Corticostriatal control of extinction in appetitive Pavlovian conditioning

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Abstract

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Animals use environmental cues to guide their behaviour to obtain desirable outcomes and avoid aversive ones. Through extinction, animals can learn to suppress learned behaviours when the expected appetitive or aversive outcome is omitted. The infralimbic cortex (IL) and its efferent projections to the nucleus accumbens shell (NAcS) and the basolateral amygdala (BLA) are thought to be critical for extinction and suppressing responding to cues that predict aversive outcomes and responding for drugs of abuse. However, fewer research has investigated whether the IL and its neural projections are important for extinction of responding to cues that predict more naturalistic appetitive outcomes.

The present thesis examined the effects of augmenting activity in the IL, the IL-to-NAcS projection, and the IL-to-BLA projection on extinction of conditioned approach to a sucrose cue. In two experimental chapters (Chapters 3 and 4) we used a renewal task, to test whether optogenetic stimulation of the IL, IL-to-NAcS, and IL-to-BLA would suppress the return of responding after extinction. Briefly, in the renewal task, rats first received Pavlovian conditioning in a specific context (Context A) to associate an auditory conditioned stimulus (CS) with the delivery of a sucrose unconditioned stimulus (US). Next, rats received extinction in a different context (Context B) by presenting the CS but omitting the expected US, leading to a reduction in conditioned responding. After extinction, renewal of responding to the CS was triggered by presenting the CS alone in the original Pavlovian conditioning context (Context A). We found that optogenetic stimulation of the IL and IL-to-NAcS projection but not the IL-to-BLA projection during the CS attenuated the renewal of appetitive Pavlovian conditioned responding.

In the last chapter (Chapter 5), we explored potential mechanisms by which stimulation of the IL-to-NAcS projection suppresses appetitive Pavlovian conditioned responding. First, we tested whether prior extinction training was necessary for IL-to-NAcS stimulation to suppress conditioned responding. Second, we tested whether stimulation during Pavlovian conditioning would lead to general suppression of responding. We found that IL-to-NAcS stimulation during

the CS suppressed conditioned responding regardless of prior extinction training. Further, IL-to-NAcS stimulation during Pavlovian conditioning did not appear to indiscriminately suppress responding, but altered the expression of conditioned responding to the CS.

In conclusion, the findings of the present thesis expand the role of the IL and IL-to-NAcS projection in extinction to appetitive Pavlovian cues. Further, we provide novel evidence that suppression of appetitive Pavlovian responding following IL-to-NAcS stimulation may not be dependent on an extinction process. Nevertheless, the IL-to-NAcS projection plays an important role in controlling conditioned responding to appetitive cues. These findings further our knowledge of how corticostriatal circuits contribute to adaptive behaviour which may be useful for understanding psychological disorders involving inhibition of learned behaviours.

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Contribution of Authors

Chapter 1: General introduction was written by Franz R. Villaruel and revised by Dr. Nadia Chaudhri and Dr. Mihaela Iordanova.

Chapter 2: General methods and all experiments were conceptualized and designed by Franz R. Villaruel and Dr. Nadia Chaudhri.

Chapter 3: Franz R. Villaruel conducted the experiments, analyzed the data, and prepared the figures. Franca Lacroix contributed to conducting the experiments. All experiments appear as a subset in Villaruel, F. R., Lacroix, F., Sanio, C., Sparks, D. W., Chapman, C. A., & Chaudhri, N. (2018). Optogenetic Activation of the Infralimbic Cortex Suppresses the Return of Appetitive Pavlovian-Conditioned Responding Following Extinction. *Cerebral Cortex*, 28(12), 4210–4221.

Chapter 4: Franz R. Villaruel conducted the experiments, analyzed the data and prepared the figures. All experiments appear in Villaruel, F. R., Martins, M., & Chaudhri, N. (2022). Corticostriatal Suppression of Appetitive Pavlovian Conditioned Responding. *Journal of Neuroscience*, *42*(5), 834–849. https://doi.org/10.1523/JNEUROSCI.1664-21.2021.

Chapter 5: Franz R. Villaruel conducted experiments, analyzed the data, and prepared the figures. Melissa Martins conducted a replication of Experiment 1 and contributed to analyzing the data. All experiments appear in Villaruel, F. R., Martins, M., & Chaudhri, N. (2022). Corticostriatal Suppression of Appetitive Pavlovian Conditioned Responding. *Journal of Neuroscience*, *42*(5), 834–849. https://doi.org/10.1523/JNEUROSCI.1664-21.2021.

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Chapter 1 - General Introduction

Animals use environmental cues to guide their behaviour and ensure survival. Paramount to adaptive functioning is also the ability to change behaviours to meet environmental demands. These phenomena can be modeled and investigated using Pavlovian or classical conditioning and extinction. Pavlovian conditioning is a process by which animals learn about the relationships between different stimuli in their environment. Conversely, extinction is a fundamental aspect of behavioral change, in which animals learn to suppress responding to stimuli when the expected outcome no longer occurs. Knowledge on the neural and psychological processes of extinction is important for understanding adaptive behaviour and pathologies involving inhibitory control. The present thesis investigates the role of the infralimbic cortex (IL) and its neural projections to the basolateral amygdala (BLA) and nucleus accumbens shell (NAcS) in extinction and suppression of conditioned responding to appetitive Pavlovian cues.

1. Pavlovian conditioning, extinction, and renewal

In Pavlovian conditioning, a conditioned response (CR) develops to a previously neutral conditioned stimulus (CS) when that CS is presented contingently on the presence of a motivationally significant unconditioned stimulus (US). After Pavlovian conditioning, the CS alone can elicit a CR, which can be taken as evidence that the organism has learned the association between the CS and the US (CS-US). The classic example from Pavlov's (1927) fundamental work is the salivation conditioned response (CR) elicited by ringing a bell (CS) after the bell was paired with the delivery of food (US). Pavlovian conditioning is thought to be the foundation of how organisms build representations of their environment to guide their behaviour (Locke, 1690/1964, as cited in Klein, 2009; Hume, 1748/1955 as cited in Klein, 2009; Pavlov, 1927).

Complementary to Pavlovian conditioning is extinction. Extinction describes the reduction in responding to a CS, when it is presented in the absence of the expected US. In Pavlov's example, this entails repeatedly presenting the bell (CS) but withholding the delivery of food (US) to reduce the salivation response (CR) previously elicited by the CS. Extinction is a principal example of behavioural change that allows animals to adapt in dynamic environments.

Extinction is thought to involve new learning, that the CS now signals the absence of the US (CS-No US) (Konorski, 1948; 1967; Mackintosh, 1975; Pearce and Hall 1980). Support for

the idea of new inhibitory learning stems from early observations that conditioned responding can spontaneously recover after a given amount of time passes following the last extinction training session (Pavlov, 1927). This finding suggests that the original CS-US association is not completely erased but remains relatively intact despite the reduction in conditioned responding observed during extinction. Although, some erasure of the original CS-US association may occur (Rescorla and Wagner, 1972; Delamater and Westbrook, 2014), the inhibitory CS-No US association established during extinction could also compete with the original conditioning association to suppress responding (Konorski, 1948; 1967; Mackintosh, 1975; Pearce and Hall 1980). Therefore, following conditioning and extinction, competing excitatory and inhibitory associations are thought to vie for the expression of behaviour (Bouton, 1993).

A memory retrieval framework can account for the competitive nature of excitatory and inhibitory associations over behaviour (Bouton, 1993; 2004). This framework holds that retrieval of an excitatory association acquired during conditioning leads to emission of the conditioned response while retrieval of an inhibitory association acquired during extinction leads to suppression of the conditioned response. Contexts are thought to play a pivotal role in moderating the competing memories learned during conditioning and extinction (Bouton, 1993; 2004). The inhibitory extinction memory is thought to be particularly context sensitive as changing the context between extinction training and test impairs extinction retrieval and triggers a return of conditioned responding (Bouton, 1993; 2004). Within this framework, Bouton (2004) argues that contexts can take on different forms and a return of responding can occur following either a change in the temporal context (spontaneous recovery), reintroduction of stimuli that was present during conditioning (reinstatement) or changes in the physical context (renewal).

Renewal is a clear demonstration of how context affects the retrieval of an inhibitory extinction memory. It is often exhibited using an ABA procedure, in which each letter refers to the conditioning, extinction, and test context, respectively. In Pavlovian conditioning, this involves pairing CS presentations with the US in one context (Context A: CS-US), and then extinguishing the conditioned response by presenting the CS alone in a different context (Context B: CS-No US). During tests, the CS is presented again in Context A which triggers a return of conditioned responding. Renewal is thought to be observed when conditioned responding is greater at test in Context A in comparison to the last extinction session or when subjects are tested again in the extinction context (Context B). It is important to note that return

of extinguished responding does not necessarily depend on returning subjects to the original conditioning context. AAB renewal can occur when conditioning and extinction occur in the same context, but test occurs in a different context (Bouton and Ricker, 1994). Alternatively, ABC renewal can also be observed, when conditioning, extinction, and test all occur in three different contexts (Bouton and Bolles, 1979).

Different ideas have been proposed to explain the renewal effect. One account suggests that the extinction context acquires inhibitory properties on its own which can protect the CS from undergoing complete extinction (Rescorla and Wagner, 1972; Delamater and Westbrook, 2014). Based on this idea, renewal occurs because the CS is released from the inhibitory constraints of the extinction context at test. Another account suggests that contexts play a more modulatory role in directing retrieval of either the CS-US conditioning association or the CS-No US extinction association (Bouton, 1993; 2004). According to this account, renewal occurs due to a failure in retrieval of the inhibitory extinction memory following a change in context. Nevertheless, the renewal phenomenon highlights the impermanence and context sensitivity of extinction.

2. Studying the neural processes of extinction

Interest in the neural processes of extinction has increased due to its principal role in adaptive function and its therapeutic applications in reducing maladaptive behaviour. Early studies investigating the neural mechanisms of extinction involved lesions and pharmacological perturbations of different brain regions and neurotransmitter systems. Moreover, electrophysiological methods were used to monitor neural activity during extinction training and retrieval. These early studies provided foundational insight into the different neural processes that are involved in extinction and the suppression of learned behaviours. Novel tools have since been developed to further probe the neural mechanisms of extinction with greater granularity.

One of these modern tools is optogenetics, a technique that allows for the manipulation of neural activity, with cell, circuit, and temporal specificity. Optogenetics works by injecting an adeno-associated viral vector (AAV) containing the transgene that codes for light-sensitive protein channels or opsins (Boyden et al., 2005; Yizhar et al., 2011). Specific promoter regions can be engineered within the transgene to allow for expression in specific cell-types. For instance, including the promoter CaMKIIa within the transgene allows for the light sensitive-

protein channels to be predominantly expressed in excitatory glutamatergic pyramidal neurons (Jones et al., 1994; Leonard et al., 1999). Stimulation is achieved through channelrhodopsin (ChR2), a light-sensitive protein channel that when activated by blue light allows for cations to enter, depolarize a neuron, and fire an action potential. Other light-sensitive protein channels such as halorhodopsin, can inhibit specific neurons by allowing chloride ions to enter and hyperpolarize a neuron when activated by yellow light. Specific wavelength of light can be delivered by a laser or light-emitting diode (LED) via an optical fiber implanted into the specific brain region with ultra-precise temporal control (Al-Hasani et al., 2015; Deisseroth, 2015). The expression of opsins following viral transfection is not specific to neurons and occurs along the processes and terminals. Thus, when light is delivered to the terminals, a projection specific gain- or loss- of function can be induced (Tye and Deisseroth, 2012). Optogenetics offers unprecedented temporal control over brain regions and circuits that allows for neural modulation at specific time points during a behavioural session such as cue or outcome presentation.

3. Infralimbic cortex and extinction

The infralimbic cortex (IL) has emerged as a central node in the brain that mediates the extinction of conditioned responses (Quirk and Mueller, 2008; Peters et al., 2009). The IL is a ventral subregion of the medial prefrontal cortex that contains unique inputs and outputs compared to other subregions (Heidbreder & Groenewegen, 2003; Hoover & Vertes, 2007). The IL is a laminar structure primarily containing glutamatergic pyramidal neurons that are found in layers 2/3 and layers 5/6. In contrast, inhibitory GABAergic interneurons are found across layers of the IL. Pyramidal neurons in layers 2 and 3 predominantly project to other cortical regions leading to a highly recurrent circuit within the cortex. In contrast, pyramidal neurons in layers in 5/6 predominantly project to thalamic and subcortical regions, which may allow the IL to exert top-down control over various behavioural processes, including extinction (Miller, 2000; Miller and Cohen 2001, Euston et al., 2012).

3.1 IL in extinction of aversive conditioned responding

Studies on the role of the IL in extinction typically use aversive Pavlovian conditioning procedures in which an auditory CS is paired with a mild foot-shock as the US. Conditioned responding is indexed by either freezing, avoidance, or suppression of ongoing behaviours

during the CS. Subsequently, during extinction, the CS is presented alone and the aversive response decreases. Neural activity in the IL positively correlates with the extinction and attenuation of conditioned freezing (Milad and Quirk, 2002; Gilmartin and McEchron, 2005, Giustino et al., 2016). Greater IL activity is observed after successful extinction training that results in the suppression of aversive conditioned responding (Santini et al., 2004; Kim et al., 2010). Extinction training also increases the excitability of IL neurons and reverses the decrease in IL intrinsic excitability induced by aversive Pavlovian conditioning (Santini et al., 2008; Cruz et al., 2014). As context plays a vital role in extinction, it is not surprising that it also gates IL activity. Presenting an aversive CS in the context in which it was extinguished induces greater activity in the IL, measured by Fos, relative to presenting the CS outside of the extinction context (Knapska and Maren, 2009, Orsini et al., 2011). In sum, there is substantial evidence that the IL is engaged during extinction learning and retrieval.

Studies disrupting IL activity through lesions or pharmacological inactivation during extinction find a consistent role for the IL in extinction retrieval. Early studies found that lesions predominantly targeting the IL preserved the acquisition of aversive conditioning and extinction but impaired subsequent extinction retrieval and delayed learning across sessions. (Morgan et al., 1993; Morgan et al., 1995; Quirk et al., 2000; Morgan et al., 2003; Lebrón et al., 2004). Consistently, pharmacological inactivation of the IL specifically during aversive conditioning did not affect acquisition of conditioned freezing (Sierra-Mercado et al., 2006). Therefore, decrements in extinction retrieval following IL lesions are not simply attributable to deficits from aversive conditioning. Further, lesions or pharmacological inactivation of the IL exclusively before extinction training did not affect initial learning but impaired retrieval, producing greater conditioned freezing in subsequent extinction sessions (Morgan et al., 2003; Sierra-Mercado et al., 2006, Laurent and Westbrook 2008; Laurent and Westbrook, 2009; Sierra-Mercado et al., 2011; Lay et al., 2020). Similar effects were also observed following the administration of glutamatergic, noradrenergic, and dopaminergic antagonists in the IL during extinction training, suggesting a complex interplay between these neurotransmitter systems in mediating extinction (Mueller et al., 2008; Mueller et al., 2010; Laurent and Westbrook, 2008). In sum, these studies suggest that the IL may not be necessary for initial extinction training but rather for retrieving an established inhibitory extinction memory to guide suppression of aversive conditioned responses.

The role of the IL in extinction retrieval can be further examined by disrupting IL function after subjects have received extinction training. However, these studies have found inconsistent effects of IL manipulations on extinction retrieval using aversive Pavlovian procedures. Consistent with the effects of IL inactivation or lesions prior to initial extinction training, pharmacological inactivation specifically during extinction retrieval impaired retrieval, resulting in heightened conditioned freezing (Laurent and Westbrook, 2009; Sangha et al., 2014). These results suggest that the IL is important for retrieving the inhibitory extinction memory. However, pharmacological inactivation of the IL using tetrodotoxin (TTX), a sodium channel blocker, prior to an extinction retrieval test paradoxically facilitated extinction retrieval and reduced conditioned freezing (Sierra-Mercardo et al., 2006). Furthermore, pharmacological inactivation of the IL using GABA receptor agonists did not affect extinction retrieval (Do Monte et al., 2015). Discrepancies in the effects of IL inactivation could be due to differences in contextual and cue conditioning procedures (Laurent and Westbrook, 2009; Do Monte et al., 2015). Context conditioning and extinction involves pairing a context with an aversive outcome and extinguishing the context alone. In contrast, cue conditioning procedures involve pairing a discrete cue with an aversive outcome and presenting the cue alone during extinction. Therefore, the role of the IL in extinction may differ depending on the conditioning procedures.

Lesions and pharmacological inactivation studies provide foundational evidence for the role of the IL in extinction. However, these manipulations disrupt IL activity across an entire session, making it difficult to assess the specific processes that the IL is involved in. For instance, it is unclear whether inactivating the IL during extinction affects processing aspects of the CS or omission of the expected US. Studies using optogenetics which can manipulate neural activity at specific events during a session could provide insight in this regard. For example, during extinction, optogenetic inhibition of the IL specifically during the CS did not affect initial extinction training but impaired extinction retrieval the next day (Do Monte et al., 2015). This finding is consistent with previous studies using lesions and pharmacological inactivation but suggests that the IL may be specifically involved in CS processing during extinction training. However, inconsistent results remain with optogenetic inhibition of the IL during the CS specifically in extinction retrieval. One study found impairments in extinction retrieval following IL optogenetic inhibition (Kim et al., 2016), while another found no effect (Do Monte et al., 2015). Differences in these findings could be due to the specific neuronal subtypes disrupted by

optogenetic inhibition. Deliberate manipulation of specific neuronal types in the IL may be necessary to parse out the role of the IL in extinction retrieval.

Enhancing IL neuronal activity has reliably been shown to suppress aversive conditioned responding during extinction training and retrieval. Augmenting IL activity, whether electrically, optogenetically, or pharmacologically, during initial extinction training or retrieval consistently suppresses conditioned freezing (Milad and Quirk, 2002; Milad et al., 2004; Vidal-Gonzalez et al., 2006; Kim et al., 2010; Thompson et al., 2010; Peters et al., 2010; Do Monte et al., 2015; Lingawi et al., 2016; Lingawi et al., 2018). Optogenetic stimulation of the IL specifically during extinction retrieval suppresses conditioned freezing and is thought to rely on prior extinction training (Do Monte et al., 2015; Kim et al., 2016). Specifically, optogenetic stimulation of the IL suppresses conditioned freezing after extinction but not after aversive Pavlovian conditioning (Kim et al., 2016). This suggests that the IL may reactivate the previously established extinction memory in order to suppress conditioned freezing. However, in many of these studies, enhancing IL activity typically reduces conditioned freezing without prior extinction training and suppression is often observed from the very first trial of extinction training (Milad and Quirk, 2002; Milad et al., 2004; Do Monte et al., 2015; Lingawi et al., 2016; Lingawi et al., 2018). Thus, extinction training does not seem to be necessary for IL stimulation to suppress aversive conditioned responding. Promoting IL function through infusions of brain derived neurotrophic factors (BDNF) can even substitute for extinction and facilitate the attenuation of conditioned freezing without the need for prior extinction training (Peters et al., 2010). These effects suggest that stimulating the IL may provide a general inhibitory signal that can suppress conditioned freezing (Heidbreder and Groenewegen, 2003; Peters et al., 2009; Gourley and Taylor, 2016). Therefore, while augmenting IL activity suppresses conditioned freezing during extinction training and retrieval, the mechanism for suppression remains unclear.

Augmenting IL activity during initial extinction training alone also facilitates extinction retrieval in subsequent sessions (Milad and Quirk, 2002; Milad et al., 2004; Vidal-Gonzalez et al., 2006, Kim et al., 2010). The facilitation of extinction seems to be temporally specific to enhancing IL activity during the CS. Electrical stimulation of the IL during the CS in extinction but not outside of the CS facilitates extinction retrieval the following day (Milad et al., 2004). These effects have been recapitulated using optogenetics in which stimulation of the IL during the CS in extinction reduces conditioned freezing and enhances subsequent extinction retrieval

(Do-Monte et al., 2015). Therefore, IL activity specifically during the CS in extinction appears to be important for extinction learning.

Improved extinction retrieval following IL stimulation also appears to be gated by context. Pharmacological activation of the IL during extinction in the same context as aversive conditioning enhances extinction retrieval in that context and enables generalization of extinction to other contexts (Thompson et al., 2010). In contrast, activating the IL during extinction in a different context from aversive conditioning does not result in the same facilitatory effects (Thompson et al., 2010). Further, IL stimulation has been shown to promote extinction retrieval in a CS specific manner (Lingawi et al., 2016). Pharmacological activation of the IL during extinction of an aversive CS facilitates extinction retrieval only if that CS has previously undergone extinction (Lingawi et al., 2016; Lingawi et al., 2018). Thus, stimulating IL activity seems to strengthen previously established extinction memory to promote extinction retrieval with a specific CS in different contexts.

3.2 IL projections to the BLA in extinction of aversive conditioned responding

IL projections to the basolateral amygdala (BLA) are thought to be involved in extinction of aversive Pavlovian conditioned responding (Peters et al., 2009; Arruda-Carvalho and Clem, 2015; Giustino and Maren, 2015). Glutamatergic neurons in the IL are thought to modulate the activity of glutamatergic pyramidal neurons in the BLA (Cho et al., 2013; Strobel et al., 2015). Extinction of aversive Pavlovian conditioned responding reduced the synaptic efficacy of IL inputs to glutamatergic neurons in the BLA, which resulted in a reduction of conditioned freezing (Cho et al., 2013). Neuronal excitability of the IL-to-BLA projection also increased after extinction in aversive Pavlovian conditioning (Bloodgood et al., 2018). The synaptic changes occurring in the IL-to-BLA projection after extinction may enable the suppression of aversive conditioned responses (Cho et al., 2013; Bloodgood et al., 2018). Consistently, chemogenetic and optogenetic inhibition of the IL-to-BLA during initial extinction training impaired subsequent extinction retrieval the next day (Bloodgood et al., 2018; Bukalo et al., 2015). Interestingly, however, IL-to-BLA optogenetic inhibition did not affect extinction retrieval of conditioned freezing to an aversive CS (Bukalo et al., 2015). These results suggest that the IL-to-BLA projection is more involved during initial extinction training rather than retrieval. However, optogenetic stimulation of the IL-to-BLA projection was sufficient in suppressing conditioned

freezing and augmenting extinction training to facilitate extinction retrieval (Bukalo et al., 2015; Bukalo et al., 2021). Therefore, although the IL-to-BLA projection appears to be more involved in initial extinction learning, augmenting its activity during extinction is sufficient to facilitate extinction retrieval.

3.3 IL in extinction of appetitive conditioned responding

The IL encodes a variety of task events during appetitive conditioning and extinction. Electrophysiological recordings show that IL activity is modulated by events such as a discriminative stimulus, operant responding that leads to the delivery or omission of an appetitive or aversive outcome, and delivery of the outcomes themselves (Moorman and Aston-Jones, 2015; Gentry and Roesch, 2018). IL neurons exhibit both excitation and inhibition to appetitive cues and operant responses for appetitive outcomes (Moorman and Aston-Jones, 2015; Gentry and Roesch, 2018). Further, IL neurons exhibit excitation and inhibition during consummatory behaviours of an appetitive outcome such as food (Burgos-Robles et al., 2013; Moorman and Aston-Jones, 2015; Barker et al., 2017). Together, electrophysiological recordings indicate that the IL encodes many aspects of appetitive conditioning, which may allow it to modulate behavior depending on environmental demands.

IL activity in appetitive tasks is associated with extinction when anticipated outcomes are omitted. Greater IL activation, measured by Fos expression, is observed following extinction training (Warren et al., 2016) and exposure to an extinction context (Marchant et al., 2010). Neurons in the IL also display dynamic modulation of activity following the omission of an expected outcome (Moorman and Aston-Jones, 2015; Barker et al., 2017; Gentry and Roesch, 2018). Specifically, IL neurons that are excited by a stimulus that predicts an appetitive outcome during operant conditioning become inhibited, and neurons that are inhibited by the stimulus become excited during extinction (Moorman and Aston-Jones, 2015). Changing contingencies from conditioning to extinction also recruits a new subpopulation of neurons in the IL that are excited by the appetitive cue only after extinction training (Gentry and Roesch, 2018). These results are consistent with the emergence of neuronal ensembles in the IL that are recruited by extinction training (Warren et al., 2016; 2019). These findings suggest that the IL fluidly encodes the expected outcome signaled by a given stimulus or operant response and may recruit new neurons to inhibit responding for appetitive outcomes during extinction.

3.3.1 IL in extinction of operant responses

The role of the IL in extinction has been heavily investigated in the framework of substance use disorders using operant drug self-administration procedures. In these procedures, rodents are typically trained to self-administer a drug by pressing on an active lever and withhold responding on an inactive lever that has no programmed outcome. During extinction, the drug reinforcer is omitted which leads to a reduction in drug-seeking as measured by active lever presses. The relapse aspect of substance use disorders is modeled using reinstatement procedures (Crombag et al., 2008). Relapse is modeled by triggering a return of drug-seeking after extinction either through the passage of time (spontaneous recovery), re-exposure to the drug or drug-associated cues (drug and cue-induced reinstatement), exposure to a stressor (stress-induced reinstatement) or changing the context from the extinction context (renewal or context-induced reinstatement) (Shaham et al., 2003, Crombag et al., 2008). Studies on the IL are usually aimed at understanding its role in attenuating drug-seeking by manipulating activity during extinction or during the return of responding tests.

The IL is thought to be a critical region involved in suppressing drug-seeking during extinction (Peters et al., 2009). This hypothesis is largely supported by seminal work with cocaine in which pharmacological inactivation of the IL disinhibits and produces a return of extinguished operant cocaine-seeking (Peters et al., 2008a). Further, pharmacological inactivation of the IL immediately after an extinction session, impairs subsequent extinction retrieval and increases cocaine-seeking (LaLumiere et al., 2010). During extinction training, optogenetic inhibition of the IL precisely after the non-reinforced response impaired extinction training and retrieval (Gutman et al., 2017), lending evidence to the idea that IL activity specifically during omission of an expected outcome is important for extinction of drug-seeking. Extinction may be dependent on glutamatergic signaling in the IL as specific blockade of glutamatergic receptors disrupted extinction consolidation of cocaine-seeking (Otis et al., 2014). Together, these results highlight the importance of the IL in mediating inhibitory extinction memories to suppress conditioned responding especially for cocaine. However, research with other drug-reinforcers have also found inconsistent results. Pharmacological inactivation of the IL does not disinhibit heroin-seeking (Bossert et al., 2011) or alcohol-seeking (Willcocks and McNally, 2013) in the extinction context. Further, pharmacological inactivation of the IL during

extinction can even lead to suppression of responding to an alcohol CS (Khoo et al., 2019). These results suggests that the role of the IL in extinction of drug-seeking may not be universal across different drug-reinforcers.

There is also mixed evidence across drug-reinforcers on the role of the IL in suppressing the return of drug-seeking after extinction. Pharmacological inactivation of the IL amplifies spontaneous recovery of cocaine-seeking after extinction, suggesting that the IL is involved in suppressing the return of responding that is triggered by a change in temporal context (Peters et al., 2008b). However, pharmacological inactivation of the IL does not seem to affect cue-induced reinstatement (McLaughlin and See, 2003), cocaine-primed reinstatement (McFarland and Kalivas, 2001), stress-induced reinstatement (Capriles et al., 2003), or renewal (Fuchs et al., 2005) of cocaine-seeking. With other drug reinforcers, IL inactivation does not affect methamphetamine-primed reinstatement, but can inhibit cue-induced reinstatement of methamphetamine-seeking (Rocha and Kalivas, 2010). Similar attenuation of cue-induced reinstatement (Rogers et al., 2008), heroin-primed reinstatement (Rogers et al., 2008), and renewal (Bossert et al., 2011; Bossert et al., 2012) of heroin-seeking have also been observed following IL inactivation. In alcohol self-administration tasks, however, IL inactivation does not affect cue-induced reinstatement (Pfarr et al., 2015) or renewal of alcohol-seeking (Wilcocks and McNally, 2013). The null effect in reinstatement of alcohol-seeking and reduction in reinstatement of heroin-seeking following IL inactivation are in stark contrast with the proposed role of the IL in suppressing drug-seeking during and after extinction (Peters et al., 2008a; 2008b; 2009). Nevertheless, the results of these studies suggest that the IL is involved in the return of responding after extinction although it may not always be in suppressing drug-seeking.

The IL has also been investigated in extinction and reinstatement using natural reinforcers such as food or sucrose. Interestingly, pharmacological inactivation of the IL does not affect extinction training or retrieval in an operant food-seeking task (Mendoza et al., 2015). Furthermore, IL inactivation following two extinction sessions does not have any disinhibitory effects on food-seeking (Warren et al., 2016). Optogenetic inhibition of the IL during extinction specifically after the non-reinforced lever press also had no effect on extinction training, extinction retrieval and subsequent cue-induced and food-primed reinstatement (Gutman et al., 2017). Therefore, the role of the IL in extinction of cocaine-seeking does not seem to generalize to operant responding for food. However, IL inactivation reduces cue-induced reinstatement

(Caballero et al., 2019) and renewal (Eddy et al., 2016) of operant sucrose-seeking. These results suggest that the IL may not be involved in suppressing the return of responding for more natural reinforcers. Similar to heroin-seeking, the IL may instead be important for driving the return of operant responding for natural reinforcers after extinction. Interestingly, pharmacological inactivation of the IL at test in the extinction context also led to an increase and disinhibition of sucrose-seeking (Eddy et al., 2016). These findings are consistent with changes in IL neural activity in response to a sucrose cue when transitioning from conditioning to extinction during a discriminative operant task (Moorman and Aston-Jones et al., 2015). Together, these data suggests that the IL may not only be involved in suppressing responding but perhaps in tracking current contingencies and coordinating both the generation and suppression of conditioned responding depending on the context.

3.3.2 IL in extinction of responding to appetitive Pavlovian cues

Fewer studies have investigated the role of the IL in extinction of appetitive Pavlovian conditioned responding. Interestingly, pharmacological inactivation of the IL during initial extinction training suppresses appetitive Pavlovian conditioned responding and does not facilitate extinction retrieval (Mendoza et al., 2015; Lay et al., 2019). These findings are inconsistent with results in aversive Pavlovian conditioning in which IL inactivation during initial extinction sessions impairs retrieval (Sierra-Mercado et al., 2006, Laurent and Westbrook 2008; Laurent and Westbrook, 2009; Sierra-Mercado et al., 2011; Do Monte et al., 2015). However, repeated inactivation of the IL across multiple extinction sessions can impair subsequent extinction retrieval in appetitive Pavlovian conditioning (Lay et al., 2019). Procedural differences in extinction of appetitive and aversive Pavlovian conditioned responding may alter the involvement of the IL. However, this finding provides some consilience that the IL is important for extinction of both appetitive and aversive Pavlovian conditioned responding.

The IL is implicated in suppressing the return of appetitive Pavlovian conditioned responding after extinction. Lesions of the IL increases food-primed reinstatement, spontaneous recovery, and renewal of conditioned responding to an appetitive Pavlovian CS (Rhodes and Killcross, 2004; 2007a). These results suggest that the IL may normally be promoting the expression of inhibitory extinction memories. However, the IL does not seem to be necessary for general encoding of inhibitory associations but rather in promoting inhibition when competing

excitatory and inhibitory associations are present within a single CS. Animals with lesions in the IL maintain the ability to suppress responding when a separate stimulus that is explicitly trained to predict the absence of reinforcement (i.e. conditioned inhibitor) is presented in conjunction with a separate CS that predicts reinforcement (Rhodes and Killcross, 2007b). In contrast, IL lesions disrupt the capability of animals to suppress responding when the same CS concurrently predicts both the presence and absence of reinforcement (i.e. extinction), leading to disinhibition of conditioned responding (Rhodes and Killcross, 2004; 2007a; 2007b). Consistently, IL inactivation hinders the ability of rats to discriminate whether a single cue signals reinforcement or non-reinforcement based on the context (Riaz et al., 2019). Therefore, the IL may play a role in using contextual information to suppress Pavlovian conditioned responding when a CS contains conflicting excitatory and inhibitory associations with a given US.

3.3.3 Functional heterogeneity of the IL

Inconsistencies in the role of the IL in extinction and return of responding may be explained by emerging evidence that different subpopulations of neurons in the IL are involved in generating and suppressing conditioned responding. This idea is supported by findings using Duan02 inactivation wherein sets of neurons or neuronal ensembles that are activated by specific stimuli or behaviours are selectively deleted (Cruz et al., 2013). Bidirectional behavioural effects have been observed following deletion of neuronal ensembles within the IL that are activated by operant self-administration or extinction. For instance, deletion of neurons activated by food self-administration decreased food-seeking, whereas deletion of neurons activated during extinction increased food-seeking (Warren et al., 2016). Similarly, ablating cocaine self-administration ensembles in the IL reduced cocaine-seeking, whereas ablating ensembles activated during extinction increased cocaine-seeking (Warren et al., 2019). Bidirectional effects have also been observed following deletion of neurons that are activated by discriminative stimuli that predict either reinforcement or non-reinforcement (Suto et al., 2016; Laque et al., 2019). However, although deletion of neuronal ensembles in the IL that are activated by a heroin-associated context reduced renewal of heroin-seeking, deletion of neurons activated by an extinction context did not affect heroin-seeking (Bossert et al., 2011). Therefore, the role of the IL in extinction and suppression of heroin-seeking remains unclear. Furthermore, in alcohol self-administration procedures, deletion of IL ensembles that are active during cue-induced

reinstatement increases further alcohol-seeking (Pfarr et al., 2015). Thus, IL neurons activated during reinstatement appears to still be primarily involved in suppressing alcohol-seeking. These emerging studies provide evidence that the IL is involved in mediating both generation and suppression of drug-seeking. However, bidirectional effects have not been demonstrated across different drug reinforcers, and the role of the IL in extinction remains unclear.

The IL also play an inhibitory role in conditioned behaviours beyond extinction. For instance, the IL is involved in encoding and retrieving inhibitory memories acquired through different procedures such as unpaired training or latent inhibition (Lingawi et al., 2016). Unpaired training involves separately presenting the CS and the US by a significant time interval within the same session (Rescorla, 1967). In latent inhibition, pre-exposure to the CS in the absence of the US is done prior to Pavlovian conditioning which delays subsequent acquisition of responding to the CS (Lubow and Moore, 1959). Inhibition learned from both procedures can be strengthened by pharmacological activation of the IL to facilitate extinction training and extinction retrieval of aversive conditioned responding (Lingawi et al., 2018). Interestingly, the facilitation of extinction by strengthening prior inhibitory memories is CS specific. Enhancing IL activity did not facilitate extinction to a CS when prior extinction or latent inhibition was conducted with a different CS (Lingawi et al., 2016; 2018). These results suggest that the IL may be a common node in which inhibitory learning is processed. This inhibitory role of the IL may also be important for suppressing irrelevant responses to effectively coordinate operant responding for appetitive outcomes. For instance, pharmacological inactivation increases operant responding to a discriminative stimulus that explicitly signals non-reinforcement (Ghazizadeh et al., 2012). Further, pharmacological inactivation of the IL disinhibits baseline operant responding for appetitive outcomes (Ishikawa et al., 2008; Keistler et al., 2015). Similarly, lesions of the IL lead to response disinhibition and increases in premature responding (Chudasama et al., 2003). Together, these results highlight the integral role of the IL in mediating different forms of inhibitory learning for a given CS, which may be useful for coordinating responses to specific cues and time points.

3.3.4 Augmenting IL activity to promoting extinction

Studies have attempted to leverage augmenting IL activity to promote extinction and suppress appetitive conditioned responding. Consistently across different drug reinforcers,

enhancing glutamatergic activity in the IL reduces the return of cocaine-, heroin-, and alcohol-seeking after extinction (Peters et al., 2008a; LaLumiere et al., 2012; Gass et al., 2014; Chen et al., 2016; Augur et al., 2016). Therefore, there appears to be consilience across different reinforcers that augmenting IL activity suppresses appetitive conditioned responses for drugs.

Enhancing IL activity is thought to suppress the return of responding by promoting the expression of inhibitory associations established during extinction. In support of this idea, augmenting IL activity reduces cocaine-seeking only after extinction training, but not after a period of abstinence (Augur et al., 2016; Müller Ewald, 2018). Furthermore, optogenetic stimulation of the IL during extinction of cocaine conditioned place preference facilitates extinction retrieval after previous extinction training (Van den Oever, 2013). Inconsistently, however, optogenetic stimulation of the IL has also been found to suppress operant food- and cocaine-seeking without prior extinction training (Do Monte et al., 2015; Cameron et al., 2019). In sum, there is compelling evidence across different procedures and reinforcers that enhancing IL activity can suppress appetitive conditioned responding. However, whether this suppression occurs due to the promotion of an inhibitory extinction memory remains unresolved.

3.4 Role of IL projections to the NAcS in extinction of drug-seeking

The NAcS has a rich history of being involved in many aspects of appetitive learning that is often attributed to dopaminergic input from the ventral tegmental area (VTA) (Schultz et al., 1997; Schultz, 1998; Berridge and Robinson, 1998; Day et al., 2007; Bromberg-Martin et al., 2010). The NAcS is primarily made up of GABAergic medium spiny neurons (MSNs) which can be divided based on their expression of D1-like (D1-MSNs) and D2-like (D2-MSNs) dopamine receptors (Gerfen et al., 1990). These D1-MSNs and D2-MSNs are thought to play antagonistic roles, with D1-MSNs promoting and D2-MSNs suppressing appetitive-related behaviours (Lobo et al., 2010; Kravitz et al., 2010; Yttri and Dudman, 2016). The NAcS receives glutamatergic inputs from various sources including the medial prefrontal cortex, the amygdala, hippocampus, and the paraventricular nucleus of the thalamus (Phillipson and Griffiths, 1985; Groenewegen et al., 1999; Britt et al., 2012). This high degree of converging inputs allows the NAcS to control many aspects of behaviour.

One of the many functions of the NAcS is in general behavioural inhibition. This idea is supported by findings in which NAcS inactivation induces behavioural disinhibition and

increases responding to an inactive lever or cue with no programmed consequences (Chaudhri et al., 2008; Peters et al., 2008a, Ambroggi et al., 2011). These disinhibitory effects of NAcS inactivation may be linked to the time-locked inhibition of the NAcS during consummatory behaviours and responding for appetitive stimuli (Nicola et al., 2004; Taha and Fields, 2006; Krause et al., 2010; Ghazizadeh et al., 2012; Reed et al., 2018). Consistently, optogenetic inhibition of glutamatergic inputs from the amygdala, thalamus, or hippocampus to the NAcS increased consummatory behaviour (Reed et al., 2018) whereas electrical stimulation of the NAcS disrupted sucrose consumption (Krause et al., 2010). Together, these studies suggest that inhibition in the NAcS is important for gating conditioned responding and increasing NAcS activity can disrupt this process.

The role of the NAcS in behavioural inhibition has been applied to extinction and suppression of responding for appetitive stimuli. Similar to the IL, pharmacological inactivation of the NAcS disinhibits extinguished alcohol-seeking (Millan et al., 2010) and cocaine-seeking (Peters et al., 2008a). These studies suggest that the NAcS is important for expression of extinction. Further, electrical stimulation of the NAcS facilitated extinction and attenuated reinstatement of cocaine-seeking (Vassoler et al., 2013). Glutamatergic signaling may be particularly important for the role of the NAcS in extinction as blocking AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptors in the NAcS produces disinhibition of extinguished alcohol-seeking in the extinction context (Millan and McNally, 2011). Together, these studies suggest that increasing glutamatergic activity in the NAcS is conducive for extinction and suppression of conditioned responding.

Glutamatergic projections from the IL to the NAcS are thought to work in concert during extinction of appetitive conditioned responding. IL neurons that project to the NAcS exhibit a decrease in activity during the onset of appetitive cues and operant responses which may be linked to gating conditioned responding (Ghazizadeh et al., 2012; Cameron et al., 2019). Electrical and optogenetic stimulation of the IL increases extracellular levels of glutamate and activates neurons in the NAcS (Quiroz et al., 2016). Interestingly, electrical and optogenetic stimulation of the IL also increases extracellular dopamine in the NAcS, suggesting that the IL may modulate dopaminergic transmission from the VTA to the NAcS (Quiroz et al., 2016). Moreover, pharmacological inactivation of the IL disrupts tonic activity of NAcS neurons involved in suppressing non-reinforced responding to inhibitory stimuli (Ghazizadeh et al.,

2012). Concurrent pharmacological inactivation of the IL and NAcS leads to disinhibition of extinguished cocaine-seeking (Peters et al., 2008a). Further, enhancing glutamatergic activity in the IL reduces cue-induced cocaine-seeking that is reversed by glutamatergic antagonists in the NAcS (LaLumiere et al., 2012). Pharmacological disconnection of the IL and NAcS also results in increased baseline responding which interferes with the capacity for an appetitive Pavlovian CS to invigorate operant conditioned responding (Keistler et al., 2015). Therefore, the IL appears to suppress appetitive conditioned responding by increasing glutamate transmission and modulating dopaminergic inputs in the NAcS.

The IL-to-NAcS projection is thought to suppress responding by promoting the expression of an inhibitory extinction memory (Peters et al., 2009). In support of this idea, IL-to-NAcS chemogenetic activation reduced cue-induced reinstatement of cocaine-seeking following extinction but had no effect after an abstinence period without extinction (Augur et al., 2016). Further, neuronal ensembles in the IL that are activated during extinction of cocaine-seeking predominantly project to the NAcS and pharmacological disconnection of these IL extinction ensembles projecting to the NAcS led to an increase in cocaine-seeking (Warren et al., 2019). However, several studies have also shown that cocaine self-administration alone and the incubation of cocaine craving during abstinence can alter glutamatergic transmission in the IL-to-NAcS projection (Ma et al., 2014; Pascoli et al., 2014; Cameron et al., 2019). Moreover, IL-to-NAcS optogenetic stimulation is sufficient in attenuating operant cocaine-seeking following abstinence without prior extinction training (Cameron et al., 2019). Therefore, it remains unclear whether augmenting IL-to-NAcS activity suppresses conditioned responding by facilitating the retrieval of an inhibitory extinction memory.

4. Overview

The infralimbic cortex (IL) is central to mediating extinction and suppression of conditioned responding. Respectively, IL projections to the basolateral amygdala (BLA) and the nucleus accumbens shell (NAcS) are thought to be paramount for extinction of conditioned responding to aversive and appetitive stimuli. However, studies on the IL, IL-to-BLA, and IL-to-NAcS projections have mostly used aversive Pavlovian conditioning procedures or operant drug-seeking procedures. As a result, little is known about the role of the IL and its projections to the BLA and NAcS in extinction of conditioned responding to appetitive Pavlovian cues.

Furthermore, the mechanism by which stimulation of the IL and its projections suppresses conditioned responding remains unclear. The present thesis investigates the role of the IL and its projections to the NAcS and BLA in suppressing appetitive Pavlovian conditioned responding using in vivo optogenetic stimulation in rats.

Chapter 2 discusses the common procedures used across experiments. Briefly, rats received appetitive Pavlovian conditioning in which an auditory conditioned stimulus (CS) was paired with the delivery of sucrose, the unconditioned stimulus (US), in a fluid port. Across conditioning, rats learned to enter the fluid port and consume the sucrose during presentations of the CS. During extinction, the CS was presented in the absence of sucrose to extinguish the conditioned CS port entry response. In the renewal procedure, rats received Pavlovian conditioning in a distinct context (Context A) and extinction in a different context (Context B). During the renewal test, the CS was presented in the original conditioning context (Context A) which triggers a return of appetitive Pavlovian conditioned responding.

Chapter 3 investigated whether augmenting IL activity could suppress the renewal of appetitive Pavlovian conditioned responding. During the renewal test, optogenetic stimulation of the IL was delivered either during the CS or in the middle of the inter-trial intervals (ITI) to examine the temporal specificity of augmenting IL activity in suppressing the return of responding. We predicted that optogenetic stimulation of the IL during the CS but not the ITI would suppress the renewal of appetitive Pavlovian conditioned responding after extinction.

Chapter 4 investigated whether specific IL projections to the NAcS or the BLA is involved in suppressing the renewal of appetitive Pavlovian conditioned responding after extinction. In two experiments, during the renewal test, optogenetic stimulation of the IL-to-NAcS or IL-to-BLA projection was delivered during the CS, to test the idea that the IL-to-NAcS specifically controls the extinction of appetitive conditioned responding. We predicted that optogenetic stimulation of the IL-to-NAcS but not the IL-to-BLA would suppress the renewal of conditioned responding to an appetitive CS.

In Chapter 5, we explored different behavioural processes that could contribute to the suppression in renewal of appetitive Pavlovian conditioned responding following IL-to-NAcS stimulation. First, we tested whether optogenetic stimulation of the IL-to-NAcS suppresses conditioned responding by facilitating the retrieval and expression of an inhibitory extinction memory. Second, we tested whether IL-to-NAcS stimulation indiscriminately suppresses

behaviour by delivering IL-to-NAcS stimulation during appetitive Pavlovian conditioning. We predicted that optogenetic stimulation of the IL-to-NAcS projection suppresses conditioned responding by promoting the expression of an inhibitory extinction memory and therefore would not affect acquisition of appetitive Pavlovian conditioned responding.

The present thesis investigates the effect of optogenetic stimulation of the IL and IL projections to the NAcS and BLA in extinction and renewal of appetitive Pavlovian conditioned responding. Our research builds on previous work in aversive Pavlovian conditioning and operant drug self-administration procedures and extends the role of the IL and its neural projections in extinction of conditioning responding to appetitive Pavlovian cues. Additionally, we provide insight into the neural and behavioural processes that may be involved in suppressing responding to appetitive cues. This work furthers our understanding of the psychological and neurobiological mechanisms that govern adaptive behaviour.

Chapter 2 - General Methods

Chapter 2 outlines common methods used in subsequent experimental chapters (Chapter 3, 4, 5).

Subjects

Male, Long-Evans rats (220-240 g on arrival, Charles River, Quebec, Canada) were pair housed on arrival and were single housed three days later. Rats were housed in polycarbonate home-cages (44.5 cm x 25.8 cm x 21.7 cm) containing Sani-Chips bedding (7090A, Envigo), a nylon bone toy (K3580, Bio-Serv), and a tunnel (K3245, Bio-Serv). Rats had unrestricted access to food (5075, Agribands) and water in their home-cage throughout the experiment. Home-cages were in a colony room with controlled temperature (21°C) and humidity (44%) on a 12 h light/dark cycle (lights on at 7:00 am). All procedures were conducted during the light phase. All procedures were approved by the Animal Research Ethics Committee of Concordia University and in accordance with the guidelines from the Canadian Council on Animal Care.

Apparatus

Behavioural procedures were conducted in six conditioning chambers (ENV-009A, Med Associates) housed in sound-attenuating, melamine cubicles. Chambers contained bar floors, a house light (75 W, 100 mA; ENV-215M, Med Associates) in the center of the left wall, and a white noise generator and speaker (5 dB above background noise; ENV-225SM, Med Associates) in the top left corner of the left wall. A fluid port (ENV-200R3AM, Med Associates) was located near the floor in the center-right of the right wall. A customized fluid port (opening height adjusted to 13.2 cm) was used in experiments in Chapters 3 and 4 to ease port access. Infrared sensors (ENV-254CB, Med Associates) flanking each side of the fluid port opening detected port entries. Polyethylene tubing (141691A, Fisher Scientific) connected the fluid port to a 20 mL syringe, which was mounted on a pump (PHM-100, Med Associates) located outside the melamine cubicle. An upward-facing house light (ENV-215M, Med Associates) near the top center-left of the left wall provided illumination. Med Associates software (Med-PC IV, Med Associates) running on a PC computer controlled all peripheral devices and recorded data.

Optical stimulation was delivered by a 150 mW, 473 nm laser (BL473T3-150, Shanghai Laser & Optics Century Co.) through a 125 μm optical fiber (FC-FCFC-MS6-2M, Fiber Optic

Cable Shop) to a unilateral optical rotary joint (FRJ-FC-FC, Doric Lenses) mounted on a modified, weighted arm (PHM-110-SAI, Med Associates) above each conditioning chamber. Each rotary joint was connected to a custom-made patch cord (Trujillo-Pisanty et al., 2015) which consisted of a 200 μm optical fiber (FT200EMT, Thorlabs) covered by heat-shrink tubing (84N583, Newark Element14), and protected by a stainless-steel compression spring in Chapter 3 (custom order, Heliplex) and stainless-steel tubing in Chapters 4 and 5 (FT05SS, Thorlabs). Each end of the optical fiber was stripped, one end was secured to a FC alloy connector (240 μm; 30126G2-240, Fiber Instrument Sales) and a 240 μm stainless alloy ferrule (F10061F240, Fiber Instrument Sales) on another end using heat curable epoxy (PFP-353ND-16OZ-A, PFP-353NC-16OZ-B, Precision Fiber Products Inc.). The protruding fibers at the ends of the patch cord were cleaved using a diamond wedge scribe (F090W, Fiber Instrument Sales) and the ends were polished using silicon carbide and diamond sheets in decreasing coarseness (LFG5P, LF6D, LF3D, LF1D, Thorlabs). During behavioural sessions, the FC connector end of the patch cord was attached to the optical rotary joint and the ferrule end was connected to the optical fiber implant on the rat using a ceramic split sleeve (F18300SSC25, Fiber Instrument Sales) covered by heat shrink tubing to minimize light leakage.

The optical fiber implant was made in house by stripping and cleaving a 300 μm optical fiber (BFH37-300, Thorlabs) and securing it inside an alloy ferrule (340 μm bore; F10061F340, Fiber Instrument Sales) using heat-cured epoxy. One end of the fiber protruded approximately 7.5 mm in Chapter 3 and 10 mm in Chapters 4 and 5 from the open end of the ferrule. The other end was cleaved to be flush with the ferrule opening and polished. Each optical fiber implant was tested to ensure high cut edge quality in the protruding end and sufficient power output. Before each test, laser output was calibrated so that light emission at the tip of the optical fiber implant was 30 ± 2 mW. Optical stimulation was delivered at a frequency of 20 Hz, with a 5 ms pulse width, in a 10.2 s pulse train programmed through a microcontroller (A000066, Arduino). These optogenetic parameters were based on preliminary data and published studies (Adamantidis et al., 2011; Stuber et al., 2011; Britt et al., 2012).

Solutions and Viruses

A 10% (w/v) sucrose solution was prepared by mixing sucrose in tap water and served as the unconditioned stimulus. Odours were prepared by diluting lemon oil (Lemon Odour;

W262528-1 KG-K, Sigma-Aldrich) or benzaldehyde (Almond Odour; B1000, ACP Chemicals) with water to make a 10% solution. In Chapter 3, viruses containing the transgene for channelrhodopsin 2 (ChR2) with an enhanced yellow fluorescent protein (eYFP) reporter (AAV2-CaMKIIa-hChR2(H134R)-EYFP, 5.1 x 10^{12} vg/ml) or eYFP alone (AAV2-CaMKIIa-EYFP, 2 x 10^{12} vg/ml) were provided by Dr. Karl Deisseroth and obtained from the University of North Carolina Vector Core. In Chapters 4 and 5, ChR2-eYFP (AAV5-CaMKIIa-hChR2(H134R)-EYFP, 1.5 x 10^{13} vg/mL, Addgene) or eYFP alone (AAV2-CaMKIIa-EYFP, 2.0 × 10^{12} Vg/mL, Addgene; AAV5-CaMKIIa-EYFP, 9.0 × 10^{12} Vg/mL, Neurophotonics) were used for optogenetic experiments.

Surgery

Rats received stereotaxic surgery starting one week after single housing. Rats were anesthetized with isoflurane (108737, CDMV) and heads were shaved to expose the scalp. Rats were then secured to the stereotaxic frame through ear bars and an incision was made along the midline of the scalp to expose the skull. Holes were placed in the skull using a stereotaxic drill (K.1070, Foredom) to allow access to target regions. A viral vector containing the transgene for either ChR2-eYFP or eYFP alone was microinfused unilaterally into the IL (right hemisphere) using a blunted 27¼ gauge needle (1482113B, Fisher Scientific). The needle was connected via polyethylene tubing (PE20, CA-63018-645, VWR) to a 10 μl Hamilton syringe (1701N, Hamilton) that was placed on a micro-infusion pump (Pump 11 Elite, 704501, Harvard Apparatus). Micro-infusion consisted of 0.5 µl of viral vector in Chapter 3 and 1 µl in Chapters 4 and 5 infused at 0.1 µl/min with 15 min diffusion per 0.5 µl. Target coordinates for the IL +2.9 mm anterior, + 0.6 mm lateral relative to bregma, and -5.1 mm ventral relative to the skull surface in Chapter 3 and +2.9 mm anterior and +3.4 mm lateral relative to bregma and -5.8 mm ventral relative to the skull surface at a 30° angle in Chapter 4 and 5. An angled approach was used in Chapter 4 and 5 to minimize spread to neighbouring brain regions and specifically stimulate neural inputs from the IL.

An optical fiber implant was lowered into the IL (+2.9 mm anterior, + 0.6 mm lateral relative to bregma, and -5.1 mm ventral relative to the skull surface) in Chapter 3 and in the NAcS (+1.2 mm anterior and +1.0 mm lateral relative to bregma and -7.5 mm ventral relative to the skull surface) or BLA (-2.5 mm posterior, +5.0 mm lateral relative to bregma and -8.5 mm

ventral relative to the skull surface) in Chapters 4 and 5 in the same hemisphere that received the virus micro-infusion. Optical fiber implants were secured in place using 4-5 jeweler's screws, Metabond (5533484, Patterson Dental), and dental acrylic (powder 525000, solvent 526000, A-M Systems). Buprenorphine (0.03 mg/kg, subcutaneous, Buprenex) was administered as an analgesic after surgery. To facilitate recovery, rats were provided with sweetened mashed food and a banana-flavored oral re-hydrator (F2351-B, PRANG, Bio-Serv) for 48 h post-surgery. Behavioural procedures began approximately 2 weeks after surgery, and tests with optical stimulation were conducted after a minimum of 4 weeks after surgery in Chapter 3 and 8 weeks after surgery in Chapter 4 and 5 to allow for transgene expression in IL cell bodies (Chapter 3) or IL terminals in the NAcS and the BLA (Chapter 4 and 5).

Behavioural Procedures

Habituation and Contexts

Rats were handled and weighed prior to each procedure. Rats were acclimated to 10% sucrose in two 24 h sessions in which a bottle containing 60-70 mL of sucrose was provided in the home-cage prior to behavioural training. In all experiments, rats subsequently received three habituation sessions conducted 24 h apart from one another. Rats were first habituated to transport, in which, their home-cages were loaded on a cart and transported to the behavioural room and remained there for 20 minutes. In the second habituation, rats were brought to the behavioural room, weighed, then returned to their home-cages and remained in the behavioural room for 20 minutes. The third habituation was a 20 min session in the conditioning chamber. During this habituation session, rats were tethered to non-functional patch cords, placed in the conditioning chamber with the house light illuminated and port entries were recorded. Conditioning chambers during this habituation session were in a default configuration consisting of bar floors, clear walls, and no administered odours (Figure 1A).

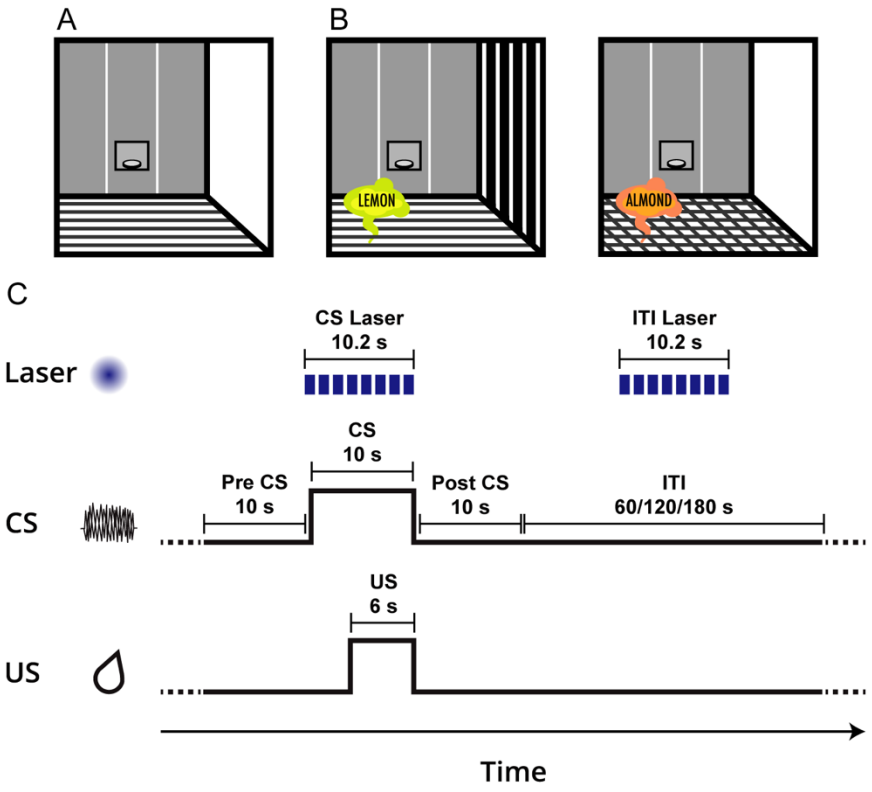
Rats in renewal experiments were matched based on average sucrose consumption and port entries made in the third habituation session and subsequently assigned to one of two context configurations to be used for conditioning and extinction (Figure 1B). Context configurations differed on visual, tactile, and olfactory modalities. Context type 1 consisted of bar floors, black and white striped walls, and a lemon odour. Context type 2 consisted of metal grid floors, clear chamber walls, and an almond odour. Context type configurations were counterbalanced to be

Context A for conditioning and Context B for extinction. Odours were applied by spraying petri dishes on waste pans located underneath the floor of the conditioning chambers. Rats in renewal experiments received two additional habituation sessions to the conditioning context, Context A and the extinction context, Context B. The order of habituation was counterbalanced across rats, such that half of the rats first received habituation in Context A and the other half in Context B. Habituation in Context A (Pavlovian conditioning context) included port training in which 0.2 mL of 10% sucrose was delivered in the port five times at 200 s intervals. This habituation was done to ensure that rats could make port entries and consume the sucrose while tethered to a patch cord. Habituation in Context B (extinction context) did not involve port training.

Pavlovian Conditioning and Extinction

Daily Pavlovian conditioning sessions (40 min) began 24 h after the last habituation session. The Med-PC program was initiated and 2 min later the house light was illuminated to indicate session onset. Each session consisted of 14 presentations of a 10 s continuous, white noise conditioned stimulus (CS) that occurred on a variable-time 120 s schedule (intertrial intervals of 60, 120 or 180 s). Pumps were activated 4 s after CS onset for a duration of 6 s to deliver 0.2 mL of sucrose into the fluid port (2.8 mL per session) for oral consumption. Ports were checked after each session to ensure sucrose consumption. During extinction sessions, CS presentations occurred just as in Pavlovian conditioning, but without sucrose delivery.

In renewal experiments, Pavlovian conditioning was conducted in Context A and extinction in Context B. Rats received extinction training until they achieved a criterion of 5 or fewer CS port entries. Rats were tested for renewal in Context A the day after the last extinction session. The renewal test was identical to Pavlovian conditioning except that no sucrose was available and optogenetic stimulation was delivered.



Chapter 2, Figure 1. Method schematic for recurring behavioural procedures. **(A)** Default conditioning chamber consisting of a fluid port, clear walls, and bar floors. **(B)** Two conditioning chamber context configurations for renewal experiments. Context type 1 (left) includes striped walls, bar floors, and a lemon odour. Context type 2 (right) includes clear walls, grid floors, and an almond odour. **(C)** Event timing diagram of Pavlovian conditioning sessions. During extinction the unconditional stimulus (US) was omitted. During tests with optogenetic stimulation, the laser was activated either during the conditioned stimulus (CS) or in the middle of the inter trial intervals (ITI).

Histology

Rats were euthanized with an overdose of sodium pentobarbital (240 mg/g, 1 mL/kg, i.p., Euthanyl) and transcardially perfused with 0.9% phosphate buffered saline (PBS) followed by 4% paraformaldehyde (4% PFA) in 0.1 M phosphate buffer. Brains were extracted and post-fixed in 4% PFA solution for 24 h followed by a 30% sucrose solution for an additional 48 h in 4 degrees Celsius. Brains were subsequently frozen at -80 degrees Celsius and coronal brain sections were collected using a cryostat (60 μm in Chapter 3 and 40 μm in a one-in-five series in Chapters 4 and 5). Brain sections were mounted onto microscope slides and processed for either Nissl staining or fluorescence microscopy with DAPI (H-1200, Vector labs) to verify optical fiber placement and transgene expression. The location of microinjectors and optical fiber implants was assessed using light microscopy (Leitz Laborlux S, Leica) in Nissl-stained sections. Transgene expression in the IL was verified using fluorescence microscopy (Nikon, Eclipse TiE, Leica). Images of transgene expression in the IL were captured using an epifluorescence microscope (Nikon, Eclipse TiE) with a 4x lens for cell bodies and 20x lens for neuron terminals. Additional images of neuron terminals were captured using a confocal microscope (Olympus Fluoview FV10i) with a 60x lens. A rat brain atlas (Paxinos and Watson, 2007) was used to approximate the location of sections relative to bregma and the images were used to model the spread of transgene expression in the IL and the placement of optical fibers at target regions (Illustrator, Adobe).

Data analysis

The primary measure of conditioned responding in behavioural experiments was a delta (Δ) CS port entry score that considered baseline levels of responding for each rat. This measure was calculated by subtracting the number of port entries that occurred during a 10 s interval immediately before each CS (Pre CS) from port entries that occurred during each of the 10 s CS (CS) (Rhodes and Killcross, 2004; 2007a; Chaudhri et al., 2010; Mendoza et al., 2015). Other behavioural data of interest included port entries made 10 s after CS offset (Post CS) and during the intertrial intervals (between Post CS offset and Pre CS onset). Intervals and variables of interest are depicted in Figure 1C. Δ CS port entries per trial was also measured and analyzed. Probability, duration, and latency of CS port entries were also collected and analyzed both as an average in the session and per CS trial to supplement Δ CS port entries. Probability was

calculated as the number of CS presentations with a port entry divided by the total number of trials (14). Duration was measured as time in the port after initiating a port entry during the CS. Latency was measured as time to initiate the first CS port entry, if a port entry was not made, a maximum latency of the duration of the CS was used. All data were organized in Microsoft Excel, visualized in Prism (Graphpad), and analyzed in SPSS (Version 23, IBM). All statistically significant interactions were further examined with Bonferroni corrected comparisons. For repeated measures ANOVA, Mauchly's test of sphericity was conducted and the Huynh-Feldt correction was applied following violations of sphericity. The alpha level for statistical significance was set to 0.05.

Chapter 3 - Optogenetic stimulation of the IL attenuated renewal of appetitive Pavlovian conditioned responding

Introduction

Extinction allows animals to suppress responding when expected outcomes are omitted. The infralimbic (IL) subregion of the medial prefrontal cortex is thought to be important for extinction. Evidence for this idea stems from aversive conditioning procedures in which pharmacological inactivation or lesioning the IL disrupted extinction training and retrieval the following day (Quirk et al., 2000; 2006, Do Monte et al., 2015). Conversely, enhancing IL activity suppressed aversive conditioned responding and facilitated extinction retrieval (Milad and Quirk 2002; Milad et al., 2004; Do-Monte et al. 2015; Kim et al. 2016). Importantly, electrical stimulation of the IL during extinction, specifically during presentations of the aversive conditioned stimulus (CS) but not outside of the CS, suppressed aversive conditioned responding and facilitated extinction retrieval (Milad et al., 2004). Therefore, in aversive Pavlovian conditioning, the IL, and its activity specifically during the CS appears to be important for response suppression and extinction learning.

Studies on the role of the IL in appetitive conditioning are typically conducted under the framework of substance use disorders. Seminal work showed that pharmacologically inactivating the IL reinstates otherwise extinguished operant responding for cocaine (Peters et al., 2008a). Subsequent work found that during extinction, optogenetic inhibition of the IL specifically after unreinforced lever pressing for cocaine impaired extinction learning and enhanced cue-induced cocaine-seeking (Gutman et al., 2017). Consistently, enhancing IL activity through micro-infusions of glutamatergic agonists (Peters et al. 2008; Chen et al. 2016) or through chemogenetics (Augur et al., 2016) attenuated cue-induced cocaine-seeking after extinction. However, studies using different reinforcers have found inconsistent results with the proposed hypothesis that the IL is important for suppressing operant responding after extinction. Pharmacological inactivation of the IL reduced rather enhanced the return of operant responding for heroin (Rogers et al., 2008; Bossert et al., 2011) and sucrose (Eddy et al., 2016) after extinction. Further, IL inactivation did not affect renewal of alcohol-seeking after extinction (Willcocks and McNally, 2013). These discrepant findings suggest that the IL may not suppress conditioned responding after extinction universally across different reinforcers.

Fewer studies have investigated the role of the IL in extinction of responses acquired through appetitive Pavlovian conditioning. Seminal work found that lesioning the IL enhanced responding to a food-predictive CS after un-signaled exposure to food (reinstatement), the passage of time (spontaneous recovery) or a change in the physical context (renewal), relative to controls with sham lesions (Rhodes and Killcross, 2004; 2007a). These findings are consistent with the role of the IL in extinction of aversive Pavlovian conditioned responding and operant responding for cocaine. The authors suggest that lesions of the IL may render inhibitory extinction memories more context-dependent, leading to greater renewal after extinction (Rhodes and Killcross, 2007a). Thus, the IL may be involved in controlling the context sensitivity of extinction memories which enables them to generalize and attenuate the return of appetitive Pavlovian responding triggered by a change in context. However, it has yet to be determined whether augmenting IL activity would be sufficient in promoting extinction and suppressing appetitive Pavlovian responding as seen with aversive stimuli.

In Chapter 3, we used a Pavlovian conditioning procedure in which a CS predicted sucrose to test the hypothesis that augmenting activity in the IL suppresses appetitive Pavlovian conditioned responding after extinction. Following Pavlovian conditioning in a distinct context (Context A) and extinction in a different context (Context B), rats were returned to the Pavlovian conditioned context (Context A) to trigger a renewal of conditioned responding. During the renewal test, we used in vivo optogenetics to stimulate the IL specifically during the CS to investigate its role in response suppression during renewal (Experiment 1). Next, we verified that IL activity during the CS was critical for response suppression by delivering optogenetic stimulation during the intertrial intervals (ITI) on the renewal test (Experiment 2). We predicted that optogenetic stimulation of the IL during the CS but not the ITI would attenuate the renewal of appetitive Pavlovian conditioned responding.

Methods

Subjects

Thirty-six, male, Long-Evans rats were used for the experiments in Chapter 3. Experiment 1 tested the effects of IL stimulation during the CS in the renewal test (ChR2, n=9; eYFP, n=9). Experiment 2 tested the effects of IL stimulation during the middle of the ITI in the renewal test (ChR2, n=9; eYFP, n=9).

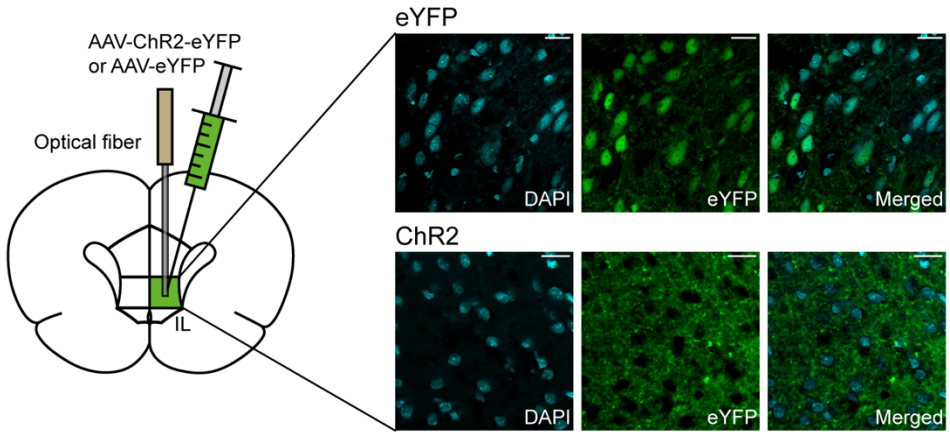
Behavioural procedures

Experiments 1 and 2 of Chapter 3 are renewal experiments and therefore Pavlovian conditioning sessions occurred in Context A and extinction sessions in Context B. Experiment 1 consisted of 13 Pavlovian conditioning sessions, and experiment 2 consisted of 12 Pavlovian conditioning sessions. In both experiments 1 and 2, at least 5 extinction sessions were conducted in Context B or until rats achieved a criterion of 5 or fewer CS port entries. Rats received a renewal test in Context A, the day after the last extinction sessions. In experiment 1, optogenetic stimulation of the IL was delivered during CS presentations at test. In experiment 2, optogenetic stimulation of the IL was delivered during the middle of the ITI at test.

Results

Histology

Figure 1 shows a method schematic and representative images for expression of ChR2 or eYFP alone transgenes in the IL. In experiment 1, two rats were excluded from analysis in the eYFP group, one due to failure to acquire Pavlovian conditioning, and another due to headcap detachment. Three rats were excluded from analysis in the ChR2 group due to headcap detachment (n = 1) and lack of transgene expression (n = 2). Final group sizes in experiment 1 were eYFP n = 7 and ChR2 n = 6. In experiment 2, rats were excluded due to misplacement of the optical fiber (eYFP n = 1, ChR2 n = 1) and lack of transgene expression (ChR2 n = 2). Final group sizes in experiment 2 were eYFP n = 8 and ChR2 n = 6.



Chapter 3, Figure 1. Method schematic and representative image of transgene expression in the IL. Viral vectors containing the transgene for ChR2 or eYFP alone was microinfused and an optical fiber was implanted into the IL. Images of ChR2 or eYFP alone transgene expression were taken using a confocal microscope with a 60x lens. Scale bar 20 μm.

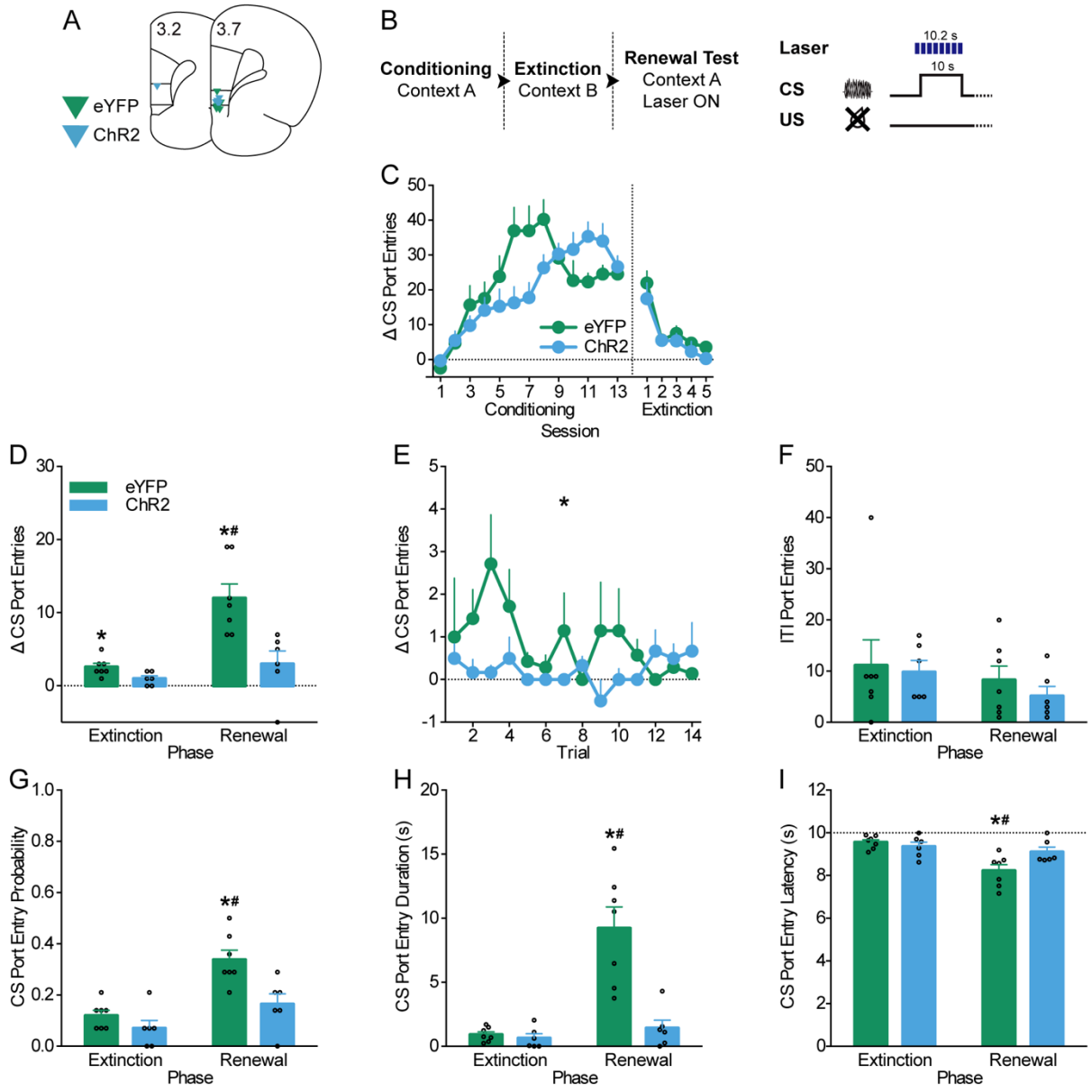
Experiment 1 - IL stimulation during the CS attenuated renewal

Experiment 1 tested whether stimulation of the IL during the CS would attenuate renewal of appetitive Pavlovian conditioned responding. Figure 2A depicts optical fiber placements in the IL of rats included in the final data analysis. Following Pavlovian conditioning in Context A and extinction in Context B, we tested renewal by returning rats to their conditioning context (Context A) and paired CS presentations with unilateral, optogenetic IL stimulation (Figure 2B).

Δ CS port entries increased equivalently in the ChR2 and eYFP group across Pavlovian conditioning sessions (Figure 2C; Session, $F(12,132) = 12.20$, $p < .001$; Virus, $F(1,11) = 1.27$, $p = .283$; Session x Virus, $F(12,132) = 3.13$, $p = .024$). Differences in Δ CS port entries were observed during sessions 6 ($p = .032$), 7 ($p = .049$), and 11 ($p = .017$). However, the eYFP and ChR2 groups did not differ in Δ CS port entries in the last two sessions of Pavlovian conditioning ($p > .05$). During extinction, Δ CS port entries decreased equivalently in the ChR2 and eYFP group across the first five sessions (Figure 2C; Session, $F(4,44) = 24.27$, $p < .001$; Virus, $F(1,11)=1.36$, $p = .267$; Session x Virus, $F(4,44) = .32$, $p = .790$). These data indicate that the ChR2 and eYFP groups similarly acquired Pavlovian conditioning and extinction.

At test, the eYFP group but not the ChR2 group displayed renewal of Δ CS port entries relative to the last extinction session (Figure 2D; Phase, $F(1,11) = 13.75$, $p = .003$; Virus, $F(1,11) = 20.50$, $p = .001$; Phase x Virus, $F(1,11) = 5.81$, $p = .035$). The eYFP group showed greater Δ CS port entries during the renewal test compared to the last extinction session, indicating renewal ($p = .001$). In contrast, in the ChR2 group, there was no significant difference in Δ CS port entries between the last extinction session and the renewal test ($p = .396$). In the last extinction, Δ CS port entries were slightly lower in the ChR2 group compared to the eYFP group ($p = .028$) without stimulation. During the renewal test, optogenetic stimulation reduced Δ CS port entries in the ChR2 group relative to the eYFP group ($p = .006$). Across trials, optogenetic stimulation of the IL in the ChR2 group attenuated renewal of Δ CS port entries compared to eYFP controls (Figure 2E; Trial, $F(13,143) = .72$, $p = .648$; Virus, $F(1,11) = 11.42$, $p = .006$; Trial x Virus, $F(13,143) = 1.06$, $p = .397$). Lastly, IL stimulation during the CS did not affect port entries made during the ITI (Figure 2F; Phase, $F(1,11) = 1.65$, $p = .226$; Virus, $F(1,11) = .35$, $p = .565$; Phase x Virus, $F(1,11) = .09$, $p = .763$). In sum, IL stimulation during CS presentations attenuated renewal of appetitive Pavlovian conditioned responding.

Additional measures of conditioned responding support that IL stimulation during the CS attenuated renewal of appetitive Pavlovian conditioned responding. Probability of CS port entries was greater at test relative to the last extinction session (Figure 2G; Phase, $F(1,11) = 16.79$, $p = .002$) and in the eYFP group relative to the ChR2 group (Virus, $F(1,11) = 17.85$, $p = .001$). However, there was no statistically significant interaction (Phase x Virus, $F(1,11) = 2.47$, $p = .145$). The eYFP group but not the ChR2 group displayed renewal at test relative to the last extinction session as measured by duration (Figure 2H; Phase, $F(1,11) = 21.22$, $p = .001$; Virus, $F(1,11) = 14.51$, $p = .003$; Phase x Virus, $F(1,11) = 18.50$, $p = .001$) and latency (Figure 2I; Phase, $F(1,11) = 21.57$, $p = .001$; Virus, $F(1,11) = 1.74$, $p = .214$; Phase x Virus, $F(1,11) = 10.15$, $p = .009$) of CS port entries. Duration ($p < .001$) was greater, and latency ($p < .001$) was shorter in the renewal test compared to the last extinction session in eYFP group. In contrast, duration ($p = .598$) and latency ($p = .341$) of CS port entries was similar in the last extinction and the renewal test in the ChR2 group. Duration ($p = .469$) and latency ($p = .379$) was similar between the ChR2 and eYFP group in the last extinction session. However, duration ($p = .002$) was greater, and latency ($p = .035$) was shorter in the eYFP group compared to the ChR2 group during the renewal test. Therefore, additional measures of conditioned responding further support that IL stimulation during the CS attenuated renewal of appetitive Pavlovian conditioning responding.



Chapter 3, Figure 2. Optogenetic stimulation of the IL during CS presentations attenuated renewal of appetitive Pavlovian conditioned responding. **(A)** Optical fiber tip placements in the IL of the eYFP (green) or ChR2 (blue) group included in the final data analysis. **(B)** Design of behavioural procedures and schematic of IL optogenetic stimulation during the CS in the renewal test. **(C)** Δ CS port entries during Pavlovian conditioning and extinction. **(D)** Δ CS port entries in the last extinction session and the renewal test. # $p < 0.05$ extinction vs renewal in the eYFP group. * $p < 0.05$ ChR2 vs eYFP in the last extinction session and renewal test. **(E)** Δ CS port entries across trials during the renewal test. * $p < 0.05$ main effect of virus group. **(F)** ITI port entries during the last extinction session and the renewal test. **(G)** Probability, **(H)** duration, and **(I)** latency of CS port entries in the last extinction session and the renewal test. **(G-I)** # $p < 0.05$ extinction vs renewal in the eYFP group. * $p < 0.05$ ChR2 vs eYFP in the renewal test. All data are mean ± SEM.

Experiment 2 - IL stimulation during the ITI did not affect renewal

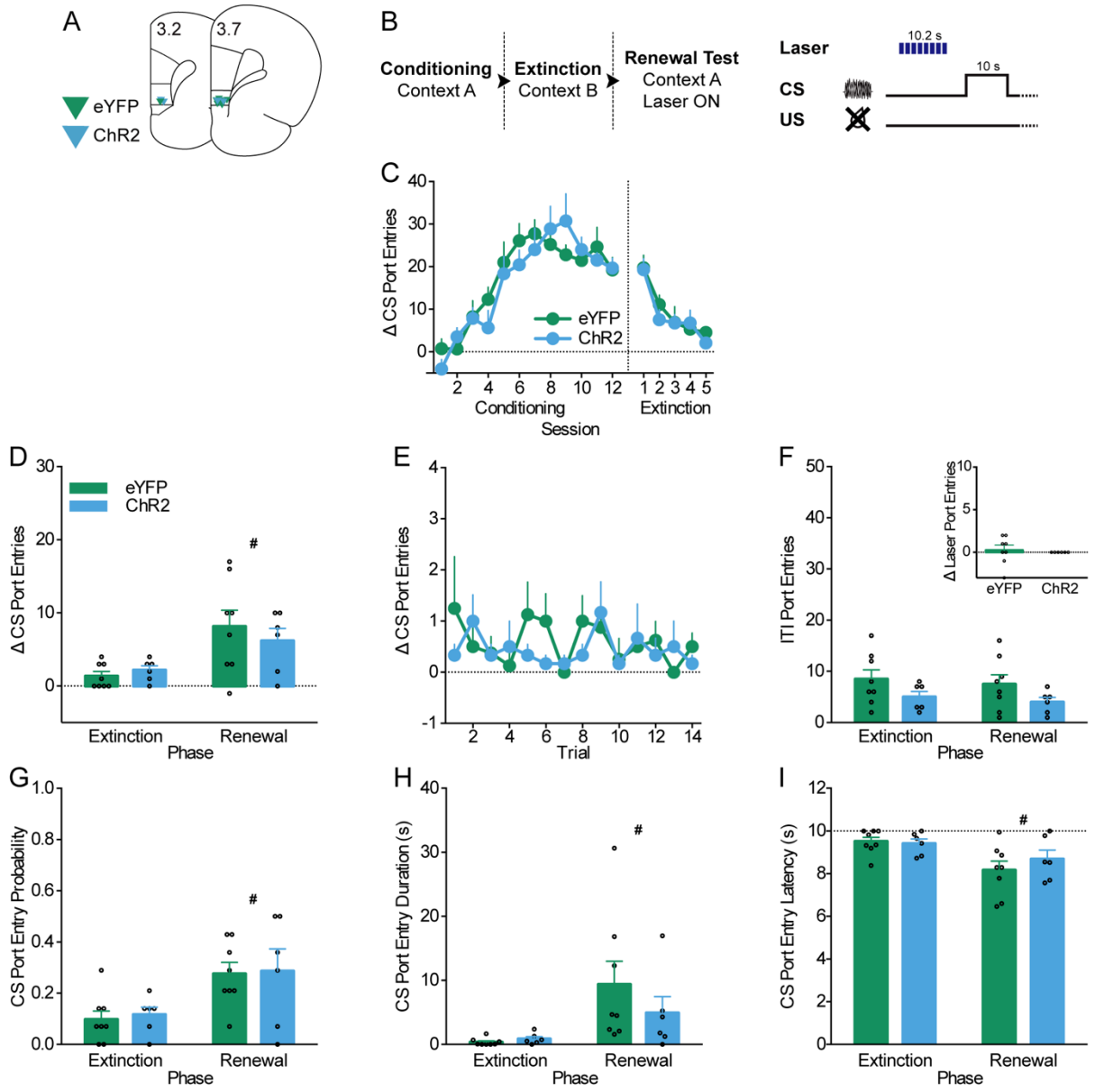
Experiment 2 tested the temporal specificity of the attenuation of renewal following optogenetic stimulation of the IL by delivering stimulation outside of the CS at test. Figure 3A depicts optical fiber placements in the IL of rats included in the final data analysis. Following Pavlovian conditioning in Context A and extinction in Context B, we tested renewal by returning rats to their conditioning context (Context A) and delivered unilateral, optogenetic IL stimulation during the middle of the ITI (Figure 3B).

Δ CS port entries increased equivalently in the ChR2 and eYFP group across Pavlovian conditioning sessions (Figure 3C; Session, $F(11,132) = 14.30$, $p < .001$) regardless of virus group (Virus, $F(1,10) = .002$, $p = .969$; Session x Virus, $F(11,132) = .78$, $p = .590$). During extinction, Δ CS port entries decreased equivalently in the ChR2 and eYFP group in the first five sessions (Figure 3C; Session, $F(4,48) = 29.24$, $p < .001$; Virus, $F(1,12) = .43$, $p = .527$; Session x Virus, $F(4,48) = .961$, $p = .424$). These results indicate that ChR2 and eYFP groups similar acquired Pavlovian conditioning and extinction.

Δ CS port entries significantly increased at test relative to the last extinction session in both ChR2 and eYFP groups, indicating renewal (Figure 3D; Phase, $F(1,12) = 10.86$, $p = .006$; Virus, $F(1,12) = .15$, $p = .705$; Phase x Virus, $F(1,12) = .71$, $p = .416$). Further, stimulation of the IL in the ChR2 group did not affect renewal of Δ CS port entries across trials (Figure 3E; Trial, $F(13,156) = .70$, $p = .726$; Virus, $F(1,12) = .42$, $p = .528$, Trial x Virus, $F(13,156) = .63$, $p = .786$). Additionally, IL stimulation during the middle of the ITI did not affect port entries made during the entire ITI period (Figure 3F; Phase, $F(1,12) = .55$, $p = .473$, Virus, $F(1,12) = 3.68$, $p = .079$; Phase x Virus, $F(1,12) = .00$, $p = 1.00$). IL stimulation also did not reduce port entries at the time of stimulation in the middle of the ITI (Figure 3F inset; $t(12) = 0.36$, $p=.723$). Together, IL stimulation during the middle of the ITI did not affect renewal and did not appear to produce general suppression of port entries.

Additional measures of conditioned responding further support that optogenetic stimulation of the IL during the middle of the ITI did not affect renewal. Probability (Figure 3G; Phase, $F(1,12) = 15.02$, $p = .002$; Virus, $F(1,12) = .06$, $p = .804$; Phase x Virus, $F(1,12) = .01$, $p = .911$) and duration (Figure 3H; Phase, $F(1,12) = 7.40$, $p = .019$; Virus, $F(1,12) = .712$, $p = .415$; Phase x Virus, $F(1,12) = 1.06$, $p = .323$) of CS port entries increased in the renewal test compared to the last extinction session in both ChR2 and eYFP groups. Further, latency (Figure 3I; Phase,

F(1,12) = 7.57, p = .018, Virus, F(1,12) = .51, p = .487; Phase x Virus, F(1,12) = .67, p = .429) of CS port entries decreased in the renewal test compared to the last extinction session in both ChR2 and eYFP groups. Together, additional measures of conditioned responding suggests that IL stimulation during the middle of the ITI did not affect renewal.



Chapter 3, Figure 3. Optogenetic stimulation of the IL during the middle of the ITI did not affect renewal of appetitive Pavlovian conditioned responding. **(A)** Optical fiber tip placements in the IL of the eYFP (green) or ChR2 (blue) group included in the final data analysis. **(B)** Design of behavioural procedures and schematic of IL optogenetic stimulation during the middle of the ITI in the renewal test. **(C)** Δ CS port entries during Pavlovian conditioning and extinction. **(D)** Δ CS port entries in the last extinction session and the renewal test. **(E)** Δ CS port entries across trials during the renewal test. **(F)** ITI port entries during the last extinction session and the renewal test. Inset graph is Δ laser port entries in the middle of the ITI. **(G)** Probability, **(H)** duration, and **(I)** latency of CS port entries in the last extinction session and the renewal test. **(D,G-I)** # $p < 0.05$ main effect of Phase. All data are mean ± SEM.

Discussion

The results of Chapter 3 showed that optogenetic stimulation of the IL specifically during the CS, suppressed the renewal of appetitive Pavlovian conditioned responding. Optogenetic stimulation of the IL during the CS attenuated renewal of conditioned responding as measured by Δ CS port entries. However, optogenetic stimulation of the IL outside of the CS, during the middle of the ITI, did not affect renewal. Therefore, the suppression of appetitive Pavlovian responding mediated by the IL appears to be temporally specific to IL activity during the CS. Together, these results highlight a critical role for the IL in suppressing the renewal of appetitive Pavlovian responding.

Optogenetic stimulation of the IL specifically during the CS attenuated conditioned responding as measured by Δ CS port entries and additional measures of CS port entry probability, duration, and latency. These results suggest that enhancing IL activity during the CS affected multiple aspects of the conditioned response during renewal. Suppression of Δ CS port entries following IL stimulation during CS presentations at the renewal test was evident from the very first trial and onwards, suggesting that IL stimulation did not just facilitate within-session extinction at test. In experiment 1, optogenetic stimulation of the IL during the CS specifically reduced the renewal of Δ CS port entries at test without affecting port entries made during the ITI. Conversely, in experiment 2, IL stimulation in the middle of the ITI did not appear to suppress port entries at the time of stimulation or the entire ITI period. Further, IL stimulation in the middle of the ITI did not seem to have any carry over effects on Δ CS port entries at test. Therefore, our results suggest that suppression of renewal was dependent on augmenting IL activity specifically during the CS.

The present findings support the proposed role of the IL in suppression of conditioned responding after extinction. The attenuation of renewal is consistent with findings that augmenting IL activity pharmacologically with AMPA micro-infusion or optogenetic stimulation during the CS reduces reinstatement and spontaneous recovery of appetitive Pavlovian responding (Villaruel et al., 2018). Further, the present findings complement studies which have found that lesioning the IL in rats enhances the return of appetitive Pavlovian responding to a food-predictive CS after extinction (Rhodes and Killcross, 2004; 2007a). The integral role of the IL in extinction has largely been studied using aversive Pavlovian conditioning procedures. For example, IL activity has been shown to correlate with effective extinction retrieval (Milad and

Quirk, 2002). Further, electrical and optogenetic stimulation of the IL specifically during the CS in extinction promotes extinction retrieval and the inhibition of aversive conditioned responding (Milad et al., 2004; Do Monte et al., 2015). Therefore, the present findings that IL stimulation suppresses renewal of appetitive Pavlovian responding, suggests that the IL plays a critical role in extinction and suppression of conditioned responding to both aversive and appetitive stimuli.

The role of the IL in response suppression for appetitive stimuli has largely been conducted under the framework of substance use disorders. For example, inhibiting IL activity disinhibits extinguished operant responding and disrupts extinction of operant responding for cocaine (Peters et al., 2008a; Gutman et al., 2017). These results highlight the importance of the IL in extinction and suppression of cocaine-seeking. However, there is mixed evidence on whether the IL is involved in suppressing the return of drug-seeking after extinction especially across different drug reinforcers. Pharmacological inactivation of the IL has been shown to have no effect on the return of cocaine-seeking (McFarland and Kalivas, 2001; McLaughlin and See, 2002; Capriles et al., 2003; Fuchs et al., 2005) or alcohol-seeking (Wilcocks and McNally, 2012; Pfarr et al., 2015) but suppresses the return of heroin-seeking after extinction (Rogers et al., 2008; Bossert et al., 2011; Bossert et al., 2012). Furthermore, pharmacological inactivation of the IL inhibits the return of operant responding for sucrose after extinction (Eddy et al., 2016; Caballero et al., 2019). Despite these inconsistent findings with disrupting IL activity, augmenting IL activity either pharmacologically or chemogenetically has consistently been shown to suppress the return of cocaine-, heroin-, and alcohol-seeking after extinction (LaLumiere et al., 2012; Gass et al., 2014; Augur et al., 2016; Chen et al., 2016). Therefore, while IL activity may not be required for suppressing the return of operant responding for drugs, activating the IL may be sufficient for inhibiting responding and promoting extinction. The results of the experiments in Chapter 3 are consistent with studies that have found that activation of the IL suppresses responding for drugs of abuse and extend this idea to Pavlovian responding for natural rewards such as sucrose.

In conclusion, Chapter 3 showed that activating the IL during a sucrose CS suppressed the renewal of appetitive Pavlovian conditioned responding. Moreover, optogenetic stimulation of the IL during the middle of the ITI did not affect renewal. Thus, IL activity specifically during the CS seems to be particularly important for mediating the suppression of renewal. The present results are consistent with other studies reporting that augmenting IL activity can inhibit

responding to aversive Pavlovian cues and drug-seeking. Therefore, the findings from Chapter 3, supports the proposed role of the IL in suppressing responding and extends it to appetitive Pavlovian conditioned responses.

Chapter 4 - Optogenetic stimulation of the IL-to-NAcS but not IL-to-BLA projection attenuated renewal of appetitive Pavlovian conditioned responding

Introduction

Different neural projections from the infralimbic cortex (IL) to the nucleus accumbens shell (NAcS) and the basolateral amygdala (BLA) are thought to mediate extinction based on the affective valence of the stimuli (Peters et al., 2009). Specifically, the IL-to-NAcS neural projection is thought to be imperative for extinction of appetitive conditioned responding especially in the context of operant drug-seeking (Peters et al., 2009), whereas the IL-to-BLA projection is implicated in the extinction of conditioned responding to aversive Pavlovian cues (Likhtik et al., 2005; Peters et al., 2009; Pape and Paré, 2010; Bloodgood et al., 2018). However, there is little research comparing the role of these two IL projections to the NAcS and the BLA in extinction of appetitive Pavlovian conditioned responses. Studies on the role of the IL-to-NAcS in extinction typically use operant conditioning procedures in which rodents must make an operant response to earn drug reinforcers. In contrast, studies on the role the IL-to-BLA in extinction are conducted using aversive Pavlovian conditioning. The use of different behavioural procedures makes comparisons between the role of the IL-to-NAcS and IL-to-BLA in extinction difficult to disentangle. For instance, the IL-to-NAcS may be important for extinction of operant responses whereas the IL-to-BLA may be imperative for extinction of Pavlovian conditioned responses regardless of affective valence.

The IL is thought to mediate extinction of aversive Pavlovian conditioned responding through its projections to the amygdala (Peters et al., 2009; Arruda-Carvalho and Clem, 2015; Giustino and Maren, 2015). Extinction of aversive Pavlovian conditioned responding modulates the synaptic properties of the IL-to-BLA projection, indicating that it may be important for extinction of aversive Pavlovian responding (Cho et al., 2013; Bloodgood et al., 2018). Chemogenetic and optogenetic inhibition of the IL-to-BLA projection during extinction impairs extinction retrieval the following day (Bloodgood et al., 2018; Bukalo et al., 2015). Conversely, IL-to-BLA optogenetic stimulation of the suppresses conditioned freezing and augments extinction training to facilitate extinction retrieval (Bukalo et al., 2015; Bukalo et al., 2021). Therefore, the IL-to-BLA projection appears to be critical for extinction of aversive conditioned

responding. However, the role of the IL-to-BLA in extinction of appetitive Pavlovian conditioned responding has yet to be explored.

Substantial evidence indicates that the IL and NAcS are involved in extinction of operant responding for appetitive stimuli, and especially cocaine (Peters et al., 2008a; LaLumiere et al., 2012; Augur et al., 2016). Extinction of cocaine-seeking induces greater Fos expression in the IL (Warren et al., 2016) and synaptic plasticity in the NAcS (Sutton et al., 2003). Further, pharmacological disconnection of the IL and NAcS results in the reinstatement of extinguished cocaine-seeking (Peters et al., 2008a). Additionally, neuronal ensembles in the IL that are activated by the extinction of cocaine self-administration predominantly project to the NAcS (Warren et al., 2019). Pharmacologically disconnecting these IL cocaine extinction ensembles and the NAcS leads to an increase in cocaine-seeking (Warren et al., 2019). Together, these results suggest that extinction engages both the IL and the NAcS and that the IL-to-NAcS projection is important for suppressing cocaine-seeking after extinction.

Inconsistently, pharmacologically disconnecting the IL and NAcS attenuates the return of heroin-seeking after extinction (Bossert et al., 2012), suggesting that the role of the IL-to-NAcS in suppressing responding may not be uniform across different reinforcers. Further, limited work has investigated the role of the IL-to-NAcS in extinction using natural reinforcers. However, pharmacologically disconnecting the IL and NAcS disrupts the capacity of a Pavlovian sucrose cue to invigorate operant responding, suggesting that the projection is involved in processing appetitive Pavlovian associations (Keistler et al., 2015). The role of the IL-to-NAcS in extinction of explicitly appetitive Pavlovian conditioned responding for natural reinforcers, however, remains unknown.

The IL may mediate extinction of responding for appetitive stimuli through glutamatergic projections to the NAcS. Concurrent pharmacological inactivation of the IL and NAcS disinhibits extinguished cocaine-seeking (Peters et al., 2008a). Further, enhancing glutamatergic activity in the IL reduced cue-induced cocaine-seeking but is reversed by glutamatergic antagonists or dopamine administration in the NAcS (LaLumiere et al., 2012). Stimulation of the IL increased extracellular levels of glutamate (Quiroz et al., 2006), and increase of glutamatergic transmission in the NAcS is associated with reduction and extinction of cocaine-seeking (Sutton et al., 2003). Moreover, pharmacological inactivation of the IL disrupts NAcS activity involved in suppressing non-reinforced responding (Ghazizadeh et al., 2012). Consistently, direct

chemogenetic stimulation of the IL-to-NAcS attenuated cue-induced reinstatement of cocaine seeking after extinction (Augur et al., 2016). Therefore, glutamatergic inputs from the IL to the NAcS may be important for suppressing responding for appetitive stimuli.

In Chapter 4, we investigated the role of the IL-to-NAcS and IL-to-BLA neural projections in extinction of appetitive Pavlovian conditioned responses using a similar renewal procedure across two experiments. First, we characterized the different IL projections to the NAcS and BLA using retrograde neural tracers (Experiment 1). Next, in separate experiments, rats received Pavlovian conditioning in Context A and extinction in a different Context B, followed by a renewal test in which rats were returned to Context A to trigger a return of responding. In the renewal test, we used in vivo optogenetics to stimulate the IL-to-NAcS projection during the CS to investigate its role in response suppression after extinction (Experiment 2). In a separate experiment, we optogenetically stimulated the IL-to-BLA projection during the CS in the renewal test to investigate its role in suppressing conditioned responses after extinction (Experiment 3). The IL-to-NAcS projection is thought to mediate extinction of responses to appetitive stimuli whereas the IL-to-BLA projection is thought to mediate extinction of responses to aversive stimuli. Therefore, we predicted that optogenetic stimulation of the IL-to-NAcS, but not the IL-to-BLA would attenuate the renewal of appetitive Pavlovian responding.

Methods

Subjects

Thirty, male, Long-Evans rats were used for the experiments in Chapter 4. Experiment 1 characterized the IL-to-NAcS and IL-to-BLA neural projection s (n=4) using retrograde tracing. Experiment 2 tested the role of the IL-to-NAcS projection in renewal of appetitive Pavlovian conditioned responding (ChR2, n = 10; eYFP, n = 10). Experiment 3 tested the role of the IL-to-BLA projection in renewal (ChR2, n = 6).

Retrograde tracing of the IL-to-NAcS and IL-to-BLA neural projections

Rats in the retrograde tracing experiment received stereotaxic surgery to unilaterally microinfuse the retrograde tracer, Cholera Toxin Subunit B (CTb; 0.5% weight/volume in 0.9% sterile saline), conjugated with either an Alexa Fluor 488 (CTb-488; Invitrogen, C34775) or Alexa Fluor 555 (CTb-555, Invitrogen, C34776) dye into the NAcS and BLA (0.3 μL, 0.1

μL/min, 10 min diffusion). Coordinates from bregma (AP and ML) and the skull surface (DV) for targeting the NAcS were AP +1.2 mm, ML +1.0 mm, DV -7.5 mm and for the BLA were AP -2.5 mm, ML -5.0 mm, DV -8.5 mm. The fluorescent label used for tracing was counterbalanced by region across rats. All rats were euthanized one week after receiving surgery and brains were processed as described in Chapter 2. Brain sections were stained with DAPI, cover slipped, and processed through fluorescence microscopy. Infusion sites were examined to ensure accurate targeting of the NAcS and the BLA using an epifluorescence microscope at 4x magnification. A confocal laser scanning microscope (Nikon C2) was used to image CTb labelled cells in the medial prefrontal cortex (4 sections per rat) using a 20x lens with 488 nm and 561 nm lasers for excitation. Images were captured with a pixel size of 2765 x 2765 and a slice depth of 24 um (6 steps, 4 um/step). Captured images were imported to Imaris Cell Imaging Software (Bitplane, Oxford Instruments) in which analysis was specifically restricted to the IL by the experimenter using a rat brain atlas (Paxinos and Watson, 2007). CTb-488 and CTb-555 labelled objects (cells) were defined using the "Blobs" tool in Imaris, and local contrast thresholding. Co-labelled cells were determined as objects labelled in one channel that had >20% of their volume also labelled in the second channel. The number of labelled and co-labelled cells was averaged across the 4 sections to get a single value for each rat. Density of labelled and co-labelled cells was calculated by dividing the average number of labelled and co-labelled by the average area of the selected quantified region across 4 brain sections.

IL-to-NAcS projection in renewal

Experiment 2 consisted of 12 Pavlovian conditioning sessions in Context A and at least three extinction sessions in Context B or until the criterion of 5 or fewer CS port entries was met. Following extinction, rats were tested in Context A and B across different days with optical stimulation delivered during the CS. Test order was counterbalanced such that half of the rats were tested in Context A first, and the other half in Context B first. Test sessions were separated by at least one extinction session or until the criterion of 5 or fewer CS port entries was met. Inter-test extinction sessions were done to mitigate any carry-over effects of optical stimulation. We found that rats in the eYFP group that received their second test in Context A did not show renewal. Therefore, after test 2, all rats received two Pavlovian re-conditioning sessions in Context A and at least two extinctions in Context B or until the criterion was met prior to

repeating the second renewal test. Final data analysis consists of collapsing the first renewal test and the repeated second test.

IL-to-BLA projection in renewal

Experiment 3 consisted of 10 Pavlovian conditioning sessions in Context A and at least three extinction sessions in Context B or until the criterion of 5 or fewer CS port entries was met. One day after the last extinction session, rats were tested for renewal in Context A with optogenetic stimulation occurring either during the CS or in the middle of the intertrial intervals (ITI). Test order was counterbalanced such that half of the rats were tested with optogenetic stimulation occurring during the CS first, and for the other half during the ITI first. Optogenetic stimulation of the IL during the ITI does not affect renewal and therefore, this group served as a within-subject control (Chapter 3; Villaruel et al., 2018). Between tests, rats received three Pavlovian conditioning sessions in Context A followed by at least two extinction sessions or until criterion was met. Final data analysis consists of the last extinction sessions prior to each test and the renewal tests in Context A collapsed across time of optogenetic stimulation delivery.

Fos Immunohistochemistry

Rats in experiments 2 and 3 received an additional test to induce c-Fos and verify that optogenetic stimulation of IL-to-NAcS and IL-to-BLA terminals expressing ChR2 had a physiological effect (Fuchikami et al., 2015; Benn et al., 2016; Wood et al., 2019). The c-Fos induction session occurred in default conditioning chambers and was identical to previous test sessions. Optogenetic stimulation was delivered for 14 trials to mimic previous tests, but in the absence of house light illumination, the white noise CS, or sucrose. In the IL-to-BLA experiment, optogenetic stimulation was omitted in half of the rats to include a non-stimulated control. Rats remained in the conditioning chambers for an additional 50 min to ensure that they were euthanized 90 min after the start of the session to maximize c-Fos expression (Muller et al., 1984; Bossert et al., 2011; Warren et al., 2016). Brain sections were processed in an anti c-Fos rabbit antibody (1:2000; Cell Signaling, 2250S) for approximately 72 h, and subsequently in a secondary solution with biotinylated goat anti-rabbit antibody (1:250; Vector Labs, BA-1000). Next, sections were placed 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. Finally, sections were rinsed in phosphate buffer, mounted on slides, and cover slipped. Images of each section were captured through a brightfield microscope (Nikon Eclipse TiE) using a 10x lens. Two sections from the IL, the NAcS, or the BLA were chosen for quantification based on location relative to bregma and image quality. A rat brain atlas (Paxinos and Watson, 2007) was used to approximate the location of sections relative to bregma and the regions of interest. Image analysis was done through ImageJ FIJI. A region of the IL, the NAcS, and BLA was selected manually for each section in both the stimulated and non-stimulated hemisphere. Quantification of the selection was done through a custom-made FIJI macro, which counted Fos positive nuclei based on colour relative to background, size, and circularity. Counts were then divided by the average area selected in FIJI to calculate density. The final Fos density for each rat consisted of the average across two sections for each hemisphere and region.

Results

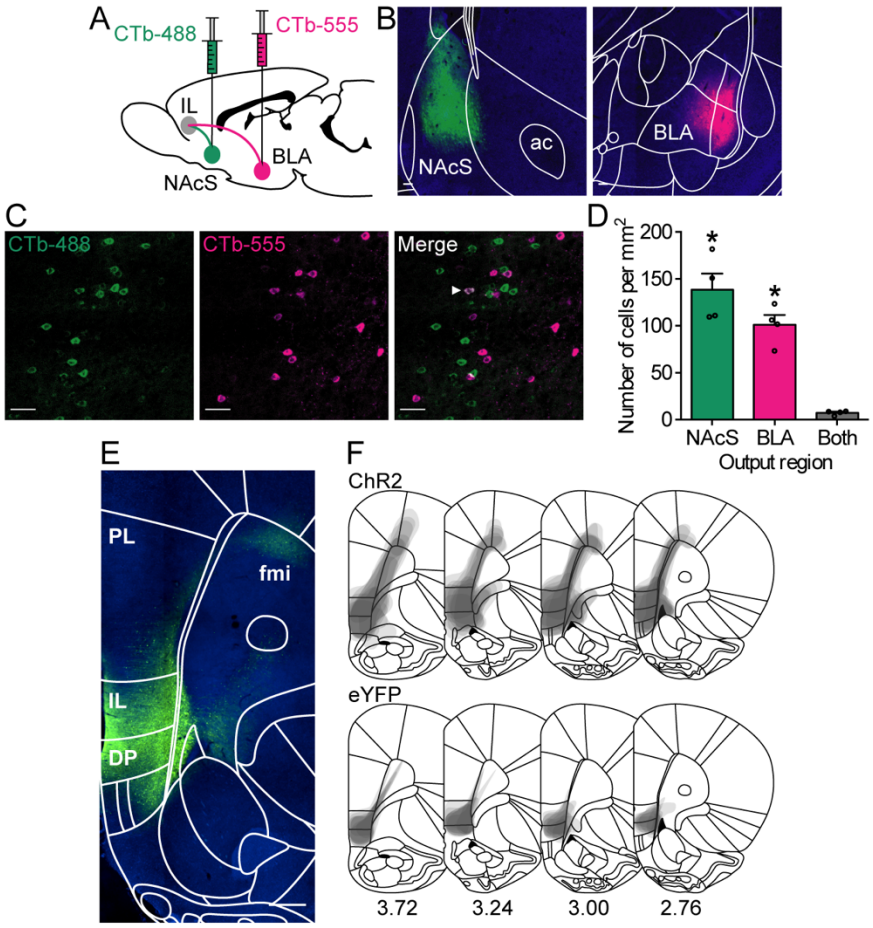
Retrograde neural tracing of IL projections to the NAcS and BLA

IL projections to the NAcS and the BLA were characterized by injecting different fluorescent labelled retrograde tracers into the NAcS and the BLA (Figure 1A-D). Neural tracing of IL projections to the NAcS and BLA revealed largely distinct, non-overlapping projections to these output regions. Only a small proportion of labelled cells were found to project to both the NAcS and the BLA (Figure 1D; $F(2,11) = 33.21$, $p < .001$). Density of labelled cells in the IL projecting to the NAcS ($p < .001$) and BLA ($p = .001$) were greater than from Both output regions. Lastly, density of labelled cells in the IL projecting either to the NAcS and BLA were similar ($p = .154$).

Histology

Figure 1E depicts the expression of the ChR2 transgene observed in the IL. The approximate spread of the transgenes for ChR2 and eYFP alone was based on rats in Experiment 2 but was consistent across all experiments (Figure 1F). The highest concentration of ChR2 expression was in the infralimbic cortex, the dorsal peduncular cortex and the ventral regions of the prelimbic cortex. Some expression of ChR2 was observed along the injector tract, in the anterior and lateral areas of the prelimbic cortex along the forceps minor of the corpus callosum and the anterior medial and ventral orbitofrontal cortex.

Rats were excluded from final behavioural data analysis due to lack of transgene expression or misplacement of optical fiber implants. In the IL-to-NAcS experiment (experiment 2), two rats (eYFP n = 1, ChR2 n = 1) were excluded due to misplaced optical fiber implants. The final group sizes for experiment 2 were ChR2 n = 9 and eYFP n = 9. One additional rat (eYFP n = 1) from experiment 2 was removed from Fos immunohistochemistry analysis due to complications with histology. The final group size for the IL-to-BLA experiment (experiment 3) was ChR2 n = 6.



Chapter 4, Figure 1. Neural tracing and optogenetic targeting of IL projections. (**A**) Method schematic for neural tracing. CTb-488 and CTb-555 retrograde tracers were injected in the NAcS and the BLA, and quantification of labelled cells was done in the IL. (**B**) Representative images of injection sites in the NAcS (left) and the BLA (right). Anterior commissure (ac). Scale bar 100 µm. (**C**) Representative images of labelled cells in the IL. Arrow in merged image shows an example of co-labelling. Scale bar 50 µm. (**D**) Quantification of labelled cells in the IL shows largely non-overlapping cells projecting to the NAcS and the BLA. Data presented as mean ± SEM. * $p < 0.05$ output region vs. Both output regions. **(E)** Representative image of ChR2 expression in the IL. Prelimbic cortex (PL), dorsal peduncular cortex (DP), forceps minor of the corpus callosum (fmi). Scale bar 500 µm. **(F)** Schematic depicting the extent of ChR2 (n = 9, top panel) and eYFP alone (n = 9, bottom panel) expression in the IL across four bregma points.

IL-to-NAcS stimulation suppressed renewal of appetitive Pavlovian conditioned responding

Experiment 2 tested whether optogenetic stimulation of the IL-to-NAcS projection would suppress the renewal of appetitive Pavlovian conditioned responding. Both ChR2 and eYFP groups similarly acquired Pavlovian conditioning in Context A as measured by Δ CS port entries (Figure 1E, Session, $F(11,176) = 14.59$, $p < .001$; Virus, $F(1,16) = .17$, $p = .684$; Session x Virus, $F(11,176) = .62$, $p = .679$) and extinguished conditioned responding in the first three sessions of extinction in Context B (Session, $F(2,32) = 16.84$, $p < .001$; Virus, $F(1,16) = 2.08$, $p = .168$; Session x Virus, $F(2,32) = 1.41$, $p = .259$). Virus groups did not differ in the last session of extinction prior to test (Virus, $F(1,16) = .51$, $p = .487$). Both ChR2 and eYFP groups similarly re-acquired (Figure 1F; Session, $F(1,16) = .004$, $p = .949$; Virus, $F(1,16) = 1.06$, $p = .319$; Session x Virus, $F(1,16) = .02$, $p = .898$) and re-extinguished (Session, $F(1,16) = 13.67$, $p = .002$; Virus, $F(1,16) = 3.16$, $p = .094$; Session x Virus, $F(1,16) = .04$, $p = .854$) conditioned responding following the first round of testing. Groups did not differ in Δ CS port entries in the last session of extinction prior to the second round of tests (Virus, $F(1,16) = .53$, $p = .476$). In sum, both ChR2 and eYFP similarly acquired Pavlovian conditioning and extinction.

Optogenetic stimulation of the IL-to-NAcS during the CS in the ChR2 group suppressed renewal of appetitive Pavlovian conditioned responding (Figure 1G). In the renewal tests, the eYFP but not the ChR2 group showed a robust renewal of conditioned responding in Context A relative to Context B (Context, $F(1,16) = 18.38$, $p = .001$; Virus, $F(1,16) = 4.96$, $p = .041$; Context x Virus, $F(1,16) = 51.04$, $p < .001$). Δ CS port entries were similarly low for both eYFP and ChR2 at test in the extinction Context B ($p = .644$). However, Δ CS port entries were greater in the eYFP group compared to the ChR2 group in Context A ($p < .001$) indicating that stimulation of the IL-to-NAcS during the CS suppressed renewal. The ChR2 group showed slightly higher levels of responding in Context B compared to A ($p = .060$). In contrast, the eYFP group showed greater Δ CS port entries at test in Context A relative to Context B ($p < .001$).

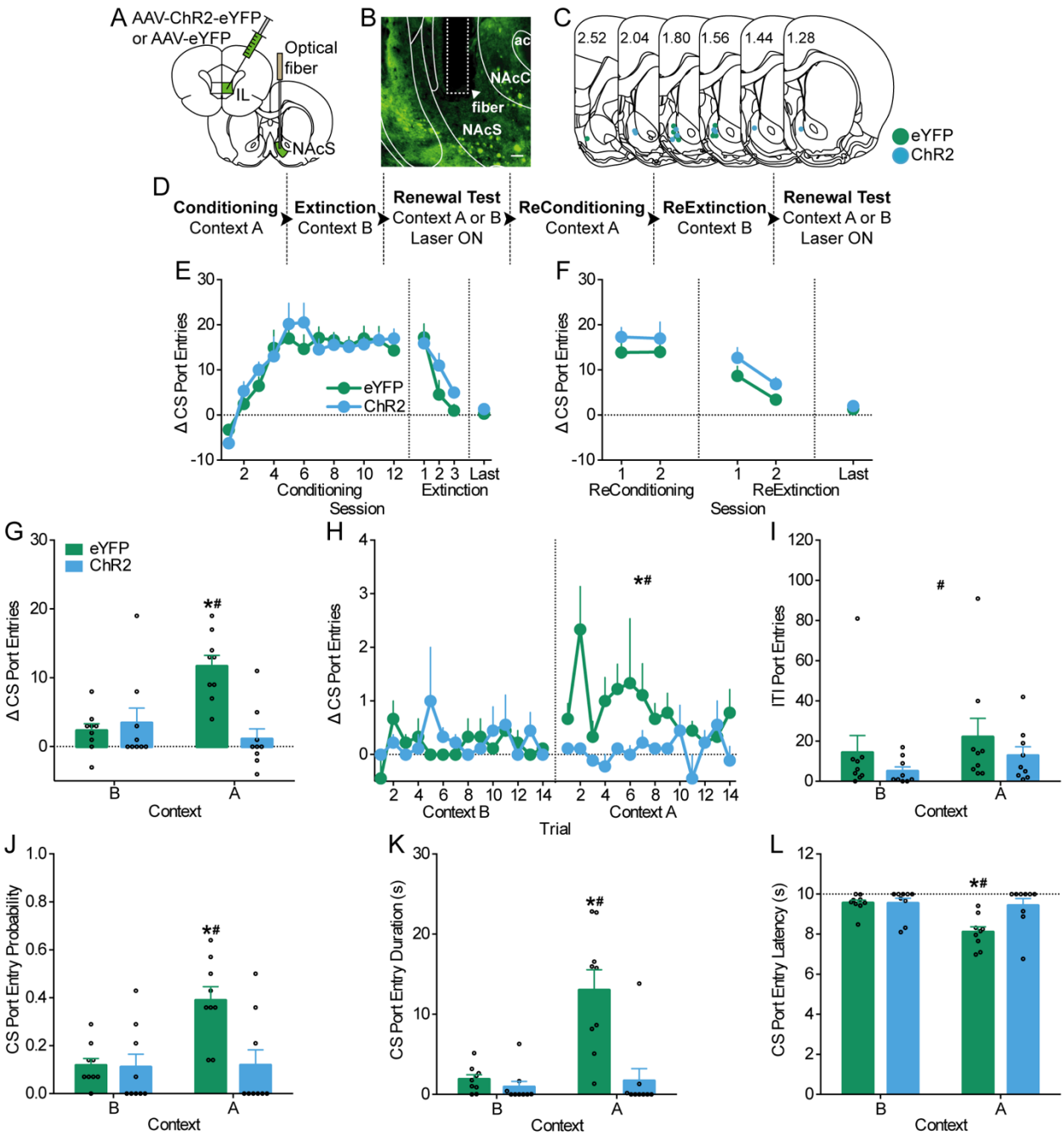
Analysis of Δ CS port entries across trials at test showed that IL-to-NAcS stimulation during CS presentations suppressed conditioned responding in all trials (Figure 1H; Context, $F(1,16) = 18.38$, $p = .001$; Virus: $F(1,16) = 4.96$, $p = .041$; Context x Virus, $F(1,16) = 51.04$, $p < .001$; Trial, $F(13,208) = 1.37$, $p = .208$; Trial x Virus, $F(13,208) = 1.49$, $p = .162$; Context x Trial, $F(13,208) = .68$, $p = .680$; Context x Trial x Virus: $F(13,208) = .83$, $p = .558$). These

results suggest that optogenetic stimulation of the IL-to-NAcS projection suppressed appetitive Pavlovian conditioned responding consistently throughout the renewal test.

Stimulation of the IL-to-NAcS did not affect port entries made during the ITI (Figure 1I). Port entries made during the ITI were greater in Context A than Context B in both ChR2 and eYFP groups (Context, $F(1,16) = 6.24$, $p = .024$; Virus, $F(1,16) = 1.05$, $p = .322$; Context x Virus, $F(1,16) < .01$, $p = .986$). Therefore, IL-to-NAcS optogenetic stimulation did not appear to produce non-specific motor effects during time intervals outside the CS and stimulation.

Additional measures of conditioned responding further support that optogenetic stimulation of the IL-to-NAcS during CS presentations suppressed renewal of appetitive Pavlovian conditioned responding (Figure 1J-L). In the renewal tests, the eYFP but not the ChR2 group displayed robust renewal in Context A relative to Context B as measured by probability of CS port entries (Figure 1J; Context, $F(1,16) = 21.647$, $p < .001$; Virus, $F(1,16) = 4.09$, $p = .060$; Context x Virus, $F(1,16) = 19.34$, $p < .001$), total duration of CS port entries (Figure 1K; Context, $F(1,16) = 20.12$, $p < .001$; Virus, $F(1,16) = 12.57$, $p = .003$; Context x Virus, $F(1,16) = 15.26$, $p = .001$), and average latency to initiate a CS port entry (Figure 1L; Context, $F(1,16) = 19.73$, $p <.001$; Virus, $F(1,16) = 3.75$, $p = .071$; Context x Virus, $F(1,16) = 14.16$, $p = .002$). Probability ($p = .918$), duration ($p = .293$), and latency ($p = .959$) were similar for both eYFP and ChR2 groups at test in the extinction Context B. However, probability ($p = .006$), and duration ($p = .001$) were higher, and latency was shorter ($p = .010$) in the eYFP group compared to the ChR2 group in Context A, indicating that stimulation of the IL-to-NAcS during the CS suppressed renewal. The eYFP group showed greater probability ($p < .001$) and duration ($p < .001$) and shorter latency ($p < .001$) of CS port entries at test in Context A relative to Context B. In contrast, the ChR2 group showed similar levels of probability ($p = .860$), duration ($p = .688$), and latency ($p = .638$) at tests in both Context A and B.

Altogether, optogenetic stimulation of the IL-to-NAcS projection during CS presentations attenuated the renewal of appetitive Pavlovian conditioned responding and did not affect port entries outside of the CS.



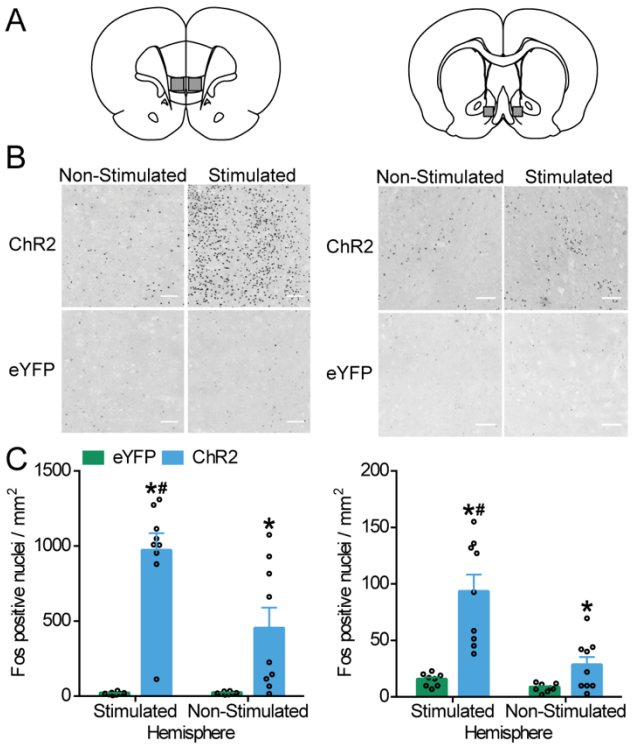
Chapter 4, Figure 2. Optogenetic stimulation of the IL-to-NAcS projection suppressed renewal of appetitive Pavlovian conditioned responding. **(A)** Method schematic of microinjections of ChR2 or eYFP alone in the IL and an optical fiber implanted in the NAcS. **(B)** Representative image depicting ChR2 expression in IL terminals and the optical fiber within the NAcS. Nucleus accumbens core (NAcC), anterior commissure (ac). Scale bar 100 µm. **(C)** Optical fiber placements in the NAcS for ChR2 (blue) or eYFP alone (green) expressing rats included in the final data analysis. Numbers are locations of sections relative to bregma. **(D)** Design of behavioural procedures. **(E-F)** Δ CS port entries during conditioning and reconditioning in Context A and extinction and re-extinction in Context B prior to tests. **(G)** Δ CS port entries at

tests in the conditioning context (Context A) for renewal relative to the extinction context (Context B). * $p < 0.05$ ChR2 vs eYFP in Context A. # $p < 0.05$ Context A vs. Context B in the eYFP group. **(H)** Δ CS port entries across trials at tests in Context A for renewal and the extinction context, Context B. * $p < 0.05$ ChR2 vs. eYFP in Context A across trials. # $p < 0.05$ Context A vs. Context B in the eYFP group across trials. **(I)** ITI port entries during tests in Context A and Context B. # $p < 0.05$ main effect of context. **(J)** Probability **(K)** duration and **(L)** latency of CS port entries during the renewal test in Context A relative to test in the extinction context, Context B. **(L)** Dashed line indicates duration of the CS and maximum latency. **(J-L)** * $p < 0.05$ ChR2 vs eYFP in Context A. # $p < 0.05$ Context A vs. Context B in the eYFP group. All data are mean ± SEM.

IL-to-NAcS stimulation induced Fos reactivity in the IL and NAcS

Fos immunohistochemistry was conducted on a subset of rats from experiment 2 (ChR2 n = 9, eYFP n = 8) to verify that optogenetic stimulation of the IL-to-NAcS projection activated the IL and the NAcS (Figure 3A). In the IL (Figure 3B, left), Fos immunoreactivity was greater in rats expressing ChR2 than eYFP alone (Figure 3C, left; Virus, $F(1,15) = 40.70$, $p < .001$) and in the stimulated hemisphere relative to the non-infected, non-stimulated, control hemisphere (Hemisphere, $F(1,15) = 9.50$, $p = .008$). Density of Fos positive nuclei in the IL showed a statistically significant interaction between virus and hemisphere (Hemisphere x Virus, $F(1,15) = 9.68$, $p = .007$). The stimulated hemisphere had greater Fos immunoreactivity than the non-stimulated hemisphere in the ChR2 group ($p < .001$) but not in the eYFP group ($p = .984$). The ChR2 group had greater Fos density than the eYFP group in both the stimulated hemisphere ($p < .001$) and the non-stimulated hemisphere ($p = .011$). These results indicate that optogenetic stimulation of IL neuron terminals in the NAcS activated the IL. Optogenetic stimulation of the ChR2-transfected hemisphere also activated the opposite, non-stimulated hemisphere.

In the NAcS (Figure 3B, right), Fos immunoreactivity was greater in rats expressing ChR2 than eYFP alone (Figure 3C, right; Virus, $F(1,15) = 20.26$, $p < .001$) and in the stimulated hemisphere relative to the non-stimulated hemisphere (Hemisphere, $F(1,15) = 27.39$, $p < .001$). A statistically significant interaction was observed in Fos density in the NAcS (Hemisphere x Virus, $F(1,15) = 17.60$, $p = .001$). Greater Fos immunoreactivity was observed in the stimulated hemisphere relative to the non-stimulated hemisphere in the ChR2 group ($p < .001$), but not in the eYFP group ($p = .486$). Fos density in the ChR2 group was also greater than the eYFP group in both the stimulated ($p < .001$) and non-stimulated hemisphere ($p = .026$). Therefore, optogenetic stimulation of IL neuron terminals in the NAcS activated the NAcS. Similar to the IL, optogenetic stimulation of the ChR2-transfected hemisphere also induced moderate activation in the non-stimulated hemisphere of the NAcS.



Chapter 4, Figure 3. Quantification of Fos positive nuclei density following optogenetic stimulation of the IL-to-NAcS projection. **(A)** Schematic depicting demarcations in the images of the IL (left panel) and NAcS (right panel) quantified for Fos positive nuclei. **(B)** Representative images of Fos positive nuclei in the IL (left panel) and NAcS (right panel) in the stimulated and non-stimulated hemisphere of rats expressing ChR2 or eYFP alone. Scale bars 100 μm. **(C)** Density of Fos positive nuclei (mean ± SEM) in the IL (left graph) and the NAcS (right graph) in rats expressing ChR2 or eYFP alone in both the stimulated hemisphere containing the optical fiber and in the non-stimulated hemisphere without an optical fiber. * $p < 0.05$ ChR2 vs. eYFP in each hemisphere. # $p < 0.05$ stimulated vs. non-stimulated hemisphere in the ChR2 group.

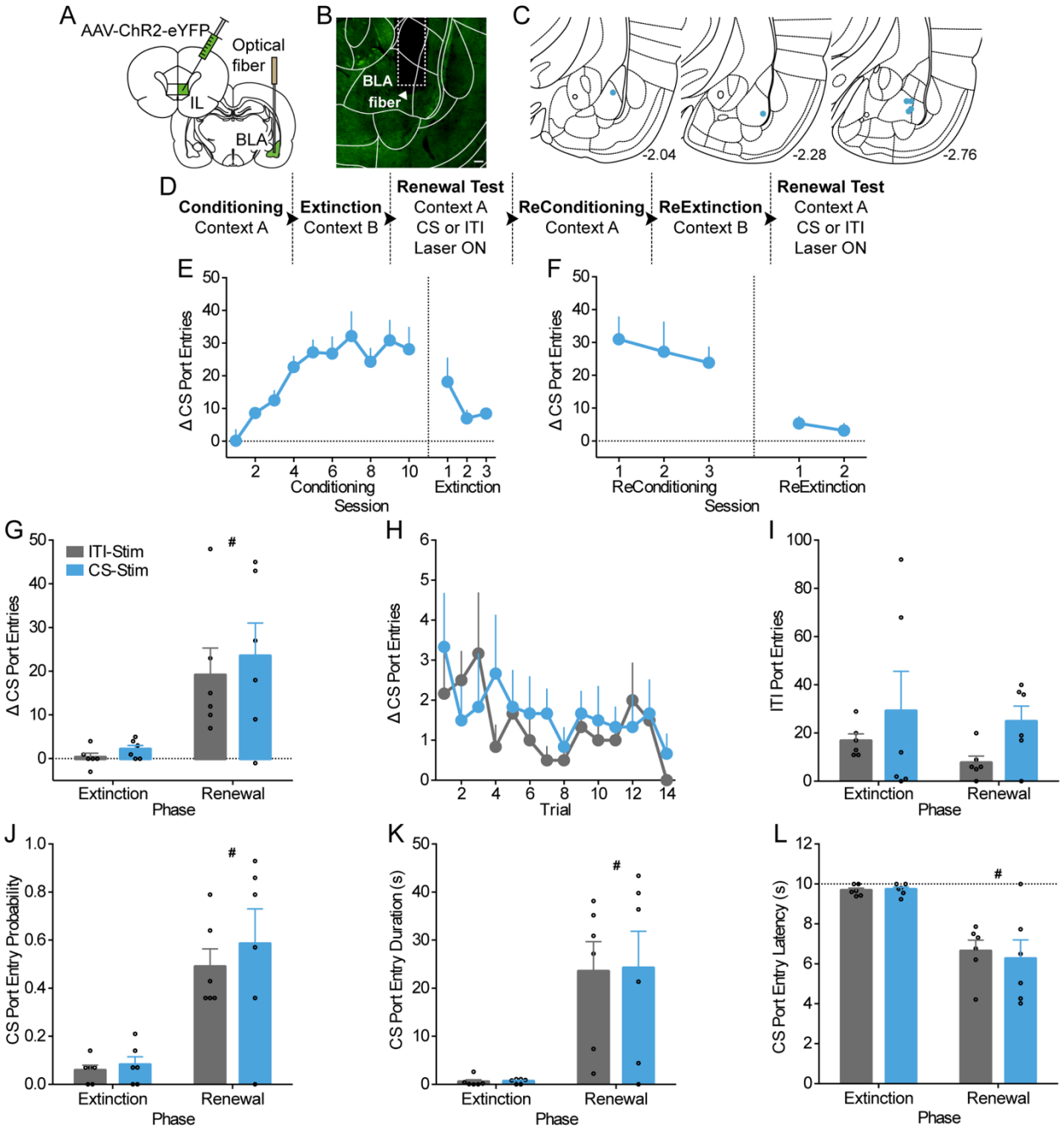
IL-to-BLA stimulation did not affect renewal of appetitive Pavlovian conditioned responding

Experiment 3 tested whether optogenetic stimulation of the IL-to-BLA projection would affect renewal of appetitive Pavlovian conditioned responding (Figure 4). Δ CS port entries increased during conditioning in Context A (Figure 4E; Session, $F(9,45) = 9.08$, $p < .001$), was lower during extinction in Context B (Session, $F(2,10) = 1.58$, $p = .254$) and remained low in the last extinction session prior to the first renewal test. Δ CS port entries remained high across three reconditioning sessions (Figure 4F; Session, $F(2,10) = 1.14$, $p = .359$), was lower during the re-extinction sessions (Session, $F(1,5) = .54$, $p = .494$) and was maintained at low levels in the last extinction session prior to the second renewal test. In sum, rats acquired both appetitive Pavlovian conditioning and extinction.

Optogenetic stimulation of the IL-to-BLA projection during CS presentations did not affect renewal of appetitive Pavlovian conditioned responding (Figure 4G). Rats exhibited renewal with greater Δ CS port entries at test relative to the last extinction session regardless of whether optogenetic stimulation of the IL-to-BLA occurred during the CS or in the ITI (Phase, $F(1,5) = 10.94$, $p = .021$; Stimulation, $F(1,5) = .81$, $p = .409$; Phase x Stimulation, $F(1,5) = .114$, $p = .749$). Further, Δ CS port entries across trials during the renewal tests were similar regardless of whether IL-to-BLA projection stimulation occurred during the CS or the ITI (Figure 4H; Stimulation, $F(1,5) = .38$ $p = .563$; Trial, $F(13,65) = 1.49$, $p = .224$; Stimulation x Trial, $F(13,65) = .68$, $p = .762$). Port entries during the ITI were also similar across extinction and renewal regardless of the time of IL-to-BLA stimulation (Figure 5I; Phase, $F(1,5) = 1.02$, $p = .359$; Stimulation, $F(1,5) = 2.94$, $p = .147$; Phase x Stimulation, $F(1,5) = .110$, $p = .754$).

Additional measures of conditioned responding further support that IL-to-BLA stimulation did not affect renewal of appetitive Pavlovian conditioned responding. Probability (Figure 4J; Phase, $F(1,5) = 29.48$, $p = .003$; Stimulation, $F(1,5) = .44$, $p = .538$; Phase x Stimulation, $F(1,5) = .27$, $p = .627$) and duration (Figure 4K; Phase, $F(1,5) = 46.72$, $p = .001$; Stimulation, $F(1,5) = .01$, $p = .948$; Phase x Stimulation, $F(1,5) = .003$, $p = .962$) of CS port entries were greater in the renewal test compared to the last extinction session regardless of whether IL-to-BLA stimulation occurred during the CS or the ITI at test. Lastly, latency to initiate a CS port entry decreased from last session of extinction compared to the renewal test and was not affected by IL-to-BLA stimulation during the CS or the ITI (Figure 4L; Phase, $F(1,5) = 30.35$, $p = .003$; Stimulation, $F(1,5) = .08$, $p = .786$; Phase x Stimulation, $F(1,5) = .25$, $p = .642$).

Altogether, optogenetic stimulation of the IL-to-BLA projection during CS presentations did not affect the renewal of appetitive Pavlovian conditioned responding.



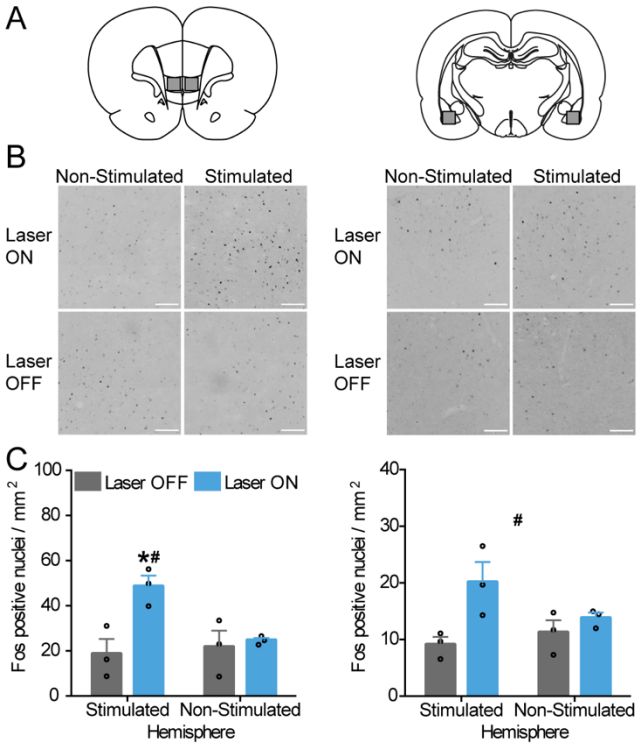
Chapter 4, Figure 4. Optogenetic stimulation of the IL-to-BLA projection did not affect renewal of appetitive Pavlovian conditioned responding. **(A)** Method schematic of microinjections of ChR2 in the IL and an optical fiber implanted in the BLA. **(B)** Representative image depicting ChR2 expression in IL terminals and the optical fiber within the BLA. Scale bar 100 µm. **(C)** Optical fiber placements in the BLA for ChR2 expressing rats included in the final data analysis. Numbers are locations relative to bregma. **(D)** Design of behavioural procedures. **(E-F)** Δ CS port entries during conditioning and reconditioning in Context A and extinction and re-extinction in Context B. **(G)** Δ CS port entries during the last session of extinction in Context B and the renewal tests in Context A with optogenetic

stimulation delivered either during the CS or the middle of the ITI. **(H)** Δ CS port entries across trials during the renewal tests. **(I)** ITI port entries during the last session of extinction and the renewal tests. **(J)** Probability, **(K)** duration, and **(L)** latency of CS port entries during the last session of extinction and the renewal test. # $p < 0.05$ main effect of phase. All data are mean ± SEM.

IL-to-BLA stimulation induced Fos reactivity in the IL and BLA

Fos immunohistochemistry was conducted to verify that optogenetic stimulation of the IL-to-BLA projection activated the IL and the BLA (Figure 5A). Density of Fos positive nuclei in the IL (Figure 5B, left) showed a statistically significant interaction between laser stimulation and hemisphere (Figure 5C, left; Hemisphere, $F(1,4) = 20.38$, $p = .011$; Stimulation, $F(1,4) = 5.00$, $p = .089$; Hemisphere x Stimulation, $F(1,4) = 34.09$, $p = .004$). Rats that received laser stimulation (Laser ON) had greater Fos immunoreactivity than rats that did not receive laser stimulation (Laser OFF) in the stimulated hemisphere containing the optical fiber ($p = .021$) but not the non-stimulated hemisphere ($p = .708$). The stimulated hemisphere containing the optical fiber had greater Fos immunoreactivity than the non-stimulated hemisphere in rats received laser stimulation ($p = .002$) but not in rats that did not receive laser stimulation ($p = .402$).

In the BLA (Figure 5B, right), density of Fos positive nuclei was greater in rats that received laser stimulation relative to rats that did not receive laser stimulation regardless of hemisphere (Figure 5C, right; Hemisphere, $F(1,4) = .96$, $p = .384$; Stimulation, $F(1,4) = 8.73$, $p = .042$; Hemisphere x Stimulation, $F(1,4) = 3.88$, $p = .120$). Overall, these results indicate that optogenetic stimulation of IL terminals in the BLA activated both the IL and BLA.



Chapter 4, Figure 5. Quantification of Fos positive nuclei density following optogenetic stimulation of the IL-to-BLA projection. **(A)** Schematic depicting demarcations in the images of the IL (left panel) and BLA (right panel) quantified for Fos positive nuclei. **(B)** Representative images of Fos positive nuclei in the IL (left panel) and BLA (right panel) in the stimulated and non-stimulated hemisphere of ChR2 expressing rats that received laser stimulation (Laser ON) or no laser stimulation (Laser OFF). Scale bars 100 μm. **(C)** Density of Fos positive nuclei (mean ± SEM) in the IL (left graph) and the BLA (right graph) in rats that received laser stimulation or no laser stimulation in both the stimulated hemisphere containing the optical fiber and in the non-stimulated hemisphere without an optical fiber. Left graph, * $p < 0.05$ laser ON vs. laser OFF in the stimulated hemisphere. # $p < 0.05$ stimulated vs. non-stimulated hemisphere in the laser ON group. Right graph, # $p < 0.05$ main effect of laser stimulation.

Discussion

The experiments in Chapter 4 investigated the role of the IL-to-NAcS and IL-to-BLA neural projections in the extinction of appetitive Pavlovian conditioned responding using a renewal procedure. In experiment 1, IL projections to the NAcS and BLA were found to be composed of distinct neural subpopulations, with only a small proportion of neurons in the IL projecting to both regions. In experiment 2, optogenetic stimulation of IL terminals in the NAcS during CS presentations suppressed the renewal of appetitive Pavlovian responding in the original conditioning context (Context A) after extinction in a different context (Context B). However, optogenetic stimulation of the IL-to-NAcS projection during the CS did not affect responding at test in the extinction context or outside of the CS. In experiment 3, optogenetic stimulation of the IL-to-BLA projection either during the CS or during the middle of the ITI did not affect renewal. Together, the results of the present chapter show that augmenting activity in the IL-to-NAcS, but not the IL-to-BLA, suppresses the renewal of appetitive Pavlovian conditioned responding.

The IL sends dense glutamatergic projections to different limbic brain regions involved in mediating motivated behaviours. The IL projection to the NAcS is thought to be important for inhibiting responding to appetitive stimuli, particularly cocaine, whereas the IL projection to the BLA is thought to contribute to extinction of responding to aversive Pavlovian cues (Peters et al., 2009). The extinction of responding to an aversive CS increases the excitability of IL neurons that project to the BLA (Bloodgood et al., 2018). In contrast, IL projections to the NAcS are recruited during extinction of operant cocaine-seeking (Warren et al., 2019). In experiment 1 of Chapter 4, IL projections to the NAcS and the BLA were found to consist of different neuronal sub-populations. Specifically, retrograde tracing revealed that different neurons in the IL projected to the NAcS and the BLA with minimal overlap. This finding is consistent with tracing studies of the same neuronal projections in mice (Bloodgood et al., 2018). Therefore, the findings of experiment 1 provide consilience across rodent species that IL projections to the NAcS and BLA largely consists of different neuronal populations. These distinct neuronal populations in the IL that project to the NAcS and BLA may allude to the different functions of these projections in suppressing responding to appetitive and aversive stimuli.

In experiment 2, we found that optogenetic stimulation of the IL-to-NAcS projection suppressed renewal of appetitive Pavlovian conditioned responding. Suppression of conditioned

responding following IL-to-NAcS stimulation was evident across all CS presentations during the renewal test in Context A. Therefore, suppression did not seem to be a result of facilitating extinction within the test. Further, suppression of renewal was evident across different measures of conditioned responding such as probability, duration, and latency of CS port entries. These results are consistent with the prevailing view that the IL and its projection to the NAcS is critical for extinction of operant cocaine-seeking (Peters et al., 2008; 2009; LaLumiere et al., 2012; Augur et al., 2016; Gutman et al., 2017). For example, concurrent pharmacological inactivation of the IL and the NAcS induced a reinstatement of extinguished cocaine-seeking (Peters et al., 2008) whereas chemogenetic activation of the IL-to-NAcS projection suppressed cue-induced cocaine-seeking after extinction (Augur et al., 2016). Our results extend these findings to appetitive Pavlovian responses using sucrose, a more natural reinforcer. Therefore, the suppression of Pavlovian responding to appetitive cues and operant cocaine-seeking after extinction may be mediated by a common neural substrate.

In contrast, in experiment 3, optogenetic stimulation of the IL-to-BLA projection during the CS did not affect renewal of appetitive Pavlovian responding. These findings are consistent with a proposed dichotomy that the IL-to-NAcS is involved in extinction of appetitive conditioned responding, while the IL-to-BLA mediates extinction of aversive conditioned responding (Peters et al., 2009). In accordance with these findings, others have reported that chemogenetic inhibition of the IL-to-BLA projection impairs extinction retrieval in aversive conditioning (Bloodgood et al., 2018). Here, we provide additional support for the functional dichotomy between IL projections to the NAcS and the BLA in suppressing conditioned responding to stimuli of different affective valence using a similar renewal procedure across experiments. However, the present results do not preclude the IL-to-BLA from playing a role in appetitive conditioned responding. An important factor to consider is the phase of extinction in which the IL-to-BLA projection may be recruited. In aversive conditioned procedures, disrupting glutamatergic transmission in the BLA often impairs initial extinction learning (Sotres-Bayon et al., 2007; Laurent et al., 2008). Therefore, it is possible that the IL-to-BLA projection may be involved in extinction of appetitive Pavlovian conditioned responding during initial extinction learning rather than during retrieval as in the renewal test.

The IL-to-NAcS and IL-to-BLA renewal experiments used slightly different procedures, which could limit a direct comparison of results. The IL-to-NAcS renewal experiment used a mixed-subjects design that included an eYFP control group. In contrast, the IL-to-BLA renewal experiment consisted of a within-subject design composed of a ChR2 group. However, the procedure used in the IL-to-BLA experiment in which optogenetic activation during the ITI served as a control has been previously used effectively to detect changes in renewal following neural manipulations (Chapter 3; Villaruel et al., 2018). Specifically, we have previously shown that activation of the IL during the CS but not the ITI suppressed the renewal of appetitive Pavlovian responding (Chapter 3; Villaruel et al., 2018). Therefore, it is unlikely that the lack of effect on renewal following stimulation of the IL-to-BLA projection is due to a difference in behavioural procedures.

Another technical consideration is the use of unilateral stimulation of the IL-to-NAcS and the IL-to-BLA projection. However, we have previously shown that unilateral optogenetic stimulation produces similar behavioural effects as bilateral pharmacological activation (Villaruel et al., 2018). Further, our Fos analysis indicated that optogenetic stimulation of the IL-to-NAcS and IL-to-BLA may have induced activation in both hemispheres, even in the hemisphere without the optical fiber. This result may be due to bilateral projections from the IL to target regions and contralateral projections between hemispheres in the IL (Hurley et al., 1991; Vertes, 2004). However, in both experiments Fos activation was greater in the hemisphere transfected with ChR2 and implanted with the optical fiber, suggesting that the IL-to-NAcS and IL-to-BLA projections are predominantly ipsilateral. Optogenetic stimulation of IL terminals in the NAcS and BLA also induced Fos in the IL, suggesting that back-propagation of action potentials may have occurred. However, if the suppression of renewal from the IL-to-NAcS experiment was only due to back-propagated activation of the IL, activation of the IL-to-BLA projection should have also attenuated renewal. We failed to see a behavioural effect on renewal following IL-to-BLA activation, making it unlikely that the suppression of renewal following IL-to-NAcS stimulation is simply due to stimulation of the IL.

In conclusion, the present chapter found that optogenetic stimulation of the IL-to-NAcS, but not the IL-to-BLA projection suppressed the renewal of appetitive Pavlovian conditioned responding. These findings extend previous research on the role of the IL-to-NAcS in suppressing operant cocaine-seeking to Pavlovian responding to a sucrose cue. Further, the

findings lend support to the proposed dichotomy between the IL-to-NAcS and IL-to-BLA in mediating suppression of conditioned responding to appetitive and aversive stimuli, respectively. Importantly, optogenetic stimulation of the IL-to-NAcS but not the IL-to-BLA suppressed renewal when tested in a similar appetitive Pavlovian conditioning task. In sum, the findings from Chapter 4 highlight that IL inputs to the NAcS are especially important for suppressing renewal and extends this role to Pavlovian conditioned responses for a natural reinforcer like sucrose.

Chapter 5 - Exploring mechanisms of suppression following optogenetic stimulation of the IL-to-NAcS projection

Introduction

Extinction is thought to involve the formation of a new inhibitory memory (Konorski, 1948; 1967; Mackintosh, 1975; Pearce and Hall 1980). The IL and NAcS may mediate response suppression by promoting the retrieval of this inhibitory extinction memory (Peters et al., 2009). This hypothesis is supported by data in both aversive Pavlovian conditioning and appetitive operant conditioning procedures. However, data is lacking in appetitive Pavlovian conditioning. IL activity increases to an aversive CS after extinction training (Milad and Quirk, 2002) and extinction of cocaine-seeking is associated with an increase in glutamatergic receptors in the NAcS (Sutton et al., 2003). Further, extinction of cocaine-seeking recruit distinct neural ensembles in the IL that predominantly project to the NAcS (Warren et al., 2016; 2019). Together, these studies indicate that extinction engages the IL and NAcS. Some studies report that prior extinction is necessary for IL (Müller-Ewald et al., 2018) and IL-to-NAcS projection (Augur et al., 2016) stimulation to suppress cocaine-seeking. These results suggest that stimulating IL-to-NAcS promotes the expression of an inhibitory extinction memory to suppress responding. However, optogenetic stimulation of the IL and the IL-to-NAcS projection have also been shown to suppress operant food-seeking and cocaine-seeking without prior extinction training (Do Monte et al., 2015; Cameron et al., 2019). Therefore, whether suppression is achieved by promoting the expression of an inhibitory extinction memory remains unclear.

Augmenting IL and NAcS activity during extinction is conducive for subsequent extinction retrieval. In aversive Pavlovian conditioning, pharmacological, electrical, and optogenetic stimulation of the IL during extinction consistently facilitated retrieval, leading to further reductions in conditioned freezing (Milad and Quirk, 2002; Milad et al., 2004; Vidal-Gonzalez et al., 2006; Kim et al., 2010; Thompson et al., 2010; Peters et al., 2010; Do Monte et al., 2015; Lingawi et al., 2016; Lingawi et al., 2018). In drug-seeking, optogenetic stimulation of the IL during extinction of conditioned place preference also facilitated extinction retrieval (Van den Oever, 2013). Further, administering glutamatergic agonists in the IL attenuated cue-induced cocaine-seeking but is reversed by glutamatergic antagonists in the NAcS (LaLumiere et al., 2012). Consistently, electrical stimulation of the NAcS promoted extinction of cocaine-seeking

(Vassoler et al., 2008). These results suggest that the IL may activate the NAcS to promote extinction. Lastly, our results from the previous chapter also indicate that augmenting IL-to-NAcS activity during CS presentations can suppress renewal. Together, these findings suggest that increasing IL and NAcS activity can strengthen extinction learning and promote extinction retrieval. However, it remains to be tested whether increasing IL-to-NAcS activity specifically during extinction training would strengthen extinction learning and retrieval in an appetitive Pavlovian conditioning procedure.

The IL and NAcS are also implicated in response suppression beyond extinction. The IL can encode inhibitory memory acquired through non-reinforced presentations of the CS prior to conditioning (i.e. latent inhibition) and non-contingent or unpaired presentations of the CS and US (Lingawi et al., 2016; 2018). Pharmacological stimulation of the IL can strengthen the inhibitory memory acquired through these different procedures to facilitate extinction with an aversive CS (Lingawi et al., 2016; 2018). These results indicate that inhibitory memory established through different means other than extinction can also be strengthened by enhancing IL activity. Furthermore, pharmacological inactivation of the IL and NAcS produce similar effects of disinhibiting responding to stimuli, time windows, and responses that signal non-reinforcement (Peters et al., 2008a; Chaudhri et al., 2008; Ambroggi et al., 2011; Ghazizadeh et al., 2012). Moreover, pharmacological inactivation of the IL alters the activity of neurons in the NAcS that respond to non-reinforced behaviours and stimuli (Ghazizadeh et al., 2012). Therefore, the role of the NAcS in response suppression may be linked to inputs from the IL. In support of this idea, pharmacological disconnection of the IL and NAcS produces similar disinhibition of inappropriate responding and disrupts the capacity for a Pavlovian cue to invigorate operant responding for sucrose (Keistler et al., 2015). Together, these studies suggest that the IL and NAcS may work in concert to mediate response suppression and coordinating operant responding to Pavlovian stimuli. However, explicit evidence for the role of the IL-to-NAcS projection in suppression of appetitive Pavlovian conditioned responses is lacking.

Chapter 5 investigates potential mechanisms that may lead to the suppression of appetitive Pavlovian conditioned responses following optogenetic stimulation of the IL-to-NAcS neural projection. In experiment 1, we adapted the experimental design from an aversive Pavlovian conditioning procedure (Lingawi et al., 2016) to test whether IL-to-NAcS stimulation would suppress appetitive Pavlovian conditioned responding by promoting the expression of an

inhibitory extinction memory. Specifically, following Pavlovian conditioning in which a CS was paired with sucrose, rats either received extinction training to establish an inhibitory memory or no extinction training. After a single re-conditioning session to establish baseline, we conducted an extinction test during which IL-to-NAcS stimulation was delivered during the CS. Approximately 24 h later, we conducted a subsequent test to determine whether IL-to-NAcS stimulation during extinction would facilitate subsequent extinction retrieval. In experiment 2, we tested whether optogenetic stimulation of the IL-to-NAcS leads to response suppression by delivering the stimulation during Pavlovian conditioning. Furthermore, we tested the expression of appetitive Pavlovian conditioned responding in the presence and absence of stimulation to investigate how IL-to-NAcS stimulation during Pavlovian conditioning may have altered conditioned responding. We predicted that optogenetic stimulation of the IL-to-NAcS projection would suppress appetitive Pavlovian conditioned responding by promoting the expression of an inhibitory extinction memory. Therefore, in experiment 1, IL-to-NAcS stimulation should suppress conditioned responding and facilitate extinction retrieval only in rats with previous extinction training. Congruently, in experiment 2, we predicted that IL-to-NAcS stimulation would not suppress conditioned responding during Pavlovian conditioning or during the expression test.

Methods

Subjects

Seventy, male, Long-Evans rats were used in the experiments in Chapter 5. Experiment 1 tested whether prior extinction training is necessary for IL-to-NAcS stimulation to suppress appetitive Pavlovian conditioned responding (ChR2, n = 26; eYFP, n = 22). Experiment 2 tested whether IL-to-NAcS stimulation indiscriminately suppresses behaviour during Pavlovian conditioning (ChR2, n = 11; eYFP, n = 11).

Behavioural Procedures

Experiment 1 was conducted in two replicates. Following habituation, rats received 10 daily sessions of Pavlovian conditioning. Next, rats were divided into either an Extinction (Ext) or No Extinction group (No Ext) matched on acquisition of Pavlovian conditioning and CS port entries in the last conditioning session. Rats in the Extinction group (ChR2 n = 13; eYFP n = 11)

received one extinction session 24 h after the last conditioning session. In contrast, rats in the No Extinction group (ChR2 n = 13; eYFP n = 11) did not receive extinction training and were instead handled and weighed in the colony room. The following day, all rats underwent a Pavlovian re-conditioning session to re-establish baseline responding. An extinction test (Test 1) was conducted the following day which was identical to an extinction session but with optogenetic stimulation delivered during the CS. An extinction retrieval test (Test 2) was conducted the next day and was identical to an extinction session without optogenetic stimulation.

In experiment 2, following habituation, rats (ChR2 n = 11, eYFP n =11) received 12 daily sessions of Pavlovian conditioning as previously described, but optogenetic stimulation was delivered during the CS. Following Pavlovian conditioning, extinction tests for expression of conditioned responding to the CS alone were conducted across two sessions approximately 24 h apart from one another. Tests were similar to Pavlovian conditioning, but the sucrose US was withheld. In one test optogenetic stimulation was present during the CS and in the other test, stimulation was withheld. Test order was counterbalanced across rats and rats were matched based on acquisition of Pavlovian conditioning measured by Δ CS port entries.

Results

Histology

In experiment 1, rats were excluded from analysis due to misplacement of the optical fiber (ChR2 n = 1, eYFP n = 1) and lack of transgene expression (eYFP n = 1). Final group sizes in Experiment 1 were ChR2-Ext n = 13, eYFP-Ext n = 10, ChR2-NoExt n = 12, eYFP-No Ext n =10). No rats were excluded from the final data analyses in Experiment 2. Final group size in Experiment 2 was ChR2 n = 11, eYFP n =11.

IL-to-NAcS stimulation suppressed appetitive Pavlovian conditioned responding regardless of prior extinction

Experiment 1 tested whether prior extinction training and the establishment of an inhibitory extinction memory were necessary for optogenetic stimulation of the IL-to-NAcS to suppress appetitive Pavlovian conditioned responding. All groups displayed an equivalent increase in Δ

CS port entries across Pavlovian conditioning sessions (Figure 1C; Session, $F(4,161) = 41.52$, $p = .001$) with no effect of virus (Virus, $F(1,41) = 1.90$, $p = .176$) or extinction group (Group, $F(1,41) = .14$, $p = .708$) and no statistically significant interactions (Session x Virus, $F(9,369) = 1.17$, $p = .326$; Session × Group, $F(9,369) = .48$, $p = .780$; Virus × Group, $F(1,41) = .03$, $p = .872$; Session × Virus × Group, $F(9,369) = 1.06$, $p = .380$). Δ CS port entries were equal between ChR2 Extinction and eYFP Extinction groups during the extinction session (Virus, $F(1,21) = .19$, $p = .670$). The ChR2 Extinction and eYFP Extinction groups had similar within-session reduction of Δ CS port entries across trials during the extinction session (Figure 1D; Trial, $F(13,273) = 7.30$, $p < .001$; Virus, $F(1,21) = .19$, $p = .670$; Trial x Virus, $F(13,273) = .66$, $p = .760$). Δ CS port entries were equivalent for all virus and extinction groups during the Pavlovian reconditioning session to re-establish baseline responding (Virus, $F(1,41) = .02$, $p = .881$; Group, $F(1,41) = .18$, $p = .675$; Virus x Group, $F(1,41) = .67$, $p = .417$). In sum, groups did not differ during Pavlovian conditioning, extinction, and re-conditioning.

In Test 1, optogenetic stimulation of the IL-to-NAcS projection during CS presentations suppressed Δ CS port entries in the ChR2 groups relative to the eYFP groups regardless of prior extinction training (Figure 1E; Virus, $F(1,41) = 18.32$, $p < .001$; Group, $F(1,41) = 3.80$, $p = .058$; Virus x Group, $F(1,41) = .02$, $p = .890$). Δ CS port entries decreased across trials within the test but were overall greater in the eYFP groups relative to the ChR2 groups (Figure 1F; Trial, $F(13,533) = 10.71$, $p < .001$; Virus, $F(1,41) = 18.32$, $p < .001$; Group, $F(1,41) = 3.80$, $p = .058$; Virus x Group, $F(1,41) = .02$, $p = .890$). There was no statistically significant interaction between trial, virus, and group (Trial x Virus, $F(13,533) = 1.66$, $p = .109$; Trial x Group, $F(13,533) = 1.04$, $p = .404$, Trial x Virus x Group, $F(13,533) = 1.45$, $p = .179$). ITI port entries was equivalent across all groups during Test 1 (Data not shown; Virus, $F(1,41) = .91$, $p = .346$; Group, $F(1,41) = .05$, $p = .829$; Virus x Group, $F(1,41) = .18$, $p = .671$). Therefore, IL-to-NAcS stimulation attenuated Δ CS port entries in the ChR2 group regardless of prior extinction training and did so from the very first trial.

Additional measures during Test 1 further support that IL-to-NAcS stimulation during CS presentations suppressed conditioned responding regardless of extinction (Figure 1I-K, left). Probability of CS port entries were attenuated in the ChR2 groups relative to the eYFP groups regardless of prior extinction training (Figure 1I, left; Virus, $F(1,41) = 17.08$, $p < .001$; Group, $F(1,41) = 1.61$, $p = .211$; Virus x Group, $F(1,41) = .002$, $p = .961$). Duration of CS port entries

were lower in ChR2 groups relative to the eYFP groups (Figure 1J, left; Virus, $F(1,41) = 36.26$, $p < .001$) and were lower in the Extinction groups relative to the No Extinction groups (Group, $F(1,41) = 9.15$, $p = .004$) with no statistically significant interaction (Virus x Group, $F(1,41) = .69$, $p = .412$). Lastly, latency to initiate a CS port entry was greater in the ChR2 group relative to the eYFP group regardless of prior extinction training (Figure 1K, left; Virus, $F(1,41) = 7.19$, $p = .011$; Group, $F(1,41) = .86$, $p = .360$; Virus x Group, $F(1,41) = .21$, $p = .651$). Together, optogenetic stimulation of the IL-to-NAcS projection suppressed appetitive Pavlovian conditioned responding during Test 1 regardless of prior extinction training.

IL-to-NAcS stimulation during extinction did not facilitate extinction retrieval

We conducted another extinction session (Test 2) the following day in the absence of optogenetic stimulation to determine if prior stimulation of the IL-to-NAcS projection during extinction training would facilitate extinction retrieval of appetitive Pavlovian responding. This prediction was based on findings in aversive Pavlovian conditioning studies in which stimulation of the IL during extinction facilitated subsequent extinction retrieval (Milad and Quirk, 2002; Milad et al., 2004; Do Monte et al., 2015; Lingawi et al., 2016; 2018).

In Test 2, Δ CS port entries were lower in rats that received prior extinction training relative to the No Extinction group (Figure 1G; Group, $F(1,41) = 8.71$, $p = .005$) with no differences between ChR2 and eYFP groups (Virus, $F(1,41) = 1.75$, $p = .193$). There was a near significant interaction (Virus x Group, $F(1,41) = 3.24$, $p = .079$), supporting a possible effect of prior IL-to-NAcS stimulation during extinction training on extinction retrieval. Exploratory simple effect comparisons showed a significant difference between ChR2 and eYFP groups within the Extinction group ($F(1,41) = 4.97$, $p = .031$) but not in the No Extinction group ($F(1,41) = .11$, $p = .740$). Visual inspection of Figure 1G showed that within the Extinction group, Δ CS port entries were higher in the ChR2 group than in the eYFP group, suggesting impaired extinction retrieval in the ChR2 Extinction group.

Δ CS port entries decreased across trials (Figure 1H; Trial, $F(13,533) = 9.06$, $p < .001$) but was greater in the No Extinction group relative to the Extinction group (Group, $F(1,41) = 11.33$, $p = .002$) with no effect of virus (Virus, $F(1,41) = 1.63$, $p = .209$, Virus x Group, $F(1,41) = 2.61$, $p = .114$). This effect was largely mediated by the difference in the eYFP Extinction and eYFP No Extinction groups, as both ChR2 groups performed similarly across trials. There were no

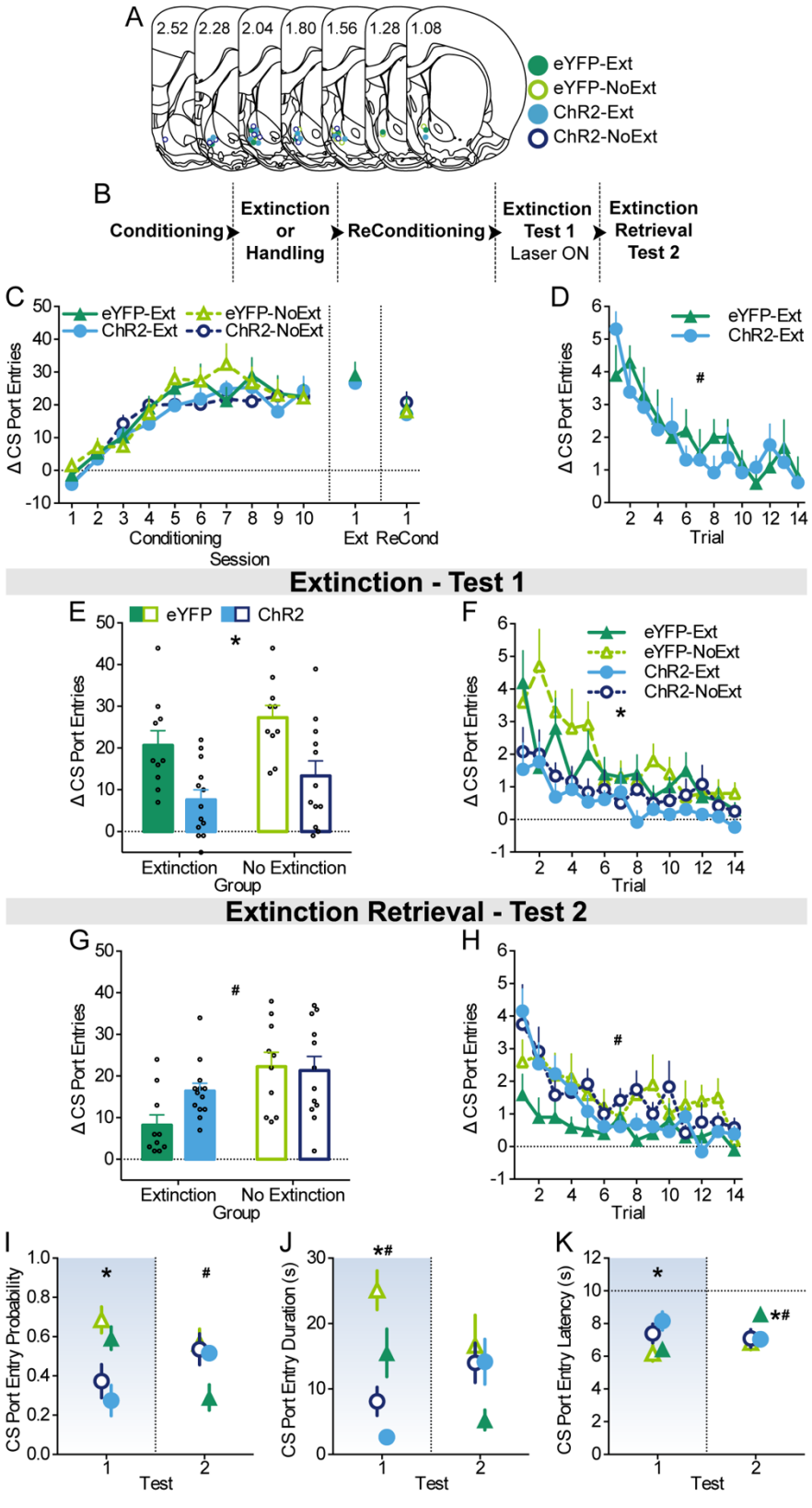
statistically significant interactions between trial, virus, and group (Trial x Virus, $F(13,533) = 1.55$, $p = .122$; Trial x Group, $F(13,533) = .50$, $p = .888$; Trial x Virus x Group, $F(13,533) = .92$, $p = .517$). Exploratory analysis comparing ChR2 and eYFP Extinction groups alone revealed that the ChR2 group made significantly more Δ CS port entries than the eYFP group at Test 2 (Virus, $F(1,21) = 7.19$, $p = .014$). Δ CS port entries decreased across Trial ($F(13,273) = 7.22$, $p < .001$), with a significant Trial x Virus interaction ($F(13,273) = 2.31$, $p = .020$). Additional post-hoc analysis indicates that within the Extinction group, Δ CS port entries were higher in the ChR2 group than in the eYFP group in Trial 1 (ChR2 vs eYFP, $p = .013$). Therefore, analysis of Δ CS port entries across trials support that stimulation during extinction training did not facilitate but perhaps impaired extinction retrieval in the ChR2 Extinction group.

ITI port entries in Test 2 were equivalent across all groups during extinction retrieval and were not affected by prior stimulation of the IL-to-NAcS during extinction training (Data not shown; Virus, $F(1,41) = .06$, $p = .803$; Group, $F(1,41) = .28$, $p = .597$; Virus x Group, $F(1,41) = 1.34$, $p = .254$).

Additional measures of conditioned responding, indicate that prior optogenetic stimulation of the IL-to-NAcS during extinction training did not facilitate extinction retrieval in the Extinction or No Extinction groups (Figure 1I-K, right). Probability of CS port entries during extinction retrieval was lower in Extinction groups relative to the No Extinction groups (Figure 1I, right; Virus, $F(1,41) = 2.20$, $p = .145$; Group, $F(1,41) = 4.89$, $p = .033$; Virus x Group, $F(1,41) = 3.68$, $p = .062$). Visual inspection suggests that this effect was largely been driven by lower probability of CS port entries in eYFP Extinction group compared to the eYFP No Extinction group. The ChR2 Extinction and ChR2 No Extinction groups had similar probability of CS port entries during extinction retrieval. Duration of CS port entries was similar across all groups during extinction retrieval (Figure 1J, right; Virus, $F(1,41) = .86$, $p = .358$; Group, $F(1,41) = 2.73$, $p = .106$; Virus x Group, $F(1,41) = 2.88$, $p = .097$). Visual inspection indicates, however, that the eYFP Extinction group had lower CS port entry durations than the eYFP No Extinction group whereas the ChR2 Extinction and ChR2 No Extinction groups had similar duration of CS port entries during extinction retrieval. Within the Extinction group, the ChR2 group showed higher duration of CS port entries relative to the eYFP group. IL-to-NAcS stimulation during extinction impaired subsequent extinction retrieval as measured by average latency to initiate a CS port entry in rats that previously received extinction training (Figure 1K, right; Virus, $F(1,41)$

= 2.19, p = .146; Group, F(1,41) = 3.83, p = .057; Virus x Group, F(1,41) = 4.22, p = .046). The eYFP Extinction group had longer CS port entry latency than the eYFP No Extinction group during extinction retrieval (p = .010). CS port entry latency was similar between ChR2 and eYFP No Extinction groups (p = .690) and between ChR2 Extinction and ChR2 No Extinction group (p = .943). However, within the Extinction groups, the eYFP group had longer CS port entry latency relative to the ChR2 group (p = .016), suggesting that prior IL-to-NAcS stimulation during extinction impaired subsequent extinction retrieval.

Together, these results suggest that prior optogenetic stimulation of the IL-to-NAcS during extinction training did not lead to a facilitation of extinction retrieval. Lastly, exploratory analyses suggest that prior stimulation may have instead impaired extinction retrieval in rats that previously received extinction training.


A
2.52
2.28
2.04
1.80
1.56
1.28
1.08
eYFP-Ext
eYFP-NoExt
ChR2-Ext
ChR2-NoExt
B
Conditioning
Extinction or Handling
ReConditioning
Extinction Test 1 Laser ON
Extinction Retrieval Test 2
C
eYFP-Ext
eYFP-NoExt
ChR2-Ext
ChR2-NoExt
Δ CS Port Entries
Conditioning
Session
Ext
ReCond
D
eYFP-Ext
ChR2-Ext
Δ CS Port Entries
Trial
Extinction - Test 1
E
eYFP
ChR2
Δ CS Port Entries
Extinction
No Extinction
Group
F
eYFP-Ext
eYFP-NoExt
ChR2-Ext
ChR2-NoExt
Δ CS Port Entries
Trial
Extinction Retrieval - Test 2
G
Δ CS Port Entries
Extinction
No Extinction
Group
H
Δ CS Port Entries
Trial
I
CS Port Entry Probability
Test
J
CS Port Entry Duration (s)
Test
K
CS Port Entry Latency (s)
Test

Chapter 5, Figure 1. Optogenetic stimulation of the IL-to-NAcS projection suppressed Δ CS port entries regardless of prior extinction and did not facilitate extinction retrieval. **(A)** Optical fiber placements in the NAcS of rats expressing either ChR2 (blue) or eYFP alone (green) in the extinction (filled) or no extinction (open) group included in the final data analysis. Numbers are locations of sections relative to bregma. **(B)** Design of behavioural procedures. **(C)** Δ CS port entries across conditioning, extinction, and reconditioning sessions. **(D)** Δ CS port entries across trials in the extinction session of rats in the Extinction group. # $p < 0.05$ main effect of trial. **(E)** Δ CS port entries during the extinction test (Test 1) with IL-to-NAcS stimulation during the CS. **(F)** Δ CS port entries across trials during the extinction test (Test 1) with IL-to-NAcS stimulation during the CS. **(E-F)** * $p < 0.05$ main effect of virus group. **(G)** Δ CS port entries during the extinction retrieval test (Test 2) without IL-to-NAcS stimulation. **(H)** Δ CS port entries across trials during the extinction retrieval test (Test 2) without IL-to-NAcS stimulation. **(G-H)** # $p < 0.05$ main effect of extinction group. **(I)** Probability, **(J)** duration, and **(K)** latency of CS port entries during the extinction test (Test 1) with optogenetic stimulation during the CS (shaded), and the extinction retrieval test (Test 2) without optogenetic stimulation. **(I, J)** * $p < 0.05$ main effect of virus group. # $p < 0.05$ main effect of extinction group. **(K)** Test 1, * $p < 0.05$ main effect of virus group. Test 2, * $p < 0.05$ ChR2 vs. eYFP in the extinction group. # $p < 0.05$ extinction vs. no extinction in the eYFP group. Horizontal dashed line indicates duration of the CS and maximum latency. All data are mean ± SEM.

IL-to-NAcS stimulation did not prevent the acquisition of Pavlovian conditioning

Experiment 2 tested whether IL-to-NAcS stimulation during the CS would lead to general response suppression and prevent the acquisition of appetitive Pavlovian conditioning. During conditioning, the US was initiated 4 seconds after CS onset. Therefore, we analyzed the effect of IL-to-NAcS stimulation on a CS only interval consisting of the first 4 seconds after CS onset, as well as on a 6 second interval encompassing the CS and US during conditioning. A Δ CS only port entry score was calculated by subtracting port entries made 4 s before CS onset from port entries made during the 4 s CS only interval. Δ CS only port entries increased equivalently across conditioning sessions in both the ChR2 and eYFP group (Figure 2C; Session, $F(11,220) = 24.56$, $p < .001$; Virus, $F(1,20) = 2.70$, $p = .116$, Session x Virus, $F(11,220) = .65$, $p = .675$). Port entries made during the remaining 6 s combining the CS and US interval were not affected by IL-to-NAcS stimulation (Figure 2D; Session, $F(11,220) = 5.55$, $p < .001$; Virus, $F(1,20) = 3.45$, $p = .078$, Session x Virus, $F(11,220) = 1.75$, $p = .143$). The trending main effect of virus is likely due to the reduced number of port entries in sessions 6-8 of Pavlovian conditioning in the ChR2 group. Interestingly, post CS port entries (10 s interval after CS offset) were greater in the ChR2 group relative to the eYFP group during Pavlovian conditioning (Figure 2E; Session, $F(11,220) = 3.49$, $p = .007$; Virus, $F(1,20) = 16.08$, $p = .001$; Session x Virus, $F(11,220) = 1.96$, $p = .097$). Together, these data indicate that IL-to-NAcS stimulation during the CS did not prevent but altered the course of appetitive Pavlovian conditioning.

ITI port entries were similar between ChR2 and eYFP groups during Pavlovian conditioning (Figures 2I, left; Session, $F(11,220) = 14.75$, $p < .001$; Virus, $F(1,20) = 2.81$, $p = .109$; Session x Virus, $F(11,220) = 1.07$, $p = .385$). IL-to-NAcS stimulation decreased the probability (Figure 2J, left; Session, $F(11,220) = 44.51$, $p < .001$; Virus, $F(1,20) = 4.49$, $p = .047$; Session x Virus, $F(11,220) = 1.14$, $p = .342$) but did not affect the duration of CS only port entries during conditioning (Figure 2K, left; Session, $F(11,220) = 37.78$, $p < .001$; Virus, $F(1,20) = 1.87$, $p = .187$; Session x Virus, $F(11,220) = 1.53$, $p = .224$). IL-to-NAcS stimulation increased the latency (Figure 2L, left; Session, $F(11,220) = 42.18$, $p < .001$; Virus, $F(1,20) = 6.73$, $p = .017$; Session x Virus, $F(11,220) = 2.03$, $p = .083$) of CS only port entries in the ChR2 group relative to the eYFP group during conditioning. These additional measures suggest that IL-to-NAcS stimulation affected some aspects of the conditioned response.

In sum, IL-to-NAcS stimulation did not prevent acquisition of appetitive Pavlovian conditioned responding but may have affected other aspects of CS responding and increased post CS port entries.

IL-to-NAcS stimulation was required for expression of appetitive Pavlovian conditioned responding

Following conditioning rats were tested in counterbalanced order for the expression of appetitive Pavlovian conditioned responding under extinction conditions. At test, removing optogenetic stimulation abolished Δ CS port entries in the ChR2 group but not the eYFP group (Figure 2F; Test, $F(1,20) = 7.62$, $p = .012$; Virus, $F(1,20) = 2.89$, $p = .105$; Test x Virus, $F(1,20) = 11.97$, $p = .002$). The eYFP group displayed an equivalent, high number of Δ CS port entries at test in the presence (Stimulation) or absence (No Stimulation) of stimulation ($p = .626$). In contrast, the ChR2 group had more Δ CS port entries when IL-to-NAcS stimulation was present during the CS compared to when stimulation was removed ($p < .001$). In the presence of IL-to-NAcS stimulation, there was no statistically significant difference in Δ CS port entries between the ChR2 and eYFP groups ($p = .229$). However, the ChR2 group made fewer port entries than the eYFP group at test when stimulation was removed ($p < .001$). Together, these results indicate that conditioned responding was reliant on the presence of optogenetic stimulation in the ChR2 group.

Analysis of Δ CS port entries per trial revealed that removing IL-to-NAcS stimulation abolished responding to the CS from the first trial and onwards in the ChR2 group (Figure 2G; Test, $F(1,20) = 7.62$, $p = .012$; Virus, $F(1,20) = 2.89$, $p = .105$); Test x Virus, $F(1,20) = 11.97$, $p = .002$). This result recapitulates the differences observed in averaged Δ CS port entries between ChR2 and eYFP groups. Within-session extinction was observed as Δ CS port entries decreased across CS presentations (Trial, $F(13,260) = 10.07$, $p < .001$). However, there were no statistically significant Trial x Virus ($F(13,260) = 1.345$, $p = .224$), Trial x Test ($F(13,260) = .89$, $p = .540$) or Trial x Test x Virus ($F(13,260) = 1.88$, $p = .050$) interactions. The near significant Trial x Test x Virus interaction is likely the result of a reduction in the number of Δ CS port entries, especially in earlier CS presentations, in the ChR2 group relative to the eYFP group when stimulation was removed. In contrast, Δ CS port entries underwent within-session extinction and decreased

equivalently across trials in both the eYFP group and the ChR2 group when stimulation was present during the CS.

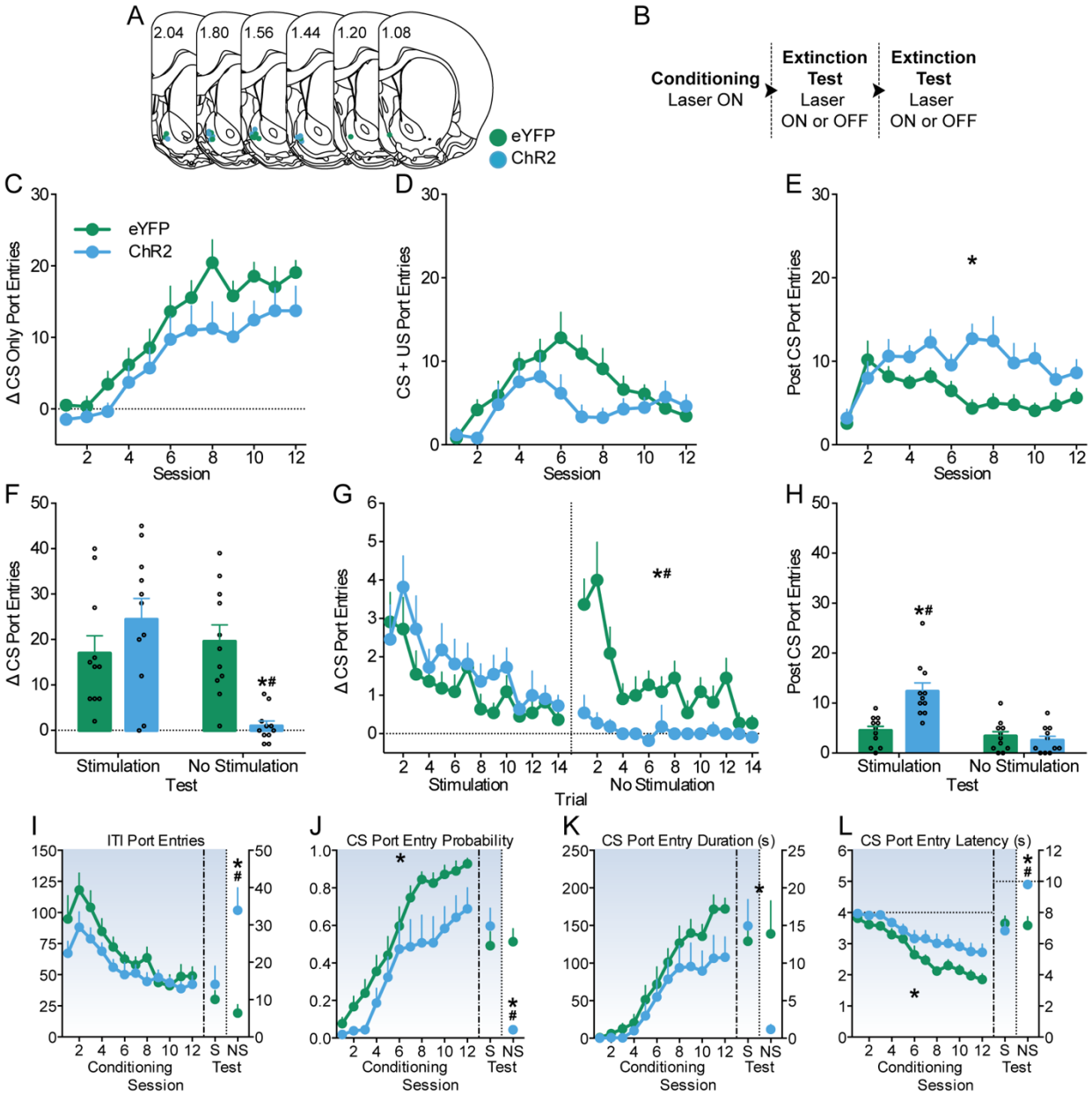
The presence of optogenetic stimulation increased post CS port entries in the ChR2 group but not in the eYFP group during the expression tests (Figure 2H; (Test, $F(1,20) = 22.32$, $p < .001$; Virus, $F(1,20) = 10.35$, $p = .004$; Test x Virus, $F(1,20) = 14.28$, $p = .001$). The eYFP group displayed similarly low post CS port entries at test in the presence or absence of stimulation ($p = .512$). In contrast, the ChR2 group made more post CS port during the Stimulation test than the No Stimulation test ($p < .001$). The ChR2 group made more post CS port entries than the eYFP group during the Stimulation test ($p < .001$) but not in the No Stimulation test ($p = .510$). Therefore, expression of post CS port entries in the ChR2 group appeared to rely on the presence of IL-to-NAcS stimulation.

At test, removing optogenetic stimulation increased ITI port entries in the ChR2 group but not in the eYFP group (Figure 2I, right; Test, $F(1,20) = 5.20$, $p = .034$; Virus, $F(1,20) = 10.84$, $p = .004$; Test x Virus, $F(1,20) = 11.14$, $p = .003$). The eYFP group showed an equivalent number of ITI port entries in the presence or absence of optogenetic stimulation ($p = .464$). In contrast, removing IL-to-NAcS stimulation in the ChR2 group increased ITI port entries relative to when stimulation was present ($p = .001$). The ChR2 group made more ITI port entries than the eYFP group during the No Stimulation test ($p < .001$) but similar ITI port entries during the Stimulation test ($p = .468$). These data suggest that appropriate responding depended on the presence of IL-to-NAcS stimulation.

Additional measures support that removal of IL-to-NAcS stimulation at test abolished conditioned responding in the ChR2 group but not the eYFP group (Figure 2J-L, right). Removal of optogenetic stimulation abolished probability of CS port entries in the ChR2 group but not in the eYFP group (Figure 2J, right; Test, $F(1,20) = 15.08$, $p = .001$; Virus, $F(1,20) = 6.42$, $p = .020$; Test x Virus, $F(1,20) = 17.43$, $p < .001$). The eYFP maintained high probability of CS port entries in the Stimulation and No Stimulation tests ($p = .839$). In contrast, the ChR2 group had lower probability of CS port entries during the No Stimulation test relative to the Stimulation test ($p < .001$). The ChR2 had lower probability of CS port entries than the eYFP group during the No Stimulation test ($p < .001$) but similar CS port entry probabilities during the Stimulation test ($p = .397$). Duration of CS port entries was lower at test in the ChR2 group compared to the eYFP group (Figure 2K, right; Test, $F(1,20) = 2.76$, $p = .113$; Virus, $F(1,20) = 4.49$, $p = .047$; Test x

Virus, $F(1,20) = 3.69$, $p = .069$). Visual inspection suggests that this effect may have largely been driven by lower duration of CS port entries in the ChR2 group relative to the eYFP group during the No Stimulation test. Lastly, removal of optogenetic stimulation increased the latency of CS port entries in the ChR2 group but not in the eYFP group (Figure 2L, right; Test, $F(1,20) = 9.94$, $p = .005$, Virus, $F(1,20) = 4.72$, $p = .042$; Test x Virus, $F(1,20) = 11.95$, $p = .002$). The eYFP group maintained similarly short latency of CS port entries during the Stimulation and No Stimulation tests ($p = .831$). In contrast, the ChR2 group had longer latency of CS port entries during the No Stimulation test relative to the Stimulation test ($p < .001$). The ChR2 group had longer latency of CS port entries than the eYFP group during the No Stimulation test ($p < .001$) but similar latency of CS port entries during the Stimulation test ($p = .533$). Together, these additional measures support the idea that proper expression of conditioned responding was dependent on the presence of IL-to-NAcS stimulation.

In sum, removing IL-to-NAcS stimulation abolished Δ CS port entries and other aspects of the conditioned response in the ChR2 group without affecting responding in the eYFP group. Further, removing IL-to-NAcS stimulation increased port entries made during the ITI in the ChR2 group without affecting responding in the eYFP group.



Chapter 5, Figure 2. Optogenetic stimulation of the IL-to-NAcS projection altered Pavlovian conditioning. **(A)** Optical fiber placements in the NAcS of rats expressing either ChR2 (blue) or eYFP alone (green) included in the final data analysis. Numbers are locations of sections relative to bregma. **(B)** Design of behavioural procedures. **(C)** Δ CS only port entries (4 sec) across Pavlovian conditioning sessions with IL-to-NAcS stimulation during the CS. **(D)** Port entries during the 6 sec overlapping CS and US interval across Pavlovian conditioning sessions. (**E**) Post CS port entries across Pavlovian conditioning sessions. * $p < 0.05$ main effect of virus group. (**F**) Δ CS port entries during the expression test under extinction conditions in the presence (Stimulation) or absence (No Stimulation) of IL-to-NAcS optogenetic stimulation during the CS. (**G**) Δ CS port entries across trials in the Stimulation

and No Stimulation expression tests. (**H**) Post CS port entries during the Stimulation and No Stimulation expression tests. (**F-H**) * $p < 0.05$ ChR2 vs. eYFP in the no stimulation test. # $p < 0.05$ stimulation test vs. no stimulation test in the ChR2 group. **(I)** ITI port entries across Pavlovian conditioning sessions and the expression tests with Stimulation (S) or No Stimulation (NS) under extinction conditions. **(J)** Probability, **(K)** duration, and **(L)** latency of CS only port entries (4 sec) across Pavlovian conditioning sessions (left) and full CS port entries (10 sec) during the expression tests (right). **(I-L)** Left Y-axis is for data during Pavlovian conditioning. Right Y-axis is for data during the expression tests. Shaded regions indicate sessions in which optogenetic stimulation was present during the CS. During Pavlovian conditioning, * $p < 0.05$ main effect of virus. **(I, J, L)** During tests, * $p < 0.05$ ChR2 vs eYFP in the no stimulation test. # $p < 0.05$ stimulation test vs no stimulation test in the ChR2 group. **(K)** During test, * $p < 0.05$ main effect of virus. All data are mean ± SEM.

Discussion

Chapter 5 explored potential mechanisms by which optogenetic stimulation of the IL-to-NAcS projection attenuates renewal of appetitive Pavlovian conditioned responding. Experiment 1 tested whether extinction training was necessary for optogenetic stimulation of the IL-to-NAcS to suppress conditioned responding. Rats were divided to either receive extinction or no extinction training prior to test. IL-to-NAcS optogenetic stimulation during the CS at test attenuated conditioned responding, regardless of prior extinction training. Furthermore, IL-to-NAcS stimulation during the extinction test, did not facilitate but seemed to impair extinction retrieval the following day without stimulation. In experiment 2, optogenetic stimulation of the IL-to-NAcS projection was delivered during CS presentations in Pavlovian conditioning to test whether stimulation would lead to general response suppression and prevent the acquisition of conditioned responding. IL-to-NAcS stimulation did not prevent acquisition of Pavlovian conditioning but altered some aspects of conditioned responding. Furthermore, in a subsequent extinction test, Δ CS port entries were maintained when IL-to-NAcS stimulation was present during the CS but was abolished when stimulation was removed. Together, the data indicate that optogenetic stimulation of the IL-to-NAcS projection can suppress conditioned responding independent of prior extinction training but does not appear to result in general response suppression.

Optogenetic stimulation of the IL-to-NAcS projection during the extinction test suppressed conditioned responding regardless of prior extinction training. Furthermore, attenuation of conditioned responding was evident from the very first trial of the extinction test, suggesting that the attenuation was not simply a facilitation of within-session extinction. These results suggest that stimulation of the IL-to-NAcS does not rely on an extinction retrieval mechanism in order to suppress appetitive Pavlovian responding. These findings are inconsistent with studies suggesting that the IL and IL-to-NAcS suppresses operant cocaine-seeking by promoting extinction retrieval (Peters et al., 2008a; Augur et al., 2016; Müller Ewald et al., 2019). Specifically, extinction training has been reported to be required for IL activation (Augur et al., 2016; Müller Ewald et al., 2019) and chemogenetic activation of the IL-to-NAcS projection to suppress cue-induced cocaine-seeking (Augur et al., 2016). This discrepancy could be related to our use of Pavlovian conditioning, whereas studies investigating the IL-to-NAcS in extinction typically use operant cocaine self-administration. Differences in the associative structures

involved in extinction of Pavlovian versus operant responding has been proposed (Trask et al., 2017) which could influence the recruitment of the IL-to-NAcS projection.

Alternatively, studies in operant cocaine-seeking used chemogenetics and stable-step function opsins which diffusely enhance IL and IL-to-NAcS activity (Augur et al., 2016; Müller Ewald et al., 2019). In contrast, we used optogenetics to stimulate the IL-to-NAcS at discrete points during behaviour. Similar application of optogenetics to stimulate the IL and IL-to-NAcS projection suppressed operant responding for food and cocaine without prior extinction (Do Monte et al., 2015; Cameron et al., 2019). Differences between discrete versus diffuse manipulations of neural activity may alter their effects on behaviour. Discrete optogenetic stimulation of IL inputs in the NAcS may disrupt time-locked inhibitory activity in the NAcS that permits consummatory behaviours (Nicola et al., 2004; Taha and Fields, 2006; Krause et al., 2010; Ghazizadeh et al., 2012; Reed et al., 2018), which is a component of the CS-elicited port entry response in our appetitive Pavlovian conditioning task.

Optogenetically stimulating the IL-to-NAcS projection during the extinction test did not facilitate extinction retrieval the following day. In fact, IL-to-NAcS stimulation during the extinction test increased CS responding during the retrieval test in rats with previous extinction training. Therefore, IL-to-NAcS stimulation during extinction seemed to weaken rather than strengthen the previously established inhibitory extinction memory. This finding contrasts with studies in aversive Pavlovian conditioning that report facilitated extinction retrieval and strengthening of inhibitory memory after enhancing IL activity during extinction (Milad and Quirk, 2002; Milad, 2004; Do Monte et al., 2015; Lingawi et al., 2016; 2018). The reason for the differences in findings between aversive and appetitive Pavlovian is unclear. One potential cause is that aversive Pavlovian conditioning studies stimulated the IL, whereas the present study stimulated the IL-to-NAcS projection. Thus, suppression of Pavlovian conditioned responding may be differentially mediated by the IL in comparison to the IL-to-NAcS. Alternatively, there are also differences in affective properties of extinction in aversive and appetitive procedures (Amsel, 1958, Gerber et al., 2014) which may alter the recruitment of the IL-to-NAcS projection.

Different neural ensembles involved in promoting and suppressing operant responding have been identified in the IL and IL projections to the NAcS (Warren et al., 2016; Warren et al., 2019). Further, pharmacologically inactivating the IL can reduce operant food- and heroin-seeking (Bossert et al., 2012; Eddy et al., 2016), suggesting that the IL is also involved in

promoting the return of responding after extinction. Therefore, our global stimulation of the IL-to-NAcS projection may have disrupted the activity of neural ensembles involved in promoting responding, thereby suppressing appetitive Pavlovian conditioned responding regardless of extinction and interfering with retrieval. Furthermore, in operant cocaine self-administration procedures, neural ensembles involved in extinction are maximally recruited after two and seven extinction sessions (Warren et al., 2016; 2019). In the present experiment, we only conducted one extinction session prior to test but observed within session extinction in rats that received extinction training and further reduction in conditioned responding compared to rats that did not receive prior extinction training. However, despite evidence for extinction learning in our Pavlovian conditioning procedure, multiple extinction sessions may be required for neural ensembles involved in extinction to be recruited in the IL-to-NAcS projection.

Optogenetically stimulating the IL-to-NAcS projection in experiment 2 did not prevent the acquisition or expression of appetitive Pavlovian conditioning, indicating that our manipulation likely did not result in general response suppression. However, stimulation of this projection affected probability and latency measures of conditioned responding and increased post CS port entries, indicating that conditioning was altered. Optogenetic stimulation of various glutamatergic inputs into the NAcS including the IL supports self-stimulation (Britt et al., 2012; Cameron et al., 2019), suggesting that stimulating this projection can also generate a perceptible stimulus. The presence of this additional stimulus may have disrupted aspects of conditioned responding during Pavlovian conditioning. Furthermore, IL-to-NAcS stimulation may have become a predictive stimulus which functioned in compound with the white noise CS to signal sucrose delivery. Interestingly, IL-to-NAcS stimulation was required for the expression of responding to the white noise CS during the subsequent expression test in extinction conditionings. Therefore, although IL-to-NAcS stimulation did not appear to impair motor function, stimulation may have altered Pavlovian conditioning by acting as an additional predictive cue for sucrose.

Removing stimulation also decreased CS responding and increased port entries during the ITI. This finding may be due to neural adaptations of the IL-to-NAcS projection following repeated stimulation during Pavlovian conditioning. Intensive optogenetic stimulation can induce plasticity that decrease neuronal excitability in order to adapt to repeated excitation (Mendez et al., 2018; Moulin et al., 2019). Therefore, in our experiment the IL-to-NAcS projection likely

downregulated its activity in order to adapt to repeated optogenetic stimulation across 12 days of Pavlovian conditioning. This is important as a decrease in basal activity of the NAcS following IL inactivation is associated with increased responding to non-reinforced cues and epochs (Ghazizadeh et al., 2012). Pharmacological inactivation of the IL and NAcS has similar effects of disinhibiting non-reinforced responding as we observed following removal of IL-to-NAcS stimulation during the expression test (Ambroggi et al., 2011; Ghazizadeh et al., 2012). These results suggest that the IL-to-NAcS projection may not only be important for suppressing CS responding but may also play a role in suppressing non-reinforced responding.

The present chapter showed that optogenetic stimulation can suppress appetitive Pavlovian conditioned responding without prior extinction training. These results indicate that IL-to-NAcS stimulation may suppress Pavlovian conditioned responding without promoting the retrieval of a previously established inhibitory extinction memory. Furthermore, IL-to-NAcS stimulation during extinction did not facilitate subsequent extinction retrieval which contrasts with findings in aversive Pavlovian conditioning. Importantly, IL-to-NAcS stimulation did not appear to generally suppress behaviour. However, IL-to-NAcS stimulation may have altered Pavlovian conditioning by potentially modulating how the white noise CS was processed and acting as an additional stimulus. Lastly, the results suggest that repeated optogenetic stimulation of the IL-to-NAcS may produce neuroadaptive changes that lead to similar behavioural effects as inactivation of these brain regions. Together, the present results highlight the complex process by which the IL-to-NAcS projection controls appetitive Pavlovian conditioned responding.

Chapter 6 - General Discussion

6.1. Summary

The present thesis investigated the role of the infralimbic cortex (IL) and its neural projections to the nucleus accumbens shell (NAcS) and basolateral amygdala (BLA) in extinction and attenuation of conditioned responding to appetitive Pavlovian cues. First, optogenetic stimulation of the IL specifically during presentations of a sucrose CS but not outside of the CS attenuated renewal of Pavlovian conditioned responding after extinction. Second, optogenetic stimulation of the IL-to-NAcS but not the IL-to-BLA projection attenuated the renewal of conditioned responding to the sucrose CS. However, extinction training was not required for optogenetic stimulation of the IL-to-NAcS circuit to attenuate responding. Furthermore, IL-to-NAcS stimulation during extinction did not facilitate but instead appeared to impair extinction retrieval. Optogenetic stimulation of the IL-to-NAcS, however did not prevent acquisition of Pavlovian conditioning, suggesting that stimulation did not simply result in general suppression of motor functions. However, IL-to-NAcS stimulation altered some aspects of the Pavlovian conditioned response and became necessary to maintain responding to the sucrose CS in a subsequent expression test. Together, the findings of the present thesis expand the role of the IL and the IL-to-NAcS neural circuit in extinction and suppression of conditioned responding to appetitive Pavlovian cues. Lastly, it provides novel insight into the processes by which organisms may achieve the suppression of appetitive Pavlovian conditioned responses.

6.2 Optogenetic stimulation of the IL during CS trials attenuated renewal

In Chapter 3, optogenetic stimulation of the IL during CS trials attenuated the renewal of conditioned responding triggered by returning rats to the conditioning context (Context A) after extinction in a different context (Context B). The suppression of renewal following IL stimulation is consistent with findings that enhancing IL activity pharmacologically or through optogenetic stimulation during the CS reduces the return of appetitive Pavlovian responding triggered by US re-exposure (reinstatement) and the passage of time (spontaneous recovery) after extinction (Villaruel et al., 2018). Furthermore, these results are consistent with findings that lesioning the IL enhances the return of appetitive Pavlovian responding after extinction (Rhodes and Killcross, 2004; 2007a). Together, the data indicates that the IL plays a critical role in suppressing appetitive Pavlovian conditioned responding during renewal.

The suppression in renewal of appetitive Pavlovian responses following IL stimulation is consistent with findings using operant drug self-administration procedures. Enhancing IL activity pharmacologically or chemogenetically suppressed the return of drug-seeking after extinction across different drug-reinforcers (LaLumiere et al., 2012; Gass et al., 2014; Augur et al., 2016; Chen et al., 2016). The IL is thought to be especially important for extinction of cocaine-seeking (Peters et al., 2009). For instance, pharmacological inactivation of the IL reinstated extinguished cocaine-seeking (Peters et al., 2008a) and optogenetic inhibition of the IL disrupted extinction and augmented cue-induced cocaine-seeking (Gutman et al., 2017). The present results highlight that the IL may play a similar role in suppressing cocaine-seeking and appetitive Pavlovian responding to natural reinforcers such as sucrose.

IL stimulation outside of the CS, during the middle of the inter-trial intervals (ITI) did not affect renewal of appetitive Pavlovian conditioned responding. This result indicates that IL activity specifically during the CS is critical for the attenuation of conditioned responding after extinction. Consistently, in aversive Pavlovian conditioning procedures, IL neural activity increased specifically in response to a tone CS after it has undergone extinction training (Milad and Quirk, 2002). Electrical and optogenetic stimulation of the IL specifically during the CS suppressed aversive conditioned responding and facilitated extinction retrieval (Milad and Quirk, 2002; Do Monte et al., 2015). In contrast, electrical stimulation of the IL outside of the CS, during the ITI, did not facilitate extinction of aversive conditioned responding (Milad et al., 2004). Similarly, during extinction, optogenetic inhibition of the IL specifically after the non-reinforced lever press but not at random epochs impaired extinction of cocaine-seeking (Gutman et al., 2017). Therefore, our results are consistent with different procedures that IL activity in extinction specifically during a non-reinforced CS or operant response is important for the suppression of conditioned responding.

6.3 Optogenetic stimulation of the IL-to-NAcS but not IL-to-BLA attenuated renewal

Different IL glutamatergic projections are thought to mediate extinction based on the affective valence of the reinforcer. The IL-to-NAcS neural circuit is thought to be involved in extinction of conditioned responding to appetitive stimuli especially in the context of operant cocaine self-administration (Peters et al., 2009). In contrast, the IL-to-BLA circuit is thought to be critical for extinction of conditioned responding to aversive stimuli (Peters et al., 2009).

Retrograde neural tracing revealed that IL projections to the NAcS and BLA are predominantly composed of separate neural subpopulations in rats, which is consistent with results from similar neural tracing done in mice (Bloodgood et al., 2018). In support of the proposed functional dichotomy, optogenetic stimulation of the IL-to-NAcS but not the IL-to-BLA neural circuit during CS trials attenuated the renewal of appetitive Pavlovian conditioned responding. These findings provide novel evidence that the IL-to-NAcS is important for extinction of appetitive Pavlovian responding. Furthermore, the results provide support for the hypothesis established in operant procedures that extinction of appetitive conditioned responding is mediated by the IL-to-NAcS circuit.

Optogenetic stimulation of the IL-to-BLA neural circuit did not affect the renewal of appetitive Pavlovian conditioned responding. These results are consistent with the hypothesis that the IL-to-BLA is specifically involved in extinction of aversive Pavlovian responses (Peters et al., 2009). In support of this idea, extinction of aversive Pavlovian responses increased the excitability of neurons projecting from the IL-to-BLA (Bloodgood et al., 2018). Furthermore, chemogenetic and optogenetic inhibition of the IL-to-BLA circuit during extinction learning impaired extinction retrieval (Bukalo et al., 2015; Bloodgood et al., 2018) whereas optogenetic stimulation facilitated extinction retrieval (Bukalo et al., 2015; 2021). These results indicate that the IL-to-BLA neural circuit is imperative for extinction of aversive Pavlovian responses. However, an important distinction is that manipulation of the IL-to-BLA circuit in these studies occurred during extinction learning whereas we stimulated the IL-to-BLA circuit during the retrieval phase in a renewal test. The IL-to-BLA may play differential roles in extinction learning and retrieval leading to our lack of an effect in renewal of appetitive Pavlovian conditioned responding. Consistently, optogenetic stimulation or inhibition of the IL-to-BLA circuit did not affect extinction retrieval of aversive Pavlovian conditioned responding (Bukalo et al., 2015). Furthermore, in aversive conditioning procedures, the BLA has been shown to be specifically involved in initial extinction learning but not in relearning extinction (Sotres-Bayon et al., 2007; Laurent et al., 2008; Laurent and Westbrook, 2010). These results suggest that the BLA may be important for the initial formation of the inhibitory extinction memory but is not imperative for extinction retrieval once the extinction memory has been established. The IL-to-BLA circuit may play a similar role in the appetitive Pavlovian conditioning in which the circuit is recruited during initial extinction acquisition but not during retrieval or renewal.

The present findings are consistent with substantial evidence suggesting that the IL-to-NAcS circuit is important for suppressing cocaine-seeking and extends this role to suppressing responding to a sucrose CS. Seminal work has shown that concurrent pharmacological inactivation of the IL and NAcS reinstates extinguished cocaine-seeking (Peters et al., 2008a). Consistently, chemogenetic activation of the IL-to-NAcS circuit attenuated cue-induced cocaine-seeking after extinction (Augur et al., 2016). Neural ensembles in the IL that are recruited for extinction of cocaine-seeking also predominantly project to the NAcS (Warren et al., 2019). Together, these results suggests that the IL-to-NAcS circuit is critical for suppression of operant cocaine-seeking after extinction. Therefore, the IL-to-NAcS circuit may be a common neural substrate that mediates extinction of conditioned responding for cocaine and natural reinforcers.

Our experiments highlight the importance of the IL-to-NAcS in extinction of appetitive Pavlovian conditioned responses but are unable to identify which neurons in the NAcS were activated or their downstream projections. The NAcS consists predominantly of medium spiny neurons (MSNs) which are traditionally thought to either be part of the direct pathway expressing D1 dopamine receptors (D1R) MSNs or the indirect pathway expressing D2 dopamine receptors (D2R) MSNs. A classic view of their function is that D1R MSNs promotes responding whereas D2R MSNS suppresses responding (Lobo et al., 2010; Kravitz et al., 2010; Yttri and Dudman, 2016). It is tempting to suggest that optogenetic stimulation of the IL-to-NAcS activated D2R MSNs which led to the suppression in renewal of appetitive Pavlovian conditioned responding. However, D1R MSNs alone can also have differential roles in extinction and renewal depending on their projections. Specifically, NAcS projections to the ventral tegmental area are involved in renewal of alcohol-seeking whereas projections to the lateral hypothalamus (LH) are involved in promoting extinction (Gibson et al., 2018). Given that IL-to-NAcS stimulation suppressed renewal of appetitive Pavlovian conditioned responding, it is possible that stimulation of this circuit led to downstream stimulation of the NAcS-to-LH circuit, leading to the suppression of renewal. However, we lack evidence for which specific neurons were affected by our optogenetic manipulation of the IL-to-NAcS circuit. Additional studies are required to delineate the specifics of the IL-to-NAcS circuit including cell types and downstream projections to examine their role in extinction of appetitive Pavlovian conditioned responding.

6.4 Potential mechanisms for suppression of appetitive Pavlovian conditioned responding following IL-to-NAcS stimulation

Optogenetic stimulation of the IL-to-NAcS circuit suppressed appetitive Pavlovian conditioned responding regardless of prior extinction training. This result suggests that IL-to-NAcS stimulation can suppress conditioned responding without relying on the promotion of an inhibitory extinction memory. Our results are inconsistent with studies using operant cocaine self-administration procedures which found prior extinction training to be necessary for stimulation of the IL and IL-to-NAcS circuit to suppress cocaine-seeking (Augur et al., 2016; Müller Ewald et al., 2019). Others have also observed extinction-independent suppression of operant responding for food and cocaine following optogenetic stimulation of the IL and IL-to-NAcS projection (Do Monte et al., 2015; Cameron et al., 2019). These inconsistent findings could be attributed to the use of discrete optogenetic stimulation at specific points during behaviour. Time-locked inhibition of neural activity has been observed in the NAcS to permit consummatory behaviours (Nicola et al., 2004; Taha and Fields, 2006; Krause et al., 2010; Ghazizadeh et al., 2012; Reed et al., 2018), which is a component of the CS-elicited port entry response in our task. Optogenetic stimulation of the IL-to-NAcS during the CS may have disrupted this time-locked inhibition, leading to suppression of conditioned responding independent of an extinction process.

Emerging studies have revealed different neural ensembles in the IL and IL-to-NAcS circuit that are involved in both promoting and suppressing conditioned responding (Bossert et al., 2012; Pfarr et al., 2015, Suto et al., 2016; Warren et al., 2016; 2019). Deletion of ensembles in the IL that are involved in expression of operant food-seeking led to the suppression of responding, whereas deletion of ensembles involved in extinction of food-seeking increased responding (Warren et al., 2016). Furthermore, in a discriminative operant conditioning procedure, deletion of IL neurons that encode a cue that predicts the availability of an appetitive reinforcer suppressed operant responding to that cue (Suto et al., 2016; Lacque et al., 2019). In contrast, deletion of IL neurons that encodes a separate cue, predicting the absence of the reinforcer, increased operant responding to that cue (Suto et al., 2016; Lacque et al., 2019). These studies indicate that different subpopulations of neurons within the IL can encode whether a cue is predictive of the presence or absence of reinforcement, and whether to initiate or suppress responding. Our optogenetic stimulation encompassing the entire IL-to-NAcS circuit,

may therefore have disrupted the activity of ensembles that are involved in promoting responding leading to response suppression regardless of extinction training. It is noteworthy, however, that neural ensembles in the IL that are involved in extinction of cocaine-seeking predominantly project to the NAcS (Warren et al., 2019), suggesting that our manipulation may be biased in activating neurons that are involved in extinction. However, in these operant conditioning procedures extinction ensembles only emerge after two or seven extinctions (Warren et al., 2016; 2019). In the present study, we only conducted one extinction session and multiple sessions may be required for extinction neurons to emerge and be susceptible to stimulation of the IL-to-NAcS circuit. Future work could leverage these new technologies that allow for targeting of specific neural ensembles involved in a specific memory or behaviour for investigating the role of the IL-to-NAcS circuits in extinction of appetitive Pavlovian conditioned responding.

In aversive Pavlovian conditioning, enhancing IL activity during extinction suppresses conditioned responding and facilitates subsequent extinction retrieval (Milad and Quirk, 2002; Milad et al., 2004; Vidal-Gonzalez et al., 2006; Kim et al., 2010; Thompson et al., 2010; Peters et al., 2010; Do Monte et al., 2015; Lingawi et al., 2016; Lingawi et al., 2018). In contrast, in our appetitive Pavlovian conditioning task, optogenetic stimulation of the IL-to-NAcS circuit during extinction did not facilitate extinction retrieval. Further, IL-to-NAcS stimulation seemed to impair extinction retrieval in rats that previously received extinction training, suggesting that stimulation may have weakened rather strengthened the previously established inhibitory extinction memory. This discrepancy could be due to separable roles of the IL alone and the IL-to-NAcS circuit in extinction. Specifically, while the IL may be involved in modulating and strengthening of inhibitory memories, the IL-to-NAcS circuit may be more involved in the expression of these memories and executing the motor programs that suppress responding. Additional work could test whether activation of the IL alone would facilitate extinction acquisition and retrieval in appetitive Pavlovian conditioning.

Alternatively, these divergent findings in extinction retrieval could highlight differences in extinction of appetitive and aversive conditioned responses. First, extinction in appetitive and aversive conditioning can have different affective properties (Amsel, 1958; Gerber et al., 2014). Specifically, the omission of the US in extinction of appetitive conditioning can lead to frustration (Adelman and Maatsch, 1956; Daly, 1974), whereas the omission of the aversive US

in extinction provides relief or negative reinforcement to an animal (Smith and Buchanan, 1954). Therefore, instead of promoting extinction per se, the IL could be enhancing the relief that is experienced following extinction in aversive conditioning procedures. Promoting relief by enhancing IL or IL-to-NAcS activity could then lead to further suppression of aversive conditioned responding during extinction retrieval. If facilitation of extinction in aversive conditioning works through an affective relief process, it could explain why we did not observe a facilitation of extinction retrieval following IL-to-NAcS stimulation in an appetitive Pavlovian conditioning task. However, additional work is necessary to test whether the IL and IL-to-NAcS circuit may mediate extinction through an affective process. Second, there are also differences in conditioning procedures with appetitive and aversive stimuli. Namely, appetitive Pavlovian conditioning procedures typically require animals to make an approach response to receive the US during CS presentations. This requirement can lead to scenarios in which US delivery is delayed and become non-contiguous with the CS, unlike aversive Pavlovian conditioning where the CS is always followed by the aversive US (e.g. foot-shock). Better control of the contiguity between the CS and the US may be required to examine the role of the IL-to-NAcS in extinction of appetitive Pavlovian conditioned responding.

Optogenetic stimulation of the IL-to-NAcS did not prevent the acquisition or expression of appetitive Pavlovian conditioning, lending some evidence that our manipulation did not simply suppress motor functions. This finding is important given that in all our previous experiments optogenetic stimulation of the IL-to-NAcS consistently suppressed conditioned responding in renewal and regardless of prior extinction training. Interestingly, however, probability and latency measures of conditioned responding were affected by IL-to-NAcS stimulation during the CS in Pavlovian conditioning. This finding could be due to the disruption of time-locked inhibition in the NAcS that is observed during appetitive-related and consummatory behaviours (Nicola et al., 2004; Taha and Fields, 2006; Krause et al., 2010; Ghazizadeh et al., 2012; Reed et al., 2018). Furthermore, we found that after Pavlovian conditioning, the presence of optogenetic maintained CS responding under extinction conditions. This finding provides additional support that IL-to-NAcS stimulation did not simply suppress responding but may differentially affect Pavlovian conditioning. These possibilities are further explored below.

6.5 IL and IL-to-NAcS contextual control of conditioned responding

The IL is thought to be important for using contextual stimuli to direct appropriate conditioned responding (Rhodes and Killcross, 2007a; Moorman and Aston-Jones, 2015; Riaz et al., 2019). The findings of the present thesis support a role for the IL and the IL-to-NAcS circuit in encoding and using contextual stimuli. Extinction is believed to be highly sensitive to context manipulations, such that changing the context results in renewal of conditioned responding (Bouton, 1993; 2004). The IL is thought to promote extinction retrieval, by reducing the context-specificity of the inhibitory extinction memory and allowing it to generalize to other contexts (Rhodes and Killcross, 2007a). In support of this idea, disrupting IL activity has been found to disinhibit responding during extinction and increase renewal (Rhodes and Killcross, 2007a; Peters et al., 2008a). These results suggest that disrupting IL activity renders the inhibitory extinction memory to be more context-sensitive, making extinction retrieval more reliant on the context. As a result, even minor changes in the extinction context can trigger a return of responding when the IL is offline. Within this framework, our present findings that stimulation of the IL and IL-to-NAcS circuit attenuated renewal, may be interpreted as a reduction in the context-sensitivity of the inhibitory extinction memory which generalized to the conditioning context.

Inconsistently, however, we found that stimulation of the IL-to-NAcS circuit suppressed conditioned responding regardless of prior extinction training. These results suggest that augmenting IL-to-NAcS activity does not suppress responding by generalizing the extinction memory to other contexts, as rats that did not receive prior extinction training had no such memory to begin with. However, our results remain consistent with the interpretation that the IL and the IL-to-NAcS projection are involved in processing and using contextual stimuli if we do not limit their control to extinction memories but extend it to conditioned responding in general. Consistently, others have found a role for the IL in discriminative responding depending on context (Moorman and Aston-Jones, 2015; Riaz et al., 2019). Therefore, in Chapter 5, the suppression of conditioned responding regardless of extinction could still be attributed to alterations in context processing. Specifically, IL-to-NAcS stimulation may have altered the perception of the context leading to impairments in generalizing the expression of conditioned responding, resulting in suppression during the extinction test. Consequently, impairments in extinction retrieval the following day could be a result of altering context processing during the extinction test with IL-to-NAcS stimulation. Further support for the idea that IL-to-NAcS

stimulation affected context processing is evident in experiment 2 of Chapter 5, in which stimulation was delivered concurrently with the CS during Pavlovian conditioning. IL-to-NAcS stimulation did not significantly alter responding during conditioning, however, during the expression test, the ChR2 only displayed evidence of Pavlovian conditioning in the presence but not in the absence of stimulation. These results could be interpreted as IL-to-NAcS stimulation altering context processing during conditioning, such that the retrieval of conditioning memory became dependent on the presence of stimulation. Therefore, while the results of Chapter 5 do not support the idea that IL-to-NAcS stimulation reduced the context sensitivity of an extinction memory, they indicate that IL-to-NAcS stimulation may affect context processing during both Pavlovian conditioning and extinction.

6.6 Technical considerations of optogenetic stimulation on extinction and suppression of appetitive Pavlovian conditioned responding

Optogenetic stimulation is perceptible to animals and can even function as a CS or US on their own (Wu et al., 2015, Saunders et al., 2018). Further, rats have been found to self-administer optogenetic stimulation of the IL-to-NAcS circuit, indicating that stimulating this circuit can also be reinforcing (Britt et al., 2012; Cameron et al., 2019). In the present thesis, it is possible that IL and IL-to-NAcS optogenetic stimulation functioned as an additional CS, which could potentially explain our various findings. The primary evidence for this idea is that IL-to-NAcS stimulation during the CS in Pavlovian conditioning, later rendered the expression of conditioned responding dependent on the presence of stimulation. Therefore, IL-to-NAcS stimulation appeared to act as another CS predicting sucrose delivery. If IL-to-NAcS stimulation functioned as an additional CS, it may have led to overshadowing, an associative learning phenomenon in which during compound conditioning, when two CSs are trained to predict a US, the more salient CS becomes the better predictor of the US (Pavlov, 1927, Rescorla and Wagner, 1972). In our experiment, the presence of the more salient IL-to-NAcS stimulation during Pavlovian conditioning may have overshadowed the white noise CS. This would explain why the expression of conditioned responding became dependent on IL-to-NAcS stimulation, as it became a better predictor of the US than the less salient white noise CS during Pavlovian conditioning. One potential test for the overshadowing effect, could be to habituate the animals to optogenetic stimulation to reduce is ability to overshadow the white noise CS.

Assuming that optogenetic stimulation of the IL-to-NAcS circuit is a salient extraneous stimulus during conditioning, it may have also functioned in a similar manner during renewal and the test for extinction-dependent suppression. The presence of salient extraneous stimuli can suppress conditioned responding in a non-associative manner through the process of external inhibition. For example, in Pavlov's experiments a loud sound or even a change in room lighting would suppress the conditioned salivation response and trigger a competing investigatory response from the animals (Pavlov, 1927, pg. 44). If optogenetic stimulation of the IL-to-NAcS circuit was acting as a salient extraneous stimulus, the suppression of conditioned responding during renewal and the extinction test may have been due to external inhibition, rather than a facilitation of an extinction memory. In further support of this argument, we observed a return of conditioned responding during extinction retrieval when optogenetic stimulation was removed. This finding is akin to the disinhibition of conditioned responding that is observed following removal of the extraneous stimulus that is causing external inhibition (Gagné, 1941).

However, while external inhibition provides a parsimonious explanation it does not completely explain all our findings. First, in Chapter 4, optogenetic stimulation of the IL-to-NAcS but not the IL-to-BLA circuit suppressed renewal, suggesting that optogenetic stimulation alone does not suppress conditioned responding. Second, in Chapter 5, experiment 1, the return of conditioned responding following optogenetic stimulation of the IL-to-NAcS circuit during extinction was observed only in rats that previously received extinction training but not in rats that did not receive prior extinction. If disinhibition occurred as a result of external inhibition, return of responding should have been observed in both groups. Lastly, in Chapter 5, experiment 2, external inhibition cannot explain why removal of IL-to-NAcS stimulation after Pavlovian conditioning increased port entries made outside of the CS, during the inter-trial intervals. Furthermore, it is important to note that in aversive conditioning, similar optogenetic stimulation of the IL facilitates extinction retrieval, indicating that optogenetic stimulation does not simply function as an external inhibitor across different behavioural procedures (Do Monte et al., 2015). Additional work is necessary to explain these findings and elucidate how the IL and the IL-to-NAcS circuit suppresses and controls appetitive Pavlovian conditioned responding. Future work using optogenetic inhibition may provide insight as to whether the observed findings are a result of external inhibition from any disruption of the IL or IL-to-NAcS circuit.

The elimination of CS responding and increase in port entries during the inter-trial intervals following removal of the IL-to-NAcS stimulation may be due to synaptic changes induced by repeated optogenetic stimulation across multiple days of Pavlovian conditioning. These behavioral effects are likely a result of repeated stimulation as we failed to see the same increase in port entries during the inter-trial intervals when IL-to-NAcS stimulation was removed following one session with stimulation during the extinction test. Chronic optogenetic stimulation of similar corticostriatal circuits across five consecutive days can alter the firing properties of neurons in the ventral striatum (Ahmari et al., 2013) and upregulate transcription factors in the NAcS that are involved in appetitive-related behaviours (Lobo et al., 2013). Furthermore, pyramidal neurons can increase inhibitory synapses and decrease excitability to adapt to intensive optogenetic stimulation (Mendez et al., 2018; Moulin et al., 2019). A similar decrease in excitability likely occurred in the IL-to-NAcS circuit to adapt to repeated optogenetic stimulation across 12 consecutive days in our experiment. This decrease in neuronal excitability is of interest as the IL is thought to modulate the basal firing rate of neurons in the NAcS that are involved in suppressing non-reinforced responses (Ghazizadeh et al., 2012). Pharmacological inactivation of the IL decreases the basal firing rate of neurons in the NAcS resulting in disinhibition of non-reinforced responding (Peters et al., 2008a; Ghazizadeh et al., 2012; Keistler et al., 2015). Given our similar findings, it is possible that repeated IL-to-NAcS stimulation led to neural adaptations that decreased the excitability of this circuit, resulting in disinhibition and an increase of port entries during the intertrial intervals when stimulation was removed. Future work could determine whether these behavioural effects are due to neuroadaptations by investigating the synaptic and electrophysical changes induced by repeated stimulation of the IL-to-NAcS circuit.

Overall, our results indicate that the IL-to-NAcS circuit can contribute to response suppression in at least two different manners, by suppressing CS responding and suppressing responding during non-reinforced epochs. This idea is supported by the findings that IL-to-NAcS stimulation during the CS suppressed renewal and responding during the extinction test, while removal of stimulation after presumably decreasing IL-to-NAcS excitability through repeated stimulation abolished CS responding and increased responding during the inter-trial interval. These results are consistent with a model proposed by Ghazizadeh et al., (2012) in which the IL is thought to suppress responding through (1) phasic feed-forward inhibition of neurons in the

NAcS involved in generating cue-elicited responses and (2) tonic excitation of neurons in the NAcS that are involved in suppressing non-reinforced responding. NAcS neurons involved in cue-elicited responses are thought to generate responding through excitation from different afferents, such as the BLA or the PL (Ghazizadeh et al., 2012). Meanwhile, the IL-to-NAcS circuit opposes this process and suppress cue-elicited responding via feed-forward inhibition of these same neurons in the NAcS. During the renewal and extinction test, IL-to-NAcS stimulation likely suppressed conditioned responding through feed-forward inhibition of neurons in the NAcS that are involved in cue-elicited responding. However, following repeated IL-to-NAcS stimulation during Pavlovian conditioning, both types of neurons in the NAcS likely underwent a decrease in excitability. Therefore, NAcS neurons involved in cue-elicited responses became less responsive to other excitatory inputs that generate responding, leading to an abolishment of CS port entries. Additionally, NAcS neurons involved in suppressing non-reinforced responding became less responsive to tonic inputs from the IL resulting in disinhibition of port entries during the inter-trial intervals. Our data is consistent with the model that the IL can suppress both conditioned responding and non-reinforced responding through two different processes in the NAcS. However, more work is needed to determine whether the abolishment of CS port entries and increase in inter-trial interval port entries following removal of stimulation is in fact due to a decrease in excitability of NAcS neurons after repeated optogenetic stimulation of the IL-to-NAcS circuit.

6.7 Sex differences in the role of the IL in renewal of appetitive conditioned responding

The present work only used male rats and is therefore unable to observe any sex differences. However, recent work has found sex differences in renewal of appetitive Pavlovian responding and the role of the IL (Anderson and Petrovich, 2017; 2018). Specifically, only male but not female rats were found to renew responding to a food CS and show increased Fos activation in the IL (Anderson and Petrovich, 2017). Furthermore, chemogenetic silencing of the IL in male rats attenuated renewal, whereas stimulation of the IL in female rats induced renewal (Anderson and Petrovich, 2018). These studies contrast our present findings that stimulation of the IL and IL-to-NAcS projection suppresses renewal, and suggests that the IL may instead generate rather than suppress the return of responding to an appetitive CS. These findings, however, are in line with other studies showing attenuation of renewal following IL inactivation

(Rogers et al., 2008; Bossert et al., 2011; Bossert et al., 2012; Eddy et al., 2016). Together, these results support the idea that the IL is involved in both the generation and suppression of conditioned responding, which may be mediated by different neural ensembles within the region (Warren et al., 2016; 2019; Suto et al., 2016; Laque et al., 2019). However, the sex differences in renewal of appetitive Pavlovian conditioning remains unclear. Moreover, they are inconsistent with early studies demonstrating the renewal effect in appetitive Pavlovian conditioning using female rats (Bouton and Peck, 1989; Bouton et al., 1993). These conflicting results on sex differences could be due to the use of different measures of conditioned responding. In earlier work showing renewal in female rats, head orientation to the CS was used as an index of conditioned responding whereas recent work used time spent in the port during the CS (Bouton and Peck, 1989; Anderson and Petrovich, 2017). Furthermore, unpublished work from our lab have found similar levels of renewal and reinstatement in both male and female rats using frequency of CS port entries as a measure of conditioned responding. Sex differences in behavioural strategies have been found using aversive Pavlovian conditioning, which may also be present in appetitive preparations and affect whether renewal is observed in different sexes (Shansky and Murphy, 2021). Therefore, future studies should use different measures of conditioned responding in order to fully capture any sex differences in renewal of appetitive Pavlovian conditioning.

6.8 Conclusion

In conclusion, the present thesis found that the IL and its projection to the NAcS play an important role in suppressing conditioned responding to a Pavlovian cue that predicts an appetitive outcome. Specifically, optogenetic stimulation of the IL and the IL-to-NAcS circuit suppressed the return of CS responding triggered by a change in context. However, response suppression following IL-to-NAcS stimulation did not seem to depend on reactivation of a previously established inhibitory extinction memory. In fact, stimulation of the IL-to-NAcS circuit may even be detrimental for extinction of conditioned responding to appetitive Pavlovian cues. Furthermore, IL-to-NAcS stimulation does not appear to simply suppress motor function but can disrupt and alter the expression of Pavlovian conditioned responding. Together, the present thesis highlights the pivotal role of the IL and IL-to-NAcS circuit in suppressing conditioned responding to appetitive Pavlovian cues.

The ability to suppress learned responses is an important aspect of adaptive behaviour. Extinction is a fundamental process by which animals learn to suppress responding to adapt to changing environmental demands. The inability to extinguish and suppress maladaptive behaviours is also a key characteristic of many mental disorders such as substance abuse, post-traumatic stress, and anxiety. Therefore, knowledge of the corticostriatal processes involved extinction and response suppression to appetitive cues will further our understanding of how organisms to adapt to dynamic environments and aid in the treatment of disorders involving inhibition of learned behaviours.

References

Adamantidis, A. R., Tsai, H.-C., Boutrel, B., Zhang, F., Stuber, G. D., Budygin, E. A., … de Lecea, L. (2011). Optogenetic Interrogation of Dopaminergic Modulation of the Multiple Phases of Reward-Seeking Behavior. *Journal of Neuroscience*, *31*(30), 10829–10835. https://doi.org/10.1523/JNEUROSCI.2246-11.2011

Ahmari, S. E., Spellman, T., Douglass, N. L., Kheirbek, M. A., Simpson, H. B., Deisseroth, K., … Hen, R. (2013). Repeated cortico-striatal stimulation generates persistent OCD-like behavior. *Science*, *340*(6137), 1234–1239. https://doi.org/10.1126/science.1234733

Al-Hasani, R., McCall, J. G., Shin, G., Gomez, A. M., Schmitz, G. P., Bernardi, J. M., … Bruchas, M. R. (2015). Distinct Subpopulations of Nucleus Accumbens Dynorphin Neurons Drive Aversion and Reward. *Neuron*, *87*(5), 1063–1077. https://doi.org/10.1016/j.neuron.2015.08.019

Ambroggi, F., Ghazizadeh, A., Nicola, S. M., & Fields, H. L. (2011). Roles of Nucleus Accumbens Core and Shell in Incentive-Cue Responding and Behavioral Inhibition. *Journal of Neuroscience*, *31*(18), 6820–6830. https://doi.org/10.1523/JNEUROSCI.6491-10.2011

Amsel, A. (1958). The role of frustrative nonreward in noncontinuous reward situations. *Psychological Bulletin*, *55*(2), 102–119. https://doi.org/10.1037/h0043125

Anderson, L. C., & Petrovich, G. D. (2017). Sex specific recruitment of a medial prefrontal cortex-hippocampal-thalamic system during context-dependent renewal of responding to food cues in rats. *Neurobiology of Learning and Memory*, *139*(23), 11–21. https://doi.org/10.1016/j.nlm.2016.12.004

Anderson, L. C., & Petrovich, G. D. (2018). Ventromedial prefrontal cortex mediates sex differences in persistent cognitive drive for food. *Scientific Reports*, *8*(1), 1–9. https://doi.org/10.1038/s41598-018-20553-4

Arruda-Carvalho, M., & Clem, R. L. (2015). Prefrontal-amygdala fear networks come into focus. *Frontiers in Systems Neuroscience*, *9*(October), 1–5. https://doi.org/10.3389/fnsys.2015.00145

Augur, I. F., Wyckoff, A. R., Aston-Jones, G., Kalivas, P. W., & Peters, J. (2016). Chemogenetic Activation of an Extinction Neural Circuit Reduces Cue-Induced Reinstatement of Cocaine Seeking. *Journal of Neuroscience*, *36*(39), 10174–10180. https://doi.org/10.1523/JNEUROSCI.0773-16.2016

Barker, J. M., Glen, W. B., Linsenbardt, D. N., Lapish, C. C., & Chandler, L. J. (2017). Habitual Behavior Is Mediated by a Shift in Response-Outcome Encoding by Infralimbic Cortex. *ENeuro*, *4*(6), ENEURO.0337-17.2017. https://doi.org/10.1523/ENEURO.0337-17.2017

Benn, A., Barker, G. R. I., Stuart, S. A., Roloff, E. v. L., Teschemacher, A. G., Warburton, E. C., & Robinson, E. S. J. (2016). Optogenetic Stimulation of Prefrontal Glutamatergic Neurons Enhances Recognition Memory. *Journal of Neuroscience*, *36*(18), 4930–4939. https://doi.org/10.1523/JNEUROSCI.2933-15.2016

Berridge, K. C., & Robinson, T. E. (1998). What is the role of dopamine in reward: Hedonic impact, reward learning, or incentive salience? *Brain Research Reviews*, *28*(3), 309–369. https://doi.org/10.1016/S0165-0173(98)00019-8

Bloodgood, D. W., Sugam, J. A., Holmes, A., & Kash, T. L. (2018). Fear extinction requires infralimbic cortex projections to the basolateral amygdala. *Translational Psychiatry*, *8*(60), 1–11. https://doi.org/10.1038/s41398-018-0106-x

Bossert, J. M., Stern, A. L., Theberge, F. R. M., Marchant, N. J., Wang, H.-L., Morales, M., & Shaham, Y. (2012). Role of Projections from Ventral Medial Prefrontal Cortex to Nucleus Accumbens Shell in Context-Induced Reinstatement of Heroin Seeking. *Journal of Neuroscience*, *32*(14), 4982–4991. https://doi.org/10.1523/JNEUROSCI.0005-12.2012

Bossert, J. M., Stern, A. L., Theberge, F. R. M., Cifani, C., Koya, E., Hope, B. T., & Shaham, Y. (2011). Ventral medial prefrontal cortex neuronal ensembles mediate context-induced relapse to heroin. *Nature Neuroscience*, *14*(4), 420–422. https://doi.org/10.1038/nn.2758

Bouton, M. E. (2004). Context and behavioral processes in extinction. *Learning & Memory*, *11*(5), 485–494. https://doi.org/10.1101/lm.78804

Bouton, M. E. (1993). Context, time, and memory retrieval in the interference paradigms of Pavlovian learning. *Psychological Bulletin*, *114*(1), 80–99. https://doi.org/10.1037/0033-2909.114.1.80

Bouton, M. E., & Bolles, R. C. (1979). Role of conditioned contextual stimuli in reinstatement of extinguished fear. *Journal of Experimental Psychology: Animal Behavior Processes*, *5*(4), 368–378. https://doi.org/10.1037/0097-7403.5.4.368

Bouton, M. E., & Peck, C. A. (1989). Context effects on conditioning, extinction, and reinstatement in an appetitive conditioning preparation. *Animal Learning & Behavior*, *17*(2), 188–198. https://doi.org/10.3758/BF03207634

Bouton, M. E., & Ricker, S. T. (1994). Renewal of extinguished responding in a second context. *Animal Learning & Behavior*, *22*(3), 317–324. https://doi.org/10.3758/BF03209840

Bouton, M. E., Rosengard, C., Achenbach, G. G., Peck, C. A., & Brooks, D. C. (1993). Effects of Contextual Conditioning and Unconditional Stimulus Presentation on Performance in Appetitive Conditioning. *The Quarterly Journal of Experimental Psychology Section B*, *46*(1), 63–95. https://doi.org/10.1080/14640749308401095

Boyden, E. S., Zhang, F., Bamberg, E., Nagel, G., & Deisseroth, K. (2005). Millisecond-timescale, genetically targeted optical control of neural activity. *Nature Neuroscience*, *8*(9), 1263–1268. https://doi.org/10.1038/nn1525

Britt, J. P., Benaliouad, F., McDevitt, R. A., Stuber, G. D., Wise, R. A., & Bonci, A. (2012). Synaptic and Behavioral Profile of Multiple Glutamatergic Inputs to the Nucleus Accumbens. *Neuron*, *76*(4), 790–803. https://doi.org/10.1016/j.neuron.2012.09.040

Bromberg-Martin, E. S., Matsumoto, M., & Hikosaka, O. (2010). Dopamine in Motivational Control: Rewarding, Aversive, and Alerting. *Neuron*, *68*(5), 815–834. https://doi.org/10.1016/j.neuron.2010.11.022

Bukalo, O., Nonaka, M., Weinholtz, C. A., Mendez, A., Taylor, W. W., & Holmes, A. (2021). Effects of optogenetic photoexcitation of infralimbic cortex inputs to the basolateral amygdala on conditioned fear and extinction. *Behavioural Brain Research*, *396*(May), 112913. https://doi.org/10.1016/j.bbr.2020.112913

Bukalo, O., Pinard, C. R., Silverstein, S., Brehm, C., Hartley, N. D., Whittle, N., … Holmes, A. (2015). Prefrontal inputs to the amygdala instruct fear extinction memory formation. *Science Advances*, *1*(6), 1–8. https://doi.org/10.1126/sciadv.1500251

Burgos-Robles, A., Bravo-Rivera, H., & Quirk, G. J. (2013). Prelimbic and Infralimbic Neurons Signal Distinct Aspects of Appetitive Instrumental Behavior. *PLoS ONE*, *8*(2), 1–7. https://doi.org/10.1371/journal.pone.0057575

Caballero, J. P., Scarpa, G. B., Remage-Healey, L., & Moorman, D. E. (2019). Differential effects of dorsal and ventral medial prefrontal cortex inactivation during natural reward seeking, extinction, and cue-induced reinstatement. *ENeuro*, *6*(5). https://doi.org/10.1523/ENEURO.0296-19.2019

Cameron, C. M., Murugan, M., Choi, J. Y., Engel, E. A., & Witten, I. B. (2019). Increased Cocaine Motivation Is Associated with Degraded Spatial and Temporal Representations in IL-NAc Neurons. *Neuron*, *103*(1), 1–12. https://doi.org/https://doi.org/10.1016/j.neuron.2019.04.015

Capriles, N., Rodaros, D., Sorge, R. E., & Stewart, J. (2003). A role for the prefrontal cortex in stress- and cocaine-induced reinstatement of cocaine seeking in rats. *Psychopharmacology*, *168*(1–2), 66–74. https://doi.org/10.1007/s00213-002-1283-z

Chaudhri, N., Sahuque, L. L., Cone, J. J., & Janak, P. H. (2008). Reinstated ethanol-seeking in rats is modulated by environmental context and requires the nucleus accumbens core. *European Journal of Neuroscience*, *28*(11), 2288–2298. https://doi.org/10.1111/j.1460-9568.2008.06517.x

Chaudhri, N., Sahuque, L. L., Schairer, W. W., & Janak, P. H. (2010). Separable roles of the nucleus accumbens core and shell in context-and cue-induced alcohol-seeking. *Neuropsychopharmacology*, *35*(3), 783–791. https://doi.org/10.1038/npp.2009.187

Chen, W., Wang, Y., Sun, A., Zhou, L., Xu, W., Zhu, H., … Liu, H. (2016). Activation of AMPA receptor in the infralimbic cortex facilitates extinction and attenuates the heroin-seeking behavior in rats. *Neuroscience Letters*, *612*, 126–131. https://doi.org/10.1016/j.neulet.2015.11.024

Cho, J. H., Deisseroth, K., & Bolshakov, V. Y. (2013). Synaptic encoding of fear extinction in mPFC-amygdala circuits. *Neuron*, *80*(6), 1491–1507. https://doi.org/10.1016/j.neuron.2013.09.025

Chudasama, Y., Passetti, F., Rhodes, S. E. V., Lopian, D., Desai, A., & Robbins, T. W. (2003). Dissociable aspects of performance on the 5-choice serial reaction time task following lesions of the dorsal anterior cingulate, infralimbic and orbitofrontal cortex in the rat: Differential effects on selectivity, impulsivity and compulsivity. *Behavioural Brain Research*, *146*(1–2), 105–119. https://doi.org/10.1016/j.bbr.2003.09.020

Gerfen, C. R., Engber, T. M., Mahan, L. C., Susel, Z., Chase, T. N., Monsma, F. J., & Sibley, D. R. (1990). D1 and D2 Dopamine Receptor-regulated Gene Expression of Striatonigral and Striatopallidal Neurons. *Science*, *250*(4986), 1429–1432. https://doi.org/10.1126/science.2147780

Crombag, H. S., Bossert, J. M., Koya, E., & Shaham, Y. (2008). Context-induced relapse to drug seeking: a review. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *363*(1507), 3233–3243. https://doi.org/10.1098/rstb.2008.0090

Cruz, E., López, A. V., & Porter, J. T. (2014). Spontaneous recovery of fear reverses extinction-induced excitability of infralimbic neurons. *PLoS ONE*, *9*(8). https://doi.org/10.1371/journal.pone.0103596

Cruz, F. C., Koya, E., Guez-Barber, D. H., Bossert, J. M., Lupica, C. R., Shaham, Y., & Hope, B. T. (2013). New technologies for examining the role of neuronal ensembles in drug addiction and fear. *Nature Reviews Neuroscience*, *14*(11), 743–754. https://doi.org/10.1038/nrn3597

Daly, H. B. (1974). Reinforcing properties of escape from frustration aroused in various learning situations. In G. H. Bower (Ed.), The psychology of learning and motivation (Vol. 8, pp. 187-232). New York: Academic Press.

Day, J. J., Roitman, M. F., Wightman, R. M., & Carelli, R. M. (2007). Associative learning mediates dynamic shifts in dopamine signaling in the nucleus accumbens. *Nature Neuroscience*, *10*(8), 1020–1028. https://doi.org/10.1038/nn1923

Deisseroth, K. (2015). Optogenetics: 10 years of microbial opsins in neuroscience. *Nature Neuroscience*, *18*(9), 1213–1225. https://doi.org/10.1038/nn.4091

Delamater, A. R., & Westbrook, R. F. (2014). Psychological and neural mechanisms of experimental extinction: A selective review. *Neurobiology of Learning and Memory*, *108*, 38–51. https://doi.org/10.1016/j.nlm.2013.09.016

Do-Monte, F. H., Manzano-Nieves, G., Quinones-Laracuente, K., Ramos-Medina, L., & Quirk, G. J. (2015). Revisiting the Role of Infralimbic Cortex in Fear Extinction with Optogenetics. *Journal of Neuroscience*, *35*(8), 3607–3615. https://doi.org/10.1523/JNEUROSCI.3137-14.2015

Eddy, M. C., Todd, T. P., Bouton, M. E., & Green, J. T. (2016). Medial prefrontal cortex involvement in the expression of extinction and ABA renewal of instrumental behavior for a food reinforcer. *Neurobiology of Learning and Memory*, *128*, 33–39. https://doi.org/10.1016/j.nlm.2015.12.003

Euston, D. R., Gruber, A. J., & McNaughton, B. L. (2012). The Role of Medial Prefrontal Cortex in Memory and Decision Making. *Neuron*, *76*(6), 1057–1070. https://doi.org/10.1016/j.neuron.2012.12.002

Fuchikami, M., Thomas, A., Liu, R., Wohleb, E. S., Land, B. B., DiLeone, R. J., … Duman, R. S. (2015). Optogenetic stimulation of infralimbic PFC reproduces ketamine's rapid and sustained antidepressant actions. *Proceedings of the National Academy of Sciences*, *112*(26), 8106–8111. https://doi.org/10.1073/pnas.1414728112

Fuchs, R. A., Evans, K. A., Ledford, C. C., Parker, M. P., Case, J. M., Mehta, R. H., & See, R. E. (2005). The Role of the Dorsomedial Prefrontal Cortex, Basolateral Amygdala, and Dorsal Hippocampus in Contextual Reinstatement of Cocaine Seeking in Rats. *Neuropsychopharmacology*, *30*(2), 296–309. https://doi.org/10.1038/sj.npp.1300579

Gagné, R. M. (1941). External inhibition and disinhibition in a conditioned operant response. *Journal of Experimental Psychology*, *29*(2), 104–116. https://doi.org/10.1037/h0054223

Gass, J. T., Trantham-Davidson, H., Kassab, A. S., Glen, W. B., Olive, M. F., & Chandler, L. J. (2014). Enhancement of Extinction Learning Attenuates Ethanol-Seeking Behavior and Alters Plasticity in the Prefrontal Cortex. *Journal of Neuroscience*, *34*(22), 7562–7574. https://doi.org/10.1523/JNEUROSCI.5616-12.2014

Gentry, R. N., & Roesch, M. R. (2018). Neural activity in ventral medial prefrontal cortex is modulated more before approach than avoidance during reinforced and extinction trial blocks. *Journal of Neuroscience*, *38*(19), 2579–17. https://doi.org/10.1523/JNEUROSCI.2579-17.2018

Gerber, B., Yarali, A., Diegelmann, S., Wotjak, C. T., Pauli, P., & Fendt, M. (2014). Pain-relief learning in flies, rats, and man: basic research and applied perspectives. *Learning & Memory*, *21*(4), 232–252. https://doi.org/10.1101/lm.032995.113

Ghazizadeh, A., Ambroggi, F., Odean, N., & Fields, H. L. (2012). Prefrontal Cortex Mediates Extinction of Responding by Two Distinct Neural Mechanisms in Accumbens Shell. *Journal of Neuroscience*, *32*(2), 726–737. https://doi.org/10.1523/JNEUROSCI.3891-11.2012

Gibson, G. D., Prasad, A. A., Jean-Richard-dit-Bressel, P., Yau, J. O. Y., Millan, E. Z., Liu, Y., … McNally, G. P. (2018). Distinct Accumbens Shell Output Pathways Promote versus Prevent Relapse to Alcohol Seeking. *Neuron*, *98*(3), 512-520.e6. https://doi.org/10.1016/j.neuron.2018.03.033

Gilmartin, M. R., & McEchron, M. D. (2005). Single neurons in the medial prefrontal cortex of the rat exhibit tonic and phasic coding during trace fear conditioning. *Behavioral Neuroscience*, *119*(6), 1496–1510. https://doi.org/10.1037/0735-7044.119.6.1496

Giustino, T. F., Fitzgerald, P. J., Maren, S., Maren, S., Phan, K., Liberzon, I., … Phelps, E. (2016). Fear Expression Suppresses Medial Prefrontal Cortical Firing in Rats. *PLoS ONE*, *11*(10), e0165256. https://doi.org/10.1371/journal.pone.0165256

Giustino, T. F., & Maren, S. (2015). The Role of the Medial Prefrontal Cortex in the Conditioning and Extinction of Fear. *Frontiers in Behavioral Neuroscience*, *9*(November), 1–20. https://doi.org/10.3389/fnbeh.2015.00298

Gourley, S. L., & Taylor, J. R. (2016). Going and stopping: Dichotomies in behavioral control by the prefrontal cortex. *Nature Neuroscience*, *19*(6), 656–664. https://doi.org/10.1038/nn.4275

Groenewegen, H. J., Wright, C. I., Beijer, A. V. J., & Voorn, P. (1999). Convergence and segregation of ventral striatal inputs and outputs. *Annals of the New York Academy of Sciences*, *877*, 49–63. https://doi.org/10.1111/j.1749-6632.1999.tb09260.x

Gutman, A. L., Nett, K. E., Cosme, C. V., Worth, W. R., Gupta, S. C., Wemmie, J. A., & LaLumiere, R. T. (2017). Extinction of Cocaine Seeking Requires a Window of Infralimbic Pyramidal Neuron Activity after Unreinforced Lever Presses. *Journal of Neuroscience*, *37*(25), 6075–6086. https://doi.org/10.1523/JNEUROSCI.3821-16.2017

Heidbreder, C. A., & Groenewegen, H. J. (2003). The medial prefrontal cortex in the rat: Evidence for a dorso-ventral distinction based upon functional and anatomical characteristics. *Neuroscience and Biobehavioral Reviews*, *27*(6), 555–579. https://doi.org/10.1016/j.neubiorev.2003.09.003

Hoover, W. B., & Vertes, R. P. (2007). Anatomical analysis of afferent projections to the medial prefrontal cortex in the rat. *Brain Structure and Function*, *212*(2), 149–179. https://doi.org/10.1007/s00429-007-0150-4

Hurley, K. M., Herbert, H., Moga, M. M., & Saper, C. B. (1991). Efferent projections of the infralimbic cortex of the rat. *Journal of Comparative Neurology*, *308*(2), 249–276. https://doi.org/10.1002/cne.903080210

Ishikawa, A., Ambroggi, F., Nicola, S. M., & Fields, H. L. (2008). Contributions of the amygdala and medial prefrontal cortex to incentive cue responding. *Neuroscience*, *155*(3), 573–584. https://doi.org/10.1016/j.neuroscience.2008.06.037

Jones, E., Huntley, G., & Benson, D. (1994). Alpha calcium/calmodulin-dependent protein kinase II selectively expressed in a subpopulation of excitatory neurons in monkey sensory-motor cortex: comparison with GAD-67 expression. *Journal of Neuroscience*, *14*(2), 611–629. https://doi.org/10.1523/JNEUROSCI.14-02-00611.1994

Keistler, C., Barker, J. M., & Taylor, J. R. (2015). Infralimbic prefrontal cortex interacts with nucleus accumbens shell to unmask expression of outcome-selective Pavlovian-to-instrumental transfer. *Learning & Memory*, *22*(10), 509–513. https://doi.org/10.1101/lm.038810.115

Khoo, S. Y., Sciascia, J. M., Pettorelli, A., Maddux, J. M. N., & Chaudhri, N. (2019). The medial prefrontal cortex is required for responding to alcohol-predictive cues but only in the absence of alcohol delivery. *Journal of Psychopharmacology*, *33*(7), 842–854. https://doi.org/10.1177/0269881119844180

Kim, H. S., Cho, H. Y., Augustine, G. J., & Han, J. H. (2016). Selective Control of Fear Expression by Optogenetic Manipulation of Infralimbic Cortex after Extinction. *Neuropsychopharmacology*, *41*(5), 1261–1273. https://doi.org/10.1038/npp.2015.276

Kim, S. C., Jo, Y. S., Kim, I. H., Kim, H., & Choi, J. S. (2010). Lack of medial prefrontal cortex activation underlies the immediate extinction deficit. *Journal of Neuroscience*, *30*(3), 832–837. https://doi.org/10.1523/JNEUROSCI.4145-09.2010

Klein, S.B. (2009). Learning: principles and applications (5th ed.). SAGE Publications Inc.

Knapska, E., & Maren, S. (2009). Reciprocal patterns of c-Fos expression in the medial prefrontal cortex and amygdala after extinction and renewal of conditioned fear. *Learning & Memory*, *16*(8), 486–493. https://doi.org/10.1101/lm.1463909

Konorski, J. (1948). Conditioned reflexes and neuron organization. Cambridge University Press.

Konorski, J. (1967). Integrative activity of the brain. University of Chicago Press: Chicago.

Krause, M., German, P. W., Taha, S. A., & Fields, H. L. (2010). A pause in nucleus accumbens neuron firing is required to initiate and maintain feeding. *Journal of Neuroscience*, *30*(13), 4746–4756. https://doi.org/10.1523/JNEUROSCI.0197-10.2010

Kravitz, A. V., Freeze, B. S., Parker, P. R. L., Kay, K., Thwin, M. T., Deisseroth, K., & Kreitzer, A. C. (2010). Regulation of parkinsonian motor behaviours by optogenetic control of basal ganglia circuitry. *Nature*, *466*(7306), 622–626. https://doi.org/10.1038/nature09159

Lalumiere, R. T., Niehoff, K. E., & Kalivas, P. W. (2010). The infralimbic cortex regulates the consolidation of extinction after cocaine self-administration. *Learning & Memory*, *17*(4), 168–175. https://doi.org/10.1101/lm.1576810

Lalumiere, R. T., Smith, K. C., & Kalivas, P. W. (2012). Neural circuit competition in cocaine-seeking: Roles of the infralimbic cortex and nucleus accumbens shell. *European Journal of Neuroscience*, *35*(3–4), 614–622. https://doi.org/10.1111/j.1460-9568.2012.07991.x

Laque, A., L. De Ness, G., Wagner, G. E., Nedelescu, H., Carroll, A., Watry, D., … Suto, N. (2019). Anti-relapse neurons in the infralimbic cortex of rats drive relapse-suppression by drug omission cues. *Nature Communications*, *10*(1), 1–15. https://doi.org/10.1038/s41467-019-11799-1

Laurent, V., Marchand, A. R., & Westbrook, R. F. (2008). The basolateral amygdala is necessary for learning but not relearning extinction of context conditioned fear. *Learning & Memory*, *15*(5), 304–314. https://doi.org/10.1101/lm.928208

Laurent, V., & Westbrook, R. F. (2009). Inactivation of the infralimbic but not the prelimbic cortex impairs consolidation and retrieval of fear extinction. *Learning & Memory*, *16*(9), 520–529. https://doi.org/10.1101/lm.1474609

Laurent, V., & Westbrook, R. F. (2008). Distinct contributions of the basolateral amygdale and the medial prefrontal cortex to learning and relearning extinction of context conditioned fear. *Learning & Memory*, *15*(9), 657–666. https://doi.org/10.1101/lm.1080108

Lay, B. P. P., Nicolosi, M., Usypchuk, A. A., Esber, G. R., & Iordanova, M. D. (2019). Dissociation of Appetitive Overexpectation and Extinction in the Infralimbic Cortex. *Cerebral Cortex*, *29*(9), 3687–3701. https://doi.org/10.1093/cercor/bhy248

Lay, B. P., Pitaru, A. A., Boulianne, N., Esber, G. R., & Iordanova, M. D. (2020). Different methods of fear reduction are supported by distinct cortical substrates. *ELife*, *9*, 1–22. https://doi.org/10.7554/eLife.55294

Lebrón, K., Milad, M. R., & Quirk, G. J. (2004). Delayed recall of fear extinction in rats with lesions of ventral medial prefrontal cortex. *Learning & Memory*, *11*(5), 544–548. https://doi.org/10.1101/lm.78604

Leonard, A. S., Lim, I. A., Hemsworth, D. E., Horne, M. C., & Hell, J. W. (1999). Calcium/calmodulin-dependent protein kinase II is associated with the N-methyl-D-aspartate receptor. *Proceedings of the National Academy of Sciences*, *96*(6), 3239–3244. https://doi.org/10.1073/pnas.96.6.3239

Likhtik, E., Pelletier, J. G., Paz, R., & Paré, D. (2005). Prefrontal control of the amygdala. *Journal of Neuroscience*, *25*(32), 7429–7437. https://doi.org/10.1523/JNEUROSCI.2314-05.2005

Lingawi, N. W., Holmes, N. M., Westbrook, R. F., & Laurent, V. (2018). The infralimbic cortex encodes inhibition irrespective of motivational significance. *Neurobiology of Learning and Memory*, *150*(February), 64–74. https://doi.org/10.1016/j.nlm.2018.03.001

Lingawi, N. W., Westbrook, R. F., & Laurent, V. (2016). Extinction and Latent Inhibition Involve a Similar Form of Inhibitory Learning that is Stored in and Retrieved from the Infralimbic Cortex. *Cerebral Cortex*, *27*(12), 5547–5556. https://doi.org/10.1093/cercor/bhw322

Lobo, M. K., Covington, H. E., Chaudhury, D., Friedman, A. K., Sun, H., Damez-Werno, D., … Nestler, E. J. (2010). Cell Type–Specific Loss of BDNF Signaling Mimics Optogenetic Control of Cocaine Reward. *Science*, *330*(6002), 385–390. https://doi.org/10.1126/science.1188472

Lobo, M. K., Zaman, S., Damez-Werno, D. M., Koo, J. W., Bagot, R. C., DiNieri, J. A., … Nestler, E. J. (2013). ΔFosB induction in striatal medium spiny neuron subtypes in response to chronic pharmacological, emotional, and optogenetic stimuli. *Journal of Neuroscience*, *33*(47), 18381–18395. https://doi.org/10.1523/JNEUROSCI.1875-13.2013

Lubow, R. E., & Moore, A. U. (1959). Latent inhibition: The effect of nonreinforced pre-exposure to the conditional stimulus. *Journal of Comparative and Physiological Psychology*, *52*(4), 415–419. https://doi.org/10.1037/h0046700

Ma, Y. Y., Lee, B. R., Wang, X., Guo, C., Liu, L., Cui, R., … Dong, Y. (2014). Bidirectional modulation of incubation of cocaine craving by silent synapse-based remodeling of prefrontal cortex to accumbens projections. *Neuron*, *83*(6), 1453–1467. https://doi.org/10.1016/j.neuron.2014.08.023

Mackintosh, N. J. (1975). A theory of attention: Variations in the associability of stimuli with reinforcement. *Psychological Review*, *82*(4), 276–298. https://doi.org/10.1037/h0076778

Marchant, N. J., Furlong, T. M., & McNally, G. P. (2010). Medial Dorsal Hypothalamus Mediates the Inhibition of Reward Seeking after Extinction. *Journal of Neuroscience*, *30*(42), 14102–14115. https://doi.org/10.1523/JNEUROSCI.4079-10.2010

McFarland, K., & Kalivas, P. W. (2001). The circuitry mediating cocaine-induced reinstatement of drug-seeking behavior. *Journal of Neuroscience*, *21*(21), 8655–8663. https://doi.org/21/21/8655 [pii]

McLaughlin, J., & See, R. E. (2003). Selective inactivation of the dorsomedial prefrontal cortex and the basolateral amygdala attenuates conditioned-cued reinstatement of extinguished cocaine-seeking behavior in rats. *Psychopharmacology*, *168*(1–2), 57–65. https://doi.org/10.1007/s00213-002-1196-x

Mendez, P., Stefanelli, T., Flores, C. E., Muller, D., & Lüscher, C. (2018). Homeostatic Plasticity in the Hippocampus Facilitates Memory Extinction. *Cell Reports*, *22*(6), 1451–1461. https://doi.org/10.1016/j.celrep.2018.01.025

Mendoza, J., Sanio, C., & Chaudhri, N. (2015). Inactivating the infralimbic but not prelimbic medial prefrontal cortex facilitates the extinction of appetitive Pavlovian conditioning in Long-Evans rats. *Neurobiology of Learning and Memory*, *118*, 198–208. https://doi.org/10.1016/j.nlm.2014.12.006

Milad, M. R., Vidal-Gonzalez, I., & Quirk, G. J. (2004). Electrical Stimulation of Medial Prefrontal Cortex Reduces Conditioned Fear in a Temporally Specific Manner. *Behavioral Neuroscience*, *118*(2), 389–394. https://doi.org/10.1037/0735-7044.118.2.389

Milad, M. R., & Quirk, G. J. (2002). Neurons in medial prefrontal cortex signal memory for fear extinction. *Nature*, *420*(6911), 70–74. https://doi.org/10.1038/nature01138

Millan, E.Z., Furlong, T. M., & McNally, G. P. (2010). Accumbens shell-hypothalamus interactions mediate extinction of alcohol seeking. *Journal of Neuroscience*, *30*(13), 4626–4635. https://doi.org/10.1523/JNEUROSCI.4933-09.2010

Millan, E. Z., & McNally, G. P. (2011). Accumbens shell AMPA receptors mediate expression of extinguished reward seeking through interactions with basolateral amygdala. *Learning & Memory*, *18*(7), 414–421. https://doi.org/10.1101/lm.2144411

Miller, E. K. (2000). The prefontral cortex and cognitive control. *Nature Reviews Neuroscience*, *1*(1), 59–65. https://doi.org/10.1038/35036228

Miller, E. K., & Cohen, J. D. (2001). An Integrative Theory of Prefrontal Cortex Function. *Annual Review of Neuroscience*, *24*(1), 167–202. https://doi.org/10.1146/annurev.neuro.24.1.167

Moorman, D. E., & Aston-Jones, G. (2015). Prefrontal neurons encode context-based response execution and inhibition in reward seeking and extinction. *Proceedings of the National Academy of Sciences*, *112*(30), 9472–9477. https://doi.org/10.1073/pnas.1507611112

Morgan, M. A., Schulkin, J., & Ledoux, J. E. (2003). Ventral medial prefrontal cortex and emotional perseveration: The memory for prior extinction training. *Behavioural Brain Research*, *146*(1–2), 121–130. https://doi.org/10.1016/j.bbr.2003.09.021

Morgan, M. A., Romanski, L. M., & LeDoux, J. E. (1993). Extinction of emotional learning: Contribution of medial prefrontal cortex. *Neuroscience Letters*, *163*(1), 109–113. https://doi.org/10.1016/0304-3940(93)90241-C

Morgan, M., & LeDoux, J. (1995). Differential Contribution of Dorsal and Ventral Medial Prefrontal. *Behavioral Neuroscience*, *109*(4), 681–688.

Moulin, T. C., Petiz, L. L., Rayêe, D., Winne, J., Maia, R. G., Lima da Cruz, R. V., … Leão, R. N. (2019). Chronic in vivo optogenetic stimulation modulates neuronal excitability, spine morphology, and Hebbian plasticity in the mouse hippocampus. *Hippocampus*, *29*(8), 755–761. https://doi.org/10.1002/hipo.23080

Mueller, D., Bravo-Rivera, C., & Quirk, G. J. (2010). Infralimbic D2 receptors are necessary for fear extinction and extinction-related tone responses. *Biological Psychiatry*, *68*(11), 1055–1060. https://doi.org/10.1016/j.biopsych.2010.08.014

Mueller, D., Porter, J. T., & Quirk, G. J. (2008). Noradrenergic signaling in infralimbic cortex increases cell excitability and strengthens memory for fear extinction. *Journal of Neuroscience*, *28*(2), 369–375. https://doi.org/10.1523/JNEUROSCI.3248-07.2008

Müller Ewald, V. A., De Corte, B. J., Gupta, S. C., Lillis, K. V., Narayanan, N. S., Wemmie, J. A., & LaLumiere, R. T. (2018). Attenuation of cocaine seeking in rats via enhancement of infralimbic cortical activity using stable step-function opsins. *Psychopharmacology*, *236*(1), 479–490. https://doi.org/10.1007/s00213-018-4964-y

Müller, R., Bravo, R., Burckhardt, J., & Curran, T. (1984). Induction of c-fos gene and protein by growth factors precedes activation of c-myc. *Nature*, *312*(5996), 716–720. https://doi.org/10.1038/312716a0

Nicola, S. M., Yun, I. A., Wakabayashi, K. T., & Fields, H. L. (2004). Cue-Evoked Firing of Nucleus Accumbens Neurons Encodes Motivational Significance during a Discriminative Stimulus Task. *Journal of Neurophysiology*, *91*(4), 1840–1865. https://doi.org/10.1152/jn.00657.2003

Orsini, C. A., Kim, J. H., Knapska, E., & Maren, S. (2011). Hippocampal and prefrontal projections to the basal amygdala mediate contextual regulation of fear after extinction. *Journal of Neuroscience*, *31*(47), 17269–17277. https://doi.org/10.1523/JNEUROSCI.4095-11.2011

Otis, J. M., Fitzgerald, M. K., & Mueller, D. (2014). Infralimbic BDNF/TrkB Enhancement of GluN2B Currents Facilitates Extinction of a Cocaine-Conditioned Place Preference. *Journal of Neuroscience*, *34*(17), 6057–6064. https://doi.org/10.1523/JNEUROSCI.4980-13.2014

Pape, H. C., & Pare, D. (2010). Plastic synaptic networks of the amygdala for the acquisition, expression, and extinction of conditioned fear. *Physiological Reviews*, *90*(2), 419–463. https://doi.org/10.1152/physrev.00037.2009

Pascoli, V., Terrier, J., Espallergues, J., Valjent, E., O'connor, E. C., & Lüscher, C. (2014). Contrasting forms of cocaine-evoked plasticity control components of relapse. *Nature*, *509*(7501), 459–464. https://doi.org/10.1038/nature13257

Pavlov, I. P. (1927). Conditioned reflexes: an investigation of the physiological activity of the cerebral cortex. Oxford Univ. Press.

Paxinos G., & Watson C. (2007). The rat brain in stereotaxic coordinates (6th ed.). Elsevier.

Pearce, J. M., & Hall, G. (1980). A Model for Pavlovian Learning: Variations in the Effectiveness of Conditioned But Not of Unconditioned Stimuli. *Psychological Review*, *87*(6), 532–552.

Peters, J., Dieppa-Perea, L. M., Melendez, L. M., & Quirk, G. J. (2010). Induction of fear extinction with hippocampal-lnfralimbic BDNF. *Science*, *328*(5983), 1288–1290. https://doi.org/10.1126/science.1186909

Peters, J., Kalivas, P. W., & Quirk, G. J. (2009). Extinction circuits for fear and addiction overlap in prefrontal cortex. *Learning & Memory*, *16*(5), 279–288. https://doi.org/10.1101/lm.1041309

Peters, J., LaLumiere, R. T., & Kalivas, P. W. (2008a). Infralimbic Prefrontal Cortex Is Responsible for Inhibiting Cocaine Seeking in Extinguished Rats. *Journal of Neuroscience*, *28*(23), 6046–6053. https://doi.org/10.1523/JNEUROSCI.1045-08.2008

Peters, J., Vallone, J., Laurendi, K., & Kalivas, P. W. (2008). Opposing roles for the ventral prefrontal cortex and the basolateral amygdala on the spontaneous recovery of cocaine-seeking in rats. *Psychopharmacology*, *197*(2), 319–326. https://doi.org/10.1007/s00213-007-1034-2

Pfarr, S., Meinhardt, M. W., Klee, M. L., Hansson, A. C., Vengeliene, V., Schonig, K., … Sommer, W. H. (2015). Losing Control: Excessive Alcohol Seeking after Selective Inactivation of Cue-Responsive Neurons in the Infralimbic Cortex. *Journal of Neuroscience*, *35*(30), 10750–10761. https://doi.org/10.1523/JNEUROSCI.0684-15.2015

Phillipson, O. T., & Griffiths, A. C. (1985). The topographic order of inputs to nucleus accumbens in the rat. *Neuroscience*, *16*(2), 275–296. https://doi.org/10.1016/0306-4522(85)90002-8

Quirk, G. J., Garcia, R., & González-Lima, F. (2006). Prefrontal Mechanisms in Extinction of Conditioned Fear. *Biological Psychiatry*, *60*(4), 337–343. https://doi.org/10.1016/j.biopsych.2006.03.010

Quirk, G. J., & Mueller, D. (2008). Neural mechanisms of extinction learning and retrieval. *Neuropsychopharmacology*, *33*(1), 56–72. https://doi.org/10.1038/sj.npp.1301555

Quirk, G. J., Russo, G. K., Barron, J. L., & Lebron, K. (2000). The role of ventromedial prefrontal cortex in the recovery of extinguished fear. *Journal of Neuroscience*, *20*(16), 6225–6231. https://doi.org/10.1523/JNEUROSCI.20-16-06225.2000

Quiroz, C., Orrú, M., Rea, W., Ciudad-Roberts, A., Yepes, G., Britt, J. P., & Ferré, S. (2016). Local Control of Extracellular Dopamine Levels in the Medial Nucleus Accumbens by a

Glutamatergic Projection from the Infralimbic Cortex. *Journal of Neuroscience*, *36*(3), 851–859. https://doi.org/10.1523/JNEUROSCI.2850-15.2016

Reed, S. J., Lafferty, C. K., Mendoza, J. A., Yang, A. K., Davidson, T. J., Grosenick, L., … Britt, J. P. (2018). Coordinated Reductions in Excitatory Input to the Nucleus Accumbens Underlie Food Consumption. *Neuron*, *99*(6), 1260-1273.e4. https://doi.org/10.1016/j.neuron.2018.07.051

Rescorla, R. A., & Wagner, A. R. (1972). A Theory of Pavlovian Conditioning: Variations in the Effectiveness of Reinforcement and Nonreinforcement. In A.H. Black & W.F. Prokasy (Eds.), Classical conditioning II: current research and theory (pp. 64-99) New York, NY: Appleton-Century-Crofts.

Rescorla, R. A. (1967). Pavlovian conditioning and its proper control procedures. *Psychological Review*, *74*(1), 71–80. https://doi.org/10.1037/h0024109

Rhodes, S. E. V, & Killcross, A. S. (2007). Lesions of rat infralimbic cortex result in disrupted retardation but normal summation test performance following training on a Pavlovian conditioned inhibition procedure. *European Journal of Neuroscience*, *26*(9), 2654–2660. https://doi.org/10.1111/j.1460-9568.2007.05855.x

Rhodes, S. E. V., & Killcross, A. S. (2007a). Lesions of rat infralimbic cortex enhance renewal of extinguished appetitive Pavlovian responding. *European Journal of Neuroscience*, *25*(8), 2498–2503. https://doi.org/10.1111/j.1460-9568.2007.05486.x

Rhodes, S. E. V., & Killcross, S. (2004). Lesions of Rat Infralimbic Cortex Enhance Recovery and Reinstatement of an Appetitive Pavlovian Response. *Learning & Memory*, *11*(5), 611–616. https://doi.org/10.1101/lm.79704

Riaz, S., Puveendrakumaran, P., Khan, D., Yoon, S., Hamel, L., & Ito, R. (2019). Prelimbic and infralimbic cortical inactivations attenuate contextually driven discriminative responding for reward. *Scientific Reports*, *9*(1), 1–13. https://doi.org/10.1038/s41598-019-40532-7

Rocha, A., & Kalivas, P. W. (2010). Role of the prefrontal cortex and nucleus accumbens in reinstating methamphetamine seeking. *European Journal of Neuroscience*, *31*(5), 903–909. https://doi.org/10.1111/j.1460-9568.2010.07134.x

Rogers, J. L., Ghee, S., & See, R. E. (2008). The neural circuitry underlying reinstatement of heroin-seeking behavior in an animal model of relapse. *Neuroscience*, *151*(2), 579–588. https://doi.org/10.1016/j.neuroscience.2007.10.012

Sangha, S., Robinson, P. D., Greba, Q., Davies, D. A., & Howland, J. G. (2014). Alterations in reward, fear and safety cue discrimination after inactivation of the rat prelimbic and infralimbic cortices. *Neuropsychopharmacology*, *39*(10), 2405–2413. https://doi.org/10.1038/npp.2014.89

Santini, E., Ge, H., Ren, K., de Ortiz, S. P., & Quirk, G. J. (2004). Consolidation of Fear Extinction Requires Protein Synthesis in the Medial Prefrontal Cortex. *Journal of Neuroscience*, *24*(25), 5704–5710. https://doi.org/10.1523/JNEUROSCI.0786-04.2004

Santini, E., Quirk, G. J., & Porter, J. T. (2008). Fear Conditioning and Extinction Differentially Modify the Intrinsic Excitability of Infralimbic Neurons. *Journal of Neuroscience*, *28*(15), 4028–4036. https://doi.org/10.1523/JNEUROSCI.2623-07.2008

Saunders, B. T., Richard, J. M., Margolis, E. B., & Janak, P. H. (2018). Dopamine neurons create Pavlovian conditioned stimuli with circuit-defined motivational properties. *Nature Neuroscience*, *21*(8), 1072–1083. https://doi.org/10.1038/s41593-018-0191-4

Schultz, W. (1998). Predictive Reward Signal of Dopamine Neurons. *Journal of Neurophysiology*, *80*(1), 1–27. https://doi.org/10.1152/jn.1998.80.1.1

Schultz, W., Dayan, P., & Montague, P. R. (1997). A Neural Substrate of Prediction and Reward. *Science*, *275*(5306), 1593–1599. https://doi.org/10.1126/science.275.5306.1593

Shaham, Y., Shalev, U., Lu, L., De Wit, H., & Stewart, J. (2003). The reinstatement model of drug relapse: History, methodology and major findings. *Psychopharmacology*, *168*(1–2), 3–20. https://doi.org/10.1007/s00213-002-1224-x

Shansky, R. M., & Murphy, A. Z. (2021). Considering sex as a biological variable will require a global shift in science culture. *Nature Neuroscience*, *24*(4), 457–464. https://doi.org/10.1038/s41593-021-00806-8

Sierra-Mercado, D., Corcoran, K. A., Lebrón-Milad, K., & Quirk, G. J. (2006). Inactivation of the ventromedial prefrontal cortex reduces expression of conditioned fear and impairs subsequent recall of extinction. *European Journal of Neuroscience*, *24*(6), 1751–1758. https://doi.org/10.1111/j.1460-9568.2006.05014.x

Sierra-Mercado, D., Padilla-Coreano, N., & Quirk, G. J. (2011). Dissociable roles of prelimbic and infralimbic cortices, ventral hippocampus, and basolateral amygdala in the expression and extinction of conditioned fear. *Neuropsychopharmacology*, *36*(2), 529–538. https://doi.org/10.1038/npp.2010.184

Smith, M. P., & Buchanan, G. (1954). Acquisition of secondary reward by cues associated with shock reduction. *Journal of Experimental Psychology*, *48*(2), 123–126. https://doi.org/10.1037/h0054508

Sotres-Bayon, F., Bush, D. E. A., & LeDoux, J. E. (2007). Acquisition of fear extinction requires activation of NR2B-containing NMDA receptors in the lateral amygdala. *Neuropsychopharmacology*, *32*(9), 1929–1940. https://doi.org/10.1038/sj.npp.1301316

Strobel, C., Marek, R., Gooch, H. M., Sullivan, R. K. P., & Sah, P. (2015). Prefrontal and auditory input to intercalated neurons of the amygdala. *Cell Reports*, *10*(9), 1435–1442. https://doi.org/10.1016/j.celrep.2015.02.008

Stuber, G. D., Sparta, D. R., Stamatakis, A. M., Van Leeuwen, W. A., Hardjoprajitno, J. E., Cho, S., … Bonci, A. (2011). Excitatory transmission from the amygdala to nucleus accumbens facilitates reward seeking. *Nature*, *475*(7356), 377–382. https://doi.org/10.1038/nature10194

Suto, N., Laque, A., De Ness, G. L., Wagner, G. E., Watry, D., Kerr, T., … Weiss, F. (2016). Distinct memory engrams in the infralimbic cortex of rats control opposing environmental actions on a learned behavior. *ELife*, *5*(DECEMBER2016), 1–12. https://doi.org/10.7554/eLife.21920

Sutton, M. A., Sutton, M. A., Schmidt, E. F., Schmidt, E. F., Choi, K. H., Choi, K. H., … Self, D. W. (2003). Extinction-induced upregulation in AMPA receptors reduces cocaine-seeking behaviour. *Nature*, *421*(6918), 70–75. https://doi.org/10.1038/nature01248.1.

Taha, S. A., & Fields, H. L. (2006). Inhibitions of nucleus accumbens neurons encode a gating signal for reward-directed behavior. *Journal of Neuroscience*, *26*(1), 217–222. https://doi.org/10.1523/JNEUROSCI.3227-05.2006

Thompson, B. M., Baratta, M. V., Biedenkapp, J. C., Rudy, J. W., Watkins, L. R., & Maier, S. F. (2010). Activation of the infralimbic cortex in a fear context enhances extinction learning. *Learning & Memory*, *17*(11), 591–599. https://doi.org/10.1101/lm.1920810

Trask, S., Thrailkill, E. A., & Bouton, M. E. (2017). Occasion setting, inhibition, and the contextual control of extinction in Pavlovian and instrumental (operant) learning. *Behavioural Processes*, *137*, 64–72. https://doi.org/10.1016/j.beproc.2016.10.003

Trujillo-Pisanty, I., Sanio, C., Chaudhri, N., & Shizgal, P. (2015). Robust optical fiber patch-cords for in vivo optogenetic experiments in rats. *MethodsX*, *2*, 263–271. https://doi.org/10.1016/j.mex.2015.05.003

Tye, K. M., & Deisseroth, K. (2012). Optogenetic investigation of neural circuits underlying brain disease in animal models. *Nature Reviews Neuroscience*, *13*(4), 251–266. https://doi.org/10.1038/nrn3171

Van den Oever, M. C., Rotaru, D. C., Heinsbroek, J. A., Gouwenberg, Y., Deisseroth, K., Stuber, G. D., … Smit, A. B. (2013). Ventromedial Prefrontal Cortex Pyramidal Cells Have a Temporal Dynamic Role in Recall and Extinction of Cocaine-Associated Memory. *Journal of Neuroscience*, *33*(46), 18225–18233. https://doi.org/10.1523/JNEUROSCI.2412-13.2013

Vassoler, F. M., White, S. L., Hopkins, T. J., Guercio, L. A., Espallergues, J., Berton, O., … Christopher Pierce, R. (2013). Deep brain stimulation of the nucleus accumbens shell

attenuates cocaine reinstatement through local and antidromic activation. *Journal of Neuroscience*, *33*(36), 14446–14454. https://doi.org/10.1523/JNEUROSCI.4804-12.2013

Vertes, R. P. (2004). Differential projections of the infralimbic and prelimbic cortex in the rat. *Synapse*, *51*(1), 32–58. https://doi.org/10.1002/syn.10279

Vidal-Gonzalez, I., Vidal-Gonzalez, B., Rauch, S. L., & Quirk, G. J. (2006). Microstimulation reveals opposing influences of prelimbic and infralimbic cortex on the expression of conditioned fear. *Learning & Memory*, *13*(6), 728–733. https://doi.org/10.1101/lm.306106

Villaruel, F. R., Lacroix, F., Sanio, C., Sparks, D. W., Chapman, C. A., & Chaudhri, N. (2018). Optogenetic Activation of the Infralimbic Cortex Suppresses the Return of Appetitive Pavlovian-Conditioned Responding Following Extinction. *Cerebral Cortex*, *28*(12), 4210–4221. https://doi.org/10.1093/cercor/bhx275

Warren, B. L., Kane, L., Venniro, M., Selvam, P., Quintana-Feliciano, R., Mendoza, M. P., … Hope, B. T. (2019). Separate vmPFC Ensembles Control Cocaine Self-Administration Versus Extinction in Rats. *Journal of Neuroscience*, *39*(37), 7394–7407. https://doi.org/10.1523/JNEUROSCI.0918-19.2019

Warren, B. L., Mendoza, M. P., Cruz, F. C., Leao, R. M., Caprioli, D., Rubio, F. J., … Hope, B. T. (2016). Distinct Fos-Expressing Neuronal Ensembles in the Ventromedial Prefrontal Cortex Mediate Food Reward and Extinction Memories. *Journal of Neuroscience*, *36*(25), 6691–6703. https://doi.org/10.1523/JNEUROSCI.0140-16.2016

Willcocks, A. L., & Mcnally, G. P. (2013). The role of medial prefrontal cortex in extinction and reinstatement of alcohol-seeking in rats. *European Journal of Neuroscience*, *37*(2), 259–268. https://doi.org/10.1111/ejn.12031

Wood, M., Adil, O., Wallace, T., Fourman, S., Wilson, S. P., Herman, J. P., & Myers, B. (2019). Infralimbic prefrontal cortex structural and functional connectivity with the limbic forebrain: a combined viral genetic and optogenetic analysis. *Brain Structure and Function*, *224*(1), 73–97. https://doi.org/10.1007/s00429-018-1762-6

Wu, G. Y., Liu, G. L., Zhang, H. M., Chen, C., Liu, S. L., Feng, H., & Sui, J. F. (2015). Optogenetic stimulation of mPFC pyramidal neurons as a conditioned stimulus supports associative learning in rats. *Scientific Reports*, *5*(September 2014), 1–11. https://doi.org/10.1038/srep10065

Yizhar, O., Fenno, L. E., Davidson, T. J., Mogri, M., & Deisseroth, K. (2011). Optogenetics in Neural Systems. *Neuron*, *71*(1), 9–34. https://doi.org/10.1016/j.neuron.2011.06.004

Yttri, E. A., & Dudman, J. T. (2016). Opponent and bidirectional control of movement velocity in the basal ganglia. *Nature*, *533*, 402–406. https://doi.org/10.1038/nature17639