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Los Angeles

Associative, Behavioral, and Computational Analysis of Sex
Differences in Defensive Behavior and Hippocampal Function

A dissertation submitted in partial satisfaction of the requirements
for the degree Doctor of Philosophy in Neuroscience

by

Jeremy Michael Trott

2022

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ABSTRACT OF THE DISSERTATION

Associative, Behavioral, and Computational Analysis of Sex
Differences in Defensive Behavior and Hippocampal Function

by

Jeremy Michael Trott

Doctor of Philosophy in Neuroscience

University of California, Los Angeles, 2022

Professor Michael S. Fanselow, Chair

Fear is an adaptive response that allows animals to respond appropriately to danger, and Pavlovian fear learning provides a mechanism that allows animals to learn about, predict, and effectively respond to dangerous situations. In the laboratory, fear conditioning is a frequently used procedure used to study the learning, expression, and underlying neurobiology of fear and emotional behavior. In such an experiment, animals learn to associate some cue/conditional stimulus (CS) or contextual location with an aversive outcome/unconditional stimulus (US), such as a footshock. As a result of this learned association, the animal will now respond to the initially neutral CS with defensive behavior, a conditional response (CR) as a result of learning. This dissertation contains two chapters which investigate different aspects of cued and contextual fear learning in male and female rodents, first with a focus on the selection

and associative nature of defensive responding, then with a focus on potential sex differences and any potential underlying mechanisms.

Chapter #1 of this dissertation presents behavioral experiments in mice that assess defensive behavior after various auditory fear conditioning procedures. Particularly, we employed a serial compound conditional stimulus (SCS), consisting of a tone followed by a white noise, training procedure which allowed us to assess differential freezing and activity burst behaviors to each component of the SCS. A number of experiments were run with various control groups to assess the associative or non-associative nature of defensive responding following such a procedure. Contrary to some conclusions in the recent literature, while associative processes had some impact on the topography of flight behavior, cue-elicited activity bursts were primarily due to nonassociative learning processes. Such activity burst behavior was viewed as being similar to a fear-potentiated startle response. The results of the experiments led to the production of a rule for defensive behavior selection: “When afraid, freeze until there is a sudden novel change in stimulation, then burst into vigorous flight attempts,” (Trott et al., 2022, p. 2).

Chapter #2 of this dissertation presents behavioral contextual fear learning experiments in rats and computational simulations of the same experiments using BACON, a conceptual and computational model of hippocampal function. Behaviorally, animals underwent a 3-day contextual fear conditioning procedure in which they received pre-exposure to a to-be-shocked context on Day 1, a shock after some placement-to-shock interval (PSI) on Day 2, and a fear test on Day 3. Experiments 1A and 1B results revealed that after shorter pre-exposure periods, male rats showed higher levels of contextual fear; however, after longer pre-exposure periods, female rats showed greater fear. In Experiment 2, when testing recently vs remotely acquired fear memories, males again showed more contextual fear. Additionally, while pre-exposure and PSI are both periods of time prior to shock, Experiment 3 revealed that they were not equivalent

to each other such that there were overall sex differences and differences in the timing of fear expression based on whether animals received the majority of pre-shock context exposure as pre-exposure or PSI. Through the use of BACON, a computational model of hippocampal function, we simulated many of our key findings and suggest a potential mechanism for such findings, that of an increased contextual feature sampling rate in males.

The experiments presented here assess both cued auditory and contextual fear learning in both rats and mice and add to a rich literature on fear conditioning and defensive behavior. The experiments and discussion within **Chapter #1** introduce important controls and considerations for the conduction and interpretation of fear conditioning experiments, particularly for a set of increasingly used fear conditioning procedures which result in different freezing and flight behavior to different cues paired with a shock US. Results from **Chapter #2** provide insight into sex differences in contextual learning and suggest a hippocampal mechanism for such differences, adding to a growing literature on sex differences in learning and hippocampal function.

The dissertation of Jeremy Michael Trott is approved.

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2022

DEDICATION

In memory of Uncle Dick. Thank you for being a wonderful role model and always encouraging me in my goals.

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LIST OF ABBREVIATIONS

ANOVA – Analysis of variance

BACON – Bayesian contextual fear learning algorithm

CA3 – *Cornu Ammonis* area 3; region of the hippocampus

CR – Conditional response

CRH – Corticotropin-releasing hormone

CS – Conditional stimulus

DG – Dentate gyrus of the hippocampus

LTP – Long-term potentiation

PAR – Peak activity ratio

PIC – Predatory (or threat) imminence continuum

PreX – Pre-exposure; context pre-exposure

PSI – Placement-to-shock interval

PTSD – Post-traumatic stress disorder

SCS – Serial compound conditional stimulus

SEM – Standard error of the mean

SSDR – Species-specific defense reaction

UR – Unconditional response

US – Unconditional stimulus

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Chapter #1 is from a manuscript that is under revision with *eLife* (2022). Figures 1-1 – 1-11 are from this manuscript. The authorship is as follows: Trott, J. M., Hoffman, A. N., Zhuravka, I., and Fanselow, M. S. Thank you Annie for helping to begin and analyze this project and Irina for assistance with behavioral aspects of the study.

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- Trott, J.M.**, Krasne, F., & Fanselow, M.S. (2022). *Sex differences in contextual fear learning and generalization: A behavioral and computational analysis of hippocampal functioning*. [Under revision]. Psychology Department, University of California-Los Angeles.

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- Trott J.M.**, Thrailkill, E.A., & Bouton, M.E. (2016). Renewal of an extinguished behavior in the context of a preceding response. Poster and 30-minute Talk presented at the March 2016 meeting of the Eastern Psychological Association (EPA) in New York, NY.
- Trott J.M.**, Adison, R., & Fanselow, M. S. (2019, October). *Sex differences in contextual fear learning and generalization*. Poster presented at the 2019 annual meeting of the Pavlovian Society in Vancouver, BC.
- Trott J.M.**, Adison, R., & Fanselow, M. S. (2019, October). *Sex differences in contextual fear learning and generalization*. Poster presented at the 2019 annual meeting of the Society for Neuroscience in Chicago, IL.
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- Trott J.M.**, Hoffman, A.N., Zhuravka, I., & Fanselow, M.S. (2021, October). *The associative nature of defensive behavior following serial conditioned stimulus fear conditioning*. Poster presented at the 2021 Annual Pavlovian Society Meeting in Ann Arbor, MI.

General Introduction

Life for any biological organism is complicated and difficult. Animals, including humans, must constantly work and adapt to survive and to thrive in a world with limited resources and plentiful danger. Animals need to be able to effectively use their environment and respond appropriately to maximize good/appetitive outcomes, like food, shelter, and sex, and to minimize bad/aversive outcomes like pain and danger. Fundamentally, animals need to learn about their environment to learn to predict the future. They need to be able to anticipate upcoming events in order to best prepare for these events.

Fortunately for us, the nervous system has evolved to allow us to learn relationships about our environment and to allow us to make predictions about the future. Here, I will focus on one of the most basic and fundamental types of learning, that of associative Pavlovian, or classical learning. Fundamentally, associative Pavlovian learning allows animals to learn relationships about different stimuli/events and to make predictions about their environment (see review by Domjan, 2005). Through the learning of associations between stimuli, animals are able to predict and anticipate upcoming, biologically-significant stimuli/events. When such biologically-significant events are well-predicted, an animal's physiological state and its behavioral patterns may change to allow for better, more efficient responses to the significant event (Domjan, 2005). If a well-predicted highly caloric meal is incoming, an animal's physiology and behavior will change to allow for the most efficient intake and digestion of the meal (Woods, 1991). If an animal is in a situation which it predicts may be dangerous, that animal's physiology and behavior will change to allow for the best chances to avoid or respond to the danger (Fanselow, 2018).

Pavlovian or classical learning allows animals to learn the relationship between stimuli. As studied in the laboratory, Pavlovian learning/conditioning typically consists of presenting

animals with a CS-US relationship during training. The conditional stimulus (CS) is typically an initially neutral cue that does not normally induce a response on its own. The unconditional stimulus (US) is some biologically-relevant stimulus that inherently is meaningful to an animal and can produce a response on its own-the unconditional response (UR). Common examples of US's used experimentally are food, drugs, and aversive footshocks. After training, these CS's are then re-presented during testing to determine if there were any changes in behavior to the initially-neutral CS as a result of CS-US pairing. The changes in response to the CS as a result of training are generally taken to be evidence of learning and are called conditional responses (CR; Domjan, 2005). These conditional responses are by-and-large adaptive responses that help an animal prepare for a potentially-upcoming, biologically relevant US. After hearing a tone (CS) which has been previously paired with food (US), animals may salivate (CR) in order to better prepare for the upcoming food. After hearing a tone (CS) which has been previously paired with shock (US), animals may freeze in a corner (CR) in order to better prepare for and avoid detection by an upcoming danger [modeled by shock].

Fear Conditioning, Freezing, and Flight

Fear conditioning is one of the most frequently used procedures to study both the behavior and neurobiology of learning, memory, and emotions (see review by Maren, 2001). In a fear conditioning procedure, a cue or a particular context/location are paired with something aversive, typically a footshock. As a result of this pairing, animals develop an association between the cue/context and the shock, such that future presentations of the cue and/or returns to the context will lead to a fear response. This fear response results in physiological changes (alterations in heart rate, respiration, temperature, analgesia; Fanselow, 1984; Iwata & LeDoux, 1988; LeDoux et al., 1988; Moberly et al., 2018; Vianna & Carrive, 2005) as well as behavioral changes (Fanselow, 1980, 1986, 1990). Animals respond to the cue/context with defensive behavior. In most situations, this fear response and resulting defensive behavior is functional

and adaptive, such that generally, the level of defensive behavior is related directly to the level of potential danger (Fanselow & Lester, 1988).

While fear and defensive behavior are fundamentally adaptive and aimed at surviving dangerous situations, it is also true that fear limits the behaviors available to an animal to its species-specific defense reactions (SSDRs), thereby precluding more flexible voluntary behavior (Bolles, 1970). The idea that limiting behavior when afraid is adaptive may seem counter-intuitive, but it is important to realize that in a real-world situation, animals at risk of predation do not have the benefit of learning particular responses through trial-and-error (Bolles, 1970; Fanselow, 2018). If an animal is at risk of being eaten by a predator, it needs to be able to perform appropriate defensive behaviors on that very first experience with a predator to allow it to survive. Thus, animals have evolved particular sets of species-specific defense reactions (SSDRs) that are appropriate for different perceived levels of potential danger, which is determined, at least in part, through fear conditioning. Species-specific defense reactions (SSDRs) are innately organized action patterns that have successfully defended members of the species during their phylogenetic history (Bolles, 1970; Fanselow, 1997). These include behaviors that maximize safety in potentially dangerous environments, behaviors that minimize detection by a predator, and behaviors that allow for the potential to escape predator contact.

That fear limits behavior to SSDRs is a major reason why Pavlovian fear conditioning is so easily and frequently studied and measured in the laboratory. These innate defensive responses (i.e., SSDRs) can be defined and measured in order to assess learning and fear levels. The vast majority of rodent studies in the last forty years of the study of fear conditioning have extensively used one of these defensive behaviors, freezing, more than any other response (Anagnostaras et al., 2010; Bouton & Bolles, 1980; Do-Monte et al., 2015; Fanselow & Bolles, 1979; Grewe et al., 2017; Kim & Fanselow, 1992; Kwon et al., 2015; Nader et al., 2000; Roy et al., 2017; Trott, Kranse, & Fanselow, 2022). Freezing is a frequently elicited defensive

behavior in the vast majority of prey species, and it is functional and adaptive in the sense that it reduces both the likelihood of detection by a predator and an attack by a predator, as movement is typically the releasing stimulus for predatory attacks (Fanselow & Lester, 1988).

While freezing is certainly a major defensive response in a rodent, it is not the only defensive response available as rodents may also display aggressive biting behavior, bursts of activity, and/or vocalizations in dangerous situations or upon experiences with a predator (Fanselow & Lester, 1988). An important question then is how particular defensive behaviors, or SSDRs, may be selected (Fanselow, 1997). In other words, under what circumstances will a rodent be likely to freeze and under what circumstances will a rodent be likely to burst into activity? Predatory (or Threat) Imminence Continuum (PIC) theory is a functional behavioristic model of SSDR selection that suggests that animals (and humans) display defensive behavior specifically appropriate for the perceived level of danger (Bouton et al., 2001; Fanselow & Lester, 1988; Mobbs, 2018). This perceived level of danger can best be thought of as a psychological distance from potential contact with a dangerous situation or a predator, and this psychological distance is based on a number of factors, including physical and temporal distance from the threat, identity of the threat, probability of attack, and the behavior of the potential threat (Fanselow & Lester, 1988).

The PIC suggests that defensive behavior can be organized into distinct modes, each associated with distinct types of activating stimuli, resulting emotions, and resulting defensive behavior. When in situations with no potential danger, animals perform behaviors according to their preferred activity patterns, but increasing levels of perceived danger result in emotional responses and defensive behavior appropriate to the level of danger. Rodent defensive behavior has been described in depth within this framework, detailed below (Fanselow & Lester, 1988, Hoffman et al., 2022). The first mode of defensive responding is labeled pre-encounter defense and occurs when a dangerous situation is possible but a specific danger has not

necessarily been detected yet. For example, when a rodent enters a foraging area in which a predator has previously been encountered, this would elicit anxiety-like pre-encounter defense responses such as increased vigilance and meal pattern reorganization (Fanselow & Lester, 1988). As the danger level increases, and particularly if the rodent detects a predator, the next mode of defensive responding occurs, labeled post-encounter defense. Post-encounter defense is characterized by emotional fear responses and is dominated by freezing behavior, especially in the rodent. Finally, as the level of danger escalates to its maximum and the predator is about to make contact, the circa-strike defense mode is activated and panic-like defensive behavior occurs, such as flight and aggression in the rodent.

In the setting of studying such defensive behavior using fear conditioning, researchers can use stimuli that represent distinct points along the PIC to elicit defensive behavior appropriate to the level of danger. Shock, which models contact with the predator, produces circa-strike panic-like activity burst behavior (Fanselow, 1982). Cues or contexts that predict shock model detection of a predator and thus produce post-encounter fear-like freezing responses (Fanselow, 1989). Importantly, within this framework, stimuli which model predator contact should produce circa-strike behaviors (activity bursts) but not post-encounter behaviors (freezing), and stimuli which model predator detection should produce post-encounter behaviors (freezing) but not circa-strike behaviors (activity bursts).

However, a number of recent reports have challenged this framework. These reports have suggested that rodents may display both freezing behavior and/or activity burst behavior to cues which predict shock. A procedure using a unique two-component serial compound conditional stimulus (SCS), consisting of a 10-second tone followed immediately by a 10-second white noise, ending with a 1-second shock, found that different behavior developed to each component of the SCS in mice (Fadok et al., 2017; Hersman et al., 2020). While freezing behavior developed to the initial tone component of the SCS, activity bursts developed to the

second noise component of the SCS. This behavioral pattern was replicated in rats (Totty et al., 2021), although there were differences in the level of overall activity bursts such that, in this study with rats, activity bursts tended to be less frequent than observed in studies with mice. Separately, using a simpler procedure in rats in which only a tone was paired with shock, activity bursts, labeled darts, developed to the tone (Colom-Lapetina et al., 2019; Gruene et al., 2015; Mitchell et al., 2022). In these studies, again the overall level of activity bursting was reduced compared to the studies in mice, but interestingly, there were sex differences in such darting behavior such that females were more likely to dart than male rats.

Each of these studies interpreted these different behaviors as a competition between “active” and “passive” defensive responding. These findings present a clear challenge to the response selection rule, from the PIC, as described above. While PIC suggests that freezing behavior and activity burst behavior occur to distinct levels of perceived threat (Fanselow, 1989), these results suggested that freezing and activity bursts were competing CRs to the same level of threat (Fadok et al., 2017; Gruene et al., 2015). Additionally, these results potentially call into question decades of results from the study of fear conditioning. If rodents, particularly female rodents, are likely to display alternative behaviors such as activity bursting, many prior studies which only measure and report freezing may have missed significant instances of learning (Gruene et al., 2015). However, some of these initial studies did not include standard control groups which allow for the assessment of the learned nature of such activity bursts (Fadok et al., 2017; Gruene et al., 2015; Colom-Lapetina et al., 2019), and those studies which do include such controls have provided mixed evidence as to the extent that such activity burst behavior is learned associatively as a result of CS-US pairings (Hersman et al., 2020; Totty et al., 2021). **Chapter #1** of this dissertation will further explore these conflicting assumptions and will carefully analyze the associative and non-associative contributions to different defensive behaviors in rodents.

Contextual Fear Learning, Anxiety Disorders, the Hippocampus, and Sex Differences

Again, emotions and defensive behavior are adaptive, and Pavlovian conditioning procedures allow us to study how emotions are learned and expressed as well as how and when animals learn to perform defensive behavior. There are, however, a number of situations that can lead to maladaptive defensive behavior. Such defensive behavior can be considered maladaptive when the level of emotion and defensive responding is inconsistent with the level of potential danger. Displaying defensive behavior and aversive emotion in response to safe situations is one of the major defining symptoms of anxiety disorders (Andreatta et al., 2015; Asok, Kandel, & Rayman, 2019; Dunsmoor & Paz, 2015). In both patients with post-traumatic stress disorder (PTSD) and animal models of the disorder for example, a prior traumatic experience can often lead to exaggerated fear responses to mildly aversive or even neutral stimuli (Fani et al., 2012; Perusini et al., 2016; Hoffman et al., 2022). Thus, in normally safe situations that an individual with an anxiety disorder and/or PTSD finds fearful, aversive emotions may be experienced and defensive behaviors will be performed. Further, fear's ability to limit available behaviors to SDRs and to prevent flexible behavior is one reason that conditions characterized by high fear levels such as anxiety disorders are so maladaptive (Fanselow, 2018).

While fear and anxiety are often adaptive, such emotional responses may be inappropriate in situations that are not dangerous. From the perspective of the organism's survival however, responding fearfully and with defensive behavior, even in safe situations, may be a 'beneficial' thing. While there is certainly a cost of showing fear and performing defensive behavior, this cost is far less than the penalty if an animal does not show proper defensive behavior in a truly dangerous situation with a predator for example. If defensive behavior is not properly performed in dangerous situations, an animal is likely to be eaten or at least injured. Thus, evolutionarily, it follows that we may be particularly susceptible to anxiety disorders.

Indeed, anxiety disorders, including PTSD, are unfortunately quite common, with up to around 30% of individuals presenting with an anxiety disorder at some point in their life (Essau et al., 2018; Kessler et al., 2005; Somers et al., 2006).

Certain aspects or symptoms of anxiety disorders are frequently studied in the laboratory using contextual fear conditioning. This procedure consists minimally of placing an animal in a conditioning chamber and giving it some aversive US, like a footshock. While there is no discrete stimulus to associate with shock, the salient features and stimuli of the conditioning chamber, or the context, are associated with the US, resulting in fear responses, such as freezing, defecation and analgesia, when later returned to the context paired with shock (Fanselow, 1980, 1984, 1986; Wiltgen et al., 2001). Again, learning and expressing fear to a dangerous place is adaptive, but it is the potential generalization of learned fear to alternate, safe environments that can be maladaptive and lead to inappropriate levels of fear and defensive responding. In rodents, contextual fear learning has been frequently studied with regard to this generalization of learned fear to safe contexts (Andreatta et al., 2015; Asok, Kandel, & Rayman, 2019; Dunsmoor & Paz, 2015; Poulos et al., 2016).

In order to effectively learn and express contextual fear, an animal needs to first form a stable representation of the context, based on the features and stimuli of the context, which can then be associated with the aversive US during conditioning (Anagnostaras, Gale, & Fanselow, 2001; Fanselow, 1980; Krasne, Cushman, & Fanselow, 2015; Rudy & O'Reilly, 1999; Yavas, Trott, & Fanselow, 2021). It is believed that as animals spend more time in the context, they are able to sample more contextual features to add to their contextual representation (Krasne, Cushman, & Fanselow, 2015). As a result, contextual fear is increased as more time is spent in the context prior to shock. Both previous non-shocked experience in the context (pre-exposure or PreX), and increases in the time in the context prior to the first shock on the day of conditioning (placement-to-shock interval; PSI) can enhance future contextual fear learning

(Fanselow, 1990; Wiltgen et al., 2001). It is known that animals need at least some time in the context prior to shock in order effectively learn contextual fear (Fanselow, 1986). If animals are given a shock very soon after entering a context (such as with very short PSIs), very little contextual fear is expressed. This effect, labeled the immediate-shock deficit, can be restored by giving the animals an opportunity to explore the context without receiving shock in a pre-exposure session prior to the actual conditioning session (Wiltgen et al., 2001).

This integration of features and stimuli to create a coherent contextual representation is thought to rely on and occur within the hippocampus. Evidence for this comes from findings that hippocampal damage or inhibition impairs fear learning and expression specifically to contextual CSs but not to discrete CSs (Asok, Kandel, & Rayman, 2019; Fanselow, 2000; Kim & Fanselow, 1992; Rudy & O'Reilly, 1999). Furthermore, the role of the hippocampus appears to be selective to contextual processing rather than the formation of context-US associations, as manipulating hippocampal function, pharmacologically or optogenetically, only during non-shocked pre-exposure is sufficient to alter contextual fear learning (Hersman et al., 2017; Matus-Amat et al., 2004).

There are notable sex differences in the frequency and severity of anxiety disorders. Women are more frequently diagnosed with nearly every anxiety disorder and tend to have more severe symptoms (Essau et al., 2018; Kessler et al., 2005). Interestingly, like anxiety disorders, there are notable sex difference in contextual fear conditioning, such that in rodents, males typically show greater contextual fear learning, and the immediate shock deficit tends to be more pronounced in females (Cossio et al., 2016; Cushman et al., 2014; Le et al., 2022; Maren, De Oca, & Fanselow, 1994; Poulos et al., 2015; Wiltgen et al., 2001; Yavas, Trott, & Fanselow, 2021). Additionally, there is growing evidence to suggest sex differences in hippocampal function. Beyond sex differences in contextual fear learning (Cossio et al., 2016; Cushman et al., 2014; Wiltgen et al., 2001; Poulos et al., 2015), sex differences have been

reported for hippocampus-dependent spatial learning tasks in both rodents and humans (Brake & Lacasse, 2018; Koss & Frick, 2017; Linn & Peterson, 1985), and there are notable sex differences in hippocampal long-term potentiation (LTP) in terms of the magnitude of LTP, the threshold to induce LTP, and the developmental trajectory of hippocampal LTP (Maren, De Oca, & Fanselow, 1994; Le et al., 2022).

Chapter #2 of this dissertation presents a series of experiments assessing the impact of pre-exposure duration, PSI length, and sex on contextual fear learning and generalization. In addition to behavioral experiments, through a collaboration with Frank Krasne, I employed BACON (Bayesian CONtext Fear Algorithm), a conceptual and computational model of hippocampal contextual learning (Krasne, Cushman, & Fanselow, 2015; Krasne et al., 2021) that successfully simulates a wide range of known properties of contextual fear learning and has previously been used to explain a number of non-intuitive properties of contextual learning (Bernier et al., 2017; Zinn et al., 2020). We used BACON to simulate hippocampal learning and fear expression using experimental designs similar to those used in the behavioral experiments. Our goal in using this model was to explain the sex differences found in our experiments as possibly being due to differences of some single underlying variable, and indeed, we revealed a potential mechanistic difference that would explain our both our behavioral results as well as findings in the general literature.

Outline of the Contained Work

Chapter #1 of this dissertation will present behavioral experiments in mice that assess defensive behavior after various auditory fear conditioning procedures. Particularly, we employed a serial compound conditional stimulus (SCS) training procedure which allowed us to assess differential freezing and activity burst behaviors (Trott et al., 2022). A number of experiments were run with various control groups to assess the associative or non-associative

nature of defensive responding following such a procedure. Results are explained with the framework of Predatory Imminence Continuum Theory.

Chapter #2 of this dissertation will present behavioral experiments in rats and computational simulations of the same experiments using BACON. The experiments and simulations generally followed the same pattern of a 3-day contextual fear conditioning procedure (Trott, Krasne, & Fanselow, 2022). On Day 1, animals were given time to explore a to-be-shocked context (pre-exposure; PreX). On Day 2, animals were returned to the context and given a single footshock US after some PSI. On Day 3, animals were either returned to the shocked context to test for contextual fear or to a novel context to test for generalization. One of the major suggestions of BACON is that the hippocampus can have distinct functional modes, one for building a contextual representation (which would mostly happen during pre-exposure) and one for retrieving that representation, prior to shock for example (which would happen during Day 2's PSI in the experiments presented here). BACON was used as a tool to help identify how males and females differ in processing contexts. The results of each chapter are further discussed in a closing general discussion section.

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Chapter #1:

**Conditional and Unconditional Components of Aversively Motivated Freezing, Flight and
Darting in Mice**

Abstract

Fear conditioning is one of the most frequently used laboratory procedures for modeling learning and memory generally, and anxiety disorders in particular. The conditional response (CR) used in the majority of fear conditioning studies in rodents is freezing. Recently, it has been reported that under certain conditions, running, jumping or darting replaces freezing as the dominant CR. These findings raise both a critical methodological problem and an important theoretical issue. If only freezing is measured but rodents express their learning with a different response, then significant instances of learning, memory, or fear may be missed. In terms of theory, whatever conditions lead to these different behaviors may be a key to how animals transition between different defensive responses and different emotional states. In mice, we replicated these past results but along with several novel control conditions. Contrary to the prior conclusions, running and darting were primarily a result of nonassociative processes and were actually suppressed by associative learning. Darting and flight were taken to be analogous to nonassociative startle or alpha responses that are potentiated by fear. Additionally, associative processes had some impact on the topography of flight behavior. On the other hand, freezing was the purest reflection of associative learning. We also uncovered a rule that describes when these movements replace freezing: When afraid, freeze until there is a sudden novel change in stimulation, then burst into vigorous flight attempts. This rule may also govern the change from fear to panic.

Introduction

Fear limits the behaviors available to an animal to its species-specific defense reactions (SSDRs), thereby precluding more flexible voluntary behavior (Bolles, 1970). This characteristic is one reason that conditions characterized by high fear levels such as anxiety disorders are so maladaptive (Fanselow, 2018). It is also one reason that Pavlovian fear conditioning is so easy to measure in the laboratory, one can simply measure innate defensive responses (i.e., SSDRs) to diagnose fear and fear-related memory. This has made fear conditioning one of the major rodent assays of learning, memory and anxiety disorders. Over the last four decades fear conditioning studies have extensively used one of these defensive behaviors, freezing, more than any other response (Anagnostaras et al., 2010; Bouton & Bolles, 1980; Do-Monte et al., 2015; Fanselow & Bolles, 1979; Grewe et al., 2017; Kim & Fanselow, 1992; Kwon et al., 2015; Nader et al., 2000; Roy et al., 2017). Freezing is a common and adaptive defensive behavior as it reduces the likelihood of detection and attack by a predator (Fanselow & Lester, 1988).

However, if rodents have multiple defensive responses, an important theoretical question is what are the conditions that select between different SSDRs (Fanselow, 1997). An influential model of SSDR selection applied to both humans and rodents is Predatory (or Threat) Imminence Continuum theory, which states that qualitatively distinct defensive behaviors are matched to the psychological distance from physical contact with a life-threatening situation (Bouton et al., 2001; Fanselow & Lester, 1988; Mobbs, 2018; Mobbs et al., 2007). Stimuli that model particular points along this continuum elicit behaviors appropriate to that level of predatory imminence. For example, rodents freeze when they detect a predator but show vigorous bursts of activity to contact by the predator (Fanselow & Lester, 1988). The former, labeled post-encounter defense, relates to fear-like states. The latter, referred to as circa-strike defense, relates to panic-like states (Bouton et al., 2001; Perusini & Fanselow, 2015). According to this account, in fear conditioning experiments the shock US models painful contact with the

predator and therefore invariably produces circa-strike activity bursts but not freezing (Fanselow, 1982). On the other hand, stimuli associated with shock such as an auditory CS, models detection of a predator and therefore invariably produces post-encounter freezing as a CR but not activity bursts (Fanselow, 1989).

Recently, there have been reports that challenge this view. Fadok et al. (2017) used a unique two component serial compound conditional stimulus (SCS) consisting of a 10-sec tone followed immediately by a 10-sec white noise ending with a 1 sec shock and found that the initial component (tone) produced freezing, while the second component (noise) produced bursts of locomotion and jumping in mice. Gruene et al. (2015) reported that in rats a tone CS resulted in a similar burst of locomotion, labelled darting, and this was replicated in subsequent studies (Colom-Lapetina et al., 2019; Mitchell et al., 2022). The results were interpreted as a competition between “active” and “passive” defenses. These findings not only challenge the above response selection rule but, also call for a “reinterpretation of rodent fear conditioning studies” because if only one SDR is measured (e.g., freezing) but the situation is characterized by a different SDR, fear and fear-related learning may be misdiagnosed (Gruene et al., 2015). Also note that contrary to Predatory Imminence Theory, Gruene et al. (2015) suggested that freezing and darting were competing CRs to the same level of threat (Fanselow, 1989).

Both previous reports concluded that these activity bursts were CRs because they increased over trials during acquisition when CS and US were paired and decreased during extinction when the CS was presented alone (Fadok et al., 2017; Gruene et al., 2015). While these behavioral patterns are certainly properties of a CR, they are not diagnostic of associative learning as these changes could also result from nonassociative processes such as sensitization and habituation (Rescorla, 1967). Additionally, no formal assessment was made of what properties of the CS led to the alternate CRs (e.g., its serial nature, the ordering of the two sounds, or stimulus modality). One subsequent study using this serial conditioning procedure in

mice has suggested that this white-noise elicited activity burst is mainly a result of the stimulus salience or intensity of the white noise and does not depend on any particular temporal relation to the US (Hersman et al., 2020). Another recent study using this procedure in rats has suggested that this flight behavior only occurs in contexts in which fear has been established and is a result of associative processes (Totty et al., 2021). Therefore, to better understand the associative nature of these flight responses, we embarked on a series of experiments to test these theoretical views and assess the validity of these concerns (**Tables 1-1 - 1-4**).

Results

Experiment 1

Experiment 1 was conducted as delineated in **Table 1-1** (see **Figure 1-1** for a schematic representation of the serial conditioned stimulus and the design for training and testing for Experiment 1). The first condition was a nearly exact replication of the conditions used by Fadok et al. (2017), using male and female mice (Replication Group). Briefly, animals received 10 pairings of footshock and the two-component stimulus (10-sec tone followed by 10-sec white noise) over 2 days before being tested on the third day with the two-component stimulus (see **Figure 1-1-figure supplement 1** to view example velocity traces from Day 1 training for one mouse in the Replication Group). We scored bursts of locomotion and jumping with a Peak Activity Ratio (PAR; Fanselow et al., 2019) and the number of darts (Gruene et al., 2015). PAR reflects the largest amplitude movement made during the period of interest, while darts reflect the frequency of large movements during the same period (see **Methods**). We included two additional groups in this experiment to test the nature of any observed behaviors. We first asked whether any observed behavior occurred to the noise specifically because it was embedded in a serial compound and/or because of the brevity of the noise (10 sec). For this group, we simply conditioned and extinguished a 10-sec white noise (CS Duration Group). In a third group

of mice, we also asked whether the noise-elicited flight behavior required the noise to be present during training. These mice were trained with a 20-sec tone, but tested with the two-component serial compound stimulus (Stimulus Change Group).

In the nearly exact replication of the conditions used by Fadok et al. (2017), using male and female mice, we obtained nearly identical results with our Replication Group (**Table 1-1, Figure 1-2**). For this and all experiments described below, no effects of sex were observed in initial comparisons/ANOVAs (see **Discussion**). Sex was thus removed as a factor in subsequent statistical analyses. In the Replication group, freezing to the initial tone progressively increased over the course of conditioning. At the beginning of training, freezing increased to the white noise but plateaued after a few trials. When freezing plateaued the noise elicited activity bursts, and this pattern maintained throughout acquisition and the beginning of extinction testing. As extinction testing continued, freezing was maintained while PAR and darting to the noise decreased.

Then, we directly asked whether the plateau in freezing and increase in activity that occurred to the noise required the noise to be a component of a serial compound stimulus. We simply conditioned and extinguished a 10-sec white noise (CS Duration Group) and found that freezing increased linearly during a 10 sec pre-noise period reflecting the acquisition of contextual fear conditioning (Kim & Fanselow, 1992; **Figure 1-2-figure supplement 1**). During testing the reaction to onset of the white noise was almost a duplicate to what we saw when the noise was embedded in the compound. In other words, activity bursts and darting in no way depended on the use of a serial compound.

To further probe the necessity of the compound and the presence of the noise during acquisition/shock-pairings, we trained a third group of mice with a 20-sec tone instead of the compound but tested them with the serial compound stimulus (Stimulus Change Group). During

these shock-free tests the noise evoked a very similar PAR and darting behavior to when training was with the compound (**Figure 1-2**). What is striking about this finding is that even though the noise was never paired with shock it still evoked an activity burst. While behavior at test generally looks very similar for the Stimulus Change and Replication groups, direct statistical comparisons reveal some minor differences. For PAR, a repeated measures ANOVA with Trial, Group, and CS type revealed a main effect of CS type [$F(1,24) = 53.121, p < .001$] as well as a Trial X Group interaction [$F(15,360) = 1.970, p = .017$] such that Stimulus Change animals showed greater PAR on trials 2 and 5 (p 's = .003, .05). Further, for only the Stimulus Change group, PAR decreased over the session ($p = .034$). For Darting, a repeated measures ANOVA with Trial, Group, and CS type revealed a main effect of Group [$F(1,24) = 4.321, p = .048$] such that the Stimulus Change group darted more than the Replication group, with no interaction with CS type. For both PAR and darting, despite unreliable interactions with CS type in the ANOVAs above, additional ANOVAs were run on responding for each CS type individually to assess potential impacts of group on tone vs noise responding specifically. In each of these ANOVAs, there were no reliable effects of group (all p 's > .05). Thus, if there are any differences between Replication and Stimulus Change groups, it is that the Stimulus Change group (no noise-shock pairing) generally show more flight than Replication group (noise-shock pairing). These findings strongly implicate nonassociative processes in the activity burst rather than conditioning.

Overall in Experiment 1, we replicated findings that different defensive behaviors develop to separate components of a serial CS (Replication Group). This pattern of behavior holds true if the noise is presented by itself during training (CS Duration group), and this pattern of behavior at testing does not require the noise to be present during training (Stimulus Change Group). Despite differences in behavioral procedures used across acquisition and extinction, we sought to examine any differences in reactivity to the noise during extinction testing between these

three groups. We directly analyzed velocity data across the three groups (**Figure 1-3**). We focused on the first four trials of extinction testing as this is when the majority of the darting behavior occurred, and we further narrowed our analyses to the 10s Noise period as all groups received at least the 10s noise at test.

A mixed model ANOVA revealed a significant effect of Time, [$F(19,361) = 8.203, p < .001$] as well as a Group X Time interaction [$F(38,361) = 1.497, p = .034$]. Generally, velocity peaked during the first bins of the noise period and then quickly decreased to more stable levels. Post-hoc analyses revealed that the Stimulus Change group trended to have the elevated velocity during the first bin of the noise period with trends for higher velocity than the CS Duration group ($p = .09$) and did have significantly higher velocity than the CS Duration group during the fifth bin (~2.5 seconds into the noise; $p = .04$).

While the noise did not need to be within a serial compound stimulus or even need to be presented during training in order to elicit flight, it is worth noting that the strongest noise-elicited flight occurred for the group that received the serial compound stimulus only at test and for which the noise was novel at test.

Experiment 2

The mice that received the 20 sec tone during training but the compound during testing showed darting to the noise embedded in the compound (**Figures 1-2, 1-3**). Since the noise was not paired with the shock, this suggests that the response to the noise was nonassociative.

However, it is possible that during the initial test trials the response to the noise occurred via second-order conditioning as the noise was paired with the previously reinforced tone. This seems unlikely because most darts were seen at the beginning of testing and decreased over the session. A second-order conditioning interpretation suggests the opposite pattern.

Nonetheless, in a second experiment, we included classic controls to directly test for the phenomenon of pseudo-conditioning (**Table 1-2**). Pseudo-conditioning is a form of sensitization whereby mere exposure to the US changes behavior to the stimulus used as a CS (Underwood, 1966), and this appears to be what was observed in Experiment 1 (Stimulus Change Group; **Figure 1-2**). Two pseudoconditioned groups of mice simply received the same shock schedule used in the prior study without any auditory stimuli (no CS). A third was merely exposed to the chamber. The final group was a conditioning group that received noise-shock pairings. All groups received tests with the 10 sec noise, except for one of the pseudoconditioning groups that was tested with the tone.

Figures 1-4 and 1-5 summarize the test results from Experiment 2 (see **Figure 1-4-figure supplement 1** for trial-by-trial data). As would be expected for a CR, freezing to the noise was greatest in the mice that received noise-shock pairings [$F(3,28) = 11.76, p < .001$]. Significant associative learning was indicated by more noise-elicited freezing in the paired group than the shock-only trained group tested with the noise. Interestingly, the No Shock group that was tested with the noise gradually increased freezing over the course of noise testing (**Figure 1-4-figure supplement 1**) suggesting that the 75dB noise itself was aversive to the mice and could support some conditioning of freezing (i.e., it was a weak US).

The test session data were very different for activity bursts (**Figures 1-4 and 1-5**). The greatest PAR occurred in the pseudoconditioned control (shock only during training) that was tested with the novel noise [$F(3,28) = 20.085, p < .001$]. The pseudoconditioned control tested with the novel noise also showed the most darting behavior. Furthermore, these results are supported by a direct analysis of velocity data during the 10s CS period at test (**Figure 1-5**).

A mixed model ANOVA on the averaged velocity measures during the CS period for the first 4 trials of the test session revealed significant effects of Group [$F(3,28) = 5.796, p = .003$] and

Time, $[F(4.06, 113.69) = 6.038, p < .001]$ as well as a Group X Time interaction $[F(12.18, 113.69) = 2.695, p = .003]$. Generally, velocity again peaked during the first bins of the noise period and then quickly decreased to more stable levels. Post-hoc analyses revealed that the Shock Only-Noise Test group had the highest velocity during the second bin of the noise period (the first second of the CS) with significantly higher velocity than the No Shock-Noise Test ($p = .03$), Shock Only-Tone Test ($p = .004$) and, importantly, the Noise Shock-Noise Test groups ($p = .007$).

Pseudoconditioning is indicated by more activity during the noise test in the previously shocked mice than the no-shock controls tested with the same noise. Note that for both of these groups the noise was novel during testing so it had no association with shock. It is worth noting that we also see indirect evidence of pseudoconditioning to the tone, such that the Shock Only Tone Test group does show an elevated PAR with respect to the non-shocked controls tested with the noise. While darting was very low in this group, it was not zero, suggesting that a novel pure tone stimulus may also support cue-elicited flight behavior in frightened animals, although to a lesser extent than a white noise stimulus, which may have inherently aversive properties (continued in **Discussion**). Another striking finding is that while the group that received noise-shock training showed an elevated PAR, the level was significantly less than the pseudoconditioning control ($p < .001$). Not only are activity bursts not conditioned, these data suggest conditioning may actually suppress such activity bursts. In other words, flight and darting are primarily a result of nonassociative processes and are likely not CRs.

Experiment 3

In a third experiment, we included a control group in which the shock and noise were explicitly unpaired to again test for the phenomenon of pseudo-conditioning but in a situation where

exposure to the CS is equated during training (**Table 1-3**). One group was again a conditioning group that received noise-shock pairings, and one group was again a pseudoconditioned group that only received shocks without any CS. A third group received equal numbers of noise and shock presentations but in an explicitly unpaired manner. An additional control group received presentations of only the white noise CS to examine whether or not the CS alone was able to support conditioning and/or activity bursts.

Acquisition and test results are summarized in **Figures 1-6 and 1-7**. As seen in the prior experiments, across training freezing to the white noise rose, and then plateaued in the Paired and Unpaired groups, at which point the noise began to elicit activity bursts. In the CS only group white noise alone supported low, but consistent levels of freezing but in the shocked groups the noise disrupted freezing to the context. During training, the Paired and Unpaired groups showed elevated PAR to the noise [$F(3,28) = 29.94$, $p < .001$ for Day 1; $F(3,28) = 75.18$, $p < .001$ for Day 2], and increased darting to the noise [$F(3,28) = 9.392$, $p < .001$ for Day 1; $F(3,28) = 29.746$, $p < .001$ for Day 2]. Interestingly, for darting, the Paired group showed elevated responding on both Day 1 ($p = .017$) and on Day 2 ($p = .004$) compared to the Unpaired group. During testing, activity bursts (measured as both PAR and darting) to the noise were elevated in all groups that received shock [$F(3,28) = 13.35$, $p < .001$ for PAR; $F(3,28) = 8.160$, $p < .001$ for darting]. Again, similar to training, darting appeared to be the most elevated in the Paired group on Trial 1 of testing.

While overall darting was elevated in the Paired group [during acquisition and on the first trial of testing], the velocity traces during testing (**Figure 1-7**) reveal that the magnitude/frequency of the initial activity burst to the noise appears to be reduced in the Paired group, and that increased levels of activity bursts during the latter portion of the CS account for any differences in overall numbers of darts. Indeed, a direct analysis of the velocity data during the 10s Noise CS period at test revealed significant effects of Group [$F(3,28) = 9.733$, $p < .001$], Time,

[$F(5.15, 144.22) = 9.614, p < .001$] as well as a Group X Time interaction [$F(15.45, 144.22) = 2.045, p = .02$]. Generally, as seen in prior experiments, velocity again peaked during the first bins of the noise period and then quickly decreased to more stable levels. In the Paired group specifically, there is an additional peak of activity in the latter half of the stimulus period. Post-hoc analyses revealed that the Unpaired group had the highest velocity during the first bin of the noise period (the first second) with significantly higher velocity than the CS Only Group ($p = .007$). Additionally, in the 16th and 17th bins towards the end of the CS period, the Paired Group showed the most activity with significantly higher velocity than the CS Only Group ($p = .002$ & $p = .001$), the Shock Only Group ($p = .001, p = .02$), and the Unpaired Group ($p < .001, p = .003$). That pairing noise and shock altered the timing of the activity bursts is an interesting fact worth considering and suggests that pairing noise and shock may have primarily resulted in a conditioned freezing response that in fact competes with/reduces any initial non-associative activity/bursting to the white noise. Taken together, this and the prior experiment using control groups to assess pseudoconditioning reveal that a large portion of the noise-elicited activity bursts observed are due to non-associative processes that result in an increase in darting behavior to the noise following shock exposure, regardless of any direct training history of the noise with shock. There does appear to be evidence that pairing noise with shock may further increase or alter the timing of this behavior, but by no means is pairing noise with shock necessary to produce these activity bursts.

Experiment 4

The experiments thus far have suggested that much of the white-noise-elicited activity bursting is a non-associative process. We have also shown that novelty of the CS at test may increase this noise-elicited activity (**Figures 1-3 & 1-4**). In a final, fourth experiment, we explicitly tested

whether habituation to the white noise stimulus prior to noise-shock training would be able to reduce noise-elicited activity bursts. If increased levels of novelty of the CS are driving noise-elicited activity bursts, then prior habituation should reduce the levels of darting to the noise CS. In this experiment, we had four groups that differed in whether they received an additional two days of habituation to the white noise stimulus (5 noise presentations each day) and whether they received noise-shock pairings during training or just shock only (**Table 1-4**). One comparison of particular interest was between the habituated or non-habituated Shock Only groups as these groups would directly compare whether prior experience with the CS would decrease darting at test compared to a group for which the CS was completely novel.

Figure 1-8 shows the results of Experiment 4 during testing (see **Figure 1-8-figure supplement 1** for trial-by-trial results for freezing, PAR, and darting across habituation, training, and testing). During the two days of habituation, interestingly, we found that within groups that received habituation, a low level of darting to the white noise alone without any shock decreased across day one [$F(4,48) = 2.887, p = .026$] and increased by the end of the second day of habituation [$F(4,48) = 2.793, p = 0.36$] (**Figure 1-8-figure supplement 1**). Concurrently, freezing to the white noise increased over habituation trials, again showing that this white noise stimulus alone can act as a US. It is interesting that darting occurred to the white noise at the start of habituation when the CS was very novel, and at the end of habituation once the white noise alone was able to support some level of fear.

Comparing the two Shock Only groups during test, the noise disrupted freezing more than tone. In this regard noise seems to act like a weak shock US (Fanselow, 1982). Like shock it disrupts freezing (**Figure 1-4-figure supplement 1**) and like shock it supports conditioning of freezing (**Figure 1-6**).

Within Paired groups (H-Paired and C-Paired), we found that throughout acquisition and particularly on the second day of training (**Figure 1-8-figure supplement 1**), prior habituation to the white noise increased freezing [$F(1,24) = 5.701, p = .025$] and decreased noise-elicited darting [$F(1,24) = 5.130, p = .033$], as predicted if prior exposure to the CS functions to reduce any partially novelty-driven darting. We again saw that freezing to the white noise initially increased during acquisition, but as the darting response begins to become more apparent, freezing decreases to medium levels. At test (**Figure 1-8 & Figure 1-8-figure supplement 1**), for freezing, we found a main effect of pairing [$F(1,24) = 11.306, p = .003$], such that animals who received white noise paired with shock froze more than animals who only received shock during acquisition, again indicative that noise-elicited freezing is a conditional behavior that results from associative learning. For darting behavior, we found a Habituation X Pairing interaction [$F(1,28) = 4.939, p = .035$] such that pairing white noise with shock increased darting within habituated animals ($p = .033$), and that habituation reduced darting within animals who only received shock during training ($p = .045$). These results reveal multiple points of interest. First, and as shown in prior experiments, the white noise acts as a US on its own and need not be paired with shock to produce darting at test. Merely experiencing the shock is enough to produce darting to the white noise at test (pseudoconditioning due to sensitization). Furthermore, prior experience with the white noise, through habituation, actually reduced this darting at test. Additionally, in this experiment, we do again show evidence that pairing white noise with shock can further increase darting behavior at test, at least within animals who have already experienced the noise during habituation. Again, as with Experiment 3 (**Figure 1-7**) the timing of the darting response in Paired groups is fundamentally altered compared to Shock Only groups (**Figure 1-8**). The magnitude/frequency of the initial activity burst to the noise appears to be reduced in the Paired groups, and increased levels of activity bursts during the latter portion of the CS account for any differences/increases in overall numbers of darts.

Indeed, a mixed model ANOVA with Pairing, Habituation, and Time as factors on the averaged velocity traces for each trial revealed significant effects of Time [$F(56,1568) = 17.420, p < .001$], a Habituation X Pairing interaction [$F(1,28) = 4.696, p = .04$], and a Pairing X Time interaction [$F(56,1568) = 3.036, p = .01$]. Generally, once again, velocity peaked during the first bins of the noise period and then quickly decreased to more stable levels. As seen in the experiments above, again, this initial peak in velocity was most apparent in the Shock Only groups, with the Paired groups showing an initially smaller peak in velocity. Post-hoc analyses revealed that the Shock Only groups had significantly higher velocity during the first three bins of the noise than the Paired groups (p 's = .02, .03, .005 respectively). Post-hoc analysis on the Pairing X Habituation interaction reveal that within the non-habituated groups, pairing noise and shock significantly reduced the velocity throughout test trials ($p < .001$). Additionally, within Shock Only groups, habituation reduced the velocity throughout test trials ($p < .001$). These results are exactly what would be predicted if exposure to the noise CS (through pre-exposure and/or through pairing CS and US) in fact reduces noise-elicited activity bursts and flight/darting behavior, that is, darting is enhanced by novelty.

All Experiments-Analysis of Dart Timing and Topography

While the majority of the data presented here suggest that cue-elicited flight or darting are due primarily to non-associative influences, we do show evidence that associative processes/pairing noise and shock alter the timing/topography of such flight behavior. Thus, we set out to further analyze these differences in dart timing, and in particular, we were interested in whether initial darts at CS onset may be functionally distinct from darts that occur later on in the CS period.

Figure 1-9 shows a detailed analysis of darting magnitude and timing collapsed across all experiments for all animals that received shock during training. First, **Figures 1-9A** and **1-9B** represent the magnitude of darts to the tone and noise stimuli during testing, as well as the

reaction to the first shock on Day 1 of training. Generally, there was an effect of stimulus on response magnitude [$W(2,123.2) = 105.3, p < .0001$]. The magnitude of response to shock was greater than to tone ($p < .0001$) and to noise ($p < .0001$), and darts to the noise tended to be stronger than darts to the tone ($p = .043$). **Figure 1-9C** shows the average magnitude of darting responses when an individual animal performs two darts within one stimulus (total $n = 65$ 'multi-darts'). On average, within a given single CS presentation, the magnitude of the response for the first dart was greater than for the second dart ($t = 2.641, df = 64, p = .01$). The magnitude of darts that occurred during the initial 3s of the 10s CS period and those that occurred during the final 7s of the 10s CS period are shown in **Figures 1-9D** (by group and stimulus) and **1-9E** (collapsed across groups and stimulus). An omnibus ANOVA with Group and Stimulus Period (Early vs Late) revealed a significant effect of Stimulus Period [$F(1,331) = 16.23, p < .0001$] with no effects of/interaction with Group. Darts that occurred early during the initial CS onset were large in magnitude than those that occurred later in the session, suggesting that these two responses may in fact be distinct types of flight behavior (see **Discussion**).

Discussion

Prior work reported that contact/pain-related stimuli (e.g., shock) disrupt post-encounter freezing and provoke panic-like circa-strike defensive behaviors (Fanselow, 1982). The current results suggest a modification of the rules governing a transition between these behavioral states. The rule is that when you are in the post-encounter mode (fear) a sudden change in stimulation, particularly the onset of an intense novel stimulus, can cause an immediate transition to the circa-strike mode (panic). Indeed, the vast majority of the activity bursts/darting behavior occurred at the onset of the stimulus (**Figures 1-3, 1-5, 1-7, 1-8**). The effectiveness of this transition depends on the qualities of the stimulus. Stronger shocks cause a greater disruption

of freezing and a longer activity burst, yet the same stronger shocks simultaneously condition more freezing to the prevailing cues (Fanselow, 1982). The current data call for an expansion of this rule to nonnociceptive stimuli. Like shock, both tone and noise disrupted ongoing freezing, the noise did so for longer than the tone (**Figure 1-10**), and noise on its own was able to support a minimal level of fear conditioning (**Figure 1-6, Figure 1-4-figure supplement 1, Figure 1-8-figure supplement 1**). The rule is: when in a state of fear (Post-encounter defense) sudden stimulus change provokes panic-like circa-strike defenses proportional to stimulus intensity and novelty.

As the majority of the experiments presented here and in most prior studies conduct both training and testing in the same context (Fadok et al., 2017; Gruene et al., 2015, Hersman et al., 2020), these animals would already be in a high state of fear or post-encounter defense (from any learned contextual fear during training), thus endowing the presentation of the white noise to be a particularly startling stimulus change that can provoke these panic-like flight responses. Novelty of the stimuli is an important factor and familiarity with the CS during conditioning and/or habituation reduced CS novelty for the test. In the experiments presented here, the mice that received noise-shock pairings and were tested with noise showed lower flight to the noise than mice trained only with shock and then received noise for the first time. Additionally, prior habituation to the noise or experience with the noise during training (i.e., paired and unpaired groups) further reduced noise-elicited flight at test.

Another important factor to consider is the timing of the activity burst with respect to CS and US onset. With poorly timed and sustained conditional responses such as freezing the CR tends to fill the entire CS-US interval and spill over beyond the time of expected US delivery (e.g., Ayres & Vigorito, 1984; Gale et al., 2004). However, shorter duration ballistic responses such as the darting response allow a clearer assessment of when the CR occurs with respect to CS and US delivery and such CRs are expected to anticipate US delivery. Hull (1934) cautioned

conditioning researchers that it is important to distinguish true conditional responses from unconditional responses to the CS, which he named alpha responses. These alpha responses occur at the onset of the CS, rather than the time of the expected US. Alpha responses have been most studied with the Pavlovian conditional eyeblink response, where the true CR is well-timed to US delivery (McCormick & Thompson, 1984, Perrett et al, 1993). Blinks that occur to CS onset are classified as alpha responses, which are considered to be nonassociative startle responses to the CS and not CRs (e.g., Gerwig et al., 2005; Nation et al., 2017; Schreurs and Alkon, 1990, Woodruff-Pak et al., 1996). Typically, in eyeblink studies alpha responses are excluded from analysis by omitting any responses that occur at the beginning of the CS. Our darting responses almost exclusively occurred at CS onset and there were rarely any US anticipatory-like responses. Thus, traditional Pavlovian analyses for ballistic CRs would have categorized darting as an unconditional alpha response and not a bona fide CR. Consistent with this analysis is that darting occurred to the noise during the first few trials of the habituation session in Experiment 4 (**Figure 1-8-figure supplement 1**).

Our interpretation that noise unconditionally elicits a ballistic activity burst bears some relationship to the unconditional acoustic startle response. Loud noises will elicit an unconditional startle response that wanes with repeated presentations of that noise (i.e., habituation; e.g., Davis, 1980; Hoffman & Fleshler, 1963; Leaton, 1976). The unconditional startle response to the loud noise can be potentiated if the loud noise is delivered in the presence of a cue or a context that has been associated with shock/fear (Brown, et al., 1951; Davis, 1989). While our 75 dB noise stimulus is less intense than the 98-120 dB noise used in typical acoustic startle studies, we observed an unconditional noise-elicited response that also decreased with habituation (Experiment 4). Furthermore, our data and those of Totty et al. (2021) indicate that these responses require a fearful context in order to occur. Fear is well known to potentiate startle responses (Brown, et al., 1951; Davis, 1989). Perhaps the low

intensity noise is below threshold to elicit a startle response on its own, but a fearful context potentiates this unconditional startle response and brings it above threshold. Additionally, there appears to be considerable overlap in the neuroanatomy that supports this circa-strike behavior and fear potentiated startle. Totty et al. (2021) found that inactivation of the Central Nucleus or the Bed Nuclei of the Stria Terminalis disrupts the flight response. These two regions have been shown to be important mediators of fear's ability to potentiate startle (e.g., Campeau & Davis, 1995; Davis & Walker, 2014). Furthermore, Fadok et al. (2017) reported that it is corticotropin releasing hormone (CRH) expressing cells, but not somatostatin expressing cells, within the Central Nucleus that support flight behavior. Again, there is extensive data implicating CRH and fear potentiated startle (Lee & Davis, 1997).

It is of note that the relationship between startle (circa-strike defense) and freezing (post-encounter defense) was described by Fanselow & Lester (1988) when accounting for how rats rapidly transitioned between these behaviors when a detected predator launches into attack. "It is as if the freezing animal is tensed up and ready to explode into action if the freezing response fails it. This explosive response probably has been studied in the laboratory for over 30 years under the rubric of potentiated startle...It seems that the releasing stimulus for this explosive motor burst is a sudden change in the stimulus context of an already freezing rat (Fanselow & Lester, 1988, p 202)."

Neither Fadok et al. (2017) nor Gruene et al. (2015) included any controls for nonassociative behavior, which is something required in order to conclude that a response is conditional (Rescorla, 1967). Both of these research groups concluded from their single group experiments that flight/darting was a CR because the behavior increased with successive shocks during the shock phase and decreased with shock omission during the test phase, likening these behavioral changes to acquisition and extinction. While acquisition and extinction are characteristics of a CR, learning theorists have never taken these as diagnostic of a CR. For

example, increases in responding with successive shocks could arise via sensitization and decreases in responding when shocks are omitted could arise from habituation. Indeed, that is exactly what we believe caused these behavioral changes that we also observed in our study. Shocks, by conditioning fear to the context, sensitize or potentiate the darting response and repeated presentations of the noise alone cause the response to habituate. The behavior of our pseudoconditioning control provides clear evidence of this. Just giving shocks conditioned fear to the context such that when the noise was presented for the first time during test it caused a strong activity burst. The behavior gradually decreased during testing because repeated presentations of the noise led to habituation of this unconditional response.

Given our argument that the flight/darting behavior is nonassociative, Totty et al.'s finding that noise-shock paired rats showed more noise elicited activity burst behavior than rats that had unpaired noise and shock requires additional comment. Since both unpaired and paired rats were exposed to noise during acquisition those exposures could lead to habituation of the unconditional response to the noise. However, it would be expected that habituation would be greater in the unpaired group because pairing a stimulus (noise in this case) with another stimulus (shock in this case) is known to reduce the magnitude of habituation (Pfautz et al., 1978). This reduction in habituation is observed even if the second stimulus is not an unconditional stimulus (Pfautz et al., 1978). Additionally, pairing a habituated stimulus with a US can also cause a return of the habituated alpha response and this loss of habituation is not observed when the two stimuli are not paired (Holland, 1977). Thus, the difference between the paired and unpaired groups reported by Totty et al. (2021) are likely due to differential habituation of the noise during training. This effect of habituation was probably enhanced by Totty et al. including a noise habituation phase prior to training.

While many of the findings presented in Totty et al. (2021) are in line with our own or can be readily explained through a non-associative lens, Figure 5 of Totty et al. presents data in which

noise-elicited flight is greater for animals that have SCS-US pairings compared to those that have received only the US during training (comparable to Paired vs Shock Only groups in Experiments 2 - 4 here). It is possible that Totty et al.'s additional habituation to the SCS in an alternate context prior to SCS or US-only training is a potential explanation for this difference. Indeed, the results here from Experiment 4 in which habituation was conducted in the training/testing context suggest that such habituation would primarily function to reduce darting, particularly in groups that only had US presentations. The use of a serial tone-noise compound may have also served to further reduce a startle response to the noise in Totty et al. It is well known that if a startle stimulus is preceded by another stimulus, the startle response is reduced in a phenomenon called pre-pulse startle inhibition (Louthan et al., 2020; Groves et al., 1974). Thus, two factors may contribute to Totty et al.'s failure to see darting behavior in the animals that did not receive SCS-Shock pairings: the use of habituation prior to conditioning and the use of a two-stimulus compound. Another factor that may be important is species. Overall, the amount of darting seen in the studies using mice (Fadok et al., 2017; Hersman et al., 2020; and the present study) was greater than in the studies using rats (Gruene et al., 2015; Totty et al., 2021).

Beyond this potential explanation, as mentioned above and detailed below, there are likely at least two different topographical types of locomotion occurring, and such cue-elicited locomotion behaviors in rats in previous studies may preferentially be 'movement' that is part of the freezing suite of behaviors, which include locomotion to an ideal thigmotactic place to freeze (e.g., Fanselow & Lester, 1988). Associative processes would be expected to alter/increase such increased locomotion as it supports/is a part of freezing behavior.

Experimenters that have examined running-like locomotion in fear conditioning situations tend to collapse the behaviors under a single label such as darting, flight or escape (Colom-Lapetina et al., 2019; Fadok et al., 2017; Gruene et al., 2015; Mitchell et al., 2022; Totty et al., 2021). These

behaviors are then thought of as “active” behaviors that compete with a “passive” freezing response (Fadok et al., 2017; Gozzi et al., 2010; Gruene et al., 2015). It is important to recognize that all such movements are not identical and often serve different functions. Here we will focus on two distinct movements we observed in the present experiments. In several instances the mice made two movements in response to stimulus presentation. The first was a very high velocity response that occurred to stimulus onset; the other was a slower velocity movement that tended to occur later on during stimulus presentation (**Figure 1-9C, D, E**). We will discuss these two behaviors within the Predatory Imminence framework and suggest that the slower movement is part of the post-encounter freezing module and the faster one is a circa-strike behavior.

Post-Encounter Movement: Elsewhere we pointed out that the dominant post-encounter behavior, freezing, is not simply immobility (Fanselow & Lester, 1988). Rather, it is an integrated behavior where rats first move to the closest, easily accessible, location appropriate for freezing (Fanselow & Lester, 1988). Typically, this location is against a wall, especially a corner with its two walls (De Oca, Minor, & Fanselow, 2007; Grossen & Kelly, 1972; Sigmundi, 1997). In other words, freezing and thigmotaxis constitute an integrated behavioral module (Fanselow & Lester, 1988). Indeed, when an especially appropriate freezing location, a dark cave, was available rats moved to the cave and froze more than when it was not (DeOca, Minor, & Fanselow, 2007). Therefore, it would be most inappropriate to characterize the movements needed to thigmotax as competing with freezing. They are an integral part of freezing. Consistent with this interpretation, the slower second movement after stimulus onset was directed at a corner and once there the animal became immobile. Since these movements are part of the freezing module and shock-associated conditional stimuli are one of the most effective ways to drive the freezing module, it is not surprising that they may be more frequent in animals that had sound-shock pairings (**Figures 1-4 – 1-8**). We conjecture that the movements seen by Totty et al.

(2021) exclusively in animals that were conditioned were largely this type of movement. Further, it is likely that the flight behavior in rats observed by other groups (Colom-Lapetina et al., 2019; Gruene et al., 2015; Mitchell et al., 2022) is this type of movement as Mitchell et al. report that the majority of their CS-elicited darting occur late in the CS presentation, about 7-10s into a 30s tone CS.

Circa-Strike Activity Bursts: Circa-strike behavior occurs at or immediately before contact with the predator and represents a vigorous evasive movement away from the predator. The most effective circa-strike eliciting stimulus in the laboratory setting is shock, as it is directly analogous to painful contact. As can be seen in **Figure 1-9A-B**, the movement to shock onset is on average the highest velocity movement we observed. The activity burst to shock is highly protean and poorly directed, sometimes looking as if the animal is bouncing off the walls (Fanselow, 1982). If directed at all it, is directed away from the predator and not toward anything in particular. We argue here that when the animal is in the post-encounter fear mode (freezing), the threshold for these bursts of activity shifts such that sudden stimuli that would not normally cause an activity burst now do so. These are the high velocity movements we see to the noise and the tone at stimulus onset (**Figure 1-9**). These are nonassociative responses as they were greatest in animals that never had the sound paired with shock and decreased with familiarity to the stimulus (**Figures 1-5, 1-7, 1-8**). **Figure 1-9A, B** shows these high velocity movement to tone, noise and shock onset. The difference between the stimuli seems quantitative, such that shock>noise>tone, but note that in the violin plot some noise elicited bursts are as fast as the fastest shock responses and many of the shock-elicited bursts overlapped in velocity with those triggered by tone. In all cases these movements seemed protean and undirected.

A final clarifying note is in order regarding stimulus type and how this impacts cue-elicited flight. As described throughout the paper and detailed above, this flight or darting behavior can and does occur to both tone and noise stimuli, regardless of whether they have been paired with

shock or not. There is a slight difference in the magnitude of the response to the tone vs the noise, with the noise having a higher proportion of particularly high-velocity movements—on par with reaction to shock at times. We have also noted that the white noise CS is not really a neutral cue and can/does support fear conditioning as if it were a US. While it is possible that these aversive properties of the white noise stimulus enhance cue-elicited flight in frightened animals, it is clearly not required as the tone can elicit similar flight. Rather than thinking about whether a cue needs to procedurally be a CS or a US to support darting, the data support the hypothesis that when an animal is afraid, any sudden change in stimulation can cause them to shift from post-encounter responses, like freezing, towards more circa-strike responses, like undirected flight. Thus, it is sudden change in stimulation, regardless of the conditional/unconditional properties of the cue, that elicits flight behavior.

An alternative explanation of the altered timing of flight behavior in animals who have had noise-shock pairings is that these animals are more accurately timing the CS-US interval and are showing better temporal discrimination and potential avoidance of an upcoming threat. While our data do not necessarily rule out this explanation, the bulk of our results suggest that the majority of the cue-elicited flight behavior observed near cue onset in our experiments was non-associative in nature. The flight behavior we observed later on in the cue presentation, which may have an associative component, may however be temporally driven. However, it is worth pointing out that this second burst of activity was also not particularly well-timed to US delivery, it tended to occur in the middle of the noise presentation (see noise-shock group in **Figure 1-5**). Future studies that vary the length of the CS in animals who have US-only vs Paired CS-US presentations during training are warranted and would help address this alternative explanation.

Initial reports suggest a sex difference in this noise-elicited flight behavior such that female rats show more of this behavior than males (Gruene et al., 2015; Mitchell et al., 2022). Within each experiment, we found no such sex differences between male and female mice for the PAR and

darting measures of flight behavior, and Totty et al. (2021) similarly found no sex differences in such behavior in male and female rats. To further increase the power of such an analysis for sex differences, we pooled all of the groups across the four experiments that received noise-shock pairings (**Figure 1-11**). In this analysis, again, we saw no sex differences in flight to the white noise across the two days of acquisition for PAR [Day1: $F(1,29) = .323$, $p = .58$; Day 2: $F(1,29) = .507$, $p = .48$]. For darting, there were no impacts of sex on Day1 [$F(1,29) = .009$, $p = .92$]. On Day 2, there was a trend for a main effect of sex [$F(1,29) = 3.752$, $p = .06$] and CS presentation X Sex interaction [$F(4,116) = 2.558$, $p = .042$], such that males darted more than females on the second CS presentation. We further observed no sex differences across testing to the white noise in extinction for both PAR [$F(1,20) = .099$, $p = .76$] and darting [$F(1,13) = 1.397$, $p = .258$]. Thus, we show no major sex differences other than a potential increase in male darting on Day 2 of acquisition. Perhaps initial reports of sex differences with more frequent darting in females could be explained by differences in handling and stress provided to females as a result of monitoring estrous phase, a potentially stressful procedure for the animals for which there is not an ideal control in males. However, results were recently replicated in which females show more darting than males in a sample of animals for which estrous cycle was not monitored (Mitchell et al., 2022). It is worth noting that Gruene et al. (2015), Colom-Lapetina et al. (2019), and Mitchell et al. (2022), generally find very low levels of darting, with a majority of animals classified as non-darters. With the procedure used here, and in mice, we generally do not observe different subpopulations of darters vs non-darters (**Figure 1-11**). While there is certainly variation in the level of darting between animals, all animals across all experiments were shown to dart at least once and would be classified as darters using the criteria set forth in Gruene et al. (2015). Finally, while we show no major differences in tone-elicited flight vs noise-elicited flight, we do show some evidence that darts to the tone may be less strong than darts to the noise (**Figure 1-9A**). Perhaps there are species-differences in such

stimulus-evoked flight behavior, such that tone-elicited flight in rats is more sensitive to impacts of sex.

Some have characterized freezing as a passive response that occurs because no other response is available (Blanchard & Blanchard, 1969; Fadok et al., 2017; Gruene et al., 2015; Yu et al., 2016). However, because motion is often the releasing stimulus for predatory attacks it is the best thing for a small mammal like a rat or a mouse to do when a predator is detected and will only be replaced if there is a change consistent with contact (Fanselow & Lester, 1988).

Rodents choose locations in which to freeze such as corners or objects (thigmotaxis) (Grossen & Kelley, 1972). The current data show that the freezing rodent also prepares to react to sudden stimulus change. There is nothing passive about freezing.

Methods and Materials

Subjects

Subjects for all experiments included 120 C57BL/6NHsd mice (Experiment 1, n = 24; Experiment 2, n = 32; Experiment 3, n = 32; Experiment 4, n = 32), aged 9-11 weeks of age and purchased from Envigo. This C57BL/6NHsd strain was chosen to match that of Fadok et al. (2017). Each group consisted of 4 male and 4 female mice. A necessary/powerful group sample size of 8 was calculated based both on years of data in our lab that suggests n = 8 is sufficient to detect such behavioral differences in fear conditioning studies and on the recent articles in the literature using this procedure. Mice were group-housed four per cage on a 12-hr light/dark cycle with ad libitum access to food and water. Across each experiment, mice in each cage were randomly assigned to one of the groups, ensuring that every group had a representative from each cage to avoid any cage effects. All experiments were conducted during the lights-on phase of the cycle. Animals were handled for 5 days prior to the start of experiments. Subjects were all treated in accordance with a protocol approved by the Institutional Animal Care and Use Committee at the University of California-Los Angeles following guidelines established by the National Institute of Health.

Apparatus and Stimuli

All experiments were conducted in standard MedAssociates fear conditioning chambers (VFC-008; 30.5 x 24.2 x 21 cm), controlled by Med Associates VideoFreeze software (Med Associates, St. Albans, VT). For each experiment, the same context was used for training and testing (see Discussion). The context was wiped down between each mouse with 70% isopropanol and 3 sprays of 50% Windex were added to the pans below the shock grid floors to provide an olfactory cue/context. The US consisted of a 1 second 0.9 mA scrambled shock delivered through a MedAssociates shock scrambler (ENV-414S). Each of the CSs were

delivered using a MedAssociates speaker (ENV-224AM-2). The tone was 7.5kHz. Both the tone and the white noise were 75dB inside the chamber. The inter-trial interval varied between 150 seconds and 210 seconds with an average length of 180 seconds.

Design and Procedure

Mice were handled for 5 days for approximately 1 minute per day prior to beginning the experiment. At the beginning of each day of the experiment, mice were transported in their home cages on a cart to a room adjacent to the testing room and allowed to acclimate for at least 30 minutes. Mice were individually placed in clean empty cages on a utility cart for transport from this room to the testing room and promptly returned to their home cages after the session was over. These transport cages were wiped down with StrikeBac in between trials/sessions.

Experiment 1 was conducted as delineated in **Table 1-1** (see **Figure 1-1** for a schematic representation of the serial conditioned stimulus and the design for training and testing for Experiment 1). The Replication group was trained on each of the two days with 5 presentations of a 10 second tone immediately followed by a 10 second noise, which was immediately followed by a 1 second shock. On Day 3 it was then tested with 16 presentations of a 10 second tone immediately followed by a 10 second noise. These parameters were chosen to match those of Fadok et al. (2017) except that we did not include a session of unreinforced CS preexposure prior to conditioning as such treatment is known to reduce conditioned behavior (Lubow & Moore, 1959; we did add such a treatment to Experiment 4 as an experimental factor). The CS Duration group was trained on each of the two days with 5 presentations of a 10 second noise, which was immediately followed by a 1 second shock. It was tested with 16 presentations of the 10 second noise. The Stimulus Change group was trained on each of the two days with 5 presentations of a 20 second tone immediately followed by a 1 second shock. It

was tested with 16 presentations of a 10 second tone immediately followed by a 10 second noise (i.e., the compound used in the replication group). Two mice were excluded from this study due to experimenter error, one female in the Replication group and one female in the Stimulus Change group.

Experiment 2 was conducted as delineated in **Table 1-2**. The Pseudoconditioned Noise and Pseudoconditioned Tone groups were trained on each of the two days with 5 presentations of a 1-sec shock without any sound using the same schedule for shocks as Experiment 1. The No Shock Control was merely allowed to explore the context for the same length of time as the other groups without receiving any shock or auditory stimuli throughout the two days of acquisition. The final Noise-Shock Conditioning group was trained on each of the two days with 5 presentations of a 10-sec noise, which was immediately followed by a 1-sec shock. As Experiment 1 revealed that similar behavior was observed in groups that received compound stimulus-shock pairings or just noise-shock pairings, we used simple noise-shock pairings in this and some of the following experiments to more specifically assess the associative nature of any white noise-driven behavior. All groups received tests with 16 presentations of the 10-sec noise in extinction, except for one of the pseudoconditioning groups that was tested with the 10-sec tone.

Experiment 3 was conducted as delineated in **Table 1-3**. The Paired Noise-Shock (Conditioning) group was trained on each of the two days with 5 presentations of a 10 second noise, which was immediately followed by a 1 second shock. The Unpaired Noise/Shock group was presented with the same number and length of noise and shocks, but they were explicitly unpaired in time. The Noise-CS Only group received 5 presentations of a 10 second noise without receiving any shocks on each of the two days. The Shock Only (Pseudoconditioning) group received 5 presentations of a 1 second shock on each of the two days. As the main behavioral responses and differences between groups occurred primarily in the first few trials of

the previous experiments, and in order to more readily complete all of the testing within one day's light cycle, for this and the following experiments we reduced the number of test trials presented to the animals. Thus, at test for this experiment, all groups received two presentations of a 10 second noise.

Experiment 4 was conducted as delineated in **Table 1-4**. Prior to training with shock, all groups underwent 2 days of additional training with either habituation to the white noise or merely exposure to the context. The habituated groups, Habituation/Shock Only (H-Shock) and Habituation/Noise-Shock Pairing (H-Paired), were trained on each of the two days with 5 presentations of a 10-second noise, while the two non-habituated groups, Context Exposure/Shock Only (C-Shock) and Context Exposure/Noise-Shock Pairing (C-Paired) received only equivalent exposure to the context. The following two days, as in the Experiments above, all groups received 10 footshocks. The Paired groups (H-Paired and C-Paired) were trained on each of the two days with 5 presentations of a 10-second noise, followed immediately by a 1-second footshock. The Shock Only groups (H-Shock and C-Shock) were trained on each of the two days with only 5 presentations of a 1-second footshock. At test, all groups received 3 presentations of the 10-second noise.

Data, Statistics and Analysis

Freezing behavior for Experiments 1-3 was scored using the near-infrared VideoFreeze scoring system. Freezing is a complete lack of movement, except for respiration (Fanselow, 1980). VideoFreeze allows for the recording of real-time video at 30 frames per second. With this program, adjacent frames are compared to provide the grayscale change for each pixel, and the amount of pixel change across each frame is measured to produce an activity score. We have set a threshold level of activity for freezing based on careful matching to hand-scoring from

trained observers (Anagnostaras et al., 2010). The animal is scored as freezing if they fall below this threshold for at least a 1-sec bout of freezing.

For Experiment 4, due to a technical error, videos for the first 4 days of the experiment could not be accurately assessed for freezing behavior using VideoFreeze. Therefore, we alternatively measured and scored freezing behavior using EthoVision. Briefly, videos were converted to MPEG, as described above, and analyzed using the Activity Analysis feature of EthoVision. Thresholds for freezing were again determined to match hand-scoring from trained observers.

Two different measures of flight were used. We scored bursts of locomotion and jumping with a Peak Activity Ratio (PAR; Fanselow et al., 2019) and the number of darts (Gruene et al., 2015). To determine PAR, we took the greatest between frame activity score during a period of interest (e.g., the first 10 s of CS presentation = During) and calculated a ratio of that level of activity to a similar score derived from a preceding control period of equal duration (e.g., 10 s before presentation of the tone = PreStim) of the form $\text{During}/(\text{During} + \text{PreStim})$. For each CS, the PreStim values were taken from the immediately-preceding 10 s period prior to the CS onset. With this measure, a 0.5 indicates that during the time of interest there was no instance of activity greater than that observed during the control period (PreStim). PARs approaching 1.0 indicate an instance of behavior that far exceeded baseline responding. This measure reflects the maximum movement the animal made during the period of interest.

Darting was assessed as in Gruene et al. (2015). Video files from VideoFreeze were extracted in Windows Media Video format (.wmv) and then converted to MPEG-2 files using Any Video Converter (AnvSoft, 2018). These converted files were then analyzed to determine animal velocity across the session using EthoVision software (Noldus), using a center-point tracking with a velocity sampling rate of 3.75 Hz. This velocity data was exported, organized, and imported to R (R Core Team, 2018). Using a custom R code, darts were detected in the trace

with a minimum velocity of 22.9 cm/s and a minimum interpeak interval of 0.8 s. The 22.9 cm/s threshold was determined by finding the 99.5th percentile of all baseline velocity data analyzed, prior to any stimuli or shock, and this threshold was validated to match with manual scoring of darts, such that all movements at that rate or higher were consistently scored as darts. See **Figure 1-1-figure supplement 1** for representative traces of velocity across Day 1 of acquisition for a mouse in the Replication group of Experiment 1. The PAR measure reflects the maximum amplitude of movement, while the dart measure reflects the frequency of individual rapid movements.

Trial-by-trial Measures of freezing and flight were analyzed with a repeated measure multifactorial analysis of variance (ANOVA) and post hoc Tukey tests. Baseline freezing and overall responding were collapsed across session when appropriate, and then analyzed with a univariate ANOVA test. To directly compare each groups' activity and the magnitude of any flight behaviors during extinction testing, velocity data was binned into .533s bins and subsequently analyzed using repeated measures ANOVA in R. Whenever violations of sphericity were found, the Greenhouse-Geisser correction was used to produce corrected degrees of freedom and p-values. For analysis of darting magnitude and timing, Welch's ANOVA test was used when assumptions of homogeneity of variance were not met. For comparisons of within-subject dart magnitude, paired-sample t-tests were performed. Significant effects and interactions were followed up with simple main effects and Bonferroni-corrected pairwise t-tests. A value of $p < .05$ was the threshold used to determine statistical reliability. For all experiments described above, no effects of sex were observed in initial comparisons/ANOVAs. Sex was thus removed as a factor in subsequent statistical analyses.

Tables

Group	Training Treatment: 10 CS-US Pairings (5 per day)	Testing Treatment: (16 on one day)
1) Replication	10-sec Tone→10-sec Noise→1-sec Shock	10-sec Tone→10-sec Noise
2) CS Duration	10-sec Noise→shock	10-sec Noise
3) Stimulus Change	20-sec Tone→shock	10-sec Tone→10-sec Noise

Table 1-1 Design of Experiment 1

Group	2 Day Training Treatment:	Testing Treatment:
1) Pseudoconditioned Noise/Shock Only Noise Test	10 Shocks (1-mA, 1-sec, 150-210s intertrial interval)	16 Noise Presentations (10-sec)
2) Pseudoconditioned Tone/Shock Only Tone Test	10 Shocks (1-mA, 1-sec, 150-210s intertrial interval)	16 Tone Presentations (10-sec)
3) No Shock Control	Context exposure Only (17-min & 15-sec per day)	16 Noise Presentations (10-sec)
4) Noise-Shock Conditioning	10 Noise (10-sec)→shock pairings	16 Noise Presentations (10-sec)

Table 1-2 Design of Experiment 2

Group	Training Treatment: 10 CS-US Pairings (5 per day)	Testing Treatment: (2 on one day)
1) Paired Noise-Shock (Conditioning)	10-sec Noise→1-sec Shock	10-sec Noise
2) Unpaired Noise/Shock	10-sec Noise & 1-sec Shock – Unpaired	10-sec Noise
3) Noise - CS Only	10-sec Noise	10-sec Noise
4) Shock Only (Pseudoconditioning)	1-sec Shock	10-sec Noise

Table 1-3 Design of Experiment 3—Paired vs Unpaired Noise-Shock

Group	Habituation Treatment: 10 CS Exposures (5 per day)	Training Treatment: 10 CS-US Pairings (5 per day)	Testing Treatment: (3 on one day)
1) Habituation/Shock Only (H-Shock)	10-sec Noise	1-sec Shock	10-sec Noise
2) Habituation/Paired Noise-Shock (H-Paired)	10-sec Noise	10-sec Noise→1-sec Shock	10-sec Noise
3) Context Exposure/Shock Only (C-Shock)	Context Exposure	1-sec Shock	10-sec Noise
4) Context Exposure/Paired Noise-Shock (C-Paired)	Context Exposure	10-sec Noise→1-sec Shock	10-sec Noise

Table 1-4 Design of Experiment 4—Testing the Effect of Habituation to the White Noise

Figures

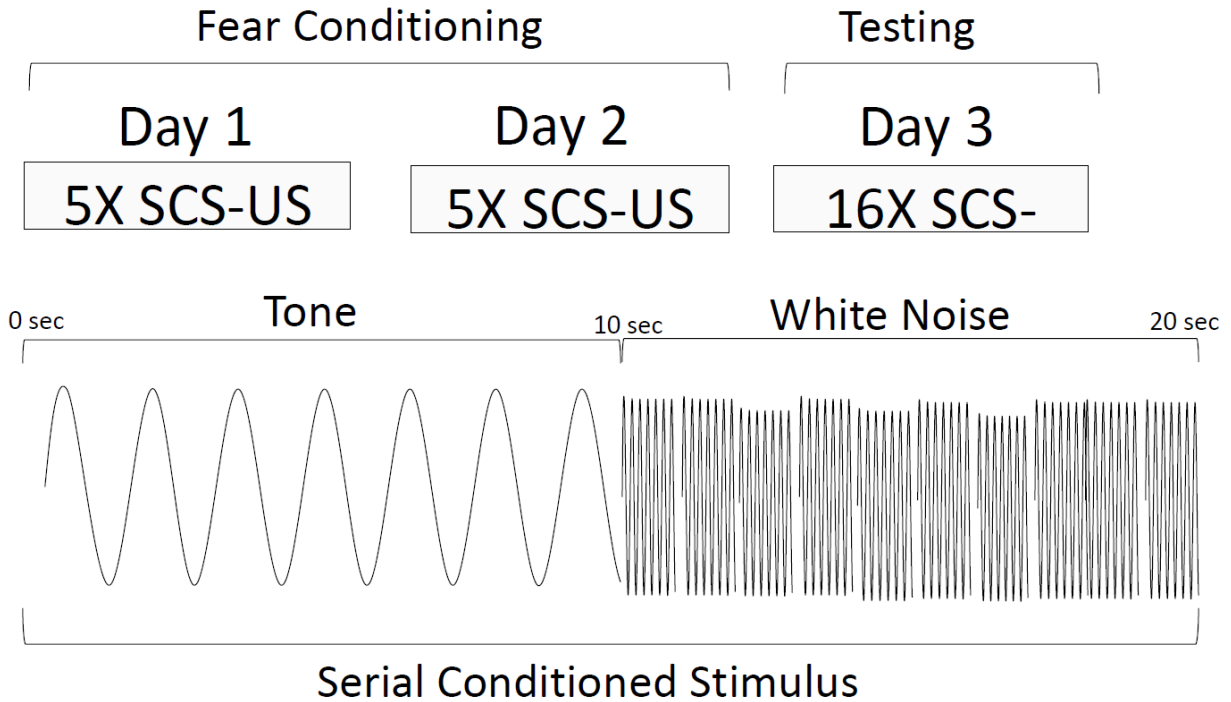


Figure 1-1 Behavioral design and schematic representation of the serial compound conditioned stimulus (SCS) used for the Replication Group in Experiment 1. During training, animals were given two days each of 5 SCS-US pairings. The SCS consisted of a 10s pure tone (7.5 kHz) followed by a 10s white noise (75 dB). Immediately upon termination of the white noise/SCS, a mild footshock US (1s, 0.9 mA) was delivered. On Day 3, the animals were tested with 16 presentations of the SCS without delivering any shocks.

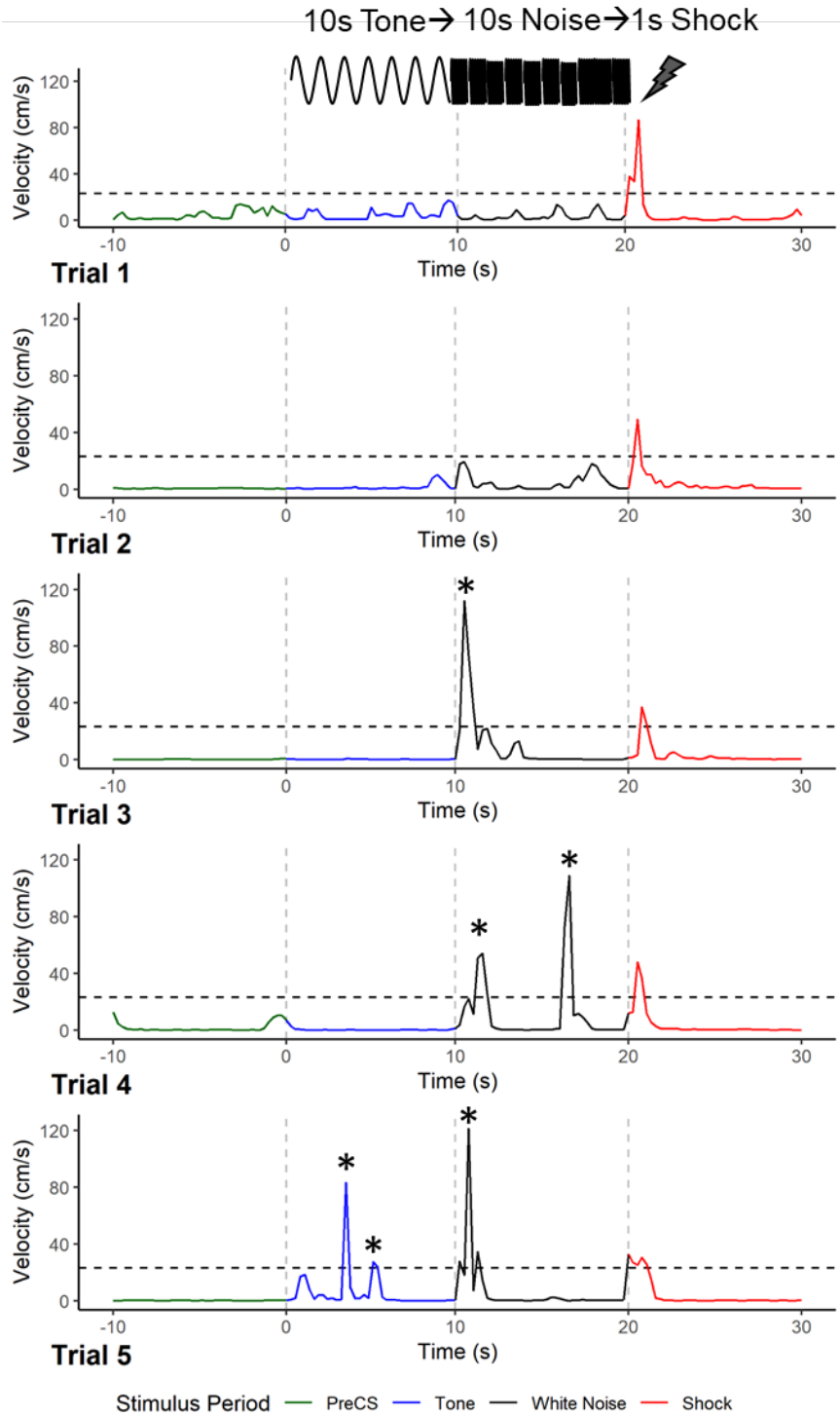


Figure 1-1-figure supplement 1 Example traces of velocity (cm/s) measurements obtained via EthoVision across five trials on the first day of training for a mouse in the Replication Group of Experiment 1. Vertical dotted lines denote stimulus onset times and the horizontal dotted line is the threshold for scoring behavior as a dart (22.9 cm/s). Darting episodes are marked with an *.

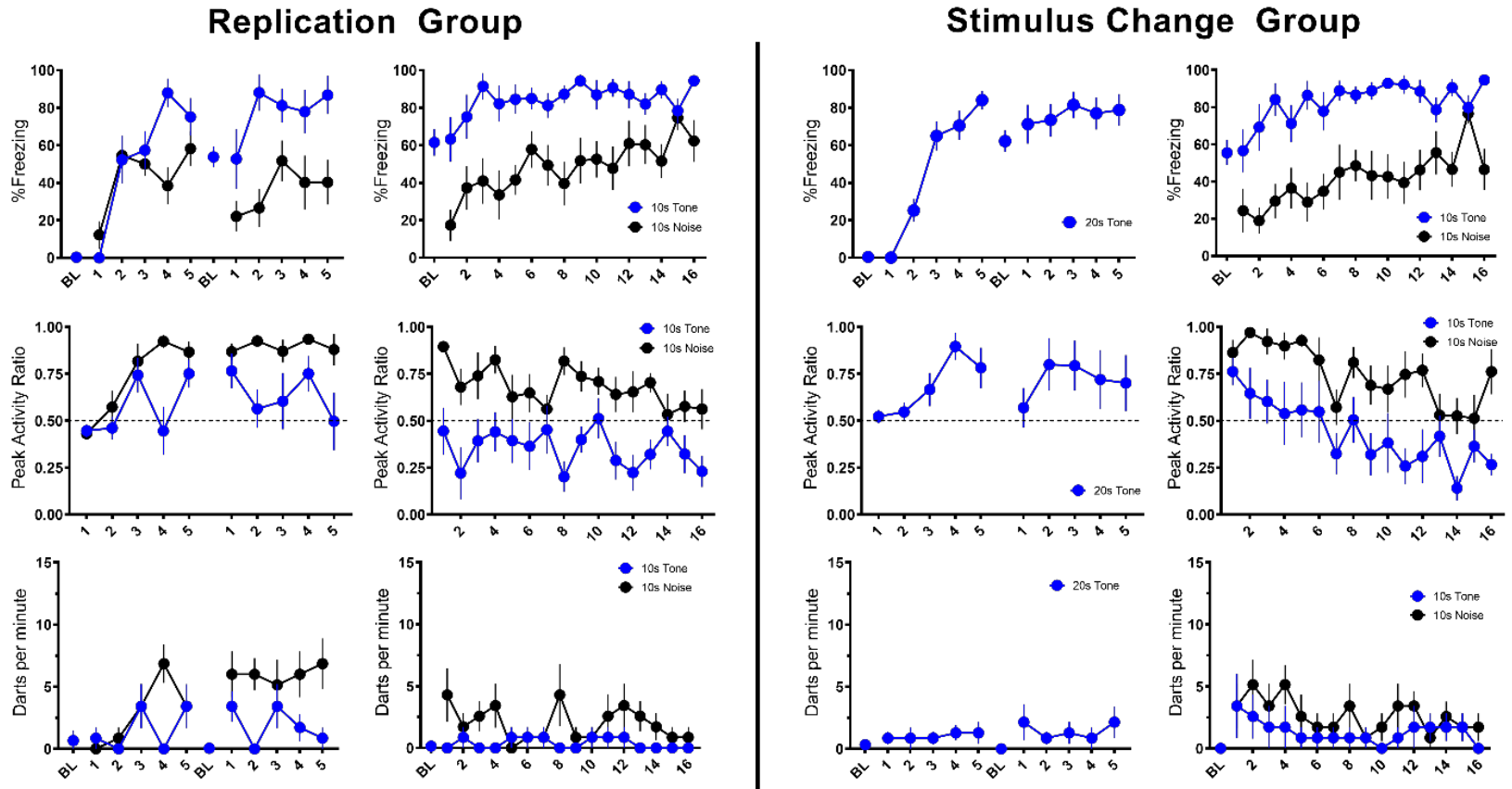


Figure 1-2 Trial-by-Trial Mean (\pm SEM) Percent Freezing, Peak Activity Ratio (PAR), and Darts per minute throughout all stimulus presentations during training (left panels) and testing (right panels) for the Replication group ($n=7$; left half of figure) and the Stimulus Change Group ($n=7$; right half of figure) in Experiment 1.

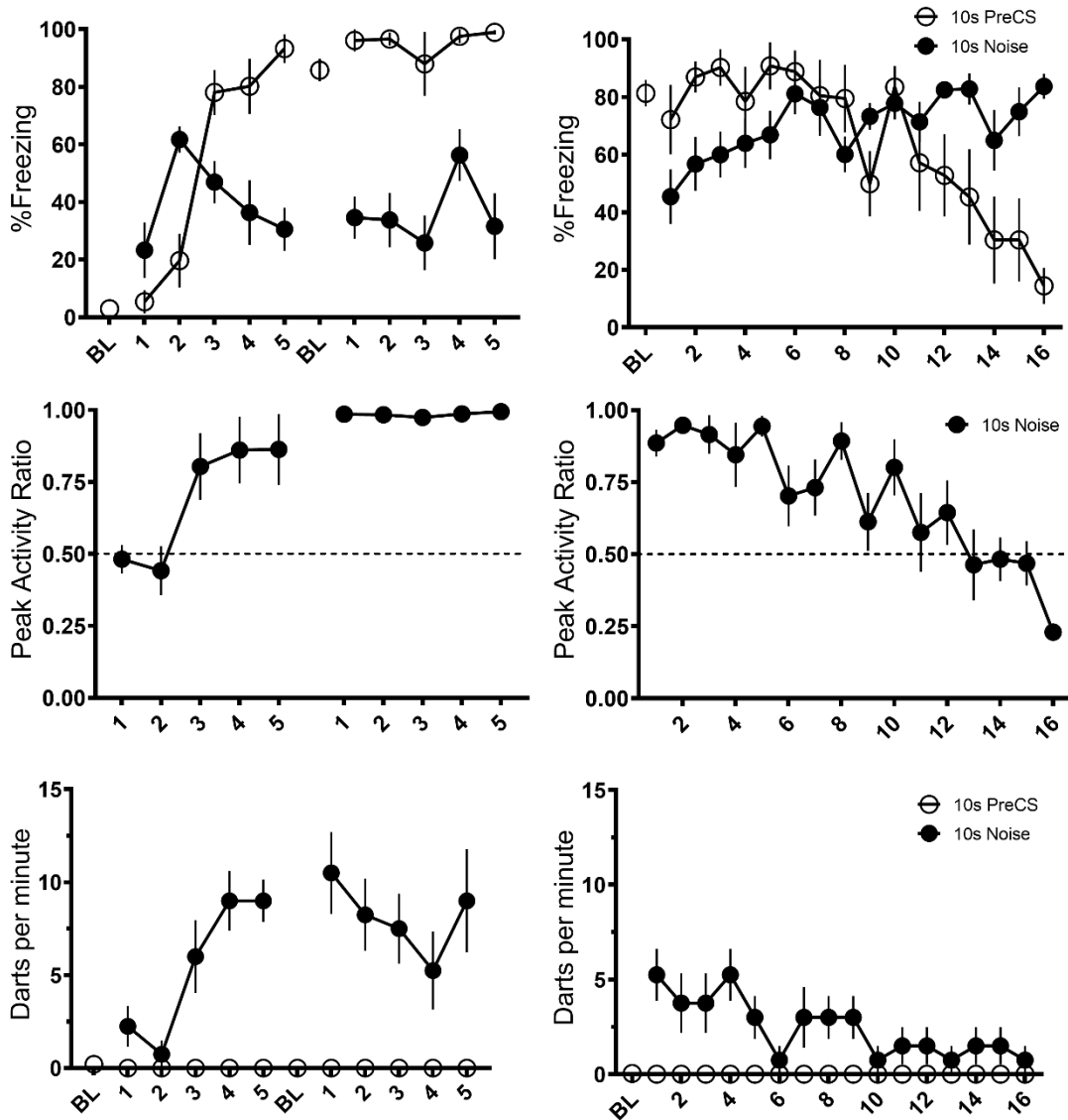


Figure 1-2-figure supplement 1 Mean (\pm SEM) Percent Freezing, Peak Activity Ratio (PAR), and Darts per minute throughout training (left panels) and testing (right panels) for the CS Duration Group ($n = 8$) of Experiment 1. Responding during the 10s preCS period is represented with open circles, responding during the noise is represented with filled in black circles.

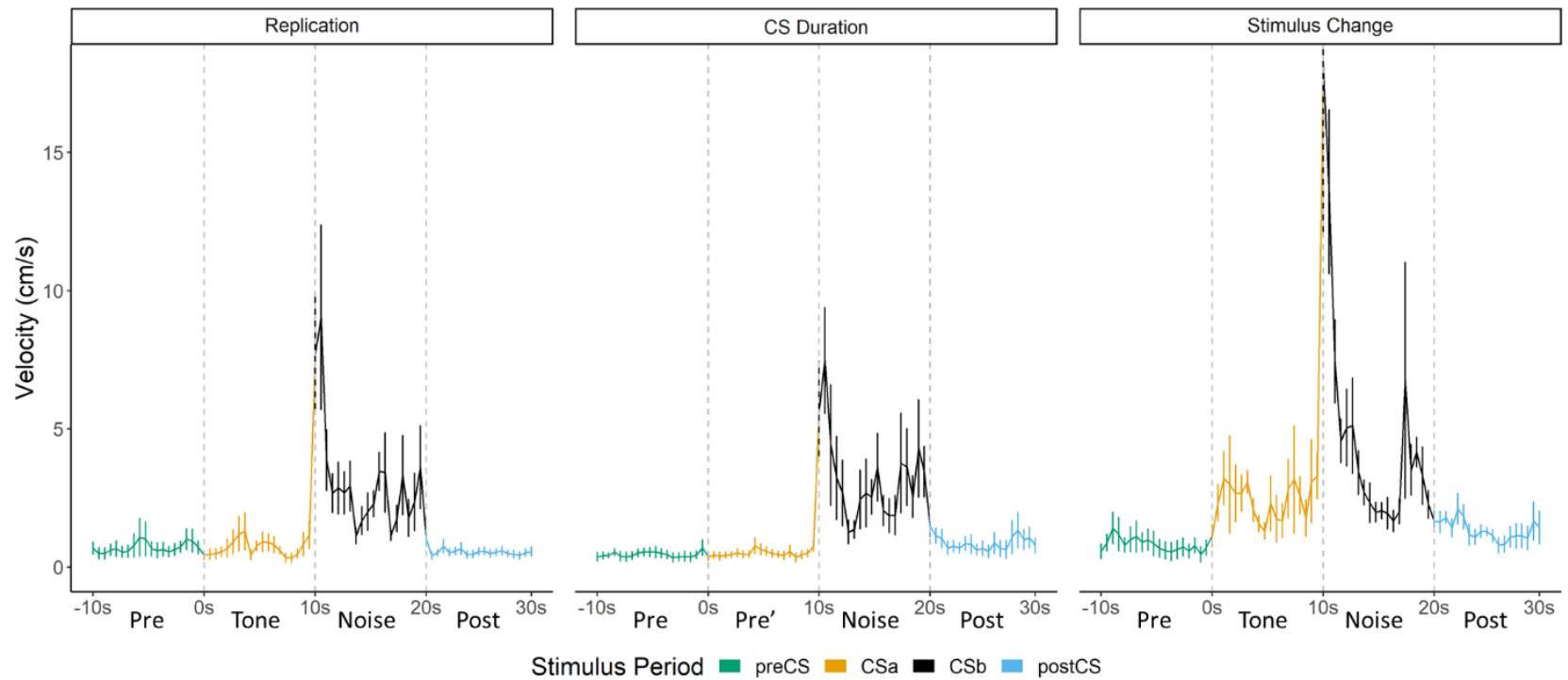


Figure 1-3 Averaged traces of velocity (cm/s) across the first 4 trials of extinction during testing for Experiment 1. Data is averaged across all animals per group and binned into ~ 0.5 s bins (0.533s) and presented as means plus/minus standard error (Mean \pm SE). These within-subject error bars are corrected for between-subject variability using methods as described in Rouder and Morey (2005). During this test, the Replication Group (n=7) and the Stimulus Change Group (n=7) received the serial conditioned stimulus (SCS) in which a 10s tone was followed by a 10s noise. The CS Duration group (n=8) was only tested with a 10s noise.

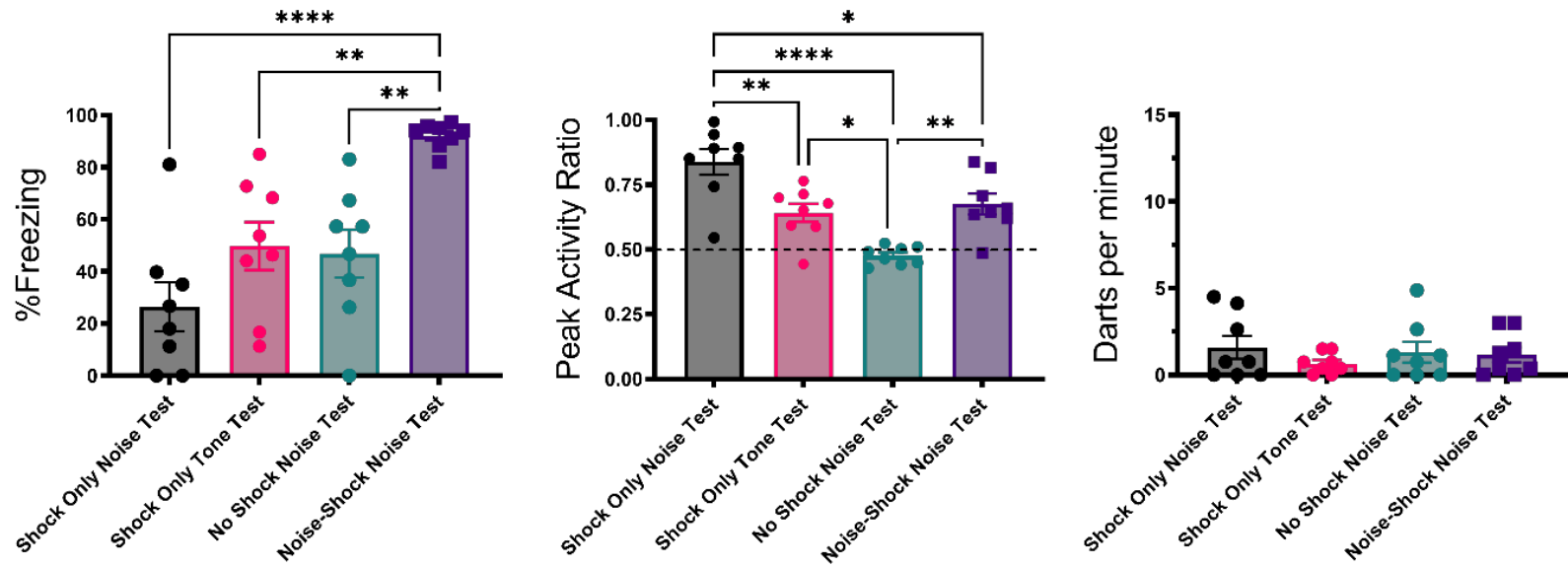


Figure 1-4 Mean (\pm SEM) Percent Freezing, Peak Activity Ratio (PAR), and Darting for the test session for Experiment 2 (n=8 per group). Values are averaged across the 16 trials of extinction during test. P-values and significance were determined through one-way ANOVA. * $p < .05$, ** $p < .01$, **** $p < .0001$

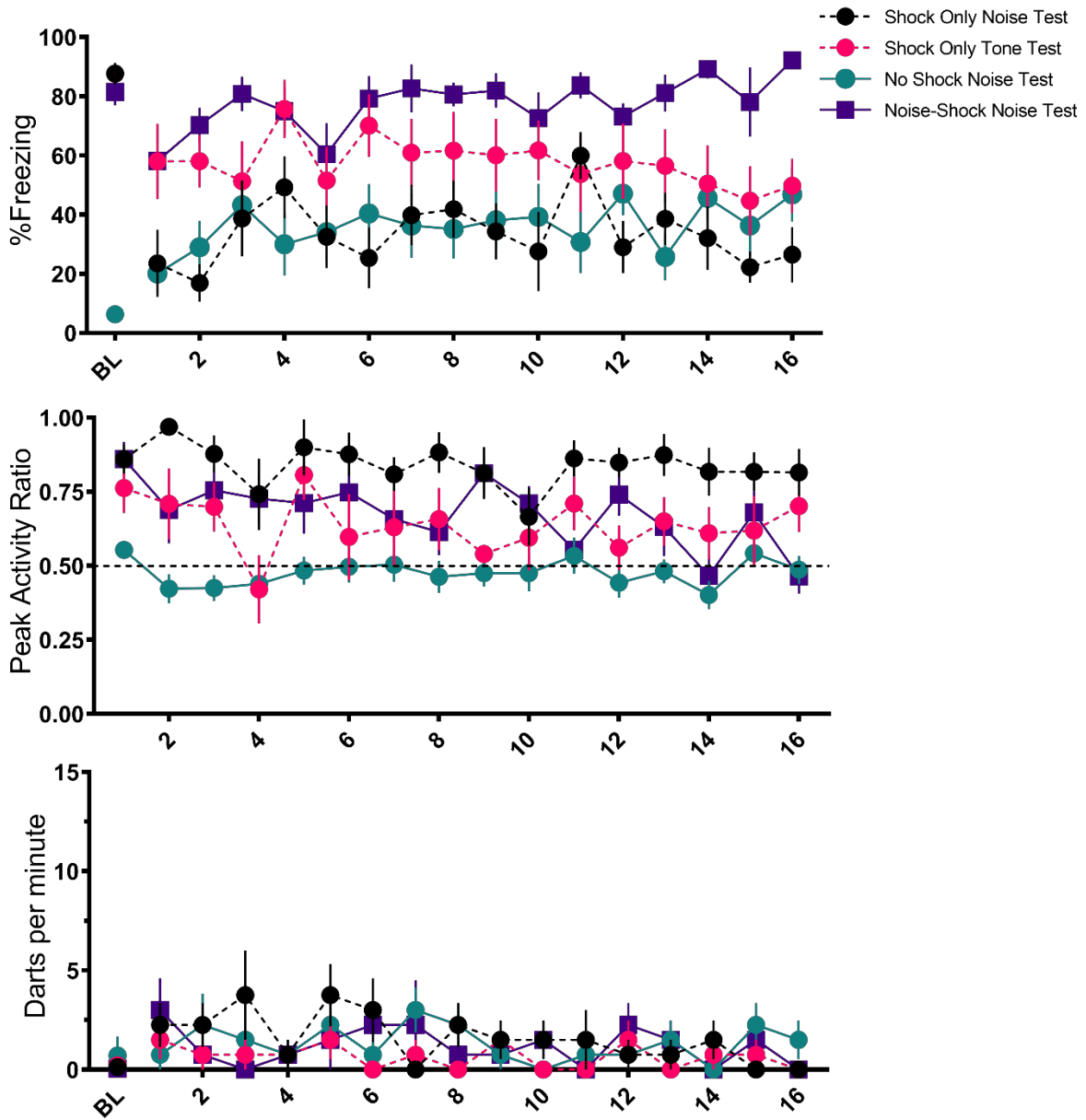


Figure 1-4-figure supplement 1 Trial-by-Trial Mean (\pm SEM) Percent Freezing, Peak Activity Ratio (PAR), and Darts per minute throughout 16 trials of testing for Experiment 2 (n = 8 per group).

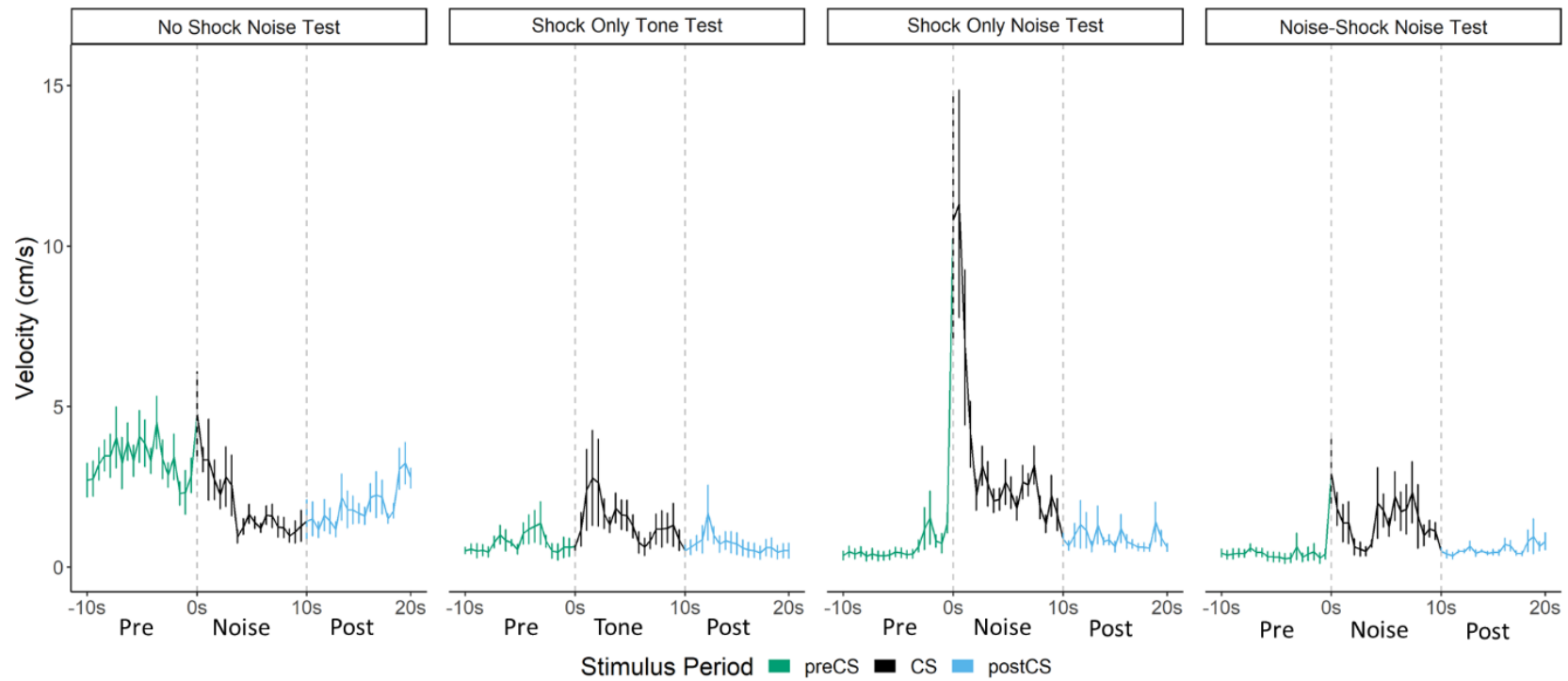


Figure 1-5 Averaged traces of velocity (cm/s) across the first 4 trials of extinction during testing for Experiment 2 (n=8 per group). Data is averaged across all animals per group and binned into ~.5s bins (0.533s) and presented as means plus/minus standard error (Mean \pm SE). These within-subject error bars are corrected for between-subject variability using methods as described in Rouder and Morey (2005). During this test, the No Shock-Noise Test, Shock Only-Noise Test, and Noise-Shock Noise Test groups were tested with a 10s noise. The Shock Only-Tone Test group was tested with a 10s tone.

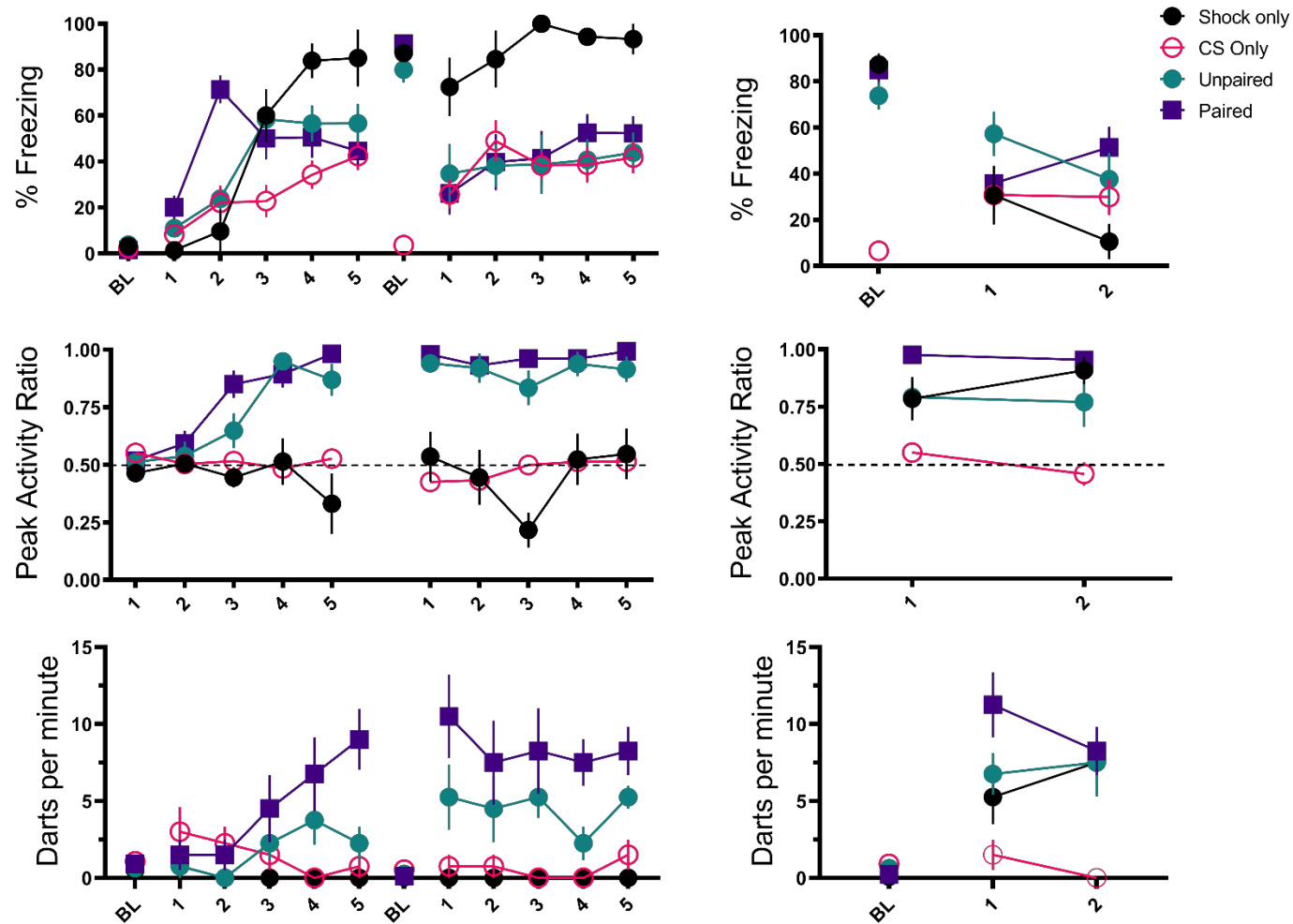


Figure 1-6 Trial-by-trial Mean (\pm SEM) Percent Freezing, Peak Activity Ratio (PAR), and Darting per minute throughout all stimulus presentations during training (left panels) and testing (right panels) for Experiment 3 (n=8 per group).

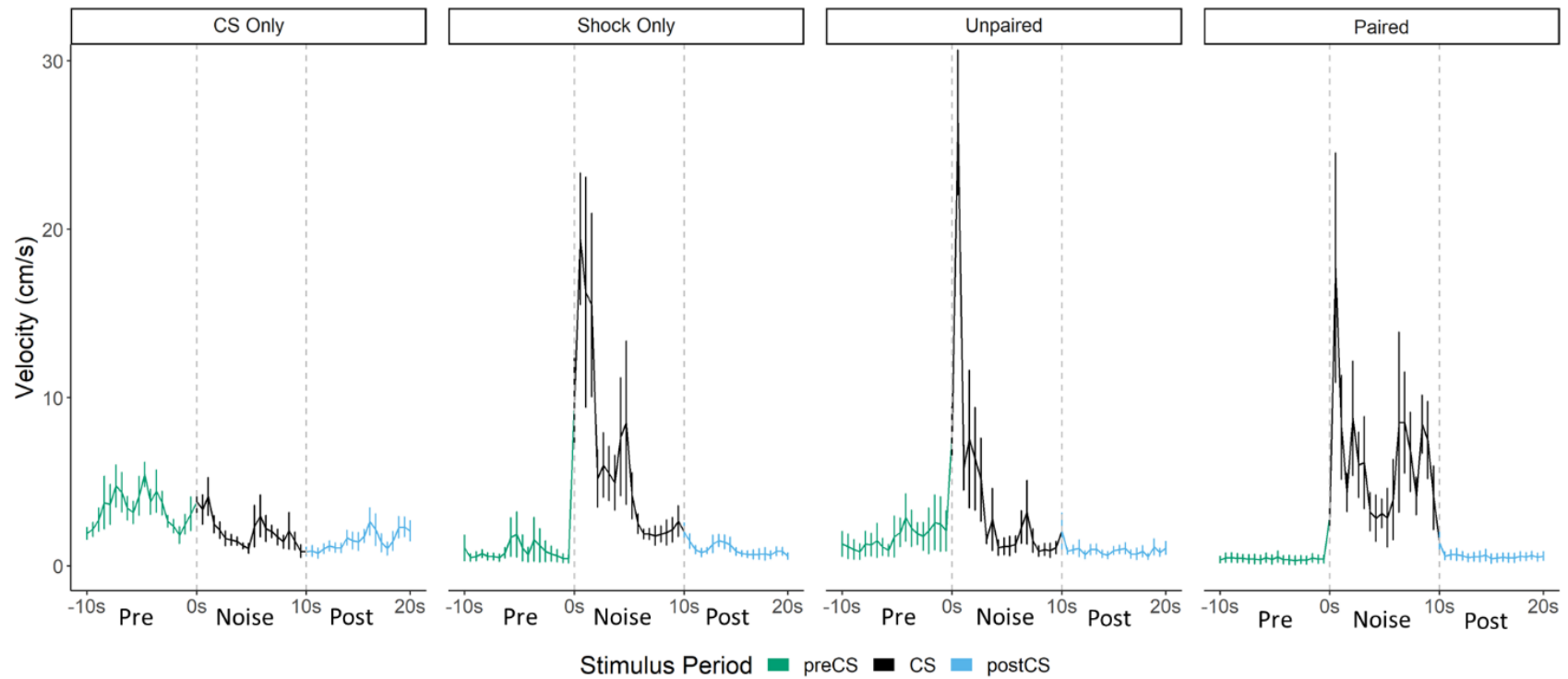


Figure 1-7 Averaged traces of velocity (cm/s) across 2 trials of extinction during testing for Experiment 3 (n=8 per group). Data is averaged across all animals per group and binned into ~.5s bins (0.533s) and presented as means plus/minus standard error (Mean \pm SE). These within-subject error bars are corrected for between-subject variability using methods as described in Rouder and Morey (2005). During this test all groups were tested with a 10s Noise CS.

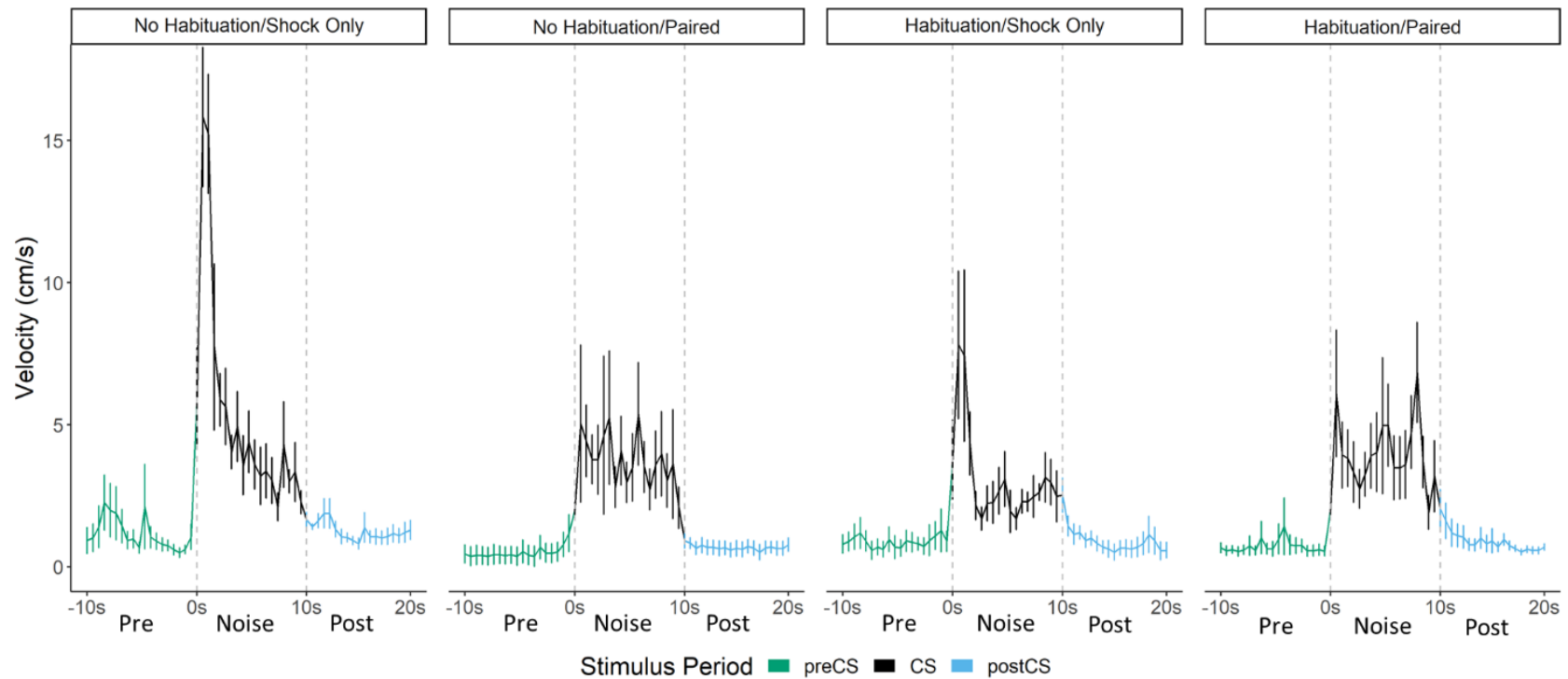


Figure 1-8 Averaged traces of velocity (cm/s) across 3 trials of extinction during testing for Experiment 4 (n=8 per group). Data is averaged across all animals per group and binned into $\sim .5$ s bins (0.533s) and presented as means plus/minus standard error (Mean \pm SE). These within-subject error bars are corrected for between-subject variability using methods as described in Rouder and Morey (2005). During this test all groups were tested with a 10s Noise CS.

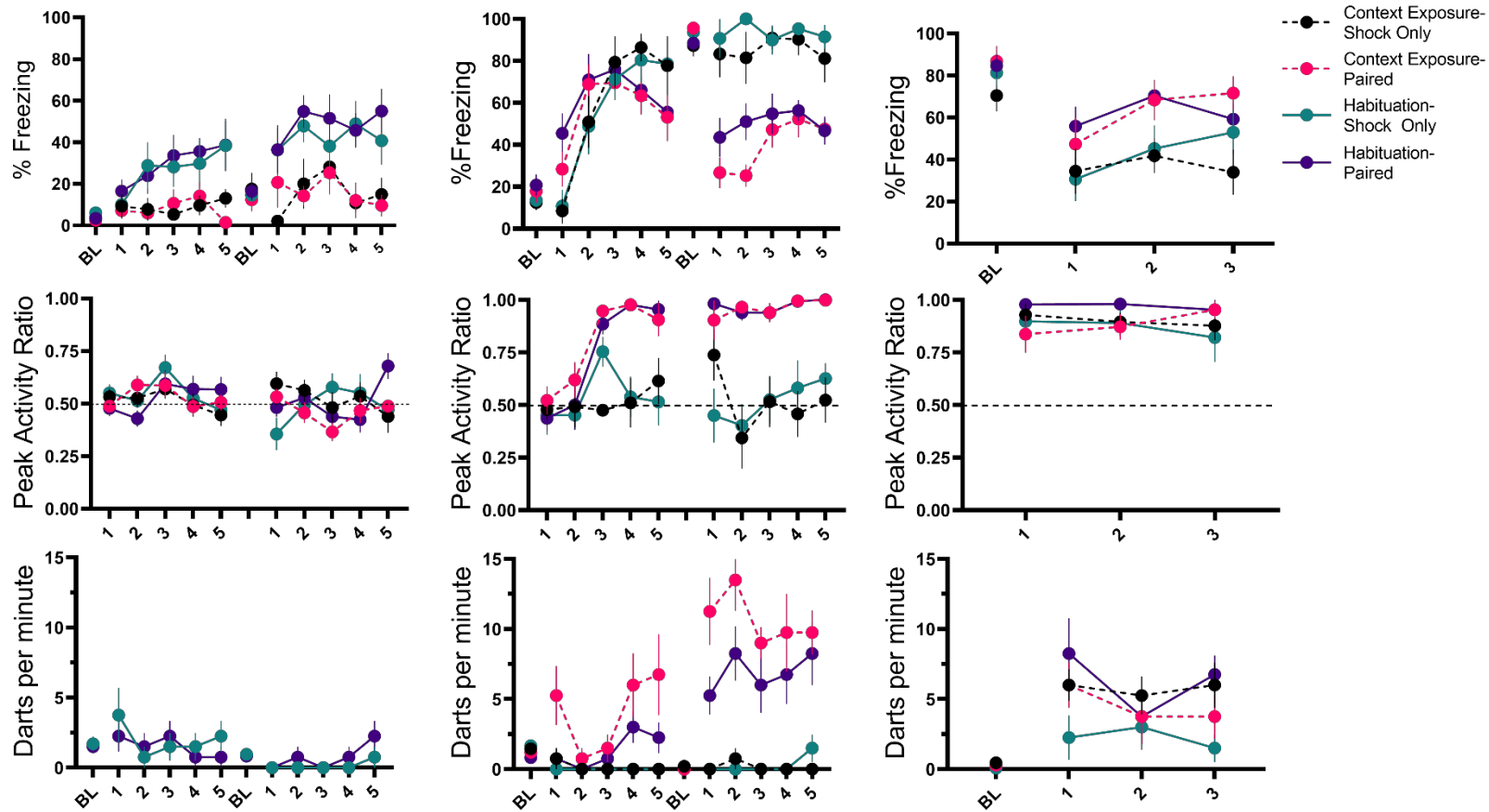


Figure 1-8-figure supplement 1 Trial-by-trial Mean (\pm SEM) Percent Freezing, Peak Ratio (PAR), and Darting per minute throughout all stimulus presentations during habituation (left panels), training (middle panels), and testing (right panels) for Experiment 4 ($n=8$ per group). For habituation, presented are the values during CS presentation for Habituation groups and values during the same 10s period for Context Exposure groups. For training, presented are the values during CS exposure for the Paired groups and values during the same 10s period for Shock Only groups. For testing, all presented values are during the 10s CS presentation.

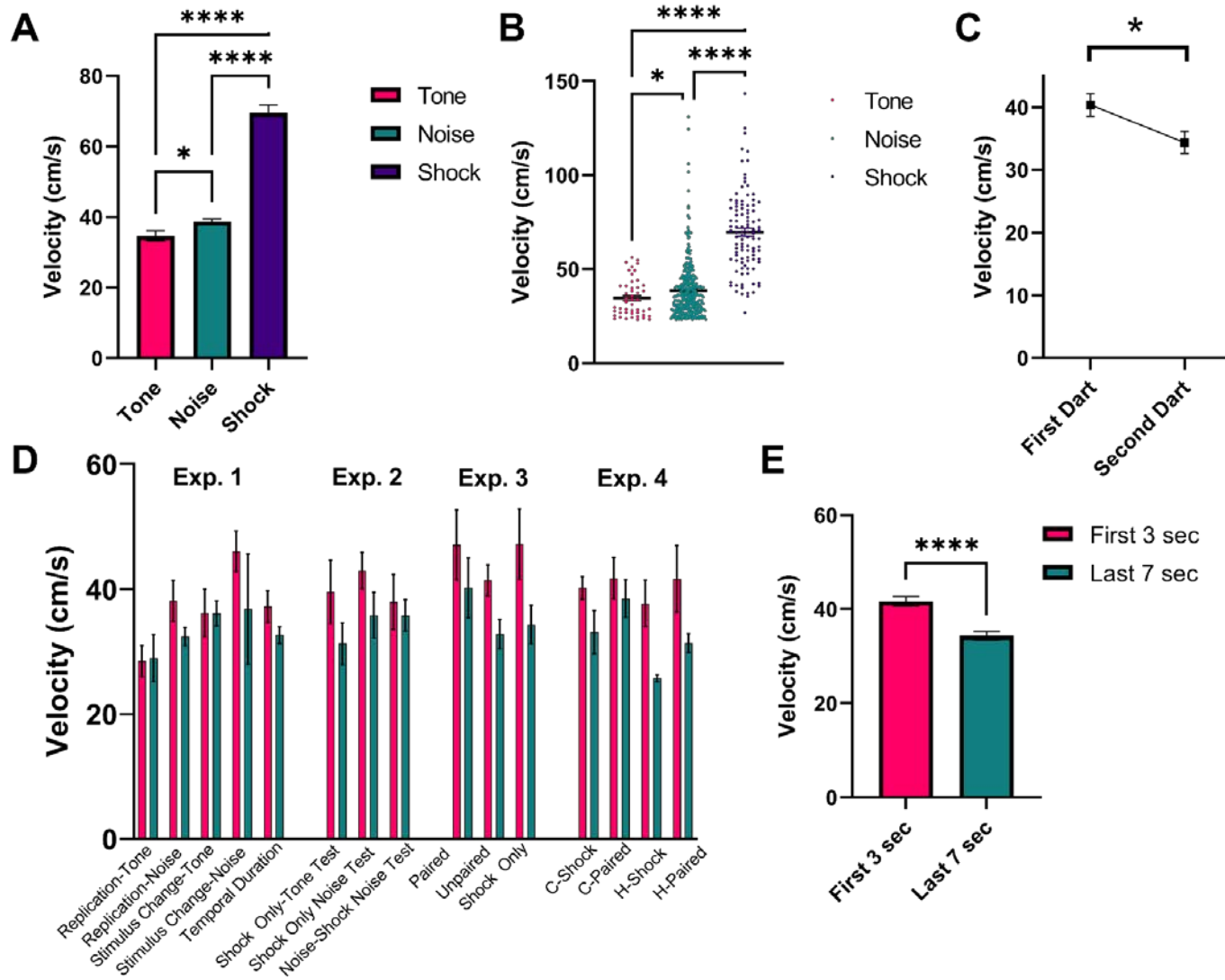


Figure 1-9 Analysis of dart timing and magnitude. **(A)** and **(B)** represent the magnitude of darts to the tone (n = 48 darts) and noise (n = 360 darts) stimuli during testing, as well as the reaction to the first shock (n = 102 shocks) on Day 1 of training. Data are presented as Mean \pm SE and come from all groups (total n = 102 animals) that received shock during training, collapsed across all experiments. P-values and significance were determined through Welch's ANOVA. *p<.05, ****p<.0001. **(C)** represents the magnitude (Mean \pm SE) of the first and second dart within a singular CS presentation for all animals across all experiments that performed 2 darts within a single 10s CS period (n = 65 'multi-darts'). P-values and significance were determined through a paired-sample t-test. *p<.05. **(D)** and **(E)** represent the magnitude of darts that occurred during the initial 3s of the 10s CS period (n = 230 darts) and those that occurred during the final 7s of the 10s CS period (n = 178 darts). Data are presented as Mean \pm SE and come from all groups that received shock during training (n= 102 animals), displayed by Group and stimulus type in **(D)** and collapsed across all experimental groups in **(E)**. P-values and significance were determined through Welch's ANOVA. ****p<.0001.

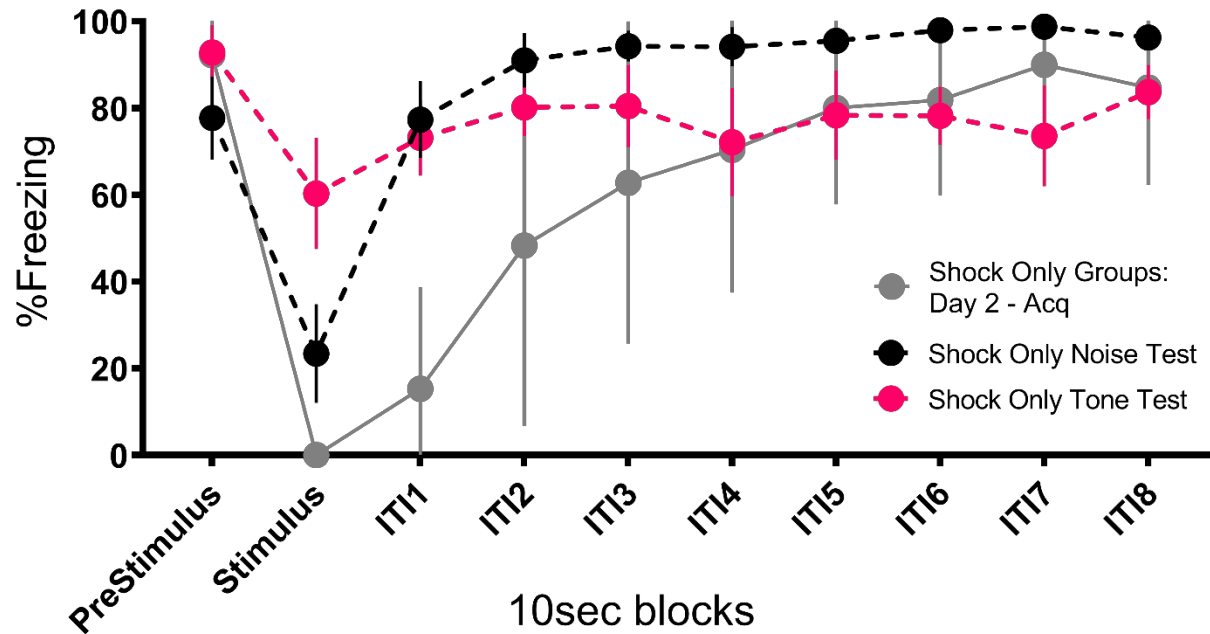


Figure 1-10 Mean (\pm SEM) Percent Freezing during extinction/testing for Experiment 2 showing that the occurrence of the stimuli at test disrupt freezing to the context and that the noise disrupts freezing to a greater extent than the tone ($n = 8$ per group). Also plotted is a similar curve showing freezing and the impact that shock presentation during training has on freezing. These data are averaged across both Shock Only groups (total $n = 16$) on Day 2 trials after fear to the context had been established, showing that shock disrupts freezing to an even greater extent than the noise.

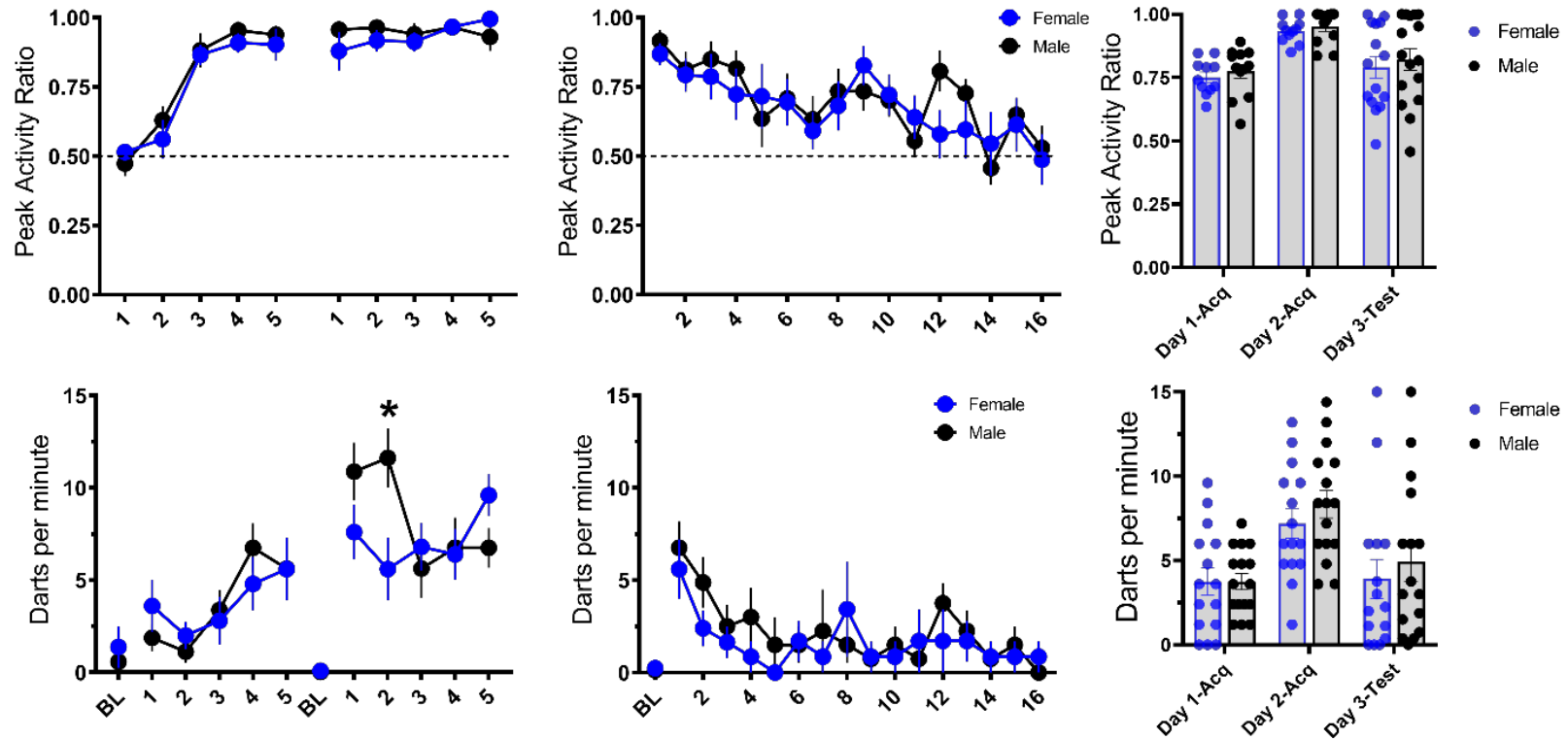


Figure 1-11 Trial-by-trial Mean (\pm SEM) Peak Ratio (PAR) and Darting per minute throughout all stimulus presentations during training (left panels), and testing (middle panels) for all groups across experiments that received Noise-Shock pairings, grouped by sex of the animal ($n = 15$ females; $n = 16$ males). The right panels show individual values for each animal's average PAR and darts per minute across training and testing. P-values and significance were determined through repeated measures ANOVA, and the interaction was followed up with pairwise t-tests. * $p < .05$.

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Chapter #2:

A Behavioral and Computational Analysis of Hippocampal Functioning and Sex Differences in Contextual Fear

Abstract

There are sex differences in anxiety disorders with regard to occurrence and severity of episodes such that females tend to experience more frequent and more severe episodes. Contextual fear learning and generalization are especially relevant to anxiety disorders, which are often defined by expressing fear and/or anxiety in safe contexts. In contextual fear conditioning, a representation of the context must first be created, and then that representation must be paired with an aversive consequence. With some variation, the experiments presented here use a 3-day procedure in which Day 1 consists of pre-exposure to the to-be-shocked context, Day 2 consists of a single context-shock pairing after some placement-to-shock interval (PSI), and Day 3 consists of testing in either the same or a novel context. With shorter pre-exposure periods, male rats showed more contextual fear, consistent with previous literature; however, after longer pre-exposure periods, female rats showed greater contextual fear. Additionally, while pre-exposure and PSI are both periods of time prior to the shock, it was found that they were not equivalent to each other. Animals with 120s of pre-exposure and a 30s PSI show a differential level and time-course of fear expression than animals who received no pre-exposure and a 150s PSI, and this further depended on sex of the rat. Additionally, an experiment comparing recently vs remotely acquired contextual fear was run. Males were again shown to have greater contextual fear at both time points, and this contextual fear incubated/increased over time in males, but not females. To facilitate identification of what processes caused sex differences, we used BaconX, a conceptual and computational model of hippocampal contextual learning. Computational simulations using this model predict many of our key findings. Furthermore, these simulations suggest potential mechanisms with regard to hippocampal computation, namely an increased feature sampling rate in males, which may account for the sex differences presented here, and in prior literature.

Introduction

Contextual fear conditioning is a procedure that can be used to study the acquisition and generalization of a fear memory. In contextual fear conditioning, the prevailing stimuli and cues of an environment, or context, are paired with some aversive unconditional stimulus (US). In the simplest versions of this procedure, an animal may be placed in a conditioning chamber and given a footshock US. As a result of this pairing of the context and the US, the animal will learn to fear that context and will display fear responses, such as freezing, defecation and analgesia, when returned to the context paired with shock (e.g., Fanselow, 1980, 1984, 1986; Wiltgen et al., 2001).

Contextual fear learning is an adaptive and functional way that animals can learn to predict danger in their environment. While learning and expressing fear to a dangerous place is adaptive, the potential generalization of learned fear to alternate, safe environments can be maladaptive and lead to inappropriate levels of fear and fear responding (Poulos et al., 2016; Yavas, Trott & Fanselow, 2021). This generalization of fear to a safe environment is a hallmark of many anxiety and stress-related disorders, and contextual fear conditioning has been used extensively in rodent models to try to understand this potential maladaptive generalization (Andreatta et al., 2015; Asok, Kandel, & Rayman, 2019; Dunsmoor & Paz, 2015). Interestingly, both contextual fear conditioning and most anxiety disorders, including post-traumatic stress disorder, have notable sex differences, such that in humans, females tend to be affected more often and more severely by the majority of anxiety disorders; whereas in rodents, contextual fear learning is typically greater in male subjects (Kessler et al., 2005; Wiltgen et al., 2001; Yavas, Trott, & Fanselow, 2021; Le et al., 2022; Cossio et al., 2016; Cushman et al., 2014; Maren, De Oca, & Fanselow, 1994; Poulos et al., 2015).

Unlike cued fear conditioning procedures, in contextual fear conditioning there is no one discrete stimulus that the animal learns to associate with shock. To effectively produce contextual fear, it is thought that an animal must be able to form a representation of the context based on the contextual features and attributes, and this representation is what becomes paired with the aversive US and is eventually able to support fear expression (Anagnostaras, Gale, & Fanselow, 2001; Fanselow, 1980; Krasne, Cushman, & Fanselow, 2015; Rudy & O'Reilly, 1999; Yavas, Trott, & Fanselow, 2021). Furthermore, while in traditional cued fear conditioning with a discrete conditional stimulus (CS), conditioning decreases as time between CS onset and US onset increases, this relationship switches such that as time between entry to the context and US onset increases, conditioning increases (Fanselow, 1986, 2010; Zinn et al., 2020).

Thus, two factors that are known to increase contextual fear learning include prior experience with the to-be-conditioned environment (pre-exposure or PreX), and the time in the context prior to the first unconditional stimulus (Placement-to-shock interval or PSI; Fanselow, 1990; Wiltgen et al., 2001). If there is inadequate time for forming a representation of the context prior to shock (such as with very short PSI's), poor conditioning results and this is called the immediate shock deficit (Fanselow, 1986). This deficit is more pronounced in female rodents and can be ameliorated by providing an opportunity to explore the context without shock prior to the conditioning session (Wiltgen et al., 2001). That the immediate shock deficit can be ameliorated by previous exposure to the context suggests that contextual representations do not necessarily need to be formed on the day of conditioning, but rather that previously formed contextual representations can be recalled and associated with shock more readily/faster than forming a new contextual representation that can then be associated with shock.

As hippocampal disruption specifically impairs conditioning to contextual CSs, but not to discrete CS, we, and others, have suggested that the hippocampus is critical for the integration process necessary for the creation of contextual representations that occurs during context

exposure (Asok, Kandel, & Rayman, 2019; Fanselow, 2000; Kim & Fanselow, 1992; Rudy & O'Reilly, 1999). This binding of contextual attributes depends on extensive interactions between the hippocampus and cortex (Bucci & Robinson, 2014; Krasne, Cushman, & Fanselow, 2015; Yavas, Gonzales, & Fanselow, 2019). Consistent with the hypothesis that the hippocampus is selectively involved in context processing as opposed to context-US association formation, manipulations of hippocampal function only during context pre-exposure will alter the benefits of pre-exposure (Matus-Amat et al., 2004). Additionally, optogenetic enhancement of hippocampal cholinergic activity during pre-exposure further facilitates later conditioning at short PSIs (Hersman et al., 2017). Furthermore, there a number of lines of research that suggest sex differences in hippocampal function, including the general sex differences in contextual fear, sex differences in spatial learning, and increased thresholds to induce hippocampal LTP in females (Wiltgen et al., 2001, Le et al., 2022; Cossio et al., 2016; Cushman et al., 2014; Koss & Frick, 2017; Maren, De Oca, & Fanselow, 1994; Poulos et al., 2015).

How differing lengths of pre-exposure and PSI impact generalization, and whether they do so in a sex-specific manner, is unknown. Additionally, while pre-exposure and PSI are both unreinforced experiences with a context prior to conditioning, it is unknown if they act upon similar processes or hippocampal mechanisms. Here we present a series of experiments assessing the impact of pre-exposure duration, PSI length, and sex on contextual fear learning and generalization.

As a framework for understanding our observations we employed BACON (BAYesian CONtext Fear Algorithm), a conceptual and computational model of hippocampal contextual learning (Krasne, Cushman, & Fanselow, 2015; Krasne et al., 2021) that successfully simulates a wide range of known properties of context fear learning and has helped to make several non-intuitive properties of such conditioning understandable (Bernier et al., 2017; Zinn et al., 2020). We particularly hoped that this model might help us explain such sex differences as were found in

our experiments as possibly being due to differences of some single underlying variable; in fact, this did turn out to be the case.

Presented here are behavioral experiments in rats and computational simulations of the same experiments using BaconX. Each experiment and simulation generally follows the same pattern of a 3-day contextual fear conditioning procedure. On Day 1, animals are pre-exposed to a to-be-shocked context. On Day 2, animals are returned to the context and given a single footshock US after some PSI. On Day 3, animals are tested for contextual fear and/or generalization. One of the major suggestions of BaconX is that the hippocampus can have distinct functional modes, one for building a contextual representation (which would mostly happen during pre-exposure) and one for retrieving that representation, prior to shock for example (which would happen during Day 2's PSI in the experiments presented here). We used BaconX as a tool to help identify how males and females differ in processing contexts.

Results

The designs of Experiments 1A-1C are presented in **Table 2-1**. Briefly, Experiments 1A and 1B use a short PSI (20s) on Day 2 after a short (2-min; Experiment 1A) or long pre-exposure session (10-min; Experiment 1B) on Day 1. Experiment 1C follows up on the results of prior experiments and assesses potential impacts of estrous cycle phase on female fear conditioning following an intermediate pre-exposure length (5-min) and a short PSI (20s). For each set of experiments presented here and below, computational simulations using the BaconX model were performed in attempts to both simulate/replicate the behavioral data as well as to reveal potential computational mechanisms underlying the effects and sex differences.

The BACON model

Before describing our experimental results, we first describe BaconX, the computational model that we used to try to understand the relationships between our various experiments and the

sex differences we found. BaconX (and its progenitor, BACON) is based on the ideas embodied in Marr's Theory of Archicortex (Marr, 1971) and experimental findings on the role of hippocampus in contextual fear conditioning (Maren & Fanselow, 1995; Kim & Fanselow, 1992). They postulate that when an animal finds itself in a new context a small group of hippocampal cells of fixed number become its hippocampal representation. These cells then become associated via Hebbian mechanisms with the cortical attribute-coding cells that the context has activated as well as with each other in such a way that thereafter exposure to just a fraction of the context's attributes will activate the full hippocampal representation, and it in turn activates cortical cells corresponding to all the known attributes of the context. In addition, the hippocampal representation neurons communicate with the amygdala where they have synapses on fear-causing cells that undergo Hebbian potentiation if a US occurs when they are active, resulting in contextual fear conditioning.

To the above, the BACON models add the idea that animals make assessments of how confident they should be that a previously established representation that has become activated in a context is in fact the right one. This assessment is then used to control aspects of learning and performance such as whether fear should become conditioned to that representation should a US occur in the context, how strongly they should express fear that was previously conditioned to the representation, and whether newly observed features of the current environment should be added to the set of features already associated the representation.

The following details of the above process are described fully in papers devoted to the BACON models (Krasne, Cushman, & Fanselow, 2015; Krasne et al., 2021), but the following description will be sufficient for our present purposes: About every half second during a visit to a context BaconX performs a computational cycle during which it randomly samples its environment, and if it observes something not previously noticed during the session it adds that attribute to working memory; the number of attributes that have been observed so far during a

visit is denoted Z_{cur} ($Z_{current}$). Whenever a new attribute is noticed, BaconX determines (or re-determines) which of its existing representations is most consistent with what has been so far observed and activates that representation. It then activates all of the attributes associated with that representation, the number of which is denoted as Z_{rec} ($Z_{recalled}$). Then, based on the number of attributes in common (" Z_{com} ") between its Z_{cur} observed attributes and its Z_{rec} recalled ones, it computes the Bayesian weight of evidence (B_{Rep} ; Kass, & Raftery, 1995) that the activated representation is the correct one (as described in detail in Krasne, Cushman, & Fanselow, 2015). *The more positive B_{Rep} is, the greater the assurance that the activated representation is correct, and the more negative, the more certain that it is not correct.*

Once B_{Rep} has been computed following an increment in Z_{cur} , B_{Rep} 's value determines several things:

- (1). If B_{Rep} of this best available representation is below a threshold value B_{new} (i.e., BaconX was very confident that it was not in fact in the active representation's context), then BaconX apparently had no representation for the current context. A new one is then constructed, and all the attributes currently in working memory become associated with it (i.e., Z_{rec} of the new representation is the value of Z_{cur} at the time of representation creation).
- (2). If B_{Rep} is greater than a value B_{cnd} , then, should a US occur, conditioning will result, and the farther B_{Rep} is above B_{cnd} , the greater that conditioning will be up to a limit. Quantitatively, the weight increase of the synapse made by an active hippocampus-amygdala pathway cell on an amygdala fear-producing cell, the "conditionability," is given by

$$\text{conditionability} = \alpha * L(B_{Rep} | B_{cnd}, B_{mxnd}) \quad (\text{Equation 1})$$

where α (alpha) is a learning rate parameter and L is a function of B_{Rep} that increases linearly starting at B_{cnd} and reaching a maximum of 1 at B_{mxcnd} .

(3). **If fear has previously been conditioned to the represented context**, then fear is expressed to an extent proportional to B_{Rep} .

Experiment 1A: Short Pre-Exposure

First, we set out on a series of experiments to assess contextual fear learning and generalization in male and female rats with a short PSI (20s) on Day 2 after a short pre-exposure (120s) on Day 1. Previous studies which allowed for comparable time exploring the context prior to shock suggested that sex differences would be likely, and males would likely show more contextual fear than females (Maren, De Oca, & Fanselow, 1994; Wiltgen et al., 2001). Following handling and transport habituation, male and female rats (n=18 each) were run through the 3-day contextual fear conditioning procedure described in the Methods section, with 120 seconds of pre-exposure to the to-be-shocked context on Day 1, a 20s PSI on Day 2, and a test for contextual fear/generalization in either the shocked context (Same) or a novel context (Different). A relatively strong footshock (2.0s, 1.5mA) was used to encourage one-trial learning. For this, and all following experiments, there was negligible levels of freezing (<5%) both to the context on Day 1 during pre-exposure and on Day 2 during the PSI interval prior to shock; and there were no group or sex differences in this or any following experiment on these baseline freezing measures (all p 's > .05). **Figure 2-1** represents the freezing scores from the test on Day 3.

A two-way ANOVA revealed a significant effect of Sex [$F(1,32) = 5.474, p = .026$] and Testing context [$F(1,32) = 7.520, p = .010$]. Males generally showed more contextual fear, and both sexes showed more fear in the training context than a novel context. In other words, both sexes

showed good levels of discrimination and low generalization of fear to a novel context. The resulting sex difference in which males show more contextual fear than females is in line with many previously reported sex differences in different contextual fear conditioning procedures in both rats and mice (Wiltgen et al., 2001; Le et al., 2022; Cossio et al., 2016; Cushman et al., 2014; Maren, De Oca, & Fanselow, 1994; Poulos et al., 2015).

BaconX interpretation of Short Pre-exposure Experiment 1A

The BaconX model suggests that at least one possible hypothesis that could account for our finding that at short PSI's (and generally throughout the literature) males show greater evidence of contextual fear than females, as well as later findings in the subsequent experiments presented here (detailed in the results and discussion below) is that females sample their environments more slowly than males. Slower sampling of contextual features in females is potentially consistent with a number of other notable findings, including the general sex differences in contextual fear, sex differences in spatial learning, and increased thresholds to induce hippocampal LTP in females (Wiltgen et al., 2001, Le et al., 2022; Cossio et al., 2016; Cushman et al., 2014; Maren, De Oca, & Fanselow, 1994; Poulos et al., 2015).

Slower sampling of attributes in females would mean that for a given duration of pre-exposure, less attributes would be associated with the representation established in that period (Z_{rec} would be less than in males). Thus, throughout the subsequent conditioning session Z_{rec} would be less. Moreover, Z_{cur} would rise more slowly during the conditioning session itself, and at the moment when the US occurred Z_{cur} would be lower than in males. Since B_{Rep} and hence conditionability increase as a function of both Z_{cur} and Z_{rec} , weaker conditioning would result. This is shown in **Figure 2-2** in which the values of B_{Rep} at the moment of conditioning are indicated by the circular markers for a case where rate of sampling is 10% slower in females than males. It should be noted that during the pre-exposure session itself, the slower sampling

would mean that BaconX would take longer in females than males to "realize" that the new context it was in was in fact new, and so the representation would have been created somewhat later in that session in females than in males. Had the session been shorter, a representation might not have been created at all.

Experiment 1B: Long Pre-Exposure

In Experiment 1B, potential sex differences in contextual fear learning and generalization were assessed following a procedure in which rats had a relatively long pre-exposure (10 min) to the to-be-shocked context on Day 1. This longer length of pre-exposure has previously been shown to abolish sex differences in contextual fear in mice (Wiltgen et al., 2001). Following handling and transport habituation, male (n=15) and female rats (n=16) were run through the 3-day contextual fear conditioning procedure, with 10 minutes of pre-exposure to the to-be-shocked context on Day 1, a 20s PSI on Day 2, and a test for contextual fear/generalization in either the shocked context (Same) or a novel context (Different) on Day 3. A slightly milder shock (1.0mA) was used in this study in hopes of avoiding potential ceiling effects after a longer opportunity to explore during pre-exposure. **Figure 2-3** represents the freezing scores from the test on Day 3.

A two-way ANOVA revealed a significant effect of Testing context [$F(1,27) = 9.191, p = .005$] and a significant Sex X Testing Context interaction [$F(1,27) = 8.192, p = .008$]. Further analyses of this interaction reveal that females showed more contextual fear in the training context (Same) compared to females tested in a novel (Different) context ($p < .001$), males tested in the training context (Same; $p = .003$), and males tested in a novel context (Different; $p = .003$).

Unlike in Experiment 1A, the sex difference was reversed here in that female rats generally showed more contextual fear and evidence of discrimination. While the sex difference reported here is inconsistent with many previous studies, it is in line with at least one study that found

that females show more contextual learning than males under certain conditions (Keiser et al., 2015).

Experiment 1C: Assessing the impact of estrous phase

The previous experiments reveal sex differences in contextual fear. As with any sex difference, it is worthwhile to consider potential impacts of estrous phase on female behavior. If estrous phase were having an impact on female learning and memory, we may expect to see increased levels of variability in the females. While a standard test for homogeneity of variance (Levene's test) reveals evidence for inequality of variance for both Experiment 1A and 1B (1A: $F = 3.214$, $p = .036$; 1B: $F = 16.178$, $p < .001$), these differences are driven by very low variability in the low levels of generalization seen across both sexes. Furthermore, in Experiment 1A, in which both males and females shows reasonable levels of contextual fear in the shocked-Same context, males were just as variable as females. However, as previous studies have shown impacts of estrous cycle on contextual and auditory fear under certain conditions (Cossio et al., 2016; Cushman et al., 2014; Milad et al., 2009; Markus & Zecevic, 1997), we sought to determine whether estrous phase had an effect on contextual fear as performed with the procedures used in these experiments.

In Experiment 1C, the potential impact of estrous phase on contextual fear learning was assessed following a procedure in which animals had an intermediate pre-exposure (5 min) to the to-be-shocked context on Day 1. Following initial handling, females were monitored for estrous cycle phase for 10 days. Animals were only run through behavioral training and testing if they showed at least two regular 4- or 5-day estrous cycles. Following this monitoring and transport habituation, female rats ($n=16$) were randomly separated into 4 groups, who would undergo each day of a modified '3-day' contextual fear procedure during a specific phase of the estrous cycle (Proestrus, Estrus, Metestrus, or Diestrus; $n=4$ each). Therefore, there were 4-5

days separating Pre-exposure, PSI and shock, and testing. Briefly all animals received 5 minutes of pre-exposure to the to-be-shocked context on 'Day 1', a 20s PSI on 'Day 2', and a test for contextual fear in the shocked context on 'Day 3'. **Figure 2-4** represents the freezing scores from the test on test 'Day 3'. A one-way ANOVA revealed no significant impact of estrous phase on contextual fear memory [$F(1,27) = 9.191, p = .005$]. Therefore, while estrous phase may play a role in other contextual fear conditioning procedures, it does not appear to play a role using the procedures reported here.

BaconX interpretation of Long Pre-exposure Experiment 1B

The finding that in males good conditioning results after a short (2-min) pre-exposure, but not after a longer (10-min) one, whereas in females the same longer pre-exposure actually facilitates subsequent conditioning, seems highly paradoxical. However, one can come to a possible explanation of it if one considers that during a very long pre-exposure, BaconX would learn a great deal about the context in which it was to be subsequently conditioned (i.e., Z_{rec} would become very high), and this would be more so in males than females if, as we have conjectured above, sampling of contextual attributes were faster in males. If a great deal is known about a given context, then BaconX will be able to discriminate it more readily from an only slightly different one. This is relevant because in reality contextual attributes are likely to change a little bit from one occasion to another. One might then suppose that after a very long pre-exposure session during which Z_{rec} became quite high, small random differences in representation attributes at a later session might cause doubts to arise about the validity of the pre-exposed representation; specifically, after sampling a moderate number of the context's attributes, B_{Rep} would fall below B_{Cnd} so no conditioning would occur. This would be more likely to happen in males than females if, as we have conjectured, Z_{rec} is higher in males than females at the end of a given length pre-exposure session.

There is, however a difficulty with this this explanation: In BACON, if the representation of the pre-exposed context were rejected (i.e., B_{Rep} went below B_{new} , which is what would probably happen in the present case) a *new (additional)* representation of the context would get created and become active, and BaconX would condition successfully to *that new representation*. In that event, BaconX would thereafter express fear (perhaps subject to some generalization decrement) whenever any representation of the conditioned context was active, since different representations of the same context, while not identical, would tend to have multiple cells in common.

However, BACON and BaconX are over-simplifications that do not take account the many remote contextual representations of various contexts that a real animal would have from its previous life experiences. In a more realistic model that takes this into account, the rejection of one representation when its B_{Rep} becomes very low would probably result in the next best existing representation being activated, and conditioning would occur to that in so far as its B_{rep} was above B_{cnd} . However, since that representation's context would probably be rather different from the context Bacon was currently in, little conditioning to it would occur (BaconREM, a model that deals with remote representations and makes these ideas explicit, is currently being developed). Conceptualized in this way, BaconX can explain the paradoxical results under discussion.

In order to make these ideas concrete, **Figure 2-5** presents such a simulation, in which BaconX is pre-exposed to a context on Day 1, returned to that context on Day 2 for a shorter session in which a shock US occurs, and then tested in that same context on Day 3, but it is assumed that a small percent (10%) of contextual features have changed between pre-exposure session on Day 1 and the conditioning session on Day 2. It is also assumed that females sample contextual features 10% more slowly. This difference in sampling leads to different levels of Z_{rec} by the end of the pre-exposure session and thus during the shock session, and **Figure 2-5** represents

these different levels of Z_{rec} as separate curves. For short pre-exposure, males are represented by the Z_{rec} 90 and females by the Z_{rec} 80 curves. For long pre-exposure, males are represented by the Z_{rec} 95 and females by the Z_{rec} 85 curves. While these differences in Z_{rec} between a short and a very long pre-exposure may seem minimal, the model assumes that BaconX sampling slows down as Z_{cur} increases [and there are fewer new attributes to sample] during the pre-exposure session. Therefore, large increases in pre-exposure time in the long condition does not necessarily reflect a much greater Z_{cur} at the end of the session.

In this situation, as described in the previous paragraphs, small differences between what BaconX remembers (Z_{rec}) and what it is now seeing (Z_{cur}) may cause its B_{Rep} go way down when returned to the pre-exposed context (see all curves in **Figure 2-5A**). The rate at which B_{Rep} will increase, and the timing for when B_{Rep} would fall depend on the levels of Z_{rec} and Z_{cur} . For males in the long pre-exposed condition (black line of **Figure 2-5A**), Z_{rec} will be very high after long pre-exposure ($Z_{rec}=95$), and Z_{cur} will be 50 at the time of shock. However, as shown in **Figure 2-5A**, at some point, around $Z_{cur}=35$, the small differences between Z_{cur} and Z_{rec} will cause B_{Rep} to plummet, preventing conditioning to the representation for context A from the prior day if the US is delivered at $Z_{cur}=50$. The overall result is that no fear would get conditioned to context A.

However, if the subject were a female, fewer attributes of context A would become associated with its representation during the long pre-exposure because it samples attributes more slowly (i.e., its Z_{rec} would be lower at the end of the session than was the case for the male), and its curve of B_{Rep} as a function of Z_{cur} during the conditioning session would look more like the green curve in **Figure 2-5A**. In this case, if the US occurred at $Z_{cur} = 45$ (10% less than the male $Z_{cur} = 50$ at the same PSI), conditioning would occur, as in fact it does in Experiment 1B. This explanation would lead to the further prediction that if one waited until much later in the session, the slight, random differences between the pre-exposure and the conditioning session might cause rejection of the representation for A in the long pre-exposed female as well as in males,

and conditioning would no longer be possible (this happens at about $Z_{cur}=65$ in the **Figure 2-5A**).

Figure 2-5B shows the potential conditionability index, defined as $B_{Rep}-B_{cnd}$ for males and females in both the short and long pre-exposure conditions. These values are taken from **Figure 2-5A**, and are denoted by red circles for females and blue circles for males. **Figure 2-5C** presents the actual behavioral data, combined from Experiments 1A and 1B, for fear to the shocked (Same) context following short or long pre-exposures in males and females, and it is presented to showcase the similarity in the patterns of the predicted/simulated conditionability with the actual fear expression of animals who underwent the same procedures.

Alternatively, beyond BACON, the particular finding of a lack of significant fear in males after 10 min of pre-exposure to a context with a short 20-s PSI could reflect something like latent inhibition. Latent inhibition refers to the phenomenon in which previous experience with/non-reinforced presentations of a neutral cue can diminish or even prevent future learning about that cue when it becomes meaningful/predictive (Lubow, 1973). While latent inhibition is not frequently observed in the case of contextual fear, and increasing exposure to a context generally is thought to enhance contextual fear learning (Wiltgen et al., 2001), there is at least one report suggesting that, in male mice, latent inhibition of contextual fear can occur as a result of prolonged pre-exposure to context prior to the conditioning day (Radulovic, Kammermeier, & Spiess, 1998). It could be the case that males are more sensitive to impacts of latent inhibition for contextual learning than females and/or that, as suggested above, males form contextual representations more quickly, thus allowing for more effective latent inhibition of later contextual learning. However, it should be recognized that on its own the term latent inhibition is an empirical observation and not an explanatory mechanism. Several theories have proposed potential processes that might underly latent inhibition but some of them do not seem readily applicable to contextual conditioning (e.g., Pearce & Hall, 1980; Mackintosh, 1975) and others

(Wagner's SOP; Wagner, 1981) suggest that latent inhibition of contexts is something that would not readily occur. In this way, the above interpretation using Bacon can be seen as a potential process explaining contextual latent inhibition.

Experiment 2: Remote vs Recent Fear

While the hippocampus is required for forming contextual representations and contextual fear learning, as time passes and the memory transitions from short to long-term memory, the hippocampus becomes less necessary for expression of a contextual fear memory (Kim & Fanselow, 1992; Kitamura et al., 2017). Recent memories require the hippocampus, whereas more remote memories do not require the hippocampus. To further experimentally assess sex differences in contextual fear memories that may or may not require the hippocampus, we tested contextual fear learning and generalization for both recent and remote memories. The design of Experiment 2 is presented as **Table 2-2**. Briefly, Experiment 2 assessed both recent (1-day old) and remote (28-days old) contextual fear memory and generalization after a slightly longer PSI (25s) and a short pre-exposure session (2-min) in male and female rats (n = 64 total; 16 of each sex tested at recent or remote timepoints, with half tested in the shocked context first). The slightly longer PSI was used in efforts to increase levels of contextual fear. This 28-day timepoint was chosen as a point when contextual memories persist but are no longer dependent on the hippocampus (Kim & Fanselow, 1992) and when incubation of contextual fear memories and generalization may occur (Houston et al., 1999; Poulos et al., 2016). This incubation is thought to reflect a loss in contextual specificity as hippocampal memories undergo systems consolidation and become more cortically-represented and dependent memories (Kim & Fanselow, 1992; Pedraza et al., 2019; Wiltgen, 2004). While incubation of fear is often difficult to produce (Pickens, Golden, & Nair, 2013), the vast majority of studies that do produce incubation of fear have only been performed in males. If the observed differences in

contextual fear learning are indeed due to differences in hippocampal functioning, one might predict that incubation of fear may have sex differences as well.

In this experiment, we tested each animal in both the shocked (Same) context and a novel (Different) context in a counterbalanced fashion. We don't typically do this as results are often difficult to interpret due to testing order effects (Keiser et al., 2017; Asok et al., 2019), but we wanted to get as much information as possible out of this longer experiment and sought to determine and describe empirically whether order effects would occur.

Results from the test sessions are presented in **Figure 2-6**. A repeated measures ANOVA across both tests (Test Order; First vs. Second), with Sex (Male vs. Female), Testing Context (Same vs. Different), and Testing Timing (Remote vs. Recent) as factors revealed a significant within-subject effect of Test order [$F(1,56) = 43.654, p < .001$]. Animals generally froze more to the first test than the second test. The ANOVA revealed a significant within-subject interactions for Test Order x Testing Context [$F(1,56) = 15.759, p < .001$], such that animals showed differential fear to the shocked (Same) context during only the second test ($p < .001$). The ANOVA also revealed a strong trend for a 3-way interaction for Test Order X Sex X Testing Timing [$F(1,56) = 3.758, p = .058$]. As the interaction is not reliable, it is not appropriate to run post-hoc comparisons, however, this trend is driven by differences between the freezing levels of males and females during the second Recent test and both Remote tests, but not during the first Recent test. Additionally, a significant main effect of Sex was found [$F(1,56) = 9.751, p = .003$], such that males generally froze more than females, and a significant Sex by Testing Timing interaction was found [$F(1,56) = 4.184, p = .046$], such that males froze more than females when tested at Remote time-points ($p < .001$), as well as a trend for females to freeze more at the recent than remote time-points ($p = .052$). These last results do provide evidence that incubation of freezing may be more apparent/easier to obtain in male than female rats.

Animals froze significantly less during the second test compared to the first, and therefore, the order in which animals were tested had a great impact on measures of discrimination and generalization. Therefore, **Figure 2-7** presents the discrimination index for each animal, which is calculated as the percent freezing in the shocked context (Same) divided by the combined percent freezing in both the shocked (Same) and novel (Different) contexts. Thus, a score of 1 means very good discrimination in which the animal only shows fear in the shocked (Same) context, and a score of 0.5 means there was no evidence of discrimination such that freezing levels were the same in each testing context. Shown in this way, it becomes clear that testing order can have a significant impact on contextual fear expression and therefore discrimination/generalization. ANOVA reveals a strong effect of test order on discrimination ratio [$F(1,56) = 41.274, p < .001$]. Animals who were first tested in the shocked context and then the novel context show good evidence of discrimination, but animals first tested in the novel context show strong generalization of contextual fear, resulting in poor discrimination.

BaconX Simulation of Testing Order

As expected, we did find evidence suggesting a very significant testing order effect in contextual fear learning and generalization. This is important to replicate as a general procedural note; and like other findings presented here, can be explained using the BaconX model. **Figure 2-8** presents a BaconX simulation of the test sessions of the Recent conditions of Experiment 3. Briefly, the automaton was exposed to 4 sessions, as in Experiment 3. The pre-exposure session was 1000 intervals, the PSI session included 150 intervals prior to US presentation (just enough time to form a representation if there was no pre-exposure), and the test sessions were 3000 intervals (all relatively comparable to our 2 minutes of pre-exposure, 25 second PSI, and 5-minute test sessions). The colored bars at the bottom show which representation was active (red=recalled conditioning context, blue= newly created novel context). The two contexts were quite similar and had 90% overlap of attributes.

In **Figure 2-8**, Expected Fear (bright green curve) and several other measures are plotted as a function of time in the test session. The blue curve plots the number of current attributes so far sampled during the session (Z_{cur}), the red lines (B_{Rep}) are BaconX's degree of confidence in the validity of its currently active representation, and the purple lines (Extinction/Inhibition) show the inhibition that is responsible for extinction and that accrues over intervals in which fear is expressed but not reinforced by a US. Overall, these simulations match quite well with what was observed in our experiments.

In the case where the automaton was tested in the shocked context A first, representation A is activated quickly, fear is expressed and fear is extinguished to the context A representation. When subsequently tested in context B, context A is activated initially; however little fear is expressed because context A fear had been extinguished in the previous session in A. One can in fact see that there is some inhibition active from the very start of the session. However, because BaconX had previously learned a lot about A during its two previous sessions there (pre-exposure + conditioning session), it quickly 'realizes' that it is not in A and creates a representation of context B which it does not fear, though a little generalized fear is expressed.

When the automaton was tested in the novel context B first, rather than second, the representation of context A is again activated initially, but in this case BaconX has not had the extra time in context A that was provided by a first test session in A; therefore, it knows less about A than when A was tested first. As a result, it takes a considerable period of time before a representation of B is created, and until that happens, expressed fear is not much different from what was expressed when the test in A itself came first. But there is somewhat less fear expression because B_{Rep} is somewhat lower due to mismatch of observed and recalled attributes, and fear expression is affected by B_{Rep} . When the test in A is second BaconX predicts that, as was found to be the case in our experiments, there is considerable fear. It should be noted that since there was considerable fear expression and within-session extinction during the

initial test while BaconX 'thought' it was in context A, one might have expected the extinction to carry over to the second test when the automaton actually was in context A. However, this does not happen in the BaconX model because within-session extinction does not consolidate unless BaconX is sure at the end of a session that it really is in the context where it got conditioned (Krasne et al., 2021). Indeed, this seems to accord well with what we observed in our experiment when the animals were actually tested in the shocked context A on the day following testing in a novel context B.

Experiment 3: Pre-exposure vs PSI

As mentioned before, both pre-exposure experience and increasing time in the context prior to the first unconditional stimulus (PSI) can increase contextual fear learning, but it is unclear whether the time spent as pre-exposure is equivalent and/or additive with time prior to shock. Additionally, this 3-day conditioning procedure has revealed novel sex differences that suggest the need for further examination of what is different or special about this procedure. Experiment 3 attempts to directly compare the impacts of pre-exposure with impacts of increasing PSI lengths. The design of Experiment 3 is presented in **Table 2-3**. BaconX and other conceptual models of the hippocampus suggest that the hippocampus can operate in different functional modes; in BaconX, the hippocampus can broadly function in two modes, a creation mode for building a contextual representation (which would happen during Day 1 pre-exposure in the experiments here) and a recall/update mode for retrieving that representation, prior to shock for example (which would happen during Day 2's PSI). In either of these modes, the hippocampus is able to form associations with USs, but the resulting learning and potential expression of learning may potentially differ depending on which mode the hippocampus is in when it experiences a new association with a US. Briefly, in this experiment all animals (n=31; 16 males, 15 females) received a total of 150s of context exposure prior to shock on Day 3, but for one group (PSI No-PreX), all 150s occur on Day 2 prior to shock, and for the other group

(PreX), 120s occur on Day 1 as pre-exposure with a 30s PSI on Day 2. Thus, the US would occur after different hippocampal modes/patterns of activity in these two cases. For the PSI group, the US would occur in a session in which the hippocampal representation is first created, and in the PreX group, the US would occur in a subsequent session in which the hippocampal representation is recalled. The resulting impact on contextual fear is presented in **Figure 2-9**.

A two-way ANOVA run on the freezing levels across the whole session (**Figure 2-9**, left panel) revealed a significant main effect of Procedure [$F(1,27) = 13.107, p = .001$] and a significant Time X Procedure interaction [$F(1,27) = 9.169, p = .005$]. Post-hoc analyses reveal an impact of sex on animals in the PreX condition ($p = .022$; replicating the sex difference from Experiment 1A with the same short pre-exposure and a similarly short PSI), and an impact of procedure only in female rats ($p < .001$). While there is no difference in overall fear in males across these two procedures, analyses of the time-course of fear expression during the test session (**Figure 2-9**; right panel) reveal an interesting impact of procedure generally across both sexes. That is, a repeated measures ANOVA revealed a significant main effect of Time [$F(4,108) = 26.959, p < .001$] and a Time X Procedure interaction [$F(4,108) = 17.838, p < .001$]. Post-hoc analyses of within-subject contrasts reveal that for animals in the PreX condition, there was a significant quadratic component to the freezing over time such that it increased initially before decreasing [$F(1,13) = 10.505, p = .006$]. However, for animals in the PSI condition, there were both significant linear [$F(1,14) = 73.800, p < .001$] and quadratic components [$F(1,14) = 57.175, p < .001$] to the freezing over time, such that freezing increased over time before plateauing towards the end of the test session. In other words, while freezing was more-or-less constant with a slight increase followed by a decrease across testing in animals with greater pre-exposure experience, freezing started out very low and increased over the 5-minute test session in animals in the PSI condition with less pre-exposure experience.

BaconX Simulation of Pre-exposure vs PSI

BaconX also provides some insight both into the PreX vs PSI (No-PreX) differences just described as well as the finding that it was specifically females that showed better evidence of conditioning when most of their experience of the conditioning context was in the session in which the shock US occurs rather than during a pre-exposure session.

Figure 2-10A presents the experimental design of BaconX simulations of Experiment 3. As in Experiment 3, subjects/automatons were exposed to three days of training/testing. On Day 1, automatons in the PreX condition receive some amount ("x") of pre-exposure, but automatons in the PSI (No-PreX) condition receive no such pre-exposure session. On Day 2, automatons in both groups are given a session where after some PSI, a shock is delivered; and on Day 3, automatons are returned to the same context to assess fear memory and expression. The PSI for the PreX group was set to be 150 intervals (just long enough to support fear conditioning). In line with the procedures of Experiment 3, in the PSI (No-PreX) condition, the PSI during the conditioning session was the duration (x) of the pre-exposure used in the PreX group + the PSI of the PreX group (i.e., x + 150 intervals). Thus, the amount of effective time that an animal experienced in the context prior to shock was equated between groups. Simulations were run using two different pre-exposure durations (150 and 400 intervals, which we refer to as Short and Long respectively).

Fear in the PreX and PSI (No-PreX) conditions is shown for the 150 interval (Short) and the 400 interval (Long) pre-exposure duration simulations in **Figures 2-10B** and **2-10C**, respectively. In the Short case and to a lesser extent in the Long one, conditioning, and thus expressed fear, was greater in the PSI (No-PreX) condition than in the corresponding PreX conditions, which is what we observed in the females, though not the males of Experiment 3 (**Figure 2-9**).

These results are expected given that the degree of conditioning in BaconX is proportional (subject to upper and lower bounds) to B_{Rep} of the active representation at the time of the US.

B_{Rep} of the active representation increases similarly as a function of both Z_{cur} and Z_{rec} (see **Figure 2-2**). In both Experiment 3 and our simulations, Z_{cur} is greater in the No-PreX condition than the PreX condition because there is more time in the context prior to the US in the No-PreX case [on the day of conditioning]. Z_{rec} is also greater in the No-PreX case because the period over which attributes contributing to Z_{rec} is equal to the pre-exposure duration (“x”) plus the 150 intervals of PSI whereas in the PreX case Z_{rec} accumulates only during the pre-exposure period (“x”) itself. This is the case because attributes newly observed during the conditioning session only add to Z_{rec} at the session's end (as explained in our above description of BaconX). So B_{Rep} is higher in the No-PreX case, and hence conditioning is more effective. Furthermore, as would be expected from these considerations, conditioning is enhanced in both the PreX and No-PreX conditions when pre-exposure duration is increased because, given the experimental design (**Figure 2-10A**), increasing pre-exposure duration increases both Z_{cur} and Z_{rec} at the time of conditioning.

That females, but not males, displayed the pattern described above deserves additional consideration. If we think of the simulation results in **Figure 2-10B** as being those of females, and we assume that, as conjectured in discussing other experiments, males sample their environments more rapidly than females, then we would expect the results of males to be somewhat more like those of **Figure 2-10C**. The faster sampling of the males would enhance both Z_{cur} and Z_{rec} and hence B_{Rep} at the time of the US relative to their values in females. Of course, in Experiment 3 there appeared to be no difference between the PreX and No-PreX conditions in males whereas there is merely a lesser difference in the simulation of **Figure 2-10C**. However, in BaconX the enhancing effect of B_{Rep} on conditionability has a limit at B_{mxcond} (see our description of BaconX and **Table 2-4 in METHODS**). If maximal conditionability (the parameter B_{mxcond}) were lowered a bit so that expressed fear were limited as indicated in the

dashed line in **Figure 2-10 B & C**, then **Figure 2-10C** would look very much like the males in our Experiment 3.

It appears that fear tends to rise throughout the course of the test session in the No-PreX group but not the Pre-X group. In BaconX, because B_{Rep} increases as a function of Z_{cur} , conditioned fear rises during a test session, until within-session extinction becomes dominant. However, for reasons similar to those discussed with regard to the decline of conditionability in males that are given very long pre-exposures, it is possible that if Z_{rec} were especially great during a test session, small random changes of attributes from session to session might cause BaconX to 'believe' that it was in a different context and stop expressing fear. And if this happened at different times in different animals, fear might fail to increase during the session and might look roughly constant, as it does in the PreX animals of **Figure 2-9B**. As described above, there are circumstances under which Z_{rec} would be higher after a pre-exposure plus a conditioning session exposure to a context than after a single No-PreX conditioning session alone, and this might provide an explanation for the relatively constant low level of fear in the PreX groups.

Discussion

We presented a series of behavioral experiments on contextual fear learning and generalization in male and female subjects together with computational simulations that we believe help to understand the nature of the sex differences that were observed. We have both replicated previous findings, such as a general increase in contextual fear in males after short pre-exposures and PSIs, as well as shown new and, initially, seemingly contradictory findings, such as an increase in contextual fear in females after long pre-exposures and a short PSI. These results have been simulated and explained with the help of BaconX theory and computations. Experiment 1A revealed that after a short 120s pre-exposure and a short 20s PSI, male rats show greater contextual fear than females and good evidence of contextual discrimination

(**Figure 2-1**). However, Experiment 1B revealed that after a long 600s pre-exposure and the same 20s PSI, females show elevated contextual fear and more evidence of contextual discrimination, but males do not (**Figure 2-3**). Experiment 1C suggested that estrous phase was not having a large impact on female contextual fear using this 3-day learning procedure (**Figure 2-4**). BaconX simulations suggested a possible mechanism that could explain both the general/typical sex difference in which males show more fear than females as well as the initially paradoxical finding that at certain longer pre-exposure lengths, females show more evidence of contextual fear, and indeed virtually all of the sex differences we have seen in the experiments of this paper. If female rats are assumed to sample attributes of their environment a bit more slowly, these contradictory results are predicted by BaconX and begin to appear lawful (**Figure 2-5**). As described above, slower sampling of contextual features in females is potentially consistent with not only these fear-based behavioral sex differences, but also the general sex differences in spatial navigation as well as increased thresholds needed to induce hippocampal LTP in females (Wiltgen et al., 2001, Le et al., 2022; Cossio et al., 2016; Cushman et al., 2014; Maren, De Oca, & Fanselow, 1994; Poulos et al., 2015). Beyond a potential difference in the rate of sampling, additional explanations building on the conceptual BACON model could include males requiring less stringent thresholds for B_{rep} to form new representations and/or condition. Males may also/instead have a different overall function of conditionability with respect to B_{rep} . Additional experiments and simulations may help tease apart such alternative explanations. The hypothesis that sampling rate is slower in females is parsimonious because this one male-female difference can explain a wide range of sex differences. However, there is really no good reason why there might not be sex differences in multiple parameters.

Experiment 2 expanded upon the prior findings and additionally assessed potential sex differences in remote vs recent memories (**Figure 2-6**). Males showed more evidence of

contextual fear than females at both recent and remote time-points, and males showed evidence of incubation of fear over time from the recent to remote time-points. Additionally, there were large testing order effects. Animals that were tested in the shocked/Same context first showed good discrimination, but animals tested in the novel/Different context first showed poor discrimination (**Figure 2-7**). These order effects are predicted by the BaconX model (**Figure 2-8**). Of note, we did not replicate our finding from Experiment 1A of greater contextual fear in males at short pre-exposures in the first test at the recent time-point of Experiment 2. This conflict may have resulted from the slightly longer PSI used in this experiment (25s vs 20s). However, we did see evidence for greater contextual fear in males at both remote tests as well as at the second recent test.

Experiment 3 found that, as predicted by BaconX, pre-exposure timing and PSI timing were not equivalent to each other. The resulting time-course of contextual fear at testing was markedly different between procedures that equated time prior to shock, but differed in whether that time was during Day 1's Pre-exposure or Day 2's PSI. Additionally, females showed greater fear with the PSI procedure than with the PreX procedure, and males showed more fear than females within the PreX procedure groups (**Figure 2-9**). BaconX would make a similar prediction, in that learning should be better in a situation for which all context exposure prior to the occurrence of the US occurs during a session in which the contextual representation is created (PSI/No-PreX) compared to animals who have had prior exposure to the context (PreX).

Why BaconX behaves this way is not entirely straight-forward. Conditionability during sessions following a session in which a representation has been created depend on the amount recalled about the context (Z_{rec}) and what has been observed during the current visit (Z_{cur}), an example of which is shown as different curves in **Figure 2-5**. However, as discussed in Krasne, Cushman, and Fanselow (2015), one could argue that when conditioning occurs in the session where a representation was created, as happens in the PSI condition of Experiment 3, an

animal knows for certain that its representation is that of the current context, and therefore conditionability should be maximal. If that were so, then one would definitely expect greater conditionability when conditioning occurred in a novel context so long a Z_{cur} was great enough to allow a representation to be created ($B_{Rep} < B_{new}$). Though, as further argued Krasne, Cushman, and Fanselow (2015), it is feasible that an animal whose representation was created in an earlier session with a given Z_{cur} probably knows at least as much about the context as an animal paced in the same context for the first time. Therefore, its conditionability should not be less than the latter animal. In reality, as fear develops gradually to a context as time before shock increases (Wiltgen et al., 2001; Fanselow, 1990), it is clear that conditionability does not in fact jump to maximum possible when a new representation is created. Therefore, BaconX assumes as well that conditionability develops gradually in a session in which a representation is created. The better conditioning in the PSI (No-PreX) condition would be even more profound if one made the simpler assumption that conditionability is maximal in the session where a representation is created.

Generally, the finding that PSI vs PreX procedures differ in resulting fear learning and expression suggests that there are different processes underlying the contextual learning during pre-exposure and during subsequent re-exposure prior to receiving a shock in a contextual fear procedure. The behavioral data and BaconX simulations further suggest that there may be sex differences in how and when these processes are activated. Additionally, incubation of fear over time in males but not females may suggest a difference in how remote memories are consolidated overtime and/or how differences in hippocampal function can impact the indexing of remotely stored cortical memories.

To explain our findings as well as past findings in the literature, we have built into our theory and computational BaconX model the assumption/hypothesis that females may sample the features of their environment more slowly than males, resulting in slower hippocampal learning. This

prediction does indeed explain much of the data, but it is worth discussing why such a mechanistic sex difference may exist. One possibility is that reduced hippocampal function/increased threshold for hippocampal learning may be some secondary by-product of unrelated behaviors or learning strategies that are functional and adaptive. A separate possibility is that higher threshold for hippocampal LTP and learning may provide advantage and reflect an adaptation that allows females to be more conservative with respect to some learning situations. In a complex environment that contains many different cues, contexts, predictors of reward and/or punishment, and other organisms, it may be beneficial to raise the threshold for learning such that only particularly important or salient events are encoded.

The experiments and simulations presented here add to a body of literature on sex differences in contextual fear learning and hippocampal functioning. Further work is needed to explore additional predictions from the BaconX model, including the prediction that with even longer pre-exposures than were used here, conditioning may be poor or even impossible. While we found no impact of estrous phase on the fear-related behavior after the procedures used in this study, such impacts are often found, and further work on the impact of estrous cycle and particularly on the impact of potential circulating sex steroids/hormones is warranted.

Materials and Methods

Animals: Naïve male and female Long Evans rats were used in all experiments. All rats were bred and maintained in an in-house breeding colony in the same building as the laboratory where all behavioral experiments took place. Animals were aged 90-135 days at the start of each experiment. All animals had free access to food and water throughout the entirety of all experiments. The Chancellor's Animal Research Committee at UCLA approved all experiments.

Apparatus: Fear conditioning experiments were conducted in standard Med Associates conditioning chambers (VFC-008; 30.5 x 24.1 x 21 cm), and all procedures were programmed

and controlled by Med Associates VideoFreeze software (Med Associates, St. Albans, VT). For these experiments, two distinct contexts were used, which differed in visual, olfactory, auditory, and tactile features as well as location within the lab. Med Associates shock scramblers (ENV 414-S) delivered scrambled shocks to the grid floors within the chambers. Near infrared cameras recorded each session, and fear to the context was assessed by measuring the time spent freezing when in the context. Freezing is defined as the absence of movement except respiration and is an ecologically valid measure of fear (Fanselow & Bolles, 1979). Using VideoFreeze software, freezing was scored as periods of time in which the animal's motion was below a certain threshold, determined from visual inspection of behavior, for at least 1 second (Perusini et al., 2016; Pennington et al., 2020).

Behavioral Training and Testing Procedures: One week prior to the beginning of each behavioral experiment, all rats were singly-housed and handled daily for 2-5 minutes per day. In the two days prior to each experiment, animals were additionally habituated to the procedures used to transport them from the vivarium to the laboratory. These experiments used a 3 or 4-day contextual fear learning procedure. In each experiment, Day 1 – Pre-Exposure consisted of pre-exposure to a to-be-shocked context, varying in length (0, 2, 5, or 10 minutes). Day 2 – PSI consisted of a single foot-shock (2s, 1.0 or 1.5 mA) after some pre-determined placement-to-shock interval (PSI; range from 20-150s). Animals were removed from the chamber 30 seconds after the shock. Day 3 – Test consisted of a contextual fear test in which animals were placed either in the shocked or a novel context for 5 minutes. For Experiment 2, this Day 3 – Test session occurred either 1 (Recent memory) or 28 (Remote memory) days following the foot-shock on Day 2 – PSI. For all other experiments, Day 3 – Test occurred 1 day following the foot-shock on Day 2 – PSI. For Experiment 3, on Day 4 – Test B animals were additionally tested for contextual fear to the alternate context as tested the day prior. As these experiments employ short sessions and the precise timing is crucial, animals were run two at a time, being

simultaneously placed in and removed from the behavioral chambers. For Experiment 1C, in which all subjects were female rats, following one week of handling, estrous cycle was monitored daily for ten days prior to any fear conditioning. Animals were run through each day of the procedure while in a distinct phase of the estrous cycle, either Proestrus, Metestrus, Diestrus, or Estrus. Therefore, there were an average of 3-4 days between each day of the procedure for this experiment.

Estrous monitoring-Experiment 1C: For experiments that assessed both male and female behavior, we opted not to monitor for estrous phase/cycle in our female rats. The act of monitoring cycle and necessary procedures to do so (vaginal lavage) can be stressful and/or stimulating in female rats in a way for which there is no ideal control in male rats, and just monitoring estrous cycle using vaginal lavage is known to impact subsequent behavior (Walker et al., 2002). Thus, when studying subtle differences in fear behavior, the potential added stress from estrous monitoring is likely to provide misleading or uninterpretable results. However, there are a number of studies that suggest that contextual fear learning can be impacted by estrous phase, but the results of these studies are conflicting, potentially due to such inability to control for stress levels in male and female rats due to cycle-monitoring (Cossio et al., 2016; Cushman et al., 2014; Milad et al., 2009; Markus & Zecevic, 1997). Therefore, in studies that employ both male and female rats, we did not monitor estrous cycle.

In a separate experiment containing only female rats, we did monitor estrous cycle daily and trained and tested animals while in a distinct phase of the estrous cycle. We monitored estrous cycle daily, taking vaginal lavage samples each morning and immediately viewing fresh, unstained samples under a microscope to assess cytology and phase of the estrous cycle (Hubscher, Brooks, & Johnson, 2005; Marcondes, Bianchi, & Tanno, 2002). Briefly, each morning, rats were transported, 4 at a time, from the vivarium to a behavioral room in the lab distinct from any of the fear conditioning rooms. One at a time, rats were transferred from their

home cages to a cart in which they were allowed to grab the metal bars of a clean cage-top with their four paws, facing away from the experimenter. The animal's tail was then lifted and a vaginal lavage was performed by using a dropper to flush sterile 0.9% NaCl into the vaginal canal. Care was taken to only flush the canal and not to actually insert the dropper any more than necessary, in order to limit stress and to avoid potential induction of pseudopregnancy. After 2-3 flushes, one drop for each rat was placed on a glass microscope slide, with a different slide for each 4 animals. When these unstained samples are viewed fresh under a brightfield microscope at 10 and/or 40x magnification (Zeiss Axioskop 40), cell morphology can be quickly, and accurately assessed by trained observers without the need for additional staining or preservation (Hubscher, Brooks, & Johnson, 2005; Marcondes, Bianchi, & Tanno, 2002). Estrous phase for each sample was determined based on the cell cytology of each sample, with respect to relative distributions of leukocytes, nucleated epithelial cells, and anucleated cornified cells. Animals were considered regular, natural cyclers, and were thus included in this experiment, only if they showed a regular pattern of a repeating 4- or 5-day estrous cycle in the 10 days prior to the start of behavioral training.

BaconX Simulations: We used BaconX to perform simulations or make interpretations for each of the experiments presented here. The features of the model that are relevant to the present work are described at the start of the Results. The parameters most relevant to our present usage are shown in **Table 2-4**, but a full list can be found in Krasne et al. (2021). Any differences from this are described in the text.

Data Analysis: Data were collected using VideoFreeze software to automatically score freezing behavior. Briefly, all data were analyzed using general linear model in SPSS. For each experiment, omnibus multifactorial ANOVA were initially performed. Higher order and simple interactions were followed up with post-hoc analyses using Bonferroni-corrected comparisons. For repeated measures ANOVA, the Huynh-Feldt correction was used when sphericity was

violated. While unadjusted degrees of freedom are presented in order to help identify group sizes, p values reflect corrections for sphericity.

Tables

Experiment 1	Day 1 Pre-Exposure Length	Day 2 PSI, Shock amplitude	Day 3 Testing Context
Exp. 1A	2 min [Short]	20 sec, 1.5 mA	A or B
Exp. 1B	10 min [Long]	20 sec, 1.0 mA	A or B
Exp. 1C [Estrous]	5 min [Intermediate]	20 sec, 1.0 mA	A

Table 2-1 Design of Experiments 1A, 1B, and 1C

Condition	Day 1 Pre-Exposure Length	Day 2 PSI, Shock amplitude	Day 3 Testing Context	Day 4 Testing Context
Recent: Tested 1 day after shock	2 min	25 sec, 1.5 mA	A or B	B or A
Remote: Tested 28 days after shock	2 min	25 sec, 1.5 mA	A or B	B or A

Table 2-2 Design of Experiment 2

Condition	Day 1 Pre-Exposure Length	Day 2 PSI, Shock amplitude	Day 3 Testing Context
PreX	120 sec	30 sec, 1.5 mA	A
PSI (No-PreX)	0 sec	150 sec, 1.5 mA	A

Table 2-3 Design of Experiment 3

Parameter	Value
Number of cortical attribute-representing cells	1,000
Number of DG and CA3 cells	10,000
Number of attributes per context	100
Number of cortical cells innervating each DG cell	60
Number of DG & CA3 cells per representation	60
B_{new}	-30
B_{cnd}	3
B_{mxnd}	12
Learning parameter α	0.59

Table 2-4 Parameters used for BaconX simulations. Details for each parameter can be found in the text.

Figures

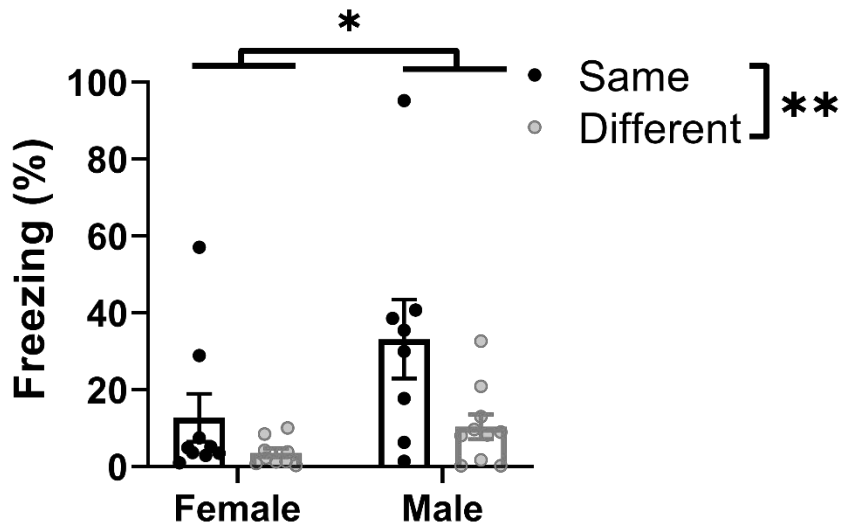


Figure 2-1 Contextual fear and generalization in males and females following short pre-exposure (2 min) on Day 1 and a short PSI (20s) on Day 2. Animals were tested on Day 3 in either the previously shocked context (Same) or a novel context (Different).

Data are presented as percent time freezing during the 5-minute test session. * $p < .05$, ** $p = .01$, all other p values $> .05$

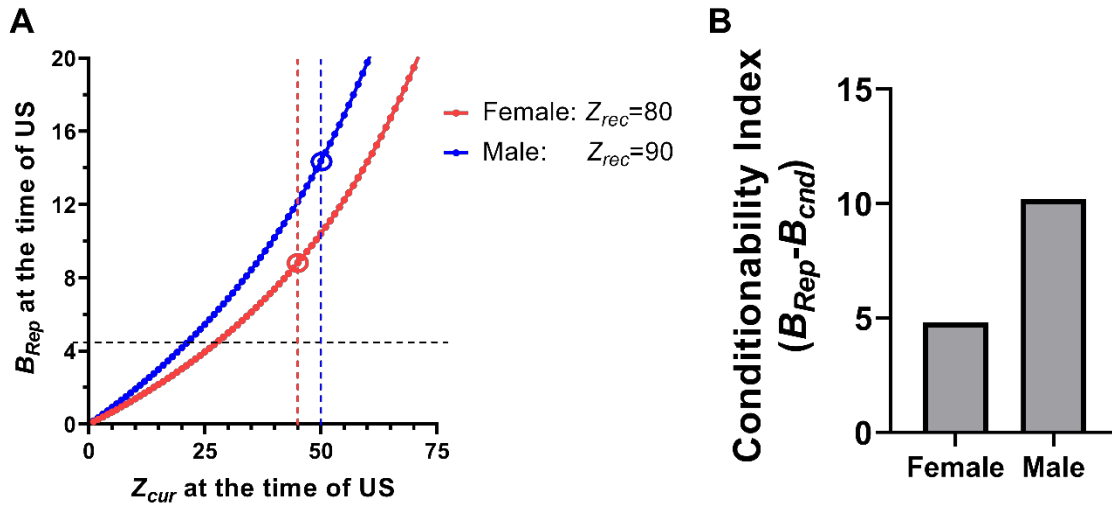


Figure 2-2 Results from a simulation in which BaconX was pre-exposed to a context, and then given a shock on the following day after some PSI in a session, as in the behavioral experiments presented here. **(A)** represents B_{Rep} as a function of Z_{cur} during a conditioning session in a context whose representation was created during a pre-exposure session. It is assumed that males sample features 10% faster, thus, the males are represented by the blue $Z_{rec}=90$ curve, and the females are represented by the red $Z_{rec}=80$ curve. The horizontal dotted black line represents B_{cnd} , the value that B_{Rep} must exceed in order to produce effective conditioning if a US occurs. The red and blue dotted lines refer to the Z_{cur} value at the time of the US for females and males respectively—the females will be at $Z_{cur}(45)$ when the males are at $Z_{cur}(50)$ due to slower sampling. The red and blue circles refer to the values pulled to create **(B)** and are the respective B_{Rep} values at the time of US. **(B)** represents the conditionability index, or $B_{Rep}-B_{cnd}$ at the time of the US for males and females.

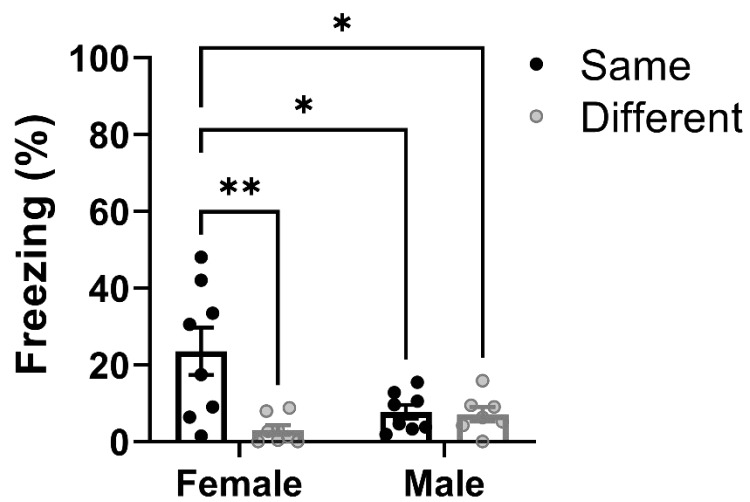


Figure 2-3 Contextual fear and generalization in males and females following long pre-exposure (10 min) on Day 1 and a short PSI (20s) on Day 2. Animals were tested on Day 3 in either the previously shocked context (Same) or a novel context (Different).

Data are presented as percent time freezing during the 5-minute test session. * $p < .05$, ** $p = .01$, all other p values $> .05$

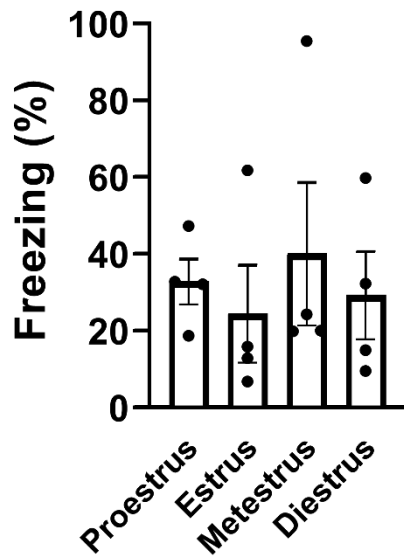


Figure 2-4 Contextual fear in females following intermediate pre-exposure (5 min) on Day 1 and a short PSI (20s) on Day 2. Animals were tested on Day 3 in the previously shocked context. Each day of the training and testing procedure occurred on a specific day of the estrous cycle for each animal.

Data are presented as percent time freezing during the 5-minute test session. * $p < .05$, ** $p = .01$, all other p values $> .05$

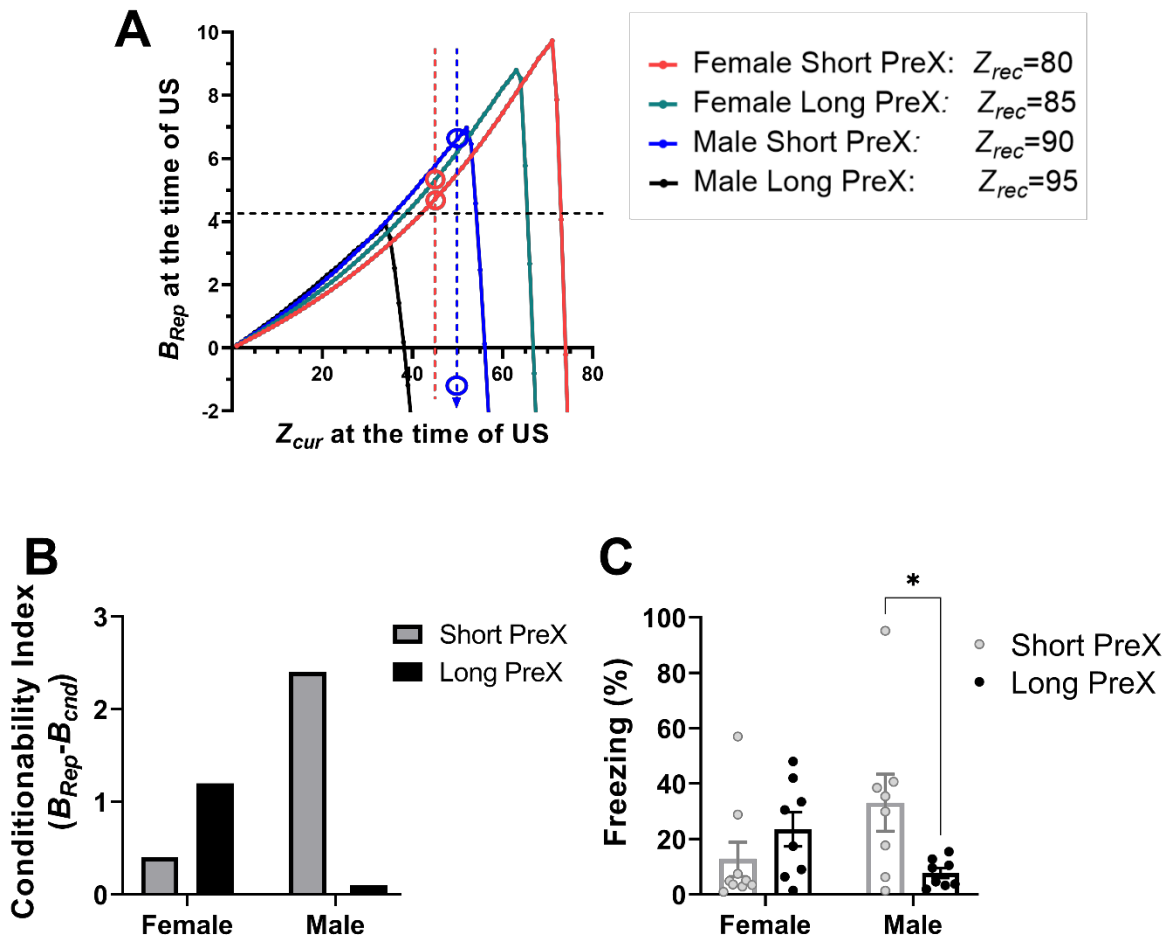


Figure 2-5 Results from a simulation in which BaconX was pre-exposed to a context for a short or long session, and then given a shock on the following day after some PSI in a session, as in the behavioral experiments presented here. **(A)** represents B_{Rep} as a function of Z_{cur} during a conditioning session in a context whose representation was created during a pre-exposure session but 10% of whose attributes have changed, as explained in the text. The dotted horizontal black line represents B_{cnd} , the value that B_{Rep} must exceed in order to produce effective conditioning if a US occurs. The vertical red and blue dotted lines refer to the Z_{cur} value at the time of the US for females and males respectively. The red and blue circles refer to the values pulled to create **(B)** and refer to the female and male values of B_{Rep} at the time of US. The lower blue circle, representing B_{Rep} for males after long pre-exposure ($Z_{rec}=95$), is shown to represent a value a B_{Rep} that has already crashed well below 0 and into negative values (not shown at this scale). **(B)** represents the conditionability index, or $B_{Rep}-B_{cnd}$ at the time of the US for males and females after short or long pre-exposure session. **(C)** shows the behavioral data from Experiments 1A and 1B for males and females tested in the shocked (Same) context. Notice the similarity in the pattern of the simulated conditionability with the actual fear expression from live animals. * $p < .05$.

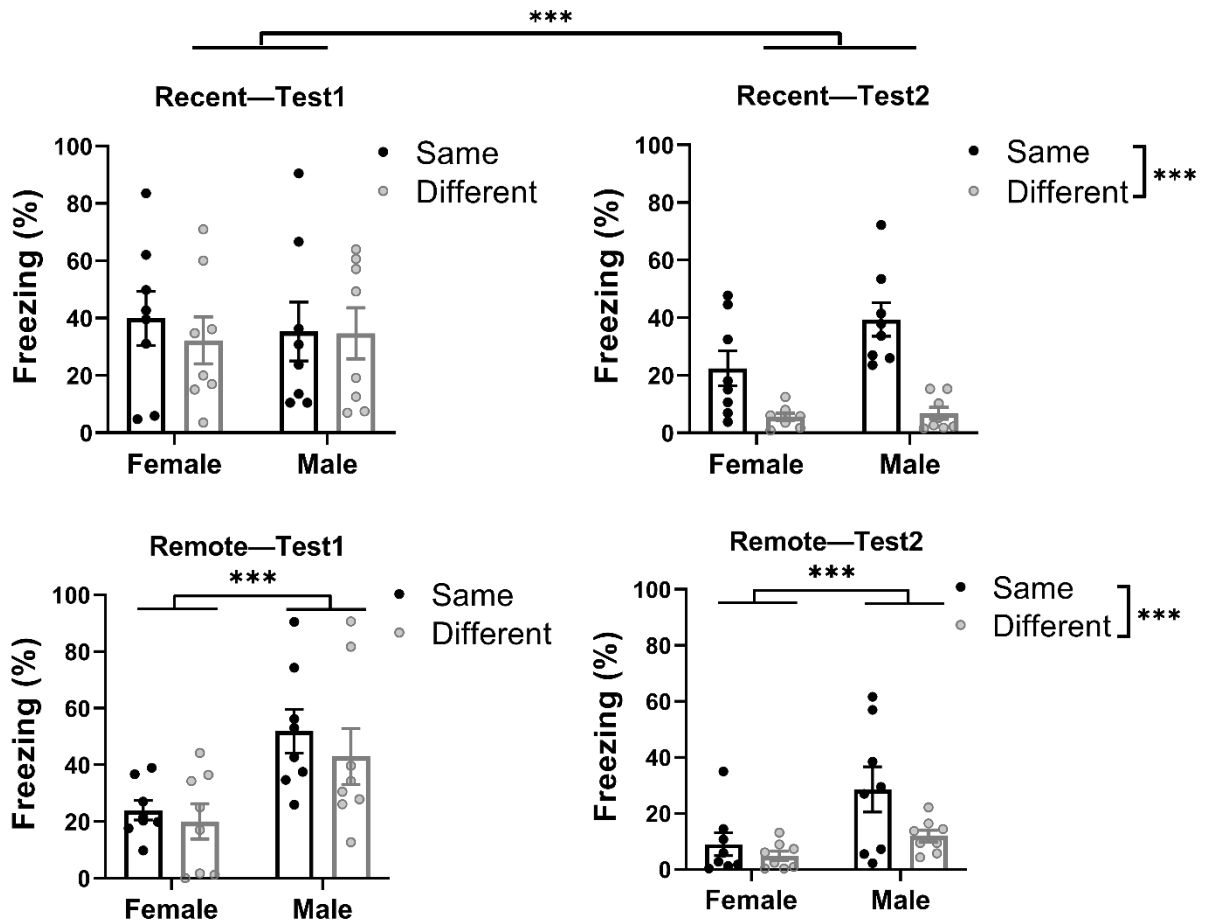


Figure 2-6 Contextual fear and generalization in male and female rats at recent (1 day) and remote (28 day) timepoints following short pre-exposure (2 min) on Day 1 and a short PSI (25s) on Day 2. Animals in the Recent condition were tested 1 day after training, on Day 3 in either the shocked context (Same) or a novel context (Different). Recent animals were then tested the following Day 4 in the opposite context as the prior day. Animals in the Remote condition were tested 28 days after training, on Day 30 in either the shocked context (Same) or a novel context (Different). Remote animals were then tested the following Day 31 in the opposite context as the prior day.

Data are presented as percent time freezing during the 5-minute test session. *** $p < .001$. Not represented here is a main effect of sex ($p = .003$).

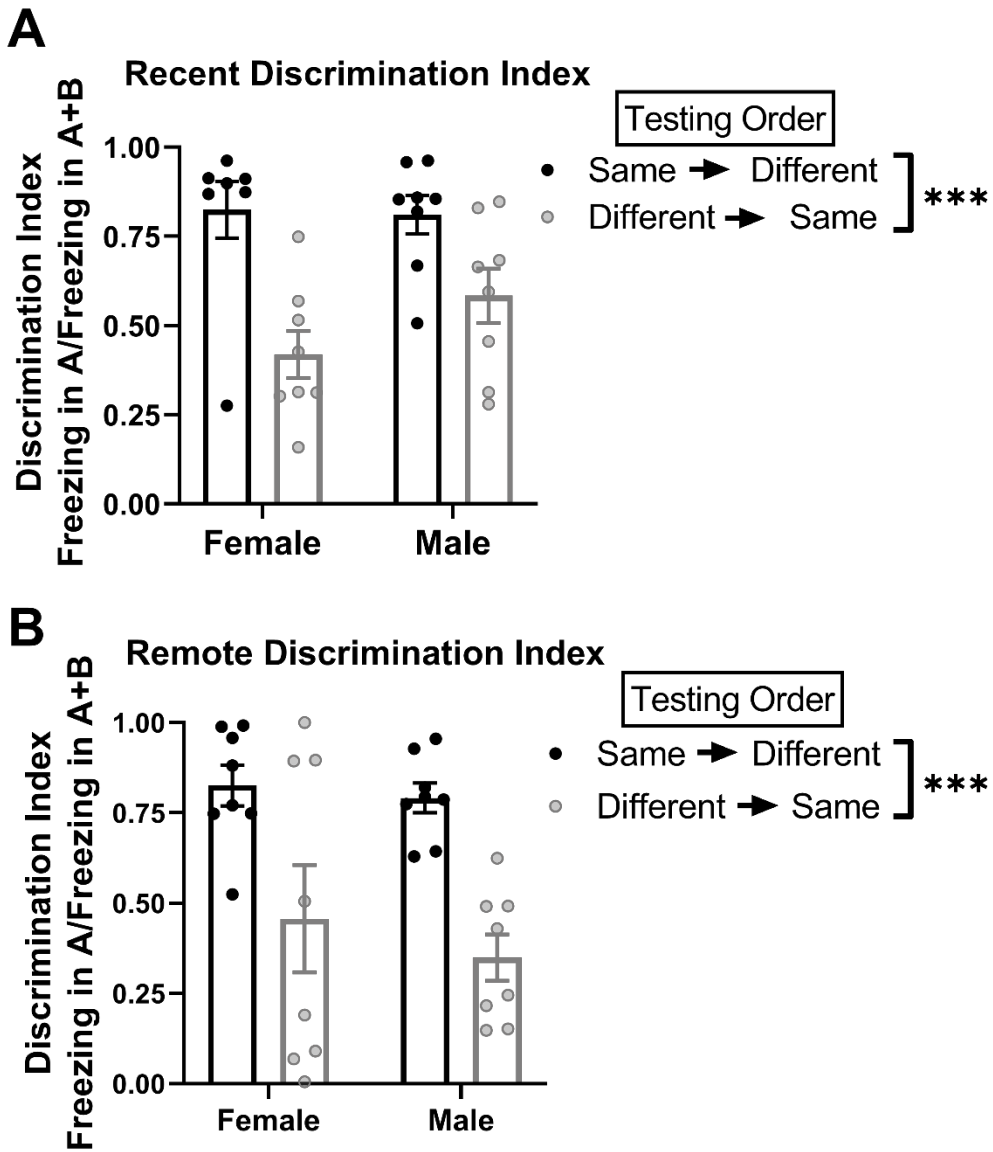


Figure 2-7 Discrimination Index in male and female rats following short pre-exposure (2 min) on Day 1 and a short PSI (25s) on Day 2, when tested at recent (**A**; 1 day later) and remote (**B**; 28 days later) time-points. Discrimination Index is calculated as percent time freezing in the shocked context (Same) divided by combined percent time freezing in both the shocked (Same) and novel (Different) contexts. Animals are grouped by testing order based on whether they were tested in the Same or Different context first. *** $p < .001$

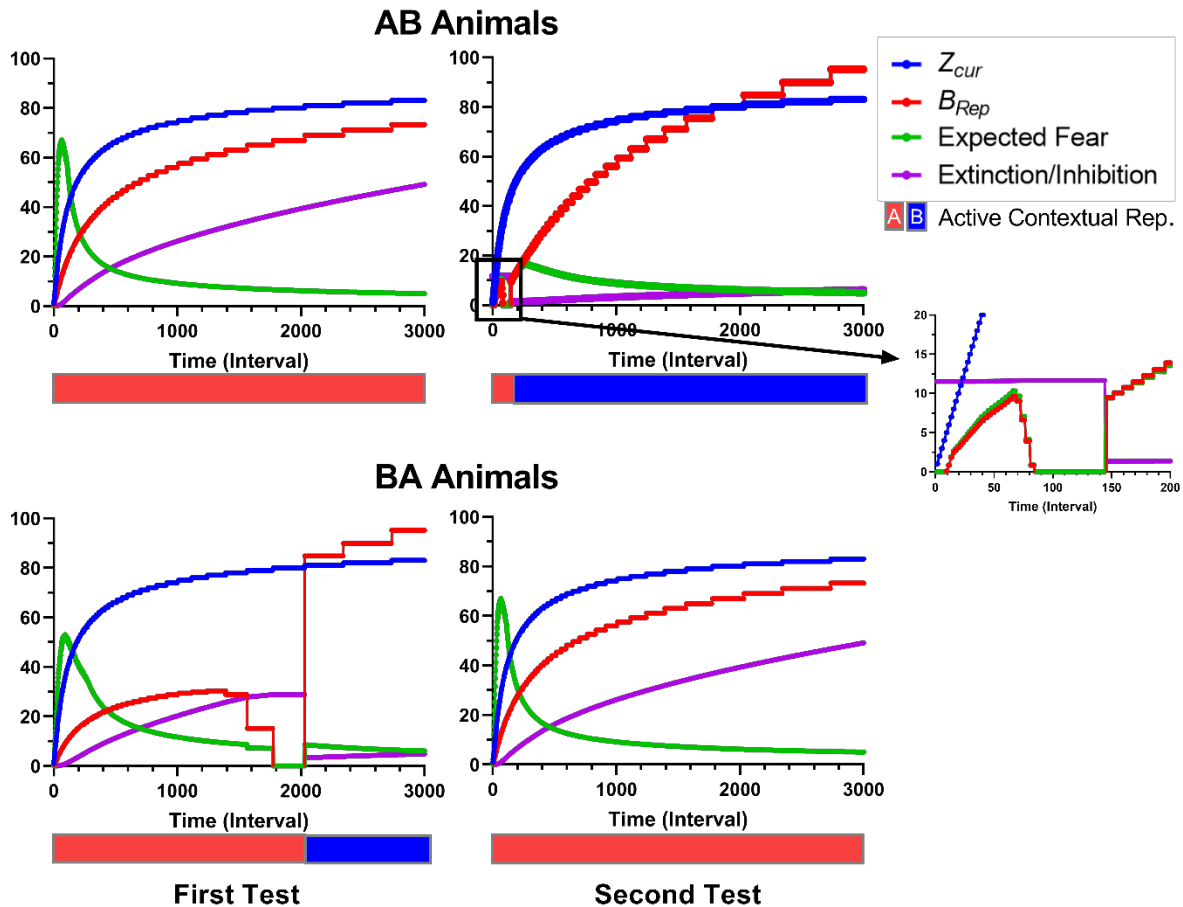


Figure 2-8 BaconX simulations of potential test order effects. Presented here are results from simulations of contextual fear learning and expression in which ‘subjects’/automatons are run through a fear conditioning procedure similar to those performed in the Recent groups of Experiment 2, including counterbalanced testing in the Shocked-A context and Novel-B context. The top panels represent the situation in which an animal is tested in A first; the bottom when tested in B first; with the left and right panels representing the first and second test respectively. The small inset plot beneath the figure legend is a zoomed-in view of the beginning of the second test for AB animals. Plotted are a number of measures calculated by BaconX across sampled time intervals on the X-axis during simulated test sessions. Z_{cur} [Blue line] = # of attributes sampled from the current context. B_{Rep} [Red line] = BaconX’s degree of confidence in the validity of its currently active representation. Expected Fear [Green line] = fear expression. Extinction/Inhibition [Purple line] = amount of extinction/inhibition accruing to the currently active representation. The red or blue bars along the bottom of the x-axis represent which contextual representation is active: A = Red (recalled representation), B = Blue (newly created contextual representation).

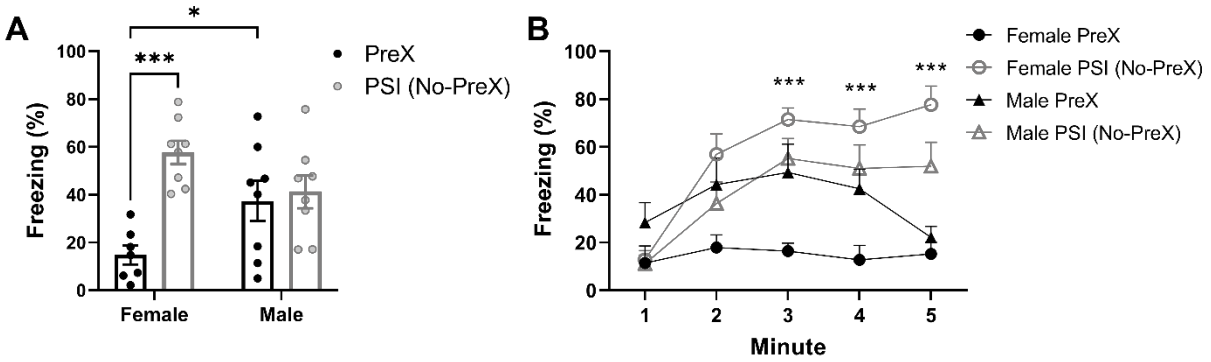
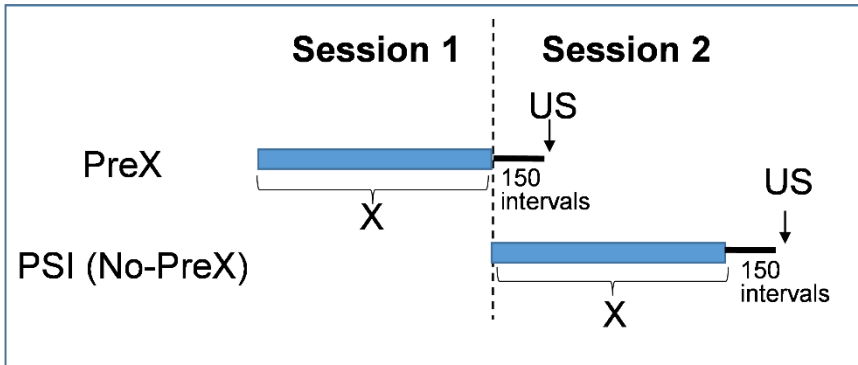
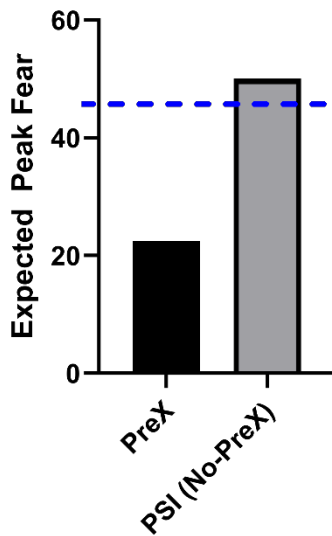


Figure 2-9 Whole Session **(A)** and by-minute **(B)** contextual fear memory testing across 5 minutes following re-exposure (PreX) or No pre-exposure (PSI No-PreX) procedures. Animals were given some amount of pre-exposure on Day 1 (120s-PreX; 0s-PSI No PreX), some amount of PSI on Day 2 (30s-PreX; 150s-PSI), and were tested in the shocked context on Day 3.

Data are presented as percent time freezing during the 5-minute test session. * $p < .05$, *** $p < .001$. Not represented here are the main effect of minute ($p < .001$) or details on the Minute X Procedure Interaction (described in the text of the Results), all other p values $> .05$.

A**B**

PreX = 150 Intervals

**C**

PreX = 400 Intervals

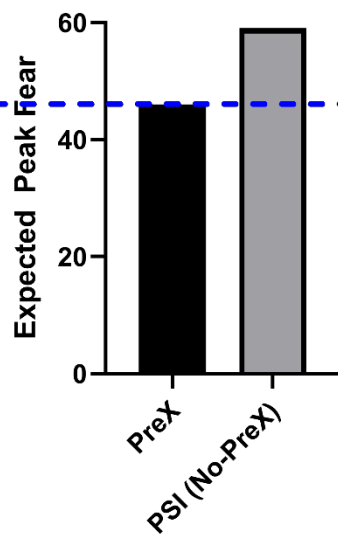


Figure 2-10 BaconX design and results from simulations of the impact of pre-exposure vs PSI. **(A)** represents the experimental design for training days of the simulations. This design is comparable to that of Experiment 3 in which all subjects receive the same total amount of context exposure prior to shock, but differing in whether this occurs only as PSI in one session or as pre-exposure and PSI across two sessions. **(B)** and **(C)** represent the resulting expected peak fear levels in a subsequent test session following two different pre-exposure lengths. The blue dashed line in panels **B** and **C** is the conjectural maximal conditioned response allowed by the BaconX if the parameter B_{mxcond} were decreased somewhat from what was actually used for the simulation (see text).

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General Discussion

Presented here are two chapters which investigate different aspects of defensive behavior using behavioral fear conditioning experiments as well as computational simulations of contextual learning. Data are discussed within the framework of the relevant literature, and a number of novel hypotheses are proposed.

In **Chapter #1**, I investigated the associative and non-associative components of defensive behavior in mice following auditory fear conditioning (Trott et al., 2022). Specifically, I initially used behavioral procedures which resulted in different types of defensive responding to distinct components of a serial compound conditional stimulus (SCS), consisting of a pure tone followed by a white noise stimulus. Following the pairing of this SCS with shock, animals develop a pattern of defensive behavior such that they typically freeze during the tone portion of the SCS and display activity bursts or darts to the white noise portion of the SCS. Follow-up experiments focused on this noise-elicited activity burst, using a number of standard learning control procedures to reveal potential associative and nonassociative components of such behavior.

I found that there appear to be distinct types of activity bursts. I observed a stronger activity burst at CS onset and a weaker, more directed activity burst occurring later in the CS. The results revealed that these peaks in activity are differentially affected by associative processes. The initial, stronger burst of activity is driven by non-associative processes and may actually be suppressed by associative learning. We describe a proposed rule regarding defensive behavior selection such that rodents will freeze when afraid, but a sudden change in stimulation can unconditionally cause a rodent to shift from a post-encounter state of freezing to a circa-strike state of activity bursting. On the other hand, this second, weaker burst of activity was indeed increased by associative learning (through pairing of the CS with shock). Careful

analysis of this second peak in activity suggests that this activity burst can best be thought of as a distinct behavior from the first unconditional activity burst to CS onset, such that this second burst is more directed and supports a rodent's ability to freeze in an ideal thigmotactic location. While the initial activity burst to CS onset tends to be undirected, including jumping and other protean behavior, the second activity burst tends to be directed, locomotive running in order to reach some corner in the conditioning box where freezing occurs.

In **Chapter #2**, sex differences in contextual fear and generalization were assessed in rats (Trott, Krasne, & Fanselow, 2022). I examined the differential impacts of pre-exposure to a context (with no shocks delivered) and time spent in the context prior to shock (placement-to-shock interval; PSI). Additionally, BaconX, a conceptual and computational model of hippocampal functioning (Krasne, Cushman, & Fanselow, 2015; Krasne et al., 2021) was used to simulate the design and results of the behavioral experiments. The goal of these simulations was to uncover potential mechanistic differences in hippocampal function that may underlie the behavioral effects I observed. With the procedure I used, the results revealed that there are indeed sex differences in contextual fear conditioning. Under most situations, males expressed more contextual fear than females, but under certain situations, such as with long pre-exposure, females showed more contextual fear than males. From these results and through simulations with BaconX, we hypothesized that these sex differences in contextual fear could be explained if it were assumed that female rats sample their environments slightly more slowly than males, requiring a bit more time in a context to form a stable contextual representation.

Chapter #1, in which I assess the associative and non-associative influences on defensive behavior, freezing, and flight in mice, adds to a growing literature suggesting there may be additional measurable fear responses other than freezing in an associative fear conditioning scenario. We found that contrary to some current conclusions, the primary factor influencing cue-induced flight behavior in mice was non-associative in nature. While associative

learning did impact the topography of cue-elicited activity bursts, the bulk of such activity bursts could best be explained as a type of non-associative fear-potentiated startle response. Our behavioral data suggest a new/updated rule of defensive behavior selection in rodents. Animals will freeze when afraid, and a sudden change in stimuli, particularly for novel stimuli, can push the animal into a panic-like state and elicit panic-like responses, which include activity bursts and 'darting'. Generally, mice that receive shock but do not experience any noise or noise-shock pairings during training show the strongest noise-elicited activity burst, suggesting that such cue-elicited activity is actually suppressed by associative noise-shock learning and is increased by the novelty of the cue in question.

It is important to note that our results are in mice and are most, but not only, relevant for the study of such behavior in mice, as detailed below. Cue-elicited activity bursts have been reported in both mice and rats. This behavior has been shown in both species of rodents using the same type of conditioning procedures used here, taking advantage of a serial compound conditional stimulus which consist of a tone and a white noise CS presented sequentially followed by shock (Fadok et al., 2017; Hersman et al., 2020; Totty et al., 2021). Darting behavior has also been reported to simple tone-shock conditioning in rats (Colom-Lapetina et al., 2019; Gruene et al., 2015; Mitchell et al., 2022). It is worth noting that regardless of the procedure used to study such activity bursts in rats, darting behavior is generally much lower than that found in mouse studies, including the one reported here. Furthermore, we show in our data that there are likely two distinct types of cue-elicited activity bursts. The first burst occurs right at/after cue onset, is stronger in magnitude, tends to be undirected, and appears to be driven solely by non-associative factors, but the second burst is delayed, occurring in the middle/toward the end of the 10s CS, is weaker in magnitude, and tends to be directed running towards a corner in order to freeze. However, this second peak in activity does appear to be affected by associative learning such that this peak is greatest in animals who receive noise-

shock pairings. We propose that this second peak in activity is best thought of as occurring as part of a directed suite of freezing behavior, as the freezing behavior module does include the locomotion required to freeze in an ideal thigmotactic place (Fanselow & Lester, 1988). When taking a deeper look at the timing of activity bursts in studies with rats (Mitchell et al., 2022; Totty et al., 2021), it seems that the timing and magnitude of the activity bursts are more in line with the second peak in activity observed in the experiments here. Generally, this does suggest that the high-activity behavior observed in the recent rat studies may, to an extent, be conditionally learned as a result of associative learning processes. However, without running the proper control conditions to assess the impact of such associative learning, it is quite likely (and especially so for studies with mice) that the behavior observed is not a result of associative learning per-se and is similar to a fear-potentiated startle response. Furthermore, our data strongly suggest that even within this 'newly-found' flight behavior, there are likely distinct types of activity bursts, and these distinct types may be differentially impacted by learning.

While such a study on the associative and non-associative influences on defensive behavior, freezing, and flight is most relevant for those studying defensive behavior directly, there are also other take-aways from these results worth considering. First, the results here provide guidance as to how a researcher may want to think about measuring behavior and whether different sexes need to be considered separately in conditioning experiments. Again, within the realm of defensive behavior, if freezing is not the only fear response, and if females may be more likely to perform different types of high-activity fear responses, this calls into question the results of many decades of study measuring fear in male and female rodents (Gruene et al., 2015). If there are sex differences in fear expression with females less likely to freeze, this casts doubt on past results which reported reduced fear learning/memory in females because they froze less than males (Cossio et al., 2016; Cushman et al., 2014; Maren, De Oca, & Fanselow, 1994; Wiltgen et al., 2021). While we, others using mice (Hersman et al., 2020),

and others using rats (Totty et al., 2021) did not find reliable sex differences, there were procedural differences that may prevent finding such reliable differences. For example, Mitchell et al. (2022) recently reported that sex differences in darting can be abolished with increased levels of training, and it is possible that the serial-compound-stimulus training used here and by others (Fadok et al., 2017, Totty et al., 2021) resulted in more overall learning/training and washed out potential sex differences. At the very least, it will continue to be important for researchers in this field and beyond to include sex as a biological variable and give the results careful consideration with respect to sex.

Furthermore, while there has been an exceptional amount of focus and study on freezing behavior, our results suggest that freezing behavior is indeed the most reliable index of the actual associative learning of fear. The procedures used here which produce such activity bursts are however quite useful to study how an animal may transition from one mode of defensive behavior to the next, i.e., from a post-encounter fear state to a circa-strike panic state (Fanselow & Lester, 1988). Indeed, such procedures which produce cue-induced activity bursts have been used to assess the impact of stress across the various modes of the predatory imminence continuum (PIC; Hoffman et al., 2022).

Second, it sheds light on a potentially troubling trend in modern neuroscience to publish single-group experiments which do not include standard control groups. This can be problematic for a number of reasons, but in this case, it is problematic when many begin to report that some new behavior is *learned* and is a good model for learned panic/learned-active-avoidance without experimentally confirming whether the behavior is learned or not. Exciting new reports about learned panic may drive research and attract funding due to such interesting translational claims. However, if, as suggested by the data here, much of this conditional panic is more 'simply' a type of fear-potentiated startle, some of this increased research and funding may lead to new, but incorrect interpretations of learning, behavior, and mental health. At the very least,

the study of defensive behavior, recently and historically, has been plagued by this idea that freezing is a “passive” response in comparison to/competition with other potentially more “active” defensive responses, such as the cue-elicited activity bursts described here (Blanchard & Blanchard, 1969; Fadok et al., 2017; Gruene et al., 2015; Yu et al., 2016). The data here, in addition to decades of research on freezing (Bouton & Bolles, 1980; Fanselow, 1980; Fanselow, Hoffman, & Zhuravka, 2019), support the notion that freezing is indeed an active behavior, such that the freezing rodent is ‘tense’ and prepared to react swiftly to sudden stimulus change. In addition, the data here suggest that even within the module of freezing behavior, there are bursts of directed locomotion which allow an animal to reach an ideal thigmotactic place to freeze.

The results of **Chapter #2** reveal a hypothesis for a potential mechanism for commonly reported sex differences in hippocampal functioning, including contextual fear and other appetitive hippocampus-dependent tasks. If females are assumed to sample contextual features more slowly than males, the results of the behavioral experiments presented here begin to look more lawful. This proposed sex difference in hippocampal sampling rate is supported not only behaviorally and with computational simulations here, but also by other work which has shown sex differences in hippocampal function, including hippocampal LTP (Koss & Frick, 2017; Maren, De Oca, & Fanselow, 1994; Le et al., 2022). Further verification and testing of this hypothesis is warranted, both in the context of contextual fear learning and appetitive contextual learning/contextual learning more generally. If there are such sex differences in hippocampal function, it may provide a framework to understand a variety of findings, including the increased incidence of anxiety disorders in females. Perhaps less contextually-rich representations in females lead to increased generalization of fear and anxiety. While the behavioral data in these experiments do not necessarily show such differences in generalization, others have indeed

reported that females are more likely and faster to generalize learned fear to safe locations (Asok, Kandel, & Rayman, 2019; Keiser et al., 2017; Lynch et al., 2013).

Overall, the data presented here in **Chapters #1** and **#2** add to a rich literature on fear conditioning and defensive behavior. The experiments presented here assess both cued auditory fear learning and contextual fear learning in both rats and mice. In **Chapter #1**, using careful experimental designs with a number of novel control conditions, I determined the extent to which different defensive behaviors are associative and proposed an update to a rule for defensive behavior selection. Behavioral data and computational simulations of hippocampal function and contextual learning from **Chapter #2** reveal a hypothesis regarding potential mechanisms underlying sex differences in contextual learning, such that females may sample contextual features more slowly than males and require more time to form and retrieve contextual representations.

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