SUPPLEMENTAL INFORMATION

Propranolol Decreases Fear Expression by Modulating Fear Memory Traces

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SUPPLEMENTAL METHODS

Genotyping

Genotyping was performed as previously described (1).

Behavioral Assays

Contextual Fear Conditioning (CFC)

A 4-shock CFC paradigm was administered as previously described (1). Briefly, mice were brought into the behavior room in a home cage, with normal lights on, and were placed in a CFC box scented with lemon (context A), to be administered 4 shocks at 180, 240, 300, and 360 s after placement into the context. Context re-exposure (RE) occurred 30 minutes or 5 days following CFC training, and each RE session lasted for 3 minutes. All sessions were scored for freezing using FreezeFrame4 (Actimetrics, Wilmette, IL).

Cued Fear Conditioning (FC)

A 4-shock cued FC was administered as previously described (2). Briefly, mice were placed in context A (identical to the CFC context) and were administered four 20 s tones (80 db, 2kHz) at 180, 260, 370, and 435 s after placement into the context. Each tone was co-terminated with a 2 s shock at 0.75 mA. The entire testing session lasted 485 s.

Five days later, to test how retrieval of cued FC is affected by prior administration of propranolol, mice were brought to the behavior room this time with red lights on, in paper buckets instead of a home cage, were injected with either saline or propranolol (10 mg/kg) and were placed in a novel context (context B) scented with anise, with a plastic floor covered with bedding, rounded walls, and cleaned with Sani-Cloth wipes. Mice were administered tones at 180, 260, 370, and 435 s. Twenty-four hours later they were tested again in context B, after drug washout,

with tones presented in the same manner, and 24 hours after this they were placed back in context A, where FC had taken place, for 8 min, without presentation of tones.

Context Fear Discrimination (CFD)

A 4-shock CFC paradigm was administered as previously described (1). Five days later, mice were administered saline or propranolol (10 mg/kg) and placed back in the aversive context A, for 3 min, and then 40 minutes later placed in context B (novel), as described above, for 3 min, to assess fear generalization. Thirty-five days after CFC mice were placed back in context A for 3 min to assess whether the prior administration of propranolol or saline before RE1 produced effects in long term memory (LTM).

Social Memory Recognition

Male mice were individually housed overnight. The next day mice were placed in a clean home cage with an ovariectomized (OVX) female mouse for 10 minutes of recorded interaction. Afterwards, each male mouse and the respective female mouse they were presented to were cohoused for 4 days, to allow formation of a consolidated social memory. After 4 days the mice were tested for social recognition. On the test day, the mice were separated and individually housed for 1.5 h, then they were administered either saline or propranolol (10 mg/kg) and immediately placed in an arena with 2 mesh cups on opposite sides, one with the familiar OVX female mouse, and one with a novel OVX female mouse. Time spent actively exploring each cup was quantified.

Elevated Plus Maze (EPM)

To test the effect of propranolol on anxiety, mice were administered with saline or propranolol before EPM testing 5 days after mice had undergone CFC. Testing was performed as previously described (3). Briefly, the maze is a plus-cross-shaped apparatus consisting of 4 arms, two open

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and two enclosed by walls, linked by a central platform at a height of 50 cm from the floor. Mice were individually placed in the center of the maze facing an open arm and were allowed to explore the maze for 5 min. The time spent in and the number of entries into the open arms was used as an index of anxiety. Videos were scored using ANY-maze behavior tracking software (Stoelting, Wood Dale, IL).

Open Field (OF)

To test the effect of propranolol on anxiety on more than one assay, mice were administered with saline or propranolol before OF testing 5 days after mice had undergone CFC. The OF assay was administered as previously described (3). Briefly, motor activity was quantified in Plexiglas open field boxes 43×43 cm² (MED Associates, Georgia, VT). Mice were individually placed in the center of the OF box and allowed to explore the field for 10 min. A periphery area and a center area were defined, with the center square consisting of 4 lines 10 cm from the wall. Distance traveled, speed, freezing, time spent in the center, time spent in the periphery, entries into the center, and entries into the periphery were quantified. Videos were scored using ANY-maze behavior tracking software (Stoelting, Wood Dale, IL).

Ovariectomy (OVX)

Female mice were OVX as described (4), at least 2 weeks before being housed with a male mouse for the social recognition task. In brief, a 5 mm transverse skin incision in the mid-dorsal thoracolumbar region of the back was created followed by a second incision halfway down the side of the abdominal wall in the dorsolateral musculature to enter the abdominal cavity in the periovarian fat pad. To exteriorize the ovary, preovarian fat was grasped using tissue forceps, the pedicle was ligated and excised between the uterine horn and fallopian tube. The procedure was performed on both sides. After surgery, mice were returned to their home cage and monitored for the following 3 days until recovery from surgery.

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Memory Trace Tagging

For all memory trace tagging experiments, mice were placed into a separate housing room in a fresh cage the night before the 4-OHT injection (Day 1). The next day, mice were injected with 4- OHT (10 mg/ml, 2 mg per mouse) and administered CFC training 5 h later (Day 2). Following the behavioral task, mice were housed in a dark room for that night and the following 3 days (Days 3-5). Mice were taken out of the dark on the morning of Day 5, cages were changed, and they were returned to the normal colony room. All precautions to prevent disturbances to the ArcCreER^{T2} x eYFP mice during dark housing were taken in order to reduce off-target labeling. Mice were re-exposed to the CFC context and euthanized 1 h following context exposure to allow for visualization of IEG (e.g., c-Fos) protein expression.

Immunohistochemistry

Mice were deeply anesthetized, and brains were processed as previously described in (1, 5, 6). Brains were then frozen in optimal cutting temperature (OCT) medium and sliced into 100 μm sections using a cryostat. An iDISCO-based immunohistochemistry protocol was performed (6). Briefly, sections were washed in 1X phosphate buffered saline (PBS) in 3 increments of 10 min each, then dehydrated in 50% MeOH for 2.5 h. Sections were then washed in 0.2% PBS with TritonX-100 (PBST) in 3 increments of 10 min each and placed in blocking solution (6% normal donkey serum (NDS) / 10% dimethyl sulfoxide (DMSO) / 84% PBST) for 2 h. After blocking, sections were washed in 3 increments of 10 min each in 1X PBS / 0.2% Tween-20 / 10 μg/ml heparin (PTwH). Sections were then incubated in a solution of primary antibody chicken polyclonal anti-GFP (1:500, Abcam, Cambridge, MA) and rabbit polyclonal IgG anti-c-Fos (1:5000 / 3% NDS / 5% DMSO / 92% PTwH, SySy, Goettingen, Germany) for 3 days at 4˚C. On day 4, sections were washed in 3 increments of 10 min each in PTwH and incubated in secondary antibody solution consisting of Alexa 647 conjugated Donkey Anti-Rabbit IgG (1:500, Life

Technologies, Carlsbad, CA) and Cy2 conjugated Donkey Anti-Chicken IgG (1:250, Jackson ImmunoResearch, West Grove, PA) in 3% NDS / 97% PTwH overnight. The next day, sections were washed in 3 increments of 10 min each in PTwH, then washed in 3 increments of 10 min each in 1X PBS. Sections were mounted on slides and allowed to dry for approximately 20 min before adding mounting medium Fluoromount G (Electron Microscopy Sciences, Hatfield, PA) and a coverslip.

Confocal Microscopy

All samples were imaged on a confocal scanning microscope (Leica TCS SP8, Leica Microsystems Inc., Wetzlar, Germany) with 2 simultaneous PMT detectors, as previously described (6). Fluorescence from Cy2 was excited at 488 nm and detected at 500–550 nm, and Alexa Fluor 647 was excited at 634 nm and detected at 650–700 nm. Sections were imaged with a dry Leica 20× objective (NA 0.70, working distance 0.5 mm), with a pixel size of 1.08 \times 1.08 μm2, a z step of 3 μm, and z-stack of 27 μm. Fields of view were stitched together to form tiled images by using an automated stage and the tiling function and algorithm of the LAS X software.

Cell Quantification

Manual cell counting

An investigator blind to treatment counted eYFP⁺ and c-Fos⁺ immunoreactive cells bilaterally in the granule cell layer (GCL) of the DG or in the pyramidal layer (PL) of CA3 throughout the entire rostro-caudal axis of the hippocampus (HPC) (2). Cells were counted bilaterally using Fiji (5) and normalized to the area of the GCL or PL. The average $eYFP +$ and c -Fos⁺ cells per mm² are presented in **Figure S11**.

Automated cell counting

Cells were automatically quantified in 3D using custom scripts in Fiji, with slight variations depending on the label. c-Fos⁺ cells were identified by first passing the image through a bandpass filter in Fourier space, subtracting the background using a rolling ball algorithm, and identifying the cells using the 3D Local Maxima Fast Filter, 3D Spot Segmentation, and 3D Manager plugins in the 3D ImageJ suite (7). eYFP⁺ cells were identified by subtracting the background, blurring the image with a Gaussian kernel, thresholding the image for the 1% brightest pixels, and using the thresholded regions as a mask for identifying the cells with the Classic Watershed plugin in the MorphoLibJ suite (8). Co-labeled cells were identified using the 3D MultiColoc plugin in the 3D ImageJ suite (9), which uses the label images created during segmentation of the individual labels to efficiently identify overlapping objects. In order to maximize the precision of the automated counts, all segmented objects were filtered by size, shape, and intensity variation. To ensure that only true co-labeled cells were identified, the co-labeled cells were additionally filtered by the amount of overlap between the objects identified in each individual channel.

Registration to an Anatomical Atlas

Immunohistochemistry-labeled coronal brain sections were aligned to an anatomical atlas using the WholeBrain package in R (10). The atlas plate most closely corresponding to each section was chosen, and WholeBrain was used to automatically align the brain section to the corresponding atlas plate. All sections were manually curated to ensure an accurate fit, and when necessary, the correspondence points automatically generated by WholeBrain were manually adjusted. In some cases, due to uneven cutting or damage to the section, different hemispheres from the same section were aligned to different atlas plates, or only regions of interest were precisely curated. In all such cases, misaligned or damaged regions were excluded from further analysis.

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Data Integration and Analysis

Cell information, including location, intensity, and size, were imported into R from Fiji and copied into the WholeBrain object corresponding to the appropriate section. WholeBrain was then used to convert the image coordinates of each cell into atlas coordinates and determine which brain region contained each cell. Data for each label and the co-labeled cells were imported separately but handled in parallel. Cells mapping to areas not expected to contain cells, such as fiber tracts and ventricles, or mapping outside the identified regions of the atlas, were excluded. Additionally, cells mapping to cortical layer 1 were also excluded, due both to this being a dendritic layer that should not contain cell bodies and to uneven antibody labeling at the edge of the sections leading to a high number of false positives in this layer. The area of each region in the original image (using the registered coordinates) was calculated using Gauss's area formula. Areas and cell counts were aggregated across layers to yield a single value per region, and aggregate areas were converted to volumes and used to normalize the aggregate cell counts for each region. Normalized counts (cells per mm³) were used in all further analyses.

Network Analysis

Cross-correlations between all pairs of regions for each label were calculated in R, using the rcorr function in the Hmisc package. Pearson's correlations were computed in all cases. Because the c-Fos and Arc-driven eYFP labels are both activity dependent, the correlations between regions are akin to functional connections, and the correlation data can be visualized and interpreted as a functional network, with regions as nodes and the correlation value determining the weight of the edges between region nodes (11, 12). Since pairwise correlations were calculated for all pairs, the initial functional network is necessarily complete, with all nodes connected to all other nodes. In order to be able to interpret the networks and discover the most salient features, correlations with an absolute R value lower than 0.5 were dropped.

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Statistical Analysis

All data were analyzed using Prism 7.0 or 8.0. and R 3.6.3. Alpha was set to 0.05 for all analyses. For the behavioral data, the effect of Drug was analyzed using a *t*-test, using two-way analysis of variance (ANOVA) to assess the effects of Drug, Time, and of their interaction where appropriate. Post-hoc Sidak's Multiple Comparison's test was used to correct for multiple comparisons when a significant effect was found in the two-way ANOVA. Data analysis of the cell count data was conducted entirely in R using the tidyverse packages to organize the data and base R functions to perform the statistical tests. The effect of Drug on average levels of c-Fos+, eYFP+ or colabeled cells was analyzed using *t*-tests. Because each of the regions was selected according to an *a priori* hypothesis regarding its involvement in extinction behavior, multiple comparisons correction is unnecessary for the count comparisons (13, 14). Pearson correlations between regions were calculated using the Hmisc package and p-values for each correlation were determined using a one-sample *t*-test. Consistent with a published study that performed similar analysis (11), no correction for multiple comparisons was done, and reported p-values are uncorrected. Networks were constructed using igraph and tidygraph packages. All statistical tests and *p* values are listed in **Tables S2-S4**.

SUPPLEMENTAL FIGURES

Figure S01. Multiple injections of propranolol do not increase the efficacy when administered post context re-exposure. (A) Experimental design. **(B)** An injection of propranolol following RE1 and an injection of propranolol following RE3 does not impact fear expression during RE2, RE3, or RE4. **(C)** Average freezing percentages do not differ between saline- and propranolol-injected mice. (n = 3-4 male mice per group). Sal, Saline; P, Propranolol; CFC, contextual fear conditioning; RE, context re-exposure; min, minutes.

Figure S02. Administration of propranolol is not anxiolytic in 129S6/SvEv mice. (A) Experimental design. Injection of propranolol prior to the EPM does not impact **(B)** total distance travelled, **(C)** time in closed arms, **(D)** time in open arms, **(E)** time in the center, **(F)** the percent of time spent in the center/total distance, **(G)** entries into the closed arms, **(H)** entries into the open arms, or **(I)** entries into the center. **(J)** Experimental design. Injection of propranolol prior to the OF does not impact **(K)** distance traveled, **(L)** speed, **(M)** freezing behavior, **(N)** time in center, **(O)** time in periphery, **(P)** entries into the center, or **(Q)** entries into the periphery. (n = 9-10 male mice per group). Sal, Saline; P, Propranolol; CFC, contextual fear conditioning; EPM, elevated plus maze; m, meters; sec, seconds; No., number; OF, open field; m/s, meters/second; min, minutes.

Figure S03. Administration of sotalol does not decrease fear expression in 129S6/SvEv mice. (A) Experimental design. **(B)** Injection of sotalol prior to RE1 does not decrease freezing behavior. **(C)** Injection of sotalol prior to RE1 does not impact average freezing behavior. (n = 9- 10 male mice per group). Sal, Saline; Sot, Sotalol; 4-OHT, 4-hydroxytamoxifen; CFC, contextual fear conditioning; RE1, context re-exposure 1; min, minutes.

Figure S04. ArcCreER^{T2} x eYFP mice experimental design for memory trace tagging. (A)

Genetic design. **(B)** Experimental design for memory trace tagging, immunolabeling, brain-wide imaging of thick sections, automated quantification, and registration to atlas**.** Tissue section labelled for **(C)** Hoechst, **(D)** eYFP, **(E)** c-Fos, and **(F)** merged image acquired at 20x magnification. Scale bar **(C-F)** = 1000 μm. Magnification of the DG labeling with **(G)** Hoechst, **(H)** eYFP, **(I)** c-Fos, and **(J)** merged image. Scale bar **(G-J)** = 100 μm.

Figure S05. Initial attempts at development of segmentation pipeline. (A) Vaa3D produced good segmentation results for c-Fos but was too difficult to incorporate with the rest of the pipeline. **(B)** After months of training, Ilastik produced comparable eYFP segmentation results to the final pipeline but was labor and computationally intensive to run. CellProfiler produced sufficient segmentation results for c-Fos **(C)** but could not quantify cells in 3D. Therefore, in order to count 3D image stacks, the stack had to be subdivided and flattened. **(D)** Further analysis showed that the number of cells identified increased depending on how the stack was subdivided (full stack vs. halves vs. thirds), making the results unreliable. $* p < 0.05$, $** p < 0.01$.

Figure S07. Filtering of eYFP counts. Initial eYFP segmentation is filtered to eliminate false positives using six metrics. Segmented objects are filtered according to size **(A)**, variance **(B)**, and shape **(C-F)**. Moments 1-4 are shape descriptors based on order 2 moments. True positive and false positive distributions were determined via manual curation of a representative subset of segmented objects. Shaded area indicates values for which objects were kept; percentages indicate proportion of objects that are maintained by the filter.

Figure S08. Segmentation of c-Fos⁺ cells. Original image (A) is pre-processed to smooth out noise **(B)** and reduce background **(C)**. 3D Fast Filters plugin is used to identify local maxima in the pre-processed image **(D)**, and 3D Spot Segmentation plugin is used to identify cells **(E)**, using the local maxima image as seeds. Each object is assigned a unique label that can be mapped to different colors. **(F)** Identified cells are indicated as a blue overlay on the original image. Scale $bar = 100 \mu m$.

Figure S09. Identification of co-labeled cells. Using the c-Fos and eYFP segmentations **(A)**, overlapping objects are calculated **(B)**, and then filtered by size and percent of overlap to identify true co-labeled cells **(C)**. In **(B-C)**, red regions indicate areas of overlap, dark grey regions are segmented c-Fos⁺ cells, and light grey regions are segmented eYFP⁺ cells. White areas are overlaps that do not correspond to co-labeled cells and are removed by the filter. **(D)** Identified co-labeled cells are outlined in white. Scale bar = $100 \mu m$.

Figure S10. Overview of complete segmentation protocol. Original image is split into individual channels and run through the segmentation process for each channel. The segmented images produced by each process, to identify eYFP⁺ and c-Fos⁺ cells, are used to determine the co-labeled population (eYFP⁺ and c-Fos⁺), which are shown here encircled in white.

Figure S11. Administration of propranolol decreases fear expression and alters memory traces in the dorsal dentate gyrus of ArcCreER^{T2} x eYFP mice as assessed by manual hand **counting.** The number of **(A)** eYFP+ cells or **(B)** c-Fos+ cells does not differ in the dDG following administration of propranolol. The percentage of **(C)** co-labeled/eYFP+ cells and **(D)** co-labeled/c-Fos⁺ cells significantly decreases in the dDG following administration of propranolol. The number of (E) eYFP⁺ cells or (F) c-Fos⁺ cells does not differ in dCA3 following administration of

propranolol. The percentage of **(G)** co-labeled/eYFP+ cells and **(H)** co-labeled/c-Fos+ cells does not differ in dCA3 following administration of propranolol. The number of **(I)** eYFP+ cells or **(J)** c-Fos⁺ cells does not differ in the vDG following administration of propranolol. The percentage of **(K)** co-labeled/eYFP+ cells and **(L)** co-labeled/c-Fos+ cells does not differ in the vDG following administration of propranolol. The number of **(M)** eYFP⁺ cells or **(N)** c-Fos⁺ cells does not differ in vCA3 following administration of propranolol. The percentage of **(O)** co-labeled/eYFP+ cells and **(P)** co-labeled/c-Fos⁺ cells does not differ in vCA3 following administration of propranolol. (n = 9 male mice per group). $* p < 0.05$, $** p < 0.01$. eYFP, enhanced yellow fluorescent protein; Sal, Saline; P, Propranolol.

Figure S12. Correlations of activity between hippocampal, prefrontal, and amygdalar regions during encoding. Correlations of the number of eYFP⁺ cells (tagged during CFC memory encoding) between brain regions. Square color reflects the Pearson correlation coefficient and asterisks represent a significant correlation. dDG, dorsal dentate gyrus; dCA1, dorsal CA1; dCA3, dorsal CA3; vDG, ventral dentate gyrus; vCA1, ventral CA1; vCA3, ventral CA3; PL, prelimbic area; ILA, infralimbic area; ACA, anterior cingulate area; LA, lateral amygdalar nucleus; BLA, basolateral amygdalar nucleus; BMA, basomedial amygdalar nucleus; PA, posterior amygdalar nucleus; CEA, central amygdalar nucleus; IA, intercalated amygdalar nucleus; FRZ, average freezing (%) during RE1. * p < 0.05

Figure S13. Correlations between memory trace reactivation and freezing levels. Memory trace reactivation levels normalized to either c-Fos⁺ or eYFP⁺ cells were correlated with freezing levels during RE1. Here, the brain regions in which there was a correlation with R>0.5 or R<-0.5 for at least one of the groups, or that had differences in c-Fos⁺ or reactivation cells between groups are shown. Correlations between memory trace reactivation and freezing levels for dDG and dCA3 in the dorsal HPC **(A-D)**, for vCA1 and vCA3 **(E-F)**, for ILA and ACA **(G-I)**, and for BLA, LA and BMA **(J-L)**. dDG, dorsal dentate gyrus; dCA3, dorsal CA3; vCA1, ventral CA1; vCA3, ventral CA3; ILA, infralimbic area; ACA, anterior cingulate area; LA, lateral amygdalar nucleus; BLA, basolateral amygdalar nucleus; BMA, basomedial amygdalar nucleus.

Table S03. Statistical analysis summary for cell quantification data.

Table S04. Statistical analysis summary for correlation analysis.

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