RESEARCH PLAN

A. Background and Preliminary Data

Mass shootings have devastated our society. Nearly one in three who witness a mass shooting will develop post-traumatic stress disorder (PTSD), not to mention the impact on the families and friends of survivors[1]. It's impossible to walk through a building or school that doesn't have an active shooter plan. Our immediate survival in these fearful situations depends on our instinctive or innate defensive responses. Our long-term survival depends on our ability to recall and adaptively respond to similar events in the future. These survival systems depend on innate and learned fear networks of the brain.

Research in the last decade has provided tremendous insight into how learned and innate fear networks of the brain operate as separate systems[2,3]. However, this "separate systems" view has limited our identification and understanding of circuits that co-control learned *and* innate fears. Understanding how these co-control circuits function and integrate into the brain-wide fear network may unlock key insights into how disorders like PTSD emerge. My research program will focus on answering three fundamental questions about co-control fear circuits in the brain:

1. What properties define co-control fear circuits of the brain?
2. How do co-control circuits influence information processing in the brain-wide fear network?
3. Do co-control circuits exhibit a unique molecular topology for different types of fear? Can key hub genes in co-control circuits reorganize the brain-wide molecular fear network to re-program fear behavior?

Building on my unpublished graduate work and past studies[4,5], my postdoctoral research discovered a major co-control fear circuit in the brain. This circuit connects the excitatory CA1 subfield of the ventral hippocampus (vCA1) – a region critical for emotional memory – with the inhibitory peri-paraventricular nucleus of the hypothalamus – a region theorized to negatively regulate the stress response[6]. Inhibitory peri-paraventricular hypothalamic neurons are unique in that they form a halo (henceforth called **Halo Cells** for simplicity) around the excitatory paraventricular nucleus of the hypothalamus (Fig. 1a). My work has leveraged a number of techniques in circuit-specific viral targeting, chemogenetics, electrophysiology, optogenetics, and fiber photometric calcium imaging to uncover how the vCA1→Halo Cell circuit acts to suppress *both* learned and innate fears. Moreover, I've discovered how the vCA1→Halo Cell circuit connects with and inhibits activity in the periaqueductal gray (PAG) – a major hub in the brain's fear network (Asok et al., *Submitted*). Taken together, my findings point to a novel co-control circuit nested within the larger brain-wide fear network. My findings also raise the question: Do Halo Cell circuits communicate with other hubs in the fear network to control different aspects of learned and innate fears?

My preliminary studies have discovered that Halo Cells synapse with three key hubs in the brain's fear network – the infralimbic medial prefrontal cortex (IL), the ventrolateral septum (vLS), and the PAG (Fig. 1b). Moreover, I have discovered that virally ablating Halo Cells with tetanus toxin suppresses learned fear while paradoxically enhancing innate fear (Fig. 3).

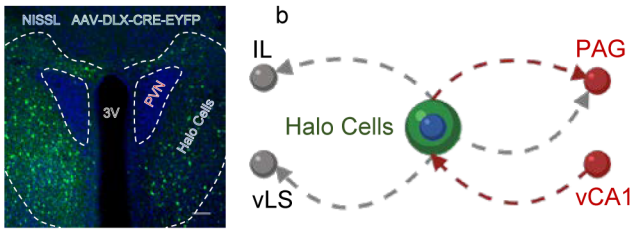

Figure 1. Halo Cells project to key nodes in the brain's threat-defense network. (a) Injection of AAV-DLX-CRE-EYFP targets Halo Cells that border the paraventricular nucleus (PVN). (b) Halo Cells project to the infralimbic medial prefrontal cortex (IL), ventrolateral septum (vLS), and periaquaductal grey (PAG). My lab will focus on dissecting how Halo Cells operate. Created with Biorender, scale bar = 100um.

B. Focus and Future Grants

During the first five years, my laboratory will focus on dissecting the electrophysiological, behavioral, and molecular function of Halo Cell co-control circuits. During this time, my laboratory will use adeno-associated and herpes simplex viruses (AAVs and HSVs) in wild-type and CRE-Cas9 mice to dissect the function of Halo Cells. In **Aim 1**, I will use micro-electrode array (MEA) technology to record from thousands of neurons across entire brain slices to identify the frequency-specific code that Halo Cell co-control circuits use to communicate with the IL, vLS, and PAG (Fig. 2). In **Aim 2**, I will use behaviorally-triggered closed-loop optogenetics to examine the frequency-specific behavioral function of each Halo Cell circuit on learned and innate fear. In **Aim 3**, I will use circuit-specific translating ribosomal affinity purification with RNA sequencing (TRAPseq) to identify circuit-specific gene networks for learned versus innate fear. I will then test how deleting key Halo Cell circuit genes influences fear behavior.

My five-year plan aims to submit two R01 applications in years 3 and 5. Aims 1 and 2 will allow me to build on my published work[7,8] to use optogenetics with multisite calcium imaging via photometry and miniscopes in order to examine how closed-loop optogenetic stimulation affects neuronal activity across the brain's fear network. The topologically-driven TRAPseq and genetic deletion studies in Aim 3 will allow me to examine how circuit-specific gene deletion alters behavior and reorganizes the brain's molecular fear network.

C. (Aim 1) Do Halo Cell Circuits use Specific Frequencies to Communicate with the IL, vLS, or PAG?

Long-range inhibitory projections may be critical for synchronizing brain networks, but their function is poorly understood[9]. Halo cell circuits are long-range inhibitory projections that synapse in the IL, vLS, and PAG (not shown). Halo Cells must communicate with each of these regions by signaling to 1) a particular GABA receptor and 2) at a specific frequency in order to explain why Halo Cell ablation can produce a bidirectional effect on learned versus innate fear (Fig. 3). That is, Halo Cells may act to entrain activity in local IL, vLS, and PAG networks to modify how information is processed during learned versus innate fears. To test this hypothesis, we will use a mixture of viral tools, large-scale MEA recordings, optogenetics, pharmacological agents, and iDISCO immunohistochemical techniques. A technically innovative strength of this Aim is the ability to register a pre-recording tissue image against both a time-dependent functional heat map (Fig. 2b-c) and post-recording cell-type specific IHC. This approach allows me to construct an "electroanatomical map" in order to identify how different populations of excitatory and inhibitory neurons function over time. Subcortical regions may prefer to communicate with cortical structures by firing at gamma frequencies[10]. Thus, I predict that Halo cells use gamma frequencies (40Hz) to influence cell-type specific activity patterns in the IL, vLS, and PAG. This aim will provide fundamental insights into how long-range inhibitory co-control circuits modulate activity in local networks of the brain-wide fear network.

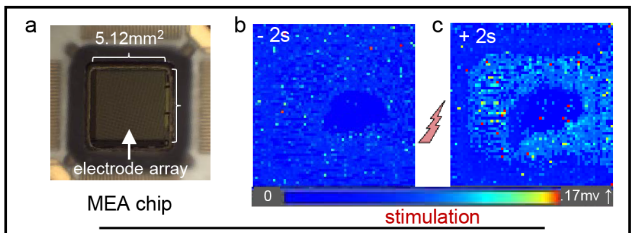

Figure 2. Micro-electrode array (MEA) approach. (a) MEA chip containing 4096 recording and 16 stimulating electrodes. (c-d) zoomed representative electroanatomical voltage variation heat map 2s before and 2s after electrical stimulation during recordings highlighting the hippocampus (HPC). 1 pixel = 1 recording electrode. Note HPC outline.

D. (Aim 2) Do Halo Cell Circuits Operate at a Specific Frequency to Control Learned and Innate Fears?

Neural circuits likely use particular frequencies to process and relay different types of information[11]. However, our knowledge of the frequency-specific function of many brain circuits is incomplete. Ablating Halo Cells reduces learned fear and paradoxically increases innate fear (Fig. 3d-g). This bidirectional effect may result from a loss of frequency-specific inputs from Halo Cells to the IL, vLS, and PAG that act to coordinate defensive behavior. A technically innovative strength of this aim is the combination of cell-type specific viruses and frequency-specific optogenetic excitation that is time-locked to software-detected episodes of behavior. By mixing these approaches with post-behavior cell-type specific cFos RNAscope, I can identify how frequency-specific circuit stimulation shifts neural activity patterns across the fear network. I predict that behaviorally-locked gamma excitation of each circuit will produce a unique pattern of cell-type specific neural activity across local IL, vLS, and PAG networks.

Although tetanus toxin ablation produces a bi-directional effect on learned versus innate fear, only three options exist for circuit-specific manipulations: an elevation, a suppression, or no effect. The post-behavior RNAscope studies will help to molecularly explain any outcome. Together, these studies will identify how long-range inhibitory neurons signal to other structures to modify defensive behavior and cell-type specific

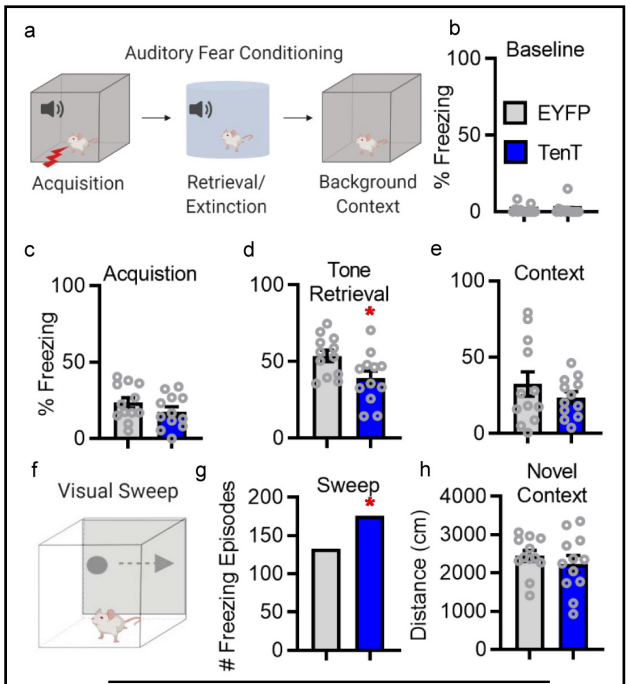

Figure 3. Tetanus toxin ablation of Halo Cells disrupts learned and innate defensive responses. (a) auditory fear/threat conditioning schematic. Halo Cell ablation did not affect (b) baseline freezing or (c) freezing at acquisition. However, it did disrupt (d) retrieval of the CS ($t(23)=2.4$, $p<.05$) with no effect on (e) background context fear. (f) Visual sweep schematic. (g) Halo Cell ablation enhanced the number of freezing episodes (chi-sq.=40.39, $p<.05$) during sweep-on periods. (h) Ablation did not affect locomotor activity. *$p<.05$ ± SEM. (n=12/group).

molecular activity. Moreover, these studies will decipher how co-control circuits bi-directionally influence defensive behaviors by shifting activity patterns across key hubs in brain's fear network.

E. (Aim 3) Can Halo Cell Molecular Networks Differentiate Between Learned and Innate Fears when Hard-wired Networks Can Not? Hard-wired brain networks contain a constantly shifting molecular environment that may contain a topological structure in much the same way as hard-wired networks. Identifying the topology of molecular networks may uncover key genes that exert master control over a molecular network and are thus critical for behavior and psychiatric dysfunction. My preliminary bulk RNAseq studies have discovered that exposure to learned and innate fears produce a different transcriptional topology in the Halo Cell↔PAG network (Fig. 4). Using a cutting-edge network analytic approach, similar to that recently used to identify novel CD8+ T-cell epitopes on HIV proteins[12], I have identified two key genes between the Halo Cell and PAG areas that may control defensive behavior to learned versus innate threats (Fig. 4). Circuit specific transcriptional profiling at cell bodies and post-synaptic targets post threat exposure may reveal critical master control genes for learned versus innate fear. By using a mixture of circuit-specific viral targeting, immunoprecipitation of mRNA from translating ribosomes, RNAseq, principles of graph and network theory, and CRISPR/Cas9 gene editing techniques, I can identify how malleable gene networks nested within the hardwired fear network influences learned versus innate fears. Given my network analytic approach is capable of identifying the most significant gene in a gene network, I expect a single gene in each Halo Cell circuit will be identified for learned versus innate fear. Moreover, I predict deletion of select genes will influence behavior. I also expect to identify novel gene subnetworks associated with physiological processes induced by threat. This aim will identify key molecules in long-range inhibitory co-control circuits that molecularly differentiate between learned and innate fear. Overall, these studies will pinpoint how circuit-specific gene networks nested within co-control circuits and the brain's larger fear network control behavior.

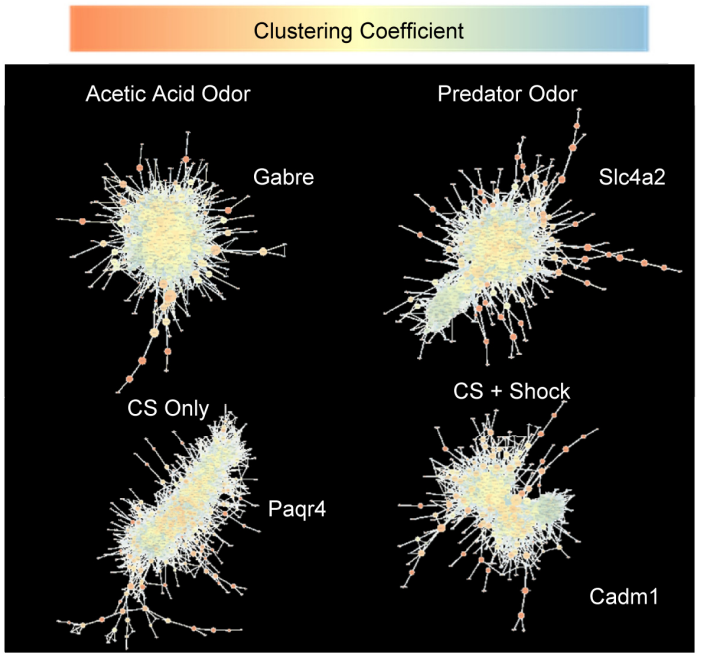

Figure 4. Network topology RNAseq analysis of the Halo Cell and PAG area 30-m after an innate threat (top) or acquisition of a learned threat (bottom) with control groups. Within group normalized gene-gene correlation analysis revealed a unique topological structure for the >7000 differentially expressed genes/condition. Remarkably, this approach, which has been applied to other biological systems, identified key hub genes for each behavior that may be critical for different defensive behaviors (n=6/group).

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