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James Park March 24, 2020

Human Medial Temporal Lobe Neuron Responses to Direct Amygdala Stimulation

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Abstract

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Forgetting memories is a common part of day to day life. In fact, the majority of memories we develop are forgotten within just a week as shown by Ebbinghaus in 1885. However, which memories are forgotten versus prioritized for long-term storage is not as obvious. Years of research have shown that emotionally arousing events are often remembered better compared to neutral events. The amygdala is one of the key structures involved in this prioritization of hippocampal-dependent declarative memory. Specifically, the basolateral amygdala (BLA) appears to be key in the enhancement of emotional memories. Moreover, recent studies in rodents and in humans have demonstrated that direct electrical stimulation of the amygdala can enhance recognition memory for neutral objects one day later without inducing subjective emotional arousal. Using a valuable data set from epilepsy patients who have depth electrodes implanted for clinical purposes, we conducted a novel analysis on medial temporal lobe single-neuron responses to amygdala stimulation. For most stimulation parameters, no significant changes to medial temporal lobe neuron firing rate occurred which parallels previous rodent experiments conducted by Bass and Manns (2015). However, an extended duration stimulation of three seconds did appear to result in an increase in firing rate at most levels of analysis. These significant differences did appear to be trivial modulations and likely non-meaningful. This study did not analyze synchronization of single neuron firing rate to low gamma oscillations as previously seen in rodent studies which will a future analysis to be conducted in human subjects.

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Introduction and Background

Introduction:

Forgetting memories is a common part of day to day life. In fact, the majority of memories we develop are forgotten within just a week as shown by Ebbinghaus in 1885 and replicated by Murre & Dros in 2015 (Ebbinghaus, 2013; Murre and Dros, 2015). However, which memories are forgotten versus prioritized for long-term storage is not as obvious. Years of research have shown that emotionally arousing events are often remembered better compared to neutral events (Cahill et al., 1995; McGaugh, 2000, 2013; Hamann, 2001; Phelps, 2004; LaBar and Cabeza, 2006). The amygdala is one of the key structures involved in this prioritization of hippocampal-dependent declarative memory (Cahill et al., 1995, 1996, 2013; Adolphs et al., 2000; McGaugh, 2000, 2013; Canli et al., 2000; Hamann, 2001; Paré, 2003; Kilpatrick and Cahill, 2003; Phelps, 2004; Richardson et al., 2004; Dolcos et al., 2004; Kensinger and Corkin, 2004; LaBar and Cabeza, 2006; Zheng et al., 2017). Specifically, the basolateral amygdala (BLA) appears to be key in the enhancement of emotional memories (McGaugh, 2000). Moreover, recent studies in rodents (Bass et al., 2012, 2014; Bass and Manns, 2015) and in humans (Inman et al., 2018b) have demonstrated that direct electrical stimulation of the amygdala can enhance recognition memory for neutral objects one day later without inducing subjective emotional arousal (Inman et al., 2018b) . However, it is unclear how amygdala stimulation impacts downstream single neuron targets in the human medial temporal lobe. The broader project this study is a part of aims to understand neuronal correlates of amygdala-mediated declarative memory enhancement in humans. Specifically, in this study, we aim to outline the neurophysiological responses of medial temporal lobe single neurons to amygdala stimulation. Using a valuable data set from epilepsy patients who have depth electrodes implanted for clinical purposes, we conducted a novel analysis on medial temporal lobe single-neuron responses to amygdala stimulation. To this date,

there have been no studies exploring how human hippocampal single neuron activity changes in function of direct electrical stimulation of the amygdala.

The Medial Temporal Lobe and Declarative Memory

The hippocampus and surrounding cortical areas serve as the core memory system for declarative memory (Squire et al., 2007). Declarative memory describes explicit memories for facts and events, and thus consists of what is colloquially considered as “memory”. Memory is not fully created and stored at the time of the event itself but instead begins neural processes at the event that cause the memory to last. Evidence indicates that over time, synaptic changes resulting from these initial processes prioritize and consolidate certain memories for long-term storage (McGaugh, 2013). Memory consolidation describes both the events at the synaptic level and the systems level (Squire et al., 2015). At the synaptic level, consolidation may involve protein synthesis while at the systems level, consolidation describes reorganization of declarative memory to become less dependent on the hippocampus over time. In either case, consolidation results in increased stability of the memory for a specific event. The amygdala is believed to interact with the medial temporal lobe to mediate these processes.

Emotional Memory and the Amygdala

Emotionally arousing events are often better remembered than neutral events (Cahill et al., 1995; McGaugh, 2000, 2013; Hamann, 2001; Phelps, 2004; LaBar and Cabeza, 2006). The amygdala has been causally implicated in emotion-dependent memory enhancement through its interaction with the medial temporal lobe – including structures such as the hippocampus and surrounding cortical areas – during the encoding phase (McGaugh, 2013), by modulating processes related to cellular consolidation (Paré, 2003). Moreover, Paré explains that direct stimulation of the basolateral amygdala (BLA) enhances memory of non-emotional events through interactions with

the hippocampus (2003). Both the hippocampus and the amygdala are key structures in emotional memory enhancement. For example, the hippocampus has been causally shown to be a necessary structure by Bass & Manns which demonstrated that hippocampal inactivation prevented the enhancement of neutral-object based recognition memory tested one day later (2014).

Numerous studies have also implicated amygdala's involvement in emotional memory. In humans, functional neuroimaging studies showed that amygdala activation during the encoding phase led to enhanced memories for emotional stimuli (Cahill et al., 1996; Hamann et al., 1999; Kilpatrick and Cahill, 2003; Dolcos et al., 2004; Kensinger and Corkin, 2004; Richardson et al., 2004). For example, a functional MRI (fMRI) study conducted by Dolcos et al. in human subjects found that blood-oxygen-level-dependent activity in the amygdala during encoding was greater and more strongly correlated for better remembered emotional pictures compared to neutral pictures (2004). Similarly, Hamann et al. conducted a positron emission topography (PET) study showing that increased energy metabolism in bilateral amygdala correlated with enhanced memory of both pleasant and aversive visual sitmuli. Beyond neuroimaging studies, patients with unilateral amygdala lesions demonstrated impaired memory for emotional sitmuli while having normal memory for neutral stimuli. Stress hormones released as a result of emotional arousal such as epinephrine, norepinephrine, and corticosterone, have been implicated in amygdala's interactions with emotional memory enhancement (McGaugh, 2000, 2013; Paré, 2003). Together, these studies make a strong case for amygdala's involvement in the enhancement of emotional memories.

Memory Enhancement through Direct Stimulation of the Basolateral Amygdala

Enhanced memory for events with emotional arousal has been a traditional focus of memory research (McGaugh, 2013). However, in recent years research has also focused on enhancing memory in neutral contexts and for neutral objects (Bass et al., 2012, 2014; Bass and Manns, 2015; Inman et al., 2018c; Ahlgrim and Manns, 2019). These studies utilize post-encoding electrical or optogenetic stimulation of the BLA in order to enhance memories for neutral objects. Specifically, previous studies in rats (Bass et al., 2012, 2014; Bass and Manns, 2015) and more recently in humans (Inman et al., 2018c) have shown that direct electrical stimulation of the basolateral amygdala can show improved memory at testing one day following the learning phase without subjective emotional arousal. Specifically, Bass & Manns found that direct electrical stimulation of the BLA can prioritize memory of specific object encounters in rats by initiating spike-timing dependent long-lasting plasticity in the hippocampus (2012, 2014, 2015). Additionally, Bass & Manns demonstrated that pharmacological inactivation of the hippocampus using muscimol, a $GABA_A$ agonist, prevents the enhancement effect from BLA stimulation – thus demonstrating the necessary role of the hippocampus (2014) in this process. Furthermore, Inman et al. showed that direct electrical stimulation of the amygdala enhances declarative memories for neutral images without patient subjective awareness (2018). These studies add to the body of literature suggesting that amgydala's role in memory modulation can be dissociable from its eliciting of emotional responses, yet still essential for enhancement of emotional memories.

Physiological Effects of BLA Electrical Stimulation

Bass & Manns demonstrated that BLA electrical stimulation results in synchronization of local field potential (LFP) low gamma oscillations (30-55Hz) across subfields in the hippocampus (2015). Additionally, this study found no significant changes in firing rate of single neurons level despite these changes in LFP oscillations. Similarly, in a study conducted by Inman et al., LFP

analyses demonstrated significant oscillatory changes throughout the medial temporal lobe during recognition of images previously stimulated in human subjects (2018). Neuronal oscillations in the hippocampus, particularly theta-modulated gamma oscillations, have been previously correlated with good memory (Tort et al., 2009; Trimper et al., 2014). For example, Trimper et al. found that rats engaging in novel object recognition tasks showed increased hippocampal subfield synchronization at the low gamma frequency range – which additionally was associated with improved recognition. While these LFP findings are illuminating, it is still unknown how direct electrical stimulation of the BLA impacts downstream medial temporal lobe single neurons in humans. Using a similar paradigm from the Inman et al. study (2018), this current study focuses on what happens at the single neuron level, in hippocampal regions, during amygdala stimulation.

Study Goals and Hypotheses

Our project is part of a larger agenda aiming to understand the specific mechanisms behind how amygdala stimulation affects other medial temporal lobe structures in order to enhance certain memories in the long-term and prioritize consolidation of these memories. Single neuron recording is an essential piece of evidence as it can be used as a direct measure of neuronal activity within a target network – the medial temporal lobe structures in this case. Furthermore, the combination with behavioral data in awake human patients is an incredibly valuable and rare opportunity to study the relationship between memory and neuronal activity. To this date, there have been no studies exploring how human single neuron activity changes and reflects the effects of amygdala modulation of memory. Our overall goal is to provide details as to how the amygdala may play a causal role in memory enhancement. Furthermore, understanding these mechanisms may be relevant for patients affected with memory-related deficits, such as dementia, traumatic brain injury or post-traumatic stress disorder.

Specifically, we aim to understand how amygdala stimulation may impact downstream single neuron activity in hippocampal regions. Given that multiple experimental paradigms were carried out (detailed in the methods section), we divided our analysis in the following three ways. (1) Firing rate was compared across all stimulation and all no-stimulation trials regardless of paradigm. (2) Firing rate was compared between different durations of stimulation (1 second vs 3 seconds). (3) Firing rate was compared between different timings of stimulation (before, during, or after stimulus presentation).

The hypotheses generated in this study draw upon conclusions from previous rodent literature. One possible hypothesis is that amygdala stimulation, regardless of the timing or duration of the stimulation, will cause a significant change in firing rate in medial temporal lobe neurons. On the other hand, the null hypothesis states that there will be no changes in firing rate in any of the analyses, in which case our study would parallel a rodent study done by Bass & Manns (2015) showing no changes in firing rate, yet increased gamma synchrony recorded in hippocampal regions following amygdala stimulation. Additionally, it is possible that a longer duration in amygdala stimulation would cause a change in firing rate not seen with a shorter stimulation. For example, previous studies have found that a one second BLA stimulation does not result in significant changes in single neuron firing rates (Bass and Manns, 2015). Lastly, the timing of the stimulation relative to image presentation may also differentially impact the firing rate of downstream medial temporal lobe neurons. Previously, one second BLA stimulation applied at the conclusion of an event resulted in memory enhancement (Bass et al., 2012, 2014; Bass and Manns, 2015; Inman et al., 2018b), but no changes to single neuron firing rates (Bass and Manns, 2015). These hypotheses and analyses aim to illuminate if and how amygdala stimulation differentially impacts medial temporal lobe neuron firing rates across different timing and durations of stimulation.

Methods

Experimental Paradigm

We examined single neuron recording data from epilepsy patients undergoing intracranial seizure monitoring via stereoelectroencephalography (SEEG), which involves neurosurgical placement of recording electrodes to specific brain networks. These are treatment-resistant epileptic patients who are temporarily implanted with depth electrodes in order to define their seizure onset zone(s) for subsequent intervention. In many of these patients, seizure onset zones are suspected to be in the medial temporal lobe, and thus implanted electrodes target the amygdala in addition to the hippocampus and other medial temporal lobe structures. This project focuses on single neuron data acquired from medial temporal lobe structures of such patients.

Patients underwent a memory task *(Figure 1)* in which they were shown a series of images for 3 seconds each, and were later tested on their memory for those images. Some of the presented images were paired with amygdala stimulation, either at different times relative to image presentation (before, during, after), or at varying durations (1s or 3s), depending on experiment subtype (“timing” or “duration” experiment subtypes). In the duration experiment subtype, there were 80 trials each of non-stimulated, one second stimulation, and three second stimulation condition. In the timing experiment subtype, there were 50 trials each of no-stimulation, before, during, and after (image presentation) stimulation timing conditions. In this experimental paradigm, “before”, “during”, and “after” refer to different conditions in which timing of stimulation relative to image presentation was varied. In contrast, we use the terms “pre-stimulation”, “during stimulation”, and “after stimulation” to refer to time periods relative to the onset of stimulation.

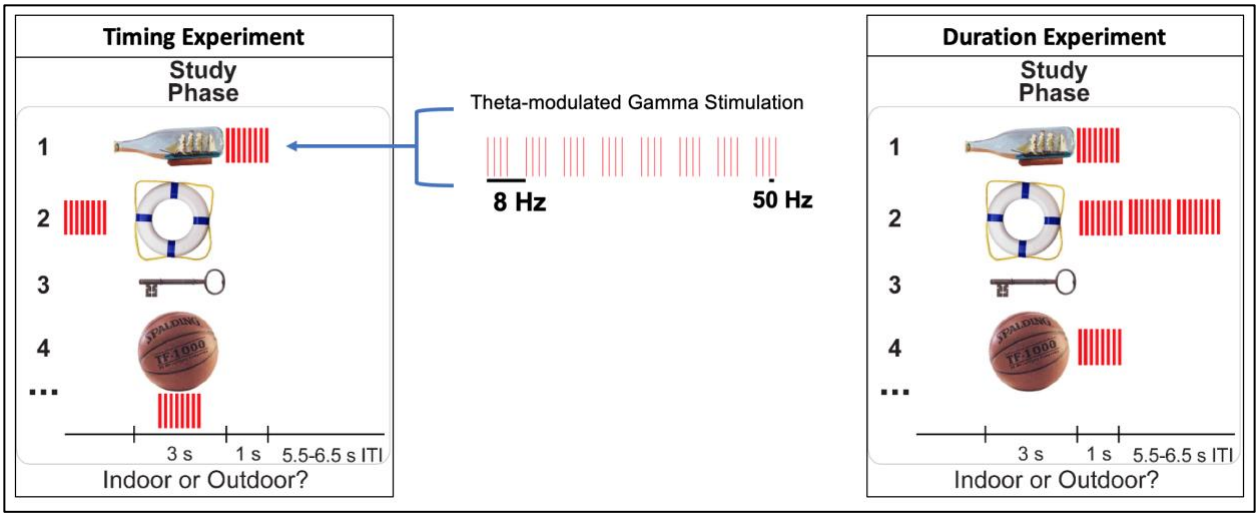

Figure 1: Experimental Paradigm. *Timing and duration experiments differ in onset and duration of stimulation, respectively. The amygdala stimulation applied is a theta-modulated gamma stimulation that parallels experiments conducted in rodents and previous studies. For the timing experiment, stimulation duration was set to one second long, and occurred either before, during, or after image presentation. For the duration experiment, stimulation duration was either one second or three seconds long and always occurred following image presentation.*

Theta-modulated gamma stimulation of the amygdala was utilized as this parallels the type of stimulation used in previous rodent and human studies (Bass et al., 2012, 2014; Bass and Manns, 2015; Inman et al., 2018b). With theta-modulated gamma stimulation, single stimulation pulses occur at a frequency of 50 Hz, in the gamma frequency range, while trains of four such pulses occur at 8 Hz, in the theta frequency range (see figure 2 right hand-side for an example of 1 second of theta-modulated gamma stimulation). As noted in the introduction section, theta-gamma co-modulation in the hippocampus is a strong correlate of memory performance (Tort et al., 2009; Trimper et al., 2014), and our stimulation pattern aims to enhance or replicate these oscillations. All patients reported that they could not tell when stimulation was occurring, which is critical to keeping subjects blinded to experimental conditions. Single neuron data is recorded at 3 points in the experiment: during the study phase, the immediate test, and the test administered one day after. This study only focused on analyzing single neuron data from the study phase, when direct BLA stimulation occurred

A total of six successful single-neuron recordings were obtained across 4 patients and 6 experiments (*Table 1*). Amygdala stimulation was applied to each hemisphere (right and left) during separate experiments for two of the patients. Only one subject participated in the duration experimental paradigm, while the other three patients participated in the timing experimental paradigm. Respective recording locations, as determined by review of spatially co-registered preoperative and postoperative imaging on a stereotactic surgery planning workstation with compared to an atlas of human brain sections (Mai et al., 2016). The number of isolated units for each recording are noted in Table 1. Five additional patients participated in experiments but the recordings yielded no isolated units, and thus they were excluded from further analysis.

Patient Code	**Stimulation Location**	**Experiment Type**	**Recording Location 1 (Study Phase Units)**	**Recording Location 2 (Study Phase Units)**
Amyg034	Left Amygdala	Duration	Entorhinal Intermediate and Lateral Rostral Subfields (8)	Parasubiculum, Entorhinal Caudal Limiting Subfield (8)
Amyg034	Right Amygdala	Duration	Entorhinal Intermediate and Lateral Rostral Subfields (8)	Parasubiculum, Entorhinal Caudal Limiting Subfield (8)
Amyg045	Left Amygdala	Timing	Presubiculum, Subiculum (0)	Parahippocampal Area TH (2)
Amyg045	Right Amygdala	Timing	Presubiculum, Subiculum (0)	Parahippocampal Area TH (2)
Amyg058	Left Amygdala	Timing	Hippocampal amygdaloid transitional area, Dorsal presubiculum, Subiculum (3)	Perirhinal Cortex BA35, 36 (3)
Amyg059	Left Amygdala	Timing	CA1/CA3/DG Hippocampal Subfields (6)	Perirhinal Cortex BA35, Entorhinal lateral rostral subfields (6)

Table 1: Summary of Recording and Stimulation Locations for Each Patient. *4 patients were tested across either the duration or timing experiments, for a total of 6 experiments run in total. There was a total of 46 single-units classified across all patients and recordings.*

Pre-Processing of the Data

Before extracting potential neuronal spikes, we removed artifacts resulting from synchronization and stimulation pulses, using MATLAB scripts developed by our laboratory. Synchronization pulses were used to synchronize neuronal recordings with the behavioral task (specifically, each pulse corresponds to onset of image presentation), while stimulation artifact is an inevitable result of direct amygdala stimulation. Stimulation and synchronization pulses must be subtracted from data because they are large amplitude artifacts that overshadow the much smaller amplitudes of actual spikes. Given that spike extraction uses a standard threshold of spike amplitudes in comparison to baseline electrical activity, having such amplitude artifacts would confound detection of spikes. Such artifacts are exemplified in *Figure* 2 where the larger amplitude stimulation pulses are prominent in comparison to the actual recording of interest. Removing stimulation and synchronization pulses allowed for smoother, more accurate extraction and sorting of spikes into units.

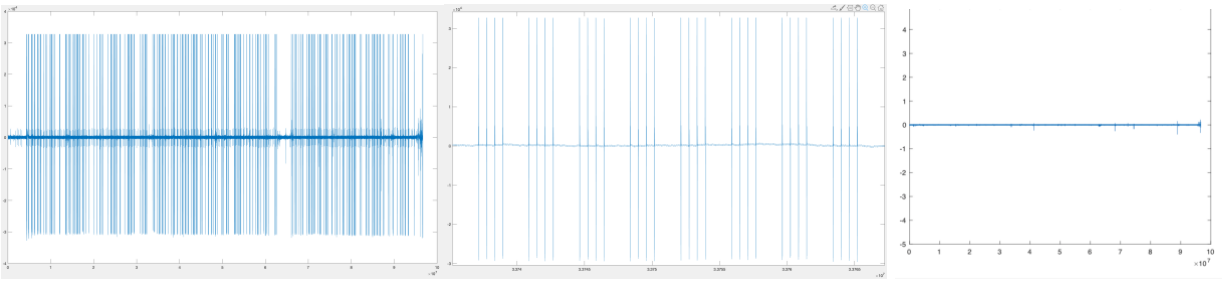

Figure 2: Example of recorded synchronization and stimulation artifacts. *Depiction of large amplitude voltage changes that were subsequently removed using code developed by our laboratory. On the left, an entire recording session is depicted with large amplitude stimulation pulses and lower amplitude sync pulses. In the middle, a zoomed-in version focuses on a single train of amygdala theta modulated gamma stimulation (in 1 second, 8 trains of 4 pulses occurring at 50Hz). On the right, the signal following removal of stimulation and synchronization pulses is shown.*

Spike Sorting

To sort our spike data into putative neurons (single-units), we used Combinato (Niediek et. al, 2016), a Python-based set of scripts built to extract and cluster units from human recordings. Combinato provides options for “spike extraction, automatic spike sorting, manual improvement of sorting, artifact rejection, and visualization of continuous recordings and spikes.” (Niediek et. al, 2016). Combinato is particularly valuable in that it is almost fully automated and thus less subjective than other spike sorting programs, such as Plexon (Plexon Inc., Texas), which rely on expertise of the researcher to sort spikes into distinct units. Here, we chose to use Combinato for sorting as it is overall easy to use and effective at processing large raw data files. Additional features for easy manual improvement of sorting and artifact rejection may make the program a better alternative to programs we previously tested, such as O-Sort (Rutishauser, Schuman, & Mamelak, 2006) and Waveclus (Wild et al., 2012).

Using Combinato, candidate spikes were extracted using a threshold superior to 6 standard deviations above baseline noise, as utilized by teams processing similar data (Tsitsiklis et al., 2020). Spikes were then sorted into different groups (single-units), using criteria such as waveform shape. Following the initial sorting process, units were not manually merged or manipulated in order to reduce subjectivity in classification (Valdez et al., 2013; Tsitsiklis et al., 2020)

Single Unit Classification Procedure

Distinguishing between artifacts, single-units, and multi-units occurred by assessment of parameters based on standard criteria previously reported by Valdez et al. (2013) and Tsitsiklis et al. (2020). Similarly to how Tsitsiklis et al. conducted their classification, single units were visually confirmed to have criteria matching single-unit requirements (*Table 2*).

Parameter	Single Unit Classification Requirements	Purpose
1. Total firing rate	Greater than 0.05 Hz (spikes/second)	Enough neural events to conduct analysis
2. Interspike Intervals	Less than 5% of spikes occurring during the 3 milliseconds refractory period.	Refractory period present
3. Population of pike peak amplitudes	Waveform peaks are significantly greater than threshold	Ability to confidently distinguish events from noise
4. Distribution of spike peak amplitudes	No bimodal spike amplitude distribution	Does not capture multiple neurons firing
5. Variability of density on waveform density plots	Generally, homogeneous variability of spike waveform throughout	Events are from one single neuron and not multiple
6. Local peaks following the main peak in spike waveform	No local peaks following main peak	Remove noisy waveforms

Table 2: Single Unit Classification Criteria. *Table 2 notes the classification criteria used for single units. Units were initially independently scored and then discussed between two co-scorers.*

These parameters include a spike firing rate greater than 0.05 Hz, less than five percent of spikes occurring within a refractory period of 3ms, peak of spikes distanced from threshold, lack of bimodal spike amplitude distribution, homogenous variability of density on spike waveform density plot, and no additional local peaks in waveforms. The classification of single units was executed by two independent scorers, then compared and discussed for internal validity. A total of 5 single units classified (11% of 46 single units classified) differed between the two scorers and were resolved following discussion.

Example Single Unit isolated through Combinato

Figure 3 depicts the Combinato interface for an example sorted single-unit. On the left-hand side, the different subgroups chosen for this particular unit can be seen. On the right-hand side, various parameters of this unit are visualized. For this unit the waveform density (A) and log density (B) plots show minimal variability throughout the length of the waveform with no local minimums outside of the main peak. Additionally, there are less than 5% of spikes occurring during the 3ms refractory period (C). The cumulative spike count appears linear showing that spiking occurred regularly and not at fixed intervals which might have indicated that the putative unit activity was the result of noise (D). Additionally, a total of 13,877 spikes attributed to this single unit far exceeds the minimum 0.05 Hz firing rate required for single unit classification. Lastly, the distribution of amplitudes shows a single peak that far exceeds the voltage threshold set by Combinato (E).

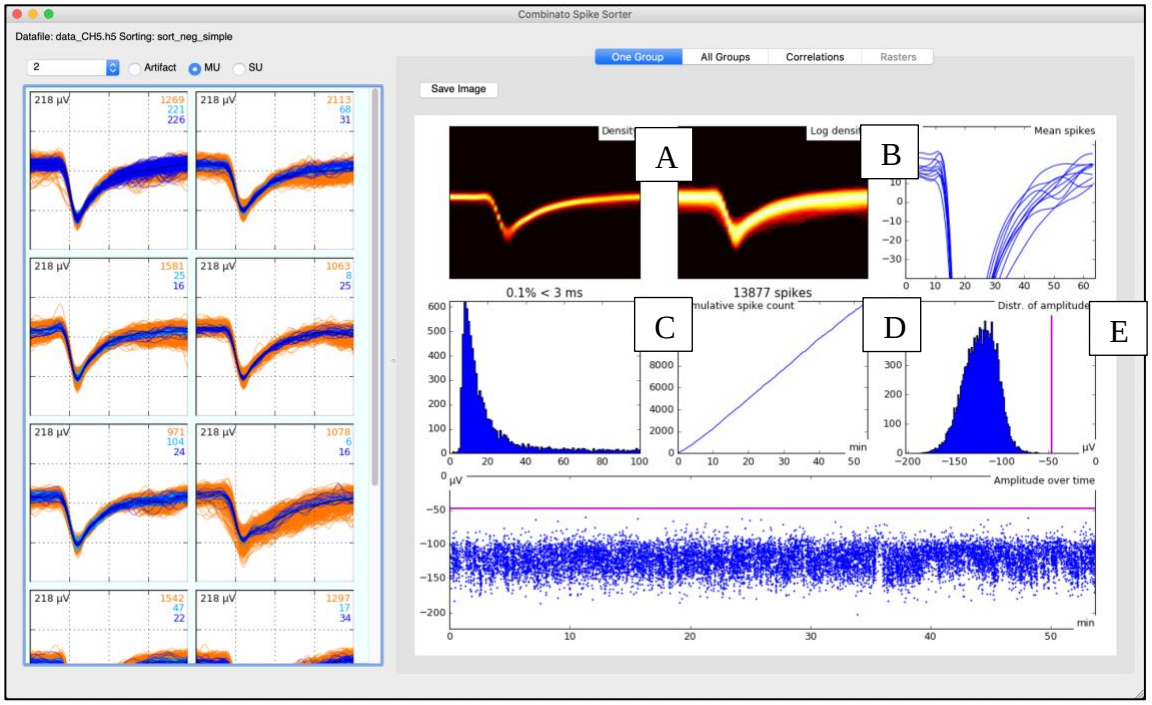

Figure 3: Example of Single Unit Classification. *Parameters of the proposed single unit are noted in the right side of the figure including waveform density plots (A, B, interspike-interval data (C), cumulative spike count (D), and information regarding spike peak amplitudes (E). On the left, numbers of spikes classified using different aspects of the sorting process are noted in orange, light blue, and dark blue.*

Analysis and Statistics

Spike-clustered data were used to examine various aspects of the recording with regards to amygdala stimulation. Raster plots and peri-event time histograms aligned to both the stimulation onset and image presentation were generated for each unit sorted along with a plot containing aligned waveforms. While firing rate data are useful to analyze across all recorded units, peri-event time histograms can serve as concrete examples for how a single unit selected by Combinato appears to react to components of the behavioral task, such as image presentation or direct amygdala stimulation.

We specifically examined how the activity of neurons in the medial temporal lobe changed during and one second following amygdala stimulation, compared to the trials where no stimulation was applied. Activity was compared within each condition as raw firing rates, difference in firing rates (stimulation – no-stimulation trials), and percent difference in firing rates. Additionally, statistical comparison by one-way analysis of variance (ANOVA) was used to evaluate changes of raw firing rates across one second pre-stimulation, during stimulation, and one second post-stimulation for each condition to see how neuronal firing rate changed in relation to stimulation.

Various control measures were applied throughout these analyses. In order to compare stimulation to no-stimulation trials, loss of spikes due to stimulation artifacts requires control for lack of spike loss in the no-stimulation trials. This was done by generating a time window template that essentially describes how stimulation looks across all trials by calculating durations of single stimulation pulses and their temporal relationships (*Figure 4)*. Each condition subsequently had non-stimulated trials controlled with respect to the stimulation condition – resulting in the non-stimulated trials being reused for each condition comparison. This template time window was then applied to all no stimulation trials in relationship to sync pulses signifying image presentation. Similarly, in order to account for spike loss due to stimulation artifacts, spikes were removed

according to stimulation time window templates applied to any analyses conducted pre-stimulation and post-stimulation. Therefore, spikes counted for each trial consisted of all spikes in the total trial duration subtracting the spikes occurring during the template control time windows.

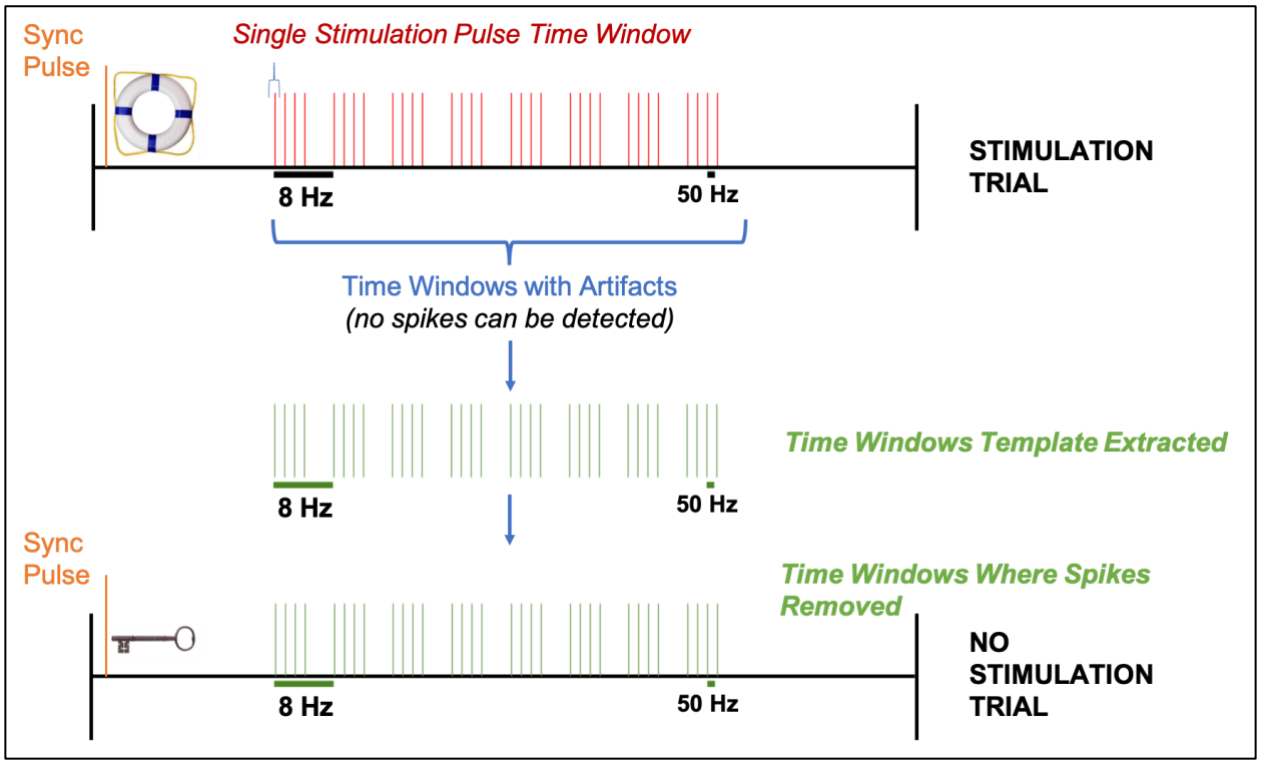

Figure 4: Spike Loss Control Procedure Diagram. *This depicts how spike loss due to stimulation artifacts was controlled for in the no-stimulation trials. A stimulation time window template was generated and applied based on all the stimulation time windows for each particular condition. Each stimulation time window template utilized the duration of each single stimulation pulse, the relationships between stimulation pulses, and the location of the stimulation pulse relative to the image presentation. This represents a general paradigm and the specific duration or timing of the time window template application differed based on the experiment type and trial condition. A similar method was utilized to control for spike loss in the pre-stimulation and post-stimulation analyses.*

Because single neuron firing rates tend to not be normally distributed, a “shuffling” procedure was coded and applied in order to compare raw firing rates (*Appendix A*). In the “shuffling” statistical analysis, there are no assumptions of normal distributions and instead a distribution is generated by randomly shuffling 10,000 random permutations of paired stimulation to no-stimulation groups or 10,000 random permutations relative to stimulation onset time. For example, for each condition the no-stimulation and stimulation values for firing rates were randomly shuffled within each trial.

Upon shuffling, the difference in means between the stimulated and non-stimulated trials was calculated and this procedure was repeated 10,000 times. When analyzing differences in raw firing rate across the stimulation timeline (pre-stimulation, during stimulation, post-stimulation), the firing rates for each unit were shuffled and a one-way ANOVA was conducted. This procedure was again repeated 10,000 times. The original value from the analysis (difference in means, f value for ANOVA) was then compared to the distribution generated through shuffling.

For the differences and percent differences in firing rates analyses, outliers were removed if outside the interquartile range by 1.5 times. Comparison across conditions of stimulation duration and onset relative to the image presentation were also examined to determine if significant differences occurred. Table 3 details the full list neuron activity analyses conducted, with their associated statistical tests. All assessments of statistical significance were conducted at an alpha value of 0.05.

STATISTICAL TESTS				
Experiment Type	**Condition**	**Period of Time Relative to Stimulation**	**Comparison Group(s)**	**Statistical Test**
Duration Experiments	One Second Stimulation	During Stimulation	Raw Stim. v Raw No Stim.	Shuffling paired t-test
			Difference (Stim-No Stim)	One sample t-test
			Percent Difference (Stim from No Stim)	
		Post Stimulation	Raw Stim. v Raw No Stim.	Shuffling paired t-test
			Difference (Stim-No Stim)	One sample t-test
			Percent Difference (Stim from No Stim)	
		Across Stimulation	Pre-Stim. v During Stim. v Post-Stim.	Shuffling One Way ANOVA
	Three Second Stimulation	During Stimulation	Raw Stim. v Raw No Stim.	Shuffling paired t-test
			Difference (Stim-No Stim)	One sample t-test
			Percent Difference (Stim from No Stim)	
		Post Stimulation	Raw Stim. v Raw No Stim.	Shuffling paired t-test
			Difference (Stim-No Stim)	One sample t-test
			Percent Difference (Stim from No Stim)	
		Across Stimulation	Pre-Stim. v During Stim. v Post-Stim.	Shuffling One Way ANOVA
	One & Three Second Stimulation	During Stimulation	One v Three second Stimulation Difference in Firing	Paired t-test
			One Second % Difference v Three Second % Difference	
		Post Stimulation	One v Three second Stimulation Difference in Firing	
			One Second % Difference v Three Second % Difference	
Timing Experiments	Before Stimulation	During Stimulation	Raw Stim. v Raw No Stim.	Shuffling paired t-test
			Difference (Stim-No Stim)	One sample t-test
			Percent Difference (Stim from No Stim)	
		Post Stimulation	Raw Stim. v Raw No Stim.	Shuffling paired t-test
			Difference (Stim-No Stim)	One sample t-test
			Percent Difference (Stim from No Stim)	
		Across Stimulation	Pre-Stim. v During Stim. v Post-Stim.	Shuffling One Way ANOVA

	During Stimulation	During Stimulation	Raw Stim. v Raw No Stim.	Shuffling paired t-test
			Difference (Stim-No Stim)	One sample t-test
			Percent Difference (Stim from No Stim)	
		Post Stimulation	Raw Stim. v Raw No Stim.	Shuffling paired t-test
			Difference (Stim-No Stim)	One sample t-test
			Percent Difference (Stim from No Stim)	
		Across Stimulation	Pre-Stim. v During Stim. v Post-Stim.	Shuffling One Way ANOVA
	After Stimulation	During Stimulation	Raw Stim. v Raw No Stim.	Shuffling paired t-test
			Difference (Stim-No Stim)	One sample t-test
			Percent Difference (Stim from No Stim)	
		Post Stimulation	Raw Stim. v Raw No Stim.	Shuffling paired t-test
			Difference (Stim-No Stim)	One sample t-test
			Percent Difference (Stim from No Stim)	
		Across Stimulation	Pre-Stim. v During Stim. v Post-Stim.	Shuffling One Way ANOVA
	Before, During, After Stim.	During Stimulation	Across Stimulation Condition Differences in Firing	One Way ANOVA
			Across Stimulation Condition Percent Differences in Firing	
		Post Stimulation	Across Stimulation Condition Differences in Firing	
			Across Stimulation Condition Percent Differences in Firing	

Table 3: Statistical Analyses *conducted to compare neuronal activity across various conditions. Duration and timing experiments were analyzed within and across conditions respective to the experiment type. For duration experiments, the two conditions were one second or three second stimulation durations. For timing experiments, the three conditions were stimulation before picture presentation, during picture presentation, and after picture presentation. Raw firing rates, differences in firing rate (stimulation – no stimulation), and percent differences in firing rates were analyzed. Additionally, one-way ANOVAs were conducted to analyze differences between conditions and across stimulation timelines. The respective statistical tests used are noted on the far right-hand side.*

Results

Example of a single unit responding to image presentation

Figure 5A shows the activity of an example single unit. For this unit, there is a sharp uptick in spiking activity around 0.5 seconds following onset of image presentation for both stimulation and no-stimulation trials. However, one important point is that this particular neuron was recorded during the timing experiment and therefore the timing of the stimulation differs across trials. Nonetheless, this figure shows that this neuron seems to respond to presentation of an image on the screen. To address the issue of differences in stimulation timing, a similar analysis was performed to analyze the effects of stimulation itself (*Figure 5B)*.

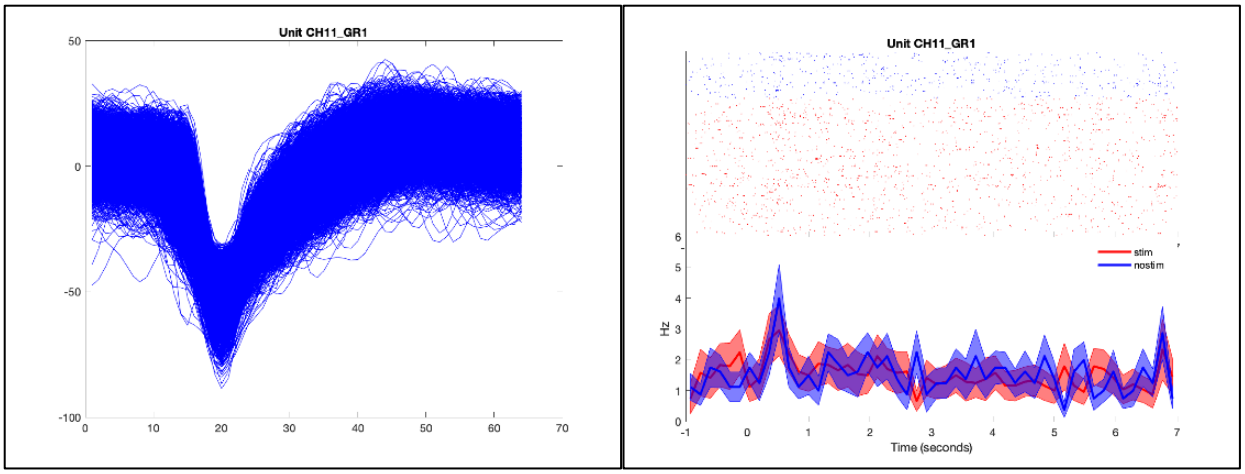

Figure 5A: Single Unit Image Presentation Response. *An ideal single unit from parahippocampal area TH (timing experiment) sorted out by Combinato. On the left, all the spike waveforms are shown aligned to the maximum amplitude. On the right, the top represents the raster plot for that single unit, each row corresponding to a trial and each dot representing an action potential. Blue shows the trials for which no stimulation was applied and red shows the trials for which stimulation was applied. Below, the mean firing rates are displayed with standard error shaded in. The zero on the spiking histogram represents the onset beginning of the image presentation.*

Example of a single unit responding to direct amygdala stimulation

The spiking histogram from *Figure 5B* shows the same neuron from *Figure 5A* with the spikes aligned to the onset of the stimulation instead of picture presentation. Given that this was a timing experiment, all stimulations occurred for the duration of one second. From this example unit, there are no clear differences in firing rate between the stimulation and no-stimulation trials. Although *Figure 5B* only depicts activity of a single neuron, this model neuron helps to visualize the raw data in a meaningful, less abstract fashion than the following analyses.

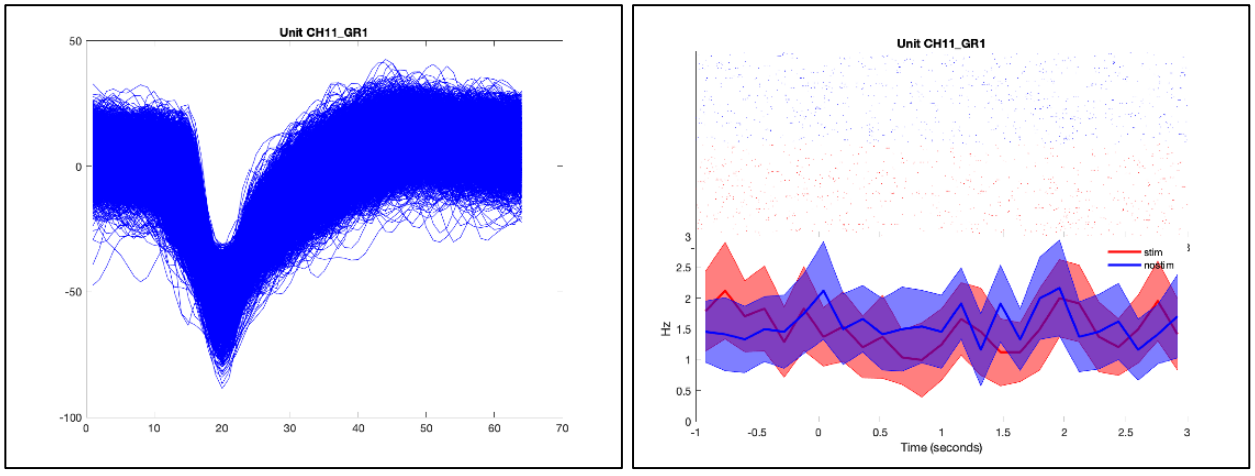

Figure 5B: Single Unit Stimulation Response: *an ideal single unit from parahippocampal area TH (timing experiment) sorted out by Combinato and its response to direct amygdala stimulation. On the left, all of the spike waveforms are shown aligned to the maximum amplitude. On the right, the top represents the raster plot for that single unit, each row corresponding to a trial and each dot representing an action potential. Blue shows the trials for which no stimulation was applied and red shows the trials for which stimulation was applied. Below, the mean firing rates are displayed with standard error shaded in. The zero on the spiking histogram represents the onset of the stimulation.*

Raw Firing Rates Analysis During and Post-Stimulation

Figures 6 and 7 provide depictions of single unit's firing rates for the duration experiments and timing experiments, respectively. The raw firing rates for the stimulated trials in each condition were compared to the non-stimulated trials. As mentioned in the methods section, significance was assessed through a "shuffled" paired t-test that does not assume a normal distribution.

For the duration experiments (*Figure 6)*, the one second and three second conditions are separated out and the firing rates for each unit are compared for the non-stimulated and stimulated trials. For the one second duration trials, there were no significant differences between stimulated and non-stimulated trials during stimulation ($p > 0.05$) and post-stimulation ($p > 0.05$). Similarly, for the three second duration trials, there were again no significant differences between stimulated and non-stimulated trials during stimulation ($p > 0.05$) and post-stimulation ($p > 0.05$).

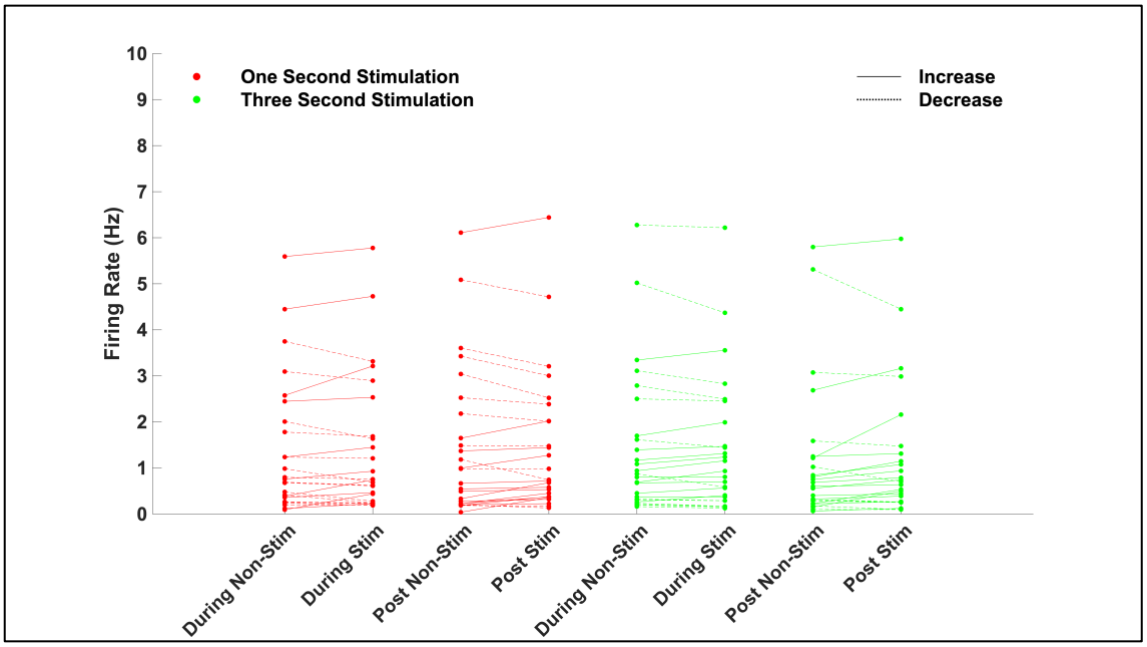

Figure 6: Duration Experiments Raw Firing Rates. *The average raw firing rates for each unit is plotted for the duration experiments for the stimulated and non-stimulated trials. The red color represents the one second stimulation condition while the green represents the three second stimulation condition. Solid lines represent a net increase in firing rate when comparing non-stimulated and stimulated trials while dashed lines represent a net decrease.*

For the timing experiments *(Figure 7)*, the different timing conditions are separated out and firing rates compared for each unit across the non-stimulated and stimulated trials. For the before condition, there were no significant differences between stimulated and non-stimulated trials during stimulation ($p > 0.05$) and post-stimulation ($p > 0.05$). Similarly, the during condition also had no significant differences between stimulated and non-stimulated trials during stimulation ($p > 0.05$) and post-stimulation ($p > 0.05$). For the after condition, there were again no significant differences between stimulated and non-stimulated trials during stimulation ($p > 0.05$) and post-stimulation ($p > 0.05$).

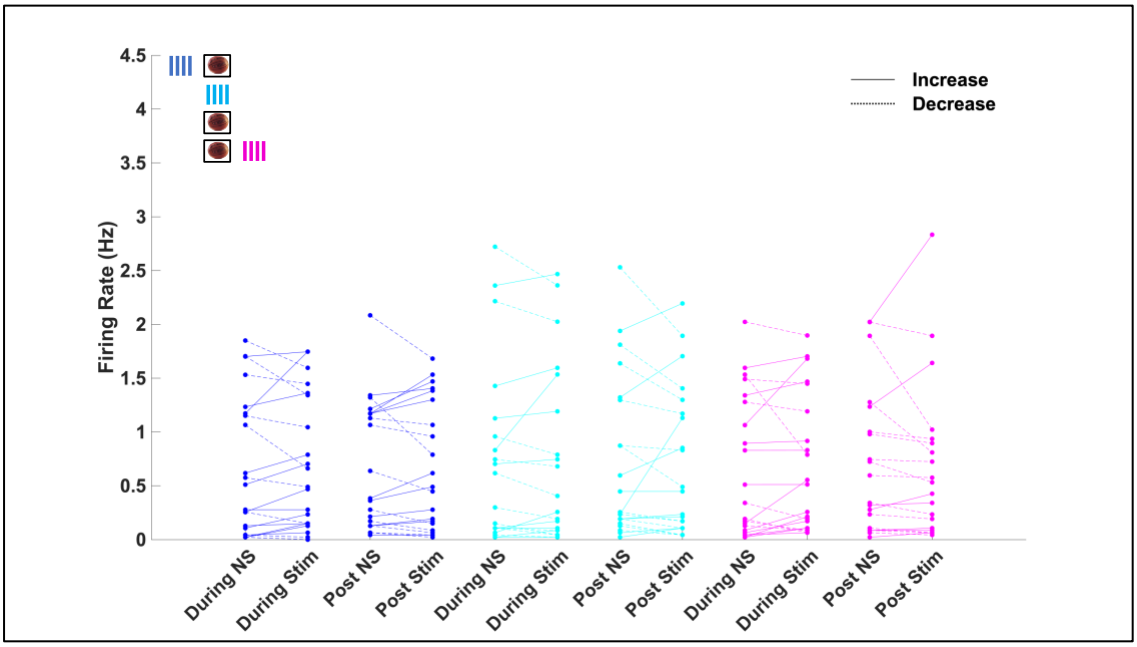

Figure 7: Timing Experiments Raw Firing Rates *The average raw firing rates for each unit is plotted for the timing experiments for the stimulated and non-stimulated conditions. The blue, teal, and magenta colors respectively represent the before, during, and after timing trials (as indicated in legend on top left corner). Solid lines represent a net increase in firing rate when comparing non-stimulated and stimulated trials while dashed lines represent a net decrease.*

Overall, when compared to non-stimulated trials, stimulation did not appear to have any significant effects on raw firing rates both during and post-stimulation across duration and timing experiments.

Change in Firing Rates Analysis During and Post-Stimulation

Figures 8 and 9 depict how the change in firing rates between stimulation and no stimulation trials for individual units appear for the duration and timing experiments, respectively. Each condition is separated out and examined both during stimulation and post-stimulation. In each case, each dot represents the difference in average firing rate for each individual unit for the stimulated trials and non-stimulated trials. As noted in the methods, a 1-sample t-test was conducted for each condition in addition to a paired t-test or one-way ANOVA comparing different conditions within each experiment.

For the duration experiments (*Figure 8)*, the medians for change in firing rate for the one second condition during stimulation and post-stimulation were respectively -0.0283 Hz and 0.0343 Hz (both non-significant, $p = 0.8247$ and p 0.9526 respectively). The medians for change in firing rate for the three second condition during stimulation and post stimulation were respectively -0.0085 Hz and 0.0586 Hz. The changes in firing rates for the three second condition were non-significant for during the stimulation ($p = 0.7244$) but were in fact significant post-stimulation ($p = 0.0352$). Additionally, a paired t-test conducted to compare differences between the one second and three second conditions within time frames found no significant differences during stimulation ($p = 0.7112$) and post-stimulation ($p = 0.2277$).

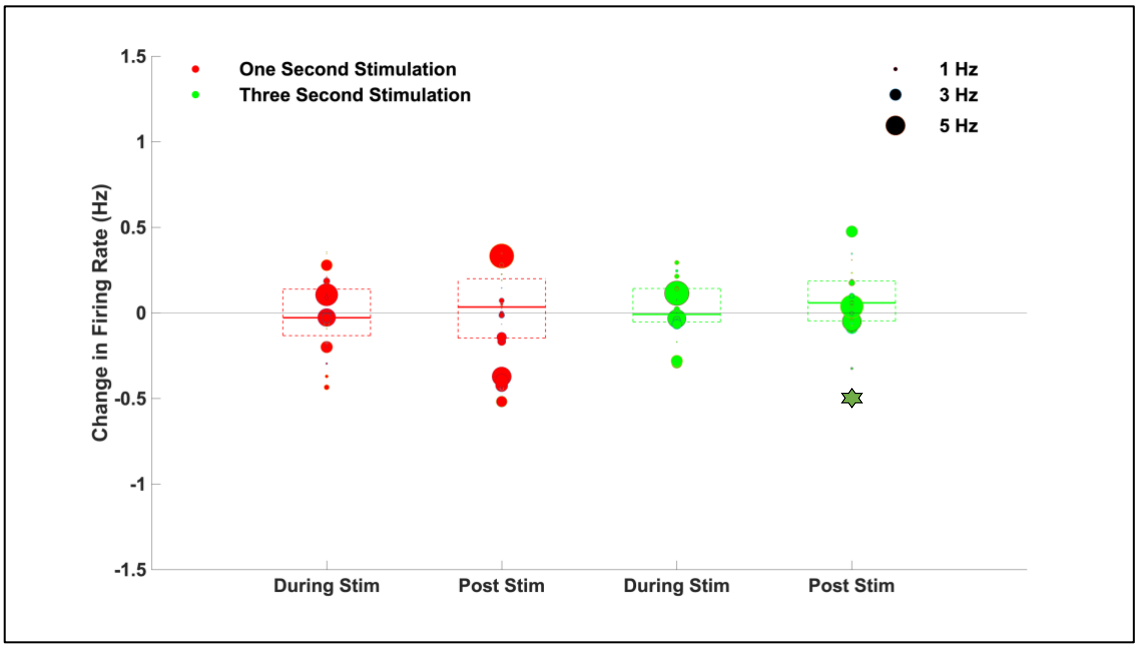

Figure 8: Duration Experiments Change in Firing Rate. *The change in firing rates between stimulated and non-stimulated trials for each unit is plotted for the duration experiments. The red color represents the one second stimulation condition while the green represents the three second stimulation condition. Each dot represents a single unit with the size of the dot representing the average firing rate of the unit across all trials (stimulated and non-stimulated). On the left and right the change in firing rate box plots (median and quantiles) for the one second and three second conditions are plotted, respectively.*

For the timing experiments (*Figure 9)*, the medians for change in firing rate for the before, during, and after conditions during stimulation respectively were 0.0107 Hz, -0.0106 Hz, and 0.0213 Hz. The medians for change in firing rate for the before, during, and after conditions post-stimulation respectively were 0.0213 Hz, -0.0532 Hz, and -0.0212 Hz. The changes in firing rates during and post-stimulation respectively were not significant for all comparisons done through one sample t-tests for the before condition ($p = 0.7099$, $p = 0.5679$), the during condition ($p = 0.3782$, 0.2000), and the after condition ($p = 0.9851$, $p = 0.0861$). Comparisons across conditions were done using a one-way ANOVA as described in the methods and no significant differences were found in the change in firing rates during stimulation ($p = 0.9693$) and post-stimulation ($p = 0.9473$).

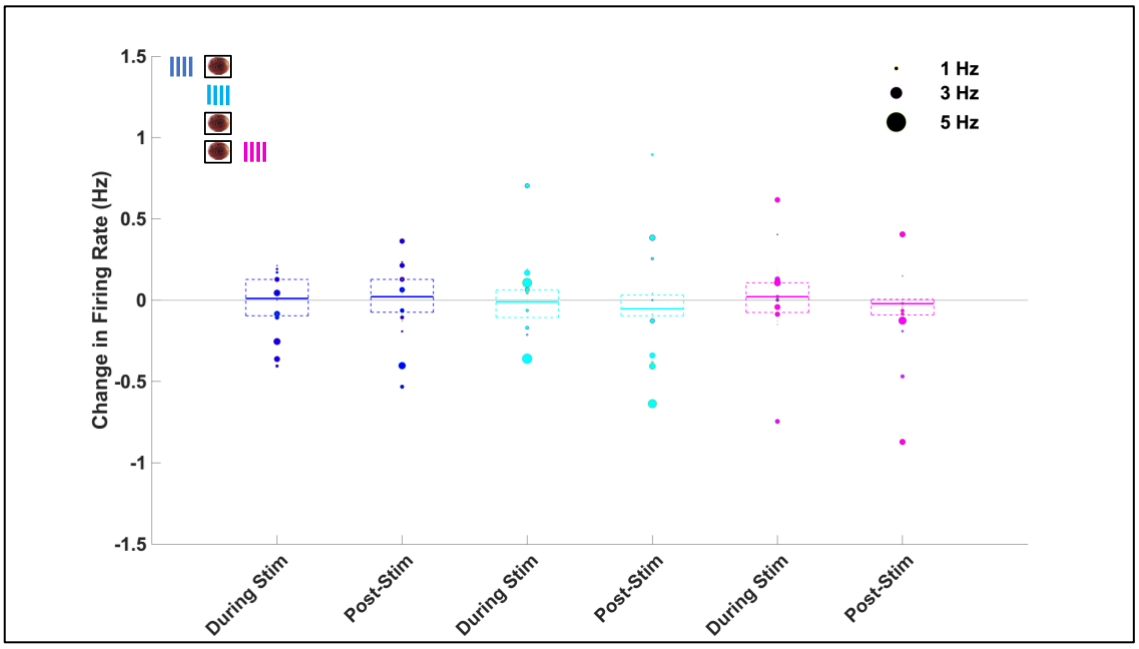

Figure 9: *The change in firing rates between stimulated and non-stimulated trials for each unit is plotted for the timing experiments. The blue, teal, and magenta colors respectively represent the before, during, and after timing conditions. Each dot represents a single unit with the size of the dot representing the average firing rate of the unit across all trials (stimulated and non-stimulated). On the left, middle, and right the change in firing rate box plots (median and quantiles) for the before, during, and after trials are plotted, respectively. Significant results at an alpha of 0.05 are denoted by a green star.*

Percent Change in Firing Rates Analysis During and Post-Stimulation

Figures 10 and 11 essentially control for the baseline firing rate of each unit by showing the percent change in firing rate when comparing stimulated and non-stimulated trials. For figures 10 and 11, each condition is separated out for the duration and timing experiments respectively. As noted in the methods section, one sample t-tests were conducted for each condition during stimulation and post-stimulation. Additionally, paired t-tests or one-way ANOVAs were used to compare percent changes in firing rate across conditions either during stimulation or post-stimulation.

For the duration experiments (*Figure 10)*, the medians for percent change in firing rate for the one second condition during stimulation and post-stimulation were -5.8308% and 0.0000% (both non-significant, $p = 0.1903$ and $p = 0.7351$ respectively). The medians for percent change in firing rate for the three second condition during stimulation and post stimulation were -1.3963% and 5.6250%

respectively. The percent change in firing rates for three second conditions were not significant during stimulation (p = 0.6854) but in fact were significant post-stimulation (p = 0.0227). One second and three second conditions percent change in firing rate were compared using a paired t-test and were found to be not significant during stimulation (p = 0.3995) and post-stimulation (p = 0.6709).

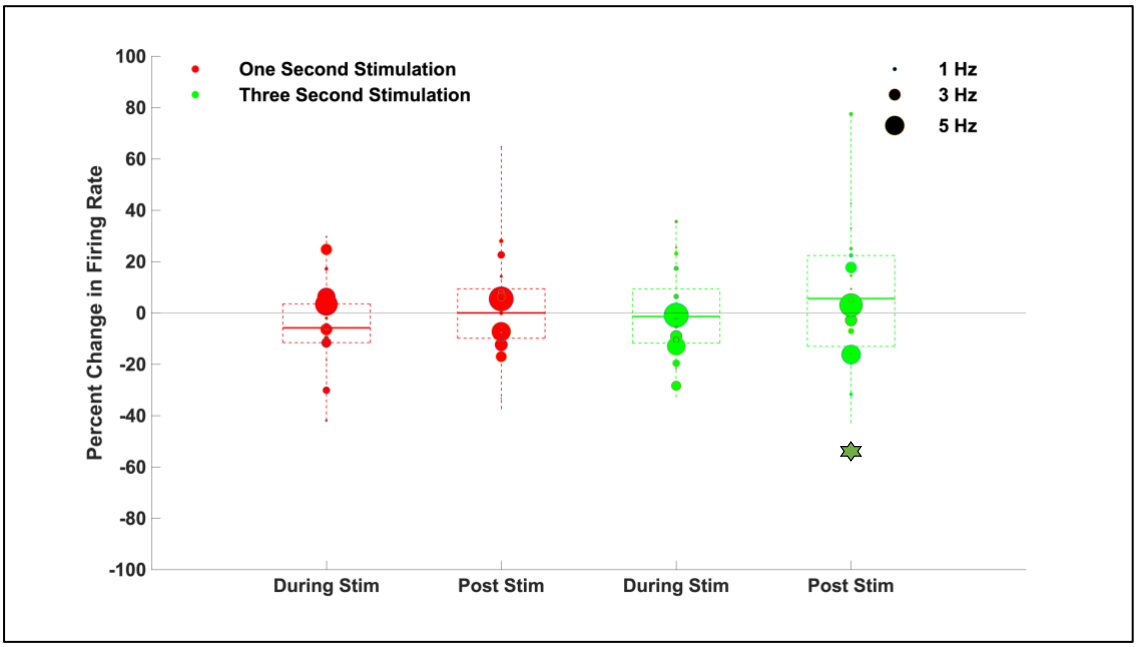

Figure 10: Duration Experiments Percent Change in Firing Rate. *The percent change in firing rates between stimulated and non-stimulated trials for each unit is plotted for the duration experiments. The red color represents the one second stimulation trials while the green represents the three second stimulation trials. Each dot represents a single unit with the size of the dot representing the average firing rate of the unit across all trials (stimulated and non-stimulated). A green star denotes a significant difference at an alpha of 0.05.*

For the timing experiments (*Figure 11)*, the median percent change in firing rate during stimulation for the before, during, and after timing conditions respectively were 0.0336%, -8.5469%, and 1.2440%. The medians for percent change in firing rate for the before, during, and after conditions post-stimulation respectively were 0.0336%, -20.7368%, and -7.5668%. The percent change in firing rates during and post-stimulation respectively were not significant for all comparisons done through one sample t-tests for the before condition (p = 0.6403, p = 0.7466), the during condition (p = 0.6264, 0.1622), and the after condition (p = 0.0694, p = 0.2082). Before, during, and after

conditions percent change in firing rates were compared against each other and were found to be non-significant both during stimulation (p = 0.4328) and post-stimulation (p = 0.4765).

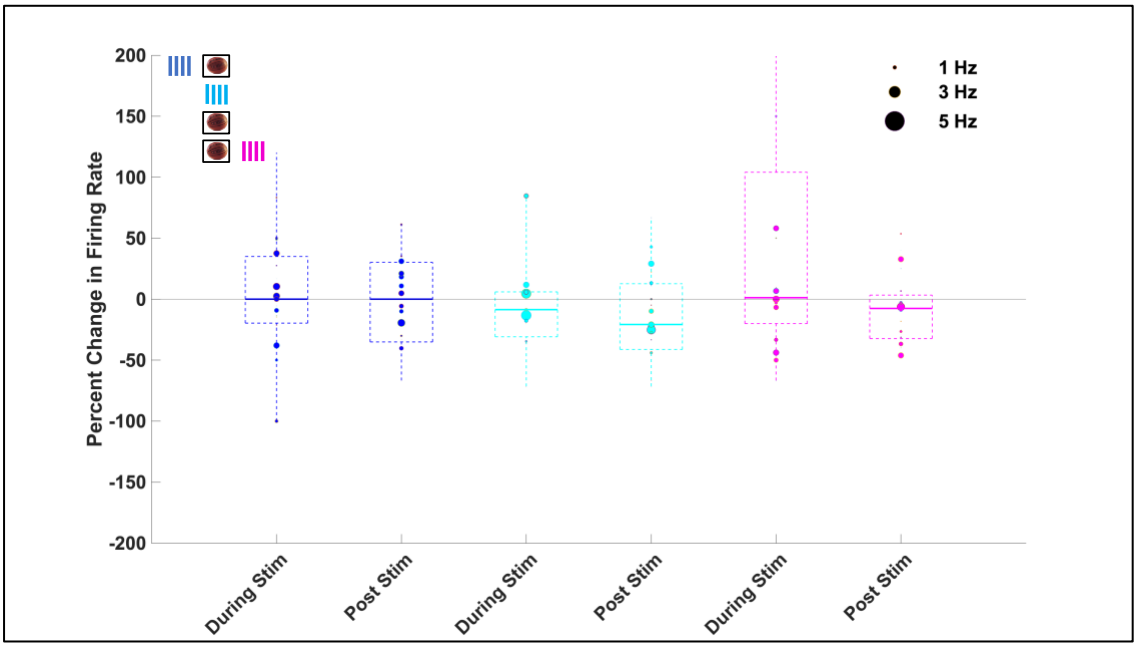

Figure 11: Timing Experiments Percent Change in Firing Rate *The percent change in firing rates between stimulated and non-stimulated trials for each unit is plotted for the timing experiments. The blue, teal, and magenta colors respectively represent the before, during, and after timing trials. Each dot represents a single unit with the size of the dot representing the average firing rate of the unit across all trials (stimulated and non-stimulated).*

Change in Firing Rates Across Stimulation Timeline

Figures 12 and 13 represent different approaches to understanding how stimulation of the amygdala impacts human medial temporal lobe single neurons. This approach looks at how the raw firing rates of each unit evolves on average from before stimulation onset, during stimulation, and post-stimulation. Figure 12 displays the change in firing rate relative to stimulation for the duration trials. A one-way ANOVA was used to analyze differences in raw firing rate which was found to be significant only for the three second condition ($p < 0.05$) but not the one second condition ($p > 0.05$).

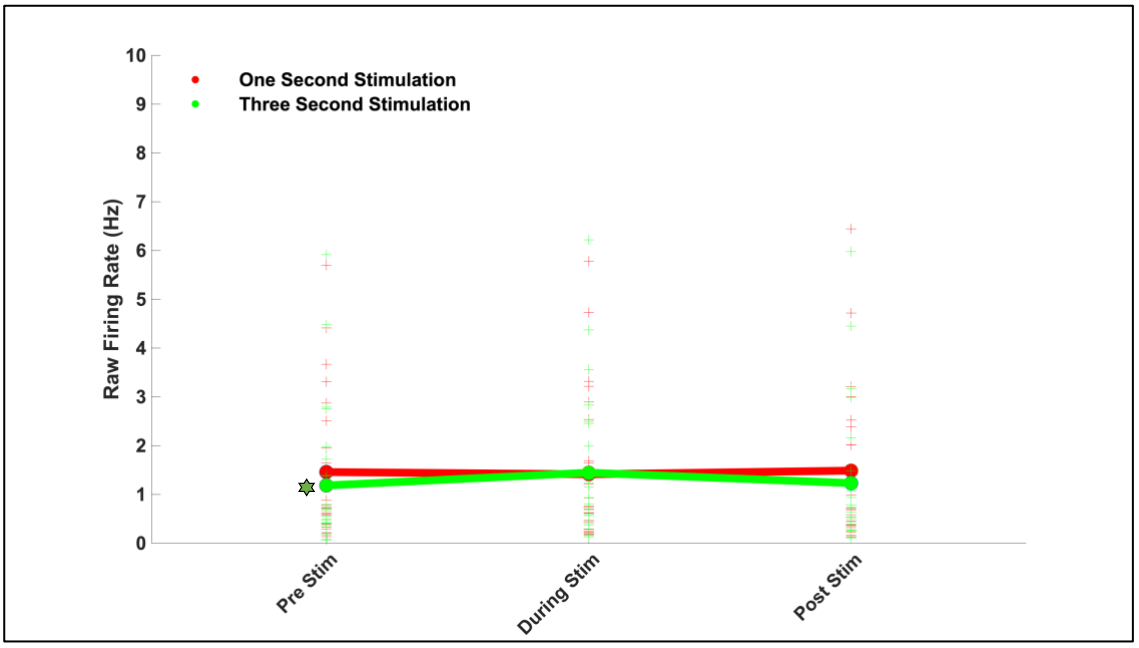

Figure 12: Change in Firing Rate Relative to Stimulation for the Duration Experiment. *The raw firing rates for the stimulation trials are plotted for the duration experiment. The red line represents the one second condition while the green line represents the three second condition. Pluses represent the firing rates of individual units at that specific relative timepoint. How the firing rate evolves relative to stimulation onset was analyzed using one-way ANOVA as noted in the methods section. A green star denotes a significant difference at an alpha of 0.05.*

Figure 13 displays the change in firing rate relative to stimulation timeline for the timing conditions. Similarly to the duration experiments, a one-way ANOVA was conducted for each condition and

found to be non-significant for the before condition ($p > 0.05$), the during condition ($p > 0.05$), and the after condition ($p > 0.05$).

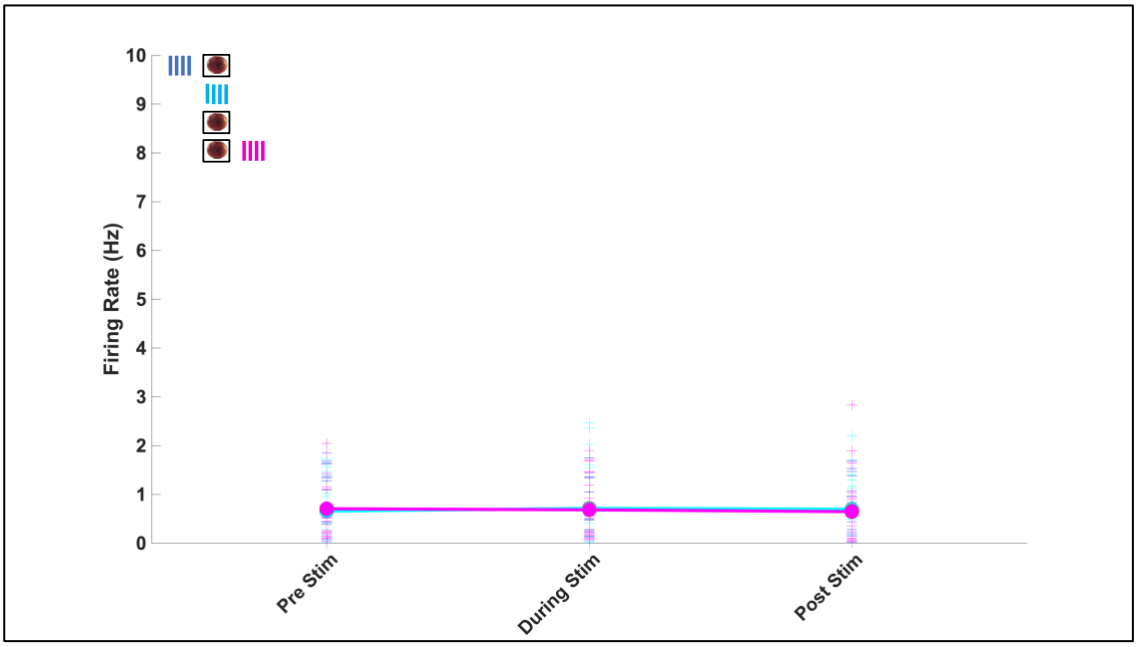

Figure 13: Change in Firing Rate Relative to Stimulation for the Timing Experiment. *The raw firing rates for the stimulation trials are plotted for the timing experiment. Blue, teal, and magenta represent the before, during, and after conditions respectively. Pluses represent the firing rates of individual units at that specific relative timepoint. How the firing rate evolves relative to stimulation onset was analyzed using one-way ANOVA as noted in the methods section.*

Discussion:

Most of the findings from this experiment largely point to amygdala stimulation having minimal to no impact on medial temporal lobe single neuron firing rate. However, the three second stimulation condition for the duration experiments did appear to be statistically significant. For example, the difference and percent difference (between stim vs no-stim conditions) in firing rate post-stimulation (*Figure 8 and 10 respectively)* for the three second stimulation in the duration experiment did appear to be significantly elevated. This significant finding was also reflected in the change in firing rate relative to three second stimulation which further emphasizes the impact (*Figure 12).* However, this represents an inconsistent finding with the raw firing rates (*Figure 6)* which was not statistically significant. Furthermore, the median change and median percent change in firing rate was around positive 0.06 Hz and 5.6% for the three second condition. This represents an extremely small modulation in firing rate which may not have a significant behavioral impact. In fact, unpublished preliminary investigations from another investigator in our laboratory found that three second stimulation does not appear to impact enhanced recognition any more than one second stimulation. In short, only a three second amygdala stimulation appeared to cause medial temporal lobe neurons to modulate firing rates, but not in a consistent or meaningful manner.

These results are consistent with parallel findings in a rodent paper which used a similar paradigm (Bass and Manns, 2015). In their study Bass and Manns utilize a neutral task involving novel object recognition paired with basolateral amygdala stimulation in rats to determine the neural correlates of enhanced memory following amygdala stimulation (2015). Following amygdala stimulation, the authors found that one second amygdala stimulation did not affect the hippocampal pyramidal neurons firing rates. Instead, differences in neuronal oscillations at the gamma frequency in the form of synchronization of hippocampal fields appeared to be triggered

as a result of amygdala stimulation. Bass and Manns did not analyze how an increase in stimulation duration would impact the single neuron activity relative to oscillatory activity and thus this would be an area for future investigation given the present results.

From a comparative point of view, the hippocampus and parahippocampal regions have been shown to have high degrees of anatomical and functional similarities across mammals (see Manns and Eichenbaum, 2006). The present study suggests lack of firing rate modulation in the medial temporal lobe by amygdala stimulation for similar conditions, thus representing a functional similarity across rodent and human species. Furthermore, a previous study conducted by Inman et al. delineates similar findings to the Bass and Manns study (Inman et al., 2018b). Inman et al. found that in human subjects, successfully recalled images show basolateral amygdala theta oscillations modulating the amplitudes of perirhinal gamma oscillations (2018b). This is similar in nature to the within hippocampus field to field synchronization of gamma oscillations, again representing functional similarities between rodent and human memory enhancement.

Some possible alternative explanations may also explain the findings from this study. Firstly, this present study controlled for spike loss by removing spikes pre-stimulation and post-stimulation in line with stimulation artifacts. While this feature is necessary to compare spike rates across stimulation timelines, *Figure 12* appears to demonstrate a similar level of spiking decrease both pre-stimulation and post-stimulation. While it is possible that longer stimulation could result in increased firing rates during stimulation, the lack of continuity across analyses of firing rates renders that interpretation less likely.

Additionally, it is possible that the basolateral amygdala stimulation used in the present study results in lack of an impact because of the recording targets used. A disproportionate number of recorded units were located within the anterior regions of the hippocampal axis and primarily from

perirhinal and entorhinal regions. However, the anterior regions of the hippocampal axis and entorhinal/perirhinal regions have been shown to have greater connectivity with the amygdala (see Strange et al., 2014). Therefore, it has been theorized (Bass and Manns, 2015) that amygdala stimulation may indirectly influence the hippocampus through the perirhinal and entorhinal cortices (Kajiwara et al., 2003; De Curtis and Paré, 2004) or directly through the anterior regions. Thus, it is unlikely that the lack of diverse anatomical sampling would have led to a null effect in firing rate noted by the present study.

Furthermore, given that this study sampled an extremely limited number of neurons it is possible that there is a subpopulation of medial temporal lobe neurons that do in fact change their firing rate in response to amygdala stimulation. These responding neurons may in fact be physiologically and behaviorally significant but represent a small minority of neurons within the broader area of interest. On a related note, we did not make distinctions between types of neurons and it is possible that certain subsets of neurons that are highly responsive to sensory input would also be more responsive to amygdala stimulation. Future studies incorporating a greater number of single-neurons into their analyses would be able to address these issues. However, this possibility is not supported by previous findings (Bass and Manns, 2015).

Conclusion and Future Directions:

This study suggests that amygdala stimulation largely does not impact the firing rate of medial temporal lobe single neurons from areas such as the entorhinal cortex, perirhinal cortex, and anterior hippocampal axis. However, three second stimulation appears to increase the firing rate of medial temporal lobe neurons and was not explored in previous studies. These findings are part of a larger attempt in identifying exactly how the amygdala appears to modulate the enhancement of memory given that previous studies have already highlighted that theta modulated gamma stimulation results in enhanced memory for neutral objects (Bass et al., 2012, 2014; Bass and Manns, 2015; Inman et al., 2018b). Furthermore, although the neuronal oscillations correlates of amygdala stimulation have already been verified in human context, human single neuron firing rate responses to amygdala stimulation have not been previously reported. Analysis along different regions of the hippocampal axis will be useful to determine if single neuron firing rate response to amygdala stimulation differs based on recording targets. Additionally, a more detailed analysis of how human neuronal oscillations specifically modulate memory consolidation is not yet known and remains a subject of interest. For example, Bass and Manns found that there were significant impacts of basolateral amygdala stimulation on interactions between neuron spiking in the CA3 hippocampal subfield and the LFP of CA1 hippocampal subfield (2015). While firing rates of single neurons remained constant, the phase in which these neurons fired became synchronized with ipsilateral stimulation of the basolateral amygdala. Synchronization of human single neurons to low gamma phase oscillations may have also occurred in our study as well and should be an area of future analysis.

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Appendix:

A. Diagram depicting the shuffling procedure. *Firstly, the original data is analyzed by taking the average of the stimulated trials firing rates for all units and the average of non-stimulated trials firing rates for all units. Following this procedure, this original value is maintained for analysis later. Secondly, the columns are randomly shuffled within each row and a "random" difference in means is calculated similarly to step one. This procedure is repeated a total of 10,000 times to generate a distribution. Finally, the original value for the difference is compared to the generated distribution in a two-tailed manner.*