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VASOPRESSIN-DEPENDENT NEURAL CIRCUITS UNDERLYING PAIR BOND FORMATION IN THE MONOGAMOUS PRAIRIE VOLE

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Abstract—Arginine vasopressin and its V1a receptor subtype (V1aR) are critical for pair bond formation between adult prairie voles. However, it is unclear which brain circuits are involved in this vasopressin-mediated facilitation of pair bond formation. Here, we examined mating-induced Fos expression in several brain regions involved in sociosexual and reward circuitry in male prairie voles. Consistent with studies in other species, Fos expression was induced in several regions known to be involved in sociosexual behavior, namely, the medial amygdala, bed nucleus of the stria terminalis, and medial preoptic area. Fos induction also occurred in limbic and reward regions, including the ventral pallidum, nucleus accumbens, and mediodorsal thalamus (MDthal). Next, we infused a selective V1aR antagonist into three candidate brain regions that seemed most likely involved in vasopressin-mediated pair bond formation: the ventral pallidum, medial amygdala, and MDthal. Blockade of V1aR in the ventral pallidum, but not in the medial amygdala or MDthal, prevented partner preference formation. Lastly, we demonstrated that the mating-induced Fos activation in the ventral pallidum was vasopressin-dependent, since over-expression of V1aR using viral vector gene transfer resulted in a proportionate increase in mating-induced Fos in the same region. This is the first study to show that vasopressin neurotransmission occurs in the ventral pallidum during mating, and that V1aR activation in this region is necessary for pair bond formation in male prairie voles. The results from this study have profound implications for the neural circuitry underlying social attachment and generate novel hypotheses regarding the neural control of social behavior.

Key words: V1a receptor, ventral pallidum, medial amygdala, mediodorsal thalamus, affiliation, reward.

The neural control of complex social behaviors has traditionally been difficult to study. The monogamous prairie vole (*Microtus ochrogaster*) is an excellent animal model for studying the neural basis of social attachment and pair bond formation (Carter et al., 1995; Insel and Young, 2001). Pair bonding between adult mates is a complex social behavior that requires a length of cohabitation anywhere between 6 and 24 h, and is facilitated by mating during the cohabitation period (Williams et al., 1992; Insel and Hulihan, 1995; DeVries et al., 1996). Previous research has implicated the neuropeptide arginine vasopressin (AVP) and its brain receptor subtype, the V1a receptor (V1aR), in the pair bonding process in male prairie voles. For example, AVP given intracerebroventricularly to male prairie voles facilitates partner preference formation, while blockade of V1aR using i.c.v. injection of a selective antagonist prevents partner preference formation (Winslow et al., 1993).

The complex nature of pair bond formation likely involves the integration of several neural processes such as olfactory memory, sociosexual stimuli, and reward pathways. It is unclear where AVP is acting to facilitate pair bonding formation; however, a recent report has identified the lateral septum as a candidate brain region (Liu et al., 2001). Two other attractive candidate brain regions are the ventral pallidum and mediodorsal thalamus (MDthal), which are both involved in reward circuitry. Monogamous prairie and pine (*M. pinetorum*) vole species both have a high density of V1aR in the ventral pallidum and MDthal, but non-monogamous voles such as montane (*M. montanus*) and meadow (*M. pennsylvanicus*) voles do not (Insel et al., 1994; Wang et al., 1997a). More recently, our laboratory has used adeno-associated viral (AAV) vector gene transfer to increase V1aR density in specific regions of adult prairie vole brains. Male prairie voles with increased V1aR expression in the ventral forebrain, including the ventral pallidum, exhibited heightened levels of social affiliation and formed partner preferences with abbreviated cohabitation with a female without mating (Pitkow et al., 2001). Another candidate brain region is the medial amygdala, which plays a critical role in social memory formation, as well as olfactory and somatosensory processing of sociosexual stimuli, and also contains V1aR in prairie voles (Insel et al., 1994; Meisel and Sachs, 1994; Ferguson et al., 2001).

Given our hypothesis that sociosexual and reward circuits underlie pair bond formation, we first examined Fos induction in these circuits during mating in male prairie voles. In the second experiment, we used pharmacological blockade of V1aR using a selective antagonist to test the necessity of V1aR in the ventral pallidum, medial amygdala, and MDthal in partner preference formation. Finally, to determine whether mating-induced Fos expression could be vasopressin-dependent, we used AAV-mediated gene transfer to over-express V1aR *unilaterally* in the ven-

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Abbreviations: AAV, adeno-associated virus; AAV-lacZ, adeno-associated viral vector containing *lacZ* gene; AAV-V1a, adeno-associated viral vector containing *V1a* gene; AVP, arginine vasopressin; BnST, bed nucleus of the stria terminalis; LDthal, laterodorsal thalamus; MDthal, mediodorsal thalamus; MeA, medial amygdala; MPOA, medial preoptic area; NAcc, nucleus accumbens; V1aR, vasopressin V1a receptor.

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tral pallidum, and looked for asymmetric Fos induction between the two sides of the brain. Our data characterize the neural activation patterns in sociosexual and reward circuits during the initial steps of pair bond formation. Our data are the first to determine the necessity of V1aR in sociosexual and reward circuits during pair bond formation. This is also the first examination of vasopressinergic neurotransmission in the ventral pallidum in prairie voles during pair bond formation.

EXPERIMENTAL PROCEDURES

Subjects

Subjects were sexually naive male prairie voles that were the F4 generation of a laboratory breeding colony originally derived from field-captured voles in Illinois. After weaning at 21 days of age, subjects were housed in same-sex sibling pairs or trios and water and Purina rabbit chow provided *ad libitum*. All cages were maintained on a 14/10 h light/dark cycle with the temperature at 20 °C. Adult males at 70–100 days of age were used as the subjects, and females of comparable ages were used as stimulus animals. For manipulations that required mating, female stimulus animals were injected with 2 μg estradiol benzoate, daily for 3 days. All experiments were approved by the institutional guidelines set by the animal care and use committee of Emory University and conformed to the guidelines set by the National Institutes of Health, and an effort was made to minimize the number of animals used and their suffering.

Experiment 1

Mating behavior. All adult male animals ($n=24$) were isolated for 24 h prior to behavioral testing. In the "mated" group, adult male prairie voles ($n=8$) were paired with receptive female prairie voles for 2 h. This time point was chosen because Fos expression has been shown to peak between 1 to 2 h after the onset of a novel event (Sagar et al., 1988; Clayton, 2000). Two control groups in which adult male prairie voles ($n=8$ per group) were exposed to either familiar co-housed siblings or isolated for 2 h were processed simultaneously. All experimental males were observed to mate within the first 30 min. Males were then killed using CO_2 and their brains processed for Fos immunocytochemistry; adjacent sections were Nissl and acetylcholinesterase stained to better delineate neuroanatomical regions of interest as previously described (Lim et al., 2004b). Sections were quantified by an experimenter blind to the treatment groups and averaged for their right and left hemispheres.

Fos immunocytochemistry. All brain sections within each experiment were processed simultaneously within 1 week after sectioning. Briefly, sections were incubated in a 1:10,000 dilution of primary c-Fos polyclonal antibody raised in rabbit (Santa Cruz Laboratories, CA, USA) and then bathed in a 1:500 dilution of secondary biotinylated goat anti-rabbit antibody (Vector Labs, CA, USA). Avidin-biotin complexes were amplified using a standard Vectastain Elite ABC kit and visualized with diaminobenzidine peroxidase staining. Fos-positive cells were visualized using light microscopy and quantified using AIS 6.0, a software program (Imaging Research, Inc., Ontario, Canada). An area was drawn freehand approximating the region of interest, using adjacent Nissl and acetylcholinesterase stained sections as a guide, and a density threshold set. Both sides of the brain for each region were measured and averaged together. All sections were counted by a rater blind to the experimental condition and each region analyzed using a one-factor ANOVA for the three experimental groups.

Experiment 2

Stereotaxic injections of V1aR antagonist. Adult male prairie voles were anesthetized with a mixture of 3:2 ratio Ketaset: Dormitor mix i.p. (0.06 ml; ketamine 100 mg/ml; Dormitor 1 mg/ml) and placed into David Kopf stereotaxic apparatus using blunt earbars to prevent eardrum puncture. Coordinates for the lateral ventricle, ventral pallidum, medial amygdala, and MDthal were determined by dye injections (lateral ventricle: AP +0.6 mm, ML ±1.0 mm, DV −2.9 mm; ventral pallidum: AP +1.2 mm, ML ±0.8 mm, DV −5.4 mm; medial amygdala: AP −1.3 mm, ML ±2.7 mm, DV −6.1 mm; MDthal: AP −1.4 mm, ML ±0.7 mm, DV −3.5 mm). The scalp was incised and a burr hole opened, whereupon a Hamilton syringe was lowered and 1 μl of V1aR antagonist was injected. The V1aR antagonist, $d(CH_2)_5$[Tyr(Me)]AVP (Bachem, CA, USA), was dissolved in lactated Ringer's solution and was previously shown to be selective and behaviorally effective in prairie voles (Winslow et al., 1993; Cho et al., 1999; Liu et al., 2001). Bilateral control injections were given in the same manner using 1 UL of lactated Ringer's solution. Bilateral injections were given using an automatic micropump (World Precision Instruments, FL, USA) at a rate of 4 nL/s. Animals were induced out of the anesthesia using an equivalent dose of Antisedan (atipamezole hydrochloride; Pfizer), a reversing agent to Dormitor, and generally awakened between 15 and 45 min after the surgery.

Partner preference test. All animals were paired with receptive females immediately upon recovery for 22 h of cohabitation prior to testing for partner preference formation. The V1aR antagonist has been previously shown to have a receptor occupancy window of 18–24 h *in vivo* in the vole brain (Winslow et al., 1993). All animals were videotaped during the cohabitation and the presence of mating recorded. All males were observed to mate within the first 10 h of cohabitation. Animals were then tested for partner preference formation in a three-chambered apparatus as previously described (Williams et al., 1992; Winslow et al., 1993). Briefly, the testing apparatus consisted of a neutral cage (20 cm high×50 cm long×40 cm wide) joined by plastic tunnels to two parallel identical cages, one housing the tethered female partner, the other with a tethered female stranger. The males moved freely throughout the apparatus, and the time spent in contact with both the partner and the stranger was recorded for 3 h using a time-lapse video cassette recorder at a 12:1 time lapse (Panasonic, NJ, USA). All videotapes were scored by an experimenter blind to the treatment groups. Partner preferences were determined if males spent significantly more time with the partner than the stranger, using a two-tailed Student's *t*-test. Control animals in all groups were observed to mate and form partner preferences with no observable differences from previous control animals, lending validity to our novel surgical technique of rapid recovery and behavioral testing within 24 h following surgery. Following testing, animals were killed with an overdose of isoflurane, their brains removed and frozen on dry ice, and sectioned for Nissl histology to verify injection placement. A total of 12 animals, eight antagonists (three in the ventral pallidum, five in the medial amygdala [MeA]) and four controls were excluded from the data analysis because of improper injection placement.

Stereotaxic injections of ^{125}I-labeled V1aR antagonist. A separate group of animals ($n=6$) were placed into a stereotax and injected bilaterally with the linear V1aR antagonist ^{125}I-*d*$(CH_2)_5$(Tyr[Me])-AVP (PerkinElmer/NEN, MA, USA) into the ventral pallidum. Injections were performed using the same pump as described above. Animals were immediately killed upon recovery and the brains removed and snap frozen on dry ice. Brain sections were sliced at 20 μm and thaw-mounted onto microscope slides and immediately apposed to Kodak MR film to determine the extent of antagonist diffusion from the injection. Film was developed 4 days later.

Experiment 3

Stereotaxic injections of AAV-V1a viral vector. Adult male prairie voles ($n=16$) were anesthetized as described above and placed into stereotax. The scalp was incised and a burr hole opened over the ventral pallidal region of the skull. Animals received a unilateral 1 μl injection of an AAV vector containing the prairie vole *V1aR* gene (AAV-V1a) driven by a neuron-specific enolase promoter, as previously described (Pitkow et al., 2001). Control injections were performed in the contralateral ventral pallidum using a viral vector for the *lacZ* gene (AAV-lacZ). Animals were counter-balanced for right and left sides receiving AAV-V1a and AAV-lacZ. Injections were given using an automatic micropump (World Precision Instruments) at a rate of 4 nL/s and at a viral titer of approximately 10^8 infectious units/μl. Following injections, animals were injected with an equivalent dose of Antisedan and recovered within 15–45 min of surgery. Animals were allowed to recover for 2 weeks following surgery to allow for ample time for the AAV-V1a to integrate and express.

Mating behavior. Animals were randomly assigned to either the experimental group (2 h of mating; $n=8$) or the control group (2 h of isolation; $n=8$). After the test, animals were killed and their brains processed for Fos-immunoreactivity as described above. Brains were quantified for Fos induction by comparing the two hemispheres of ventral pallidum, instead of averaging the two values as previously described. This allowed for a within-subjects design, with each animal serving as its own control. Adjacent brain sections were also Nissl stained for histological verification of the injection sites. One animal from the mated group was dropped from data analysis because of improper injection placement.

V1aR autoradiography for unilateral AAV-V1a injection. A separate group of animals ($n=6$) was injected in the same manner as described above (one side AAV-V1a, the other side AAV-lacZ), and processed their brains for receptor autoradiography using the linear ligand ^{125}I-*d*$(CH_2)_5$(Tyr[Me])-AVP in order to verify that the AAV-V1a was successfully expressing. Animals were allowed to recover for 2 weeks before killing with an overdose of CO_2. Their brains were removed, snap frozen on dry ice, and sliced on a cryostat at 20 μm on Superfrost Plus slides (Fisher, PA, USA). Slides were processed for V1aR autoradiography using a 50 pM concentration of the ^{125}I-labeled antagonist as previously described (Young et al., 1997). The slides were then apposed to Kodak Biomax MR film for 4 days.

RESULTS

Experiment 1: which neural circuits show Fos induction during mating in monogamous prairie voles?

We chose to examine Fos induction in several brain regions involved in sociosexual behavior and reward circuitry. A schematic of the chosen candidate brain regions and their connectivities is shown in Fig. 1A and Fig. 1B. It is interesting to note that several of these regions, such as the medial amygdala, bed nucleus of the stria terminalis (BnST), medial preoptic area (MPOA), ventral pallidum, MDthal, and laterodorsal thalamus (LDthal), express V1aR in the prairie vole (Insel et al., 1994; Wang et al., 1997a). The LDthal was included because it is not hypothesized to be involved in sociosexual or reward circuitry, but has arguably the densest V1aR expression of all the regions, and was thus quantified as a control region.

As predicted, after 2 h of mating, Fos induction was observed in brain regions in both sociosexual and reward

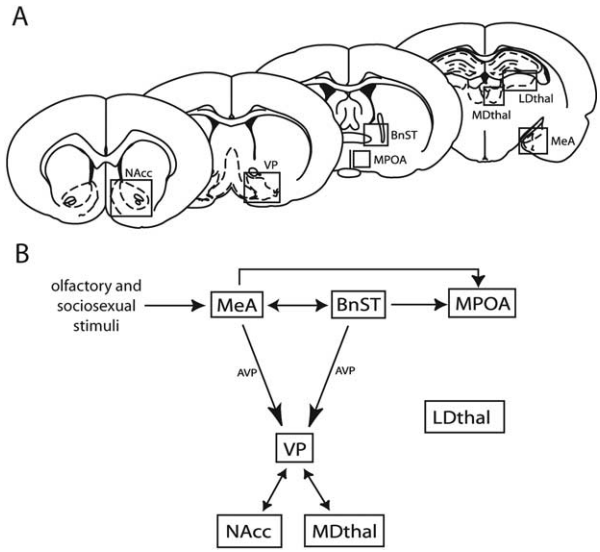

Fig. 1. (A) Neuroanatomical areas analyzed for Fos immunoreactivity after mating in male prairie voles. The boxes approximate the regions quantified for Fos expression in all three experimental groups. Adapted from a rat atlas (Paxinos and Watson, 1998). VP, ventral pallidum. (B) A schematic illustrating the flow of information from sensory systems during a sociosexual encounter into the rodent brain. Vasopressinergic projections from the MeA and BnST are thought to activate V1aR in the ventral pallidum, which projects to other reward-related regions (NAcc and MDthal), during pair bonding in prairie voles. All brain regions depicted, except the NAcc, show V1aR binding in prairie voles.

circuits, but not in the LDthal (Fig. 2). Fos expression was significantly higher in the medial amygdala, BnST, and MPOA, as well as in the ventral pallidum and nucleus accumbens (NAcc) in the mated group compared with both the sibling and the isolated groups ($P<0.05$, Kruskal-Wallis ANOVA for MeA, BnST, MPOA followed by Dunn's post hoc; single factor ANOVA for ventral pallidum and NAcc followed by Holms-Sidak post hoc). Fos expression did not significantly differ in the LDthal between any of the groups ($P>0.05$, single factor ANOVA). Interestingly, Fos expression in the MDthal was only significantly different between the mated and isolated groups, but not between the mated and sibling groups ($P<0.05$, Kruskal-Wallis ANOVA followed by Dunn's post hoc). Representative sections of Fos-immunoreactivity in the ventral pallidum are shown in Fig. 3, while Fos induction in the medial amygdala and MDthal are shown in Fig. 4. Because the ventral pallidum and NAcc were difficult to distinguish in the more rostral sections stained for Fos, we stained adjacent sections with Nissl and acetylcholinesterase to better delineate their neuroanatomical boundaries, as previously shown (Lim et al., 2004a).

Since Fos induction occurred in these brain regions with mating, and many of these brain areas express V1aR, these regions could potentially be involved in vasopressin-induced pair bond formation. We chose three candidate regions to infuse V1aR antagonist to test necessity for partner preference formation. The ventral pallidum and MDThal were both chosen for their involvement in reward circuitry, which is one of the putative cognitive mechanisms underlying pair bond formation (Insel and Young,

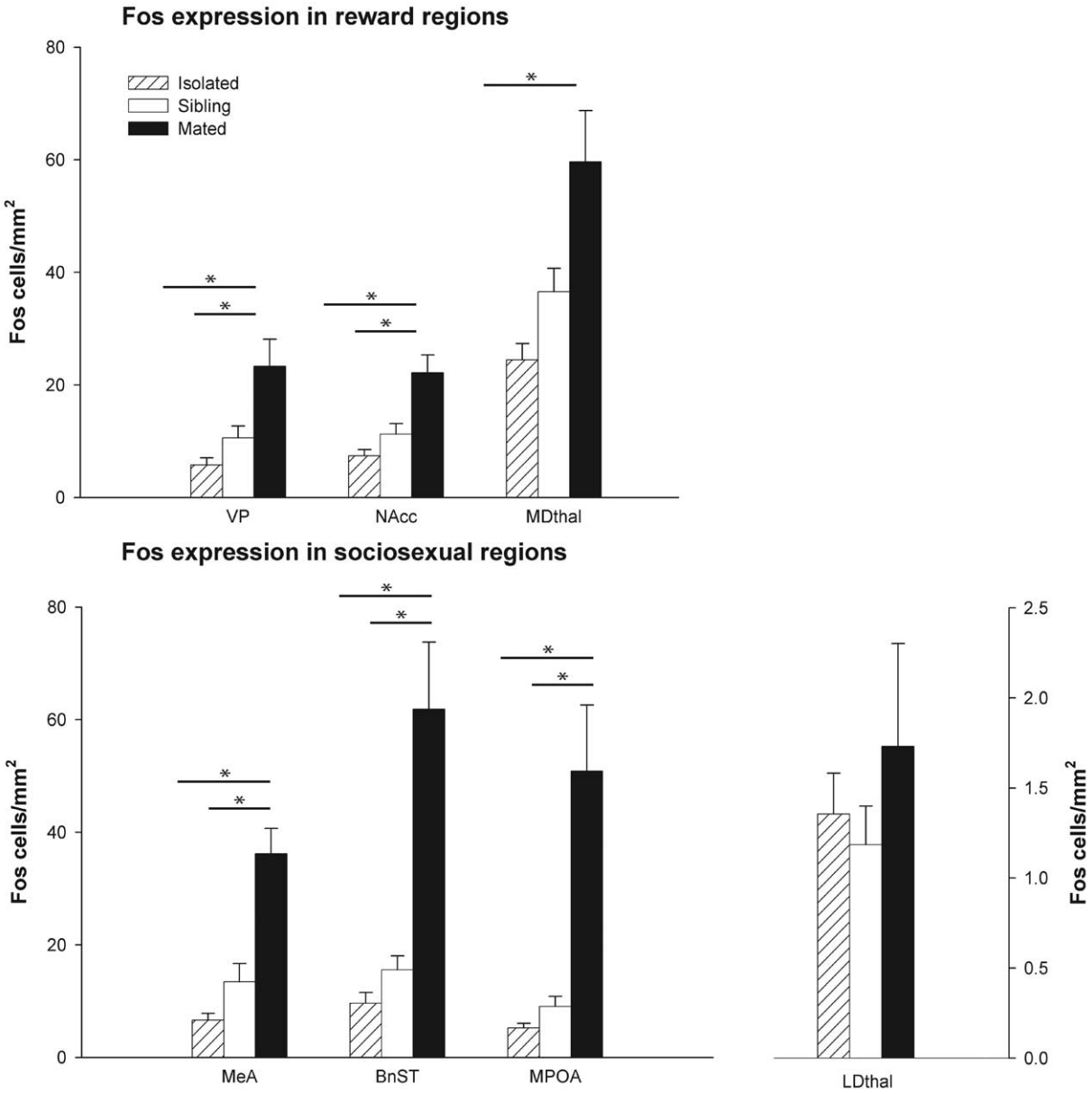

Fig. 2. Neural activation in several brain regions in different neural circuits for pair bonding. VP, ventral pallidum. Significant Fos induction was observed in the VP, NAcc, MDthal, MeA, BnST, and MPOA between the mated and isolated groups. Significant Fos induction was observed in the VP, NAcc, MeA, BnST, and MPOA between the mated and sibling-exposed groups. No significant differences in Fos expression were observed in the LDthal, despite this region showing dense V1aR expression. * $P<0.05$, one-way ANOVA.

2001; Aragona et al., 2003). The medial amygdala was also chosen because it is implicated in social memory and sociosexual sensory processing, which is a critical component for recognition of the partner (Ferguson et al., 2001).

Experiment 2: in which brain regions are V1aR necessary for partner preference formation?

We chose a site-specific dose, 0.05 ng/μl, for the V1aR antagonist injections based on previous reports using this antagonist in prairie voles (Winslow et al., 1993; Liu et al., 2001). To ensure that this dose was not effective i.c.v. for pair bond formation, animals were infused with 0.05 ng/μl into the lateral ventricles. These animals spent significantly more time with the partner than the stranger ($P<0.05$, Student's *t*-test; $n=9$). Next, animals were infused with 0.05 ng/μl bilaterally into the medial amygdala, MDthal, and the ventral pallidum. Infusions into the medial amygdala and MDthal failed to block partner preference formation, with animals preferring to spend more time with the partner over the stranger ($P<0.05$, Student's *t*-test; $n=14$ and $n=9$, respectively). However, animals that received V1aR antagonist infusion into the ventral pallidum did not show a preference for the partner or the stranger ($P>0.23$, Student's *t*-test; $n=7$). Control animals that received site-specific injections of lactated Ringer's into all

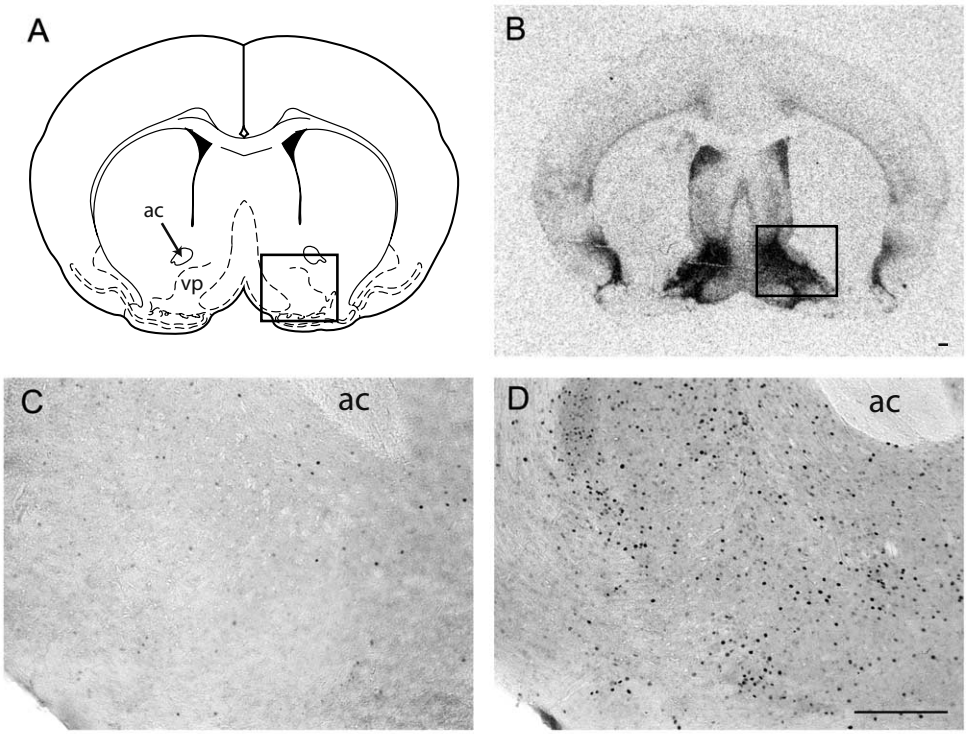

Fig. 3. Fos expression after mating in the ventral pallidum of male prairie voles. (A) Schematic adapted from the rat atlas showing the region of interest, the ventral pallidum (see box). ac, anterior commissure; vp, ventral pallidum. (B) Receptor autoradiography for V1aR in prairie vole brain at the same level as the rat atlas schematic (see box). Note the dense V1aR labeling in the ventral pallidum, but not the NAcc. (C) Fos immunoreactivity in the ventral pallidum in an animal isolated for two hours. (D) Fos induction in the ventral pallidum in an animal that mated for 2 h. Borders for the ventral pallidum were approximated using acetylcholinesterase and Nissl-stained adjacent sections for neuroanatomical guides (not shown). Scale bar=250 μm.

three regions readily spent more time with their partner than the stranger ($P<0.05$, Student's *t*-test; $n=20$, pooled). When the dose of V1aR antagonist was lowered 10-fold to 0.005 ng/μl, infusions into the ventral pallidum failed to block partner preference formation ($P<0.05$, Student's *t*-test; $n=4$). All results are shown in Fig. 5. Though there might appear to be a trend toward significance for decreased overall partner contact time with the antagonist injections in the medial amygdala and the MDthal, compared with the controls, this was not statistically significant ($P>0.65$, single factor ANOVA).

In order to determine if the injections hit the ventral pallidum, medial amygdala, and MDthal, brain sections were Nissl stained through the injection sites. A representative section of the ventral pallidum is shown in Fig. 6A. In addition, the extent of diffusion of the antagonist can be inferred in Fig. 6B, where bilateral infusions of ^{125}I-labeled antagonist appear confined to the ventral pallidum. Representative sections of the medial amygdala and MDthal are shown in Fig. 6C and 6D, respectively.

Experiment 3: does mating induce vasopressinergic neurotransmission in the ventral pallidum?

Since V1aR in the ventral pallidum, but not the medial amygdala or the MDthal, were found necessary for partner preference formation, we chose to examine vasopressinergic neurotransmission in the ventral pallidum during mating. Fos expression was used as a marker of V1aR-specific activation, following the AAV-vector mediated over-expression of V1aR in one hemisphere of the ventral pallidum. We replicated our results from experiment 1 showing that mated animals had significant Fos induction in the ventral pallidum compared with unmated animals. In addition, here we also show that mated animals ($n=7$) showed asymmetric Fos expression in the ventral pallidum: They showed a significant increase in Fos expression on the side that over-expressed V1aR from the AAV-V1a injection ($P<0.002$, Wilcoxon rank-sum test; Fig. 7). On average, mated animals showed an increase of 20 Fos cells per square mm on the side that had the increased V1aR. Isolated animals ($n=8$) did not show a difference in Fos expression between the AAV-V1a- and the AAV-lacZ-infected sides of the brain, suggesting that the observed increase in Fos on the AAV-V1a side was not an artifact of viral vector gene transfer itself.

In addition, in order to verify that the viral vector was successfully expressing, we injected a separate group of animals in the same manner as described above (one side AAV-V1a, the other side AAV-lacZ), and processed their brains for receptor autoradiography using the linear V1aR antagonist ^{125}I-*d*$(CH_2)_5$(Tyr[Me])-AVP. A representative section from these brains is shown in Fig. 7B. Because there are currently no viable antibodies to the V1aR in vole, a separate group of animals was used to obtain fresh-frozen sections to verify the over-expression of V1aR in the

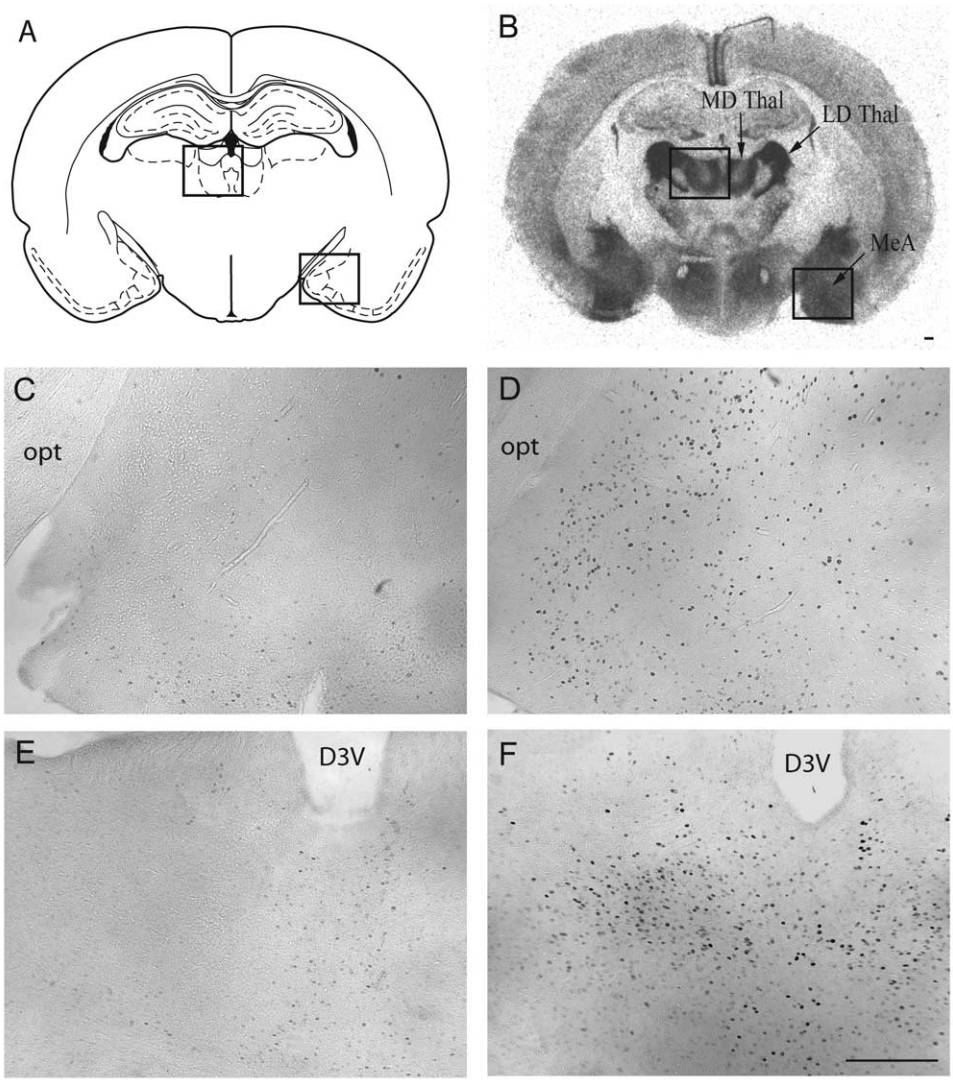

Fig. 4. Fos expression after mating in the MeA and MDthal of male prairie voles. (A) Schematic adopted from the rat atlas showing both regions of interest (see boxes). (B) V1aR autoradiography in prairie vole brain at the same level as the rat atlas schematic. Note the dense V1aR labeling in the MeA, MDthal, and LDthal. (C) Fos immunoreactivity in the MeA in an isolated animals. (D) Fos immunoreactivity in the MeA in a mated animal. (E) Fos immunoreactivity in the MDthal in an isolated animal. (F) Fos immunoreactivity in the MDthal in a mated animal. opt, optic tract, D3V, dorsal third ventricle. Scale bar=250 μm.

ventral pallidum. This provided a general idea as to how much V1aR was over-expressed on one side versus the control side, which on average was about two-fold higher (Fig. 7B). This level of over-expression is consistent with what was observed previously using AAV-V1a infection in the prairie vole (Pitkow et al., 2001).

DISCUSSION

These experiments show the neural activation patterns in sociosexual and reward-related brain regions during the first 2 h of pair bond formation in the prairie vole. Our data are the first to determine the necessity of V1aR in sociosexual and reward circuits during partner preference formation, and we demonstrate a critical role for vasopressinergic neurotransmission in the ventral pallidum, but not the medial amygdala or the MDthal, for pair bonding in the male prairie vole.

Mating-induced Fos expression

Our experiments were undertaken in the context of a hypothesis of the integration of specific neural circuits involved in pair bonding, including reward circuits, the olfactory processing of sociosexual stimuli, and social memory. In this proposed circuit, olfactory input projects to the medial amygdala, which in turn projects to the BnST and MPOA (Meisel and Sachs, 1994). The medial amygdala and BnST contain extrahypothalamic vasopressin cell bodies, which project to the forebrain, including the ventral pallidum (De Vries and Buijs, 1983). The ventral pallidum has extensive interconnections with reward regions such as the NAcc and the MDthal (Mogenson et al., 1987; Zahm and Heimer, 1990; Groenewegen et al., 1993), and is itself critical for conditioned place preference (Gong et al., 1996, 1997). We examined Fos induction in each of these regions as a consequence of mating, dem-

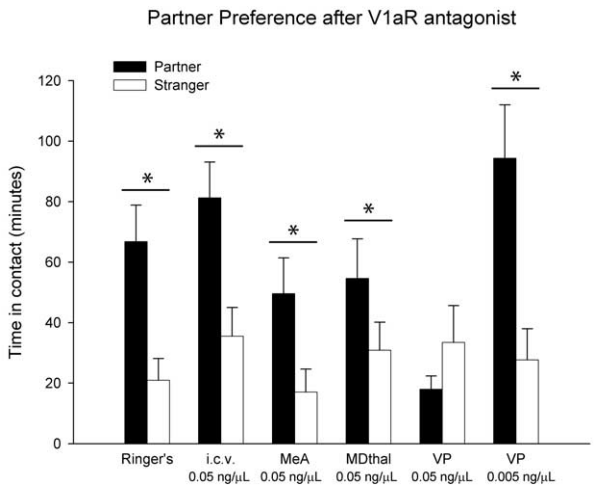

Fig. 5. Partner preference formation after V1aR antagonist injection into various brain regions. Control animals that received lactated Ringer's solution readily formed partner preferences, as well as animals that received 0.05 ng/μl of the V1aR antagonist i.c.v. Animals that received 0.05 ng/μl antagonist into either the MeA or the MDthal also spent significantly more time with the partner than the stranger. However, bilateral infusions of 0.05 ng/μl antagonist into the ventral pallidum prevented partner preferences. In contrast, animals that received a 10-fold lower dose of antagonist, 0.005 ng/μl, into the ventral pallidum formed partner preferences. * $P<0.05$, Student's *t*-test.

onstrating that several V1aR-expressing limbic and reward regions are activated preferentially with mating.

Several studies have clearly established the pattern of Fos induction as a consequence of male sexual behavior in a variety of species, including hamsters, rats, macaques, rabbits, and musk shrews (Baum and Everitt, 1992; Fernandez-Fewell and Meredith, 1994; Coolen et al., 1997; Newman et al., 1997; Gill et al., 1998; Michael et al., 1999; Reyna-Neyra et al., 2000). Another study has established the pattern of Fos induction in prairie voles after 1 h of cohabitation in males (Cushing et al., 2003), as well as 6 h of cohabitation in female prairie voles (Curtis and Wang, 2003). Consistent with Fos patterns in these studies, we also observed Fos induction in the medial amygdala, BnST, and MPOA. The fact that these brain regions were activated in both mating and cohabitation behaviors across many species suggests that these regions may be involved in more general aspects of sociosexual behavior, rather than specific aspects of pair bond formation. These past reports did not report activation in the ventral pallidum or NAcc, so it is unclear what Fos expression pattern would appear there.

It should be noted that the Fos activation observed in this study is likely a result of a combination of many events, and is not simply limited to just mating behavior, per se. Thus, additional control groups that might address the more detailed aspects of sociosexual interactions and mating behavior were not included in this study, since it is not known exactly which of these aspects truly contribute to the formation of the pair bond. All that is known is that mating and cohabitation with a member of the opposite sex can facilitate the pair bond in prairie voles (Williams et al., 1992; Insel et al., 1995). Since it is possible for males to form partner preferences with unreceptive females (Cho et al., 1999), but they have not been reported to form partner preferences with their same-sex siblings, we chose to include only the control group with exposure to their same-sex sibling. We did not include a control group with males exposed to a non-receptive female because we were not interested in the Fos activation observed during mating behavior alone. In addition, unreceptive females have been observed to fight with pursuant males, which would confound any conclusions drawn from Fos induction. Thus, many sensory, motor, and cognitive processes are engaged during the initial steps of pair bond formation, and all of these processes could potentially contribute to Fos induction in these brain regions.

In particular, neural activation in the ventral pallidum, medial amygdala, and MDthal confirmed our initial hypothesis that these three regions might be the critical sites for vasopressin-dependent pair bond formation in voles. All three brain regions highly express V1aR in prairie voles, but not in non-monogamous montane voles (Insel et al., 1994; Wang et al., 1997a). Pair bond formation can be dissociated into several cognitive processes, including the learned association between the olfactory/sociosexual characteristics of the mate and reward. The ventral pallidum and the MDthal are both involved in reward processes, and are directly connected to each other (Bielajew and Fouriezos, 1985; Mogenson et al., 1987; Kalivas et al., 1999). We hypothesized that the potential disruption of reward circuitry by V1aR antagonism in these regions might block pair bond formation. Additionally, the medial amygdala is thought to be a site of sensory integration of social stimuli, with projections from the accessory olfactory bulb and vomeronasal organ (Meisel and Sachs, 1994). It is also a crucial brain region for social memory and processing of social stimuli (Ferguson et al., 2001). For these reasons, we hypothesized that the potential disruption of sensory processing by V1aR antagonism in the medial amygdala would block pair bonding.

Ventral pallidal V1aR are necessary for pair bonding

Our results show that selective V1aR blockade in the ventral pallidum will prevent partner preference formation, suggesting that receptors in this region are necessary for pair bonding. Prairie voles are not the only monogamous species with highly abundant V1aR in the ventral pallidum; this same receptor pattern is seen in another monogamous vole species, the pine vole (*M. pinetorum*). Interestingly, other monogamous species across distantly related taxa also show this dense V1aR expression in homologous regions of the ventral forebrain. For example, the monogamous marmoset monkey and the monogamous California mouse (*Peromyscus californicus*) both have receptors here, but the non-monogamous rhesus monkey and the non-monogamous white-footed mouse (*P. leucopus*) do not (Wang et al., 1997b; Bester-Meredith et al., 1999; Young, 1999; Young et al., 1999). It is possible that the presence of V1aR in the ventral pallidum evolved convergently as a proximate mechanism for monogamous social structure in several different species.

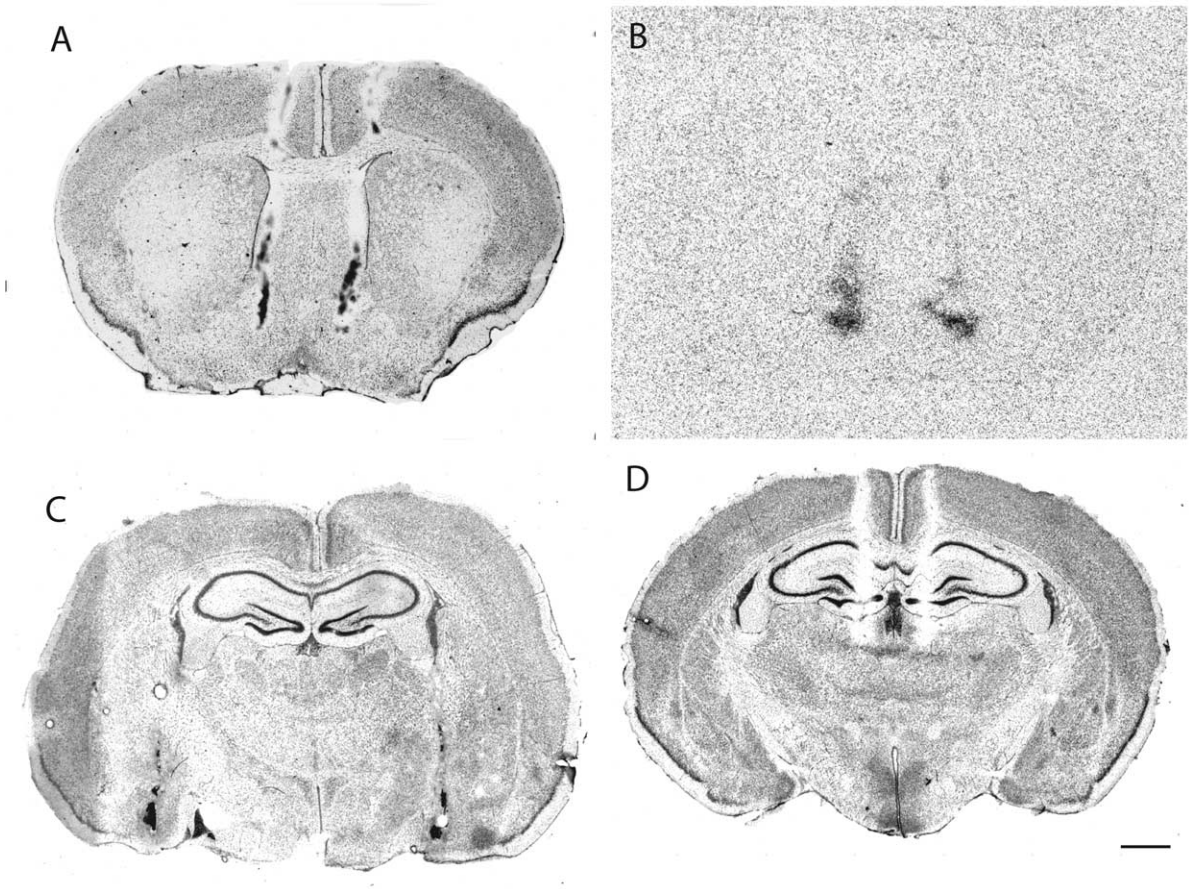

Fig. 6. Histology of V1aR antagonist injections. (A) Nissl stain of a representative brain section showing bilateral placement of V1aR antagonist injection in the ventral pallidum. (B) An infusion of ^{125}I-labeled V1aR antagonist into the ventral pallidum shows that the spread of injection is confined to a small area and is not diffusing to other brain regions. (C) Nissl stain of a representative brain section showing bilateral placement in the medial amygdala. (D) Nissl stain showing placement in the MDthal. Scale bar=1 mm.

Our results are also consistent with the hypothesis that pair bond formation is, at least in part, mediated by cognitive mechanisms of reinforcement. Recent studies have found that dopaminergic neurotransmission in the NAcc is both necessary and sufficient for partner preference in both male and female prairie voles (Gingrich et al., 2000; Young et al., 2001; Aragona et al., 2003). Since the ventral pallidum is heavily interconnected with the NAcc and both are key relay nuclei in the mesolimbic dopamine reward pathway, the ventral pallidum is anatomically and functionally poised to integrate V1aR activation with the dopaminergic reward aspects of pair bonding.

However, our results show that selective V1aR blockade of two efferent and afferent projects of the ventral pallidum, the medial amygdala and the MDthal, failed to prevent partner preference formation, suggesting that V1aR activation in these regions is not necessary for pair bond formation. The dose of V1aR antagonist used here was the same effective dose for the ventral pallidum. It is possible that the medial amygdala and MDthal might require a slightly higher dose of antagonist than the ventral pallidum; however, this is unlikely because a dose just 10-fold higher is effective i.c.v, and therefore not useful for site-specific injections. One study suggests that both V1aR and oxytocin receptors might be involved in male partner preference formation (Cho et al., 1999). It is possible that the administration of *both* an oxytocin receptor and V1aR antagonist into the medial amygdala and MDthal might then block partner preference. In the mouse, social memory formation in the medial amygdala has been shown to require oxytocin receptors (Ferguson et al., 2001). A recent study reported that vasopressin in the lateral septum regulated pair bonding in voles (Liu et al., 2001). Since vasopressin in the lateral septum is essential for social memory in other rodents, this is consistent with our hypothesis that social memory circuits are involved in the process of pair bond formation (Engelmann et al., 1996; Landgraf et al., 2003). In fact, vasopressin fibers course through the ventral pallidum, possibly releasing peptide through synaptic boutons *en passant,* en route to the lateral septum (De Vries and Buijs, 1983; Lim et al., 2004a). In the MDthal, the Fos induction pattern indicates that mating activity does not significantly differ from exposure to a same-sex sibling. This suggests that the MDthal activity may reflect general social interaction (and perhaps the rewarding aspects of), as opposed to pair bond-specific interaction.

These results do not imply that the medial amygdala and MDthal are not involved at all in pair bonding, but that *vasopressin* in these regions is not heavily involved. It is

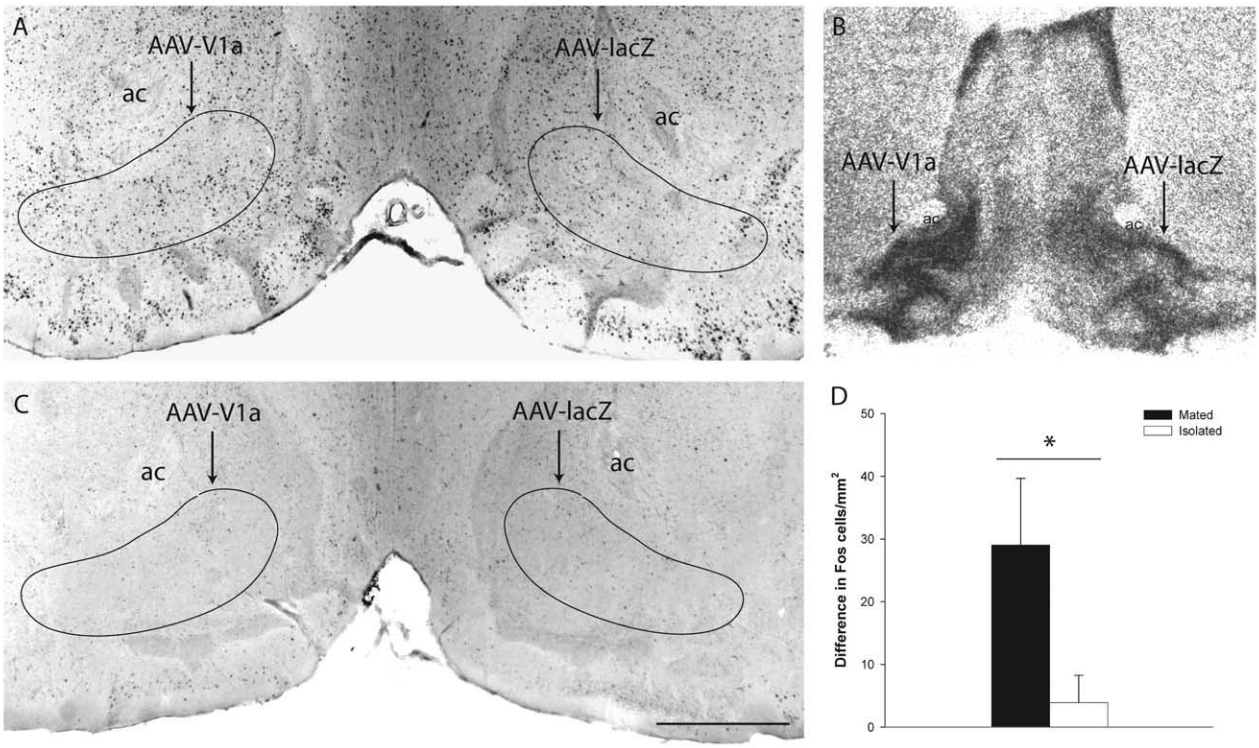

Fig. 7. Unilateral AAV-V1a increases mating-induced Fos in the ipsilateral ventral pallidum. (A) Mated animals show significantly higher Fos expression in the side that received the AAV-V1a virus than the side which received the control virus, AAV-lacZ. (B) V1aR autoradiography showing the unilateral over-expression of V1aR on the left ventral pallidum, with the control virus, AAV-lacZ on the contralateral side. (C) Animals that have been isolated for 2 h show no difference in Fos expression between the AAV-V1a and AAV-lacZ injected sides. (D) A bar graph showing the average difference in Fos expression between the two sides of the ventral pallidum, mated versus isolated animals. * $P<0.002$, Wilcoxon rank-sum test. Scale bar=1 mm.

also interesting to consider the fact that prairie voles show extraordinary individual variability in the density of V1aR in the medial amygdala and MDthal, with some animals showing only very weak binding (Phelps and Young, 2003). In contrast, the ventral pallidum shows very little individual variation in V1aR binding between prairie voles, consistent with the notion that there would be little variability in an important brain region that regulates species-typical behavior such as monogamy.

Vasopressinergic neurotransmission in the ventral pallidum

In the third experiment, our results show that the mating-induced Fos in the ventral pallidum was, in fact, due to V1aR activation, because upregulation of V1aR results in a significant increase in Fos in the same region. Previously, there has been no direct evidence of vasopressinergic neurotransmission in the ventral pallidum as a consequence of mating in the prairie vole. Given the recent literature on accumbens dopamine in pair bonding, it would be interesting to investigate the interaction between vasopressin and dopaminergic systems in the ventral forebrain in prairie voles (Aragona et al., 2003). Perhaps the combination of both these neurotransmitter systems within the ventral forebrain in prairie voles creates the foundation for social attachment.

In conclusion, our results show that vasopressinergic neurotransmission in the ventral pallidum, but not in other sociosexual or social memory circuits, is critical for pair bond formation in the prairie vole. This study provides support for reward circuitry in pair bonding and generates novel hypotheses about the role of vasopressin receptors in the integration of reward learning processes and social attachment. Ultimately, additional studies of the neurobiology underlying social attachment may lead to a better understanding of the neural control of social behavior and associated behavioral disorders.

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