The Influence of the Basolateral Amygdala-medial Prefrontal Cortex Circuitry in Appetitive Cue Learning and Valuation

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THE INFLUENCE OF THE BASOLATERAL AMYGDALA-MEDIAL PREFRONTAL CORTEX CIRCUITRY IN APPETITIVE CUE LEARNING AND VALUATION

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Abstract: Environmental cues that are neutral in respect to hunger and feeding can come to predict food through Pavlovian appetitive conditioning. These learned cues can drive food seeking and eating independent of physiological hunger leading to overeating and obesity. However, the food outcome, and thus the value of the cues, can change due to environmental alterations. A change in the values of learned cues requires altering behavioral responses to accurately reflect the cue's new outcome. This behavioral flexibility is necessary to respond appropriately to changes in the environment and, as such, is an adaptive trait. The aim of this dissertation was to determine critical neural mechanisms specifically within the basolateral amygdala (BLA) and also with its interactions with the medial prefrontal cortex (mPFC) during behavioral flexibility when outcomes of learned appetitive cues change using the appetitive reversal learning paradigm. The main focus was on the BLA (Chapter 2) and its connection with the mPFC (Chapters 3 and 4) since both of these areas are critical in appetitive cue learning and valuation and subsequent behavioral modifications.

The first study in this dissertation examined if separate neuronal ensembles within the BLA respond to different learned cues, a cue that signals

food availability and a cue that does not. Additionally, we investigated if these potentially distinct neuronal ensembles are necessary during periods of behavioral flexibility when the value of the specific learned cues are changed during reversal learning. We determined that there are distinct neuronal ensembles within the BLA that respond to different learned cues, and that the cue-specific ensembles are necessary for updating the value of each specific cue (Chapter 2). Next, we examined a projection target of the BLA, the mPFC, to determine if BLA-projecting neurons are activated during learning (Chapter 3). Using retrograde tract tracing combined with Fos detection, we found recruitment of the anterior BLA to prelimbic area of the mPFC across cue-food learning, signifying that the BLA can inform the mPFC of the value of learned cues. Then to establish that communication between the BLA and mPFC is necessary for cue value learning and updating (Chapter 4), we functionally disconnected communication between these regions and examined appetitive learning using discriminative conditioning, reversal learning, and devaluation paradigms. We found impairments in cue value recall and subsequent updating of the cues' values during reversal learning. Together, these studies indicate the BLA may be important in informing the mPFC of the value of learned cues, and their interaction is critical to optimally guide behavioral responding. The findings from these experiments are valuable for our understanding of the neural mechanisms that motivate eating behavior under the control of learned food cues and to understand the mechanisms necessary for behavioral flexibility when the outcomes of learned cues are changed.

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To my daughter, Vanessa Keefer –

From the intricacies of the brain to the expanse of the universe, may you never lose your sense of wonder

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Chapter 1: General Introduction

Through associative learning, hunger and eating can be controlled by learned environmental cues. External, environmental factors associated with food can override internal, physiological signaling, leading to a non-homeostatic motivation to obtain and consume food when satiated (Weingarten, 1983; Birch et al., 1989; for reviews see Saper et al., 2002; Petrovich & Gallagher, 2003; Holland & Petrovich, 2005; Petrovich, 2013)*. Indeed, cues repeatedly associated with food can increase the motivation to obtain food (i.e., lever presses; Estes, 1948) and increase consumption despite satiation (e.g. Weingarten, 1983; Birch et al., 1989; Reppucci & Petrovich, 2012; for review, see Petrovich, 2013). This increase in eating due to learned environmental cues is considered maladaptive in our current environment and has been associated with the increase in the prevalence of obesity in developed countries (Schachter, 1968; Saper et al., 2002; Stroebele & De Castro, 2004; Levitsky, 2005; Popkin et al., 2005; Berthoud, 2007; Petrovich, 2013). There has been significant insight into the neural mechanisms underlying overeating due to learned cues (Holland et al., 2002; Petrovich et al., 2002; Petrovich et al., 2005; Petrovich et al., 2007; Petrovich et al., 2012; Cole et al., 2015b; for review, see Petrovich 2013); however, the neural substrates for the initial cue-food associative learning are understudied. Determining the fundamental neural substrates necessary for learning and updating the values of learned cues is essential to further our understanding of the control of feeding by learned food cues.

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*References within the Introduction are included in the References list after Chapter 5: General Discussion.

In the laboratory, a typical paradigm used to study cue-food associative learning is Pavlovian conditioning (Pavlov, 1927). In this paradigm, an initially neutral cue from the environment (e.g. a discrete tone or light; conditioned stimulus, CS) is repeatedly followed by food (unconditioned stimulus, US), which conjures an innate behavioral response (e.g. approach to the location of the food and feeding; unconditioned response, UR). Following repeated CS-US pairings, the CS comes to predict the US and can induce the same behavior without the US (e.g. approach to the food cup; conditioned response, CR). During training, an increase in the CR during presentation of the CS signifies the subject is learning the CS-US association. Additionally, to further assess that the CR is specific to the CS being learned, a second, distinct environmental cue is presented alone, unaccompanied by any other stimuli. As a result, subjects must discriminate between the cue signaling the delivery of food (CS+) and the cue signaling nothing (CS-). This discrimination results in two separate behaviors: an increase in CR to the CS+ and an inhibition of CR to the CS-. Since these behaviors are distinct from each other, they could be mediated by distinct neural mechanisms.

The outcomes, and thus the values, of learned appetitive cues are not always static and can change based on environmental variations. This change in the outcome of learned cues requires updating the value of the cues to produce an appropriate behavioral response, which can be assessed using two distinct behavioral paradigms: reversal learning and devaluation. After successful two cue discriminative conditioning, reversal learning entails a switch of the cues'

outcomes. The original CS+ that was associated with the food is now no longer followed by food (reversal CS-; rCS-), and the cue that was previously followed by nothing (CS-) is now always followed by the delivery of food (reversal CS+; rCS+). Successful reversal learning is observed by two distinct behaviors: the inhibition of CR to the cue previously associated with food since the cue now signals the absence of food (CS+ now rCS-), and the initiation of CR to the cue now signaling food delivery (CS- now rCS+). Reversal learning requires the subject to update the value of the cues based on the change in the cues' outcomes and entails behavioral flexibility to respond appropriately.

Devaluation is another behavioral paradigm used to assess the valuation of learned cues by decreasing the value of the outcome (US) and thus any cues previously associated with it (e.g. CS). In these preparations, the value of the US (e.g. food) can be altered by illness through conditioned taste aversion (CTA) or by satiety. During CTA, consumption of the US is by followed by illness (e.g. lithium chloride injections to cause malaise), causing the illness to be strongly associated with the food. Next, since the value of the US has changed, subjects are tested on CR to the CSs to evaluate if the CSs are also devalued. This devaluation is evident by a decrease in CR to the CSs that are associated with the devalued food. Unlike reversal learning where the outcomes of the learned cues are only switched, devaluation results in a negative association (e.g. illness) with the food. Using the devaluation procedure, we can examine the neural mechanisms necessary for behavioral inhibition due to a decrease in the valuation of food and food-associated cues.

The goal of this dissertation was to examine the neural circuitry between the amygdala and medial prefrontal cortex and the specificity and necessity of this circuitry in behavioral flexibility during appetitive cue learning and valuation updating. We focused on the amygdala, a telencephalic structure located in the ventral temporal lobe, because it is important in various associative learning paradigms. It is involved in attending to biologically relevant events and is well positioned to inform other brain regions via distinct functional connections (e.g. Weiskrantz, 1956; Swanson & Petrovich, 1998). Based on developmental, structural, and connectivity evidence, the amygdala is a heterogeneous structure comprised of several cortical and striatal regions. Notorious for its involvement in various associative learning paradigms, the amygdala can also be defined based on its various sub-regions governing different aspects of learning. Within this dissertation, we focused on one part of the cortical amygdala – the basolateral nucleus of the amygdala (BLA). It is considered an early processor of relevant sensory information during appetitive associative learning (Piette et al., 2012; Cole et al., 2013), and after learning, it is necessary to use the acquired associations to motivate new behavior (Hatfield et al., 1996; Schoenbaum et al., 1999; Blundell et al., 2001; Setlow et al., 2002; Nomura et al., 2004; Corbit & Balleine, 2005; Ishikawa et al., 2008; for reviews, see Holland et al., 2001, 2002; Everitt et al., 2003; Holland & Petrovich 2005, Petrovich, 2013; Wassum & Izquierdo, 2015). Furthermore, the BLA is involved in updating the value of learned cues when the cue contingencies are reversed (Scheonbaum et al., 1999) and when the outcome is omitted (Tye et al., 2010) or altered, such as

during conditioned taste aversion (Grossman et al., 2008) and devaluation (Blundell et al., 2003; Ostlund & Balleine, 2008; Coutureau, et al., 2009; Johnson et al., 2009).

These studies suggest the BLA is important when the outcome and value of food-associated cues change; however, the underlying neural mechanism is unclear. We hypothesized that BLA neuronal ensembles that are activated during the recall of a learned cue are necessary when cue valuation is needed to form a new association. To test this premise, in Chapter 2 we used an innovative method, the Daun02 inactivation procedure, to specifically ablate neuronal ensembles that are activated in response to a stimulus. In *Fos-lacZ* transgenic rats, the transgene *lacZ* produces the protein β-galactosidase (β-gal), and its expression is controlled by the induction of an immediate early gene, *c-fos* (Koya et al., 2009; Cruz et al., 2013). These activated neurons that produce β-gal can be inactivated by infusions of Daun02, which is catalyzed by β-gal into daunorubicin, resulting in a decrease in neuronal excitability (Santone et al., 1986; Engeln et al., 2016). Using this method, rats were presented with the CS+ or CS- and infused with either Daun02 or a vehicle solution to examine if these same neuronal ensembles within the BLA are necessary when new associations are formed due to a change in outcomes during reversal learning.

Importantly, the BLA has extensive connections with another cortical region implicated in adaptive behaviors and executive control – the medial prefrontal cortex (mPFC). Similar to the BLA, the mPFC is critical in driving motivation specifically during learned feeding behavior. Two sub-regions of the

mPFC, the prelimbic (PL) and infralimbic (ILA) areas, are recruited when cues are robustly associated with food (Burgoes-Robles et al, 2013; Cole et al., 2015a; Moorman & Aston-Jones, 2015; Warren et al., 2016) and mediate the homeostatic (Mena et al., 2011, 2013; Land et al., 2014) and non-homeostatic drive to eat (Petrovich et al., 2007; Blasio et al., 2014; Cole et al., 2015b). Additionally, the current evidence suggests the mPFC may have a less critical role in reversal learning (Ragozzino et al., 1999; Salazar et al., 2004; Boulougouris et al., 2007; Floresco et al., 2008; Coutureau et al., 2009); however, the role of the mPFC and its communication with the BLA in cue value updating is unknown.

Since the mPFC is well known for executive function and can also drive feeding, the functional connection between the BLA and mPFC was necessary to investigate during appetitive learning. The BLA has extensive, topographically organized connections with the mPFC (Sesack et al., 1989; Kita & Kitai, 1990: Swanson & Petrovich, 1998; Petrovich et al., 2001; Dalley et al., 2004; Hoover & Vertes, 2007; O'Doherty, 2011; Little & Carter, 2013; Reppucci & Petrovich 2016). The anterior part of the BLA sends more dense projections to the dorsal regions of the mPFC, while the posterior part the BLA sends more dense projections to the ventral regions of the mPFC (Kita & Kitai, 1990; Hoover & Vertes, 2007; Reppucci & Petrovich, 2016). The anterior BLA is the first forebrain region selectively activated during early cue-food conditioning and maintains activation throughout conditioning (Cole et al., 2013). Meanwhile, the posterior BLA, PL, and ILA are activated when the cue-food association is well-learned

(Cole et al., 2013; Cole et al., 2015a), suggesting learning-induced plasticity within the circuitry of interest. Other studies have shown the BLA can directly alter mPFC activity (Sotres-Bayon et al., 2012; Sun & Laviolette, 2012), and inhibition of the BLA-mPFC pathway decreases conditioned reward seeking (Fuchs et al., 2007; Mashhoon et al., 2010; Stefanik et al., 2013) and learning (Churchwell et al., 2009). However, inhibition of this pathway may not interfere with devaluation (Coutureau et al., 2009), but further testing with additional cue valuation paradigms is needed.

These previous studies signify functional connectivity between the BLA and mPFC during appetitive associative learning. Chapters 3 and 4 examined if communication between the BLA and mPFC is critical for cue value learning and updating. As previously mentioned, the anterior and posterior BLA are differentially recruited during different phases of cue-food learning (Cole et al., 2013; Cole et al., 2015a) and send differential, topographically organized projections to the mPFC (e.g. Reppucci & Petrovich, 2016). Thus, Chapter 3 examined if distinct pathways that originate in these BLA nuclei that project to the mPFC are differentially activated during early and late stages of learning and if there is learning-induced plasticity within these pathways. We accomplished this using retrograde tract tracing in combination with Fos induction analysis to determine how activation of the BLA-mPFC pathways changes from early to well-learned appetitive associative learning.

The results from Chapter 3, together with prior work, provided necessary background for Chapter 4. The goal of Chapter 4 was to examine if

communication between the BLA-mPFC circuitry is necessary for appetitive learning and behavioral flexibility. To accomplish this, we disrupted communication within this circuitry using the contralateral lesion design. This method lesions the BLA in one hemisphere and the mPFC in the other hemisphere, functionally disconnecting communication between the structures in both hemispheres but allowing one of each region to interact within other circuitries. We then examined if this disruption altered discriminative conditioning of appetitive cues and subsequent cue value updating during reversal learning and devaluation (as described above). The disconnection of the BLA-mPFC circuitry allowed us to assess how this circuitry mediates appetitive cue learning as well as behavioral flexibility due to a change in the value of food and food-associated cues (Chapter 4).

The studies in this dissertation explored the causal role of the BLA and its interaction with the mPFC in appetitive cue learning and valuation. Understanding how these regions interact when learning about neutral cues that come to signal food as well as when the value of these cues change is important to further our understanding of the neural substrates necessary for appetitive learning and memory, especially when these cues control eating behavior.

Chapter 2: Investigation of cue-specific neuronal ensembles within the basolateral amygdala during appetitive reversal learning

ABSTRACT: Through Pavlovian appetitive conditioning, environmental cues can come to predict food. However, the food outcome, and thus the value of the associated cues, can change based on environmental variations. This change in outcome necessitates updating of the value of the cue to appropriately alter behavioral responses to these cues. The basolateral amygdala (BLA) is critical in updating the outcomes of learned cues; however, it is unknown if the same BLA neuronal ensembles that are involved in the initial associative memory are required when the new cue-outcome association is formed during reversal learning. The current study used a method that enables selective targeting of activated neurons. In *Fos-lacZ* transgenic rats, Fos activation in response to a stimulus initiates *lacZ* transcription resulting in the protein product β-gal. Infusions of Daun02 selectively inactivate β-gal expressing neurons, resulting in inactivation of neuronal ensembles responsive to a specific stimulus. Using this method, we tested whether the same BLA neuronal ensembles that were activated during memory recall of a learned cue (CS+ or CS-) at the end of discriminative conditioning are necessary to learn new associations when the cue outcomes are changed during reversal learning. *Fos-lacZ* transgenic rats were implanted with bilateral cannulas in the BLA and underwent appetitive discriminative conditioning in which rats had to discriminate between two auditory stimuli (tone; white noise). One stimulus co-terminated with food delivery (CS+),

and the other stimulus was unrewarded (CS-; counterbalanced). Rats were then tested for CS+ or CS- memory retrieval and infused with either Daun02 or a vehicle solution into the BLA to inactivate neuronal ensembles that are activated during that test. Then, to assess if the same neuronal ensembles are necessary for learning new associations when the outcomes are changed, rats underwent reversal learning: the CS+ is no longer followed by food (reversal CS-, rCS-), and the CS- is now always followed by food (reversal CS+; CS+). The group that received Daun02 following CS+ session showed a decrease in conditioned responding to the rCS- (previously CS+) throughout reversal learning. This indicates that neuronal ensembles that are activated during the recall of the CS+ memory are the same neuronal ensembles needed for learning the new outcome of the same CS. Additionally, the group that received Daun02 following CS- session was slower to respond to the rCS+ (previously CS-) during reversal learning. This indicates that neuronal ensembles that are activated during the recall of the CS- memory are the same neuronal ensembles needed for learning the new outcome of the same CS. These results indicate different neuronal ensembles within the BLA mediate memory recall of CS+ and CS- cues and reactivation of each cue-specific neuronal ensemble is necessary to update the value of that specific cue to respond appropriately during reversal learning.

1. Introduction

Environmental cues can become strongly associated with food if they frequently occur together, and subsequent presentation of these learned cues can lead to food procurement and consumption when internal hunger cues are absent (Weingarten, 1983; Birch et al., 1989; for reviews see Saper et al., 2002; Petrovich & Gallagher, 2003; Holland & Petrovich, 2005; Petrovich, 2013). However, the outcomes, and thus the values, of associated cues are not always static and can change based on environmental variations. This change in the outcome of a learned cue requires updating the value of the cue to produce appropriate behavioral responses.

One paradigm in the laboratory used to assess this valuation updating is reversal learning, which entails switching the outcomes of previously learned cues. After successful discriminative conditioning between a cue that signals food delivery (conditioned stimulus; CS+) and cue that signals nothing (CS-), the CS+ is now no longer followed by food (reversal CS-; rCS-), and the CS- is now always followed by the delivery of food (reversal CS+; rCS+) during reversal learning. Successful reversal learning is observed by two distinct behaviors: the inhibition of conditioned responding to the initial food-associated cue (CS+) since the cue now signals the absence of food (now rCS-), and the initiation of conditioned responding to the cue now signaling food delivery (CS- now rCS+). Reversal learning necessitates updating the value of the cues based on the change in the outcomes and requires behavioral flexibility to respond correctly.

The basolateral nucleus of the amygdala (BLA) is a critical forebrain region necessary for associative conditioning and is an early processor of appetitive learning (Piette et al., 2012; Cole et al., 2013). The BLA is critically involved in appropriate behavioral responding when the values of learned appetitive cues are changed (Schoenbaum et al., 1999; Nomura et al., 2004; Corbit & Balleine, 2005; Tye et al., 2010) or additional cues are incorporated to update the value of learned appetitive cues (Blundell et al., 2001; Hatfield et al., 1996; Setlow et al., 2002; Ishikawa et al., 2008; for reviews, see Holland et al., 2001, 2002; Everitt et al., 2003; Holland & Petrovich 2005, Petrovich, 2013; Wassum & Izquierdo, 2015). *In vivo* recording studies have shown that BLA neurons respond to appetitive cues, but then change their response profiles when cue outcomes are reversed (Scheonbaum et al., 1999) and when the reward is omitted (Tye et al., 2010), such as when the CS+ switches to the rCS-. These studies suggest BLA neurons can alter their response to food predictive cues when the outcome changes; however, it is unknown if the same or different BLA neuronal ensembles that are activated during the recall of learned cues are necessary for updating the values of these cues to form a new association when the outcomes change.

The current study used a chemogenetic inactivation procedure, the Daun02 inactivation method, to inactivate BLA neuronal ensembles that are selectively activated by either a CS+ or CS- to determine if these specific neuronal ensembles are necessary for reversal learning. In *Fos-lacZ* transgenic rats, the transgene *lacZ* produces the protein β-galactosidase (β-gal), and its

expression is controlled by the induction of an immediate early gene, *c-fos,* a well-known marker of neural activation (Curran et al., 1985; Müller et al 1984). Thus, neurons that produce *c-fos* in response to a stimulus also produce β-gal (Koya et al., 2009; Cruz et al., 2013). These specific neurons can be inactivated by infusions of Daun02, which is catalyzed by β-gal into daunorubicin, resulting in a reduction in neuronal excitability (Santone et al., 1986; Engeln et al., 2016) and eventually in cell death by apoptosis (Pfarr et al., 2015). After at least three days (optimal time for Daun02 inactivation [Koya et al., 2009]), rats can be tested on the behavioral paradigm of interest. This method has been effective in examining the role of specific neuronal ensembles in various appetitive learning paradigms for rewards, including food (Warren et al., 2016; Whitaker et al., 2017), drugs (Koya et al., 2009; Fanous et al., 2012; Cruz et al., 2014; Funk et al., 2016; Caprioli et al., 2017; Xue et al., 2017), and alcohol (Pfarr et al., 2015; de Guglielmo et al., 2016; George & Hope, 2017).

With the Daun02 method, we inactivated BLA neuronal ensembles that were activated by either the CS+ or CS- memory to examine if either the CS+ or CS- neuronal ensembles or both are needed to learn new cue associations when the outcomes are changed. Specifically, *Fos-lacZ* transgenic rats underwent discriminative conditioning and were then infused with Daun02 or a vehicle solution into the BLA after presentation of either the CS+ or CS- to inactivate the responsive neuronal ensembles. Afterwards, rats underwent either one or fifteen sessions of reversal learning to observe how BLA neuronal ensemble inactivation

affected conditioned responding to the initial memory recall of the CSs and during complete reversal learning, respectively.

We hypothesized that separate BLA neuronal ensembles are activated during CS+ and CS- memory recall, and inactivating the neuronal ensembles that respond to a particular CS will only alter the memory of that CS and not the other CS. We also predicted that neuronal ensembles that are activated by a particular CS will be necessary to learn the new associations to the same CS when the outcome is changed throughout reversal learning.

2. Materials and Methods

2.1. Subjects. Experimentally naïve male and female *Fos-lacZ* transgenic rats bred in the animal facility at Boston College were used. Rats had *ad libitum* access to food (standard laboratory chow) and water and were grouped housed until ~2 days prior to surgical procedures when they were individually housed and acclimated to experimenter handling (~2 months old). The colony room was maintained on a 12-hour light/dark cycle with lights on at 06:00. All procedures complied with the National Institutes of Health *Guidelines for Care and Use of Laboratory Animals* and were approved by the Boston College Animal Care and Use Committee.

2.2. Surgical Procedure. For surgeries, rats were anesthetized with either isoflurane (1-3%) or a mixture (1 ml/kg body weight) of ketamine (50 mg/ml) and xylazine (10 mg/ml). Rats were bilaterally implanted with 22- or 26-gauge guide cannulas (Plastics One Inc.) targeting the BLA. The flat-skull coordinates from bregma were 2.7-3.0 mm anteroposterior, ±4.6-4.8 mm

mediolateral, and -7.1 mm dorsoventral. Cannulas were anchored to the skull with screws and dental cement. Obturators were inserted into the guide cannulas where they remained throughout the experiment, except during microinfusions. Triple antibiotic cream was applied around the cannula cap, and rats were given analgesic Rimadyl (Henry Schein; 50 mg/ml) in a sterile saline solution (4.4 mg/kg) the day of surgery and chewable Rimadyl tablets (Bio Serv; 2 mg/1 tablet/100 g bodyweight) for two days after surgery. Rats were allowed to recover for at least 5 days post-surgery prior to behavioral training and were monitored and weighed daily.

2.3. Intracranial Infusions. For microinfusions, obturators were removed, and injectors were inserted into the guide cannula. Injectors that projected 1 mm ventral to the tip of the guide cannula were connected via polyethylene tubing to 10 µl Hamilton syringes and mounted onto an infusion pump. Either Daun02 or vehicle solution were infused at a rate of 0.5 µl/side over 1 min with an additional 1 min post-injection diffusion time. Following infusions, obturators were reinserted, and rats were returned to their home cage.

Daun02 (Sequoia Research Products) was dissolved in 5% dimethyl sulfoxide (Sigma-Aldrich), 6% Tween-80, and 89% phosphate buffered saline. Vehicle solution consisted of the same solution without Daun02.

2.4. Apparatus. All habituation and training occurred within the same set of behavioral chambers (30 x 28 x 30 cm; Coulbourn Instruments) located in a separate room from the colony room. The top and sides of the chambers were aluminum, and one side contained a recessed food cup (3.2 x 4.2 cm). The front

and back of the chamber were transparent Plexiglas, and the front panel was hinged. The floor was composed of 5 mm stainless-steel rods spaced 15 mm apart. Each chamber was contained within an isolation cubicle (79 x 53 x 53 cm; Coulbourn Instruments) composed of monolithic rigid foam walls and a ventilation fan (55 dB). On the rear wall of each isolation cubicle was a video camera connected to a recording system (Coulbourn Instruments).

The conditioned stimuli (CSs) were a 10s 75dB, 2kHz tone and a 10s 75dB white noise. The unconditioned stimulus (US) was two food pellets (TD pellets; formula 5TUL, 45 mg: Test Diets) and were delivered into the food cup of each chamber when applicable. GraphicState 3.0 software system (Coulbourn Instruments) controlled all stimuli.

2.5. Behavioral Training Procedure. Fig. 2.1 outlines the experimental design. Rats were food restricted to maintain 90% of their maximum recorded body weight throughout training. Two days prior to training, rats received 1g of the US in their home cage to reduce novelty to the training food. The following day rats underwent a ~30 min magazine training in the behavioral chambers where they were given random deliveries of the US to familiarize them with eating from the food cup.

***2.5.1. Discriminative Conditioning*.** Rats received ten 30 min training sessions over 5 days (2 sessions/day). Each session consisted of intermixed presentations of two different auditory cues, each presented six times. One auditory CS (e.g. tone) was immediately followed by delivery of 2 TD pellets (CS+), and the other CS (e.g. noise) was presented alone (CS-). Auditory cues

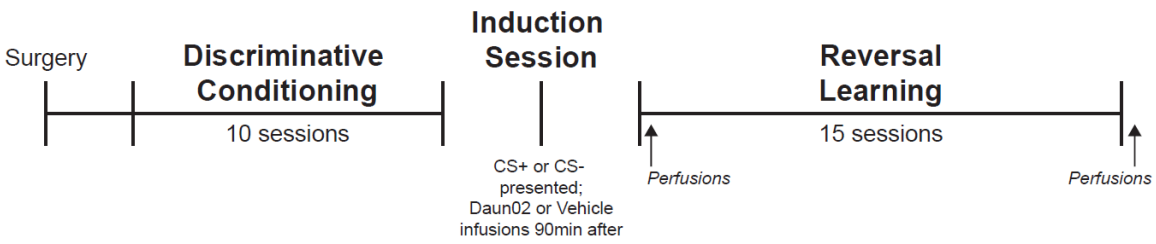

Figure 2.1 Experimental Design. Prior to training, all rats received bilateral cannulas aimed at the BLA. Behavioral training included 10 sessions of discriminative conditioning (2 sessions/day), and in each session, rats received intermixed presentations of 6 CS-US pairings (CS+; e.g. tone-food) and 6 CS- presentations (e.g. white noise alone). During the induction session, rats were given 6 presentations of either the CS+ or CS- without food delivery, and then infused with either Daun02 or Vehicle into the BLA. Three days later, rats underwent reversal learning for either 1 or 15 sessions (1-2 sessions/day). Rats were perfused 90 min after the end of the test sessions for analysis of Fos and β-Gal induction.

that served as CS+ and CS- were counterbalanced. The inter-trial intervals (ITIs) were between 60-219s, and ITIs and CS order varied randomly across training sessions.

2.5.2. Induction session. Following successful discriminative conditioning, rats underwent an induction session in which they were given six presentations of either the CS+ or CS- (without pellets) to induce Fos and thus β-gal in neuronal ensembles activated during that session and thus specific to one CS. Ninety minutes following the beginning of the induction session when Fos expression is optimal, rats were briefly anesthetized with isoflurane and received infusions of either Daun02 or vehicle.

2.5.3. Reversal Learning. At least three days following induction session, rats began reversal learning. These sessions were similar in length and number of CS presentations as the discriminative conditioning sessions;

however, the outcomes of the CS+ and CS- were reversed. The original cue that was associated with the TD pellets (CS+) was now no longer followed by the pellets (reversal CS-; rCS-), and the cue that was previously followed by nothing (CS-) was now always followed by the delivery of pellets (reversal CS+; rCS+). A subset of rats underwent only 1 reversal session to observe responding to the initial memory recall of the CSs and for induction of Fos and β-gal, and the rest of the rats underwent 15 sessions of reversal learning (1-2 sessions/day).

2.6. Behavioral Measures. The primary measure of learning was conditioned responding to the food cup during the presentations of the CSs. This behavior was defined as rats standing in front of and directly facing the food cup or demonstrating distinct nose pokes into the food cup. Trained observers unaware of group allocation recorded rats' behavior every 1.25 seconds during each 10 second CS, as well as during 10 seconds prior to the onset of the CS as a measure of baseline responding (pre-CS). Only one behavior was recorded at each observation (food cup or nothing). The total number of identified food cup observations for each CS during each period was summed and converted into a percentage of total time rats spent in the food cup, which was used to calculate the mean value for each group. CS elevation was calculated by subtracting pre-CS responding from CS responding (CS minus pre-CS) then used to calculate the mean value for each group.

Latency was measured as the time elapsed from the onset of the CS until the rat approached the food cup within 20s after the CS onset (10s CS plus 10s to collect the US, if applicable). After this time, behavior was considered

unspecific to the presentation of the CS, and a maximum latency of 20s was assigned to any trial that surpassed this time without a response. For each rat, latency for each trial was used to calculate the average latency responding for each CS during each session and then used to calculate the mean value for each group.

2.7. Histological Procedures and Immunohistochemistry. Ninety minutes following either the first or 15th reversal session, rats were perfused, and brain tissue was collected for analysis of Fos and β-gal induction and cannula placement. Rats were given a lethal dose of tribromoethanol (1.5ml/100g bodyweight; i.p.) and transcardially perfused with 0.9% saline followed by ice cold 4% paraformaldehyde in 0.1M borate buffer. Brains were stored overnight (18-24hr) in the paraformaldehyde solution with 12% sucrose at 4°C, and then rapidly frozen in hexanes cooled in dry ice and stored at -80°C.

Frozen brains were sliced into 30 μm coronal slices using a sliding microtome (Leica Biosystems) and collected into four serially adjacent series. One series of tissue was mounted from a potassium phosphate-buffered saline solution (KPBS) onto gelatin-coated slides, dried at 45°C, dehydrated through graded alcohols, stained with thionin, cleared in xylenes, and coverslipped with DPX Mountant. The thionin stain allowed for identification of neuroanatomical borders and cannula placements, which were examined under a light microscope and mapped using a rat brain atlas (Swanson, 2004).

Another series of tissue was stained for identification of Fos and β-gal induction to verify the Daun02 inactivation method (i.e. decreased β-gal levels in

Daun02 infused groups compared to vehicle infused groups). Brain tissue was incubated with anti-*c-fos* primary antibody raised in rabbit (1:10,000, ABE457; Millipore, Temecula, CA, USA) and anti-β-gal primary antibody raised in mouse (1:2,000, sc-65670; Santa Cruz, Dallas, TX, USA) in a solution containing KPBS, 2% normal donkey serum (NDS; Jackson ImmunoResearch, West Grove, PA, USA), and 0.3% Triton X-100 (Sigma-Aldrich) for 72 hours at 4°C. Tissue was then rinsed with KPBS and incubated with secondary fluorescent antibodies: Alexa 488 anti-rabbit raised in donkey (1:200, A21206; Invitrogen, Carlsbad, CA, USA) and Alexa 594 anti-mouse raised in donkey (1:200, A21203, Invitrogen) in KPBS, NDS, and Triton X-100 for 1 hr in semi-darkness. Tissue was rinsed again and mounted onto Superfrost slides in semi-darkness, air-dried, coverslipped with Vectashield Hardset Mounting Medium with DAPI (4',6-diamidino-2-phenylindole; H-1500; Vector Laboratories), and stored at 4°C until analysis.

2.8. Image Acquisition and Analysis. Images throughout the BLA were acquired with a Zeiss Axio Image Z2 fluorescence microscope (Carl Zeiss Microscopy GmbH, Jena, Germany) and attached Hamamatsu ORCA-R2 camera (Bridgewater, NJ, USA) using Zen software. Images were pseudo-colored with green for Fos, red for β-gal, and blue for DAPI (nuclear counterstain). Single Fos-labeled and single β-gal-labeled neurons were manually counted from acquired images and summed for each stain for each rat to calculate the total number of Fos-labeled neurons and the total number of β-gal-labeled neurons. Each count was then averaged within groups to determine mean counts for each group.

2.9. Statistical Analysis. Behavioral data were analyzed using a mixed design analysis of variance (ANOVA) with Group (CS+Daun02, CS-Daun02, Vehicle) as a between-subjects factor and type of CS Elevation (CS+, CS- or rCS+, rCS-) and conditioning sessions (discriminative conditioning 10, induction session, or reversal learning session 1, 5, 10, and 15) as within-subjects repeated factors. The dependent variable was the percentage of time rats displayed conditioned responding and latency (in seconds) for all behavioral training. For the neural data analysis, the dependent variable was the number of β-gal-labeled neurons across experimental groups. A significance value of $p < 0.05$ was used for all analyses, except for Group *post-hoc* analyses in which Bonferroni adjusted alpha level was used ($p = 0.05/3 = 0.017$). SPSS software was used for all statistical analyses.

Daun02 groups were treated as separate groups due to the nature of the hypothesis – the CS+ and CS- are mediated by separate BLA neuronal ensembles and inactivating the neuronal ensembles that respond to a particular CS will only alter responding of that specific CS and not the other CS. Furthermore, in order to confirm Daun02 did not have a general drug effect regardless of CS induction, statistical analyses comparing all Daun02 infused rats compared to Vehicle infused rats confirmed no overall differences caused specifically by the drug for all measures recorded (p's > 0.05). This analysis shows that any observed behavioral differences in either Daun02 group is not generally due to the drug itself but is instead due to the specific CS neuronal ensemble inactivation caused by Daun02 (see Pfarr et al., 2015).

Additionally, groups that received Vehicle infusions following either CS+ or CS- induction session were combined into one group for analyses. The rationale was that responding should be similar between these groups since no neural deficits occurred due to infusing the vehicle solution, which was statistically confirmed for all measures recorded (*p*'s > 0.05), except Reversal session 5 rCS difference (t(7) = -2.52, *p* = 0.04), which may be a small artifact due to a small sample size in the CS+Vehicle group (n=2).

2.10. Exclusion. Thirteen rats were excluded due to improper cannula placements, and two rats were excluded due to extensive damage caused by infection. Additionally, 8 rats were excluded due to failure to discriminate between CSs (CS+ responding minus CS- responding < 15% food cup responding), and 4 rats were excluded due to failure to consume food pellets during discriminative conditioning.

3. Results

3.1. Histology. All groups received similar cannula placements as shown in Fig 2.2. Cannula placements were within or directly above the BLA. Final group numbers based on proper cannula placements were CS+Daun02 (n=9 total; n=3 for Reversal Session 1 [R1]; n=6 for Reversal Session 15 [R15]), CS-Daun02 (n=8 total; n=2 for R1 rats; n=6 for R15), and Vehicle groups were combined for analyses, except for induction session (n=14 total; n=3 for R1 CS+; n=2 for R1 CS-; n=2 for R15 CS+; n=7 for R15 CS-).

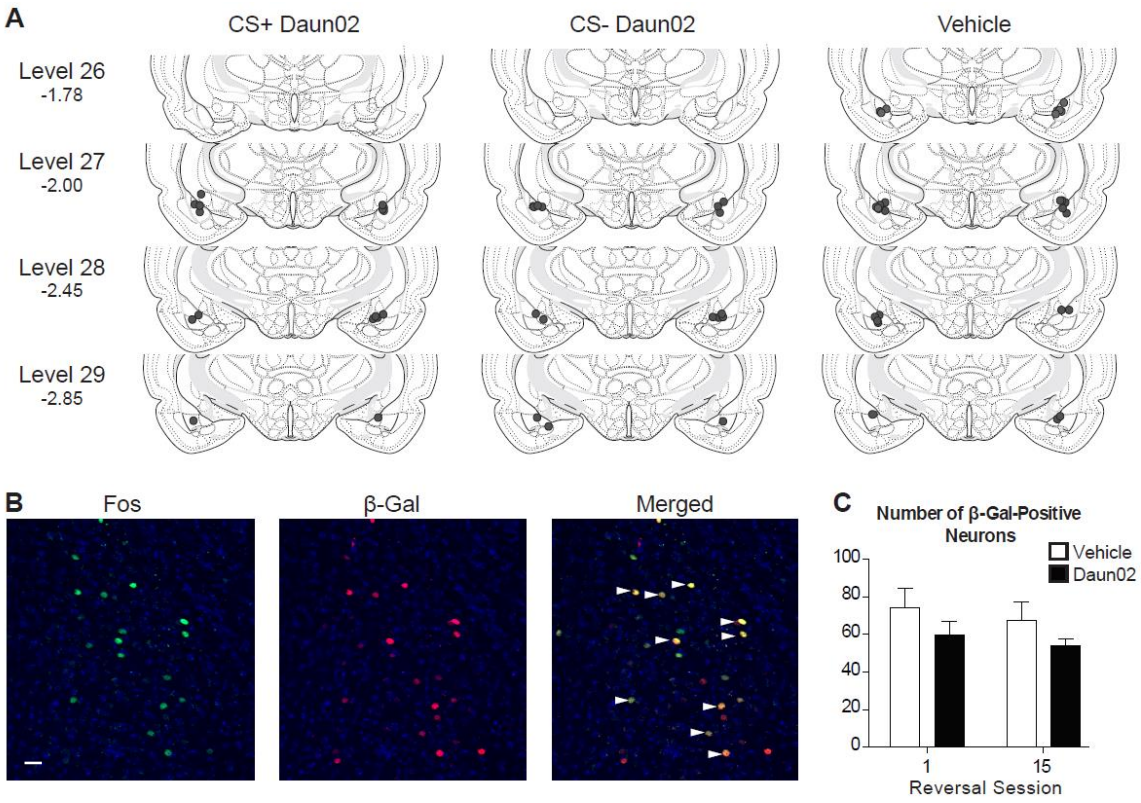

Fig. 2.2 Cannula placements and Fos and β-Gal induction. (A) All cannula placements were within atlas levels 26-29 of the BLA (-1.78 to -2.85mm from Bregma). (B) Representative images showing Fos, β-Gal, and colocalization (white arrows). (C) There was a general reduction in β-Gal-labeled neurons in groups that received Daun02, but it did not reach significance ($F(1,25) = 2.08$, $p = 0.16$). Scale bar = 50 μm.

There was a general decrease in the number of β-Gal-labeled neurons in rats that received the Daun02 compared to Vehicle, but this effect did not reach significance ($F(1,25) = 2.08$, $p = 0.16$; Fig 2.2B,C).

3.2. Discriminative Conditioning. All groups successfully discriminated between the CS+ and CS-, as shown by higher conditioned responding during the CS+ compared to the CS- during the tenth training session (Fig. 2.3A). These results were expected since no drug was given during training, and group allocation was based on drug treatment after the induction session. A

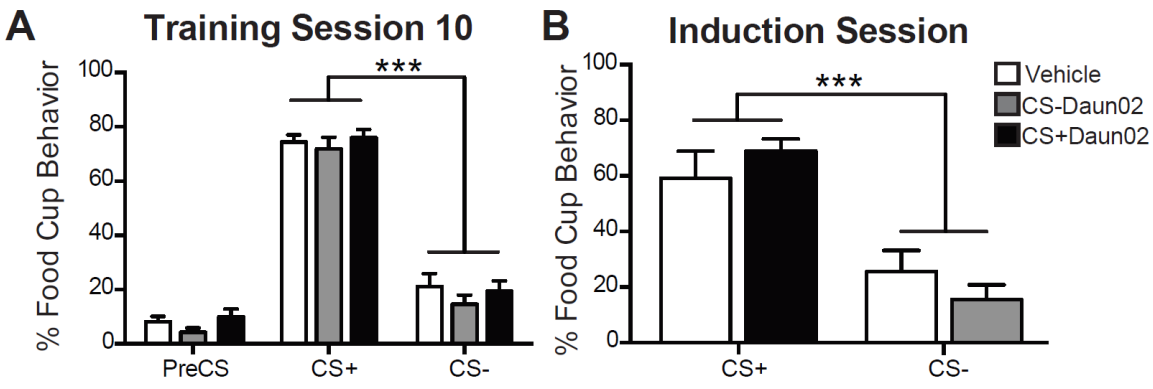

Figure 2.3 Conditioned responses during the final discriminative conditioning session and induction session. Average percentage of time (mean ± SEM) rats expressed food cup behavior during PreCS, CS+, and CS- during training session 10 (A) and during either presentation of CS+ or CS- during the induction session (B).
*** indicates $p < 0.001$.

group (CS+Daun02, CS-Daun02, Vehicle) X CS Elevation (CS+, CS-) repeated measures ANOVA showed an effect of CS ($F(1,28) = 338.29$, $p < 0.001$) but no Group effect or interaction (F's < 1).

3.3. Fos & β-gal Induction Session. After discriminative conditioning, rats underwent a session with either presentation of the CS+ or CS- to induce Fos in the BLA in response to the respective CS. Conditioned responding was similar to the last conditioning session with higher responding in the CS+ induction groups compared to the CS- induction groups (Fig. 2.3B). A Drug (Daun02, Vehicle) X CS Elevation (CS+, CS-) ANOVA during the induction session confirmed a CS effect ($F(1,27) = 42.26$, $p < 0.001$) but no drug effect or interaction (F's < 2.3, p's > 0.1). These results were expected since drug infusions occurred following the induction session.

3.4. Reversal Learning. For behavioral analysis, all rats were included in analysis for reversal session 1 (Section 3.4.1), and only rats that underwent 15 sessions of reversal training were included in analysis for reversal sessions 1-15 (Section 3.4.2).

3.4.1. Reversal Session 1. The group that received Daun02 following CS+ presentations during the induction session (CS+Daun02), and thus CS+ neuronal ensemble inactivation, had lower conditioned responding to the same CS, now rCS-, during reversal session 1 (Fig. 2.4). Analysis of conditioned responding to the CSs above the baseline (Elevation: CS minus pre-CS; Fig. 2.4A) with a Group X CS repeated measures ANOVA showed a significant effect of CS ($F(1,28) = 223.24$, $p < 0.001$) but no effect of Group ($F < 1.5$, $p > 0.1$) or interaction ($F < 2.4$, $p = 0.11$). Simple effect analyses showed a Group effect on average responding to the rCS- ($F(2,28) = 3.36$, $p = 0.049$), and using adjusted Bonferroni alpha levels of p = 0.017, there was a nearly significant effect between the CS+Daun02 and CS-Daun02 ($p = 0.018$), but not between the CS+Daun02 and Vehicle ($p = 0.066$) or between the CS-Daun02 and Vehicle ($p > 0.1$).

Additionally, we analyzed latency to approach the food cup after the onset of each cue and found rats approached the food cup faster during the rCS- compared to the rCS+, as expected since the rCS- was previously the CS+. A Group X CS ANOVA showed an effect of CS on overall average latency to respond to the food cup ($F(1,28) = 103.81$, $p < 0.001$; Fig. 2.4B) showing rats approached the food cup faster during the rCS- compared to the rCS+, but there was no Group effect or interaction (F's < 1). Separate follow-up analyses showed

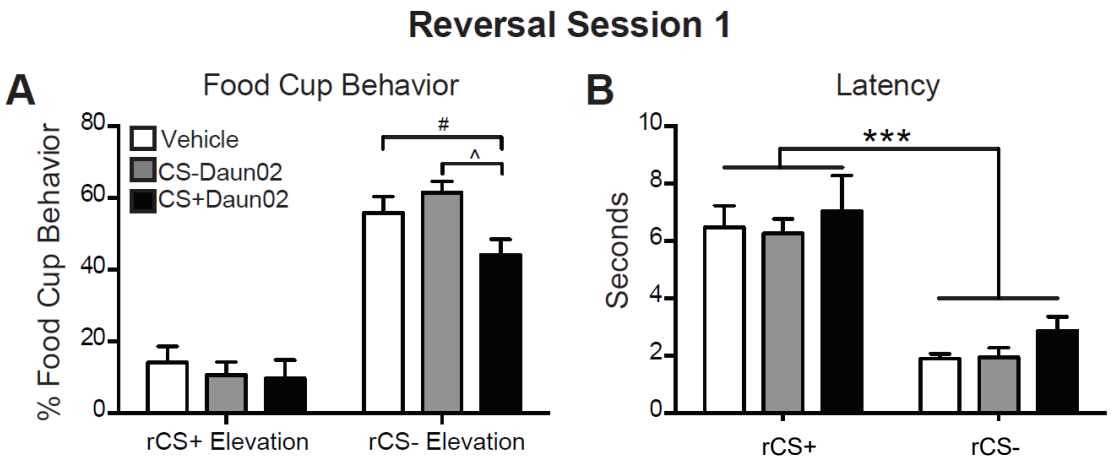

Figure 2.4 Conditioned responding during Reversal Session 1. (A) Average food cup responding (mean ± SEM) during rCS+ and rCS- during the session. Data shown as elevation score (CS responding minus pre-CS [baseline] responding). (B) Average latency to approach the food cup (mean ± SEM) during the rCS+ and rCS- during the session.
***$p < 0.001$; ^ $p = 0.018$; # $p = 0.066$

a nearly significant effect of Group for rCS- ($F(2,28) = 2.78$, $p = 0.079$) but no Group effect for the rCS+ ($F < 1$).

3.4.2. Reversal Learning Across Sessions. We then analyzed responding in a group of rats that underwent 15 sessions of reversal learning to determine if CS-specific inactivation of BLA neuronal ensembles interfered with reversal learning across sessions. The group that received Daun02 following CS+ induction showed a decrease in conditioned responding to the same CS, now the rCS-, throughout reversal learning (Fig 2.5B). Analysis of responding to CSs above the baseline (Elevation: CS minus pre-CS) with a Group X CS X Session repeated measures ANOVA showed a CS X Group interaction ($F(2,18) = 7.33$, $p < 0.01$), a main effect of Session ($F(3,54) = 5.23$, $p < 0.01$), CS ($F(1,18) = 40.82$, $p < 0.001$), and a Session X CS interaction ($F(3,54) = 81.38$, $p < 0.001$), but no other effects or interactions (F's < 2, $p > 0.1$). Follow-up analyses

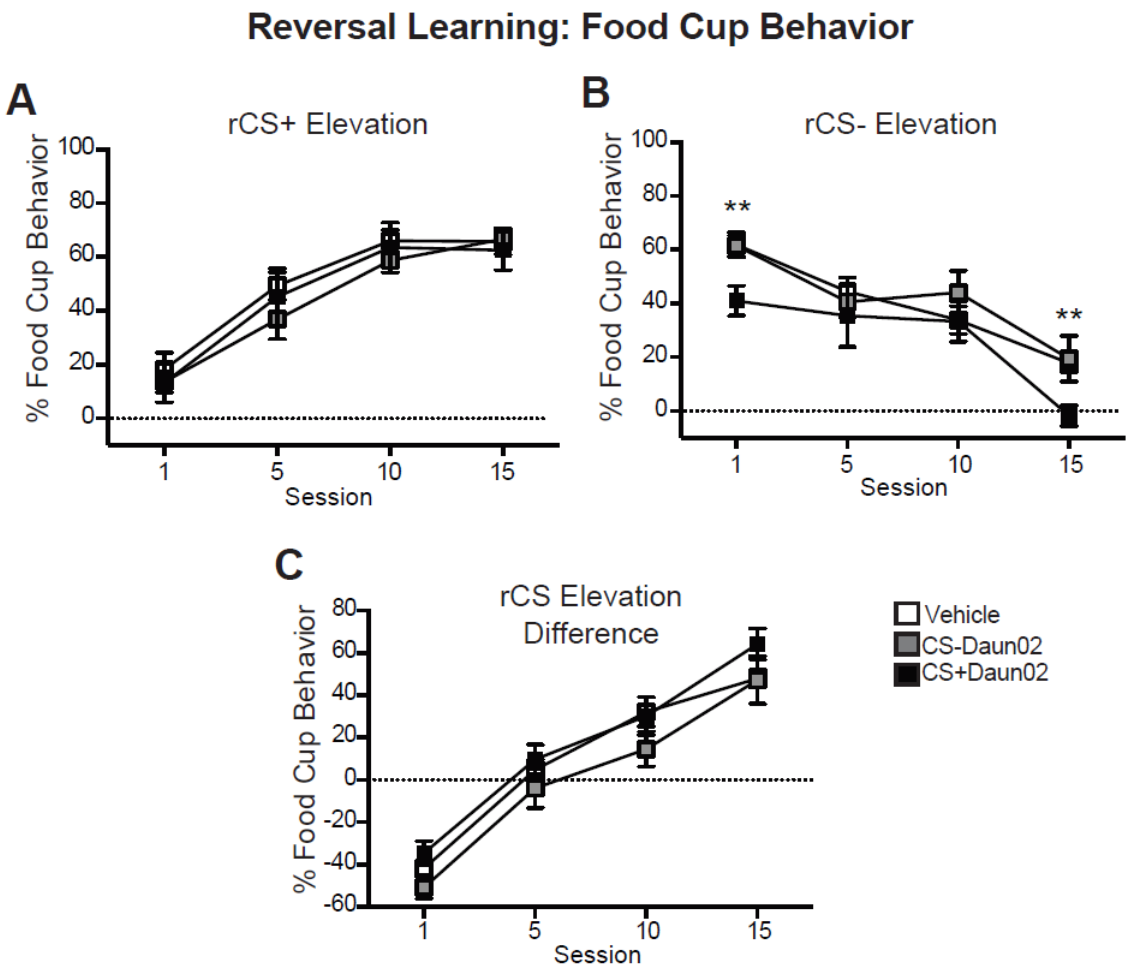

Figure 2.5 Conditioned responding throughout reversal learning. Average percentage of time (mean ± SEM) rats expressed food cup behavior during each session represented as an elevation score (CS responding minus pre-CS [baseline] responding) during the rCS+ (A), rCS- (B), or the difference in responding between rCS+ and rCS- (C).
** $p < 0.017$ (correct alpha level) CS+Daun02 vs CS-Daun02 and Vehicle

confirmed a significant effect of Group on conditioned responding to the rCS- ($F(2,18) = 5.08$, $p < 0.05$). After Bonferroni corrected alpha levels, the CS+Daun02 group had significantly lower responding to the rCS-, which was previously their CS+, compared to the Vehicle and CS-Daun02 groups (p's < 0.017) across reversal learning. Follow up analyses showed a Group effect specifically for session 1 ($F(2,20) = 5.61$, $p < 0.05$), similar to the effect found in

Reversal Session 1 (Section 3.4.1), and also showed a Group effect for session 15 ($F(2,20) = 4.71$, $p < 0.05$). The CS+Daun02 group had significantly lower responding compared to the Vehicle and CS-Daun02 groups during both sessions (p's < 0.017).

Additionally, we analyzed the difference in specific responding (rCS+ Elevation minus rCS- Elevation; Fig 2.5C) and found an effect of Group ($F(2,18) = 7.33$, $p < 0.01$) and Session ($F(3,54) = 81.38$, $p < 0.001$). *Post-hoc* analyses showed the CS+Daun02 group responded significantly higher on this measure, due to their lower conditioned responding to the rCS-, compared to the CS-Daun02 group ($p < 0.017$) and compared to the Vehicle group ($p = 0.04$). Additionally, the CS-Daun02 group responded lower on this measure, due to their lower conditioned responding to the rCS+, compared to the Vehicle group, but this failed to reach significance ($p = 0.06$).

The analyses on latency responding showed the group that received Daun02 following CS- induction was slower to respond to the food cup after the same cue presentation, now rCS+, during reversal learning (Fig 2.6A). A Group X CS X Session repeated measures ANOVA found a main effect of Session ($F(3,54) = 14.51$, $p < 0.001$) and CS ($F(1,18) = 6.85$, $p < 0.02$), and a Session X CS interaction ($F(3,54) = 40.79$, $p < 0.001$), but no Group effects or interaction (F's < 1.5, p's > 0.1). Simple effect analyses showed an effect of Group on responding to the rCS+ during session 5 ($F(2,20) = 7.39$, $p < 0.01$) and session 10 ($F(2,20) = 4.21$, $p < 0.05$). The CS-Daun02 group had significantly longer latencies to the rCS+ compared to the Vehicle group during session 5 and 10 (p's

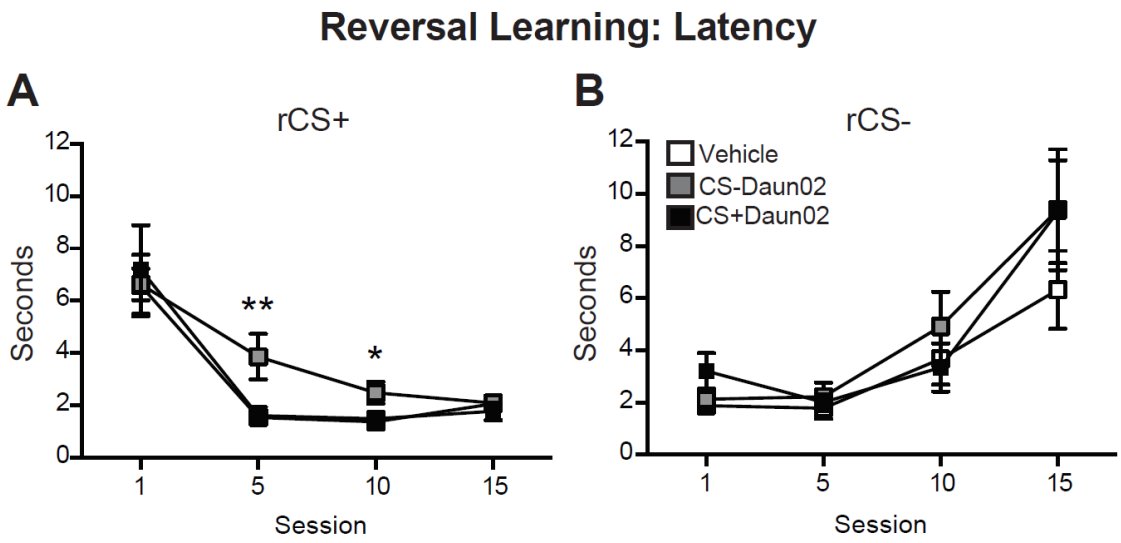

Figure 2.6 Latency responding throughout reversal learning. Average latency (mean ± SEM) to approach the food cup during reversal learning after rCS+ onset (A) and rCS- onset (B).
** $p < 0.017$ (correct alpha level) CS-Daun02 vs CS+Daun02 and Vehicle
* $p < 0.017$ CS-Daun02 vs Vehicle and $p = 0.037$ CS-Daun02 vs CS+Daun02

< 0.017) and compared to the CS+Daun02 group during session 5 ($p < 0.01$) and

10 ($p = 0.037$). No differences were found in latency responding to the rCS- ($F <$

2; Fig. 2.6B).

4. Discussion

The current study examined if separate BLA neuronal ensembles are activated during CS+ and CS- memory recall. This was accomplished by testing if inactivating the neuronal ensembles that respond to a particular CS altered the memory of that specific CS and not the other CS. Additionally, we examined if neuronal ensembles that are activated by a particular CS are necessary to learn the new associations to the same CS when the outcome is changed during reversal learning. Using the Daun02 inactivation method, we specifically inactivated BLA neuronal ensembles that were activated by either a CS that was previously associated with food (CS+) or a CS not paired with food (CS-), and then observed conditioned responding when the outcomes of the cues were switched during reversal learning. We found that the group that received the Daun02 following the CS+ session (CS+Daun02 group) showed a decrease in conditioned responding to the same CS, now the rCS-, during the first session and throughout reversal learning. This decreased conditioned responding to the rCS- indicates that the CS+ neuronal ensembles were necessary to recall the previously learned outcome of the cue during the first reversal session and to incorporate this information during reversal learning. Second, we found that the group that received Daun02 following CS- induction (CS-Daun02 group) was slower to respond to the food cup (i.e. longer latency) after the same cue presentation, now the rCS+, during reversal learning. Together, these results support our hypotheses that separate BLA neuronal ensembles mediate CS+ and CS- memory recall, and reactivation of each cue-specific neuronal ensemble

is necessary for updating the value of that specific learned cue to respond appropriately during reversal learning.

Importantly, the observed impairments in conditioned responding were specific to the CS to which the neuronal ensembles were inactivated and did not cause general impairment in behavioral responding. The CS+Daun02 was impaired on responding to rCS-, previously the CS+, and CS-Daun02 was impaired on responding to rCS+, previously the CS-. This suggests the Daun02 inactivated separate neuronal ensembles between the drug treated groups, which impaired subsequent, CS-specific reversal learning. This is in agreement with prior work that found specific effects of neuronal ensembles inactivation by Daun02 (see Pfarr et al., 2015). Additionally, other studies have shown altered reward-seeking behaviors due to specific neuronal ensemble inactivation with this method (Koya et al., 2009; Fanous et al., 2012; Cruz et al., 2014; de Guglielmo et al., 2016; Funk et al., 2016; Warren et al., 2016; Caprioli et al., 2017; George & Hope, 2017; Whitaker et al., 2017; Xue et al., 2017).

The results of the current study are in agreement with previous studies that showed separate BLA neuronal ensembles respond to distinct learned cues. Previous studies have shown that ~60% of BLA neurons respond to a distinct learned cue during appetitive learning, and then half of these neurons alter their responding when the outcomes are switched during reversal learning in rats (Schoenbaum et al., 1999) and primates (Paton et al., 2006). Interestingly, in both studies the neurons responded selectively to specific cues prior to correct behavioral performance, indicating BLA neurons are tracking the outcome and

the value of learned cues to guide behavior. Similarly, another study showed a subset of BLA neurons (~10%) respond to a well-learned reward predictive cue, but then distinctly alter their responses when food is no longer delivered during extinction (“reinforcement-omission” neurons; Tye et al., 2010), suggesting this subset of neurons may be tracking the outcome of the learned cues. This indicates that BLA neurons can alter their responses based on environmental changes, signifying neural plasticity.

Additionally, the current results indicate that the BLA regulates the updating of the outcome and value of learned appetitive cues. This was confirmed for both groups that received cue-specific neuronal ensemble inactivation by Daun02: the CS+Daun02 group had lower conditioned responding to the same CS during reversal learning (rCS-), and the CS-Daun02 group was slower to respond to the food cup after presentation of the same CS during reversal learning (rCS+). Indeed, previous studies have shown that an intact BLA is needed to access the value of the learned cue in order to appropriately update the value and alter necessary behavioral responding when the outcome is changed (as reviewed in Wassum & Izquierdo, 2015). In brief, the BLA encodes the value of the cues during learning (Uwano et al., 1995; Schoenbaum et al., 1999; Tye & Janak, 2007; Piette et al., 2012; Cole et al., 2013; Parkes & Balleine, 2013; Esber & Holland, 2014) and is involved in appetitive cue discrimination (Ambroggi et al., 2008; Ishikawa et al., 2008) and reversal learning (Churchwell et al., 2009). However, several studies have shown the BLA may not be critical for initial acquisition of cue value learning (Hatfield et al., 1996;

Parkinson et al., 2000; Holland et al., 2002; Balleine et al., 2003; Corbit & Balleine, 2005), but it is critical to encode and assess the representation of the learned associations to alter subsequent behavioral motivation and learning (Hatfield et al., 1996; Blundell et al., 2001; Holland et al., 2002; Setlow et al., 2002; Corbit & Balleine, 2005; Tye & Janak et al., 2007; Ostlund & Balleine, 2008; Coutureau et al., 2009; Johnson et al., 2009; Galarce et al., 2010; for reviews, see Holland et al., 2001, 2002; Everitt et al., 2003; Holland & Petrovich 2005, Petrovich, 2013; Wassum & Izquierdo, 2015). This suggests a specific role for the BLA in reward value representation when appetitive learning is altered.

In the current study, the goal was to induce Fos selectively within neuronal ensembles responsive to one learned cue (CS+ or CS-) without neural activation from the food or the other CS that would confound the results. For that reason, only one cue was given without food delivery during the induction session. As a result, extinction learning could have occurred, resulting in the observed lower conditioned responding in the CS+ group; however, we did not see a decrease in responding in the CS+ group compared to the CS- group that received the vehicle solution (see Section 2.9).

Additionally, it is possible the induction session activated distinct neurons responsible for memory recall and extinction learning. As previously mentioned, a subset of BLA neurons responded to a cue that predicts reward during learning and responded to that same cue without reward during extinction (“reinforcement-omission” neurons). Additionally, a subset of neurons responded only to the unexpected empty port during extinction learning (“extinction-only”

neurons; Tye et al., 2010). It is possible that since no reward was presented during the induction session, the current study could have induced Fos in a combination of these ensemble types. However, Tye and colleagues (2010) showed a direct relationship between the amount of neural activation of "extinction-only" neurons and extinction learning: more activation of these neurons resulted in better extinction learning, as observed by lower conditioned responding. In the current study, we also observed a decrease in conditioned responding to the cue no longer followed by food (i.e. extinction conditions of that cue; CS+ now rCS-) after CS+ ensemble inactivation, whereas Tye and colleagues (2010) showed more decreased responding with increased activation. Additionally, another study showed inactivation of the BLA impaired extinction learning as observed by maintained high conditioned responding (McLaughlin & Floresco, 2007), whereas the current study showed lower conditioned responding after BLA inactivation manipulations. Based on the patterns we observed, the manipulation in the current study inactivated the BLA neuronal ensembles responsible for the memory of the CS+ resulting in the improper recall of the learned value of the CS during the first reversal session. It does not appear that we inactivated "extinction-only" neurons; however, we cannot rule out the possibility that a small proportion of the induced Fos was in neuronal ensembles responsible for extinction learning.

The current results and previous studies signify that separate neuronal ensembles within the BLA can respond to distinct stimuli during appetitive learning. Tye and colleagues (2010) showed the heterogeneity of neural

responding in the BLA with about half of recorded neurons showing a response selective to at least one stimuli across appetitive learning and extinction, such as to a learned cue regardless of outcome, to the sucrose outcome itself, to port entries that either contained sucrose or not, and to all food port entries regardless of reward. The heterogeneity of the response profiles of the BLA neurons during appetitive learning may reflect the distinct, and presumably slightly overlapping, outputs to other regions to regulate value updating (i.e the medial prefrontal cortex, orbital cortex, gustatory area, and nucleus accumbens) and to regulate behavioral output (i.e. the central amygdala and lateral hypothalamus). Indeed, the BLA sends topographically organized pathways to the medial prefrontal cortex (mPFC; Kita & Kitai, 1990; Swanson & Petrovich, 1998; Hoover & Vertes, 2007; Reppucci & Petrovich, 2016), and the interactions between the BLA and mPFC are important for reward learning (Fuchs et al., 2007; Mashhoon et al., 2010; Stefanik et al., 2013; Keefer & Petrovich, 2017) and behavioral impulsivity (Churchwell et al., 2009). The BLA also has reciprocal connections with another part of the prefrontal cortex, the orbital area (McDonald, 1991; McDonald et al., 1996; Hoover & Vertes, 2011; Murphy & Deutch, 2018), and communication between these areas is critical in value representation during learning (Baxter et al., 2000; Saddoris et al., 2005; Schoebaum et al., 2003a, 2003b; Rudebeck et al., 2013; Zeeb & Winstanley, 2013). Additionally, palatability processing and reward value representation depend on communication from the BLA to the gustatory area (Piette et al., 2012; Parkes & Balleine, 2013) and neighboring agranular insular area (Nasser et al., 2018).

Apart from these cortical structures, BLA projections to the nucleus accumbens (McDonald, 1991; Wright et al., 1996) are critical for behavioral responding during reward seeking and reward learning (Cador et al., 1989; Setlow et al., 2002; Di Ciano & Everitt, 2004; Kelley, 2004; Ambroggi et al., 2008; Shiflett & Balleine, 2010; Stuber et al., 2011). Lastly, the BLA has critical direct outputs to the lateral hypothalamus as well as indirect connections through the central amygdala (Krettek & Price, 1978; Ono & Nhmi, 1985; Petrovich & Swanson, 1997; Petrovich et al., 2001; Hahn & Swanson, 2010, 2012, 2015; Reppucci & Petrovich, 2016), and these functional connections are important for appetitive motivation and behavioral output in the control of feeding behavior (Petrovich et al., 2002; Petrovich et al., 2005). It would be important for future work to determine how these differential BLA outputs are distributed to guide cue valuation and behavior, and indeed several studies are currently investigating the dissociation between these differential projections in associative learning (Namburi et al., 2015; Beyeler et al., 2016; 2018).

Conclusions

The current study investigated the plasticity across a learning paradigm that requires value updating. We found that inactivation of the BLA neuronal ensembles responsive to a specific learned cue resulted in impaired conditioned responding to the same cue during reversal learning without interfering with responding to the other learned cue. These results show separate neuronal ensembles within the BLA are activated during specific cue memory recall and are necessary to update the value of that cue during reversal learning.

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Chapter 3: Distinct recruitment of basolateral amygdala-medial prefrontal cortex pathways across Pavlovian appetitive conditioning * ^

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ABSTRACT: Associative learning can enable environmental cues to signal food and stimulate feeding, independent of physiological hunger. Two forebrain regions necessary in cue driven feeding, the basolateral area of the amygdala and the medial prefrontal cortex, communicate via extensive, topographically organized connections. The basolateral nucleus (BLA) sends extensive projections to the prelimbic cortex (PL), and our aim here was to determine if this pathway was selectively recruited during cue-food associative learning. The anterior and posterior basolateral nuclei are recruited during different phases of cue-food learning, and thus we examined whether distinct pathways that originate in these nuclei and project to the PL are differently recruited during early and late stages of learning. To accomplish this, we used neuroanatomical tract tracing combined with the detection of Fos induction. To identify projecting neurons within the BLA, prior to training, rats received a retrograde tracer, Fluoro-Gold (FG) into the PL. Rats were given either one or ten sessions of tone-food presentations (Paired group) or tone-only presentations (Control group). The Paired group learned the tone-food association quickly and robustly and had

greater Fos induction within the anterior and posterior BLA during early and late learning compared to the Control group. Notably, the Paired group had more double-labeled neurons (FG + Fos) during late training compared to the Control group, specifically in the anterior BLA. This demonstrates selective recruitment of the anterior BLA-PL pathway by late cue-food learning. These findings indicate plasticity and specificity in the BLA-PL pathways across cue-food associative learning.

1. Introduction

Cues that signal food can increase the motivation to procure and consume food in the absence of hunger across species (e.g., Weingarten, 1983; Birch et al., 1989; for reviews see Petrovich & Gallagher, 2003; Holland & Petrovich, 2005; Petrovich, 2013). Environmental cues can gain this ability through associative learning, such as during Pavlovian appetitive conditioning. In this preparation, a neutral cue from the environment (conditioned stimulus, CS) is repeatedly followed by food (unconditioned stimulus, US), which innately evokes feeding behaviors (unconditioned response, UR). The CS then becomes the predictor of the US and ultimately drives the same behaviors (conditioned response, CR). These acquired abilities are well established behaviorally; however, much less is known about the neural plasticity, particularly at a circuit level, that underlies cue-food learning.

The amygdala, specifically the basolateral area, is important for appetitive associative learning and subsequent behaviors (Corbit & Balleine, 2005; Cole et al., 2013; for reviews see Gallagher & Schoenbaum, 1999; Everitt et al., 2003; Holland & Petrovich, 2005; Crombag et al., 2008; Wassum & Izquierdo, 2015), and its function is conceptualized to involve ‘tagging’ biologically relevant incoming stimuli and then informing other brain systems via complex and distributed connectional networks (e.g., Weiskrantz, 1956; Swanson & Petrovich, 1998). The amygdala is a heterogeneous structure (Swanson & Petrovich, 1998), and recent work found that distinct nuclei within the basolateral area (containing the lateral, basolateral [BLA] and basomedial nuclei) were differentially recruited

during early and late cue-food learning (Cole et al., 2013). Specifically, the anterior basolateral nucleus (BLAa; defined by its magnocellular morphology; Swanson, 2004) was the only amygdalar nucleus that displayed a significant increase in activation (measured with Fos induction) during early learning, which was maintained throughout training. The posterior basolateral nucleus (BLAp; defined by its parvocellular morphology; Swanson, 2004), along with other amygdalar nuclei that are connected with the BLAa, was recruited during late training. These results demonstrate specificity in the recruitment of amygdalar nuclei, and the differential recruitment across early and later learning suggests plasticity within the BLAa and, potentially, with its connectional targets.

The BLA has extensive connections with the medial prefrontal cortex (Kita & Kitai, 1990; Hoover & Vertes, 2007; Reppucci & Petrovich, 2016), which is important for the executive function and control of feeding and other motivated behaviors (Swanson & Petrovich, 1998; Dalley et al., 2004; O'Doherty, 2011). Specifically, the ventromedial prefrontal cortex, including the prelimbic (PL) and infralimbic (ILA) areas, is critical in appetitive cue learning (Ashwell & Ito, 2014; Baldwin et al., 2000, 2002; Burgos-Robles et al., 2013; Cole et al., 2015a; Corbit and Balleine, 2003). This area is necessary for feeding driven by learned food cues (Petrovich et al., 2007; Cole et al., 2015b), can be stimulated to drive food intake (Blasio et al., 2014; Land et al., 2014; Mena et al., 2011) and alters activity in downstream neural regions mediating feeding behaviors (Mena et al., 2013). Furthermore, disruption of the BLA-mPFC pathway attenuates reward-seeking driven by learned contextual and discrete cues (Fuchs et al., 2007; Mashhoon et

al., 2010; Stefanik et al., 2013). Nevertheless, the functional connectivity of the BLA-PL pathways has not been investigated during the acquisition of cue-food associations.

Within the medial prefrontal cortex, the BLA most densely innervates the PL, with topographically distinct pathways originating in the BLAa and BLAp (Kita & Kitai, 1990; Hoover & Vertes, 2007; Reppucci & Petrovich, 2016). The BLAa and BLAp are recruited during different phases of cue-food learning (Cole et al., 2013), suggesting that the BLAa-PL and BLAp-PL pathways may also be differently engaged. The goal of the current study was to determine whether the BLA neurons that send direct projections to the PL are selectively activated during cue-food learning and whether distinct pathways that originate in the BLAa and BLAp are differentially recruited during early and late learning of cue-food associations.

2. Materials and Methods

2.1. Subjects. Male Long-Evans rats (Charles River Laboratories, Portage, MI) were approximately two months of age and 250-275g upon arrival. Rats were individually housed and maintained on a 12-hour light/dark cycle (lights on 06:00). During a one-week acclimation period to the colony room, rats had *ad libitum* access to standard laboratory chow and water and were handled by the experimenter. Following acclimation, rats underwent surgery to receive neuroanatomical tracer injections and were allowed approximately 1 week to recover before food deprivation and subsequent behavioral procedures began.

Body weights were recorded daily throughout the experiment starting two days prior to surgery. All procedures complied with the National Institutes of Health *Guidelines for Care and Use of Laboratory Animals* and were approved by the Boston College Animal Care and Use Committee.

2.2. Surgical Procedures. Surgeries were performed under aseptic conditions. Subjects were briefly anesthetized with isoflurane gas and given intramuscular injections of ketamine (50 mg/ml)/xylazine (10 mg/ml) mix (0.1 ml/100 g bodyweight). Glass micropipettes (50 µm tip diameter; 50611; Stoelting Company, Wood Dale, IL) were backfilled with 3% Fluoro-Gold (FG; Fluorochrome, LLC, Denver, CO) in 0.9% saline. Using a stereotaxic frame (Kopf Instruments, Tujunga, CA), FG was unilaterally injected into the right PL (coordinates: anteroposterior, +3.40 mm; mediolateral, +0.70 mm, and dorsoventral, -3.80 mm; Swanson, 2004). FG was delivered via iontophoresis for 10 minutes with 5-second on/off, 5 mA pulses. After surgery, the scalp was closed with clips and coated with triple antibiotic ointment. Subjects were given chewable Rimadyl tablets (Bio Serv, Frenchtown, NJ; 2 mg/1 tablet/100 g bodyweight) for the first 48 hours.

2.3. Apparatus. Habituation and training occurred in the same set of eight identical behavioral chambers (30 x 28 x 30 cm; Coulbourn Instruments, Allentown, PA), which were located in a room different from the colony housing room. The behavioral chambers were composed of aluminum top and sides with one side containing a recessed food cup (3.2 x 4.2 cm), a transparent Plexiglas

front with a hinge, a transparent Plexiglas back, and a stainless steel rod floor composed of 5 mm wide rods spaced 15 mm apart. Each chamber was contained in an isolation cubicle (79 x 53 x 53 cm; Coulbourn Instruments, Allentown, PA) composed of monolithic rigid foam walls and a ventilation fan (55 dB). A video camera was mounted onto the rear wall of the isolation cubicle and was attached to a recording system (Coulbourn Instruments, Allentown, PA). A 10 second tone (75 dB, 2 kHz) was used as the conditioned stimulus (CS). Two food pellets (formula 5TUL, 45 mg: Test Diets, Richmond, IN) were the unconditioned stimulus (US) and delivered into the food cup of each chamber for the appropriate training group. GraphicState 3.0 software system (Coulbourn Instruments, Allentown, PA) was used for controlling the stimuli.

2.4. Behavioral Training. All behavioral training occurred between 9:00 and 13:00. Prior to behavioral training, subjects were food restricted to gradually reach 85% of their post-surgery body weight and maintained at this weight throughout training. Two days prior to training rats were given two-30 minute habituation sessions to acclimate them to the behavioral chambers (no CS or US were given). All subjects were given 1 g of the food pellets (US) in their home cages one day prior to the start of training to familiarize them with the pellets. For behavioral training, subjects were divided into two temporally distinct groups: early training and late training groups. To examine the neural substrates involved in early training, half of the rats received only one session of training (S1). From this group, half of the animals received eight presentations of the tone (CS), each followed by immediate delivery of two food pellets (US) during 34-minute training

sessions (Paired S1 group). The other half of the early training group was given the same presentations of the CS but no food pellets were delivered (Control S1 group). These two groups were perfused 90 minutes after the cessation of the training session, and brain tissue was collected. To examine the neural substrates involved in late training, the other half of the rats experienced ten training sessions (one session per day; S10). From this group, half of the animals received the eight presentations of the CS-US pairings with random inter-trial intervals between presentations (110-326 seconds), which varied randomly across trials and sessions (Paired S10 group). The Control S10 group received similar, random eight presentations of the CS within the behavioral chambers, and then received the food pellets in their home cage (placed onto the bedding) at varying intervals from 30-270 minutes after training sessions 1-9 and did not receive pellets after the tenth training session prior to perfusion. All rats were perfused 90 minutes after the cessation of their respective training session for detection of Fos induction. The timing was chosen to match the peak of Fos induction (occurring 60-120 minutes following a stimulus; Curran et al., 1985; Müller et al., 1984).

2.5. Behavioral Observations. The primary measure of learning was the expression of approach behavior towards the food cup ('food cup behavior') during the CS (i.e., prior to food delivery). The 'food cup behavior' refers to the following behaviors: rats standing in front of and directly facing the food cup or demonstrating distinct nose pokes into the food cup. To assess the amount of time rats spent expressing food cup behavior, trained observers unaware of

group conditions recorded rats' behavior every 1.25 seconds during each 10 second CS as well as during 10 seconds immediately preceding the onset of the CS (pre-CS period, without stimuli). At each observation only one behavior was recorded ('food cup' or 'nothing'). For each rat these observations were summed and the percentage of time rats spent displaying food cup behavior during the pre-CS and CS was calculated. These percentages were then used to calculate a mean value for each group.

2.6. Brain Tissue Collection and Preparation. Ninety minutes after the end of the training sessions, rats were briefly anesthetized with isoflurane and given a lethal injection of tribromoethanol (375mg/kg) intraperitoneally. They were then transcardially perfused with 0.9% saline followed by ice cold 4% paraformaldehyde in 0.1 M borate buffer. Brains were immersed in the paraformaldehyde solution with 12% sucrose at 4°C and stored for 18-24 hours. Brains were rapidly frozen in hexanes cooled in dry ice and stored at -80°C until further processing. Using a microtome, brains were cut into 30 µm coronal sections and collected into four serially adjacent sets. One series of tissue was processed for FG detection, and another series of tissue was processed for combined detection for FG and Fos. One series from each set was mounted from a potassium phosphate-buffered saline solution (KPBS) onto gelatin-coated slides and stained with thionin for the identification of cytoarchitectonic borders as defined in Swanson's rat brain atlas (Swanson, 2004).

2.6.1 FG detection with Single-label immunohistochemistry.

Immediately following slicing, the tissue was incubated for 1 hour at room temperature in a blocking solution (KPBS, Triton X-100 [Sigma-Aldrich, St. Louis, MO], normal goat serum [NGS; Vector Laboratories, Burlingame, CA], and milk), followed by incubation with the anti-FG antibody raised in rabbit (1:20K; AB153; Millipore, Billerica, MA) in the blocking solution for 72 hours at 4°C. Tissue was then rinsed in a solution containing KPBS, NGS, and milk and incubated with biotinylated secondary antibody against rabbit (1:500, BA-1000; Vector Laboratories, Burlingame, CA) in the blocking solution for 45 minutes. After several KPBS rinses, tissue was immersed in an avidin-biotin complex (ABC, PK-6100; Vector Laboratories, Burlingame, CA) for 45 minutes, rinsed in KPBS, and reacted with 3, 3'-diaminobenzidine (SK-4100; Vector Laboratories, Burlingame, CA) for visualization of FG. After KPBS rinses, sections were mounted onto SuperFrost slides (Fisher Scientific, Pittsburgh, PA), dried at 45°C, dehydrated through graded alcohols, cleared in xylenes, and coverslipped with DPX Mountant (Electron Microscopy Services, Hatfield, PA).

2.6.2. Combined FG and Fos Detection with Double-Label Fluorescent Immunohistochemistry.

Brains with confirmed injection locations (see below) in the area of interest (n=36) were further processed for detection of FG and Fos. Immediately following slicing, tissue was incubated with KPBS, Triton X-100, normal donkey serum (NDS; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA), anti-FG antibody raised in rabbit (1:10K), and anti-*c-fos* antibody raised in goat (1:2K; SC-52-g; Santa Cruz Biotechnology

Inc., Santa Cruz, CA) for 72 hours at 4°C. Tissue was then rinsed in KPBS and incubated in the dark for 1 hour with KPBS, Triton X-100, NDS, and secondary fluorescent antibodies: Alexa 488 anti-rabbit raised in donkey (1:200; A21206; Invitrogen, Carlsbad, CA) and Alexa 546 anti-goat raised in donkey (1:200; A11056; Invitrogen, Carlsbad, CA). Tissue was then immediately mounted in semi-darkness onto SuperFrost slides, air-dried, coverslipped with Vectashield HardSet Mounting Medium with DAPI (4',6-diamidino-2-phenylindole; H-1500; Vector Labs, Burlingame, CA), and stored at 4°C until analysis.

2.7. Image Acquisition and Analysis. For identification of FG injection sites, tissue was examined using the 10X objective on an Olympus BX51 light microscope attached to an Olympus DP72 camera using DP2-BSW software (Olympus America Inc, Center Valley, PA). Location and extent of injections were determined based on adjacent thionin-stained tissue and were drawn onto templates from the rat atlas (Swanson, 2004). The location and extent of all FG injections were analyzed, and subjects that had insufficient or misplaced deposits (>50% outside PL) were excluded from analyses (n=36). The brains with well-placed injections in the PL were further processed for double-label fluorescent immunohistochemistry. Images of the double-labeled processed tissue were captured using the 20x objective on a Zeiss Axioplan II fluorescence microscope with an attached Hamamatsu ORCA ER camera (Bridgewater, NJ) controlled through Micro-Manager software (Edelstein et al., 2010). Images were acquired on the ipsilateral side of the FG injection throughout the rostro-caudal extent of the amygdalar anterior basolateral nucleus (BLAa; atlas levels 26-29) and

posterior basolateral nucleus (BLAp; atlas levels 28-30; Swanson, 2004). Two images were acquired for each atlas level. Amygdalar nuclei were identified using the thionin-stained tissue, and borders and nomenclature were based on the Swanson rat brain atlas (Swanson, 2004). Images were pseudo-colored with green for FG, red for Fos, and blue for DAPI (nuclear counterstain). Observers unaware of the experimental conditions manually counted neurons from the triple-merged images, and single images were consulted as needed for cell- and stain-type confirmation. Three types of neurons were identified and counted: single-labeled FG-positive, single-labeled Fos-positive, and double-labeled FG- and Fos-positive neurons (FG + Fos). FG-positive neurons were identified by distinct cytoplasmic staining that surrounded a clearly visible nucleus (identified with DAPI), while Fos-positive neurons were identified by distinct nuclear staining. Neurons were counted as double-labeled if they depicted both the cytoplasmic-FG and nuclear-Fos labeling. Cell counts from all images acquired across the rostro-caudal extent of the region of interest (BLAa; BLAp) were summed to calculate the total number of FG-positive neurons, the total number of Fos-positive neurons, and the total number of double-labeled (FG + Fos) neurons for each rat. Counts were then averaged within groups to determine mean counts of FG-positive, Fos-positive, and double-labeled neurons for each group.

2.8. Statistical Analysis. Behavioral data (food cup behavior) was analyzed using repeated measures analysis of variance (ANOVA) followed by *t*-Tests or Fisher's LSD *post hoc* analysis when appropriate. For immunohistochemical data analysis, the total number of FG-positive neurons,

Fos-positive neurons, and double-labeled (FG + Fos) neurons were analyzed with two-way ANOVAs (Condition x Session) and *post hoc t*-Tests or Fisher's LSD when applicable. A significance value of $p < 0.05$ was used for all analyses. SPSS software was used for all statistical analyses.

3. Results

3.1. Behavioral Analysis

3.1.1. Early Training. During early training (Session 1), the Paired group displayed increasingly more food cup behavior during CSs throughout the session compared to the Control group, as shown by more food cup behavior during the second half of the session, signifying learning (Figure 1A). Repeated measures ANOVA (Training group x CS) found a significant effect of CS ($F_{(1,18)}$=2.713, P<0.05), but no effect of training group ($F_{(1,18)}$=2.793, P>0.05), or interaction ($F_{(1,18)}$=1.239, P>0.05). Further analysis revealed the Paired group displayed more food cup behavior during the last four CSs compared to their responding during the first four CSs (P<0.05) and compared to the Control group (P<0.05; Figure 1B). There were no differences between the groups during the first four CSs (P>0.05) or during pre-CS intervals (P>0.05).

3.1.2. Late Training. Over ten sessions of training, the Paired group showed an increase in food cup behavior during the CSs, while the Control group displayed minimal and non-specific food cup behavior throughout training. Repeated measures ANOVA (Training group x Session) revealed a significant

effect of training group ($F_{(1,14)}$=139.018, P<0.0001), a significant effect of session ($F_{(1,14)}$=6.968, P<0.001) and a significant interaction across sessions ($F_{(1,14)}$=9.781, P<0.001). During session 2, the Paired group had higher food cup responding compared to the Control group (P<0.05; Figure 1C), but similar responding during the pre-CS and CS intervals (P>0.05). Throughout sessions 3-10, the Paired group showed high responding specifically to the CS compared to their pre-CS responding (P<0.05) and compared to the behavior of the Control group during the CS (P<0.05). During the last session of training (session 10), repeated measures ANOVA (Training group x Time period [CS or pre-CS]) found

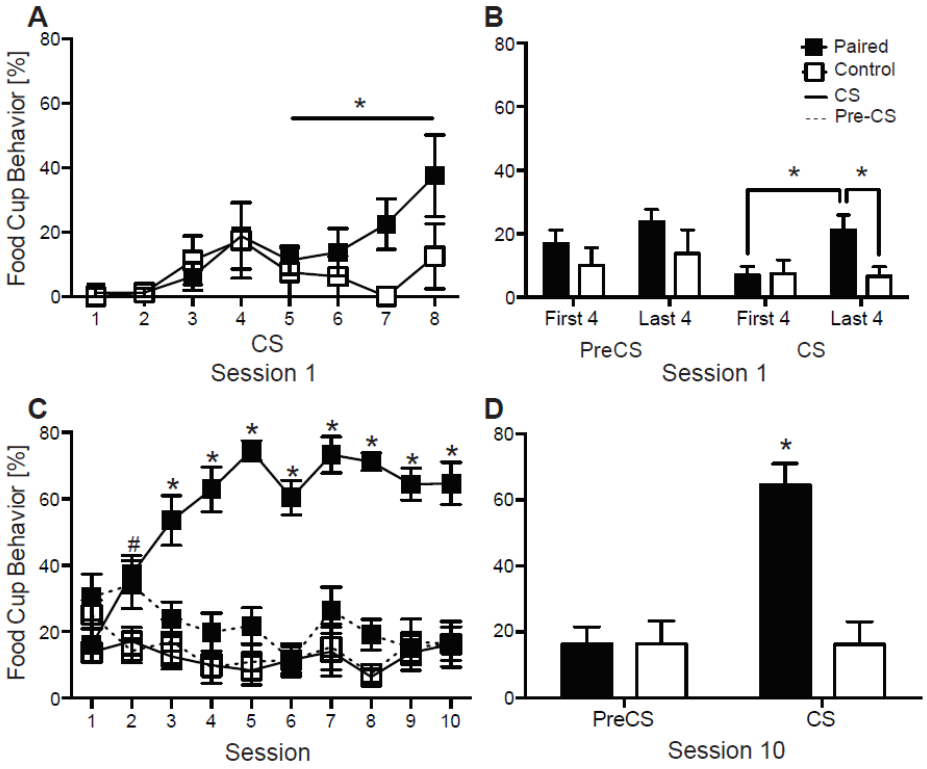

Figure 3.1. Conditioned responses during training. Percentage of time rats expressed food cup behavior (mean ± SEM) during each CS presentation (**A**) and during the first and last four pre-CSs and CS (**B**) during session 1. Expression of food cup behavior during the pre-CS and CS across ten sessions of training (**C**) and during session 10 (**D**). *P<0.05; #P<0.05 Paired pre-CS = Paired CS > Control CS.

a significant effect of training group ($F_{(1,14)}$=8.287, P<0.05), a significant effect of CS vs Pre-CS time period ($F_{(1,14)}$=63.816, P<0.0001), and a significant interaction ($F_{(1,14)}$=64.858, P<0.0001). The Paired group showed higher food cup behavior during the CS than the Control group (P<0.001) with no difference in pre-CS behavior between the groups (P>0.05; Figure 1D).

3.2. Neural Analysis.

The location and spread of FG injection sites were analyzed throughout the rostro-caudal extent of the prelimbic cortex (PL) based on the Swanson brain atlas (Swanson, 2004). Acceptable injections (see Supplemental Materials) were confined predominantly within the PL (n=36) and were centered within the mid rostro-caudal extent of the PL (Figure 2; Levels 6, 7 and 8; +4.2, +3.6, and +3.2mm from Bregma respectively). The final group numbers were S1 Paired (n=10), S1 Control (n=10), S10 Paired (n=8), and S10 Control (n=8). Importantly, the total numbers of retrogradely-labeled neurons were similar across groups (Figure 3B), confirmed by two-way ANOVAs (Training group x Session) in the BLAa (Trainin g group: $F_{(1,32)}$=2.477, P>0.05; Session: $F_{(1,32)}$=0.585, P>0.05) and BLAp (Training group: $F_{(1,32)}$=0.542, P>0.05; Session: $F_{(1,32)}$=0.119, P>0.05), signifying that any differences found in the number of double-labeled (FG + Fos) neurons are not due to variances in the number of FG-labeled neurons.

3.2.1. Fos-only Activation. Representative images of Fos and FG labeled neurons in the BLAa are shown in Figure 3A. Fos induction in the BLA neurons was examined during early (session 1; S1) and late (session 10; S10) tone-food conditioning. Within the BLAa, the Paired group had more Fos-positive

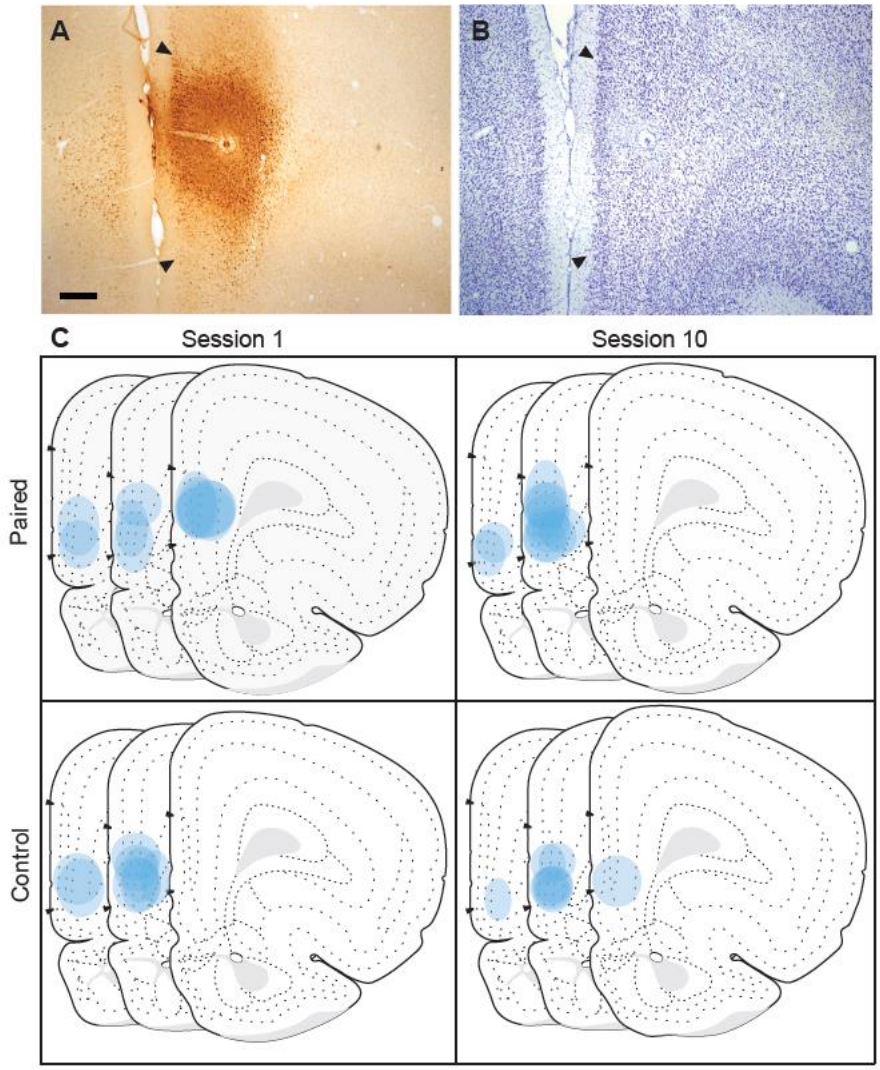

Figure 3.2. Fluoro-Gold (FG) injection sites in the prelimbic cortex (PL). A photomicrograph of a representative FG injection in the PL (**A**) with adjacent thionin-stained section (**B**) used to demarcate PL borders based on a rat atlas (Swanson, 2004). Illustration of all FG injections in the PL for each training group shown on modified Swanson atlas templates (atlas Levels 6, 7 and 8; +4.2, +3.6, and +3.2mm from Bregma respectively; **C**). Scale bar = 100 μm.

neurons than the Control group during S1 and S10 (Figure 3B). The two-way ANOVA (Training group x Session) revealed a significant effect of training group ($F_{(1,32)}$=16.722, P<0.01), but no effect of session ($F_{(1,32)}$=0.609, P>0.05), or interaction ($F_{(1,32)}$<0.000, P>0.05). *Post hoc* analysis confirmed the Paired group

had significantly more Fos-positive neurons than the Control group during S1 (P<0.01) and S10 (P<0.05), replicating previous findings using this protocol (Cole et al., 2013).

There was a similar pattern within the BLAp of higher Fos induction in the Paired group compared to Control group, but there was also an overall decrease in Fos induction across training (Figure 3C). Within the BLAp, a two-way ANOVA (Training group x Session) of Fos induction found a significant effect of training group ($F_{(1,32)}$=11.279, P<0.01) and a significant effect of session ($F_{(1,32)}$=6.369, P<0.05), but no interaction ($F_{(1,32)}$=0.378, P>0.05). The Paired group had more Fos-positive neurons than the Control group during S1 (P<0.001) and a trend towards significance during S10 (P=0.087). Overall, there were more Fos-positive neurons in the S1 groups compared to the S10 groups (P<0.05).

3.2.2. Pathway Activation. To examine the activation of the BLA-PL pathways, the total number of double-labeled neurons (FG + Fos) within the BLAa and BLAp was quantified (see Supplemental Materials for specifications) and compared across groups and sessions. We found selective Fos induction in the PL projecting BLAa neurons, but not BLAp neurons, in the Paired group during S10. In the BLAa, two-way ANOVA (Training group x Session) of Fos induction in BLAa neurons that project to the PL revealed a significant effect of training group ($F_{(1,32)}$=7.818, P<0.01), but no effect for session ($F_{(1,32)}$=0.123, P>0.05), or interaction ($F_{(1,32)}$=0.290, P>0.05). *Post hoc* analysis confirmed the

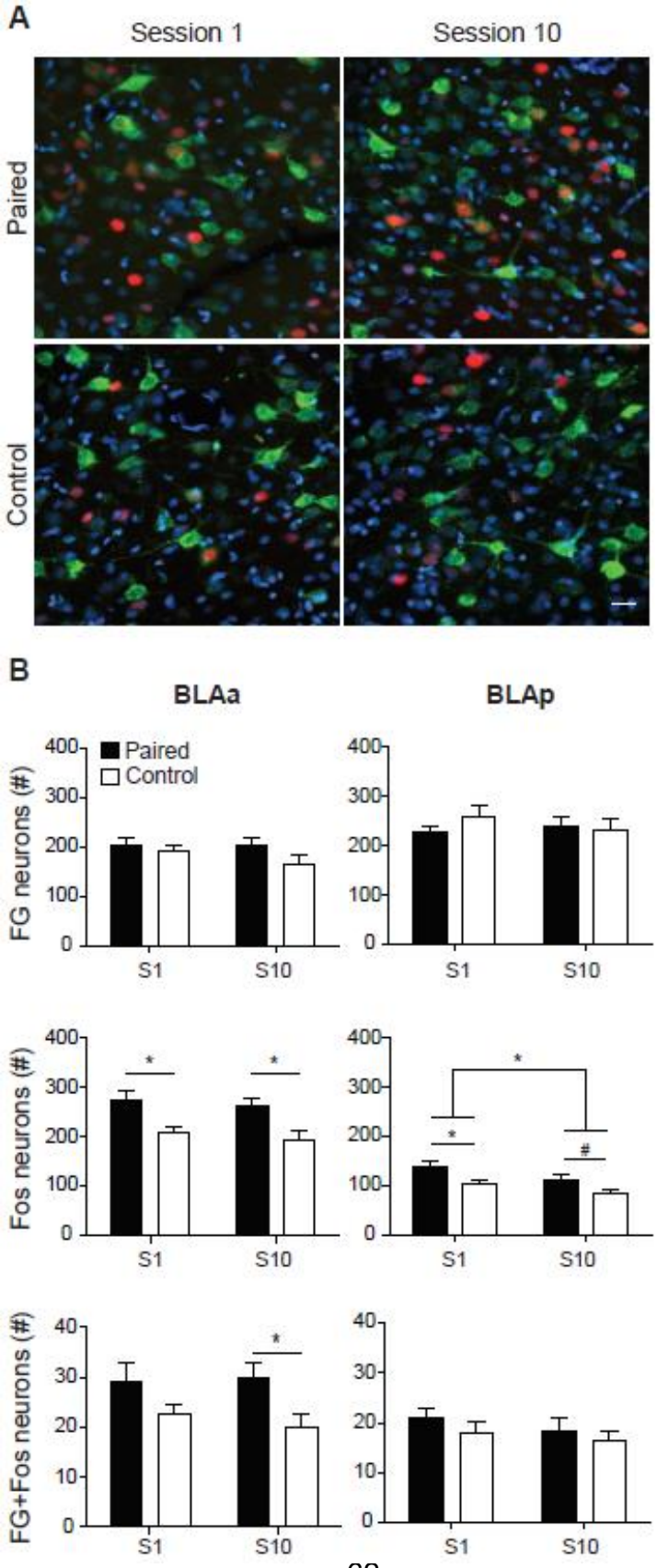
A
Session 1
Session 10
Paired
Control
B
BLAa
BLAp
Paired
Control
FG neurons (#)
Fos neurons (#)
FG+Fos neurons (#)
S1
S10
*
#

Figure 3.3. Fos induction in BLA-PL projecting neurons during early and late cue-food learning. Representative images from the BLAa from each training group depicting FG-positive neurons (green), Fos-positive neurons (red), and DAPI, a nuclear counterstain (blue). Scale bar = 25μm (**A**). Total number of FG-positive neurons, Fos-positive neurons, and double-labeled (FG+Fos) neurons (mean ± SEM) during the first (Session 1; S1) and last (Session 10; S10) training sessions in the BLAa (*left*) and BLAp (*right*; **B**). **P*<0.05; #*P*=0.087.

Paired S10 group had more double-labeled neurons than the Control S10 group (*P*<0.05), but a difference between S1 groups was not statistically significant (*P*>0.05; Figure 3D). In the BLAp, there were no differences in the number of activated projecting neurons between the Paired and Control groups during S1 or S10 (Training group: $F_{(1,32)}$=1.127, *P*>0.05; Session: $F_{(1,32)}$=0.730, *P*>0.05; Figure 3E).

4. **Discussion**

In the current study, we examined the functional activation of the BLA-PL pathways during the acquisition of Pavlovian appetitive conditioning. We found significantly more Fos induction in BLAa-to-PL projecting neurons in the Paired group compared to the Control group. This effect was statistically reliable specifically during the late training, but not during the early training. This finding demonstrates recruitment of the BLAa-PL pathway across training, suggesting plasticity during cue-food associative learning. Interestingly, Fos induction in projecting neurons within the BLAp was similar between training groups throughout tone-food conditioning, demonstrating activation of the BLAp-PL pathway was similar in the Paired and Control groups throughout learning. Together, these results show that only the BLAa-PL pathway, but not the BLAp-

PL pathway, is activated during well-learned cue-food associations. Additionally, we analyzed total Fos induction in the BLAa and BLAp and found higher overall induction in the Paired groups compared to the Control groups during both phases of learning. This difference between overall activation and the activation of specific BLA-to-PL projecting neurons highlights the importance of identifying how specific neurons are recruited within a critical neural circuitry underlying behavior.

Here, the retrograde tracer injections were aimed at the PL, an area substantially innervated by the BLAa. Our focus was on the BLAa, because that was the only amygdalar cell group recruited during early cue-food training, suggesting it is informing its connectional targets during appetitive conditioning (Cole et al., 2013). Nevertheless, the BLAa and BLAp have distinct connections with the medial prefrontal cortex, and while the BLAa has dense connections with the PL and the anterior cingulate area, the BLAp is connected more heavily with the ILA compared to the PL (Sesack et al., 1989; Kita & Kitai, 1990; Swanson & Petrovich, 1998; Hoover & Vertes, 2007; Little & Carter, 2013; Reppucci & Petrovich, 2016). In accordance, our injections in the PL resulted in labeling and analysis only within the rostral half of the BLAp, and thus the current study did not capture the more substantial projections from the BLAp to the ILA. Given the ILA was also recruited during late learning of cue-food associations, similar to the PL (Cole et al., 2015a), it is possible the BLAp-ILA pathway may be important during appetitive associative learning. Furthermore, the current study found more overall Fos induction in the BLAp (total Fos induction in both projecting and non-

projecting neurons) during early and late training in the Paired group, whereas Cole and colleagues (2013) found recruitment of the BLAp only during late learning. A methodological difference in sampling is a potential reason why these results differ. Cole and colleagues (2013) examined the entire extent of the BLAp (the entire dorso-ventral and rostro-caudal area within the nucleus), while in the current study the total Fos was counted within the area of substantial PL projection (only the rostral portion of the BLAp).

In addition to the current functional differences and aforementioned distinct connections with the mPFC, the BLAa and BLAp also differ in their connections with other forebrain areas. Within the amygdala, the BLAp sends substantial direct pathways to the central amygdala, while the BLAa reaches it indirectly through its connections to the BLAp (Savander et al., 1995; Swanson & Petrovich, 1998). Based on its additional forebrain connections, the BLAa was characterized as a part of the frontotemporal system, and it projects to the nucleus accumbens and caudoputamen and has bidirectional connections with the frontal and parietal somatosensory-motor areas (Kita & Kitai, 1990; Swanson & Petrovich, 1998). Additionally, it does not send direct projections to hippocampal formation, the hypothalamus, or the bed nuclei of the stria terminals (Swanson & Petrovich, 1998; Dong et al 2001; Petrovich et al., 2001). The BLAp was characterized as a part of the main olfactory system, and it projects to the nucleus accumbens and the substantia innominata, as well as the hippocampal formation, the hypothalamus, and bed nucleus of the stria terminalis, (Swanson & Petrovich, 1998; Petrovich et al., 2001; Reppucci & Petrovich, 2016).

The findings from the current study and previous work support the notion that the BLAa is a critical early ‘processor’ during appetitive associative learning. Here, we found recruitment of the BLAa during early learning in agreement with Cole and colleagues (2013). The BLAa was the only forebrain region to show selective activation during early learning, while the amygdalar and forebrain targets of its inputs were recruited during late training (Cole et al., 2013, 2015a). Furthermore, electrophysiological recordings also provide evidence that the BLA precedes and influences other cortical processing. Single-unit recordings found that the BLA is activated prior to the activation of the gustatory cortex during palatability processing (Grossman et al., 2008), and BLA inactivation can alter gustatory cortex responses (Piette et al., 2012). This early processing function of the BLA may capture its role in tasks with reward predictive cues, including cue-potentiated eating (Holland et al., 2002), discriminative stimulus responding (Ishikawa et al., 2008), second-order conditioning (Hatfield et al., 1996), devaluation (Hatfield et al., 1996), and Pavlovian to instrumental transfer (Blundell et al., 2001; for review see Wassum & Izquierdo, 2015). The current study suggests that the BLAa processing is relayed to the PL during acquisition, potentially enabling this pathway to later control cue driven reward behaviors. Indeed, inhibition of the BLA-PL pathway decreased conditioned reward seeking (Fuchs et al., 2007; Mashhoon et al., 2010; Stefanik et al., 2013), and BLA inactivation caused a disinhibition of the PL activity during reward seeking, resulting in a deficit in conditioned place preference for morphine (Sun & Laviolette, 2012).

The BLAa is a cortical part of the amygdala (Swanson & Petrovich, 1998), and its output from pyramidal neurons can influence the PL through monosynaptic (McDonald, 1992; Sotres-Bayon et al., 2012) and polysynaptic pathways involving inhibitory interneurons (Perez-Jaranay & Vives, 1991; Gabbott et al., 2006; Floresco & Tse, 2007; Sun & Laviolette, 2012; Dilgen et al., 2013). Inactivation of the BLA decreased PL pyramidal neuron activity, suggesting a monosynaptic pathway (Sotres-Bayon et al., 2012). Alternatively, BLA stimulation increased activity within interneurons, which inhibited PL pyramidal neurons, suggesting a polysynaptic pathway (Dilgen et al., 2013). Through these pathways the BLA input can critically control PL activity, either through excitation or inhibition, and ultimately control behavioral outcomes.

5. Conclusions

In conclusion, we found plasticity and selectivity within the BLA-PL pathways across Pavlovian appetitive conditioning. The BLAa-PL, and not the BLAp-PL, pathway was selectively recruited during cue-food learning and, importantly, this recruitment suggests plasticity in BLAa and PL communication across training. These results suggest the BLA is important during initial appetitive learning, and its communication with the medial prefrontal cortex increases throughout learning as a cue becomes predictive of food to control behavior.

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Chapter 4: The basolateral amygdala-medial prefrontal cortex circuitry regulates behavioral flexibility during appetitive reversal learning

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ABSTRACT: Environmental cues can become predictors of food through Pavlovian appetitive conditioning. Two forebrain regions important in this associative learning are the basolateral amygdala (BLA) and medial prefrontal cortex (mPFC). Recently, we showed the BLA-mPFC pathway is activated when a single cue reliably signals food, suggesting the BLA informs the mPFC of the cue's value. The current experiment tested this hypothesis after discriminative conditioning by altering the value of two cues during reversal learning and devaluation by conditioned taste aversion (CTA). Rats received contralateral, ipsilateral, or sham excitotoxic lesions of the BLA-mPFC, and then received ten sessions of discriminative conditioning in which two auditory stimuli (tone; white noise) were each presented six times within each session. One stimulus co-terminated with the delivery of two palatable food pellets (CS+), and the other stimulus was unrewarded (CS-; counterbalanced). All groups successfully discriminated between the two auditory stimuli, demonstrating this learning does not require BLA-mPFC communication. Next, the outcomes of the stimuli were reversed: the CS+ was now unrewarded (reversal CS-; rCS-), and the CS- was now rewarded (reversal CS+; rCS+). During 15 sessions of reversal learning, the rats that received the contralateral disconnection of the BLA-mPFC showed increased responding to the CSs, especially to the rCS+ during the first session,

compared to the other groups, suggesting impaired cue memory recall and impaired behavioral inhibition. Next, half of the rats in each lesion group underwent CTA (food-LiCl) and were then tested for devaluation through assessment of conditioned responding to each CS without reward. All groups successfully learned CTA. However, there was no immediate evidence of cue devaluation, and there were no differences between groups. Interestingly, the non-devalued contralateral group was still responding more to the rCS- compared to the devalued contralateral group, especially during test 8, while there were no differences within the sham or ipsilateral groups. These results suggest BLA-mPFC communication is necessary for appropriate behavioral responding during periods of behavioral flexibility when the outcomes, and thus the values, of learned cues are altered.

1. Introduction

Behavioral flexibility is the ability to appropriately alter one's behavior in response to a change in the environment (Brown & Tait, 2014). To respond appropriately, the subject must remember previously learned information about the stimuli and incorporate that memory with the current environmental demands. One adaptive example of learning that requires constant behavioral flexibility is attending to and deciphering between cues that signal the availability of food. Within the laboratory, two appetitive learned paradigms often used to assess behavioral flexibility when the outcomes, and thus the values, of previously learned cues change are reversal learning and devaluation. After discriminative conditioning where one cue signals food availability (conditioned stimulus, CS+) and one cue signals nothing (CS-), reversal learning involves switching the outcomes of the cues: the CS+ is no longer presented with food (reversal CS-; rCS-), and the CS- is now always followed by food delivery (reversal CS+; rCS+). Successful reversal learning is indicated by two distinct behaviors: inhibition of conditioned responding to the rCS-, which was previously the CS+, since the cue now signals the absence of food, and initiation of conditioned responding to the rCS+, which was previously the CS-, since the cue now signals the availability of food.

Appetitive cue devaluation is another paradigm used to assess behavioral flexibility. After successful CS-US associative learning, the value of the US (e.g. food) is decreased by illness through conditioned taste aversion (CTA), or by satiety. During CTA, illness is induced artificially by injections of lithium chloride

after US consumption to cause malaise, resulting in an association between the illness and the food, and thus “devaluation” of the US. CTA is confirmed with a decrease in US consumption. Since the representation of the value of the US has changed, subjects are then tested on their responding to the CSs that were previously associated with the US to evaluate if the CSs are also devalued. This devaluation is evident by a decrease in conditioned responding to the learned cues that now signal the devalued food. Using the reversal learning and devaluation procedures, we can examine if distinct neural mechanisms are necessary for behavioral inhibition due to a change in valuation of food and food-associated cues.

The current study focused on determining if communication between two forebrain regions important in associative learning and decision-making, the basolateral area of the amygdala (BLA) and medial prefrontal cortex (mPFC), respectively, is necessary during appetitive cue value learning and subsequent value updating. A plethora of studies have shown the BLA is critical for various associative learning paradigms. Specifically, our laboratory has shown the anterior part of the BLA is the first forebrain region activated during learning (Cole et al., 2013). Interestingly, even though the BLA is activated early, it does not appear to be critical for the acquisition of appetitive conditioning (Hatfield et al., 1996; Holland et al., 2002; Parkinson et al., 2000; Balleine et al., 2003; Corbit & Balleine, 2005). However, it is necessary when the acquired cues motivate new learning (Hatfield et al., 1996; Schoenbaum et al., 1999; Blundell et al., 2001; Setlow et al., 2002; Nomura et al., 2004; Corbit & Balleine, 2005; Ishikawa

et al., 2008; for reviews, see Holland et al., 2001, 2002; Everitt et al., 2003; Holland & Petrovich 2005, Petrovich, 2013; Wassum & Izquierdo, 2015). Related to the current study, BLA neurons respond to cue value alterations (Tye et al., 2010) and are necessary for successful reversal learning (Schoenbaum et al., 2003; Churchwell et al., 2009). Additionally, several studies have also shown the BLA is needed for cue value updating during devaluation (Hatfield et al., 1996; Balleine et al., 2003; Corbit & Balleine, 2005; Ostlund & Balleine, 2008; Coutureau et al., 2009; Johnson et al., 2009; Parkes & Balleine, 2013). Based on these previous findings, the BLA is necessary for driving behavioral responding when the value of the cue changes.

The mPFC is critical in adaptive behaviors and executive control (Dalley et al., 2004; O'Doherty, 2011) and has topographically organized and reciprocal projections with the BLA (Sesack et al., 1989; Kita & Kitai, 1990; Swanson & Petrovich, 1998; Petrovich et al., 2001; Hoover & Vertes, 2007; Reppucci & Petrovich, 2016). The mPFC mediates the homeostatic drive to eat (Mena et al., 2011, 2013; Land et al., 2014), as well as the non-homeostatic drive to eat influenced by learned food cues (Petrovich et al., 2007; Blasio et al., 2014; Cole et al., 2015b). Two sub-regions of the mPFC, the prelimbic (PL) and infralimbic (ILA) areas, are activated in well-learned cue-food associations (Burgos-Robles et al., 2013; Cole et al., 2015a; Moorman & Aston-Jones, 2015; Warren et al., 2016). Less is known about the role of the mPFC in updating the value of learned appetitive cues, but evidence suggests it may be less critical in reversal learning (Ragozzino et al., 1999; Salazar et al., 2004; Boulougouris et al., 2007; Floresco

et al., 2008; Churchwell et al., 2009; Coutureau et al., 2009) and devaluation (Corbit & Balleine, 2003; Killcross & Coutureau, 2003; Ostlund & Balleine, 2005). Only one study has shown the mPFC is important for altering behavioral responding during satiety-induced devaluation (Coutureau et al., 2009), suggesting the mPFC may be needed to incorporate previously learned information with current cue valuation to appropriately alter behavior.

Previously, our laboratory has shown differential activation of the BLA and mPFC and the connection from the BLA to mPFC during cue-food learning. The anterior BLA was recruited during the early acquisition of learning and the mPFC was recruited during the expression of learning (Cole et al., 2013; Cole et al., 2015a). From these results along with the discussed neuroanatomical topography, we previously examined if the BLA-mPFC pathways are involved in cue-food learning. We found that neurons in the BLA that directly communicate with the mPFC are activated when cue-food associations are well-learned, suggesting that the BLA can inform the mPFC of the associative value of a conditioned cue (Keefer & Petrovich, 2017). Furthermore, other studies have shown the BLA can directly alter activity within the mPFC (Sotres-Bayon et al., 2012; Sun & Laviolette, 2012), and inhibition of the BLA-mPFC pathway decreases conditioned reward seeking (Fuchs et al., 2007; Mashhoon et al., 2010; Stefanik & Kalivas, 2013) and learning (Churchwell et al., 2009). However, inhibition of this pathway may not interfere with satiety-induced devaluation (Coutureau et al., 2009).

Therefore, the current study investigated if communication between the

BLA and mPFC is necessary in appetitive discriminative cue learning and behavioral flexibility when learned information about the cues is altered during reversal learning and devaluation by CTA. We hypothesized disconnection of the BLA-mPFC circuitry would not alter the initial discriminative conditioning since these structures are not necessary for the initial learning of cue-food associations, but it would interfere with cue value updating during reversal learning and devaluation.

2. Materials and Methods

2.1. Subjects. Experimentally naïve, male Long-Evans rats (250-275 g upon arrival) were obtained from Charles Rivers Laboratories and individually housed and maintained on a 12-hour light/dark cycle. For one week, rats had *ad libitum* access to food (standard laboratory chow) and water and were acclimated to the colony room and experimenter handling prior to surgical and behavioral procedures. All procedures were in accordance with the National Institutes of Health *Guidelines for Care and Use of Laboratory Animals* and approved by the Boston College Animal Care and Use Committee.

2.2. Surgical Procedures. Under aseptic conditions, subjects were briefly anesthetized with isoflurane gas and given a mixture (1 ml/kg bodyweight; intramuscularly) of ketamine (50 mg/ml) and xylazine (10 mg/ml) prior to stereotaxic frame (Kopf Instruments) placement. Rats received a 0.3 µl injection into one mPFC (coordinates from bregma: anteroposterior, +3.0mm; mediolateral, ±0.7mm; dorsoventral, -4.5mm) and two 0.1 µl injections into either the contralateral or ipsilateral BLA depending on group allocation (coordinates

from bregma: anteroposterior, -2.3-2.5mm; mediolateral, ±5.0mm; dorsoventral, -8.4-8.7mm) using a 1 µl 32-gauge Hamilton Neuros syringe driven by a Quintessential Stereotaxic Injector (Stoelting) at a rate of 0.1 µl/min. Excitotoxic lesions were induced by injecting 0.15M N-methyl-D-asparatate (NMDA; Sigma-Aldrich) in a phosphate buffered saline solution (PBS), whereas sham lesions received contralateral injections of PBS alone. The syringe was left in place for 4 min after each infusion to allow for diffusion of solution. After infusions, the scalp was clipped closed and coated with triple antibiotic cream, and rats were given chewable Rimadyl tablets (Bio Serv, Frenchtown, NJ; 2 mg/1 tablet/100 g bodyweight) for two days after surgery. Rats recovered for at least one week prior to behavioral testing and were monitored and weighed daily.

2.3. Apparatus. All habituation and training occurred within the same set of behavioral chambers (30 x 28 x 30 cm; Coulbourn Instruments) located in a separate room from where the colony was housed. The chambers had aluminum top and sides, and one side contained a recessed food cup (3.2 x 4.2 cm). The front and back of the chambers were clear Plexiglas with a hinged front. The floor was stainless-steel rods 5 mm wide and 15 mm apart. Each chamber was contained within an isolation cubicle (79 x 53 x 53 cm; Coulbourn Instruments) composed of monolithic rigid foam walls and a ventilation fan (55 dB). Video cameras were attached to the rear wall of each isolation cubicle and connected to a recording system (Coulbourn Instruments).

The conditioned stimuli (CSs) were a 10s 75dB, 2kHz tone and a 10s 75dB white noise. The unconditioned stimulus (US) was two food pellets (formula

5TUL, 45 mg: Test Diets), delivered into the food cup of each chamber. GraphicState 3.0 software system (Coulbourn Instruments) controlled all stimuli.

2.4. Behavioral Training Procedure. Experimental design is described in Table 4.1. Rats were food restricted to maintain 85% of their post-surgery recovery body weight throughout training. A day prior to training, all rats received a 30 min habituation session to the behavioral chambers without stimuli, and then received 1g of the US in their home cage to familiarize them with the pellets.

2.4.1. Discriminative Conditioning. Rats received ten 30 min training sessions with two cues, and each session consisted of six intermixed presentations of each cue. One auditory cue (e.g. white noise) was immediately followed by the delivery of the US, two palatable food pellets (CS+), and the other CS (e.g. tone) was presented alone (CS-). Cues that served as the CS+ and CS- were counterbalanced, and the inter-trial intervals (ITIs) were between 60-219s. ITIs and CS order varied across training sessions.

2.4.2. Reversal Learning. Following successful discriminative conditioning, reversal learning commenced for 15 sessions. Reversal sessions were similar in length and number of CS presentations as during discriminative conditioning; however, the outcomes of the CS+ and CS- were reversed. The previous CS+ was now not followed by the delivery of food and referred to as the reversal-CS- (rCS-), and the previous CS- was now followed by the delivery of food and referred to as the reversal-CS+ (rCS+).

2.4.3. Devaluation.

2.4.3.1. *Conditioned Taste Aversion*. Following reversal

Table 4.1 Experiment Design.

Discriminative Conditioning	Reversal Learning	Devaluation	
		CTA (x2)	Tests
CS+ → US CS- → (nothing)	→ rCS- → (nothing) → rCS+→ US	Devalued: US → LiCl	rCS+ rCS-
		Non-devalued: US; LiCl	

CS: Conditioned Stimulus (tone or white noise); rCS: Reversal Conditioned Stimulus; US: Unconditioned Stimulus (food); LiCl: Lithium Cloride injection (i.p.)
Grey arrows indicate same CS used but outcome changed

learning, half of each group received conditioned taste aversion (CTA) in 2 trials over 4 days. All rats were given access to 5g of the US in a ceramic dish in their home cage for 10 min (a measure of baseline consumption), and then half of the rats received a LiCl injection (0.3M LiCl in 0.9% sterile saline; 5 ml/kg; i.p.) to induce malaise immediately following access to the US (Devalued group). The control, Non-devalued group received the same LiCl injections, but 24 hours after US access, and thus did not form the US-illness association. Twenty-four hours later the same procedure was repeated to induce stronger CTA. Rats were tested for US consumption the day following each CTA session (consumption test 1 and 2), and successful CTA is evident by a decrease in consumption.

2.4.3.2. *CS Devaluation Testing*. Subsequently, rats received eight sessions with six presentations of each CS alone, without US, to test if their responding to the CS changed due to the devalued US.

2.4.3.3. *Final Consumption Test*. Following CS testing, all rats underwent another consumption test with the US in their home cage to confirm the CTA memory remained intact throughout testing.

2.5. Behavioral Measures. The primary measure of learning was

conditioned responding to the food cup ("food cup behavior") during the presentations of the CSs. Food cup behavior refers to the rats standing in front of and directly facing the food cup or demonstrating distinct nose pokes into the food cup. Trained observers unaware of group allocation recorded rats' behavior every 1.25s during each 10s CS and during the 10s prior to the onset of the CS as a measure of baseline responding (pre-CS). The total number of identified food cup observations during each period was summed and converted to a percentage of total time rats were in the food cup during each CS. Specific learned responding (Elevation) was calculated by subtracting responding during the pre-CS period from responding during the CS (CS minus pre-CS) for both CS+ and CS-, which was used to calculate the mean value for each group.

For CTA, the total amount of the US consumed during each consumption test was measured in grams and averaged for each group.

2.6. Histological Procedures and Immunohistochemistry. After behavioral testing, rats were perfused, and brain tissue was collected to examine the accuracy and extent of the lesions. Rats were given a lethal dose of tribromoethanol (1.5ml/100g bodyweight; i.p.) and transcardially perfused with 0.9% saline followed by ice cold 4% paraformaldehyde in 0.1M borate buffer. Brains were stored overnight in the paraformaldehyde solution with 12% sucrose at 4°C, and then rapidly frozen in hexanes cooled in dry ice and stored at -80°C until sectioning and analysis.

Brains were sliced into 40 μm coronal slices using a sliding microtome (Leica Biosystems) and collected into 3 serially adjacent series. Brain tissue from

one series was processed for NeuN detection to verify lesion placements. Tissue was incubated for 1 hour at room temperature in a blocking solution (potassium phosphate-buffered saline solution [KPBS] containing normal horse serum [NHS], Triton X-100, and milk), followed by incubation with anti-NeuN antibody raised in mouse (1:1000, MAB377; Millipore) in the blocking solution for 72hr at 4°C. Tissue was rinsed in a solution containing KPBS, NHS, and milk followed by incubation with biotinylated secondary antibody against mouse (1:500, BA-2001; Vector Laboratories) in the blocking solution for 45 min. After KPBS rinses, tissue was incubated in an avidin biotin complex (ABC, PK-6100; Vector Laboratories) for 45 min, rinsed with KPBS, and treated with nickel-intensified 3, 3'-diaminobenzidine (SK-4100; Vector Laboratories) for color visualization of neurons labeled for NeuN. Sections were rinsed again, mounted onto SuperFrost slides (Fisher Scientific), dried at 45°C, dehydrated through graded alcohols, cleared in xylenes, and coverslipped with DPX Mountant (Electron Microscopy Services).

A second series of tissue sections was mounted from KPBS onto gelatin-coated slides and stained with thionin for the identification of neuroanatomical borders as defined in Swanson's rat brain atlas (Swanson, 2004).

2.7. Image Acquisition and Analysis. Verification and extent of lesions within the mPFC and BLA were analyzed using a 10X objective on an Olympus BX51 light microscope attached to an Olympus DP72 camera using DP2-BSW software (Olympus America Inc, Center Valley, PA). Extent and location of BLA and mPFC lesions were determined based on analysis of NeuN-

stained tissue, using adjacent thionin-stained tissue to identify nuclear borders and were drawn onto templates from the rat atlas (Swanson, 2004). Acceptable lesions ablated more than 60% of the PL and ILA within the mPFC and more than 60% of the anterior and posterior BLA. Subjects with less than 60% damage of either structure were excluded.

2.8. Statistical Analysis. Data were analyzed using a mixed design analysis of variance (ANOVA) with the following between-subjects factors: Lesion group (Contralateral, Ipsilateral, Sham) and devaluation group (Devalued, Non-devalued), and within-subjects factors: type of CS (CS+, CS- or rCS+, rCS-) and conditioning sessions within each paradigm (discriminative conditioning sessions 1, 5, and 10; reversal learning sessions 1, 5, 10, and 15; consumption tests 1-4; or devaluation tests 1, 4, and 8). The percentage of conditioned responding was the dependent variable for all experiments, except for CTA in which pellet consumption (in grams) was analyzed. A significance value of $p < 0.05$ was used for all analyses, except for Lesion group *post-hoc* analyses in which Bonferroni adjusted alpha level was used ($p = 0.05/3 = 0.017$). SPSS software was used for all statistical analyses.

3. Results

3.1. Histology. The location and extent of lesions were analyzed throughout the rostro-caudal span of the mPFC and BLA based on the Swanson brain atlas (Swanson, 2004; Fig. 4.1). Acceptable mPFC lesions ablated at least 60% of prelimbic cortex (PL; Levels 6-9 [+4.2 to +2.8 mm from Bregma]) and infralimbic cortex (ILA; Levels 8-9 (+3.2 to +2.8 mm from Bregma]) and in the

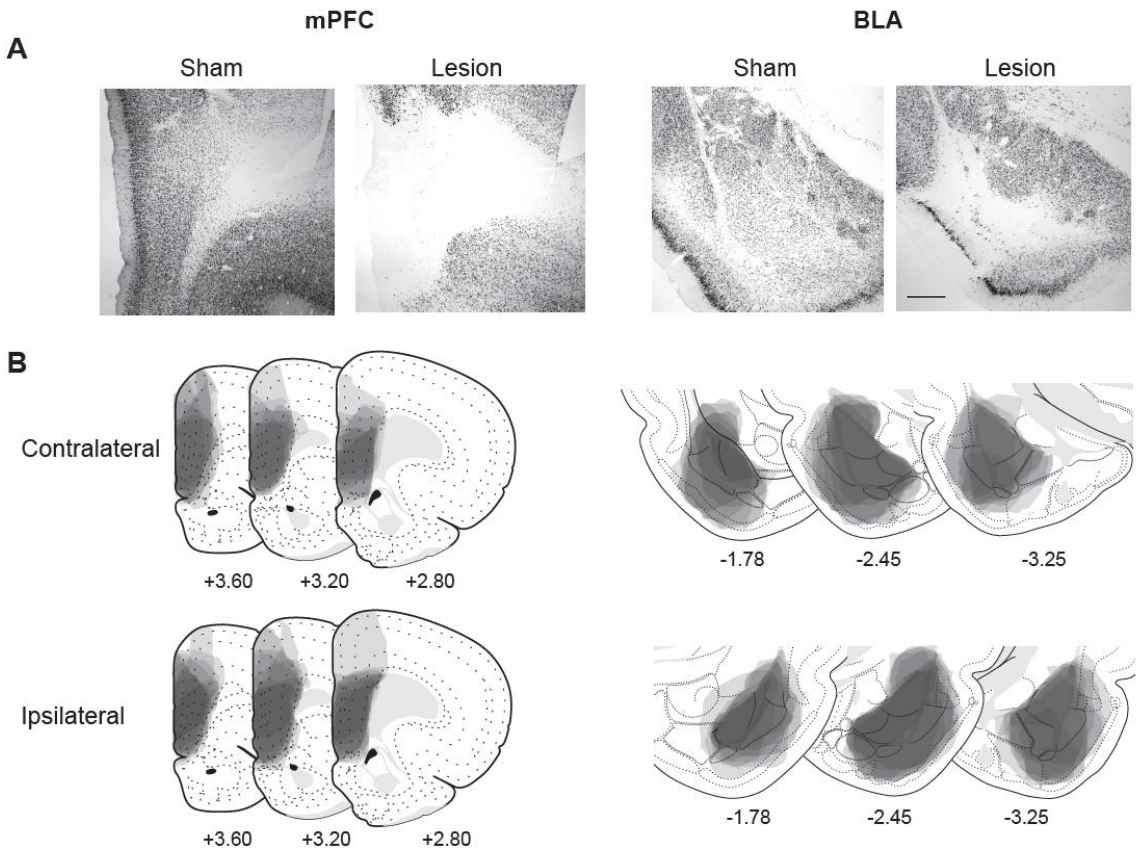

Figure 4.1 Extent of lesions identified by NeuN immunohistochemistry. (A) Images show NeuN-stained tissue from mPFC sham (*first panel*) and NDMA lesion (*second panel*) and BLA sham (*third panel*) and lesion (*fourth panel*). (B) Lesion extent of all included subjects with contralateral (*top*) and ipsilateral (*bottom*) lesions, drawn with 10% opacity in Adobe Illustrator CS6. Numbers below each schematic refers to distance (in mm) from Bregma and correspond to the following Swanson brain atlas levels: +3.60, Level 7; +3.20, Level 8; +2.80, Level 9; -1.78, Level 26; -2.45, Level 28; and -3.25, Level 30. Scale bar = 100 µm.

majority of cases, there was, on average, 50% damage to the dorsal anterior cingulate area. Acceptable BLA lesions ablated at least 60% of the anterior BLA (BLAa; Levels 25-29 [-1.53 to -2.85 mm from Bregma]) and the posterior BLA (BLAp; Levels 28-32 [-2.45 to -3.90 mm from Bregma]) with additional damage (~60%) to the lateral amygdala in acceptable brains (Levels 28-30 [-2.45 to -3.25

mm from Bregma]). Eleven subjects were excluded due to inadequate damage to the mPFC or BLA (less than 60% of either structure damaged), resulting in a total of 25 subjects included in analyses (Sham, n=8; Ipsilateral, n=9; and Contralateral, n=8).

3.2. Discriminative Conditioning. All groups successfully discriminated between the CSs by the end of training, with minimal differences between lesion groups (Fig. 4.2, top). A Lesion Group (sham, ipsilateral, contralateral) X CS (CS+Elevation, CS-Elevation) X Session (1, 5, 10) repeated measures ANOVA found a main effect of Session ($F(2,44) = 148.32$, $p < 0.001$) and CS ($F(1,22) = 31.14$, $p < 0.001$) and a Session X CS interaction ($F(2,44) = 23.95$, $p < 0.001$), but no effect of Lesion Group ($F(2,22) = 0.92$, $p > 0.05$) or any other interactions (F's < 2.0, p's > 0.05).

A Lesion Group X Session repeated measures ANOVA on CS+ elevation responding showed a main effect of Session ($F(2,44) = 135.43$, $p < 0.001$), but no effect of Lesion Group or interactions (F's < 2, p's > 0.05), confirming all lesion groups similarly increased conditioned responding to the CS+ across training sessions (Fig 4.2A).

Additionally, all groups showed a change in conditioned responding during the CS- (Fig 4.2B). A Lesion Group X Session repeated measures ANOVA on CS- elevation responding showed a main effect of Session ($F(2,44) = 51.79$, $p < 0.001$), but no effect of Lesion Group or interactions (F's < 2, p's > 0.05). Follow-up analyses during the first training session showed a main effect of Group ($F(2,22) = 3.97$, $p < 0.05$), and individual CS one-way ANOVAs showed a

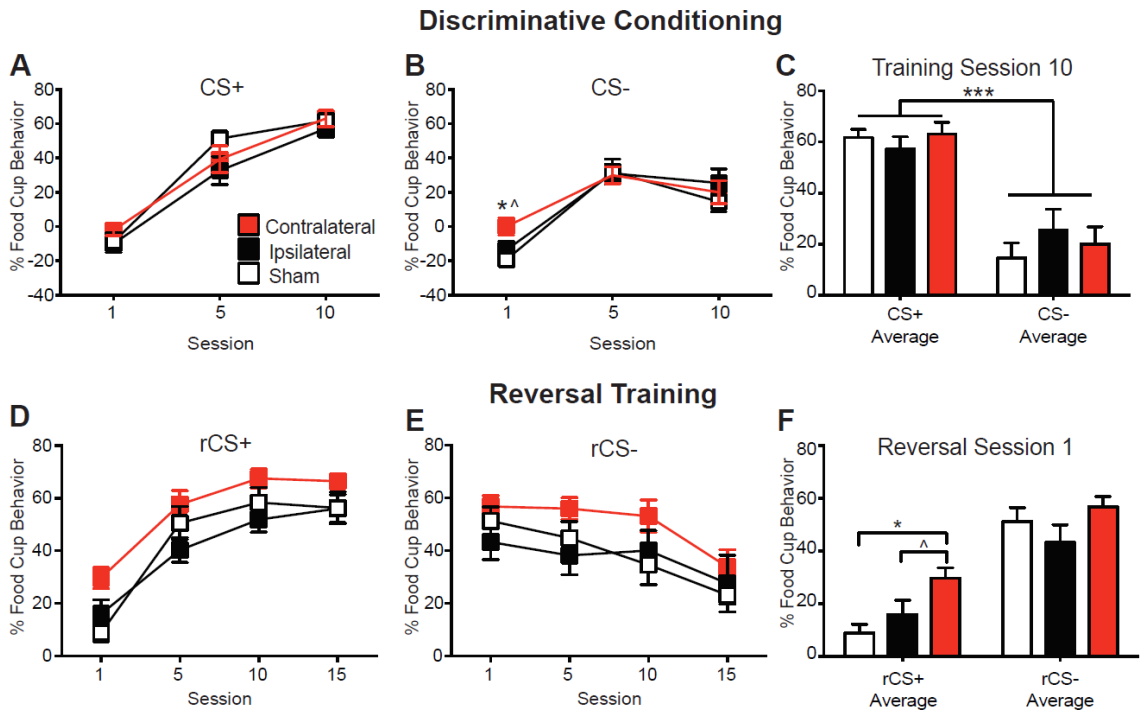

Figure 4.2 Conditioned responding throughout discriminative conditioning (*top row*) and reversal learning (*bottom row*). Percentage of time (mean ± SEM) rats expressed food cup behavior across discriminative conditioning in response to the CS+ (A) and CS- (B) and average responding during session 10 (C). Percentage of time (mean ± SEM) rats expressed food cup behavior across reversal learning in response to the rCS+ (D; previously the CS-) and rCS- (E; previously the CS+) and average responding during session 1 (F). *** $p < 0.001$; * $p < 0.01$ Contralateral vs Sham; ^ $p = 0.04$ Contralateral vs Ipsilateral.

significant Group effect on CS- responding during the first session ($F(2,24) = 5.00$, $p < 0.05$). *Post-hoc* analyses showed the Contralateral lesion group was responding more to the CS- compared to the Sham group ($p < 0.017$) and Ipsilateral Lesion group ($p = 0.036$).

To confirm successful discrimination at the end of training, follow-up analyses on training session 10 showed a main effect of CS ($F(1,22) = 73.67$, $p < 0.001$) with no differences between Lesion groups or interactions (F's < 1, p's >

0.05), confirming higher food cup responding to the CS+ compared to the CS- in all groups (Fig 4.2C).

3.3. Reversal Learning. All rats successfully learned the new outcomes during reversal learning; however, the Contralateral lesion group consistently responded higher to the CSs throughout learning (Fig. 4.3, bottom). A Lesion Group X reversal CS (rCS+, rCS-) X Session (1, 5, 10, 15) repeated measures ANOVA found an effect of Lesion Group ($F(2,22) = 4.11$, $p < 0.05$), CS ($F(1,22) = 4.27$, $p = 0.05$), Session ($F(3,66) = 12.13$, $p < 0.001$), and a CS X Session interaction ($F(3,66) = 49.18$, $p < 0.001$). *Post-hoc* analysis showed the Contralateral lesion group had increased responding throughout reversal learning compared to Ipsilateral lesion group ($p < 0.017$) and Sham group ($p = 0.035$).

To examine CS simple effects, a Lesion Group X Session repeated measures ANOVA on the rCS+ (Fig. 4.3D) showed a main effect of Lesion Group ($F(2,22) = 5.15$, $p < 0.05$) and Session ($F(3,66) = 67.13$, $p < 0.01$), but no interaction ($F < 2$, $p > 0.05$), showing that all groups increased responding to the rCS+ throughout reversal learning. Additionally, the Contralateral lesion group consistently responded higher to the rCS+ compared to the Ipsilateral lesion group ($p < 0.017$) and Sham group ($p = 0.023$).

A Lesion Group X Session repeated measures ANOVA on the rCS- (Fig. 4.3E) showed a main effect of Session ($F(3,66) = 8.96$, $p < 0.01$), with no effect of Lesion Group ($F(2,22) = 2.17$, $p = 0.14$) or interaction ($F < 1$, $p > 0.05$), showing that all groups similarly decreased responding to the rCS- throughout reversal learning.

Follow-up analyses on individual reversal sessions found a significant effect during session 1. A rCS X Lesion Group repeated measures ANOVA on reversal session 1 showed a main effect of Lesion group ($F(2,22) = 5.48$, $p < 0.05$) and CS ($F(1,22) = 53.93$, $p < 0.001$). *Post-hoc* analyses showed an effect of Lesion group during the rCS+ ($F(2,24) = 5.51$, $p < 0.05$), but not during the rCS- ($F(2,24) = 1.53$, $p > 0.05$). The Contralateral lesion group had higher conditioned responding to the new rCS+ compared to the Sham ($p < 0.017$) and Ipsilateral lesion group ($p = 0.038$) during reversal session 1 (Fig 4.2F).

3.4. Devaluation. Devaluation testing commenced following reversal learning. For devaluation testing, half of each lesion group received LiCl-US pairings ("Devalued") and half received LiCl and US unpaired ("Non-devalued"), resulting in Sham: Non-devalued (n=4) and Devalued (n=4); Ipsilateral: Non-devalued (n=6) and Devalued (n=3); and Contralateral: Non-devalued (n=4) and Devalued (n=4).

3.4.1. Conditioned Taste Aversion. All rats that received the LiCl-US pairings (Devalued group) showed a decrease in food consumption across tests with no differences between Lesion groups (Fig. 4.3). A Lesion Group X Devaluation X Consumption Test repeated measures ANOVA showed a main effect of Test Session ($F(2,38) = 21.16$, $p < 0.001$) and Devaluation ($F(1,19) = 16.36$, $p < 0.001$) and a Test Session X Devaluation interaction ($F(2,38) = 31.71$, $p < 0.001$). *Post-hoc* analyses showed no differences between Lesion Groups or Devaluation groups (F's < 1, p's > 0.05) during the initial consumption measured (baseline consumption), as expected since LiCl administration followed this

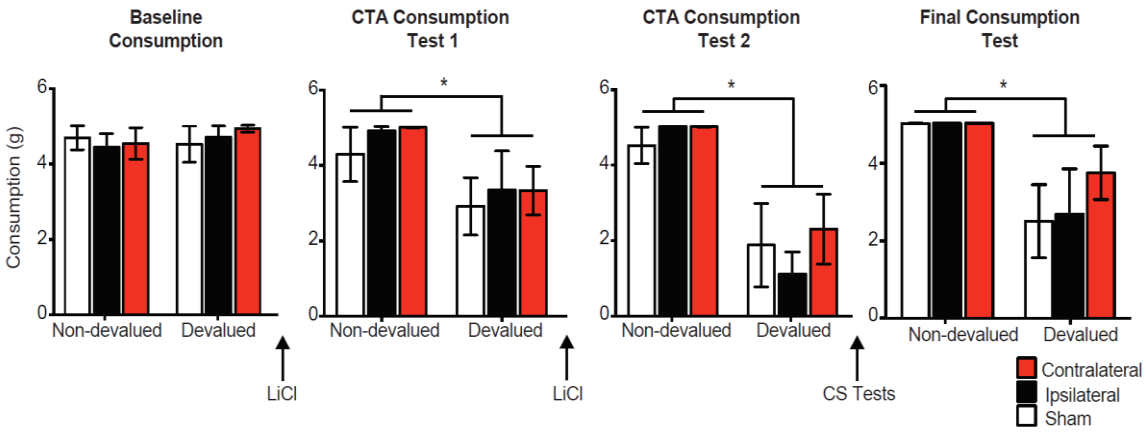

Figure 4.3 Consumption during Conditioned Taste Aversion procedures. Prior to LiCl administration, all rats received an initial consumption test (baseline). Then, rats were given LiCl injections (i.p.) either immediately following consumption (Devalued) or 24 hours later (Non-devalued). 24 hours following LiCl injections, rats were given another consumption test (Test 1) followed again by LiCl injections either immediately or 24 hours later. Rats were given a third consumption test (Test 2) prior to CS testing. Rats were given a final consumption test to examine the maintenance of the CTA memory after CS testing.

session. Following CTA, there was an effect of Devaluation during the first ($F(1,19) = 14.27$, $p < 0.01$) and second ($F(1,19) = 36.39$, $p < 0.001$) consumption tests, but no effect of Lesion Group or interaction (F's < 1, p's > 0.05), confirming all devalued groups, regardless of lesion condition, decreased consumption of the US.

3.4.2. CS Devaluation Testing. Following CTA, all rats were presented with the CSs (without US). Overall, there was no evidence of devaluation to the CSs and minimal differences between lesion groups (Fig 4.4). A Lesion Group X Devaluation X CS X Session repeated measures ANOVA showed main effects of CS ($F(1,19) = 174.05$, $p < 0.001$) and Session ($F(2,38) = 33.71$, $p < 0.001$) and a CS X Devaluation interaction ($F(1,19) = 5.71$, $p < 0.05$)

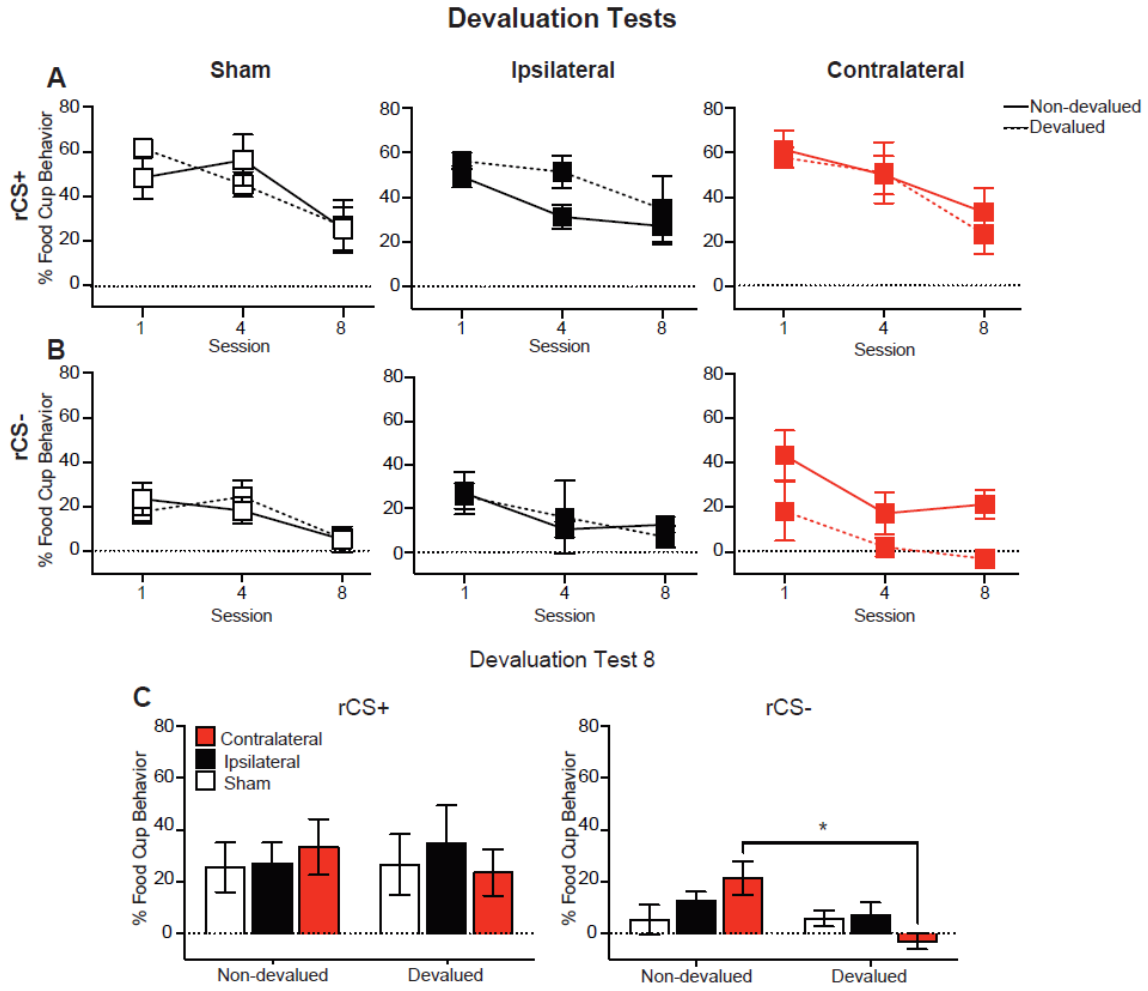

Figure 4.4 Conditioned responding throughout CS devaluation testing without the delivery of food. Percentage time (mean ± SEM) rats expressed food cup behavior to the rCS+ (A) and rCS- (B) across testing and during the last test session (C). * $p < 0.01$

and a marginally significant CS X Session interaction ($F(2,38) = 2.66$, $p = 0.08$), but no other effects or interactions (F's < 2, $p > 0.05$). Follow-up analyses on conditioned responding to the rCS- showed an effect of session ($F(2,38) = 10.50$, $p < 0.001$) and a marginally significant Lesion Group X Devalued interaction ($F(2,19) = 2.62$, $p = 0.099$). Additional analyses on test session 8 showed an effect of Devaluation ($F(1,19) = 6.72$, $p < 0.05$) and a Lesion Group X

Devaluation Interaction ($F(2,19) = 3.94$, $p < 0.05$). Independent *t*-tests showed the Contralateral Non-devalued group was responding higher to the food cup compared to the Contralateral Devalued group (t (6) =3.44, $p < 0.05$), with no other differences between lesion and devalued groups (p's > 0.05).

3.4.3. Final Consumption Test. After CS devaluation testing, rats were given a final consumption to confirm the CTA memory of the US, and, indeed, all rats that received CTA maintained decreased consumption of the US (Fig 4.3). A Lesion Group X Devalued ANOVA on US consumption during the final test showed a Devaluation effect ($F(1,19) = 19.44$, $p < 0.001$), but no effect of Lesion group or interactions (F's < 1).

4. Discussion

The current study examined if communication between the BLA and mPFC is necessary in appetitive cue learning and behavioral flexibility. First, we found discriminative conditioning between a cue paired with food (CS+) and a cue not paired with food (CS-) was similar across all lesion groups. These results indicate communication between the BLA and mPFC is not necessary for discriminative learning. However, when the outcomes of the cues were reversed, the group that had disconnection of communication between the BLA and mPFC had higher conditioned responding during reversal learning, particularly to the cue that previously did not predict food, the rCS+, during session 1. These results demonstrate that communication between the BLA and mPFC is necessary to incorporate previously learned information during new learning. Furthermore, disconnection of the BLA-mPFC circuitry had no effect on CTA and

minimally affected CS devaluation, even though the control groups (shams and ipsilateral groups) did not show evidence of devaluation. Together, these results show communication between the BLA and mPFC is not necessary for discriminative learning or CTA but is necessary for new learning when outcomes of learned cues change.

Contralateral disconnection of the BLA-mPFC circuitry resulted in sustained, increased responding across reversal learning, evident by more conditioned responding to the cue that previously did not signal food (rCS+), especially during the first session of reversal, compared to the ipsilateral and sham groups. The behavior during the first session reflects the recall of the memory of the cue's learned value, as acquired during discriminative conditioning. The rCS+ was previously the CS- throughout discriminative conditioning, and the reversal session 1 was the first time that cue was presented with food. All groups should have maintained low responding during early reversal learning since the CS- reliably predicted the absence of food. However, the contralateral disconnection group had increased responding, suggesting their ability to inhibit behavioral responding was impaired. The increased conditioned responding in the contralateral disconnection group signifies that they are not appropriately recalling the learned outcome of the cue that is needed to be incorporated during learning of the new outcomes and respond appropriately. These results demonstrate that communication between the BLA and mPFC is necessary for cue value recall to learn new associations and to guide behavioral responding (for review, see Wassum & Izquierdo, 2015).

Another explanation for the increased conditioned responding during the first reversal session in the contralateral disconnection group is that communication between the BLA and mPFC is needed for behavioral inhibition during learning. Indeed, previous studies have shown inactivation of the BLA and mPFC impaired behavioral inhibition and, as a result, increases impulsive behavior for rewards. After bilateral BLA or mPFC or contralateral inactivation by muscimol infusions, rats spent less time waiting for a high value reward and decreased preference for that reward, suggesting less behavioral inhibition and more impulsivity (Churchwell et al., 2009). The same study showed rats with bilateral BLA, but not mPFC, inactivation had more impairments in appetitive reversal learning and took longer to successfully learn (Churchwell et al., 2009). Additionally, rats with mPFC lesions showed increased responding during early reversal learning and took longer to display successful reversal learning (Salazar et al., 2004), but showed minimal impairments during early appetitive learning and discrimination (Salazar et al., 2004; Petrovich et al., 2007). Collectively, prior and current results suggest the BLA-mPFC circuitry is needed to appropriately recall and assess the value of learned cues when the outcomes and values of those cues change. The recalled value of the cues is then used to guide and control behavioral inhibition and impulsivity when appropriate.

To further investigate the role of the BLA-mPFC circuitry in cue value updating, rats were tested in a CS devaluation paradigm after reversal learning. Half of each lesion group underwent food devaluation by receiving LiCl-induced illness after food consumption (conditioned taste aversion; CTA) and were then

tested on the associated learned cues (rCS+, rCS-) in the absence of food. All groups that received the US-LiCl pairings successfully learned CTA, regardless of lesion group, as shown by a decrease in consumption of the US, indicating communication between the BLA and mPFC is not necessary for CTA. This is in agreement with previous studies that have shown that independently the BLA and mPFC are not necessary for the acquisition of CTA (Bahar et al., 2003; Johnson et al., 2009). However, other studies have shown the mPFC and BLA are activated during the maintenance of the CTA memory (Mickley et al., 2005; Xin et al., 2014), and NDMA receptor signaling (Akirav et al., 2006) and alterations in BDNF gene expression (Xin et al., 2014) within the mPFC and BLA impairs the maintenance of the CTA memory. These results suggest that independently the BLA and mPFC are not necessary in the acquisition of CTA but are necessary in the recall and maintenance of CTA.

In the current study, we hypothesize that the rats that received contralateral disconnection of this circuitry successfully learned CTA, because they still had one of each region intact. The contralateral disconnection method lesioned the BLA in one hemisphere and the mPFC in the other hemisphere, functionally disconnecting communication between the structures in both hemispheres but allowing one of each region to interact within other circuitries. The intact unilateral BLA and mPFC and their connections with other regions (e.g. the central amygdala [Yamamoto et al., 1992; Bahar et al., 2003] and nucleus of the solitary tract [Houpt et al., 1994; Schafe and Bernstein, 1996; Sakai and Yamamoto, 1997; Spray & Bernstein, 2004, for review, see Jahng &

Lee, 2015]) could be sufficient to learn and recall CTA.

The CS devaluation tests examined how rats responded to the learned cues for food after CTA devalued the food. Successful CS devaluation is evident by a decrease in conditioned responding to the CS since the associated US was devalued; however, we did not find robust evidence of CS devaluation, even in the sham groups. All groups maintained increased responding to the rCS+ compared to the rCS- after CTA likely due to extensive training and experience with the CSs and US (25 sessions of learning prior to devaluation). Interestingly, we did find a small interaction between BLA-mPFC disconnection and devaluation. The non-devalued contralateral lesion group still had increased conditioned responding during the presentation of the rCS- compared to the devalued contralateral group, specifically during the last session of testing. This increased responding reflects similar impairments in behavioral inhibition that we found in reversal learning. These results suggest rats with BLA-mPFC disconnection may not be incorporating previously learned information, including that the rCS- signals the absence of food and the US is now devalued, to update the value of the cue to appropriately inhibit behavior. However, these results should be interpreted with caution since the sham groups did not show CS-devaluation, and the group sizes were small. Notably, another study showed that disconnecting the BLA-mPFC circuitry did not interfere with satiety-induced devaluation (Coutureau et al., 2009).

One limitation of the disconnection preparation is that it does not provide information about the directionality of communication needed. Therefore, we

cannot determine if disruption of communication from the BLA to the mPFC or mPFC to BLA or both were responsible for the impairments in the current study. It is possible each pathway is involved in distinct aspects of the investigated behavioral paradigms: the BLA could inform the mPFC of the cue's value (e.g. Keefer & Petrovich, 2017) and the mPFC could have top-down control onto the BLA to guide behavioral inhibition (e.g. Likhtik et al., 2005). Indeed, there is evidence for anatomical bidirectional communication (Sesack et al., 1989; Takagishi & Chiba, 1991; Kita & Kitai, 1990; Swanson & Petrovich, 1998; Petrovich et al., 2001; Vertes, 2004; Gabbott et al., 2005; Hoover & Vertes, 2007; Hirai et al., 2012; Little & Carter, 2013; Reppucci & Petrovich, 2016), and each region has been shown to directly alter activity within the other region (Rosenkranz & Grace, 2001; Likhtik et al., 2005; Nowak et al., 2012; Sotres-Bayon et al., 2012; Sun & Laviolette, 2012; Little & Carter, 2013; Xin et al., 2014) to guide reward seeking and learning (Fuchs et al., 2007; Churchwell et al., 2009; Mashhoon et al., 2010; Stefanik et al., 2013; Keefer & Petrovich, 2017). Future work is necessary to delineate the differences between the involvement of these pathways in appetitive cue learning and value updating.

5. Conclusions

The current study examined if communication between the BLA and mPFC is necessary for appetitive cue learning and behavioral flexibility when the outcome of learned cues change. Rats that received disconnection of the BLA-mPFC successfully discriminated between appetitive cues; however, they displayed increased conditioned responding to the cues throughout reversal

learning. Additionally, disconnection of the BLA-mPFC circuitry did not interfere with CTA but slightly altered CS devaluation responding. These results demonstrate that communication between the BLA and mPFC is necessary to respond appropriately when the outcomes, and thus the values, of learned cues that predict food availability have changed.

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Chapter 5: General Discussion

The main goal of the research in this dissertation was to determine the influence of the basolateral amygdala (BLA) and its connections with the medial prefrontal cortex (mPFC) in appetitive cue learning and valuation. First, we determined there are separate neuronal ensembles within the BLA that respond to different learned cues, and that these distinct neuronal ensembles are necessary for updating that value of that specific cue (Chapter 2). Next, we determined that neurons within the BLA that project to the mPFC were differently activated across cue-food learning (Chapter 3), indicating plasticity and specificity within this pathway. Lastly, we determined that communication between the BLA and mPFC is necessary for accurate cue value recall and subsequent value updating of learned appetitive cues (Chapter 4).

In Chapter 2, we used a chemogenetic method to selectively inactivate neurons that respond to a specific stimulus and examined if the same BLA neuronal ensembles that are involved in the initial associative memory are required when the new cue-outcome association is formed during reversal learning. After these selective inactivations, we observed behavioral impairments specific to the cue to which the neuronal ensembles were inactivated. These results demonstrated that separate neuronal ensembles within the BLA are responsive to different learned cues, and these cue-specific neuronal ensembles are necessary to update the value of that cue. As discussed in Chapter 2, previous studies have shown that separate BLA neuronal ensembles mediate responding to distinct cues and switch their responding during periods of

necessary behavioral flexibility (Schoenbaum et al., 1999; Paton et al., 2006; Tye et al., 2010). The experiment in Chapter 2 extended these findings by showing that these cue-specific neuronal ensembles are necessary to appropriately recall and update the value of the learned cues.

To determine a circuitry BLA circuitry, in Chapter 3 we investigated a specific target of the BLA that could receive information about the cues' values – the mPFC. Anatomical studies have shown well-organized topographical connections from the BLA to mPFC in that the anterior BLA has more dense projections to the PL while the posterior BLA has more dense projections to the ILA (e.g. Reppucci & Petrovich, 2016). Functionally, our laboratory has shown distinct temporal recruitment of these areas. The anterior BLA is recruited during early cue-food learning, and the posterior BLA, PL, and ILA are recruited when the association is well-learned (Cole et al., 2013; 2015a). Given this distinct temporal activation of these topographically connected areas, we focused on the recruitment of specific BLA-mPFC pathways during cue-food learning. We determined that the pathway specifically originating from the anterior BLA neurons that project to the prelimbic area (PL) of the mPFC was recruited when cue-food associations were well-learned, indicating plasticity of this pathway across learning.

Based on the activation of the BLA to mPFC pathway we found during cue value learning, in Chapter 4 we examined if the BLA-mPFC circuitry is necessary for cue value learning and subsequent updating of the value of the learned cues. Rats received contralateral, ipsilateral, or sham excitotoxic lesions of the BLA-

mPFC, and then underwent discriminative conditioning, reversal learning, and devaluation by CTA. Contralateral disconnection of this circuitry did not alter initial discriminative conditioning. This was expected since previous studies have shown that bilateral inactivation of the BLA (Hatfield et al., 1996; Parkinson et al., 2000; Holland et al., 2002; Balleine et al., 2003; Corbit & Balleine, 2005) or bilateral mPFC (Petrovich et al., 2007) does not significantly impair the initial acquisition of reward learning. Furthermore, disconnection of the BLA and mPFC resulted in an increase in behavioral responding throughout reversal learning, signifying impairment in appropriate responding when behavioral flexibility is needed. These results suggest communication between the BLA and mPFC are necessary to recall the value of learned cues and update the value of the cues to respond appropriately. Other studies have shown similar behavioral impairments during reward valuation paradigms (Salazar et al., 2004; Churchwell et al., 2009). Together, along with the results from Chapters 2 and 3, we propose that the BLA is necessary to inform and update the mPFC about cues' values to order to alter behavioral responding for learned cues.

Influence of the BLA inputs onto mPFC neurons

Chapters 3 and 4 indicated the involvement of communication between the BLA and mPFC during appetitive learning, and anatomical evidence indicates the BLA can alter mPFC activity through direct inputs via pyramidal neurons. Indeed, stimulation of the BLA excited the mPFC through monosynaptic, glutamatergic transmission (Perez-Jaranay & Vives, 1991; McDonald, 1992; Sotres-Bayon et al., 2012), and inactivation of the BLA decreased activity of PL

pyramidal neurons (Sotre-Bayono et al., 2012). However, several studies have also found polysynaptic pathways from BLA pyramidal neurons via inhibitory interneurons within the mPFC (Perez-Jaranay & Vives, 1991; Gabbott et al., 2006; Floresco & Tse, 2007; Sun & Laviolette, 2012; Dilgen et al., 2013). BLA stimulation results in stimulation of the interneurons within the mPFC resulting in inhibition of the mPFC pyramidal neurons (Perez-Jaranay & Vives, 1991; Gabbott et al., 2006; Dilgen et al., 2013), while BLA inactivation leads to decreased activation of the interneurons resulting in disinhibition of the mPFC pyramidal neurons (Sun & Laviolette, 2012). Thus, the BLA could modulate mPFC via multiple pathways either by activating or inhibiting pyramidal neurons resulting in a change of communication to regions the mPFC projects to during learning (discussed below). In Chapters 2 and 4, inactivation of BLA neurons and disconnection of the BLA and mPFC pathway, respectively, would have altered BLA communication to the mPFC, and thus, changed mPFC output to downstream regions critical for modulating behavioral responding (discussed below).

mPFC top-down control of behavior

In Chapter 4, the contralateral disconnection procedure eliminated reciprocal communication between the BLA and mPFC. As a result, along with abolishing communication from the BLA to mPFC, mPFC inputs to the BLA were also abolished. Thus, a limitation of this method is that it cannot provide information about the directionality of communication needed for the appetitive learning paradigms that were investigated. The mPFC is critical for regulating the

top-down control of behavior (e.g. Likhtik et al., 2005), and disconnection of the circuitry may have interfered with the control of the mPFC on the BLA and other downstream regions during behavioral inhibition. Indeed, there is evidence that the mPFC can directly alter activity within the BLA; however, research on the specific mechanisms on how the mPFC alters activity has shown opposing findings. The majority of studies indicate the mPFC pyramidal neurons preferentially synapse onto the BLA pyramidal neurons resulting in BLA excitation (Brinley-Reed et al., 1995; Smith et al., 2000; Likhtik et al., 2005). However, one study showed the mPFC synapses onto and stimulates BLA interneurons resulting in BLA inhibition (Rosenkranz & Grace, 2001), but this finding has been debated (for discussion, see Quirk et al., 2003).

Together, these findings suggest the mPFC can directly control BLA activity to ultimately control behavioral output. Investigations of this pathway within appetitive learning is scarce. The mPFC to BLA pathway is not necessary for alcohol seeking (Keistler et al., 2017), and, stimulation of mPFC terminals within the BLA (specifically of mPFC neurons that contain dopamine 1 receptor) increased food intake (Land et al., 2014). This control of food intake and behavior likely arises through different BLA outputs – the lateral hypothalamus (LHA) and other regions (discussed below).

BLA and mPFC inputs to the LHA are critical for behavioral motivation

Importantly, both the BLA and mPFC communicate with the LHA - an area critical for homeostatic feeding (Wise, 1974; Krettek & Price, 1978; Ono et al., 1985; Sesack et al., 1989; Hurley et al., 1991; Petrovich & Swanson, 1997;

Risold et al., 1997; Elmquist et al., 1999; Petrovich et al., 2001; Hahn & Swanson, 2010, 2012, 2015; Jennings et al., 2013; Reppucci & Petrovich, 2016) and appetitive learning (e.g. Cole et al., 2015a; Sharpe et al., 2017). The LHA contains neuronal populations that express the neuropeptide orexin/hypocretin (ORX; de Lecea et al., 1998; Sakurai et al., 1998), which can stimulate feeding (e.g., Sakurai et al., 1998; Rodgers, 2000; Clegg et al., 2002), are necessary for wakefulness and arousal (e.g. de Lecea et al., 1998; Chemelli et al., 1999; Berridge et al., 2010), and mediate other motivated behaviors driven by food and drug rewards (e.g. Petrovich et al., 2012; Cole et al., 2015b; Keefer et al., 2016; Sharpe et al., 2017; Cole et al., in prep; for reviews, see Sakurai, 2014; Mahler et al., 2014; Moorman, 2018). Importantly, similar to the recruitment of the mPFC and posterior BLA, when cue-food associations are well-learned (Cole et al., 2015a), LHA ORX neurons are also recruited when the cue is well-learned (Cole et al., 2015a), supporting the evidence of plasticity of activation within this proposed circuitry.

Indeed, previous studies have shown the BLA and mPFC inputs to the LHA are critical for driving behavior in relation to reward seeking. Stimulation of the mPFC, particularly by μ-opioid receptor stimulation, increased food intake, locomotion, and LHA ORX neuron activation (Mena et al., 2013), while another study showed increased activation of the ILA to LHA pathway during behavioral inhibition in reward extinction (Marchant et al., 2010). Additionally, the mPFC and BLA pathways to the LHA are activated during cue-induced feeding (Petrovich et al., 2005), and disconnection of the BLA-LHA (Petrovich et al., 2002) and mPFC-

LHA (Cole et al., in prep) pathways interfere with that behavior. Thus, the BLA and mPFC pathways to the LHA are necessary in appetitive learning and motivation that influence behavioral output.

In Chapter 2, critical inputs from the BLA to the LHA could have been altered due to inactivation of BLA neuronal ensembles that communicate with the LHA which could have contributed to the observed decrease in conditioned behavior in both Daun02 infused groups. In Chapter 4, abolished communication between the BLA and mPFC could have altered activation of the LHA. The contralateral disconnection method unilaterally lesions the mPFC in one hemisphere and the BLA in the other hemisphere. As a result, LHA activation may have been altered since the LHA in each hemisphere only received communication from the mPFC or the BLA, but not both, due to the contralateral lesion design, resulting in changes in behavioral responding.

PL versus ILA influence in appetitive motivation

Importantly, the mPFC has several sub-regions, including the PL and infralimbic (ILA) areas (Heidbreder & Groenewegen, 2003; Swanson, 2004) that have similar roles in appetitive learning. Chapter 3 specifically analyzed projections from the anterior and posterior nuclei of the BLA to the PL and did not include BLA projections to the ILA, the ventral region of the mPFC. Similar to the PL, previous studies in our laboratory have shown significant activation of the ILA when a cue reliably predicts food (Cole et al., 2015a). The ILA receives more dense projections from the posterior BLA and less dense projections from the anterior BLA (McDonald, 1987; Hoover & Vertes, 2007; Reppucci & Petrovich,

2016). It is highly probable the posterior BLA to the ILA pathway is recruited when a cue is well-learned, similar to activation of the anterior BLA to the PL pathway we found in Chapter 3. This would suggest that overall the BLA can inform different mPFC regions during appetitive learning in a topographically organized way.

Additionally, in Chapter 4, lesions of the mPFC encompassed both the PL and ILA, and as a result, we could not compare the potentially differential involvement of each area during cue value learning and updating; however, there is much evidence that they have similar roles in the behavioral paradigms investigated. Indeed, previous findings showed that PL and ILA neurons similarly maintain responding between reward-predictive cues and cues that no longer predicts food even when behavioral responses decrease (Moorman & Aston-Jones, 2015). These activation patterns correlated to behavioral responses: increased activation was related to increased responding during cue learning and then maintained increased activation during behavior inhibition during cue extinction (Mulder et al., 2003; Moorman & Aston-Jones, 2015), suggesting that the mPFC is critically guiding behavioral responses based on monitoring the outcome or “representation” of the cue.

Similarly, the PL and ILA are both involved in appetitive learning (Ishikawa et al., 2008a, 2008b; Moorman & Aston-Jones, 2015; Cole et al., 2015a), but may mediate distinct aspects of learning. The PL responds to the delivery of food, while the ILA responds during food collection (Burgos-Robles et al., 2013; Moorman & Aston-Jones, 2015). Some evidence from appetitive tasks supports

the notion of a distinct dichotomy between the PL and ILA in behavioral initiation and inhibition, respectively, but there is stronger evidence for this dichotomy in behavioral control during aversive paradigms (“go-stop” dichotomy; Gourley & Taylor, 2016). Evidence from appetitive studies show, indeed, PL lesions, but not ILA lesions, inhibit early responding to discrete cues associated with reward and during discriminative conditioning (Ashwell & Ito, 2014; Corbit & Balleine, 2003; Rhodes & Killcross, 2004, 2007a), suggesting the “go” role of the PL. Conversely, ILA inactivation increased behavioral responding to a cue that was not associated with food (Ishikawa et al., 2008b), increased spontaneous recovery (Rhodes & Killcross, 2004), and increased responding during appetitive renewal (Rhodes & Killcross, 2007a). Additionally, ILA stimulation enhanced extinction learning (Peters & De Vries, 2013).

However, there has been controversy in the literature regarding appetitive studies for the “go-stop” dichotomy of the PL and ILA (e.g. Moorman & Aston-Jones, 2015). ILA inactivation increased (Ishikawa et al., 2008b) but has also decreased responding (Ashwell & Ito, 2014) to cues that signal the absence of food. The differences between these two findings could be due to the food-associated cues being discrete (Ishikawa et al., 2008b) versus contextual (Ashwell & Ito, 2014), signifying the PL and ILA may alter behavioral responding depending on the type of appetitive cue. Indeed, several studies have shown the mPFC is involved in discrete cue processing (Holland & Petrovich, 2005; Cole et al., 2015a, 2015b, in prep) and in contextual processing (Petrovich et al., 2007; Bossert et al., 2011; Willcocks & McNally, 2017; Anderson & Petrovich, 2015,

2017, 2018a, 2018b; Moorman & Aston-Jones, 2015; Eddy et al., 2016) during appetitive learning and motivation, but investigations in potential differences between cue processing between the PL and ILA is needed.

Furthermore, other studies showed inactivation of the ILA, but not PL, results in better extinction learning shown by more inhibition of behavior (Mendoza et al., 2015; Moorman & Aston-Jones, 2015), suggesting contradictory evidence of the ILA being a behavioral inhibition regulator. If the ILA is intact, more behavioral inhibition should occur, but these studies found more behavioral inhibition with the ILA inactivated. Additionally, other studies have found no effect on extinction learning with inactivation of the ILA (Rhodes & Killcross 2004, 2007a).

The topographical organization of connections between the BLA and mPFC along with evidence that the PL and ILA may mediate different aspects of appetitive learning suggest separate but parallel pathways could regulate distinct aspects of appetitive learning and behavioral regulation. Neural circuitries involving the PL and ILA could differentially regulate behavioral initiation and inhibition, respectively. In agreement within the ILA being needed for behavioral inhibition, one appetitive study showed increased activation of the ILA to LHA pathway, but not the PL to LHA pathway, during behavioral inhibition in reward extinction (Marchant et al., 2010). Stronger evidence for the importance of this pathway is shown in aversive learning paradigms. Connectivity from the mPFC, specifically the PL, to the BLA is strengthened after fear learning (Arruda-Carvalho & Clem, 2014), but weakens after fear extinction (Cho et al., 2013),

suggesting this pathway is critical in modulating behavioral responding during aversive learning. Additionally, stimulation of the mPFC to BLA pathway enhances fear extinction learning (Bukalo et al., 2015), while inhibition of this pathway impaired extinction learning (Bloodgood et al., 2018). Given these findings in the aversive literature, we propose this pathway is similarly critical for behavioral control in appetitive paradigms; however, future research in appetitive learning is necessary to define similarities and differences between PL and ILA functional connections to output regions that modify behavioral responding.

Other outputs of the mPFC and BLA

The overarching finding of the research in this dissertation shows neural plasticity within the BLA (Chapter 2) and its communication with the mPFC (Chapters 3 and 4) are critical for cue valuation, but it is possible communication from these regions to other regions can change during learning in order to update the value of appetitive cues and subsequent behavior.

The central amygdala (CEA) a critical output region of the amygdala, and the mPFC and BLA can alter activity within the CEA resulting in alterations in behavioral responding (Paré et al., 1995; Pitkänen et al., 1997; Quirk et al., 2003). Indeed, stimulation of the mPFC exclusively inhibited activity within the CEA (Quirk et al., 2003), either from direct inputs from the ILA (Hurley et al., 1991) or indirectly through the BLA or through the GABAergic intercalated cells (Sesack et al., 1989; McDonald et al., 1996) that synapse onto and inhibit CEA neurons (Paré & Smith 1993; Royer et al., 1999). Thus, activation of the mPFC can indirectly alter behavioral output through stimulation of BLA pyramidal

neurons that project to CEA GABAergic neurons. In Chapter 4, disconnection of this mPFC to BLA pathway could have interfered with communication to the CEA and as a result, altered behavioral.

The mPFC and BLA also interact with other regions necessary for appetitive motivation. Another critical region necessary for appetitive learning and motivation is the nucleus accumbens that receives projections from the BLA and mPFC (Sesack et al., 1989; McDonald, 1991; Brog et al., 1993; Wright et al., 1996; Voorn et al., 2004), and these connections are critical for behavioral responding during reward learning (Cador et al., 1989; Setlow et al., 2002; Di Ciano & Everitt, 2004; Kelley, 2004; Ambroggi et al., 2008; Shiflett & Balleine, 2010; Stuber et al., 2011; Beyeler et al., 2016). Additionally, the BLA and mPFC have reciprocal connections with another part of the prefrontal cortex, the orbital cortex (McDonald, 1991; McDonald et al., 1996; Vertes, 2004; Hoover & Vertes, 2011; Murphy & Deutch, 2018), and connections between the BLA and orbital cortex is critical in value representation during learning (Baxter et al., 2000; Saddoris et al., 2005; Schoebaum et al., 2003a, 2003b; Rudebeck et al., 2013; Zeeb & Winstanley, 2013). Palatability processing and reward value representation depend on communication from the BLA to the gustatory area (Piette et al., 2012; Parkes & Balleine, 2013) and neighboring agranular insular cortex (Nasser et al., 2018). Additionally, projections from the gustatory area to the mPFC is also involved in palatability processing (Jezzini et al., 2013). Furthermore, the paraventricular nucleus of the thalamus (PVT) is critical for feeding and appetitive motivation (Hamlin et al., 2009; Stratford & Wirtshafter,

2013; Matzeu et al., 2014, 2015; Cole et al., 2015b; for reviews, see Kirouac, 2015; Millian et al., 2017), communicates extensively with the mPFC and BLA (Li & Kirouac, 2012; Vertes, 2004), and receives input from the mPFC during reward seeking (Marchant et al., 2010; Anderson & Petrovich, 2018b). It is probable that communication between the mPFC and BLA and these areas changes when the contingencies of the appetitive cues also change warranting necessary behavioral modification.

Conclusions

In summary, the conclusions from the experiments completed in this dissertation add to the increasing knowledge and understanding of the neural mechanisms necessary for appetitive learning and memory. We determined the BLA and its connections with the mPFC undergoes neural plasticity in order to update the values of learned appetitive cues due to alterations in cue outcome. This knowledge is valuable for guiding future clinical investigations in understanding the neural mechanisms that drive eating behavior and overeating under the influence of food cues, which are abundant in developed countries.

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Appendix

Orexin/hypocretin receptor 1 signaling mediates Pavlovian cue-food conditioning and extinction *

**Published Manuscript*
Keefer, S.E., Cole, S., & Petrovich, G.D. (2016) Orexin/hypocretin receptor 1 signaling mediates Pavlovian cue-food conditioning and extinction. Physiology of Behavior, 162:27-46.

ABSTRACT: Learned food cues can drive feeding in the absence of hunger, and orexin/hypocretin signaling is necessary for this type of overeating. The current study examined whether orexin also mediates cue-food learning during the acquisition and extinction of these associations. In Experiment 1, rats underwent two sessions of Pavlovian appetitive conditioning, consisting of tone-food presentations. Prior to each session, rats received either the orexin 1 receptor antagonist SB-334867 (SB) or vehicle systemically. SB treatment did not affect conditioned responses during the first conditioning session, measured as food cup behavior during the tone and latency to approach the food cup after the tone onset, compared to the vehicle group. During the second conditioning session, SB treatment attenuated learning. All groups that received SB, prior to either the first or second conditioning session, displayed significantly less food cup behavior and had longer latencies to approach the food cup after tone onset compared to the vehicle group. These findings suggest orexin signaling at the 1 receptor mediates the consolidation and recall of cue-food acquisition. In Experiment 2, another group of rats underwent tone-food conditioning sessions (drug free), followed by two extinction sessions under either SB or vehicle

treatment. Similar to Experiment 1, SB did not affect conditioned responses during the first session. During the second extinction session, the group that received SB prior to the first extinction session, but vehicle prior to the second, expressed conditioned food cup responses longer after tone offset, when the pellets were previously delivered during conditioning, and maintained shorter latencies to approach the food cup compared to the other groups. The persistence of these conditioned behaviors indicates impairment in extinction consolidation due to SB treatment during the first extinction session. Together, these results demonstrate an important role for orexin signaling during Pavlovian appetitive conditioning and extinction.

1. Introduction

The motivation to seek and consume food is essential for survival. One neural substrate mediating this motivation is the neuropeptide orexin/hypocretin (for reviews, see [1-3]), which is synthesized within the lateral hypothalamus [4,5], a brain region critical for feeding [6,7]. Specifically, orexin-A is important for appetitive motivation [1] and binds to both orexin receptors, orexin 1 (OX1R) and orexin 2 receptors; however, OX1R has a higher affinity for orexin-A than for orexin-B [5,8]. Indeed, manipulations that disrupt OX1R signaling interfere with the consumption of standard chow [9-11], as well as binge eating for highly palatable foods [12]. OX1R blockade decreases the motivation to work for and seek high fat food [9,13-15], sucrose [16,17], and saccharin [18]. Similarly, orexin knockout mice consume smaller amounts of sucrose [19] and are less motivated to work for food [15]. These studies clearly demonstrate orexin is necessary for the motivation to obtain food.

However, food consumption is not only driven by internal, physiological signals, but can also be induced by external, environmental signals through associative learning. Cues previously associated with food can later increase the motivation to obtain and consume food independent of physiological hunger across species [20-24]. We recently demonstrated that such non-homeostatic, cue-driven consumption also requires orexin signaling [25]. Additionally, orexin neurons are recruited during late Pavlovian cue-food conditioning when cues reliably signal food delivery [26], and by environmental cues previously

associated with food [9,27,28]. Nevertheless, whether orexin signaling is necessary during the initial formation of cue-food associations remains unknown.

Here, we used Pavlovian appetitive conditioning to examine if orexin mediates the initial cue-food acquisition and the extinction of these associations. Employing a pharmacological approach, we systemically blocked OX1Rs with the selective antagonist SB-334867 (SB) during the two initial sessions of either acquisition or extinction in two separate experiments. Using a crossover design, we monitored learning in subjects that received either vehicle or SB prior to one or both sessions. This approach allowed assessment of the role of orexin during various phases of learning – the initial acquisition, the consolidation phase, and the recall of the memory. Furthermore, acquisition and extinction are expressed through different behaviors, an increase in responding to a reward and a decrease in responding in the absence of a reward, respectively. Thus, examination of both types of learning allowed for an assessment of orexin signaling function in learning independent of the direction of the behavior and whether the reward was present or not.

2. Materials and Methods

2.1. Subjects

Sixty-four, experimentally naïve, male Long-Evans rats (300-325 g) obtained from Charles Rivers Laboratories were used. Rats were individually housed and maintained on a 12 h light/dark cycle (lights on at 06:00). Behavioral testing was conducted during the light phase between 09:00 and 13:00. Rats

were given one week to acclimate to the colony room with *ad libitum* access to water and food (standard laboratory chow) and were handled and weighed daily. All experiments were in accordance with the National Institute of Health *Guidelines for Care and Use of Laboratory Animals* and were approved by the Boston College Institutional Animal Care and Use Committee.

2.2. Apparatus

Habituation, acquisition, and extinction occurred in the same set of identical behavioral chambers (30 x 28 x 30 cm; Coulbourn Instruments, Allentown, PA), located in a room different from the colony housing room. Behavioral chambers were composed of an aluminum top and sides with one side containing a recessed food cup (3.2 x 4.2 cm), a transparent Plexiglas front with a hinge, a transparent Plexiglas back, and a black Plexiglas floor, and were illuminated with a house light (4W). Each chamber was contained in an isolation cubicle (79 x 53 x 53 cm; Coulbourn Instruments, Allentown, PA) composed of monolithic rigid foam walls, which contained a ventilation fan (55 dB). A video camera located on the rear wall of each isolation cubicle recorded subjects' behavior during the sessions. The conditioned stimulus (CS) was a 10 s tone (75 dB, 2 kHz), and the unconditioned stimulus (US) was two food pellets (formula 5TUL, 45 mg: Test Diets, Richmond, IN) delivered into the food cup. A computer located in an adjacent room controlled the stimuli and video cameras (GraphicState 3.0, Coulbourn Instruments, Allentown, PA).

2.3. Drugs

SB-334867 (SB; Tocris Bioscience, Ellisville, MO, USA) was suspended in a solution consisting of 2% dimethylsulfoxide and 10% 2-hydroxypropyl-*b*-cyclodextrin (Sigma-Aldrich, St. Louis, MO, USA) in sterile water. SB was administered via intraperitoneal injection (i.p.) at a volume of 2 ml/kg and concentration of 20 mg/kg. SB or vehicle was given 30 min prior to each acquisition session (Experiment 1) or prior to each extinction session (Experiment 2).

2.4. Experiment 1: Effect of SB on the acquisition of Pavlovian appetitive conditioning

Experimental design is shown in Fig.1A. Rats were food restricted to gradually reach 85% of their *ad libitum* body weight, which was maintained throughout the experiment. Prior to acquisition, rats were given one 30 min habituation session to acclimate them to the behavioral chambers. During that session all subjects had access to 1 g of the food pellets (US) in the food cup to familiarize them with the pellets.

Acquisition training commenced the following day. All groups received two identical acquisition sessions on two separate days. During each 34 min session, rats received eight CS-US pairings, where presentations of the CS were immediately followed with delivery of the US. The inter-trial intervals (ITIs) were of variable duration (2-6 min) and were randomly distributed during the sessions. Thirty minutes prior to each session, rats received an injection (i.p.) of either SB or vehicle in a crossover design resulting in four groups: Vehicle/Vehicle

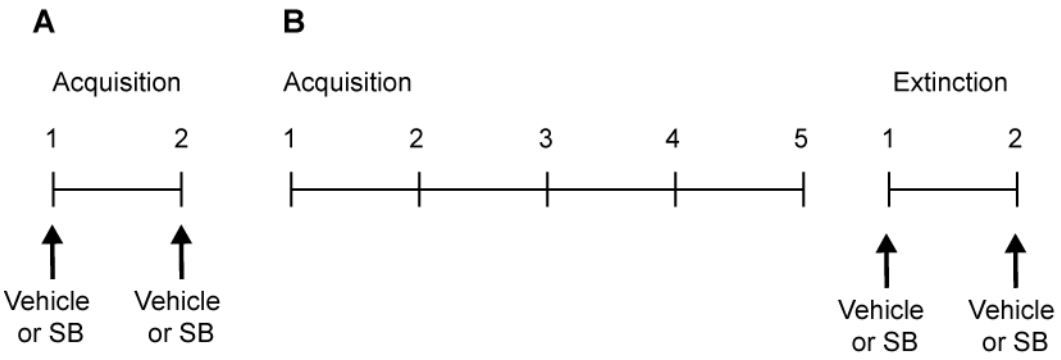

Fig. 1. Experimental design. (**A**) Experiment 1 included two tone-food acquisition sessions, under the orexin 1 receptor antagonist SB-334867 (SB) or vehicle treatment. (**B**) Experiment 2 included five drug-free, tone-food acquisition sessions, and two extinction (tone only) sessions, under SB or vehicle. Arrows indicate administration of either SB or vehicle 30 min prior to the session.

(Veh/Veh), Vehicle/SB (Veh/SB), SB/Vehicle (SB/Veh), and SB/SB (n=8/group). The SB/Veh and Veh/SB groups were included to dissociate the impact SB may have on initial learning and consolidation, from memory recall and expression, respectively. The two acquisition sessions were separated by 48 h to eliminate any potential residual drug effects from the first to the second session.

2.5. Experiment 2: Effect of SB on the extinction of Pavlovian appetitive conditioning

Experimental design is shown in Fig.1B. A separate group of rats underwent the same food restriction and habituation session as described above. Rats then underwent five, drug free, acquisition sessions, each consisting of eight CS-US pairings occurring at random ITIs (2-6 min). Following acquisition, rats received two extinction sessions, each with eight CS-only presentations. Thirty minutes prior to each extinction session rats received an injection (i.p.) of

either SB or vehicle in a crossover design resulting in the same four groups as described for Experiment 1: Veh/Veh, Veh/SB, SB/Veh, and SB/SB (n=8/group). Extinction sessions occurred 48 h apart to eliminate the possibility of residual drug effects.

2.6. Behavioral Observations

Observations were made from recordings of animals' behavior during all acquisition and extinction sessions by trained observers unaware of group allocation. The primary measures of learning were expression of food cup behavior and latency to approach the food cup following CS onset. Food cup behavior was defined as standing in front of and directly facing the food cup or displaying distinct nosepokes into the recessed food cup. Observations of animals' behavior were recorded every 1.25 s during the pre-CS, CS, and post-CS periods. The pre-CS period was the 10 s immediately prior to the onset of the CS, and the post-CS period was the 10 s immediately after the cessation of the CS. The number of food cup responses observed was separately summed for each period (pre-CS, CS, and post-CS), converted to a percentage of total time during each period, and averaged for each trial block (two trials per block) and session, for each group. Latency was the time elapsed from the CS onset until the rat approached the food cup during the 10 s CS and 10 s post-CS. After this time, behavior was considered unspecific to the presentation of the CS, and a maximum latency of 20 s was assigned to any trial in which a response was made later or did not occur. Latency for each CS trial block (two trials per block)

and session was averaged for each group. Additionally, to ensure SB did not impact overall arousal, potentially confounding results, rats' behavior was scored every 15 s during the ITIs for sessions with drug treatment. Recorded behaviors included sitting, sniffing, walking, rearing, grooming, and food cup behavior. The number of times each behavior occurred was summed and converted to a percentage of the total number of observations.

2.6. Data analysis

Behavioral data were analyzed using one-way (Session 1 Treatment; first day of acquisition or extinction) or two-way (Session 1 Treatment by Session 2 Treatment; second day of acquisition or extinction) repeated measures analysis of variance (ANOVA), with CS trial block or pre-CS, CS, and post-CS period as repeated measures where appropriate. *Post hoc t*-Tests were used for any subsequent analyses. SPSS (v.21) software was used for statistical analyses, and the significance value was set at $p < 0.05$.

3. Results

3.1. Experiment 1: Effect of SB on the acquisition of Pavlovian appetitive conditioning

3.1.1. Acquisition 1 Pretreatment with SB did not alter CS-US learning compared to the vehicle group during the first acquisition session (Fig. 2). All rats increased food cup behavior across CS presentations as the session progressed (Fig. 2A), as shown by a group (SB, Veh) by CS repeated measures ANOVA effect of CS

(F (1, 30) = 5.68, p < 0.001). Both groups had similar food cup behavior during the pre-CS, CS, and post-CS periods (ps > 0.05; Fig. 2B).

Similarly, pretreatment with SB did not affect latency to approach the food cup. Both groups had shorter latencies to the food cup as the session progressed (Fig. 2C), as shown by a group by CS repeated measures ANOVA effect of CS (F (1, 30) = 10.88, p < 0.001). Both groups had similar average latency responding (p > 0.05; Fig. 2D).

Additionally, SB treatment did not affect overall arousal, as there were no differences across any behaviors measured during the ITIs, including sitting, sniffing, walking, rearing, grooming, or food cup behavior compared to the vehicle group (all p values > 0.05; Table 1).

3.1.2. Acquisition 2 SB treatment affected conditioned responses during the second session of acquisition. All groups increased food cup responding during each CS across the session (Fig. 3A). An increase in responding to the CS was confirmed with a significant CS effect in a two- way repeated measures ANOVA (F (3, 28) = 12.33, p < 0.001; Fig 3A). However, all groups treated with SB had significantly attenuated food cup behavior compared to the Veh/Veh group, specifically during the CS (Fig. 3B). The ANOVA found a main effect of Acquisition 2 Treatment (F (3, 28) = 4.76, p < 0.05), but no effect for Acquisition 1 Treatment or interaction (ps > 0.05). *Post hoc* analyses revealed groups given SB prior to Acquisition 1 (SB/Veh, SB/SB) and Acquisition 2 (Veh/SB, SB/SB) had lower food cup responding during the CS presentations compared to the

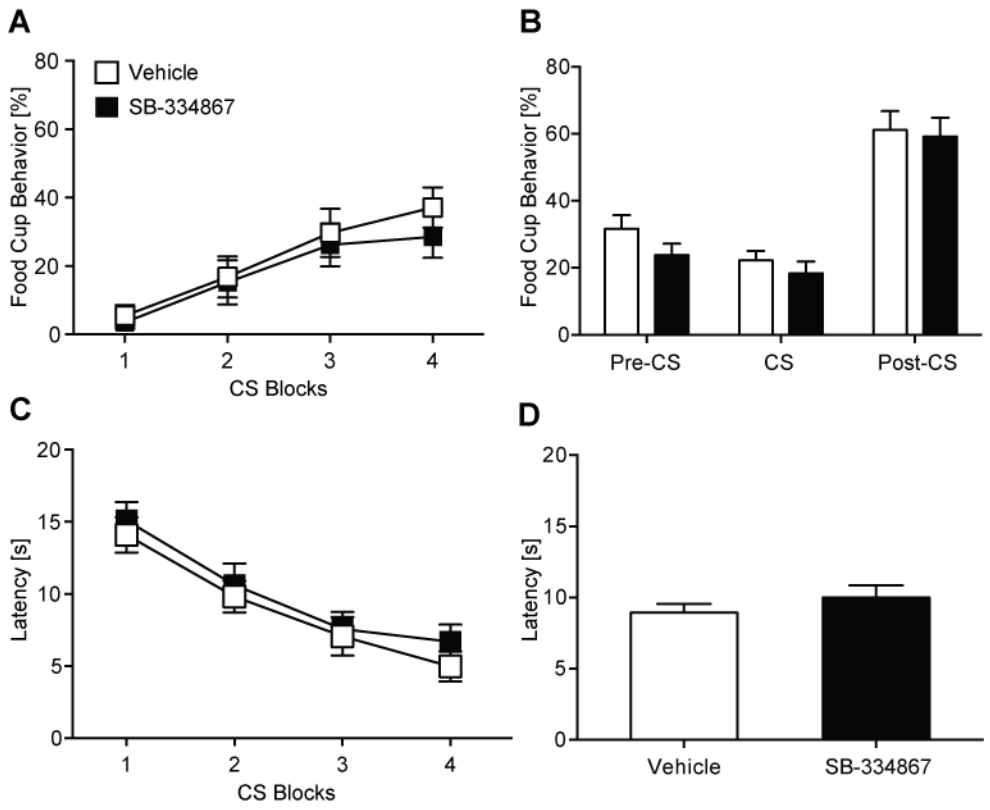

Fig. 2. Conditioned responses during acquisition session 1 in Experiment 1 in SB-334867 or vehicle treated groups. (**A**) Percentage of time (mean ± SEM) rats expressed food cup behavior during CS presentations across the session. Data shown in blocks of 2 trials. (**B**) Average responding (mean ± SEM) during the pre-CS, CS, and post-CS periods during the session. (**C**) Latency (mean ± SEM) to the food cup after CS onset, shown across 2-trial blocks. (**D**) Average latency during the session (mean ± SEM).

Veh/Veh group (*p*s < 0.05; Fig 3B). There were no differences between SB groups (*p*s > 0.05). All groups responded similarly during the pre-CS and post-CS periods (*p*s > 0.05).

All groups significantly decreased latency to approach the food cup as the session progressed (Fig. 3C). The main effect of CS was confirmed by a two-way repeated measures ANOVA (F (3, 28) = 8.42, p < 0.001). However, groups treated with SB had overall greater latencies to approach the food cup compared

to the Veh/Veh group during the session (Fig.3D). The two-way ANOVA found a main effect of Acquisition 1 Treatment (F (3, 28) = 7.79, p < 0.01), an approaching main effect of Acquisition 2 Treatment (F (3, 28) = 3.95, p = 0.057), but no interaction (p > 0.05). *Post hoc* analyses revealed groups given SB prior to Acquisition 1 (SB/Veh, SB/SB) and Acquisition 2 (Veh/SB, SB/SB) were slower to approach the food cup compared to the Veh/Veh group (ps < 0.05). Similar to Acquisition 1, all groups spent similar amounts of time sitting, sniffing, walking, grooming, and expressing food cup behavior during the ITIs (Table 1). A minimal difference was found in rearing between two groups. In the two-way ANOVA, there was a main effect of Acquisition 1 Treatment (F (3, 28) = 4.29, p < 0.05), but no effect of Acquisition 2 Treatment or interaction (ps > 0.05). Further analysis showed the Veh/SB group had less rearing compared to the SB/SB group (p < 0.05) with no other differences between groups (ps > 0.05).

3.2. Experiment 2: Effect of SB on the extinction of Pavlovian appetitive conditioning

A separate cohort of rats underwent five acquisition sessions, drug free. All rats robustly learned the CS-US association across the five sessions of acquisition. These findings were expected given all groups underwent conditioning drug free, and allocated groups are based on drug treatment during extinction. Repeated measures ANOVA confirmed a significant increase in food cup responding, specifically during the CS periods across sessions (F (3, 28) =

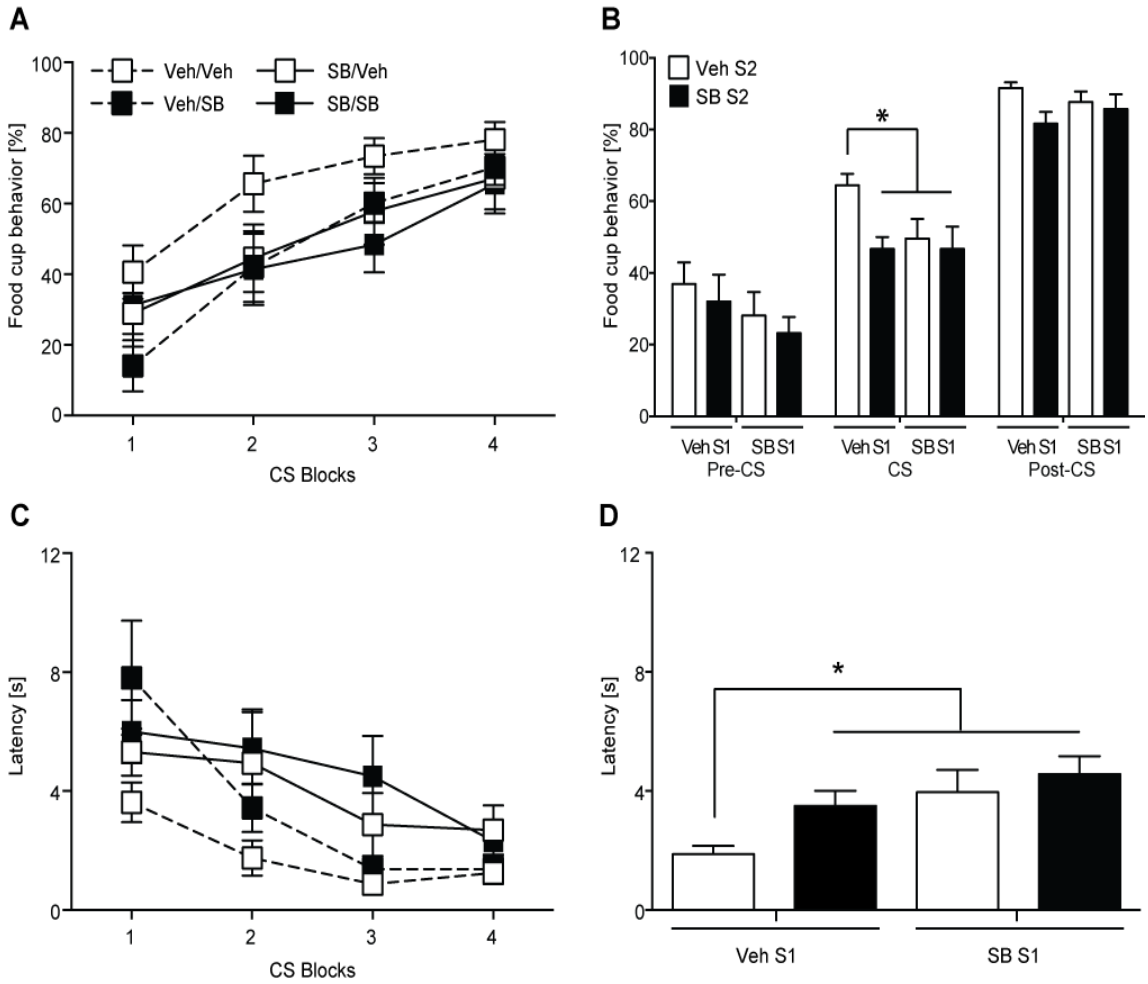

Fig. 3. Conditioned responses during acquisition session 2 in Experiment 1 in SB or vehicle (Veh) treated groups. (**A**) Percentage of time (mean ± SEM) rats expressed food cup behavior during CS presentations across the session. Data shown in blocks of 2 trials. (**B**) Average responding (mean ± SEM) during the pre-CS, CS, and post-CS periods during the session. (**C**) Latency (mean ± SEM) to the food cup after CS onset, shown across 2-trial blocks. (**D**) Average latency during the session (mean ± SEM). S1 and S2 indicate session 1 and session 2 administration, respectively. * indicates $p<0.05$ compared to each group.

90.42, $p < 0.001$; Fig. 4A). All groups had similar responding during the pre-CS, CS, and post-CS periods during the last session (ps > 0.05; Fig 4B).

Additionally, all rats showed a significant decrease in latency across the five sessions (data not shown). A repeated measures ANOVA found a significant

decrease in latency to approach the food cup (F (3, 28) = 146.88, p < 0.001), and all groups had similar latencies to approach the food cup during each session (ps > 0.05).

3.2.1. Extinction 1 Pretreatment with SB did not affect extinction learning compared to the vehicle group during the first extinction session (Fig. 5), evident by a similar decrease in food cup responding during the CS presentations (Fig. 5A). A group by CS repeated measures ANOVA found a significant effect of CS (F (1, 30) = 3.13, p < 0.01). There were no differences between groups during the pre-CS, CS, and post-CS periods (ps > 0.05; Fig. 5B).

Latency to approach the food cup after CS onset increased across Extinction 1 (Fig. 5C), confirming extinction learning. A group by CS repeated measures ANOVA confirmed an effect of CS (F (1, 30) = 2.83, p < 0.01). There were no differences between groups (p > 0.05; Fig. 5D).

Furthermore, SB treatment did not affect any of the behaviors measured during the ITIs compared to the vehicle group (ps > 0.05; Table 2).

3.2.2. Extinction 2 Pretreatment with SB affected the expression of extinction learning during the second extinction session, specifically during the post-CS periods when pellets were previously delivered during conditioning (Fig. 6). A two-way ANOVA confirmed a main effect of Extinction 1 Treatment (F (3, 28) =

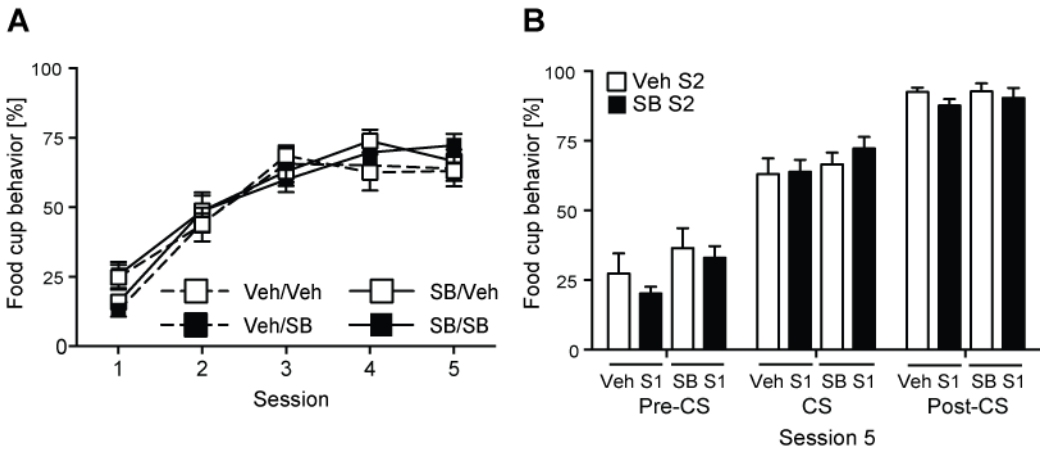

Fig. 4. Conditioned responses during acquisition in Experiment 2. Percentage of time (mean ± SEM) rats expressed food cup behavior. (**A**) Average responding during the CS presentations during each session. (**B**) Average responding (mean ± SEM) during the pre-CS, CS, and post-CS periods for the last acquisition session.

4.93, $p < 0.05$) during the post-CS period, with no effect of Extinction 2Treatment or interaction (*p*s > 0.05). *Post hoc* analyses confirmed the SB/Veh displayed more food cup behavior compared to the Veh/Veh and Veh/SB groups (*p*s < 0.05) and the difference approached significance with the SB/SB group ($p = 0.07$; Fig. 6B). There were no differences between the other three groups during the post-CS periods (*p*s > 0.05), and no differences between any groups during the pre-CS or CS periods (*p*s > 0.05). We also assessed conditioned responding during the first block of the post-CS (Fig. 6A), and a two-way ANOVA confirmed a main effect of Extinction 1 treatment (F (3, 28) = 6.64, $p < 0.05$) and a main effect of Extinction 2 treatment (F (3, 28) = 6.14, $p < 0.05$). *Post hoc* analysis revealed the SB/Veh group had higher responding compared to the Veh/SB ($p < 0.05$) and Veh/Veh ($p < 0.10$) groups, while the Veh/SB group had lower responding than the Veh/Veh group ($p < 0.10$).

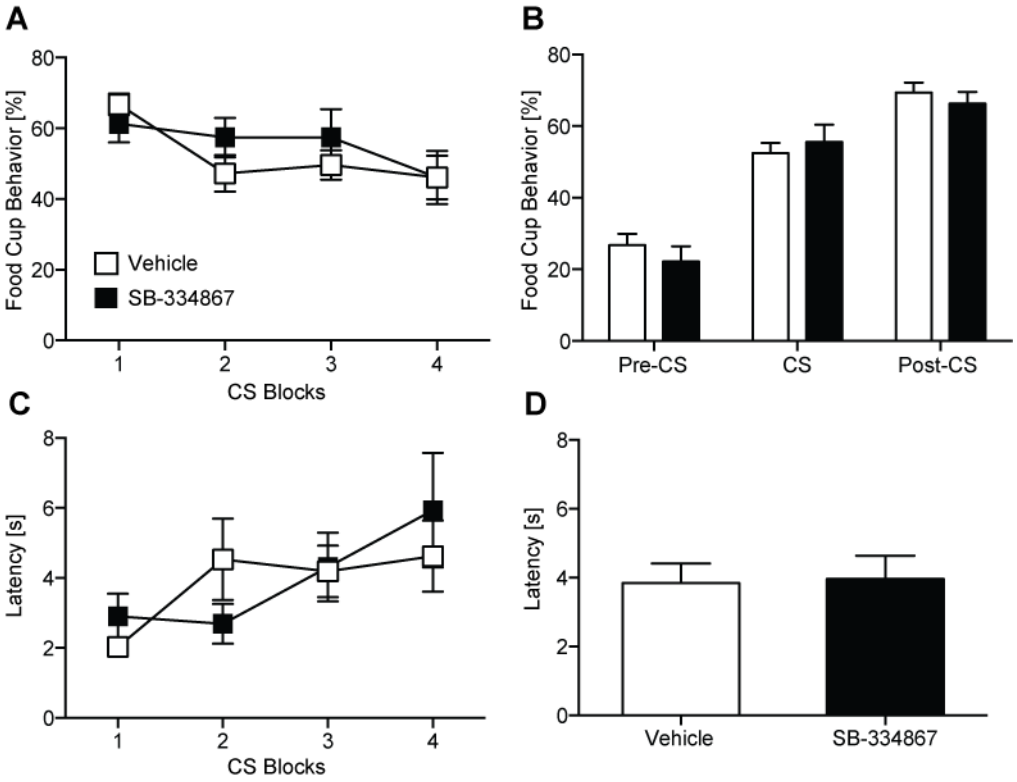

Fig. 5. Conditioned responses during extinction session 1 in Experiment 2 in SB-334867 or vehicle treated groups. (**A**) Percentage of time (mean ± SEM) rats expressed food cup behavior during CS presentations across the session. Data shown in blocks of 2 trials. (**B**) Average responding (mean ± SEM) during the pre-CS, CS, and post-CS periods during the session. (**C**) Latency (mean ± SEM) to the food cup after CS onset, shown across 2-trial blocks. (**D**) Average latency during the session (mean ± SEM).

The SB/Veh group maintained faster latencies to respond to the food cup compared to other groups, which showed evidence of extinction learning with longer latencies (Fig. 6C). A two-way repeated measures ANOVA found a significant effect of CS (F (3,28) = 7.44, $p < 0.05$) and a CS by Extinction 2 Treatment interaction (F (3,28) = 7.24, $p < 0.05$) on latency. *Post hoc* analyses revealed groups given SB prior to Extinction session 2 (Veh/SB and SB/SB) significantly increased their latency during the last two CSs compared to the first

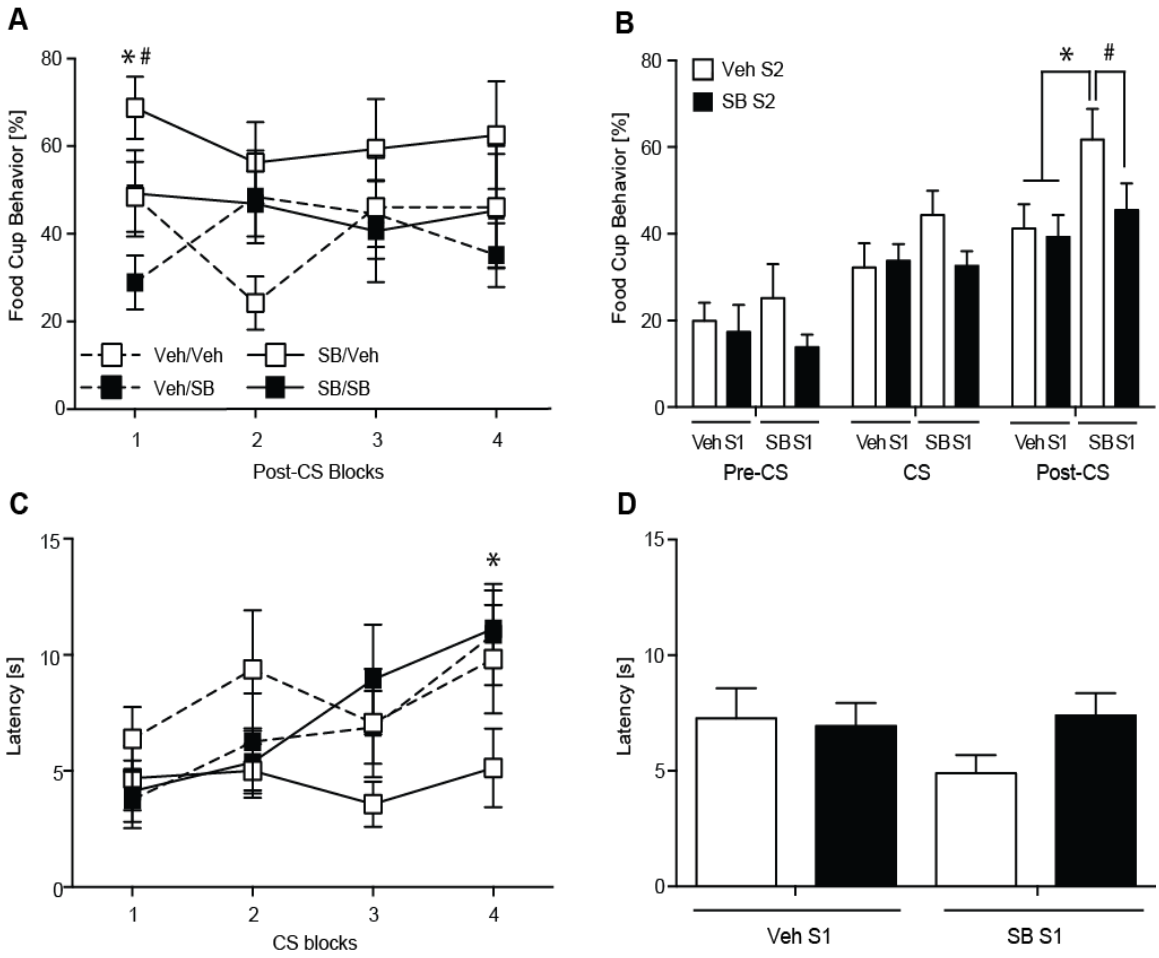

Fig. 6. Conditioned responses during extinction session 2 in Experiment 2 in SB or vehicle (Veh) treated groups. (**A**) Percentage of time (mean ± SEM) rats expressed food cup behavior during post-CS periods across the session. Data shown in blocks of 2 trials. *SB/Veh > Veh/SB, $p<0.05$; #SB/Veh > Veh/Veh > Veh/SB, ps <0.10. (**B**) Average responding (mean ± SEM) during the pre-CS, CS, and post-CS periods during the session. *$p<0.05$; #$p<0.10$ (**C**) Latency (mean ± SEM) to the food cup after CS onset, shown across 2-trial blocks. *SB/Veh < Veh/SB and SB/SB ps<0.05. (**D**) Average latency during the session (mean ± SEM). S1 and S2 indicate session 1 and session 2 administration, respectively.

two CSs (ps < 0.05; Fig. 6C). The groups given vehicle prior to Extinction 2 (SB/Veh and Veh/Veh) maintained similar latency responding across the session (ps > 0.05). Additionally, the SB/Veh group was faster to approach the food cup during the last two CSs compared to the Veh/SB and SB/SB groups (p = 0.05

and $p < 0.05$, respectively) but not the Veh/Veh group ($p > 0.05$). No differences were found between the other three groups during the last two CSs (ps > 0.05), or during the average latency responding (ps > 0.05, Fig. 6D).

All groups expressed similar behavior during the ITIs, except for sniffing (Table 2). A two-way ANOVA found an Extinction 1 Treatment by Extinction 2 Treatment interaction (F (3,28) = 5.35, $p < 0.05$). *Post hoc* analyses revealed the Veh/SB group spent a higher percentage of time sniffing compared to the SB/SB group ($p < 0.05$) and close to significant compared to the Veh/Veh group ($p = 0.06$), but not different from the SB/Veh group ($p > 0.05$).

4. Discussion

The current study found that systemic administration of the OX1R antagonist, SB-334867 (SB), attenuated the acquisition and extinction of Pavlovian appetitive conditioning. In Experiment 1, administration of SB prior to the first session of acquisition had no effect on the expression of learning—food cup behavior or latency to approach the food cup—during that session. However, both measures of learning were attenuated during the second session in groups that received SB prior to either the first or second session of acquisition. In Experiment 2, we found a similar pattern, in that SB had an effect during the second, but not the first, extinction session. Specifically, the group that received SB prior to the first session and vehicle prior to the second session showed impaired extinction during the second session. These results demonstrate orexin signaling via the OX1R mediates the acquisition and extinction of cue-food associations.

The learning impairments observed in the current study were not simply due to non-specific changes caused by SB administration either in locomotor activity or in reduced consumption of the training food pellets. The current study used the highest known dose that does not impair locomotor abilities (20mg/kg), yet is effective in appetitive learning studies (e.g. [25]). Accordingly, the current study found no effects on several measures of locomotor activity, including sitting, sniffing, walking, rearing, grooming and food cup behavior during the ITIs (with two small exceptions, see Results 3.1.2. and 3.2.2. for details). Additionally,

we found that groups pretreated with SB decreased food cup behavior during acquisition, but maintained food cup behavior at the acquired high levels during extinction. This demonstrates the effects of SB were specific to the expression of learning in the direction distinct to the learning paradigm, rather than changes in locomotion or general arousal. Notably, in the extinction sessions the cue is presented without food pellets, and therefore SB administration specifically interfered with the cue-no reward learning. Finally, during the acquisition sessions all rats retrieved and consumed all delivered food pellets, indicating SB did not interfere with food consumption, even though SB has been shown to decrease consumption [10,11].

To our knowledge, this is the first study to demonstrate that orexin signaling is necessary for optimal acquisition of Pavlovian cue-food associations. Our findings suggest that orexin signaling is specifically required for the consolidation and recall of cue-food learning. Orexin blockade during the first acquisition session did not affect conditioned responding within the session, but selectively decreased conditioned responding during the second session when vehicle was administered, suggesting a selective impairment in the consolidation occurred after the first session. SB circulates in the brain and blood for at least 4 hours post-injection [29], indicating a plausible time course to affect consolidation. Impairments in the recall of the acquired learning from the first session were evident by a significant decrease in conditioned responding in the group that received SB during the second session, but received vehicle prior to the first session of acquisition.

The studies here were conducted under food restricted conditions, and the results from Experiment 1 are particularly interesting since all SB groups displayed lower conditioned responding, even though the motivation to seek food should be increased due to food restriction. During periods of hunger, the stomach-derived hormone, ghrelin [30,31] increases the motivation to eat and seek food [32-34] and the neural mechanisms of ghrelin involve action on orexin neurons [32,35,36]. In the current study specific reduction in conditioned responding caused by OX1R blockade may have interfered with the interaction between ghrelin and orexin.

The current results are in agreement with prior appetitive and aversive behavioral studies that demonstrated an important role for orexin in the acquisition of learning. In appetitive tasks, orexin signaling via OX1R was necessary for instrumental learning [15] and taste preference learning [37]. Additionally, the acquisition and recall of conditioned place preference activated orexin neurons and required signaling at the OX1R for other rewards, including morphine [27,38-41] and cocaine [27], but not alcohol [42]. In a spatial learning task, central administration of SB impaired the acquisition, consolidation, and recall of Morris water maze learning [43,44], while orexin-A administration also impaired learning of this task [45]. In aversive paradigms, orexin signaling during fear conditioning and during the consolidation period following training was necessary for successful learning and memory [46-48], and orexin also mediates the fear potentiated startle response [49]. Interestingly, orexin blockade enhanced taste aversion learning [37], and central administration of orexin-A

enhanced within session avoidance learning, the consolidation of avoidance learning, and the retrieval of this learning [50-52].

A prior study [13] interestingly, found no differences in conditioned approach behavior between groups repeatedly administered SB or vehicle across seven sessions of cue-food conditioning. Several methodological differences could explain why the current findings differ, including differences in drug concentration and administration timing, the length of training, and procedural differences. Our study used a slightly higher concentration of SB (20mg/kg versus 15mg/kg), and the drug was administered 30 min (versus 15 min) prior to behavioral training. There were also differences in the training protocol, including the number of training sessions (2 versus 7), the number of CS-US presentations per session (8 versus 30), the CSs (tone versus tone and light), and the behavioral measures of learning (percentage of food cup behavior during the CS and latency versus proportion of nosepokes during the CS relative to total nosepokes during the session). Finally, the crossover design in our study enabled comparisons across four different treatment conditions that revealed specific consolidation and recall effects, which would not be possible to assess with fewer groups.

In addition to the SB effects on acquisition, our findings demonstrated OX1R blockade interfered with appetitive extinction learning. Orexin blockade during the first extinction session did not affect conditioned responding within the session; however, when vehicle was administered prior to the second session,

conditioned responding was maintained at high levels indicating impaired extinction. These findings suggest SB did not impair the initial extinction learning during the first session, but interfered with the consolidation of that learning. There was no overall effect on recall; however, the Veh/SB group had lower responding during the first block of the second extinction session. This transient effect may reflect better recall of extinction learning or may reflect impaired conditioned responding of recall. It is important to note that this impairment in the SB/Veh group cannot be attributed to a state-dependent learning effect, since the Veh/SB group, which was also in a different state from the first session, did not show a similar overall deficit during the second extinction session. Interestingly, the group that received SB prior to both extinction sessions had similar conditioned responding compared to the Veh/Veh group. One interpretation of these results is that the behavior of the SB/SB group reflects the summation of the SB effects on consolidation and on recall, which were in opposite directions - SB/Veh maintained high conditioned responding, while Veh/SB had transient low conditioned responding.

These findings are in agreement with prior evidence for the role of orexin in extinction. Extinction of lever pressing for sucrose was impaired in female rats by OX1R blockade [17]. Activation of orexin neurons (measured by Fos induction) in response to conditioned cues for food [9,16,27,28], or drugs [27,53-55] during tests conducted without rewards might also reflect a function in extinction. Similar to appetitive tasks, orexin manipulations also interfered with extinction in aversive tasks, however the effects observed were opposite. For

example, orexin receptor antagonism facilitated, while orexin-A administration attenuated, fear extinction, and the effects occurred particularly during the consolidation period [46].

Orexin function during learning could reflect its suggested role in mediating motivation and attention towards biologically relevant events [2,3,56-58]. Impaired learning under SB treatment in the current study could therefore reflect a decrease in motivation, attention, or both during learning. Indeed, orexin signaling is necessary for the motivation to initially seek food [13,15-18,59] and drugs ([59-66] for reviews, see [1,3,67]), and the motivation to seek reward during extinction [65,68,69]. In addition, orexin signaling blockade decreased attention during a signal detection task [70]. Attentional processing in associative learning tasks was impaired by unilateral orexin saporin lesions, which destroyed a majority of orexin neurons within the lateral hypothalamus [71]. Furthermore, central administration of orexin-B, which has a lower affinity for orexin 1 receptors than orexin-A [5], enhanced accuracy on an attention task [72].

In the current study orexin signaling at OX1R was blocked systemically, and therefore our results do not indicate the critical neural sites where it acts to mediate the effects observed on appetitive acquisition and extinction. Nevertheless, recent work has identified specific cell groups within the amygdala, the medial prefrontal cortex, and the lateral hypothalamus that are recruited during the acquisition of cue-food associations [26,73]. These regions contain OX1R [74-76] and receive projections from orexin neurons [77] making them

primary regions of interest for orexin signaling during learning. Additional forebrain regions may be important sites for orexin modulation during food intake and learning, including the paraventricular nucleus of the thalamus [25] and hindbrain regions, including the locus coeruleus [47,48] and nucleus of the solitary tract [78]. Neural mechanisms of appetitive extinction have been minimally explored, but based on differences in recall between acquisition and extinction observed here, other regions, in addition to the aforementioned areas, could be critical in appetitive extinction learning (for review, see [79]). For that reason, the recall of extinction may be sufficiently mediated by a brain region without OX1Rs, which would not be affected by SB administration, and would function optimally in the SB treated recall groups, as supported by the current results. Future studies are needed to identify critical neural circuitries where orexin signaling mediates appetitive associative learning and memory.

5. Conclusions

In summary, the current study demonstrated OX1R signaling mediates cue-food acquisition and extinction learning, and may be necessary for optimal consolidation and recall of learning. These findings are important for understanding the mechanisms underlying food cue driven behaviors. Notably, food cues can drive feeding in the absence of physiological hunger, and that overeating depends on orexin [25]. The evidence provided here that orexin is also critical during the initial acquisition and extinction learning of these food cues, conducted under food deprivation, suggests a common mechanism may

mediate the initial encoding and subsequent motivation to overeat in the presence of food cues independent of physiological hunger state.

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Table 1 Locomotor behaviors during acquisition sessions. Values represent percentage (mean ± SEM) of all observed behaviors during the inter-trial intervals for SB-334867 (SB) and vehicle (Veh) treated groups.

	Acquisition 1		Acquisition 2			
	Veh	**SB**	**Veh/Veh**	**Veh/SB**	**SB/Veh**	**SB/SB**
Sitting	10.16±1.9	12.86±3.0	15.17±3.9	19.08±2.9	13.01±4.1	14.48±5.4
Sniffing	21.13±1.5	23.17±2.2	14.77±1.3	22.19±4.5	17.66±2.1	19.78±3.2
Walking	16.16±1.8	18.10±1.8	16.72±3.3	11.67±1.5	19.59±3.3	17.83±2.9
Rearing	9.55±1.1	10.30±1.3	10.13±2.1	7.24±1.0*	12.61±2.4	13.47±2.6*
Grooming	5.37±0.7	4.12±0.7	2.77±0.4	4.58±1.1	1.89±0.4	4.30±1.8
Food Cup	36.46±2.6	29.98±2.9	39.25±2.6	34.50±6.0	33.76±4.5	28.68±3.3

*p<0.05 between groups

Table 2 Locomotor behaviors during extinction sessions. Values represent percentage (mean ± SEM) of all observed behaviors during the inter-trial intervals for SB-334867 (SB) and vehicle (Veh) treated groups.

	Extinction 1		Extinction 2			
	Veh	**SB**	**Veh/Veh**	**Veh/SB**	**SB/Veh**	**SB/SB**
Sitting	29.72±2.9	33.50±4.2	33.53±3.5	33.22±4.6	28.42±4.6	37.09±4.2
Sniffing	20.15±2.9	17.55±1.9	16.76±3.5^	24.64±1.8*^	19.09±3.8	13.70±1.8*
Walking	9.97±1.1	9.58±1.1	8.52±1.0	11.05±0.7	9.38±1.5	8.45±1.4
Rearing	12.08±1.0	9.55±2.4	13.71±2.0	7.72±1.4	10.08±2.5	9.93±2.0
Grooming	3.63±0.5	4.99±0.6	7.03±1.2	5.79±1.2	6.49±1.6	10.56±3.2
Food cup	23.20±2.9	23.70±3.8	19.66±4.0	16.69±3.6	25.57±5.6	18.30±3.1

*p<0.05 between groups

^p=0.06 between groups