
Mapping the Neuronal Ensembles Responsible for Fear Learning and Fear Extinction

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Abstract

Nearly every animal on this planet portrays behaviors associated with fear. However, the underlying neural mechanisms controlling this behavior remain partially unexplored. There are two very distinct types of information processing related to these behaviors, fear learning, and fear extinction. A wide source of evidence indicates that the amygdala, a brain region of the medial temporal lobe, is closely associated with multiple fear behaviors. In order to investigate the populations of neurons responsible for fear learning and extinction in the basolateral complex of the amygdala, this study used the well-known technique of evaluating neural activation through immediate early gene induction (*c-fos* and *npas4* genes), in conjunction with a novel tool known as the Robust Activity Marker (RAM) synthetic molecular construct. The combination of these two molecular markers allowed the detection of neurons related to states associated with learned fear, and fear extinction in the same rat brains. This paper determined there is significant overlap in the neuronal ensembles responsible for fear learning and fear extinction in the basolateral amygdala of rats, suggesting that the mechanisms of 'forgetting' about a fearful stimulus appears to engage the recall of older fear-related memories. Future studies will need to replicate these observations in larger groups of animal

Introduction

Throughout the animal kingdom several mental functions prevail, fear lies chief among them. This study seeks to investigate the neural mechanisms responsible for governing fear related behaviors. Mapping the neuronal ensembles responsible for learning about, and extinguishing, fear has significant implications in terms of mental health. A greater understanding of the neuronal populations responsible for processing a given fear related behavior, may offer great insight into anxiety disorders (Felix-Ortiz, et al., 2013; Tye, et al., 2011). Developing treatments which specifically target the proper cell populations may offer a drastic improvement on the less than satisfactory success of treating stress and fear related mental health issues the likes of PTSD.

Evidence suggesting the amygdala's role in the processing of emotion reaches deep into the roots of neuroscience. Kluver-Bucy syndrome was an early demonstration of the amygdala's impact on emotional processing. Rhesus monkeys which had undergone bilateral temporal lobectomies, the region containing the amygdala, presented with significant alterations to their emotional predispositions. Further research indicated the amygdala played a vital role in the process of fear learning; experiments by Bruce Kapp demonstrated that neurons in the amygdala were activated by a fear conditioned stimulus. Later experiments by Joseph Ledoux demonstrated that lesions in the basolateral amygdala (BLA) complex prevented the visceral responses to learned fear (Bear, Connors, & Paradiso, 2016). Furthermore, research also indicates the BLA's role in learned fear (Duvarci & Pare, 2014), and it has been shown to specifically impact auditory and contextual learning of fear (Goosens & Maren, 2001; Scott, et al., 1997). The BLA's role in this case seems to be in the learning of the association of the CS and the US. The culmination of research to this point strongly suggests the BLA's role in fear learning, by directing and mediating the valence of various fear related stimuli (Baxter, et al.,

2000; Beyeler, et al., 2016; Burgos-Robles, et-al., 2017). As a result, furthering the understanding of the BLA is vital in understanding the processing of fear learning.

In terms of this project there are two distinct theories which may explain the way in which fear extinction occurs. The first model of fear extinction suggests it is the learning of a new opposite association of the CS and the US. However, the other model of fear extinction suggests that it is the forgetting of the previous association between CS and the US. The former would suggest that the same cells are responsible for fear learning and fear extinction. While the latter would suggest that different neuronal ensembles are responsible for each of the given tasks.

In terms of fear extinction, evidence exists for the significance of the BLA in the processing of this information as well (Zhang, Kim, & Tonegawa, 2020). The gross inhibition of the BLA decreases fear extinction (Kim & Richardson, 2008), and the encoding of fear learning lies in a genetically distinct population of neurons in the BLA (Haubensak, et al., 2010). Research has shown that inactivation of NMDA receptors in the BLA, receptors well known for their role in learning, inhibits fear extinction (Sotres-Bayon, Bush, & LeDoux, 2007). The gross inhibition of the BLA decreases fear extinction (Kim & Richardson, 2008), and optogenetic activation, or inhibition of a specific subclass of cells within the BLA may enhance or inhibit fear extinction (Zhang, Kim, & Tonegawa, 2020). Together this information suggests that a greater understanding of the neuronal ensembles in the BLA would further knowledge of fear related behavior.

In terms of this project there are two distinct theories which may explain the way in which fear extinction occurs. The first model of fear extinction is that it is the learning of a new opposite association of the CS and the US. However, the other model of fear extinction is that it is the forgetting of the previous association of the CS with the US. The former would suggest

that the same cells are responsible for fear learning and fear extinction. While the latter would suggest that different neuronal ensembles are responsible for each of the given tasks.

The development of new molecular tools now allows the visualization of individual neurons, and their projections. The processing of positive and negative cues is confined to distinct neural networks within the BLA (Namburi, et al., 2015). Multiple recent studies of the BLA employing molecular fluorescent microscopic signals suggest the projections from these distinct amygdala networks to the prefrontal cortex assist in determining the valance of conflicting cues (Burgos-Robles, et al., 2017; Baxter, et al., 2000). Some of these methods have been employed to distinguish the neuronal populations responsible for fear learning, as well as fear extinction in mice (Beyeler, et al., 2018; Haubensak, et al., 2010; Zhang et al,. 2020). The current study was designed to evaluate whether the same BLA neuronal population responsible for fear learning is also responsible for fear extinction, in rats. This assessment was conducted in the rat BLA using a combination of molecular techniques that allow the tagging of active neurons during two distinct test sessions within the same rats, with two different fluorescent activity markers. Neural activity in the form of changes in membrane potentials (e.g., action potentials) and other activity signals (e.g., calcium influx, growth factors) is frequently associated with the rapid induction and expression of immediate-early genes, such as the proto-oncogenes *c-fos* and *Npas4* (Bartel, Sheng, Lau & Greenberg, 1989; Sheng, Dougan, McFadden & Greenberg, 1988; Sun & Lin, 2016). The visualization of active neurons has been achieved for almost 30 years when the brains of experimental animals are processed for the detection of immediate-early genes shortly after an inducing stimulus (Herrera & Robertson, 1996). Only recently has the activity occurring during two distinct test sessions been able to be distinguished within the same animals over a significant period of time (He, Wang, Hu, 2019). One of these successful techniques in rats involved the

development of an immediate-early gene-dependent Robust Activity Marker (RAM) that, when expressed in the brain of experimental animals, leads to the production of a lasting fluorescent protein (mKate, a red fluorescent protein – Sorenson et al., 2016). The activity of the RAM construct relies on the activity immediate early genes to produce the mKate protein. RAM constructs production of this lasting mKate protein provides the significant difference between the technique of c-fos and RAM, and is what provides this tool its value. The proteins produced by c-fos and Npas4 fade from existence 150 minutes after the given activity while the mKate protein can last for 14 days. The RAM construct was further designed to be expressed under specific temporal control by employing a doxycycline-dependent Tet-Off system; thus, while rats are kept on a doxycycline-ladden diet, expression of the RAM construct is repressed. However, when doxycycline is removed from the diet, the RAM construct becomes available for activation by immediate-early genes, but this temporal window can be closed by doxycycline reintroduction. This method therefore allows for an initial behavioral test to be performed during RAM availability (off doxycycline), and a second test to be performed in the same animals using endogenous immediate-early genes once doxycycline is reintroduced.

In order to use the RAM construct it must first be inserted into a cell, in order to interact with the specified genes. This is achieved via stereotaxic surgery, during which the RAM construct is injected into the desired location in the brain. The RAM construct consists of an activity dependent transcription component, and an effector gene within an adeno-associated virus (AAV). By injecting the RAM construct by viral vector infection, it can be integrated into the host genome, and then produces mKate in response to activation of immediate early genes. Using this combination of RAM and immediate-early gene immunohistochemistry, the BLA cells active during an initial test, after a fear conditioning paradigm were tagged using the RAM

construct, and the BLA cells active during a second test following a fear extinction procedure were tagged via immunohistochemical staining of Fos/Npas4. By tracking the expression of immediate early genes one can track the function of the brain in relation to a given task. Using these tools in conjunction, at 2 different time periods allowed this study to assess the subsets of BLA neurons respectively responsible for these 2 different stimulus representations, e.g., a fear conditioned stimulus, and the same stimulus after an extinction task. We expected to see that the groups exposed to the fear conditioning task would express significant levels of mKate during the first test day, as this would indicate the function of neurons responsible for fear learning (Duvarci & Pare, 2014; Goosens & Maren, 2001); the cells responsible for fear extinction would express high levels of Fos and Npas4 during test day 2 (Bartel, Sheng, Lau & Greenberg, 1989; Sheng, Dougan, McFadden & Greenberg, 1988; Sun & Lin, 2016). The major conclusion this study sought to investigate was whether distinct populations of cells fluoresce in respect to each task, or if the cells responsible for each task were the same, i.e. they were co-localized.

Methods

Subjects

Twenty adult male Long Evans rats, age two-three months were used in the study (Envigo, Madison, WI). After arrival at CU Boulder's Wilderness facility the rats were housed as pairs in cages. The cages were made of plastic and measured 48 cm x 26 cm x 20 cm (Allentown Caging Equipment Company, Allentown, NJ). The rats were kept in an isolated room where the temperature and humidity were controlled to 22±1°C and 30% humidity. At all points in the study the cages contained 7093 Teklad shredded aspen bedding (Envigo) and the rats were able to consume tap water. However, as their diet changed according to the study, their standard food

(8640 Rodent Diet - Envigo) was replaced with the antibiotic containing food (200mg/kg doxycycline hyclate from BioServ) at times. Over the entirety of the study the rats were on a 12h:12h light dark cycle. The rats' care and procedures followed the guidelines put forth in *Guide for the Care and Use of Laboratory Animals* (DHHS Publication No. [NIH] 80-23, revised 2010 eighth edition) approved and regulated by the Animal Use and Care Committee at the University of Colorado. Prior to any experimental procedures, the rats were handled 2 minutes each day for 4 days.

Surgery

To use the RAM construct requires stereotactic surgery, in order to inject the construct in the desired location. A construct expressing enhanced green fluorescence protein (eGFP) was added to the RAM construct solution (0.5 ul eGFP/5ul RAM) to help in identifying the injection sites, since mKate is not expressed except in cells that are active, and would make determination of the injection sites impossible on their own (Sorensen, et al., 2016). The subjects were anesthetized with 1-3% Isoflurane via inhalation. After they were unconscious their heads were shaved and they were mounted on the stereotaxic instrument for precise delivery of the viral RAM construct. At this point an incision was made along the sagittal plane spanning from slightly more than the distance from bregma to lambda. The dorsal-ventral measurements of bregma and lambda were then equalized, and full measurements of bregma were taken, (anterior-posterior, medial-lateral, dorsal-ventral). At this point, the location of the BLA was calculated by the following: AP:-3.0, ML: ± 5.1, DV -8.8 mm from bregma. The tip of the injector was brought to the coordinates predicted by ML, and AP. A drill was then used to create a small hole through the cranium for the injection at this location. The injecting needle containing the RAM construct and an additional viral marker expressing eGFP, both carried by viral vectors, was then

inserted into the hole and lowered to the BLA at coordinates predicted by the DV calculation. The viruses were then injected at a rate of 100 ηL per minute, for 10 minutes, (total 1µL) at a dilution of 1.09x10^13. The injector was then left in place for 10 minutes before removing from the brain. Following the bilateral injections, the incision was then closed with metal wound clips, and the rats received injections of long-acting, slow-release buprenorphine .5mg/kg, and meloxicam 2mg/kg for pain management. They were then single housed and left to recover for the allotted period of time (9 days) prior to any additional manipulations.

Experimental design

What are the neuronal populations responsible for fear learning and fear extinction?

In order to achieve this aim, the established methods of *c-fos* tagging were employed, in conjunction with a relatively novel tool, the RAM (immediate early gene Robust Activity Marker) construct. The RAM construct requires surgical injection into the region of interest. Prior to RAM injections all rats were switched to doxycycline containing food 2 days prior to surgery, which is employed to inhibit the activation of RAM. The surgeries to inject RAM bilaterally in the basolateral amygdala (BLA), were then performed on all rats (n=20).

After 9 days of post-surgical recovery, a fear conditioning task was employed in which a conditioned stimulus (CS, 3000 kHz, 90 dB, 30-second tone) was paired with an unconditioned stimulus (US, electrical foot shocks, 0.80mA, 2-second co-terminating with the tone) through a classical conditioning procedure, using Med Associates Inc fear conditioning hardware and software (#MED-VFC2_USB-R). The fear conditioning session consisted of a 5-min pre-period and the presentation of 3 tone alone trials at an interstimulus interval of 90-120 seconds. This provided a context extinction of the tone and the apparatus. the conditioning portion fo the

experiment then occurred where 3 additional tones co-terminating with a 2 second foot shock, with an interstimulus interval ranging from 90-120 seconds; the entire session lasted 20 min. Control rats received only the CS with no US; this served as a negative control for the RAM, as there should be less activation of the cellular population associated with fear, as they received no shocks to learn about. Following this conditioning task the rats were taken off the doxycycline-laden chow for 3 days prior to the first fear test to allow most of the doxycycline to be eliminated from the body (Sorensen et al., 2016), except for another group of control rats. These control rats again served as a negative control for the RAM, as the doxycycline should block the RAM construct from expressing the fluorescent mKate protein.

Test day 1 (day 14) was then conducted in the same Med Associate Inc conditioning boxes with the exception that the house lights were turned on, and the grid floor was covered with a white plastic floor (context removal), and all rats were switched back to doxycycline chow for the remainder of the study to inhibit further expression of RAM. Test day 1 consisted of a 15 minute session in which the same tone CS was presented 3 times after an initial 5-min preexposure in the testing box, at an interstimulus interval ranging from 90-120 sec. During the test sessions, the behavior of rats was video recorded and later analyzed off-line to determine the percentage of time the rats spent "freezing" during a 30-sec period immediately before presentation of the tone CSs, or during each of the 30-sec CS presentation periods. Freezing assessments were done automatically using the Video Freeze® software program (Med Associates Inc., Fairfax, VT). Freezing behavior is characterized by the suppression of all movement except that required for respiration (Bolles, 1970).

Two days after test 1, two days of fear extinction took place (day 16 and 17). The fear extinction session consisted of 15 tone CSs presented in the same way at the same interval for

the context and CS extinction group during 45-min sessions. The context-only group simply sat in the experimental apparatus for 45 min. On the following day (day 18) test 2 took place, followed 90 minutes later by euthanasia. Test day 2 consisted of an identical experiment to test day 1. After fixing the extracted brain tissue via cardiac perfusion and freezing them, the tissue was sectioned via cryostat. At this point immunohistochemistry was performed, in order to detect Fos and Npas4 using a fluorescent secondary antibody. The slices were then visualized via microscopy, and cell counts were performed.

c-fos and Npas4

C-fos and *Npas4* are immediate early genes expressed in response to cellular activity generated by the experience of a given stimulus. Immediate early genes code for proteins, in this case proteins that contribute to processes such as long-term potentiation and the strengthening of neural connections. Thus, activated cells that express *c-fos/Npas4* provide a way to visualize stimulus-dependent cellular/gene activity within single cells, and therefore their possible association with cellular functions and learning. To make use of these immediate early genes, the subjects were euthanized and their tissues were fixed within 90 minutes of the second test day of the experiment. Ninety minutes has been shown to be the optimal time for the expression of these immediate early gene proteins (Hoffman, Smith & Verbalis, 1993). This allows for the high levels of Fos/Npas4 related to the second test to be visualized, before these protein levels degrade. Then by using immunofluorescence the cells expressing Fos/Npas4 can be visualized. When used in conjunction with the RAM construct it can be used to provide cellular activity information at two time points.

RAM

The RAM construct works via similar mechanisms as *Fos/Npas4* in that its expression is related to increased neural activity/immediate early genes. The RAM construct itself is a synthetic activity-regulated promotor construct that specifically responds to the induction and expression of the immediate-early genes *c-fos* and *Npas4*, thereby displaying neuronal activity sensitivity. One of the unique characteristics of the RAM construct is that it maintains expression of the mKate fluorescence level for an extended period of time, i.e. not degrading as immediate early genes do. This allows for another test to be ran days later. Therefore, using RAM in conjunction with Fos/Npas4 creates results at 2 different time points, during potentially 2 different activities.

This technique then produced results in which both the immediate early gene activity tracked by the RAM, and the immediate early gene activity tracked via Fos/Npas4 fluorescent detection show up in the visualization of the specimens, even if they overlap.

The RAM construct must be injected into the brain, *in vivo*. By way of a viral vector, the RAM construct infects the cells nearby the injection site, as any typical viral infection. At this point the RAM is transcribed as an enhancer would be transcribed in response to an increase in neural activity. However, this conversion to the RAM construct from its precursor, can be blocked by doxycycline. The antibiotic blocks TRE tTA from binding TRE, and creating the enhancers, of which RAM is one. Therefore the expression of RAM can be temporally controlled to a given task via a diet including food containing the antibiotic.

Sacrifice and tissue collection

Ninety minutes post-test day 2 (day 18), the subjects were injected with fatal plus, and euthanized. By euthanizing the animals in this time frame the expression of relevant Fos/Npas4 activity may be captured to detect neural activity during the second test day. The thoracic cavity was opened by making a transvers incision to open the abdomen, followed by sagittal incisions on either side of the specimen. The superficial layer of the rats' abdomen was lifted up exposing the diaphragm, which was cut away revealing the thoracic cavity. After exposing the heart, a needle was inserted into the left ventricle and up into the ascending aorta. At this point a saline solution was pumped through the subjects' circulatory system (100 mLs), followed by a solution of 4% paraformaldehyde in 0.1M sodium buffer (pH: 7.4, 500 mLs). This fixes and hardens the brain and makes it suitable for tissue sectioning. After perfusion, the brain was extracted and placed in a vial containing the same 4% paraformaldehyde solution overnight, which was then switched to a 20% sucrose solution in 01.M sodium phosphate buffer for 4-6 days for later cryoprotection.

Sectioning

The tissue was placed in a sucrose solution after the paraformaldehyde, and then prepared for sectioning via cryostat. After removing the tissue from the sucrose solution, brains were rapidly frozen and placed in a -80^{o}C freezer. The cryostat (Leica CM 1900, Buffalo Grove, IL) was set to -20^{o}C and slices of 30 micrometers thickness were collected serially into 4 wells. To prepare the tissue for sectioning, the brains were attached to a base plate with OCT compound and covered in a mounting media (M1), which protected the brain from unwanted damage during sectioning. The slices were then taken via a roll plate and placed into a cryoprotectant solution and kept at -20^{o}C until further processing.,

Immunohistochemistry

Immunofluorescence was performed using Fos (SC-52; rabbit or goat polyclonal Fos primary antibody, Santa Cruz Biotechnology, Dallas, TX) and Npas4 (rabbit monoclonal Npas4 primary antibody, Activity Signaling, San Diego, CA) antibodies, on tissue sections from the study. An immunobuffer diluent was made with 5% donkey normal serum (DNS, 2 mLs); 1% of Bovine Serum Albumin (BSA, 0.4g); 0.5% of Triton x-100, (200 µL), to a volume of 40 mLs of 1x phosphate buffered saline (PBS). The Fos antibody was used in a 1:8000 dilution, and the Npas4 antibody was used in a 1:1000 dilution.

An initial procedure for the dual detection of Fos and Npas4 was then carried out as follows: the brain sections were rinsed 6x in 1X PBS for 5 min each and incubated for 1 hour in immuno buffer; all these steps were carried out at room temperature under moderate agitation. Sections were then placed into 8 mLs of immuno buffer, in addition to respective antibody additions and placed at 4^{o}C with gentle agitation. Twenty-four hours later primary antibodies were washed off with 6x 5 minutes washes in 1X PBS, and incubated for 4 hours into the secondary fluorescent antibodies (Jackson ImmunoResearch Labs, Westgrove, PA), both donkey antibodies one against goat (705-545-147, conjugated to Alexa Fluor 488 for green fluorescence - Fos), one against rabbit (711-585-152, conjugated to Alexa Fluor 594 for deep red fluorescence – Npas4) at room temperature, with gentle agitation and light protection. Sections were then washed 6x in 1X PBS for 5 minutes each at room temperature. Sections were then kept in 1X PBS at 4^{o}C until mounted on glass slides and cover slipped with Vectashield Vibrance (Vector Labs, Burlingame, CA) mounting media.

For the detection of eGFP, mKate and the two immediate-early genes, the endogenous fluorescence of eGFP and mKate were detected in combination with detection of Fos/Npas4 with

their respective rabbit polyclonal and monoclonal antibodies, with detection using an anti-rabbit CY5-conjugated antibody for both Fos and Npas4 (711-175-152), allowing overall immediate-early gene detection with a far red filter set.

Microscopy

Microscopic observations were performed on a Zeiss Axio Imager Z1 widefield upright microscope equipped with an MRm monochrome camera (Zeiss) for acquisition of fluorescence signals, filters for the detection of DAPI, CY3/eGFP, and mCherry/mKate fluorescence and the AxioVision software for image acquisition. A second Zeiss Axio Imager Z1 system equipped with a monochrome infrared camera and the Zen blue software image acquisition system was employed to capture images with CY3/eGFP, mCherry/mKate, and CY5 fluorescence. Cells from each rat brain deemed to have appropriate viral BLA injections were counted from 3 different anterior-to-posterior BLA regions (approximately 2.1, 2.8 and 3.6 mm posterior to Bregma), counted from single sections in these respective regions for each brain.

Cell Counting

The cell counts were performed manually after capturing the various images. The BLA was outlined and all cells fluorescing above threshold were counted within this region of interest (ROI). Cells failing to reach fluorescence threshold, or with only partial cellular profiles were not counted. To analyze the images the hue, and contrast was adjusted to clearly distinguish cells from background, a clear example of this can be found in Figure 4. The only structures visible in that image are the fluorescent cells, which were counted, while the background was completely black. Again, cell counts were performed manually, and a cell counting program may be used in future experiments to eliminate human error.

Statistical analysis

All results in this study were analyzed using the R (ver. 3.6.3)/RStudio (ver, 1.2.5033) statistical packages. An overall factorial repeated measures ANOVA was performed on the freezing percentages on the two test days, followed by factorial repeated measures ANOVA on each separate day. Each significant test day's factor or interaction effects ($p \leq 0.05$) were followed by multiple means comparisons using the Bonferonni correction to isolate the source of significant differences, where appropriate. Similar factorial repeated measures ANOVA were employed to evaluate the cell counts for eGFP, mKate, Fos/Npas4, and the colocalization of mKate and Fos/Npas4 at different anterior-posterior BLA levels. Significant factor or interaction effects on cell counts were also followed by multiple means comparisons using the Bonferroni correction to isolate the source of significant differences, where appropriate.

Results

Behavioral analysis

A factorial repeated-measures ANOVA over the two test days freezing results (test day 1, test day 2 – within-subjects), the stimuli (pre-CS vs. CS periods – within-subjects), and the three test groups (CS-shock, context extinction, Cs-shock, CS+context extinction, and CS, context extinction – between-subjects) revealed overall group ($F_{1,144} = 20.86$, $p < 0.05$) and stimuli ($F1,144 = 6.71$, $p < 0.05$) effects, but not significant interactions (all p's > 0.05). Freezing behaviors were further analyzed on the two distinct test days. The first test day evaluated the success of the fear conditioning parameters, the pairing of a tone (conditioned stimulus, CS) with a foot shock (unconditioned stimulus, US). The success of the task was measured by the amount of time the subjects froze in response to the CS. Freezing represents a fear response for the

chosen subjects, and freezing when the tone alone was presented, post conditioning, indicates successful fear learning. Test day 2 investigated the success of a fear extinction paradigm. In other words the learning that the CS no longer predicts the US. A successful fear extinction paradigm would result in the subjects no longer exhibiting a fear response, i.e. not freezing when the tone was presented.

For this experiment the subjects were broken into 3 groups. Group 1 received the CS-shock pairings and underwent context extinction. Group 2 received CS-shock pairings as in group 1, but group 2 received CS and context extinction prior to test day 2. Finally group 3 received no classical conditioning of the tone with the foot shock and received a context extinction task prior to test day 2. During test day 1, Group 1 (paired, no extinction)exhibited a significant level of freezing, as shown in Figure 1. Group 2 (paired, extinction) also exhibited a high level of freezing. However group 3 (unpaired, no extinction) exhibited a very low level of freezing. These results were supported by a factorial repeated measures ANOVA on the percent freezing results on day 1, which revealed significant group ($F_{1,72} = 11.69$, $p < 0.05$) and stimulus condition ($F_{1,72} = 4.30$, $p < 0.05$) effects, with a multiple means comparisons indicating significant differences between the two groups that received CS-shock pairings compared to the CS no shock group, respectively ($p < 0.05$, Bonferroni correction). The results of test day 1 indicated reliable and specific fear conditioning to the tone CS; groups 1 and 2 (paired groups) both exhbited a significant fear response, while group 3 (unpaired) exhibited very little fear response.

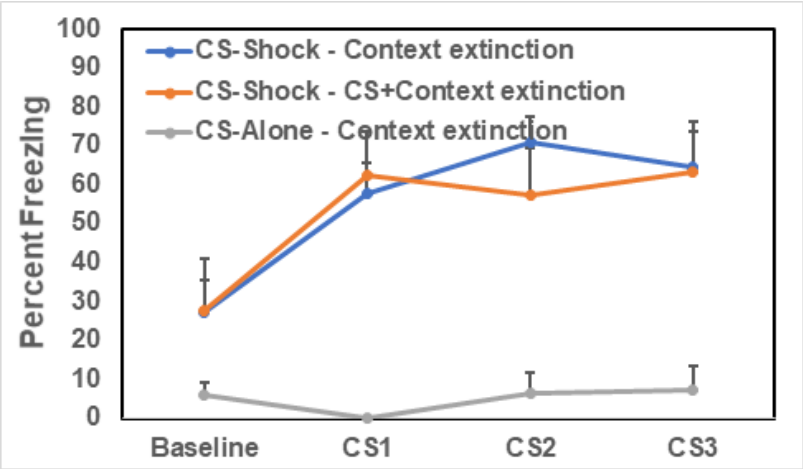

Figure 1: graphical depiction of Test day 1 results. The graph shows that animals receiving the classical conditioning paradigm, pairing a shock with a tone, exhibited a greater fear response to the tone alone on test day 1, as opposed to the control.

During test day 2, Group 2 (paired, extinction) and 3 (unpaired, no extinction) both exhibited reduced freezing time as compared to group 1 (paired, no extinction), which still exhibited significant freezing responses, as displayed in Figure 2. These results were supported by a factorial repeated measures ANOVA on the percent freezing results on day 2, which revealed a significant group ($F_{1,72} = 11.69$, $p < 0.05$) effect, with a multiple means comparisons indicating significant differences between group 1 (paired noextinction) as compared to the CS no shock and CS extinguished groups ($p < 0.05$, Bonferroni correction). The results of test day 2 indicated the success of the extinction task. Group 2 (paired, extinction)had previously behaved in accordance with the fear conditioning paradigm during test day 1, now behaved as if they had never received tone and footshock pairings, i.e. group 3.

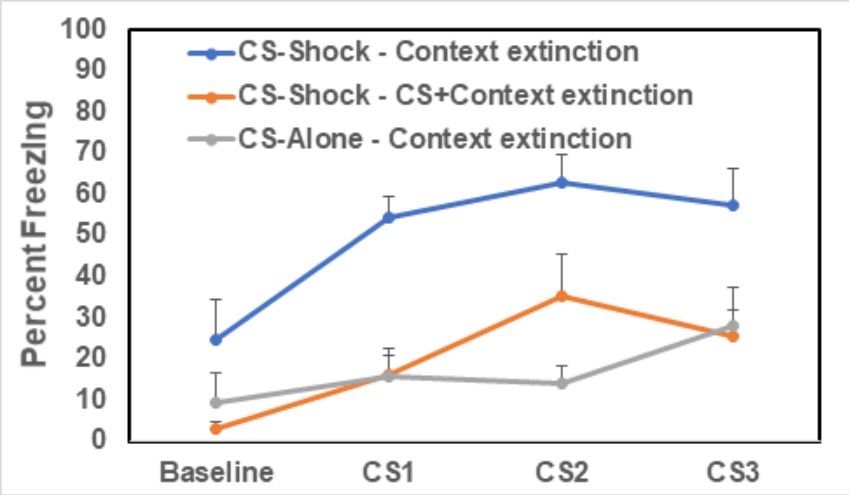

Figure 1: graphical depiction of test day 2 results. The graph shows the group receiving the CS extinction task exhibited a lower fear response than the group receiving just a context extinction task.

The behavioral results as a whole served to confirm the hypotheses that classical conditioning of a tone CS reliably induces fear, and CS extinction significantly reduces fear responses. The behavioral data suggested that the histological brain results should reflect these different states between the two test days.

Microscopy data

The microscopy data serves to visually depict the neuronal ensembles responsible for the behavioral state induced by a given task. The first step in this process was confirming the success of the distribution of the RAM construct, and the success of the surgical injection. eGFP was employed to investigate injection success. eGFP was expressed in all cells wherever it reached, displaying the spread of the RAM construct, as well as accuracy of injection, or if an injection occurred.

eGFP

The imaging of eGFP indicated there was a repeated issue where animals received no, or unilateral injections. 6 of the 20 injections failed. However, all animals displayed cannula tracks, bilaterally. A significant number of the remaining animals underwent injections which did not

arrive in the BLA, however the dispersion of the RAM construct allowed cells in the BLA to present eGFP. Twelve of the 20 injections induced eGFP expression in the BLA even though only 7 of the 20 injections actually landed in the BLA. Therefore the microscopy results were limited to the animals expressing eGFP in the BLA (n=2, group 1, n=3, group 2, n=1, group 3). Figure 3 graphically depicts the number of cells expressing eGFP in the various groups, as well as a visual depiction of eGFP expressing cells in the BLA. Based on these rats, the number of eGFP-expressing cells in the BLA indicated a significant effect of group ($F_{1,6} = 7.26$, $p < 0.05$), with a significant difference only between groups 2 and 3 ($p < 0.05$, Bonferroni correction). Based on these findings, it is expected that infection of BLA cells by the RAM construct should be relatively similar in groups 1 and 2, with the rat from group 3 having relatively higher RAM infection.

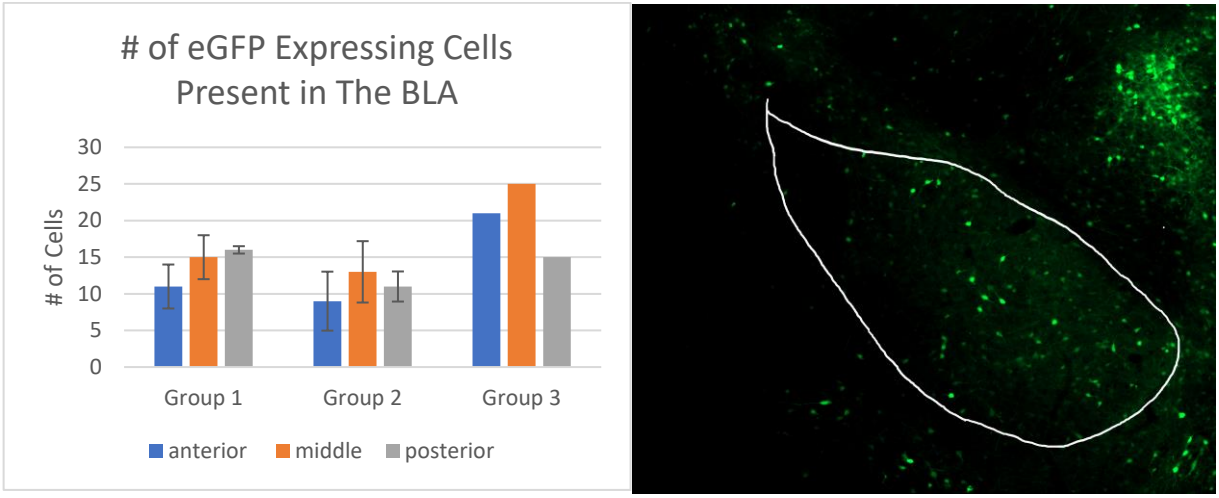

Figure 2: left: graphical depiction of the number of infected cells in the BLA of each group; right: image of the BLA (outlined in white) showing infected cells (eGFP expressing) in green (subject 13, in group 2).

mKate

Microscopic analysis revealed significant numbers of cells expressing mKate in the BLA of groups 1 and 2, the groups which underwent fear conditioning. Additionally, the control group

of subjects which remained on doxycycline the entire time expressed no mKate, and group 3, which did not experience fear conditioning, expressed no mKate, refer to figure 4. The number of mKate-expressing cells in the BLA indicated a significant effect of group ($F_{1,6} = 19.06$, $p < 0.05$), but no significant differences were obtained from the multiple mean comparisons with Bonferroni correction. This was likely due to the small n's in the groups and the lack of variability in group 3 due to the absence of mKate-expressing cells. No differences in mKate expression were anticipated between groups 1 and 2 because both groups received CS-footshock pairings and were behaviorally similar based on the freezing results presented above.

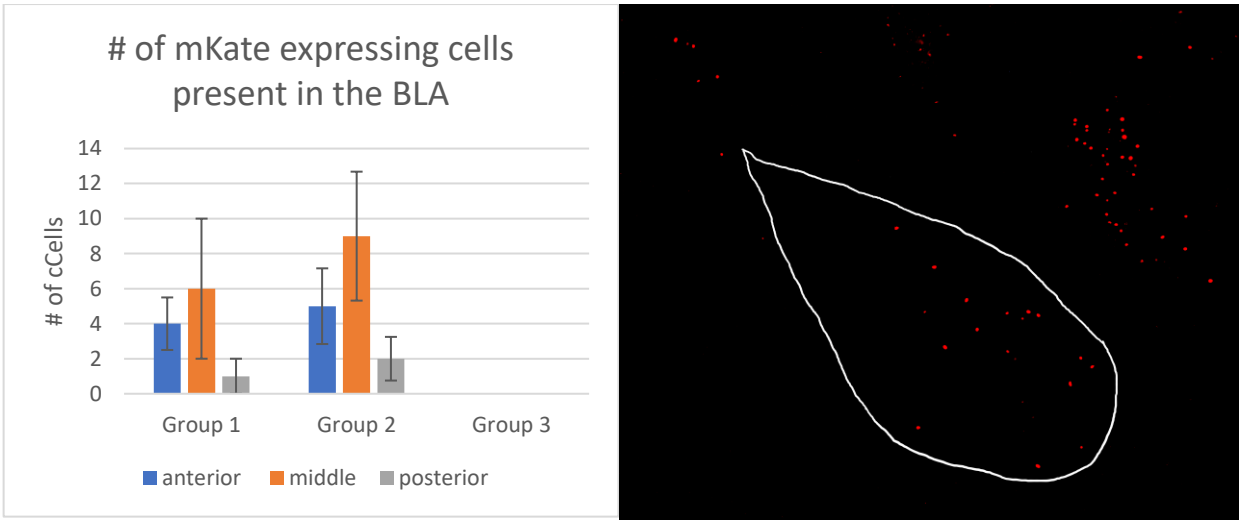

Figure 3: left; graphical depiction of the number of cells expressing mKate in each group right: image of the BLA outlined in white and the mKate expressing cells shown in red, (subject 13 in group 2).

Fos/Npas4

To further investigate these results, the two immediate-early gene proteins were studied alone with different color fluorescent markers. These results showed there was relatively more Npas4 expression than Fos, and approximately ½ of these cells were colocalized. The n of this investigation was small as only several sections of one subjects' brain were evaluated. Figure 5 depicts the number of cells in each group.

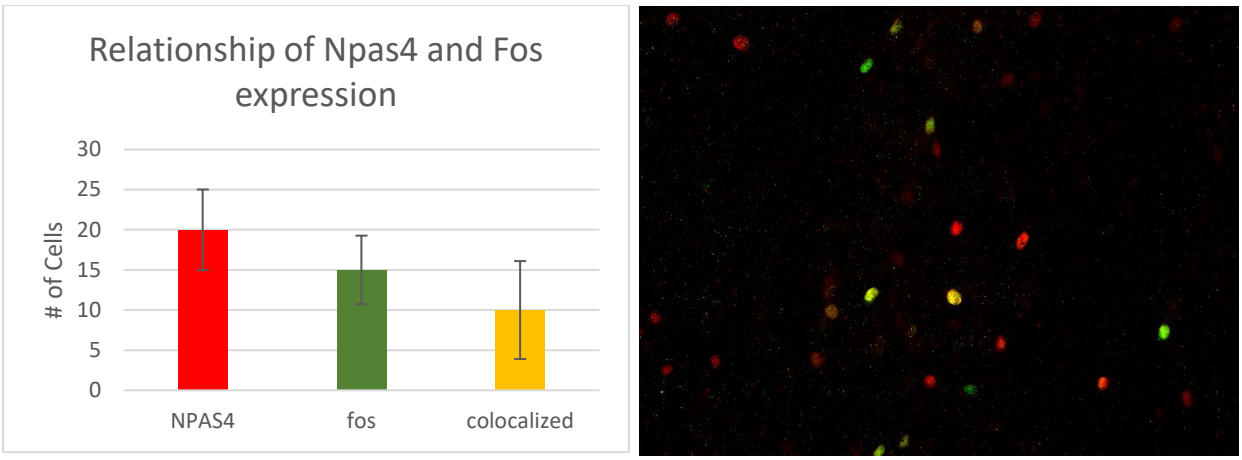

Figure 5: left; graphical depiction of the average number of fos and Npas4 cells; Right; image displaying Npas4 cells in red and Fos expressing cells in green. Colocalization of Fos and Npas4 fluorescence gives rise to yellowish color, as shown in the image at right.

In test day 2 all groups expressed significant levels of Fos/Npas4, regardless of the learning and extinction paradigm they had experienced, as seen in figure 6. Both proteins were detected with the same fluorescent marker, and are shown in Figure 6 as white regardless of which protein is detected.

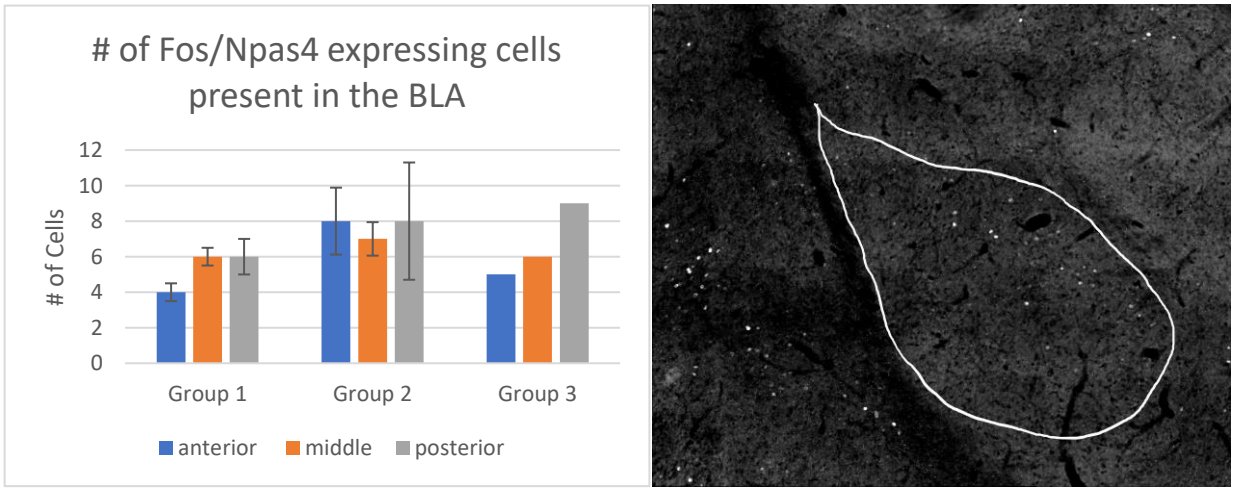

Figure 6: left; graphical representation of the numbers of cells expressing Fos/Npas4 in each group; right; image of the BLA containing Fos/Npas4 expressing cells shown in white (subject 13 in group 2).

Colocalization of mKate and Fos/Npas4

The populations of mKate and Fos/Npas4 expressing cells were then evaluated in respect to each other. The analysis indicated there was significant colocalization of the cells responsible for fear learning and fear extinction. This was suggested by the double labeling of cells as Fos/Npas4 and mKate expressing as seen graphically in figure 7 and visually in figure 8. In the factorial repeated measures ANOVA on colocalization of Fos/Npas4 in mKate positive cells, (group = between-subjects factor; 1, 2, 3), (BLA area = repeated-subjects factor; Anterior, middle, posterior), there was not effect of group or area reported by this analysis (all p's > 0.05), likely due to the small number of rats per group.

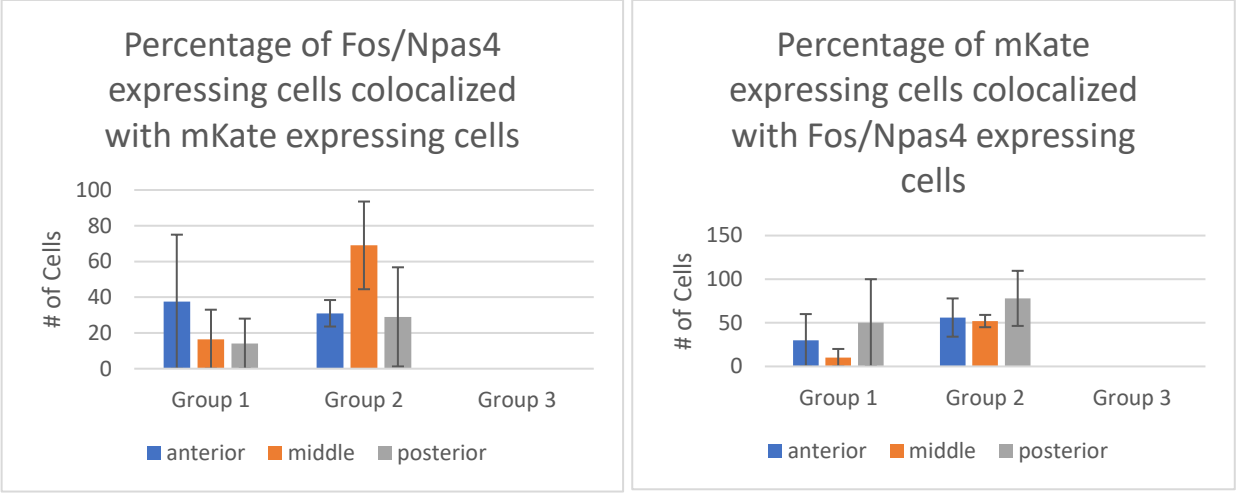

Figure 4: Percentage of colocalized cells with respect to each cell type

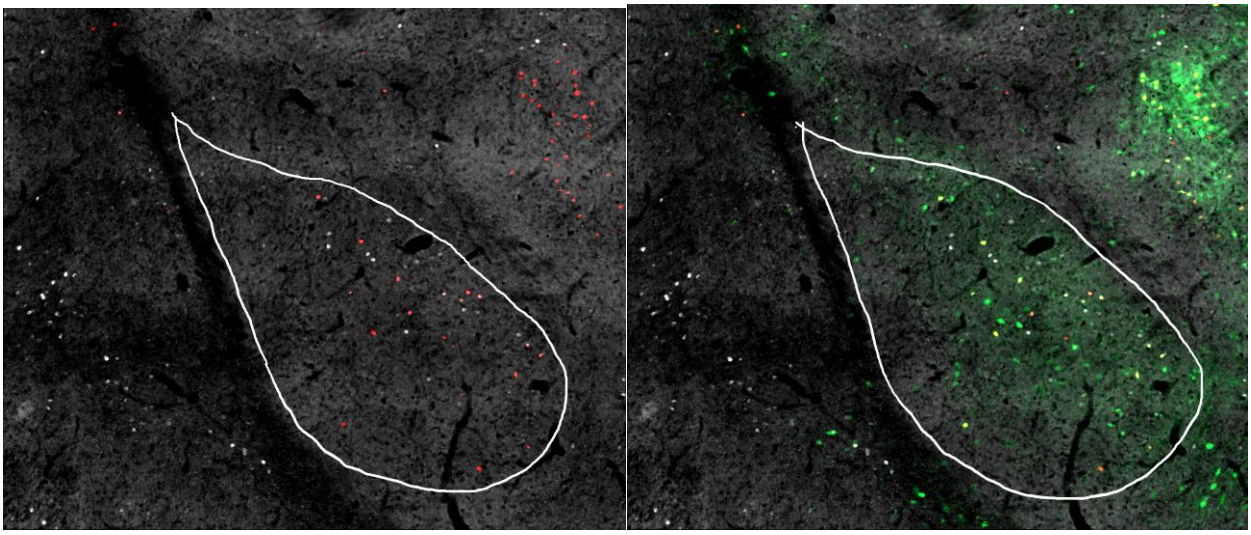

Figure 5: Left; image displaying both mKate expressing cells (red) and Fos/Npas4 expressing cells (white); Right; image displaying eGFP expressing cells (green), mKate expressing cells (red), and Fos/Npas4 expressing cells (white) (Subject 13 in group 2).

Discussion

Surgery

The most substantial issue with the implementation of the RAM technology lies in the surgical injection. As depicted in the results, the surgeries suffered the repeated misfortune of a failed injection. In total 6 of the 20 injections failed completely. Several steps can be taken to correct this issue. Cleaning the tip of the injector with a saline solution after measurements and between injections, may ensure no dried blood blocks the injection of the viral solution. Furthermore, ensuring a good fit of the injector pin within the syringe may ensure substantial pressure is created to push the viral solution into the brain, as the process of inserting a syringe may cause brain matter to block the tip of the injector. Despite these setbacks the injection of the RAM construct and the mechanism of its distribution (viral vector) were relatively successful in a small number of rats. Additionally the spread of the ability for the construct to disperse allows for some error in injection accuracy as it spreads throughout the local area.

Behavior

The behavioral portion of the study confirmed the previous understanding of fear learning and fear extinction. The fear conditioning was successful and resulted in significant freezing behaviors during presentations of the CSs on test day 1, as would be expected from the groups receiving pairings of the CS and footshock. This response was specific to the CS based on the significant stimulus effect obtained on test day 1, indicating that freezing was significantly increased in response to the CS. Furthermore, the results obtained on test day 2 strongly suggested that the conditioned group that underwent CS extinction (group 2) displayed lower levels of freezing as compared to the context only extinction rats (group 1). Based on these behavioral observations, the differential behavioral outcome was expected to lead to differential BLA neuronal ensembles partly responsible for a state of increased fear as compared to a state of reduced fear.

RAM

Relevance

The ability for the RAM construct to tag relevant cells was investigated in correlation with the behavioral results. The behavioral results indicated that groups 1 and 2, had undergone a successful fear learning paradigm due to their level of freezing during the first fear test. Group 3 which had not undergone a fear conditioning paradigm exhibited no freezing. Therefore we would expect to see active cells in the BLA in groups 1 and 2 but not in group 3 (Zhang, Kim, & Tonegawa, 2020). The microscopy results displayed significant expression of mKate fluorescent protein in groups 1 and 2 but not in group 3. This demonstrated the efficacy of the RAM construct in that it tagged cells of expected neuronal ensembles.

Temporal Specificity

After evaluating the success of injection of the RAM construct, and the relevance of mKate expressing cells, the temporal specificity was tested. The temporal specificity of the RAM construct relies on doxycycline's ability to inhibit transcription of the enhancer/reporter mKate complex. Subjects of a group which remained on doxycycline during test day 1 (n=2, data not shown) failed to express any mKate, whereas subjects of the same group which did not remain on doxycycline during test day 1 expressed significant levels of mKate. This served to demonstrate the efficacy of doxycycline as an inhibitor of the RAM construct, and the constructs' temporal selectivity.

Fos/Npas4

The Fos/Npas4 expression was found to be relatively equal across all groups, and approximately 50% colocalization between mKate expressing and Fos/Npas4 expressing cells. However, the cells responsible for fear extinction are genetically and spatially separate (Zhang, Kim, & Tonegawa, 2020). The report by Zhang and collaborators (2020) was published midway through the current project, and a prior understanding of the subject matter was lacking until that point. The Zhang et. al., paper had the same goal, but used slightly different methods, they only used Fos as a marker for activity. Therefore, there may be a specificity issue with dual labeling of Fos and Npas4, specifically Npas4, because the colocalization of the two immediate-early genes fell short of 100%. This technical difference could explain the differential results obtained in the 2 studies.

To investigate the potential specificity issue, the relationship between Fos/Npas4 was studied. Each protein was tagged with their own fluorescent marker. The results indicated that

the two are not interchangeable markers, as a significant portion of the cells expressing a given protein may not colocalize with the other protein. Fos when used as the primary activity marker to investigate the neuronal ensembles responsible for fear learning and extinction may suggest that the neuronal populations are distinct and separate for each respective task (Zhang, Kim, & Tonegawa, 2020), however, due to the variance in the expression of each protein further investigation must be done to understand the role of Npas4. While Npas4 and Fos are proteins made in response to the activation of immediate early genes, and can be used to track neural activity, their expression may vary. Suggesting that only using one of the markers does not fully depict the activity occurring in the brain. Furthermore, the RAM construct interacts with Npas4, and understanding its expression and relationship with Fos offers significant value.

Conclusion and implications

The results of this study indicate the success of the RAM construct, while its largest shortcomings lie in the efficacy of Fos vs Npas4. This study suggests that there are a significant number of cells responsible for fear learning that are also responsible for fear extinction, and vice versa. This evidence opposes the current understanding of these neuronal ensembles, which are understood to be functionally and spatially separate (Zhang, Kim, & Tonegawa, 2020). The implications of this would be to further investigate the role of Npas4 expression and its relationship to Fos expression.

A firm understanding of the neuronal ensembles responsible for governing fear behavior has significant implications in the treatment of fear related disorders. The ability to selectively modulate the cells responsible for fear learning and extinction would offer massive innovation in the realm of mental health treatment. The specificity and efficiency of new therapies based on this knowledge would drastically improve the current state of the mental healthcare system.

Acknowledgements.

This journey started almost three years ago as I walked into my first neuroscience class, which happened to be with Dr. Campeau. After that semester I began emailing numerous professors hoping to find an opportunity to work in a lab. Eventually Dr. Campeau offered me a spot, and to my utter amazement I was a brain surgeon within a few months.

Working on this project over the past year has shed much light on what it means to do research. I was presented with the opportunity to collaborate with brilliant minds while developing a project, for that I am grateful to have met Kayla Siletti, Dr. Johnathan Grizzell, and Dr. Michael Saddoris. This work was generously supported by the National Institutes on Drug Abuse, and I would not have been able to visualize any of my results had it not been for Dr. Spencer's generosity with his equipment.

I appreciate the time and energy that, Dr. David Root, Dr. Michael Saddoris, and Dr. Andrew Chapman, have spent working with me as members of my thesis committee and providing the opportunity to show them what I have accomplished. What the neuroscience department has done for me reaches outside the realm of academia.

Lastly, I want to thank Dr. Campeau for the last 3 years, as he has played the most significant, and consistent role in my academic experience at CU. Without him I would not have done brain surgery, or open heart surgery (perfusions). He has provided me with guidance whenever I needed it during this process, but also gave me the freedom to take things into my own hands. Through Dr. Campeau's mentorship and the efforts of all who helped me in this project, I have been given a glimpse of what it is like to feel ownership of, and produce a thesis.

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