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Neural and Behavioral Correlates of Adaptive and Maladaptive Behavior

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy in Psychology

by

Alexander Corby Lamparelli

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ABSTRACT OF THE DISSERTATION

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Adaptive and Maladaptive Behavior

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Alexander Corby Lamparelli

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Professor Kate Wassum, Chair

For many years, researchers have worked to understand the nuances of behavior, from understanding how the brain fundamentally makes decisions to meet our needs, to how our behavior can become maladaptive, leading us to forgo our basic needs and develop dependence on drugs. Understanding the basic neural systems that underlie reward-related decision making, including how reward-paired stimuli inform reward pursuit decisions, can help clarify what changes occur in the brain following opioid exposure, and how those changes drive drug pursuit and decision making biased towards drugs and away from adaptive rewards. The research presented here discusses potential pathways involved in reward valuation and decision making, and a behavioral model to study decision making between natural and drug rewards.

Using pathway-specific chemogenetic inactivation during a memory retrieval task, I have shown that projections between the anterior cingulate cortex (ACC) and basolateral amygdala (BLA) may be critical for using environmental stimuli to make reward value predictions to guide reward behavior. Rats with inactivation of BLA axon terminals in the ACC, and ACC terminals in

the BLA, showed deficits in using the recently updated value of one of two food rewards to guide their behavior during an extinction probe test. Further investigation of these pathways using alternative methods, including optogenetic and/or dual viral intersectional chemogenetic approaches, will clarify the roles of these pathways.

Pathways involved in reward valuation for adaptive rewards (e.g. food) likely contribute to drug reward valuations and, in turn, contribute to decision making processes that may drive the development of substance dependence. Further, stress and trauma are also known to contribute to substance use disorders. To study how trauma may influence drug-related behavior, I developed a novel food vs. opioid (fentanyl) decision making task to overcome several limitations of canonical drug self-administration studies, combined with stress-enhanced fear learning (SEFL), a well-established rodent model of posttraumatic stress disorder (PTSD). While I did not detect effects of SEFL on opioid behavior, the results suggest several future directions to address the known human PTSD-opioid use disorder comorbidity in preclinical models that can then inform development of behavioral interventions and pharmacotherapies.

The dissertation of Alexander Corby Lamparelli is approved.

Alicia Izquierdo Edler

Andrew M. Wikenheiser

Edythe Danick London

Catherine Marie Cahill

Kate Wassum, Committee Chair

University of California, Los Angeles

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Vita

Education

University of California, Los Angeles — 2018 - present
PhD Candidate, Department of Psychology

University of California, Los Angeles — 2018 - 2019
Master of Arts, Psychology

Binghamton University — 2010 - 2015
Bachelor of Science, Neuroscience, cum laude

Awards and Honors

NIDA Ruth L. Kirschstein National Research Service Award (NRSA - F31)	2021 - 2024
Graduate Summer Research Mentorship, UCLA	2021
NIDA T32 Translational Neuroscience of Drug Abuse Fellowship	2019 - 2021
Graduate Summer Research Mentorship, UCLA (declined)	2019
UCLA Stone Fellowship	2018

Selected Publications

Sias, A. C., Jafar, Y., Goodpaster, C. M., Ramirez-Armenta, K., Wrenn, T. M., Griffin, N. K., Patel, K., **Lamparelli, A. C.**, Sharpe, M. J., Wassum, K. M. (2024). Dopamine projections to the basolateral amygdala drive the encoding of identity-specific reward memories. *Nature Neuroscience.*

Freda, P. J., Ghosh, A., Zhang, E., Luo, T., Chitre, A. S., Polesskaya, O.,… **Lamparelli, A.C.**, ... & Moore, J. H. (2023). Automated quantitative trait locus analysis (AutoQTL). *BioData Mining*, *16*(1), 14.

Wright, S. N., Leger, B. S., Rosenthal, S. B., Liu, S. N., Jia, T., Chitre, A. S.,…**Lamparelli, A.C.**, ... & Palmer, A. A. (2023). Genome-wide association studies of human and rat BMI converge on synapse, epigenome, and hormone signaling networks. *Cell reports*, *42*(8).

Chitre, A. S., Polesskaya, O., Munro, D., Cheng, R., Mohammadi, P., Holl, K.,… **Lamparelli, A.C.,** ... & Palmer, A. A. (2023). An exponential increase in QTL detection with an increased sample size. *Genetics*, *224*(2), iyad054.

Lichtenberg, N. T., Sepe-Forrest, L., Pennington, Z. T., **Lamparelli, A. C.**, Greenfield, V. Y., & Wassum, K. M. (2021). The medial orbitofrontal cortex–basolateral amygdala circuit regulates the influence of reward cues on adaptive behavior and choice. *Journal of Neuroscience*, *41*(34), 7267-7277.

King, C. P., Tripi, J. A., Hughson, A. R., Horvath, A. P., **Lamparelli, A.**, Holl, K. L., & Meyer, P. J. (2021). Sensitivity to food and cocaine cues are independent traits in a large sample of heterogeneous stock rats. *Scientific reports*, *11*(1), 1-17.

Chitre, A. S., Polesskaya, O., Holl, K., Gao, J., Cheng, R., Bimschleger, H., **Lamparelli, A.C.**, ... & Solberg Woods, L. C. (2020). Genome-Wide Association Study in 3,173 Outbred Rats Identifies Multiple Loci for Body Weight, Adiposity, and Fasting Glucose. *Obesity*, *28*(10), 1964-1973.

Chapter 1: Introduction to the dissertation

We live in a complex world filled with a flood of information showing us the many possible realities we can experience; sights, smells, tastes, sounds, and textures interact to give us a rich and detailed understanding of our environment that we can use to learn how best to meet our needs. Stimuli in the environment, for example, are specific combinations of these sensory features that contain detailed information for us to use, enabling us to reflect on our past experiences when making decisions and to consider where we want to see ourselves in the future (J. J. Clark et al., 2012). When feeling hungry, for example, we can use the logos of nearby restaurants to remind us what options are available, how we have experienced them in the past, and thus how we expect to feel upon obtaining the food. One may encounter the McDonald's golden arches, remember that the meal may not feel good to consume, and thus choose the alternative Sweetgreen salad option that will feel better. This fundamental process of using stimuli to generate expectations of the consequences of our actions is critical to decision making, in that it allows us to direct our energy toward things that will meet our needs and away from what will be detrimental to our goals. Disruptions in this process of using expected value of our reward seeking to guide behavior can lead to and perpetuate psychiatric conditions marked by maladaptive decision making, i.e. decisions that do not aid, and often impede, achieving our goals. Substance use disorders (SUDs), including opioid use disorder (OUD), are marked by such aberrant reward valuation, manifest in decision making that often does not align with one's stated priorities (Badger et al., 2007). Further, psychiatric comorbidities can exacerbate the extent of maladaptive decision making. For example, posttraumatic stress disorder (PTSD) confers greater risk for developing OUD (Dahlby & Kerr, 2020), and PTSD can further degrade the ability to generate accurate reward expectations, preventing people from being able to execute behaviors to meet their needs (Nawijn et al., 2015). Yet, there is still much to be uncovered about how the healthy brain generates and uses reward expectations, and further, how these brain systems become disrupted

to produce the maladaptive phenotypes observed in people with conditions such as PTSD and opioid use disorder. Gaining a clearer understanding of these brain systems both in health and disease is crucial for understanding how to manage and treat psychiatric conditions marked by maladaptive decision making.

Associative learning – Pavlovian and instrumental processes

When making decisions, we have many alternatives to consider where to direct our behavior. To use our cognitive resources efficiently, we use information in the environment, along with our past experiences, to envision the consequences of our actions and then use this information when making a decision (Jeremy J Clark et al., 2012; Fareri et al., 2008). Through learned associations, many things in our environment signal the potential or explicit availability of a specific outcome. A famous example is described by Pavlov, whose lab serendipitously discovered that hungry dogs salivate upon seeing a light that preceded noncontingent food delivery, revealing that the dogs used the light as predictive information relevant to their hunger (Pavlov, 1906). Through a process termed Pavlovian conditioning, the previously neutral meaning of the light interacted with the need-meeting experience of the food such that the light gained meaning of its own. Because the food was a biologically significant outcome, it served as an unconditioned stimulus (US) that, via learned association, enabled the previously neutral light to take on meaning itself. Thus the light becomes a conditioned stimulus (CS) because of the new connection it has to the biologically relevant food outcome (US). This represents what is defined as a stimulus-outcome (S-O) relationship, as the association between the stimulus (S) elicits a motivational state for the food outcome (O). Further, CSs produce a conditional response reflecting the expectation of reward delivery, ranging from the salivation response in Pavlov's dogs to approaching a food port and awaiting reward in rodent studies of Pavlovian conditioning (Galarce et al., 2007). Conditioned stimuli are ubiquitous in our world and are used constantly to direct our behavior in specific ways, often to meet our needs (Domjan, 2005).

Along with stimuli, we also form associations between the actions we perform and the rewards those actions produce. Appetitive instrumental conditioning, also known as operant conditioning, refers to the process by which the performance of an action results in reward delivery, and this rewarding experience increases the probability of (i.e., reinforces) performing that action again in the future. Similarly to stimuli, the ability to perform a given reward-earning action can aid in reward memory retrieval, trigger an enhanced motivational state for the reward, and elevate performance of the action (Staddon & Cerutti, 2003). For example, one may have a stronger desire to buy a pint of beer when passing a pub on the street vs walking by a bakery, because exchanging money is more likely to yield the beer reward in the former scenario. Working with this example, behavior is guided by action-outcome (A-O) associations, wherein performing the specific action (A) of giving money to a bartender has previously resulted in delivery of the desirable beer outcome (O), such that the ability to perform the specific action can engender a motivational state for the outcome. In reality, reward pursuit is complex in that most rewarding situations, if not all, contain both Pavlovian stimuli and available instrumental actions that jointly predict reward. Nevertheless, Pavlovian and instrumental processes can be dissociated in the laboratory setting to isolate the specific behavioral and neurobiological underpinnings of each (Cartoni et al., 2016; Delamater & Oakeshott, 2007).

Pavlovian to instrumental transfer

Conditioned stimuli (CSs) in our environment provide us information about the expected experience of their associated rewards. They also enable us to retrieve information from memory about the behaviors we performed in the past to access those rewards, thus facilitating the process of obtaining future rewards and further maintaining efficient and adaptive control over behavior (Estes, 1948; Holmes et al., 2010). This ability of stimuli to promote reward-seeking actions, Pavlovian-to-instrumental transfer (PIT), manifests both in general motivational and reward specific ways. For example, presenting animals with a CS previously paired with reward

can enhance motivation for rewards broadly (Estes, 1943). While CSs can induce a general enhanced motivational state for rewards, they can also specifically bias behavior toward a particular reward. When arbitrating among potential reward-earning actions, both animals and humans use stimuli available to infer the reward most likely to occur and use this to selectively perform actions aimed at earning that specific reward (Blundell et al., 2001; Corbit & Balleine, 2005). Using the PIT task, we can understand how animals use information in the environment to control decision making, and thus probe the neurobiological substrates that enable the stimulus-outcome associations to retrieve reward representations that guide appropriate action selection.

Pavlovian/Instrumental devaluation

Along with linking specific CSs with the actions that can be used to earn rewards, a critical component of the information signaled by a CS is the expected experience of the outcome (e.g., a reward) associated with the stimulus. We use expected outcome value to guide our behavior to ensure our actions meet our needs. Upon encountering a given reward-paired stimulus, both animals and humans elevate pursuit of the associated reward, reflecting increased expected value of the reward. While this is somewhat due to the general arousing properties of reward paired stimuli (Rule & Nesdale, 1976), numerous studies have manipulated reward value after learning and found that the Pavlovian conditioned response is sensitive to changes in expected value, suggesting animals and humans alike adjust reward-pursuit behavior in line with value (Allman et al., 2010; Holland & Straub, 1979). Performance of instrumental reward-seeking actions is also sensitive to the expected value of the reward (Adams & Dickinson, 1981; Balleine & Dickinson, 1991). The process of forecasting the specific outcome value and using this to direct our behavior is known as goal-directed decision making, a thoughtful cognitive process that contrasts with habitual decision making (Rescorla, 1987).

In habitual decision making, your past history of experiencing reward along with a given stimulus reinforces the performance of the behavior you executed to get the reward, and repeated past reinforcement enables the stimulus itself to cause you to perform reward-earning actions absent any consideration of the outcome (Balleine & O'doherty, 2010). This has also been shown for reward-earning actions in absence of discrete Pavlovian stimuli (Balleine & Ostlund, 2007; Delamater, 2007).

Basolateral amygdala function in appetitive behavior

There is a rich body of literature examining the neural bases of both Pavlovian and instrumental associative learning and memory. The amygdala is a highly conserved brain structure in the limbic region and has canonically been associated with fear and aversive learning (Holland & Gallagher, 1999; Janak & Tye, 2015). The amygdala is composed of several subdivisions, including the basolateral amygdala (BLA) (Swanson & Petrovich, 1998). While its role in aversive learning and memory has been well established, recent work has revealed critical roles for the BLA in appetitive behaviors as well (Janak & Tye, 2015; LeDoux, 1993; Wassum, 2022; Wassum & Izquierdo, 2015). Our lab (Lichtenberg et al., 2017; Lichtenberg & Wassum, 2017) and others (Balleine et al., 2003; Baxter & Murray, 2002; Hatfield et al., 1996; Johnson et al., 2009; Murray, 2007; Pickens et al., 2003) have shown that rather than simply assigning emotional valence to predictive events, the BLA is critical for detailed reward associative memories, i.e. the link between a stimulus or available reward-earning behavior and the unique rewarding event with which it is associated. Interestingly, the BLA is not needed for simple Pavlovian approach behavior, but becomes critical when animals must arbitrate between two distinct stimulus-reward memories during decision making and reward pursuit (Corbit & Balleine, 2005). The BLA is particularly important when animals must make reward value considerations in absence of observable information about the reward, via the association between a given reward and the stimuli/actions that have been associated with the reward in the past (Johnson et al., 2009; Pickens et al., 2003). For example,

the BLA supports adaptive behavior after a negative shift in an expected reward's value (Johnson et al., 2009; Malvaez et al., 2019; Wassum et al., 2009; Wellman et al., 2005) and supports decision making between two alternatives when the positive value of one reward has been temporarily reduced. Thus, understanding the function of the BLA, and other regions with which it communicates, in appetitive learning and memory is critical for understanding how both animals and humans are using value considerations to guide their decision making.

Anterior cingulate cortex function in appetitive behavior

Many cortical subregions are also critical for appetitive learning and memory, serving nuanced and sometimes overlapping roles in adaptive control of behavior (Cardinal et al., 2002; Everitt et al., 2003). The anterior cingulate cortex (ACC) is a subregion of the frontal cortex often described as an integrator of cognition and emotion (Allman et al., 2001; Bush et al., 2000). The ACC has a well-established role in enabling effort allocation. ACC lesions prevent animals from pursuing high effort alternatives and instead bias behavior toward low effort/low reward options (Holec et al., 2014). In humans, ACC activity is correlated with effort expenditure on behavioral tasks (Klein-Flügge et al., 2016; Mulert et al., 2005). Further, the ACC is critical for using subjective value comparisons to allocate effort and thus invigorate pursuit of a specific reward (Hart et al., 2017), and using the value of anticipated rewards to guide behavior (Gourley et al., 2016). Apart from effort, other work has also uncovered the role of ACC in Pavlovian conditioning (Cardinal et al., 2002). Like the BLA, the ACC is not necessary for simple Pavlovian conditioned approach, but is implicated when animals must discriminate between two Pavlovian stimuli to guide reward pursuit behavior (Cardinal et al., 2003). Further, similarly to the BLA, the ACC enables value considerations for guiding motivated behavior. Activity in human and primate ACC reflects subjective value of expected rewards (Cai & Padoa-Schioppa, 2012; Yee et al., 2021). By tracking outcome values, the ACC enables primates to reflect on recent reward histories, allowing for behavioral adjustment under changing reward availability circumstances (Walton et al., 2007).

Thus, the ACC is a potential node for using outcome values to guide behavior like the BLA, and may collaborate with the BLA to achieve these functions.

BLA-ACC collaboration in appetitive behavior

Given the overlapping roles of the BLA and ACC in behavior, and their neuroanatomical positioning with monosynaptic bidirectional projections (Jhang et al., 2018; Shao et al., 2021), it is possible the BLA and ACC directly collaborate in Pavlovian learning and memory. Some work has begun to investigate this putative role of BLA-ACC circuits. In the appetitive motivation literature, work has been done to investigate the individual nuanced roles of both the BLA and the ACC in cost-benefit decision making (Hosking et al., 2014) and decision making under uncertainty (Stolyarova et al., 2019), and how synchrony in electrophysiological activity between the regions modulates decision making (Cao et al., 2016; Xu et al., 2015). Disconnection of the BLA and ACC prevents animals from exerting higher effort to highly rewarding options (Floresco & Ghods-Sharifi, 2007), suggesting the BLA supports the ACC in its role in effort allocation during decision making. While some work has directly interrogated the monosynaptic projections between the BLA and ACC in aversive conditioning (Jhang et al., 2018; Ortiz et al., 2019; Shao et al., 2021), little is known of how these direct connections contribute to appetitive learning.

Stress and decision making – models of posttraumatic stress disorder and substance use disorder

Reward valuation is a central component to reward pursuit considerations, in that we generally pursue higher value rewards more frequently and vigorously than lower value alternatives (Peters & Büchel, 2010). In this way, we prioritize our behavior so that we appropriately meet our needs. However, disruptions in this process may underlie a number of psychiatric conditions marked by maladaptive decision making. A deeper understanding of the manifestation of reward valuation

dysfunction is needed to tailor behavioral and pharmacological treatment plans for alleviating decision making deficits in psychiatric disease, including stress and substance use disorders.

Opioid use disorder (OUD) is a chronic relapsing substance use disorder characterized by escalating and uncontrollable opioid use, even in the face of negative consequences created by ongoing use, and causes a physiological withdrawal syndrome upon cessation of use (Boscarino et al., 2015). Along with physical symptoms, opioid withdrawal produces a negative affective state that can perpetuate ongoing use via negative reinforcement mechanisms (Monroe & Radke, 2023; Redmond Jr & Krystal, 1984). One theory posits that opioid use in OUD is perpetuated by inappropriately inflated incentive value during withdrawal, such that the expected subjective value of consuming opioids is disproportionately higher than the realized value upon using (Hutcheson et al., 2001). In fact, in acute opioid withdrawal, animals assign inflated value to rewards that is discordant with the emotional experience of consuming the reward (Hutcheson et al., 2001; Wassum et al., 2016). Similarly, humans overvalue rewards associated with opioid use over nondrug alternatives as a function of time in acute withdrawal, with value inflation positively correlated with time since last opioid consumption (Biernacki et al., 2022). This accords with the reported experience of opioid craving, wherein patients will experience intense urges to seek and obtain the drug, overvaluing the experience of the drug compared to periods outside of acute craving (Badger et al., 2007; Kleykamp et al., 2019).

Opioid use disorder shares high comorbidity with other psychiatric conditions, including posttraumatic stress disorder (PTSD) (Fareed et al., 2013). PTSD can emerge following the experience of one or several traumatic events, and causes patients to experience intrusive thoughts, nightmares, flashbacks, severe anxiety, and uncontrollable thoughts about the traumatic event(s) (Nemeroff et al., 2006). PTSD also causes deficits in reward information processing that impairs decision making. Many people with PTSD report anhedonia, the loss of

interest in previously pleasurable activities and the inability to experience positive emotion (Nawijn et al., 2015; Olson et al., 2018). Further, PTSD patients report significantly lower subjective ratings of pleasant stimuli, along with reduced activation of multiple brain regions associated with appetitive motivation (Elman et al., 2018). Interestingly, despite this diminished motivation for rewards, there is still high comorbidity between PTSD and opioid use disorder, the latter driven by excessive opioid reward seeking suggesting enhanced motivation. Some work has investigated how reward processing dysfunction is manifest in comorbid PTSD-SUD, revealing that there is much still unknown of how this comorbidity develops. Counterintuitively, a number of studies have found decreased drug self-administration following trauma exposure (Vujanovic et al., 2017), discordant with the self-medication hypothesis, i.e. that PTSD patients may consume drugs of abuse to alleviate negative symptoms (Chilcoat & Menard, 2003). Thus, much remains unknown about the behavioral and neurobiological manifestations of comorbid PTSD-OUD.

Modeling psychiatric disorders in experimental animals

To understand the causal relationship between PTSD and the development of OUD, we must turn to animal models. Rodent models offer the ability to understand psychiatric disorders through systematic study of how a disorder arises, the behavioral and neurobiological correlates of a disorder, and potential treatments to alleviate symptoms, while minimizing ethical concerns that naturally preclude such study in humans (Geyer & Markou, 1995). However, animal models are historically difficult to develop, in that it is often challenging to create analogous scenarios for rodents that capture the complexity of human psychology. Arguments have also been made for moving away from analyzing behavioral data, focusing more on the neurobiological abnormalities that underlie human psychiatric disease, as such abnormalities are more likely to be conserved across species than specific behavioral phenotypes (Kaiser & Feng, 2015), though neural data collected from ethologically irrelevant or poorly controlled behavioral designs can produce misinformed conclusions that ultimately complicate our understanding of the brain (Niv, 2021).

Therefore, we must combine rigorous approaches from neurobiological and behavioral bodies of literature to accurately understand psychiatric diseases using animal models.

There are numerous rodent models of PTSD, each with their advantages and disadvantages. In each model, rodents are exposed to a stressor (e.g. electric shock, restraint, underwater trauma, single prolonged stress, chronic unpredictable stress, social defeat, predator stress) and the behavioral and neurobiological consequences are investigated to understand how PTSD develops and presents in humans (Verbitsky et al., 2020). Often, rodents will exhibit behaviors analogous to human PTSD symptomatology. For example, the stress-enhanced fear learning (SEFL) paradigm uses electric shock stress, giving animals several unsignaled shocks in one context that causes a sensitization of the fear response in other contexts, analogous to the exaggerated responses to mild stressors in humans with PTSD (Rau et al., 2005). Along with shock-induced sensitization, SEFL produces many physiological and behavioral phenotypes that reflect the human experience of PTSD. SEFL causes long-lasting fear sensitization (90 days) that does not require explicit memory of the traumatic event, in that rodents will still show this sensitization even when administered protein synthesis inhibitors following the SEFL procedure that would preclude consolidation of the fear memory. SEFL also produces sensitized fear to to other aversive stimuli besides the shock stimulus used to drive trauma, dysregulates basal diurnal corticosterone (CORT) levels, increases alcohol consumption, and can produce anxiety- and depressive-like behavioral phenotypes (Perusini et al., 2016). Thus, the SEFL model is a powerful tool for investigating the behavioral and neural correlates of PTSD in rodents, such that we can better understand the presentation and treatment of PTSD in humans.

Similarly, there are many rodent models of substance use disorders (SUDs) that can be leveraged to understand aspects of SUD in humans. Many models of SUD involve experimenter administration of drugs to animals to induce behavioral and neurobiological phenotypes that are

thought to parallel the human presentation of the SUD being studied (Kuhn et al., 2019). While helpful for capturing the neurobiological consequences of substance exposure, the lack of agency over drug taking is in stark contrast to how SUD develops in humans, which itself may cause known or unknown differential neurobiological changes compared to passive administration, complicating the interpretation of work using experimenter-administered drug paradigms. In fact, a number of studies have found distinct neurobiological profiles following self- vs experimenter-administered drugs, likely reflecting the cognitive processes involved in decision making for drugs that is present in the former but not latter experimental design (Jacobs et al., 2003; Stefanski et al., 1999). Thus, for understanding the cognitive processes underlying decision making in drug pursuit, self-administration paradigms are essential. Animal self-administration models of SUD, including OUD, have been shown to recapitulate many aspects of SUD seen in humans. Like humans, rodents readily self-administer numerous mu-opioid receptor agonists (i.e. opioid drugs). Interestingly, the self-administration patterns in rats across different types of opioids mirrors the positive subjective ratings and abuse liability of these drugs in humans, demonstrating the predictive validity of rodent models (O'Connor et al., 2011).

Despite their widely accepted utility, animal models of psychiatric disease always have limitations, in that specific aspects of the human condition are difficult to replicate in animals. One such limitation in opioid self-administration studies is the unavailability of alternative rewards. The majority of rodent opioid self-administration studies use singly housed animals in poorly enriched environments whose only task is to perform an action to receive a dose of a drug, thus precluding engaging in other rewarding behaviors (e.g. social interaction, nest building, knowing/object interaction) both during and between self-administration sessions (Campbell & Carroll, 2000). Not only do the rewarding behaviors available in enriched environments attenuate opioid consumption (Hofford et al., 2017; Imperio et al., 2018), they also preclude understanding of how animals make decisions between adaptive rewards (e.g. food) vs drugs when both rewards are concurrently

available. A hallmark of OUD is choosing opioid drugs at the expense of more adaptive rewards (e.g. going to work, time with friends/family, money, etc.), yet not many animal models are not designed to capture decision making between drugs and nondrug alternatives. Several choice models have been developed using food (Reiner et al., 2020) and social interaction (Venniro & Shaham, 2020) as alternative rewards, yet the design parameters used in these studies, such as reward salience and magnitude, bias animals to voluntarily abstain from drugs. While highly useful for understanding the behavioral and neurobiological underpinnings of how adaptive rewards can attenuate drug pursuit, the tendency for the vast majority of animals to abstain from drug use in these choice models precludes the ability to capture how dynamic environmental circumstances, such as need state and trauma exposure, may alter decision making for drugs vs adaptive rewards.

Interacting neurobiology of PTSD and OUD

Understanding how stress, especially a history of trauma, affects opioid use is critical in treating opioid use disorder. Stress increases opioid use in experimental animals and in humans, and self-reports from opioid users often cite stress as a contributing factor to their use (MacLean et al., 2019). Further, the rate of OUD in people with PTSD is significantly higher than the general population, reflecting the comorbidity between the two disorders (Danovitch, 2016). PTSD causes a series of neurobiological changes that may facilitate the development of OUD. For example, trauma exposure in the SEFL model produces neurobiological changes in both the basolateral and central amygdala, including dysregulation of the diurnal cycling of the stress hormone corticosterone (CORT) (Poulos et al., 2014) and elevated insertion of GluA1-AMPA receptor subunits (Perusini et al., 2016). The stress-induced enhancement of both self-administration and the psychomotor effects of opioids are associated with and dependent on CORT (Carmack et al., 2022; Deroche et al., 1992). Further, increased expression of GluA1-AMPA receptor subunits in the central amygdala facilitates opioid reward learning (Cai et al., 2013) and increases self-

administration of morphine (Hou et al., 2020). These findings present a potential neurobiological mechanism by which PTSD may enhance opioid learning, memory, and maladaptive decision making leading to OUD, and may explain the high comorbidity between PTSD and OUD. Animal models that integrate PTSD and opioid use are needed to understand the behavioral and neurobiological underpinnings of this comorbidity.

Chapter 2: The basolateral amygdala collaborates with the anterior cingulate cortex in using reward memories to guide behavior

Introduction

Every day we engage in various behaviors to satisfy our needs and desires. These behaviors are often informed by environmental stimuli that signal available rewards. Stored stimulus-outcome associative memories allow us to use environmental stimuli to predict the specific rewarding events that might occur in the face of those stimuli and inform decision making accordingly (Wassum, 2022; Wassum & Izquierdo, 2015). In addition to information like reward identity, the value of an anticipated reward is also a critical component of these calculations, allowing individuals to flexibly adjust their responses to predictive stimuli after changes in the reward experience (Schreiner et al., 2021). For instance, after something rewarding becomes less valuable, one will know to modulate their responding to cues that predict that less valuable reward in the future (Pool et al., 2023; Sood & Richard, 2023). An inability to retrieve such stimulus-outcome memories or use them to update behavior when reward value has changed can lead to ill-informed motivation and maladaptive or compulsive behavior. This is characteristic of the cognitive symptoms underlying many psychiatric diseases, including substance use disorder (Byrne et al., 2019; Hogarth & Field, 2020). Yet very little is known of the neural circuitry enabling stimuli to retrieve reward memories and even less about the systems that allow these memories to adapt following a negative shift in value of the expected reward. Such knowledge is vital to understanding how the brain uses stimuli to guide adaptive behavior and for exposing the brain vulnerabilities that may drive maladaptive reward pursuit in psychiatric disease.

While commonly known for its role in aversive learning, the **basolateral amygdala (BLA)** also mediates appetitive emotional learning (Janak & Tye, 2015; LeDoux, 1993). Our lab (Lichtenberg et al., 2017; Lichtenberg et al., 2021; Lichtenberg & Wassum, 2017) and others (Balleine et al.,

2003; Baxter & Murray, 2002; Hatfield et al., 1996; Johnson et al., 2009; Murray, 2007; Pickens et al., 2003) have shown that rather than simply assigning emotional valence to predictive events (Janak & Tye, 2015; LeDoux, 1993), the BLA is critical for detailed and specific stimulus-outcome associative memories, i.e. the link between a specific stimulus and the unique rewarding event with which it is associated. Accordingly, the BLA supports adaptive behavioral control after a negative shift in the value of an expected reward (Johnson et al., 2009; Wellman et al., 2005). The specific inputs and outputs via which the BLA achieves this function are largely unknown and will be investigated here.

The BLA is well networked with many cortical subregions (Price, 2007) and may regulate stimulus-outcome memory through its connections with the **anterior cingulate cortex (ACC)**. The ACC is implicated in discriminating between Pavlovian stimuli to guide reward pursuit behavior (Cardinal et al., 2003). ACC neurons track features of Pavlovian stimuli to generate prediction errors that drive learning and decision making (Garrison et al., 2013). Further, the ACC is critical when using subjective value comparisons to invigorate pursuit of a specific reward (Hart et al., 2017) and using the value of anticipated rewards to guide behavior (Gourley et al., 2016). While some work has investigated how the BLA and ACC interact in fear behavior (Hakamata et al., 2020; Jhang et al., 2018), little is known of how these pathways may contribute to appetitive decision making.

To address this gap in knowledge, we used pathway-specific chemogenetic inactivation in male and female rats to clarify the role of both BLA→ACC and ACC→BLA direct projections in using stimuli to generate reward expectations to maintain adaptive control of reward pursuit. Using Pavlovian and instrumental conditioning, we trained rats to encode specific stimulus-outcome and action-outcome memories, and then tested their ability to use these memories in two probe tests designed to assess how rats use reward-paired stimuli to guide behavior. We used an outcome-specific version of the Pavlovian-to-instrumental transfer task to test the ability of rats to infer the

appropriate reward-seeking action when in the presence of specific reward-paired stimuli, followed by an outcome-specific satiety devaluation task to assess how rats use specific stimuli to anticipate the value of the reward associated with that stimulus and modulate their behavior accordingly. Procedures were identical to our prior work interrogating the functions of projections between other cortical subregions and the BLA (Lichtenberg et al., 2017; Lichtenberg et al., 2021; Malvaez et al., 2019) to integrate these findings with the existing literature.

Results

Projections from the BLA→ACC are not needed for stimuli to guide action selection

We used pathway-specific chemogenetic inactivation combined with behavioral tasks developed from associative learning theory to assess the contribution of BLA→ACC activity in using stimulus-reward memories to guide behavior. To target BLA→ACC projection neurons, we infused rats with a virus encoding the inhibitory DREADD (i.e. designer receptor exclusively activated by designer drugs) hM4Di with an mCherry fluorophore tag in the BLA, driving expression of the DREADD and mCherry in BLA cell bodies and their terminals. Then, we implanted bilateral guide cannulas over the ACC for later infusion of clozapine-N-oxide (CNO), the ligand for hM4Di, to silence activity of only the BLA inputs into ACC while leaving all other circuits and regions intact (Figure 2.1). Control animals were infused with a virus expressing the mCherry fluorophore but not hM4Di to control for potential off-target effects of CNO and fluorophore expression on behavior. Control tissue was histologically processed identically to hM4Di tissue and checked to ensure normal tissue health, but is not shown here. We have previously validated this inactivation approach both *in vivo* and *ex vivo* (Collins et al., 2019; Lichtenberg et al., 2017; Malvaez et al., 2019)

We trained food-restricted rats in Pavlovian conditioning to associate 2 auditory stimuli (white noise and clicker; 2-min duration, 3-min average random inter-trial interval, 4 of each conditioned

stimulus (CS) per session in pseudorandom order; 5 sessions) each with a distinct food reward, 20% sucrose or grain pellets; delivered on average every 30s during CS as described in our prior work (Lichtenberg et al., 2017; Lichtenberg et al., 2021; Lichtenberg & Wassum, 2017). In training, rats form two distinct stimulus-outcome memories, linking each CS with the specific outcome it signals (Estes, 1943; Kruse et al., 1983). We confirmed rats in both the mCherry and hM4Di groups acquired the Pavlovian associations, in that the percent food-port dwell time during the Pavlovian stimuli increased over days during each stimulus, analyzed as an elevation ratio to correct for baseline dwell time (Figure 2.3 A-B; day 1 mCherry: 0.477 +/- 0.037 SEM; day 1 hM4Di: 0.516 +/- 0.019 SEM; day 5 mCherry 0.724 +/- 0.017 SEM; day 5 hM4Di 0.709 +/- 0.015 SEM; Day: $F_{(3.192,73.42)} = 31.73$, $p < 0.0001$; Virus: $F_{(1,23)} = 0.1671$, $p = 0.6865$; Day x Virus: $F_{(4,92)} = 0.8193$, $p = 0.5161$).

We then gave rats instrumental training to learn to press on 2 levers, each earning one of the two food rewards given during Pavlovian conditioning (e.g. left lever→20% sucrose and right lever→grain pellets; ultimately random-ratio 10 reinforcement schedule), thus forming 2 action-outcome associative memories (Bouton & Swartzentruber, 1991). We confirmed acquisition of the instrumental respons as press rates increased each day (Figure 2.3 C-D; day 1 mCherry: 2.91 presses +/- 0.52 SEM; day 1 hM4Di: 2.83 presses +/- 0.31 SEM; day 9 mCherry: 21.77 presses +/- 3.11 SEM; day 9 hM4Di: 24.90 presses +/- 2.12 SEM; Day: $F_{(3.085,70.96)} = 51.69$, $p<.0001$; Virus: $F_{(1,23)} = 0.01557$, $p=0.9018$; Day x Virus: $F_{(8,184)} = 0.5869$, $p=0.7879$). We did not perform any manipulations during either Pavlovian or instrumental conditioning. Following the instrumental phase, we gave rats an additional Pavlovian conditioning session as a reminder preceding the retrieval tests.

To assess the role of BLA→ACC projections in using associative reward memories to guide decision making, we first tested rats in two Pavlovian-to-instrumental transfer (PIT) tests,

counterbalanced for stimulus order. 5 min prior to each test, we infused rats with CNO (1mM, 0.3 uL/hemisphere) into the ACC to selectively inhibit BLA→ACC activity. In this task, both the left and right levers were available to the rats in the same conditioning chambers where they formed the stimulus-reward and action-reward associations during training. However, in this test, pressing the levers did not earn rewards. In this way, we assessed how the rats were using a mental representation of unobservable information (i.e. of the reward itself) to guide reward-directed decision making. Throughout the task, we gave rats 4 presentations of each CS (2-min duration, 3-min fixed intertrial interval) to assess the influence of each stimulus on lever-press responding. Critically, because levers were not available in Pavlovian conditioning, and the Pavlovian cues never presented during instrumental conditioning, the rats never had an experience during training to link the reward-paired stimuli with the same actions that would earn those rewards. Thus, if rats selectively increase lever-press responding on the lever associated with the same reward as is signaled by the current stimulus, they must be using the stimulus to retrieve a mental representation of the specific outcome to guide lever pressing accordingly. Rats with BLA→ACC inactivation showed this specific PIT effect, indicated by increased lever pressing on the lever that earns the same outcome as is signaled by a given auditory CS (i.e. “CS-Same” pressing) vs pre-CS baseline and vs pressing on the other lever (i.e. CS-Different) (Figure 2.4; Virus: $F_{(1,22)} = 1.302$, $p = 0.2662$; CS/lever congruence: $F_{(1,22)} = 14.22$, $p = 0.001$; CS-on: $F_{(1,22)} = 29.66$, $p < 0.0001$; Virus x CS/lever congruence: $F_{(1,22)} = 0.8127$, $p = 0.3771$; Virus x CS-on: $F_{(1,22)} = 0.097$, $p = 0.7584$; CS/lever congruence x CS-on: $F_{(1,22)} = 16.44$, $p = 0.0005$; Virus x CS/lever congruence x CS-on: $F_{(1,22)} = 4.773$, $p = 0.0399$). Although we did detect a 3-way interaction (virus by cs-congruence by cs-on), posthoc analyses revealed this was due to control subjects not showing the PIT effect, likely due to that group being underpowered to detect the effect.

Projections from the BLA→ACC are needed for using stimuli to generate reward value expectations that guide behavior

The PIT effect relies on rats to use two distinct stimulus-reward memories to arbitrate between two reward-earning actions, and that rats expressed PIT indicates that BLA→ACC projections are not needed for retrieving specific stimulus-reward memories or using them to select the appropriate reward-earning action. However, our lab and others have shown that after selectively manipulating the value of a specific rewarding option (i.e. outcome-specific devaluation), projections from the BLA to nearby prefrontal cortical regions (medial OFC, lateral OFC) (Lichtenberg et al., 2017; Lichtenberg et al., 2021) enable the use of this reward-specific updated value to maintain adaptive, value-based control over reward pursuit behaviors. Further, the expression of specific PIT is not sensitive to devaluation of the outcome (Holland, 2004), thus presenting the possibility that BLA→ACC activity, while not needed for PIT, may be needed to modify behavioral responses after outcome value changes. Whether the ACC also receives this reward-specific updated value information from the BLA is not known.

To that end, we tested the necessity of BLA→ACC activity during two outcome-specific devaluation probe tests (counterbalanced across conditions for order), following selective devaluation of one of the two trained outcomes. First, we retrained rats on 1 Pavlovian and 2 instrumental conditioning sessions to remind them of the stimulus-outcome and action-outcome associations learned in training, because of extinction in the prior PIT test. Next, we used a sensory-specific satiety devaluation procedure to devalue one of the two rewards. We gave rats *ad libitum* access to one of the two trained outcomes for 1h, to allow rats to consume the outcome until reaching a satiety point. In this way, the subjective value of the pre-fed rewarding outcome was lowered, while the value of the non-pre-fed outcome remained unchanged. No manipulations were made during the 1h prefeeding period, to allow for normal devaluation learning. Rats in both the control and experimental conditions in both pathways ate similar amounts during pre-feeding

(Figure 2.5; BLA→ACC control 13.325g +/- 0.954g; BLA→ACC hM4Di 12.48g +/- 0.75g; $t_{(22)}$ = 0.671; p = 0.510).

Following prefeeding, we intracranially infused rats with CNO into the ACC, similarly as prior to PIT, and 5 min later placed them in the behavioral chambers for the combined instrumental and Pavlovian devaluation probe test. First, in the instrumental phase, we gave rats access to both levers for 5 min, allowing them to press at will, but with no reward deliveries. Removing the reward meant that rats could not use the current reward experience itself, but rather a mental representation of the reward they expect to earn upon lever pressing, and its expected value, to decide which lever(s) to press and how much to press them. Rats pressed much less on the lever associated with the devalued reward than that of the valued reward (Virus: $F_{(1,21)}$ = 2.170, p = 0.1555; Value: $F_{(1,21)}$ = 12.68, p = 0.0018; Virus x Value: $F_{(1,21)}$ = 0.2972, p = 0.5914), indicating that they were using current reward value to guide their instrumental reward-seeking actions.

Next, the levers retracted into the chamber wall and the Pavlovian devaluation probe phase began. Rats were presented with each of the reward paired stimuli 4 times (8 trials total), in absence of levers, and the conditional food port response was analyzed as percent time spent in the food-port magazine before and during each stimulus presentation. The amount of time spent in the food-port on a given trial is considered to be positively correlated with the subjective value of the expected reward. Rats in both the control and BLA→ACC inactivation condition showed insensitivity to devaluation in the conditioned food-port response, indicated by a nonspecific increase over baseline in food-port dwell time during the stimulus associated with the valued and the devalued (i.e. pre-fed) outcome, indicating that animals were unable to use the expected value of the reward signaled by the stimulus to guide approach behavior. Interestingly, we also detected a main effect of virus, in that animals with BLA→ACC inactivation showed overall elevated responding throughout the Pavlovian phase compared to controls (Virus: $F_{(1,18)}$ = 8.081, p =

0.0108; CS-period: $F_{(1.725, 31.04)} = 20.56$, $p < 0.0001$; Virus x CS-period: $F_{(2,36)} = 1.514$, $p = 0.2337$). This suggests that BLA→ACC inactivation has a disinhibitory effect on behavior, perhaps through enabling more impulsive reward-directed responding.2

Projections from the ACC→BLA are not needed to use stimuli to select optimal reward-earning actions

We next investigated the function of the reciprocal pathway, projections from the ACC→BLA, in retrieving stimulus-outcome memories to guide behavior. The behavioral training and test procedures for this experiment were identical to the interrogation of BLA→ACC projections described above, with the exception of the specific pathway targeted. Here, we infused a virus encoding an inhibitory DREADD (hM4Di; AAV8-CaMKII-hM4Di-mCherry, 3.8×10^{12} vg/ml; Addgene; 0.3 µL) in the ACC, driving hM4Di receptor expression in ACC cell bodies and axons of ACC projection neurons. Then, we implanted bilateral infusion cannulae over the BLA to allow for later CNO infusion, to selectively inactivate the ACC→BLA pathway while leaving all ACC targets intact (Figure 2.2). Control tissue was histologically processed identically to hM4Di tissue and was checked to ensure normal tissue health, but is not shown here. As in the prior experiment, rats received an intracranial infusion of CNO prior to two PIT tests and two outcome-specific devaluation probe tests. While intracranial infusion of CNO and other DREADD ligands into BLA has not been shown to affect behavior (Lichtenberg et al., 2017; Lichtenberg et al., 2021; Malvaez et al., 2019), control subjects were infused with virus encoding the fluourophore mCherry but not hM4Di (mCherry; AAV8-CaMKII-mCherry, 3.9×10^{12} vg/ml; Addgene; 0.3 µL), to control for potential off-target effects of CNO and unexpected consequences of fluorophore expression. Rats across both groups showed evidence of successful Pavlovian conditioning as indicated by increasing time spent in the food-port magazine during each of the stimuli (control mean day 5: 0.741 +/- 0.020; hM4Di mean day 5: 0.716 +/- 0.028; virus: $F_{(1,80)} = 0.001$, $p = 0.9758$; day: $F_{(4,80)} = 38.31$, $p < 0.0001$; virus x day: $F_{(4,80)} = 0.8684$, $p = 0.4867$), and acquired the two instrumental

lever-food associations. We detected a slight pre-existing group difference between the control and hM4Di groups, with controls pressing slightly more than hM4Di subjects (control day 9: 28.272 presses/min +/- 2.844 SEM, hM4Di day 9: 26.434 +/- 1.855; virus: $F_{(1,144)}$ = 8.443, p = 0.0042; day: $F_{(8,144)}$ = 36.82, p < 0.0001; virus x day: $F_{(8,144)}$ = 0.2897, p = 0.9685). Because the DREADD surgeries occurred before any training, we were unable to counterbalance the viral conditions based on training performance.

Like the BLA→ACC pathway, the ACC→BLA pathway is also not needed for using stimuli to guide appropriate reward-seeking actions. After intracranial infusion of CNO (1mM; 0.5 uL/hemisphere) into the BLA, rats were placed in the operant chamber and given a PIT test, a test in extinction wherein the reward-associated levers were available to press and each reward-paired stimulus was serially presented to assess the influence of each stimulus on reward-directed lever pressing. During a given stimulus, both control and ACC→BLA inactivation subjects selectively elevated responding on the lever associated with the same outcome as the stimulus (i.e. “CS-same pressing”) and not on the alternate lever (“CS-different pressing”) (Figure 2.6; Virus: $F_{(1,16)}$ = 0.6748, p = 0.4235, CS/lever congruence: $F_{(1,16)}$ = 4.058, p = 0.0611; CS-on: $F_{(1,16)}$ = 38.39, p <0.0001; Virus x CS/lever congruence: $F_{(1,16)}$ = 0.0225, p = 0.8827; Virus x CS-on: $F_{(1,16)}$ = 0.1686, p = 0.6868; CS/lever congruence x CS-on: $F_{(1,16)}$ = 5.424, p = 0.0333; Virus x CS/lever congruence x CS-on: $F_{(1,16)}$ = 0.0403, p = 0.8434). Because animals never directly experienced the Pavlovian stimuli with performing the instrumental actions during training, this pattern of results is consistent with the interpretation that animals used the specific stimuli to infer the action most likely to be reinforced, thus preferentially pressing the lever associated with the same outcome as signaled by the CS.

Projections from the ACC→BLA enable stimulus-reward value memories to maintain adaptive control of conditional reward responding

Similarly to the BLA→ACC pathway, we were interested in investigating how ACC→BLA projections may be important for using value expectations to guide behavior. Thus, we also tested these animals on an outcome-specific devaluation probe test, performed identically to the BLA→ACC inactivation experiment, with the exception of inactivating the reciprocal pathway (ACC→BLA) instead. Rats were given 1-hour prefeeding period on one of the two trained outcomes, eating similar amounts regardless of manipulation condition (Figure 2.7; ACC→BLA control mean 12.184g +/- 0.873g SEM; ACC→BLA hM4Di 12.831g +/- 1.324g; $t_{(16)} = 0.3567$, $p = 0.726$). Rats then received an intra-BLA infusion of CNO similarly as prior to PIT, immediately after pre-feeding and were placed in the test chamber 5 min later. Then, both levers were available in absence of the Pavlovian cues to assess the motivation for each of the two rewards. Rats across both conditions demonstrated sensitivity to instrumental devaluation as indexed by pressing more on the lever that would earn the reward that was not just pre-fed, as that reward is believed to hold more subjective value during the test than the reward on which they had just sated (Figure 2.7A; virus: $F_{(1,16)} = 0.1593$, $p = 0.6951$; value: $F_{(1,16)} = 13.81$, $p = 0.0019$; virus x value: $F_{(1,16)} = 1.264$, $p = 0.2774$).

In contrast, during the Pavlovian phase of the probe test, animals in both groups showed food-port approach responses insensitive to devaluation of the outcome, and instead increased food-port dwell time over baseline regardless of the stimulus presented (Figure 2.7-B, Virus: $F_{(1,16)} = 1.840$, $p = 0.1938$; CS-period: $F_{(1.467,23.47)} = 16.56$, $p = 0.0001$; Virus x CS-period: $F_{(2,32)} = 1.414$, $p = 0.2580$). However, this result must be interpreted with caution. Upon examining behavior of individual subjects, denoted by traces on the bar graphs, some subjects across both groups were able to modulate their CS-food port response based on expected outcome value, while others could not. This variability may be due to the intra-BLA infusions, that we later found produced

intracranial swelling that may have impacted behavior in unclear ways. Thus, it is possible that the ACC→BLA pathway is not needed for stimulus-outcome value memory retrieval, but simply that the untoward effects of the CNO infusion disrupted normal performance across both groups. However, one recent study has implicated this pathway in reward devaluation, albeit for water rewards and using a caspase ablation strategy that may engage compensatory mechanisms more than acute DREADD activation (Yuan et al., 2023). More work must be done to clarify the role of ACC→BLA projections using other manipulation methods, e.g. optogenetics, that do not risk damaging tissue as observed with intra-BLA CNO infusions.

Discussion

In the present study, we assessed the role of both BLA→ACC and ACC→BLA projection neurons in using environmental cues to generate prospective reward evaluations to maintain adaptive control of behavior. Using pathway-specific chemogenetics and behavioral assays rooted in learning theory, we found that activity in BLA→ACC neurons may be critical for using stimuli to generate reward value expectations that help inform the vigor of conditional reward responding, but not for using stimuli to guide reward-seeking actions. Interestingly, we found the same pattern of results in studying ACC→BLA projections, finding that they too are needed for stimulus-generated reward value expectations.

Activity in projections from BLA→ACC is necessary for stimuli to guide value-based approach behavior. Inactivating BLA→ACC projections following a value shift in one of two rewarding outcomes produced a nonspecific increase in responding to both stimuli regardless of the value of the associated outcome. However, rats did modulate their instrumental responding following devaluation, pressing much more on the lever associated with the reward that was not just devalued. These results suggest animals retrieved specific outcome memories when presented with a given action, and used expected reward value to guide behavior, but that using stimuli to

guide this process was disrupted, showing that the BLA→ACC pathway is not needed for reward value representations broadly, but specifically as they relate to Pavlovian stimuli. These results must be interpreted with caution, however, as the control subjects showed a similar pattern of results. Surprisingly, we found that animals with BLA→ACC inactivation responded more overall in every phase of the Pavlovian probe compared to controls. While this behavior is highly variable and this may contribute to the effect, it is possible that BLA→ACC inactivation disinhibited responding in a way that is meaningful when considering the role of the pathway in reward-related behavior. ACC inactivation produces enhances impulsive responding in the 5-choice serial reaction time task (Robbins, 2002), and promotes premature reward responding (Hosking et al., 2014), another measure of impulsivity. It is therefore possible that the activity of BLA inputs into the ACC exerts inhibitory control over behavior in response to stimuli, and their inactivation increased reward-directed responding regardless of the CS-period of the task.

We found no contribution of the BLA→ACC pathway to using stimulus-outcome memories to select the appropriate reward-earning action. During a given stimulus presentation, animals with BLA→ACC inactivation showed a specific increase in lever-press responding on the lever that would earn the same outcome as is associated with the stimulus, indicating an ability to use the distinct outcome representations to select the appropriate lever press action to earn reward. However, we did not observe similar effects in control subjects that were not expressing the hM4Di receptor, so these results must be interpreted with caution. PIT is a highly variable behavior and this control group was underpowered to detect the PIT effect. Yet, it is also possible that BLA→ACC inactivation enhanced expression of PIT in unknown ways, perhaps by mitigating the effects of extinction that occur in PIT. More work on the function of this pathway is needed to clarify its role in stimulus-guided behavior.

Our work shows that activity in ACC→BLA projections is not necessary to express PIT, but may be critical for Pavlovian devaluation, i.e., adjustment of cue responding after outcome value has changed. During the devaluation probe test, rats responded to both reward-paired CSs, regardless of the value of the outcome associated with them. While needed for stimuli to retrieve reward value expectations, we did not find that ACC→BLA projections are involved in the sensitivity of the instrumental response to devaluation. ACC→BLA inactivation did not prevent rats from using outcome value to reduce lever pressing for only one of the two rewards, i.e. demonstrating sensitivity to devaluation. Taken together, these findings provide evidence that the ACC and BLA collaborate to enable stimuli to retrieve updated reward value.

These findings complement other results from our lab and others investigating how the BLA works with other cortical regions to maintain adaptive behavioral control, adding more information to the circuit mapping of appetitive behavior. Projections from the BLA to the lateral orbitofrontal cortex (lOFC) and medial orbitofrontal cortex (mOFC) are needed to use reward-paired stimuli to guide value-based behavior (Lichtenberg et al., 2017; Lichtenberg et al., 2021)., In contrast, ACC projections to the BLA investigated here are not needed for action selection, while inputs from medial OFC into the BLA are needed for using stimuli to select reward-seeking actions (Lichtenberg et al., 2021). Understanding this nuance of medial prefrontal-amygdala contributions to behavior is essential for a comprehensive understanding of the interacting circuits enabling adaptive control of behavior.

Unfortunately, ACC→BLA inactivation with CNO resulted in unexpected tissue damage and illness in a number of subjects, and this may confound our results. Many subjects in the ACC→BLA group were thus only able to complete 1 of each PIT and outcome-specific devaluation test, vs 2 of each in the BLA→ACC group and our prior work, so data from just 1 of each test are shown. Because so few animals in the ACC→BLA group were able to complete 2 iterations of

each test, the group was underpowered to detect any effects using data from both of each test, so we chose to analyze and present data only from the first of each test. We were therefore unable to average over two tests, and spurious effects owing to individual differences, such as preference, may be more present in these data. This may contribute to the variability we observed and may explain why control subjects did not perform as we have seen previously. More work is needed to understand the role of ACC→BLA projections in these and other reward-related behaviors. Pathway specific manipulations that do not require intracranial infusion, such as a dual-viral intersectional approach with systemic CNO administration or optical manipulations, may be better suited for testing experimental questions in ACC→BLA projections.

Conclusions

Using pathway-specific chemogenetic manipulation, I have shown that ACC inputs to the BLA and BLA inputs to the ACC may both be important for using reward-paired stimuli to generate reward value expectations that guide decision making and reward pursuit behavior. Now having identified a potential role for these pathways in stimulus-guided appetitive behavior, several future directions can clarify the extent to which these pathways affect behavior. Monitoring endogenous activity of ACC to BLA and BLA to ACC neurons may provide insight into how these reciprocal pathways affect behavior by analyzing changes in activity with respect to discrete aspects of behavioral tasks. This can provide information about the neural underpinnings of the specific psychological processes used to maintain adaptive control of reward pursuit. Calcium imaging can provide proxy information about the activity in a region during specific behavioral events, while miniature microscope calcium imaging can track how individual cells contribute to behavior to understand how different cells from the same region act differently to enable adaptive control of behavior (Siciliano & Tye, 2019).

We already know the BLA plays a substantial role in reward learning and memory (Wassum & Izquierdo, 2015), and these findings add to the map of neural circuits underlying reward behavior, for example, how VTA-dopamine projections to the BLA modulate ACC activity during reward value considerations, or how ACC inputs into the BLA change striatal activity to guide action execution. Along with contributing to adaptive behavioral control, the BLA and ACC also contribute to opioid learning and memory (Cai et al., 2013; Hou et al., 2020). Further, the BLA contains dense amounts of mu-opioid receptors (Mansour et al., 1994) and BLA contributions to reward learning and memory depend on mu-opioid signaling (Lichtenberg & Wassum, 2017; Wassum et al., 2011). This provides a potential neural substrate for drug-directed behavior after opioid exposure. Clarifying how these regions and the direct connections between them contribute to opioid behavior will add to our understanding of poor decision making in the context of substance use disorders, including with comorbid trauma.

Methods

Subjects

Male and female Long Evans rats aged 10–12 weeks at the start of the experiment (Charles River Laboratories) were pair housed before surgery and then subsequently housed individually in a vivarium with regulated temperature (68–79°F) and humidity (30–70%). Rats were provided with filtered tap water *ad libitum* in the home cage and were maintained on a food-restricted 12–14 g daily diet (LabDiet) to maintain ~85–90% free-feeding body weight. Rats were handled for ~3 d before experiment onset. Separate groups of naive rats were used for each experiment. Experiments were performed during the dark phase of a 12:12 h reverse dark/light cycle (lights off at 7 A.M.). All procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the University of California, Los Angeles Institutional Animal Care and Use Committee.

Surgery

Standard surgical procedures, described previously (Lichtenberg et al., 2017; Lichtenberg et al., 2021; Malvaez et al., 2015; Malvaez et al., 2019) were used for all surgeries. Rats were anesthetized with isoflurane (4–5% induction, 1–2% maintenance), and a nonsteroidal anti-inflammatory agent (Carprofen 5mg/ml at 1ml/kg) was administered before and after the operation to minimize pain and discomfort. Rats also received carprofen and saline (2mL, s.c.) for 2 days after surgery and antibiotic-enriched home chow for 7 days during recovery (Irradiated Uniprim Diet, Envigo).

Chemogenetic inhibition of BLA→ACC projections

Prior to behavioral training, rats received an intracranial surgery for later manipulations. Rats (*N* = 24, final) were infused bilaterally with AAV expressing the inhibitory designer receptor human M4 muscarinic receptor (hM4Di; AAV8-CaMKII-hM4Di-mCherry, 4.8×10^{12} vg/ml; Addgene; 0.5 µL) or control virus lacking hM4Di but expressing the same mCherry fluorophore (AAV8-CaMKII-mCherry, 3.7×10^{12} vg/ml, Addgene) at a rate of 0.1 µL/min into the BLA (AP: -3.0, ML: ±5.0, DV: −8.6 mm from bregma) using a 28 gauge injector (Protech international). Injectors were left in place for an additional 10 min, and then withdrawn from the brain slowly (1mm per min) to ensure adequate diffusion and to minimize off-target spread along the injector tract. A bilateral twenty-two gauge stainless-steel guide cannula was implanted above the ACC (AP: +3.2, ML: ±0.6, DV: −1.6 mm from bregma). Rats recovered for ∼16 d before the onset of behavioral training. Behavioral tests that followed the training began 6–7 weeks following surgery to ensure anterograde transport and robust expression in BLA axons and terminals in the ACC.

Chemogenetic inhibition of ACC→BLA projections

Surgery occurred before onset of behavioral training. Rats (*N* = 18, final) were infused bilaterally with AAV expressing the inhibitory designer receptor hM4Di (AAV8-CaMKII-hM4Di-mCherry, 4.8 × 10^{12} vg/ml; Addgene; 0.3 µL) or control virus expressing mCherry without hM4Di (AAV8-CaMKII-mCherry, 3.7 × 10^{12} vg/ml) at a rate of 0.1 µL/min into the ACC (AP: +3.2, ML: ±0.6, DV: −2.6 mm from bregma) using a 28 gauge injector. Injectors were left in place for an additional 10 min. Twenty-three gauge stainless-steel guide cannulas were implanted bilaterally above the BLA (AP: -3.0, ML: ±5.0, DV: −7.0 mm from bregma). Rats recovered for ∼16 d before the onset of behavioral training. Behavioral testing began 6–7 weeks following surgery to ensure anterograde transport and robust expression in ACC axons and terminals in the BLA.

Behavioral procedures

Apparatus

Training took place in conditioning chambers (Med Associates) housed within sound- and light-attenuated boxes, described previously (Malvaez et al., 2015, 2019; Lichtenberg and Wassum, 2017; Wassum et al., 2016; Lichtenberg et al., 2017; Collins et al., 2019). Each chamber contained two retractable levers that could be inserted to the left and right of a recessed food-delivery port in the front wall. A photobeam entry detector was positioned at the entry to the food port. Each chamber was equipped with a syringe pump to deliver 20% sucrose solution in 0.1 ml increments through a stainless-steel tube into one well of the food port and a pellet dispenser to deliver single 45 mg grain food pellets (Bio-Serv) into another well of the same food port. Both a clicker and white noise generator were attached to individual speakers on the wall opposite the levers and food port. A 3 W, 24 V house light mounted on the top of the back wall opposite the food-delivery port provided illumination, and a fan mounted on the outer chamber provided ventilation and external noise reduction. Behavioral procedures were similar to those we

described previously (Malvaez et al., 2015; Lichtenberg and Wassum, 2017; Lichtenberg et al., 2017).

Pavlovian conditioning

Rats first received five sessions of Pavlovian training (one session/day) to learn to associate each of two auditory conditional stimuli (CS; 80–82 dB, 2 min duration), white noise or clicker (10hz), with a specific food reward, sucrose (20% in filtered water, 0.1 ml/delivery) or grain pellets (one 45 mg pellet/delivery; Bio-Serv). CS-reward pairings were counterbalanced across sex and virus type at the start of each experiment. For half the subjects, white noise was paired with sucrose and clicker with pellets, with the other half receiving the opposite arrangement. Each session consisted of six tone and six white noise presentations. During each 2 min CS the associated reward was delivered on a 30 s random-time schedule, resulting in an average of four CS–reward pairings per trial. CSs were delivered in pseudorandom order with a variable 2–4 min (mean = 3 min) intertrial interval.

Instrumental conditioning

Rats were then given 9 days minimum of instrumental training. They received two separate training sessions per day, one with the left lever and one with the right lever, separated by at least 1 hour. Each action was reinforced with a different outcome (e.g., left press→grain pellets; right press→sucrose; counterbalanced with respect to the Pavlovian contingencies, virus, and sex). Each session terminated after 30 outcomes had been earned or 30 min had elapsed. The reinforcement schedule was escalated ultimately to random ratio (RR)-10 (on average 10 presses required to obtain reward delivery). Rats received 1 training day (minimum) in which their actions were continuously reinforced, then a minimum of 2 training days on RR-2, 2 days on RR-5, and 3 days on RR-10 schedule for at least 3 days. To be escalated to the next schedule, rats had to

earn at least 24 outcomes within 30 min. This requirement had to be met on the final RR-10 schedule for at least 2 training days before subjects were advanced to testing.

Outcome-specific Pavlovian-to-instrumental transfer test

Following Pavlovian and instrumental conditioning, rats received a set of outcome-specific Pavlovian-to-instrumental transfer (PIT) tests. One day prior to the first of two PIT tests, rats were given a single 20 min extinction session during which both levers were available, but pressing was not reinforced to establish a low level of responding. The following day, during the PIT test, both levers were continuously present, but pressing was not reinforced. After 5 min of lever-press extinction, each 2 min CS was presented separately four times in pseudorandom order, separated by a fixed 4 min intertrial interval. Rewards were not delivered during CS presentation or at all during the session. Prior to each test, all rats were intracranially infused with clozapine-N-oxide (CNO), selectively inactivating BLA→ACC activity in subjects expressing hM4Di in BLA neurons, while controlling for any potential off-target effects of CNO in controls only expressing mCherry. Rats in the BLA→ACC group received two of each test, counterbalanced for order. Between each test, rats were given two retraining sessions to serve as a reminder for each instrumental association (two sessions/day for 2 d) and one Pavlovian retraining session. Owing to technical problems with intra-BLA infusions leading to illness, rats in the ACC→BLA group only received one PIT test. Rats in this group that became ill within 24 hours of this test were excluded from analysis.

Outcome-selective devaluation test

Rats were given a set of two outcome-specific devaluation tests to probe associative reward value memories. Immediately prior to each test, rats were given 1 h *ad libitum* access to either the sucrose solution or grain pellets in preexposed feeding chambers to establish a sensory-specific satiety and thus selectively devalue the pre-fed reward, but not the alternate reward. The test

consisted of two phases. First, rats were able to press on both levers in extinction for 5 min. The levers then retracted to start the Pavlovian test phase in which each 2 min CS was presented serially 2 times each in alternating order and separated by a fixed 3 min intertrial interval. Rats in the BLA→ACC group received two of each test, to assess behavior after both grain pellets and sucrose were devalued. Owing to technical problems with intra-BLA infusions leading to illness, rats in the ACC→BLA group only received one devaluation test. Rats in this group that became ill within 24 hours of this test were excluded from analysis.

After prefeeding and immediately before the test, rats were infused with CNO. Test order and the pre-fed outcome relationship were counterbalanced across subjects and with respect to the CS–reward and lever-reward relationships. For both the BLA→ACC and ACC→BLA groups, there were no significant differences in the amount consumed during prefeeding between mCherry controls and those expressing hM4Di. Successful devaluation was confirmed by a post-test consumption choice test, wherein animals were given 10g of each outcome simultaneously in the feeding chamber and able to freely consume either. Animals were excluded from analyses if they consumed more of the devalued (recently pre-fed) outcome than the valued outcome, as this indicates unsuccessful devaluation. Animals in both pathway groups and across both virus conditions ate significantly less of the devalued food than the valued food. We excluded 1 BLA→ACC rat from the devaluation test dataset because it failed the posttest choice consumption test, consuming similar amounts of the devalued and valued outcome, thus indicating unsuccessful outcome devaluation. Data from 3 more rats in the BLA→ACC condition were not shown due to technical difficulties resulting in loss of the magazine dwell time data. Rats were given 1 day without behavioral training or testing after each devaluation test to reestablish hunger and then received two retraining sessions for each instrumental association (two sessions/day for 2 d) and one Pavlovian retraining session in between tests.

Chemogenetic inhibition of BLA→ACC projections

For the BLA→ACC group, chemogenetic inhibition was used to inactivate hM4Di-expressing BLA axons and terminals in the ACC before each PIT test and after prefeeding before each devaluation test. We selected chemogenetic inhibition to ensure circuit inhibition throughout all of each test. CNO (HelloBio) was dissolved in artificial cerebral spinal fluid (aCSF) to 1 mM concentration, and 0.3 µL was intracranially infused over 1.5 min bilaterally into the ACC as previously described (Lichtenberg et al., 2017; Malvaez et al., 2019). Injectors were left in place for at least 1 additional minute to allow for drug diffusion. Controls without hM4Di expression were also given CNO to control for potential off-target effects of the drug. We have previously shown these procedures to effectively attenuate the activity of BLA terminals in a nearby subregion of the prefrontal cortex, the orbitofrontal cortex (OFC), and have found that intra-OFC infusion of this dose of CNO does not affect reward-related behavior, OFC activity, or BLA terminal activity in the OFC in the absence of hM4Di (Lichtenberg et al., 2017). Thus, we expect the manipulation has similar effects along BLA→ACC pathways. Three rats were excluded from this group because of cannula clog and thus the inability to infuse CNO.

Chemogenetic inhibition of ACC→BLA projections

In the ACC→BLA group, chemogenetic inhibition was used to inactivate hM4Di-expressing ACC axons and terminals in the BLA before each PIT test, and after pre-feeding but before each devaluation test. Procedures were identical to those above with the exception that CNO or aCSF was instead infused bilaterally into the BLA (0.5 µL over 2.5 min) 5-10 min prior to behavioral testing. Using these procedures, we have previously demonstrated effective inactivation of cortical (OFC) axons and terminals in the BLA both *in vivo* and *ex vivo* (Lichtenberg et al., 2017; Malvaez et al., 2019). We have also shown that this dose of CNO when infused into the BLA has no effect on reward-related behavior, BLA activity, or OFC terminal activity in the BLA in the absence of the hM4Di receptor (Lichtenberg et al., 2017; Malvaez et al., 2019). Nonetheless, rats

expressing mCherry without the hM4Di receptor were used to control for potential off-target effects of CNO infusion. In this experiment, we found that intracranial infusions of CNO into the BLA produced pronounced negative side effects causing a number of subjects to become severely ill and unable to finish the experiment. Animals showing untoward behavioral characteristics within 24h of a CNO infusion were excluded from analyses for that test and all future tests.

Histology

Rats were deeply anesthetized with pentobarbital and transcardially perfused with PBS followed by 4% paraformaldehyde. Brains were removed and postfixed in 4% paraformaldehyde overnight, placed into 30% sucrose (in PBS) solution, then sectioned into 40 µm slices using a cryostat (Leica) and stored at -20° in liquid cryoprotectant. Free-floating coronal sections containing the ACC and the BLA were mounted onto slides and coverslipped with ProLong Gold mounting medium with DAPI (Invitrogen). The signal for BLA axonal expression of mCherry in the ACC (or ACC axonal expression of mCherry in the BLA) was immunohistochemically amplified using antibodies directed against mCherry. Floating coronal sections were washed two times in 1 × PBS for 10 min and then blocked in a solution of 5% normal goat serum (NGS) and 1% Triton X-100 dissolved in PBS for 1–2 h at room temperature. Sections were then washed three times in PBS for 15 min and then incubated in blocking solution containing rabbit anti-DsRed antibody (1:1000; EMD Millipore) with gentle agitation at 4°C for 18–22 h. On the second day, sections were rinsed three times in the blocking solution and incubated in Alexa Fluor 594-conjugated (red) goat secondary antibody (1:500; Invitrogen) for 2 h.

Fluorescence imaging was used to visualize amplified mCherry ACC-projecting BLA cells, BLA-projecting ACC cells, and guide cannula placements. Images were acquired using a Keyence BZ-X710 microscope with a 4×,10×, and 20× objective (CFI Plan Apo), (ACC→BLA, *N* = 18;

BLA→ACC, *N* = 24). Accuracy of virus and guide cannula placements were verified for each subject and any subjects with missed placements were removed from analyses. Additionally, many subjects from the ACC→BLA group had extensive tissue damage and were removed from analyses.

Data analysis

Behavioral analysis

Behavioral data were extracted from raw MedAssociates output files using the MedPC to Excel tool, and further processed in Microsoft Excel. Left and/or right lever presses and/or entries into the food delivery port were collected continuously for each training and test session. For the last day of Pavlovian training, we compared the time spent in the food port between the CS-probe (after CS onset, before reward delivery) and the preCS baseline periods. Data were averaged across trials for each CS and then averaged across the two CSs. Press rates on the last day of instrumental training were averaged across levers. For the PIT test, the baseline lever-press rate (presses/min) averaged across both levers during the 2 min periods immediately before the onset of each CS was compared with that during the CS periods. During the CS periods, lever pressing was aggregated by presses on the lever that during training, earned the same outcome as was associated with the current stimulus (CS-Same presses) versus presses on the other available lever (CS-Different presses). Data were averaged across trials for each CS and then averaged across the two CSs. Time spent in the food delivery port was also compared between the baseline and CS periods, averaged across both CSs. For the instrumental phase of the devaluation test, the lever-press rate was compared between the lever associated with the pre-fed food outcome (i.e., the devalued outcome) and the other lever, associated with the non-pre-fed (valued) outcome. During the Pavlovian phase the time spent in the food-delivery port was compared between the 2 min preCS baseline periods and the CS periods, which were separated by the CS that predicted the devalued outcome versus the CS that predicted the valued outcome.

Statistical analysis

Datasets were analyzed by two-tailed, paired Student's *t* tests, one- or two-way repeated-measures ANOVA, as appropriate (GraphPad Prism, GraphPad; IBM SPSS). *post hoc* tests used the Bonferroni correction. All data were tested for normality before analysis with ANOVA, and the Greenhouse-Geisser correction was applied to mitigate the influence of heterogenous variance between conditions if the assumption of sphericity was not met. Alpha levels were set at $p < 0.05$.

Rigor and reproducibility

Group sizes were estimated a priori based on prior work using male and female Long Evans rats in this behavioral task (Malvaez et al., 2015; Lichtenberg and Wassum, 2017; Lichtenberg et al., 2017) and to ensure accurate counterbalancing of CS-reward and lever-reward pairings across sex and virus conditions. Investigators were blinded to condition when possible, with the exception of the surgeon being aware of hM4di-expressing- vs control virus infusion during surgery. All behaviors were scored using automated software (MedPC). Each behavioral experiment included one replication cohort, and cohorts were balanced by sex, viral group, CS-reward and lever-reward pairings, and drug test order, before the start of the experiment.

Data and Code Availability

All data supporting the findings of this study are available from the corresponding author on request.

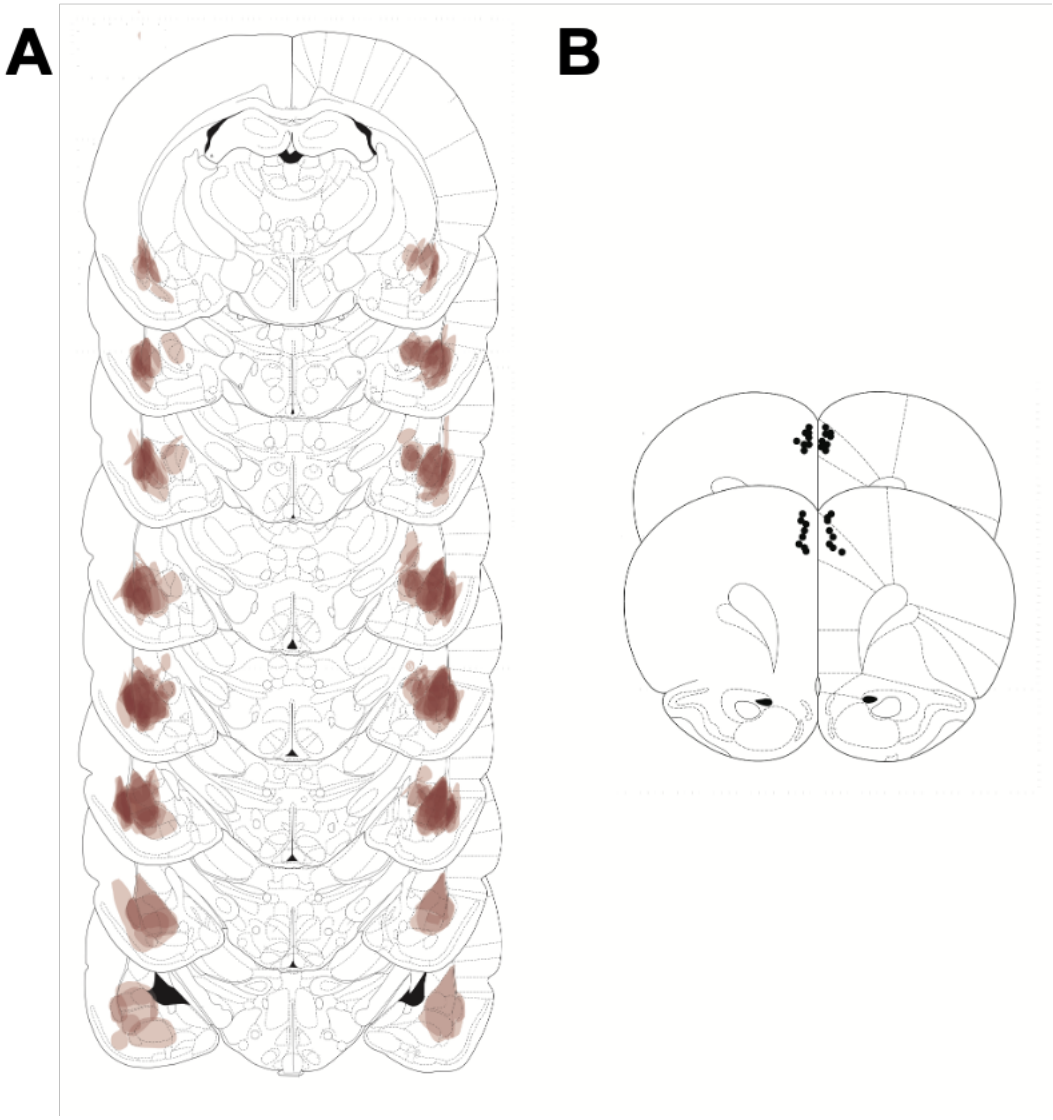


Figure 2.1. Histological verification of BLA viral expression and ACC infusion cannula placements. Rats were infused with AAV8-CaMKII-hM4Di-mCherry in the BLA (**A**) to drive hM4Di expression in BLA glutamatergic neurons and implanted with infusion cannulas over the ACC (**B**) for later infusion of CNO.

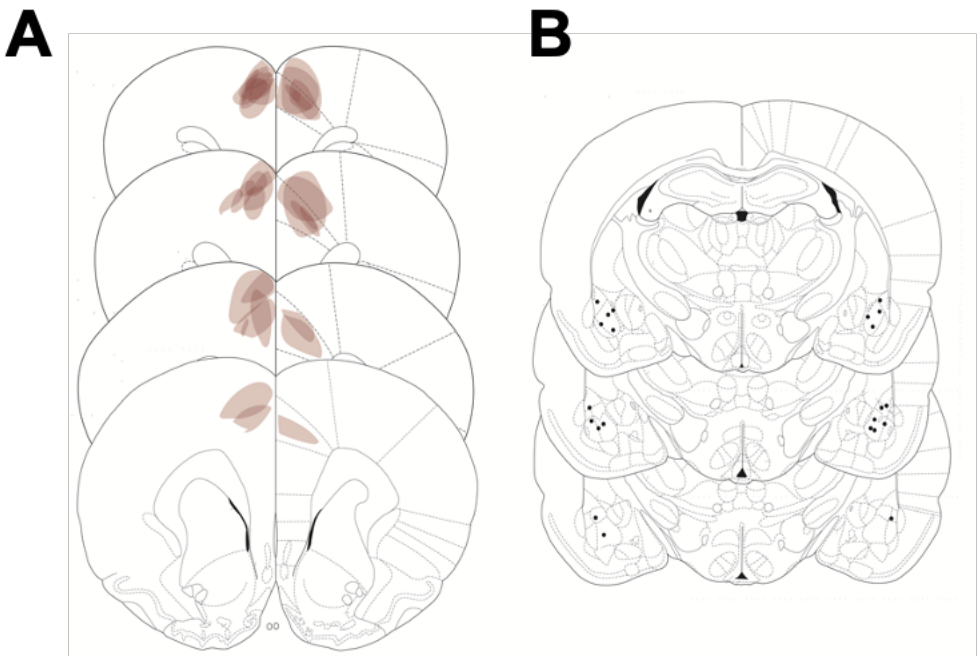


Figure 2.2. Histological verification of ACC viral expression and BLA infusion cannula placements. Rats were infused with AAV8-CaMKII-hM4Di-mCherry in the ACC (**A**) to drive hM4Di expression in ACC glutamatergic neurons and implanted with infusion cannulas over the ACC (**B**) for later infusion of CNO.

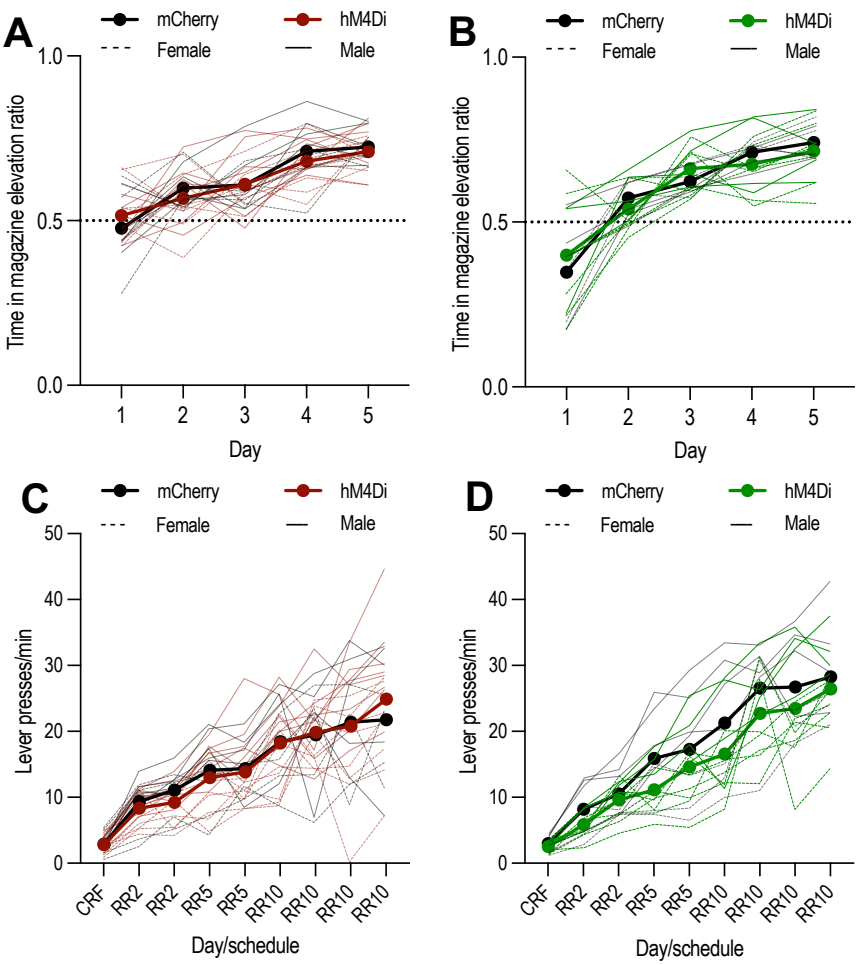


Figure 2.3. Pavlovian and instrumental training. Following intracranial surgery for later inactivation of BLA→ACC or ACC→BLA projections, animals were trained for 5 days in absence of manipulation to associate two distinct auditory stimuli (clicker or white noise) with two food outcomes (grain pellets or sucrose solution). Rats show evidence of Pavlovian conditioning as food-port magazine dwell time during the stimuli vs baseline increased over days, with data disaggregated by future manipulation group, BLA→ACC (**A**) (Virus: $F_{(1,23)} = 0.1671$, $p = 0.6865$; Day: $F_{(3.192,73.42)} = 31.73$, $p < 0.0001$; Virus x Day: $F_{(4,92)} = 0.8193$, $p = 0.5161$) and ACC→BLA (**B**) (Virus: $F_{(1,16)} = 0.0006$, $p = 0.9810$; Day: $F_{(4, 64)} = 44.99$, $p < 0.0001$; Virus x Day: $F_{(4,64)} = 1.02$, $p = 0.4039$). Following Pavlovian training, the same rats were trained for 9 days to associate two instrumental actions (left/right lever press) with the grain pellets and sucrose solution outcomes learned about in prior Pavlovian conditioning. All rats acquired these instrumental associations, as shown by escalating lever pressing over training in both the BLA→ACC group (**C**) (Virus: $F_{(1,23)} = 0.0156$, $p = 0.9018$; Day: $F_{(3.085,70.96)} = 51.59$, $p < 0.0001$; Virus x Day: $F_{(8,184)} = 0.5869$, $p = 0.7879$) and the ACC→BLA group (**D**) (Virus: $F_{(1,16)} = 1.566$, $p = 0.2288$; Day: $F_{(3.618,57.88)} = 81.62$, $p < 0.0001$; Virus x Day: $F_{(8,128)} = 0.6423$, $p = 0.7409$). Critically, no levers were present during Pavlovian training, no Pavlovian stimuli present during instrumental training, and no neural manipulations were given in training.

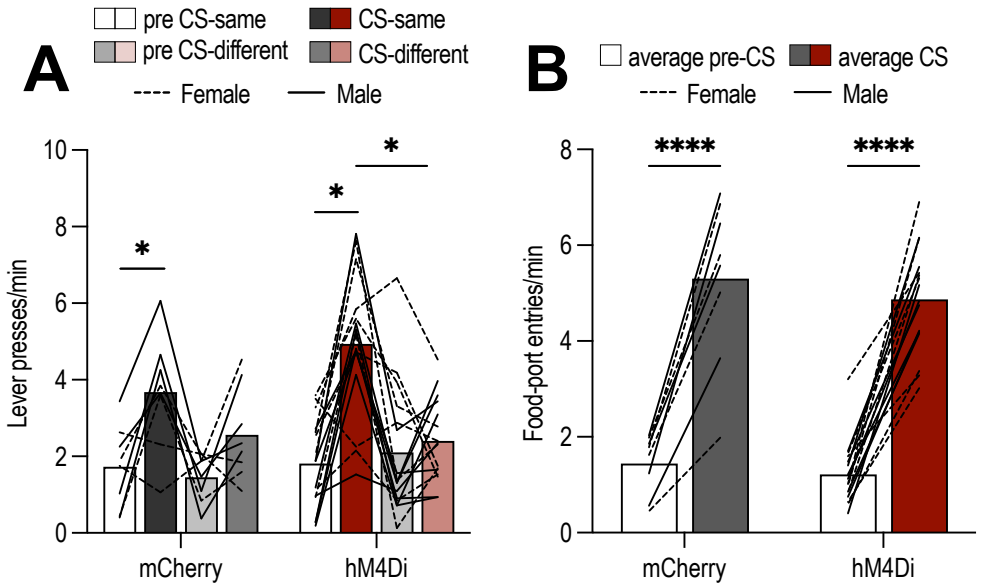


Figure 2.4: BLA→ACC contribution to outcome-specific Pavlovian-to-instrumental transfer. Rats were given an outcome-specific Pavlovian-to-instrumental transfer test, where each reward-paired lever was available and each stimulus serially presented to assess the extent to which each stimulus engendered responding on each lever. Inactivating projections from the BLA→ACC does not disrupt animals' ability to select the relevant reward-seeking action when presented with a specific reward-associated stimulus (**A**), indexed by selective increases in lever pressing on the lever associated with the same reward as the stimulus is (i.e. "CS-same") vs the alternate lever ("CS-different")(Virus: $F_{(1,22)}$ = 1.302, p = 0.2662; CS/lever congruence: $F_{(1,22)}$ = 14.22, p = 0.001; CS-on: $F_{(1,22)}$ = 29.66, p < 0.0001; Virus x CS/lever congruence: $F_{(1,22)}$ = 0.8127, p = 0.3771; Virus x CS-on: $F_{(1,22)}$ = 0.097, p = 0.7584; CS/lever congruence x CS-on: $F_{(1,22)}$ = 16.44, p = 0.0005; Virus x CS/lever congruence x CS-on: $F_{(1,22)}$ = 4.773, p = 0.0399). Animals in both experimental conditions retain reward delivery expectations in the task as indexed by general increases in discrete food-port entries during each stimulus compared to baseline (**B**) (Virus: $F_{(1,22)}$ = 0.762, p = 0.3921; CS-on: $F_{(1,22)}$ = 215.4, p < 0.0001; Virus x CS-on: $F_{(1,22)}$ = 0.1687, p = 0.6853). *p < 0.05, **** p < 0.0001, Bonferroni correction.

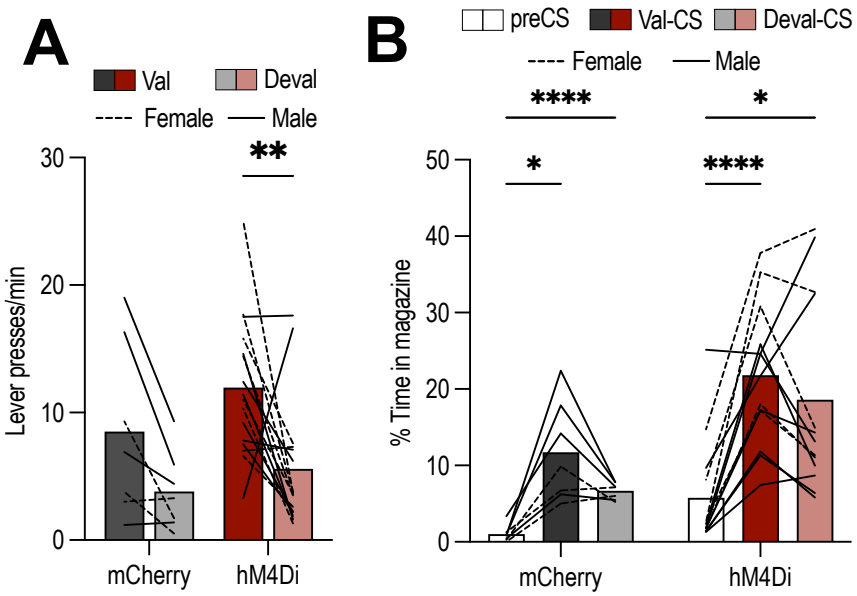


Figure 2.5: BLA→ACC contribution to outcome-specific devaluation. Rats were given a combined instrumental and Pavlovian devaluation probe test to assess how they are using reward value memories to guide behavior. After pre-feeding of one food outcome, thus reducing its value, rats were able to lever press in extinction on the reward-paired levers, and then, the Pavlovian stimuli were serially presented to assess how outcome-specific devaluation affected the Pavlovian conditioned approach response to each stimulus. Selectively inactivating projections from the BLA→ACC does not disrupt the ability to use expected reward value to guide instrumental reward seeking (**A**)(Virus: $F_{(1,21)} = 2.170$, $p = 0.1555$; Value: $F_{(1,21)} = 12.68$, $p = 0.0018$; Virus x Value: $F_{(1,21)} = 0.2972$, $p = 0.5914$), but does prevent animals from adjusting their food-port approach response to a specific stimulus associated with now-devalued reward (**B**), in that responding to both CSs is elevated above baseline. Animals with BLA→ACC inactivation responded more overall than mCherry-expressing control subjects (Virus: $F_{(1,18)} = 8.081$, $p = 0.0108$; CS-period: $F_{(1.725, 31.04)} = 20.56$, $p < 0.0001$; Virus x CS-period: $F_{(2,36)} = 1.514$, $p = 0.2337$). *$p < 0.05$, **$p < 0.01$, **** $p < 0.0001$, Bonferroni correction.

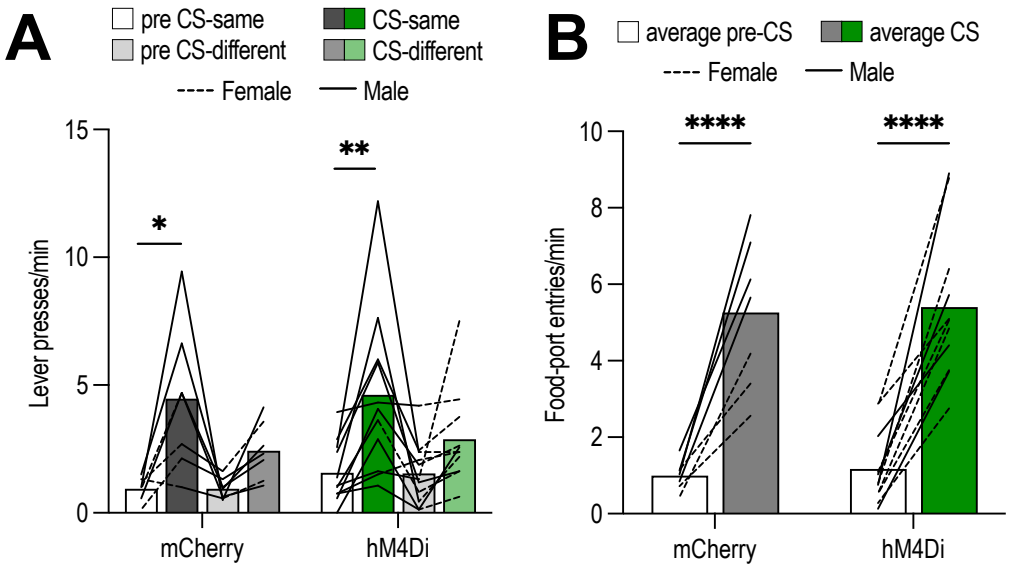


Figure 2.6 ACC→BLA contribution to outcome-specific Pavlovian-to-instrumental transfer. Rats were given an outcome-specific Pavlovian-to-instrumental transfer test, where each reward-paired lever was available and each stimulus serially presented to assess the extent to which each stimulus engendered responding on each lever. Despite ACC→BLA projection inactivation, animals are still able to select the appropriate reward-seeking action when presented with a reward-paired stimulus, indicated by selective increases in lever pressing on the lever that would earn the same outcome as is associated with the stimulus presented (i.e. "CS-same" pressing) (**A**) (Virus: $F_{(1,16)}$ = 0.6748, p = 0.4235, CS/lever congruence: $F_{(1,16)}$ = 4.058, p = 0.0611; CS-on: $F_{(1,16)}$ = 38.39, p <0.0001; Virus x CS/lever congruence: $F_{(1,16)}$ = 0.0225, p = 0.8827; Virus x CS-on: $F_{(1,16)}$ = 0.1686, p = 0.6868; CS/lever congruence x CS-on: $F_{(1,16)}$ = 5.424, p = 0.0333; Virus x CS/lever congruence x CS-on: $F_{(1,16)}$ = 0.0403, p = 0.8434). Animals also showed evidence of reward expectation generally, maintaining conditioned food-port responses to CSs during the task (**B**) (Virus: $F_{(1,16)}$ = 0.073, p = 0.7904; CS-period: $F_{(1,16)}$ = 94.37, p < 0.0001; Virus x CS-period: $F_{(1,16)}$ = 0.0013; p = 0.9712). *p < 0.05, **p < 0.01, **** p < 0.0001, Bonferroni correction.

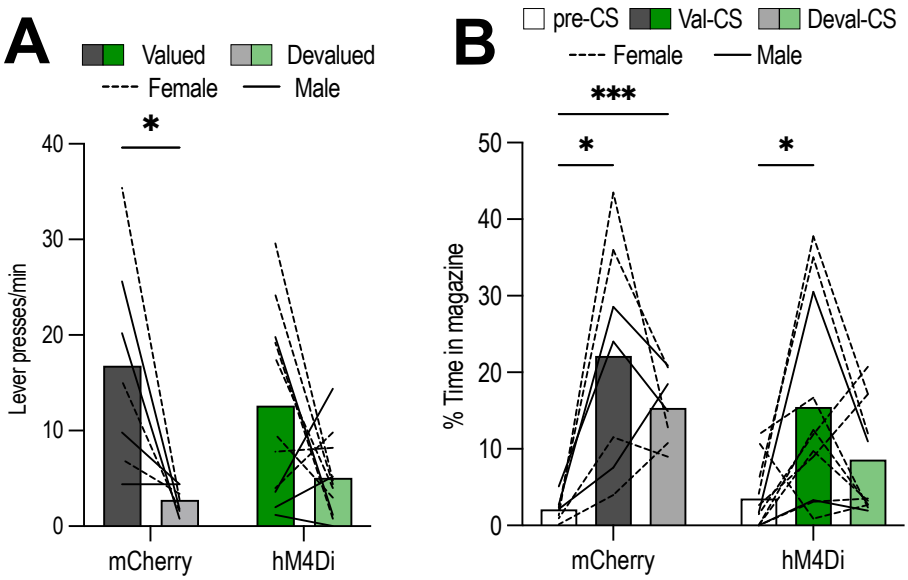


Figure 2.7: ACC→BLA projections in outcome-specific devaluation. Rats were given a combined instrumental and Pavlovian devaluation probe test to assess how they are using reward value memories to guide behavior. After pre-feeding of one food outcome, thus reducing its value, rats were able to lever press in extinction on the reward-paired levers, and then, the Pavlovian stimuli were serially presented to assess how outcome-specific devaluation affected the Pavlovian conditioned approach response to each stimulus. Under ACC→BLA projection inactivation, animals are still able to modulate lever press responding based on the value of the outcome associated with each lever (**A**) (Virus: $F_{(1,16)} = 0.1593$, $p = 0.6951$; Value: $F_{(1,16)} = 13.81$, $p = 0.0019$; Virus x Value: $F_{(1,16)} = 1.264$, $p = 0.2774$). However, the manipulation rendered the Pavlovian conditioned approach response insensitive to the current value of the outcome associated with the stimulus (**B**) (Virus: $F_{(1,16)} = 1.840$, $p = 0.1938$; CS-period: $F_{(1.467,23.47)} = 16.56$, $p = 0.0001$; Virus x CS-period: $F_{(2,32)} = 1.414$, $p = 0.2580$). *$p < 0.05$, *** $p < 0.001$, Bonferroni correction.

Chapter 3: Stress-enhanced fear learning (SEFL) is not associated with canonical trauma effects on opioid behavior

Introduction

Traumatic life events influence risk for psychiatric disorders, including both the development of posttraumatic stress disorder and opioid use disorder. Posttraumatic stress disorder (PTSD) is a chronic psychiatric disease marked by symptoms such as intrusive thoughts, nightmares, flashbacks, severe anxiety, and uncontrollable thoughts about the event (Nemeroff et al., 2006). Further, people with PTSD show exaggerated fear responses to novel stimuli (Shvil et al., 2013). Importantly, there is high comorbidity between PTSD and other psychiatric disorders, including opioid use disorder (OUD) (Fareed et al., 2013). Opioid use disorder (OUD) emerges from a problematic pattern of opioid use, that persists through processes of withdrawal, craving, and loss of control of opioid use (Gressler et al., 2019). OUD encompasses cognitive, behavioral, and physiological symptoms of continued opioid use despite significant related problems (Reps et al., 2020). OUD is also associated with an increased risk of mood and anxiety disorders, including PTSD (Martins et al., 2011; Cragg et al., 2019). While much work has revealed the high rate of comorbidity between OUD and PTSD (Dahlby & Kerr, 2020; Takemoto et al., 2020; Upadhyay et al., 2022), little is known of the fundamental decision making processes and behavioral strategies that engender OUD in people who are diagnosed with PTSD.

To study how the neurological changes of psychiatric disorders manifest and produce associated phenotypes, the gold-standard strategy is to examine these conditions in a meticulously controlled environment, using laboratory animals to model a psychiatric condition and then probe the behavioral and neural underpinnings of each disorder to gain insight as to how these behavioral and neurological changes are occurring in humans (Geyer & Markou, 1995). Animal models have been employed to gain insights into the behavioral and neurobiological manifestations of PTSD.

A critical limitation of such studies is the difficulty in modeling the many aspects of the human experience that may contribute to the development of PTSD following a traumatic stressful event. One way to model a critical aspect of PTSD in experimental animals is to use a stress-enhanced fear learning (SEFL) paradigm, wherein animals are given a series of shocks that constitute a traumatic stressor. Following exposure to this traumatic stressor, animals show an exaggerated fear response in subsequent fear conditioning preparations that do not support substantial fear learning in animals that did not experience the traumatic stressor (Perusini et al., 2016; Rau et al., 2005). In this way, SEFL is intended to model how exaggerated fear responses emerge in situations where one should not experience fear, a hallmark of PTSD. In addition to the behavioral effects, much work has been done to understand the neurobiological changes induced by the SEFL paradigm. SEFL produces neurobiological changes in both the basolateral and central amygdala, including dysregulation of the stress hormone corticosterone (CORT) (Poulos et al., 2014) and elevated insertion of GluA1-AMPA receptor subunits (Perusini et al., 2016). Further, increased levels of GluA1-AMPA receptor subunits in the central amygdala facilitate opioid reward learning (Cai et al., 2013) and increase self-administration of morphine (Hou et al., 2020). Thus, it is possible that these neurobiological changes exhibited in PTSD may be a substrate for enhanced opioid learning, memory, and maladaptive decision making and may explain the high comorbidity between PTSD and OUD.

Like the SEFL model, models of OUD also have limitations, failing to capture many critical aspects of the opioid use experience in humans. In some cases, rodents are passively given opioid drugs by an experimenter to induce opioid dependence, but such experimental preparations bypass the voluntary nature by which humans choose to take drugs, thus introducing some interpretational limitations. More recent work has employed drug self-administration strategies, which allow experimental animals to have agency over the amount and timecourse of drug intake and can offer interesting behavioral insights (O'Connor et al., 2011; Panlilio & Goldberg, 2007). However,

such studies often occur in animals living in a very poorly enriched environment, including socially isolated housing in traditionally highly social animals, where the only activity apart from living in the homecage is to press a lever in an operant box for a drug infusion, thus in absence of negative consequences. While this approach has proven helpful for many different research questions in understanding OUD, this model leaves out several critical behavioral factors that define OUD, namely, that drug use in OUD is marked by choosing the drug at the expense of other adaptive rewards, e.g. family, career, money, etc. (Boscarino et al., 2015). While there is emerging work to capture reward comparison strategies using choice tasks (Reiner et al., 2020; Venniro & Shaham, 2020), these models use highly rewarding non-drug alternatives (e.g. large amounts of otherwise inaccessible palatable food, social reward in single-housed animals) that bias animals toward stable voluntary abstinence. While this work is critical for understanding distinct aspects of substance use disorders, such designs preclude the neurobiological interrogation of dynamic (vs stable) food vs drug decision making processes. To gain insight into how comorbid PTSD-OUD develops, persists, and can be treated, an animal model that more closely encapsulates how PTSD affects decision making processes in OUD is needed.

Here, we developed a behavioral model to understand opioid-related decision making and assess how PTSD may alter these processes, to enable later interrogation of the neural underpinnings of PTSD-OUD comorbidity. Building upon traditional opioid paradigms, we used fentanyl self-administration combined with other appetitive behavioral tasks to investigated how a preparation used to model aspects of PTSD impacted later opioid learning and decision making in rats. We trained male and female rats to self-administer food pellets, an adaptive natural reward. Then, some were given a traumatic stressor using the stress-enhanced fear learning (SEFL) paradigm, a rodent model that captures aspects of PTSD. Following this, all rats were trained to intravenously self-administer infusions of fentanyl, and then were given a series of food vs fentanyl choice tests, where choosing one reward type meant forgoing the other reward for that

trial. In this way, we created a model to assess willingness to choose the drug over more adaptive rewards that is featured in OUD and absent in many other animal models. We also tested willingness to exert effort for fentanyl using progressive ratio self-administration, to model escalating efforts to obtain the drug, and tested fentanyl reinstatement following a subthreshold stressor to understand how prior experience with a traumatic stressful event interacts with an acute mild stressor to alter opioid pursuit behavior.

Results

Similarity between appetitive and traumatic stressor contexts impacts the expression of learned fear following trauma

To test how PTSD impacts decision making between maladaptive (opioid) and adaptive (food) rewards, we first trained animals to self-administer palatable food pellets. Over 7 days, rats were trained in operant chambers to lever press for 45-mg purified diet pellets in 2-hour sessions. Lever pressing was reinforced on a fixed-ratio 2 schedule, meaning every 2 presses resulted in delivery of a pellet. Pellet delivery was accompanied by the lever retracting into the chamber wall and presentation of discrete visual cue, a stimulus light above the lever that either flashed at 2Hz or remained illuminated for the 20-second presentation. Rats acquired this instrumental behavior quickly, reaching stable levels of responding after several days (Figure 3.1; Experiment 1: (Day: $F_{(2.851,85.54)} = 13.94$, $p < 0.0001$; Sex: $F_{(1,30)} = 5.519$, $p = 0.0256$; Day x Sex: $F_{(6,180)} = 0.4442$, $p = 0.8484$); Experiment 2: (Day: $F_{(2.883,86.48)} = 15.50$, $p < 0.0001$; Sex: $F_{(1,30)} = 0.0935$, $p = 0.7618$; Day x Sex: $F_{(6,180)} = 3.084$, $p = 0.0067$). Data are separated by the first and second iteration of the experiment, due to changes made to the operant context to distinguish it from the trauma context where shocks are administered.

Following food training, rats were exposed to a traumatic stressor as part of the SEFL model. Rats were placed in shock chambers and given 15 1-second, 1mA unsignaled shocks with 2-4

min variable intershock interval over a 93 min session. Control subjects were placed in identical boxes and not shocked. The following day, rats were returned to the shock chambers and fear conditioning was assessed by analyzing freezing behavior over an 8 min session to confirm successful administration of the traumatic stressor. In Experiment 1, animals received food training in operant boxes sharing similar tactile floors as the shock chambers prior to trauma. We did not detect freezing in female subjects, and found lower levels of freezing in males than are typically observed in studies using the SEFL model (Perusini et al., 2016; Rajbhandari et al., 2018; Rau et al., 2005), (Figure 3.2, Sex: $F_{(1,27)} = 7.791$, $p = 0.0095$; Trauma: $F_{(1,27)} = 13.89$, $p = 0.0009$; Sex x Trauma: $F_{(1,27)} = 8.872$, $p = 0.0061$). Because we observed much lower freezing than in other studies using the SEFL model, in Experiment 2, we changed the floors of the operant context to be more distinct from the shock chambers. Here we found that rats in both sexes showed freezing to the trauma context, with males freezing more than females (Sex: $F_{(1,27)} = 12.89$, $p = 0.0013$; Trauma: $F_{(1,27)} = 64.14$, $p < 0.0001$; Sex x Trauma: $F_{(1,27)} = 11.29$, $p = 0.0023$). This suggests that rats in Experiment 1 generalized between the appetitive and aversive contexts resulting in disrupted fear learning and expression, that was mitigated by making the contexts as distinguishable as possible in Experiment 2, indicating that contextual distinguishability is a critical consideration when combining assays of appetitive and aversive behavior.

Prior experience of traumatic stressful event does not alter opioid learning

In Experiment 1, following the traumatic stressful event, rats were trained to self-administer intravenous fentanyl. Rats were returned to the operant chambers and for 12 days were able to lever-press for infusions of fentanyl (0.2mL, 3.2ug/kg/infusion) in 2-hour sessions, using the opposite side lever as was used in food training. Lever presses were reinforced on a fixed-ratio 2 schedule with an intravenous infusion of fentanyl and illumination of the stimulus light above the lever for 20s, either solid or flashing 2Hz, whichever pattern was not that of the food lever cue. While both groups increased self-administration over the 12 days, we detected no differences in

fentanyl self-administration behaviors between the trauma and control conditions, with rats earning a similar number of infusions across groups (Figure 3.3A; trauma mean 32.31 infusions +/- 2.07 SEM, control mean 31.73 +/- 1.90 SEM, $p = 0.84$; day: $F_{(5.621, 163.0)} = 4.374$, $p = 0.0005$; trauma: $F_{(1,29)} = 0.2796$, $p = 0.601$; day x trauma: $F_{(11,319)} = 1.192$, $p = 0.2913$). Because of contextual similarities between the shock chambers and the operant chambers, in Experiment 2 we used distinct floor inserts in the operant boxes to mitigate generalization between the fear and appetitive contexts. Despite this change, rats in Experiment 2 also earned similar amounts of fentanyl infusions regardless of trauma condition (Figure 3.3B; trauma mean 32.10 infusions +/- 3.01 SEM, control mean 27.73 +/- 1.32 SEM, $p = 0.11$; day: $F_{(4.741, 136.6)} = 2.763$, $p = 0.0227$; trauma: $F_{(1,29)} = 0.0422$, $p = 0.8386$; day x trauma: $F_{(11,317)} = 2.866$, $p = 0.0013$). Here, we did detect a trauma by day interaction. Post-hoc analyses determined control subjects earned statistically more infusions on day 3 vs day 1, and day 5 vs day 3, with no differences in the trauma condition, but we do not consider this meaningful for the interpretation of the results. We collapsed results across sex as we did not detect any differences across sex within or between the trauma and control conditions in Experiment 1 nor Experiment 2.

Trauma does not alter decision making between food vs drug rewards regardless of need state

Following 12 days of self-administration training, rats were given 4 days of food vs fentanyl choice tests to assess decision making between food vs drug rewards. Rats were able to lever press to earn either a food pellet or a fentanyl infusion, where earning one reward prevented earning the other reward for that trial. Sessions started with 4 forced-choice trials to offer sampling of both alternatives prior to 8 free-choice trials. Free choice behavior was quantified and analyzed as percent of trials when a fentanyl infusion was chosen. We did not detect any differences in choice behavior between trauma and control conditions in either Experiment 1 (Figure 3.4A; $t_{(29)} = 1.498$, $p = 0.1449$) or Experiment 2 (Figure 3.4B; $t_{(29)} = 0.1417$, $p = 0.8883$). Results were collapsed

across sex as we did not detect any sex differences when using ANOVA to examine sex by trauma interactions.

We then wanted to explore how animals make food vs drug decisions following a motivational shift in need state. To that end, we food restricted animals to 85% free-feeding weight and repeated the choice tests, to examine how the hungry state, in which food should be more valuable than in the sated state, affects choice behavior. We did not detect any sex differences in food vs fentanyl choice between trauma conditions in Experiment 1 (Figure 3.4C; $t_{(29)} = 1.284$, $p = 0.2094$) or Experiment 2 (Figure 3.4D; $t_{(29)} = 0.1237$, $p = 0.9024$).

Extended fentanyl self-administration training does not alter self-administration behavior or food vs drug choice

Given that we detected no differences in self-administration or choice behavior, we considered that animals may not have had enough fentanyl self-administration experience for any differences induced by the traumatic experience to emerge, thus we gave rats in Experiment 2 an additional 12 days of self-administration following the sated and hungry choice tests. Here we detected a day by trauma interaction (Figure 3.5A; day: $F_{(7.028,\ 202.9)} = 3.565$, $p = 0.0012$; trauma: $F_{(1,29)} = 0.4005$, $p = 0.5318$; day x trauma: $F_{(23,664)} = 2.247$, $p = 0.0008$), but post-hoc analyses determined this was due to variability in behavior on random days, rather than a consistent trend across days, and thus was not meaningful for interpretation of the results. We also repeated the choice tests, giving rats 2 food vs fentanyl choice sessions, to assess how extended experience with fentanyl might also change choice behavior. Despite extended experience, there were still no differences in food vs fentanyl decision making across conditions (Figure 3.5B; $t_{(29)} = 0.4209$, $p = 0.6769$).

Trauma does not affect effort allocation for fentanyl

While we had not yet detected any differences between the trauma and control conditions across multiple self-administration measures, we wanted to understand if the prior traumatic experience would affect how much effort animals were willing to expend for fentanyl infusions, given that willingness to exert great amounts of effort for opioids is a criterion for OUD (Kleykamp et al., 2019) and may be a mechanism of PTSD-facilitated OUD. To that end, we tested fentanyl self-administration behavior used a progressive ratio schedule of reward delivery to assess effort allocation. Here, each fentanyl infusion required 50% more lever presses to earn delivery than the previous infusion (e.g. 1, 2, 3, 5, 8 presses, etc.), and the last completed response requirement was designated as the animal's breakpoint, thus a higher breakpoint indicates more effort expended for fentanyl. Surprisingly, we again did not detect any differences in behavior, with trauma and control animals reaching similar breakpoints in the task (Figure 3.6; Experiment 1: $t_{(27)} = 0.9775$, $p = 0.337$; Experiment 2: $t_{(28)} = 0.6843$, $p = 0.4994$).

Trauma does not alter extinction of fentanyl responding

Following progressive ratio for fentanyl, we were interested in understanding how persistent opioid pursuit was in animals that received the SEFL treatment. A hallmark of substance use disorders is to continue engaging in drug-directed behaviors despite the fact that such behaviors are no longer rewarding (Hyman & Malenka, 2001). To that end, animals in Experiment 1 were given 3 days of fentanyl extinction, where lever presses were not reinforced with fentanyl or any associated cues. Animals in Experiment 2 received 7 days of extinction due to their extended fentanyl experience. Lever-press responding for fentanyl decreased within each session and across each day, with no differences evident between the trauma and control conditions in either Experiment 1 (Figure 3.7A; Day: $F_{(1.776, 51.50)} = 53.91$, $p < 0.0001$; Trauma: $F_{(1,29)} = 0.1128$, $p = 0.7393$; Day x Trauma: $F_{(2,58)} = 0.8025$, $p = 0.4531$) or Experiment 2 (Figure 3.7B; Day: $F_{(1.509, 42.24)}$

= 71.45, $p < 0.0001$; Trauma: $F_{(1,28)} = 0.0013$, $p = 0.9719$; Day x Trauma: $F_{(6,168)} = 0.0803$, $p = 0.998$)

Trauma does not change how subthreshold stressor impacts reinstatement of opioid seeking

A critical feature of SEFL model is that animals that received the SEFL treatment show enhanced fear responses to a stressor that normally is not sufficient to produce fear in control animals (Perusini et al., 2016). We wanted to capitalize on this to understand how the experience of such an event could then motivate animals to seek fentanyl, in a way that may mirror stress-induced drug seeking in humans. First, animals were exposed to a mild stressor as part of the SEFL model. Rats were placed in shock chambers that were contextually distinct from the chambers where they received trauma or non-shocked exposure and given one 1-second, 1mA shock ~3 min later, and were then removed from the chamber ~30s after shock. Freezing shown to the one-shock context on the following day is shown to indicate the magnitude of stress induction that occurred prior to reinstatement. Immediately following the one-shock administration, we moved the rats from the shock chambers to the operant chambers and tested animals in cue-induced fentanyl reinstatement. Animals were able to lever press for fentanyl, and 2 presses earned the fentanyl-associated cue and lever retraction, but not an infusion of fentanyl, to assess how the recent stressful experience interacted with fentanyl cues to reinvigorate fentanyl lever pressing following the prior extinction training. There were no group differences in fentanyl reinstatement between the trauma and control conditions, however, we did detect a sex difference in Experiment 1 (Figure 3.9A; Sex: $F_{(1,27)} = 7.273$, $p = 0.0119$; Trauma: $F_{(1,27)} = 0.3545$, $p = 0.5565$; Sex x Trauma: $F_{(1,27)} = 0.4944$, $p = 0.488$). Females showed higher reinstatement of fentanyl pursuit compared to males, across both the control and trauma conditions. However, this was not observed in Experiment 2 (Figure 3.9B; Sex: $F_{(1,10)} = 1.428$, $p = 0.2597$; Trauma: $F_{(1,10)} = 0.5466$, $p = 0.4767$; Sex x Trauma: $F_{(1,10)} = 0.5466$, $p = 0.4767$). Finally, the following day, rats were returned to the

one-shock context to assess how the mild stressor impacted fear learning. We measured freezing in an 8.5 min session and found that rats exposed to trauma froze significantly more than controls, verifying that the traumatic stressor rats had experienced prior did in fact enhance subsequent fear learning (Figure 3.8; Experiment 1: Sex: $F_{(1,27)} = 7.163$, $p = 0.0125$; Trauma: $F_{(1,27)} = 31.89$, $p < 0.0001$; Sex x Trauma: $F_{(1,27)} = 8.398$, $p = 0.0074$; Experiment 2: Sex: $F_{(1,25)} = 1.542$, $p = 0.2259$; Trauma: $F_{(1,25)} = 15.55$, $p = 0.0006$; Sex x Trauma: $F_{(1,25)} = 0.7212$, $p = 0.4038$). This suggests that the enhancement of fear learning observed in the SEFL model does not affect reinstatement to fentanyl seeking.

Discussion

Rates of OUD are higher in PTSD patients than in the general population (Fareed et al., 2013). Understanding the behavioral manifestations of OUD in PTSD, and the underlying neurobiology that facilitates development of OUD after trauma, is critical to developing behavioral and pharmacotherapies to treat people with both diagnoses. To that end, we combined a well-established rodent model of PTSD, stress-enhanced fear learning (SEFL), with classical opioid self-administration procedures and a novel food vs. opioid decision making task to assess how PTSD affects opioid behavior, with the ultimate goal of understanding both the behavioral and neurobiological underpinnings of comorbid PTSD-OUD. Surprisingly, we did not detect any effects of trauma on any canonical opioid behavioral measures, including total intake, escalation of intake, effort allocation, extinction, or reinstatement. Despite this, the comorbidity between PTSD and OUD is well known (Fareed et al., 2013; Upadhyay et al., 2022), thus it is possible the methods used in this work were insufficient to model these two psychiatric diseases.

A number of animal models strive to capture specific aspects of PTSD observed in humans, such that we can understand behaviorally how PTSD develops and manifests after trauma.

Additionally, perhaps more importantly, animal models also enable rigorous investigation of the underlying neurobiology that is not possible in humans, both due to ethical concerns and due to the level of experimental control one has in human vs animal studies. Here, we used the stress-enhanced fear learning (SEFL) model as a model of PTSD to then test how PTSD affected opioid learning and decision making, as OUD arises from aberrant decision making for opioid rewards. We selected the SEFL model because it replicates a number of features of PTSD in humans, including long-lasting sensitization of fear that does not require explicit memory of the traumatic event (i.e. nonassociative fear enhancement), generalization of exaggerated fear to other aversive stimuli not present during trauma, dysregulates basal diurnal corticosterone (CORT) levels, increases alcohol consumption, and produces anxiety- and depressive-like behavioral phenotypes in rodents (Perusini et al., 2016). However, we did not detect any effects of the SEFL procedure on any classical opioid behavioral assays nor on a novel food vs fentanyl decision making task. This pattern of results, however, does provide insights into model design considerations that may help improve testing hypotheses about comorbid PTSD and OUD in the future.

There are several potential reasons why we did not find any impact of trauma modeled by SEFL on opioid behavior. First, it is possible that the changes produced by SEFL in animals do not capture the aspects of human PTSD that lead to facilitated development of OUD. While animal models are powerful tools for understanding human disease, each model has its limitations, namely that no one model can encapsulate every aspect of a disease. Other animal stress paradigms that mirror PTSD by inflicting some form of physical trauma might better address this question, including single-prolonged stress, restraint stress, and underwater trauma. There are also paradigms that rely more on stressors that are ethologically relevant to rodents, such that experimenters can attempt to replicate the psychological state of humans with PTSD in animals, rather than simply applying strong physical traumatic experiences and presuming the resulting

psychological state is similar across species. These include psychosocial stress models such as housing instability, social instability, and early life stress, along with predator scent stress (Borghans & Homberg, 2015; Verbitsky et al., 2020). It is possible that another model of PTSD would produce in animals the conditions that facilitate OUD development in humans, so that deeper interrogation of the underlying neurobiology is possible.

It is also possible that our model of opioid self-administration did not provide a comparable amount and pattern of opioid exposure vs what humans experience to be able to detect any effects of trauma. We trained rats to self-administer fentanyl infusions for 2-hour sessions per day for 12 days. In the substance use/addiction literature, this would be considered a “short access” schedule, in contrast to other models that enable rats to self-administer drugs for 6-12 hours (O’Connor et al., 2011). This schedule was selected to enable high-throughput investigation and to understand if the PTSD-like phenotype produced by SEFL would produce any differences in self-administration even in conditions that normally do not produce addiction-like phenotypes (e.g. escalation of opioid taking, increased effort allocation, resistance to extinction, accelerated reinstatement). Other models of intravenous drug self-administration that produce an OUD-like phenotype, including long access (6-23 hours of continuous drug access) or intermittent access (short bouts of drug access with long timeout periods, totaling 6-12 hours) may be better for understanding how PTSD interacts with opioid use to confer higher risk of OUD, as these designs have been shown to induce addiction-like behavioral phenotypes indicative of dependence. It is possible that the PTSD-OUD comorbidity is driven by effects of trauma on dependence and withdrawal that could not have been observed with the short access schedule used here.

Conclusions

Here, I found that SEFL, a rodent model of PTSD, is not sufficient to produce canonical trauma-associated phenotypes in opioid behavior. More work investigating the impact of trauma, namely

PTSD, on opioid behavior is needed to understand how severe trauma affects the development of OUD in people with PTSD. Further, understanding the neural consequences of PTSD in reward pathways that may facilitate OUD development could help develop targeted pharmacotherapies for OUD in people with comorbid PTSD patients. A different stress induction protocol such as chronic unpredictable stress or single prolonged stress (Borghans & Homberg, 2015), and/or extended access to opioids may better reproduce behavioral deficits seen in OUD. We can then compare animals with poor decision making (i.e. bias for drugs) to healthy controls to examine how the circuitry involved in adaptive behavioral control becomes hijacked to lead to maladaptive decision making. Information from basic systems neuroscience and associative learning theory can thus greatly improve the design and interpretation of models of substance use disorders. Causal evidence from preclinical research can then help develop clinical behavioral and pharmacological interventions to improve substance use disorder outcomes.

Methods

Food self-administration

To study how rats make decisions between adaptive (food) and maladaptive (drug) rewards, we first trained rats to lever press for food pellet deliveries. Rats were trained in operant boxes (MedAssociates) described previously (Collins et al., 2019; Lichtenberg et al., 2017; Lichtenberg et al., 2021; Lichtenberg & Wassum, 2017; Malvaez et al., 2015; Malvaez et al., 2019; Wassum et al., 2016). Each chamber contained two retractable levers that inserted to the left and right of a recessed food-delivery port in the front wall, with a small stimulus key light above each lever. A photobeam entry detector was positioned at the entry to the food port. Each chamber had a pellet dispenser to deliver single 45 mg grain food pellets (Bio-Serv) into the central well of the food port. Both a clicker and white noise generator were attached to individual speakers on the wall opposite the levers and food port. A 3 W, 24 V house light mounted on the top of the back wall opposite the food-delivery port for chamber illumination, and a fan mounted on the outer chamber

provided ventilation and external noise mitigation. Behavioral procedures were similar to those described previously (Malvaez et al., 2015; Lichtenberg and Wassum, 2017; Lichtenberg et al., 2017).

In Experiment 1, the operant chambers contained a wire-grid floor, and in Experiment 2, this was changed to a Plexiglas floor to maximize differences between the operant context and subsequent shock contexts. Rats were given a fixed-ratio 2 (FR2) schedule, whereby every 2 lever presses resulted in reward delivery and the delivery of a 20s visual stimulus (solid light or 2hz flashing light above the lever). This schedule, used both for food pellets and subsequent fentanyl self-administration, was used during training and later food vs fentanyl choice tests. We chose this FR2 schedule to ensure intentional pressing for the food reward that is critical for later phases of the experiment. We trained rats in food self-administration for 7 days, each session lasting 2h each with no reward limit, to match the future conditions of fentanyl self-administration.

Jugular vein catheterization surgery

Following food self-administration training, we implanted rats with a jugular catheter for subsequent intravenous fentanyl self-administration. Rats were anesthetized with isoflurane (4–5% induction, 1–2% maintenance), and a nonsteroidal anti-inflammatory agent (carprofen 5mg/ml s.c. at 1ml/kg) was administered before and after the operation to minimize pain and discomfort. After shaving the upper back and right-side ventral neck region, a 6cm incision was made on the animal's back, starting at and perpendicular to the midline, ~2cm caudal to the shoulders, and connective tissue under the skin surface was cleared to allow for subdermal routing of the catheter from the dorsal back to ventral neck region of the animal. Then, the vascular access button with a custom pre-attached polyurethane catheter (Instech) was inserted under the surface of the skin on the animal's back. A syringe with heparinized saline was attached to the button using specialized tubing and the catheter was filled with the solution to prevent introduction of air into

the catheter. The animal was then inverted, the location of the right jugular vein was visually identified on the neck, and a shallow 5cm incision was made to find and isolate the vein beneath the connective tissue of the neck. Once isolated, two silk sutures were placed under the vein for later ligation of the vein around the catheter. Then, a probe with eye tool was inserted into the incision site and pushed back to the dorsal-side incision, where the catheter tip was then inserted into the probe eye, and the tool was pulled back through the throat incision to bring the catheter from the back surface to the throat. Then, a small opening was made in the ventral side of jugular vein using a 19G needle, a vein pick (Braintree Scientific) inserted in the opening, and finally the catheter tip inserted into the jugular vein via the vein pick. The catheter was inserted to 3cm from the tip, where a bead affixed to the catheter prevented further insertion and served as an anchor point for ligation to the vein. Successful insertion of the catheter was verified by applying mild back-pressure to the heparinized saline syringe and visualization of blood quickly coming up through the catheter at the throat incision site. This blood was then flushed back in to prevent clotting in the catheter, and the catheter was secured to the vein using the two silk sutures placed under the vein, with knots surrounding the upper and lower ends of the bead affixed to the catheter. Finally, the throat and back incisions were sutured and rats were allowed to recover for 7+ days before subsequent training. Rats were given carprofen, saline (2mL s.c.) for 2 days, and fed antibiotic chow (Irradiated Uniprim Diet, Envigo) for 7 days to aid recovery. The timing of the surgery with respect to behavioral training was selected to minimize catheter patency loss over time and minimizing time between the traumatic stressful experience and further behavioral training.

Stress-enhanced fear learning (SEFL)

Next, we put rats through a stress-enhanced fear learning protocol to induce aspects of PTSD-like behaviors in some subjects. Two contexts, Context A and Context B, were used throughout the experiment, differing in illumination, background sound, and odor, and first context (A vs B)

was counterbalanced across sex and trauma conditions. The fear conditioning chambers housed in the contexts differed in shape, size, and texture. In Context A, the light source was a single red 30 W incandescent bulb, and the humming of the shock-scrambler motor (65 dB) was used as background noise. Context A chamber (28×21×10.5 cm; Lafayette Instrument Co.; Lafayette, IN) floors were composed of 17 stainless steel rods (4 mm in diameter) that were staggered into two rows spaced 1 cm apart vertically and 2.6 cm apart horizontally. Grid floors were wired to a shock generator and scrambler (Lafayette Instrument Co.; Lafayette, IN). Chambers were modified by inserting two Plexiglas panels that divided the chamber diagonally from floor to ceiling on both sides. Chambers were washed with a 1% acetic acid solution and dried before and after each subject was run in the box. The same solution was placed in stainless steel pans underneath the chambers. Animals were transported to and from the context in an opaque rubber box that was partitioned into four equivalent areas (20×15×25 cm). A lid was placed on the box so that animals were transported in darkness.

In Context B, two ceiling mounted 6-ft fluorescent bulbs lit the room and a ventilation fan was used for background noise (65 dB). Context B chambers (28×21×10.5 cm; Lafayette Instrument Co.; Lafayette, IN) had floors composed of 18 stainless steel rods (4 mm in diameter), spaced 1.5 cm center to center, that were wired to a shock generator and scrambler (Lafayette Instrument Co.; Lafayette, IN). Chambers were washed with ammonium hydroxide (5%) and dried before and after each subject was run in the box, and the same solution was placed in stainless steel pans underneath the chambers. Animals were transported to and from the context in their homecages, which were placed in a metal carrier secured onto a cart. In both contexts, the front door of each chamber was made up of clear Plexiglas allowing a video camera to record the animals' behavior for automated behavioral scoring of freezing. The two contexts were designed with multimodally distinguishable features to facilitate interpretation of the fear conditioning taking place within the SEFL paradigm.

All experimental procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (7th Edition, National Academy Press, Washington, D.C., 1996) and were approved by the Chancellor's Animal Research Committee of the University of California, Los Angeles Office for the Protection of Research Subjects.

Shock pre-exposure on day 1 in the first context (either A or B due to counterbalancing) consisted of 15 spaced shocks (1 mA, 1 s) with a variable shock interval of 240–480 s. Non-shock control animals were placed in the same chambers for the same amount of time as shocked animals, 93 min, with no cues or shocks delivered. On day 2, animals were returned to this same context without shock to assess fear conditioning to the context that occurred the day prior, to confirm sufficient learning, over a period of 8 min and 32 sec. Freezing, a reliable measure of learned fear (Fanselow, 1994), was assessed the day after shock pre-exposure (day 2) to confirm successful fear conditioning. Freezing was defined as the absence of all movement except that necessary for respiration (Fanselow, 1980) and was measured with automated behavioral analysis of video data.

Fentanyl self-administration

Following SEFL, we trained rats in intravenous fentanyl self-administration following jugular catheter implant surgery. Similarly to food self-administration, rats were trained in the same operant boxes to lever press for fentanyl on an FR2 schedule, using the opposite-side lever as was used in food training (i.e. left vs right lever). Every 2 presses yielded an intravenous infusion of fentanyl (3.2ug/kg/inf) and presentation of a 20s visual stimulus above the lever, either solid or 2Hz, whichever was opposite the pattern for the food cue (e.g. food→20s solid light; fentanyl→20s 2Hz light). Rats underwent fentanyl self-administration 2h per day for 12 days prior to food vs fentanyl choice probe testing. In Experiment 2, rats had further fentanyl self-administration sessions for 12 more days after completing 6 choice tests.

Food vs fentanyl choice

To assess decision making for adaptive vs maladaptive reward, we tested rats using a food vs. fentanyl choice probe test. We placed rats in the operant chamber where they had to complete 4 forced choice trials, 2 for each reward. Only one of the two levers was presented and rats had 6 min to make a response, or else an omission was recorded from that trial. In this way, rats were able to sample each of the two rewards before free choice. After these 4 forced choice trials, both the food- and fentanyl-associated levers were available. Two consecutive presses on either lever yielded delivery of either a food pellet or fentanyl infusion, and the levers retracted into the chamber wall for a 6-min intertrial interval. Thus, choosing one reward comes at the expense of the other reward. We gave rats 8 free choice trials and then analyzed their choice behavior using percent of trials with drug chosen. After 4 choice probe tests, rats were food restricted to 85% free feeding weight and 2 more tests were repeated in the hungry state, to assess the impact of a motivational shift on choice behavior.

Fentanyl progressive ratio

After choice tests, rats were tested in progressive ratio for fentanyl, an assay of effort allocation. Rats were placed in the operant chamber and able to press for infusions of fentanyl. Rather than the previous FR2 requirement, each infusion required ~1.5x as many presses as the previous (e.g. 1, 2, 3, 5, 8., etc) and effort was determined using breakpoint, the last completed reinforcement requirement before session termination. Sessions lasted until a new response requirement was not met in 20 min.

Subthreshold/mild stressful event (one-shock test)

To assess the impact of the traumatic stressful event on future fear learning and reward seeking, all animals were given a 1-shock fear conditioning test. Rats were given one shock in their second context (either A or B, whichever was not the context where they received the prior traumatic

stressor). The shock (1 mA, 1s) was given 192 s after placement in the chamber, and animals were removed from the chambers 32 s later. Non-shocked controls were exposed to the chamber for an equivalent amount of time, 3 min 44 s, without shock. The following day, animals were given an 8 min 32 s context test in this second context.

Freezing during the 192 s baseline period prior to the single shock in the second context was assessed to rule out contextual fear generalization that may emerge from the shock pre-exposure, and the mean (±SEM) percentage of freezing was calculated. We detected minimal baseline freezing in the second context, with no group exceeding 10% freezing (not shown). Further analysis using ANOVA on these observations confirmed that shock pre-exposure in the first context had no effect on baseline freezing in the second context. Freezing was also assessed the day after the one-shock test, to confirm effective stress-enhanced fear learning produced by the earlier shock pre-exposure phase.

Reinstatement following subthreshold stressor

Immediately following the 1-shock test, rats were brought from the fear conditioning chambers to the operant chambers where they completed food and fentanyl self-administration training and were tested in reinstatement. Only the fentanyl-associated lever was available, and every 2 presses on the lever resulted in the fentanyl-associated cue and lever retraction, but no fentanyl infusion. The number of completed cycles of lever pressing/cue presentation was measured and a reinstatement score was calculated, by subtracting the lever press rate from the last extinction day from the reinstatement press rate.

Drugs

Fentanyl solution for self-administration was made by dissolving fentanyl citrate (Spectrum Chemicals) in saline to create a stock solution. Then, we weighed rats daily and used their body

weight to adjust the concentration of the solution for each animal, by adding an appropriate amount of saline to reach the target concentration. In this way, each rat received a 3.2 ug/kg/infusion over the same 2s delivery period, with heavier rats thus receiving a more concentrated, but dose-matched infusion compared to less heavy rats.

Data analysis

While we did detect sex differences in several assays, sex differences were not present in the majority of behaviors we assessed. Thus, results for most phases of the experiment were collapsed across sex for analysis, while results with sex difference include sex as a factor. Behavioral was analyzed with unpaired t-tests and ANOVA where applicable. A mixed-effects restricted maximum likelihood (REML) model was used in analyses where individual subject data points were lost due to technical errors. The Greenhouse-Geiser correction was applied in analyses where there was heterogeneity of variance across groups.

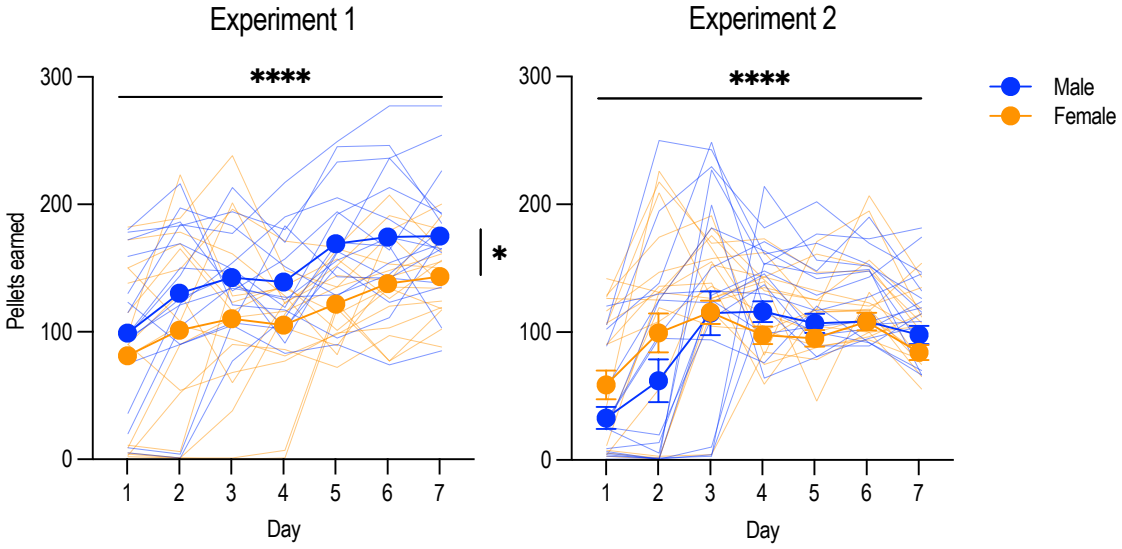


Figure 3.1. Food pellet self-administration. Rats were trained to self-administer food pellets made from purified rodent diet, a palatable reward. In experiment 1, the operant context used shared tactile features with the subsequent shock chambers used later in the project. Thus, rats in experiment 2 were trained in a more distinguishable operant context. Rats were placed in the operant chamber with a lever (left or right side) and lever pressing was reinforced with a purified food pellet on a fixed-ratio 2 schedule. Food delivery into the food-port magazine was accompanied by the presentation of a visual cue, a stimulus light over the lever that illuminated for 20s, either constantly lit or flashing at 2Hz. Over 7 days, rats acquired this instrumental association, with a sex difference in pellets earned detected in experiment 1 (Day: $F_{(2.851,85.54)}$ = 13.94, p <0.0001; Sex: $F_{(1,30)}$ = 5.519, p = 0.0256; Day x Sex: $F_{(6,180)}$ = 0.4442, p = 0.8484) that was not seen in experiment 2 (Day: $F_{(2.883,86.48)}$ = 15.50, p < 0.0001; Sex: $F_{(1,30)}$ = 0.0935, p = 0.7618; Day x Sex: $F_{(6,180)}$ = 3.084, p = 0.0067). **** p < 0.0001, Bonferroni correction.

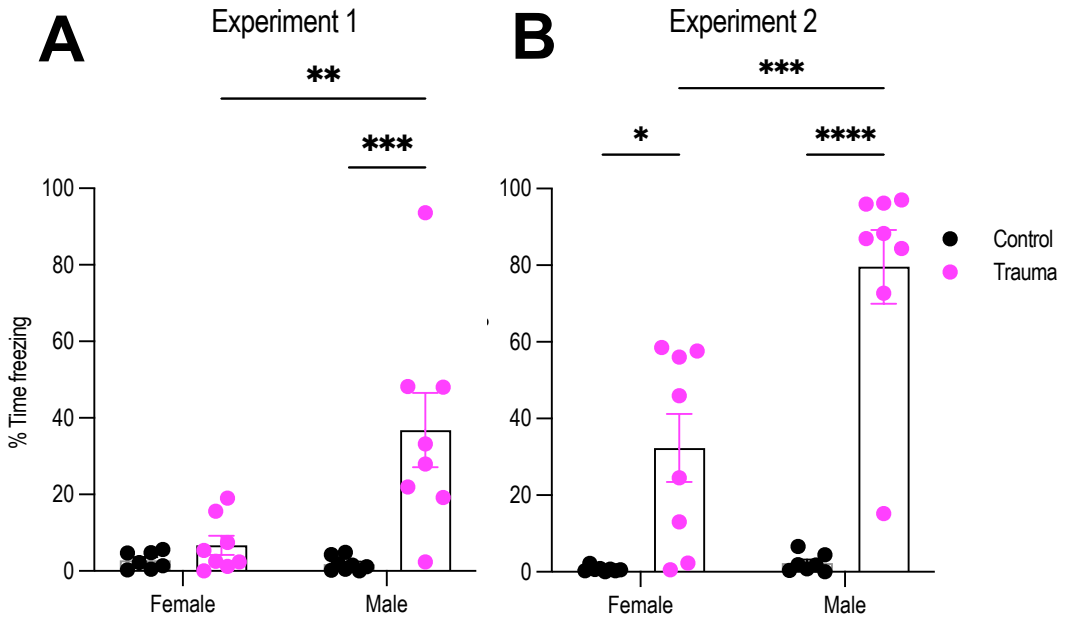


Figure 3.2. Exposure to traumatic stressor using the stress-enhanced fear learning protocol. After food self-administration training, rats were given a traumatic stressful experience. Rats were placed in shock chambers and given 15 1-second, 1mA unsignaled shocks in a 93 min session. Control subjects were placed in identical boxes and not shocked. The following day, rats were returned to the shock boxes where fear conditioning, to validate successful administration of the traumatic stressor, was assessed by analyzing freezing behavior over an 8 min session. In experiment 1, where animals received food training in operant boxes sharing similar tactile floors as the shock chambers prior to trauma, we did not detect freezing in female subjects, and found lower than expected levels of freezing in males (Sex: $F_{(1,27)} = 7.791$, $p = 0.0095$; Trauma: $F_{(1,27)} = 13.89$, $p = 0.0009$; Sex x Trauma: $F_{(1,27)} = 8.872$, $p = 0.0061$). Thus in experiment 2, we changed the floors of the operant context to be more distinct from the shock chambers, and found that rats in both sexes showed freezing to the trauma context, with males freezing more than females (Sex: $F_{(1,27)} = 12.89$, $p = 0.0013$; Trauma: $F_{(1,27)} = 64.14$, $p < 0.0001$; Sex x Trauma: $F_{(1,27)} = 11.29$, $p = 0.0023$). *$p < 0.05$, **$p < 0.01$, *** $p < 0.001$, ****$p < 0.0001$, Bonferroni correction.

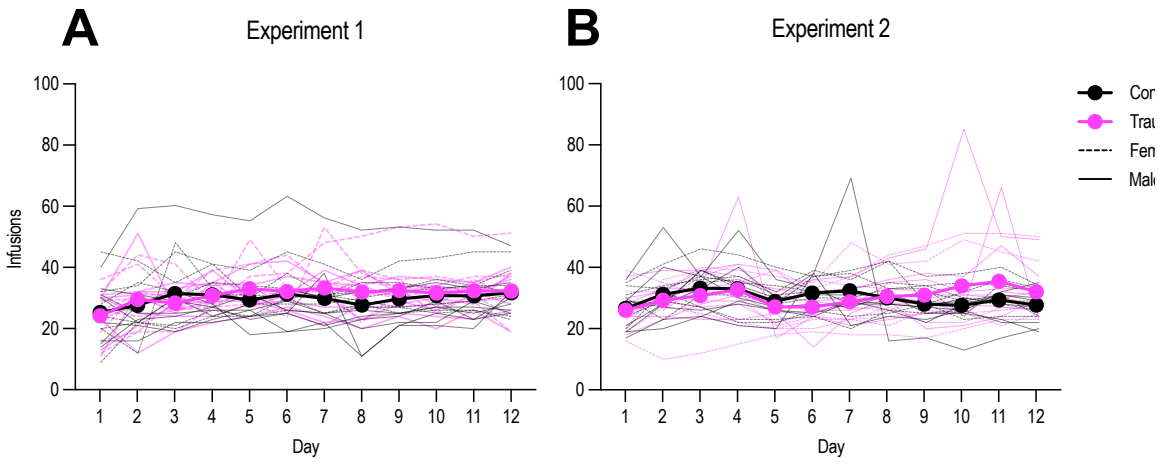


Figure 3.3. Intravenous fentanyl self-administration. Rats were trained to self-administer intravenous infusions of fentanyl in 2-hour sessions over 12 consecutive days in an operant context sharing features of the trauma context (**A**) or in an operant context distinct from the trauma context (**B**). In daily 2h sessions, rats were trained to lever-press for fentanyl on a fixed-ratio 2 schedule. The lever used was whichever lever was not used for food training (left vs right, counterbalanced), and infusion delivery was accompanied by 20s illumination of the stimulus light above the lever, either solid or flashing, again whichever was opposite the cue pattern in food training. All rats acquired fentanyl self-administration and demonstrated similar patterns of intake across experimental conditions, with no differences in self-administration behavior owing to trauma nor the different operant contexts across both experiment 1 (Day: $F_{(5.621,\ 163)} = 4.374$, $p = 0.0005$; Trauma: $F_{(1,29)} = 0.2796$, $p = 0.601$; Day x Trauma: $F_{(11,319)} = 1.192$, $p = 0.2913$) and experiment 2 (Day: $F_{(4.741,136.6)} = 2.763$, $p = 0.0227$; Trauma: $F_{(1,29)} = 0.0422$, $p = 0.8386$; Day x Trauma: $F_{(11,317)} = 2.866$, $p = 0.0013$). Individual performance for each subject is shown along with summary data for control and trauma conditions.

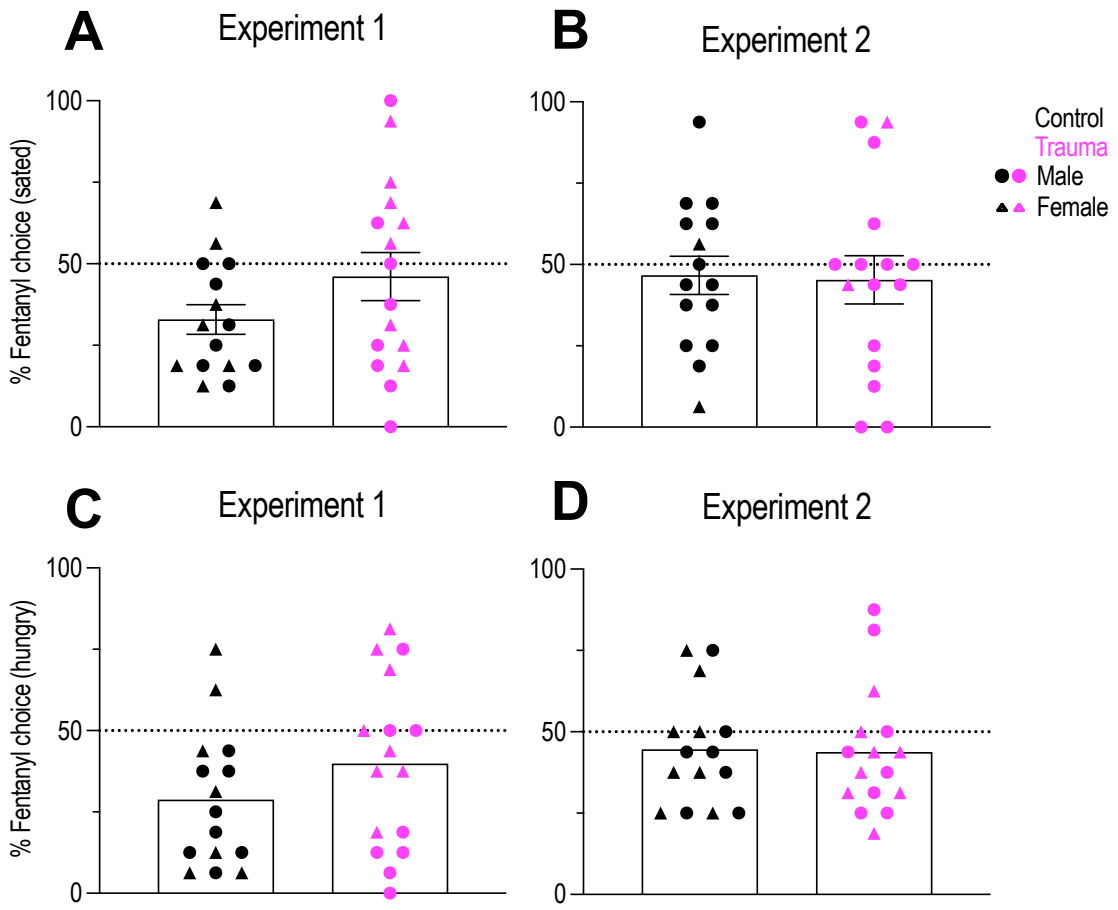
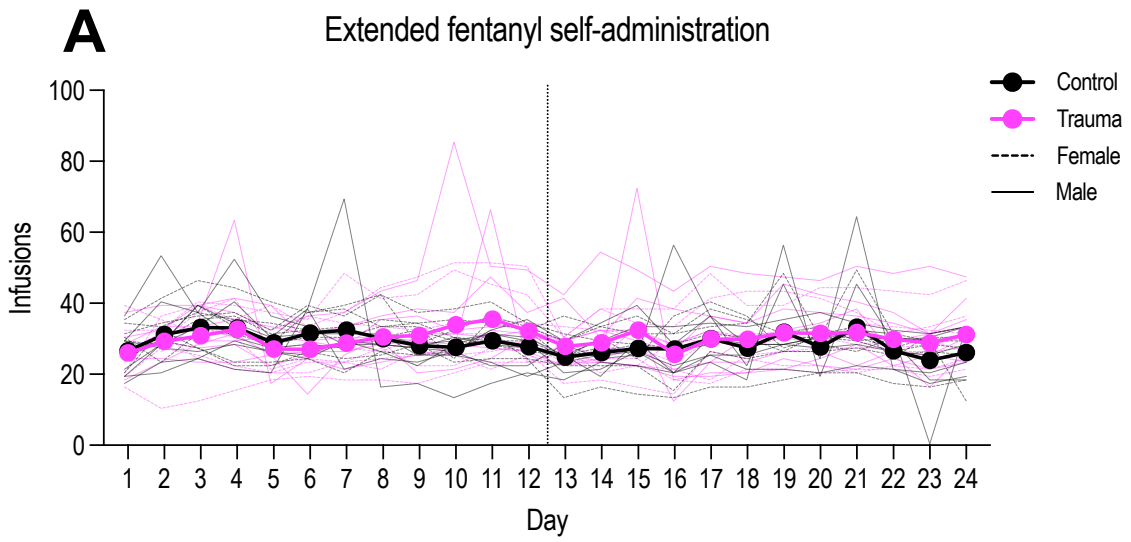


Figure 3.4. Food vs fentanyl choice tests. After learning to lever press for food pellets and fentanyl infusions, rats were given a series of choice tests to assess decision making for adaptive (food) vs maladaptive (fentanyl) rewards. Rats were placed in the operant chambers with both levers present, given 4 "forced choice" sampling trials, 2 per outcome, and then 8 free choice trials where 2 presses on either lever resulted in delivery of the associated outcome (food or fentanyl), followed by a 6-min intertrial interval. Prior trauma experience did not impact the choice behavior between food in fentanyl in experiment 1 (**A**) ($t_{(29)}$ = 1.498, p = 0.1449) or experiment 2 (**B**) ($t_{(29)}$ = 0.1417, p = 0.8883). We then food restricted animals to 85% free feeding weight and repeated the choice tests in the hungry state. Interestingly, this shift in motivational state did not change food vs fentanyl decision making behavior (**C-D**). We did not detect any differences in choice behavior between trauma and control conditions in either experiment 1 ($t_{(29)}$ = 1.284, p = 0.2094) or experiment 2 ($t_{(29)}$ = 0.1237, p = 0.9024).



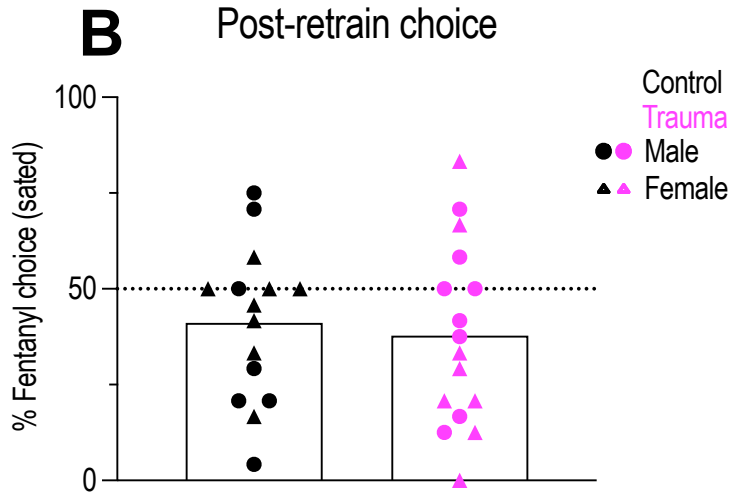


Figure 3.5. Extended fentanyl self-administration experience and choice re-testing. After completing 12 days of fentanyl self-administration training and food vs fentanyl choice tests, we gave rats in experiment 2 extended fentanyl experience for 12 additional days (**A**). Despite this extended training, we did not observe any changes in fentanyl taking across any of the experimental conditions (day: $F_{(7.028, 202.9)} = 3.565$, $p = 0.0012$; trauma: $F_{(1,29)} = 0.4005$, $p = 0.5318$; day x trauma: $F_{(23,664)} = 2.247$, $p = 0.0008$). Similarly, we did not detect any differences in choice behavior upon repeating the food vs fentanyl choice test following extended opioid self-administration training (**B**) ($t_{(29)} = 0.4209$, $p = 0.6769$).

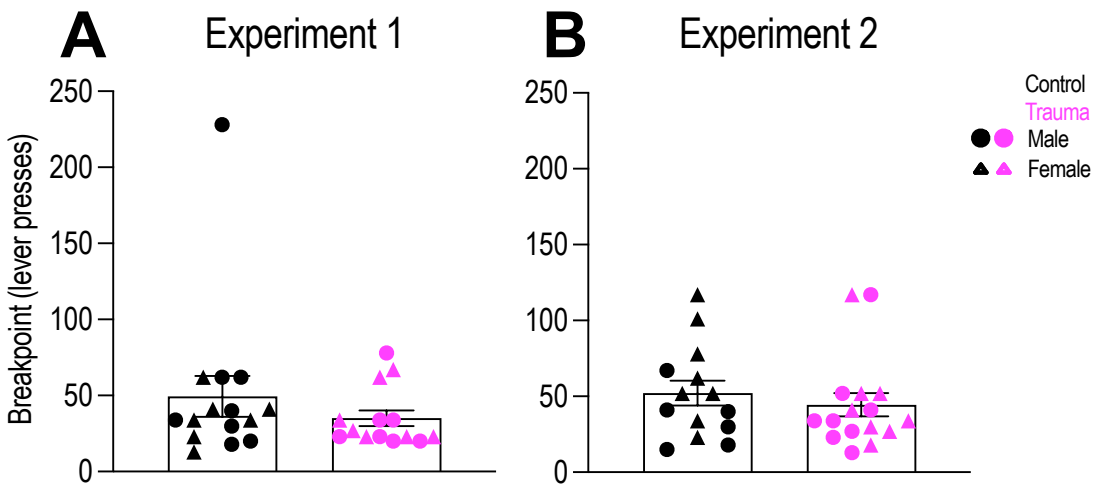


Figure 3.6. Effort allocation for fentanyl. Trauma does not impact willingness to exert effort for fentanyl. We tested rats' willingness to work for fentanyl infusions using a progressive ratio schedule of reinforcement wherein each infusion required 50% more presses than the prior infusion. Breakpoint represents the last completed reinforcement requirement, i.e. the number of presses the final fentanyl infusion required for each subject. We did not detect any effects of prior trauma on effort allocation as determined by breakpoint in experiment 1 (**A**) ($t_{(27)}$ = 0.9775, p = 0.337) or experiment 2 (**B**) ($t_{(28)}$ = 0.6843, p = 0.4994).

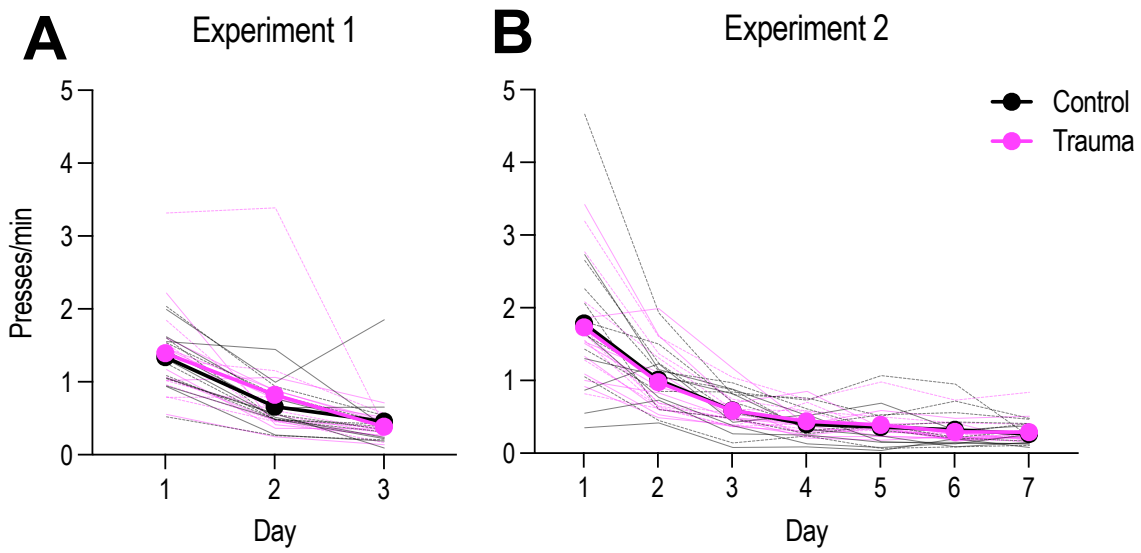


Figure 3.7. Extinction of fentanyl responding. Trauma does not alter extinction of fentanyl responding. Rats were placed in the operant chamber for a 1hr extinction session where presses on the fentanyl-associated lever were measured but had no programmed consequences. Because of extended fentanyl training in experiment 2, we gave 4 more sessions of extinction than in experiment 1. Fentanyl responding extinguished in all animals, and we did not detect any differences across the experimental conditions in experiment 1 (**A**) (Day: $F_{(1.776, 51.50)} = 53.91$, $p < 0.0001$; Trauma: $F_{(1,29)} = 0.1128$, $p = 0.7393$; Day x Trauma: $F_{(2,58)} = 0.8025$, $p = 0.4531$) or experiment 2 (**B**) (Day: $F_{(1.509, 42.24)} = 71.45$, $p < 0.0001$; Trauma: $F_{(1,28)} = 0.0013$, $p = 0.9719$; Day x Trauma: $F_{(6,168)} = 0.0803$, $p = 0.998$).

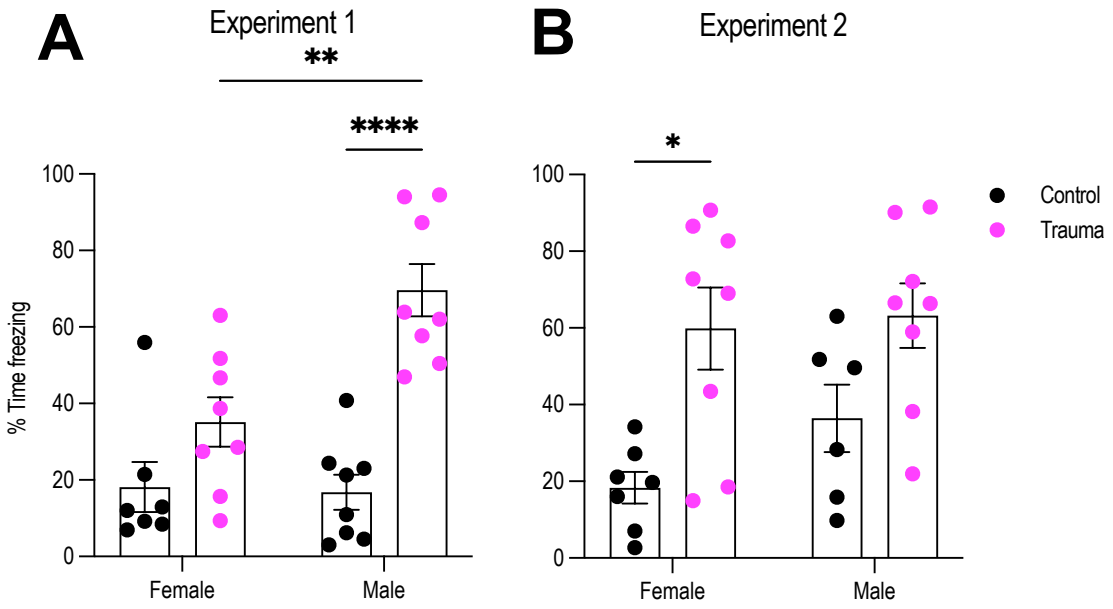


Figure 3.8. Subthreshold stressor exposure phase of stress-enhanced fear learning: one-shock test. After extinction of fentanyl responding, animals were exposed to a mild stressor as part of the SEFL paradigm. Rats were returned to shock chambers, though these chambers featured contextual differences to distinguish them from the original trauma context (context A vs B, counterbalanced across sex and trauma conditions), and administered 1 1-second, 1mA shock during a 4-min session. The rats were returned to the one-shock context the following day and freezing behavior was measured. Rats in experiment 1, with similar operant and trauma contexts, showed freezing in the one-shock context, and this was enhanced in animals that had experienced trauma prior, with trauma-exposed males freezing more than trauma-exposed females (Sex: $F_{(1,27)} = 7.163$, $p = 0.0125$; Trauma: $F_{(1,27)} = 31.89$, $p < 0.0001$; Sex x Trauma: $F_{(1,27)} = 8.398$, $p = 0.0074$). Rats in experiment 2 with the distinct operant and shock contexts also showed freezing in the one-shock context, with trauma-exposed animals freezing more than controls (Sex: $F_{(1,25)} = 1.542$, $p = 0.2259$; Trauma: $F_{(1,25)} = 15.55$, $p = 0.0006$; Sex x Trauma: $F_{(1,25)} = 0.7212$, $p = 0.4038$). *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$, Bonferroni correction.

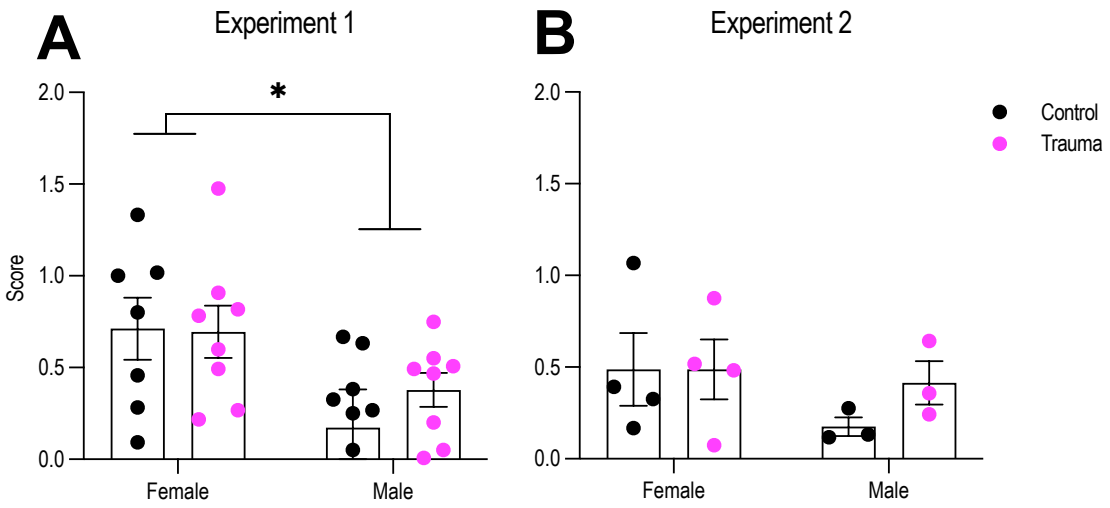


Figure 3.9: Fentanyl cue-induced reinstatement following mild stressor. Immediately following the one-shock test phase of the SEFL paradigm, rats were moved from the shock chambers to the operant chambers for fentanyl cue-induced reinstatement. The fentanyl lever was available in the chamber, and every 2 presses was reinforced with the fentanyl visual cue and lever retraction, but no fentanyl infusion. Reinstatement was measured using a reinstatement score, calculated by subtracting the lever press rate from the final fentanyl extinction session from the reinstatement press rate. While we did not detect any effects of prior trauma on reinstatement following the mild stressor, we did detect a sex difference in experiment 1 (Sex: $F_{(1,27)} = 7.273$, $p = 0.0119$; Trauma: $F_{(1,27)} = 0.3545$, $p = 0.5565$; Sex x Trauma: $F_{(1,27)} = 0.4944$, $p = 0.488$). However, this was not observed in experiment 2 (Sex: $F_{(1,10)} = 1.428$, $p = 0.2597$; Trauma: $F_{(1,10)} = 0.5466$, $p = 0.4767$; Sex x Trauma: $F_{(1,10)} = 0.5466$, $p = 0.4767$). *$p < 0.05$, Bonferroni correction.

Chapter 4: General discussion

Here I investigated the behavioral and neural processes underlying adaptive decision making and developed a new behavioral model to study how animals arbitrate between adaptive and maladaptive rewards in the context of psychiatric disease. In chapter 2, I found that projections between the ACC and BLA are critical for distinct aspects of reward pursuit. Chemogenetic inactivation during memory retrieval tasks revealed that projections from the BLA→ACC may be needed for stimuli to generate reward value expectations to guide behavior, but not for using stimuli to guide the appropriate reward-earning action. Similarly, projections from ACC→BLA are also needed for stimuli to generate reward value expectations, and not for selecting the appropriate action.

In separate work described in chapter 3, I hypothesized that PTSD would interact with opioid learning and decision making mechanisms to augment opioid self-administration behavior and potentially produce OUD-like phenotypes. Surprisingly, despite the known high comorbidity between PTSD and OUD, using a number of canonical opioid self-administration assays and a novel decision making task, I found that PTSD modeled using the stress-enhanced fear learning (SEFL) model does not cause any differences in opioid learning, decision making between drug and nondrug rewards, extinction of drug pursuit, effort allocation for opioids, or reinstatement to opioid seeking following extinction.

BLA→ACC projections enable stimuli to retrieve reward value memories to guides reward pursuit

Chemogenetic inactivation of BLA→ACC projections during outcome-specific Pavlovian-to-instrumental transfer (specific PIT) did not produce significant deficits in using environmental stimuli to make reward pursuit decisions. During the PIT tests, animals increased lever pressing

on the lever that would earn the same food outcome as was also signaled by the stimulus presented. However, subjects in the control condition also elevated responding on the alternate lever, something not observed in the BLA→ACC inactivation condition. The lack of a clear expression of specific PIT in the control condition is unexpected, but potentially suggests that BLA→ACC inactivation enhanced PIT, which may provide more insight into how the pathway contributes to appetitive behavior. It is also possible that both the behavioral variability in this task and the fact that the control group was underpowered to detect significant differences between pressing the same vs different levers during a given CS led to controls not showing PIT. All animals in this experiment received intracranial infusions of CNO that necessitate restraining the animal for a period of ~ 3-4 minutes that could potentially cause stress. Signs of stress during and following all intracranial infusions were monitored and any animals experiencing visible distress were removed from analyses, though we did not detect such stress in this experiment. Animals are also given ~5-10 min post-infusion for the drug to take effect, thus also providing some time for acute stress to dissipate before engaging in the task. Nevertheless, it is possible that undetectable stress experienced by some subjects contributed to variability and the difficulty in detecting effects in the control condition.

We also tested the role of BLA→ACC activity in using stimuli to generate reward value expectations needed for adaptive control of behavior. While not needed to express PIT, we detected that the Pavlovian conditioned response was insensitive to devaluation of the outcome associated with each stimulus when BLA→ACC activity was inhibited. Surprisingly, we observed that animals in the BLA→ACC inactivation condition showed overall higher levels of reward-directed responding, in that they spent more time in the food-port magazine than control subjects in all phases of the test (both at baseline and during the stimuli). While behavior in this task is normally highly variable and this may contribute to the effect, it is possible that BLA→ACC inactivation disinhibited responding in a way that is meaningful when considering the role of the

pathway in reward-related behavior. ACC inactivation during the 5-choice serial reaction time test, a test of attentional control, produces significant increases in impulsive responding (Robbins, 2002), and in effortful choice tasks promotes premature reward responding, i.e. when rewards are not available (Hosking et al., 2014), another measure of impulsivity. It is therefore possible that the activity of BLA inputs into the ACC exerts inhibitory control over behavior in response to stimuli, and their inactivation increased responding regardless of the CS-period of the task. Though we did not detect such increases in responding during PIT, perhaps this is due to distinct psychological processes occurring within each task and may give insight into the nuances of this circuitry. In Pavlovian devaluation, the behavioral options are to approach the food port, or not to, whereas in PIT, the options are to arbitrate between two specific instrumental actions and to approach the food port or not. The relative simplicity of Pavlovian devaluation, absent consideration of instrumental actions, thus might encourage impulsive responding in ways that the specific PIT task does not.

ACC→BLA projections enable using environmental stimuli to generate reward value expectations

We have shown that activity in ACC→BLA projections is not necessary for using stimulus-outcome memories for action selection needed to express PIT, but is critical for using Pavlovian stimuli to retrieve a mental representation of the value of a specific rewarding outcome. During the devaluation probe test, rats elevated responding to both reward-paired CSs, regardless of the value of the outcome associated with them. While critical for using stimuli to make reward value predictions, we did not find that ACC→BLA projections contribute to these reward valuations when considering instrumental reward-directed actions. Despite inactivating ACC→BLA projections, rats were able to use the recently changed outcome value to selectively reduce lever pressing for only one of the two rewards, i.e. demonstrating sensitivity to devaluation. Taken together with our

data, this suggests that the ACC and BLA may directly collaborate within their roles in appetitive behavior to enable stimulus-generated reward value expectations.

These findings complement other results from our lab and others investigating how the BLA works with other cortical regions to maintain adaptive behavioral control, adding more information to the circuit mapping of appetitive behavior. It has recently been shown that direct projections from the ACC→BLA control devaluation for water rewards following selective caspase ablation of the pathway (Yuan et al., 2023). Additionally, we have previously found that ascending projections from the BLA to other cortical regions such as lateral orbitofrontal cortex (lOFC) and medial orbitofrontal cortex (mOFC) are needed to communicate value information when animals must use reward-paired stimuli to guide behavior (Lichtenberg et al., 2017; Lichtenberg et al., 2021). While inputs from the nearby medial OFC into the BLA have been demonstrated as critical for using stimuli to select reward-seeking actions (Lichtenberg et al., 2021), ACC projections to the BLA investigated here are not needed for action selection. Understanding this nuance of medial prefrontal-amygdala contributions to behavior is essential for a complete understanding of the neural circuits controlling appetitive behavior.

The interrogation of ACC→BLA projections resulted in a number of unexpected aversive consequences that constrain the interpretation of the results. We found in many animals that intra-BLA infusion of CNO may have contributed to deficits in PIT and outcome value sensitivity both in controls and hM4Di-expressing subjects. Several subjects exhibited severe malaise within 24 hours of a CNO infusion, and any animals with this profile were removed from analyses. Fortunately, no animals in the BLA→ACC pathway experienced any untoward effects of CNO infusion, leading us to believe that the BLA as an infusion target was problematic, given that the same CNO batch was used across all animals. Because of this, many subjects in the ACC→BLA group were only able to complete 1 of each PIT and outcome-specific devaluation test, vs 2 of

each in the BLA→ACC group and our prior work. Completing 2 of each test allows for proper counterbalancing of test order with respect to the stimulus-outcome and action-outcome relationships, and first outcome devalued, that allows for any potential stimulus- or outcome-specific effects (e.g. salience, preference) on behavior to be compensated for across both tests. Because so few animals in the ACC→BLA group were able to complete 2 iterations of each test, the group was underpowered to detect any effects by using data from both of each test, so we chose to analyze and present data only from the first of each test. Thus, the imperfect counterbalancing may contribute to variability in behavior and may explain why control subjects were not able to execute the behaviors as we have seen in our prior work.

Further, it is possible that tissue damage that did not affect observable wellness occurred in some subjects, and this could have impacted behavior in unknown ways. Upon histological analysis, some subjects (including those removed from analyses) had ventricular swelling that certainly caused intracranial pressure on many regions including the BLA itself. This likely disrupted both BLA function specifically and the general well-being of the rats, both of which understandably would disrupt normal performance of behaviors in controls and experimental animals. Finally, it is also possible the inability to detect successful Pavlovian devaluation is also owing to the low number of subjects in the control condition. Taken together, intra-BLA infusion of CNO (and likely other compounds) may produce a series of undesirable effects that preclude understanding of behavioral results. Further investigation into this phenomenon in the ACC→BLA pathway using alternative manipulation methods, e.g. a dual-viral intersectional approach with systemic CNO administration or an optical manipulation approach, will shed more light on the role of this pathway in stimulus-driven reward seeking

Stress-enhanced fear learning as a model for PTSD in the context of comorbid PTSD-OUD

Here we investigated how PTSD impacts learning, memory, and decision making for opioid drugs using a rodent model of PTSD combined with fentanyl self-administration paradigms. We also developed a novel choice test for assessing how rats make decisions between adaptive and maladaptive rewards. Surprisingly, we did not detect effects of trauma on any of the opioid behaviors we studied here. Nevertheless, these findings contribute many insights into how best to model comorbid PTSD and OUD in rodents, and considerations for future experimental design.

We considered several factors when selecting the combination and order of behavioral paradigms to assess interactions between PTSD and OUD. While there are multiple animal models of PTSD, we selected SEFL because it captures many aspects of human PTSD, and the behavioral and neurobiological consequences of the SEFL paradigm are quite well understood (Perusini et al., 2016), giving insight into what we might expect to see during opioid behavioral training. Yet, we did not detect any effects of the trauma induced by the SEFL paradigm on any opioid-related behaviors. While SEFL has replicated numerous aspects of human PTSD in animals, no single model can fully capture every aspect. It is possible that the aspects of PTSD that facilitate the development of OUD are not the same aspects that SEFL reproduces. Thus, other models of traumatic stress may be better suited for understanding how PTSD facilitates the transition from casual opioid use to OUD.

The order that animals experienced different phases of the experiment may also contribute to our results. We carefully considered the order animals would experience different phases of training and how each training experience could affect subsequent assays. We selected to give rats operant training for food pellets prior to experiencing trauma, because we were concerned that trauma might have impeded the acquisition of food self-administration behavior. Further, this order enabled us to test the impact of prior appetitive training on the experience of the trauma

phase of the SEFL model. Indeed, the first group of animals we ran (Experiment 1) trauma-exposed subjects froze much less following the 15-shock exposure session than other research groups have found using SEFL (Perusini et al., 2016; Rajbhandari et al., 2018; Rau et al., 2005). We then hypothesized that the wire-grid floors of the operant chamber where food self-administration occurred were too similar to the wire-grid floors in the shock boxes, and that rats may have generalized the safety of the operant context to the shock context, reducing freezing. Thus, in Experiment 2, we replaced the wire-grid floors of the operant boxes with Plexiglas floors, to further distinguish the appetitive and aversive contexts rats would be trained in. This change produced levels of freezing similar to what we had expected to see in the prior iteration of the experiment, thus indicating to us that distinct contexts for appetitive and aversive behavior are needed when combining SEFL with reward-related paradigms. We also did not counterbalance order of food training vs fentanyl training first, to ensure optimal catheter patency once surgeries were completed. It is possible that drug self-administration prior to trauma would have different effects on SEFL than food self-administration.

Despite very low levels of freezing in Experiment 1 animals, we continued training them through the rest of the behavioral assays to test whether the trauma exposure still produced any effects on behavior, absent the expression of freezing in the trauma context test. Interestingly, trauma-exposed animals showed the enhancement of fear learning expected after the 1-shock mild stressor exposure, despite not showing strong evidence of fear conditioning the day after trauma. This suggests a few possibilities, including that freezing during the initial trauma context test is not necessarily predictive of behavior in the one-shock context, and/or perhaps experience with fentanyl altered how animals respond to the one-shock mild stressor. It is also worth noting that animals in this project received the one-shock stressor 25 days (Experiment 1) or 48 days (Experiment 2) after trauma exposure, supporting the assertion that the SEFL procedure induces long-lasting sensitization of fear, even after fentanyl exposure.

Another key decision that may have impacted results was the selection of a short-access 2-hour schedule for fentanyl self-administration. This was selected to enable high-throughput behavioral training and testing, and due to limitations on accessing shared laboratory equipment. While short-access schedules are not typically shown to induce drug dependence or addiction-like behaviors, the design allowed us to detect potential effects of trauma that overcome the aspects of shorter access schedules that normally prevent development of dependence criteria. In attempt to overcome the lack of effects on opioid self-administration in Experiment 1, we gave rats in Experiment 2 extended fentanyl self-administration training, to test if more experience with fentanyl taking would enable effects of trauma to emerge both on self-administration behavior and decision making for food vs fentanyl. Despite this extra training, we still did not detect any effects of trauma. It is possible that longer schedules of access that produce OUD phenotypes, including continuous long access (6-23h sessions) or intermittent access (many short bins of drug access separated by long timeouts over 6-12 hours) (Ahmed, 2018), may be better tools for understanding interactions between PTSD and OUD behavior and neurobiology. It is possible that trauma could change the development and experience of dependence and withdrawal, and that these effects could explain the comorbidity observed in humans, thus using longer access schedules that are known to produce addiction-like phenotypes may be better for these questions.

Finally, we expected that opioid behavior after a PTSD-inducing trauma would be most sensitive to trauma effects following experience of a mild stressor. Further, we were interested in this from a translational perspective, in that acute experiences of stress can motivate drug pursuit (Mantsch et al., 2016), and trauma may enhance this. We thus chose to test how the mild stressor would affect reinstatement of drug seeking after the behavior had been extinguished. While we did not detect effects of trauma itself, we did detect a sex difference in that females showed more reinstatement, in line with prior work showing that females are more susceptible to reinstatement

than males after short-access fentanyl training (Malone et al., 2021). Some other studies have also found females reinstate to opioid seeking more than males (D'Ottavio et al., 2023; Fulenwider et al., 2020), yet those results are confounded by females consuming more opioids during training than males, potentially due to stress experienced during estrous cycle monitoring that is not possible to control for in males. Technical problems resulted in losing reinstatement data for half the subjects in Experiment 2, which precluded detecting effects that may have been present. Furthermore, it is possible that giving the one-shock treatment and test prior to a different phase of the experiment may have yielded different results. For example, it would be interesting to test food vs fentanyl decision making after the one-shock exposure, as the process is more cognitively complex than reinstatement, with our choice model requiring reward comparisons and cost-benefit analyses to complete. Thus, it is possible that the effects of trauma interacting with a mildly stressful experience could have emerged during decision making. Taken together, by altering some parameters of the experimental design used in this project, it is possible that a comprehensive battery of behavioral paradigms can be used together in a way that captures how PTSD facilitates OUD to then understand the intersectional neurobiology of both disorders.

Conclusions and future directions

This work has demonstrated a new role for the bidirectional projections between the BLA and the ACC. Using pathway-specific chemogenetic manipulation, I have shown that ACC inputs to the BLA and the reciprocal BLA inputs to the ACC may both be important for generating reward value expectations to guide behavior. Now having identified a causal role for these pathways in stimulus-guided appetitive behavior, there are several exciting avenues of research that naturally follow. Monitoring endogenous activity of neurons that comprise these pathways during complex behavioral paradigms that leverage learning theory can provide insight into the nuanced contribution of the circuit to adaptive behavior. Such data will add to our understanding of the neural underpinnings of the specific psychological processes used to maintain adaptive control of

reward pursuit. Bulk calcium fiber photometry of genetically- and projection-defined cell types can provide information about the population activity of groups of cells in a region during specific behavioral events, while miniscope calcium imaging can track the contributions of individual cells to understand how different cells within the same region may act differently to achieve the function of the studied pathway (Siciliano & Tye, 2019).

Because the BLA acts as a hub for both adaptive and maladaptive reward learning and memory (Wassum & Izquierdo, 2015), these findings can be used to construct a more thorough map of the neural circuits underlying appetitive behavior, for example, how VTA-dopamine input into the BLA modulates ACC activity during reward valuation, or how ACC inputs into the BLA modulate striatal activity to guide action execution. Along with their roles in adaptive behavior, both the BLA and ACC have also been implicated in opioid learning and memory (Cai et al., 2013; Hou et al., 2020). Further, while the ACC has some mu-opioid receptor expression, the BLA is highly enriched with mu-opioid receptors (Mansour et al., 1994) and the roles of the BLA in reward learning and memory are dependent on mu-opioid receptor signaling (Lichtenberg & Wassum, 2017; Wassum et al., 2011), providing a potential neural substrate for maladaptive decision making after opioid exposure. Further investigation of how these regions, and the direct connections between them, contribute to opioid behavior can also add to our understanding of maladaptive decision making, especially after experiencing trauma. To do this, we first need better models of dynamic adaptive vs maladaptive decision making so that specific neurobiological hypotheses can be tested.

Similarly, more work investigating the impact of trauma, namely PTSD, on opioid learning and decision making is needed to understand the behavioral factors that facilitate the transition from casual opioid use to OUD in people with PTSD. Further, understanding how PTSD changes the reward neural circuitry to change the response to opioids, and facilitate OUD development, could

prove essential in developing targeted pharmacotherapies to treat, and perhaps prevent, OUD in trauma and PTSD patients. By using a different stress induction protocol such as chronic unpredictable stress or single prolonged stress (Borghans & Homberg, 2015), and/or more extended access to opioids, behavioral models of adaptive vs maladaptive choice have potential to capture decision making deficits seen in OUD, and further, the neurobiological changes that occur to cause behavior to become maladaptive. Using circuit mapping of the healthy brain in reward learning and decision making, we can understand the precise ways that different substances of abuse, and experiences of trauma, hijack normal brain systems to cause different psychiatric symptoms and disorders to emerge. It is therefore necessary to integrate the rich literature of basic systems neuroscience and associative learning theory with models of substance use disorders. Causal evidence from these preclinical lines of research is essential for developing clinical behavioral and pharmacological interventions to improve the lives of people living with substance use disorders and posttraumatic stress disorder.

References

Adams, C., & Dickinson, A. (1981). Actions and habits: Variations in associative representations during instrumental learning. *Information processing in animals: Memory mechanisms*, 143-165.

Ahmed, S. H. (2018). Trying to make sense of rodents' drug choice behavior. *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, *87*, 3-10.

Allman, J. M., Hakeem, A., Erwin, J. M., Nimchinsky, E., & Hof, P. (2001). The anterior cingulate cortex: the evolution of an interface between emotion and cognition. *Annals of the New York Academy of Sciences*, *935*(1), 107-117.

Allman, M. J., DeLeon, I. G., Cataldo, M. F., Holland, P. C., & Johnson, A. W. (2010). Learning processes affecting human decision making: An assessment of reinforcer-selective Pavlovian-to-instrumental transfer following reinforcer devaluation. *Journal of Experimental Psychology: Animal Behavior Processes*, *36*(3), 402.

Badger, G. J., Bickel, W. K., Giordano, L. A., Jacobs, E. A., Loewenstein, G., & Marsch, L. (2007). Altered states: The impact of immediate craving on the valuation of current and future opioids. *Journal of health economics*, *26*(5), 865-876.

Balleine, B., & Dickinson, A. (1991). Instrumental performance following reinforcer devaluation depends upon incentive learning. *The Quarterly Journal of Experimental Psychology*, *43*(3), 279-296.

Balleine, B. W., Killcross, A. S., & Dickinson, A. (2003). The effect of lesions of the basolateral amygdala on instrumental conditioning. *J Neurosci*, *23*(2), 666-675.

Balleine, B. W., & O'doherty, J. P. (2010). Human and rodent homologies in action control: corticostriatal determinants of goal-directed and habitual action. *Neuropsychopharmacology*, *35*(1), 48-69.

Balleine, B. W., & Ostlund, S. B. (2007). Still at the choice-point: action selection and initiation in instrumental conditioning. *Annals of the New York Academy of Sciences*, *1104*(1), 147-171.

Baxter, M. G., & Murray, E. A. (2002). The amygdala and reward. *Nat Rev Neurosci*, *3*(7), 563-573. https://doi.org/10.1038/nrn875

Biernacki, K., Lopez-Guzman, S., Messinger, J. C., Banavar, N. V., Rotrosen, J., Glimcher, P. W., & Konova, A. B. (2022). A neuroeconomic signature of opioid craving: How fluctuations in craving bias drug-related and nondrug-related value. *Neuropsychopharmacology*, *47*(8), 1440-1448.

Blundell, P., Hall, G., & Killcross, S. (2001). Lesions of the basolateral amygdala disrupt selective aspects of reinforcer representation in rats. *Journal of Neuroscience*, *21*(22), 9018-9026.

Borghans, B., & Homberg, J. R. (2015). Animal models for posttraumatic stress disorder: an overview of what is used in research. *World journal of psychiatry*, *5*(4), 387.

Boscarino, J. A., Hoffman, S. N., & Han, J. J. (2015). Opioid-use disorder among patients on long-term opioid therapy: impact of final DSM-5 diagnostic criteria on prevalence and correlates. *Substance abuse and rehabilitation*, 83-91.

Bush, G., Luu, P., & Posner, M. I. (2000). Cognitive and emotional influences in anterior cingulate cortex. *Trends in cognitive sciences*, *4*(6), 215-222.

Cai, X., & Padoa-Schioppa, C. (2012). Neuronal encoding of subjective value in dorsal and ventral anterior cingulate cortex. *Journal of Neuroscience*, *32*(11), 3791-3808.

Cai, Y.-Q., Wang, W., Hou, Y.-Y., Zhang, Z., Xie, J., & Pan, Z. Z. (2013). Central amygdala GluA1 facilitates associative learning of opioid reward. *Journal of Neuroscience*, *33*(4), 1577-1588.

Campbell, U. C., & Carroll, M. E. (2000). Acquisition of drug self-administration: environmental and pharmacological interventions. *Experimental and Clinical Psychopharmacology*, *8*(3), 312.

Cao, B., Wang, J., Zhang, X., Yang, X., Poon, D. C.-H., Jelfs, B.,…Li, Y. (2016). Impairment of decision making and disruption of synchrony between basolateral amygdala and anterior cingulate cortex in the maternally separated rat. *Neurobiology of learning and memory*, *136*, 74-85.

Cardinal, R. N., Parkinson, J. A., Hall, J., & Everitt, B. J. (2002). Emotion and motivation: the role of the amygdala, ventral striatum, and prefrontal cortex. *Neurosci Biobehav Rev*, *26*(3), 321-352.

Cardinal, R. N., Parkinson, J. A., Marbini, H. D., Toner, A. J., Bussey, T. J., Robbins, T. W., & Everitt, B. J. (2003). Role of the anterior cingulate cortex in the control over behavior by Pavlovian conditioned stimuli in rats. *Behav Neurosci*, *117*(3), 566-587. https://doi.org/10.1037/0735-7044.117.3.566

Carmack, S. A., Vendruscolo, J. C., Adrienne McGinn, M., Miranda-Barrientos, J., Repunte-Canonigo, V., Bosse, G. D.,…Hinrich, A. J. (2022). Corticosteroid sensitization drives opioid addiction. *Molecular Psychiatry*, *27*(5), 2492-2501.

Cartoni, E., Balleine, B., & Baldassarre, G. (2016). Appetitive Pavlovian-instrumental transfer: a review. *Neuroscience & Biobehavioral Reviews*, *71*, 829-848.

Chilcoat, H. D., & Menard, C. (2003). Epidemiological investigations: Comorbidity of posttraumatic stress disorder and substance use disorder.

Clark, J. J., Hollon, N. G., & Phillips, P. E. (2012). Pavlovian valuation systems in learning and decision making. *Current opinion in neurobiology*, *22*(6), 1054-1061.

Clark, J. J., Hollon, N. G., & Phillips, P. E. (2012). Pavlovian valuation systems in learning and decision making. *Curr Opin Neurobiol*, *22*(6), 1054-1061. https://doi.org/10.1016/j.conb.2012.06.004

Corbit, L. H., & Balleine, B. W. (2005). Double dissociation of basolateral and central amygdala lesions on the general and outcome-specific forms of pavlovian-instrumental transfer. *J Neurosci*, *25*(4), 962-970. https://doi.org/10.1523/JNEUROSCI.4507-04.2005

D'Ottavio, G., Reverte, I., Ragozzino, D., Meringolo, M., Milella, M. S., Boix, F.,…Caprioli, D. (2023). Increased heroin intake and relapse vulnerability in intermittent relative to continuous self-administration: Sex differences in rats. *British journal of pharmacology*, *180*(7), 910-926.

Dahlby, L., & Kerr, T. (2020). PTSD and opioid use: implications for intervention and policy. *Substance abuse treatment, prevention, and policy*, *15*, 1-4.

Danovitch, I. (2016). Post-traumatic stress disorder and opioid use disorder: A narrative review of conceptual models. *Journal of addictive diseases*, *35*(3), 169-179.

Delamater, A. R. (2007). The Role of the Orbitofrontal Cortex in Sensory-Specific Encoding of Associations in Pavlovian and Instrumental Conditioning. *Annals of the New York Academy of Sciences*, *1121*(1), 152-173.

Delamater, A. R., & Oakeshott, S. (2007). Learning about multiple attributes of reward in Pavlovian conditioning. *Annals of the New York Academy of Sciences*, *1104*(1), 1-20.

Deroche, V., Piazza, P. V., Casolini, P., Maccari, S., Le Moal, M., & Simon, H. (1992). Stress-induced sensitization to amphetamine and morphine psychomotor effects depend on stress-induced corticosterone secretion. *Brain research*, *598*(1-2), 343-348.

Domjan, M. (2005). Pavlovian conditioning: A functional perspective. *Annu. Rev. Psychol.*, *56*, 179-206.

Elman, I., Upadhyay, J., Langleben, D. D., Albanese, M., Becerra, L., & Borsook, D. (2018). Reward and aversion processing in patients with post-traumatic stress disorder: functional neuroimaging with visual and thermal stimuli. *Translational psychiatry*, *8*(1), 240.

Estes, W. K. (1943). Discriminative conditioning. I. A discriminative property of conditioned anticipation. *Journal of Experimental Psychology*, *32*(2), 150.

Estes, W. K. (1948). Discriminative conditioning. II. Effects of a Pavlovian conditioned stimulus upon a subsequently established operant response. *Journal of experimental psychology*, *38*(2), 173.

Everitt, B. J., Cardinal, R. N., Parkinson, J. A., & Robbins, T. W. (2003). Appetitive behavior: impact of amygdala-dependent mechanisms of emotional learning. *Annals of the new York Academy of Sciences*, *985*(1), 233-250.

Fareed, A., Eilender, P., Haber, M., Bremner, J., Whitfield, N., & Drexler, K. (2013). Comorbid posttraumatic stress disorder and opiate addiction: a literature review. *Journal of addictive diseases*, *32*(2), 168-179.

Fareri, D. S., Martin, L. N., & Delgado, M. R. (2008). Reward-related processing in the human brain: developmental considerations. *Development and psychopathology*, *20*(4), 1191-1211.

Floresco, S. B., & Ghods-Sharifi, S. (2007). Amygdala-prefrontal cortical circuitry regulates effort-based decision making. *Cerebral cortex*, *17*(2), 251-260.

Fulenwider, H. D., Nennig, S. E., Hafeez, H., Price, M. E., Baruffaldi, F., Pravetoni, M.,…Schank, J. R. (2020). Sex differences in oral oxycodone self-administration and stress-primed reinstatement in rats. *Addiction biology*, *25*(6), e12822.

Galarce, E. M., Crombag, H. S., & Holland, P. C. (2007). Reinforcer-specificity of appetitive and consummatory behavior of rats after Pavlovian conditioning with food reinforcers. *Physiology & behavior*, *91*(1), 95-105.

Geyer, M. A., & Markou, A. (1995). Animal models of psychiatric disorders. *Psychopharmacology: the fourth generation of progress*, 787-798.

Gourley, S. L., Zimmermann, K. S., Allen, A. G., & Taylor, J. R. (2016). The Medial Orbitofrontal Cortex Regulates Sensitivity to Outcome Value. *J Neurosci*, *36*(16), 4600-4613. https://doi.org/10.1523/JNEUROSCI.4253-15.2016

Hart, E. E., Gerson, J. O., Zoken, Y., Garcia, M., & Izquierdo, A. (2017). Anterior cingulate cortex supports effort allocation towards a qualitatively preferred option. *European Journal of Neuroscience*, *46*(1), 1682-1688.

Hatfield, T., Han, J. S., Conley, M., Gallagher, M., & Holland, P. (1996). Neurotoxic lesions of basolateral, but not central, amygdala interfere with Pavlovian second-order conditioning and reinforcer devaluation effects. *J Neurosci*, *16*(16), 5256-5265.

Hofford, R. S., Chow, J. J., Beckmann, J. S., & Bardo, M. T. (2017). Effects of environmental enrichment on self-administration of the short-acting opioid remifentanil in male rats. *Psychopharmacology*, *234*, 3499-3506.

Holec, V., Pirot, H. L., & Euston, D. R. (2014). Not all effort is equal: the role of the anterior cingulate cortex in different forms of effort-reward decisions. *Frontiers in behavioral neuroscience*, *8*, 12.

Holland, P. C., & Gallagher, M. (1999). Amygdala circuitry in attentional and representational processes. *Trends Cogn Sci*, *3*(2), 65-73. https://doi.org/10.1016/s1364-6613(98)01271-6

Holland, P. C., & Straub, J. J. (1979). Differential effects of two ways of devaluing the unconditioned stimulus after Pavlovian appetitive conditioning. *Journal of Experimental Psychology: Animal Behavior Processes*, *5*(1), 65.

Holmes, N. M., Marchand, A. R., & Coutureau, E. (2010). Pavlovian to instrumental transfer: a neurobehavioural perspective. *Neuroscience & Biobehavioral Reviews*, *34*(8), 1277-1295.

Hosking, J. G., Cocker, P. J., & Winstanley, C. A. (2014). Dissociable contributions of anterior cingulate cortex and basolateral amygdala on a rodent cost/benefit decision-making task of cognitive effort. *Neuropsychopharmacology*, *39*(7), 1558-1567.

Hou, Y.-Y., Cai, Y.-Q., & Pan, Z. Z. (2020). GluA1 in central amygdala promotes opioid use and reverses inhibitory effect of pain. *Neuroscience*, *426*, 141-153.

Hutcheson, D. M., Everitt, B. J., Robbins, T. W., & Dickinson, A. (2001). The role of withdrawal in heroin addiction: enhances reward or promotes avoidance? *Nat Neurosci*, *4*(9), 943-947. https://doi.org/10.1038/nn0901-943

Imperio, C. G., McFalls, A. J., Hadad, N., Blanco-Berdugo, L., Masser, D. R., Colechio, E. M.,…Vrana, K. E. (2018). Exposure to environmental enrichment attenuates addiction-like behavior and alters molecular effects of heroin self-administration in rats. *Neuropharmacology*, *139*, 26-40.

Jacobs, E. H., Smit, A. B., de Vries, T. J., & Schoffelmeer, A. N. (2003). Neuroadaptive effects of active versus passive drug administration in addiction research. *Trends in pharmacological sciences*, *24*(11), 566-573.

Janak, P. H., & Tye, K. M. (2015). From circuits to behaviour in the amygdala. *Nature*, *517*(7534), 284-292. https://doi.org/10.1038/nature14188

Jhang, J., Lee, H., Kang, M. S., Lee, H.-S., Park, H., & Han, J.-H. (2018). Anterior cingulate cortex and its input to the basolateral amygdala control innate fear response. *Nature communications*, *9*(1), 1-16.

Johnson, A. W., Gallagher, M., & Holland, P. C. (2009). The basolateral amygdala is critical to the expression of pavlovian and instrumental outcome-specific reinforcer devaluation effects. *J Neurosci*, *29*(3), 696-704.

Kaiser, T., & Feng, G. (2015). Modeling psychiatric disorders for developing effective treatments. *Nature medicine*, *21*(9), 979-988.

Klein-Flügge, M. C., Kennerley, S. W., Friston, K., & Bestmann, S. (2016). Neural signatures of value comparison in human cingulate cortex during decisions requiring an effort-reward trade-off. *Journal of Neuroscience*, *36*(39), 10002-10015.

Kleykamp, B. A., De Santis, M., Dworkin, R. H., Huhn, A. S., Kampman, K. M., Montoya, I. D.,…Turk, D. C. (2019). Craving and opioid use disorder: A scoping review. *Drug and Alcohol Dependence*, *205*, 107639.

Kuhn, B. N., Kalivas, P. W., & Bobadilla, A.-C. (2019). Understanding addiction using animal models. *Frontiers in behavioral neuroscience*, *13*, 262.

LeDoux, J. E. (1993). Emotional memory systems in the brain. *Behav Brain Res*, *58*(1-2), 69-79.

Lichtenberg, N. T., Pennington, Z. T., Holley, S. M., Greenfield, V. Y., Cepeda, C., Levine, M. S., & Wassum, K. M. (2017). Basolateral Amygdala to Orbitofrontal Cortex Projections Enable Cue-Triggered Reward Expectations. *J Neurosci*, *37*(35), 8374-8384. https://doi.org/10.1523/JNEUROSCI.0486-17.2017

Lichtenberg, N. T., Sepe-Forrest, L., Pennington, Z. T., Lamparelli, A. C., Greenfield, V. Y., & Wassum, K. M. (2021). The Medial Orbitofrontal Cortex–Basolateral Amygdala Circuit Regulates the Influence of Reward Cues on Adaptive Behavior and Choice. *Journal of Neuroscience*, *41*(34), 7267-7277.

Lichtenberg, N. T., & Wassum, K. M. (2017). Amygdala mu-opioid receptors mediate the motivating influence of cue-triggered reward expectations. *European Journal of Neuroscience*, *45*(3), 381-387.

MacLean, R. R., Armstrong, J. L., & Sofuoglu, M. (2019). Stress and opioid use disorder: A systematic review. *Addictive behaviors*, *98*, 106010.

Malone, S. G., Keller, P. S., Hammerslag, L. R., & Bardo, M. T. (2021). Escalation and reinstatement of fentanyl self-administration in male and female rats. *Psychopharmacology*, *238*(8), 2261-2273.

Malvaez, M., Shieh, C., Murphy, M. D., Greenfield, V. Y., & Wassum, K. M. (2019). Distinct cortical-amygdala projections drive reward value encoding and retrieval. *Nat Neurosci*, *22*(5), 762-769. https://doi.org/10.1038/s41593-019-0374-7

Mansour, A., Fox, C. A., Burke, S., Meng, F., Thompson, R. C., Akil, H., & Watson, S. J. (1994). Mu, delta, and kappa opioid receptor mRNA expression in the rat CNS: an in situ hybridization study. *Journal of Comparative Neurology*, *350*(3), 412-438.

Mantsch, J. R., Baker, D. A., Funk, D., Lê, A. D., & Shaham, Y. (2016). Stress-induced reinstatement of drug seeking: 20 years of progress. *Neuropsychopharmacology*, *41*(1), 335-356.

Monroe, S. C., & Radke, A. K. (2023). Opioid withdrawal: role in addiction and neural mechanisms. *Psychopharmacology*, 1-17.

Mulert, C., Menzinger, E., Leicht, G., Pogarell, O., & Hegerl, U. (2005). Evidence for a close relationship between conscious effort and anterior cingulate cortex activity. *International Journal of Psychophysiology*, *56*(1), 65-80.

Murray, E. A. (2007). The amygdala, reward and emotion. *Trends Cogn Sci*, *11*(11), 489-497. https://doi.org/10.1016/j.tics.2007.08.013

Nawijn, L., van Zuiden, M., Frijling, J. L., Koch, S. B., Veltman, D. J., & Olff, M. (2015). Reward functioning in PTSD: a systematic review exploring the mechanisms underlying anhedonia. *Neuroscience & Biobehavioral Reviews*, *51*, 189-204.

Nemeroff, C. B., Bremner, J. D., Foa, E. B., Mayberg, H. S., North, C. S., & Stein, M. B. (2006). Posttraumatic stress disorder: a state-of-the-science review. *Journal of psychiatric research*, *40*(1), 1-21.

Niv, Y. (2021). The primacy of behavioral research for understanding the brain. *Behavioral Neuroscience*, *135*(5), 601.

Olson, E. A., Kaiser, R. H., Pizzagalli, D. A., Rauch, S. L., & Rosso, I. M. (2018). Anhedonia in trauma-exposed individuals: functional connectivity and decision-making correlates. *Biological psychiatry: cognitive neuroscience and neuroimaging*, *3*(11), 959-967.

Ortiz, S., Latsko, M. S., Fouty, J. L., Dutta, S., Adkins, J. M., & Jasnow, A. M. (2019). Anterior cingulate cortex and ventral hippocampal inputs to the basolateral amygdala selectively control generalized fear. *Journal of Neuroscience*, *39*(33), 6526-6539.

O'Connor, E. C., Chapman, K., Butler, P., & Mead, A. N. (2011). The predictive validity of the rat self-administration model for abuse liability. *Neuroscience & Biobehavioral Reviews*, *35*(3), 912-938.

Pavlov, I. P. (1906). The scientific investigation of the psychical faculties or processes in the higher animals. *Science*, *24*(620), 613-619.

Perusini, J. N., Meyer, E. M., Long, V. A., Rau, V., Nocera, N., Avershal, J.,…Fanselow, M. S. (2016). Induction and expression of fear sensitization caused by acute traumatic stress. *Neuropsychopharmacology*, *41*(1), 45-57.

Peters, J., & Büchel, C. (2010). Neural representations of subjective reward value. *Behavioural brain research*, *213*(2), 135-141.

Pickens, C. L., Saddoris, M. P., Setlow, B., Gallagher, M., Holland, P. C., & Schoenbaum, G. (2003). Different roles for orbitofrontal cortex and basolateral amygdala in a reinforcer devaluation task. *J Neurosci*, *23*(35), 11078-11084.

Poulos, A. M., Reger, M., Mehta, N., Zhuravka, I., Sterlace, S. S., Gannam, C.,…Fanselow, M. S. (2014). Amnesia for early life stress does not preclude the adult development of posttraumatic stress disorder symptoms in rats. *Biological psychiatry*, *76*(4), 306-314.

Rajbhandari, A. K., Gonzalez, S. T., & Fanselow, M. S. (2018). Stress-enhanced fear learning, a robust rodent model of post-traumatic stress disorder. *JoVE (Journal of Visualized Experiments)*(140), e58306.

Rau, V., DeCola, J. P., & Fanselow, M. S. (2005). Stress-induced enhancement of fear learning: an animal model of posttraumatic stress disorder. *Neuroscience & biobehavioral reviews*, *29*(8), 1207-1223.

Redmond Jr, D., & Krystal, J. (1984). Multiple mechanisms of withdrawal from opioid drugs. *Annual review of neuroscience*, *7*(1), 443-478.

Reiner, D. J., Lofaro, O. M., Applebey, S. V., Korah, H., Venniro, M., Cifani, C.,…Shaham, Y. (2020). Role of projections between piriform cortex and orbitofrontal cortex in relapse to fentanyl seeking after palatable food choice-induced voluntary abstinence. *Journal of Neuroscience*, *40*(12), 2485-2497.

Rescorla, R. A. (1987). A Pavlovian analysis of goal-directed behavior. *American Psychologist*, *42*(2), 119.

Robbins, T. (2002). The 5-choice serial reaction time task: behavioural pharmacology and functional neurochemistry. *Psychopharmacology*, *163*, 362-380.

Rule, B. G., & Nesdale, A. R. (1976). Emotional arousal and aggressive behavior. *Psychological Bulletin*, *83*(5), 851.

Shao, D., Cao, Z., Fu, Y., Yang, H., Gao, P., Zheng, P., & Lai, B. (2021). Projection from the basolateral amygdala to the anterior cingulate cortex facilitates the consolidation of long-term withdrawal memory. *Addiction Biology*, *26*(6), e13048.

Siciliano, C. A., & Tye, K. M. (2019). Leveraging calcium imaging to illuminate circuit dysfunction in addiction. *Alcohol*, *74*, 47-63.

Staddon, J. E., & Cerutti, D. T. (2003). Operant conditioning. *Annual review of psychology*, *54*(1), 115-144.

Stefanski, R., Ladenheim, B., Lee, S. H., Cadet, J. L., & Goldberg, S. R. (1999). Neuroadaptations in the dopaminergic system after active self-administration but not after passive administration of methamphetamine. *European journal of pharmacology*, *371*(2-3), 123-135.

Stolyarova, A., Rakhshan, M., Hart, E. E., O'Dell, T. J., Peters, M., Lau, H.,…Izquierdo, A. (2019). Contributions of anterior cingulate cortex and basolateral amygdala to decision confidence and learning under uncertainty. *Nature communications*, *10*(1), 4704.

Swanson, L. W., & Petrovich, G. D. (1998). What is the amygdala? *Trends in neurosciences*, *21*(8), 323-331.

Venniro, M., & Shaham, Y. (2020). An operant social self-administration and choice model in rats. *Nature protocols*, *15*(4), 1542-1559.

Verbitsky, A., Dopfel, D., & Zhang, N. (2020). Rodent models of post-traumatic stress disorder: behavioral assessment. *Translational psychiatry*, *10*(1), 132.

Vujanovic, A. A., Wardle, M. C., Smith, L. J., & Berenz, E. C. (2017). Reward functioning in posttraumatic stress and substance use disorders. *Current opinion in psychology*, *14*, 49-55.

Walton, M. E., Croxson, P. L., Behrens, T. E., Kennerley, S. W., & Rushworth, M. F. (2007). Adaptive decision making and value in the anterior cingulate cortex. *Neuroimage*, *36*, T142-T154.

Wassum, K., Ostlund, S., Maidment, N., & Balleine, B. (2009). Distinct opioid circuits determine the palatability and the desirability of rewarding events. *Proceedings of the National Academy of Sciences*, *106*(30), 12512-12517.

Wassum, K. M. (2022). Amygdala-cortical collaboration in reward learning and decision making. *Elife*, *11*, e80926.

Wassum, K. M., Cely, I. C., Balleine, B. W., & Maidment, N. T. (2011). μ-Opioid receptor activation in the basolateral amygdala mediates the learning of increases but not decreases in the incentive value of a food reward. *Journal of neuroscience*, *31*(5), 1591-1599.

Wassum, K. M., Greenfield, V. Y., Linker, K. E., Maidment, N. T., & Ostlund, S. B. (2016). Inflated reward value in early opiate withdrawal. *Addict Biol*, *21*(2), 221-233. https://doi.org/10.1111/adb.12172

Wassum, K. M., & Izquierdo, A. (2015). The basolateral amygdala in reward learning and addiction. *Neurosci Biobehav Rev*, *57*, 271-283. https://doi.org/10.1016/j.neubiorev.2015.08.017

Wellman, L. L., Gale, K., & Malkova, L. (2005). GABAA-mediated inhibition of basolateral amygdala blocks reward devaluation in macaques. *J Neurosci*, *25*(18), 4577-4586. https://doi.org/10.1523/JNEUROSCI.2257-04.2005

Xu, X., Cao, B., Wang, J., Yu, T., & Li, Y. (2015). Decision-making deficits associated with disrupted synchronization between basolateral amygdala and anterior cingulate cortex in rats after tooth loss. *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, *60*, 26-35.

Yee, D. M., Crawford, J. L., Lamichhane, B., & Braver, T. S. (2021). Dorsal anterior cingulate cortex encodes the integrated incentive motivational value of cognitive task performance. *Journal of Neuroscience*, *41*(16), 3707-3720.

Yuan, Z., Qi, Z., Wang, R., Cui, Y., An, S., Wu, G.,…Li, A. (2023). A corticoamygdalar pathway controls reward devaluation and depression using dynamic inhibition code. *Neuron*, *111*(23), 3837-3853. e3835.