



Rewritable fidelity: How repeated pairings and age influence subsequent pair-bond formation in male prairie voles

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ABSTRACT

The prairie vole has proven a valuable animal model for the neurobiological study of social monogamy and pair bonding. Previous research has focused almost exclusively on virgin prairie voles forming pair-bonds for the first time – a paradigm with limited relevance to human social behavior. In the present study, we used stud males to assess the impact of repeated pair-bond formation and dissolution on the behaviors and neurobiology relevant to subsequent pair-bond formation. Stud males were tested for behavioral and neurobiological effects of repeated pair-bonding after the 1st, 5th, and 10th pairing. Aged breeder males that experienced minimal pair-bond dissolution were included to control for the effects of aging. Results showed that male prairie voles readily form new pair-bonds after repeated pair-bond dissolution. In terms of social monogamy, old age was associated with males spending less time in close social contact with unfamiliar females. There were no effects of age nor number of lifetime pairings on depressive-like behavior or paternal behavior toward pups. Within the brain, the patterns of oxytocin (OTR) and vasopressin type 1a (V1aR) receptors were largely unaffected, with the following exceptions: 1) males with only a single pairing had higher OTR densities in the paraventricular thalamus and bed nucleus of the stria terminalis; 2) there was an age-related increase in the density of OTR in the caudate putamen and an age-related decline in the density of V1aR in the cortical amygdala. The present findings have translational relevance to human social behavior in the context of aging and social experience.

1. Introduction

The neurobiology of social bonding has great public health relevance given the protective effects of stable social bonds on health outcomes, the prevalence of dysfunctional social behavior in psychiatric disorders, and the significance of social bonds in healthy human life. These phenomena have been extensively studied using a variety of animal models. The prairie vole (*Microtus ochrogaster*) is one such model organism, studied most often for its social monogamy – a trait shared with humans but rare among mammals (~5% (Carter and Perkeybile, 2018; Kleiman, 1977)). Among other neurotransmitter systems, the neuropeptides oxytocin (OXT) and vasopressin (AVP) have been found to be important for prairie vole pair-bonding (Carter, 2017; Tabbaa et al., 2016), and these findings have spurred similar research in other species, including humans (Feldman, 2017).

Experiences that influence the OXT and AVP systems, such as adverse treatment in early life, also have the ability to influence social bonding (Perkeybile and Bales, 2017; Perkeybile et al., 2018). Whereas

experiences in early life have received a great deal of research attention, we know relatively little about experiences in adulthood that could influence pair-bonding. In the wild, prairie voles experience significant mortality, with 36.8% of pair-bonded females experiencing pair-mate loss at some point (Getz and McGuire, 1993). After disruption of the original pair-bond, 80.6% of wild male prairie voles and 80.9% of wild females do not form another pair-bond (Carter et al., 1995; Getz and McGuire, 1993). Studies in both the field (Getz and McGuire, 1993) and lab (Thomas and Wolff, 2004) have found that females that lose their mate typically fail to re-pair and form a bond with another male due to their rejection of novel males. The purpose of this study was to investigate whether repeated pair-bond formation/dissolution affects behavior and/or neurophysiology in male prairie voles.

There are several potential factors at play when a male prairie vole is repeatedly forms new pair-bonds. First, there is the issue of the underlying neurobiology of learning and memory. If the pair-bond is analogous to most other memories, then it ought to show plasticity and be able to be re-assigned to a subsequent partner. On the other hand, if

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the pair-bond is more akin to other types of learning, such as imprinting on a caregiver (another oxytocin-dependent bonding process), or the highly emotional over-exaggerated learning of trauma, then the erasure of a pair-bond and subsequent re-writing of a new bond would likely be less successful. Secondly, the experience of pair-bond disruption and dissolution may introduce a high degree of stress and negative affect, the accumulating, compounding effects of which may influence subsequent behavior. Lastly, there is the potential for aging to influence social bonding in later life, either by adaptive changes in reproductive strategy or via maladaptive dysfunctions that accrue with age.

The neurobiology of prairie vole social bonding has been studied predominantly using virgin animals forming an initial pair-bond. However, the impact of prior experience has not been addressed. Many contemporary human relationships occur in the context of repeated pair-bonding experiences. In the present study, we used breeder males (multiple matings with one female) and stud males (multiple matings with multiple females) in order to investigate how repeated pair-bond formation and dissolution experiences affected subsequent pair-bonds. Males were tested for their tendency to form new pair-bonds after their 1st, 5th, or 10th pair-bonding experience. Alongside the testing of partner preference formation, we also assayed the males' affective state using measures of anxiety-like and depressive-like behavior, and investigated the distribution of OXT receptors (OTR) and AVP type 1a receptors (V1aR) within the brain.

2. Methods

2.1. Experimental design

For this study on the effects of repeated pair-bond formation and dissolution, we used stud and breeder males from our colony as a sample of convenience. All subjects were adult male prairie voles (*Microtus ochrogaster*), descendants of a wild-caught stock captured near Champaign, Illinois. Breeding pairs were housed in large polycarbonate cages (44 cm × 22 cm × 16 cm) and same sex offspring pairs were housed in smaller polycarbonate cages (27 cm × 16 cm × 16 cm) after weaning on postnatal day (PND) 20 (date of birth: PND0). Animals were given food (high-fiber Purina rabbit chow) and water ad libitum, cotton nestlets for nesting material in breeding cages, and were maintained on a 14:10 light:dark cycle. Behavioral testing took place between 12:00 and 15:00.

Subjects were aged 60–1036 days old, consisting of three groups of stud males and one group of aged breeder males with varying social experiences (see experimental design in Fig. 1). Stud males were repeatedly paired with novel females for the purpose of generating litters for other studies in our lab. A group of breeder males was included in

the present study to control for the confound of age that necessarily accompanied repeated pairings. Whereas breeder males remained with a single female mate and produced multiple litters, stud males were separated from their mate after each litter. Typically, pups were removed at weaning age (20 days), though in a minority of cases, pups were removed within the first 7 days postnatal. After weaning, stud males were separated from their mate and left isolated for at 1–2 weeks before pairing with a novel female. Behavioral observations of affect and tissue were collected at the conclusion of the 1 week of separation following either the 1st, 5th, or 10th pairing experiences. In the group of breeder males (“Aged”), subjects were re-paired with a novel female following 1 week of separation from their original mate, hence they were considered to have two total lifetime pairings. The approximate age ranges for males of each condition are as follows: 1st, 115–150 days; 5th, 375–425 days; 10th, 640–700 days; and Aged, 500–1036 days. All new pairings occurred with adult females aged 60–90 days old.

Social monogamy was examined via partner preference formation 24 h after pairing with a novel female using the Partner Preference Test (PPT). Paternal caregiving behavior was assessed in a partially overlapping set of stud males with 1–9 pairing experiences during postnatal days 1–3. To assess the socio-emotional effects of repeated pair-bond formation and dissolution, subjects were observed in an Open Field Test (OFT) for anxiety-like behavior and the Forced Swim Test (FST) for depressive-like behavior on subsequent days one week after separation from their mate. At the conclusion of behavioral testing, subjects were euthanized for tissue collection.

All subjects were given food (high-fiber Purina rabbit chow) and water ad libitum, and nesting material in breeding cages, and were maintained on a 14:10 light:dark cycle. All procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Indiana University Institutional Animal Care and Use Committee.

2.2. Partner Preference Test

Subjects were paired with an opposite sex conspecific (henceforth referred to as the Partner) and tested 24 h later in the PPT according to previously published methods (Kenkel and Carter, 2016; Williams et al., 1992). During the PPT, each subject was free-ranging while the Partner and a novel opposite sex, age-matched conspecific (henceforth referred to as the Stranger) were each tethered in separate but adjoining cages (Fig. 2A); testing lasted 3 h. Subject, Partner, and Stranger locations and movements were analyzed using idTracker (Perez-Escudero et al., 2014) and subsequently analyzed using custom Matlab code. Measures included total distance traveled and total time calmly spent in close

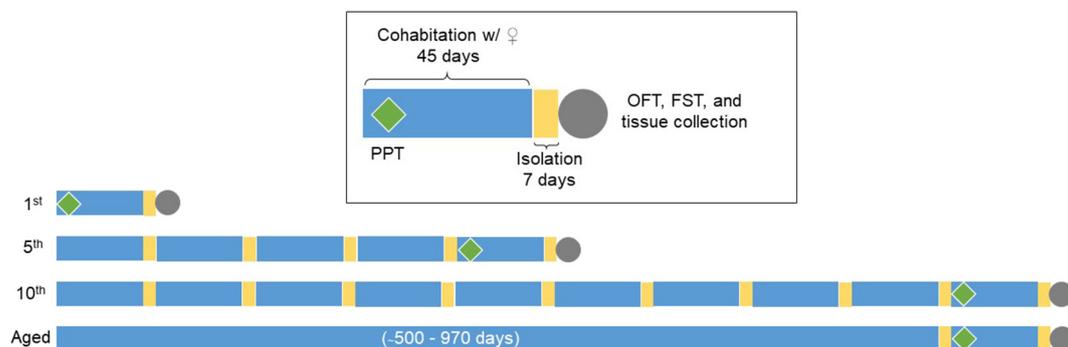


Fig. 1. Experimental design: adult male prairie voles were paired with novel adult females for the purpose of generating litters either 1 time, 5 times, 10 times, or left with the same female to continually produce litters (colony breeders of the ‘Aged’ group). Upon the dissolution of each pairing, males were kept isolated for seven days (yellow) before being re-paired with a new 60–90 day old female (blue). After either the 1st, 5th, 10th pairing, or 2nd pairing in the case of the Aged group, subjects were tested for partner preference in the Partner Preference Test (PPT, green diamond symbol). Following dissolution of the pair and isolation for seven days, subjects were tested for affective behaviors in the Open Field Test (OFT) and Forced Swim Test (FST) then euthanized for tissue collection (gray circle symbol). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

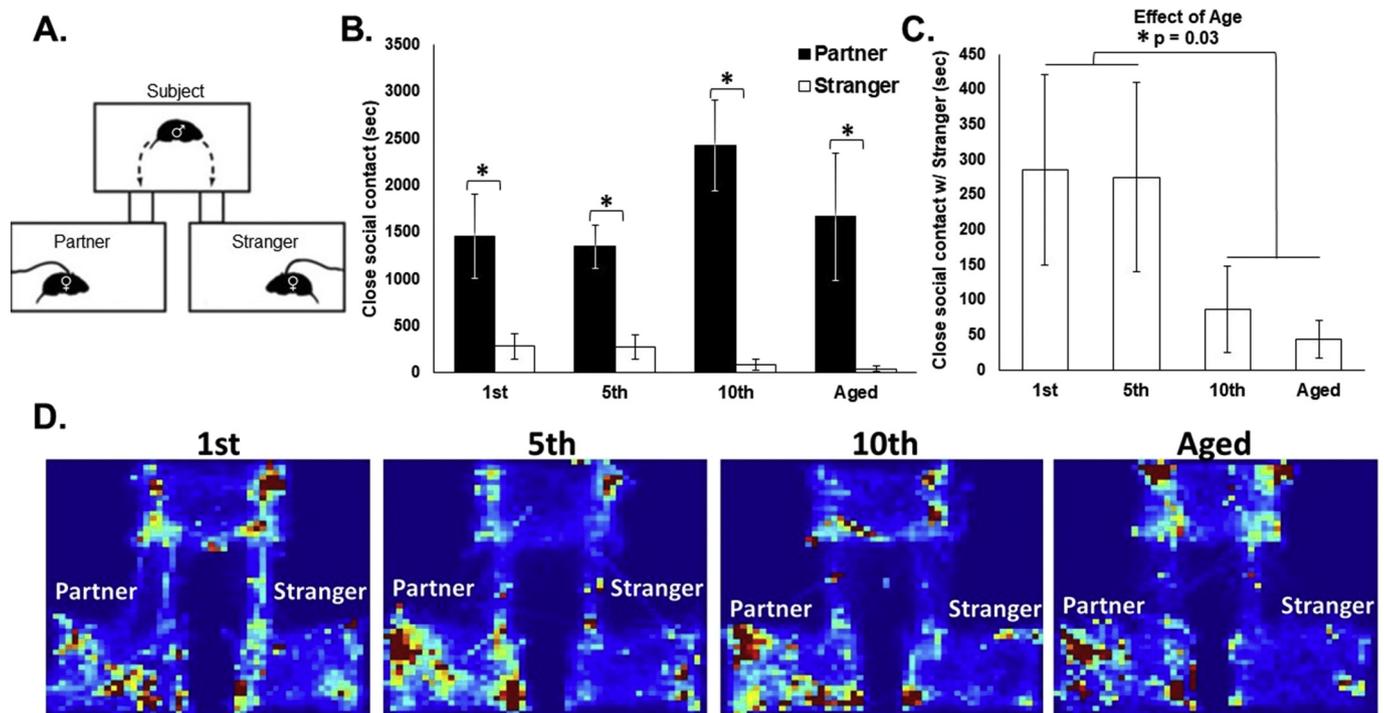


Fig. 2. The Partner Preference Test (PPT). A.) Schematic of the PPT featuring a free-moving Subject and tethered Partner and Stranger. B.) Males of all four conditions showed preferences for the familiar Partner over the novel Stranger, as evidenced by more time spent in close social contact (asterisks indicate $p < 0.05$ for all comparisons). For the purposes of visualization, heat maps are presented so that Partners are consistently on the left. C.) Older-age males (those of the 10th and Aged conditions) spent less time in close social contact with novel Stranger females ($F_{1,56} = 4.91, p = 0.03$). D.) Heatmaps showing the location of subjects during the 3-hour PPT, with warmer colors indicating increased time spent at that location.

social contact with either the Partner or Stranger. We used a combination of proximity and immobility (a relative lack of movement within 6 s) as parameters to approximate the traditional PPT measure of ‘side by side contact’. This approach correlated with hand-scored measures with $R = 0.978$. Sample sizes were as follows: 1st pairing: $n = 13$; 5th pairing: $n = 25$; 10th pairing: $n = 15$; Aged: $n = 7$.

2.3. Paternal caregiving

Subjects were observed in the natal nest in the presence of their mate for a morning and afternoon observation on two of the first three postnatal days. Subjects were observed for 20 min for each of the four observations and behaviors were scored by a trained observer using Behavior Tracker (www.behaviortracker.com) according to previously published methods (Perkeybile et al., 2013). Measures consisted of total time spent in/on the nest, huddling over pups, nest-building, licking and grooming of pups, retrieving pups, other non-huddling contact with pups, and auto-grooming. A total of 63 subjects were observed, ranging from 1 to 9 pairing experiences.

2.4. Open Field Test

The OFT was conducted according to previously published methods (Kenkel et al., 2017). Subjects were placed in one corner of a 40×40 cm arena and observed for 20 min. Subject location and movement was tracked using idTracker (Perez-Escudero et al., 2014) and subsequently analyzed using custom Matlab code. Measures consisted of total distance traveled and total time spent in the center of the arena, with the latter measure taken as indicative of a less anxious-like state. Sample sizes were as follows: 1st pairing: $n = 16$; 5th pairing: $n = 24$; 10th pairing: $n = 14$; Aged: $n = 14$.

2.5. Forced Swim Test

The FST was conducted according to previously published methods (Grippe et al., 2008). Subjects were placed in a clear, cylindrical plexiglas tank (diameter 20 cm) filled with tap water ($23\text{--}25^\circ\text{C}$) and observed for 5 min. Behaviors were video recorded and scored by trained observers using Behavior Tracker. Measures consisted of total time spent: swimming, clamoring at the wall, diving, and passively floating, with the latter measure taken as indicative of a depressive-like state. Sample sizes were as follows: 1st pairing: $n = 9$; 5th pairing: $n = 17$; 10th pairing: $n = 14$; Aged: $n = 9$.

2.6. Tissue collection and autoradiography

Within 24 h after completing the FST, subjects were euthanized for tissue collection, at which point brains were removed and immediately set on dry ice. Tissue was then stored at -80°C until processed. Autoradiographic analyses of OTR and V1aR densities were carried out according to previously published methods (Perkeybile et al., 2015). Briefly, brains were sectioned coronally at $20\ \mu\text{m}$ and mounted onto super-frost slides. For OTR binding [^{125}I]-ornithine vasotocin analog [^{125}I]OVTA [vasotocin, $\text{d}(\text{CH}_2)_5[\text{Tyr}(\text{Me})^2, \text{Thr}^4, \text{Orn}^8, (^{125}\text{I})\text{Tyr}^9\text{-NH}_2]$; 2200 Ci/mmol] was used (NEN Nuclear, Boston, MA, USA). For V1aR binding [^{125}I]-lin-vasopressin [^{125}I -phe-nylacetyl-D-Tyr(ME)-Phe-Gln-Asn-Arg-Pro-Arg-Tyr-NH₂] (NEN Nuclear) was used. Slides were exposed to Kodak BioMaxMR film (Kodak, Rochester, NY, USA) with ^{125}I microscale standards (American Radiolabeled Chemicals, Inc., St., Louis, MO, USA). Slides were exposed for 168 h for OTR binding and 96 h for V1aR binding.

Images of slides were digitally scanned at 1800dpi. Three images of each subject, matched for anterior-posterior position, were then registered to one another using Photoshop (Adobe Systems Inc., San Jose, CA). A measure of non-specific binding (NSB) was also taken for each section from a gray matter region where minimal binding is detected.

The NSB value was subtracted from the binding value for each section and a mean was then calculated for each section. Optical density was measured within each atlas-defined ROI using custom designed Matlab code and a slightly modified version of the prairie vole brain atlas we originally developed for magnetic resonance imaging (Yee et al., 2016). Results were calculated as regional averages of optical density (which corresponds to receptor density). Images were then consolidated into group composites for the purpose of visualizing group differences. Heat maps were generated in Matlab for each group-by-group comparison by subtracting one group composite from another.

2.7. Statistics

Number of pairings were considered as a continuous variable (1, 2, 5, or 10), while age was considered as a broad categorical variable, owing to incomplete records. Subjects in the 1st and 5th groups were considered ‘adult’ and subjects in the 10th and Aged groups were considered ‘old-age’. Thus, for the PPT, OFT, and FST, behavioral measures were compared using a two-way ANOVA (age and number of lifetime pairings). Post-hoc analyses were carried out using Tukey’s HSD testing. Because this work took place secondary to other efforts in our lab, a minority of subjects did not receive the full complement of testing described above, and thus sample sizes vary somewhat. These inconsistencies were randomly distributed throughout the dataset.

Autoradiographic analyses of OTR and V1aR densities were compared on a regional basis, first by bilaterally averaging each subject’s measures, then using a repeated measures linear mixed effects model, so as to accommodate the repeated sampling of each brain region (two slices). Outlying autoradiographic data points were detected and flagged automatically using Grubb’s test and then manually confirmed. Parental behavior was summed and compared to total number of lifetime pairings using a two-way ANOVA that included number of lifetime pairings and maternal behavior, although these observations occurred randomly throughout the subjects’ repeated pairings, as opposed to the set milestones shown in Fig. 1. All measures were collected either by objective, automated approaches (OFT, PPT, autoradiography) or by observers blind to group (FST, parental behavior). All statistical analyses were carried out in R.

3. Results

Prairie vole stud and breeder males from our colony were examined for the consequences of repeated pair-bond formation/dissolution experiences. In total, these data represent 48+ cumulative years of vole life. Social monogamy was measured via behavior in the PPT, which showed that male prairie voles retain the ability to form selective partner preferences with novel females up to at least 10 pairing experiences (Fig. 2). Males from all four conditions showed significant preferences for Partner over Stranger ($p < 0.05$; Fig. 2B). There was a main effect of age ($F_{1,56} = 4.91$, $\eta^2 = 0.029$, $p = 0.03$), such that males of the two older-age conditions, 10th and Aged, exhibited significantly less time in close social contact with the Stranger (Fig. 2C). There was a trend toward an effect of number of pairings on the amount of time subjects spent not near either Partner nor Subject ($p = 0.0782$), with more pairings leading to less time spent far from stimulus females. There was also a trend toward number of pairings leading to less total distance traveled in the PPT ($p = 0.0875$), largely driven by the Aged males traveling longer distances.

There was a significant effect of the number of pairings on amount of time spent in the center of the OFT ($F_{1,64} = 10.32$, $\eta^2 = 0.138$, $p = 0.002$), with post-hoc analyses revealing that Aged males spent significantly more time in the center as compared to males from the 5th ($d = 0.80$, $p = 0.026$) and 10th ($d = 0.84$, $p = 0.036$) pairing groups (Fig. 3A). However, there were no differences between males of the 1st pairing group and any other ($p > 0.05$ for all comparisons). In the FST, there were no differences in passive floating (Fig. 3B, $p > 0.05$ for all

comparisons). Likewise, we observed no relationship between number of pairings and paternal caregiving ($p = 0.76$). There was however a significant inverse relationship between paternal caregiving and maternal care ($R = -0.36$, $p = 0.022$).

Autoradiographic analyses of OTR and V1aR densities revealed several differences related to aging and pairing experience. In terms of OTR, within the bed nucleus of the stria terminalis (BNST), there was a significant main effect of the number of pairings ($F_{1,40} = 15.27$, $p = 0.0004$), such that more pairings led to less OTR density. Post-hoc analyses revealed that 1st pairing males had greater BNST OTR than all other groups (Fig. 4, $d = 1.03$ – 1.20 , $p < 0.01$ for all comparisons). Within the caudate putamen ($F_{1,40} = 5.92$, $p = 0.0196$) and core of the nucleus accumbens ($F_{1,41} = 5.72$, $p = 0.0215$), there were significant main effects of age on OTR density, with older-age males having higher densities than adult males. However only a single post-hoc comparison was significant (Aged males had greater OTR in the core of the nucleus accumbens compared to 5th pairing males, Fig. 5, $d = 0.80$, $p = 0.0483$). In terms of V1aR, there were main effects of the number of pairing on the cingulate cortex ($F_{1,33} = 5.13722$, $p = 0.03$) and paraventricular thalamus ($F_{1,33} = 4.10$, $p = 0.0511$), though no post-hoc analyses were significant. There was a significant main effect of age within the cortical amygdala ($F_{1,33} = 5.42$, $p = 0.0262$), such that 1st pairing males had greater V1aR density than Aged males ($d = 0.77$, $p = 0.0262$). There was also a similar trend in the paraventricular thalamus ($p = 0.0593$), with adult males having greater V1aR densities than old-age males. Lastly, there were interaction effects of age and pairings on V1aR density in the retrosplenial cortex ($F_{1,33} = 5.22$, $p = 0.0289$), such that 1st pairing males had greater V1aR density than Aged males ($d = 0.97$, $p = 0.048$), as well as on OTR density in the paraventricular thalamus ($F_{1,37} = 6.19$, $p = 0.0174$), such that 1st pairing males had greater OTR density than 5th pairing males ($d = 1.01$, $p = 0.0254$).

4. Discussion

Our results show that male prairie voles retain the ability to form new pair-bonds in the face of repeated pair-bond dissolution. Males of all conditions exhibited selective partner preferences, indicative of social monogamy. Aged males (sires from colony breeding pairs and stud males with 10 pairing experiences) spent less time in close social proximity with novel females. Aged breeder males showed less anxiety-like behavior in the OFT, while there were no effects of pairing experience nor age on depressive-like behavior. Total number of lifetime pairings had no effect on paternal behavior. Within the brain, the densities of OTR and V1aR were largely similar across groups, although males with a single pairing experience had higher OTR densities in the paraventricular thalamus and bed nucleus of the stria terminalis. There was also an age-related increase in the density of OTR in the caudate putamen and an age-related decline in the density of V1aR in the cortical amygdala. Increased number of lifetime pairings led to declines in OTR in the BNST and in V1aR within the paraventricular thalamus and cingulate cortex. The age-related change in OTR and V1aR densities may have contributed to the old-age males spending less time in close proximity to unfamiliar females. However, the neurobiological changes associated with the number of lifetime pairings did not match the observed patterns in behavior, which suggests the possibility of further, as of yet unexplored, behavioral differences related to either age or experience.

These findings have relevance to the behavioral ecology of prairie voles, human pair-bonding, and prairie vole husbandry/lab practice. By showing that male prairie voles retain the tendency to form new pair-bonds, we observe a contrast with females, that remain aggressive toward new males and thereby inhibit the formation of new pair-bonds (Thomas and Wolff, 2004). Previous work has shown that males retain a partner preference after two weeks of separation, but not after four (Sun et al., 2014). Male prairie voles retain old bonds and can evidently form

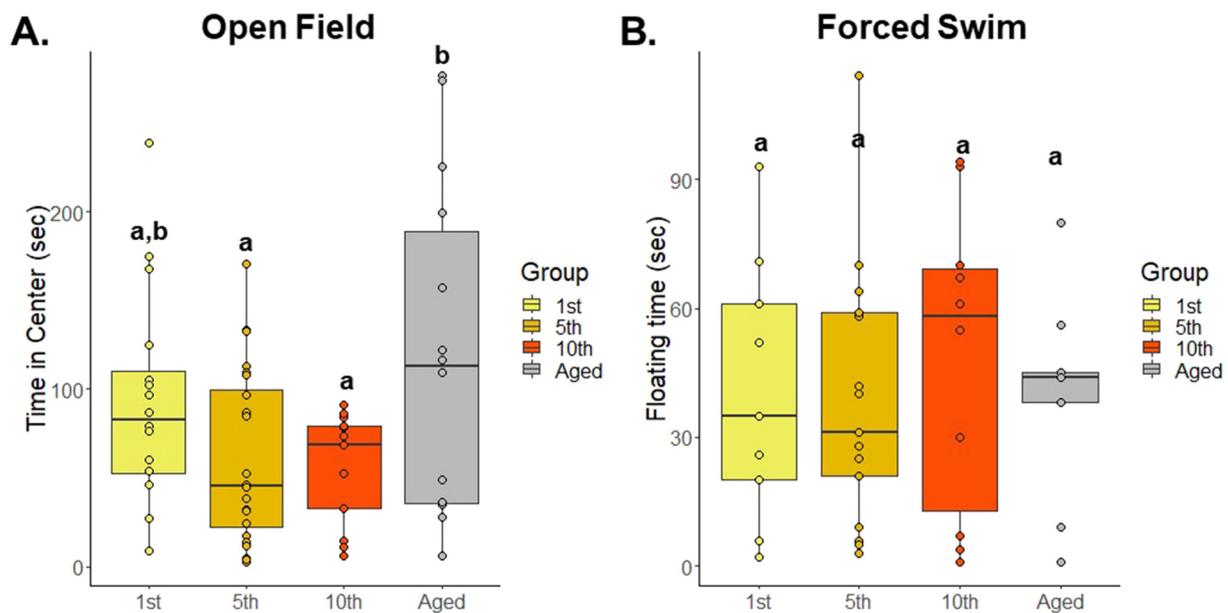


Fig. 3. Affective Behavior in the Open Field Test (OFT) and Forced Swim Test (FST). A.) Anxiety-like behavior is shown as less time in the center of the OFT. Anxiety-like behavior was greater in the males of the 5th and 10th pairing conditions as compared to those of the Aged condition (two-way ANOVA, $F_{1,64} = 10.32$, $p = 0.002$; post-hoc $p = 0.026$ and 0.036 , respectively; different letters denote significant differences). B.) Depressive-like behavior is shown as more time floating in the FST. There were no differences between conditions in terms of depressive-like behavior.

new pair-bonds, but we do not know if the formation of a new bond overwrites the previous.

In the wild, only 19% of adult prairie voles acquire a new pair-mate following dissolution of a previous pair-bond (Getz and McGuire, 1993). 63.6% of males that lose their pair-mates typically abandon the nest and adopt a wandering strategy (Getz and McGuire, 1993). This may relate to the observation that males survive longer if they adopt a single, wandering strategy as opposed to remaining within a pair (57.8 ± 10.2 days vs. 41.5 ± 1.5 days, respectively (Getz and McGuire, 1993)). At any given time, approximately 45% of males have adopted this wandering strategy (Getz and McGuire, 1993). In the monogamous pine vole (*Microtus pinetorum*), separation from an initial pair-mate for 0, 7, or 14 days does not affect subsequent reproduction in terms of producing offspring or re-pairing with a subsequent mate (Renfro et al., 2009).

Previous work has established that the loss of a partner induces negative affect in socially monogamous species (Pohl et al., 2018). In male prairie voles, the loss of a bonded partner induces an increase in basal plasma corticosterone and depressive-like behavior in the FST after a week of separation (Bosch et al., 2008). The loss of a partner also induces heightened anxiety-like behavior and exacerbated pain responsiveness in male prairie voles during the first week of separation (Osako et al., 2018). After four weeks of separation from a partner, males experience heightened anxiety-like and depressive like behavior, increased plasma corticosterone, and increased oxytocin and vasopressin immunoreactivity within the paraventricular nucleus of the hypothalamus (Sun et al., 2014). In the present study, males were isolated for 1 week prior to testing, and there were no males that did not undergo pair-bond dissolution/isolation. Thus, we do not know if pair-bond disruption was anxiogenic in the present work as it has been previously reported.

Previous work has also found that acute pair-bond dissolution decreases OTR in the nucleus accumbens (Bosch et al., 2016). In the present study, Aged males had high levels of OTR in the nucleus accumbens, and several Aged males had exceptionally high levels of OTR in the caudate putamen. Previous work has also identified a decline in OT synthesis in the paraventricular nucleus of the hypothalamus (Bosch et al., 2016). Because the current study focused exclusively on receptor

distribution, changes in ligand regulation cannot be excluded. Based on the accumulated behavioral and neurobiological evidence from this work in which all subjects experienced partner-loss, we can conclude that the tenth such experience seems similar to the first.

Cross-cultural comparisons reveal that within contemporary human societies, 70–80% of people re-marry following divorce (Fisher, 1989). In 1992, the National Health and Social Life Survey asked American adults about their sexual histories (Laumann, 2000). The plurality of respondents reported 2–10 lifetime sexual partners across age groups from 20 to 50 years of age. The proportion reporting 11+ lifetime sexual partners ranged from 8.3% in the 20-year-old sample, to 24.1% in the 50-year-old sample. According to the National Survey of Family Growth, as of 2015, the median number of lifetime sexual partners for men and women aged 25–44 years was 6.1 and 4.2 respectively; and 21.1% of men aged 25–44 reported having 15+ sexual partners over the course of their lifetime (CDC, 2017). It is likely that not all of these sexual partners reflect social bonds, however. Among American college students who engaged in uncommitted sexual encounters, 51% reported doing so in order to initiate a romantic relationship (Garcia and Reiber, 2008). Moreover, in a survey of 1042 American adults, 40% of men and 24% of women reported that they had experienced an uncommitted sexual encounter turning into a long-term committed romantic relationship (Garcia and Fisher, 2015). The translational implications of the present work suggest that repeated pair-bonding is unlikely to affect the formation of subsequent bonds if the neurobiology of pair-bonding is similar between humans and prairie voles. Although there is deep homology between transcriptomic profiles in the brains of monogamous vertebrates (Young et al., 2019), there is still a wide gulf in our understanding of the behavior of humans and rodents.

This work also has implications for animal husbandry and the use of voles in the lab as research subjects. Our lab initially began to use stud males due to their higher success at achieving impregnation. The present results suggest that the use of stud males does not introduce a compounding social stress beyond the disruption of the initial pair-bond. This work is also the first to utilize idTracker to automate the analysis of behavior in the PPT. idTracker is free, open-source software that reliably identifies individual animals without labeling. The use of automated procedures permitted this study, which was conducted using

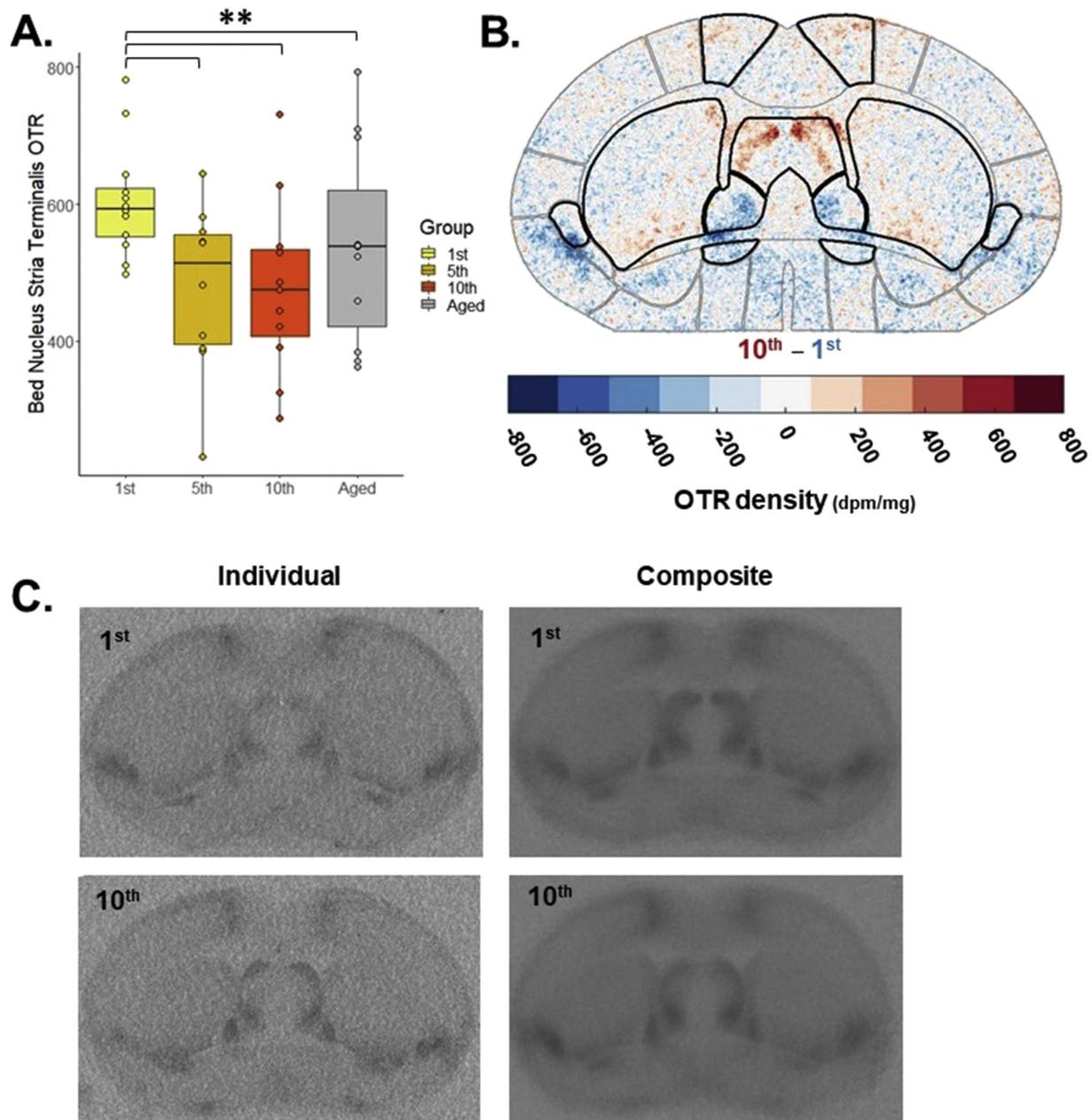


Fig. 4. OTR density at the level of the lateral septum and bed nucleus of the stria terminalis (BNST) measured in decays per minute/mg (dpm/mg). A.) There was a main effect of pairing on BNST OTR ($F_{1,39} = 17.31$, $p = 0.0002$) such that males of the 1st pairing condition showed higher OTR density relative to all other conditions (double asterisks indicate $p < 0.01$ for all comparisons). B.) A heatmap illustrating the difference between OTR density across the brains of males from the 1st and 10th pairing conditions. Blue colors represent regions where males of the 1st pairing condition had relatively greater OTR density, red colors represent regions where males of the 10th pairing condition had relatively greater OTR density. C.) Representative photomicrographs on the left and, on the right, group-wise composite images across a single slice for the 1st pairing (top, $n = 12$) and 10th pairing (bottom, $n = 11$) conditions. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

samples of convenience ancillary to our lab's primary studies. Using idTracker to capture voles' positions and Matlab to analyze voles' relative proximities and stillness, we were able to analyze 180 h of video observations with minimal human effort. Furthermore, this approach permits a far greater level of precision, an objective definition of close social proximity, greater potential for visualization of data, and the ability to retroactively re-analyze video, as we did in the current study when the heatmaps of subjects' activity suggested that males with fewer pairings might be spending more time away from both Partner and Stranger in the PPT. Similarly, the use of a semi-automated approach to quantifying autoradiography permits a broader examination of the brain – one that is atlas-based and has greater potential for data

visualization.

The prairie vole has proven very useful for the neurobiological study of social bonds. In order to continue using this animal model, we must make certain we understand the processes and constraints of social bonding. The present findings suggest male prairie voles are resilient to repeated pair-bond dissolution and readily form new bonds. Such findings are relevant to laboratory practices that include the use of stud males, field studies that take into account the high level of predation/mortality on prairie voles, and possibly also to contemporary human society, where repeated pair-bonding experiences have become increasingly common over the course of the last century.

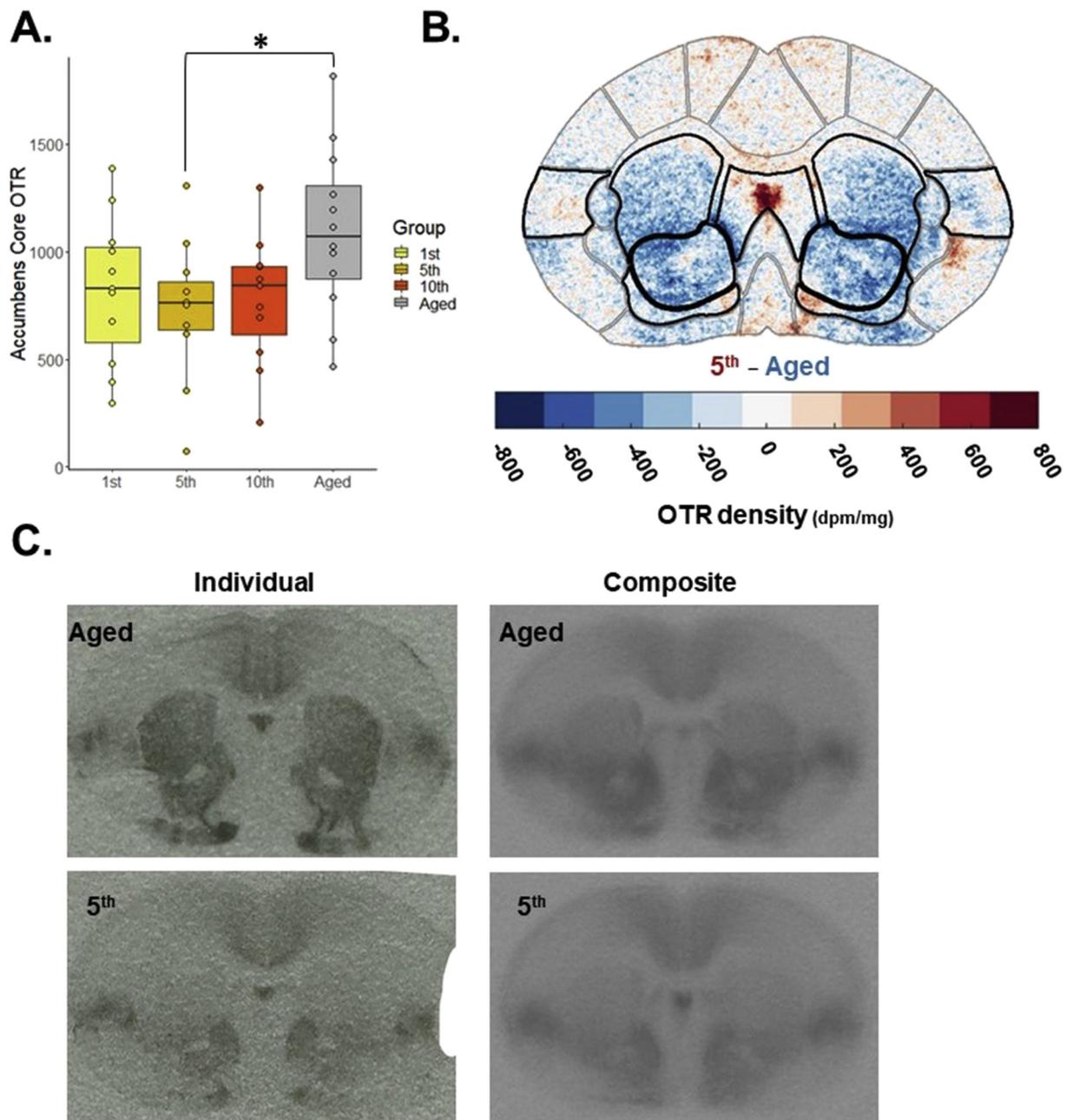


Fig. 5. OTR density at the level of the nucleus accumbens measured in decays per minute/mg (dpm/mg). A.) There was a main effect of age on OTR density within the core of the nucleus accumbens ($F_{1,41} = 5.72$, $p = 0.021$) such that males of the Aged condition showed higher OTR density relative to the 5th pairing condition (asterisk indicates $p < 0.05$). B.) A heatmap illustrating the difference between OTR density across the brains of males from the Aged and 5th pairing conditions. Blue colors represent regions where males of the Aged condition had relatively greater OTR density, red colors represent regions where males of the 5th pairing condition had relatively greater OTR density. C.) Representative photomicrographs on the left and, on the right, group-wise composite images across a single slice for the Aged (top, $n = 12$) and 5th pairing (bottom, $n = 10$) conditions. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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