Immunoreactivity of Central Vasopressin and Oxytocin Pathways in Microtine Rodents: A Quantitative Comparative Study

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ABSTRACT

The genus Microtus includes several closely related species of voles with diverse patterns of social organization. Comparative studies of these species have previously tested hypotheses related to the evolution of monogamy and affiliation. In earlier studies, monogamous voles have been reported to differ from closely related nonmonogamous voles in the neural distribution of oxytocin and vasopressin receptors. These receptors have also been implicated in the behavioral differences relevant to monogamy, as oxytocin and vasopressin influence pair-bond formation in the monogamous species. In the current study, two monogamous and two nonmonogamous vole species were compared for the distribution of oxytocin and vasopressin immunoreactivity. Contrary to our predictions, gender dimorphisms in vasopressin immunoreactivity were as evident in the monogamous as in the nonmonogamous species. Also, species differences in oxytocin and vasopressin staining were subtle relative to the profound species differences previously reported for receptor binding. These results are consistent with the hypothesis that neuroendocrine systems may evolve by changes in receptor distribution rather than by restructuring the presynaptic pathway.

Indexing terms: voles, monogamy, sexual dimorphism

Microtine rodents with their remarkable diversity in social organization have proven useful for comparative behavioral studies. At least two of these species, the prairie vole (Microtus ochrogaster) and the pine vole (M. pinetis), appear highly affiliative and monogamous, in that males and females pair bond and both parents exhibit parental care (McGuire and Novak, 1984; Oliveras and Novak, 1986; Shapiro and Dewsbury, 1990; Carter and et al., 1993). By contrast, meadow voles (M. pennsylvanicus) and montane voles (M. montanus) appear nonmonogamous, with males and females inhabiting isolated burrows and only females showing parental care (McGuire and Novak, 1984; 1986; Shapiro et al., 1991). These species differences in social organization are particularly intriguing because so many aspects of nonsocial behavior appear similar across this genus (Shapiro et al., 1991). These species differences in social organization are particularly intriguing because so many aspects of nonsocial behavior appear similar across this genus (McGuire and Novak, 1984; 1986; Shapiro et al., 1991).

Recent studies have investigated the possibility that these species differences in social organization are associated with differences in neuroanatomy. A widespread observation in comparative zoology is that monogamous mammals exhibit less sexual dimorphism in appearance than nonmonogamous species (Kleiman, 1977). Accordingly, a morphometric study of the sexually dimorphic regions of the hypothalamus reported no evidence of male–female differences in the volume or cell density of either the anteroventral–periventricular nucleus or the sexually dimorphic nucleus of the preoptic area in monogamous prairie voles (Shapiro et al., 1991). As expected from studies with other rodents, both areas appeared sexually dimorphic in the promiscuous montane vole (Shapiro et al., 1991). In addition, monogamous and promiscuous voles have been reported to differ in the size of the hippocampus (Jacobs et al., 1990).

Perhaps the most profound species differences in these microtine rodents have been observed in studies of neuropeptide receptor distribution. As detected with in vitro and in vivo procedures, oxytocin and vasopressin receptors have been shown to be developmentally regulated and to decline in abundance as a function of social isolation (McKee et al., 1995; 1996). These studies suggest that these species differences in social organization are associated with differences in neuropeptide receptor distribution.

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receptor autoradiography, the telencephalic maps of both oxytocin (OT) and vasopressin (AVP) receptors are virtually nonoverlapping in monogamous and promiscuous voles (Insel and Shapiro, 1992; Insel et al., 1994). For both neuropeptides, the receptors show few species differences in terms of kinetics or specificity, but the patterns of regional expression are markedly different in the two monogamous compared to the two promiscuous voles. And these differences may be specific to OT and AVP; maps of μ opiate or benzodiazepine receptors are not different across the four vole species, even in regions expressing OT or AVP receptors (Insel and Shapiro, 1992).

On the basis of these results, one might hypothesize that the species differences in OT and AVP receptor distribution indicate that these hormones are influencing distinct target fields in the brains of monogamous and promiscuous voles and that these peptides might therefore have markedly different functional roles across vole species. Pharmacological studies suggest that OT and AVP may indeed be critical to the species differences in social organization. In male prairie voles, central administration of AVP promotes pair bonding behaviors such as partner preference formation, mate guarding, and paternal care, whereas a selective AVP antagonists blocks or reduces each of these behaviors (Winslow et al., 1993; Wang et al., 1994a). In female prairie voles, OT facilitates the development of a partner preference, an OT antagonist prevents this important early step in pair-bond formation (Williams et al., 1992, 1994; Insel and Hulihan, 1995).

The current study was undertaken to investigate species differences in the distribution of OT and AVP cells and fibers. Previous studies have reported that male prairie voles (monogamous) have more AVP fibers in the lateral septum than male meadow voles (nonmonogamous) (Wang, 1995). After mating and cohabitation with a female, AVP staining in the lateral septum decreases and AVP mRNA in the bed nucleus of the stria terminalis increases in the male prairie vole, whereas AVP mRNA does not change in the male meadow vole (Bamshad et al., 1994; Wang et al., 1994b). The current study extended these earlier reports by examining four species instead of two for both AVP and OT staining. We hypothesized that, consistent with the receptor maps, the two monogamous species would exhibit different patterns of OT and AVP staining relative to the two nonmonogamous species. In addition, we hypothesized that the sexually dimorphic AVP staining previously reported in rats would be less apparent in the two monogamous vole species.

**MATERIALS AND METHODS**

**Subjects and tissue preparation**

Subjects were F3 or F4 generation of the laboratory breeding colony that started with field-captured animals. Prairie (Microtus ochrogaster), pine (M. pinetorum), and meadow (M. pennsylvanicus) voles were from the Department of Psychology, University of Massachusetts at Amherst. The subjects of each species were housed in same-sex pairs in plastic cages (26 × 30 × 51 cm) which contained peat moss, wood chips, and 10 cm hay covering. Montane voles (M. montanus) were from the NIH Animal Center in Poolesville, Maryland. They were also housed in same-sex pairs in the plastic cages which contained ceder chips as bedding with 10 cm alfalfa cover. For all subjects, food and water were provided ad libitum. All cages were maintained on a 14:10 light-dark photoperiod. The temperature was kept about 18°C.

At 60–90 days of age, male and female pairs of each species were made and put into the same cage for 3 days. After that, 15 prairie (seven males and eight females), 11 pine (seven males and four females), 17 meadow (eight males and nine females), and 11 montane (six males and five females) voles were deeply anesthetized with chloropent (0.03 ml/10 g body weight) and perfused through the ascending aorta with 0.9% saline followed by 5% acrolein in 1 M phosphate buffer (PBS). The brains were removed, and 35-μm transverse sections were cut with a Vibratome.

**AVP immunocytochemistry**

One set of floating sections at 70-μm intervals was processed for AVP immunocytochemistry as described previously (Bamshad et al., 1994). Briefly, sections were incubated in 1) 20% goat serum in 0.05 M Tris-NaCl with 0.3% Triton (Tri-Triton), 15 minutes; 2) anti-AVP serum (ICN, Immunobiologicals) 1:8,000 in Tris-Triton with 2% goat serum (Tritrigo), 90 minutes at 37°C; 3) biotinylated goat-anti-rabbit in Tritrigo, 45 minutes at room temperature; and 4) ABC complex in Tris-NaCl, 45 minutes. Sections were stained by nickel-intensified diaminobenzidine (DAB). To reduce variability in the background, sections from all subjects were processed concurrently for AVP immunocytochemistry. Control sections were incubated with rabbit anti-AVP that was pretreated with 50 μM AVP, which eliminated specific staining. A previous study using the same staining conditions demonstrated that this antibody did not cross-react with 50 μM oxytocin (Bamshad et al., 1993).

**OT immunocytochemistry**

An alternative set of brain sections from the same subjects were processed for OT immunocytochemistry. Similar to AVP immunoreactivity (ir) staining, floating sections were incubated in 1) 10% normal goat serum in PBS with 0.3% Triton, 15 minutes; 2) rabbit-anti-OT (gift from Dr. Mariana Morris, Bowman Gray School of Medicine), 1:50,000 in PBS with 0.5% goat serum and 0.3% Triton, 90 minutes at 37°C; 3) biotinylated goat-anti-rabbit, 1:200 in PBS with 0.5% goat serum and 0.3% Triton, 60 minutes at room temperature; and 4) ABC complex in PBS.
Fig. 1. A-F: Camera lucida drawing of vasopressin (AVP) immunoreactive fibers in a male pine vole. Coronal sections paced at 280 μm intervals are shown with (A) most rostral and (F) most caudal. The most intense staining is evident in the inferior aspects of the lateral septum (ls) where AVP immunoreactive fibers form a dense plexus. Large fibers are evident in the hypothalamic-hypophysial tract (D,E). Consistent staining for fibers and terminals could be detected in the lateral habenula (lh) and the medial amygdala (ma).
with 0.5% goat serum and 0.3% Triton, 30 minutes. Sections were stained by DAB with H₂O₂ in PBS. Control sections were incubated with rabbit-anti-OT that was pretreated with 50 μM oxytocin, which eliminated specific staining. Conversely, pretreatment with 50 μM AVP did not reduce staining.

Data quantification and analysis

The density of AVP-ir fibers in the lateral septum (LS), lateral habenular nucleus (LH), and the paraventricular nucleus of thalamus (PVNT) was examined using computerized gray-level thresholding (IMAGE 1.55 developed by Wayne Rasband at NIH). The number of pixels representing images of the fibers were determined in 250 × 300-μm² sampling area for the LS, 200 × 400-μm² area for the LH, and 300 × 350-μm² area for the PVNT, respectively. For the LS and LH, AVP-ir fibers were counted bilaterally in two consecutive sections that spanned the area with the highest density of AVP-ir fibers. AVP-ir fibers in the PVNT were counted in two consecutive sections. Slides were coded so that the experimenter was blind to the identity of the specimens.

For each subject in sections through the LS and LH, we chose the section with the highest density of AVP-ir fibers on each side of the brain, and the average from both sides was used for data analysis. The higher density of AVP-ir fibers in the PVNT was chosen from each subject for data analysis. The data were analyzed with a two-way analysis of variance (ANOVA) with species and sex as between-subject variables. Significant species differences and species-sex interactions were further evaluated with the Newman Keul post-hoc test (SNK).

OT-ir cells in brain sections were counted (200×) on coded slides. In the medial preoptic nucleus (MPO), preoptic periventricular nucleus (POPVN), paraventricular nucleus of hypothalamus (PVN), supraoptic nucleus (SON), and the bed nucleus of the stria terminalis (BST), OT-ir cells were counted bilaterally in two consecutive sections. In the median preoptic nucleus (MEPN), OT-ir cells were counted in two consecutive sections. The higher number of OT-ir cells on each side of the brain was chosen, and the average from both sides was used for data analysis. In the lateral hypothalamic area (LHA), OT-ir cells were counted bilaterally in one section. Data were analyzed using a two-way analysis of variance with species and sex as between-subject variables. Significant species differences were further evaluated by a SNK test.

RESULTS

AVP Immunoreactivity

As expected, dense clusters of AVP-ir cells were found in the supraoptic (SON), paraventricular (PVN), and suprachiasmatic (SCN) nuclei of the hypothalamus. The distribution of these cells did not differ between species, nor was the overall pattern distinct from previous descriptions in other rodents (Buijs, 1978, 1983; Sofroniew and Weindl, 1981). In each of the vole species, AVP-ir cells were also observed in small clusters in the preoptic area of the anterior hypothalamus.

The distribution of AVP-ir fibers showed few species differences but marked gender differences irrespective of species. The generic male pattern is illustrated in Figure 1. A plexus of fine fibers was observed in the lateral septum, particularly in the inferior aspects rostrally continuous with the diagonal band and caudally continuous with staining in the bed nucleus of the stria terminalis (BST) and medial preoptic area (MPO). Fibers were also observed in the lateral habenular nucleus, the paraventricular nucleus of the thalamus, and the medial nucleus of the amygdala. Of note, regions which we have previously reported as abundant in AVP receptors in the prairie vole (Insel et al., 1994), including the diagonal band and the laterodorsal thalamus, appeared conspicuously unstained for AVP fibers or terminals.

A striking difference in the density of AVP-ir fibers in the lateral septum was observed across all species, comparing males and females (F(1,46) = 34.75, P < .0001, Figs. 2a, 3). In each case, fiber density was greater in males. A species difference was also detected, in which male pine voles exhibited more staining than males of the other three species (F(3,46) = 4.24, P < .01, Figs. 2a, 3). In addition, there was a sex–species interaction (F(3,46) = 4.07, P < .05) owing to a greater male–female difference in pine voles relative to other species. In the lateral habenular
Fig. 3. Photomicrographs displaying vasopressin immunoreactive fibers in the lateral septum (ls) of a prairie vole male (A) and female (B); pine vole male (C) and female (D); montane vole male (E) and female (F); and meadow vole male (G) and female (H). Scale bar = 100 μm.
Fig. 4. Photomicrographs displaying vasopressin immunoreactive fibers in the lateral habenular nucleus of a prairie vole male (A) and female (B); pine vole male (C) and female (D); montane vole male (E) and female (F); and meadow vole male (G) and female (H). Boundaries of photomicrographs are shown by box in camera lucida insert. hc, hippocampus; opt, optic tract. Scale bar = 100 µm.
Fig. 5. A–F: Camera lucida drawing of oxytocin (OT)-immunoreactive cells in a male pine vole. Coronal sections spaced at 280-μm intervals are shown with (A) most rostral and (F) most caudal. Each dot represents three cells. Fibers are shown as dashed lines. Note rostral extension of cells ventrally and pattern of periventricular expression reminiscent of other rodents.
areas, such as the lateral septum, with clear differences in OT receptor binding (Insel and Shapiro, '92). Across species, no gender difference in OT-ir cells was observed. Prairie voles had more OT-ir cells than both montane voles and meadow voles (F(3,45) = 6.69, P < .01, Figs. 2c) in which prairie voles had less staining than the other three species and pine voles had more staining than montane or meadow voles.

OT immunoreactivity

Although abundant OT-ir cells were detected in all of the species, the pattern of distribution showed subtle, but significant variation. The basic pattern, from a male pine vole, is shown in Figure 5. As expected, OT-ir cells were found in the medial preoptic nucleus (MPO), preoptic periventricular nucleus (POPVN), paraventricular nucleus of hypothalamus (PVN), suprachiasmatic nucleus (SON), the bed nucleus of the stria terminalis (BST), the median preoptic nucleus (MEPN), and the lateral hypothalamic area (LHA) in four species of voles. Bars with different letters (a or b) are significantly different from each other. Error bars indicate standard errors of the means.

The current study examined immunoreactivity of AVP and OT in these same four species to determine if 1) gender dimorphisms were less apparent in monogamous species and 2) differences in presynaptic distribution matched the species-typical patterns of receptor distribution. On both counts, our results failed to confirm predictions.

DISCUSSION

Microtine rodents provide a useful model for comparative study because of their close taxonomic relationship and profound differences in social organization and behavior. In autoradiographic studies, monogamous prairie and pine voles show similar patterns of AVP and OT receptor distribution, with little overlap with the patterns observed in nonmonogamous meadow and montane voles (Insel and Shapiro, 1992; Insel et al., 1994). These patterns, from our previous publications, are summarized in Table 1. The explanation for these apparent gender-specific behavioral effects is not obvious. Certainly the absence of gender differences in OT-ir or OT receptors does not provide a ready explanation for the selective responses to OT in females. The profound regional differences in AVP innervation between males and females (Fig. 2) would seem to be consistent with the evidence for effects in males but not females, but the absence of a corresponding receptor differ-
ence is troublesome. In the prairie vole, there is relatively little binding of either $^3$H-AVP or $^{125}$I-sarc-AVP in the lateral septum of either males or females. The dense innervation in this region suggests that either 1) AVP released in the lateral septum acts at a distant site which is responsive only in males or 2) there is a lateral septum receptor for AVP in males that is not detected with current methods. The first explanation is not supported by the absence of gender differences in other brain sites. The second possibility may be addressed with in situ hybridization studies examining AVP receptor mRNA in the lateral septum. It is possible that these studies will detect receptors that have been obscured in binding studies by the high concentration of endogenous peptide in males.

It is also worth considering that the immunocytochemical results reported here were biased because the animals were studied after 3 days of cohabitation with the possibility of mating. In an earlier study, sexually naïve prairie voles had a denser AVP-ir projection in the lateral septum than sexually naïve meadow voles (Wang, 1995). After 3 days of cohabitation (and probable mating) with a female, male prairie voles showed decreased AVP-ir staining in the lateral septum and increased AVP mRNA in the BST, consistent with release of peptide in the septum and a compensatory increase in synthesis (Bamshad et al., 1994; Wang et al., 1994b). Most important, corresponding changes in AVP mRNA was not evident in the promiscuous meadow vole (Wang et al., 1994b). Thus, it is possible that the housing conditions used in the current experiment selectively reduced the intensity of staining in the male prairie vole. That a marked gender difference persists even with conditions that should reduce staining in the male only underscores the evidence for a gender dimorphism in the monogamous species.

**Species differences**

The absence of greater species differences in either AVP-ir or OT-ir may seem surprising given the marked species differences in both AVP and OT receptors. Species
Fig. 8. Photomicrographs displaying OT immunoreactive cells in the bed nucleus of the stria terminalis (bst) in a male prairie (A), pine (B), montane (C), and meadow (D) vole. ac, anterior commissure. Scale bar = 100 μm.
TABLE 1. Receptor Distribution in Prairie and Montane Voles

<table>
<thead>
<tr>
<th>Region</th>
<th>Oxytocin</th>
<th>Vasopressin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nucleus accumbens</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Diagonal band</td>
<td>0</td>
<td>+</td>
</tr>
<tr>
<td>Lateral septum BST</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>Thalamus (centromedial)</td>
<td>+ + +</td>
<td>0</td>
</tr>
<tr>
<td>Lateral habenular</td>
<td>0</td>
<td>++</td>
</tr>
</tbody>
</table>

A mismatch of terminals and receptors is no longer surprising in the study of neuropeptides (Herkenham, 1987). There is no reason, a priori, to assume that neuropeptides and their receptors should be regulated by the same transcription factors; studies of both AVP and OT and their respective receptors demonstrate diverse patterns of regulation (Johnson et al., 1989; Crowley et al., 1995). Nor is there clear registration of OT or AVP terminals with their postsynaptic receptors, except in a few autonomic nuclei (Buijs, 1978; Sofroniew and Weindl, 1981; Raggenbass et al., 1989). While one might assume therefore that these systems work via a paracrine mechanism, the major argument for “action at a distance” in the brain is mostly the lack of evidence for terminal-receptor apposition. In any case, the current results suggest that the evolution of the OT and AVP neural pathways are predominantly at the receptor and not the peptide-synthesizing cells. In other words, species differences in OT and AVP functions appear to have evolved by alterations in the targets for these neuropeptides, not by developing new sources. In ongoing studies we are investigating whether the vole species differences in OT and AVP receptors are due to heterochronic patterns of development or variations in promoter sequences in the receptor genes.

In summary, comparative immunocytochemical studies of OT and AVP were undertaken to follow up on previous results demonstrating species differences in OT and AVP receptor distribution. The results indicate relatively few species differences in patters of OT or AVP staining. Terminals were not detected in many of the regions with receptors, and some of the regions with dense staining for AVP fibers and terminals showed little if any receptor binding in earlier studies. Contrary to a prediction of reduced sexual dimorphism in the monogamous species, in both monogamous and nonmonogamous voles, the AVP projection to the lateral septum and lateral habenula was greater in males than females.

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