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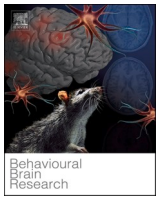

Research article

Hypothalamic vasopressin neural densities are higher in male Mongolian gerbils after separation from a pair bond partner and may facilitate behavior to form a new bond

B.A. Fricker [1], J. Jiang [1], C.J. Esquilin-Rodriguez, M.L. Dowling, A.M. Kelly [*]

Department of Psychology, Emory University, 36 Eagle Row, Atlanta, GA, USA

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ABSTRACT

Although pair bonding has been studied for several decades, only somewhat recently have researchers began studying the neural consequences of separation from a pair bond partner. Here we examined the impact of partner separation on the socially monogamous Mongolian gerbil. Using a within-subjects design, we assessed nonsocial, nonreproductive, and reproductive behavior in male gerbils pre- and post- either 4 weeks of cohabitation with or separation from a pair bond partner. We then conducted an immediate early gene study to examine the influence of partner separation on hypothalamic oxytocin and vasopressin neural responses to interactions with a novel, opposite-sex conspecific.

1. Introduction

Pair bonding, a social attachment between mates, is a hallmark of socially monogamous species and conveys several benefits, such as shared-parenting, stress-buffering, and increased fitness [38,47]. Although socially monogamous species are typically able to form a new pair bond after loss of a former partner [14,21], studies show that there are a suite of consequences associated with partner separation [37]. In voles, separation from a pair bond partner increases anxiety-like behavior, depressive-like behavior, and passive stress-coping, as well as circulating levels of adrenocorticotropic hormone and corticosterone [2,4,27,41].

In addition to behavioral and hormonal changes associated with partner loss, studies have also examined the consequences of partner separation on the brain. For example, a recent study in prairie voles demonstrated that prolonged partner separation erodes transcriptomic signatures of pair bonding in the nucleus accumbens [36]. Additionally, studies examining the nonapeptide system (i.e., oxytocin, OT; vasopressin, VP) of prairie voles revealed that partner separation increases OT neuronal densities in the paraventricular nucleus of the hypothalamus (PVN), rescuing a pair bond-induced decrease in OT neurons in this region [7,41]. Because OT has anxiolytic effects in mammals [23,45], it is possible that an increase in PVN OT production may help an animal cope after partner loss. Nonapeptide-producing neuronal populations of the PVN are of particular interest in the context of pair bonding and partner loss because peptides in this region modulate social behaviors as well as the stress response [3,28,34,42]. Further, studies show that PVN VP and OT cell groups are not only exceptionally plastic during development, but are also flexible in adulthood [7,18], and can thus change in response to major life events, enabling an animal to adapt to the loss of a partner and alter behavior to successfully form a new pair bond.

Thus far, most studies examining consequences of partner separation have been conducted in prairie voles. Whether other socially monogamous species exhibit behavioral and neural responses to partner loss in a similar manner remains largely unknown. Although the literature in prairie voles holds substantial translational value, taking a comparative approach and examining the consequences of partner separation in other species can generate insight into the generalizability of findings from a single rodent species. To that end, here we use the socially monogamous Mongolian gerbil to examine the consequences of partner separation on social behavior and PVN nonapeptide function and neuroanatomy. Mongolian gerbils live in small family groups comprised of an adult male and female and 1–3 litters of offspring [12,35]. Although male gerbils are typically aggressive toward novel, same-sex conspecifics [30], they are highly affiliative with mates and exhibit a preference for a familiar female partner over a novel opposite-sex

* Corresponding author.
E-mail address: aubrey.kelly@emory.edu (A.M. Kelly).
[1] Joint first authorship

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conspecific after 48 hours of cohabitation with the partner [24,43].

In the present study, we used a within-subjects design to determine the consequences of partner separation on reproductive and nonreproductive behavior in male gerbils. Additionally, after completion of behavioral testing, we conducted an immediate early gene (IEG) study to examine whether PVN nonapeptide neuronal responses to a novel, opposite-sex conspecific (i.e., a potential new mate) differ between males that were pair bonded and those that had been separated from their partner for 4 weeks. Lastly, we examined the number of PVN OT and VP cells in paired vs. separated males. We predicted that, if PVN nonapeptide neuronal densities respond to partner separation similarly in prairie voles and gerbils, we would observe higher PVN OT densities in separated male gerbils as previously observed for separated male prairie voles [7].

2. Methods

2.1. Animals

15 adult male Mongolian gerbils (*Meriones unguiculatus*) were used as subjects and 15 adult female Mongolian gerbils were used as pair bond partner stimuli for the male subjects. Subjects were between PND80–120 at the start of the experiment. Females were not used as subjects here because they were dedicated for use in a later study. All animals were obtained from our breeding colony using breeders purchased from Charles River. Sex was defined by external genitalia. Prior to the start of the experiment, animals were co-housed with 2–3 other same-sex siblings in standard rat polycarbonate cages (40.64 cm×20.32 cm×20.32 cm). All cages were lined with Sani-Chips bedding and included nesting material, chewing blocks, and shelter tubes. Food and water were provided ad libitum. Animals were kept on a 14 L:10D cycle, with ambient temperatures maintained at 24 ± 2°C. One subject in the Separation condition was prematurely euthanized prior to the IEG study. The experiment complies with ARRIVE guidelines and was carried out in accordance with the National Research Council's Guide for the Care and Use of Laboratory Animals. All procedures were approved by the Institutional Animal Care and Use Committee of Emory University.

2.2. Experimental design

Male subjects were randomly assigned to one of two groups: (1) Paired or (2) Separated. Subjects were then housed in the same cage with an adult female for 1 week, with a barrier separating the male and the female. This was done to prime the gerbils so that they would become familiar with each other and more readily accepting pairing once the barrier was removed. After this week of priming, the barrier was removed and male subjects and the females were allowed to cohabitate for 14 days prior to the beginning of testing. Previous studies have demonstrated that 48 hours of cohabitation is sufficient for male gerbils to form a partner preference [43]. Confirmation of an established pair bond in subjects was noted by huddling in the home cage, which was observed in all subjects within 4 days after removing the barrier. In our colony, if pairing is not accepted, we observe intense female aggression toward the male and separate the animals immediately. All animals in the present study accepted pairing. After 14 days of cohabitation with the pair bond partner, males underwent an initial series of behavioral tests (Timepoint 1) in a randomized order over 3 days. Tests included an open field test, a nonreproductive social interaction test, a social approach test, and a resident-intruder test. Of the 4 tests, the resident-intruder test was run as a single test for a day to avoid any potential stress of the test influencing behavior in other tests. 2 tests were conducted, 1 in the morning and 1 in the afternoon, on the other 2 testing days. After Timepoint 1 behavioral testing, males in the Paired condition remained co-housed with their pair bond partners, whereas males in the Separated condition were single-housed for 4 weeks. After 4 weeks, subjects underwent the same battery of tests a second time (Timepoint 2), again in a randomized order. 3–5 days after Timepoint 2 behavioral testing, subjects were run through an IEG study and brains were collected for subsequent analyses.

2.3. Behavioral tests

Subjects were tested twice (Timepoint 1 and Timepoint 2 as described above) in a series of tests in both nonreproductive and reproductive contexts to determine if separation from a pair bond partner globally influences a variety of types of social and nonsocial behavior or specifically influences reproductive social behaviors. All behavioral tests were video recorded using Sony Handycam HDR-CX405 1080p Camcorders (Sony).

2.3.1. Open field test

To determine whether pair bond status influences exploratory behavior in a novel environment, we tested subjects in an open field test similar to that used for numerous rodent species [22,31,46]. Subjects were transferred from their homecage via a plastic beaker to the center of a large open field chamber (120 cm X 120 cm X 60 cm), where they were allowed to freely explore for 10 min. In Ethovision XT (Noldus, Information Technology, Netherlands), the arena was subdivided into a center region (38 cm X 38 cm), an intermediate zone, and a border region along the periphery of the chamber (24 cm X 50 cm on all four sides). The time spent along the periphery and in the center zone of the chamber was quantified. Additionally, the distance traveled throughout the 10 min test was also quantified.

2.3.2. Nonreproductive social interaction test

To determine whether pair bond status influences behavior with same-sex conspecifics outside the context of mating, we conducted a nonreproductive social interaction test. The subject and an age- and weight-matched novel, same-sex conspecific were placed under plastic beakers in a clean, novel standard rat cage (i.e., neutral territory). Both the subject and stimulus animal were released simultaneously and allowed to freely interact for 10 min. The following behavior exhibited by the subject was quantified using BORIS [5]: investigative behavior (head, flank, and rear investigation), prosocial behavior (huddling and allogrooming), aggressive behavior (pinning, lunging/attacking, biting, chasing), and non-overt behaviors (time alone, jumping, and autogrooming). For similar social interaction tests in gerbils see [9].

2.3.3. Social approach test

To determine whether pair bond status influences the rate at which a male gerbil approaches a novel, opposite-sex conspecific (i.e., a potential mate), we conducted a social approach test. Subjects were first placed into a testing chamber (81 cm×40.5 cm X 38 cm) and allowed to acclimate for 3 min. After acclimation, subjects were then contained under a plastic beaker at one end of the chamber. A novel, opposite-sex conspecific was placed under a wire mesh container at the opposite end of the chamber. The subject was then released and allowed to freely explore for 5 min. The latency for the subject to make physical contact with the stimulus container was recorded. For similar social approach tests in prairie voles see [19]; for other forms of social approach tests that have been conducted in gerbils see [40,46].

2.3.4. Resident-intruder test

To determine whether pair bond status influences how a male interacts with a novel, opposite-sex conspecific (i.e., a potential mate) on their home territory, we conducted a resident-intruder test similar to resident-intruder tests conducted in prior studies [17,20]. Subjects were tested in their homecage. Prior to testing, the female pair bond partner was removed from the homecage and placed into a clean, novel rat cage. Chewing blocks and shelter tubes were also removed from the homecage so that only nesting material and the male subject remained. An

age-matched novel, opposite-sex conspecific was transferred into the subject's homecage via a plastic beaker; the subject and stimulus animal were allowed to freely interact for 10 min. Behavior was scored using BORIS as described above for the social interaction test.

2.4. Reproductive social interaction immediate early gene study

An IEG study was conducted to determine whether males that had been separated from their pair bond partner for 4 weeks would exhibit differential behavioral and neural responses to a novel, opposite-sex conspecific (i.e., a potential new mate). Similar to previous IEG studies in gerbils [17], subjects were placed into a clean, novel rat cage (i.e., neutral territory) and allowed to acclimate for 20 min. A novel, opposite-sex conspecific was then transferred via a plastic beaker into the test cage with the subject. The subject and stimulus animals were allowed to freely interact for 30 min. At that time, the stimulus animal was removed, and the subject remained in the test cage for an additional 60 min. Subjects were then immediately perfused in order to capture Fos responses to exposure to the novel, opposite-sex conspecific. The first 10 min of the reproductive social interaction was scored using BORIS as described above for the nonreproductive social interaction test; this time period most closely corresponds to the Fos responses quantified in brain tissue.

2.5. Histology and immunohistochemistry

At the end of the IEG study, subjects were euthanized by isoflurane overdose and transcardially perfused with 0.1 M phosphate buffered saline (PBS) and 4 % paraformaldehyde dissolved in 0.1 M borate buffer (pH 9.5). Brains were then extracted and post-fixed overnight in 4 % paraformaldehyde prior to cryoprotection in 30 % sucrose for 48 hours. Brains were frozen in Tissue-Tek O.C.T. Compound (Sakura Finetek) in Peel-A-Way molds and stored at −80°C until cryosectioning. Brains were sectioned into 3 series on a Leica cryostat at 40μm.

Hypothalamic tissue from one series was dedicated to immunofluorescent staining of VP and Fos, whereas tissue from a second series was stained for OT and Fos following previously published protocols [6,9,17]. Tissue was rinsed 5 times for 10 min in 0.1 M PBS (pH 7.4) prior to incubation in a blocking solution (PBS, 10 % normal donkey serum, and 0.03 % Triton X-100) for 1 hour at room temperature. Tissue was then incubated for 48 hours at 4°C in primary antibodies diluted in PBS containing 5 % normal donkey serum and 0.03 % Triton X-100. Primary antibodies for the first series of tissue were guinea pig anti-vasopressin (1:1000; Peninsula Laboratories) and rabbit anti-Fos (1:500; Synaptic Systems). Primary antibodies for the second series of tissue were rabbit anti-oxytocin (1:250; Abcam) and guinea pig anti-Fos (1:1000). After the primary incubation, tissue was rinsed in PBS for 30 min twice. For the first series of tissue staining for VP, tissue was incubated in a biotinylated donkey anti-guinea pig secondary antibody (1:125; Jackson Immunoresearch) for 1 hour at room temperature to amplify signal of the guinea pig anti-VP antibody which can otherwise exhibit faint signal. After the biotin step, tissue was rinsed in PBS twice for 15 min.

Tissue for both the first and second series was then incubated in a secondary antibody solution diluted in PBS containing 5 % normal donkey serum and 0.03 % Triton X-100 for 2 hours at room temperature in the dark. Secondary antibodies for the first series of tissue were streptavidin conjugated to Alexa Fluor 488 (1:300; ThermoFisher) and donkey anti-rabbit conjugated to Alexa Fluor 594 (1:200; ThermoFisher). Secondary antibodies used for the second series of tissue were donkey anti-rabbit conjugated to Alexa Fluor 488 (1:300; ThermoFisher) and donkey anti-guinea pig conjugated to Alexa Fluor 594 (1:200; ThermoFisher). Finally, tissue was rinsed twice for 30 min in PBS prior to mounting on microscope slides (TruBond 380) and cover-slipped with Prolong Gold antifade with DAPI (ThermoFisher).

2.6. Neural quantification

Images of the PVN were acquired using a Zeiss AxioImage II microscope outfitted with an AxioCam Mrm, z-drive, and an Apotome optical dissector (Carl Zeiss Inc.). Z stack images were flattened and processed with ZenPro software (Carl Zeiss Inc.). Designation of the PVN was based on the Mongolian gerbil brain atlas [32]. One rostral and one caudal image of the PVN were acquired and the number of VP-immunoreactive [26] cells, OT-ir cells, as well as the total number of VP-ir and OT-ir cells colocalized with Fos were quantified for both rostral and caudal sections. Cell counts were then averaged across rostral and caudal sections. Cell counts were conducted in FIJI [39].

2.7. Statistics

Repeated measures general linear models (rmGLM) were used to analyze behavioral data from Timepoint 1 and Timepoint 2 testing. Posthoc pairwise comparisons were adjusted using Sidak correction. Data were not normally distributed and thus Mann Whitney U-tests were used to analyze brain and behavioral data, and Pearson's correlations were used to examine relationships between brain and behavioral data, from the IEG study. All statistics were analyzed using SPSS 29 (IBM Analytics) and graphs were made using Prism 10 (GraphPad).

3. Results

3.1. Partner separation does not influence boldness or nonreproductive social behavior

We first examined whether 4 weeks of separation from a pair bond partner influenced boldness in an open field test. A rmGLM with Pair Bond Status (Paired or Separated) as a fixed factor and Time (Timepoint 1 vs. Timepoint 2) as a repeated measure revealed no effects or interactions, such that males that were pair bonded did not exhibit differences in time spent along the periphery (all $p > 0.57$) or in the center (all $p > 0.98$) of the open field chamber compared to males that were separated. However, we found a main effect of Time for velocity ($p < 0.01$; $F_{(1,13)} = 10.69$), as well as the distance moved during the open field test ($p < 0.01$; $F_{(1,13)} = 10.48$), showing that male gerbils traveled faster and for a greater distance during the second round of testing. Together, this suggests that the experience of being tested in the open field chamber significantly influences some aspects of exploratory behavior.

We next examined whether partner separation influenced social behavior during interactions in a nonreproductive context (i.e., with a novel, same-sex conspecific). A rmGLM with Pair Bond Status as a fixed factor and Time as a repeated measure revealed no effects or interactions for investigation (all $p > 0.26$), non-overt behavior (all $p > 0.36$), or aggressive behavior (all $p > 0.08$). Although we did not observe an effect of Pair Bond Status or an interaction between Pair Bond Status and Time for prosocial behavior (all $p > 0.07$), we did observe a main effect of Time for prosocial behavior, such that all subjects exhibited less prosociality at Timepoint 2 compared to Timepoint 1 ($p = 0.02$; $F_{(1,12)} = 7.92$).

3.2. Partner separation influences social behavior in a reproductive context

To determine if partner separation influenced social behavior in a reproductive context, male subjects were tested in a social approach test with a novel, opposite-sex conspecific and in a resident-intruder test with a novel, opposite sex conspecific in the subject's homecage. For the social approach test, a rmGLM with Pair Bond Status as a fixed factor and Time as a repeated measure revealed no effects or interactions for the latency to approach the stimulus animal (all $p > 0.94$). Similarly, during the resident-intruder test, a rmGLM revealed no effects or interactions

for prosocial behavior (all $p > 0.39$; Fig. 1A), non-overt behavior (all $p > 0.06$), or aggression (all $p > 0.07$; Fig. 1B). However, for investigation during the resident-intruder test, while we observed no effects of Time or Pair Bond Status on the time spent investigating the novel, opposite-sex intruder ($p = 0.42$), we found a significant interaction ($p < 0.01$; $F_{(1,13)} = 9.65$) between Pair Bond Status and Time. Sidak-corrected posthoc analyses showed that paired and separated males did not exhibit differences in investigation at Timepoint 1 or Timepoint 2 (all $p > 0.10$); yet, within separated males only, investigation increased from Timepoint 1 to Timepoint 2 ($p = 0.02$; MD = 54.07; Fig. 1C). This suggests that separated males may be more receptive to a potential new mate after 4 weeks of separation from their previous pair bond partner.

We also examined interactions with a novel, opposite-sex conspecific on neutral territory in the final IEG test (i.e., only a single test and not repeated measures). A Mann Whitney-U test revealed a main effect of Pair Bond Status on investigation of ($p = 0.01$; $Z = -2.45$; Fig. 2A) and prosocial behavior toward ($p = 0.02$; $Z = -2.38$; Fig. 2B) the novel, opposite-sex conspecific, showing that separated males were more investigative of and prosocial with the novel female compared to paired males. We observed no differences in aggressive or non-overt behavior in the reproductive social interaction IEG test (all $p > 0.16$).

3.3. Partner separation does not influence PVN OT neuronal densities or responses

We examined whether partner separation influenced PVN OT neural densities or responses to an interaction with a novel opposite-sex conspecific during the IEG test. Mann Whitney U-tests showed no difference in PVN OT-ir cell numbers ($p = 0.44$; $Z = -0.78$) or PVN OT-Fos colocalization ($p = 0.16$; $Z = -1.42$) between males in the paired and separated conditions. These findings suggest that PVN OT may be robust to pair bond status in male gerbils.

Although we did not observe any differences in PVN OT neural densities between paired and separated males, because the reproductive social interaction IEG test was conducted as the final test immediately prior to perfusion of subjects, and thus behavior during this test most accurately reflected the "state" of the brain captured at perfusion, we examined relationships between behavior in the final IEG study and PVN OT cell densities and PVN OT-Fos colocalization of all subjects combined. Pearson's (for normally distributed data) and Spearman's (for non-normally distributed data) correlations did not reveal any significant relationships between PVN OT neuron number and any type of behavior (all $p > 0.30$). Similarly, PVN OT-Fos colocalization did not relate to investigative or prosocial behavior (all $p > 0.63$). However, we observed a significant relationship between PVN OT-Fos colocalization and aggression, such that males that had greater PVN OT-Fos colocalization exhibited more aggression toward a novel, opposite-sex conspecific ($p = 0.01$; Spearman's $r(14) = 0.66$; Fig. 3). Notably, only 5 (2 separated males; 3 paired males) of the 14 subjects exhibited any aggression in the reproductive social interaction IEG test.

3.4. Partner separation influences PVN VP neuronal densities but not responses

To determine if 4 weeks of partner separation influenced PVN VP expression, we examined the number of VP-ir cells in the PVN. A Mann-Whitney U test revealed that males that were separated from their partners had significantly greater PVN VP neuronal densities compared to males that remained pair bonded with their partner ($p < 0.01$; $Z = -2.58$; Fig. 4A). We next examined PVN VP colocalization with Fos in response to interacting with a novel, opposite-sex conspecific on neutral territory. A Mann-Whitney U test yielded no difference in PVN VP-Fos colocalization between paired and separated males ($p = 0.12$; $Z = -1.55$; Fig. 4B), suggesting that the responsiveness of PVN VP to a novel, opposite-sex conspecific is not significantly influenced by partner separation and/or pair bond status.

Next, we related PVN VP neural densities to behavior exhibited during the IEG study, in which the subject interacted with a novel, opposite-sex conspecific. PVN VP cell numbers did not correlate with prosocial, non-overt, or aggressive behavior (all $p > 0.11$). However, we did find a significant correlation between PVN VP cell number and investigation ($p = 0.04$; Pearson's $R = 0.55$; Fig. 5). This suggests that, regardless of pair bond status, PVN VP may promote investigative behavior in a reproductive context in male gerbils. Lastly, we observed no significant relationships between PVN VP-Fos colocalization and any behavior (all $p > 0.30$).

4. Discussion

In the present study, we found that partner separation in male Mongolian gerbils did not significantly influence exploratory behavior or nonreproductive social behavior but did impact social behavior in reproductive contexts. When on their home-territory (i.e., the resident-intruder test), males increased investigation of a novel, opposite-sex conspecific only in the Partner Separation condition, suggesting that males may be more receptive to an intruding female after they have been separated from their former partner for 4 weeks. Similarly, on neutral territory (i.e., reproductive social interaction test), separated males were not only more investigative but also more prosocial with a novel, opposite-sex conspecific compared to males that remained paired with their partners. Thus, while partner separation may not globally influence all types of social and nonsocial behavior in male gerbils, 4 weeks of separation from one's partner does increase affiliative and investigative behavior toward novel, opposite-sex conspecifics, potentially to facilitate receptivity of forming a new pair bond. While PVN VP and OT neuronal responses to a novel, opposite-sex conspecific did not differ between paired and separated males, males that had been separated from their partner exhibited significantly more PVN VP cells than paired

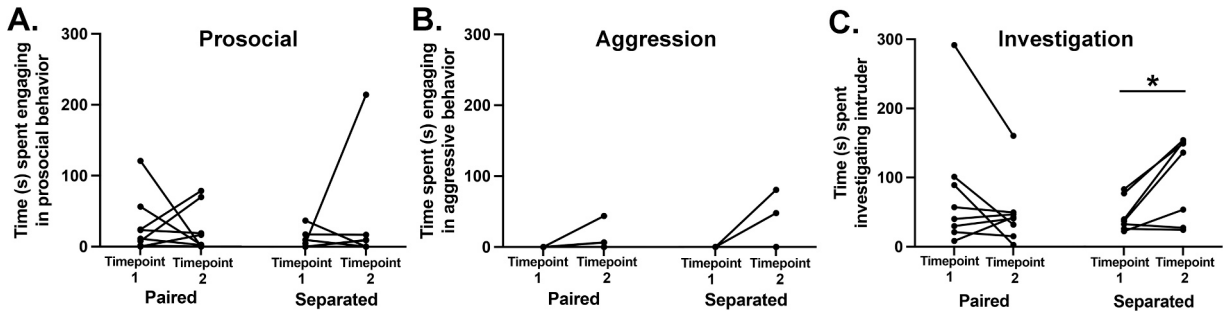

Fig. 1. Resident-intruder test. (A) Prosocial and (B) aggressive behavior exhibited toward a novel, opposite-sex intruder did not differ based on Pair Bond Status or Time. (C) Investigation of a novel, opposite-sex intruder increased between the time males in the Separated condition were pair bonded (Timepoint 1) and after they had been separated from their partner for 4 weeks (Timepoint 2). Investigative behavior did not differ from Timepoint 1 to Timepoint 2 for paired males. * indicates $p < 0.05$.

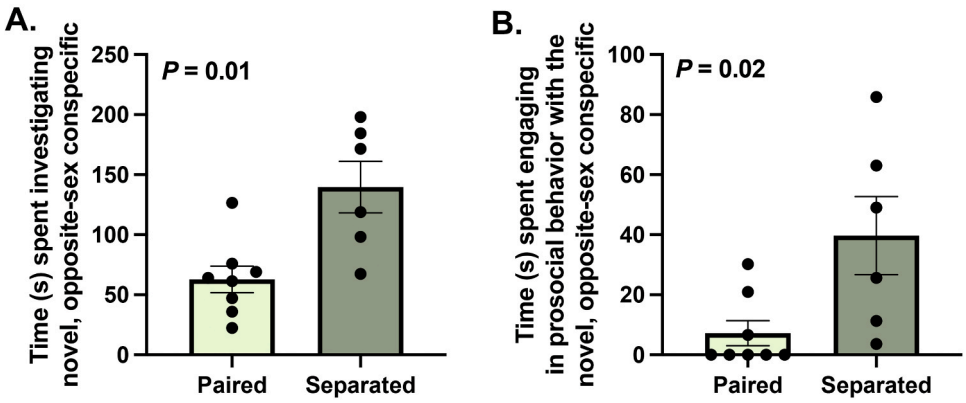

Fig. 2. Reproductive social interaction test. Males that had been separated from their pair bond partner (dark green) for 4 weeks spent more time (A) investigating and (B) engaging in prosocial behavior with a novel, opposite-sex conspecific on neutral territory compared to males that had remained paired with their pair bond partner (light green). Data represented as mean ± SEM. Dots represent individual data points.

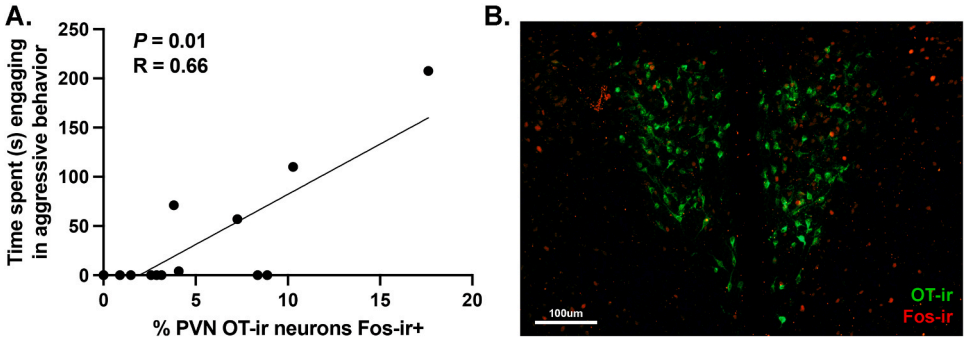

Fig. 3. PVN OT neural responses relate to aggression. (A) Regardless of Pair Bond Status or Time, PVN OT neural responses positively correlate with the time spent exhibiting aggressive behavior toward a novel, opposite-sex conspecific on neutral territory (i.e., during the reproductive social interaction IEG study). Dots represent individual data points. (B) A representative image from a male gerbil of OT-ir (green) and Fos-ir (red) labeling in the PVN.

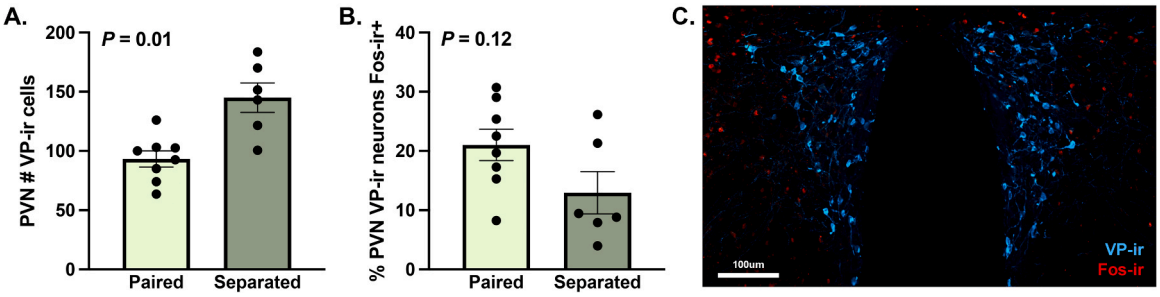

Fig. 4. PVN VP neuroanatomy and function. (A) Males that had been separated from their pair bond partner (dark green) for 4 weeks exhibited significantly more VP-ir neurons in the PVN compared to males that had remained paired with their pair bond partner (light green). (B) PVN VP-Fos colocalization in response to an interaction with a novel, opposite-sex conspecific did not differ between males in the Paired and Separated conditions. Data represented as mean ± SEM. Dots represent individual data points. (C) A representative image from a male gerbil of VP-ir (cyan) and Fos-ir (red) labeling in the PVN.

males. Further, investigative behavior of a novel, opposite-sex conspecific positively correlated with the number of VP cells in the PVN. Together, these findings suggest that PVN VP neuronal densities may increase in male gerbils in response to partner separation, thereby facilitating investigative behavior of potential new mates to increase the likelihood of forming a new pair bond. However, it should be noted that we were unable to control for the potential of behavioral and neural effects being due to social isolation; follow-up studies are required to disentangle the influence of social isolation and partner separation on behavior and the brain of gerbils.

4.1. Effects of partner separation on behavior in socially monogamous rodents

Studies have examined the impact of pair bond disruption on nonsocial behaviors using field-standard tests to examine anxiety- and depression- like behaviors. For example, separation from a pair bond partner for just 4–5 days resulted in increased passive stress-coping in male prairie voles as assessed via forced swim and tail suspension tests [1,2]. Similar to our paradigm in the present study, other research in voles has also examined the consequences of partner separation following longer periods of partner separation. Mandarin vole males that had been separated from their partners for 2 weeks spent less time

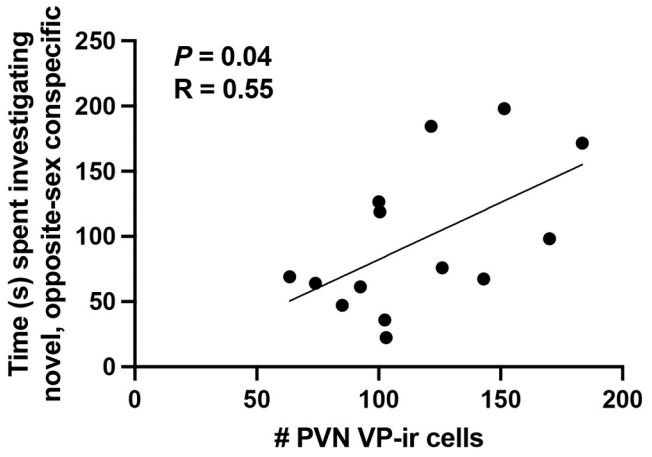

Fig. 5. Brain-behavior relationship. (A) The total number of PVN VP-ir cells positively correlated with the time spent investigating a novel, opposite-sex conspecific on neutral territory (i.e., during the reproductive social interaction IEG study). Dots represent individual data points.

in the central zone of an open field test compared to paired males [4]. Additionally, separated males also spent less time in the light box of a light-dark box test and exhibited more immobility in a forced swim test than paired males [4]. Similar findings have been observed in male prairie voles, such that males separated for 4 weeks spent less time in the center of an open field chamber [29] and less time in the light area of a light-dark box [41]. To our knowledge, only one other study has examined the consequences of partner separation on behavior in Mongolian gerbils. Contrary to what has been observed in voles, and similar to our findings here, no differences in exploratory behavior in an open field test were observed between male gerbils that were paired and males that had been separated from a pair bond partner for 4 weeks [15]. Together these studies suggest that exploratory and/or anxiety-like behavior of voles may be more susceptible to the influence of partner separation than for gerbils. An advantage of not exhibiting a decrease or impairment in exploratory behavior post-separation is that greater exploratory behavior may be more likely to lead to finding a new mate/partner. Whether gerbils form a new pair bond after partner separation more quickly than prairie voles is unknown.

Pair bond disruption has been shown to not only influence nonsocial behaviors but also different aspects of social behavior. Male prairie voles separated from a partner for 4 weeks were more affiliative and less aggressive with a novel, same-sex conspecific compared to paired males, suggesting that the stereotypical selective aggression associated with pair bonding decreased in males as the bond eroded over 4 weeks [41]. Here we failed to observe any significant influence of partner separation on prosocial, aggressive, investigative, or non-overt behaviors during a social interaction with a novel, same-sex conspecific. However, in our study, male gerbils, regardless of pair bond status, were less prosocial during the second round of testing. This effect could be due to experience with the test and/or reflect consequences of partner loss/social isolation (for separated males) and a deepening pair bond (for paired males) – both of which may result in a male gerbil being less affiliative with a strange male. Although previous studies have observed high levels of aggression in nonreproductive contexts in gerbils [13,30,35], we observed low degrees of aggression in the present study, mirroring previous findings from nonreproductive interactions in gerbils from our lab [9,17,20]. Notably, because the behavioral ecology of Mongolian gerbils is to be aggressive/territorial toward same-sex conspecifics, for ethical reasons we restrict nonreproductive social interactions in the lab to 10 min; it is possible that if the test were extended, we would observe more aggressive behavior. Interestingly, though, the same-sex interaction in male prairie voles from Sun et al. used a 10 min test [41], suggesting that prairie voles may be more territorial and/or exhibit more pronounced pair bond-induced selective aggression than gerbils.

Similar to behavior in a nonreproductive context, Sun et al. found that male prairie voles that had been separated from their partner were also less aggressive toward a novel, opposite-sex conspecific (i.e., a potential new mate) compared to paired males [41], again suggesting a decrease in selective aggression toward conspecifics as a bond erodes. Interestingly, in a similar study in prairie voles, we previously observed no behavioral differences toward a novel, opposite-sex conspecific in males that were paired and males that had been separated from their partner for 4 weeks [7]. In fact, in this study we failed to observe selective aggression in males and found that only female prairie voles exhibited selective aggression associated with pair bonding [7]. Whether these differences in male prairie vole reproductive behavior after partner separation is due to differences in labs/testing paradigms, natural variation, and/or genetic differences in source populations remains unknown. Notably, previous studies have demonstrated that there is substantial variation in the strength of pair bonds in prairie voles [44]. Conversely, while data in prairie voles suggests that males become more receptive to novel females after partner separation, a previous study using gerbils found that, compared to pair bonded males, males separated from a partner for 4 weeks exhibited less anogenital sniffing of a female [15]. Unfortunately, due to the language/writing of this paper, it is unclear whether the female stimulus animal was the former partner or a novel female. This result is contradictory to our observations in the present study, which showed that separated male gerbils exhibited more investigative, as well as prosocial, behavior with a novel female. Here we defined investigative behavior as sniffing the head, flank, *and* rear of the stimulus animal, which could potentially account for discrepancies between findings of the previous study and our findings, assuming the stimulus females in the Hendrie et al. study were novel to the male subjects. Further, the study by Hendrie et al. paired males and females for 5 weeks prior to bond disruption in comparison to our pairing of 14 days in the current study; it is feasible that partner separation may have a more substantial detrimental impact on males that had been bonded for a longer period of time. Indeed, studies in prairie voles have shown that the length of time after partner separation can significantly influence the brain and behavior [14,36]; notably, similar to our study, studies in voles also use a 4 week separation timeline demonstrating that 4 weeks of partner separation is sufficient to observe behavioral and neural changes associated with partner separation [7,14,36,41]. Alternatively, the male subjects in Hendrie et al. were vasectomized prior to the start of the study, and therefore differences in reproductive social behavior from our study may be due to very different circulating hormonal profiles. However, similar to prior findings in male prairie voles, our data suggest that male gerbils that have lost a partner are more investigative and prosocial with, and thus arguably more receptive to, a novel female and exhibit a behavioral repertoire that could potentially lead to the formation of a new pair bond.

Importantly, it is worth considering that behavioral consequences of partner separation could be due to social isolation instead of or in addition to the consequences of bond loss. Indeed, studies have demonstrated that female voles exhibit depressive-like behaviors when separated from same-sex siblings [10]. However, while male prairie voles display increased passive stress-coping when separated from a female pair bond partner, no such effect was observed when males were separated from a same-sex sibling, suggesting that, at least for male prairie voles, separation from a pair bond partner yields unique outcomes compared to social isolation in the absence of bond loss [2]. Consistent with this, social isolation from a same-sex sibling in male and female prairie voles also does not result in differences in aggressive behavior toward a novel, same-sex conspecific compared to voles that were housed with same-sex siblings [11]. Therefore, while social isolation may be stressful for socially monogamous species, isolation from a nonreproductive bond is distinct from separation from a reproductive bond as has been previously discussed (see [36]).

4.2. Effects of partner separation on PVN oxytocin and vasopressin

Although several studies have examined the impact of partner separation on behavior, fewer studies have specifically investigated consequences of bond disruption on nonapeptide expression and function. One of, if not the, first studies that examined the influence of partner separation on nonapeptide expression found that male prairie voles separated from their partners for 4 weeks exhibited more PVN OT neurons than males that remained paired with their pair bond partners [41]. This study concluded that partner separation increased PVN nonapeptide cell densities. As a follow up to this study, we recently conducted a similar study in prairie voles but added additional control groups such that prairie voles were either separated from a pair bond partner, separated from a same-sex sibling, remained co-housed with their pair bond partner, or remained co-housed with a same-sex sibling. With the non-pair bond control groups, we found that pair bonding decreases PVN OT cell densities, and that partner separation rescues this pair bond-induced decrease, returning the brain to a 'baseline' state. Specifically, male and female prairie voles that were separated from their pair bond partners exhibited similar PVN OT cell densities as voles that were separated from a sibling and co-housed with a sibling, whereas voles that were pair bonded had fewer PVN OT neurons compared to all other groups [7].

Although males separated from their pair bond partners exhibited more PVN OT cells in the two studies using prairie voles discussed above, here we found that pair bond status did not significantly influence PVN OT cell densities in male gerbils. Further, we did not observe an influence of partner separation on PVN OT-Fos colocalization, suggesting that partner separation also does not influence the responsiveness (measured via Fos) of this cell group to an interaction with a novel, opposite-sex conspecific. To our knowledge, no other studies have examined whether partner separation influences PVN OT neural responses to any type of social stimuli. However, we previously found that PVN OT-Fos colocalization positively correlates with aggression and negatively correlates with prosocial behavior in male and female gerbils in response to an interaction with a novel, *same*-sex conspecific [9]. This is consistent with our findings in the current study, such that PVN OT-Fos colocalization positively correlated with aggression during an interaction with a novel, *opposite*-sex conspecific. These findings suggest that PVN OT promotes aggression with novel conspecifics in gerbils. Therefore, we may not have observed an influence of partner separation on PVN OT neural responses in the current study because partner separation did not significantly impact male aggression in a reproductive (or nonreproductive) context. Lastly, because PVN OT has anxiolytic properties [23,45], higher PVN OT cell densities in partner separated prairie voles may reflect a stress-reducing impact of being pair bonded [8]. Unfortunately, studies have not been conducted in Mongolian gerbils to determine whether pair bonding incurs stress-buffering effects as has been observed for prairie voles. Further studies are required to determine if there are species differences in PVN OT social functions and/or whether pair bonding influences stress responses differently in gerbils and voles.

Although we did not observe an influence of partner separation on PVN OT neural densities or responses in male gerbils, we did find that males separated from their partners exhibited more PVN VP neurons compared to paired males, consistent with previous findings in prairie voles [41]. Because chronic stress increases PVN VP expression [16,25], it is feasible that separated male voles and gerbils exhibit higher densities of PVN VP compared to pair bonded males due to stress – either from social isolation and/or from partner separation. However, PVN VP neurons have diverse projections throughout the brain in addition to projections to the pituitary where they are classically known to modulate a stress response [33]. In our study, we did not observe any differences between paired and separated male gerbils in non-overt behaviors (i.e., stereotypies, autogrooming) during social interactions or exploratory/boldness behaviors in the open field test, suggesting that separated gerbils were at least not outwardly stressed in a manner that was detectable in our behavioral tests. In fact, the most notable behavioral difference between separated and paired gerbils was that separated males were more investigative and prosocial with a novel, opposite-sex conspecific (i.e., a potential new mate and/or future pair bond partner). Interestingly, regardless of pair bond status, we found that PVN VP cell densities positively correlated with investigative behavior during a reproductive social interaction, and males that had been separated from their partners exhibited greater numbers of PVN VP neurons. Together, these results suggest that PVN VP promotes investigative behavior in male gerbils, at least in a reproductive context, and that a bond-loss induced increase in PVN VP may help facilitate social behaviors that can lead to the formation of a new pair bond.

5. Conclusion

Here we examined how partner separation influenced the brain and behavior of male Mongolian gerbils. Partner separation did not globally influence multiple behaviors, and we did not observe differences in behavior between paired and separated males in nonreproductive social contexts or in an open field test. However, males that were separated from their partner exhibited more prosocial and more investigative behavior toward a novel female, suggesting that after 4 weeks of losing a partner, a male gerbil may be more amenable toward exploring a potential new mating opportunity. This increase in investigative behavior may be the result of an increase in PVN VP neurons, which positively relate to investigation of a novel, opposite-sex female. These results suggest that PVN VP neuronal densities may increase in male gerbils in response to partner separation, thereby facilitating investigative behavior of potential new mates to increase the likelihood of forming a new pair bond. Alternatively, an increase in PVN VP densities may also reflect the stress of social isolation and/or partner separation, however PVN VP-mediated investigative behavior could serve to alleviate such stress by driving an animal toward a social encounter to avoid isolation. Lastly, our study identified both similarities and differences in the consequences of partner separation on the brain and behavior of prairie voles and Mongolian gerbils – two socially monogamous species used for research examining neural mechanisms of pair bonding and bond disruption. These discrepancies in findings stress the importance of using a comparative approach to determine aspects of social bonds that may be generalizable across species and potentially hold translational insight for humans.

CRediT authorship contribution statement

Brandon A. Fricker: Writing – review & editing, Supervision, Methodology, Investigation, Conceptualization. **Jinrun Jiang:** Methodology, Investigation. **Christian Esquilin-Rodriguez:** Methodology, Investigation. **Mikala Dowling:** Investigation. **Aubrey Kelly:** Writing – original draft, Supervision, Project administration, Funding acquisition, Formal analysis, Data curation, Conceptualization

Declaration of Competing Interest

None.

Data availability

Data will be made available on request.

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