Population and Projection-Specific Segregation of Fear and Reward in the Hippocampus

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Population and Projection-Specific Segregation of Fear and Reward in the Hippocampus

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A Thesis in the Field of Biology

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Abstract

The hippocampus is involved in a variety of mnemonic computations, including processing spatial-temporal dimensions of memory, as well as regulating stress-responses and processing emotional stimuli. Recent studies have demonstrated vast structural and functional heterogeneity along the dorsal-ventral axis of the hippocampus, and while much is known about how the dorsal hippocampus processes spatial-temporal content, much less is known about whether or not the ventral hippocampus (vHPC) contains defined populations and circuits capable of parsing out discrete emotional experiences. Here, we combine transgenic and all-virus based activity-dependent tagging strategies, as well whole brain clearing techniques, to provide evidence that the vHPC recruits two partially segregated populations in response to rewarding or aversive stimuli. While optogenetic manipulation of tagged cell bodies in vHPC is not sufficient to drive behavior, tagged vHPC terminals projecting to the amygdala and nucleus accumbens, but not the prefrontal cortex, have the ability to drive preference and aversion, as well as to “switch” or “reset” their capacity to drive either. Moreover, using an RNA Sequencing approach, we find that vHPC fear and reward cells upregulate genes associated with Alzheimer’s Disease and neuroprotection, respectively. We conclude that the vHPC contains genetically, anatomically, and behaviorally distinct populations processing fear and reward. Together, our findings suggest that separable monosynaptic vHPC outputs are functionally malleable and point to their genetic landscape as unique targets for intervening with neurodegenerative diseases.
Dedication

To my parents, Faina Shpokene and Antanas Shpokas, for always supporting and believing in me; for pushing me to reach my fullest potential pursuing the American dream. To my sister, Maria Shpokayte, for being an inspiration and a role model. This thesis is dedicated to them.
Acknowledgments

I would like to thank both of my thesis directors, Dr. Steve Ramirez and Dr. Amar Sahay for their invaluable support and guidance to this project. Without their help these findings and developments would have never been possible; thank you for believing in my abilities as a scientist and a colleague. I would also like to thank Dr. James Morris for guiding me through the thesis process and helping me achieve this major milestone. And last, but not least, I am thankful and grateful for the support and motivation I received from the Extension School overall from the faculty, staff, and students.
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Chapter I

Introduction

An elusive and unanswered question in neuroscience is how a distinct memory is formed and stored in the brain (Liu, 2012). In the last decade a substantial amount of progress has been made to identify the cellular basis of a specific memory, or an engram. New techniques combining genetic tools with optogenetics, chemogenetics, and revolutionary cellular imagining techniques have helped bring new scientific revelations to light. Understanding maladaptive states that lead to malfunctions in memory processing is key for the development of new treatments for both psychiatric and neurodegenerative disorders. Deficiencies in memory have been associated with disorders like Generalized Anxiety, Major Depressive Disorder, Post-traumatic stress disorder (PTSD), Schizophrenia, Addiction, and Alzheimer’s Disease just to name a few. Treatment options for these diseases are problematic not only in regards to side effects, but also in efficacy. For example, SSRIs (selective-serotonin reuptake inhibitors) are still the most prescribed form of antidepressant drugs and yet work for only a little more than half of patients and include side-effects such as suicide and memory loss; thus a new wave of understanding and treatment of psychiatric disorders is long overdue. The proper understanding of the neural circuits underlying these disorders may illuminate a new avenue for the development of more targeted treatment options.

The circuits implicated in mood disorders and memory modulation are in abundance, though their causal neuronal basis remains elusive. The basolateral amygdala (BLA) and the hippocampus have long been known to play a critical role in the encoding of both positive and negative memories. Nonetheless, how emotional valences are stored
and incorporated into memories is poorly understood. The ability to appropriately recognize stimulated imbued with either positive or negative valence is critical for survival as well as mental health (Beyeler, 2016). For instance, normally, anxiety is an adaptive state of increased apprehension that helps an animal avoid potential danger. However, inappropriate dysfunctions in these circuits have been known to cause various pathologies such as anxiety, depression, PTSD, and addiction (Padilla-Coreano, 2016).

The amygdala is known to be an important structure in the mediation of emotions such as anxiety, fear, pleasure, and flight responses. Bilateral ablation of the amygdala in primates has been shown to produce emotional blunting (Weiskrantz, 1956). In humans, lesions of the amygdala produces a “calming effect”, (Balasubramaniam, 1970) whereas electrical stimulation elicits feelings of fear and confusion (Feindel, 1954). Although, the amygdala circuit is implicated in anxiety in both human and rodent models, the way in which the neural circuit functions and mediates anxiety requires a mechanistic dissection. More specifically, a major circuit that is poorly understood is the monosynaptic connection between ventral CA1 (vCA1) to the BLA—a clear and strong connection between the emotional center of the brain, the amygdala, and the memory center of the brain, the hippocampus.

vCA1 and BLA are bidirectionally interconnected both anatomically and functionally (Ciocchi, 2015). The ventral hippocampus (vHPC) modulates memories associated with fear, anxiety, motivation and pleasure (Spruston, 2015). Moreover, valence associated with a particular memory appears to be malleable in the hippocampus but not in the BLA (Guenthner, 2013). Our initial data has demonstrated that vCA1-BLA terminals are plastic and have the capacity to switch valences. Does this phenomenon hold in vCA1
cell bodies or is this specific only to vCA1 terminals? If it is specific to the terminals, do other vCA1 connections, like Nucleus Accumbens (NAcc) and PFC also hold similar plastic qualities? It is possible that the capacity and malleability of vHPC to process not just different valences, but also their capacity to change, may be controlled by activity-dependent signaling pathways that mediate gene expression. To analyze the gene expression that occurs in these vHPC cells, we will pull down cells associated with either valence using Fluorescence Activated Live Cell Sorting (FACs) and process them for RNA-Seq. Sequencing these valence associated cells will allow for a various set of analyses from protein expression to DNA-Methylation that would aid in our understanding of cell signaling pathways and genes involved in vHPC memory storage. There is a strong therapeutic value and potential interest that may be associated if we were to identify a “fear” protein, gene, or receptor that is associated with this biological system.

The idea of an engram, the physical substrate of a memory, was first introduced by Richard Semon over a 100 years ago (Denny, 2014; Semon, 1925). Since then, scientists have been hunting for the elusive and mysterious engram with controversial success. Recently, several groups demonstrated that specific hippocampal cells, that are activated during memory encoding, are both sufficient (Liu, 2012; Ramirez S. L., 2013; Semon, 1925) and necessary (Cowansage K. K., 2014; Ramirez S. L., 2015) for driving future recall of a contextual fear memory and, therefore, is a representative component of a memory engram. These findings suggest a new way of studying memory with its findings leading to possible psychiatric treatments. The way in which activity dependent defined cells interact with psychiatric-disease related states, at the neuronal and systems level, remains elusive.
The brain structures associated with memory encoding, consolidation, and have been heavily studied. However, the way in which a memory is stored on a physical and molecular level is unknown. Memories are hypothesized to be stored and encoded in structural changes at synaptic junctions. (Tonegawa, 2015) Ramon Cajal suggested that memory storage is associated with synaptic strengthening. But it was Hebb that proposed that neuronal assemblies linked by adaptable synaptic connection could encode informational content in the brain. (Hebb, 1949) Memories and synaptic plasticity have many overlapping causalities, however, how the two are directly related is still complicated, as Susumu Tonegawa has hypothesized:

“… memory may be stored in a specific pattern of connectivity between engram cell ensembles distributed in multiple brain regions and this connectivity pattern is established during encoding and retained during consolidation in a protein synthesis-independent manner” (Tonegawa, 2015)

To take on the challenge of understanding the engram, technology has been developed fusing IEGs and optogenetics. c-Fos is a popular neuronal marker of activity whose promoter is used for activity-dependent inducible optogenetics. This technique allows for ChR2 targeting to only neurons active only during a very specific time period of the scientists choosing. In 2012, Liu et al. used this inducible optogenetic approach to directly reactivate neurons encoding a memory in a distinct cell population in the hippocampus. Activation of the neurons was sufficient to recall a previously formed fear memory. The approach and methods described in this work provide a powerful tool for mapping and manipulating the various components comprising an engram. In 2015, Ramirez et al. using and activity-dependent optogenetics system, chronically reactivated cell bodies associated
with a positive memory in the dentate gyrus (DG) of the hippocampus, which was able to rescue stress-induced behavioral impairments in mice. The authors speculate that:

“…acute behavioral changes reflect the degree to which directly stimulating positive-memory-engram-bearing cells might bypass the plasticity that normally takes antidepressants weeks or months to achieve, thereby temporarily suppressing the depression-like state” (Speer, 2017)

Megan E. Speer and Mauricio R. Delgado applied this theory to human studies without invasive techniques. They found that stressed individuals who chronically recalled positive memories were able to recruit reward-related neural circuits and served as an effective way to reduce stress (Felix-Ortiz, 2013).

The circuitry that modulates anxiety-related behaviors relies heavily on the synaptic dialog within the amygdala, its bidirectional connections with the ventral hippocampus (Speer, 2017). Optogenetic reactivation or inhibition of BLA axon terminals projecting to the vCA1 can increase and reduce anxiety related behaviors, respectively (Ciocchi, 2015). Targeting the opposite circuit, vCA1 to the BLA has not yet been fully characterized. The malleability of a valence in this circuit has been touched on but not dissected by Redondo et al. The authors were able to switch and permanently change the capacity the DG but not in the BLA to drive appetitive or aversive behaviors (Guenthner, 2013). This finding suggests that a hippocampal engram is reversible and plastic, however, the BLA is hardwired to drive either fear or reward memories rather than both. The authors did not investigate the effects of terminal manipulation within this circuit. It is possible that downstream effects from the hippocampus are causing the BLA to hardwire in response to gene upregulation, changes in strength in synaptic transmission, or anatomical changes in dendritic spines.
Here, we hypothesize that the cell bodies and projections from the vCA1 to the BLA may be anatomically and functionally segregated. Genetic markers profiling positive and negative memory associations have been identified in the BLA. Kim. Et al. showed that $Rspo2^+$ and $Ppp1r1b^+$ neurons are anatomically separated into anterior and posterior BLA subfields respectively. (Kim, 2016) $Rspo2^+$ BLA neurons are activated by stimuli that elicit negative behaviors, while $Ppp1r1b^+$ BLA neurons are activated by stimuli that elicit positive behaviors. The distinct projection targets from $Rspo2^+$ and $Ppp1r1b^+$ neurons may reveal divergent brain structures that mediate positive and negative behaviors. Another study has shown that positive and negative valence associated neurons are spatially intermingled and heterogeneous throughout the BLA. (Ramirez S. L., 2013) The authors suggested that valence characterization in the BLA depends solely on where the projections are coming from: NAc or Central Amygdala (CeA). Understanding the anatomical population of fear and rewarding cells both in vCA1 and the BLA will add to the scientific community’s understanding of how valence is stored in both brain regions. Taking a similar perspective as Kim et al., cells in vCA1 may not only be anatomically segregated but there may also be a gradient similar to that of the BLA. These cell population may also have distinct projections to the BLA where rewarding vCA1 cells project to the posterior BLA and fear vCA1 cells project predominantly to the anterior BLA in a $Ppp1r1b^+$ and $Rspo2^+$ dependent manner.

There are multiple theories as to how and where memory is stored ranging from synaptic connectivity to RNA storage, neither of which has yet to be proven nor disproven. In 1984, Francis Crick made a valid speculation as to how memory is stored in the brain:

“It would be sensible to look carefully for modifications to synaptic proteins and for the enzymes which modify them, in case one of these enzymes should have
peculiar characteristics. If such an enzyme were to be found it might prove to be a pointer to the seat of long-term memory” (Tognini, 2015) There are many proteins thought to be involved in memory storage and consolidation ranging from methylation markers like Dnmt1 and Dnmt3a (Feng, 2010) to neuroplasticity and transcription factors markers like CREB, HDAC, AP1, CLOCK, and NK-kB (Mcclung, 2007). Experience dependent plasticity is the capacity of neuronal circuits to remodel themselves and undergo modifications based on changes in activity and sensory inputs. These events are controlled by activity dependent signaling pathways that mediate gene expression by modifying the activity, location, and/or expression of transcriptional-regulatory enzymes in combination with alterations in chromatin structure in the nucleus. (Hebb, 1949) Further studies assessing DNA methylation and histone posttranslational modifications in a more cellular and activity dependent manner is necessary. Single cell analysis using laser capture microdissection and fluorescence activated cell sorting (FACs) will help yield necessary findings. Understanding epigenetic mechanisms behind the processing of positive and negative valences and their switch will potentially provide novel directions to create better therapeutic interventions for disorders afflicted by improper memory storage and recall like Alzheimer’s disease and PTSD.
Chapter II

Methods

Subjects

FosCreER (Jax stock: #021882) and Wildtype male C57BL/6 mice (2-3 months of age; Charles River Labs) were housed in groups of 5 mice per cage. The animal vivarium was maintained on a 12:12-hour light cycle (lights on at 0700). Mice were placed on a diet containing 40 mg/kg doxycycline (Dox) for a minimum of 48 hours prior to surgery with access to food (doxycycline diet) and water ad libitum. (Liu, 2012; Ramirez S. L., 2013) Mice were allowed to recover for a minimum of ten days after surgery. Dox-containing diet was replaced with standard mouse chow (ad libitum) 48 hours prior to behavioral tagging to open a time window of activity-dependent labelling (Ramirez S. L., 2015). All subjects were treated in accord with protocol 17-008 approved by the Institutional Animal Care and Use Committee at Boston University.

Stereotaxic Surgery and Optic Implant

Stereotaxic injections and optical fiber implants follow methods previously reported (Liu, 2012; Ramirez S. L., 2013). All surgeries were performed under stereotaxic guidance and subsequent coordinates are given relative to Bregma (in mm) dorsal ventral injections were calculated and zeroed out relative to the skull. Mice were placed into a stereotaxic frame (Kopf Instruments, Tujunga, CA, USA) and anesthetized with 3% isoflurane during induction and lowered to 1-2% to maintain anesthesia (oxygen L/min) throughout the surgery. Ophthalmic ointment was applied to both eyes to prevent corneal
desiccation. Hair was removed with a hair removal cream and the surgical site was cleaned three times with ethanol and betadine. Following this, an incision was made to expose the skull. Bilateral craniotomies involved drilling windows through the skull above the injection sites using a 0.5 mm diameter drill bit. Coordinates were -3.16 anteroposterior (AP), ±3.1 mediolateral (ML), and -4.6 dorsoventral (DV) for vCA1; -1.8 AP, ± 3.1 ML, and -4.7 DV for the BLA; -2.0 AP, ±1.3 ML, and -2.7 DV for the DG; 1.25 AP, ±1.0 ML, and -4.7 DV for the NAcc; 1.70 AP, ±0.35 ML, and -2.8 DV for the PFC. All mice were injected with a volume of 0.300 μl of cocktail per site at a control rate of 100 μl min⁻¹ using a mineral oil-filled 33-gage beveled needle attached to a 10 μl Hamilton microsyringe (701LT; Hamilton) in a microsyringe pump (UMP3; WPI). The needle remained at the target site for five minutes post-injection before removal. For all targets, bilateral fiber optics were placed 0.5 DV above the injection site. Jewelry screws secured to the skull acted as anchors. Layers of adhesive cement (C&B Metabond) followed by dental cement (A-M Systems) were spread over the surgical site. Mice received 0.1 mL of 0.3 mg/ml buprenorphine (intraperitoneally) following surgery and placed on a heating pad during recovery. Histological assessment verified viral targeting and fiber placement. Data from off-target injections were not included in analyses.

Activity-dependent viral constructs

pAAV9-cFos-tTA, pAAV9-TRE-eYFP and pAAV9-TRE-mCherry were constructed as previously described (Ramirez et al., 2015). pAAV9-c-Fos-tTA was combined with pAAV9-TRE-eYFP or pAAV9-TRE-mCherry prior to injection at a 1/1 ratio. This
cocktail was further combined in a 1:1 ratio with rAAV5/Efla-DIO-bReaChEs-TS-EYFP or AAV2/9 CAG-Flex-tdTomato.

Optogenetic Method

Optic fiber implants were plugged into a patch cord connected to a 473 or 638 nm laser diode controlled by automated software (Doric Lenses). Laser output was tested at the beginning of every experiment to ensure that at least 15 mW of power was delivered at the end of the patch cord (Doric lenses).

Behavior Tagging

When animals were off Dox, as previously reported (1,2), Dox diet was replaced with standard lab chow (ad libitum) 48-hours prior to behavioral tagging. Female exposure: One female mouse (PD 30-40) was placed into a clean home cage with a clear cage top. The experimental male mouse was then placed into the chamber and allowed to interact freely for two hours. Fear exposure: Mice were placed into a conditioning chamber and received four 0.5mA foot shocks over a 8 minute training session. Following tagging, Dox was reintroduced to the diet and the male mice was returned to their home cage with access to Dox diet. (Liu, 2012; Ramirez S. L., 2013) For 4-OHT tagging, 40mg/kg was administered I.P. in FoscreER mice one hour following behavior and were left undisturbed for 72 hours.
Behavioral Assay

All behavior assays were conducted during the light cycle of the day (0700–1900). Mice were handled for 3–5 days, 5-10 minutes per day, before all behavioral experiments.

The testing chamber consisted of a custom-built rectangular box with a fiber optic holder (38 x 23.5 x 42 cm). Red tape divided the chamber down the middle, creating two halves, each with unique designs on each wall. Right and left sides for stimulation were randomized. Day 1 was used to assess baseline levels, during which the mouse was given 10 minutes to freely explore the arena. The following day, mice received light stimulation (15 ms pulses at 20-Hz) upon entry in the designated side of the chamber (counterbalanced across groups) over a 10-minute test period. Once the mouse entered the stimulated side, a TTL signal from the EthoVision software via a Noldus USB-IO Box triggered a stimulus generator (STG-4008, Multi-channel Systems). A video camera (Activeon CX LCD Action Camera) recorded each session and an experimenter blind to treatment conditions scored the amount of time on each side. Statistical analyses involved a one-way ANOVA comparing group difference scores [time (in seconds) on stimulated side minus time on unstimulated side].

Immunohistochemistry

Immunohistochemistry follows protocols previously reported [15, 16, 18]. Mice were overdosed with 3% isoflurane and perfused transcardially with cold (4° C) phosphate-buffered saline (PBS) followed by 4% paraformaldehyde (PFA) in PBS. Brains were extracted and stored overnight in PFA at 4°C. Fifty μm coronal sections were collected in
serial order using a vibratome and collected in cold PBS. Sections were blocked for 1 hour at room temperature in PBST and 5% normal goat serum (NGS) on a shaker.

Sections were transferred to wells containing primary antibodies (1:1000 rabbit anti-c-Fos [SySy]; 1:1000 rabbit anti-RFP [Rockland]; 1:5000 chicken anti-GFP [Invitrogen]) and allowed to incubate on a shaker overnight at 4°C. Sections were then washed in PBST for 10-min (x3), followed by 2-hour incubation with secondary antibody (1:200 Alexa 555 anti-rabbit [Invitrogen]; 1:200 Alexa 488 anti-chicken [Invitrogen]).

Following three additional 10-min washes in PBST, sections were mounted onto micro slides (VWR International, LLC). Vectashield Hart Set Mounting Medium with DAPI (Vector Laboratories, Inc) was applied, slides were cover slipped, and allowed to dry overnight.

Cell Counting

Only animals that had accurate bilateral injections were selected for counting. Fluorescence images were acquired using a microscope (Zeiss LSM800, Germany) with a 10X objective. All animals were sacrificed 90 minutes post-assay or optical stimulation for immunohistochemical analyses. The number of c-Fos-immunoreactive neurons in the BLA, mPFC, and NAcc were counted to measure the number of active cells in the respective area of terminal stimulation and in the vCA1 the number of eYFP- or c-Fos-immunoreactive neurons were counted to measure the number of active cells during defined behavioral tasks in 3– 5 coronal slices (spaced 50 um from each other) per mouse. The number of eYFP-positive, c-Fos-positive, and DAPI-positive cells in a set region of interest were quantified with ImageJ (https://imagej.nih.gov/ij/) and averaged
within each animal. To calculate the percentage of overlapping cells we counted the number of eYFP-positive cells and divided by the total number of DAPI-positive cells. Statistical chance was calculated by multiplying the observed percentage of eYFP-single-positive cells by the observed percentage of c-Fos-single-positive cells; overlaps over chance were calculated as observed overlap divided by chance overlap: the percentage of double-labeled neurons ([eYFP and cFos]/DAPI) were analyzed against overlap expected by chance ([eYFP/DAPI] x [cFos/DAPI]) using paired t tests.

RNA Sequencing and FACs

Generation of single cell suspension from mouse hippocampal tissue: Five-week old male mice labeled with ChR2-YFP transgene (Liu et al., 2012) after conditioning are euthanized by isoflurane. Mouse brains were rapidly extracted, and the hippocampal regions were isolated by microdissection. Eight mice were pooled by each experimental condition. Single cell suspension was prepared according to the guideline of Adult Brain Dissociation Kit (Miltenyi Biotec, Cat No: 13-107-677). Briefly, the hippocampal samples were incubated with digestion enzymes in the C Tube placed on the gentleMACS Octo Dissociator with Heaters with gentleMACS Program: 37C_ABDK_01. After termination of the program, the samples were applied through a MACS SmartStrainer (70 μm). Then a debris removal step and a red blood cell removal steps were applied to obtain single cell suspension.
Isolation of YFP-positive single cell by FACS: The single cell suspension was subject to a BD FACS Aria cell sorter according to the manufacture’s protocol to isolate YFP-single cell population.

Preparation of RNA-seq library: The RNA of FACS isolated YFP-positive cells was extracted by using Trizol (Life Technologies) followed by Direct-zol kit (Zymo Research) according to manufacturer’s instructions. Then the RNA-seq library was prepared using SMART-Seq® v4 Ultra® Low Input RNA Kit (TaKaRa).

Analysis of RNA-seq data: The resulting 40 bp single-end reads from Illumina had good quality by checking with FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Reads were mapped to MM10 using STAR (Dobin et al., 2013), which was indexed with Ensembl GRCm38.91 Gene Annotation. The read counts were obtained using featureCounts (Liao et al., 2014) function from Subread package (Liao et al., 2013) with strandness option as -r 2. Reads were normalized with DESeq2 (Love et al., 2014). The biological replicates in the neuron samples processed at different time periods, was corrected with removeBatchEffect from limma (Smyth, 2004). Raw data along with gene expression levels were deposited to NCBI Gene Expression Omnibus GSE.

Passive CLARITY

Perfusion: C57BL6 mice were injected and tagged with viruses and experiences respectively. Mice were perfused, one mouse at a time, with 30mL of PBS first and
immediately followed by cold (4*) hydrogel mixture containing 40% acrylamide and 2% bisacrylamide. It is important to keep the hydrogel solution cold and on ice for as long as possible to prevent the solution from crosslinking prior to polymerization. Immediately following the completion of the perfusion, the brain is extracted as quickly as possible without damaging and placed into 50ML tubes with additional hydrogel, placed back on ice. Brains are left in hydrogel solution for 24-48 hours at 4*C.

Polymerization: Tubes were placed in a vacuum chamber and allowed to reach room temperature. The tube lids are then removed and the tubes are vacuumed for 30 minutes to remove all oxygen from the chamber. The chamber is then flooded with nitrogen gas and the tube tops are quickly replaced. Tubes were placed in 37*C for 3-5 hours.

Clearing: The brains are removed from the hydrogel, which should still be liquid but lightly viscous, and washed in a new tube with 4% SDS with the intention to remove all hydrogel from the sample. Samples were placed into new tube containing 4% SDS and placed on a shaker at 37*C. Clearing took about 2-3 weeks where new SDS every other day. Sections are washed in PBST for 1-2 days and are then processed and prepared for imaging.

Imaging: Samples were imaged in a 65% TDE immersion with a LaVision UltraMicroscope light sheet microscope. Images were acquired using the ImSpector software suite (LaVision Biotec) and stitched using the Stitching plugin in Fiji. Images were visualized using Imaris software (Bitplane) and cell counts were completed using Fiji.
Quantification and Statistics

Sampling strategy. Subjects were randomly assigned to groups. No statistical methods were used to determine sample size; the number of subjects per group were based on those in previously published studies and are reported in figure captions.

Image Integrity. Acquired image files (.czi) were opened in ImageJ. Processing of images in Figure 1 involved maximizing intensity, removing outlier noise, and adjusting contrast of images.

Data Analysis. Data were analyzed using Prism (GraphPad Software, La Jolla, CA) and Statistica 13 data analysis software (TIBCO Software, Inc., Palo Alto, CA). Data were analyzed using paired t-tests (two factors), unpaired t-tests, one-way or two-way ANOVAs with repeated measures ANOVAs (more than two factors), where appropriate. Post-hoc analyses (Tukey’s multiple comparisons test) were used to characterize treatment and interaction effects, when statistically significant alpha set at p< 0.05, two-tailed). Statistical analyses are reported in figure captions.
Chapter III

Results

An outstanding question in the hippocampal literature is whether or not cells active during aversive or rewarding experiences are segregated or co-mingled in the hippocampus in terms of their molecular landscape, anatomical location, and projection-specific element. The hippocampus has been traditionally under the lens of spacial and contextual memory, as opposed to events imbued with valence. To tackle these questions, I developed a novel dual-memory tagging strategies and combined that with well-established behavioral tasks, imaging techniques, and gene sequencing approaches to sparse out fear and reward in the hippocampus and its projections to the amygdala, prefrontal cortex, and nucleus accumbens.

Recent studies have successfully visualized and manipulated defined sets of cells previously active during a variety of mnemonic processes, including hippocampus and amygdala-mediated fear memorie, as well as documenting both population and projection-specific cells sufficient to drive fear and reward. Moreover, the hippocampus has been recently shown to contain functionally plastic cell bodies capable of “switching” their capacity to drive behaviors, in contrast to amygdala cells that preferentially process fear or reward. However, ample evidence suggests that the ventral hippocampus (vHPC) in particular processes both spatial-temporal components of memory, as well processes and relays emotional information monosynaptically to downstream targets contingent on mnemonic content. To that end, we intersected various activity-dependent tagging strategies, delivered optical perturbation of discrete sets of cells, and performed large-scale RNA sequencing approach to investigate the extent to which vHPC contains
molecularly distinct populations that preferentially process experiences of varying emotion, while simultaneously testing for their causal role in a projection-specific manner in driving and resetting behavioral outputs.

Figure 1

Ventral hippocampal cell bodies recruit segregated population of valence encoding neurons, however, they do not have the capacity to drive preference or aversion. To label and reactive valence encoding cells within the vHPC a viral cocktail of AAV9-TRE-ChR2-EYFP and AAV9-cFos-tTA was used paired with a optical fiber implant directly above the viral injection location. This approach directly couples the cFos promotor TRE to the tetracycline transactivator (tTA), which is dependent on doxycycline (Dox), creating an activity inducible system dependent on cFos. The system is dependent on Dox, meaning, that as long as molecule is present in the animal’s body, tTA cannot bind to its targeted tetracycline-responsive element (TRE), and prevents the driving of the expression of ChR2-EYFP. However, in the absence of Dox, after a 48 hour period of normal chow, opens up a window of time to allow for the activity dependent expression of ChR2 to occur. Fig.1a shows the expression of the viral cocktail in vCA1 of the vHPC. The number of cells tagged in vCA1 in response to fear and reward experiences recruits a similar ensemble size (Fig. 1b.).

To test the capability of vCA1 ChR2 and EYFP-only control labeled neurons to drive preference or aversion, the mice were tested in an real-time optogenetic place memory task (Fig 1E-H) On Day 1, the mice went through a habituation process, where
they were connected with the patch cord but no light or stimulation was emitted. There was no significant difference between EYFP and ChR2 positive animals during baseline. After baseline, the mice were taken off of doxycycline by changing their cage and replacing a normal chow. During the preinduction (PreI) animals showed no difference in aversion or preference. Induction was defined by Redondo et al, 2014. The procedure is used to investigate whether the valence of the memory can be reversed.

For the process of induction, the mouse is exposed to the opposite valence while receiving optogenetic stimulation for 10 minutes at 20Hz. So, the mice that had been previously fear conditioned were exposed to a female mouse for 10 minutes while receiving optogenetic stimulation, (Fig 1c.) and the mice that had previous female exposure, were fear conditioned to the same parameters as the other cohort, while receiving optogenetic stimulation (Fig 1e.). In the original fear group, there was no significance between EYFP and ChR2 groups driving preference or aversion when vCA1 cell bodies are stimulated, Pre or Post Induction. (Fig 1d.) Following induction, we failed to observe a significant difference across groups in driving preference or aversion when vCA1 cell bodies were stimulated (Fig. 1d, f.)

Following histology, we confirmed the injection location and viral expression of the mice. The tissue was stained for cfos where some animals were exposed to a female mouse or shocked 90 minutes before perfusions. Surprisingly, the subsequent histological analysis suggested that vCA1 cells processing two fearful or two rewarding experiences displayed significantly higher rates of overlapping activity compared to cells that processed fear and reward (Fig. 1g-l.), thus raising the possibility that the vCA1
nonetheless allocates emotionally-relevant experience to two partially non-overlapping sets of cells.

Figure 2

The valence associated with the terminals from vCA1 to the BLA and NAcc drive preference or aversion and have the capacity to change the valence in which they drive post-induction. Recent studies suggest that computations along the axons of a given cell body can differentially drive behaviors contingent on the downstream target. Accordingly, we reasoned that, consistent with studies suggesting that vCA1 preferentially routes content-specific information in a target-specific manner, perhaps the terminals from vCA1 to the basolateral amygdala (BLA), nucleus accumbens (NAcc), or prefrontal cortex (PFC) are sufficient drive preference or aversion. Accordingly, we tested the capacity of vCA1 terminals to switch or reset their behaviorally-relevant outputs post-induction. Animals were injected into the vCA1 and optic fibers were placed over the BLA, NAcc, or PFC (Fig. 2A). Mice were injected with a virus cocktail of AAV9-c-Fos-tTA and AAV9-TRE-EYFP into vCA1 and optic fibers were placed over the BLA, Nacc, or the PFC. (Fig. 2a.). Real-Time Place Preference schematic is presented in Fig 2B. The fear to reward experimental protocol (Fig 2c.) helps dissociate an animal’s percent preference for terminal stimulation. The subjects received stimulation of BLA, NAcc, or PFC terminals originating from vCA1 ((Fig 2d.) n = 7 subjects for EYFP, n = 8 subjects for BLA, n = 7 for NAcc, and n = 7 for PFC, **P = 0.0018, ***P = 0.0006, repeated measures one-way ANOVA followed by Tukey’s multiple comparison test) Reward to Fear experimental protocol. Animal’s percent preference for reward to
fear protocol is shown in Fig 2e. in which the subjects received stimulation of BLA, NAcc, or PFC terminals originating from vCA1 ((Fig 2f.)n = 7 subjects for EYFP, n = 8 subjects for BLA, n = 7 for NAcc, and n = 7 for PFC, **P = 0.0032, ****P < 0.0001, repeated measures one-way ANOVA followed by Tukey’s multiple comparison test). We observed that stimulations of vCA1 terminals over the BLA and the NAcc were sufficient to drive preference and aversion; remarkably, these terminals also had the capacity to “switch” or “reset” the behaviors they drove when stimulated during an experience of opposite valence. In contrast, the PFC did not drive preference or aversion and neither did the control EYFP animals, (Fig 2c-f).

To show terminal functionality, (Fig 2g-o.), we stimulated the terminals for 10 minutes in the animals home cage 90 minutes prior to perfusions. Afterwards, the tissue was stained for cfos and cfos positive cells were counted over the region of terminal stimulation. All regions, BLA, NAcc, and mPFC had significantly increased levels of cfos as compared to control No Stim animals. This provides evidence that ChR2 was sufficiently trafficked to the terminals and were functional in its ability to increase cfos levels in the cell body region of interest. Representative images of ChR2-EYFP labeling in BLA, NAcc, and PFC terminals and c-Fos after light reactivation (Fig.2g, l, m.). Percent cFos/DAPI of BLA, NAcc, and PFC non stim vs stim groups ((Fig 2i, l, o.) **P =0.0014, ****P < 0.0001 unpaired Student’s t-test).
While most studies to date have tagged and manipulated discrete sets of cells active during a defined period of time, we next sought to create a “dual memory tagging system” such that we could leverage two points in time to tag and manipulate two discrete sets of cells. To that end, we utilized a Fos-based transgenic animal under the control of 4-Hydroxy-Tamoxifen (4-OHT) paired with an all-virus Fos-based strategy under the control of Dox. Combining two activity inducible systems, allowed for the tagging of two discrete experiences in vCA1, DG, and BLA. It also further supported that fear and reward are anatomically segregated. Using this novel “dual-memory tagging” approach, we utilize both blue- and red-shifted activity-dependent channelrhodopsins to tag fear and reward in a within-subject manner and successfully demonstrate bi-directional control of behavior by activating two discrete projections to the amygdala and nucleus accumbens, as well as in the dorsal dentate gyrus and amygdala to demonstrate the robust nature of the system.

Our dual memory tagging system allowed us to visualize and corroborate our previous findings that vCA1 recruits at least two populations of cells for fear and reward, such that cells processing similar emotional experiences recruited levels of overlap significantly above chance (Fig. 3b,c. t=5.544 df=12, p value = 0.0001) When compared the chance opposite valence storing cells were significantly higher suggesting the possibility of salient cells(Fig. 3d,e. t=2.328 df=34, p value=0.0260). Further, when comparing same vs different valence of observed cells, same valence cells had a
significantly higher overlap and cell recruitment when compared to different valences (Supplemental Fig. 3. t=5.455 df=23, p value = <0.00001). Interestingly, when we compared cells recruited by an aversive or rewarding experience, we still observed a significant increase in overlap but to a lesser extent than when two similar experiences were tagged, which we speculate reflects hippocampal processing of salience. Our results were supported by combining the brain-clearing technique CLARITY with our dual labeling strategy (Fig. S1.), in which we observed largely non-overlapping sets of vCA1 cells processing fear or reward; and, interestingly, we observed a notably higher number of overlapping cells in the lateral entorhinal cortex.

Next we sought to provide evidence that this dual memory system has the capability of within animal optogenetic memory manipulations. We first chose two brain regions that are known how to have direct projections to one another, BLA and DG. We used a red-shifted channelrhopsin (bReaCh-ES) and the standard blue wavelength (ChR2) in order to avoid potential spectral overlap. (Fig 3E-F) By targeting a rewarding memory with ChR2 and a fear memory with bReaCh-ES we were able to drive preference and aversion within the same animal (RM Anova: F= 69.86, P value= 0.0001, Adjusted P values: Baseline vs Reward= 0.0058, Baseline vs Aversion = 0.0642, Reward vs Aversion: 0.0002).

By providing a proof of concept that the dual memory tagging system works, we injected an activity-dependent bReaCh-ES and TRE-ChR2 construct into vCA1 and placed the optic fibers over the terminals in the BLA and NAcc. (Fig. 3F). Despite using two different wavelength-shifted opsins to stimulate the terminals in the
BLA and the NAcc, optogenetic activation of each was sufficient to drive preference and aversion. While our observed effect was modest in comparison with DG and the BLA, (Fig. 3K) there was a significant difference between fear and reward during terminal stimulation in a place preference tasks across days (Fig. 3H). (One-Way Anova: F= 8.269, p value = 0.0021; Adjusted P Values: Baseline vs Preference: 0.0041, Baseline vs Aversion: 0.5525 Preference vs Aversion: 0.0122) (Fig. 3D)

Figure 4
To characterize the genetic and molecular landscape of vHPC reward and fear processing cells, we performed RNA-Seq experiments to get at the question whether or not reward and fear cells are genetically distinct from one another. The RNA-Seq experiments used nuclei isolated from these cells by FACS (Supplemental Figure 2A). Both fear (negative) and reward (positive) cells showed distinct transcriptomes compared to mock hippocampal neurons as shown by the principle component analysis in Supplemental Figure 2B. Top 20 differentially expressed genes identified between negative vs. mock (Fig. a.) and positive vs. mock (Fig. b.) showed no overlap with each group, suggesting fear and reward cells are transcriptionally distinct populations. We also compared the gene expressions between positive and negative, and identified top 30 differentially expressed genes as shown in Figure C. These candidate genes will be of great interest to investigate the plasticity of fear and reward cells in the following study.

To gain more insight on the molecular signature of transcriptomes associated with fear and reward cells, we grouped a set of genes with defined neuroprotective function (Supplemental Table 1) and another set of genes involved in Alzheimer’s disease, a
neurodegenerative disorder (Supplemental Table 1). Gene Enrichment Set Analysis showed that the neuroprotective gene set is only enriched and upregulated in reward cells, but not enriched in the fear cells (Fig d.). The Alzheimer’s disease gene set is enriched in fear cells but not in reward cells (Fig. e.). Interestingly, some Alzheimer’s disease genes were downregulated in reward cells. These observations suggest these two anatomically and transcriptionally distinct cell populations as potential therapeutic targets for neurodegeneration diseases and putative biomarkers for the emergence of neuroprotective phenotype.
Figure 2

a. Diagram showing neural circuitry with labels BLA, NAcc, PFC.

b. 3D diagrams of brain structures with different colors and labels.

c. Bar chart showing preference data with error bars.

d. Bar chart showing preference data with error bars.

e. Bar chart showing preference data with error bars.

f. Bar chart showing preference data with error bars.

g. Images of tagged experience and light reactivation with c-fos staining.

h. Images of no reactivation with c-fos staining.

i. Bar chart showing change in c-fos activity with error bars.

j. Images of NAcc with c-fos staining.

k. Images of PFC with c-fos staining.

l. Bar chart showing change in c-fos activity with error bars.

m. Images of BLA with c-fos staining.

n. Images of NAcc with c-fos staining.

o. Bar chart showing change in c-fos activity with error bars.
Figure 3
Figure 4

(A) Negative vs. mock

(B) Positive vs. mock

(C) Positive vs. Negative

D Neuroprotective Gene Set

E Alzheimer Gene Set
Supplemental Figure 1:

A. [Graph A]

B. [Graph B]

Video 1: Dual Memory CLARITY
Supplemental Figure 2

A

Supplemental Table 1

RNA-Seq Data

<table>
<thead>
<tr>
<th>Top 10 Genes Negative vs Positive:</th>
<th>Top 10 Genes Positive vs Negative:</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1qb</td>
<td>Complement component 1, q subcomponent, beta polypeptide [Source:MGI Symbol:Acc:MGI:88224]</td>
</tr>
<tr>
<td>Slc1a3</td>
<td>Solute carrier family 1 (glutamate transporter), member 3 [Source:MGI Symbol:Acc:MGI:99917]</td>
</tr>
</tbody>
</table>
Supplemental Figure 3

![Graph showing overlap between N-PObserved and N-NObserved](image-url)
Chapter IV
Discussion

Here we have shown that the ventral hippocampus processes fear and reward in a populations of cells that are partially distinct at the molecular and anatomic levels, as well as in their capacity to drive behaviors through functionally plastic projection-specific terminals. We further show that these same cells project to distinct areas in the brain, BLA, NAcc, and mPFC. We show that these terminals projecting from vCA1, but not vCA1 cell bodies, have the capacity to drive preference and aversion. There is previous precedent that cell body stimulation did not cause a behavioral output but terminal stimulation did (Warden, 2012). While cell body stimulation was not sufficient to drive preference or aversion despite showing heightened levels of overlapping activity in response to two experiences of similar valence, our data suggest that vCA1-BLA and vCA1-NAcc axonal outputs each perform computations sufficient to drive behavior and to respectively “switch” or “reset” their capacity to drive such behaviors, in line with the notion that vCA1 axonal outputs preferentially route independent features of a given behavior. The basis by which this switch happens has not yet been investigation and opens up the potential for future studies.

Moreover, previous studies have demonstrated that the dorsal hippocampus contains defined sets of functionally plastic cell bodies capable of driving fear or reward, while the BLA contains fixed populations that drive fear or reward contingent on their anatomical locations along the anterior-posterior axis as well as on their projection-specific elements. Our data provide evidence that monosynaptic connections between the ventral hippocampus and BLA can be artificially stimulated to bi-directionally drive
behaviors in a functionally plastic manner as well. It is interesting that when it comes to driving valence, the terminals that drive aversion, when the “switch” happens they return to a baseline level and not to preference. This suggests that fear is difficult to change and is possibly hard-wired evolutionarily to be harder to change than pleasurable experiences or emotions; the storage of fear may be stronger than pleasure, further electrophysiological may be able to shed some light on this weakening or strengthening of terminals regarding valence.

Our immunohistochemical data suggest that vCA1 contains at least three populations of neurons: two subsets which preferentially respond to positive or negative stimuli, and a population which responds to both, perhaps reflecting a biological predilection for salience. Evidence is provided through cFos histology overlap and the development of the dual memory tagging system. The development of this strategy will potentially illuminate many future studies to come. As of now, studies have been limited to one activity dependent system, here, I have provided proof of concept that not only is dual memory tagging in a single animal possible, but the memories can be functionally manipulated for a strong behavioral output. Further, by intersecting these approaches with clearing techniques and genetic sequencing strategies, these approaches enable the tagging, manipulation, and molecular documentation of cells processing fear and reward, which opens the possibility of cataloguing topographical similarities and differences between the two in a brain-wide manner. Upcoming studies will be using the dual memory strategy to tag two discrete neural ensembles, expand the whole brain using expansion microscopy, and clearing the tissue to develop a whole brain wide schematic of various behavioral experiences.
The dual memory strategy combined with expansion microcopy will allow visualization of axons, terminals, and spinal densities. It is important to note that in the current study, terminal manipulation did not have as strong of a behavioral output as the stand alone cell body manipulations, this may be due to spectral effects of red and blue wavelengths overlapping. In order to parse apart this potential phenomenon we recommend doing slice electrophysiology to attack this question on a precise cellular level. Whereas, when we reactivated in the DG or BLA in opposite wavelengths, there was no change compared to the baseline. Being able to manipulate two memories within a single animal will reduce the amount of animals used in studies saving cost of resources and animals.

Another potential next step will be to replicate the RNA-Seq findings using FACs to isolate red and green, within animal, cells. The RNA-Seq generated some very interesting findings related to the genetic differences between the cellular populations, but also in the types of genes that were upregulated. There was an upregulation of Alzheimer’s related genes, specifically Ttr and APOE were of very strong interest. Ttr is associated with familial Alzheimer’s disease; there are therapeutic targets already created to treat Alzheimer’s patients with a dysfunctional Ttr gene, it would be interesting if the same drugs could be used to change recall or extinction following fear conditioning. Further, the reward cells upregulated many mitochondria related genes and neuroprotective ones like BDNF. Using large scale genetic sequencing and single cell RNA-Seq in the future, we may be able to hone in on the cell specificity and develop potential therapies are that information.
Research Limitations

When studying phenomenon such as anxiety and depression, working with animals instead of humans, limits our abilities to properly communicate and ask questions. Mice cannot explain to us their feelings; we must use rigorous experimental techniques that are tried and true. Fear is a primal behavior that can be studied in rodents by measuring levels of freezing behavior. Pleasure is also a primal behavior that can be assessed in mice by preference, such as sucrose preference. However, anxiety and depression models are harder to study because these psychiatric diseases are much more complicated than fear or pleasure. Therefore, further studies will need to be followed up with human patients, as in the Megan E. Speer and Mauricio R. Delgado study.

Another limitation of the suggested experiments is that optogenetics is an artificial manipulation of neurons. The way the animal behaves post stimulation isn’t necessarily a natural phenomenon. While 20hz is utilized on the basis of Liu et al. 2012, this firing frequency most likely fails to recapitulate the endogenous neural firing patterns and sequence of cell activity in areas like the dentate gyrus and downstream regions as well. To overcome this barrier, electrophysiology must be done both in-vitro and in-vivo to assess the normal firing patterns of these positive and negative valence associated cells. Electrophysiology is beyond my area of expertise but may be an interesting avenue to pursue in collaboration with another lab in the future. It is likely that positive and negative valence associated neurons fire at different currents.

The last limitation to discuss is the immediate early gene (IEG) cfos. Neurons communicate effectively by means of electrical and chemical signals. In these experiments, active neurons upregulate IEGs like cfos after 1.5 hours. However, cfos is not the only IEG
that becomes upregulated or downregulated; there are many others such as *Erg*, *Arc*, and *Zif* that are found commonly in the hippocampus. There is another inducible optogenetics system paired with Arc that may be another possible method to study engrams and compare the outcomes to the *cfos* system.


Time, and Adult Neurogenesis. *Neuron*, 83(1), 189-201. doi:10.1016/j.neuron.2014.05.018


