

Facilitation of Affiliation and Pair-Bond Formation by Vasopressin Receptor Gene Transfer into the Ventral Forebrain of a Monogamous Vole

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Behaviors associated with monogamy, including pair-bond formation, are facilitated by the neuropeptide vasopressin and are prevented by a vasopressin receptor [V1a receptor (V1aR)] antagonist in the male prairie vole. The neuroanatomical distribution of V1aR dramatically differs between monogamous and nonmonogamous species. V1aR binding is denser in the ventral pallidal region of several unrelated monogamous species compared with nonmonogamous species. Because the ventral pallidum is involved in reinforcement and addiction, we hypothesize that V1aR activation in this region promotes pair-bond formation via a mechanism similar to conditioning. Using an adeno-associated viral vector to deliver the *V1aR* gene, we

increased the density of V1aR binding in the ventral pallial region of male prairie voles. These males exhibited increased levels of both anxiety and affiliative behavior compared with control males. In addition, males overexpressing the V1aR in the ventral pallidal region, but not control males, formed strong partner preferences after an overnight cohabitation, without mating, with a female. These data demonstrate a role for ventral pallidal V1aR in affiliation and social attachment and provide a potential molecular mechanism for species differences in social organization.

Key words: vasopressin; V1a receptor; ventral pallidum; social attachment; affiliation; viral vector; monogamy; pair bond

Affiliative behavior and social bonding are essential components of human society, yet little is known of the neural circuitry regulating these complex behavioral processes. Disruptions in these processes may be associated with psychiatric diseases characterized by social deficits, such as autism. Voles provide a useful animal model for investigating the neural mechanisms underlying these behaviors (Carter, 1998; Young et al., 1998; Insel and Young, 2001). Vole species display a wide range of social behavior, ranging from being highly social and monogamous to being solitary and promiscuous. Both field and laboratory studies have demonstrated that prairie voles (*Microtus ochrogaster*) are a highly gregarious species that forms enduring, selective social bonds between mates (Shapiro and Dewsbury, 1990; Carter and Getz, 1993).

Pharmacological studies in prairie vole males have demonstrated that the neuropeptide arginine vasopressin (AVP) increases affiliative behavior and is critical for the formation of the pair bond (Winslow et al., 1993; Cho et al., 1999; Young et al., 1999a). In the laboratory, the formation of the pair bond is assessed using a partner-preference paradigm in which the subject chooses to associate with either the partner or a novel female of similar stimulus value. An AVP antagonist selective for the V1a receptor (V1aR) subtype blocks the development of the partner preference when given centrally before mating (Winslow et al., 1993; Cho et al., 1999). Conversely, central infusions of AVP

facilitate partner-preference formation even in the absence of mating (Winslow et al., 1993). AVP infusions do not alter affiliative behavior in the nonmonogamous montane vole (Young et al., 1999a). These data suggest that AVP released during social interactions and during mating bouts activates neural circuitry that regulates these behaviors in a species-specific manner.

The neuroanatomical distribution of the V1aR differs dramatically among closely related species that differ in their social structure, providing a potential explanation for the species-specific effects of AVP (Insel et al., 1994). The monogamous prairie (*Microtus ochrogaster*) and pine (*M. pinetorum*) voles, California mouse (*Peromyscus californicus*), and common marmoset (*Callithrix jacchus*) each have high densities of V1aR binding in a region of the ventral forebrain containing the ventral pallidum and substantia innominata. In this same region, relatively little V1aR binding is detectable in related nonmonogamous montane (*M. montanus*) and meadow (*M. pennsylvanicus*) voles, white-footed mice (*Peromyscus leucopus*), and rhesus monkeys (Insel et al., 1994; Bester-Meredith et al., 1999; Young, 1999; Young et al., 1999b).

Because the ventral pallidum has been associated with reward and conditioned place preference (McBride et al., 1999), we hypothesized that V1aR in this area may facilitate affiliation and partner-preference formation through its reinforcing actions in the ventral pallidum. If this is correct, animals with high densities of V1aR in this region would exhibit higher levels of affiliative behavior and be more likely to form social attachments. To test this hypothesis, we used an adeno-associated viral (AAV) vector to selectively increase V1aR expression in the ventral pallidal area of male prairie voles and examined the behavioral consequences, including anxiety, affiliation, and partner-preference formation.

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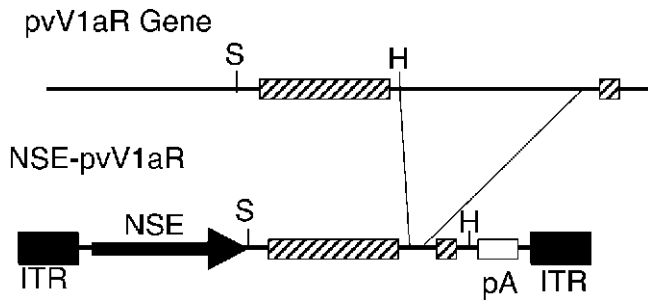


Figure 1. The NSE-*pvV1aR* plasmid was constructed by inserting a modified genomic prairie vole *V1aR* clone between the NSE promoter (arrow) and the SV40 small t intron and poly(A) signal (*pA*) (open box). The *V1aR* sequence used in the plasmid spanned from +20 relative to the transcription start site to the stop codon, with all but 287 bp of the intron deleted. The striped box indicates *V1aR* exons. *S*, *SalI*; *H*, *HindIII*; *ITR*, viral iterated terminal repeats.

MATERIALS AND METHODS

Animals and treatment

Subjects were 2- to 5-month-old sexually naive male prairie voles (*M. ochrogaster*) reared in our laboratory from field-caught specimens. Animals were housed with one to two same-sex litter mates in a 14/10 hr light/dark cycle and were provided food (Purina Rabbit Chow; Purina Mills, St. Louis, MO) and water *ad libitum*. Littermates housed together were assigned to different treatment groups to control for variability within litters and within cages. All experiments were performed in compliance with the rules and oversight of the Emory Institutional Animal Care and Use Committee.

Three groups of male prairie voles were used in this study. The experimental group [*V1aR*-ventral pallidum (VP); $n = 13$] received bilateral infusions of the neuron-specific enolase (NSE)-prairie vole *V1aR* (*pvV1aR*) viral vector into the ventral pallidum area. One control group [*V1aR*-caudate putamen (CP); $n = 9$] received identical injections into the caudate putamen, a region that does not express the endogenous *V1aR*. A second control group (*lacZ*-VP/CP, $n = 8$) received a control vector expressing the *lacZ* gene rather than the *V1aR*, into either the ventral pallidum or the caudate putamen.

AAV infusions were performed under ketamine anesthesia in a stereotaxic apparatus fitted with an Ultra Micro Pump II (World Precision Instruments, Sarasota, FL) apparatus with a Hamilton syringe adapter. The injection coordinates were as follows (in mm): ventral pallidum, 1.3 anterior, 1.0 lateral, and 5.5 ventral; caudate putamen, 1.3 anterior, 2.0 lateral, and 4.0 ventral. Bregma was used as the anterior–posterior and medial–lateral reference point, and the top of the skull at the anterior–lateral coordinate site was used as the dorsal–ventral reference point. Once the Hamilton syringe was lowered to the injection site, the recombinant AAV (rAAV) preparation (1 μ l/site at 10^8 infectious units/ μ l) was infused at a rate of 3–5 nl/sec. The needle was left in place for 2 min after the injection. All injections were bilateral. Animals recovered for 12 d before behavioral testing.

After the behavioral studies, brains were collected and analyzed for *V1aR* expression and accuracy of injection.

AAV production

The prairie vole *V1aR* viral vector (NSE-*pvV1aR*) consisted of a modified *V1aR* genomic clone with the majority of the intron removed, spliced downstream of a neuron-specific enolase promoter (Fig. 1). The *V1aR* cassette was created by first excising the sequence downstream of the *HindIII* site located 123 bp 3' of the exon–intron boundary. The *HindIII* end was destroyed and the plasmid was recircularized to facilitate subsequent cloning steps. The second exon was amplified by PCR using a 5' primer located 133 bp upstream of the second exon and a 3' primer that contained the stop codon and a *HindIII* restriction site. The amplified fragment was spliced downstream of the destroyed *HindIII* site. At the 5' end of the clone, a *SalI* site was inserted into the *SacI* site located 20 bp downstream of the putative transcription initiation site and 227 bp upstream of the translation initiation codon. The resulting *V1aR* sequence, containing a 227 bp 5' untranslated region and the first and second exons separated by a 287 bp intron, was cloned into the *SalI* and

HindIII sites of an AAV plasmid derived from pSSV9, a genomic clone of AAV-2 (originally provided by R. J. Samulski, University of North Carolina, Chapel Hill, NC). The AAV coding sequences of pSSV9 were first excised and replaced with a 0.44 kb simian virus 40 (SV40) DNA fragment (SVpA) containing the SV40 small t intron and poly(A) signal as well as several unique cloning sites. The viral iterated terminal repeats required for packaging remained intact in this plasmid. A PCR-amplified NSE promoter fragment (1 kb in length) and the *pvV1aR* sequence were then inserted 5' to the SVpA segment. Transduction by AAV-2 vectors into the brain is primarily confined to neurons (Bartlett et al., 1998), but the NSE promoter further restricts expression to neurons (Peel et al., 1997). The *lacZ* vector was prepared in similar manner, incorporating a PCR-amplified 0.6 kb cytomegalovirus-immediate early promoter and the 3.7 kb *Escherichia coli lacZ* gene sequence. Packaging of all recombinant AAV plasmids was done according to standard protocols described previously, with some modifications, by co-complementation with the AAV *trans*-acting factors provided on a separate plasmid and an adenovirus helper (Wu et al., 1998). AAV vector stocks were titrated by real-time PCR using an Applied Biosystems Prism 7700 Sequence Detection System from Perkin-Elmer Applied Biosystems (Foster City, CA). Titers of AAV vector preparations as determined by this technique average 10^{11} /ml.

Behavior testing

Elevated plus maze. The elevated plus maze testing was performed in an isolated behavior room at 23–24°C. Animals were brought to the testing room 30 min before testing began to acclimate to the environment. The plus maze apparatus consisted of an open plank intersecting a walled plank, each measuring 1.4 m in length and elevated 1 m above the ground. The subject was placed in the center of the apparatus and its location was recorded for 10 min.

Affiliation test. The affiliation test was conducted in a novel cage between 3:00 and 5:00 P.M. in a quiet room with a temperature of 23–24°C. After a 30 min acclimation, a 29- to 31-d-old juvenile male prairie vole was placed in the cage and behavior was recorded for 10 min. Each juvenile was used for no more than two tests per day.

Partner-preference test. Partner-preference tests were performed immediately after a 17 hr cohabitation with a nonestrous adult female. The pair were allowed to interact freely but mating did not occur during the cohabitation period because the females were not in behavioral estrus. Under these conditions, male prairie voles typically do not develop a partner preference in <24 hr of cohabitation unless mating occurs (Insel and Hulihan, 1995; Insel et al., 1995). The experimental male was placed in the center, neutral chamber of a three-chambered testing arena in which the partner was tethered in one chamber and a novel (stranger) female was tethered in a second chamber. The experimental animal was free to move throughout these chambers via Plexiglas connecting tubes. During the 3 hr test, the location and proximity of the male to each female was recorded. Partner preference was defined as spending more than twice as much time in the partner's cage relative to the stranger's cage.

Receptor binding analysis

V1aR binding was localized by receptor autoradiography using a 125 I-linear AVP antagonist (NEX 310; DuPont NEN, Boston, MA) as described previously (Young et al., 1997). Receptor density on autoradiographic film was quantified using NIH Image software on a Macintosh computer. Bilateral measurements from three sections per animal were taken, and a mean density measurement was obtained by subtracting background. Optical densities were converted to dpm/mg tissue equivalents using 125 I microscalers (Amersham Pharmacia Biotech, Arlington Heights, IL) as standards.

RESULTS

The animals receiving the NSE-*pvV1aR* vector into the ventral pallidum region (*V1aR*-VP) had nearly a 100% increase in receptor density in the ventral pallidum area compared with the control groups (Fig. 2) [ANOVA, $F_{(2,24)} = 6.99$, $p < 0.004$; Fisher's least significant difference (LSD) *post hoc* test, $p < 0.003$ and $p < 0.01$ compared with *V1aR*-CP and *lacZ*-VP/CP, respectively]. Those animals receiving similar injections into the caudate putamen (*V1aR*-CP) also had significantly more *V1aR* binding in the caudate relative to the *V1aR*-VP and the *lacZ*-VP/CP groups

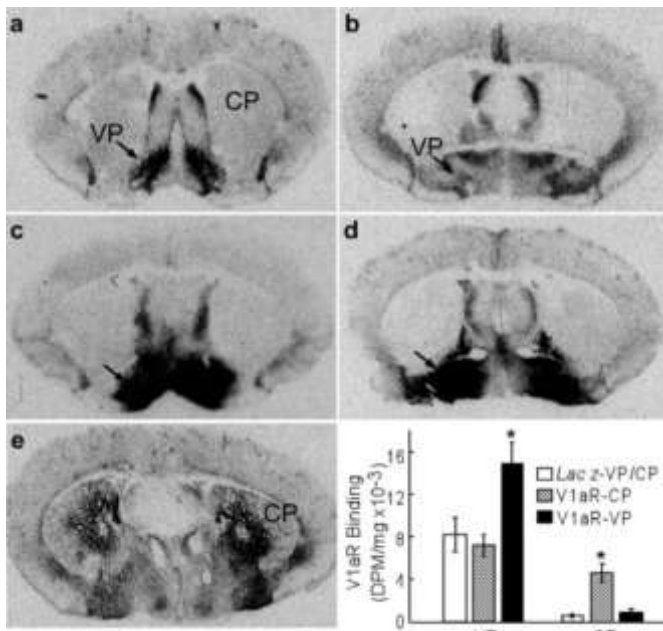


Figure 2. V1aR binding autoradiograms illustrating ¹²⁵I-linear AVP antagonist binding in *lacZ*-VP/CP males (*a*, *b*) and *V1aR*-VP males (*c*, *d*) at the level of the VP (arrows). Sections *a* and *c* are slightly rostral to those of *b* and *d*. The V1aR binding field in control animals is most intense in the more rostral section of the ventral pallidum. V1aR binding in the caudate putamen of the *V1aR*-CP males is illustrated in *e*. The quantitative analysis (mean ± SEM) of the V1aR binding in the ventral pallidum and caudate putamen of each group is presented in the bottom right panel. **p* < 0.01.

(Fig. 2) (ANOVA, $F_{(2,24)} = 22.6$, $p < 0.001$; Fisher's LSD *post hoc* test, $p < 0.001$ compared with each of the other groups). V1aR binding was not limited exclusively to the ventral pallidum in all animals because there was some spread of the virus beyond the boundaries of this nucleus; however, the ventral pallidum showed a consistent increase in V1aR binding in all of the *V1aR*-VP animals.

Because AVP has been shown to increase anxiety in rats via a V1aR-dependent mechanism (Landgraf et al., 1995; Liebsch et al., 1996), the infected voles were first tested on an elevated plus maze to measure general anxiety. The *V1aR*-VP group spent significantly less time on the open arms of the plus maze compared with the control groups (Fig. 3*a*) (ANOVA, $F_{(2,23)} = 11.43$, $p < 0.0001$; Fisher's LSD *post hoc* test, $p < 0.001$ compared with each control group). This suggests that increasing V1aR in the ventral pallidum results in increased anxiety in this testing paradigm.

The *V1aR*-VP group also displayed elevated levels of affiliative behavior relative to the control groups. Specifically, when placed in a novel cage and then presented with a novel juvenile male for a 10 min exposure, *V1aR*-VP animals spent significantly more time investigating and huddling (side-by-side contact) with the juvenile compared with the control groups (Fig. 3*b*) (ANOVA, $F_{(2,27)} = 4.85$, $p < 0.004$; Fisher's LSD *post hoc* test, $p < 0.007$ and $p < 0.04$ compared with *pvV1a*-CP and *lacZ*-VP/CP, respectively). Within the *V1aR*-VP group, there was no significant correlation between the time spent in the open arms of the elevated plus maze and affiliative behavior.

To determine whether overexpression of V1aR altered pair-bond formation, we tested for the development of a partner preference, measured as time spent with the female partner versus a

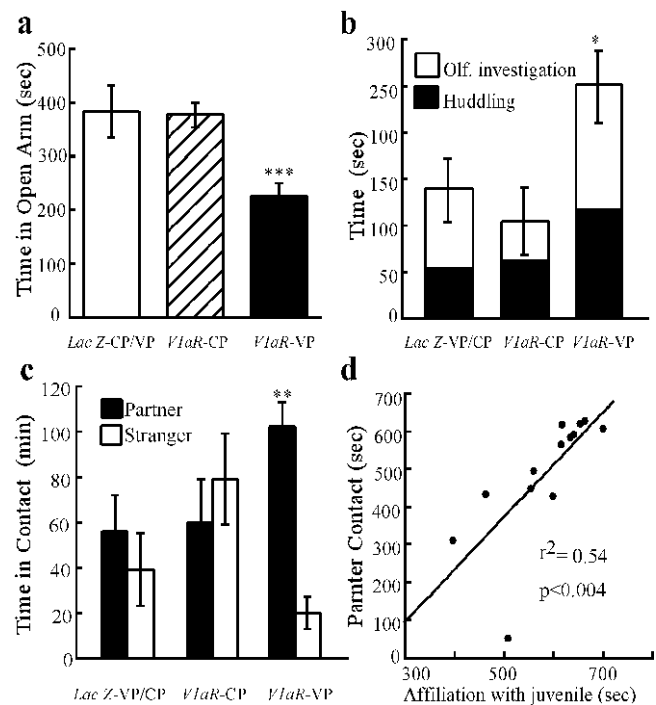


Figure 3. The effects of *V1aR* gene transfer on anxiety, affiliative behavior, and partner-preference formation in male prairie voles. *a*, Animals with increased *V1aR* expression in the ventral pallidum area (*V1aR*-VP) spent significantly less time on the open arms of the elevated plus maze compared with animals injected with the *lacZ* control virus (*lacZ*-VP/CP) or the *pvV1aR* vector into the caudate putamen (*V1aR*-CP). *b*, *V1aR*-VP males engaged in more affiliative behavior, defined as either olfactory investigation (filled bar) or side-by-side contact (open bar), during a 10 min encounter with an unfamiliar juvenile male. *c*, After a 17 hr cohabitation with a nonreceptive female, *V1aR*-VP males exhibited a partner preference as indicated by spending significantly more time in side-by-side contact with the partner than with the stranger in a 3 hr partner-preference test. This pattern was not observed in either control group. *d*, Among the *V1aR*-VP males, there was a significant correlation between the duration of social interactions in the affiliation test and the time spent in side-by-side contact with the partner in the partner-preference test. * $p < 0.05$; ** $p < 0.005$; *** $p < 0.0001$.

novel female in a simple 3 hr choice test. The sexually naive males were housed with nonestrous females for 17 hr during which mating did not occur. The *V1aR*-CP and the *lacZ*-VP/CP males did not consistently display a partner preference, defined as spending twice as much time in the cage with the partner than with the novel female. Of the 17 animals in these groups, 5 exhibited a preference for the partner, 6 exhibited a preference for the stranger, and 6 did not reach the criteria for a preference for either animal. In contrast, 12 of the 13 *V1aR*-VP males exhibited a partner preference. In addition, as a group, the *V1aR*-VP animals spent significantly more time in side-by-side contact with their partner than with the stranger (Fig. 3*c*) ($p < 0.005$, Wilcoxon signed rank test). This was not the case for the control groups. In the *V1aR*-VP group, but not in the control groups, there was a positive correlation between the duration of social interactions in the affiliation test with the juvenile and the time spent in contact with the partner in the partner-preference test (Fig. 3*d*) ($r^2 = 0.54$; $p < 0.004$).

DISCUSSION

The results from this study also confirm a role for AVP and the V1aR in the regulation of anxiety, affiliative behavior, and social

attachment. This report is also the first demonstration that complex social behaviors, such as social attachment, can be facilitated by viral vector gene transfer. In addition, the results demonstrate that animals expressing relatively high levels of V1aR in the ventral pallidum display higher levels of affiliative behavior and are more likely to form a pair bond than animals with lower levels of receptor in this region. The results are consistent with the hypothesis that species differences in V1aR expression may explain species differences in social organization, particularly because several monogamous species have higher densities of V1aR in the ventral pallidum than related nonmonogamous species. These data also imply that individual differences in the expression of the *V1aR* gene could account for individual differences in social behavior and attachment and could have important implications for psychiatric conditions such as autism.

Dense networks of AVP immunoreactive fibers are found in the lateral septum and extend ventrally into the ventral pallidal area of the male prairie vole (Wang et al., 1996). In rats, the septal AVP innervation arises from the bed nucleus of the stria terminalis and the medial amygdala (DeVries and Buijs, 1983). It has been demonstrated recently that AVP infused into the septal area facilitates the formation of partner preferences in the male prairie vole (Liu et al., 2001). Together with the present results, these data suggest that vasopressinergic neurons located in the bed nucleus of the stria terminalis and the medial amygdala regulate partner-preference formation through the release of AVP into the lateral septal and ventral pallidal area, resulting in the activation of ventral pallidal V1aR.

There are two caveats worth noting in our present results. First, V1aR binding increased in a diffuse region of the medioventral forebrain, sometimes including the portions of diagonal band, ventral lateral septum, and nucleus accumbens. We have focused on the ventral pallidum because this region showed increased binding in all of the experimental animals and because this is the region that expresses the endogenous *V1aR* gene in prairie voles. A second issue is the possibility that regions other than the ventral pallidum are involved in the AVP-dependent regulation of social behavior and pair bonding. V1aRs are also found in the amygdala, thalamus, cingulate cortex, and olfactory bulb. Although our results do not rule out an involvement of these areas, they do demonstrate that increased levels of V1aR in the ventral pallidum facilitate partner-preference formation.

The V1aR is a G-protein-coupled, seven transmembrane domain receptor. Hepatic V1aR is coupled to $G_{\alpha q11}$, which activates phospholipase C, thereby increasing inositol triphosphate and intracellular calcium (Wang et al., 1991). Biochemical studies have suggested that the V1aR is also associated with other G_{α} subunits as well as with phospholipase A (Strakova et al., 1997). Presumably, the behavioral effects of V1aR activation in the ventral pallidal area are the result of the activation of a specific set of second messenger pathways, and are therefore not necessarily specific for AVP or the V1aR. It would be interesting to express in the ventral pallidum other G-protein-coupled receptors that are coupled to the same effector systems in conjunction with the appropriate ligand administration to determine whether the behavioral specificity lies in the V1aR itself or in the downstream second messenger systems.

Microdialysis studies in rats have demonstrated that vasopressin is released in the brain under certain stressful situations such as after a social defeat or during a forced swim test (Wotjak et al., 1996, 1998). Decreasing V1aR expression using antisense oligonucleotides or infusion of a V1aR antagonist decreases anxiety in rats, as measured by increased time in the open arms of the

elevated plus maze (Landgraf et al., 1995; Liebsch et al., 1996). The present results are consistent with these observations because animals with elevated V1aR expression in the ventral pallidum exhibited a decrease in time spent in the open arms of the elevated plus maze. These same animals exhibited higher levels of affiliation as measured by increased time investigating and huddling with a juvenile. It is plausible that heightened levels of affiliation are attributable to the increased anxiety. For example, animals that are more anxious may tend to seek the comfort of social contact. However, the lack of a correlation between time spent in the open arms of the plus maze and the duration of affiliative behavior in the *V1aR*-VP group suggests that the increases in anxiety and affiliation are probably regulated by different mechanisms.

The *V1aR*-VP males exhibited a strong partner preference after the 17 hr cohabitation without mating. It is important to note that in previous studies from our group, male prairie voles that cohabitated with a female for 24 hr did form partner preferences if mating occurred, but typically did not if mating did not occur (Insel and Hulihan, 1995; Insel et al., 1995). Thus, it seems that by increasing the density of V1aR in the ventral pallidum, the amount of social stimulation required to form a partner preference was decreased. Also, the probability of forming a pair bond during this short exposure period could have been affected by increased affiliative behavior displayed by the males. Among the *V1aR*-VP males, there was a correlation between the duration of affiliative behavior with the juvenile and the time spent in contact with the partner. Perhaps the increased social interest expressed in these animals resulted in increased social stimulation, thereby facilitating the formation of the pair bond.

This is the first study to implicate the ventral pallidum in the regulation of social behavior and attachment. It should be noted that the field of V1aR binding in the prairie vole ventral forebrain is not restricted to the ventral pallidum but likely extends into the ventral lateral septum and substantia innominata. Thus we cannot rule out a role for these structures in the regulation of AVP-dependent behaviors. However, functional anatomical studies of the ventral pallidum make it a particularly interesting candidate site for a role in pair-bond formation. The ventral pallidum is a major relay of the shell of the nucleus accumbens and, like the nucleus accumbens, receives dopaminergic input from the ventral tegmental area (Klitenick et al., 1992). This striatopallidal system is an important neurobiological substrate for the rewarding and reinforcing properties of natural stimuli and psychostimulants (McBride et al., 1999). Infusion of psychostimulants directly into the ventral pallidum leads to the development a conditioned place preference for the environment in which the injections were experienced (Gong et al., 1996). Given the abundance of V1aR in the prairie vole ventral pallidum and its role in conditioned place preference, we hypothesize that AVP released during social interactions or mating activates V1aR in the ventral pallidum. Activation of this reward circuitry then reinforces this behavior, leading to an increase in social interactions. In a mating pair, the reinforcement is powerful enough to lead to a conditioned partner preference in the monogamous prairie vole and thereby initiates the formation of a pair bond. The lack of V1aR in the ventral pallidum of nonmonogamous vole species may explain their inability to form partner preferences after mating. There are most certainly other genetic, neurochemical, or anatomical differences between monogamous and nonmonogamous species that contribute to their diverse social behavior; however, the viral vector approach provides an opportunity to test this hypothesis. It should be possible to elevate V1aR expression in the ventral forebrain of nonmonogamous montane or

meadow voles and determine whether partner preferences are formed.

The role of AVP in facilitating pair bonding in the male prairie vole is remarkably parallel to that of oxytocin in the female prairie vole. In the female it is oxytocin, not vasopressin, that facilitates the formation of the pair bond with the mate (Insel and Hulihan, 1995). Oxytocin antagonist infused into the nucleus accumbens prevents partner-preference formation in the female (Young et al., 2001). In addition, prairie voles have much higher concentrations of oxytocin receptors in the nucleus accumbens than do nonmonogamous vole species (Insel and Shapiro, 1992). Dopamine D2 receptor antagonists infused into the nucleus accumbens also prevent partner-preference formation (Gingrich et al., 2000). Thus it appears that pair bonding is facilitated in a sex-specific manner, by two different neuropeptide systems acting at two separate points in a common neural circuit.

Our results are consistent with the hypothesis that the striato-pallidal reward circuitry facilitates certain aspects of affiliation and social attachment, implying common neural pathways for social attachment and the reinforcing effects of drugs of abuse (Panksepp, 1998). A recent functional magnetic resonance imaging study examined the pattern of brain activation and deactivation in human subjects as they viewed photographs of individuals with whom they reported to be romantically in love. The regions of activation were strikingly similar to those activated in studies of cocaine- and μ opioid agonist-induced euphoria (Bartels and Zeki, 2000). Although the role of vasopressin in human social attachment is unclear and the distribution of V1aR in the human brain has not been fully described, it is intriguing to consider that plasma vasopressin levels are elevated during sexual arousal in the human male (Murphy et al., 1987). Although increases in plasma AVP released from the posterior pituitary do not necessarily correspond to releases in the brain, these findings raise the possibility that similar neural mechanisms may underlie pair-bond formation in rodents and human males.

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