Vassar College| URSI Symposium|2021

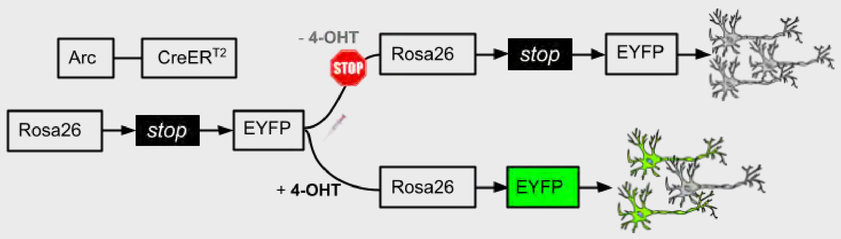
IMAGING NEURONAL ENSEMBLES UNDERLYING FEAR MEMORY

Diego Scala Chavez '22, Hannah Mae Thompson '23 and Dr. Hadley C. Bergstrom
Department of Psychological Science, Neuroscience and Behavior Program

INTRODUCTION

- Post-traumatic stress disorder (PTSD) is characterized by overgeneralization of traumatic memories.
- Generalization is the transfer of learned responses to stimuli that are similar to, but not the same as, the original stimuli.
- We hypothesize that sparse neurons in the brain, known as "neuronal ensembles," may mediate fear generalization
- Our approach attempts to characterize these neuronal ensembles in mice using tamoxifen-dependent Cre recombinase expression (Cre-human estrogen receptor fusion protein construct, or CreERT2) driven by the Arc/arg3.1 (Arc) promoter.

EXPERIMENTAL DESIGN



METHODS

Subjects

- (B6.Cg-Tg(Arc-Cre/ERT2)MRhn/CdnyJ (JAX no. 022357) X B6.129X1-Gt(ROSA)26Sor/J (JAX no. 006148) mice.
- ArcCreERT2 and ROSA positive mice were crossbred. Offspring was genotyped using a PCR assay.

PCR

- DNA samples were collected via tail biopsy then incubated at 55 degrees C overnight
- Samples were mixed with ArcCre and ROSA "supermixes" and aliquoted into 20 ul tubes.
- Samples were placed in the thermocycler and allowed to run
- Following thermocycling, samples were placed in an agarose gel and allowed to run for 20-30 minutes at 130 V.
- Samples were imaged to determine which were ROSA and Cre positive.

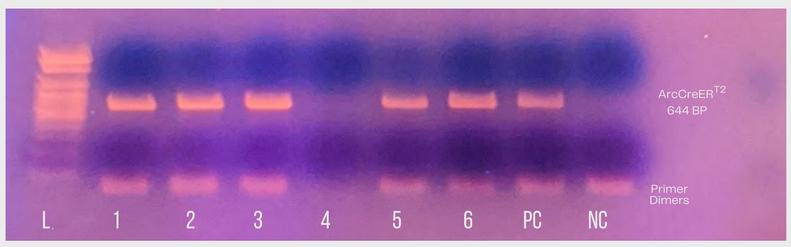


Figure 1a: Gel electrophoresis results for the ArcCre gene. The bottom bands are primer dimers, present in any sample with DNA. All mice in this figure except Mouse 4 are Cre positive.

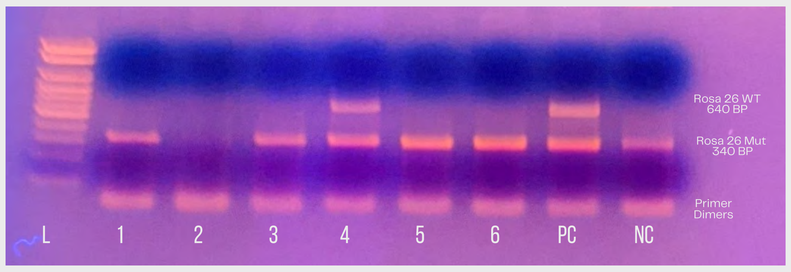


Figure 1b: Gel electrophoresis results for the EYFP (ROSA) gene. The bottom bands are primer dimers, present in any sample with DNA. The middle band is the mutant type ROSA gene, the target gene for EYFP expression. The top band is the wild type ROSA gene.. Either the wild type or mutant ROSA gene is present in all mice. Mice that only have the wild type ROSA band are not EYFP positive. All mice in this figure except Mouse 2 are EYFP positive. Mice 4 and 7 are heterozygous for the EYFP gene.

Fear Conditioning

- ArcCreERT2 X EYFP positive mice were dark housed for 24 hrs prior to behavior testing.
- Mice injected with 4-OHT to permanently genetically label cells during fear conditioning with Green Fluorescent Protein (GFP).
- 5 hours following 4-OHT injection, mice were fear conditioned with 3 presentations of a 5-kHz auditory stimulus (CS; ~75 dB, 20s) followed by a footshock (US; 0.5s, 0.6 mA, 20-03P)

Fear Recall

- A disguised context (Context B) was used during CS re-exposure
- Mice were re-exposed to either the 5-kHz tone (target stimulus) or a 3-kHz tone (novel stimulus) to assess generalization. A group that received no tone (0khz) was used to control for baseline locomotor activity.
- Re-exposure occurred either 6 days (recent) or 30 days (remote) following original CS exposure.

Immunohistochemistry

- Animals were perfused and brains were sectioned into 40 micron thick sections
- GFP chicken polyclonal primary antibodies (1:10000, ab13970) and Goat anti-chicken IgY-H-L secondary antibodies (1:500, #A32931) were used to label the original tagging event (EYFP expressing neurons, Green), alongside Arc polyclonal primary antibodies (1:5000, 156 003) and Donkey anti-rabbit secondary antibodies (1:1000, #A32754) which were used to label the retrieval event (Red).

Imaging

- A Leica confocal microscope was used to image the EYFP and Arc expressing neurons in the medial prefrontal cortex.
- Resolution is 1024 x 1024 at 1x zoom. Images taken show a total of 32 z-stacks, at 0.79 microns per stack, and analysis width of 25.28 microns.

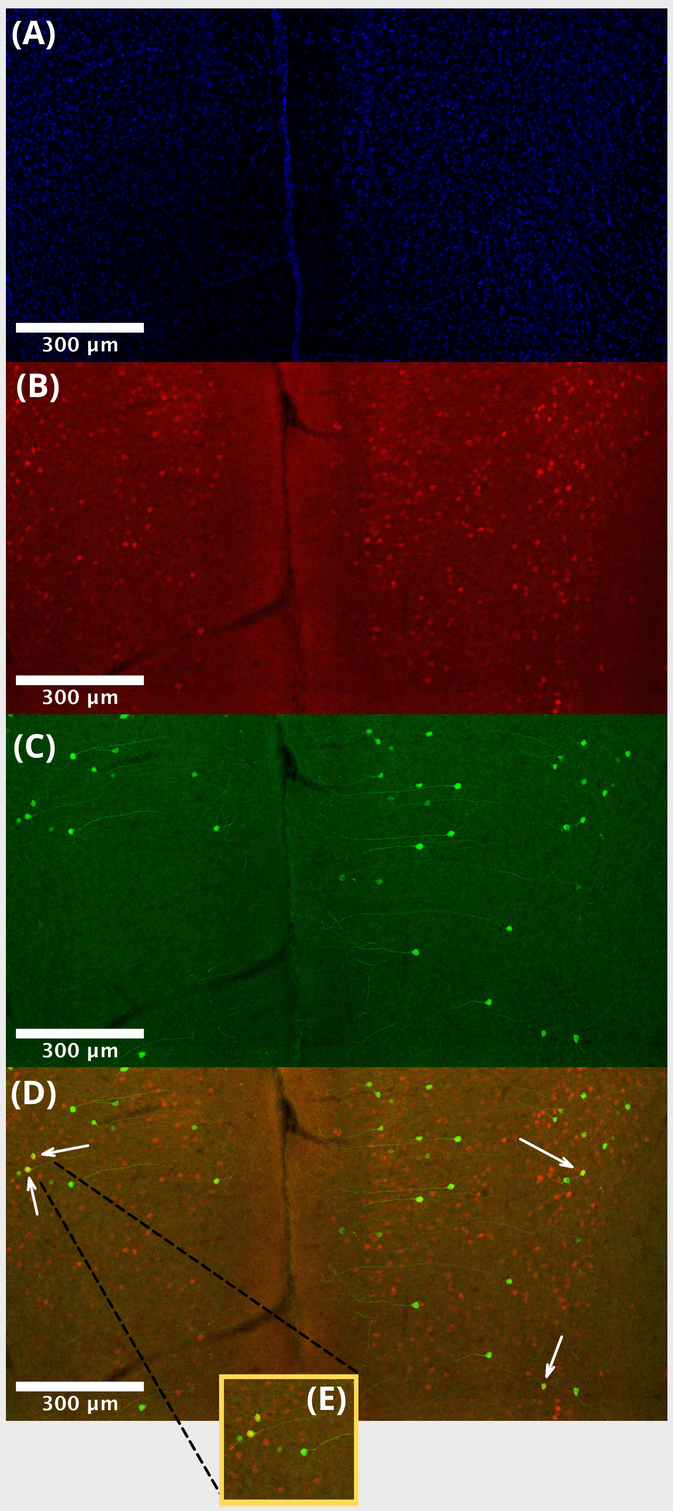


Figure 2. Visualizing active neurons using fluorescent imaging. Images taken using a Leica confocal microscope at 20x objective power. The female mouse brain shown was treated with 4-OHT before fear conditioning. All areas shown are approximately 1.64 bregma, and show an area of 1550 x 826 microns. (A) DAPI showing blue-fluorescent DNA staining. (B) Arc expressing neurons which were activated shortly before brain was extracted, when mouse was reintroduced to auditory cue. (C) GFP staining of EYFP expressing neurons active during cued fear conditioning. (D) Merged channel of Arc and GFP show colocalized neurons active at both the original conditioning event and the recall event. Colocalized neurons are labeled with arrows, and are shown in detail in (E).

Statistical Analysis

- A RMANOVA was used to compare freezing percentages between mice from the 3hz, 5hz and 0hz groups during conditioning, context B habituation and retrieval
- All statistical tests used an alpha level of 0.05 to test for significance, and graphs report mean freezing percentages ±SEM

RESULTS

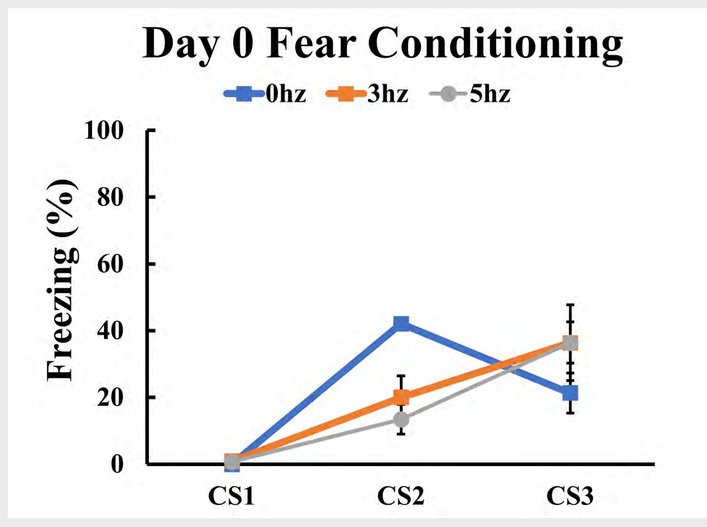


Figure 3. Mice showed increase in freezing response over time. CS = conditioned stimulus. There is a significant relationship between CS freezing and time [$F(2, 28) = 17.192$, $p < 0.001$]. N = 2 for 0khz, n = 6 for 3khz and n = 9 for 5khz. All results are reported as mean ±SEM, with an alpha level of 0.05.

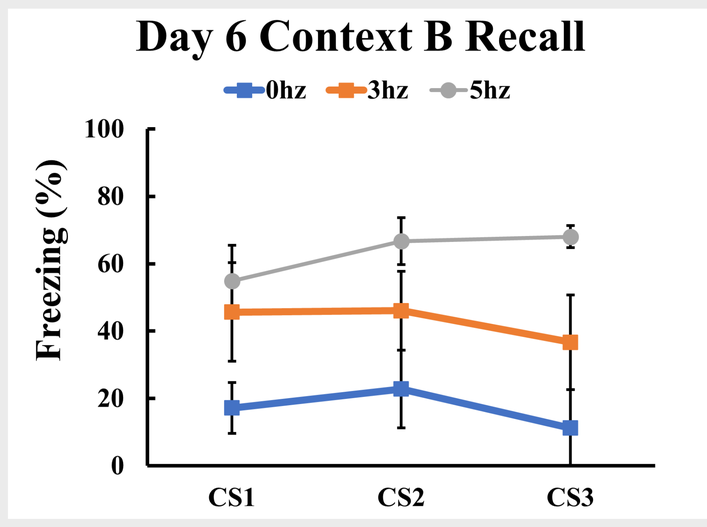


Figure 4. Mice from the 3khz group show a trend towards distinguishing auditory cues. There were no significant differences between groups due to low sample sizes, however there is a trend of reduced freezing in the 3khz recent group, indicating distinction between the two auditory cues. N = 2 for 0khz, N = 6 for 3khz and N = 8 for 5khz. All results are reported as mean ±SEM, with an alpha level of 0.05.

DISCUSSION AND FUTURE DIRECTIONS

- The refinement of the PCR protocol allows us to accurately identify ArcCre and EYFP positive mice.
- We tested different dilutions for the Arc and GFP primary and secondary antibodies to achieve optimal fluorescent imaging
- The co-labeled neurons in Figure 2e are potentially part of a neuronal ensemble underlying the conditioned fear memory.
- These neurons give us an insight into how fear memory is formed and stored in the infralimbic cortex of the medial prefrontal cortex
- Future studies will aim at characterizing neuronal ensembles underlying fear memory discrimination and generalization
- In continuation with this project, we plan to test the same conditioning and recall assay on a remote group, 30 days after conditioning.

ACKNOWLEDGEMENTS

We would like to thank Hadley C. Bergstrom, Bojana Zupan, David Lewis and Kate Carter for their mentorship, as well as the Gin E and Fon Li Memorial Fund, the NIH, Vassar College, and the entire URSI community for funding and for allowing us to do this research.