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Neural mechanisms of grouping behavior

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**Abstract**

Neural mechanisms of grouping behavior
By Brandon A. Fricker

Highly social, group-living species have the ability to accomplish impressive feats. For many species, including humans, interactions with groups occur on a daily basis. Behaviors that may be exhibited during group interactions can range from affiliative to aggressive or cooperative to simply tolerant. For this dissertation, grouping behavior is broadly defined as any type of behavior that occurs in groups, focusing not only on the behavior of the group as a whole unit but also on individuals within the group. Although there are numerous species that live in large groups, we know surprisingly little about the neurobiological mechanisms underlying grouping behaviors in mammalian species. We can further our understanding of why and how animals display the dazzling array of grouping behaviors we observe in the wild by examining how neural mechanisms facilitates basic drives such as the preference to affiliate with large groups and the ability to exhibit social recognition. To study mammalian grouping behavior and relevant underlying neural mechanisms, I used the spiny mouse (*Acomys dimidiatus*). I have previously shown that spiny mice prefer affiliating in large groups, indiscriminately approach any conspecific, regardless of context, and exhibit high degrees of prosociality and low levels of aggression. In this dissertation, I examine how neural mechanisms facilitate the preference to affiliate with a large group as well as how these circuits allow an animal to distinguish between different types of conspecifics within a group. In Chapter 1 I use immediate early gene studies, neural tracing techniques, and circuit-specific chemogenetic manipulations to identify a neural circuit involved in the modulation of peer group size preferences. In Chapter 2 I then examine differences in spiny mice behavior with distinct types or conspecifics and neural processing of

kinship and familiarity during dyadic interactions. Together, this body of work provides new insight to a growing field on the neuroscience of grouping behavior.

Neural mechanisms of grouping behavior

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# General Introduction

Choosing an appropriate organism to answer specific scientific questions has been a valued approach in the animal behavior community for many decades. Indeed, scientists have not limited themselves to using only domesticated, laboratory rats and mice for experiments but include prairie voles[1] and zebra finches[2] for studying pairbonding, and African striped mice[3], and deer mice[4] for biparental care. While we know a fair amount about hormonal and neural mechanisms underlying flocking in birds[5,6], and a few recent studies have begun to examine the neural mechanisms underlying grouping behavior in fish and insects[7–9], we know surprisingly little about how neural mechanisms modulate mammalian grouping behavior. This is likely due to the lab tractability and access to neural manipulations offered by more common, but solitary or small group-living, rodent species, such as mice or rats. The result is literature focused on bonding between two individuals in reproductive contexts, such as parent-offspring or mating bonds[10–12]. Yet, prior studies indicate that reproductive and nonreproductive contexts are differentially processed in the brain[13], and thus we cannot assume that neural circuits underlying reproductive bonding will similarly regulate nonreproductive affiliation. To expand upon our understanding of the mechanisms underlying mammalian grouping behavior, we use the spiny mouse (*Acomys dimidiatus)* – a highly gregarious rodent that can be readily bred and maintained in the lab.

Spiny mice are a communally breeding rodent native to Africa, the Middle East, and southern Asia[14–16]. Prior to their use in behavioral neuroscience, spiny mice were primarily used in studies of obesity[17,18], tissue regeneration[19,20], and reproductive biology due to having a menstrual cycle[21,22]. However, they are especially attractive for grouping behavior research because they can be group housed with about 30 individuals in a lab setting[23], allowing for the

study of various grouping behaviors. Indeed, we have previously reported that spiny mice exhibit a preference to affiliate with large over small groups of conspecifics, are highly affiliative, and exhibit little aggression in multiple social contexts[24,25]. How the brain drives the preference to affiliate with larger peer groups has yet to be studied.

Unlike many other species that are tractable for lab studies, spiny mice readily accept unrelated newcomers into same- and mixed- sex established groups[23,26]. This is likely a reflection of their communal breeding system. While field studies that systematically characterize the behavioral ecology of spiny mice are still needed, most communally breeding systems have high rates of male dispersal with females typically representing the philopatric sex. Notably, there is variation in dispersal and philopatry across species[27], and thus group composition of sex and kin may vary in spiny mice in the wild, however, field studies are needed to determine natural variation in group composition. Given that established same- and mixed- sex groups of spiny mice will accept unrelated newcomers in the lab[26], it is possible that both males and females of this species may disperse from the natal home, albeit likely at varying rates, and are likely to be subsequently welcomed into a new group in the wild. Although field studies are needed to confirm dispersal and philopatry patterns in spiny mice, the highly social nature of spiny mouse groups in the lab lends this system to the study of variation in group dynamics and social networks. Because spiny mice naturally live in complex groups, they are also an excellent organism for exploring the importance of identity and social recognition in complex social environments. Early studies from the 1980s showed that, spiny mice use odor cues associated with nursing to identify kin[28,29] and exhibit more food sharing with kin[30], demonstrating the ability to distinguish kin from non-kin. While it's clear spiny mice can discriminate between

individuals, how that information influences behavior remains an open question. Further, neural mechanisms of social recognition in spiny mice remains unexplored.

In this dissertation, I present research that examines how neural circuits facilitate the preference to affiliate with a large group as well as how these circuits allow an animal to distinguish between different types of conspecifics within a group. Chapter 1 uses immediate early gene studies, neural tracing techniques, and circuit-specific chemogenetic manipulations to identify a neural circuit involved in the modulation of peer group size preferences. Chapter 2 then examines differences in spiny mice behavior with distinct types or conspecifics and neural processing of kinship and familiarity during dyadic interactions. Together, this body of work provides new insight to a growing field on the neuroscience of grouping behavior.

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# 1 CHAPTER I. Cingulate to septal circuitry facilitates the preference to affiliate with large peer groups.

This chapter takes the form of a manuscript prepared for submission to a journal for review.

## Summary

Despite the prevalence of large group-living across the animal kingdom, no studies have examined the neural mechanisms that make group living possible. Spiny mice, *Acomys dimidiatus*, evolved to live in large groups and exhibit a preference to affiliate with large over small groups. Here, we determine neural circuitry that facilitates the drive to affiliate with large groups. We first identify an anterior cingulate cortex (ACC) to the lateral septum (LS) circuit that is more responsive to large than small groups of novel, same-sex peers. Using chemogenetics, we then demonstrate that this circuit is necessary for both male and female group investigation preferences, but only for the males' preference to affiliate with larger peer groups over smaller ones. Further, inhibition of the ACC-LS circuit specifically impairs social, but not nonsocial, grouping preferences. These findings reveal a key circuit for the regulation of mammalian peer group affiliation.

**Cingulate to septal circuitry facilitates the preference to affiliate with large peer groups.**

## Introduction

For many species, including humans, environmental pressures have led to a high degree of sociality associated with living in large groups. Indeed, large group-living provides species with numerous advantages, such as collective traveling [1,2], reduced predation [3–5], enhanced offspring survival[6–8], and more effective homeostatic regulation[9,10] and thus has evolved numerous times across taxa, including in insect[11,12], avian[13,14], and mammalian species[15–17]. However, Spiny mice (*Acomys dimidiatus*) are one of the only lab tractable mammals in which it is possible to study the neural mechanisms of grouping behavior because they naturally evolved to live in large groups. Unlike other mammals often studied in the laboratory, spiny mice exhibit little aggression and are highly prosocial with conspecifics, regardless of novelty, familiarity, genetic relation, or reproductive/non-reproductive context[15,18,19]. Further, spiny mice exhibit a preference to affiliate with larger groups over smaller ones[15,19] and readily accept newcomers into established groups[20]. Spiny mice are thus an ideal model for inquiries about brain adaptations that arose to support the fundamental building blocks of prosociality and grouping behaviors that are precursors to even more complex social behaviors, including cooperation.

The lateral septum (LS) is emerging as a hub for numerous social behaviors potentially important for group living[21] . To date, it has been implicated in social recognition in rats[22,23] and spiny mice[18], as well as affiliative behaviors in voles and birds[13,24,25]. The LS receives inputs from several subcortical regions, positioning the LS to integrate sensory information critical for getting along with others in a group and then organize context-appropriate social output[26]. Socially-relevant information related to sociality may originate in cortical regions; recent studies

examining group social communication between familiar bats revealed unique neural representations for specific individuals within the frontal cortex[27], and other studies have demonstrated a role for the frontal cortex, specifically the anterior cingulate cortex (ACC), in complex, prosocial behaviors that likely facilitate group-living, such as consolation[28]. Further, the evolution of highly prosocial phenotypes in group-living species may have not only been associated with the development of circuits that enhance prosocial responsiveness, but also circuits that inhibit the aggression many animals typically show towards conspecifics in several, if not most contexts, which the LS is also poised to do. Specifically, the primarily GABAergic[26,29] LS may inhibit activity in downstream, aggression-promoting regions of the brain. LS circuitry could thereby allow species like spiny mice to engage in prosocial interactions with large groups of conspecifics, regardless of their novelty, relatedness, or the reproductive/non-reproductive context.

Here, we contribute novel insights to the field of social neuroscience by moving beyond the delineation of circuits that mediate traditional dyadic interactions and affiliative preferences between two individuals. Specifically, we identify neural circuitry that modulates the drive to affiliate with large groups of novel peers in a nonreproductive context in male and female spiny mice. Using a combination of neural tracing techniques and immediate early gene (IEG) studies, we first identify a circuit that potentially mediates peer grouping preferences – neuronal projections from the ACC to the LS. Next, we use chemogenetics to demonstrate that this circuit directly modulates spiny mice affiliative and investigative preferences towards larger peer groups compared to smaller ones. Further, additional tracing and IEG studies reveal that the LS may suppress aggressive behavior with novel, same-sex conspecifics via action in the lateral hypothalamus, thereby enabling cohesive peer groups. Together, our data demonstrate, for the

first time in a mammal that, like humans, evolved to live in groups, that the ACC-LS circuit is an integral mediator of peer group affiliation responses that are likely critical for the formation and possibly cohesion of complex mammalian societies.

## Results

*The LS differentially responds to group size.*

We first aimed to identify whether the LS differentially responds to exposure to small versus large groups. Additionally, we also examined two other brain regions that have been previously implicated in a variety of social behaviors – the medial preoptic area (mPOA; crucial for parental care[30,31]) and the BNST (facilitates grouping in birds and social vigilance in California mice[32–34]). We performed an IEG study in which six male spiny mice were exposed to a single novel, same-sex conspecific and another six to a group of seven novel same-sex conspecifics for 30 min, followed by an additional 30 min of isolation. Using immunohistochemistry (IHC), we quantified the number of Fos-ir+ cells for each subject in the mPOA, BNST, and LS.

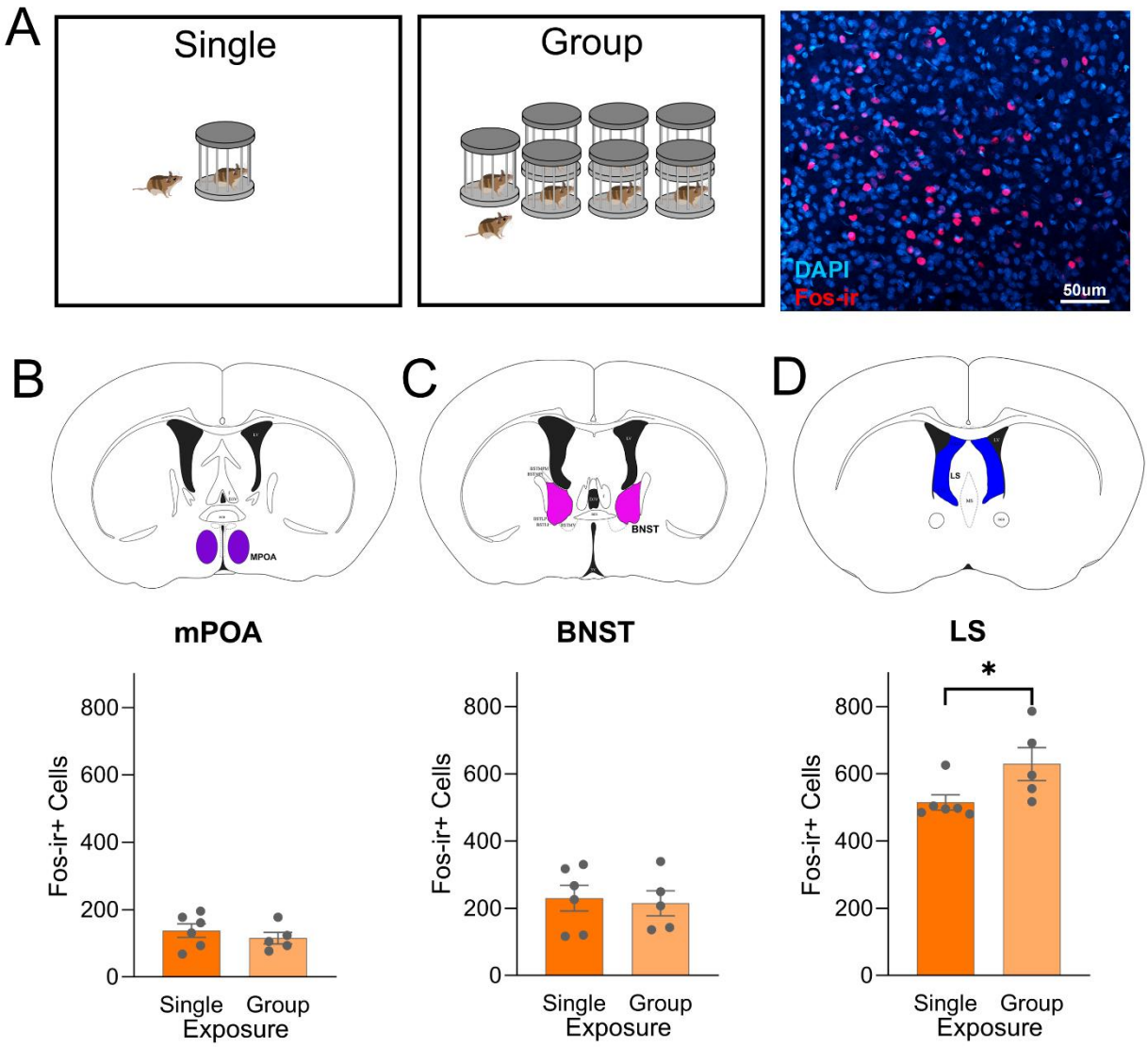



**Figure 1-1. The lateral septum differentially responds to group size.**

(A) Left, illustration of single exposure condition for group size preference in the IEG study. Middle, illustration of group exposure condition for group size exposure IEG study. Right, representative histological image depicting DAPI nuclear stain (blue) and Fos-ir (red) staining within the LS.
(B)Top, schematic depicting mPOA region used for analysis. Bottom, mPOA showed no difference in Fos-ir+ cells based on group size exposure, independent t-test, $p = 0.429$.
(C) Top, schematic depicting BNST region used for analysis. Bottom, BNST showed no difference in Fos-ir+ cells based on group size exposure, independent t-test, $p = 0.793$.
(D) Top, schematic depicting LS region used for analysis. Bottom, LS had significantly more Fos-ir+ cells when subjects were exposed to a large group compared to a single novel, same-sex conspecific. Independent t-test, $p = 0.050$ after multiple comparison corrections.
Data represented as mean ± SEM.

For both the mPOA ($t_{(9)} = -0.828$, $p = 0.429$) and BNST ($t_{(9)} = -0.270$, $p = 0.793$), there were no differences in the number of Fos-ir+ cells between the single exposure and group exposure

conditions (**Figure 1A,B**). However, for the LS, there was a significant difference between exposure conditions such that spiny mice exposed to the group exhibited significantly more Fos-ir+ cells than those exposed to a single conspecific ($t_{(9)} = -2.267$, $p = 0.05$; **Figure 1C**). This initial IEG study identified the LS as a region that differentially responds based on peer group size and, therefore, might play a role in regulating peer group preference in spiny mice.

*LS-projecting neurons within the ACC differentially respond to peer group size.*

We next sought to identify regions upstream of the LS that may be differentially modulated by exposure to large groups compared to a single novel conspecific. To identify such regions, we repeated the IEG study design above with the addition of retrograde tracing in 12 male and 12 female spiny mice. Seven days prior to the IEG study, we performed an intracranial injection of red Lumafluor retrobeads into the LS of one hemisphere (**Figure 2A**). The use of retrobeads allowed for colocalizing Fos-ir expression in cell bodies of neurons projecting to the LS. This co-labeling enabled us to calculate a percentage of LS-projecting neurons that were Fos-ir+ in a given region (**Figure 2C**). With Exposure Type (single conspecific or peer group) and Sex as fixed factors, we analyzed the percentage of retrobead labeled neurons that were Fos-ir+ as well as the average number of Fos-ir+ neurons in several regions associated with social behavior in other rodents. Brains were examined for retrobead labeling from the olfactory bulb through the ventral tegmental area (VTA). Brain regions that expressed robust retrobead labeling and are well-known to modulate aspects of social behavior were selected for analyses. The brain regions analyzed include the anterior cingulate c ACC (implicated in consolation behavior and contains emotional mirror neurons[28,36]), the anterior olfactory nucleus (AON; critical for social recognition[37] and social odor cue processing[38]), the basolateral amygdala (BLA; responds to

social novelty[39]), the ventral tegmental (VTA; regulates reward[40] and aggression[29]) and the piriform cortex (PC; involved in processing of social odor cues[41]). Due to differences in the number of retrobead labeled neurons across all sections of a brain region, analyses were conducted on both rostral and caudal portions of each brain region separately. Additionally, we analyzed the percentage of retrobead labeled neurons that were Fos-ir+ across all tissue sections of a region. For Fos-ir expression, only an average across the region was quantified.

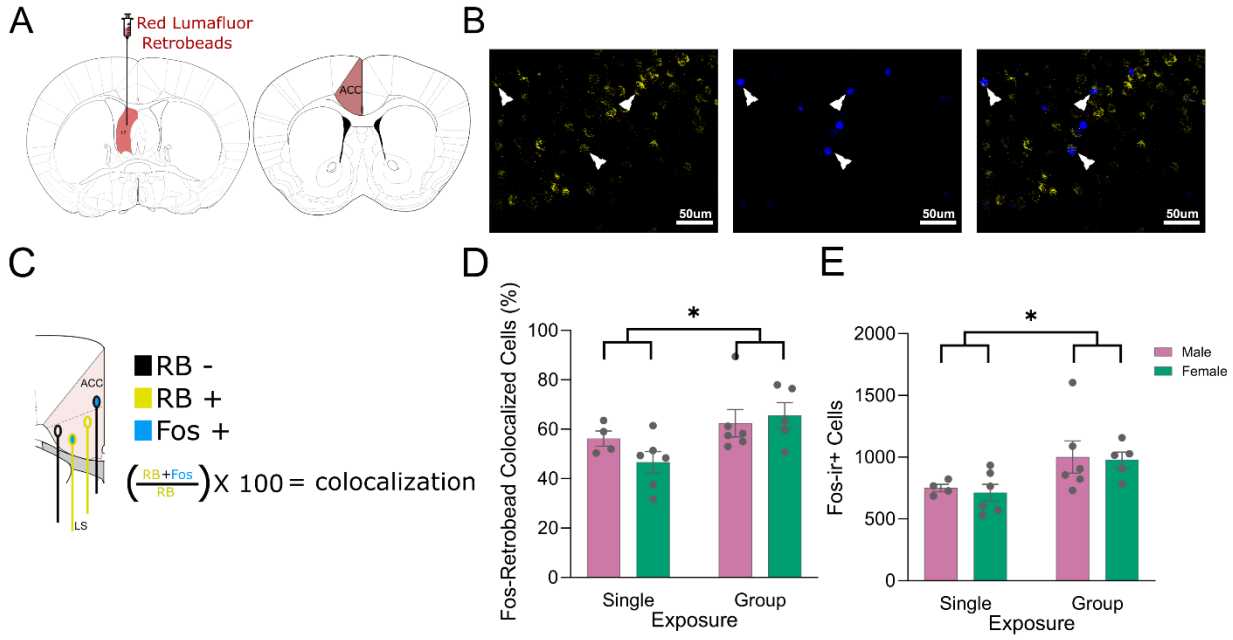



**Figure 1-2. Lateral septum-projecting neurons within the anterior cingulate cortex differentially respond to group size.**
(A) Left, schematic of red retrobead injection location within the LS. Right, example schematic of area considered ACC for cell counting.
(B) Representative histological images depicting retrobead (left; pseudocolored yellow), Fos-ir (middle; blue), and retrobead-Fos colocalization (right) staining within the ACC. White arrows indicate retrobead-Fos colocalized cells.
(C) Left, schematic depicting possible combinations of retrobead positive or negative and Fos positive or negative neurons. Right, formula depicting how figure 2D values were calculated.
(D) The rostral ACC showed a greater percentage of retrobead-labeled neurons colocalized with Fos-ir in subjects exposed to a large group compared to a single novel, same-sex conspecific for both sexes. GLM with Exposure Type and Sex as fixed factors. Sex $p = 0.416$, Exposure Type $p = 0.020$, interaction $p = 0.210$.
(E) The average of all ACC tissue sections quantified showed significantly higher Fos-ir expression in subjects exposed to a large group compared to a single, same-sex conspecific for both sexes. GLM with Exposure Type and Sex as fixed factors. Sex $p = 0.566$, Exposure Type $p = .028$, interaction $= 0.305$.

Data represented as mean ± SEM.

For the AON and PC (**Figure S2**), analyses yielded no differences nor any interactions for the percentage of retrobead-labeled neurons that were Fos-ir+ as well as the average number of Fos-ir+ neurons (All $p > 0.057$; **Table S1 and S1**). However, analysis of the rostral ACC yielded a significant difference across Exposure Type for the percentage of retrobead-labeled neurons that were Fos-ir+ ($F_{(1, 21)} = 6.529$, $p = 0.02$). Similarly, the average of Fos-ir expression across all ACC sections significantly differed ($F_{(1,21)} = 5.791$, $p = 0.028$), such that both the percentage of retrobead-labeled neurons that were Fos-ir+ and the average Fos-ir expression across all ACC sections were higher for the group exposure condition compared to the single exposure condition (**Figure 2D**). We observed no effects or interactions with Sex for retrobead-Fos colocalization or Fos-ir expression in the ACC (all $p > 0.527$). Although there were no effects of Exposure Type or Sex observed for analyses of the BLA or VTA, we found a significant interaction between Exposure Type and Sex for BLA retrobead-Fos colocalization averaged across all sections quantified($F_{(1,21)} = 4.610$, $p = 0.045$) and for rostral VTA retrobead-Fos colocalization ($F_{(1,19)} = 5.113$, $p = 0.031$; **Figure S1**, **Table S1**); however, *post-hoc* analyses with corrections did not yield any significant differences (all $p > 0.097$). These results suggest that peer group size is processed in ACC neurons that project to the LS.

*Inhibition of the ACC-LS reverses large peer group preference in male, but not female, spiny mice.*

The IEG study paired with retrograde tracing identified a circuit – the ACC-LS – that responded to variation in group size. Thus, we hypothesized that the ACC-LS circuit facilitates the preference to affiliate with large groups in spiny mice. To test this hypothesis, we utilized cre-dependent inhibitory chemogenetics to determine the direct contribution of this circuit to peer group size preference.

To confirm that activation of cre-dependent inhibitory designer receptors exclusively activated by designer drugs (DREADDs) within the ACC decreases neural activity, we first ran an initial between-subjects validation study in eight male spiny mice. Half of the subjects received a uni-lateral intracranial injection of cre AAVs into the LS and an injection of the cre-dependent DREADD Dio-hM4Di into the ACC, ensuring that only ACC neurons that project to the LS will express DREADDs. The other half of subjects received the same cre injection within the LS and an mCherry AAV into the ACC (**Figure S2A**), serving as a viral control group. After five weeks of incubation, all subjects received an intraperitoneal (IP) injection of 10mg/kg Clozapine N-oxide (CNO) 30 min prior to a 30 min social interaction test with a novel, same-sex conspecific. This social interaction was followed by an additional 30 min of isolation and then perfusion to capture Fos responses to exposure to the novel, same-sex conspecific after CNO injection. Brain tissue from each subject underwent IHC to visualize colocalization of Fos-ir with Dio-hM4Di or mCherry cell body expression (**Figure S2B**). Analysis revealed a significant difference between groups ($t_{(22)} = 6.617$, $p < 0.001$) such that the Dio-hM4Di condition ($M = 23.352$) had a significantly lower percentage of colocalized neurons than the mCherry condition ($M = 83.305$;

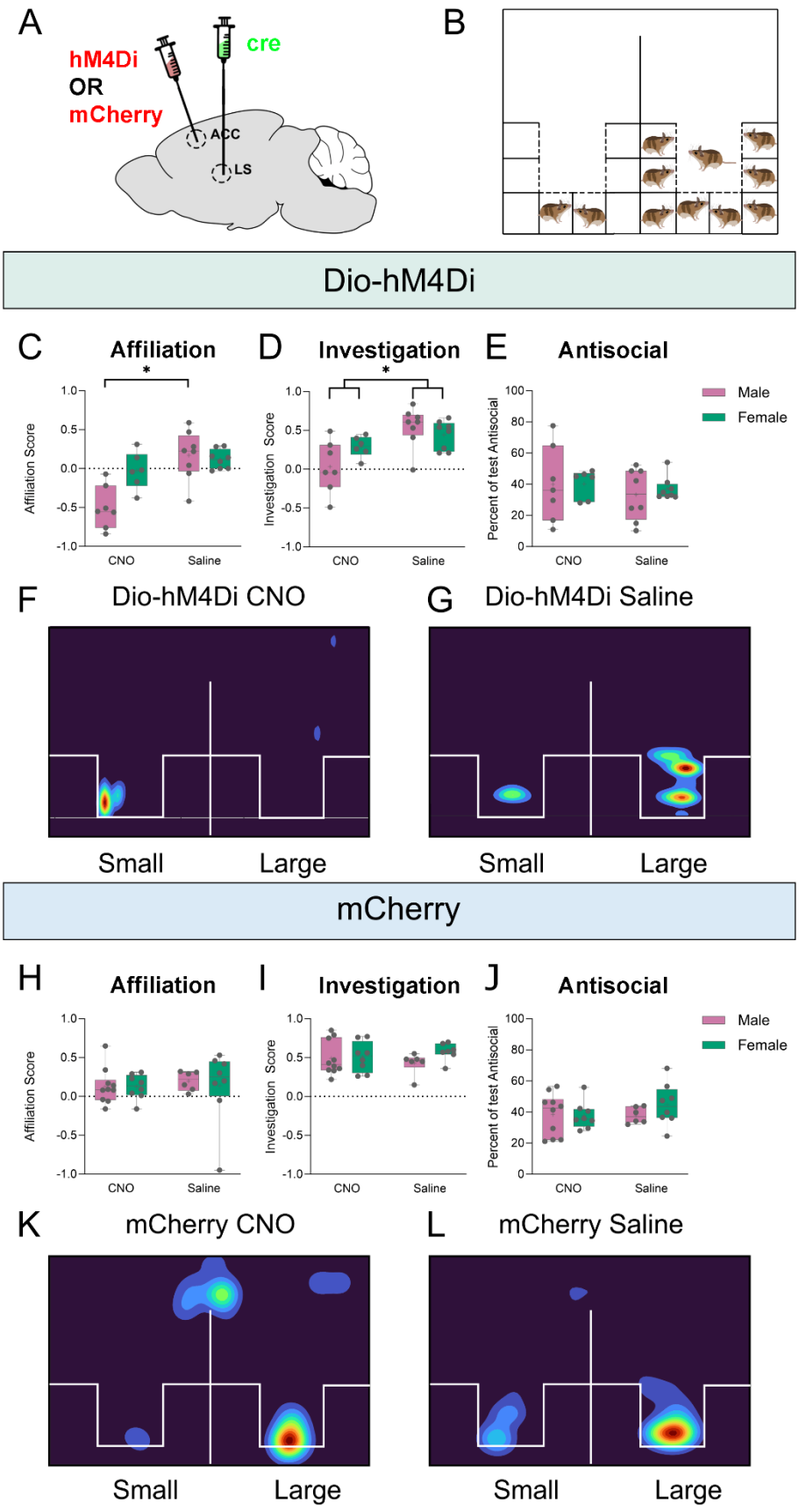
MD = 58.953), confirming for the first time the efficacy of cre-dependent inhibitory DREADDs in spiny mice (**Figure S2C)**.

In previous studies examining group size preference in spiny mice, we used a single-chambered apparatus that allowed subjects to view both the large and small groups simultaneously [15,19]. To force more of a choice on subjects, we created a new, modified testing chamber, which required subjects to enter two distinct subchambers to access the stimulus groups. Subjects had physical access to stimulus animals via 0.312 cm diameter holes in the subchamber walls. We utilized the validated cre-dependent inhibitory DREADDs to inhibit the ACC-LS circuitry during this modified peer group size preference test to obtain causal evidence of the circuit's contribution to behavior when an animal has a choice to investigate and affiliate with a large or small group of novel peers. Thirty-two male and 32 female spiny mice were randomly assigned to one of four conditions: (1) Dio-hM4Di + saline, (2) Dio-hM4Di + CNO, (3) mCherry + saline, or (4) mCherry + CNO. Subjects underwent bi-lateral intracranial injections in which cre AAVs were injected into the LS for all subjects, and half of the subjects received an injection of cre-dependent Dio-hM4Di AAVs into the ACC, whereas the other half received an injection of an mCherry AAV into the ACC (**Figure 3A**). Five weeks after the intracranial injections, all subjects underwent two behavioral tests with an hour in between each test – a social peer group size preference test and a nonsocial group size preference test; the order of tests was counter-balanced across all four groups. For the peer group size preference test, one subchamber had two novel, same-sex conspecifics and the other had eight novel, same-sex conspecifics (**Figure 3B**). All stimulus animals were unrelated to the subject. During the nonsocial group size preference test, however, the conspecifics were replaced with novel, spiny mouse-sized rubber ducks (**Figure 4B**). 30 minutes prior to the first test, half of the Dio-hM4Di and mCherry subjects received an

IP injection of saline, whereas the other half received CNO. For behavioral scoring, we quantified the amount of time each subject spent near each group (henceforth referred to as affiliation) and investigating each group. We divided the chamber into four quadrants (**Figure S4A**), with the quadrants closest to each group considered as "near" the group (i.e., affiliation), whereas investigation was considered as pressing one's nose up to a chamber containing a stimulus animal.

We previously showed that both male and female spiny mice preferentially investigate and affiliate with a large peer group over a small peer group in a group size preference test, reflecting their behavioral ecology to live in large groups in the wild[15,19]. To validate that this preference was retained in our new dual-chambered apparatus, we compared the time all control subjects (i.e., all subjects excluding the hM4Di+CNO condition) affiliated with and investigated the large group compared to the small group of novel, same-sex conspecifics. There was a significant effect of Group for affiliation ($F_{(1,92)} = 26.755$, $p < 0.001$) and investigation ($F_{(1, 92)} = 93.457$, $p < 0.001$) such that, for both sexes, spiny mice spent more time affiliating with (MD = 59.893, $p < 0.001$) and investigating (MD = 66.468, $p < 0.001$) the large group of peers compared to the small group (**Figure S4B,D**). For investigation, there was also a significant effect of sex ($F_{(1, 92)} = 4.823$, $p = 0.031$) such that females investigated groups less than males (MD = 15.100, $p = 0.031$; **Figure S4D**). These results provide further support for the observation that spiny mice

prefer larger peer groups and show that our new testing chamber does not significantly impact group preference.


A
hM4Di
OR
mCherry
cre
ACC
LS
B
Dio-hM4Di
C
Affiliation
Affiliation Score
CNO
Saline
D
Investigation
Investigation Score
E
Antisocial
Percent of test Antisocial
Male
Female
F
Dio-hM4Di CNO
G
Dio-hM4Di Saline
Small
Large
mCherry
H
I
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mCherry CNO
L
mCherry Saline

**Figure 1-3. The anterior cingulate cortex to lateral septum circuit modulates social peer group size preference in a sex-specific manner.**
(A) Schematic of cre (LS) and hM4Di (ACC) AAV injection locations.
(B) Schematic depicting a top-down view of the group size preference testing during a social peer group size preference test.
(C) For Dio-hM4Di subjects, CNO-induced inhibition of ACC-LS circuitry reversed the affiliative preference for larger social peer groups in male, but not female, spiny mice. GLM with Injection Type (CNO or saline) and Sex (male or female) as fixed factors. Sex $p = 0.032$, Injection Type $p < 0.001$, interaction $p = 0.009$. Female-injection interaction $p = 0.298$, male-injection interaction $p < 0.001$.
(D) For Dio-hM4Di subjects, CNO-induced inhibition of ACC-LS circuitry resulted in less investigation of the large group for male and female spiny mice. GLM with Injection Type (CNO or saline) and Sex (male or female) as fixed factors. Sex $p = 0.395$, Injection Type $p = 0.001$, interaction $p = 0.057$.
(E) For Dio-hM4Di subjects, CNO-induced inhibition of ACC-LS circuitry did not affect the percentage of test time spent away from either group (i.e., antisocial behavior). GLM with Injection Type (CNO or saline) and Sex (male or female) as fixed factors, $p = 0.427$.
(F),(G) Representative heatmaps of male subject location during the social peer group size preference test for the Dio-hM4Di + CNO and Dio-hM4Di + saline Conditions.
(H) For mCherry subjects, CNO administration did not affect affiliative preference. GLM with Injection Type (CNO or saline) and Ssex (male or female) as fixed factors, all $p > 0.092$.
(I) For mCherry subjects, CNO administration did not affect investigative preference. GLM with Injection Type (CNO or saline) and Sex (male or female) as fixed factors, all $p > 0.232$.
(J) For mCherry subjects, CNO administration did not affect the percentage of test time spent away from either group (i.e., antisocial behavior). GLM with Injection Type (CNO or saline) and Sex (male or female) as fixed factors, all $p < 0.415$.
(K),(L) Representative heatmaps of male subject location during the social peer group size preference test for mCherry + CNO, and mCherry + saline conditions.
Data represented as box plots with a median line and mean +.

To determine whether neural circuits influence behavior specifically in a social context, we also examined spiny mouse group size preferences for rubber ducks (i.e., a nonsocial context). For all control subjects (i.e., all subjects excluding the hM4Di+CNO condition), males and females did not significantly affiliate with one group of ducks more than the other ($F_{(1,92)} = 0.088$, $p = 0.768$), but did investigate the large group of ducks more than the small group of ducks ($F_{(1,92)} = 56.159$, MD = 30.349, $p < 0.001$; **Figure S4C,E).** This suggests that spiny mice have a preference to investigate more of an object, social or nonsocial.

To test the hypothesis that the ACC-LS circuit facilitates the preference to affiliate with large groups in spiny mice, we compared three normalized behavioral scores across the Dio-hM4Di

and mCherry conditions using the group size preference tests just described: (1) An affiliation score, which was based on all time spent in close proximity to the large group compared to the small group, (2) an investigation score, which was based on the time spent investigating the large group compared to the small group, and (3) an antisocial score, which was the percentage of the test the subject spent in one of the two quadrants not near a group. Positive affiliation and investigation scores reflect more affiliation/investigation with the large group, whereas negative scores reflect more affiliation/investigation with the small group. While investigation is likely a key component of affiliation, we separated these measures for analyses in this experiment to better detect if any changes associated with circuit inhibition were due to investigation specifically or preference altogether.

In the peer group size preference test, the mCherry condition did not differ across Injection Type or Sex (All $p < 0.092$; **Figure 3H-L**). However, for the Dio-hM4Di conditions, there was an effect of Injection Type for investigation score ($F_{(1,25)} = 13.670$, $p = 0.001$, $\eta_p^2 = 0.354$) and affiliation score ($F_{(1,25)} = 19.089$, $p < 0.001$, $\eta_p^2 = 0.433$), and an effect of Sex for affiliation score ($F_{(1,25)} = 5.149$, $p = 0.032$, $\eta_p^2 = 0.171$). There was also a significant interaction between Injection Type and Sex for affiliation score ($F_{(1,25)} = 8.031$, $p = 0.009$, $\eta_p^2 = 0.243$), and a trend for investigation score ($F_{(1,25)} = 3.982$, $p = 0.057$, $\eta_p^2 = 0.137$). Post-hoc analysis revealed that, while CNO administration reversed investigation preference for both sexes compared to saline administration (MD = 0.336, $p = 0.001$), CNO reversed the affiliation score for male (MD = 0.672, $p <0.001$) but not female (MD = 0.143, $p = 0.298$) spiny mice (**Figure 3C-G**). The antisocial score was not significantly different across injection types for the mCherry ($F_{(1,25)} = 0.687$, $p = 0.415$) or Dio-hM4Di ($F_{(1,25)} = 0.651$, $p = 0.427$) conditions. For both the Dio-hM4Di (all $p > 0.104$) and the mCherry (all $p > 0.228$) there were no effects of Injection Type nor any

interactions between Injection Type and Sex on the velocity of subjects during the behavior testing. Together, these findings indicate that activation of the ACC-LS circuit in male spiny mice is necessary for their ethologically-relevant preference to affiliate with a large peer group. Furthermore, inactivation of this circuit did not induce a preference to be antisocial, but instead affected social preferences in a sex-specific manner.

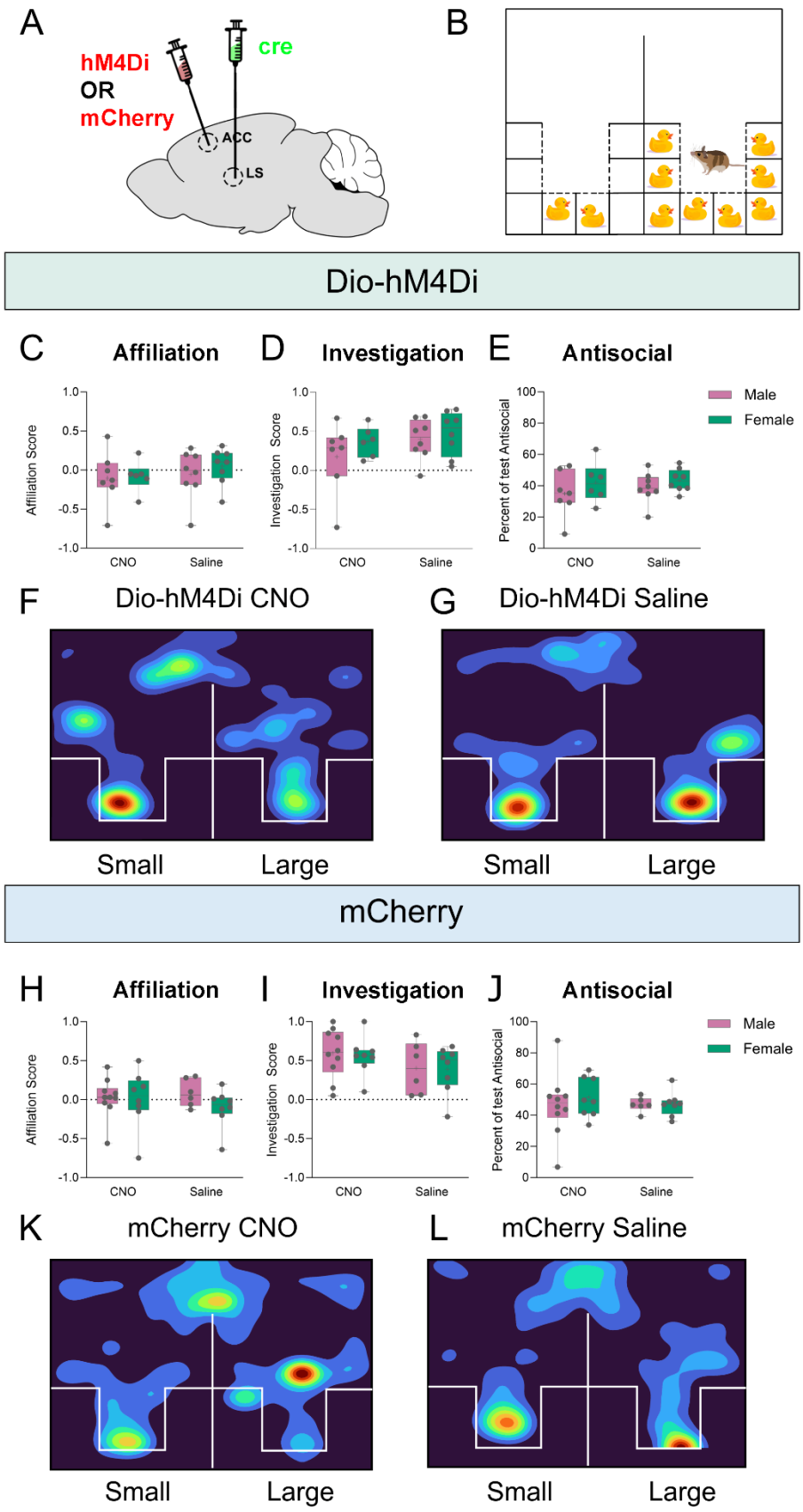
A
hM4Di
OR
mCherry
cre
ACC
LS
B
Dio-hM4Di
C
Affiliation
D
Investigation
E
Antisocial
Affiliation Score
Investigation Score
Percent of test Antisocial
CNO
Saline
Male
Female
F
Dio-hM4Di CNO
G
Dio-hM4Di Saline
Small
Large
mCherry
H
Affiliation
I
Investigation
J
Antisocial
K
mCherry CNO
L
mCherry Saline

**Figure 1-4. The anterior cingulate cortex to lateral septum circuit does not influence behavior during nonsocial group size preference tests.**
(A) Schematic of cre (LS) and Dio-hM4Di (ACC) AAV injection locations.
(B) Schematic depicting a top-down view of the group size preference testing during a nonsocial group size preference test, with rubber ducks acting as a nonsocial stimulus.
(C) For Dio-hM4Di subjects, CNO-induced inhibition of ACC-LS circuitry did not affect affiliative preferences during a nonsocial group size preference test. GLM with Injection Type (CNO or saline) and Sex (male or female) as fixed factors, all $p > 0.358$.
(D) For Dio-hM4Di subjects, CNO-induced inhibition of ACC-LS circuitry did not affect investigative preferences during a nonsocial group size preference test. GLM with Injection Type (CNO or saline) and Sex (male or female) as fixed factors, all $p > 0.188$.
(E) For Dio-hM4Di subjects, CNO-induced inhibition of ACC-LS circuitry did not affect the percentage of test time spent away from either group (i.e., antisocial behavior). GLM with Injection Type (CNO or saline) and Sex (male or female) as fixed factors, $p = 0.095$.
(F),(G) Representative heatmaps of male subject location during the social peer group size preference test for the Dio-hM4Di + CNO and Dio-hM4Di + saline Conditions.
(H) For mCherry subjects, CNO administration did not affect affiliative preferences. GLM with Injection Type (CNO or saline) and Sex (male or female) as fixed factors, all $p > 0.274$.
(I) For mCherry subjects, CNO administration did not affect investigative preferences. GLM with Injection Type (CNO or saline) and Sex (male or female) as fixed factors, all $p > 0.114$.
(J) For mCherry subjects, CNO administration did not affect the percentage of test time spent away from either group (i.e., antisocial behavior). GLM with Injection Type (CNO or saline) and Sex (male or female) as fixed factors, all $p > 0.614$.
(K),(L) Representative heatmaps of male subject location during the social peer group size preference test for mCherry + CNO, and mCherry + saline conditions.
Data represented as box plots with a median line and mean +.

*Inhibition of the ACC-LS does not alter behavior during a nonsocial group size preference test.*

To determine whether the effects of inhibiting the ACC-LS were specific to a social context or rather affect general responses to large quantities of objects, all male and female spiny mice also underwent a nonsocial group size preference test, for which, instead of novel, same-sex conspecifics as stimuli, the two stimulus groups were comprised of novel, spiny mouse-sized rubber ducks (**Figure 4B**). We found no significant differences for the mCherry and the Dio-hM4Di conditions (all $p > 0.173$; **Figure 4**). These results for the nonsocial group size preference test indicate the ACC-LS's modulation of peer group preference is restricted to a social context.

*The lateral hypothalamus – a downstream target of the LS – responds to peer group size in a sex-specific manner.*

Finally, because social group size preference and related behaviors likely require complex interactions between neural circuits spanning more than two regions, we sought to identify brain regions downstream from the LS that respond to variation in peer group size. In the retrograde group size IEG study described earlier, in addition to an injection of red retrobeads, subjects also received a uni-lateral injection of green, high molecular weight dextran, an anterograde tracing molecule that labels downstream synaptic terminals, into the LS (**Figure 5A,B**). Due to dextran labeling synaptic terminals rather than cell bodies, we were unable to colocalize dextran labeling with Fos-ir. We therefore quantified the average number of Fos-ir+ cells across three sections that spanned the rostral to caudal portions of regions previously implicated in rodent social behavior. Brains were examined for retrobead labeling from the olfactory bulb through the VTA. Areas that expressed robust dextran labeling and are well-known for modulating aspects of social behavior were selected for analyses. These regions included: the lateral hypothalamus (LHa; involved in aggression and social dominance [42,43]), the lateral habenula (LHb; facilitates social preferences [44]), the lateral preoptic nucleus (lPOA; responds to pup retrieval and parenting behavior [45]), the median preoptic nucleus (MnPO; shown to have oxytocin producing neurons in spiny mice [46]), the mPOA (crucial for parental care[30,31]), the nucleus accumbens (NAc; promotes social reward[47]), the paraventricular nucleus of the hypothalamus (PVN; major source

of oxytocin and vasopressin within mammals[48,49]), and the supramammillary nucleus (SuM; influences social recognition and social memory[50,51]).

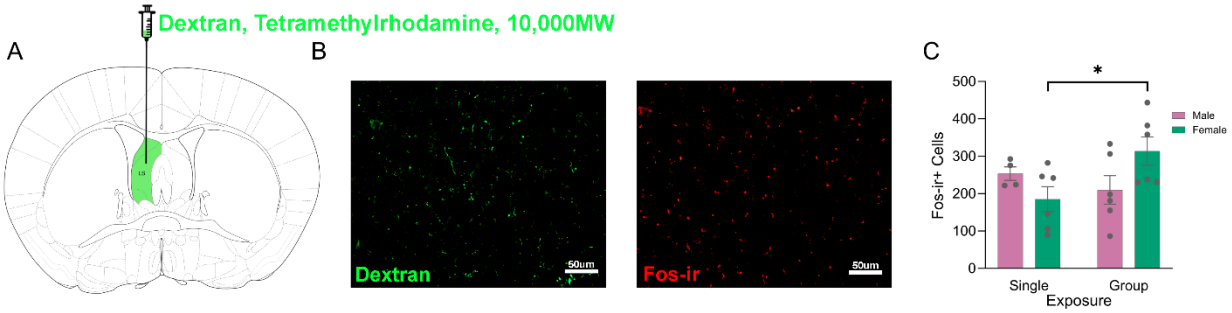


**Figure 1-5.The lateral hypothalamus receives projections from the lateral septum and differentially responds to group size in a sex-specific manner.**
(A) Schematic of high molecular weight dextran injection location within the LS.
(B) Representative histological images depicting dextran (right) and Fos-ir (left) staining within the LHa.
(C) The LHa had significantly higher Fos-ir in subjects exposed to a large group compared to a single, same-sex conspecific for females, but not males. GLM with Exposure Type and Sex as fixed factors. Sex p = 0.339, Exposure Type p = 0.252, interaction p = 0.028. Female-exposure p = 0.015, male-exposure p = 0.428. Data represented as mean ± SEM.

The majority of regions analyzed, such as the LHb, NAc, lPOA, mPOA, SuM, PVN, and MnPO showed no differences between single conspecific and peer group exposure conditions (all p > 0.069; **Table S3**; **Figure S5**). However, we identified a significant interaction for Fos-ir in the LHa ($F_{(1, 18)} = 5.725$, p = 0.028), with females (MD = 128.556, p = 0.015) but not males (MD = 43.50, p = 0.428) exhibiting a significantly larger number of Fos-ir+ cells in the peer group exposure compared to the single conspecific exposure (**Figure 5C**). Together, our results suggest that while ACC-LS circuitry modulates investigation of larger peer groups in both sexes, the same circuit only promotes the preference to affiliate with larger peer groups in males. Further, the activity within the ACC-LS circuitry may alter the downstream activity of the LHa in a sex-specific manner, potentially differentially influencing affiliative behavior and/or preferences in males and females.

## Discussion

Here we show for the first time in a group-living mammal that the ACC-LS regulates grouping preferences. Through IEG and tracing studies, we showed that neurons in the ACC responded more to larger than smaller groups of novel, same-sex conspecifics. This higher response in the ACC was also observed specifically in neurons projecting to the LS. Through chemogenetic inhibition of the ACC-LS circuit, we observed a significant reversal in spiny mouse species-typical investigative and affiliative preferences during a social peer group size preference test. Further, ACC-LS modulation of group size preference was specific to a social context, as inhibition of this circuit did not influence behavior in a nonsocial group size preference test.

To our knowledge, this is the first example of a specific neural circuit modulating group size preferences, particularly in a non-reproductive context. For species that live in large groups, including humans, the majority of social interactions individuals engage in on a daily basis are likely to be non-reproductive. To further our understanding of peer group interactions, including their dysregulation, it is paramount for us to have a solid understanding of the circuits driving the base motivation to non-reproductively associate with others. Although no other researchers have specifically manipulated the ACC-LS circuit in social group size contexts, several studies have examined functions of these brain regions separately from each other. Indeed, both the LS and ACC have both been implicated in numerous social behaviors across species. The LS is involved in grouping in birds[13] and kin/social recognition in rodents[18,22–24]. Similarly, the ACC promotes consolation[28] and helping[52–54] behavior and regulates different forms of attention[56,57], which may affect how subjects attend to, assess the numbers of, and interact with conspecifics. For an organism to display a social preference, they must (1) attend to social stimuli consisting of at least two choices, (2) process these stimuli and discriminate between them, and (3) affiliate with

the most contextually relevant stimulus. Because affiliating with a large group is ecologically-relevant for spiny mice, the ACC may function to direct attention toward large groups as they likely afford more benefits compared to smaller groups. Additionally, given that the LS is involved in social recognition in rats[22] and promotes affiliation in voles and finches[13,24], the LS in spiny mice may facilitate social discrimination of small vs. large peer groups and/or promote via specific neural activity affiliation with the most beneficial group. Together, the ACC-LS circuit may modulate group size preference by regulating attention to, discrimination between, and behavioral outputs toward large groups in spiny mice.

We've previously shown that both male and female spiny mice exhibit a robust preference to affiliate with and investigate larger over smaller groups[15,19]. Here, the preference to investigate larger peer groups was reversed via inhibition of the ACC-LS in both sexes, but only in males did inhibiting this circuit reverse their affiliative preference towards larger peer groups. Notably, these group size preference tests were conducted with novel, same-sex conspecifics. In a former study, we showed that during social preference tests *both* males and females prefer to affiliate with novel males over novel females[15]. While in the current study spiny mice were given a choice to affiliate only with same-sex conspecifics, the drive to be affiliative with same-sex peers may have been greater in males than in females because females did not have the option to affiliate with their preferred sex while males did. Indeed, in the current study, the sex difference for ACC-LS inhibition is confounded with the sex of the stimulus animal, as males were presented with males and females with females. The ACC-LS circuit may have evolved to promote social investigation in both sexes and was subsequently co-opted in male spiny mice to also drive affiliation preferences. Several species, such as the African striped mouse[58], form bachelor groups during different phases of their life history, and thus in some species the drive to

affiliate with same-sex peers may be greater in males than in females. Although field studies of grouping are lacking in spiny mice, which are similar to striped mice in their behavioral ecology and habitat[59,60], male spiny mice may also form bachelor groups like striped mice do, potentially driven by the ACC-LS circuit. While inhibition of the ACC-LS did not influence affiliative grouping preferences in female spiny mice, our studies have shown that, if given a choice, females do prefer to affiliate with a large group over a small group of female peers. Therefore, circuitry outside of the ACC-LS must modulate this preference in females.

Downstream from the LS, the LHa exhibited greater Fos-ir expression in response to a large group compared to a small group of same-sex peers in female, but not male, spiny mice. The LHa has been implicated in aggression in several rodent species[61,62] as well as social dominance in C57/BL6J mice[42]. Notably, female spiny mice are dominant over males and same-sex aggression is more common in female-female co-housed cages than in male-male cages[63]. Consistent with this difference, female spiny mice are also highly discriminatory over the types of males they interact and mate with[64], and menstrual cycle state (spiny mice are the only known rodent to menstruate[65–67]) greatly effects female's receptivity to males[68]. At least some of this sex-specific dominance and discrimination may develop early in life as a consequence of maternal care given that spiny mouse mothers block male, but not female, pups from leaving the nest, allowing females to be more exploratory and engage in more social interactions throughout development[69]. Thus, while male spiny mice may find a large group of same-sex conspecifics simply rewarding, females may have a more complex response to the same context, resulting in increased focus on social hierarchy or potential threats. Alternatively, the sex effect of chemogenetic inhibition of the ACC-LS on affiliative preferences here could be due to

differences in incentive values in male and female spiny mice. Although we observed no significant differences in Fos expression based on exposure condition in either the NAc or VTA, the LS sends axonal projections to the NAc and receives them from the VTA. This interconnectivity with reward regions could potentially influence reward-processing.

Unfortunately, we are currently unable to parse subregion and cell type specificity of projections in spiny mice. However, once a chemoarchitectural map of the spiny mouse LS like those available in mice[21] is developed, future studies can parse the function of individual cell types and LS subregions in social peer group preferences. Furthermore, because chemogenetic inhibition of the ACC-LS circuit did not influence behavior in the nonsocial group size preference test, this circuit is likely not involved in the subject's ability to process numerosity[79,80] or global attention to any type of stimuli. Indeed, this circuit appears to be specific to social stimuli and may potentially be involved in social attentional processes.

The drive to affiliate with a large peer group is likely a critical precursor to other, more complex, grouping behaviors. Indeed, without an initial preference to form groups, advantageous behaviors such as group foraging, co-parenting, organized defense, or homeostatic regulation may not frequently occur in natural environments. This initial preference may be innate but could equally be learned early in life. Although large groups in the wild are often comprised of mixed-sex individuals, disentangling the motivation to mate from general peer affiliation can help elucidate distinct mechanisms that contribute toward effective grouping and enable cooperative behaviors between same-sex peers. By revealing neural underpinnings of the preference to investigate and affiliate with large groups, we have identified brain regions of interest that may influence other more complex grouping behaviors. Further, by using an ethologically-relevant organism for the study of grouping behavior we uncovered a neural circuit dedicated to social

preference that functions in a sex-specific manner. Our results highlight the ACC-LS as a promising circuit for the regulation of complex social behaviors in large group-living species.

## STAR Methods

### Resource Availability

*Lead Contact*

Further information and requests for resources and reagents should be directed to, and will be fulfilled by, the lead author, A.M. Kelly (aubrey.kelly@emory.edu).

*Materials availability*

This study did not generate unique reagents.

*Data and code availability*

- IHC cell counts and behavioral data have been deposited at Dryad and are publicly available as of the date of publication. Accession numbers are listed in the key resources table.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

### Experimental model and study participant details

Spiny mouse, *Acomys dimidiatus* (formerly known as *Acomys cahirinus*)

66 male and 46 female spiny mice (post-natal day (PND) 106-496, only 3 animals were older than 380 days, median age was PND 186) were used for behavioral testing, immunohistochemistry (IHC), and viral validation. Subject sex was assigned based on external sexual organs, and littermates of the same sex were randomly assigned to experimental conditions. All procedures were approved by the Institutional Animal Care and Use Committee of Emory University (PROTO201900126). All methods were conducted in accordance with relevant ARRIVE guidelines and regulations. All methods were performed in accordance with the relevant guidelines and regulations. All animals were obtained from our breeding colony; breeders were from the captive-bred colony of Dr. Ashley W. Seifert (University of Kentucky). All animals were group-housed (2–4) in a standard rat polycarbonate cage (40.64 × 20.32 × 20.32 cm) lined with Sani-Chips bedding. Animals were provided with nesting material, rodent igloos, and shepherd shacks and were able to obtain food and water *ad libitum*. Spiny mice were kept on a 14-h light: 10-h dark cycle with an ambient temperature of 24 ± 2 °C.

**Method Details**

*Stereotaxic injections*

Retrograde and anterograde tracing was achieved via two 350nL intracranial injections into the LS (coordinates: 2.3mm anterior, 0.44mm lateral, depths 3.2 and 2.8mm) into a single hemisphere. The injection consisted of a 1:1 mixture of undiluted Green Dextran, Tetramethylrhodamine, 10,000 MW (Invitrogen) and Lumafluor Red Retrobeads that had been diluted 1:1 with sterile saline. For chemogenetic experiments, conditional expression of hM4Di gene was achieved by two 200nL injections of retrograde AAV cre (retroAAV.pENN.AAV.hSyn.HI.eGFP-Cre.WPRE.SV40 , titer: 2.6 x$10^{13}$Gc/mL ) into the LS (Coordinates: 2.20mm anterior, 0.44mm lateral, depths 3.3 and 3.2mm) and encoding a double-

floxed inverted open reading frame (DIO) of the hM4Di gene. The hM4Di AVV (AAV8-pAAV-hSyn-DIO-hM4D(Gi)-mCherry, titer: 2.2 x$10^{13}$Gc/mL) or mCherry serotype (AAV8-pAAV-hSyn-mCherry, titer: 2.6 x$10^{13}$Gc/mL) were intracranially injected twice at volumes of 150nL into the ACC (coordinates: 2.30mm anterior, 0.6mm lateral, depths 2.1 and 1.9mm).

For all intracranial injections, subject spiny mice received a 1.5mg/kg dose of meloxicam orally 30 minutes prior to anesthetization with isoflurane (4% for induction, 2% for maintenance). Subjects were placed into a stereotaxic setup (Kopf), and all AAVs were delivered through a pulled glass pipette at a rate of 1nl/sec using a nanosyringe (Drummond Nanoject III). Pipettes were held at the lowest injection site for 5 min and the final injection site for 10 min following AAV release. Coordinates were slightly adjusted based on mouse age and size, and viral vectors were allowed to express for 1 week for tracing injections and at least 5 weeks for chemogenetic injections before behavioral testing. During this time, all subjects were group-housed with up to 3 other same-sex littermates.

**Behavioral Assays**

*Immediate Early Gene (IEG) studies*

We ran two identical IEG studies to first identify an initial region within the rodent social brain network (SBN) that differentially responds to peer group size in 12 male spiny mice, and then determine upstream and downstream regions from the LS also differentially respond to peer group size in 12 male and 12 female spiny mice. For both IEG studies, all subjects were placed in a large Plexiglas testing chamber (60.96 x 45.72 x 38.1 cm) with either a single, novel, same-sex conspecific or a group of 7 novel, same-sex conspecifics confined under their own wire mesh container in the center of the testing chamber. Subjects were allowed to investigate the stimulus animals for 30 min before being transferred to a clean, standard rat polycarbonate cage (40.64 x

20.32 x 20.32 cm) for an additional 30 min prior to undergoing a perfusion to capture Fos-ir+ expression in response to the stimulus exposure. All interactions were recorded. For the second IEG to examine upstream and downstream regions of the LS, all subjects received their cranial injections 1 week prior to the IEG.

*Group size preference tests*

We developed a new group size preference test chamber based on our previous behavioral test[15] for use in the social peer group size preference test and the nonsocial group size preference test. The testing chamber consists of an initial acrylic chamber (15.24 x 15.24 x 45.72 cm) that releases subjects into a large, opaque acrylic chamber (55.88 x 71.12 x 45.72 cm) divided into two identical sections. Each section can hold up to 8 stimulus animals or objects, separated from the subject by clear acrylic with 0.12 cm diameter holes. When the subject is in one section, their view of the other section is entirely obstructed (**Fig. 3B**).

The order of social and nonsocial group size preference tests was counterbalanced across subjects. Subjects were given an intraperitoneal (ip) injected with either saline or 10mg/kg of CNO 30 minutes prior to testing. Subjects were then released from the initial holding chamber into the larger chamber and allowed to affiliate with and investigate both sides of the chamber, one side of which contained a small (2) group and the other side a large (8) group of novel, same-sex conspecifics for the social peer group size preference test or a small (2) and large (8) group of novel, spiny mouse-sized rubber ducks for the nonsocial group size preference test. The location of the small and large groups were counterbalanced. After 20 min, the subjects were removed from the chamber and returned to their home cage. Roughly 1 hr later, subjects were tested again for group size preference (social or nonsocial, depending on test type for the first test).

The first 10 min of each test were hand scored by a single observer, blind to experimental treatment, using BORIS[72]. We quantified the amount of time each subject spent near each group and investigating each group. The chamber was divided into four quadrants, where the quadrants closest to each group were considered "near" the group, and investigation was considered as pressing one's nose up to a chamber containing a stimulus animal. Using these values, we calculated three behavioral scores: (1) an affiliation score, which was a normalized score based on the time near the large group minus the small group, (2) an investigation score, which was a normalized score based on the time spent investigating the large group minus the small group, and (3) an antisocial score, which was the percentage of the test time that the subject spent in one of the two quadrants not near either group. Additionally, the velocity of each animal during the behavioral tests were obtained with automated tracking via Ethovision XT 17 (Noldus, Leesburg, VA, USA).

*Histology and immunohistochemistry*

All histology and immunohistochemistry followed our previously published protocol[73]. For both IEG studies, subjects were immediately euthanized at the end of the test by isoflurane overdose and were transcardially perfused with 0.1 M phosphate buffer saline (PBS) followed by 4% paraformaldehyde. Brains were extracted, post-fixed overnight in 4% paraformaldehyde, and underwent cryoprotection in 30% sucrose dissolved in PBS for 48 h. Brains were frozen in tissue-tek O.C.T. compound and stored at -80 °C before sectioning coronally at 40 for the first or 30µm (due to the retrobead protocol) for the second IEG using a Leica cryostat, with every third section saved for use in the present study. Tissue sections were immunofluorescently stained for Fos (the protein of the immediate early gene cFos; Synaptic Systems rabbit c-Fos 1:1000 dilution).

For targeting confirmation of the chemogenetic experimental animals, transcardial perfusions, post-fixing, and sectioning occurred identically to how the IEG study brains were processed. However, no immunofluorescent staining took place. Instead, sectioned tissue was mounted directly onto slides after rinsing in PBS.

*Neural Quantification*

Photomicrographs were obtained using a Zeiss AxioImager II microscope fitted with an apotome. For all regions quantified, we took 10 × images and quantified the average number of Fos-ir+ and retrobead labeled (when appropriate) cells and the number of cells that co-expressed retrobeads and Fos across 3 consecutive tissue sections that spanned the rostral-to-caudal axis. This totaled 120 or 90µm for the first and second IEG respectively, and the initial anterior-posterior coordinates from bregma are reported in Tables S1, S2, and S3. Due to differences in retrobead staining across consecutive sections, the percent retrobead labeled neurons that were Fos-ir+ measure was analyzed for each individual section as well as the average across all three, while the Fos-ir+ measure was analyzed for the average across sections. FIJI[74] was used to create standard regions of interest (ROIs) for all regions, and a Cell Profiler [75] pipeline was created to automatically count fluorescent cells and nuclei and to identify colocalized neurons. For retrograde tracing analysis, the Fos-retrobead colocalization was calculated separately due to differences in retrobead abundance from rostral to caudal portions of each region. For all other analyses, however, the Fos-ir values across all sections were averaged together.

For tracing studies, subjects were included in the analysis if the injection site was less than 90µm anterior to the merged anterior commissure (2.3mm anterior to bregma) and between 1.5 and 3.25mm from the top of the cortex. For chemogenetic studies, subjects were included in the analysis if the cre injection was less than 120µm anterior to the merged anterior commissure

(2.3mm anterior to bregma) and between 1.5 and 3.25mm from the top of the cortex. Additionally, the hM4Di/mCherry injection site was required to be between 1.38 and 2.3mm from bregma and not fall below the corpus collosum. All figure schematics displaying injection locations are adapted from Paxinos The Mouse Brain in Stereotaxic Coordinates[76].

**Quantification and Statistical Analysis**

Behavioral measurements for each test were analyzed using SPSS 29 (IBM Analytics). Tests used include independent t-tests when comparing two means for experiments detailed in figures 1 and S2, as well as general linear models (GLM) that include exposure type and sex for experiments detailed in figures 2,5, S1, and S5, group and sex for figure S4, and injection type and sex for figures 3 and 4 as fixed factors. To correct for multiple comparisons, all *post-hoc* pairwise comparisons were adjusted using Sidak corrections. The tests used for specific analyses are detailed in the figure legends. We screened for outliers for each individual test, defined as 3 standard deviations outside the mean, but none were found.

**KEY RESOURCES TABLE**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|---|---|
| Antibodies | | |
| Rabbit anti-c-Fos | Synaptic Systems | Cat# 226003; RRID:AB_2231974; lot 5-69 |
| Bacterial and Virus Strains | | |
| AAV8.pAAV-hSyn-DIO-hM4D(Gi)-mCherry | Krashes et al., 2011 | Addgene; cat# 44362-AAV8; RRID:Addgene_44362 |

| | | |
|---|---|---|
| AAV8.pAAV-hSyn-mCherry | Karl Deisseroth | Addgene; cat# 114472-AAV8; RRID:Addgene_114472 |
| retroAAV.pENN.AAV.hSyn.HI.eGFP-Cre.WPRE.SV40 | James M. Wilson | Addgene; cat# 105540-AAVrg; RRID:Addgene_105540 |
| Chemicals, Peptides, and Recombinant Proteins | | |
| Clozapine N-oxide (CNO) dihydrochloride (water soluble) | Hello Bio | Cat# HB6149; |
| Invitrogen Dextran, Tetramethylrhodamine, 10,000 MW, Neutral | Thermo Fisher Scientific | Cat# D1816 |
| Red Retrobeads | Lumafluor | https://lumafluor.com/shop/ols/products/xnred-retrobeads-100-l-o9a1330r |
| Software and Algorithms | | |
| GraphPad Prism | GraphPad | RRID: SCR_002798 |
| FIJI | Schindelin et al. 2012 | http://fiji.sc/; RRID: SCR_002285 |
| Cell Profiler | Stirling et al., 2021 | https://cellprofiler.org/; RRID:SCR_007358 |
| SPSS 29 | IBM | RRID:SCR_002865 |
| Behavioral Observation Research Interactive Software (BORIS) project | Friard and Gamba, 2016 | https://www.boris.unito.it/; RRID:SCR_021434 |

## Supplementary Material

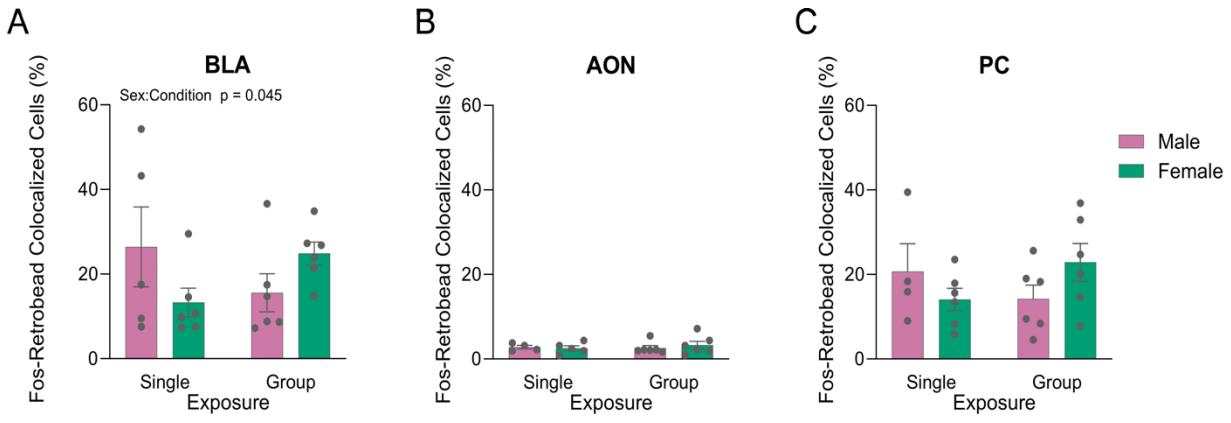


**Figure S 1-1 Fos-retrobead colocalization for regions upstream of the lateral septum.**
(A) BLA neurons projecting to the LS showed differences in the percentage of Fos-ir when subjects were exposed to a large group compared to a single novel, same-sex conspecific depending on the subject's sex.

GLM with Exposure Type and Sex as fixed factors. Sex $p = 0.714$, Exposure Type $p = 0.943$, interaction $p = 0.045$. No significant post-hoc analyses, $p > 0.124$.
(B) AON neurons projecting to the LS showed no differences in the percentage of Fos-ir when subjects were exposed to a large group compared to a single novel, same-sex conspecific. GLM with Exposure Type and Sex as fixed factors, all $p > 0.527$.
(C) PC neurons projecting to the LS showed no differences in the percentage of Fos-ir when subjects were exposed to a large group compared to a single novel, same-sex conspecific. GLM with Exposure Type and Sex as fixed factors, all $p > 0.082$.
Data represented as mean ± SEM.

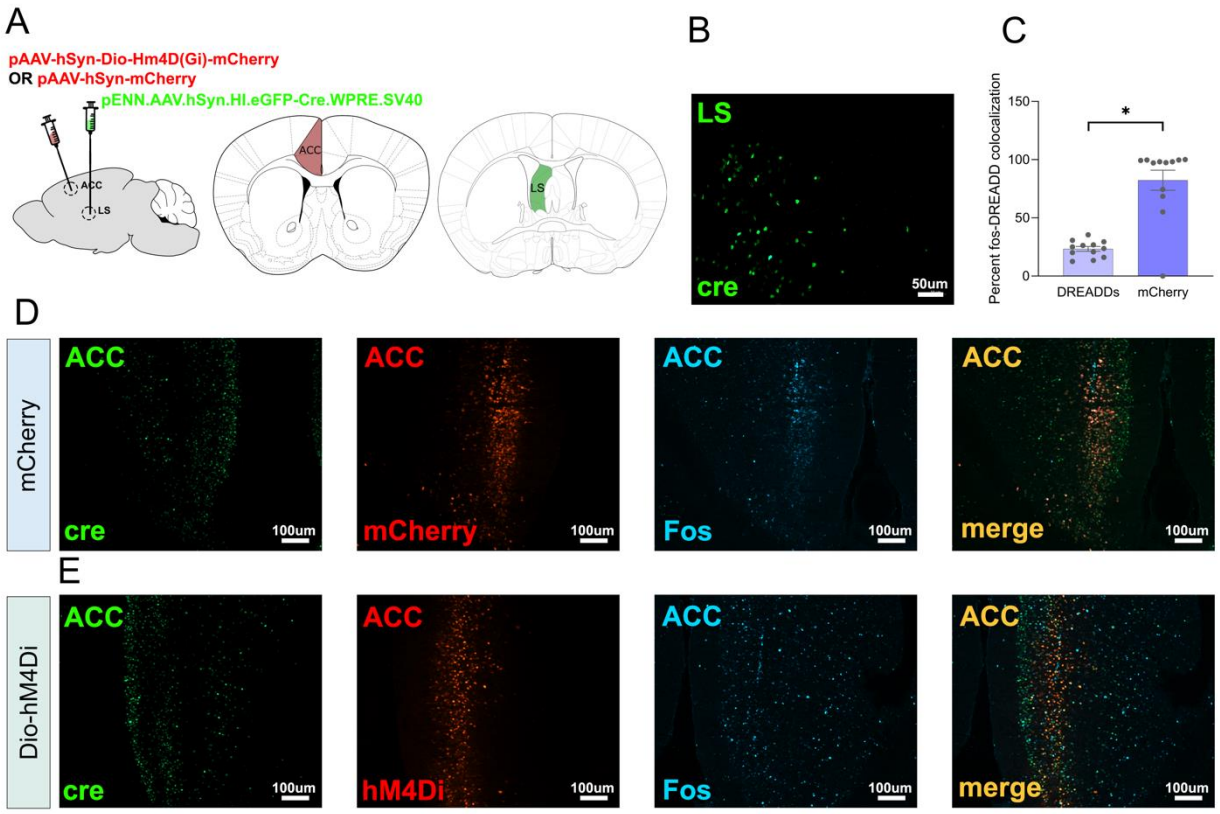


**Figure S 1-2. Efficacy of inhibitory cre-dependent DREADDs in spiny mice.**
(A) Left, Schematic of Dio-hM4Di (ACC) and of cre (LS) AAV injection locations. Middle, Schematic of ACC region analyzed. Right, Schematic of LS region analyzed.
(B) Representative histological images depicting cre injection site within the LS.
(C) CNO administration yielded a lower percentage of Fos-virus colocalization for the Dio-hM4Di compared to the mCherry condition. Independent t-test, $p < 0.001$.
(D) Representative histological images for mCherry subjects depicting cre, mCherry, Fos-ir, and colocalization staining respectively within the ACC.
(E) Representative histological images for Dio-hM4Di subjects depicting cre, Dio-hM4Di, Fos-ir, and colocalization staining respectively within the ACC.
Data represented as mean ± SEM.

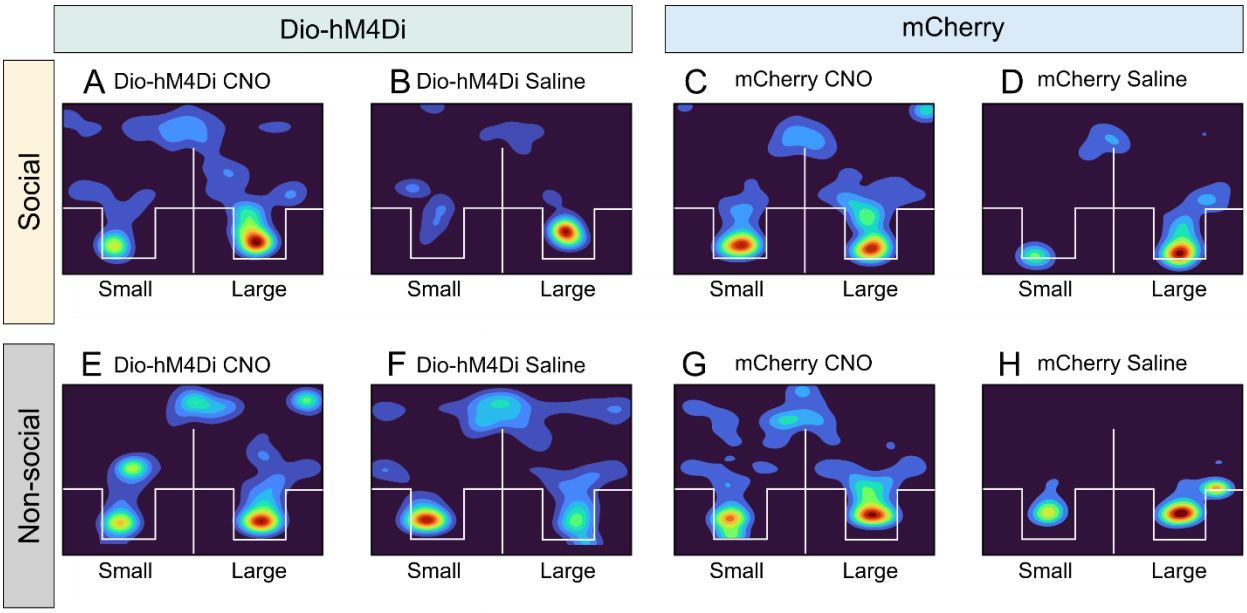


**Figure S 1-3. Representative heatmaps for females during group size preference tests.**

(A) – (D) Representative heatmaps of female subject location during the social peer group size preference test for the Dio-hM4Di + CNO, Dio-hM4Di + saline, mCherry + CNO, and mCherry + saline conditions respectively.
(E) – (H) Representative heatmaps of female subject location during the nonsocial group size preference test for the Dio-hM4Di + CNO, Dio-hM4Di + saline, mCherry + CNO, and mCherry + saline conditions respectively.

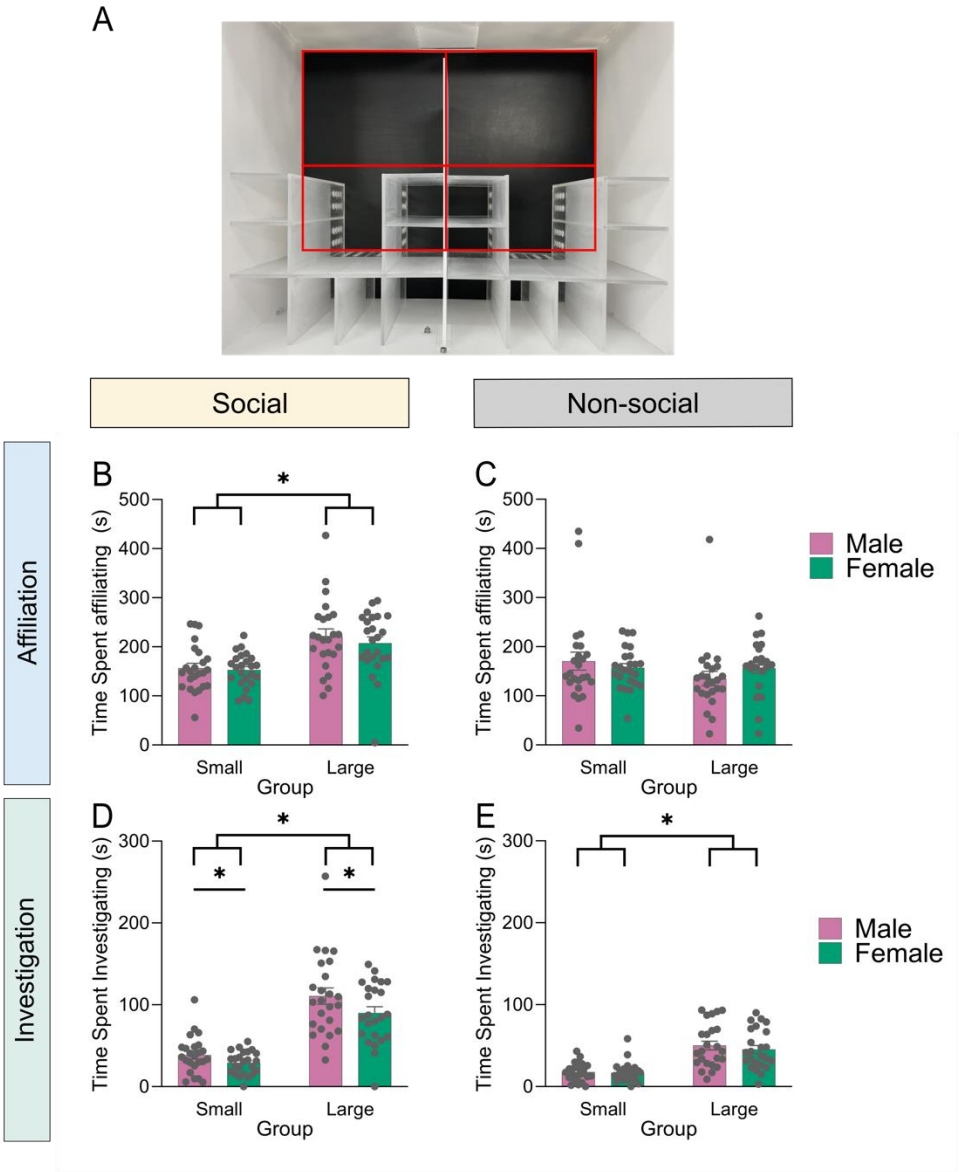


**Figure S 1-4. Validation of social and nonsocial group size preferences in a modified group size preference chamber.**

(A) Image depicting group size preference testing apparatus with red outlines indicating subdivisions for behavioral scoring.
(B) Male and female control spiny mice (i.e., all subjects excluding those in the Dio-hM4Di + CNO condition) spent significantly more time affiliating with the large group of peers compared to the small group. GLM with Group and Sex as factors. Sex $p = 0.426$, Group $p < 0.001$, interaction $p = 0.653$.
(C) Male and female control spiny mice (i.e., all subjects excluding those in the Dio-hM4Di + CNO condition) did not exhibit a preference for affiliating with a large or small group of rubber ducks. GLM with Group and Sex as factors, $p > 0.404$
(D) Male and female control spiny mice spent significantly more time investigating the large peer group compared to the small peer group of same-sex conspecifics, with a significantly stronger effect in males. GLM with Group and Sex as fixed factors. Sex $p = 0.031$, Group $p < 0.001$, interaction $p = 0.425$.

(E) Male and female control spiny mice spent significantly more time investigating the large group compared to the small group of rubber ducks. GLM with Group and Sex. Sex $p = 0.493$, Group $p < 0.001$, interaction $p = 0.625$.

Data represented as mean ± SEM.

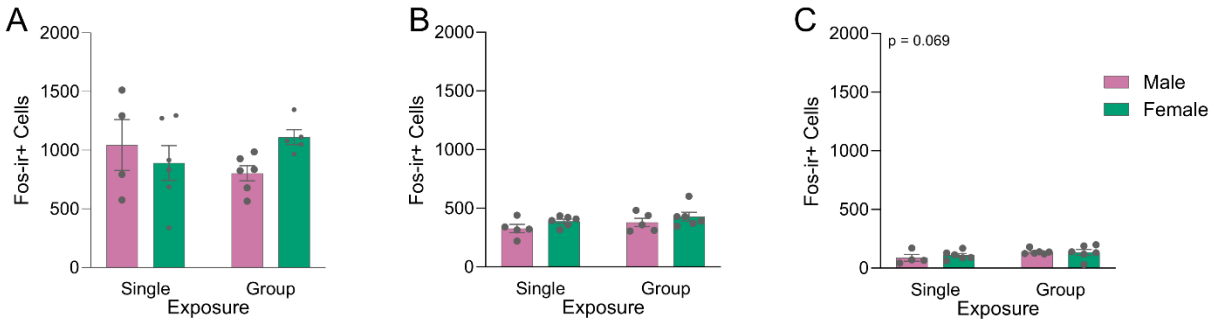


**Figure S 1-5. Fos-ir+ cell counts for regions downstream of the lateral septum.**

(A) NAc Fos-ir  expression did not differ across exposure conditions. GLM with Exposure Type and Sex as fixed factors, all $p < 0.089$.
(B) PVN Fos-ir expressiondid not differ across exposure conditions. GLM with Exposure Type and Sex as fixed factors, all $p < 0.102$.
(C) MnPO Fos-ir expression did not differ across exposure conditions. GLM with Exposure Type and Sex as fixed factors, all $p < 0.069$.
Data represented as mean ± SEM.

**Table S 1-1. Statistics for Retrobead-Fos colocalization.**

| Region | Section | Test | F | P |
|---|---|---|---|---|
| **ACC** | 1 | Exposure | 6.529 | 0.020* |
| | | Sex | 0.416 | 0.527 |
| | | Exposure:Sex | 1.696 | 0.210 |
| | 2 | Exposure | 0.471 | 0.502 |
| | | Sex | 0.058 | 0.813 |
| | | Exposure:Sex | 2.541 | 0.129 |
| | 3 | Exposure | 2.746 | 0.116 |
| | | Sex | 0.215 | 0.649 |
| | | Exposure:Sex | 1.865 | 0.190 |
| | Average | Exposure | 3.624 | 0.074 |
| | | Sex | 0.010 | 0.923 |
| | | Exposure:Sex | 2.759 | 0.115 |
| **AON** | 1 | Exposure | 1.438 | 0.247 |
| | | Sex | 0 | 0.988 |
| | | Exposure:Sex | 0.004 | 0.953 |
| | 2 | Exposure | 1.128 | 0.303 |
| | | Sex | 0.027 | 0.871 |
| | | Exposure:Sex | 1.128 | 0.303 |
| | 3 | Exposure | 1.181 | 0.292 |
| | | Sex | 1.181 | 0.292 |
| | | Exposure:Sex | 1.176 | 0.293 |
| | Average | Exposure | 0.138 | 0.714 |
| | | Sex | 0.138 | 0.714 |
| | | Exposure:Sex | 0.401 | 0.535 |
| **BLA** | 1 | Exposure | 0.052 | 0.823 |
| | | Sex | 0.003 | 0.955 |
| | | Exposure:Sex | 0.304 | 0.588 |
| | 2 | Exposure | 0.260 | 0.616 |
| | | Sex | 0.521 | 0.479 |
| | | Exposure:Sex | 0.754 | 0.396 |
| | 3 | Exposure | 1.449 | 0.244 |
| | | Sex | 0.514 | 0.482 |
| | | Exposure:Sex | 0.021 | 0.885 |
| | Average | Exposure | 0.005 | 0.943 |
| | | Sex | 0.138 | 0.714 |
| | | Exposure:Sex | 4.610 | 0.045* |
| **PC** | 1 | Exposure | 0.608 | 0.446 |
| | | Sex | 0.498 | 0.498 |
| | | Exposure:Sex | 0.495 | 0.491 |
| | 2 | Exposure | 0.516 | 0.482 |
| | | Sex | 4.142 | 0.057 |
| | | Exposure:Sex | 2.672 | 0.119 |

| | | | | |
|---|---|---|---|---|
| | 3 | Exposure | 0.550 | 0.468 |
| | | Sex | 2.748 | 0.115 |
| | | Exposure:Sex | 1.769 | 0.200 |
| | Average | Exposure | 0.075 | 0.787 |
| | | Sex | 0.061 | 0.808 |
| | | Exposure:Sex | 3.381 | 0.083 |
| **VTA** | 1 | Exposure | 0.021 | 0.887 |
| | | Sex | 0.105 | 0.750 |
| | | Exposure:Sex | 5.113 | 0.036* |
| | 2 | Exposure | 0.240 | 0.629 |
| | | Sex | 0.782 | 0.388 |
| | | Exposure:Sex | 1.370 | 0.256 |
| | 3 | Exposure | 0.863 | 0.364 |
| | | Sex | 1.426 | 0.247 |
| | | Exposure:Sex | 0.819 | 0.377 |
| | Average | Exposure | 0.279 | 0.603 |
| | | Sex | 1.110 | 0.305 |
| | | Exposure:Sex | 0.360 | 0.556 |

* denotes significance

**Table S 1-2. Statistics for average Fos-ir expression in regions upstream of the LS.**

| Region | Test | F | P |
|---|---|---|---|
| **ACC** | Exposure | 5.791 | 0.020* |
| | Sex | 0.343 | 0.566 |
| | Exposure:Sex | 1.119 | 0.305 |
| **AON** | Exposure | 3.766 | 0.069 |
| | Sex | 0.312 | 0.584 |
| | Exposure:Sex | 0.009 | 0.925 |
| **BLA** | Exposure | 0.359 | 0.556 |
| | Sex | 0.087 | 0.771 |
| | Exposure:Sex | 2.396 | 0.138 |
| **PC** | Exposure | 0.312 | 0.583 |
| | Sex | 0.330 | 0.573 |
| | Exposure:Sex | 2.478 | 0.133 |
| **VTA** | Exposure | 0.241 | 0.629 |
| | Sex | 1.420 | 0.248 |
| | Exposure:Sex | 0.094 | 0.763 |

* denotes significance

**Table S 1-3. Statistics for average Fos-ir+ in regions downstream from LS.**

| Region | Test | F | P |
|---|---|---|---|
| **LHa** | Exposure | 1.399 | 0.252 |
| | Sex | 0.245 | 0.626 |
| | Exposure:Sex | 5.725 | 0.028* |
| **LHb** | Exposure | 1.773 | 0.199 |
| | Sex | 0.585 | 0.454 |
| | Exposure:Sex | 2.552 | 0.127 |
| **lPOA** | Exposure | 0.960 | 0.341 |
| | Sex | 2.169 | 0.159 |
| | Exposure:Sex | 0.807 | 0.382 |
| **MnPO** | Exposure | 3.754 | 0.069 |
| | Sex | 0.194 | 0.665 |
| | Exposure:Sex | 0.396 | 0.537 |
| **mPOA** | Exposure | 0.397 | 0.537 |
| | Sex | 3.212 | 0.091 |
| | Exposure:Sex | 2.872 | 0.108 |
| **NAc** | Exposure | 0.006 | 0.938 |
| | Sex | 0.364 | 0.554 |
| | Exposure:Sex | 3.234 | 0.090 |
| **PVN** | Exposure | 1.986 | 0.176 |
| | Sex | 2.962 | 0.102 |
| | Exposure:Sex | 0.045 | 0.835 |
| **SuM** | Exposure | 0.038 | 0.848 |
| | Sex | 0.071 | 0.793 |
| | Exposure:Sex | 0.077 | 0.784 |

* denotes significance

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# 2 CHAPTER II. Biased brain and behavioral responses towards kin in males of a communally breeding species.

## Author's contribution and acknowledgement of reproduction

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The dissertation author designed the study, conducted IEG behavioral tests, scored behavioral videos, cryosectioned brains, conducted immunohistochemistry, microscopy and cell counts, analyzed the data, and wrote the manuscript. Deborah Ho conducted group interaction behavioral tests and scored behavioral videos. Ashley W. Seifert edited and reviewed the manuscript. Aubrey M. Kelly designed the study, analyzed the data, wrote the manuscript, and as principal investigator obtained funding.

**Conflicts of Interest:**

The authors have no conflicts of interest to disclose.

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## Abstract

In complex social environments, individuals may interact with not only novel and familiar conspecifics but also kin and non-kin. The ability to distinguish between conspecific identities is crucial for most animals, yet how the brain processes conspecific type and how animals may alter behavior accordingly is not well known. We examined whether the communally breeding spiny mouse (*Acomys cahirinus*) responds differently to conspecifics that vary in novelty and kinship. In a group interaction test, we found that males can distinguish novel kin from novel non-kin, and preferentially spend time with novel kin over familiar kin and novel non-kin. To determine whether kinship and novelty status are differentially represented in the brain, we conducted immediate early gene tests, which revealed the dorsal, but not ventral, lateral septum differentially processes kinship. Neither region differentially processes social novelty. Further, males did not exhibit differences in prosocial behavior toward novel and familiar conspecifics but exhibited more prosocial behavior with novel kin than novel non-kin. These results suggest that communally breeding species may have evolved specialized neural circuitry to facilitate a bias to be more affiliative with kin, regardless of whether they are novel or familiar, potentially to promote prosocial behaviors, thereby facilitating group cohesion.

**Biased brain and behavioral responses towards kin in males of a communally breeding species.**

## Introduction

Large group-living is phylogenetically widespread and is advantageous for numerous species, including insects [1,2], birds [3,4], and mammals [5–7], providing benefits such as collective traveling [8,9], more effective homeostatic regulation [10,11], predation reduction [12–14], and enhanced offspring survival [15–17]. Large group-living inherently yields complex social environments, such that conspecifics an individual encounters will vary in the degree of novelty (strangers, acquainted, etc.) and their kinship status to a much greater extent than for small group-living species. In such dynamic environments, accurately recognizing whether an individual is familiar or related is likely highly adaptive and promoted through unique sensory cues [18,19] that facilitate context-appropriate behavioral choices [20–23]. Indeed, social recognition likely promotes successful group identification [22,24], accurate navigation of social hierarchies [25,26], and incest avoidance [1,27].

Communal breeding is a social structure with a highly complex social environment. Communal breeders, like Degus, *Octodon degus* [7,28], can have several breeding pairs of mixed relation, and most group members engage in parenting [29]. Further, there is high group member turnover in Degus, resulting in a frequent influx of social novelty[28]. Because there are varying degrees of genetic relationships in communally breeding groups, the ability to distinguish between kin and non-kin, regardless of familiarity, is especially important to avoid incest and promote navigation of the social environment in an optimal manner. For example, individuals that disperse to a

neighboring group that contains novel, older relatives would need to rely on kin recognition more than familiarity for accurate social recognition of kin.

In large group-living, communally breeding species, social recognition may be dampened to promote indiscriminate parental care among the entire population, thereby facilitating group cohesion. However, this strategy would likely result in high rates of incest and unviable offspring. Alternatively, then, social recognition abilities may indeed be high, but social motivation systems may have evolved to deprioritize genetic relation specifically for raising offspring but not for mating. The conflict between high rates of socially novel encounters and indiscriminate parental care raises the question as to whether the ability to distinguish between kin and non-kin or between novel and familiar individuals is dampened in communally breeding species. Further, whether these distinct types of social recognition are modulated via similar mechanisms, to our knowledge, remains unknown.

The spiny mouse, *Acomys cahirinus,* is a communally breeding species primarily found in the deserts of Africa, the Middle East, and Southeast Asia [33]. Spiny mice naturally live in large groups [33]; in the lab they are highly prosocial and prefer affiliating with large groups over small groups [5]. Additionally, spiny mice readily accept a newcomer into an established group [34]. Thus, spiny mice's social structure provides a particularly complex and challenging environment for social recognition. This naturally complex social landscape allows us to explore preferences and recognition abilities for distinguishing between kin and non-kin, while controlling for familiarity and novelty, in ethologically valid ways that are often challenging in other species. Thus far, spiny mice have been shown to successfully identify direct and cross-fostered littermates through olfactory cues and phenotype matching based on the female they nursed from

[19], and we have previously shown they accurately recognize a new conspecific after repeated exposure to a different individual in a social recognition test [5]. However, how novelty and kinship may interact to influence behavior in a group and whether the neural underpinnings of these distinct types of social recognition differ have yet to be explored.

One potential brain region contributing to the neural underpinnings of social recognition in spiny mice may be the lateral septum (LS). In recent years, the LS has become an increasingly examined region for social behaviors, such as aggression [35], social exploration [36], flocking [3], prosocial behavior[37] , and social recognition [38,39]. While studies have examined contributions of the LS to social novelty recognition, fewer studies have specifically sought to determine whether the LS also differentiates kin from non-kin, especially while controlling for novelty [36,40,41]. Clemens et al. (2020) showed that lesioning of the LS disrupts a preference for siblings and identified a neural topographic mapping of kinship in the LS of Long Evans rats. However, while these results position the LS as a promising region for regulating social recognition, and specifically kin recognition, in spiny mice, the study did not control for the familiarity of littermates compared to novel non-siblings. Thus, it is unclear not only if the LS holds a similar role for kin recognition in spiny mice as it does for a highly selected strain of domestic rat with low levels of social motivation [42], but also whether the LS differentiates between novelty and familiarity in this species.

Here we examined whether spiny mice appear to recognize and behave differently based on both the novelty and the kinship status (kin versus non-kin) of individuals they interact with in group and dyadic social contexts. Because we previously found that spiny mice exhibit no preference

for affiliating with or investigating novel or familiar conspecifics, we hypothesized their behavior would not differ during social interactions with novel and familiar non-kin conspecifics but would differ between novel kin and novel non-kin. Further, to determine if context-specific social recognition occurs via processing in the same brain region, we used immediate early gene (IEG) studies to examine whether the LS distinguishes novelty and familiarity as well as kinship status. Because of previous findings revealing a topographic map for kinship in rats within the LS [39], demonstrating how subregions within the LS can have separate functions [35,43], we hypothesized that the LS would differentially process exposure to novel kin versus novel non-kin, potentially in a spatially distributed manner. Lastly, because communal breeders frequently interact with novel and familiar conspecifics, and because spiny mice show no preference for affiliating with or investigating novel or familiar conspecifics [5], we hypothesized that the LS would not show differentiated responses between novel and familiar non-kin.

**Results**

*Male spiny mice differentiate between kinship and familiarity and preferentially affiliate with novel kin in a novel, same-sex group*

Here we aimed to examine whether spiny mice recognize and behave differently with conspecifics based on the novelty and kinship status of the individuals they interact with within a group. 5 groups of 8 male spiny mice were placed into a novel arena for a 1-hr group interaction test. Each subject was familiar with only one other conspecific - their sibling and cagemate. For each subject, 2 others were novel kin, and the remaining 4 were novel non-kin. Novel kin were at least 2 litters removed from the subject spiny mice. Time spent investigating and positively affiliating with each stimulus type (familiar kin, novel non-kin, and novel kin) were sampled for each subject every 5 min. A Poisson generalized linear mixed model (GLMM) analysis with

conspecific type as a fixed factor, group as a random factor, and subject as a random factor to account for within-subject testing found that the frequencies of investigation ($X^2_{(2,120)} = 35.281$, $p < 0.001$; **Figure 2-1A**) differed based on stimulus type. Specifically, Sidak-corrected post hoc analyses revealed that male spiny mice investigated novel non-kin more than familiar kin (N = 40, MD = 1.24, $p < 0.001$) and novel kin (N = 40, MD = 0.91, $p < 0.001$). Additionally, a Poisson GLMM analysis yielded a main effect of affiliation frequencies ($X^2_{(2,120)} = 19.423$, $p < 0.001$; **Figure 2-1B**), with Sidak-corrected post hoc analyses showing that males affiliated more with novel kin than novel non-kin (N = 40, MD = 0.48, $p = 0.012$) and more with novel kin than familiar kin (N = 40, MD = 0.68, $p < 0.001$). These results suggest spiny mice accurately differentiate between kin and non-kin regardless of the conspecific's novelty status and that spiny mice opt to investigate novel non-kin more; this may be to gather more information about unknown individuals. Further, affiliation preferences upon forming a new group may be biased toward interacting with novel kin.

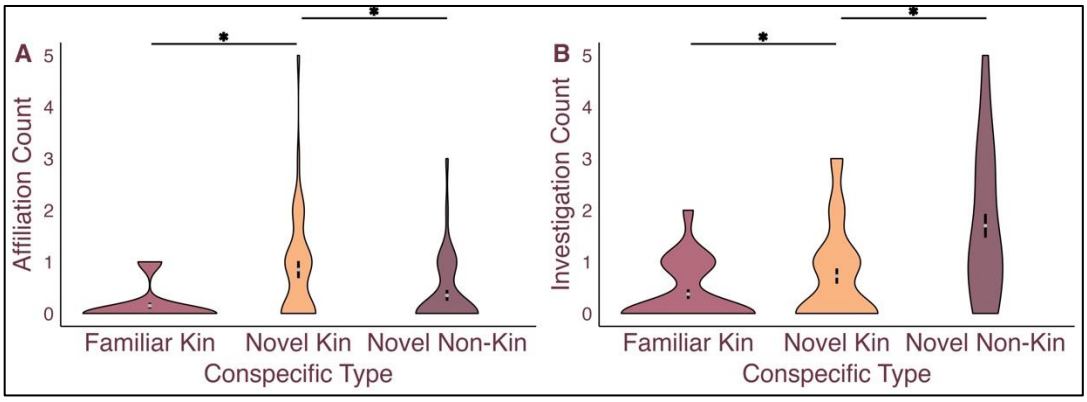


**Figure 2-1. Male spiny mice differentially investigated and affiliated with peers based on conspecific type in a group interaction test.**

Male spiny mice mean frequencies represented in a violin plot with the mean (± SEM) represented for **(A)** affiliation and **(B)** investigation during a group social interaction. Spiny mice spent significantly more time

investigating novel kin (orange) over familiar kin (wine) and novel non-kin (plum) and preferred to affiliate with novel kin. Dots represent individual data. * Indicate $P \leq 0.05$.

The findings from the group social interaction test show that male spiny mice discriminate between novel kin, novel non-kin, and familiar kin and can distinguish novel kin from novel non-kin, even if they preferentially affiliate with novel kin. These results suggest kinship status and novelty may interact in a complex and nuanced way when making social decisions in the communally breeding spiny mouse and begs the question whether distinct types of social discrimination are differentially reflected in brain regions important for social behavior. The LS has recently been proposed as a hub for processing social information [44] and a recent study in Long Evans rats demonstrated that kin versus non-kin responsive cells may be topographically organized [39]; however, this paper did not control for social novelty. Thus, we next conducted IEG studies to examine whether neurons in the LS differentially process a) novelty and familiarity when controlling for kinship, as well as b) kin and non-kin when controlling for novelty. To specifically examine neural responses in the LS, we quantified NeuN-immunoreactive (ir+) cells (a neuronal marker) colocalized with Fos, a marker of neural activity.

*LS NeuN-Fos colocalization does not differentiate between familiar and novel conspecifics*

To identify whether neurons in the LS differentially respond to novelty, we conducted an IEG study where 16 male spiny mice were allowed to freely interact with either a same-sex familiar non-kin conspecific or a same-sex novel non-kin conspecific for 30 min. We analyzed the percentage of NeuN-ir+ cells that were also Fos-ir+ for both the dorsal and ventral LS. A GLM with condition (novel non-kin or familiar non-kin) and LS region (dorsal or lateral) as fixed factors yielded no main effects for condition ($F_{(1,28)} = 2.031$, $p = 0.165$), or region ($F_{(1,28)} = 0.018$,

p = 0.893) nor an interaction ($F_{(1,28)}$ = 0.069, p = 0.795) (**Figure 2-2A**). These results suggest the LS does not show differentiated responses to familiarity when controlling for kinship.

*Spiny mice investigate different areas of the body on novel and familiar conspecifics*

Despite a lack of neural response differences within the LS as measured by NeuN-Fos colocalization, behavioral differences during interactions with novel and familiar non-kin may still be present. Therefore, we analyzed behavior from the first 5 min of the IEG study to determine if spiny mice interacted differently with novel and familiar non-kin conspecifics. The overall behavioral breakdown for each condition was compared via Friedman's test and post hoc Wilcoxon ranked sum tests. For both conditions, time spent engaging in prosocial, aggressive, and non-overt behaviors differed ($\chi^2(2) = 16$, and $p < 0.001$ for both) (**Table S1**). Between overt (i.e., interactive) behaviors, spiny mice in both conditions were more prosocial than aggressive (all Z = -2.521, p = 0.012, r = 0.63) (**Table S2-1**).

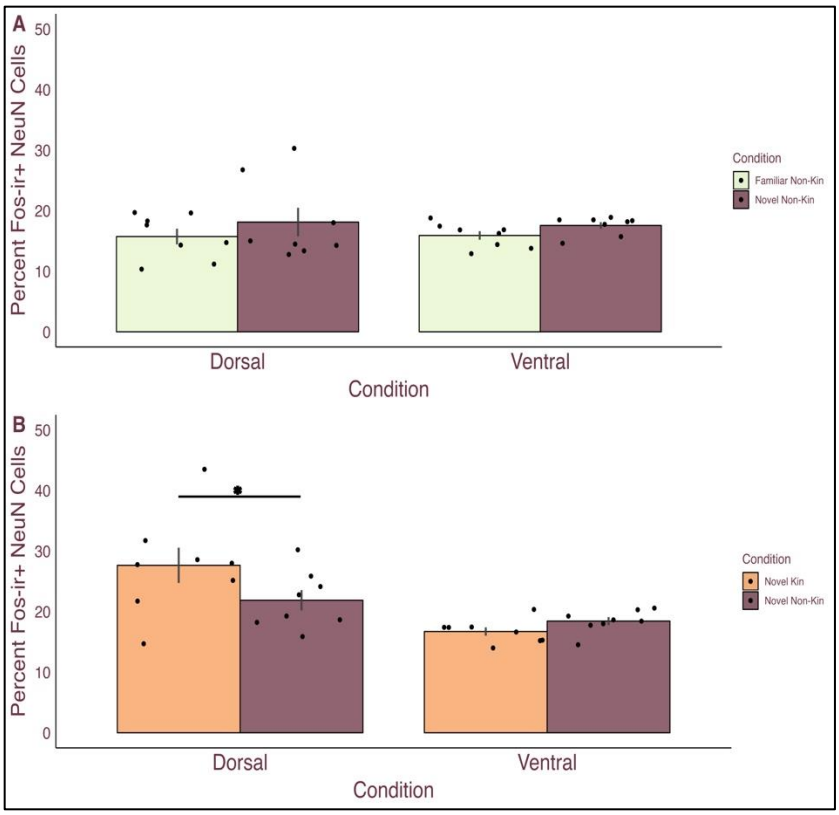


**Figure 2-2. Lateral septum neural responses differed between novel kin and novel non-kin conspecific exposure.**

**(A)** Male spiny mice mean (± SEM) percentage of NeuN-Fos colocalized cells within the dorsal and ventral lateral septum for familiar non-kin (mint) and novel non-kin (plum). Neither region nor condition significantly differed. Dots represent individual data. **(B)** Male spiny mice mean (± SEM) percentage of NeuN-Fos colocalized cells within the dorsal and ventral lateral septum (LS) for novel kin (orange) and novel non-kin (plum). Neural responses were greater in response to interactions with novel kin than non-kin in the dorsal, but not ventral LS. Dots represent individual data. * Indicate $P \leq 0.05$.

In addition to overall behavioral breakdowns, because rodents are primarily olfactory communicators, we sought to determine whether differences in investigation time could be detected based on the bodily location of stimulus animal. A Friedman's test revealed that subjects that interacted with novel non-kin conspecifics did not differentially investigate the head, flank, or rear of stimuli ($\chi^2(2) = 1.750$, $p = 0.417$). However, subjects that interacted with familiar non-kin stimulus animals differentially investigated distinct areas of the stimulus' body ($\chi^2(2) =$

7.750, p = 0.021) (**Figure S2-1**). Specifically, after false discovery rate correction on a Wilcoxon Signed Ranks test results, males investigated the flank more than the head of familiar non-kin conspecifics (Z = -2.521, p = 0.036, r = 0.63) (**Figure S2-1**). Although we observed no significant differences in LS responsivity to novel vs. familiar non-kin, our behavioral data suggests that male spiny mice discriminate between novel non-kin and familiar non-kin.

*LS NeuN-Fos colocalization differentiates between novel kin and novel non-kin*

We next aimed to determine if neurons within the LS differentially process kinship. We conducted an IEG study where 16 male spiny mice were allowed to freely interact with either a novel kin conspecific or a novel non-kin conspecific for 30 min.

The percentage of NeuN-ir+ cells that were also Fos-ir+ for both the dorsal and ventral LS was analyzed via a GLM with condition (novel kin versus novel non-kin) and LS region (dorsal or lateral) as fixed factors. While there was no main effect of condition ($F_{(1,28)} = 1.334$, $p = 0.258$), we found a main effect of LS region, such that Bonferroni post hoc analysis revealed the dorsal LS had a higher percentage of colocalized cells than the ventral LS ($F_{(1,28)} = 16.332$, $p < 0.001$). Additionally, we observed a significant interaction ($F_{(1,28)} = 4.573$, $p = 0.041$) with Bonferroni post hoc analysis identifying that the dorsal, but not ventral, LS had a higher percentage of colocalized cells in the novel kin condition (M = 27.65% ) compared to the novel non-kin condition (M = 21.878%; MD = 5.77, SEM = 2.48, p = 0.027, d = 0.69) (**Figure 2-2B**). This finding suggests that the dorsal LS may process kinship status.

*Spiny mice are more prosocial toward novel kin than novel non-kin*

Similar to the previous IEG study, the overall behavioral breakdown for each condition was compared via Friedman's test and post hoc Wilcoxon ranked sum tests. For both conditions, time spent engaging in prosocial, aggressive, and non-overt behaviors differed ($\chi^2(2) = 16$, and $p < 0.001$ for both) (**Table S2-2**). Between overt (i.e., interactive) behaviors, males in both conditions were more prosocial than aggressive (all $Z = -2.521$, $p = 0.012$, $r = 0.63$) (**Table S2-2**). Between conditions, male spiny mice that interacted with novel kin spent more time engaging in prosocial behaviors ($U(n_1 = 8, n_2 = 8) = 8$, $Z = -2.521$, $p = 0.012$, $r = 0.63$) and less time in non-overt behaviors ($U(n_1 = 8, n_2 = 8) = 8$, $Z = -2.521$, $p = 0.012$, $r = 0.63$) compared to males that interacted with novel non-kin conspecifics (**Figure 2-3A**). Additional analyses revealed no difference in males for their investigation in the novel kin condition compared to the novel non-kin condition ($U(n_1 = 8, n_2 = 8) = 14$, $Z = -1.890$, $p = 0.059$, $r = 0.50$) (**Figure 2-3B**).

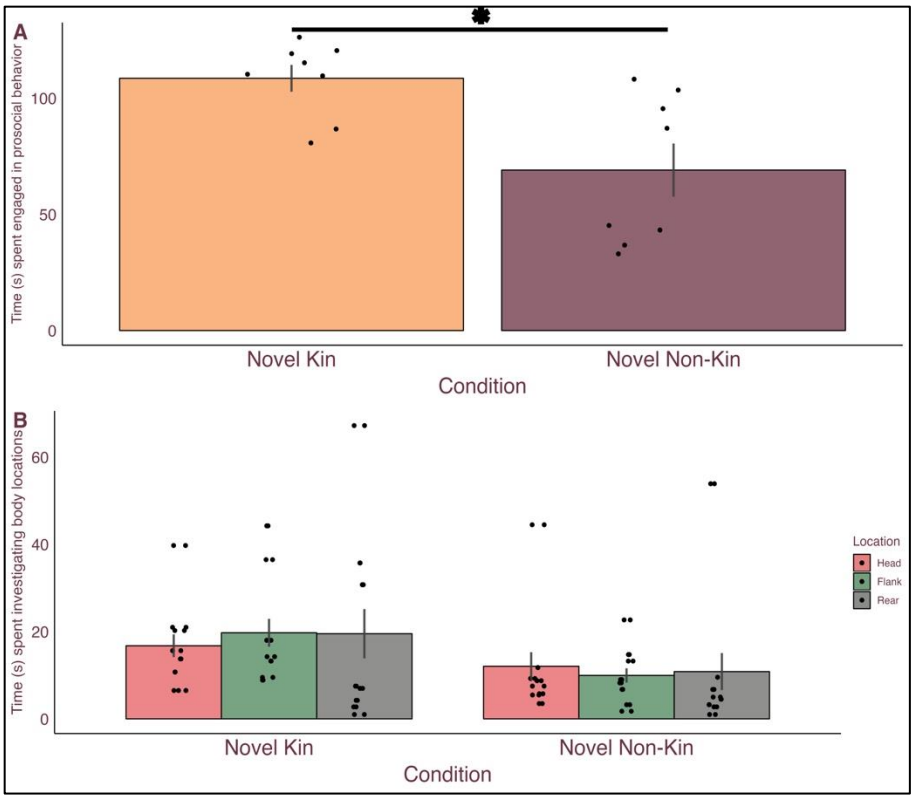


**Figure 2-3. Male spiny mice engaged in more prosocial behavior with novel kin than novel non-kin but did not investigate them differently.**

Male spiny mice mean (± SEM) time in seconds (s) engaged in **(A)** prosocial behavior and **(B)** investigation. Male spiny mice spent more time engaged in prosocial behavior with novel kin (orange) than novel non-kin (plum). Spiny mice displayed a trend towards investigating novel kin more than novel non-kin specifically at the flank (green). Dots represent individual data. * Indicate $P \leq 0.05$.

*Ventral, but not dorsal, LS neural responsivity relates to behavior*

To examine brain-behavior relationships, we conducted Pearson's correlation analyses between the percentage of NeuN-Fos colocalized cells and investigation as well as time spent engaging in prosocial behavior with stimulus animals. We did not observe any significant brain-behavior correlations in males that were exposed to familiar conspecifics (all $P > 0.128$). However, we found a positive correlation between percentage of NeuN-Fos colocalization in the ventral LS and total investigation time ($r = 0.703$, $p = 0.052$) of novel non-kin. Further, we observed

significant correlations between ventral LS NeuN-Fos colocalization and investigation of the flank of stimuli for males exposed to novel non-kin ($r = 0.757$, $p = 0.03$; **Figure S2-2A)** as well as those exposed to novel kin ($r = 0.727$, $p = 0.041$; **Figure S2-2B**). Together, these findings suggest that the ventral LS may play a particularly important role in investigative behavior of novel conspecifics.

## Discussion

Properly recognizing the kinship and novelty status of individuals is an important component of navigating the social environment, especially for large group-living, communally breeding species that encounter a wide variety of conspecifics. Here we showed that spiny mice alter their behavior in group and dyadic contexts based on the novelty and kinship status of conspecifics. As has been shown for many species (e.g. [45–47]), we demonstrated that spiny mice recognize novel kin from novel non-kin; additionally, we showed that males differentially investigate bodily locations of conspecifics based on identity. Further, in a novel group interaction and in dyadic interactions, males preferentially affiliate and engage in prosocial behaviors with novel kin over novel non-kin and familiar kin, suggesting that a general drive to affiliate with novel relatives may have evolved to promote group cohesion in communally breeding species. Even though encountering a novel conspecific involves uncertainty and potentially risk, only kinship status was differentially represented in neural responses within the LS of spiny mice. Together these findings suggest that male spiny mice may have evolved specialized neural circuitry to distinguish kin and behave more prosocially toward them, perhaps to facilitate behaviors such as nepotism.

*Affiliation and conspecific identity in groups*

For species that live in complex, large groups, differentially affiliating with specific individuals based on their identity may confer distinct benefits related to fitness and survival [48–52]. The affiliative preference for novel kin observed in the present study adds new context to our previous findings in which spiny mice did not show a preference in time spent with novel non-kin or familiar kin in a 2-choice test despite altering their behavior based on novelty in a social recognition test [5]. Together, these findings reinforce that male spiny mice exhibit strong social recognition abilities and are not neophobic and demonstrate that social preferences arise when animals are allowed to freely interact. Recognizing kin regardless of familiarity is likely highly beneficial for avoiding inbreeding. Similar to our findings here, female meerkats can discriminate between odors of kin and non-kin and spend more time investigating scents from related than unrelated novel individuals [53]. After an individual recognizes a novel conspecific as kin, behaving more prosocially toward them may confer fitness benefits. Indeed, affiliation and prosociality with even distantly related kin have likely contributed to the fitness of other large group-living species. For example, meerkats [54–56] and prairie dogs [57,58] live in large groups primarily comprised of kin and engage in mobbing and sentinel behavior to protect the group from predators. Favoring kin can thus aid in protection and potentially recruit alloparents for assistance with rearing offspring. Therefore, although animals that live in large groups may affiliate with kin and non-kin, exhibiting a bias to behave more prosocially with kin likely enhances fitness, perhaps without a direct cost to the group as a whole.

*Kinship and prosociality*

Dyadic social interactions allow for more detailed analysis of how individuals interact with specific types of conspecifics. Interestingly, spiny mice did not differ in the amount of time they spent engaged in general prosocial, aggressive, or non-overt behaviors between familiar and novel non-kin conspecifics but did spend significantly more time engaged in prosocial behaviors with novel kin versus novel non-kin. Similar to spiny mice, female Cape ground squirrels, *Xerus inauris*, are a cooperatively breeding species and show similar patterns in kin and non-kin odor discrimination and investigation tasks [47]. Further, Belding's ground squirrels, *Urocitellus beldingi*, also discriminate between novel kin, familiar kin, novel kin, and novel non-kin [45,46]. This trend in discriminating across components of conspecific identity suggests there are important components of familiarity and novelty that are separable.

It is yet unclear why kin status drove differences in the time spiny mice spent engaged in prosocial behavior. One possibility is that novel kin are particularly salient because they are less frequently encountered compared to novel non-kin members. Supporting this possibility, investigation accounted for most of the subjects' prosocial behavior time. Recognition of novel kin in addition to familiar kin can optimize foraging behavior [59], decrease stress [60], and allow for nepotism [61]. Indeed, kin selection is commonly evoked to explain these benefits, as any genetic relation is a potential opportunity for shared genes to move to future generations [62,63]. It is no surprise, then, that many scientists consider kin recognition directly when discussing kin selection [18], though social learning has also been suggested as an alternative mechanism fostering kin recognition [18,64,65]. Regardless, how kin relation alters other behaviors in spiny mice is an open question that future studies should aim to address.

*Pheromonal communication and bodily location of glands*

Rodents produce informational odors and pheromones through glands located across their bodies. For example, mice scent mark with their urine [66], the greater long-tailed hamster, *Tscheskia triton*, and golden hamster, *Mesocricetus auratus*, have left and right flank and midventral glands used for flank marking [67,68], and Mongolian gerbils, *Meriones unguiculatus*, have harderian glands on their face [69] and mid-ventral sebaceous glands for scent and pheromone release [70]. While spiny mice exhibit phenotype matching [19], it is currently unknown where scent glands are located on spiny mice, but most rodents have glands on their face, flanks, and anogenital region (referred to here as "rear") that emit olfactory and pheromonal cues. It is likely that individual glands provide specific cues that vary in salience to conspecifics based on their identity. In our study, male spiny mice investigated the flanks of familiar non-kin conspecifics more than their head and showed a statistical trend towards investigating novel kin members more than novel non-kin both overall and at their flanks. An alternative to olfactory cues, however, is whisker-to-whisker contact, often referred to as "social facial touch" seen in rodents, such as rats [71]. Social facial touch may provide additional information about a conspecific, such as their identity or their recent behavior. Any face investigation with familiar conspecifics in spiny mice may be due to similar motivations. However, more work is needed to locate scent glands on spiny mice and determine their effects on behavior as well as to differentiate head investigation for olfactory cues versus whisker communication.

*The LS and social discrimination*

The LS is increasingly considered a critical region for regulating social behavior [38,43,44], and was recently shown to be topographically mapped for responsiveness to kinship in Long Evans rats [39]. Rats had more non-kin responsive neurons within the dorsal LS and more kin responsive neurons in the ventral LS [39]. In contrast, we found that the spiny mice dorsal LS exhibited greater neural responsivity to novel kin compared to novel non-kin and that the LS did not distinguish between novel and familiar non-kin. These results run counter to those of Clemens et al. (2020) and suggest that there may be species differences in the functional organization of the LS. Additionally, our study controlled for familiarity, while the Long Evans rat study used familiar littermates and mothers as the kin stimuli and novel individuals as the non-kin stimuli. Furthermore, we examined IEG responses from freely behaving animals, which lack the temporal resolution of the single-cell patch clamping of head-fixed animals used in the Clemens et al. (2020) study. Based on these details, differences between our studies may instead reflect methodological discrepancies. Regardless, the dorsal LS appears to play a critical role in social recognition, particularly for distinguishing kinship status in spiny mice.

In our study, the novel kin condition was the only condition that showed both an increase in neural responsivity as well as an increase in prosocial behavior. While we cannot rule out that it was prosocial behavior rather than kin recognition that drove greater neural responses within the dorsal LS, we found no correlation between dorsal LS neural responses and prosocial behavior, which would be expected if prosocial behavior was the primary driver of the differences in neural response. Additionally, because Clemens et al. (2020) found that cells within the dorsal LS distinguish between kin and non-kin, it is likely that our findings in spiny mice also reflect

processing of kinship status rather than greater prosocial responses. Further studies, ideally neuro-manipulative in nature, are required to determine the direct role the dorsal LS and connected regions play in kin recognition.

Our results also show distinct differences in responsiveness to kinship between the dorsal and ventral LS, as well as differences in how neural responses in these subregions of the LS relate to behavior. Interestingly, neural responses in the ventral LS positively correlated specifically with investigation in male spiny mice that interacted with novel conspecifics (both kin and non-kin), but not familiar conspecifics. This brain-behavior relationship suggests that the ventral LS may be particularly important for processing tactile and olfactory information of novel individuals. Although ventral LS neurons may be important for either promoting social investigation or processing novel social information, it is possible that we did not observe global ventral LS neural responsivity differences between animals exposed to novel vs. familiar conspecifics because other social behaviors directed toward the conspecifics did not significantly differ (i.e., we found no differences in prosocial or aggressive behaviors). These findings add to the growing literature depicting the LS as a highly heterogeneous region made up of several subregions. Indeed, while the LS is mostly GABAergic [35,72], there are robust structural and genetic differences between the various subregions [35,43,44,72,73]. These differences have already been shown to have functional consequences outside of social recognition. For example, a small population of neurons within the LS of C57BL/6J mice receives oxytocin from the VTA and promotes aggressive behavior [35], and ventral LS mGlu2/3 receptors promote stress resilience in male C57BL/6J mice [74]. Furthermore, subdivision of the LS is not unique to mammals; neurochemical examination of the LS in finches and waxbills yielded multiple

chemoarchitectonic subzones within the LS, with neurochemical profiles suggesting the LS is involved in an array of social behaviors. Indeed, studies have shown that the LS regulates prosocial behavior [75–78] as well as aggression [35,43,79,80]. Together, these findings highlight the importance of exploring how subregions and cell types within the LS respond to different social contexts rather than treating the LS as a monolithic structure.

*Conclusion*

In the present study, we demonstrated that spiny mice modify their behavior based on the familiarity and kinship status of individuals. Spiny mice exhibit strong social recognition abilities in a novel group setting and preferentially affiliate with novel kin over novel non-kin or familiar kin. This affiliative preference in a group interaction coupled with the exhibition of more prosociality with novel kin over novel non-kin during dyadic interactions suggests a strong bias toward kin even though kin and non-kin maintain positive social relationships in this species. We further showed that the LS distinguishes between kinship, but not familiarity, status, demonstrating that there are distinct types of social recognition that are differentially represented in the brain. Together, our study highlights the complex dynamics of social recognition in the communally breeding spiny mouse and lays a foundation for future studies that may seek to identify the neural underpinnings of novel vs. familiar social discrimination or explore the involvement of motivational systems in social recognition and preferences.

## Materials and methods

### *Animals*

Forty adult male *Acomys cahirinus* (post-natal day (PND) 95-220) were used for behavioral testing and 32 adult male *A. cahirinus* (PND 60-512) were used for immediate early gene (IEG) studies. All animals were obtained from our breeding colony; breeders were from the captive-bred colony of Dr. Ashley W. Seifert (University of Kentucky). Dr. Seifert's colony has been maintained for 10+ years. All animals were group-housed (2-4) in either a standard rat polycarbonate cage (40.64 x 20.32 x 20.32 cm) or a larger two-level polycarbonate cage (32 x 38 x 40 cm) lined with Sani-Chips bedding. Animals were provided with nesting material, rodent igloos, and shepherd shacks and were able to obtain food and water ad libitum. Spiny mice were kept on a 14-h light: 10-h dark cycle with an ambient temperature of 24 ± 2°C. All procedures were approved by the Institutional Animal Care and Use Committee of Emory University (PROTO201900126). All methods were conducted in accordance with relevant ARRIVE guidelines and regulations. **Al**l methods were performed in accordance with the relevant guidelines and regulations. Due to a lack of female spiny mouse availability in our colony, we were only able to conduct this study in males. Future studies will be needed to examine the influence of novelty/familiarity and kinship on spiny mouse female behavior.

### *Experimental Design*

To identify if male spiny mice recognize and behave differently with individuals based on novelty and kinship status, we ran 1 cohort of males through a group social interaction test and a different cohort of males through dyadic social interaction IEG tests. Stimulus mice were color

coded with a small amount of animal-safe marker for unique identification. All tests were video recorded using Sony Handycam HDR-CX405 1080p Camcorders (Sony) for subsequent scoring using Behavioral Observation Research Interactive Software [81] or a modified hand-scoring method. At the end of each group interaction, the testing arena was cleaned with Virkon-S, followed by water and then towel dried (fresh towel each time) to eliminate odors from the previous group. All IEG tests were conducted in separate clean cages.

*Group Social Interaction*

To determine if male spiny mice investigate and affiliate with different conspecific types at different rates in a dynamic social environment, 5 groups of 8 males (PND 80-200) were placed into a novel arena (58L x 120W x 60H cm) containing two transparent rodent igloos and were allowed to freely interact for 1 hr. Each subject was familiar with only one other conspecific - their sibling/cagemate. 2 conspecifics were novel kin, and the remaining 4 animals were novel non-kin. Novel kin were at least 2 litters removed from the subject spiny mice. Time sampling behavioral observations were recorded for each subject every 5 min to obtain frequencies of social contact (i.e., investigation and positive affiliation (i.e., positive side-by-side contact and huddling)) with different conspecific types.

*Immediate Early Gene Studies*

To identify differences in the neural response of the LS based on conspecific identity, 32 male spiny mice (PND 60-512) underwent 1 of 2 IEG studies: (1) males engaged in a social interaction with a novel kin conspecific or a novel non-kin conspecific (kin vs non-kin IEG) or (2) males engaged in a social interaction with a novel non-kin conspecific or a familiar non-kin

conspecific (novel vs familiar IEG). All novel non-kin conspecifics were at least 2 litters apart, ensuring they had never interacted prior to the IEG. Familiar non-kin stimulus animals were obtained by rehousing subjects in a large two-level cage with a divider installed. The cage divider was a Plexiglas barrier that contained 1cm holes to allow for tactile and olfactory communication. Two subjects (i.e., siblings) were housed on one side, and 2 non-kin conspecifics were housed on the other side. Subjects and stimulus animals cohabitated with the divider installed for 7 days prior to undergoing the IEG study.

For the IEG tests, subjects were placed simultaneously into a standard rat polycarbonate cage (40.64 x 20.32 x 20.32 cm) with 1 of 3 possible conspecific types (novel kin, novel non-kin, familiar non-kin). Subjects interacted with the stimulus conspecific for 30 min before being transferred to a second, clean rat cage for an additional 30 min prior to undergoing a perfusion to capture Fos-ir+ expression in response to the stimulus exposure. All interactions were recorded, and the first 5 min scored for prosocial (allogrooming, positive side-by-side contact, huddling, head investigation, flank investigation, and rear investigation), aggressive (biting, chasing, lunging, pinning, rearing, and aggressive side-by-side contact) and non-overt (all remaining time of scored recording) behaviors (**Table S3**). For social investigation, we analyzed behavior by bodily location (flank, head, or rear) as well as all bodily locations of investigation together. The two IEG tests (kin vs. non-kin and novel vs. familiar) were conducted separately, and thus were analyzed as separate tests.

*Histology and Immunohistochemistry*

At the end of both IEG tests, subjects were immediately euthanized by isoflurane overdose and were transcardially perfused with 0.1 M phosphate buffer saline (PBS) followed by 4% paraformaldehyde. Brains were extracted, post-fixed overnight in 4% paraformaldehyde, and underwent cryoprotection in 30% sucrose dissolved in PBS for 48 hrs. Brains were frozen in Tissue-Tek O.C.T. compound and stored at -80°C before sectioning coronally at 40 μm using a Leica cryostat, with every third section saved for use in the present study. Tissue sections were immunofluorescently stained for Fos (the protein of the immediate early gene *cFos;* Synaptic Systems rabbit c-fos 1:1000 dilution) and NeuN, a neuron-specific nucleus marker (Millipore mouse NeuN 2:1000 dilution).

*Neural Quantification*

Photomicrographs were obtained using a Zeiss AxioImager II microscope fitted with an apotome. For LS cell counts, we took 10x images and quantified the total number of Fos-immunoreactive (-ir) and NeuN-ir cells and the number of NeuN-ir neurons that co-expressed Fos across 6 tissue sections for the novel vs familiar IEG and 7 sections for the kin vs non-kin IEG in both the dorsal and ventral LS (note that the tissue section number discrepancy was due to tissue availability, and that data from the 2 IEG studies are analyzed separately) (**Fig. 4**). These tissue sections spanned a-p coordinates of +1.5 mm To +1.78 mm from bregma and d-v coordinates of -4.4 to -3.0 mm from the top of cortex based on a recent stereotaxic atlas [82] Note that hodological evidence of the division of the LS is lacking in spiny mice, however studies in numerous species have demonstrated that there is anatomical and functional subdivision of the LS with strong consistency in dorsal and ventral subdivision in rodents [83]. Thus, while we

capture the dorsal and ventral portions of the LS of spiny mice, we acknowledge that future studies may further refine these subdivisions. FIJI [84] was used to create standard ROIs for all dorsal and ventral LS images, and a cell profiler [85] pipeline was created to automatically count fluorescent cells and nuclei and identify colocalized neurons. The values across all sections were summed and a percentage of the number of NeuN-ir cells that expressed Fos-ir across was used to account for individual differences in cell number. **Tables S4** and **S5** provide group averages for NeuN-ir cell counts. Significant statistical differences were identified between the dorsal and ventral LS and were thus analyzed separately.

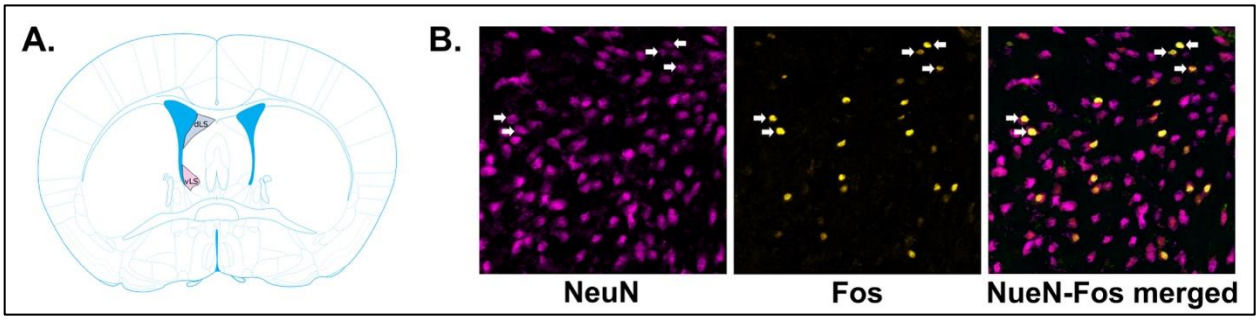


**Figure 2-4. Dorsal and ventral lateral septum regions and representative images of NeuN-Fos colocalization.**

**(A)** Illustration of a mouse coronal section showing the subdivision of the dorsal (dLS; blue) and ventral (vLS; pink) lateral septum (LS) used for cell counts (image edited from Paxinos & Franklin (2001)). **(B)** 40x images in spiny mice of NeuN (left), Fos (center), and merged NeuN-Fos colocalization (Right). White arrows indicate colocalization.

*Statistical Analysis*

Behavioral measurements for each test were analyzed using SPSS 28 (IBM Analytics). For the group social interaction, investigation and affiliation frequencies were transformed to z-scores for each subject by conspecific type to account for the greater number of opportunities to interact with novel non-kin than novel or familiar kin. The use of parametric or non-parametric tests was based on the distribution of the data and Shapiro-wilks tests. Tests used include Poisson

generalized linear mixed model (GLMM) with condition as a fixed factor and subject and group as random factors, general linear models (GLM) with condition and region (ventral versus dorsal) as fixed factors, Friedman's tests, Mann-Whitney U-tests, and Wilcoxon ranked-sum tests. All *post-hoc* pairwise comparisons were adjusted using either the Bonferroni, Sidak, or false discovery rate correction, depending on the statistical test used. The tests used for specific analyses are detailed in the Results. Pearson's correlations were calculated between percentage of colocalization cell counts and behavioral measures. Outliers for each individual test were 3 standard deviations outside the mean and were removed from analyses. Effect sizes for normally distributed data were calculated and reported as Cohen's *d*, whereas effect sizes for nonparametric analyses were reported as r where $r = \frac{z}{\sqrt{N}}$.

**Data availability**

Data are available upon request from the corresponding author.

## Supplementary Materials

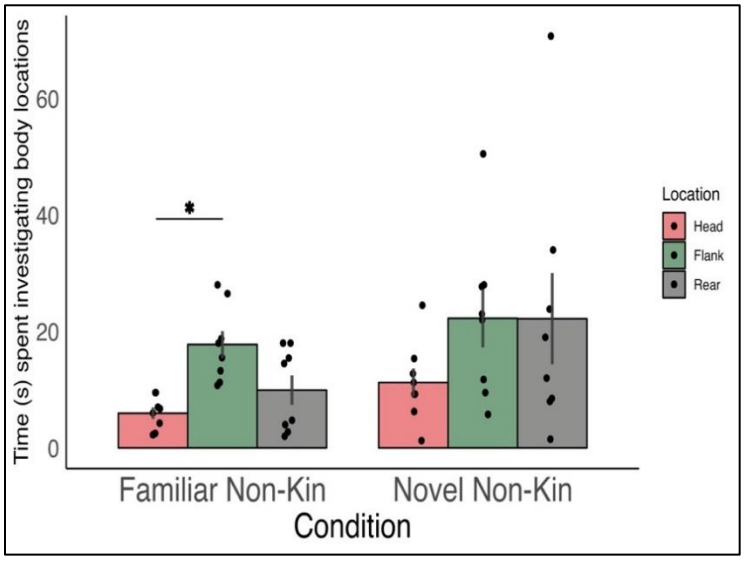


**Figure S 2-1. Male spiny mice differentially investigated parts of the body of familiar but not novel non-kin conspecifics.**

Male spiny mice mean (± SEM) time in seconds (s) for investigating the flank (green), head (pink), and rear (grey) of familiar (left) and novel (right) non-kin conspecifics. Male spiny mice significantly investigated the flank more than the head of familiar non-kin but did not differentiate the bodily location of investigation for novel non-kin. Dots represent individual data. * Indicate $P \leq 0.05$.

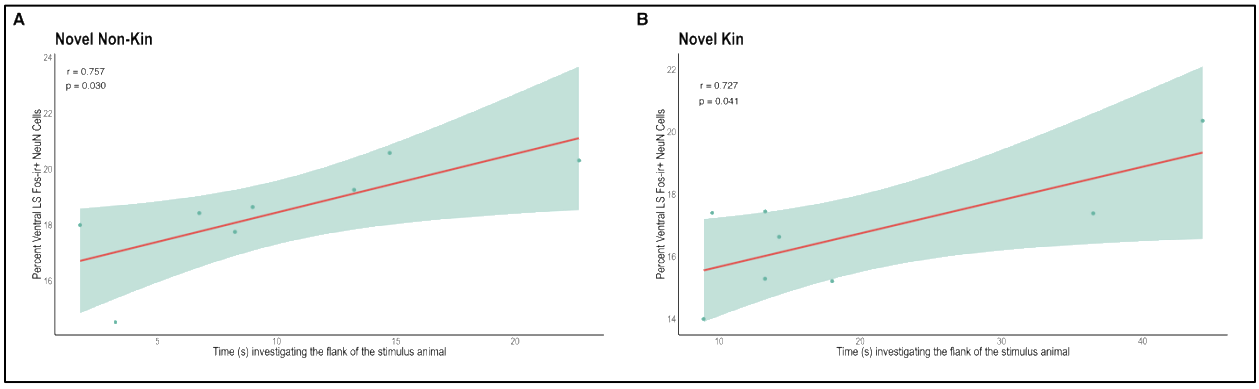


**Figure S 2-2. Neural responses in the ventral lateral septum correlate with investigation of novel kin and non-kin.**

Correlations (± 95% CI in green) between the percentage of NeuN-Fos colocalized cells in the ventral lateral septum (LS) and time in seconds (s) engaged in **(A)** flank investigation of novel non-kin and **(B)** flank investigation of novel kin. Dots represent individual data.

**Table S 2-1. Breakdown of behavior during the novel non-kin vs familiar non-kin social interaction immediate early gene test.**

| Conspecific | Comparison | Test | χ2/Z | P | r |
|---|---|---|---|---|---|
| **Novel Non-Kin** | Overall | Friedman's test | 16 | <0.01* | |
| | Overt Prosocial* vs Overt Aggression | Wilcoxon | -2.521 | 0.012* | 0.88 |
| | Overt Prosocial vs Non-Overt* | Wilcoxon | -2.521 | 0.012* | 0.88 |
| | Overt Aggression vs Non-Overt* | Wilcoxon | -2.521 | 0.012* | 0.88 |
| **Familiar Non-Kin** | Overall | Friedman's test | 16 | <0.01* | |
| | Overt Prosocial* vs Overt Aggression | Wilcoxon | -2.521 | 0.012* | 0.88 |
| | Overt Prosocial vs Non-Overt* | Wilcoxon | -2.521 | 0.012* | 0.88 |
| | Overt Aggression vs Non-Overt* | Wilcoxon | -2.521 | 0.012* | 0.88 |

Note: under comparison * means greater time.

**Table S 2-2. Breakdown of behavior during the novel kin vs novel non-kin social interaction immediate early gene test.**

| Conspecific | Comparison | Test | χ2/Z | P | r |
|---|---|---|---|---|---|
| **Novel Non-Kin** | Overall | Friedman's test | 16 | <0.01* | |
| | Overt Prosocial* vs Overt Aggression | Wilcoxon | -2.521 | 0.012* | 0.88 |
| | Overt Prosocial vs Non-Overt* | Wilcoxon | -2.521 | 0.012* | 0.88 |
| | Overt Aggression vs Non-Overt* | Wilcoxon | -2.521 | 0.012* | 0.88 |
| **Novel Kin** | Overall | Friedman's test | 16 | <0.01* | |
| | Overt Prosocial* vs Overt Aggression | Wilcoxon | -2.521 | 0.012* | 0.88 |
| | Overt Prosocial vs Non-Overt* | Wilcoxon | -2.521 | 0.012* | 0.88 |
| | Overt Aggression vs Non-Overt* | Wilcoxon | -2.521 | 0.012* | 0.88 |

Note: under comparison * means greater time.

**Table S 2-3. Ethogram for immediate early gene dyad social interactions.**

| | Behavior | Description |
|---|---|---|
| **Prosocial** | Head Investigation | Subject sniffing or positively investigating the stimulus animal's head. |
| | Flank Investigation | Subject sniffing or positively investigating the stimulus animal's flanks. |

| | | |
|---|---|---|
| | Rear Investigation | Subject sniffing or positively investigating the stimulus animal's rear. |
| | Allogrooming | Subject grooms the stimulus animal. |
| | Huddling | Subject and stimulus are either touching flanks or criss-crossed on top of each other. |
| | Positive Side-by-Side Contact | Subject showing positive, prosocial contact side-by-side with the stimulus that is not specifically huddling. |
| **Aggressive** | Biting | Subject biting at the stimulus animal, mouth making contact with the stimulus animal's body. |
| | Chasing | Subject aggressively chasing the stimulus animal. Initiator is chaser for entire event. |
| | Pinning | Pinning the stimulus down |
| | Rearing | Subject rearing up on hind paws for either offense or defense. |
| | Aggressive Side-by-Side Contact | Subject and stimulus are touching flanks but in an aggressive manner. May be between aggression bouts. |
| Non-overt | All behavior instances not included in Prosocial or Aggressive. | Subject is not making contact with or exhibiting behavior direct toward stimulus animal. |

**Table S 2-4. Breakdown of NeuN and Fos+ NeuN cells by Conspecific condition and LS subregion for the Novel vs Familiar Non-kin IEG.**

| Conspecific | Subregion of LS | Average Fos+ NeuN | Average NeuN | % of Fos+ NeuN Cells |
|---|---|---|---|---|
| **Novel Non-Kin** | Dorsal | 127.17 | 712.31 | 18.12 |
| | Ventral | 159.56 | 911.875 | 17.56 |
| **Familiar Non-Kin** | Dorsal | 124.73 | 789.71 | 15.73 |
| | Ventral | 104.94 | 661.90 | 15.91 |

Note: Values are rounded to the second decimal place based on descriptive analysis output.

**Table S 2-5. Breakdown of NeuN and Fos+ NeuN cells by Conspecific condition and LS subregion for the Novel Non-Kin vs Novel Kin IEG.**

| Conspecific | Subregion of LS | Average Fos+ NeuN | Average NeuN | % of Fos+ NeuN Cells |
|---|---|---|---|---|
| **Novel Non-Kin** | Dorsal | 138.86 | 635.54 | 21.88 |
| | Ventral | 116.43 | 630.46 | 18.44 |
| **Novel Kin** | Dorsal | 178.70 | 631.73 | 27.65 |
| | Ventral | 114.75 | 689.09 | 16.72 |

Note: Values are rounded to the second decimal place based on descriptive analysis output.

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# General Discussion

The aim of this dissertation was to examine how the brain facilitates large group affiliative preferences and distinguishes between different types of conspecifics within a group. For that purpose, Chapter 1 described a series of experiments using immediate early genes (IEGs), neural tracing, and chemogenetics to identify a neural circuit involved in the modulation of peer group size preferences. Chapter 2 then examined how the brain processes kinship and familiarity during dyadic interactions. These chapters together provide new foundational insights for the growing field of the neuroscience of grouping behavior.

## Summary of Findings

In chapter 1, anterior cingulate cortex (ACC) neurons that project to the lateral septum (LS) were identified via IEG and neural tracing studies to have a larger response, as measured by Fos, to exposure to a large peer group compared to a single same-sex conspecific. Spiny mice typically prefer to affiliate and investigate larger peer groups over smaller ones. However, inhibition of this ACC to LS circuit resulted in a reversal in male spiny mice's group affiliative and investigative preferences. Chapter 2 found that the LS of male spiny mice differentially processes kinship but not familiarity, as measured by Fos during IEG studies. Within the LS, the dorsal, but not ventral, region showed an increase in Fos-ir to novel kin compared to novel non-kin. Behaviorally, male spiny mice also engaged in more prosocial behavior with novel kin compared to novel non-kin in same-sex dyadic interactions. The combined findings of these chapters indicate that a) neural activity within the ACC-LS circuit modulates male spiny mice

peer group preferences, b) the LS is involved in distinguishing conspecific identity, and c) conspecific kinship and familiarity status may alter how spiny mice engage with peers in groups.

## Social Context influences grouping behavior

The social environment and context heavily influence the nonsocial and social behaviors animals engage in. This is true for grouping behaviors as well. For example, to a territorial species, like the small-group living gerbil, exposure to a large group does not result in affiliative behavior with that group[1], while it reliably does so in the large group-living spiny mice[1,2] as well as other gregarious mammals[3–5] and birds[6,7]. To different species that vary in species-typical group size, a group of same-sex conspecifics likely means very different things. For example, to a territorial gerbil, even a small group of novel, same-sex conspecifics would be perceived as a threat and induce behaviors such as social avoidance and/or aggression. Whereas for a colonial spiny mouse, a group of novel, same-sex conspecifics could be an opportunity of a new group to join, which would convey benefits such as group foraging, co-parenting, and group defense from predators. When studying grouping behaviors, it is critical to consider the behavioral ecology of the species of interest. The importance of social context for grouping behavior is not limited to inter-species comparisons. In chapter 1, I noted a sex effect such that, while control animals of both sexes preferred larger peer groups over smaller peer groups, inhibition of the ACC-LS only reversed affiliation preferences in males. One possible explanation for this sex difference could be that the same social context- a large group of novel, same-sex conspecifics- influences grouping behavior differently for male and female spiny mice. In a former study, we showed that during social preference tests *both* males and females prefer to affiliate with novel males over novel females[2], and same-sex aggression has been shown to be more common in female spiny mice[8]. Thus, groups of novel, same-sex conspecifics may induce different behavioral outputs

for male and female spiny mice. Taken together, inhibition of the ACC-LS circuit in a different social context than that used in chapter 1, such as with novel, *opposite-sex* conspecifics, may influence affiliative grouping preferences in females.

Similarly, the effects of the kinship and familiarity status of novel same-sex conspecifics on male spiny mice social behavior detailed in chapter 2 likely play a role in group preferences for *both* sexes. Spiny mice may show increased preferences for groups if they consist of kin rather than non-kin, while familiarity may have more subtle influences on grouping behavior. Regardless, it is clear that the composition of a group influences aspects of grouping behavior.

### The lateral septum bridges social recognition and group preferences

The LS played a crucial role in both chapter 1 and chapter 2. In chapter 1, the LS was central to a circuit that appeared responsive to differences in group size. Further, inhibiting inputs to the LS from the ACC reversed affiliative preferences in males from a preference for larger peer to smaller groups. Chapter 2 identified differences in how the LS processed kinship and familiarity, two aspects of identity that likely play a critical role in group preferences and grouping behavior.

The LS is gaining recognition as a hub for the modulation of social behavior. To date, the region been linked to aggression[9], social exploration[10], flocking[6], prosocial behavior[11,12], and social recognition[13,14] as well as numerous other behaviors. The LS may also be topographically mapped based on kinship in some rodent species[15], further highlighting its role in social recognition. A single region influencing multiple behaviors may be beneficial as a method to promote efficient integration or interaction across related behaviors. In the case of social recognition and group preferences, the composition of individuals within a group is information that is likely taken into account during the neural computations leading to the display of a social

preference. In chapter 1, I suggest that for an organism to display a social preference, they must (1) attend to a social stimuli consisting of at least two choices, (2) process these stimuli and discriminate between them, and (3) affiliate with the most contextually relevant stimulus. Here, the familiarity and kinship status of the individuals obtained via social recognition likely influence the results of step 2. Social behavior hubs like the LS will likely be key to disentangling the neural circuitry of a variety of grouping behaviors.

## Conclusion

Peer group preferences and social recognition skills are key drivers of grouping behavior in large group-living species. My research implicates an cingulate to septal circuit for regulating affiliative peer group preferences in male spiny mice, and I further report a difference in neural response within the dorsal LS of male spiny mice based on the kinship status of novel same-sex conspecifics they are exposed to. These chapters act as a foundation for which future studies within spiny mice can be used to advance our understanding of grouping behaviors.

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