REMOTE CONTEXTUAL FEAR RETRIEVAL ENGAGES ACTIVITY FROM SALIENCE NETWORK REGIONS IN RATS

Short title: Remote fear memory engages the salience network in rats

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ABSTRACT

The ability to retrieve contextual fear memories depends on the coordinated activation of a brain-wide circuitry. Transition from recent to remote memories seems to involve the reorganization of this circuitry, a process called systems consolidation that has been associated with time-dependent fear generalization. However, it is not known whether emotional memories acquired under different levels of stress can undergo different systems consolidation processes. Here, we explored the activation pattern and functional connectivity of key brain regions associated with contextual fear conditioning (CFC) retrieval after recent (2 days) or remote (28 days) memory tests performed in rats submitted to strong (1.0mA footshock) or mild (0.3mA footshock) training. We used brain tissue from Wistar rats from a previous study, where we observed that increasing training intensity promotes fear memory generalization over time, possibly due to an increase in corticosterone levels during memory consolidation. Analysis of Fos expression across 8 regions of interest (ROIs) allowed us to identify coactivation between them at both timepoints following memory recall. Our results showed that strong CFC elicits higher Fos activation in the anterior insular and prelimbic cortices during remote retrieval, which was – along with the basolateral amygdala – positively correlated with freezing. Rats trained either with mild or strong CFC showed a broad functional connectivity at the recent timepoint whereas only animals submitted to the strong CFC showed a widespread loss of coactivation during remote retrieval. Our findings suggest that increasing training intensity results in differential processes of systems consolidation, possibly associated with increased post-training corticosterone release, and that strong CFC engages activity from areas associated with the salience network during remote retrieval.

KEYWORDS: systems consolidation; stress; basolateral amygdala; anterior insular cortex; prelimbic cortex

INTRODUCTION

Traumatic events may induce pathological forms of fear memory, including overgeneralized fear, which contribute to symptoms of stress and anxiety-related disorders together with post-traumatic stress disorder (PTSD) (Mahan and Ressler, 2012). Recent advances suggest that long-term contextual fear memory retrieval seems to be dependent on the coordinated activation of a brain-wide engram circuit (Wheeler et al., 2013). Furthermore, the transition of a recent to remote memory is thought to involve a reorganization of this circuit, a process called systems consolidation (DeNardo et al., 2019; Moscovitch et al., 2016). Traditionally, recent contextual fear conditioning (CFC) retrieval is associated with high contextual specificity and neuronal activity in the hippocampal formation, especially its dorsal portion (dHPC). On the other hand, activity in neocortical regions have been associated with generalized, remote contextual memories (Frankland et al., 2004; Winocur et al., 2010). Nonetheless, evidence also suggests that the dHPC may be engaged in remote retrieval (Barry and Maguire, 2019; Goshen et al., 2011; Tanaka et al., 2014), whereas the medial pre-frontal cortex (mPFC) may also be involved in the retrieval of precise remote memories (Matos et al., 2019; Moscarello and Maren, 2018). Hence, the mechanisms by which the temporal reorganization of the engram circuit occurs is still controversial.

Parallel to the research into systems consolidation and time-dependent fear generalization, other studies also suggest that recognition, spatial or emotional memory engram cells are allocated during acquisition in several brain regions other than the hippocampus (Roy et al., 2016). Some of these regions are the amygdala (Redondo et al., 2014; Wang et al., 2009), the anterior insular cortex (aIC) (Sano et al., 2014) and the mPFC, especially the prelimbic cortex (PL) (Zhang et al., 2011). These regions are part of the so-called Salience Network (SN) and are highly responsive to arousing situations in humans (Hermans et al., 2014b) and in mice (Mandino et al., 2021; Zerbi et al., 2019). Strong, aversive events are thought to prompt a shift of memory encoding dependence from a hippocampal-dominated engram circuit to SN regions during memory acquisition and consolidation, favoring the encoding of emotional memories (Arnsten, 2009; Hermans et al., 2014b). In contrast, there is evidence that CFC in rats is associated with decreased functional connectivity between the retrosplenial cortex (RSC) – a region considered part of the “Default-mode-like network” (DMN) in rodents – to the aIC (Ji et al., 2018; Upadhyay et al., 2011). Furthermore, a comprehensive neuroimaging study using human data showed that generalized fear is associated with decreased activation in nodes of the default mode network, especially the HPC, and increased activation of the aIC, mPFC and ACC (Webler et al., 2021). Hence, this shift in memory systems resultant from higher stress and engagement of different neurocognitive networks elicited by strong aversive situations may trigger different processes of systems consolidation.

Several studies have also linked increasing levels of arousal and stress hormones with strengthened contextual fear memories and to an acceleration of the rate of time-dependent fear generalization/systems consolidation (Dos Santos Corrêa et al., 2021, 2019; Pedraza et al., 2016). More recently, studies showed that post-training activation of the noradrenergic and glucocorticoid systems modulates contextual fear memory specificity (Atucha et al., 2017; Dos Santos Corrêa et al., 2021; Gazarini et al., 2021; Roozendaal and Mirone, 2020). Therefore, it is possible that interfering with arousal levels by changing training parameters may affect contextual memory accuracy and the process of time-dependent fear generalization/systems consolidation through changes in the engagement of neurocognitive networks (Schwabe, 2017). To explore this question, a recent study from our laboratory investigated the rate of time-dependent fear memory generalization following increasing intensities of CFC training. Our data showed that mild-footshocks (0.3mA) elicited lower corticosterone (CORT) levels and accurate remote contextual memories while, on the other hand, strong footshocks (1.0mA) increased CORT release during memory consolidation, which was associated with remote generalized fear (Dos Santos Corrêa et al., 2019). These results indicate that increasing levels of arousal elicited different appraisal of the aversive experience, promoting fear memory generalization possibly due to an increase in glucocorticoid activity during memory consolidation.

In the present study we examined the expression of the immediate early gene product Fos following memory retrieval in the same rats used in the previous study (Dos Santos Corrêa et al., 2019). Here, we explored whether there were changes in functional connectivity of key brain regions associated with the SN, the DMN-like or both networks (Mandino et al., 2021; Zerbi et al., 2019). We hypothesized that increasing footshock intensity during CFC training would elicit different processes of systems consolidation, which can be observed through changes in functional connectivity between these regions.

METHODS

1 Subjects and behavioral task

For the present study we used a subset of animals from a previously published experiment (Dos Santos Corrêa et al., 2019) and the behavioral data from these animals was extracted from the previously published dataset ([dataset] dos Santos Corrêa et al., 2019). These animals were three-month old male Wistar rats, obtained from *Instituto Nacional de Farmacologia* (INFAR-UNIFESP (total n = 42; weighing between 275-385g at time of training). All rats were kept in controlled conditions of temperature (23 ± 2°C) and maintained on a 12 h light/dark cycle with free access to food and water. Behavioral experiments were conducted during the light phase of the cycle, from 11 am to 4 pm, during the rat's nadir of corticosterone circadian rhythm. All procedures were conducted

according to the guidelines and standards of CONCEA - *Conselho Nacional de Controle de Experimentação Animal* (Brazilian Council of Animal Experimentation) and were previously approved by the Ethics Committee on Animal Use - UFABC (CEUA - protocol numbers 5676291015 and 7479070916)

Contextual fear conditioning experiments were conducted as described before (Dos Santos Corrêa et al., 2019) in a windowless room containing the conditioning chamber (32 cm wide, 25 cm high and 25 cm deep, Med-Associates VFC-008). Rat freezing behavior was monitored via a frontal near infra-red-light camera. Freezing was assessed using an automated scoring system (Video Freeze, Version 1.12.0.0, Med-Associates), which digitized the video signal at 30 Hz and compared frame by frame movement to determine the amount of time spent freezing. The context was characterized by a grid floor composed of 20 stainless steel rods (diameter: 4.8 mm), top and front walls made of transparent polycarbonate, a back wall made of white acrylic, stainless-steel sidewalls and a drop pan below the floor grid. The light in the conditioning box remained on, and a background noise was emitted during the training and test sessions. The chamber was cleaned with alcohol 10% before and after each rat.

During training, rats were placed in the conditioning chamber for four minutes. After two minutes they were presented with three unsignalled footshocks (1 s duration, 0.3 or 1.0 mA, 30 seconds apart). Following the last footshock rats remained in the chamber for another minute, and then were returned to their home cage. Control rats underwent the same training procedure but did not receive the footshocks during the conditioning session (Context Only – C.O.). After each training condition, separate groups of rats were tested either 2 days (C.O.|Recent, n = 5; 0.3mA|Recent, n = 6; 1.0mA|Recent, n = 6) or 28 days (C.O.|Remote, n = 5; 0.3mA|Remote, n = 6, 1.0mA|Remote, n = 6) later. During testing, rats were placed back in the training context for five minutes and freezing time was assessed. No footshock was delivered during test sessions. Another control group of rats was only handled but did not undergo CFC training or testing (Homecage, Recent, n = 4, Remote, n = 4).

2 Perfusion and histology

Ninety minutes following the end of the test session, rats were deeply anesthetized with 30% urethane and then perfused transcardially with 100 mL saline followed by 500 mL of ice-cold 4% paraformaldehyde (PFA) dissolved in phosphate buffer saline. The brains were removed, fixed for at least 2 hours in PFA, then transferred to 30% sucrose solution and stored at 4˚C. After dehydration, these brains were frozen in isopentane on dry ice and stored at -80 ˚C. Frozen coronal sections (40 μm) were cut in a microtome with dry ice and stored in 5 serial sets. One set was collected in glass slides and Nissl-stained to cytoarchitectural delineation of brain areas, and another set was used for Fos immunolabeling.

3 Immunohistochemistry and cell counting

Free-floating sections underwent antigen retrieval (10mM Sodium Citrate, ph 8.5) for 30 minutes at 80 ºC, and peroxidase blocking (0.3% hydrogen peroxide in 0.02M PBS) followed by indirect antibody staining protocols aiming Fos labeling by avidin-biotin reaction. Sections were incubated at 4 ºC for 72 hours with rabbit anti-cfos polyclonal antibody (1:10,000 AbCam) in 2% normal goat serum, 0.3% Triton X-100 and 0.02M PBS. After washes, incubation with secondary antibody (anti-rabbit Ab from goat diluted in 1:200 – BA1000, Vector, in 0.02M PBS and 0.3% Triton X-100) took 90 minutes in room temperature. The secondary antibody was biotinylated in the avidin-biotin-complex solution (1:200, ABC Kit, VectaStain Elite, Vector) in 0.02M PBS and 0.3% Triton X-100. The peroxidase complex was visualized using the chromogen diaminobenzidine 3,3-tetrachloride (DAB Kit, Vector). Finally, sections were mounted on gel-coated slides and left to dry for at least 48 h. After drying, sections were diaphanized, coverslipped with DPX mountant medium (Sigma 06522) and left to dry for at least a week.

Fos expression was analyzed in 8 regions of interest (ROIs, Table 1). Images from each ROI were acquired using either a Zeiss microscope model Axio image 2 at 20x magnification or a Leica microscope model DM5500 at 10x magnification, following the same frame size, image size and area for each microscope. One ROI (anterior retrosplenial cortex) was smaller than the standard area and was delimited using the ImageJ software (NIH, Washington, United States). The anatomical delimitation of each ROI was based on Paxinos and Watson rat brain atlas (Paxinos and Watson, 2007) and on adjacent Nissl-stained sections. Table 1 shows the information regarding anteroposterior coordinates of slices, image size, amplification and number of bilateral sections for each ROI. ImageJ® (1.52a) was used to count cells automatically following parameters of size, circular shape and contrast level adapted to each image but within a pre-established range to ensure consistency. Experimenters were blinded to the experimental group. Cell density of each photo was obtained dividing the number of cells to the total area of the image. Mean Fos density of the photos from each animal, either for recent or remote timepoints, was normalized by the averaged Fos density of homecage rats that were euthanized in the same days of the other groups. The experimental unit was the cell Fos density normalized by the homecage group for each animal. One case from the 0.3mA|Remote group was discarded due to issues after multiple immunohistochemical essays, lowering the number of cases in this group down to 5. Counts from sections with tissue damage were excluded from analysis.

4 Statistical Analysis

4.1 Behavior and Fos density for each ROI

All descriptive and inferential statistical analysis for behavior and Fos density were done using JAMOVI (version 1.8) and Python. The conditioned fear response to context was quantified as the percent time the animal spent freezing during re-exposure to the training context. Behavioral results are expressed as the group mean percent freezing time ± standard error of the mean (S.E.M). Immediate early gene activation results are expressed as the group mean normalized Fos density ± S.E.M. All data were checked for the assumptions of normality and homogeneity of variances with the tests of Kolmogorov-Smirnov and Levene.

Behavioral data and Fos differences for each brain region were analyzed using ANOVAs, with the between subject factors being Footshock and Timepoint. The Tukey post hoc test was further used to identify significant differences when applicable. Significance for all tests described in this section was set at $p \leq 0.05$. Effect sizes for ANOVAs (η^2p) and post hoc tests (Cohen'd) are reported only when the test was found significant ("η^2p" values above 0.14 are considered large effects; values between 0.06 and 0.14 are considered moderate; and below 0.06, small) or in the Supplementary Material. Associations between freezing times and Fos density for each region were calculated using bicaudal Pearson correlations.

4.2 Fos Coactivation analysis

In experimental animal studies, neuronal activity is usually measured by evaluating changes in expression of immediate early genes such as *c-fos* or their products. Functional connectivity for these measures is assessed by calculating covariance across subjects, rather than within subjects (Careaga et al., 2019; Coelho et al., 2018; Worley et al., 2020). Within each of the six experimental groups of animals (retrieval test at recent or remote timepoints; Context Only, 0.3 and 1.0 mA), all possible pairwise correlations between the Fos signal within the eight brain regions analyzed were determined by computing Spearman correlation coefficients (totaling 384 correlations). Each set of correlations was calculated from a vector of size 5-6, and were displayed as color-coded correlation matrices using Python. Fos coactivation (i.e., functional connectivity) was considered when the Spearman's rank correlation coefficient was below a threshold of one-tailed significance level of $p < 0.05$. To determine how Fos coactivation correlations differed by footshock intensity and timepoint, we contrasted mean r values between all ROIs for each group using a one-way Welch ANOVA. Games-Howell post hoc tests were used for multiple comparisons between groups. Significance for the ANOVA and post hoc tests described in this section was set at $p \leq 0.05$. To investigate effects of footshock intensity and timepoint on individual ROI by ROI correlations (i.e. is PrL more strongly correlated with BLA at the Remote timepoint versus Recent for each footshock intensity?), we

contrasted the correlation of each condition to the others (all possible comparisons) using the Fisher r to z transformation and unicaudal z test to determine *p* values for each contrast. The set of p-values were then globally adjusted to correct for multiple comparisons using Benjamini and Hochberg's false discovery rate procedure (Worley et al., 2020) maintaining a false discovery rate of 25%. Pairwise ROI by ROI comparisons identified as significant using this method are summarized in Table 3.

Table 1

Description of microscopy for each region of interest (ROI).

Region	Microscope	Bregma Range	Number of bilateral frames per ROI	Size (pixels) per photo	µm per pixel	Lens	Median bilateral sections per animal
Hippocampus - Dentate Gyrus (DG)	Zeiss	[-2.28,-3.72]	2	1388:1040	0,5	20x	2
Hippocampus - CA3	Zeiss	[-2.28,-3.72]	1	1388:1040	0,5	20x	2
Hippocampus - CA1	Zeiss	[-2.28,-3.72]	3	1388:1040	0,5	20x	2
Prelimbic (PrL)	Leica	[4.2,2.52]	1	3072:2204	0,3	10x	3
Basolateral Amygdala (BLA)	Zeiss	[-1.56,-3.24]	1	1388:1040	0,5	20x	2
Anterior Retrosplenial Cortex (aRSC)	Leica	[-2.28,-3.72]	1	2410:1807	0,3	10x	3
Anterior Cingulate Cortex (ACC)	Zeiss	[3.24,2.52]	1	1388:1040	0,5	20x	2
Anterior Insular Cortex (aIC)	Zeiss	[3.24,0.72]	1	1388:1040	0,5	20x	3

Note: The frames were taken from an interval of bregma coordinates that ranged from the most anterior to the most posterior for each brain region (column “Bregma”). Brain regions larger than the microscope’s standard area were obtained in more than one frame (column “Number of bilateral frames per ROI”). Each animal had at least 1 and up to 3 bilateral brain sections to capture frames from; the column “Median bilateral sections per animal” shows the median number of bilateral sections per animal taken from all experimental groups in both recent and remote timepoints.

RESULTS

1. Remote memory retrieval is associated with higher neocortical functional connectivity

The brains of a subset of rats used in a previous study (Dos Santos Corrêa et al., 2019) were used for analyses of the *c-fos* activity-dependent neuronal expression. Behavioral data of this subset of animals is representative of the main results previously published (Figure 1a). Increasing footshock intensity elicited higher freezing times in both recent [Footshock ($F(2,14) = 53.0$, $p < .001$, $\eta^2p = 0.88$)] and remote timepoints [Footshock ($F(2,13) = 24.60$, $p < .001$, $\eta^2p = 0.79$)]. In both timepoints, rats from the C.O. groups showed significantly lower freezing when compared to the 0.3 (Recent, $p = 0.01$; Remote, $p = 0.02$) and 1.0 mA groups (Recent and Remote, $p < .001$). In addition, rats from the 0.3 mA group showed significantly lower freezing times when compared to the 1.0 mA group in both timepoints (Recent, $p < 0.001$; Remote, $p = 0.003$, see table S1 for all post hoc results and effect sizes). These results were associated with remote contextual discrimination after low intensity training (0.3 mA) and remote contextual generalization after high intensity training (1.0 mA; DOS SANTOS CORRÊA et al., 2019). Post-training plasma CORT levels for each of the three footshock intensities in this subset of animals and inferential statistics can be seen in the supplementary material Fig S1 e Table S2.

Among the 8 brain regions analyzed, two-way ANOVAs revealed interaction effects for the aIC, PrL and aRSC. Figure 1 (b, c) shows quantification of Fos cells in the aIC and PrL, respectively. In the aIC we observed an increase of Fos signal only in the 1.0 mA|Remote group. The ANOVA showed significant effects for the Timepoint factor: $F(1,27) = 10.55$, $p < 0.01$, $\eta^2p = 0.28$ and Footshock * Timepoint interaction: $F(2,27) = 5.84$, $p < 0.01$, $\eta^2p = 0.30$, but not for the Footshock factor: $F(2, 27) = 2.40$, $p = 0.11$. Post hoc tests showed that the group of rats trained with 1.0 mA intensity and tested on the remote timepoint had a significant increase of immunoreactive cells when compared to rats trained with the same intensity and tested at the recent timepoint ($p < 0.001$, d = 2.75 95%CI = [1.34-4.16]) and to rats only exposed to the context during training and tested at the recent ($p = 0.003$, d = 2.59 95%CI = [1.15-4.03]) or remote ($p = 0.032$, d = 1.98 95%CI = [0.62-3.34]) timepoints. All other comparisons were not significant ($p > 0.09$, Fig. 2b). A similar increase was seen in the PrL. The ANOVA showed significant effects for the Footshock factor: $F(2, 26) = 3.89$, $p = 0.033$, $\eta^2p = 0.23$; Timepoint factor: $F(1,26) = 9.22$, $p < 0.01$, $\eta^2p = 0.26$ and Footshock * Timepoint interaction: $F(2,26) = 6.58$, $p < 0.01$, $\eta^2p = 0.34$. Post hoc tests confirmed that the group of rats trained with 1.0 mA intensity and tested on the remote timepoint had a significant increase of immunoreactive cells when compared to all other groups ($p < 0.042$) whereas all other pairwise comparisons were not significant ($p > 0.70$, Fig. 2c). Table S3 and S4 report all post hoc comparisons for the aIC and PrL ANOVAs, with their effect sizes and 95%CI. Even though the aRSC ANOVA showed an interaction effect [$F(2,26) = 3.77$, $p = 0.04$, $\eta^2p = 0.23$, Table 2], post hoc tests did not

confirm any differences between groups ($p > 0.10$, Table S5). The Footshock and Timepoint factors for the aRSC ANOVA were also not significant ($p > 0.29$, Table 2). Among the other 5 ROIs, two-way ANOVAs revealed main effects of Timepoint for the DG and CA1, but no main effect of Footshock or interactions (see Table 2 for full statistics). The ANOVAs also did not show any significant effects for the BLA, CA3 and ACC (Table 2).

To verify associations between freezing times and Fos activity for each ROI, we ran 16 bicaudal Pearson correlation tests for the recent and remote timepoints (8 per timepoint). The correlation tests did not reveal any significant correlation for the recent timepoint ($p > 0.07$, see Fig. 1d) but revealed significant positive correlations for the BLA, PrL and aIC during remote retrieval ($p = 0.024$, 0.034 and 0.022, respectively, see Fig. 1d,e). All other correlation tests for the remote timepoint were not significant ($p > 0.43$).

In summary, regional immunoreactivity analyses showed an increased in Fos expression during the remote retrieval test in the aIC and PrL for the strong CFC. On the other hand, there was an overall increase in Fos cells for all groups during the remote CFC test in the DG and CA1 hippocampal subregions, regardless of footshock intensity. The correlation tests also suggest that Fos activity in the aIC, PrL and BLA during remote retrieval seems to be positively associated with higher freezing levels.

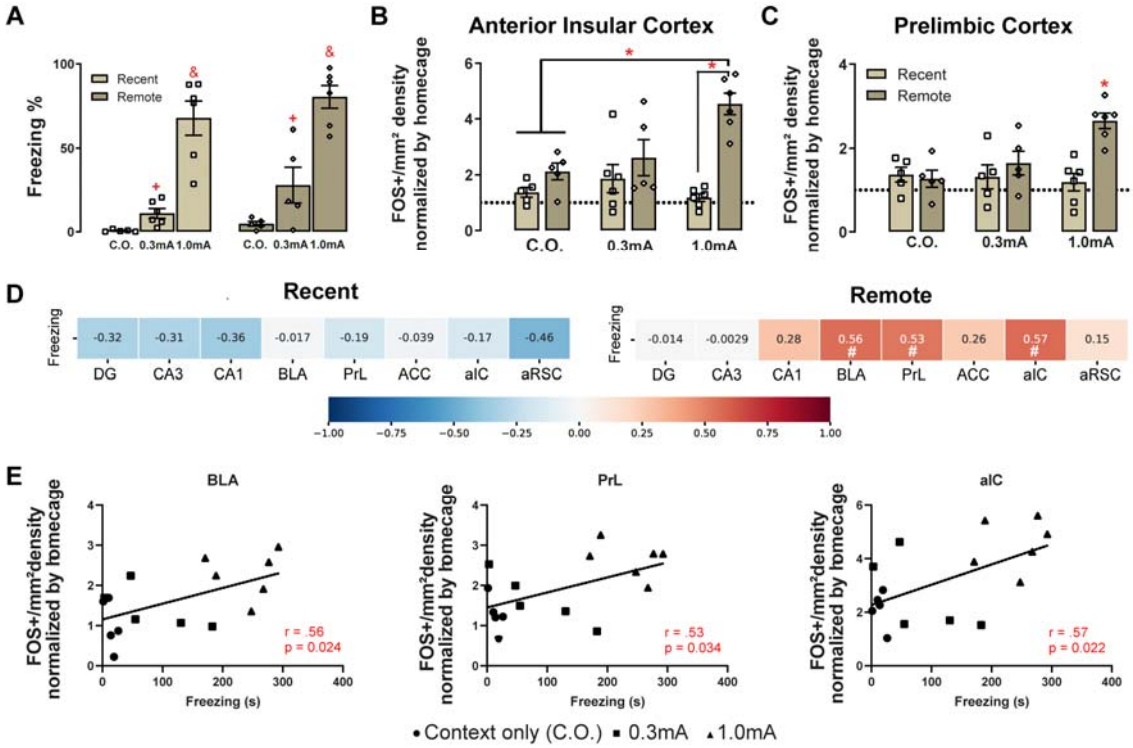

Figure 1: Remote strong contextual fear conditioning retrieval is associated with increased Fos activity in the Anterior Insular and Pre limbic cortices. *(a) Mean percentage of freezing time ± Standard Error of the Mean (S.E.M) for rats submitted to the mild (0.3 mA footshocks) or strong (1.0 mA) contextual fear conditioning (CFC) training and tested at the recent and remote timepoints (2 or*

28 days after training, respectively). Control animals were exposed to the context but did not receive any footshocks during training and were tested in the same timepoints (Context only, C.O.). Points represent raw data for each rat. N=5 for the 0.3mA|Remote and C.O. groups, N=6 for all other groups. (+) $p < 0.05$ when compared to the C.O. group in the same timepoint. (&) $p < 0.05$ when compared to the 0.3mA and C.O. groups in the same timepoint. (b, c) Mean Normalized Fos density ± S.E.M. in the aIC and PrL, respectively. (*) $p < 0.05$. Symbols show each animal's raw data. (d) Color-coded heatmap for Spearman correlations between freezing times and Fos activation in all ROIs during recent or remote retrieval. Red color represents strong, positive correlations, whereas blue color represents negative ones. (#) significant bicaudal Pearson correlation $p < 0.05$. (e) Correlations between freezing times during remote test sessions and Fos activation in the BLA, PrL and aIC; triangles correspond to the 1.0mA|Remote group, squares to the 0.3mA|Remote group and circles to the C.O.|Remote group.

2. Pairwise co-variance between ROIs

To map neuronal coactivity between brain areas implicated in either recent or remote memory retrieval, Fos expression was analyzed in the ROIs from each animal, and interregional Spearman correlations were calculated for each group (split by footshock intensity and timepoint, Figure 2a) (Careaga et al., 2019; Coelho et al., 2018; Worley et al., 2020). This analysis allows identifying pairs of brain regions in which normalized Fos density co-varies between subjects. Only those with high correlation were considered functionally connected (rho ≥ 0.81). Results showed that Context Only groups show little to no coactivation between the ROIs in both timepoints. On the other hand, for the conditioned groups tested in the recent timepoint, many ROIs showed coactivation between themselves, in both footshock intensities. It is of note that the BLA and PrL show significant coactivation with all hippocampal subregions at this timepoint. Additionally, during remote retrieval, the 0.3mA group shows sustained high coactivation between ROIs, similar to the pattern shown by the recent group, but with weakened coactivation between the BLA and PrL with hippocampal subregions. In contrast, the 1.0mA|Remote group showed lessened coactivation between ROIs, having significant results only between the aIC and the DG-CA1.

To determine if coactivation between areas differed between experimental groups, we compared the averaged "r" values of all 8 ROIs using a Welch 1-way ANOVA (Worley et al., 2020). The ANOVA showed a significant effect of Group [$F(5,73.3) = 23.2$, $p < 0.001$, $\eta^2p = 0.42$ Fig. 2b]. Averaged across all ROIs, we found more correlated activity in the 0.3mA|Recent, 0.3mA|Remote, 1.0mA|Recent when compared to the C.O.|Recent, C.O.|Remote and 1.0mA|Remote groups (Fig. 2b, for all post hoc results and effect sizes see Table S6). To further investigate specific differences in coactivation between the shocked groups from the recent to the remote timepoint, data from each correlation was contrasted between timepoints using the Fisher r to z transformation and z test (Table 3). The test for the 0.3mA groups indicated a significant increase in coactivation over time between the BLA-aRSC and from the DG-CA1. On the other hand, there was a significant decrease in connectivity in the 1.0mA groups between the DG-ACC, BLA-aRSC as well as between the aRSC-

ACC and aRSC-BLA (Table 3). P-values of all z tests contrasts can be seen in Table S7. Z tests contrasting footshocks in the same timepoint were not done.

In summary, these results show that increasing footshock intensity in the CFC task elicited different trends in Fos coactivation between cortical and subcortical regions associated with contextual fear memories during remote retrieval, with a significant decrease of overall connectivity between ROIs in the 1.0mA|Remote group compared to all other conditioned groups. Results from this group also show stronger decreases in coactivation between the aRSC and DG to the BLA and ACC when comparing the two timepoints. On the other hand, the 0.3mA footshock intensity seems to maintain high connectivity between ROIs over time.

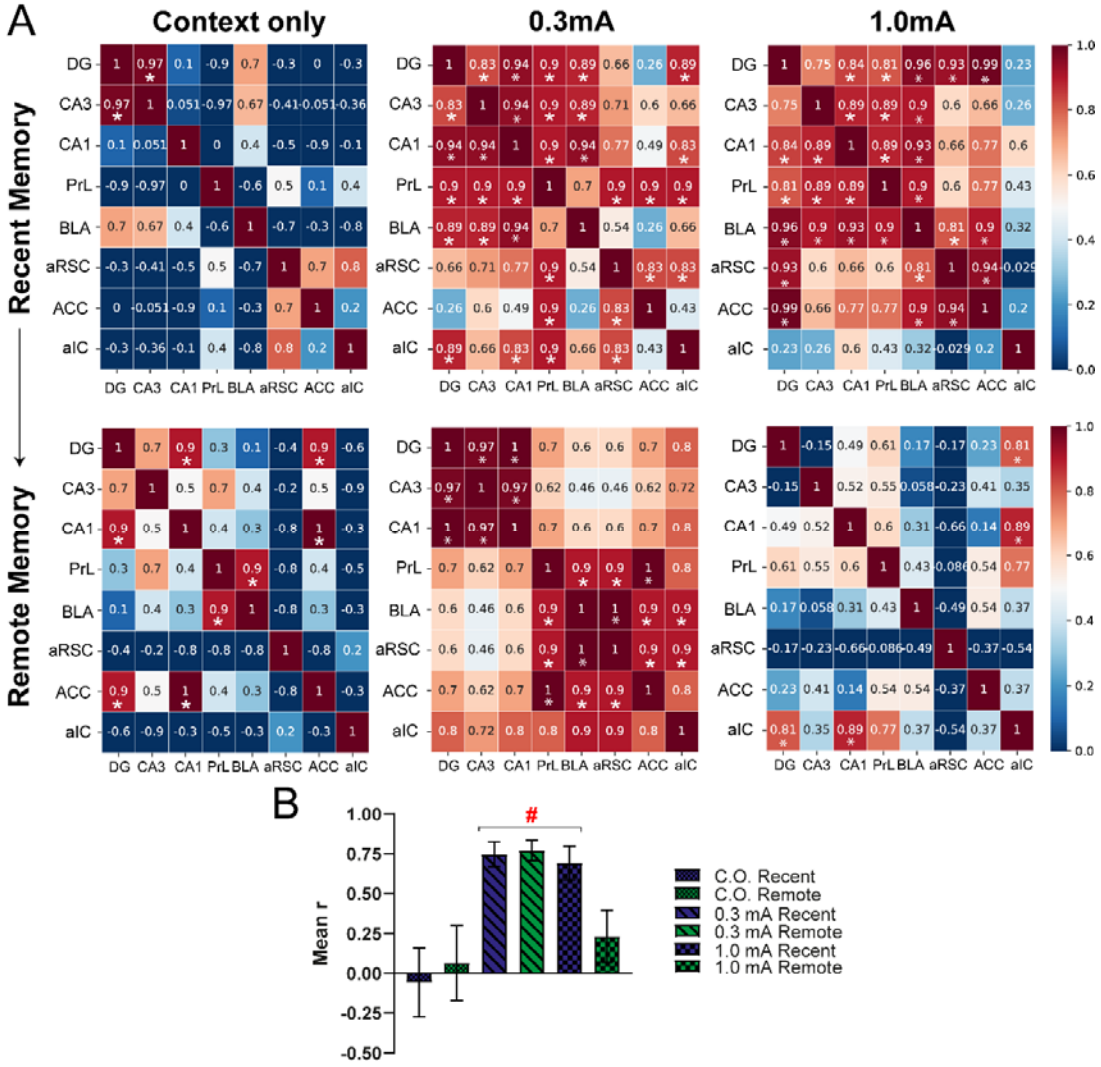

Figure 2: Increasing footshock intensity elicits different trends of functional connectivity between contextual memory brain regions during remote retrieval. *(A) Correlation matrices showing pairwise co-variance between Regions of Interest (ROIs), or functional connectivity. Rats were submitted to the mild (0.3 mA footshocks) or strong (1.0 mA) contextual fear conditioning (CFC) training and tested at the recent and remote timepoints (2 or 28 days after training, respectively). Control animals were exposed to the context but did not receive any footshocks during training and were tested in the same timepoints (Context only, C.O.). Each correlation test compared vectors sized 5-6 (number of animals). Red color represents strong positive correlations, whereas blue color represents strong negative correlations (*) $p < 0.05$ in the unicaudal Spearman correlation test. (B) Mean r values ± 95% confidence interval across all ROIs per group. (#) $p < 0.001$ in comparison to the 1.0mA|Remote and Context Only groups (recent and remote timepoints).*

Table 2

Descriptive (mean normalized Fos density ± S.E.M) and inferential statistics (ANOVAs) for other analyzed brain regions.

Brain Region		Context-only		0.3mA		1.0mA		ANOVA			
		Recent	Remote	Recent	Remote	Recent	Remote	Factor	F	df	*p*-value
DG	Mean	1.50	1.94	1.31	1.74	0.877	2.22	Footshock	0.119	2,27	0.888
	S.E.M ±	0.415	0.549	0.515	0.528	0.204	0.280	Timepoint	4.485	1,27	**0.044***
	N	5	5	6	5	6	6	Footshock*Timepoint	0.796	2,27	0.461
CA3	Mean	1.44	1.29	1.12	0.948	0.922	1.48	Footshock	0.326	2,27	0.725
	S.E.M ±	0.459	0.553	0.388	0.509	0.310	0.172	Timepoint	0.062	1,27	0.805
	N	5	5	6	5	6	6	Footshock*Timepoint	0.552	2,27	0.582
CA1	Mean	1.74	2.23	1.38	1.78	0.889	3.63	Footshock	0.501	2,27	0.612
	S.E.M ±	0.528	0.886	0.508	0.904	0.217	0.915	Timepoint	4.517	1,27	**0.043***
	N	5	5	6	5	6	6	Footshock*Timepoint	1.898	2,27	0.169
BLA	Mean	1.11	1.03	1.13	1.43	1.11	2.29	Footshock	2.27	2,27	0.123
	S.E.M ±	0.407	0.276	0.153	0.238	0.424	0.238	Timepoint	3.48	1,27	0.073
	N	5	5	6	5	6	6	Footshock*Timepoint	2.30	2,27	0.120
aRSC	Mean	1.52	0.971	1.38	1.65	1.00	2.03	Footshock	0.557	2,26	0.580
	S.E.M ±	0.175	0.111	0.285	0.437	0.194	0.310	Timepoint	1.151	1,26	0.293
	N	5	4	6	5	6	6	Footshock*Timepoint	3.772	2,26	**0.036***
ACC	Mean	0.893	0.837	0.703	0.836	0.846	1.71	Footshock	2.21	2,27	0.129
	S.E.M ±	0.155	0.233	0.200	0.275	0.243	0.370	Timepoint	2.12	1,27	0.157
	N	5	5	6	5	6	6	Footshock*Timepoint	1.76	2,27	0.192

Note: (*) refers to a significant effect.

Table 3

Summary of significant pairwise ROI by ROI comparisons.

Footshock	ROI – ROI	Contrast	*p*-value
0.3mA	BLA-aRSC	REC < **REM**	<0.001
	DG-CA1	REC < **REM**	0.01
1.0mA	DG-ACC	**REC** > REM	<0.01
	DG-BLA	**REC** > REM	0.02
	DG-aRSC	**REC** > REM	0.01
	BLA-aRSC	**REC** > REM	0.02
	ACC-aRSC	**REC** > REM	<0.01

Note: REC = recent timepoint; REM = remote timepoint. The timepoint that had higher correlation between ROIs is shown in bold.

DISCUSSION

Our laboratory recently showed that stronger CFC trainings (3x 0.6 or 1.0mA footshocks) elicited time-dependent fear generalization, whereas mild CFC trainings (3x 0.3mA footshocks) prompted remote contextual discrimination (Dos Santos Corrêa et al., 2019). We suggested that these results were due to different systems consolidation processes that occur after a mild or strong CFC, which is now further confirmed here. We also observed that higher CORT levels were associated with time-dependent fear generalization. Now, we demonstrate that strong CFC training elicits higher Fos activation of the aIC and PrL during remote retrieval, and Fos activity in these regions and in the BLA at this timepoint is associated with freezing response to the training context. Rats trained with the strong CFC protocol are also the ones with higher levels of post-training corticosterone (Fig. S2). Also, remote retrieval after mild CFC training seems to induce sustained high functional connectivity, whereas strong CFC training elicits lower functional connectivity between the ROIs at this timepoint. Lastly, strong CFC remote retrieval seems to have diminished connectivity between the aRSC and DG to the BLA and ACC compared to the same training intensity in recent retrieval, suggesting a possible decrease in engagement of the retrosplenial/hippocampus network overtime after stronger trainings. The overall data suggest that increasing training intensity during memory acquisition results in differential processes of systems consolidation that can be further associated with either fear discrimination after mild CFC or generalized fear after strong CFC (Dos Santos Corrêa et al., 2019). Fos levels reported here are similar to those conveyed in past studies that have analyzed neuronal activity after recent and remote contextual fear memory retrieval (Silva et al., 2019; Tayler et al., 2013).

Wheeler and colleagues (2013) showed that there is increased co-variance between neocortical and hippocampal regions after remote retrieval, in contrast to what is found after recent

retrieval when co-variance seems to be restricted to a hippocampal-centered network (Wheeler et al., 2013). Earlier lesion studies indicated that remote retrieval becomes independent of the hippocampus, and the memory trace seems to lose some of the contextual details that are necessary for discriminative retrieval in this process (Frankland and Bontempi, 2005). Nonetheless, this hypothesis has been challenged by several studies showing that the hippocampus remains engaged during remote memory retrieval (Albo and Gräff, 2018; Sutherland and Lehmann, 2011). The connectivity studies mentioned above strengthen this hypothesis and our results agree with them. Our data suggest that some neocortical regions become more engaged during memory retrieval at remote timepoints (but only for strong CFC) and the hippocampus may maintain its functional connectivity to other ROIs (mild CFC) at this same timepoint. It is possible that, in the earlier lesion studies, the strengthening of neocortical functional connectivity over time compensated for the absence of a functional hippocampus during remote retrieval, and may be the reason why temporarily inactivating the hippocampus before remote retrieval tests did not impair freezing levels (Quillfeldt, 2019).

Our results suggest an association between freezing behavior and Fos activity in the PrL, BLA and aIC during remote CFC retrieval, possibly indicating that engram cells allocated in these regions during encoding may mature over time into a “contextual fear network”. Interestingly, these regions are associated with the SN (with some divergence over which region specifically, the ACC or PrL, is indeed part of the network in rodents) (Hermans et al., 2014a; Seeley, 2019). The dHPC, aRSC and ACC in rodents are associated with the DMN-like network, an analogous of the DMN in humans, which is inversely correlated with SN activation (Zerbi et al., 2015). Therefore, we hypothesize that strong contextual fear memory engrams are allocated during the encoding phase in SN associated regions, especially in the aIC and PrL, via increased noradrenergic and glucocorticoid activity in these regions (Schwabe, 2017).

Neuroimaging studies in humans have associated increased coherence between the aIC, mPFC and amygdala in response to stressful events or treatment with noradrenergic or glucocorticoid agonists (Quaedflieg and Schwabe, 2018). Correspondingly, recent studies with PTSD patients have indicated that they show an exacerbated processing of interoceptive/arousal stimuli in the aIC (Nicholson et al., 2020), increased connectivity between the ACC and the aIC (Caseras et al., 2013) and also that these brain regions are associated with generalized threat detection in these patients (Berg et al., 2020). Webler and colleagues (2021) described that an increasing gradient of generalization is associated with higher connectivity between the aIC, dorsomedial PFC (the analogous of the rodent PrL), dorsal ACC along with other regions, and also with a decrease in connectivity in nodes of the DMN as generalization increased (Webler et al., 2021). Therefore, it is possible that strongly arousing events (i.e. strong CFC training) produce an enduring memory engram in neocortical regions associated with the SN, which are initially silent and mature over time (Josselyn

and Tonegawa, 2020), undergoing systems consolidation. On the other hand, a different process seems to occur after mildly arousing events, maintaining memory dependence on DMN-like regions.

Recent studies in rodents showed that noradrenergic activation of the BLA during memory consolidation influences neural plasticity in the aIC and PrL and enhances recognition memory (Barsegyan et al., 2019). Likewise, activation of glucocorticoid receptors in the aIC is associated with enhanced contextual fear memory consolidation (Fornari et al., 2012). These results suggest that aversive events may recruit these regions during memory encoding. However, is this recruitment arousal-dependent? It seems likely to be. The engagement of the aIC in the retrieval of CFC is intensity dependent, as its post-training functional inhibition impairs recent retrieval after stronger trainings (Alves et al., 2013) but not weaker ones (de Paiva et al., 2021). This dependence on the training intensity was also reported in the PrL in mice (Matos et al., 2019). These results together agree with ours and suggest that the aIC and PrL may be recruited differentially for the consolidation of contextual memories after mild or stronger aversive events. Lastly, the increase in functional connectivity between DG and CA1 in the 1.0mA|Remote group to the aIC is intriguing, considering that there are few input and output projections to and from the aIC and the hippocampus (Gehrlach et al., 2020). Possibly, functional connectivity between these two regions may be explained by their mutual projections to the medial prefrontal cortex, suggesting that a mPFC-aIC pathway is engaged during strong CFC remote retrieval. Barsegyan and colleagues (2019) provide evidence that the PrL may serve as a site of integration between the hippocampal and insular systems (Barsegyan et al., 2019) and this circuit might also be relevant for generalized remote CFC retrieval.

A limitation of our study is that we have only quantified Fos from animals that were tested in the training context and not in a novel one. More experiments are needed to investigate neuronal activation in the ROIs that are engaged during exposure to novel contexts after mild or strong CFC. It is possible that the fear network investigated here is engaged only when the animal is exposed to the training context, therefore all associations between generalized fear and loss of functional connectivity between the ROIs studied here are second-order inferences. Moreover, the lack of differences between rats trained with or without footshocks for most ROIs is noteworthy. Even though we expected to see differences in activation in the hippocampal and amygdalar regions, this difference was not confirmed by the data. Another study that also quantified Fos expression has found a lack of difference between these groups in the dorsal hippocampus at the remote timepoint (Silva et al., 2019) but this study and others have found significant differences in neocortical and amygdalar regions that we did not, especially in the ACC and aRSC (Aceti et al., 2015; Silva et al., 2019). These studies, however, used several different parameters that could account for the divergent results, such as the use of a different species (mice), stronger training parameters (5 or 30 footshocks) or a different behavioral task (inhibitory avoidance).

To conclude, we found correlational evidence between an increase of Fos activation in regions associated with the SN after strong CFC remote retrieval. Hence, we infer that increasing arousal levels (with contiguous increased glucocorticoid signaling in the brain) during memory encoding elicit different processes of systems consolidation, resulting in the recruitment of different clusters of brain regions in response to contextual fear conditioning. Strong CFC elicits the release of higher levels of CORT (Dos Santos Correa et al., 2019), and maybe noradrenaline, possibly recruiting regions associated with the SN (PrL and aIC). On the other hand, mild CFC training would not reach the necessary levels of arousal that allow engram allocation in these areas, preserving contextually-specific remote retrieval (Dos Santos Corrêa et al., 2021; Pedraza et al., 2016) and the forementioned dependence on the DMN-like (Tonegawa et al., 2018). The highest footshock intensity training was previously associated with time-dependent fear generalization, implicating a possible relationship between increased neuronal activity in the SN to generalized freezing (Dos Santos Corrêa et al., 2019). Overgeneralization of fear is one of the most debilitating symptoms of PTSD and generalized anxiety disorder, consequently our results indicate the necessity of targeting the SN in future neuromodulatory treatments for these disorders.

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Declaration of Competing Interests

The authors report no conflicts of interest in this work.

Research data for this article

The data used for all analyses in this study can be found at https://data.mendeley.com/datasets/kztbft87y6.

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Author Statement

MdSC helped conceptualizing, doing experiments, curating data, doing formal analysis and experiments, planning methodology and writing the original draft. GDVG, IAFF, and TSAT helped doing experiments and reviewing the manuscript. PAT, TLF and RVF helped conceptualizing, acquiring funds, planning methodology, reviewing the manuscript, and supervising the project.

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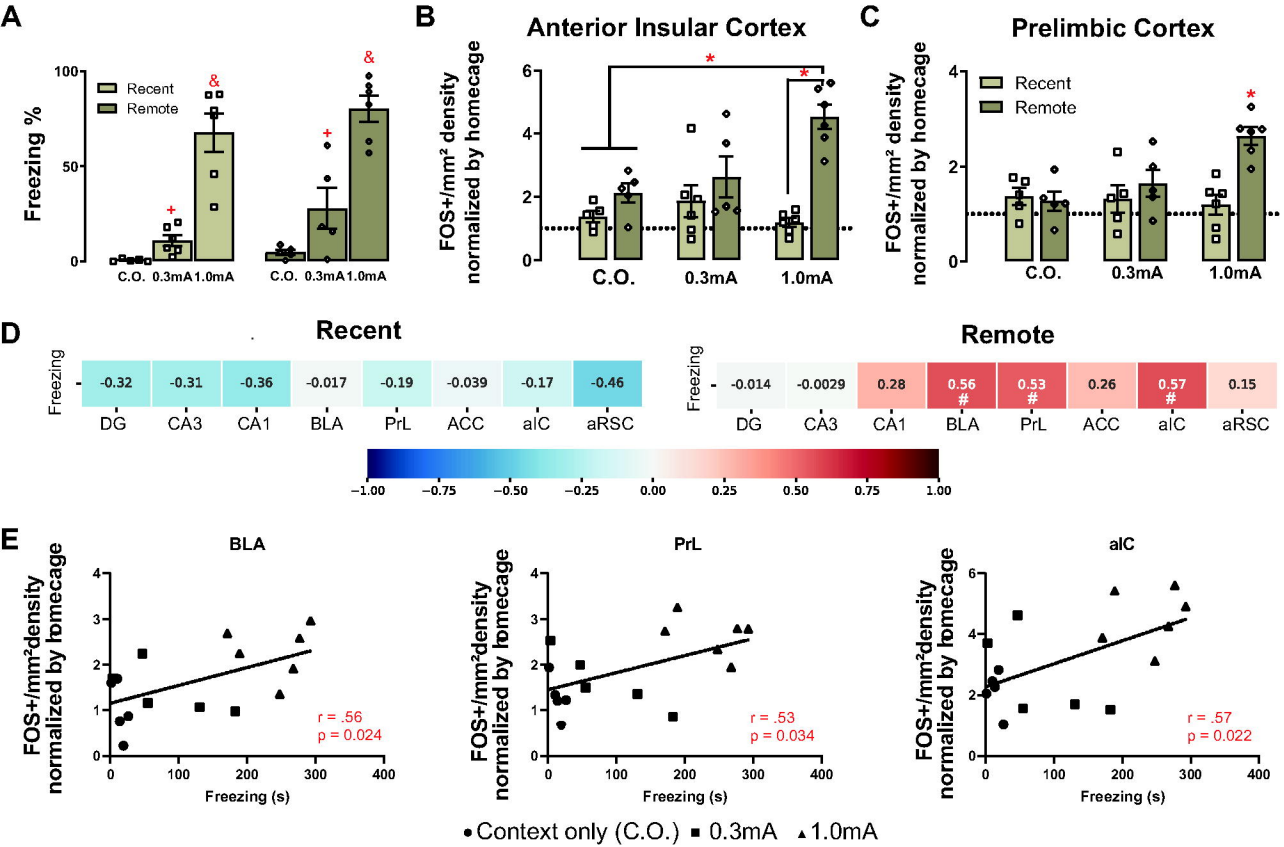
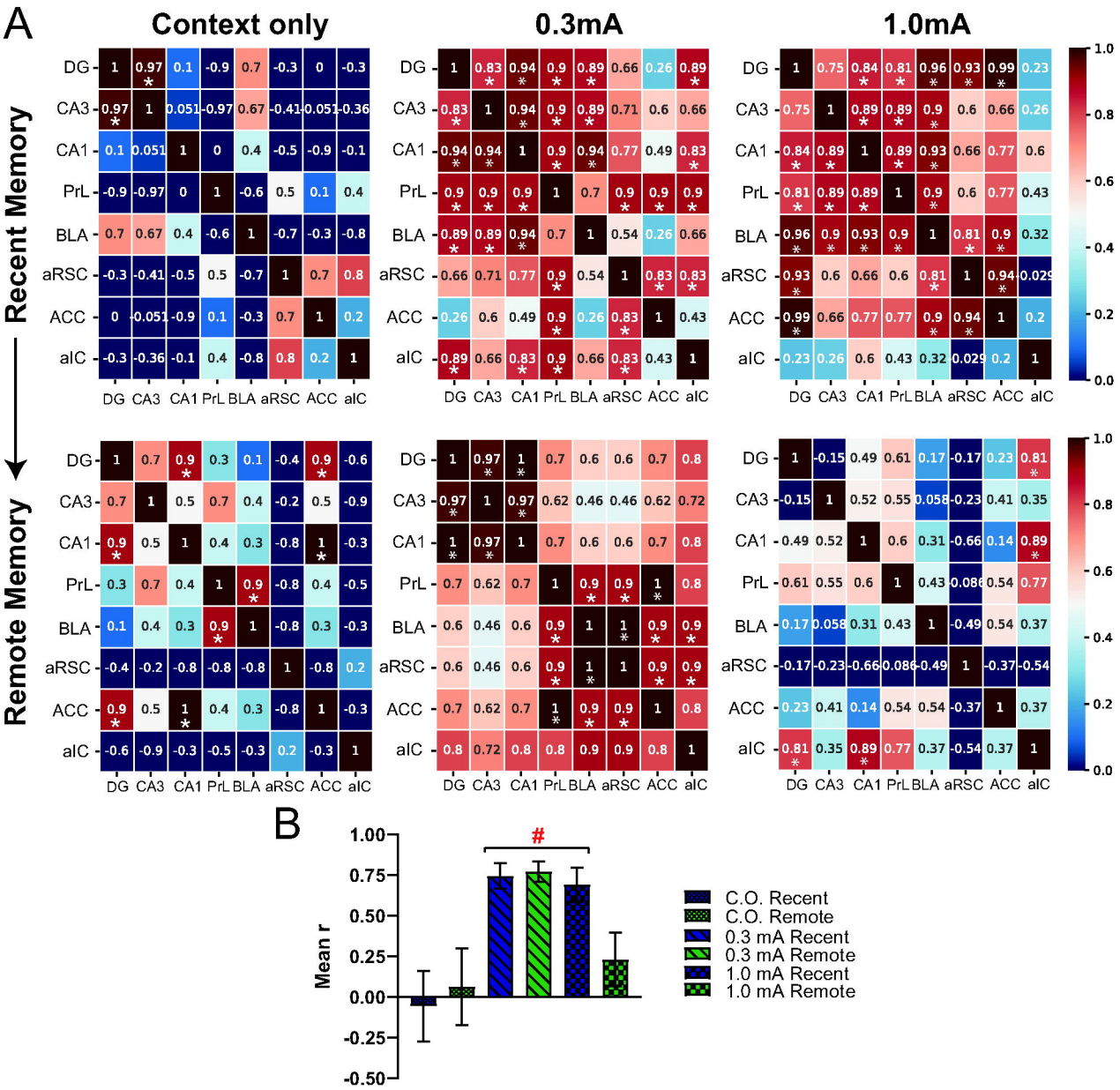

A
Freezing %
Recent
Remote
C.O. 0.3mA 1.0mA
C.O. 0.3mA 1.0mA
B
Anterior Insular Cortex
FOS+/mm² density normalized by homecage
C.O.
0.3mA
1.0mA
C
Prelimbic Cortex
FOS+/mm² density normalized by homecage
Recent
Remote
C.O.
0.3mA
1.0mA
D
Recent
Freezing
DG CA3 CA1 BLA PrL ACC aIC aRSC
-0.32 -0.31 -0.36 -0.017 -0.19 -0.039 -0.17 -0.46
Remote
Freezing
DG CA3 CA1 BLA PrL ACC aIC aRSC
-0.014 -0.0029 0.28 0.56 # 0.53 # 0.26 0.57 # 0.15
E
BLA
r = .56
p = 0.024
PrL
r = .53
p = 0.034
aIC
r = .57
p = 0.022
FOS+/mm²density normalized by homecage
Freezing (s)
Context only (C.O.) 0.3mA 1.0mA


A
Context only
0.3mA
1.0mA
Recent Memory
Remote Memory
DG CA3 CA1 PrL BLA aRSC ACC aIC
B
Mean r
#
C.O. Recent
C.O. Remote
0.3 mA Recent
0.3 mA Remote
1.0 mA Recent
1.0 mA Remote