Monogamy Evolves through Multiple Mechanisms: Evidence from V1aR in Deer Mice

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Associate editor: Adriana Briscoe

Research article

Abstract

Genetic variation in *Avpr1a*, the locus encoding the arginine vasopressin receptor 1A (V1aR), has been implicated in pair-bonding behavior in voles (genus *Microtus*) and humans, raising the possibility that this gene may contribute commonly to mating-system variation in mammals. In voles, differential expression of V1aR in the brain is associated with male partner–preference behavior in a comparison of a monogamous (*Microtus ochrogaster*) and promiscuous (*Microtus montanus*) species. This expression difference is correlated, in turn, with a difference in length of a 5′ regulatory microsatellite in *Avpr1a*. Here, we use a combination of comparative sequencing of coding and regulatory regions, analysis of neural expression patterns, and signaling assays to test for differences in V1aR expression and function among eight species of deer mice (genus *Peromyscus*). Despite well-documented variation in *Peromyscus* social behavior, we find no association between mating system and length variation in the microsatellite locus linked to V1aR expression in voles. Further, there are no consistent differences in V1aR expression pattern between monogamous and promiscuous species in regions of the brain known to influence mating behavior. We do find statistical evidence for positive selection on the V1aR coding sequence including several derived amino acid substitutions in a monogamous *Peromyscus* lineage, yet these substitutions have no measurable effect on V1aR signaling activity. Together, these results suggest that mating-system variation in rodents is mediated by multiple genetic mechanisms.

Key words: mating system, monogamy, *Peromyscus*, social behavior, vasopressin, vasopressin receptor.

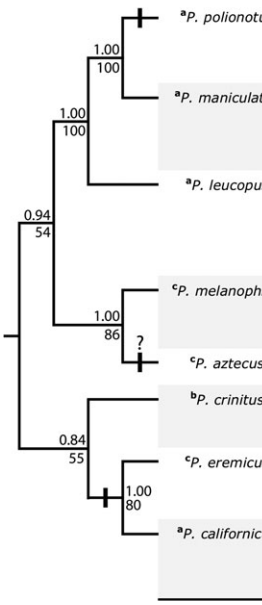
Introduction

Extensive variation in social behavior exists within and between species. Social behavior, particularly in the context of reproduction, can be a major determinant of organismal fitness, and thus, understanding its genetic regulation is of great interest to evolutionary biologists. One important open question is whether social behavior is controlled by the same genes in different taxa (Robinson et al. 2008).

Monogamy is rare in mammals; only 3% of species form exclusive pair bonds during their reproductive period (Kleiman 1977), and further, parentage analysis has revealed that many of these socially monogamous species are not genetically monogamous (Cohas and Allaine 2009). Monogamy comprises a set of complex behavioral components, including pair bonding in males and females, parental care, and increased aggression in the context of home range defense. The few truly monogamous species are dispersed across the mammalian phylogeny, indicating that monogamy has likely evolved multiple times in mammals. Comparing the genetic basis of monogamy in distant mammalian taxa provides an opportunity to examine whether evolution of complex social behavior occurs through conserved mechanisms.

The neurohypophyseal hormone arginine vasopressin (AVP), largely mediated through the AVP receptor 1A (V1aR), plays a central role in mammalian social behavior (Goodson and Bass 2001). Genetic variation in *Avpr1a*, which encodes V1aR, has been linked to male pair-bonding behavior in voles (genus *Microtus*, reviewed in Nair and Young 2006) and in humans (Walum et al. 2008). It has been hypothesized that V1aR may be an important contributor to mating-system variation across mammals more generally—increases or decreases in its expression in certain brain regions could act as a molecular switch turning on and off pair-bonding behavior—and thus has been referred to as a "monogamy gene."

V1aR's role in mating behavior has been studied most extensively in voles. Variation in the length of a microsatellite repeat in the 5′ promoter region of *Avpr1a* is associated with differences in V1aR expression in several regions of the brain (Young et al. 1999), which in turn contribute to variation in male reproductive behavior (i.e., pair bonding, paternal care, and aggression) both within and between *Microtus* species (Hammock and Young 2004, 2005). These results have been confirmed by functional assays: overexpression of *Avpr1a* constructs from a monogamous male



Species	Mating system	Evidence: Range overlap	Evidence: Reproductive organs	Evidence: Reproductive Behavior	Evidence: Reproductive characteristics	Evidence: Genetics	Reference
[a]*P. polionotus*	M	√	√	√	√	√	Kleiman 1977; Dewsbury 1981; Foltz 1981; Bree and Hoekstra, unpub. data
[a]*P. maniculatus*	P	√	√	√	√	√	Birdsall and Nash 1973; Dewsbury 1981; Ribble and Millar 1996; Bree and Hoekstra, unpub. data
[a]*P. leucopus*	P	√	√	√	√	√	Mineau and Madison 1977; Dewsbury 1981; Wolff 1989; Xia and Millar 1991; Bree and Hoekstra, unpub. data
[c]*P. melanophrys*	P		√	√	√		Dewsbury 1981; Ferkin 1987; Bree and Hoekstra, unpub. data
[c]*P. aztecus*	U		√ (M)				Demas and Nelson 1998
[b]*P. crinitus*	P				√	√	Dewsbury 1981; Shurtliff, Pearse, and Rogers 2005
[c]*P. eremicus*	M		√	√	√		Kleiman 1977; Dewsbury 1981; Bree and Hoekstra, unpub. data
[a]*P. californicus*	M	√	√	√	√	√	Kleiman 1977; Dewsbury 1981; Ribble 1990, 1991; Bree and Hoekstra, unpub. data

Fig. 1. Mating systems and phylogenetic relationships of eight *Peromyscus* species. Mating system (M = monogamous, P = promiscuous, and U = uncertain) is shown, with check marks to indicate the available evidence for each species. Evidence includes range overlap in natural populations, weight of reproductive organs (i.e., testes, epididymis, vas deferens, prostate, and seminal vesicles), reproductive behavior (i.e., allogrooming and paternal care), reproductive characteristics (i.e., sexual dimorphism, intromission latency, number of ejaculations, Coolidge effect, presence of mating plug, and litter size), and genetics (i.e., paternity analysis in natural populations). The Bayesian/ML phylogeny topology is shown to the left, rooted with the outgroup (*Onychomys torridus*). Posterior probabilities are indicated above and bootstrap values below each node. Vertical black bars indicate when monogamy evolved, based on parsimony.

vole in the ventral forebrain of a promiscuous male can induce pair-bonding behavior (Lim et al. 2004). Furthermore, male laboratory mice transgenic for the monogamous vole *Avpr1a* locus show V1aR expression patterns and pair-bonding behavior typical of these voles (Young et al. 1999). This similar behavioral response in mice, which diverged from voles 22–25 Ma (Steppan et al. 2004), suggests that there may be common neural mechanisms controlling rodent mating behavior. However, a recent survey of *Avpr1a* variation in 21 *Microtus* species revealed that variation in the 5′ microsatellite locus is not consistently associated with mating-system differences (Fink et al. 2006).

In addition to V1aR expression differences, a comparison of *Microtus* and other mammals revealed extensive variation in both the amino acid sequence and length of the V1aR. It has been proposed that these structural changes also may contribute to variation in mating system (Fink et al. 2007). Comprehensive analysis of variation in both V1aR expression and structure in additional taxa that differ in mating system is needed to determine whether AVP signaling through V1aR represents a universal genetic mechanism controlling male reproductive behavior in mammals.

To this end, we tested the role of the AVP-V1aR pathway in governing mating behavior in deer mice of the genus *Peromyscus*. Monogamy has evolved independently at least twice in *Peromyscus* (fig. 1); thus, the genetic basis of mating behavior can be compared among closely related lineages as well as between *Peromyscus* and other mammals. Further, differences in AVP binding in several brain regions have been reported for *Peromyscus* species (Insel et al. 1991; Bester-Meredith et al. 1999; Bester-Meredith and Marler 2001). To assess the contribution of V1aR variation to mating system differences in *Peromyscus*, we analyzed patterns of sequence and expression variation in eight species representing a range of mating systems and phylogenetic distance. First, we tested for a role of V1aR expression on mating system by both assaying V1aR expression among species and genotyping two *Avpr1a* microsatellites, including one known to affect V1aR expression. Second, we sequenced the V1aR coding region and tested the functional relevance of derived amino acid substitutions. Together our results suggest that monogamy can evolve through multiple genetic mechanisms in rodents.

Materials and Methods

DNA Extraction, Amplification, and Sequencing

We sampled 26 individuals from eight *Peromyscus* species for sequence and/or microsatellite genotyping analysis (supplementary table S1, Supplementary Material online). We extracted DNA from tissue samples (liver, kidney, or tail) using a DNeasy Blood and Tissue kit (Qiagen, Valencia, CA). To confirm phylogenetic relationships among species, we sequenced a 1,213-bp region of the mitochondrial genome (including *COIII* and *ND3*) using published primers (Hoekstra et al. 2004).

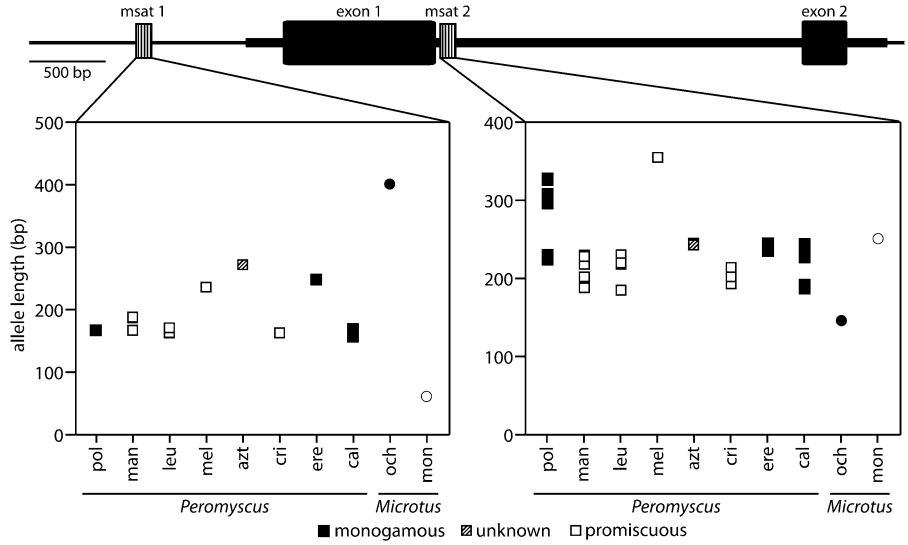


FIG. 2. Microsatellite variability in *Avpr1a*. Gene structure is shown: The sequenced flanking region is indicated by a thin line; hatched boxes indicate microsatellites (msat1 and msat2); and filled boxes indicate coding regions. Data from microsatellite 3 are not shown. Graphs below show allele sizes in *Peromyscus* (squares)—*Peromyscus polionotus* (*pol*), *P. maniculatus* (*man*), *P. leucopus* (*leu*), *P. melanophrys* (*mel*), *P. aztecus* (*azt*), *P. eremicus* (*ere*), and *P. californicus* (*cal*). Size of the homologous repeats in *Microtus* voles (circles)—*M. ochrogaster* (*och*) and *M. montanus* (*mon*)—are inferred from alignment of primers to GenBank sequences (AF069304, AF070010) for comparison. Alleles from monogamous species are shown in black, promiscuous species in white, and alleles from species whose mating system is uncertain are hatched.

To assess regulatory and coding sequence variation in *Avpr1a*, we first used a genome-walking approach (Universal Genome Walker Kit, Clontech, BD Biosciences) to capture most of a 5.7-kb region encompassing 1.7 kb upstream of the start codon and ending 400 bp downstream of the stop codon in two divergent species, *Peromyscus polionotus* and *Peromyscus californicus* (fig. 2). Based on these sequences, we designed primers to amplify and sequence two regions (supplementary table S2, Supplementary Material online): the 1.7 kb region upstream of *Avpr1a* (the putative 5′ regulatory region) in three additional species and the entire 1.3-kb coding region (2 exons) in six additional species. We cloned amplicons of the promoter region (TOPO-TA, Invitrogen, Carlsbad, CA) and directly sequenced polymerase chain reaction (PCR) products from the coding region. We performed cycle sequencing using BigDye terminator (v. 3.1, Applied Biosystems) and ran products on an ABI 3100 automated sequencer. We checked base calls by eye and assembled contigs in SEQUENCHER (Gene Codes, Ann Harbor, MI). Haplotypes were inferred computationally using PHASE (v 2.1.1, Stephens et al. 2001; Stephens and Donnelly 2003). We aligned nucleotide sequences using MUSCLE (v. 3.6, Edgar 2004). Using DNASP (v. 4.20.2, Rozas et al. 2003), we performed a sliding window analysis of average pairwise sequence differences in *Avpr1a*. Sequences were deposited in GenBank (accession numbers GU254538–GU254609).

Phylogeny Construction

Because existing phylogenies are sometimes inconsistent (e.g., Turner and Hoekstra 2006; Bradley et al. 2007), we constructed Bayesian and maximum likelihood (ML) phylogenies to determine relationships among the eight species in this study. Data include 1,213 bp of mitochondrial sequence (from this study and Turner et al. 2008) and 1,201 bp from two nuclear genes (*Mc1r* and *Lcat*, Turner and Hoekstra 2006) for three individuals from each *Peromyscus* species and one outgroup, *Onychomys torridus*. We determined the appropriate substitution model (general time reversible [GTR] + *I* + *G*) using MrModeltest (v. 2.3 Nylander 2004). In MrBayes (v. 3.1.2, Huelsenbeck and Ronquist 2001), we performed two runs of Markov chain Monte Carlo for 10 million generations (average standard deviation of split frequencies <0.001), discarding the first million generations of each run as burn-in. We generated the ML tree using heuristic search in PAUP* (v. 4b10, Swofford 2002) and performed 1,000 bootstrap replicates using GARLI (v. 0.96b Zwickl 2006).

Microsatellite Genotyping

To test for associations between mating system and length variation in the *Avpr1a* regulatory region, we genotyped two microsatellite loci in 24 individuals representing eight *Peromyscus* species (supplementary table S1, Supplementary Material online) using primers designed based on our sequence alignments (supplementary table S2, Supplementary Material online). A CAG tag (5′ CAGTCGGGCGTCATCA 3′) was added to the 5′ end of forward primers to allow for fragment analysis using a single fluorescently labeled complementary probe (Boutin-Ganache et al. 2001). The amplicons were then run on an ABI 3130 or 3730 sequencer and assigned genotypes using Genemapper (v. 3.7, Applied Biosystems).

V1aR Autoradiography

We measured neural V1aR expression in four sexually mature individuals, two males and two females, from each of five *Peromyscus* species and two subspecies each of *Peromyscus maniculatus* and *P. polionotus*. Animals were asphyxiated by CO_2, and their brains were removed and stored at −80 °C. We sectioned five sets of slices (20-μm thickness and 100-μm spacing) on a CM 1900 cryostat (Leica Microsystems, Germany), starting at the base of the olfactory bulb and extending caudally until approximately the level of the posterior commissure. Sections were thaw mounted onto Superfrost slides (Fisher Scientific, Pittsburgh, PA) and stored at −80 °C.

We performed V1aR autoradiography on one complete rostrocaudal set for each individual, using 50 pM ^{125}I-linear-AVP (PerkinElmer, Waltham, MA; Young et al. 1997). Binding specificity of this ligand has been confirmed previously in two *Peromyscus* species (*Peromyscus leucopus* and *P. californicus*; Bester-Meredith and Marler 2001) and in other rodents (e.g., *Rattus*, Barberis et al. 1995; *Microtus*, Young et al. 1997; and *Scotinomys*, Campbell et al. 2009). To identify neuroanatomical boundaries, we counterstained for acetylcholinesterase (AChE) on one set of replicate slides for each taxon following Lim et al. (2004), with the following modifications: a 20-min fixation in 4% paraformaldehyde followed by two 5-min washes in 1× phosphate buffered saline were performed prior to staining, ethopropazine was omitted from the enzymatic reaction mixture, and silver intensification solution was not used. Finally, after dehydration in ascending ethanol, we rinsed slides in Citrisolv (Fisher Scientific) and mounted them using Permount (Fisher Scientific).

To quantify binding, we measured average pixel density readings of autoradiograms using NIH ImageJ software version 1.37 (http://rsb.info.nih.gov/ij/) in three adjacent sections of the ventral pallidum (VP) and lateral septum (LS). As a control, we measured nonspecific binding in the caudate putamen in each section. To calculate specific binding values, we subtracted averaged nonspecific binding from mean binding values for each region. Using a standard curve based on ^{125}I-labeled autoradiographic standards, we transformed average pixel densities into disintegrations per minute/milligram tissue equivalent.

We pooled binding data for the sexes because we did not find any sex effects ($P > 0.05$, two-way analysis of variance [ANOVA] testing species, sex, and interaction), although with the small sample size this result should not be interpreted as evidence for no sex-based differences in binding. We also treated each species independently because we did not find evidence for phylogenetic autocorrelation using a test for serial independence (Abouheif 1999) on species means performed using the program Phylogenetic Independence (Reeve and Abouheif 2003). We tested for significant differences in binding in each brain region using one-way ANOVA and compared species means using a post hoc Tukey's honestly significant difference technique (HSD) test. Statistical analyses were performed in JMP (v. 5, SAS, Cary, NC) or Stata (v. 8, Stata, College Station, TX).

V1aR Signaling Assays

To conduct signaling assays, we first amplified and cloned the two *Avpr1a* coding exons from *P. californicus*, *Peromyscus crinitus* and *P. maniculatus*. Next, using a PCR-based overlapping fragment mutagenesis approach, we joined the exons and introduced epitope-tags (N-terminal hemagglutinin and C-terminal FLAG epitopes). We then generated a chimeric construct (termed "maximum") by PCR-based site-directed mutagenesis and restriction fragment replacement, which included all amino acid substitutions found in *P. californicus*. Finally, we subcloned the amplicons into the mammalian expression vector pcDps and confirmed construct sequences by restriction analysis and sequencing.

To compare the receptor function of different alleles, we measured the accumulation of D-*myo*-inositol phosphate 1 (IP1), a downstream product of V1aR-mediated signaling. COS-7 cells were cultivated in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a humidified 7% CO_2 incubator. COS-7 cells were split into 24-well plates (55,000 cells/well) and 24 h later transfected with 0.3-μg expression plasmid per well using Lipofectamine 2000 (Invitrogen). The next day, we performed IP1 accumulation assays, according to the manufacturer's protocol, in the presence of 0–10 μM AVP (Sigma, Munich, Germany). The IP1 contents of cell extracts were determined using a nonradioactive competitive immunoassay (IP-One enzyme-linked immunosorbent assay, Cisbio US, Bedford, MA) (Trinquet et al. 2006). This assay is based on the competition between free IP1 and IP1-HRP (horseradish peroxidase) conjugate for a limited number of binding sites on an anti-IP1 monoclonal antibody. The IP1 accumulation data were analyzed using GraphPad Prism program (v. 5.01 for Windows, San Diego, CA).

Results

Phylogeny

Based on our multilocus data, trees constructed using Bayesian and ML methods were identical in topology. Relationships among species are consistent with published species trees, with the exception of the deepest split. Our results show the *P. polionotus*/*P. maniculatus*/*P. leucopus* clade clusters with the *Peromyscus melanophrys*/*Peromyscus aztecus* clade, albeit with low bootstrap support (fig. 1). Published trees show the *P. melanophrys*/*P. aztecus* clade grouped with the *P. crinitus*/*P. eremicus*/*P. californicus* clade (based on two nuclear loci; Turner and Hoekstra 2006) or the *P. polionotus*/*P. maniculatus*/*P. leucopus* clade grouped with the *P. crinitus*/*P. eremicus*/*P. californicus* clade (based on a single mtDNA locus; Bradley et al. 2007). Uncertainty in the relationship among the three clades does not affect interpretation of the V1aR data presented here because parsimony mapping of monogamy does not differ among these topologies.

Variation in Noncoding Regions of *Avpr1a*

In five *Peromyscus* species, the 5′ flanking region of *Avpr1a* is similar in sequence and length: 75% of sites are identical

(1,198/1,594 sites, excluding two repetitive regions), and the largest indel is only 18 bp and polymorphic within *P. leucopus* (supplementary fig. S1, Supplementary Material online). There are no obvious structural differences (e.g., insertions, deletions, and inversions) differentiating monogamous species (*P. polionotus* and *P. californicus*) from the most closely related promiscuous species (*P. maniculatus* and *P. crinitus*, respectively, fig. 1). Because of the association between repeat polymorphism and social behavior in voles and humans, we focused on identifying repetitive regions in the 5′ flanking sequence. Specifically, the microsatellite locus associated with male affiliative behavior in *Microtus* is conserved in *Peromyscus*, located ~980 bp upstream of the start codon (microsat 1, fig. 2). We genotyped a second microsatellite in the *Avpr1a* intron, located just after the 5′ splice site, which is also present in *Microtus* and variable in *Peromyscus* (microsat 2, fig. 2). Finally, we identified a repeat region (microsat 3, located 1.7 kb upstream of the start codon) with no clear motif.

Overall, we found no association between mating system and allelic variation at any of the three microsatellite loci examined. For microsatellites 1 and 2, the ranges of allele length for monogamous and promiscuous taxa overlap (fig. 2), and several alleles are shared. For example, *P. californicus* and *P. crinitus* have a common allele (163 bp) at microsatellite 1, and *P. polionotus* and *P. maniculatus* share three alleles (224, 228, and 230 bp) at microsatellite 2. Variation in microsatellite 3 correlated with phylogenetic distance rather than mating system—repeats were shorter in *P. californicus*/*P. crinitus* (18–28 bp) than in *P. polionotus*/*P. maniculatus*/*P. leucopus* (43–80 bp).

Variation in Neural V1aR Expression

We next examined variation in V1aR expression among *Peromyscus* species in the VP and LS, two regions of the brain with important roles in pair bonding in *Microtus* (Insel et al. 1994; Young et al. 1997). V1aR expression level in the VP does not vary significantly among species (fig. 3). In contrast, expression in the LS is strongly differentiated (ANOVA $F_{6,36} = 15.84$, $P < 0.0001$), confirming that the binding assay is sensitive enough to detect interspecific variation. We did not, however, find consistent differences in expression between monogamous and promiscuous species. For example, expression is lower in monogamous *P. polionotus* than in its promiscuous sister species, *P. maniculatus* (Tukey HSD, $P < 0.05$), but not significantly different from another closely related promiscuous species, *P. leucopus*. Moreover, two closely related monogamous species, *P. californicus* and *P. eremicus*, have significantly different expression levels in the LS (Tukey HSD, $P < 0.05$). Thus, directional changes in V1aR expression in the LS are not consistently associated with changes in mating behavior.

Coding-Sequence Variation

V1aR amino acid sequence is relatively conserved in *Peromyscus*: 6% (27/426) of sites are variable (fig. 4A) compared with 3–29% of sites in 15 other *Peromyscus* proteins (Turner and Hoekstra 2006; Turner et al. 2008). However, 12 of 27 variable sites have derived substitutions in the monogamous *P. californicus*/*P. eremicus* lineage. These derived sites, and variable sites in *Peromyscus* overall, are concentrated in the N-terminal extracellular domain of V1aR (fig. 4B). A sliding window analysis shows a peak in both d_N and d_N/d_S in the first ~45 aa positions and that d_N/d_S values are greater than 1 in this region, consistent with positive selection (fig. 4C).

Functional Characterization of V1aR Variants

To determine if these amino acid changes affect receptor function, we performed V1aR signaling assays. V1aR mediates signal transduction via G_q/phospholipase C and subsequent formation of IPs; therefore, we measured IP levels as a proxy for receptor signaling activity. Specifically, we compared the function of four alleles: a naturally occurring *P. californicus* allele, a maximum allele containing all amino acid substitutions observed in *P. californicus*, an allele from *P. crinitus* (the most closely related promiscuous species to *P. californicus*), and an allele from *P. maniculatus* that is nearly identical to the *Peromyscus* consensus sequence (tested alleles are shown in fig. 4). As expected, all the V1aR alleles showed concentration-dependent receptor activation in response to stimulation with the natural agonist AVP (fig. 5). However, we found no difference in either agonist potency (half maximal activity, EC_{50}) or efficacy (maximum activity, EC_{max}) among alleles (supplementary table S3, Supplementary Material online). Thus, the amino acid changes we tested—the derived excess in the monogamous *P. californicus*—have no measurable effect on V1aR function.

Discussion

The neuropeptide vasopressin, or its nonmammalian homologue vasotocin, is present in all vertebrate classes and plays an essential role in a broad range of social behaviors, including vocalization, aggression, and reproductive interactions. The pervasive importance of vasopressin has lead some to hypothesize that convergent evolutionary changes in social strategies, such as transitions from promiscuous to monogamous mating systems, may be caused by convergent changes in vasopressin signaling mechanisms (Goodson and Bass 2001). Associations between male pair-bonding behavior and variation in repeat length at *Avpr1a* microsatellite loci in both voles and humans suggests that there even may be a common molecular mechanism for evolutionary change in reproductive behavior mediated by the AVP–V1aR pathway.

The lack of correlation between *Peromyscus* mating system and repeat length at any of three identified *Avpr1a* microsatellite loci suggests that the precise molecular mechanism causing divergence in mating behavior is not the same as that proposed for *Microtus* and humans. This result may not be particularly surprising because control of V1aR expression may differ even within *Microtus*—for example, the simple association between repeat length and

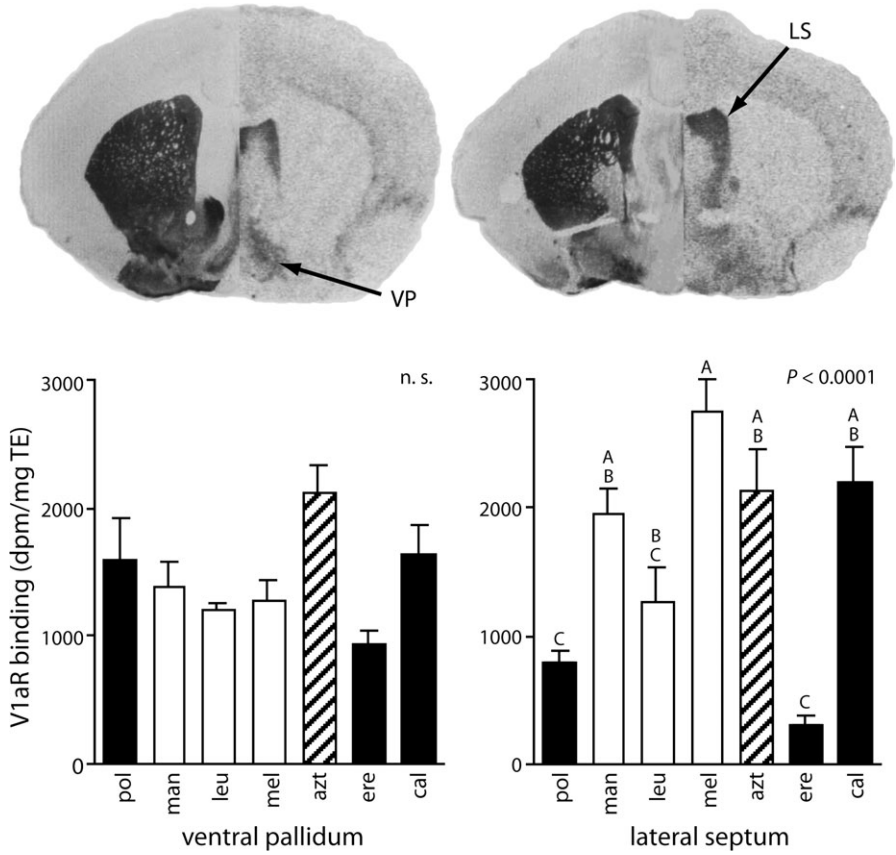


Fig. 3. Neural V1aR expression patterns. Autoradiograms of *Peromyscus* brains are shown: Left side of images shows sections stained with AChE to identify anatomical features, and right side highlights V1aR expression in the VP and LS. Graphs show V1aR expression level in VP and LS of *P.* polionotus (*pol*), *P. maniculatus* (*man*), *P. leucopus* (*leu*), *P. melanophrys* (*mel*), *P. aztecus* (*azt*), *P. eremicus* (*ere*) and *P. californicus* (*cal*). Species are ordered by phylogenetic relationship (see fig. 1). Monogamous species are in black, promiscuous in white, and uncertain are hatched. Significance of the association between species and expression level (ANOVA) is indicated for each brain region. Letters above each bar for LS indicate results of post hoc comparisons of species means (Tukey HSD); species without shared letters are significantly different ($P < 0.05$).

mating system differences between *Microtus montanus* and *Microtus ochrogaster* (Young et al. 1999) was not consistent among a larger sample of *Microtus* species (Fink et al. 2006).

It is still possible that monogamy evolved through a convergent process in *Peromyscus*—a change in V1aR neural expression pattern, but a different molecular switch caused regulatory divergence. Although we found extensive variation in V1aR expression among *Peromyscus* species in one brain region, we found neither the pattern reported in *Microtus* (higher expression in monogamous species in the VP and lower expression in the LS) nor any other correlation between expression and mating system. Hence, a convergent role for V1aR regulatory change in mating system divergence in *Peromyscus* is unlikely. We cannot, however, rule out the possibility that small changes in V1aR expression level or expression in other regions of the brain cause social evolution in *Peromyscus*. *In vivo* manipulation of V1aR expression, as performed in voles (Lim et al. 2004), is needed to directly test for effects of site-specific increases or decreases in expression on male reproductive behavior.

For the LS, our results are consistent with a previous study that reported higher AVP binding in *P. californicus* than in *P. leucopus* (Bester-Meredith et al. 1999). This expression pattern, opposite to the one observed in voles, appears correlated with differences in aggressive behavior; behavioral responses to neural infusions of AVP and V1aR antagonists provide further evidence for a role of V1aR in aggression in *Peromyscus* and other rodents (Ferris et al. 1986; Bester-Meredith et al. 2005). Goodson and Bass (2001) noted that the apparent discrepancy between voles and *Peromyscus* could be explained if V1aR expression in the LS is associated with social spacing rather than pair bonding—species that maintain exclusive territories (e.g., *P. californicus* [Ribble and Salvioni 1990] and *M. montanus* [Tamarin 1985]) have higher V1aR expression in the LS than species with greater range overlap (e.g., *P. leucopus* [Wolff 1985] and *M. ochrogaster* [Getz et al. 1993]). This hypothesis is particularly intriguing because arginine vasotocin, an AVP homologue, modulates territorial behavior in birds (Goodson and Bass 2001), and V1aR expression in several brain regions is correlated with communal living in female tuco-tucos (Beery et al. 2008) and with social spacing in singing mice (Campbell et al. 2009). The results presented here from additional *Peromyscus* species, however, do not support an association between V1aR expression

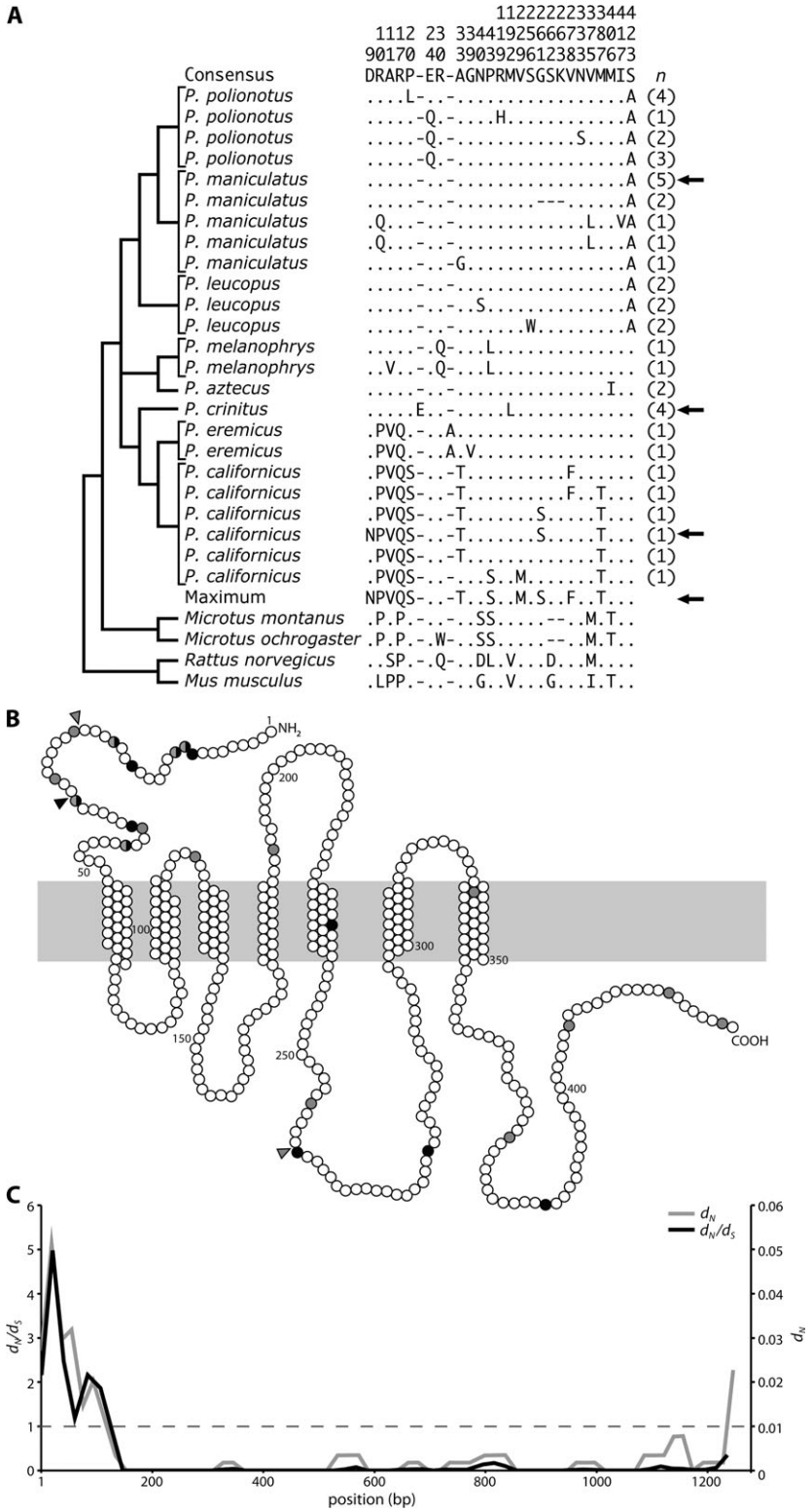


Fig. 4. V1aR coding sequence evolution in *Peromyscus*. (*A*) Alignment of variable amino acid sites. Dots indicate identity with the consensus sequence. Amino acids for *Microtus montanus* (AF070010), *Microtus ochrogaster* (AF069304), *Rattus norvegicus* (NM_053019), and *Mus musculus* (NM_016847) are provided below for the sites variable in *Peromyscus*. Alleles tested in IP-one assays are indicated with arrows. The maximum allele is a chimeric construct containing all *P. californicus* sequence variants, created for functional analysis (see Materials and Methods). (*B*) V1aR structure indicating transmembrane (gray bar), extracellular (above) and intracellular (below) regions. Variable sites in *Peromyscus* are in black (derived substitution in *P. californicus*/*P. eremicus* lineage), gray (derived substitution in another lineage), or both (independent substitutions in *P. californicus*/*P. eremicus* and another lineage). Arrowheads indicate sites of insertions/deletions. (*C*) A sliding window analysis of sequence divergence at nonsynonymous sites (d_N, in gray) and the ratio of nonsynonymous-to-synonymous divergence (d_N/d_S, in black) for an alignment of *Avpr1a* coding sequence from eight *Peromyscus* species (d_N—35 sites, step size 14 sites, d_N/d_S—50 sites, step size 20 sites).

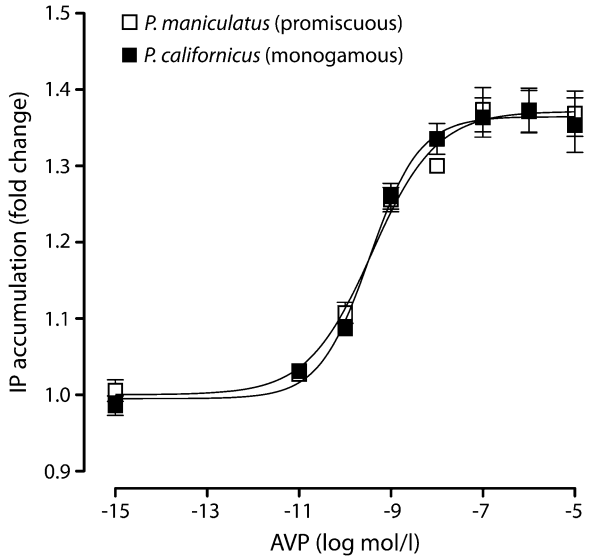


Fig. 5. V1aR signaling assay for *Peromyscus*. *P. maniculatus* (promiscuous, open squares) and *P. californicus* (monogamous, black squares), transiently expressed in COS-7 cells, are shown. Intracellular IP accumulation (shown as fold change over unstimulated *P. maniculatus*) was measured in response to increasing concentrations of AVP. Data are given as mean ± standard error of two independent assays, each performed in duplicate.

and social spacing. For example, home ranges in *P. maniculatus* are sometimes highly overlapping (Wolff 1985), as in *P. leucopus*, yet V1aR expression level in the LS in P. *maniculatus* is similar to expression in *P. californicus* (fig. 3). Evidence is lacking more generally for any clear, consistent relationship between V1aR expression pattern and social behavior in *Peromyscus*.

V1aR Structure and Function

V1aR signaling may be mediated through changes in expression level or changes in the receptor itself—increased signaling may be achieved, at least theoretically, through an increase in receptor number or alternatively receptor signaling potential. A recent study comparing *Avpr1a* coding sequence from *Microtus* species and a phylogenetically diverse sample of mammals revealed high amino acid substitution rates, extensive length variation and a peak in d_N/d_S in the N-terminal region of V1aR (Fink et al. 2007). However, the functional significance of amino acid sequence variation in V1aR had not been investigated.

As in voles, *Peromyscus* shows a strong signal of positive selection in the N terminus of V1aR. Furthermore, multiple derived amino acid substitutions occurred in the monogamous *P. californicus*/*P. eremicus* lineage, raising the possibility that they caused changes in receptor signaling and thus mating behavior in this *Peromyscus* lineage. However, IP1 signaling assays revealed no detectable effect of these amino acid substitutions (including six amino acid substitutions and an indel in the N-terminal region of V1aR, where d_N/d_S exceeds 1) on V1aR function, underscoring the importance of performing functional tests to validate inferences of positive selection based on statistical analyses of sequence data (e.g., Dean and Thornton 2007; Nozawa et al. 2009).

Why then are there so many amino acid changes in the N-terminal region of V1aR? This extracellular domain may be subject to species-specific selection for altered function unrelated to signaling. An alternative explanation to positive selection is relaxed functional constraint (Hughes 2007). Functional studies of V1aR in laboratory rats (Wheatley et al. 2007) and of many other rhodopsin-like G-protein-coupled receptors (e.g., MC1R, Schioth et al. 1997) show that deletions of large portions of the N-terminal domain do not have substantial effects on signaling function. Further, only a few amino acids of the N terminus, located close to the junction with the transmembrane domain, are essential in V1aR signaling (Wheatley et al. 2007). Similar functional analyses in *Microtus* and other mammals are needed before we can conclude that V1aR evolves adaptively at the amino acid sequence level.

Potential Mechanisms of Mating System Divergence in *Peromyscus*

Identifying and considering both conserved and novel genetic determinants of the behavioral components that constitute mating systems in *Peromyscus*, *Microtus*, and other taxa can yield an insight into the evolution of social behaviors. Although we have shown that changes in V1aR expression, structure, and function are not consistently associated with changes in mating behavior in *Peromyscus*, this certainly does not rule out different changes in signaling through the AVP–V1aR pathway. Other elements of this pathway, such as AVP dynamics (e.g., rates of production and breakdown, timing, and location of release) or downstream responses to V1aR-mediated signaling may be important in behavioral evolution. Alternatively, other pathways may be involved. For example, the role of corticosterone, another hormone affecting male pair-bonding behavior (Carter et al. 1995), should be considered.

Importantly, transitions in mating system require changes in the social behavior of both males and females. Although the AVP–V1aR pathway influences mating system by regulating male reproductive behavior, female reproductive behavior is modulated primarily through oxytocin and the oxytocin receptor (OT–OTR pathway). OTR's role in female pair-bonding behavior is well characterized in voles (Donaldson and Young 2008), and differences in OTR abundance between a promiscuous and a monogamous *Peromyscus* species have been reported (Insel et al. 1991). Detailed analysis of OTR in additional *Peromyscus* species, similar to this study of V1aR, may reveal whether evolution of monogamous behavior in females occurred through convergent or distinct mechanisms.

Conclusion

We find that changes in V1aR expression and sequence are neither necessary nor sufficient to explain mating system variation among *Peromyscus* species. Our results do not rule out a role for V1aR in the evolution of monogamy

in *Peromyscus*, but it is unlikely to be a simple molecular switch that turns on male pair-bonding behavior, as reported in some vole species. Consequently, the genetic determinants of monogamy in mammals may be more complex than we had imagined; similar behavior can evolve through multiple mechanisms. These results contribute to our growing understanding of the genetic mechanisms responsible for social evolution and the conservation of these pathways among species.

Supplementary Material

Supplementary tables S1–S3 and supplementary figure S1 are available at *Molecular Biology and Evolution* online (http://www.mbe.oxfordjournals.org/).

Acknowledgments

We thank C. Eizaguirre for statistical advice, P. Krumbholz and C. Stäubert for technical assistance, members of the Hoekstra laboratory for useful discussion, and two anonymous reviewers for comments on an earlier version of this manuscript. M. Kalcounis-Rueppell, K. Nutt, J. Storz, A. Suazo, the M.L. Bean Life Science Museum (Brigham Young University), the Museum of Texas Tech University, the *Peromyscus* Genetic Stock Center, and the Museum of Vertebrate Zoology (University of California, Berkeley) kindly provided tissue samples. This research was supported by funds from the Deutsche Forschungsgemeinschaft (SFB 610) and the IZKF Leipzig to T.S., NSF (IOS-0316451) to S.M.P., and an Arnold and Mabel Beckman Foundation Young Investigator Award to H.E.H.

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