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Prefrontal ensembles represent freezing of conspecifics

Abbreviated title: MPFC cells represent freezing of conspecifics

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Abstract

Social buffering is a process in which the presence of other members of the same species (conspecifics) decreases stress reactions during threat exposure. In parallel, prior reports showed that groups of individuals display synchronous actions in non-threatening situations. However, it is unknown if individuals also synchronize actions when facing threats. Prior studies showed social buffering of freezing in pairs of subjects. We now expand these findings and show social buffering of freezing during exposure to a predator and a shock grid in mice of both sexes in a larger group of four conspecifics. Furthermore, mice also displayed synchronized freezing. The presence of freezing synchrony suggests the existence of a neural representation of conspecific freezing. We reasoned that this encoding might be in the medial prefrontal cortex (mPFC), as this region has been implicated in social behaviors and freezing. Strikingly, mPFC neural activity represented the average level of freezing across conspecifics. MPFC ensembles displayed differential encoding of self and conspecific freezing, and during predator exposure the mPFC encoded the number of conspecifics freezing and conspecific freezing bout duration. These data show, for the first time, neural representation of conspecific freezing, and suggest that the mPFC may coordinate behavioral responses to threat across conspecifics.

Significance Statement

Social animals display attenuated threat-elicited responses when encountering threats as a group. However, it is unknown how freezing signals from conspecifics are encoded by the brain when facing threats, as prior work has been limited to studies of pairs, or assays without social interactions. We identify a neural population in the medial prefrontal cortex (mPFC) that represents conspecific freezing during grouped threat exposure, and these cells dynamically represent the duration of other animals' freezing bouts. Strikingly, the activity of these ensembles also represents the number of conspecifics freezing. These findings uncover a previously unknown function of the mPFC to encode conspecific freezing.

Introduction

Social interactions are ubiquitous throughout much of the animal kingdom; among conspecifics – that is, members of the same species – social interactions vary widely in their complexity, ranging from minimal encounters such as brief breeding engagements such as in bears, to intricate societies, such as in humans (Krause and Ruxton, 2002; Rocha et al., 2021; Anon, 2024). Evolutionary perspectives suggest that sociality confers distinct survival advantages whereby conspecific behaviors influence and tune individual responses that minimize the risk of predation and other threats (Rubenstein, 1978; Strodl and Schausberger, 2012; Guindre-Parker and Rubenstein, 2020). Interestingly, threat-elicited responses are reduced in the presence of conspecifics in a phenomenon known as social buffering (Taylor, 1981; Kikusui et al., 2006; Flannery et al., 2017). This effect of social buffering has been documented in mice (Parise et al., 2022), zebrafish (Faustino et al., 2017), insects (Sanmartín-Villar, 2023), non-human primates (Sanchez et al., 2015) and humans (Yirmiya et al., 2020; Qi et al., 2021). However, it is unknown how animals encode conspecific information when experiencing threat exposure.

In the presence of conspecifics, individuals display coordinated actions at levels higher than expected by chance (Hasson and Frith, 2016; Murakami et al., 2022), indicative of group coordination. The simplest form of group coordination is the temporally aligned co-incidence of behavior, or behavioral synchrony (Mayo and Gordon, 2020). Behavioral synchrony (Feldman and Rimé, 1991; Koul et al., 2023) is present during sharing of affective states (Fuhrmann et al., 2014), or emotional mirroring (Vartanov et al., 2023). However, most assays interrogating synchrony measure unidirectional sharing of states between pairs of subjects (Pärnamets et al., 2020). This limits our understanding of how synchrony is represented when a larger group of conspecifics encounter a threat.

83

84 Social buffering and synchronized freezing responses presumably require the representation of conspecific
85 freezing. The medial prefrontal cortex (mPFC) is a particularly promising candidate for this role, as it regulates
86 social processes such as social novelty preference (Liu et al., 2025) and supports conspecific recognition through
87 its reciprocal connections to the nucleus reuniens (Chen et al., 2024). Moreover, excitation of socially-activated
88 prefrontal neurons induces social buffering of threat-induced behavior (Gutzeit et al., 2020). This region also
89 encodes key social information such as hierarchical rank within a group (Padilla-Coreano et al., 2022). However,
90 previous studies have not uncovered if mPFC cells represent conspecific freezing. This gap is potentially due to
91 the lack of an assay where multiple conspecifics are simultaneously exposed to the same aversive stimulus.

92

93 Here, we present a novel multi-animal experimental paradigm where four mouse conspecifics are simultaneously
94 exposed to a live predator, which is a highly ethologically relevant situation. Grouped mice displayed social
95 buffering of freezing that was not explained by interruptions due to conspecific social interaction, and their
96 freezing responses were synchronized at levels greater than expected by chance. These findings did not require
97 prior familiarity and were seen in mice of both sexes. We further show that mPFC neurons differentially encoded
98 freezing of conspecifics and self freezing behavior. Cortical activity also encoded the duration of conspecific
99 freezing bouts and the number of conspecifics freezing in real-time. This report defines a previously
100 unrecognized role of the mPFC in encoding conspecific freezing, suggesting this structure may possibly have a
101 function in using social information to guide behavior in threatening situations.

102

103 **Methods**

104 All procedures were approved by the University of California, Los Angeles Institutional Animal Care and Use
105 Committee, protocols 2017-011 and 2017-075.

106

107 **Mice**

108 Mice (*Mus musculus*) of the C57BL/6J strain of both sexes (Jackson Laboratory stock No. 000664) between 2
109 and 6 months of age were used for all experiments. Mice were maintained on a 12 hr reverse light-dark cycle
110 with food and water ad libitum. Mice were handled for a minimum of 5 days prior to any behavioral task. All mice,
111 including mice that were exposed to each assay alone as part of the "single" cohort, were group-housed in cages
112 containing four mice. Accordingly, "grouped" mice were group-housed cage-mates of four mice for all behavioral
113 assays. All cages were composed of mice of the same sex. Cages were randomly assigned to be part of the
114 single or grouped conditions. For the miniscope experiments, one mouse per cage was randomly assigned to
115 be implanted with the miniscope.

116

117 **Number of mice used and exclusion criteria**

118 Rat and toy rat: n = 24 single mice, 9 grouped sessions, 4 mice per grouped session (36 unique grouped mice
119 in total).

120 Shock grid: n = 10 single, 8 grouped sessions, 4 mice per grouped session (32 unique grouped mice in total).

121 Miniscope recording 6 mice, 653 cells, one rat session recording per mouse.

122 All mice were exposed to either the rat or the shock grid assay only once. The same mice were exposed first to
123 toy rat and then live rat, 24 hours later.

Animals were excluded due to mistargeting of the miniscope lens implant or due to unexpected deterioration in mouse health. No mice were excluded from any group.

Rats

Male Long-Evans rats (250-400 g) were obtained from Charles River Laboratories and were individually housed on a standard 12 hr reverse light-dark cycle with food and water *ad libitum*. Rats were only used as a predatory stimulus presentation.

Surgeries

Eight-week-old mice were anesthetized with 1.5-3.0% isoflurane and affixed to a stereotaxic apparatus (David Kopf Instruments). A scalpel was used to open an incision along the midline to expose the skull following fur removal and site preparation by alternating Betadine and 70% isopropyl alcohol. After performing craniotomy, 250 nl of AAV9-CaMK2 α -GCaMP6f was injected into the mPFC (coordinates from skull surface): AP 1.9 mm, ML $\pm$ 0.35mm, and 2.2mm DV. Five days after virus injection, animals underwent a second surgery in which two skull screws were inserted and a microendoscope was implanted above the injection site. A 0.5 mm diameter, ~4 mm-long gradient refractive index (GRIN) lens (Inscopix, Palo Alto, CA) was implanted above the mPFC at 2.0mm DV. The lens was fixed to the skull with cyanoacrylate glue and adhesive cement (Metabond; Parkell, Edgewood, NY, USA). The exposed end of the GRIN lens was protected with transparent Kwik-seal glue and animals were returned to a clean cage. Two weeks later, a small aluminum base plate was cemented onto the animal's head on top of the previously formed dental cement.

Behavior and miniscope recordings

All behavior videos were captured at 30 frames/s in standard definition (640 x 480) using a Logitech HD C310 webcam. For miniscope experiments, videos were captured using a custom-built, head mounted UCLA miniscope. Open-source UCLA Miniscope software and hardware (<http://miniscope.org/>) were used to capture and synchronize the neural and behavioral videos. There were 6 mice in which miniscope recordings from mPFC cells were obtained. Mice were only exposed to the rat once, so only one rat session per mouse was used, and in total 653 cells were recorded, over 6 sessions (1 session per unique mouse). All cells were used in all the analyses, including the GLMs. The median number of cells recorded in each session was 108. Recordings were only obtained in the rat assay and not in the shock grid or toy rat assays. Three mice of each sex were used for miniscope recordings. This sample size does not allow identification of sex-specific effects, when considering the individual animal as the independent biological replicate.

Rat exposure assay

For rat experiments, mice were habituated to an empty white rectangular box (75 cm length, 30 cm width, 45 cm height) for 2 days in periods lasting 10 minutes. Mice used in the single cohort were habituated alone while mice from the grouped cohorts were habituated with their cage mates. On the following day, mice were exposed to the same environment for 10 minutes but with a toy rat. Twenty-four hours later, mice were exposed to an adult rat in this same environment. The rat was secured by a leash tied to one of the walls and could not freely ambulate outside of a limited area. Rats were handled for several weeks prior to use, and were screened for low aggression to avoid attacks on mice. Rats were gently tied with a leash connected to the upper corner of the box. The leash allows all normal rat ambulatory motions, but in a perimeter restricted by the leash. The leash is

not too tight, as this makes the rat stressed, inducing it to vocalize and make high acceleration movements to break free, which in turn create erratic behavior in mice across sessions. We only used well-handled docile rats that were able to comfortably rest and explore during the entire session, as we have done previously (Reis et al., 2021, 2024; Wang et al., 2021; La-Vu et al., 2022; Tobias et al., 2023; Torossian et al., 2025). Mice were placed near the wall opposite the rat and freely explored the environment. No separating barrier was placed between the mice and the rat, allowing for close naturalistic encounters that can induce a variety of defensive behaviors. The same mice were exposed to the toy rat and rat. All mice were exposed to each of these stimuli only once. Different mice were exposed to the shock grid.

Non-cagemate rat exposure assay

For the non-cagemate rat exposure assay, grouped mice were habituated with their cage-mates to an empty white rectangular box (75 cm length, 30 cm width, 45 cm height) for 2 days in periods lasting 10 minutes. On day 3, mice were placed in groups of four with mice not from their home cage and habituated to the enclosure for 10 minutes. On day 4, mice were placed in new groups of four with mice not from their home cage or from their final habituation day, and were exposed to a toy rat placed on one end of the enclosure. On day 5, mice were again placed in new groups of non- cage mates and exposed to a live rat tethered to one end of the enclosure.

Shock grid assay

We used a modified version of the standard contextual conditioning method to better evaluate a broader species-specific defense repertoire that may differ between single and grouped mice. Conditioning was performed in a chamber (dimensions 77 × 15 cm with 40 cm high walls), with a 16.5 × 15 cm shock grid placed at one end of the chamber. All sessions of this experiment were held for 10 min. Mice were given two habituation days to acclimate to the enclosure. On shock day, mice freely explored the environment and received a 0.6 mA shock when mice touched the grid. A 10 min retrieval day without shock was given 24 h later.

Behavior detection

To extract the pose of freely behaving mice in the described assays, we implemented Social LEAP Estimates Animal Poses (SLEAP) (Pereira et al., 2022) and DeepLabCut (Mathis et al., 2018), both open-source deep learning toolboxes that allow for the markerless tracking of mouse nose, ear, and tail base x and y- coordinates in each recorded video frame. These coordinates were then used to calculate speed and position at each time point. These toolboxes were selected for their ability to track multiple animals within the same session simultaneously, a recent development in the pose estimation field.

'Freezes' were defined as epochs when head and tail base velocities fell below 0.25 cm/s for a period of at least 0.33 s.

'Approaches' were defined as epochs for which (1) the mouse moved toward the threat at a speed exceeding a minimum threshold of 3 cm/s and (2) the distance traveled between approach onset and offset exceeded 5 cm.

'Escapes' were defined as epochs for which (1) the mouse moved away from the threat at a speed exceeding a minimum threshold of 3 cm/s, (2) movement away from the threat was initiated at a minimum distance-from-threat of 40 cm and (3) the distance traversed from escape onset to offset exceeded 5 cm.

'Stretches' were defined as epochs for which (1) the distance between mouse nose and tail base exceeded a distance of approximately 1.2 mouse body lengths and (2) mouse tail base speed fell below 1 cm/s.

'Rears' were defined as epochs for which the mouse's head exceeded the enclosure floor boundaries at a duration greater than 0.5 seconds.

'Social investigations' were defined as epochs for which a mouse's nose xy coordinate was less than 1 cm away from any tracked body part of another mouse (nose, both ears, sides, and tail base).

t-Distributed Stochastic Neighbor Embedding (t-SNE)

Unsupervised dimensionality reduction of behavioral states was performed using the MATLAB implementation of t-Distributed Stochastic Neighbor Embedding (t-SNE) to characterize behaviors as ethologically distinct actions repeated throughout each session. As t-SNE preserves small pairwise distances found in high-dimensional space when embedding to low-dimensional space, its structure therefore retains local neighborhoods of close points. The features used in the t-SNE process were mouse x position, x velocity, distance to rat and distance between mouse tail base and mouse nose.

Synchrony

Binary freeze traces from each mouse were obtained after scoring freezing as detailed in "Behavioral Detection" above. The four freeze traces of each mouse in a session were added together. Timepoints with zero, one, two, three, or four mice freezing together were identified. Synchronous freezing was defined as $100 \times (\# \text{ of timepoints with 2 or more mice freezing}) / (\# \text{ of total timepoints with freezing})$. Additionally, we also quantified synchrony of behavior by calculating the pairwise Pearson correlation of binary freeze traces across all pairs of mice in a session. The average of these values was the mean pairwise correlation of freezing for a given session. These procedures were repeated to calculate synchrony between other variables, such as escape and approach behavior. To determine a null distribution of synchrony, each freeze trace was shifted by at least 200 samples, and then the synchrony measures described above were calculated for this shuffled data. This was repeated 1000 times to generate a circular shifted shuffled null distribution. A session was considered to have significant freezing synchrony if the observed synchrony was higher than the 95th percentile of the shuffled distribution.

Mutual information

Mutual information is an information theory-derived metric reflecting the amount of information obtained for one variable by observing another variable. In the case of the multi-animal behavioral analysis, the related variables were the behavior indices (freezing, approach-avoidance score, stretch-attending, rearing) between a pair of conspecific mice. Mutual information was calculated using custom Matlab code for all samples. Calculating mutual information requires computing the marginal distribution and joint distribution for behavioral indices of both mice in a pair. This distribution was calculated using a histogram count after discretizing mouse pair behaviors each into 20 bins. The product between the joint probability and the log-ratio of the marginal and joint

probabilities was calculated for all possible values, and their summation indicates the amount of mutual information shared in bits. This same procedure was completed for pseudo pairs of mice for each behavior.

Approach-avoidance score

To calculate the continuous approach-avoidance score for each assay, the distance from safety was calculated as the distance from the safe wall and normalized such that it ranged from 0 to 1, as described in prior work (Reis et al., 2021). A binarized direction value was also assigned to each timepoint, indicating if the mouse was moving toward (+1) or away from (−1) the threat. To incorporate categorized behaviors, the approach-avoidance score for freeze samples equaled the minimum score of −1.

To calculate the score at each timepoint:

Score while approaching threat: approach-avoidance score = distance to safety × direction

Score while avoiding threat: approach-avoidance score = [1−distance to safety] × direction

Perfusion and histological verification

Mice were anesthetized with Fatal-Plus and transcardially perfused with phosphate-buffered saline followed by a solution of 4% paraformaldehyde. Extracted brains were stored for 12 hr at 4°C in 4% paraformaldehyde. Brains were then placed in sucrose for a minimum of 24 hr. Brains were sectioned in the coronal plane in a cryostat, washed in phosphate-buffered saline, and mounted on glass slides using PVA-DABCO. Images were acquired using a Keyence BZ-X fluorescence microscope with a 10 or 20× air objective.

Miniscope post-processing

Calcium imaging videos were processed using the open-source MiniAn package for Python (<https://github.com/denisecailab/minian>), which includes motion correction, background subtraction, and spatial footprint and temporal calcium calculations using constrained non-negative matrix factorization. Parameters were consistent across all mice, and each extracted cell was manually confirmed prior to conversion to a MATLAB-readable format for subsequent analysis.

z-Scoring and high-frequency noise removal

Cell activity was z-scored prior to being used in any analysis. A Gaussian kernel was convolved with each neural trace to attenuate high-frequency noise while preserving real calcium dynamics. The kernel is given by:

$$G[k] \propto \exp\left(-\frac{k^2}{2\sigma^2}\right),$$

for $k = -M, \dots, M$, where M defines half of the kernel width. The standard deviation σ was defined as:

$$\sigma = \frac{f_s}{2\pi f_{cutoff}},$$

where f_s is the sampling rate and f_{cutoff} is the cutoff frequency. The kernel was normalized to sum to 1 to preserve signal amplitude before convolution with the fluorescence trace.

Ridge regression decoder using raw (non-residualized) data

To evaluate whether population-level calcium imaging traces encode behavioral states, we implemented a linear decoding pipeline using ridge regression. Raw GCaMP fluorescence traces were converted into inferred spike rates using an autoregressive AR(1) model to correct for indicator decay dynamics. Behavioral target variables to be decoded were self freezing (S-freeze), conspecific freeze (C-freeze), and freeze of individual mice #s 2, 3, and 4. To prevent bias from unbalanced behavioral states, target distributions were balanced via stratified binning into equal intervals. Decoders were trained and evaluated using 5-fold cross-validation. To prevent temporal autocorrelation artifacts inherent to calcium imaging data, a 150-sample (10 seconds) buffer gap was enforced between the training and testing sets. We used nested cross-validation to choose the regularization factor lambda. Hyperparameter optimization for ridge regularization (lambda) was performed via an inner 3-fold cross-validation loop embedded within each outer training fold. Candidate lambda values were tested across a base 10 logarithmic range from 10^{-3} to 10^5 (9 values total). The optimal lambda for each outer fold was selected as the value that minimized the mean squared error (MSE) averaged across the inner validation folds. The final model was then trained on the full outer training set using the selected lambda and evaluated on the held-out test set. Decoder performance was quantified using the Pearson correlation coefficient (r) between observed and predicted behavioral traces in the testing dataset. To confirm that decoding performance reflected true time-locked neural representation rather than general temporal structure or slow baseline drifts, we performed circular shifting. To calculate confidence intervals for each mouse's decoding performance, the Pearson correlation coefficients obtained across the 5 cross-validation folds were used. For each mouse, the mean correlation and its standard error of the mean (SEM) were computed from the fold-level correlation values. The 95% confidence intervals were then calculated using a normal approximation, defined as $1.96 \times \text{SEM}$. This same decoder was also used to predict mouse freeze using rat behavior as predictors (rat x position, rat velocity, rat acceleration, distance between rat headbase and tailbase, and rat approach).

GLM with rat behavior as predictors for mouse freeze

To investigate the relation between rat behavior and mouse freezing, we constructed one GLM per grouped mouse recording session using rat behavior as a predictor for freezing. The predictors used were angle between the rat head and mouse, rat x position, rat velocity, rat acceleration, distance between rat headbase and tailbase, and rat approach. A rat approach event was registered when a mouse met all of the following conditions relative to the rat's head: (1) proximity within 15 cm, (2) rat head-mouse body center angle < 20 degrees, (3) a forward approach rat velocity of at least 5 cm/sec projected toward the target mouse, and (4) a spatial distance reduction of at least 3 cm over a lookback window of 5 frames. If multiple mice satisfied these criteria simultaneously, the closest target was selected. Rat approaches were required to persist for a minimum duration of eight frames. A rat approach event was terminated if the rat-to-mouse distance exceeded 1.5 times the proximity threshold or if heading alignment deviated beyond 30 degrees (1.5 times the angle threshold) following the minimum duration. Two approaches could only be scored if they were at least 1 second apart from each other.

Ridge regression using residualized neural activity and residualized C-freeze

To test whether neural calcium imaging data encodes conspecific freezing (C-freeze) independently of spatial and motor confounds, we implemented a regularized GLM decoding pipeline with blocked cross-validation. Because freezing may be affected by physical movement, spatial positions, and social interactions, we

constructed a comprehensive matrix containing numerous behavioral and kinematic covariates (23 variables in total). These included: individual velocities and x and y positions for all mice and the rat, distance between the miniscope implanted mouse and the rat, rat acceleration, miniscope-implanted mouse acceleration, two vectors containing binary score of receiving or performing social investigations, angle between the miniscope-implanted mouse's head and the rat's body, angle between the rat's head and the miniscope mouse. To isolate the unique neural representation of conspecific freezing, multiple linear regression was used to regress out (residualize) the effects of these covariates from both the target behavior (C-freeze) and the neural fluorescence traces. The resulting residualized C-freeze and residualized neural data were utilized for all subsequent decoding analyses. These residualized data have been stripped of influences from the 23 behavioral covariates listed above. Since we used residualized neural activity to decode residualized C-freeze, the resulting decoding accuracy cannot be accounted for by the covariates listed above. To prevent temporal autocorrelation from artificially inflating decoding performance, data were partitioned into 3 continuous outer folds. A separation gap (5 seconds) was enforced around the test fold boundaries, completely excluding adjacent training samples to prevent information leakage between the training and testing sets. For each outer fold, prediction of held-out testing residualized C-freeze was performed using regularized linear regression (via MATLAB's lasso function with $\alpha = 0.0001$, approximating ridge regression). Features were z-scored within the training set, and the scaling parameters were applied to the test set. Optimal regularization parameters (λ) were determined in each outer fold via internal 5-inner fold cross-validation by selecting the λ that minimized the mean squared error across a log-spaced grid of 50 custom λ values ranging from 10^{-5} to 10^{-1} . Decoding accuracy for each outer fold was quantified using the Pearson correlation coefficient (r) between the observed and predicted residualized C-freeze.

To evaluate the statistical significance of the decoding performance, real decoding accuracy was compared against two baseline null models. To implement a block shuffled null model C-freeze was segmented into contiguous blocks of 200 samples and randomly permuted. This preserves local temporal structure while destroying time-locking with the neural data. To implement circular shift shuffle C-freeze was circularly shifted in time by a random offset larger than 100 samples over multiple iterations. Decoding performance with the original data was compared to these shuffled controls to determine if decoding was higher than chance.

Generalized linear model (GLM) using behavior as predictors for df/F

To classify cells as being self or conspecific freeze-responsive, a GLM was fit for each cell. Each GLM mapped behavior variables to the cell's calcium activity. In total, there were eight behavior variables used: rat position, rat speed, mean conspecific centroid x-position, mean conspecific speed, mean conspecific freezing, self mouse centroid x-position, self mouse speed, and self mouse freezing. 'Self' variables indicate the imaged mouse, while 'conspecific' variables indicate the average (mean) of the three other mice within the same session. All predictor variables were z-scored, and no other processing was done prior to using them in the GLM. Behavior variables were then modeled to produce the cell's calcium fluorescence as:

$$y = \beta_1 x_1 + \beta_2 x_2 + \dots + \beta_m x_m + c$$

where β_i is the coefficient for the i th behavior variable. The Matlab function *glmfit* was used for this and all other GLMs. These predictors and procedures were used for all GLMs to fit neural data.

Encoding of C-freeze Bout-Length

Raw calcium fluorescence traces from individual neurons were deconvolved into inferred spike rates or events at 15 Hz using autoregressive AR(1) temporal filtering. C-freeze bouts were separated into those longer or shorter than 1.0 second. Bouts longer than 1.0 second were excluded from the training data. The remaining timepoints, containing C-freeze=0 or =1, were used to fit a separate GLM on each individual neuron. GLM

predictors were rat velocity, self x position, mean conspecific x position, S-freeze and C-freeze. All predictors were z-scored. Neurons were classified as C-freeze-activated (C-freeze+) or C-freeze-inhibited (C-freeze-) based on significant GLM regression coefficients ($p < 0.05$) corresponding to C-freeze. To evaluate neural encoding properties independent of the training set, only the long social freezing bouts (bouts longer than 1.0 second) were subsequently analyzed. For each classified cell, we calculated the Pearson correlation between average activity in long bouts (longer than 1 sec) against the duration of these long bouts. The correlations of all C-freeze+ cells in a mouse were averaged. The same was done for C-freeze- cells from a given mouse. To test whether population-level correlation coefficients deviated significantly from zero across recording sessions while properly accounting for hierarchical session-to-session variance, Linear Mixed-Effects Models (LMM) were fitted using restricted maximum likelihood estimation. Specifically, session-aggregated mean correlation coefficients were modeled against an intercept with mouse ID as a random intercept.

Encoding of number of conspecifics freezing

To identify neural populations encoding the number of conspecifics freezing, for each neuron a GLM was trained using data restricted strictly to timepoints where zero or one other conspecific mouse was freezing. Samples with more than 1 conspecific freezing were excluded. The GLM predictors were: rat speed, S-speed, C-speed, C-x position, S-x position, S-freeze and C-freeze.

Neurons were classified into functional populations based on significant GLM regression coefficients ($p < 0.05$). Cells were classified as C-freeze+ if they had a positive GLM weight for C-freeze, with $p < 0.05$ for that weight, and higher activity during one mouse freezing compared to zero mice freezing. Conversely, C-freeze- cells had a negative GLM coefficient and lower mean activity during one mouse freezing than 0 mice freezing.

Following classification, neural activity of the classified cells was averaged across all cells within each recording session for zero, one, and two freezing conspecific conditions. To test for significant population-level differences specifically between the one-mouse and two-mouse freezing conditions while properly accounting for session-to-session baseline variability, Linear Mixed-Effects Models (LMM) were fitted using restricted maximum likelihood estimation, modeling session-averaged activity against condition with recording session included as a random intercept.

Shuffled df/F

Cell activity was shuffled by circular shifting by a randomly chosen value between 1500 and 6000 samples using the Matlab function circshift.

Statistical analyses

For all main behavioral analysis in grouped mice conditions, effects were first averaged across mice in a single session and then averaged across sessions. Thus, the group, or session, was considered the independent sample. Similarly, for neural data analysis, effects were first average across cells in one mouse and then averaged across mice. Nonparametric Wilcoxon rank-sum tests were used, unless otherwise stated. Welch's t-test with unequal variances is considered a better option than the rank sum test when comparing two groups with large differences in sample size (Krzywinski and Altman, 2014). We thus used Welch's t-test with unequal variances across groups when comparing single mouse rat sessions with grouped mouse rat sessions ($n=24$ single sessions and $n=9$ grouped sessions, respectively). Two-tailed tests were used with $\alpha = 0.05$. Standard error of the mean was plotted in each figure as an estimate of variation of the mean. Correlations were calculated using Pearson's method. All statistical analyses were completed with custom MATLAB scripts. Unless otherwise stated, all data is represented as mean $\pm$ SEM. See complete statistical information for all main figures in **Table S1**.

Data, Materials, and Software Availability

The Matlab code and miniscope data are available at <https://doi.org/10.5061/dryad.wdbrv164n>

Results

Mice show social buffering of freezing during exposure to a live predator

In order to investigate social buffering of freezing in mice, we exposed both grouped (4 cage mates) and single mice to a live predatory rat (**Fig. 1a**), and used markerless pose estimation to score behavior in both cohorts (**Fig. 1b**). Both single and grouped mice showed avoidance of the rat (**Fig. 1c**). Further behavioral analysis showed that the scored behaviors (escape, approach, stretch-attend postures and freeze) formed clusters in a low dimensional space (**Fig. 1d**), indicating these actions have consistent pose and kinematic differences. Relative to single mice, grouped mice showed reduced freezing (**Fig. 1e**) (% time freezing for single mice is 11.99 ± 1.39 and for grouped mice it is 6.95 ± 0.6 ; Ranksum $p=0.002$), suggesting social buffering of freezing. These two groups did not significantly differ in other metrics, such as percent time facing the rat, number of escapes, distance to the rat, number of approaches or rears (**Fig. 1g-n**) (Ranksum test $p > 0.05$). These results were observed for both sexes (**Fig. S1**). For this, and all other assays, mice of both sexes were used, and all mice were grouped housed, including mice that were presented with threat alone. Importantly freezing and avoidance were significantly higher during exposure to the rat compared to a toy rat, indicating the live predator was inducing threat-induced actions in mice (**Fig. S2c-d**). Additionally, exposure to the toy rat did not desensitize mice to the live rat (**Fig. S2e-f**). The same mice were first exposed once to the toy rat, and once to the live rat, for both single and grouped mice.

We sought to determine if the lower amount of freezing seen in grouped mice was due to freezing bouts being interrupted or prematurely ended by the freezing mouse being socially investigated (SI) by conspecifics (**Fig. 2a**). SI occurrence was suppressed during freezing, showing that conspecifics perform less SI to a freezing mouse (**Fig. 2b-c**). Furthermore, the majority of freezing bouts did not contain any SIs within a 1 second window centered at freeze bout offsets (**Fig. 2e**, left panel). The presence or absence of SI near freeze offset did not alter freeze bout duration (**Fig. 2e**, right panel) (freezing bout with SI duration was 1.81 ± 0.13 and bout duration for No SI bouts was 1.71 ± 0.06 , Signrank $p=0.86$). This result suggests that freeze bouts were not being prematurely ended by SIs (**Fig. 2d-e**). Furthermore, the correlation between SI and freezing was modest ($r=-0.03 \pm 0.01$ (mean $\pm$ SEM)) (**Fig. 2f-g**), consistent with our result showing that the majority of freezing happens during time periods without SI (**Fig. 2e**). To rule out the possibility that simple proximity to conspecifics reduces freezing, we partitioned freeze bouts based on whether the freezing mouse was near (<10 cm) or far (>10 cm) from the closest conspecific at the freeze bout offset (**Fig. 2h**). There was no statistically significant difference in the duration of freeze bouts (**Fig. 2i**) (freezing bout duration near and far from other mice was, respectively, 1.61 ± 0.06 and 1.82 ± 0.1 , Signrank $p=0.14$). Additionally, there was no significant correlation between freeze bout duration and distance from the closest mouse at freeze offset (**Fig. 2j-l**). These results confirm that the reduced freezing observed in grouped mice is not a consequence of investigation or conspecific proximity artificially terminating freezes. Social buffering of freezing was also not caused by other mice blocking visual detection of the rat, as freezing levels were not significantly different when comparing timepoints with and without visual occlusion (Signrank test $p=0.45$) (**Fig. S3**).

Freezing is synchronized across conspecifics

We next investigated if freezing behaviors were synchronized in grouped mice. To calculate freezing synchrony, we added the binary freeze traces from the four mice during rat exposure (**Fig. 3a-b**). Some timepoints showed coincident freeze occurrences, where more than 1 mouse was freezing (**Fig. 3b**, red). We next calculated synchronized freezing as the percentage of timepoints with freezing that had more than 1 mouse freezing (29% in the example in **Fig. 3b**). We then repeated the procedure after circular shuffling to obtain a null distribution. In the example circular shuffle shown in **Fig. 3d**, only 13% of synchronized freezing was observed. The observed synchronized freezing (red arrowhead in **Fig. 3e**) was then compared to a distribution created by 1000 circular shuffles (**Fig. 3e**, grey). All rat sessions showed synchronized freezing above the 95th percentile of the circular shuffle (**Fig. 3f-h**). Freezing synchrony was higher in observed data than in the 95th percentile of the circular shuffle (**Fig. 3g**) (observed synchrony was 22.77 ± 1.83 and 95th percentile shuffle synchrony was 14.96 ± 1.0 , Wilcoxon test $p=0.0039$). In contrast, escape and approach behaviors did not consistently display synchronization higher than the 95th percentile of the null distribution (**Fig. 3j,l**). To validate behavioral synchrony with another approach, we calculated the average pairwise correlations and mutual information between freeze traces and compared them to a null distribution generated by circular shuffle. These approaches also revealed higher synchrony than chance for freezing, but not other behaviors (**Fig. S4**).

These data show that cagemate conspecifics show higher than chance freezing synchrony. Interestingly, social buffering of freezing and freezing synchrony was also observed when unfamiliar groups of mice were exposed to a live rat (**Fig. S5**), demonstrating these effects are independent of prior familiarity.

It is noteworthy that changes in rat behaviors are shared by mice in the same session. It is thus possible that mouse freezing synchrony occurred not due to social information, but instead were elicited by synchronous exposure to the same rat actions, which in turn induced freezing synchrony. Rats mostly laid approximately perpendicularly oriented to mice (rat head to mouse angle = 86.0 ± 6.51 degrees) (**Fig. 4a**). Additionally, as rats were constrained by a leash, they showed restricted movements (**Fig. 4b-c**), and low speed (**Fig. 4d**). Rat kinematics also were not found to be significantly correlated with freezing (**Fig. 4e**). Freezing was also not significantly different in periods of high or low rat speeds (**Fig. 4f**) (% time freezing when rat speed was < 5 cm/s and > 5 cm/s was respectively 6.97 ± 0.61 and 8.72 ± 1.94 ; Signrank test $p=0.30$). Similarly, freezing was not significantly different when the rat was facing toward or away from mice (**Fig. 4g**) (% time freezing when rat was not and was facing the mouse was respectively 7.05 ± 0.63 and 6.40 ± 0.89). Furthermore, none of the variables in a GLM using rat behavioral predictors had a distribution of weights that was significantly different from zero (**Fig. 4h**). Additionally, we could not decode freezing above chance using rat behaviors as predictors (**Fig. 4k**). Taken together, we did not find evidence indicating that rat behavior strongly affected freezing, suggesting it is unlikely that rat behavior produced synchronous freezing in grouped mice. Additionally, we did not detect differences in rat behavior between grouped and single sessions that could potentially drive synchronized freezing (**Fig. 4l**).

Though we failed to find results suggesting that rat behavior affected freezing, it is possible that unmeasured variables, such as changes in rat smell or vocalizations may synchronize freezing. To address this confound, we exposed mice to a non-dynamic, static shock grid (**Fig. 5a**). Mice were exposed only once to the shock grid. Social buffering of freezing was also observed in the shock grid assay (**Fig. 5c**). Additionally, escape velocity was higher in single mice (**Fig. 5d**). Single and grouped conditions did not differ significantly in # of stretches, # of escapes, # of approaches or distance from the shock grid (**Fig. 5e-h**). In the shock assay, there was no significant difference in freeze bout duration associated with the freezing mouse being socially investigated (**Fig. 5j**). These findings are similar to the results seen in the rat assay (**Fig. 2e,i**), and suggest that the observed decrease in freezing in grouped mice is not artifactually induced by other mice prematurely ending freeze bouts of the freezing mouse. Mice also displayed significantly higher freezing correlation and showed higher % of

synchronized freezing than expected by chance (**Fig. 5m-n**). Additionally, freezing did not differ in non-shocked mice during shock delivery (**Fig. 5o-p**).

mPFC ensembles represent conspecific freezing

The presence of freezing synchrony (**Fig. 3h**) suggests that mice have a neural representation of conspecific freezing. We reasoned that the mPFC may encode this variable because this structure controls freezing (Morgan and LeDoux, 1995; Burgos-Robles et al., 2009) and also represents social information (Bicks et al., 2015; Chavez and Heatherton, 2015; Demolliens et al., 2017). To analyze how mPFC activity encodes conspecific freezing, we obtained GCaMP6f-expressing excitatory CamK2 α cell activity recordings from the dorsal mPFC of one mouse from each group (**Fig. 6a-b**) (see histology in **Fig. S6a**). This was achieved by implanting miniature head-mounted microscopes (miniscopes) above the mPFC of one mouse during rat exposure (**Fig. 6d**). We hereafter refer to freezing by the miniscope-implanted mouse as self-freezing (S-freeze), and we named the average conspecific freezing of the other three mice as conspecific freezing (C-freeze) (**Fig. 6e**). We show that a ridge regression decoder can predict C-freeze in non-overlapping testing data (**Fig. 6f-i**), indicating that mPFC cells represent freezing in conspecifics (Observed Mean decoding accuracy $r: 0.31 \pm 0.04$ and Shuffled Mean: 0.013 ± 0.03 ; Wilcoxon Signrank Test $p=0.03$) (**Fig. 6i**). It is noteworthy that C-freeze may be related to and influenced by various behavioral and kinematic variables, such as mouse position and rat velocity, among others. It is thus possible that mPFC encoding of C-freeze (**Fig. 6i**) may be due to neural activity representing not C-freeze, but covariates that are related to C-freeze. To account for this possibility, we regressed out a large collection of covariates from both C-freeze and neural data, obtaining residualized versions of C-freeze and mPFC activity. In total, 23 covariates were regressed out, including S-freeze, the velocities, x and y positions of all four mice and the rat, rat and mouse head direction and acceleration, among other covariates (see “**Methods**”). This procedure generated df/F and C-freeze with these covariates regressed out (i.e., residualized versions of df/F and C-freeze). We then used a ridge regression decoder to decode residualized C-freeze using residualized df/F. Example traces of measured and predicted residualized C-freeze suggest the decoder performed well (**Fig. 6j**). Importantly, the regularization parameter lambda was selected within each fold (**Fig. 6k**) to prevent overfitting of the training data and optimize generalization. Decoder accuracy was above chance relative to both block shuffled data and circular shuffled data (Signrank $p=0.031$) (**Fig. 6m**), showing that the mPFC encoded C-freeze even after regressing out S-freeze and numerous other covariates. Furthermore, decoder performance did not become significantly impaired when using only timepoints without self-freeze (S-freeze=0) (**Fig. S6b**). Interestingly, cells could decode the freezing of individual conspecifics, and thus are not only representing aggregate conspecific freezing (**Fig. S6b**). Taken together, these data suggest that mPFC ensembles can represent conspecific freezing (C-freeze). Importantly, we did not find significant behavioral differences in mice with miniscopes compared with other mice (**Fig. S7**). Additionally, groups in which neural recordings were obtained did not display significantly different behaviors from un-surgerized groups (**Fig. S7**). These comparisons suggest that there were no overt behavioral differences in sessions with miniscope-implanted mice that may confound the analysis of results of the neural data.

Conspecific and self-freezing are encoded differentially in the mPFC

We next investigated whether mPFC ensembles represent C-freeze and S-freeze with differential dynamics. Indeed, the activity of a representative cell was positively correlated with C-freeze and negatively correlated with S-freeze, suggesting that these two variables may be differentially encoded (**Fig. 7a-b**). To further investigate how mPFC cells represented kinematic and behavioral variables, we performed a GLM on each cell (**Fig. 7c-e**). For a representative mouse, we show the correlation of C-freeze GLM weights for each cell with the correlation of that cell's df/F with various features, including S-freeze GLM weight (**Fig. 7f**), C-freeze (**Fig. 7g**), S-freeze (**Fig. 7h**), walking speed (**Fig. 7i**) and x position (**Fig. 7j**). In this example mouse, C-freeze GLM weight only showed a strong correlation with the correlation of df/F and C-freeze (**Fig. 7g**). In average, C-freeze and S-freeze

GLM weights were not significantly correlated, suggesting differential representation of conspecific and self-freezing (mean Pearson correlation of C-freeze and S-freeze GLM weights was 0.02 ± 0.10) (**Fig. 7k**). C-freeze GLM weights were correlated with the correlation of each cell's neural activity and C-freeze, validating the C-freeze GLM weights (**Fig 7k**) ("C" is used as an abbreviation for "conspecifics" in all these variable names. For example, C-speed is the average speed of the three conspecifics). These results indicate differential encoding of C-freeze from S-freeze, as the GLM weights for these variables were not significantly correlated (**Fig. 7k**).

Neural representation of the number of conspecifics freezing

We next investigated if mPFC activity represented the number of conspecifics freezing and the length of C-freeze bouts, as shown by a representative cell (**Fig. 8a-c**). To determine if the population of cells encoded the number of conspecifics freezing, we performed a GLM (GLM1) on each cell using the same predictors as before in (**Fig. 7**), but this time excluding all time points with more than one conspecific mouse freezing (light blue, in **Fig. 8e**). The cells classified as activated by C-freeze (C-freeze+) with GLM1 were selected based on activation to one conspecific freezing compared to no (zero) conspecific freezing. Remarkably, these C-freeze+ cells displayed higher activation when 2 conspecifics were freezing compared to 1 (observed modulation was 0.13 ± 0.05 and shuffle data modulation was 0.01 ± 0.0 , Significance was determined using a linear mixed-effects model comparing 1 versus 2 mice freezing with session as a random effect $p = 0.019$) (**Fig. 8f, h**). This higher activation of C-freeze+ cells in bouts with 2 conspecifics freezing was found even though samples with two conspecifics freezing were excluded from the model used to classify them as C-freeze+ cells. In contrast, C-freeze- cells did not show increased modulation when two mice were freezing compared to one mouse freezing (**Fig. 8h**). These data indicate that C-freeze+ cells monotonically scale neural activity with the number of conspecifics freezing.

Next, we investigated if cells encoded the duration of C-freeze bouts. To do so, we performed a second GLM (GLM2) for each cell using the same predictors as before, but excluding all C-freeze bouts longer than 1 second (light green in **Fig. 8j**). Thus, C-freeze+ and C-freeze- cells were now classified only using short C-freeze bout lengths. The activity of these cells was then correlated with bout lengths using only bouts longer than 1 second. We observed that C-freeze+ and C-freeze- cells classified using only short bout lengths showed significantly positive and negative correlations with bouts longer than 1 second (**Fig. 8i**), suggesting mPFC ensembles encode C-freeze bout length. Importantly, neither the representation of number of conspecifics freezing nor C-freeze bout length was seen using cells classified as C-freeze+ after circular shifting (Linear Mixed-Effects Model, Wald t-test, $p=0.018$ and $p=0.022$ for C-freeze+ and C-freeze- cells, respectively) (gray bars in **Fig. 8h, i**). These data show mPFC cells encode the duration of conspecific freezing bouts, as well as the number of conspecifics freezing.

Discussion

We investigated how conspecific freezing is represented by the medial prefrontal cortex (mPFC) using a novel multi-animal assay that recruits four mice facing threats. We show that mice tend to freeze synchronously and that the mPFC dynamically represents conspecific freezing, including features such as the number of conspecifics freezing.

Freezing synchrony and social buffering of freezing occur in grouped mice

We show that the presence of conspecifics suppresses freezing. This social buffering of freezing was not an artifact of either social investigation by other mice or of proximity to other mice, in agreement with reports showing that the presence of conspecifics can attenuate threat-induced behaviors (Kiyokawa et al., 2014; Mulej Bratec

et al., 2020; Qi et al., 2020). Additionally, freezing buffering and synchrony were present in unfamiliar mice, showing that these effects do not require prior familiarity.

Reduced freezing and social buffering of freezing was seen during both rat and shock grid assays. This replication is important because it shows the effects are generalizable across two distinct threat-inducing situations. The live predator induces innate responses, while the shock grid elicits freezing due to associative learning. Consequently, these two assays depend on unique neural circuitry (Fanselow, 2010; Silva et al., 2013; Tobias et al., 2023). Additionally, the assays have different features, as relative to the shock grid, the rat assay is more naturalistic, but less well-controlled, due to the rat's behavior.

We did not detect any significant correlations between the rat's behavior on mouse freezing (**Fig. 4h,k**). We also did not detect any significant differences between the rat's behavior across single and group mice conditions that may have suppressed freezing in grouped mice (**Fig. 4l**). It is important to emphasize that the rat did not attempt to aggressively attack or eat mice, and it spent most of the time facing in a direction approximately perpendicular to the mice (**Fig. 4b**), displaying relatively low speed (**Fig. 4d**). The freezing displayed by the mouse was thus not induced by aggressive rat attacks, and was instead most likely caused by the presence of a large foreign animal combined with the presence of predator odor, which is a stimulus known to cause freezing even in the absence of a predator (Wallace and Rosen, 2000; Otsuka, 2017).

mPFC neurons encode conspecific freezing

The mPFC is well-positioned to represent social and freezing conspecific information considering its extensive connections with brain areas that regulate social behaviors (Choi et al., 2024) and emotional valence (Park et al., 2018; Chen et al., 2022). Excitatory mPFC projections to the basolateral amygdala regulate social novelty preference (Liu et al., 2025), and optogenetic excitation of mPFC-projecting cells in the basolateral amygdala increases anxiety (Gutzeit et al., 2020). Additionally, reciprocal connections between the mPFC and the thalamic nucleus reuniens are necessary for conspecific recognition (Chen et al., 2024), and inputs from the ventral hippocampus to mPFC support social memory (Wang et al., 2025). When compared to the nucleus reuniens and ventral hippocampus, mPFC neurons consistently display more stable encoding of social information. Lastly, individual populations of mPFC neurons encode competitive actions of a partner during social dominance (Kingsbury et al., 2019), suggesting this region has a key role in representing conspecific actions. We add to this growing body of work, finding that mPFC cell activity encodes conspecific freezing. Furthermore, cells encoded the number of conspecifics freezing, providing a potential mechanism for the observation that group size modulates social buffering of freezing (Kiyokawa et al., 2018; Tedeschi et al., 2021).

These results support the view that the mPFC may potentially act as a multisensory integrator that underlies behavioral adaptations when responding to environmental cues. Conspecific freezing in threatening contexts represents a complex signal that contains both social and freezing information. The mPFC may use conspecific freezing information to regulate self-freezing, potentially by recruiting downstream structures necessary for inducing freezing, such as the amygdala (Garcia et al., 1999; McGarry and Carter, 2017) and periaqueductal gray (Rozeske et al., 2018; Skog et al., 2024).

In summary, our study reveals a unique function of the mPFC to represent conspecific freezing in a manner that follows the synchronous freezing of group members. By designing a novel assay and performing single-cell analyses of mPFC neurons, we show that cells represent conspecific freezing, expanding upon the function of

the mPFC as a hub that delineates complex internally and externally generated social actions. Though we observed both social buffering of freezing and freezing synchrony, it is unclear if these effects occur independently or if they affect each other. Future studies in which these features can be separately manipulated are needed to test their causal relationship with each other. Future studies may also investigate whether the encoding of threats is altered in grouped mice compared to single mice, among other interesting questions.

Author Contributions

BAM performed data analysis, data interpretation and wrote the manuscript. AT conceptualized the project, performed surgeries and behavioral assays, designed experiments and interpreted data. FMCVR performed miniscope surgeries. NAP, XAL, EEW, ELH and RP assisted in behavioral assays and histology. AA contributed to experimental design, data interpretation, miniscope data analysis and wrote the manuscript.

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Figures Legends

Figure 1. Grouped mice freeze less than single mouse during exposure to predator. (a) Schemes of (top) single mice assay and (bottom) grouped mouse assay. The rat was restrained by a short lead (shown in red) attached to the upper enclosure edge and cannot move outside of the colored area. Groups in the grouped rat exposure assay were composed of four cagemates of the same sex. **(b)** Kinematic tracking was completed using multi-animal 2D markerless pose estimation algorithms that track individual mouse and rat body parts for both (top) single mice and (bottom) grouped mice. **(c)** Representative heatmaps of mouse centroid positions for both

(top) single and (bottom) grouped mice. Mice in both cohorts predominantly avoided the area containing the rat demarcated by the dashed red boundary line. **(d)** tSNE was done using kinematic (velocity, position, etc) and pose (distance from tailbase to nose, etc) variables, showing that the main scored behaviors form clusters. **(e)** Compared to single mice, grouped mice showed significantly less freezing in the rat assay (t-test; $p = 0.002$; $*p < 0.05$, $**p < 0.01$) **(f)** Top: example mouse exploration track at top. Bottom: distribution of freeze bouts relative to distance to the rat. In both cohorts, freeze bouts were more frequent far from the rat. There was no difference between single and grouped mice in time facing rat (t-test; $p = 0.26$) **(g)**, distance to rat (t-test; $p = 0.95$) **(h)**, number of escapes (t-test; $p = 0.26$) **(i)**, escape velocity (t-test; $p = 0.62$) **(j)**, number of approaches (t-test; $p = 0.24$) **(k)**, approach velocity (t-test; $p = 0.76$) **(l)**, number of stretch attend postures (t-test; $p = 0.42$) **(m)** and number of exploratory rears **(n)**. **(d-n)** $n = 24$ single mice, 9 grouped cages ($9 \times 4 = 36$ grouped mice in total). Data are plotted as mean $\pm$ SEM.

Figure 2. Freezing bouts in grouped mice are not being prematurely ended by social interaction. **(a)** Scheme showing social investigation (SI) or no social investigation (no SI). SI here is defined as a non-freezing mouse socially investigating a freezing mouse. **(b)** Top: probability of social investigation relative to freeze bout onset. Bottom: probability of social investigation relative to time of freeze bout offset for real pairs of mice in the rat assay. Note that social investigation by the non-freezing mouse increased after the freeze bout ends, indicating that being socially investigated was not causing freeze bouts to end. **(c)** Social investigations were suppressed during freezing. Wilcoxon signrank test (Wilcoxon Signrank $p = 0.008$). **(d)** If an SI occurred within a 1 second window centered at freeze bout offset, that bout was designated as an “SI freeze bout” (top), while instances where SI did not occur within this window were “no SI freeze bouts” (bottom). **(e)** Left: The majority of freeze bouts did not have SI in a 1 second window centered at freeze bout offset (Wilcoxon Signrank $p = 0.0004$). Right: Bouts with SI within 1 second around bout offset were the same average length to no SI bouts, indicating that the presence of social interactions was not prematurely ending freeze bouts (Wilcoxon Signrank $p = 0.86$). **(f)** Example plot showing freezing and social investigation (SI) binary indices. **(g)** Correlation between SI and freezing across mice (Wilcoxon signrank test, $p = 0.004$). **(h)** Scheme showing classifications of being near or far from the closest mouse (< 10 cm or > 10 cm distance between mice body centers, respectively). **(i)** Freeze bout durations did not differ in bouts in which mice are near or far from the closest other mouse (Wilcoxon Signrank $p = 0.14$). **(j)** Left: Example scatter plot showing no correlation between freeze bout length and proximity to other mice (each point represents a freeze bout, pooling bouts across all mice). Right: There was no correlation between duration of freeze bout length and proximity to other mice (Wilcoxon Signrank $p = 0.57$). **(k)** Example scatter plot showing lack of correlation between freeze bout length and proximity to the closest other mouse (each point is a freeze bout from a single example mouse). **(l)** There was no correlation between freeze bout duration and distance to the nearest mouse (Wilcoxon Signrank $p = 0.78$). **(a-l)** $n = 9$ grouped cages ($9 \times 4 = 36$ grouped mice in total). Data are plotted as mean $\pm$ SEM.

Figure 3. Mice show synchronized freezing during exposure to a live rat. **(a)** Representative freezing traces of four grouped mice during the rat assay. **(b)** Sum of the four example traces in **(a)**. Samples with more than one mouse freezing are plotted in red and samples with 1 mouse freezing are shown in blue. Synchronized freezing was quantified as the % of freezing samples in which more than one mouse is freezing ($\text{synch. freeze} = 100 \times (\# \text{ of red timepoints} / \# \text{ of total timepoints with freeze} > 0)$). In this example, 29% of the freeze samples displayed synchronized freeze. **(c)** Example circular shuffle of the four traces shown in **(a)**. **(d)** Plot of the sum of the circular shifted shuffled traces. In this example circular shuffle for the same data shown in **(a-b)**, the amount of synchronous freeze was 13%. Note that circular shuffling decreased synchronized freezing. **(e)** Distribution of % synchronized freezing obtained after multiple circular shuffles of the data in **(a)** are shown in grey. The red arrowhead indicates synchronous freezing calculated in the observed, un-shuffled real data. **(f)** Bar plot showing for each session, the percentile for the circular shuffle that was seen for the observed (unshuffled) data. For all sessions, the amount of synchronized freezing was above the entire range of the shuffled distribution ($p = 0.0020$, Wilcoxon Signrank right-tailed test comparing the 95th percentile of the shuffle with the observed synchrony). **(g)** Bar plot showing the amount of synchronized freezing seen in the real data (red) and the 95th percentile of the circular shuffle. **(h)** Difference in synchronized freezing between the observed data and the 95th percentile of the

circular shuffle. **(g-h)**, $p=0.0039$, Wilcoxon Signrank test). **(i-j)** Same as **(e-f)**, but for escape behavior (**j**, $p=0.99$, right tailed Wilcoxon Signrank test). **(k-l)** Same as **(e-f)**, but for approach (mice voluntarily approaching the rat) (**j,l**, $p=0.99$, right tailed Signrank test). **(j,l)** Note that the real observed synchronized freezing is not significantly above the 95th percentile of the circular shuffle for escape and approach behavior. **(f,g,h,j,l)** ($n=9$ sessions, 4 mice per session, 36 unique mice in total, exposed to the rat only once). Data are plotted as mean $\pm$ SEM.

Figure 4. Relation between rat behavior and mouse freezing. **(a)** Example histogram showing distribution of angles between the head of a rat and the center of a mouse. Zero degrees corresponds to the rat's head pointedly directly at the mouse without any angular offset. Ninety degrees corresponds to the rat's head being oriented perpendicularly relative to the mouse. **(b)** Average angle between rat head and mouse **(a)**. **(c)** Example data showing that rat movement (red) along the x axis is much more restricted than the mouse (blue). **(d)** Median rat speed across sessions ($n=9$ rats). **(e)** Correlation of mouse freezing with rat speed ($p=0.07$ Signrank test) and acceleration ($p=0.1$ Signrank test). **(f)** Freezing was not significantly different during moments with high or low rat speed ($p=0.30$, Signrank test). **(g)** Freezing was not significantly different when the rat was facing the mice or not ($p=0.42$, Signrank test). **(h)** GLMs was performed using rat behavioral features to reconstruct mouse freezing. None of the measured behaviors had a distribution of weights significantly different from zero ($p>0.05$, Signrank test). **(i)** A decoder using ridge regression and the rat behaviors shown in **(h)** was used to predict mouse freezing. Example data showing observed and predicted freezing. Note the poor correspondence between the two time series. **(j)** Representative plot showing inner-loop validation mean squared error (MSE) as a function of candidate penalty values lambda. The decoder was 5-fold cross-validated. Within each of these 5 outer folds, the optimal regularization parameter lambda was chosen as the value yielding the minimum MSE across a 3-fold inner cross-validation on the training set. **(k)** Difference between the correlation of observed and predicted freeze for real and circular shifted shuffled data. Decoding using unshuffled data was not significantly above chance levels ($p=0.13$, Signrank test). **(l)** A wide variety of rat behavioral measures were quantified during the rat assay for grouped and single mice conditions. None of the plotted measures showed significant differences across conditions, including angle (t-test $p=0.68$), x position (t-test $p=0.58$), velocity (t-test $p=0.12$), # of stretches (t-test $p=0.07$) and # of rat approaches (t-test $p=0.18$). Angle measures the angle between rat head and mouse body center. Stretch measures the distance from rat head to tailbase. Rat approaches are exploratory approaching movements rats made towards mice. **(d-h, k,l)** $n=9$ grouped cages ($9 \times 4=36$ grouped mice in total). Data are plotted as **(d-h, k, l)** $n=9$ grouped cages ($9 \times 4=36$ grouped mice in total). Data are plotted as mean $\pm$ SEM.

Figure 5. Grouped mice freeze less than single mice in shock grid contextual conditioning. **(a)** Scheme of assay. Mice were placed in a corridor with a shock grid in one end, either with cagemates (grouped) or by themselves (single). **(b)** Representative occupancy heatmaps showing that both grouped and single mice avoided being near the shock grid. Relative to grouped mice, single mice displayed higher freezing (Wilcoxon Ranksum test, $p = 0.043$) **(c)** and escape velocity (Wilcoxon Ranksum test, $p = 0.0013$) **(d)**. No significant differences were found in # of stretch-attend postures (Wilcoxon Ranksum test, $p=0.09$) **(e)**, # of escapes (Wilcoxon Ranksum test, $p=0.57$) **(f)**, # of approaches (Wilcoxon Ranksum test, $p=0.91$) **(g)**, or mean distance from the shockgrid (Wilcoxon Ranksum test, $p=0.89$) **(h)**. **(i)** The majority of grouped mice freezing bouts did not have social investigation (no SI) occurring within 1 second of freezing bout offsets. SI in this figure indicates a non-freezing mouse socially investigating a freezing mouse (Wilcoxon Signrank test, $p = 0.007$). **(j)** Freeze bout duration was not different for bouts that did or did not have SI within 1 second of freeze bout offset. (Wilcoxon Signrank test, $p=0.94$) **(k)** Freeze bout durations were similar regardless of whether the closest non-freezing mouse was near (<10 cm distance between mouse body centers) or far (>10 cm) from the freezing mouse. (Wilcoxon Signrank test, $p=0.81$) **(l)** There was a significant, but very modest, negative correlation between SI and freeze ($r=-0.03 \pm 0.01$, Wilcoxon Signrank test, $p = 0.02$). **(m)** Freezing was more strongly correlated across mouse pairs compared to circular shuffled data. ($n=24$ mouse pairs) (Wilcoxon Ranksum test, $p= 0.03$). **(n)** Across sessions, the observed synchronous freezing was higher than that of the 95th percentile of circular shuffled data (Wilcoxon Signrank test, $p=0.039$). **(o-p)** For each delivered shock, a 10-second window centered at shock onset was determined. Freezing **(o)** and synchronized freezing **(p)** by the other 3 non-shocked mice was measured in all other time points (non-shock samples) and during these shock

delivery windows.. (o, $p=0.11$, Signrank test, p, $p=0.54$ Signrank test). (c-p) $n=8$ shock grid sessions, 4 unique mice per session. Data are plotted as mean $\pm$ SEM.

Figure 6. mPFC encodes conspecific freezing. (a) Representative image showing expression of GCamP6f in CamK2 α glutamatergic cells in the mPFC, along with the track from the implanted GRIN lens used for miniaturized microscopy. (b) Representative spatial footprints of extracted mPFC cells. (c) Example fluorescence traces of GCamP6f-expressing mPFC CamK2 α cells. (d) Cage mates were exposed to a predatory rat. One mouse per cage had an implanted miniaturized microscope to record neural activity of mPFC cells. (e) Self freeze (S-freeze) is the freezing of the miniscope-implanted mouse. Freezing traces of the other 3 cagemate mice were averaged to calculate mean conspecific freezing (C-freeze). (f) Representative data shows observed C-freeze (light brown) overlaid with the reconstructed prediction generated by a ridge regression decoder (blue). The correspondence between the two time series suggests that mPFC activity encodes C-freeze. (g) Representative plot showing inner-loop validation mean squared error (MSE) as a function of candidate penalty values lambda. The decoder was 5-fold cross-validated. Within each of these 5 outer folds, the optimal regularization parameter lambda was chosen as the value yielding the minimum MSE across a 3-fold inner cross-validation on the training set. (h) Decoder performance was quantified as the Pearson correlation coefficient (r) between observed and predicted behavioral values across held-out testing folds. Decoder performance for individual mice showing 95% confidence intervals. (i) Correlation between real and predicted C-freeze in testing data for decoder using observed and shuffled data ($p=0.03$, Wilcoxon Signrank Test). (j-m) To ensure neural activity genuinely encodes conspecific freezing (C-freeze) rather than correlated kinematics, 23 behavioral covariates (including S-freeze, position and speed for all mice and the rat, and head direction for the rat, among other features) were regressed out from both the neural data and C-freeze prior to decoding. A regularized ridge regression decoder was trained on these regressed out (i.e., residualized) neural traces and residualized C-freeze using a blocked cross-validation scheme and compared against block-shuffled and circular shifted null models. (j) Example traces showing observed and predicted residualized C-freeze. (k) Example internal fold regularization curve. (l) Decoder performance for individual mice (mean $\pm$ SEM) (m) The decoder performance was significantly higher compared to block-shuffled or circular shuffled data ($*p=0.031$, Signrank test). Data is plotted as mean $\pm$ SEM. ($n=6$ mice in their own grouped rat assay sessions, 653 mPFC cells).

Figure 7. mPFC uses differential encoding to represent self and conspecific freezing (a) Example cell showing positive correlation between neural activity and mean conspecific freeze (C-freeze). (b) This same cell's activity was negatively correlated with self-freeze (S-freeze) during the rat exposure assay. (c) Example cell trace (gray) overlaid with its generalized linear model (GLM)-reconstructed activity trace (red). (d) Histogram showing distribution of correlation of observed df/F and GLM-reconstructed GLM df/F for all cells. (e) Matrix showing pairwise GLM weight correlations across all variables used in the GLM. Variables starting with "C-" and "S-" respectively indicate "conspecific" and "self". For example, "C-x pos." is the average x position of the 3 conspecific mice, while "S-x pos." is the x position of the mouse with the miniaturized microscope. (f) Scatterplot showing that C-freeze and S-freeze GLM weights were not positively correlated in an example mouse. (g) Scatterplot showing that C-freeze GLM weight was positively correlated to the correlation of cell activity with C-freeze in an example mouse. (h-j) C-freeze GLM weights were not correlated with the correlation of neural activity and S-freeze (h), C-speed (i), or C-x position (j) in a representative mouse. (k) Average correlation across mice for the example scatterplots in (f-j) ($n=6$ mice in their own grouped rat assay sessions, 653 mPFC cells). Data are plotted as mean $\pm$ SEM.

Figure 8. mPFC cells represent the number of conspecific mice freezing in rat assay. (a) Trace of an example mPFC cell (green), with mean conspecific freeze (C-freeze) overlaid (blue). (b) Scatterplot showing the activity of this example cell was positively correlated with C-freeze bout length in rat assay. (c) This example cell showed higher activity when a larger number of conspecifics was freezing. (d) Distribution of behavioral bouts with 0, 1 or 2 conspecifics freezing. (e) GLM1 was performed for each cell after excluding time points in which the number of conspecifics freezing was higher than 1. Cells with significant positive and negative scores were classified as C-freeze+ and C-freeze-cells. Excluding bouts with more than one conspecific freezing allows

954 testing if cells classified as C-freeze+ based on activation during 1 mouse freezing show higher activity when 2
955 mice were freezing, even though those timepoints were excluded from GLM1 input. This GLM1 was used for the
956 analysis shown in **(f-h)**. **(f)** GLM1-classified C-freeze+ activity was higher when 2 mice were freezing compared
957 to 1 mouse freezing (n=6 mice, 74 C-freeze+ cells, Linear Mixed effects Wald t-test p=0.024). **(g)** GLM1 C-
958 freeze- cell activity was not significantly different between samples with 1 and 2 mice freezing (n=6 mice, 186 C-
959 freeze- cells, Linear Mixed effects Wald t-test p=0.30). **(h)** Bar graph showing the difference in neural activity
960 between 2 and 1 conspecific freezing. A positive number indicates higher activity when 2 conspecifics were
961 freezing compared to 1 other mouse freezing. GLM1-classified C-freeze+ cell activity was higher when 2
962 conspecifics were freezing compared to 1 conspecific freezing. Shuffled cells that were classified as C-freeze+
963 and C-freeze- cells after circular shifting did not show increased modulation in bouts with 2 conspecifics freezing.
964 **(f-h)** Data were evaluated using a Linear Mixed-Effects Model to test for significant differences between 1-mouse
965 and 2-mouse freezing conditions while considering session ID as a random effect. (n=6 mice, 74 C-freeze+ cells,
966 186 C-freeze- cells). **(i)** Distribution of C-freeze bout lengths across all mice. **(j)** GLM2 was performed for each
967 cell after excluding C-freeze bouts with duration longer than 1 second. Cells with significant positive and negative
968 scores were classified as GLM2 C-freeze+ and GLM2 C-freeze- cells. Excluding bouts longer than 1.0 second
969 allows testing if cells classified as GLM2 C-freeze+ based on activation during short C-freeze bouts show higher
970 activity during longer bouts, even though those timepoints were excluded from the GLM2 input. GLM2 was used
971 for the analysis shown in **(k-l)**. **(k)** Representative cell classified as C-freeze+ after excluding C-freeze bouts
972 longer than 1.0 second. Scatterplot showing correlation of neural activity of this representative cell with C-freeze
973 bout length. **(l)** The activity of cells activated by C-freeze+ and C-freeze- cells was, respectively, positively and
974 negatively correlated with C-freeze bout length, which was calculated only using bouts longer than 1.0 second.
975 Shuffled cells that were classified as C-freeze+ and C-freeze- cells after circular shifting did not show correlation
976 with C-freeze bout length (n=99 C-freeze+ cells, 302 C-freeze- cells, *p<0.05, Linear Mixed-Effects Model, Wald
977 t-test p=0.018 and 0.022 for C-freeze+ and C-freeze- cells, respectively). (n=6 mice in their own grouped rat
978 assay sessions, 653 mPFC cells). Data are plotted as mean $\pm$ SEM.















