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The V1a Vasopressin Receptor Is Necessary and Sufficient for Normal Social Recognition: A Gene Replacement Study

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Summary

Vasopressin modulates many social and nonsocial behaviors, including emotionality. We have previously reported that male mice with a null mutation in the *V1a receptor* (*V1aR*) exhibit a profound impairment in social recognition and changes in anxiety-like behavior. Using site-specific injections of a V1aR-specific antagonist, we demonstrate that the lateral septum, but not the medial amygdala, is critical for social recognition. Reexpressing V1aR in the lateral septum of V1aR knockout mice (V1aRKO) using a viral vector resulted in a complete rescue of social recognition. Furthermore, overexpression of the V1aR in the lateral septum of wild-type (wt) mice resulted in a potentiation of social recognition behavior and a mild increase in anxiety-related behavior. These results demonstrate that the V1aR in the lateral septum plays a critical role in the neural processing of social stimuli required for complex social behavior.

Introduction

Vasopressin (AVP) is a peptide produced in the hypothalamus and is both a neurohypophysial peptide released into circulation by the posterior pituitary, where it affects renal and vascular function, and a neuromodulator produced and released in specific regions throughout the brain, where it affects a wide range of behaviors (Landgraf et al., 1998). AVP was one of the first peptides to be characterized, and its peripheral roles in fluid homeostasis and blood pressure control have been extensively investigated. The specific behavioral roles for central AVP, the first of which was originally described in the 1960s by David de Wied, are still murky and under active investigation (reviewed in van Wimersma Greidanus et al., 1983; Engelmann et al., 1996). AVP has been associated with a wide number of behaviors including flank-marking behavior, paternal behavior, social recognition, aggression, affiliation behavior, and anxiety-like behavior (Ferris et al., 1984, 1986; Le Moal et al., 1987; Winslow et al., 1993; Landgraf et al., 1995; Bamshad and Albers, 1996; Liebsch et al., 1996; Everts and Koolhaas, 1997; Young et al., 1999; Parker et al., 2001; Pitkow et al., 2001). The existence of three major receptor types for AVP and the extensive distribution of AVP throughout the brain present a challenge for determining the neuroanatomical and neurochemical specifics of AVP in particular behaviors.

While AVP binds to three major receptors, only two exist in the central nervous system, the V1a and V1b receptor subtypes. The V1aR is located in many regions throughout the brain, while the V1bR is located primarily in the pituitary and in several other discrete areas of the brain including the amygdala (Johnson et al., 1993; de Vries and Miller, 1998; Hernando et al., 2001). Pharmacological manipulations using intracerebroventricular (icv) and intraseptal injections of V1aR-specific antagonists have been shown to affect both social behaviors and anxiety-like behaviors in rats (Dantzer et al., 1987; Engelmann and Landgraf, 1994; Everts and Koolhaas, 1999). Furthermore, administration of V1aR antisense oligos into the septal region resulted in impaired social recognition and altered anxiety-like behaviors (Landgraf et al., 1995).

While most of the evidence for AVP's effects on behavior has focused on the V1aR, there is mounting evidence that the V1bR may also play a role in these behaviors. Anxiety-like behavior was found to be altered after treatment with an orally active V1bR antagonist; however, characterization of a V1bR knockout mouse found no difference in anxiety-like behavior, but a significant effect on aggression and a mild impairment of social behavior (Griebel et al., 2002; Wersinger et al., 2002). It is possible that the different AVP receptors facilitate distinct behaviors.

We have previously reported that mice with a null mutation in the *V1aR* display a profound impairment in social recognition and a marked reduction in anxiety-like behaviors (Bielsky et al., 2004). Site-specific treatment with a V1aR viral vector into the lateral septum of normal rats resulted in an increase in V1aR binding of around 300% and a significant potentiation of social discrimination. Subsequent administration of a V1aR antagonist abolished this effect (Landgraf et al., 2003). Oxytocin, a closely related neuropeptide, has also been shown to be important in the regulation of social recognition in mice, particularly in the medial amygdala (Ferguson et al., 2000, 2001). Given the pharmacological and behavioral findings from all of these studies, the V1aR appears to be an important target for the study of AVP and social behaviors. Furthermore, the site-specific social effects of V1aR and oxytocin manipulations in the lateral septum and medial amygdala make these areas of particular interest in the study of social recognition. It is possible that V1aR-specific activation in either the lateral septum or the medial amygdala is critical for normal social recognition.

We attempt to address these possibilities by testing

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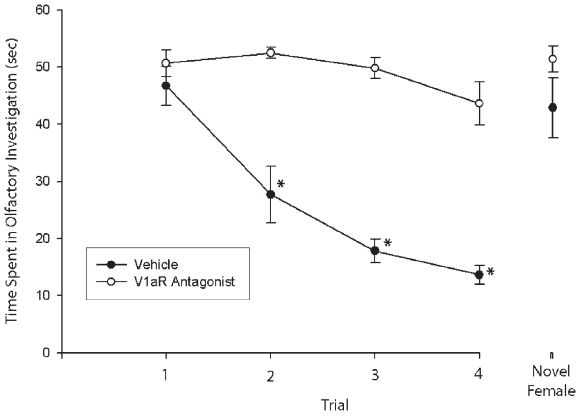

Figure 1. Social Recognition and Lateral Septum V1aR Antagonist Injections

Social recognition effects of lateral septum V1aR antagonist treatment (open circles; n = 9) and vehicle treatment (closed circles; n = 8) in wt mice was measured as a difference in olfactory investigation of the same ovariectomized female during each of four successive 1 min trials (ITI = 10 min). A fifth dishabituation trial depicts the response to a novel female in a 1 min pairing 10 min after the fourth trial. Wt mice treated with lateral septum vehicle injections showed a significant decrease in olfactory investigation after repeated pairings of the same female on trials 2, 3, and 4. Wt mice treated with lateral septum V1aR antagonist injections never showed a decrease in olfactory investigation. *p < 0.001. Error bars represent ±SEM. Asterisks represent a significant decrease between each trial as compared to the first trial.

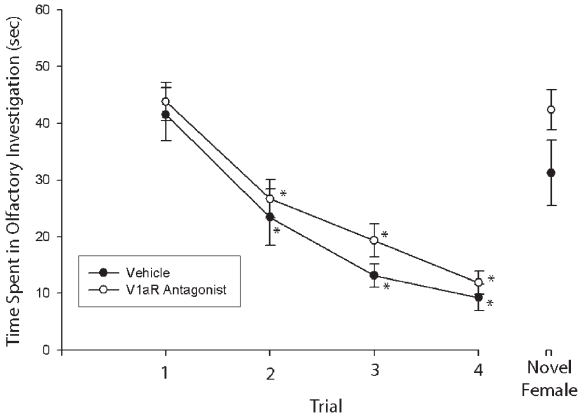

Figure 2. Social Recognition and Medial Amygdala V1aR Antagonist Injections

Social recognition effects of medial amygdala V1aR antagonist treatment (open circles; n = 9) and vehicle treatment (closed circles; n = 8) in wt mice was measured as a difference in olfactory investigation of the same ovariectomized female during each of four successive 1 min trials (ITI = 10 min). A fifth dishabituation trial depicts the response to a novel female in a 1 min pairing 10 min after the fourth trial. Wt mice treated with both medial amygdala vehicle and V1aR antagonist injections showed a significant decrease in olfactory investigation after repeated pairings of the same female on trials 2, 3, and 4. *p < 0.001. Error bars represent ±SEM. Asterisks represent a significant decrease between each trial as compared to the first trial.

the effects of site-specific V1aR antagonists on the social recognition behaviors of wild-type (wt) male mice and the effects of viral vector V1aR expression in both V1aRKO and wt mice. We chose both the lateral septum and the medial amygdala as site-specific targets for the V1aR antagonists and then assessed the effects of these bilateral injections on social recognition in order to determine the importance of both the specific receptor subtype and the neuroanatomical specificity of receptor activation on normal social recognition behavior.

We then used the results from these antagonist studies to determine which regions should be targeted with the V1aR viral vector in both V1aRKO and wt mice. The V1aRKO mice treated with the V1aR viral vector had renewed V1aR binding in only those areas targeted, while the wt mice had increased V1aR bindings in the specific areas targeted. Social recognition and anxiety-related behavior was then assessed in these groups in order to determine the neuroanatomical specificity and behavioral dependence on V1aR binding in discrete brain regions. These studies demonstrate that viral vector gene transfer used in conjunction with transgenic mouse technology provides a valuable method for the study of the role of localized gene expression in complex behaviors.

Results

Antagonist Studies

V1a Receptor Antagonist in Lateral Septum

Wt mice bilaterally injected with V1aR antagonist into the lateral septum showed a significant deficit in social recognition. Significant main effects for trial [$F(4, 15) = 26.276$; $p < 0.001$] and treatment [$F(1, 15) = 38.170$; $p < 0.001$] were detected for time spent in olfactory investigation of a stimulus female. A significant interaction effect was also detected [$F(4, 15) = 14.702$; $p < 0.001$]. Post hoc analysis revealed that wt mice bilaterally injected with vehicle into the lateral septum showed a significant decrease in social olfactory investigation upon subsequent presentations of the same female in trials 2 ($p < 0.001$), 3 ($p < 0.001$), and 4 ($p < 0.001$) as compared to trial 1. The mice treated with the antagonist had significantly impaired social recognition and never demonstrated a significant reduction in olfactory investigation in any of the trials (Figure 1). The olfactory investigation decrease seen in the vehicle-treated wt subjects in trials 2, 3, and 4 was not due to a general decrease in olfactory investigation, because presentation of a novel female during trial 5 resulted in a similar amount of investigation as trial 1 with the original female.

V1a Receptor Antagonist in Medial Amygdala

Wt mice bilaterally injected into the medial amygdala with either V1aR antagonist or vehicle showed no deficits in social recognition. There was no significant main effect for treatment [$F(1, 13) = 1.357$; $p = 0.265$] and no significant interaction effect [$F(4, 13) = 1.436$; $p = 0.235$]. There was a significant main effect for trial [$F(4, 13) = 75.960$; $p < 0.001$]. Post hoc analysis revealed that both medial amygdala treatment groups showed a significant decrease in social olfactory investigation upon subsequent presentations of the same female in trials 2 ($p < 0.001$), 3 ($p < 0.001$), and 4 ($p < 0.001$) as compared to trial 1. The wt mice in both groups showed

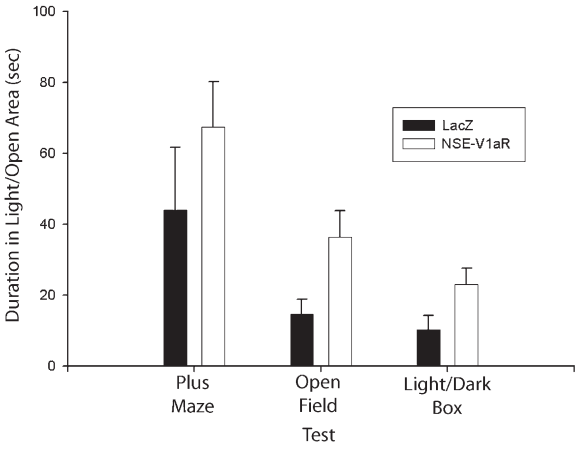

Figure 3. Anxiety-Related Behaviors in V1aRKO

Anxiety-related behaviors for LacZ virus-treated (filled bars; n = 8) and NSE-V1aR viral vector-treated (open bars; n = 15) V1aRKO male mice. There was no difference between the LacZ- and NSE-V1aR-treated mice on any of the anxiety-related behavior tests, as they spent similar time in the open arm of the plus maze test, the open area of the open field test, and the light box in the light/dark box test. Error bars represent SEM.

a typical dishabituation to a novel female as measured by an increase in olfactory investigation in trial 5 as compared to trials 2 ($p < 0.05$), 3 ($p < 0.001$), and 4 ($p < 0.001$) (Figure 2). Therefore, it is clear that the olfactory investigation decrease seen in both medial amygdala treatment groups in trials 2, 3, and 4 was not due to a general decrease in olfactory investigation.

Reexpression of V1aR in V1aRKO

Anxiety-Related Behaviors

There was no difference between the V1aRKO mice treated with the NSE-V1aR and LacZ viral vectors in the lateral septum on any of the tests for anxiety-like behavior. NSE-V1aR- and LacZ-treated V1aRKOs spent similar time in the open arm in the elevated plus maze ($p = 0.314$), in the open area of the open field test ($p = 0.208$), and in the light box in the light/dark box test ($p = 0.272$) (Figure 3). There was also no significant treatment effect in overall locomotor activity in the elevated plus maze as measured by the number of dark arm entries ($p = 0.651$) and the distance traveled in the open field test ($p = 0.06$).

Social Recognition and Olfactory Habituation

V1aR reexpression using the NSE-V1aR viral vector in the lateral septum resulted in a complete rescue of social recognition in the V1aRKO males. The V1aRKO males that were treated with the control LacZ virus showed the significant deficit in social recognition previously reported for untreated V1aRKO mice (Bielsky et al., 2004). Significant main effects for treatment [$F(1, 21) = 25.053$; $p < 0.001$] and trial [$F(4, 21) = 26.611$; $p < 0.001$] were detected for time spent in olfactory investigation of a stimulus female. A significant interaction effect was also detected [$F(4, 21) = 15.141$; $p < 0.001$]. Post hoc analysis revealed that NSE-V1aR viral vector-treated mice showed a significant decrease in olfactory investigation upon subsequent presentations of the same female in trials 2 ($p < 0.001$), 3 ($p < 0.001$), and 4($p < 0.001$) as compared to trial 1.V1aRKO treated with the control LacZ virus had significantly impaired social recognition and never demonstrated a reduction in olfactory investigation in any of the trials (Figure 4A). The olfactory investigation decrease seen in the NSE-V1aR-treated subjects in trials 2, 3, and 4 was not due to a general decrease in olfactory investigation, because presentation of a novel female during trial 5 resulted in a similar amount of investigation as trial 1 with the original female.

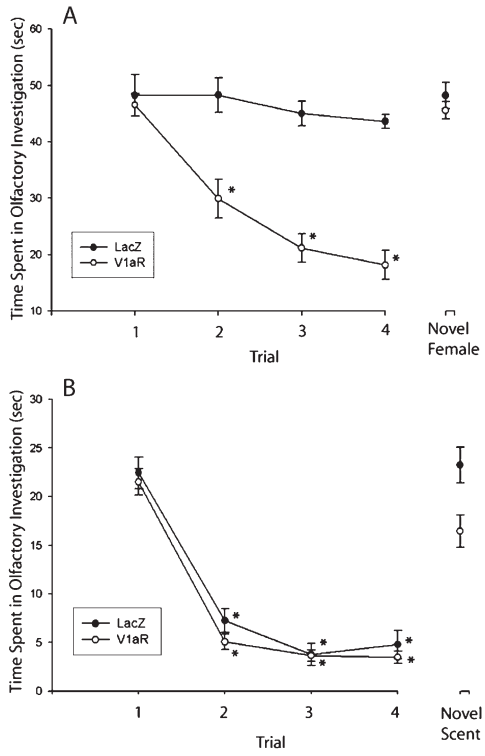

Figure 4. Social Recognition and Olfactory Habituation

(A) Social recognition in NSE-V1aR viral vector-treated (open circles; n = 15) and LacZ virus-treated (closed circles; n = 8) V1aRKO mice was measured as a difference in olfactory investigation of the same ovariectomized female during each of four successive 1 min trials (ITI = 10 min). A fifth dishabituation trial depicts the response to a novel female in a 1 min pairing 10 min after the fourth trial. NSE-V1aR viral vector-treated V1aRKO mice showed a significant decrease in olfactory investigation after repeated pairings of the same female on trials 2, 3, and 4. LacZ virus-treated V1aRKO mice never showed a decrease in olfactory investigation. (B) Olfactory habituation was measured as a difference in olfactory investigation of an anise-scented cotton ball during each of four successive 1 min trials (ITI = 10 min). The fifth, dishabituation trial depicts the response to a coconut-scented cotton ball in a 1 min exposure 10 min after the fourth trial. Both LacZ-treated (n = 8) and NSE-V1aR-treated (n = 15) mice showed a decrease in investigation upon subsequent presentations of the same-scented cotton ball on trials 2, 3, and 4. *$p < 0.001$. Error bars represent ±SEM. Asterisks represent a significant decrease between each trial as compared to the first trial.

The lack of social recognition in the LacZ-treated V1aRKO was not due to a general deficit in olfaction, as both the NSE-V1aR and LacZ virus-treated V1aRKO subjects habituated to repeated presentations of a same-

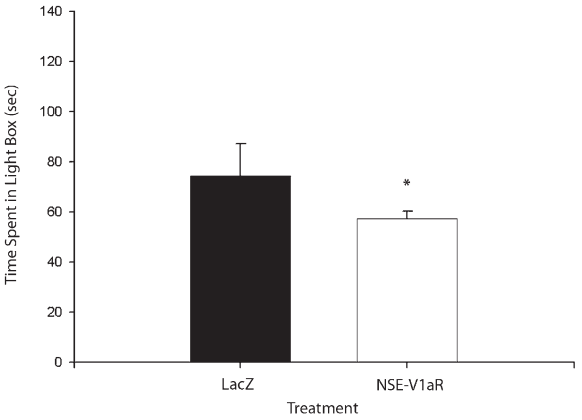

Figure 5. Anxiety-Related Behaviors in Wt

Anxiety-related behaviors for LacZ virus-treated (filled bars; n = 10) and NSE-V1aR viral vector-treated (open bars; n = 12) wt male mice. Lateral septum NSE-V1aR viral vector-treated wt mice spent significantly less time in the light box of the light/dark box as compared to the lateral septum control LacZ virus-treated wt mice (A). There was no difference between the NSE-V1aR- and LacZ-treated wt mice in the number of light box entries (B). Error bars represent SEM. *p < 0.05.

scented cotton ball. A significant effect for trial [$F(4, 21) = 130.905$; $p < 0.001$] but not treatment [$F(1, 21) = 3.653$; NS] was detected for the time spent in olfactory investigation. Post hoc analysis revealed that both NSE-V1aR- and LacZ-treated V1aRKO mice showed a significant decrease in the time spent in olfactory investigation of a same-scented cotton ball on trials 2, 3, and 4 ($p < 0.001$) (Figure 4B). Again, this was not due to a general decrease in olfactory investigation, because presentation of a novel-scented cotton ball on trial 5 resulted in a similar amount investigation as trial 1 with the original scent. There was no treatment difference in the amount of initial investigation in either test.

Receptor Autoradiography

There was no effect on V1aR binding of the LacZ virus treatment in the V1aRKO. The LacZ-treated V1aRKO had a complete lack of V1aR binding throughout the brain including the lateral septum (Figure 7C). The NSE-V1aR viral vector treatment into the lateral septum resulted in very high levels of V1aR binding in the lateral septum; however, there was some spread into the surrounding regions including the medial septum (Figure 7D). All other regions lacked V1aR binding.

Overexpression of V1aR in Wt Mice

Anxiety-Related Behavior

Overexpression of the V1aR in the lateral septum of wt mice resulted in an increase in anxiety-related behavior as measured by the light/dark box test. NSE-V1aR viral vector-treated wt mice spent significantly less time in the light part of the light/dark box as compared to the control LacZ virus-treated wt mice ($p < 0.05$) (Figure 5A). There was no significant treatment effect in overall locomotor activity as measured by the number of light box entries ($p = 0.467$) (Figure 5B).

Social Discrimination

V1aR overexpression using the NSE-V1aR viral vector in wt mice resulted in a significant improvement in social discrimination as compared to control LacZ virus-treated wt. After IEI of 0.5 hr ($p < 0.001$), 2 hr ($p < 0.001$), 6 hr ($p < 0.001$), and up to 24 hr ($p < 0.01$) (Figures 6A–6D, respectively) the NSE-V1aR-treated wt mice were able to recognize a previously encountered female, as they spent significantly less time investigating the same female and significantly more time investigating the novel female. The LacZ-treated wt mice were able to recognize a previously encountered female only after an interexposure interval (IEI) of 0.5 hr ($p < 0.001$). After IEI of 2 hr, 6 hr, and 24 hr, the LacZ-treated wt mice spent similar amounts of time investigating the same and novel females, indicating a lack of social discrimination. There was no difference in the time spent in the initial investigation of the same female between the NSE-V1aR and LacZ viral vector-treated animals for any of the IEIs.

Receptor Autoradiography

V1aR overexpression in the lateral septum of wt mice was confirmed using V1aR autoradiography. The wt mice treated with the NSE-V1aR viral vector in the lateral septum had an average V1aR binding (dpm/mg) in the lateral septum of 4631.28 ± 328.3 (Figure 7A), while wt mice treated with the LacZ control virus in the lateral septum had an average V1aR binding (dpm/mg) in the lateral septum of 2036.78 ± 113.1 (Figure 7B). The overexpression of the V1aR using the NSE-V1aR viral vector was statistically significant ($p < 0.001$) and resulted in an almost 100% increase in V1aR binding in the lateral septum of wt mice; however, there was some spread into the surrounding regions including the medial septum.

Discussion

The results of this study definitively establish a role for the lateral septum in V1aR-regulated social recognition in male mice. Furthermore, these findings suggest that, while both the lateral septum and medial amygdala have been previously shown to be important regions for social recognition, it is V1aR in the lateral septum and not in the medial amygdala that is critical to social recognition, and the social behavior effects of a null mutation in the *V1aR* gene can be reversed by V1aR activation in the lateral septum alone. In addition, these findings support our previous report on the profound social recognition deficits in mice with a null mutation in the *V1aR* gene. Given the results of this current study, it appears that disruption of social recognition in the V1aRKO mice may have been due to the specific lack of V1aR activation in the lateral septum.

While the hypothalamus is the major source of AVP production, there is also an extrahypothalamic AVP system, and this sexually dimorphic system plays a critical role in the central and behavioral effects of AVP (de Vries and Miller, 1998). AVP is synthesized in the medial amygdala and the bed nucleus of the stria terminalis, and these AVP neurons project to forebrain structures including but not limited to the lateral septum. However, the lateral septum contains a dense

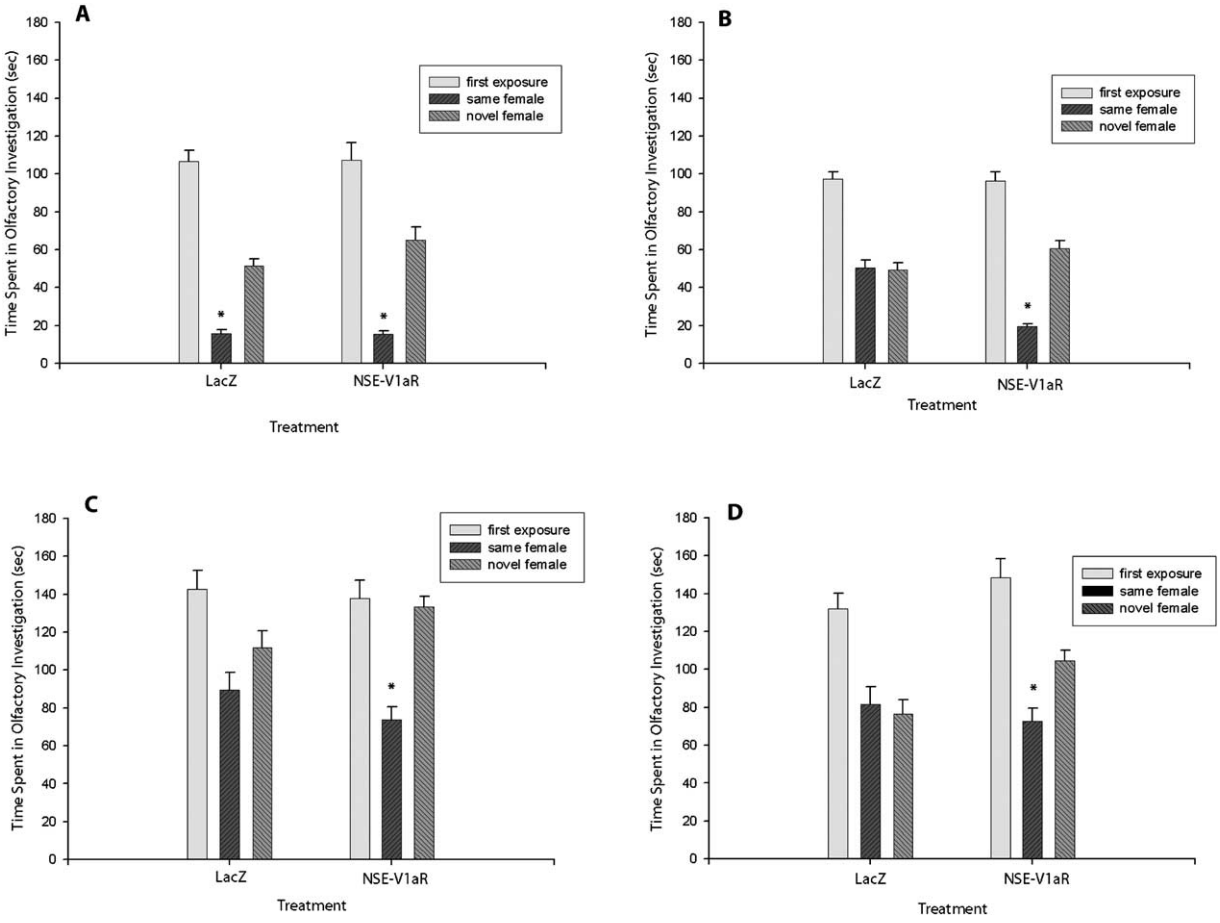

Figure 6. Social Discrimination

Social discrimination of NSE-V1aR viral vector-treated (open circles; n = 12) and LacZ virus-treated (closed circles; n = 10) wt mice as measured by the olfactory investigation times of a previously encountered female (same) and a novel female (novel) during a simultaneous exposure of 5 min, with varying interexposure intervals (IEI) of (A) 0.5 hr, (B) 2 hr, (C) 6 hr, and (D) 24 hr, after the initial 5 min exposure to the (same) female. After an IEI of 0.5 hr, both groups spent more time investigating the novel female. After 2 hr, 6 hr, and 24 hr, only the NSE-V1aR viral vector-treated wt males spent significantly more time investigating the novel females. Error bars represent SEM. *$p < 0.001$, **$p < 0.01$.

plexus of AVP-containing fibers (de Vries and Miller, 1998). V1aR binding autoradiograms show very dark and concentrated binding in the lateral septum, suggesting that this area is rich in V1aR and may be an important area in AVP-controlled behaviors (Barberis and Tribollet, 1996; Bielsky et al., 2004). Furthermore, the lateral septum projects to many hypothalamic regions, including the medial preoptic area, that are involved in the regulation of social behaviors, including male sexual behaviors (Sheehan and Numan, 2000). The lateral septum is also capable of receiving information from the olfactory vomeronasal system, which would be critical to the ability of an animal to use scent for social recognition (Risold and Swanson, 1997). The circuitry and neurochemistry of the lateral septum make an attractive target for the study of social recognition in male mice.

Social Recognition

The ability to recognize a familiar conspecific is the foundation for all social relationships, including parent-offspring relationships, pair bonding in voles, dominant-subordinate hierarchies in hamsters, social status in fish, and courtship and aggression in birds, and while these behavioral examples vary in both expression and species, they all share the involvement of AVP or its nonmammalian analog AVT (Ferris et al., 1986; Bamshad and Albers, 1996; Chu et al., 1998; Goodson and Adkins-Regan, 1999; Parker and Lee, 2001; Semsar et al., 2001; Lim and Young, 2004). The role for AVP in social cognition appears to be heavily conserved across species, as does the presence of AVP and the V1aR in the lateral septum. While the importance of species-typical binding and expression patterns of V1aR and AVP have been demonstrated in some of the above behaviors, including pair bonding in voles, the existence of septal V1aR does not differ greatly and thus may be responsible for the underlying social recognition that is the foundation for the complex social behaviors.

Social recognition tasks were originally introduced as nonaversive alternatives to the traditional avoidance tests

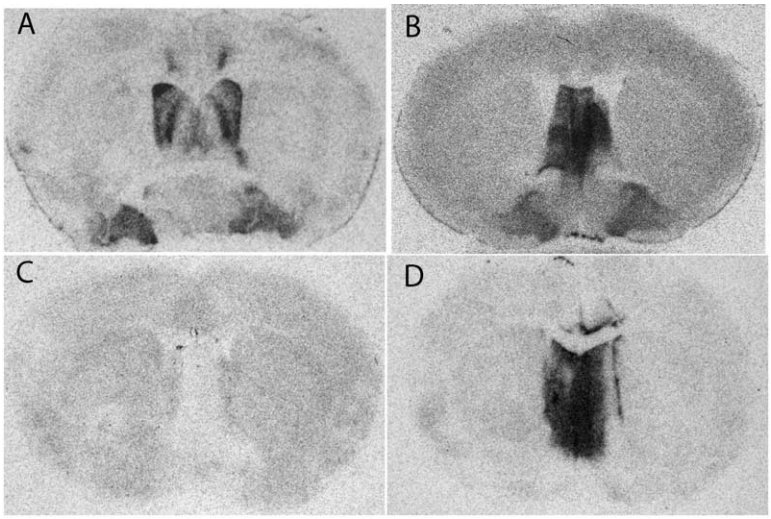

Figure 7. V1aR Binding Autoradiograms

V1aR binding autoradiograms illustrating ^{125}I V1aR antagonist binding in LacZ virus-treated wt (A) and V1aRKO (C) and NSE-V1aR viral vector-treated wt (B) and V1aRKO (D) at the level of the lateral septum injection site.

for learning and memory (reviewed in van Wimersma Greidanus et al., 1983). However, more recent investigations have demonstrated that social recognition is a unique form of learning and memory that utilizes distinct neural mechanisms that are specific to social cognition (van Wimersma Greidanus and Maigret, 1996). These paradigms allow investigation of a critical component of the social brain. Mice have been shown to demonstrate distinct forms of social memory, one transient and the other more enduring. The transient social memories, like those tested in the current studies, are normally held for up to an hour and seem geared more toward the differentiation of conspecifics over short interactions or durations and involve the ability to distinguish between two similar but different olfactory signatures (reviewed in Ferguson et al., 2002). More enduring, long-term social memories were not tested in these studies.

There is considerable experimental evidence that the lateral septum is involved in the control of social recognition behaviors. Lesions of the lateral septum resulted in altered social behavior, and AVP injections directly into the lateral septum resulted in enhanced social recognition in male rats (Clarke and File, 1982; Dantzer et al., 1988). Treatment with AVP antagonists and V1aR-specific antagonists injected in the lateral septum have both been found to significantly disrupt social recognition as well as treatment with anti-AVP serum into the septal area (Dantzer et al., 1988; van Wimersma Greidanus and Maigret, 1996; Everts and Koolhaas, 1997, 1999). V1aR antisense oligos have also been shown to disrupt normal social recognition and alter anxiety-related behavior in male rats when injected into the lateral septum (Landgraf et al., 1995). In contrast, social recognition in female rats is independent of vasopressin transmission, which is probably due, in part, to the sexual dimorphism of the extrahypothalamic AVP system, which is markedly reduced in females (Bluthe and Dantzer, 1990; de Vries and Miller, 1998). While the importance of the lateral septum in vasopressinergic social recognition has been well established in rats, the lack of septal research in mice necessitated the current studies. Furthermore, the lateral septum is not the only extrahypothalamic region rich in AVP that has been shown to affect social recognition in both rats and mice.

Like the lateral septum, the BNST and medial amygdala are regions rich in both AVP and V1aRs. The majority of extrahypothalamic AVP is produced by the neurons in the medial amygdala and BNST, a part of the extended amygdala, and project to the lateral septum (de Vries and Miller, 1998). Lesions of the medial amygdala in neonatal rats resulted in impaired social recognition and social play; however, rhesus macaques with bilateral lesions of the medial amygdala demonstrated normal social interactions with conspecifics (Daenen et al., 2002; Amaral et al., 2003). Investigations into the behavioral effects of a null mutation in the *oxytocin* gene (OTKO) revealed that the medial amygdala is critical to normal social recognition in male mice (Ferguson et al., 2001). Oxytocin is a nonapeptide also produced in the hypothalamus that differs from AVP by only two amino acids. OTKO male mice demonstrated a complete disruption of social recognition similar to that seen in V1aRKO mice (Ferguson et al., 2000). This deficit also resulted in decreased neuronal activation, using c-*fos* as a marker, in the medial amygdala but not the lateral septum. Social recognition was rescued in these mice after treatment of oxytocin directly into the medial amygdala (Ferguson et al., 2001). AVP treatment in the OTKO mice did not restore normal social recognition (Ferguson et al., 2000). These findings suggest that the medial amygdala is involved in the regulation of social recognition but it was unclear whether AVP transmission is required in this area. The current findings suggest that V1aR activation is not required in the medial amygdala and that V1aR expression is required only in the lateral septum for normal social recognition to occur. The role of the BNST in vasopressin effects on social recognition is still unclear and was not investigated in the current studies.

A mechanism to increase the duration of social memory would be critical to establishing dominant-subordinate relationships and pair bonding in monogamous species, which are examples of long-term social mem-

ories that have an emotionally salient aspect. It is possible that emotional or stressful situations could increase the saliency of a social encounter and lead to a potentiation of social recognition or conversion to a more long-lasting social memory. The examples above and the findings in hamsters, that males who lose a fight form long-term social memories, lend support to this hypothesis (Lai and Johnston, 2002). The current paradigms used a largely innocuous exposure to a nonreceptive female and thus may not be the most potent initiator of social recognition. It is possible that increased levels of AVP release and thus V1aR activation may increase the duration of social memory. Not surprisingly, stressful and sexual encounters have been shown to cause increases AVP release, and thus such significant encounters would result in a facilitation of social memory needed to remember a mate or an adversary/predator (Wotjak et al., 1996; J.C. Morales et al., 2004, Soc. Neurosci., abstract; Wigger et al., 2004).

The prairie vole NSE-V1aR viral vector used in the current mouse studies has been used successfully in several other species. Ventral pallidum injections in prairie and montane voles resulted in increased affiliative behaviors in both species (Pitkow et al., 2001; Lim et al., 2004). Septal injections of the same viral vector in male rats resulted in a significant facilitation of social discrimination similar to, but not as sustained as, that seen in the current study (Landgraf et al., 2003). These findings support the current studies, that V1aR expression in discrete brain regions is involved in social and anxiety-like behaviors. The use of a viral vector to induce sight-specific expression of a single gene is not without problems, however. It is difficult to tightly control the spread of the viral injection, and it is not unusual to get expression in the adjacent brain regions. Furthermore, the viral vector used in this study has a neuron-specific promoter but not a V1aR neuron-specific promoter; thus, it is probable that the V1aR is being expressed in genes that would not normally express this gene; however, it is probably not expressed in glia. Even in light of these shortcomings, the current NSE-V1aR viral vector has been used with great efficacy and provides an important model for the study of the behavioral effects of regional changes in gene expression.

Anxiety-Like Behaviors

Pharmacological evidence for the role of AVP and the V1aR in anxiety-related behavior in rats has historically been contradictory. One group has reported an anxiolytic effect of AVP administration and no effect of AVP antagonists, while others have reported no effect of AVP administration but an anxiolytic effect of V1aR antagonist (Liebsch et al., 1996; Appenrodt et al., 1998). Still another group reported an anxiogenic-like effect after treatment with an AVP receptor antagonist (Everts and Koolhaas, 1999). Treatment with an orally active V1bR antagonist resulted in a decrease in anxiety-like behavior in rats; however, a study of V1bRKO mice reported no effect of the null mutation in anxiety-related behavior (Griebel et al., 2002; Wersinger et al., 2002). Furthermore, septal injection of a selective V1bR antagonist resulted in antidepressant, but not anxiolytic, behavior in rats (Stemmelin et al., 2005). Nonpharmacological studies have shown that V1aR antisense oligos injected into the lateral septum have an anxiolytic-like effect on male rats, and our previous findings with the V1aRKO showed a decrease in anxiety-like behavior in the V1aRKO as compared to the wt (Landgraf et al., 1995; Bielsky et al., 2004).

The current findings suggest that, while V1aR expression in the lateral septum may contribute to the normal anxiety-like behavior, reexpressing V1aR in V1aRKO in the lateral septum is not sufficient to alter such behavior. While expression of V1aR in the V1aRKO mice did not alter anxiety-like behaviors, increased expression of V1aR in wt mice did result in increased anxiety-like behaviors. The current anxiety-related behavior data suggest that, while the V1aR is important for anxiety-related behavior, it must rely on other developmental, neurochemical, or neuroanatomical factors. The V1aRKO mice developed with a total absence of V1aR expression and activation both in the periphery and centrally, and thus it is not surprising that reexpression in a single area did not affect anxiety-like behavior in these mice. The increase in anxiety-related behavior in response to overexpression of V1aR in the wts demonstrates that the V1aR expression modulates anxiety-like behavior but that gene replacement during adulthood is not sufficient to overcome constitutive lack of V1aR, which could be critical in other regions as well or be responsible for downstream neuronal or chemical connections that are not formed in the V1aRKO mice.

A correlation between performance on anxiety-related tasks and hippocampal-dependent learning has been established and thus must be addressed given the anxiety-like effects of V1aR re- and overexpression (Belzung and Griebel, 2001; Bannerman et al., 2004; Packard and Wingard, 2004; Deacon and Rawlins, 2005). The initial behavioral phenotyping of the V1aRKO mice found no effect of the null mutation on hippocampal-dependent learning as assessed by the Morris water maze (MWM) and found that the non-hippocampal-dependent impairment in social recognition was concurrent with a decrease in anxiety (Bielsky et al., 2004). These findings cannot be accounted for by a correlation between decreased anxiety-like behavior and improvement in hippocampal-dependent learning, as the relationship in the V1aRKO mice was found to be the opposite. Furthermore, the current findings demonstrate a facilitation of social recognition in both the V1aRKO and wt animals that received V1aR viral vector injection but opposing effects on anxiety-like behavior. The V1aRKO mice that received V1aR reexpression showed a trend toward decreased anxiety (although this was not significant), while the wt mice that received V1aR overexpression showed an increase in anxiety. These opposing effects on anxiety-related measures taken in concert with the effects on social memory and the previous MWM findings therefore suggest that the relationship between hippocampal-dependent learning and anxiety is not relevant here.

The current findings suggest that the lateral septum may provide a convergence or relay point in the social olfactory circuit by receiving olfactory information from the conspecifics and delivering necessary information to the hypothalamus, with its role in sexual behaviors, and the hippocampus, with its role in learning and

memory, both of which are integral parts of behavioral responses to social stimuli. AVP via activation of the V1aR may be the modulator required for normal circuitry functioning in the lateral septum, thus social cognition and aspects of anxiety-like behavior are dependent on V1aR activation in the lateral septum of male mice. These findings do not rule out the possible importance of V1bR and OTR in cognition and emotionality, but the localizations of actions are still under investigation. Finally, the use of viral vector technology in concert with transgenic mice in the current studies provides an innovative model for targeting the role of a single gene in very complex behaviors.

Experimental Procedures

Animals

All subjects were 2- to 5-month-old sexually naive male mice. The animals were maintained in a 14/10 hr light/dark cycle and were provided food and water ad libitum (Purina mouse chow; Purina Mills, St. Louis, MO). Animals were housed in groups of three to five for the anxiety testing and then individually housed for the remainder of the experiments. All experiments were performed in compliance with the rules and oversight of the Emory Institutional Animal Care and Use Committee.

Wt Mice

Subjects were male C57/BL6 mice ordered from Jackson Laboratory (Bar Harbor, ME). Four groups of wt male mice were used in the antagonist studies: experimental groups receiving a V1aR antagonist treatment into the lateral septum (n = 9) or the medial amygdala (n = 8), and control groups receiving vehicle into the lateral septum (n = 8) or medial amygdala (n = 7). Two groups of wt male mice were used in the viral vector studies: an adeno-associated NSE-V1aR viral vector treatment group (NSE-V1aR) (n = 11) or a control LacZ viral vector treatment group (LacZ) (n = 10).

V1aRKO Mice

Subjects were knockout mice (–/–) (V1aRKO) bred in our laboratory. Briefly, the mice were produced by standard homologous recombination in which neo gene replacement deleted a 1.5 kb fragment of the *V1aR* gene starting at 31 bp downstream of the translation start site and ending at 244 bp downstream of exon #I. Chimeric males were generated and mated to C57/BL6 females, and germline transmission was achieved. These animals were backcrossed to 129/SvJ for five generations, and experimental mice were produced from heterozygote crossings (Hu et al., 2003). The genotype of all animals was assessed using a PCR-based genotyping protocol as previously described (Bielsky et al., 2004). Two groups of V1aRKO male mice were used in these studies: an adeno-associated NSE-V1aR viral vector treatment group (NSE-V1aR) (n = 15), and a control LacZ viral vector treatment group (LacZ) (n = 8).

Antagonist Injections

On the afternoon before behavior testing, animals were site specifically injected bilaterally into either the lateral septum or the medial amygdala with either 5 ng of V1aR antagonist or vehicle (Ringer's solution). Antagonist dose was chosen based on previous findings using site-specific antagonist injections (Lim and Young, 2004). Animals were anesthetized with isoflurane anesthesia administered using an anesthesia airflow machine (Vetequip, CA) and placed into Kopf stereotaxic apparatus using blunt earbars to prevent eardrum puncture. Coordinates for the lateral septum and medial amygdala were determined by dye injections (lateral septum: AP +0.6 mm, ML ±0.4 mm, DV –4.0 mm from dura; medial amygdala: AP –1.8 mm, ML ±2.6 mm, DV –5.4 mm from dura). The scalp was incised, and a dental drill was used to create a 1 mm^3 hole in the skull, whereupon a Hamilton syringe was lowered, and 0.5 μl of V1aR antagonist was injected. The V1aR antagonist [$d(CH_2)_5^1$,$Tyr(Me)^2$,Arg^8]-Vasopressin (Bachem, CA) was dissolved in lactated Ringer's solution (Fisher Scientific, Pittsburgh, PA) and was previously shown to be selective and behaviorally effective in mice (Boccia et al., 1998). Bilateral control injections were given in the same manner using 0.5 μl of lactated Ringer's solution. Bilateral injections were given using an automatic micropump (World Precision Instruments, FL) at a rate of 1 nl/s. After surgery, animals were removed from the anesthesia setup and generally awakened between 2 and 7 min after the surgery. Behavioral testing was performed the morning after surgery in an isolated behavior room at 23°C–24°C. The V1aR antagonist has been shown previously to have a receptor occupancy window in vivo of 18–24 hr (Winslow et al., 1993). Animals were brought to the testing room at 07:00, an hour before testing began, to acclimate to the environment. Behavior testing occurred at 08:00, 16 hr after surgery, during the first half of the light part of the light/dark cycle. Immediately after testing, animals were sacrificed, and the brains were removed, flash frozen, sectioned at 20 μm, and slide mounted. A visual inspection of slices was used to confirm correct injection placement. A total of nine animals, four antagonist (one in the lateral septum, three in the medial amygdala) and five controls, were excluded from the data analysis because of illness due to surgery or improper injection placement. Correct lateral septum injection was defined by location of the injection tip within the lateral septal complex, medial to the lateral ventricles, ventral to the dorsal peduncular cortex, and lateral to the medial septum. The lateral septal complex includes the dorsal (LSD), intermediate (LSI), and ventral (LSV) parts. Correct medial amygdala injection was defined by location of the injection tip within the medial amygdala complex, ventral and medial to the central amygdala and basolateral amygdala nuclei. The medial amygdala complex includes the basomedial amygdala nucleus, the posterodorsal medial amygdala nucleus, and the posteroventral medial amygdala nucleus. Any injections outside these parameters were excluded.

Viral Vector Injections

V1aRKO and wt animals were site specifically injected bilaterally into the lateral septum with either adeno-associated V1aR viral vector (NSE-V1aR) or control LacZ viral vector. Animals were anesthetized with isoflurane anesthesia administered using an anesthesia airflow machine (Vetequip, CA) and placed into Kopf stereotaxic apparatus using blunt earbars to prevent eardrum puncture. Coordinates for the lateral septum were determined by dye injections (lateral septum: AP +0.6 mm, ML ±0.4 mm, DV –4.0 mm from dura). The scalp was incised, and a dental drill was used to create a 1 mm^3 hole in the skull, whereupon a Hamilton syringe was lowered, and 0.5 μl/side of NSE-V1aR was injected. The production of the NSE-V1aR has been previously described in detail (Pitkow et al., 2001). Briefly, the neuron-specific enolase (NSE) prairie vole NSE-V1aR consists of a *V1aR* genomic clone with the majority of the intron removed, spliced downstream of a rat NSE promoter. The *V1aR* modified sequence contains the first and second exons separated by a 287 bp intron and a 227 bp 5′-untranslated region. The NSE promoter restricts expression to neurons, and transduction by AAV-2 vectors into the brain is usually confined to neurons (Peel et al., 1997; Bartlett et al., 1998; Nair and Young, 2002). The LacZ virus was prepared very similarly, by incorporating a 0.6kb cytomegalovirus-immediate early promoter and the 3.7kb *Escherichia coli lacZ* gene sequence. The packaging of AAV plasmids was performed using the standard methods previously described. AAV vector stock was titrated using real-time PCR and averaged 1×10^{11}/ml. The viral vector was completely free of the contaminating helper virus and only contained the gene of interest with no viral genes. The NSE-V1aR was dissolved in lactated Ringer's solution so that each animal received a total of 0.5 μl/side. Bilateral control injections were given in the same manner using 0.5 μl/side of LacZ viral vector. Bilateral injections were given using an automatic micropump (World Precision Instruments, FL) at a rate of 1 nl/s. After surgery, animals were removed from the anesthesia setup and generally awakened between 2 and 7 min after the surgery. Animals were allowed to recover for at least 7 days before beginning behavioral testing. A total of four animals, all wt V1aR treated, were removed from analysis due to illness or improper injection placement, which was defined as described above for the lateral septum.

Behavioral Testing

All testing was performed in an isolated behavior room at 23°C–24°C. Animals were brought to the testing room an hour before testing began, to acclimate to the environment. All behavior testing occurred in the first half of the light part of the light/dark cycle. All behaviors were videotaped and scored later by a single trained observer blind to genotype using a computer-assisted data acquisition system (Stopwatch+; http://www.cbn-atl.org/organization/stopwatch.html). For all anxiety tests, the light in the room measured 300–400 lux. Each behavioral test was separated by at least 24 hr, and tests were performed in the order presented. Animals were group housed during anxiety testing and individually housed for olfactory and social testing. Anxiety tests took place on separate days and preceded olfactory and social testing where applicable.

Elevated Plus Maze

The elevated plus maze apparatus consisted of two open arms and two closed arms, each measuring 30.5 × 7.6 cm and elevated 81 cm above the ground. The subject was placed in the center of the apparatus facing the open arm, and its location was recorded for 5 min. An arm entry was defined as the mouse having all four paws into the arm (Fernandes and File, 1996). The number of dark arm entries was also recorded in order to assess general locomotion behavior.

Open Field

The open field apparatus consisted of a clear-walled, Plexiglas, rectangular open arena with a length of 45.5 cm and a width of 40.5 cm. This arena is attached to an automated photo-beam activity system that tracks movement by recording photo-beam breaks (San Diego Instruments, Flex Field Photo-beam system) The subject was placed in the arena, along the wall, and its location was recorded for 5 min. The photo-beam system records the number of beam breaks and where the beam breaks occur. This measure directly correlates to the distance traveled, which is a measure of general locomotor activity. The critical anxiety-like behavior measure was the time spent in the inner area of the arena (>5 cm away from any wall), which was scored by hand.

Light/Dark Box

The light/dark box apparatus consisted of a light, open-topped, opaque, Plexiglas box connected to a dark, closed-topped, opaque, Plexiglas box, each measuring 27.5 × 17.5 × 12.5 cm. The boxes were connected by a small opening that allowed the subject to cross between them. The subject was placed in the light box, and its location was recorded for 5 min. The time spent in the light side of apparatus was considered the primary measure of the anxiety because of its sensitivity to anxiolytics (Hascoët et al., 2001). Light box entry was defined as the mouse having all four paws into the light box.

Olfactory Habituation

Animals were individually housed for at least 10 days before testing, and olfactory habituation was performed 7 days after social recognition. The procedure was used previously by Ferguson et al. (2000). Briefly, a small plastic cartridge was packed with cotton scented with 10 μl of anise extract. This was placed in the home cage of the subject for 1 min over four trials, with an intertrial interval (ITI) of 10 min. In the fifth dishabituation trial, the subject was presented with a cotton ball scented with 10 μl of coconut extract. The time spent in olfactory investigation for each trial was recorded. Olfactory investigation was defined as direct nasal contact with the cartridge.

Social Recognition

Animals were individually housed for 10 days before testing to permit the establishment of a home cage territory. The procedure was the same as previously used in Ferguson et al. (2000). Briefly, the subject was exposed to the same ovariectomized adult female mouse for 1 min over four trials with an ITI of 10 min. During the fifth dishabituation trial, the subject was exposed to a novel ovariectomized female for 1 min. The time spent in olfactory investigation for each trial was recorded. Olfactory investigation was defined as direct, active, olfactory exploration of the stimulus female, specifically nosing and sniffing of the head and anogenital regions, closely following, and pursuit. Each female was used only once each day. Mice displaying normal social recognition show a decline in investigation upon subsequent exposure to the same individual, and in the dishabituation trial, investigation times are similar to the initial exposure to the first individual.

Social Discrimination

The animals were individually house for 10 days before testing to allow the establishment of a home territory. The social discrimination paradigm has been described previously (Ferguson et al., 2002; Bielsky and Young, 2004). Briefly, the test animal is exposed to the stimulus ovariectomized female animal and then, after an ITI, simultaneously presented with the same stimulus female and a novel stimulus ovariectomized female. Normally, the test animal will spend significantly more time investigating the novel stimulus female as compared to the original stimulus female. Four different ITIs were used: 0.5 hr, 2 hr, 6 hr, and 24 hr. This allowed for the testing of possible social discrimination facilitation.

Data and Statistical Analysis

For the plus maze, open field, and light/dark box, the time spent in the open arm, open field, and light box was compared between treatment groups using a Student's t test. The number of closed arm entries in the plus maze and the distance traveled in the open field were compared between treatment groups using a Student's t test. For the social recognition and olfactory habituation tests, two-way ANOVAs, with treatment group and trial as factors, with repeated measures on trial were performed. For the social discrimination test, a two-way ANOVA with stimulus type (novel female versus same female) and treatment as factors with repeated measures on stimulus type was performed for each ITI time point. If a significant main or interaction effect was detected, a Neuman-Keuls post hoc analysis was performed (GraphPad Prism 4). The initial time spent investigating the female was compared between treatment types for each ITI using a Student's t test ($\alpha = 0.05$).

Receptor Autoradiography

To confirm V1aR viral vector expression and injection placement, radioligand receptor binding was performed. Immediately after completion of behavior testing, animals were sacrificed, and the brains were removed and flash frozen on dry ice, sectioned at 20 μm, and slide mounted into two serial sets for each experimental animal. One set was used for V1aR binding, and the other was archived. Sections were processed for receptor autoradiography using ^{125}I-labeled (Phenylacetyl^{1},0-Me-D-Tyr^{2}, [^{125}I-Arg^{6}]-) vasopressin (linear), V-1A antagonist (Perkin Elmer Life Analytical Sciences, Boston, MA) for the V1aR as described previously (Young et al., 1997). Slides were exposed to BioMax MR film (Kodak) for 48 hr. A visual inspection of the V1aR autoradiogram was used to confirm correct injection placement.

Data and Statistical Analysis

Film autoradiograms were analyzed using the AIS program (Imaging Research Inc., St. Catharines, ON, Canada). Optical densities were converted to dpm/mg of tissue equivalents using ^{125}I microscales (Amersham, Arlington Heights, IL). Specific binding in the lateral septum was obtained by subtracting the nonspecific binding from the total binding for each section. All sections were analyzed by a trained experimenter blind to treatment, and each region of interest was measured bilaterally from at least two sections. Values from V1aR- and LacZ-treated wt subjects were compared using Student's t test ($\alpha = 0.05$).

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