Patterns of Brain Vasopressin Receptor Distribution Associated with Social Organization in Microtine Rodents

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Central vasopressin pathways have been implicated in the mediation of paternal behavior, selective aggression, and affiliation in monogamous prairie voles. Here we demonstrate markedly different patterns of brain vasopressin receptor binding in the monogamous prairie vole and the congeneric nonmonogamous (promiscuous) montane vole. Vasopressin binding was assessed with both ^{3}H-vasopressin and ^{125}I-sarc-AVP using receptor autoradiography. The specificity of binding was consistent with a V_{1a} receptor, the saturation kinetics were similar in the two species, and neither species showed evidence of sexual dimorphisms. In the prairie vole, highest specific binding was observed in the accessory olfactory bulb, diagonal band, laterodorsal thalamus, and superior colliculus. In the montane vole, specific binding was observed in the accessory olfactory bulb and superior colliculus as well, but in several other regions with high levels of binding in the prairie vole, binding was low or undetectable in the montane vole. In this nonmonogamous species, specific binding was high in lateral septum. Functional studies demonstrated the induction of phosphoinositol by AVP in the septum of the montane vole but not in the prairie vole. The pattern of ^{125}I-sarc-AVP binding to lateral septum may reflect the social organization of these two species, as similar differences in AVP receptor distribution in the lateral septum were also observed in two related species, pine voles and meadow voles, which are monogamous and nonmonogamous, respectively. These results, along with earlier studies of AVP's effects on pair bonding, suggest the importance of this neuropeptide for the mediation of behaviors related to social organization.

[Key words: vasopressin, AVP, vole, septum, thalamus, phosphoinositol, monogamy]

Central administration of the neuropeptide arginine-vasopressin (AVP) has been associated with a variety of effects from enhanced learning to antipyresis (reviewed in de Wied et al., 1993). One cluster of central effects of AVP that has received recent attention might be best described as the facilitation of species-specific social behaviors. AVP in the golden hamster induces flank marking (Ferris et al., 1984), in the adult rat facilitates social memory (Dantzer et al., 1987), in the infant rat

modulates the isolation call (Winslow and Insel, 1993), and in the squirrel monkey alters the behavioral response to social separation (Winslow and Insel, 1991a). Remarkably, many of the AVP effects on social behavior have been reported exclusively in males and appear androgen dependent (Bluthé et al., 1990; Winslow and Insel, 1991b; Ferris, 1992). Perhaps the most ambitious claims for AVP as a peptide facilitating male social behavior come from recent studies with a monogamous rodent, *Microtus ochrogaster*, the prairie vole. In the male of this species, AVP has been implicated in mate guarding, paternal behavior, and the development of a preference for one specific mate—all critical to the process of pair bonding that characterizes monogamous social organization (Winslow et al., 1993; Wang et al., in press).

If AVP were essential for the mediation of pair bonding, one might predict that neural circuits for this peptide would differ in monogamous and polygynous species, as pair bonding is observed in the former and not the latter. Voles of the genus *Microtus* display diverse patterns of social organization and may provide some useful comparisons in which to test this prediction. For instance, prairie voles and montane voles (*M. montanus*), two species that are similar in appearance and many aspects of nonsocial behavior, display marked differences in social affiliation (Shapiro and Dewsbury, 1990). In their natural habitat, prairie voles frequently form long-term monogamous relationships and show high levels of parental care (Dewsbury, 1981; Getz et al., 1981; Getz and Hofman, 1986). Even in the laboratory, adult prairie voles usually sit side by side with a mate, attack strange adults, and display intense maternal and paternal care (Oliveras and Novak, 1986; Carter et al., 1990; Shapiro and Dewsbury, 1990). In contrast, montane vole adults live in isolated burrows and show no evidence of monogamy (Jannett, 1980). In the laboratory, montane voles appear minimally parental and generally spend little time in contact with conspecifics (McGuire and Novak, 1986; Shapiro and Dewsbury, 1990). Species differences in social behavior are evident very early in life, as prairie vole young show marked increases in ultrasonic calls and glucocorticoid secretion in response to social isolation, whereas montane vole pups show little, if any, behavioral or physiologic response to separation from the nest (Shapiro and Insel, 1990). It appears that an inherent difference in the propensity for social contact or affiliation underlies the differences in social organization.

We have previously described species differences in the distribution of oxytocin receptors in prairie and montane voles (Insel and Shapiro, 1992). These differences were of interest

because (1) related species selected for similar contrasts of social organization showed analogous differences in binding, (2) no species differences were detected for opiate or benzodiazepine receptors, and (3) in the montane vole female, the pattern of binding changed postpartum to resemble the pattern in the prairie vole, with the emergence of maternal behavior. In addition, oxytocin was found to facilitate selective affiliation in the female prairie vole (Williams et al., in press). The present study investigated the distribution of vasopressin receptors in both prairie and montane voles. To examine the coupling of prairie and montane vole receptors to a second-messenger system, regional induction of phosphoinositol by vasopressin was measured in both species. Finally, to examine if species differences in receptor distribution were related to patterns of social organization, the comparison of receptor binding was extended to two other congeneric species, pine and meadow voles, which are believed to be monogamous and nonmonogamous, respectively.

Materials and Methods

Animals. Both male and female prairie and montane voles from the NIH breeding colonies in Poolesville, MD, were studied. Male pine and meadow voles were obtained from the breeding colonies of the University of Massachusetts Department of Zoology. All animals were weaned at 21 d of age and housed with same-sex littermates until 60–90 d of age, when they were used for autoradiographic experiments. All animals were sexually inexperienced. Housing was on a 12:12 light:dark schedule with food and water available ad libitum.

Receptor autoradiography. Following decapitation, brains were quickly removed, frozen on dry ice, and stored at $-70°C$ until sectioned. Cryostat-cut 20 μm slide-mounted sections (at 120 μm intervals) were used for the receptor binding assay. Two ligands were used. Initial studies employed ^{3}H-AVP (from New England Nuclear–Dupont, Boston, MA); subsequent studies used ^{125}I-sarc-AVP, a selective, high-affinity V_{1a} receptor antagonist previously characterized in rat and hamster brain sections (Phillips et al., 1988; Ferris et al., 1993).

For ^{3}H-AVP binding, sections were preincubated for 10 min in 50 μM GTP in Tris-HCl buffer with 100 mM NaCl to remove endogenous ligand, and then washed twice for 10 min in Tris-HCl buffer to remove NaCl and GTP. Sections were then placed in incubation buffer consisting of 50 mM Tris-HCl (pH 7.4) with 10 mM $MgCl_2$, 0.1% BSA, 0.05% bacitracin for 90 min at 24°C containing 5 nM ^{3}H-AVP. In adjacent sections, 0.5 μM unlabeled AVP was added to the incubation buffer to define nonspecific binding. Following incubation, all sections were washed twice for 4 min in 50 mM Tris-HCl with 10 mM $MgCl_2$ at 4°C, and then immediately dried under a stream of cool air.

For ^{125}I-sarc-AVP binding, sections were preincubated for 5 min in 50 μM GTP in 100 mM NaCl (this step was eliminated from later studies without affecting the results) followed by 2 × 5 min rinses in Tris-HCl (pH 7.4). Incubation buffer was the same as described above for ^{3}H-AVP. For ^{125}I-sarc-AVP, incubation lasted 60–75 min and a tracer concentration of 30–50 pM was used. In initial studies it was found that nonspecific binding (i.e., background label) with this ligand was high but reversible with extensive washes. The following wash protocol was found to minimize the nonspecific binding without reducing the specific binding of ^{125}I-sarc-AVP: dip in incubation buffer to remove excess tracer, 4 min in 0.1% paraformaldehyde (to preserve tissue integrity) in Tris-HCl with 10 mM $MgCl_2$, 4–6 × 4 min in Tris-HCl with 10 mM $MgCl_2$ buffer, 4 min in same buffer diluted 1:1 with D.I. H_2O, dip in D.I. H_2O (to remove buffer salts). All washes were performed at 4°C and followed immediately by drying under stream of cool air. Nonspecific binding was defined by incubating adjacent sections with approximately 0.5 μM [1-(β-mercapto-β,β-cyclopentamethylene propionic acid),2-(O-methyl)tyrosine]-Arg^8-AVP—a selective V_{1a} ligand (Kruszynski et al., 1980), here referred to as d(CH_2)$_5$[Tyr(Me)]AVP.

When dry, slides were apposed to Hyperfilm (Amersham, Arlington Heights, IL) along with ^{125}I plastic standards (Amersham) for 2–3 d (^{125}I-sarc-AVP) or ^{3}H plastic standards for 12 weeks (^{3}H-AVP). The resulting autoradiograms were analyzed with the IMAGE program for the Macintosh, permitting the conversion of optical density to dpm/tissue equivalents using a third-order polynomial regression. Nonspecific binding was subtracted from total binding to yield values for specific binding.

Based on previous experience comparing optical density quantification to binding values obtained in homogenate assays with other iodinated ligands, we recommend that the absolute values obtained as dpm/tissue equivalent be treated as relative numbers for comparison within an experiment.

Saturation and competition studies. In previous studies with rat and hamster, ^{125}I-sarc-AVP appeared to bind specifically to a vasopressin rather than an oxytocin receptor, as AVP was roughly 1000 times more potent than oxytocin for displacing ^{125}I-sarc-AVP binding in brain sections (Phillips et al., 1988; Ferris et al., 1993). To compare the specificity of ^{125}I-sarc-AVP binding in prairie and montane voles, 20 adjacent 10 μm sections through the laterodorsal thalamus (prairie vole) and the lateral septum (montane vole) were exposed to ^{125}I-sarc-AVP using the binding protocol described above. In each region, the first and last sections were compared to ensure consistent binding along the 200 μm in the rostral–caudal plane. Specificity of ^{125}I-sarc-AVP binding (70 pM) was determined in competition studies using nine concentrations of the selective V_{1a} ligand d(CH_2)$_5$[Tyr(Me)]AVP ranging from 10^{-12} to 10^{-6} or five concentrations of Thr^{4}Gly^{7}OT (oxytocin) and [d(CH_2)5,D-Phe^2,Ile^4,Ala^9-NH$_2$]-AVP (V_2) ranging from 10^{-9} to 10^{-6}. Each of these studies compared adjacent sections from a single animal with three to four animals used for each displacer. Displacer peptides were obtained from Peninsula (Burlingame, CA).

A similar strategy was used to investigate the saturation kinetics of ^{125}I-sarc-AVP binding in 20 adjacent 10 μm sections through the laterodorsal thalamus (prairie vole) and the lateral septum (montane vole). For each species, sections from three or four animals were exposed to 1 of 10 concentrations of ^{125}I-sarc-AVP, ranging from 2 to 1000 pM. Both species were represented in every study. At each concentration, blanks were generated by adding 1 μM d(CH_2)$_5$[Tyr(Me)]AVP. To facilitate image analysis, slides exposed to the highest concentrations of ^{125}I-sarc-AVP were exposed to film for 24 hr, while lower concentrations received either 5, 7, or 12 d exposures. Once again, the reader is cautioned against overinterpreting the numbers resulting from image analysis, particularly because sections were 10 μm thick whereas 20 μm standards were used for converting optical density to dpm/mg tissue equivalent.

Iodination of sarc-AVP. As previously described, synthetic sarc-AVP was iodinated through reaction with chloramine-T (Ferris et al., 1993). Sarc-AVP (4 μg) and 0.5 mCi of Na^{125}I were mixed in 50 μl of phosphate buffer (0.5 M, pH 7.5). The reaction was initiated by the addition of 40 μl of chloramine-T (0.2 mg/ml in 0.5 phosphate buffer; Sigma Chemical). After mixing, 0.5 ml of 0.1% bovine serum albumin (in phosphate buffer) was added. The ^{125}I-sarc-AVP was purified by high-pressure liquid chromatography using a Bondapak C-18 column (3.9 × 300 mm) equilibrated at 1.5 ml/min with 0.1% trifluoroacetic acid (TFA). Two minutes after sample injection, a 60 min linear gradient was run to 40% acetonitrile in 0.1% TFA. Fractions (1.5 ml) were collected every minute from the time of injection. The major peak of monoiodinated sarc-AVP appeared in fractions 47–48, while a smaller peak of diiodinated peptide appeared in fractions 51–52. Fractions 47–48 were diluted in 0.1 M acetic acid with 0.1% bovine serum albumin and stored at $-80°C$. Specific activity was approximately 2400 Ci/μg of peptide. Unlabeled sarc-AVP was generously provided by Prof. Z. Grzonka (University of Gdansk, Gdansk, Poland).

Phosphoinositol hydrolysis. To determine if receptors in both species were functionally coupled to the phosphoinositol-diacylglycerol cycle, we measured the generation of phosphoinositol following AVP incubation with tissue slices. Male prairie and montane voles were decapitated and brains were rapidly dissected to harvest the septum, dorsal thalamus, and corpora quadrigemina. Dissected tissue was immediately placed into ice-cold, freshly oxygenated (95% O_2, 5% CO_2) buffer containing (mM) 118.0 NaCl, 4.8 KCl, 1.3 $CaCl_2$, 1.2 $MgSO_4$, 5.0 $MgCl_2$, 1.2 KH_2PO_4, 11.0 glucose, 25.0 NaHCO$_3$ (final pH adjusted to 7.4 with 0.1 N HCl). Tissue was cross-chopped at 0.35 mm interval with a McIlwain tissue chopper (Brinkman, Westburg, NY). The slices were equilibrated in 25 ml of buffer with O_2/CO_2 by bubbling for 45 min at 37°C with one intermediate change of buffer. After equilibration, the buffer was removed by aspiration and 0.3 μM ^{3}H-inositol was added for 1 hr with continuous O_2/CO_2 perfusion and dispersion of slices with a Pasteur pipette every 15 min. Following incubation with tracer, slices were washed three times with 15 ml of buffer and then 40 μl of gravity-packed slices was pipetted into 4 ml vials containing 10 mM LiCl. Following preincubation with LiCl for 20 min in O_2/CO_2 atmosphere, vehicle (buffer) or agonist (0.5 or 5.0 μg AVP) was added to each tube.
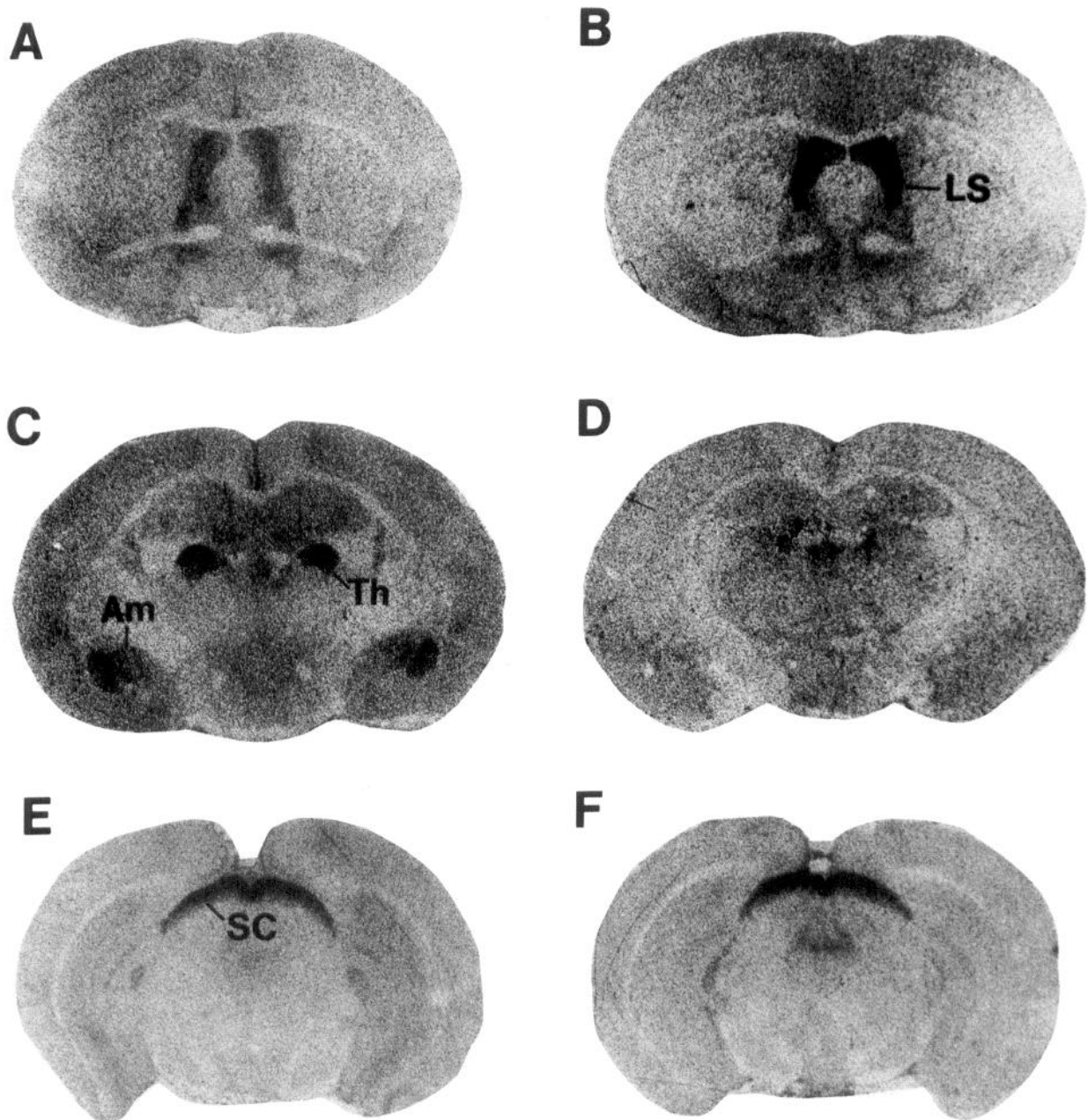
Figure 1. Binding of ³H-AVP in prairie and montane voles. Pattern of ³H-AVP binding is shown in bright-field images from prairie vole (*A, C,* and *E*) and montane vole (*B, D,* and *F*). Quantification is provided in Table 1. Note increased binding to dorsolateral septum (*LS*) in montane vole and binding to laterodorsal thalamus (*Th*) and both central and basolateral nuclei of amygdala (*Am*) in prairie vole. Both species show binding to superior colliculus (*SC*). ³H-AVP is not an ideal ligand—it binds with relatively low affinity to AVP receptors and binds with slightly lower affinity to oxytocin receptors. Therefore, the patterns of binding observed here cannot be unambiguously attributed to AVP receptors.

Following a 45 min incubation in O_2/CO_2 atmosphere at 37°C, the reaction was terminated by the addition of $CHCl_3$:MeOH (1:2). The samples were vortexed vigorously and centrifuged at 3000 rpm for 5 min at 4°C, and 1 ml of the aqueous phase was applied to a Dowex-1-Resin column. ³H-IP₁ was eluted with 5 ml of 0.2 M ammonium formate/0.1 N formic acid. Samples were corrected for tissue volume with counts from the dried organic phase. All samples were run in triplicate, with 10 male sexually naive voles used for each species in each experiment. Each experiment included both species and was replicated twice (total of 30 voles for each species).

Data analysis. For autoradiographic studies, all slides from a given region were processed simultaneously with each run, including at least three males and three females of each species for ³H-AVP. Analysis by region included a two-way ANOVA with main factors of species and gender. For ¹²⁵I-sarc-AVP binding, four or five males of each species

were compared. Sections from females (*n* = 3 for each species) were run independently. Anatomic regions were defined from stained slides, with anatomic boundaries adopted from Paxinos and Watson (1986). Generally, three sections were measured in each individual for each region to provide an individual mean value. Differences at the *p* = 0.05 level were considered significant.

Results

Distribution of ³H-AVP binding

Specific binding of ³H-AVP was found in several regions in both species (Fig. 1). As shown in Table 1, prairie voles had higher levels of ³H-AVP binding in the laterodorsal thalamus and amygdala (both central and basolateral nuclei), whereas mon-

Table 1. ³H-AVP binding in prairie and montane voles

Region	Prairie vole	Montane vole	F (species)	F (gender)
Lateral septum	2044 ± 271	6137 ± 443	237.9*	1.5
Laterodorsal thalamus	1767 ± 469	261 ± 97	16.2*	5.2
Basolateral amygdala	1770 ± 236	492 ± 92	24.8*	1.7
Superior colliculus	2967 ± 623	2381 ± 319	0.9	2.5

Values represent mean ± SEM dpm/mg tissue equivalent from six voles of each species.
* $p < 0.01$ for main effects by two-factor (gender × species) ANOVA.

tane voles had much higher levels of ³H-AVP binding in the lateral septum. ³H-AVP binding to superior colliculus was high in both species. There were no overall effects of gender on ³H-AVP binding in any of the regions examined. Nonspecific binding was high but homogeneous in both species (nonspecific binding = 2331 ± 365 dpm/mg tissue equivalent), representing roughly half of total binding in an area labeled in both species, such as the superior colliculus.

Distribution of ¹²⁵I-sarc-AVP binding

With ¹²⁵I-sarc-AVP, several regions that were not detected with ³H-AVP binding appeared to show clear specific binding (Table 1). Nonspecific binding was lower than observed for ³H-AVP

and was consistently homogeneous across sections. In the forebrain, mean (±SEM) binding in the presence of an excess of the V_1 displacer was 1561 ± 195 dpm/mg tissue equivalent for the prairie vole ($n = 5$) and 938 ± 167 dpm/mg tissue equivalent for the montane vole ($n = 5$). These values represent 7.7% and 7.0% of total binding in the superior colliculus in prairie and montane voles, respectively.

Beginning with the most rostral sections in the prairie vole (Fig. 2), specific binding was high in the accessory olfactory bulb including the adjacent granule cell layer. In the main olfactory bulb, modest binding was observed in the internal granular layer with an inconsistent beaded appearance in the outer part of this layer. Although this latter pattern could be specific to the mitral
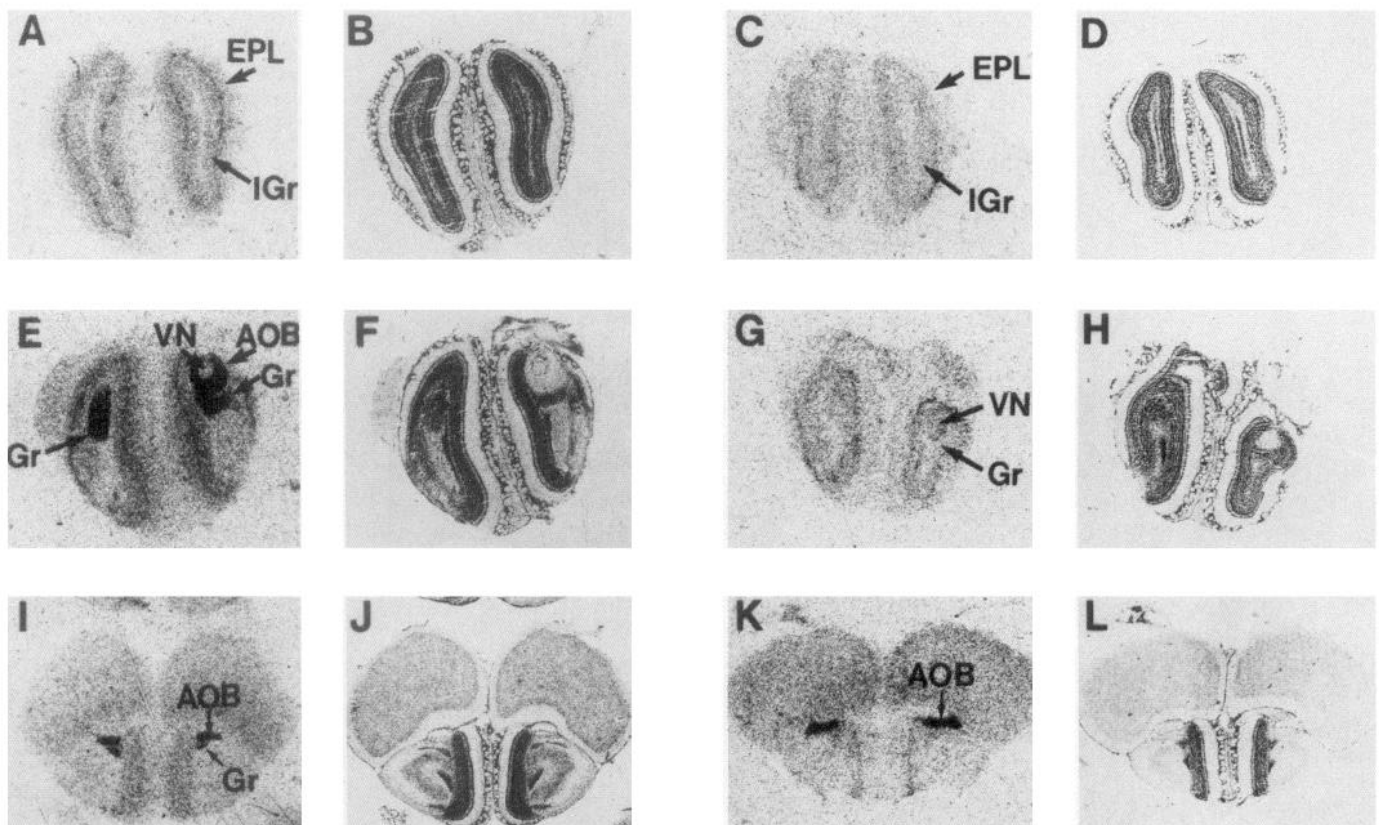
Figure 2. Binding of ¹²⁵I-sarc-AVP to olfactory bulb in prairie and montane voles. Bright-field images of ¹²⁵I-sarc-AVP binding to olfactory bulb in prairie voles (*A, E,* and *I*) and montane voles (*C, G,* and *K*) are shown adjacent to Nissl-stained sections (*B, F,* and *J* for prairie vole; *D, H,* and *L* for montane vole). In both species there is modest binding in the internal granular layer (*IGr*), with local foci detected in the outer margin, possibly indicating binding to the mitral cell layer (*A* and *C*). Specific binding could not be detected consistently in the external plexiform layer (*EPL*) of either species. In the prairie vole, but not the montane vole, there is intense binding to the accessory olfactory bulb (*AOB*), including the granule cell layer (*Gr*), but not the vomeronasal nerve (*VN*) (*E* and *G*). In the montane vole, binding is detectable in the more posterior part of the AOB (*K*), although not in the granule cell layer.
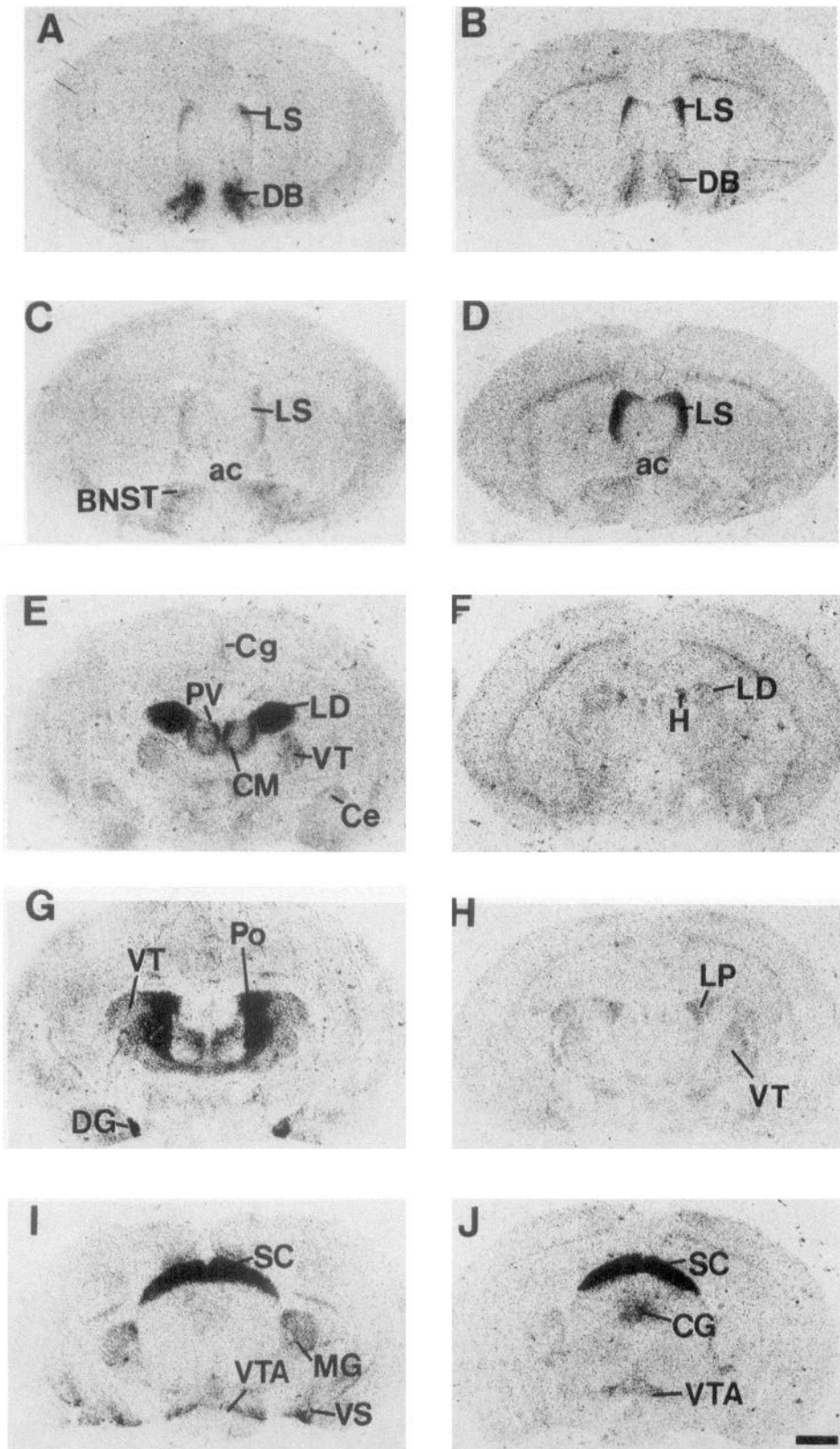
Figure 3. Binding of ^{125}I-sarc-AVP to forebrain and midbrain in prairie and montane voles. Bright-field images of ^{125}I-sarc-AVP binding to coronal sections through prairie vole (*A, C, E, G,* and *I*) and montane vole (*B, D, F, H,* and *J*) are shown. Abbreviations are given in Table 2 (except for *ac*, anterior commissure), where quantitative values appear statistically different in virtually every region except for superior colliculus (*SC*). Scale bar, 1.0 mm.

cell layer, we were unable to determine this precisely with either film or emulsion autoradiography. In contrast to the pattern of oxytocin receptors in the prairie vole (Witt et al., 1991), no specific binding was evident in the main olfactory nuclei with ^{125}I-sarc-AVP. In the ventral forebrain (Fig. 3), specific binding was evident throughout the descending arms and horizontal limb of the diagonal band and in the ventral rostral division of the bed nucleus of the stria terminalis. Binding was not observed in medial preoptic area and only light label was evident in the lateral septum, mostly in the rostral dorsal division with the
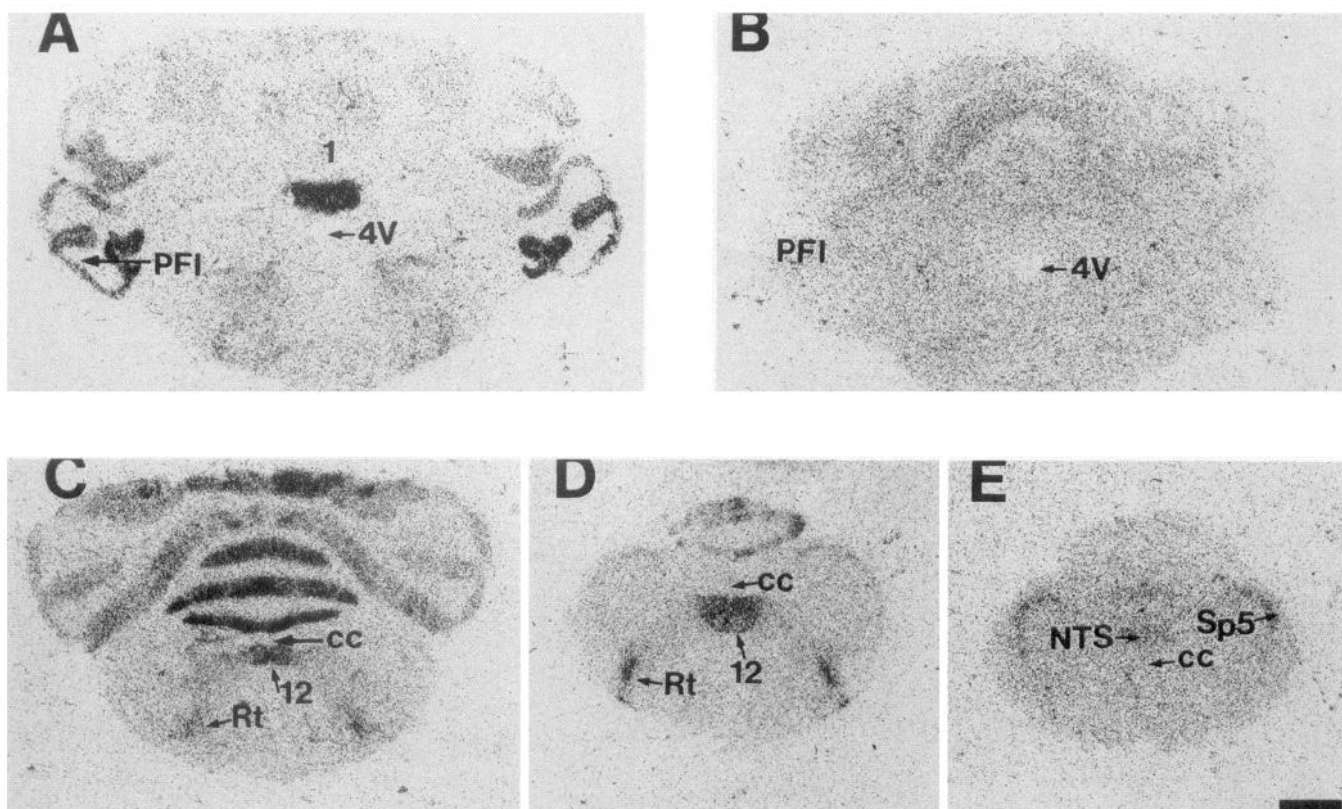
Figure 4. Binding of ^{125}I-sarc-AVP to brainstem in prairie and montane voles. In contrast to forebrain pattern, in which prairie voles generally show more ^{125}I-sarc-AVP binding than montane voles, in the brainstem and cerebellum it is the montane vole that shows more intense labeling. *A, C,* and *D* show the pattern of ^{125}I-sarc-AVP binding in montane vole, with label most intense in first lobule of cerebellum (*1*) and in paraflocculus (*PFl*) molecular layer. In addition, binding can be seen throughout the extent of the hypoglossal nucleus (*12*) and in the reticular nucleus (*Rt*) at the level of the nucleus ambiguus. In the prairie vole, specific binding was undetectable in the cerebellum or pons (*B*), although low levels of label could be discerned in the medulla (*E*) through the spinal tract of V (*Sp5*) and in the nucleus of the solitary tract (*NTS*). Scale bar, 1.0 mm.

ventral division [a site of dense staining for AVP terminals in the male prairie vole (Bamshad et al., 1993)] conspicuously unlabeled. Several thalamic nuclei showed intense specific binding in most (but not all) animals, particularly the laterodorsal and paraventricular, but also the centromedial, posterior, and ventral posterior nuclei. Moderate levels of binding were consistently found in the medial geniculate. In contrast to the pattern observed in the rat, guinea pig, and hamster (Phillips et al., 1988; Dubois-Dauphin et al., 1990; Tribollet et al., 1992; Ferris et al., 1993), there was no evidence of specific binding in the hippocampus or neocortex, except in three highly localized regions: intense binding was observed in the most posterior tip of the dentate gyrus at the ventral surface of the brain; light, diffuse label was evident in the ventral subiculum; and fine stippled label was found deep in the cingulate cortex at the rostral–caudal level of the thalamus. Only very modest binding was observed in the amygdala (note contrast to Fig. 1), exclusively in the central nucleus. In the midbrain, we found intense binding in the superior colliculus and faint label in the ventral tegmental area near the supramammillary nucleus. In the brainstem (Fig. 4), there was faint but consistent binding in the nucleus of the solitary tract and in the spinal tract of V, but no other detectable specific binding was notable in the prairie vole.

In the montane vole, specific binding of ^{125}I-sarc-AVP was just as high as the prairie vole in the main and accessory olfactory bulbs and superior colliculus, but in virtually all other

regions there was a markedly different pattern of label (Figs. 2–4). Even within these regions, subtle species differences were apparent. For instance, the granule cell layer of the accessory olfactory bulb that was labeled in the prairie vole showed much less specific binding in the montane vole. In the diagonal band, bed nucleus of the stria terminalis, and most thalamic nuclei specific binding was significantly less in the montane vole. However, in a few discrete regions, ^{125}I-sarc-AVP binding was greater in this species. This species difference was most evident in the lateral septum, especially the intermediate division, in which binding was approximately fourfold greater in the montane vole. Specific binding of ^{125}I-sarc-AVP was also greater in the montane vole in one fine point of the lateral habenula, in the ventral subiculum, and in the central gray. In the brainstem, where binding was unremarkable in the prairie vole, several nuclei were labeled in the montane vole. The highest density of binding was found throughout the nucleus of the 12th nerve, including the prepositus division. Specific binding of ^{125}I-sarc-AVP was also observed in the reticular nucleus at the level of the nucleus ambiguus and in specific regions of the cerebellum, especially in the paraflocculus. Binding in the cerebellum was entirely restricted to the molecular layer.

No sex differences were found with ^{125}I-sarc-AVP binding in either species. Three females of each species were compared to three males of each species in a separate experiment with equivalent results to those shown in Table 2 (data not shown).

Table 2. Comparison of prairie and montane voles: ^{125}I-sarc-AVP binding (mean ± SEM dpm/mg tissue equivalent)

Region	Abbrev.	Prairie	Montane
Olfactory (main and accessory)			
Internal granular layer	IGL	1276 ± 98	1210 ± 102
External plex. layer	EPL	ND	ND
Ant. olf. nucleus	AOP	681 ± 319	475 ± 71
Vomeronasal nerve	VN	ND	ND
Granule cell layer (accessory)	Gr	4538 ± 193	1131 ± 192**
Accessory olf. bulb	AOB	4919 ± 567	5508 ± 1056
Forebrain and midbrain			
Diagonal band	DB	5576 ± 586	1388 ± 418**
Nuc. accumbens	NAcc	452 ± 81	548 ± 295
Lateral septum	LS	1510 ± 166	5785 ± 810**
Bed nuc. stria term. (ventral)	BNST	3441 ± 571	906 ± 287**
Cingulate cortex	Cg	1678 ± 690	281 ± 200*
Ventral subiculum	VS	657.2 ± 203	1934 ± 332**
Dentate gyrus (post.)	DG	3140 ± 847	70 ± 47**
Amygdala (central nuc.)	Ce	1359 ± 391	315 ± 105*
Habenula (lateral)	H	815 ± 342	2516 ± 910*
Thalamus (laterodorsal)	LD	11,234 ± 1665	945 ± 331**
Thalamus (centromedial)	CM	3489 ± 310	859 ± 327**
Thalamus (paraventricular)	PV	4193 ± 1153	ND**
Thalamus (ventral posterolat.)	VT	1760 ± 313	2025 ± 985
Thalamus (posterior)	Po	5383 ± 1060	644 ± 446**
Medial geniculate	MG	2538 ± 214	598 ± 198**
Superior colliculus	SC	10,550 ± 705	12,417 ± 562
Ventral tegmental area	VTA	542 ± 184	379 ± 153
Central gray	CG	469 ± 147	2085 ± 497**
Brainstem and cerebellum			
Reticular nucleus	Rt	601 ± 173	2944 ± 380**
Hypoglossal nucleus	12	ND	4780 ± 698**
Nucleus of solitary tract	NTS	1867 ± 385	ND**
Spinal tract of V	Sp5	1529 ± 312	134 ± 61**
Paraflocculus	PFI	ND	1648 ± 247**
Cerebellum (molecular)	Cer-M	ND	3467 ± 732**
Cerebellum (granule)	Cer-G	ND	ND

Values represent mean dpm/mg tissue equivalent from four or five males in each species.

* $p < 0.05$.

** $p < 0.01$; comparing means by Student's t test.

Characteristics of ^{125}I-sarc-AVP binding

To determine if ^{125}I-sarc-AVP was binding to a similar receptor in the two species, 10 μm sections through the laterodorsal thalamus for the prairie vole and lateral septum for the montane vole were compared in both saturation and competition studies. Although ^{125}I-sarc-AVP was displaced by nanomolar concentrations of the selective V_{1a} antagonist d(CH$_2$)$_5$[Tyr(Me)]AVP in both species (IC$_{50}$ = 8.8 nM for prairie voles and 3.8 nM for montane voles), neither species showed appreciable displacement of binding when sections were coincubated with Thr[4]Gly[7]OT (OT specific) or [d(CH$_2$)$_5$,D-Phe[2],Ile[4],Ala[9]-NH$_2$]-AVP (V$_2$ specific) concentrations up to 1 μM (Fig. 5A). This pharmacologic characterization resembles several previous reports of binding to AVP receptors in rat brain (see, e.g., Phillips et al., 1988; Shewey and Dorsa, 1988). Furthermore, exposing sections from both species to incremental concentration of ^{125}I-sarc-AVP resulted in a similar pattern of association with 50% of receptors occupied at 183.1 nM for prairie vole laterodorsal thalamus and 180.6 nM for montane vole lateral septum (Fig. 5B).

Phosphoinositol hydrolysis

Phosphoinositol hydrolysis was examined in three regions for both prairie and montane voles (Table 3). In the montane vole septum, the induction of phosphoinositol increased 10.7 ± 1.2% and 17.3 ± 0.3% over baseline after 0.5 and 5.0 μg AVP, respectively. In the prairie vole septum, AVP did not significantly increase phosphoinositol. Conversely, in the prairie vole thalamus, AVP increased phosphoinositol by 15.7 ± 2.3%, whereas no change was evident in the same region from the montane vole. In both species, AVP was ineffective at inducing phosphoinositol in the superior colliculus.

Comparative studies

If AVP receptor distribution were functionally related to social organization, a similar pattern should be evident in related species with analogous differences in behavior. Therefore, we next
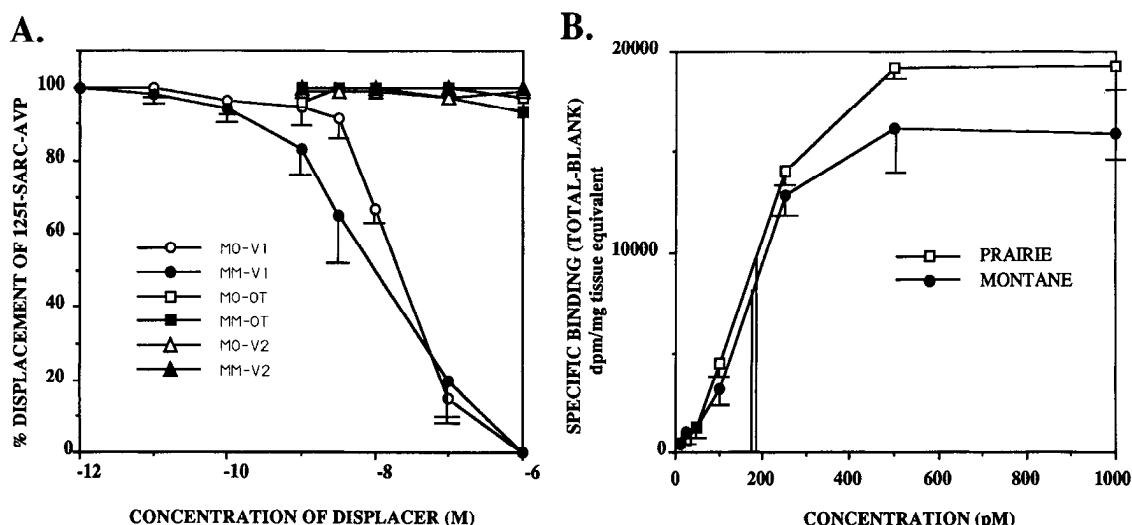
Figure 5. Competition and saturation studies with ^{125}I-sarc-AVP. *A,* Sections through prairie laterodorsal thalamus and montane vole lateral septum were compared for their profiles of ^{125}I-sarc-AVP displacement. In both species, ^{125}I-sarc-AVP was displaced by the selective V_1 ligand d(CH$_2$)$_5$[Tyr(Me)]AVP, with an IC$_{50}$ in the low nanomolar range. Neither the oxytocin ligand Thr^{4}Gly^{7}OT or the V_2 ligand [d(CH$_2$)$_5$,D-Phe^2,Ile^4,Ala^9-NH$_2$]-AVP showed appreciable displacement at concentrations up to 1 μM. Data are means from three studies in each species. *B,* Saturation curves obtained by incubating sections in incremental concentrations of ^{125}I-sarc-AVP were compared in laterodorsal thalamus (prairie vole) and lateral septum (montane vole). Values represent specific binding after subtracting nonspecific binding [adjacent sections incubated in same concentration of ^{125}I-sarc-AVP with 1 μM of d(CH$_2$)$_5$[Tyr(Me)]AVP]. Point at which 50% of receptors are occupied appears nearly identical in two species. Data are means ($\pm$SEM) from three experiments.

examined receptor distributions in two additional species of *Microtus* with similar (although not identical) behavioral differences, the monogamous pine vole (*M. pinetorum*) (FitzGerald and Madison, 1983) and the promiscuous meadow vole (*M. pennsylvanicus*) (Madison, 1980). As with prairie and montane voles, ^{125}I-sarc-AVP binding in the lateral septum was intense in the nonmonogamous species and relatively sparse in the monogamous species (meadow voles = 6298 ± 1644; pine voles = 708 ± 184 dpm/mg tissue equivalent; $t(4) = 3.38, p < 0.03$) (Fig. 6). Meadow voles also showed more binding in the brainstem in the reticular nucleus, just at the region of the nucleus ambiguus, and in the hypoglossal nucleus (Fig. 6). Conversely, the most intense concentration of ^{125}I-sarc-AVP binding in the pine vole brain began in the posterior division of the cingulate cortex and extended into retrosplenial cortex, where intense labeling was evident in layers I and III (5741 ± 946 dpm/mg

tissue equivalent). Specific binding was not detectable in this region in the meadow vole. These species also differed in the superior colliculus (569 ± 333 vs 6010 ± 1506 in pine and meadow voles, respectively), but not in several other regions such as the diagonal band, thalamus, and dentate gyrus, where prairie and montane voles were found to show contrasting patterns of ^{125}I-sarc-AVP binding.

Discussion

Based on previous behavioral studies, we hypothesized that central pathways for AVP would differ in closely related monogamous and nonmonogamous voles. The results demonstrate differences in the distribution of both ^{3}H-AVP and ^{125}I-sarc-AVP binding in prairie and montane voles. These differences (1) appear to reflect local numbers of AVP receptors (based on competition and saturation data), (2) are associated with regional differences in the functional response to AVP, and (3) are partially replicated in congeneric species. In a previous study, we demonstrated analogous species differences in the distribution of OT receptors (Insel and Shapiro, 1992). It does not appear that voles show unusual species variability in the expression of all membrane binding proteins, as comparative studies across four vole species found no differences in the distribution of μ-opiate or benzodiazepine receptors (Insel and Shapiro, 1992). Differences in AVP receptor distribution are of particular interest because AVP has been recently implicated in the mediation of pair bonding and paternal behavior in the male prairie vole, essential aspects of monogamous social organization (Winslow et al., 1993; Wang et al., in press).

Several aspects of these binding studies require comment. First, the reader should note that certain regions showed considerable variability. In particular, the laterodorsal and midline thalamic nuclei, labeled so clearly in the prairie vole with ^{125}I-sarc-AVP (Fig. 3), were labeled inconsistently. A few animals showed little detectable binding in these regions, even though

Table 3. Regional induction of phosphoinositol: mean ± SEM percentage induction

Region	Prairie vole	Montane vole
Septum		
AVP (0.5 μg)	4.3 ± 4.3	10.7 ± 1.2*
AVP (5.0 μg)	10.0 ± 6.1	17.3 ± 0.3*
Thalamus		
AVP (0.5 μg)	ND	ND
AVP (5.0 μg)	15.7 ± 2.3*	3.0 ± 3.3
Colliculus		
AVP (0.5 μg)	ND	4.5 ± 4.5
AVP (5.0 μg)	ND	1.5 ± 1.5

Values represent means of three experiments (two for sup. coll.) with 10 animals of each species in each experiment. ND, not detectable (AVP values < buffer values).

* $p < 0.05$ compared to control (buffer).
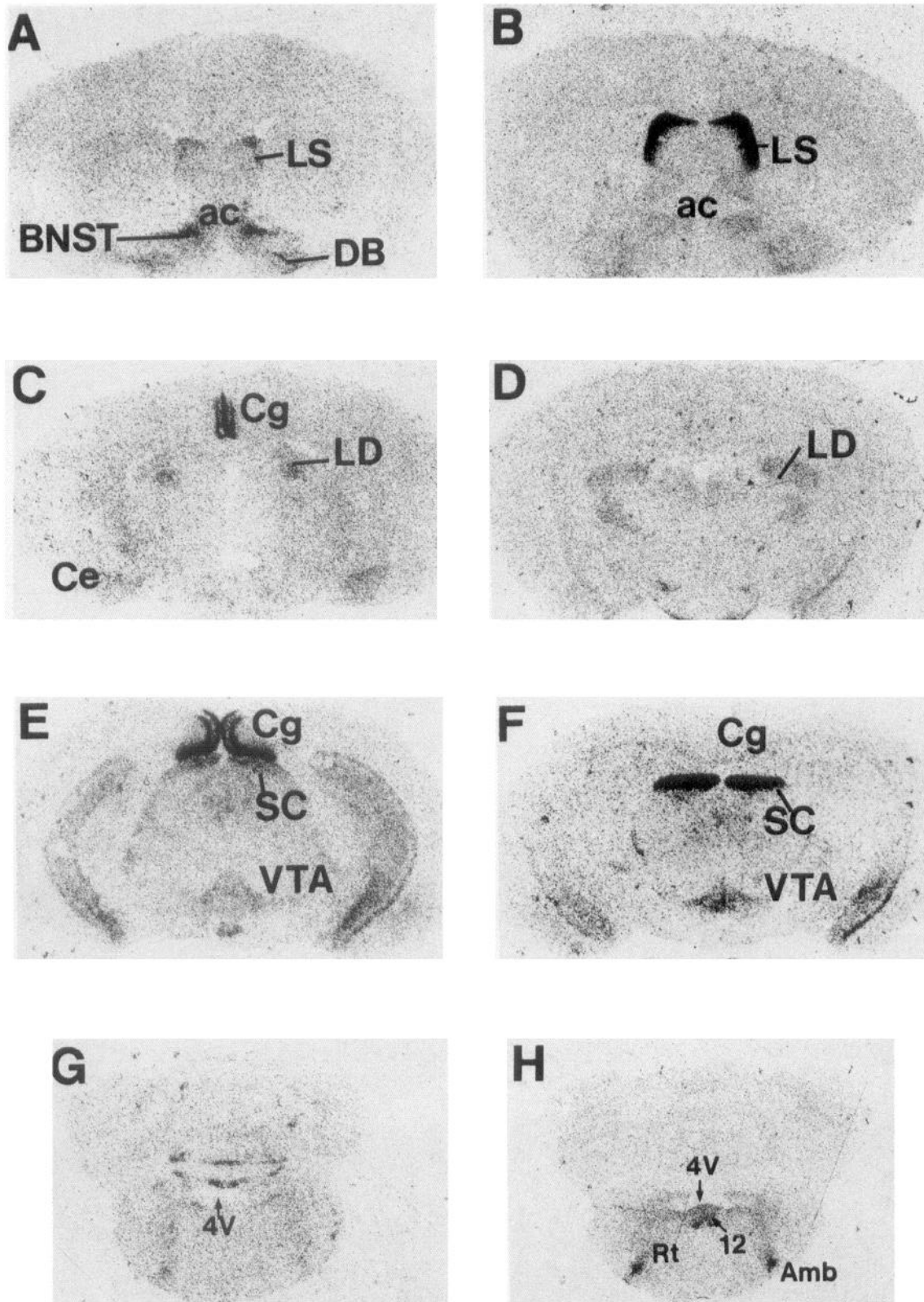
Figure 6. Binding of ¹²⁵I-sarc-AVP in pine and meadow voles. To extend the species comparison to other voles selected for patterns of social organization, the pattern of ¹²⁵I-sarc-AVP binding was compared in monogamous pine voles (*A, C, E,* and *G*) and polygynous meadow voles (*B, D, F,* and *H*). Bright-field images shown here differ from the maps shown for prairie and montane voles (Fig. 3) except in the lateral septum, for which binding was low in pine vole and high in meadow vole. In the pine vole, ¹²⁵I-sarc-AVP binding was particularly high in the posterior cingulate cortex, extending back into the retrosplenial cortex (here labeled *Cg*), but conspicuously low in the superior colliculus (*SC*). Binding was not detectable in the cingulate or retrosplenial cortex of the meadow vole, but was high in the superior colliculus (compare *E* and *F*). ¹²⁵I-sarc-AVP binding was also high in the hypoglossal nucleus (*12*), the reticular nucleus (*Rt*), and nucleus ambiguus (*Amb*) of the meadow vole, but not in the pine vole (compare panels *G* and *H*). All abbreviations and quantitative values provided in Table 4. Scale bar, 1.0 mm.

Table 4. Comparison of pine and meadow voles: ^{125}-I-sarc-AVP binding (mean ± SEM dpm/mg tissue equivalent)

Region	Abbrev.	Pine	Meadow
Olfactory (main and accessory)			
Internal granular layer	IGL	340 ± 131	168 ± 168
External plex. layer	EPL	ND	ND
Ant. olf. nucleus	AOP	ND	ND
Vomeronasal nerve	VN	ND	ND
Granule cell layer	Gr	876 ± 131	497 ± 287
Accessory olf. bulb	AOB	1270 ± 146	931 ± 183
Forebrain			
Diagonal band	DB	1747 ± 367	1292 ± 613
Nuc. accumbens	NAcc	ND	ND
Lateral septum	LS	708 ± 184	6298 ± 1644*
Bed nuc. of stria terminalis	BNST	1829 ± 375	1220 ± 288
Cingulate cortex	Cg	5741 ± 946**	ND
Ventral subiculum	VS	1204 ± 423	674 ± 168
Dentate gyrus (post.)	DG	ND	ND
Amygdala (central nuc.)	Ce	284 ± 151	92 ± 92
Habenula (lateral)	H	ND	ND
Thalamus (laterodorsal)	LD	216 ± 216	1170 ± 1092
Midbrain			
Superior colliculus	SC	569 ± 333	6010 ± 1506*
Ventral tegmental area	VTA	412 ± 232	210 ± 105

Values represent mean dpm/mg tissue equivalent from three males in each species.
* $p < 0.05$.
** $p < 0.01$; comparing means by Student's t test.

in these same brains ^{125}I-sarc-AVP binding to other forebrain regions appeared unaffected. The reason for the variability in thalamic binding remains unclear at this time. In addition, binding to the central nucleus of the amygdala of the prairie vole, clearly detectable with ^{3}H-AVP, was barely evident with ^{125}I-sarc-AVP. The difference may reflect the tendency of ^{3}H-AVP to bind to both oxytocin and AVP receptors (Tribollet et al., 1988), as the central amygdala in the prairie vole is labeled by ^{125}I-OTA, a selective oxytocin ligand (Witt et al., 1991; Insel and Shapiro, 1992). The alternative explanation, that ^{125}I-sarc-AVP binds to non-AVP receptors, seems less plausible as, consistent with previous studies (Phillips et al., 1988; Ferris et al., 1993), we found high selectivity for this ligand with as much as 93% displacement by a selective V_1 ligand and no evident displacement by a selective oxytocin agonist. Finally, the apparent absence of gender differences in either prairie or montane voles is surprising given the previously reported androgen-dependent behavioral responses to AVP (see above), the gender difference in AVP receptors in the golden hamster (Dubois-Dauphin et al., 1991), and the profound gender differences in the patterns of AVP innervation in the prairie vole (Bamshad et al., 1993).

Functional studies of second-messenger activation generally support the regional differences in ligand binding in that AVP induced phosphoinositol in the prairie vole thalamus and the montane vole septum. The magnitude of phosphoinositol induction in the septum was somewhat less than previous reports (Shewey and Dorsa, 1988; Poulin and Pittman, 1993b), probably due to the relatively small percentage of tissue with AVP receptors in each sample. In these studies, the need for large tissue volumes was offset by the importance of quickly delivering the tissue into an oxygenated buffer. We felt that the rapid dissection of 10 brains was the temporal limit for this method

and 10 vole brains provided just enough tissue for three sets of triplicate tubes (two doses of AVP in addition to buffer). Therefore, we were unable to perform extensive dose–response studies to determine the optimal concentration of AVP for the induction of phosphoinositol. Nevertheless, a consistent increase in phosphoinositol could be detected in those regions with abundant ^{125}I-sarc-AVP binding. The one conspicuous exception to this rule was the superior colliculus, which showed intense binding but no evidence of phosphoinositol induction. AVP receptors in this region may be coupled to a different effector system (Thibonnier, 1992) and thus play an important role in the processing of visual information. Even in the pine vole, which showed a relative absence of binding to the superior colliculus, there is a striking emergence of binding in the retrosplenial cortex—consistent with a role for AVP in the visual system. However, without positive data regarding cellular effects of AVP in either the superior colliculus or the retrosplenial region, one should be guarded about presuming any such function. Indeed, it seems equally plausible from our data that these binding sites are not functional but remain as an evolutionary vestige of the arginine-vasotocin (AVT) innervation of the visual system of the amphibian (Boyd et al., 1992), possibly related to the AVT receptor found in the avian optic tectum (Voorhuis et al., 1988).

What accounts for the species differences in receptor distribution? As noted above, these species do not differ in their distribution of two other neuroreceptors that have been examined, so the patterns with AVP and oxytocin cannot be attributed to some general species difference in chemical neuroanatomy. It seems possible that the species differences might reflect alterations in the selectivity for the ligand, but our competition data revealed similar patterns of displacement in both species, with both showing the expected pattern of $V_1 \gg V_2, OT$.

Although our rather primitive saturation experiments describe similar kinetics in prairie vole thalamus and montane vole lateral septum, these experiments do not rule out the possibility that (1) ^{125}I-sarc-AVP is binding to different receptors in the two species or (2) ^{125}I-sarc-AVP is binding to a family of receptors with variable species expression. We hope that current attempts to clone the cDNAs for these receptors in these different species will provide some clarity on each of these possibilities. We have also entertained the 'possibility that there is only a single V_1 receptor across these various species, but that species differences in either genomic DNA regulatory elements or local transcription factors lead to the observed patterns of regional expression.

As prairie voles show pair bonding and paternal behavior and montane voles do not, which of the receptor fields in the prairie vole are likely to be responsible for these important species differences in behavior? The most consistent species differences across the four species was the increased binding in the lateral septum of the nonmonogamous voles relative to the monogamous voles. Several lines of evidence suggest the importance of this region for AVP's behavioral effects. AVP facilitates social memory in rats (Dantzer et al., 1988), flank marking in hamsters (Irvin et al., 1990), and paternal behavior in prairie voles (Wang et al., in press) following microinjection into the lateral septum. Moreover, AVP appears to have both excitatory effects and to induce long-term potentiation in rat lateral septum slices *in vitro* (van den Hooff et al., 1989; Van den Hooff and Urban, 1990). The vasopressinergic innervation of the lateral septum has been shown to be sexually dimorphic in the rat (De Vries et al., 1985) and the prairie vole (Bamshad et al., 1993) and to be associated with both aggressive behavior (Compaan et al., 1993) and paternal care (Bamshad et al., 1993). As territorial defense and paternal care are critical aspects of monogamous social organization, one would predict that monogamous males should show intense AVP receptor binding in the lateral septum. Contrary to this prediction, we found few AVP receptors in the lateral septum from prairie and pine voles, the two monogamous species. The reduced functional response to AVP in the prairie vole septum, relative to the montane vole septum, further underscores this paradox.

We do not have a simple explanation for the apparent paucity of AVP receptors in the prairie vole lateral septum but we have one additional observation that may resolve this problem. Sexually naive prairie voles (used in the present study) do not respond to centrally administered AVP with an increase in aggression unless (1) AVP is given chronically via a minipump or (2) the vole is given extensive mating experience (believed to release AVP into the lateral septum) (J. Winslow and T. Insel, unpublished observations). As AVP administration has been shown recently to sensitize the lateral septum to subsequent AVP (as measured by the induction of phosphoinositol) (Poulin and Pittman, 1993a), it seems plausible that the AVP receptors in the lateral septum of the sexually naive prairie (and pine) vole are immature until after mating when the repeated release of endogenous AVP facilitates the coupling of the receptor to intracellular second-messenger systems. Unfortunately, this hypothesis does not explain why montane voles, which are less territorial and less paternal, show a rich expression of functionally coupled AVP receptors in the naive state. If montane and meadow voles lack the AVP innervation of the lateral septum that is so prominent in male (but not female) prairie voles or if AVP is not released in these species in response to mating or pup stimuli, then the abundance of receptors in this region will

not subserve pair bonding or paternal behavior. We are currently investigating these possibilities with comparative studies of AVP projections in these same four species.

Comparative studies are by their very nature correlational. Nevertheless, these kinds of studies in conjunction with experimental studies using selective agonists and antagonists provide a converging picture of AVP as an important mediator of social signals for males. How does AVP release translate into complex behaviors such as pair bonding and paternal care? The present results can only begin to answer this question by suggesting the likely targets for AVP's central effects in monogamous males. The next step will require a careful analysis of what aspects of these complex behaviors are affected by AVP. In other species, there are convincing data for AVP effects on social recognition, regulation of body temperature (possibly promoting huddling and nest building), and responses to social separation (reviewed in de Wied et al., 1993). The intense binding evident in the accessory olfactory system suggests that AVP could also modulate the processing of vomeronasal signals. Receptors in the thalamus may modulate information flow from brainstem projections through relay nuclei. Further research will be necessary to link the species-specific patterns of receptors shown here to discrete aspects of behavioral responses.

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