THE FEAR CONDITIONED RESPONSE UNDER HEAD FIXATION

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Dedication

Dedicated to my parents for which I owe everything to. The words on this page can never do justice to the love you have given me and the sacrifices you have made.

Abstract

In classic measures of fear induced freezing behavior, animals stop all movement except breathing. Many large-scale recording techniques in modern neuroscience such as wide-field and two-photon fluorescent imaging require animals be in a head-fixed preparation. Here we demonstrate that it is possible to measure freezing behavior in head-fixed mice based on video measurements of motion, the pupillary response and electromyography of neck muscles. Animals were either conditioned to form an association between a tone (conditioned stimulus; CS) and a footshock (unconditioned stimulus; US) or were presented both tones and footshocks explicitly unpaired. Animals who were conditioned to form an association showed less movement, and a stronger pupil response when presented with the CS. We outline key considerations which are likely important in establishing a strong fear response in a head-fixed preparation.

Acknowledgements

I would like to present my absolute gratitude to Dr. Majid Mohajerani for giving me an opportunity only a privileged fraction of people get to enjoy – to study and learn about the inner workings of the brain. Thank you for your continued support and giving the guidance to pursue big questions. Thank you Dr. Rob Sutherland for helping me guide the ship of science through the treacherous waters of my masters' project. You have helped me question the status quo, and think critically of what is often accepted as "fact" in textbooks and papers. I would like to wholeheartedly thank Dr. Ian Whishaw for his insights, assistance, and entertaining stories. It is an honor to be able to work with you on a project, and see how you can expertly deconstruct animal behaviour. I hope that we will have many more collaborations in the future. I would like to thank my committee member Dr. Bruce McNaughton for his feedback in meetings and posing important questions. I would like to thank Javad Karimi for all his support and for teaching me so many skills and techniques in recording and analysis. You have been a role model of professionalism and academic rigor. There are many others who have given me a helping hand in the lab including Naomi Cramer, Mojtaba Nazari, Navvab Afrashteh, Dr. JianJun Sun, HaoRan Chang, Surjeet Singh, David Tomas Cuesta, Sean Lacoursiere, Di Shao, Karim Ali, Isabelle Gauthier, Karen Dow-Cazal, Moira Holley, Dr. Michael Kyweriga, Dr. Jogender Mehla, Dr. Edgar Bermudez, Rebeca Leon, Dr. Samsoon Inayat, Dr. Hardeep Ryait, Adam Neumann, Megan Okuma, and Dr. Ingrid De Miranda Esteves to name just a few. To all the friends I made in Lethbridge, thank you for making me laugh and making my time in this town that much more enjoyable. To my fellow graduate students, thank you for the often profound and sometimes silly lunchtime conversations on morality, ethics, the philosophy of science, intelligence, and consciousness. We often went in circles, but I thoroughly enjoyed the conversations. Above all, I

would like to thank my parents for supporting me every step of the way and their limitless

compassion & selflessness.

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List of Abbreviations

AMPA	Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic Acid
BLA	Basolateral Amygdala
CEA	Central Nucleus of the Amygdala
CR	Conditioned Response
CS	Conditioned Stimulus
dHPC	Dorsal Hippocampus
EMG	Electromyography
FC	Fear Conditioning
GABA	Gamma aminobutyric acid
HPC	Hippocampus
iGluSnFR	Intensity-based Glutamate Sensing Fluorescent Reporter
ITC	Intercalated Discs
LA	Lateral Amygdala
LFP	Local Field Potential
mPFC	Medial Prefrontal Cortex
NMDA	N-methyl-D-aspartate
PAG	Periaqueductal Gray
PFC	Prefrontal Cortex
PTSD	Post Traumatic Stress Disorder
ROI	Region of Interest
POR	Pupil Orienting Reflex
RSC	Retrosplenial Cortex
SWR	Sharp Wave Ripple
US	Unconditioned Stimulus
vHPC	Ventral Hippocampus
VBA	Virtual Burrow Assay
WT	Wildtype

Introduction

1. Genetic tools for surveying neural circuits

The development of genetically encoded fluorescent indicators of neural activity, along with optical imaging advances has allowed researchers unprecedented access to specific and large-scale neural circuits (Shen et al., 2020; Stringer et al., 2019; W. Yang & Yuste, 2017) . Recording from such a large number of cells has previously not been possible using traditional electrophysiology measures (Deisseroth & Schnitzer, 2013; Peron et al., 2015). Here, I focus on behavioral characterization under head-fixed fear conditioning (FC) of mouse transgenic lines which express iGluSnFR and GCaMP6. These transgenic animals are widely used and are useful in studying large scale communication patterns in the brain.

2. Pavlovian Fear Conditioning

For animals to adapt their behaviour flexibly to their environments for survival, they need to be able to form and utilize associations (Rudy, 2014). For example, using cues from their surroundings to predict when a threat or predator is likely to be nearby. This adaptive response based on previously sampled information is the key evolutionary purpose for learning and memory systems. Pavlovian fear conditioning (FC) is a powerful tool for studying memory processes, in which an aversive unconditioned stimulus (US) such as a footshock is paired with a previously neutral conditioned stimulus (CS) such as a tone (Maren, 2001) . A single block of a few CS-US pairings is sufficient to create a long-lasting memory in which the CS presented alone will result in the conditioned response (CR) of freezing to the perceived threat. In cued

delay FC, a specific auditory, visual, odor, or other perceptual cue terminates with the US. In cued trace FC, the CS is followed by the US after some time interval (Buccafusco, 2009). Typically, in cued FC, the CR is tested for in a different context from conditioning, and sometimes includes pre-exposure to the conditioning context. This is to ensure that animals are responding to the tone, not the context, and that the context does not predict the US (Weiner, 1990). In contextual FC, the CR is evaluated in the original conditioning context. In the simplest designs, contextual FC includes no other stimuli other than context to predict the US in both conditioning and retention tests. Occasionally both cued and contextual FC is tested in the same animals, in which animals are tested for the CR in response to the conditioning context or the tones in a novel context (Mehla et al., 2018).

Along with the freezing CR, there is an autonomic physiological response that includes increased heart rate, increased blood pressure, increased blood directed to peripheral muscles, and pupil dilation (Ashe et al., 1978; Oleson et al., 1972; Rudy, 2014) . There is a release of stress hormones from the adrenal gland, and release of norepinephrine from the locus coeruleus to many brain areas including the amygdala which promotes plasticity (Giustino & Maren, 2018; Rudy, 2014). Midbrain structures such as the lateral hypothalamus are responsible for the autonomic response, and the periaqueductal gray (PAG) for expression of freezing (Amorapanth et al., 1999; Tovote et al., 2015). The signal for the behavioural response is relayed to the midbrain via the central nucleus of the amygdala (CEA). Information regarding the CS is thought to enter the lateral amygdala (LA) via inputs from the medial geniculate nucleus of the thalamus, and higher order areas such as the hippocampus (HPC), and auditory cortex (Tovote et al., 2015). It is unclear how the "representation" or expectation of the US enters the amygdala, however this may be computed locally in the LA. The LA projects to the CEA both directly and indirectly via

the basal nucleus (BA) and a disinhibitory mechanism involving GABAergic intercalated cells (Rudy, 2014). Introducing NMDA receptor antagonists such as APV in the LA or blocking NMDA receptor mediated upregulation of AMPA receptors in the LA, prevents the acquisition of CS-US associations (Bauer et al., 2002; Huang & Kandel, 1998; Kim et al., 1991; Miserendino et al., 1990; Rumpel et al., 2005). Thus, long term potentiation (LTP) in the LA is an important component of forming CS-US associations.

Since experimenters are able to present the CS at early time points (~ <24h) and remote time points following conditioning, one particularly fruitful application of FC is to study memory consolidation (Axmacher & Rasch, 2017; Sutherland et al., 2010). Following initial cellular consolidation on the order of seconds to hours, there is thought to be some large scale re-organization of the memory which continues over the entire lifetime of the animal. This process is implicated in generalization whereby animals have a CR to similar but distinct stimuli to the CS at remote but not recent time points (Pearce, 1987; Pollack et al., 2018). More generally however, many have suggested that consolidation is a necessary feature of mammalian memory systems to integrate information with past experiences and generate "schematized" representations (McClelland et al., 1995). As an example, it's been found that sensory cortical areas, the mPFC, and the RSC have a greater role in fear memory retrieval at remote rather than recent time points (Arruda-Carvalho & Clem, 2015; Courtin et al., 2013; Peters et al., 2009; Sacco & Sacchetti, 2010; Sotres-Bayon & Quirk, 2010). Even within brain areas such as the prelimbic cortex, different ensembles of neurons are responsible for fear memory retrieval at remote vs recent fear memories (DeNardo et al., 2019). One area of interest for many researchers is hippocampal-cortical communication which is thought to underlie much of the consolidation

process (Axmacher & Rasch, 2017). The hippocampus is involved in both context FC and cued FC (Quinn et al., 2008; Sutherland et al., 2008).

In the standard model of memory consolidation, episodic and semantic memories are thought to become hippocampal independent over time (Axmacher & Rasch, 2017) . This has been refuted by a number of studies that show retrograde amnesia (RA) for the CS, at both recent and remote time points with complete hippocampal lesions after conditioning (Sutherland et al., 2010). In contrast, in multiple trace theory, over time the hippocampal-cortical connections corresponding to the original engram expand with repeated retrieval or reactivations. Predictions of this theory include a flat retrograde amnesia gradient with complete hippocampal lesions, but also a temporally graded retrograde amnesia with partial lesions (where remote memories are less affected). In fact, the opposite finding has been found, where remote memories are disrupted by lesions more than recent memories (Sutherland et al., 2020). The hippocampus' role in representing and integrating new and old information is an area of active debate that will require further experiments. More generally a fine-grained understanding of memory consolidation will require large scale recordings of cortical and subcortical areas.

The fear memory association in rodents may also be used as a model to study disorders related to trauma, stress and anxiety (Maren, 2001).This includes but is not limited to post-traumatic stress disorder (PTSD), panic attacks, and phobias which are debilitating to those who experience them (Briscione et al., 2014; Johnson, 2016; Johnson et al., 2012). Generalization, extinction learning, and reconsolidation of the CS continues to be thoroughly explored by researchers to help develop therapies.

As previously mentioned, generalization occurs when subjects have a CR to stimuli which were never originally paired with the US (Jasnow et al., 2017). The analogue of this in humans

perhaps may be found in PTSD where "triggers" can illicit anxiety and emotions associated with a stressful event. Extinction is the process by which repeated exposures of the CS not reinforced with the US will cause attenuation or cessation of the CR (Milad & Quirk, 2012). In most cases, extinction is thought to not constitute "erasure" of the original association, but rather new learning that has distinct neural pathway in the amygdala (Rudy, 2014). The analogue of this phenomenon in humans may be considered exposure therapy whereby subjects learn to overcome their phobias with controlled presentations of stimuli (Cain et al., 2003; Milad & Quirk, 2012; Paredes & Morilak, 2019). In reconsolidation, presentation of the CS is thought to reactivate the original CS-US memory, rendering it labile and more open to manipulation (Alberini, 2005; McKenzie & Eichenbaum, 2011; Nader, 2015; Nader et al., 2000). In one classic study, infusing protein inhibitors into the basolateral amygdala (BLA) immediately after presentation of the CS, attenuated the CR on subsequent retrieval tests (Nader et al., 2000). These processes have been translated into human studies to develop behavioral therapies (Kroes et al., 2016). In one study, giving human participants propranolol (an anti-anxiety medication) after presenting the CS (when the memory is thought to have become labile), reduced the CR in a follow up retrieval test (Kindt et al., 2009). Further research in this area will likely help develop interventions that usefully integrate both drugs and therapy.

FC in rodents has helped shed light on the fundamental neural circuitry that underlies fear responses, learning and memory consolidation, and emotional memories. These experiments are greatly aided by advanced modern recording techniques and transgenic fluorescent markers.

3. Fear Conditioning Under Head Fixation

The inherent difficulty in carrying out FC experiments under head fixation is the fact that we are not able to directly measure the classical definition of freezing which is the complete cessation of

movement except for breathing (Maren, 2001). Some have worked around this by first having animals undergo a retention test in a free roam setting to confirm the presence of the CR, before they are head-fixed and presented with the CS under a brain imaging apparatus to identify neural correlates (Ross & Fletcher, 2018; Wood et al., 2020). The added CS presentations in this paradigm present the risk that the CR is more likely to undergo extinction. If there is a distinct activation pattern that would accompany only the first or first couple CS presentations, then these would be missed. If not enough time is passed between the freely behaving recall test and the CS presentation under head-fixation, than you may put the animal into a different arousal state which would confound results (McGinley et al., 2015; Reimer et al., 2016). Lastly, recording the CR under freely moving behavior does not necessarily mean that the animal is undergoing a similar sort of memory retrieval under head-fixation unless it is directly measured. Particularly if there is a strong contextual component to the fear memory not present during head-fixation, animals may freeze in the freely roaming context and not during head-fixation.

To measure the CR under head-fixation, previous FC studies have used lick suppression as an index of a CR (Ahmed et al., 2020; Kaifosh et al., 2013; Lovett-Barron et al., 2014; Rajasethupathy et al., 2015). In this paradigm, mice are deprived of water and trained to lick a spout to get water. A decrease in the number of licks or licking rate is used as the measure of CR instead of freezing. One issue with this approach is that it is measuring the cessation of a behavior that was never originally present during conditioning. It is not a direct measure of the animals' fear response but rather an indirect one based on trained behavior. In addition, the water restriction and associated stress are likely to have a significant effect on the brain state and the animal's level of arousal. As animals consume water and become satiated to differing levels over

time, the added confound of motivation must be considered in lick suppression and differences we observe in the brain signal. More generally whenever animals must be trained for a task, different levels of proficiency between animals can add variability to the underlying neural dynamics.

The best measure of the CR therefore is one which most closely resembles the animals natural fear response to the US, devoid of any tasks or external rewards, punishments, or extra manipulations. One such recent attempt is the Virtual Burrow Assay or VBA (Fink et al., 2019). In the VBA, the CR is measured as the ingress of head-fixed mice into a tube which they can move with their feet. Even in this measure however, the CR is a closer measure to avoidance or escape behavior rather than freezing and is likely recruiting alternative neural circuits. With repeated presentations and diminished responses, experimenters cannot dissociate learned helplessness (from repeatedly not being able to escape) with extinction.

 The closest natural measure of freezing under head-fixation has come from Gehrlach and colleagues who define freezing as periods of pupil dilation, concurrent with a relative lack of orofacial movements measured through video (Gehrlach et al., 2019). In this measure of freezing, animals showed more freezing to the CS after conditioning as opposed to pre-conditioning CS presentations. The main limitation in their study is the lack of a proper separate control group that does not undergo conditioning. We cannot preclude the possibility that the mere presentation of footshocks independent of any association is responsible for the differences they observed (Rescorla, 1967).

Here we demonstrate using yoked control animals, that it is possible to measure the freezing CR in a delay FC paradigm under head-fixation, using simple video recordings. Consistent with free roaming conditions, experimental animals have less movement. This finding is mirrored in

electromyography recordings in neck muscles which indicate an inhibition of movement in response to the CS, which has also previously been found in freely behaving rodents (Steenland & Zhuo, 2009). Consistent with higher levels of fear and arousal, experimental animals have a stronger pupil dilation response and a more static gaze. Lastly, we show that hippocampal local field potential (LFP) recordings exhibit a distinct signature in response to the CS, when comparing the two groups.

Material and methods

1. Animals

A total of 28 transgenic mice were used in the study (15 experimental, 13 control). 12 of these animals expressed GCaMP6s; an intracellular calcium-sensing fluorescent reporter protein (6 experimental, 6 control). 16 transgenic mice expressed iGluSnFr; a glutamate-sensing fluorescent reporter protein (9 experimental, 7 control). Among these animals, 10 animals (5 experimental, 5 control) were an Emx-CaMKII-Ai85 strain and expressed iGluSnFR in excitatory neurons in all layers of the neocortex. 4 animals (2 experimental, 2 control) were a Ras-CaMKII-Ai85 strain expressing iGluSnFR in excitatory neurons in layers 2/3 of the neocortex. 26 of the 28 animals underwent a yoked control design in which every experimental animal had a control-matched pair. Each pair underwent the exact same procedures on the same days in short succession of one another. 2 additional animals - one Ras-CaMKII-Ai85, and one C57 wildtype (WT) mouse with iGluSnFR expression induced via adenoviral injection, underwent the experimental conditioning protocol without a control match. Similar to the Emx-CaMKII-Ai85 strain, adenoviral injection produced iGluSnFR expression in all layers of the neocortex.

2. Surgical Preparation

All animals were injected with 0.5 gr/Kg buprenorphine, and were anesthetized half an hour later with 1-2% isoflurane. A flap of skin from the head was removed, and animals were implanted with a bipolar LFP electrode targeting pyramidal layer of the CA1 subfield of the dorsal hippocampus. Another pair of electrodes was implanted into neck muscles to measure EMG activity. One WT C57 mouse was injected with a viral vector (50 ul of AAV2 CAG-

SF.iGluSnFR-S72A) into its tail vein to induce expression of iGluSnFr (Marvin et al., 2018). The 16 iGluSnFr animals kept an intact skull, whereas the 12 GCaMP6 animals had 5mm of the skull removed over the cortex and covered with a glass window in preparation for another set of experiments. A thin and transparent layer of metabond (Parkell, Inc) was applied to exposed skull, and a metal head plate was attached to the skull to keep animals head-fixed during recordings. Animals were given 1- 2 weeks to recover from surgery before beginning any experiments.

3. Habituation

After recovering from surgery, all animals were habituated to experimenters, the recording setup and head-fixation for ~2 weeks (figure 1a). Animals were gradually exposed to experimenter handling and head-fixation over longer intervals of time until they were judged to be relatively comfortable during hour long recording sessions. Experimenters judged comfort and stress as the animals' lack of struggle accompanied by moderate levels of grooming which is associated with periods of calmness (Fernández-Teruel & Estanislau, 2016; Nazareth Veloso et al., 2016). During the entire habituation period, animals were handled for a minimum of 2 minutes per day to get acclimatized to getting picked up and transferred by experimenters. On the first habituation session, animals were allowed to freely explore the recording rig environment for 15 minutes, without being head-fixed. On the second day of habituation, animals were head-fixed for only 5 minutes. On each subsequent session, the head-fixation period increased by 5 minutes until 20 minutes of head-fixation was reached. At this point, each subsequent head-fixation period was extended by 10 minutes until 60 minutes of head-fixation was reached. The length of subsequent sessions was not extended if animals were significantly distressed (as judged by vocalizations, and excessive movement). During the first 3-4 days of habituation, bedding

material from the home cage was placed in and around the head-fixation setting with the intent of introducing an odour that the animal is familiar and comfortable with (Duke et al., 2001; Schondelmeyer et al., 2006). Once animals reached 60 minutes of head-fixation comfortably, they were head-fixed for at least two more 60-minute sessions. To reduce anxiety as much as possible, mice were always handled by either one of two experimenters. Mice were transferred by allowing them to first enter an acrylic tube that is native to their homecage, avoiding picking them up directly (Buccafusco, 2009; Hurst & West, 2010). Mice were never picked up by their tail. The transfer cage was filled with paper towels, and included their homecage acrylic tube to give mice dark enclosed spots to hid in and avoid bright open spaces which can induce stress (Buccafusco, 2009).

To keep animals as comfortable as possible, all head-fixation occurred in a low-noise warm environment slightly above room temperature (20 to 25 degrees Celsius). Paper towel padding was taped to the floor, and was heated with a heating pad before animals were head-fixed. In several cases, animals were calm enough to the point of falling asleep under head-fixation.
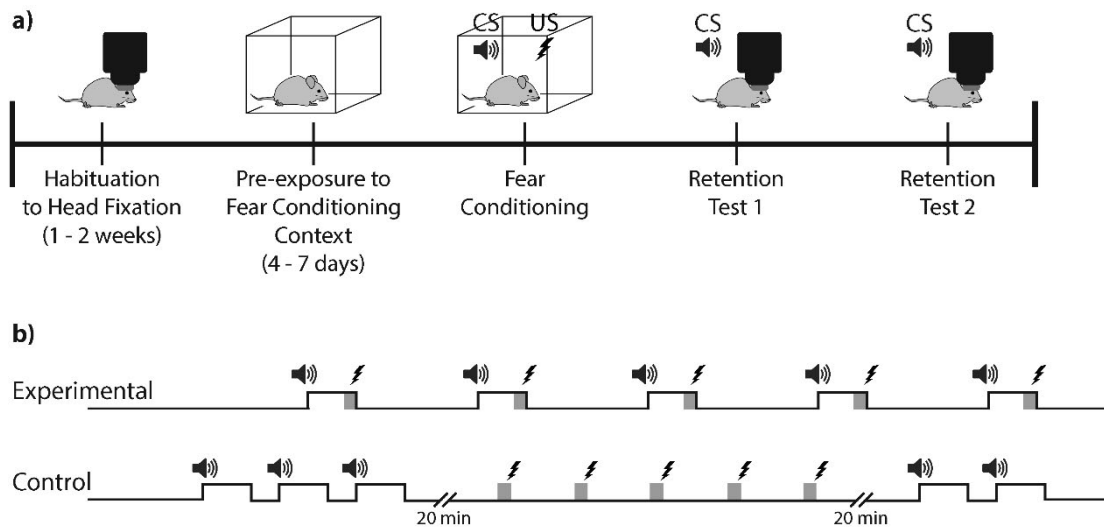
Figure 1: Experimental design.
 a) Experiment timeline: After recovery from surgery, all animals were habituated to experimenters, the recording setup and head-fixation for 1 – 2 weeks. For 4 – 7 days prior to fear conditioning, animals were pre-exposed to context for 20 minutes per day. On the fear conditioning day, both experimental and control animals were exposed to 5 tones and 5 shocks. A day after, both experimental and control animals undergo a retention test, where they are exposed to either 3 or 2 tones. Four or seven days later, after the first retention test, all animals undergo an identical second retention test, and are exposed to the same number of tones they were exposed to in the first retention test. **b) Tone Shock Pairing.** All animals were acclimated to the box for 2 minutes before being presented tones or shocks. Experimental animals (n = 15) were conditioned with five 20 second presentations of 2700 Hz 85 dB tones coterminating with 2-second long 0.5 mA footshocks. Each CS-US presentation was separated by an intertrial interval of 2 minutes (ITI = 2min). Control animals (n=12) were exposed to the same intensity tones and shocks, separated in time by two 20 minute wait periods to avoid the possibility of a CS-US association being made. All animals were left in the context for an additional two minutes before being returned to homecages.

4. Delay Fear Conditioning

During the habituation period and 4 to 7 consecutive days leading up to the day of FC, animals were pre-exposed to the conditioning box for 20 minutes per day (figure 1a). This was to attenuate the effect of forming a contextual fear memory on the day of conditioning. The

conditioning context is a 33x33x25cm box with black and white acrylic walls, and transparent plastic ceiling. The floor in which the shocks would later be delivered during conditioning were 64 stainless steel rods at 2mm diameter, spaced 5 mm apart connected to a shock generator. Before and after placing animals in the conditioning box during both pre-exposure and conditioning sessions, the walls and floor of the conditioning box was thoroughly cleaned with 1% Virkon and 70% ethanol, and allowed to dry.

Delay conditioning consisted of five 20 second-long presentations of a 2700 Hz 85 dB tones co terminating with 2 second-long 0.5 mA foot shocks (figure 1b). Each CS-US presentation was separated by an intertrial interval (ITI) of 2 minutes. All animals could explore the conditioning context for 2 minutes prior to the first presentation of the tone and after the last presentation of the tone, before being returned to their home cages. Control animals were presented with an equal number of explicitly unpaired shocks and tones in a manner such that tones did not predict shock. In the control condition, the session consisted of a block of 3 tones followed by a block of 5 shocks, ending with a block of 2 tones where each block was separated by 20 minutes. In the control condition, tones were presented with an ITI of 2 minutes and shocks were presented with an ITI of 2 minutes and 18 seconds to mimic the time difference in the experimental condition. All animals were presented the CS 24 hours after conditioning under head-fixation in the first retention test. GCaMP6 animals were presented 2 tones (n = 12, 6 experimental, 6 control), and iGluSnFr animals were presented 3 tones (n = 16, 9 experimental, 7 control). During retention tests, animals were head restrained for 20 to 70 minutes in a quiet environment and presented with the CS during periods of calmness, as judged in real-time video by experimenters. The first CS was presented after at least 5 minutes of recordings. CS presentations were separated by a minimum of 2 minutes, and recordings continued for a minimum of 5 minutes after the final CS

presentation. A second retention test with the same number of CS presentations was held 7 days after the first retention test for iGluSnFr animals and 4 days after for GCaMP6 animals.

Fear tones were delivered through miniaturized speakers mounted near the top of the conditioning box during conditioning. The same speakers were mounted roughly 8 to 15 cm in front, and to the right of the mouse's snout during retention tests. Audio files were generated via a matlab script and calibrated to the recording & conditioning setups using a decibel meter to ensure that the sound being delivered was at the same intensity in all environments. To synchronize with LFP recordings and record the exact moment current was delivered to speakers to generate sound, a custom-built speaker system was used. The audio jack used to deliver the signal was cut, and plastic insulation removed from the tip such that one set of wires would be connected to the speakers and another set of wires connected to a digital acquisition system. From the waveform signal, tone onset and offset were detected using an algorithm that utilizes the findpeaks function in matlab to find where the crest exceeded 12 standard deviations above the mean signal (figure 2). All waveform signals were visually checked to ensure the accuracy of detection.
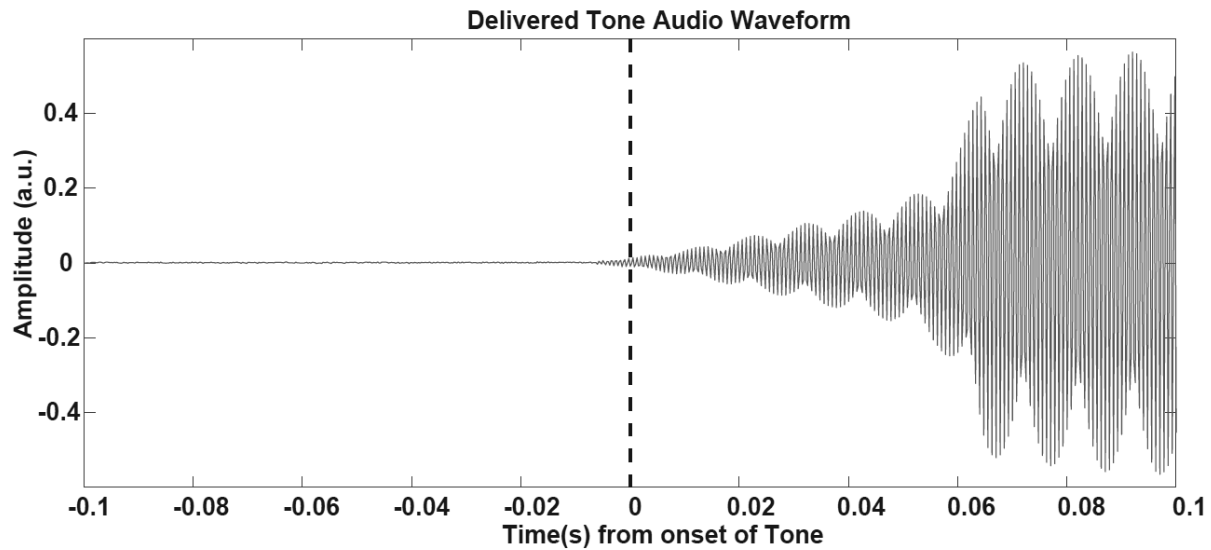
Figure 2: Audio waveform and detection of tone onset
Example of an audio waveform captured by digital acquisition system around the onset of a tone presentation. Vertical dashed line is the detected time of tone onset.

5. Electrophysiological and Video Recordings:

During retention tests, motion and pupillary changes were tracked with a raspberry pi "pycam" camera video at 25 frames per second (figure 3). Simultaneously, LFP in CA1 of the dorsal hippocampus and neck EMG was amplified, filtered (0.1-1000 Hz), and sampled at 2000 Hz. In order to align behavioral video recordings with the onset of the CS as accurately as possible, a python script running on the raspberry pi captured time stamps of all video frames in a table. The recorded audio waveform (figure 2) which captured the amount of time elapsed since the beginning of the recording to the onset of the tone was used to find the closest behavioural video frame listed in the table. After careful inspection of time stamps, we found that frames were consistently not being recorded at fixed time intervals likely due to limitations in the internal memory buffer of the hardware. These "missed frames" were interleaved back into the time

15

stamps to get the precise video frame of tone onset. With this correction, there was little to no

delay between the recorded onset of the tone and the animals reaction and was therefore used for
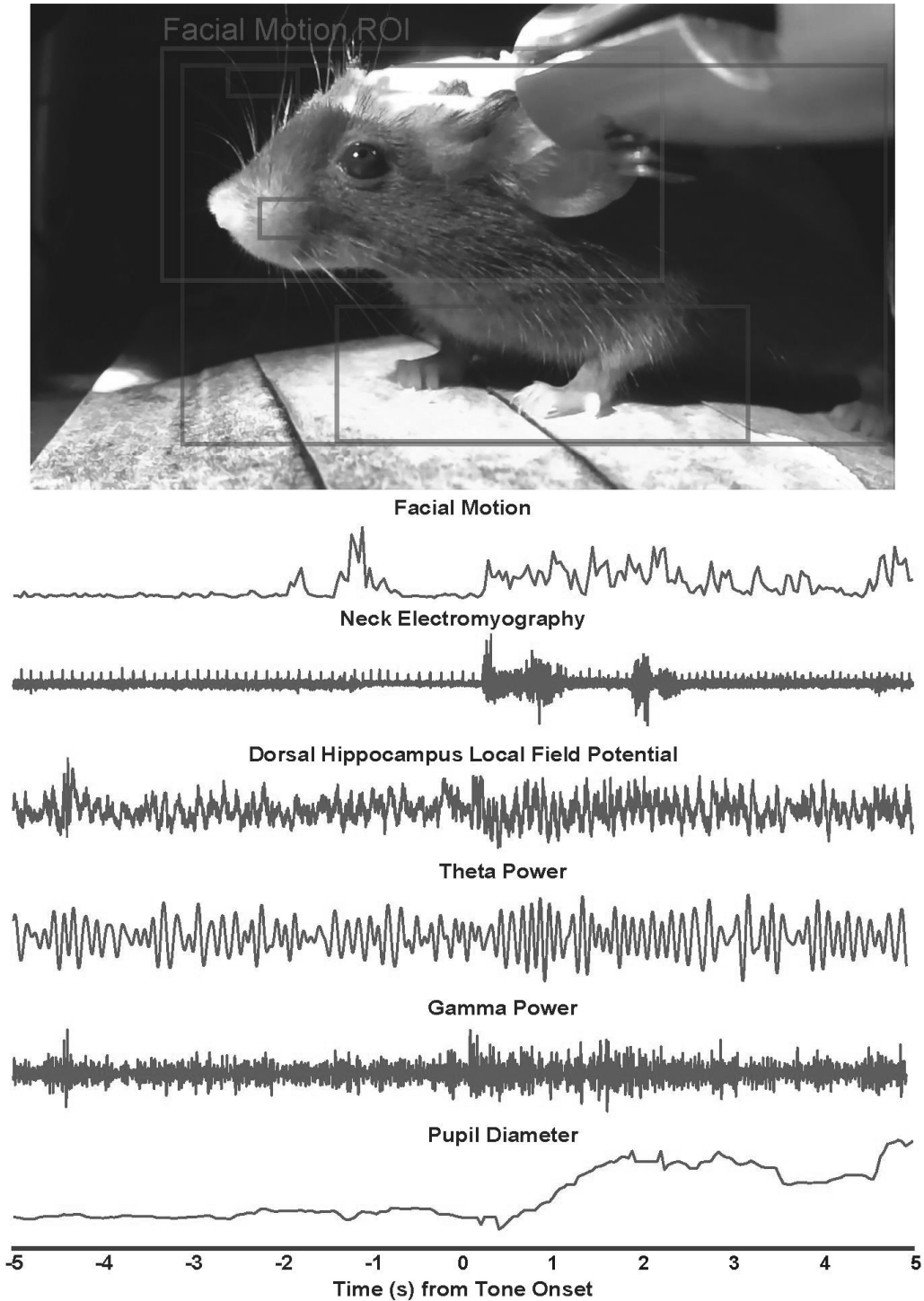
all analyses.

Figure 3: Video image and raw signal traces.
Red outline in image indicates typical position of ROI to track facial movements, as plotted below. Purple outlines indicate positions of other ROIs (whole body, forepaws, whisker pad, whiskers). Videography recorded motion of different body parts, and was used to measure pupil

diameter and gaze location. Electrodes placed in the neck muscle tracked contraction. Bipolar electrodes in CA1 of the dorsal hippocampus tracked changes in LFP. From the LFP, different frequencies could be extracted such as theta (4-12 Hz) and gamma (20 – 110 Hz).

Motion and pupil changes were extracted using FaceMap as previously described (Stringer et al., 2019). Briefly, the absolute motion energy at each timepoint was calculated by subtracting consecutive frames in the specified region of interest and taking the absolute value of the difference. Motion signals were computed for the entire face, the whisker pad, the whiskers hovering above the snout, the forepaws, as well as the entire mouse's body in the camera's field of view (figure 3). To calculate pupil area, an ellipse was fitted to the outline of the pupil in relation to the estimated pupil center (figure 4b). Videos captured of GcaMP6 animals had to be color inverted first using a custom python script to make pupil pixels dark enough to be detected by the FaceMap algorithm (figure 4a). In most recordings, there was a significant glare on the mouse's eye that would obstruct part of the pupil and could affect the accuracy of pupil detection. These were corrected via interpolation to predict pixel values, had the glare not been present.
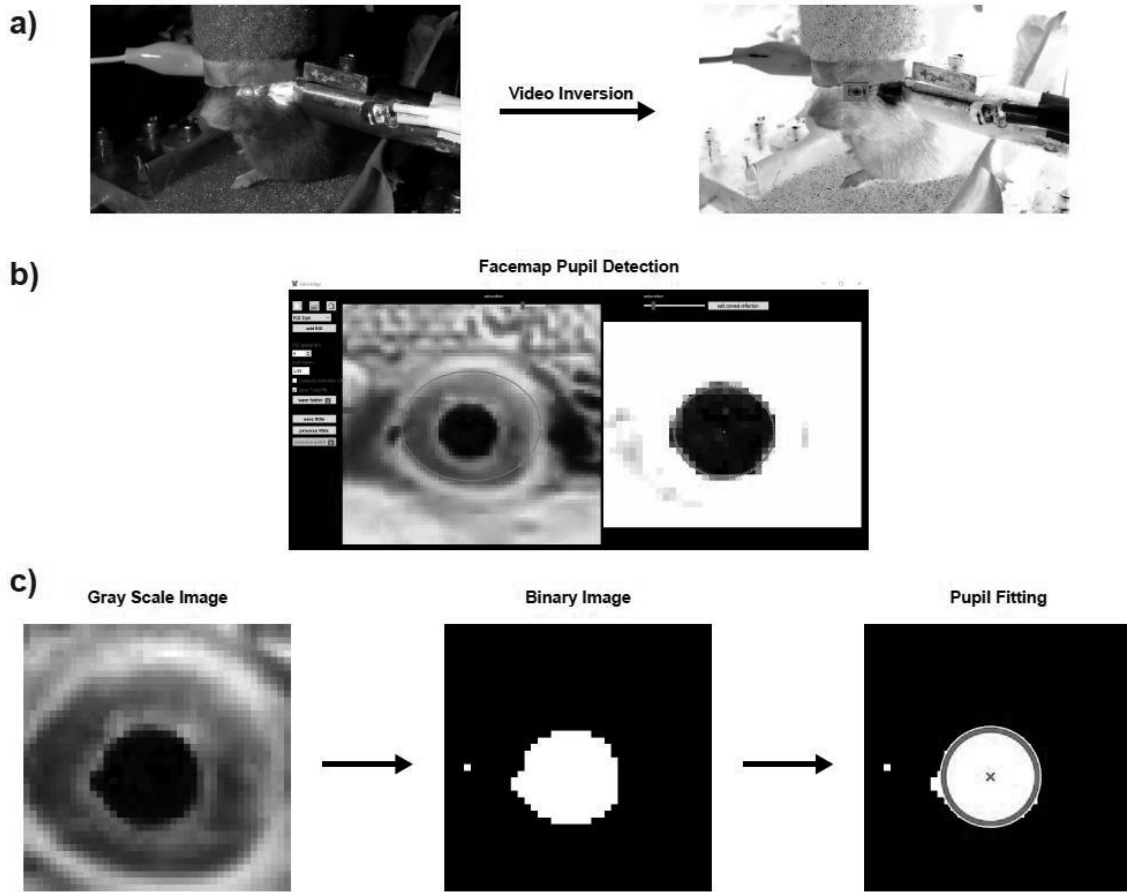
Figure 4: Pupil measurement pipeline.
Videos of GcaMP6 animals were inverted in order to have the pupil dark enough for detection. Videos collected of iGluSnFR animals were not inverted, since pupils were already dark. **b) Facemap Pupil Detection.** Using the Facemap GUI, the pupil is detected by defining a region of interest, and setting a threshold for the darkest pixels which correspond to the pupil. From these values, the center is estimated and an iterative calculation fits an ellipse to the outline of the pupil. This calculation is based on a multi-variate gaussian maximum likelihood method **c) Custom Matlab Pupil Detection.** If pupil detection was not accurately detected in Facemap, a simpler algorithm implemented in matlab was used. Grayscale images of the eye were thresholded to include only the pupil. Pupil center and diameter was then calculated by the imfindcircles function in matlab.

All video recordings were visually examined and in recordings where the FaceMap pupil

detection algorithm was failing to fit an accurate ellipse, a custom matlab script was used (figure

4c). In these instances, video recordings were cropped around the eye, converted into grayscale,

thresholded to only include pixels dark enough to be the pupil, and fit with a circle using a

circular hough transform based method (available as the imfindcircles function in matlab). If

multiple pupils were detected in a frame, then a sensitivity parameter in the function was

iteratively reduced by 10% until only one was detected. Pupil area was calculated in this way for

3 GCaMP6 animals (experimental animals) in the first retention test, and 5 GCaMP6 animals (3

experimental, 2 control) in the second retention test.

Five animals in the first retention test (2 experimental, 3 control), and three animals in the second

retention test (2 experimental, 1 control) were excluded from pupil analyses due to pupil

obstructions (residue on eye, extensive video glare, poor video quality etc).

6. Analyses and Statistics

All motion and pupil signals were normalized by the interquartile range (IQR) (the difference

between the 25th and 75th percentile) of the entire recording session under head-fixation. For

each tone presentation, the signal was further normalized by the median of the signal 3 seconds

prior to the onset of the tone. The IQR of motion during the presentation of the tone was used as

the measurement of the range of body motion and eye movement. The median pupillary area

during the presentation of the tones was used as the dilation response to each tone.

For all comparisons, differences were considered significant if the null hypothesis was rejected at

P-value < 0.05 using a Wilcoxon rank-sum test. Animals were a priori predicted to move less,

make less eye movements, and dilate pupils more in the experimental group (in line with

descriptions of freezing and heightened arousal), thus one tailed tests were used for statistical

hypothesis testing on motion-related and pupillary responses. On the other hand, a two-tailed

version of the Wilcoxon rank-sum test was used for comparison of the power of different frequency bands present in the hippocampal LFP.

Results

1. CS Startle Reactions

Videos of all mice were systematically reviewed to identify trends associated with the reaction to the tone. Figure 5 illustrates changes in facial expression in response to the first presentation of the CS in three experimental animals. Whiskers erect forward, and begin moving at a different frequency. The nose and ears change position, and eyelids open. The eyes saccade either forward or backward and the pupil dilates. Similar reactions displaying one or more of these characteristics were typical across all experimental and control animals. After the onset of the CS, animals had varying levels of movement from slight adjustments in forepaw position to large erratic movements. To quantify these measurements, timeseries of pupil diameter, gaze, and bodily motion were extracted from videos.
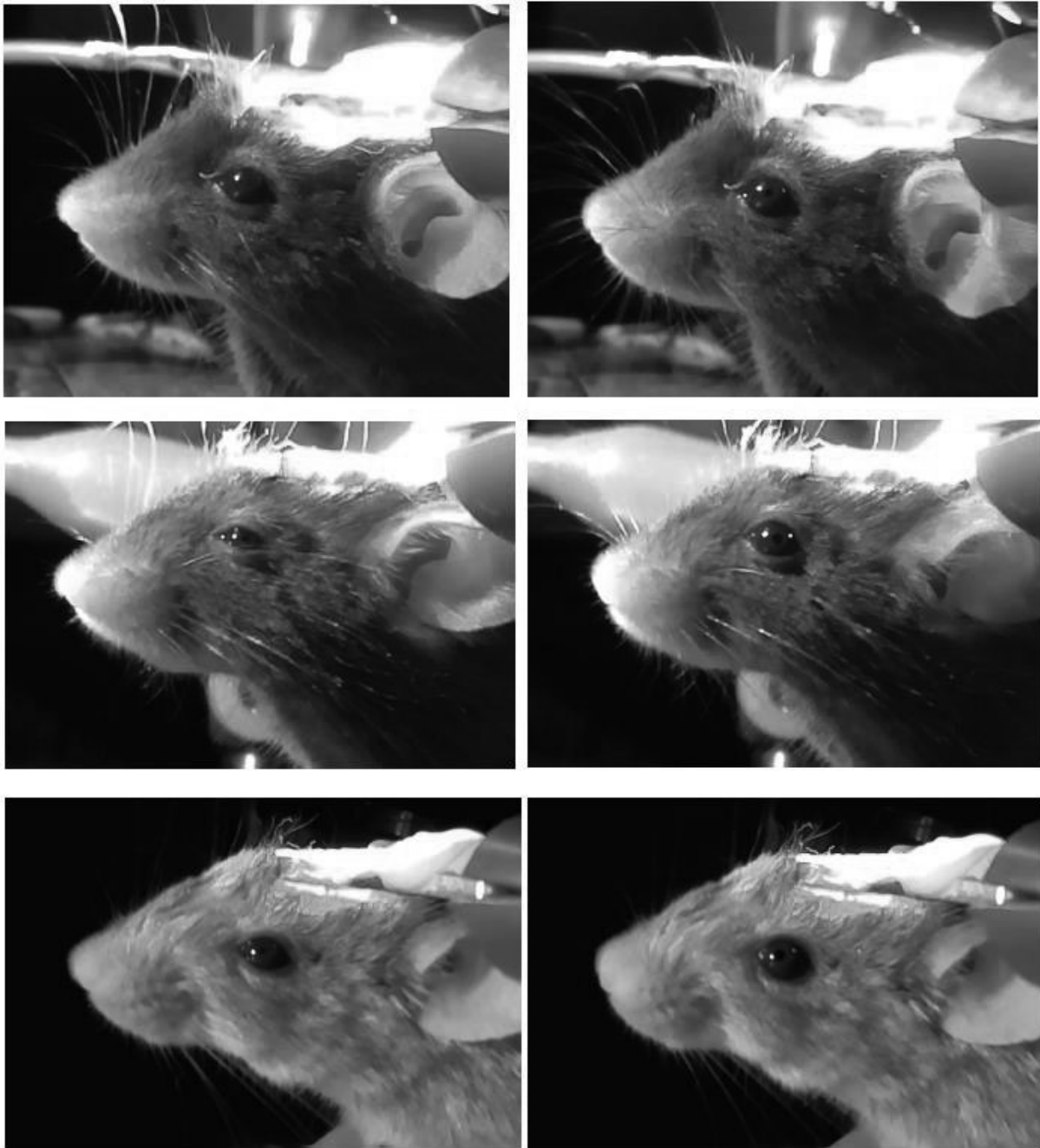
1 Second Before CS Onset   **1 Second After CS Onset**

Figure 5: Facial characteristics upon fear tone presentation.
Three conditioned animals responses shown in each row. Left column is the 1 second before CS onset, and right column is 1 second after CS onset. Pupils dilate and move, eyelids expand, whiskers and snout change position, and face twitches.

2. Video Motion

Animals had a lot of variability in the range of movement, "twitchiness", pupil fluctuations or irritability outside the presentation of any CS, and this could obfuscate any differences observed in the response to tones. For example, an animal that regularly has large movements may appear to show a large reaction to the CS, regardless of any association. Therefore, signals were divided by the IQR of the entire recording for normalization. Within these timeseries, we focused on individual epochs that immediately preceded and proceeded the CS since these are when changes in behavior are likely to be the most pronounced. For each CS presentation, the median of the three seconds immediately before the presentation of the CS served as the baseline and all activity was subtracted from that baseline. During this baseline, there is a relative lack of movement and animals are calm because that was the a priori criteria used to determine whether experimenters presented the CS. All animals are roughly in the same arousal state, and all movements were largely initiated after the onset of the CS (there was no large sequence of movements that started before the CS and continued into the onset of the CS). Because of this, the CS was likely intrinsically startling for most animals. Figure 6 is a comparison between whisker pad movements in an experimental and control animal on the first retention test, 24 hours after conditioning. For visualization purposes, the signal has been smoothened with a 400 ms sliding averaging window.

a)

Whisker Pad Motion in Response to Tone 1

b)

Whisker Pad Motion in Response to Tone 2

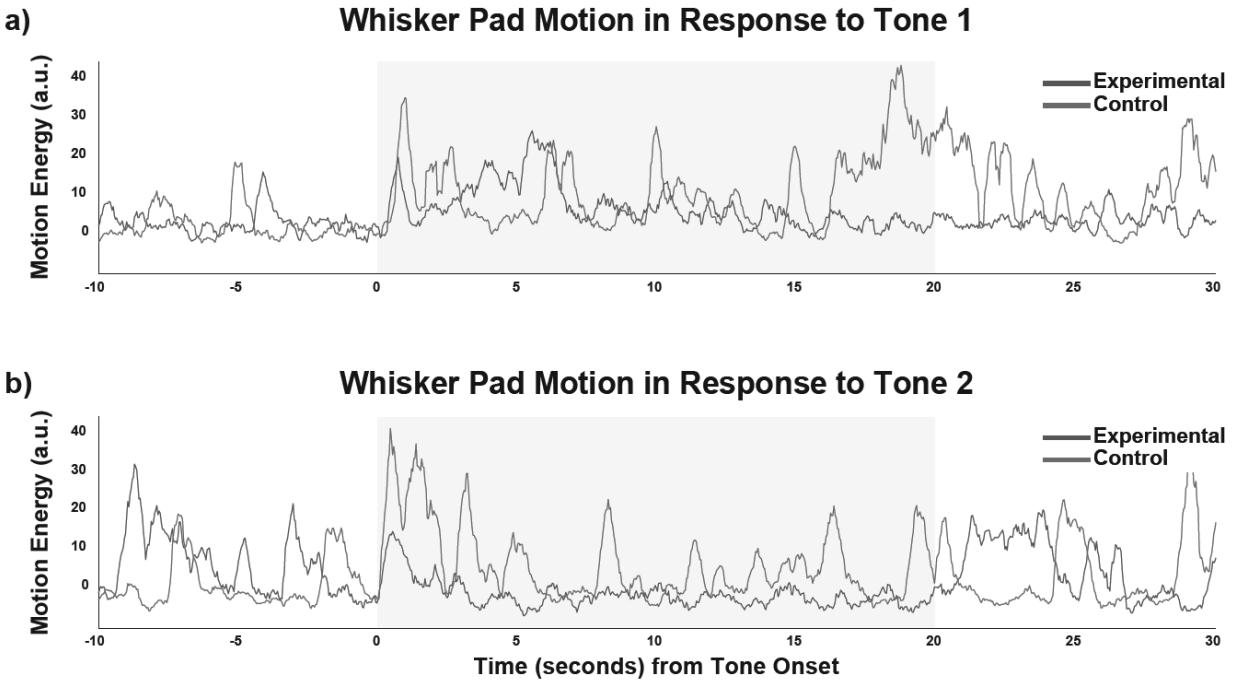**Time (seconds) from Tone Onset**

Figure 6: Example of experimental and control whiskerpad movement in first two cs presentations in first retention test.
Whiskerpad motion energy of conditioned animal is in blue, and pseudo-conditioned animal in red. Light red highlighting indicates the presentation and duration of CS. Signal was smoothened with a 400 ms sliding averaging window. **a) Whiskerpad motion during first CS presentation. b) Whiskerpad motion during second CS presentation.**

In this example, both animals show an immediate reaction to the tone, and whisker pad movements are roughly similar in amplitude. After this initial response however, the control animal has many more bouts of movement during the entire 20 second presentation of the CS. Consistently we found the greatest differences between control and experimental animals was observed to be during the actual presentation of the tone.

 Since the amplitude of these motion signals is not as informative as the fluctuations within them, a measure of variability was used as each animals' response to the CS. Motion signals follow a skewed distribution (figure 7) and have very large outliers, so IQR is a more accurate measure of the variance than standard deviation. IQR captures the sustained movements during the entire presentation of the tone as opposed to the average which may be affected by a very large initial

startle to the CS onset. In addition to the whisker pad and whiskers, we were interested in the

motion response of the snout, and forepaws as well as motion that encompassed the entire face

and entire body (figure 3). We hypothesized that experimental animals would show less motion

due to an inhibition of movement, consistent with freezing. Moreover, since freezing likely

involves the coordinated action of many body parts, we expected to see this in all ROI
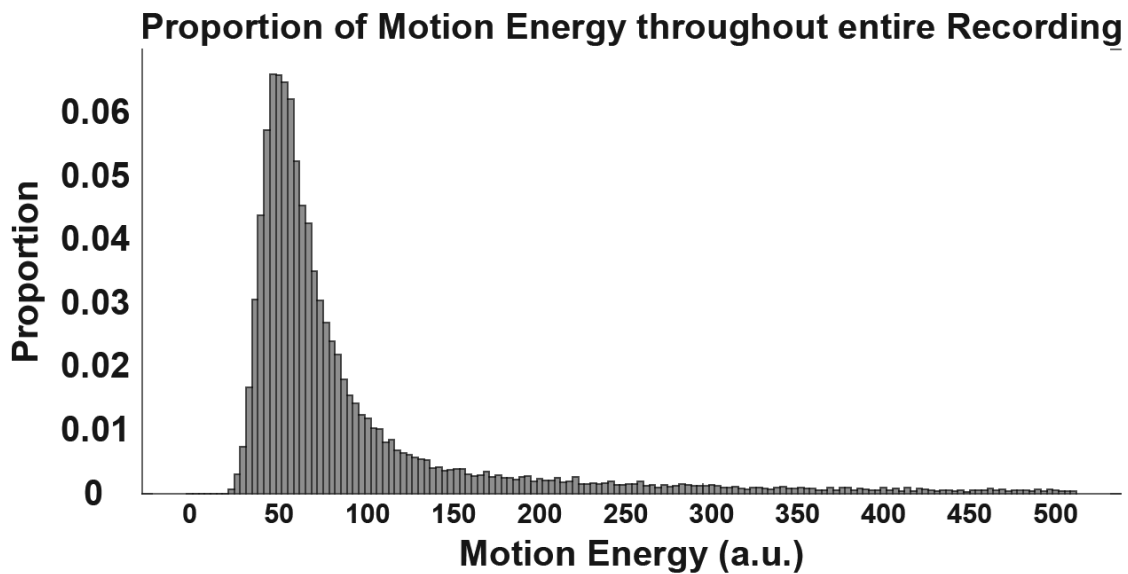
measurements.

Figure 7: Motion energy recordings follows a skewed distribution.
The frequency of different motion energy values throughout an entire video recording. Motion
energy values above the 95th percentile are not included for visualization purposes.

Apart from whisker specific movements in the first retention test, control animals had a higher

degree of motion than experimental animals in both retention tests (figure 8) ($P < 0.05$, ranksum
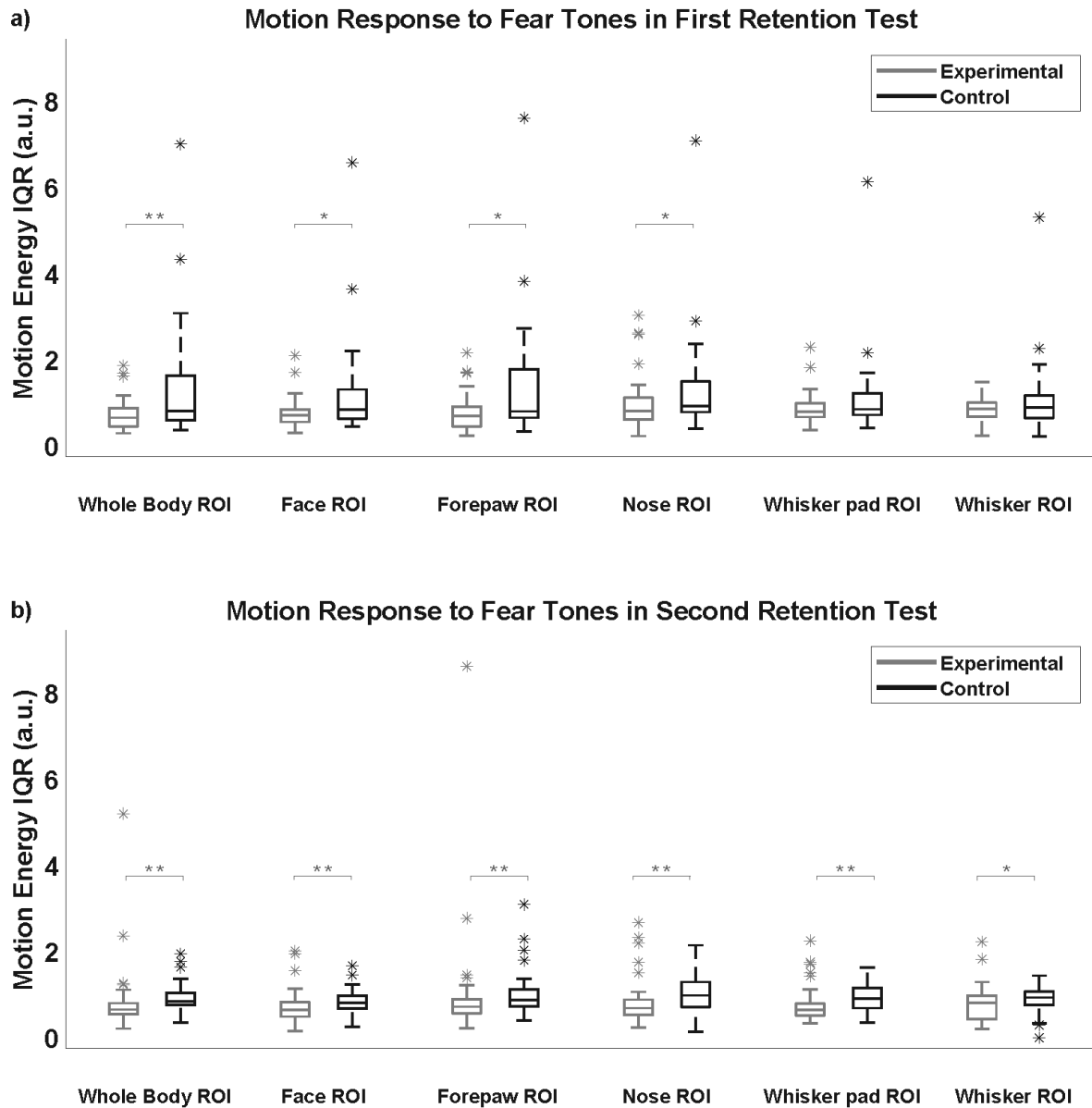
test).

Figure 8: Control move more in response to fear tones
Boxplot of IQR of various motion signals during presentations of fear tones. Red asterisk indicates statistical significance in one-tailed Wilcoxen rank-sum test (p < 0.05). Double asterisk indicates high statistical significance (p < 0.01) . **a) Retention test 1.** All measurements with the exception of whiskers show greater motion in control animals. **b) Retention test 2.** Control animals show more motion in every body movement measurement.

3. Electromyography

Given that there was greater motion in control animals, we then looked to see if this could be replicated in neck EMG recordings. EMG activity was normalized and processed in the same way as the video motion recordings – normalized by the IQR of the entire recording, baseline subtracted and expressed as the IQR during tone presentation. Consistent with video motion results, control animals had a greater amount of muscle tone than experimental animals (figure 9).
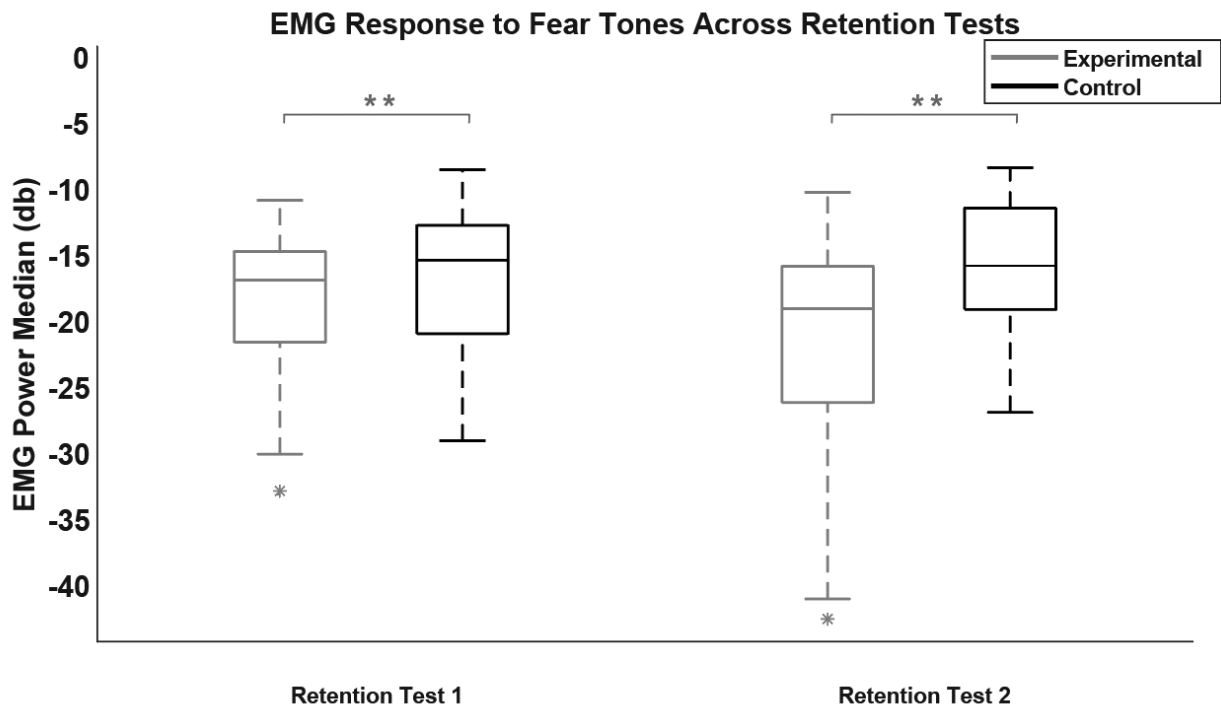

Figure 9: Control show greater muscle tone in response to fear tones.
Boxplot of median muscle tone in response to fear tones. Red asterisk indicates statistical significance in one-tailed Wilcoxen rank-sum test (p < 0.05). Double asterisk indicates high statistical significance (p < 0.01) .

4. Eye Movements

In video recordings, many control and experimental animals appeared to shift their gaze during the presentation of the tone (figure 5). After this initial reaction, we expected experimental animals to have a frozen gaze as part of the fear response, and control animals to continually move their eyes. Figure 10 shows the eye position of an example experimental and control animal during all presentations of the tone within the first retention test. To visualize the eye position, the centre of the pupil from the video is plotted on a 2D cartesian plane. This representation is not entirely accurate as it is a 2D projection from the 3D spherical eye, and we cannot be sure what the animal is looking at. Nonetheless it provides a rough approximation. Whereas the experimental animal appears to have three large clusters where gaze is focused, the control animal's gaze appears to be much more widely dispersed.
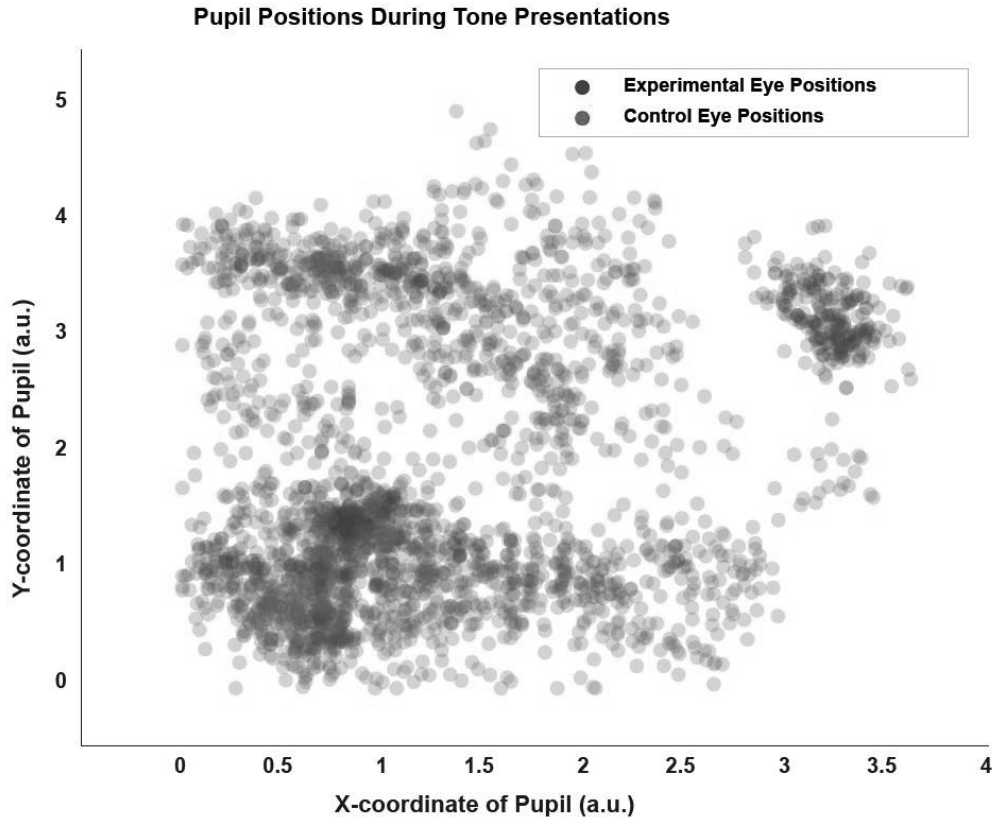

Pupil Positions During Tone Presentations

Figure 10: Pupil coordinate positions of an example experimental and control animal during all three presentations of the CS
Blue coordinates are pupil positions from a conditioned animal, and red coordinates are pupil positions from a pseudo-conditioned control animal. Eye positions for the conditioned animal appears to be clustered at three points, and more spatially distributed in the pseudo-conditioned animal.

By calculating the distance between eye positions in consecutive frames of the video we were able to extract pupil movements during the CS. Eye movements were normalized as mentioned previously, and the IQR taken as the CS response. Control animals had a wider range of eye movements than experimental animals, in the first retention test but not the second (figure 11).
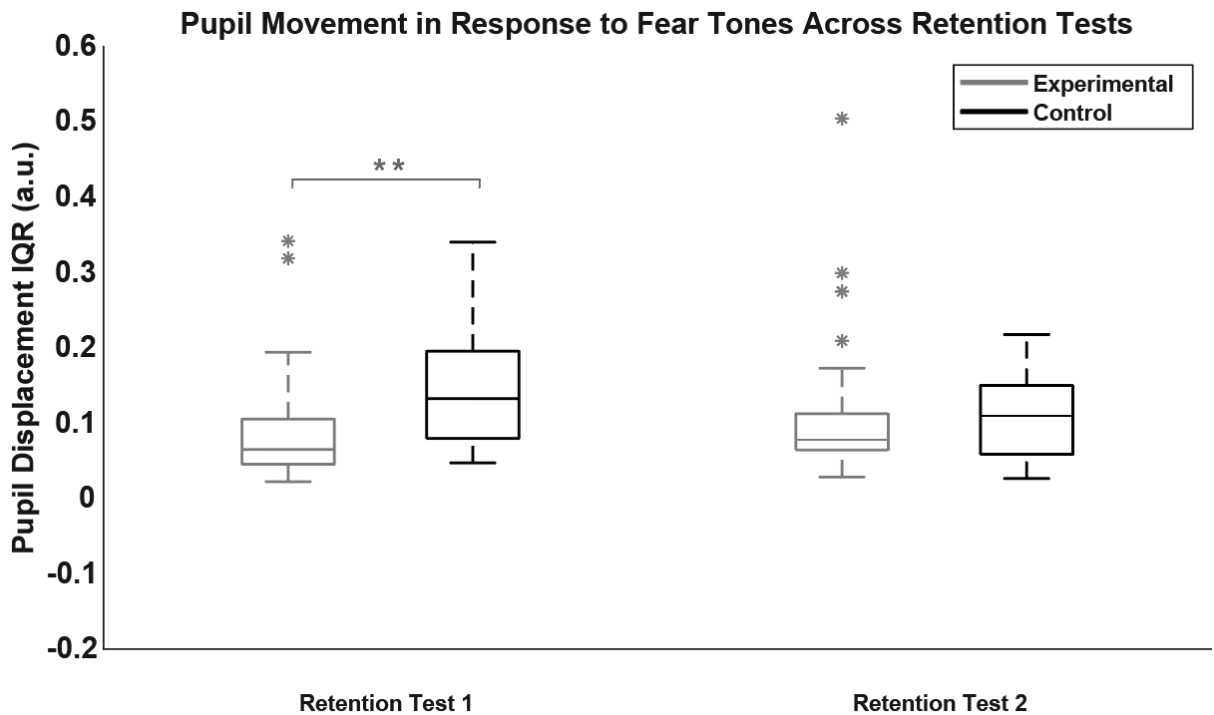

Figure 11: Experimental animals have a more a static eye gaze in response to fear tones in first retention test but not second.
Boxplot of the IQR pupil movements in response to fear tones. Red asterisk indicates statistical significance in one-tailed Wilcoxen rank-sum test ($p < 0.05$). Double asterisk indicates high statistical significance ($p < 0.01$).

5. Pupil Dilation

We expected a larger magnitude in the pupil dilation response to the CS in experimental animals consistent with a greater level of fear, and the CS carrying more emotional valence (Lennartz & Weinberger, 1992; Oleson et al., 1972). Pupil measurements were normalized as previously mentioned and the response of two pairs of animals in the first retention test are shown in figures 12 & 13. In figure 12 the experimental animal displays greater pupil dilation than the control for all three CS presentations in the session. There is a larger initial reaction, and the greatest difference between the two is during the CS, however the pupil size remains bigger even long after the CS offset. In the second and third presentation of the CS, the control animals pupil remains fairly stable or even shows constriction in response to the CS.
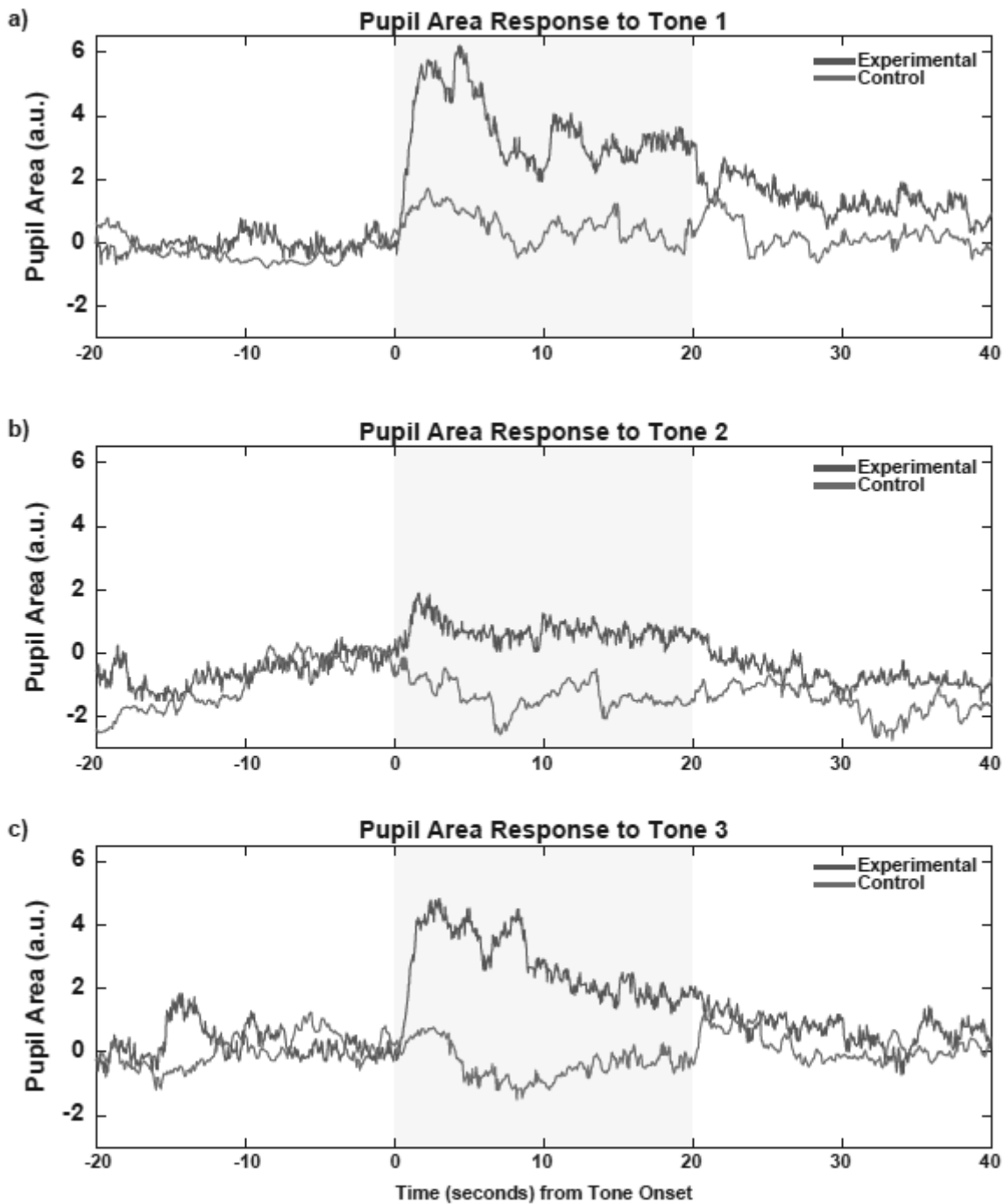
Figure 12: Figure 12: Example of an experimental and control animal that show large differences in pupil dilation response upon onset of CS in first retention test.
Pupil area of conditioned animal is in blue, and pseudo-conditioned animal in red. Light red highlighting indicates the presentation and duration of CS. Conditioned animal shows more

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dilation in initial response, and sustains larger pupil area. **a) First CS presentation. b) Second CS presentation. c) Third CS presentation.**
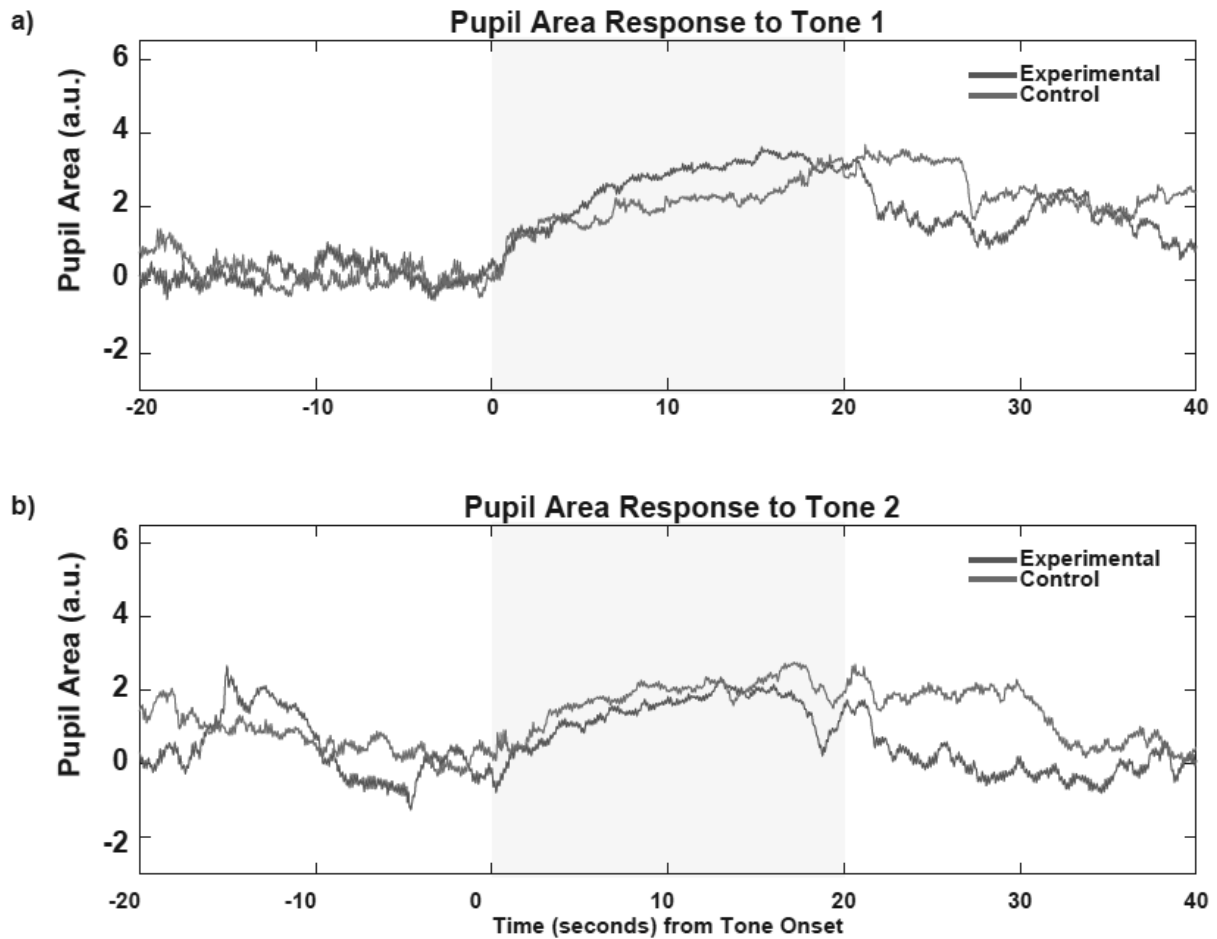
Figure 13: Example of an experimental and control animal that show minimal differences in pupil dilation response upon onset of CS in first retention test.
Pupil area of conditioned animal is in blue, and pseudo-conditioned animal in red. Light red highlighting indicates the presentation and duration of CS. Both animals show a similar response to the CS presentation. **a) First CS presentation. b) Second CS presentation**

 Although many pairs of animals showed this trend, there was a great deal of variability between

animals and even CS presentations. Figure 13 shows another pair of experimental and control

animals that show dilation in response to the CS. In this instance the pupil response is much

slower, taking many seconds before plateauing and there is no clear difference in their responses.

It has been previously reported in cats that the dilation associated with the pupillary orienting reflex (POR) in some animals to an auditory tone can be as strong as the pupillary response to a shock (Lennartz & Weinberger, 1992). Given this intersubject variability, is not surprising that some control animals could show a strong response to an unconditioned tone. Despite this variability we expected that across all animals, experimental animals to show a greater response than control animals. The median pupil diameter during the presentation of the CS was taken as the pupillary dilation response to each tone. Experimental animals displayed a stronger pupil dilation response across both the first and second retention tests (figure 14).
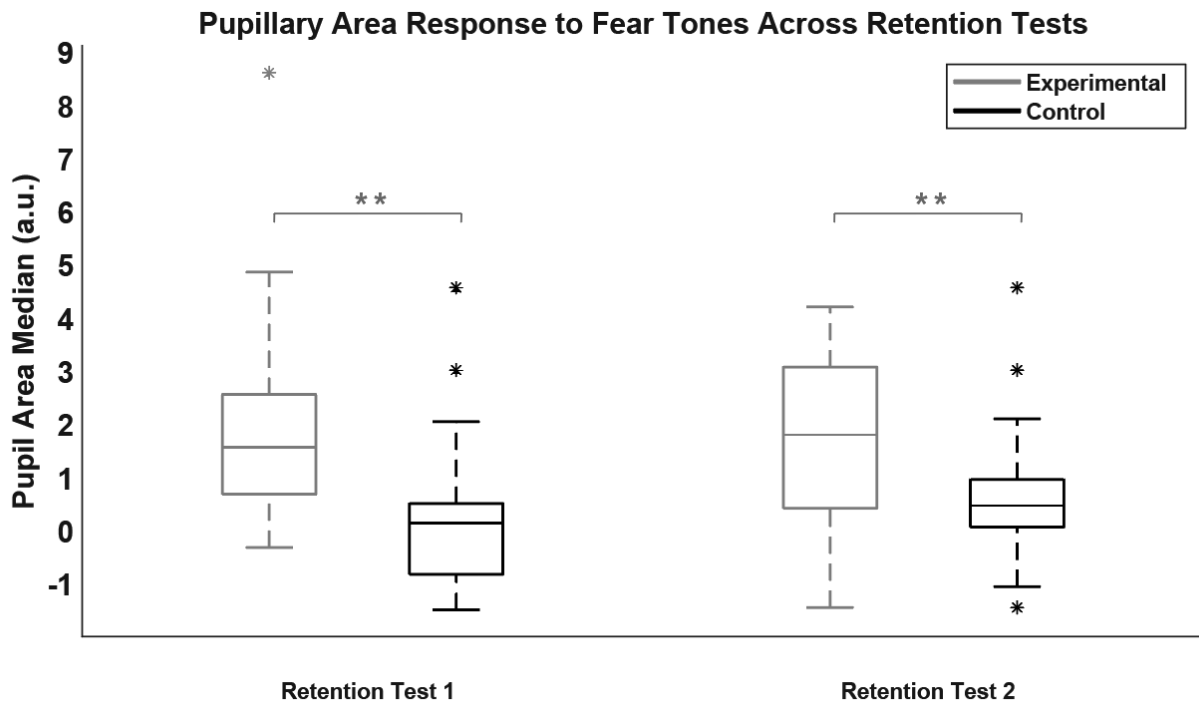

Figure 14: Experimental animals show more pupil dilation in response to fear tones.
Boxplot of median pupil area measurements in response to fear tones. Red asterisk indicates statistical significance in one-tailed Wilcoxen rank-sum test (p < 0.05). Double asterisk indicates high statistical significance (p < 0.01).

6. Hippocampus Local Field Potential

The last measurement that we looked at was LFP recordings in the dorsal hippocampus during the onset of the CS. The median power spectrum of theta (4 -12 Hz), low gamma (20 -55 Hz) and, high gamma (70 – 110 Hz), and ripple (120 – 250 Hz) frequencies was compared between experimental and control animals. Experimental animals had higher power in the theta frequency band and lower power in the high gamma frequency band across both retention tests (figure 15). There was no difference observed between experimental animals and control animals in the low gamma or ripple frequency bands.
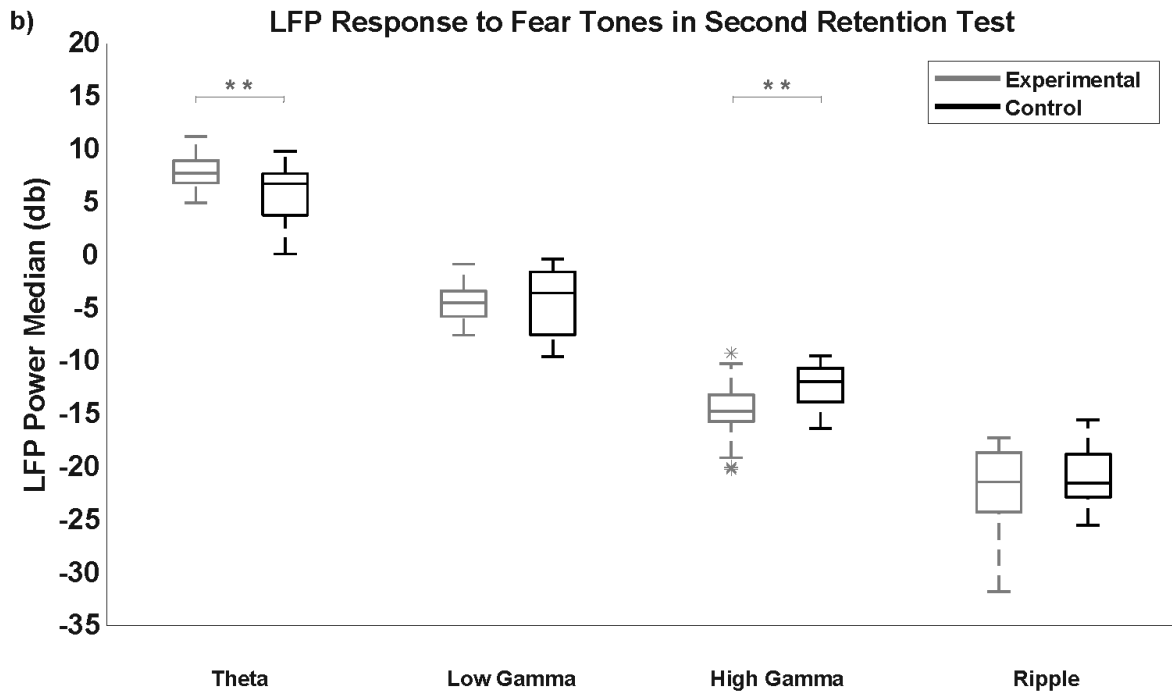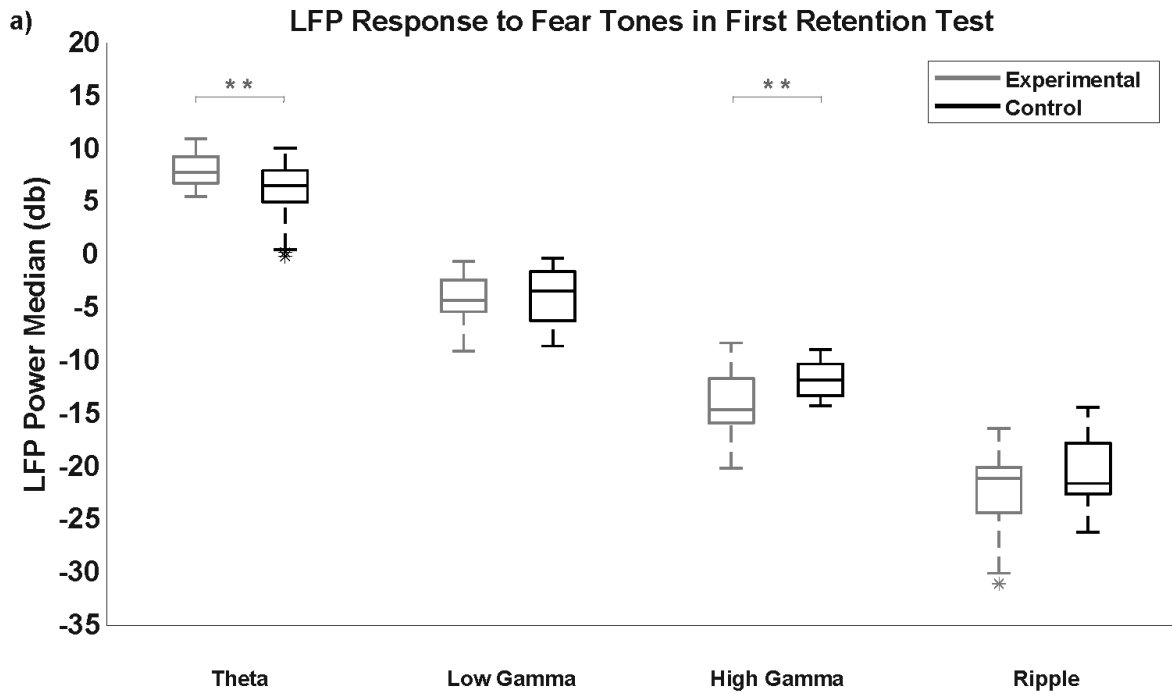
Figure 15: Local field potential frequency profile in response to fear tones in a) first and b) second retention test.
Experimental animals show higher power in theta frequency band (4 -12 Hz) in both first and second retention tests. Control animals show higher power in high gamma frequency band (70 –

110 Hz) in both the first and second retention tests. There was no difference detected in low gamma (20 -55 Hz) and ripple (120 – 250 Hz) frequency bands in either retention tests.

Discussion

Under head fixation, experimental animals responded to tones with less bodily motion than controls and a stronger pupil dilation response. Given that the pupil dilation response in mice is positively correlated with body and eye movement (McGinley et al., 2015; Reimer et al., 2016) and that experimental animals moved less it is likely that this pupil response is indicative of arousal (McCormick et al., 2020) . Pupils dilate in response to fearful stimuli (Ashe et al., 1978; Oleson et al., 1972), and in experimental animals the greater dilation is likely indication of the CS-US association being recalled. Similarly, the inhibition of movements seen in experimental animals is consistent with traditional measures of freezing in rodents quantified under freely roaming conditions (Maren, 2001). Here we show that this inhibition of movement in response to the CS is reflected in the movements of the entire body, the face, the forepaws, as well as twitches of the nose and potentially whiskers. Each of these measurements may be considered different CRs, some of which could be considered more "reflexive" and others more "volitional" (Lennartz & Weinberger, 1992; Shumake & Monfils, 2015). The fact that even diminutive measurements of motion in the nose reflect a CR, is indicative that freezing behavior is expressed in muscles throughout the entire body of the mouse. Whiskers showed no significant difference in the first retention test, however showed less movement in the second retention test. Aside from natural variance in the data, this could be due to experimental animals using whisking as an attempt to sample information from their environment (Arakawa & Erzurumlu,

2015; Carvell & Simons, 1990). Whisking in some animals may stem from fear memory retrieval as part of the preparation for survival-related actions such as escape.

Experimental animals gaze remained fixated more than control animals during the presentation of the CS in the first retention test. This may be indicative of selective information sampling of the environment and heightened arousal (Hopkins et al., 2015). A previous study in humans found that eye gaze froze more in anticipation of a shock when the subjects knew that they would be able to avoid it (akin to escape behavior in animals) (Rösler & Gamer, 2019). Despite being under head-fixation, mice might still engage in such preparatory behaviors for escape. The fact that eye gaze freezing did not persist into the second retention test, whereas movement inhibition did, suggest distinct roles for these behaviors. Immobility of animals is adaptive for avoiding predatory detection and eye movements are useful for detecting threats (M. Fanselow & Lester, 1988). The differential expression of different CRs such as immobility and eye movements point to different neural pathways in the expression of fear (Lennartz & Weinberger, 1992).

Experimental animals had higher theta power in the hippocampus than control animals during the presentation of the CS across both retention tests. This is in line with previous studies which have shown an increase of hippocampal theta during conditioned immobility in rats (Kramis et al., 1975; Sainsbury et al., 1987; Vanderwolf, 1969; Whishaw & Vanderwolf, 1973). Theta synchronization between the hippocampus, the BLA and the mPFC has been implicated in fear memory retrieval in rodents (Seidenbecher et al., 2003; Stujenske et al., 2014). It is suggested that this synchrony is to facilitate communication between different brain areas, and has a role in timing related computations such as modulating excitability of neurons and plasticity (Colgin, 2013).

Control animals displayed higher amounts of high gamma activity than experimental animals, and no difference was observed in low gamma. This finding was surprising given that gamma is implicated in enhancing communication between nearby regions, and perceptual binding (Fries, 2009). Previously, increased power in low gamma in the amygdala has been associated with spontaneous recovery of the CR after extinction (Courtin et al., 2013). In recordings of the rat auditory cortex, increase in gamma activity and greater gamma phase-locking in multi-unit activity was correlated with the CR (Headley & Weinberger, 2013). Given a fear memory network that would encompass the amygdala, hippocampus and auditory cortex, one might expect a similar result from LFP recordings of the HPC. In contrast, one research group has found no difference in either high gamma or low gamma power in the dorsal or ventral HPC between unconditioned and conditioned stimuli, and/or weak/strong CRs (Stujenske et al., 2014). Instead, they report an increase of fast gamma in the BLA, and increased theta-gamma coupling with the mPFC in the absence of the CR. Although they report in an increase in high gamma in the BLA, rather than the HPC, the discrepancy in our results and theirs could be attributed to methodological differences. In their study, mice were conditioned over a period of three days with 15-18 CS-US presentations whereas we had 5 CS-US presentations in a single session. The length and distribution of training episodes is likely to have a strong effect on the hippocampal role in fear memory retrieval (Lennartz & Weinberger, 1992; Quinn et al., 2008; Sutherland et al., 2020; Wotjak, 2019). An area of further inquiry that would be worth exploring is the extent to which different learning parameters (length of CS, length of US, number of training sessions, etc) affect oscillations.

Lastly, there was no difference observed in the ripple power spectrum between experimental and control animals. One might expect experimental animals that have a CS-US association to show

more ripple activity given its role in memory reactivation (Buzsáki, 2015; Ólafsdóttir et al., 2018). However, both the experimental and control animals were exposed to the CS an equal number of times, and both have some experience associated with it, and it is unclear the extent to which ripples have a causal role in fear memory retrieval. It is worth noting that the analysis is on ripple power and not on the frequency of sharp wave ripples (SWRs) which contain large amplitude polarity deflections (40 – 100ms) originating from CA1 (Buzsáki, 2015). SWRs during the CS duration occurred too infrequently in order to substantially analyze.

The findings here demonstrate that numerous signals from head-fixed animals can be used as a measure of the CR without any additional intervention or training on another task. The procedure was run on 4 different commonly used mice strains in imaging experiments (all expressing some variation of either GCaMP6s or iGluSnFr). A number of precautions we took are likely important in detecting these CRs, and are worth noting. The first is that a proper control needs to be established and although in theory there are no perfect controls (Rescorla, 1967), a group needs to be established in which the CS has no associative strength. The benefit of the explicitly unpaired control design in this study is that it allows experimenters to conclude that differences in either behavior or brain activity between groups is due to the CS-US association, and not because the control group was not exposed to the CS or US, or exposed to them in a significantly different way. Even a single exposure to a stimulus such as a shock or a loud tone may have long lasting effects on neurophysiology and behavior (M. S. Fanselow & Bolles, 1979). One concern with our explicitly unpaired control design is that it may introduce an association between the CS and the absence of any US, whereby the CS becomes a cue for safety from the US. We consider this unlikely in our experiment because we used a loud tone as the CS (85 dB) that is intrinsically startling, somewhat aversive, and is unlikely to signal safety for the animal. An additional benefit

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to using a startling tone during retention tests of the CS, is that animals are more likely to begin fidgeting or reflexively reacting to the tone. The inhibition of movement in the freezing response would therefore be easier to detect.

 An alternative design that has been used in other studies is to present experimental animals with two distinct tones, one of which is followed by or terminates with footshock (CS+) and another which is not (CS-) (Ahmed et al., 2020; Wood et al., 2020). The benefit of this design is that each animal serves as its own control, and any difference in freezing between the CS+ and CS- can only be explained by recall of the US and not differences between groups of animals. This protocol is useful in studying fear memory generalization, where animals typically have more difficulty discriminating between the CS+ and CS- at remote tests rather than recent tests (Asok et al., 2019; Lopresto et al., 2016; Pollack et al., 2018). This generalization however is exactly the limitation as well. Any amount of generalization of the CS+ to the CS-, has the potential to diminish our ability to measure the CR particularly in head-fixation where the animals available actions are limited. More importantly perhaps, generalization would confound our ability to study discrete CS-US associations without interference from CS- associations. Another within-subjects design which has been used, is to present and record animals response to the CS both before and after conditioning, to study the effect of forming an association with the US (Gehrlach et al., 2019). With pre-exposure to the CS however, the CS becomes less predictive of the US and conditioning of the animal is reduced (Lubow & Moore, 1959). Post conditioning presentations of the CS may elicit neural dynamics that are associated with pre-exposure rather than conditioning. We conclude that presenting only one type of tone that either is or is not associated with shocks in separate animals (as we have done) is likely better suited to study discrete associative memories.

One last major key consideration in our study was to make the CS as salient as possible during conditioning and retention tests. Animals were extensively pre-exposed to the conditioning context and experimenters, so the CS and US were the only novel events in their environment. Animals were habituated to head-fixation, to achieve a high level of calmness and low level of arousal during long periods of recordings. If animals are agitated, they will likely pay less attention to the CS, and the CR will be obstructed by stress related movements. Understanding real time activity of the brain involved in retrieval of a CR over time and repeated presentations will provide a better understanding of the neural circuits involved in fear memories, and memory consolidation. Past investigations into large scale activity patterns have used post-mortem labeling techniques which lack temporal resolution (Wheeler et al., 2013). Having a measure of the CR under head-fixation will allow researchers to investigate various brain mechanisms involved with greater precision.

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