The Psychological and Neural Bases of Extinction Learning

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Abstract

The Psychological and Neural Bases of Extinction Learning

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Extinction is a fundamental learning and memory process that enables humans and animals to survive in the face of shifting environmental conditions. The context-specific nature of extinction learning is demonstrated by the renewal phenomenon, in which responding returns following a change in context after extinction. Pavlovian fear conditioning procedures have primarily been used to investigate the psychological and neural processes mediating extinction. However, compared to passive defensive strategies, the mechanisms governing the extinction of active defensive strategies are not well understood. This thesis examined the psychological processes mediating the extinction of both active and passive defensive responses using the shock-probe defensive burying task. We found robust ABA renewal and less marked ABC and AAB renewal of passive coping behaviours. Active coping strategies linked to conditioned defensive burying did not display renewal, indicating that passive coping strategies are more prone to renewal than active coping strategies. These findings have important implications for understanding how context influences the extinction of different defensive responses to aversive stimuli. Moreover, this thesis employed an appetitive Pavlovian conditioning procedure to investigate the neural mechanisms mediating the extinction of responding to a discrete sucrose cue. Using Fos immunohistochemistry and correlational network analysis, we identified the neural correlates and networks associated with the recall vs extinction of responding to a sucrose-predictive Pavlovian cue. Our findings are consistent with those obtained using Pavlovian fear and operant reward-seeking procedures, which have demonstrated a functional dichotomy between the prelimbic (PL) and the infralimbic (IL) cortices of the medial prefrontal cortex. Namely, our results were consistent with the idea that the PL promotes the expression, while the IL mediates the extinction of conditioned responding. Additionally, we found that the paraventricular nucleus of the thalamus (PVT) plays a role in the recall of appetitive Pavlovian responding, and a neural network including the IL and PVT is active during extinction but not recall, suggesting that IL projections to the PVT may be involved in appetitive Pavlovian extinction. In support of this hypothesis, additional experiments found that optical stimulation of the IL-to-PVT pathway completely blocked appetitive Pavlovian renewal, while stimulation of the PL-to-PVT pathway had only modest effects on renewal. In the same experiments, stimulation of the IL-to-PVT, but not the PL-to-PVT, pathway supported self-stimulation, suggesting that this pathway has a reinforcing property. Together, these findings provide novel insights into the neural mechanisms underlying the extinction of responding to appetitive Pavlovian cues, and they point to the PVT as a critical node in the neural circuitry underlying the extinction of appetitive Pavlovian conditioned responding.

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Contribution of Authors

Chapter 2:

- Alexa Brown and Dr. Nadia Chaudhri conceptualized and designed the experiment.
- Alexa Brown collected and analyzed the data, generated the figures, and wrote the manuscript.
- Isabelle Richards and Melissa Martins assisted with data collection.
- Dr. Nadia Chaudhri contributed to the analysis strategy, figure design, and revised the manuscript.
- Dr. C. Andrew Chapman contributed to figure design and revised the manuscript.

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- Alexa Brown and Dr. Nadia Chaudhri conceptualized and designed the experiment.
- Alexa Brown collected and analyzed the data, generated the figures, and wrote the manuscript.
- Ingrid Matei, William Edmiston, and Melissa Martins assisted with histology.
- Dr. Franz Villaruel assisted with the creation of network connectivity graphs and revised the manuscript.
- Dr. Nadia Chaudhri contributed to the analysis strategy.
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- Alexa Brown and Dr. Nadia Chaudhri conceptualized and designed the study.
- Alexa Brown conducted the surgeries, collected and analyzed the data, generated the figures, and wrote the manuscript.
- Dr. Nadia Chaudhri contributed to the analysis strategy and figure design.
- Dr. C. Andrew Chapman contributed to figure design and revised the manuscript.

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Chapter 1: General Introduction

Psychological mechanisms of Pavlovian and operant extinction and renewal

Pavlovian and operant conditioning are fundamental learning processes critical to survival. During Pavlovian conditioning, an animal learns that a discrete conditioned stimulus (CS) predicts the occurrence of an unconditioned stimulus (US), while in operant conditioning, an animal learns that an instrumental response results in an outcome (Fig. 1A). Extinction refers to the learning process that results in a decline in conditioned responding when the expected US or outcome is omitted. Extinction is a fundamental learning process that is conserved across species and is important because it allows a learned behaviour to adjust to changing environmental contingencies. Extinction learning does not erase the original conditioned memory, but rather results in a new learned association that the CS or response no longer results in reinforcement (Bouton, 2002; Bouton et al., 2006). In preclinical research, extinction has predominantly been studied using Pavlovian fear conditioning procedures in which a rat or mouse learns to associate a CS (e.g., a tone) with an aversive US (e.g., footshock), which results in CS-elicited freezing. During extinction, the CS is presented in the absence of the US, and CS-elicited freezing diminishes. Conversely, appetitive extinction has predominantly been studied using operant drug self-administration procedures in which a rat learns to perform an operant response (e.g., lever-press) to receive a drug reward (e.g., cocaine). During extinction, the operant response is not reinforced by the drug reward, which results in a decrease in the lever-pressing response (Bouton et al., 2012; Khoo et al., 2017).

Since Pavlov (1927), it was known that extinguished behaviour can suddenly return with the passage of time, a phenomenon known as "spontaneous recovery." Therefore, the original CS-US (Pavlovian) or response-outcome (operant) association remains intact after extinction even though the learned behaviour has diminished. Research on extinction has grown significantly since then, and we now know that conditioned responding can also return following exposure to the US after extinction, referred to as "reinstatement" and following a change from the extinction context, referred to as "renewal" (Bouton & Bolles, 1979; Rescorla, 2004). These phenomena support the idea that extinction does not depend on the erasure of the original learned association, but rather on the creation of a new inhibitory "CS - no US" (Pavlovian) or "response - no outcome" (operant) association (Bouton, 2002; Bouton et al., 2006, 2021). Since the original conditioned memory is retained following extinction, the CS (Pavlovian) or the response (operant) has two available memories relating to reinforcement after extinction. The context determines which memory is retrieved and expressed. In the extinction context, the inhibitory memory formed during extinction is retrieved and the conditioned response is suppressed. Removal from the extinction context, on the other hand, reduces the retrieval of the inhibitory memory and favours the retrieval of the original conditioned memory, which results in the expression of the conditioned response.

The renewal effect is the return of conditioned responding following a change from the extinction context (Fig. 1B). Context is a term used frequently in preclinical research to describe distal environmental cues such as visual, olfactory, tactile, auditory, and spatial modalities. Changing the distal environmental cues where the training takes place is the main way that context is manipulated. The ABA renewal design is the most common form of renewal (Bouton & Bolles, 1979). In this design, conditioning takes place in context A, extinction occurs in a distinct context B, and, once responding has diminished, animals are returned to context A. A strong restoration of conditioned responding, known as ABA renewal, can occur after returning

to the original training context A. A second renewal design known as ABC renewal occurs in a novel context C after conditioning in context A and extinction in context B. AAB renewal is a third type of renewal design that takes place when conditioning and extinction are carried out in the same context A and renewal occurs in a novel context B. Both ABC and AAB renewal, which take place after the removal from the extinction context without the return to the original training context, are considered evidence in favour of the commonly held theory that extinction creates a new inhibitory memory that is highly context specific.

In operant drug self-administration experiments, ABA renewal has been demonstrated for heroin (Bossert et al., 2004), cocaine (Hamlin et al., 2008), a heroin-cocaine mixture (Crombag & Shaham, 2002), and alcohol (Chaudhri et al., 2008a, 2009; Hamlin et al., 2007; Zironi et al., 2006). Thus, renewal of operant drug-seeking has become an important model for investigating the role of contextual cues in relapse. The ABC and AAB renewal designs are less often used compared to the ABA renewal design, and several studies have been unable to detect either ABC or AAB renewal of operant drug-seeking for heroin, cocaine, a heroin-cocaine mixture, and alcohol (Bossert et al., 2004; Crombag & Shaham, 2002; Fuchs et al., 2005; Zironi et al., 2006). Furthermore, ABA, ABC, and AAB renewal of operant responding have been demonstrated for natural rewards, such as food and sucrose (Bouton et al., 2011; Bouton & Schepers, 2015; Nakajima et al., 2000; Todd, 2013; Todd et al., 2012; Trask et al., 2017a; Zironi et al., 2006). Like drug rewards, it appears that AAB renewal of operant responding for natural reinforcers is less robust than ABA renewal (Bouton et al., 2011; Nakajima et al., 2000).

Using Pavlovian conditioning procedures, ABA (Bouton & Bolles, 1979; Bouton & King, 1983; Bouton & Peck, 1989), ABC (Bouton & Bolles, 1979; Bouton & Brooks, 1993), and AAB renewal (Bouton & Ricker, 1994) of conditioned fear have been observed. Although less often studied, ABA renewal of Pavlovian responding for appetitive reinforcement has also been reported for alcohol and sucrose (Chaudhri et al., 2008b, 2010, 2013; Khoo et al., 2020; Sciascia et al., 2014). In appetitive Pavlovian learning designs, an animal learns to associate a CS (e.g., a tone) with an appetitive US (e.g., sucrose), which results in CS-elicited port entries. During extinction, the CS is presented in the absence of the US, and CS-elicited responding diminishes. In both appetitive and aversive Pavlovian conditioning procedures, ABA renewal has been found to be a more robust effect than ABC and AAB renewal (Bouton & King, 1983; Khoo et al., 2020; Tamai & Nakajima, 2000).

Although Pavlovian fear conditioning procedures have been used extensively to study the psychological and neural mechanisms mediating the extinction of passive freezing (Bouton et al., 2021; Maren, 2001), little is known about the psychological processes underlying the extinction of active types of avoidance. In nature, rodents have a natural, unlearned, tendency to actively avoid noxious stimuli in their environment (De Boer & Koolhaas, 2003). Active avoidance is often investigated in preclinical research using a combination of Pavlovian and operant learning procedures. The animal initially learns that a Pavlovian CS (e.g., a tone) predicts an aversive US (e.g., shock). The animal then learns that an operant response, such as pressing a lever, shuttling to a separate compartment of the conditioning chamber, or stepping onto a platform, can prevent (rather than produce) the presentation of the aversive stimulus. ABA, ABC, and AAB renewal have been reported using the signaled shuttle box task (Nakajima, 2014). In this task, rats are conditioned to cross a shuttle box's midline when an aversive CS is presented to avoid being shocked. Then, crossings decrease once shocks are no longer delivered during extinction. When rats are removed from the extinction context, renewal occurs.

The inability to directly compare active and passive avoidance responses is a limitation of commonly used aversive conditioning tasks, such as Pavlovian fear conditioning and the signaled shuttle box task. The shock-probe defensive burying (SPDB) task offers the opportunity to measure and directly compare the extents of active and passive defensive responses. In the SPDB task, rats are conditioned in a chamber with an electrified shock-probe protruding from one of the chamber walls. Deliberate contact with the shock-probe results in shock, which elicits an array of passive and active defensive behaviours. Rats can remain immobile or passively avoid the side of the chamber containing the shock-probe and avoid contact with the probe. Moreover, they can engage in active defensive burying, a species-typical response in which rats use their paws or snouts to push bedding from the chamber floor onto the shock-probe to avoid shock. The shock-probe is unarmed and ceases to deliver shocks during extinction, and both passive and active defensive behaviours decrease. Chapter 2 of this thesis assessed whether renewal is differentially expressed for passive vs active coping strategies in the SPDB task. In addition, ABA, ABC, and AAB renewal were directly compared to assess the role of context in mediating the extinction of defensive responses in the SPDB task.

Neural mechanisms mediating aversive Pavlovian extinction

The brain areas and pathways implicated in the recall and extinction of Pavlovian fear conditioning have been delineated (Fig. 1C). The amygdala, which regulates the acquisition, expression, and extinction of conditioned fear, is a crucial node in this neural circuitry (Herry et al., 2010; Maren & Quirk, 2004; Myers & Davis, 2002; Orsini & Maren, 2012; Pape & Pare, 2010; Quirk & Mueller, 2008). Infusion of an N-methyl-D-aspartate (NMDA) glutamate receptor antagonist into the amygdala impairs fear extinction learning (Falls et al., 1992). More specifically, infusion of an NMDA receptor antagonist into the basolateral amygdala (BLA), but not the central amygdala (CEA), disrupts fear extinction learning (Zimmerman & Maren, 2010). Together, these results suggest that NMDA receptor activation in the BLA is necessary for extinction learning using a Pavlovian fear conditioning procedure.

The BLA is important for fear extinction learning, but it is not implicated in the consolidation of fear extinction memories (Laurent & Westbrook, 2008). The infralimbic cortex (IL), often also referred to as the ventromedial prefrontal cortex (vmPFC) in rodents, plays a key role in the consolidation of fear extinction memories. In support of this idea, neural activity in the IL is increased during fear extinction retrieval (Knapska & Maren, 2009; Orsini et al., 2011, 2013). Moreover, stimulation of the IL enhances fear extinction learning and retrieval (Adhikari et al., 2015; Do-Monte et al., 2015a; Milad et al., 2004; Vidal-Gonzalaz et al., 2006). Conversely, inhibition of the IL impairs extinction memory consolidation (Burgos-Robles et al., 2007; Do-Monte et al., 2015a; Laurent & Westbrook, 2008; Sierra-Mercado et al., 2011; Sotres-Bayon et al., 2009). Similarly, lesions of the IL potentiate freezing during extinction retrieval (Farrell et al., 2010; Lebrón et al., 2004). Interestingly, lesions encompassing both the IL and the prelimbic cortex (PL), also commonly referred to as the dorsomedial prefrontal cortex (dmPFC) in rodents, does not affect the retrieval of fear extinction memories (Garcia et al., 2006). Specific inactivation of the PL disrupts the expression of conditioned fear but does not affect extinction learning or retrieval (Choi et al., 2010; Laurent & Westbrook, 2009; Sierra-Mercado et al., 2011). Together, these findings have led to the popular hypothesis that within the medial prefrontal cortex (mPFC), the PL functions to drive the expression of conditioned fear while the IL mediates fear extinction (Peters et al., 2009).

The functional dichotomy between the PL and IL in mediating the expression and extinction of fear, respectively, is thought to be maintained through distinct projections to the BLA. The PL and IL both project to the BLA (Vertes, 2004). Moreover, Fos expression, a metric of neural activity, is elevated in PL inputs to the BLA during the retrieval of fear conditioned memories (Quiñones-Laracuente et al., 2021). Consistently, inhibition of the PL-to-BLA pathway impairs the retrieval of fear conditioned memories, supporting a role of this neural pathway in promoting the expression of conditioned fear (Do-Monte et al., 2015b). On the other hand, IL neurons that project to the BLA show increased Fos expression during fear extinction retrieval (Orsini et al., 2011). Consistently, activation of IL inputs to the amygdala enhances fear extinction learning and retrieval, while inhibition of the IL-to-BLA pathway impairs fear extinction learning and retrieval (Adhikari et al., 2015; Bloodgood et al., 2018; Bukalo et al., 2015). Together, these results suggest that PL inputs to the BLA promote the expression of conditioned fear, while IL inputs to the BLA are critical for the learning and consolidation of fear extinction memories.

The paraventricular nucleus of the thalamus (PVT), a midline thalamic nucleus, has also been implicated in Pavlovian fear conditioning, and may act as a key node in cortico-amygdalar networks (Do-Monte et al., 2015b). The PVT is a heterogenous brain region, and recent evidence has uncovered functionally distinct neuronal subtypes present along the anteroposterior axis of the PVT (Gao et al., 2020). Galanin is expressed in the anterior PVT (aPVT) but not in the posterior PVT (pPVT), and the DRD2 gene which encodes dopamine D_2 receptors is expressed in the pPVT but not the aPVT (Gao et al., 2020). These distinct neuronal subtypes are referred to as: type I neurons that are contained mainly in the pPVT, and type II neurons, which are predominantly expressed in aPVT. The middle PVT (mPVT) contains comparable proportions of both types of neurons. The PL and IL both send dense glutamatergic projections to the PVT, but the PL projects primarily to the pPVT, while the IL predominantly projects to the aPVT (Gao et al., 2020; Li & Kirouac, 2012; Vertes, 2004). Both the aPVT and pPVT send projections to the BLA, but the pPVT does so more densely (Vertes & Hoover, 2008). Fos expression in the pPVT, and in PL inputs to the middle-to-posterior PVT, is increased during the retrieval of fear conditioned memories (Do-Monte et al., 2015b; Quiñones-Laracuente et al., 2021). Consistently, inhibition of PL inputs to the middle-to-posterior PVT disrupts the retrieval of conditioned fear (Do-Monte et al., 2015b). These results are consistent with the role of the PL in promoting the expression of conditioned fear and suggests that the role of the PL is maintained through its projections to the pPVT. Chemogenetic inhibition of pPVT inputs to the amygdala also disrupts fear conditioning (Penzo et al., 2015). Therefore, cortico-thalamic-amygdalar connectivity including the PL, pPVT and amygdala may play a role in Pavlovian fear conditioning and the retrieval of fear conditioned memories. Conversely, inhibition of IL inputs to the middle-to-posterior PVT impairs fear extinction retrieval, suggesting that the role of the IL in mediating extinction retrieval is maintained through its projections to the pPVT (Tao et al., 2021). Moreover, inhibition of pPVT inputs to the amygdala also disrupts fear extinction retrieval (Tao et al., 2021). These findings suggest that fear extinction retrieval may recruit a cortico-thalamic-amygdala circuit composed of the IL, pPVT, and amygdala. Altogether, these results support a functional dichotomy within the mPFC in which the PL promotes the expression of conditioned fear, while the IL mediates the extinction of conditioned fear, and this functional distinction is maintained through distinct projections to both the BLA and pPVT. Moreover, the pPVT appears to be a critical node in the cortico-amygdala circuity mediating Pavlovian fear conditioning and extinction.

Neural mechanisms mediating appetitive operant extinction

Models of drug relapse predominate in the research of operant extinction and have provided significant insights into the neural mechanisms mediating the recall and extinction of operant drug-seeking (Fig. 1D). These operant models of drug-seeking also seem to be influenced by the opposing roles of the PL and IL in regulating the expression and extinction of conditioned responding, respectively (Peters et al., 2008, 2009). Inactivation of the PL disrupts the reinstatement and renewal of operant cocaine-, methamphetamine-, heroin-, alcohol-, and food-seeking, suggesting a role of the PL in promoting the expression of operant responding for appetitive reinforcement (Calu et al., 2013; Capriles et al., 2003; Eddy et al., 2016; Fuchs et al., 2005; McFarland & Kalivas, 2001; McLaughlin & See, 2003; Rocha & Kalivas, 2010; Rogers et al., 2008; Willcocks & McNally, 2013). Conversely, chemogenetic or optogenetic stimulation of the IL attenuates the reinstatement of operant cocaine-seeking (Augur et al., 2016; Müller Ewald et al., 2019). Similarly, infusion of an α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) glutamate receptor agonist in the IL disrupts the reinstatement of operant cocaine-seeking (LaLumiere et al., 2012; Peters et al., 2008). Consistently, inactivation of the IL impairs extinction learning and retrieval of operant cocaine- and food-seeking (Eddy et al., 2016; Gutman et al., 2017), and potentiates the reinstatement of operant cocaine-seeking (Peters et al., 2008). Together, these results support a functional distinction in the mPFC, where the PL drives the expression of operant reward-seeking, and the IL mediates the extinction of operant reward-seeking.

The PL and IL are thought to exert opposing functions through downstream projections to the nucleus accumbens (NAc), which is a functionally heterogeneous brain area that is highly linked to operant responding and reward (Carelli, 2002, 2004). Specifically, the NAc core (NAcC) is thought to promote operant reward-seeking, while the NAc shell (NAcSh) mediates the extinction of operant reward-seeking (Peters et al., 2009). In support of this idea, inactivation of the NAcC attenuates the reinstatement and renewal of operant cocaine-, methamphetamine-, and alcohol-seeking, suggesting a role of the NAcC in promoting drug-seeking after extinction (Chaudhri et al., 2008; Fuchs et al., 2008; McFarland & Kalivas, 2001; Rocha & Kalivas, 2010; Rogers et al., 2008). Similarly, injection of a dopamine D_1 receptor antagonist in the NAcC attenuates ABA renewal of operant alcohol-seeking (Chaudhri et al., 2009). Conversely, stimulation of the NAcSh attenuates the reinstatement of sucrose- and cocaine-seeking, suggesting a role of the NAcSh in promoting appetitive operant extinction retrieval (Guercio et al., 2015; Vassoler et al., 2008). Consistently, inactivation of the NAcSh disrupts extinction expression and promotes the reinstatement of operant cocaine- and alcohol-seeking (Chaudhri et al., 2008; Fuchs et al., 2008; Millan et al., 2010; Peters et al., 2008). These findings collectively provide evidence for functional heterogeneity in the NAc subregions, where the NAcC and NAcSh, respectively, enhance the expression and extinction of operant reward-seeking.

This functional dichotomy between the NAcC and NAcSh may result from distinct projections from the mPFC. Both the PL and IL project to the NAcC and the NAcSh (Vertes, 2004), but PL projections to the NAcC are thought to promote appetitive operant conditioned responding while IL projections to the NAcSh mediate appetitive operant extinction (Peters et al., 2009). In support of this hypothesis, optogenetic inhibition of the PL-to-NAcC pathway attenuates the reinstatement of operant cocaine-seeking (Stefanik et al., 2013, 2016). Conversely, chemogenetic excitation of the IL-to-NAcSh pathway attenuates the reinstatement of operant cocaine-seeking (Augur et al., 2016). Similarly, simultaneous unilateral inactivation of the IL and NAcSh promotes the reinstatement of operant cocaine-seeking (Peters et al., 2008).

Interestingly, inhibition of the IL-to-NAcSh pathway *attenuates* ABA renewal of heroin-seeking, suggesting that different neural mechanisms may mediate heroin vs cocaine-seeking (Bossert et al., 2012). Together, these results suggest that separable cortico-striatal projections, namely the PL-to-NAcC and the IL-to-NAcSh, mediate the expression and extinction of operant drug-seeking, respectively. However, there is some evidence that suggests responding for heroin vs cocaine is mediated by distinct neural pathways.

In addition to the mPFC and the NAc, there is evidence that the BLA is implicated in appetitive operant conditioning and extinction. In the BLA, Fos expression is increased during ABA renewal of operant alcohol-, cocaine-, and sucrose-seeking, suggesting that neural activity in the BLA is associated with the expression of operant reward-seeking (Hamlin et al., 2006, 2007, 2008; Marinelli et al., 2007). Consistently, inactivation of the BLA attenuates ABA renewal of cocaine- and alcohol-seeking (Fuchs et al., 2005; Marinelli et al., 2010). Evidence suggests that projections from the prefrontal cortex to the BLA mediate the expression of operant reward-seeking. Inactivation of projections from both the PL and the orbitofrontal cortex (OFC) to the BLA attenuates ABA renewal of operant cocaine-seeking (Fuchs et al., 2007; Lasseter et al., 2011). Interestingly, the BLA, and its efferent projections to the NAc, are also implicated in appetitive operant extinction. Inactivation of the BLA impairs extinction learning in animals trained to lever-press for food (McLaughlin & Floresco, 2007). Similarly, inactivation of the BLA-to-NAcSh pathway disrupts extinction expression and promotes the renewal of operant alcohol-seeking (Millan & McNally, 2011). Together, these findings imply that, depending on the activity in distinct sets of afferent and efferent projections, the BLA may play a role in both the expression and extinction of operant reward-seeking.

The PVT also appears to be a key node in the neural circuitry driving the expression of operant drug-seeking. Neurons along the anteroposterior axis of the PVT are activated during the reinstatement and renewal of operant cocaine- and alcohol-seeking (Dayas et al., 2008; Hamlin et al., 2009; James et al., 2011; Perry & McNally, 2013). Consistently, lesions or inactivation of the middle-to-posterior PVT attenuates the reinstatement of cocaine-seeking and ABA renewal of alcohol-seeking (Hamlin et al., 2009; James et al., 2010; Matzeu et al., 2015). Interestingly, when the pPVT is pharmacologically or chemogenetically inactivated, heroin-seeking is unaffected; yet, when the pPVT is chemogenetically activated, chronic food restriction-induced heroin-seeking is suppressed (Chisolhm et al., 2020). These conflicting and even contradictory behavioural outcomes raise questions about how precisely the PVT affects reward processing.

The IL projects mainly to the aPVT while the PL primarily projects to the pPVT, and the NAcC receives more dense projections from the aPVT than pPVT, while the NAcSh is similarly innervated by both the aPVT and pPVT (Vertes, 2004; Vertes & Hoover, 2008). Thus, the PVT is well situated to act as a key node in the cortico-striatal circuity regulating the expression and extinction of operant drug-seeking. Consistent with the role of the PL in promoting the expression of operant drug-seeking, inhibition of PL inputs to the middle-to-posterior PVT attenuates the reinstatement of operant cocaine-seeking (Giannotti et al., 2018). Interestingly, inhibition of PL inputs to the middle-to-posterior PVT has no effect on the reinstatement of operant sucrose-seeking, suggesting that PL inputs to the PVT drive the expression of drug, but not natural, reward-seeking (Giannotti et al., 2018). Downstream projections from the PVT along the anteroposterior axis to the NAcSh have been shown, using a combination of retrograde tracing and Fos immunohistochemistry, to be active during ABA renewal of alcohol-seeking (Hamlin et al., 2009). The role of the IL-to-PVT pathway in appetitive extinction learning and retrieval has not yet been studied. Additionally, it is unknown whether activation of PL and IL

inputs to the PVT is sufficient to increase and decrease appetitive Pavlovian conditioned responding, respectively. The experiments provided in Chapter 4 of this thesis, however, offer a basis for future studies to pursue this question.

Neural mechanisms mediating appetitive Pavlovian extinction

In comparison to the extinction of appetitive operant and aversive Pavlovian responding, much less is known about the neural mechanisms mediating the extinction of appetitive Pavlovian responding. This led to the central question posed in Chapter 3 of this thesis: What are the neural correlates and networks associated with the recall and extinction of appetitive Pavlovian conditioned responding?

The IL is a common node in the neural circuitry mediating the extinction of appetitive operant and aversive Pavlovian conditioned responding, and there is some evidence to support the role of the IL in mediating the extinction of appetitive Pavlovian responding. Optogenetic stimulation of the IL suppresses ABA renewal of responding to a discrete sucrose-predictive CS (Villaruel et al., 2018). Consistently, lesions or inactivation of the IL disrupt extinction retrieval in rats trained in a Pavlovian task with food or sucrose reinforcement and potentiate ABA renewal of CS-elicited responding for food (Lay et al., 2019; Rhodes & Killcross, 2004, 2007). However, inconsistent results also show that inactivation of the IL *facilitates* the extinction of appetitive Pavlovian conditioned responding for sucrose (Lay et al., 2019; Mendoza et al., 2015). Recent evidence from our laboratory has also shown that optogenetic stimulation of the IL-to-NAcSh pathway attenuates ABA renewal of appetitive Pavlovian responding, although *not* through an extinction mechanism (Villaruel et al., 2022). Together, these results suggest that the IL may mediate appetitive Pavlovian extinction, but the role of the NAcSh is unclear because of inconsistencies in the available evidence.

The PVT may also play a role in mediating the extinction of responding to appetitive Pavlovian cues. The aPVT is activated by Pavlovian cues that predict rewarding conditions (Igelstrom et al., 2010). Moreover, the aPVT, but no the pPVT, is activated by Pavlovian cues that have gained incentive saliency (Flagel et al., 2011). Consistent with a role of the aPVT in mediating appetitive Pavlovian responding, the aPVT, but not the pPVT, is active during ABA renewal of appetitive Pavlovian responding (Anderson & Petrovich, 2017). However, findings have also been shown that are inconsistent with the idea that the aPVT, but not the pPVT, mediates appetitive Pavlovian responding. Yager and colleagues (2015) found that the pPVT is activated by incentive stimuli previously paired with reward. Moreover, Haight and colleagues (2017) found dissociable roles of the aPVT and pPVT, showing that pPVT, but not aPVT, projections to the NAc are activated by cues that have gained incentive saliency (Haight et al., 2017). Similarly, calcium signalling in the pPVT, but not the aPVT, predicts responding to a sucrose-predictive CS (Choi et al., 2019). In addition, the mPVT is activated by reward predictive cues, and inhibition of the mPVT disrupts appetitive Pavlovian conditioning and extinction (Zhu et al., 2018). Consistently, activity in the middle-to-posterior PVT is associated with the reinstatement of appetitive Pavlovian responding (Dayas et al., 2008). These inconsistent results regarding the roles of distinct PVT subregions in appetitive Pavlovian responding may be the result of inconsistent boundaries being used to define the different subregions or a lack of targeting specificity. Moreover, it has been shown that the distinct cell populations in the PVT transition gradually along the anterior-posterior gradient rather than abruptly based on specific coordinates, which may also lead to inconsistent results (Gao et al., 2020; McGinty & Otis, 2020). Another hypothesis is that the PVT signals salience rather than

valence, which may also explain why studies have found that the PVT has a role in behaviours that are appetitively motivated along its anteroposterior gradient (Zhu et al., 2018). Experiments in Chapter 3 of this thesis therefore assessed whether the distinct PVT subregions (anterior, middle, posterior) are differentially activated during the recall vs extinction of appetitive Pavlovian conditioned responding to a sucrose-predictive CS.

Sex differences in extinction and renewal

Sex may be an important factor influencing the neural and behavioural mechanisms of extinction. Sex differences have been observed in the extinction of Pavlovian and contextual fear conditioning, as evidenced by lower freezing levels during extinction and greater extinction rates in females compared to males (Daviu et al., 2014; Gupta et al., 2001; Maren et al., 1994). These sex differences may be influenced by hormonal status. Female rats in proestrus show more rapid fear extinction and greater fear extinction retrieval relative to female rats in metestrus or male rats (Chang et al., 2009; Milad et al., 2009).

Few studies have investigated sex differences in the extinction of appetitive conditioned responding. One study found no difference between females and males in the extinction of operant food-seeking (Beatty & O'Briant, 1973), while another found that females respond more than males throughout the extinction of operant cocaine-seeking (Perry et al., 2008). Females have also been shown to extinguish more slowly than males on the first and second day of extinction of CS-elicited responding for sucrose (Hammerslag & Gulley, 2014). Inconsistently, however, others have reported no sex differences in the extinction of responding to a CS that predicts food (Anderson & Petrovich, 2015, 2017).

Sex differences have been observed in ABA renewal of Pavlovian responding for a food reward, as evidenced by a failure to detect renewal in female, but not male, rats (Anderson & Petrovich, 2015, 2017), and there is some evidence to suggest that renewal in female rats may be related to estradiol (Anderson & Petrovich, 2015). Interestingly, sex differences in ABA renewal of CS-elicited responding for food is associated with differential recruitment of the IL, PL, and aPVT in male and female rats (Anderson & Petrovich, 2017). Thus, a pertinent question that is relevant to Chapter 4 of this thesis is: Are there sex differences in the extinction and renewal of responding to a sucrose-predictive CS? Moreover, are there variations between females and males in the sufficiency of IL and PL inputs to the PVT to promote and attenuate ABA renewal of appetitive Pavlovian responding, respectively?

Conclusions

A popular hypothesis is that within the mPFC, the PL and IL have distinct functional roles in promoting the expression and extinction of responding, respectively (Peters et al., 2009). This functional distinction within the mPFC is thought to be maintained through efferent projections to the NAc and BLA. Specifically, the PL-to-NAcC pathway is thought to drive the expression of operant drug-seeking, while the IL-to-NAcSh mediates the extinction of operant drug-seeking (Peters et al., 2009). Conversely, the PL-to-BLA pathway is thought to drive the expression of Pavlovian fear conditioning, whereas the IL-to-BLA pathway mediates the extinction of Pavlovian fear conditioning (Peters et al., 2009). Therefore, the projections originating in the mPFC are thought to diverge to distinct downstream targets in the NAc or BLA based on the valence of the reinforcer. However, it is also evident that the types of learning in both preparations differ. Operant learning procedures have been used predominantly to study appetitive motivation, while Pavlovian learning procedures have been primarily used to study

aversion. Operant and Pavlovian extinction learning processes depend on different psychological mechanisms; Pavlovian extinction is thought to rely on an occasion-setting mechanism, while operant extinction depends on context-response associations (Bouton et al., 2021). Thus, rather than the valence of the reinforcer, the type of learning may instead be important for the recruitment of specific neural circuits in extinction. Very little is known about the neural circuits mediating the extinction of responding to appetitive Pavlovian cues. Therefore, investigating the neural correlates and circuits involved in appetitive Pavlovian extinction may help disambiguate whether the function of different brain regions and pathways implicated in extinction is due to the valence of the reinforcer or the type of learning employed.

In addition, evidence indicates that the PVT receives direct projections from the PL and IL and sends inputs to the NAc and BLA (Vertes, 2004; Vertes & Hoover, 2008), suggesting that the PVT may be an important node in the neural circuitry mediating the expression and extinction of conditioned responding in both appetitive and aversive learning models. Findings support a role of the PVT in responding to both aversive and appetitive cues, as well as reward cues that have gained incentive saliency (Do-Monte et al., 2015b; Flagel et al., 2011; Igelstrom et al., 2010). However, little is known about the role of IL and PL inputs to the PVT in appetitive extinction, and to date, these pathways have not been investigated using appetitive Pavlovian conditioning and extinction procedures.

Overview

In Pavlovian conditioning, extinction refers to the progressive decline in responding to a conditioned stimulus (CS) when it is no longer followed by the expected unconditioned stimulus (US). It is believed that extinction does not erase the original CS-US association, but rather results in the formation of a new inhibitory "CS - no US" association (Bouton, 2002; Bouton et al., 2006, 2021). Support for this idea comes from findings that extinguished responding can return following the passage of time (spontaneous recovery), exposure to the US (reinstatement), or a change in context after extinction (renewal) (Bouton & Bolles, 1979; Rescorla, 2004; Rescorla & Heth, 1975). Since a change from the extinction context can renew extinguished responding, extinction is believed to be highly context-specific (Bouton, 2002; Bouton et al., 2006, 2021). Studies investigating the behavioural and neural mechanisms of extinction have predominantly used aversive Pavlovian conditioning procedures in which a passive freezing response to a CS paired with foot shock is used as a measure of aversive conditioning (Corcoran & Maren, 2001; Hobin et al., 2003, 2006; Knapska & Maren, 2009; Maren, 2001). This model has been widely used to investigate the psychological and neural mechanisms of fear and is considered an important model for understanding anxiety. Rats, however, are known to use both active and passive behavioural coping strategies to environmental threats, and this raises concerns about the ethological validity of typical Pavlovian fear conditioning procedures. Active avoidance is less well understood in the context of renewal, and it is not well understood how these active defensive strategies may interact with mechanisms of extinction learning and renewal. Conversely, the neural mechanisms mediating the extinction of responding for appetitive reinforcement have primarily been studied using operant learning procedures, and little is known about the neural underpinnings of extinction of responding to discrete appetitive Pavlovian cues.

In Chapter 2, one of the behavioural procedures for the thesis, the shock-probe defensive burying (SPDB) task, was used to evaluate the renewal of active and passive coping strategies following extinction to an aversive stimulus. In the SPDB task, rats must deliberately contact an

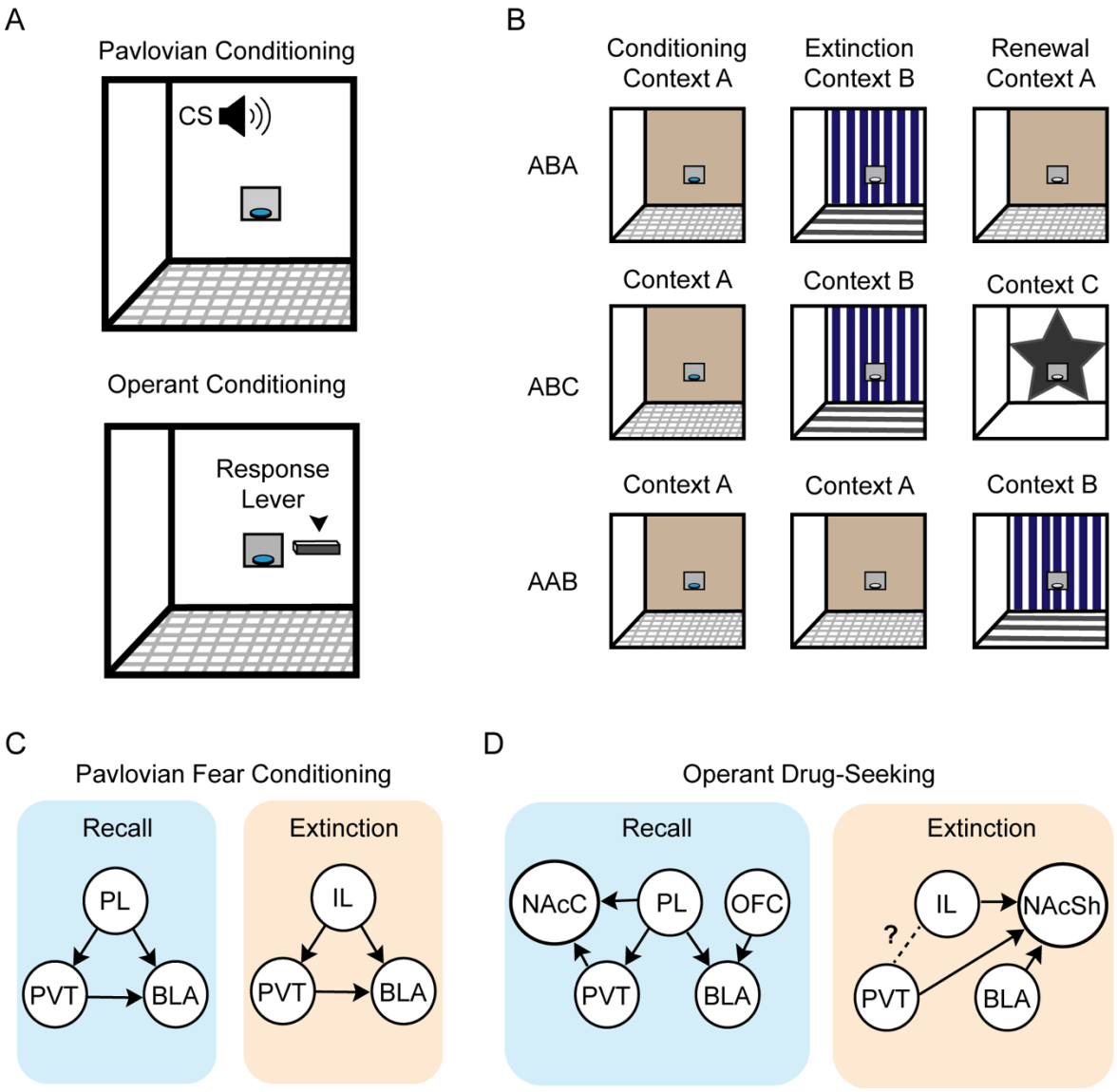
electrified shock-probe to receive a shock during conditioning (Pinel & Treit, 1978). This task allows for a wide array of behavioural responses to be observed, including active defensive burying, in which rats actively bury a noxious stimulus in their environment with available bedding from the chamber floor. In addition, rats decrease their interactions with the shock-probe once they learn that it is electrified and spend increasing amounts of time on the side of the chamber that does not contain the shock-probe, a behaviour termed passive avoidance. Therefore, rats display an array of behavioural responses to the electrified shock-probe including both active and passive coping responses. During extinction, when the shock-probe is no longer electrified and no shocks are delivered, rats demonstrate a reduction in both active defensive burying and passive avoidant coping strategies (Pinel et al., 1985). Using this task, the first objective of this thesis was to investigate whether active defensive burying, as well as passive avoidant responses recorded during the SPDB task, undergo renewal. Also in Chapter 2, the psychological mechanisms of renewal were examined by directly comparing three renewal designs: ABA, ABC and AAB renewal, where consecutive letters refer to the context in which conditioning, extinction and renewal occur. The behavioural data from Chapter 2 generated new information about the capacity of the original training context to renew passive, but not active, coping behaviours using the SPDB task. Moreover, the finding that ABA renewal was more robust compared to ABC and AAB renewal suggests that renewal in this task does not depend only on a change from the extinction context. Instead, this finding is consistent with the interpretation that context A retains a residual excitatory association with the US (shock) that summates with the residual excitatory strength of the CS (shock-probe) at test, and results in greater ABA renewal compared to ABC and AAB renewal (Delamater & Westbrook, 2014; Polack et al., 2013; Rescorla & Wagner, 1972).

In Chapter 3, the involvement of a set of cortical, striatal, thalamic, and amygdalar brain regions in the extinction of appetitive Pavlovian conditioned responding was investigated. Specifically, the extents of neural activation in these regions associated with the extinction of responding to a discrete sucrose-predictive CS were examined using Fos immunohistochemistry. Moreover, the unique networks of correlated neuronal activation in these regions that were associated with extinction to a discrete sucrose CS were explored using a network connectivity analysis approach. Collectively, the results from Chapter 3 demonstrated that the recall of conditioning was associated most strongly with increased neural activity in the prelimbic cortex (PL), nucleus accumbens core (NAcC), and anterior, middle, and posterior paraventricular nucleus of the thalamus (PVT). In contrast, the extinction of responding was associated with sustained neural activity during both recall and extinction in the infralimbic cortex (IL), medial orbitofrontal cortex (mOFC), and nucleus accumbens shell (NAcSh). Interestingly, only the aPVT and mPVT were found to have a special role in the recall of responding to the discrete sucrose CS *paired* with the US (vs *unpaired* with the US). All other areas examined were similarly recruited by the CS and the contextual cues associated with the training chamber as evidenced by similar levels of Fos expression in the paired and unpaired groups. Further, we found a distinct neural network including the IL and mPVT that was active only during extinction of responding to the discrete sucrose CS in the paired group, that was not present during recall. Chapter 3 thus outlined the neural correlates and networks of extinction and recall of conditioned responding to a discrete sucrose cue.

In Chapter 4, the sufficiency of activity in IL and PL inputs to the PVT to inhibit and promote responding to a discrete sucrose CS was examined using an ABA renewal procedure. Using optogenetics, either the IL or PL projections to the PVT were stimulated specifically

during CS presentations in separate groups of rats to test whether this activation was sufficient to reduce or increase renewal of responding. This excitatory circuit-specific optogenetic approach was validated using Fos immunohistochemistry and immunofluorescence. The results suggested that stimulation of IL inputs to the PVT was sufficient to attenuate ABA renewal of responding to a discrete sucrose CS. Though neural activity in the PL and the PVT was found to be associated with the recall of responding to a discrete sucrose CS in Chapter 3, optogenetic stimulation of PL inputs to the PVT was not sufficient to elevate renewal of responding to a discrete sucrose CS. Next, the reinforcing properties of optogenetic stimulation of IL and PL inputs to the PVT were examined using a self-stimulation procedure in the absence of contextual or discrete cues. Rats self-administered optical stimulation of the IL, but not the PL, inputs to the PVT, suggesting that the IL, but not the PL, may project to a distinct cell population within the PVT that is involved in reward.

The Fos immunohistochemical and optogenetic experiments in Chapters 3 and 4 contributed to identifying the neural underpinnings of extinction and renewal of responding to a discrete sucrose CS. Neural activity in the IL was associated with extinction, while neural activities in the PL and PVT were associated with the recall of responding to a discrete sucrose CS. Moreover, neural activities in the IL and PVT were correlated, suggesting that they form a unique neural network that is active during extinction of responding to a discrete sucrose CS. Further, activation of the glutamatergic projection from the IL to the PVT was found to be sufficient to inhibit renewal of responding to a discrete sucrose CS in the original training context. However, activation of the glutamatergic projection from the PL to the PVT is not sufficient for the elevation of renewal of responding to a sucrose CS in the original training context. Collectively, this thesis contributes to the understanding of how context influences the extinction of appetitive Pavlovian conditioned responding and identifies a glutamatergic circuit that underlies the extinction of responding to a discrete sucrose CS.



Chapter 1 Figure 1. Behavioural procedures used to investigate the psychological mechanisms of extinction and renewal, and the neural pathways associated with the recall and extinction of conditioned responding. **A** A schematic diagram illustrating Pavlovian (top panel) and operant (bottom panel) conditioning procedures. In Pavlovian conditioning, a conditioned stimulus (CS; e.g., a tone) predicts the availability of an unconditioned stimulus (US; e.g., sucrose). In operant conditioning, the outcome (e.g., sucrose) is contingent on performing an operant response (e.g., lever-pressing). **B** A schematic diagram illustrating ABA (top panel), ABC (middle panel), and AAB (bottom panel) renewal designs. The letters refer to the context in which conditioning, extinction, and the renewal test occur. Contexts are typically composed of distinct visual, tactile, olfactory, and auditory modalities. **C** Simplified schematic diagrams indicating neural pathways mediating the recall (left panel) and extinction (right panel) of Pavlovian fear conditioning, and **D** operant drug-seeking. BLA: basolateral amygdala; IL: infralimbic cortex; NAcC: nucleus accumbens core; NAcSh: nucleus accumbens shell; OFC: orbitofrontal cortex; PL: prelimbic cortex; PVT: paraventricular nucleus of the thalamus.

Chapter 2: Context-induced renewal of passive but not active coping behaviours in the shock-probe defensive burying task

Introduction

In Pavlovian conditioning, extinction refers to the progressive decline in responding to a conditioned stimulus (CS) that is no longer followed by an unconditioned stimulus (US). Extinction does not erase the original CS-US association, but rather results in new inhibitory learning (Pavlov, 1927). The inhibitory learning that occurs in extinction is thought to be context-specific, since a change in context after extinction can trigger a return of extinguished conditioned responding, termed 'renewal' (Bouton et al., 2006; Bouton & Bolles, 1979; Bouton & King, 1983). Context-dependent renewal can be observed in ABA, ABC and AAB designs, in which consecutive letters refer to the different contextual configurations present during conditioning, extinction, and the renewal test (Bouton & Bolles, 1979; Bouton & Ricker, 1994). Renewal is most robust in the ABA design, in which an increase in conditioned responding occurs upon a return to the original conditioning context (context A) following extinction in a different context (context B) (Bouton & Bolles, 1979; Bouton & King, 1983; Bouton & Peck, 1989). However, renewal can also occur when conditioning, extinction and renewal test are all conducted in distinct contexts (ABC renewal; Bouton & Bolles, 1979; Bouton & Brooks, 1993), or when conditioning and extinction are conducted in the same context and the renewal test is conducted in a different context (AAB renewal; Bouton & Ricker, 1994).

Renewal of aversive conditioned responding has predominantly been investigated using aversive Pavlovian conditioning procedures in which a CS (e.g., a tone) is paired with an aversive US (e.g., footshock), and passive freezing in response to the CS is used as a measure of aversive conditioning (Corcoran & Maren, 2001; Hobin et al., 2003, 2006; Knapska & Maren, 2009; Maren, 2001). The conditioned suppression task has also been used, in which animals first learn to perform an operant response (e.g., lever-press) for appetitive reinforcement (e.g., food pellet), and then a CS (e.g., a tone) is paired with an aversive US (e.g., footshock). Animals learn to suppress ongoing behaviour in response to the CS and this results in a suppression of the food-seeking response, which is used as a measure of aversive conditioning (Bouton & Bolles, 1979; Bouton & Ricker, 1994). In both procedures, the CS-footshock association is extinguished by repeatedly presenting the CS in the absence of shock, which causes responding to diminish. During the renewal test, animals are either returned to the original training context (ABA) or removed from the extinction context (ABC and AAB), which triggers the return of conditioned responding. Similar renewal effects have been observed when comparing ABA with either ABC or AAB renewal (Corcoran & Maren, 2004), although others have found ABC and AAB renewal to be substantially weaker than ABA renewal (Bouton & King, 1983; Tamai & Nakajima, 2000; see also Crombag & Shaham, 2002; Khoo et al., 2020; Nakajima et al., 2000; Zironi et al., 2006, for failures to detect ABC or AAB renewal in other conditioning preparations).

An alternative animal model of aversive conditioning is the shock-probe defensive burying (SPDB) task which can elicit both active and passive conditioned responding (Pinel & Treit, 1978). In this task, rats freely explore a behavioural chamber in which an electrified shock-probe mounted on one of the walls above the surface of the bedding delivers an electric shock upon contact. Rats employ passive coping behaviours to avoid the shock-probe by spending more time away from the shock-probe (passive avoidance), making fewer contacts with the shock-probe, and by remaining immobile (freezing). Rats also display active coping behaviours (De Boer & Koolhaas, 2003; Fucich & Morilak, 2018; Pinel & Treit, 1978) including 'defensive

burying' in the SPDB task, in which the shock-probe is buried by pushing bedding from the floor forward using thrusting movements with the forepaws or snout (Pinel et al., 1980). Rats also use defensive burying for other aversive stimuli including flash bulbs, plastic tubing that delivers a burst of air, and mousetraps (Terlecki et al., 1979). During extinction, when the shock-probe is no longer electrified, rats cease to bury the probe (Pinel et al., 1985). Therefore, the SPDB task can be used to study differences in the acquisition, extinction, and renewal of both passive and active coping behaviours in response to an aversive stimulus.

The SPDB task is thought to be a more ethological model of aversive learning than traditional aversive Pavlovian conditioning procedures in which the shock administered by the experimenter is unavoidable (Rodgers et al., 1997; Treit, Pinel & Fibiger, 1981), and the observation of conditioned responses is usually limited to freezing (Gruene et al., 2015). The SPDB task allows the aversive stimulus to be actively or passively avoided as seen in naturalistic environments (Owings & Cross, 1977), and defensive burying may be analogous to the active burying of entrance holes to underground burrows that wild rats use to avoid predators (De Boer & Koolhaas, 2003). The SPDB task is also not dependent on appetitive motivation, since it does not involve food or drug reinforcement which are present in some aversive conditioning procedures involving approach-avoidance conflict or conditioned suppression (Treit & Pesold, 1990; Treit, Pinel & Fibiger, 1981).

Although the SPDB task allows a more ethological spectrum of passive and active coping responses to be observed, renewal has not been investigated using the SPDB task, and the capacity of different coping responses to return after extinction is unknown. In the present experiment, we used the SPDB task to investigate ABA, ABC and AAB renewal of active and passive coping responses. During conditioning, rats were placed in a distinct context, consisting of tactile, visual, olfactory, and auditory stimuli (context A), and the shock-probe delivered a constant 3-mA shock upon contact. During extinction, rats were either placed in the same context used in training (context A), or in a different context (context B), but contacts with the shock-probe did not result in shock. Renewal in the ABA group was tested in the original training context (context A), and renewal in the ABC group was tested in a novel context (context C). Finally, the AAB group underwent extinction in the same context as training, and renewal was tested in a different context (context B). We predicted the strongest renewal of both active and passive coping responses in the ABA design, and a stronger renewal of passive versus active coping responses in each of the three groups.

Methods

Animals

Subjects were 37 experimentally naïve, male Long-Evans rats (Charles River, St. Constant, Quebec, Canada; 220-240 g upon arrival). Rats were maintained in a climate-controlled (21°C) room on a 12-h light/dark cycle with lights turned on at 7:00 h. All procedures occurred during the light phase. Rats were individually housed in standard cages (44.5 cm × 25.8 cm × 21.7 cm) containing beta chip bedding (Aspen Sani chips; Envigo, Indianapolis IN) with unrestricted access to water and food (Agribands, Charles River). Each cage contained a nylabone toy (Nylabones; Bio-Serv, Flemington, NJ), a polycarbonate tunnel (Rat Retreats, Bio-Serv) and shredded paper for enrichment. During a nine-day acclimation period to the colony room, rats were handled, and body weight was recorded daily. Following acclimation, rats were assigned into one of three groups: ABA, ABC and AAB matched according to body weight. Rats were excluded for failure to extinguish if they spent >65 % of the session length avoiding the

side of the chamber containing the shock-probe during the final extinction session (ABA, n=1; ABC, n=1; AAB, n=2). The final number of rats in each group was 11. All procedures followed the guidelines of the Canadian Council for Animal Care and were approved by the Concordia University Animal Research Ethics Committee.

Apparatus

The SPDB task, adapted from Pinel and Treit (1978), was conducted using three identical Plexiglas chambers (40 cm × 30 cm × 40 cm). Each chamber was encased in a sound-attenuating melamine cubicle. On the left sidewall of the chamber, 5 cm above the floor of the chamber, and 1 cm above the surface of the bedding, was a hole through which the removable shock-probe could be inserted. The shock-probe (12.7 cm in length and .97 cm in diameter) was constructed of ABSplus-P430 thermoplastic using a 3D printer (Stratasys Dimension uPrint). The shock-probe was wrapped with a 18AWG bare, tinned copper bus bar wire (Beldon). Two cameras were used to videotape the sessions for offline behavioural scoring. One camera was mounted to the top of the melamine cubicle pointing downwards, and the second camera was positioned 30 cm from the front of the conditioning chamber.

Three contextual configurations were used which differed in visual, tactile, olfactory, and auditory elements and were in different laboratory rooms. Context 1 consisted of .5 inch black and yellow vertical striped walls, ¼ inch corncob bedding (7097 Teklad; Envigo, Madison, WI), lemon odor, and no background noise. Context 2 consisted of white walls with a large black star centered on each wall, dry cellulose bedding (7070C Teklad Diamond; Envigo), almond odor, and a fan within the melamine cubicle. Context 3 consisted of white walls with small red polka-dots (2 cm in diameter), aspen woodchip bedding (7093 Teklad, Envigo), cedar wood odor, and a fan within the laboratory room. Odors were prepared by diluting lemon oil (W262528-1, Sigma Aldrich), benzaldehyde (B1000, ACP Chemicals) or cedar wood oil (8000-27-9, Fisher Chemicals) with water to make a 10% solution.

Procedure

Prior to each session, a new layer of bedding was added to the chamber floor and was smoothed to a height of 4 cm. The appropriate odor was sprayed (2.6 ml) onto the bedding. Rats were placed in the conditioning chamber on the side of the chamber opposite the shock-probe, facing away from the probe (Pinel & Treit, 1978). Prior to conditioning, all rats received three daily 10 min habituation sessions on consecutive days in the absence of the shock-probe. Rats were habituated once to each contextual configuration and the order of context habituations was counterbalanced across rats.

Rats received three daily 15 min conditioning sessions beginning 24 h after the final habituation session. The shock-probe was present during the conditioning phase and delivered a constant 3 mA shock upon contact. The context used for conditioning was counterbalanced across groups and was referred to as context A.

At 24 h after the final conditioning session, rats received five daily 15 min extinction sessions. Extinction sessions were identical to conditioning sessions except that the shock-probe was not electrified. In the AAB group, extinction occurred in the conditioning context (context A). In the ABA and ABC groups, extinction occurred in a different context from conditioning (context B).

At 24 h after the final extinction session, rats received two counterbalanced test sessions on consecutive days, in which the shock-probe was not electrified. A control test was conducted

on one day, in which rats were tested in the same context as extinction, and the renewal test was conducted on the other day. In the ABA group, the renewal test occurred in the conditioning context (context A), and in the ABC and AAB groups the renewal test occurred in a different context (context C and B, respectively).

Data analysis

Selected behavioural variables (Table 1) were scored from video recordings using Behavioral Observation Research Interactive Software (BORIS) v6.3.1 (Friard & Gamba, 2016) by two experimenters who were blind to the experimental conditions. Inter-rater reliability between the two coders was assessed for all behavioural variables (r=.88 - .92). The acquisition and extinction of conditioned responding was assessed separately using mixed analyses of variance (ANOVA) with group (ABA, ABC or AAB) and session as factors. Renewal of conditioned responding was assessed using a mixed ANOVA with group (ABA, ABC or AAB) and context (renewal or extinction) as factors. Greenhouse-Geisser corrections are reported following violations of Mauchly's test of sphericity. Post-hoc analyses were corrected for multiple comparisons using the Bonferroni adjustment. All data analyses were conducted using IBM SPSS v21.0 (IBM Corp., Armonk, NY). Results were considered statistically significant at $p<.05$.

Variable (measure)	Definition	Presumed psychological process
Passive avoidance (duration)	Time spent on the side of the chamber opposite the shock-probe in the absence of freezing	Passive coping
Freezing/immobility (duration)	The absence of any movements, excluding those required for respiration	Passive coping
Shock-probe contact (frequency, duration, latency)	Direct contact made with the shock-probe	Passive coping
Defensive burying (duration, latency)	Burying the shock-probe using available bedding from the chamber floor	Active coping
Height of bedding (cm)	Height of the peak of accumulated bedding surrounding the shock-probe	Active coping
Shock reactivity (intensity scale)	Reactivity to shock measured using a 4-point scale (*1=flinch involving head or paw, 2=whole-body flinch with or without slow ambulation away from probe, 3=whole-body flinch or jumping followed by immediate ambulation away from probe, 4=whole-body flinch or jumping, accompanied by the rat running to the opposite end of the chamber*) (Shah & Treit, 2004).	Reactivity

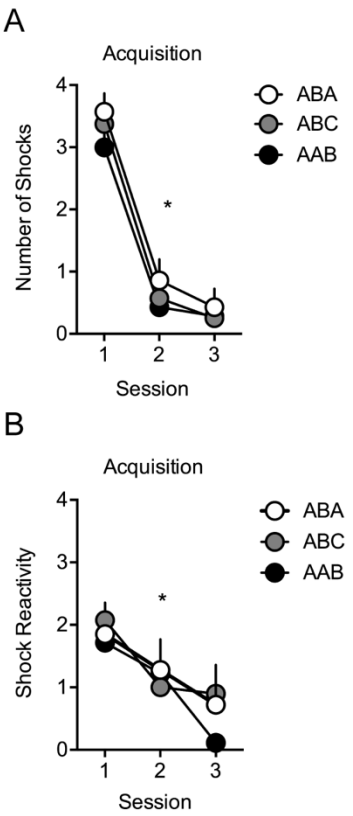
Table 1. Behavioural variables in the shock-probe defensive burying task and associated presumed psychological processes (De Boer & Koolhaas, 2003).

Results

Number of shocks and shock reactivity

When the shock-probe was electrified during conditioning, all groups received a similar number of shocks (Fig. 1A; Group, $F_{2,30}$=2.51, p=.098, η_p^2=.143; Group x Session, $F_{4,60}$=.45, p=.772, η_p^2=.029) and the number of shocks decreased significantly across sessions (Session, $F_{2,60}$=227.46, p<.001, η_p^2=.883). Reactivity to shock was measured using a 4-point scale (1=flinch involving head or paw, 2=whole-body flinch with or without slow ambulation away from probe, 3=whole-body flinch or jumping followed by immediate ambulation away from probe, 4=whole-body flinch or jumping, accompanied by the rat running to the opposite end of the chamber) (Shah and Treit, 2004). Groups showed comparable reactivity to shocks during acquisition (Fig. 1B; Group, $F_{2,30}$=.44, p=.646, η_p^2=.029; Group x Session, $F_{2.9,42.9}$=.62, p=.596, η_p^2=.040), and reactivity decreased significantly across conditioning sessions (Session, $F_{1.4,42.9}$=8.68, p=.002, η_p^2=.224).

After reacting to contacting the armed shock-probe in context A, animals in all groups displayed freezing/immobility. The duration of freezing was low in the first conditioning session (ABA, 6.4 ± 2.9 s; ABC, 2.3 ± .8 s; AAB, 2.6 ± .9 s), and decreased across sessions similarly in all groups (Data not shown; Session, $F_{1.0,31.1}$=10.63, p=.002, η_p^2=.262; Group, $F_{2,30}$=1.61, p=.216, η_p^2=.097; Group x Session, $F_{2.1,32.1}$=1.47, p=.245, η_p^2=.089). Freezing was not observed during extinction or during renewal and extinction tests when the shock-probe was unarmed.

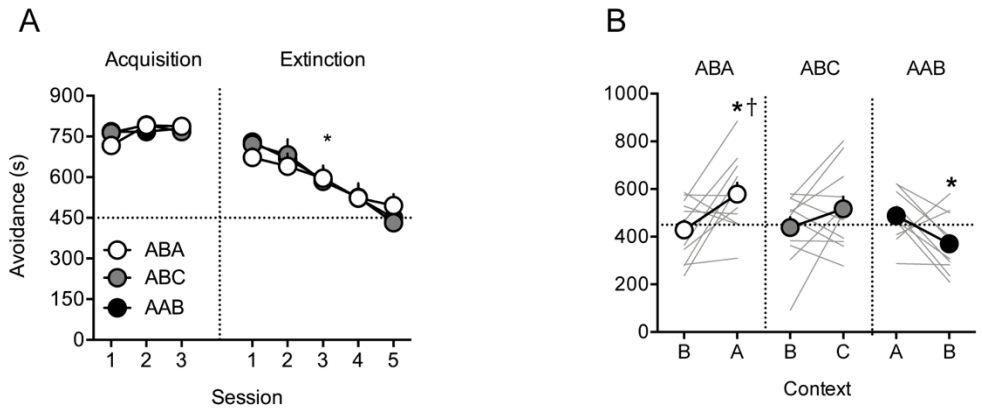


Chapter 2 Figure 1. Average number of shocks and shock reactivity during acquisition in the ABA, ABC and AAB groups. **A** Average number of shocks received during the three conditioning sessions of the acquisition phase. **B** Average shock reactivity scores during the three conditioning sessions. * $p<.05$, main effect of session (A, B). Error bars indicate the standard error. n=11 per group.

Passive avoidance

Acquisition and extinction. Passive avoidance was measured as time spent on the side of the chamber opposite of the shock-probe. Passive avoidance during conditioning was high across groups (Fig. 2A; Group, $F_{2,30}$=.07, p=.937, η_p^2=.004; Session, $F_{2,60}$=2.09, p=.132, η_p^2=.065; Group x Session, $F_{4,60}$=1.07, p=.380, η_p^2=.066), and decreased similarly across groups during extinction (Fig. 2A; Session, $F_{2.7,82.2}$=24.97, p<.001, η_p^2=.454; Group, $F_{2,30}$=.01, p=.989, η_p^2=.001; Group x Session, $F_{5.5,82.2}$=.50, p=.790, η_p^2=.032). Extinction was similar in context A and B when data was collapsed across groups (See Appendix A Fig. 1). Time spent on both sides of the chamber was roughly equivalent at the end of extinction. Therefore, rats in all groups learned to passively avoid the shock-probe during conditioning, and extinguished passive avoidance during extinction.

Renewal test. Renewal of passive avoidance was observed in the ABA group, but not in the ABC or AAB groups (Fig. 2B; Group, $F_{2,30}$=1.40, p=.261, η_p^2=.086; Context, $F_{1,30}$=1.43, p=.242, η_p^2=.045; Group x Context, $F_{2,30}$=6.66, p=.004, η_p^2=.307). Passive avoidance was significantly increased in the renewal context compared to the extinction context in the ABA group (p=.009, η_p^2=.206) but did not reach statistical significance in the ABC group (p=.153, η_p^2=.067). In contrast, passive avoidance was significantly reduced in the renewal context compared to the extinction context in the AAB group (p=.036, η_p^2=.138). Therefore, we observed renewal of passive avoidance in the ABA group, and a suppression of avoidance in the AAB group.



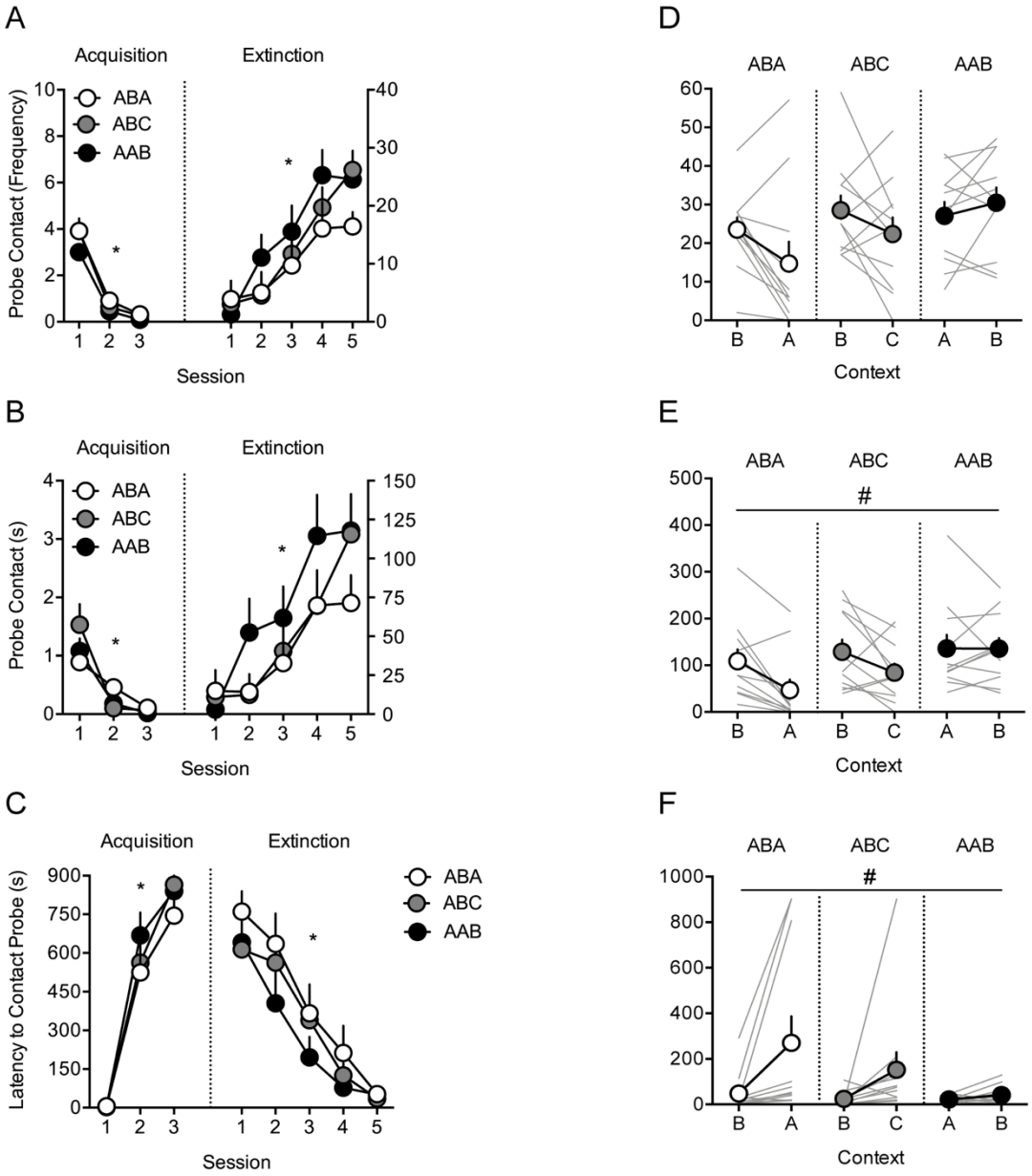
Chapter 1 Figure 2. ABA renewal, but no ABC, and reverse AAB renewal of passive avoidance in rats. The horizontal dotted line represents half of the session length. **A** Acquisition and extinction of avoidance in the ABA, ABC and AAB groups. Data represent time in seconds on the side of the chamber that did not contain the shock-probe. * $p<.05$, main effect of session. **B** Each rat was tested once in the extinction context and once in the renewal context. Passive avoidance was significantly increased in the renewal context compared to the extinction context in the ABA group and was significantly decreased in the renewal context compared to the extinction context in the AAB group. In the ABC group, there was no significant difference between the extinction and renewal contexts. * $p<.05$, extinction vs. renewal context. † $p<.05$, ABA vs. AAB in the renewal context. Error bars indicate the standard error and data shown for each individual rat are overlaid on the graph. n=11 per group.

Shock-probe contact

Acquisition and extinction. Measures of shock-probe contact showed similar changes during conditioning in all groups, and during the five extinction sessions. During acquisition, the frequency of shock-probe contacts decreased significantly in all groups (Fig. 3A; Session, $F_{1.7,49.8}$=147.11, p<.001, η_p^2=.831; Group, $F_{2,30}$=2.71, p=.083, η_p^2=.153; Group x Session, $F_{3.3,49.8}$=.77, p=.528, η_p^2=.049), the duration of shock-probe contacts was reduced (Fig. 3B; Session, $F_{1.3,38.1}$=44.10, p<.001, η_p^2=.595; Group, $F_{2,30}$=.59, p=.559, η_p^2=.038; Group x Session, $F_{2.5,38.1}$=2.74, p=.065, η_p^2=.155) and the latency to contact the shock-probe was increased (Fig. 3C; Session, $F_{1.4,42.5}$=106.17, p<.001, η_p^2=.780; Group, $F_{2,30}$=.92, p=.409, η_p^2=.058; Group x Session, $F_{2.8,42.5}$=.47, p=.692, η_p^2=.031).

Responses in all three groups were also similar during extinction, in which the frequency of shock-probe contacts was increased (Fig. 3A; Session, $F_{2.7,80.4}$=32.88, p<.001, η_p^2=.523; Group, $F_{2,30}$=1.07, p=.357, η_p^2=.066; Group x Session, $F_{5.4,80.4}$=1.43, p=.220, η_p^2=.087), the duration of shock-probe contacts was increased (Fig. 3B; Session, $F_{2.3,68.6}$=24.42, p<.001, η_p^2=.449; Group, $F_{2,30}$=1.39, p=.265, η_p^2=.085; Group x Session, $F_{4.6,68.6}$=1.36, p=.253, η_p^2=.083) and the latency to contact the shock-probe was reduced (Fig. 3C; Session, $F_{4,120}$=38.40, p<.001, η_p^2=.561; Group, $F_{2,30}$=1.21, p=.312, η_p^2=.075; Group x Session, $F_{8,120}$=.54, p=.828, η_p^2=.034). Therefore, rats across all renewal groups learned that the shock-probe was aversive and avoided contact during conditioning, and increased contact during extinction when the probe was unarmed.

Renewal test. Renewal of shock-probe contact was expressed by shock-probe contact durations and latencies to contact the shock-probe. Shock-probe contact duration was significantly reduced in the renewal context compared to the extinction context across groups (Fig. 3E; Context, $F_{1,30}$=8.92, p=.006, η_p^2=.229; Group, $F_{2,30}$=1.73, p=.194, η_p^2=.104; Group x Context, $F_{2,30}$=2.35, p=.113, η_p^2=.136), and the latency to contact the shock-probe was increased in the renewal context compared to the extinction context across groups (Fig. 3F; Context, $F_{1,30}$=8.39, p=.007, η_p^2=.218; Group, $F_{2,30}$=1.99, p=.155, η_p^2=.117; Group x Context, $F_{2,30}$=1.94, p=.162, η_p^2=.114). However, the frequency of shock-probe contacts did not differ significantly between the renewal context and the extinction context (Fig. 3C; Context, $F_{1,30}$=2.89, p=.099, η_p^2=.088; Group, $F_{2,30}$=1.81, p=.182, η_p^2=.107; Group x Context, $F_{2,30}$=2.63, p=.089, η_p^2=.149). Therefore, we observed renewal of passive coping measured by shock-probe contact duration and latency, and these effects appeared to be primarily driven by the ABA group, followed by the ABC group, and weak renewal in the AAB group.



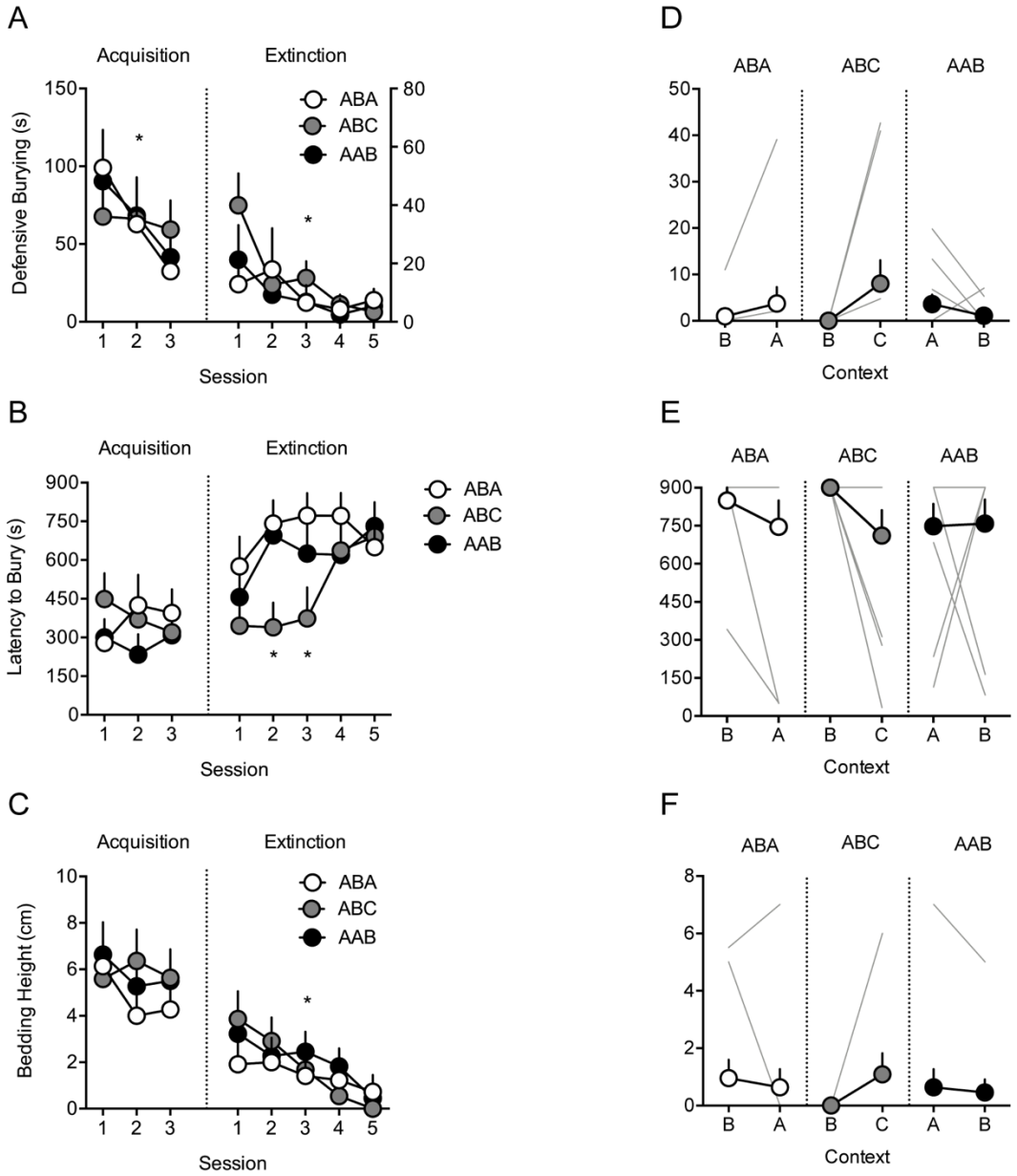
Chapter 2 Figure 3. Renewal of shock-probe contact duration and latency across groups, but no renewal of shock-probe contact frequency. **A** Average frequency of shock-probe contacts, **B** Average duration of shock-probe contacts, and **C** Average latency to initiate shock-probe contact during the acquisition and extinction phases in the ABA, ABC and AAB groups. * $p<.05$, main effect of session (A, B, C). **D** Each rat was tested once in the extinction context and once in the renewal context. There was no difference between extinction and renewal contexts for the frequency of shock-probe contacts. **E** The duration of shock-probe contacts was significantly decreased in the renewal context compared to the extinction context. **F** The latency to initiate shock-probe contact was significantly increased in the renewal context compared to the extinction context. # $p<.05$, main effect of context (E, F). Error bars indicate the standard error and data shown for each individual rat are overlaid on the graph. n=11 per group.

Active defensive burying

Acquisition and extinction. Measures of active defensive burying during conditioning showed similar changes in all groups. The duration of defensive burying decreased across sessions in all groups (Fig. 4A; Session, $F_{2,60}$=5.22, p=.008, η_p^2=.148; Group, $F_{2,30}$=.00, p=.996, η_p^2<.001; Group x Session, $F_{4,60}$=.92, p=.457, η_p^2=.058). However, there were no significant changes in the latency to initiate defensive burying (Fig. 4B; Session, $F_{2,60}$=.00, p=.999, η_p^2<.001; Group, $F_{2,30}$=.50, p=.614, η_p^2=.032; Group x Session, $F_{4,60}$=1.15, p=.342, η_p^2=.071) or in the height of the accumulated bedding surrounding the shock-probe (Fig. 4C; Session, $F_{2,60}$=1.10, p=.341, η_p^2=.035; Group, $F_{2,30}$=.39, p=.681, η_p^2=.025; Group x Session, $F_{4,60}$=.71, p=.587, η_p^2=.045).

During extinction, all groups ceased to bury the shock-probe. The duration of defensive burying decreased across sessions in all groups (Fig. 4A; Session, $F_{2.1,64.2}$=8.20, p=.001, η_p^2=.215; Group, $F_{2,30}$=.45, p=.642, η_p^2=.029; Group x Session, $F_{4.3,64.2}$=1.83, p=.130, η_p^2=.109), and the latency to initiate defensive burying was increased (Fig. 4B; Session, $F_{3.1,93.5}$=5.09, p=.002, η_p^2=.145; Group, $F_{2,30}$=2.06, p=.145, η_p^2=.121; Group x Session, $F_{6.2,93.5}$=2.27, p=.041, η_p^2=.132). The ABC group had shorter latencies to bury during extinction session 2 compared to the ABA (p=.015, *d*=1.37) and AAB (p=.035, *d*=1.18) groups, and during extinction session 3 compared to the ABA group (p=.044, *d*=1.20). However, all groups reached similar latencies by the final extinction session. The height of the peak of accumulated bedding surrounding the shock-probe decreased comparably across sessions in all groups (Fig. 4C; Session, $F_{4,120}$=8.47, p<.001, η_p^2=.220; Group $F_{2,30}$=.19, p=.827, η_p^2=.013; Group x Session, $F_{8,120}$=1.10, p=.369, η_p^2=.068). Therefore, rats in all groups showed defensive burying behaviour during conditioning and reduced defensive burying during extinction.

Renewal test. Measures of defensive burying behaviour did not show significant renewal in any group. There was no significant difference between the renewal and extinction contexts in all groups for the duration of defensive burying (Fig. 4D; Context, $F_{1,30}$=1.91, p=.177, η_p^2=.060; Group, $F_{2,30}$=.21, p=.813, η_p^2=.014; Group x Context, $F_{2,30}$=2.32, p=.115, η_p^2=.134), the latency to initiate defensive burying (Fig. 4E; Context, $F_{1,30}$=2.34, p=.137, η_p^2=.072; Group, $F_{2,30}$=.20, p=.816, η_p^2=.013; Group x Context, $F_{2,30}$=.88, p=.425, η_p^2=.055), or the height of the accumulated bedding (Fig. 4F; Context, $F_{1,30}$=.43, p=.515, η_p^2=.014; Group, $F_{2,30}$=.08, p=.923, η_p^2=.005; Group x Context, $F_{2,30}$=2.25, p=.123, η_p^2=.130). Therefore, we did not observe renewal of active coping strategies measured by duration, latency, and height of the peak of accumulated bedding surrounding the shock-probe.



Chapter 2 Figure 4. There was no renewal of active defensive burying in the ABA, ABC or AAB groups. **A** Average duration of defensive burying, **B** Average latency to initiate defensive burying, and **C** Average height of the peak of accumulated bedding material surrounding the shock-probe during the acquisition and extinction phases. * p<.05, main effect of session (A, C). *p<.05, significantly different responding in the ABC group compared to the ABA and AAB groups in extinction session 2, and significantly different responding in the ABC vs. ABA group in extinction session 3 (B). **D** Each rat was tested once in the extinction context and once in the renewal context. There was no difference between extinction and renewal contexts for the duration of defensive burying. **E** There was no difference between extinction and renewal contexts for the latency to initiate defensive burying. **F** There was no difference between extinction and renewal contexts for the height of the peak of accumulated bedding surrounding the shock-probe. Error bars indicate the standard error and data shown for each individual rat are overlaid on the graph. n=11 per group.

Discussion

The present experiment investigated ABA, ABC and AAB renewal of active and passive coping behaviours using the shock-probe defensive burying (SPDB) task in rats to determine whether these coping strategies are differentially subject to renewal. During conditioning, rats in all three groups passively avoided the side of the chamber containing the shock-probe, reduced contact with the probe, and actively buried the shock-probe using bedding from the chamber floor. During extinction, all groups showed similar reductions in passive avoidance, an increase in shock-probe contact, and a reduction in active defensive burying. Increased passive avoidance in the renewal test was significant for the ABA group but did not reach statistical significance in the ABC group. In contrast, in the AAB group, rats spent less time passively avoiding the shock-probe in the novel context as compared to the extinction context. Moreover, at test, we detected renewal of passive coping measured by shock-probe contact duration and latency, which appeared to be primarily driven by the ABA group, and less robust renewal in the ABC and AAB groups. Our observation of context-dependent renewal of passive coping strategies, in the absence of renewal of active coping behaviours associated with defensive burying, suggests that passive coping behaviours are more prone to renewal compared to active coping behaviours, and that context is a critical factor in the renewal of passive coping strategies in the SPDB task (Bouton, 2004).

Acquisition and extinction

The SPDB task is an ethological model of aversive conditioning that allows for the expression and measurement of multiple naturalistic behaviours (De Boer & Koolhaas, 2003; Pinel & Treit, 1978). Other aversive conditioning tasks, such as Pavlovian fear conditioning and conditioned suppression, allow assessment of only passive coping behaviours such as freezing (Bouton & Bolles, 1979; Bouton & King, 1983; Corcoran & Maren, 2001; Hobin et al., 2003). The SPDB task allows assessment of passive coping through measures of shock-probe contact and time spent avoiding the side of the chamber containing the shock-probe, and allows assessment of active coping including the duration, latency, and height of the peak of accumulated bedding surrounding the shock-probe, which is analogous to natural responses to predation (De Boer & Koolhaas, 2003).

During acquisition in context A, we found that rats in the three groups similarly expressed passive and active coping behaviours. Freezing was only observed during acquisition, as a response to the shock, and was less frequent than passive avoidance. Similar levels of extinction of passive and active behaviours were observed in all three groups when the shock-probe was unarmed. Our finding that extinction was similar in the same context as conditioning (AAB) as when extinction was conducted in a different context as conditioning (ABA and ABC) is consistent with reports in Pavlovian learning where switching the extinction context after conditioning has no considerable effect on responding to the CS (Bouton & Brooks, 1993; Bouton & King, 1983). Moreover, Rosas and colleagues (2007) demonstrated that extinction of a taste aversion response is similarly expressed when extinction occurs in the conditioning context (AAB) and following a switch in context after conditioning (ABA), suggesting that across a variety of aversive learning procedures switching the extinction context after conditioning does not affect extinction. Together, the acquisition and extinction of passive and active coping responses allow for the observation of renewal outside of the extinction context.

Renewal of passive coping strategies

Renewal involves the return of conditioned behaviours after removal from the extinction context. In studies of Pavlovian fear conditioning and conditioned suppression, renewal is expressed most strongly when animals are re-exposed to the original conditioning context (Bouton & King, 1983; Tamai & Nakajima, 2000), reflecting the importance of contextual cues for renewal (Bouton, 2004). We assessed the role of context in the SPDB task by comparing renewal in the ABA design, in which renewal should be most robust, with the ABC and AAB renewal designs in which renewal occurs in a novel context. Comparisons of passive coping behaviours in the renewal context vs the extinction context at test indicated that the ABA group showed renewal through an increase in the latency to initiate probe contact, reduced probe contact duration, and increased passive avoidance characterised by increased time spent on the side of the chamber opposite the shock-probe. This finding of robust ABA renewal of passive coping strategies is consistent with Pavlovian freezing and conditioned suppression studies (Bouton & Bolles, 1979; Bouton & King, 1983; Bouton & Peck, 1989; Corcoran & Maren, 2001, 2004; Hobin et al., 2006). Renewal of freezing behaviour was not observed, and this may be due to the low levels of freezing observed just following reactions to probe contacts during conditioning. Low levels of freezing have been observed in both ethological and experimental studies, which show that rats freeze for less than ~10% of the session length (De Boer & Koolhaas, 2003; Tao et al., 2017).

Renewal of passive coping strategies in the SPDB task was much less marked in the ABC and AAB groups. A subset of rats in the ABC group showed a renewal of passive avoidance but there was no significant renewal in the ABC group ($p=.153$). Studies directly comparing ABA and ABC renewal in aversive learning procedures are sparse. ABA and ABC renewal have been shown to be similar in magnitude using a conditioned suppression task (Thomas et al., 2003) and a signaled shuttle box task (Nakajima, 2014). However, in appetitive conditioning procedures, ABA renewal has been shown to be a more robust effect compared to ABC renewal (Khoo et al., 2020; Zironi et al., 2006). It is possible that we did not detect a significant ABC renewal effect here because pre-exposure to context C during the habituation phase may have interfered with the rats' ability to detect context C as a novel context. However, we do have reservations about this hypothesis considering that others have detected ABC renewal despite pre-exposure to context C (Nakajima, 2014; Thomas et al., 2003).

We also observed the opposite of a renewal effect in the AAB group such that passive avoidance was reduced in the renewal context compared to the extinction context. AAB renewal has been observed in a variety of learning tasks (Bouton et al., 2011; Bouton & Ricker, 1994; Rescorla, 2007, 2008; Rosas et al., 2007; Todd et al., 2013), but other studies have been unable to detect AAB renewal (Bossert et al., 2004; Crombag & Shaham, 2002; Fuchs et al., 2005; Khoo et al., 2020; Nakajima et al., 2000). Moreover, AAB renewal has been found to be much weaker than ABA and ABC renewal in fear conditioning (Thomas et al., 2003).

Our observation of ABA, but not ABC or AAB renewal of passive avoidance, is consistent with the interpretation that context A retains a residual excitatory association with the US (i.e., shock) after extinction, which summates with the residual excitatory strength of the CS (i.e., shock-probe) at test, and results in greater ABA renewal compared to ABC and AAB renewal (Delamater & Westbrook, 2014; Polack et al., 2013; Rescorla & Wagner, 1972).

AAB renewal is not easily explained by many learning theories (e.g., Pearce, 1987; Rescorla & Wagner, 1972) and AAB suppression of conditioned responding has only been previously observed using an appetitive Pavlovian conditioning procedure (Khoo et al., 2020).

Khoo and colleagues (2020) suggested that AAB suppression may be due to the AAB group experiencing extinction of the stimulus and of context A, presumably resulting in an inhibitory memory that is strong enough to prevent renewal in context B.

Similarly, Laborda and colleagues (2011) have suggested that AAB renewal is a weak effect because the AAB group undergoes "deeper extinction" resulting in weaker AAB renewal compared of ABC renewal. Moreover, the switch from the conditioning/extinction context A to the novel renewal context B may have resulted in greater exploratory behaviour, which may explain why rats spent more time on the side of the chamber containing the shock-probe in context B compared to context A at test. Future studies are needed to replicate the AAB suppression effect using different aversive conditioning procedures.

Renewal of active coping strategies

Context-induced renewal of active coping strategies measured by defensive burying was not observed in any of the groups, even in the ABA group that showed renewal of passive avoidance. Several procedural variables that are known to affect the variability of defensive burying were considered prior to testing and are unlikely to have contributed to the lack of renewal of active defensive burying. We used Long-Evans rats that are known to bury more than Wistar rats (Tarte & Oberdiek, 1982) and used a chamber size that is typical in SPDB tasks (Degroot et al., 2001; Pesold & Treit, 1992; Shah & Treit, 2004; Tao et al., 2017; Trent & Menard, 2013). The intensity of the shock administered in our experiment (3 mA) is also like that used in other studies that have observed robust defensive burying (Tao et al., 2017; Trent & Menard, 2013).

The absence of renewal of defensive burying is more likely due to the variability in the degree to which rats employed the defensive burying response strategy. We observed robust defensive burying like burying observed in other studies (Pesold & Treit, 1992; Shah & Treit, 2004) and, on average, defensive burying represented about 10% of the conditioning session length (85.7 $\pm$ 16.0 s). Burying was highly variable between rats however and ranged from 1.3 to 299.3 s. This is in line with previous research showing a high degree of variability in defensive burying both within- and between- studies, with defensive burying representing, on average, 3 to 30% of the observation time in standard duration test (10-15 min) (De Boer & Koolhaas, 2003). The tendency of some rats to show low degrees of defensive burying may have contributed to the absence of significant renewal.

Variability in defensive burying can also be attributed to the high degree of flexibility over behaviour in the SPDB task. Active defensive states such as defensive burying are metabolically costly (McEwan et al., 2015) and may even increase the likelihood of shock if rats approach too close to the shock-probe (De Boer & Koolhaas, 2003). In contrast, passive coping responses require little metabolic investment and animals can avoid the shock altogether (De Boer & Koolhaas, 2003). This idea is consistent with the predatory imminence continuum (Fanselow & Lester, 1988; Perusini & Fanselow, 2015), which indicates that the perceived distance from contact with a threatening stimulus determines the selection of either passive or active responses. When rats employ a passive avoidance response by spending time on the side of the chamber opposite the shock-probe, they become spatially distanced from the threat, and this may reduce active defensive burying by reducing the perceived imminence of danger. Active coping responses such as defensive burying may therefore be most actively employed after receiving the shock during conditioning when the perceived threat is imminent, but not after extinction in the renewal context when it can be passively avoided. Therefore, the lack of a

renewal effect may be due to animals employing a passive coping strategy that negated the necessity for more active coping strategies.

Conclusions

Our results show renewal of passive coping behaviours, but not renewal of active defensive burying, in the SPDB task. Consistent with renewal of passive freezing in aversive Pavlovian conditioning (Bouton & King, 1983; Tamai & Nakajima, 2000), renewal of passive coping was predominantly observed in the ABA group and less so for the ABC and AAB groups. We did not detect renewal of active defensive burying in any group. These results are the first to investigate differences in context-induced renewal of passive and active coping strategies using the SPDB task. Our results provide novel evidence suggesting that active coping responses are less susceptible to renewal compared to passive coping responses. Active coping responses may be less susceptible to renewal because they are more energetically demanding and are less likely to be expressed when danger can be passively avoided. These findings highlight the importance of studying multiple indices of aversive conditioned responding.

Chapter 3: Neural correlates of recall and extinction in a rat model of appetitive Pavlovian conditioning

Introduction

Through associative learning, animals and humans can adapt their behaviour in response to changes in their environment to survive. In Pavlovian conditioning, organisms learn to associate a conditioned stimulus (CS; e.g., white noise) with an unconditioned stimulus (US; e.g., sucrose). As a result, the CS comes to elicit conditioned responding. Alternatively, in operant conditioning, organisms learn to perform an operant response (e.g., lever press) to receive an outcome (e.g., sucrose). During extinction, responding can be reduced in bother Pavlovian and operant preparations by withholding the US or outcome. Instead of erasing the original learning, extinction is thought to involve new brain plasticity that encodes a "CS-no US" (Pavlovian) or "response-no outcome" (operant) association (Bouton et al., 2006). Conditioning- and extinction-related memories are thought to be encoded separately (Bouton, 2002; Bouton & Swartzentruber, 1991; Lacagnina et al., 2019). As a result, even after extensive extinction training, the memory formed during conditioning is retained and can provide a powerful basis for relapse (Bouton, 2002). Studies investigating the neurobiology of conditioning and extinction typically use Pavlovian fear conditioning and appetitive operant conditioning procedures (Corcoran & Maren, 2004; LaLumiere et al., 2012; Marchant et al., 2010; Maren, 2001; Milad & Quirk, 2002; Moorman et al., 2015; Peters et al., 2008; Warren et al., 2016, 2019). In contrast, studies using appetitive Pavlovian procedures typically focus on the neural mechanisms involved in the acquisition of the conditioned response (Keefer & Petrovich, 2017; Saddoris et al., 2009). Therefore, less is known about the brain areas and networks that mediate the extinction of appetitive Pavlovian conditioned responding.

Extinction is mediated by various interconnected brain regions. The infralimbic (IL) subregion of the medial prefrontal cortex (mPFC) is thought to be a critical brain area mediating the extinction of Pavlovian fear conditioning and operant reward-seeking (Milad & Quirk, 2002; Peters et al., 2009; Quirk et al., 2000). In support of this hypothesis, IL neurons are active during fear extinction retrieval, and during extinction learning and extinction expression of operant drug- and natural reward-seeking (Knapska & Maren, 2009; Marchant et al., 2010; Orsini et al., 2011, 2013; Warren et al., 2016). Moreover, activation of the IL with the glutamate receptor agonist AMPA, or with the AMPA potentiator PEPA, suppresses the reinstatement of operant drug-seeking, suggesting that enhanced excitatory synaptic activity in the IL inhibits operant drug-seeking (LaLumiere et al., 2012; Peters et al., 2008). Similarly, stimulation of the IL enhances fear extinction learning and retrieval (Adhikari et al., 2015; Do-Monte et al., 2015a; Milad et al., 2004; Vidal-Gonzalaz et al., 2006). Consistent with a role of the IL in inhibiting responding, inactivation of the IL disinhibits operant drug-seeking and promotes reinstatement (Gutman et al., 2017; Peters et al., 2008). Consistently, inhibition of the IL impairs fear extinction memory consolidation and potentiates freezing (Burgos-Robles et al., 2007; Do-Monte et al., 2015a; Farrell et al., 2010; Laurent & Westbrook, 2008; Lebrón et al., 2004; Sierra-Mercado et al., 2011; Sotres-Bayon et al., 2009). Together, these results support a similar role of the IL in mediating the extinction of operant reward-seeking and Pavlovian conditioned fear.

The IL also appears to play a similar role in the extinction of appetitive Pavlovian conditioned responding. Optogenetic stimulation of the IL suppresses the reinstatement, spontaneous recovery, and renewal of cue-elicited responding for sucrose after extinction (Villaruel et al., 2018). Consistently, lesions of the IL disrupt extinction retrieval and promote

the reinstatement and renewal of cue-elicited responding for food after extinction (Rhodes & Killcross, 2004, 2007). These results parallel findings from appetitive operant conditioning studies and support a central role for the IL in extinction. In contrast, however, inactivation of the IL *facilitates* the initial extinction of cue-elicited responding for sucrose (Mendoza et al., 2015). This result may be due to the dual roles of the IL in both the expression and extinction of conditioned responding (Warren et al., 2016, 2019).

A functional dichotomy in the medial prefrontal cortex (mPFC) has been suggested in which the IL mediates the extinction of conditioned responding, while the prelimbic cortex (PL) drives the expression of responding (Peters et al., 2009). The differential roles of the PL and IL in conditioning and extinction are thought to be due to their downstream projections. Specifically, PL projections to the basolateral amygdala (BLA) and the nucleus accumbens core (NAcC) are thought to drive the expression of conditioned fear and operant reward-seeking, respectively (Peters et al., 2009). In contrast, the neural pathways from the IL to the BLA and nucleus accumbens shell (NAcSh) are thought to mediate the extinction of Pavlovian fear conditioning and operant reward-seeking, respectively (Peters et al., 2009). Support for this hypothesis comes from the findings that PL inactivation disrupts the expression of conditioned fear but does not affect extinction learning or retrieval (Choi et al., 2010; Laurent & Westbrook, 2009; Sierra-Mercado et al., 2011). Moreover, Fos expression is increased in PL inputs to the BLA during the retrieval of fear conditioned memories, while inhibition of the PL-to-BLA pathway impairs the retrieval of fear conditioned memories (Do-Monte et al., 2015b; Quiñones-Laracuente et al., 2021). Similarly, inhibition of the PL-to-NAcC pathway attenuates the reinstatement of operant drug-seeking, suggesting that this pathway is involved in the expression of conditioned responding (Stefanik et al., 2013, 2016). Conversely, chemogenetic activation of the IL-to-NAcSh pathway suppresses the reinstatement of operant drug-seeking, while simultaneous inhibition of the IL and NAcSh drives the reinstatement of operant drug-seeking (Augur et al., 2016; Peters et al., 2008). Likewise, activation of IL inputs to the amygdala enhances fear extinction learning and retrieval, while inhibition of the IL-to-BLA pathway impairs fear extinction learning and retrieval (Adhikari et al., 2015; Bloodgood et al., 2018; Bukalo et al., 2015). Together, these results support a functional distinction within the mPFC such that the IL and PL mediate the extinction and expression of conditioning responding, respectively, and these functions are maintained through distinct projections to the BLA and NAc based on the valence of the reinforcer.

The paraventricular nucleus of the thalamus (PVT) is another region implicated in both the expression and extinction of conditioned responding. The PVT expresses Fos during the renewal and reinstatement of operant drug-seeking (Dayas et al., 2008; Hamlin et al., 2009; James et al., 2011) and during the renewal of cue-elicited responding for food (Anderson & Petrovich, 2017). Consistently, inactivation of the PVT attenuates the renewal and reinstatement of operant drug-seeking (Hamlin et al., 2009; James et al., 2010; Matzeu et al., 2015). Furthermore, inactivation of the PVT impairs fear memory retrieval, supporting a role of the PVT in promoting the expression of learned fear (Do-Monte et al., 2015b; Penzo et al., 2015). The PVT receives inputs from both the PL and IL (Vertes, 2004), and the functional dichotomy between the PL and IL has been found to be maintained through projections to the PVT. Inhibition of the IL-to-PVT pathway impairs fear extinction retrieval, supporting a role of this pathway in extinction (Tao et al., 2021). Moreover, activation of the IL-to-PVT, but not the PL-to-PVT, pathway attenuates the renewal of cue-elicited responding for sucrose (Brown & Chaudhri, 2022). Interestingly, inhibition of the PL-to-PVT pathway attenuates the reinstatement

of operant drug-seeking, but not sucrose-seeking, suggesting that the PL-to-PVT pathway may be specifically implicated in the seeking of drug, but not natural, rewards (Giannotti et al., 2018). Therefore, the PVT appears to be an important region for mediating the expression and extinction of conditioned responding via distinct projections from the PL and IL, respectively.

Other regions such as the orbitofrontal cortex (OFC) and the BLA have also been widely implicated in motivated behaviours and may therefore be involved in both the expression and extinction of appetitive Pavlovian learning. Inactivation of the OFC disrupts the extinction of responding to a food-predictive cue (Panayi & Killcross, 2014), as well the extinction of operant food-seeking in non-human primates (Butter et al., 1963). Inconsistently, however, others have demonstrated no effect of OFC inactivation on the extinction of responding to a sucrose-predictive cue (Burke et al., 2008, 2009). Thus, while the role of the OFC in extinction is unclear, other reports suggest that OFC inputs to the BLA may be important for both the expression and suppression of conditioned responding. Inactivation of the BLA impairs the extinction of operant food-seeking (McLaughlin & Floresco, 2007). Moreover, single-unit recordings have shown that the BLA represents learning in over-expectation with a sucrose reinforcer via inputs from the OFC (Lucantonio et al., 2015). Over-expectation produces a decline in Pavlovian conditioned responding, like that observed in extinction, but through lowering the outcome's value, rather than omitting the outcome. However, inactivation of the BLA, or the OFC-to-BLA pathway, also disrupts the renewal of drug-seeking, suggesting that the OFC and BLA may also be implicated in driving the expression of conditioned responding for drug rewards (Fuchs et al., 2005, 2007; Lasseter et al., 2011; Marinelli et al., 2010). Together, these data implicate both the BLA and OFC in the expression of conditioned drug-seeking, and in behavioural inhibition during extinction or over-expectation with natural rewards.

The current study examines the neural basis of appetitive Pavlovian extinction by assessing the extent of activation in multiple key brain regions during recall and following extinction. We examined a set of cortical, striatal, thalamic, and amygdalar brain regions, and assessed brain activation by measuring differences in Fos density, an immediate early gene widely used for brain activity mapping (Franceschini et al., 2020). Based on these data, we computed inter-regional correlations of Fos density to produce functional network activation graphs (Silva et al., 2019) for the recall and extinction of appetitive Pavlovian conditioned responding.

Methods

Animals

Subjects were 38 experimentally naïve, male Long-Evans rats (Charles River, St. Constant, Quebec, Canada; 220-240 g upon arrival). Rats were maintained in a humidity (40-45%) and climate controlled (21 °C) room on a 12-h light/dark cycle with lights turned on at 7:00 h. All procedures occurred during the light phase. Rats were individually housed in standard cages containing beta chip bedding (Aspen Sani chips; Envigo, Indianapolis IN) with unrestricted access to water and food (Agribands, Charles River). Each cage contained a nylabone toy (Nylabones; Bio-Serv, Flemington, NJ), a polycarbonate tunnel (Rat Retreats, Bio-Serv) and shredded paper for enrichment. During a seven-day acclimation period to the colony room, rats were handled, and body weight was recorded daily. All procedures followed the guidelines of the Canadian Council for Animal Care and were approved by the Concordia University Animal Research Ethics Committee.

Apparatus

Experiments were conducted in sound-attenuating melamine cubicles that each contained a conditioning chamber (Med Associates, ENV-009A, St-Albans, VT, USA). Each conditioning chamber consisted of stainless-steel bar floors, and a house light (75 W, 100 mA, ENV-215M) was in the center of the left wall. A white noise generator and speaker (ENV-225SM) in the top left corner of the left wall produced the white noise conditioned stimulus (CS) 5 dB above the background noise of an exhaust fan mounted inside the cubicle. A 10% sucrose solution was delivered to a fluid port (ENV-200R3AM) located 2 cm above the floor in the center of the right wall. Infrared sensors (ENV-254CB) lined both sides of the port opening to detect port entries. Solutions were delivered into the fluid port via a polyethylene tube (Fisher Scientific, 141 691 A) connected to a 20 ml syringe in a pump (Med Associates, PHM-100, 3.33 rpm) located outside the cubicle. All events were controlled and recorded by Med Associates software (Med-PC IV) on a computer in the experimental room.

Behavioural procedures

Rats were acclimated to the taste of 10% sucrose in the home-cage for 48 h, and subsequently assigned into one of three experimental conditions: paired (n=14), unpaired (n=12) and home-cage (n=12). Groups were matched according to body weight, sucrose preference, and sucrose consumption. All rats were then habituated to the experimental chambers on one day (20 min session) during which the house light turned on after a 1 min delay and total port entries were recorded.

Rats were then trained in a Pavlovian conditioning task for 8 days (57 min sessions). During Pavlovian conditioning, the house-light turned on after a 2 min delay to signal the start of each session and shut off to signal the end of the session. For the paired condition, Pavlovian conditioning sessions consisted of 10 pairings of an auditory CS (20 s white noise) that co-terminated with the delivery of .3 ml of 10% sucrose solution into a fluid port (10 s; 3 ml per session). The variable inter-trial interval (ITI) averaged 240 s (120, 240, or 360 s). Ports were checked after each session to ensure consumption.

Rats in the unpaired and home-cage conditions received identical sessions except that the sucrose was not delivered contingent to the CS. Rather, rats in the unpaired condition were presented with the same number of white noise presentations and sucrose deliveries as the paired condition, but sucrose was delivered to the fluid port mid-way during the ITI. Rats in the home-cage condition received the same number of white noise presentations as the paired and unpaired conditions and following the same ITI schedule, however, sucrose was delivered in the home-cage at a random time 1-4 h after the session. These control groups helped to determine whether Fos expression was specific to Pavlovian conditioned responding. In contrast to the home-cage control condition, where only the white noise stimulus was delivered, the CS and US were presented at intervals that prevented the establishment of an excitatory CS-US association for the unpaired control condition.

Following Pavlovian conditioning, rats were assigned to one of two experimental groups: recall (n=19; n=7 paired, n=6 unpaired, n=6 home-cage) and extinction (n=19; n=7 paired, n=6 unpaired, n=6 home-cage). Groups were matched according to ΔCS port entries (CS minus pre-CS port entries) during the training phase. Rats in the recall group received 1 extinction session (57 min session) prior to collection of tissue for Fos immunohistochemistry, while rats in the extinction group received 6 days of extinction training (57 min sessions). Extinction sessions were identical to Pavlovian conditioning sessions except that sucrose delivery was withheld.

Fos immunohistochemistry and quantification

To quantify Fos expression induced by recall or extinction, rats were sacrificed 90 min after the start of either the first or sixth extinction session (Warren et al., 2016). Rats were anesthetized with sodium pentobarbital (100 mg/kg, intraperitoneal) and transcardially perfused with 0.1 M phosphate buffered saline (PBS), followed by 4% paraformaldehyde (PFA) in 0.1 M PBS. Brains were extracted and post fixed for 24 h in 4% PFA, before transfer to a 30% sucrose solution in water for 48 h. Coronal sections (40 µm) were obtained with a cryostat and collected in 0.1 M phosphate buffer (PB). Sections were blocked for 1 h in 0.3% PBS-Triton-X-100 with 6% normal goat serum (NGS; Vector Labs, S-1000), followed by incubation for 72 h at 4 ºC with anti-cFos rabbit primary antibody (1:2000; Cell Signalling, 2240). Sections were washed 3 x 10 min with PBS, and then incubated in a biotinylated goat anti-rabbit secondary antibody (1:250; Vector Labs, BA-1000) for 1 h in 0.3% PBS-Triton-X-100 and 3% NGS. Sections were washed 3 x 10 min with PBS and incubated in a tertiary of avidin and biotinylated horseradish peroxidase (1:1000; ABC kit, Vector Labs, PK-6100) and stained with a 3, 3'-diaminobenzidine (DAB) solution. Sections were washed in PB, mounted on slides and cover slipped.

Selected brain regions of interest (Table 1) were chosen a priori for statistical comparisons. A bright field microscope (Nikon Eclipse TiE) captured images at 20 × magnification. Images were imported into Fiji (ImageJ) software, and the number of Fos-positive neurons in each region of interest was quantified using a custom-made cell-counting Fiji macro, which quantified Fos-positive cells based on contrast with background, size, and circularity. A rat brain atlas (Paxinos & Watson, 2007) was used to approximate the location of sections compared to bregma. We quantified the number of Fos-positive neurons at the following bregma coordinates: 3.24, 3.00, 2.76 for the IL; 4.68, 4.20, 3.24 for the PL; 4.68, 4.20 for the mOFC; 4.20, 3.24 for the lOFC and vOFC; 2.28, 2.04, 1.20 for the NAcC and mNAcSh; 2.04, 1.20 for the lNAcSh; -1.56, -1.80, -2.76, -3.24 for the BLA; -1.80, -2.04 for the aPVT; -2.64, -2.76 for the mPVT, and -3.00, -3.24 for the pPVT. The area used for quantification in all brain regions was selected manually and was consistent across all rats. All cells within that area were quantified for each section. Cell counts were then divided by the area selected in Fiji to calculate density. The density of Fos-positive neurons was averaged across sections for each rat. Density counts for the paired and unpaired conditions were normalized to the average density in the home-cage condition. This normalization was used to account for baseline Fos activity, and previous studies have similarly used a home-cage control as a baseline for Fos activity (Barbosa et al., 2013; Lopez et al., 2018).

Data analysis

Behavioural data of interest included port entries made during the 20 s intervals before CS onset (pre-CS) and ΔCS port entries (port entries made during the 20 s CS minus port entries made during the pre-CS period). ΔCS port entries were used as the measure of conditioned responding because it takes into consideration variation in baseline activity (Brown & Chaudhri, 2022; Rhodes & Killcross, 2004, 2007; Villaruel et al., 2018). During acquisition, pre-CS and ΔCS port entries were assessed separately using mixed analyses of variance (ANOVA) with group (recall or extinction), condition (paired, unpaired or home-cage), and session as factors. On the first extinction session, pre-CS and ΔCS port entries were assessed separately using ANOVAs with group (recall or extinction) and condition (paired, unpaired or home-cage) as factors. Across the six extinction sessions, pre-CS and ΔCS port entries were assessed separately in the extinction groups only using mixed ANOVA with condition (paired, unpaired or home-

cage) and session as factors. Collapsed across the two Fos induction sessions (extinction session 1 or 6), pre-CS and ΔCS port entries were assessed separately using ANOVAs with group (recall or extinction) and condition (paired, unpaired or home-cage) as factors.

Fos density in each region of interest was analyzed separately using ANOVAs with group (recall or extinction) and condition (paired, unpaired or home-cage) as factors.

Greenhouse-Geisser corrections are reported following violations of Mauchly's test of sphericity. Post-hoc analyses were corrected for multiple comparisons using the Bonferroni adjustment. All data analyses were conducted using IBM SPSS v21.0 (IBM Corp., Armonk, NY). Results were considered statistically significant at $p<.05$.

Network activation graphs were constructed using Fos density and Pearson correlation coefficients (Silva et al., 2019). Within each experimental group and condition (e.g., home-cage recall), Fos densities were averaged across bregma coordinates for each rat, thus each rat was an n=1 for each brain region. For all 12 brain regions analyzed, Pearson correlation coefficients were then calculated for Fos density between all pairwise combinations (Silva et al., 2019). Each node represents one of the 12 brain regions examined in this study. Node sizes in the paired and unpaired conditions are proportional to the Fos density increase for each brain region compared to the Fos densities in the corresponding home-cage control condition (Silva et al., 2019). Each edge connecting two nodes represents a Pearson correlation between brain regions that had a $p<.05$ and an $r \geq .05$ (adapted from Silva et al., 2019). Edge thickness reflects the r value of the correlation between the two brain regions, with thicker edges indicating a greater correlation in Fos densities in the two regions. Negative correlations are not represented in the network activation graphs. Correlations were displayed as a colour-coded correlation matrix using GraphPad Prism (Version 7; La Jolla, CA). The NetworkX (v2.7.1) package (Hagberg et al., 2008) in Python was used to visualize network activation graphs.

Abbreviation	Region name
aPVT	Anterior paraventricular nucleus of the thalamus
BLA	Basolateral amygdala
IL	Infralimbic cortex
lNAcSh	Lateral nucleus accumbens shell
lOFC	Lateral orbitofrontal cortex
mNAcSh	Medial nucleus accumbens shell
mOFC	Medial orbitofrontal cortex
mPVT	Middle paraventricular nucleus of the thalamus
NAcC	Nucleus accumbens core
PL	Prelimbic cortex
pPVT	Posterior paraventricular nucleus of the thalamus
vOFC	Ventral orbitofrontal cortex

Chapter 3 Table 1. Abbreviations of brain areas

Results

Behavioural paradigm to assess Fos expression during recall and extinction

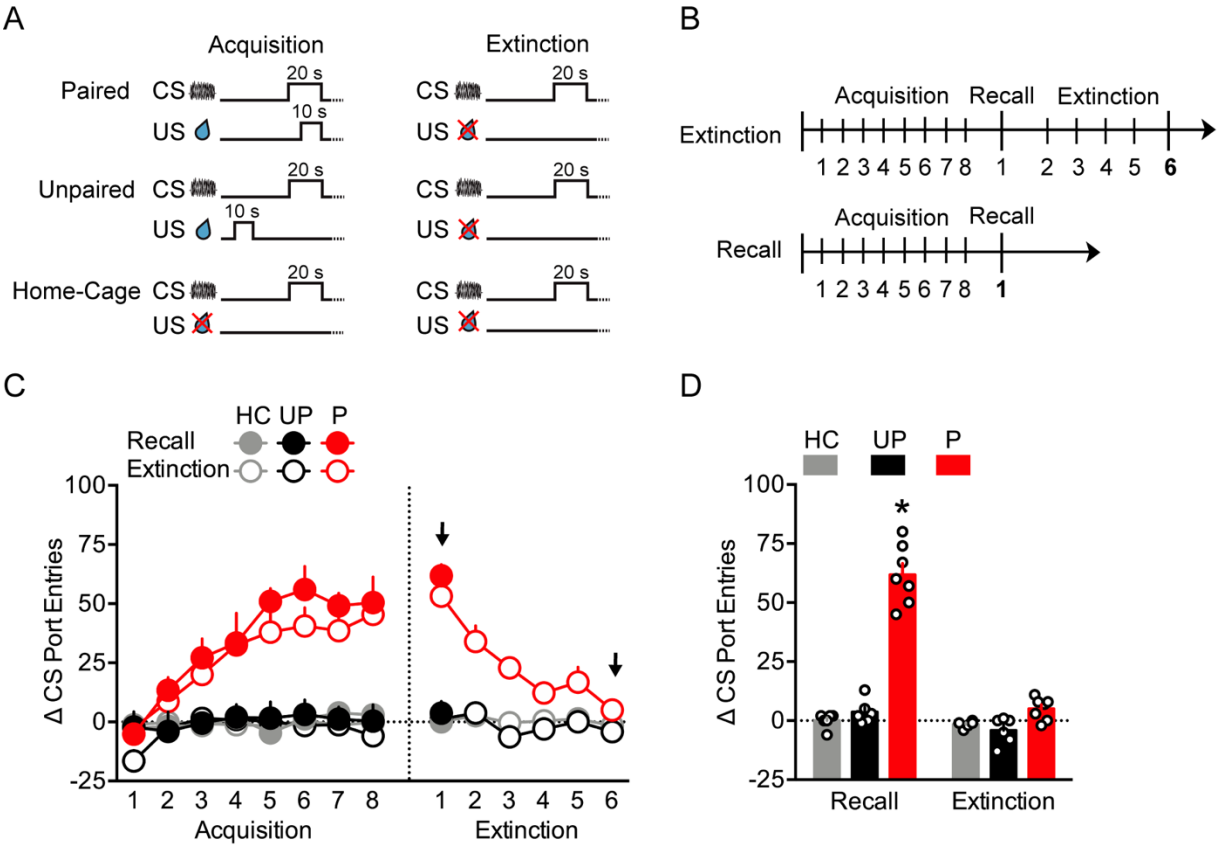
To assess brain activation patterns during recall and extinction of appetitive Pavlovian conditioned responding, we established a behavioural paradigm that could efficiently condition and extinguish responding to a Pavlovian CS (Fig. 1A). Rats in the paired condition received presentations of a 20 s white-noise CS that was paired with a 10% sucrose US, while rats in the unpaired and home-cage conditions received sucrose either during the ITI or in the home-cage after the session. Following training, rats in the recall groups received 1 extinction session, while rats in the extinction groups received 6 extinction sessions (Fig. 1B). Brains were extracted 90 minutes following the start of either the first or sixth extinction session and analyzed for Fos expression.

During conditioning, ΔCS port entries increased across sessions in the paired groups and remained low for the unpaired and home-cage groups (Fig. 1C; Condition, $F_{2,32}$=60.77, p<.001; Session, $F_{3.5,112.2}$=14.80, p<.001; Condition x Session, $F_{7.0,112.2}$=9.38, p<.001). There was no significant difference in ΔCS port entries during conditioning between the recall and extinction groups that were formed by matching based on ΔCS port entries during conditioning (Group, $F_{1,32}$=1.63, p=.211; Group x Condition x Session, $F_{7.0,112.2}$=.61, p=.748). The increase in ΔCS port entries across sessions in the paired groups but not the unpaired and home-cage controls do not appear to be driven by differences in responding during the pre-CS period. Pre-CS port entries showed no significant changes across conditioning sessions (Session, $F_{4.9,157.4}$=.50, p=.772), and there were no significant differences between the recall and extinction groups (Group, $F_{1,32}$=.05, p=.819; Group x Condition x Session, $F_{9.8,157.4}$=1.09, p=.375). Pre-CS port entries were greater for the paired (p=.001) and unpaired (p<.001) groups relative to the home-cage control group, but there was no difference between the paired and unpaired groups (p=1.000) (Data not shown; Condition, $F_{2,32}$=12.21, p<.001). Therefore, paired recall and extinction groups similarly acquired Pavlovian conditioned responding to the CS compared to the unpaired recall and extinction groups and home-cage controls.

During the first extinction session, ΔCS port entries were greater for the paired recall and paired extinction groups, compared to the unpaired and home-cage conditions (Fig. 1C; Condition, $F_{2,32}$=68.67, p<.001; Group, $F_{1,32}$=.63, p=.434; Condition x Group, $F_{2,32}$=.34, p=.718). Responding was significantly increased in the paired groups compared to the unpaired (p<.001) and home-cage (p<.001) groups with no difference between the unpaired and home-cage groups (p=1.000). Moreover, responding was similar for all groups during the pre-CS interval (Data not shown; Condition, $F_{2,32}$=2.07, p=.143; Group, $F_{1,32}$=.92, p=.345; Condition x Group, $F_{2,32}$=1.34, p=.275). Together, the data indicate that during the first extinction session, paired recall and extinction groups similarly expressed conditioned responding, relative to the unpaired and home-cage control groups.

Across the 6 extinction sessions, ΔCS port entries decreased for the paired extinction group, while the unpaired and home-cage extinction groups showed stable, low levels of responding (Fig. 1C; Condition, $F_{2,16}$=30.39, p<.001; Session, $F_{2.7,44.0}$=8.68, p<.001; Condition x Session, $F_{2.7,44.0}$=5.73, p<.001). Responding during the pre-CS showed no change across extinction sessions in all groups (Data not shown; Condition, $F_{2,16}$=3.13, p=.071; Session, $F_{2.8,44.6}$=.31, p=.803; Condition x Session, $F_{5.6,44.6}$=.68, p=.658). This data indicates that the paired extinction group extinguished responding to the CS compared to the unpaired and home-cage controls.

Collapsed across both Fos induction sessions, ΔCS port entries were significantly greater for the paired recall group compared to all other groups (Fig. 1D; Condition, $F_{2,32}$=113.86, p<.001; Group, $F_{1,32}$=102.91, p<.001; Group x Condition, $F_{2,32}$=68.02, p<.001). There were no significant differences between the paired extinction, unpaired extinction, and home-cage extinction groups (all p>.05). However, responding was significantly increased in the paired recall group compared to the paired extinction group (p<.001). Furthermore, during the Fos induction sessions, there was no differences between groups during the pre-CS period (Data not shown; Condition, $F_{2,32}$=.84, p=.443; Group, $F_{1,32}$=2.62, p=.115; Group x Condition, $F_{2,32}$=.63, p=.541). Therefore, only rats in the paired conditions learned to respond by entering the fluid port during the CS during conditioning, and only rats in the paired extinction group extinguished DCS port entries across extinction sessions.

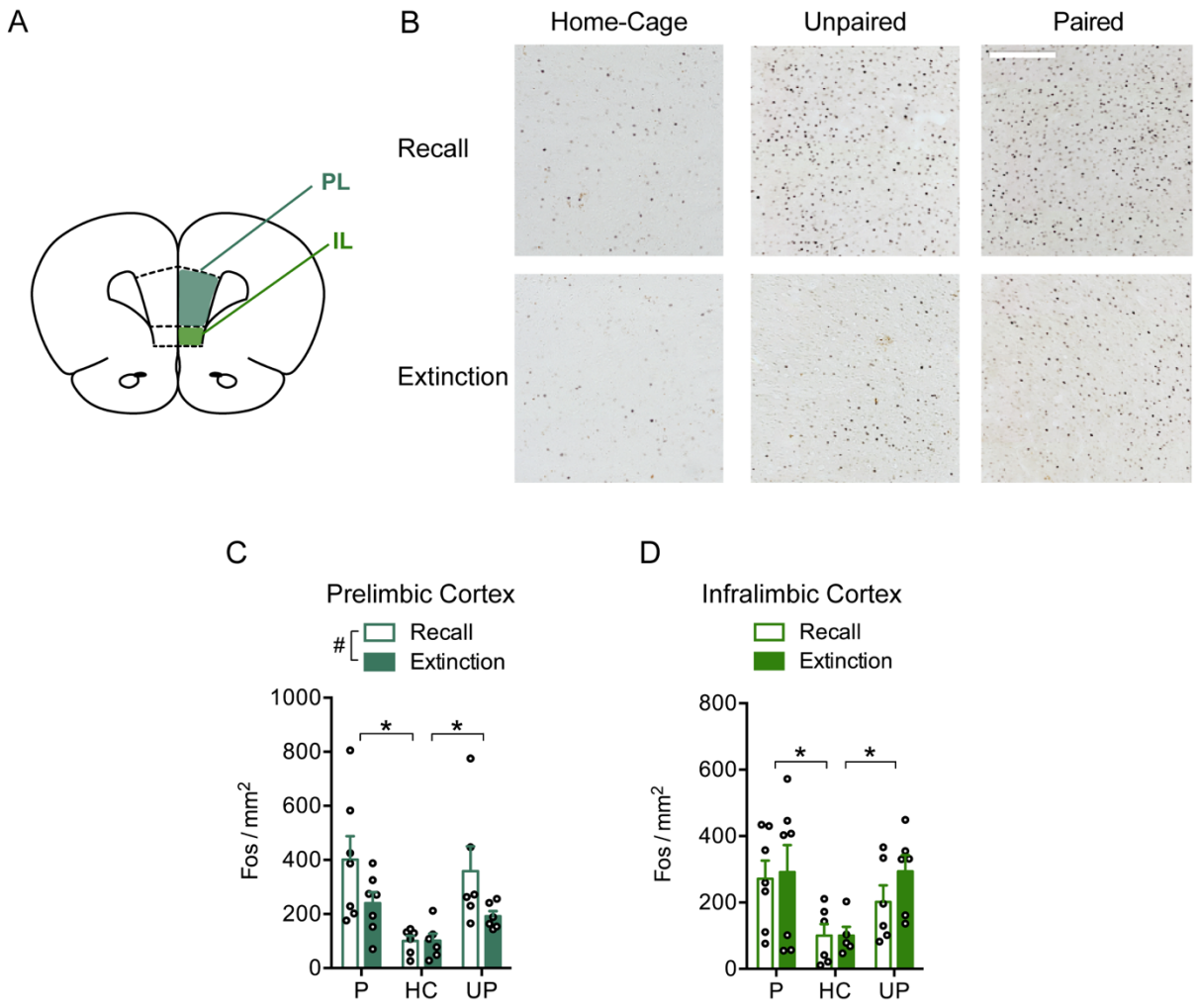


Chapter 3 Figure 1. Acquisition and extinction of conditioned responding to a discrete sucrose CS. **A** Schematic representation of the experimental paradigm. During acquisition, the paired condition received pairings of a 20 s white noise CS that co-terminated with delivery of a sucrose US. The unpaired and home-cage conditions received white noise presentations that were not contingent with sucrose delivery and received sucrose either during the ITI or in the home-cage. During extinction, the white noise was presented as in acquisition, but sucrose was withheld. **B** Schematic diagram indicating the timing of acquisition and extinction sessions. Tissue was collected for Fos analysis 90 min after the first extinction session in the recall groups, and after the sixth extinction session in the extinction groups. **C** Changes in ΔCS port entries during acquisition show the development of Pavlovian conditioned responding only in the paired groups. Responding was maintained in both paired groups after one extinction session (recall) and responding was extinguished over the 6 extinction sessions in the paired extinction group. **D** ΔCS port entries collapsed across the two Fos induction sessions were increased for the paired recall group compared to the paired extinction, unpaired recall and home-cage recall groups. The data displayed in D are the same as those indicated by the arrows in C, which show sessions after which tissue was collected for Fos analysis. *p<.05, recall vs extinction in the paired condition. P: paired; UP: unpaired; HC: home-cage. Here and in subsequent figures, data are depicted as mean ± SEM, and individual data points are overlaid on the bar graphs.

Fos immunoreactivity

Prelimbic and infralimbic cortices. In the prelimbic cortex (PL), there was a main effect of condition, in which both the paired and unpaired groups showed markedly greater Fos density relative to the home-cage condition (Fig. 2A, B, C; Condition, $F_{2,32}$=7.96, p=.002). Fos density did not differ significantly between the paired and unpaired conditions (p=1.000), but Fos density was greater in both the paired (p=.002) and unpaired (p=.018) conditions relative to the home-cage control. There was also a significant main effect of recall vs extinction, in which Fos density was greater during recall as compared to extinction (Group, $F_{1,32}$=5.33, p=.028), but there was no significant Group x Condition interaction ($F_{2,32}$=1.30, p=.286), indicating that the lower Fos density in the extinction groups did not depend upon history of conditioning.

In the infralimbic cortex (IL), Fos density was similar during recall and extinction (Fig. 2A, D; Group, $F_{1,31}$=.66, p=.423). Like results for the PL, Fos density in the IL was markedly greater in the paired and unpaired groups relative to the home-cage groups (Condition, $F_{2,31}$=5.68, p=.008). Fos density was not significantly different between the paired and unpaired conditions (p=1.000) but was greater in the paired (p=.009) and unpaired (p=.049) conditions relative to the home-cage condition. There was no Group x Condition interaction ($F_{2,31}$=.36, p=.702).

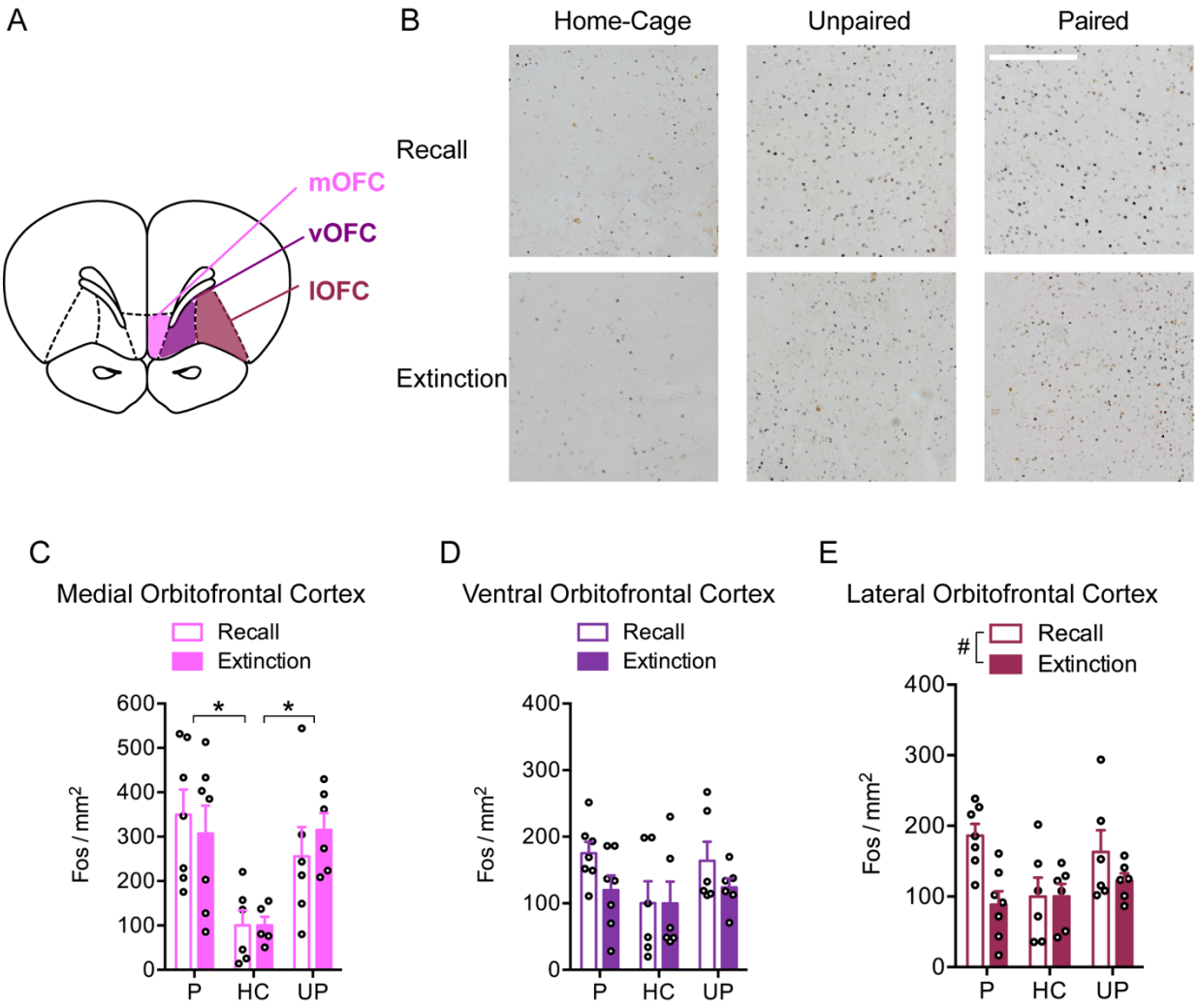


Chapter 3 Figure 2. Fos density in the prelimbic (PL) and infralimbic (IL) cortices. **A** A schematic diagram indicates regions where Fos was quantified in the PL and IL. **B** Representative Fos photomicrographs in the PL. Scale bar is 250 µm. **C** Fos density in the PL was greater following recall relative to extinction, and for the paired and unpaired groups relative to home-cage groups. **D** Fos density in the IL was not different between recall and extinction and was greater for the paired and unpaired groups relative to home-cage groups. #p<.05, recall vs extinction main effect. *p<.05, post hoc comparisons following main effect of training condition.

Orbitofrontal cortex. In the medial orbitofrontal cortex (mOFC), Fos density was greater in the paired and unpaired groups relative to the home-cage groups (Fig. 3A, B, C; Condition, $F_{2,31}$=10.39, p<.001). Fos density was not significantly different between the paired and unpaired conditions (p=1.000) but was greater in the paired (p<.001) and unpaired (p=.005) conditions relative to the home-cage condition. Like results in the IL, Fos density was similar during recall and extinction (Group, $F_{1,31}$=.02, p=.894), and there was no Group x Conditioned interaction (Group x Condition, $F_{2,31}$=.50, p=.610).

In contrast to other cortical regions, in the ventral orbitofrontal cortex (vOFC), there was no significant difference in Fos density between the recall and extinction groups, and there were no significant differences between the paired, unpaired, and home-cage conditions (Fig. 3A, D; Group, $F_{1,32}$=2.42, p=.130; Condition, $F_{2,32}$=2.18, p=.129; Group x Condition, $F_{2,32}$=.65, p=.527).

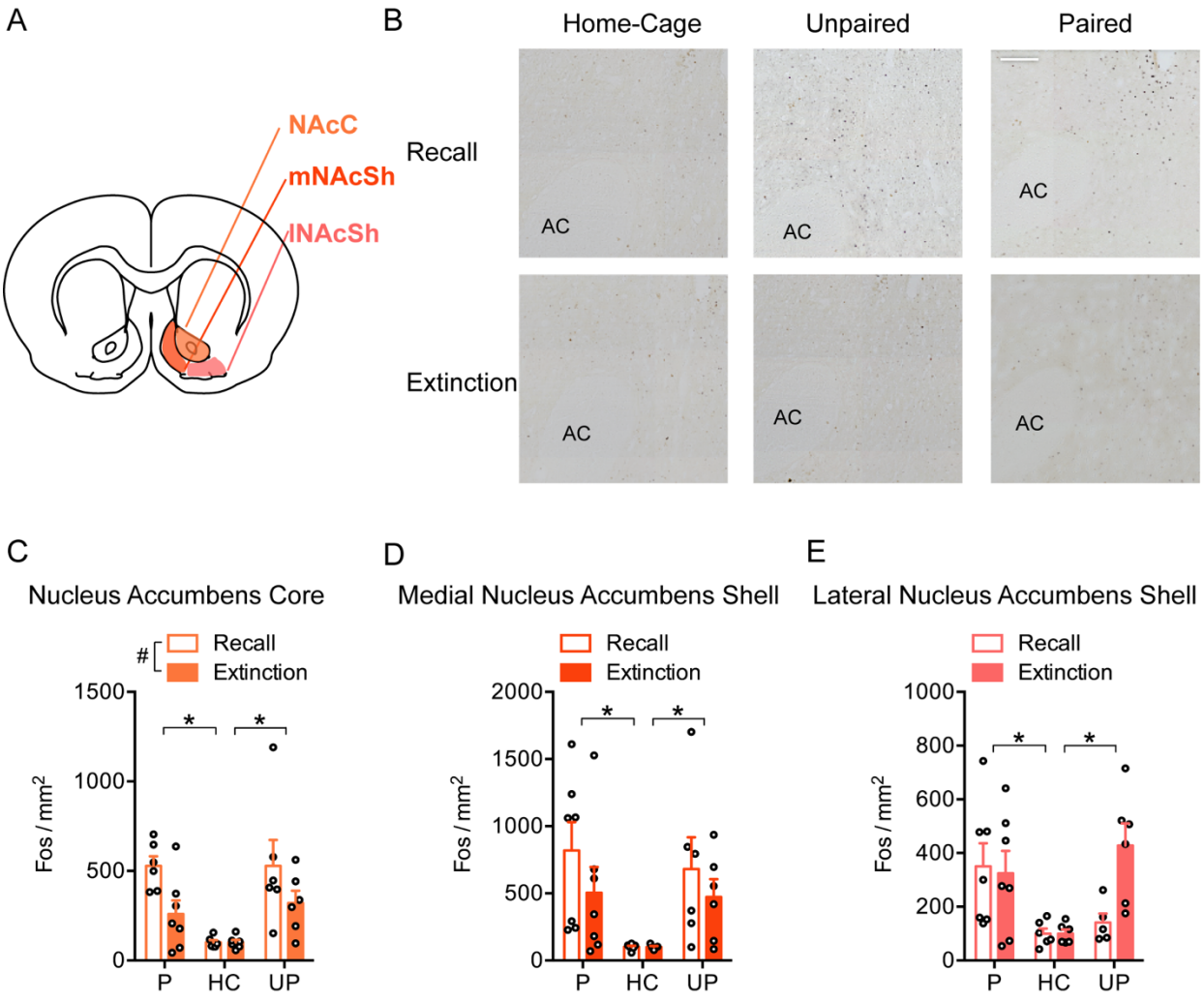
In the lateral orbitofrontal cortex (lOFC), there was no significant differences between paired, unpaired, and home-cage conditions (Fig. 3A, E; Condition, $F_{2,32}$=2.38, p=.109). There was a significant main effect of recall vs extinction, in which Fos density was greater during recall as compared to extinction (Group, $F_{1,32}$=7.23, p=.011), but there was no significant Group x Condition interaction ($F_{2,32}$=2.81, p=.075), indicating that the lower Fos density in the extinction groups did not depend upon history of conditioning.



Chapter 3 Figure 3. Fos density in the medial orbitofrontal cortex (mOFC), ventral orbitofrontal cortex (vOFC), and lateral orbitofrontal cortex (lOFC). **A** A schematic diagram indicates regions where Fos was quantified in the mOFC, vOFC, and lOFC. **B** Representative Fos photomicrographs in the mOFC. Scale bar is 250 µm. **C** Fos density in the mOFC was not different between recall and extinction and was greater for the paired and unpaired conditions relative to the home-cage condition. **D** Fos density in the vOFC was not different between recall and extinction and was similar between the paired, unpaired, and home-cage conditions. **E** Fos density in the lOFC was greater following recall relative to extinction, and was similar between the paired, unpaired, and home-cage conditions. #p<.05, recall vs extinction main effect. *p<.05, post hoc comparisons following main effect of training condition.

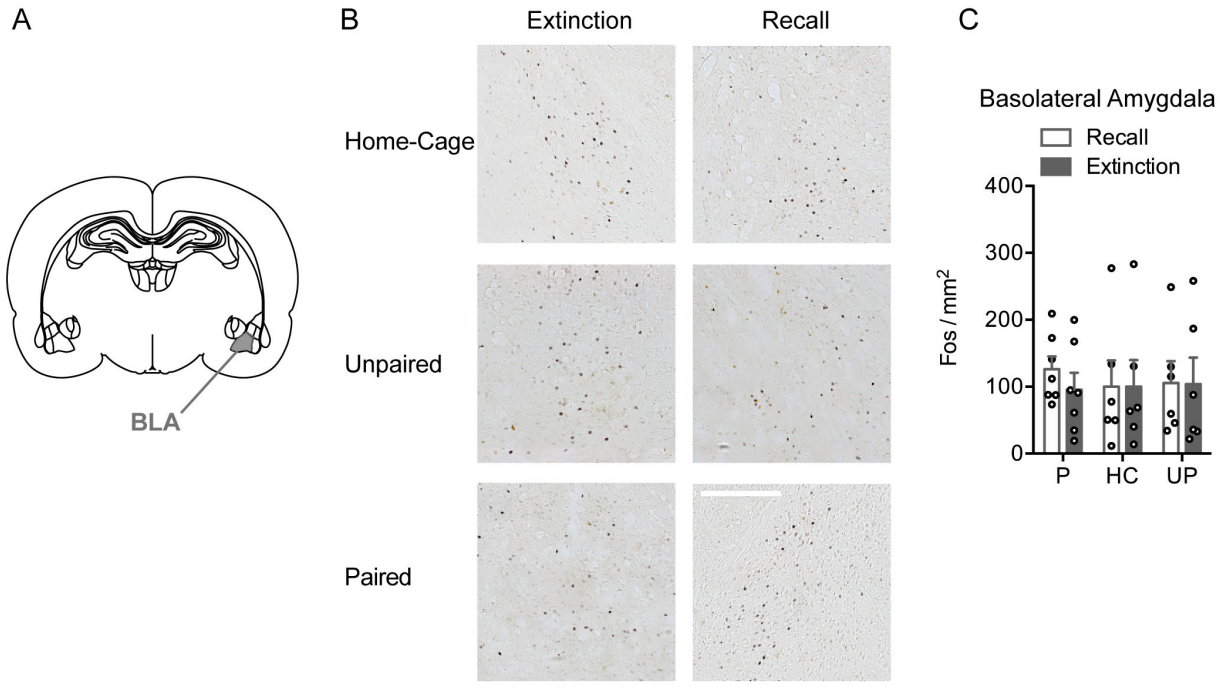
Nucleus accumbens. In the nucleus accumbens core (NAcC), there was a main effect of condition in which the paired and unpaired groups showed greater Fos density compared to the home-cage controls (Fig. 4A, B, C; Condition, $F_{2,31}$=10.86, p<.001). There was no significant difference between the paired and unpaired conditions (p=1.000), but Fos density was markedly greater in the paired (p=.002) and unpaired (p=.001) conditions relative to the home-cage condition. Like results in the PL, there was a main effect of recall vs extinction, in which Fos density was greater during recall compared to extinction (Group, $F_{1,31}$=6.49, p=.016), but there was no Group x Condition interaction ($F_{2,32}$=1.70, p=.199).

Fos density in the medial and lateral nucleus accumbens shell (mNAcSh and lNAcSh), was found to be greater in the paired and unpaired conditions relative to the home-cage condition, but there was no difference between recall and extinction groups. In the mNAcSh, there was a main effect of condition (Fig. 4A, D; Condition, $F_{2,31}$=5.88, p=.007) indicating that Fos density was no different between the paired and unpaired conditions (p=1.000) but was markedly greater in the paired (p=.008) and unpaired (p=.036) conditions relative to the home-cage controls. There was no significant difference between recall and extinction groups (Group, $F_{1,31}$=1.53, p=.225) and no significant Group x Condition interaction ($F_{2,31}$=.43, p=.658). Similarly, in the lNAcSh, Fos density was similar between the paired and unpaired conditions (p=1.000) but was greater in the paired (p=.003) and unpaired (p=.037) conditions relative to the home-cage condition (Fig. 4A, E; Condition, $F_{2,31}$=7.10, p=.003). There was no significant main effect of recall vs extinction (Group, $F_{1,31}$=2.52, p=.123), and no Group x Condition interaction ($F_{2,31}$=3.19, p=.055).



Chapter 3 Figure 4. Fos density in the nucleus accumbens core (NAcC), medial nucleus accumbens shell (mNAcSh), and lateral nucleus accumbens shell (lNAcSh). **A** A schematic diagram indicates regions where Fos was quantified in the NAcC, mNAcSh, and lNAcSh. **B** Representative Fos photomicrographs in the NAcC. Scale bar is 200 µm. Anterior commissure (AC). **C** Fos density in the NAcC was greater following recall relative to extinction, and for the paired and unpaired conditions relative to the home-cage condition. **D** Fos density in the mNAcSh, and **E** lNAcSh, was not different between recall and extinction, and was greater for the paired and unpaired conditions relative to the home-cage condition. #p<.05, recall vs extinction main effect. *p<.05, post hoc comparisons following main effect of training condition.

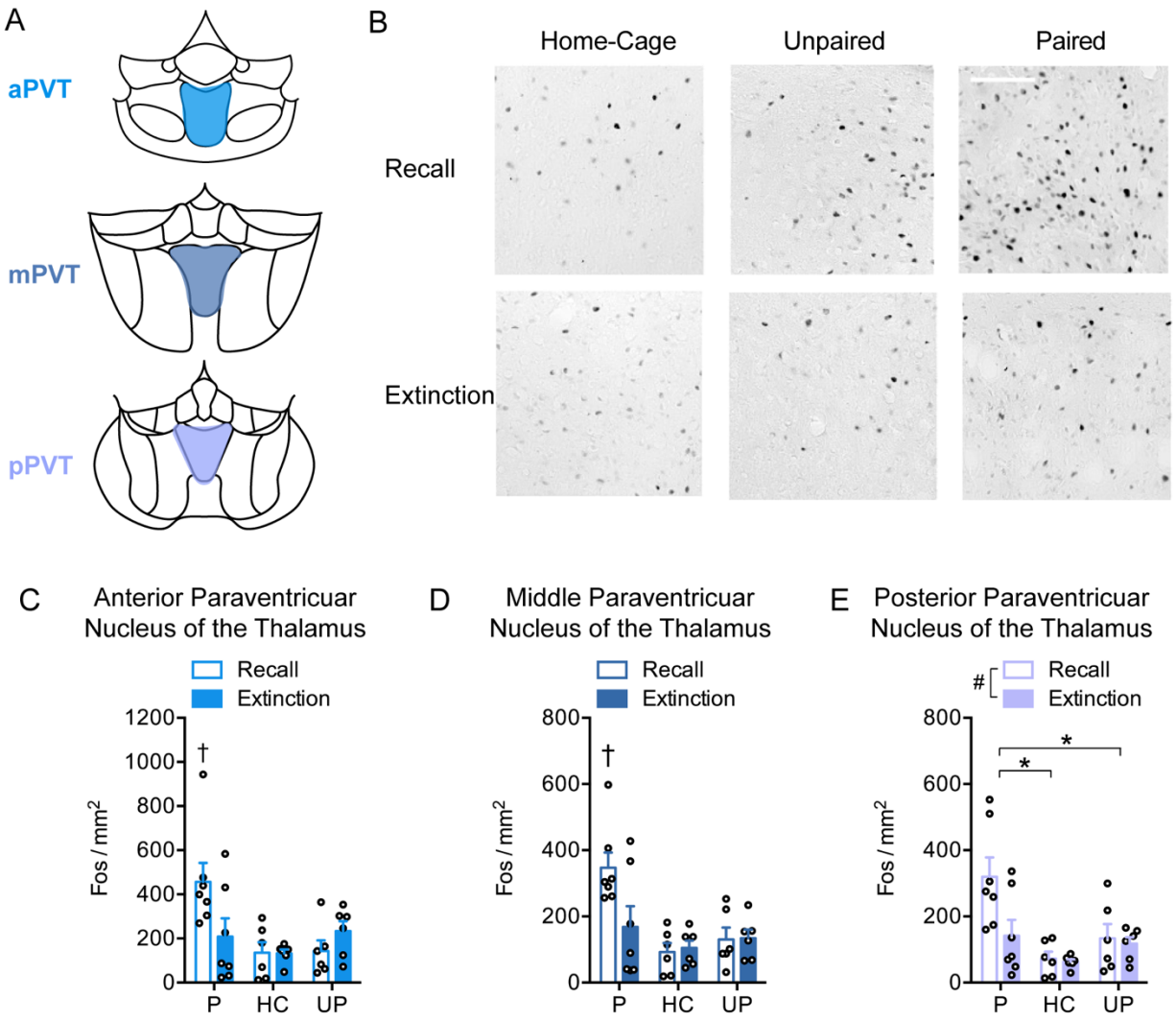
Basolateral amygdala. In contrast to other brain regions, the basolateral amygdala (BLA) showed no significant differences in Fos density between the recall and extinction groups, as well as no differences between the paired, unpaired, and home-cage conditions (Fig. 5A, B, C; Group, $F_{1,32}$=.17, p=.687; Condition, $F_{2,32}$=.06, p=.944; Group x Condition, $F_{2,32}$=.15, p=.863).



Chapter 3 Figure 5. Fos density in the basolateral amygdala (BLA). **A** A schematic diagram indicates region where Fos was quantified in the BLA. **B** Representative Fos photomicrographs in the BLA. Scale bar is 250 µm. **C** Fos density in the BLA was not different between recall and extinction, and was similar between the paired, unpaired, and home-cage conditions.

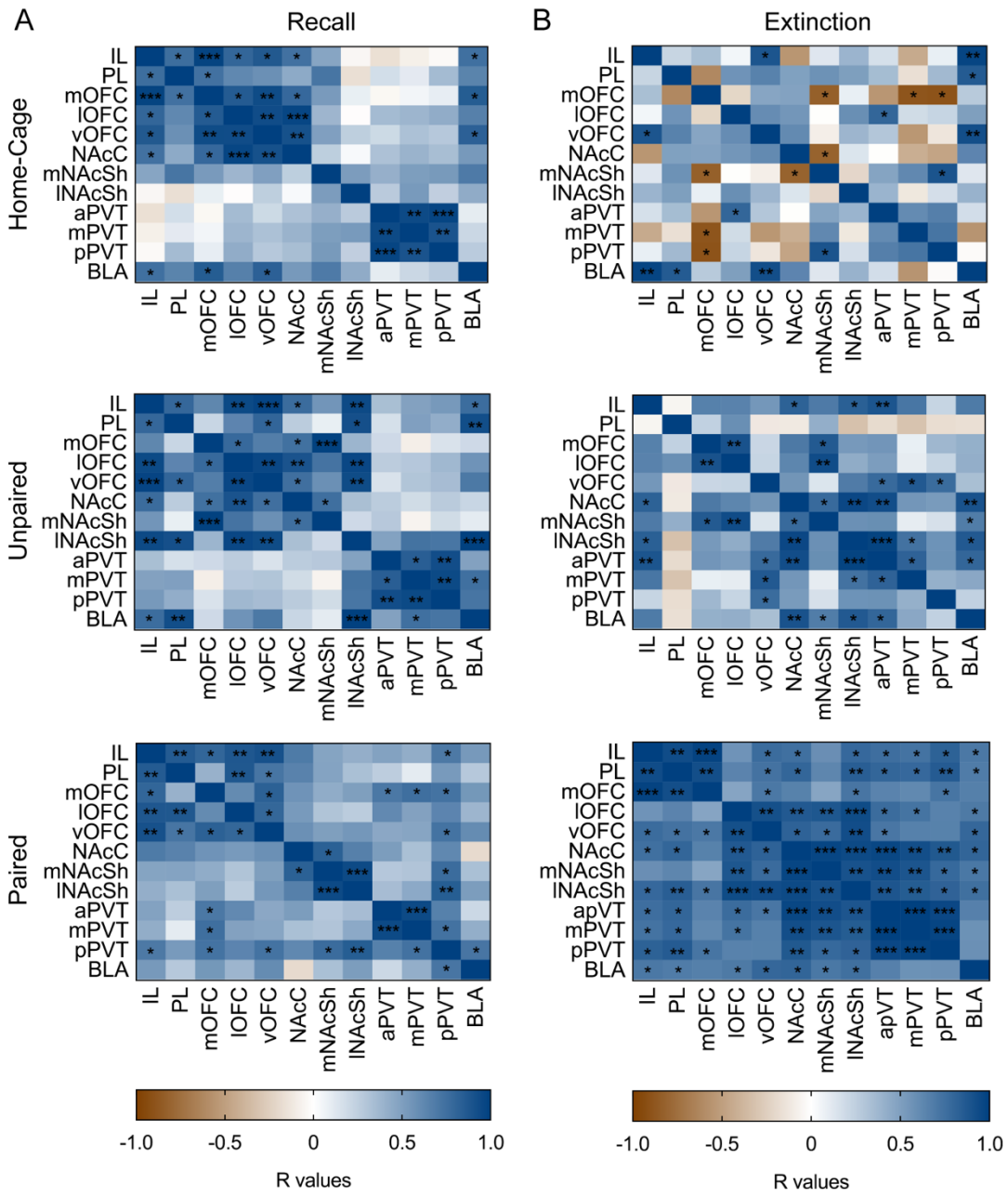
Paraventricular nucleus of the thalamus. Fos density in the anterior and middle paraventricular nucleus of the thalamus (aPVT and mPVT) in the recall groups was found to be much greater in the paired condition relative to the unpaired and home-cage conditions, but this effect was not observed in the extinction groups. In the aPVT, Fos density was greater for the paired recall group compared to the paired extinction group (p=.006). Moreover, Fos density was greater for the paired recall group compared to the unpaired recall (p=.004) and home-cage recall groups (p=.003). All other post-hoc comparisons were not significant (p>.05) (Fig. 6A, B, C; Group, $F_{1,32}$=1.09, p=.304; Condition, $F_{2,32}$=5.51, p=.009; Group x Condition, $F_{2,32}$=3.99, p=.028). Similarly, in the mPVT, Fos density was greater for the paired recall group compared to the paired extinction group (p=.003). Further, Fos density was greater for the paired recall group relative to the unpaired recall (p=.002) and home-cage recall groups (p<.001). No other statistical post-hoc comparisons were significant (p>.05) (Fig. 6A, D; Group, $F_{1,32}$=2.56, p=.119; Condition, $F_{2,32}$=8.61, p=.001; Group x Condition, $F_{2,32}$=3.57, p=.040).

A similar pattern of results occurred in the posterior paraventricular nucleus of the thalamus (pPVT), but the interaction between Group and Condition did not reach statistical significance (Fig. 6A, E; Group x Condition, $F_{2,32}$=3.00, p=.064). Fos density was greater during recall compared to extinction (Fig. 6F; Group, $F_{1,32}$=4.36, p=.045; Condition, $F_{2,32}$=8.96, p=.001). Fos density was greater for the paired condition compared to the unpaired (p=.036) and home-cage conditions (p=.001) but was not different between the unpaired and home-cage conditions (p=.493).

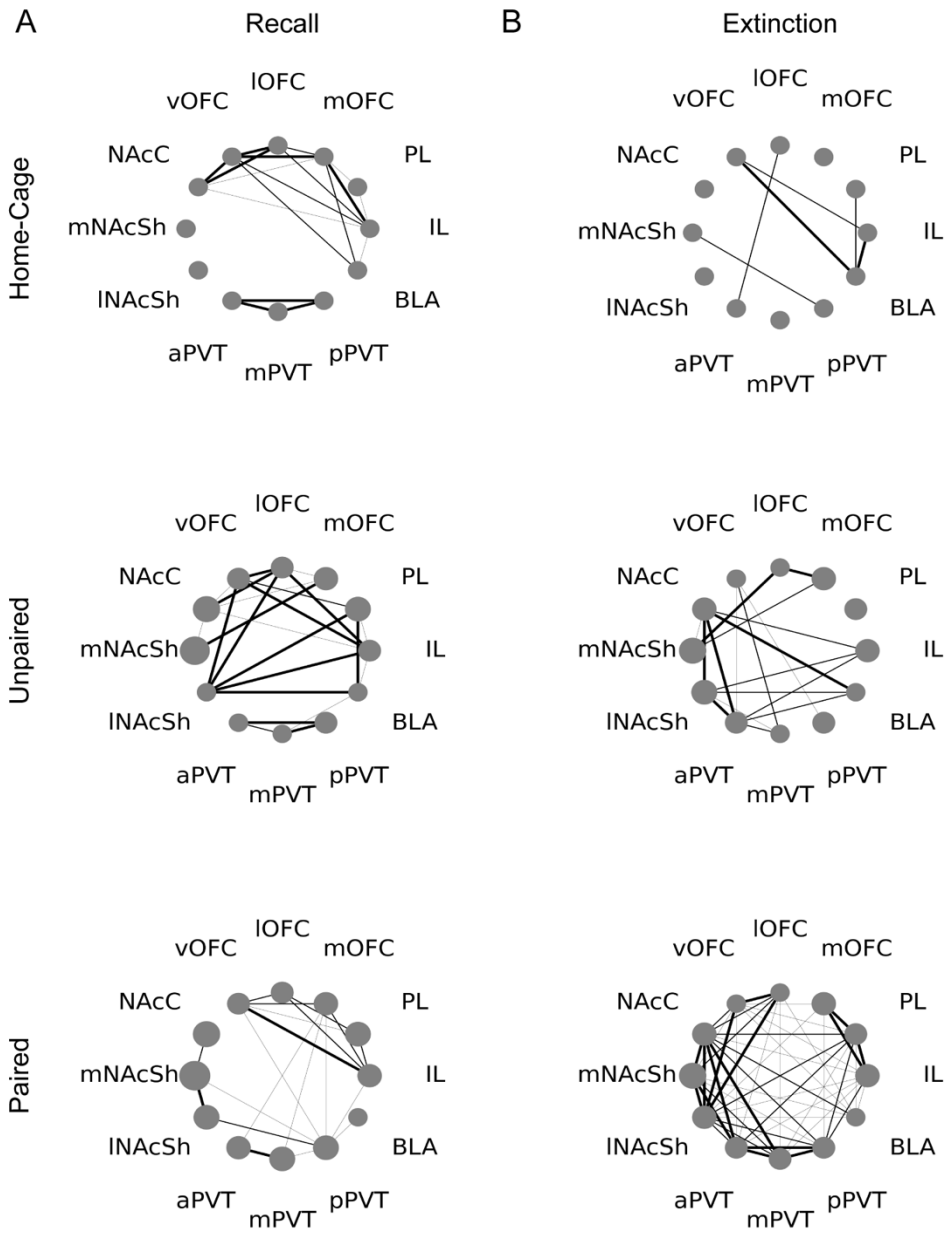


Chapter 3 Figure 6. Fos density in the anterior paraventricular nucleus of the thalamus (aPVT), middle paraventricular nucleus of the thalamus (mPVT), and posterior paraventricular nucleus of the thalamus (pPVT). **A** A schematic diagram indicating regions where Fos was quantified in the aPVT, mPVT and pPVT. **B** Representative Fos photomicrographs in the aPVT. Scale bar is 100 µm. **C** Fos density in the aPVT, and **D** mPVT, was greatest for the paired recall group compared to the paired extinction, unpaired recall and home-cage recall groups. **E** Fos density in the pPVT was greater following recall relative to extinction, and for the paired group relative to the unpaired and home-cage groups. †$p<.05$, significant group x condition interaction. #$p<.05$, recall vs extinction main effect. *$p<.05$, post hoc comparisons following main effect of training condition.

Correlational network analysis. To investigate the functional co-activation of the regions of interest, we computed correlations for each pair of regions within each group of animals. We then created inter-regional correlation matrices (Fig. 7A, B) and network activation graphs for each experimental group following recall and extinction (Fig. 8A, B). Network activation graphs display only strong and significant correlations ($r \geq .5$, $p < .05$). The number of inter-regional correlations was greater for the paired extinction group compared to the paired recall group, with sparse connectivity in the home-cage and unpaired control conditions. Cortical sites showed an increase in connectivity during extinction and less so in recall. Moreover, we observed high correlations within related areas such as the different PVT subregions, and between the IL and PL, which were amongst the most widely correlated structures. Moreover, within the different PVT subregions, we observed increased node sizes in the paired recall, but not unpaired recall network activation graphs, reflecting an increase in Fos density compared to the home-cage controls. Interestingly, we found paired extinction specific network correlations with activity correlations between the IL and the mPVT, and between the mNAcSh and the aPVT and mPVT that were absent in the other groups.



Chapter 3 Figure 7. Cross-correlation analysis of Fos density in the home-cage (top panel), unpaired (middle panel), and paired (bottom panel) during recall and extinction. **A** Pearson correlation matrices showing inter-regional correlations for Fos density during recall, and **B** extinction. Axes represent brain regions. Colours reflect Pearson correlation coefficients and labels within squares correspond to P values of correlations. *p<.05; **p<.01; ***p<.001.



Chapter 3 Figure 8. Network activity analysis of Fos density in the home-cage (top panel), unpaired (middle panel), and paired (bottom panel) conditions during extinction and recall. **A** Network activation graphs indicate the strongest correlations ($r \geq .5$, $p < .05$) between brain areas during recall, and **B** extinction. Node size is proportional to the change of Fos density relative to the home-cage control condition. Connecting line transparency represents correlation strength.

Discussion

The present study investigated the neural correlates of appetitive Pavlovian conditioning recall and extinction learning using Fos immunohistochemistry and correlational network connectivity analysis. We established a behavioural paradigm that allowed for the observation of differential Fos expression induced by the recall versus the extinction of responding to an appetitive Pavlovian CS. We found greater Fos density during recall compared to extinction in the PL, lOFC, NAcC, aPVT, mPVT, and pPVT. In contrast, Fos density was similarly elevated for the recall and extinction conditions in the IL, mOFC, mNAcSh and lNAcSh. Furthermore, Fos density was greater for the paired condition relative to the unpaired condition in the aPVT, mPVT, and pPVT, suggesting that these regions are specifically recruited during responding elicited by the CS. However, there were no differences between the paired, unpaired, and home-cage conditioned for the BLA, vOFC, and lOFC, suggesting that these regions might not be implicated in appetitive Pavlovian recall or extinction. In all other brain areas investigated (IL, PL, mOFC, mNAcSh, and lNAcSh), we found greater Fos density for the paired and unpaired conditions relative to the home-cage condition, and no difference between the paired and unpaired conditions. This suggests that many of these structures may be similarly recruited in both paired and unpaired conditions. Lastly, we observed high correlations of Fos density across investigated brain regions especially in the paired extinction group compared to all other groups, which revealed recall- and extinction-specific neural networks. Together, our results provide novel network activation graphs of recall and extinction in appetitive Pavlovian conditioning and emphasize the role of the PVT in responding to discrete appetitive Pavlovian cues.

Acquisition and extinction

The present study used an appetitive Pavlovian learning design to identify neural correlates of recall and extinction instead of the predominantly used Pavlovian fear conditioning and operant reward-seeking models (Bouton et al., 2021; Hamlin et al., 2009; Perry & McNally, 2013; Warren et al., 2016). During conditioning, rats in the paired conditions showed an increase in ΔCS port entries across sessions indicating that they learned to associate sucrose reward with the CS, and rats in the unpaired and home-cage conditions did not acquire a conditioned response. After one extinction session, the paired recall and the paired extinction groups displayed greater ΔCS port entries compared to the control groups, and after six extinction sessions, ΔCS port entries were reduced in the paired extinction group, while responding in the unpaired and home-cage groups remained low. Therefore, our behavioural paradigm efficiently conditioned and extinguished responding to a Pavlovian CS in the paired condition and allowed for the comparison of brain activation induced by the CS on the first extinction (recall) versus the last extinction session.

Interestingly, in several brain regions investigated, including the PL, IL, mOFC, and subregions of the NAc, Fos density was similarly higher for both the paired and unpaired conditions relative to the home-cage controls. Similar results have been found using an appetitive Pavlovian conditioning task with a food reinforcer where Fos expression in the IL, PL and OFC were no different for paired and unpaired conditions (Yager et al., 2015). One argument could be that rats in the unpaired condition learned an association between sucrose delivery and the contextual stimuli present during training (i.e., a context-US association; Reppucci & Petrovich, 2012). Therefore, brain regions may have been activated both by responding elicited by the CS (paired condition) and by the appetitive context (unpaired condition) (Reppucci & Petrovich, 2012). Consistent with this idea, both the IL and PL integrate contextual information to guide

behavioural responses (Hyman et al., 2012; Moorman & Aston-Jones, 2015). Therefore, similar Fos densities in the paired and unpaired conditions may be due to different contextual associations that induce levels of neural activation similar to that induced by the CS-US association.

Region activation patterns

Cortical regions. In the IL, Fos density was similar during recall and extinction, suggesting that IL was active during both induction periods. IL activation during extinction is consistent with the idea that the IL is involved in extinction learning and inhibiting conditioned responding. For example, activation of the IL attenuates renewal, while IL lesions enhance the reinstatement of extinguished appetitive Pavlovian conditioned responding for natural rewards (Rhodes & Killcross, 2004; Villaruel et al., 2018). Similarly, IL inactivation disinhibits operant drug-seeking, promotes the reinstatement of operant drug-seeking, and disrupts the retrieval of extinction memories (Gutman et al., 2017; Peters et al., 2008). However, we also observed activation in the IL during recall of appetitive Pavlovian conditioning. One potential interpretation is that the recall session may have functioned as a first extinction session, and IL activity was maintained throughout early and extensive extinction learning. Consistently, the IL has been shown to be active during early and late extinction of operant food-seeking (Warren et al., 2016). Alternatively, the IL could also be involved in the recall of appetitive Pavlovian conditioned responding. For example, previous work from our laboratory found that IL inactivation during the first extinction session reduces responding to a sucrose cue, suggesting that the IL may contribute to appetitive Pavlovian recall (Mendoza et al., 2015). Further, others have also shown that neural activity in the IL is similar during both extinction and the renewal of operant drug-seeking (Perry & McNally, 2013). Therefore, the IL may mediate both the expression and extinction of appetitive Pavlovian conditioning. Consistently, recent work has shown that discrete neural ensembles within the IL are involved in the expression and inhibition of conditioned responding for both natural and drug rewards (Suto et al., 2016; Warren et al., 2016, 2019). Thus, in the present study, the similar activation in the IL during recall and extinction of appetitive Pavlovian conditioned responding, may have been due to separate ensembles. This question could be addressed by determining whether the same neuronal ensembles remain active from recall to extinction, and whether recall and extinction induce activation in overlapping or distinct neuronal ensembles (Josselyn & Tonegawa, 2020).

In the PL, we found elevated Fos density in the recall group compared to the extinction group. This finding is consistent with the prominent idea that the PL is important for promoting the expression of conditioned responding but not extinction (Peters et al., 2009). Support for this hypothesis comes from studies showing that PL inactivation disrupts operant reward-seeking and attenuates renewal and reinstatement for both natural and drug rewards (Corbit & Balleine, 2003; Fuchs et al., 2005; McLaughlin & See, 2003; Trask et al., 2017a). Further, like our results, Fos expression in the PL is greater during renewal as compared to extinction of responding to a Pavlovian CS paired with food (Anderson & Petrovich, 2017). Moreover, inactivation of the PL disrupts the expression of conditioned fear but does not affect extinction learning or retrieval (Choi et al., 2010; Laurent & Westbrook, 2009; Sierra-Mercado et al., 2011). Our results, however, are inconsistent with the finding that inhibition of the PL during the first extinction session does not affect Pavlovian conditioned responding for sucrose (Mendoza et al., 2015). Thus, further studies are needed to determine what the PL encodes during recall of appetitive Pavlovian conditioned responding. Overall, our results support a majority of the literature

suggesting that the PL contributes to the expression rather than the extinction of conditioned responding.

In the OFC, we observed different patterns of neural activation between subregions, which is consistent with functional heterogeneity between distinct OFC subregions (Heilbronner et al., 2016). Like the IL, Fos density in the mOFC was comparable during recall and extinction. This finding suggests that the IL and mOFC may play similar roles during recall and extinction of appetitive Pavlovian responding. Consistently, the mOFC and IL have been shown to respond similarly to Pavlovian cues that predict sucrose in decision-making and behavioural inhibition tasks (Bradfield et al., 2015; Hardung et al., 2017; Verharen et al., 2020). In the lOFC, Fos density was greater following recall than extinction. This finding appears consistent with studies showing that lOFC inactivation impairs the reinstatement of operant drug-seeking and supports a role of the lOFC in the expression of conditioned responding (Arinze & Moorman, 2020; Fuchs et al., 2004). However, this effect is unclear because Fos densities in the paired and unpaired groups did not significantly differ from home-cage controls. Lastly, in the vOFC, we found no difference in Fos density between conditions or as a function of recall vs extinction, suggesting that the vOFC was not associated with recall or extinction of conditioned responding. Together, our results are consistent with findings that vOFC and lOFC inactivation have no effect on the acquisition or extinction of responding to a Pavlovian CS that predicts sucrose (Burke et al., 2008, 2009). Further, the lOFC has been implicated in behavioural inhibition in Pavlovian over-expectation but not extinction (Lay et al., 2020; Takahashi et al., 2009). Together, our results suggest that the mOFC may play a similar role as the IL in appetitive Pavlovian recall and extinction, whereas the ventral and lateral OFC play lesser roles.

Striatal structures. The differential pattern of neural activity observed in the core and shell subregions of the NAc is consistent with evidence that these structures play distinct functional roles in learning and memory. In the NAcC, we found greater Fos density during recall relative to extinction, while in the mNAcSh and lNAcSh, Fos density was similar during recall and extinction. Like our findings, Fos expression in the NAcC is greater during renewal compared to extinction in an operant drug-seeking task, whereas Fos expression in the mNAcSh and lNAcSh is similar during extinction and the renewal of operant drug-seeking (Perry & McNally, 2013). Our findings, however, are inconsistent with work showing that inactivation of mNAcSh drives reinstatement of operant drug-seeking and is therefore involved in extinction memory retrieval (Peters et al., 2008). This difference could be due to distinct neurons within the mNAcSh that mediate expression and extinction of conditioned responding depending on the output regions (Gibson et al., 2018). Together, these results suggest that the NAcC is involved in recall, whereas the lNAcSh and mNAcSh may be involved in both recall and extinction of appetitive Pavlovian conditioned responding.

Amygdala. In the BLA, we did not detect a significant difference in Fos density in either the paired or unpaired groups compared to the home-cage control, or between recall and extinction. However, the BLA has been shown to have a prominent role in extinction learning using aversive Pavlovian conditioning (Knapska & Maren, 2009; Laurent & Westbrook, 2008; Lingawi et al., 2019; Zimmerman & Maren, 2010) and operant reward-seeking procedures for both natural and drug rewards (Fuchs et al., 2005; Fuchs & See, 2002; McLaughlin & Floresco, 2007; McLaughlin & See, 2003). Interestingly, others have shown using single-unit recordings that the BLA is involved in learning during over-expectation, although inactivation of the BLA

has no effect on responding in over-expectation using a sucrose reward (Haney et al., 2010; Lucantonio et al., 2015). Therefore, despite Fos density in the BLA not being associated with the recall or extinction of appetitive Pavlovian conditioned responding, it may be that the technique used here was unable to detect the BLA's involvement in these learning processes.

Thalamic nuclei. Our results indicate that PVT neurons were selectively activated by the recall of appetitive Pavlovian conditioned responding and showed reduced activation to the CS following extinction. Subregions across the anterior-posterior axis of the PVT are composed of distinct neuronal subtypes (Gao et al., 2020) and are thought to have separate roles in reward learning (Barson et al., 2020). We found that Fos density in the aPVT and mPVT was significantly greater for the paired recall group vs the unpaired recall group. However, this greater Fos density in the paired vs unpaired recall groups did not reach significance in the pPVT. It has similarly been reported that neurons in the aPVT, but not the pPVT, regulate operant sucrose-seeking during recall (Do-Monte et al., 2017). Interestingly, in all subregions of the PVT, Fos densities were greater for the paired condition relative to the unpaired condition, suggesting that this region is specifically recruited during responding to an appetitive Pavlovian CS. Consistently, an incentive stimulus previously paired with either a food or opioid reward has been shown to active cells in the PVT in a paired, but not unpaired, training condition (Yager et al., 2015). Moreover, the PVT has previously been shown to be activated by cues that predict natural rewards, and by incentive stimuli previously paired with drug or natural rewards (Flagel et al., 2011; Haight et al., 2017; Igelstrom et al., 2010; Yager et al., 2015). Therefore, the PVT, and especially the aPVT and mPVT subregions may be specifically recruited by Pavlovian cues during recall.

Network analysis

To investigate the functional co-activation of the regions of interest, we computed correlations for each pair of regions within each group of animals. Moreover, increases in Fos density in the paired and unpaired conditions relative to the home-cage controls were also computed for both the recall and extinction groups and were reflected by the node sizes in the network connectivity graphs. Consistently across groups, we found high correlations of Fos density within the different cortical, striatal, and thalamic subregions. This finding may reflect the anatomical connections between subregions of a given brain area (Vertes, 2004; Vertes & Hoover, 2008). Overall, we also found greater inter-regional connectivity in the paired and unpaired groups compared to the home-cage controls, suggesting that multiple brain regions were synchronously engaged following behavioural training. Surprisingly, in the unpaired recall group, we observed strong correlations between regions that appeared to have weakened in the unpaired extinction group. The significance of these inter-regional correlations is unclear but could be due to presentations of the sucrose US in the ITI during Pavlovian conditioning, which allowed context-US associations to develop. Notably, within the paired group, we observed greater and stronger inter-regional correlations in the extinction group compared to the recall group. Importantly, the paired extinction group also had the greatest degree of inter-region connectivity in comparison to all other groups. This finding suggests that extinction of appetitive Pavlovian conditioning depended on concurrent activation of multiple brain regions, perhaps to update the original Pavlovian conditioning memory and suppress responding.

The IL is thought to be a critical brain region that mediates extinction through its downstream projections to the NAcSh and the BLA in appetitive operant procedures, and

aversive Pavlovian conditioning, respectively (Peters et al., 2009). However, in the paired extinction group of our appetitive Pavlovian conditioning task, we did not find correlated Fos expression between the IL and mNAcSh and only a slight correlation between the IL and BLA. These results suggest that these circuits may not be as critical in extinction of appetitive Pavlovian conditioned responding. Consistently, recent evidence from our laboratory using a similar appetitive Pavlovian conditioning procedure suggests that the IL-to-mNAcSh pathway does not suppress renewal through an extinction mechanism (Villaruel et al., 2022). Interestingly, the paired extinction group showed strong correlations between the IL and PL, and the PL in turn had strong co-activation with striatal structures such as the NAcC and the lNAcSh. Recent work has shown that the PL can be involved in extinction of Pavlovian fear through inputs from the IL (Marek et al., 2018). Therefore, the IL could also be involved in extinction of appetitive Pavlovian conditioning via feed-forward inputs to the PL. Further work is necessary to determine how the IL contributes to the extinction of appetitive Pavlovian conditioned responding.

The PL and particularly its projection to the NAcC, is thought to be important for the expression of appetitive operant responding (Peters et al., 2008). However, we did not detect a significant correlation in Fos density between the PL and NAcC in our paired recall group. This difference may be due to the use of an appetitive Pavlovian learning procedure. Alternatively, the use of sucrose as the US may also play a role in detecting co-activation between the PL and NAcC during recall. In an operant conditioning task, the PL-to-NAcC pathway was found to be recruited during reinstatement of operant cocaine-seeking but not sucrose-seeking (McGlinchey et al., 2016). Therefore, the PL and NAcC may be differentially recruited depending on the type of US. Furthermore, we found PL activation to be correlated with the NAcC in the paired extinction group, suggesting that the PL and NAcC may instead be involved in extinction learning of appetitive Pavlovian conditioning. More work is necessary to identify the role of the PL and its projections to the NAcC in recall and extinction of appetitive Pavlovian conditioned responding.

The PVT has been heavily implicated in appetitive Pavlovian responding especially through its projections from the PL and IL. We found a relatively strong correlation in Fos density between the PL and PVT, particularly in the aPVT and pPVT in the paired extinction but not the paired recall group. These results suggest that a functional network between the PL and subregions of the PVT may not be important for recall and expression of responding to a sucrose-predictive cue. Consistently, we have recently shown that optogenetic stimulation of the PL-to-PVT pathway does not affect renewal of responding to a sucrose cue (Brown & Chaudhri, 2022). This finding is consistent with other reports that the PL-to-PVT may be implicated in conditioned responding for drugs, but not natural rewards, like sucrose (Giannotti et al., 2018). The IL was also found to have significant correlations in Fos density with the aPVT and the mPVT in the paired extinction group that was absent in the paired recall group. This finding is consistent with previous research demonstrating a role of the IL-to-PVT pathway in fear extinction retrieval (Tao et al., 2021) and in extinction memory retrieval using an appetitive Pavlovian conditioning procedure (Brown & Chaudhri, 2022). Together, our results suggest that the PL-to-PVT pathway may not be critical for recall but instead may work alongside the IL-to-PVT pathway in extinction of appetitive Pavlovian conditioned responding for sucrose.

The PVT, and especially the aPVT and mPVT were also found to have strong activation correlations with striatal structures that was specific to the paired extinction but not paired recall group. This finding is consistent with work using operant drug-seeking which demonstrated that

extinction learning fundamentally alters the function of the PVT-to-NAc pathway (Giannotti et al., 2021). Moreover, it has been suggested that the PVT-to-mNAcSh pathway may deepen extinction of morphine memories (Keyes et al., 2020). Furthermore, we observed a strong correlation of Fos density between the aPVT and mNAcSh, which is consistent with a previous report that the aPVT-to-NAcSh pathway is important for suppressing operant sucrose-seeking in extinction conditions (Do Monte et al., 2017). Together, our results support a role for the neural circuit between the anterior-to-middle PVT and ventral striatum subregions in extinction of appetitive Pavlovian conditioned responding.

The OFC is another key region that has been implicated in conditioned responding for rewarding outcomes (Howard & Kahnt, 2021; Izquierdo, 2017). Particularly, the OFC is thought to interact with the BLA for expression of drug-seeking in a renewal task (Lasseter et al., 2011). However, we did not detect a significant correlation in Fos density between OFC subregions and the BLA in our paired recall group. Instead, we found strong correlations between the OFC and striatal subregions in the paired extinction group that were not present in the paired recall group. This finding is in contrast with work showing that the OFC is typically involved in over-expectation but not extinction of appetitive conditioned responding (Lucantonio et al., 2015; Lay et al., 2020). Our result suggests that the OFC, and particularly the vOFC and lOFC may interact with the NAc to mediate extinction of appetitive Pavlovian conditioned responding. The behavioural inhibition that is necessary in both over-expectation and extinction procedures may result in the common recruitment of the OFC.

Conclusions

We analyzed Fos density to evaluate the roles of multiple brain structures in recall and extinction of appetitive Pavlovian conditioned responding. We found that the IL, mOFC, mNAcSh and lNAcSh showed similar activation following both recall and extinction, implicating these regions in both the expression and suppression of appetitive Pavlovian responding. In contrast, the PL, NAcC and subregions of the PVT showed greater activation during recall than extinction, consistent with the selective roles of these structures in the expression of conditioned responding. Only the PVT was found to show enhanced activation in the paired versus the unpaired condition, suggesting that the PVT may play a special role in responding to a discrete Pavlovian CS. Lastly, analysis of inter-region coactivation patterns revealed increased connectivity in the paired extinction group compared to all other groups, implying that extinction of appetitive Pavlovian responding requires the coordinated activity of various brain regions. The present study provides novel evidence on the neural correlates and connectivity of multiple brain regions underlying the expression and suppression of conditioned responding to appetitive stimuli.

Chapter 4: Optogenetic stimulation of infralimbic cortex projections to the paraventricular thalamus attenuates context-induced renewal

Introduction

In Pavlovian conditioning, extinction is the decline of conditioned responding when the conditioned stimulus (CS) is no longer followed by the expected unconditioned stimulus (US). Renewal is a phenomenon in which conditioned responding returns following a change in context after extinction, and it is typically modeled in the laboratory using the ABA renewal design (Bouton & Bolles, 1979). In this design, conditioning occurs in a distinct context (Context A), followed by extinction in a different context (Context B), and lastly, a renewal test in the original training context (Context A). ABA renewal is demonstrated when there is a significant increase in non-reinforced conditioned responding in the renewal Context A as compared to the extinction Context B. Our current understanding of the neural mechanisms of extinction and context-induced renewal is based primarily on findings obtained from Pavlovian fear conditioning and appetitive operant drug-seeking procedures (for reviews see Bouton et al., 2021; Crombag et al., 2008). However, little is known about the neural mechanisms that mediate extinction and renewal processes within appetitive Pavlovian conditioning procedures.

Extinction and renewal of conditioned responding are modulated by interconnected brain regions including the prelimbic cortex (PL) which is thought to drive the expression of conditioned responding, and the infralimbic cortex (IL) that is thought to suppress conditioned responding after extinction (Peters et al., 2009). In rats, activation of the PL increases the expression of conditioned responding (Vidal-Gonzalez et al., 2006), while inactivation of the PL reduces conditioned responding and renewal (Corcoran & Quirk, 2007; Fuchs et al., 2005; Trask et al., 2017a). Conversely, activation of the IL promotes extinction and attenuates renewal (Do-Monte et al., 2015a; Milad & Quirk, 2002; Vidal-Gonzalez et al., 2006; Villaruel, et al., 2018; Warren et al., 2016), while inactivation of the IL impairs extinction learning and recall, and enhances renewal (Morgan & LeDoux, 1995; Peters et al., 2008; Quirk et al., 2000; Rhodes & Killcross, 2004, 2007; Sierra-Mercado et al., 2011). These findings support a functional distinction in the roles of the PL and IL in mediating the expression and suppression of conditioned responding after extinction. The efferent projections of the PL and IL that mediate these distinct functions, however, are less well understood.

The paraventricular nucleus of the thalamus (PVT) is a central neural locus that mediates emotional processing and is involved in both reward-seeking and the expression of conditioned fear (Barson et al., 2020; Do-Monte et al., 2015b). The PVT is activated by cues and contexts that predict rewarding conditions in rats (Brown et al., 1992; Igelstrom et al., 2010; Wedzony et al., 2003) and in mice (Rhodes et al., 2005; Zhu et al., 2018). Furthermore, in rats, the PVT is activated by incentive stimuli previously paired with reward (Flagel et al., 2011; Haight et al., 2017; Yager et al., 2015), and during the renewal and reinstatement of conditioned responding for rewards (Anderson & Petrovich, 2017; Dayas et al., 2008; Hamlin et al., 2009; James et al., 2011; Perry & McNally, 2013). Consistently, inactivation of the PVT attenuates appetitive renewal and reinstatement in rats (Hamlin et al., 2009; James et al., 2010; Matzeu et al., 2015), and impairs fear memory retrieval in rats (Do-Monte et al., 2015b) and mice (Penzo et al., 2015). Together, these findings strongly support a role of the PVT in promoting the expression of learned fear and reward-seeking.

The PL and IL are thought to contribute to the expression and extinction of cue-motivated behaviours in part through their distinct projections to different subregions of the PVT (Campus

et al., 2019; Kirouac, 2015). Recent evidence in mice has uncovered distinct neuronal subtypes present along the anteroposterior axis of the PVT in which galanin is expressed in anterior PVT (aPVT) but not in the posterior PVT (pPVT), and in which the DRD2 gene, which encodes dopamine D_2 receptors is expressed in the pPVT but not the aPVT (Gao et al., 2020). The middle PVT (mPVT) contains comparable proportions of both types of neurons. In rats, the PL and IL both send dense glutamatergic projections to the PVT (Vertes & Hoover, 2008), but in both rats and mice the PL projects primarily to the pPVT while the IL predominantly projects to the aPVT (Gao et al., 2020; Li & Kirouac, 2012). Evidence suggests that inputs from the PL to the PVT drive the expression of conditioned fear and operant drug-seeking. In rats, specific inhibition of PL inputs to the pPVT attenuates cue-induced reinstatement of cocaine-seeking (Giannotti et al., 2018). Moreover, inhibition of PL inputs to both the aPVT and the middle-to-posterior PVT attenuates cue-induced reinstatement of cocaine-seeking in sign-tracker but not in goal-tracker rats (Kuhn et al., 2021). Together, these findings support the role of PL projections to the PVT in driving the expression of drug-seeking after extinction and suggest that this pathway may be particularly important for promoting drug-seeking in rats with a propensity to attribute incentive salience to drug cues. Moreover, in Pavlovian fear conditioning, inhibition of PL inputs to the middle-to-posterior PVT impairs the retrieval of conditioned fear in rats (Do-Monte et al., 2015b; Quiñones-Laracuente et al., 2021). Thus, consistent with the role of the PL in promoting conditioned responding, inputs from the PL to PVT seem to be involved in maintaining fear conditioning and operant drug-seeking memories. In contrast, specific inactivation of IL inputs to the middle-to-posterior PVT in mice impairs fear extinction retrieval (Tao et al., 2021). This finding is consistent with the role of the IL in mediating extinction processes and suggests that the IL suppresses conditioned fear after extinction through its connections with the PVT. Together, the PL and IL may provide top-down control over the expression and extinction of cue-motivated behaviours through distinct inputs to the PVT (Campus et al., 2019; Kirouac, 2015). However, the specific involvement of the PL-to-PVT and IL-to-PVT pathways in the extinction and renewal of conditioned responding to appetitive Pavlovian cues remains unknown.

The current study investigated the effects of optogenetic stimulation of PL and IL projections to the PVT during ABA renewal of responding to a sucrose-predictive CS in male and female rats. Rats were trained to associate a CS with the delivery of sucrose in Context A, and then underwent extinction in a different Context B. At test, rats were returned to Contexts A and B, and CS presentations were paired with optogenetic stimulation of inputs from either the PL or the IL to the PVT. We predicted a reduction in ABA renewal following stimulation of the IL-to-PVT but not PL-to-PVT pathway. The role of PL and IL inputs to the PVT in positive reinforcement was also assessed by determining if stimulation of these pathways would support optogenetic self-stimulation. Lastly, we used Fos immunohistochemistry to assess whether optogenetic stimulation was sufficient to induce neural activation in the PL, IL, and PVT.

Methods

Animals

Subjects were 60 experimentally naïve, male and female Long-Evans rats (Charles River, St. Constant, Quebec, Canada; eight weeks old on arrival). Rats were maintained in a humidity (40-45%) and climate controlled (21 ºC) room on a 12-h light/dark cycle with lights turned on at 7:00 h. All procedures occurred during the light phase. Rats were individually housed in standard cages containing beta chip bedding (Aspen Sani chips; Envigo, Indianapolis IN) with unrestricted access to water and food (Agribands, Charles River). Each cage contained a

nylabone toy (Nylabones; Bio-Serv, Flemington, NJ) and a polycarbonate tunnel (Rat Retreats, Bio-Serv) for enrichment. During a seven-day acclimation period to the colony room, rats were handled, and body weight was recorded daily. Rats were excluded due to lack of virus expression (n=1), missed optic-fiber placement (n=2), or loss of optical implant (n=1). The final number of rats in the study was 56 (29 females, 27 males). All procedures followed the guidelines of the Canadian Council for Animal Care and were approved by the Concordia University Animal Research Ethics Committee.

Viruses and surgery

Experiments used adeno-associated viruses (AAVs) containing Channelrhodopsin-2 with an enhanced yellow fluorescent protein tag (ChR2-eYFP; Addgene) or eYFP alone (Neurophotonics) under a CAMKIIα promoter. Viral titers were 1.5 x 10^{13} particles/ml for channelrhodopsin (AAV5-CAMKIIα-hChR2(H134R)-eYFP), and 2 x 10^{12} particles/ml for control (AAV5-CAMKIIα-eYFP). Rats expressing only eYFP in the PL or IL were used to control for nonspecific effects of viral infection and optical stimulation. Viruses were stored in a -80 ºC freezer until the day of surgery. During stereotaxic surgery, rats were anesthetized with isoflurane (5% for induction, 2.5% for maintenance). The viral vector containing ChR2-eYFP or eYFP alone was injected unilaterally into the PL (AP, +2.9 mm; ML, +0.6 mm; DV, -3.6 mm) or the IL (AP, +2.9 mm; ML, +3.4 mm; DV, -5.8 mm at a 30º angle). One µl of virus was infused at a rate of 0.1 µl/min (Harvard Apparatus, Pump 11 Elite) using a blunted 27 ¼ gauge needle connected via polyethylene tubing (VWR, CA-63 018-645) to a 10 µl Hamilton syringe (Hamilton, 1701N). The injector was kept in place for an additional 20 min to reduce backflow. An optical fiber implant was inserted into the PVT (AP, -2.6 mm; ML, +2.0 mm; DV, -6.0 mm at a 20º angle) in the same hemisphere for illumination of PL or IL terminals in the PVT. The optical fiber was fixed to the skull using five jeweller's screws (Patterson Dental, Metabond, 5 533 484) and dental acrylic (A-M Systems; powder 525 000, solvent 526 000). Buprenorphine (0.03 mg/kg) was administered as an analgesic after surgery. Tests using optogenetic stimulation occurred 8 weeks following surgery to allow time for virus expression.

Apparatus

Experiments were conducted in six sound-attenuating melamine cubicles that each contained a conditioning chamber (Med Associates, ENV-009A, St. Albans, VT, USA). Each conditioning chamber consisted of stainless-steel bar floors, and a house light (75 W, 100 mA, ENV-215M) was in the center of the left wall. A white noise generator and speaker (ENV-225SM) in the top left corner of the left wall produced the white noise conditioned stimulus (CS) 5 dB above the background noise of an exhaust fan mounted inside the cubicle. A 10% sucrose solution was delivered to a customized fluid port (ENV-200R3AM, opening height of 13.2 cm) located 2 cm above the floor in the center of the right wall. Infrared sensors (ENV-254CB) lined both sides of the port opening to detect port entries. Solutions were delivered into the fluid port via a polyethylene tube (Fisher Scientific, 141 691 A) connected to a 20 ml syringe in a pump (Med Associates, PHM-100, 3.33 rpm) located outside the cubicle. All events were controlled and recorded by Med Associates software (Med-PC IV) on a computer in the experimental room.

Two distinct contexts were created by modifying the interiors of the conditioning chambers. One context consisted of vertical black-and-white striped walls, lemon odor, and bar floors. The second context had uncovered cubicle walls, an almond odor, and a wire mesh floor placed on top of the bar floor. Odors were prepared by diluting lemon oil (Sigma Aldrich,

W262528-1) or benzaldehyde (ACP Chemicals, B1000) with water to make a 10% solution that was applied into a petri dish under the floor of the conditioning chamber. The two contexts were counterbalanced across rats with respect to their roles as contexts A and B.

Optogenetic stimulation of the PL-to-PVT and IL-to-PVT pathways was done using a laser (473 nm; Shanghai Laser & Optics Centuery Co., BL473-150) connected to a rotary joint (Doric Lenses, FRJ-FC-FC, Quebec, Canada) via a 125 µm optical fibre (Fiber Optic Cable Shop, FC-FCFC-MS6-2M, Richmond, CA, USA). A custom-made patch cord containing a 200 µm fiber (Trujillo-Pisanty et al., 2015) connected the rotary joint to a custom-made optical fiber implant containing a 300 µm fiber. Stimulation parameters were based on previous studies from our laboratory (Villaruel et al., 2018; 2022). Laser intensity was calibrated to provide approximately 10 mW for each optical fiber implant, and 5 ms duration optical stimulation pulses were delivered at a frequency of 20 Hz. For renewal tests, the laser was activated 0.2 s prior to CS onset and persisted during the 10 s CS presentation. For the self-stimulation and stimulation induced Fos tests, the laser was activated for 10.2 s in the absence of the CS.

ABA renewal procedure

Rats were acclimated to the taste of 10% sucrose in the home-cage for 48 h. Rats were then habituated to the experimental chambers in the absence of any contextual cues on one day (20 min session), followed by two days (20 min sessions) in which they were habituated to contexts A and B in a counterbalanced order. During the habituation sessions, the house light turned on after a 1 min delay and total port entries were recorded.

Rats were trained in a Pavlovian conditioning task in context A for 12 days (40 min sessions). During Pavlovian conditioning, the house-light turned on after a 2 min delay to signal the start of each session and shut off to signal the end of the session. Pavlovian conditioning sessions consisted of 14 pairings of an auditory CS (10 s white noise) that co-terminated with the delivery of 0.2 ml of 10% sucrose solution into a fluid port (6 s; 2.8 ml per session). The variable inter-trial interval (ITI) averaged to 140 s (80, 140, or 200 s). Ports were checked after each session to ensure consumption.

Extinction was conducted (40 min sessions) in context B and was identical to Pavlovian conditioning but with the absence of sucrose delivery. Rats underwent extinction until the criterion of < 5 CS port entries was met with. This required a minimum of 2 sessions and a maximum of 5 sessions.

Each rat was tested in the renewal context (context A), and the extinction context (context B). The order of the two tests was counterbalanced across rats to control for test order effects. At tests, CS presentations were paired with optical stimulation of IL inputs to the PVT (ChR2, n=16, 8 female and 8 male; eYFP, n=13, 7 female and 6 male) or PL inputs to the PVT (ChR2, n=14, 7 female and 7 male; eYFP, n=13, 7 female and 6 male). The first test was conducted 24 h following the final extinction session, in which rats received a 40 min test session under extinction conditions. Rats then received 2-3 sessions of Pavlovian re-training in context A, extinction to criterion in context B, which required a minimum of 2 sessions and a maximum of 4 sessions, and a second test session under extinction conditions. Rats were tethered to patch cords throughout the entire experiment, including the habituation sessions.

Optical self-stimulation

To determine if the stimulation delivered during renewal tests was reinforcing, a subset of rats in which the IL-to-PVT pathway (ChR2 n=7, eYFP n=10) or PL-to-PVT pathway (ChR2

n=14, eYFP n=13) was targeted were then tested for 3 days (40 min sessions) for optical self-stimulation in the absence of the CS, contextual cues, and sucrose. Rats were placed into conditioning chambers containing two nose-poke apertures each with a yellow light at the back of the opening (Med Associates, ENV-114BM). Apertures were 2.8 cm above the rod floor and an infrared detector lined the opening to detect nose-pokes. The house-light signaled the start and end of each session. The first nose-poke made during the first session determined the active nose-poke aperture for each rat and resulted in the delivery of optical stimulation identical to that used during the renewal tests (unilateral, 5 ms pulses of 473 nm, 10 mW light at 20 Hz for 10.2 s) paired with presentation of a flashing light at the back of the aperture. Inactive nose-pokes were recorded but had no consequence. There was a 2 s time-out period following stimulation in which the lights in each aperture turned off, and nose-pokes were recorded but resulted in no stimulation. After each time-out, the aperture lights turned on to signal the availability of optical stimulation.

Optical stimulation induced Fos expression

We tested if the optogenetic parameters used in the renewal and self-stimulation tests were sufficient to induce Fos in the IL, PL, and PVT in a subset of rats in which the IL-to-PVT pathway (ChR2 n=6, eYFP n=10) and the PL-to-PVT pathway (ChR2 n=8, eYFP n=8) were targeted. Rats were habituated to the conditioning chambers for one 90 min session with the house-light illuminated and in the absence of the CS, contextual cues, and sucrose. Fos induction was conducted 24 h later and was identical to habituation but with 14 trials of optical stimulation of the IL-to-PVT or PL-to-PVT pathway in the first 40 min of the session. Optogenetic parameters were identical to the renewal and self-stimulation tests. Rats remained in the chamber for an additional 50 min to maximize Fos expression (Müller et al., 1984; Warren et al., 2016).

Histology and Fos immunohistochemistry

Rats were anesthetized with sodium pentobarbital (100 mg/kg, intraperitoneal) and transcardially perfused with 0.1 M phosphate buffered saline (PBS), followed by 4% paraformaldehyde (PFA) in 0.1 M PBS. Brains were extracted and post fixed for 24 h in 4% PFA, before transfer to a 30% sucrose solution in water for 48 h. Five series of coronal sections (40 μm) were obtained with a cryostat and collected in 0.1 M phosphate buffer (PB). One series of sections was mounted onto microscope slides and Nissl stained to verify optical fibre placements. A second series of sections was processed for fluorescence microscopy with DAPI (Vector labs, H-1200). Images of transgene expression were taken using an epifluorescent microscope (Nikon Eclipse TiE) at 4 × magnification. The images were used to model the spread of transgene expression in the IL and PL (Adobe Illustrator) guided by a rat brain atlas (Paxinos & Watson, 2007) to approximate location of sections relative to bregma.

For Fos immunohistochemistry, the third series of sections was blocked for 1 h in 0.3% PBS-Triton-X-100 with 6% normal goat serum (NGS; Vector Labs, S-1000), followed by incubation for 72 h at 4 ºC with anti-cFos rabbit primary antibody (1:2000; Cell Signalling, 2240). Sections were washed 3 x 10 min with PBS, then incubated in a biotinylated goat anti-rabbit secondary antibody (1:250; Vector Labs, BA-1000) for 1 h in 0.3% PBS-Triton-X-100 and 3% NGS. Sections were washed 3 x 10 min with PBS and incubated in a tertiary of avidin and biotinylated horseradish peroxidase (1:1000; ABC kit, Vector Labs, PK-6100) and stained with a 3, 3'-diaminobenzidine (DAB) solution. Sections were washed in PB, mounted on slides and cover slipped. A bright field microscope (Nikon Eclipse TiE) captured images at 10

× magnification. Images were imported into Fiji (ImageJ) software, and the number of Fos-positive neurons in the IL, PL and PVT were quantified using a cell-counting macro. The number of Fos-positive neurons were averaged across 3 or 4 sections per rat. Cell counts were then divided by the area selected in Fiji to calculate density.

Data analysis

The acquisition and extinction of ΔCS port entries (CS minus pre-CS port entries) was assessed separately using mixed analyses of variance (ANOVA) with virus (eYFP or ChR2), sex (female or male) and session as factors. ΔCS port entries during the final extinction session were assessed using univariate ANOVA with virus (eYFP or ChR2) and sex (female or male) as factors. At tests, ΔCS port entries, CS port entry latency, duration, probability, and ITI port entries were assessed separately using mixed ANOVAs with virus (eYFP or ChR2), sex (female or male) and context (A or B) as factors. Latency was measured as the average time to initiate the first CS port entry across the session. Duration was measured as the total time in the port after initiating a port entry during the CS across the session. Probability was calculated as the number of trials with a port entry divided by the total number of trials. ITI port entries were measured as the average number of port entries made during the inter trial intervals across the session.

The frequency of nose-pokes during the first self-stimulation test was analyzed using mixed ANOVA with virus (eYFP or ChR2) and aperture (active or inactive) as factors. The cumulative frequency of nose-pokes during the first self-stimulation test was analyzed using mixed ANOVA with virus (eYFP or ChR2), aperture (active or inactive) and time bin (10, 20, 30 or 40 min) as factors. The frequency of nose-pokes across the three sessions was analyzed using mixed ANOVA with virus (eYFP or ChR2), aperture (active or inactive) and session as factors.

Fos density in the IL and PL were analyzed separately using mixed ANOVAs with virus (eYFP or ChR2) and hemisphere (stimulated or non-stimulated) as factors. Fos density in the PVT was analyzed using t tests (ChR2 vs. eYFP).

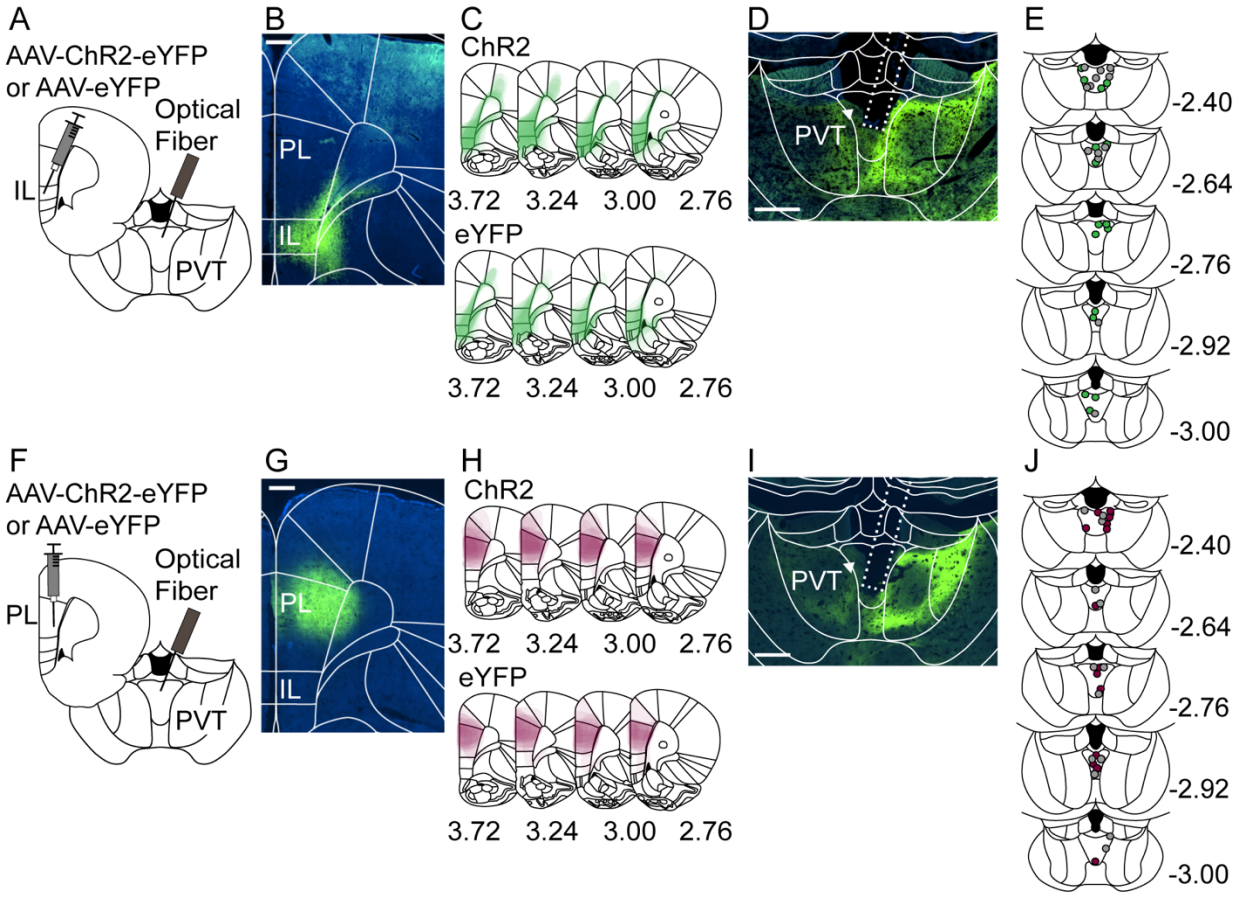
Greenhouse-Geisser corrections are reported following violations of Mauchly's test of sphericity. Post-hoc analyses were corrected for multiple comparisons using the Bonferroni adjustment. All data analyses were conducted using IBM SPSS v21.0 (IBM Corp., Armonk, NY). Results were considered statistically significant at $p<.05$.

Results

Histology

We injected AAV-ChR2-eYFP or AAV-eYFP alone in the IL or PL and implanted an optical fiber in the PVT to target the IL-to-PVT and PL-to-PVT projections (Fig. 1A, F). The expression of the ChR2 transgene observed in the IL and PL is depicted in Figures 1B and 1G respectively, and the spread of the transgenes for ChR2 and eYFP alone is shown in Figures 1C and 1H, respectively. For rats injected in the IL, the greatest concentration of ChR2 expression was in the IL and the dorsal peduncular cortex. Some expression of ChR2 was observed along the injector tract, along the forceps minor of the corpus collosum and the anterior medial and ventral orbitofrontal cortex. For rats injected in the PL, the highest concentration of ChR2 expression was in the PL, and some expression was observed in the cingulate area 1. The expression of the ChR2 transgene and the placement of the optical fiber in the PVT in rats injected in the IL and PL are depicted in Figures 1D and 1I respectively, and the approximate placements of optical fibers in the PVT in the ChR2 and eYFP groups are shown in Figures 1E

and 1J respectively. The placement of optical fiber implants was in the mPVT for the majority of rats. Four rats in which the IL-to-PVT pathway was targeted, and three rats in which the PL-to-PVT pathway was targeted, were found to have optical fiber implants near AP level -3.0, which is at the tail-end of the mPVT and is considered the start of the pPVT.



Chapter 4 Figure 1. A Schematic of microinjections of ChR2-eYFP or eYFP in the IL and optical fiber implant in the PVT. **B** Representative image of ChR2 transgene expression in the IL. Scale bar is 500 µm. **C** Schematic depicting expression of ChR2 and eYFP transgene expression in the IL. Numbers indicate mm anterior to Bregma. **D** Representative image of ChR2 transgene expression and optical fiber implant in the PVT in a rat injected in the IL. Scale bar is 500 µm. **E** Optical fiber placements in the PVT for rats injected with ChR2 (green) or eYFP (gray) in the IL. **F** Schematic of microinjections of ChR2-eYFP or eYFP in the PL and optical fiber implant in the PVT. **G** Representative image of ChR2 transgene expression in the PL. Scale bar is 500 µm. **H** Schematic depicting expression of ChR2 and eYFP transgene expression in the PL. Numbers indicate mm anterior to Bregma. **I** Representative image of ChR2 transgene expression and optical fiber implant in the PVT in a rat injected in the PL. Scale bar is 500 µm. **J** Optical fiber placements in the PVT for rats injected with ChR2 (magenta) or eYFP (gray) in the PL. IL: infralimbic cortex; PL, prelimbic cortex; PVT: paraventricular nucleus of the thalamus.

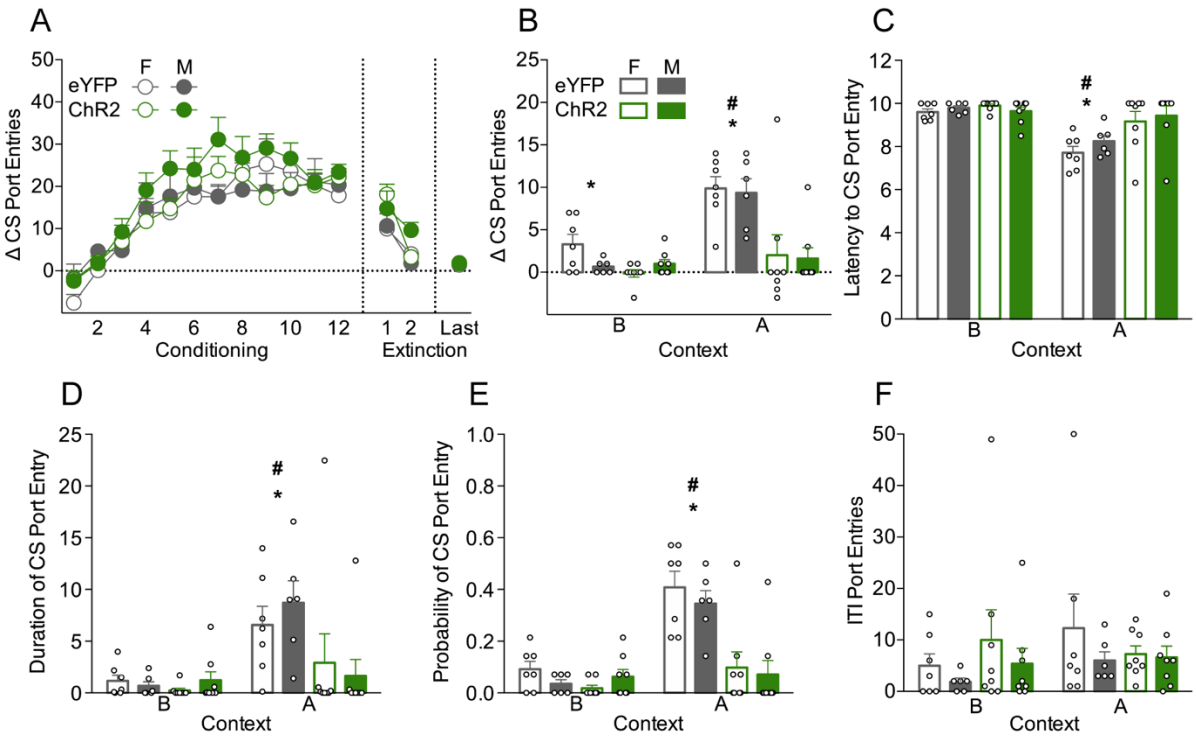
Stimulation of IL inputs to the PVT attenuates appetitive Pavlovian renewal

Acquisition and extinction. During conditioning in context A, ΔCS port entries increased across sessions (Fig. 2A; Session, $F_{5.2,130.9}$=34.15, p<.001) similarly in both groups (Group, $F_{1,25}$=2.09, p=.161; Group x Session, $F_{5.2,130.9}$=.74, p=.600), and there was no main effect of sex or interactions with sex (See Appendix B Table 1). During extinction in context B, ΔCS port entries decreased across sessions (Fig. 2A; Session, $F_{1,25}$=58.17, p<.001) comparably in both groups (Group, $F_{1,25}$=4.11, p=.053; Group x Session, $F_{1,25}$=3.75, p=.064) with no significant main effect of sex or interactions with sex. By the final extinction session, all rats made very few ΔCS port entries (1.7 ± 0.4 port entries) and responding was similar in both groups (Fig. 2A; Group, $F_{1,25}$=.06, p=.805) with no significant main effect of sex or interactions with sex. Therefore, rats across groups and sexes learned to respond by entering the fluid port during the CS during conditioning, extinguished ΔCS port entries during extinction, and showed negligible responding during the final extinction session.

Renewal test. ABA renewal of ΔCS port entries was observed in the eYFP group, but not in the ChR2 group (Fig. 2B; Group, $F_{1,25}$=18.95, p<.001; Context, $F_{1,25}$=28.47, p<.001; Group x Context, $F_{1,25}$=13.72, p=.001). ΔCS port entries were significantly greater in context A compared to context B in the eYFP group (p<.001) but not in the ChR2 group (p=.234). Moreover, ΔCS port entries were significantly greater in the eYFP group compared to the ChR2 group in context A (p<.001) and context B (p=.035). There was no significant main effect of sex or interactions with sex. These results indicate that optogenetic stimulation of IL inputs to the PVT attenuated renewal of appetitive Pavlovian conditioned responding, and this effect is independent of sex.

Additional measures of conditioned responding support the finding that stimulation of IL inputs to the PVT attenuated renewal of appetitive Pavlovian conditioned responding. The eYFP group, but not the ChR2 group, displayed renewal in context A relative to context B as measured by the reduced latency to initiate the first CS port entry (Fig. 2C; Group, $F_{1,25}$=8.07, p=.009; Context, $F_{1,25}$=35.76, p<.001; Group x Context, $F_{1,25}$=11.53, p=.002), increased total duration of CS port entries (Fig. 2D; Group, $F_{1,25}$=5.56, p=.027; Context, $F_{1,25}$=14.75, p=.001; Group x Context, $F_{1,25}$=5.77, p=.024), and increased probability of CS port entries (Fig. 2E; Group, $F_{1,25}$=18.94, p<.001; Context, $F_{1,25}$=49.33, p<.001; Group x Context, $F_{1,25}$=27.77, p<.001). There were no significant main effects of sex or interactions with sex for all measures. The eYFP group showed shorter latency (p<.001), and greater duration (p<.001) and probability (p<.001) of CS port entries in context A relative to context B. However, the ChR2 group showed comparable latencies (p=.065), duration (p=.292) and probability (p=.201) in contexts A and B. Moreover, in context B, latency (p=.615), duration (p=.758) and probability (p=.324) of CS port entries was comparable in the eYFP and ChR2 groups. However, in context A, the CS port entry latency was shorter (p=.003), and the duration (p=.021) and probability (p<.001) were greater in the eYFP group compared to the ChR2 group.

Port entries during the ITI were similar in contexts A and B in both the eYFP and ChR2 groups (Fig. 2F; Group, $F_{1,25}$=.13, p=.727; Context, $F_{1,25}$=1.13, p=.297; Group x Context, $F_{1,27}$=1.92, p=.178), and there was no significant main effect of sex or interactions with sex. Therefore, optogenetic stimulation of IL inputs to the PVT did not produce non-specific effects on responding outside of the CS presentations. Altogether, these results suggest that independent of sex, optogenetic stimulation of IL inputs to the PVT suppressed renewal of appetitive Pavlovian conditioned responding without affecting responding outside of the CS presentations.



Chapter 4 Figure 2. Optogenetic stimulation of IL inputs to the PVT attenuates renewal without affecting ITI port entries. **A** Both sexes and virus groups acquired Pavlovian conditioned responding similarly in Context A as measured by ΔCS port entries. All rats extinguished responding similarly in the first two extinction sessions in Context B. There were no sex or virus differences on the last extinction session. **B** In both sexes, the eYFP group, but not the ChR2 group, displayed renewal with increased ΔCS port entries in Context A compared to Context B. ΔCS port entries in Context A were increased in the eYFP group compared to the ChR2 group. *p<.05 ChR2 vs. eYFP in Context A and Context B, # p<.05 Context A vs. Context B in the eYFP group. **C** In both sexes, the eYFP group, but not the ChR2 group, showed decreased CS port entry latency, **D** increased CS port entry duration, and **E** increased CS port entry probability in Context A relative to Context B at test. (C, D, E) *p<.05 ChR2 vs. eYFP in Context A, # p<.05 Context A vs. Context B in the eYFP group. **F** ITI port entries were equivalent at test in both contexts across virus groups and sexes. All data are presented as mean ± SEM. Here and in subsequent figures, individual data points are overlaid on the bar graphs.

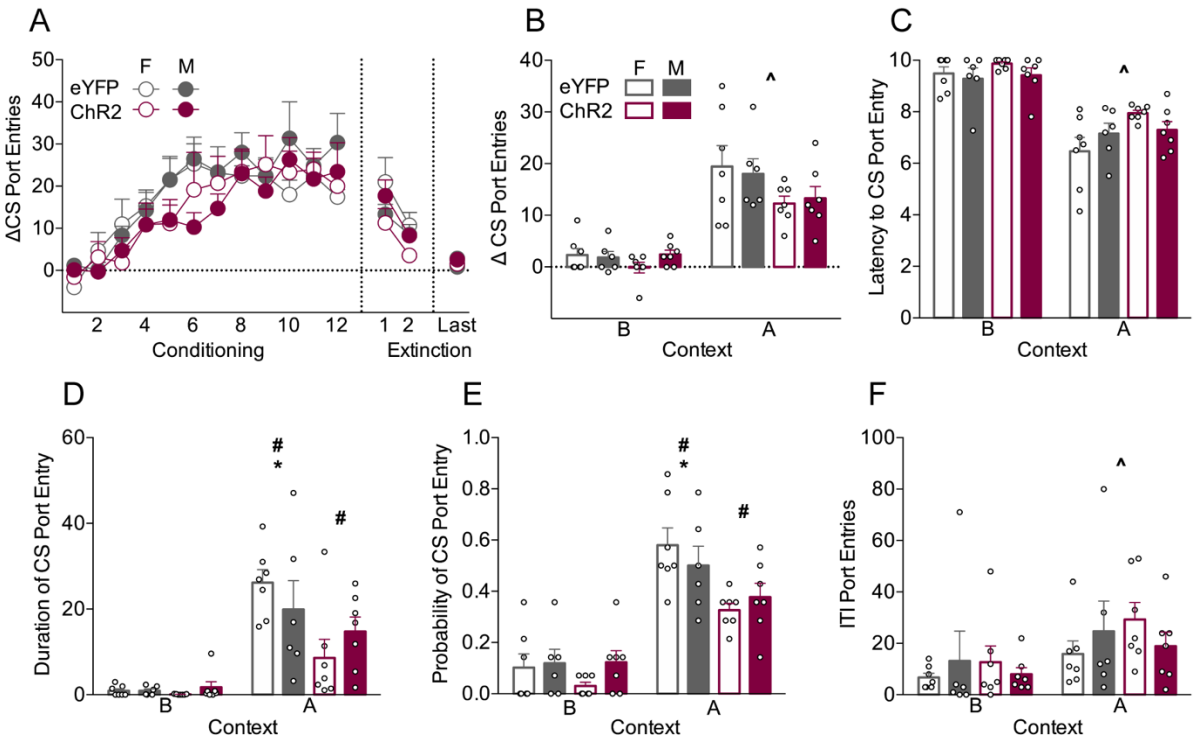
Stimulation of PL inputs to the PVT does not block appetitive Pavlovian renewal

Acquisition and extinction. During conditioning in context A, ΔCS port entries increased across sessions (Fig. 3A; Session, $F_{3.9,90.4}$=26.17, p<.001) similarly in both eYFP and ChR2 groups (Group, $F_{1,23}$=1.33, p=.261; Group x Session, $F_{3.9,90.4}$=1.23, p=.303) and there was no significant main effect of sex or interactions with sex (See Appendix B Table 2). During extinction in context B, ΔCS port entries decreased across sessions (Fig. 3A; Session, $F_{1,23}$=15.59, p=.001). There was no significant main effect of group (Group, $F_{1,23}$=1.74, p=.200) or sex (Sex, $F_{1,23}$=.135, p=.717). There was a significant interaction (Group x Sex x Session, $F_{1,23}$=5.06, p=.034), which indicated that in extinction session 1, ΔCS port entries were greater for eYFP females compared to ChR2 females (p=.019). Although, by the second and final extinction session, there were no significant differences between ChR2 and eYFP females (p>.05). By the final extinction session, ΔCS port entries were low for all rats (2.0 $\pm$ 0.5 port entries) and were similar in both groups (Fig. 3A; Group, $F_{1,23}$=.06, p=.815) with no significant main effect of sex or interactions with sex. Therefore, rats across groups and sexes similarly acquired and extinguished conditioned responding in Contexts A and B and showed low levels of responding during the final extinction session.

Renewal test. ABA renewal was evidenced by increased ΔCS port entries in context A relative to context B (Fig. 3B; Context, $F_{1,23}$=115.64, p<.001), in both the eYFP and ChR2 groups (Group, $F_{1,23}$=3.98, p=.058; Group x Context, $F_{1,23}$=3.63, p=.069). There was no significant main effect of sex or interactions with sex. In addition, ABA renewal was evidenced by reduced latencies to initiate the first CS port entry in context A relative to context B (Fig. 3C; Context, $F_{1,23}$=123.13, p<.001), in both the eYFP and ChR2 groups (Group, $F_{1,23}$=4.24; p=.051; Group x Context, $F_{1,23}$=1.79, p=.194). There were no significant main effects of sex or interactions with sex.

Group differences were observed, however, in the degree of ABA renewal for the duration of port entries (Fig. 3D; Context, $F_{1,23}$=57.96, p<.001; Group, $F_{1,23}$=6.05, p=.022; Group x Context, $F_{1,23}$=6.88, p=.015) and probability of CS port entries (Fig. 3E; Context, $F_{1,23}$=120.31, p<.001; Group, $F_{1,23}$=7.07, p=.014; Group x Context, $F_{1,23}$=5.49, p=.028). There were no significant main effects of sex or interactions with sex for both measures. Responding in context B was similar for the eYFP and ChR2 groups for the duration (p=.982) and probability (p=.453) of CS port entries. However, the duration (p=.017) and probability (p=.005) of CS port entries were greater in context A in the eYFP group compared to the ChR2 group. Importantly, both groups showed greater duration (eYFP, p<.001; ChR2, p=.002) and probability (eYFP, p<.001; ChR2, p<.001) of CS port entries in context A relative to context B indicating that ABA renewal occurred in both groups.

Port entries during the ITI were greater in context A compared to context B (Fig. 3F; Context, $F_{1,23}$=28.67, p<.001), and were comparable in both groups (Group, $F_{1,23}$=.11, p=.746; Group x Context, $F_{1,23}$=.57, p=.457). There was no significant main effect of sex or interactions with sex. Therefore, optogenetic stimulation of PL inputs to the PVT did not have any non-specific effects on responding outside of the CS presentations. Altogether, these results indicate that independent of sex, optogenetic stimulation of PL inputs to the PVT suppressed some measures of conditioned responding but did not affect renewal of ΔCS port entries or responding outside of the CS presentations.



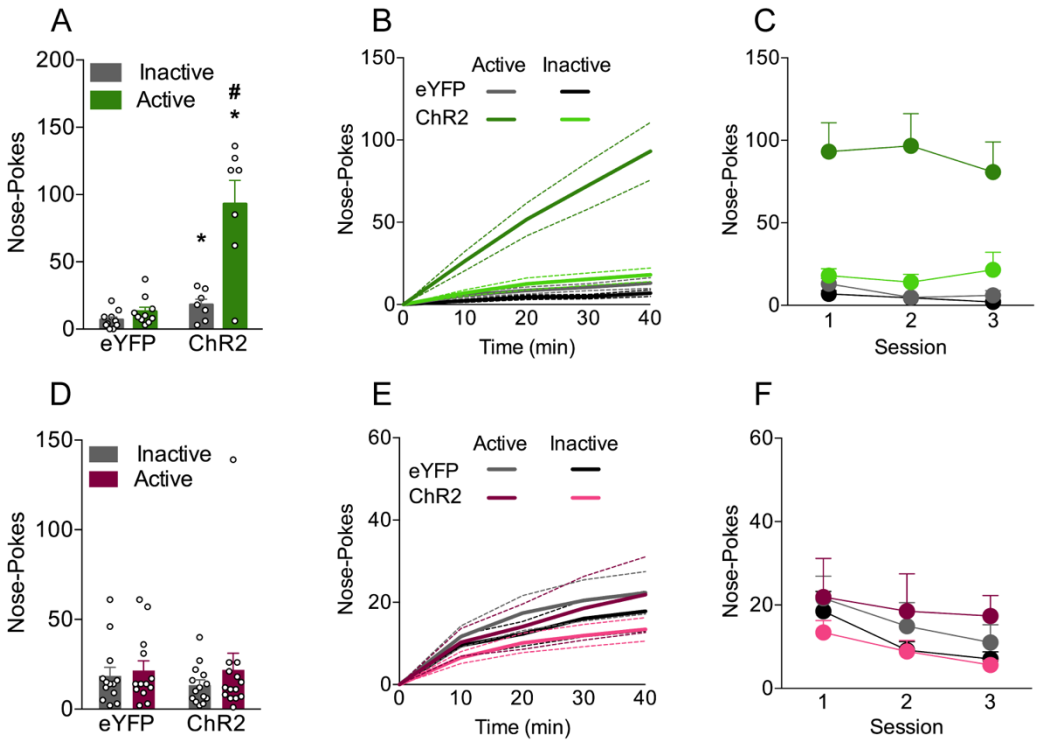
Chapter 4 Figure 3. Optogenetic stimulation of PL inputs to the PVT does not block renewal nor ITI responding. **A** Both sexes and virus groups acquired Pavlovian conditioned responding similarly in Context A as measured by ΔCS port entries. Rats extinguished responding in the first two extinction sessions in Context B. There were no sex or virus differences on the last extinction session. **B** Both groups and sexes displayed renewal with increased ΔCS port entries, and **C** decreased CS port entry latency in Context A compared to Context B. (B, C) ^p<.05, main effect of Context. **D** In both sexes, CS port entry duration, and **E** probability were increased in Context A compared to Context B and were lower in the ChR2 group compared to the eYFP group in Context A. (D, E) *p<.05 ChR2 vs. eYFP in Context A, #p<.05 Context A vs. Context B in both groups. **F** Both groups and sexes displayed renewal with increased ITI port entries in Context A compared to Context B. ^p<.05, main effect of Context. All data are presented as mean ± SEM.

Stimulation of IL, but not PL, inputs to the PVT supports optical self-stimulation

Subsets of rats in the IL-to-PVT and PL-to-PVT pathway experiments were tested for optical self-stimulation to determine whether stimulation of these pathways is reinforcing. We repeated the test on three consecutive days to assess if these effects persist across sessions. Sex differences were not analyzed because of the lack of sex differences in the effects of optogenetic stimulation on renewal.

In rats in which the IL-to-PVT pathway was targeted, the ChR2 group made significantly more active ($p<.001$), and inactive ($p=.021$) nose-pokes compared to the eYFP group during the first test. Moreover, the ChR2 group made more active nose-pokes relative to inactive nose-pokes ($p<.001$), while the eYFP group did not ($p=.447$) (Fig.4A; Group, $F_{1,15}=24.80$, $p<.001$; Active/Inactive, $F_{1,15}=44.54$, $p<.001$; Group x Active/Inactive, $F_{1,15}=32.17$, $p<.001$). Time series analysis of self-stimulation during the first test revealed that the ChR2 group made more active ($p<.01$) and inactive ($p<.05$) nose-pokes compared to the eYFP group at all time bins, and the ChR2 group made more active nose-pokes compared to inactive nose-pokes at all time bins ($p<.001$) while the eYFP group did not ($p>.05$) (Fig. 4B; Group, $F_{1,15}=22.99$, $p<.001$; Active/Inactive, $F_{1,15}=45.44$ $p<.001$; Time, $F_{1.1,17.2}=45.96$, $p<.001$; Group x Inactive/Inactive x Time, $F_{1.2,18.4}=28.07$, $p<.001$). Self-stimulation was maintained in the ChR2 group over the three sessions on consecutive days (Fig. 4C; Group, $F_{1,15}=29.73$, $p<.001$; Active/Inactive, $F_{1,15}=34.67$, $p<.001$; Group x Active/Inactive, $F_{1,15}=28.62$, $p<.001$). There was no main effect of session (Session, $F_{2,30}=1.71$, $p=.198$), and no other significant interactions.

Conversely, optogenetic stimulation of the PL-to-PVT pathway did not support self-stimulation as there was no significant difference in the frequency of active and inactive nose-pokes between ChR2 and eYFP groups (Fig. 4D; Group, $F_{1,25}=.11$, $p=.745$; Active/Inactive, $F_{1,25}=2.16$, $p=.154$; Group x Active/Inactive, $F_{1,25}=.30$, $p=.591$). Nose-pokes increased across the session (Fig. 4E; Time, $F_{1.1,27.4}=24.91$, $p<.001$) in both the ChR2 and eYFP groups (Group, $F_{1,25}=.23$, $p=.634$) but there was no difference between active and inactive nose-pokes (Active/Inactive, $F_{1,25}=2.42$, $p=.132$). There were no significant interactions. Increases in nose-pokes were therefore not related to optogenetic stimulation, suggesting that PL inputs to the PVT do not support optical self-stimulation. Across the three sessions, there were more active than inactive nose-pokes (Active/Inactive, $F_{1,25}=4.28$, $p=.049$), however nose-pokes were similar in both groups (Fig. 4F; Group, $F_{1,25}=.01$, $p=.937$), and decreased across sessions (Session, $F_{2,50}=7.91$, $p=.001$). There were no significant interactions. Altogether, these results indicate that stimulation of IL, but not PL, inputs to the PVT supports optical self-stimulation, and this effect persists across sessions.

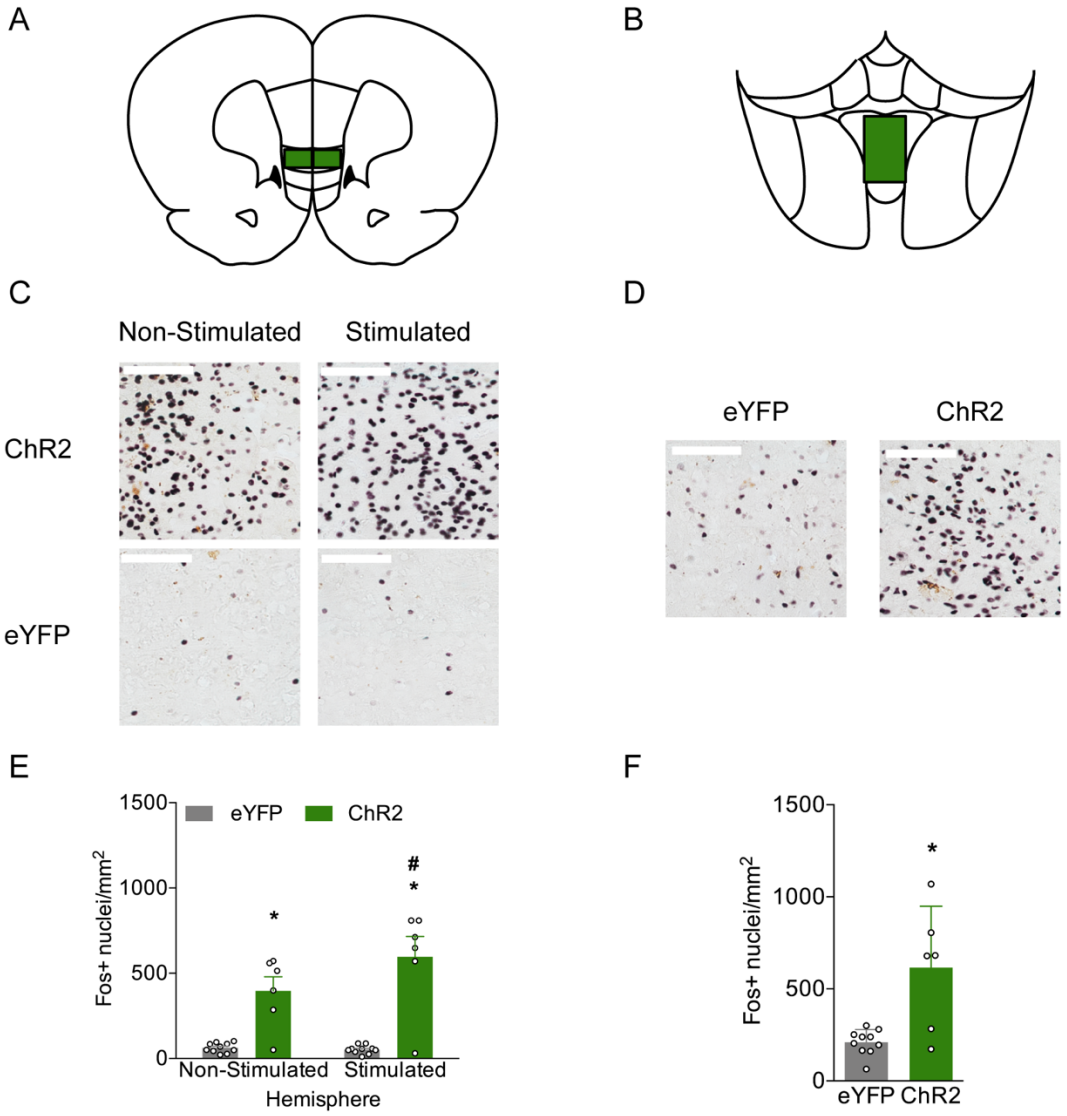


Chapter 4 Figure 4. Stimulation of IL, but not PL, inputs to the PVT supports optogenetic self-stimulation. **A** In rats in which the IL-to-PVT pathway was targeted, the ChR2 group made more active nose-pokes compared to the eYFP group in the first session, **B** made more cumulative active nose-pokes compared to the eYFP group across the session, and **C** made more active nose-pokes compared to the eYFP group across three sessions. (A) *p<.05, ChR2 vs. eYFP, #p<.05 active vs. inactive nose-pokes in the ChR2 group. **D** In rats in which the PL-to-PVT pathway was targeted, there was no difference between groups for the frequency of active and inactive nose-pokes in the first session, **E** the cumulative frequency of nose-pokes across the session, and **F** the frequency of active and inactive nose-pokes across three sessions. All data are presented as mean ± SEM. In B and E, solid lines indicate the mean and dashed lines indicate the SEM.

Increased Fos expression in the IL, PL and PVT following optical stimulation

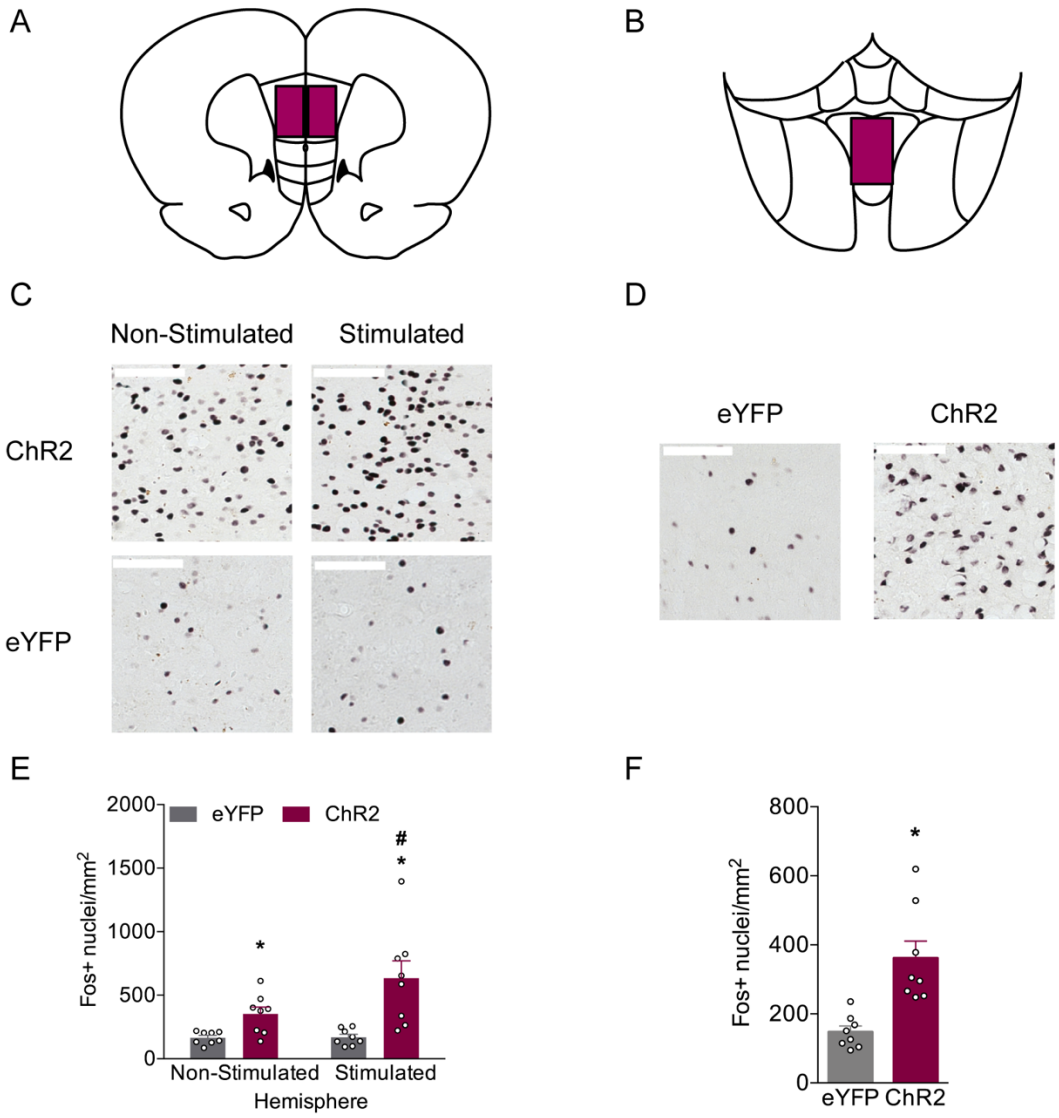
We analyzed the density of Fos expression in the IL, PL and PVT following optical stimulation of IL or PL inputs to the PVT in a subset of rats to assess whether optogenetic stimulation in the absence of conditioned or contextual stimuli induced neural activation. Sex differences in Fos density were not analyzed because of the lack of sex differences in the effects of optogenetic stimulation on renewal.

In rats in which the IL-to-PVT pathway was targeted (Fig. 5 A, B), we found increased Fos density in the IL in the ChR2 group in both the stimulated ($p<.001$) and non-stimulated ($p<.001$) hemispheres compared to the eYFP group. Further, Fos density in the IL was greater in the stimulated hemisphere relative to the non-stimulated hemisphere in the ChR2 group ($p=.001$), but not in the eYFP group ($p=.779$). (Fig. 5C, E; Group, $F_{1,14}=37.57$, $p<.001$; Hemisphere, $F_{1,14}=9.03$, $p=.009$; Group x Hemisphere, $F_{1,14}=11.25$, $p=.005$). Given that the PVT is a midline thalamic nucleus, hemispheric differences were not analyzed. In the PVT, Fos density was increased in the ChR2 group relative to the eYFP group (Fig. 5D, F; Group, $F_{1,14}=14.39$, $p=.002$). Therefore, our optical parameters were sufficient in activating neurons in the IL and PVT.



Chapter 4 Figure 5. Stimulation of the IL-to-PVT pathway induces Fos expression in the IL and PVT. **A** Schematic depicting the region of Fos quantification in the IL, **B** and in the PVT. **C** Representative image of Fos in the non-stimulated hemisphere of the IL from a rat in the ChR2 (top left) and eYFP (bottom left) group, and in the stimulated hemisphere of the IL from a rat in the ChR2 (top right) and eYFP (bottom right) group. Scale bar is 100 µm. **D** Representative image of Fos in the PVT from a rat in the eYFP (left) and ChR2 (right) group. Scale bar is 100 µm. **E** Increased Fos density in the stimulated and non-stimulated hemispheres of the IL in the ChR2 group relative to eYFP alone controls, and increased Fos density in the stimulated hemisphere relative to the non-stimulated hemisphere of the IL in the ChR2 group. *p<.05, ChR2 vs. eYFP in both hemispheres, #p<.05, stimulated vs. non-stimulated hemisphere in the ChR2 group. **F** Increased Fos density in the PVT in the ChR2 group relative to eYFP controls. *p<.05 ChR2 vs. eYFP.

In rats in which the PL-to-PVT pathway was targeted (Fig. 6A, B), Fos density in the PL was greater in the stimulated (p=.005) and non-stimulated (p=.006) hemispheres in the ChR2 group relative to the eYFP group. Moreover, Fos density in the PL was greater in the stimulated hemisphere compared to the non-stimulated hemisphere in the ChR2 group (p=.004), but not in the eYFP group (p=.963). (Fig. 6C, E; Group, $F_{1,14}$=13.49, p=.003; Hemisphere, $F_{1,14}$=6.15, p=.026; Group x Hemisphere, $F_{1,14}$=5.82, p=.030). In the PVT, Fos density was greater in the ChR2 group relative to the eYFP group (Fig. 6D, F; Group, $F_{1,14}$=16.86, p=.001). Altogether, these results suggest that the optic parameters used to stimulate both IL and PL inputs to the PVT were sufficient to activate neurons in the IL, PL, and the PVT.



Chapter 4 Figure 6. Stimulation of the PL-to-PVT pathway induces Fos expression in the PL and PVT. **A** Schematic depicting region of Fos quantification in the PL, **B** and in the PVT. **C** Representative image of Fos in the non-stimulated hemisphere of the PL from a rat in the ChR2 (top left) and eYFP (bottom left) group, and in the stimulated hemisphere of the PL from a rat in the ChR2 (top right) and eYFP (bottom right) group. Scale bar is 100 µm. **D** Representative image of Fos in the PVT from a rat in the eYFP (left) and ChR2 (right) group. Scale bar is 100 µm. **E** Increased Fos density in the stimulated and non-stimulated hemispheres of the PL in the ChR2 group relative to eYFP alone controls, and increased Fos density in the stimulated hemisphere relative to the non-stimulated hemisphere of the PL in the ChR2 group. *p<.05, ChR2 vs. eYFP in both hemispheres, #p<.05, stimulated vs. non-stimulated hemisphere in the ChR2 group. **F** Increased Fos density in the PVT in the ChR2 group relative to eYFP controls. *p<.05 ChR2 vs. eYFP.

Discussion

The present study investigated the roles of IL and PL inputs to the PVT in ABA renewal of appetitive Pavlovian conditioned responding in male and female rats. Optogenetic stimulation of IL inputs to the PVT during CS presentations blocked renewal in both sexes, as evidenced by a marked suppression of ΔCS port entries, increased latency, and decreased duration and probability of CS port entries in the ChR2 group relative to the eYFP group. However, stimulation of PL inputs to the PVT had minimal effects on renewal. We found robust renewal in both the ChR2 group and eYFP alone controls in Context A compared to Context B as evidenced by increased ΔCS port entries, duration, and probability of CS port entries and decreased latency to CS port entries. Further, neither stimulation of IL nor PL inputs to the PVT affected port entries during the inter trial interval, suggesting that stimulation of these pathways did not have non-specific effects on responding. Optogenetic stimulation of the IL-to-PVT and PL-to-PVT pathways both induced Fos expression indicating that light stimulation activated presynaptic and postsynaptic neurons in the targeted input pathways. Additionally, we found that stimulation of IL, but not PL, inputs to the PVT supported optical self-stimulation, indicating that specific activation of the IL-to-PVT pathway has reinforcing properties. These results provide novel evidence that stimulation of the IL-to-PVT pathway suppresses the renewal of appetitive Pavlovian conditioned responding after extinction in a sex-independent manner.

We found that stimulation of IL inputs to the PVT during CS presentations attenuated renewal of appetitive Pavlovian conditioned responding. The finding that stimulation of IL inputs to the PVT attenuates renewal is consistent with results from an array of learning procedures showing that the IL is a key node in the circuitry mediating extinction of conditioned responding. In both Pavlovian fear conditioning and operant drug-seeking preparations, stimulation of the IL enhances extinction learning and retrieval (Do-Monte et al., 2015a; LaLumiere et al., 2012; Lay et al., 2020; Marchant et al., 2010; Peters et al., 2008; ; Tao et al., 2021; Vidal- Gonzalez et al., 2006; Warren et al., 2016), while inactivation or lesions of the IL disrupt extinction retrieval and promote reinstatement and renewal (Bossert et al., 2011; Do-Monte et al., 2015a; Gutman et al., 2017; Laurent & Westbrook, 2009; Peters et al., 2008; Pfarr et al., 2015; Quirk et al., 2000; Warren et al., 2019). Moreover, our findings parallel those of a Pavlovian fear conditioning study in which inactivation of IL inputs to the middle-to-posterior PVT pathway impaired fear extinction retrieval (Tao et al., 2021). Combined with the present results, this suggests that the IL-to-PVT pathway is important for extinction and suppression of responding to Pavlovian cues that signal either appetitive or aversive outcomes. The role of the IL-to-PVT pathway therefore appears to be independent of valence.

Our findings are consistent with the very limited existing research investigating the role of the IL in mediating appetitive Pavlovian extinction learning. Stimulation of the IL suppresses renewal of Pavlovian conditioned responding for sucrose (Villaruel et al., 2018). Moreover, lesions or inactivation of the IL disrupt extinction retrieval during reinstatement and renewal of Pavlovian conditioned responding for food or sucrose (Lay et al., 2019; Rhodes & Killcross, 2004, 2007). The use of selective optogenetic stimulation techniques in the present study provides evidence that specific projections from the IL-to-PVT promote appetitive Pavlovian extinction retrieval during renewal.

Stimulation of PL inputs to the PVT caused modest reductions in the duration and probability of CS port entries but did not significantly affect ABA renewal of appetitive Pavlovian conditioned ΔCS port entries or latency to CS port entries. This contrasts with Pavlovian fear conditioning studies showing that PL stimulation increases conditioned

responding after extinction (Vidal-Gonzalez et al., 2006), and with operant drug-seeking studies showing that inactivation of the PL attenuates conditioned responding after extinction (Capriles et al., 2003; McFarland et al., 2004; McFarland & Kalivas, 2001). It is possible that an increase in renewal was not observed here because we used specific optogenetic targeting of PL inputs to the PVT. The PL and aPVT have been implicated in appetitive Pavlovian renewal using a food reward, and both the PL and aPVT are more active following renewal of conditioned responding to a food-predictive cue relative to extinction (Anderson & Petrovich, 2017, 2018). Moreover, silencing PL inputs to the pPVT attenuates cue-induced reinstatement of cocaine-seeking (Giannotti et al., 2018). Furthermore, silencing PL inputs to both the aPVT and the middle-to-posterior PVT attenuates cue-induced reinstatement of cocaine-seeking in sign-trackers but not in goal-trackers (Kuhn et al., 2021). However, silencing PL inputs to the pPVT has been shown to have no effect on cue-induced reinstatement of operant sucrose-seeking (Giannotti et al., 2018). Similarly, we found that augmenting the activity of PL inputs to the PVT had relatively minimal effects on renewal of responding to a sucrose CS. These results suggest a role for the PL-to-PVT pathway in promoting drug-seeking after extinction, but not in the seeking of natural reward after extinction. The majority of rats used in this study received mPVT-targeting optical fiber implants, and this prevented us from assessing the functional role of distinct PVT subregions in appetitive Pavlovian extinction retrieval using the renewal model. The role of IL and PL projections to the PVT along its anterior-posterior axis in the renewal of responding for natural and drug rewards will require further investigation.

Stimulation of the IL-to-PVT, but not PL-to-PVT pathway, supported optical self-stimulation, suggesting that the IL-to-PVT pathway is involved in positive reinforcement. This finding is consistent with evidence that intracranial self-stimulation is supported by electrode placements in the PVT, suggesting a role of the PVT in reward (Clavier & Gerfen, 1982; Cooper & Taylor, 1967). However, our finding suggests that this effect may be input specific. IL and PL projections to the PVT may differentially activate cell populations in the PVT that are specifically involved in reward (McGinty & Otis, 2020). The PVT is composed of two functionally distinct cell types referred to as type I and type II PVT neurons (Gao et al., 2020). These two distinct PVT neurons are differentially innervated by the PL and IL; the PL preferentially innervates type I neurons mostly contained in the pPVT, and the IL mainly targets type II neurons located in the aPVT (Gao et al., 2020). Supporting a role of the pPVT in signalling aversive states, type I neurons in the pPVT are more likely to express the DRD2 gene than type II neurons in the aPVT (Gao et al., 2020). The mechanism through which optogenetic stimulation of the IL-to-PVT produced positive reinforcement is unknown. Perhaps the IL-to-PVT pathway promotes reward by innervating non-D_2-expressing neurons in the PVT. Another possibility is that stimulation of IL inputs to the PVT may activate downstream projections to the nucleus accumbens (NAc) to induce reward. Stimulation of PVT inputs to the NAc supports optical self-stimulation (Lafferty et al., 2020), and the rewarding effects of stimulation of the IL-to-PVT pathway may result from preferential activation of PVT neurons that then project to the NAc. Therefore, a serial IL-PVT-NAc circuit may mediate the rewarding effects of optical self-stimulation of the IL-to-PVT pathway.

The rewarding effects of optogenetic stimulation of IL inputs to the PVT, which could reinforce nose-poking behaviour, are unlikely to bear directly on the interpretation of the renewal results. We used a Pavlovian behavioural paradigm to study renewal, which was not contingent on performance of an operant response, and the stimulation began .2 s prior to CS onset and lasted for the duration of the CS. Moreover, others have found similar suppression of behaviour

due to activation of IL inputs to the NAc despite the finding that stimulation of this pathway supports optical self-stimulation (Cameron et al., 2019). Likewise, Lafferty and colleagues (2020) found that stimulation of PVT inputs to the NAc suppresses behavior while also supporting optical self-stimulation.

We analyzed Fos density in the IL, PL and PVT following optical stimulation of IL-to-PVT and PL-to-PVT pathways to assess whether our optogenetic parameters induced neural activation in the targeted brain areas. Importantly, we found that stimulation of IL and PL inputs to the PVT induces Fos expression in the PVT and either the IL or PL, which indicates that our optical stimulation parameters selectively elicited neural activity in the IL-to-PVT and PL-to-PVT pathways. Therefore, it is unlikely that the absence of substantial effects of PL-to-PVT stimulation on renewal and self-stimulation was due to insufficient activation of this pathway.

In the IL-to-PVT experiment, ChR2 expression was predominantly in the IL, however, we did observe some spread in the anterior medial and ventral orbitofrontal cortex (OFC) and along the forceps minor of the corpus callosum. Further, in the PL-to-PVT experiment, although ChR2 expression was primarily in the PL modest spread was observed in the cingulate area 1. Additionally, we observed ChR2 expression in the medial thalamic areas surrounding the PVT. Thus, optogenetic stimulation of inputs from regions surrounding the PL and IL, such as the OFC, which sends projections to the medial and central regions of the mediodorsal thalamic nucleus (Bay & Çavdar, 2013), may have contributed to our findings. However, extinction of responding to a Pavlovian CS that predicts sucrose is unaffected by OFC lesions or inactivation (Burke et al., 2008, 2009), suggesting that the observed results are unlikely due to activation of the OFC. The IL and PL also project to the mediodorsal thalamic nucleus (Vertes, 2004) which surrounds the PVT, and observed effects may therefore be due to activation of these projections. However, we only included rats with optical fibers aimed at the PVT, and rats with optical fibers located outside of the PVT were excluded from the study. Moreover, our findings are consistent with studies demonstrating a role of the IL and the IL-to-PVT pathway in extinction (Milad & Quirk, 2002; Peters et al., 2008; Tao et al., 2021; Villaruel et al., 2018), as well as the finding that the PL-to-PVT pathway is not necessary for the reinstatement of sucrose-seeking (Giannotti et al., 2018), suggesting that we successfully targeted the IL-to-PVT and PL-to-PVT pathways.

We did not detect any sex differences in ABA renewal of conditioned responding using a sucrose reinforcer. Few studies have systematically investigated differences between the sexes in renewal, even in studies that included both male and female subjects (Bouton & Ricker, 1994; Campese & Delamater, 2013). However, the present results are consistent with several studies showing ABA renewal of conditioned responding using a food or sucrose reward in female rats (Bouton et al., 2011; Bouton & Schepers, 2015; Schepers & Bouton, 2017; Todd et al., 2012, 2013). Renewal for a grain reward has also been found with female Carneau pigeons, suggesting that females across species demonstrate context-induced renewal (Rescorla, 2008). There is some evidence to suggest that renewal in female rats may be related to estradiol (Anderson & Petrovich, 2015), but the present study did not find either sex differences in renewal, or markedly greater variability in renewal among female rats.

Conclusions

In conclusion, stimulation of IL, but not PL, inputs to the PVT blocked ABA renewal of appetitive Pavlovian conditioned responding using a sucrose reinforcer in male and female rats. Our findings provide further evidence of a functional distinction in which the IL, but not the PL, suppresses the expression of conditioned responding after extinction. These findings highlight an

important role of different prefrontal cortex projections to the PVT in controlling responding to an appetitive Pavlovian cue after extinction, and provides novel evidence that functional differences between the IL and PL are maintained with their projections to the PVT. These findings should improve our understanding of the fundamental neural mechanisms underlying extinction memory retrieval and contextual processing in appetitive associative learning.

Chapter 5: General Discussion

Research on the behavioural, psychological, and neurophysiological basis of associative learning has focused greatly on the acquisition of appetitive and aversive conditioned responses, but, in the past 20 years, a growing body of research has focused on the mechanisms of extinction learning (Milad & Quirk, 2012). The clinical implications of extinction for the treatment of numerous psychiatric disorders, such as post-traumatic stress disorder and addiction, has renewed interest in extinction and the fundamental mechanisms of this form of learning and memory (Milad & Quirk, 2012). Extinction learning can be assessed with renewal, reinstatement, and spontaneous recovery paradigms. Extinguished behaviour returns when cues are encountered outside the extinction context in renewal, following presentation of the unconditioned stimulus in reinstatement, and following the passage of time in spontaneous recovery. These effects provide support for the generally accepted belief that extinction is a new form of learning as opposed to the erasure of the initial memory formed during conditioning (Bouton, 2002; Bouton et al., 2006). It is believed that conditioning and extinction memories are largely separate from each other (Bouton, 2002; Bouton & Swartzentruber, 1991), and may coexist in distinct neural circuits and ensembles (Lacagnina et al., 2019; Warren et al., 2016, 2019). Importantly, extinction memories are strongly affected by contextual cues, which provide a way for animals to modify their behaviours in adaptive ways (Bouton & Bolles, 1979).

This thesis has focused on three aspects of extinction learning that have each provided important insights into the behavioural, psychological, and neural mechanisms underlying extinction and the role of context in the extinction of aversive and appetitive conditioned behaviour. The ABA, ABC and AAB renewal models are three distinct designs that have been shown to produce renewal (Bouton & Bolles, 1979; Bouton & King, 1983; Bouton & Ricker, 1994). The ABC and AAB models emphasise the importance of removal from the extinction context in triggering renewal, whereas the ABA model proposes that returning to the initial training environment stimulates the renewal of conditioned responding. According to research that explicitly compares ABA, ABC, and AAB renewal and finds equal levels of renewal in all three designs, the removal from the extinction context, and not the return to the initial training environment, is the triggering factor for renewal (Bouton, 2002; Bouton et al., 2006). This has led theorists to believe that in Pavlovian conditioning procedures, the extinction context acts as a negative occasion setter that disambiguates the current meaning of the CS (Bouton et al., 2021). Removal from the extinction context releases the suppression of responding elicited by the context, producing renewal in ABA, ABC and AAB designs. Conversely, studies that show that ABA renewal is a stronger effect than ABC or AAB renewal imply that the renewal context A retains an excitatory association with the US that sums with the residual excitatory association with the CS, resulting in a stronger ABA renewal effect than ABC or AAB renewal (Delamater & Westbrook, 2014; Polack et al., 2013; Rescorla & Wagner, 1972).

Very few studies have directly compared all three renewal designs – ABA, ABC, and AAB – using aversive learning procedures. The magnitudes of ABA and ABC renewal have been found to be similar in a conditioned suppression task, however AAB renewal seems to be weaker than ABA or ABC renewal (Thomas et al., 2003). Similarly, using appetitive conditioning procedures, ABA renewal is more robust than ABC renewal, and several studies have been unable to detect AAB renewal (Bossert et al., 2004; Crombag & Shaham, 2002; Fuchs et al., 2005; Khoo et al., 2020; Nakajima et al., 2000; Zironi et al., 2006;). Only one study has compared ABA, ABC and AAB renewal of *active* defensive behaviour and shown equivalent

ABA, ABC and AAB renewal effects using the signaled shuttle box task (Nakajima, 2014). The experiment in Chapter 2 examined how contexts control the differential renewal of passive vs active defensive responses to an aversive stimulus using the shock-probe defensive burying (SPDB) task. Moreover, the underlying psychological mechanisms controlling the extinction of active vs passive coping responses were probed by explicitly contrasting ABA, ABC, and AAB renewal. The results showed that passive coping strategies are more susceptible to renewal as opposed to active coping strategies, and ABA renewal is more robust compared to ABC and AAB renewal. These results suggest that, as opposed to the proposed occasion setting mechanism of extinction (Bouton et al., 2021), direct context associations with either the US or the response may mediate renewal in this task.

At the neural level, the mechanisms mediating extinction have predominantly been studied using Pavlovian fear conditioning procedures. Relative to fear extinction, there are few studies on the neural mechanisms mediating appetitive extinction. The available evidence on the neural mechanisms of appetitive extinction has predominantly been conducted using operant models of relapse to drug-seeking, and far less is known about the neural mechanisms mediating the extinction of responding to appetitive Pavlovian cues. Investigating the function of multiple brain regions in extinction using an appetitive Pavlovian conditioning approach is essential in determining the neural underpinnings of extinction. In Chapter 3, we identified the neural correlates and networks associated with the recall vs extinction of responding to a discrete sucrose CS. Neurons in the prelimbic cortex (PL) and paraventricular nucleus of the thalamus (PVT) were preferentially activated during recall, while neurons in the infralimbic cortex (IL) were similarly activated during recall and extinction, suggesting that distinct neuronal ensembles within the IL may be separately recruited by the recall and extinction of appetitive Pavlovian conditioned responding. Moreover, we identified a neural network of coactivated neurons in the IL and mPVT that was specifically recruited during extinction, but not during recall. These results prompted the experiments in Chapter 4, which assessed whether activation of the PL-to-PVT and the IL-to-PVT neural pathways was sufficient to promote or inhibit appetitive Pavlovian renewal, respectively. Using an optogenetic stimulation technique that allowed selective activation of neurons that give rise to these pathways, we found that activation of the IL-to-PVT neural pathway during CS presentations suppressed ABA renewal of responding to a discrete sucrose CS, whereas activation of the PL-to-PVT neural pathway had only minor effects on renewal. Activation of both pathways induced Fos expression in the targeted brain areas, however, only activation of the IL-to-PVT pathway supported optogenetic self-stimulation, suggesting that neurons projecting from the IL to the PVT are implicated in reward. Altogether, our findings provide novel insights into the behavioural, psychological, and neural underpinnings of extinction using innovative behavioural paradigms, and have provided further insights into the contextual modulation of extinction in aversive and appetitive learning preparations.

Psychological mechanisms mediating renewal of active vs passive defensive behaviours

Chapter 2 of this thesis examined the psychological mechanisms involved in the renewal of active and passive coping responses using the SPDB task. The psychological mechanisms underlying extinction learning have primarily been studied using Pavlovian fear conditioning procedures, which have been critical for understanding the mechanisms of fear and anxiety (LeDoux & Pine, 2016). Comparing the renewal of defensive behaviours using ABA, ABC, and AAB designs is crucial for understanding the ability of environmental contexts to modulate the extinction of aversive conditioned responding. If the extinction context acts as a negative

occasion setter, as proposed by Bouton and colleagues (2021), then renewal should be comparable in the ABA, ABC and AAB designs since the removal from the extinction context releases the suppression of responding elicited by the extinction context. However, if renewal in the ABA design is greater in magnitude than in the ABC or AAB designs, this would suggest that the conditioning context A retains a direct association with the US formed during conditioning, which at test summates with the residual excitatory strength of the CS (Delamater & Westbrook, 2014; Rescorla & Wagner, 1972). These predictions are based on theories of Pavlovian conditioning; however, operant conditioning is thought to be context-dependent since after acquisition, a change in context causes a reduction in responding, suggesting that during acquisition, the context is an important component underlying operant conditioning (Bouton et al., 2011, 2014; Thraillkill & Bouton, 2015; Trask et al., 2017b). This effect of the context change contrasts with findings from the Pavlovian literature, in which there is often little effect of a context switch after acquisition (Harris et al., 2000; Bouton & King, 1983; Bouton & Peck, 1989). Moreover, in operant tasks, ABA renewal is more robust compared to AAB renewal, and this difference is not due to a direct association between the acquisition context and the reinforcer (Bouton et al., 2011). Instead, in operant learning, a direct context-response association is thought to mediate responding in acquisition and extinction (Bouton et al., 2011, 2021; Todd, 2013). Together, these theories have posited different psychological mechanisms mediating extinction in Pavlovian and operant conditioning procedures, which can be investigated by comparing renewal in the ABA, ABC and AAB designs.

It has been proposed that both Pavlovian and operant associations have an impact on responding in the SPDB task (Maren, 2003). In this task, associations can be formed between the shock-probe CS and the shock US in a Pavlovian manner, as well as between contacting the shock-probe and receiving shocks in an operant manner (Maren, 2003). The influence of both Pavlovian and operant associations provides an interesting opportunity to investigate the psychological mechanisms mediating extinction and renewal in this task. We found robust ABA renewal of passive avoidance of the side of the chamber containing the shock-probe, but there was no such renewal in the ABC or AAB designs, and surprisingly, we found a *suppression* of passive avoidance in the AAB group. Further, renewal of probe contact duration and latency was detected across groups. Visual inspection of the data revealed robust ABA, and modest ABC and AAB renewal effects for these measures, although the group by context interaction did not reach statistical significance. Moreover, we did not detect a renewal effect for any measure of active coping linked to defensive burying. The present thesis's finding that passive coping behaviours are renewed following a return to the original training context supports the widely accepted theory that extinction depends on new learning rather than on the erasure of the conditioned memory (Bouton, 2002). Additionally, the finding of robust ABA renewal of passive coping strategies is consistent with findings from Pavlovian fear conditioning and conditioned suppression experiments, which have demonstrated robust ABA renewal of a passive coping responses (Bouton & Bolles, 1979; Bouton & King, 1983; Bouton & Peck, 1989; Corcoran & Maren, 2001, 2004; Hobin et al., 2006). Our results do not support the notion that renewal relies on an occasion setting mechanism since we found robust ABA renewal, compared to relatively weaker ABC and AAB renewal. Given the renewal effects observed in the ABA group, it is unlikely that the rats' inability to distinguish between the various contexts prevented the detection of robust ABC or AAB renewal. Rats generalise fear responses across contexts if they are not sufficiently distinct (Thomas et al., 2003). However, this notion is refuted by our observation of ABA renewal and makes it unlikely that the rats were unable to distinguish

between the contexts used in this study. Furthermore, responding easily transferred from the acquisition context to novel contexts since extinction was similar in context A (AAB) and in context B (ABA and ABC) for all behaviours (Chapter 2; Figure 2A, 3A, 3B, 3C, 4A, 4B, 4C; see also Appendix A Fig. 1). This would not be expected if the context enters a direct association with the US and/or the response during acquisition, which would weaken responding following a context change (Bouton et al., 2021; Delamater & Westbrook, 2014). Therefore, the precise mechanism mediating extinction learning in this task remains unclear, but perhaps an interplay of different processes may be involved depending on the experiment's training phase.

The finding that passive coping strategies are more prone to renewal than active coping strategies is another important result from Chapter 2 of this thesis. Due to the diminished perceived imminence of threat following extinction, which is consistent with the concept of a predatory imminence continuum, we may not have been able to detect the renewal of active defensive burying (Fanselow & Lester, 1988; Perusini & Fanselow, 2015). According to this theory, the choice of defensive behaviours is controlled by an animal's perception of the imminence of a threat, which is influenced by its physical and psychological distance from the predator. In addition, 'pre-encounter', 'post-encounter', and 'circa-strike' periods are thought to be three phases into which defensive behaviours can be divided (Timberlake & Lucas, 1989). Anxiety, low predatory imminence, and defensive tactics such as increased attention, data gathering, and risk assessment are characteristics of pre-encounter behaviour. During the post-encounter phase, in which the threat is perceived to be imminent, fear is prevalent, and passive freezing is the primary defensive behaviour employed to minimise detection. Finally, the threat is encountered during the circa-strike stage, which is characterised by panic and includes active defensive behaviours such as jumping. Circa-strike behaviours are thought to be unlearned, automatic reactions to danger. However, findings demonstrating that rats bury a probe that shocked them more than a control probe that had not shocked them suggests that defensive burying can be conditioned (De Boer & Koolhaas, 2003; Pinel & Treit, 1978). In the SPDB task, rats that spend time on the side of the chamber that is opposite the shock-probe passively avoid the threat by moving away from it spatially. This may diminish active defensive burying by reducing the perception of an impending threat. Active coping strategies like defensive burying may therefore be used most vigorously after experiencing the shock during conditioning but not after extinction in the renewal context, when the threat may be passively avoided, and the perceived imminence of threat is low. In addition, there may not have been a renewal effect because rats may have preferred use of passive coping strategies which are less metabolically costly than active coping strategies (McEwan et al., 2015).

In conclusion, the findings of Chapter 2 of this thesis indicate that in the SPDB task, renewal of passive coping was most notable in the ABA group and was less marked in the ABC and AAB groups. Stronger ABA renewal compared to ABC and AAB renewal suggests that a direct context-US and/or context-response association mediates renewal in this task. However, we did not see a significant weakening of responding following a context switch after acquisition, which would be expected if context A enters an associative structure during acquisition. Therefore, the results create uncertainty about the precise psychological mechanisms that underlie extinction learning in this task, and further research is required to gain a better understanding of these mechanisms. Moreover, passive coping behaviours undergo renewal, but active defensive burying does not. Active coping strategies, which require more energy, may be less likely to be expressed and less susceptible to renewal when perceived danger is low and may be avoided passively.

The neural correlates and networks associated with appetitive Pavlovian extinction

The neural underpinnings of extinction have predominantly been studied using Pavlovian fear conditioning procedures. These studies identified the infralimbic cortex (IL) of the medial prefrontal cortex (mPFC) as a critical node mediating extinction memory consolidation, as well as a functional dissociation with the more anterior subregion of the mPFC, the prelimbic cortex (PL), which was found to drive the expression of Pavlovian conditioned freezing (Burgos-Robles et al., 2007; Milad & Quirk, 2002; Santini et al., 2004; Sierra-Mercado et al., 2011; Vidal-Gonzalez et al., 2006). Furthermore, IL projections to the basolateral amygdala (BLA) and paraventricular nucleus of the thalamus (PVT) were found to be critical for Pavlovian fear extinction (Bloodgood et al., 2018; Bukalo et al., 2015; Tao et al., 2021). Relative to fear extinction, there are few studies on the neural mechanisms of appetitive extinction. The available evidence, however, has predominantly been conducted using operant models of relapse to drug-seeking, and has demonstrated converging evidence for a dichotomy between the IL and PL in the extinction and promotion of operant reward-seeking, respectively, as well as a role of the BLA in appetitive extinction (McLaughlin & Floresco, 2007; Peters et al., 2008, 2009). Moreover, the orbitofrontal cortex (OFC), as well as IL projections to the nucleus accumbens shell (NAcSh) appear critical for the extinction of operant reward-seeking (Augur et al., 2016; Butter et al., 1963; Peters et al., 2008). In comparison to the extinction of Pavlovian fear and operant reward-seeking, little is known about the neural mechanisms mediating the extinction of responding to appetitive Pavlovian cues. Investigating the function of multiple brain regions in the extinction of responding using an appetitive Pavlovian conditioning approach is essential in determining the neural underpinnings of extinction.

Chapter 3 of the current thesis used Fos immunohistochemistry and correlational network connectivity analysis to identify the neural correlates and brain activation networks that are associated with the extinction vs recall of responding to a discrete sucrose CS. We developed a behavioural paradigm to compare Fos density induced by the extinction vs recall of responding to an appetitive Pavlovian CS (paired condition) with responding in a putatively appetitive context (unpaired condition) and exposure to the white noise stimulus alone (home-cage condition). Environmental cues that predict rewards can cause responses in the unpaired condition (Bouton, 2002), and so the addition of the home-cage condition and its use as a baseline for Fos density was a crucial consideration. We found greater Fos density for the paired and unpaired conditions relative to the home-cage condition, and no difference between the paired and unpaired conditions, in most brain regions examined including the IL and PL. This result suggests that responses generated by the discrete sucrose CS and the appetitive context stimulate neural activity in these regions in a similar manner (Repucci & Petrovich, 2012). Similar findings have been found using a food reinforcer (Yager et al., 2015), and are consistent with the well-established roles of the IL and PL in mediating context-specific learning processes (Fuchs et al., 2005; Hyman et al., 2012; Moorman & Aston-Jones, 2015; Perry & McNally, 2013; Trask et al., 2017a; Warren et al., 2016).

In the PVT, particularly in the anterior and middle subregions of the PVT (aPVT and mPVT), Fos density was significantly greater for the paired condition relative to the unpaired condition during recall, suggesting that these regions are specifically recruited during the recall of responding elicited by the discrete sucrose CS. The current findings emphasise the PVT's special function in responding to a discrete appetitive Pavlovian CS, which is line with the finding that neurons in the PVT are activated by cues that predict reward and by cues that have

gained incentive saliency (Flagel et al., 2011; Haight et al., 2017; Igelstrom et al., 2010; Yager et al., 2015). Moreover, correlational network connectivity analysis revealed an extinction-specific network composed of the IL and mPVT that was active during extinction but not recall in the paired condition, suggesting that a neural circuit composed of the IL and mPVT may be implicated in appetitive Pavlovian extinction. In Chapter 4 of this thesis, this hypothesis was investigated. The findings support the idea that the IL-to-PVT pathway plays a functional role in appetitive Pavlovian extinction memory retrieval by suppressing the renewal of responding to a discrete sucrose CS.

When comparing Fos density induced by the recall vs extinction of responding, we found similar levels of Fos density in the IL, medial OFC, and medial and lateral subregions of the NAcSh. These results raise the possibility that distinct neuronal ensembles within various brain regions may be recruited by the recall and extinction of responding. Warren and colleagues (2016, 2019) have shown that distinct neuronal ensembles within the IL encode operant food-seeking and extinction memories, supporting this theory. Additionally, distinct neuronal ensembles within the IL promote or inhibit operant reward-seeking in response to discriminative stimuli that predict the availability or omission of rewards, respectively (Suto et al., 2016). Also in support of this hypothesis, a neuronal ensemble within the IL encodes the learned association between heroin and the conditioning context, which when re-activated after extinction in a different context promotes the renewal of heroin-seeking (Bossert et al., 2011). These findings suggest that distinct neuronal ensembles within the IL encode the original operant conditioning memory and extinction memories. Therefore, neuronal ensembles are an important element to consider in future analysis on the neural mechanisms of appetitive Pavlovian extinction. Future research conducted using the Daun02 inactivation method (Koya, 2009) can help determine whether distinct neuronal ensembles within the IL are recruited by the recall and extinction of appetitive Pavlovian conditioned responding.

In the PL, NAcC, and all PVT subregions, Fos density was greater during recall than extinction, suggesting that certain brain regions are selectively activated during the recall of responding. These results are consistent with research demonstrating that these regions promote the expression of operant drug-seeking and promote the renewal and reinstatement of extinguished responding (Dayas et al., 2008; Fuchs et al., 2005, 2008). Additionally, prior studies have shown that the PL-to-NAcC pathway plays a role in promoting appetitive operant conditioned responding (Stefanik et al., 2013, 2016). More recent findings have also shown that PL inputs to the PVT play a role in the reinstatement of operant cocaine-seeking, but not sucrose-seeking (Giannotti et al., 2018; Kuhn et al., 2021). Based on these results, Chapter 4 investigated the sufficiency of the PL-to-PVT neural pathway in promoting the renewal of extinguished appetitive Pavlovian responding. The results showed that optically activating PL inputs to the PVT had only modest effects on renewal of responding to a discrete sucrose CS, suggesting that direct inputs from the PL to PVT might not be implicated in promoting the renewal of responding for natural rewards like sucrose.

IL inputs to the PVT are sufficient to inhibit renewal of responding to a discrete sucrose CS

Building upon the findings obtained in Chapter 3 of this thesis, Chapter 4 investigated the neural circuitry sufficient to promote or inhibit the renewal of responding to a discrete sucrose CS in the original training context. Immunofluorescence was used to validate the excitatory circuit-specific optogenetic technique, and Fos immunohistochemistry verified that laser activation increased neuronal activity in the IL and PVT, and PL and PVT following optical

stimulation of either the IL-to-PVT or PL-to-PVT pathways. Optogenetic activation of the PL-to-PVT pathway did not affect the latency to initiate CS port entries or ΔCS port entries in the renewal context. However, compared to the eYFP control group, activation of the PL-to-PVT pathway resulted in minor decreases in the probability and duration of CS port entries. In contrast, activation of the IL-to-PVT pathway decreased the probability and duration of CS port entries, increased the latency to initiate CS port entries, and attenuated the renewal of ΔCS port entries. Collectively, optogenetic activation of IL neurons projecting to the PVT inhibited all measures of CS-elicited responding during renewal, whereas optogenetic activation of PL inputs to the PVT had only minor effects on renewal.

The finding that activation of IL inputs to the PVT attenuated the renewal of appetitive Pavlovian conditioned responding is consistent with the well-established role of the IL in mediating extinction. In both operant drug-seeking and Pavlovian fear conditioning experiments, IL stimulation enhances extinction learning and retrieval, whereas IL inactivation or lesions impede extinction retrieval and promote reinstatement and renewal (Laurent & Westbrook, 2009; Peters et al., 2008; Quirk et al., 2000; Vidal-Gonzlez et al., 2006). Moreover, our results are consistent with the sparse amount of research that has been done to date on the IL's potential to mediate appetitive Pavlovian extinction learning. The renewal of Pavlovian conditioned responding to a sucrose predictive CS is suppressed by IL stimulation (Villaruel et al., 2018). Moreover, the reinstatement and renewal of Pavlovian conditioned responding for food or sucrose are potentiated by lesions or inactivation of the IL (Lay et al., 2019; Rhodes & Killcross, 2004, 2007). To date, the role of the IL-to-PVT pathway in extinction has only been reported using an aversive conditioning procedure (Tao et al., 2021). The results of Chapter 4 provide novel evidence that the functional role of the IL in mediating extinction is maintained through its projections to the PVT using an appetitive Pavlovian conditioning procedure.

Within the medial prefrontal cortex (mPFC), a popular hypothesis is that the IL mediates extinction, while the PL functions to drive the expression of conditioned responding. Pavlovian fear conditioning experiments demonstrate that PL stimulation increases conditioned responding after extinction, and operant drug-seeking studies have shown that PL inactivation reduces conditioned responding after extinction (Capriles et al., 2003; McFarland et al., 2004; McFarland & Kalivas, 200; Vidal-Gonzalez et al., 2006). It is likely that we did not see an increase in renewal because we used optogenetic targeting of PL inputs to the PVT. It has been demonstrated that cue-induced reinstatement of operant sucrose-seeking is unaffected by PL-to-pPVT inactivation (Giannotti et al., 2018). The aPVT has been linked to operant sucrose-seeking (Do-Monte et al., 2017), whereas the pPVT regulates relapse to operant drug-seeking (Matzeu et al., 2015). Moreover, the pPVT plays a role in signalling aversive states (Penzo et al., 2015; Zhu et al., 2018). Interestingly, optogenetic inhibition of the PL-to-mPVT impairs the retrieval of conditioned fear at late time points (after 7 days), but not at early time points (after 24h) (Do-Monte et al., 2015b). Thus, consistent with the role of the PL in promoting conditioned responding, the PL-to-mPVT pathway seems to be involved in maintaining fear conditioning memories, but in a time-sensitive manner. Discrepant findings in the roles of different PVT subregions are likely due to the heterogeneous population of neurons in the PVT (Gao et al., 2020). Additionally, few studies have investigated the role of the mPVT, and most studies examining the role of the PVT in motivated behaviours distinguish between the aPVT and pPVT, which are not necessarily consistent across studies. We discovered that increasing the activity of PL inputs mainly targeting the mPVT had only a small impact on the renewal of responding to sucrose CS. Future studies are required that further investigate the functional role of distinct PVT

subregions in appetitive extinction. The role of IL and PL projections to the PVT along its anteroposterior axis in renewal of responding for natural vs drug rewards also need additional study.

The role of the IL-to-PVT neural pathway in reward processing

We questioned if IL inputs to the PVT may also drive reinforcement, despite being implicated in behavioural suppression (Gao et al., 2020; Tao et al., 2021), given that PVT input to the NAc can suppress behaviour in some circumstances while reinforce self-stimulation (Lafferty et al., 2020). To test the reinforcing properties of the IL-to-PVT pathway, we used a self-stimulation procedure in rats that had ChR2 expressed in IL neurons and fiber optic implants targeting the PVT. When optical stimulation was made dependent on active nose-pokes, IL input to the PVT readily supported self-stimulation behaviour, showing that acute IL-to-PVT pathway stimulation can reinforce operant behaviour. On the other hand, optical stimulation did not reinforce operant behaviour when the PL-to-PVT pathway was targeted. Therefore, operant behaviour can be reinforced by brief stimulation of IL inputs to the PVT.

Early reports have shown that electrode placement in the PVT supports intracranial self-stimulation (Cooper & Taylor, 1967; Clavier & Gerfen, 1982). However, more recent reports have also implicated the pPVT, as well as PL inputs to the middle-to-posterior PVT, in aversive states (Bhatnagar et al., 2002; Penzo et al., 2015; Do-Monte et al., 2015b). These discrepancies may be due to the distinct cell populations in the PVT and their differential efferent and afferent projections. The PVT is a heterogeneous brain area that has recently been shown to be composed of two functionally distinct cell types referred to as type I and type II PVT neurons (Gao et al., 2020). These two distinct PVT neurons are differentially innervated by the PL and IL; the PL preferentially innervates type I neurons mostly contained in the pPVT, the IL mainly targets type II neurons located in the aPVT, and the mPVT is a transitional heterogeneous subregion composed of similar proportions of both types of neurons (Gao et al., 2020). Supporting a role of the pPVT in signalling aversive states, type I neurons in the pPVT are more likely to express dopamine D_2 receptors than type II neurons in the aPVT (Gao et al., 2020). Overall, our results support the idea that, possibly through their innervation of different cell populations in the PVT, IL and PL inputs to the PVT make distinct contributions to operant reward-seeking and appetitive Pavlovian conditioned responding. The IL-to-PVT pathway may be implicated in reward through the innervation of non-D_2-expressing neurons in the PVT by the IL.

Sex differences in preclinical models of relapse to sucrose use

Investigating sex differences in conditioned responding for sucrose is important because, to date, the results in both human and animal studies using food or sucrose rewards have generally been conflicting and ambiguous. Some studies have suggested that women experience greater food cravings than men, especially for sweet foods (Weingarten & Elston, 1991; Zeller et al., 1999). Although, the gender difference in sweet cravings was found to be mediated by cultural differences rather than physiological variations (Cepeda-Benito et al., 2003). Yet, women have been found to consume more sugary foods during the luteal phase of their menstrual cycle, pointing to the possibility that sex hormones may contribute to gender differences in sucrose consumption (Asarian & Geary, 2006, 2013). Research from animal models can offer crucial new insights into these differences.

In Chapter 4 of this thesis, we investigated potential sex differences in the acquisition, extinction, and renewal of Pavlovian conditioned responding employing a 10% liquid sucrose

reinforcer, and no sex differences were found. This finding is consistent with other studies that have reported no significant sex differences in the acquisition (Cox et al., 2013) or extinction (Zhou et al., 2015) of conditioned responding for sucrose in rats. However, mixed results have also been found, with some studies showing that females respond more than males, and others showing the opposite effect (Grimm et al., 2022; Hammerslag & Gulley, 2014; Zhou et al., 2015). Few studies have thoroughly investigated sex differences in renewal even in studies with both male and female subjects (Bouton & Ricker, 1994; Campese & Delamater, 2013). The findings from Chapter 4, however, are in line with several studies showing ABA renewal of conditioned responding in female rats using food or sucrose as a reinforcer (Bouton et al., 2011; Bouton & Schepers, 2015; Schepers & Bouton, 2017; Todd et al., 2012, 2013). Female Carneau pigeons have also shown renewal in response to a grain incentive, indicating that females of various species do as well (Rescorla, 2008). Although there is some indication that renewal of responding to a food cue in female rats may be influenced by estrogen (Anderson & Petrovich, 2015), neither sex differences in renewal nor noticeably higher variability in renewal among female rats were observed. Females are, however, disproportionately underrepresented in preclinical models of addiction, with the great majority of studies utilising solely male subjects (Beery & Zucker, 2011; Shansky, 2019). This is a significant continuing issue in neuroscience research, and future research is needed that investigates sex differences in preclinical models of relapse to sucrose use.

Conclusions

Experiments presented within this thesis have provided novel insights into the behavioural, psychological, and neurobiological basis of extinction. Chapter 2 characterized the renewal of active and passive coping strategies in an aversive conditioning paradigm and indicated that passive coping responses are more susceptible to renewal than active coping responses. Chapters 3 and 4 focused on the roles of brain structures in extinction and renewal using novel analytical and experimental techniques. The results of Chapter 3 are consistent with a functional distinction within the mPFC, such that the PL promotes the expression of conditioned responding and the IL mediates the extinction of conditioned responding (Peters et al., 2009). Additionally, the results emphasize the unique function of the PVT in responding to a discrete appetitive sucrose CS. In Chapter 4, it was demonstrated that, in both male and female rats, stimulation of IL inputs to the PVT suppressed ABA renewal of responding to a discrete sucrose CS. Whereas, stimulation of the PL-to-PVT pathway only had modest effects on renewal. Altogether, this thesis offers new insights into the behavioural, psychological, and neural mechanisms underlying extinction, a fundamental learning and memory process that has been conserved across species, and which enables humans and animals to adapt to shifting environmental conditions to survive.

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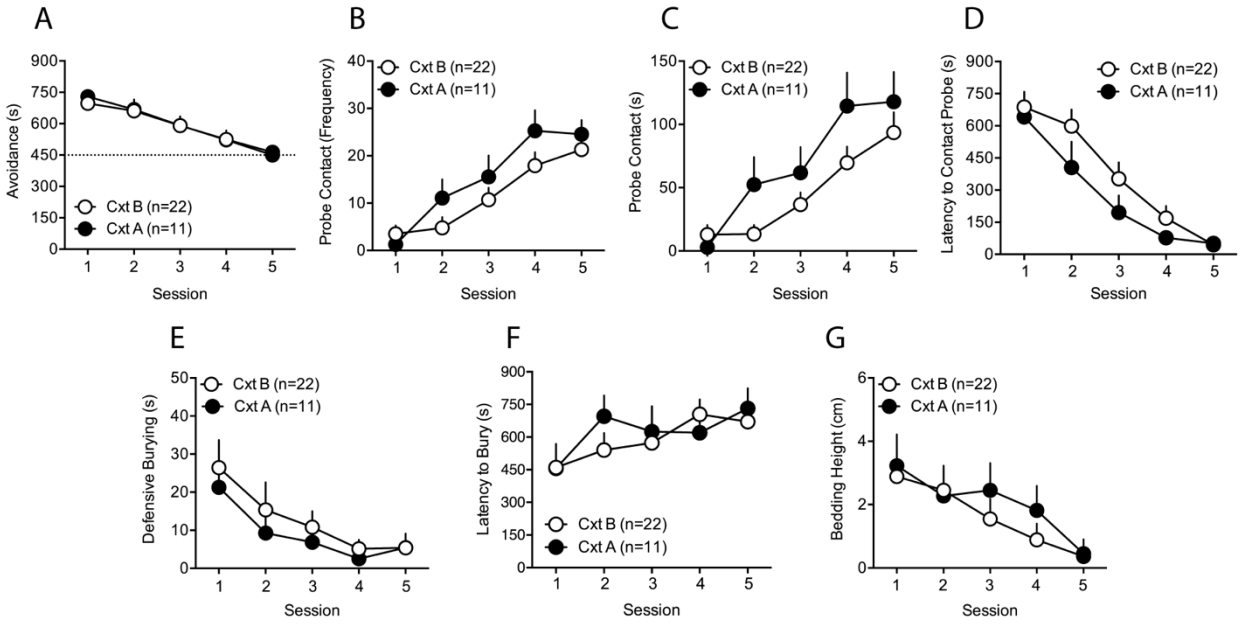
Appendix A



Appendix A Figure 1. Comparing extinction of responding across 5 sessions in context A (AAB group) vs context B (collapsed across the ABC and ABA groups). **A** The duration of passive avoidance significantly decreased across extinction sessions comparably in contexts A and B (Session, $F_{2.7,84.9}$=23.47, p<.001; Context, $F_{1,31}$=.02, p=.900; Session*Context, $F_{2.7,84.9}$=.16, p=.909). The dotted line indicates half the session length. The **B** frequency and **C** duration of shock-probe contacts significantly increased, and the **D** latency to initiate shock-probe contact significantly decreased across extinction sessions comparably in contexts A and B (Frequency: Session, $F_{2.7,82.6}$=31.25, p<.001; Context, $F_{1,31}$=1.55, p=.222; Session*Context, $F_{2.7,82.6}$=1.43, p=.241; Duration: Session, $F_{2.3,71.8}$=23.93, p<.001; Context, $F_{1,31}$=2.58, p=.119; Session*Context, $F_{2.3,71.8}$=1.63, p=.199; Latency: Session, $F_{4,124}$=33.77, p<.001; Context, $F_{1,31}$=1.75, p=.195; Session*Context, $F_{4,124}$=.84, p=.503). The **E** duration of defensive burying significantly decreased, the **F** latency to bury significantly increased, and **G** the height of accumulated bedding surrounding the shock-probe significantly decreased across extinction sessions comparably in contexts A and B (Duration: Session, $F_{2.1,66.2}$=6.32, p=.003; Context, $F_{1,31}$=.32, p=.576; Session*Context, $F_{2.1,66.2}$=.14, p=.880; Latency: Session, $F_{3.1,95.7}$=4.20, p=.007; Context, $F_{1,31}$=.12, p=.727; Session*Context, $F_{3.1,95.7}$=.96, p=.417; Bedding Height: Session, $F_{4,124}$=7.24, p<.001; Context, $F_{1,31}$=.26, p=.614; Session*Context, $F_{4,124}$=.44, p=.783).

Appendix B

Pathway	Measure	Phase	Factor	*F*	*p*
IL-to-PVT	ΔCS	Acquisition	Sex	1.48	.235
			Group*Sex	1.07	.312
			Session*Sex	.38	.873
			Session*Group*Sex	1.24	.292
IL-to-PVT	ΔCS	Extinction	Sex	.15	.699
			Group*Sex	.54	.468
			Session*Sex	.24	.629
			Session*Group*Sex	.33	.571
IL-to-PVT	ΔCS	Test	Sex	.31	.581
			Group*Sex	.83	.372
			Context*Sex	.03	.861
			Context*Group*Sex	1.14	.296
IL-to-PVT	Latency	Test	Sex	.56	.462
			Group*Sex	.54	.468
			Context*Sex	1.40	.248
			Context*Group*Sex	.06	.813
IL-to-PVT	Duration	Test	Sex	.08	.775
			Group*Sex	.17	.683
			Context*Sex	.01	.934
			Context*Group*Sex	1.29	.266
IL-to-PVT	Probability	Test	Sex	.49	.492
			Group*Sex	.89	.354
			Context*Sex	.59	.450
			Context*Group*Sex	.40	.531
IL-to-PVT	ITI	Test	Sex	1.58	.221
			Group*Sex	.13	.723
			Context*Sex	.01	.926
			Context*Group*Sex	.58	.453

Appendix B Table 1. F statistics and p values of comparisons between virus groups (eYFP vs ChR2) and sexes (male vs female) as a factor of training session during acquisition and extinction phases for ΔCS port entries and as a factor of context (B vs A) during the test phase for ΔCS port entries, latency to CS port entries, duration of CS port entries, probability of CS port entries and ITI port entries in rats targeting the IL-to-PVT pathway.

Pathway	Measure	Phase	Factor	*F*	*p*
PL-to-PVT	ΔCS	Acquisition	Sex	.05	.821
			Group*Sex	.42	.525
			Session*Sex	1.41	.239
			Session*Group*Sex	.52	.721
PL-to-PVT	ΔCS	Extinction	Sex	.14	.717
			Group*Sex	4.06	.056
			Session*Sex	.29	.598
			Session*Group*Sex	5.06	.034
PL-to-PVT	ΔCS	Test	Sex	.06	.808
			Group*Sex	.63	.435
			Context*Sex	.23	.633
			Context*Group*Sex	.01	.911
PL-to-PVT	Latency	Test	Sex	.37	.548
			Group*Sex	2.36	.138
			Context*Sex	.69	.416
			Context*Group*Sex	1.75	.198
PL-to-PVT	Duration	Test	Sex	.03	.867
			Group*Sex	2.30	.143
			Context*Sex	.04	.841
			Context*Group*Sex	1.55	.226
PL-to-PVT	Probability	Test	Sex	.29	.594
			Group*Sex	1.45	.240
			Context*Sex	1.02	.322
			Context*Group*Sex	.14	.713
PL-to-PVT	ITI	Test	Sex	.00	.996
			Group*Sex	1.39	.250
			Context*Sex	.14	.712
			Context*Group*Sex	.81	.378

Appendix B Table 2. F statistics and p values of comparisons between virus groups (eYFP vs ChR2) and sexes (male vs female) as a factor of training session during acquisition and extinction phases for ΔCS port entries or as a factor of context (B vs A) during the test phase for ΔCS port entries, latency to CS port entries, duration of CS port entries, probability of CS port entries and ITI port entries in rats targeting the PL-to-PVT pathway.