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Complex selection on a regulator of social cognition: Evidence of balancing selection, regulatory interactions and population differentiation in the prairie vole *Avpr1a* locus

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Abstract

Adaptive variation in social behaviour depends upon standing genetic variation, but we know little about how evolutionary forces shape genetic diversity relevant to brain and behaviour. In prairie voles (*Microtus ochrogaster*), variants at the *Avpr1a* locus predict expression of the vasopressin 1a receptor in the retrosplenial cortex (RSC), a brain region that mediates spatial and contextual memory; cortical V1aR abundance in turn predicts diversity in space use and sexual fidelity in the field. To examine the potential contributions of adaptive and neutral forces to variation at the *Avpr1a* locus, we explore sequence diversity at the *Avpr1a* locus and throughout the genome in two populations of wild prairie voles. First, we refine results demonstrating balancing selection at the locus by comparing the frequency spectrum of variants at the locus to a random sample of the genome. Next, we find that the four single nucleotide polymorphisms that predict high V1aR expression in the RSC are in stronger linkage disequilibrium than expected by chance despite high recombination among intervening variants, suggesting that epistatic selection maintains their association. Analysis of population structure and a haplotype network for two populations revealed that this excessive LD was unlikely to be due to admixture alone. Furthermore, the two populations differed considerably in the region shown to be a regulator of V1aR expression despite the extremely low levels of genomewide genetic differentiation. Together, our data suggest that complex selection on Avpr1a locus favours specific combinations of regulatory polymorphisms, maintains the resulting alleles at population-specific frequencies, and may contribute to unique patterns of spatial cognition and sexual fidelity among populations.

KEYWORDS
adaptation, behaviour/social evolution, gene regulation, population genetics—empirical, sexual fidelity

1 | INTRODUCTION

The fields of neurobiology and cognitive ecology seek to understand individual differences in complex behaviours, but are limited by a lack of data on the genetic mechanisms and evolutionary processes that shape intraspecific variation. Individual differences in social behaviour are particularly common, ranging from the continuous variation that characterizes "personality" in great tits (Naguib, van Rooij, Snijders, & van Oers, 2016) to the dramatic differences that define alternative reproductive tactics (Taborsky, Oliveira, &

Brockmann, 2008). Among spadefoot toads, for example, population density influences whether individuals adopt a cannibalistic morphology and social foraging aggregations, which are thought to be shaped by both genetic variation and environmental cues (Bazazi, Pfennig, Handegard, & Couzin, 2012; Pfennig, 1992). Similarly, the territorial behaviour of male side-blotched lizards consists of three discrete phenotypes that vary in aggressiveness, allowing each to dominate for brief periods before being displaced by an alternative, with population dynamics that have been compared to a game of rock–paper–scissors (Sinervo & Lively, 1996). Indeed, classic game theoretic approaches, more recent models and a substantial body of empirical data suggest frequency- and density-dependent selection should be major contributors to variation in social behaviour (Kokko & Rankin, 2006; Slatkin, 1979; Smith & Price, 1973; Sokolowski, Pereira, & Hughes, 1997). Such adaptive variation in behaviour must surely rest to some degree on heritable variation in gene function within the nervous system; nevertheless, we know little about heritable variation in the brain, its relationship to social behaviour or its interaction with the evolutionary forces of selection and drift. Here, we examine the population genetics of the *Avpr1a* locus, a gene extensively implicated in vertebrate social behaviour, and which is known to be causally related to male pair-bonding in the socially monogamous prairie vole, *Microtus ochrogaster* (Barrett et al., 2013; Caldwell, Lee, Macbeth, & Young, 2008; Hammock, Lim, Nair, & Young, 2005; Ophir, Phelps, Sorin, & Wolff, 2008; Phelps & Young, 2003; Winslow, Hastings, Carter, Harbaugh, & Insel, 1993). By doing so, we hope to better understand how selection has shaped individual differences in the brain and behaviour.

Prairie voles are well known for forming enduring pair-bonds characterized by shared territory defence, biparental care of young and selective attachment between mates (Getz, Carter, & Gavish, 1981). Although monogamy is clearly the modal mating system, a significant number of individuals mate outside the pair-bond. Multiple-sired litters have been reported by different laboratories (Ophir, Wolff, & Phelps, 2008; Solomon, Keane, Knoch, & Hogan, 2004), and ~25% of young are sired outside the pair-bond (Ophir, Campbell, Hanna, & Phelps, 2008). Moreover, populations undergo drastic changes in population density annually, ranging from densities that are near zero to as many as 625 animals per hectare (Getz, Hofmann, McGuire, & Dolan, 2001). These changes are also accompanied by changes in extra-pair encounter rates and extra-pair fertilizations (Blondel et al., 2016; McGuire, Pizzuto, & Getz, 1990; Solomon et al., 2004). This particular natural history suggests a number of opportunities for frequency- or density-dependent selection to shape the mechanisms of fidelity.

The gene *Avpr1a* codes for the vasopressin 1a receptor (V1aR), the predominant vasopressin receptor in the central nervous system, a protein critical to male social behaviour in many taxa (Bachner-Melman et al., 2005; Donaldson & Young, 2008; Goodson & Bass, 2000; Goodson, Schrock, Klatt, Kabelik, & Kingsbury, 2009; van Kesteren et al., 1996). Among prairie voles, expression of V1aR in regions of the brain that coordinate reward contributes to the ability of male prairie voles to form pair-bonds (Lim & Young, 2004). Expression of *Avpr1a* in reward centres is uniformly high among prairie voles (Phelps & Young, 2003), a finding consistent with the idea that selection favours the capacity to form pair-bonds (Ophir, Campbell, et al., 2008; Ophir, Phelps, et al., 2008; Ophir, Wolff, et al., 2008; Phelps & Ophir, 2009). Interestingly, *Avpr1a* expression in other brain regions, including the retrosplenial cortex (RSC, Figure 1a), varies tremendously among individuals (Insel, Wang, & Ferris, 1994; Phelps & Young, 2003). The RSC is a critical node in a circuit that coordinates complex memory and navigation (Todd & Bucci, 2015; Troy & Whishaw, 2004). Moreover, the abundance of V1aR in the RSC is predictive of differences in territorial intrusion rates and extra-pair paternity among males (Ophir, Wolff, et al., 2008).

We recently demonstrated that individual differences in V1aR abundance and *Avpr1a* mRNA within the RSC are well predicted by a set of four single nucleotide polymorphisms (SNPs) in the *Avpr1a* locus (Figure 1a). These SNPs colocalize with markers of regulatory DNA: one SNP occurs within a DNAse I hypersensitive site 5′ of the *Avpr1a* locus, a marker of open, regulatory chromatin; two additional SNPs fall within a putative enhancer located within the *Avpr1a* intron (Figure 2a, top). Field experiments suggest that the two alternative allele classes, which we refer to as HI and LO RSC alleles, are under opposing selection when environments favour intrapair or extra-pair fertilization (Okhovat, Berrio, Wallace, Ophir, & Phelps, 2015).

In this study, we examine polymorphisms both at the *Avpr1a* locus and throughout the genome to more fully characterize the effects of selection on standing variation at the locus. Our previous results compared variation at the *Avpr1a* locus to three putatively neutral loci and suggest that the variation in *Avpr1a* was actively maintained by balancing selection; here, we augment this approach with genomewide polymorphism data to more rigorously assess whether *Avpr1a* diversity is the product of balancing selection. Next, we characterize patterns of nucleotide diversity, linkage disequilibrium (LD) and recombination rate along the *Avpr1a* locus to ask whether evidence of balancing selection—such as enhanced levels of nucleotide diversity and LD—is localized to putative regulatory elements within the *Avpr1a* locus. We then test the hypothesis that the SNPs associated with RSC-V1aR abundance are linked due to selection favouring specific combinations of polymorphisms. First, we ask whether the extent of LD among the SNPs that define HI and LO RSC alleles is greater than expected based on overall levels of linkage at the *Avpr1a* locus; next, we use haplotype reconstructions and genomewide polymorphism data from two prairie vole populations to test the alternative hypothesis that the linkage of the SNPs is due to a more recent introduction of one of the alleles. Lastly, we compare patterns of polymorphism across two populations of prairie voles to test whether selection has favoured different frequencies of *Avpr1a* alleles in the two populations. Together, these data follow up on our previous work by more systematically examining patterns of variation at the *Apr1a* locus, comparing it to data from throughout the genome and examining such data across populations. The results

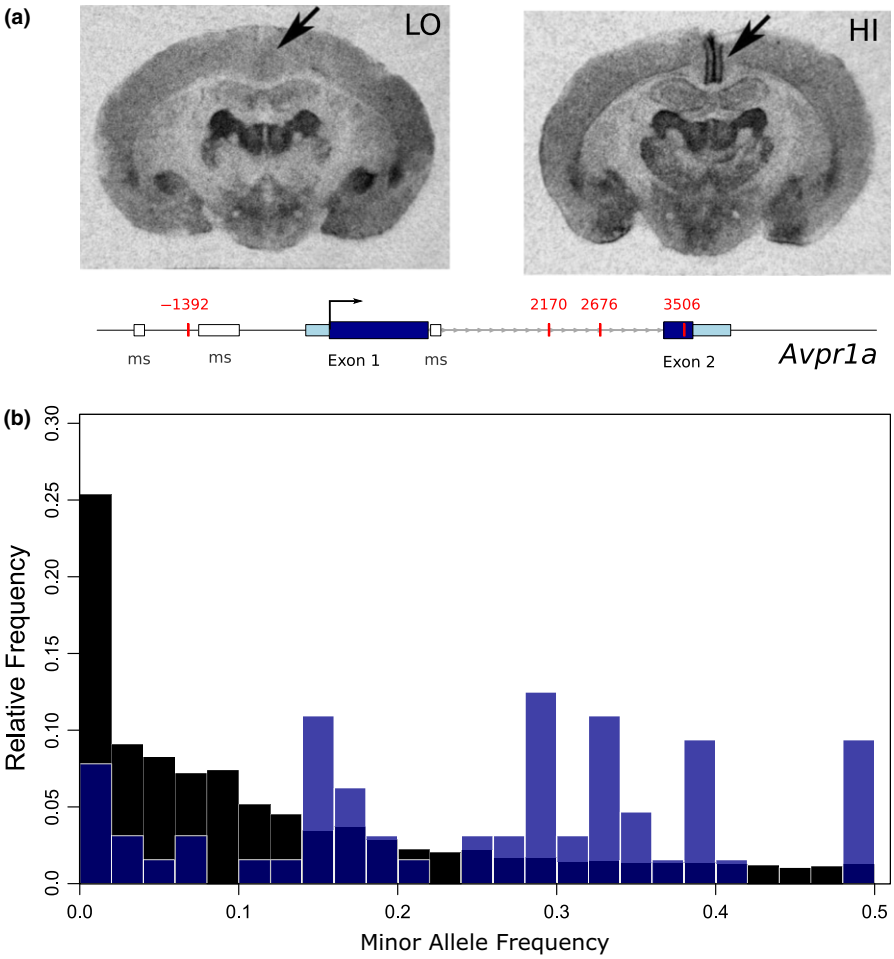

FIGURE 1 Evidence of balancing selection on the *Avpr1a* locus. (a). Low and high V1aR-expressing phenotypes in the retrosplenial cortex (RSC). *Avpr1a* locus contains two exons (blue, cds; light blue, UTRs) and three microsatellite sequences (white). Highly linked single nucleotide polymorphisms (SNPs) associated with RSC-V1aR expression are represented as red vertical lines, along with position with respect to the translation start site. (b). Relative frequency distribution of minor allele frequencies observed for genomewide loci (black bars) and SNPs at the *Avpr1a* locus (blue bars) [Colour figure can be viewed at wileyonlinelibrary.com]

should deepen our understanding of how selection shapes an important regulator of social behaviour and their neural substrates.

2 | MATERIALS AND METHODS

2.1 | Population sampling and DNA amplification

To assess intraspecific variation in prairie voles, we extended our analysis of 32 individuals that were previously collected in Champaign County, IL (Okhovat et al., 2015), and added a new population of 29 individuals that were collected in Jackson County, IL, approximately 320 km south of Champaign Co. A total of 61 genomic DNA extractions were prepared from liver samples using the QIAGEN DNEasy kit for tissue and blood following the manufacturer's protocol.

We amplified and Sanger sequenced a region targeting the *Avpr1a* locus in each of these populations. These sequences spanned a conserved DNAse I hypersensitive site 5′ of the *Avpr1a* locus, both exons, the 3′ UTR and four SNPs implicated in cortical V1aR expression and associated behaviour variation (Figure 1; see also Okhovat et al., 2015). Our focal population is in Champaign County, IL; amplicons from this population spanned ~7.5 kb and have been previously published (Okhovat et al., 2015). Samples from Jackson County, IL, were amplified at a shorter ~5.5 kb length that nevertheless spanned the same features described for the longer amplicons. For simplicity, the Champaign County sequences were trimmed to match the sequence available from Jackson County samples. Figures 1b, 2 and 4 depict the shorter span common to both samples.

2.2 | Haplotype determination

Determining the phase of alleles can be performed empirically by cloning, with substantial effort, or computationally based on LD, with the limitation that inferred phases may sometimes be mistaken. We cloned full-length amplicons (7.2 kb in Champaign Co, 5.5 kb Jackson Co) for any animals that were heterozygous for the HI and LO alleles or who showed extensive tracts of heterozygosity ($n = 8$ in Champaign, $n = 3$ Jackson). In addition, we randomly selected an additional 14 individuals from our focal population (Champaign Co) and cloned an amplicon that spanned the ~2.5-kb intron and some flanking coding sequence (see Supporting Information for primers), allowing us to empirically determine the phase of many intron polymorphisms and reducing the number of phases that needed to be estimated computationally.

To clone PCR products, amplicons were diluted in water and then ligated and cloned using the StrataClone PCR Cloning kit and the recommended protocol from the manufacturer (Agilent

Technologies). Briefly, ligation reactions were transformed into chemically competent StrataClone SoloPack cells and plated onto LB agar plates supplemented with kanamycin and spread with X-Gal at 2% (Fisher Scientific). At least three transformed white colonies were transferred to a tube with 5 ml of LB broth to grow overnight at 37°C. Plasmid DNA was isolated using the QIAprep spin miniprep kit (Qiagen). The insert size was confirmed by restriction digestion with EcoRI and visualization in a 1% agarose gel. Sanger sequencing was conducted at the University of Texas at Austin Institute for Cellular and Molecular Biology (ICMB).

In a sample of 29 individuals from the Jackson County and 32 from Champaign County, there were 64 SNPs with a minor allele frequency greater than 0.05. We entered empirically determined phases from our cloning and estimated remaining phase data needed for haplotype reconstruction. We used the default settings in the statistical package PHASE v2.1 (Stephens, Smith, & Donnelly, 2001). Next, we aligned both populations using the reconstructed haplotypes and a *Avpr1a* reference sequence (GenBank: DP001225.1) in the program GENEIOUS version 6.1 (Kearse et al., 2012).

To illustrate the relationships among haplotypes and examine the origin of the HI alleles, a haplotype network of the *Avpr1a* locus was constructed using the median-joining network algorithm (Bandelt, Forster, & Röhl, 1999) implemented in PopART v1.7 (Leigh & Bryant, 2015, http://popart.otago.ac.nz); the resulting network was edited in Inkscape v0.91. For our first analysis, we used 5.5 kb corresponding to sequences obtained from both populations (as depicted in Figures 1, 2, S1 and described above). To assess whether haplotype network estimation was influenced by phase estimation, we repeated our analysis using empirically determined haplotypes. These were restricted to cloned sequences spanning the *Avpr1a* intron and some adjacent coding sequence (see Figure S1).

2.3 | Linkage disequilibrium

To identify pairwise LD among SNPs across the *Avpr1a* locus in our focal population from Champaign County, we computed LD values (R^2) using the "linkage format" supported in HAPLOVIEW v4.2 (Barrett, Fry, Maller, & Daly, 2005), excluding microsatellite loci. These values were used to evaluate a sliding-window track of LD across the *Avpr1a* locus by evaluating at least three SNPs within a window of 300 bp. Windows with fewer than three SNPs were treated as missing data. To test whether the set of polymorphisms defining HI and LO alleles exhibited higher LD than expected by chance, we first regressed pairwise LD values (R^2) against distances between sites and then calculated the average residual R^2 for each of six pairwise comparisons of the four strongly linked sites. To estimate the null distribution of this statistic, we randomly sampled four SNPs (excluding the four that define HI/LO alleles) 10,000 times and calculated the average residual R^2 based on their nucleotide distance. For an alternative analysis, we randomly selected a focal SNP and noted the position of all other SNPs with an LD as great as the minimum observed in the HI/LO SNPs; we then calculated the distance along the *Avpr1a* locus that this randomly selected linkage group spanned. SNPs with no other strongly linked SNPs were assigned a span of 1. Because there were a small number of such linkage groups (40), we fit an exponential distribution to the range of spans, and estimated the *p*-value for observing a span of 4,898 bp from the cumulative distribution function. 4,898 bp is the distance between two most distant sites that define the HI/LO alleles (see Figure 1a). Because the mean span (430 bp) was much smaller than the observed span of the HI/LO alleles, we also calculated the *p*-value using the upper bound of the 95% confidence interval as our estimate of the distribution mean (604 bp). Alternative distributions (Poisson, Gamma) were weaker fits to the locus data and gave less conservative *p*-value estimates.

2.4 | Calculation of population genetic summary statistics

We focused our summary statistics of population genetics on our samples from Champaign County, for which we had more sampling data across the whole county area than in Jackson County. We used the package DNASP v5.1 (Librado & Rozas, 2009) to compute *nucleotide diversity* (π) in 300-bp sliding windows. The recombination rate *rho* was computed with the program *RECSLIDER* (http://genapps.uchicago.edu/labweb/index.html) within a window size of 10 variable sites. A preliminary estimate of $\rho = 0.0044$ was based on the average recombination rate between sites, obtained from *DnaSP*.

2.5 | RAD library construction and sequencing

We previously found differences in the frequency spectrum distribution of the *Avpr1a* locus and three putatively neutral loci, a pattern suggesting balancing selection on *Avpr1a* (Okhovat et al., 2015). To test whether the frequency of *Avpr1a* was indeed unusual, we sought to estimate the frequency spectrum of polymorphisms sampled randomly throughout the genome. To do so, we applied a restriction enzyme-based genotyping-by-sequencing method known as 2bRAD-seq. Type IIB enzymes excise 36-bp fragments alongside the recognition site to allow detection of genetic variants. Vole DNA was treated with the enzyme BcgI (NEB) to produce sufficient genome fragmentation; fragments were ligated to Illumina adapters, amplified for 10 cycles and then purified according to the protocol designed by Wang, Meyer, McKay, and Matz (2012). Final library preparations were pooled and sequenced by University of Texas at Austin Genome Sequencing and Analysis Facility (GSAF). Quality control and fragment distribution were examined before sequencing using the Agilent Bioanalyzer. The pooled sample was sequenced using a 50-bp single-read strategy on the Illumina HiSeq4000 platform at the GSAF facility, which generated a total of 277,180,409 reads (3,903,949 reads per individual on average). The quality of the reads was examined and approved by visualizing the FastQC output for each sample. The reads were filtered for the correct read structure and base call quality (PHRED score >20 at more than 90% of bases), deduplicated based on the ligated degenerate tag and mapped to *M. ochrogaster* genome using *bowtie2*, and genotypes were called using customized *GATK* pipeline (Broad Institute).

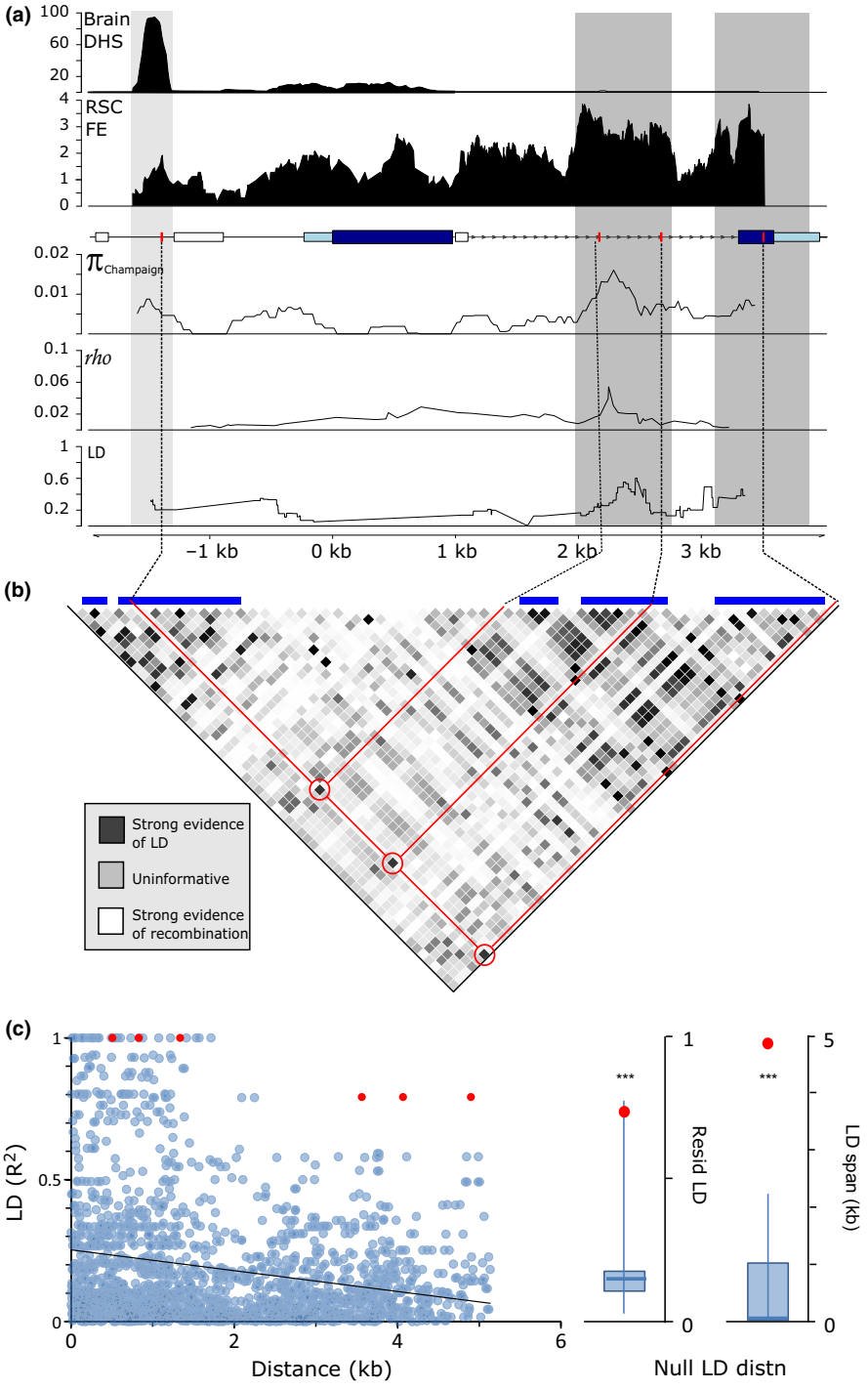

FIGURE 2 Patterns of diversity, recombination and linkage at the *Avpr1a* locus. (a) *Top panels*, tracks of two noncoding DNA elements, a conserved DNase I hypersensitivity site (DHS) in mammals from mouse brain (Encode), and ChIP-H3Kme1 site revealing fold enrichment of a mark for enhancers in the prairie vole RSC (Okhovat et al., 2015); these define the putative regulatory elements highlighted in panels below (light and dark grey, respectively). *Middle*, structure of the *Avpr1a* locus, red lines correspond to single nucleotide polymorphisms (SNPs) associated with V1aR expression in RSC; *lower panels*, sliding-window analysis of nucleotide diversity (π) in animals from Champaign County, IL; recombination rate (*rho*); linkage disequilibrium (LD, R^2). (b) Heatmap depicts significant evidence of linkage (black) and recombination (white) between pairs of SNPs. Four SNPs linked to cortical V1aR abundance are strongly linked with each other (red circles), but poorly linked to surrounding sequences. Five LD blocks are depicted in blue. (c) Left panel depicts LD decay with distance (in thousands of bases, kb). Red dots correspond to LD between pairs of SNPs linked to RSC expression; blue dots correspond to comparisons between SNPs not linked to RSC. Right panels demonstrate that the average residual LD for RSC-linked SNPs (red) is much larger than four randomly sampled SNPs at the locus and that the span of linked SNPs defining HI/LO alleles is significantly longer than the span of equally linked SNPs chosen at random. Box-and-whisker plot depicts median, quartiles and range of null distributions. ***$p < .001$ [Colour figure can be viewed at wileyonlinelibrary.com]

Average read count post deduplication and quality filtering was 373,289 reads per sample. After this initial quality check, one individual sample (out of $n = 61$) was dropped due to low sequencing depth.

2.6 | Genome-guided RAD genotyping and variant discovery

Trimmed reads were aligned to the prairie vole draft genome assembly using bowtie2 (Langmead & Salzberg, 2012); mapping efficiency was >95% for all the samples according to the flagstat report implemented in SAMTOOLS software (Li et al., 2009). The program GATK (McKenna et al., 2010) was used to identify genetic variants. UnifiedGenotyper tool was run twice followed by base quality score recalibration using the script GetHighQualVcfs.py to score SNPs with a quality percentile of 75 or higher. The last recalibration step was performed using VQSR process in GATK, producing a total output of 132,573 SNPs, of which 4,955 passed a reproducibility test that was run in four sets of replicate samples. The false discovery rate (FDR) was estimated from each portion of SNPs (i.e., tranche) based on the difference between the estimated and expected transition (Ti)/transversion (Tv) ratio of 2.41. This ratio was estimated from 4,955

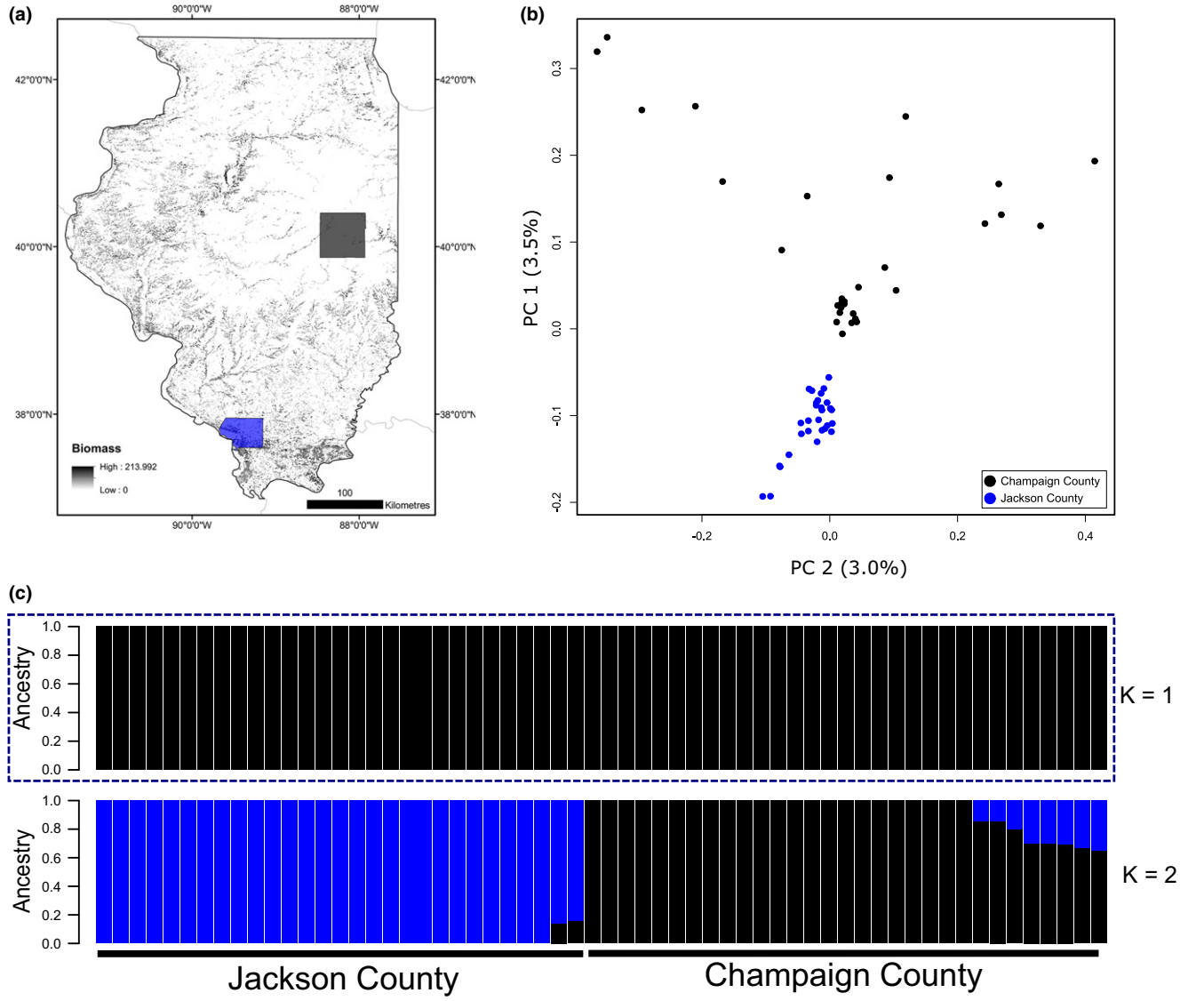

FIGURE 3 Population structure analysis of prairie voles in central and southern Illinois. (a) Prairie voles were sampled in Champaign (black) and Jackson (blue) counties. Shading depicts biomass variation reflecting vegetation differences across sites (southern forest vs. central prairie; Bailey, 1995). (b) Principal components analysis (PCA) generated on the basis of individual genotypes from 2bRAD data. PC1 and PC2 represent eigenvectors that accounted for 3.5% and 3.0% of the total genetic variation. (c) Admixture cluster analysis representing the inferred ancestry from *K* ancestral populations. Blue dashed box highlights the cluster (*K*) with the smallest error [Colour figure can be viewed at wileyonlinelibrary.com]

polymorphic sites that were fully consistent and highly reproducible among replicate samples. This model only considers tranches with a Ti/Tv ratio that is higher than expected due to a deficiency of false positives. The 35,630 SNPs that passed this final filter were recorded in a VCF file. Current versions of step-by-step 2bRAD library preparation and bioinformatics protocols are maintained at https://github.com/z0on/2bRAD_GATK.

2.7 | Population structure

We thinned the resulting VCF file using the script thinner.pl, available in https://github.com/z0on/2bRAD_GATK, which reduced the density of SNPs to one polymorphism per cutting site (24,800 SNPs). We further pruned this file using the function snpgdsLDpruning, implemented in the R library SNPRelate v1.6.4 (Zheng et al., 2012), to reduce and equalize the physical linkage between sites. With the resulting 13,013 pruned SNPs, we conducted a principal components analysis (PCA) using the function "snpgdsPCA" from the SNPRelate library to examine the distances between individual samples and populations. Alternatively, we estimated genetic clusters using ADMIXTURE v1.3 (Alexander, Novembre, & Lange, 2009), which evaluates the ancestry in a model-based manner from SNP data sets. Finally, to estimate global F_{ST}, we used BayeScan to identify F_{ST} outliers among the SNPs that passed our quality controls (Foll & Gaggiotti, 2008), from which no outliers were identified at $q < 0.1$. Then, we measured global F_{ST} using the Weir and Cockerham method implemented in Vcftools

(Danecek et al., 2011) after excluding these outliers. We used 4P (Benazzo, Panziera, & Bertorelle, 2015) to calculate genomewide diversity per population from the VCF file and custom scripts in R (R Core Team 2015) to calculate the global genome average of D_{xy} between populations. From all RAD sites, 139,204 (89%) had at least two or more reads mapped in at least two individuals. To calculate a null distribution of population differentiation for a sample size of the *Avpr1a* locus, we sampled 5.4 kb from the genome (150 RAD sites of 36 bp each) and calculated their mean F_{ST} and D_{xy}. Similarly, we randomly sampled 11 RAD sites (396 bp) to obtain an estimate of differentiation based on a window size comparable to a sliding window size used to examine differentiation of the *Avpr1a* locus. The resulting null distributions were based on 10,000 randomly sampled sequences.

2.8 | Population differentiation at *Avpr1a*

To examine population differences at the *Avpr1a* locus, we used the *Gene Flow and Genetic Differentiation* tool in *DnaSP* to compute F_{ST} and D_{xy} for the entire locus (~5.5 kb). 300-bp sliding-window analysis of F_{ST} and D_{xy} was generated using the program SLIDER (http://genapps.uchicago.edu/labweb/index.html). To determine whether D_{xy} was elevated in *Avpr1a* compared to genomewide levels, we used the 2bRAD data described above. To this end, we randomly sampled 2bRAD windows from our data, concatenating them to produce sequences of equivalent size to the region of interest (i.e., 5.5 kb and 300 bp), and calculated D_{xy} in the concatenated sequence. Then, we generated D_{xy} distributions by drawing 10^5 of these pseudosamples. From these distributions, we obtained empirical *p*-values for the D_{xy} levels observed in the region of interest (i.e., the proportion of pseudosamples with D_{xy} equal or greater to the value observed). We used a similar approach for the levels of nucleotide diversity in the Champaign population.

3 | RESULTS

3.1 | Evidence of balancing selection in the *Avpr1a* locus

We examined the frequency spectrum of genomewide SNPs and at the *Avpr1a* locus among wild-caught samples from Champaign County, IL. As expected from the neutral distribution of allele frequencies near mutation–drift equilibrium (Luikart, Allendorf, Cornuet, & Sherwin, 1998; Nei, Chakraborty, & Fuerst, 1976), we found that the *Avpr1a* locus was strongly skewed towards an excess of intermediate-frequency alleles compared with the rest of the genome (Figure 1b), a typical signature of balancing selection (Kolmogorov–Smirnov, $D = 0.44$, $p < 9.6e{-}6$).

3.2 | Patterns of polymorphism and LD at the *Avpr1a* locus

When performing haplotype reconstructions, we found that 3,858 of 3,904 (99%) of all sites were assigned a phase with high confidence (>95% posterior probabilities). The haplotypes were used for a variety of downstream analyses. To characterize local patterns of polymorphism at *Avpr1a*, we used our reconstructed haplotypes from the Champaign population of wild-caught voles. From these, we estimated nucleotide diversity (π), LD (R^2) and recombination (rho). A sliding-window analysis shows local peaks of nucleotide diversity and LD within a known putative intron enhancer (Figure 2a; Okhovat et al., 2015). We found that average nucleotide diversity for the *Avpr1a* locus ($\pi_{Avpr1a} = 0.004$) was significantly higher than across the rest of the genome (mean $\pi = 0.0012$; empirical $p < 10^{-5}$). *Avpr1a* diversity values ranged from 0 to 0.015, with one main peak ($\pi > 0.01$) spanning roughly 200 bp (between positions 4515 and 4790).

On average, LD is low—even between adjacent sites—and decays with distance (0.252–0.037*kb). There is, however, considerable variation in LD across the region. This heterogeneity in LD patterns is apparent in our recombination estimates, which suggest the presence of a hotspot between the two RSC-predictive SNPs of the intron enhancer (Figure 2a), a finding also reflected in the localized evidence of recombination evident in patterns of LD evident in within the intron enhancer (Figure 2b). Additionally, a set of linked SNPs predictive of RSC variation are more highly linked than four SNPs chosen at random, even after correcting for the decay of LD with distance (Figure 2b,c; $p = .0002$). Similarly, the span of LD for the SNPs that define the HI and LO alleles is much greater (4,898 bp) than expected from a sample of SNP groups with comparable linkage (mean span = 430 bp, CI = 321,604; assuming a mean of 430, $p = 1.1 \times 10^{-5}$, and assuming a mean of 604, $p = 3.0 \times 10^{-4}$). Indeed, outside of the HI/LO SNPs, the maximum span of a strongly linked group was 2,248 bp.

3.3 | Genomewide population structure

We found very weak genomewide structure between two populations of prairie voles separated by ~320 km (Figure 3a). A PCA suggested that, while it represents the largest source of variance, between-population structure accounts for only 3.5% of genetic variation in our sample (PC1, vertical axis in Figure 3b). Moreover, our admixture model confirms very low population structure, which favours a model with a single population. Cluster one (K1) exhibited less cross-validation error than clusters K2 and K3 (CV error [$K = 1$]: 0.52 vs. CV error [$K = 2$]: 0.55 vs. CV error [$K = 3$]: 0.60) (Figure 3b). In addition, genomewide values of absolute (D_{xy}) and relative nucleotide divergence (F_{ST}) indicate that these two populations are extremely similar (BayeScan $F_{ST} = 0.059$, Weir and Cockerham weighted average F_{ST} estimate: 0.027, Global $D_{xy} = 0.0054$).

3.4 | Haplotype network of the *Avpr1a* locus

To evaluate the origin of the HI/LO RSC alleles, we examined the haplotype structure of the *Avpr1a* locus by reconstructing a neighbour-joining haplotype network. We sampled 93 haplotypes from a total of 61 wild-caught prairie voles collected in Champaign and

Jackson County. As in our genomewide data, the general pattern of our median-joining network analysis suggests that *Avpr1a* haplotypes have common origins across populations, although the haplotype diversity is quite high (Figure S2). LO alleles were extremely diverse in both populations—we observed 91 distinct LO haplotypes (average $\pi = 0.00406$ across all LO alleles). The HI alleles, in contrast, were far less diverse (average $\pi = 0.00003$) with only two haplotypes. Both HI alleles clustered together on the haplotype network. A more restricted analysis of empirically phased introns in which low-frequency SNPs (but not singletons) were included (Figure S2b) also revealed a common origin of the HI allele in each population and indicate a high level of haplotype diversity. Lastly, none of the *Avpr1a* haplotypes clustered near a known *Avpr1a* pseudogene haplotype, confirming the specificity of our primers.

3.5 | Patterns of population differentiation at the *Avpr1a* locus

Overall, relative differentiation between populations at the intron of the *Avpr1a* locus was considerably elevated compared to the rest of the genome ($F_{ST} = 0.22$; greater than 99% of SNPs genomewide). Relative sequence divergence was maximum at a site within a putative enhancer of the *Avpr1a* intron ($F_{ST} = 0.26$), thought to shape cortical gene expression and sexual fidelity (Okhovat et al., 2015). This pattern of relative differentiation was similar to the absolute differentiation across the *Avpr1a* locus, which showed a significant peak in the putative enhancer ($D_{xy} = 0.02$; empirical $p = .007$). Both measures of population differentiation within *Avpr1a* roughly coincided with local levels of nucleotide diversity (π) in the pooled sample (Figure 4b, top track). Overall absolute differentiation (D_{xy}), however, was not significantly increased at the locus compared to genomewide levels ($D_{xy\text{-}Avpr1a} = 0.006$; empirical $p = 0.289$).

4 | DISCUSSION

Prairie voles are socially monogamous rodents that exhibit biparental care and territory defence, but also display considerable individual differences in territorial behaviour, space use and sexual fidelity (Carter, Getz, & Cohen-Parsons, 1986; Getz, McGuire, & Pizzuto, 1993; Phelps & Ophir, 2009; Solomon et al., 2004). Four SNPs reside in *cis*-regulatory elements at the *Avpr1a* locus and predict expression of V1aR in the RSC (Figure 1a); RSC expression in turn predicts aspects of space use and sexual fidelity (Okhovat et al., 2015; Ophir, Wolff, et al., 2008). In the present study, we examined patterns of variation at the *Avpr1a* locus to understand the origin and evolution of its regulatory diversity.

We found that the *Avpr1a* locus had an excess of intermediate-frequency alleles when compared to the frequency spectrum obtained from across the genome (Figure 1b). These data are consistent with a more limited examination of putatively neutral loci and indicate that the unusual diversity at the *Avpr1a* locus is likely due to the active maintenance of alternative *Avpr1a* alleles through balancing selection (Okhovat et al., 2015). Similarly, patterns of linkage within the *Avpr1a* locus revealed a local peak in nucleotide diversity and LD within a transcriptional enhancer previously identified in the intron of the locus (Figure 2a). This local excess of polymorphism and linkage is another classic signature of balancing selection (Braverman, Hudson, Kaplan, Langley, & Stephan, 1995; Charlesworth, 2006; Luikart et al., 1998; Nei et al., 1976). The fact that this signature coincides with the location of HI/LO allele SNPs—regulatory variation previously associated with cortical V1aR abundance and sexual fidelity (Okhovat et al., 2015)—strengthens the interpretation that selection has actively maintained individual differences in *Avpr1a* function within the prairie vole brain. Although behavioural and theoretical data suggest balancing selection might be common in social phenotypes, our results are among a small number of molecular examples. Lonn et al. (2017) recently found evidence of balancing selection at both oxytocin and vasopressin receptors in bank voles. Researchers in evolutionary psychology have speculated that genetic contributions to human personality might be under balancing selection (Keller & Miller, 2006), but initial reports have not strongly supported this prediction (Verweij et al., 2012). Systems in which the heritable variation in social behaviour has been documented, such as great tits, sparrows and wild mice (Benus, Bohus, Koolhaas, & van Oortmerssen, 1991; Dingemanse, Both, Drent, van Oers, & van Noordwijk, 2002; Thomas et al., 2008), suggest promising models for testing whether balancing selection may indeed be common among social phenotypes.

We previously noted that the SNPs that define HI and LO RSC alleles were localized to distinct regulatory regions at the *Avpr1a* locus and were strongly linked to one another. Here, we find that the extent of linkage among these SNPs was much greater than expected based on their positions within the locus (Figure 2b,c). These regulatory SNPs were unlinked to adjacent sites (Figure 2b) and were much more strongly linked to one another than predicted based on the number of nucleotides that separated them (Figure 2c). This inference of excess linkage was consistent across a variety of measures. We believe there are two plausible explanations for this high linkage across sites. One hypothesis is that selection has actively favoured specific combinations of regulatory polymorphisms to produce appropriate allele-specific cortical expression patterns. An alternative hypothesis is that the high-expressing alleles arose in a third population and were introduced into our focal populations. This would suggest the presence of admixture in our populations with some external source (Ardlie, Kruglyak, & Seielstad, 2002; Bürger & Akerman, 2011). Our *Avpr1a* locus and genomewide data allowed us to address these alternatives.

To explore the structure of prairie vole populations more thoroughly, we conducted 2bRAD on a second population of prairie voles located ~320 km from our original source, and we examined genomewide polymorphisms from both populations for evidence of admixture (Figure 3a). Interestingly, although a PCA revealed limited differentiation between the two populations, an ADMIXTURE

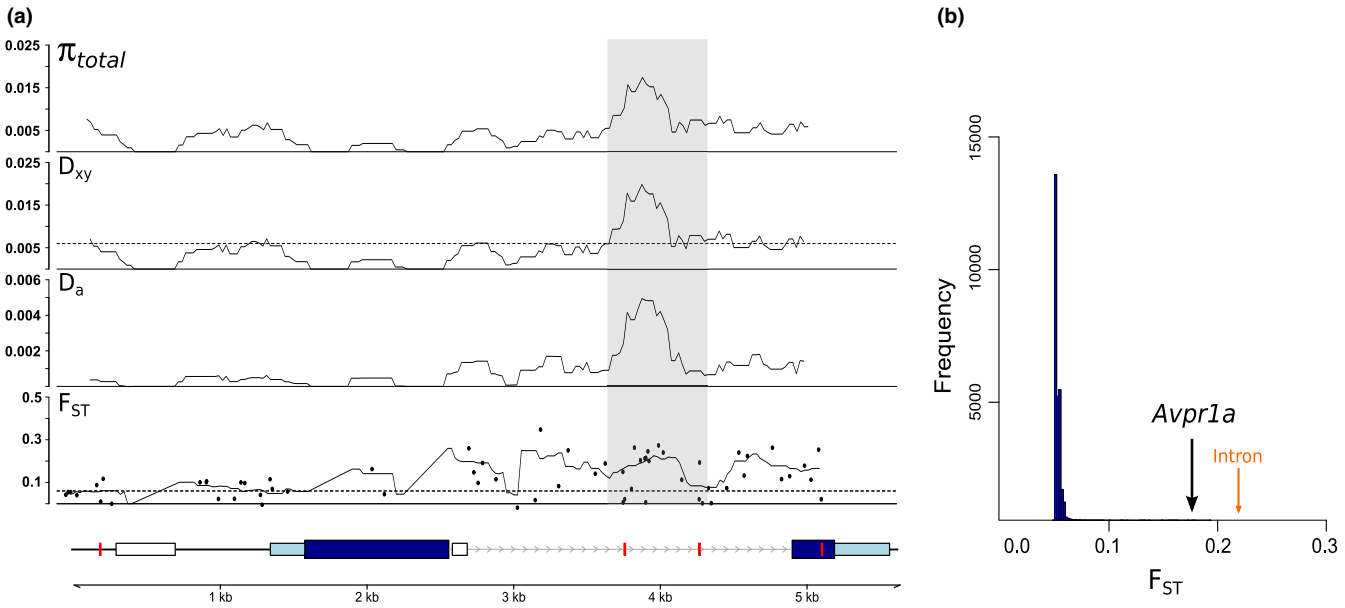

FIGURE 4 Patterns of nucleotide diversity and population differentiation. (a) From top, a sliding-window analysis of nucleotide diversity (π) across populations; relative differentiation (F_{ST}) of the two populations; absolute differentiation (D_{xy}); and the "net" population differentiation D_a. Dashed lines represent genomewide averages for F_{ST} and D_{xy}. Location of the putative intron enhancer is highlighted in grey, and RSC-associated single nucleotide polymorphisms are in red (Okhovat et al., 2015). (b) Distribution of F_{ST} between Champaign and Jackson County populations per site across the prairie vole genome (blue) as well as for the entire *Avpr1a* locus (black) and its intron (orange) [Colour figure can be viewed at wileyonlinelibrary.com]

analysis suggested that a model with a single population was slightly better than a model with two populations. Similarly, the very low genomewide F_{ST} of the two populations confirmed the lack of genomewide differentiation. Moreover, the origin of the HI or LO alleles from an outside source would require that there was a third population that could be detected in the current sample—a model that was not supported by our data. Lastly, the absence of secondary peaks or modes at intermediate frequencies forming a U-shape within our genomewide SNP frequency spectrum (Figure 1b) suggests that recent admixture with a distant population has not had a major influence on the distribution of variation within the prairie vole genome (Caicedo et al., 2007; Theunert & Slatkin, 2017). Therefore, admixture alone is unlikely to explain the unusual pattern of LD and nucleotide diversity at the *Avpr1a* locus.

An examination of the haplotype network (Figure S2) reveals that the HI allele is less diverse, presumably more recent than the LO allele, and has a common origin in both populations. While our admixture and frequency spectrum analysis refute the hypothesis that the unusual linkage and recent origin of the HI-allele SNPs was a simple by-product of gene flow, more complex scenarios remain possible. For example, it is possible that the HI allele was recently introduced into both of our focal populations and rose to intermediate frequencies through selection at the locus. Such a scenario would require the HI allele to be old enough or its origins distant enough from our current sample that the resulting signatures of admixture were not visible to our analyses in 2bRAD-seq data and *Avpr1a* locus. An alternative approach would be to test for evidence of epistatic selection more directly. For example, it may be possible to examine rare but naturally occurring recombinants among the SNPs that define HI and LO alleles to ask whether recombinants occur significantly less often than predicted by chance. Similarly, one could ask whether such recombinants or artificially induced mutations alter the evolutionary fitness in a field setting, or the behaviour driven by changes in the magnitude of RSC expression of the *Avpr1a* locus.

Because gene products interact with one another, epistasis is likely to be common in biology. Nevertheless, it is difficult to detect because it requires simultaneous examination of multiple sites of variation. Well-known examples include interactions among protein-coding sequences in histocompatibility loci (Gregersen et al. 2006; Trowsdale & Knight 2013) and coat-colour mutations (Marklund et al. 1998). With respect to brain and behaviour, some unusual patterns of haplotype structure have been identified in the context of genes relevant to behaviour. For example, serotonin-related genes HTR2A and SLC6A4 display unusual patterns of haplotype structure and adaptive evolution in both human and chimpanzee populations (Claw, Tito, Stone, & Verrelli, 2010). Similarly, there are signatures of positive selection that seem to maintain LD in the *DRD4* locus in four populations of great tits (Mueller et al., 2013). A few recent studies suggest epistatic interactions may be common between regulatory and coding loci (Anderson, McKeown, & Thornton, 2015; Lappalainen, Montgomery, Nica, & Dermitzakis, 2011; Phillips, 2008), but fewer still have examined epistasis in *cis*-regulatory variants (Lagator, Igler, Moreno, Guet, & Bollback, 2016).

In our case, selection may have favoured haplotypes in which a SNP in a DNAse I hypersensitive site is in phase with SNPs in an

intron enhancer. This 5′ hypersensitive site contains a highly conserved CTCF binding site, and the polymorphism occurs within a flanking conserved sequence that shows evidence of enhancer activity within the brain (Crawford, 2005; Song & Crawford, 2010). Thus, the putative epistasis could reflect a need for coordinated function across enhancers, binding of interacting transcription factors at different parts of the *Avpr1a* locus, or interactions between topological domains and enhancer sequences (Gertz, Gerke, & Cohen, 2010; Jolma et al., 2015; Pombo & Dillon, 2015). Indeed, these possibilities echo the likely sources of epistasis at any pair of regulatory polymorphisms, and investigating the hypothesized epistasis at the *Avpr1a* locus could serve as a model for *cis*-regulatory interactions more generally.

While epistatic selection may have favoured specific combinations of *Avpr1a* polymorphisms, and balancing selection seems to have maintained variation in the locus, the ecological parameters that drive frequency- or density-dependent selection may also vary across populations. In that case, selection on the *Avpr1a* locus would also differ between our populations, and we would expect *Avpr1a* to show stronger differentiation between populations than observed in the genomewide background. We found elevated F_{ST} throughout the *Avpr1a* locus, and particularly at its intronic enhancer. These results would be surprising if balancing selection was equivalent in both populations, as balanced polymorphisms are typically associated with reduced F_{ST} (Hohenlohe, Phillips, & Cresko, 2010). A sliding-window analysis revealed that elevated values for both relative differentiation (F_{ST}) and absolute divergence (D_{xy}) occur in the vicinity of the intron enhancer (Figure 4b). The intron enhancer was also elevated in D_a, a measure that quantifies population divergence after subtracting background levels of polymorphism (Cruickshank & Hahn, 2014). Thus, the intron enhancer seems to be more differentiated between populations than expected by chance, and this differentiation is not simply an artefact of increased polymorphism. These results suggest that balancing selection operating on the intron enhancer may favour different frequencies of HI and LO alleles in these two populations.

It is not clear what drives population differences in *Avpr1a* HI/LO frequencies, but the populations differ in the severity of winter and the relative abundance of suitable grassland habitat, either of which might contribute to fluctuations in population density and resource availability. Population density alters extra-pair encounter rates and sexual fidelity among prairie voles (Blondel et al., 2016; McGuire et al., 1990; Streatfeild, Mabry, Keane, Crist, & Solomon, 2011) and is generally among the strongest predictors of mammalian mating systems (Lukas & Clutton-Brock, 2013). Given our prior data showing that HI and LO alleles predict cortical V1aR abundance, sexual fidelity and influence fitness through either intrapair (HI) or extra-pair paternity (Okhovat et al., 2015), it seems plausible that population-specific fluctuations in the defensibility of females could influence the strength and direction of selection on HI and LO alleles. Direct tests of this hypothesis will require experimental manipulations of population density in natural settings.

Our data reveal a complex pattern of selection on the *Avpr1a* locus, including evidence of balancing selection, epistasis, and spatially varying selection concentrated in the vicinity of regulatory elements implicated in cortical *Avpr1a* expression, space use and sexual fidelity. The decision to focus on a single candidate gene does not, of course, indicate that variation in *Avpr1a* is the only or even the principle regulator of behavioural variation. Nevertheless, by combining a well-motivated candidate gene approach with genomewide assessments of population structure, we gain deeper insight into the forces that have shaped both the candidate gene and the genome as a whole. Our approach explores more deeply the many ways in which individual differences in brain and behaviour may be maintained by selection. Lastly, we hope that our focus on variation with regulatory elements serves as a model for researchers in other systems interested in understanding how transcriptional regulation contributes to natural variation in complex behaviours.

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DATA ACCESSIBILITY

2bRAD sequence data presented in this manuscript are available in SRA-NCBI (BioProject ID PRJNA382962). Scripts for analysing 2bRAD data are currently available in the GitHub of one of the authors (https://github.com/z0on/2bRAD_GATK), other custom R scripts and data sets containing genotype scores, and sequence alignments are publicly available in Dryad Digital Repository (accession no. https://doi.org/10.5061/dryad.2d7b8).

AUTHOR CONTRIBUTIONS

A.B. and S.M.P. contributed equally to the project. A.B and S.M.P. designed the study and wrote the manuscript. S.M.P. oversaw the project. A.B., M.O. collected DNA and Sanger sequencing data from clones and wild prairie voles. A.B. and G.M.A. prepared the 2bRAD libraries. A.B. analysed 2bRAD-seq. M.V.M. oversaw 2bRAD sequencing and analysis. A.B. and R.F.G. analysed and discussed. A.B., R.F.G, M.V.M. and S.M.P helped revise the manuscript.

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