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Amygdala-cortical circuits in associative reward memory retrieval

A dissertation submitted in partial satisfaction of the

requirements for the degree of Doctor of Philosophy

in Psychology

by

Nina Teresa Lichtenberg

2019

ABSTRACT OF THE DISSERTATION

Amygdala-cortical circuits in associative reward memory retrieval

by

Nina Teresa Lichtenberg

Doctor of Philosophy in Psychology

University of California, Los Angeles, 2019

Professor Kate Wassum, Chair

Adaptive decision making requires the accurate anticipation or expectation of rewarding events. To survive in our environment, we must retrieve and use detailed associative memories of reward-predictive cues and actions taken to reach a goal to inform and guide our decisions. Often times, this cognitive process and underlying neural mechanisms can go awry, leading to maladaptive reward representation and improper choice behavior. Here, we elucidate the basic brain mechanisms of reward-expectation guided behaviors by employing neuroanatomical tracing alongside targeted pharmacological and chemogenetic manipulations of neural circuitry. The data presented here reveal novel contributions of a basolateral amygdala (BLA) opioid receptor system and of specific amygdala-cortical projection pathways to cue-guided behavior.

First, we reveal that the endogenous activation of mu-, but not delta-opioid receptors in the BLA are needed for a reward-predictive cue to guide action selection. BLA mu-opioid receptor antagonism did not disrupt the ability of a reward itself to influence actions, suggesting a selective role for this receptor in mediating cue-outcome memory retrieval. Next, we sought to understand the role of the BLA within a larger neural network, so we first used anterograde and retrograde

tract tracers to anatomically map populations of BLA projection neurons to the medial (mOFC) and lateral (lOFC) orbitofrontal cortices, and also identified reciprocal overlap in BLA-OFC (orbitofrontal) circuitry within the frontal cortex. We found spatially distinct populations of BLA→mOFC and BLA→lOFC neurons and dense overlap between OFC cell bodies and BLA terminals in the mOFC and lOFC. Thereafter, we causally manipulated pathways within the BLA-OFC network using a novel projection-specific chemogenetic approach during a series of behavioral tasks designed to assess the retrieval and use of cue- and action-outcome memories. BLA→lOFC projections were required for cue-guided action selection and responding according to a reward's current value, while BLA→mOFC projections were only required for the latter. Much like the BLA→lOFC pathway, mOFC→BLA projections were needed for reward-predictive cues to guide action selection, and for such cues to influence responding according to a reward's current value. lOFC inputs to the BLA were not needed for cue-guided action selection, and no projection was found to be necessary for action selection based on a reward's current value. Taken together, these data provide evidence that distinct projection pathways in the BLA-OFC network coordinate unique and overlapping aspects of reward expectation-guided behaviors, particularly when these behaviors are informed by reward-predictive environmental cues. Often times, mental illness is characterized by improper reward expectation or foresight because patients are deficient in mentally representing anticipated rewards and in reward valuation. Therefore, the findings presented in this work may contribute to our understanding and treatment of psychiatric disease, such as addiction, and suggest that they may arise due to dysfunctional amygdala-OFC circuits.

The dissertation of Nina Teresa Lichtenberg is approved.

Avishek Adhikari

Michael S. Fanselow

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2019

Dedicated to my mom Ella Lichtenberg.

Thank you for your love, positivity, spirit, and support in every moment.

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Burton, A.C., Bissonette, G.B., **Lichtenberg, N.T.**, Kashtelyan, V., & Roesch, M.R. (2014). Ventral striatum lesions enhance stimulus and response encoding in dorsal striatum. *Biological psychiatry*, *75* (2), 132-139.

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Oral presentations *(abbreviated)*

Lichtenberg, N.T. "Examining the role of amygdala-cortical connections and opioid receptor activation in cue-biased decision making." PSYCH201 seminar presentation (2016).

Lichtenberg, N.T. "A bottom-up amygdala-cortical circuit controls cue-triggered reward-expectation." UCLA Integrative Center for Learning and Memory Young Investigator seminar presentation (2017).

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Chapter 1: General Introduction

Efficient and adaptive decision making is ubiquitous in our daily lives. Making an optimal choice among options requires careful consideration and accurate anticipation of potential rewarding events. Initially, positive outcomes become associated with the environmental cues and/or deliberate actions preceding such rewards. These relationships are encoded as detailed associative memories. For example, the smell of ground coffee beans in the morning or motions taken to pour a cup of coffee become linked to the reward: consuming one's favorite brew. To survive in an ever-changing environment both humans and animals must utilize information in their current surroundings, such as stimuli or avaliable actions, to retrieve detailed memories of associated outcomes (Dickinson & Balleine, 1994; Balleine & Ostlund, 2007). In the current state, these associative memories inform expectations of the future, or what we believe may happen, and allow us to choose the most appropriate behavior to reach our goal. On a basic level, the ability to envision future positive events is beneficial. It helps us navigate around the grocery store to procure food or plan and execute daily activities, such as brewing and consuming coffee each morning. However, the ability to accurately represent rewarding events and any related negative consequences can become dysregulated in psychiatric diseases, including drug addiction, schizophrenia, depression, and social anxiety disorder (Everitt & Robbins, 2005; Ostlund & Balleine, 2008b; Hogarth *et al.*, 2012; Everitt & Robbins, 2016; Radulescu & Niv, 2019). How associative memories are represented in the brain at the functional and neural circuit level and used to inform everyday decisions is fundamental to our understanding of both normal and aberrant choice behavior.

1.1 Associative and behavioral processes

Goal-directed behavior, associative memory retrieval, and reward expectancy

Goal-directed behavior is an adaptive capacity, allowing us and other animals to control our environment in the service of our needs and desires. Early theoretical accounts of goal-directed behavior suggest that rather than simply being controlled by situational cues, behavior is often

influenced by *incentive motivation*, which requires a representation or expectation of the consequences of a behavioral response (Tolman, 1951). Over the last half century, the study of the psychological processes controlling performance of goal-directed, instrumental behavior by basic motivational states has evolved quite extensively.

Decades of converging evidence suggests that appropriate decision making in both humans and animals depends upon two distinct learning processes; one encoding the relationship between actions and their consequences and another involving the formation of reflexive stimulus-response associations (Balleine & O'Doherty, 2010). These cognitive constructs control goal-directed and habitual behavior, respectively. Goal-directed behavior is mediated by an association between representations of the response and efficacy of this action in procuring an outcome, *i.e.* response-outcome (R-O) contingency (Balleine & Dickinson, 1998a; Balleine & O'Doherty, 2010). Habitual behavior is governed by learned stimulus-response (S-R) associations without any link to the outcomes of those actions. Thus, goal-directed actions require forethought about the consequences of chosen actions, while habitual actions are automatic and performed in response to preexisting stimuli in the environment.

It is commonly argued that for a behavior to be considered goal-directed, an action must satisfy two criteria: the goal criterion and contingency criterion (Dickinson & Balleine, 1993; Balleine & Dickinson, 1998a). The *goal criterion* is met if the performance of an action depends on the desirability of the outcome or consequences it produces. Thus, action performance should be sensitive to motivational changes in outcome value. An animal must be hungry, or in a relevant need state, to learn to press a lever for food. In a future state, the animal must also be hungry and/or the outcome desirable, in order to exhibit goal-directed actions, otherwise lever pressing would be obsolete. The *contingency criterion* is met if the performance of an action is mediated by knowledge of the causal relationship between the action and its outcome, which is conceptualized as an expectation or belief (Tolman, 1951; Bolles, 1972; Dickinson & Balleine, 1993; Dickinson & Balleine, 1994). Suppose that during training, a hungry animal learns that there

is a relationship (R-O contingency) between an action and outcome (*e.g.,* a lever press produces food). To make an optimal choice in a future state, the animal must know, or expect, that pressing the lever will guarantee food delivery.

Apart from a known R-O contingency, reward expectancy can be informed by stimuli present in the environment that were previously associated with the outcome (*i.e.,* a conditional stimulus, CS). According to stimulus-outcome (S-O) theory, associative stimuli come to evoke an expectation of food, which will elicit natural stereotypical behaviors that prepare the animal for goal-approach responding (Balleine & Ostlund, 2007; Balleine & O'Doherty, 2009). For example, when presented with a tone in a current state, an animal previously trained to associate a tone with a food outcome (*i.e.,* S-O relationship) should approach the food receptacle because it anticipates food delivery. The S-O account of goal-approach behavior, however, may not be so simple. Ostlund and Balleine (2007) argue that encoding the approach-food R-O relationship would also increase receptacle approach behavior because the animal may infer that approach *produces* food, and not because of food anticipation (Balleine & Ostlund, 2007). Further, the influence of Pavlovian stimuli on action choice depends on the predictive status of the cue. Delamater (1995) selectively degraded one stimulus-outcome contingency prior to tests of goal approach and cue-guided action selection, and found that the degraded stimulus failed to elicit approach behavior and abolished action choice when behavior was guided by cues (*i.e.,* Pavlovian-to-instrumental transfer, see below), suggesting that cues with a high predictive validity (*i.e.,* those that provide clear information about their specific outcomes) more readily bias choice (Delamater, 1995).

Despite being seemingly at odds, the R-O and S-O accounts both depend on the idea that outcome representation, or the capacity to mentally simulate outcome relationships with the environment, governs action selection (Balleine & Ostlund, 2007). The ability to predict and control our behaviors not only puts our mind at ease, but is ecologically advantageous. Across species, organisms show a strong preference for predictable conditions, even in the face of known

undesirable consequences (Badia *et al.*, 1976). Thus, elucidating the psychological processes underlying reward prediction is essential to understanding how we survive.

Behavioral tests of reward-expectation guided behaviors

In the laboratory, reward-expectation guided behaviors can be modeled in rodents using several standardized behavioral paradigms. The paradigms described below provide evidence that action selection and conditional responding can be mediated by expectations informed by specific stimulus- or action-outcome representations, or stored memories. It is, in fact, the ability to represent future events *in detail* that enables us to optimize our decisions.

Pavlovian-to-instrumental transfer

Cues in our environment can powerfully control behavior by signaling the presence or absence of desirable outcomes (Estes & Skinner, 1941; Doya, 2008). The phenomenon of specific Pavlovian-to-instrumental transfer (PIT) is used to study goal-directed action selection. In specific-PIT, the presentation of a stimulus previously associated with a certain outcome biases choice towards actions expected to earn the same unique reward (Kruse *et al.*, 1983; Holmes *et al.*, 2010; Corbit & Balleine, 2015). During the transfer test, the presentation of conditional stimuli (CSs) have the ability to *selectively* enhance the action with which each stimulus shares a previously trained rewarding outcome. This transfer phenomenon can be interpreted from a cognitive perspective. Theories suggest that Pavlovian cues have the ability to elicit an expectation of their predicted outcome, which then motivates the selection of actions associated with that same outcome (Rescorla, 1994; Balleine & Ostlund, 2007).

Outcome devaluation

Outcome devaluation is a process by which a negative change in the value of an outcome results in a decrease in the action or approach response associated with that outcome (Colwill &

Rescorla, 1990). Outcomes can be devalued permanently by pairing an outcome with sickness induced by lithium chloride administration (Adams & Dickinson, 1981). Findings from this study provided some of the earliest evidence that lever pressing in rats, as implied by performance observed in mazes and runways (Tolman, 1951), is goal-directed and influenced by the organism's knowledge of reinforcer value. Transient devaluation is produced by feeding animals to satiety on one outcome, which reduces performance of its paired action immediately after satiety (Balleine & Dickinson, 1998b). Habitual actions are inflexible and insensitive to devaluation (Dickinson & Balleine, 1994), while goal-directed actions are flexible and typically reduced by the devaluation procedure. In a choice scenario, when two outcomes are trained, performance of the devalued action is compared to performance of the nondevalued action. Instrumental devaluation is most commonly used to study how action-outcome representations guide motivated behavior. The influence of stored stimulus-outcome (S-O) information on goal-approach behavior after a shift in value can also be assessed using a Pavlovian devaluation procedure (Holland & Straub, 1979; Johnson *et al.*, 2009).

Reinstatement

It is well known that after a period of extinction following both instrumental (Ostlund & Balleine, 2005) and Pavlovian conditioning (Donegan *et al.*, 1977), noncontingent delivery of the trained outcome will reinstate or reinvigorate performance of the instrumental action or conditional response, respectively. Until recently, much was unknown about the specific function of the reinstated outcome. Evidence from a series of experiments by Ostlund and Balleine (2007) suggest that, rather than being dependent upon motivational components of the outcome, reinstatement is mediated by the discriminative properties of the outcome through an S_O-R association (Ostlund & Balline, 2007). That is, the outcome itself is encoded as a discriminative stimulus during training and, in the current state, its mere presence acts to retrieve stored associations. Importantly, the reinstatement paradigm assesses the ability of a physically present

outcome to retrieve stored associations, and therefore, can be used as a comparison to interpret findings from measures of associative memory retrieval in unobservable scenarios (*i.e.*, PIT, devaluation).

Goal-directed behavior, reward expectation, and addiction

Drug addiction, like numerous other mental illnesses, can be characterized by the inability to accurately mentally represent future rewarding events (Everitt & Robbins, 2005; Hogarth *et al.*, 2012). According to psychological accounts of addiction, chronic drug use results from deficits in retrieving the identity of rewarding outcomes (Hogarth *et al.*, 2007; Hogarth *et al.*, 2012). Initially, drug outcomes become linked to drug seeking responses and drug-predictive stimuli, forming R-O and S-O contingencies, respectively. One account of addiction argues that the disease results from a transition in behavioral control by these associations to S-R habit and S-O (incentive value) associations (Ostlund & Balleine, 2008b), rendering behavior under the control of the reflexive habit system (Hogarth *et al.*, 2013). Indeed, drug related cues (*e.g.*, paraphernalia, people, contexts, etc.) are especially powerful in that they can contribute to relapse, even in the face of known negative consequences (Robinson & Berridge, 2001). Furthermore, a core criterion of the clinical diagnosis for dependence is that drug-seeking is resistant to the intention to quit, suggesting that drug-seeking is habitual or automatic (Hogarth & Chase, 2011).

Another account of addiction claims that drug-seeking is mediated by craving and expectations of positive events, rendering this behavior intentional and goal-directed (Hogarth & Chase, 2011). Support for this theory comes from the finding that individual differences in drug dependence are correlated with the value addicts assign to their drug of choice (Hursh & Silberberg, 2008; Hogarth & Chase, 2011). Dual-process theories claim that drug-seeking is mediated by dissociable goal-directed and habitual controllers in parallel, with each process predominating under different freely elected/choice and cue-controlled conditions, respectively

(Balleine & O'Doherty, 2010). Elucidating the psychological mechanisms underlying addiction may aid in the development of novel behavioral treatment approaches.

1.2 Neural substrates and circuitry of reward expectation-guided behavior

Basolateral amygdala (BLA)

The amygdala has traditionally been implicated in innate and learned fear (Blanchard & Blanchard, 1972; Spevack *et al.*, 1975). This view became widely accepted and studies of aversive learning, such as those using the Pavlovian fear conditioning paradigm, greatly advanced our understanding of amygdala function (LeDoux, 1993a; Maren & Fanselow, 1996; Davis *et al.*, 1997; LeDoux, 2000; Fanselow & Wassum, 2015). However, in the past decade the amygdala, particularly the basolateral nucleus (BLA), has emerged as a key structure in appetitive, goal-directed behavior and decision making. Across species, the BLA complex includes the lateral amygdala, basal and basomedial nuclei. Given that it is rather difficult to individually target amygdalar nuclei, a majority of studies on both fear and reward processing have examined both the lateral and basal nuclei as a single structure. Thus, in all proceeding text, BLA refers to both subnuclei.

The BLA has been known to process emotionally significant events regarding both fear (LeDoux, 1993b) and reward (Balleine & Killcross, 2006). In contrast to aversive conditioning studies (LeDoux, 2000), findings from appetitive conditioning experiments claim that the BLA and central amygdala (CN) nucleus function in parallel to mediate distinct aspects of emotion separate from other structures and brain networks, suggesting that the BLA plays a unique role in processing emotional information (Balleine, 2005; Balleine & Killcross, 2006).

The BLA resides within a highly interconnected cortical-subcortical network that allows information flow and exchange. Based on its anatomical connections, the BLA is thought to mediate associations between predictive stimuli and sensory-specific features of biologically significant events (Wassum & Izquierdo, 2015). BLA circuitry is remarkably conserved across

species (Janak & Tye, 2015). In rodents and nonhuman primates, the BLA is composed primarily of glutamatergic principle neurons and inhibitory interneurons, including GABAergic intercalated cell clusters located in between the BLA and CN and along the perimeter of the BLA (Millhouse, 1986; Royer *et al.*, 2000; Marowsky *et al.*, 2005). Across species, the BLA is known as a "cortical" amygdala nucleus because it receives dense glutamatergic projections carrying sensory information from the frontal cortex and thalamus (McDonald, 1998; Wassum & Izquierdo, 2015). These projections primarily target the lateral amygdala, which projects within the BLA to the basal and basomedial nuclei, and to the nearby CN. The BLA is reciprocally connected with cortical regions, including the orbitofrontal cortex, the hippocampus, and sensory association areas (McDonald, 1998; 2003). The BLA sends unidirectional outputs to the dorsomedial striatum (DMS), nucleus accumbens (NAc), bed nucleus of the stria terminalis (BNST) and CN (McDonald, 1991b). The BLA is anatomically well positioned to mediate motivated behavior by processing and integrating sensory information, but the precise role of the BLA in reward processing remains elusive.

Appetitive conditioning

Despite being an "associative hub" of incoming sensory information, studies using traditional interference methods (*i.e.*, lesions and transient inactivation) have revealed that the BLA is not needed for many measures of Pavlovian and instrumental associative encoding (Hatfield *et al.*, 1996; Parkinson *et al.*, 2000; Corbit & Balleine, 2005), nor reward-related instrumental discrimination (Schoenbaum *et al.*, 2003). As previously mentioned, a critical aspect of expectation-guided decision making is the use of stored stimulus- or action-outcome associations in guiding responding. The BLA is needed for this process, particularly when *specific* outcome representations guide choice. Studies using the PIT paradigm have supported this notion. The BLA is not required for general-PIT (Corbit & Balleine, 2005), thus does not mediate the general motivating properties of CSs over actions. However, BLA lesions disrupt the expression of

specific-PIT, a process in which *specific* outcome identities are used to guide actions (Corbit & Balleine, 2005; Balleine & Killcross, 2006). Indeed, the BLA is distinct from the adjacent CN, which is not required for specific-PIT, nor for instrumental or Pavlovian devaluation (Corbit & Balleine, 2005).

Further, evidence suggests that the BLA is needed when multiple outcomes are encoded and represented. Johnson, Gallagher, and Holland (2009) showed that post-training BLA lesions did not disrupt the performance of outcome-specific devaluation when a single outcome was trained, but rendered rats insensitive to devaluation when multiple outcomes were used in both Pavlovian and instrumental conditioning (Johnson *et al.*, 2009). These data also support the notion of *selective* motivation. When multiple associations are learned, one must distinguish between two outcomes by relying on distinct, sensory-specific features of each reward to *selectively* motivate and guide behavior. This idea contrasts to the underlying processes of general-PIT, where animals must rely on the general motivational properties of a single conditioned reward-predictive stimulus (CS) to motivate and guide behavior.

Interestingly, pre-training BLA lesions also disrupt outcome-specific devaluation (Hatfield *et al.*, 1996; Balleine *et al.*, 2003; Blundell *et al.*, 2003). In these studies, BLA-lesioned rats fail to distinguish between stimuli associated with the devalued and nondevalued outcomes, whereas non-lesioned controls showed a clear elevation in conditional responding for the nondevalued outcome. The ability to update outcome value, however, remained intact in BLA-lesioned animals because both groups consumed less of the devalued outcome post extinction (Hatfield *et al.*, 1996). Thus, results from devaluation studies further support the idea that the BLA is needed for representing and utilizing outcome-specific information in the current state.

Incentive learning

As mentioned prior, the BLA mediates the emotional or motivational significance of specific rewards. This includes the process of *incentive learning*. Studies of incentive learning seek to

evaluate the impact of motivational shifts on performance in pursuit of a reward previously experienced in a relevant need state (Balleine, 2001). For example, an increase in a state such as hunger typically increases actions that gain access to a food, but only after the individual has previously experienced food in a hungry state (Balleine *et al.*, 1995; Balleine & Dickinson, 1998b). The BLA is important for encoding the incentive value of outcomes. For example, in reward devaluation, the BLA is needed during the actual "devaluation experience" (*e.g.,* sensory-specific satiety). Parkes and Balleine (2013) showed that BLA inactivation prior to, but not following, the satiety experience makes rats insensitive to instrumental devaluation (Parkes & Balleine, 2013). Further, the BLA is required for encoding a post-training shift in value. Studies have shown that the BLA mu-opioid receptor is needed for detecting and performing actions after a positive shift in value (Wassum *et al.*, 2009; Wassum *et al.*, 2011). Thus, the BLA, and mu-opioid receptors therein, may be necessary for linking incentive value to specific outcome representations. Overall, these studies support the notion of the BLA as an integrator of incoming sensory information; the BLA may link changes in value to information provided by meaningful, or predictive, sensory stimuli.

The BLA endogenous opioid receptor system

The endogenous opioid system consists of three classes of receptors, mu, delta, and kappa, which are all recruited in response to natural rewards and drugs of abuse (Le Merrer *et al.*, 2009). All three receptors are widely expressed in the brain, especially in the cortex and limbic areas, and have long been implicated in reward-related behavior (Le Merrer *et al.*, 2009). Early studies have shown that nonspecific opioid receptor agonists are reinforcing, and antagonists induce aversive states (Mucha & Iversen, 1984; Mucha & Walker, 1987; Laurent *et al.*, 2015). Based on early studies of reward processing, this system emerged as the 'hedonic mediator' of the CNS, in that it assigns emotional or pleasurable values to reward-related stimuli (Koob & Le Moal, 1997; Berridge & Robinson, 2003).

Recent evidence suggests that delta- and mu-opioid receptors make dissociable contributions to goal-directed action selection (Laurent *et al.*, 2015). In a recent study, mice with a global knockout of the delta, but not the mu-opioid receptor, were impaired in specific-PIT (Laurent *et al.*, 2012). Mu-knockout mice, however, showed reduced sensitivity to instrumental outcome devaluation. Laurent et al. (2012) also found that antagonism of the delta-opioid receptor in the NAc shell impaired specific PIT, and that antagonism of the mu-opioid receptor in the NAc core impaired devaluation (Laurent *et al.*, 2012). Thus, the NAc core mu-opioid receptor mediates outcome value-based choice, and the NAc shell delta-opioid receptor is required for outcome identity to guide action selection. These dissociable contributions suggest a functional difference in the role of specific opioid receptors across brain areas.

Both delta- and mu-opioid receptors are densely expressed in the BLA (Mansour *et al.*, 1994a; Mansour *et al.*, 1994b). Much like the BLA itself, the BLA mu-opioid receptor is not needed for general-PIT (Mahler & Berridge, 2012), but is needed when specific outcome associations are modified to encode a positive shift in value (*i.e.*, incentive learning) (Wassum *et al.*, 2009; Wassum *et al.*, 2011). Wassum et al. (2009) found that activation of opioid receptors in the BLA is required for *encoding* an increase in incentive value, but not for *retrieving* and utilizing value related information to guide behavior (Wassum *et al.*, 2009). Also, opioid receptor blockade had no effect on increases in reward palatability, or the hedonic experience, induced by this value shift. Further, Wassum, Cely, Balleine, and Maidment (2011) extended this finding by showing that activation of the BLA mu-, but not delta- or kappa-, opioid receptor mediates the encoding of a positive shift in reward value during revaluation (Wassum *et al.*, 2011). Rather than encoding incentive value, Wassum et al. postulated that the BLA mu-opioid receptor may be needed for mediating the association between "hunger-state cues" and heightened affect, or that mu-antagonism may have altered the Pavlovian incentive value of the context. Therefore, the BLA mu-opioid receptor may play a role in both incentive learning and in Pavlovian processes.

Taken together, these findings suggest that BLA mu-opioid receptor activation may gate access to precise outcome-related contingencies. Indeed, the BLA mu-opioid receptor is located presynaptically on GABAergic neurons, where it is well positioned to regulate the excitability of cells within the BLA (Millhouse, 1986; Finnegan *et al.*, 2005; Likhtik *et al.*, 2008). Distributions of mu-opioid receptor mRNA expression compared to receptor binding is much lower, which suggests that within the BLA, the mu-opioid receptor may play a larger role in modulating input signals from the frontal cortex or sensory areas (Mansour *et al.*, 1994b). Alternatively, within the BLA, the mu-opioid receptor may modulate GABAergic inputs onto BLA projection cell bodies, thereby altering their response to incoming glutamate signals, which have been implicated in specific-PIT (Malvaez *et al.*, 2015).

Orbitofrontal cortex (OFC)

The orbitofrontal cortex (OFC) has been broadly implicated in using outcome expectancies, or predictions informed by stored associative memories, to guide behavior. However, the function of this cortical structure in such behaviors has fairly recently come to light. For decades, the OFC was thought of as the core neural substrate for response inhibition because animals and humans with OFC damage lose inhibitory control and become more impulsive in their actions (Damasio, 1996; Bechara *et al.*, 2000; Berlin *et al.*, 2004; Torregrossa *et al.*, 2008; Schoenbaum *et al.*, 2009; Stalnaker *et al.*, 2015). The proposed role of the OFC quickly expanded to include flexible stimulus-outcome encoding, inferring value, prediction error signaling, and numerous other "higher-order" cognitive functions, creating a quite exhaustive list of OFC functions across a wide array of cognition and behavior. Perhaps the best explanation of OFC function, however, is the most parsimonious. Much like the BLA, the OFC is needed when behavior is guided by mental representations of consequences not previously experienced (Balleine *et al.*, 2011; Stalnaker *et al.*, 2015). All of the functions listed above require this process.

Cue-guided behavior

Evidence suggests that the OFC plays a vital role in using Pavlovian stimulus-outcome associations to guide goal-directed behavior. Pre-training OFC lesions impair Pavlovian devaluation, but not the acquisition of stimulus-outcome contingencies (Gallagher *et al.*, 1999). Post-training lesions of the OFC prior to and after devaluation (Pickens *et al.*, 2003b; Pickens *et al.*, 2005) also make rats insensitive to devaluation. Based on these findings, the OFC may be necessary for retrieving stored stimulus-outcome associations. In support of this notion, studies using outcome-specific PIT have shown that post-training, but not pre-training, OFC lesions impair PIT performance (Ostlund & Balleine, 2007a; Scarlet *et al.*, 2012). Interestingly, in this study OFC lesions disrupted specific-PIT when they occur after, but not prior to, training. Ostlund and Balleine (2007) suggest that when the OFC is nonfunctional, specific-PIT is mediated by other regions with which the OFC interacts, which may include the BLA (Ostlund & Balleine, 2007b; a).

Studies using instrumental devaluation suggest that the OFC is *not* needed for instrumental performance. Ostlund and Balleine (2007) found that pre- and post-training OFC lesions do not disrupt instrumental choice performance after outcome devaluation (Ostlund & Balleine, 2007a). Taken together, the OFC is needed exclusively for reward-paired cues to provide predictive information about rewarding outcomes, and subsequently, to guide action choice.

Outcome expectancy-guided behavior

The OFC has been heavily implicated in using outcome representations, or expectations, to guide behavior. Findings from *in vivo* electrophysiological studies suggest that OFC enables flexible responding because OFC activity corresponds to the anticipation of outcomes (Schoenbaum *et al.*, 1998b; Tremblay & Schultz, 2000; Feierstein *et al.*, 2006; Padoa-Schioppa & Assad, 2008). Indeed, neurons in the OFC fire in response to the anticipation of reward delivery, rather than exclusively at the time of reward receipt (Schoenbaum *et al.*, 2009). Outcome expectancies also involve the representation of the value of an anticipated outcome. As mentioned

above, the OFC plays a unique role in devaluation when outcome-related information is provided by cues in the environment. Schoenbaum, Saddoris, and Stalnaker (2007) argue that, because devaluation does not require the formation of new S-O associations, deficits in Pavlovian devaluation reflect an important OFC function in modulating, or integrating, previously acquired associations in the current state (Schoenbaum *et al.*, 2007), perhaps provided by downstream brain regions.

OFC subnuclei and associative memory retrieval

Recent evidence, largely from nonhuman primate work, has suggested that the medial (mOFC) and lateral (lOFC) OFC subnuclei have specialized roles in using reward representations to guide behavior (Elliott *et al.*, 2000; Rudebeck & Murray, 2011a; Noonan *et al.*, 2017). In the rodent, these OFC subregions are anatomically distinct (Izquierdo, 2017), and recent findings suggest that the lOFC and mOFC may mediate different aspects of outcome-guided behavior. The lOFC may broadly facilitate sensory integration and choice behavior, whereas the mOFC may specialize in value-guided decision making and goal selection (Noonan *et al.*, 2010; Bradfield *et al.*, 2015; Murray *et al.*, 2015; Izquierdo, 2017; Noonan *et al.*, 2017; Bradfield *et al.*, 2018).

Although relatively unexplored in the rodent, converging evidence suggests that the mOFC is critical maintaining reward representations and for reward value evaluation. Bradfield, Dezfouli, van Holstein, Chieng, and Balleine (2015) identified a critical role for the mOFC in using action-outcome representations to guide behavior when outcomes are unobservable (*i.e.*, not present). Pre-training mOFC lesions and chemogenetic inactivation prior to testing abolished instrumental devaluation and specific-PIT. However, rats receiving mOFC lesions and inactivation prior to tests in which lever press actions were rewarded (*i.e.*, in observable task situations) did not show such deficits. Further, mOFC lesions did not affect instrumental reinstatement nor instrumental contingency degradation, suggesting that the mOFC may be needed for representing *unobservable* outcomes associated with specific actions in guiding choice behavior (Bradfield *et*

al., 2015). Moreover, in support of these findings, the mOFC is implicated in probabilistic discrimination and reversal learning (Dalton *et al.*, 2016), tasks which require accurate reward representation. Lastly, mOFC neurons fire in response to cues predicting decreases, but not increases, in reward value (Burton *et al.*, 2014; Lopatina *et al.*, 2016), thus, in addition to representing action-outcome contingencies, the mOFC may also enable the mental representation of the value of cue-predicted rewards.

BLA-OFC interactions and expectation-guided behavior

The OFC and BLA are remarkably similar in function. As outlined above, damage to either the BLA or OFC causes similar deficits when rewards must be predicted (Gallagher *et al.*, 1999; Pickens *et al.*, 2003a; Izquierdo *et al.*, 2004; Wellman *et al.*, 2005; Ostlund & Balleine, 2007a; 2008a; West *et al.*, 2011; Jones *et al.*, 2012; Rhodes & Murray, 2013; Malvaez *et al.*, 2015). Given their overlapping functions, it is perhaps not surprising that both the lOFC and mOFC share dense and reciprocal connections (Krettek & Price, 1977; Kita & Kitai, 1990; McDonald, 1991a; b; Ghashghaei & Barbas, 2002). A majority of published studies have focused on investigating connections between the BLA and lateral subregion of the OFC, so the discussion below will focus on BLA-lOFC interaction in outcome expectancy-guided behavior.

Evidence suggests that the OFC and BLA must interact to guide reward-related behavior. Disconnection of the lOFC and BLA causes deficits in reinforcer devaluation and cost-benefit decision making (Baxter *et al.*, 2000; Zeeb & Winstanley, 2013), both of which involve detailed reward anticipation. Further, associative encoding in one region has been shown to be altered by lesions to the other (Schoenbaum *et al.*, 2003; Saddoris *et al.*, 2005; Schoenbaum & Roesch, 2005; Lucantonio *et al.*, 2015). Using *in-vivo* electrophysiology, Schoenbaum et al. (2003) recorded from lOFC neurons in BLA-lesioned rats during learning and reversal of odor discriminations (Schoenbaum *et al.*, 2003). BLA lesions did not alter firing after responding in anticipation of outcome delivery, but decreased the proportion of cue-selective neurons in the

lOFC and abolished the activation of these during cue sampling. That is, BLA lesions had no effect on outcome-expectancy, but hindered the formation of stimulus-outcome associations in the lOFC during learning. What about signaling from the lOFC to the BLA? Given this finding, perhaps one would expect the opposite result if the lOFC were lesioned. In support of this notion, Saddoris et al. (2005) showed that lOFC lesions disrupted anticipatory outcome-expectant firing in the BLA, and BLA neurons became cue-selective more slowly, suggesting that the expectancy signal was generated in the lOFC (Schoenbaum & Roesch, 2005). Therefore, these studies suggest that the BLA is vital for acquiring and representing associative information, while the lOFC is involved in using this information (perhaps provided by the BLA) to generate reward expectancies and to inform and guide behavior (Schoenbaum & Roesch, 2005). Broadly speaking, the studies above suggest that BLA-lOFC interaction seems to be necessary when task parameters require the acquisition and use of stimulus-outcome information. In support, damage to either the BLA or lOFC alters the ability of a cue to become a conditioned reinforcer (Parkinson *et al.*, 2001; Pears *et al.*, 2003).

Wassum, Tolosa, Tseng, Balleine, Monbouquette and Maidment (2012) inactivated the OFC while monitoring glutamate concentration changes in the BLA during a self-paced instrumental reward-seeking task (Wassum *et al.*, 2012). They found that glutamate transient frequency was attenuated when rats were engaged in the task sequence. This suggests that lOFC-BLA interaction, specifically top-down signals from the lOFC to BLA, is needed for establishing reward value and for the use of this value information in guiding actions (Holland & Gallagher, 2004). Given that this instrumental task inherently involves cues (*e.g.* context, insertion of lever, etc.) an alternate possibility is that lOFC inputs are needed for stimulus-outcome learning. Malvaez (2015) extended on these findings by recording glutamate during specific-PIT, and found that glutamate release correlated with the PIT effect. Though they did not manipulate OFC inputs, this glutamate signal was hypothesized to arise from the OFC (Malvaez *et al.*, 2015). Building upon this finding, distinct OFC inputs to the BLA were recently found to be involved in reward value encoding and

retrieval. By using chemogenetic and optogenetic manipulations, Malvaez, Shieh, Murphy, Greenfield, and Wassum (2019) found that lOFC→BLA projections were necessary and sufficient to drive value encoding of a positive change in reward value, whereas mOFC→BLA inputs were needed and sufficient for retrieving reward value from memory (Malvaez *et al.*, 2019).

1.3 Why study neural circuits?

Our current understanding of the structure and function of the nervous system is based on foundational principles established in the nineteenth century. The neuron doctrine postulated that the nervous system is composed of individual units, called neurons, and their supporting structures (y Cajal, 1888). Single neurons connect with one another via synapses to form circuits, a concept originally proposed in the late nineteenth century (Foster & Sherrington, 1897). Today, we know that coordinated activity within these neural circuits enables all brain function, from basic perception to complex behavioral phenomena.

In recent years, technological advances that enable causal and astoundingly precise investigations of neural circuits have allowed scientists more comprehensively understand the neural mechanisms underlying cognition and mental disease. Indeed, modern approaches have revealed novel roles of brain regions and pathways in control of behaviors typically altered in states of psychopathology, including reward expectation-guided behaviors, in rodents (Lüthi & Lüscher, 2014; Kravitz *et al.*, 2015; Saunders *et al.*, 2015; Fettes *et al.*, 2017), and have greatly advanced our understanding of the human condition (Volkow *et al.*, 2013; Gordon, 2016). This has contributed to the emergence of new circuit models of psychiatric diseases, including addiction (Lüscher, 2016), and a prevalent desire across all domains of neuroscience to investigate and treat the underlying causes of brain disease with circuitry in mind.

1.4 Technical and methodological approaches to identify and investigate the neural circuitry of behavior

"Traditional techniques" in elucidating neural circuits

Although densely interconnected, manipulating single regions within the brain can (and has been extensively used to) reveal how specific structures and circuits control behavior. One classic method for determining the behavioral function of a given region is to permanently lesion it surgically, electrolytically, or chemically. Lesions are useful for examining loss-of-function, but not ideal for disentangling learning from expression, nor for repeated testing in certain behavioral paradigms. Reversible silencing of brain regions was previously achieved by local cooling or pharmacologically by administering GABA receptor antagonists, neurotransmitter receptor antagonists, or sodium channel blockers at desired behavioral time points. Local intracranial infusions of these agents, however, are limited by infusion number and require significant hardware that is often difficult to maintain, thus also not ideal for studying learning or expression over long time periods. Strategic use of lesions and/or pharmacological manipulations can provide useful information about how brain regions interact to guide behavior (Corbit *et al.*, 2013; Leung & Balleine, 2013). For example, two regions can be disconnected contralaterally by lesioning or antagonizing one region in each hemisphere. Further, neuroanatomical retrograde tract tracing combined with markers of immediate early gene expression (*e.g.*, c-Fos) enable a "snapshot" of activated projection neurons during specific behavioral measures, such as during associative memory retrieval (Leung & Balleine, 2013; Jin & Maren, 2015; Leung & Balleine, 2015).

Despite obstacles, findings from studies employing these techniques have led to fundamental insights into the role of defined brain structures and circuits. Precise control over the activity of specific neurons and neural circuits, as well as the measurement of neural activity within a circuit, can only be achieved by a new generation of genetically encoded tools.

Modern approaches in elucidating neural circuits

Advances in technology used to investigate the neural circuitry of behavior have expanded at an exponential rate over the last decade. As powerful new genetic tools used to target neural circuits are introduced, our ability to answer a wide range of complex questions about how the brain controls behavior will be more easily achievable now than ever before.

A primary first step to studying the function of circuits is to map neuronal connectivity at the gross level ("tract tracing"). Animal connectivity models, compared to human brain tissue, are particularly useful because they provide fast and decisive results in terms of precise connectivity of brain networks, and tissue does not suffer from fixation issues. Transport-based neuroanatomical tracing methods, such as anterograde (*i.e.*, forward-traveling) and retrograde (*i.e.*, backward-traveling) tracing, are most commonly used to visualize long-range projections. These methods have been utilized for nearly half a century, and can provide useful insight into the function of interconnected brain regions and allow for optimal targeting of an entire structure or subnuclei during causal manipulations.

Chemogenetics (DREADDs)

Chemogenetics is a process by which macromolecules can be engineered to interact with inert small molecules (Urban & Roth, 2015). Commonly abbreviated as DREADDS (*Designer Receptors Exclusively Activated by Designer Drugs*), this lock-and-key approach uses synthetically derived receptors and selective ligands to transiently activate or inactivate targeted neuronal populations. DREADDs are modified muscarinic receptors made insensitive to exogenous ligands but become activated by the otherwise inert ligand clozapine-n-oxide (CNO). The most widely used variants (and those that will be discussed below) are the inhibitory hM4Di, which is an engineered version of the M4 muscarinic acetycholine receptor, and excitatory hM3Dq, an engineered M3 muscarinic receptor. Other variants exist, such as the new inhibitory Gi-coupled kappa opioid receptor (KORD), which is activated by salvinorin B (SalB), but not the endogenous kappa ligand dynorphin (Marchant *et al.*, 2016). Once introduced into neural tissue

using viral mediated gene transfer, receptors can be manipulated by systemic or local administration of the activating ligand at desired behavioral time points.

Chemogenetic targeting of neural circuits

To investigate cell-type specific behavioral effects, DREADDs can be expressed in specific brain regions using a DREADD-expressing virus, transduction of which can be limited to a specific cell population by using certain promoters (Roth, 2016; Smith *et al.*, 2016). Alternatively, DREADD receptor expression can be restricted to genetically distinct cells by using a recombinase-dependent (*e.g.,* Cre) DREADD-encoding virus in a Cre-driver transgenic mouse or rat line (Urban & Roth, 2015; Smith *et al.*, 2016). To target neural circuits in a projection-specific manner, one could use the dual-virus "Retro-DREADD" approach (Urban & Roth, 2015). Retrogradely transported viral vectors can be delivered into terminal regions, or a recombinase-encoding virus can be expressed in terminals (*e.g.*, to express CAV-cre), while a second DREADD-encoding virus is delivered to cell body regions. Thus, administration of CNO results in projection-specific DREADD activation. Alternatively, after driving DREADD receptor expression in cell bodies, anterogradely-transported DREADDs expressed in axon terminals can be activated by local administration of CNO via an intracranial infusion. This approach has been successfully used to silence (Mahler *et al.*, 2014; Stachniak *et al.*, 2014; Zhu & Roth, 2014; Lichtenberg *et al.*, 2017; McGlinchey & Aston-Jones, 2018; Malvaez *et al.*, 2019) and excite (Vazey & Aston-Jones, 2014; Mahler *et al.*, 2019) local terminal activity. The most modern approach, although not widely used, would be to use an hM4Di variant capable of selectively silencing axons and axon terminals can be used to achieve the same goal (Stachniak *et al.*, 2014).

Advantages

The use of DREADDs in behavioral neuroscience has many advantages. Perhaps the greatest advantage is the ability to manipulate systems and circuits noninvasively via systemic injection of

CNO or orally (*i.e.*, via drinking water) (Rogan & Roth, 2011; Smith *et al.*, 2016). After initial intracranial surgery to deliver viral constructs, as well as time to allow for sufficient receptor expression, neurons expressing the DREADD receptor can be transiently inhibited or activated following an intraperitoneal (i.p.) injection of CNO. The drug typically exerts effects over prolonged timescales (*e.g.*, minutes to hours) (Roth, 2016), and due to the ease of i.p. injections, manipulations can occur in many animals at once and without invasive hardware or tethering. Often, for behavioral sessions lasting up to an hour, continuous excitation or inhibition of neurons over prolonged timescales is crucial. Additionally, DREADDs are spatially advantageous in that they can be used to transiently change activity within large brain regions or in several regions at once, which may be difficult to target with fiberoptics or intracranial infusions. DREADDs are also useful for transient manipulation over many days. Permanent lesions and transient intracranial microinjections are not ideal due to several aforementioned limitations. Thus, chemogenetics is especially well-suited for studying learning over time, and for testing behavioral acquisition versus expression effects (*i.e.*, learning versus performance) (Smith *et al.*, 2016). Despite these advantages, as with any new technique, DREADDs should be used only when experimentally necessary, and results interpreted cautiously.

Limitations, caveats, and considerations

The ligand CNO was chosen as an agonist because it easily penetrates the blood-brain barrier and was shown to be pharmacologically inert in mice (Alexander *et al.*, 2009; Krashes *et al.*, 2011; Zhu & Roth, 2014). However, recent evidence suggests that systemically-administered CNO is metabolized via back-transformation to clozapine. Once converted, *clozapine* crosses the blood-brain barrier and binds to DREADD receptors with a higher affinity than CNO itself, and may also bind to endogenous GPCRs, resulting in undesirable behavioral effects (*e.g.*, hypotension, sedation, etc.) (Gomez *et al.*, 2017). To resolve this, CNO can be administered at the lowest dose possible (generally 0.1-3mg/kg systemically) and appropriate controls should be used (*i.e.*,

administration of CNO to DREADD-negative control animals expressing an irrelevant fluorescent protein) (Mahler & Aston-Jones, 2018). Another option would be to use new non-CNO chemical actuators (*e.g.*, Compound 21 or perlapine, (Chen *et al.*, 2015; Thompson *et al.*, 2018), which cannot be back-metabolized in mammals (Roth, 2016).

An additional concern relates to receptor desensitization and downregulation. Following repeated administration of CNO, diminished responses may be observed as with known GCPRs due to desensitization and internalization (Roth, 2016). However, when expressed at higher levels than native GPCRs, desensitization is not of concern. On the other hand, overexpression is also an issue, especially of the hM4Di variant, which has been shown to alter the biophysical properties of neurons and expression of existing GPCRs (Saloman *et al.*, 2016).

Mechanisms of action: DREADD-mediated neuronal silencing

An important consideration in chemogenetics (and optogenetics – see below) is mechanism of action. Although the precise mechanism of action remains unclear, hM4Di appears to induce neuronal silencing via two mechanisms: induction of hyperpolarization by Gβ/γ-mediated activation of G-protein inwardly rectifying potassium channels (GIRKs) (Armbruster *et al.*, 2007) and via inhibition of presynaptic release of neurotransmitters (Stachniak *et al.*, 2014). Thus, unlike opsins, which silence neurons via a strong hyperpolarization within milliseconds (see below), DREADDs create a *weak* hyperpolarization at the soma and *strong* inhibition of presynaptic neurotransmitter release in the seconds to hours timescale (Roth, 2016).

Recent studies claim that hM4Di attenuates synaptic neurotransmitter release and does *not* affect action potential firing (Mahler *et al.*, 2014; Stachniak *et al.*, 2014; Zhu & Roth, 2014). Indeed, GIRK channels are not the only downstream actuators of $G\alpha_{i/o}$ signaling, and possibly do not play a role in attenuating transmitter release (Wiegert *et al.*, 2017). Other mechanisms may mediate the suppression of presynaptic neurotransmitter release, such as inhibition of N-type Ca^{2+} channels, and disruption of other components (Wiegert *et al.*, 2017). Whether hM4Di

silences presynaptic neurotransmitter release or reduces firing at cell bodies, one must consider differential mechanisms in cell bodies and at terminals when interpreting and comparing behavioral effects of DREADD-mediated inhibition.

Optogenetics

Optogenetic approaches to investigating brain region and circuit function have rapidly expanded alongside chemogenetics (Aston-Jones & Deisseroth, 2013). Optogenetics involves the introduction of genes encoding light-sensitive transmembrane ion conductance regulators (*i.e.,* opsins) to allow excitation or inhibition of specific neurons (Deisseroth, 2011; Aston-Jones & Deisseroth, 2013; Adamantidis *et al.*, 2015). The most commonly employed opsins are the excitatory channelrhodopsins (ChR2), light-sensitive cation channels that rapidly depolarize neurons in response to blue light (460nm), and inhibitory halorhodopsins (eNpHR3.0), light-sensitive inward chloride pumps that silence neuronal firing in response to yellow light (580nm). Alternative methods of silencing neurons include opsins Archaerhodopsin-3 (Arch) and Mac, proton pumps that move protons out of the neuron, thus decreasing the current normally generated by an action potential. Newly engineered channelrhodopsin variants have recently emerged, such as those in the ChETA family and ChIEF, which can be used to evoke faster firing frequencies in fast-spiking neurons (Gunaydin *et al.*, 2010; Tye & Deisseroth, 2012). Much like DREADDs, opsins can be introduced into neurons using viral vectors encoding opsin genes, or by using transgenic animals that express these proteins. Once expressed, opsins are activated by direct *in vivo* light stimulation at corresponding wavelengths.

Optogenetic targeting of neural circuits

Similar to chemogenetics, the use of transgenic animal lines and dual-viral approaches allows for cell-type and projection-specific targeting of opsins. Rather than activating inserted receptors pharmacologically, neuronal cell bodies or axonal processes can be directly stimulated by

implanting an optic fiber to deliver light into the opsin-expressing cell body or terminal region, respectively (Tye & Deisseroth, 2012). Like chemogenetics, Cre-recombinase and retrovirus approaches can be used to target specific projections. However, in this case, the cell bodies of neurons projecting to the Cre-injected area can be manipulated via light stimulation. Intriguingly, another advantage of optogenetics is that it allows for combinatorial manipulations at cell bodies or projection neurons. That is, the cell bodies of multiple regions projecting to the same target region can be transfected with opsins with different "activation spectra" (*i.e.,* sensitive to different wavelengths), and projections can be stimulated (or inhibited) at the common shared terminal region via multiple wavelengths of light (Tye & Deisseroth, 2012), allowing for bi-directional control of signaling and behavioral output (Nieh *et al.*, 2013).

Advantages

Optogenetic approaches have several advantages over chemogenetics and traditional interference methods. Perhaps the most significant advantage is precise temporal control: opsins can modify neural activity within milliseconds of stimulation (Rein & Deussing, 2012). With precisely times light stimulation, it is possible to mimic naturalistic firing rates or patterns of neural activity, and thus, to manipulate neural activity in a time-locked manner specific to environmental events during behavior. For example, this temporal specificity allows for precise stimulation during fine motor movements, stimulus presentation, or reward delivery (Nieh *et al.*, 2013; Saunders *et al.*, 2015). Optogenetics is also spatially specific. The ability to locally target light delivery ensures that stimulation occurs within the virally transfected brain region and/or neuronal population of interest. Further, much like chemogenetics, optogenetic manipulations can be repeated for many days without concern of opsin degradation over time, making this approach well-suited for learning experiments and repeated testing.

Limitations, caveats and considerations

Despite widespread use in behavioral neuroscience, one clear disadvantage of optogenetics is the invasive method of light delivery. For the delivery of light, surgery to implant an optical fiber is required above the brain region of interest. Despite being successfully used in freely-moving animals, hardware and tethering may limit movement during important experimental time points and optical fiber implants cause tissue damage. Wireless optogenetic tools alleviate this problem (Shin *et al.*, 2017) but are uncommon. Another important consideration is heat production and phototoxicity with light delivery. When using high powered stimulation or extended cycles, the light-induced heating may alter neuronal activity in an undesirable manner and affect cell health (Tye & Deisseroth, 2012). Overexpression of the microbial opsin is also of concern. Like chemogenetics, light-only control animals and frequent light source assessment can be employed to control for behavioral effects of heating and overexpression of the opsin.

A fundamental issue with both optogenetic and chemogenetic approaches relates to the physiological and/or behavioral relevance of these manipulations (Saunders *et al.*, 2015). Chemogenetics induces changes over extended time periods, and optogenetics activates or inhibits neurons in "bulk synchrony" and at high frequencies, both of which are not representative of endogenous, natural circuit rhythms (Saunders *et al.*, 2015). A movement towards more naturalistic, 'closed-loop' circuit manipulations may bring us closer to mimicking endogenous signaling (Häusser, 2014; Jackman *et al.*, 2014). Despite caveats, circuit-specific manipulation approaches are incredibly powerful tools. The advent of modern genetics and viral-mediated gene transfer has enabled neuroscientists across disciplines to develop and test causal hypotheses about the necessity and sufficiency of specific cells and pathways in the brain, thereby greatly advancing our understanding of the complex neural networks in control of behavior.

Chapter 2: Amygdala mu-opioid receptors mediate the motivating influence of cue-triggered reward expectations

2.1 Abstract

Environmental reward-predictive stimuli can retrieve from memory a specific reward expectation that allows them to motivate action and guide choice. This process requires the basolateral amygdala (BLA), but little is known about the signaling systems necessary within this structure. Here we examined the role of the neuromodulatory opioid receptor system in the BLA in such cue-directed action using the outcome-specific Pavlovian-to-instrumental transfer (PIT) test in rats. Inactivation of BLA mu-, but not delta-opioid receptors was found to dose-dependently attenuate the ability of a reward-predictive cue to selectively invigorate the performance of actions directed at the same unique predicted reward (*i.e.,* to express outcome-specific PIT). BLA mu-opioid receptor inactivation did not affect the ability of a reward itself to similarly motivate action (outcome-specific reinstatement), suggesting a more selective role for the BLA mu-opioid receptor in the motivating influence of currently unobservable rewarding events. These data reveal a new role for BLA mu-opioid receptor activation in the cued recall of precise reward memories and the use of this information to motivate specific action plans.

2.2 Introduction

Environmental stimuli that signal forthcoming reward can motivate reward seeking, influence action planning, and guide choice. Typically this is adaptive, but disruptions can lead to the cognitive symptoms underlying myriad psychiatric disorders. One primary way reward cues direct action is by triggering the recall of a precise memory of their specific predicted reward. This reward expectation biases choice towards and selectively motivates performance of those actions that earn the same unique reward (Kruse *et al.*, 1983; Colwill & Motzkin, 1994; Corbit & Balleine, 2015). The basolateral amygdala (BLA) is required for this cognitive process (Blundell *et al.*, 2001;

Corbit & Balleine, 2005; Ostlund & Balleine, 2008a; Malvaez *et al.*, 2015), but little is known about the signaling systems necessary within this structure.

The neuromodulatory endogenous opioid system has long been implicated in reward-related behavior (Le Merrer *et al.*, 2009) and all three opioid receptor subtypes are expressed in the BLA (Mansour *et al.*, 1994a). Delta- and mu-opioid receptors have been especially implicated and shown to make dissociable contributions (Laurent *et al.*, 2015). Indeed, reward-predictive cues are unable to selectively motivate action in mice with a global knockout of the delta-opioid receptor, while mu-knockout mice have no such deficit, though are impaired in using changes in the value of anticipated rewards to guide choice (Laurent *et al.*, 2012). Therefore, here we tested the hypothesis that BLA delta- and mu-opioid receptor activation are differentially involved in cue-directed action by evaluating the influence of BLA delta- or mu-opioid receptor inactivation on outcome-specific Pavlovian-to-instrumental transfer (PIT).

In this task, rats are trained to associate two auditory stimuli (conditioned stimuli; CSs) with two distinct food rewards and then to earn each of those two rewards by responding on independent levers. In the critical PIT test, both levers are available and CS presentation will selectively enhance performance of the action with which it shares a rewarding outcome. Because the CSs are never associated with the instrumental actions, this test assesses the rats' ability to, upon CS presentation, retrieve a stored memory of the specific predicted reward and use this expectation to guide and motivate reward-seeking actions. Under these conditions the expected reward is not observable, but rather must be cognitively represented by the subject. Data from the PIT test were, therefore, compared to choice performance influenced by presentation of a fully observable reward using the outcome-specific reinstatement task.

2.3 Materials and Methods

Subjects

Male, Long Evans rats (Experiment 1: n=35, Experiment 2: n=8, Charles River Laboratories, Wilmington, MA) weighing between 300-360 g were pair housed with no additional enrichment in a temperature (68-79 °F) and humidity-regulated (30-70%) vivarium. Training and testing took place during the dark phase of the 12:12 hr reverse dark:light cycle. Rats had *ad libitum* access to filtered tap water in the home cage and were maintained on a food-deprived schedule whereby they received 12-14 g of their maintenance diet (Lab Diet, Brentwood, MO) daily to maintain ~85-90% free-feeding body weight. All procedures were conducted in accordance with the NIH Guide for the Care and use of Laboratory Animals and approved by the UCLA Institutional Animal Care and Use Committee.

Behavioral training

Subjects were handled for 3 days prior to training. Training and testing took place in a set of 16 Med Associates (East Fairfield, VT) operant chambers, described previously (Wassum *et al.*, 2016).

Pavlovian training. Each of the 8 daily sessions consisted of 8 tone (1.5 kHz) and 8 white noise CS presentations (75 db, 2-min duration), during which either sucrose solution (20%, 0.1 ml/delivery) or grain pellets (45 mg; Bio-Serv Frenchtown, NJ), were delivered on a 30-s random-time schedule into the food-delivery port, resulting in an average of 4 stimulus-reward pairings per trial. For half the subjects, tone was paired with sucrose and noise with pellets, with the other half receiving the opposite arrangement. CSs were delivered pseudo-randomly with a variable inter-trial interval (2-4 min, mean=3 min). Entries into the food-delivery port were recorded for the entire session. Comparison of anticipatory entries during the CS-probe periods (interval between CS onset and first reward) to entries during baseline periods (2-min period prior to CS onset) provided a measure of Pavlovian conditioning.

Instrumental training. Rats were given 11 days of instrumental training, receiving 2 separate training sessions per day, one with the lever to the left of the food-delivery port and one with the right lever. Each action was reinforced with a different outcome, either grain pellets or sucrose

solution (counterbalanced with respect to the Pavlovian contingencies). Each session terminated after 30 outcomes had been earned or 30 min had elapsed. Actions were continuously reinforced on the first day, and then escalated to a random-ratio 20 schedule. The rate of responding on each lever was measured throughout training.

Surgery

After training, rats were implanted with guide cannula (22-gauge, 7 mm-length, stainless steel, Plastics One, Roanoke, VA) targeted bilaterally 1 mm above the BLA (AP -3.0 mm, ML ±5.1 mm, V -7.0 mm relative to bregma). Standard aseptic surgical procedures were used under isoflurane anesthesia (5% induction, 1-2% maintenance). The nonsteroidal anti-inflammatory agent Carprofen was administered pre- and post-operatively to minimize pain and discomfort. Following surgery rats were individually housed and allowed to recover for ~5-7 days.

Experiment 1: Pavlovian-to-instrumental transfer

After recovery, rats received 2 retraining sessions for each instrumental association (2 sessions/day for 2 days) and then one Pavlovian retraining session. On the day prior to each PIT test rats were given a single 30-min extinction session during which both levers were available, but pressing was not reinforced to establish a low level of responding. Rats were also given this retraining between each PIT test.

Rats were split into two groups, one (n=20) group receiving bilateral infusions of 0, 0.5, or 1 μg/side of the selective delta-opioid receptor antagonist naltrindole into the BLA and another (n=15) receiving 0, 0.5, or 1 μg/side of the selective mu-opioid receptor antagonist CTOP, immediately prior to the onset of the PIT test. Each rat was given 3 total PIT tests to allow within-subject drug dose comparisons (test order counterbalanced). During each PIT test, both levers were continuously present, but pressing was not reinforced. After 5 min of extinction, each 2-min CS was presented separately 4 times each in pseudorandom order, separated by a fixed 4-min

inter-trial interval. No rewards were delivered during CS presentation. The 2-min prior to each CS presentation served as the baseline control period.

Experiment 2: Outcome-specific reinstatement

Following recovery and retraining, each rat was given two reinstatement tests, one each following intra-BLA infusion of CTOP (1 μg/side) or vehicle, with intervening retraining. During each reinstatement test, both levers were continuously present, but pressing was never reinforced. After 5 min of extinction, rewards were presented in 8 separate reward-presentation periods (4 sucrose, 4 pellet periods, in pseudorandom order) separated by a fixed 4-min inter-trial interval. Each reward presentation period was 2-min in duration and began with 2 deliveries of the appropriate reward, separated by 6 s. The 2-min period prior to each reward-delivery period served as the baseline.

Drug administration

Naltrindole (Tocris Bioscience, Sterling Heights, MI) and CTOP (Tocris Bioscience; Sigma-Aldrich, St. Louis, MO) were chosen based on their selective affinities for the delta- and mu-opioid receptor, respectively (Pelton *et al.*, 1986; Portoghese *et al.*, 1988; Hyytia & Kiianmaa, 2001). The dose range for each drug was selected based on relative affinities and on previous research demonstrating an influence on reward-related behavior when infused into the BLA (Hyytia & Kiianmaa, 2001; Wassum *et al.*, 2011; Wassum *et al.*, 2016).

Drugs were dissolved in sterile saline and infused in a volume of 0.5 μl as described previously (Malvaez *et al.*, 2015; Wassum *et al.*, 2016). Previous work in which infusions were made into the adjacent amygdala central nucleus suggests that these infusion parameters restrict diffusion to the BLA (Wassum *et al.*, 2009). Testing commenced within 5 min following infusion.

Data Analysis

Data were processed with Microsoft Excel (Redmond, WA) then analyzed with GraphPad Prism (La Jolla, CA) and SPSS (IBM Corp, Chicago, IL). For all hypothesis tests, the α level for

significance was set to $P<0.05$. Analyses included repeated-measures ANOVAs (Geisser-Greenhouse correction) with Bonferroni and Dunnets post-hoc analyses used to clarify main effects and interactions, post-hoc linear regression, and Bayes factor analysis for use in supporting a null hypothesis (Gallistel, 2009; Rouder *et al.*, 2009).

For both experiments, data were analyzed for the rate of both lever pressing and entries into food-delivery port. All data were averaged across trials. For the results of Experiment 1, lever presses during the baseline period was collapsed across levers because there was no significant effect of Lever (Delta Group: $F_{1,19}=1.15$, $P=0.30$; Mu Group: $F_{1,14}=0.25$, $P=0.63$), or Lever x Drug dose interaction (Delta Group: $F_{2,38}=0.70$, $P=0.50$; Mu Group: $F_{2,28}=0.64$, $P=0.54$) on baseline press rate. This baseline pressing was compared to pressing during the CS periods, which was separated by presses on the lever that, during training, earned the same outcome as the cue predicted (*i.e.,* CS-Same presses) versus those on the other available lever (*i.e.,* CS-Different presses). Initial analyses detected no significant effects of either Cue-reward pairing, Lever-reward pairing, or Test order (Delta Group: F's=0.01-1.16, P's=0.93-0.34; Mu Group: F's=0.10-1.00, P's=0.79-0.57) and no significant interaction between these variables and Drug dose (Delta Group: F's=0.25-2.06, P's=0.73-0.17; Mu Group: F's=0.10-5.06, $P=0.80$-0.16) on lever pressing during the PIT test, so these variables were not included in the primary analyses presented below. To focus on the selective elevation in responding induced by CS presentation, in an additional analysis a difference score was computed by subtracting the baseline response rate (thereby normalizing for local response tendencies) from lever pressing during the CS period. These data were then compared across action.

The results of Experiment 2 were analyzed similarly, with reward-period presses separated for those on the lever that previously earned the same outcome as the presented reward (*i.e.,* Reinstated presses) versus those on the alternate lever (*i.e.,* Non-reinstated). Baseline response rates did differ slightly between levers during for this experiment (main effect of Lever: $F_{1,8}=21.65$, $P=0.002$), but, importantly, this did not differ between drug conditions (no Lever x Drug

interaction: $F_{1,8}$=0.43, P=0.53). During the baseline period, responding was lower on the to-be-reinstated lever than the to-be-non-reinstated lever for both the Vehicle (Non-reinstated baseline: 15.49$\pm$4.47 s.e.m.; Reinstated baseline: 12.21$\pm$3.64) and CTOP (Non-reinstated baseline: 11.06$\pm$2.55; Reinstated baseline: 5.06$\pm$1.47) conditions. Again we detected no main effect of Lever-reward pairing or Test order (Fs=0.06-0.13, Ps=0.81-0.74) and no significant interaction between these variables and Drug (Fs=0.11-4.42, P=0.76-0.09) on lever pressing during the reinstatement test and so did not include these variables in the primary analysis.

Histology

Histological verification of infusion locations was conducted as described previously (Malvaez *et al.*, 2015; Wassum *et al.*, 2016) and is presented in Figure 2-1. Three subjects were removed from Experiment 1 and 4 from Experiment 2 due to cannula misplacement and/or tissue damage.
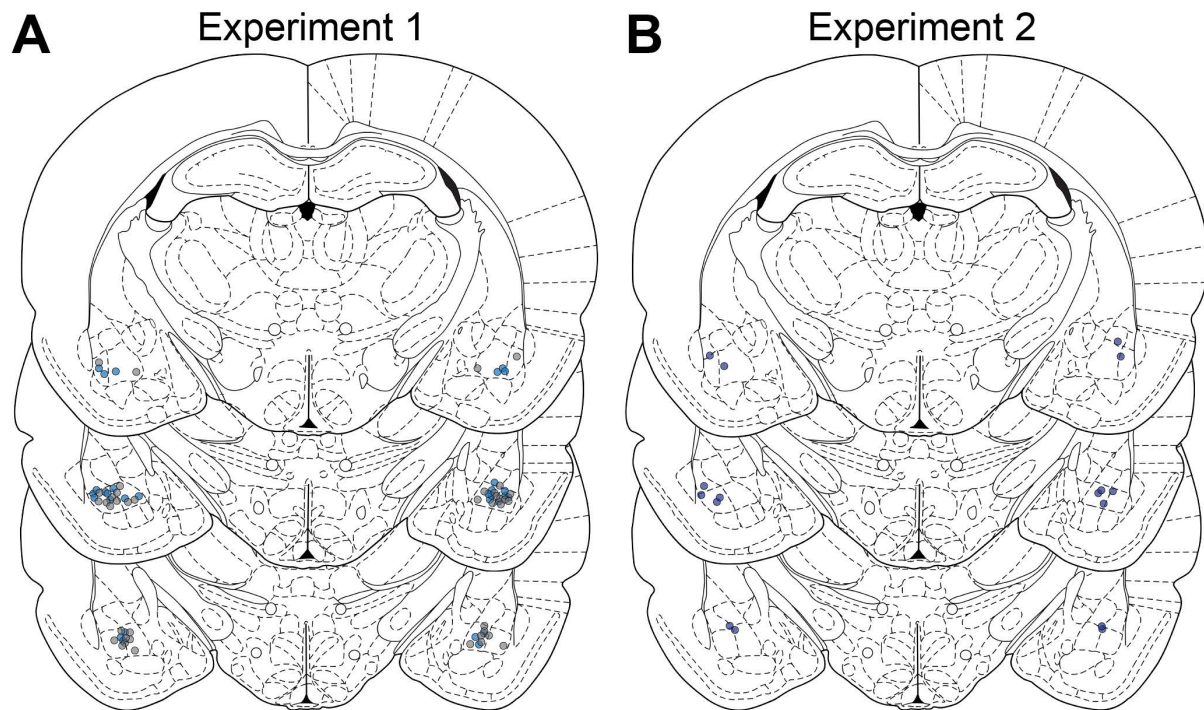
Figure 2-1. Histological verification of BLA cannula placements. A,B. Schematic representation of microinfusion injector tips for Experiment 1 **(A)** gray, delta group; blue, mu group) or Experiment 2 **(B)**. Line drawings of each section taken from (Paxinos & Watson, 1998) -2.8 – 3.3 mm posterior from bregma.

2.4 Results

Effect of BLA mu- or delta-opioid receptor inactivation on Pavlovian-to-instrumental transfer

Pavlovian conditioning was used to pair each of two distinct auditory stimuli with delivery of one of two unique, but relatively equally valued, food rewards. During the final Pavlovian session rats entered the food-delivery port significantly more during the CS probe period (Delta group: average entry rate 26.16 ± 1.38 s.e.m.; Mu group: 28.31 ± 2.47) than during the baseline period (Delta group: 14.68 ± 0.99; Mu group: 15.32 ± 1.97) and this did not differ between future drug groups (CS: $F_{1,33}=152.9$, $P<0.001$; Group: $F_{1,33}=0.40$, $P=0.53$; Group x CS: $F_{1,33}=0.58$, $P=0.45$). Rats were then trained to instrumentally earn those same food rewards by responding on independent levers. There were also no pre-existing group differences in final average press rate (Delta group: 43.01 ± 2.81; Mu group: 44.20 ± 2.95; $t_{33}=0.29$, $P=0.77$).

At the PIT test, both levers were simultaneously present and lever pressing was not rewarded. Each CS was presented 4 times in pseudorandom order (also without accompanying reward), with intervening CS-free, baseline periods. In this test, CS presentation triggers retrieval of a stored memory of the specific predicted reward, which then guides and motivates action performance in the novel choice scenario. Rats were given 3 PIT tests, one each following bilateral intra-BLA infusion of either 0 (vehicle), 0.5, or 1 µg of the selective delta-opioid receptor antagonist naltrindole (Delta group) or the selective mu-opioid receptor antagonist CTOP (Mu group).

As is clear from Figures 2-2A and B, we detected differential effects of BLA delta- and mu-opioid receptor blockade on the selective-invigorating influence of the reward-predictive cues over instrumental activity (*i.e.*, expression of outcome-specific PIT). Inactivation of BLA delta-opioid receptors did not significantly alter PIT performance (Figure 2-2A). ANOVA on these data detected a significant main effect of CS ($F_{2,38}=13.68$, $P<0.0001$), with neither an effect of Naltrindole dose ($F_{2,38}=0.37$, $P=0.69$), nor a Dose x CS interaction ($F_{4,76}=0.76$, $P=0.55$). Corrected post-hoc comparisons revealed that under each drug dose CS presentation elevated press rate

selectively on the lever that, in training, earned the same predicted reward (CS-Same) relative to both baseline press rate and pressing during the CS on the alternate available lever (CS-Different; P<0.05-0.001). Blockade of BLA mu-opioid receptors did, however, disrupt expression of outcome-specific PIT (Figure 2-2B). ANOVA on these data detected a significant effect of CS ($F_{2,28}$=5.36, P=0.01), no effect of CTOP dose ($F_{2,28}$=0.39, P=0.68), and a marginally not significant Dose x CS interaction ($F_{4,56}$=2.14, P=0.09). Robust PIT was demonstrated under vehicle control conditions; the CS elevated performance of the CS-Same action relative to both baseline (P<0.05) and CS-Different responding (P<0.01). This effect was not apparent following intra-BLA CTOP (P>0.05, in all cases) and CS-Same responding was lower following infusion of the high dose of CTOP relative to vehicle control (P<0.01). Indeed, isolated analysis of PIT performance following vehicle v. the high dose of CTOP detected a significant Drug x CS period interaction ($F_{2,28}$=3.81, P=0.048), with no main effect Drug ($F_{1,14}$=0.49, P=0.49) and a marginally not significant effect of CS Period ($F_{2,28}$=2.57, P=0.09). Bayesian analysis further supported the lack of specific PIT expression following the high CTOP dose; the null hypotheses of no difference between CS-Same pressing and either baseline or CS-Different pressing was found to be 3.46 and 3.76 times more likely, respectively, than the alternate hypothesis.

Under conditions of either BLA delta- or mu-opioid receptor blockade rats were able to show Pavlovian conditioned food-port approach responding. Entries into the food-delivery port were significantly elevated during the CS relative to the baseline period at all Naltrindole doses (Figure 2-2C). ANOVA on these data detected a significant main effect of CS ($F_{1,19}$=89.04; P<0.0001), with neither an effect of Naltrindole dose ($F_{2,38}$=0.64; P=0.53), nor a Dose x CS interaction ($F_{2,38}$=0.27; P=0.76). For the Mu group, ANOVA detected a main effect of CS ($F_{1,14}$=53.49; P<0.0001) on food-port entries, as well as an effect of CTOP dose ($F_{2,28}$=3.71; P=0.04) and a Dose x CS interaction ($F_{2,28}$=5.92; P=0.007; Figure 2-2D). Food-port entries were elevated during the CS relative to the baseline period in all conditions (P<0.001, in all cases), but were lower during the CS following intra-BLA CTOP infusion, relative to vehicle control (P<0.001).
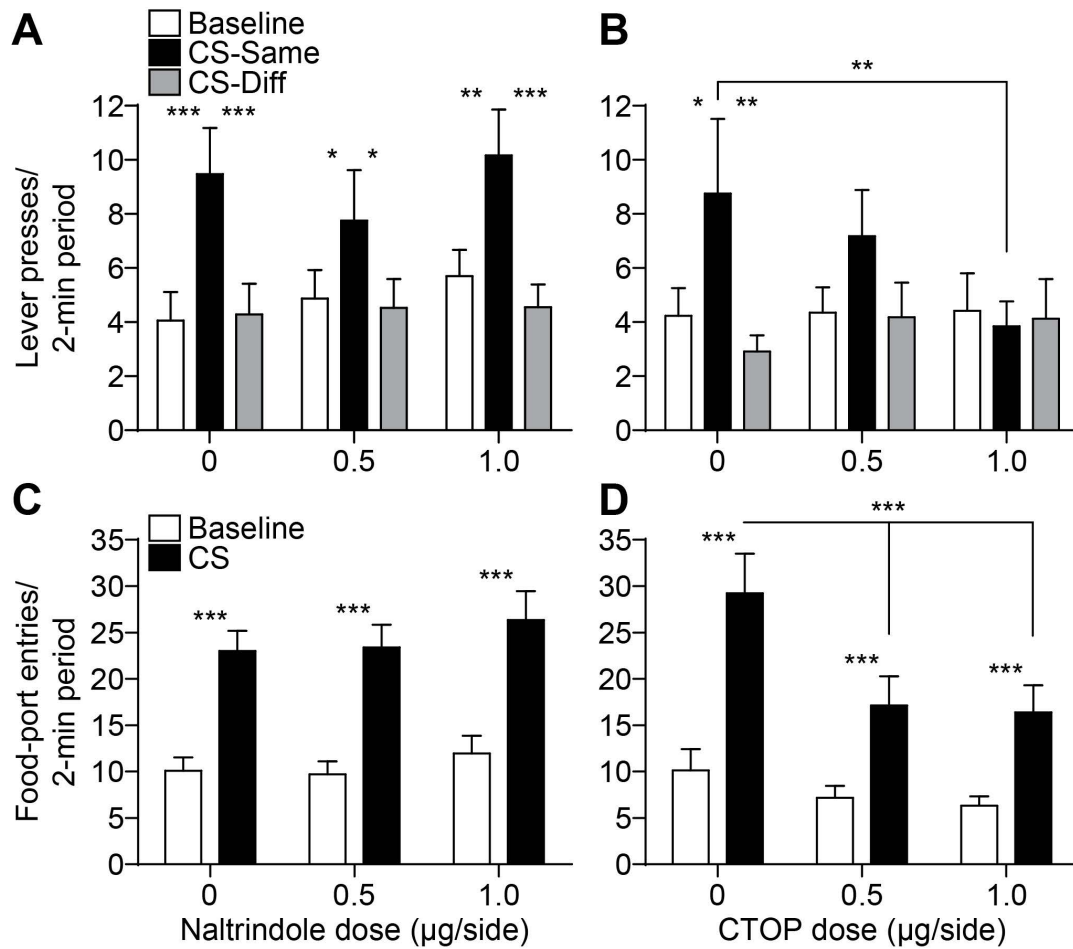
Figure 2-2. Effect of BLA delta- or mu-opioid receptor inactivation on Pavlovian-to-instrumental transfer. A, B. Trial-averaged lever presses per 2-min period averaged across both levers during the baseline periods compared to pressing during the CS separated for presses on the lever that, in training, delivered the same outcome as predicted by the CS (CS-Same) and pressing on the other available lever (CS-Diff) for the delta- **(A)** or mu-opioid receptor antagonist **(B)** group. **C, D**. Trial-averaged entries into the food-delivery port during the baseline and CS periods for the delta- **(C)** or mu-opioid receptor antagonist **(D)** group. Error bars ±s.e.m. $*P<0.05$, $**P<0.01$, $***P<0.001$.

To further clarify the effect of CTOP on the selective elevation of instrumental responding produced by the reward-predictive cues, we computed the CS-induced change in pressing by subtracting baseline press rate from both CS-Same and CS-Different pressing (Figure 2-3). ANOVA on these data exposed a main effect of Action (Same v. Different: $F_{1,14}=4.29$, $P=0.057$), no effect of CTOP dose ($F_{2,28}=1.17$, $P=0.32$), but an Action x Dose interaction ($F_{2,28}=3.20$, $P=0.056$). CS presentation caused an elevation in responding on Action Same relative to Action Different ($P<0.05$) following vehicle infusion, but this was blocked by intra-BLA infusion of CTOP at the highest dose ($P<0.05$). Highlighting the effect of drug dose, there was a significant downward linear trend for the change in Action Same performance ($R^2=0.12$, $P=0.02$) with increasing CTOP dose, which was not detected for Action Different ($R^2=0.05$, $P=0.18$).
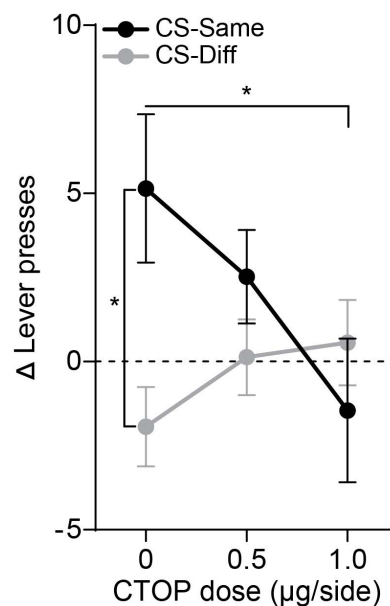
Figure 2-3. Effect of BLA mu-opioid receptor inactivation on cue-induced change in lever pressing during Pavlovian-to-instrumental transfer. CS-induced change (CS – Baseline) in lever pressing on action Same v. Different. Dashed line indicates no change from baseline. Error bars ±s.e.m. *$P<0.05$.

Effect of BLA mu-opioid receptor inactivation on outcome-specific reinstatement

The data show that blockade of BLA mu-, but not delta-opioid receptors disrupts the ability of a reward-predictive cue to selectively invigorate the performance of actions directed at the same unique reward. This phenomenon requires that the cue is able to retrieve from memory an expectation of its specific predicted reward, information that is currently unobservable. BLA mu-opioid receptor activation may, therefore, participate in this cognitive representation of specific rewards. Conversely, the BLA mu-opioid receptor may simply be needed for a reward, whether observable or not, to motivate action performance. To test between these possibilities, we evaluated the effect of intra-BLA CTOP infusion on outcome-specific reinstatement.

A separate group of rats was trained to instrumentally earn one of two unique, but relatively equally valued food rewards by responding on independent levers (average press rate: 43.26 ± 1.62). During the reinstatement test, both levers were simultaneously present and lever pressing was never rewarded. Each reward was non-contingently presented 4 times in pseudorandom order, with intervening baseline periods. In this task, reward presentation will selectively reinstate performance of the action that earns the same reward. Each rat was tested twice, once following intra-BLA infusion of vehicle and once following infusion of CTOP (1 µg). If BLA mu-opioid receptor activation is selectively required for the motivating influence of cue-elicited expectations of unobservable rewards, then BLA mu-opioid receptor inactivation should have little effect. If however, BLA mu-opioid receptor activation is required for a reward to direct action regardless of its physical presence, then inactivation of this receptor should impair performance.
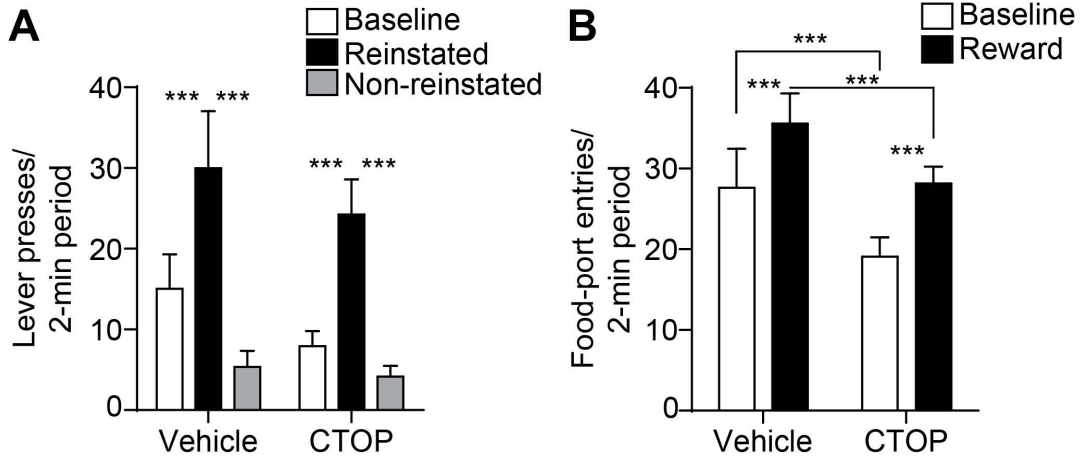
Figure 2-4. Effect of BLA mu-opioid receptor inactivation on outcome-specific reinstatement. A. Trial-averaged lever presses per 2-min period averaged across both levers during the baseline periods compared to pressing during the 2-min periods following reward delivery, separated for presses on the lever that, in training, delivered the same outcome as the presented reward (Reinstated) and pressing on the other available lever (Non-reinstated). **B.** Trial-averaged entries into the food-delivery port during the baseline and reward periods. Error bars ±s.e.m. ***$P<0.001$.

The data provide support for the former. As can be seen in Figure 2-4A, BLA mu-opioid receptor inactivation was without effect on reinstatement performance. ANOVA on these data detected a significant main effect of Reward delivery ($F_{2,14}=16.63$, $P<0.001$), with neither an effect of Drug ($F_{1,7}=1.57$, $P=0.25$), nor a Reward x Drug interaction ($F_{2,14}=0.94$, $P=0.41$). Following intra-BLA infusion of either vehicle or CTOP reward presentation selectively elevated press rate on the lever that, in training, earned the same unique reward (Reinstated) relative to both baseline press rate and pressing on the alternate available lever (Non-reinstated; $P<0.001$, in all cases). Entries into the food-delivery port were also elevated by reward delivery under both conditions, though there was an overall attenuation of this behavior following intra-BLA mu-opioid receptor blockade, similar to that detected during the PIT test (Figure 2-4B). ANOVA on these data detected significant main effects of Reward delivery ($F_{1,7}=8.38$, $P=0.02$) and of Drug ($F_{1,7}=8.02$, $P=0.03$), with no interaction between these factors ($F_{1,7}=0.4$, $P=0.55$). Food-port entries were elevated during the reward period relative to the baseline period in all conditions ($P<0.001$, in

both cases), but lower following intra-BLA CTOP infusion relative to vehicle control ($P<0.001$, in both cases).

2.5 Discussion

One major source of reward-seeking motivation is the cognitive expectation of specific rewards, information that is often provided by environmental cues. Here we show that endogenous activation of mu-, but not delta-opioid receptors in the BLA is required for a reward-predictive cue to selectively invigorate the performance of actions directed at the same unique predicted reward. Though we note that these effects should be considered in the context on the high variability in PIT performance under control conditions. BLA mu-opioid receptor activation was found not to be required for a reward itself to similarly motivate action. These data reveal a new role for BLA mu-opioid receptor activation in the cued recall of precise reward memories and the use of this information to motivate the execution of specific action plans.

The data demonstrate differential roles for BLA delta- and mu-opioid receptor activation in the expression of outcome-specific PIT. Surprisingly, this was in the opposite direction to that expected based on behaviors observed after globally knocking out these receptors. Delta-opioid receptor knockout mice are unable to show PIT, an effect that has been localized to the nucleus accumbens shell (Laurent *et al.*, 2012), and shown here not to require BLA delta-opioid receptor activity. Conversely, mu-opioid receptor knockout leaves PIT intact (Laurent *et al.*, 2012). The current finding of attenuated PIT following blockade of BLA mu-opioid receptors suggests, therefore, the presence of compensatory mechanisms for this behavior in the mu-knockout mouse, or perhaps differing functions for mu-opioid receptor activation across brain regions.

The BLA is required for the selective motivation of action elicited either by reward-predictive cues (Blundell *et al.*, 2001; Corbit & Balleine, 2005; Ostlund & Balleine, 2008a) or by physically present rewards (Ostlund & Balleine, 2008a). The data here reveal that BLA mu-opioid receptor activation is only needed for the former. BLA mu-opioid receptor activation was required when

the subject had to, upon cue presentation, retrieve a specific reward expectation from memory, information that was previously observed, but was not presently observable, and was not required when this information was fully observable. Disruption of the retrieval of specific reward memories could also explain the slight attenuation of goal-approach responding in both tasks, which may have to a more limited extent been motivated by such information. The lack of an impairment in outcome-specific reinstatement also suggests that BLA mu-opioid receptor activation is not required for rats to access knowledge of the specific consequences of their instrumental actions. It is also unlikely that the BLA or mu-opioid receptor activation therein is required for the decision-making process itself. Were this the case, BLA mu-opioid receptor inactivation would have resulted in a non-specific CS-induced increase in instrumental responding, indicating an inability to select between actions on the basis of the CS-provided specific reward expectation. Instead, BLA mu-opioid receptor blockade only attenuated the selective motivating influence of CSs, similar to the effect of BLA inactivation (Corbit & Balleine, 2005; Ostlund & Balleine, 2008a; Malvaez *et al.*, 2015).

The BLA is thought to encode motivationally-salient, precise reward memories (Wassum & Izquierdo, 2015). Indeed, neither the BLA (Corbit & Balleine, 2005), nor BLA mu-opioid receptor activation (Mahler & Berridge, 2012) is needed for the expression of the more general form of PIT, in which less precise, more gist-like reward memories can non-discriminately motivate action. Interestingly, BLA mu-opioid receptor activation is also required when the memory of a specific reward is modified to encode a positive shift in value (Wassum *et al.*, 2009; Wassum *et al.*, 2011). Together, these data suggest that BLA mu-opioid receptor activation may regulate access to these specific reward memories, perhaps by modulating GABAergic inputs onto BLA projection cells (Finnegan *et al.*, 2006), thereby altering their response to the incoming glutamate signals shown previously to encode these precise reward memories (Malvaez *et al.*, 2015). This speculation is consistent with the proposed function of the GABAergic, mu-expressing intercalated cells to gate the influence of afferent sensory input over BLA projections (Millhouse, 1986; Likhtik *et al.*, 2008;

Asede *et al.*, 2015). Intra-BLA CTOP infusion here likely disrupted activity at both these mu receptors and those expressed, albeit more sparsely, in the BLA itself (Ding *et al.*, 1996; Zhang *et al.*, 2015).

In summary, these findings support a role for BLA mu-opioid receptor activation in use of cue-recalled precise reward expectations to motivate specific action plans. Deficits in this cognitive process have been associated with several psychiatric disorders, including depression, schizophrenia, and drug addiction (Seymour & Dolan, 2008; Hogarth *et al.*, 2013; Morris *et al.*, 2015). These data, therefore, have implications for the understanding and treatment of these and related conditions. They may also help to explain the clinical efficacy of naltrexone, an opioid receptor antagonist with affinity for mu-opioid receptors in humans (Toll *et al.*, 1998) that has been shown to reduce cue-induced urges to use drug in smokers (Hutchison *et al.*, 1999) and alcoholics (Monti *et al.*, 1999; Rohsenow *et al.*, 2000; O'Malley *et al.*, 2002).

Chapter 3: Neuroanatomical explorations of amygdala-cortical circuitry

3.1 Introduction

With recent technological advances in the study of neural circuitry, neuroscientists can now map complex anatomical circuits onto behavior. The precise anatomical identification of neuronal connections is a fundamental first step to experimentally determine how brain networks control cognition and behavior. Across species, the basolateral amygdala (BLA) and orbitofrontal cortex (OFC) not only share behavioral function, but are densely interconnected (Krettek & Price, 1977; Kita & Kitai, 1990; McDonald, 1991b; a; Ghashghaei & Barbas, 2002; Hoover & Vertes, 2011; Reppucci & Petrovich, 2016). Apart from a handful of retrograde and anterograde tract tracing experiments, reciprocal connections between the BLA and subnucleai of the OFC remain relatively unmapped.

The BLA sends outputs widely throughout frontal cortical regions and its excitatory projections are particularly dense to the midline and orbital prefrontal cortices (Kita & Kitai, 1990; Janak & Tye, 2015). The BLA complex includes the lateral amygdala, basal, and basomedial nuclei, each of which contain projection cells that often cluster based on output target (Kita & Kitai, 1990; Reppucci & Petrovich, 2016). For example, using retrograde tract tracing Reppucci and Petrovich (2016) showed that BLA projections to the medial OFC (mOFC) are located primarily within the basal amygdalar nucleus (Reppucci & Petrovich, 2016). The organization of BLA projections to the lateral OFC (lOFC) are less explored. Older studies have anatomically mapped these output neurons retrogradely, but in these studies tracer loci extended beyond lOFC boundries into the agranular insula (AI) (McDonald, 1991a; 1992). In addition, quantitative comparisons between distinct populations of BLA outputs to multiple subnuclei of the orbitofrontal cortex in the same subject are lacking. Furthermore, collateralization is a defining feature of projection neurons, and synaptic inputs from one population of projection cells onto different targets may support diverse behaviors. Apart from a few studies, evidence of BLA

collaterals bifurcating to multiple frontal cortical targets remains paltry (Sarter & Markowitsch, 1984).

Broadly speaking, the BLA receives sensory information about the external environment from the thalamus and various sensory cortices, including the frontal cortex, which projects densely to the lateral nucleus of the amygdala (McDonald, 1998; Wassum & Izquierdo, 2015). Much like BLA afferents, BLA inputs from frontal regions are primarily principle glutamatergic neurons. The mOFC sends excitatory projections throughout several limbic areas, including the basolateral and central amygdala (Gabbott *et al.*, 2005; Hoover & Vertes, 2011). Similarly, a substantial population of neurons in the lOFC also project to the BLA (Gabbott *et al.*, 2005; Price, 2007; Rempel-Clower, 2007). By using anterograde tracers, researchers have also spatially mapped BLA terminal spread in the mOFC and lOFC (Kita & Kitai, 1990; Malvaez *et al.*, 2019a). These studies, however, did not identify reciprocal anatomical overlap within a single circuit between cell bodies in one region and terminals arising from a distal region. Thus, whether or not there is reciprocal overlap between projections within BLA-OFC circuits is unknown.

The BLA sends afferents to both the mOFC and lOFC, so the first aim of this study was to map and quantify the proportion of BLA output neurons projecting to the mOFC or lOFC, as well as to identify and quantify BLA collateral neurons projecting to both orbital subregions. The second aim was to anatomically visualize overlap between mOFC→BLA and lOFC→BLA projection cell bodies and terminals arising from the BLA in the mOFC and lOFC. To do so, we used a modern dual-virus tracing approach which involved injecting a mixture of an anterograde and retrograde virus, each conjugated to a distinct fluorophore (*i.e.*, mCherry or EGFP), into the BLA and then imaging OFC subregions for reciprocal anatomical overlap.

3.2 Materials and Methods

Subjects

Male and female Long Evans rats (Experiment 1: n=4, Experiment 2: n=3, Charles River Laboratories, Wilmington, MA) weighing between 280-400g were pair housed on a 12:12 hr reverse dark:light cycle with access to *ad libitum* access to filtered tap water and chow (Lab Diet, Brentwood, MO). All procedures were conducted in accordance with the NIH Guide for the Care and use of Laboratory Animals and approved by the UCLA Institutional Animal Care and Use Committee.

Viral constructs

For Experiment 2, to identify overlap between terminals of neurons projecting from BLA to the OFC and cell bodies of neurons projecting from these subregions back to the BLA, we used an anterograde and retrograde viral cocktail (Figure 3-2). The anterograde adeno-associated virus (AAV) expressing the fluorophore mCherry under the human synapsin promoter (AAV8-hsyn-mCherry, viral concentration: 4.6×10^{12} vg/ml; University of North Carolina Vector Core, Chapel Hill, NC) was mixed with the retrograde virus (AAVrg-hsyn-EGFP, viral concentration: 7.4×10^{12} vg/ml; Addgene) in equal proportions and infused into the BLA.

Surgery

For both experiments, standard aseptic surgical procedures were used under isoflurane anesthesia (5% induction, 1-2% maintenance). For Experiment 1, 33-gauge injectors were lowered into the mOFC (AP +4.1 mm, ML ±0.7 mm, DV -5.0 mm) and lOFC (AP +3.0 mm, ML ±3.2 mm, DV -5.8 mm from bregma). AlexaFluor-594 and AlexaFluor-488 conjugated cholera toxin B (CTb) (5μg/μL; Life Technologies, San Diego, CA) were ipsilaterally infused at a volume of 0.4 μL (mOFC) or 0.6 μL (lOFC) at a flow rate of 0.1 μL/min. Injectors were left in place for an additional 10 min to allow diffusion of CTb. Following surgery rats were individually housed and allowed to recover for 2 weeks post-op.

For Experiment 2, ipsilateral virus injections were made into the BLA (AP -3.0 mm, ML ±5.1 mm, DV -8.6 mm relative to bregma). A cocktail of both AAV-mCherry and AAVrg-EGFP was

infused into the BLA at a rate of 0.1 µL/min at a volume of 0.5 µL. Injectors were left in place for 10 min post-infusion to allow for viral diffusion. Rats were individually housed and allowed to recover for ~7-8 weeks to allow for sufficient retrograde transport and terminal expression of fluorophores. For both experiments, the nonsteroidal anti-inflammatory agent Carprofen was administered pre- and post-operatively to minimize pain and discomfort.

Image Analysis

In Experiment 1, BLA images ipsilateral to the hemisphere containing CTb injections were captured. For each subject, 4 images per AP plane of the anterior (-2.3 to -2.56 mm posterior to bregma), middle (-2.8 to -3.14 mm posterior to bregma), and posterior (-3.3 to -3.6) BLA were acquired for quantification. All images were taken at 20X magnification using a Keyence microscope (BZ-X710; Osaka, Japan). For each BLA image, single- and double-labeled neurons for each fluorophore were counted using a custom written script in ImageJ (National Institutes of Health, Bethesda, MD, version 1.50i). For Experiment 2, images were captured at 4X and 20X magnification, and no cell quantification was performed.

Histology

For both experiments, after sufficient tract tracer and viral anterograde and retrograde transport, rats were deeply anesthetized with Nembutal and transcardially perfused with PBS followed by 4% paraformaldehyde. Brains were extracted and post-fixed overnight, placed into 30% sucrose solution, then sectioned into 40 µm slices and stored in PBS or cryoprotectant. For Experiment 2, the signal for axonal expression of mCherry in the OFC was immunohistochemically amplified using antibodies directed against mCherry. Floating coronal sections were washed 2 times in 1X PBS for 10 min and then blocked in a solution of 5% normal goal serum (NGS) and 1% Triton X-100 dissolved in PBS for 1-2 hrs at room temperature. Sections were then washed 3 times in PBS for 15 min and then incubated in blocking solution containing rabbit anti-DsRed antibody (1:1000; EMD Millipore, Billerica, MA) with gentle agitation at 4°C

for 18-22 hrs. Sections were next rinsed 3 times in the blocking solution and incubated in Alexa Fluor 594-conjugated (red) goat secondary antibody (1:500; Invitrogen) for 2 hr. Sections were washed 3 times in PBS for 30 mins, mounted on slides, and coverslipped with ProLong Gold mounting medium with DAPI (Invitrogen, Carlsbad, CA). For Experiment 1, we verified CTb placement within boundaries of the mOFC and lOFC (Figure 3-1A). Data and images from subjects in which CTb or AAV-mCherry + AAVrg-EGFP could not be confirmed as on target were excluded from analysis.

Data Analysis

For Experiment 1, data were processed with Microsoft Excel (Redmond, WA) then analyzed with GraphPad Prism (La Jolla, CA).
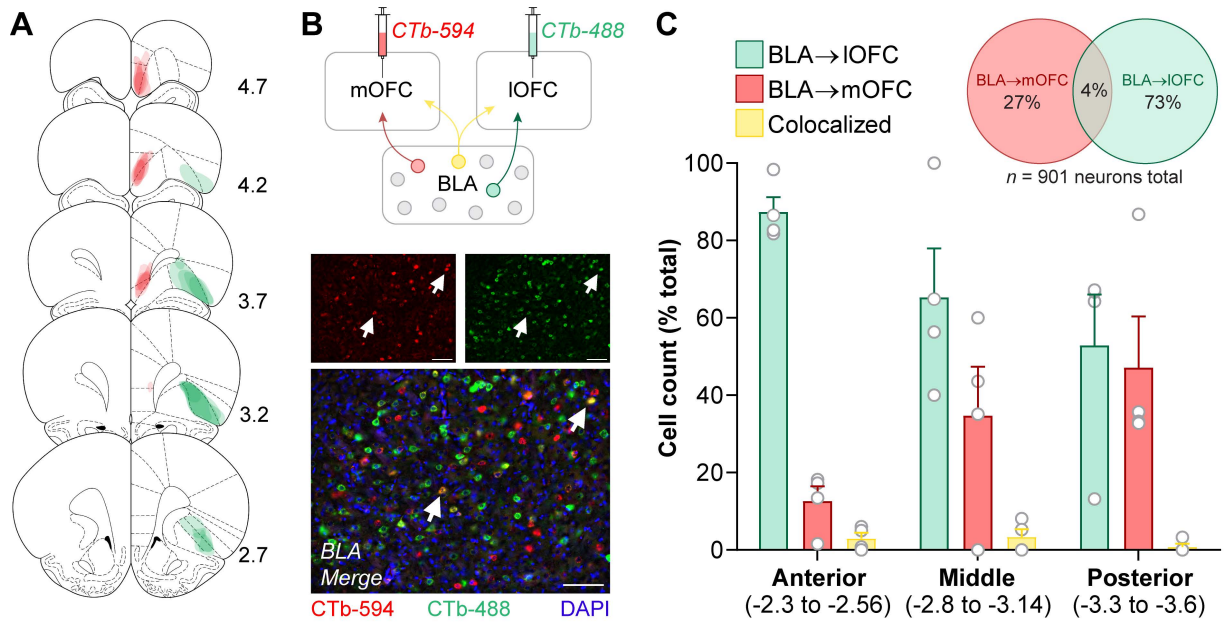
Figure 3-1. Alexa fluor conjugated cholera toxin B (CTb) infusion sites within the mOFC and lOFC and BLA projection neurons. A. Schematic representation of CTb spread in mOFC (red) and lOFC (green). Line drawings of each section taken from (Paxinos & Watson, 1998) 2.7 – 4.7 mm anterior from bregma. **B.** Experimental schematic (top) and representative fluorescent image of CTb-labeled BLA→mOFC projecting neurons (red), BLA→lOFC projecting neurons (green), and co-labeled dual-projecting neurons (mOFC + lOFC; yellow, white arrows). Scale bars = 100 μm. **C.** Average percentage of BLA projection neurons to the lOFC (green), mOFC (red), or dual-projecting (yellow) within the anterior, middle, and posterior BLA. Inset – Venn diagram representing the percentage of total labeled BLA neurons projecting to the lOFC, mOFC, or both orbital regions. Error bars represent s.e.m.

3.4 Results and Discussion

The BLA and OFC are two densely connected and functionally similar brain regions. Precise mapping of anatomical connections within the BLA-OFC circuit alongside causal experimental projection-specific manipulations may enable a more comprehensive understanding of how this neural network governs behavior. Here, by using retrograde tract tracers to quantify distinct populations of BLA projection cells to the mOFC and lOFC we found that the organization of projection cells differed along the anterior-posterior axis. We identified very few collateral neurons (4%, 38/901 neurons) projecting from the BLA to both mOFC and lOFC (Figure 3-1C). Further, using a combined anterograde-retrograde viral tracing approach, we found reciprocal anatomical overlap between OFC→BLA projection cell bodies and terminals of neurons arising from the BLA, suggesting that connections within BLA-OFC circuits may work in concert to guide behavioral output.

Experiment 1 revealed spatially distinct populations of BLA projections to the mOFC and lOFC (Figure 3-1). Overall, we observed a larger proportion of BLA→lOFC projections compared to BLA→mOFC projections in anterior and middle portion of the BLA. In posterior BLA sections, we observed a similar proportion of BLA neurons projecting to either orbital subregion (Figure 3-1C). From anterior to posterior sections, counts of BLA→lOFC projections decreased, while counts of BLA→mOFC projections increased. Across all anterior-poster planes, approximately 27% (656/901) of labeled cells in the BLA projected to the mOFC and 73% (656/901) projected to the lOFC (Figure 3-1 - inset). Surprisingly, BLA collateral neurons bifurcating to both mOFC and lOFC were sparse. Only about 4% (38/901) of total labeled cells were identified as collaterals, perhaps suggesting these cells may not be responsible for any particular shared behavioral function, however, this notion remains unexplored.

Notably, we refrain from drawing conclusions regarding the proportion of neurons in the BLA projecting to the mOFC and lOFC, as well as those that send collaterals to both orbital subregions, given that (1) tracers often have limited efficacy in retrograde transport and (2) CTb injections

differed in volume between OFC subregions (see methods). Nevertheless, these data provide useful visualization and quantitative data needed to precisely target projection cells in causal manipulation or recording experiments. The mere existence of distinct populations of BLA→OFC projections is intriguing. Indeed, evidence across species suggests that the medial and lateral subdivisions of the OFC are functionally dissociable (Noonan *et al.*, 2010; Rudebeck & Murray, 2011a; b; Murray *et al.*, 2015; Izquierdo, 2017; Noonan *et al.*, 2017), thus, distinct BLA projections to various frontal regions may serve unique functions in guiding reward-related behaviors.

In Experiment 2, axon terminals of neurons originating in the BLA were observed in the vicinity of both mOFC and lOFC neurons projecting to the BLA (Figure 3-2). In this study, we limited imaging to reciprocal anatomical overlap in the OFC. Future studies examining reciprocal overlap in the opposing direction, between BLA→OFC projections and OFC terminals, is essential to comprehensively map connectivity within BLA-OFC circuits. Overall, these data demonstrate clear overlap in BLA-OFC circuitry, suggesting that at the behavioral level, interactions between the BLA and subregions of the OFC may be critical for coordinating specific aspects of behavioral output.
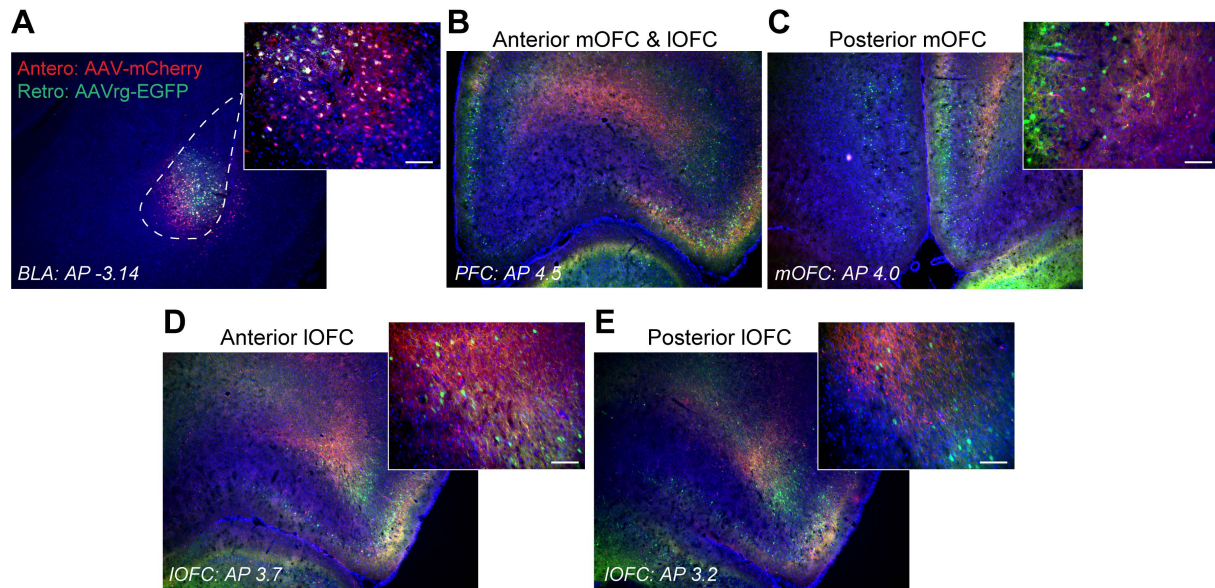
Figure 3-2: Reciprocal overlap between lOFC and mOFC neurons projecting to the BLA and BLA terminals. A. Representative image of AAV-hsyn-mCherry + AAVrg-hsyn-EGFP placement in the BLA. **B-C.** Representative fluorescent images of anterior mOFC and lOFC **(B)** and posterior mOFC **(C)**. **D-E.** Representative fluorescent images of anterior **(D)** and posterior **(E)** lOFC. All OFC images **(B-E)** depict projection neuron cell bodies (green) surrounded by BLA terminals (red). Scale bars = 100 µm.

Chapter 4: Basolateral amygdala to orbitofrontal cortex projections enable cue-triggered reward expectations

4.1 Abstract

To make an appropriate decision one must anticipate potential future rewarding events, even when they are not readily observable. These expectations are generated by using observable information (*e.g.*, stimuli or available actions) to retrieve often quite detailed memories of available rewards. The basolateral amygdala (BLA) and orbitofrontal cortex (OFC) are two reciprocally-connected key nodes in the circuitry supporting such outcome-guided behaviors. But there is much unknown about the contribution of this circuit to decision making, and almost nothing known about the whether any contribution is via direct, monosynaptic projections, or the direction of information transfer. Therefore, here we used designer receptor-mediated inactivation of OFC→BLA or BLA→OFC projections to evaluate their respective contributions to outcome-guided behaviors in rats. Inactivation of BLA terminals in the OFC, but not OFC terminals in the BLA, disrupted the selective motivating influence of cue-triggered reward representations over reward-seeking decisions as assayed by Pavlovian-to-instrumental transfer. BLA→OFC projections were also required when a cued reward representation was used to modify Pavlovian conditional goal-approach responses according to the reward's current value. These projections were not necessary when actions were guided by reward expectations generated based on learned action-reward contingencies, or when rewards themselves, rather than stored memories, directed action. These data demonstrate that BLA→OFC projections enable the cue-triggered reward expectations that can motivate the execution of specific action plans and allow adaptive conditional responding.

4.2 Introduction

Appropriate decision making requires the accurate anticipation of potential rewarding outcomes. Often these rewards are not present or noticeable in the immediate environment. So

one must use information that can be observed, such as the presence of stimuli or available actions, to enable the mental representation of the critical information needed to make a choice: future possible outcomes. Indeed, stored knowledge of specific stimulus-outcome or action-outcome relationships permits recollection of the detailed reward memories that facilitate the outcome expectations that influence conditional responses, reward seeking, and decision making (Balleine & Dickinson, 1998a; Delamater & Oakeshott, 2007; Delamater, 2012; Fanselow & Wassum, 2015). Detailed reward predictions enable adaptive behavior by allowing individuals to rapidly adjust to environmental changes and to infer the most advantageous option in novel situations. But disruptions in this process can lead to the cognitive symptoms underlying many psychiatric diseases.

The orbitofrontal cortex (OFC) and basolateral amygdala (BLA) are two identified key nodes in the circuitry supporting outcome-guided behaviors. Damage to either region causes performance deficits when specific rewarding events must be anticipated (Gallagher *et al.*, 1999; Blundell *et al.*, 2001; Pickens *et al.*, 2003a; Izquierdo *et al.*, 2004; Pickens *et al.*, 2005; Wellman *et al.*, 2005; Machado & Bachevalier, 2007; Ostlund & Balleine, 2007a; 2008a; Johnson *et al.*, 2009; West *et al.*, 2011; Jones *et al.*, 2012; Scarlet *et al.*, 2012; Rhodes & Murray, 2013; Malvaez *et al.*, 2015). These regions share dense and reciprocal direct connections (Carmichael & Price, 1995; Price, 2007) and associative encoding in one region has generally been shown to be altered by lesions of the other (Schoenbaum *et al.*, 2003; Saddoris *et al.*, 2005; Hampton *et al.*, 2007; Rudebeck *et al.*, 2013; Lucantonio *et al.*, 2015; Rudebeck *et al.*, 2017). The unique contribution of each region is still a matter of debate, but there is some evidence to suggest that the BLA might acquire reward representations, while the OFC is more important for using this information to generate the expectations that guide action (Pickens *et al.*, 2003a; Wellman *et al.*, 2005; Wassum *et al.*, 2009; Jones *et al.*, 2012; Parkes & Balleine, 2013; Takahashi *et al.*, 2013; Gore *et al.*, 2015). The OFC may be especially needed when critical determining elements of future possible states (*e.g.*, potential rewarding outcomes) are not readily observable (Wilson *et al.*, 2014; Bradfield *et*

al., 2015; Schuck *et al.*, 2016). But understanding of BLA-OFC function in reward seeking and decision making is limited by the fact that the contribution of direct, monosynaptic projections and the direction of information transfer are unknown. Therefore, here we used designer receptor-mediated inactivation of OFC→BLA or BLA→OFC monosynaptic projections to evaluate their respective contributions to the ability to use detailed reward expectations to influence reward seeking and decision making. Follow-up tests focused on the specific contribution of BLA→OFC projections.

4.3 Materials and Methods

Subjects

Subjects were male, Long Evans rats (*n*=60 total, Charles River Laboratories, Wilmington, MA) weighing between 300-390 g (age ~3 months) at the beginning of the experiment. Rats were pair housed and handled for ~5 days prior to the onset of the experiment. Training and testing took place during the dark phase of the 12:12 hr reverse dark:light cycle. Rats had *ad libitum* access to filtered tap water in the home cage and were maintained on a food-restricted schedule whereby they received 12-14 g of their maintenance diet (Lab Diet, Brentwood, MO) daily to maintain ~85-90% free-feeding body weight. All procedures were conducted in accordance with the NIH Guide for the Care and use of Laboratory Animals and approved by the UCLA Institutional Animal Care and Use Committee.

Viral constructs

Transduction of OFC or BLA neurons with the inhibitory Designer-Receptor-Exclusively-Activated-by-Designer-Drug (DREADD) hM4Di was achieved with an adeno-associated virus (AAV) driving the hM4Di-mCherry sequence under the human synapsin promoter (AAV8-hSyn-hM4Di-mCherry, viral concentration 7.4×10^{12} vg/ml; University of North Carolina Vector Core, Chapel Hill, NC). A virus lacking the hM4Di DREADD gene (AAV8-hSyn-mCherry; viral concentration 4.6×10^{12} vg/ml; University of North Carolina Vector Core) was used as a control.

For ex vivo electrophysiology experiments, hM4Di and the excitatory opsin, channelrhodopsin (ChR2; AAV5-CAMKII-ChR2-EYFP; viral concentration 6.2×10^{12} vg/ml; University of North Carolina Vector Core), were co-expressed in either the OFC or BLA using a cocktail of both viruses. A separate control group received only the ChR2 virus. Behavioral testing began between 6-8 weeks post viral injection to allow anterograde transmission and robust axonal expression in terminal regions.

Surgical procedures

Standard aseptic surgical procedures were used under isoflurane anesthesia (5% induction, 1-2% maintenance). Bilateral virus injections were made via 33-gauge, stainless steel injectors inserted into either the BLA (AP -3.0 mm, ML ±5.1 mm, DV -8.0 or -8.5 mm relative to bregma) or OFC (AP +3.0, ML ±3.2, DV -6.0 mm). Viruses were infused in a volume of 0.6 (BLA) or 0.8 (OFC) µl per hemisphere at a flow rate of 0.1 µL/min. Injectors were left in place for an additional 10 min to ensure adequate diffusion and to minimize viral spread up the injector tract. For rats in the behavioral experiments, during the same surgery, 22-gauge, stainless steel guide cannulae (Plastics One, Roanoke, VA) were implanted bilaterally targeted 1 mm above the BLA (AP -3.0 mm, ML ±5.1 mm, DV -7.0 mm) for the OFC viral injection group, or the OFC (AP +3.0, ML ±3.2, DV -5.0 mm) for groups receiving viral injections into the BLA. A nonsteroidal anti-inflammatory agent was administered pre- and post-operatively to minimize pain and discomfort. Following surgery, rats were individually housed and allowed to recover for ~16 days prior to the onset of any behavioral training.

Behavioral training

Training and testing took place in a set of 16 Med Associates (East Fairfield, VT) operant chambers, described previously (Wassum *et al.*, 2016).

Pavlovian training. Each of the 8 daily sessions consisted of 8 tone (1.5 kHz) and 8 white noise conditional stimulus (CS) presentations (75 db, 2-min duration), during which either

sucrose solution (20%, 0.1 ml/delivery) or grain pellets (45 mg; Bio-Serv, Frenchtown, NJ), were delivered on a 30-s random-time schedule into the food-delivery port, resulting in an average of 4 stimulus-reward pairings per trial. For half the subjects, tone was paired with sucrose and noise with pellets, with the other half receiving the opposite arrangement. CSs were delivered pseudo-randomly with a variable 2-4 min inter-trial interval (mean=3 min). Entries into the food-delivery port were recorded for the entire session. Comparison of anticipatory entries during the CS-probe periods (interval between CS onset and first reward) to entries during baseline periods (2-min period prior to CS onset) provided a measure of Pavlovian conditioning.

Instrumental training. Rats were then given 11 days of instrumental training, receiving 2 separate training sessions per day, one with the lever to the left of the food-delivery port and one with the right lever. Each action was reinforced with a different outcome, either grain pellets or sucrose solution (counterbalanced with respect to the Pavlovian contingencies). Each session terminated after 30 outcomes had been earned or 30 min had elapsed. Actions were continuously reinforced on the first day, and then escalated to a random-ratio 20 schedule. The rate of responding on each lever was measured throughout training.

Pavlovian-to-instrumental Transfer Test

4 groups of subjects received PIT tests: $OFC_{hM4Di} \rightarrow BLA$ (n=10), $BLA_{hM4Di} \rightarrow OFC$ (n=10), $OFC_{mCherry} \rightarrow BLA$ (n=11), and $BLA_{mCherry} \rightarrow OFC$ (n=12). On the day prior to each PIT test, rats were given a single 30-min extinction session during which both levers were available, but pressing was not reinforced to establish a low level of responding. Each rat was given 2 PIT tests, one following infusion of vehicle and one following infusion of the otherwise inert hM4Di ligand, clozapine-n-oxide (CNO), into the BLA ($OFC_{hM4Di} \rightarrow BLA$ and $OFC_{mCherry} \rightarrow BLA$ groups) or OFC ($BLA_{hM4Di} \rightarrow OFC$ and $BLA_{mCherry} \rightarrow OFC$ groups). Test order was counterbalanced across subjects. During each PIT test, both levers were continuously present, but pressing was not reinforced. After 5 min of lever-pressing extinction, each 2-min CS was presented separately 4 times each in pseudorandom order, separated by a fixed, 4-min inter-trial interval. No rewards were delivered

during CS presentation. The 2-min period prior to each CS presentation served as the baseline. Rats were given 2 retraining sessions for each instrumental association (2 sessions/day for 2 days) and 1 Pavlovian retraining session in between PIT tests.

Outcome-specific devaluation test

Following training, a second cohort of $BLA_{hM4Di} \rightarrow OFC$ rats (n=9) was given a series of two outcome-specific devaluation tests. Prior to each test, rats were given 1-hr, unlimited access to either sucrose solution or food pellets in pre-exposed feeding chambers such that the pre-fed reward would become devalued, while the other reward would remain valued. Immediately after this pre-feeding, rats received infusions of either vehicle or CNO into the OFC and were then tested. The test consisted of two phases. In the first, both levers were available and non-reinforced lever pressing was assessed for 5 min. The levers were then retracted, which started the second, Pavlovian, test phase, in which each 2-min CS was presented, without accompanying reward, separately 2 times each in alternating order, separated by a fixed, 4-min inter-trial interval. The 2-min period prior to each CS presentation served as the baseline. Successful devaluation of the earned outcome was confirmed by post-test consumption of each food reward, in which rats ate significantly less of the devalued reward type (Average: 1.81 g $\pm$ 0.43 s.e.m.) relative to the valued reward (5.38$\pm$0.7; t_{17}=4.05, P=0.0008).

After the first test, rats remained in their home cage for 2 days and were then given 2 retraining sessions for each instrumental association (2 sessions/day for 2 days) and 1 Pavlovian retraining session, prior to the second outcome-specific devaluation test. For the second test, rats were pre-fed on the opposite food reward (*e.g.*, pellets if sucrose had been pre-fed on Test 1), and infused with the opposite drug (*e.g.*, CNO, if they had previously received vehicle). Thus, each rat experienced 2 devaluation tests to allow a within-subject drug-treatment design, one following vehicle and one following CNO infusion, counterbalanced for order. Because in the absence of the hM4Di receptor CNO itself was found to have no effect on the expression of PIT, which requires *both* action-outcome and stimulus-outcome associative information, empty-vector controls were

not included for this experiment in which the use of *either* action-outcome or stimulus-outcome associations was assessed.

Outcome-specific reinstatement test

Rats then received 4 days of instrumental retraining prior to outcome-specific reinstatement testing. On the day prior to each reinstatement test, rats received a 30-min lever-pressing extinction session. Each rat was given 2 reinstatement tests, one following intra-OFC vehicle infusion and one after CNO infusion, counterbalanced for order. Rats were given instrumental retraining in between the two reinstatement tests. During each reinstatement test, both levers were continuously present, but pressing was never reinforced. After 5 min of extinction, rewards were presented in 8 separate reward-presentation periods (4 sucrose, 4 pellet periods, in pseudorandom order) separated by a fixed 4-min inter-trial interval. Each reward presentation period was 2 min in duration and began with 2 deliveries of the appropriate reward, separated by 6 s. The 2-min period prior to each reward-delivery period served as the baseline.

Drugs

For behavioral experiments, CNO (Tocris Bioscience, Sterling Heights, MI) was dissolved in aCSF to 1 mM and was intracranially infused over 1 min in a volume of 0.25 μL into the OFC or 0.5 μL into the BLA. Injectors were left in place for at least 1 additional min to allow for drug diffusion. Behavioral testing commenced within 5-10 min following infusion. CNO dose was selected based on evidence of both its behavioral effectiveness and ability to inactivate terminal activity when intracranially infused over hM4Di-expressing terminals (Mahler *et al.*, 2014). CNO was dissolved in aCSF to 100 μM for *ex vivo* electrophysiology experiments (Stachniak *et al.*, 2014).

Ex vivo electrophysiology

Whole-cell patch clamp recordings were performed in brain slices from ~5-6 month-old rats (*n*=8 rats) 8-13 weeks following AAV injection. To prepare brain slices, rats were deeply

anesthetized with isoflurane and perfused transcardially with an ice-cold, oxygenated NMDG-based slicing solution containing (in mM): 30 NaHCO3, 20 HEPES, 1.25 NaH2PO4, 102 NMDG, 40 glucose, 3 KCl, 0.5 CaCl2-2H2O, 10 MgSO4-H2O (pH adjusted to 7.3-7.35, osmolality 300-310 mOsm/L). Brains were extracted and immediately placed in ice-cold, oxygenated NMDG slicing solution. Coronal slices (350 μm) were cut using a vibrating microtome (VT1000S; Leica Microsystems, Germany) and transferred to an incubating chamber containing oxygenated NMDG slicing solution warmed to 32-34°C and allowed to recover for 15 minutes before being transferred to an aCSF solution containing (in mM): 130 NaCl, 3 KCl, 1.25 NaH2PO4, 26 NaHCO3, 2 MgCl2, 2 CaCl2, and 10 glucose) oxygenated with 95% O2-5% CO2 (pH 7.2-7.4, osmolality 290-310 mOsm/L, 32-34°C). After 15 minutes, slices were moved to room temperature and allowed to recover for an additional ~30 min prior to recording. All recordings were performed using an upright microscope (Olympus BX51WI, Center Valley, PA) equipped with differential interference contrast optics and fluorescence imaging (QIACAM fast 1394 monochromatic camera with Q-Capture Pro software, QImaging, Surrey, BC, Canada).

Whole-cell patch clamp recordings in voltage-clamp mode were obtained from postsynaptic BLA (OFC$_{hM4Di/ChR2}$→BLA: n=7 cells, or OFC$_{ChR2}$→BLA: n=5 cells) or OFC (BLA$_{hM4Di/ChR2}$→OFC: n=5 cells, or BLA$_{ChR2}$→OFC: n=5 cells) neurons using a MultiClamp 700B Amplifier (Molecular Devices, Sunnyvale, CA) and the pCLAMP 10.3 acquisition software. Visible eYFP-expressing terminals were identified in the OFC or BLA and recordings were obtained from cells located only in highly fluorescent regions. The patch pipette (3-5 MΩ resistance) contained a Cesium methanesulfonate-based internal recording solution (in mM): 125 Cs-methanesulfonate, 4 NaCl, 1 MgCl2, 5 MgATP, 9 EGTA, 8 HEPES, 1 GTP-Tris, 10 phosphocreatine, and 0.1 leupeptin; pH 7.2 with CsOH, 270-280 mOsm). Biocytin (0.2%, Sigma-Aldrich, St. Louis, MO) was included in the internal recording solution for subsequent postsynaptic cell visualization and identification.

After breaking through the membrane, recordings were obtained from cells while holding the membrane potential at −70 mV. Electrode access resistances were maintained at <30 MΩ. Blue

light (470 nm, 5 ms pulse, 8 mW; CoolLED Ltd, Andover, UK) was delivered through the epifluorescence illumination pathway using Chroma Technologies filter cubes to activate ChR2 and stimulate BLA terminals in the OFC, or OFC terminals in the BLA. All voltage-clamp recordings were performed in the presence of $GABA_A$ receptor antagonists, bicuculline or gabazine (10 μM, Tocris, R&D Systems, Minneapolis, MN). Optically-evoked excitatory post-synaptic currents (EPSCs) were recorded both prior to and after CNO bath application (100 μM; 20 min). As an additional control, recordings were made with identical timing, but without CNO bath application (n=4 cells).

Histology

Rats in the behavior experiments were deeply anesthetized with Nembutal and transcardially perfused with PBS followed by 4% paraformaldehyde. Brains were removed and post-fixed in 4% paraformaldehyde overnight, placed into 30% sucrose solution, then sectioned into 30-40 μm slices using a cryostat and stored in PBS or cryoprotectant. To visualize hM4Di-mCherry expression in BLA or OFC cell bodies, free-floating coronal sections were mounted onto slides and coverslipped with ProLong Gold mounting medium with DAPI (Invitrogen, Carlsbad, CA). The signal for axonal expression of hM4Di-mCherry in terminal regions was immunohistochemically amplified using antibodies directed against mCherry as done prior (see methods Chapter 3). All images were acquired using a Keyence (BZ-X710; Osaka, Japan) microscope with a 4X or 20X objective (CFI Plan Apo), CCD camera, and BZ-X Analyze software. Data from subjects for which hM4Di-mCherry expression could not be confirmed bilaterally in the target region were omitted from the analysis. We also confirmed that cannula placement was in the target region and coincided with labeled axon terminals.

Following *ex vivo* recordings, brain slices were fixed in 4% PFA for 24 hrs. Slices were then washed with 1x PBS, permeabilized with 1% Triton overnight at 4°C, and incubated for 2 hrs with streptavidin-Marina Blue (365 nm, ThermoFisher) at room temperature. Fluorescent images

were taken of both recorded cells and eYFP or mCherry-expressing terminals using a Zeiss Apotome (Göttingen, Germany) equipped with 20x and 40x objectives.

Experimental design and statistical analysis

Data were processed with Microsoft Excel (Redmond, WA) and then analyzed with GraphPad Prism (La Jolla, CA) and SPSS (IBM Corp, Chicago, IL). For all hypothesis tests, the α level for significance was set to $P<0.05$. The behavioral data of primary interest were statistically evaluated with repeated-measures ANOVAs (Geisser-Greenhouse correction). For well-established behavioral effects (PIT, devaluation, reinstatement), multiple pairwise comparisons (paired t-test, two-tailed) were used for *a priori* posthoc comparisons, as advised by (Levin *et al.*, 1994) based on a logical extension of Fisher's protected least significant difference (PLSD) procedure for controlling familywise Type I error rates. Bonferroni or Dunnet's corrections were used for posthoc analyses of all drug effects. Electrophysiological data were analyzed with unpaired *t*-tests.

Behavioral data were analyzed for the rate of both lever pressing and entries into food-delivery port. Both drug and test phase were within-subject factors. All data were averaged across trials. For the PIT tests, lever pressing was averaged across levers for the 2-min baseline period and compared to that during the CS period, which was separated for presses on the lever that, during training, earned the same outcome as the cue predicted (*i.e.*, CS-Same presses) versus those on the other available lever (*i.e.*, CS-Different presses). Data from the reinstatement test were analyzed similarly, with reward-period presses separated for those on the lever that previously earned the same outcome as the presented reward (*i.e.*, Reinstated presses) versus those on the alternate lever (*i.e.*, Non-reinstated). For the PIT tests, entries into the food-delivery port were compared between the baseline and CS periods. Food-delivery port entries were analyzed similarly for the Pavlovian phase of the devaluation test; baseline entry rate was compared to entries during presentation of each CS separated for the cue that predicted the valued versus devalued reward type. Lever pressing during the instrumental phase of the devaluation test was separated for actions on the lever that, in training, earned the currently devalued v. valued reward.

To specifically examine how CS presentation *changed* behavior during PIT and the Pavlovian devaluation test, in addition to these analyses, we also evaluated cue-induced change in lever pressing (PIT test) or food-port entries (Pavlovian devaluation test) by calculating an elevation ratio [CS responses/ (CS responses+ Baseline responses)].

For electrophysiological data optically-evoked EPSC amplitudes following CNO application were expressed as a percentage of the evoked response prior to CNO for comparison between AAV groups (hM4Di+ChR2 v. ChR2 only).

4.4 Results

Pathway-specific chemogenetic OFC-BLA manipulations

We used a chemogenetic approach (Armbruster *et al.*, 2007; Smith *et al.*, 2016) to manipulate monosynaptic OFC→BLA or BLA→OFC projections by taking advantage of the fact that DREADDs are trafficked to axon terminals where when hM4Di is activated by its otherwise inert exogenous ligand, CNO, it can attenuate presynaptic activity (Mahler *et al.*, 2014; Stachniak *et al.*, 2014). We first validated presynaptic suppression by terminal hM4Di activation with *ex vivo* electrophysiology. The G_i-coupled DREADD hM4Di and the excitatory opsin ChR2 were co-expressed in either the OFC (Figure 4-1A) or BLA (Figure 4-1D) and whole-cell patch clamp recordings were obtained from postsynaptic cells in the ChR2 and hM4Di-expressing terminal regions (Figure 4-1B,C). EPSCs were evoked by blue light activation of ChR2 in both the BLA (Figure 1E) and OFC (Figure 4-1F) and the amplitude of these responses was markedly attenuated in the presence of CNO. The CNO-induced change in the optically-evoked EPSC was significantly lower in both BLA (t_8=5.68, *P*=0.0005) and OFC (t_{10}=5.41, *P*=0.0003) slices expressing hM4Di relative to ChR2-only controls lacking this receptor (Figure 4-1G). Identically-timed recordings without CNO application indicated <10% rundown of evoked EPSCs due to time alone (Average response = 98.31% $\pm$ 4.60 s.e.m.).

For behavioral experiments, a synapsin-driven AAV yielding hM4Di expression was injected into either the OFC (OFC$_{hM4Di}$→BLA group) or BLA (BLA$_{hM4Di}$→OFC group), yielding robust hM4Di expression (visualized by the mCherry fluorescent reporter protein; Figure 4-2A-B, and 2E-F). Guide cannulae were implanted over either the BLA (for OFC$_{hM4Di}$→BLA group) or OFC (for BLA$_{hM4Di}$→OFC group) terminal fields in close proximity to the area of axonal expression (Figure 4-2C-D and 2G-H) to allow CNO infusion to selectively inactivate OFC terminals in the BLA or BLA terminals in the OFC. We focused on the lateral OFC subregion, which is densely connected with the BLA (Kita & Kitai, 1990; Carmichael & Price, 1995; Ongür & Price, 2000) and

heavily implicated in outcome-guided conditional responding and action (Schoenbaum *et al.*, 1998a; Ostlund & Balleine, 2007a; Lucantonio *et al.*, 2015).
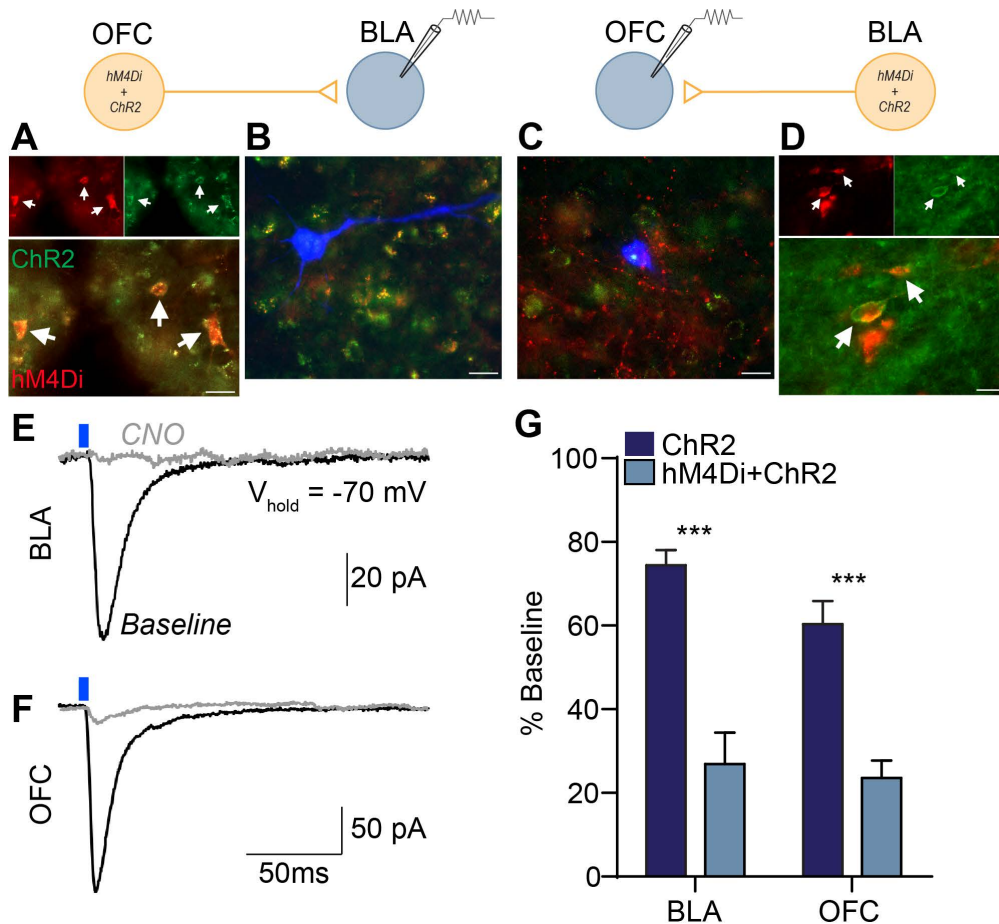
Figure 4-1. Effect of CNO-hM4Di inactivation of OFC→BLA or BLA→OFC projections on postsynaptic responses. *hM4Di-mCherry* and/or *ChR2-EYFP* were expressed in either the BLA or OFC and whole-cell patch clamp recordings in voltage-clamp mode were obtained from postsynaptic BLA (OFC$_{hM4Di/ChR2}$→BLA: *n*=7 cells; OFC$_{ChR2}$→BLA: *n*=5) or OFC cells (BLA$_{hM4Di/ChR2}$→OFC: *n*=5; BLA$_{ChR2}$→OFC: *n*=5) before and after CNO application. **A.** Representative fluorescent image of hM4Di-mCherry/ChR2-eYFP expression in OFC cell bodies. Arrows mark co-expressing cells. **B.** Representative florescent image of biocytin-filled cell (blue) surrounded by ChR2-eYFP and hM4Di-mCherry terminals in BLA. **C.** Representative fluorescent image of biocytin-filled cell surrounded by ChR2-eYFP and hM4Di-mCherry terminals in OFC. **D.** Representative fluorescent image of hM4Di-mCherry/ChR2-eYFP expression in BLA cell bodies. Scale bars = 20 μm. **E.** Sample traces (average of 2-3 sweeps) of evoked EPSCs in BLA in response to optical stimulation of OFC terminals (blue line: 470 nm, 5 ms pulse, 8 mW) prior to (black) and after (gray) CNO application. **F.** Sample traces of evoked EPSCs in OFC in response to optical stimulation of BLA terminals. **G.** Average optically-evoked EPSC response following CNO, expressed as a percent of pre-CNO baseline responses, compared between subjects expressing hM4Di and ChR2 to ChR2-only controls for recordings made in the BLA or OFC. Error bars ± s.e.m. ***P<0.001.
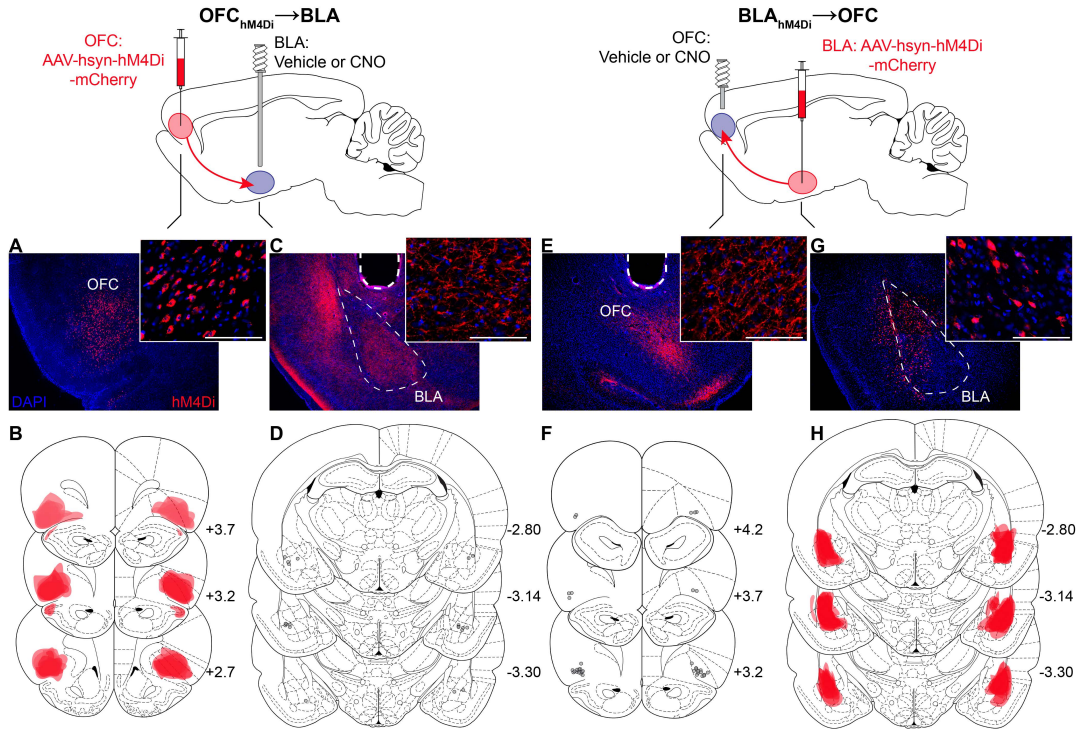
Figure 4-2. Viral expression and cannula placements. A-D. OFC$_{hM4Di}$→BLA rats (*n*=10). Bilateral *hsyn-hM4Di-mCherry* injections were made into the OFC and guide cannulae were implanted above the BLA, such that CNO infusion would inactivate OFC terminals in the BLA. **A.** Representative fluorescent image of hM4Di-mCherry expression in the OFC. Scale bars = 100 μm. **B.** Schematic representation of hM4Di-mCherry maximal viral spread in the OFC for all subjects. Numbers to the lower right of each section represent distance anterior to bregma. Coronal section drawings taken from (Paxinos & Watson, 1998). **C.** Representative immunofluorescent image of hM4Di-mCherry expression in the BLA. Dashed line demarcates guide cannula track. **D.** Schematic representation of microinfusion injector tips in the BLA. E-H: BLA$_{hM4Di}$→OFC rats (*n*=19). Bilateral *hsyn-hM4Di-mCherry* injections were made into the BLA and guide cannulae were implanted above the OFC, such that CNO infusion would inactivate BLA terminals in the OFC. **E.** Representative immunofluorescent image of hM4Di-mCherry expression in the OFC. **F.** Schematic representation of microinfusion injector tips in the OFC. **G.** Representative fluorescent image of hM4Di-mCherry expression in the BLA. **H.** Schematic representation of hM4Di-mCherry maximum viral spread in the BLA for all subjects.

Contribution of OFC→BLA and BLA→OFC projections to outcome-specific Pavlovian-to-instrumental transfer

Using this approach, we examined the contribution of OFC→BLA and BLA→OFC projections to the ability to retrieve a stored memory of a specific predicted reward and to use this information to influence reward-seeking decisions during outcome-specific Pavlovian-to-instrumental transfer (PIT; Figure 4-3A). Rats were trained to associate two auditory CSs with two distinct food rewards and then to earn each of those two rewards by pressing on independent levers. Rats demonstrated acquisition of the Pavlovian associations by entering the food-delivery port significantly more during the CS probe periods (Average entry rate on the final training session OFC$_{hM4Di}$→BLA group: 11.05 entries/min $\pm$1.25 s.e.m.; BLA$_{hM4Di}$→OFC group: 11.89$\pm$1.51) than during the baseline periods (OFC$_{hM4Di}$→BLA group: 4.52$\pm$0.50, t_9=5.72, P=0.0003; BLA$_{hM4Di}$→OFC group: 6.70$\pm$1.44, t_9=4.92, P=0.0008). All rats also acquired the instrumental behavior (Final average press rate OFC$_{hM4Di}$→BLA group: 21.13 presses/min $\pm$1.37; BLA$_{hM4Di}$→OFC group: 21.45$\pm$1.54). At the critical PIT test, both levers were present, but lever pressing was not rewarded. Each CS was presented 4 times (also without accompanying reward), with intervening CS-free baseline periods, to assess its influence on action performance and selection in the novel choice scenario. Because the CSs are never associated with the instrumental actions, this test assesses the rats' ability to, upon CS presentation, retrieve a stored memory of the specific predicted reward and to use this information to motivate performance of those actions known to earn the same unique reward (Kruse *et al.*, 1983; Colwill & Motzkin, 1994; Gilroy *et al.*, 2014; Corbit & Balleine, 2015).

CNO-hM4Di inactivation of OFC terminals in the BLA did not alter the expression of outcome-specific PIT (Figure 4-3B; Main effect of CS Period: $F_{2,18}$=10.18, P=0.001; Drug: $F_{1,9}$=0.45, P=0.52; CS x Drug interaction: $F_{2,18}$=0.04, P=0.96). Following either vehicle or CNO infusion, CS presentation elevated press rate selectively on the lever that, in training, earned the same

predicted reward (CS-Same) relative to both pressing during the CS on the alternate available lever (CS-Different) and baseline press rate (P=0.001 - 0.002).

CNO-hM4Di inactivation of BLA terminals in the OFC did, however, attenuate PIT expression (Figure 4-3C; CS Period: $F_{2,18}$=15.64, P=0.0001; Drug: $F_{1,9}$=0.63, P=0.45; CS x Drug: $F_{2,18}$=3.54, P=0.05). Robust PIT was demonstrated under vehicle-infused control conditions; the CS elevated performance of the CS-Same action relative to both baseline (P=<0.001) and CS-Different pressing (P=0.002). Following CNO infusion, there was no significant difference between CS-Same and either CS-Different (P=0.15) or baseline pressing (P=0.09) and CS-Same performance was lower following CNO relative to vehicle (P=0.01). The result was similar when the CS-induced elevation in performance on each action choice was evaluated (Figure 4-4C-inset). Under control conditions the CS induced a greater elevation in performance on action Same than action Different (t_9=3.08, P=0.01), but following CNO infusion there was no significant difference between actions (t_9=0.10, P=0.92). The effect of inactivating BLA terminals in the OFC was restricted to cue-influenced action; lever pressing during the baseline period was not altered by CNO (P=0.90). CNO-hM4Di inactivation of BLA terminals in the OFC consistently attenuated PIT expression across trials (Drug x CS x Trial: $F_{6,54}$=1.61, P=0.20).

Inactivation of neither OFC terminals in the BLA (Figure 4-3D; CS Period: $F_{1,9}$=95.95, P=<0.0001; Drug: $F_{1,9}$=1.62, P=0.23; CS x Drug: $F_{1,9}$=0.08, P=0.78), nor BLA terminals in the OFC (Figure 4-3E; CS Period: $F_{1,9}$=106.30, P=<0.0001; Drug: $F_{1,9}$=0.26, P=0.62; CS x Drug: $F_{1,9}$=0.49, P=0.50) altered Pavlovian conditional food-port approach responding. In all cases, CS presentation significantly elevated entries into the food-delivery port (P=<0.0001 - 0.001).

CNO had no effect on lever pressing during PIT in subjects lacking the hM4Di receptor when it was infused into either the BLA (OFC$_{mCherry}$→BLA group; Figure 4-4A; CS Period: $F_{2,20}$=7.07, P=0.005; Drug: $F_{1,10}$=1.04, P=0.33; CS x Drug: $F_{2,20}$=0.20, P=0.82) or OFC (BLA$_{mCherry}$→OFC group; Figure 4-4B; CS Period: $F_{2,22}$=34.21, P=<0.0001; Drug: $F_{1,11}$=0.31, P=0.59; CS x Drug: $F_{2,22}$=0.04, P=0.96).
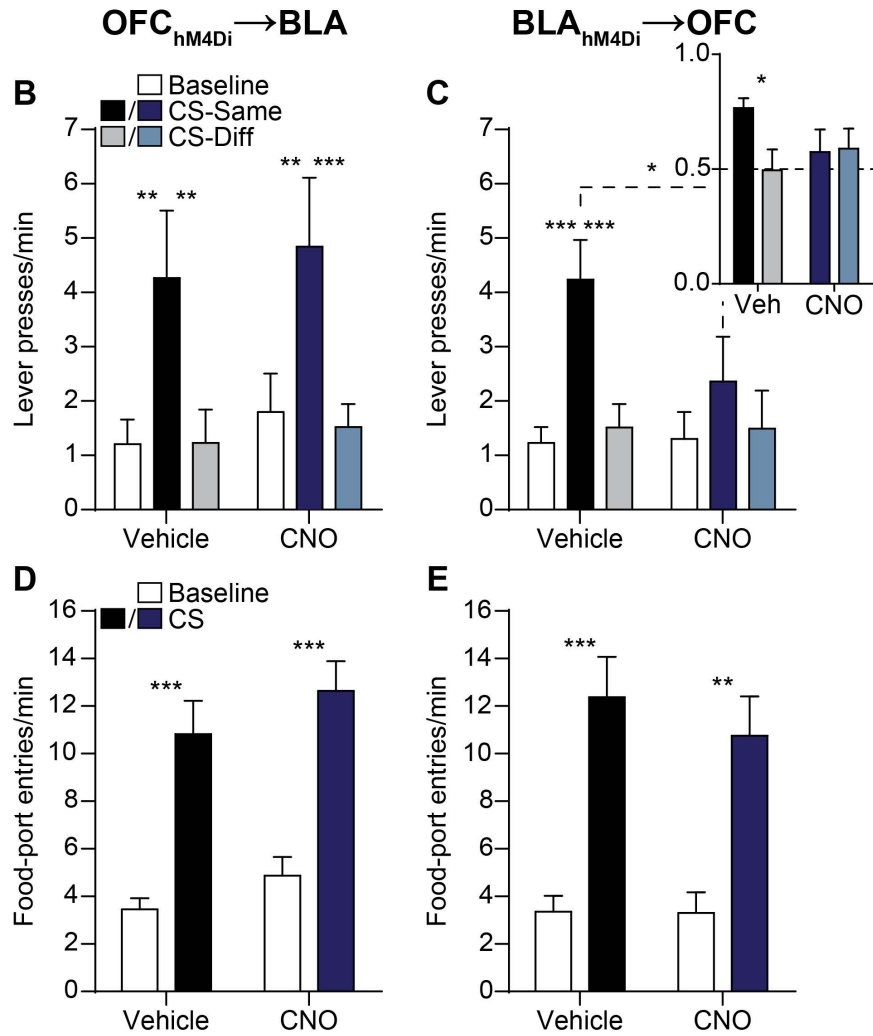
Figure 4-3. Effect of inactivating OFC→BLA or BLA→OFC projections on Pavlovian-to-instrumental transfer. **A.** Task design, see text. CS, conditional stimulus; O; outcome/reward; A, action. **B-C.** Trial-averaged lever presses per 2-min period averaged across both levers during the Baseline periods compared to pressing during the CS periods separated for presses on the lever that, in training, delivered the same outcome as predicted by the CS (CS-Same) and pressing on the other available lever (CS-Diff) for OFC$_{hM4Di}$→BLA (**B**; n=10) or BLA$_{hM4Di}$→OFC (**C**; n=10) groups. Inset- CS-induced elevation in responding [CS presses/ (CS presses + Baseline presses)] on action Same v. Different for the BLA$_{hM4Di}$→OFC group. **D-E.** Trial-averaged entries into the food-delivery port during the Baseline and CS periods for the OFC$_{hM4Di}$→BLA (**D**) and BLA$_{hM4Di}$→OFC (**E**) groups. Error bars ± s.e.m. *P<0.05, **P<0.01, ***P<0.001.
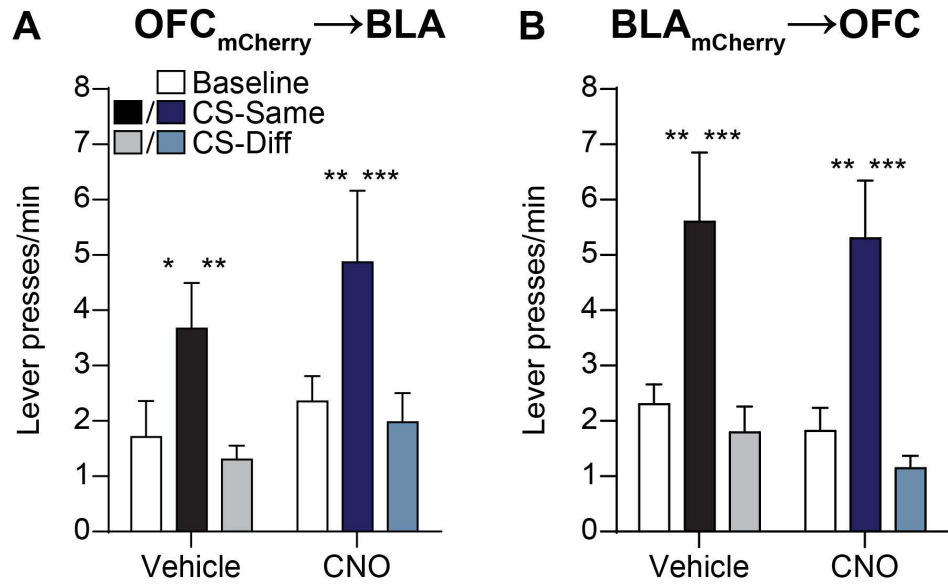
Figure 4-4. Effect of CNO infusion in subjects lacking hM4Di receptors. **A-B.** Trial-averaged lever presses per 2-min period averaged across both levers during the Baseline periods compared to pressing during the CS periods separated for presses on the lever that, in training, delivered the same outcome as predicted by the CS (CS-Same) and pressing on the other available lever (CS-Diff) for OFC$_{mCherry}$→BLA (**A**; *n*=11), BLA$_{mCherry}$→OFC (**B**; *n*=12) groups. Error bars ± s.e.m. *$P<0.05$, **$P<0.01$, ***$P<0.001$.

Contribution of BLA→OFC projections to the sensitivity of instrumental actions and Pavlovian conditional responses to outcome-specific devaluation

The above data suggest that BLA→OFC, but not OFC→BLA projections are required for a reward-predictive cue to selectively motivate performance of an action that results in the same rewarding outcome. This capacity relies upon retrieval of a representation of the specific shared reward (*i.e.,* outcome) encoded in both the previously learned Pavlovian stimulus-outcome and instrumental action-outcome associations (Dickinson & Balleine, 2002; Corbit & Janak, 2010). The BLA is required for both types of associations (Blundell *et al.*, 2001; Balleine *et al.*, 2003; Ostlund & Balleine, 2008a; Johnson *et al.*, 2009). Therefore, we next asked whether BLA→OFC projections are required for reward representations triggered by either Pavlovian reward-predictive stimuli, by the rats' own knowledge of available action-outcome contingencies, or both (Figure 4-5A).

A separate group of BLA$_{hM4Di}$→OFC rats were trained as described above. These subjects demonstrated acquisition of the Pavlovian associations by entering the food-delivery port significantly more during the CS probe periods (12.22$\pm$1.08) than the baseline periods (5.03$\pm$0.62; t_8=7.24, P=<0.0001) and acquired the instrumental behavior (final average press rate 20.54$\pm$1.48). Prior to test, one of the food rewards was devalued by sensory-specific satiety. Rats were then given a brief unrewarded instrumental choice test followed by a test of conditional food-port approach responding, in which levers were retracted and each CS was presented 2 times (without accompanying reward), with intervening CS-free, baseline periods. Infusions were made after the sensory-specific satiety procedure, but prior to test to evaluate the influence of inactivation of BLA terminals in the OFC on the retrieval of reward representations, rather than on devaluation learning *per se*. If rats are able to recall the learned action-outcome contingencies, then, during the instrumental phase of the test, they should be able to select the action that earns the valued reward, downshifting responding on the action that earns the devalued reward. Similarly, if the Pavlovian cues trigger the recall of a memory of their specific predicted reward,

then rats should show robust conditional food-port approach responding to the cue signaling the valued reward, but attenuated responding to the cue signaling the devalued reward. Because, in both cases, a specific reward expectation is needed to influence behavior, this test provided an opportunity to evaluate the contribution of BLA→OFC projections to the generation of detailed reward expectancies.

CNO-hM4Di inactivation of BLA terminals in the OFC was without effect on the sensitivity of instrumental choice performance to reward devaluation (Figure 4-5B; Devaluation: $F_{1,8}$=13.50, P=0.006; Drug: $F_{1,8}$=0.81, P=0.39; Devaluation x Drug: $F_{1,8}$=0.31, P=0.60). Conversely, this did impair rats' ability to adjust their Pavlovian conditional food-port approach responding according to the current value of each specific predicted reward (Figure 4-5C). The CS-induced elevation in food-port approach responding (Figure 4-5C-inset; Devaluation: $F_{1,8}$=2.78, P=0.13; Drug: $F_{1,8}$=0.30, P=0.60; Devaluation x Drug: $F_{1,8}$=5.50, P=0.047) was higher when the CS signaled a valued reward relative to a devalued reward in the vehicle-infused condition (P=0.047), but responding was equally elevated by both CSs following CNO infusion (P=0.36). Indeed, following vehicle infusion, rats' food-port entries were significantly elevated above baseline by presentation of the CS previously associated with the valued reward (P=0.006), but were not significantly elevated when the CS predicting the devalued reward was presented (P=0.40). Conversely, following CNO infusion rats', food-port approach responding was elevated above baseline during both CSs (Valued: P=0.03, Devalued P=0.04; Figure 4-5C- main; Devaluation: $F_{2,16}$=25.21, P=<0.0001; Drug: $F_{1,8}$=0.42, P=0.53; Devaluation x Drug: $F_{2,16}$=1.65, P=0.22).

A.

Pavlovian conditioning	Instrumental conditioning	Sensory-specific satiety	Devaluation test *Vehicle or CNO*
CS1 – O1 CS2 – O2	A1 – O1 A2 – O2	O1 (1hr)	Inst A1 v. **A2** ----- Pav CS1: Entries CS2: **Entries**

B *Instrumental*

C *Pavlovian*
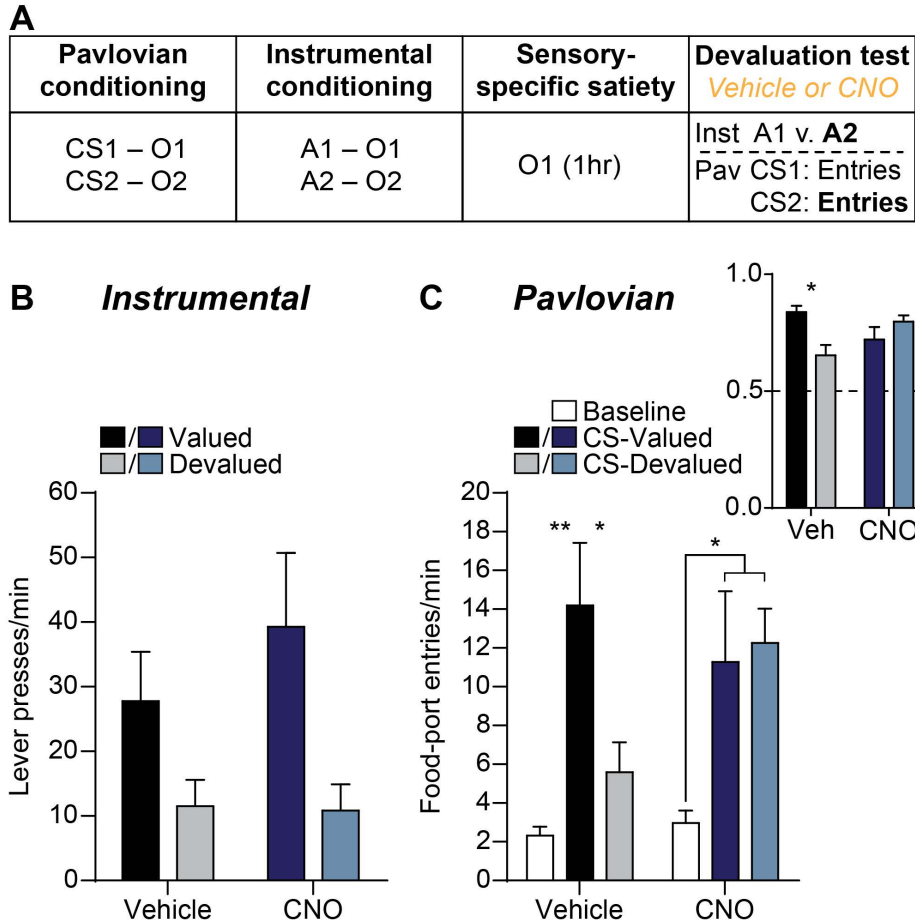
Figure 4-5. Effect of inactivating BLA→OFC projections on sensitivity to outcome-specific devaluation. **A.** Task design, see text. Only one devaluation condition shown. **B.** Average lever-press rate during the devaluation test. Presses separated for those that, in training, earned the currently devalued v. valued reward type. **C.** Trial-averaged entries into the food-delivery port during the Baseline and CS periods separated by the CS predicted the valued v. devalued reward. Inset- CS-induced elevation in responding [CS entries/ (CS entries + Baseline entries)]. (*n*=9) Error bars ± s.e.m. *P<0.05, **P<0.01.

Contribution of BLA→OFC projections to outcome-specific reinstatement

The data show that activity in BLA→OFC projections is required when a cue-triggered reward representation is used to either selectively motivate instrumental action or to direct adaptive conditional goal-approach responding. In both cases, the critical information, a predicted food reward, is not physically available, but rather must be expected based on previously learned associations. That is, the information was previously observed, but is not currently observable. BLA→OFC projections may, therefore, participate in this reward expectation. Conversely, these projections may simply be needed for a reward, whether observable or not, to influence action. The BLA is itself required for both (Ostlund & Balleine, 2008a). To test between these possibilities, we evaluated the effect of inactivation of BLA terminals in the OFC on outcome-specific reinstatement (see Figure 4-6A).

Rats were retrained on the instrumental contingencies (final average press rate: 31.77 ± 2.26) and then given a reinstatement test that was similar in structure to the PIT test, but with rewards themselves rather than CSs presented. During this test, rats hold the reward identity in working memory long enough to drive responding on the correct action without requiring access to a stored memory. As a result, reward presentation will selectively reinstate performance of the action that earns the same unique reward. If BLA→OFC projections are selectively required for the motivating influence of cue-elicited expectations of unobservable rewards, then inactivation of these projections should have little effect in this task. If, however, these projections are required for a reward to selectively motivate action regardless of its physical presence, then inactivation of this pathway should impair performance.

The data support the former. CNO-hM4Di inactivation of BLA terminals in the OFC did not significantly affect the expression of outcome-specific reinstatement (Figure 4-6B; Reward delivery: $F_{2,16}=5.49$, $P=0.02$; Drug: $F_{1,8}=0.15$, $P=0.71$; Reward x Drug: $F_{2,16}=0.37$, $P=0.70$). Following either vehicle or CNO infusion reward presentation selectively elevated press rate on the lever that, in training, earned the same reward type (Reinstated) relative to both pressing on

the alternate available lever (Non-reinstated) and baseline press rate (P=0.0002 - 0.006). There was also no effect on food-port entries in this task (Figure 4-6C; Reward delivery: $F_{1,8}$=19.32, P=0.002; Drug: $F_{1,8}$=0.03, P=0.86; Reward x Drug: $F_{1,8}$=1.59, P=0.24).

A	Instrumental conditioning	Reinstatement test *Vehicle or CNO*
	A1 – O1 A2 – O2	O1: **A1** v. A2 O2: A1 v. **A2**
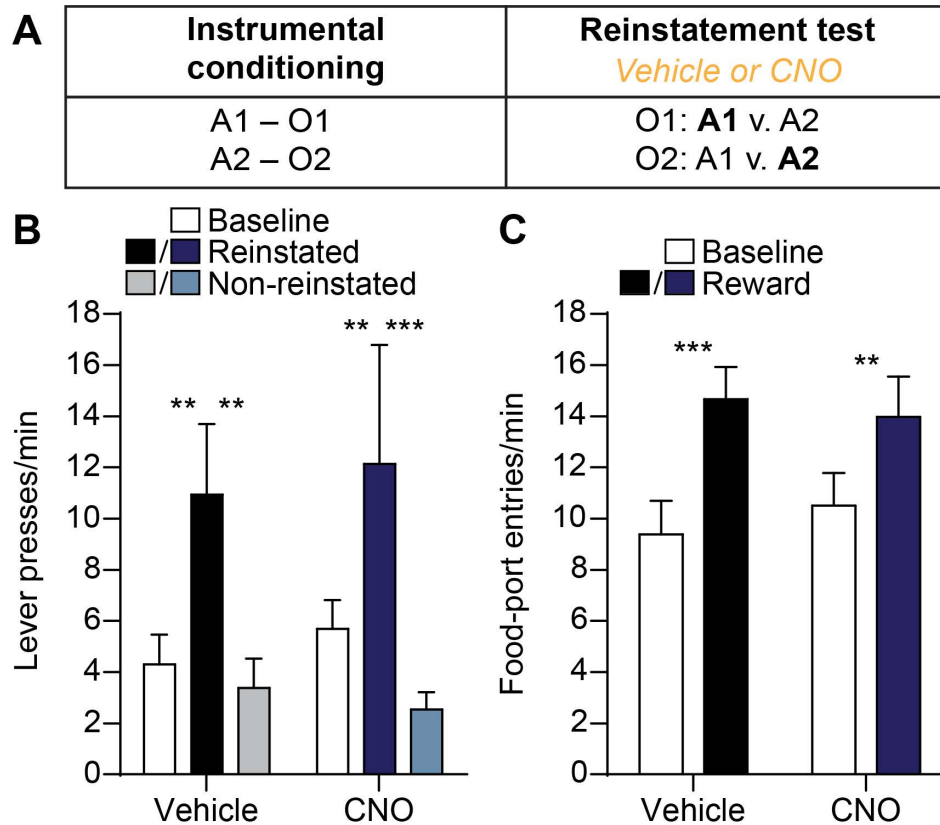
Figure 4-6. Effect of inactivating BLA→OFC projections on outcome-specific reinstatement. **A.** Task design, see text. **B.** Trial-averaged lever presses per 2-min period averaged across both levers during the Baseline periods compared to pressing during the 2-min Reward periods following reward delivery, separated for presses on the lever that, in training, delivered the same outcome as the presented reward (Reinstated) and pressing on the other available lever (Non-reinstated). **C.** Trial-averaged entries into the food-delivery port during the Baseline and Reward-delivery periods. (n=9) Error bars ± s.e.m. **P<0.01, ***P<0.001.

4.5 Discussion

Here we evaluated the contribution of OFC→BLA and BLA→OFC projections to outcome-guided behaviors. Inactivation of BLA terminals in the lateral OFC was found to disrupt the influence of cue-generated reward expectations over both instrumental action choices and Pavlovian goal-approach responses. Activity in these projections was not required when actions were guided by reward expectations based on stored action-outcome contingencies, or when rewards themselves directed action selection. BLA→OFC projections, therefore, enable the cue-triggered reward expectations that can motivate the execution of specific action plans and allow adaptive conditional responding.

BLA→OFC, but not OFC→BLA projections mediate the selective motivating influence of reward cues over action

Chemogenetic inactivation was used to evaluate the function of monosynaptic, direction-specific connections between the BLA and OFC. CNO-hM4Di activation was found to suppress terminal output through presynaptic inhibition, consistent with similar findings in other pathways (Stachniak *et al.*, 2014; Yang *et al.*, 2016; Zhu *et al.*, 2016). Projection inactivation was temporally restricted to specifically assess contribution to online behavioral control. CNO-hM4Di inactivation of BLA→OFC, but not OFC→BLA projections attenuated expression of outcome-specific PIT. In particular, BLA→OFC inactivation blunted the cues' ability to selectively invigorate actions directed at the same unique reward. That this manipulation did not cause the cues to non-discriminately increase action performance and did not alter discrimination between outcomes during reinstatement argues against a simple deficit in discriminating between the CSs. Rather, activity in BLA→OFC projections was found to be necessary for a reward cue, by way of retrieving a representation of a specific predicted reward, to motivate specific action plans.

This result is generally consistent with findings that surgical BLA-OFC disconnection disrupts outcome-guided choice behavior (Baxter *et al.*, 2000; Zeeb & Winstanley, 2013; Fiuzat *et al.*, 2017) and specifically implicates monosynaptic, bottom-up BLA→OFC projections. It does,

however, contrast to data showing that OFC→BLA, but not BLA→OFC projections are necessary for cue-induced reinstatement of cocaine seeking (Arguello *et al.*, 2017), perhaps indicating that cocaine alters recruitment of OFC→BLA projections. An intact OFC is required for BLA neurons to develop associative encoding of cue-predicted rewards (Saddoris *et al.*, 2005). OFC→BLA projections may, therefore, be important for stimulus-outcome encoding, but not normally required once those associations have been well formed. This hypothesis warrants further investigation.

BLA→OFC projections mediate cue-triggered reward expectancies

Successful PIT requires retrieval of both the previously learned action-outcome and stimulus-outcome associations. Two pieces of evidence here suggest that BLA→OFC projections are *not* required for rats to access knowledge of the specific consequences of their instrumental actions. First, inactivation of BLA terminals in the OFC did not affect the ability to use the current value of specific anticipated rewards to influence instrumental choice. Second, it also left unaffected the ability of reward delivery to selectively reinstate performance of the action known to earn the same unique reward. These results could be interpreted as inconsistent with findings that BLA-OFC disconnection lesions disrupt the sensitivity of choice behavior to outcome-specific devaluation (Baxter *et al.*, 2000; Zeeb & Winstanley, 2013; Fiuzat *et al.*, 2017). But, in these previous studies, OFC-BLA connectivity was disrupted throughout both the devaluation learning opportunity and the choice test (and, in some cases, the whole of training and test), unlike the present study in which, to focus on memory retrieval, BLA→OFC projections were inactivated after devaluation just prior to test. While BLA→OFC projections are not needed for value-guided instrumental choice, BLA-OFC connectivity might be necessary for learning about changes in value. This possibility is consistent with evidence that the BLA is required for value encoding (Wassum *et al.*, 2009; Wassum *et al.*, 2011; Parkes & Balleine, 2013; Wassum *et al.*, 2016).

BLA→OFC projections were, however, required for cue-triggered outcome expectations to influence behavior. In support of this, inactivation of BLA terminals in the OFC prevented subjects from modulating their Pavlovian conditional goal-approach responding according to the current value of the specific cue-predicted reward. The PIT deficit, therefore, resulted from an inability of the cue to engender a reward expectation based on a stored stimulus-outcome memory. This could also explain why BLA-OFC disconnection lesions disrupt the sensitivity of instrumental choice behavior to devaluation, given that task demands in these experiments likely required stimulus-outcome information (Baxter *et al.*, 2000; Zeeb & Winstanley, 2013; Fiuzat *et al.*, 2017). That BLA→OFC projections are vital for cue-triggered reward expectations is consistent with evidence that reward cues activate BLA neurons (Paton *et al.*, 2006; Tye & Janak, 2007; Ambroggi *et al.*, 2008; Sangha *et al.*, 2013; Beyeler *et al.*, 2016) and that the OFC specializes in stimulus-outcome representations (Ostlund & Balleine, 2007a; b; Rudebeck *et al.*, 2008; Camille *et al.*, 2011; Rudebeck *et al.*, 2017).

These projections were not, however, necessary for the general, non-specific motivational influence of the cue. During PIT, the cue-induced elevation in goal-approach responding, which did not require a specific reward expectation because there was a single shared food port, was unaffected by inactivation of BLA→OFC projections. Moreover, following devaluation food-port entries were elevated by the reward-predictive cue regardless of whether the specific predicted reward was devalued or not. This is consistent with evidence that the BLA is not required for expression of the general form of PIT, in which cues non-discriminately motivate action (Corbit & Balleine, 2005; Mahler & Berridge, 2012).

The BLA has been suggested to encode motivationally-salient, precise reward representations (Schoenbaum *et al.*, 1998a; Fanselow & Wassum, 2015; Wassum & Izquierdo, 2015). Such information is needed to generate expectations about the current and potential future states, or situations, that guide decision making. Both the expression of outcome-specific PIT and the sensitivity of Pavlovian conditional responses to devaluation are consistent with the subject using

an internally-generated state of the environment to guide behavior. In the devaluation test in particular, appropriate responding requires an understanding that, although things have not perceptually changed (*e.g.*, CS presence), the state is nonetheless different because the specific anticipated reward is no longer valuable. The data here can, therefore, be interpreted as evidence that BLA→OFC projections are required when one must use a cue to generate a state expectation when the critical information, the reward, is not currently observable. In further support of this, these projections were not needed when the reward was itself present to direct action.

While BLA→OFC projections appear to facilitate decision making, they are unlikely to mediate the actual decision-making process itself. Were this the case, inactivation of BLA terminals in the OFC during PIT would have resulted in a non-specific cue-induced increase in performance of both Same and Different actions, indicating an inability to select between actions on the basis of the cue-provided expectation. Rather, BLA projections may relay currently unobservable reward-specific information to the OFC for use in making predictions about future states. Indeed, the OFC has been suggested to be important for using reward expectations to guide action (Izquierdo *et al.*, 2004; Delamater, 2007; Balleine *et al.*, 2011; Schoenbaum *et al.*, 2016; Sharpe & Schoenbaum, 2016) perhaps by influencing downstream decision circuits (Keiflin *et al.*, 2013), and lesions to this region do cause non-specific cue-induced increases in instrumental activity during PIT (Ostlund & Balleine, 2007a). Moreover, activity in the OFC of humans (Gottfried *et al.*, 2003; Klein-Flügge *et al.*, 2013; Howard *et al.*, 2015; Howard & Kahnt, 2017), non-human primates (Rich & Wallis, 2016), and rodents (McDannald *et al.*, 2014; Farovik *et al.*, 2015; Lopatina *et al.*, 2015) can represent detailed information about unobservable anticipated events. Correspondingly, OFC lesions or inactivations cause deficits in using anticipated rewarding events to guide behavior (Gallagher *et al.*, 1999; Pickens *et al.*, 2003a; Izquierdo *et al.*, 2004; Pickens *et al.*, 2005; Ostlund & Balleine, 2007a; West *et al.*, 2011; Jones *et al.*, 2012; Bradfield *et al.*, 2015; Murray *et al.*, 2015). If, as proposed (Wilson *et al.*, 2014; Schuck *et al.*, 2016), the OFC represents the current, not fully observable state, then the results here suggest

that projections from the BLA enable reward-predictive cues to provide the OFC with detailed expectations of potential rewards available in that state. In concordance with this, an intact BLA is needed for neuronal encoding of anticipated outcomes in the OFC in rats (Schoenbaum *et al.*, 2003; Rudebeck *et al.*, 2013), non-human primates (Rudebeck *et al.*, 2013; Rudebeck *et al.*, 2017), and humans (Hampton *et al.*, 2007).

Implications

Evidence suggests the cognitive symptoms underlying many psychiatric diseases result from a failure to appropriately anticipate potential future events. Indeed, deficits in the cognitive consideration of potential rewarding events have been detected in patients diagnosed with addiction (Hogarth *et al.*, 2013), schizophrenia (Morris *et al.*, 2015), depression (Seymour & Dolan, 2008), and social anxiety disorder (Alvares *et al.*, 2014). Disrupted amygdala and OFC activity and connectivity have also been associated with these diseases (Ressler & Mayberg, 2007; Price & Drevets, 2010; Goldstein & Volkow, 2011; Passamonti *et al.*, 2012; Liu *et al.*, 2014; Sladky *et al.*, 2015). These data, therefore, have important implications for the understanding and treatment of these psychiatric conditions, and suggest that they might arise, in part, from disrupted transmission of reward information from the BLA to the OFC.

Chapter 5: Projections within the amygdala-medial orbitofrontal circuit cooperatively mediate cue-guided behavior

5.1 Abstract

Making an optimal choice in a changing environment requires careful consideration and accurate anticipation of potential rewarding events. Often, we rely on the retrieval of detailed reward memories to generate mental representations of future events and to inform our decisions. The medial OFC (mOFC) and basolateral amygdala (BLA) are two essential nodes in the circuitry supporting outcome-guided behaviors. But, mOFC function in reward seeking behavior is relatively unexplored, and how mOFC-BLA circuitry underlies decision making is not known. Therefore, here we used chemogenetic inactivation of mOFC→BLA or BLA→mOFC projections to evaluate their unique contributions to reward seeking and decision making in rats. Transient inactivation of the mOFC→BLA pathway disrupted both the ability of cues to influence action choice and Pavlovian conditional approach responding after a shift in reward value. Inactivation of BLA→mOFC projections, however, only disrupted the latter, leaving cue-guided action selection intact. Neither projection was needed when behavior was guided by action-outcome memories. These data suggest that mOFC-BLA projections may mediate specific aspects of cue-guided behavior: the mOFC→BLA pathway may be necessary for cue-outcome memory retrieval, enabling associative cues to broadly influence choice and adaptive responding, while BLA→mOFC projections enable the retrieval of current value information related to cue-predicted rewards.

5.2 Introduction

Efficient and adaptive decision making relies on the accurate anticipation of rewarding events. During learning, these events become linked to environmental stimuli and actions taken to reach a goal; these relationships are encoded as cue-outcome and action-outcome associative memories, respectively. Often times, rewards are not physically present, requiring one to mentally envision

future positive events. In novel scenarios, the retrieval of stored memories, as well as awareness of internal states, enables appropriate mental representation. If this memory process is intact everyday reward seeking and decision making is adaptive (Balleine & Dickinson, 1998a; Delamater, 2012; Fanselow & Wassum, 2015). However, this cognitive process can go awry in states of psychopathy, resulting in maladaptive behavior.

The basolateral amygdala (BLA) and orbitofrontal cortex (OFC) are two interconnected nodes in the circuitry supporting outcome-guided behaviors. Combined evidence across studies suggests that BLA-OFC interaction is needed for associative encoding and for specific rewarding events to be anticipated (Schoenbaum *et al.*, 2003; Saddoris *et al.*, 2005; Hampton *et al.*, 2007; Rudebeck *et al.*, 2013; Lucantonio *et al.*, 2015; Lichtenberg *et al.*, 2017; Rudebeck *et al.*, 2017). These studies, however, focused on BLA connections with the lateral subdivision of the OFC (lOFC). The medial subregion of the OFC (mOFC) has been far less studied, but emerging evidence across species suggests that this region may specialize in outcome representation, value-guided decision making, and goal selection (Noonan *et al.*, 2010; Bradfield *et al.*, 2015; Murray *et al.*, 2015; Izquierdo, 2017; Noonan *et al.*, 2017; Bradfield *et al.*, 2018). Much like other OFC subregions, the mOFC may be especially needed when determining future rewarding events when outcomes are not observable (Wilson *et al.*, 2014; Bradfield *et al.*, 2015; Schuck *et al.*, 2016; Bradfield *et al.*, 2018). The mOFC and BLA share dense and reciprocal direct connections (Kita & Kitai, 1990; Carmichael & Price, 1995; Hoover & Vertes, 2011; Reppucci & Petrovich, 2016), suggesting that these regions interact to inform reward seeking decisions. Apart from one study (Malvaez *et al.*, 2019a), the contribution of distinct projection pathways within the mOFC-BLA circuit to reward seeking behavior is unknown, and their respective role in decision making remains completely unexplored. Therefore, here we used chemogenetic inactivation of mOFC→BLA or BLA→mOFC monosynaptic projections to evaluate their contributions to the ability to retrieve and utilize detailed associative reward memories to influence reward seeking and decision making.

5.3 Materials and Methods

Subjects

Adult male, Long Evans rats (n=53 total, Charles River Laboratories, Wilmington, MA) weighing between 310-420 g (age ~3 months) at the beginning of the experiment were pair housed in a temperature (68-79 °F) and humidity-regulated (30-70%) vivarium. Rats had *ad libitum* access to filtered tap water in the home cage and were maintained on a food-restricted schedule whereby they received 12-14 g daily of their maintenance diet (Lab Diet, Brentwood, MO) to maintain ~85-90% free-feeding body weight. Rats were handled for ~3 days prior to the onset of the experiment. Training and testing took place during the dark phase of the 12:12 hr reverse dark:light cycle. All procedures were conducted in accordance with the NIH Guide for the Care and use of Laboratory Animals and approved by the UCLA Institutional Animal Care and Use Committee.

Viral constructs

Rats were infused bilaterally with an adeno-associated virus (AAV) expressing the *human M4 muscarinic receptor* hM4Di (AAV8-hSyn-hM4Di-mCherry, viral concentration 4.8×10^{12} or $3.7 \times 10^{\wedge}12$ vg/ml; Addgene). A virus lacking the hM4Di gene (AAV8-hSyn-mCherry; viral concentration 4.6×10^{12} vg/ml; University of North Carolina Vector Core) was used as a control. Behavioral testing began between 6-8 weeks post viral injection to ensure anterograde transport and robust axonal expression of the receptor in axon terminals.

Surgical procedures

Standard aseptic surgical procedures were used under isoflurane anesthesia (5% induction, 1-2% maintenance) as described previously (Lichtenberg et al., 2017). 33-gauge, stainless steel injectors were inserted into either the BLA (AP -3.0 mm, ML ±5.1 mm, DV -8.6 mm relative to bregma) or medial OFC (AP +4.1, ML ±0.5, DV -5.2 mm relative to bregma). Then, viruses were infused at a flow rate of 0.1 μL/min into the BLA (0.4 μL) or mOFC (0.3 μL). Injectors were left

in place for an additional 10 min to ensure virus diffusion. Bilateral guide cannulae (22-gauge stainless steel; Plastics One, Roanoke, VA) were implanted 1 mm above the BLA (AP -3.0 mm, ML ±5.1 mm, DV -7.0 mm from skull) for the mOFC viral injection group. Thinner bilateral guide cannulae (23-gauge stainless steel; Plastics One, Roanoke, VA) targeted the mOFC (AP +4.1, ML ±0.7, DV -2.8 mm from dura) for groups that received viral injections into the BLA. A nonsteroidal anti-inflammatory drug was administered pre- and post-operatively to minimize pain and discomfort. Following surgery, rats were individually housed and allowed to recover for ~14-16 days prior to the onset of behavioral training.

Behavioral training

Training and testing took place in a set of 16 Med Associates (East Fairfield, VT) operant chambers, described previously (Wassum *et al.*, 2016).

Pavlovian training. Each of the 8 daily sessions consisted of 8 tone (1.5 kHz) and 8 white noise conditional stimulus (CS) presentations (75-80 db, 2-min duration), during which either sucrose solution (20%, 0.1 ml/delivery) or grain pellets (45 mg; Bio-Serv, Frenchtown, NJ), were delivered on a 30-s random-time schedule into the food-delivery port, resulting in an average of 4 stimulus-reward pairings per trial. For half the subjects, tone was paired with sucrose and noise with pellets, and the other half received the opposite pairing. CSs were delivered pseudo-randomly with a variable inter-trial interval (2-4 min ITI, mean=3 min). Entries into the food-delivery port were recorded for the entire session. Pavlovian conditioning was measured by comparing anticipatory entries during the CS-probe periods (interval between CS onset and first reward delivery) to entries during baseline periods (2-min period prior to CS onset).

Instrumental training. Rats then received 11 days of instrumental training, which included 2 separate training sessions per day, one with the lever to the left of the food-delivery port and one with the right lever. Each action was reinforced with either grain pellets or sucrose solution (counterbalanced with respect to Pavlovian contingencies). Each session terminated after 30 outcomes had been earned or 30 min had elapsed. Actions were continuously reinforced on the

first day, and then escalated to a random-ratio 20 schedule. The rate of responding on each lever was measured throughout training sessions.

Pavlovian-to-instrumental Transfer Test

Two groups received PIT tests: $mOFC_{hM4Di} \rightarrow BLA$ (n=10) and $BLA_{hM4Di} \rightarrow mOFC$ (n=9). On the day prior to each PIT test, rats were given a single 30-min extinction session during which both levers were available, but pressing was not reinforced to establish low response levels. Each rat was given 2 PIT tests, one following infusion of aCSF vehicle and one following infusion of the otherwise inert hM4Di ligand, clozapine-N-oxide (CNO), into the BLA ($mOFC_{hM4Di} \rightarrow BLA$ group) or mOFC ($BLA_{hM4Di} \rightarrow mOFC$ group). Test order was counterbalanced across subjects. During each PIT test, both levers were continuously present, but pressing was non-reinforced. After 5 min of lever extinction, each 2-min CS was presented separately 4 times each in pseudorandom order, separated by a fixed, 4-min inter-trial interval. No rewards were delivered during CS presentation. The 2-min period prior to each CS presentation served as the baseline. Rats were given 2 retraining sessions for each instrumental association (2 sessions/day for 2 days) and 1 Pavlovian retraining session prior to the second PIT test. Empty vector controls were not included in this behavioral test because we previously verified that CNO has no effect on similar instrumental tasks used in the lab when infused into mOFC terminals in the BLA (Malvaez *et al.*, 2019a), nor PIT performance when infused into BLA terminals in the adjacent lateral OFC (lOFC) region (Lichtenberg *et al.*, 2017).

Outcome-specific devaluation test

The two active virus groups were given a series of two outcome-specific devaluation tests. In addition, immediately following training, an additional cohort of rats lacking the hM4Di receptor, $BLA_{mCherry} \rightarrow mOFC$ group (n=9), received devaluation tests. Prior to each test, rats were given 1-hr, unlimited access to either sucrose solution or grain pellets in pre-exposed feeding chambers such that the pre-fed reward would become devalued or undesirable, while the other reward

would remain valued. Immediately after this pre-feeding, rats received infusions of either vehicle or CNO into the BLA or mOFC and were then tested. The test consisted of two phases. In the first, both levers were available and non-reinforced lever pressing was assessed for 5 min. The levers were then retracted, which started the second phase. During the Pavlovian phase, each 2-min CS was presented, without accompanying reward, separately 2 times each in alternating order, separated by a fixed, 4-min inter-trial interval. The 2-min period prior to each CS presentation served as the baseline. Successful devaluation of the earned outcome was confirmed by post-test consumption of each food reward, in which rats ate significantly less of the devalued reward type (Average: 1.77 g $\pm$ 2.39 s.e.m.) relative to the valued reward (7.05$\pm$4.11; t_{55}=7.55, P<0.0001).

After the first test, rats remained in their home cage for 1-2 days. Then they were given 2 instrumental retraining sessions for each lever-press association (2 sessions/day for 2 days) and 1 Pavlovian retraining session prior to the second test. For the second test, rats were pre-fed on the opposite food reward (*e.g.*, pellets if sucrose had been pre-fed on Test 1), and infused with the opposite drug (*e.g.*, CNO, if they had previously received vehicle). Thus, each rat experienced 2 devaluation tests to allow a within-subject drug-treatment design, counterbalanced for order. After the second devaluation test, the active virus groups were given the second PIT test. The first PIT test was given prior to devaluation testing.

Drugs

CNO (Tocris Bioscience, Sterling Heights, MI) was dissolved in aCSF to 1 mM and was intracranially infused over 1 min in a volume of 0.3 μL into the mOFC or 0.5 μL into the BLA. Injectors were left in place for at least 1 min to allow for drug diffusion. Behavioral testing commenced within 5-10 min following infusion. CNO dose was selected based on evidence of behavioral and pharmacological effectiveness at hM4Di-expressing terminals (Mahler *et al.*, 2014; Lichtenberg *et al.*, 2017).

Histology

At the conclusion of the experiment, rats were deeply anesthetized with Nembutal and transcardially perfused with PBS, and then 4% paraformaldehyde. Brains were removed and post-fixed in 4% paraformaldehyde overnight, placed into 30% sucrose solution, then sectioned into 40 μm slices and stored in cryoprotectant. To visualize hM4Di-mCherry expression in BLA or mOFC cell bodies, free-floating coronal sections were mounted onto slides and coverslipped with ProLong Gold mounting medium with DAPI (Invitrogen, Carlsbad, CA). Axonal expression of hM4Di-mCherry in terminals, signal was immunohistochemically amplified. Floating coronal sections were washed 2 times in 1X PBS for 10 min and then blocked in a solution of 5% normal goal serum (NGS) and 1% Triton X-100 dissolved in PBS for 1-2 hrs at room temperature. Sections were then washed 3 times in PBS for 15 min and then incubated in blocking solution containing rabbit anti-DsRed antibody (1:1000; EMD Millipore, Billerica, MA) with gentle agitation at 4°C for 18-22 hrs. On the second day, sections were rinsed 3 times in the blocking solution and incubated in Alexa Fluor 594-conjugated (red) goat secondary antibody (1:500; Invitrogen) for 2 hr. Sections were washed 3 times in PBS for 30 mins, mounted on slides, allowed to dry, and coverslipped with ProLong Gold mounting medium with DAPI. All images were acquired using a Keyence (BZ-X710; Osaka, Japan) microscope with a 4X or 20X objective (CFI Plan Apo), CCD camera, and BZ-X Analyze software. Subjects with off target hM4Di-mCherry expression were omitted from the analysis. For each included subject, we also confirmed that cannula placement was in the target terminal region and coincided with labeled axon terminals.

Experimental design and statistical analysis

Data were processed with Microsoft Excel (Redmond, WA) then analyzed with GraphPad Prism (La Jolla, CA) and SPSS (IBM Corp, Chicago, IL). For all hypothesis tests, the α level for significance was set to $P<0.05$. The behavioral data of primary interest were statistically evaluated with repeated-measures ANOVAs (Geisser-Greenhouse correction). For well-established behavioral effects (PIT, devaluation, reinstatement), multiple pairwise comparisons (paired t-test, two-tailed) were used for *a priori* posthoc comparisons, as advised by (Levin *et al.*, 1994)

based on a logical extension of Fisher's protected least significant difference (PLSD) procedure for controlling familywise Type I error rates. Bonferroni or Dunnet's corrections were used for posthoc analyses of all drug effects.

Behavioral data for all cohorts were analyzed for the rate of both lever pressing and entries into food-delivery port. Both drug and test phase were within-subject factors. All data were averaged across trials. For the PIT tests, lever pressing was averaged across levers for the 2-min baseline period and compared to that during the CS period, which was separated for presses on the lever that, during training, earned the same outcome as the cue predicted (*i.e.*, CS-Same presses) versus those on the other available lever (*i.e.*, CS-Different presses). For the PIT tests, entries into the food-delivery port were compared between the baseline and CS periods. Food-delivery port entries were analyzed similarly for the Pavlovian phase of the devaluation test; baseline entry rate was compared to entries during presentation of each CS separated for the cue that predicted the valued versus devalued reward type. Lever pressing during the instrumental phase of the devaluation test was separated for actions on the lever that, in training, earned the currently devalued v. valued reward.

5.4 Results

Pathway-specific chemogenetic mOFC-BLA manipulations

We used a chemogenetic approach (Armbruster *et al.*, 2007; Smith *et al.*, 2016) to manipulate mOFC→BLA or BLA→mOFC projections by injecting an AAV carrying a synapsin-driven transgene allowing hM4Di expression in the mOFC (mOFC$_{hM4Di}$→BLA group) or BLA (BLA$_{hM4Di}$→mOFC group). Then, we implanted guide cannulae over the BLA (mOFC$_{hM4Di}$→BLA group) or mOFC (BLA$_{hM4Di}$→mOFC group) for local CNO inactivation of mOFC terminals in the BLA or BLA terminals in the mOFC, as performed and validated prior (Lichtenberg *et al.*, 2017; Malvaez *et al.*, 2019). This approach yielded robust hM4Di-mCherry expression in cell bodies

(Figure 5-1A-B) and axon terminals (Figure 5-1E-F) and injector tip locations were in close proximity to axonal expression (Figure 5-1C-D, G-H).
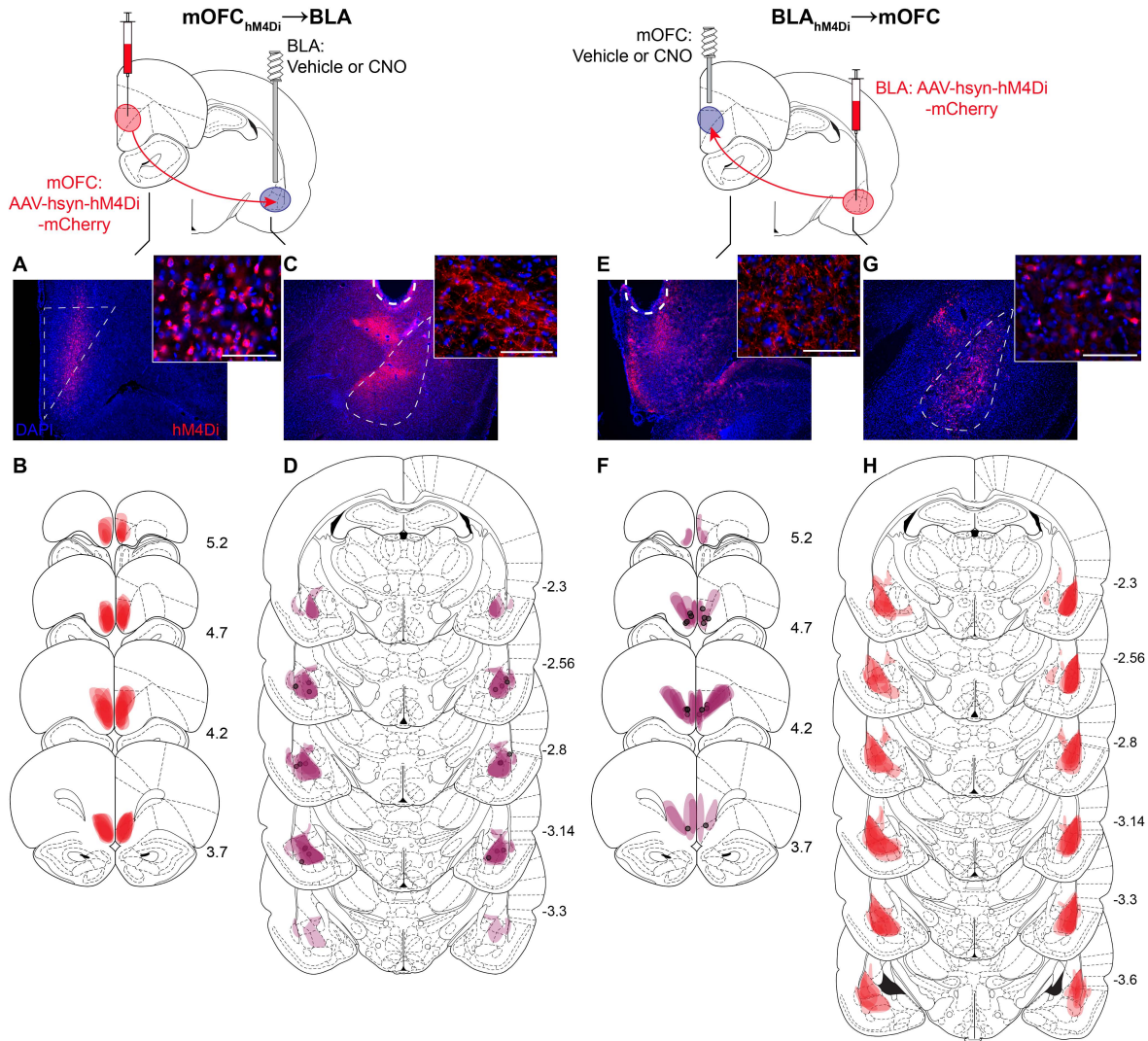
Figure 5-1. hM4Di viral expression and cannula placements. **A-D:** mOFC$_{hM4Di}$→BLA rats (*n*=10). Bilateral *hsyn-hM4Di-mCherry* injections were made into the mOFC and guide cannulae were implanted above the BLA, such that CNO infusion would inactivate mOFC inputs to the BLA. **A.** Representative fluorescent image of hM4Di-mCherry expression in the mOFC. Dashed line indicates boundaries of the mOFC. Scale bars = 100 μm. **B.** Schematic representation of hM4Di-mCherry maximal viral spread in the mOFC for all subjects (red). Number labels to the right of each coronal represent distance anterior to bregma. Coronal section drawings taken from (Paxinos & Watson, 1998). **C.** Representative image of hM4Di-mCherry expression in the BLA. Dashed line demarcates guide cannula track and outlines the BLA. **D.** Schematic of microinfusion injector tips in the BLA and mOFC terminal spread (purple). **E-H:** BLA$_{hM4Di}$→mOFC rats (*n*=9). Bilateral *hsyn-hM4Di-mCherry* injections were made into the BLA and guide cannulae were implanted above the mOFC. **E.** Representative immunofluorescent image of hM4Di-mCherry expression in the mOFC. **F.** Schematic representation of microinfusion injector tips in the mOFC and BLA terminal spread (purple). **G.** Representative fluorescent image of hM4Di-mCherry expression in the BLA. **H.** Schematic representation of hM4Di-mCherry maximum viral spread in the BLA for all subjects (red).

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Contribution of mOFC→BLA and BLA→mOFC projections to outcome-specific Pavlovian-to-instrumental transfer

Using a chemogenetic approach, we examined the contribution of mOFC→BLA and BLA→mOFC projections to the ability to retrieve a stored associative memory of distinct predicted rewards and to use this information to influence reward-seeking decisions during outcome-specific Pavlovian-to-instrumental transfer (PIT; Figure 5-2A). Rats were trained to learn that two auditory CSs predicted two distinct food rewards and then to earn each of those two rewards by pressing on independent levers. During the final Pavlovian session, rats learned that CSs were predictive of rewards because they entered the food-delivery port significantly more during the CS probe periods (Average entry rate on the final training session mOFC$_{hM4Di}$→BLA group: 18.8 entries/min $\pm$ 1.58 s.e.m.; BLA$_{hM4Di}$→mOFC group: 15.45$\pm$1.64; BLA$_{mCherry}$→mOFC group: 15.45$\pm$1.64) compared to baseline periods (mOFC$_{hM4Di}$→BLA group: 8.08$\pm$0.96, t_9=12.12, P=<0.0001; BLA$_{hM4Di}$→mOFC group: 5.71$\pm$1.02, t_9=4.92, P=0.0008; BLA$_{mCherry}$→mOFC group: 5.71$\pm$1.02, t_8=7.04, P=0.0001). Rats in all groups also acquired the instrumental behavior (Final average press rate mOFC$_{hM4Di}$→BLA group: 18.49 presses/min $\pm$ 1.41 s.e.m.; BLA$_{hM4Di}$→mOFC group: 19.85$\pm$1.26; BLA$_{mCherry}$→mOFC group: 21.71$\pm$2.02).

At the PIT test, both levers were presented at once, but lever pressing was not rewarded. Each CS was presented 4 times (without reward delivery), with intervening CS-free baseline periods, to assess its influence on action choice in the novel scenario. Because the CSs are never associated with the instrumental actions in training, this test assesses the rats' ability to, upon CS presentation, retrieve a stored memory of the specific predicted reward and to use this information to bias behavioral responses towards actions known to earn the same reward (Kruse *et al.*, 1983; Colwill & Motzkin, 1994; Gilroy *et al.*, 2014; Corbit & Balleine, 2015).

Chemogenetic inactivation of mOFC terminals in the BLA attenuated expression of outcome-specific PIT (Figure 5-2B; Main effect of CS Period: $F_{2,18}$=10.46, P=0.001; Drug: $F_{2,18}$=13.42, P=0.005: CS x Drug: $F_{2,18}$=3.34, P=0.059). Rats demonstrated robust PIT under vehicle-infused

control conditions; post-hoc comparisons revealed that presentation of the CS elevated responding selectively on the lever that, in training, earned the same predicted reward (CS-Same) relative to the alternate lever (CS-Different) (P=0.0005) and baseline performance (P=<0.0001). After CNO infusion, there was no significant difference between CS-Same and CS-Different (P=0.117) or baseline responding (P=0.171). Further, CS-Same performance was lower following CNO infusion (P=0.0001) than following vehicle. Importantly, inactivating mOFC terminals in the BLA was restricted to cue-influenced action during the PIT test; although there were differences between CS-Same responding when comparing the two treatment conditions, lever pressing during the baseline period was unaffected by CNO (P=0.22).

Contrary to this finding, CNO-hM4Di inactivation of mOFC terminals in the BLA did not disrupt the expression of PIT (Figure 5-2C; Main effect of CS Period: $F_{2,16}$=63.71, P<0.0001; Drug: $F_{1,8}$=0.26, P=0.625; CS x Drug interaction: $F_{2,16}$=0.22, P=0.803). Following either vehicle or CNO infusion, rats demonstrated robust PIT; presentation of the CS elevated performance of the CS-Same action relative to both baseline (P=<0.0001) and CS-Different performance (P=<0.0001).

Pavlovian conditional food-port approach responding was not altered by chemogenetic inactivation of mOFC terminals in the BLA (Figure 5-2D; CS Period: $F_{1,9}$=58.31, P=<0.0001; Drug: $F_{1,9}$=10.43, P=0.01; CS x Drug: $F_{1,9}$=3.17, P=0.109), nor BLA terminals in the mOFC (Figure 5-2E; CS Period: $F_{1,8}$=83.04, P=<0.0001; Drug: $F_{1,8}$=0.04, P=0.856; CS x Drug: $F_{1,8}$=0.06, P=0.813). In both groups under vehicle and CNO treatment conditions, CS presentation significantly elevated food-port entries (P=<0.0001). Thus, the retrieval and use of cue-outcome memories to guide goal-approach responding was intact. These findings suggest that mOFC→BLA, but not BLA→mOFC projections, are needed for reward-predictive cues to influence action selection, perhaps resulting from a failure to properly retrieve cue- and/or action-outcome memories.

A

Pavlovian conditioning	Instrumental conditioning	PIT Test *Vehicle or CNO*
CS1 – O1 CS2 – O2	A1 – O1 A2 – O2	CS1: **A1** v. A2 CS2: A1 v. **A2**
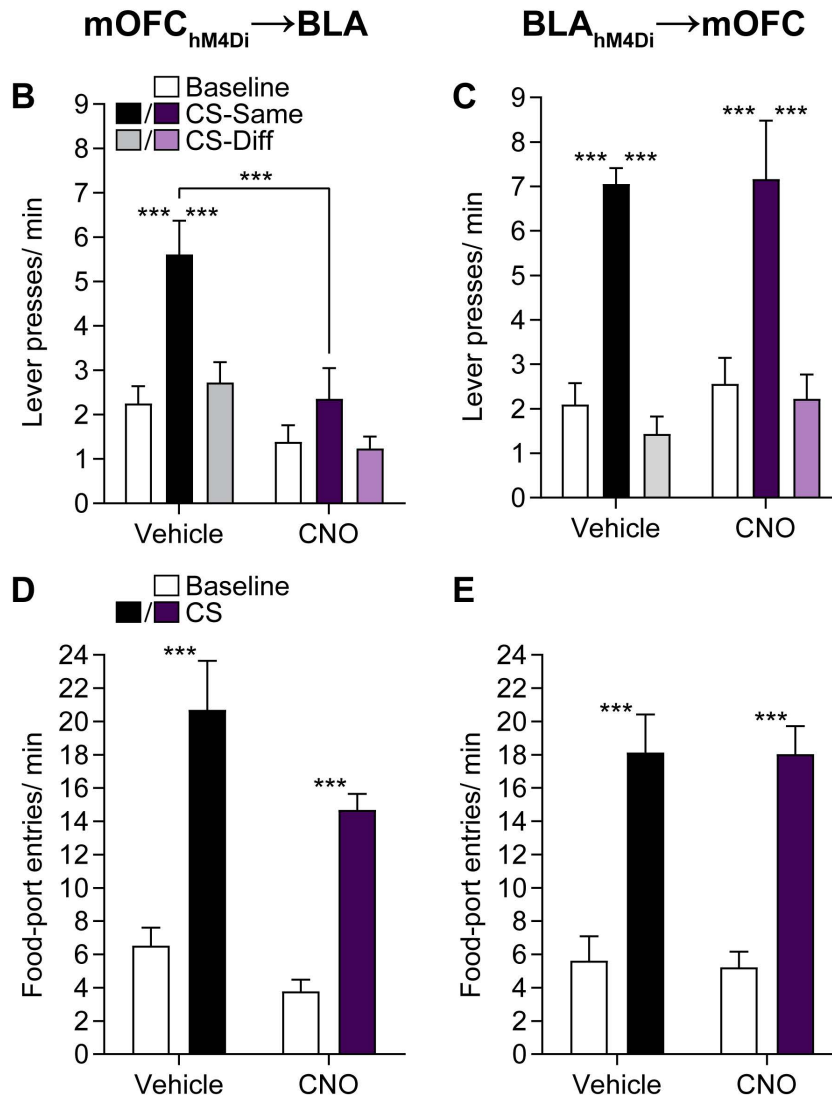
Figure 5-2. Effect of inactivating mOFC→BLA or BLA→mOFC projections on Pavlovian-to-instrumental transfer. **A.** Task design - see text. CS, conditional stimulus; O; outcome/reward; A, action. **B-C.** Trial-averaged lever presses per 2-min period averaged across both levers during the Baseline periods compared to pressing during the CS periods separated for presses on the lever that, in training, delivered the same outcome as predicted by the CS (CS-Same) and pressing on the other available lever (CS-Diff) for mOFC$_{hM4Di}$→BLA (**B**; *n*=10) or BLA$_{hM4Di}$→mOFC (**C**; *n*=9) groups. Inset- CS-induced difference score (CS presses - Baseline presses) on action Same v. Different. **D-E.** Trial-averaged entries into the food-delivery port during the Baseline and CS periods for the mOFC$_{hM4Di}$→BLA (**D**) and BLA$_{hM4Di}$→mOFC (**E**) groups. Error bars ± s.e.m. *$P<0.05$, **$P<0.01$, ***$P<0.001$.

Contribution of mOFC→BLA and BLA→mOFC projections to the sensitivity of instrumental actions and Pavlovian conditional responses to outcome-specific devaluation

The current data show that mOFC→BLA, but not BLA→mOFC, projections are necessary for cue-guided action selection. As described prior, this behavior relies on the retrieval of a representation of specific shared rewards (*i.e.*, outcomes) previously encoded in both Pavlovian stimulus-outcome and instrumental action-outcome associations (Dickinson & Balleine, 2002). The BLA is required for using both types of associations to guide performance (Blundell *et al.*, 2001; Balleine *et al.*, 2003; Ostlund & Balleine, 2008a; Johnson *et al.*, 2009), and the mOFC is required to retrieve unobservable instrumental action-outcome associations to guide choice (Bradfield *et al.*, 2015; Bradfield *et al.*, 2018). Given this functional dissociation, we next sought to determine the role of mOFC→BLA and BLA→mOFC projections in the use of stimulus- and action-outcome contingencies separately in guiding behavior after a shift in value (Figure 5-3A).

Similar to our previous investigations of the lOFC-BLA circuit (Lichtenberg *et al.*, 2017), after the first PIT test, we also tested the same cohort of rats on their ability to use action-outcome (*i.e.*, instrumental devaluation) and cue-outcome (*i.e.*, Pavlovian devaluation) memories to influence behavior after a shift in reward value. Prior to test, one of the prior trained food rewards was devalued by sensory-specific satiety. Rats were then given an unrewarded instrumental choice test followed by a test of food-port approach responding, during which levers were retracted and each CS was presented 2 times. As done prior, infusions of vehicle or CNO were made after sensory-specific satiety, but prior to test to assess the retrieval of instrumental and Pavlovian reward representations, rather than on devaluation encoding during satiety (Lichtenberg *et al.*, 2017)

CNO-hM4Di inactivation of mOFC terminals in the BLA nor BLA terminals in the mOFC did not affect the sensitivity of instrumental choice performance to reward devaluation (mOFC$_{hM4Di}$→BLA group; Figure 5-3B; Devaluation: $F_{1,9}$=15.32, P=0.004; Drug: $F_{1,9}$=0.20,

P=0.668; Devaluation x Drug: $F_{1,9}$=0.47, P=0.512; BLA$_{hM4Di}$→mOFC group; Figure 5-3C; Devaluation: $F_{1,8}$=41.08, P=0.0002; Drug: $F_{1,8}$=1.01, P=0.345; Devaluation x Drug: $F_{1,8}$=0.622, P=0.453).

Pavlovian conditional food-port approach responding, however, was impaired following CNO in both projection pathway groups after shifting the current value of each specific predicted reward (Figure 5-3D,E). CNO impaired the CS-induced elevation in food-port approach responding when infused into the BLA (mOFC$_{hM4Di}$→BLA group; Figure 5-3D; Devaluation: $F_{2,18}$=20.47, P=<0.0001; Drug: $F_{1,9}$=1.32, P=0.281; Devaluation x Drug: $F_{2,18}$=8.07, P=0.003) and mOFC (BLA$_{hM4Di}$→mOFC group; Figure 5-3E; Devaluation: $F_{2,16}$=9.86, P=0.002; Drug: $F_{1,8}$=2.98, P=0.123; Devaluation x Drug: $F_{2,16}$=5.01, P=0.021). Indeed, after vehicle infusion, post-hoc comparisons revealed that rats demonstrated higher food-port approach responding when the CS signaled a valued reward (CS-valued) relative to a devalued reward (CS-devalued) (mOFC$_{hM4Di}$→BLA group: P=0.001; BLA$_{hM4Di}$→mOFC group: P=0.001) and baseline entries (mOFC$_{hM4Di}$→BLA group: P=0.0002; BLA$_{hM4Di}$→mOFC group: P=0.006). Following CNO infusion, for both projection pathway groups, there was no significant difference between CS-valued and CS-devalued entries (mOFC$_{hM4Di}$→BLA group: P=0.1008; BLA$_{hM4Di}$→mOFC group: P=0.315). Further, food-port entries were lower during presentation of CS-valued following CNO infusion (mOFC$_{hM4Di}$→BLA group: P=0.001; BLA$_{hM4Di}$→mOFC group: P=0.0007). The null virus control BLA$_{mCherry}$→mOFC group (n=9) did not demonstrate any clear behavioral deficits in lever press nor food-port approach responding during the devaluation test (data not reported). Overall, these data suggest that after a shift in reward value, both projection pathways were not needed for behavior guided by action-outcome memories, but were necessary for the retrieval of the current value information related to cue-predicted rewards.
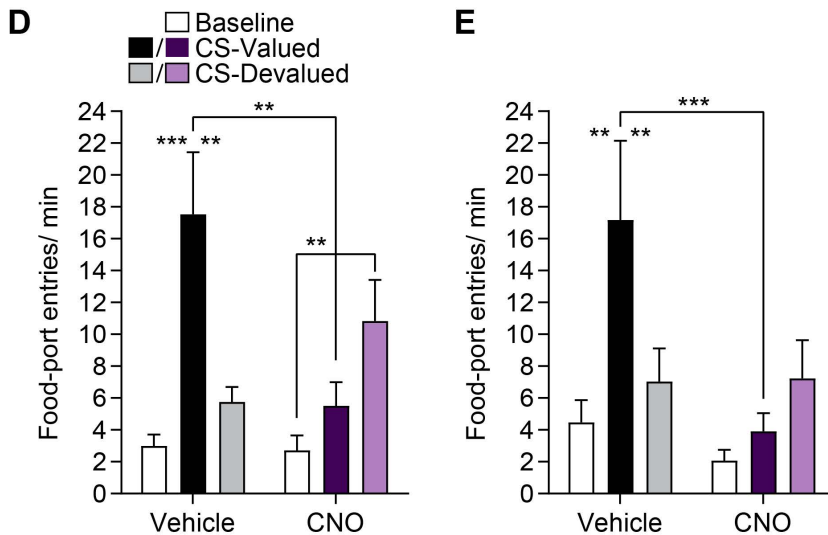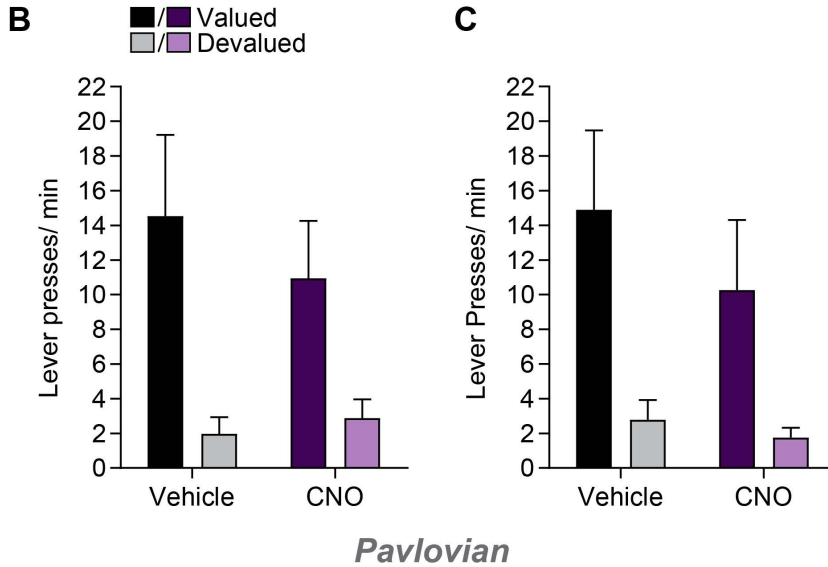
Figure 5-3. Effect of inactivating mOFC$_{hM4Di}$→BLA and BLA$_{hM4Di}$→mOFC projections on sensitivity to outcome-specific devaluation. **A.** Task design (see text). Only one devaluation condition shown. **B-C.** Average lever-press rate during the devaluation test. Presses separated for those that, in training, earned the currently devalued v. valued reward type for mOFC$_{hM4Di}$→BLA (**B**; n=10) or BLA$_{hM4Di}$→mOFC (**C**; n=9) groups. **D-E.** Trial-averaged entries into the food-delivery port during the Baseline and CS periods separated by the CS predicted the valued v. devalued reward for the mOFC$_{hM4Di}$→BLA (**D**) or BLA$_{hM4Di}$→mOFC (**E**) groups. Error bars ± s.e.m. *$P<0.05$, **$P<0.01$, ***$P<0.001$.

5.5 Discussion

Reward-seeking motivation is driven by expectations of future rewards, a cognitive process that is informed by associative memories linked to specific rewarding events. Here, we evaluated the contribution of mOFC→BLA and BLA→mOFC projections to outcome-guided behaviors. Chemogenetic inactivation of mOFC terminals in the BLA disrupted the ability of cues to guide instrumental action choices (*i.e.*, outcome-specific PIT) and Pavlovian conditional approach responding following a shift in outcome value (*i.e.*, Pavlovian devaluation). Inactivation of BLA terminals in the mOFC only disrupted the latter, leaving PIT intact. Neither pathway was required when responding was guided by action-outcome memories alone. Projections within the mOFC-BLA circuit, therefore, may mediate specific aspects of cue-guided behavior: the mOFC→BLA pathway may be necessary for cue-outcome memory retrieval, thus enabling reward-predictive cues to broadly influence both choice and adaptive value-based responding, while BLA→mOFC projections enable the retrieval of value information related to cue-predicted rewards.

mOFC→BLA projections mediate the influence of reward cues over action and value-guided Pavlovian responding

CNO-hM4Di inactivation of mOFC→BLA projections attenuated the expression of cue-guided action selection, *i.e.* outcome-specific PIT. Activity within mOFC→BLA projections was found to be necessary for a reward cue to motivate specific actions directed towards unique cue-predicted rewards. It is hypothesized that the OFC is involved in representing and using information that is not readily observable in the environment to guide behavior (Sharpe *et al.*, 2015; Schuck *et al.*, 2016). Consistent with the current finding, studies have shown that the mOFC is required for outcome-specific PIT, instrumental devaluation, and instrumental reversals (Gourley *et al.*, 2010; Bradfield *et al.*, 2015; Gourley *et al.*, 2016), tasks in which subjects must use multiple outcome representations to guide actions. Successful PIT requires subjects to use stored cue- and action-outcome memories, so mOFC→BLA projections may facilitate the retrieval and/or representation of this associative information when subjects must make a choice when outcomes are

unobservable. Furthermore, it is unlikely that the mOFC→BLA pathway is involved in CS discrimination nor the decision-making process itself. Were this the case, mOFC→BLA inactivation would result in a non-specific cue-induced increase in performance of both Same and Different actions, representing a deficit in choice between actions. Rather, CNO-hM4Di inactivation only attenuated the selective motivating influence of CSs, similar to behavioral effects observed after mOFC or BLA lesions, supporting the idea that the mOFC may convey unobservable reward-related information to the BLA for use in predicting future states (Ostlund & Balleine, 2008a; Bradfield *et al.*, 2015; Malvaez *et al.*, 2015; Bradfield *et al.*, 2018).

As mentioned prior, the performance of PIT requires subjects to retrieve previously learned cue- and action-outcome memories. Here, two main findings suggest that mOFC→BLA projections are required for cue-triggered, rather than action-based, reward expectancies to guide behavior. First, in addition to PIT, we found that the mOFC→BLA pathway is needed for Pavlovian devaluation. Inactivation of mOFC terminals in the BLA prevented subjects from modulating their Pavlovian conditional goal-approach behavior according to the current value of cue-predicted rewards. Thus, the PIT deficit likely resulted from an inability to retrieve and/or utilize stored cue-outcome memories to guide action selection. Second, we found that mOFC→BLA projections were *not* needed for instrumental devaluation, a behavior reliant upon stored action-outcome associations. Coherent with this finding, in mice, post-training mOFC lesions leave instrumental devaluation intact (Gourley *et al.*, 2010). Recent reports suggest that lesioning the anterior, but not posterior, mOFC disrupts devaluation (Bradfield *et al.*, 2018), indicating that anatomical distinctions within the mOFC are key. In support of these findings, mOFC→BLA projections are densest in the posterior mOFC (Hoover & Vertes, 2011; Bradfield *et al.*, 2018), thus, these output cells were likely spared resulting in no instrumental devaluation impairment.

BLA→mOFC projections are required for cues to modulate value-guided Pavlovian responding

Interestingly, the current data suggest that BLA→mOFC projections allow cues to guide Pavlovian responding based on the current value of anticipated rewards. Two main findings support this claim. First, we found that inactivation of BLA terminals in the mOFC did not affect outcome-specific PIT, a process mediated by the use of previously learned associative memories in a context in which the value of rewards remains unaltered. If BLA→mOFC projections were simply needed for cue-outcome memory retrieval, PIT would be disrupted. Furthermore, cue-induced elevation in goal-approach responding during PIT, which requires the retrieval of cue-outcome associations to motivate food-port entries, was also intact. Second, chemogenetic inactivation of this pathway did not affect the ability of subjects to use the current value of rewards to influence instrumental choice, *i.e.* instrumental devaluation.

BLA→mOFC projections are not required to retrieve cue-outcome memories; if this were the case, PIT would be impaired. Projections from the BLA to the mOFC enable cues to trigger the retrieval of outcome value information used to guide behavior. Indeed, reward cues activate BLA neurons (Paton *et al.*, 2006; Tye & Janak, 2007; Ambroggi *et al.*, 2008; Sangha *et al.*, 2013; Beyeler *et al.*, 2016), and an intact BLA is needed for Pavlovian devaluation (Johnson *et al.*, 2009). Much like BLA neurons, single-unit studies have shown that mOFC neurons are also activated by reward cues, but specifically fire in response to cues predictive of a change, in particular a decrease, in reward value (Burton *et al.*, 2014; Lopatina *et al.*, 2016). Further, findings across species suggest that the mOFC is implicated in mediating value-guided behaviors in rats (Bradfield *et al.*, 2015; Münster & Hauber, 2018), monkeys (Noonan *et al.*, 2010), and humans (Camille *et al.*, 2011; Noonan *et al.*, 2017). In particular, the mOFC may be needed for value updating during scenarios in which outcome value is uncertain (Murray *et al.*, 2015; Izquierdo, 2017) such as during reversal learning (Gourley *et al.*, 2010; Dalton *et al.*, 2016). Therefore, much like the BLA and mOFC alone, BLA→mOFC projections are required to guide behavior when value information of cue-predicted rewards is needed to inform behavior, such as when value is in flux.

The BLA is required for reward value encoding (Wassum *et al.*, 2009; Wassum *et al.*, 2011; Parkes & Balleine, 2013; Wassum *et al.*, 2016). Moreover, BLA neurons encode associative reward memories (Redondo *et al.*, 2014; Beyeler *et al.*, 2016) and affective value in general (Schoenbaum *et al.*, 1998b; Shabel & Janak, 2009; Beyeler *et al.*, 2016), thus, the BLA may act as a storage hub for this information. During both PIT and Pavlovian devaluation, subjects must use an internally-generated state of the environment to guide behavior. Outcome value information, however, is most relevant in the devaluation scenario. One possibility is that cues may activate specific memories (*i.e.*, outcome value) in the BLA, which are then sent to cortical areas specializing in further processing this information for use in decision execution. In support of this notion, in the nonhuman primate, BLA neurons fire in response to reward-predictive cues in a manner that reflects the anticipated value of the outcome (Belova *et al.*, 2008). Indeed, the mOFC has emerged as a critical locus for processing reward value-related information (Malvaez *et al.*, 2019) and in the nonhuman primate, mOFC neurons are sensitive to sensory-specific satiety (Bouret & Richmond, 2010).

Interestingly, the mOFC→BLA pathway was required for *two* different novel scenarios or states in which successful performance was reliant upon stored outcome information. This projection, therefore, may broadly mediate the retrieval of state-dependent reward memories. In support of this idea, using identical chemogenetic inactivation methods, Malvaez *et al.* (2019) found that mOFC→BLA projections facilitate the retrieval of reward value, specifically when a state-dependent reward value had been previously encoded (*i.e.*, during incentive learning) (Malvaez *et al.*, 2019). In the current study, upon reward-predictive cue presentation, the mOFC may provide the BLA with the most relevant outcome information necessary to perform the task at hand.

Both projection pathways within the mOFC-BLA circuit were needed for tasks in which outcomes were unobservable, *i.e.* situations in which cue-outcome memories had to be used to mediate behavior. Additional experiments may be necessary to conclude any exclusive role of

these projections in unobservable task states. In support of the current findings, the mOFC is *not* required to mediate behavior in scenarios in which rewards are present (*i.e.*, during outcome-specific reinstatement) (Bradfield *et al.*, 2018). In contrast, however, an intact BLA is required for physically present rewards to guide behaviors dependent on action-outcome memories (Ostlund & Balleine, 2008a). Whether or not the mOFC-BLA circuit is needed when rewards are physically present to guide behavior requires further exploration.

One critical remaining question is how mOFC→BLA projections interact with BLA neurons projecting back to the mOFC, and vice versa, to mediate memory retrieval processes. Projections within the BLA-mOFC circuit anatomically overlap quite extensively (see Chapter 2, Figure 2-3), making mOFC→BLA projection neurons well positioned to gate the retrieval of outcome-related information stored in the BLA, perhaps via direct connections to BLA→mOFC back-projections or perhaps indirectly by synapsing onto neighboring excitatory projections or interneurons. At the physiological level, studies have identified strong reciprocal connections between the medial prefrontal cortex and BLA (Little & Carter, 2013). Elucidating the function of synaptic connections within the mOFC-BLA circuit warrants future investigation.

The cognitive symptoms underlying several psychiatric disorders result from a failure to properly foresee potential future rewarding events (Hogarth *et al.*, 2013; Radulescu & Niv, 2019). Disrupted amygdala and mOFC activity and connectivity has been observed in patients with bipolar disorder (Linke *et al.*, 2012), social anxiety disorder (Sladky *et al.*, 2015), major depressive disorder (Keedwell *et al.*, 2005), and obsessive-compulsive disorder (Milad & Rauch, 2012). The current data provide insight into how amygdala-cortico dysfunction underlies these disorders and may contribute to future applications of therapeutic brain stimulation targeted at this circuitry in patients.

Chapter 6: Conclusions and general discussion

The psychological processes underlying reward-related decision making are governed by expansive pharmacologically diverse, interconnected brain networks. The data presented in this work uncover a novel function of an endogenous basolateral amygdala (BLA) opioid receptor system and elucidate the role of specific projection pathways within BLA-OFC circuitry in cue-mediated behaviors. In the following text, major findings supporting this claim and their significance is discussed.

The BLA mu-opioid receptor in cue-guided behavior

First, by pharmacologically targeting opioid receptors in the BLA we found that endogenous activation of the BLA mu-, but not delta-opioid receptor was needed for reward-predictive cues to guide action selection (*i.e.*, outcome-specific PIT). BLA mu-opioid receptor antagonism did not disrupt the ability of a reward itself to motivate action, suggesting a selective role for this receptor in cue-mediated memory retrieval processes.

The BLA is thought to encode motivationally-salient, precise reward memories (Wassum & Izquierdo, 2015), and BLA mu-opioid receptor activation is required during incentive learning, specifically when the memory of a reward is modified to encode a positive shift in value (Wassum *et al.*, 2009; Wassum *et al.*, 2011). Therefore, the BLA mu-opioid receptor may regulate access to these specific reward memories. Within the BLA, the mu-opioid receptor is located both presynaptically and postsynaptically (Finnegan *et al.*, 2006; Likhtik *et al.*, 2008), making this receptor well positioned to act on incoming and outgoing signals to and from the BLA. Perhaps mu-opioid receptors modulate GABAergic inputs onto BLA projection cells (Finnegan *et al.*, 2006), thereby altering their response to incoming glutamate signals known to encode precise reward memories (Wassum *et al.*, 2012; Malvaez *et al.*, 2015). This speculation agrees with the function of GABAergic, mu-expressing intercalated cells (ITCs) surrounding the BLA. ITCs are thought to gate the influence of afferent sensory input over BLA projections (Millhouse, 1986; Likhtik *et al.*, 2008; Asede *et al.*, 2015). Prefrontal regions and auditory cortices project robustly

to BLA ITCs (Strobel *et al.*, 2015), and in the nonhuman primate, the OFC sends direct projections to amygdalar ITC cells (Barbas *et al.*, 2011). During cue-informed behaviors such as PIT, it is possible that the BLA mu-opioid receptor modulates incoming sensory information from frontal cortical regions via ITCs to enable cue-outcome memory retrieval. In the current study, intra-BLA CTOP likely disrupted the activity of both ITC mu-opioid receptors and those expressed, albeit more sparsely, within the BLA itself (Ding *et al.*, 1996; Zhang *et al.*, 2015). How BLA mu-opioid receptors control cue-mediated behavior at the circuit level is a critical remaining question for future exploration.

A BLA-OFC network model of cue-guided behavior

Second, we sought to understand BLA-OFC circuitry in reward expectation-guided behaviors, so we used retrograde tract tracers to anatomically map BLA projection neurons to the mOFC and lOFC, and found distinct populations of BLA→mOFC and BLA→lOFC projectors. By combining anterograde and retrograde viral tracers, we also visually identified gross reciprocal overlap between OFC projection cell bodies and terminals arising from the BLA in frontal cortical regions, suggesting that pathways within the BLA-OFC network may work in concert to facilitate behavioral output.

Next, to determine a causal role of specific BLA-OFC projection pathways in outcome-guided behaviors, we optimized and applied an innovative pathway-specific chemogenetic approach to transiently inactivate projections during reward expectation-guided behaviors via tests of associative reward memory retrieval. The series of experiments presented in this work reveal novel functions of projections within the BLA-OFC network in mediating cue-guided behaviors. BLA→lOFC projections were required for cue-guided action selection (*i.e.*, outcome-specific PIT) and Pavlovian goal-approach responding according to a reward's current value (*i.e.*, Pavlovian devaluation), while BLA→mOFC projections were only required for the latter. Therefore, we conclude that BLA→lOFC outputs may relay general cue-outcome information for use in a variety

of behavioral states, whereas BLA→mOFC projections transmit specific information related to outcome value, particularly when reward value is uncertain or newly altered (Figure 6-1A).
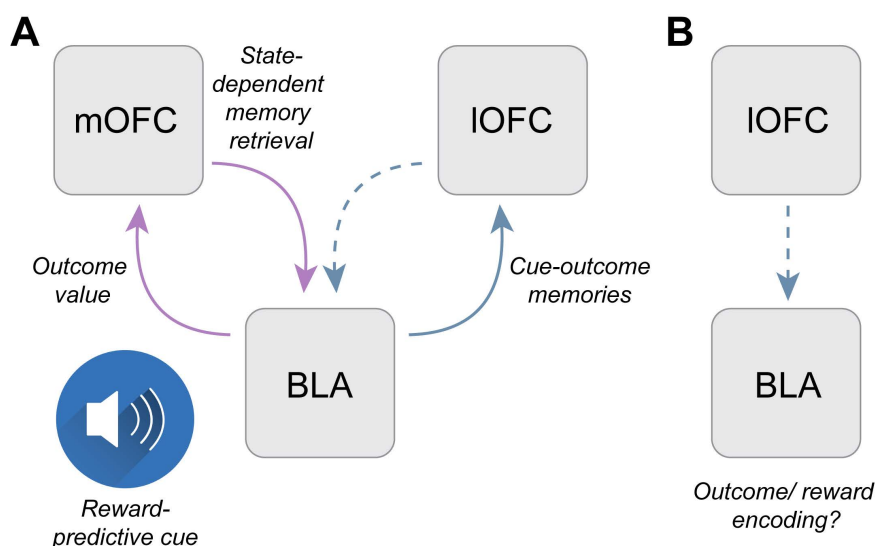
Figure 6-1. Proposed BLA-OFC network model of cue-guided behavior. A. BLA→ lOFC projections mediate cue-guided action selection (*i.e.*, outcome-specific PIT) and goal-approach responding according to a reward's current value (*i.e.*, Pavlovian devaluation), while BLA→ mOFC projections mediate outcome value retrieval. The mOFC→BLA projection pathway is required for both PIT and devaluation, thus, may mediate state-dependent memory retrieval **B.** Hypothesized role of lOFC→BLA projections in reward value and cue-outcome memory encoding (right).

Decades of evidence from studies in both the fear and appetitive domain suggest that the BLA is an "associative hub" for incoming sensory information (Cardinal *et al.*, 2002; Fanselow & Wassum, 2015; Wassum & Izquierdo, 2015), receiving input from an array of brain regions, including the cortex, hypothalamus, hippocampus, and brain stem (Sah *et al.*, 2003; Pessoa, 2011). Thus, the BLA is well positioned to determine the overall state of an organism by linking incoming environmental information, particularly sensory properties of stimuli, to their affective valence (Fanselow & LeDoux, 1999; Ghashghaei & Barbas, 2002; Morrison & Salzman, 2010; Janak & Tye, 2015). As mentioned prior, reward cues activate BLA neurons (Paton *et al.*, 2006; Tye & Janak, 2007; Ambroggi *et al.*, 2008; Sangha *et al.*, 2013; Beyeler *et al.*, 2016). Therefore, the amygdala may be critical for associative cues to access, or engage stored reward memories

within BLA-OFC circuitry. But how exactly does the BLA participate in the BLA-OFC network to allow cue-guided behavior?

One interpretation is that the BLA may act as a storage hub for previously encoded reward-related information. BLA neurons encode associative reward memories (Redondo *et al.*, 2014; Beyeler *et al.*, 2016) and affective value in general (Schoenbaum *et al.*, 1998b; Shabel & Janak, 2009; Beyeler *et al.*, 2016). BLA neurons activated by an appetitive associative learning event can be "reactivated" during memory retrieval (Redondo *et al.*, 2014). In the current state or behavioral task (*i.e.*, PIT or devaluation), reward-predictive cues may reawaken a particular population of previously active "encoding" BLA projection neurons, which then relay the most useful cue-triggered information to distinct OFC subregions for further processing, decisions, and action execution. It is also possible that reward-predictive cues may activate a separate set of "retrieval" neurons, or an intermingled population of "encoding" and "retrieval" cells. This question remains to be further explored in appetitive memory retrieval processes. Nevertheless, according to this theory, the BLA itself may play an integral role in state detection and in linking cues to specific reward memories, or in assigning value to cue-reward memories.

A second possibility is that the mOFC and lOFC store specific outcome information, while the BLA may allow reward-predictive cues to access these memories via OFC inputs. In contrast to the above speculation, this notion fits with the idea that the BLA is the site for "low resolution information" providing quick detection of sensory stimuli important for survival (John *et al.*, 2013). Perhaps sensory stimuli are assigned a general "affective valence" which is then relayed to cortical regions for further refinement (Wassum & Izquierdo, 2015). Therefore, rather than the BLA, the OFC may play a more active role in linking associative cues to their predicted rewards, as well as in formulating the detailed reward representations needed for decision making. Which OFC subregion "wins out" may depend on the current state. During PIT, BLA→lOFC projections may be activated because, broadly, the lOFC is efficient at sensory integration, detailed stimulus-outcome representation, and choice (Stalnaker *et al.*, 2015; Izquierdo, 2017). Congruently, the

lOFC, but not the (posterior) mOFC is needed for specific-PIT (Bradfield *et al.*, 2018). In devaluation, both the mOFC and lOFC are needed to process reward value and stimulus-outcome identity information in the current state, respectively, so BLA projections to both subregions may come online.

Notably, both of the above speculations fit with emerging evidence suggesting that the mOFC and lOFC are functionally dissociable. Although nearly impossible to define singular functions of OFC subregions, evidence primarily from nonhuman primate literature suggests that the lOFC may broadly facilitate sensory integration and choice behavior (but not value-guided choice) (Stalnaker *et al.*, 2015), while the mOFC is responsible for value-guided decision-making and goal selection (Noonan *et al.*, 2010; Rudebeck & Murray, 2011a; Murray *et al.*, 2015; Noonan *et al.*, 2017). In nonhuman primates, mOFC neurons are sensitive to sensory-specific satiety and self-initiated Pavlovian responses, suggesting that this region plays a rather unique role in internal state detection (Bouret & Richmond, 2010), information that may be provided by the BLA. Additionally, others have postulated that the lOFC influences choice behavior by "encoding a rich representation" of cue-predicted outcomes, rather than via representing reward value (Ostlund & Balleine, 2007b; Stalnaker *et al.*, 2015). The lOFC may process incoming associative information for use in both PIT and devaluation, tasks dependent on detailed cue-outcome memories, while the mOFC may be responsible for computing the value comparisons required for proper goal-approach responding in reward devaluation.

How do OFC inputs to the BLA fit into this network model? Using identical pathway-specific chemogenetic inactivation methods and optogenetics, Malvaez et al. (2019) demonstrated that mOFC→BLA projections are needed for reward value retrieval, but not value encoding, when this state-dependent reward value had been encoded prior (*i.e.*, during incentive learning) (Malvaez *et al.*, 2019). The data presented here suggest that mOFC→BLA projections may also be needed for two additional behavioral states (*i.e.*, PIT and Pavlovian devaluation) informed by the retrieval of previously encoded reward-related information. Therefore, we propose that the mOFC→BLA

pathway may be necessary for state-dependent memory retrieval (Figure 6-1A). This idea is consistent with evidence that the mOFC itself mediates effort allocation according to reward value (Gourley *et al.*, 2016), and other components of reward-related decision making (Stopper *et al.*, 2014; Dalton *et al.*, 2016).

Lastly, we found that lOFC inputs to the BLA were *not* required for cue-guided action selection as assessed by outcome-specific PIT. If this projection pathway is not needed for outcome-related memory retrieval, what is it necessary for? lOFC neurons encode reward identity information (McDannald *et al.*, 2014; Howard *et al.*, 2015), and lOFC lesions disrupt anticipatory outcome-expectant firing in the BLA and hinder cue-selectivity (Saddoris *et al.*, 2005), suggesting that lOFC input to the BLA may facilitate the anticipation of cue-predicted rewards during learning. Therefore, we suspect that lOFC→BLA projections are involved in stimulus-outcome encoding, however, this theory is largely based on preliminary data. In line with the notion that this pathway supports the encoding of reward features, lOFC→BLA projections are necessary for and sufficient to drive reward value encoding, but do not mediate the retrieval of state-dependent reward value memories (Malvaez *et al.*, 2019). Importantly, we did not identify a single pathway needed for action selection based on the reward's current value (*i.e.*, instrumental devaluation), demonstrating a unique role of the BLA-OFC network in cue-mediated behaviors.

Scientific implications and limitations

In Chapters four and five, we used a novel projection-specific chemogenetic approach to inhibit hM4Di-expressing terminals of projection neurons within BLA-OFC circuits (Figures 4-1 and 5-1). By microinjecting CNO locally, we precisely targeted projection neurons in a direction-specific manner without concern of off-target effects and back metabolism of CNO to clozapine (Mahler *et al.*, 2014; Zhu & Roth, 2014; Mahler & Aston-Jones, 2018). Additionally, collateralization (*i.e.*, a single cell body bifurcating to multiple regions) is a defining feature of projection neurons, and synapses of one projection-defined population onto different target regions may support diverse behavioral effects. BLA neurons are known to collateralize to many

downstream brain regions (Shinonaga *et al.*, 1994; Beyeler *et al.*, 2016), so inhibiting or stimulating the cell bodies of one projection pathway, for example with a dual-virus "Retro-DREADD" approach (Urban & Roth, 2015; Campbell & Marchant, 2018) may have a different impact on behavior compared to manipulating hM4Di-expressing terminals. By localizing CNO to terminal regions, we avoided this caveat. Therefore, the data presented in this work demonstrate the remarkable specificity that can be achieved with DREADD technology.

Current theories of memory encoding and retrieval posit that memories are widely distributed in the brain at the network level (McIntosh, 2000; Josselyn *et al.*, 2015). Rather than working independently, BLA-OFC projections may enable precise reward memory retrieval by working as a cooperative network. Novel genetic 'capture' studies allow one to tag populations of neurons that are active during memory encoding, and then causally manipulate these engrams at later times, thus elucidating memory retrieval processes (Josselyn *et al.*, 2015). But, to study how projections and circuits contribute to memory retrieval in this manner, one must combine activity dependent genetic tagging (*e.g.*, TRAP, other genetic tools) with causal manipulation techniques (*e.g.*, chemogenetics, optogenetics), viral tracing, and brain-wide activity analysis (Luo *et al.*, 2018). In the future, this multifaceted approach may help reveal how the BLA-OFC network as a whole supports cue-outcome memory retrieval.

Importantly, although the BLA-OFC network model proposed here may implicate the BLA, mOFC, and lOFC as associative "storage loci" of reward-related information, the experiments in this work do not directly address this notion. Furthermore, during any behavioral assessment, we measure performance or behavioral output, and not necessarily neurons responsible for memory retrieval *per se*. Neurons activated by or required to drive behavioral output are not necessarily the same cells that are solely responsible for retrieving stored memories. Rather, we conclude that that BLA projections to the mOFC and lOFC, as well as mOFC inputs to the BLA, facilitate the retrieval of specific reward-related memories for use in generating expectations necessary for guiding cue-mediated behaviors.

Future directions

The BLA shares dense and reciprocal connections with the mOFC and lOFC (Krettek & Price, 1977; Kita & Kitai, 1990; McDonald, 1991b; a; Hoover & Vertes, 2011; Reppucci & Petrovich, 2016), and here we identified gross reciprocal overlap within the frontal cortex (Chapter 3, Figure 3-2). The current data suggest shared behavioral functions of BLA-OFC projection pathways in mediating cue-guided behaviors; for example, chemogenetic inhibition of BLA-mOFC projections in both directions, as well as BLA→lOFC projections produced deficits in Pavlovian goal-approach responding according to a reward's current value. Furthermore, taking any pathway offline (except for lOFC→BLA projections) produced deficits in behaviors guided by reward-predictive cues. This begs the question, were these deficits all simply the result of taking a single projection offline, or were they produced by a small perturbation in the BLA-OFC network? Does the BLA-OFC network work cooperatively to process memories elicited by reward-predictive cues?

It is feasible that these projections directly interact with one another to control these processes. One critical remaining question ripe for future exploration is whether or not mOFC→BLA projection pathways gate the activity of BLA projections back to the mOFC, or to the lOFC, to control cue-guided behaviors. By using anterograde and retrograde tracers, slice electrophysiology studies have shown that that BLA projections to the PFC preferentially contact reciprocally connected neurons in the PFC (Little & Carter, 2013; McGarry & Carter, 2016), and that PFC inputs also selectively synapse onto BLA back projections to the PFC (McGarry & Carter, 2017). Future studies are needed to investigate the synaptic connectivity and functional relation (see below) between BLA and OFC long-range projections *in vivo.* Synaptic tracing can be accomplished *in vivo* by harnessing powerful viral-based methods for trans-synaptic labeling. Retrogradely traveling rabies viruses, which label direct synaptic inputs by traveling from postsynaptic to presynaptic neurons, have been commonly used to monosynaptically trace neural circuits (Lerner *et al.*, 2015; Luo *et al.*, 2018). Using this approach to identify and potentially manipulate or monitor mOFC→BLA projections synaptically-connected to BLA outputs *ex vivo*

may give meaning to the reciprocal overlap we anatomically observed in the BLA-OFC network. Once identified, determining the local network effects of inhibiting or exciting inputs to reciprocal output neurons within the BLA or OFC may also be interesting.

Here, we anatomically identified and manipulated BLA-OFC circuit pathways to reveal their roles in cue-mediated behavior. This leaves one clear question: what about the activity of these projections? Is pathway activation correlated with events such as cue presentation during PIT or devaluation? Identifying the temporal nature of signaling within the BLA-OFC network may reveal how specific pathways work together (or independently) to guide behavior. The role of these projections can be further elucidated by monitoring projection-specific activity *in vivo* using calcium imaging technologies, such as fiber photometry or miniaturized microscopes (Cui *et al.*, 2013; Gunaydin *et al.*, 2014; Resendez & Stuber, 2015). Simultaneous *in vivo* calcium or electrophysiological recording of the BLA and OFC, or combining this approach with causal manipulations of inputs to recording loci via projection-specific chemogenetics or optogenetics may provide further insight into how each projection participates in the broader BLA-OFC circuit, or how the BLA and OFC work congruently, to guide cue-mediated behaviors.

In Chapters 4 and 5, we used an inhibitory projection-specific chemogenetic approach to reveal novel functions of various pathways within the BLA-OFC circuit in cue-mediated behaviors. CNO-hM4Di inhibition of activity in specific projections produced nearly identical deficits in cue-motivated lever pressing and goal-approach responding during PIT and devaluation, respectively (Chapters 4 and 5, Figures 4-3, 4-5, 5-2, 5-3). A major question remains: would enhancing neural activity in these pathways augment measures of cue-mediated behavior? This notion is relatively unexplored in the BLA-OFC circuit, but a recent study showed that optogenetic excitation of lOFC→BLA projections during reward value encoding enhances subsequent lever press responding, and excitation of lOFC→BLA projections enhances reward value retrieval in a state-dependent manner (Malvaez *et al.,* 2019).

Rather than an excitatory opsin, projection-specific excitation can be achieved by expressing an excitatory hM3Dq DREADD (see Chapter 1), which would be an interesting future direction of study. Findings from projection-specific chemogenetic excitation experiments, however, need be interpreted cautiously. Although we hypothesize that enhancing projection pathway signaling would augment measures of cue-guided behavior, it is entirely possible that we would see deficits similar to those observed during CNO-hM4Di inhibition. By using an hM3Dq dual-virus or a local terminal projection-specific excitation approach some studies report enhancements (Boender *et al.*, 2014; Mahler *et al.*, 2019), while others report reductions in reward-seeking behaviors (Augur *et al.*, 2016; Verharen *et al.*, 2018). As alluded to prior, normal memory retrieval and decision-making may depend on cells and circuits working in cooperation, and any positive or negative deviation from basal activity may result in improper information processing.

If employing a projection-specific excitatory manipulation in the BLA-OFC network effectively augments measures of cue-mediated behaviors, coupled with the current findings, these data may be translationally valuable. Mental illness, particularly drug addiction, is often characterized by deficits in envisioning future rewards and improper reward valuation (Hogarth *et al.*, 2013). One day, perhaps such behavioral deficiencies in human patients can be reversed or rescued by targeting imbalanced neural circuits noninvasively, or perhaps even chemogenetically (English & Roth, 2015; Urban & Roth, 2015).

Therapeutic implications and final notes

Elucidating the neuromodulatory systems and neural circuits in support of reward-expectation guided behavior is essential to advancing our scientific knowledge of the basic brain mechanisms in control of our everyday decisions. Appropriate encoding, retrieval, and use of associative reward memories allows us to mentally represent the future with great detail. This cognitive function is essential for survival, and perhaps less imperatively, necessary for making coffee each morning. Notably, studying the neural basis of these processes may shine light on how circuit dysfunction may underlie the behavioral deficits characteristic of mental illness (Volkow

et al., 2013; Lüscher, 2016). In humans, amygdala and OFC activity and connectivity is dysfunctional in patients diagnosed with addiction (Volkow & Fowler, 2000; Goldstein & Volkow, 2011), depression (Keedwell *et al.*, 2005), anxiety (Ressler & Mayberg, 2007), and schizophrenia (Liu *et al.*, 2014). Therefore, exposing circuit-based vulnerabilities in the brain that contribute to maladaptive decision making may aid in treating the behavioral and neural abnormalities exhibited by patients.

Modern circuit-based approaches enable great precision. The work presented here demonstrates the astounding specificity that can be achieved by combining novel, multifaceted behavioral and technological approaches to elucidate the function of amygdala-cortical circuits. Furthermore, given the rising scientific and public interest in mapping brain connectivity and convergence between psychiatry and basic neuroscience (Gordon, 2016; Sakurai, 2017; Gordon *et al.*, 2019), this work may help inform future clinical behavioral and circuit-based therapies in the human population.

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