Thinking outside the conditioning box: ethological paradigms for studying fear, anxiety and risky decision-making in rodents

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Abstract

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Traditional behavioral neuroscience paradigms assessing fear, anxiety and risky decision-making in rodents typically utilize restrictive environments that severely limit the animal's behavioral repertoire. Additionally, such paradigms take a hyper-focused approach by studying few behavioral variables over brief periods, effectively producing "snapshots" of neurobehavioral phenomena. Finally, these paradigms largely ignore animals' innate fear and anxiety-related behavior toward non pain-inducing environmental stimuli and instead commonly rely on conditioned stimuli to instigate defensive behavior. Ethological paradigms of fear, anxiety and risky decision-making, which seek to engender naturalistic scenarios within a laboratory setting, rectify many of these limitations by providing goal-directed tasks in more permissive environments, examining both innate and conditioned fear/anxiety-related behavior, studying the brain and behavior across a wider timescale and by studying many variables (a

holistic approach). This dissertation presents specific experiments conducted in ethological paradigms of fear, anxiety and risky decision-making in both rats and mice that exemplify the utility and advantages of ethological paradigms as a whole. First introduced is the longitudinal, semi-naturalistic "Risky Closed Economy" paradigm (RCE). The first study details how the RCE can be used to comprehensively investigate the impact of chronic threat on day-to-day behavior in mice. The following study conducted in rats shows how the holistic approach afforded by the RCE can facilitate interpretation of nuclei function; specifically, it is shown that the lateral habenula participates in appetitive behavior involving natural reinforcers (food pellets) and not approach-avoidance conflict involving chronic, unpredictable threat. Lastly, the "Approach Food-Avoid Predator" paradigm (AFAP) is introduced; an ethological foraging paradigm that simulates predator-prey interaction. The final study covered shows how the AFAP produces a unique neural signature of fear and risky decision-making in mice relative to standard fear conditioning and innate fear paradigms lacking naturalistic approach-avoidance conflict.

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Chapter 1. Background and Introduction

1.1 Traditional Paradigms of Fear, Anxiety and Risky Decision-making

Traditional rodent paradigms implementing threat have been used to understand a variety of processes, including defensive behavior, learning and decision-making. Perhaps the oldest and well-known threat-based paradigm is classical, or "Pavlovian" fear conditioning (Pavlov, 1927). In the standard "delay" fear conditioning procedure, the subject receives a presentation of a neutral stimulus (conditioned stimulus; CS) that co-terminates with a subsequent aversive stimulus (unconditioned stimulus; US). The US—typically an electric shock delivered to the animals paws via a shock-grid floor—reliably elicits a reflexive defensive response (unconditioned response; UR). After the animal has learned the association between the CS and US from one or more pairings, presentation of the CS alone elicits defensive behavior. This CS-alone evoked defensive response is called the "conditioned response" (CR). Traditional rodent fear conditioning studies are conducted in small, enclosed chambers. In this setting, the predominate CR is "freezing," a defensive behavior in rodents defined as the lack of movement except for respiration (M. S. Fanselow, 1980). Using this reductionist paradigm, researchers have made great progress in understanding the molecular and neural circuit dynamics of learned fear and its importance in psychology and neuroscience is well deserved.

Avoidance learning paradigms expand upon standard fear conditioning protocols, implementing both a classical conditioning and operant conditioning component. In the most common form, avoidance learning begins with CS and shock US pairings, as in fear conditioning. Unlike fear conditioning, the apparatus in avoidance learning provides the animal with a means of either escaping the US (terminating the shock after it has begun) or avoiding the US altogether by performing a correct response upon CS presentation. In active avoidance

paradigms, animals are required to *emit* a response to escape or avoid the US. A prominent example of this procedure is the Shuttle Box paradigm, wherein animals are required scale a small barrier upon CS presentation to escape or avoid footshock US (Warner, 1932).

In inhibitory (passive) avoidance paradigms, animals are required to *omit* a response to escape/avoid the US. A commonly used inhibitory avoidance paradigm is the Step-Through task (Jarvik & Kopp, 1967). In this task, the apparatus consists of two compartments separated by a door: a brightly lit compartment and a darkly lit compartment. Animals are first placed into the brightly lit compartment, which is mildly aversive. After some elapsed time, the door separating the compartments open and the animal is free to enter the darker (preferred) compartment. Once the animal enters the darker compartment, the door closes and shortly thereafter the animal receives an inescapable footshock US. In this way, the context of the dark compartment becomes the CS which is associated with the footshock US. During the test phase, the animal is placed back into the brightly lit compartment with the door open and must withhold movement into the darkly lit compartment. Freezing during the test is rarely observed in Step-Through inhibitory avoidance and the contextual CS/US association can be acquired in one trial (Ögren & Stiedl, 2010). Avoidance during the test phase is associated with an anxiety-like state (J. W. Deakin & F. G. Graeff, 1991). Using avoidance learning paradigms, distinct circuits apart from those involved in classical conditioning have been implicated in the generation of defensive behavior (LeDoux et al., 2017).

Approach-avoidance conflict paradigms integrate classical and operant conditioning principles as in avoidance learning, but more closely resemble a naturalistic situation by pitting the animal's drives to explore and obtain sustenance with its drive to minimize exposure to threat (Lima & Dill, 1990). This latter feature allows for a more nuanced assessment of decision-

making under risk, or "risky decision-making." Different approach-avoidance conflict paradigms examine conflict between approach behavior toward an incentive and either conditioned or unconditioned (innate) defensive behavior. Rodents and other organisms reflexively exhibit innate, or "instinctive," fear and anxiety toward certain evolutionarily reliable signals of danger without having to first learn through previous experience—as required in classical conditioning—that such stimuli predict danger (Blanchard & Blanchard, 1989b; Kim & Jung, 2018).

An example of an approach-avoidance conflict paradigm investigating conditioned defensive behavior is the Vogel Conflict Test. In this paradigm, water deprived animals are placed into an enclosed chamber containing a sipping tube dispensing water and after every 20 licks the animal is punished with a mouth shock delivered from the tube (Vogel et al., 1971). Similarly, in the Geller-Seifter Conflict Test (Geller et al., 1962), food-deprived animals trained to lever press in operant chambers for food receive a shock if they press during the presence of a tone. The degree of approach/avoidance in gauged by the amount of shocks received from the tube or the lever response rate, respectively. Like the Geller-Seifter Conflict Test, the Conditioned Suppression task involves hungry animals lever pressing for food, but intermittent presentations of a shock-associated CS (followed by a subsequent shock) are presented and response rates examined (Estes & Skinner, 1941).

The Elevated Plus Maze is a popular approach-avoidance conflict task that relies on rodent's drive to explore new environments and innate aversion toward open, bright spaces and heights, as opposed to conditioned defensive behavior (Handley & Mithani, 1984). Animals are placed in the center of a plus-shaped maze elevated above the ground that contains two arms enclosed by walls and two arms without walls. The ratio of approach to avoidance is

operationalized through the number of entries/time spent in the open arms versus the enclosed arms. The innate avoidance response of rodents in this task has been linked to psychological construct of innate anxiety, primarily driven by findings that show anxiolytic drugs reduce the time spent in the enclosed arms (Walf & Frye, 2007). Like avoidance tasks that provide the animal with a safe location, such as the Step-Through task, "stretched attend posture" can be observed in the Elevated Plus Maze. Stretched attend posture is a believed to be a risk-assessment behavior (Blanchard et al., 1991; Grant & Mackintosh, 1963) and is similarly attenuated in animals administered anxiolytics (Kaesermann, 1986).

1.2 Limitations of Traditional Paradigms

While traditional fear conditioning, avoidance learning and approach-avoidance conflict paradigms have proven to be useful tools, they are not without limitations. In typical fear conditioning studies, animals are enclosed in small chambers without a means to escape aversive stimuli, which ultimately results in a severe restriction of their behavioral repertoire and therefore limits what can be observed and measured (Pellman & Kim, 2016). Overt defensive behavior is limited to freezing, or in extremely aversive conditions, panic-like, "bursting behavior" (Fanselow & Lester, 1988). Additionally, standard fear conditioning does not readily address innate fear toward non pain-inducing discrete stimuli. In fact, innate fear toward non pain-inducing stimuli has been postulated to be the primary mechanism of fear-based defensive behavior; from an evolutionary perspective, it is superior to the trial-based, potentially fatal fear learning mechanisms (Bolles, 1970).

Traditional avoidance learning paradigms, while they slightly broaden behavioral options, likewise do not consider innate defensive behavior and instead focuses on classical and operant conditioning as mediators. In fact, whether or not defensive behavior in certain

avoidance learning tasks is natural or merely a laboratory artifact has been questioned. Similar to findings that demonstrate that not every neutral stimulus can be readily classically conditioned (Garcia et al., 1974; Garcia & Koelling, 1966), Bolles (1970) contends that not every type of response can be readily conditioned in avoidance learning paradigms; only those responses that are natural to the animal become readily conditioned, such as fleeing to safety. This idea was initially framed within the context of learning and motivation theory, however, the notion that researchers need to consider the evolutionary history of the subject and their behavior in nature if one is to obtain an accurate understanding of their behavior within laboratory is a valid and important point applicable to animal research in general.

Traditional approach-avoidance conflict paradigms better approximate a naturalistic scenario. The procedure is goal-oriented; for example, obtain food, water or explore a novel environment. As such, the animal is engaged in purposive behavior (Tolman, 1932), which can facilitate a more accurate interpretation of behavioral results. Moreover, foraging and exploration in nature is not without risk and requires that animals make decisions that carefully balance reward and costs (Krebs, 1980; Lima & Dill, 1990; MacArthur & Pianka, 1966). In this regard as well, approach-avoidance paradigms are inherently more naturalistic than the simpler fear conditioning and avoidance learning paradigms and allows one to examine the influence of cognition on defensive behavior. Innate anxiety can also be examined in the case of the Elevated Plus Maze, however, the utility of this paradigm is often limited to a single test session because animals explore the open arms progressively less with each exposure, presumably because they learn that the potentially dangerous arms contain no rewards (La-Vu et al., 2020).

Nonetheless, traditional approach-avoidance conflict paradigms suffer a limitation applicable to all of the above-mentioned: testing is brief, effectively yielding only “snapshots” of

a given phenomenon. Encounters with dangerous stimuli in the wild can produce long-lasting changes that re-organize the animal's day-to-day routine and decision-making (Lima & Bednekoff, 1999; Lima & Dill, 1990; Stephens & Krebs, 1986). In a similar vein, exposure to trauma in humans can cause long-lasting alterations in cognition, emotion and behavior (Foa et al., 2006)—and in the case of posttraumatic stress disorder, cause profound impairment in day-to-day functioning and sleep disturbance (American Psychiatric Association, 2013). In other words, these traditional paradigms do not adequately address the temporal aspect of fear and anxiety (Pellman & Kim, 2016). Also in line with the above-mentioned, traditional approach-avoidance conflict paradigms take a "hyper-focused" approach by measuring few behavioral variables and utilize physically restrictive testing environments. While traditional threat paradigms retain their utility and have undoubtedly provided important insights, overreliance on these paradigms yields an incomplete understanding of the brain's defense system and thus diminishes translational potential.

1.3 Ethological Paradigms of Fear, Anxiety and Risky Decision-making

Historically, semi-naturalistic threat paradigms have occupied a small niche in fear and anxiety neurobehavioral research in relation to the predominance of fear conditioning. Early ethologically-relevant threat paradigms in behavioral neuroscience used live predators to instigate innate fear and anxiety/defensive behavior. For example, early experiments in rodents characterized their innate defensive behavior when exposed to an innately aversive, visual threat: a live cat (D. C. Blanchard & R. J. Blanchard, 1972; R. J. Blanchard & D. C. Blanchard, 1972; Blanchard & Blanchard, 1989a). Rodents in these studies were placed in apparatus with varying degrees of escape route/shelter from which to escape the threat source, which ultimately permitted the observation and measurement of a greater variety of defensive responses when

compared to the typical fear conditioning, avoidance learning or approach-avoidance conflict environment. From these studies, it was determined that both distance from the predator and the features of the testing environment strongly influence innate defensive behavior; flight dominates at distances of 1-5 meters if an escape route is possible (otherwise freezing occurs) and active attack occurs near contact with the predator (Blanchard, Blanchard, et al., 1990b). In other words, by using this ethological approach in tandem with a more expansive, permissive environment, the authors were able to gain a better understanding on the spatial component of fear. It is worth noting that in rats provided with a burrowing apparatus that provides shelter, a single brief exposure (sans harm) to a cat predator in this familiar environment leads to robust suppression of daily non-defensive behaviors, such as foraging (Blanchard & Blanchard, 1989a).

Partial predator stimuli have been demonstrated to be viable alternatives to live predators. A common ethobehavioral paradigm is the use of predator odorants. In rodents, cat fur/saliva and bobcat, weasel, fox and ferret urine/feces have been shown to elicit innate defensive behavior (Dielenberg et al., 2001; Ferrero et al., 2011). In the paradigms simplest form, animals are placed within an enclosed chamber that contains a localizable source of the predator odorant and avoidance and risk-assessment behaviors measured. Avoidance is typically measured by the time spent near and number of contacts with the threat source and risk-assessment with stretch-attend posture toward the threat source (Apfelbach et al., 2005; Blanchard, Blanchard, Weiss, et al., 1990; Papes et al., 2010). In apparatus without shelter form the threat source, freezing can also be observed (Sullivan & Gratton, 1998). Similar to the Step-Through paradigm, predator odor supports contextual fear learning and inhibits movement into the odor-associated zone when tested again after the initial exposure (McGregor et al., 2002).

Another partial predator stimulus-based ethological paradigm involves the use of overhead, two dimensional "looming" stimuli, which produce rapid innate defensive responding in both rats and mice (Yilmaz & Meister, 2013; Zambetti et al., 2019). These evoked "looming defense responses" include fleeing or freezing, once again depending on the presence or absence of an escape route (shelter), respectively (Edut & Eilam, 2003; Yilmaz & Meister, 2013; Zambetti et al., 2019). The most common variant of the paradigm used in behavioral neuroscience research involves placing subjects (predominantly mice) in small enclosed chambers outfitted with an overhead monitor capable of displaying the two-dimensional looming stimuli a short distance above, the most widely used being a rapidly expanding black disc or sweeping black bar (De Franceschi et al., 2016; Shang et al., 2018; Wallace et al., 2013; Wei et al., 2015; Yilmaz & Meister, 2013). The expanding black disc and sweeping black bar are intended to simulate a rapidly descending or approaching "cruising" predator, respectively.

Recently, Zambetti et al. adapted this paradigm for use in male and female rats and made it more naturalistic (2019). In this study, hunger-motivated male and female rats were allowed to forage for food pellets in a large, open arena comprised of two zones: a smaller "Nest Zone" and a larger, outwardly fanning "Foraging Zone." During testing, animals left the safety of the Nest Zone to procure a food pellet placed fixed distance away from the nest opening in the Foraging Zone. Upon reaching the location of the food pellet, looming stimuli were presented overhead on the ceiling and innate looming defense responses examined. In another condition, a three-dimensional, life-like plastic owl programmed to surge downward toward the animal via pneumatic actuator was used as the aversive stimulus.

By incorporating an ethologically relevant approach-avoidance conflict, using a larger arena and by presenting partial and full looming predator stimuli at heights more plausible in

nature, a more comprehensive understanding of innate defensive behavior toward these looming stimuli was achieved. It was determined that in rats, the two-dimensional stimuli produced only moderate innate fear responses that transiently interfered with foraging behavior; though more females failed to obtain the pellet than males upon first exposure, both sexes rapidly habituated to the two-dimensional stimuli by the next test session. In contrast, the three-dimensional owl produced robust, long-lasting fear in both sexes, with females again being more affected. This more ethological preparation also helps clarify the predominance of flight versus freezing (freezing was never observed) in response to the stimuli, as the standard looming paradigm produces mixed results (De Franceschi et al., 2016; Yilmaz & Meister, 2013). The observed sex-differences in fear behavior were also opposite of that obtained from standard fear conditioning protocols (Graham et al., 2009; Maren et al., 1994), and thus align better with human data that show women have higher incidences of anxiety and posttraumatic stress disorder than men (Olff, 2017).

1.4 The Utility of Ethological Paradigms

This dissertation is primarily concerned with detailing the utility of ethological, semi-naturalistic paradigms to study fear, anxiety and risky decision-making in rats and mice and how they may be used to rectify the limitations associated with traditional paradigms investigating these processes. To do so, the proceeding chapters will cover experiments and their findings conducted in ethological paradigms specialized for longitudinal or acute behavioral analyses, as well as how such findings exemplify the merits of ethological paradigms. Chapter 2 introduces and describes in-depth the "Risky Closed Economy," a longitudinal, ethological paradigm used for measuring circadian and infradian behavior. This chapter also presents data from the first ever Risky Closed Economy study in mice that shows how chronic, unpredictable threat shapes

their day-to-day behavior and how they are a viable alternative to rats in this paradigm. Chapter 3 highlights the Risky Closed Economy's ability to clarify the behavioral role of targeted nuclei in studies using irreversible inactivation methods. Specifically, the chapter focuses on the effects of lateral habenula lesions in rats living in the Risky Closed Economy. Finally, chapter four introduces the "Approach Food-Avoid Predator" paradigm, an ethological paradigm that simulates a predator-prey encounter and is specialized for analyzing the effects of innate fear on defensive behavior and acute decision-making. This chapter further describes a novel, mouse-adapted version that expands upon the original paradigm in rats and presents findings pertaining to the neural signature of innate fear of mice tested in this paradigm.

Chapter 2. The Risky Closed Economy: A Holistic, Longitudinal Approach to Studying Fear and Anxiety in Rodents (Schuessler et al., 2020)

2.1 Introduction

Neuroscience techniques are becoming exponentially more sophisticated, allowing researchers to measure and manipulate the brain at larger scales with more precision and specificity. However, in rodent fear and anxiety research, what appears to remain static is the use of a limited set of behavioral paradigms in which these new technologies are being employed (Kim & Jung, 2018; Mobbs & Kim, 2015). Examples include Pavlovian (classical) fear conditioning and the freezing response (Michael S. Fanselow, 1980), the Elevated Plus Maze and time spent in open arms (Pellow et al., 1985), and inhibitory (passive) avoidance and latency to enter a shock-associated dark compartment (J. F. Deakin & F. G. Graeff, 1991; Venable & Kelly, 1990). While such paradigms have yielded invaluable insights, they are usually short in duration (typically minutes) and measure a narrow range of behaviors, in effect providing only a "snapshot" of a given phenomenon (Pellman & Kim, 2016). For example, this brief sampling of behavior excludes temporal aspects of fear and anxiety, including how fear and anxiety-related behavior vary over time, as well as how fear and anxiety affect circadian/infradian rhythms and long-term, risky decision-making.

On one hand, the continued use of these customary paradigms allows researchers to focus on thoroughly mapping and characterizing the neurocircuitry of a small set of well-known, predictable behaviors. On the other hand, their overreliance leads to gaps in knowledge regarding the behaviors and neurophysiology associated with fear and anxiety in rodents and thus a restriction on translational potential (Pellman & Kim, 2016). What is needed in addition are

paradigms that can more comprehensively model in rodents the complexities of normative fear and anxiety. One such paradigm that provides the foundation for a more holistic approach to studying rodent behavior is the "closed economy"; a paradigm in which animals obtain their daily food exclusively through operant responding and typically live in the operant chambers themselves (Collier, 1983; Collier et al., 1972). By introducing an aversive component to the closed economy—namely, context-dependent, unpredictable footshock lasting several weeks (Fanselow et al., 1988)— a unique, naturalistic chronic approach/avoid conflict is engendered; "The Risky Closed Economy" (RCE). With the addition of modern animal tracking and automation technologies (Kim et al., 2014), the RCE allows for fear and anxiety-related behavior to be expansively studied.

2.2 Historical Origins

In economic terms, a closed economy refers to an ideal state in which daily consumption is the result of the equilibrium of supply and demand. As it applies to animal research, a closed economy refers to a scenario in which the animal's consumption of food (demand) results solely from its interaction with schedules of reinforcement (supply); that is, the animal controls its total food intake via operant responding without experimenter food supplementation (Hursh, 1980). This contrasts with an "open economy", where food is supplemented outside of the operant session and thus behavior within the session is independent of total daily consumption of the reinforcer (Hursh, 1980, 1984). Also characteristic of animal closed economy experiments are long measurement sessions—typically 23 hours per day over several days—sufficient within-session reward densities suitable for survival and deprivation levels that are determined by the animal's within-session food intake (Posadas-Sanchez & Killeen, 2005; Timberlake & Peden, 1987) (**Figure 2.1A**). Collier et al. (1972) were the first to characterize rats' foraging behavior

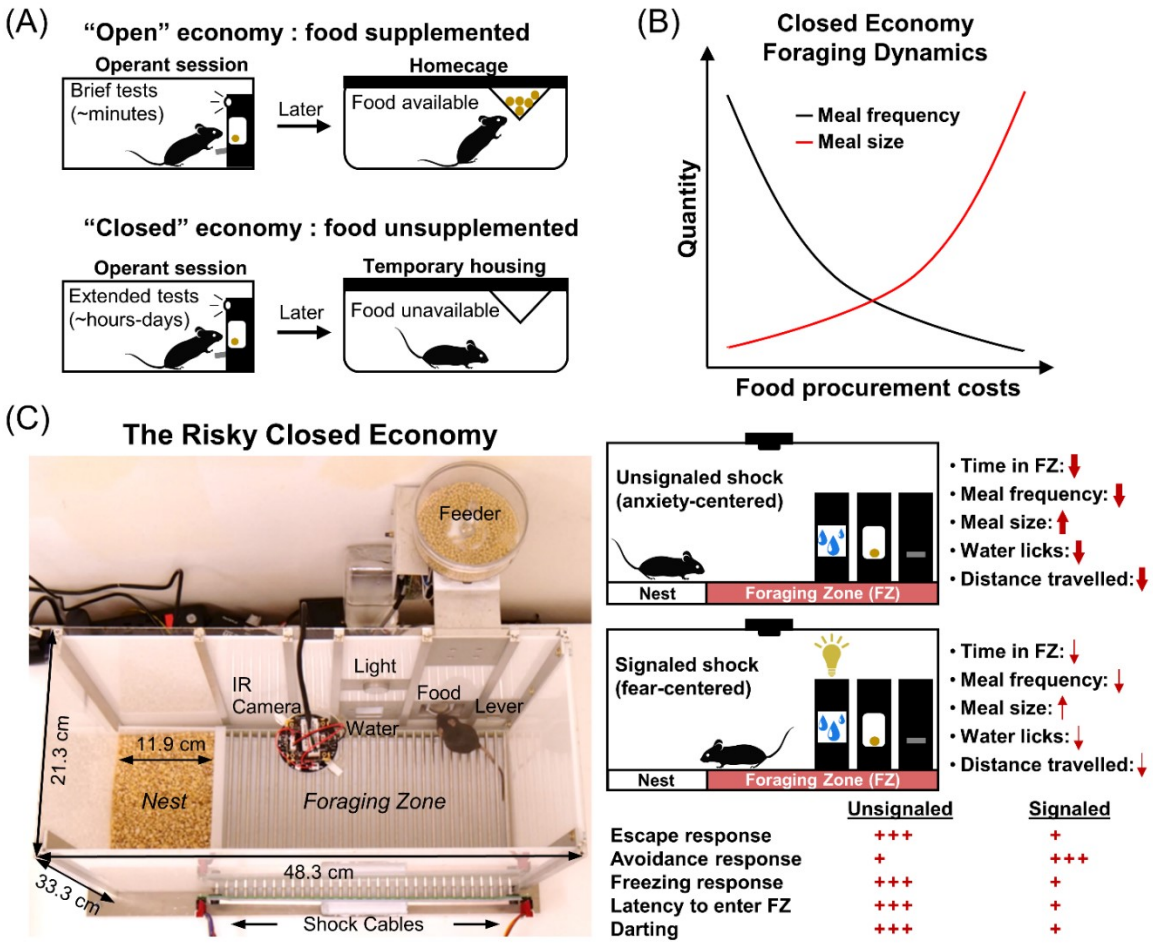

Figure 2.1. Traditional closed economy concepts and The Risky Closed Economy (RCE). **(A)** In "closed economies," food and/or water is not supplemented after testing. Closed economy experiments also typically utilize long testing periods. This contrasts with "open economies" where food is supplemented after brief tests. **(B)**. The use of chained schedules of reinforcement in closed economy experiments (e.g., fixed ratio-continuous reinforcement) allows for discrete eating bouts ("meals") and the number of pellets per eating bout ("meal size") to be measured. Closed economy animals characteristically decrease the frequency of meals and increase meal size in response to increasing food procurement costs. **(C)** In the RCE, pseudo-random footshock is integrated into a closed economy framework as a means to model naturalistic risky foraging with predation threat. Use of an unsignaled (no cue) or signaled (cued) shock allows one to investigate the effects of diffuse, anxiety-evoking or imminent, fear-evoking threat, respectively, on circadian and infradian behavior. Red arrow thickness and quantity of red plus signs represent the impact of shock condition on the listed behavioral variables under optimal experimental parameters (i.e., from rat studies mentioned in the text).

as a function of effort in a closed economy system and showed that male rats exhibit robust operant responding for food pellets at unusually high reinforcement schedules when daily food consumption was made entirely contingent on the animals' behavior, as would be the case in the animals' natural environment. They additionally demonstrated that rats tend to decrease eating bouts while increasing pellets obtained per eating bout in response to increasing schedule demands, in accordance with optimal foraging strategy, which postulates that animals strive to maximize caloric intake while minimizing energy and time costs (MacArthur & Pianka, 1966) (**Figure 2.1B**). Importantly, these results contrast with studies that show that animals are far less willing to engage similar schedules of reinforcement when food is supplemented outside the testing session, emphasizing the notion that the animal's total reinforcer economy and their experience outside of the testing session are crucial determinants of operant behavior within the testing session (Hursh, 1978; Hursh, 1980; Kearns, 2019; but see Timberlake & Peden, 1987). Since these original studies, much research has been dedicated to exploring the influences of open economies versus closed economies on operant behavior in a variety of species (Kearns, 2019; Posadas-Sanchez & Killeen, 2005).

2.3 Naturalistic Qualities

The closed economy approximates a naturalistic foraging scenario. As mentioned above, the animal alone dictates its daily nutrition but must exert effort to obtain sustenance. This effort component arises from a chained schedule of reinforcement, which is used to model the time/energy costs associated with initially procuring the food item and the subsequent energy/time costs associated with manipulating and consuming the food item while foraging (Collier, 1983). In completing the procurement phase of the schedule (first chain component; e.g., fixed-ratio 50, FR50), the animal initiates a "meal" and then transitions into the

consumption phase (second chain component; e.g., continuous reinforcement, CRF), where it may obtain food as long as it continues to emit operant responses. A meal ends when the animal fails to respond after a set amount of time in the consumption phase, which resets the schedule and ends the meal. The amount of food obtained during a meal is referred to as the "meal size." Therefore, in response to shifting foraging constraints, the animal can choose to alter its foraging strategy by changing parameters such as daily meal frequency, meal size and response rate (Collier, 1983).

The closed economy is further made naturalistic when a risk component is added to the foraging experience. In nature, animals must often leave the safety of their nests to forage in potentially dangerous locations (Lima & Valone, 1986). Fanselow et al. (1988) housed female rats in operant chambers that included a safe "Nest Zone", comprised of saw dust bedding and a water bottle, and a risky "Foraging Zone", which contained a shock grid floor and the operant lever/food port. After a baseline foraging and activity period, unsignaled but escapable footshocks were administered in the Foraging Zone for several weeks to model an additional cost associated with naturalistic foraging: predation (Krebs, 1980). These unpredictable shocks were then terminated for a "Post-Shock" ("Extinction") assessment. Rats responded to this persistent threat by decreasing meal frequency but compensated caloric intake by increasing meal size (**Figure 2.1B**). This strategy, coupled with a strong avoidance of the risky Foraging Zone during the "Shock" phase, allowed animals to continue to gain weight and minimized the amount of daily footshock received. The results of this study and future studies expanding on this paradigm (Helmstetter & Fanselow, 1993; Kim et al., 2014; Pellman et al., 2015; Pellman et al., 2017) support the ethological theory that animals integrate the risk of predation in their daily foraging and activity decisions (Lima & Dill, 1990). Note that footshock is not intended to represent

predatory encounter per se, but is used to broadly model the risk of predation while foraging. The incorporation of risk into the closed economy framework and longitudinal design form the basis of the RCE and enhances the paradigm to achieve greater ethological relevance (**Figure 2.1C**). In the RCE, the need to acquire food and water while avoiding unpredictable threat is integrated into the animals' lives—an ubiquitous scenario in nature (Mobbs & Kim, 2015). The naturalistic qualities and longitudinal design of the RCE provide unique benefits compared to traditional methods (**Table 2.1**) as discussed in the proceeding sections.

2.4 Utility in Fear and Anxiety Research

When shocks are delivered unpredictably, the Shock phase of the RCE most suitably evokes anxiety (Fanselow et al., 1988; Helmstetter & Fanselow, 1993; Kim et al., 2014; Pellman et al., 2015; Pellman et al., 2017) (**Figure 2.1C**, top right). According to Predatory Imminence theory, which proposes that organisms' momentary perception of predation risk determines their defensive behavioral topography (Fanselow & Lester, 1988), the subtle changes in meal patterns and avoidance resulting from these shock parameters in the RCE resemble "pre-encounter" defensive reactions to threat (Fanselow et al., 1988; Helmstetter & Fanselow, 1993). The pre-encounter phase is defined as a situation in which there is a possibility of harm but is low in probability or distant and is accompanied by anxiety-like reactions such as avoidance, risk assessment and vigilance meant to decrease the chances of encountering danger (Perusini & Fanselow, 2015). The diffuse and unpredictable nature of shock using these parameters and the observed defensive behavior also align well with the "sustained fear" concept of anxiety, where the defensive behavior maintains long after the aversive stimulus is removed (Davis et al., 2010).

In a general sense, this paradigm shares features with traditional punishment-based approach-avoid conflict paradigms used to screen anxiolytics, such as the Vogel Conflict Test

Table 2.1. Advantages of the Risky Closed Economy paradigm relative to traditional fear and anxiety paradigms, such as Pavlovian (classical) fear conditioning, the elevated plus maze, and inhibitory (passive) avoidance paradigms.

The Risky Closed Economy	Traditional Fear/Anxiety Paradigms
Longitudinal; 23 hrs/day data collection for several weeks.	Brief tests offering only "snapshots" of behavior.
Multitude of behavioral variables (holistic approach).	Few behavioral variables (hyper-focused approach).
Naturalistic. A risky-foraging scenario requiring effort and decision-making; the need to acquire food and water while avoiding unpredictable threats is integrated into the animals' lives. The ethologically-relevant, goal-oriented (purposive) task facilitates interpretation of behavior.	Less naturalistic. Food and water provided and/or restricted by experimenter. Small chambers and short test duration constrain the animals' behavioral repertoire.
Minimal experimenter interaction.	Increased experimenter interaction (handling, feeding, frequent transport).

(Vogel et al., 1971) and Geller-Seifter Test (Geller et al., 1962). It therefore may be useful for studies investigating the longitudinal effects of anti-anxiety medications on factors such as avoidance, decision-making, feeding behavior and sleep/wake cycles. The RCE also shares qualities with the platform-mediated avoidance paradigm (Bravo-Rivera et al., 2014) where rats are trained to lever press for food then receive tone-shock pairings, such that when the tone is presented animals escape the shock-grid to a nearby platform. Likewise, when a signaled shock is utilized in the RCE, the paradigm contains elements found in conditioned suppression tasks, where the presence of a cue paired with shock terminates lever-pressing behavior (Estes & Skinner, 1941). However, these acute behavioral paradigms do not afford a comprehensive picture of the effects of threat on day-to-day behavior, measure fewer variables over shorter periods, and involve food restriction/supplementation (open economy) which can affect operant behavior (Hursh, 1980) (**Table 2.1**).

The use of a discrete cue preceding shock in the Foraging Zone, such as a light, may be used to invoke fear (**Figure 2.1C**, bottom right). Fear is typically conceptualized as a defensive state resulting from imminent, predictable threat with behaviors and neural substrates dissociable from that of anxiety (Davis, 1998; Perusini & Fanselow, 2015; Robinson et al., 2019). Indeed, when threat cues are utilized within the RCE, the foraging and activity level changes seen in the standard unsignaled shock condition are near absent, as active avoidance take precedence over passive avoidance responses (Pellman et al., 2015). The paradigm may therefore be used to study continuously the development of Pavlovian instrumental transfer under more naturalistic conditions, or in the case of consecutive unsignaled to signaled Shock phases, whether a neural manipulation disrupts both contextual and/or discrete cue learning.

The RCE affords researchers a means to study facets of fear and anxiety-related behavior typically not feasible in the predominant paradigms mentioned above. One facet includes the spatiotemporal dimension of fear and anxiety. In segmenting the apparatus into distinct "risky" versus "safe" zones and by continuously measuring the animal's behavior for extended periods, one can investigate how context and prolonged exposure to aversive stimuli interact to shape the animal's day-to-day behavior. For example, it has been shown that threat associated with the Foraging Zone during the dark portion of the dark/light cycle can change rats' foraging and overall activity to occur primarily during the light portion of the dark/light cycle, essentially reversing the animals' typical circadian activity patterns (Pellman et al., 2015). The paradigm may therefore be of use in research centered on fear and anxiety's disruptive effects on circadian rhythm, which is known to be dysregulated in human anxiety and mood disorders (Amir & Stewart, 1998; American Psychiatric Association, 2013; Roybal et al., 2007; Tapia-Osorio et al., 2013). Given that anxiety disorders emerge early on in life (Pine, 2007), the RCE could also be used in developmental research investigating the impact of chronic, unpredictable threat or cyclic threat on anxiety, fear and decision-making behavior in different age groups, or as an initial screening for individual differences in trait anxiety. Finally, due to the delineation of safe versus risk zones, risk assessment behaviors such as a stretched, attentive posture toward the source of threat (Blanchard, Blanchard, et al., 1990a; Choi & Kim, 2010) may also be examined in addition to standard freezing and avoidance metrics (**Figure 2.1C**).

The naturalistic qualities of the RCE facilitate the assessment of fear and anxiety on decision-making (Mobbs et al., 2018), a form of executive functioning (Robinson et al., 2013). Aside from the aforementioned changes in meal patterns and avoidance, other forms of decision-making under risk can be examined with creative use of the operant devices and reinforcement

contingencies used to simulate the work component of foraging. Kim et al. (2014) incorporated two operant levers; one located close to the safe Nest Zone and another located on the same wall of the apparatus but at the distal end of the Foraging Zone. Results indicated that amygdala lesioned rats with an initial preference for the farther lever failed to switch to the closer, safer lever during the Shock phase unlike sham lesioned controls. In this study, the authors varied lever distance to probe the animals' distance gradient of fear and its influence on appetitive behavior, but similar methods can be used to study a variety of other cognitive processes. For example, one could utilize two levers equidistant from the nest area that offer either high reward at low probability or low reward at high probability, respectively, to examine how risk of shock influences impulsivity under closed economy conditions (Green & Myerson, 2004). In a broad sense, the RCE can also be used to examine in rodents human behavioral economic principles, such as those outlined in Kahneman and Tversky's Prospect Theory (1979), which proposes that decisions under risk are subject to influence of past outcomes and cognitive biases that promote or inhibit risk taking behavior. For example, one can examine how differing levels of anxiety/fear interact with differing levels of weight loss over time to promote risky foraging or avoidance.

2.5 Viability in Mice

Studying fear and anxiety using the RCE is also feasible in mice, thus opening the door for use of transgenic mouse models. **Figure 2.1C** (left) depicts the mouse-adapted RCE. Animal position is tracked via mounted infrared camera connected to a central computer running ANY-maze tracking software (RRID:SCR_014289). The software also quantifies lever presses/licks, triggers the pellet dispenser (ENV-310W, ENV-251M, ENV-203M-45; Med Associates, Fairfax, VT) to deliver 20 mg dustless precision food pellets (F0163; Bio-Serv, Flemington, NJ) and

triggers the precision animal shocker to deliver shocks to Forging Zone steel grid floor (H13-15, H10–11M–XX–SF; Coulbourn, Holliston, MA). One central computer with ANY-maze software and interface accommodates four chambers. Mice (N = 7) proceeded through Lever Shaping, Baseline, Shock, and Extinction phases as outlined in **Figure 2.2A**. **Figure 2.2B-G** shows adult (3 months) male C57BL/6 mouse (IMSR Cat# CRL_27, RRID:IMSR_CRL:27) data. Custom Python scripts were used for data aggregation and the formation of custom variables. Parametric data were analyzed with one factor repeated measure ANOVAs followed by Bonferroni-corrected Dunnett's post-hoc comparisons in Prism (GraphPad Prism, RRID:SCR_002798). Greenhouse-Geisser corrected degrees of freedom were used when the sphericity assumption was violated (Mauchly's test). Non-parametric data were analyzed with rank-based repeated measure ANOVAs followed by Bonferroni-corrected multiple comparisons using the R package nparLD (Noguchi et al., 2012). All statistical tests were performed with an alpha level set to 0.05.

The introduction of unsignaled, pseudo-random (~2/hour) footshocks (0.5 mA, 10 seconds or until escape to Nest Zone; 48 shocks/day max) significantly reduced time spent per day ($F_{4, 24} = 17.89$, $p < 0.0001$; **Figure 2.2B**) in the Foraging Zone during both Shock and Extinction phases relative to Baseline (p's < 0.0001). Footshock also reduced the daily meal frequency ($F_{2.425} = 2.802$, $p < 0.05$; **Figure 2.2C**) and food pellets consumed ($F_{1.521, 9.125} = 11.11$, $p < 0.01$; **Figure 2.2D**) during the first week of shocks (p's < 0.05), which recovered to Baseline levels by week two of the Shock phase. Footshock significantly decreased the amount of water licks per day ($F_{2.557} = 11.191$, $p < 0.0001$; **Figure 2.2E**) throughout the Shock phase and first week of Extinction (p's < 0.0001) but was not significantly different from Baseline by Extinction

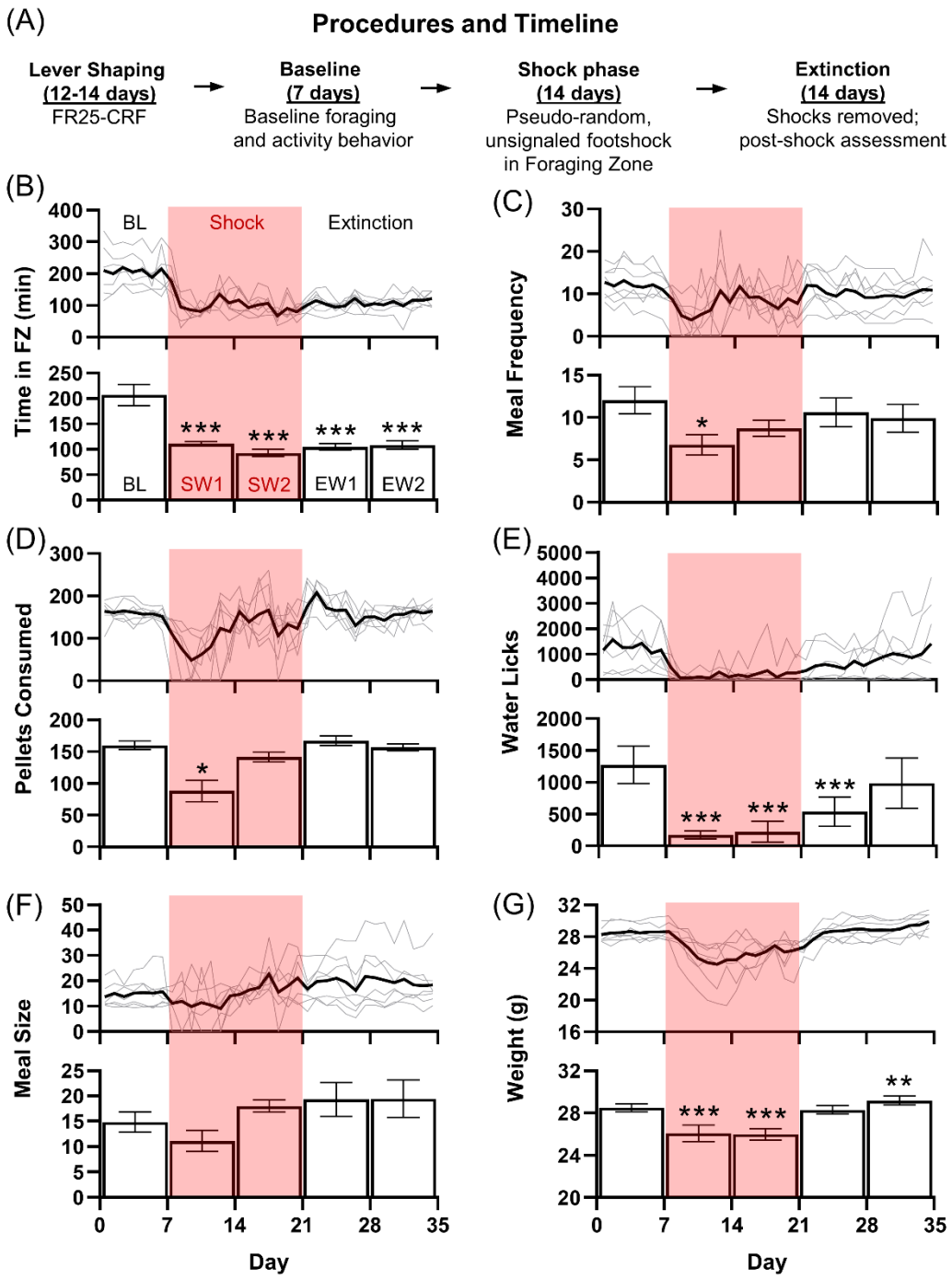

Figure 2.2. Mice are a viable alternative to rats in Risky Closed Economy experiments. **(A)** Upon arrival, animals were immediately housed in Risky Closed Economy Chambers distributed between two rooms (4 chambers/room). Mice were then shaped to lever press for food at a fixed ratio 25-continous chained schedule of reinforcement (FR25-CRF), wherein each press beginning at FR25 results in one pellet/press. The schedule resets after 1 minute of lever inactivity. A "meal" occurs when the FR threshold is met and continues until the schedule resets. Shaping begins at FR1 and doubles every two days until FR25 (except FR16 transitions to

FR25). Baseline foraging and activity level assessment follows until seven days of stable behavior is obtained. Unsignaled, pseudo-random (~2/hour) footshocks (0.5 mA, 10 s or until escape to Nest Zone; 48 shocks/day max) are introduced in the Foraging Zone for a two-week Shock phase. Finally, shocks are terminated for a two-week Extinction phase. **(B-G)** shows the daily average (black line) with individual mouse data (gray lines) (top) and weekly average ± SEM (bottom) for daily total time spent (minutes) in the Foraging Zone (**B**), meal frequency (or number of meals/day) (**C**), food pellets consumed (**D**), water lick meter beam breaks (**E**) meal size (food pellets/meal) (**F**) and animal weight (grams) (**G**) of adult male C57BL/6 mice (3 months old upon arrival; N=7) across Baseline (BL), Shock (S) and Extinction (E) phases (* $p <$ 0.05, ** $p <$0.01, *** $p <$ 0.0001 versus Baseline).

week 2. There were no significant changes in meal size ($F_{1.556, 9.337} = 2.744$, $p = 0.1224$; **Figure 2.2F**).

Finally, footshock depressed weight ($F_{1.884} = 33.228$, $p < 0.0001$; **Figure 2.2G**) during the Shock phase, which returned to Baseline levels during the first week of Extinction and exceeded Baseline levels by Extinction week 2 (p's < 0.01). Ultimately, mice behaved similarly to adult female Long-Evans rats in the RCE that experienced comparable shock densities (Pellman et al., 2017); mice did not increase the amount of pellets consumed per eating bout to offset the decreased eating bouts per day during the Shock phase, lost weight, and did not extinguish avoidance of the Foraging Zone after shock was removed. The shock density, selected based off the performance of male rats in previous studies conducted in our laboratory, proved too aversive for our male mice and likely prevented them from displaying the abovementioned meal alterations (Helmstetter & Fanselow, 1993). Given that footshock strongly inhibited foraging, the reduction in aversion resulting from avoidance likely negatively reinforced the behavior to a degree that prevented extinction of avoidance when shock was terminated (Mowrer, 1939). Thus, the data presented here may not reflect an optimized version of the task; future experiments adjusting lever contingencies and/or shock intensity and density are warranted.

2.6 Discussion

The RCE's unique longitudinal design and ethological qualities serve to expand both the animal's behavioral repertoire and what can be measured in a controlled laboratory setting. Data obtained from such goal-oriented (Tolman, 1932), "big picture" analyses can be used to further refine our understanding of rodent fear and anxiety and subsequently aid in mapping their behavior onto human behavior to enhance translational relevance. Similarly, by studying neural

mechanisms under more ethological conditions, a more accurate understanding of how these mechanisms naturally operate may be achieved. Understanding how these mechanisms operate in situations they likely evolved to handle can pave the way for understanding how they malfunction in mental illness. With recent advances in tracking software, reversible, time-specific neural manipulation techniques, wireless recording/optogenetics technologies and increased feasibility of big data analysis, the RCE has the potential to generate a wealth of knowledge regarding the neural circuitry of fear and anxiety-related behavior.

The RCE concept and apparatus offers benefits applicable to behavioral testing in general. Our design (**Figure 2.1C**) allows for automated acquisition and scoring of behavioral variables, reducing experimenter biases that negatively impact a study's validity and reproducibility (Barber & Silver, 1968). This automation further assists in the RCE's "hands off" approach that minimizes experimenter-subject interaction. For example, in our procedure, animals are removed from their chambers for only one hour/day for apparatus maintenance and health checks; animals are left undisturbed the remaining 23 hours of the day. Importantly, limiting experimenter-subject interaction reduces potential stress on the animals (Hurst & West, 2010; Sorge et al., 2014), which improves overall animal wellbeing and reduces study variability. Unlike other relatively chronic tasks in rodents, such as "touchscreen" paradigms (Delotterie et al., 2014), no training is required to perform the task, as animals are autoshaped to procure food and water and no food deprivation is imposed. Lastly, in light of the fact that the animal's home cage is the testing apparatus itself and behavior is measured nearly continuously for extended periods, post-surgery changes in baseline behavior can be screened prior to testing animals under new experimental conditions. This helps clarify test results and interpretations and

is especially relevant to research incorporating irreversible neural manipulations, such as electrolytic, chemical or genetic lesions.

Although we encourage the implementation of the RCE in neuroscience research, we acknowledge that the paradigm has limitations that make it impractical for certain research projects. The most obvious is that by design, RCE experiments take a substantial amount of time to complete (**Figure 2.2A**). Thus, for those seeking to adopt the paradigm, it is advisable that multiple chambers be constructed (at least 8). We also recognize that in its current configuration (**Figure 2.1C**), the RCE introduces social isolation as a factor. This can be partially ameliorated by having clear, perforated acrylic chamber walls where animals can both see and smell each other and by group-housing animals during the daily one-hour removal period. The longitudinal aspect and enclosed chamber also make the use of certain tools, like tethered optogenetics and electrophysiological recording, challenging. However, careful planning and wireless alternatives can overcome this obstacle to provide future studies a powerful means by which to study the neural mechanisms of complex behaviors over time. For example, pharmacological and/or chemogenetic tools such as Designer Receptors Exclusively Activated by Designer Drugs (Armbruster et al., 2007) are suitable for use in RCE experiments; treatment may be given on alternating days (A-B-A-B design) either manually through injections/infusions or remotely with programmable minipumps (see iPRECIO Programmable Infusion Pump; ALZET Osmotic Pumps, Cupertino, CA). Finally, given the viability of mice in the RCE, transgenic strains may also be taken advantage of. In sum, the RCE provides both unique advantages and opportunities relative to more traditional fear and anxiety paradigms and general benefits applicable to other subfields within neuroscience research.

Chapter 3. A Holistic Examination of the Effects of Lateral Habenula Lesions in Rats

3.1 Introduction

The habenular complex, or habenula, is a critical component of the Dorsal Diencephalic Conduction system, integrating and processing emotional and behavioral state information from the limbic forebrain and basal ganglia to influence midbrain monoamine release throughout the brain (Fakhoury, 2018). Evolutionarily, it is highly conserved among vertebrate species and is divided into two major divisions: the lateral habenula (LHb) and medial habenula (MHb) (Bianco & Wilson, 2009). The habenula has long been implicated in avoidance learning following experience with aversive stimuli. Early experiments demonstrated that destruction of the habenula, or its afferent or efferent projections, led to deficits in avoidance learning using active avoidance tasks (Ross & Grossman, 1977; Thornton & Bradbury, 1989; Thornton et al., 1994; Wilcox et al., 1986).

Later studies with more precise targeting and/or advanced manipulation methods have painted a more nuanced picture of the habenula and avoidance behavior. For example, stimulation of the LHb after successfully avoiding a footshock US produces a deficit in the rate of active avoidance acquisition using a shuttle box procedure (Shumake et al., 2010), whereas optogenetic excitation of the LHb during conditioned place preference training leads to passive avoidance of the stimulation paired chamber the following day during a preference test (Lammel et al., 2012). Similarly, when animals are allowed to move freely between the two compartments in a real-time place preference paradigm, they exhibit passive avoidance of the LHb stimulation-paired compartment (Stamatakis & Stuber, 2012). The LHb also plays a role in standard fear conditioning paradigms, as it has been demonstrated that pharmacological inhibition of D1

receptor-containing neurons impairs acquisition of context and tone conditioning (Chan et al., 2017). Deficits in tone CS extinction following fear conditioning has also been reported following electrolytic lesion of the LHb, although this study did not find deficits in CS acquisition (Song et al., 2017).

Less is known about the MHb's contribution to associative learning involving aversive stimuli (and the contribution of the MHb to cognition and behavior in general), however, silencing of the dorsal habenula, the MHb analog in zebra fish, enhances freezing during fear conditioning training and retrieval sessions (Agetsuma et al., 2010). Similarly, silencing bed nucleus of the anterior commissure afferents to the MHb enhances freezing to footshock and increases passive avoidance using the step-through inhibitory avoidance paradigm in mice (Yamaguchi et al., 2013). Finally, genetically ablating cholinergic MHb neurons slows the rate of extinction to an auditory CS in mice (Zhang et al., 2016). Collectively, the results of the abovementioned LHb and MHb studies raise the intriguing possibility that these adjacent subnuclei exert opposite influence on fear and avoidance learning/behavior.

Despite a substantial amount of evidence suggesting the LHb plays a role in avoidance learning/behavior, less is known about the LHb's involvement when there is competing motivation to approach or avoid a stimulus or location—i.e., an approach-avoidance conflict. A recent study investigated the effects of LHb inactivation on defensive and appetitive behavior using two variations on the Conditioned Suppression task (Velazquez-Hernandez & Sotres-Bayon, 2021). In this study, food deprived animals trained to lever press for food pellets within an enclosed operant chamber experienced tone-shock pairings, then underwent extinction of tone-induced CRs. Twenty-four hours after extinction training, animals were placed back into the operant chamber and presented the tone once more. Inactivation of the LHb during the final tone

test following extinction (but not during other phases of the task) impaired renewal of fear compared to control animals as measured by freezing and lever suppression ratios, which the authors suggested is due to impairments in conflict resolution when threat and safety cues are simultaneously present. In a similar vein, LHb inactivation enhanced latent inhibition of freezing and lever pressing behavior. Finally, they found that LHb inactivation impairs platform-mediated active avoidance of shock associated tone CS when a safe platform was available for escape or avoidance behavior in the operant chamber, such that LHb animals avoided shock less and spent more time lever pressing for food pellets relative to controls.

Another recent study examined the LHb's role in approach-avoidance conflict—albeit from a different angle than typical approach-avoidance studies— by using a novel foraging task that forgoes pain-inducing stimuli (i.e., foot shock) in favor of a different motivator; a physical barrier that required scaling to obtain a high value reward (Sevigny et al., 2021). In this study, rats in a T-maze were allowed to choose between a "high effort" arm that contained a 30 cm ramp they had to scale in order to obtain two sucrose pellets (high reward), or a "low effort" arm containing no ramp but a single sucrose pellet (low reward). Pharmacological inhibition of LHb in this effort-based, approach-avoidance conflict paradigm resulted a significant decrease in preference for the high effort, high reward arm relative to baseline preferences. In contrast to Velazquez-Hernandez & Sotres-Bayon (2021), inhibition of the LHb did not bias the animal toward approach behavior when conflict was present. Obvious differences in procedures and stimuli likely explain the disparate findings, however, the results of both studies indicate that LHb helps shape decisions made under conflict and can bias approach *or* avoidance behavior in a context and task dependent manner.

Finally, there is but a few studies that demonstrate that the LHb participates in purely appetitive and consummatory behavior. For example, silencing lateral hypothalamic area (LHA) terminals in the LHb enhances preference for a palatable liquid (Ensure) in a two-bottle choice task, but does not alter aversion toward bitter solutions (Stamatakis et al., 2016). Disconnecting LHA to LHb connection likewise increased voluntary ethanol intake and preference in an intermittent ethanol access procedure, but curiously did not affect the rate of lever pressing at a fixed ratio-3 schedule of reinforcement for ethanol (Sheth et al., 2017). Chemogenetic silencing of actual LHb neurons leads to increased lever pressing for cocaine, but not for standard food pellets at a continuous reinforcement schedule (Nair et al., 2020). Such studies support the general notion that the LHb serves to negatively regulate appetitive and/or consummatory behavior in tasks without overt aversive stimuli, although its participation may dependent on the specific rewards and the means of which they are obtained. In line with Sevigny et al. (2021), the differing results of LHb inhibition on operant responding for rewards could be due to the LHb's putative role in weighing the incentive salience of the reward with the effort needed to obtain the reward.

The present study sought to clarify the involvement of the LHb in naturalistic appetitive behavior under risk-free and risky conditions using the Risky Closed Economy (RCE) paradigm in rats (**Figure 3.1A**) (Kim et al., 2014; Pellman et al., 2015; Pellman et al., 2017; Schuessler et al., 2020). Specifically, the RCE allowed us to comprehensively examine the consequences of LHb loss-of-function on day-to-day foraging behavior and approach-avoidance conflict—two areas lacking knowledge as mentioned above. As the need to obtain sustenance and avoid threat is integrated into the RCE, more cognitively involved forms of risky decision-making, such meal pattern re-organization, could be measured along with traditional avoidance measures typical of

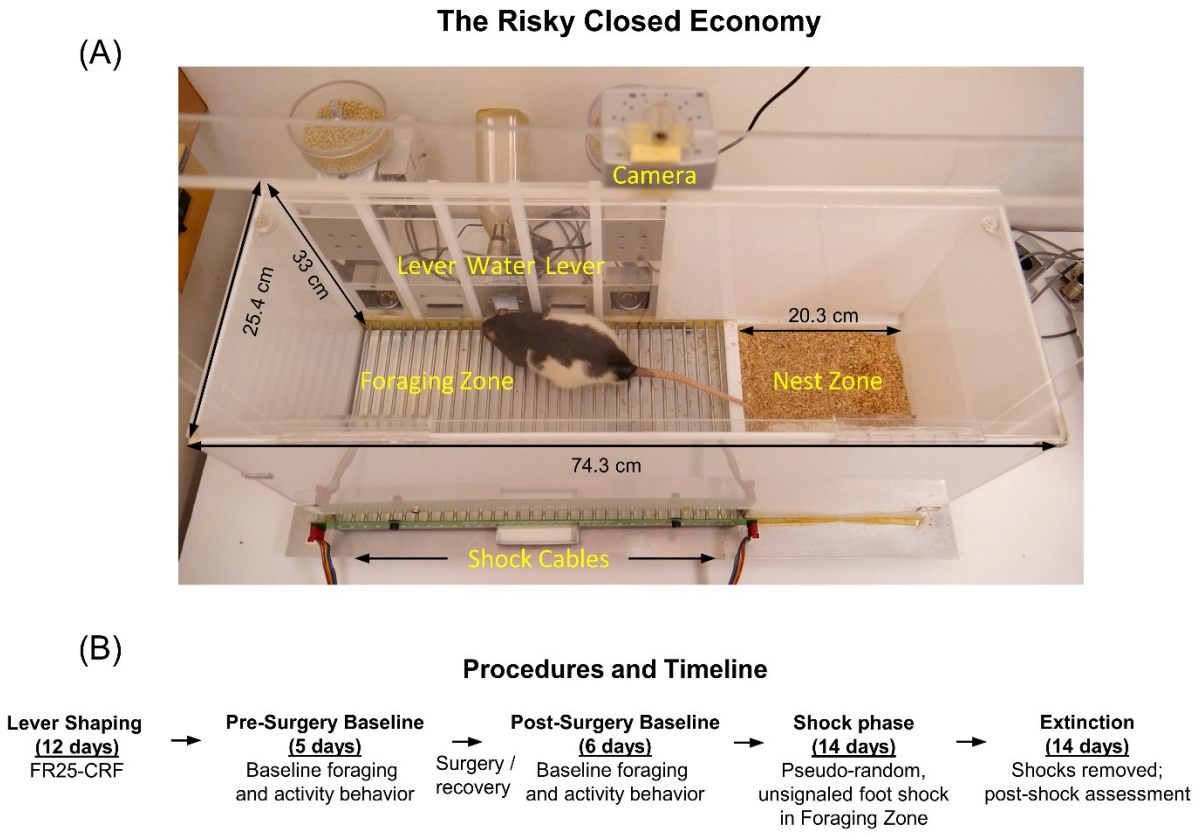

Figure 3.1. The rat Risky Closed Economy (RCE) and experimental procedures/timeline. **(A)** The rat RCE apparatus with measurements. **(B)** Experimental procedures and timeline detail. Animals recovered from surgery in RCE chambers.

approach-avoidance conflict studies. This feature also allowed us to test the well-evidenced theory that the LHb is crucial for behavioral flexibility in dynamic environments (Baker et al., 2022; Baker et al., 2015; Hones & Mizumori, 2022; Mizumori & Baker, 2017).

It was hypothesized that electrolytic lesions of the LHb would not disrupt baseline foraging behavior given the lack of effects of LHb inactivation (or its afferents) on operant responding for standard food pellets. However, it was of interest to determine if this holds true when appetitive behavior was measured uninterrupted for extended periods—i.e., nearly continuously for days-to-weeks rather the short, hourly testing as employed by the studies this hypothesis was predicated on (Nair et al., 2020; Sheth et al., 2017; Stamatakis et al., 2016). To assess this, rats living in RCE chambers and trained to obtain food under a fixed ratio-25-continous (FR25-CRF) schedule of reinforcement first underwent a "Pre-Surgery Baseline" period prior to surgery, then a subsequent "Post-Surgery Baseline" before entering the remaining "Shock" and "Extinction" phases (**Figure 3.1B**). It was also hypothesized that LHb lesions would result in decreased avoidance, meal pattern re-organization and foraging suppression during the "Shock" phase of the experiment, where pseudo-random, unsignaled foot shocks were introduced in the Foraging Zone for an extended period, relative to sham operated controls. It follows that during Extinction, LHb lesioned animals will have behavior similar to that of their Post-Surgery Baseline, whereas sham operated controls will continue to display the typical altered meal pattern that develops during the Shock period due to negative reinforcement; decreased meals (eating bouts) and increased meal size (Pellman et al., 2017; Schuessler et al., 2020).

3.2 Methods

Subjects

Adult male Long-Evans rats, initially aged 7-8 weeks (Charles River, Wilmington, MA) were immediately singled housed in RCE chambers distributed between two rooms in Guthrie Hall, Department of Psychology at the University of Washington, Seattle, upon arrival (accredited by the Association for Assessment and Accreditation of Laboratory Animal Care). Animals were housed under reverse 12-hour light/dark cycle. Animals were randomly assigned to receive electrolytic lesion of the lateral habenula (n = 6) or sham surgery (n = 6). Experimental groups were counterbalanced between both Risky Closed Economy rooms.

Rat Risky Closed Economy paradigm

The rat RCE (**Figure 3.1A**) chamber measured 74.3 cm L x 25.4 cm W x 33 cm H (length x width x height). The chamber was comprised of two general areas; a "Nest Zone" (20.3 cm L x 25.4 cm W) that contained saw dust bedding and a "Foraging Zone" (54 cm L x 25.4 cm W) that contained a shock grid floor with 32 stainless steel rods (4.5 mm diameter; Coulbourn Instruments, Allentown, PA), two operant levers/food hoppers and a lick meter (Med Associates, Fairfax, VT). The shock grid was connected to a precision animal shocker (Coulbourn Instruments, Allentown, PA) for the delivery of foot shock within the Foraging Zone. Mounted atop the apparatus was a FireWire camera (Fire-I B/W Board camera; Unibrain Inc., San Ramon, CA) connected to a desktop computer running ANY-maze software (Stoelting, Wood Dale, IL) which continuously tracked animal position. Additionally, the software was used to count lever presses, dispense food 45 mg food pellets (#F0165, Bio-Serv, Flemington, NJ) through chamber food dispensers (Med Associates, Fairfax, VT), count lick meter beam breaks, trigger foot shock and generate white noise (70 dB) to mask background noise. Subject data were collected

continuously for 23 hours/day; during the last hour of the light phase (hour 24), animals were removed from the chambers and individually housed in the Guthrie Hall central vivarium for RCE chamber cleaning, maintenance, and food and water replenishment. While in the central vivarium, animals were weighed, and importantly, had access only to water in their temporary housing. Food was not supplemented at any point in the experiment except if body weight dropped below 85% the subject's pre-Shock phase weight.

Immediately upon arrival, animals underwent an acclimation and shaping phase lasting 12-14 days. During this period, they were gradually shaped to procure food pellets at a FR25-continuous chained schedule of reinforcement (FR25-CRF). Under this schedule, animals had to initially press the lever 25 times to break the FR threshold, which initiated the beginning of a "meal", or eating bout. Every press thereafter resulted in one pellet. Importantly, absence of lever presses for one minute at any point in the FR25-CRF schedule restarted the schedule. After breaking the FR25 threshold, a schedule reset served as the endpoint for a meal. The total amount of pellets earned during a meal constituted the "meal size." During shaping, the FR schedule was doubled every two days, apart from the last increase, which went from FR16 to FR25. A five day "Pre-Surgery Baseline" period ensued after the shaping period. After, animals underwent surgery and were placed back into RCE chambers for a 5-6 day recovery period. This was followed by a six day "Post-Surgery Baseline."

After the Post-Surgery Baseline, unsignaled foot shock was introduced in the Foraging Zone for a two week "Shock" period. Shocks (0.8 mA; 1s) occurred pseudo-randomly throughout the day (approximately 2 shocks/hr; maximum of 48 shocks/day). Finally, after the two week Shock period, shocks were terminated and a subsequent two week "Extinction" period was initiated. **Figure 3.1B** lists the experimental timeline. At the end of the experiment, animals

were killed with an overdose with Beuthanasia, then transcardially perfused with physiological saline/fixative and their brains harvested for histology.

Surgery and histology

Animals were anesthetized with a ketamine/xylazine cocktail (30 mg/kg ketamine, 2.5 mg/kg xylazine) and placed into stereotaxic frames for bilateral electrolytic (1 mA; 7s constant current) lesion of the lateral habenula (from bregma: AP -3.3; ML ± 0.8; DV -5.2) (Paxinos & Watson, 1998) or sham surgery (electrodes lowered 1 mm above lateral habenulae without passing current). Lesions were made using epoxy coated insect pins (#00, 0.5 mm exposed tip) connected to a precision lesion making device (Grass Medical Instrument, Quincy, MA). At the end of the experiment, animals were overdosed with Beuthanasia and transcardially perfused with 0.9% saline followed by 10% buffered formalin. Brains were then extracted and left overnight in the fixative at 4°C. The following morning, the fixative was replaced with 30% sucrose solution. Brains remained in the sucrose solution until fully sunk before being sectioned by a microtome (Leica SM2010R; Leica Biosystems, Nußloch, Germany). Transverse, 50 µm cuts were collected and mounted onto gelatin-subbed slides for subsequent cresyl and Prussian blue staining, which was used to visualize the extent of lesion damage. Coordinates and reconstruction of lesion damage (**Figure 3.2**) was accomplished using the The Rat Brain in Stereotaxic Coordinates (Paxinos & Watson, 1998). **Figure 3.3** shows representative histology of the LHb lesion.

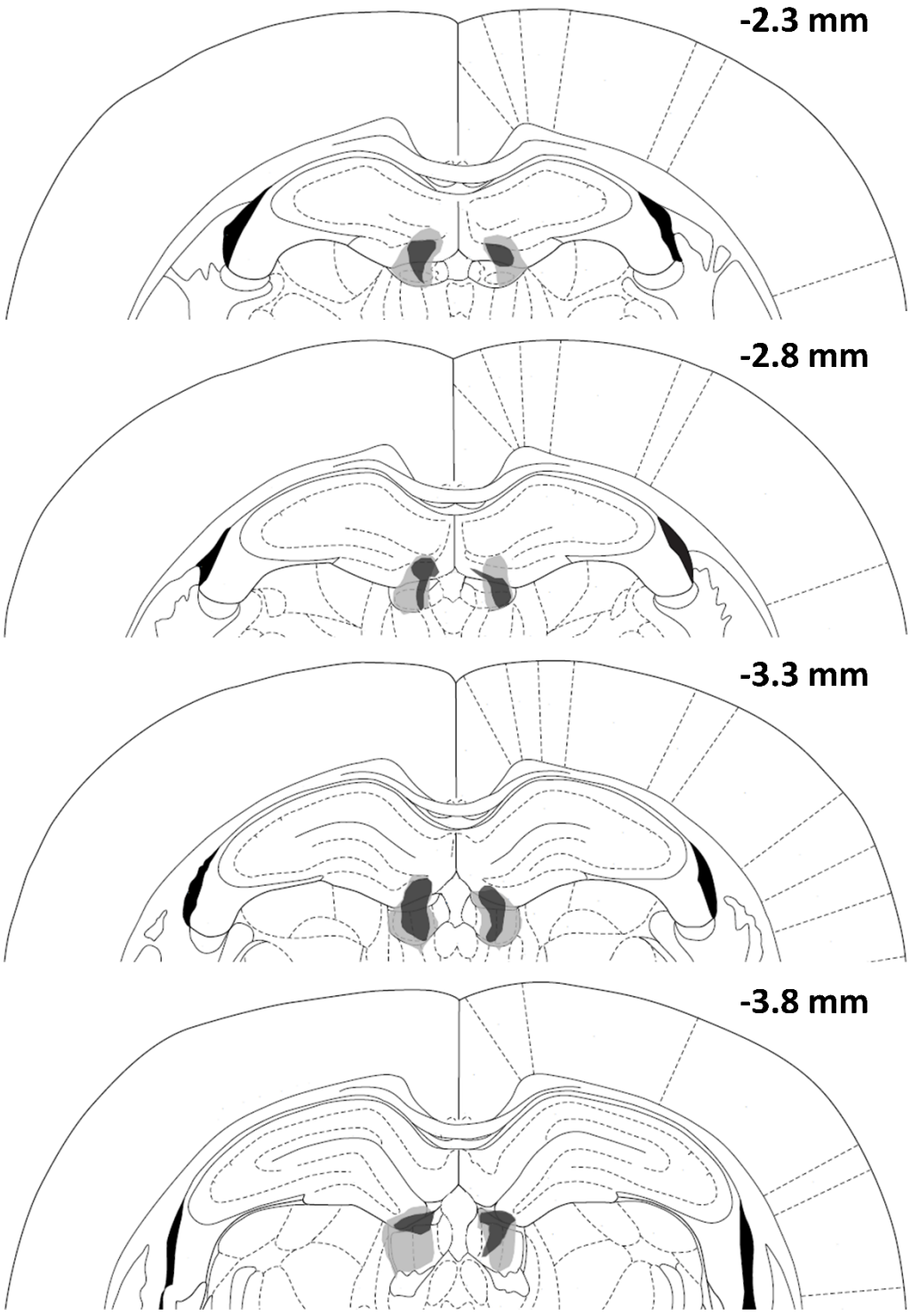

Figure 3.2. Histological reconstruction of the smallest (dark-shaded) and largest (light-shaded) lesions of the lateral habenula. Numbers indicated the mm posterior to bregma. Plates adapted from Paxinos and Watson (1998).

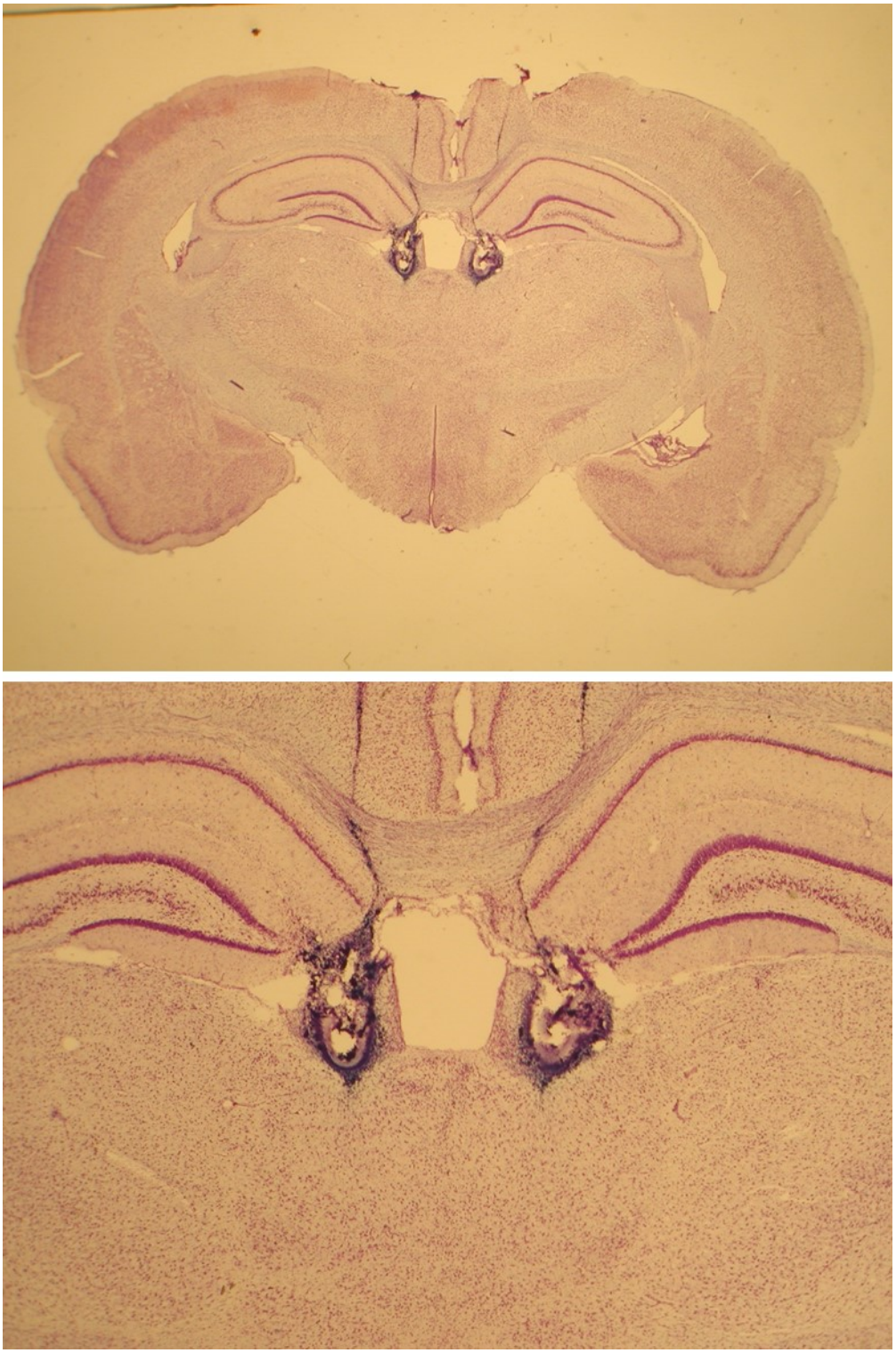

Figure 3.3. Representative histology of lateral habenula electrolytic lesion. Coordinates from bregma: AP -3.3; ML ± 0.8; DV -5.2 (Paxinos & Watson, 1998).

Statistics

Normality for each variable was assessed using the Shapiro-Wilk test and by examination of QQ-plots. Bonferroni-corrected, one-sample t-tests for both groups were used to assess the initial percent change in Pre-Surgery Baseline behavior following surgery. For parametric variables, comparisons between groups across the experimental phases were conducted using mixed factor ANOVAs, with a between subjects factor of group (Lesion or Intact) and a within subjects factor of experiment phase (Pre-Surgery Baseline, Post-Surgery Baseline, Shock week 1, Shock week 2, Extinction week 1, Extinction week 2) using SPSS (SPSS Inc., Chicago, IL).

Greenhouse-Geisser corrected degrees of freedom were used when sphericity was violated (Mauchly's Test). Parametric post hoc comparisons of group at each time point were conducted with one way between subjects ANOVAs, whereas within subjects post hoc comparisons were conducted with paired t tests. For non-parametric data, comparisons between groups across the experimental phases were conducted using rank-based mixed factor ANOVAs using the R package nparLD (Noguchi et al., 2012). Non-parametric post hoc comparisons of group at each time point were conducted with Wilcoxon rank sum tests, whereas within subjects post hoc comparisons were conducted with rank-based repeated subjects ANOVAs. All statistical tests were performed two-tailed with the alpha value set to 0.05 and all post hoc tests were Bonferroni-corrected. Nine animals were excluded from analysis due to incomplete, misplaced or unilateral lesions of the lateral habenula.

3.3 Results

There was not a significant difference between those destined for electrolytic lesion of the LHb ("Lesion group"; n = 6) and those in the sham operated control group ("Intact group"; n = 6) on average daily meal frequency during the Pre-Surgery Baseline phase. Following surgery,

however, there was a significant escalation in the Lesion group that was not seen in the Intact animals (Group: $F_{1, 9.932} = 7.7$, $p = 0.02$; Phase: $F_1 = 4.645$, $p = 0.031$; Group*Phase: $F_1 = 5.429$, $p = 0.02$; Post Hoc: Lesion pre-surgery v. post-surgery, $F_1 = 15.625$, $p < 0.001$; Intact pre-surgery v. post-surgery, $F_1 = 0.032$, $p > 0.999$; Lesion v. Intact pre-surgery, $W = 10.5$, $p = 0.523$; Lesion v. intact post-surgery, $W = 0$, $p = 0.004$; **Figure 3.4A**, left). As there was substantial Pre-Surgery Baseline variability in meal patterns, likely associated with modest meal procurement costs [FR25; avg. daily meal frequency median = 10.1, max. = 17.8, min. 7.2; avg. daily meal size (pellets/meal) median = 80.39, max. = 113.1, min. = 40.8] daily meal frequency, meal size and total pellet intake for each animal were additionally normalized to their respective Pre-Surgery Baseline averages to facilitate interpretation of surgery effects with respect to animals' individual baseline differences (Kim et al., 2014). One sample t-tests (Bonferroni corrected) confirm the unique escalation of the average daily meal frequency following bilateral destruction of the LHb (Lesion pre-surgery v. post-surgery: $t_5 = 3.68$, $p = 0.029$; Intact pre-surgery v. post-surgery $t_5 = 0.133$, $p > 0.999$; **Figure 3.4A**, middle) with animals in LHb lesion group having a 40% increase from their Pre-Surgery Baseline average.

There was only a main effect of experiment phase on Pre-Surgery Baseline versus Post-Surgery Baseline average daily meal size (pellets/meal), although there was a trend for a group by experimental phase interaction (Group: $F_{1, 9.935} = 2.848$, $p = 0.123$; Phase: $F_1 = 3.927$, $p = 0.048$; Group*Phase: $F_1 = 2.727$, $p = 0.099$; **Figure 3.4B**, left). However, one-sample t-tests assessing individual percent change from their Pre-Surgery Baseline indicates that those in the Lesion group experienced a significant decrease in average daily meal size (Lesion pre-surgery v. post-surgery: $t_5 = 4.197$, $p = 0.017$; Intact pre-surgery v. post-surgery: $t_5 = 0.001$, $p > 0.999$;

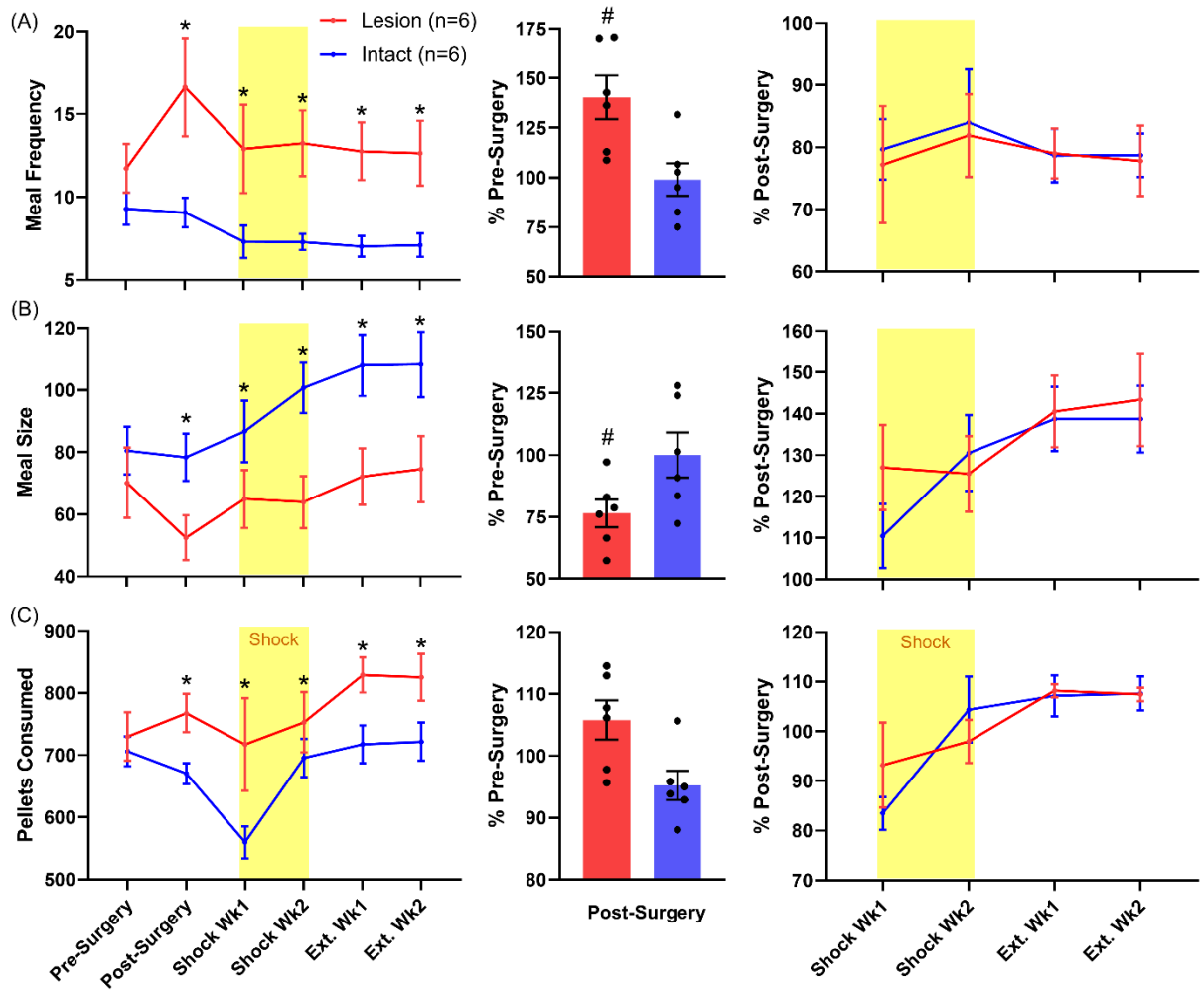

Figure 3.4. Foraging variables. **(A-C)** each row depicts data for average **(A)** daily meal frequency (number of meals/day), **(B)** meal size (average food pellets/meal/day) and **(C)** pellets consumed. The leftmost columns shows the raw data for each respective variable across all phases of the experiment. Middle columns show the average percent change from Pre-Surgery Baseline during the Post-Surgery Baseline for respective row variables (all animals normalized to their Pre-Surgery Baseline average; individual data represented by the black circles in bar graphs). To better visualize the impact of foot shock given Post-Surgery Baseline differences, the rightmost columns show the average percent change from Post-Surgery Baseline across Shock weeks 1-2 and Extinction weeks 1-2 for respective row variables (all animals normalized to their Post-Surgery Baseline average). * indicates significant group difference at a given time point ($p < 0.05$). # indicates significant percent change from Pre-Surgery Baseline ($p < 0.05$). Data are presented as mean ± SEM.

Figure 3.4B, middle), with lesioned animals having a 24% decrease from their Pre-Surgery Baseline average and Intact animals having a less than 0.001% decrease.

There was a significant interaction between group and experimental phase in the average daily pellet intake between Pre-Surgery Baseline and Post-Surgery Baseline. Post hoc analyses reveal that despite no significant changes in average daily pellet intake for each group across phases, there was nonetheless a significant difference at the group level during the Post-Surgery Baseline (Group: $F_{1, 10} = 2.505$, $p = 0.145$; Phase: $F_{1, 10} = 0.006$, $p = 0.938$; Phase*Group: $F_{1, 10} = 7.003$, $p = 0.024$; Post Hoc: Lesion pre-surgery v. post-surgery, $t_5 = -1.743$, $p = 0.284$; Control pre-surgery v. post-surgery, $t_5 = 2.058$, $p = 0.19$; Lesion v. Intact pre-surgery, $F_{1, 10} = 0.269$, $p = 0.615$; Lesion v. Intact post-surgery, $F_{1, 10} = 7.776$, $p = 0.019$; **Figure 3.4C**, left). One sample t-tests confirms a non-significant percent change from Pre-Surgery Baseline in both groups following surgery (Lesion pre-surgery v. post-surgery: $t_5 = 1.84$, p = 0.25; Control pre-surgery v. post-surgery, $t_5 = 2.022$, p = 0.198; **Figure 3.4C**, middle).

Given the escalation in meal frequency in LHb lesioned animals, it was of interest to measure the efficiency of their operant behavior following lesions as well. Earning a meal requires not only effort to complete the FR25 component of the schedule, but sustained attention directed toward pressing, as the schedule resets if the lever is inactive for more than one minute. To assess animals' daily "operant efficiency," or their ability to follow through with the FR25 requirement in order to enter the continuous reinforcement component of the schedule, the ratio of non-reinforced lever presses (lever presses outside of CRF; total lever presses – total pellets) to the absolute minimum required non-reinforced lever presses given the number of obtained meals (meal frequency * 24 required non-reinforced lever presses), was calculated. Thus, having a higher total number of non-reinforced lever presses (numerator) than what is required given the

total meals one obtained (denominator) results in a greater percent of "excess" lever presses. There was no significant effects of group nor a group by experimental phase interaction, but a main effect of experiment phase indicating that both groups improved their operant efficiency during the Post-Surgery Baseline (Group: $F_{1, 10} = 0.715$, $p = 0.418$; Phase: $F_{1, 10} = 11.97$, $p = 0.006$; Group*Phase: $F_{1, 10} = 0.631$, $p = 0.446$; **Figure 3.5**, left). One-sample t-tests reveal a significant percent change from Pre-Surgery Baseline only in the Intact group, however (Lesion pre-surgery v. post-surgery, $t_5 = 1.787$, $p = 0.268$; Intact pre-surgery v. post-surgery, $t_5 = 3.190$, $p = 0.049$; **Figure 3.5**, middle).

There were no statistically reliable differences in average daily distance travelled (locomotor behavior) between groups during the Pre-Surgery Baseline nor Post-Surgery Baseline (Group: $F_{1,10} = 0.066$, $p = 0.802$; Phase: $F_{1, 10} = 1.555$, $p = 0.241$; Group*Phase: $F_{1, 10} = 7.930$, $p = 0.018$; Post Hoc: Lesion pre-surgery v. post-surgery, $t_5 = -2.481$, $p = 0.112$; Intact pre-surgery v. post-surgery, $t_5 = 1.367$, $p = 0.460$; Lesion v. Intact pre-surgery: $F_{1, 10} = 2.09$, $p = 0.179$; Lesion v. Intact post-surgery: $F_{1, 10} = 2.296$, $p = 0.161$; **Figure 3.6A**, left). One sample t-tests of the percent change from Pre-Surgery Baseline confirms this outcome (Lesion pre-surgery v. post-surgery: $t_5 = 2.565$, $p = 0.101$; Intact pre-surgery v. post-surgery; $t_5 = 1.545$, $p = 0.37$; **Figure 3.6A**, middle). Both groups spent more time in the Foraging Zone during the Post-Surgery Baseline than the Pre-Surgery Baseline, with no significant differences between groups (Group: $F_{1, 10} = 0.006$, $p = 0.941$; Phase: $F_{1, 10} = 10.209$, $p = 0.01$; Group*Phase: $F_{1, 10} = 0.102$, $p = 0.756$; **Figure 3.6B**, left). One sample t-tests on percent change from Pre-Surgery Baseline shows that specifically Intact animals experienced significant change from the Pre-Surgery Baseline phase (Lesion pre-surgery v. post-surgery: $t_5 = 1.789$, $p = 0.267$; Intact pre-surgery v. post-surgery: $t_5 = 3.308$, $p = 0.043$; **Figure 3.6B,** middle).

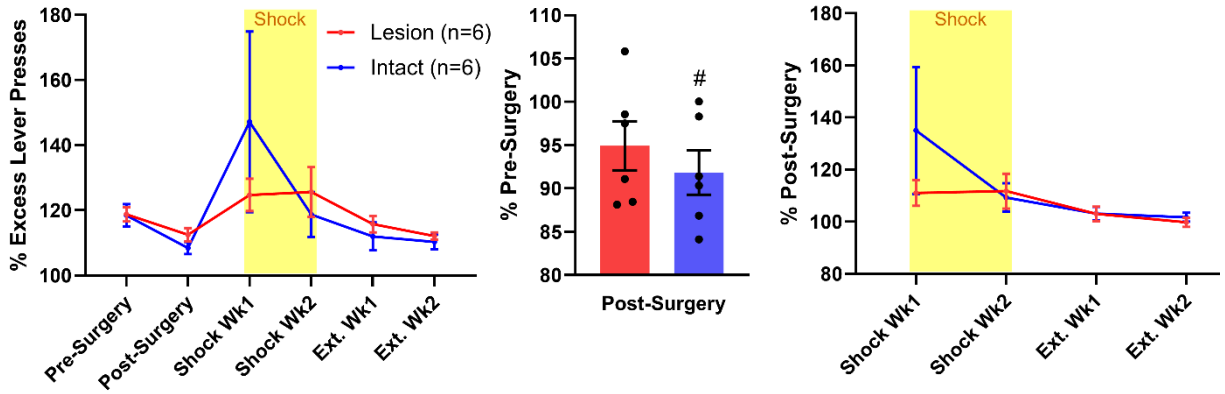

Figure 3.5. Operant Efficiency. To gain access to the continuous reinforcement (CRF) component of the meal schedule, animals must lever press a minimum of 25 times (animals reinforced on 25th press; FR25). However, the schedule resets if one minute elapses without pressing. If while lever pressing the schedule resets before a meal is initiated, animals must start over, resulting in excess, non-reinforced lever presses. "Operant Efficiency" captures the percent of excess, non-reinforced lever presses relative to the absolute minimum required, given the amount of meals obtained. It is calculated as follows: total lever presses outside of CRF (total lever presses – total pellets) / minimum required non-reinforced lever presses given meals (24 presses * number of meals obtained). The leftmost graph shows the raw data across all phases of the experiment. The middle graph shows the average percent change from Pre-Surgery Baseline during the Post-Surgery Baseline (all animals normalized to their Pre-Surgery Baseline average; individual data represented by the black circles in bar graphs). To better visualize the impact of foot shock given Post-Surgery Baseline differences, the rightmost graph shows the average percent change from Post-Surgery Baseline across Shock weeks 1-2 and Extinction weeks 1-2 for respective row variables (all animals normalized to their Post-Surgery Baseline average). * indicates significant group difference at a given time point ($p < 0.05$). # indicates significant percent change from Pre-Surgery Baseline ($p < 0.05$). Data are presented as mean ± SEM.

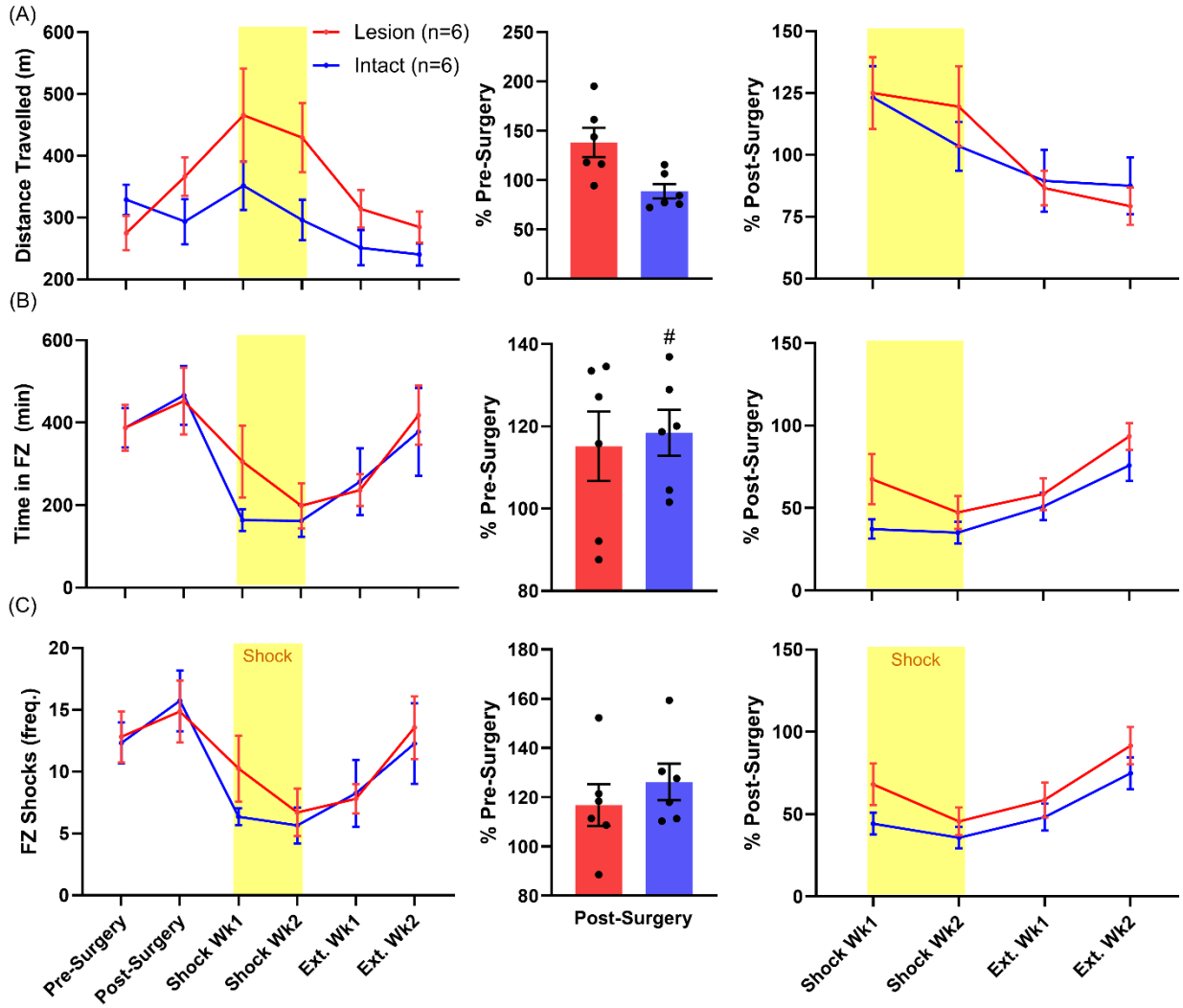

Figure 3.6. Movement-related variables. **(A-C)** each row depicts data for average **(A)** daily distanced travelled (meters), **(B)** Time spent in the Foraging Zone (minutes) and **(C)** frequency of foot shocks received in the Foraging Zone (note that shocks received during Pre and Post-Surgery Baselines are theoretical; shocks triggered but no cables were attached to grid). The leftmost columns shows the raw data for each respective variable across all phases of the experiment. Middle columns show the average percent change from Pre-Surgery Baseline during the Post-Surgery Baseline for respective row variables (all animals normalized to their Pre-Surgery Baseline average; individual data represented by the black circles in bar graphs). To better visualize the impact of foot shock given Post-Surgery Baseline differences, the rightmost columns show the average percent change from Post-Surgery Baseline across Shock weeks 1-2 and Extinction weeks 1-2 for respective row variables (all animals normalized to their Post-Surgery Baseline average). Data are presented as mean ± SEM. * indicates significant group difference at a given time point ($p < 0.05$). # indicates significant percent change from Pre-Surgery Baseline ($p < 0.05$).

Analysis of the average daily meal frequency from the Post-Surgery Baseline through the remaining phases (Shock week 1- Extinction week 2) indicates that LHb lesioned animals continued to have more frequent meals than Intact animals, regardless experimental phase (Group: $F_{1, 9.787} = 0.003$; Phase: $F_{2.67} = 6.796$, $p < 0.001$; Group*Phase: $F_{2.67} = 0.738$, $p = 0.514$; Post Hoc: post-surgery v. shock wk1, $F_1 = 9.763$, $p = 0.007$; post-surgery v. shock wk2, $F_1 = 10.707$, $p = 0.004$; post-surgery v. extinction wk1, $F_1 = 26.\ 506$, $p < 0.001$, post-surgery v. extinction wk2, $F_1 = 25.487$, $p < 0.001$; **Figure 3.4A**, left). That both groups experienced a significant decrease in meal frequency from Shock week 1 onwards, as shown by the main effect of experimental phase, suggests the impact of pseudo-random, unsignaled foot shocks in Foraging Zone during the Shock phase affected meal frequency in both groups similarly (**Figure 3.4A**, right).

Similar to meal frequency, there was a significant main effect of group and experimental phase, but no significant group by experimental phase interaction, in average daily meal size, with the Lesion group showing a significantly decreased average daily meal size relative to Intact animals from the Post-Surgery Baseline through the end of Extinction. Both groups increased their meal size relative to their Post-Surgery Baseline's beginning week 2 of Shock, which lasted throughout the Extinction phase (Group: $F_{1, 9.952} = 7.062$, $p = 0.024$; Phase: $F_{2.884} = 11.122$, $p < 0.001$; Group*Phase: $F_{2.884} = 0.854$, $p = 0.46$; Post Hoc: post-surgery v. shock wk1, $F_1 = 2.267$, $p = 0.528$; post-surgery v. shock wk2, $F_1 = 13.636$, $p < 0.001$; post-surgery v. extinction wk1, $F_1 = 31.869$, $p < 0.001$; post-surgery v. extinction wk2, $F_1 = 33.197$, $p < 0.001$; **Figure 3.4B,** left/right).

Analysis of the average daily total pellet intake detected only a main effect of group and experimental phase, again showing that LHb lesioned animals obtained more pellets overall

across Post-Surgery Baseline through Extinction periods relative to intact animals. The analysis also indicates that both groups were able to maintain their Post-Surgery Baseline level of pellet intake during Shock, and that both groups increased their pellet intake during the Extinction phase (Group: $F_{1, 7.259} = 6.730$, $p = 0.034$; Phase: $F_{2.403} = 7.244$, $p < 0.001$; Group*Phase $F_{2.403} = 1.549$, $p = 0.208$; Post Hoc: post-surgery v. shock wk1, $F_1 = 4.735$, $p = 0.118$; post-surgery v. shock wk2, $F_1 = 0.005$, $p > 0.999$; post-surgery v. extinction wk1, $F_1 = 13.138$, $p = 0.001$; post-surgery v. extinction wk2, $F_1 = 13.888$, $p = 0.001$; **Figure 3.4C**, left/right). There was only a main effect of experimental phase on operant efficiency, which shows that during the shock phase, both groups had an increase in excess lever presses relative to the Post-Surgery Baseline, which recovered to Post-Surgery Baseline levels during the Extinction phase (Group: $F_{1, 7.685} = 2.07$, $p = 0.19$; Phase: $F_{2.27} = 6.91$, $p < 0.001$; Group*Phase: $F_{2.27} = 0.674$, $p = 0.528$; Post Hoc: post-surgery v. shock wk1, $F_1 = 22.25$, $p < 0.001$; post-surgery v. shock wk2, $F_1 = 13.956$, $p < 0.001$; post-surgery v. extinction wk1, $F_1 = 1.535$, $p = 0.861$; post-surgery v. extinction wk2, $F_1 = 0.541$, $p > 0.999$; **Figure 3.5**, left/right).

There were no statistically reliable changes in average daily distanced travelled in both groups from the Post-Surgery Baseline onward, although there was a trend for a significant main effect of group (Group: $F_{1, 10} = 3.708$, $p = 0.083$; Phase: $F_{2.261, 22.613} = 8.535$, $p = 0.001$; Group*Phase: $F_{2.261, 22.613} = 0.827$, $p = 0.463$; Post Hoc: post-surgery v. shock wk1, $p = 0.112$; post-surgery v. shock wk2, $p > 0.999$; post-surgery v. extinction wk1, $p = 0.184$; post-surgery v. extinction wk2, $p = 0.064$**; Figure 3.6A**, left/right). The introduction of foot shock in the Foraging Zone led to significant decreases in average daily time spent in the Foraging Zone in both groups, from Shock week 1 to Extinction week 1, with values recovering to Post-Surgery Baseline levels by Extinction week 2. There was no group by experimental phase interaction

(Group: $F_{1, 9.776} = 0.543$, $p = 0.478$; Phase: $F_{2.457} = 18.09$, $p < 0.001$; Group*Phase: $F_{2.457} = 1.056$, $p = 0.358$; Post Hoc: post-surgery v. shock wk1, $F_1 = 22.761$, $p < 0.001$; post-surgery v. shock wk2, $F_1 = 35.294$, $p < 0.001$; post-surgery v. extinction wk1, $F_1 = 23.954$, $p < 0.001$; post-surgery v. extinction wk2, $F_1 = 5.805$, $p = 0.064$; **Figure 3.6B**, left/right). Likewise, both groups significantly reduced the amount of shocks they received relative to their theoretical amounts received during the Post-Surgery Baseline (ANY-maze triggered shock during both Pre and Post-Surgery Baseline but shock cables were not connected to chambers) from Shock week 1 to Extinction week 1, with values recovering to Post-Surgery Baseline levels by Extinction week 2 (Group: $F_{1, 9.737} = 0.337$, $p = 0.575$; Phase: $F_{2.674} = 15.96$, $p < 0.001$; Group*Phase: $F_{2.674} = 0.576$, $p = 0.611$; Post Hoc: post-surgery v. shock wk1, $F_1 = 29.19$, $p < 0.001$; post-surgery v. shock wk2, $F_1 = 54.045$, $p < 0.001$; post-surgery v. extinction wk1, $F_1 = 31.197$, $p < 0.001$; post-surgery v. extinction wk2, $F_1 = 6.184$, $p = 0.52$; **Figure 3.6C**). As typical avoidance studies employ a limited time frame for assessing avoidance behavior, examination of the time spent and shocks received in the Foraging Zone on the very first day of Shock was also examined. There were no differences between groups on the first day of Shock regarding time spent ($t_{10} = 0.59$, $p = 0.569$) or shocks received ($t_{10} = 0.204$, $p = 0.842$) in the Foraging Zone.

3.4 Discussion

Using the RCE in rats, which affords a comprehensive, ethological approach to behavioral testing, the present study shows that the LHb is implicated in day-to-day naturalistic appetitive behavior. Specifically, lesions of the LHb led to baseline changes in daily meal pattern. Most striking was the substantial increase in meal frequency, or "eating bouts" per day during the Post-Surgery Baseline phase. This increase in meal frequency was accompanied by a simultaneous decrease in daily meal size, or the average pellets obtained per meal within a day. This was evident when considering animals' individual Pre-Surgery Baseline differences by normalizing their meal size data during the Post-Surgery Baseline to their respective Pre-Surgery Baseline averages, and in subsequent analyses incorporating the Post-Surgery Baseline, Shock and Extinction phases. Lesioned animals also consumed more food pellets compared to Intact animals following surgery, an effect that lasted throughout the experiment.

In addition to the observed altered foraging pattern, it was also of interest to know whether lesioned animals in this experiment likewise had attentional disturbances that could have further affected their foraging behavior. It has been shown that habenula lesions cause attentional disturbances in rats, as measured by the 5-choice serial reaction time task (Lecourtier & Kelly, 2005). In this task, animals must attend to a brief light stimulus that alternates between one of five food recesses and correctly nose-poke into the food recess where the stimulus had just occurred. Habenula lesioned rats made more errors in this task compared to sham operated controls. One way in which attention deficits could affect pellet procurement is by preventing sustained lever pressing that is required to complete the FR25 component of the FR25-CRF chained schedule of reinforcement, as the schedule resets if the lever is inactive for more than one minute. So, if an animal is not committed to completing the FR component of the schedule,

and only sporadically presses, the schedule could reset and the animal will have to restart the FR component if it wishes to obtain pellets, leading to excess non-reinforced lever presses. Interestingly, LHb lesioned animals were no different than Intact animals throughout the experiment, and both groups showed an increase amount of excess non-reinforced lever pressed during the Shock phase that later recovered during Extinction. There was also no statistically reliable effect of lesions on overall locomotor activity.

Following the introduction of pseudo-random, unsignaled foot shocks in the Foraging Zone during the Shock phase, both Lesion and Intact animals significantly decreased their meal frequency while significantly increasing their meal size, consistent with previous closed economy experiments in rats (Fanselow et al., 1988; Helmstetter & Fanselow, 1993; Pellman et al., 2017). Less frequent meals minimize the exposure to shock in the Foraging Zone, while the simultaneous increase in meal size prevents compromising pellet intake resulting from fewer meals. That this strategy persisted into the Extinction phase is likely due to negative reinforcement (Mowrer, 1939). Analysis also shows that animals overall were able to maintain their Post-Surgery Baseline levels of pellet intake during the shock phase. Despite this, however, LHb lesioned animals continued having significantly higher meal frequencies and significantly lower meal sizes relative to the Intact group during the Shock and subsequent Extinction phases.

In terms of avoidance behavior, both groups were identical during the Shock and Extinction phases; both groups significantly decreased their time spent in the Foraging Zone during the Shock phase, thereby minimizing the number of actual shocks received and returned to Post-Surgery Baseline levels of time spent in the Foraging Zone by week 2 of Extinction. There was no main effect of group as there was with the foraging variables. Even when investigating the first day of Shock, which more closely matches typical avoidance learning

paradigms in terms of task time frame and number of shock-context pairings, there was no difference in the number of shocks received nor time spent in the Foraging Zone on that first day. Altogether, this suggests that the LHb is not crucial for the acquisition of passive avoidance behavior in chronic, approach-avoid conflict scenarios involving unsignaled foot shock, nor the presumed extinction of anxiety toward the Foraging Zone and subsequent discontinuation of passive avoidance behavior.

In sum, the overall pattern of results suggests that destruction the LHb disrupts baseline appetitive foraging behavior, but not passive avoidance of unsignaled threat during approach-avoid conflict (**Table 3.1**). Second, like their Intact counterparts, LHb lesioned animals similarly adapted to shock by re-organizing meal patterns, which shows that the LHb is not implicated in all forms of behavioral flexibility. The aberrant foraging behavior was characterized by more frequent but less substantial meals. One possible mechanism to explain this aberrant foraging behavior is that without the LHb, there is ineffectual relay of appetitive drive information from the LHA to the mesocorticolimbic dopaminergic system, which results in a failure of such information to appropriately modulate the incentive saliency of the food cues (e.g., the operant lever) and approach behavior toward them. The following passage describes the logic of this hypothesis.

The LHA receives internal state information regarding hunger or satiety by way of the arcuate nucleus, which it uses to either facilitate or suppress food seeking behavior, respectively (Qualls-Creekmore & Munzberg, 2018). As stated in the introduction, glutamatergic LHA-to-LHb activity appears to negatively regulate appetitive behavior (especially toward highly rewarding reinforcers, e.g., drugs of abuse and/or high caloric solutions). It is well known that

Table 3.1. Summary of lateral habenula lesion effects relative to sham operated control animals.

Dependent Variable	Lesion Effect (v. Intact)
Meals	↑
Meal Size	↓
Pellets Consumed	↑
Distance Travelled (locomotion)	—
Time spent in Foraging Zone	—
Shocks in Foraging Zone	—
Overall effect of shock	—

Lateral habenula lesions significantly increased the frequency of meals but decreased meal size (average pellet/meal). Lesions also increased the overall number of pellets obtained. This altered foraging pattern subsisted throughout all phases of the experiment. Despite this baseline change in foraging pattern, the effect of pseudo-random, unsignaled foot shock in the Foraging Zone during the Shock phase of the experiment affected both groups equally; both groups decreased the meal frequency and increased their meal size to minimize exposure and both groups similarly reduced their time spent in the Foraging Zone during Shock. This strategy led to both groups equally avoiding the number of foot shocks received.

activation of the LHb inhibits ventral tegmental area (VTA) dopamine firing by exciting GABAergic interneurons within the rostromedial tegmental nucleus (Jhou et al., 2009; Matsumoto & Hikosaka, 2007), which in turn can reduce the assignment of incentive salience to reward related cues and reduce approach behavior via the mesocorticolimbic pathway (Berridge, 2012). Indeed, a previous study lesioned the fasciculus retroflexus, the primary efferent fiber bundle of the habenula, and found that rats had exaggerated "sign-tracking" behavior in a Pavlovian autoshaping paradigm—a behavior which is dependent on dopaminergic activity and is thought to arise from high incentive saliency being placed on cues the predict reward (Danna et al., 2013).

Thus, perhaps one mechanism by which the LHA can suppress inappropriate appetitive behavior (given the internal hunger/satiety state signaled by arcuate nucleus) is by indirectly inhibiting dopaminergic firing in the VTA via the LHB, which functionally results in decreased incentive saliency of stimuli associated with food reward and decreased approach behavior toward those stimuli—e.g., engagement with the operant lever. Therefore, when the LHb "node" is removed, the instruction from the LHA to inhibit foraging behavior does not reach the mesocorticolimbic dopamine circuit, and animals engage in more appetitive, meal seeking behavior (**Figure 3.7**). Moreover, that LHb lesioned had consistently decreased meal size suggests that they may be compensating for this compulsive meal seeking behavior, and that perhaps consummatory mechanisms that prevent over-eating are *fairly* intact (LHb lesioned animals still consumed slightly more pellets than Intact animals overall). Alternatively, LHb destruction could have affected homeostasis itself, which necessitated frequent, low pellet density meals.

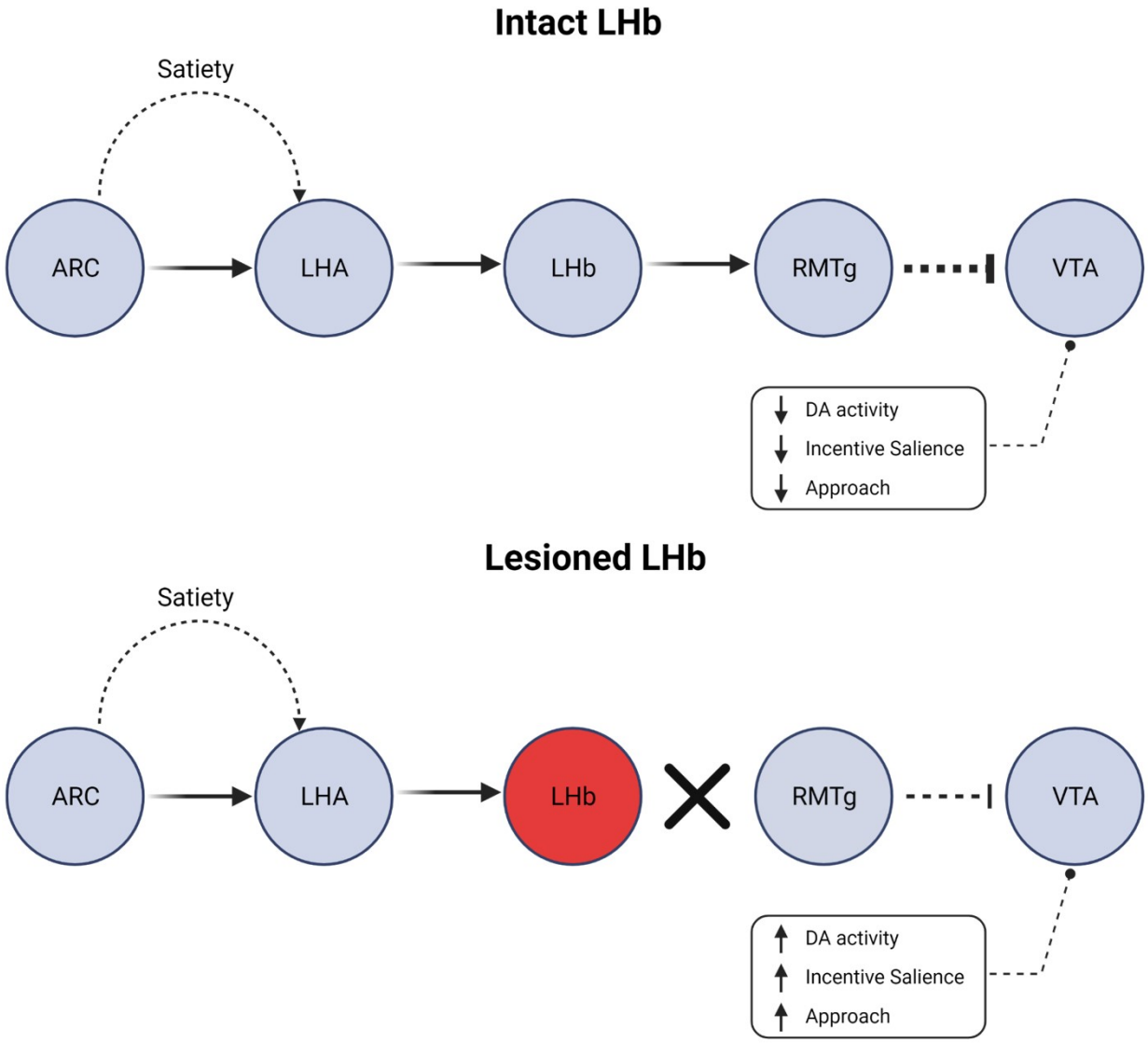

Figure 3.7. Hypothetical mechanism of lateral habenula lesion effects. (Top) The lateral hypothalamic area (LHA) receives satiety instruction from the arcuate nucleus of the hypothalamus. During satiety, LHA glutamatergic neurons excite neurons in the lateral habenula (LHb), which in turn excite GABAergic cells within the rostromedial tegmental nucleus (RMTg). Finally, the RMTg inhibits the ventral tegmental area (VTA), resulting in decrease dopamine (DA) expression in the mesocorticolimbic pathway and functionally, decreased incentive salience and approach toward food cues/sources. (Bottom) Destruction of the LHb prevents satiety instruction from the hypothalamic nuclei from reducing VTA activity, functionally resulting in inappropriate incentive salience of food cues/sources and food seeking behavior.

This study is of course not without its limitations. First and most obvious is the fact that the study had a relatively small sample size, which likely interacted with variation in lesion damage among the Lesion group to produce a substantial degree of behavioral variability in the study. Second, an extended Post-Surgery Baseline period could have been used to observe whether the increase in meal frequency and decrease in meal size in lesioned animals eventually subsided, or perhaps became more exaggerated with time before proceeding to the Shock phase. Also, permanent destruction of the LHb through electrolytic lesion likely disrupted fibers of passage, including those of the MHb (Zahm & Root, 2017). Temporary inactivation methods in future studies are needed to verify the current studies results. Similarly, all animals had partial damage to the dorsal hippocampus.

Despite these limitations, the study nonetheless highlights the utility of the RCE and ethological paradigms in general. For example, because behavior was measured nearly continuously and for extended periods, the unexpected finding that LHb loss-of-function indeed caused changes in appetitive behavior involving standard foot pellets—contrary to previous reports (Nair et al., 2020)—was detected. Equally contributing to this finding is the naturalistic conditions surrounding food procurement in the RCE. This includes the schedule of reinforcement designed to simulate food procurement costs (FR component) and food consumption costs (CRF component) associated with foraging in the wild, and in the fact that food is not supplemented outside of the testing environment, leaving animals in charge of their daily nourishment. Another unique attribute of the RCE is the ability and ease to screen baseline changes in a variety of behaviors following lesions that could influence the interpretation of behavioral results during a later test session—not only that, but you can assess baseline changes in behaviors in the exact same context in which the animal will later be tested. This study's

findings especially apply; by examining changes in baseline foraging behavior under risk-free conditions during the Post-Surgery Baseline, this permitted the conclusion that although lesioned animals *continued* to have more frequent, less pellet dense meals during the Shock phase compared to sham-operated controls, they nonetheless re-organized their meals in a similar fashion to adapt to this new challenge. The paradigm also can screen for baseline changes in behaviors adjacent to those of interest during a test period but can nonetheless influence interpretation of test results. For example, the RCE can detect whether destruction of a brain area led to overall increases in locomotor activity in the apparatus under risk-free conditions. If so, this could increase the amount of time spent in the Foraging Zone during the Shock phase, a measure of avoidance behavior and anxiety. Having the Post-Surgery Baseline locomotor data could help clarify whether the lesioned structure is truly involved in avoidance/anxiety behavior.

Chapter 4. The Neural Signature of Innate Fear in Mice Facing a Terrestrial Predator Threat

4.1 Introduction

From early studies in ethology, it has been shown that animals instinctually exhibit defensive behavior toward evolutionarily reliable signals of danger—that is, they possess genetically hardwired "innate fears" of certain stimuli that in their evolutionary history have been consistent sources of threat. The most well-known—albeit controversial—ethological study to examine this phenomena was conducted by Konrad Lorenz and Nikolaas Tinbergen (Lorenz, 1939; Tinbergen, 1939), who reported that young, naïve fowl exhibited fear toward an overhead black cardboard silhouette, that when flown over the chicks in one direction resembled a goose (long neck and short tail) and when flown over the other resembled a hawk (short neck and long tail); a natural avian predator of fowl. The hawk configuration was stated to uniquely elicit innate fear. However, future replications in many types of fowl, both free ranging and those raised in captivity, have shown that they innately fear *any* overhead "looming" type stimulus, regardless of shape, and that other parameters such as size and speed of the stimulus are primary "releasers" of the defensive behavior (Schleidt et al., 2011). As mentioned in Chapter 1, overhead looming stimuli have been shown to elicit innate defensive behavior in laboratory rodents as well.

Most important, however, was that these ethology experiments clearly demonstrated that animals did not have to have prior experience with the stimuli in order to fear them, which went against the prevailing behavioristic view in psychology that fear of any stimulus was learned (Hull, 1929; Watson & Rayner, 1920). Although the study of learned fear and the use of the Pavlovian fear conditioning paradigm has ultimately dominated psychology and neuroscience for more than a century, more researchers are beginning to "think outside the conditioning box" and

are exploring the neurobehavioral underpinnings of innate fear using a variety of paradigms (Beckers et al., 2013; Kim & Jung, 2018; Pellman & Kim, 2016). Contemporary rodent studies investigating innate defensive behavior toward three-dimensional predators (as opposed to partial predator stimuli, e.g., predator odors, overhead 2D shapes) use more controlled stimuli than the historically used live predator. For example, Svodboda et al. (2012) placed male and female rats in a circular, enclosed arena with scattered food pellets and a moving robot. When the animal came within a set distance of the robot, it received an electric shock to their back by means of subcutaneous implanted wire. In this task, they found that females exhibited greater defensive behavior toward the robotic threat than males, confirming previous studies investigating sex differences in innate fear of visual predator stimuli in rats (Shepherd et al., 1992). Another study used a robotically controlled laser beam to "chase" rats, simulating a predator-prey interaction (Wilson et al., 2015).

In addition to simply studying innate fear/defensive behavior toward predatory stimuli, researchers are taking additional inspiration from ethology by studying innate fear of predatory stimuli within the context of naturalistic, foraging tasks involving risky decision-making. This is due to the recognition that defensive behavior and decision-making share overlapping neural circuits (Mobbs & Kim, 2015) and that by studying these circuits under conditions they likely evolved to handle, one can gain a more accurate and complete understanding of their specific functions and dynamics, as well as gain a broader understanding of fear and anxiety as mechanisms intended to keep organisms alive. This in turn can pave the way for the development of more effective treatments for fear and anxiety-related disorders in humans. One such paradigm that investigates both innate fear and risky decision-making in the context of a

naturalistic foraging scenario is the Approach Food-Avoid Predator (AFAP) paradigm, first developed by Choi & Kim (2010).

In all variations of the paradigm, the AFAP apparatus is characterized by a large arena sectioned into a smaller, enclosed "Nest Zone" and larger, open "Foraging Zone." In this arena, hunger-motivated rodents begin the task by being placed into the Nest Zone, and upon opening of a gate, are allowed to procure and consume a food pellet located in the Foraging Zone at a varying distance from the Nest Zone opening. After a baseline period assessing risk-free foraging behavior, during the test session, a predator stimulus is situated at the distal end of the Foraging Zone. Each time the animal attempts to procure the food pellet, the predator stimulus surges toward the animal then resets to its original position. In the original AFAP experiment, the predator stimulus was a programmable LEGO Mindstorms robot, the "Robogator," complete with eyes, a tail and mechanical snapping jaws (Choi & Kim, 2010). As mentioned in Chapter 1, another variation uses 2D (computer generated shapes) and 3D overheard aerial predator stimuli (a life-like plastic owl) instead of the terrestrial Robogator (Zambetti et al., 2019). Predator stimuli in the AFAP paradigm are highly controllable, moving at user defined speeds and distances and as such produce reliable and quantitative interactions with the subjects (Choi & Kim, 2010).

Unlike the longitudinal Risky Closed Economy (RCE), the AFAP paradigm engenders a more *acute* approach-avoidance conflict, with testing occurring on a shorter time scale, and is specialized to predominately elicit and measure "post-encounter" and "circa-strike" behavior. According to the Predatory Imminence theory, post-encounter behavior in prey occurs when prey detects the predator and perceives threat as highly imminent, and is characterized by behaviors that reduce the chance of being detected (e.g., freezing), whereas circa-strike behavior occurs

when the predator begins to actively pursue the aware prey with the intention of capture and consumption and is characterized by the prey fleeing or fighting if threat is inescapable (Fanselow & Lester, 1988; Mobbs & Kim, 2015). A multitude of behavioral variables are still able to measured, including those related to the risky decision-making. Namely, one can evaluate the interaction between fear motivation and hunger motivation by varying the distance of the pellet from the Nest Zone opening during the test session. This manipulation taps into animals the "spatial gradient of fear," whereby pellets located closer to the nest (farthest from the predator) are more easily obtained than those farthest (closest to the predator) (Choi & Kim, 2010; Kim et al., 2018; Kim et al., 2015; Kong et al., 2021). Contextual (associative) fear and passive avoidance can also be measured in the AFAP paradigm. This is accomplished by measuring the latency to leave the Nest Zone on the first, predator-free baseline trial the following the day after the first predator test session.

The AFAP paradigm in rats has yielded unique findings relating to fear and decision-making that were not obtainable using traditional paradigms. It has been shown that amygdala is crucial for producing innate fear of the predator and subsequently inhibiting maladaptive foraging behavior (Choi & Kim, 2010). Further, stimulating the dorsal periaqueductal gray (dPAG) generates fleeing behavior in the AFAP arena sans predator, but that same dPAG stimulation evokes only activity bursts followed by freezing in a traditional Pavlovian fear conditioning chamber (Kim et al., 2013). This shows that brain stimulation-evoked behavior is modulated by context. A series of studies have shown that Predator-responsive basal amygdala cells interact with dorsal CA1 hippocampal place cells to form a spatial gradient of fear in the arena (Kim et al., 2015; Kong et al., 2021). Finally, using the AFAP, it has been demonstrated

that lateral amygdala and prelimbic cells signal impending and immediate threat, respectively (Kim et al., 2018).

The present study sought to characterize the neural signature of innate fear in male and female mice using a mouse-adapted AFAP paradigm (**Figure 4.1**). Adult male and female C57BL/6 mice underwent two days of habituation to the food pellets and Nest Zone, which was followed by a baseline, risk-free foraging assessment period lasting six days. The following day after the last baseline session, animals experienced a single test session. For half of the animals, the test session was composed of four risk-free baseline trials. For the other half, the test session was composed of three risk-free baseline trials and a final trial that included a surging predator; a taxidermy weasel attached to a frame with wheels. Surging was accomplished using a software-activated pneumatic actuator. Group differences in neural activity during the test trial was examined by measuring the protein expression of the immediate early gene c-Fos, a marker for neural activation (Dragunow & Faull, 1989) **(Figure 4.1B**). Specifically, neural activity was measured in the amygdala [basolateral complex (BLA) and central nucleus (CeA)], dorsal periaqueductal gray [dorsomedial (dmPAG) and dorsolateral columns (dlPAG)], dorsal hippocampus CA1 area (proximal and distal CA1); areas contributing to innate fear and risky decision-making behavior in rats as detailed above. In addition, neural activity of the ventral hippocampus CA1 area in a subset of animals was measured, as the structure has been shown to be involved in in arbitrating approach-avoidance decisions (Bryant & Barker, 2020). It was hypothesized that all regions of interest would should greater neural activity following predator exposure.

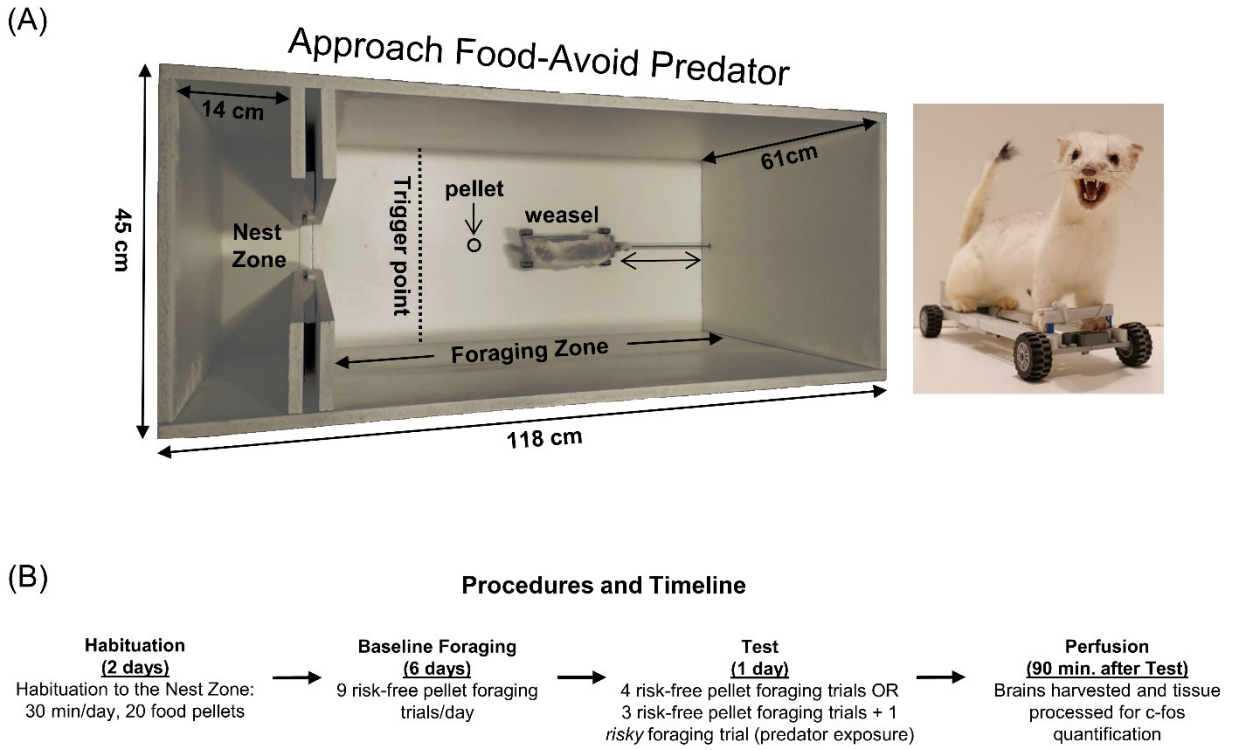

Figure 4.1. The mouse-adapted Approach Food-Avoid Predator (AFAP) paradigm and experimental procedures and timeline. **(A)** AFAP apparatus with measurements and predator stimulus. The predator was a taxidermy weasel attached to a plastic frame with wheels. Predator surging was accomplished via software-controlled pneumatic actuator attached to the plastic frame. **(B)** Experimental procedures and timeline.

4.2 Methods

Subjects

Adult male and female C57BL/6 mice, aged 3 months upon arrival (Charles River, Wilmington, MA) to the Guthrie Hall, Department of Psychology central vivarium at the University of Washington, Seattle (accredited by the Association for Assessment and Accreditation of Laboratory Animal Care), were initially housed in groups of four. Animals were allowed to acclimate to the central vivarium for seven days. On day five of vivarium acclimation, animals were single housed. After the one-week acclimation period, animals were food restricted to 85% of their free feeding weight and maintained at this level for the remainder of the experiment. Animals were randomly assigned to the Foraging-Only control group (male n = 5, female n = 4) or the Predator-Exposure group (male n = 4, female n = 5). Animals were housed under a reverse 12-hour light/dark cycle (lights on at 7 pm). The experiment was performed during the dark phase of the cycle in strict compliance with the University of Washington Institutional Animal Care and Use Committee guidelines. All animals were handled for 2 minutes/day until the beginning of habituation.

Approach Food-Avoid Predator paradigm

The mouse-adapted AFAP apparatus was a custom built rectangular, elevated arena measuring 118 cm L x 45 cm W x 61 cm H (length x width x height) and consists of two compartments: a small, enclosed "Nest Zone" and a larger, open "Foraging Zone" (**Figure 4.1A**). The two compartments are separated by an automated gate. The nest and foraging area measure 14 cm and 104 cm long, respectively. Animals were tested under dim white light (2.2 lux in nest area; 5.0 lux in foraging area) and with 72 dB white noise to mask background noise. Each day before behavioral experiments (and in between sexes), the arena was wiped with

Clidox-S disinfectant (Pharmacal Research Laboratories Inc., Waterbury, CT) followed by 70% ethanol. In between each animal, the arena was cleaned with 70% ethanol. After reaching a stable 85% body weight, subjects first underwent habitation to the nest area. During habituation, animals were confined to the nest area for 30 minutes/day for two days with 20, 45 mg dustless precision food pellets (#F0165, Bio-Serv, Flemington, NJ). After two days of habituation, animals began a baseline pellet foraging period lasting six days. During each baseline session, animals began confined in the nest area with two food pellets. After consuming the pellets, the first trial was initiated. A trial began with the automated gate lowering, at which point they were allowed to leave the nest area to procure and consume a food pellet located a fixed distance from the nest opening. A trial ended at the instance the mouse began consuming the pellet. The pellet was first located a short distance from the nest opening (12.7 cm) and progressively moved to a medium (25.4 cm) and long (38.1 cm) distance away from the nest opening during a baseline session. Animals were given three trials/fixed distance, for a total of nine baseline trials per baseline session. During the baseline foraging period, there was no trial time limit imposed.

After the baseline period, animals underwent a single test session. Testing began with three baseline trials with the pellet located at the long, medium or short distance, respectively. Baseline trials during the test session were identical to those during the baseline foraging period, except that animals were given a maximum of 180s to procure the pellet. After the last baseline trial, all animals were given a single test session. For those in the Foraging-Only control group, the test trial was identical to the long distance, predator-free baseline trial. Animals in the Predator-Exposure group were tasked with retrieving the pellet located at the long-distance location in the presence of an automated, surging predator: a taxidermy weasel mounted atop a frame with wheels (27.94 cm L x 10.2 cm H x 16.5 cm H). At the start of the test trial, the weasel

remained stationary at its starting position against the end of the foraging area back wall. Each time an animal reached the trigger location (12.7 cm before the pellet), the predator surged a fixed distance (30 cm; 60 cm/s) via pneumatic actuator then immediately retracted to its starting position. The test trial duration was a maximum of 180s for both groups. Animals that did not procure the pellet within the 180s test trial were given the pellet in the nest area, such that all animals received the same amount of reinforcement on the test day. All animals were promptly returned to the vivarium after testing.

Brain tissue collection and processing

Ninety minutes after the start of the test trial, animals were sacrificed with Beuthanasia then transcardially perfused with phosphate buffered saline (PBS) followed by 4% paraformaldehyde. Brains were then extracted and left overnight in the fixative at 4°C. Afterwards, brains were incubated with 30% sucrose solution at 4°C for two days. Brains were then mounted and 40 µm thick transverse sections collected via microtome (Leica SM2010R; Leica Biosystems, Nußloch, Germany). The following nuclei were selected for c-Fos analysis: amygdala (basolateral complex and central nucleus) and dorsal hippocampus CA1 (proximal and distal), -1.31 to -2.15 mm AP bregma; ventral hippocampus CA1, -2.91 to -3.15 mm AP bregma and periaqueductal gray (dorsomedial and dorsolateral nuclei), -3.79 to -4.71 mm AP bregma (Paxinos & Franklin, 2019). Within each range, sections spaced 160 µm apart were used for staining/c-Fos cell counting analysis. Sections were placed in cryoprotectant solution and stored at -20°C until immunofluorescence staining.

Immunofluorescence staining, Nissil staining and microscopy

Sections were first rinsed for 10 minutes in 0.1M phosphate buffered saline (PBS) solution three times. Sections were then rinsed for 10 minutes in a PBS and TritonX solution

(PBST; 0.3% TritonX stock solution, Sigma-Aldrich, Inc, St. Louis, MO) before a two-hour incubation in a blocking solution [0.5 PBST (1:1 PBST + PBS) and 5% normal goat serum (NGS; Vector Laboratories, Burlingame, CA)]. Sections were hereafter covered from light. Next, sections were incubated in a primary antibody solution [0.5 PBST, 2.5% NGS and 1:500 c-Fos Rabbit IgG monoclonal antibody (Cell Signaling, Danvers, MA)]. After three, 10-minute PBS rinses, sections were then incubated in the secondary antibody solution [0.1M PBS, 2.5% NGS and 1:500 Alexa Flour 488 goat-anti-rabbit IgG (Cell Signaling, Dangers, MA)]. After, sections were rinsed twice for 10 minutes with PBS and mounted onto slides, covered from light and left to dry overnight. All immunofluorescence staining was done at room temperature. The following day, sections were coverslipped with Fluoromount-G with DAPI (Invitrogen, Waltham, MA) to non-specifically label neuronal soma. Each mounted, stained section was photographed via Keyence BZ-X800E fluorescent microscope (Keyence, Itasca, IL) with fixed exposure settings.

C-Fos quantification

All acquired images were identified and matched to individual plates in the Paxinos and Franklin Mouse Brain Atlas (Paxinos & Franklin, 2019), which was used to determine the region of interest (ROI) size and placement. **Table 4.1** list the ROI parameters. All images were then manually contrasted to remove background staining using the Keyence Analyzer software. Images were subsequently analyzed using Image J (U.S. National Institutes of Health, Bethesda, MD). Each scanned image contained an overlaid scale bar from the Keyence Analyzer software, which was used to calibrate the scale (μm units) within Image J necessary for accurate and automated cell counting. In image J, c-Fos only images (eGFP tagged) were overlaid with their respective Nissil stain (DAPI tagged) overlay mask to assist in ROI placement with reference to the Paxinos and Franklin Mouse Brain Atlas (Paxinos & Franklin, 2019).

Table 4.1. Regions of interest and the c-Fos quantification borders.

Region of Interest	Parameters
Proximal Dorsal Hippocampus CA1	400 x 200 µm rectangle
Distal Dorsal Hippocampus CA1	400 x 200 µm rectangle
Ventral Hippocampus CA1	600 x 300 µm rectangle
Basolateral Amygdala	Manual trace
Central Amygdala	400 x 400 µm circle
Dorsomedial periaqueductal gray	350 x 200 µm rectangle
Dorsolateral periaqueductal gray	180 x 180 µm square

Images were then cropped to the ROI border, de-speckled and smoothed to further reduce background noise before conversion to 8-bit grayscale. After conversion, cropped images were auto-thresholded using the default image J thresholding method to isolate fluorescent puncta and fully remove background staining. Automated cell counting was then performed with particle size set to 15-400 μm and particle circularity set to 0.8 using the Analyze Particle function by experimenters blind to each animal's group participation. Counted particles were verified to be within cells by referencing the image's Nissil overlay mask. Counts were taken bilaterally from each serial section within that brain region's specified anterior-posterior range and averaged to produce a single, average c-Fos positive count for that brain region (Comoli et al., 2003; Dallaporta et al., 2007; Lelos & Good, 2012). Ventral hippocampus CA1 sections were available only in a subset of animals (Foraging-Only group, n = 7; Predator-Exposure group, n = 8).

Statistics

Normality for each variable was assessed using the Shaprio-Wilk test and by examination of QQ-plots. Non-parametric data with a within subjects factor of time (baseline day or trial-type) were analyzed with rank-based mixed factor ANOVA using the R package nparLD (Noguchi et al., 2012), with sex and/or group as between subjects factor where applicable, followed by post-hoc between subjects comparisons using Wilcoxon rank sum tests and within subjects post-hoc comparisons using repeated measures Wilcoxon signed rank tests. Parametric data with a within subjects factor of time (baseline day or trial-type) were analyzed using a mixed factor ANOVA in GraphPad Prism (GraphPad Software, San Diego, CA), with sex and/or group as between subjects factor where applicable. For comparisons strictly between groups and/or sexes, the Wilcoxon rank sum, two-sample independent t-test or two-factor between

subjects ANOVA were used. All statistical tests were performed two-tailed with the alpha value set to 0.05. All post-hoc analyses were Bonferroni corrected.

4.3 Results

Baseline foraging period

On the first day of baseline, there were no significant differences between groups ($F_{1, 14}$ = 1.40, p = 0.256), sexes ($F_{1, 14}$ = 3.434, p = 0.085), nor a group by sex interaction ($F_{1, 14}$ = 0.719, p = 0.411) in latency to leave the nest area on the first trial, indicating no statistically reliable differences among animals in spatial neophobia (rank transformed two factor ANOVA; **Figure 4.2**). Only a main effect of baseline day was present with animals' average total distance travelled (locomotor activity; $F_{2.997}$ = 15.384, p < 0.001) and no main effect of group ($F_{1, 7.481}$ = 0.029, p = 0.87), sex ($F_{1, 7.481}$ = 1.217, p = 0.304), nor a group by sex ($F_{1, 7.481}$ = 0.055, p = 0.820), group by baseline day ($F_{2.997}$ = 1.926, p = 0.123), sex by baseline day ($F_{2.997}$ = 0.724, p = 0.537) or group by sex by baseline day interaction ($F_{2.997}$ = 0.768, p = 0.512). There was a stable reduction of exploratory behavior after the initial baseline session (day 1 v. 3, V = 171, p < 0.001; day 3 v. 6, V = 134, p = 0.068; **Figure 4.3A**). Similarly, there were no significant main effects of group ($F_{1, 9.793}$ = 0.012, p = 0.914), sex ($F_{1, 9.793}$ = 0.554, p = 0.474), nor any interactions involving group, sex or baseline day in their average latency to consume food pellets (group by sex, $F_{1, 9.793}$ = 0.414, p = 0.535; group by baseline day, $F_{2.843}$ = 1.614, p = 0.186; sex by baseline day, $F_{2.843}$ = 0.743, p = 0.52; group by sex by baseline day, $F_{2.843}$ = 0.374, p = 0.761). However, there was a significant main effect of baseline day ($F_{2.843}$ = 27.306, p < 0.001). Post hoc analysis reveals that all animals steadily reduced their average latency to consume food pellets over the course of the baseline period (day 1 v. 3, V = 171, p < 0.001; day 3 v. 6, V = 142, p = 0.024; **Figure 4.3B**). As there were also no statistically reliable sex differences in any

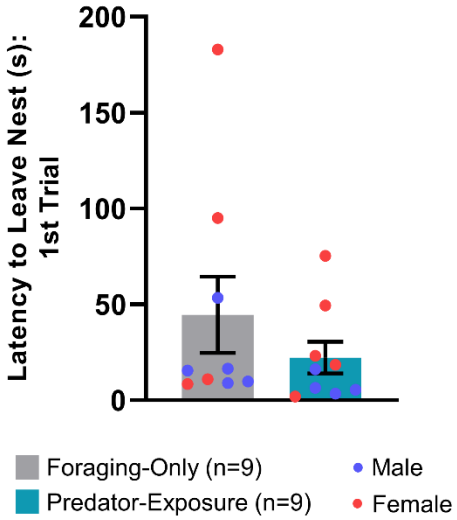

Figure 4.2. Spatial neophobia. Graph depicts the latency to leave to the Nest Zone (seconds) on the first trial of the first baseline session. All data presented as means ± SEM. Gray bars represent the Foraging-Only control group. Teal bars represent the Predator-Exposure group. Individual data for males (blue dots) and females (red dots) are presented in bar graphs.

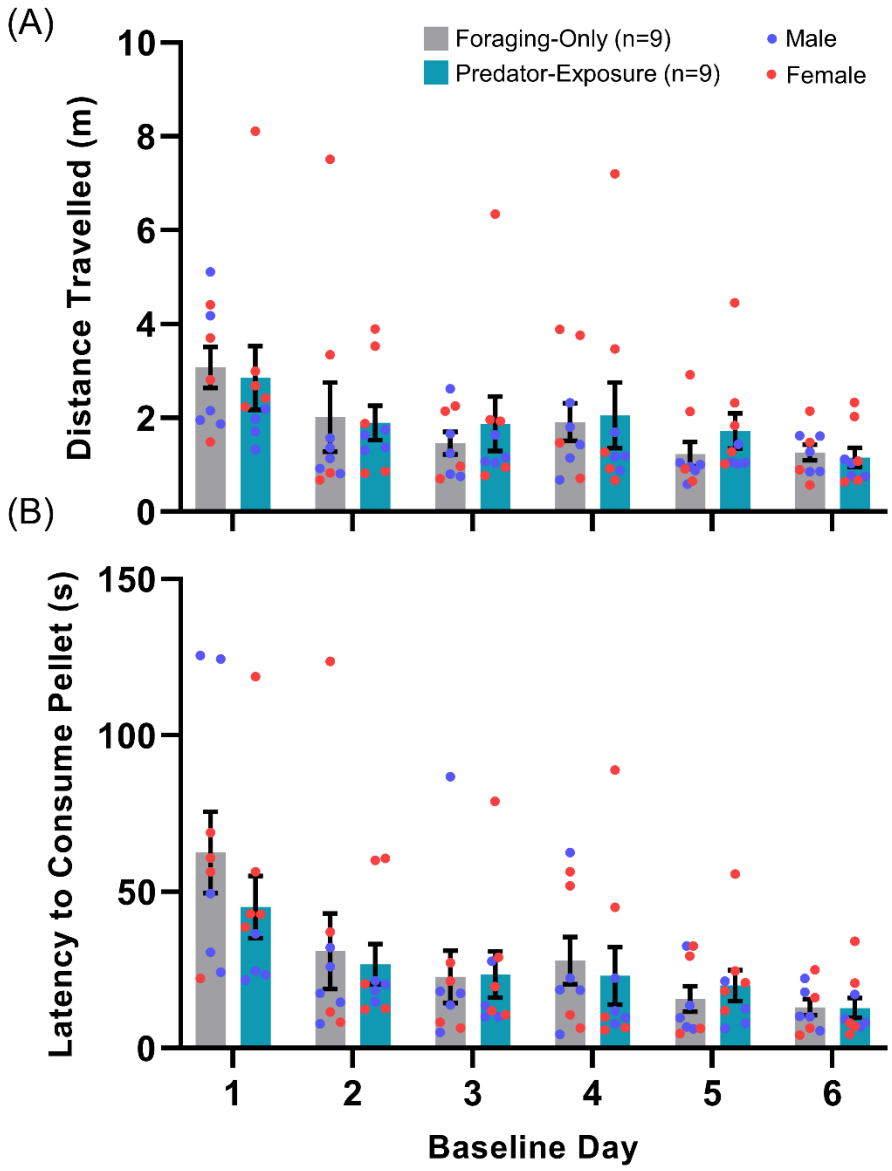

Figure 4.3. Reduction in exploratory behavior and latency to consume food pellets across the baseline period. **(A)** The average distance travelled (meters) per trial/baseline day. **(B)** The average latency to procure the food pellet (seconds) per trial/baseline day. There were no sex differences detected, thus sex was collapsed across group. Gray bars represent the Foraging-Only control group. Teal bars represent the Predator-Exposure group. Individual data for males (blue dots) and females (red dots) are presented in bar graphs. All data presented as means ± SEM

dependent variable during the testing period, sexes were pooled in each group for test day analysis.

Testing

On test day, there was a significant group by trial type interaction on animals' latency to leave the nest area upon trial start ($F_1 = 32.212$, $p < 0.001$). Namely, there was no difference among animals in latency to leave the nest during the test session's baseline trials ($W = 47.5$, $p > 0.999$), but those in the predator exposure group had significantly higher latencies to leave the nest at the start of the test trial ($W = 7$, $p = 0.007$; **Figure 4.4A**). There was also a significant group by trial type interaction for the animals' latency to reach the trigger zone after emerging from the nest ($F_1 = 15.407$, $p < 0.001$). Again, there were no group differences during the initial baseline trials ($W = 27$, $p = 0.501$), but a significant group difference during the test, such that animals in the Predator-Exposure group had longer latencies to reach the trigger zone than the Foraging-Only control group after emerging from the nest ($W = 4$, $p = 0.003$; **Figure 4.4B**). There was a significant interaction between group and trial type on animals' latency to consume food pellets during the test session ($F_1 = 35.514$, $p < 0.001$). Specifically, there was no difference between groups during the initial baseline trials ($W = 40$, $p > 0.999$), but a significant group difference during the test ($W = 0$, $p < 0.001$; **Figure 4.4C**). In fact, all animals in the Predator-Exposure group failed to achieve the pellet within the 180s test trial. Both male and females in the Predator-Exposure group made several failed attempts to procure the pellet, however, and there was no significant differences between them ($t_7 = 1.398$, $p = 0.205$). There was a main effect of trial type on the total distance travelled ($F_1 = 14.920$, $p < 0.001$) and no main effect of group ($F_{1, 13.108} = 1.865$, $p = 0.195$) nor a group by trial type interaction ($F_1 = 2.197$, $p = 0.138$), indicating that all animals, regardless of group, had significantly increased locomotor behavior

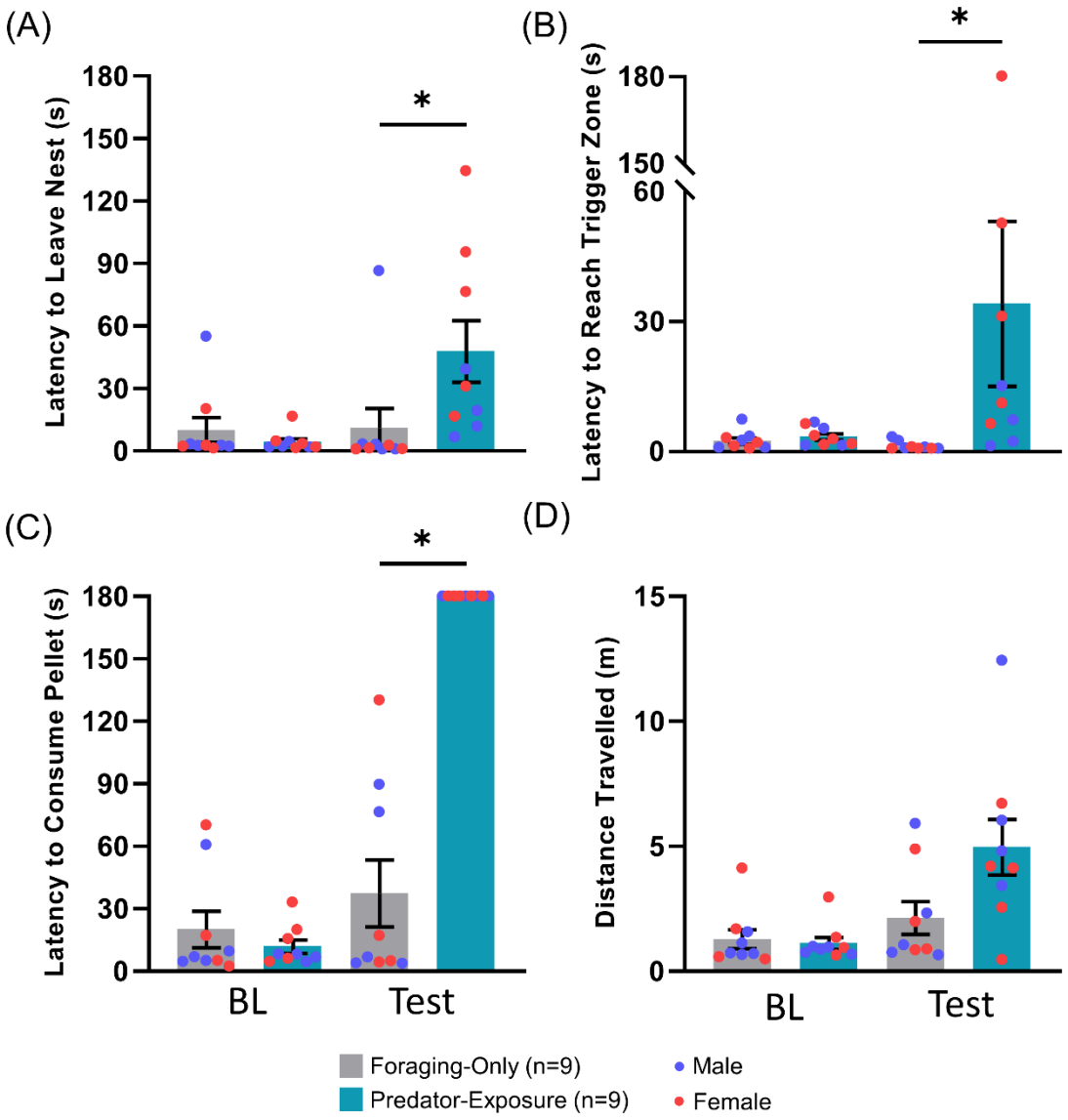

Figure 4.4. Test day behavior. **(A-D)** compares the baseline trial average versus test trial outcome between Foraging-Only controls and the Predator-Exposure group for **(A)** latency to leave the nest area **(B)** latency to reach the trigger zone after emerging from the nest (25.4 cm from nest opening), **(C)** latency to consume the food pellet (placed 38.1 cm from nest opening) and **(D)** total distance travelled. There were no sex differences detected, thus sex was collapsed across group. Gray bars represent the Foraging-Only control group. Teal bars represent the Predator-Exposure group. Individual data for males (blue dots) and females (red dots) are presented in bar graphs. * denotes group difference at test trial (p <0.05). Data are presented as mean ± SEM.

during the last trial (**Figure 4.4D**).

C-Fos expression

Acute exposure to the naturalistic, surging predator in the Predator-Exposure group produced a different pattern of neural activity in the selected nuclei relative to their Foraging-Only control counterparts. No sex differences in c-Fos expression were detected, thus sex was collapsed across group. There was no difference between groups ($F_{1, 16} = 2.251$, $p = 0.153$), nor a group by region interaction ($F_{1, 16} = 0.314$, $p = 0.583$), in c-Fos expression in the dorsal CA1 hippocampus. There was, however, a main effect of CA1 region ($F_{1, 16} = 22.790$, $p < 0.001$), such that in all animals, the proximal region of the CA1 showed greater c-Fos activity than the distal CA1 region (**Figure 4.5A**). Those in the Predator-Exposure group had increased c-Fos levels in the ventral CA1 hippocampus ($t_{13} = 2.323$, $p = 0.037$), however (**Figure 4.5A**). In the amygdala, predator exposure lead to increased c-Fos expression in the basolateral complex ($t_{10.73} = 2.639$, $p = 0.024$; Welch corrected t), but not in the central nucleus ($t_{16} = 1.027$, $p = 0.320$; **Figure 4.5B**). Finally, in the dorsal periaqueductal gray, those in the Predator-Exposure group had elevated c-Fos expression in the dorsomedial ($t_{16} = 2.825$, $p = 0.012$), as well as the dorsolateral columns ($t_{16} = 3.268$, $p = 0.005$; **Figure 4.5C**). **Figures 4.6-4.8** show representative histology for both groups.

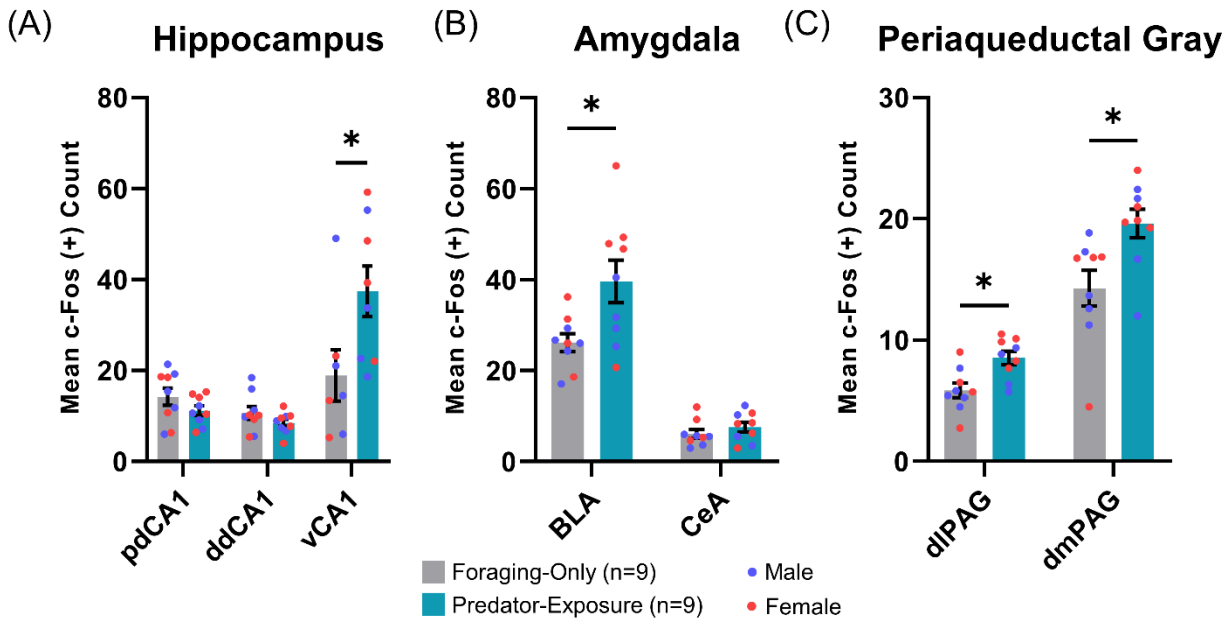

Figure 4.5. Mean c-Fos expression between Foraging-only controls and the Predator-Exposure group following the test trial. **(A)** Proximal dorsal hippocampus CA1 (pdCA1), distal dorsal hippocampus CA1 (ddCA1) and ventral hippocampus CA1 (vCA1; N subset: Foraging-Only n=7, Predator-Exposure n=8). **(B)** Basolateral amygdala (BLA) and central nucleus of the amygdala (CeA). **(C)** Dorsolateral periaqueductal gray (dlPAG) and dorsomedial periaqueductal gray (dmPAG). There were no sex differences detected, thus sex was collapsed across group. Teal bars represent the Predator-Exposure group. Individual data for males (blue dots) and females (red dots) are presented in bar graphs. * denotes significant group difference ($p < 0.05$). Data are presented as mean ± SEM.

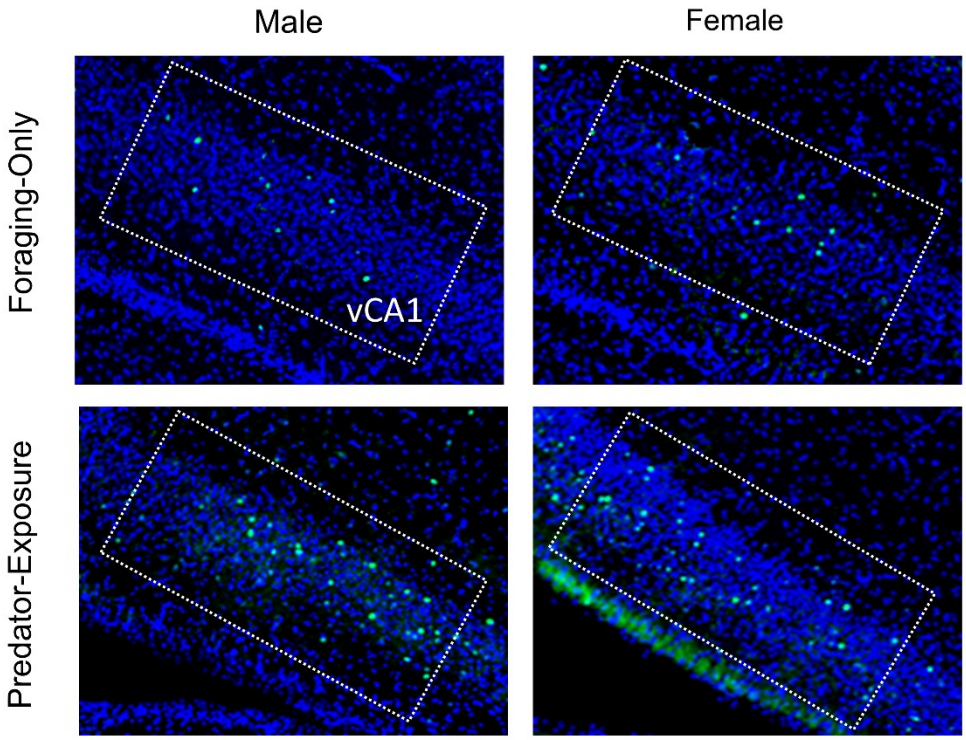

Figure 4.6. Representative histology of ventral hippocampus CA1 c-Fos expression in Foraging-Only and Predator-Exposure groups. Coordinates from bregma: -2.91 mm AP (Paxinos & Franklin, 2019).

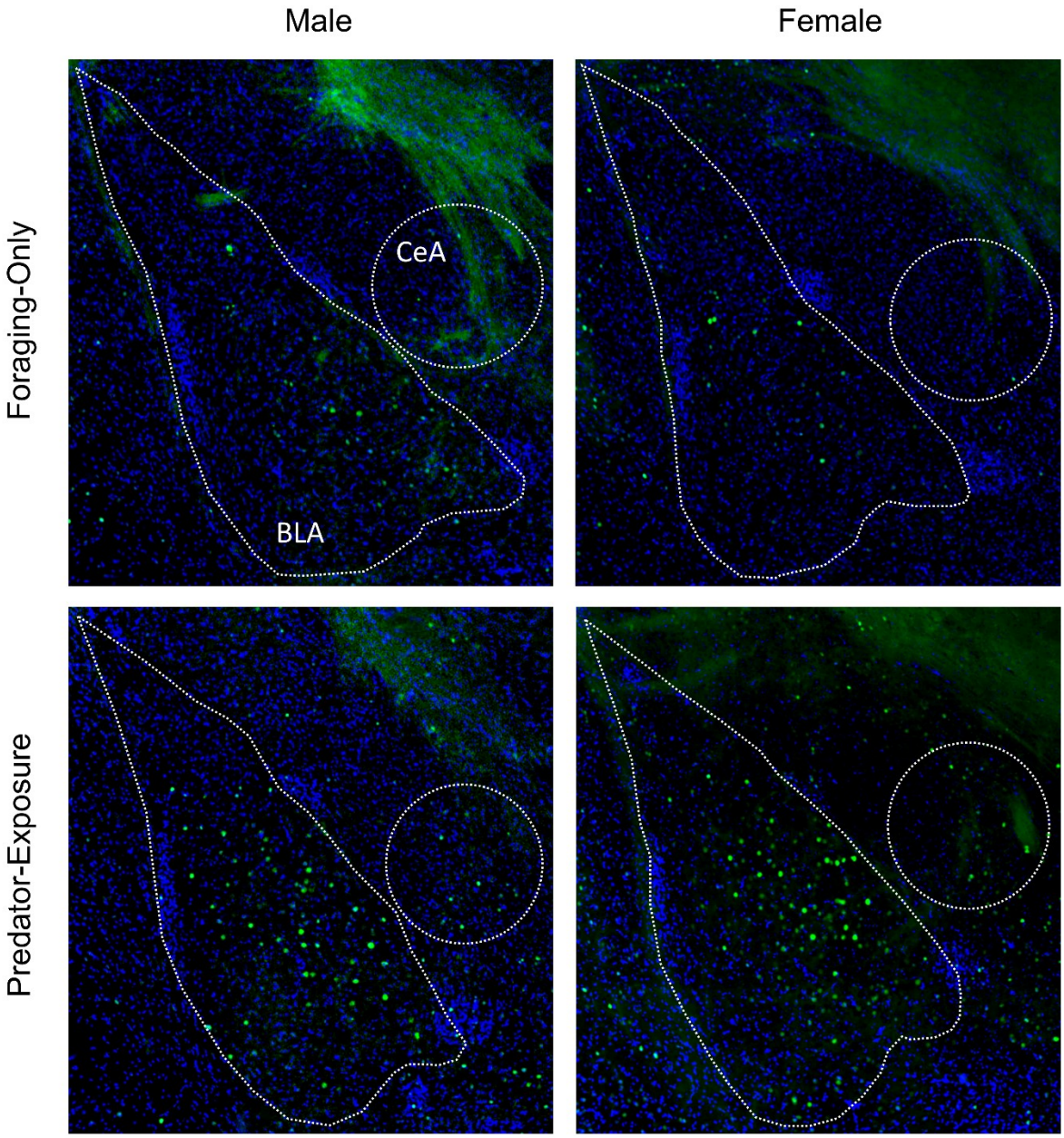

Figure 4.7. Representative histology of amygdala [basolateral complex (BLA) and central nucleus (CeA)] c-Fos expression in Foraging-Only and Predator-Exposure groups. Coordinates from bregma: -1.55 mm AP (Paxinos & Franklin, 2019).

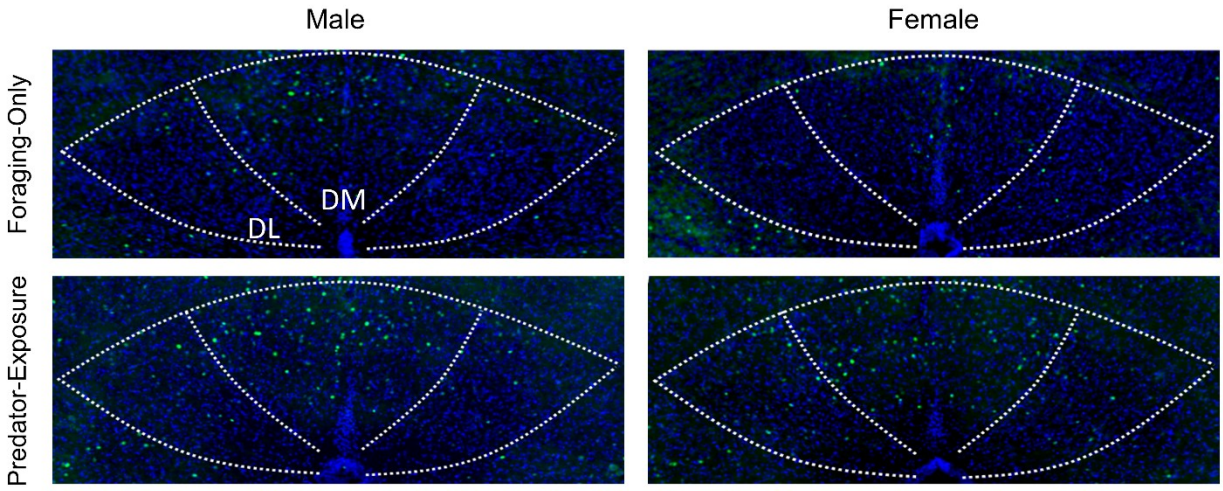

Figure 4.8. Representative histology of dorsal periaqueductal gray [dorsomedial (DM) and dorsolateral (DL) columns] c-Fos expression in Foraging-Only and Predator-Exposure groups. Coordinates from bregma: -4.35 mm AP (Paxinos & Franklin, 2019).

4.4 Discussion

During baseline, all animals steadily reduced their latency to consume pellets and exploratory behavior, such that by the end of the baseline period they were procuring and consuming the food pellets under 15 seconds of the gate opening on average. The present study was primarily concerned with capturing the neural activity of structures active during the initial innate fear experience. Therefore, to minimize the influence of other processes associated with repeated testing (e.g., habituation to the predator) on c-Fos expression in our regions of interest, testing was limited to a single trial. As expected, there were no differences among the groups on any variable of interest during the baseline trials on the test day. For those in the Predator-Exposure group, the introduction of the predator caused an immediate defensive reaction; animals showed a robust hesitancy to leave the nest in the presence of the stationary weasel, and once exiting, took an appreciable amount of time to enter the area containing the pellet, which subsequently triggered the predator surge. Both males and females in the Predator-Exposure group nonetheless made multiple failed attempts to retrieve the pellet, triggering the predator surge multiple times. There were no significant differences among sexes in terms of failed attempts. No animal in the Predator Exposure was able to procure the pellet within the allotted 180 seconds, however.

Overall, the taxidermy weasel served as an effective aversive stimulus. This terrestrial predator stimulus is considerably more realistic than the previous robotic variants used in the rat AFAP paradigm. While evidence suggests that laboratory raised rodents display innate anxiety toward predator odors (Fendt, 2006), it unclear whether laboratory rodents possess the ability to innately “recognize” their predators from non-predator stimuli using visual information (Fanselow, 2018; Guimaraes-Costa et al., 2007). It is unlikely that genes supply the organisms

with detailed information about their predators, rather, they likely encode for detection and defensive behavior toward simple stimuli, such as general looming objects (Kim & Jung, 2018). Nonetheless, a future study could examine the generalization gradient between the weasel stimulus and similar stimuli with reduced features to further test this idea. For example, measuring the defensive response to the stationary weasel stimulus lacking eyes, teeth, etc. (Ruxton et al., 2008).

There were no statistically reliable differences between sexes in terms of neural activity in any of the regions of interest. The surging predator in the Predator-Exposure group led to an increase in neural activity in the BLA, but not the CeA, relative to the Foraging-Only control group. This pattern of amygdalar c-Fos activity is consistent with previous studies examining amygdalar c-Fos activity following predator exposure in rats (Kim et al., 2016; Martinez et al., 2011). This also supports the conception that the CeA is important for the initial learning and subsequent expression of fear toward formally neutral stimuli associated with pain, but not for the detection and/or behavioral expression of fear toward non-pain inducing, innately aversive stimuli (Gross & Canteras, 2012; Silva et al., 2016). Nonetheless, CeA c-Fos expression in mice has been shown to be elevated following predator odor exposure (Francesconi et al., 2020; Gross & Canteras, 2012; Kolter et al., 2021), which necessitates more experiments defining the role of the CeA in innate fear and anxiety-related behavior. Also consistent with past literature is the increased c-Fos expression in the both the dmPAG and dlPAG following predator exposure in rats (Canteras & Goto, 1999); areas associated with the generation of panic-like defensive responses to predators and general imminent threat (Bandler & Depaulis, 1991; Kim et al., 2013; Lefler et al., 2020).

Although we previously have shown increased c-Fos expression in the rat dorsal CA1 following exposure to the Robogator in the AFAP paradigm (Kim et al., 2016), the present study did not detect any neural activity differences between the Predator-Exposure group and the Foraging-Only controls. This may well be a genuine species difference, or perhaps result from differences in c-Fos protocols (peroxidase immunochemical versus immunofluorescence method) (Kim et al., 2016). To date, no study has investigated c-Fos expression in the mouse dCA1 following exposure to a visual, terrestrial predator threat. Two sites within the dCA1 were chosen for c-Fos quantification; the proximal dCA1 (near subiculum) and distal dCA1 (near CA2/CA3) areas. While the proximal dCA1 areas appears to encode general context, the distal dCA1 is more selective toward objects and specific features of an environment (Igarashi et al., 2014). Therefore, it was surprising that the distal dCA1 was not more active in those exposed to the novel, aversive predator. Given that the importance of the dCA1 in contextual fear learning (Kim & Fanselow, 1992; Phillips & LeDoux, 1992) and that c-Fos expression in the mouse dorsal hippocampus is elevated during contextual fear retrieval using standard Pavlovian fear conditioning (Strekalova et al., 2003), it is likely that increased c-Fos expression in the dCA1 would be exclusively detected in Predator-Exposure animals if they were not sacrificed for histology after the first predator exposure, but rather re-exposed to the AFAP arena the following day after predator exposure and then sacrificed.

In contrast to the dCA1, vCA1 c-Fos expression was significantly higher in the Predator-Exposure group relative to Foraging-Only controls. This is consistent with the ventral hippocampus' role in approach-avoidance conflict (Bryant & Barker, 2020). For example, inhibition of vCA1 terminals in the medial prefrontal cortex reduced avoidance of open arms in the Elevated Plus Maze, biasing the animal toward exploratory behavior (Padilla-Coreano et al.,

2016). Excitotoxic lesions of the ventral hippocampus also lead to an increased preference for a context with mixed valence (cues predicting shock and sucrose) over a context with neutral valence, whereas sham operated control animals avoid the mixed valence context and prefer the neutral valenced context (Schumacher et al., 2016). In a study using a live predator (cat), ibotenic acid lesions of the ventral hippocampus did not affect innate fear toward the predator, but reduced contextual fear of the predator context when placed back in the arena the following day without the predator (Pentkowski et al., 2006). Notably, this task did not have an approach-avoidance conflict; animals were simply placed in a chamber with plexiglass divider from which to view the predator in an adjacent chamber, highlighting the potential selective role of the ventral hippocampus is approach-avoidance conflict and not innate defensive behavior per se.

This study provides foundation for future mouse AFAP studies. Overall, the study indicates considerable overlap of innate fear/decision-making circuits with rats (Kim et al., 2016). The elevated c-Fos expression in the vCA1 provides a novel target for in-vivo electrophysiology or manipulation methods within the AFAP paradigm, which can clarify the structure's role in risky decision-making in the face of imminent predatory threat. This study also highlights the benefits of using the AFAP paradigm—and ethologically relevant tasks in general—to probe the neural circuitry underlying fear and risky decision-making behavior. For example, using the mouse-adapted AFAP paradigm, it was shown that c-Fos expression in the BLA, but not the CeA, was increased in animals experiencing visual predator threat relative to control animals. This is at odds with fear conditioning data showing that context pre-exposed animals have increased c-Fos expression in both the BLA and CeA following a later, single-trial context and/or tone-shock pairing relative to context pre-exposed animals who simply re-experienced the context without shock (Radulovic et al., 1998). Worth noting is that animals in

the current study were very familiar with the context as well by the time they experienced the predator. In sum, the AFAP scenario invoked a different pattern of activity in the same nuclei implicated in another form of fear. This suggests that these nuclei may have distinct patterns of activity under more naturalistic scenarios, and at the very least, supports the need to expand behavioral testing beyond simple fear conditioning if we wish to comprehensively understand the neural underpinnings of fear and anxiety-related behavior. In a similar vein, by incorporating an ethological approach-avoidance conflict component to our task, we've shown that the vCA1 was active above and beyond controls, which is at odds with another study that likewise used a visual predator stimulus but did not have the additional ethological approach-avoidance conflict component that supports decision-making (Pentkowski et al., 2006).

Chapter 5. General Conclusions

5.1 Chapter Summary

The purpose of this dissertation was to present and describe in detail ethological paradigms used to study fear, anxiety and risky decision-making in rodents and how they overcome limitations associated with traditional, predominant paradigms assessing those functions. Equally important, the dissertation was focused on how use of ethological alternatives can expand our knowledge of behavior and their neural substrates. Chapter 2 introduced a longitudinal ethological paradigm that is suited to address the impact of fear and anxiety on day-to-day behavior: the Risky Closed Economy (RCE). In addition to outlining the history of closed economies, the RCE's naturalistic qualities and utility in neuroscience research, the chapter demonstrated that the RCE is viable for use in mice. Chapter 3 demonstrated the RCE's capacity to comprehensively investigate the impact of irreversible neural manipulations on appetitive behavior under risk-free and risky conditions. This chapter specifically focused on the effects of lateral habenula (LHb) lesions in rats. Because of the naturalistic qualities of the RCE and the ease of which to measure pre and post-surgery baseline changes in behavior, a novel finding related to LHb function was uncovered. Specifically, LHb lesions disrupted daily foraging (appetitive) behavior without disrupting passive avoidance of a chronic, unpredictable threat (foot shock).

Finally, Chapter 4 introduced the Approach Food-Avoid Predator (AFAP) paradigm; an acute ethological paradigm that simulates predator-prey encounter and can be used to investigate the interaction of innate fear and risky decision-making. Along with describing the history and utility of the paradigm in rats, the chapter presented data on the behavior and neural signature of innate fear in mice exposed to a naturalistic, surging predator: a taxidermy weasel on wheels

connected to a pneumatic actuator. Namely, the chapter characterized c-Fos expression in the hippocampus, amygdala and dorsal periaqueductal gray between mice simply foraging for food pellets and those tasked with foraging in the face of predation. For those exposed to the predator, c-Fos was significantly elevated in the basolateral complex of the amygdala, the ventral CA1 area of the hippocampus and both columns of the dorsal periaqueductal gray. The predator experience, which robustly elicited innate fear-related behaviors in the mice, did not elevate c-Fos expression in the central nucleus of the amygdala above and beyond control animals. This is in contrast to fear conditioning, where both the basolateral complex and central nucleus show increased neural activity following context/tone-shock pairing. This exemplifies the idea that circuits/nuclei involved in associative fear have distinct patterns of activity under more naturalistic scenarios.

5.2 Feasibility of the RCE and AFAP Paradigms

Although the RCE and AFAP paradigms are custom-built, both are generally feasible in their costs and construction. In terms of costs, they are far less expensive than commercially bought apparatus—and with a bit of creativity and time, their total costs can pale in comparison. For example, the casing of the mouse-adapted RCE chamber was built with affordable acrylic and fabricated at one of University of Washington's free student workshops. The overall design was drafted in Autodesk Inventor, offered to students for free (there are also many open source computer aided drafting software available). Metal rails were fabricated for a reduced price via a campus workshop. Commercially bought specialty devices such as operant levers, shock grids, etc., contributed most to the cost of the mouse-adapted RCE chambers. The mouse-adapted AFAP apparatus was likewise affordable to build, with almost all materials coming from local home-improvement stores and fabrication done at reduced price on campus. With this paradigm

in particular, commercial behavior tracking software is not nearly as crucial, and may be accomplished “offline” via free software such as DeepLabCut (Mathis et al., 2018). Actual constructing of the RCE and AFAP paradigms required only basic knowledge of carpentry and electronics. Thus, not only can ethological paradigms be used to overcome limitations of reductionist behavioral paradigms and generate novel findings, they can be used to save funds.

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