INCREASED NEURAL ACTIVITY IN THE PREFRONTAL CORTEX DURING FEAR SUPPESSION TO A SAFETY SIGNAL

by

Ka Ho Ng

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STATEMENT OF COMMITTEE APPROVAL

Dr. Susan Sangha, Chair
Department of Psychological Sciences

Dr. Julia A. Chester
Department of Psychological Sciences

Dr. Edward L. Bartlett
Department of Biological Sciences

Dr. Richard M. Van Rijn
Department of Medicinal Chemistry Molecular Pharmacology

Approved by:
Dr. David Rollock

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ABSTRACT

Persistent and maladaptive fear in the absence of a threat can be disruptive because it decreases an organism's opportunity to seek life-sustaining substances. Learned safety signaling can suppress fear and encourage reward-seeking behavior, thus freeing the organism from fear induced immobilization. The infralimbic (IL) region of the prefrontal cortex is important for recalling fear extinction memories and for suppressing fear via learned safety signals. Neurons in the IL show an excitatory response to an extinguished fear cue. We thus hypothesized that neurons in the IL would encode safety by showing an excitatory response during active fear suppression to a learned safety signal.

To assess global changes in IL activity, we monitored IL multi-unit activity to different cues while training animals in a fear-reward-safety discrimination task (Sangha, Chadick, & Janak, 2013). During the discrimination task, male rats learned that the reward cue predicted liquid sucrose, the fear cue predicted footshock and the joint presentation of both the fear and safety cues resulted in no footshock. We also counterbalanced the modality of fear and safety cues (auditory vs visual) with two separate groups of animals to control for potential sensory modality effects. Male rats showed high levels of freezing to the fear cue, and significantly reduced levels of freezing to the combined fear+safety cue. Male rats also showed high levels of port activity to the reward cue. There was no significant difference in the learning rate between the two counterbalanced conditions.

Our multi-unit-data showed an increase in IL neuronal firing to the fear+safety cue across training sessions. This effect was consistent between the two counterbalanced conditions. We also examined single-unit activity from all animals that received light as the safety cue (n=8). This allowed us to examine the population response profile with a subset of the total animals. Although not statistically significant, our preliminary single-unit data demonstrated a decrease in the percentage of neurons that showed an inhibitory response to the fear+safety cue, but no change in the percentage of neurons that showed an excitatory response to the fear+safety cue. There was also no change in the magnitude of averaged firing rate in fear+safety excitatory or inhibitory neurons across training. Taken together, the decreased inhibition of single-unit activity in the IL may drive the increased excitation in multi-unit activity in the IL during behavioral fear suppression to a safety signal.

INTRODUCTION

Environmental cues can signify the availability of life-sustaining resources, threat or safety. In natural settings where conflicting cues are present in the same environment, organisms often have to decide between foraging for resources and shelter-seeking behaviors. Displaying persistent fear when there is no threat is maladaptive because it prevents the organism from seeking resources that are necessary for survival. Understanding the safety circuit is important because it can regulate both approach and avoidance behaviors (Dickinson & Michael, 1979; Dickinson & Pearce, 1977; Gray, 1987). A learned safety signal can rescue the organism from this fear induced state of immobilization and result in a resumption of foraging behavior. Since current literature has mostly focused on investigating fear and reward circuits separately (Janak & Tye, 2015), there is a knowledge gap in understanding how the brain uses safety signals to inhibit fear behavior and encourage reward-seeking behavior. The purpose of the current research is to investigate how the brain encodes safety during active fear suppression and how the same region of the brain encodes the threat and reward signifying cues as safety learning emerges in the animal.

An Overview of Defensive Behaviors

Defensive behaviors can occur in response to an immediate threat or to an anticipated threat. The response to an immediate threat is a type of unconditioned response because it does not require any prior learning. In humans, artificial threats could be generated with an air blast to the throat (Jovanovic et al., 2009), a loud sound (Morriss, Christakou, & Van Reekum, 2015), or a finger shock (Milad et al., 2009) in laboratory settings. The defensive behaviors can then be measured with changes in pupil dilation (Morriss et al., 2015), skin conductance (Milad et al., 2009), startle response (Jovanovic et al., 2009) and self-reports (Jovanovic et al., 2009). In contrast, rodent models typically use artificial threats such as a footshock (Milad & Quirk, 2002), predator odor (Wang et al., 2012), a loud sound (Falls & Davis, 1995), open space (Rogan, Leon, Perez, & Kandel, 2005), or elevation from ground (Bananej, Karimi-Sori, Zarrindast, & Ahmadi, 2012). The defensive behaviors can then be measured with changes in pupil dilation, blood pressure (LeDoux, Iwata, Cicchetti, & Reis, 1988), fear potentiated started (Falls & Davis, 1995), ultrasonic vocalization (Frysztak & Neafsey, 1991), conditioned freezing (Blanchard & Blanchard, 1969;

Fendt & Fanselow, 1999), darting (Colom-Lapetina, Li, Pelegrina-Perez, & Shansky, 2019), suppression of behavior (Rescorla, 1969), and willingness to explore open areas (Rogan et al., 2005). Even though there are many methods for evaluating fear in animal models, using freezing to assess fear is a common approach among rodent studies partly because conditioned freezing is easy to measure and can be manually scored by humans in real-time without the need for any prior surgeries or specialized instruments. This is unlike other approaches that rely on instruments to measure changes in blood pressure, filtering sound frequencies to identify ultrasonic vocalizations, or measuring the changes in velocity from video footage to identify darting behavior. In addition, learning to freeze in anticipation to a threat can be acquired quickly in animal models, allowing researchers to study learning of defensive behaviors with only a few trials of threat exposure. This is unlike investigating fear using suppression of lever-pressing behavior because animals would have to be pre-trained to lever press days prior to actually learning fear. Thus, investigating animal models of threat anticipation using conditioned freezing provides a reasonable approach for modeling Post Traumatic Stress Disorders (PTSD) in humans when the exaggerated fear learning is typically learned in only a few presentations.

Different Approaches to Learning Safety

There are primarily three different training procedures for producing cued safety learning (Figure 1). These include within-subject fear/safety discrimination training, between-subject fear/safety discrimination training and fear extinction training (Christianson et al., 2012; Sangha, Diehl, Bergstrom, & Drew, 2020). In within-subject fear/safety discrimination training, the same animals are trained to associate a fear cue with an aversive outcome and a safety cue with the omission of the aversive outcome in the same training context. This results in the animal producing more fear responses to the fear cue than to the safety cue at the end of training (e.g.(Jovanovic et al., 2009; Sangha et al., 2013)). This paradigm is beneficial when assessing neural responses to cues because the learning-related changes in neural activity to the safety cue can be directly compared against the learning-related changes in neural activity to the fear cue (Sangha et al., 2020). In between-subject fear/safety discrimination, one group of animals are trained to associate a fear cue with an aversive outcome. A separate group of animals receive the unpaired presentations of a safety cue and an aversive outcome so that the safety cue never occurs alongside the aversive outcome (e.g.(Ostroff, Cain, Bedont, Monfils, & Ledoux, 2010; Ronovsky et al.,

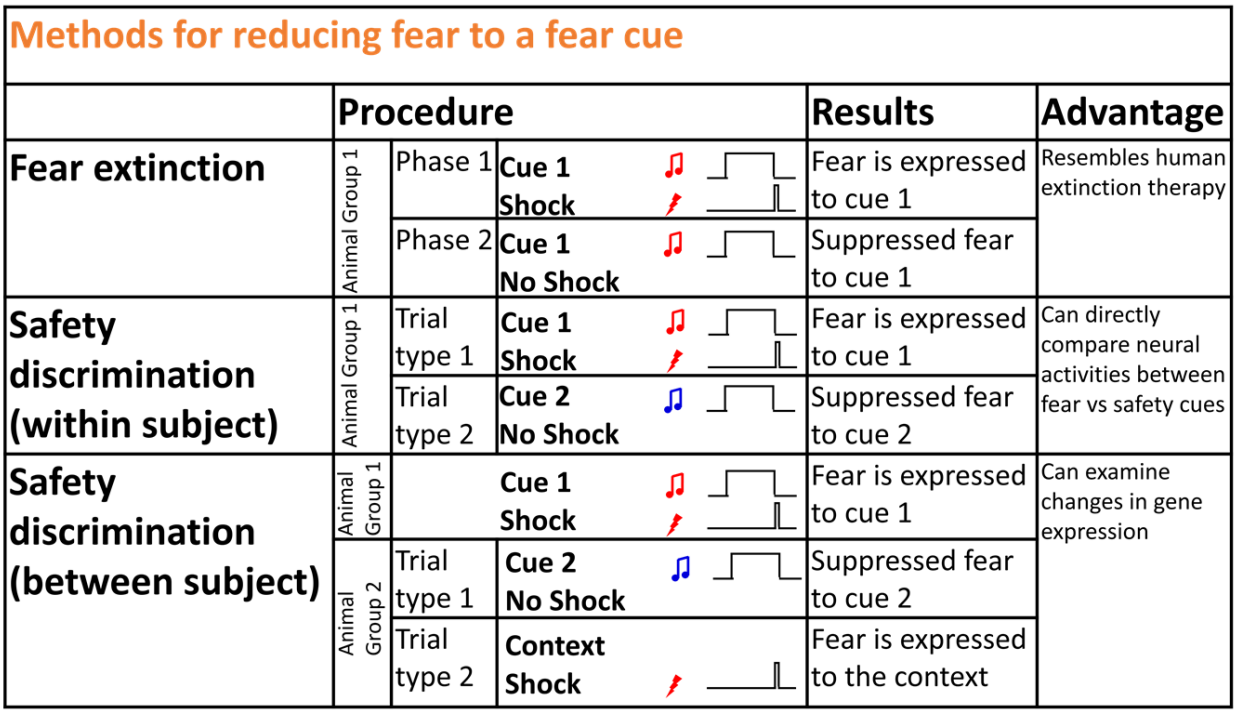


Figure 1. Different ways to learn cued fear suppression in animals and humans. In animals the aversive outcome is typically a footshock or loud sound. In humans, the aversive outcome is typically a finger shock, wrist shock, air blast, or aversive sounds.

2019)). As a result, the level of fear to the safety cue from animals with unpaired training will be lower than the level of fear to the fear cue from animals with paired training. This training procedure can be problematic because it tends to produce a high level of fear to the training context in the safety learning condition. It is nevertheless still useful for assessing changes in gene expression in the brain. Since animals from each group would only be exposed to one of the cues, the changes in gene expression observed in each group can be attributed to either the safety cue or fear cue. Another way to learn cued safety learning is through extinction training. Animals are first trained on fear learning to associate a fear cue with an aversive outcome. Then, the animals are presented with the fear cue but without the aversive outcome. Consequently, animals show an increased fear to the fear cue during fear learning, and this is followed by a gradual loss in fear responses to the extinguished fear cue (Sangha et al., 2020). One advantage of using the fear extinction protocol to study safety learning is that fear extinction training resembles human extinction-based exposure therapy in clinical settings.

Distinguishing Between Safety and Fear Extinction

Despite the fact that learning safety through either an explicit safety cue or fear extinction procedure involves fear suppression behaviors, fear extinction still does not equate to learned safety. In fear extinction, the fear suppression is limited to the aversive conditioned stimulus and is usually context-specific (Sangha et al., 2020). The extinguished fear is usually recoverable by spontaneous recovery, reinstatement, and renewal (Sangha et al., 2020). In contrast, learning safety through an explicit safety cue can provide a reinforcing and broader antidepressant effect in addition to producing fear suppression behaviors (Kong, Monje, Hirsch, & Pollak, 2014; R. A. Rescorla, 1969). The reinforcing properties of the safety signal can encourage approach behaviors and facilitate the learning of operant behaviors. For example, safety signals can encourage the animal to explore the aversive center of the open field (Rogan et al., 2005), overcoming their innate fear. Safety signals can also encourage social exploration after inescapable tail shock exposure (Christianson et al., 2008). Furthermore, our lab has previously identified basolateral amygdala (BLA) neurons that respond to the reward cue, safety cue, and the combined fear+safety cue (Sangha et al., 2013), showing that the BLA has overlapping safety and reward neural circuits which may allow the safety signal to produce reinforcing properties. Taken together, fear extinction provides a method to assess the role of IL in fear suppression. However, we still need

to assess the role of IL in the context of safety learning using an explicit safety cue when the effect of fear suppression can be applied more broadly and the reinforcing effect of the safety signal can be examined concurrently.

Overview of Neural Circuitry for Cued Fear Learning (Figure 2)

Cued fear learning usually involves the repeated pairing of a conditioned stimulus (CS) such as an auditory tone with an aversive unconditioned stimulus (US) such as a mild electric footshock. The repeated pairing of the CS and US consequently leads to an increase in the probability of the conditioned fear response to the fear CS (Watson & Rayner, 1920). During cued fear learning, auditory CS information enters the lateral amygdala (LA) from the medial geniculate nucleus and auditory cortex (Tsvetkov, Carlezon, Benes, Kandel, & Bolshakov, 2002). In contrast, information for the aversive US enters the LA from the spinal/trigeminal dorsal horn through the periaqueductal gray (PAG) (Herry & Johansen, 2014). The CS and US pathways then converge onto LA principal neurons (Pape & Pare, 2010), which are also important for cue discrimination (Grosso, Santoni, Manassero, Renna, & Sacchetti, 2018).

Fear conditioning induces plasticity in the CS pathway in multiple ways. It induces long term potentiation of the synaptic input going from auditory cortex to the LA by increasing the probability of presynaptic neurotransmitter release (Tsvetkov et al., 2002). It also increases LA responses to the auditory CS (Maren, 2000). LA field potential amplitude and slope increases with fear conditioning and decreases with safety conditioning (Rogan et al., 2005). Consequently, the facilitation of CS input to the LA during fear conditioning aids the CS in the activation of the LA neurons (Quirk, Repa, & LeDoux, 1995), allowing the CS-alone presentation to produce a conditioned response in the absence of US input to the LA. The LA's direct projections to the central amygdala (CEA) (Li et al., 2013) and indirect projections to the CEA through the basal amygdala (BA) and intercalated cells (ITC) (Pape & Pare, 2010) produce the conditioned fear behavior. The central amygdala (CEA) in turn projects to the PAG (LeDoux et al., 1988). The activation of PAG in turn leads to defensive behaviors such as conditioned freezing (Deng, Xiao, & Wang, 2016).

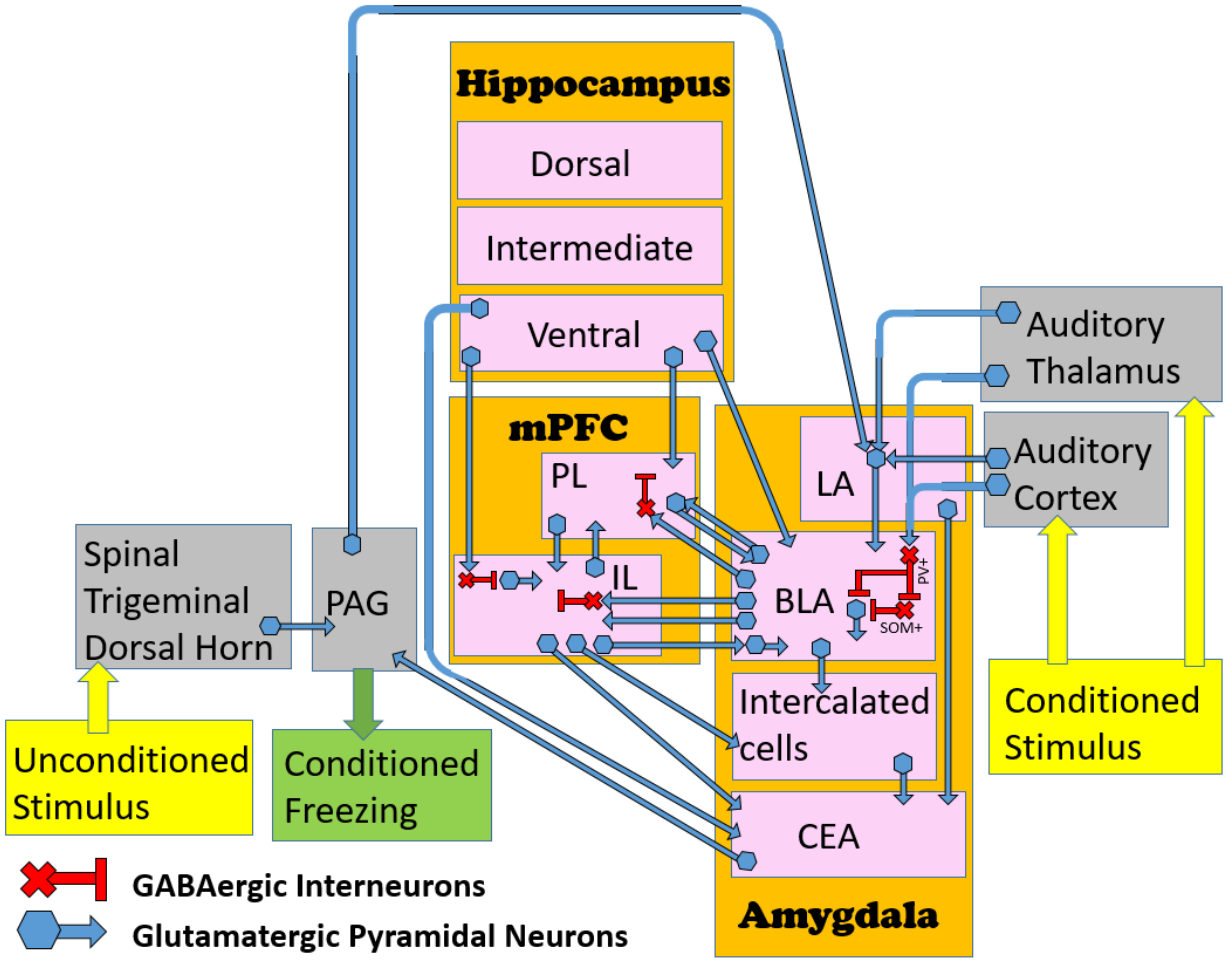


Figure 2. Wiring diagram for fear behavior and fear suppression behavior. Successful fear suppression behavior requires the infralimbic cortex, amygdala and hippocampus

Overview of Prefrontal Cortex and Amygdala Neural Circuitry for Cued Fear Suppression

Cued fear suppression primarily engages the prefrontal cortex and amygdala (Sangha et al., 2020). Fear suppression is impaired in people with high PTSD symptoms (Jovanovic et al., 2009). Moreover, PTSD patients have impaired fear extinction memory recall and they have lower fMRI activity in the ventral medial prefrontal cortex (vmPFC) to the extinguished fear cue (Milad et al., 2009). The basolateral amygdala (BLA) which is comprised of both the basal amygdala (BA) and lateral amygdala (LA) regulates fear along with the medial prefrontal cortex (mPFC) (Sangha et al., 2020). The mPFC receives direct projections from BA fear neurons and also has bidirectional projections with BA fear extinction neurons (Herry et al., 2008). Both the mPFC and BLA show increased synchronization in the theta frequency range to the safety cue after discriminative safety learning (Likhtik, Stujenske, Topiwala, Harris, & Gordon, 2014). Thus, the activity between the mPFC and BLA are important in mediating fear suppression behavior.

The mPFC is subdivided into the prelimbic cortex (PL) and infralimbic cortex (IL), which have reciprocal connections with each other (Marek, Xu, Sullivan, & Sah, 2018) and with the BLA (Senn et al., 2014; Vertes, 2004). PL and IL also have inhibitory interneurons that receive projections from the BLA (Cheriyan, Kaushik, Ferreira, & Sheets, 2016). mPFC pyramidal neurons in layer 2 and layer 5 receive excitatory projections from BLA neurons. PL neurons in layer 2 have reciprocal excitatory projections with BLA neurons. IL neurons send excitatory projections to BLA pyramidal neurons which is important for fear extinction (Strobel, Marek, Gooch, Sullivan, & Sah, 2015). IL neurons in layer 5 that receive excitatory projections from the BLA also send projections to the PAG (Cheriyan et al., 2016). The IL also has excitatory projections to the ITC (Berretta, Pantazopoulos, Caldera, Pantazopoulos, & Paré, 2005) as well as to the CEA (Hurley, Herbert, Moga, & Saper, 1991), which are important for regulating fear extinction behaviors (Amano, Unal, & Paré, 2010; Likhtik, Popa, Apergis-Schoute, Fidacaro, & Paré, 2008).

The PL and IL have separate roles in the regulation of fear behaviors. The PL is needed for fear expression and neurons in the PL show a sustained increase in firing rate to the fear cue that correlates with the time course of freezing behavior with second-to-second resolution (Burgos-Robles, Vidal-Gonzalez, & Quirk, 2009). The PL also receives projections from fear neurons in the BA (Senn et al., 2014). In contrast, the IL is needed for fear suppression (Sangha, Robinson,

Greba, Davies, & Howland, 2014) and receives projections from fear extinction neurons in the BA (Senn et al., 2014). The optical inactivation of BA-to-IL projections weakens fear extinction memory and the optical inactivation of BA-to-PL projections strengthens extinction memory (Senn et al., 2014). This suggests that the activity balance between PL and IL is important in regulating fear suppression behavior. The reverse direction of IL-to-BLA projections may also be important for safety learning. Among animals that learned safety discrimination, BLA firing activity was phase-locked to theta activity from the mPFC during the safety cue presentation suggesting that the mPFC leads the firing of the amygdala during fear suppression (Likhtik et al., 2014). This is further supported by experiments with functional manipulations. The optical activation of IL-to-BLA glutamatergic projections during fear extinction facilitates fear extinction recall memory (Bukalo et al., 2015). Conversely, the optical inactivation of IL-to-BLA projections impaired fear extinction recall memory (Bukalo et al., 2015). Taken together, the bidirectional connection between IL and BLA is important for fear suppression behavior.

Overview of Hippocampus Neural Circuitry for Cued Fear Suppression

The hippocampus encodes the traumatic context and emotional value of the context (Sangha et al., 2020). For example, place cells in hippocampal CA1 encode place fields following fear learning (Wang et al., 2012), which remap during fear extinction (Wang, Yuan, Keinath, Ramos Álvarez, & Muzzio, 2015), demonstrating the influence of context in fear extinction. It is also well established that changing the context where extinction took place leads to recovery of fear to the previously extinguished cue (Hobin, Goosens, & Maren, 2003).

The hippocampus mediates context dependent fear suppression through its projection to the IL. The ventral portion of the hippocampus (vHPC) in both mice and humans show differential responses during fear memory recall and cued fear suppression (Meyer et al., 2019). The vHPC projects to the IL (Cenquizca & Swanson, 2007) and inhibits IL pyramidal neurons through PV (parvalbumin) expressing interneurons (Marek, Jin, et al., 2018). This vHPC-to-IL projection is important for the relapse of fear due to the change in the fear extinction context (Marek, Jin, et al., 2018). The vHPC also projects to the CeA and BA (Cenquizca & Swanson, 2007). It has been shown that neurons in the ventral hippocampus send projections to fear, but not fear extinction, neurons in the BA. (Herry et al., 2008). Lastly, safety learning has been shown to require hippocampal neurogenesis in a between-subjects design where the safety group received unpaired

fear conditioning (Pollak et al., 2008). Thus, the hippocampus is important for encoding the context and mediates fear and fear suppression behaviors through its projection to the IL and BLA.

General Overview of the Infralimbic Cortex in Fear Suppression Behavior

The infralimbic cortex is necessary for learning fear extinction (Sierra-Mercado, Padilla-Coreano, & Quirk, 2011) and for suppressing fear in the presence of a safety signal (Sangha et al., 2014). Animals that performed well in fear extinction memory recall also showed a higher level of IL neuronal activity to the extinguished fear cue than animals that did not perform well in fear extinction memory recall (Milad & Quirk, 2002). Neurons in the IL also showed learning-related changes in excitability and increased spike burst activity in response to fear extinction training (Santini, Quirk, & Porter, 2008). The electrical stimulation of the IL during the presentation of fear cues during fear extinction facilitated fear extinction learning and fear extinction memory recall the next day (Milad & Quirk, 2002). This evidence shows that IL is important for fear extinction. Even though the IL has also been shown to be necessary for fear suppression to a safety signal (Sangha et al., 2014), it is unclear how the IL encodes safety signals.

SPECIFIC AIMS

The infralimbic region of the prefrontal cortex (IL) is necessary for fear/safety discrimination behavior (Sangha et al., 2014) and it has reciprocal connections (Senn et al., 2014; Vertes, 2004) with the basolateral amygdala (BLA), which is a site of convergence of the fear, reward, and safety circuits in rats that also contain safety-specific neurons (Sangha et al., 2013). The IL-to-BLA projection is necessary for the recall of fear extinction memory (Bloodgood, Sugam, Holmes, & Kash, 2017). These data suggest that IL may provide critical safety information to the BLA to inhibit fear behavior. However, safety-specific neurons in the IL have yet to be identified. The overall objective of this thesis was to investigate changes in IL activity during fear suppression behavior and to categorize safety specific activity in the IL during training. The central hypothesis was that the learning-related increase in IL firing rate would gradually develop as the animals learn to suppress fear behavior in the presence of a safety signal. These experiments would allow future research to better target IL activity at different phases of learning to manipulate fear suppression behavior. We tested our central hypothesis with the following aims.

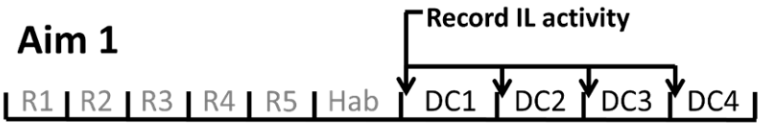
Specific Aim 1: Demonstrate That IL Shows Changes in Neural Activity to the Safety Cue

A previous study from our lab showed that reversibly inactivating the IL impaired fear/safety discrimination behavior (Sangha et al., 2014). This showed that the IL is necessary for processing learned safety in our training paradigm and we thus hypothesized that the IL encodes safety information. We monitored IL multi-unit activity with multi-array electrodes, as male rats were being trained with a safety, fear, and reward cue discrimination learning (SFRDL) paradigm that was well established in our laboratory (Greiner, Müller, Norris, Ng, & Sangha, 2019; Ng, Pollock, Urbanczyk, & Sangha, 2018; Sangha et al., 2013, 2014). Assessing multi-unit activity allowed the inclusion of neurons with very low firing rates or neurons with incomplete data due to threshold cutoffs that would otherwise be omitted from single unit analysis. Including a large number of suboptimal quality cells benefited multi-unit analysis by dramatically increasing the number of contributing neurons, resulting in a more comprehensive view of the global IL response. We predicted that the IL would show increased multi-unit activity to both the fear+safety cue and to the safety cue as animals acquired the safety association during discrimination training (Figure

3). This prediction was drawn based on a previous study that categorized safety neurons in the BLA which identified safety neurons that were responsive to the fear + safety cue and to the safety cue (Sangha et al., 2013). These data in the BLA indicated that there may be potential safety neurons in the IL that would be responsive to both the fear + safety cue and safety cue.

Specific Aim 2: Demonstrate That Safety Learning Leads to Alternations in Population Recruitment in the IL

Learning related changes in IL multi-unit activity could be attributed to plasticity occurring within the IL or changes in input from other brain structures that project to the IL. One way to confirm plasticity within a brain region is to examine the change in cell recruitment during learning. We hypothesized there would be a change in the proportion of cells that responded to the fear + safety cue and safety cue across training sessions. Single-unit activity from animals that received light as the safety cue (n = 8) were classified based on their excitatory or inhibitory responses to the fear + safety cue relative to their precue baseline. We predicted that the IL would show an increase in the percentage of neurons that showed excitatory responses to the fear+safety cue across training sessions.



Cues	Predictions for IL activity
Fear	No change in firing rate
Fear + Safety	Increases in firing rate Short Latency or Sustained Safety Cue Safety Cue
Safety	Increases in firing rate Short Latency or Sustained Safety Cue Safety Cue
Reward	No change in firing rate

Figure 3. Prediction of IL multi-Unit activity during discrimination training. The IL should show an increase in firing to the fear + safety cue and to the safety cue

MATERIALS AND METHODS

Subjects

Sixteen male Long Evans rats (Blue Spruce; Envigo, Indianapolis) weighing 300-350 g were singly housed under a 12 hr light/dark cycle (lights on 09:00) and handled for 1 week before commencing experiments. All procedures were performed during the light cycle and approved by the Purdue Animal Care and Use Committee. Rats had *ad libitum* access to food and water up until the first training session, at which point they were restricted to 20 g of food per day for the remainder of the experiment.

Apparatus

The training chamber was a Med Associates Plexiglas box (28 cm length x 21 cm width x 35 cm height) encased in an electrically shielded sound-attenuating chamber (Med Associates, ST Albans, VT). 10% liquid sucrose (100μL) was delivered through a recessed port located in the center of one wall, containing an infrared beam for detecting port entries and exits. There were two lights (28 V, 100 mA), one on each side of the port for delivering the 20s continuous light cue, and a house light (28v, 100 mA) located at the top of the wall opposite to the port for providing a constant background illumination. Next to the house light, there was a "tweeter" speaker (ENV-224BM) for delivering auditory cues. Footshocks were delivered through the grid floor by a constant current aversive stimulator (ENV-414S). A side-view video camera located on the door of the sound-attenuating chamber recorded the rat's behavior for offline video analyses.

DC Behavioral Training Procedure

The three stimuli used as cues were a 20 s continuous 3 kHz tone (70 dB), a 20 s pulsing 11 kHz tone (200 ms on, 200 ms off; 70 dB), and a 20 s continuous light (28 V, 100 mA). The 20s continuous 3kHz tone (70 dB) was reserved for the reward cue for all animals. The remaining two cues were counterbalanced for fear and safety cues between two groups of animals (n = 8 each group).

Animals first received 5 sessions of reward training across 5 days (R1-R5). Each session consisted of 25 pairings (ITI, 90-130 s) of the reward cue with a 3s delivery of 10% liquid sucrose

(100μL pseudorandomly presented 10-20s after reward cue onset) into a port. Animals then received one session of habituation training (HAB), which consisted of 25 trials of the reward cue paired with liquid sucrose (100μL pseudorandomly presented 10-20s after reward cue onset), 5 trials of the future fear cue presented alone, and 5 trials of the future safety cue presented alone (ITI, 90-130 s). This habituation procedure reduces any baseline freezing present to the novel cues and the number of trials presented is not sufficient to produce latent inhibition (Sangha et al., 2013). Animals then received 4 sessions of discriminative conditioning (DC1-4) across 4 days. Each session consisted of 15 trials of the reward cue paired with liquid sucrose (100μL presented 18s after reward cue onset), 4 trials of the fear cue paired with footshock (0.5s, 0.45 mA at cue offset), 15 trials of the safety cue and fear cue presented concurrently without footshock, and 10 trials of the safety cue presented alone without footshock (44 trials total, ITI 100-140 s).

In Vivo Electrophysiology

Each electrodes array was composed of 50μm stainless steel wires in a 2 by 4 arrangement and spaced 250 μm apart (NeuroBiological Laboratories). During surgery, two sets of electrode arrays were implanted bilaterally to the IL (AP = + 2.4mm; ML = +/- 0.5 mm; DV -3.9; 8 wires per side) while the rats were deeply anesthetized with isoflurane. The animals had 7-10 days of surgical recovery with *ad libitum* access to food and water.

During training, the implanted electrode array was connected to a suspended headstage cable. The recorded neuronal signals passed through a headstage amplifier, a commutator, and a programmable amplifier (Plexon) to be amplified and filtered (0.4 and 5kHz). The spike threshold was set to 2.5 standard deviations from the mean of peak amplitude distribution that was customized to the individual channel before the start of each session (Omniplex and PlexControl; Plexon). Discrimination of multi-unit activity from background noise was performed with an offline multichannel spike sorter (Offline Sorter; Plexon) using peak amplitude, valley amplitude and principal component analysis on waveform shapes. The video frame rate was in sync with cue onset by external timestamp signals (CinePlex; Plexon), so neural activity could be analyzed in parallel with behavior.

Histology (Figure 4, Figure 11)

After recordings were completed, rats were deeply anesthetized with sodium pentobarbital. A 15 s 20 μA current was passed through each wire to mark each electrode tip. Rats were then perfused with PBS and 10% formalin containing 3% potassium ferrocyanide. Brains were soaked in 30% sucrose-containing formalin and cryo-sectioned at 50 μm. Sections were stained with cresyl violet and examined under a light microscope to verify placements. 229 wires were confirmed to be in the IL and only these wires were included in the analyses.

Data Analyses

Behavioral Analyses

Fear behavior was assessed manually offline from videos by measuring freezing, defined as complete immobility with the exception of respiratory movement, which is an innate defensive behavior (Blanchard & Blanchard, 1969; Fendt & Fanselow, 1999). The amount of time spent freezing within a 20 s interval during cue presentation was quantified and expressed as percentages. Reward behavior was assessed manually by quantifying the amount of time the animals spent inside the port or having their nose positioned at the port entrance, and was expressed as percentages. The person performing the manual behavioral scoring and electrode placements had a Pearson's correlation of at least $r = 0.8$ with other scorers in the same laboratory for freezing and reward behaviors. The behavioral data were analyzed with two-way repeated-measures ANOVAs with *post hoc* Dunnett's multiple comparisons in GraphPad Prism. The Dunnett's test was used because it limits the comparisons to a reference cue. Thus, it can have better statistical power for detecting differences. To further quantify fear suppression learning, fear suppression ratios were calculated for each animal by dividing the percentage of time spent freezing to fear+safety cue by the percentage of time spent on freezing to fear cue alone. Fear suppression ratios of less than one would indicate that the animal was showing less fear to the fear+safety cue than the fear cue alone.

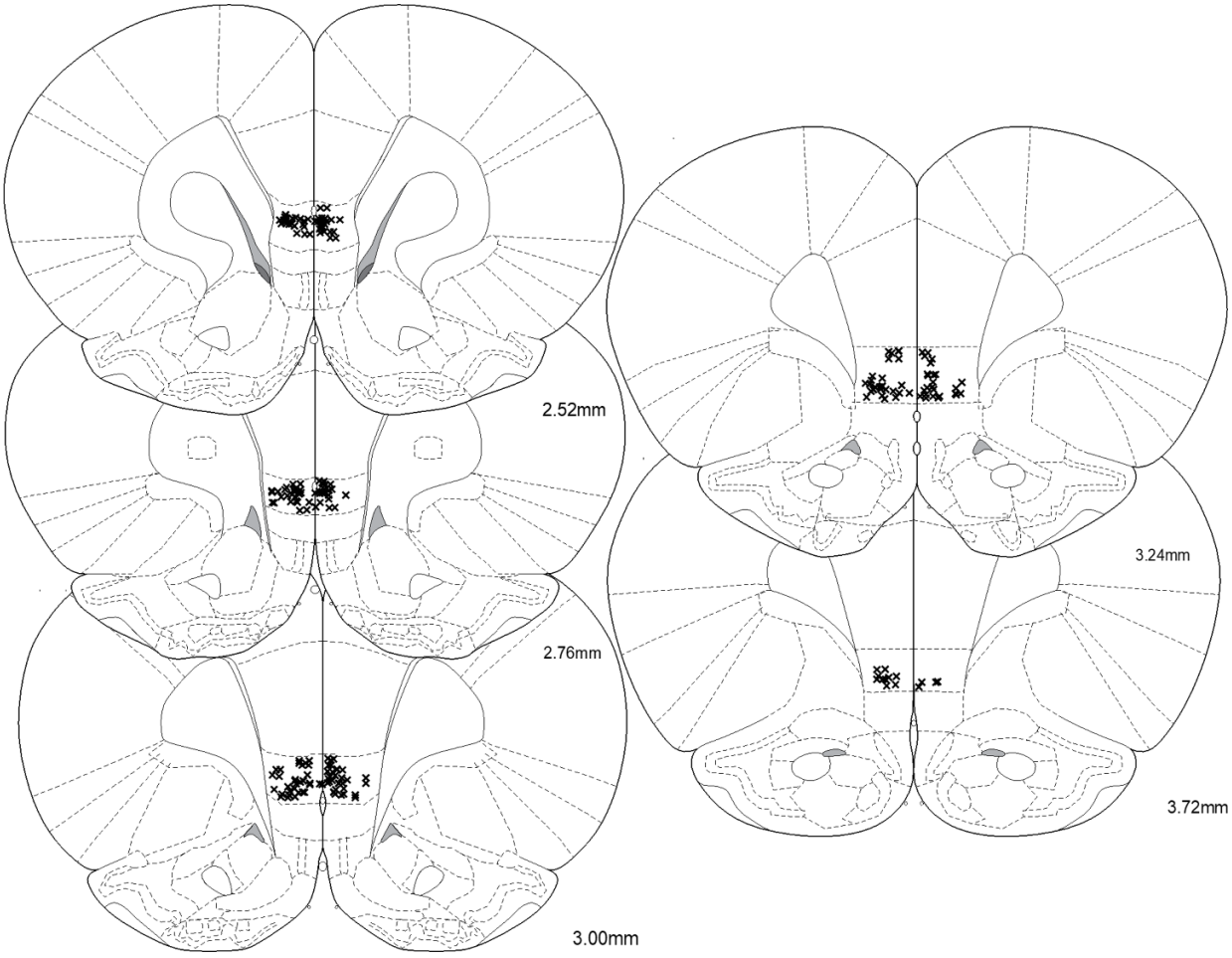


Figure 4. Placement of electrode tips for multi-unit activity from 16 animals. The “x” are marking electrode tips with confirmed hits in the IL. Data from these electrodes were used in multi-unit analysis.

Multi-Unit Analyses

After isolating multi-unit signals using Offline Sorter, peri-event histograms were generated for each of the wires for their responses to the four cues. All trials were included regardless of the level of freezing behavior. 10,000 round permutation analyses were used to compare the averaged count per bin (50 ms/bin) during the 2 second pre-cue baseline against the 1 second post-cue onset to determine if there was a significant response to a given cue (Sangha et al., 2013). To examine the differences in the magnitude of neural responding to each cue across DC training sessions, the activity for each wire during each cue type (i.e. fear cue, reward cue, etc) was normalized against its individual 2 second pre-cue baseline firing rate. Then the area under the curve was calculated for the 1 and 4 seconds post-cue onset using MATLAB. The maximum peak amplitude in the first second of the cue for each wave was calculated in MATLAB. This 1 second window was chosen to reflect the same time window for the brief cue onset activity that was observed with the averaged unit response during DC1. The areas under the curve and maximum peak amplitudes were examined with two-way RM ANOVAs followed with *post hoc* Tukey's multiple comparisons test in GraphPad Prism. The Tukey's test was used because it allows all possible comparisons for cue effects and session effects.

Preliminary Single-Unit Analyses

Neural data from animals with a light as the safety cue (n = 8) during DC 1, 3 and 4 were used to examine the preliminary population activity at a single neuron level. DC1 neural data was examined because the animals had not yet demonstrated any fear suppression learning to the fear+safety cue. DC3 and 4 neural data were examined because the animals were showing high levels of fear suppression to the fear+safety cue. After isolating single-unit signals using Offline Sorter, peri-event histograms were generated for each of the wires for their responses to the four cues. 10,000 round permutation analyses were used to compare the averaged count per bin (50 ms/bin) during the 2 second pre-cue baseline against the 1 second post-cue onset to determine if there was a significant response to a given cue (Sangha et al., 2013). All trials were included regardless of the level of freezing behavior. Neurons that showed significant changes in activity to any of the cues during the first second of cue onset relative to its two seconds pre-cue baseline

were counted as responsive neurons. To examine the population response profile, neurons that showed significant excitation or inhibition to the fear+safety cue during the first second of fear+safety cue onset were counted and divided by the total number of responsive neurons for that DC session. Neurons that showed significant excitatory or inhibitory responses to the fear+safety cue were averaged to assess the magnitude of change in firing rate across DC training sessions. The counts of fear+safety responsive neurons were used in chi-square tests to investigate if there was any relationship between fear+safety cue-excitation/inhibition across the three DC training sessions.

RESULTS

Similar Learning Rates to the Auditory Fear Cue and Visual Fear Cue (Figure 5)

To account for potential differences in behavioral responses to cue modality, half the animals (n = 8) had a light as a safety cue, and a tone as a fear cue; the other half of the animals had the opposite. A two-way RM ANOVA was performed to examine the freezing percentages across 16 fear trials for the 2 counterbalanced conditions during the 4 DC sessions to examine fear learning over time. There was no significant trial by condition interaction ($F(15, 210) = 1.515$, $p = 0.1019$) indicating that the two counterbalanced groups learned fear at a similar rate. There was a significant main effect of trial number ($F(6.175, 86.45) = 17.68$, $p < 0.0001$), indicating that both conditions showed an increase in freezing across fear trials. There was no significant main effect of condition ($F(1, 14) = 4.565$, $p = 0.0508$), indicating that the two counterbalanced conditions had a similar level of freezing to the fear cue overall. A two-way RM ANOVA was performed to examine the freezing percentages across 60 combined fear + safety trials for the 2 counterbalanced conditions during the 4 DC sessions to examine safety learning over time. There was no significant effect of trial by condition interaction ($F(59, 826) = 0.8744$, $p = 0.7372$), indicating that both conditions learned fear suppression at a similar rate. There was a significant main effect of trial ($F(9.756, 136.6) = 2.343$, $p = 0.0146$), indicating that both counterbalanced conditions showed a change in the freezing percentages across fear+safety trials. There was a significant main effect of condition ($F(1, 14 = 9.366$, $p = 0.0085$), indicating that animals with the light as a safety cue showed an overall higher freezing percentages than animals with the tone as a safety cue throughout fear+safety trials. A two-way RM ANOVA was performed to examine the port percentages across 60 reward trials throughout the 4 DC sessions. There was no significant trial by condition interaction ($F(59, 826) = 0.8235$, $p = 0.8251$), indicating that the two counterbalanced conditions learned reward at a similar rate There was no significant main effect of trial ($F(10.62, 148.7) = 0.9250$, $p = 0.5159$), indicating that there was no further reward learning during DC sessions. This occurred because animals had already experienced five sessions of reward pre-training prior to DC sessions. There was no significant main effect of condition ($F(1, 14) = 0.4165$, $p = 0.5291$), indicating that there was no difference in the overall level of port percentages between the two different counterbalanced conditions during DC training. Overall, our data showed that

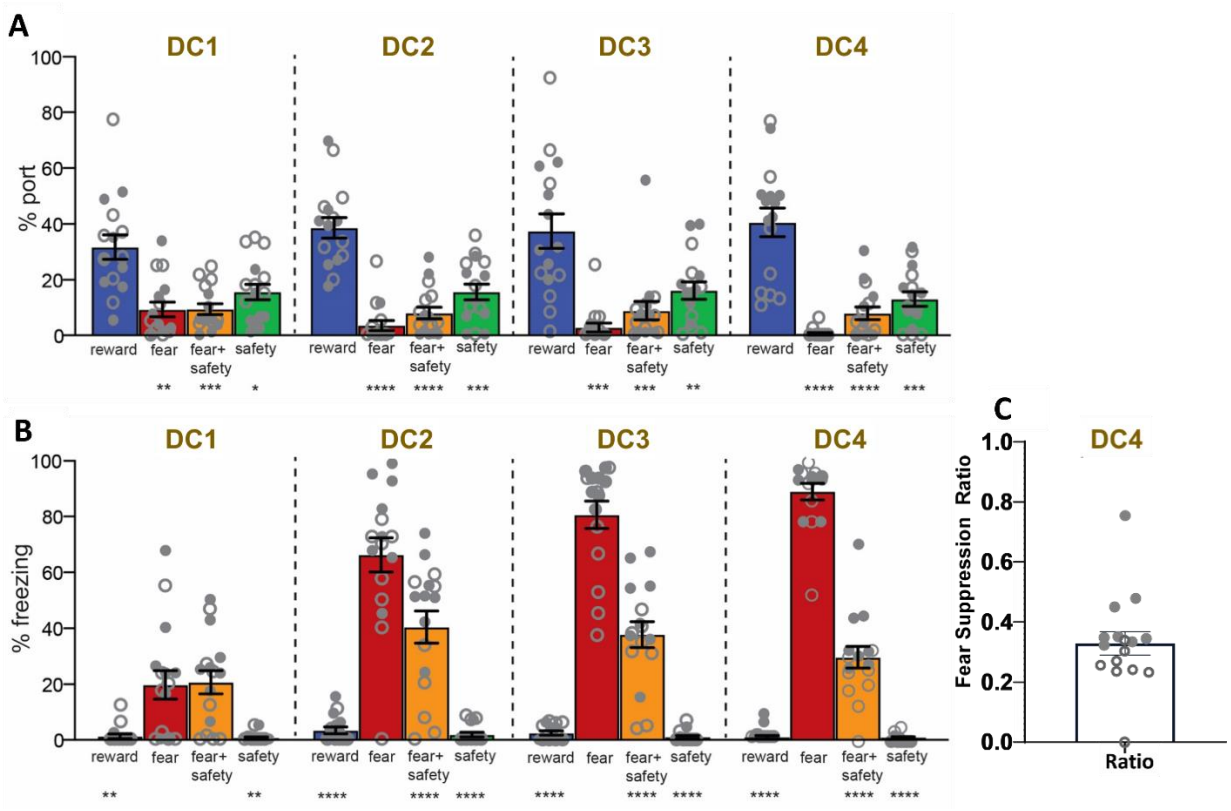


Figure 5. Combined behavioral data from the two counterbalanced groups showing that animals (n = 16) learned to discriminate between fear, reward and safety (open circles represent animals that had tone as the safety cue and grey circles represent animals that had light as the safety cue (A) % Port; Animals showed high levels of port activity to the reward cue. (B) % Freezing; Animals showed high levels of freezing to the fear cue and reduced freezing level to the fear + safety cue. (C) Fear suppression ratio for DC4. Fear suppression ratio = (% freezing to fear + safety cue) / (% freezing to fear cue).

there was no significant difference in the learning rate for freezing to the fear cue, fear suppression to the combined fear + safety cue, or port activity to the reward cue between the two counterbalanced conditions. All data from the two counterbalanced conditions were combined for subsequent analysis.

Normal Fear Learning, Safety Learning, and Reward Learning During DC Sessions

A two-way RM ANOVA was performed to examine port percentage to the 4 cues across 4 DC sessions. There was no significant cue by session interaction ($F(9, 180) = 1.243$, $p = 0.2715$). There was a significant main effect of cue ($F(3, 60) = 48.04$, $p < 0.0001$), but no significant main effect of session ($F(2.706, 162.3) = 0.1057$, $p = 0.9448$). Post hoc Dunnett's multiple comparisons to the reward cue showed that animals had a significantly higher level of port percentages to the reward cue than all other cues across all 4 DC sessions ($p < 0.0001$). These data indicated that animals had a higher level of selective port activity to the reward cue starting at DC1 even though there were no further changes in port activity throughout DC training.

A two-way RM ANOVA was performed to examine freezing percentage to the 4 cues across 4 DC sessions. There was a significant cue by session interaction ($F(9,180) = 34.91$, $p < 0.0001$). There was a significant main effect of cue ($F(3, 60) = 128.3$, $p < 0.0001$), and a significant main effect of session ($F(2.757, 165.4) = 56.30$, $p < 0.0001$). Post hoc Dunnett's multiple comparisons to the fear cue showed that animals had a significantly higher level of freezing to the fear cue than to the reward and safety cues from DC1 to DC4 ($p < 0.05$) indicating animals learned to freeze to the fear cue starting at DC1. Animals had a significantly higher level of freezing to the fear cue than to the combined fear + safety cue from DC2 to DC4 ($p < 0.0001$) indicating animals learned to suppress freezing to the combined fear + safety cues starting at DC2.

Categorizing Cue Responsive Neural Activity (Figure 6, Figure 7)

10,000 round permutations were performed to compare multi-unit activity during a 2 second pre-cue baseline against the first 1 s of cue onset to the four cues for both DC1 and DC4. There were 229 electrodes with confirmed placements in the IL. DC1 had 80 electrodes with significant cue responses and 149 electrodes without significant cue responses. DC4 had 132 electrodes with significant cue responses and 97 electrodes without significant cue responses.

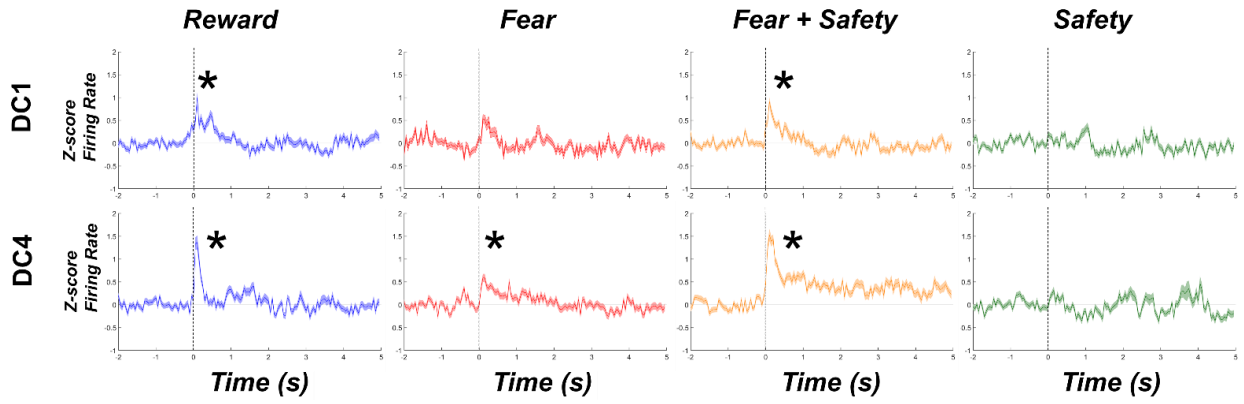


Figure 6. IL multi-unit activity from 16 animals (229 electrodes) for cue onset traces during DC1 and DC4. The asterisks (*) indicate that there was a significant increase in multi-unit activity to the 1 second of cue onset relative to the 2 second pre-cue baseline.

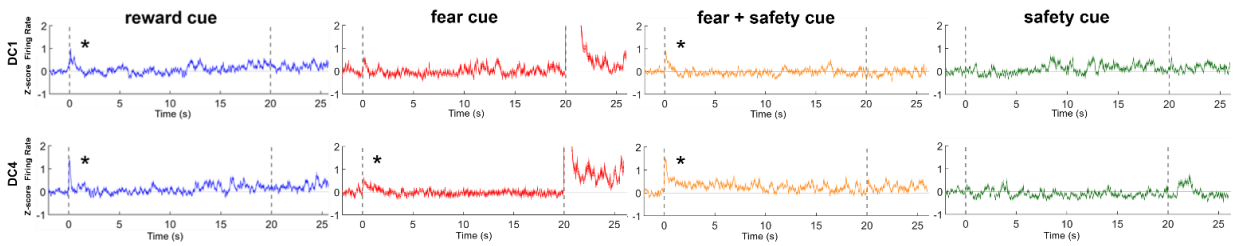


Figure 7. IL multi-unit activity from 16 animals (229 electrodes) for the entire duration of the 20 second cue during DC1 and DC4. The asterisks (*) indicate that there was a significant increase in multi-unit activity within 1 second of cue onset relative to the 2 second pre-cue baseline. Towards the end of the fear cue, a footshock was delivered which lead to measurement artifacts. Overall, there were no learning related changes in multi-unit activity observed for cue offsets.

Electrodes without cue responses were included in the analysis because we wanted to capture IL global changes during learning. Including all electrodes also allowed our analysis to be sensitive to change in overall population recruitment during learning. For DC1, there was a significant increase in cue driven multi-unit activity to the combined fear + safety cue ($p < 0.0001$) and to the reward cue ($p < 0.0001$), but not to the fear cue alone or safety cue alone. For DC4, there was a significant increase in cue driven multi-unit activity to the fear cue ($p < 0.0001$), fear + safety cue ($p < 0.0001$) and reward cue ($p < 0.0001$), but not to the safety cue alone.

Short-Latency Multi-Unit Activity for the Combined Fear + Safety Cue Increased Across Sessions and was Larger Than all Other Cues During DC4 (Figure 8)

To compare the differences in area under the curve for short-latency multi-unit responses, a 1 s time window after cue onset was examined. There was a significant cue by session interaction ($F(3, 684) = 26.00$, $p < 0.0001$), as well as significant main effects of cue ($F(3, 684) = 56.66$, $p < 0.0001$) and session ($F(1, 228) = 14.38$, $p < 0.001$). Post hoc Tukey's multiple comparisons test showed that in DC1, the area was not different between the reward cue and the combined fear + safety cue ($p = 0.9988$). Both the reward cue and the fear + safety cue had a larger 1s area under the curve than the fear cue and the safety cue ($p < 0.001$). In DC4, the fear+safety cue had a larger area than the reward cue ($p < 0.0001$), fear cue ($p < 0.0001$) and the safety cue ($p < 0.0001$). There was a significant increase in area from DC1 to DC4 for the fear + safety cue ($p < 0.0001$) and the fear cue ($p < 0.05$), but not to the reward cue ($p = 0.9679$) or the safety cue ($p = 0.7556$)

Sustained Multi-Unit Activity to the Combined Fear + Safety Cue Increased Across Sessions and was Larger Than all Other Cues During DC4 (Figure 8)

To be more inclusive with the later occurring sustained learning-related multi-unit responses, a 4 second time window after cue onset was examined. There was a significant cue by session interaction ($F(3, 684) = 22.08$, $p < 0.0001$), as well as significant main effects of cue ($F(3, 684) = 22.19$, $p < 0.0001$) and session ($F(1, 228) = 39.29$, $p < 0.0001$). Post hoc Tukey's multiple comparison tests showed that in DC1, the area was not different between the reward and the combined fear + safety cue ($p = 0.9965$). Both the reward cue and the fear + safety cue area did not differ with the area from the fear cue ($p = 0.6386$; $p = 0.9655$) and the safety cue ($p = 0.339$; $p = 0.8050$). The lack of significant differences in the area under curve between responses to the fear

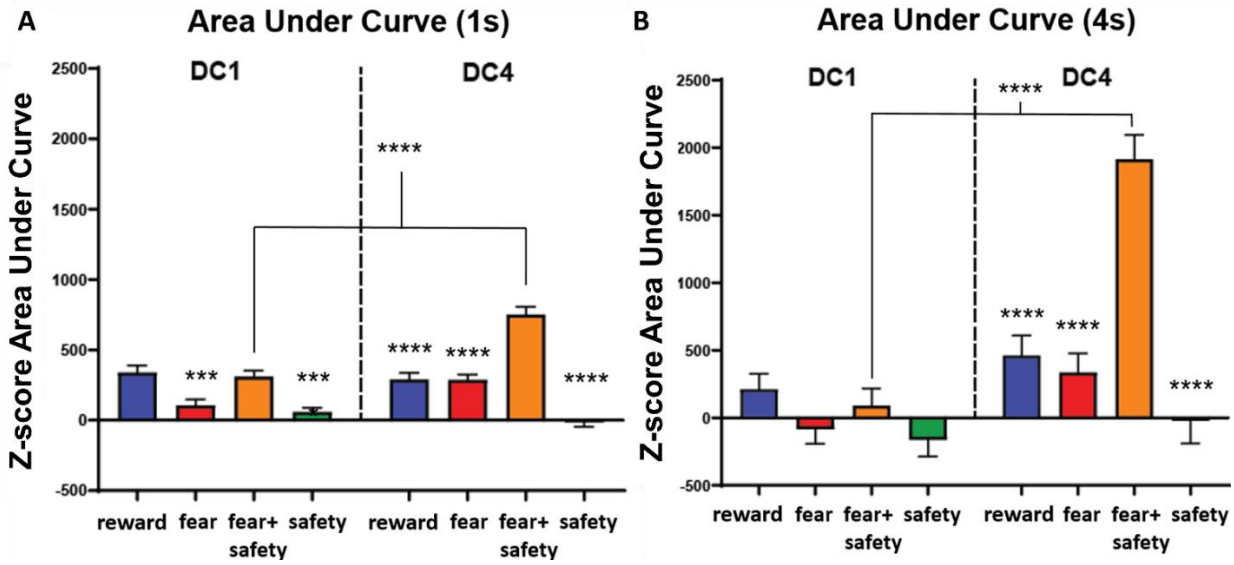


Figure 8. IL Multi-unit activity from 16 animals (229 electrodes) for cue onset area under curve. (A) Area under curve for 0-1 s of cue onset. (B) Area under curve for 0-4 s of cue onset. The asterisks (*) are showing within session comparisons for cues against the fear+safety cue and between sessions comparison of fear+safety cue. (***$p < 0.001$, ****$p < 0.0001$)

+ safety cue versus other cues in DC1 after increasing the analysis window from 1 second to 4 seconds may be because the long analysis time window is masking the brief signal. This may have occurred because the brief 1 second signal is now being averaged with the subsequent 3 seconds when the signal had returned back to baseline level. In DC4, the fear + safety cue has a larger area than the reward cue ($p < 0.0001$), fear cue ($p < 0.0001$) and the safety cue ($p < 0.0001$). There was a significant increase in area from DC1 to DC4 for the fear + safety cue ($p < 0.0001$), but not to the fear cue ($p = 0.1852$), reward cue ($p = 0.8085$) or safety cue ($p = 0.9915$).

Maximum Peak Amplitude for the Fear + Safety Cue Increased With Learning and was Higher Than all Other Cues During DC4 (Figure 9, 10)

To examine maximum cue evoked responses in the IL, we calculated the highest point within 1 second of cue onset for each electrode wire. There was a significant cue by session interaction ($F(3, 684) = 13.87$, $p < 0.0001$), and significant main effects of cue ($F(3, 684) = 45.41$, $p < 0.0001$) and session ($F(1, 228) = 8.557$), $p < 0.01$). Post hoc Tukey's multiple comparisons tests showed that in DC1, the maximum amplitude between the combined fear + safety cue and reward cue did not differ from each other ($p = 0.999$). Both the fear + safety cue and the reward cue had a significantly higher maximum amplitude than the fear and safety cues alone ($ps < 0.05$). During DC4, the maximum amplitude for the combined fear+ safety cue was significantly higher than the reward cue ($p < 0.05$), fear cue ($p < 0.05$) and safety cue ($p < 0.05$). From DC1 to DC4, there was a significant increase in maximum amplitude for the combined fear+ safety cue ($p < 0.05$), but not for the reward cue ($p = 0.1069$), fear cue ($p = 0.9807$) and safety cue ($p = 0.0937$)

No Change in Baseline Firing Across Training

A paired T-test was performed on the 2 seconds of pre-cue baseline raw firing rates for DC1 and DC4. There was no significant change in baseline raw firing rates between the two DC sessions ($t(228) = 1.75$, $p = 0.082$).

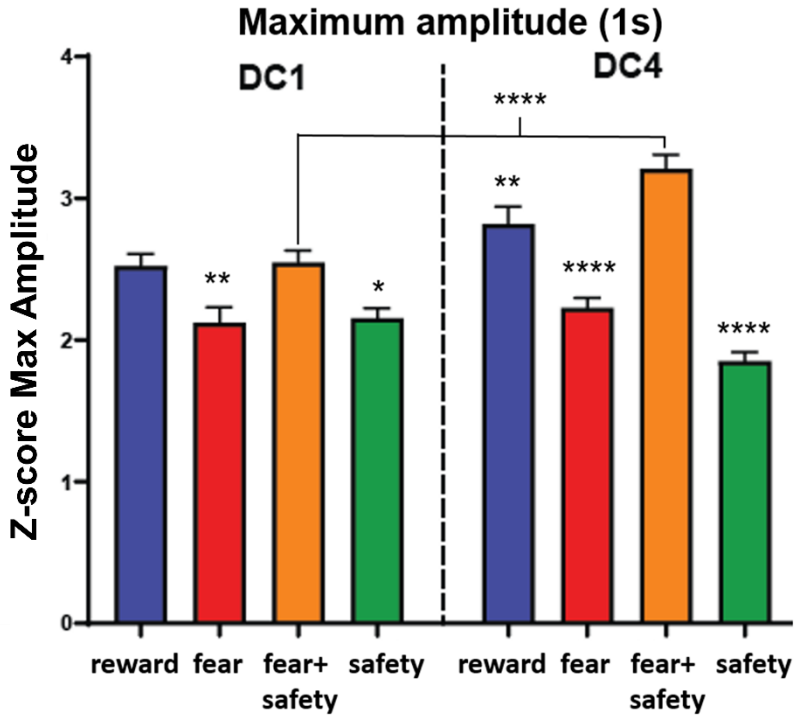


Figure 9. IL multi-unit activity from 16 animals (229 electrodes) for maximum peak amplitude. The asterisks (*) are showing within session comparisons of cues against the fear+safety cue and between sessions comparisons of the fear+safety cue with itself. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$)

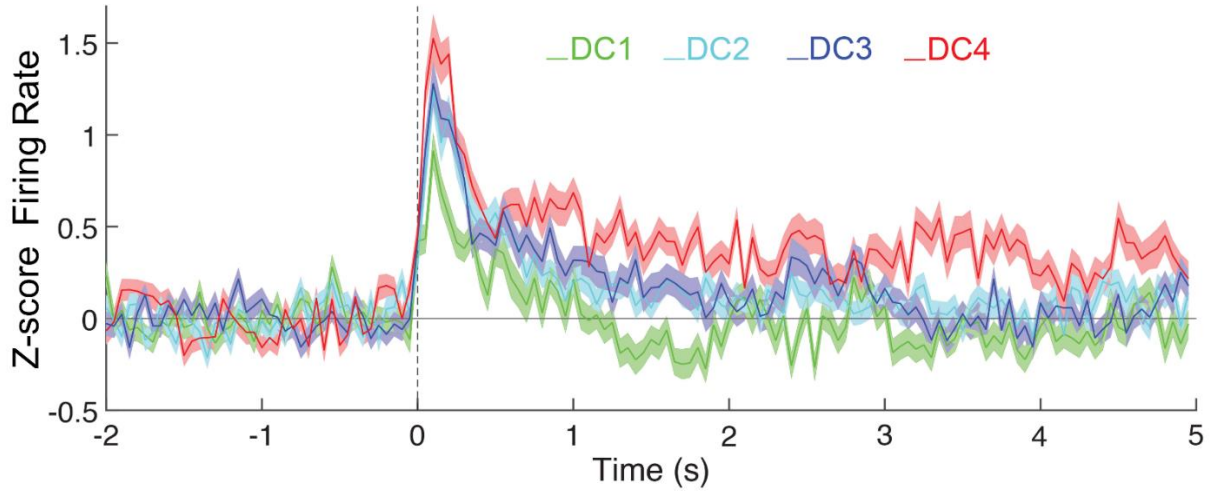


Figure 10. IL multi-unit response from 16 animals (229 electrodes) to the fear+ safety cue during each DC session.

Preliminary Single Unit Data

Preliminary single-unit data from animals that received light as the safety cue was analyzed (n = 8) (Figure 11). When the single-unit analysis was restricted to the same neurons that stayed throughout all of DC1, 3 and 4, there were 56 neurons (Figure 12). DC1, 3 and 4 had 66%, 71% and 64% nonresponsive neurons, respectively. A chi-square test of independence was performed on the cell count of fear+safety responsive neurons to examine the relationship between the directions of response (excitation/inhibition) across training sessions. There was a drop in the percentage of neurons that showed inhibition to the fear+safety cue across the training sessions. However, the chi-square test did not reveal a significant relationship between response direction and training session with our sample size X^2 (2, N = 34) = 0.219, p = 0.896). When the single unit analysis included all possible neurons for all sessions, there were 74 neurons in DC1, 77 neurons in DC3 and 86 neurons in DC4 (Figure 13). By including all possible neurons, the statistical power can be improved for detecting differences with our small sample size. DC1, 3 and 4 had 73%, 71% and 70% nonresponsive neurons, respectively. A chi-square test of independence was performed again on the cell count of fear + safety responsive neurons to examine the relationship between the directions of response and training sessions. There was a drop in the percentage of neurons that showed inhibition to the fear + safety cue across training sessions. However, the chi-square test did not reveal a significant relationship between response direction and training session with our sample size X^2 (2, N = 42) = 1.384, p = 0.501).

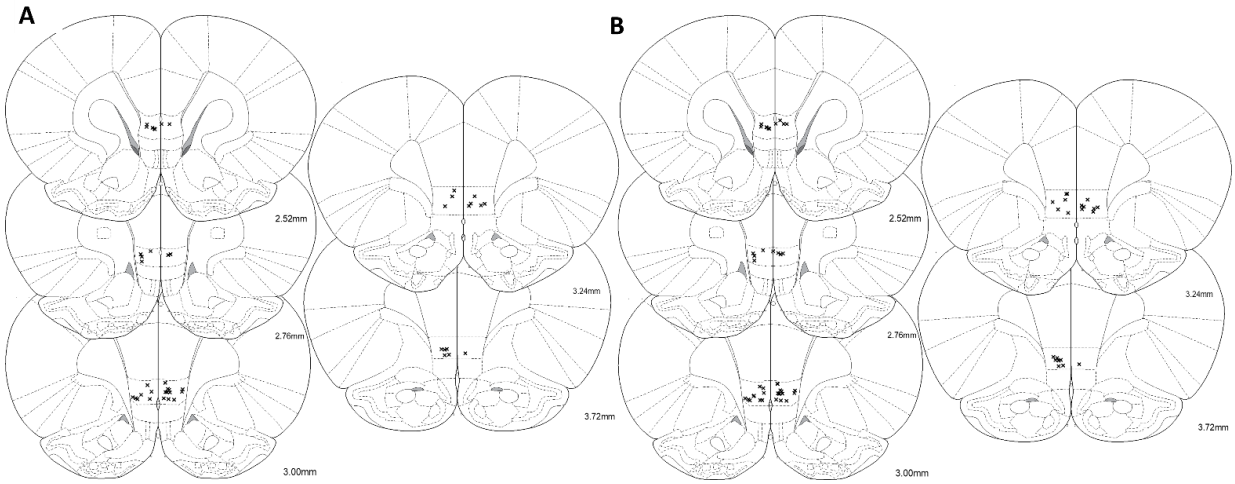


Figure 11. Electrode placements for single-unit analysis from 8 animals. (A) Electrode placements for single-units that sustained throughout DC1, 3 and 4. (B) Electrode placements for all available single-units.

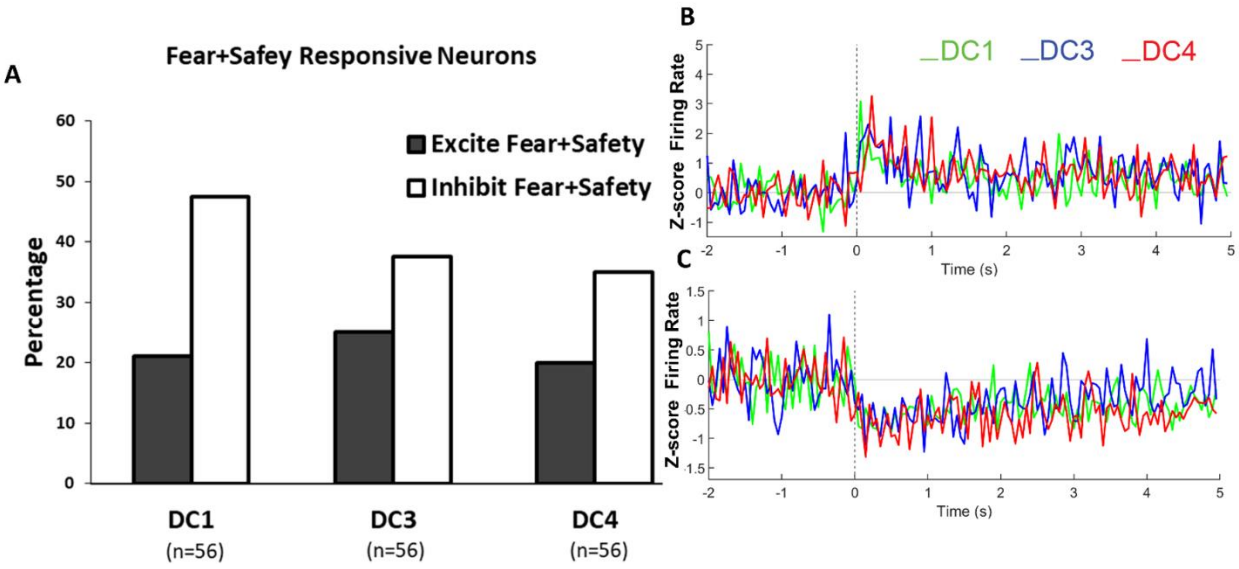


Figure 12. Single-unit response profile of IL neurons from 8 animals during DC1, DC3 and DC4. Only the same units (*n* = 56) that stayed throughout DC1, DC3 and DC4 were included in the analysis. (A) Percentage of excitatory or inhibitory fear + safety neurons out of all responsive neurons in that training session. (B) Averaged magnitude of neurons that showed an excitatory response to the fear + safety cue. (C) Averaged magnitude of neurons that showed an inhibitory response to the fear + safety cue.

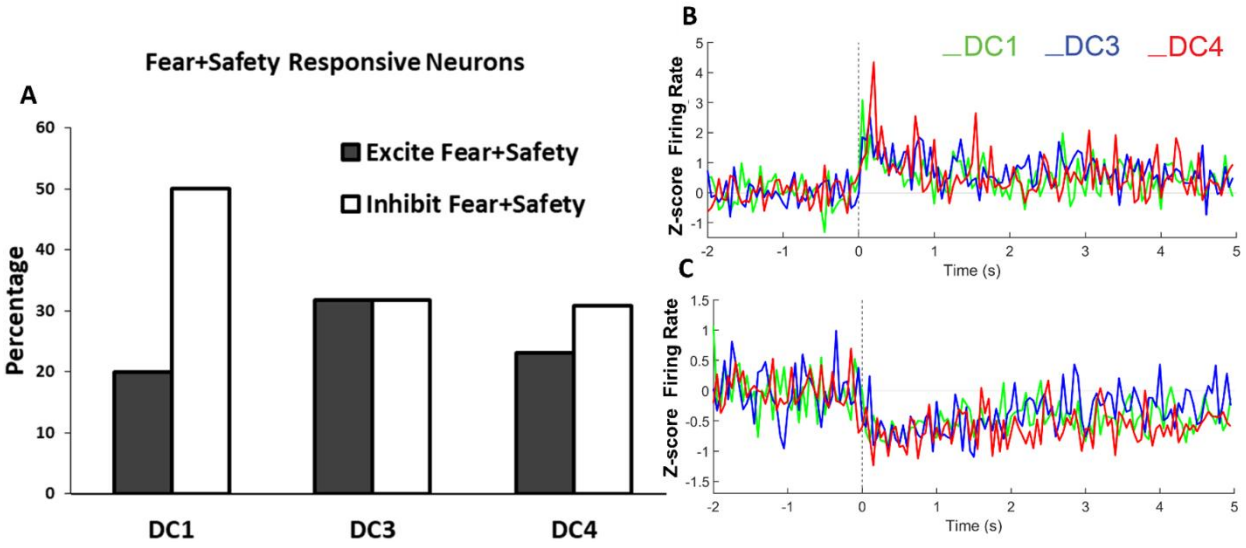


Figure 13. Single-unit response profile of IL neurons from 8 animals during DC1, DC3 and DC4. All available units (DC1: *n* = 74, DC3: *n* = 77, DC4: *n* = 86) that appeared in any of the DC sessions were included. (A) Percentage of excitatory or inhibitory fear + safety neurons out of all responsive neurons in that training session. (B) Averaged magnitude of neurons that showed an excitatory response to the fear + safety cue. (C) Averaged magnitude of neurons that showed an inhibitory response to the fear + safety cue.

DISCUSSION

Neural recordings within the IL showed increased activity to the fear + safety cue that developed across training sessions (Figure 10). This increase in the multi-unit response to the combined fear + safety cue onset across DC training sessions was quantified using the area under the curve and the maximum amplitude. These increases in the area under the curve were observable for both the 1 second and 4 second analysis window, while the increase in maximum amplitude was observable for the 1 second analysis window. During the last DC session, the area under the curve and the maximum peak amplitude of multi-unit activity were largest to the combined fear + safety cue compared to all other cues. This indicates that DC training resulted in the development of both a short-lasting response (1 second) and a more sustained response (4 seconds) to the combined fear + safety cue in the IL. These increases in IL multi-unit activity in our study indicates the IL overall produces an excitatory response during fear suppression. We also analyzed single-unit activity from animals that received light as a safety cue. Although not statistically significant, there was a decrease in the percentage of neurons that showed inhibitory responses to the fear + safety cue while there was no change in the percentage of neurons that showed excitatory responses to the fear + safety cue. There was also no change in the magnitude of firing rate for excitatory or inhibitory fear + safety responsive neurons. These suggest that the increased multiunit response in IL during fear suppression learning may be driven by the decreased amount of inhibitory fear + safety response neurons.

One strength with our current study was the use of both single-unit analysis and multi-unit analysis to demonstrate IL's engagement during cued fear suppression behavior. The commonly performed single-unit analysis is advantageous for developing activity profiles of neurons in a given brain region. However, this approach restricts analysis to neurons with certain baseline firing rates because of the unit-sorting analysis procedure. The amount of baseline firing needed for a neuron to be included could vary based on the analysis procedure and analytical skill of the investigator. Single-unit analysis may also restrict analysis to neurons that produce high voltage amplitudes during data collection. As a result, the single-unit analysis could underrepresent neurons with low baseline firing rates and smaller units with partial cutoffs from voltage thresholds during data collection. In contrast, multi-unit analysis has the advantage of better representing averaged population activity by including additional neurons that would have been rejected with

single-unit analysis. These include the low firing rate neurons and smaller units with low voltage amplitudes. Unlike single-unit analysis, multi-unit analysis has the disadvantage of blending the effects of different neuronal subtypes and can even cancel neural responses when excitatory and inhibitory responses are averaged together. Nevertheless, averaging the excitatory and inhibitory responses in multi-unit analysis could still be beneficial in some settings when assessing the overall downstream effects of a brain region. Thus, it is important to use both analyses to compensate for each other's disadvantage.

Overall, our multi-unit results are consistent with previous studies (Milad & Quirk, 2002; Sierra-Mercado et al., 2011). Here, we demonstrated that the IL showed an increase in multi-unit activity in response to the combined fear + safety cue during the last training session. Using the same DC training paradigm, previous data from our lab demonstrated that the IL is necessary for suppressing fear in the presence of a safety signal (Sangha et al., 2014). Thus, our previous study might have impaired fear suppression behavior by interrupting the necessary increase in IL activity during learning. Also, we found an increase in multi-unit activity within the first second of the cue onset specific to the combined fear + safety cue. This is consistent with a previous study that found an increase in IL activity to the fear cue onset during the recall of fear extinction memory (Milad & Quirk, 2002). The IL may encode fear suppression during the early portion of the cue onset. Our finding supports the hypothesis that the IL would show an increase in firing to the combined fear + safety cue. We observed two types of facilitation resulting from DC training. There was an increase in the short-lasting multi-unit activity that was captured in the 1 second analysis window, which may be responsible for initiating fear suppression response. There was also an increase in the sustained activity that was captured in the 4 second analysis window, which may allow the maintenance of fear suppression throughout the entire duration of the safety cue. One disadvantage with multi-unit analysis is the restriction in our hypothesis in examining changes in firing rate. A previous study had found that neurons in the frontal cortex can encode properties of the conditioned stimuli and animal behavior with interspike interval coding in addition to the traditional rate coding that was used in the current study (Insanally et al., 2019). Hence, our rate coding from the multi-unit analysis may not fully explain fear suppression behavior at the trial level. Another disadvantage of using multi-unit analysis with the current study is the inability to confirm the cell types responsible for fear suppression within the IL. Nevertheless, the cells that showed learning-related changes in the current study were likely IL glutamatergic neurons because

a previous study that found enhancement of fear extinction behavior following optical activation of IL was performed using a CaMKII promotor that targeted pyramidal neurons (Do-Monte, Manzano-Nieves, Quiñones-Laracuente, Ramos-Medina, & Quirk, 2015). Future studies could confirm this by using an optogenetic approach with the same CaMKII promotor to inactivate pyramidal neurons in the IL after animals have acquired fear suppression behavior. This manipulation should impair fear suppression only when the IL pyramidal neurons are optically inactivated.

The increase in IL in multi-unit activity during safety learning may be driven by the decrease in IL inhibition at single-unit level. Our preliminary single-unit data from animals that received light as a safety cue showed a visible but not statistically significant decrease in the percentage of neurons that showed an inhibitory response to the fear + safety cue while showing no change in the averaged magnitude of excitatory or inhibitory response to the fear + safety cue. This change in cell recruitment in the IL provides supporting evidence that plasticity is occurring within the IL rather than simply having BLA providing more input to the IL to drive up the multi-unit activity. The time course of the loss in inhibition observed in our study is also consistent with current literature. The increase in IL activity does not emerge right away during the initial training of fear extinction (Milad & Quirk, 2002). Instead, it emerges the next day after the fear extinction memory has had time to consolidate overnight (Milad & Quirk, 2002). This change in cell recruitment in the single-unit level in our study may reflect a gradual shift in the leading role between the IL and BLA during safety learning (Lesting et al., 2013). Data looking at theta synchrony found that the firing of IL neurons leads the firing of neurons in the LA and CA1 during the recall of a successfully learned extinction memory. This IL-to-BLA projection may be the dominant route for fear suppression behavior. Optical activation of IL during the CS period has been shown to facilitate fear extinction and subsequent retrieval of fear extinction (Do-Monte et al., 2015). On the contrary, the optical inactivation of IL during the CS period impairs the retrieval of fear extinction (Do-Monte et al., 2015). Future studies could test the time course of the IL-to-BLA pathway's involvement by using retro-DREADDs to inactivate the IL-to-BLA pathway at different time points during fear suppression learning. Inactivating IL-to-BLA pathway in early training should not have any impact in fear suppression levels in the last training session. In contrast, inactivating IL-to-BLA pathway in middle or late training should impair fear suppression levels in the last training session.

The two components of learning related changes in the IL multiunit activity in our study are similar to those observed in the BLA. The BLA contains principal neurons that can develop two separate components during training. These include a short-lasting increase in firing to the cue onset and a later occurring component that mirrors the timing of the CR. These occur to both aversive and rewarding valences (Kyriazi, Headley, & Pare, 2018). During fear suppression, the short-lasting increase in IL activity may function to initiate fear suppression behavior and to suppress the short-lasting cue evoked response in the amygdala. In contrast, the sustained increase in IL activity may function to maintain fear suppression behavior and to suppress the more variable amygdala CR activity that drives fear expression. Future studies could test this hypothesis by using optogenetics to inactivate the IL during the first second of cue onset or inactivating the IL during the later time window of the cue onset, in order to examine the impact on conditioned freezing during the recall of the fear +safety cue. Alternatively, a future study could also vary the presentation time of the safety signal relative to the fear signal, to provide a better understanding of the function of the cue onset facilitation in the IL. For example, the short-lasting IL activity to the fear cue may increase in cue onset duration when the safety cue presentation is delayed with respect to the fear cue presentation during the fear + safety trial. This would provide additional support for the role of short-lasting activity in the initiation of the fear suppression behavior.

We also found the fear cue to induce an unexpected increase in the short-lasting multi-unit response of the area under the curve but no increase in maximum amplitude during the last DC session. This occurred because the cue evoked response took longer to return to baseline after DC training. Safety learning may drive the increase in IL response duration because the animal may be anticipating the occurrence of the safety signal. In real-world settings, it is almost impossible to have the safety signal and the aversive signal to start at the same time. This short-lasting increase in IL activity to the fear cue may be responsible for anticipating the expected safety cue to start fear suppression behavior. Future studies could test this hypothesis by introducing a delay of the safety cue after the fear cue presentation. A longer delay for safety cue onset should increase the fear cue onset duration of IL.

Contrary to our original hypothesis, we did not observe any cue evoked response to the safety cue alone throughout DC training. This lack of response to the safety cue was consistent among the animals that had a light as a safety cue and animals that had a tone as safety cue. These data suggest that the IL is not encoding the stimulus property of a safety signal. The IL may only

be involved during active fear suppression when the fear cue is also present. In contrast, neurons in the BA showed the same response to both the fear + safety cue and safety cue alone, suggesting that the BA may encode stimulus properties of a safety signal (Sangha et al., 2013). This discrepancy between IL and BLA in safety encoding may be due to the BLA having a heavier role in sensory processing. Neurons in the BLA have been shown to be necessary for tone discrimination (Grosso et al., 2018). If the BLA encodes the stimulus properties before passing the information to IL, it might be redundant for the IL to encode the same stimulus again.

Taken together, our study has established that the IL encodes fear suppression. We have identified that the IL showed changes in both short-lasting and sustained multi-unit activity during trials when both the fear cue and safety cue were presented together. This increase in multi-unit activity during fear suppression may be driven by the decrease in the percentage of single-unit inhibitory responses in the IL. The increase in the IL multi-unit response may in turn lead to fear suppression behavior through its projection to the BLA (Figure 14). The BLA contains neurons that show decreased activity to the fear cue after extinction (Hobin et al., 2003), as well as safety neurons that are responsive to the fear +safety cue (Sangha et al., 2013). The excitatory IL input may suppress fear behavior through BLA interneurons. There are two primary types of interneurons in the BLA. These include PV interneurons that expresses parvalbumin and SOM interneurons that express somatostatin. Normally, the auditory fear CS excites PV interneurons and then PV interneurons in turn disinhibit BLA principal neurons by inhibiting SOM interneurons (Wolff et al., 2014). We hypothesize that the increase in IL activity should lead to an increase in SOM interneuron activity and a decrease in BLA principle neuron activity, with the ultimate result of fear inhibition. Future studies could train animals in the same fear and safety discrimination task while monitoring BLA single-unit activity and selectively silencing SOM interneurons or principal neurons using transgenic methods.

BLA also communicates with the IL for fear suppression learning. The BLA contains both excitatory and inhibitory fear + safety responsive neurons that increase in responding across training sessions (Sangha et al., 2013) and the BLA projection to IL is necessary for fear extinction memory recall (Senn et al., 2014). Thus, it is expected to also see changes in cell recruitment in the BLA during fear suppression learning. We predict that safety discrimination training would lead to an increase in the percentage of excitatory fear + safety response neurons and no change in the percentage of inhibitory fear + safety response in the BLA. The increase in excitatory fear +

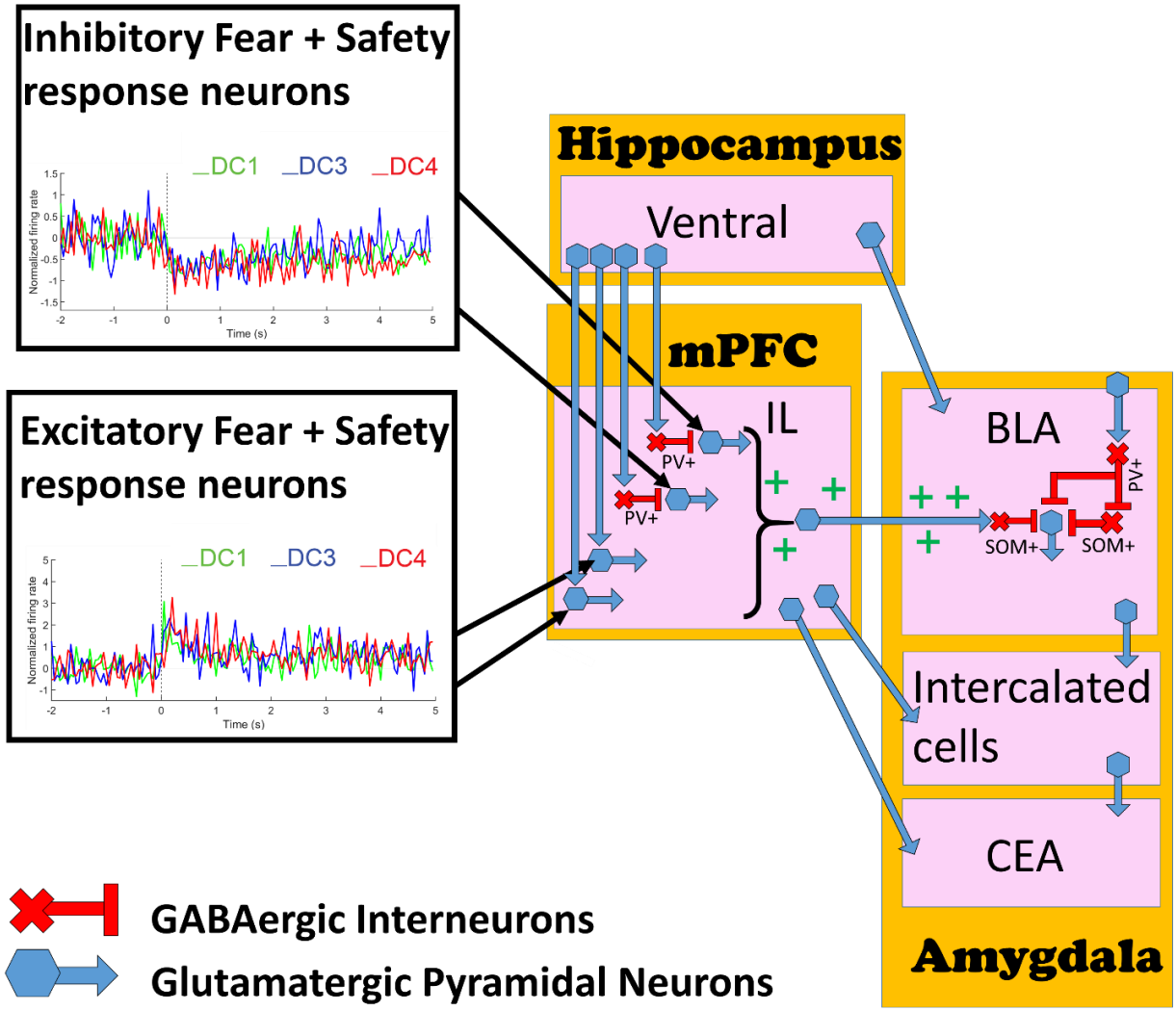


Figure 14. Proposed wiring diagram for IL changes during cued fear suppression learning. The ventral hippocampus may suppress the firing of inhibitory fear + safety response neurons through IL PV+ interneurons. The number of inhibitory fear+ safety response neurons that gets silenced may increase with DC training. This results in an overall excitatory effect in IL output at the population level. The increase in overall excitation in the IL may increase the strength of excitatory input to BLA SOM interneurons and inhibit BLA principal neurons. The decrease in BLA principal neurons would lead to a reduction in CEA output and reduced conditioned freezing behavior to the learned safety signal.

safety responsive cells may provide feedback to the IL to enhance overall IL activity during memory consolidation.

Dopamine signaling may modulate safety learning through existing dopamine receptors in IL and BLA (Boyson, McGonigle, & Molinoff, 1986). Dopamine producing neurons in the ventral tegmental area (VTA) are known to be important for encoding reward prediction errors (Montague, Dayan, & Sejnowski, 1996). These neurons increase in firing when an obtained reward is higher than expected, and they decrease in firing when an obtained reward is less than expected (Tobler, Fiorillo, & Schultz, 2005). Optical activation of VTA dopamine neurons that mimicked prediction error during reward delivery has been shown to increase cued reward seeking behavior (Steinberg et al., 2013). Since learned fear suppression to a safety signal is a form of negative reinforcement in which the shock is omitted when the safety cue is present, this unexpected shock omission outcome may drive dopamine neurons to respond during fear suppression learning. There is evidence that dopamine signaling could modulate fear suppression behavior through the BLA. For example, a previous study from our lab has shown that stimulating dopamine D1 receptor activity in the BLA impairs fear suppression behavior to a safety signal (Ng et al., 2018). Future studies could use dopamine receptor agonists and antagonists to examine the role of IL dopamine receptors in fear suppression learning. This could dissect the direction of dopamine modulation between IL and BLA.

Since PTSD patients have lower levels of vmPFC activity during fear extinction recall (Milad et al., 2009), therapeutic targets will likely involve neurons that excite IL principal neurons. This is consistent with our current finding that the decrease in IL inhibitory responses may lead to a global excitation of IL activity during fear suppression to a safety signal. Thus, alterations in IL neurons with inhibitory responses may be a potential mechanism for the impaired cued fear suppression in PTSD patients. Future studies could test this hypothesis in animal models of PTSD by monitoring IL neural activity while training animals in cued safety learning, followed by a predator odor trauma, and then safety memory recall. Animals that still showed high levels of fear during safety memory recall should have a higher percentage of neurons showing an inhibitory response to the safety cue than animals that showed low levels of fear during safety memory recall.

A potential source of IL inhibition could be supplied by the hippocampus (Figure 14) since the hippocampus encodes the trauma context (Wang et al., 2012) and fear extinction context (Wang et al., 2015). The vHPC has projections to IL principal neurons through PV interneurons, which are important for fear renewal (Marek, Jin, et al., 2018). Thus, the excitatory input from vHPC could suppress inhibitory fear + safety responding IL neurons through IL PV interneurons, resulting in the reduced number of inhibitory fear + safety responding neurons in the IL. Alternatively, the vHPC may also influence IL activity through its input to the PL. In both mice and humans, vHPC neurons that project to the PL show differential responding during fear memory recall and cued fear suppression (Meyer et al., 2019). The PL could in turn relay that input to the IL through their reciprocal connections (Marek, Xu, et al., 2018). The competing hippocampal input between safety and trauma representations into the IL may alter the excitation balance within the IL. Future studies could train animals in fear and safety discrimination learning while recording from the IL. This can be followed by activity dependent tagging of the fear neurons in the hippocampus during cue recall. After that, both fear suppression behavior and IL activity could be assessed with the concurrent optical inactivation of hippocampus-to-IL projecting fear neurons. We hypothesize that IL projecting fear neurons from the hippocampus during the fear+safety cue presentation should have lower activity in animals that learned fear suppression well compared to animals that did not learned fear suppression well. The subsequent optical inactivation of IL projecting fear neurons from the hippocampus should improve fear suppression to the safety signal and increase IL multi-unit activity in the same animals.

In terms of clinical significance, potential PTSD treatments can focus on weakening the fear input from the hippocampus entering the IL. Human behavioral therapy could use multiple contextual cues to reactivate hippocampal dependent traumatic memories while monitoring IL activity using fMRI. If a given trauma context elicits a large alteration of IL activity, this could be immediately followed by drugs that interrupt memory reconsolidation. This procedure would weaken the trauma contextual representation from the hippocampal input entering the IL and allowed the safety contextual representation from the hippocampus to be unblocked. This method of using IL activity to identify critical contextual trauma cues have several advantages. By concurrently monitoring IL activity with fMRI, it allows the assessment of the relative strength of the fear encoding hippocampal input. It is likely that different trauma contexts will

have different magnitudes of disruptions in IL activity and the corresponding fear suppression behavior. The effective context would be different for each individual with PTSD because it depends on which aspect of the scenery was salient at the moment when the traumatic experience occurred. For example, some retired veterans may selectively find the scenery of burning items to induce a larger alterations in IL activity, while other veterans may selectively find the scenery of being under water to induce a larger alterations in IL activity. Thus, it is important to identify traumatic scenery cues that are clinically relevant to the individual. In addition, concurrently monitoring IL activity would allow identification of subcomponents of the contextual cues. There may be incidences when different contextual cues would separately elicit a high level of alteration in IL activity. For example, a retired veteran may find the scenery of weapons, aircraft, and blood to induce high levels of IL alteration when presented separately because those items were encoded together in the same trauma experience. Trying to interrupt the collective scenery with all three items presented together might be ineffective because the individual may have already re-experienced the event repeatedly through dreams and memory recall resulting in the collective trauma context being over consolidated which makes it resistant to disruption from medication that targets memory reconsolidation. However, targeting memory reconsolidation by presenting the same fear eliciting context separately may provide an alternative approach in disrupting the hippocampal input to IL when the original context which includes all the contextual cues may be resistant to disruption.

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