Sex differences in social synchronization of conditioned fear

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Running title: Emotional synchrony differs between sexes

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ABSTRACT

Coordinated behaviors are prevalent in the animal kingdom as they promote the survival of social specie. Meanwhile, the distinct social roles of males and females predict sex differences in behavioral coordination. However, because of its complexity, behavioral coordination and the underlying circuits have not been studied extensively in laboratory animals. Recently, we established a mouse model of social synchronization of conditioned fear and identified the ventral hippocampus to the amygdala pathway as the integrator of the affective and social information. We also found that males synchronized freezing bouts more strongly than females. Here, we investigated sex differences further by manipulating the animals' social cues or emotional states. First, familiarity with social cues increased freezing synchrony in male but not female dyads. Second, the emotional distress from brief immobilization had a sex-dimorphic effect by disrupting freezing synchrony in males and enhancing it in females. Finally, inactivation of the ventral hippocampus abolished fear synchrony in both sexes but elicited negative synchrony in females only. In addition, we evaluated the coordination of snout movement during periods of non-freezing. While males and females did not differ in snout velocities, only females coordinated snout movement, and stress diminished that coordination. These findings reveal that conspecific identity and emotional state are sex-specific modulators of social synchrony, suggesting sex-specific functions within the socio-emotional integrator, which includes the ventral hippocampus and yet unknown circuits.

INTRODUCTION

Coordination of behaviors provides an evolutionary advantage to social organisms. In particular, coordinated responses to threats are crucial for the survival of a social group. The sex differences in behavioral coordination have been reported in humans (Weitz 1976, Lafrance and Ickes 1981, Tronick and Cohn 1989, Fujiwara, Kimura et al. 2019) and proposed to reflect the distinct social roles of males and females, particularly when dealing with external threats (Van Vugt, De Cremer et al. 2007). The coordinated threat responses rely on the affective and social cognition systems. The brain circuits that process affective and social information are presumably intertwined: while social cues modulate emotional state, the emotional state influences the perception of social signals (Taylor 1981, Sandi and Haller 2015, Muroy, Long et al. 2016). However, there are virtually no studies on the neuronal mechanisms of coordinated threat response and possible sex differences, despite ample evidence that social information is a potent modulator of the threat response in rodents (Morozov and Ito 2019)). For example, exposure to a recently stressed conspecific enhances subsequent contextual fear learning (Knapska, Mikosz et al. 2010). Observing the conspecific receiving electrical footshocks acts as an unconditioned stimulus (Chen, Panksepp et al. 2009, Jeon, Kim et al. 2010). Meanwhile, a non-fearful conspecific suppresses the acquisition and expression of conditioned fear (Kikusui, Winslow et al. 2006, Kiyokawa, Takeuchi et al. 2007, Guzman, Tronson et al. 2009).

While these works demonstrated social modulation of fear, they did not study the temporal coordination of response to a specific threat between conspecifics. We recently

established such a paradigm: the animals in dyads coordinate temporal patterns of freezing bouts of one another when exposed to a continuous conditioned stimulus. Because CS is continuous, the dynamic coordination of freezing bouts relies exclusively on the cues from the partner. At the same time, CS is required for freezing, which makes the paradigm suitable for investigating how affective and social factors influence behavioral synchronization.

Given the sex differences in either social or emotional processing (Taylor, Klein et al. 2000, Reyes, Valentino et al. 2008, Bangasser, Reyes et al. 2013, Bangasser and Cuarenta 2021), we also predicted sex differences in the interaction between the social and emotional system, resulting the differences in the fear synchrony. Accordingly, our initial study revealed that males synchronized conditioned fear more strongly than females (). To test that further, we modulated social information using familiar or unfamiliar animals or the emotional state by giving mild stress before the test. We also inactivated vHPC, a potential site for socio-emotional integration. The effects of all these manipulations on fear synchrony were different between males and females, which points to the sex differences in socio-emotional integration and their potential mechanisms.

RESULTS

Social cues modulate freezing in males more strongly than in females

First, we examined how the presence of conspecific during fear retrieval affects fear level. The mice were fear conditioned and then tested one day later twice, together with a partner ("pair") and individually ("single"). To minimize the effects of repeated testing, the interval between two tests was 5 hours, and the order of testing was counter-balanced between "pair -> single" and "single -> pair" sequences (Fig. 1A). There were no sex differences in freezing levels, regardless whether the animals were tested alone or with a partner. The partners' freezing levels correlated when they were tested in the same chamber, and the correlation was more significant in males ($p=0.00019$) than in females ($p=0.02$) (Fig. 1B left). However, there was no correlation when the partners were tested in separate chambers (Fig. 1B right). The freezing differences between male and not female partners were significantly lower when they were tested together than separately ($p=0.04$) (Fig. 1C). These data indicate that social cues modulate fear levels in males more strongly than in females. The mean freezing in the combined male and female group was moderately lower when mice were tested with partners than in isolation (freezing, singles: 51.1±2.8, pairs: 43.4±2.5, $p=0.03$, paired t-test), indicating moderate fear buffering; however, when data were split between sexes, the buffering effects were no longer significant (males: $p=0.08$, females: $p=0.23$) (Fig. 1D).

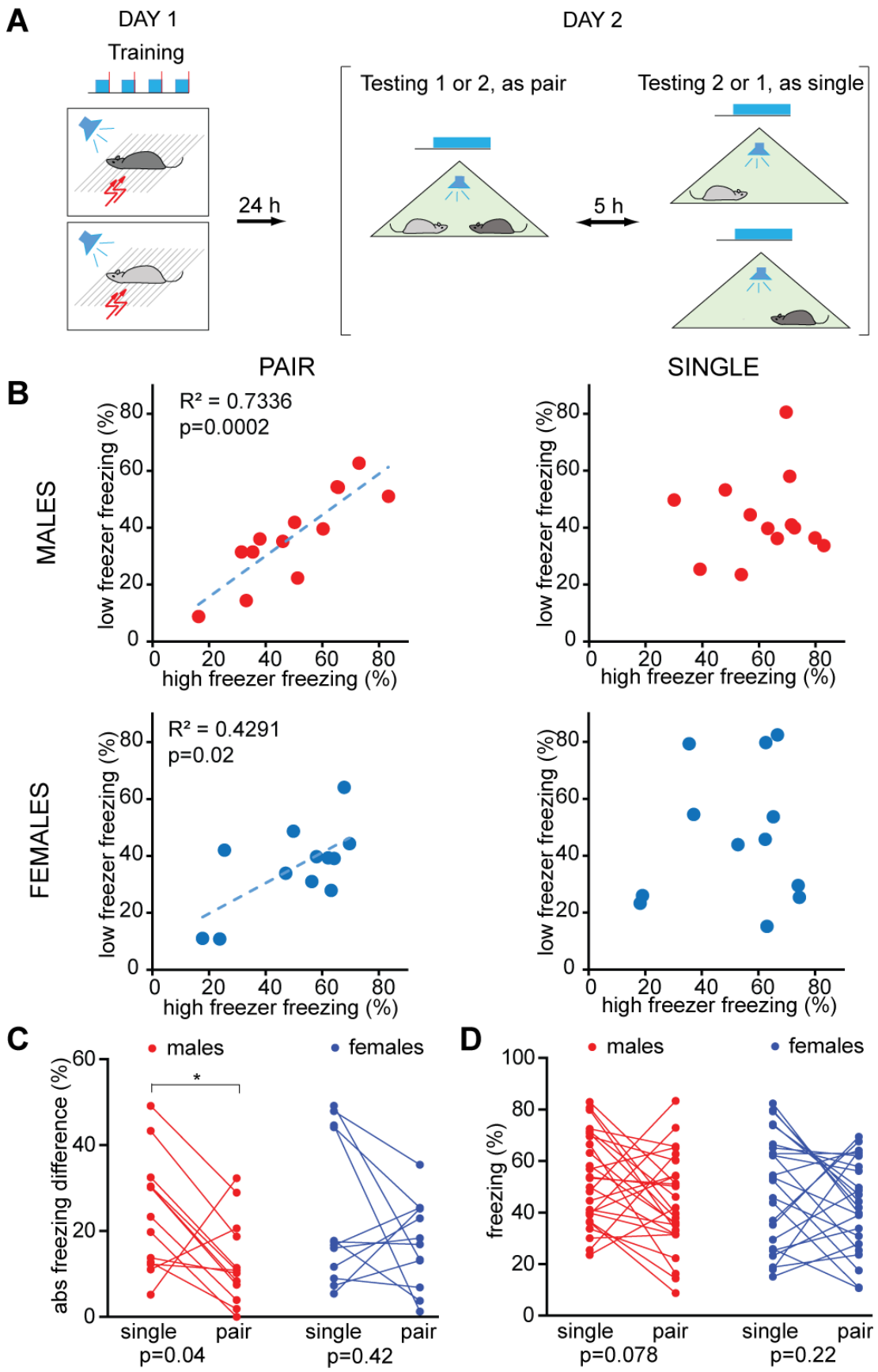
A
DAY 1
Training
24 h
DAY 2
Testing 1 or 2, as pair
5 h
Testing 2 or 1, as single
B
PAIR
SINGLE
MALES
FEMALES
R² = 0.7336
p=0.0002
R² = 0.4291
p=0.02
low freezer freezing (%)
high freezer freezing (%)
C
males
females
abs freezing difference (%)
*
single
pair
p=0.04
p=0.42
D
freezing (%)
p=0.078
p=0.22

Fig1. Males coordinate the level of freezing more strongly than females.

A) Scheme for testing social modulation of freezing. Fear conditioning on Day 1, followed by sequential fear testing in the pair->single and single->pair configurations on Day 2.
B) Freezing of partners correlates when they are tested together (PAIR) but not separately (SINGLE), and the correlation is more significant in males than in females. The animals are assigned as the high and low freezers of the dyad when tested together.
C) Freezing differences between partners tested individually (single) and together (pair). Significant equalization of freezing in males (p=0.04, n=13) but not in females (p=0.43, n=12) tested together. Wilcoxon matched pairs test.
D) Freezing of individual animals tested alone (single) or with a partner (pair). Males tend towards lower freezing when tested with a partner (p=0.08, Wilcoxon matched pairs test, n=26) but females do not (p=0.22, n=24)

Familiarity enhances social synchronization of fear in males but not in females

Next, we asked whether the identity of conspecific affects the synchrony of fear. Several studies demonstrated that familiar rodents modulate threat response more strongly than unfamiliar ones (Jeon, Kim et al. 2010, Kiyokawa, Honda et al. 2014, Pisansky, Hanson et al. 2017), and in one study, familiar males transmitted fear more strongly than familiar females (Pisansky, Hanson et al. 2017). To examine the effects of familiarity, we tested four independent groups of dyads composed of familiar or unfamiliar males or females. Synchrony was significant in all four groups; synchrony was higher in familiar than unfamiliar males (Fig. 2A), but there was no such difference in females (Fig. 2I). Average freezing did not differ between familiar and unfamiliar dyads in both sexes (Fig. 2E, M). The correlation between freezing and synchrony was not significant in all males and in unfamiliar females (Fig. 2B, C, F, G, N, O), whereas in familiar females, synchrony correlated with freezing of the lower freezer but not the high freezer of the dyad (Fig. 2J, K). The correlation suggests that the females with stronger emotional responses exhibit stronger social modulation. The correlation between the freezing levels of the partners was significant in familiar males and all females (Fig. 2D, L, P), but there was no such correlation in unfamiliar males (Fig. 2H).

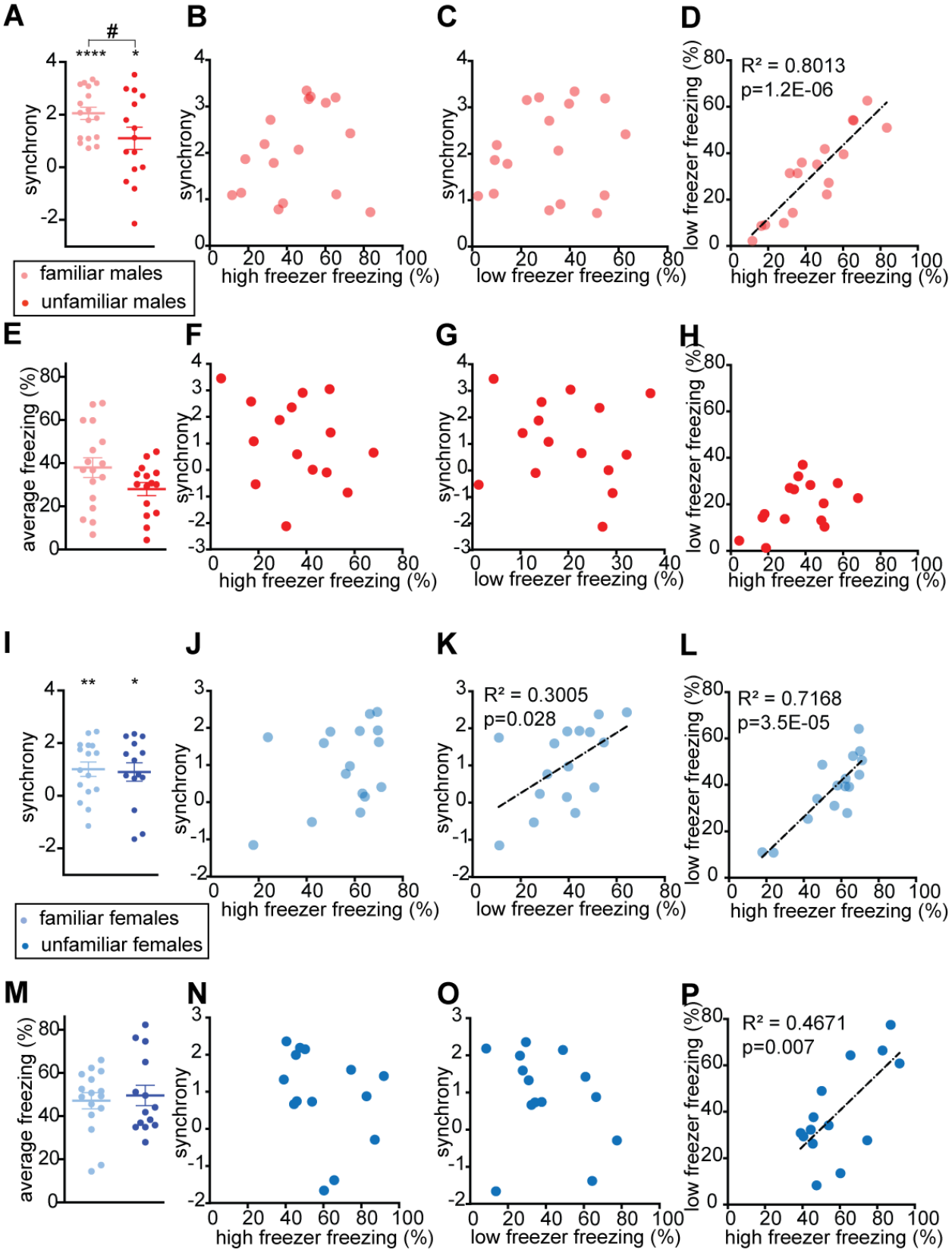
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
#
****
*
**
synchrony
average freezing (%)
low freezer freezing (%)
high freezer freezing (%)
familiar males
unfamiliar males
familiar females
unfamiliar females
R² = 0.8013
p=1.2E-06
R² = 0.3005
p=0.028
R² = 0.7168
p=3.5E-05
R² = 0.4671
p=0.007

Fig2. Familiarity increases freezing synchrony in males but not in females

A, I) Synchrony in males (A) and females (I), familiar (light symbols) and unfamiliar (dark symbols).

E, M) Average freezing in male (E) and female (M) dyads.

B, F, J, N) Scatter plots of synchrony as the function of freezing in the high freezer animal of the dyad in familiar males (B), unfamiliar males (F), familiar females (J), and unfamiliar females (N).

C, J, K, O) Scatter plots of synchrony as the function of freezing in the low freezer animal of the dyad in familiar males (C), unfamiliar males (J), familiar females (K), and unfamiliar females (O).

D, H, L, P) Scatter plot of freezing of the low freezer as the function of freezing of the high freezer of the dyad in familiar males (D), unfamiliar males (H), familiar females (L), and unfamiliar females (P).

The synchrony and freezing data for familiar males and females have been published () and are included for comparison. *$p<0.05$, **$p<0.01$, ****$p<0.0001$, one sample t-test compared to 0; # $p<0.05$, two-sample t-test. The regression lines, correlation coefficients (R^2) and p-values are shown for statistically significant correlations only.

Brief stress exerts opposing effects on fear synchrony in males and females

To examine the effects of emotional state on fear synchrony, we modulated emotional state by brief (5 min) restraint stress (Lolait, Stewart et al. 2007, Newson, Pope et al. 2013) 1 h before testing synchrony (Fig. 3A). For synchrony, the two-way ANOVA detected a strong stress*sex interaction ($F_{(1,60)} = 22.75$, $p<0.001$). In stressed males, the synchrony was not different from 0 and lower than in unstressed males ($p<0.0001$) (Fig. 3B left). In contrast, in stressed females, synchrony was positive ($p=0.0024$) and higher than in the non-stressed females ($p=0.03$) (Fig. 3E left). For freezing, the two-way ANOVA detected no significant stress*sex interaction, and freezing did not differ significantly between stressed and non-stressed females, although there was a tendency for higher freezing after Stress (Fig. 3E, right). In stressed animals of either sex, synchrony did not correlate with freezing (Fig. 3C, F), unlike in non-stressed females (Fig. 2K). There was a notable sex difference in correlation between freezing of stressed partners. The correlation was significant in females (Fig. 3G) but not in males (Fig. 3D), unlike in non-stressed animals, in which the correlation was significant in both sexes (Fig. 2D, L). The data indicate that brief stress weakens the social modulation of freezing in males and strengthens it in females

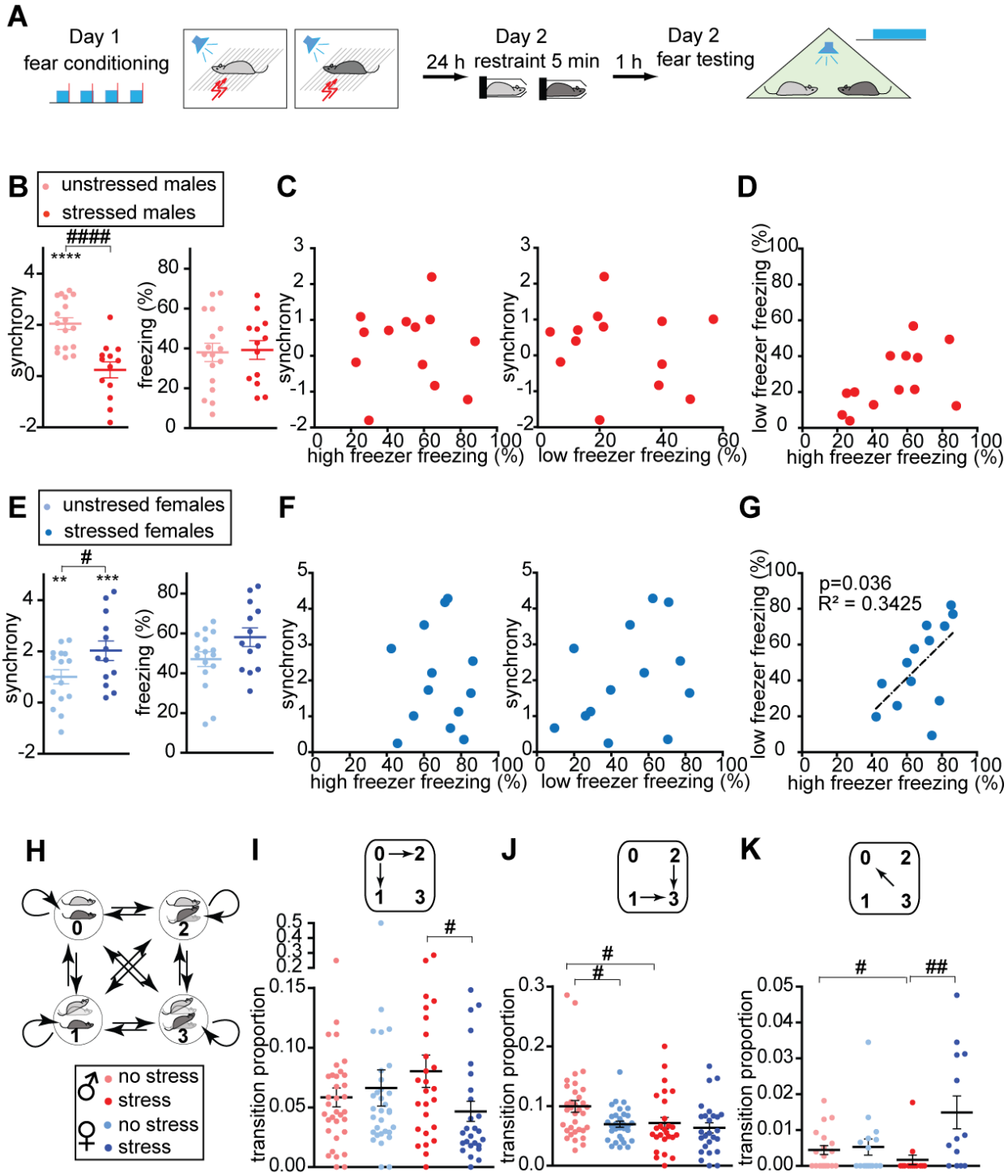

Fig3. Sex-dimorphic effect of stress on fear synchrony.

A) Experimental timeline: fear conditioning training on Day 1 and the 5 min restraint followed by fear testing on Day 2.

B, E) Summary diagrams for synchrony (left) and freezing (right) in males (B) and females (E). Published data for non-restrained animals (submitted) are included for comparisons.

C, F) Correlation between freezing of the high freezer (left) or low freezer (right) and synchrony in males (C) and females (F).
D, G) Scatter plots of freezing of the low freezer as the function of freezing of the high freezer of the dyad in stressed males (D) and females (G).
H) Four behavioral states of a mouse dyad (0,1,2,3): horizontal and angled symbols represent freezing and moving mice, respectively. Arrows depict all possible transitions among the four states.
I-K) Transition proportions diagrams for: 0->1/0->2 (K), 11->3/2->3 (L), and 3->0 (M). Sex and treatments are shown by color.
**p<0.01, ***p<0.001, ****p<0.0001, one sample t-test compared to 0; # p<0.05, ## p<0.01, #### p<0.0001, two-sample t-test for synchrony and Mann-Whitney test for transitions. The regression line, correlation coefficient (R^2) and p are shown for statistically significant correlations only.

To better understand the mechanism of fear synchrony and its modulation, we characterized the dynamics of dyads as transitions among four behavioral states: 0, 1, 2, and 3. For a dyad composed of animals A and B, in state 0, A and B freeze together; in state 1, A freezes and B moves; in state 2, A moves and B freezes; and in state 3, both animals move (Fig. 3J). We define the states 0 and 3 as congruent and states 1 and 2 as incongruent. The one-step transitions from incongruent to congruent states and between congruent states increase synchrony, whereas the opposite transitions decrease synchrony. We computed the proportion of every transition as a fraction of all transitions from the same initial state and found significant effects of sex and stress. The 0->1/0->2 transition, which decreases synchrony, did not differ between unstressed males and females, but in the stressed group, it was higher in males than females (Fig. 3K), consistent with the higher synchrony in stressed females. The 1->3/2->3 transition, which increases synchrony, was higher in non-stressed males than in non-stressed females (Fig. 3L), consistent with the higher synchrony of non-stressed males. However, there was no sex difference in stressed animals. The 3->0 transition is a simultaneous onset of freezing in two animals. However, since there is no external signal that can trigger a simultaneous behavioral change in two animals, the 3->0 transition likely consists of rapid sequences of the 3->1, 1->0 or the 3->2, 2->0 transitions that occur within the 0.25 s video frame interval and represents a very rapid response to the partner's state change. The 3->0 transition was lower in stressed than non-stressed males but higher in stressed than non-stressed females (Fig. 3M), consistent with the higher synchrony in stressed males and lower synchrony in stressed females.

Sex-dependent coordination of non-freezing behaviors

To examine non-freezing behaviors, we measured the velocity of the animal snout as an indicator of movement during the non-freezing periods. If partners in the dyad coordinate movement, the partners' snout velocities are expected to correlate. The mean velocities in males did not correlate in all groups (Fig. 4A, B, C). However, in females, the velocities correlated in the familiar and unfamiliar groups (Fig. 4E, G) but not in the stressed group (Fig. 4F). stress also decreased the velocities in both males (p=0.004) and females (p=0.011) (Fig. 4D, H). However, there were no sex differences within the

non-stressed and stressed groups. The two-way ANOVA revealed a significant sex*familiarity interaction ($F(1, 111)=5.9$, $p=0.017$), and the Turkey multiple comparisons revealed a higher velocity in unfamiliar males than females ($p<0.001$).

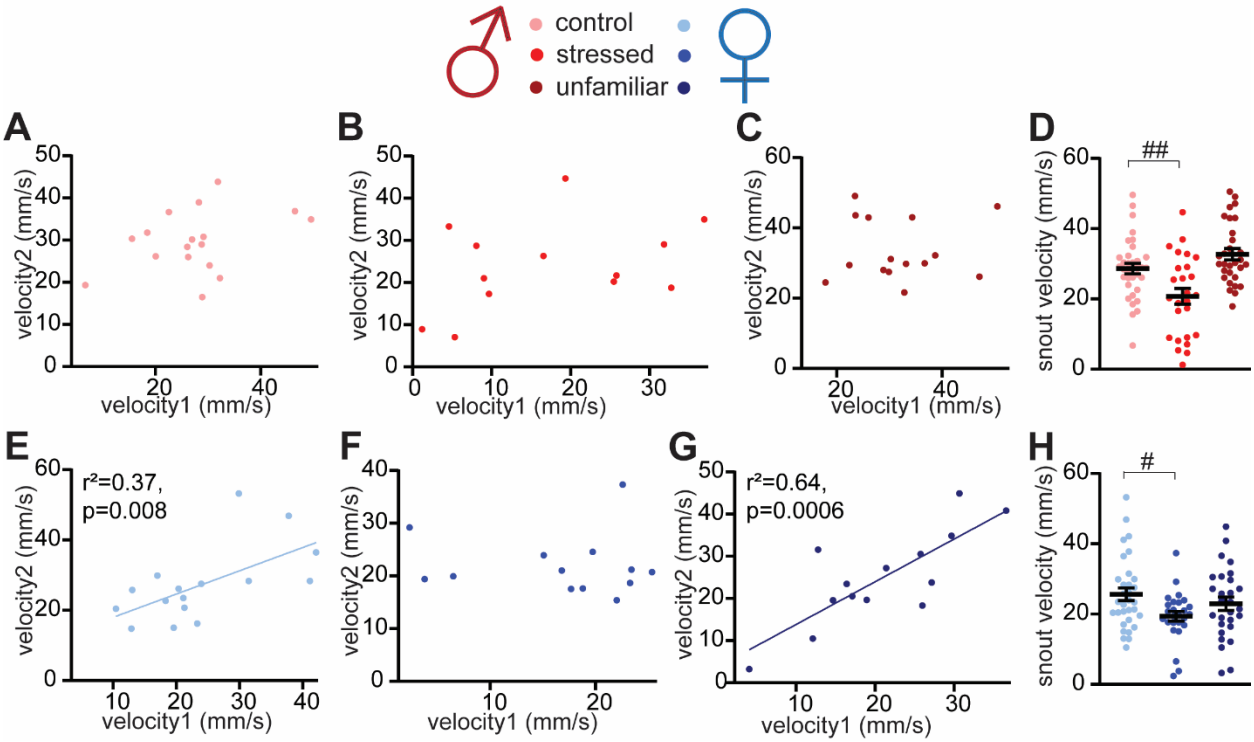

Fig4. Sex-dependent coordination of snout velocity

A-C) Scatter plots of the partners' mean snout velocities of dyads with the control (A), stressed (B), and unfamiliar (C) mice.

D) Male snout velocities comparison diagram.

E-G) Data from female dyads as in A-C.

H) Data from female dyads as in D.

[#] $p<0.05$, [##] $p<0.01$, two-sample t-test. The regression line, correlation coefficient (R^2) and p are shown for statistically significant correlations only.

Copying behavior as a determinant of fear synchrony

Animals achieve perfect synchronization when they rapidly copy each other's actions. Copying can be described as a transition between congruent states 0 and 3, mediated by a non-congruent state 1 or 2, the duration of which is the copying delay (Fig. 5A). Copying delay was higher in females than in males. It was also higher in unfamiliar and stressed males, consistent with their lower synchrony. The data suggest that stress and unfamiliarity with partners attenuate fear synchrony in males by lengthening copying delay. Surprisingly, in females, copying delay was the same in the regular and stressed animals despite the differences in fear synchrony (Fig. 5B). It suggests that stress alters fear synchrony in males and females via non-symmetrical mechanisms.

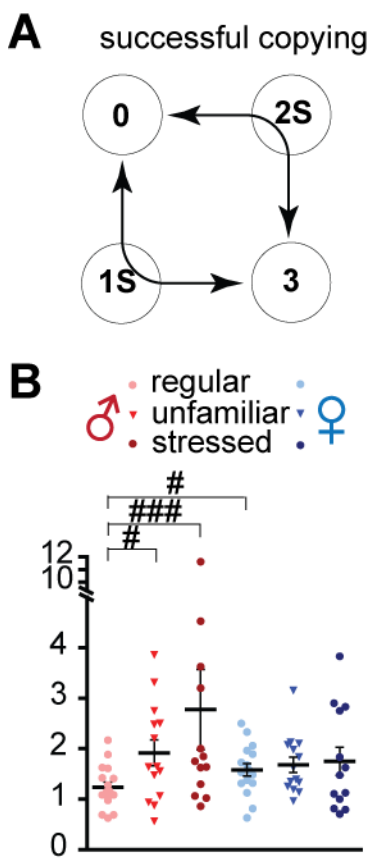

Fig5. Stress and familiarity affect copying delay in males but not in females.
A) Scheme of the copying behavior as transitions between states 0 and 3 via intermediate states 1 and 2.
B) Copying delays as the duration of states 1 and 2. # $p<0.05$, ### $p<0.001$, Mann-Whitney test. Means and SEM are shown by horizontal bars.

Inactivation of ventral hippocampus in females elicits incongruent behaviors

Earlier, we reported that muscimol inactivation of vHPC in males diminishes fear synchrony to zero but has no significant effect on freezing (Fig. 6C, D). Here, we inactivated vHPC in females (Fig. 6A, B). Unlike in males, the mean synchrony in muscimol-injected females was significantly below zero (Fig. 6E), and freezing was significantly lower than in control females (Fig. 6F). Thus, inactivation of vHPC in females not only abolished synchronization of freezing but caused incongruent responses to CS: when one animal of the dyad was moving, another one was freezing. Also, unlike males, females exhibited two significant correlations between freezing and synchrony. In the vehicle-injected females, there was a positive correlation between synchrony and freezing of the lower freezer of the dyad (Fig. 6J, upper), but not the high freezer (Fig. 6I, upper). Surprisingly, in the muscimol-injected females, there was a negative correlation between synchrony and freezing of the high freezer of the dyad (Fig. 6I, middle), but not the low freezer (Fig. 6J, middle). The negative correlation indicates that fear increases

incongruent defensive responses in females with inactivated vHPC. The between-partners correlation of freezing was significant in the vehicle-injected males and females (Fig. 6J, I, lower), replicating the findings shown in Fig. 1D, L. The muscimol-injected males did not show a correlation (Fig. 6H, low). By contrast, the muscimol-injected females did (Fig. 6J, low), suggesting that vHPC inactivation in females does not interfere with the ability to respond to social cues.

We also identified three behavioral transitions affected by muscimol inactivation (Fig. 6O, P, Q). The proportions of the 0->1/0->2 transition, which breaks synchrony, were higher in the muscimol groups of each sex than in the vehicle groups, whereas the proportions of the reverse 1->0/2->0 transition were lower, which explains the lower synchrony in both sexes with inactivated vHPC. Furthermore, only in females the proportion of the 1->2/2->1 transition, which requires opposing actions at the same time, was higher in the muscimol groups than in the vehicle control; it explains the negative synchrony of the muscimol-injected females. The findings suggest that vHPC is necessary for fear synchronization in both males and females. However, while in males, inactivation of vHPC abolishes social modulation of fear, in females, it switches the response to social cues from congruent to incongruent.

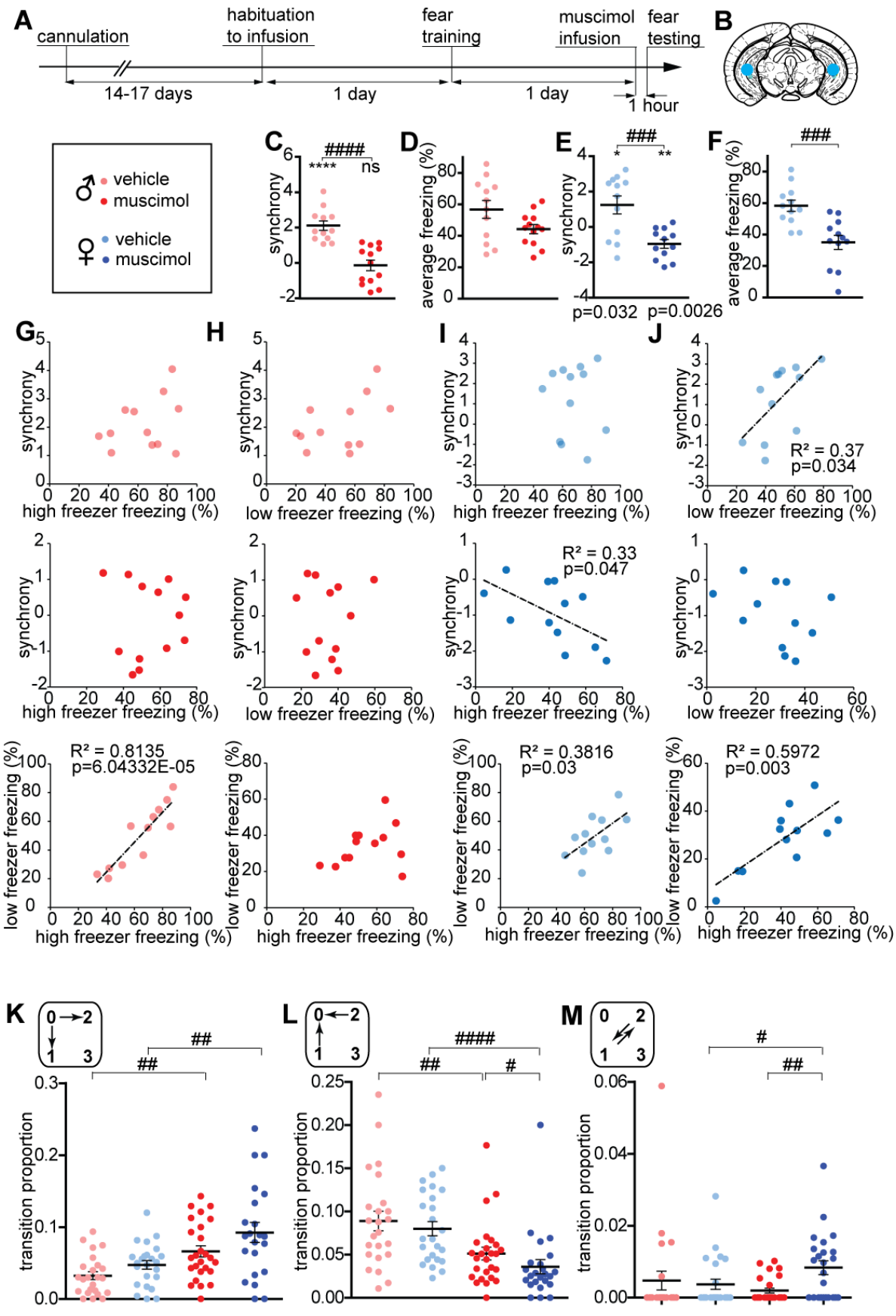
A
cannulation
habituation to infusion
fear training
muscimol infusion
fear testing
14-17 days
1 day
1 day
1 hour
B
vehicle
muscimol
vehicle
muscimol
C
synchrony
D
average freezing (%)
E
synchrony
p=0.032
p=0.0026
F
average freezing (%)
G
H
I
J
synchrony
high freezer freezing (%)
low freezer freezing (%)
R² = 0.37
p=0.034
R² = 0.33
p=0.047
R² = 0.8135
p=6.04332E-05
R² = 0.3816
p=0.03
R² = 0.5972
p=0.003
low freezer freezing (%)
K
L
M
transition proportion

Fig6. Freezing synchrony after vHPC inactivation
A) Experimental timeline.
B) Muscimol injection in vHPC, the target area is shown as blue circles.
C-F) Summary diagrams of synchrony (C, E) and average freezing (D, F) in males, n=12/13 for vehicle/muscimol, and females (E, F), n=12/12 for vehicle/muscimol.
G-J) Scatter plots of synchrony as the function of freezing of the high freezer of the dyad (G, I) and the low freezer of the dyad (H, J) in males (red symbols) and females (blue symbols) injected with vehicle (upper row, light color) or muscimol (lower row, dark color). Linear regression, R^2 and p-values are shown for significant correlations.
K-M) State transition diagrams for: 0->1/0->2 (K), 11->3/2->3 (L), and 3->0 (M).
*p<0.05, **p<0.01,****p<0.0001, one sample t-test compared to 0; # p<0.05, ### p<0.001, ## p<0.01, #### p<0.0001, two-sample t-test for C, E, R, K and Mann-Whitney test for L, M. The correlation coefficient (R^2) is shown for statistically significant correlation only.

MATERIALS AND METHODS

All experiments were performed according to a Virginia Tech IACUC-approved protocol.

Animals

Breeding trios of one C57BL/6N male and two 129SvEv females produced 129SvEv/C57BL/6N F1 hybrid male and female mice weaned at p21 and housed four littermates per cage of the same sex as described (Ito, Erisir et al. 2015). Subjects underwent tests at p75-p90. 7-10 days before testing, mice were split two per clean cage with a quarter of the nesting material from the originating cage.

Fear conditioning

Mice in each dyad were trained independently in two separate conditioning chambers (Med Associates, St. Albans, VT) as described (Pan, Vautier et al. 2008) and then tested together as dyads in a single chamber. Each animal spent the first 2 min in the chamber without stimuli and then received four pairings of the conditioned stimulus (CS) and unconditioned stimulus (US) given in variable intervals (60-180 s). The CS was a 30 s, 8 kHz, 80 dB tone, and the US was a 0.5 mA 0.5 s electrical shock co-terminated with CS. Mice returned to the home cage 30 s after the last CS-US pairing. Cued fear was tested once one day later. First, the animals spent 1 min in a new context without CS and 2 min with CS. Videos were recorded at four frames per second, exported as AVI files with MJPEG compression using the Freezeframe system (Actimetrics, Wilmette, IL), and then converted to the mp4 format using a Python script.

Quantification of freezing synchrony

Freezing synchrony was defined as the standardized difference between the observed and chance freezing overlaps and quantified as previously described (Ito, Palmer et al. 2023). Briefly, annotators, unaware of the treatment of the animals, manually recorded the first and last video frames of each freezing bout using a Python script. A freezing bout was defined as the lack of movement except for respiration for at least four consecutive video frames. From the annotation, another Python script calculated the freezing duration for each animal, freezing overlap, chance overlap, and freezing synchrony for each dyad.

Quantification of transitions among behavioral states

The behavioral states were sampled with every video frame (4 per second), giving 480 samples and 479 transitions for the 2 min session. The occurrence of the behavioral transition of interest was calculated by dividing the counts of that transition by the total number of all transitions from the corresponding initial state, including the transitions to that initial state.

Animal tracking and estimation of snout velocity

For each video frame, the X-Y coordinate of the snout projection on the chamber floor was estimated using a triangular similarity-based formula, the pixel coordinates of the snout, the three-chamber corners, and the actual coordinates for each corner. Ground velocity in cm/s was calculated for every 0.25 s interval as the distance between the projection points from the consecutive video frames divided by the interframe interval of 0.25 s. The mean velocity during CS was calculated only for the periods of movement.

Intracranial infusion of muscimole

Cannulation. Ten to 14 days before fear conditioning, mice received implantation bilaterally with guide cannulas (Plastic One, Roanoke, VA) to target vHPC (from bregma: -3.3 and -3.2 mm posterior for males and females, respectively, ±3.3 mm lateral, from the brain surface -2.8 mm ventral). Dummy cannulas were placed in the guide cannulas to prevent clogging.
Intracranial infusion. One day before fear conditioning training, mice were habituated to infusion using the vehicle: (in mM) 150 NaCl, 10 D-glucose, 10 HEPES, 2.5 KCl, 1 MgCl2, pH 7.35 (Stachniak, Ghosh et al. 2014) in the home cage in the presence of the partner. The infusion cannula extended 1 mm over the guide cannula. The infusion volume was 150 nL per site, and the infusion rate was 75 nL/min. Muscimol (1.17 mM) or vehicle were infused one hour before fear testing. Both mice in the same dyad received identical infusions.
Cannula position verification. Mice were anesthetized with 2.5% Avertin, received an intracranial infusion of Chicago Sky Blue (0.2 %), followed by transcardial perfusion with 4% paraformaldehyde. Visible light microscopy identified the site of cannulation.

Data analysis

Statistical analyses were performed using GraphPad Prism 5 (GraphPad Software, La Jolla, CA). Normality was tested using the Shapiro–Wilk test. Datasets with normal distribution were compared using the one-sample or two-sample t-test. The datasets with non-normal distribution were compared using the Wilcoxon signed-rank test and Mann–Whitney test. All the tests were two-sided. The two-tailed p-value was calculated for the Spearman correlation analysis. The effects were deemed significant with $p < 0.05$.

Data and code availability

All primary data, including video files, are available from authors upon reasonable request. In addition, codes for data analysis and statistics are provided with example data as part of the replication package. It is available at https://github.com/wataruito/codes_in_Emotional_sync_Ito_et_al.

DISCUSSION

The key finding of this study is that the interaction between social and emotional systems of the brain differs between males and females but relies on the ventral hippocampus in both sexes. We investigated those interactions using a recently established mouse paradigm for social synchronization of auditory-conditioned fear (Ito, Palmer et al. 2023). We measured emotional response to conditioned stimulus as the function of social cues and responses to social cues as the function of emotional state.

To study social modulation of emotional response, we varied two social parameters: the presence of conspecific and conspecific familiarity and measured freezing and freezing synchrony. In both sexes, freezing correlated between partners tested together but not in separate chambers (Fig. 1B). It indicates that social cues modulate emotional response in both sexes. However, the correlation was more significant in males, and a related measure, reduction in freezing differences when tested together, was significant only in males (Fig. 1C). It suggests that males coordinate freezing levels more strongly than females, which explains the higher fear synchrony in males. The Blanchards' lab reported lower sociability in females (Defensor, Pearson et al. 2011), which is a plausible explanation of a weaker social modulation of fear in this study. However, several other studies revealed no sex differences in sociability (Yang, Clarke et al. 2009, Silverman, Tolu et al. 2010). Another social parameter, the familiarity of conspecific, influenced fear coordination in males but not females. In unfamiliar males, fear synchrony was lower (Fig. 2A), and freezing levels did not correlate between partners (Fig. 2H). By contrast, in unfamiliar females, synchrony was the same as in familiar females (Fig. 2I) and freezing levels correlated between partners (Fig. 2P). It is unclear why unfamiliar males exhibited weaker coordination of freezing. However, it is consistent with the studies on observational fear learning, fear conditioning by proxy, and fear buffering, in which social modulation of threat response was more robust in familiar conspecific (Jeon, Kim et al. 2010, Kiyokawa, Honda et al. 2014, Pisansky, Hanson et al. 2017, Agee, Jones et al. 2019). Interestingly, in this study, familiarity had no effect on synchrony in females, which may reflect sex differences in emotional perception of

unfamiliar conspecific. Perhaps, males, more than females, perceive unfamiliar animals of the same sex as competitors and a threat, which may interfere with the coordination of fear response.

To study the reciprocal process and emotional modulation of the response to social cues, we modified the emotional state by the restraint Stress and measured fear synchrony. In males, stress abolished fear synchrony (Fig. 3B). The loss of synchrony in stressed males coincided with longer copying delay (Fig. 4B) and fewer simultaneous transitions from movement to freezing (Fig. 3K), and loss of freezing correlation between partners (Fig. 3D). It indicates that stress impairs the ability of males to respond to social cues. By contrast, in females, stress increased fear synchrony (Fig. 3E) and did not disrupt the correlation of freezing between partners (Fig. 3G). While the mechanism of the sexually dimorphic effects of stress remains unknown, there are signaling molecules whose behavioral effects differ between males and females (reviewed in (Bangasser and Cuarenta 2021). For example, oxytocin, induced by Stress (Takayanagi and Onaka 2021), enhances prosocial behaviors in females but anxiety-like behaviors in males (Li, Nakajima et al. 2016). In addition, the sex dimorphic effects of acute stress on the hippocampal areas CA1, CA2, and CA3, in which an indicator of neuronal activity pCREB declines in males but not females (Lin, Ter Horst et al. 2009), could explain the male-specific loss of synchrony because fear synchrony requires neuronal activity in vHPC.

Besides the coordination of freezing, mice also coordinated the snout velocities, which represent movement. However, only females exhibited this form of coordination. Notably, stress had opposite effects on two forms of behavioral coordination in females: it diminished the coordination of movement but enhanced the coordination of freezing. It suggests that separate brain circuits, distinguished by differential stress modulation, coordinate freezing and movement.

Other prosocial phenomena disrupted by stress in males include social approach (Zain, Pandy et al. 2019) and emotional contagion of pain (Martin, Hathaway et al. 2015); however, females have not been tested in those experiments. Additional evidence that aversive emotions strengthen social synchronization in females but not males comes from analyzing the correlation between freezing and synchrony. Because freezing is a proxy measure of the aversive emotions, that correlation in non-stressed females suggests that the emotional state in females determines their ability to respond to conspecific (Fig. 2K). At the same time, there was no such correlation in males. The stressed females no longer showed that correlation, possibly because stress brought their aversive emotional state to the ceiling.

We previously found that vHPC is required for fear synchrony in males (Ito, Palmer et al. 2023) (Fig. 5C). Here, we confirmed that vHPC functions in females (Fig. 5E). However, the females with inactivated vHPC had three functional differences from males. First, they exhibited negative synchrony; when one animal in a dyad was freezing, another was moving (Fig. 5M). A plausible explanation for this non-congruent behavior is the perception of conspecific movement as a threat. Second, the negative synchrony correlated with freezing (Fig. 5I, middle), suggesting that fear in females strengthens the response to social cues. Third, (Fig. 5H, bottom) muscimol did not disrupt the correlation between partners' freezing (Fig. 5J, bottom), indicating that females without vHPC can coordinate freezing levels. By contrast, vHPC inactivation in males abolished synchrony and coordination of freezing levels. These findings suggest that while females possess

hippocampus-dependent and hippocampus-independent pathways for social modulation of fear, males rely mainly on the hippocampus.

Finally, vHPC inactivation in females significantly reduced freezing in addition to modulating fear synchrony. In males, the reduction did not reach significance. The weaker effect on freezing in males is consistent with our recent finding that disconnection of BLA from vHPC attenuates conditioned freezing in females but not in males (Ito, Palmer et al. 2023). However, the published reports about vHPC role in cued fear expression are conflicting and limited to rats. Thus, Sierra-Mercado et al. found a decrease in the cued freezing following the inactivation of vHPC in male rats (Sierra-Mercado, Padilla-Coreano et al. 2010). In contrast, Chen et al. found no such effect (Chen, Foilb et al. 2016), and there have been no published comparisons between sexes.

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