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The Effect of Lateral Periaqueductal Grey on Non-Associative and Associative Learning Processes

A dissertation submitted in partial satisfaction of the

requirements for the degree Doctor of Philosophy

in Psychology

by

Sara Markowitz

2023

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ABSTRACT OF THE DISSERTATION

The Effect of Lateral Periaqueductal Grey on Non-Associative and

Associative Learning Processes

by

Sara Markowitz

Doctor of Philosophy in Psychology

University of California, Los Angeles, 2023

Professor Melissa Sharpe, Co-Chair

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The present thesis investigated neural correlates of Stress Enhanced Fear Learning (SEFL) in the periaqueductal grey (PAG). In the first set of experiments, we found that SEFL is transmodal. Animals that have received a stressful experience in the form of foot shock, they will demonstrate enhanced fear conditioning in the future to a white noise. Further, we found that white noise is not significantly stressful enough to cause SEFL. We demonstrated that footshock causes activity burst, a “circa-strike” behavior, as well as “post-encounter” freezing. In contrast, white noise at most caused freezing. This allowed us to use noise as a stress control when examining neural correlates of SEFL in order to verify that activity was not elicited by any fear or freezing *per se.*

In our next set experiments, we investigated neural correlates of SEFL in PAG subregions using the activity marker cFos. We compared activity resulting from either shock stress or noise stress. In central positions of PAG, the lateral periaqueductal gray (lPAG) and ventral-lateral PAG (vlPAG) both showed a graded response to stress, such that there was the greatest amount of cFos to shock stress, then noise stress, and finally low levels in homecage controls. We also saw this pattern in caudal vlPAG. However, in caudal lPAG, only shock stress caused significantly more expression than homecage controls, suggesting it is specifically severe stress that recruits the causal lPAG. Therefore, caudal lPAG may uniquely contribute to the ability of stress to cause SEFL.

In our final set of experiments, we tested the necessity of lPAG for SEFL using optogenetics. lPAG was inhibited during each shock presentation that occurs during shock stress. Surprisingly, we found that lPAG was not necessary for SEFL. Next, we asked whether lPAG might be important instead for the associative components of fear. To test this, we optogenetically inhibited lPAG during the shock in a Pavlovian fear conditioning procedure. Inhibiting lPAG during each shock presentation caused enhanced fear to both the shock-paired tone and context. Taken together, our work suggests that lPAG signals predictive teaching information about the shock during associative learning.

This dissertation of Sara Markowitz is approved.

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2023

TABLE OF CONTENTS

Chapter 1: Stress Enhanced Fear Learning, Defensive Behavior, and the Periaqueductal Grey 1

Early Studies of Fear Learning 1

Species Specific Defense Response 4

The Predatory Imminence Continuum 7

Neural Correlates Across the Predatory Imminence Continuum 10

The Midbrain Periaqueductal Grey and Defense Response 14

The Midbrain Periaqueductal Grey and Associative Learning 19

Stress Enhanced Fear Learning: a non-associative model of fear 22

Chapter 2: Behavioral characterization of the transmodality of the SEFL effect 26

Stress Enhanced Fear Learning Dictated by Non-Associative Process 26

General methods 29

2.1 Experiment 1: Transmodal SEFL 37

2.2 Experiment 2: Noise SEFL 41

2.3 Experiment 2, Noise SEFL: Analysis of Behavior during Severe Stress 46

2.4 Discussion 50

Chapter 3: SEFL increases cFos expression in lPAG and vlPAG 54

General Methods 57

3.1 Characterization of stress across the noise and shock conditions 63

3.2 Rats receiving shock stress show significantly more cFos expression in lPAG and vlPAG 67

3.3 Discussion 70

Chapter 4. Inhibiting lPAG is not necessary for Non-Associative SEFL, But Does Enhance Associative Fear Learning 79

General Methods 82

4.1 Inhibiting lPAG During Stress Does Not Attenuate SEFL 92

4.2 Inhibiting lPAG During Acquisition increases Associative Learning to cues and contexts 95

4.3 Discussion 102

Chapter 5: General Discussion 108

5.1 Summary of Findings 108

The Caudal lPAG Shows Unique Differences In cFos Expression 110

Inhibiting Caudal lPAG Does Not Attenuate SEFL 113

Inhibiting Caudal lPAG Affected Associative Fear Learning 113

5.2 The lPAG's role in an Analgesic Negative Feedback Learning Circuit: Signaling Positive Prediction Error 116

5.3 Concluding Remarks 121

References 123

LIST OF FIGURES

Chapter 2

Figure 1.1: Procedure to test for SEFL transmodality 38

Figure 1.2: SEFL is Transmodal 40

Figure 1.3: Procedure for Noise SEFL 42

Figure 1.4: A noise stressor does not produce SEFL 44

Figure 1.5: No difference in reaction to noise during the hearing test 45

Figure 1.6: Activity during Day 1 Shock Stress and Noise Stress

1.6A 47

1.6B 49

1.6C 50

Chapter 3

Figure 2.1: Procedure for SEFL cFos experiment 62

Figure 2.2: Behavior across Shock and Noise Stress 65

Figure 2.3A: Average cFos count in subregions of central PAG between animals that received Shock Stress, Noise Stress or Homecage 66

Figure 2.3B: Average cFos count in subregions of caudal PAG between animals that received Shock Stress, Noise Stress, or remained in Homecage 69

Chapter 4

Figure 3.1: Figure 3.1. Design for inhibition of lPAG during Shock Stress in the SEFL procedure 91

Figure 3.2: Inhibiting lPAG during Stress does not attenuate SEFL 92

Figure 3.3: lPAG and Associative Learning Experiment 98

Figure 3.4: Inhibiting lPAG during enhances associative fear to the context and tone 101

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Chapter 1: Stress Enhanced Fear Learning, Defensive Behavior, and the Periaqueductal Grey

Early Studies of Fear Learning

Fear learning is often conceptualized in adverse terms. It is "negatively valent", a juxtaposition to "positively valent" appetitive learning. And yet, it is by this very process that an organism is able to survive danger, and to learn to avoid future noxious scenarios. Unpleasant emotional sensations such as fear, anxiety, and panic are as strict a teacher as the physical sensations of hunger or reward. These unpleasant emotions serve to motivate the animal towards avoiding these feelings for its own benefit. The following chapter outlines a model of fear learning that accounts for evolutionary-driven defensive behaviors. This chapter will also address the brain regions contributing to these defense behaviors across a danger severity continuum and how the periaqueductal grey (PAG) in particular appears to contribute in specific ways to aversively-motivated learning and behavior.

The origins of associative learning

Ivan Pavlov (1927) showed that a normally reflexive response to an unconditioned stimulus could be trained to become associated with a previously neutral cue. The trained cue could elicit the same response without the presence of the unconditioned stimulus; said response would therein become a "conditional response." In Pavlov's landmark study, dogs learned to associate a tone cue with imminent arrival of food. Food as an unconditional stimulus naturally elicits salivation. After pairing of the tone with food, dogs would then salivate to the tone alone, showing a conditioned response to the tone by virtue of its ability to predict the food. Learning to associate the stimulus with the cue in order to make the conditional response was dubbed "classical conditioning" (McSweeny & Bierley, 1984), in the context of appetitive learning.

In the Little Albert study, Watson and Rayner (1920) sought to classically condition fear responses rather than appetitive responses. The human infant "Albert" was exposed to a white rat stuffed animal, which was followed by a loud noise to evoke a fear response. Watson and Reyner were able to condition the boy to show fear to the white rat when it was presented alone, without the loud noise. Albert also showed a conditioned fear response to other white fluffy objects, indicating that this conditional fear response can be generalized (Watson & Rayner, 1920). Watson and Reyner thereby extended Pavlov's (1927) classical conditioning procedures to the aversive realm.

The origins of operant conditioning

A second major contributor to the early study of associative learning was the process of operant conditioning. While classical conditioning refers to the process of learning to associate an unconditioned stimulus (e.g., food) with a neutral cue (e.g., a tone), operant conditioning is the process of shaping a particular behavioral response via consequential reinforcement. For example, Thorndike (1911) conducted a puzzle-box experiment wherein a cat had to learn a particular response to escape the box in order to receive a food reward. While the cat was free to choose any behavior while in the box, once it learned which behavior was needed to escape (such as turning a latch) it would neglect any other action in favor of this response to escape. From these studies, Thorndike's Law of Effect (1911) was developed and postulated that reward could be used to reinforce a desired behavior in the presence of a stimulus (i.e., a stimulus (S)-Response (R) association; here, seeing a latch and it), while other possible behaviors would be weakened, or extinguished.

Skinner (1938) built on this Law of Effect by showing that a behavioral response could be strengthened with reinforcement (that is, a net positive outcome) or extinguished with

punishment (that is, a net negative outcome). Importantly, Skinner demonstrated that a behavior could be reinforced by administering an appetitive outcome (such as food) or by removing an aversive outcome (such as shock). For example, lever pressing response in rats can be reinforced with the appetitive outcome of food. During early training, the rat may incidentally press the lever while exploring the context, which will cause a magazine to release a food pellet. As no other behavior leads to receiving the food outcome, the rat will orient its behavior towards lever pressing over time. Once this stimulus-response association is learned, the rat will constrain its behavior to pressing the lever, much like the cat constrained its behavior to turning the latch to escape the puzzlebox and receive food in Thorndike's (1911) experiment. This is an example of "positive reinforcement". In contrast, "Negative reinforcement" occurs when the desired response causes the removal of an unpleasant stimulus. For example, a rat placed into a brightly lit context will find the light to be aversive. Once it learns that pushing the lever turns the light off, then its behavior will become constrained to pushing the lever whenever the light switches back on (Skinner, 1938).

Fear as a reinforcer

These contingencies of instrumental learning would go on to spawn many theories about its mechanisms. Hull (1943) and Spence (1956) both theorized that behaviors are internally motivated by drive and incentive. Briefly, an animal is motivationally driven by intrinsic biological needs, such as food, water, safety, or sex (Seward, 1956). Its incentive towards one of these drives depends on how far the animal is from homeostasis; for instance, a food-deprived animal is incentivized towards fulfilling its hunger drive (Seward, 1956). Thus, a food outcome for this animal will best motivate an animal to learn a desired response to receive the food. While the drive itself is biologically innate*, its utility as a reinforcer for a desired response should be*

non-specific. That is, any type of behavioral response that serves this homeostatic drive should be learned at the same rate, if the same reinforcer is used.

Due to its motivational attributes, fear elicited by shock or by a cue-predictive shock was initially understood to be an aversive force that could drive conditioned instrumental response (Miller, 1948). Fear, much like reward, should serve as a non-specific reinforcer such that any given fear-reducing behavior should become the dominant learned response concurrent with reduction of fear (Mowrer, 1951). For example, in a negatively reinforced fear avoidance task, a rat may be instrumentally trained to run in a wheel in order to avoid shock. If the rat learns that light is a cue for the shock, the light will elicit fear. By running in the wheel when presented with the light, its fear drive evoked by the light should diminish, as the light is no longer followed by shock (e.g., Morris, 1974; Mowrer, 1960). If fear serves as a non-specific reinforcer, then the same rate of learning should be observed for negatively reinforcing a different behavior to the light cue, such as pressing a lever or jumping in a shuttle. This theory assumes that the natural behavioral response to fear is: a) one learned over time from previous experience, and b) the dominant response due to it being the most reinforced (i.e., removal of the fear-evoking stimulus). Thus, a prey animal will freeze when it hears the sound of a predator because this behavior has been previously reinforced by the animal managing to avoid further exposure to the predator.

Species Specific Defense Response Theory

However, problems with animals' capability to learn the avoidance task started to crop up. For example, while Miller and Mowrer were able to successfully demonstrate dogs' ability to learn avoidance of one side of a box in their shuttle box task, and rodents could demonstratively learn to run in a wheel to terminate shock (e.g., Myers, 1965; Bolles, et al., 1966), the same

could not be said of other responses. Specifically, a large proportion of rats could not reliably learn to press a lever in the presence of a cue that was already paired with a shock in order to avoid the shock, sometimes even after thousands of training trials (e.g. Meyer, et al., 1960; D'Amato & Schiff, 1964). For a time, experiments were dedicated towards reducing the number of inevitable "non-learner" subjects, whether it be by selecting a particular strain of rat (Schaeffer, 1959), increasing intensity of the shock in an effort to increase motivation (D'Amato & Schiff, 1964), manipulating interval of shock (D'Amato et al., 1965), ITI (Brush, 1962), or the apparatus (Baum, 1965). Indeed, these "non-learners" were not restricted by species, having been recorded in dogs and in some cases, during shuttle avoidance tasks (Brush & Levine 1966; Solomon & Wynne, 1953).

If, as was believed at the time, enacting the fear-terminating behavior should reinforce said behavior, then why were certain avoidant behaviors relatively easy to learn, while others were not, even after thousands of trials? If all behavioral responses are equally likely to be enacted in a laboratory setting, shouldn't all responses be equally learnt? Yet, there was a clear tendency for laboratory subjects to show faster learning of particular responses in these aversive settings. For example, while rats would fail to learn to lever press after thousands of trials, learning to jump to one side of a shuttle box could take as many as 100, while learning to flee down a corridor as little as six (Bolles, 1970).

Bolles (1970) argued that fear behaviors, rather than being trained based on prior experience, are an innate and inflexible set of Species-Specific Defense Responses (SSDR). Fear does not serve to reinforce any arbitrary behavior; rather, it triggers an adaptive response which has, over the course of its species' evolution, best reduced the probability of predation (Bolles, 1970). Thus, an animal will instinctually freeze when hearing the sound of its predator even

upon first occurrence. If a rat's constrained set of SSDRs were to flee, freeze, or fight, it becomes readily apparent why they are able to learn running and jumping avoidance behaviors. Alternatively, lever-pressing becomes nearly impossible if movement becomes suppressed (freezing) during a fear-evoking cue. Not only did "non-learners" exist because lever-pressing is obviously not an innate response to danger, Bolles argued, but furthermore the so-called "learners" were not showing an operantly-conditioned pressing response either. Rather, in the small confines of an inescapable box, a rat's SSDR would be constrained to freezing. If the rat gained the ability to freeze while touching the lever, it would still be able to reflexively push the lever, thereby still terminating the shock punishment (Bolles & McGillis, 1968). Thus, even when non-specific behaviors are learnt in an aversive setting, it was argued this was because it was learnt serendipitously in the context of an SSDR.

However, there were elements of Bolle's (1970) SSDR theory that did not always bear out in experimental manipulations. Specifically, Bolles (1970) argued that a given SSDR is selected out of a species' repertoire based on functionality. Bolles (1970) gives the example of running behavior not being readily learned as an operant response in all situations, despite running very apparently being a SSDR that rats may use as a means to escape danger. The key word here is "escape" because when placed in an inescapable box or shuttle, running becomes futile. Being able to jump out of a box or run down a corridor to flee are scenarios in which running in response to a cue for shock is more easily learned. According to Bolles, when escape is impossible, the SSDR response to the cue may instead be to freeze (Bolles, 1970). However, Bolles' students, Fanselow and Lester (1988), subsequently set up an experiment wherein escape from a shock conditioning chamber was possible by traversing through an obstacle. They found that even when escape was a possibility, the animals still froze to the shock-paired cue. This

suggested that Bolles's (1970) SSDR theory did not capture some of the nuances seen in laboratory setting, where an animal may not select the most adaptive SSDR to avoid an aversive stimulus.

The Predatory Imminence Continuum

The SSDR theory changed the way we think about fear learning. In particular, it brought our attention to thinking about the complexities of how an animal responds in fearful situations. But if an animal has several distinct SSDRs and does not always choose the most adaptive response, what other factors contribute to which defense response is selected? Fanselow and Lester (1988) extended Bolle's (1970) theory to postulate that selection of a specific defense response is determined by the perceived distance between predator and prey. Perceived speed of the predator's approach is also a deciding factor, as approach at an angle elicits shorter flight distance than approach straight on (Thomson et al., 1884). In order to best describe the transition between defensive states as it relates to these two factors, they coined the term *Predatory Imminence Continuum*.

According to the Predatory Imminence Continuum, there are three main defensive states, each with their own distinct selective behaviors. It is important to note that the closer the perceived oncoming danger appears and the more extreme the threat, the more inflexible these behaviors become (Fanselow, 2022). On the least-extreme end of the continuum is *pre-encounter*. An animal enters this state whenever it leaves a completely safe environment. It engages in behavior to reduce the chances of potential predation, but no actual predator is detected. It may reorganize its meal pattern to take fewer trips into a food-rich area that carries a risk of danger (Fanselow & Lester, 1988; Fanselow & Helmstetter, 1993). It may stay close to

the walls of a context (Simon, Dupius & Constantin, 1993), and show avoidance of wide-open spaces (Suarez & Gallup, 1981). Introducing mildly aversive stimuli, such as bright light or sudden noise, may also cause a pre-encounter response by motivating the animal to move to a less stressful environment (Godsil & Fanselow, 2004). Within a human framework, pre-encounter can be likened to anxiety; a more generalized caution of a non-specific danger (Fanselow, 2022; Mobbs et al., 2009).

The next state in the continuum is *post-encounter*. In post-encounter, the animal has detected a predator in its vicinity (Fanselow & Lester, 1988). Previously, freezing to an aversive stimulus was thought to have occurred when the animal was at its most fearful state, and in a context in which escape was impossible, rendering them “paralyzed by fear” (Bolles, 1970). But rats froze to a fear-evoking cue even in a context in which escape was possible by traversing through an obstacle (Fanselow & Lester, 1988). Furthermore, rats would not always immediately freeze to the cue but, rather, briefly move to reach a corner, wall, or more familiar safe context before freezing (DeOca et al., 2007). Rather than lack of movement to the fear cue being analogous to tonic immobility, it appeared that the animal was engaging in behaviors that reduce its chances of being detected by the predator in turn, or if already detected, will reduce the likelihood of being attacked (Fanselow, 2022). Movement suppression (i.e., freezing) is often most auspicious in achieving this in a wide variety of species, ranging from rodents (Fanselow, 1984), to fish (Fu et al., 2019) to humans (Mobbs et al. 2009; Gladwin et al., 2016; Roelofs, 2017). Within a human framework, post-encounter can be likened to fear; a more specific adaptive response to an oncoming danger which is not yet proximal enough to make contact (Fanselow, 2022; Mobbs et al., 2009).

On the most extreme end of the continuum is the *circa-strike* state. This occurs when the predator attacks and makes contact, or is about to make contact, with the prey animal (Wendt et al., 2017). The prey animal is now in a life-or-death situation, and its survival is contingent on behaviors that successfully allow it to escape the predator (Fanselow & Lester, 1988). Common circa-strike defense behaviors include escape (i.e., flight, jumping or activity burst), and aggression towards the threat (i.e., fight; Fanselow & Lester, 1988). It is argued that this state of response to life-or-death situations is best analogous to panic in humans (Fanselow, 2022; Mobbs et al., 2009).

Importantly, in lab settings, if the aversive stimulus contains a tangible element (such as a shock) or a perceived oncoming tangible element (such as an approaching looming stimulus) it elicits a stronger immediate reflexive circa strike response (i.e., flight; Fanselow, 1980; Fanselow, 1982; Franscecho et al., 2016; Kim & Choi, 2010). Freezing may also occur, but it is expressed after the presentation of the stimulus, or when the stimulus is more distal to the animal (e.g., looming disk is placed further away). For example, Fanselow (1995) specifically noted in an early study relating to predatory imminence that rats would always show activity bursts and attempts to escape (both circa-strike behaviors) for the duration of the shock itself and immediately afterwards. Rats would revert to freezing after the shock had terminated. According to Fanselow, this was indicative of the rat shifting from a circa-strike to a post-encounter state. The behavior during the shock was analogous to attempting to escape an attacking predator, while the behavior after the shock was analogous to detecting a predator in its vicinity (Fanselow, et al., 1995). Compare this to an aversive stimulus with no tangible element, such as predator odor, which as most evokes avoidance or freezing (Belzung et al., 2001) or sudden loud noise, which as most evokes a potentiated startle (Davis, 1986). This both supports that defense

response is dictated by real or perceived imminence of danger, as well as provides a general lab-based model for which neural mechanisms related to these behaviors can be studied.

Neural Correlates Across the Predatory Imminence Continuum

Anxiety-like behavior has been linked to prefrontal and limbic regions, including the medial prefrontal cortex (Jinks & McGregor, 1997; Lacroix et al., 2000; Shah & Treit, 2003), ventral hippocampus (Kjelstrup et al., 2002), and the Bed Nucleus of the Stria Terminalis (BNST; Duvarci et al., 2009; Kim, et al, 2013a; Walker et al., 2009). Specifically, these studies show dissociation between effects on anxiety (i.e., pre-encounter) and fear (i.e., post-encounter) behavior. Lacroix et al. (2000) found that lesions to the mPFC increased exploration of open field and open arms of elevated plus maze, which indicate the mPFC's necessity for anxiety- like behavior. In contrast, it largely left conditional fear intact, if not exaggerated (Lacroix et al., 2000). Kjelstrup et al. (2002) similarly found that lesioning the ventral hippocampus caused increased time in the open arms of an elevated plus maze, but did not attenuate contextual fear learning (Kjelstrup et al, 2002). Similarly, Duvarci et al. (2009) directly compared conditional fear and anxiolytic traits after lesioning the BNST. They found that while lesioned rats still showed conditional fear to the shock-predicting cue, they showed better fear discrimination towards a non-shock cue and more time in the open arms of an elevated plus maze compared to sham lesion controls (Duvarci et al., 2009). Thus, these prefrontal and limbic regions are thought to be involved in pre-encounter forms of aversive behavior, which is generally conceptualized as comparable to anxiety-like behaviors.

In contrast, the amygdala is considered to be a fear epicenter for both innate and conditional fear responses. Lesioning the amygdala abolishes instinctive fear of predators and

also conditioned fear to a shock-predictive cue (Amaral, 2002; Blanchard & Blanchard, 1972; Choi & Kim, 2010; Fox & Sorenson, 1994; Helmstetter, 1992; LeDoux et al., 1988; Roozendaal et al., 1991). Stimulating the amygdala produces multiple post-encounter fear behavior, including increased respiration, cardiovascular changes, and most notably, freezing (Davis, 1992; Graeff, et al., 1986; Hitchcock & Davis, 1986). For example, in an ethobehavioral study, Choi and Kim (2010) pharmacologically activated GABA receptors in the amygdala. This caused rats to show no fear in a food foraging task where a robotic predator guarding the food pellet would lunge at them once they were in close radius. Rats without amygdala function would largely ignore the predator in favor of procuring the pellet. When testing these same rats the next day while drug-free, natural fear was reinstated and the rats fled from the lunging predator before freezing in a safer area. However, when the pellet was placed closer to the entrance of the arena (and thus farther away from the predator) the rats were able to retrieve the pellet (Choi & Kim, 2010). This indicates that the amygdala regulates natural defense response towards a predator, and this response is dictated by predator distance (i.e., predatory imminence).

Thus, within the Predatory Imminence Continuum spectrum, the amygdala's role is not dissociable. While it necessary for post-encounter response (Amaral, 2002; Blanchard & Blanchard, 1972; Choi & Kim, 2010; Fox& Sorenson, 1994; Philips, 1968) it has also been linked to pre-encounter anxiety behaviors (Davis et al., 2010; Moreira et al., 2007; Tye et al., 2011; Tovote et al., 2015). For example, Tye et al. (2011) used optogenetic methods to dissect a microcircuit that controlled anxiety behavior in the amygdala. They found that stimulating terminals projecting onto the lateral nucleus of the central amygdala (which normally inhibits the fear-activating medial nucleus) increased time in open arms of an elevated plus maze and in the center space of an open field, both of which indicate a decrease in pre-encounter anxiety (Tye et

al., 2011). Thus, the amygdala is directly involved in regulating both pre-encounter and post-encounter response.

The amygdala is also involved in the processes of conditional fear learning. The dominant model of fear learning argues that the basolateral amygdala is where information about cues and information about aversive stimuli converge into learned associations (Fanselow & Kim, 1994; LeDoux, 2000). The lateral part of the basolateral amygdala receives sensory information from primary sensory cortices and the thalamus about stimuli of all modalities (Bordi & LeDoux, 1994; LeDoux, et al., 1991) and is thought to be the sight for long term potentiation of cue-stimulus associations (Chapman et al., 1990; Maren, 2001; Maren& Quirk, 2004; Fanselow & Poulos, 2005; Quirk, et al, 1995). For example, Quirk and colleagues used single unit recording in the lateral amygdala to record firing rate of neurons to tone during pre-exposure, before the tone was paired with shock. They recorded firing rate in the same neurons to tone during extinction training, after the tone was paired with shock. Neurons showed greatest firing rate during early extinction trials, when the association between tone and shock was strongest. This implies that activity is contingent on the current status of the cue-shock association (Quirk et al., 1995). Thus, the lateral amygdala is thus thought to be a major driver of learned fear behavior.

The lateral amygdala also receives information about shock prediction during learning. Using electrophysiological single cell recordings in rats, Johansen et al. (2010) found that neurons in the lateral amygdala show stronger firing rate to shock during early fear conditioning trials, when the animal had not yet learned the cue for shock and so the shock is more surprising. Neuronal responding in the LA to the shock decreased in later conditioning blocks when the cue was learned, which was also behaviorally demonstrated by the rats freezing at asymptote to the tone. However, these neurons showed renewed activity when presented with a surprising shock

that was not preceded by the shock-predictive cue, indicating that the decrease in firing rate over the course of fear learning wasn't due to habituation (Johansen et al., 2010). This indicates that the lateral amygdala shock-responding neuron activity is dependent on predictive learning of the shock.

While the basolateral amygdala is thought to be a "fear processing center" for association between aversive stimulus and cue (LeDoux, 2000), the central amygdala is specifically responsible for sending output commands to elicit fear behavior (Cardinal et al., 2003; Davis, 1992; Tovote et al., 2015). Stimulating the central amygdala produces innate fear response (Ciocchi et al., 2010; Duvarci et al., 2011; Poulin et al., 2013; Wilensky et al., 2006) while inhibiting the central nucleus suppresses fear (Kalin et al., 2004). For example, Ciocchi et al. (2010) reliably induced freezing in freely behaving mice by optogenetically exciting the central amygdala. Furthermore, locally injecting a GABA agonist into central amygdala during test reduced conditioned freezing to a shock-paired tone. (Ciocchi et al., 2010). This is consistent with an idea that conditional freezing is elicited via central amygdala output (but see: Killcross et al., 1997).

Freezing evoked by central amygdala is likely caused by its projections to midbrain, hypothalamus and brainstem, all of which regulate autonomic behavioral fear response (Misslin et al., 2003; Sah et al., 2003; Tovote et al., 2016). Of particular relevance is its projections to the periaqueductal grey (De Oca et al., 1998; Fanselow, 1994; Kim et al., 1993; LeDoux et al., 1998; Ozawa et al., 2017; Tovote et al., 2016). For example, Ozawa et al., (2017) used retrograde viral tracing to find that central amygdala "freezing" cells projected axon terminals onto the ventral lateral periaqueductal grey (Ozawa et al., 2017). The periaqueductal grey is thought to be specifically involved in the more extreme end of the predatory imminence continuum, which is

the post-encounter fear (Assareh et al., 2016; De Oca et al., 1987; Gross and Canteras, 2012; LeDoux, 2000; Maren, 2001; Ozawa et al., 2017; Tovote, 2016) and circa-strike panic (Assareh et al., 2016; Bittencourt et al., 2004; Deng et al., 2016; Di Scala et al., 1987, Kim et al, 2013; Schenberg et al., 1990; Viana et al, 2003).

The Midbrain Periaqueductal Grey and Defense Response

The midbrain periaqueductal grey (PAG) is a tubular region surrounding the cerebral aqueduct that dictates behavioral response to predatory threat via projections from limbic and prefrontal regions (Carrive, 1993; Gross& Cantares, 2012). The PAG can be divided longitudinally across the dorsal-ventral axis into dorsal-medial (dmPAG), dorsal-lateral (dlPAG) lateral (lPAG) and ventral-lateral (vlPAG) subregions (Bandler and Keay, 1996; Carrive, 1993). In some cases, the separate subregions mediate oppositional modes of motor behavior: freezing, defined as an aroused but physically immobile state; and escape, defined by quick bursts of activity, flight, or jumping (Fanselow, 1995).

Early studies using the PIC postulated that post-encounter response such as freezing is mediate by the vlPAG, while circa-strike escape responses such as flight, jumping and activity burst are mediated by dorsal and lateral regions (De Oca et al., 1989; Fanselow, 1991; Fanselow, 1994; Fanselow et al., 1995; Helmstetter, 1992; Ledoux et al., 1988). For example, Fanselow et al. (1995) compared the effects of lesions to vlPAG or dorsal/lateral PAG (d/lPAG) on post-encounter and circa-strike responses. They used footshock to evoke these responses, as they argued that shock is analogous to a tactile predatory attack. Sham-lesioned rats, when given footshock at a spaced schedule (60 second ITI), will demonstrate circa-strike escape behavior such as activity burst. After the shock, rats will freeze, which is analogous to the post-encounter

response rats demonstrate when a predator is in its immediate vicinity. Alternatively, rats given footshock at a massed schedule (3 second ITI) will have more activity bursts, and as a result, freeze less overall during the session. Fanselow and colleagues (1995) administered footshock in a spaced or massed schedule to rats with vlPAG lesions or rats with d/lPAG lesions. They measured time freezing between each shock. Rats with vlPAG lesions showed a significant reduction in freezing during both the massed and spaced shock schedules compared to sham lesion controls. In comparison, rats with d/lPAG lesions showed a marked enhancement in freezing to the massed shock schedule, which should have instead been characterized by activity bursts (Fanselow et al., 1995). Together, this indicated that vlPAG is necessary for freezing, and therefore for the post-encounter response, while the d/lPAG was necessary for activity bursts, and therefore for circa-strike response. d/lPAG lesioned rats showing enhanced freezing also implied that d/lPAG is suppressing the post-encounter response in the circa-strike state.

Further evidence supports vlPAG being the mediator for freezing behavior (Assareh et al., 2016; De Oca et al., 1987; Gross and Canteras, 2012; LeDoux, 2000; Maren, 2001; Ozawa et al., 2017; Tovote, 2016). For example, Assareh and colleagues (2016) optogenetically excited lPAG or vlPAG and analyzed behavior that occurred during the 10-second stimulation onset. During the duration of high intensity vlPAG stimulation, rats showed an increase in the amount of observations of freezing. In comparison, strongly stimulating the lPAG caused an immediate increase in observations of flight and jumping (Assareh et al., 2016). This is consistent with the idea that the vlPAG elicits post-encounter freezing, while the d/lPAG is involved in the circa-strike flight response.

The dmPAG and dlPAG (often referred to as one combined dPAG region) as well as lPAG are less dissociable by behavior alone than the vlPAG, as both these regions elicit escape

(Assareh et al., 2016; Bittencourt et al., 2004; Deng et al., 2016; Di Scala et al., 1987, Kim et al, 2013a; Schenberg et al., 1990; Viana et al, 2003). For example, Bittencourt et al. (2004) directly compared the behavioral effects of stimulating dPAG and lPAG with electrical pulse or microinfusion of excitatory neurotransmitter NMDA. Electrical stimulation of both dPAG and lPAG caused nearly identical defensive behavioral response probability patterns of running and jumping, which was activated at the same stimulation intensity and frequency thresholds. Chemical infusion of NMDA did reveal some difference in response curves, with lPAG needing a higher effective dose to elicit these escape behaviors compared to dPAG (Bittencourt et al., 2004). Therefore, dPAG has a lower threshold for causing escape compared to lPAG based on excitatory NMDA receptor proliferation, which may be indicative that it is the primary subregion that activates escape. However, many recent studies of defensive behavior that use more sophisticated methods such as optogenetics, viral tracing, single cell recording or calcium imagine either combine dPAG and lPAG into one region (eg, Ozawa et al., 2017; Tovote et al., 2016), or only target dPAG (eg., Deng et al., 2020; Carvalho et al., 2015; Evans et al., 2018; Lefler et al., 2020; Yeh et al, 2021) making it difficult to dissect any further intricacies between regions in response to aversive stimuli or external threat.

While it is parsimonious to consider that the vlPAG to be involved in post-encounter behaviors, and d/lPAG stimulation in circa-strike, studies show that this dissociation is not quite as clear cut. Specifically, while d/lPAG stimulation causes immediate flight or an activity burst, it subsequently causes freezing, a post-encounter behavior (Assareh et al., 2016; Bittencourt, et al., 2004; Carvalho et al., 2015; Deng et al., 2016; Di Scala et al., 1987; Viana et al, 2001a; Vianna et al., 2001b). For example, Carvalho et al., (2015) electrically stimulated rats in dPAG past threshold necessary to cause escape behavior, defined as running or jumping. Behavior was

recorded for eight minutes after stimulation. Rats froze near ceiling level during the post-stimulation time period (Carvalho et al., 2015). Assareh et al. (2016) reported similar behavior when optogenetically stimulating lPAG. During the stimulation itself, rats exhibited an increase of observations of running and jumping, both of which are escape behaviors. However, in the 10-second interval after lPAG stimulation, the rats transitioned to showing an increase in the freezing response (Assareh et al., 2016). Importantly, freezing occurred after the stimulation, and not during the stimulation itself.

Logically, circa-strike, as the most extreme state, is meant to be brief. From an evolutionary perspective, being in an extended fight-or -flight mode would consume a large amount of energy. Indeed, as soon as the animal successfully escapes danger, it reverts back to the post-encounter state (Blanchard & Blanchard, 1989; Choi & Kim, 2010). Emerging research on cellular activity in the dPAG paints a more precise picture of its role in this pattern of circa-strike behavior. Distinct cell populations in dPAG are involved in risk assessment of predatory threat (Bindi et al, 2022; Deng et al., 2016; Evans et al., 2018; Masferrer et al., 2020; Reis et al., 2021). Evans et al. (2018) used a looming disk stimulus to calculate an escape behavioral threshold in mice. When the disk looked smaller, and thus farther away, the mice were in a post-encounter fear state. However, as the disk grew larger and appeared to come closer (resembling an oncoming swooping predator) the mice would switch to a circa-strike state and flee. Evans first found that optogenetically inhibiting glutamatergic dPAG cells caused the mice to continue to freeze to the "close" looming stimulus, implying that these cells were necessary for triggering escape. They next used calcium imaging in freely behaving mice to find that these dPAG neurons were only active during, and not before, escape. Finally, they optogenetically stimulated dPAG at incremental intensities and found a steep "all or nothing" curve- that is, dPAG needed to be

stimulated past a certain intensity, after which escape was initiated (Evans et al., 2018). Taken together, this indicates that dPAG gates circa strike behavior and does not allow release unless the saliency and/or proximity of danger reaches a threshold.

This would in turn explain why stimulating dPAG first causes flight, and afterward, freezing. The onset of stimulation would have passed said threshold, causing activity burst. Once stimulation ceased, the dPAG was no longer excited past threshold, and so competing processes took over. These competing processes may originate from more distal regions such as the ventral medial hypothalamus (Masferrer et al., 2020) or from more local vlPAG neurons (Johnson et al., 2004; Tovote et al., 2016). For example, Tovote et al. (2016) investigated *in vivo* neural correlates of freezing in mice using electrophysiological single unit recordings of vlPAG neurons. Neuronal firing rate in vlPAG during a shock-predicting cue revealed two separate populations of neurons: excitatory glutamatergic neurons, and inhibitory GABAergic neurons. Excitatory glutamatergic neurons caused freezing when optogenetically excited. Inhibitory GABAergic neurons showed lower firing rates during freezing compared to baseline, indicating that freezing may be caused by an inhibitory feedback loop. That is, the GABAergic neurons are tonically inhibiting the glutamatergic neurons that cause freezing. Tovote and colleagues (2016) next used cre-dependent viral tracing to find that excitatory d/lPAG neurons enervate the GABAergic vlPAG neurons (Tovote et al., 2016). Together, this indicates an intricate flight-freezing mediating pathway wherein the d/lPAG inhibits vlPAG to prevent freezing while simultaneously causing flight. This reflects the behavior exhibited by d/lPAG stimulation (e.g., Evans, et al., 2018), wherein the initial stimulation passes the threshold necessary to cause circa-strike escape activity such as flight and jumping. After the stimulation ceases, post-encounter freezing processes mediated by the vlPAG are again free to resume.

The Midbrain Periaqueductal Grey and Associative Learning

The PAG was initially assumed to be a behavioral output center for not only innate defensive responses, but also for learned responses. Specifically, the central amygdala projections to the vlPAG were thought to dictate learned fear responses (i.e., freezing) to a shock-paired cue (Fanselow, 1991; Carrive et al., 1999, Vianna et al., 2001a). Within the PIC framework, conditional freezing and "innate" freezing to threat are both considered to be post-encounter responses mediated by the same circuitry (Fanselow et al., 1994). And indeed, the vlPAG is involved in freezing as both a defense response to a predator (De Oca et al., 1987; Fanselow et al., 1994; Tovote et al., 2016) and a learned conditional response to a cue (Ozawa et al., 2017; McDannald, 2010; Tovote et al., 2016; Viana et al., 2001b). For example, Tovote and colleagues (2016) optogenetically inhibited glutamatergic cells in vlPAG in mice while playing a learned shock-predicting tone cue. They found that inhibiting these cells reduced freezing to both the tone and context that the mice were fear conditioned in. Importantly, photoinhibition also dramatically reduced freezing to an oncoming looming stimulus meant to evoke fear of a swooping predator (Tovote et al., 2016). This is evidence that vlPAG is necessary for both innate freezing as a defense response and conditional freezing to a shock-signaling cue. In the same study, Tovote and colleagues (2016) found that vlPAG was sufficient for freezing by optogenetically exciting glutamatergic cells in freely behaving mice (Tovote et al., 2016). This may indicate that freezing response to both conditional cue and predator threat activates the same pathway.

More recent evidence suggests that vlPAG is not only a behavioral output center for conditional freezing to a shock-paired cue. vlPAG also signals information about the shock-predicting cue (McNally et al., 2005; Walker et al., 2020; Wright et al., 2019; Wright & McDannald, 2010). Wright and colleagues (2019) used single cell in-vivo electrophysiological

recording in vlPAG while presenting three cues: a 'certain danger' cue that always predicted shock, a 'certain safety' cue that always predicted no shock, and an 'uncertain' cue that was only sometimes followed by shock (p=.375). Fear learning was measured as reward nose poke suppression ratio (freezing) during cue presentation. The rats demonstrated good discrimination between cues, as the suppression ratio for certain danger was high, for certain safety was low, and for uncertain danger intermediate between the other two cues. If vlPAG neurons are signaling information about the probability of a cue predicting threat, then they should show the most activity to the certain danger cue. And indeed, Wright and colleagues (2019) found that this population of vlPAG neurons showed bias towards firing to the certain danger cue during cue onset. This bias towards the certain danger cue was strong, as there was greatest difference in firing rate between the certain danger cue and the other two cues (Wright et al., 2019). This indicates that these vlPAG neurons were specifically signaling which cue had the greatest probability of predicting danger.

While dPAG and lPAG both mediate the same defense behaviors, there is evidence that they convey different informational aspects during fear learning. The dPAG directly projects sensory information about aversive stimuli (Heinricher et al.,1987; Keay et al., 1997) to threat-mediating nuclei in the thalamus and hypothalamus (Gross & Canteras, 2012; Keay & Bandler, 2015). While there are not many direct projections from dPAG to amygdala (Otterson, 1981), functional behavioral studies show that dPAG manipulations affect amygdala-dependent associative learning and plasticity (Di Scala et al., 1987; Kim et al., 2013; Yeh et al., 2021). For example, Kim et al. (2013) electrically stimulated dPAG, vlPAG, or basolateral amygdala (BLA) in rats at the end of a tone presentation Only rats in the dPAG stimulation group froze to both the tone and the context in which the fear learning took place. However, stimulating the dPAG while

pharmacologically inactivating BLA with muscimol did not produce fear learning, indicating that the learning itself was amygdala-dependent (Kim et al., 2013). As dPAG stimulation was able to cause fear learning to tone and context, and BLA was necessary for this fear learning, this indicates that dPAG has an upstream effect on associative learning plasticity in BLA.

There are relatively few studies that specifically investigate whether stimulating lPAG is sufficient for fear learning, although it does receive direct projections from the trigeminal and spinal dorsal horn (Keay & Bandler, 2001) and there is evidence that lPAG receives and processes information about noxious and nociceptive stimuli from these projections (Bandler et al., 2000; Keay & Bandler, 2015). Specifically, lPAG is activated in response to escapable, painful stimuli. In one study, Keay and Bandler (2001) exposed rats to a variety of stressors, including opiate withdrawal, formalin-induced muscle pain, cutaneous neck clipping, radiant heat, and non-physical exposure to a cat. Keay and Bandler (2001) stained for cFos in the PAG to compare expression between subregions. Importantly, cutaneous pain caused high expression of the activity marker cFos in the lPAG. In comparison, visceral and muscular pain caused high expression in vlPAG, and predator exposure caused high expression in dPAG (Keay & Bandler, 2001). Thus, it is likely that lPAG is upstream from even other PAG subregions when signaling about shock, and in doing so contributes to fear-mediated neural plasticity.

Possible role of PAG in non-associative learning

One other question that remains is whether PAG is also involved in other types of learning, such as non-associative learning. Habituation and sensitization are generally considered the main non-associative learning processes. Habituation and sensitization refer to a change in response to an unconditioned stimulus without use of any explicit cues that serve to predict those

stimuli. Specifically, Habituation refers to a decrease in response to repeated presentation of a stimulus (Thompson & Spencer, 1966) and sensitization occurs wherein presentation of a stimulus (for our purposes, an aversive one such as shock) causes an enhanced response (Groves and Thompson, 1970). For example, administering foot shocks repeatedly to a rat may push it to a generalized sensitized state, such that it will freeze to an innocuous unpaired tone cue afterward (Harris, 1943). This enhanced fear of the tone is non-associative, as it is a neutral stimulus instead of a learned cue for shock.

There is some evidence that dPAG stimulation can support plasticity that causes non-associative stress sensitization (Almeida et al., 2006; Carvalho et al., 2015; Carvalho et al., 2018). For example, administering a single session of electrical stimulation to dPAG that causes escape behavior in a particular context, caused a decreased amount of time spent in the open arms of an elevated plus maze in a different context 24 hours later (Carvalho et al., 2015). This indicates that briefly stimulating dPAG is sufficient to cause a long-lasting anxiogenic sensitization effect. However, it is still unknown whether dPAG is sufficient for stronger forms of fear sensitization, and unclear whether there is involvement from other PAG subregions.

Stress Enhanced Fear Learning: a non-associative model of fear.

One behavioral model which causes strong non-associative fear sensitization is Stress Enhanced Fear Learning (SEFL). In this model, a significant stress ("Stress"; 15 unsignaled footshocks) in Context A causes robust enhancement of mild fear conditioning to novel Context B (Rau et al., 2005). Much research has been conducted to examine whether SEFL is the result of sensitization rather than due to generalized fear. If SEFL were due to generalization, then the enhanced fear response would be due to the summation of residual fear of the Stress context and

the conditioned fear of the novel context (Nishimura et al., 2022). To test this, Rau and colleagues (2005) conditioned rats in the novel Context B using a single shock a day before administering the15-shock stress in Context A. If the SEFL effect was due to fear summation, then order of experience of the Stress and fear conditioning should not matter. However, they found that the rats did not show enhanced fear of Context B during test when returned to it after the stress in Context A. Thus, the Stress must occur prior to the mild fear conditioning in Context B in order for SEFL to occur (Rau et al., 2005). This indicates that the elevated conditioning seen in Context B in the standard SEFL design is not due to generalization of the fear to the novel Context B.

Another factor that would support a generalization account of SEFL would be if it was dependent on the association between the stress and the context in which it occurred (i.e., Context A). However, SEFL persists even if there is no memory of the stress event itself (Poulos et al., 2014; Rau et al., 2005). Specifically, associative memory is dependent on NMDA receptor-mediated plasticity in the amygdala (Bliss & Collingridge, 1993; Huang & Kandel, 1998; Maren & Fanselow, 1995), and so NMDA antagonists reduce associative learning (Fanselow et al., 1994; Kim et al., 1992). Rau and colleagues (2005) took advantage of this to examine whether infusion of an NMDA antagonist into the basolateral amygdala during the stress experience in Context A would influence the SEFL effect. Critically, Rau et al. (2005) found that this manipulation did not attenuate SEFL fear conditioning to the novel Context B (Rau et al., 2005). This provides additional evidence that stress caused sensitization that was not dependent on the recollection of the traumatic memory.

Finally, if SEFL is associative, then it should be mitigated by fear extinction of the Stress Context A prior to fear conditioning in Context B. Yet SEFL is not prevented by fear extinction

of the context that the trauma was experienced in (Amir & Fanselow, 2011; Hassien et al., 2020; Long & Fanselow, 2011). In one study, the anxiogenic drug midazolam was administered in rats during Stress in Context A. This greatly reduced freezing to Context A when tested 24 hours later. However, the treatment did not attenuate enhanced fear conditioning to Context B (Long & Fanselow, 2010). This is further proof that SEFL is not dependent on associative mechanisms, as the effect is not reliant on fear of the stress context. This is consistent with the notion that SEFL is non-associative fear sensitization.

We will therefore use SEFL as a means of testing the neural correlates within the PAG to reveal their role in non-associative fear learning. Much of the previous work investigating the PAG's role in learning focuses on associative learning involving aversive events (e.g. Fanselow, 1991; Carrive et al., 1999; Johansen et al., 2010; Ozawa et al., 2017; McDannald, 2010; Vianna et al., 2001a; Yeh et al., 2021). This research has indicated that the dPAG signals information about the aversive unconditioned stimulus (i.e., absolute magnitude of shock; (Di Scala et al., 1987; Kim et al., 2013; Yeh et al., 2021), and the vlPAG signal information about the cue as a predictor of the aversive conditioned stimulus (McDannald, 2010; McNally et al., 2005; Ozawa et al., 2017; Walker et al., 2020; Wright et al., 2019; Wright & McDannald, 2010). In comparison, we know less about how the PAG may contribute to non-associative fear sensitization that is produced by severe stress, which is not dependent on acquisition and recall of an associative framework (Groves and Thompson, 1970). Thus, we can use SEFL as a means of understanding how PAG may contribute to non-associative fear sensitization states. This is important because it will help us understand how the PAG contributes to different kinds of fear behaviors. Further, given the SEFL procedure and fear sensitization more generally share some of the characteristics with post-traumatic stress disorder (PTSD) in humans (Glover et al., 2015;

Paige et al., 1990; Orr et al., 2000) it will help us to understand the neural corelates of this disorder.

Chapter 2: Behavioral characterization of the transmodality of the SEFL effect

One symptom of PTSD is hyperarousal and enhanced reactivity to stress (American Psychiatric Association, 2022), which may lead to a heightened susceptibility for maladaptive fear learning. For example, a patient with PTSD may show exaggerated response to a sudden loud noise, and subsequently fear the context in which it occurred- in spite of this new context not containing any triggers related to the initial trauma. Stress Enhanced Fear Learning (SEFL) is a rodent model of this wherein exposure to acute Stress will cause subsequent enhanced fear conditioning in a novel context. This phenomenon has been found in rats (e.g. Rau et al., 2009) and mice (e.g. Hassien et al., 2020) of both sexes (Poulos et al., 2015). There is some debate as to whether SEFL is due to fear generalization of the Stress (an associative learning process) or general fear sensitization (a non-associative learning process). There is evidence which indicates that SEFL is not reliant on fear memory of the Stress, and is instead due to a heightened arousal state (Aimir & Fanselow, 2011; Nishimura et al., 2022; Poulos et al., 2014; Rau & Fanselow, 2005; Rau & Fanselow, 2009). This indicates that SEFL is a non-associative fear sensitization process, which is not due to generalization, and shares some of the characteristics seen in people with PTSD. As such, understanding the neural correlates of SEFL will help us to understand the neural regions that may be changed in humans with PTSD.

Stress Enhanced Fear Learning Dictated by Non-Associative Process

While SEFL appears to be due to non-associative fear sensitization, we still have not examined all aspects of SEFL that make it like sensitization. In particular, as mentioned above, the effects of sensitization in the context of PTSD are not constrained to the sensitizing stimulus; rather, a sensitized response will be seen to different types of stimuli (Glover et al., 2011; Paige

et al., 1990; Orr et al., 2000). For the majority of studies that have used the SEFL model, shock is used for both the stressor in Context A and the conditioning stimulus in Context B (Barkell et al., 2022; Conoscenti et al., 2015; Fanselow & Long, 2012; Hoffman et al., 2022; Poulos et al., 2014; Parekh et al., 2022; Poulos et al., 2015; Rajbhandari et al., 2018; Rau et al., 2005). However, given that Perusini et al. (2016) found that SEFL causes an upregulation of excitatory GluA1 receptors in the basolateral amygdala, it is possible that the exposure to shock stress sensitizes the amygdala to future aversive stimuli. This would essentially modify the threshold for fear conditioning such that a normally weak stress of any modality may now support it (Perusini et al., 2016). Thus, amygdala sensitization should produce enhanced fear learning that is non-specific and can be generated to any stimulus that is used for conditioning in Context B.

If this is indeed non-associative sensitization, then the modality of Stress in Context A and conditioning stimulus in Context B need not be the same. Although there has been some investigation into enhanced response to other stressors such as the open space of an elevated plus maze (Poulos et al., 2014) or sudden noise (Hassien et al., 2020; Hoffman et al., 2022), these studies focus on anxiolytic and fear responses to the stimulus when it is presented. To our knowledge, it is yet unknown whether said enhanced fear *responses* to transmodal stimuli is strong enough to support fear *learning* to Context B in rats.

An appropriate alternative conditioning stimulus is a white noise burst. White noise may be used as a milder stressor to study startle response after exposure to shock (Carvalo et al., 2021; Davis, 1986; Walker & Davis, 2002; Russo & Parsons, 2021). White noise has also been used as an aversive stimulus in associative fear conditioning paradigms (LeBar & LeDoux, 1996; Ledgerwood et al., 2005). Importantly, fear conditioning to both noise and shock are amygdala dependent (LeBar & LeDoux, 1996). If SEFL is due to amygdala sensitization, as has been

postulated by Perusini et al., (2016), then fear conditioning using noise after experience with a shock stressor should similarly enhance conditioning to Context B. The utility of using noise on stressed animals within the SEFL paradigm is already known. Unsignaled noise has previously been used in SEFL rat studies to measure sensitized response to novel aversive stimuli (Hoffman et al., 2022). Thus, using noise as a transmodal conditioning stimulus extends upon these studies by further measuring whether this sensitized *response* is strong enough to support fear *learning*.

Using white noise also presents an opportunity to test whether it could function as a "control stimulus" for a Stressor. That is, not all acute stressors are significant enough to cause PTSD (e.g., Norris & Slone, 2007). Previous works that have studied susceptibility to SEFL used a model wherein 4 footshocks were used as Stress instead of 15 footshocks (Gonzalez et al., 2021). Four shocks still produced a bimodal distribution wherein some subjects still showed SEFL, while others did not (Gonzalez et al., 2021). Thus, we have yet to find a stimulus to use as a stressor that cannot produce SEFL but which is still aversive enough to elicit fear responding. Rats may fear condition to white noise presentations at higher volumes (Ledgerwood et al., 2005) albeit at a slower rate than shock (e.g., LaBar & LeDoux, 1996) indicating that it is a weaker fear-producing aversive stimulus. White noise and shock can also be delivered in the same manner (i.e., 15 times around the same variable inter-trial interval). This eliminates confounding variables that might be associated with different kinds of stressors that are a uniquely different experience, such as restraint, predator urine, or low bedding.

In the following experiments, we will test SEFL transmodality in rats by presenting shock as a stressor in Context A and then white noise as the conditioning stimulus in Context B. Showing enhanced fear conditioning to noise, a different and novel stimulus, will strengthen the argument that SEFL is a non-associative process. We also investigate the utility of noise as a

good control stimulus for stress. Specifically, if Noise Stress elicits fear without producing the same sensitization effect that shock does, it may be further used as a stress control to investigate whether there are any dissociable brain regions necessary specifically for the non-associative sensitization evoked by SEFL, and not freezing behavior *per se*.

General Methods

Subjects

Male and female Long-Evans rats, approximately 90 days of age at the start of the experiment (Envigo, Indianapolis, IN) were single housed in a temperature and humidity regulated vivarium. Rats were provided food and water ad libitum. The rats acclimated for two weeks in the vivarium, during which they were handled by the investigator for 2 minutes/day over the course of the seven days directly prior to the beginning of the experiment. All cohorts were naïve animals with no prior conditioning. The experiments took place during the light phase of the 12:12 light/dark cycle. All procedures were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the UCLA Institutional Animal Care and Use Committee.

For *Experiment 1: Transmodal SEFL*: we administered Stress in Context A and then fear conditioning in Context B with either a mild shock or noise. This would test whether SEFL is transmodal. Rats were assigned to a 2x3 factorial with the first factor being Stress (Stress, No Stress) and the second factor being Conditioning (1 Shock, 1 Noise Burst, or 2 Noise Bursts). A near-equal number of animals of both sexes (male=20, female=19) were assigned to each group (Stress 1 Shock=4, Stress 1 Noise=8, Stress 2 Noise=8, No Stress 1 Shock=3; No Stress 1 Noise=8, No Stress 2 Noise=8; *n*=39 total).

For *Experiment 2: Noise SEFL*: we compared Shock and Noise as Stress in Context A and also conditioned the rats in Context B to either mild shock or noise. This would examine whether noise is sufficient to produce SEFL. Rats were assigned in a 2x2 factorial with the first level being Stress (Stress, No Stress) and the second level being Modality (Shock, Noise). An equal number of male and female animals (*n*=16) were assigned to each group (Shock Stress=8 Noise Stress=8 Shock No Stress= 8 Noise No Stress= 8; *n*=32 total).

<u>*Behavioral Procedure*</u>

Apparatus

All behavioral experiments took place in Med Associates Conditioning chambers and recorded using infrared cameras sent to Med Associates VideoFreeze software. Each box (30x25x25 cm) was in its own sound attenuating chamber. The side walls of each chamber were aluminum and the back wall was solid white plastic. There were two contexts, “Context A” and “Context B”. For Context A, the room was illuminated by white 65 W bulbs and each chamber was illuminated from above by a single white house light. The fans were switched off and did not provide sound. The floor grid consisted of 16 stainless steel rods (4.8 mm) 1.6 cm apart. A metal pan beneath each grid was sprayed with a thin layer of diluted Windex solution (1:1 water dilution), and the walls were wiped with Windex as well. For Context B, the was illuminated by a single red 30 W incandescent bulb. The conditioning chamber itself was not illuminated. Fans mounted within each chamber provided a 65 dB uninterrupted sound. A metal pan beneath each grid were sprayed with a thin layer of acetic acid, and the walls were wiped with acetic acid as well. A black acrylic 20x20 cm insert was placed on the upper portion of the conditioning box such that the ceiling of the box was at a 60° acute angle.

<u>*Experiment 1: Transmodal SEFL*</u>

Rats were randomly assigned to receive either "Stress" or "No Stress" in Context A and 1 Shock, 1 Noise, or 2 Noises for fear conditioning in Context B.

On **Day 1**, rats were transported in groups of 4 to Context A in their homecages. Session duration was 90 minutes (5400s). Stress animals experienced 15 (1s, 1mA) shocks given in a semi-random, unpredictable interval with a variable ITI of 3 to 7 minutes. No Stress animals remained in the chamber for the duration of the trial without exposure to any stressors for all experiments. After the 90-minute session the subjects were removed and returned to their home cages.

On **Day 2**, context fear was measured in Context A. Rats were transported in their homecages. Session duration was 8 minutes (480s) during which freezing was recorded and scored using MedAssociates VideoFreeze software. After the 8-minute session the subjects were removed and returned to their home cages.

On **Day 3**, rats were transported to novel Context B in a 76x76cm black tub that contained different bedding than what was used for their homecage. After a 3-minute (180s) ITI subjects were administered the conditioning trial. An equal number of animals received 1 (1 mA, 1s) footshock, 1 (120dB 1s) noise burst or 2 (120 dB, 1s, 10s ISI) noise bursts. 30 seconds after termination of the stimulus, rats were removed from the chamber and returned to their home cages.

On **Day 4**, context fear was measured in Context B. Animals were transported to the context in the black tubs. Session duration was 8 minutes (480s) during which freezing was recorded and scored using MedAssociates VideoFreeze software. After the 8-minute session the subjects were removed and returned to their home cages.

Experiment 2: Noise SEFL

An equal amount of male and female rats were assigned to receive either Shock Stress (*n*=8), Noise Stress (n=8) or No Stress (*n*=16; Shock No Stress=8, Noise No Stress=8) in Context A. All stressed rats were fear conditioned in Context B to the same modality of stressor experienced in Context A. No Stress control animals were evenly split to receive either Shock or Noise in Context B.

On **Day 1**, rats were transported in groups of 4 to the Context A conditioning chambers and placed in individual chambers. Rats were transported in their home cages. Session duration was 90 minutes (5400s). Shock Stress animals experienced 15 (1s, 1mA) shocks given in a semi-random, unpredictable interval with a 3 to 7 minute ITI. Noise Stress animals experienced 15 (1s, 120 dB) white noise bursts given at the same semi-random interval as Shock Stress animals. No Stress animals remained in the chamber for the duration of the trail without exposure to any stressors for all experiments. After the 90-minute session the subjects were removed and returned to their home cages.

On **Day 2**, fear of Context A was measured. Rats were transported in their home cages to Context A and placed in the same individual chambers as Day 1. Session duration was 8 minutes (480s) during which freezing was recorded and scored using MedAssociates VideoFreeze software. After the 8-minute session the subjects were removed and returned to their home cages.

On **Day 3**, rats were transported to novel Context B in a novel 76x76cm black tub that contained different bedding than what was used for their homecage, to avoid any generalization from the prior two days of training. After a 3-minute (180s) ITI subjects were administered mild fear conditioning. Animals that received Shock Stress received 1 (1mA, 1s) footshock and animals that received Noise Stress received 1 (120dB, 1s) noise burst. An equal number of no

stress controls received 1 footshock or 1 noise burst. 30 seconds after termination of the stimulus, rats were removed from the chamber and returned to their home cages.

On **Day 4**, context fear conditioning was measured in Context B. Animals were once more transported in the black tubs. Session duration was 8 minutes (480s) during which freezing was recorded and scored using MedAssociates VideoFreeze software. After the 8-minute session the subjects were removed and returned to their home cages.

Hearing Test

In order to ensure that Noise animals did not experience hearing loss from 15 presentations of 120 dB noise bursts, a hearing test was conducted after the SEFL test for a cohort of animals (*n*=8) that had been assigned to Noise Stress during the Noise SEFL experiment. Animals assigned to the No Stress condition were used as control subjects, with a "Noise Control" group (*n*=8) having experienced a single noise burst for conditioning and a "Shock Control" group (n=8) having experienced a single foot shock for conditioning in Context B. Animals assigned to Shock Stress were excluded as controls to Noise Stress due to concern of sensitization, as startle response would be the measurement used to indicate hearing. Noise Stress and Control animals were restrained in a plastic cylinder for 15 minutes. 35 1-second noise bursts varying randomly between 60-120 decibels (60 dB, 70 dB, 80 dB, 90 dB, 100 dB, 110 dB, 120 dB) were played, with each decibel level being presented 5 times across the entire session. Hearing acuity was measured by maximum movement amplitude of startle to each noise burst.

Statistics

Behavioral data was extracted from VideoFreeze and processed using Microsoft Excel. The dependent variable was Percent Time Freezing. Video was taken at 30 frames/second. The

dependent variable was Percent Time Freezing. Freezing was determined by the program calculating whether the number of pixels which moved were below a set threshold (< 50 for longer than 1s). All significant main effects and significant interaction effects were further analyzed for simple main effects in SPSS (IBM).

Experiment 1: Transmodal SEFL

On **Day 2**, total percent time freezing in Context A was analyzed using a two-way ANOVA for the independent variables of Stress (Stress or No Stress) and Sex (Male or Female). For baseline freezing on **Day 3**, a two-way ANOVA for Stress (Stress or No Stress) and Sex (Male or Female) was used to analyze percent time freezing during the 3-minute ITI during the conditioning session in Context B.

For context fear on **Day 4**, when analyzing total percent time freezing in Context B, we used a three-way ANOVA for the independent variables of Stress (Stress or No Stress), Conditioning Stimulus (1 Shock, 1 Noise, or 2 Noises), and Sex (Male or Female).

Experiment 2: Noise SEFL

For the stress on **Day 1** in Context A, we calculated the proportion of observations in which the rats were freezing in the 30s before the shock, and the 30s after the shock. This allowed us to calculate the percent of freezing spent prior to the shock and the percent freezing after the shock on each session. To do this, separate repeated measures ANOVAs assessing effect of Stress Stimulus (Shock or Noise) and Sex (Male or Female) was used for the 30s percent time freezing before each stressor, 30s after each stressor, and for the calculated proportion between pre- and post- stressor.

For the context fear test on **Day 2**, total percent time freezing in Context A was analyzed using a two-way ANOVA for the independent variables of Type of Stress (Shock Stress, Noise Stress, or No Stress) and Sex (Male or Female).

For baseline freezing on **Day 3**, a two-way ANOVA for Type of Stress (Shock Stress, Noise Stress, or No Stress) and Sex (Male or Female) was used to analyze percent time freezing during the 3-minute ITI before the Conditioning session in novel Context B.

For the context fear test on **Day 4**, total percent time freezing for was analyzed using a three-way ANOVA for the independent variables of Stress (Stress or No Stress), Modality (Shock or Noise), and Sex (Male or Female).

For the **Hearing Test**, reaction to noise was measured by maximum velocity. Noise presentations at varying decibels (60 dB, 70 dB, 80 dB, 90 dB, 100 dB, 110 dB, 120 dB) (duration: 1s) were played in random order across a 35-minute session. Each decibel level was played a total of 5 times. Animals that experienced Noise Stress (experiencing 15 120 dB noise bursts in Context A and an additional noise burst in Context B) were compared to controls that did not experience any Stress on Day 1 in Context A (and experienced 1 presentation of 120 dB noise in Context B, or 1 presentation of footshock in Context B). Animals that experienced Shock Stress on Day 1 were excluded because of concerns of sensitization. A repeated measures ANOVA was conducted for the independent variables of Decibel Level, Presentation Order (T1-T5), and Stress Experience.

Rigor and Reproducibility

All analyses were conducted using SPSS (IBM). Simple main effect analysis was used for all significant effects. For *Experiment 1: Transmodal SEFL*, animals assigned to be fear conditioned with shock had small group numbers based on capacity limitations. Previous results

from our lab, both published and unpublished, indicated that there would be a robust significant effect apparent between stressed and unstressed animals fear conditioned with 1 shock in Context B (e.g., Rau et al., 2009). Group sizes for all noise conditioned groups (n=8) were estimated using prior work with Long-Evans rats in Stress Enhanced Fear Learning studies, which used 8 subjects per group (Rau et al 2007, Long & Fanselow 2011, Poulos et al 2015). (Stress 1 Shock=4; Stress 1 Noise=8; Stress 2 Noise=8; No Stress 1 Shock=3; No Stress 1 Noise=8; No Stress 2 Noise=8; *n*=39 total).

For Experiment 2: Noise SEFL there were an equal number of males and females per group (*n*=4) and an equal amount of animals total per group (*n*=8).

For *Experiment 1: Transmodal SEFL* formal post-hoc power analysis using G*Power 3.1 was conducted on the data elicited by the lPAG SEFL experiment. The average partial η^2 of our critical effects of Stress groups was ~0.9 which revealed a high estimated power (1-β, .99) with a type 1 error rate (α) below .05 with the sample size used in this study. For *Experiment 2: Noise SEFL* average partial η^2 of the critical effects of our Modality groups was ~.95, which revealed a high degree of power (1-β, .99) with a type 1 error rate (α) below .05. This demonstrates that our sample size was sufficient to detect critical effects with low likelihood of type 1 (α) or type 2 (β) errors. All behaviors were scored using automated software (VideoFreeze). At least one fully powered replication was conducted for each experiment.

2.1 Experiment 1: Transmodal SEFL

There is evidence that exposure to shock Stress sensitizes the amygdala to future aversive stimuli. This was demonstrated by Perusini et al. (2016) using Western blot for the basolateral amygdala of rats. Rats that were administered Stress expressed an upregulation of excitatory GluA1 receptors in this region (Perusini et al., 2016). This upregulation of excitatory receptors in

the basolateral amygdala may be modifying the thresholds for fear conditioning such that a normally weak stress of any modality may now support it (Perusini et al., 2016). If this is indeed non-associative sensitization, then the modality of stimulus used for Stress in Context A and fear conditioning in Context B need not be the same. Therefore, noise was compared to shock as the conditioning stimulus. An additional conditioning group using 2 Noises was added, as it was unclear whether a single noise burst would be sufficient to cause a significant effect, much like how in Poulos et al.'s (2015) study, .33 mA shock, but not .25 mA shock, produced a SEFL effect when used as a mild conditioning stimulus.

Results

In this experiment, we examined the effect of Stress on fear conditioning to Context B using a stimulus of a different modality. Rats were assigned to receive either Stress or No Stress in Context A. Rats were then further assigned to be fear conditioned to either 1 Shock, 1 Noise Burst, or 2 Noise Bursts in Context B. Learned fear of Contexts A and B were measured used an 8-minute context fear test. Fear was measured by freezing, defined as immobile posture with no movement besides for respiration.

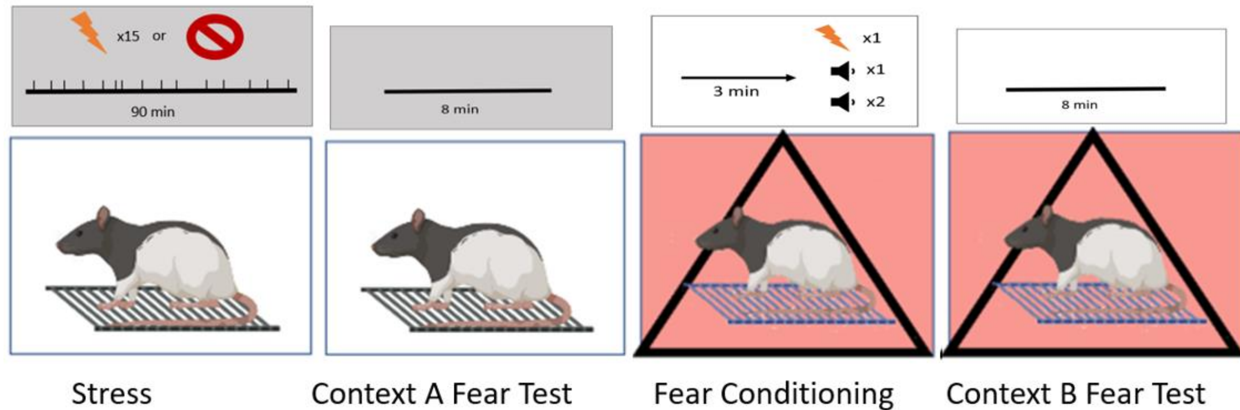

Figure 1.1 Procedure to Test for SEFL Transmodality. On Day 1 rats were placed into Context A and received either Shock Stress (footshock x15) or No Stress. On Day 2, rats were placed back into Context A for a context fear test. On Day 3, animals were next placed into novel Context B, where rats received either 1 Shock (footshock x1), 1 Noise (white noise x1) or 2 Noises (white noise x2) for conditioning. On Day 4, rats were placed back into Context B for a final context fear test to measure the SEFL effect.

On **Day 1**, rats assigned to Shock Stress were administered 15 (1mA 1s) footshocks at semirandom interval over a 90-minute session in Context A. Rats assigned to No Stress groups were placed in Context A for the 90-minute session without being administered any stressors (*Figure 1.1*).

On **Day 2**, animals were returned to Context A for an 8-minute context fear test (*Figure 1*). As expected, Stress animals froze significantly more to the context compared to No Stress animals (*Figure 1.2A*). A 2-way ANOVA looking at Stress (Stress or No Stress) and sex (male or female) found that animals administered Stress froze significantly more than No Stress controls (Stress: $F_{(1,38)}$= 237.145, p<.001). This confirms that Shock Stress was effective. There was no significant effect of Sex ($F_{(1,38)}$=.403, p=.527) and no significant interaction between Sex and Stress ($F_{(1,38)}$=.700, p=.405).

On **Day 3** for fear conditioning, rats were placed into novel Context B. After a 3-minute ITI, animals were administered either one (1mA, 1s) foot shock (“1 Shock”), one (120 dB, 1s)

noise burst ("1 Noise") or two (120 dB, 1s) noise bursts ("2 Noises") (*Figure 1.1*). Freezing remained low across groups in Context B before administering the conditioning trial (Fig 2B). A 2-way ANOVA for the effects of Stress (Stress or No Stress) and sex (Male or Female) for percent time freezing during the 3-minute baseline reveals that all groups froze at similarly low levels. There were no significant main effects or interaction effects between groups (Stress*:* $F_{(1,38)}$= 1.407, p=.247; Sex: : $F_{(1,38)}$= .09, p=.766; Stress*Sex: $F_{(1,38)}$= .898, p=.352) (*Figure 2B*). This demonstrates no generalized fear of Context B.

On **Day 4**, animals were placed back into Context B for an 8-minute context fear test (*Figure 1.1*). While Stress animals administered 1 Shock conditioning froze significantly more than all other groups, Stress animals in the 1 Noise conditioning group showed significantly enhanced freezing to Context B compared to its No Stress controls (*Figure 1.2C*). A 3-way ANOVA comparing Stress treatment in Context A (Stress or No Stress), stimulus used as conditioning in Context B (1 Shock, 1 Noise, or 2 Noises) and sex (Male or Female) found that animals that received Stress in Context A froze significantly more in Context B than No Stress controls, and that this effect was most pronounced in animals that received 1 Shock for conditioning (Stress: $F_{(1,38)}$= 33.843, p<.001; Conditioning: $F_{(2,37)}$= 9.024, p<.001; Stress*Conditioning: $F_{(2,37)}$= 6.675, p<.01). An analysis of simple effects revealed that while Stress did strongly enhance freezing for 1 Shock animals ($F_{(1,38)}$=30.633, p<.001), Stressed animals that received 1 Noise also showed a significant enhancement ($F_{(1,38)}$=7.46, p<.01). This provides evidence that SEFL is indeed transmodal, as shock Stress enhanced fear conditioning to a white noise.

Curiously, there was no such trend of enhanced fear in Context B for Stress animals that received 2 Noise bursts ($F_{(1,38)}$=1.582, p=.212). While the mean for the 1 Noise No-Stress control

($\bar{x}$=1.942) was lower than the mean of the 2 Noise No-Stress Control ($\bar{x}$=8.113) and 1 Shock No-Stress control ($\bar{x}$=8.835), analysis of simple effects revealed no significant difference between average percent time freezing in the No-Stress groups ($F_{(1,38)}$=.556, p=.576). This indicates that the significant effect for the Stress 1 Noise group was not due to freezing in the No Stress 1 Noise group being significantly lower than the other No Stress control groups. Thus, only the 1 Noise burst produced transmodal SEFL.

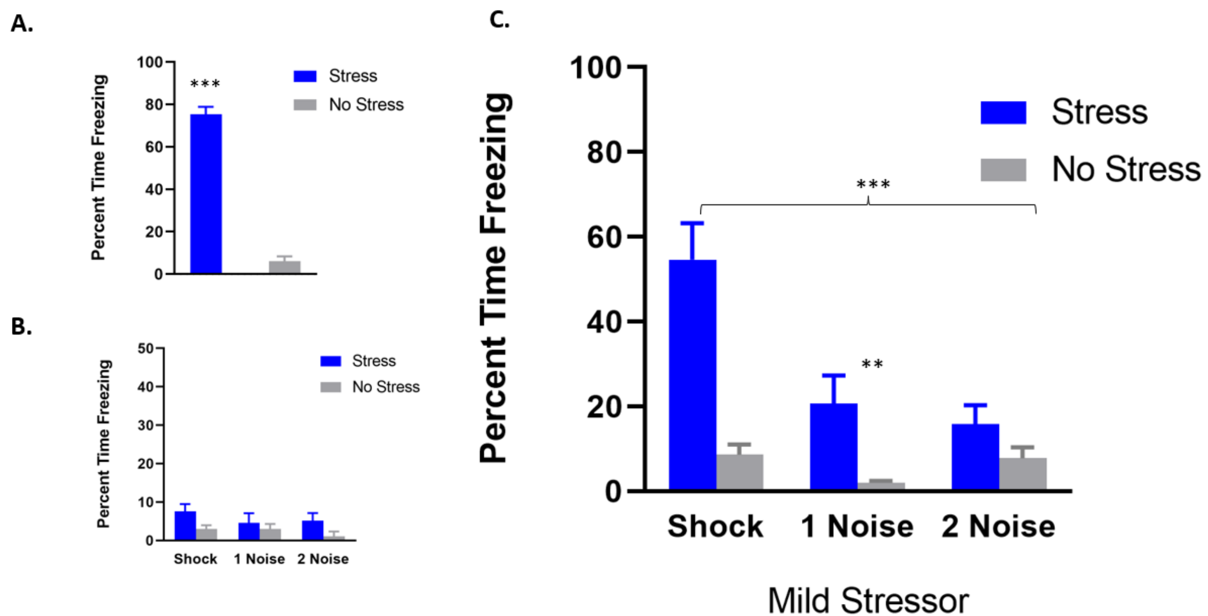

Fig 1.2. SEFL is transmodal. A). On Day 2, rats were placed back into Context A for a context fear test as measured by total percent time freezing. Shock Stress showed significantly more freezing (p<.001) to Context A compared to No Stress. **B).** On Day 3, rats were placed in novel Context B. Baseline percent time freezing in the 3 minutes prior to receiving conditioning to rule out context fear generalization. There was no significant difference in freezing between groups. **C).** On Day 4, rats were placed back into Context B for a context fear test as measured by percent time freezing. Stressed animals conditioned to 1 Shock froze significantly more compared to all other groups (p<.001), and Stressed animals conditioned to 1 Noise froze significantly more than No-Stress controls (p<.01). All error bars represent SEM. **p<.01, ***p<.001

2.2 Experiment 2: Noise SEFL

We now know that SEFL is transmodal. Animals showing enhanced fear learning in Context B to 1 Noise burst, but not 2 Noise bursts (*Figure 1.2C*) may further indicate that increasing the number of noise presentations does not produce the same kind of stress sensitizing effect as seen in shock. Finding a suitable non-sensitizing stress control may be helpful to investigate which areas of the brain are necessary for SEFL in future experiments, as different stressor-dependent defensive behaviors utilize different neural pathways (Fanselow, 2018; Fanselow, 2022; Fanselow & Kim, 1994; Hoffman, et al., 2022). Therefore, we conducted another experiment to examine if the noise burst stimulus was in itself able to support SEFL. That is, we examined if Noise Stress would produce enhanced fear conditioning to Context B with a single noise burst.

<u>*Results*</u>

In this experiment, we investigated whether white noise could produce SEFL. An equal number of male and female rats (*n*=16) were administered either Shock Stress, Noise Stress or No Stress on Day 1. For rats that received Shock Stress or Noise Stress on Day 1, Modality of assigned stressor remained consistent when fear conditioned to Context B on Day 3. No Stress animals were evenly divided to receive either shock or noise conditioning on Day 3.

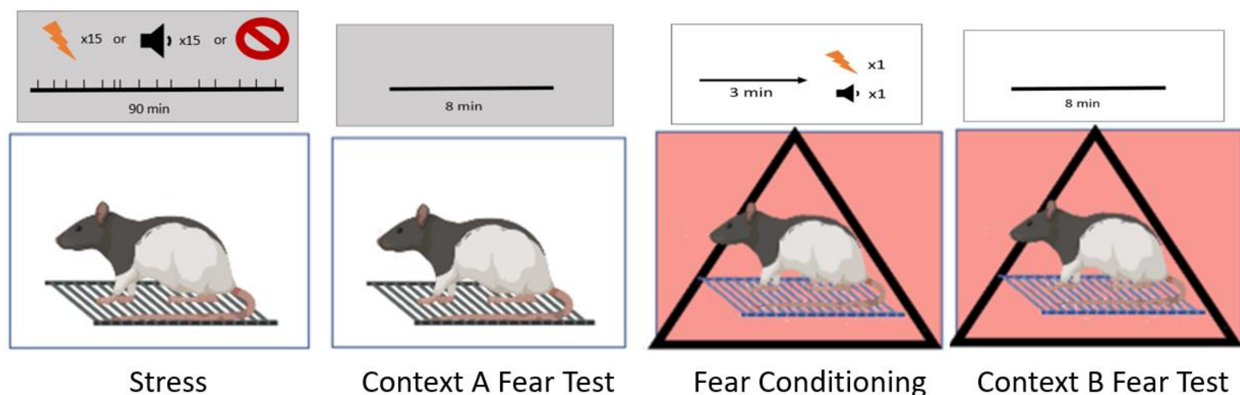

Fig 1.3. Procedure for Noise SEFL. On Day 1 rats were placed into Context A and received either Shock Stress (footshock x15), Noise Stress (white noise x15) or No Stress. On Day 2, rats were placed back into Context A for a context fear test. On Day 3, animals were next placed into novel Context B, where in the Shock Stress group was fear conditioned with a mild shock (footshock x1), while rats that received Noise Stress in Context A were fear conditioned with with 1 noise burst (white noise x1). No Stress rats were equally divided to be fear conditioned with shock or noise. On Day 4, rats were placed back into Context B for a final context fear test to measure the SEFL effect.

On **Day 1**, rats assigned to Shock Stress were administered 15 (1mA 1s) footshocks at semirandom interval over a 90-minute session in Context A. Rats assigned to Noise Stress were administered 15 (120 dB, 1s) white noise bursts at the same semirandom interval over a 90-minute session in Context A. Rats assigned to No Stress groups were placed in Context A for the 90-minute session without being administered any stressors. (*Figure 1.3*).

On **Day 2**, animals were returned to Context A for an 8-minute Context Fear Test (*Figure 1.3*). We found that Shock Stress animals froze significantly more compared to Noise Stress and No Stress groups, regardless of sex (*Figure 1.4A*). In order to compare fear conditioning to Context A, we analyzed the total percent time freezing using a 3 x 2 ANOVA to investigate the effects of Type of Stress (Shock Stress, Noise Stress or No Stress) and Sex (Male or Female). There was a main effect of Type of Stress ($F_{(2,30)}$=76.711, p<.001) ; an analysis of simple effects

revealed that Shock Stress froze significantly more than Noise Stress and No Stress ($F_{(2,30)}$=70.656, p<.001) but Noise Stress did not cause significantly more freezing than No Stress ($F_{(2,30)}$=4.957, p=.059). This indicates that Shock Stress, but not Noise Stress, caused fear conditioning to Context A.

On **Day 3** for fear conditioning, all animals were placed in Context B. After a 3-minute ITI, all Shock animals received one (1mA, 1s) footshock and all Noise animals received one (120 dB, 1s) white noise burst, regardless of whether they had been assigned Stress or No Stress for Day 1 (*Figure 1.3*). Freezing remained low across groups in Context B before administering the first conditioning trial (*Figure 1.4B*). A 2-way ANOVA analyzed the effects of Type of Stress (Shock Stress, Noise Stress or No Stress) and Sex (Male or Female) on percent time freezing during the 3-minute baseline period. There were no differences between groups and no interaction effects prior to receiving the conditioning trial in Context B (Type of Stress: $F_{(2,30)}$=1.552, p=.211; Sex: $F_{(1,28)}$=1.578, p=.214; Type*Sex: $F_{(2,30)}$=1.589, p=.202). This indicates there was no baseline generalized fear of Context B.

On **Day 4**, all animals were placed back into Context B for an 8-minute context fear test to examine the effect of conditioning to Context B (*Figure 1.3*). Shock Stress, but not Noise Stress, caused significantly enhanced freezing to Context B (*Figure 1.4C*). A 3-way ANOVA comparing Stress (Stress or No Stress), Modality (Shock or Noise) and Sex (Male or Female) found a significant main effect of Stress ($F_{(1,31)}$=18.041, p<.001) and Modality ($F_{(1,31)}$=23.236, p<.001) and a significant interaction between Stress and Modality ($F_{(1,31)}$=18.674, p<.001). Simple main effect analysis confirms that this driven by the Shock Stress group freezing significantly more than all other groups ($F_{(1,31)}$=35.461, p<.001). This indicates that Shock, but not Noise, is significantly stressful enough to cause SEFL.

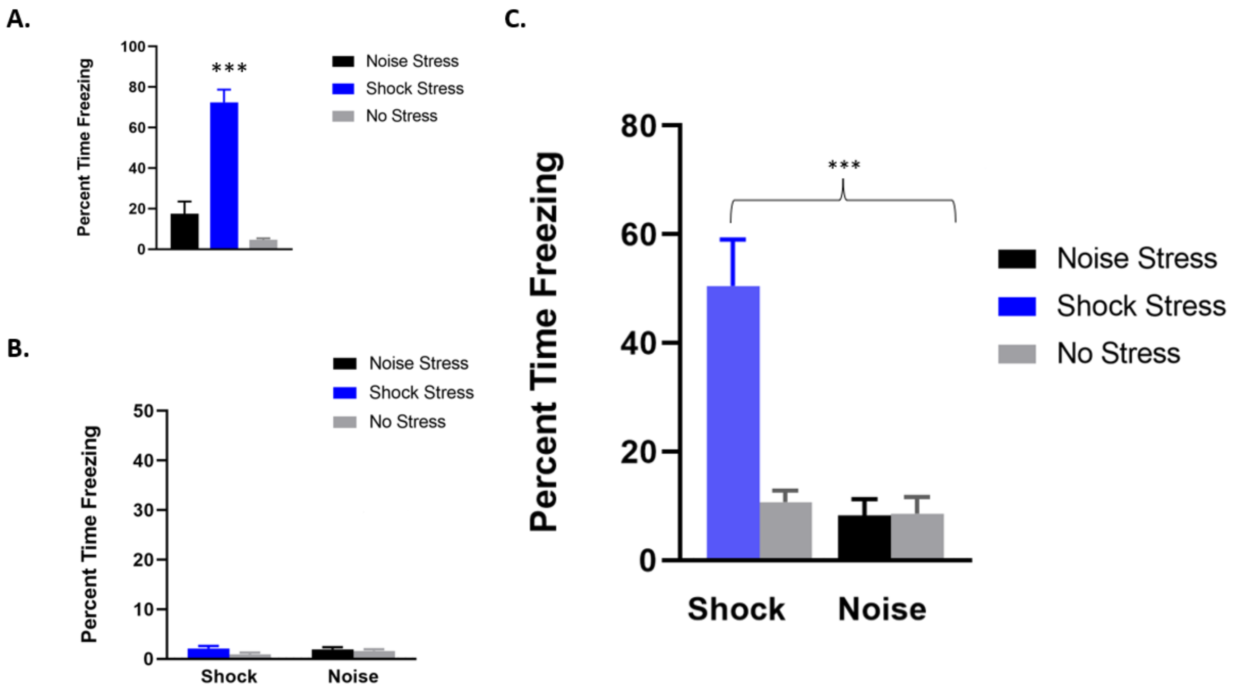

Fig 1.4. A noise stressor does not produce SEFL. A). On Day 2, rats were placed back into Context A for a context fear test as measured by total percent time freezing. Shock Stress caused significantly more freezing ($p<.001$) to Context A compared to Noise Stress and No Stress. **B).** On Day 3, rats were placed in novel Context B. Baseline percent time freezing in the 3-minutes prior to receiving conditioning demonstrates there was no fear generalization as there was no significant difference in freezing between groups. **C).** On Day 4, rats were placed back into Context B for a context fear test as measured by percent time freezing. Shock Stress froze significantly more compared to all other groups ($p<.001$), with no difference between Noise Stress and Noise No Stress. All error bars represent SEM.

Hearing Test

To ensure that Noise Stress rats were not freezing less due to loss of hearing to the 15 presentations of 120 dB white noise, the hearing for this group (n=8) was tested and compared to the No Stress groups, which had only experienced 1 presentation of Noise in Context B ("Noise Control"; *n*=8) or 1 presentation of Shock in Context B ("Shock Control"; *n*=8). Shock Stress animal hearing was not tested, as there was the chance their fear sensitization could produce

enhanced startle. Animals were restrained in a plastic cylinder for 15 minutes. Thirty-five 1-second noise bursts varying randomly between 60-120 decibels were played, with each decibel level playing a total of 5 times. Hearing acuity was measured by maximum movement amplitude of startle to each noise burst. *Figure 1.5* shows that while Maximum Velocity significantly varied between decibel levels ($F_{(6,120)}$= 35.75, p<.001), animals exposed to 15 white noise bursts for Stress on Day 1 did not show significantly less response compared to control groups that experienced no stress on Day 1. A repeated measures ANOVA found no significant main effect between groups (Noise Stress, Noise Control, Shock Control; $F_{(1,20)}$= .194, p=.825) and no significant within-subject interaction effects (Group*Decibel Level: $F_{(12,120)}$= .685, p=.763; Group*Time: $F_{(8,80)}$= 1.676, p=.146). There was no indication of hearing loss in the Noise Stress group.

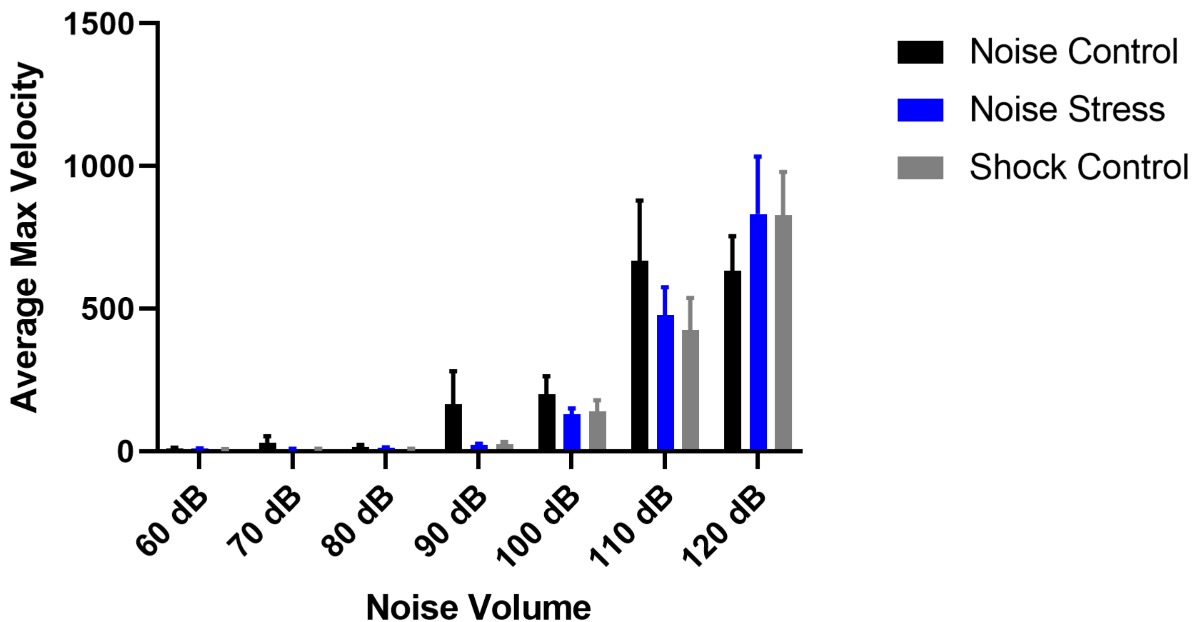

Fig 1.5. No difference in reaction to noise during the hearing test. Rats were placed in a tube within a chamber and randomly presented with noise varying 60-120dB in volume. Noise Stress animals did not react significantly less compared to groups that did not experience 15 120dB white noise bursts. Error bars represent SEM.

2.3 Experiment 2, Noise SEFL: Analysis of Behavior during Severe Stress

Shock Stress, but not Noise Stress, is significant enough of a stressor to cause SEFL. However, when used as a Stressor *(see 2.2),* Noise Stress seemed aversive enough to cause comparable rates of freezing to Shock Stress by the end of the 90-minute session. For this reason, we further investigated behavior elicited by Shock Stress and Noise Stress.

On **Day 1** of the Noise SEFL experiment, rats (n=8 per group) were first exposed to the stressor. This stressor differed between groups and was either a shock (Shock Stress group; 1mA, 1s) or a white noise (Noise Stress group; 120 dB, 1s). These stressors were given 15 times across 90-minute session at a semi-random ITI, which varied between 3 minutes and 7 minutes (*Figure 1.3*). We first analyzed the development of freezing across this session by scoring the 30s prior to each stressor session. A repeated measures ANOVA found that rats in the Noise Stress and Shock Stress groups differed in the percent time that they spent freezing across the session, with rats in the Shock Stress group increasing freezing at a faster rate (Figure 1.6A: time: $F_{(14,392)}$=13.852, p<.001; group: $F_{(1,28)}$= 17.468, p<.001; time*group $F_{(14,392)}$ =3.01, p<.001). Simple main effects analyses showed that rats in the Shock Stress group exhibited significantly more freezing early on the session, where a significant difference in the Shock Stress and Noise Stress groups was present on sessions 3 through 6 (trial: 3:$F_{(1,28)}$= 44.74, p<.001; trial 4: $F_{(1,28)}$= 30.8, p<.001; trial 5: $F_{(1,28)}$= 8.798,p<.01; trial 6: $F_{(1,28)}$= 8.877, p<.01) (*Figure 1.6A*). There was no consistent significant difference in percent time freezing 30s before each stimulus between Noise Stress and

Shock Stress animals by the second half of the session. This indicates that Noise Stress was eventually able to elicit a comparable level of fear in the later phase of the stress session.

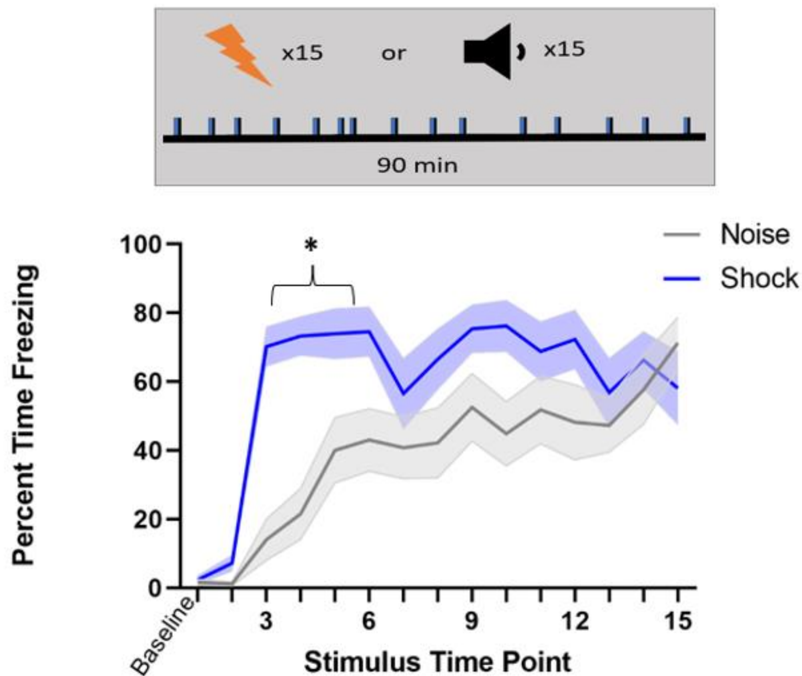

Fig 1.6A. **Activity during Day 1 Shock Stress and Noise Stress.** Data are shown as the percent time freezing 30 seconds prior to the 15 presentations of each stimulus across the session. Shock Stress animals reached freezing asymptote and froze significantly more ($p<.05$) starting at the 3rd presentation, but this difference freezing diminished over time as Noise Stress animals reached asymptote at a slower rate. Error bars present SEM.

We found that Shock Stress rats showed considerably more pre-session freezing across the stressor session relative to Noise Stress rats. This indicates that Shock Stress caused more fear than Noise Stress over the total duration of the session. While it is possible that manifesting SEFL is dependent on the total amount of time that a stress pushes an animal into the fear state, an alternative explanation is that Shock Stress causes SEFL because it elicits the more extreme emotional response of panic. However, if the Shock stressor is actually producing panic, we

would predict that post-session freezing could look quite different. Specifically, rats are likely to show activity burst directly after a shock (Fanselow & Lester, 1988; Fanselow & Kim, 1994), and according to the PIC, this indicates that the shock brings the animal into circa-strike mode. This can be identified by examining freezing for the 30s period after the shock. That is, an activity burst would correlate negatively to freezing, as has been suggested in previous works (De Oca et al., 1998; Fanselow, 1994; Hersman et al., 2020). As predicted, we found that rats in Shock Stress group showed significantly less freezing in the 30s post-session, relative to rats in the Noise Stress group (*Figure 1.6B*). A repeated measures ANOVA found that Noise Stress animals showed a higher rate of freezing post-noise burst across the session (time: $F_{(14,392)}$= 13.686, $p<.001$; group: $F_{(1,28)}$= 17.355, $p<.001$; time*group $F_{(14,420)}$= 2.975, $p<.001$). Simple main effects analysis found that Shock Stress animals froze significantly less than Noise Stress animals directly after foot shock at timepoints throughout the session (*Figure 1.6B*, denoted by asterisks; trial 5: $F_{(1,28)}$=8.213, $p=.001$; trial 7: $F_{(1,28)}$=14.033, $p<.001$; trial 8: $F_{(1,28)}$=5.245, $p<.05$; trial 9: $F_{(1,28)}$=12.934, $p=.001$; trial 11; $F_{(1,28)}$=9.68, $p<.01$, trial13: $F_{(1,28)}$=15.251, $p<.001$; trial 14: $F_{(1,28)}$=4.816, $p<.05$). The only outlier of this trend was during trial 3, where Shock Stress animals showed significantly more freezing post-shock (Trial 3: $F_{(1,30)}$=35.657, $p<.001$).

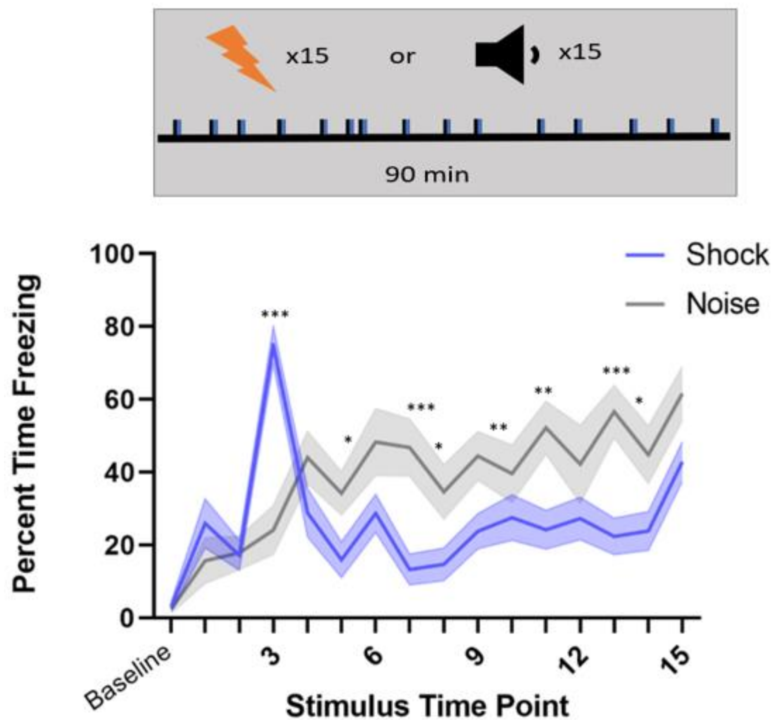

Figure 6B. Activity during Day 1 Shock Severe Stress and Noise Severe Stress. Data are shown as percent time freezing 30 seconds after the 15 presentations of each stimulus across the session. Shock animals froze significantly less than Noise animals throughout most of the session. Significant differences denote by asterisk (*<.05, **<.01, ***p<.001). Error bars represent SEM.

One way to examine the interplay between the freezing response before the session (post-encounter) and the post-session freezing (post-encounter, circa strike) is to take a difference score between these two timepoints on each session. This is important because it provides evidence to show that the absence of freezing seen in the Shock Stress group after the session is not due to the absence of fear. That is, if the post-session response is an activity burst and not an absence of fear, we should see a marked difference in freezing pre- and post-session freezing. To examine this, we subtracted the freezing during the 30s pre- and post-session periods. Here, we found that rats in the Shock Stress group showed a negative difference in their freezing pre- and post-session, which indicated significantly greater freezing pre-session than post-session. This

was not seen in the Noise Stress group, which showed very little difference between these measures on each session (*Figure 1.6C*). Statistical analyses confirmed these observations, with a repeated measures ANOVA showing a significant effect of time ($F_{(14,392)}$ = 8.973, p<.001) as well as group ($F_{(1,28)}$=95.91, p<.001), and a significant interaction between time and group ($F_{(14,392)}$= =4.674, p<.001). Simple main analyses showed that the interaction was due to significant difference in the difference scores between groups in the middle of the session (e.g., trial 9: $F_{(1,28)}$=22.097, p<.001). This suggests that there was a different phenotype between rats in the Shock Stress group and the Noise Stress group and is consistent with data showing that the Shock stressor with these parameters produces an activity burst post-session (e.g., Fanselow & Lester, 1988; Fanselow & Kim, 1994).

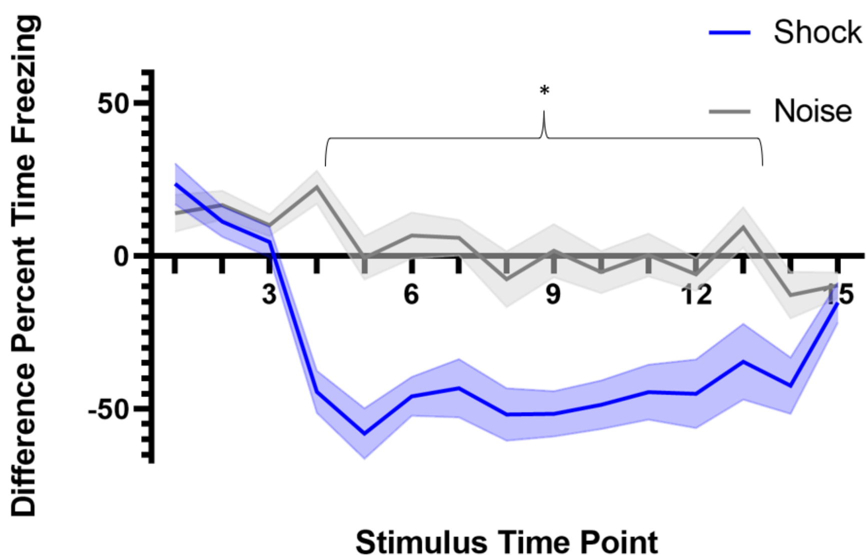

Figure 1.6C. The difference in freezing before and after stimulus presentations across the stress session on Day 1. Shock animals showed a significant decrease in freezing after each shock across the session, while Noise animals showed little change in freezing after each white noise burst. *p<.05.

2.4. Discussion

Fear of aversive or dangerous stimuli, and subsequent learning of fearful contexts they were experienced in, is an adaptive defensive behavior so long as the fear response is

proportional to the extremity of the stressor. In the SEFL model, animals sensitized to stress show a maladaptive, exaggerated response to mild stress that causes enhanced fear learning (Rau et al., 2005). The SEFL process is thought to be non-associative sensitization to stress; if so, it should generalize across modalities. Thus, the stimulus used for stress and conditioning should not have to be the same. Indeed, here we found that the typical 15-shock stressor in Context A produced enhanced fear conditioning to Context B using either a 1 shock (1 mA, 1s) or 1 noise burst (120 dB, 1s; *Figure 2C*). The ability of shock Stress to produce enhanced fear conditioning to a noise stimulus indicates that SEFL is transmodal. This is consistent with recent work that similarly shows that mice exposed to footshock trauma will show a subsequent sensitized fear *response* to an aversive auditory stimulus in a novel context (Hassien et al., 2020), with our results further confirming that this sensitized response supports fear *learning* to the context. This transmodality and enhancement of weak stress supports SEFL being a non-associative process.

Curiously, while subjects showed SEFL to 1 Noise burst, Stressed animals that received 2 Noise bursts for fear conditioning did not show significant enhanced fear learning (*Figure 1.5C*). One possible explanation is that multiple presentations of noise confirmed that there was no oncoming tactile component, as opposed to what might have been experienced when being ambushed by a predator in the wild (i.e. a cue- no outcome association). As outlined below, this may have influenced the animals' assessment of danger being in more distant vicinity. However, the exact reason for lack of SEFL to 2 noise bursts is yet to be known.

That the 2 Noise group did not show SEFL may indicate that it does not have a sensitizing effect in the same way that shock produces a sensitizing component. To test this, we next examined the effects of white noise when used as a Stress. Animals were assigned to either Shock Stress (1 mA, 1s, footshock, x15), Noise Stress (120 dB, 1s, white noise, x15) or No

Stress. Animals assigned to Noise Stress did not show enhanced fear learning (*Figure 1.2C*). Lack of an enhanced fear response to Context B was not due to hearing loss that may have potentially occurred during the Noise Stress treatment (*Figure 1.3*). Noise Stress animals could presumably hear the single noise burst during the conditioning session just as well as No Stress animals. Thus, the noise stressor was not sufficient to produce SEFL.

According to the Predatory Imminence Continuum, animals will be pushed into defensive states of increasing severity, which is dependent on the perceived distance between itself and a predator (Fanselow & Lester, 1988). An animal will enter the post-encounter state when it perceives a predator to be in the immediate area; a common behavior associated with this state in rats is freezing (Fanselow, 1984). The animal will be further pushed into the more severe state of circa-strike when the predator is about to, or has, made aggressive contact. A common behavior associated with circa-strike in rats is flight, often measured as activity bursts (Fanselow & Lester, 1988). During the Stress session of Experiment 2, presentation of each noise burst did not disturb the arrested freezing state to cause activity burst, as could be observed in rats given the Shock Stress (*Figure 1.6C*). This may indicate that Shock Stress pushes the animal from a post-encounter to a circa-strike defensive state. Noise Stress, containing no tactile component, nor any other indication of a predator in the very immediate vicinity, could not do the same. This suggests that shock is capable of driving a panic-like state, which may underlie its ability in driving SEFL in a way white noise cannot.

While Noise Stress was not significantly stressful enough to cause SEFL, or indeed to push the animal into the circa-strike state, the noise stressor increased freezing over the 90-minute session (*Figure 1.6A*). Noise Stress animals freeze at comparable rates to Shock Stress, but context fear learning was not supported when tested 24 hours later (*Figure 1.4A*). This may

be related to previous findings that the strength of an aversive stressor proportionately enforces learning of a cue (Maren et al., 2005). In this case, Noise Stress was significantly stressful enough to cause fear, but too weak to support context fear learning. Thus, Noise Stress may still work as a good stress control for further study comparing innate fear and panic responses.

In the Predatory Imminence model, post-encounter and circa-strike utilize some different areas of the brain. Post-encounter defensive behavior such as freezing utilizes the amygdala (Ciocchi et al., 2010; Johansen et al., 2011; LeDoux, 2000) which has already been shown to undergo long-term plasticity after SEFL (Perusini et al., 2016). One other region recruited during both post-encounter and circa-strike is the periaqueductal grey (PAG). Specifically, the vlPAG is both necessary and sufficient for post-encounter freezing (Assareh et al., 2016; DeOca et al., 1998; Fanselow, 1989; Viana et al., 2001b). In contrast, the d/lPAG is necessary and sufficient for circa-strike activity burst (Deng et al., 2016; Di Scala et al., 1987; Kim et al, 2013; Schenberg et al., 1990;Viana et al, 2001a). If it is indeed necessary for the Stress to cause a circa-strike response in order for SEFL to occur, then Shock Stress should recruit the d/lPAG, more so than Noise Stress, which could underlie the differences in associative and non-associative fear. In the next chapter, we investigated whether there are differences in activity between these two subregions for SEFL using noise or shock stimuli to help understand how the PAG differentially regulates the associative and non-associative components of fear learning.

Chapter 3: SEFL increases cFos expression in lPAG and vlPAG

In Chapter 2, we found that while Noise Stress elicited a comparable amount of freezing to Shock Stress, only Shock Stress was capable of producing SEFL. This allows Noise Stress to serve as a control condition for the investigation of the neural substrates involved in the SEFL effect. That is, it allows us to compare the neural substrates involved in freezing (evoked by both Shock Stress and Noise Stress) with those involved in the SEFL sensitization effect (evoked by Shock Stress). In order to begin to understand the regions of PAG that may be important for the sensitization effect of SEFL, we examined expression of the activity marker cFos in the PAG immediately following noise or shock stress.

In Chapter 2, we found that while both Noise stress and Shock stress produced freezing, only Shock Stress produced activity bursts during shock. Evidence supports that the fear (freezing) response is controlled by the vlPAG (Assareh et al., 2016; Fanselow, 1984; De Oca, et al., 1998 Viana et al., 2001b). Lesioning the vlPAG reduces freezing, while stimulating this subregion initiates freezing (Assareh et al., 2016; De Oca et al., 1998; Tovote et al., 2016). For example, Assareh et al. (2016) optogenetically stimulated channelrhodopsin-expressing neurons in the vlPAG at different levels of intensity. They quantified the observations of different behaviors (grooming, rearing, freezing and activity burst) during the stimulation, and for the ten seconds immediately following stimulation. Exciting vlPAG at the highest intensity only elicited an increase in observed freezing behavior both during and immediately following stimulation. In comparison, stimulating lPAG at high intensity caused a sharp increase in activity bursts during stimulation, indicating a circa-strike response (Assareh et al., 2016). Thus, the vlPAG is a dissociable subregion in that it only causes freezing, even to highly stressful stimuli which can elicit activity bursts when exciting other regions of PAG (such as shock). Therefore, we should

expect to see an indication of an increase of activity in vlPAG for both noise and shock stress, as both caused freezing.

The dorsal periaqueductal grey (dPAG) has been specifically implicated in panic-like responses in rodents, differentiating it from vlPAG. Behaviorally, this includes aimless vertical jumping and freezing behavior altered by flight (Bittencourt et al., 2004; Buenoe et al.,2005; Fanselow, 1984; Lefler, et al., 2020). Stimulating the dPAG initiates unconditional escape behavior (Bueno et al., 2005; Kim et al., 2013; Oliveriara et al., 2004), while inactivating it inhibits escape behavior (Evans et al., 2018) indicating that dPAG is both necessary and sufficient for circa-strike defense response. However, the dPAG is responsible for more than simple execution of behavior. There is also evidence that dPAG plays a role in calculating vicinity of a threat, which is a determinant of whether or not to initiate the circa-strike flight, jumping, or escape response (Deng et al., 2016; Evans et al., 2018; Reis et al., 2021). For example, Evans et al. (2018) used a looming disk stimulus, meant to emulate a swooping predator, on freely behaving mice. They observed that when the disk was smaller and thus appeared to be far away, the mice froze; as the size of the disk widened and it appeared to be coming close, the mice fled. Evans and colleagues (2018) used cholinergic extracellular recording to find distinct populations of neurons in the dPAG: “threat” neurons, which activated right before the onset of fleeing from the looming stimulus, and “flight” neurons, which activated when the mice reached maximum velocity. Both types of neurons appeared to be activated by a threshold-like mechanism, meaning that they only significantly fired when the stimulus caused the mice to transition into circa-strike mode (Evans et al., 2018). This indicates that dPAG activity is reliant on perceived proximal threat. Shock contains a painful tactile component, which may signal indication of a proximal predator (Fanselow et al., 1995). In comparison noise

burst, while aversive, contains no component indicating a threat within close proximity. Therefore, we would expect shock stress to cause indication of significantly more activity in dPAG, compared to Noise Stress. Only shock causes activity bursts- an escape behavior controlled by the dPAG- and only shock has a tactile component that is indicative of a proximal threat, which may activate the dPAG.

The dPAG can further be divided into dorsal-medial (dmPAG) and dorsal-lateral (dlPAG) subregions, owing to some differences in functionality between the two. For example, stimulating the dlPAG specifically appears to elicit and activity burst followed by freezing (Deng et al., 2016; Kim et al., 2013), suggesting correlation between activity in this subregion and circa-strike response. The dmPAG meanwhile plays a greater role in social interaction and aggression (Gross & Canteras, 2012; Xie et al., 2023). For example, chemogenetically activating distinct cell populations in dmPAG that received projections from ventral hypothalamus increased fighting behavior in male mice (Xie et al., 2023). For this reason, dmPAG and dlPAG will be analyzed separately to more accurately pinpoint PAG subregions necessary for SEFL.

The lateral periaqueductal grey (lPAG) has also been implicated in circa-strike behavior such as flight and jumping (Assareh et al., 2016; Bittencourt et al., 2004; Schenberg et al., 1990). There is some indication that dPAG and lPAG have different flight-initiating thresholds; for example, Bittencourt et al. (2004) directly compared the behavioral effects of chemically stimulating dPAG and lPAG with microinfusions of NMDA. dPAG and lPAG showed difference in response curves, with lPAG needing a higher effective dose to elicit circa-strike responses such as jumping, running, galloping and defecation compared to dPAG (Bittencourt et al., 2004). Therefore, dPAG has a lower threshold for causing escape compared to lPAG based on excitatory NMDA receptor proliferation, which may be indicative that it is the primary subregion that

activates escape. However, many previous experiments studying the PAG during defense behaviors do not specifically target the lPAG, instead either focusing on dPAG and vlPAG for stronger segregation between post-encounter and circa-strike areas (e.g. Borelli et al., 2005; Vianna et al., 2001a; Vianna et al., 2001b; Watson et al., 2016) or drawing no distinction between d/lPAG (eg Ozawa et al., 2017; Tovote et al., 2016). Compared to dPAG and vlPAG, whether and how the lPAG functionally differs in response to threat or stress remains largely unknown.

Overall, the PAG is a good candidate for comparing activity between fear- and panic-eliciting stimuli. Shock Stress, but not Noise Stress, may pass a threshold needed to activate the dPAG and/or lPAG in order to trigger the panic response. If panic is indeed necessary for future SEFL to occur, then shock should cause significantly higher activity in these regions compared to noise. A good method to investigate neural activity is to measure expression of early indicator gene cFos in the cells within a given brain region. Fos protein is rapidly expressed during exposure to noxious stimuli (Keay & Bandler, 1993) and live predator exposure (De Paula et al., 2022; Sukikara et al., 2006) in the PAG. Thus, we stained for cFos in the PAG of animals that underwent Shock Stress, Noise Stress, or remained in their Homecage in order to reveal a neural locus that may be specifically necessary for the SEFL effect (Shock Stress), rather than freezing *per se* (Shock and Noise Stress). Thus, we expect that a region specifically involved in the SEFL effect will show selective induction of cFos activity to shock stress and not noise stress. To facilitate this analysis, we segregated our analyses of the cFos counts across the different subregions of PAG that likely subserve different functions.

General Methods

<u>Subjects</u>

Male and female Long-Evans rats, approximately 90 days of age at the start of the experiment (Envigo, Indianapolis, IN) were single housed in a temperature and humidity regulated vivarium. Rats were provided food and water *ad libitum.* The rats acclimated for two weeks in the vivarium, during which they were handled by the investigator for 2 minutes/day over the course of the seven days directly prior to the beginning of the experiment. All cohorts were naïve animals with no prior conditioning. The experiments took place during the light phase of the 12:12 light/dark cycle. All procedures were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the UCLA Institutional Animal Care and Use Committee.

Rats were assigned to one of three stress conditions (Shock Stress (n=7), Noise Stress (n=10), Homecage (n=10)). While an equal number of male and female rats were assigned within each stress group, previous behavioral data analysis of Noise Stress and Shock Stress did not indicate any significant effect of sex. Therefore, sex was not included as part of our power analysis. Rats assigned to the Shock Stress or Noise Stress received 15 presentations of shock or noise burst in Context A. Rats assigned to the Homecage group remained in the vivarium. All animals were perfused and their brains extracted 90 minutes after the end of the behavioral procedure.

<u>*Behavioral Procedure*</u>

Apparatus

All conditioning took place in Med Associates Conditioning chambers and recorded using infrared cameras sent to Med Associates VideoFreeze software. Each box (30x25x25 cm) was in its own sound attenuating chamber. The side walls of each chamber were aluminum and the back wall was solid white plastic. The room was illuminated by white 65 W bulbs and each

chamber was illuminated from above by a single white house light. The fans were switched off and did not provide sound. The floor grid consisted of 16 stainless steel rods (4.8 mm) 1.6 cm apart. A metal pan beneath each grid was sprayed with a thin layer of diluted Windex solution (1:1 water dilution), and the walls were wiped with Windex as well.

Stress Treatment

Noise Stress and Shock Stress rats were transported to Context A in their homecages. Shock Stress animals experienced 15 (1s, 1mA) shocks in semi-random, unpredictable interval. ITI varied between 3 and 7 minutes. Noise Stress animals experienced 15 (1s, 120dB) white noise bursts given in the same semi-random interval as the Shock Stress group. After the 90-minute trial the subjects were removed and returned to their home cages. Homecage animals remained in vivarium for the duration of the trial.

Histology

All animals were deeply anesthetized with isoflurane and transcardially perfused first with phosphate buffered saline (PBS) followed by 4% paraformaldehyde (PFA) until the tissue became fixed and rigid. Brains were extracted and post-fixed in 4% PFA overnight, before being treated with a 30% sucrose solution over the course of approximately three days. 40 µm coronal slices of the brain (Interaural 0.7 mm :1.37 mm) were collected via cryostat and placed in wellplates of PBS.

Immunohistochemistry

Floating coronal slices were washed in 1xPBS three times for ten minutes each. Slices were then incubated in a blocking solution (3% Normal Goat Serum (NGS), 3% Triton-X in 1xPBS solution) for 1 hour at approximately 140 rpm. Slices were then washed again for two 10-minute cycles. In order to tag cFos expression in PAG neurons, slices were incubated overnight

with 12 μL Primary antibody (Rabbit polyclonal anti-cFos (AbCam) in blocking solution, 140 rpm) for 18 hours at 4°C. Slices were then washed for three 10-minute cycles. In order to enhance immunofluorescence of c-Fos expressing cells, slices were then incubated in 24 μL Secondary antibody (Goat anti-Rabbit IgG H+L (AbCam)) for 2 hours at room temperature. Slices were once more washed for three 10-minute cycles before being mounted on slides, treated with Prolong Gold mounting medium with DAPI, and cover-slipped.

Statistics

Behavioral data was extracted from VideoFreeze and processed using Microsoft Excel. Video was taken at 30 frames/second. The dependent variable was Percent Time Freezing. Freezing was determined by the program calculating whether the number of pixels which moved were below a set threshold (< 50 for longer than 1s). For cFos expression, slides were imaged using a Keyence BZ-X710 microscope (Keyence, El Segundo, CA), CCD camera using GFP light channel at 4x and 10x objectives. c-Fos expressing cells were quantified using BZ-X Analyze software, after images were adjusted by a human observer to remove tagging of debris. All significant main effects and significant interaction effects were further analyzed for simple main effects in SPSS (IBM).

Behavior

For the Stress in Context A, we calculated the proportion of observations in which the rats were freezing in the 30s before the shock, and the 30s after the shock. This allowed us to calculate the percent of freezing spent prior to the shock and the percent freezing after the shock on each session. To do this, separate repeated measures ANOVAs assessing effect of Stress Stimulus (Shock Stress or Noise Stress) were used for the 30s percent time freezing before each

stressor, 30s after each stressor, and for the calculated proportion between pre- and post- stressor. Freezing was defined as an alert, immobile posture with no movement besides for respiration.

cFos expression

Average cell count of central (AP -7.5 : -7.8) and caudal (AP -7.8: -8.3) PAG was divided into the following subregions: dorsal medial, dorsal-lateral, lateral, and ventral-lateral as defined by *The Rat Brain In Stereotaxic Coordinates* (Paxinos & Watson, 1997). Analysis between posterior and central PAG sections was separate due to the disparate sizes of each given subregion (*Figure 1B-C*). Cell count within subregions (dorsal-medial (dm), dorsal-lateral (dl), lateral (l), and ventral-lateral (vl)) was compared between Stress groups (Shock Stress, Noise Stress, and Homecage) using two-way ANOVA (SPSS).

Rigor and Reproducibility

All analyses were conducted using SPSS (IBM). Simple main effect analysis was used for all significant effects. Group sizes were estimated using prior work with Long-Evans rats in Stress Enhanced Fear Learning studies, which used 8 subjects per group (Rau et al 2007, Long & Fanselow 2011, Poulos et al 2015). Due to mold contamination, brain slices and slides from other animals initially used in this study were discarded. Noise Stress (n=10) and Homecage (n=10) had more than 8 subjects; while Shock Stress (n=7) did not. However, we conducted a formal post-hoc power analysis using G*power 3.1 on the data elicited from our study. The average partial η2 elicited from our critical effects of Stress on cFos expression was ~.7 with a high degree of power (1-β, .99) and a type 1 error rate (α) below .05 with the sample size used in this study. This demonstrates that our sample sizes were sufficient to detect our critical effects with a low likelihood of type 1 (α) or type 2 (β) errors. Investigators were blind to stress

treatment when analyzing cFos data. All behaviors were scored using automated software (VideoFreeze). Fluorescent cell count was scored using automated software (BZ-X).

3.1 Characterization of stress across the noise and shock conditions

Results

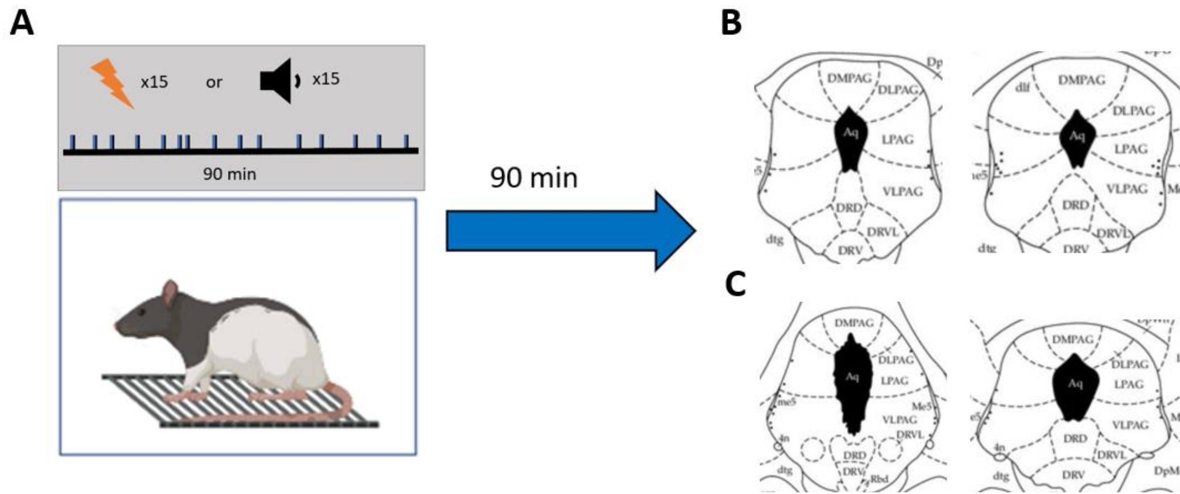

Figure 2.1. Procedure for SEFL cFos experiment. A. Rats were placed into Context A and received either Shock Stress (footshock x15) or Noise Stress (white noise x15) session. A third group remained in their Homecage (not shown). 90 minutes after the end of the session, all rats in Shock Stress, Noise Stress, and Homecage groups were perfused and their brains extracted. **B.** Example figures of slices within Central PAG coordinates (AP: -7.5 : -7.8) stained for cFos. **C.** Example figures of slices within Caudal PAG coordinates (AP:-7.9: -8.3) stained for cFos. Images from Paxinos and Watson (1997).

Rats were administered Shock Stress or Noise Stress in Context A, and then sacrificed 90 minutes after the end of the session. Rats were first exposed to the stressor. This stressor differed between groups and was either a shock (Shock Stress group; 1mA, 1s) or a loud white noise (Noise Stress group; 120 dB, 1s). These stressors were given 15 times across 90 mins at a semi-random ITI which varied between 3 minutes and 7 minutes (*Figure 2.1A*). We first analyzed the development of freezing across this session by scoring the 30s prior to each stressor session (*Figure 2.2A, top)*. A repeated measures ANOVA found that rats in the Noise Stress and Shock

Stress groups increased freezing across the session, with rats in the Shock Stress group freezing significantly more than those in the noise group (*Figure 2.2B*: time: $F_{(14,210)}$= 15.382, p<.001; group: $F_{(1,15)}$= 6.801, p<.05). Simple main effects analyses showed that rats in the Noise Stress group did not freeze significantly less than Shock Stress by the later part of the session, where a significant difference in the Shock Stress and Noise Stress groups was present on trials 3, 4, 7 and 10 (trial 3:$F_{(1,15)}$= 9.784, p<.001; trial 4: $F_{(1,15)}$= 6.339, p<.05; trial 7: $F_{(1,15)}$= 5.525, p<.05; trial 10: $F_{(1,15)}$= 5.151, p<.05) (*Figure 2.2B*). There was no consistent significant difference in percent time freezing 30s before each stimulus between Noise Stress and Shock Stress animals by the last third of the session. This indicates that noise was eventually able to cause a comparable level of freezing as shock in Context A during the Stress session.

We next analyzed freezing for the 30s period after the stressors to once more show that Shock Stress animals displayed both freezing and activity burst, an indicator of panic (Fanselow & Lester, 1988; Fanselow & Kim, 1994) (*Figure 2.2A, bottom)*. Activity bursts directly after shock correlate negatively to freezing, as has been suggested in previous works (De Oca et al., 1994; Fanselow, 1994; Hersman et al., 2020). As predicted based on the findings of the Noise Stress SEFL experiment, we found that rats in the Shock Stress group showed significantly less freezing in the 30s post-trial, relative to rats in the Noise Stress group (*Figure 2.2C*). A repeated measures ANOVA found that rats in the Noise Stress group showed a higher rate of freezing post-trial across the session (trial: $F_{(14,210)}$= 2.187, p<.01; group: $F_{(1,15)}$=5.017 p<.05; trial*group $F_{(14,210)}$= 2.838, p<.001). An analysis of simple effect revealed that Shock Stress froze significantly less throughout the session (*Figure 2.2C*, denoted by asterisks: trial 4: $F_{(1,15)}$=5.016, p<.05; trial 5: $F_{(1,15)}$=8.765, p=.01; trial 6: $F_{(1,15)}$=10.516, p<.01; trial 9: $F_{(1,15)}$=6.763, p<.05; trial 13: $F_{(1,15)}$=9.969, p<.01 trial 14: $F_{(1,15)}$=11.894, p<.01). The only outliers was at trial 3 where

Shock Stress froze significantly more than Noise Stress (trial 3: $F_{(1,15)}$=11.229, p<.01). This confirms that, much like in the previous Noise SEFL experiment, Shock Stress and Noise Stress are eliciting different behaviors, with only Shock Stress interrupting the freezing state to cause activity bursts for the majority of presentations.

Finally, we examined the interplay between the freezing response before each stimulus (post-encounter) and the post-stimulus freezing (post-encounter, circa strike) by taking the difference score between these two trials on each session, as has been done in previous works (e.g. Assareh, 2016). If the 30 second post-trial response is an activity burst and not an absence of fear, we should see a marked difference in freezing pre- and post-trial freezing. To examine this, we subtracted the freezing during the 30s pre- and post-trial periods. We once more found that rats in the Shock Stress group showed a negative difference in their freezing pre- and post-trial, which indicated significantly greater freezing pre-trial than post-trial. This was not seen in the Noise Stress group, which showed very little difference between these measures on each trial (*Figure 2D*). Statistical measures using a repeated measures ANOVA confirmed that Shock Stress animals showed a significant difference in pre- and post-shock freezing activity compared to Noise Stress across the session (Trial: $F_{(14,210)}$= 7.773, p<001; Group: $F_{(1,15)}$=41.822, p<.001; Trial*Group: : $F_{(14,210)}$=2.049, p<.05). A simple effects analysis shows that this difference is observable across the session (*Figure 2D*, denoted by asterisks: trial 4: $F_{(1,15)}$=13.241, p<.01; trial 5: $F_{(1,15)}$=28.823, p<.001; trial 6: $F_{(1,15)}$=32.908, p<.001; trial 7:$F_{(1,15)}$=10.411, p<.01; trial 9: $F_{(1,15)}$=7.567. p<.05; trial 10: $F_{(1,15)}$=19.901, p<.001; trial 13: $F_{(1,15)}$=11.126, p<.01; trial 14: $F_{(1,15)}$=17.786, p<.001). Altogether, these results successfully replicated those from Day 1 of the

Noise Stress SEFL experiment to verify that Shock Stress and Noise Stress produce dissociable behavioral response phenotypes.

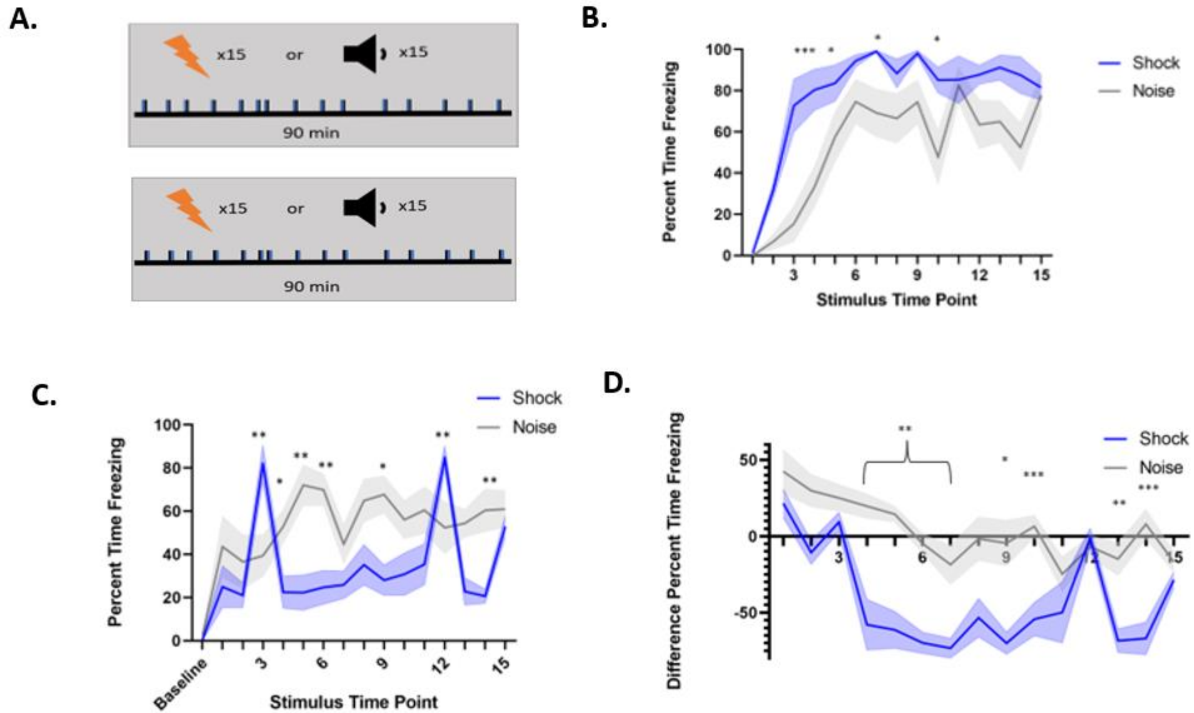

Figure 2.2: Behavior across Shock and Noise Stress. A. Schematic of data extraction points throughout the Stress session. Time points were taken 30 seconds before each stimulus*(Top) and* 30 seconds after each stimulus *(Bottom)* **B.** Percent time freezing 30 seconds prior to the 15 presentations of each stimulus across the session Shock Stress animals reached freezing asymptote and froze significantly more ($p<.05$) starting at the 3rd presentation, but this difference freezing diminished over time as Noise Stress animals did eventually reach asymptote. Freezing to Noise after the 6th presentation showed some vacillation but stabilized by the last third of the session. **C.** Percent time freezing 30 seconds after the 15 presentations of each stimulus across the session Shock animals froze significantly less than Noise animals at points throughout the session. **D.** The calculated difference in freezing Pre-Post stimulus for each of the 15 Shock and Noise stimulus presentations across the Severe Stress Session on Day 1. Shock animals showed a significant decrease in freezing after each shock across the session, while Noise

3.2 Rats receiving shock stress show significantly more cFos expression in lPAG and vlPAG

Results

We next sought to investigate whether these differences in behavior reflected differences in cFos expression within the subregions of the PAG. 90 minutes after the end of the Stress trial, brains of animals from Shock Stress, Noise Stress and Homecage were perfused and extracted. Slices from the PAG region were stained with primary antibody (Rabbit polyclonal anti-cFos (AbCam) in blocking solution, 140 rpm) and fluorescent secondary antibody (Goat anti-Rabbit IgG H+L (AbCam)) cFos expression was quantified in dorsal medial (dm), dorsal lateral (dl), lateral (1) and ventral-lateral (vl) regions in the central (*Figure 2.1B*) and caudal PAG (*Figure 1C*). We chose this range specifically based on previous studies on the role of dissociable defensive behaviors within subregions of the PAG from Fanselow and colleagues (DeOca et al., 1998; DeOca & Fanselow, 2004; Fanselow et al., 1995).

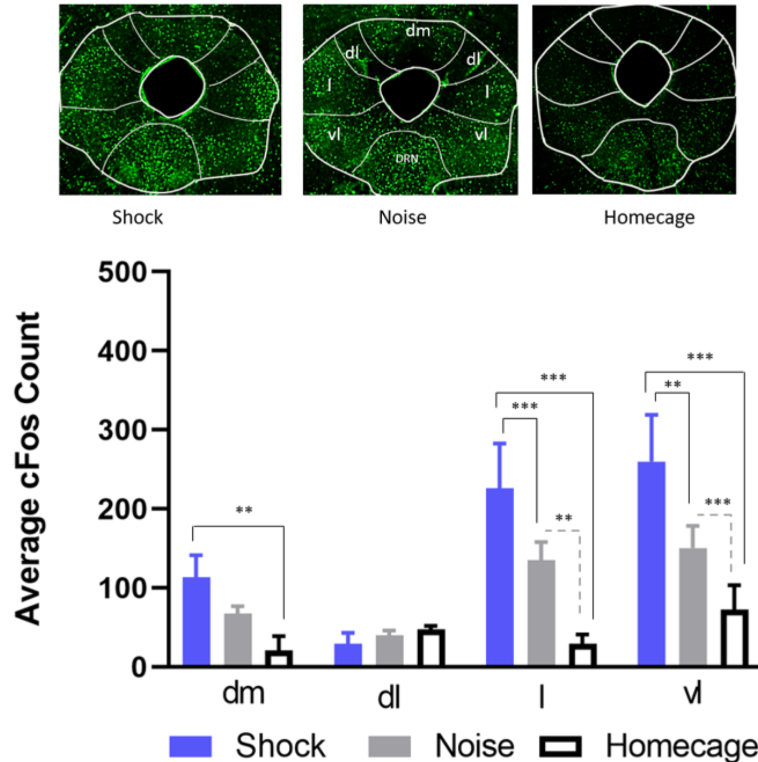

Figure 2.3A. Average cFos count in subregions of central PAG between animals that received Shock Stress, Noise Stress, or remained in Homecage. Shock Stress animals expressed significantly more cFos in lPAG and vlPAG compared to Noise Stress and Homecage (solid black lines). Noise Stress expressed significantly more than Homecage in lPAG and vlPAG (grey dotted lines). Error bars represent SEM. *p<.05, **p<.01, ***p<.001. dm: dorsal medial dl: dorsal lateral l: lateral vl: ventral lateral DRN: dorsal raphe nucleus

In central PAG (AP: -7.5: -7.8), Shock Stress animals expressed significantly more cFos than both Noise Stress and Homecage in lateral PAG and ventral-lateral PAG (*Figure 2.3A*). A two-way ANOVA looking at the effects of Stress (Shock, Noise and Homecage) and subregion (dm, dl, l and vl) revealed a significant main effect of Stress across subregions (Stress: $F_{(2,84)}$=27.597, p<.001; Subregion: $F_{(3,84)}$=14.138, p<.001; Stress*Subregion : $F_{(6,84)}$=4.269, p<.001). An analysis of simple effects reveals that Shock Stress cFos count was higher than Homecage in dorsal-medial PAG ($F_{(2,84)}$=3.593, p<.001). Shock Stress cFos count was higher than Noise Stress and Homecage in lateral ($F_{(2,84)}$=16.37, p<.001) and ventral-lateral regions ($F_{(2,84)}$=16.236,

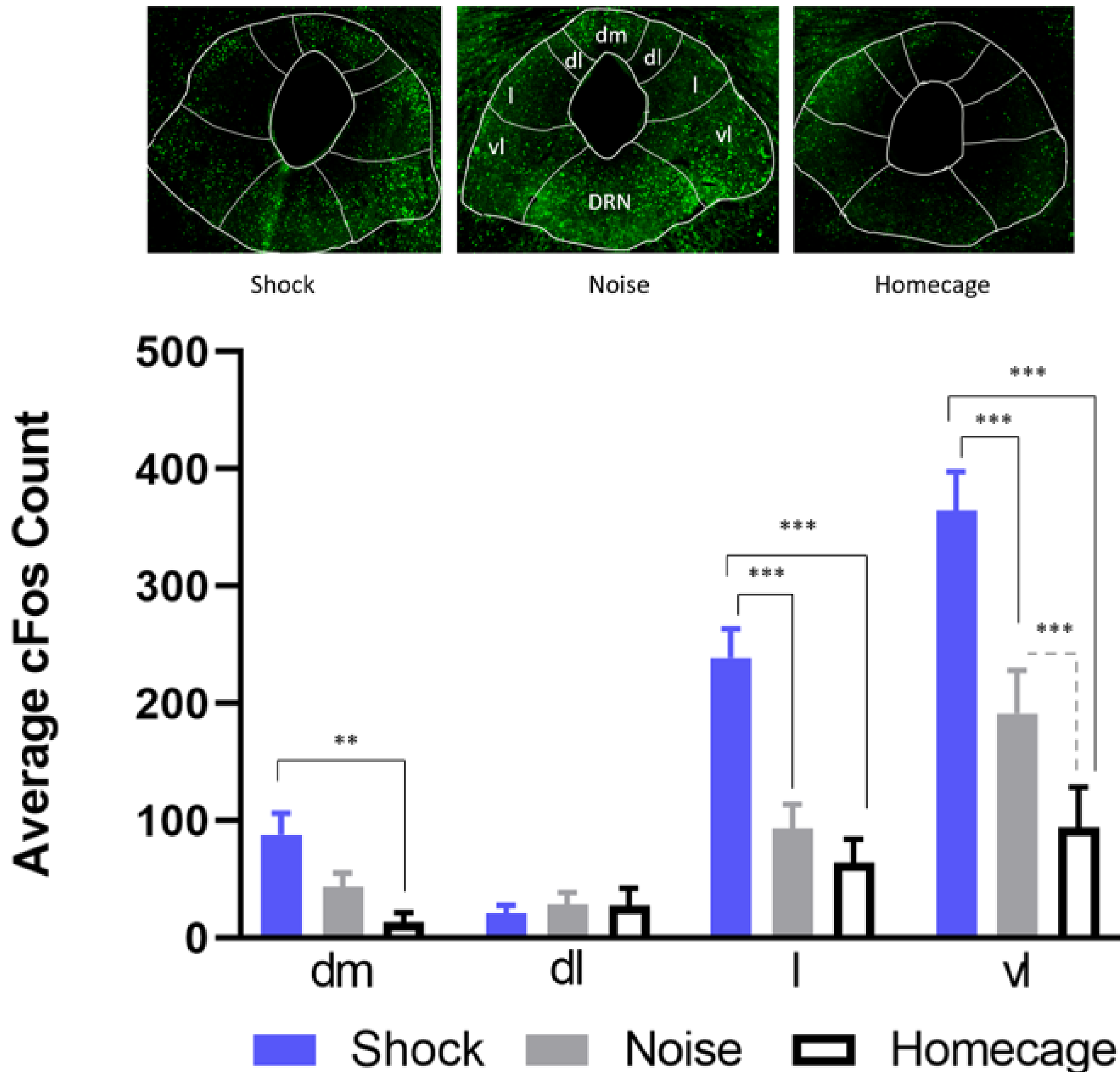

Figure 2.3B. Average cFos count in subregions of caudal PAG between animals that received Shock Stress, Noise Stress, or remained in Homecage. Shock Stress animals expressed significantly more cFos in lPAG and vlPAG compared to Noise Stress and Homecage (solid black lines). Noise stress expressed significantly more than Homecage in vlPAG, but not lPAG (grey dotted lines). Error bars represent SEM. *p<.05, **p<.01, ***p<.001. dm: dorsal medial dl: dorsal lateral l: lateral vl: ventral lateral DRN: dorsal raphe nucleus

p<.001). There was also a relationship between severity of stress and cFos count, as Noise Stress count was higher than Homecage in lateral ($F_{(2,84)}$=16.37, p<.01) and ventral and vlPAG show a graded response to stress, with highest cFos activity to the shock stress, then lateral ($F_{(2,84)}$=16.236, p<.001) regions as well (*Figure 2.3A*). This indicates that central lPAG noise stress, and finally homecage.

This trend carried over to caudal PAG (AP: -7.9 : -8.3) as well, with Shock Stress once more showing significantly more expression than Noise Stress and Homecage in lPAG and vlPAG (*Figure 2.3B*). A two-way ANOVA looking at the effects of Stress (Shock, Noise and Homecage) and subregion (dm, dl, l and vl) revealed a significant main effect of Stress across subregions (Stress: $F_{(2,84)}$=32.321, p<.001; Subregion: $F_{(3,84)}$=47.951, p<.001; Stress*Subregion : $F_{(6,84)}$=6.606, p<.001). An analysis of simple effects reveals that Shock cFos count was higher than Homecage in dorsal medial PAG ($F_{(2,84)}$=2.632, p<.01). Shock Stress expressed more than Noise Stress and Homecage in lateral ($F_{(2,84)}$=16.915, p<.001) and ventral-lateral regions ($F_{(2,84)}$=32.832, p<.001), with Noise Stress showing more expression than Homecage in ventral-lateral PAG ($F_{(2,84)}$=32.832, p<.001) further indicating lateral and ventral-lateral PAG as subregions of interest (*Figure 2.3B*). An analysis of simple effects reveals that unlike in central PAG, Noise Stress was not significantly higher than Homecage in lateral PAG ($F_{(2,84)}$=16.915, p=.282). The lateral region of caudal PAG is the only region where Shock shows significantly more expression than the other groups without Noise Stress also showing significantly more expression than Homecage. It may therefore be a particular region of interest for further investigation into which regions of the brain are necessary for SEFL.

3.3 Discussion

We discovered that Noise Stress elicits comparable levels of freezing as Shock Stress, without eliciting the sensitizing effects that lead to SEFL. We therefore used Noise Stress as a control for Shock Stress to dissect the neural correlates of stress strong enough to cause SEFL and not just freezing. Our initial aim in examining PAG subregions was based on the observation that Shock Stress, which can cause SEFL, elicits activity bursts. Activity bursts denote the most

extreme defense response analogous to panic, which activates different subregions of the PAG than fear (Fanselow, 1991; Fanselow & Lester, 1989).

Within the PAG, dorsal and lateral subregion activity correlates to panic, and ventral subregions to fear (Assareh et al., 2016; De Oca et al, 1998; Fanselow, 1994; Fanselow et al., 1995; Viana et al., 2001b). Higher expression of cFos within a given PAG subregion may imply that this subregion must pass a certain activity threshold in order for SEFL to occur. We therefore expected that rats in the Shock Stress group would show significantly more expression in dorsal and/or lateral subregions compared to Noise Stress and Homecage animals.

Figure 2 shows analyzed behavior of Shock Stress and Noise Stress animals during the Stress session. Overall, we were successfully able to replicate the results of our previous Noise SEFL experiment (p. 48-51, *Fig 1.6A-C*) We extracted percent time freezing 30 seconds before and 30 seconds after each of the 15 stressor presentations. Shock Stress animals reached freezing asymptote earlier on in the session, with Noise Stress animals reaching comparable levels by the end of the session (*Figure 2.2A*). Shock Stress, but not Noise Stress animals showed significantly lower freezing immediately after each stressor for the majority of presentations, imply not a sudden lack of fear but rather activity bursts associated with panic (*Figure 2.2B*). This is further illuminated by plotting the differences in freezing 30 seconds pre- and 30 seconds post-stressor. Shock Stress animals showed a steep decrease in freezing, indicating that Shock Stress interrupted their fear state to cause the panic response. The difference in Noise Stress animals' freezing remaining around 0 indicates that their freezing state was not interrupted during each white noise presentation (*Figure 2.2C*). This indicates that only Shock Stress was able to push animals into the circa-strike state.

There were some differences in this behavioral data compared to the Noise SEFL experiment in Chapter 2. For 30 seconds pre-, freezing asymptote in Noise Stress animals rose at an overall sharper rate by the 5th noise presentation. Diminishing differences by the middle of the session do not denote a steady increase in freezing, but rather a vacillation between higher freezing and lower freezing. This may be because sample size for this experiment was smaller. Due to mold contamination found in samples during the histology process, subject brains from the initial pool had to be discarded. For accuracy's sake, behavioral data shown here reflect only those animals whose brains survived up to the cFos cell quantification process. Smaller sample size leads to greater variation between subjects. There may have also been differences in prior experience to stressors between cohorts. All rats were delivered from an outside facility and, depending on transport, may have experienced prior stress. In spite of these differences, however, animals still followed the general trend wherein Shock Stress causes more overall freezing across the session than Noise Stress, with noise taking longer to reach comparable levels of freezing.

Ninety minutes after the end of the Stress session, animals from all groups were perfused and their brains extracted. We stained central (AP: -7.5: -7.8) and caudal (AP: -7.9: -8.3) 40 µm PAG slices for cFos expression. In the central PAG, we found that the Shock Stress animals showed significantly more expression than Homecage in the dorsal-medial PAG (dmPAG), lateral PAG (lPAG) and ventral-lateral PAG (vlPAG). Shock Stress caused significantly more expression than Noise Stress in lPAG and vlPAG. Noise Stress caused significantly more expression than Homecage in lPAG and vlPAG *(Figure 2.3A*). This pattern of activity for Shock Stress was also reflected in caudal PAG. Noise Stress caused significantly more expression than Homecage in vlPAG, but not lPAG (*Figure 2.3B*). Therefore, caudal lPAG was unique in that

Shock Stress, but not Noise Stress, caused significantly more expression than Homecage. (*Figure 2.3A-B*).

Shock Stress caused significantly more cFos expression than Homecage, but not Noise Stress, in dmPAG. This was reflected in both central and caudal PAG sections (*Figure 2.3A-B*). The dmPAG is involved in social aggression (Xie et al, 2023) and anxiogenic avoidance behaviors (Borelli & Brandao, 2008). Corticotropin-releasing factor (CRF) is a neuropeptide responsible for coordinating a broad range of autonomic and behavioral responses to stress and anxiety (Dunn & Berridge, 1990; Koob et al., 1993). Borelli & Brandao (2008) injected a non-specific CRF agonist into dmPAG, dlPAG, or lPAG in rats. They found that only the dmPAG group exhibited anxiogenic behavior such as avoidance of open arms in elevated plus maze (Borelli & Brando, 2008). Interestingly, CRF release has been linked to SEFL (Perusini et al., 2016) but we did not observe significant differences in expression between Shock Stress and Noise Stress. Therefore, this expression in the dmPAG subregion may reflect a more general response to different kinds of stress.

cFos expression in both central and caudal vlPAG appears to be dependent on strength of stress. Shock Stress expressed significantly more than Noise Stress and Homecage, and Noise Stress expressed significantly more than Homecage (*Figure 2.3A-B*). This graded pattern of expression may relate to the amount of freezing that each condition caused. Shock Stress caused a high level of freezing early on in the session, and this freezing remained at ceiling level (*Figure 2.2A)*. Noise Stress also caused freezing, but not as much as in total as Shock Stress (*Figure 2.2A*). Homecage, being the no-stress condition, did not cause any freezing and so levels of expression in vlPAG remained low. Activating vlPAG causes an increase in freezing (Assareh et al., 2016; Borelli et al., 2005; Tovote et al., 2016) while inhibiting it attenuates freezing (De Oca

et al., 1989; Fanselow, 1989). For example, Tovote et al. (2016) optogenetically stimulated glutamatergic cells in vlPAG of freely behaving mice, which reliably caused an increase in freezing during stimulation. In order to test the necessity of glutamatergic vlPAG cells for conditional freezing, Tovote and colleagues next trained mice to associate a tone cue with footshock. Optogenetically inhibiting these cells when playing the tone during extinction training attenuated conditional freezing to it. Furthermore, photoinhibition of vlPAG also dramatically reduced freezing to an oncoming looming stimulus meant to evoke fear of a swooping predator (Tovote et al., 2016). This implies that the freezing state for both conditioned and innate fear is directly linked to vlPAG, which is reflected in our own results.

We observed this same type of stress-dependent graded expression in central lPAG as well. Shock expressed significantly more cFos than Noise and Homecage, and Noise expressed significantly more than Homecage (*Figure 2.3A*). The lPAG's function does differ across the rostral-caudal plane (Bandler & Depaulis, 1988; Comoli et al., 2005; Depaulis et al., 1992; Depaulis et al., 1994). For example, Depaulis et al. (1994) found that chemically exciting the central lPAG with EAA caused "backward defense" behaviors in rats (facing towards and backing away from a perceived threat) and freezing, while exciting caudal lPAG induced escape behavior (Depauli et al., 1994). If a range of defense behavior is organized across the rostal-caudal plane of lPAG, then perhaps central lPAG activity corresponds to a post-encounter "distal" threat. This would explain why central lPAG cFos expression showed a similar trend of expression to vlPAG, which is also involved in post-encounter response.

Consistent with differences in the function of the lPAG across the rostral-caudal plane, we found differences in the pattern of cFos activation in central and caudal lPAG. Specifically, while we saw a graded pattern of cFos expression in central lPAG, in caudal lPAG we saw

greater levels of cFos selectively in the Shock Stress group and not the Noise Stress or Homecage group. This differed from other regions we examined in caudal PAG, including vlPAG, central lPAG, and central vlPAG, where we found that Noise Stress evoked significantly more cFos than Homecage (*Figure 2.3A-B*). This suggests that in caudal lPAG, only the most severe Shock Stress is able to elicit more activity than the no-stress Homecage group. This provides a neural locus that may be specifically involved in SEFL.

These cFos analyses yielded some unexpected results. Expression in dlPAG remained the lowest out of all subregions across groups in both central and caudal PAG, which differs from previous cFos PAG studies on defensive behaviors (Aguiar et al., 2014; Corelli et al., 2005). However, the prior studies utilized a single no-contact exposure to a live predator, which differs from our Noise and Shock stress treatments. Indeed, a study directly comparing cFos expression in PAG between foot shock and no-contact predator exposure found that the predator group showed significantly more expression in the most dorsal PAG regions compared to the shock group (Baisley et al., 2011). This may explain why we saw lower levels of cFos expression in dlPAG in this current study. That is, the differences in the tactile components present in our study, relative to the no-contact predator-like stress in previous studies may have produced differences in the amount of cFos seen across these studies.

Nevertheless, it still cannot be ignored that the dlPAG has been heavily implicated in panic response to shock (Deng et al., 2016; Evans et al., 2018; Masferrer et al., 2020). Evans et al. (2018) for example demonstrated via calcium imaging that there is correlation between amount of activity in the dPAG and severity of defensive response, with more activity correlating to the panic-like escape behavior (Evan et al., 2018). However, these studies largely used methods such as calcium imaging and single cell recording to study dynamic cell activity,

whereas here we analyzed static cFos results. It is possible that we could have found evidence for behaviorally-relevant activity in dlPAG had we used activity recording methods, which was not sufficient to generate indicators of activity using our cFos technique.

Peak expression of cFos occurs approximately 90-120 minutes after neuron stimulation (Hudson, 2018). We extracted brains 90 minutes after the end of the SEFL session, which was itself 90 minutes long. Therefore, our cFos results most likely reflect activity in PAG for the later trials of the session rather than changes in activity to the stress stimulation across the session. Changes in subregion activity across the session is of particular relevance because the interplay of activity between subregions during stress is itself quite dynamic. It has previously been discovered that a vlPAG-dlPAG mutually inhibitory microcircuit keeps freezing and flight in check (Graeffe et al., 2015; Johnson et al. 2004; Tovote et al., 2016; Walker & Carrive, 2003). For example, injecting excitatory amino acid (EAA) into dlPAG ordinarily causes escape behavior. However, injecting serotonin agonist 8-OHDPAT into dlPAG prior to administering EAA reduces escape behavior (Becket & Marsden, 1997; Johnson et al., 2004). This indicates that dlPAG-induced escape behavior is tonically inhibited by serotonin. Although there have been no viral tracing studies that have found vlPAG → dlPAG serotinergic projections, electrically stimulating the medial vlPAG causes a significant increase of 5HT in the dlPAG (Johnson et al., 2004). While indirect, these findings together indicate the possibility that the vlPAG can inhibit dlPAG activity. The increase of cFos we observed in vlPAG may therefore, in part, explain why there was so little expression in the dlPAG compared to other subregions. Indeed, Noise Stress animals showed higher activity in the dlPAG than Shock Stress and Homecage animals, although not significantly so. As Shock Stress expressed significantly more activity in vlPAG compared to Noise Stress, this may hint at more vlPAG→dlPAG inhibition in

Shock Stress animals. Interestingly, if this were the case, these data would indicate that there is no such vlPAG-reliant inhibition of lPAG activity, as lPAG activity was still overall higher in Shock animals. Future studies are needed to better understand the interplay between these neural substrates in defensive behaviors.

While we did not quantify expression in the dorsal raphe nucleus (DRN) it may be noted that expression within this region was high for rats in the Shock Stress and Noise Stress groups. We did not investigate this subregion because we were investigating PAG regions involved with defensive responses and traditionally the DRN and serotonin neurons are thought to regulate appetitive behaviors and reward (Fischer et al., 2017; Li et al., 2016; Luo et al., 2015). Freezing has been specifically linked to the lateral “wings” of ventral PAG (Cocchi et al., 2013; Ozawa et al, 2017; Yeh et al., 2021). For example, Coicchi et al (2013) used viral tracing to find that freeze-activating neurons in the central amygdala projected specifically to vlPAG. However, it is possible that the DRN regulates panic responses, as dlPAG activity is inhibited by serotonin as mentioned above (Becket et al., 1997; Hammack et al., 2002; Johnson et al., 2004; Maier et al., 1993; Miguel et al., 2010). As a matter of fact, Miguel et al. (2010) found that chemically stimulating the DRN increased release of 5HT in the dPAG which, in turn, attenuated escape behavior. This increased DRN expression may therefore also relate to the low cFos expression seen in the dlPAG and is an interesting direction for future research.

Based on the cFos data, caudal lPAG is the best candidate for further study into which regions are necessary for SEFL because we saw that activity in this region was specifically elevated for the Shock Stress group and not the Noise Stress or Homecage groups. This is consistent with the literature showing that lPAG has been found to be involved with panic (Assareh et al., 2016; Bittencourt et al., 2004; Buenoe et al., 2005; De Oca, et al., 1998;

Fanselow, 1984; Kim et al., 2013; Lefler et al., 2020; Viana et al., 2001b). Thus, in the next chapter we explicitly focused on investigating a causal role for the caudal lPAG in SEFL, given our ability to isolate this as a region specifically involved in shock stress and not noise stress.

Chapter 4: Inhibiting lPAG is not necessary for Non-Associative SEFL, But Does Enhance Associative Fear Learning

In Chapter 3, we compared cFos expression in PAG subregions for animals that received Shock Stress, Noise Stress, or no stress (Homecage). Noise Stress had been used as an additional control condition, as it is stressful enough to elicit freezing but not strong enough to produce SEFL. We found that both the central vlPAG and lPAG showed a graded increase in cFos expression depending on the strength of the stressor. Specifically, both these regions showed high levels of cFos induction in the Shock Stress groups, followed by Noise Stress groups, and finally the homecage control. This suggested that central vlPAG and lPAG are involved in fear behaviors exhibited by both these stressor conditions. In contrast, we revealed that the caudal lPAG was the only region where cFos induction was seen only for the Shock Stress group, which we know is the only group to show SEFL. This suggests that only the sensitizing stress inherent in the Shock Stress condition which causes SEFL recruits this region. Therefore, in the next experiments, we tested the necessity of caudal lPAG activity during the Shock Stress for the subsequent SEFL effect.

lPAG activity may be linked to the activity burst escape behavior observed by rats in the Shock Stress group, indicating a panic-like response. Indeed, lPAG stimulation at stronger intensities causes escape behavior such as flight, jumping, and activity bursts (Assareh et al., 2016; Bittencourt et al., 2005; Schenberg et al., 1990). Assareh and colleagues optogenetically excited cells in lPAG or vlPAG and analyzed behavior that occurred during the 10-second stimulation onset. They quantified activity by percent of observations of freezing (defined as a crouching immobile posture with no movement besides for respiration) and flight (activity bursts

and escape attempts) during the duration of each trial. During the duration of high frequency lPAG stimulation, rats showed a significant increase in amount of observed flight responses. In comparison, strongly stimulating the vlPAG only caused an increase in freezing (Assareh et al., 2016). This indicates that lPAG, and not vlPAG, will cause circa-strike related behavior such as activity burst when it is strongly excited. This is consistent with our data indicating that only Shock Stress is sufficient to produce the sensitizing effects of SEFL induced cFos activity in caudal lPAG and supports the notion that this region might be necessary for SEFL.

SEFL is a form of non-associative, long-term fear sensitization (Aimir & Fanselow, 2011; Poulos et al., 2014; Rau, et al. 2005; Rau, et al., 2009) but studies on the neural correlates of long-term fear sensitization component of SEFL are sparse. Most focus on correlative plasticity and changes in circuitry following the sensitizing event (Kaylnchuk et al., 2001; Perusini et al., 2016). For example, in a study specifically looking at SEFL-induced plasticity which may contribute to fear sensitization, Perusini et al. (2016) used Western blot to find that Stress caused an upregulation of GluA1 AMPA receptors in the basolateral amygdala. They hypothesized that this causes increased excitability in the basolateral amygdala and that this excitability contributes to enhanced fear conditioning seen after stress (Perusini et al., 2016), but did not demonstrate a causal role for amygdala in the SEFL effect.

There are some causal studies that examine the role of distinct neural structure in SEFL, which are generally areas that are considered associative fear-related structures. For example, activity in the basolateral amygdala, dorsal hippocampus, and infralimbic cortex during the stress component of SEFL have been found to be necessary for the SEFL effect (Hersman et al., 2019; Pennigton et al., 2017; Perusini et al., 2016). However, some methods used caused the effects to last during the entire stress session (rather than during each shock). Other methods would have

lasted for the duration of the entire SEFL experiment, meaning the region was also inactivated during fear conditioning and fear recall in Context B. For example, Pennington et al. (2017) used excitotoxic lesions of the infralimbic cortex prior administering the Stress on Day 1 of the SEFL procedure. While lesioned rats that received Stress on Day 1 did show some enhanced fear learning to Context B compared to no stress controls, this effect was significantly reduced compared to non-lesioned Stressed rats. (Pennigton et al., 2017). Because the lesion inhibited infralimbic activity for the entire four-day experiment, it is difficult to pinpoint whether this region is involved in causing fear sensitization, or whether it is involved in processing the context. And indeed, rats with lesioned infralimbic cortex did not show a reduction of SEFL when a discreet tone cue was used for fear conditioning (Pennington et al., 2017). Similarly, Hersman and colleagues (2019) infused scopolamine, a cholinergic antagonist, in dorsal hippocampus just prior to the Stress on Day 1. They found that pharmacologically inhibiting cholinergic signaling in dorsal hippocampus during Stress attenuated fear learning to Context A. It also prevented sensitized fear conditioning to Context B, but the subsequent sensitized fear learning seen to a cue that predicted shock was kept intact (Hersman et al., 2019). This suggests that this disrupted some components of the SEFL effect, but not the general sensitization caused by SEFL. These studies highlight the difficulty of pinpointing which brain regions are specifically involved in causing the fear sensitization effect during stress, as multiple processes (such as context encoding) are simultaneously taking place. Precisely inhibiting a target brain region in a temporal-specific manner during each shock presentation would better dissociate neural correlates of SEFL-causing stress processing.

While there are a lack of studies investigating whether lPAG is necessary for fear sensitization, as is seen in SEFL, there is evidence that lPAG sends shock-related information to

the lateral amygdala, and is necessary for fear learning (Heinricher et al., 1987; Johansen et al., 2010; Keay & Bandler, 2001; Keay & Bandler, 2015) For example, Johansen and colleagues (2010) used electrophysiology to record neuron firing in the lateral amygdala (LA) while presenting tone-shock pairings to rats. Specific populations of LA neurons fired preferentially to shock during early acquisition, and this activity decreased across trials. This indicated that the neurons were signaling information about the shock during fear learning. Inactivating the lPAG with muscimol during tone-shock pairing caused these LA neurons to no longer respond to the shock. Furthermore, rats with inhibited lPAG exhibited low freezing to the tone during test (Johansen et al., 2010). This indicates that lPAG is signaling information about the shock necessary for fear learning.

It is possible that the lPAG plays a similar role in driving non-associative learning plasticity in the amygdala. There is no direct causal link between amygdalar plasticity that causes fear sensitization and lPAG activity. However, the functional role of shock signaling neurons in lPAG, in addition to our cFos data shows that only Shock Stress causes increased expression in caudal lPAG, indicate it as a good region for further investigation. In the following experiment, we tested the necessity of lPAG activity during Stress for SEFL. We inhibited lPAG in rats in a temporally-specific manner in using optogenetics. Specifically, we briefly inhibited lPAG neurons for 2.5s over the 15 shocks administered during a Shock Stress session. Then, we examined how this impacted on the degree of fear conditioning seen to a mild foot shock in a different context (i.e. the SEFL effect).

General Methods

Subjects

We used experimentally-naïve Male Long-Evans rats (*n*=25) that were approximately 120 days old at the start of this experiment (Charles River, MA). These rats were single housed in a temperature and humidity regulated vivarium. Rats were provided food and water *ad libitum*. Rats acclimated for four weeks in the vivarium prior to the beginning of the study. Rats were each handled for 2 minutes/day of the course of the seven days directly prior to surgery. The experiments took place during the light phase of the 12:12 light/dark cycle. All procedures were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the UCLA Institutional Animal Care and Use Committee.

Surgery

Rats were anesthetized with isoflurane and treated with carprophen (5 mg/kg) and localized buprovicaine injection before being bilaterally injected with 1 µL adeno-associated virus (AAV) carrying either inhibitory halorhodopsin (pAAV-CaMKII-NpHR3.0-eGFP) or control virus without opsin (pAAV-CaMKII-eGFP) into lPAG (AP: -8.0, DV: -4.0, ML: ±1.85 at 10° towards midline). Optic fibers were bilaterally implanted at lPAG (AP: -8.0, DV: -3.5, ML: ±1.85 at 10° towards midline). Co-ordinates were determined by prior pilot studies, which indicated appropriate placement into the caudal lPAG. Rats were given 4-6 weeks to recover from surgery and to allow for sufficient time for the virus to incubate. Recovery care was based on AAALAC guidelines.

For *Experiment 1: lPAG and SEFL*, we administered shock (1mA, 1s, x15) as Stress in Context A and then fear conditioned them in Context B to test whether lPAG activity during Shock Stress was necessary for SEFL. Male rats were assigned to a 2x2 factorial with the first factor being Stress (Stress, No Stress) and the second factor being Virus (NpHR or eYFP). We used male rats as there was concern that the females in our colony were unusually small and

would struggle to maintain the optogenetic hardware. An equal number of animals were assigned to each group. Over the course of the study, 4 animals needed to be omitted (2 animals for self-removing headcaps, 2 for incorrect virus infusion placement), which produced the final sample sizes for each group (Stress NpHR= 6, Stress eYFP=5, No Stress NpHR= 6, No Stress eYFP=4). A formal post-hoc power analysis using G*Power 3.1 was conducted on the data elicited by the lPAG SEFL experiment. The average partial η^2 of our critical effects of Stress groups was ~0.85 which revealed a high estimated power (1-β, .99) with a type 1 error rate (α) below .05 with the sample size used in this study. This demonstrates that our sample size was sufficient to detect critical effects with low likelihood of type 1 (α) or type 2 (β) errors.

For *Experiment 2: lPAG and Associative Learning,* we used the rats in the No Stress NpHR and No Stress eYFP groups from *Experiment 1,* which yielded a final n of 6 and 4, respectively. We used a formal post-hoc analysis of the data elicited by the lPAG associative learning experiment using G*Power 3.1. The average partial η^2 of the critical effects of our NpHR and eYFP groups was ~.65, which revealed a high degree of power (1-β, .95) with a type 1 error rate (α) below .05. This demonstrates that our sample size was sufficient to detect critical effects with low likelihood of type 1 (α) or type 2 (β) errors.

<u>*Behavioral Procedures*</u>

Apparatus

There were two contexts, “Context A” and “Context B”. Context A took place in Med Associates conditioning chambers and recorded using BlueIris infrared cameras. The room and the chamber were unilluminated and the fans switched on to provide 65 dB uninterrupted noise. The floor grid consisted of 16 stainless steel rods (4.8 mm) 1.6 cm apart connected to a shock

scrambler. A metal pan beneath each grid was sprayed with a thin layer of diluted Windex solution (1:1 water dilution), and the walls were wiped with Windex as well. Two armored fiber optic patch cords were connected to a rotary joint dual connector (Doris Lenses, Quebec, Canada), which relayed our optic fiber to to DPSS lasers (532 nm, Shanghai Lasers and Optics Century Co., Shanghai, China). Light leakage from the laser was prevented using 5 cm long black shrink tubing to cover the connecting point of the patch cord and cannula ferrules. All equipment was controlled using MED-PC V programs and Med Associates Software. Context B took place in Med Associates Conditioning chambers and recorded using infrared cameras sent to Med Associates VideoFreeze software. Each box (30x25x25 cm) was in its own sound attenuating chamber. The room was illuminated by white 65 W bulbs and each chamber was illuminated from above by a single white house light. The fans were switched off and did not provide sound. A metal pan beneath each grid was sprayed with a thin layer of acetic acid, and the walls were wiped with acetic acid as well. A black acrylic 20x20 cm insert was placed on the upper portion of the conditioning box such that the ceiling of the box was at a 60° acute angle.

4.1 Experiment 1: lPAG and SEFL

Male rats, bilaterally infused in lPAG with either inhibitory NpHR virus (*n*=12) or a control eYFP virus (*n*=9), were assigned to receive either the Stress or No Stress in Context A (Stress NpHR= 6, Stress eYFP=5, No Stress NpHR= 6, No Stress eYFP=4 ; *n*=21 total).

On **Day 1,** rats were transported to Context A in their homecages. Session duration was 90 minutes (5400s). Stress animals experienced 15 (1s, 1mA) shocks given in a semi-random, unpredictable interval. The ITI varied between 3 and 7 minutes. No Stress animals remained in the chamber for the duration of the trail without exposure to any stressor. Green light pulses (532nm nm, 16-18 mW) were delivered during the shock, beginning 500ms prior to shock

delivery, and continuing for 2.5s. These parameters were chosen because our prior work has shown that this suppresses neural activity without producing brief inhibition that would mimic a prediction error (Chang et al., 2018; Sharpe et al., 2017). No Stress animals received light pulses during the same timepoints as Stress animals. After the 90-minute session the subjects were removed and returned to their home cages.

On **Day 2**, context fear was measured in Context A. Rats were transported in their homecages. Session duration was 8 minutes (480s) during which freezing was recorded using BlueIris software and hand scored. Freezing was defined as an alert, immobile posture with no movement besides for respiration. After the 8-minute session the subjects were removed and returned to their home cages.

On **Day 3**, rats were transported to novel Context B in their homecages, covered by a novel black plastic bag covering. After a 3-minute (180s) ITI subjects were administered 1 (1 mA, 1s) footshock. 30 seconds after termination of the stimulus, rats were removed from the chamber and returned to their home cages. Behavior was recorded and scored using MedAssociates Videofreeze software.

On **Day 4**, context fear was measured in Context B. Animals were transported to the context covered by the plastic bag. Session duration was 8 minutes (480s) during which freezing was recorded and scored using MedAssociates VideoFreeze software. After the 8-minute session the subjects were removed and returned to their home cages.

Experiment 2: lPAG and Associative Learning

Four weeks after the end of Experiment 1, the rats from the No Stress NpHR(*n*=6) or No Stress eYFP (*n*=4) condition were trained with tone-shock Pavlovian conditioning in Context A.

On **Day 1**, rats were re-exposed to Context A. Rats were transported in their homecages. Session duration was 30 minutes (1800s) during which freezing was recorded using BlueIris software and handscored. Freezing was defined as an alert, immobile posture with no movement besides for respiration. After the 30-minute session the subjects were removed and returned to their home cages.

On **Day 2,** rats were returned to Context A for tone-shock fear acquisition. Session duration was 25 minutes (1500). Three presentations of a tone cue (10s) were followed 1 second later by footshock (.6 mA, 1s) at variable ITI of 5 to 7 minutes. Green light pulses (532 nm, 16-18 mW) were delivered across each shock, beginning 500ms prior to shock onset and continuing for 2.5s. the gap between the cue and shock was to ensure that inhibition of lPAG neurons could occur during the shock and dissociated from cue presentation, as we have done previously (Chang et al., 2018; Sharpe et al., 2017). Freezing was recorded using BlueIris software and handscored. After the 25-minute session, animals were removed and returned to their homecages.

On **Day 3,** rats were placed into Context A for a context fear test. Rats were transported in their homecages. Session duration was 30 minutes (1800s) during which freezing was recorded using BlueIris software and handscored. After the 30-minute session the subjects were removed and returned to their home cages.

On **Day 4,** rats were placed into Context A for tone fear extinction test. Session duration was 37 minutes (2220s). Five presentations of tone (10s) were played at variable ITI of 5 to 7 minutes. These ITIs were all different than those used on Day 2. Freezing was recorded using BlueIris software and handscored. After the 37-minute session, animals were removed and returned to their homecages.

On **Day 5**, rats were placed into Context A for a second tone fear extinction test. Session duration was 75 minutes (4500s). Ten presentations of tone (10s) were played at variable ITI of 5 to 7 minutes. These ITIs were all different than those used on Day 2 and Day 4. Freezing was recorded using BlueIris software and handscored. After the 75-minute session, animals were removed and returned to their homecages.

Histology

All animals were deeply anesthetized with isoflurane and transcardially perfused first with phosphate buffered saline (PBS) followed by 4% paraformaldehyde (PFA) until the tissue became fixed and rigid. Brains were extracted and post-fixed in 4% PFA overnight, before being treated with a 30% sucrose solution over the course of approximately three days. 40 µm coronal slices of the brain (Interaural 0.7 mm :1.37 mm) were collected via cryostat and mounted on slides, treated with Prolong Gold mounting medium with DAPI, and cover-slipped. Slides were imaged using a Keyence BZ-X710 microscope (Keyence, El Segundo, CA), CCD camera using GFP light channel at 4x objective to verify virus infusion and optic fiber placement.

Statistics

For Context A, behavior was recorded using BlueIris software and percent time freezing was handscored. Freezing was defined as an alert, immobile posture with no movement besides for respiration. For Context B, behavioral data was extracted from VideoFreeze and processed using Microsoft Excel. Analyzed parameter was Percent Time Freezing per component.

For *Experiment 1: lPAG and SEFL,* the final sample sizes for each group were Stress NpHR= 6, Stress eYFP=5, No Stress NpHR= 6, No Stress eYFP=4.

For *Experiment 2: lPAG and associative learning,* we used the rats in the No Stress NpHR and No Stress eYFP groups from *Experiment 1.*Group sample sizes were NpHR= 6 and eYFP=4.

All significant main effects and significant interaction effects were further analyzed for simple main effects in SPSS (IBM).

Experiment 1: lPAG and SEFL

For context fear on **Day 2**, total percent time freezing in Context A was analyzed using a two-way ANOVA for the independent variables of Stress (Stress or No Stress) and Virus (NpHR or eYFP).

For baseline freezing on **Day 3**, a two-way ANOVA for Stress (Stress or No Stress) and Virus (NpHR or eYFP) was used to analyze percent time freezing during the 3-minute ITI before shock for the fear conditioning session in Context B.

For context fear on **Day 4**, when analyzing total percent time freezing in Context B, we used a two-way ANOVA for the independent variables of Stress (Stress or No Stress) and Virus (NpHR or eYFP).

Experiment 2: lPAG and Associative Learning

For context fear on **Day 1**, total percent time freezing in Context A was analyzed using a one-way ANOVA for the effect of virus (NpHR or eYFP).

For fear acquisition on **Day 2**, time freezing during each 10-second cue in Context A was analyzed using a repeated measures ANOVA for Virus (NpHR or eYFP) across tone presentations (Baseline, CS1, CS2, and CS3). Time freezing for the 30 seconds before tone

presentation was analyzed using a repeated measures ANOVA for Virus (NpHR, eYFP) across time (Pre1, Pre2, and Pre3).

For context fear on **Day 3** in Context A, we used a repeated measures ANOVA compare total percent time freezing over the 30-minute session. The session was divided into 5-minute bins and analyzed using a repeated measures ANOVA for Virus (NpHR or eYFP) across Time (Bin 1, Bin 2, Bin 3, Bin 4, Bin 5). Additionally, we used a repeated measures ANOVA to compare total percent time freezing before acquisition on DAY 1 and after acquisition on DAY 3 between Virus groups (NpHR or eYFP).

For tone extinction on **Day 4,** time freezing during each 10-second cue in Context A was analyzed using a repeated measures ANOVA for Virus (NpHR or eYFP) across tone presentations (CS1, CS2, CS3, CS4, and CS5).

For tone extinction on **Day 5,** time freezing during each 10-second cue in Context A was analyzed using a repeated measures ANOVA for Virus (NpHR or eYFP) across ten tone presentations (CS1-CS10).

Rigor and Reproducibility

All analyses were conducted using SPSS (IBM). Simple main effect analysis was used for all significant effects. For *Experiment 1: lPAG and SEFL* formal post-hoc power analysis using G*Power 3.1 was conducted on the data elicited by the lPAG SEFL experiment. The average partial η^2 of our critical effects of Stress groups was ~0.85 which revealed a high estimated power (1-β, .99) with a type 1 error rate (α) below .05 with the sample size used in this study. For *Experiment 2: lPAG and associative learning* average partial η^2 of the critical effects of our NpHR and eYFP groups was ~.65, which revealed a high degree of power (1-β, .95) with

a type 1 error rate (α) below .05. This demonstrates that our sample size was sufficient to detect critical effects with low likelihood of type 1 (α) or type 2 (β) errors. Freezing in Context B was analyzed using automated Videofreeze software. Observers were blind when scoring for freezing in Context A.

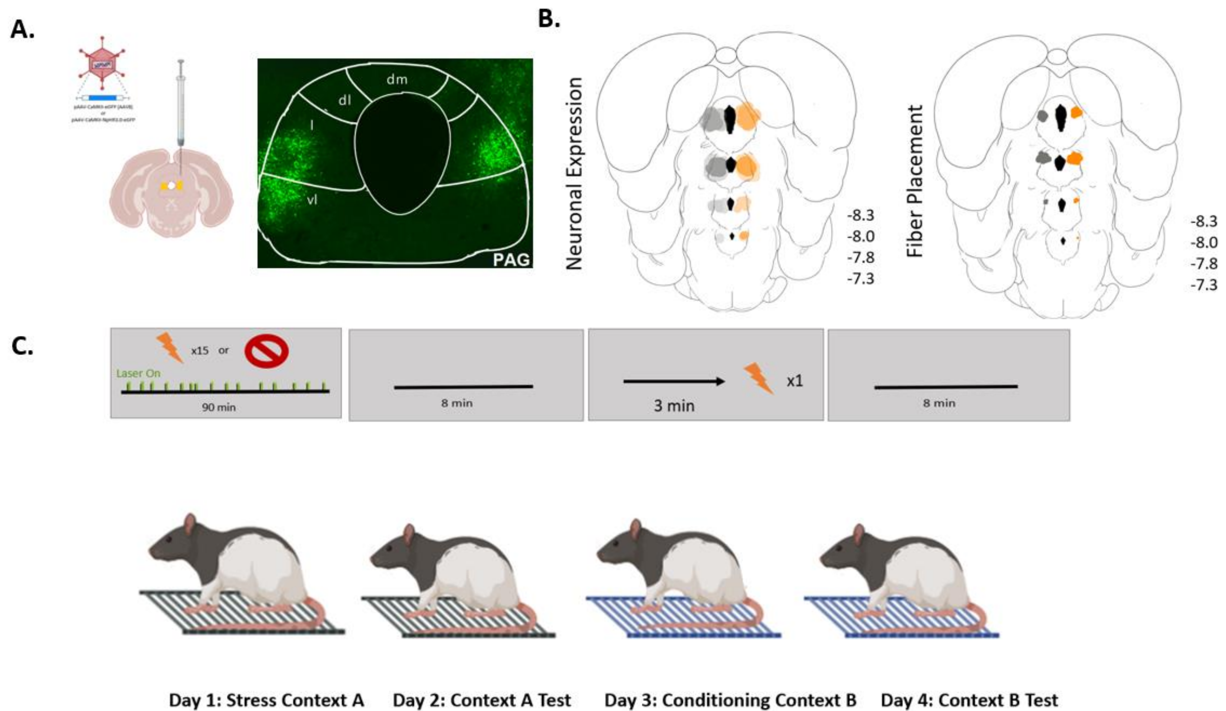

Figure 3.1. Design for inhibition of lPAG during Shock stress in the SEFL procedure. A. *Left*: Rats were injected bilaterally in lPAG with either inhibitory opsin (NpHR, n=12) or control eYFP (n=9) and then implanted with fiber optic cannula. *Right*: example of bilateral viral expression in lPAG neurons. **B.** *Left:* Unilateral representation of bilateral viral expression in lPAG for NpHR (orange) and eYFP (grey) in lPAG. *Right:* Unilateral representation of approximate bilateral fiber optic tip placement for NpHR (orange) and eYFP (grey) in lPAG. **C.** Experimental design. On Day 1, animals in Context A received Stress (x15 shocks) or No Stress. Green light was delivered for 2.5s across each shock timepoint for both Stress and No Stress groups. On Day 2, animals were returned to Context A for a context fear test. On Day 3, animals were placed in novel Context B for fear conditioning consisting of a single footshock. On Day 4, animals were placed back into Context B for a context fear test to measure SEFL.

4.1 Inhibiting lPAG During Stress Does Not Attenuate SEFL

Results:

We bilaterally injected Long Evans rats with an adeno-associated virus (AAV) carrying either inhibitory halorhodopsin (pAAV-CaMKII-NpHR3.0-eGFP, n=12) or eYFP control (pAAV-CaMKII-eGFP n=9) into the lPAG and bilaterally implanted optic fibers directly above the lPAG (*Figure 3.1A-B*). This would allow us to precisely inhibit lPAG during each shock presentation of the Stress session. Four weeks after surgery, rats underwent the SEFL procedure.

On **Day 1** in Context A, animals assigned to the Stress condition received 15 pseudorandom unpredictable footshock presentations over a 90-minute session period. No Stress animals remained in Context A for 90 minutes without receiving footshock. Green light was on for 2.5s during each footshock presentation or at the time when the foot shock was presented in no stress groups (*Figure 3.1C*).

On **Day 2**, animals were placed back into Context A for an 8-minute context fear test (*Figure 3.1C)*. Both NpHR Stress and eYFP Stress froze more than No-Stress controls (*Figure 3.2A*). A two-way ANOVA looking at the effect of Stress (Stress, No Stress) and Virus (NpHR, eYFP) found a main effect of Stress ($F_{(1,17)}$=143.173, p<.001), but no effect of Virus ($F_{(1,17)}$=.523, p=.48) or interaction between Stress and Virus ($F_{(1,17)}$=.693, p=.417). This indicates that inhibiting lPAG during Stress did not impact on fear learning to Context A.

On **Day 3**, animals were placed into novel Context B. After a 3-minute ITI, all animals received a single footshock (1mA, 1s) as fear conditioning (*Figure 3.1C).* Freezing remained low across groups before administering the shock (*Figure 3.2B*). A two-way ANOVA looking at the effect of Stress (Stress, No Stress) and Virus (NpHR, eYFP) for percent time freezing during the

3-minute baseline found no significant effects (Stress: $F_{(1,17)}$=.227, p=.639; Virus: $F_{(1,17)}$=.311, p=.584; Stress*Virus: $F_{(1,17)}$=.893, p=.356). This indicates that there was no fear generalization to Context B.

On **Day 4**, animals were placed back into Context B for an 8-minute context fear test (*Figure 3.1C)*. All shock Stress groups showed SEFL relative to our No Stress groups in Context B, and there was no impact of virus (*Figure 3.2C*). A two-way ANOVA looking at effects of Stress (Stress, No Stress) and Virus (NpHR, eYFP) found a significant main effect of Stress ($F_{(1,17)}$=7.031, p<.05), but no significant effect of virus or interaction of stress and virus (Virus: $F_{(1,17)}$= .198, p=.661; Stress*Virus: $F_{(1,17)}$=1.494, p=.237). Thus, inhibiting lPAG during Stress in Context A did not attenuate enhanced fear learning to Context B. The somewhat inflated freezing to Context B seen in eYFP controls was due to lack of movement during the last minute of the session, which is more indicative of lack of exploration than to freezing.

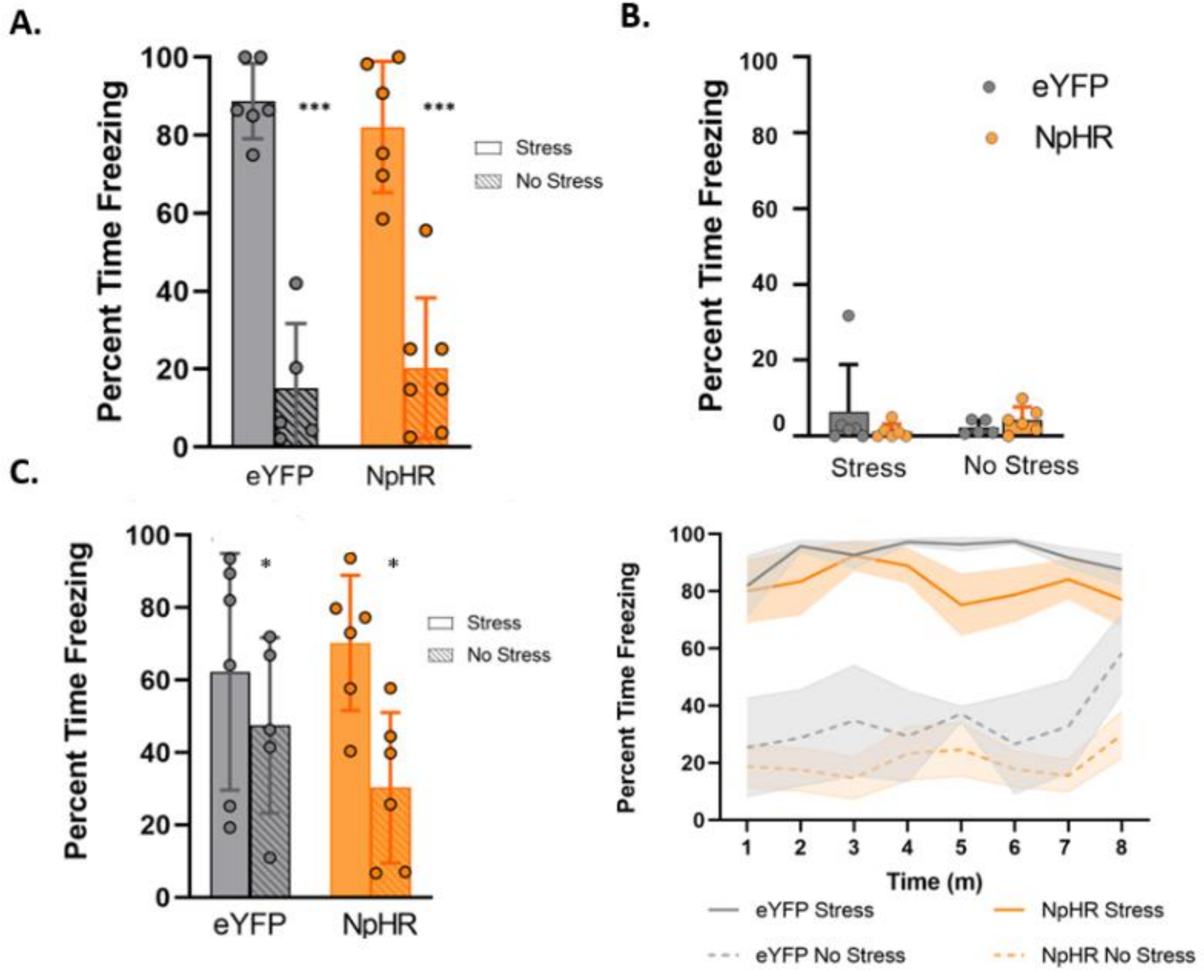

Figure 3.2: Inhibiting lPAG during Stress does not attenuate SEFL. A. On Day 2, animals were returned to Context A for a context fear test as measured by total percent time freezing. While the main effect of Stress greatly increased fear of Context A (p<.001), there was no effect of Virus, indicating that inhibiting lPAG during Stress did not inhibit fear learning of the context. **B.** On Day 3, rats were placed in novel Context B. Baseline percent time freezing in the 3 minutes prior to receiving the fear conditioning trial to rule out context fear generalization. No significant difference in freezing between groups. **C.** On Day 4, animals were placed back into Context B for a context fear test. *Left:* Total percent time freezing. There was no difference between Stress groups, indicating that inhibiting lPAG during Stress in Context A did not attenuate fear conditioning to Context B. *Right:* Percent time freezing across the session. *p<.05 *** p<.001 Error bars represent SEM.

4.2 Inhibiting lPAG During Acquisition increases Associative Learning to cues and contexts

As detailed above, inhibiting lPAG during Stress did not attentuate non-associative SEFL. This still raises the question as to why, in our cFos study, there was significantly more expression in lPAG after Shock Stress, which causes SEFL, compared to Noise Stress, which does not. The answer may relate to there being both non-associative and associative processes occurring during Stress. That is, while the sensitization occurring during SEFL is non-associative, rats are also learning that the shock occurs in Context A. Thus, it is possible that we saw an induction of cFos in the lPAG during the Shock Stress reflected these associative components rather than the non-associative sensitization processes.

During associative fear learning, a previous innocuous cue- such as a context, or a discreet stimulus such as a tone- comes to be associated with an aversive stimulus, such as a shock (LeDoux, 2000). Inputs providing information about the aversive stimulus and the cue stimulus converge in the basolateral amygdala, where learning plasticity connecting the cue and shock occurs (Bordi & LeDoux, 1994; Kim & Fanselow, 1994; LeDoux et al., 1991). There is evidence that the PAG provides information during learning which generates fear responding (Deng et al., 2016; Johansen et al., 2013; Kim et al, 2013; McNally et al., 2011; Yeh et al., 2021). Much like there is dissociable function in PAG subregions regarding innate responses to threat, so too there are differences relating to associative fear conditioning.

The dPAG is necessary for fear acquisition (Di Scala et al., 1987; Deng et al., 2016; Herry & Johansen, 2014; Kim et al., 2013; Yeh et al., 2021). For example, Yeh et al. (2021) optogenetically inhibited either dPAG or vlPAG of mice in tandem with shock during tone-shock pairings. Mice with inhibited dPAG during acquisition showed significantly less freezing to the

tone during test. Mice with inhibited vlPAG during shock did not show this same deficit in fear learning (Yeh et al., 2021). Thus, the dPAG specifically appears to be necessary for conveying information about innately aversive stimuli during learning. While there is no evidence that dPAG receives direct projections from the dorsal horn, it does receive information about aversive stimuli and threat from thalamic and hypothalamic nuclei (Gross & Canteras, 2012) which it may then signal to other regions such as amygdala.

In contrast, there is evidence that vlPAG signals information about the cue during associative learning (McNally et al., 2005; Walker et al., 2019; Wright et al., 2019; Wright & McDannald, 2010). For example, Wright and colleagues (2019) used single cell in-vivo electrophysiological recording in vlPAG while presenting three cues: a 'certain danger' cue (p=1.00) that always predicted shock, a 'certain safety' cue that always predicted no shock (p=.00), and an 'uncertain' cue that was only sometimes followed by shock (p=.375). Fear learning was measured as reward nose poke suppression during cue presentation. The rats demonstrated good discrimination between cues, as the suppression ratio score for certain danger was high, for certain safety was low, and for uncertain danger intermediate between the other two cues. If vlPAG neurons are signaling information about the probability of a cue predicting threat, then they should show the most activity to the certain danger cue. And indeed, Wright and colleagues found that this population of vlPAG neurons showed bias towards firing to the certain danger cue during cue onset. This bias towards the certain danger cue was strong, as vlPAG neurons significantly greater activity to it compared to the other two cues (Wright et al., 2019). This indicates that vlPAG signals information about which stimulus has the best certainty of predicting threat.

There is some evidence that lPAG plays a role in conveying predictive information about shock during associative learning. Johansen and colleagues (2010) used electrophysiology to record single cells in lPAG during tone-shock acquisition training in rats. A population of cells in lPAG exhibited a greater firing rate during the earliest tone-shock pairing trials. This firing rate decreased during mid- and late-acquisition stages as rats learned that the tone was a cue for shock, as exhibited by time freezing. However, these shock-responding lPAG neurons regained their original levels of robust activity when an uncued shock was presented (Johansen et al., 2010). Together, this indicates that lPAG is specifically signaling about the predictability of the shock during learning (i.e., aversive prediction errors; Johansen et al., 2010).

Johansen and colleagues (2010) had found neurons in the lateral amygdala (LA) which exhibited the same activity patterns in response to the predictability of the shock as lPAG. These LA neurons showed the highest firing rate when the shock was unpredictable, and this firing to shock decreased during mid- and late-acquisition stages as rats learned that the shock was predicted by the cue. Importantly, inactivating the lPAG with muscimol during tone-shock fear acquisition caused the shock-responsive LA neurons to stop responding to shock altogether. Furthermore, rats with lPAG inhibited by muscimol during tone-shock fear acquisition exhibited a decrement of freezing to the tone during test (Johansen et al., 2010). Together, this indicates that the LA is receiving teaching signals about the shock from lPAG necessary for fear learning. However, muscimol would have inhibited the lPAG for the entire duration of fear acquisition, when other information about cue and context were also relayed to the amygdala. Therefore, while the electrophysiological data indicates that lPAG signals predictive information about shock, it is still unknown whether this specific type of signaling is necessary for fear learning.

Thus, here we tested in activity in the lPAG is necessary for associative fear learning.

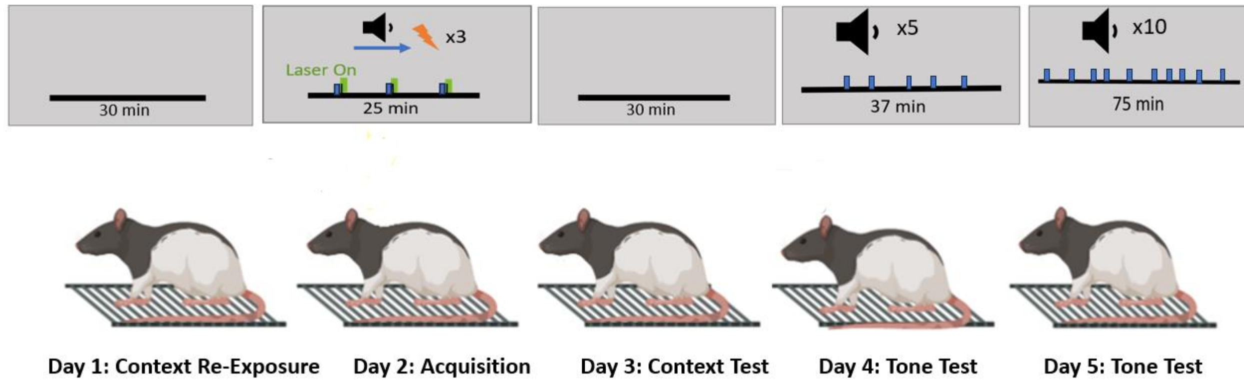

Figure 3.3. lPAG and Associative Learning Experiment. Procedure of experiment. Animals from the No Stress condition from Experiment 1 were used (NpHR=6, eYFP=4). On Day 1, animals were re-exposed to Context A. On Day 2, animals received presentation of a tone cue followed by shock (x3). Green light was delivered for 2.5s across each shock presentation. On Day 3, animals were placed back into Context A for a context fear test. On Day 4, animals received presentation of tone alone (x5) in Context A for a tone fear extinction test. On Day 5, animals again received presentation of tone alone (x10) in Context A for a second tone fear extinction test.

Results

On **Day 1**, animals were returned to Context A for 30 minutes of context re-exposure (*Figure 3.3)*. NpHR and eYFP rats froze at similarly low levels to the context (*Figure 3.4B*, 'Pre'). A one-way ANOVA found no difference between groups ($F_{(1,8)}$= .036, p=.855). While these animals did have prior experience with the single shock in Context B, this did not appear to cause generalized fear to Context A.

On **Day 2,** all rats received three tone-shock pairings. Green light was delivered for 2.5s across each footshock presentation (*Figure 3.3C)*. There was no difference in rate of fear acquisition to the tone *(Figure 3.4A*). A repeated measures ANOVA of percent time freezing during the cue looking at the effects of Virus (NpHR, eYFP) across presentations found a significant increase in freezing across the acquisition session ($F_{(3,24)}$= 29.759, p<.001), but no differences between virus groups (Virus: $F_{(1,8)}$= .155, p=.704; Virus*Time: $F_{(3,24)}$= .388, p=.763).

This indicates that there is strong evidence of fear acquisition in all rats and that inhibiting lPAG did not appear to overtly affect fear acquisition to the tone. A repeated measures ANOVA of percent time freezing before the cue looking at effects of Virus (NpHR, eYFP) across presentations found a significant increase in freezing across the acquisition sessions ($F_{(2,16)}$= 15.32 , p<.001) but no differences between Virus groups (Virus: $F_{(1,8)}$=3.248, p=.109; Virus*Time: $F_{(2,16)}$= .479, p=.628). This indicates inhibiting lPAG did not appear to affect fear acquisition to the context.

On **Day 3**, rats were returned to the context for a 30-minute context fear test (*Figure 3.3)*. NpHR animals froze to the context more than eYFP animals (*Figure 3.4B*). A repeated measures ANOVA percent time freezing to the context across five-minute bins found a significant main effect of Virus (Virus: $F_{(1,8)}$=8.951, p<.05) but there was no effect of Time ($F_{(5,40)}$)=.922, p=.477) or interaction effect between Time and Virus ($F_{(5,40)}$=.48, p=.789) (*Figure 3.4B, right*).This indicates that inhibiting lPAG caused enhanced associative contextual fear in NpHR animals. However, neither group showed fear extinction to the context over the duration of the session. A repeated measures ANOVA comparing percent time freezing to the context before and after acquisition (DAY 1, DAY 3) between virus groups (NpHR, eYFP) found more pronounced freezing in NpHR animals after acquisition on Day 3 compared to eYFP animals (Day: $F_{(1,8)}$= 35.229, p<.001; Virus: $F_{(1,8)}$= 6.271, p<.05; Day*Virus: $F_{(1,8)}$= 5.512, p<.05) (*Figure 3.4B, left*). An analysis of simple effects confirmed that this significant difference was driven by freezing on Day 3 ($F_{(1,8)}$=9.768, p<.05). This further indicates that inhibiting lPAG during shock enhanced fear of the context.

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On **Day 4**, rats received 5 presentations of unpaired tone to test fear to the cue (*Figure 3.3)*. There was no fear extinction to the tone by the end of the session (*Figure 3.4C).* A repeated measures ANOVA of percent time freezing showed no decrease in freezing across the session and no differences between groups (Time: $F_{(4,32)}$=1.561, p=.209; Virus: $F_{(1,8)}$=.091, p=.77; Time*Virus: $F_{(4,32)}$=2.343, p=.164).

Therefore, on **Day 5** rats were subsequently exposed to 10 more tone presentations in order to generate extinction (*Figure 3.3)*. During this session, we found that NpHR animals had stronger resistance to fear extinction of the tone compared to eYFP animals (*Figure 3.4C)*. A repeated measures ANOVA of percent time freezing found a significant main effect of tone presentations across the session (Time: $F_{(9,72)}$=12.52, p<.001) and main effect of virus (Virus: $F_{(1,8)}$=129.87, p<.001) such that the NpHR group froze for a longer amount of time for more tone presentations compared to the eYFP group (Time*Virus: $F_{(9,72)}$=3.945, p<.001). An analysis of simple effects reveals that while both groups froze equally to the first tone presentation (CS1*:* $F_{(1,8)}$=.096, p=.765), the eYFP group froze for significantly less time than the NpHR group by the third presentation (CS3*:* $F_{(1,8)}$=28.646, p<.001). The NpHR group froze for a significantly longer amount of time to tone up until the nineth presentation (*Fig 3.4C*, denoted by asterisks, ex CS6: $F_{(1,8)}$=16.118, p=.004). This indicates that inhibiting lPAG during acquisition caused stronger associative fear learning to the tone, which was more resistant to extinction.

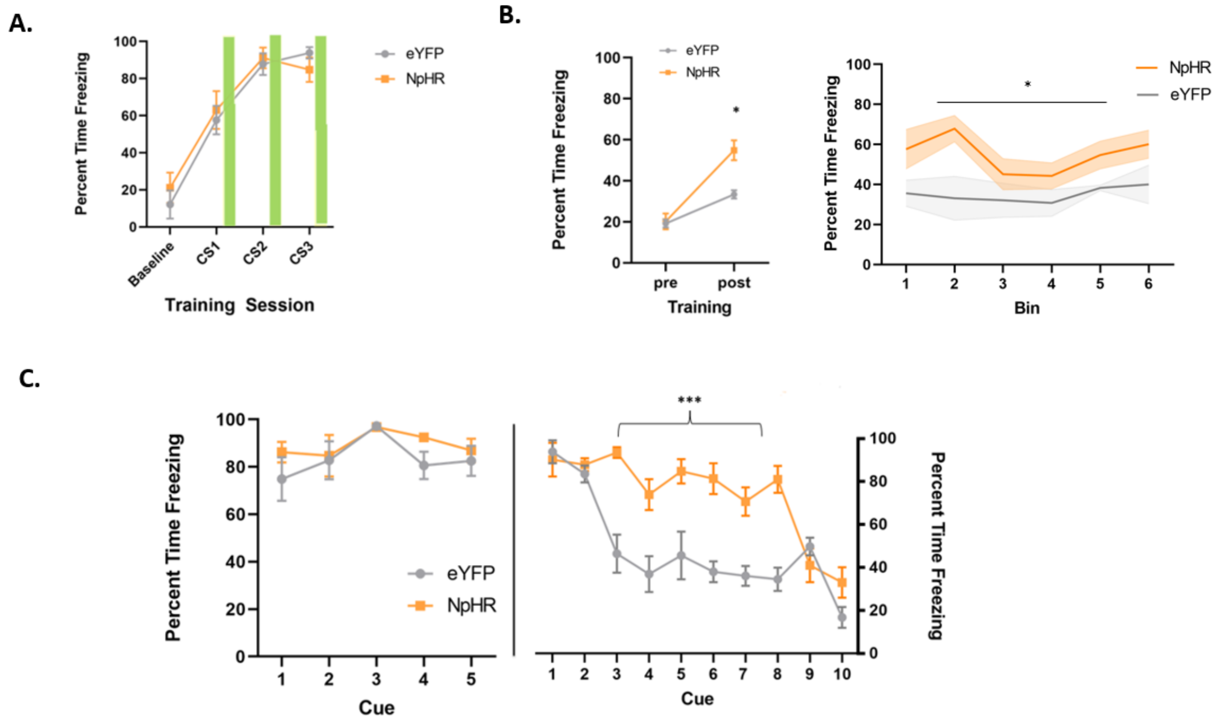

Figure 3.4: Inhibiting lPAG during enhances associative fear to the context and tone. A. On Day 2, animals received three tone-shock pairings, with light delivery inhibiting lPAG during each shock (represented by yellow bars). Fear to each tone (CS) measured by percent time freezing. Both groups showed similar rates of fear acquisition to the tone on Day 2. **B.** *Left:* Fear of the context, as measured by percent time freezing, compared between Day 1 before acquisition (pre) and Day 3 after acquisition (post). NpHR animals froze significantly more to the context (p<.05) on Day 3 compared to eYFP controls. *Right:* Percent time freezing across the session on Day 3 **C.** *Left:* On Day 4, animals were presented with tone alone (x5) for a tone fear extinction test, measured by percent time freezing. Neither group extinguished by the end of the session. *Right:* On Day 5, animals were presented with tone alone (x10) for a second tone fear extinction test. NpHR animals showed a significantly slower rate of fear extinction to the tone compared to eYFP controls (p<.001). *p<.05 ***p<.001 Error bars represent SEM.

4.3 Discussion

In this study, we sought to investigate whether activity in the lPAG was necessary during shock Stress in order for SEFL to occur. We based this assumption off of data from our cFos study which showed that Shock Stress, but not Noise Stress, caused significantly more cFos expression in caudal lPAG compared to Homecage controls. As only Shock Stress causes SEFL, we reasoned that this may have indicated that caudal lPAG is uniquely necessary for SEFL. Here, we sought to precisely inhibit lPAG with optogenetics during presentation of shock to investigate whether this would attenuate SEFL.

Our first study found that inhibiting lPAG during Shock Stress did not attenuate SEFL or result in any overt impact to context freezing seen during the SEFL procedure. Specifically, we found that rats without caudal lPAG activity during each footshock presentation still showed strong, ceiling-level freezing to Context A, where the Stress occurred (*Figure 3.2A*). Further, the NpHR Stress rats also showed enhanced fear conditioning to Context B (*Figure 3.2C*). From this, we may conclude that activity in lPAG due to the shock experienced during Shock Stress is not necessary for SEFL.

There may be several explanations for this outcome, in spite of caudal lPAG showing uniquely high cFos expression during SEFL-inducing stress. While Shock Stress causes non-associative fear sensitization, it is also supported fear learning to the context. Noise Stress did not support this same long-term fear learning. Therefore, the differences in cFos expression for Shock Stress and Noise Stress in caudal lPAG may be indicative of associative fear learning rather than the non-associative sensitization. The results from our experiment inhibiting lPAG during associative learning (*Figure 3.4B-C*) indicate that inhibiting lPAG enhances fear to both

tone and context, and are further discussed below. While we might have expected a role for the lPAG in the associative components of SEFL to be evident in the Context A fear seen to all rats in Experiment 1 (*Figure 3.2A*), as all rats were exhibiting freezing levels at ceiling, it may have been occluded in this particular procedure.

While caudal lPAG may be uniquely activated by Shock Stress, these results indicate that activity in this region is not necessary for the sensitization effect. While there is evidence that dPAG signals information about aversive stimuli to the amygdala, and is necessary for associative fear learning (Kim et al., 2013; Yeh et al., 2021) we did not find a significant increase of cFos expression in dPAG for Shock Stress. The thalamus, hypothalamus and cortex also signal to the amygdala through fear and stress-related pathways (Das et al., 2005; Gross & Canteras, 2012; Liang et al., 2020) and the pontine parabrachial nucleus (PBN) directly projects nociceptive information to amygdala, thalamus and hypothalamus in a stress pathway parallel to the PAG (Jaramillo et al., 2021; Nagase et al., 2019; Sarhan et al., 2005; Sato et al., 2015; Todd, 2010). For example, Sato et al., (2015) optogenetically stimulated PBN axon terminals in the central amygdala of mice after playing a tone cue. Stimulating PBN to central amygdala projections were sufficient to support fear learning to the tone, as demonstrated by a significant increase in freezing to the tone compared to GFP controls during test (Sato et al., 2015). This indicates that PBN activity elicits fear learning plasticity in the central amygdala, likely by signaling aversive information. It may similarly support amygdala plasticity necessary for fear sensitization. SEFL induced sensitizing plasticity of excitatory GluA1 receptors have thus far only been detected in the basolateral amygdala (Perusini et al, 2016), which the PBN does not directly project to (Nagase et al., 2019). However, this is not in itself evidence that PBN activity is not necessary for SEFL, as it may also be caused by other as-yet unknown neuroplasticity.

Activity from stress-signaling regions such as PBN during Shock Stress may have compensated for the lack of lPAG activity, and may even hint at parallel central amygdala plasticity sufficient for SEFL.

One other limitation of this study is that only males were used for this study, which may have affected results. Studies on the interaction between sex and SEFL show no difference between the sexes (Poulos et al., 2014), and we found no impact of sex when analyzing our own behavioral procedures. However, some studies show sex differences in both contextual and cued fear learning (i.e., Gresack et al., 2009; Gruene et al., 2015; Maren et al., 1994; Poulos et al., 2015; Pryce et al., 1999) in terms of both severity and behavior. Males have been particularly noted to show stronger context fear conditioning compared to females (e.g. Davui et al., 2014). This may have affected the contextual fear results seen in *Experiment 2*. Furthermore, it is important to include females in future studies to make them more applicable to human studies. This is especially important for anxiety-related studies, as females generally report higher rates of anxiety disorders (Kessler et al., 2003; Seeman, 1997).

While SEFL itself appears to be non-associative, multiple learning processes are likely to occur during the shock Stress session. Given that we did not see any involvement of lPAG in SEFL itself, it is possible that the high cFos expression seen in lPAG to Shock Stress reflected the associative components of learning that are also taking place during the Shock Stress session. As discussed above, this would reflect the work of Johansen and colleagues (2010), which demonstrated that neurons in the lPAG signal teaching information about shock prediction to the amygdala, which is necessary for learning (Johansen et al., 2010).

Therefore, we next investigated whether lPAG activity was necessary for associative fear learning. We inhibited lPAG in tandem with shock presentation during acquisition of a tone-shock association (*Figure 3.3C*). We did not find any differences between groups in the degree of freezing seen to the tone across conditioning, nor in the degree of freezing measured prior to each tone. This indicated that inhibiting lPAG did not affect fear acquisition to cue or context (*Figure 3.4A)*. This is in line with previous works studying the effects of PAG inactivation on the fear acquisition stage (Arico et al., 2017; Lowery-Gionta et al., 2018). For example, inactivating the vlPAG using Gi DREADDs during fear learning did not affect rate of acquisition, despite causing increases in context freezing during test (Arico et al., 2017). However, analyses of the freezing for context fear test and tone extinction test indicated differences in the strength of the association that developed during conditioning.

While rats without activity in the lPAG during the shock did not show a change in acquisition of fear to the tone compared to controls, we saw that this produced a more enduring fear memory to both the context and the cue. Specifically, rats in the NpHR group showed enhanced freezing to the context the following day (*Figure 3.4B*). Further, NpHR rats also showed greater resistance to fear extinction of the tone compared to eYFP controls (*Figure 3.4C*), which indicating stronger fear learning of the tone.

This enhancement of fear learning indicates that lPAG is signaling predictive information about the shock, as has been previously demonstrated in correlational studies (Johansen et al., 2010). Johansen and colleagues (2010) used electrophysiological single unit recording of lPAG cells during a tone-shock conditioning session. They found that lPAG neurons responded preferentially to shock during earlier presentations, when the shock was most surprising. As the rats learned that the tone predicts the shock, activity in the neurons decreased. Shock predicting

neurons in the lateral amygdala, a known area for learning plasticity (Maren & Quirk, 2004) similarly showed highest activity to unpredictable shock and lower activity to predicted shock. Critically, this study also revealed that pharmacologically inhibiting lPAG prevented neurons in the lateral amygdala from responding to the shock. This provides correlative evidence to suggest that the lPAG functions as an aversive prediction error to drive associative fear learning.

In this light, our data can be interpreted as disrupting prediction-error signaling during aversive conditioning, which is necessary for associative fear learning. Prediction-updating models of learning postulate that the rate of learning is highest during the earliest stages, when the oncoming aversive stimulus (e.g., the shock) is the most unpredictable. The rate of learning decreases as the predictive cue is associated with the shock- thus, the strength of the shock as a reinforcer decreases as it becomes predictable (Rescorla & Wagner, 1972). Accordingly, if the shock remains unpredictable, then its strength as a reinforcer should not decrease and learning will continue beyond asymptote. If lPAG is indeed conveying predictive information about the shock to the amygdala during learning, then inhibiting it when the shock is presented may interfere with this process. Thus, the shock would keep its strength as a reinforcer across the learning session, leading to stronger fear learning to all present elements, in this case the context and the tone cue.

Importantly, it has been shown that inactivation of lPAG with muscimol during acquisition inhibits fear learning, rather than enhances it (Johansen et al. 2010). However, pharmacological inactivation would have lasted for the duration of the entire session. In contrast, our experiments precisely inhibited lPAG during shock. Thus, in our study the prediction error to the aversive shock would be disrupted, but the acquisition of any cue-evoked response across learning could still be intact. However, in the prior study (Johansen et al., 2010) both signals

would be abolished. Together, these data suggest that the lPAG is likely necessary for both the prediction error and the cue-evoked response that develops across time and is relayed to the amygdala to generate the long-term fear memory.

In this study, we found that inhibiting lPAG enhanced associative fear learning to both context and the shock-predictive tone. We did not see this effect on SEFL when inhibiting lPAG during shock Stress, although this may be due to the Stress being severe enough on its own to induce ceiling-level freezing. This enhancement of associative fear learning may be due to the lPAG's role in sending predictive information about the shock to upstream aversive learning structures such as the amygdala. As an often overlooked region, much about the lPAG is still unknown, and future studies may follow up these findings by investigating possible projections to and from the lPAG to known aversive learning structures such as the amygdala, BNST, and hypothalamus.

Chapter 5: General Discussion

In this present dissertation, we set out to find neural correlates of Stress Enhanced Fear Learning (SEFL). Specifically, we first set out to investigate whether SEFL is non-associative by examining whether it is transmodal. From this, we were able to find a non-sensitizing stress which can be used as a control in order to examine neural substrates specifically involved in SEFL, rather than during any stress *per se.* Next, we used a cFos approach to search for regions in the periaqueductal grey (PAG) which may be specifically involved in SEFL. On the basis of our cFos results, we used optogenetics to inhibit the caudal lPAG during Stress in the SEFL procedure. Specifically, we precisely inhibited caudal lPAG during each footshock presentation. Surprisingly, we found that caudal lPAG is not necessary for this non-associative effect, but rather, contributed in critical ways to associative fear learning.

5.1 Summary of Findings

We first demonstrated that the SEFL to fear conditioning in Context B is transmodal. While there is a proliferation of evidence that SEFL is non-associative sensitization (Amir & Fanselow, 2011; Hassien et al., 2020; Long & Fanselow, 2010; Nishimura et al., 2022; Poulos et al., 2014; Rau et al., 2005; Rau et al., 2009), we specifically investigated whether Stress in Context A caused a sensitizing effect to different types of stimuli used for fear conditioning in Context B. We used shock for Stress in Context A and compared the effects of using one shock, one noise burst, or two noise bursts for fear conditioning in Context B. We found that the stress sensitization effect was transmodal, as stressed rats showed significantly more fear conditioning to a single white noise burst in Context B compared to no-stress controls. Curiously, stressed rats

did not show SEFL to two white noise bursts. We speculated that this may be indicative that the noise as a stressor did not have a sensitizing effect.

Noise Stress as a Non-Sensitizing Control

To further investigate potential sensitizing effects of noise, we used footshock ("Shock Stress"; 1 mA, 1s, x15) or white noise ("Noise Stress"; 120 dB, 1s, x15) as stress in Context A. Only Shock Stress animals froze in Context A when tested for context fear 24 hours after stress. Therefore, Shock Stress, but not Noise Stress, supported fear conditioning to Context A. Furthermore, Shock Stress, but not Noise Stress, produced enhanced fear learning (i.e., SEFL) to Context B. Specifically, rats that received Shock Stress showed enhanced fear conditioning to one shock in Context B, while rats that received Noise Stress did not freeze more than no-stress controls when conditioned with one noise in Context B. Therefore, only Shock Stress was significantly stressful enough to cause both associative fear learning and non-associative fear sensitization. In order to find potential causes for this, we analyzed behavioral response to shock and noise during the stress session.

We found that over the course of the stress session, Noise Stress was able to elicit a comparable level of freezing to Shock Stress. This indicated that Noise Stress was still stressful enough to cause fear. As Noise Stress did not support fear conditioning to Context A, this observed fear is likely an innate response to aversive stimuli, rather than evidence of associative fear learning. Taken together with the results that Noise Stress did not support SEFL in Context B, this indicates that Noise Stress does not induce long-lasting plasticity. Noise Stress could therefore be used as a type of control to further investigate neural processes that specifically occur during significantly stressful, SEFL-inducing events. That is, we could now differentiate

between freezing *per se* (produced by both Noise Stress and Shock Stress), from the more severe stress that is produced by the Shock Stress condition that produces SEFL.

One other difference found between Shock Stress and Noise Stress is that only Shock Stress was able to elicit activity bursts. According to the Predatory Imminence Continuum, freezing and activity bursts correspond to different defensive and emotional states (Fanselow & Lester, 1988). Freezing is a post-encounter behavior enacted to avoid detection by a nearby predator (Fanselow, 2022). Activity burst, like flight and jumping, is a circa-strike behavior triggered as a means to escape a proximal predator (Fanselow & Lester, 1988). We postulated that the stress must push the animal to the most extreme circa-strike state, as indicated by the escape behavior, in order for the strong, long-lasting fear sensitization effect seen in SEFL to occur. As such, Shock Stress should be exciting escape-mediating brain regions more than Noise Stress.

The Caudal lPAG Shows Unique Differences In cFos Expression

The periaqueductal grey (PAG) is a midbrain region that mediates both freezing and escape, and these two states are regulated in separate columnar subregions (Bandler, 1982; Bandler & Depaulis, 1988; Blanchard et al., 1981; Di Scala, 1984; LeDoux et al. 1988).

The dPAG mediates escape (Deng, et al., 2016; Di Scala et al., 1987, Evans et al., 2018; Kim et al, 2013; Schenberg et al., 1990; Viana et al, 2001a). For example, Evans and colleagues (Evans et al., 2018) reliably caused flight by optogenetically exciting glutamatergic dPAG cells. When exposed to a looming disk stimulus meant to mimic a swooping predator, mice freeze when the disk is far away and flee when the disk appears to be close. When optogenetically inhibiting dPAG cells, mice froze to the close looming stimulus instead switching to flight

(Evans et al., 2018). Taken together, this indicates that dPAG is both necessary and sufficient for escape, with lack of dPAG activity during a perceived danger causing a takeover by regions that cause freezing.

Activating lPAG is also sufficient for escape (Assareh et al., 2016; Bittencourt et al., 2004; Di Scala et al., 1987). For example, Assareh and colleagues (2016) optogenetically excited cells in lPAG or vlPAG and analyzed behavior that occurred during the 10-second stimulation onset. During the duration of high frequency lPAG stimulation, rats showed an immediate escape response. In comparison, strongly stimulating the vlPAG only caused freezing (Assareh et al., 2016). What this may indicate, along with dPAG manipulation studies (eg, Evans et al., 2018), is that a stimulus that elicits escape behavior, such as shock, is able to activate dPAG and lPAG. However, once these regions are no longer excited past a threshold, the behavioral response shifts to freezing, which is caused by vlPAG activity.

Other studies corroborate that vlPAG mediates freezing (Assareh et al., 2016; Bittencourt et al., 2005; Tovote et al, 2016; Viana et al., 2001b). Tovote et al. (2016) optogenetically stimulated excitatory glutamatergic vlPAG cells in mice. They found that stimulating these cells caused immediate freezing. Tovote and colleagues next optogenetically inhibited glutamatergic cells in vlPAG while playing a shock-predicting tone cue. They found that inhibiting these cells reduced freezing to both the tone and context that the mice were fear conditioned in. Importantly, photoinhibition also dramatically reduced freezing to an oncoming looming stimulus meant to evoke fear of a swooping predator (Tovote et al., 2016). This is evidence that vlPAG is necessary for both innate freezing as a defense response and conditional freezing to a shock-signaling cue. As both Shock Stress and Noise Stress caused freezing, we expected to see indication of

significantly more activity in vlPAG of both of these stress groups compared to a no-stress control group.

We compared cFos expression in PAG subregions (dmPAG, dlPAG, lPAG and vlPAG) after administering Shock Stress, Noise Stress (non-sensitizing stress control) or keeping animals in Homecage (no-stress control). We found that in *central* lPAG and vlPAG, cFos expression showed a graded increase dependent on the strength of the stress. Shock Stress elicited the most expression, followed by Noise Stress, then Homecage. This suggests that both central lPAG and vlPAG are involved in behavior induced by Shock Stress and Noise Stress. Surprisingly, we observed the least amount of expression in dorsal PAG regions, in spite of its known role in signaling aversive information about stimuli.

In contrast, we found that in *caudal* lPAG, Shock Stress, but not Noise Stress, caused significantly more expression compared to Homecage. Caudal lPAG may be recruited only during stress that causes SEFL. That is, caudal lPAG activity may therefore be uniquely necessary for this stress-induced sensitization and only activated by the most severe stress that produced SEFL. Indeed, there is evidence that central lPAG and caudal lPAG serve different functions. Caudal lPAG specifically seems to mediate escape (Bandler et al., 2000; Keay & Bandler, 2001). For example, Bandler and colleagues (2000) injected excitatory amino acid into caudal lPAG or rostral lPAG of male rats to chemically stimulate these regions. Chemically stimulating rostral lPAG caused aggressive behaviors a male rat might exhibit towards an intruder rat, such as rearing in an upright position with front paws making “boxing” motions. Chemically exciting caudal lPAG caused flight (Bandlet et al., 2000). This reflects our own results which show that flight-inducing Shock Stress specifically recruited caudal lPAG.

Inhibiting Caudal lPAG Does Not Attenuate SEFL

In order to investigate caudal lPAG's necessity for SEFL, we bilaterally injected either an inhibitory halorhodopsin virus (NpHR) or a control virus (eYFP) into this region. Rats in the Stress condition received unsignaled footshocks, with green light inhibition being delivered across each shock. Rats in the No Stress control condition received light stimulation at the same semirandom intervals as rats in the Stress condition. When tested for fear learning in Context A, both NpHR Stress rats and eYFP Stress rats froze at ceiling level, indicating that inhibiting lPAG during shock did not attenuate associative context fear conditioning.

All animals were fear conditioned in novel Context B using a single footshock, and tested for fear learning to Context B 24 hours later, as has been described in other works (eg, Rau et al., 2005). If lPAG activation by Shock Stress is necessary for SEFL, then inhibiting it during shock should attenuate SEFL to Context B. Surprisingly, SEFL was not attenuated. Specifically, Stressed animals froze at similar high rates in Context B during the context fear test regardless of virus. Our results indicated that caudal lPAG activity is not necessary for this non-associative fear sensitization.

Inhibiting Caudal lPAG Affected Associative Fear Learning

During the Stress session, two processes took place: the non-associative sensitization effects that cause SEFL, and associative fear learning to Context A. As detailed above, we noted during our experiment comparing Shock Stress and Noise Stress that Noise Stress did not cause fear conditioning to Context A. This could explain the results of our cFos study, which showed that only Shock Stress showed significantly more cFos expression in caudal lPAG compared to the Homecage group. In our previous experiment using optogenetic inhibition, inhibiting caudal

lPAG during Shock Stress did not appear to affect fear learning to Context A. However, stressed animals froze at ceiling level in Context A, which was likely due to the severity of the stress. We might observe differences when using a less extreme fear learning paradigm.

There is evidence that lPAG activity affects associative fear learning. For example, Johansen et al. (2010) used single unit neuronal recordings in rats to test if lPAG neurons responsive to shock during the tone-shock fear acquisition stage. Importantly, lPAG neurons showed higher response during early trials, when the shock was surprising, compared to later trials when rats learned that the shock could be predicted by the tone cue. This indicated that lPAG were specifically signaling predictive information about the shock. Johansen and colleagues (2010) compared activity of these shock-responsive lPAG neurons to shock responsive neurons in the lateral amygdala (LA), a known site of fear learning plasticity (Maren & Quirk, 2004). They found that during fear acquisition, shock responsive lPAG neurons and LA neurons behaved in an identical manner, with robust signaling during early fear learning, when the shock was less predictable due to not yet having strong association to the tone cue. Firing rate to shock decreased during later stages of fear acquisition, as the rats reached freezing asymptote to the tone. That is, these neurons responded less to shock preceded by a learned cue. This decrease in response was not due to habituation, as presenting an unsignaled shock restored robust activity (Johansen et al., 2010). This indicates that both lPAG and LA were signaling information about the predictability of the shock. The lPAG receives nociceptive information about shock via direct projections from the dorsal horn (Bandler & Keay, 2015). It is therefore possible that lPAG is upstream from LA in signaling this information.

Johansen and colleagues (2010) provided further evidence that lPAG signals shock prediction information to LA during learning. They inhibited lPAG with microinjections of

muscimol during tone-shock fear acquisition while recording neuron firing activity in the LA. Inhibiting lPAG with muscimol caused shock-responding LA neurons to be unresponsive to both predictable and unpredictable shock, and prevented fear learning to the tone (Johansen et al., 2010). However, muscimol has a long-lasting effect, meaning that lPAG was inactive during presentation of both the tone cue and the shock during fear learning. Furthermore, while the majority of injection sites were in lPAG, or right along the border of lPAG and vlPAG (Johansen et al., 2010) muscimol has a tendency to travel upward through the infusion lines. There is therefore a very strong possibility that both lPAG and dPAG were inactivated. We used a more precise method by optogenetically inhibiting lPAG only during each shock presentation. If lPAG sends aversive information about the shock, as dPAG does, then inhibiting lPAG should attenuate fear learning to the tone.

To test the role of lPAG in associative fear learning, we paired a tone cue with shock. Rats received three presentations of tone with shock, with green light inhibition presented across each shock. We found that NpHR rats froze for significantly longer than eYFP controls during the context fear test. Additionally, NpHR rats took longer to extinguish fear to the tone cue compared to control eYFP rats. This indicated that inhibiting lPAG enhanced fear learning to the context and caused stronger associative fear learning to the tone, as indicated by resistance to extinction.

This fear enhancement due to inhibiting lPAG during shock differs from the results of studies which optogenetically inhibit dPAG during shock. For example, Yeh et al. (2021) optogenetically inhibited either dPAG or vlPAG of mice in tandem with shock during tone-shock pairing, much as we have done here. Mice received light pulses during the shock, after the shock (‘Offset’ control) or during the shock with non-inhibitory virus (GFP control). Mice with

inhibited dPAG during shock showed less freezing to the tone compared to GFP virus controls and Offset controls during extinction training. In contrast, inhibiting vlPAG had no effect on fear acquisition (Yeh et al., 2021). Therefore, while dPAG and lPAG may share behavioral function overlap by both causing escape behavior, they have dissociated roles during fear learning.

The lPAG's role in an Analgesic Negative Feedback Learning Circuit: Signaling Positive Prediction Error

In the Rescorla-Wagner behavioral model, learning will only occur when there is a discrepancy in what is predicted (based on a cue such as a tone) and actual outcome (such as shock) (Rescorla & Wagner, 1972). Freezing behavior across trials during fear acquisition reaching asymptote is demonstrative of this; once the shock becomes completely predictable by the tone cue, there is no discrepancy between prediction and outcome, and therefore no more increase in fear learning. Johansen et al. (2010) provided evidence for this prediction-dependent plasticity by recording neurons in the lateral amygdala (LA) during fear learning to a discreet tone cue. Shock-responding LA neurons showed the most activity during early acquisition when the cue was not yet learned. During subsequent trials over fear acquisition training, as the rats showed increased freezing to the tone, these LA neurons expressed a decrease in firing rate. The neurons showed increased activity again to an unsignaled shock. Therefore, these LA neurons were not as excited by the shock once it became predictable (Johansen, 2010). Too little excitation in these cells during late-stage fear acquisition likely prevents any further learning plasticity.

The Rescorla-Wagner model states that amount of fear learning is also determined by the perceived strength of the aversive stimulus (Rescorla & Wagner, 1972). In the case of shock, this

translates as nociception. Early theories postulated that the strength of shock as a learning modifier decreases in proportion to its predictability due to the shock-predicting cue (such as tone) triggering a pre-emptive analgesic response (Bolles & Fanselow, 1980; Fanselow, et al., 1994; Young & Fanselow, 1992). Young and Fanselow (1992) demonstrated that the teaching strength of shock is reliant on perceived nociception. Rats were injected with opioid antagonist naloxone or saline and placed in a context they had been fear conditioned to using low-strength shock. Therefore, while the rats had learned that they would experience shock in the context, the learning asymptote was quite low. Administering a higher-intensity shock should increase fear learning to the context, should there be no interfering analgesic processes elicited by the context. Rats injected with saline showed attenuated fear learning to subsequent mid-strength shock, while naloxone-injected rats showed a higher rate of fear conditioning (Young & Fanselow, 1992). This indicated that conditional analgesia weakens fear learning to shock, and that perceived strength of the shock as a learning modifier is dampened by opioid-dependent processes.

The vlPAG mediates conditional responses to shock-predicting cues, one of which is analgesia (Fanselow, 1994). This vlPAG-controlled conditional analgesia modifies the perceived strength of the shock at the as part of a sensory negative feedback loop during learning (Cole & McNally, 2007; Herry & Johansen, 2014; McNally & Cole, 2006; McNally et al., 2011). For example, blocking experiments are meant to exemplify the effect of expectation on new associative learning. If a learned shock-predicting cue A is paired with novel cue B during a second stage of fear acquisition, rats will not show new fear learning to cue B when cue B is tested on its own. Because A is already a cue for shock, there is no discrepancy between prediction and outcome when A and B are presented together. Therefore, no new learning can

occur. Using the predictive negative feedback model, this may be explained as cue A evoking an analgesic response to weaken the shock, thereby preventing the necessary excitation in lateral amygdala to support learning to cue B. McNally and Cole (2006) found evidence that this analgesic process is regulated at the level of vlPAG. Rats injected with opioid receptor antagonist CTAP in vlPAG during the second stage of blocking, when learned cue A is paired with cue B, exhibited freezing to cue B alone (McNally and Cole, 2006). This indicates that the vlPAG modifies the strength of the shock signal in response to the cue via analgesic pathways, attenuating fear learning.

The vlPAG receives signals from projections in the central amygdala to initiate preparatory response to the shock-predicting cue (Ciocchi et al., 2010; LeDoux, 2000; Ozawa et al., 2017; Tovote et al., 2016). Ozawa et al. (2017) confirmed that a CeA-vlPAG pathway activated by a shock-predicting cue contributed to the negative feedback loop during learning. Ozawa and colleagues (2017) used in-vivo single cell recordings in mice of shock-predicting neurons in the LA to confirm that these cells were most active during early learning, when the shock was surprising. They then optogenetically inhibited CeA terminals that projected to cells in the vlPAG while training an already learned tone-shock pairing. If these analgesic vlPAG cells activated antinoiception in response to a learned aversive cue, then inhibiting them should inhibit the conditioned analgesic response that weakens the aversive strength of shock. The shock should then, in turn, register to LA cells as “surprising” and restore their activity. Ozawa and colleagues found this to be the case. Furthermore, by restoring the shock to its full “surprising” strength due to CeA-vlPAG inhibition, rats showed enhanced fear learning to a cue that had already reached fear asymptote (Ozawa et al., 2017). This indicates that once predictive information about a cue is learned, the vlPAG is a part of an inhibitory feedback loop that

weakens further learning to shock. The changes in learning to tone-shock during late acquisition would be incredibly small as a result, as is modelled by Rescorla and Wagner (1972). Importantly, Ozawa and colleagues (2017) also recorded cell activity in lPAG when optogenetically inhibiting this CeA-vlPAG pathway during learned tone-shock presentations. Shock-predicting lPAG neurons, like the LA neurons, showed an increase in activity in response to the shock as if it were surprising when the CeA-vlPAG pathway was inhibited (Ozawa et al., 2017).

The lPAG is upstream from the amygdala, receiving nociceptive information about the shock (Keay & Bandler, 2001; Keay & Bandler, 2015). Keay and Bandler (2001) demonstrated that PAG has subregion sensory specificity by exposing rats to a variety of aversive stimuli, including formalin-induced muscle pain, visceral pain by means of 5HT injection, cutaneous neck clipping, and radiant heat. Keay and Bandler stained for cFos in the PAG to compare expression between subregions. Stimuli that caused cutaneous pain caused high expression in the lPAG. In comparison, visceral and muscular pain caused high expression in vlPAG (Keay & Bandler, 2001). Therefore, while both vlPAG and lPAG process sensory afferents, this is consistent with lPAG receiving pain information, like shock. Together with the results from Ozawa et al., (2017), this demonstrates that the lPAG is receiving input about the changing magnitude of the shock during fear learning. It is therefore responding with prediction teaching signals.

The lPAG is transmitting these prediction teaching signals to the lateral amygdala. Johansen and colleagues (2010) compared activity of these shock-responsive lPAG cells to shock responsive cells in LA using in vivo single cell recording in rats. They found that during fear acquisition, shock responsive lPAG cells and LA cells behaved in an identical manner, with

robust signaling during early fear learning, when the shock was less predictable. During late stage acquisition, when the tone was a learned cue for the shock, lPAG showed decreased response to the shock. Importantly, Johansen et al. (2010) further demonstrated inhibiting lPAG with muscimol caused these LA cells to be unresponsive to both predictable and unpredictable shock (Johansen et al., 2010). This indicates that the LA, a known site of learning plasticity (Maren & Quirk, 2004) is receiving these prediction teaching signals from the lPAG to update learning.

Our results showing that inhibition of lPAG during shock presentations caused stronger fear learning to the tone and context supports the notion that the lPAG is involved in signaling of aversive prediction errors. During the first tone-shock pairing, the tone would not have elicited a pre-emptive analgesic response for the shock. This signaling about diminishment in the shock's aversive strength would only come during subsequent pairings. Learning during the first tone-shock pairing can be driven by other regions of PAG, which is likely due to dPAG signaling aversive information about the shock to the amygdala (Di Scala et al., 1987; Kim et al., 2013; Yeh et al., 2021). However, after the first tone-shock pairing, the lPAG would usually evoke a diminished signal to the shock as it now becomes expected by the tone. Thus, our inhibition of lPAG during the shock would disrupt this prediction error process, and prevent the decline in the efficacy of the shock to support fear learning. As a result, learning would be increased to all available stimuli, which would produce the enhancement in fear learning to the context and the tone that we have saw in our experiment.

We are therefore hypothesizing that lPAG signals predictive information during learning by calculating how well the rat has learned that particular stimuli (like a tone) predicts the shock. The dPAG (Kim et al., 2013; Yeh et al., 2021) signals aversive information about the shock to the

lPAG. The vlPAG, as part of an inhibitory analgesic feedback loop with CeA, signals the efficacy of the tone predicting the shock. This was demonstrated by Ozawa et al (2017) preventing the decrease in perceived potency of the shock across learning by silencing the CeA-vlPAG pathway. Further, silencing the CeA-vlPAG pathway caused shock-responding lPAG neurons to show an increase in firing, which drove further fear learning to the tone cue (Ozawa et al, 2017). This is consistent with our idea that the lPAG is calculating prediction error by being a point of convergence for which to integrate information about shock and cue from dPAG and vlPAG and therefore drives associative plasticity in BLA during learning.

We have demonstrated in our own data that only stimuli capable of driving associative fear learning (here, shock) was able to uniquely drive cFos expression in caudal lPAG. In our procedures, noise was sufficient to drive freezing but not learning about the context. This may have been why we saw selective cFos expression in caudal lPAG to shock and not noise stress. That is, it may have revealed the role of caudal lPAG in associative fear learning, rather than SEFL as was originally hypothesised. Further, our finding that inhibition of this region during shock enhanced, rather than attenuated learning indicates the integration of cue- and shock-related information to lPAG during learning, likely via dPAG and vlPAG.

Concluding remarks

Our results are indicative of a dissociation between associative and non-associative learning processes which occur during SEFL-producing Stress. The results of our cFos study implicated caudal lPAG as being uniquely active during SEFL-inducing Stress. However, the results of our optogenetic inhibition experiments indicate that lPAG activity affects BLA learning plasticity, but it does not affect the BLA sensitization which supports SEFL. If it did, then

inhibiting lPAG during shock should have enhanced the SEFL effect. However, we observed that the Stressed rats with inhibited lPAG did not demonstrate higher fear conditioning to Context B compared to Stressed control rats. Previous literature has demonstrated that weakening or preventing associative learning during Stress does not attenuate the SEFL effect (e.g., Amir & Fanselow, 2011; Hassien et al., 2020; Long & Fanselow, 2010). Here, we demonstrate a "double dissociation" by providing evidence that reinforcing associative learning does not strengthen the SEFL effect. In addition to further demonstrating that SEFL is not reliant on recalling the associative representation of Context A, this also indicates that processes supporting learning plasticity between tone and shock in BLA do not necessarily recruit the neural substrates of fear sensitization within the same brain region. That is, lPAG inhibition affected the updating of predictive signals about the aversive shock, but this did not facilitate further upregulation of excitatory receptors in BLA that cause SEFL. Together, our results indicate two processes occurring in BLA: one of which generates the specific associations in order to best prepare for oncoming stress, and the other which generates non-selective sensitized responses in order to enhance the learning of cues to future, unknown stress. While both processes are reliant on the strength of the stressor, only the former is reliant on predictive signaling of the stress from lPAG.

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