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Partner-seeking and limbic dopamine system are enhanced following social loss in male prairie voles (*Microtus ochrogaster*)

Erika M. Vitale[1] | Adrianna Kirckof[2] | Adam S. Smith[1,2]

[1]Department of Pharmacology and Toxicology, School of Pharmacy, University of Kansas, Lawrence, Kansas, USA

[2]Program in Neuroscience, School of Pharmacy, University of Kansas, Lawrence, Kansas, USA

Correspondence
Adam S. Smith and Erika M. Vitale, Department of Pharmacology and Toxicology, School of Pharmacy, University of Kansas, Lawrence, KS 66045, USA.
Email: adamsmith@ku.edu and evitale@ku.edu

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Abstract

Death of a loved one is recognized as one of life's greatest stresses, and 10%–20% of bereaved individuals will experience a complicated or prolonged grieving period that is characterized by intense yearning for the deceased. The monogamous prairie vole (*Microtus ochrogaster*) is a rodent species that forms pair bonds between breeding partners and has been used to study the neurobiology of social behaviors and isolation. Male prairie voles do not display distress after isolation from a familiar, same-sex conspecific; however, separation from a bonded female partner increases emotional, stress-related, and proximity-seeking behaviors. Here, we tested the investigatory response of male voles to partner odor during a period of social loss. We found that males who lost their partner spent significantly more time investigating partner odor but not non-partner social odor or food odor. Bachelor males and males in intact pairings did not respond uniquely to any odor. Furthermore, we examined dopamine (DA) receptor mRNA expression in the anterior insula cortex (aIC), nucleus accumbens (NAc), and anterior cingulate (ACC), regions with higher activation in grieving humans. While we found some effects of relationship type on DRD1 and DRD2 expression in some of these regions, loss of a high-quality opposite-sex relationship had a significant effect on DA receptor expression, with pair-bonded/loss males having higher expression in the aIC and ACC compared with pair-bonded/intact and nonbonded/loss males. Together, these data suggest that both relationship type and relationship quality affect reunion-seeking behavior and motivational neurocircuits following social loss of a bonded partner.

KEYWORDS
dopamine receptor 1, dopamine receptor 2, *Microtus ochrogaster*, motivation, pair bond, prairie vole, social attachment, social loss

1 | INTRODUCTION

Each year in the United States, approximately 8 million individuals suffer from the loss of a close family member, and spousal loss alone creates over 800,000 new widows and widowers.[2] Bereaved individuals are at a higher risk of having a complicated or prolonged grieving period following the death of an intimate partner,[1,2] suggesting that relationship type is a major predictor of grief outcome. In particular, loss of a high-quality, intimate relationship is related to the core grief symptom of "yearning,"[3,4] which may be related to differences in both

the feelings evoked within the individual (i.e., higher compassion, joy, passion and love ratings) and in the function of brain areas that regulate the salience of different relationships. Indeed, neuroimaging studies in humans have shown that an intimate, romantic partner evokes higher activity of the ventral tegmental area (VTA), nucleus accumbens (NAc), anterior cingulate (ACC) and insula (IC) compared with a close friend.[5] These are mesocorticolimbic regions which use the neurotransmitter dopamine (DA) to control sensations of pleasure, attachment and reward as well as emotional pain.[6,7] Studies on human grief have revealed that many of these same brain regions are active when bereaved individuals view photos of their deceased loved one.[8,9] However, details of the circuitry and neurotransmitter systems that are responsible for processing relationship salience and their connection to social loss have been difficult to assess using laboratory rodents, as the most used species (rats and mice) do not establish adult bonds.

The prairie vole is a monogamous rodent species that has been used to study the neurobiological mechanisms of social behaviors for over three decades.[10–13] Pair bonding in males and females is behaviorally documented by a selective affiliation for a social partner over other conspecifics, intruder-directed aggression, and social proximity-seeking following partner separation.[10,14] The formation of these bonds involves coordination of neurochemical systems including DA, oxytocin, corticotrophin-releasing hormone (CRH), vasopressin and endogenous opioids.[15,16] Prairie voles also form conditioned place preference for their opposite-sex mates but not for social peers, suggesting that mate relationships are more rewarding than peer relationships.[17,18] Thus, prairie voles may be particularly useful for studying the effects of losing specific types of relationships on partner-seeking and motivation for reunion. In support of this, extensive data has demonstrated that NAc DA, a neurochemical system involved in reward and motivation, is involved in the regulation of pair-bonding behavior in male and female prairie voles.[19–21] Thus, the mesocorticolimbic reward system, consisting of DA cell bodies in the VTA that terminate and release DA into the ventral striatum (i.e., NAc) and cortex (i.e., ACC, IC) may also be involved in processing the loss of salient social relationships and contribute to behavioral outcomes related to motivation for reunion.[22–24] Interestingly, while the prairie vole model has been used to assess behavioral outcomes of social loss, most studies have focused on changes in anxiety-like and stress-coping behavior, which are both higher in male voles that are separated from a female partner compared with males that are separated from a same-sex cage mate. These studies have shown that CRH receptors are increased while oxytocin receptors are decreased in the NAc following partner loss, and the stress-coping alterations resulting from loss are blocked by pharmacologically inhibiting CRH receptors or activating oxytocin receptors in the NAc.[25–27] Thus, the NAc, in coordination with other higher order brain regions, may comprise a circuit through which the "weight" of the loss of a particular social relationship is evaluated to dictate the emotional and behavioral responses.

To determine whether prairie voles display motivation for reunion with a conspecific from which they were separated, we designed an odor preference test (OPT) where the subjects are given access to two odor cues representing different motivational states—bedding scented with their partner and bedding scented with their regular chow. The design of this test was inspired by the "social vs. food" test originated by Reppucci et al.[28,29] and allowed for examination of the individual's social-motivational state during social loss without physically reuniting them with the lost conspecific. Furthermore, male and female prairie voles are capable of distinguishing individual differences in the odors contained in soiled bedding from male and female conspecifics and can also use such odor stimuli to discriminate between mates and non-mates.[30] Thus, we hypothesized that male prairie voles separated from a familiar social conspecific would spend more time investigated an odor cue from their partner or cage mate than those that remained with their social companion. We also predicted that the salience of the relationship that was lost would dictate how much time separated individuals spent investigating companion odor, with those losing an opposite-sex partner expected to show more olfactory investigation than those that lost a same-sex cage mate. Furthermore, we assessed whether the strength of a bonded opposite-sex relationship predicted this "reunion-seeking" behavior by screening for pair-bond status, as a small proportion of prairie voles (~30%) do not establish a partner preference even after extended cohabitation with an opposite-sex mate,[31,32] and "relationship quality" has previously been found to predict anxiety-like behavior and pain sensitivity using the prairie vole social loss model.[33] Finally, we investigated components of the central dopaminergic system, particularly in striatal and cortical regions that are involved in salience processing and reward, following social loss.

2 | METHODS

2.1 | Animals

All prairie voles were lab-bred and descended from a population captured in southern Illinois. Voles were weaned at 21 ± 3 days of age and housed in same-sex non-sibling pairs in microisolator cages (29.2 L × 19.1 W × 12.7 H cm) with corn cob bedding, crinkle nesting material and ad libitum access to food (Tekland global rabbit diet 2030) and water. Colony rooms were maintained at 21 ± 1C with a 14 L:10D photoperiod (lights on at 0600 h). Male subjects were between 90 and 120 days of age at the start of the experiment. To remove the confound of pregnancy in the opposite-sex pairs, female prairie voles from the colony that were used as partners were tubal ligated and allowed to recover for at least 1 week before pairing. Based on the previous experiments in the lab and published work from other vole groups, tubal ligation of the female partner does not negatively influence pair-bond formation or mating.[34] All behavior testing was performed between 0900 and 1700 h and all procedures were conducted in accordance with the National Institutes of health Guide for the Care and Use of Laboratory Animals and the Institutional Animal Care and Use Committee at the University of Kansas.

2.2 | Experimental design

The timeline for each behavioral experiment is presented in Figure 1.

Experiment 1. Relationship type affects partner odor-seeking behavior in male prairie voles following 1 week of social loss.

Male subjects ($N = 40$; $n = 10$/group) were either moved to a new cage and introduced to a tubal ligated female partner for 1 week (opposite-sex groups; OS) or were moved to a new cage with their current same-sex, non-sibling cagemate (same-sex groups; SS) to control for handling effects. Cages were changed on experimental day 4 of pairing to allow for sampling of 3-day-old home cage bedding on day 8. Immediately following bedding collection on day 8, half of the subjects in each relationship type group were removed from their partner or cage mate and placed into a new clean cage (loss groups) while the other half were placed into a new cage with their partner/cage mate (intact groups). Bedding was frozen at -20°C until use in the OPT (see below).

Another cohort of male prairie voles ($N = 18$; $n = 9$/group) were housed with a tubal ligated female following the same cage changing and loss protocol as above. This cohort was then tested in the OPT on experimental day 15, only the partner odor was replaced by soiled bedding from the home cage of a novel male–female pair (food-scented bedding remained unchanged).

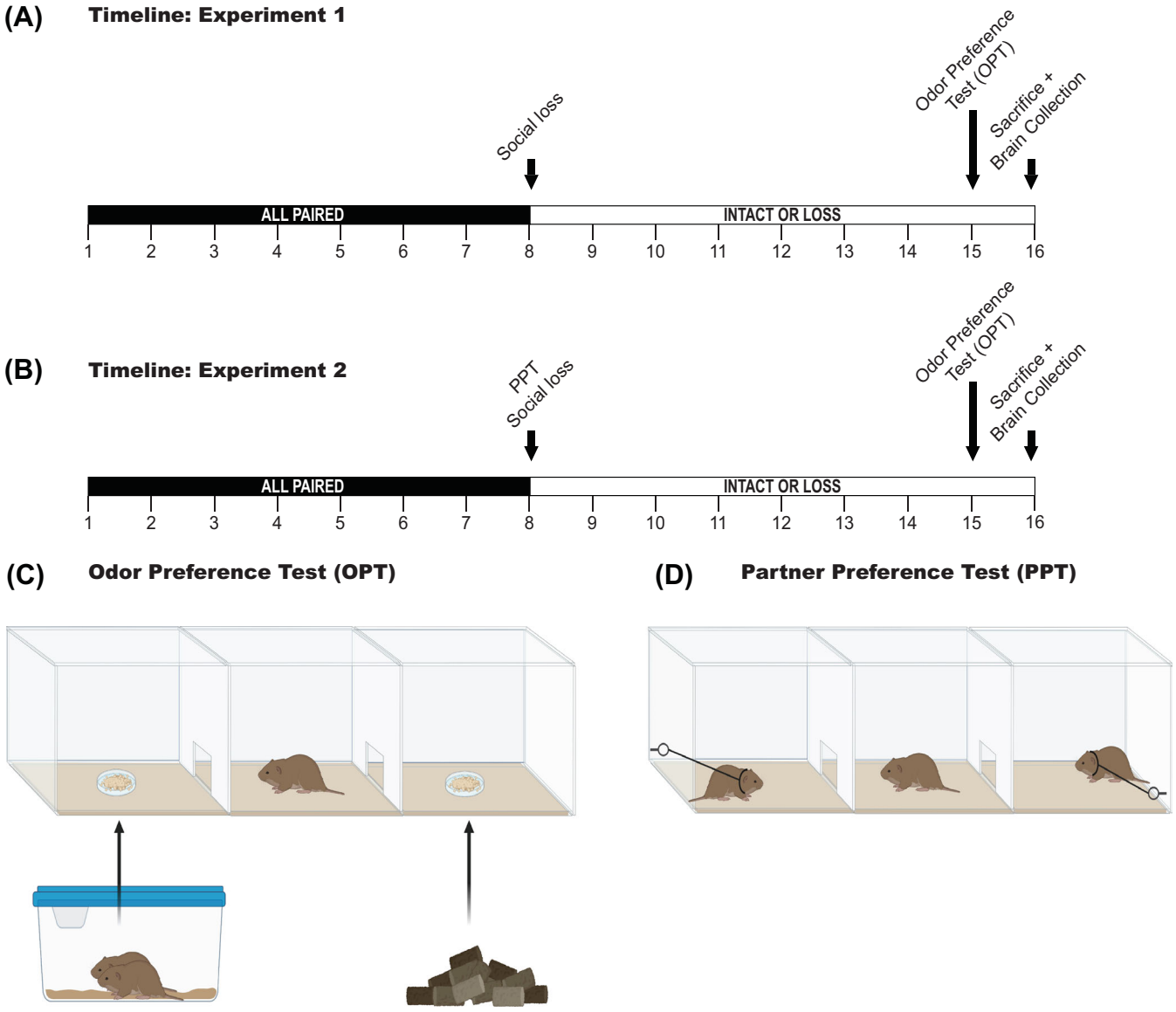

FIGURE 1 Experimental timelines. (A) Timeline for Experiment 1 examining the effects of relationship type (same-sex cage mate vs. opposite sex partner) on partner odor preference following social loss. (B) Timeline for Experiment 2 examining the effects of relationship quality (pair-bonded or non-bonded) on partner odor preference following social loss. (C) Diagram of OPT. (D) Diagram of partner preference test (PPT).

Experiment 2. Relationship quality affects partner odor-seeking behavior after loss.

Male subjects ($N = 24$; $n = 8$/group) were housed with tubal ligated female partners for 1 week. Subjects received a cage change on experimental day 4 and then were screened for partner preference formation on day 8. Partner preference test (PPT) videos were scanned to determine bond status (pair-bonded or non-bonded, with PB status determined by displaying a 3:1 ratio of affiliation behaviors toward the partner over the stranger). All subjects in the nonbonded group were removed from their partner on day 8 (NB loss), while half of the bonded group were removed from their partner (PB loss) and the other half were moved to a new cage with their partner (PB intact). Home cage bedding was collected immediately prior to separation, then subjects were tested in the OPT on testing day 15 (after 1 week of loss).

3 | BEHAVIOR

3.1 | OPT

The OPT was developed to assess motivation of the subject for reunion with their lost social companion without physical reunion. The testing arena was composed of three chambers (75 L × 20 W × 25 H cm) with the outer 2 chambers each containing a 60 x 15 mm petri dish taped to the center of the arena floor. A thin layer of corn cob bedding covered the bottom of the arena and petri dish, and subjects were allowed to habituate to the chamber and the petri dishes for 10 min. After the 10-min habituation period, animals were corralled to the center chamber while an experimenter added the odor stimuli to each petri dish. The odor stimulus was either 3-day-old bedding or clean bedding mixed with 300 mg food particulates. After odorants were added to fill the dishes (counterbalancing the side placement of the social and the food bedding), chamber barriers were lifted, and animals were recorded for 10 min using Logitech web cameras recording at 30 frames per second.

3.2 | PPT

The PPT is a well-documented assessment of pair bonding in prairie voles.[10] Briefly, estrogen-benzoate-primed (EB primed) stimulus females were collared with a beaded zip-tie threaded through jewelry wire and clipped to an eye screw in one of the outer chambers of the 3-chamber arena used above (counterbalanced across groups). Female partners were similarly collared and clipped to the opposite side of the arena. Subjects were placed in the center of the arena with barriers in place to keep them contained in the center chamber. Once the test started, barriers were removed to allow the subject access to all chambers, and animals were video recorded for 2 hours.

3.3 | Biological characterization

3.3.1 | Sacrifice and brain collection

Twenty-four hours after the OPT, subjects were taken directly from the colony and rapidly decapitated to minimize disturbance and/or stressors that could impact mRNA expression.[35] Twenty-four hours were chosen to ensure that mRNA expression changes occurring as a result of behavioral manipulation in the OPT were given time to decay and return to baseline.[36] Brains were flash-frozen on dry ice and stored at −80°C until processing for qRT-PCR.

3.3.2 | qRT-PCR

Brains were sectioned with a cryostat at 200 microns, and unilateral tissue punches (1.0 mm diameter) containing the anterior cingulate cortex (ACC), NAc, and anterior insular cortex (aIC) were collected. These regions were selected for their role in grief and yearning in human fMRI work.[8,9] Tissue punches were homogenized and RNA was extracted and purified using a Qiagen RNEasy mini kit following manufacturer's instructions. RNA was quantified using a Qubit 3 fluorometer and a high sensitivity RNA quantification kit (Thermo Fisher Scientific, Waltham, MA). About 20–100 ng of mRNA was converted to cDNA using a high-capacity reverse transcription kit (Applied Biosystems, Foster City, CA) per the manufacturer's instructions. mRNA for dopamine type-1 (DRD1) and dopamine type-2 (DRD2) receptors were analyzed. Hypoxanthine-guanine phosphoribosyltransferase (HPRT) was used as the comparison "housekeeping" gene based on its relatively constant expression in cells independent of experimental conditions.[37,38] qRT-PCR for each target was run in triplicate for every subject in one experiment on one plate with wells containing 5 ng cDNA, SYBR green PCR Master Mix (Applied Biosystems, Foster City, CA) and 200 nM of each forward and reverse primer (DRD1: forward - AGT TGA CCG TAG AAG CGC C, reverse - ATG TAT CAC GAT GCC CGC TC; DRD2: forward - AAG ACC CCA CTC AAG GGC AA, reverse - CCA TTC TCC GCC TGT TCA CT; HPRT: forward - CCC AGC GTC GTG ATT AGT GA, reverse - TCG AGC CAG TCT TTC AGT CC). A ThermoFisher StepOnePlus PCR plate reader was used for quantification. A dissociation curve was generated for each sample and used to confirm that only a single product was transcribed. The deldelCT method was used to calculate the fold differences between groups.[39]

3.3.3 | Data analyses

Dixon's outlier tests were performed for each group in each analysis, and in cases where a significant outlier was found, the datapoint was removed from the analysis. Results for Experiment 1 behavior and brain data were obtained by performing Two-Way Analysis of Variance tests to assess the main effects of companion sex, loss condition and their interaction, with significant ANOVA results followed by LSD

post-hoc comparisons. For Experiment 2, behavior and brain data were analyzed using a One-Way Analysis of Variance test. LSD post-hoc comparisons followed significant ANOVA effects for the behavior. Results for the behavior from Experiment 2 led us to perform planned simple contrasts between both loss groups and the intact group for DRD1 and DRD2 mRNA in the ACC, aIC and NAc. Significant differences were determined by a p-value of <0.05.

4 | RESULTS

4.1 | Excluded animals

Five animals were excluded from mRNA analyses in Experiment 1—1 each from the SS Intact and SS Loss group did not have sufficient RNA quantity to analyze, and an additional 1 each from the SS Intact, SS Loss and OS Intact groups were statistical outliers. In Experiment 2, one animal from the PB Intact was excluded from behavior and mRNA analyses due to no performance in the OPT.

Experiment 1. Relationship type affects partner odor-seeking behavior in male prairie voles the following 1 week of social loss.

In the OPT, OS males spent significantly more time sniffing bedding from their partner compared with SS males ($F^{1,35} = 12.62$, $p = 0.001$) and there was a significant interaction between companion sex and loss condition on partner odor investigation ($F = 9.67$, $p = 0.003$). Post hoc pairwise comparisons between the four experimental groups revealed that OS loss males, specifically, showed significantly higher partner odor investigation compared with all other groups (Figure 2A). Furthermore, this difference in partner odor

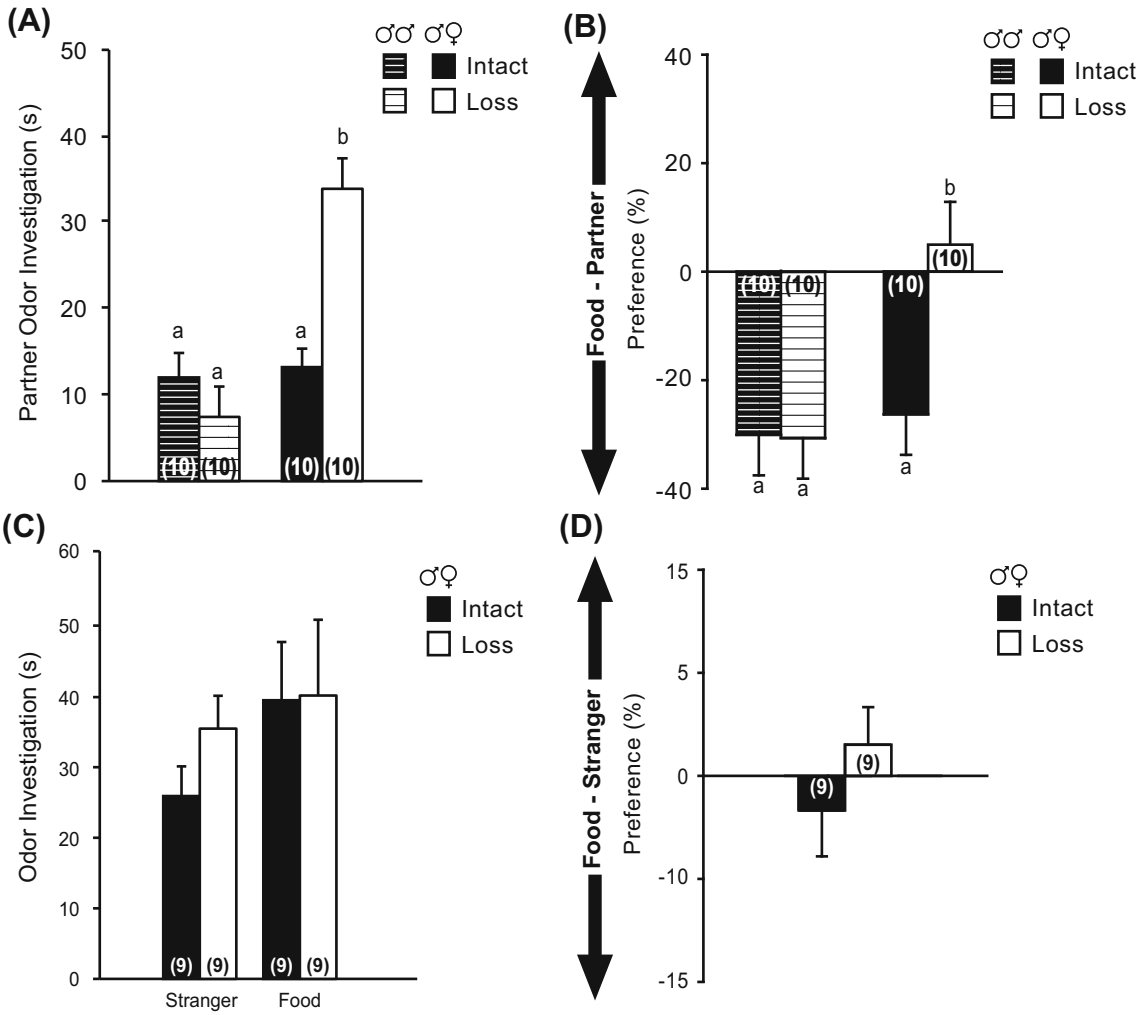

FIGURE 2 Relationship type influences partner odor preference following social loss. (A) Male prairie voles that lost a female partner for 1 week (OS Loss) displayed a positive preference (shifted toward partner investigation) compared with SS Intact, SS Loss and OS Intact groups. (B) Shifted preference in OS Loss group is explained by the significant increase in partner odor investigation that is not present in the other groups. (C) Social odor preference is specific to the partner odor cue, as there are no significant differences in preference (%) when the social odor option is bedding from a stranger cage. (D) There are no significant differences in odor investigation of the stranger-scented bedding or the food-scented bedding in OS Intact versus OS Loss groups. Different letters denote significant differences between groups ($p < 0.05$).

investigation resulted in significantly different preference ratios for OS loss subjects, which were the only group to display a positive preference score (i.e., more interaction with partner bedding than food bedding), with the other three groups showing a strong negative preference score (male vs. female companion: $F = 6.72$, $p = 0.01$; intact vs. loss: $F = 4.08$, $p = 0.05$; interaction: $F = 4.40$, $p = 0.04$; Figure 2A, B).

To determine whether loss of an opposite-sex partner affects general social odor investigation, a separate cohort of OS intact and loss subjects were given a choice between food-scented bedding and bedding from the home cage of a novel male–female pair (in place of partner odor). There were no significant differences in olfactory investigation of the social odor ($t = 2.1$, $p = 0.17$), food odor ($t = 0.42$, $p = 0.525$) or preference score ($t = 1.69$, $p = 0.21$) between

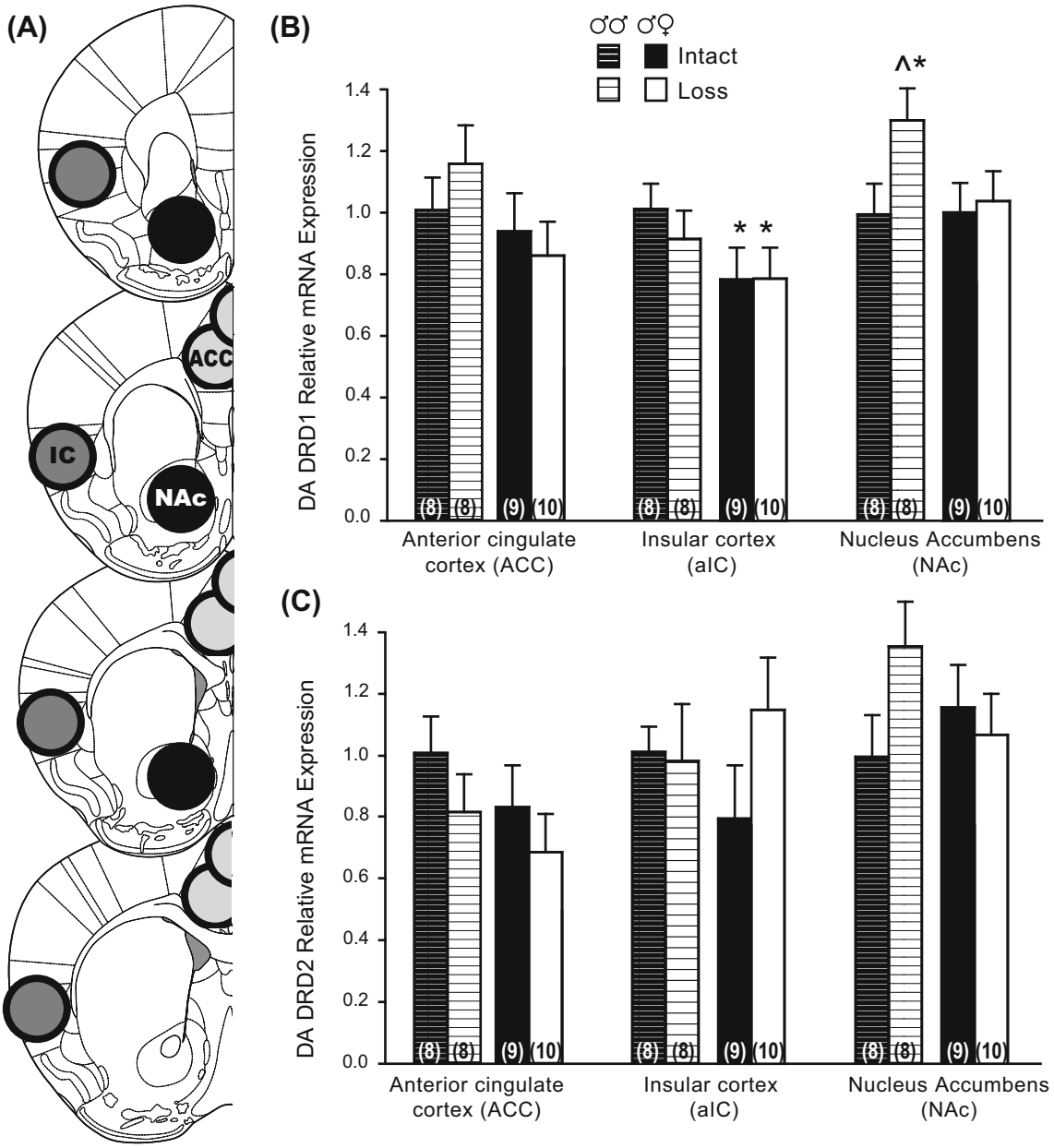

FIGURE 3 Relationship type and social loss influence DA receptor mRNA expression in brain regions associated with human grief. (A) Diagram of atlas plates referencing the location of punches for the ACC, aIC and NAc. (B) Males with female partners show significantly lower DRD1 mRNA expression, regardless of loss condition, in the aIC compared with males with male cage mates. Loss of a familiar companion, regardless of companion sex, is associated with significantly higher DRD1 mRNA expression in the NAc. Pair-wise comparisons showed that this loss effect was only present when comparing males with male cage mates. (C) There were no significant effects of companion sex or social loss on DRD2 mRNA expression in the ACC, aIC or NAc. **denotes significant main effect of companion sex ($p < 0.05$), ^*denotes significant effect of loss within specific companion sex group ($p < 0.05$).

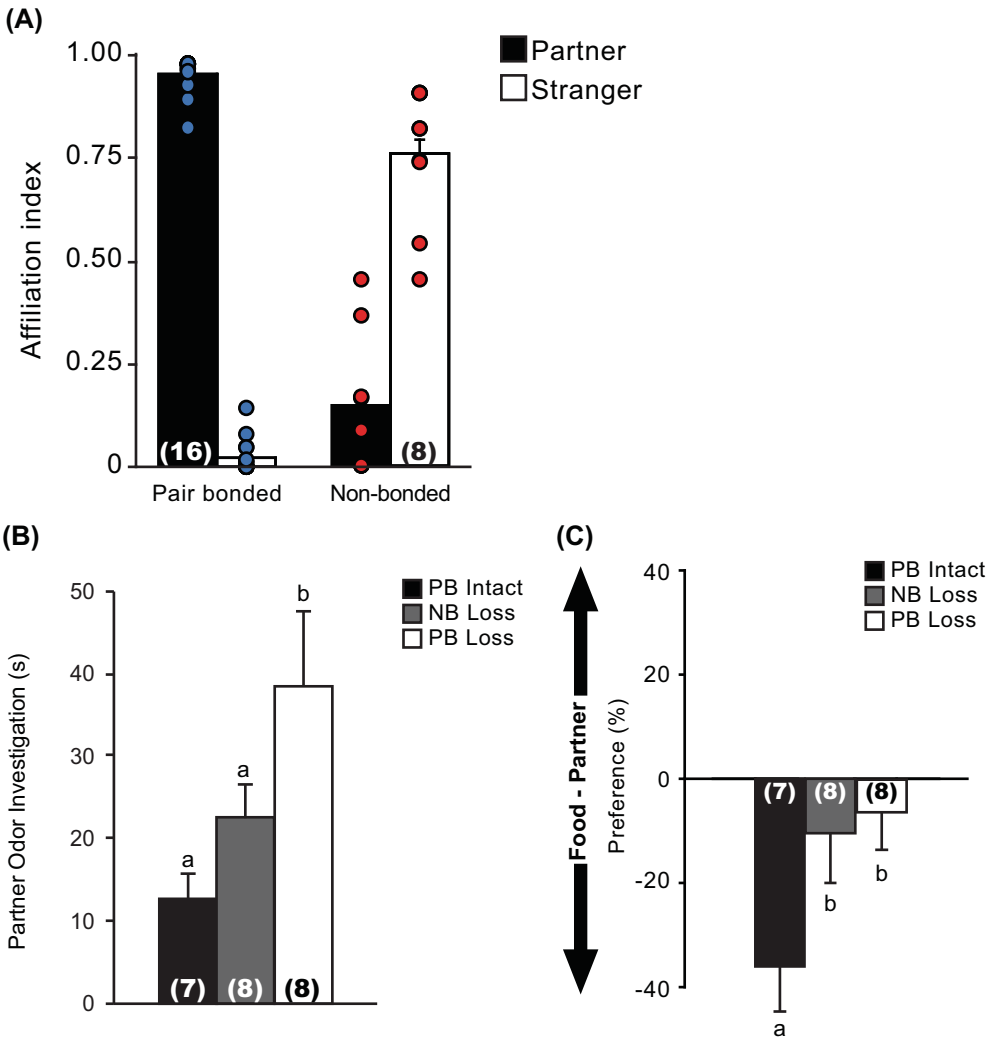

FIGURE 4 Relationship quality influences partner odor investigation following social loss of an opposite-sex partner. (A) Following the PPT, males were divided based on partner affiliation index, which measures the proportion of total affiliation that is displayed toward their partner versus a novel female. Males were determined to be "Pair bonded" if they had a partner affiliation index greater than or equal to 0.75. (B) Pair-bonded males remaining with their partner showed a strong negative preference score (strong food preference) compared with both loss groups. (C) PB Loss males spent significantly more time investigating their partners' odor compared with PB Intact and NB Loss groups. Different letters denote significant differences between groups ($p < 0.05$).

OS intact and loss groups with this comparison, suggesting that the differences found in Experiment 1.1 were specific to the partner versus food choice and was not present when a nonfamiliar social versus food choice was given (Figure 2C, D).

mRNA analyses for DA receptor expression in the ACC, aIC and NAc revealed several region and receptor-specific effects that were dependent on companion sex, loss group and specific combinations of the two (Figure 3). In the aIC, DRD1 receptor expression was significantly lower in both OS groups compared with both SS groups ($F = 4.21$, $p = 0.05$) with no main effect of social loss ($F = 0.12$, $p = 0.73$) while there were no differences between groups on DRD2 expression (companion sex: $F = 3.26$, $p = 0.08$; social loss: $F = 0.01$, $p = 0.93$) or DRD2 (companion sex: $F = 0.12$, $p = 0.73$; social loss: $F = 0.83$, $p = 0.37$). In the NAc, companion sex had no effect on DRD1 mRNA expression ($F = 1.65$, $p = 0.21$); however, subjects that experienced social loss did show significantly higher DRD1 expression ($F = 4.15$, $p = 0.05$). Post-hoc analyses determined that the social loss main effect was driven by a significant difference in DRD1 expression between SS Intact and SS Loss subjects ($p = 0.04$), specifically. There were no group differences in DRD2 receptor expression in the NAc (companion sex: $F = 0.02$, $p = 0.89$; social loss: $F = 0.91$, $p = 0.35$) and no effects on DRD1 (companion sex: $F = 2.53$, $p = 0.12$; social loss: $F = 0.41$, $p = 0.53$) or DRD2 (companion sex: $F = 1.28$, $p = 0.27$; social loss: $F = 2.17$, $p = 0.15$) mRNA in the ACC.

Experiment 2. Relationship quality affects partner odor-seeking behavior after loss.

In the PPT, males that were categorized as pair bonded ($n = 16$) displayed a significantly higher frequency of affiliative behaviors (side-by-side, huddling, allogrooming) toward their partner compared with the stranger ($F = 31.73$, $p < 0.001$), while males that were categorized as non-bonded ($n = 8$) displayed higher affiliation toward the stranger ($F = 9.18$, $p = 0.006$). Furthermore, affiliation toward the partner was significantly higher when comparing pair bonded and nonbonded males ($F = 21.78$, $p < 0.001$), while affiliation with the stranger was significantly higher in nonbonded compared with bonded males ($F = 13.11$, $p = 0.001$). These data validate that the subjects were appropriately categorized into their bond status groups and shows

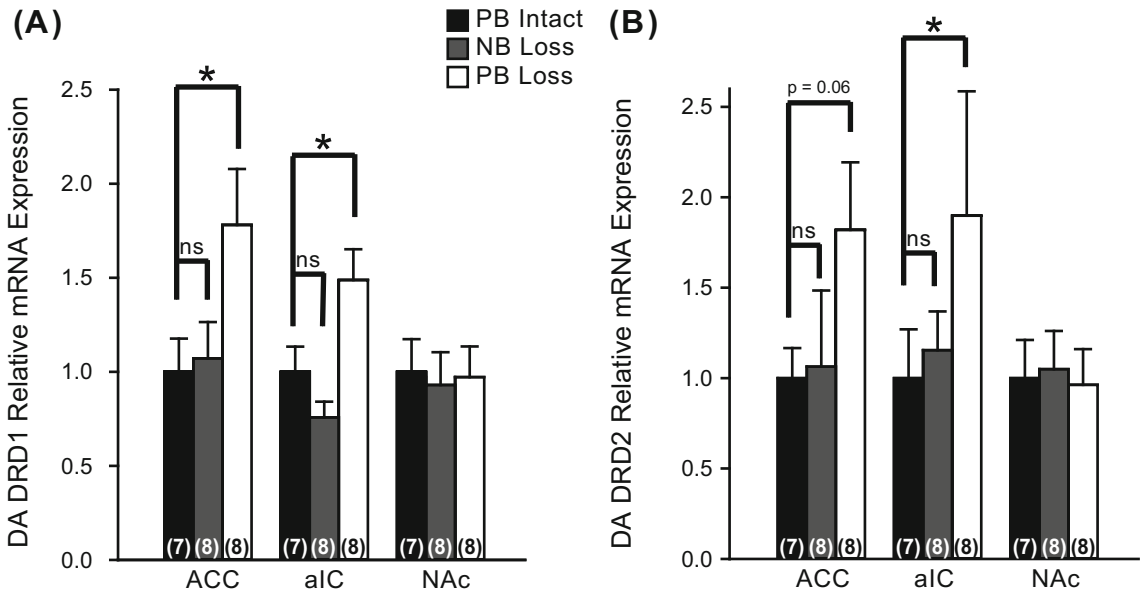

FIGURE 5 Loss of a high-quality, opposite-sex relationship influences DA receptor mRNA expression in brain regions associated with human grief. (A) When comparing both loss groups to the intact group, only PB Loss males showed significantly higher DRD1 mRNA expression in the ACC and aIC while NB Loss males did not differ from the intact group. (B) Similarly, PB Loss males showed higher DRD2 mRNA in the ACC and aIC compared with PB Intact males, while NB Loss males did not differ from the intact group. $^{*}p < 0.05$.

that male prairie voles do display variation in partner- and stranger-directed affiliation in the PPT (Figure 4A).

In the OPT, ANOVA revealed significant group differences in the time spent in close proximity to the partner odor cue ($F = 5.09$, $p = 0.02$), partner odor olfactory investigation ($F = 4.41$, $p = 0.03$) and preference ratio ($F = 3.66$, $p = 0.04$). Post-hoc comparisons showed that PB/loss males spent significantly more time in proximity to their partner's odor and spent more time sniffing the partner odor compared with both the PB/intact and NB/loss groups. Post-hoc analysis of preference ratio showed that both loss groups, regardless of PB status, had a similar partner/food preference and were both significantly higher (i.e., shifted toward partner) than the PB intact group (Figure 4B, C).

mRNA analyses for DA receptor expression revealed that pair-bond status does influence DRD1 and DRD2 expression in a region-dependent manner (Figure 5). In the ACC, a one-way ANOVA using simple planned contrasts (because our a priori hypothesis was that only the pair bonded/loss subjects would differ from the intact subjects and the non-bonded/loss subjects would not differ from intact subjects) found that DRD1 expression was significantly higher when comparing PB/loss group with the PB/intact group ($p = 0.046$) and no differences were found between PB/intact and NB/loss ($p = 0.99$). DRD2 mRNA expression in the ACC tended to be higher in the PB/loss group compared with the PB/intact group ($p = 0.065$), and again did not differ between NB/loss and PB/intact ($p = 0.97$). DA receptor mRNA expression followed the same pattern in the aIC, with DRD1 being significantly higher and DRD2 tending to be higher in the PB/loss group compared with the PB/intact group (DRD1: $p = 0.029$, DRD2: $p = 0.060$), and no such differences were found comparing NB/loss subjects to PB/intact subjects (DRD1: $p = 0.20$, DRD2: $p = 0.87$). In the NAc, DRD1 and DRD2 mRNA expression did not differ between any groups.

5 | DISCUSSION

This series of experiments represents the first examination of the impact of both relationship type and relationship quality on social motivation-related outcomes following companion loss. Furthermore, the development of a novel behavioral test to assess social motivation using partner odor cues allows for assessing reunion-seeking behavior without physically reuniting the subject with the lost companion, providing a flexible approach to study the time course of loss resolution with regards to social motivation.

Using the prairie vole model, we assessed the effects of companion sex on social loss outcome and found that male prairie voles losing an opposite sex mate display a preference for their partner's odor over food odor in an odor choice test, while males losing the same-sex cage mate did not differ from those that remained intact with their same- or opposite-sex companion. This result is due to an increase in olfactory investigation of partner odor, specifically, in the OS Loss group with no change in food odor olfactory investigation. We argue that this indicates a shift in the socio-motivational state of male prairie voles after 1 week of partner loss, which we term "partner-seeking" behavior. Moreover, there was no difference between OS intact and OS loss males in their social odor preference when the social option was bedding from a novel male–female pair, suggesting that the OS loss subjects are, indeed, displaying "partner-seeking"

behavior and not general social-seeking. Additionally, the OPT revealed differences in partner-seeking behavior based on the "quality" as determined by categorization of males as pair bonded or not bonded to their opposite-sex partner based on their display of bond-typical behavior in the partner preference test.

Like other social loss researchers, we focused our attention on the mesolimbic dopamine system, with the NAc being at the center of prior research due to its essential role in pair bonding in prairie voles. We also expanded our focus to include the IC and ACC due to their roles in emotional processing, emotional pain and reward salience. These regions, in addition to the NAc, also show heightened activation when grieving humans are viewing photos of a deceased loved-one, suggesting that they may be involved in grief processing.[8,9] All three brain regions express DA receptors, with prior studies describing a role for DA receptors in these regions in various social, emotional and reward-related behaviors.[40–43] In prairie voles, specifically, DA receptors in the NAc are important for pair-bond formation and maintenance. For instance, DRD1 binding is increased in the NAc core and shell in males paired with females for 2 weeks compared with sexually naïve, same-sex housed males, with no change in DRD2-like binding.[20] Conversely, DRD1 are not upregulated when new prairie vole peer relationships are formed,[44] confirming that reorganization of NAc DA receptors is related to the display of specific relationship types. Interestingly, we did not find a difference in DRD1 or DRD2 mRNA expression in the NAc of male–female paired versus same-sex paired subjects in our study. However, there is not always a match between mRNA and protein expression, as differences in protein translation could affect the turnover of mRNA to protein without affecting mRNA transcription.[45] Alternate explanations for the high binding during pair bonding that our mRNA results did not confirm also include a reduction in receptor internalization following receptor activation, and/or an increase in receptor sensitivity for binding DA or DA-like ligands. These possibilities could be further explored using receptor binding techniques employed by previous studies, or by performing western blot analysis for DA receptors as well as for proteins that are responsible for initiating receptor internalization. We did find that males separated from male cage mates showed significantly higher DA receptor mRNA compared with males that remained pair-housed, while there was no loss-dependent effect in the OS subjects. Short-term isolation is known to affect social behavior and social recognition,[46] so this finding corroborates previous literature examining the effects of short-term social isolation. The lack of effect in OS subjects could indicate a suppression mechanism through which general social motivation may be dampened to facilitate partner-seeking behavior.

This study is the first to assess DA system-related changes in the IC following opposite-sex cohabitation in prairie voles. Indeed, we did find significantly less DRD1 mRNA expression in the IC of males with female partners compared with males with same-sex cage mates, regardless of loss experience. DA receptors in the IC are involved in a diverse set of behaviors, including impulsive decision-making,[47] stimulus salience,[48] and nociception.[49] In support, there is significant DA, but not glutamate, GABA, or norepinephrine, release in the IC in mice during presentation of novel tastes or objects measured by in vivo microdialysis.[50,51] Of note, novel taste presentations induced strong DA release in the IC regardless of the valence of the stimuli,[50] suggesting that the insula may be responsible for detecting all relevant sensory stimuli, perhaps to relay the information to the appropriate downstream targets for display of the appropriate behavioral response. Indeed, DA terminals in the rostral agranular IC have been found in close contact with GABAergic interneurons that influence IC projections to the amygdala and NAc.[24] In addition, optogenetically activating the VTA-IC DA pathway facilitates object recognition memory, while blocking DRD1 prevents this activation-induced object discrimination.[52] Although DRD1 expression is lower in OS-housed males compared with SS-housed males, expression is higher if OS males have formed a pair bond with their female partner prior to loss compared with those that did not. This result could indicate a shift in valence processing capabilities of the IC during loss of a high-quality relationship. Further research will be needed to fully understand the role of IC DA and its receptors in pair-bond formation and/or maintenance in prairie voles, though it is well-situated to influence the salience of social conspecifics. Experiments are currently underway to determine whether the IC exhibits relationship-specific neural activity patterns during social interaction.

Surprisingly, we did not find significant loss or companion-sex specific changes in DA receptor mRNA in the ACC, although loss of a pair-bonded partner resulted in higher DRD1 and DRD2 mRNA in Experiment 2. In prairie voles, the ACC is involved in partner consolation behavior following a stressor, suggesting that it is sensitive to the stress state of social conspecifics. Moreover, this consolation behavior is only displayed when the stressed companion is a mate or a sibling and is not displayed toward stressed strangers,[53] suggesting that neural circuits that process relationship value may influence function of the ACC to drive the display of emotional behaviors. Interaction with stressed partners also increased cFos immunoreactivity in GABA neurons in the ACC of male and female mandarin voles (a *Microtus* species found in Asia which exhibits pair bonding and social monogamy similar to that of prairie voles), while site-specifically infusing a $GABA_A$ antagonist reduced consolation behavior.[54] Furthermore, when mandarin voles were physically or emotionally stressed themselves, they showed less consolation behavior toward their stressed partner and significantly lower DRD2 protein expression in the ACC.[55] Infusing a DA agonist into the ACC restored consolation behavior. Excitatory/inhibitory tone appears to be particularly important for the processing of, and the subsequent behavioral response, to noxious stimuli, and DA inhibits both excitatory and inhibitory synaptic transmission in the ACC via postsynaptic GPCR activation. Interestingly, inflammatory pain reduces the ability of DA to inhibit excitatory but not inhibitory responses, showing that DA dynamically modulates E/I transmission in the ACC during "normal" and aversive states.[56] The role of DA on valence state processing by the ACC is likely influenced by motivational state, as reward and motivation are primary functions of the mesocorticolimbic DA system.

In Experiment 2, loss of an opposite-sex mate with whom the subject was pair bonded (determined by PPT assessment) resulted in

more robust effects on DA receptor expression, with both DRD1 and DRD2 mRNA expression being >50% higher in the IC and ACC of pair-bonded males that lost their partner compared with pair-bonded males that remained with their partner. Importantly, male prairie voles that lost a non-pair-bonded mate did not differ from the intact group, implying that this receptor expression effect is dependent on the loss of a high-quality relationship. These data may appear to contradict Experiment 1, which found no DA receptor changes specific to the OS loss group although these males still displayed increased partner-seeking behavior in the OPT. One possibility for this could be a higher-than-average number of nonbonded males in the OS loss group from Experiment 1, resulting in nonsignificant DA mRNA effects. While the average proportion of bonded to nonbonded animals is 70/30, our lab has seen this ratio closer to 50/50 in some experiments. Because we did not assess partner preference in Experiment 1, we cannot definitively say what proportion of males were pair bonded in our OS-paired groups. It is also worth pointing out that a higher proportion of non-bonded males in Experiment 1 could have affected the PCR data and not the OPT data due to the difference in magnitude of these effects. NAc DA receptor mRNA was not affected by loss of a high-quality relationship, matching what we found with the relationship type experiment (Experiment 1).

In sum, loss of a social companion affects motivation-related behavior depending on the salience of the social partner. This suggests the neurobiological mechanisms that govern social valence during the formation of a social relationship may also play a role in maintaining this salience when the relationship is lost and underlie "yearning" or reunion-seeking behavior. Furthermore, changes in the mesocorticolimbic DA system were found alongside the behavioral display of partner-seeking behavior following social loss. Ongoing work will continue to explore the function of DA and its receptors in the striatum and higher-order cortical regions (i.e., NAc, ACC and aIC) as they are particularly sensitive to partner-associated cues in grieving individuals. Taken together, these findings, along with the previous research showing increased anxiety-related behavior and altered stress-coping behavior[26,27,33] demonstrate that the prairie vole social loss model can be a powerful tool to examine the neurobiological processes underlying these loss-associated behaviors.

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CONFLICT OF INTEREST STATEMENT

The authors declare no competing financial interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author, AS.

ORCID

Adam S. Smith https://orcid.org/0000-0003-2837-6205

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