunoreactivity.

The possibility that VP staining in the lateral septum of male spotted hyenas is bimodally distributed is quite interesting given the unusual social hierarchy of this species. Reproductively mature male spotted hyenas in the wild fall into two categories: immigrant males, which are born outside the clan, and natal males, which are sexually mature but have not dispersed (Smale et al., 1997). Immigrant males have high plasma testosterone but are subordinate to all females and their offspring, whereas natal males, which have low testosterone levels, are dominant to immigrant males and are relatively aggressive (Holekamp and Smale, 1998). The animals in the present study were from a captive colony, and males could not disperse as they would in nature. However, the two males lacking septal VP had been removed from their peer groups and housed in isolation prior to sacrifice (see Table 1). It would be interesting to examine VP innervation in spotted hyenas from wild populations, with the prediction that VP staining in the lateral septum would be lower in immigrant than in natal males. If so, then one might also expect a negative correlation between testosterone and septal VP immunoreactivity, as suggested by our data. Finally, because the level of VP in cerebrospinal fluid correlates with a history of aggression in humans (Coccaro et al., 1998), it would be of interest to determine whether cerebrospinal VP levels correlate with dominance status in feral spotted hyenas living within a social hierarchy.

![Fig. 5. Frontal sections of VP-ir and galanin-ir fibers in the lateral septum of male 75 (A, B, respectively), and male 67 (C, D, respectively). The lateral edge of the septum is on the left in each view. E) Galanin-ir cell bodies (arrows) and fibers in the BST of male 67. Scale bar in A = 50 μm for views A–D; scale bar in E = 200 μm.](image)
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LITERATURE CITED


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