Materials and Methods

Cell culture assays

Plasmid construction. The generation of the short prairieA luciferase plasmid has been described elsewhere [“prairie-luc”; (S1)]. The longer prairieB-luc construct was created by cloning a common long microsatellite allele from our prairie colony. Briefly, genomic DNA was amplified as described in the genotyping section below. Nested PCR with primers containing modified Nde I sites (5’-CATATGTCCTTGGTTGCGTCCCTTCGCC-3’, and 5’-CATATGGTTTCCACAGCAGCAGGGTGTCG-3’) were used to amplify the microsatellite from the first round of PCR. The nested PCR product was subcloned into a TA cloning vector (pCRII-TOPO, Invitrogen, Carlsbad, CA). Then the microsatellite allele was digested with Nde I from the TOPO vector and ligated into a “no cassette” vector (S1). All clones were sequenced to verify orientation and amplification fidelity. Plasmids were purified for experiments with Qiagen mini and maxipreps (Qiagen, Inc., Valencia, CA).

Cell culture. The A7r5 cell line was purchased from ATCC (Manassas, VA) and grown in DMEM supplemented with 10% FBS in a 37°C incubator with 5% CO2. All cell culture reagents were purchased from Mediatech (Herndon, VA). All maintenance plastic-ware was purchased from Corning (Corning, NY).

Transient transfections and luciferase assays. Cells were seeded in 12-well plates (Greiner Bio-One; Frickenhausen, Germany) at a density between 1 and 10 x 10^3 cells/well. Twenty-four hours later, each well was transiently transfected with 1 µg of a firefly luciferase test plasmid (prairieA-luc or prairieB-luc) and 0.1 µg of a renilla luciferase plasmid (phRL-tk, Promega; Madison, WI) with a 3:1 ratio of fuGene transfection reagent (Roche; Mannheim, Germany). At the time of transfection, 50% of the media was changed. The cells were fed the following day. Forty-eight hours after transfection, the cells were harvested and assayed according to the Dual-Luciferase Reporter Assay (Promega) in a TD-2020 luminometer (Turner BioSystems, Sunnyvale, CA). For each experiment, n = 6 wells per group. Each sample was a ratio of firefly luciferase relative light units (RLU) to renilla luciferase RLU. For statistical analysis, these ratios were normalized to the average activity of the prairieA-luc construct. In three independent experiments, two-tailed Student’s t tests were used to compare the group means of the two allele constructs.
avpr1a microsatellite genotyping

To select animals for breeding based on the length of the avpr1a microsatellite, we used PCR with primers specific to the prairie vole avpr1a microsatellite region to amplify the polymorphic microsatellite. Template DNA was extracted from ear punches with a commercially available kit (Gentra Systems, Minneapolis, MN) and resuspended in 10 mM Tris-EDTA buffer. About 10 ng of genomic DNA was used in a 50 µl reaction with Expand HiFi PCR kit (Roche, Mannheim, Germany). Reactions were pre-mixed on ice and amplified in an Eppendorf thermal cycler with an annealing temperature of 58°C for 25 cycles: 94°C, 2 min; 10x (94°C, 15 s; 58°C, 30 s; 72°C, 45 s); 15x (94°C, 15 s; 58°C, 30 s; 72°C, 45 s + 5 s each round); 72°C, 7 min; 4°C hold. The forward and reverse primers were 5'GTATTGCCACAAATAGACCAACG and 5'GTAAGGATGACAGGCGTTACTG, respectively. About 100 ng of PCR product was loaded onto 2.3% SFR agarose (Amresco Inc, Solon, OH) and run in 1x TBE buffer at 100 V, 4°C for 8 hours with 3 changes of cold 1x TBE. The gels were stained in ethidium bromide after completion of the gel run. PCR product lengths of each avpr1a allele for each individual were estimated using 100 bp (Promega, Madison, WI) and 20 bp (Cambrex, East Rutherford, NJ) ladders as standards on a UV light box with Eagle Eye Image Analysis system (Stratagene, La Jolla, CA). Products were reliably resolved with about 5 bp resolution. The microsatellite itself is about 600 bp, but the PCR process generates slightly larger fragments because the primers bind 5' and 3' of the microsatellite. All sequences were aligned using Align X software (VectorNTI suite, Informax/Invitrogen, Carlsbad, CA) with a ClustalW alignment algorithm.

Animals

We genotyped 80 prairie voles from our regular laboratory breeding colony. This screen resulted in 25 viable pairs of males and females homozygous for either longer or shorter than the colony average. Care was taken to make sure that the pairs were outbred to a distance of at least the level of second cousin. Males and females were sexually naïve at pairing. The male and female pairs were placed into a cage together, although they were initially separated by a Plexiglas divider so the females could come into estrous. After 5 days of cohabitation without contact, the dividers were removed. Mating was observed in all cages within 30 min. Gestation is about 22 days in voles. Several days prior to the expected delivery of pups, the backs of the males were shaved to allow differentiation of the males from the females during behavioral observations. Cages were monitored closely for pup delivery. Six to 8 hours after pup delivery, entire litters were cross-fostered to reduce environmental confounds. Briefly, the entire litter was removed from the birth cage, cleaned with a damp paper towel and wrapped in soiled bedding of their adoptive parents. We collected 3 litter cohorts from the breeder pairs. The first and third litter cohorts were cross-fostered, while the second litter cohort was not cross-fostered. The breeder pairs were maintained in an isolated cubicle on a 14:10 L:D cycle, and provided with water and rabbit chow (PMI Nutrition International; Brentwood, MO) ad libitum. All animal experiments were approved by the Institutional Animal Care and Use Committee of Emory University, Atlanta, GA, USA.
Behavioral phenotyping

Observation of breeder pairs. We collected data on the parental care of the breeder pairs over the first 10 post-natal days after the birth of the first litter cohort. We used a scanning observation methodology which resulted in about 4000 “frames” of observations. Briefly, we visually scanned and recorded the behavior in each cage 10 times spread across a 1-hour period. We performed such scanning twice a day, including an hour session at 0500h (lights on) and an hour session at 1900h (lights “off”, 1-2 lux to allow for observation). The frequency of each behavior (out of 10 possible) was tabulated for each hour session. The frequency per hour (20 hours total) was then averaged for each parent. Mann-Whitney statistics were used on the average hourly frequency of each behavior to assess genotype differences. Nest behavior included time on the nest by both parents simultaneously (huddled) or by either parent individually (male alone or female alone). Active nursing postures included arch-backed nursing and the upright crouch. The passive nursing posture occurred when the female was lying on her side. Pup licking and grooming was defined as any oral stimulation of any part of the pup (perioral, anogenital, flank, etc).

Phenotyping of F₁ male offspring. All male and female offspring were weaned at post-natal day 20 (day of birth = PND 0). At weaning, animals were weighed and ear clipped for identification. Males and females were weaned into separate cages and all weanlings were housed in groups of two or three. Animals were combined from separate litters when necessary. At 45 days of age, males were tested for their social approach behavior towards a novel social odor as described in the text. Only litter 1 males were used in the nonsocial odor task. At 3 months of age, males were individually-housed for the subsequent duration of the behavioral phenotyping (2 weeks). All behaviors were both videotaped and scored real-time when possible. Tests were performed in an isolated room around mid-day at temperatures between 23°C and 25°C under fluorescent lights. Social engagement and pair bonding behaviors [reviewed in (S2)] were assessed with juvenile affiliation and partner preference tests. Trait anxiety was measured with the elevated plus maze (S3, S4) and the open-field test (S4, S5).

Social and nonsocial odor test. One day before each of three test days, soiled bedding was collected from a single cage containing 3 females, unrelated to any of the test males. The soiled bedding was thoroughly mixed by shaking in a plastic bag. The bedding was distributed in about 1 gram aliquots into Histosette cartridges (Fisher Scientific International, Pittsburgh, PA). Test males were placed in a clean empty cage for a 2 minute acclimation. After 2 min, the odor cartridge was placed into the center of the cage, and the male’s behavior was recorded for 5 min. To test if this behavioral difference was specific to social odor, we tested a subset of the animals (litter 1 males) in the same apparatus but instead of soiled bedding, we used 1 ml of a banana-like odor on a cotton ball inside the cartridge (0.000001% amyl acetate; Acros Organics, Geel, Belgium).

Juvenile affiliation. A 15- to 20-day-old juvenile prairie vole was placed in the test animal’s home cage for 10 min. We measured the approach latency of the test subject to the juvenile, as well as the frequency and duration of time the test subject spent sniffing, allogrooming, and huddling with the juvenile. Because of the large number of
test subjects, each stimulus juvenile was used several times. Genotype differences in behavior were analyzed with nonparametric Mann-Whitney test, with $P$ value set at 0.05.

**Partner preference.** Partner preference behavior was assayed in a 3-hour test after overnight cohabitation with mating (18 hours) with a novel, unrelated sexually receptive female. Females were made receptive with 25 $\mu$g estradiol benzoate i.p., 3 times over 3 days. After 24 hours of cohabitation with mating, at least 80% of male prairie voles form a partner preference (unpublished observations). We therefore chose a truncated period of cohabitation in an effort to increase the variation in the behavior. We observed mating by the test males within the first 15 to 30 min of cohabitation. The partner preference test chamber is a 3 cage apparatus, where the test male’s “partner” is tethered in one cage, a novel “stranger” female of equal stimulus value is tethered in a separate cage, and the two females’ cages are connected by a third empty “neutral” cage. The males have free access to all three chambers via Plexiglas tubes and must choose between their partner, the stranger female and the neutral cage. The amount of time the male spent in each portion of the three-chambered apparatus, as well as the amount of time the animal spends in direct contact with the 2 females was quantified. This behavior was videotaped and scored time-lapsed at a compression ratio of 12:1 (i.e., the 3-hour test was condensed to 15 min). Group means of time spent with partner versus time spent with stranger were analyzed with a two-way ANOVA, with preference and genotype as factors. Bonferroni corrected planned $t$ tests were used. Observed rates of partner preference formation were compared to expected rates (random preference) with Chi-square analysis.

**Elevated Plus Maze.** The elevated plus maze test was conducted in an apparatus measuring 108 cm by 108 cm, raised to a height of 60 cm. Animals were transported to the maze from their home cage in a narrow box and placed in the center of the maze. The amount of time the animal spent in the center, open and closed arms and the number of crosses through the center was recorded for 10 min. The criterion for time in open arms was met when the animal had all four paws in the open arms. Genotype differences in behavior were analyzed with either Student’s $t$ test or Mann-Whitney, with $P$ value set at 0.05.

**Open-field test.** Animals were placed in the center of a 40 cm by 40 cm Plexiglas arena for 20 min. We videotaped and scored the amount of time subjects spent in the periphery and the center of the apparatus. The center was defined as the region of the testing chamber that was 2 animal widths away from the chamber walls (about 8 cm). The animal was scored as being in the center when all four paws left the periphery. Genotype differences in behavior were analyzed with either Student’s $t$ test or Mann-Whitney, with $P$ value set at 0.05.

**Autoradiography and quantification**

Animals were deeply anesthetized with isoflurane prior to decapitation. Their brains were removed and snap-frozen on dry ice and stored at $-80^\circ\text{C}$. Brains were cryosectioned in the coronal plane from the olfactory bulb to the periaqueductal grey at the level of the hindbrain. Each brain was sectioned at 20 $\mu$m in a Leica CM 1800 cryostat (Wetzlar, Germany) and thaw-mounted onto Superfrost/ Plus slides (Fisher Scientific International, Inc). Six serial sets of sections were taken for each individual, such that each adjacent
section on a slide was 120 µm apart. V1aR autoradiography was performed on one complete rostro-caudal set for each animal as previously described (S6) in 50 pM 125I-labeled linear V1aR antagonist 125I-Phaa-d-Tyr(Me)-Phe-Gln-Asn-Arg-Pro-Arg-Tyr-NH2, PerkinElmer, Boston, MA). The binding was carried out for each litter cohort separately due to the large number of slides. The sections were apposed to Biomax MR Film (Kodak) along with 125I autoradiography standards (Amersham Biosciences, Buckinghamshire, UK), for 4 days and then developed in a Kodak automatic developer. The same sections used for radio-labeling were then post-processed for acetylcholinesterase histochemistry (S7). V1aR binding was quantified against the 125I radiography standards using AIS 6.0 software (Imaging Research, Inc, St. Catharines, Ontario) with the acetylcholinesterase-stained sections and a rat brain atlas (S8) as neuroanatomical guides. Two to four sections per brain region were bilaterally quantified to give an average binding value for that brain region. Background binding levels were obtained by quantifying fiber tracts (corpus callosum and anterior commissure). The average binding value for fiber tracts was subtracted from the binding value for each brain region for each individual. These “background adjusted” data were used for the analysis. Three separate binding assays we performed, one for each litter cohort. For each cohort, the binding value for each brain region for each individual was normalized to the average binding value of that brain region of the short-alleled group. This normalization allowed for the assessment of fold differences in expression between the two genotype groups independent of any experimental variation among binding assays.

### Primate genetic analysis

The human AVPR1A gene has been cloned and characterized previously (S9). We identified the chimpanzee AVPR1A from a nucleotide-nucleotide BLAST (S10) of the coding region for the human AVPR1A. This identified a highly homologous locus on chromosome 12 of the chimpanzee genome that corresponds to the putative chimpanzee AVPR1A (predicted gene accession number XM_509185). The human AVPR1A also resides on human chromosome 12. The alignment of the chimpanzee shotgun sequence (NW_114825) with the human AVPR1A revealed a highly conserved 5’ noncoding region, with the exception of the 360bp in and around the –3625 microsatellite that was absent in the chimpanzee sequence. Considering that bonobos (Pan paniscus) have intriguing social bonding behavior, we decided to investigate the bonobo sequence at this uniquely human locus to determine whether it was more similar to humans or to chimpanzees. The genome sequence of Pan paniscus is not available, so we obtained blood samples from a bonobo (“P-suke”) at Georgia State University (Atlanta, GA). The DNA was extracted with a kit (Genta Systems; Minneapolis, MN) using standard methods. Specific primers flanking the microsatellite region were used to amplify the bonobo sequence (forward: 5’-ATCGATCTAGATATGCACCTCATACTGTAAGC; reverse: 5’-ATCGATGGGATGAGTTAACAATGT). The letters in bold indicate primer modifications with Cla I sites for use in downstream applications. Using a high fidelity Taq polymerase (ExpandHiFi, Roche; Mannheim, Germany), these primers produced a PCR product that was much closer to the human size than the chimpanzee size. This bonobo PCR product was TA-cloned into a TOPO vector (Invitrogen; Carlsbad, CA) and sequenced. The bonobo, human and chimpanzee sequences were aligned with AlignX software (VectorNTI suite, Informax/Invitrogen, Carlsbad, CA) with a ClustalW alignment algorithm.
Note

Avpr1a is the gene name for the entire locus encoding and regulating (in cis) the expression of the arginine vasopressin 1a receptor. We have previously used v1ar as the gene name in voles (Microtus spp.). By changing the name to avpr1a, we are now using the same name that is used for this locus in many other species (mouse, rat, human, chimp, sheep, etc.). Other researchers studying this receptor use V1aR to identify the protein and avpr1a to identify the gene.

Supporting References

S4. E. Hammock, M. Lim, H. Nair, L. Young, Genes Brain Behav (in press).
Fig. S1. In the elevated plus maze (A), long-alleled males spent more time in the center (Student’s t test, $P < 0.05$) and less time in the closed arms (Student’s t test, $P < 0.05$) compared to the short-alleled males, but there were no differences in the time spent by the two genotypes out in the open arms (Student’s t test, $P > 0.05$), or in the total number of crosses through the center (Mann-Whitney, $P > 0.05$). In the open-field test (B), there were no genotype differences in duration in the center of the field or freezing (Student’s t test, $P > 0.05$), or rearing frequency (Mann-Whitney, $P > 0.05$). However, long-alleled males had a lower grooming duration (Student’s t test $P < 0.05$) compared to short-alleled males.