Modulating endocannabinoid signaling to treat methamphetamine relapse

by

Troy Douglas Fort

B.A., Southwestern College, 2018
M.S., Kansas State University, 2022

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Psychological Sciences
College of Arts and Sciences

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Abstract

Overdose deaths due to methamphetamine (meth) have risen significantly in the last decade. One system of therapeutic interest is the endocannabinoid (eCB) system. The eCB, anandamide (AEA), has previously been shown to decrease cue-induced drug craving when intracranially administered. The current project took a different therapeutic approach by inhibiting the metabolism of AEA via the fatty-acid amide hydrolase (FAAH)-inhibiting drug, URB597. Administration of URB597 has been shown to attenuate cue-induced craving for both cocaine and nicotine. We aimed to evaluate whether the efficacy of URB597 extends to cue-induced methamphetamine craving. To test this question, we administered URB597 (vehicle, 0.3, or 1.0 mg/kg; ip) daily during abstinence to extended-access meth self-administration. We then assessed cue-induced methamphetamine seeking at Withdrawal Day 2 (WD2) and Withdrawal Day 28 (WD28). Animals were sacrificed following the WD28 cue-test to assess changes in cannabinoid and glutamate receptor expression via western blot and enzyme-linked immunosorbent assay (ELISA).

We did not observe that either dose of URB597 was effective in attenuating cue-induced craving at either WD2 or WD28. Results of western blot analysis indicated that meth abstinence significantly impacted the expression of cannabinoid receptor-1 and metabotropic glutamate receptor-5 in the dorsal hippocampus (dHipp), as well as the dorsomedial (dmPFC) and ventromedial prefrontal cortex (vmPFC). Finally, ELISA measurements of AEA indicated that meth did not significantly lower AEA levels as predicted. Taken together, these results further implicate the involvement of the eCB system in meth-use. However, different eCB mechanisms of action should be explored in the development of meth relapse therapeutics.

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Dedication

To my wife, Quinlan,

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Chapter 1 - Introduction

Background and Significance

Overdose deaths due to psychostimulant use have increased dramatically over the last decade. In the span between 2014 and 2020, overdose deaths due to psychostimulant use (primarily methamphetamine) have increased from 5,716 to 23,837 (NIDA Trends and Statistics). This sobering statistic clearly illustrates the ramifications of widespread methamphetamine use. While a number of potential therapeutic targets for preventing relapse to methamphetamine have been proposed, they have shown little success in the development of new and effective treatment strategies. This project aims to fill that gap by evaluating the efficacy of modulations in endocannabinoid (eCB) signaling as a therapeutic approach to lower cue-induced methamphetamine-seeking. Further, this work further characterizes specific neurobiological mechanisms implicated in methamphetamine self-administration and abstinence.

Abstinence and Relapse: The Systemic Cycle of Substance Use

Substance-use disorder (SUD) is a chronic disorder characterized by repeated episodes of abstinence and relapse. The drug-craving that ultimately leads to relapse remains a key obstacle in treatments designed to attenuate relapse and SUDs. Drug craving is often induced through drug-associated cues which increase the motivation to obtain drug-associated outcomes, referred to here as cue-induced craving. Even after long periods of drug abstinence, drug-associated cues are still able to induce relapse, thus perpetuating addiction. Exacerbating this issue is the finding that cue-induced drug craving increases concurrently with drug abstinence. This relationship between the increase in the motivational impact of drug-associated cues and drug abstinence is

referred to as *incubation of drug craving* (Pickens et al., 2011; Grimm, Hope, Wise & Shaham, 2001; Wolf, 2016).

Though incubations in drug craving have been reported in human clinical settings (Hunt et al., 1971; Pickens et al., 2011), rodent models of addiction are often used to assess the degree to which drug-craving incubates or intensifies during abstinence. In such studies, animals are trained to self-administer a drug by performing an operant response (e.g. a nose poke or lever press) and are rewarded with an infusion of the drug reinforcer. As such, the learned association at this phase of learning, between the operant response and outcome, represents early recreational drug-taking (Everitt & Robbins, 2005). Under these conditions, the outcome (drug reward) reinforces the action and thus increases the probability of the same action in the future and the subsequent formation of a response-outcome (R-O) relationship. However, response-outcome (R-O) relationships can only explain a portion of the issue at hand in substance-use. Stimuli and environmental cues can also become associated with drug reinforcement and accrue associative strength due to incentive salience (Robinson & Berridge, 1993; Flagel, Watson, Robinson, & Akil, 2007). According to the incentive salience theory posited by Robinson and Berridge (1993), these conditioned cues stimuli elicit arousal states that increase drug-seeking.

In animal models, the craving that is elicited by drug-conditioned cues is then assessed via a cue-seeking test in which active-lever pressing typically results in the presentation of the discrete drug-paired cues but no delivery of the drug itself. In some cases, the behavior will be extinguished after acquisition. The ability of discrete drug-paired cue to elicit drug-seeking behavior is then assessed via a cue-induced reinstatement test. Such paradigms are commonly referred to as “extinction-reinstatement” models. It is worth noting that though extinction training is decreasing the expression of the previously conditioned behavior, this should not be

interpreted as “forgetting” the response-outcome relationship. Instead, extinction can be seen as the formation of new associations and not a permanent erasure of the previous engram which would otherwise result in “forgetting” (Bouton, 1994; Berman & Dudai, 2001). Rather than “forgetting”, extinction training is introducing the new contingency that the relevant stimulus no longer elicits the previous outcome.

In other cases, the response-outcome relationship is never extinguished. Instead, following acquisition during self-administration, animals enter an abstinence period in which they are not allowed access to the drug, self-administration chambers, or drug-associated cues. The ability of the cue to elicit drug-seeking behaviors is then assessed via a cue-induced relapse test in which active lever pressing results in the presentation of drug-associated cues, but no delivery of the drug itself (Wolf, 2016). A wealth of research has shown that the impact that these drug-associated cues have on the motivation to perform drug-seeking behaviors generally increases during abstinence using both the reinstatement and relapse models of drug craving (Pickens et al., 2011; Grimm, Hope, Wise & Shaham, 2001; Wolf, 2016). While this craving does not continue indefinitely during abstinence, the asymptote of craving and decreases in cue-induced craving show variability across different drugs of abuse (Grimm, et al., 2001; Mead, et al., 2007; Shalev, et al, 2001; Airavaara, et al., 2011; Abdolahi, et al, 2010; Bienkowski, et al., 2004).

Though reinstatement and relapse models present valuable models to assess the craving, they are not without key shortcomings. In such models, it can often be difficult to delineate the craving that is elicited by contextual cues versus that which is elicited by discrete cues present during the infusion of the drug during self-administration. For instance, during typical relapse/reinstatement paradigms, the presentation of any tone-light discrete cues that were

previously paired with the drug infusion are contingent on the first lever press during the session. As such, one could argue that the craving that is initially assessed in such sessions is the craving that is elicited by the contextual information as any previously drug-paired discrete cues are contingent on lever-responding. This conundrum makes disentangling the effect of contextual and discrete cues on driving drug-seeking behavior quite difficult.

Kruzich et al. (2001) used different stimulus presentation formats to assess the effect that these had on reinstatement to cocaine-seeking. To do so, the authors subjected rats to one of five stimulus presentation formats during cocaine self-administration. In Group 1 (CC Only), animals self-administered cocaine without any discrete stimuli during Sessions 1-4, 6-9, and 11-14. However, during Sessions 5 and 10, the levers were retracted and animals received noncontingent cocaine infusions (the number of infusions was determined by the average number of infusions earned during the two previous sessions) that were paired with a short-delay presentation of a light-tone compound stimulus. In Group 2 (Random control), animals self-administered cocaine without other programmed stimuli during Sessions 1-4, 6-9, and 11-14. Likewise, the levers were retracted on Sessions 5 and 10 and animals received noncontingent cocaine infusions. However, the light-tone compound stimulus was presented randomly to Group 2 animals in order to remove predictive contingencies between the light-tone stimulus and cocaine infusions. Differing from the others, Group 3 (LT maintenance and CC) animals received response-contingent presentations of the tone-light stimulus that was presented for 5 seconds during the cocaine infusion. On Session 5 and 10, Group 3 animals received noncontingent infusions of cocaine that were paired with a short-delay presentation of the tone-light stimulus. In Group 4 (LT maintenance and control), animals received response-contingent stimulus presentations that were paired with the cocaine infusion (same as Group 3). However,

during Sessions 5 and 10, Group 4 animals received random presentations of the tone-light stimulus similar to Group 2. Animals in Group 5 (Novelty) did not receive any light-tone stimulus presentations during Sessions 1-4, 6-9, and 11-14. On Sessions 5 and 10, Group 5 animals received passive infusions of cocaine but were not presented with an stimuli during these sessions.

Following 6 days of extinction training, all groups underwent a noncontingent reinstatement test session in which animals received a presentation of the tone-light stimulus every 10 minutes during the three-hour test session. Ultimately, this test aimed to determine the whether the Pavlovian-conditioned light-tone stimulus (trained during Sessions 5 and 10) could elicit cocaine-seeking. Following two days of extinction training, all animals underwent a contingent reinstatement session where lever-presses resulted in the presentation of the tone-light stimulus. This test allowed the experimenters to determine whether the tone-light stimulus could drive drug-seeking as a secondary reinforcer. Results from the noncontingent reinstatement test showed that responding was significantly higher in the first hour of the three-hour long test, suggesting probable extinction due to the lack lever-contingent reinforcement. Importantly, there was no significant difference in the active-lever responding of Groups 1-5 and all noncontingent reinstatement responding was comparable to the responding during the previous extinction training session. As far as active-lever responding during the contingent reinstatement test, responding was significantly higher for CC-only, LT maintenance + CC, and LT maintenance + control groups when compared to responding on the extinction test the day prior and the day following the contingent reinstatement test. When comparing the stimulus presentation groups' responding during contingent reinstatement, the CC-only, LT maintenance + CC, and LT maintenance + control groups responding significantly more when compared to the random

control and novelty groups. The very fact that the CC-only group showed such elevated contingent reinstatement responding suggests that drug-conditioned stimuli can facilitate responding for the same cues when they are contingent on operant responding. Given that this light-tone stimulus was only paired with noncontingent cocaine infusions during Sessions 5 and 10 for the CC-only condition (Group 1), this suggests that the light-tone stimulus transferred secondary reinforcing properties. Likewise, contingent reinstatement responding was also significantly elevated in LT maintenance + CC and LT maintenance + control groups suggesting that the prior association between the light-tone stimulus and cocaine also produce secondary reinforcing effects.

Overall, these results demonstrate that Pavlovian conditioned stimuli may likely acquire their associative value through conditioned reinforcement and that operant responding can be maintained by these stimuli. In support of this, other work has shown that random presentations of drug associated stimuli do not maintain operant responding. However, conditioned reinforcement does promote operant responding, thus suggesting that drug-related stimuli are rewarding and motivate drug-seeking behavior (Di Ciano & Everitt, 2003; Grimm, Kruzich, & See, 2000).

While both the reinstatement and relapse models are valid models of addiction and cue-seeking, the relapse model, in which the operant drug-seeking response is never extinguished, has a number of advantages over the former model of drug self-administration. First, the relapse model shows greater translational validity when compared to the reinstatement model. For instance, human subjects suffering from SUD rarely receive extinction training for specific drug-cues during in-patient, or out-patient drug rehabilitation (Monti & MacKillo, 2007; Murray, Lacoste & Belin, 2016). Rather, rehabilitation typically requires individuals to maintain drug

abstinence and then return to society, often presented with a myriad of drug-associated cues. Second, extinction is not well-suited to understanding the underlying the plasticity that maintains drug-craving during abstinence, as extinction training itself has been shown to induce changes in plasticity of neural circuity associated with reward-based processing (Wolf, 2016).

Escalation of Intake as a Catalyst for Substance Abuse

Increases or escalations in the frequency of drug intake have become a key diagnostic criterion for SUD, as outlined in the *Diagnostic and Statistical Manual of Mental Disorders* (American Psychiatric Association, 2013). This shift could result from changing disease states during the time course of SUD. Namely, early in the SUD disease state, drug taking tends to be recreational and impulsive in nature. However, as the time course of SUD progresses, drug-intake tends to increase both in frequency, and transitions from impulsive to compulsive in nature (Edwards & Koob, 2013). It is this shift from impulsive to compulsive decision making that clinically demarcates recreational from pathological drug use. While the focus of this project lies in mitigating cue-induced relapse to methamphetamine, the usage of translationally valid models of drug intake are critical to accurately model the nuance of substance use and relapse to use.

This shift towards pathological drug seeking likely involves the interaction between two separable and opposing motivations, namely, the balance between positive and negative reinforcement. As to the former, positive reinforcement occurs when conditioned stimuli increase the probability of responses associated with obtaining the reward. The primary framework for positive reinforcement in the context of SUD comes from the incentive salience model of addiction described previously (Robinson & Berridge, 1993). Here, cues that have

come to be associated with drug-taking lead to a maladaptive strengthening of behaviors associated with obtaining drug reinforcement rather than other more adaptive behaviors associated with other natural rewards (Berridge & Robinson 1993; Volkow et al., 2011). As for the second motivational state, negative reinforcement involves drug use to combat negative or aversive physical or emotional states (self-medication), which can be induced by withdrawal, dysphoria, or anxiety (Edwards & Koob, 2014). These motivational states exist in a negative feedback loop, often described by behavioral scientists as an opponent-process model. In such models, stimulus-derived emotional states are countered (or "opposed") by the opposite emotional state to help keep the organism at some level of hedonic homeostasis (Edwards & Koob, 2014; Poulos & Cappell, 1991; Koob & Le Moal, 2008).

How drug-taking changes across the addiction cycle can be studied in animal models using intravenous self-administration, often seen as the gold standard in translational validity to human drug-use. Ahmed and Koob (1998) suggested that this increase in drug intake reliably accompanies protracted exposure to drugs of abuse. Specifically, they modeled differential access to cocaine by allowing rats to self-administer cocaine for either 1 or 6 hours per session. They found that though both short- and extended-access rats adjusted their responding to the different cocaine doses tested during self-administration, only extended-access animals (6 hours per session), increased their responding over the course of self-administration. They suggested that escalation of cocaine-intake occurs due to a shift in the hedonic set-point of the drug, wherein drug-intake must increase in order to elicit the hedonic effects of the drug. This seminal research has been consistently supported by reports showing that extended-, but not short-access drug self-administration is required to elicit escalations in drug-taking during self-administration paradigms. Since this model has been shown to reliably induce escalated drug-intake with

extended access periods, the usage of extended access self-administration periods is of particular interest to the proposed research.

Neurobiology of Cue-Induced Craving and Relapse

A number of drugs of abuse stimulate the mesocorticolimbic pathway, a pathway intimately involved in reward processing and learning. Everitt and Robbins (2005) have suggested that the mesocorticolimbic circuit is responsible for demarcating the transition from substance use to substance abuse through the influence of intervening stimuli associated with drug-taking. Therefore, it seems that the neural properties that make this system well-suited to associative learning also contribute to maladaptive drug-seeking behaviors.

The role that dopamine (DA) plays in addiction and drug-seeking has long been a topic of investigation in the field of addiction science (Berridge & Robinson, 1998; Kelley, 2004). The DA release, elicited from drugs of abuse, ultimately results increased reward learning and drives subsequent drug-seeking behaviors. The pervasive nature of chronic relapse in addicts is largely a result of neuroadaptations of dopaminergic axons in the mesocorticolimbic circuit (Kalivas, 2009). However, the last decade has a seen a shift in focus towards increasing understanding of the neurocircuity governing dopaminergic signaling within the mesocorticolimbic pathway. Particularly, the study of glutamatergic neurotransmission in the mesocorticolimbic circuit is a burgeoning field for understanding how disruptions or alterations in glutamate and its associated mechanisms can lead to drug-seeking. This shift is largely due to the perspective that the pervasive changes is dopaminergic signaling and concomitant increases in drug-seeking behavior are a consequence of long-term changes in the neurocircuitry that house these dopamine axons (Kalivas, 2009).

While mesocorticolimbic projections are implicated in generating learned behaviors (e.g. drug-taking), these same projection neurons are also responsible for helping to modulate behavior in response to changes in the environment (Kelley, 2004). In the case of pathological substance-use, this behavioral phenotype can be seen as an inability to control drug-seeking in response to drug-associated cues. Kalivas (2009) and other colleagues have suggested that this pathology could arise from two primary sources in the mesocorticolimbic system. First, SUD could arise from a maladaptive strengthening of drug-seeking behaviors. Second, SUD could also arise from deficiencies in the ability to control drug-seeking behaviors in response to drug cues. While these two potential sources of addiction point to different structures in the mesocorticolimbic system, both are likely implicated in the initiation and maintenance of SUD.

Disruptions in glutamatergic signaling in the medial prefrontal cortex (mPFC), nucleus accumbens (ACb), ventral tegmental area (VTA), orbitofrontal cortex (OFC), hippocampus, and the amygdala have been shown to contribute to the initiation and maintenance of cue-induced craving. The ACb primarily (90-95%) consists of GABAergic medium spiny projection neurons (MSNs) (Wolf, 2016). These medium spiny neurons help to integrate glutamatergic inputs, which play a pivotal role in generating motivated behaviors, and relaying information to regions of the basal ganglia tasked with execution of motor movements associated with that behavior. In this light, the ACb serves as a gateway for information derived from limbic subcircuitry to elicit activation in the motor circuit (Yin and Knowlton, 2006) and is therefore critical for turning motivation into action (Wolf, 2016; Sesack & Grace, 2010; Mogenson, Jones & Yim, 1980). When novel stimuli that predict appetitive outcomes are presented, the limbic circuitry attend to information pertaining to the stimulus. Due to the integration of the ACb, this information then elicits activation in the motor circuit, and ultimately to the execution of motor movements

targeted to the attainment of the reinforcer. As stimuli continue to predict the presentation of a particular reinforcer, neuronal activity in the limbic subcircuit decreases and neuronal activity is primarily driven by the motor subcircuit (Barnes, Kubota, Hu, Jin & Graybiel, 2005; Yin & Knowlton, 2006). This motor compensation ultimately drives behaviors associated with the attainment of that reinforcer to become increasingly habitual (Yin and Knowlton, 2007). However, if stimuli or behavior no longer elicit the presentation of the reinforcer (e.g. extinction training) then the limbic subcircuit takes over control of behavior. The proper functioning of the limbic subcircuitry should make behavioral changes more amenable to changes in the environmental contingencies associated with that reinforcer (Barnes, Kubota, Hu, Jin & Graybiel, 2005; Doya, 2008). In this light, addiction can occur from an inability to adopt new behaviors in response to changes in environmental contingencies. According to this model, compulsive drug relapse occurs from the inability of the limbic subcircuitry to inhibit behavior in the presence of negative environmental contingencies (Kalivas, 2009). Instead, the model suggests that the maladaptive cycle of behavior in addiction suffers from overactivity of the motor subcircuit, ultimately driving habitual and compulsive drug-seeking.

Collectively, the prefrontal cortex (PFC) is linked to cognitive, emotional, and motivational processes that can help guide behavior such as: attention, inhibition, and decision-making. However, while much of the work in addiction has tended to focus on the motivational aspects, addiction is undoubtedly a disorder that involves a disruption of the balance between motivation and self-regulation (Moorman, James, McGlinchey & Aston-Jones, 2015). As I have previously indicated, inhibiting drug-seeking in response to the presentation of drug-associated cues is one of the primary hurdles in treating addiction. Activity of the PFC has been found to be positively correlated with individuals' self-reported craving of cocaine, nicotine, and alcohol

(Bonson et al., 2002; Yalachkov et al., 2009). Importantly, Volkow et al. (2010) has shown that the PFC is involved in modifying behavior in response to drug-cues. Control participants were not instructed to inhibit their craving and showed PFC activity that positively correlated with their self-reported craving levels. Conversely, instructing addicts to suppress their craving in response to cocaine-related images resulted in a significant reduction in the activation of the right OFC, a structure responsible for processing visual cues, and significant activation of the right inferior frontal gyrus, a structure linked to inhibitory control (Goldstein & Volkow, 2011). However, the PFC contributes to numerous different aspects of addiction, from motivation and craving to behavioral restraint in response to drug-craving. This diversity is largely due to the heterogeneous organization of the PFC, with different subregions contributing to separate aspects of addiction (e.g. motivation, attention, restraint, etc.).

The rodent medial prefrontal cortex (mPFC) receives dopaminergic input from the VTA and other limbic structures such as the amygdala and hippocampus. These inputs help the mPFC to monitor the motivational significance and salience of drug-related stimuli, thus making it a crucial component of the mesocorticolimbic pathway (Kalivas, 2009; Lasseter et al., 2010). The mPFC then uses this information to control and direct cue-induced drug-seeking via the ACb and motor structures of the basal ganglia, particularly the caudate and putamen of the dorsal striatum (Kalivas, 2009; Lasseter et al., 2010; Vertes, 2004). Previous research has shown that cocaine infusions to mPFC increases cocaine-seeking behaviors while inactivating the mPFC can attenuate cocaine-seeking in both cocaine reinstatement and conditioned place-preference paradigms (McFarland & Kalivas, 2001; Capriles et al., 2003). However, inactivation of the mPFC has also been shown to hasten the acquisition of cocaine-taking during operant self-

administration, further demonstrating the complex nature of the mPFC circuitry (Weissenborn et al, 1997).

The heterogenous functioning of the rodent mPFC can be linked to the dissociable organization of the two primary subsections of the mPFC, the dorsomedial prefrontal cortex (dmPFC) which consists of the prelimbic (PL) and anterior cingulate (ACC) cortices, and the ventromedial prefrontal cortex (vmPFC) consisting of the infralimbic (IL) and orbitofrontal (OFC) cortices. These two regions are highly dissociable due to differences in their afferent projections to other brain areas associated with reward-based processing, particularly the ACb (Vertes, 2004). The majority of PL afferents project to the ACb core while IL afferents project to the ACb shell. Both PL and IL regions also show divergent behavioral roles with the PL being pivotal for the execution of behavior, while the IL is vital for response inhibition (Gass & Chandler, 2013; Van den Oever et al., 2010). The role of PL in addiction was elucidated by the finding that pharmacological inactivations (i.e. muscimol, baclofen) of PL generally decrease cocaine-seeking, thus demonstrating that PL plays a pivotal role in drug-seeking (Di Pietro et al., 2006). Additionally, lesioning PL has been shown to decrease reinstatement of drug-seeking following drug abstinence (Pelloux et al., 2013). The control that PL exerts over reinstatement is due to glutamatergic projection neurons to the ACb core. Indeed, cocaine-primed reinstatement has shown to increase glutamate concentrations within the ACb core, an effect which is inhibited by inactivation of PL (Baker et al., 2003; McFarland et al., 2003). This evidence further corroborates the link between PL- ACb core projections and the reinstatement of drug seeking. However, unlike PL, inactivation of IL does not affect cue-induced reinstatement to methamphetamine (Hiranita et al., 2006). While many could take this as evidence that IL is not involved in drug-seeking, IL has shown to be an integral component in suppressing drug-

seeking. Inactivation of IL increases lever pressing and spontaneous recovery of extinguished cocaine self-administration following 28 days of forced abstinence while activation of IL engendered significant reductions in cocaine-induced reinstatement (Peters et al., 2008). These behavioral effects are corroborated by changes in the cellular physiology of IL-ACb shell projections. Formation of silent synapses in IL-ACb shell projections occurs following withdrawal of cocaine self-administration (Ma et al., 2014).

In addition to the ACb and mPFC, recent addiction theories have started to account for the involvement of limbic regions, such as the hippocampus. This is due to the role of the hippocampus in forming contextual memories. In the case of SUD, the hippocampus is crucial in forming the craving that is elicited from the reintroduction to contexts in which the animal has previous exposure to the drug (Castilla-Ortega et al., 2016). Two key paradigms that have been used to illustrate the role of the hippocampus in the cue-induced drug seeking are the operant self-administration and conditioned place preference (CPP) paradigms. To briefly summarize, operant self-administration paradigms utilize an operant conditioning chamber to allow the animal to perform a behavioral response to obtain reinforcement via a drug infusion. In the case of CPP paradigms, a three-compartment apparatus is typically used in which the outer compartments have distinctive characteristics to allow animals to discriminate between the two contexts. During training, animals are injected with the experimental drug and placed in the drug-paired compartment. The following day, the animal will receive a vehicle injection and placed into the opposite outer compartment. This alternation will happen several times to increase the number of drug- or vehicle-paired trials. Finally, the test day commences by placing the animal into the center compartment (never received drug- or vehicle-pairings) and allowed to freely move between the chambers, measuring the amount of time that the animal spends in each

outer compartment. Conditioned-place preference exists when the animal spends more time in the drug-paired compartment compared to the vehicle-paired compartment.

Unsurprisingly, the hippocampus is intimately involved in both the acquisition of drug-stimulus associations and the maintenance of cue-induced drug-seeking/preference. For instance, temporary inactivation or lesioning of the subiculum (primary hippocampal output) precludes the acquisition of both cocaine-seeking using CPP paradigms (Hernandez-Rabaza et al., 2008; Meyers et al., 2003) and cocaine self-administration (Caine et al., 2001). Similar evidence suggests that the hippocampus is required for both the retrieval of drug-associated memories in both self-administration (Black et al., 2004) and CPP paradigms (Otis et al., 2014; Raybuck & Lattal, 2014). Additionally, the hippocampus is necessary for the extinction of cocaine self-administration (Szalay et al., 2013) and for the reinstatement of extinguished cocaine-seeking (Fuchs et al., 2007; Fuchs et al, 2005; Lasseter et al., 2010).

Molecular Bases for the Incubation of Drug Craving

Glutamate Homeostasis

The incubation of drug-craving in response to drug-associated cues that occurs during drug abstinence is largely a consequence of maladaptive neuroplasticity in the ACb (Wolf, 2016). The behavioral increase in the motivational impact of drug-associated cues is corroborated by an increase in the number of ACb neurons that respond to drug-associated cues. Indeed, drug-associated cues have been shown to elicit higher activation of neurons in the ACb core on Withdrawal Day 30 than on Withdrawal Day 1 (Hollander & Carelli, 2005), further corroborating a behavioral and neuronal link to the incubation of drug craving that occurs during

drug abstinence. The ACb core has been found to contribute to incubation of drug-seeking, while the ACb shell has generally been found to contribute to escalation of drug intake (Wolf, 2016).

As discussed in the previous section, the last ten to fifteen years have seen a shift toward understanding the effects of glutamatergic signaling in the mesocorticolimbic pathway. However, this view has since expanded further still by implicating the action of glial cells in the release and elimination of glutamate from the synaptic and extrasynaptic space. This shift largely resulted from the finding that in *in vivo* microdialysates, extracellular glutamate levels were unaffected by pharmacological blockade of Na^+ and Ca^{2+} voltage-gated channels at the synapse (Timmerman & Westerink, 1997). This seminal finding changed a number of ways that researchers would begin to see glutamate in the mesocortioclimbic pathway. First, the fact that extracellular glutamate remained homeostatic, despite antagonism of synaptic iGluRs, indicated that extracellular glutamate arises and is eliminated primarily through glial sources. Second, glial-derived reuptake mechanisms protect iGluRs at the synapse from exposure to extrasynaptic glutamate spillover. Third, perisynaptic metabotropic glutamate autoreceptors (mGluRs) are predominately stimulated by glial-derived extrasynaptic glutamate (Kalivas, 2009). As such, this finding reframed the traditional view of glutamatergic neurotransmission, which had seen this neurotransmission occurring via a systemic cycle of pre-synaptic glutamate release, binding of the ligand to iGluRs located on the post-synaptic cell, and elimination of residual glutamate via Na^+-dependent glutamate transporters (Herman & Jahr, 2007; Diamond & Jahr, 1997). This shift ultimately led to what is now known as the, "Glutamate Homeostasis Hypothesis of Addiction" (Kalivas, 2009).

The role of the mGluR5 receptor in the incubation of methamphetamine has been supported by the finding that inhibition of mGluR5 has been effective at lowering both

methamphetamine self-administration and relapse (Gass et al., 2009; Murray et al., 2021). Though this treatment was shown to be effective, Murray et al. (2021) did not show that surface mGluR5 expression in the ACb core was decreased following abstinence to methamphetamine. However, the scope of the potential dysregulations in mGluR5 expression in other relevant brain areas, such as the mPFC and hippocampus, are under-characterized in response to methamphetamine abstinence.

The Endocannabinoid System

More recently, research in the field of SUD has started evaluating the role of the endocannabinoid (eCB) system. The eCB system is composed of a myriad of chemical messengers and are distinctive in their neurotransmission, primarily operating as retrograde messengers (Karimi-haghighi et al., 2023). The action of eCB signaling is intimately involved in motor function, cognition, memory, sleep, stress, analgesia, and reward processing (Cristino et al., 2020). Though simplified, the eCB system can be understood as the eCB compounds themselves (primarily lipid neurotransmitters), the receptors responsible for binding those eCB ligands, and the enzymes responsible for the hydrolysis and recycling of eCB-constituent molecules. The two primary CB receptors are the cannabinoid receptor-1 (CB_1) and cannabinoid receptor-2 (CB_2) receptors. Though serving distinctive behavioral functions, both CB_1 and CB_2 receptors are G-protein coupled receptors, linking to inhibitory G-proteins (Matsuda et al., 1990; Munro, Thomas, and Abu-Shaar, 1993). Through this action, CB_1 receptors inhibit presynaptic Ca^{2+} channels and activate inwardly-flowing K^+ channels, thus facilitating repolarization of the presynaptic neuron (Mackie, Westenbroek, and Mitchell, 1995; Twitchell, Brown, and Mackie, 1997). The expression of CB_1 receptors in the CNS is mainly localized to neurons throughout the

brain, while CB_2 mRNA expression is mainly immune cells in the peripheral nervous system (Mackie, 2006).

Two of the most ubiquitous eCBs are anandamide (AEA) and 2-arachidonyl glycerol (2-AG). Anandamide is the endogenous ligand for the CB_1 receptor and shows high affinity for binding to CB_1 in the CNS while 2-AG primarily binds to the CB_2 receptor (Parsons and Hurd, 2015). Perhaps more important are the dissociable routes of metabolism that exist for AEA and 2-AG. First, AEA is primarily hydrolyzed by the enzyme, fatty-acid amide hydrolase (FAAH). However, 2-AG is primarily broken down by monacylglycerol lipase (MAGL). In addition to differential metabolic roles, FAAH and MAGL share distinct neuroanatomical localization with most FAAH being localized in post-synaptic neurons while MAGL tends to predominate in presynaptic axon terminals (Parson and Hurd, 2015).

As far as how AEA and 2-AG are synthesized, both molecules again show distinct routes of formation. Though the complete synthesis is not completely understood, it is known that the enzyme *N*-acyl-phosphatidylethanolamine-specific phospholipase D (NAPE-PLD) converts NAPE to AEA. Synthesis of 2-AG occurs via the hydrolytic metabolism of 1,2-diacylglycerol (DAG) by one of two diacylglycerol lipase (DAGL) enzyme subtypes, DAGLα or DAGLβ (Parsons & Hurd, 2015).

Integration of Endocannabinoid Signaling and Glutamate Homeostasis

Recent evidence has shown a unique interplay between glutamate and eCB signaling within the mesocortiolimbic pathway. Perhaps the most understood mechanism for the regulation of glutamate by eCB signaling exists between metabotropic glutamate receptor-5 (mGluR5) and CB_1. In this pathway, binding of glutamate to postsynaptic mGluR5 initiates a signaling cascade

that triggers the release of eCBs (such as AEA) from the postsynaptic neuron. These eCBs are then trafficked in a retrograde fashion back across the synapse to bind CB_1 receptors on the presynaptic neuron (Li et al., 2018). This binding ultimately inhibits presynaptic glutamate release and precludes the long-term potentiation required to elicit cue-induced drug craving. In this way, optimal glutamatergic tone at mGluR5 receptors permits long-term depression (LTD) of glutamatergic synapses via this mGluR5-eCB-CB_1 signaling cascade (Moussawi et al., 2009; Kalivas, 2009). Given that synaptic strengthening is a prerequisite for incubations in craving (Wolf, 2016), this system provides one mechanism for attenuating cue-induced craving. The importance of this LTD system is illustrated by the finding that the administration of the mGluR5 antagonist, MPEP, eliminates LTD effects, an effect which was present in both control animals and animals that received a mGluR5 agonist (Robbe, et al., 2002). In total, mGluR5-eCB-CB_1 mediated synaptic depression is a vital mechanism for LTD, particularly at glutamatergic synapses.

This idea is bolstered by the finding that intracranial infusions of AEA into the nucleus accumbens is sufficient to both restore glutamate homeostasis and lower cue-induced cocaine craving (Zhang et al., 2021). The specific role of mGluR5-CB_1 mediated synaptic depression within the ACb core has been shown to be implicated in the rising phase of the incubation of methamphetamine craving (Murray et al., 2021). A simplified depiction of this mGluR5-eCB-CB_1 mechanism is shown below in Figure 1.

Dysregulations in Endocannabinoid System Induced by Drug Exposure

Numerous drug classes disrupt various stages of eCB regulation (Parsons and Hurd, 2015). This leads to the persistent neuroplasticity that may permit increases in drug-craving

during abstinence due to the eCB system's integral role in various forms of inhibitory synaptic transmission and short- and long-term depression (Lovinger, 2008; Heifets, and Castillo, 2009). However, the patterns in eCB signaling disruption amongst psychostimulants is less clear. Previous evidence has shown that cocaine has shown to increase extracellular AEA and 2-AG levels within the dorsal hippocampus and ACb during cocaine-primed reinstatement testing (Bystrowska et al., 2019). However, AEA and 2-AG levels have been shown to decrease basal levels of AEA and 2-AG in the ACb during amphetamine-induced locomotor sensitization (Thiemann et al., 2008).

Decreases in basal AEA levels in response to amphetamine could present one mechanism for subsequent cue-induced psychostimulant craving. In this light, a lack of eCB tone on presynaptic CB_1 receptors would occur due to systemically low AEA levels. Given that mGluR5-eCB-CB_1 LTD is amongst the most commonly used mechanisms for LTD at glutamatergic synapses, the corruption of this system could present a mechanism for increased cue-induced craving during abstinence. To our knowledge, there are currently no studies which have directly assessed the effects of methamphetamine on brain AEA levels, thus illustrating the need in this research area.

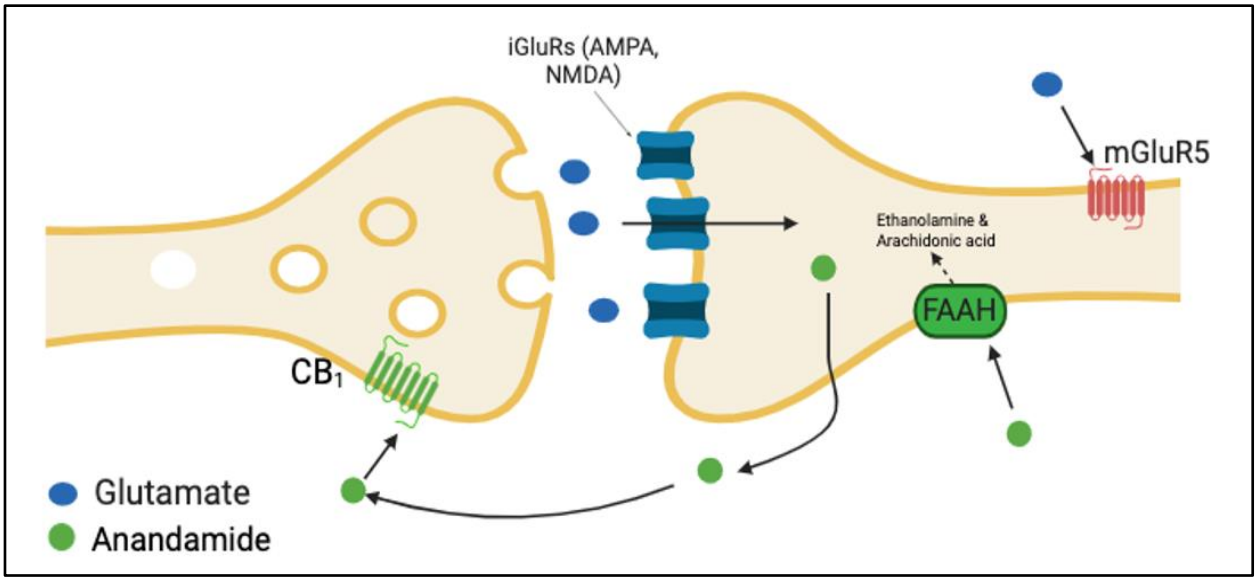


Figure 1. Simplified depiction of the mGluR5-eCB-CB_1 mediated long-term synaptic depression. Glutamate is available to bind post-synaptically located mGluR5, eliciting the retrograde trafficking of anandamide cross the synapse to bind CB_1 and halting presynaptic glutamate release. In this system, anandamide is broken down FAAH located on the post-synaptic membrane.

The Endocannabinoid System as a Pharmacological Target for Methamphetamine Cue-Induced Relapse

Though a number of potential therapeutic targets for preventing relapse to methamphetamine-use have been proposed, they have had little success in the development of effective preventative treatments. Manipulations in the eCB system presents a new therapeutic target for treating methamphetamine relapse. One specific application of this idea is evident in the finding that intracranial infusions of AEA into the ACb core have been effective in lowering cue-induced relapse following abstinence to cocaine self-administration (Zhang et al., 2021).

However, though this treatment strategy has been shown to be effective, it is relatively limited in its translational potential given its invasive nature.

Currently, pharmacological approaches designed to modulate CB_1 receptor binding through the inhibition of either FAAH or MAGL have emerged as a preferred route for treatments targeting the eCB system. One target of specific interest is FAAH, the enzyme responsible for hydrolyzing AEA. Inhibition of FAAH breakdown of AEA blunts methamphetamine-induced decreases in tyrosine hydroxylase, a putative marker for dopamine (Nader et al., 2014). Though these results implicate FAAH and AEA in response to methamphetamine, the effect of subtoxic doses of methamphetamine on these systems is less understood. This same approach of inhibiting the hydrolysis of AEA, through inhibition of FAAH activity has started gathering support in treating relapse to a number of drugs of abuse. For instance, the FAAH inhibitor, URB597, has been shown to decrease cue-induced reinstatement to nicotine and cocaine (Chauvet et al., 2014; Forget, Coen, & Foll, 2009). However, the potential efficacy of FAAH inhibition in the context relapse to methamphetamine has not been explored. Importantly, the findings of FAAH treatment precluding methamphetamine-induced dopaminergic neurotoxicity likely support the involvement of FAAH-eCB signaling as a therapeutic target for lowering relapse to methamphetamine-use (Nader et al., 2014).

Therefore, this experiment evaluates the efficacy of the FAAH inhibitor, URB597, in lowering cue-induced methamphetamine-craving following abstinence to extended-access methamphetamine self-administration. To test this, I examine cue-induced relapse at Withdrawal Day 2 and Withdrawal Day 28. These timepoints are consistent with both the incubation profile

of methamphetamine (Murray et al., 2019; Funke et al., 2023; Scheyer et al., 2016) and URB597 efficacy in attenuating cue-induced reinstatement to cocaine (Chauvet et al., 2014).

Chapter 2 - Methods and Materials

Animal Husbandry

Forty-eight male Sprague-Dawley rats were obtained from Charles River Laboratories. All rats weighed approximately 250 grams when arriving at Kansas State University. Group numbers were determined via power analysis in the statistical program G*Power. For this analysis, the alpha level was set at 0.05, the power level was set at 0.95, and the effect size was set to 0.35. All animals were pair-housed in plastic shoebox cage containing bedding and wire tops. All animals were handled weekly during scheduled cage changes. Animals were maintained under a 12-hour reverse light-dark cycle for the entirety of the experiment. All animals were given *ad libitum* access to food and water throughout the experiment and the colony room temperature and humidity were maintained at approximately 22°C and 30-50%, respectively. All experimental procedures were conducted in accordance with the Institutional Animal Care and Use Committee at Kansas State University and the NIH guidelines for the care and use of laboratory animals. Figure 2 shows the group sizes and assignments for all animals that met the criteria to be included in the analyses for the current study.

URB597 Dose (ip)			
	0.0 mg/kg	**0.3 mg/kg**	**1.0 mg/kg**
METH	**10**	**10**	**10**
SAL	**6**	**6**	**6**

Figure 2. Animal numbers and group sizes. Numbers in red reflect methamphetamine self-administering animals while numbers in blue reflect saline self-administering animals.

Apparatus

Operant Self-Administration Chambers

All training and testing was conducted in standard operant chambers (Med Associates, St. Albans, Vermont). Chambers are enclosed within ventilated sound-attenuating chambers. Each chamber contained two metal retractable levers, two 3 cm cue-lights (one above each lever; illuminated during infusion), house light (illuminated during timeout period), and a tone generator. Intravenous methamphetamine was delivered via a variable-speed syringe pump (PHM-100, Med Associates) connected to a 10 mL syringe. To intravenously self-administer methamphetamine solutions, tubing from the syringe was connected to a swivel and metal extension spring (to prevent chewing or accidental punctures of tubing). The extension spring tubing was connected to the rats' back mount to allow free movement throughout each extended-access session.

Drugs and Solutions

Heparinized Saline

Heparin (10 IU/mL) was dissolved in 0.9% sterile saline. Each day, 0.1 mL of heparinized saline was intravenously administered via the catheter to maintain catheter patency.

Baytril (Enrofloxacin*)*

The fluoroquinolone antibiotic, Enrofloxacin was dissolved in 0.9% sterile saline to yield a stock concentration of 8 mg/mL. Each day, 0.1 mL of Baytril was intravenously delivered to reduce the possibility of infection. Baytril was obtained from the Veterinary Pharmacy at Kansas State University.

Metacam (Meloxicam)

To alleviate pain and distress following surgery, the non-steroidal anti-inflammatory, Meloxicam, was used. Meloxicam was delivered at a dose of 2 mg/kg (dissolved in 0.9% sterile saline) subcutaneously immediately following surgery, and then 1 mg/kg doses were given on the two recovery days immediately following surgery.

Methamphetamine

Methamphetamine (Sigma Aldrich, MO, USA) was dissolved in 0.9% sterile saline to a stock concentration of 1 mg/mL. When administered intravenously during self-administration, methamphetamine was delivered at a dose of 0.1 mg/kg/infusion.

URB597 (FAAH Inhibitor)

The FAAH inhibitor URB597 (Focus Biomolecules, Plymouth Meeting, Pennsylvania) was dissolved in a vehicle containing 15% dimethyl sulfoxide (DMSO), 3% Tween-80, and 0.9% saline. Stock solutions (1 mg/mL and 0.3 mg/mL) were mixed via an ultra sonicator bath until the URB597 powder was solubilized. Once mixed, 1 mg/mL and 0.3 mg/mL URB597 stock solutions were transferred to individual sterile aliquots and frozen at -20°C for daily use. This minimized repeated freezing and thawing of stock solutions. Each day, 0.3 mg/mL and 1.0 mg/mL URB aliquots were removed and then used to administer intraperitoneal injections each day during the forced abstinence period.

Procedures

Surgical Procedures

Upon arrival at Kansas State University, animals were allowed to acclimate to the colony room for 1 week prior to any experimental manipulation. Following habituation, rats were deeply

anesthetized with isoflurane (~ 5%) and prepped for surgery by shaving incision sites on the animal's dorsal side and the ventral side of the neck. All incision sites were then scrubbed with chlorhexidine and isopropyl alcohol. Following preoperative care, a 3 cm horizontal incision was made approximately 6-8 cm below the animal's scapulae. A second incision was made approximately 4 cm above the initial horizontal incision which served as a point for the catheter port to exit the animal's back. A final superficial incision was made on the ventral surface of the neck, just above and perpendicular to animal's jugular vein. Polyurethane catheters (12 cm in length, 0.2 mm internal diameter: SAI Infusion Technologies; Lake Villa, IL) were inserted through the dorsal incision in the animal's back, tunneled under the skin and into the animal's left jugular vein. The jugular vein was isolated inside the neck incision and a small lateral incision was made in the jugular vein using vein scissors. The catheter tubing was then inserted into the jugular vein and secured in place via suture thread. The neck incision was closed using 3M VetBond surgical glue. The horizontal incision on the back of the animal was closed via suture thread and surgical glue.

Catheter tubing was subcutaneously connected to a 22-gauge back-mounted cannula (Plastics One: Roanoke, VA) and sutured to surgical mesh (Biomedical Structures; Warwick, RI). The catheter cannula was protected by a cannula cap. To prevent animals from chewing or damaging back mounts, a stainless-steel bolt was threaded onto the end of the back mount. During the first 2 days of recovery rats were treated with Meloxicam (2.0 mg/kg after surgery;1.0 mg/kg thereafter) in order to alleviate pain. Baytril® (Enrofloxacin, 2.5% (25 mg/ml) diluted with sterile saline to a final concentration of 6-8 mg/ml administered in a 0.1 ml infusion volume) was used to prevent infection. Propofol (Methohexital 10 mg/ml @ 0.1 ml infusion) was used to check catheter patency. At this dose propofol administered intravenously causes rats to

lose muscle tone and become lethargic within a few seconds of the infusion. Rats that did not display signs of a functional catheter were excluded from further analyses. No rats were excluded due to loss of catheter patency.

Extended-Access Methamphetamine Self-Administration

Following 4-5 days of post-surgical recovery, all animals began 12, 6-hour long fixed-ratio 1 (FR1) self-administration sessions for methamphetamine (Sigma Aldrich, MO, USA) during the dark phase of their light cycle. During these sessions, active lever pressing resulted in a 0.1 mg/kg infusion of methamphetamine or saline administered over ~5.9 sec and the concomitant illumination of the cue-light located above the active lever. Illumination of the cue-light terminated at the completion of the drug infusion. Following the infusion and cue-light presentation, the house light illuminated signaling a 20-sec timeout period during which active lever presses were recorded but were inconsequential. Similarly, inactive lever presses were recorded but had no programmed consequence. Catheter patency was re-checked before the first and following the last self-administration session via propofol infusion (10 mg/ml; 0.1-0.15 ml, i.v.). To achieve stable responding, animals were required to average 20 or more methamphetamine infusions across the final 6 self-administration sessions. No animals were excluded due to this stability criterion. There were no stability criteria for saline self-administering animals.

Forced Abstinence and URB597 Treatment Period

Following completion of 12 self-administration sessions, all animals entered a forced abstinence period. During the abstinence period all animals remained in their home cages and

were not exposed to the drug, self-administration chamber, or drug-associated cues. Animals received daily intraperitoneal injections of either the FAAH inhibitor, URB597 (Focus Biomolecules, Plymouth Meeting, Pennsylvania) at either 0.3 mg/kg or 1 mg/kg (ip; dissolved in 15% DMSO, 5% Tween-80 and sterile saline) or vehicle (ip; 15% DMSO, 5% Tween-80 and sterile saline). Stock solutions of URB597 were prepared in large volumes in advance to minimize batch-to-batch variability and were pipetted into separate 2 mL aliquots and frozen at -20°C. When preparing to inject each abstinence day, one aliquot of each dose was removed and thawed to room temperature. All other aliquots were maintained at -20°C to ensure stability of the URB597 stock solutions over the duration of the abstinence period. These doses have previously been demonstrated to be effective in attenuating cue-induced reinstatement and relapse to cocaine and nicotine (Chauvet et al., 2014; Forget, Coen, & Foll, 2009).

Cue-Induced Relapse Testing

Cue-induced relapse was assessed at Withdrawal Day 2 (animals received first URB597 dose on Withdrawal Day 1) and Withdrawal Day 28 using a within-subjects manipulation. Animals did not receive injections of URB597 on cue-test days, consistent with methods described in Chauvet et al. (2014). During the cue-induced relapse test, the motivational impact of drug-associated cues was evaluated by reinforcing active lever presses with presentations of the previously associated drug-cue but no delivery of the drug itself. All relapse tests were 2 hours in duration and active lever presses resulted in the illumination of the previously associated cue light and houselight. There was no timeout period during the cue-induced relapse tests as no drug infusions were administered. Inactive lever presses were recorded but had no programmed consequence. All experimental methods are summarized in Figure 3.

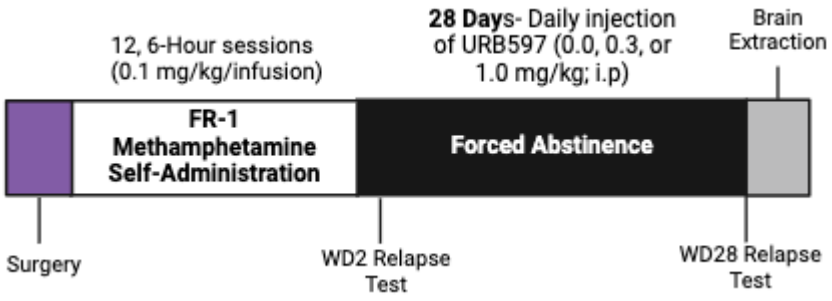


Figure 3. Overview of experimental timeline.

Tissue Dissection and Protein of Interest Analysis

The following sections describe the details and methods involved in the quantification of eCB and glutamate receptor expression via immunoblotting, and anandamide (AEA) levels via enzyme-linked immunosorbent assays (ELISAs).

Tissue Preparation

Immediately following the cue-test on WD28, animals were rapidly decapitated to allow for dissection of the dorsomedial prefrontal cortex (dmPFC), ventromedial prefrontal cortex (vmPFC), nucleus accumbens (ACb), and dorsal hippocampus (dHipp). Anesthetic agents were not used prior to decapitation as previous work has shown that sevoflurane and isoflurane significantly lowers anandamide levels and FAAH activity (Schelling et al., 2006; Patel et al., 2003). One mm coronal sections were collected using a brain matrix and 2 mm biopsy punches were used to isolate each region of interest. Tissue punches from each region of interest were immediately transferred to aliquots and flash frozen at -80°C. Punches from the dmPFC, vmPFC, and NAc were transferred to 1 mL of sucrose harvest buffer containing 10 µL of protease inhibitor cocktail (ThermoScientific Protease Cocktail). Samples were then homogenized via sonication, incubated on ice for 15-20 minutes, and then frozen at -80°C until further use. Figure 4 shows the punching locations for each region of interest.

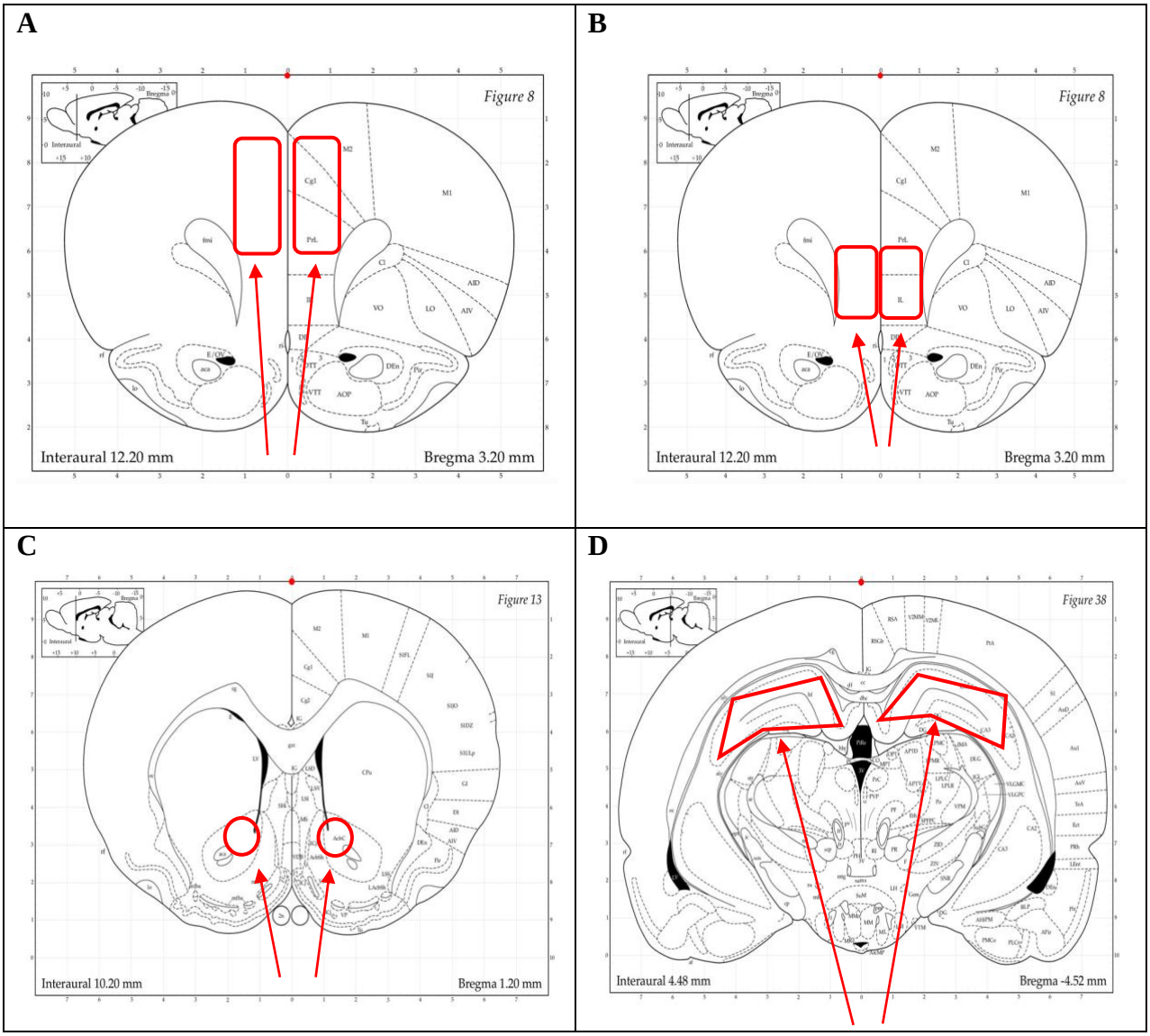


Figure 4. Anatomical location of tissue collection. A) Punching area for the dorsomedial prefrontal cortex. B) Punching area for the ventromedial prefrontal cortex. C) Punching area for the nucleus accumbens. D) Punching area for the dorsal hippocampus.

Tissue Fractionation

Tissue homogenates were removed from the freezer and thawed to room temperature. Given that all receptors of interest (CB_1, FAAH, and mGluR5) were membrane-bound, the cellular membrane fraction was targeted. To achieve the membrane fraction, the initial thawed sample was transferred to a refrigerated 4°C centrifuge and spun for 5 minutes[1] at 1,000g. The pellet (P_1) from this initial spin removed the nuclear fraction. The supernatant (S_1) was then transferred to a new aliquot and contains the crude membrane and cytosolic fractions. This S_1 fraction was then for another 20 min at 10,000g. The resultant supernatant (S_2) was pipetted into a new aliquot and 2 µL of protease inhibitors were added. This S_2 fraction contains the cytoplasmic fraction and was used to quantify AEA levels via ELISA. The pellet produced by the second spin (P_2) contains the cellular membrane fraction, the fraction used for quantification of all membrane-bound proteins. Pellets from the dHipp, and NAc were re-suspended in 200 µL of NP-40 lysis buffer and 2 µL of protease inhibitors and briefly vortexed. Pellets from the dmPFC and vmPFC were re-suspended in 175 µL of NP-40 lysis buffer and 2 µL of protease inhibitors and briefly vortexed. The resultant samples were incubated on ice for 15 minutes and then transferred to –80 C for long term storage.

Pierce Bicinchoninic Acid (BCA) Total Protein Assay

Total protein concentrations were measured via the Pierce BCA total protein assay to inform total protein concentrations prior to western blotting and ELISA testing. Briefly, the Pierce BCA assay relies on two reactions. First, the peptide bonds present in the protein reduce

[1] Due to lower total protein yield in the dmPFC and vmPFC, longer centrifugation times were required to produce adequate pelleting.

the Cu^{2+} ions in the bicinchoninic acid working reagent to Cu^{+} ions. Second, the two molecules of bicinchoninic acid chelate to a Cu^{+} ion. This reaction creates the purple color characteristic of the Pierce BCA assay. The amount of Cu^{+} that is chelated is directly proportional to the amount of protein in the sample. As such, the more protein there is in the sample, the darker the purple hue of the sample (Thermo Scientific).

Thermo Scientific albumin standards were diluted according to manufacturer specifications. This will yield albumin standards ranging from 25-2,000 ug/mL to generate the standard curve. Once albumin standards were prepared, 200 µL working reagent was pipetted into a 96 well-plate. Next, 25 µL of each albumin standard or unknown sample were added to each well, as well as a blank that contained the sample buffer (NP-40 lysis buffer or sucrose harvest buffer). Following complete loading of the plate, samples were gently agitated on an orbital shaker and incubated at 37°C for 30 minutes. Following 30 minutes of incubation, total absorbance was measured at a wavelength of 562 nm. The absorbance reading of all unknown samples was compared against the standard curve of known albumin standards. The absorbance readings of all unknown samples were run in duplicate and the average of the measurements was used to inform total protein adjustments for western blotting. These total protein assessment methods were used for both the pellets used in future western blot analysis and the supernatants used in future ELISA measurements.

Given that I did not predict any individual differences with regard to protein of interest expression, tissue homogenates were then pooled within their respective treatment conditions. These pooling procedures are consistent with previous literature when there are no specific predictions regarding individual differences in protein expression (Reich, Taylor, & McCarthy, 2009; Taylor & Posch, 2014; Hudson et al., 2019). As such, three samples from each treatment

condition were combined to give two pools of analysis for each treatment condition (6 total treatments; 12 pool samples). These pooled samples were then run through the Pierce BCA procedure detailed above to obtain the total protein concentration and equal protein loading for the western blot portion of this experiment. These pooling procedures were identical for the supernatant samples used in the anandamide ELISA analysis.

Western Blot

One mm thick precast acrylamide gel (4-20% bisacrylamide) were obtained from Bio Rad. Ten µg of total protein from the dHipp and NAc were added to loading buffer containing Lamelli buffer and beta-mercaptoethanol. Due to lower sample protein concentrations in the dmPFC and vmPFC, 7 µg of total protein from the dmPFC and vmPFC was added to the loading buffer. A total of 30 µL of diluted sample was loaded into the SDS-PAGE gel. Samples within the same self-administration and treatment conditions were pooled prior to loading. Proteins were separated under electrophoresis conditions of 100 volts for a total of 90 minutes. The electrophoresis tank was surrounded with ice to prevent excessive heat buildup over the 90-minute electrophoresis phase. Following 90 minutes, the gel was removed and rinsed with deionized water and placed in transfer buffer while the transfer sandwich and apparatus are prepared. The transfer sandwich was constructed in the following order from the black side to clear side of the transfer cassette: a sponge, two pieces of filter paper, the gel, PVDF membrane, two pieces of filter paper, and a sponge. Once constructed the transfer cassette was loaded into the Bio Rad protein transfer apparatus and run at 80 volts for 120 minutes. Again, the transfer tank was kept in a larger mouse cage containing ice to keep the apparatus cool.

For the immunoblotting phase, all assays began by blocking the membrane with 3% non-fat dry milk containing TBST for 30 minutes under gentle agitation on a plate rocker at room temperature. Prior to primary antibody incubation, the blocking solution was poured off the membrane and the primary antibody solution was added to the membrane and left on the plate rocker to incubate overnight at 4°C. Primary antibodies (CB_1 , rabbit polyclonal, Cell Signaling, ~60kDa, 1:500; FAAH, rabbit monoclonal, Cell Signaling, ~63 kDA, 1:200; mGluR5, rabbit monoclonal, Cell Signaling, ~260 kDa, 1:5,000) were dissolved in 3% non-fat dry milk +TBST. The mGluR5 band at 260 kDa was analyzed as the band from 260-300 kDa comprises the functional subunits of this receptor (Jiangami et al., 2003; Murray et al., 2021). Table 1 contains information regarding the vendor, product number and dilution of all primary antibodies used. All primary antibody concentrations were optimized for this procedure. The next morning, the primary antibody solution was removed, and the membrane was washed every 10 minutes with TBST for 1 hour. During this time, secondary antibody solutions were prepared by diluting goat anti-rabbit (ab205718) conjugated with horseradish peroxidase in a 5% non-fat dry milk solution. The secondary antibody was then applied to the membrane and left rocking for 1 hour at room temperature. Following secondary antibody incubation, the membrane was washed again with TBST, exchanging the TBST every 10 minutes for 1 hour. Immunoblotting was visualized by binding the horseradish peroxidase-conjugated secondary antibody with chemiluminescent substrate (WesternSure; LiCor). Prior to imaging, the bands of present in the lane containing the protein standard ladder (Precision Plus Dual Color; Bio Rad) were marked with the WesternSure standard pen (LiCor) containing toulene to react under the chemiluminescent matrix. One and a half milliliters of ECL solution was applied to each membrane for approximately 4-5 Minutes prior to imaging. Visualization of the blot was conducted using a LiCor C-Digit blot imager. All

exposure times were optimized for each protein of interest. The signal of each image was measured via densitometric analysis in ImageJ. The total signal of each protein of interest was normalized to the loading control, Calnexin (~ 90kDa; rabbit polyclonal, Abcam, 1:20,000) which was applied to each membrane.

Table 1. *Summary of primary antibodies used.*

Antibody	**Dilution (TBST+3% NFDM)**	**Vendor**	**Identifier**
Rabbit anti-mGluR5	1:5,000	Cell Signaling	55920
Rabbit anti-FAAH1	1:250	Cell Signaling	9179
Rabbit anti-CB_1	1:500	Cell Signaling	93815
Rabbit anti-Calnexin	1:20,000	Abcam	ab22595

Enzyme-Linked Immunosorbent Assay (ELISA)

Previous work has shown that incranial infusions of AEA into the ACb is effective in restoring glutamate homeostasis and for reducing cue-induced cocaine seeking (Zhang et al., 2021). Given these findings, we examined AEA in the ACb in order to assess the effect of methamphetamine abstinence and URB597 treatment on AEA. Commercial competitive ELISA kits (MyBioSource: MBS7254942) were used to determine the AEA concentrations within the ACb. As previously described in the tissue fractionation methods, the S_2 supernatant was used for ELISA analysis as this fraction contains the cytoplasmic fraction. Prior to performing the

ELISA, total protein concentrations of the S_2 fractions was determined using the previously described Pierce BCA assay method. These total protein concentrations were used to inform the sample dilutions utilized in the ELISA to ensure that sample concentrations fell within standard curve produced by the six standards provided in the ELISA kit. All reagents were prepared according to kit procedures provided.

Prior to beginning the assay, the appropriate number of pre-coated wells were removed and secured in the microtiter plate (unused pre-coated wells were returned the sealed package and stored at 4° C until use). Once secured, 100 µL of each standard or sample was added to the precoated plates and 100 µL of PBS was added as a blank control well. All standards, blanks, and samples were run in duplicate. Next, 10 µL of balance solution was to the unknown samples and mixed by gently pipetting up and down three times. Following the balance solution, 50 µL of the enzyme conjugate solution was added to the standard and sample wells but not the blank control well. Again, wells were mixed by pipetting up and down several times. The plate was covered and left to incubate for 1 hour at 37° C. After a 1 hour incubation period, the microtiter plate was manually washed by emptying the plate contents into a waste container, and each well was filled with 1X wash solution and then removed. The microtiter plate was rinsed a total of five times. Following the final wash, 50 µL of Substrate A and 50 µL of Substrate B were added to all of the wells in the plate (standards, samples, and blanks) and the plate was left to incubate for 20 minutes at 37° C. Following incubation, 50 µL of stop solution was added to all plate wells to terminate the enzymatic reaction. Immediately after adding the stop solution, the plate was transferred to a microplate reader (BioTek) and optical densities (OD) were measured at 450 nm.

Each of the duplicate OD readings for each of the six AEA standards were averaged and subtracted from the mean of the blank control well. A standard curve was constructed by linearizing the standard curve by taking the natural log of the concentration plotted against the natural log of the absorbance reading (450 nm). This linear function was used to calculate the concentration of all unknown sample concentrations, which was expressed in ng/mL. Finally, all sample AEA concentrations were normalized to the total protein present in the sample by dividing the AEA concentration by the total protein value from the Pierce BCA assay. All AEA concentrations are thus expressed as ng of AEA per 1 µg of total protein.

Data Analysis

Self-Administration

Responding over the course of the 12, extended-access methamphetamine self-administration sessions was analyzed using linear multilevel modeling. The number of active lever presses, excluding those that occurred during the 20 second timeout period, served as the primary dependent variable for all analyses. Self-administration multilevel models specified the following fixed effects: the drug that was self-administered (meth or saline), session (continuous variable), and a drug x session interaction. Models utilizing different random effect structures were compared using Akaike's Information Criterion (AIC) to determine optimal model selection.

Cue-Induced Relapse Tests

Active-lever responding during the WD2 and WD28 relapse tests was also assessed using linear multilevel modeling. Importantly, there was no timeout period during the cue-induced relapse test as no drug was infused during these sessions. As such, all active lever-responding during these sessions were quantified as active-lever presses regardless of when they occurred during the session. The self-administration condition (methamphetamine or saline), dose of URB597 (0.3 mg/kg, 1.0 mg/kg, or vehicle), Withdrawal Day (WD2 or WD28), and all possible two- and three-way interactions were coded as fixed effects. Models utilizing different random effect structures were compared using Akaike's Information Criterion (AIC) to determine optimal model selection.

Western Blotting

Expression of all proteins of interest was normalized to Calnexin expression. Following normalization, expression of each protein of interest for each region of interest was analyzed via separate 3 (3 doses of URB597) x 2 (Meth or Saline) full factorial ANOVA. Tukey's HSD pairwise comparisons were used where appropriate. Significance levels were set to $\alpha = .05$ for all analyses conducted.

Anandamide (AEA) ELISA

AEA concentrations in the ACb were normalized to the total protein concentration in that sample and expressed as ng of AEA per ug of total protein. These sample concentrations were the analyzed using a 3 (3 doses of URB597) x 2 (Meth or Saline) factorial ANOVA. Tukey's

HSD pairwise comparisons were used where appropriate. Significance levels were set to $\alpha = .05$ for all analyses conducted.

Chapter 3 - Hypotheses

Self-Administration

Overall, I predicted greater responding in methamphetamine self-administering animals when compared to saline self-administering animals. Additionally, there is a wealth of evidence suggesting reliable escalations in drug-intake for extended-access (6-8 hour access/session) methamphetamine self-administration (Murray et al., 2019; Funke et al., 2023; Scheyer et al., 2016). As such, I predicted that methamphetamine self-administration, as measured by the number of infusions earned, would significantly increase over the course of the 12 self-administration sessions.

Cue-Induced Relapse and Incubation of Craving

Previous evidence has shown that peak craving for methamphetamine occurs around 28-30 days of abstinence to methamphetamine (Murray et al., 2019; Funke et al., 2023; Scheyer et al., 2016). Additionally, previous evidence showing the efficacy of URB597 treatment in attenuating cue-induced cocaine-seeking was tested at Withdrawal Day 28. Generally, I predicted that methamphetamine-craving would significantly increase from WD2 to WD28 consistent with reports of incubations in craving in response to extended-access methamphetamine self-administration. I predicted that treatment with URB597 would significantly lower cue-induced methamphetamine responding at WD28 when compared to vehicle-treated animals. Given that I predicted low levels of cue-induced methamphetamine craving at WD2, I did not expect that URB597 treatment would be effective in lowering relapse at WD2. Additionally, I predicted that treatment with URB597 would preclude incubations in craving from WD2 to WD28.

Immunoblotting

The following subsections breakdown individual hypotheses for mGluR5, CB_1, and FAAH expression changes following 28 days of abstinence to methamphetamine. Given that I did not predict URB597 administration to be effective in lowering relapse at the WD2 timepoint, tissue was not collected to assess changes during acute abstinence. The first two proteins of interest were chosen in light of the involvement of mGluR5 and CB_1 in the negative feedback regulation of presynaptic glutamate described previously. Expression of FAAH is of interest given that it is the primary mechanism for URB597 treatment and AEA metabolism.

MGluR5

Given that previous research has shown that surface ACb core mGluR5 expression is not shown to be affected by prolonged abstinence to methamphetamine (Murray et al., 2021), I did not predict that mGluR5 would be disrupted in the ACb, mPFC, or dHipp. However, it is important to note that specific characterizations of mGluR5 expression in the response to methamphetamine abstinence have not been previously conducted in the mPFC or dHipp and are therefore, of interest to the proposed study. Given the role of mGluR5 in facilitating LTD at glutamatergic synapses, I predicted that treatment with URB597 would dose-dependently increase the expression of mGluR5 in the rats that previously self-administered methamphetamine. Such a finding would be consistent with other previous literature showing that URB597 is effective in removing AMPA subunits from the synapse (Wu et al., 2020).

CB_1

Consistent with previous research showing that repeated administration of psychostimulants produces increases in CB_1 receptor expression (Blanco et al., 2015), I predicted that I would see increased surface-level expression of CB_1. I predict that this increased expression will be seen in all brain areas for animals exposed to methamphetamine. Though theoretical, I propose that increased CB_1 receptor expression during abstinence to methamphetamine serves as a compensatory response for decreased AEA concentrations that occur in response to psychostimulants (Thiemann et al., 2008).

Further, prior literature has shown that six days of URB597 (0.5 mg/kg) administration decrease CB_1 receptor expression in the ACb and Hipp (Marco et al., 2009). As such, I predicted that I would observe similar decreases in CB_1 expression induced by URB597 in methamphetamine-naïve rats across all brain areas. In the context of my theoretical framework, I propose that the increased AEA levels produced by URB597 treatment would attenuate the compensation in CB_1 expression that occurs in the absence of URB597 treatment.

FAAH

Previous research has shown decreased AEA concentrations in the ACb in response to chronic amphetamine (Thiemann et al., 2008). Additionally, chronic cocaine exposure has been shown to increase expression of FAAH, further suggesting low resting levels of AEA in response to psychostimulants (Blanco et al., 2015). Overall, I predicted that methamphetamine would increase expression of FAAH (thus leading to perpetually low AEA levels). Generally, I predicted that treatment with URB597 would attenuate these increases in FAAH expression (presumably by restoring AEA levels).

With regard to the effect of URB597 of FAAH expression, previous work has shown that acute treatment with URB597 (0.3 mg/kg; ip) produces modest decreases in FAAH expression in the Hipp (Rivera et al., 2015). Consistent with this research, I predicted that both doses of URB597 would significantly decrease FAAH expression in the Hipp for methamphetamine-naïve rats. However, given that Rivera et al. (2015) did not observe changes in the striatum following repeated treatment with URB597, I did not predict that URB597 would significantly alter FAAH expression in the ACb.

Anandamide Concentrations

To our knowledge, this is the first study of its kind to assess AEA levels following subtoxic dosing of methamphetamine. I predicted that AEA concentrations in the ACb would be significantly lowered by previous exposure to methamphetamine, similar to previous reports using amphetamine (Thiemann et al., 2008). The theoretical basis and significance of this prediction stems from my hypothesis that decreased AEA levels engender a significant upregulation in CB_1 in order to restore a homeostatic eCB tone. I propose that exposure previous exposure to methamphetamine produces systemic decreases in AEA and increased cue-induced craving during abstinence. Given that URB597 inhibits the metabolism of AEA via FAAH, I predicted that URB597 treatment would significantly increase AEA concentrations in the ACb for both methamphetamine and saline self-administering animals.

Chapter 4 - Results

Cohort Active-Responding During 6-Hr Self-Administration

Given that the number of animals required to complete the study exceeded the number of available operant boxes, four separate cohorts of animals underwent extended-access methamphetamine self-administration. Therefore, the cohort that the animal was in was included as a factor in preliminary analyses of methamphetamine self-administration. This preliminary analysis specified both the intercept and session slope of each rat as random effects. The self-administration cohort of each rat (1-4) was specified as the sole fixed effect in the multilevel model. This multilevel analysis was conducted solely on methamphetamine self-administering animals using data from the second week of self-administration. Results of this preliminary multilevel model indicated that there were no significant differences in active-lever responding between any methamphetamine cohorts during the second week of self-administration, $F(3, 150) = 2.38$, $p = .07$. Active-lever responding during the second week of self-administration is shown in Figure 5.

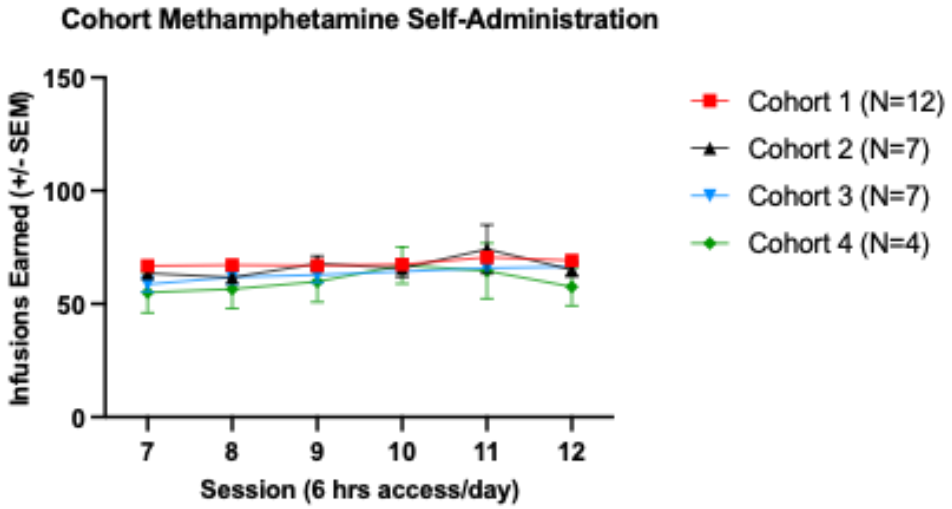


Figure 5. Mean (± SEM) methamphetamine infusions earned (0.1 mg/kg/infusion) during the second week of extended-access FR-1 self-administration training.

Responding During Extended-Access Methamphetamine Self-Administration

Linear multilevel modeling was used to examine differences in responding during the 12 extended-access sessions. Preliminary models were run to determine optimal random effect structure. Aikaike's information criterion (AIC) was used to compare models consisting of different random effect structures. In self-administration Model 1, only the intercept was specified as a random effect. This analysis yielded an AIC value of 4742. In self-administration Model 2, both the intercept and session slope of each subject were specified as random effects. This random effect structure yielded a modest decrease in AIC, ultimately yielding a value of 4737. As such, future self-administration analyses will specify both the intercept and subject slope as random effects. Results of these two models are summarized in Tables 2 and 3. Results of the linear multilevel model examining active-lever responding during self-administration found a significant main effect of the drug self-administration condition (Meth or Saline) such that average active lever responding was greater in methamphetamine animals when compared to saline animals, $B = 20.76$, $SE = 0.65$, $t(572) = 32.17$, $p < .001$. Additionally, results indicated a significant fixed effect of the self-administration session, such that responding increased across the 12 self-administration sessions, $B = 1.03$, $SE = 0.19$, $t(572) = 5.52$, $p < .001$. Finally, there was a significant two-way interaction between the drug self-administration condition (Meth or Saline) and the self-administration session, $B = 1.03$, $SE = 0.21$, $t(572) = 4.96$, $p < .001$. Post-hoc probing of this interaction indicated a significant escalation in active-lever responding for methamphetamine animals when compared to saline animals. These results are shown in Figure 6 and model parameter estimates are shown in Table 3.

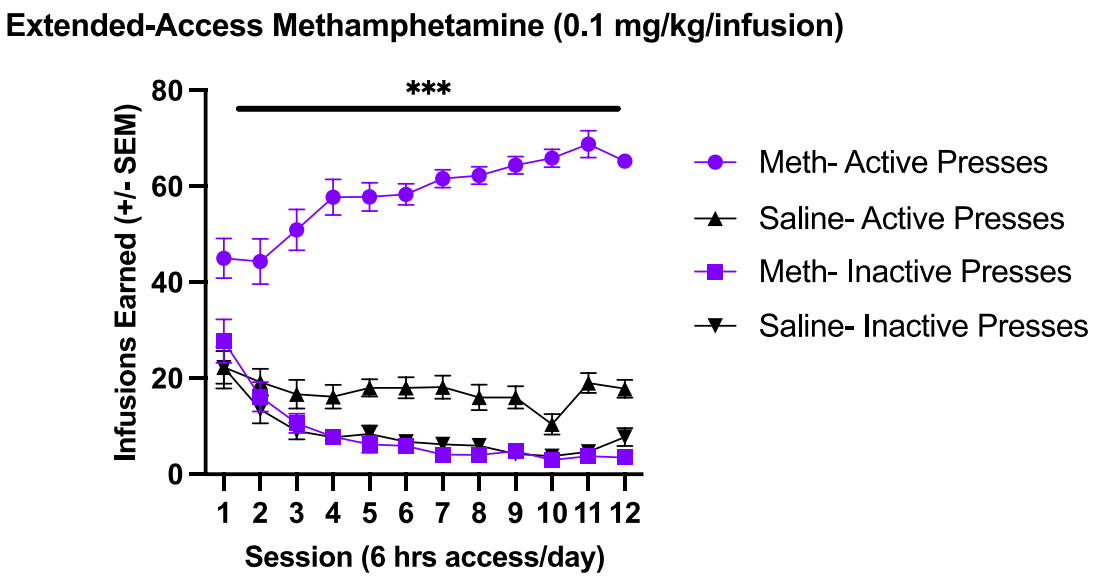


Figure 6. Average infusions earned (± SEM) during extended access self-administration. Results indicated that active-lever responding was significantly greater in methamphetamine animals compared to saline ($p < .001$). Methamphetamine-intake significantly increased over the course of self-administration ($p < .001$).

Table 2. *Fixed Effects and Model Fit Statistics for Self-Administration Multilevel Model Using Only the Intercept Random Effect*

					Model Statistics		
	B	*SE*	*t*	*p*	R^2	$R^2_{adjusted}$	*AIC*
Intercept	32.05	1.31	24.37	**< .001**	0.66	0.66	4742
Drug (Meth/Sal)	20.76	0.65	32.17	**< .001**			
Session	0.97	0.19	5.21	**< .001**			
Drug * Session	1.27	0.19	6.81	**< .001**			

Note: Significance is indicated by boldfaced font

Table 3. *Fixed Effects and Model Fit Statistics for Self-Administration Multilevel Model Using Both Intercept and Subject Slope of Each Session as Random Effects*

					Model Statistics		
	B	*SE*	*t*	*p*	R^2	$R^2_{adjusted}$	*AIC*
Intercept	31.67	1.31	24.18	**< .001**	0.67	0.67	4737
Drug (Meth/SAL)	20.75	0.64	32.42	**< .001**			
Session	1.03	0.19	5.52	**< .001**			
Drug * Session	1.03	0.21	4.96	**< .001**			

Note: Significance is indicated by boldfaced font

Cue-Induced Relapse Responding

Linear multilevel modeling was used to assess active-lever responding during the cue-induced relapse sessions on Withdrawal Day 2 and Withdrawal Day 28. The self-administration condition (methamphetamine or saline), dose of URB597 (0.3 mg/kg, 1.0 mg/kg, or vehicle), Withdrawal Day (WD2 or WD28), and all possible two- and three-way interactions were coded as fixed effects while the intercept was specified as a random effect. Results of this multilevel indicated that there was a significant main effect of the self-administration condition (methamphetamine or saline), such that relapse responding was significantly higher in methamphetamine animals when compared to saline self-administering animals, $F(1, 86) = 90.20$, $p < .001$. Additionally, there was a marginally significant main effect of the Withdrawal Day (WD2 or WD28), such that responding was significantly higher on WD28 when compared to WD2, $F(1, 86) = 3.31$, $p = .07$ (see Figure 7). No other main effects or interactions, including treatment with URB597, were found to be significant predictors of relapse responding. Model statistics are shown in Table 4.

To assess further differences in responding that may have occurred during the relapse test sessions, an exploratory analysis was conducted in which only the data during the first 30

minutes of the WD2 and WD28 relapse test sessions was analyzed. This exploratory analysis used the same fixed and random effect structures described above. Results of this second relapse test multilevel model again indicated that there was a significant main effect of the drug self-administration condition, such that relapse responding during the first 30 minutes of each test session was significantly higher in methamphetamine self-administering animals compared to saline, $F(1, 86) = 79.86$, $p < .001$. There was a significant main effect of the Withdrawal Day with relapse responding increasing on Withdrawal Day 28 when compared to Withdrawal Day 2, $F(1, 86) = 5.22$, $p = .02$ (see Figure 8). These results are summarized in Table 5.

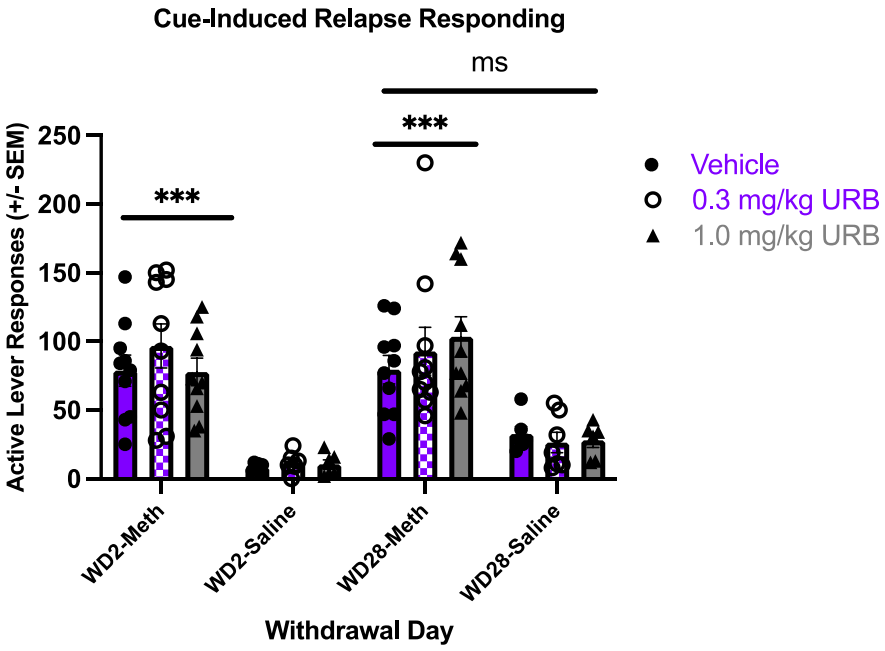


Figure 7. Mean active-lever responses (+/- SEM) during the WD2 and WD28 cue tests. Results showed that relapse responding was significantly greater in animals that previously self-administered methamphetamine (*** $p < .001$). There was marginally significant increase in responding on the WD28 cue-test when compared to the WD2 cue-test (ms, $p = .07$).

Table 4. *Fixed Effects and Model Fit Statistics for Cue-Induced Relapse Responding (Full session)*

				Model Statistics		
	F	*DF*	*p*	R^2	$R^2_{adjusted}$	*AIC*
Drug (Meth/Sal)	90.20	1	**< .001**	0.53	0.47	938
URB Dose	0.35	2	.71			
Withdrawal Day (WD)	3.31	1	.07			
Drug*URB Dose	0.50	2	.61			
Drug*WD	0.61	1	.44			
URB Dose*WD	0.42	2	.66			
Drug*URB Dose*WD	0.46	2	.64			

Note: Significance is indicated by boldfaced font

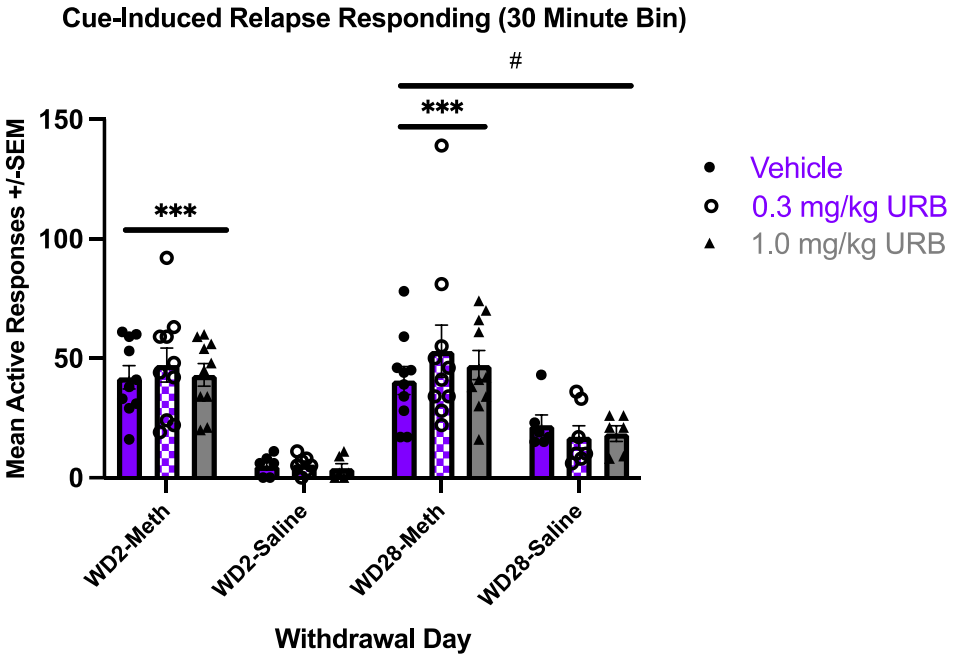


Figure 8. Mean active-lever responses (+/- SEM) during the first 30 minutes of the WD2 and WD28 cue tests. Results showed that relapse responding was significantly greater in animals that previously self-administered methamphetamine (*** $p < .001$). There was significant increase in responding on the WD28 cue-test when compared to the WD2 cue-test (# $p = .02$).

Table 5. *Fixed Effects and Model Fit Statistics for Cue-Induced Relapse Responding (30 Minute Bin)*

				Model Statistics		
	F	*DF*	*p*	R^2	$R^2_{adjusted}$	*AIC*
Drug (Meth/Sal)	79.86	1	**< .001**	0.51	0.45	825
URB Dose	0.28	2	.74			
Withdrawal Day (WD)	5.22	1	**.02**			
Drug*URB Dose	0.72	2	.49			
Drug*WD	2.34	1	.13			
URB Dose*WD	0.01	2	.99			
Drug*URB Dose*WD	0.24	2	.79			

Note: Significance is indicated by boldfaced font

Expression Changes in Cannabinoid Receptor-1 (CB_1)

Separate two-way factorial ANOVA was used to assess CB_1 expression changes in the dHipp, ACb, dmPFC, and vmPFC. The dependent variable in each ANOVA was the amount of relative CB_1 expression after being normalized to the loading control, Calnexin, on that given membrane.

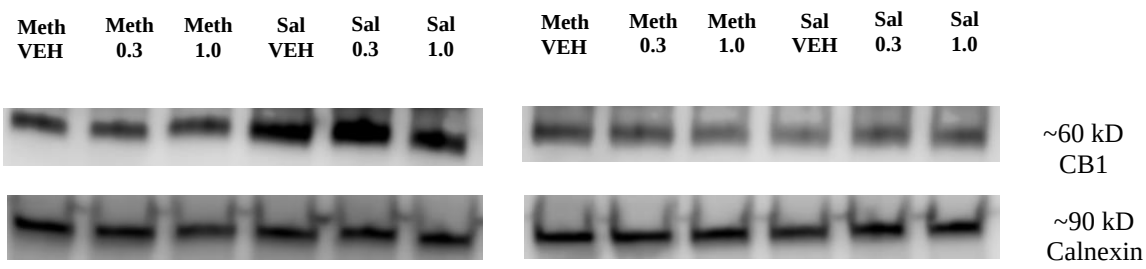
In the dHipp, the factorial ANOVA did not indicate that there were significant effects of either the drug self-administration condition, URB597 treatment, or their two-way interaction. These results are show in Figure 9 and summarized in Table 6.

The factorial ANOVA examining CB_1 receptor expression in the ACb did not indicate that there were significant main effects of either the drug self-administration condition or the URB597 treatment condition, and no significant drug * treatment interaction (See Figure 10 and Table 7).

In the vmPFC, results indicated that there was a marginally significant main effect of the drug self-administration condition, such that methamphetamine self-administering animals

showed marginally lower CB_1 expression when compared to saline control animals, $F(1, 6) = 5.88$, $p = .051$ (see Figure 11 and Table 8).

In the dmPFC, the two-way ANOVA indicated that there was a significant main effect of URB597 treatment, $F(2, 6) = 8.82$, $p = .02$. Post-hoc probing of this effect indicated that both the 1 mg/kg ($t(6) = 4.66$, $p = .01$) and 0.3 mg/kg ($t(6) = 3.10$, $p = .04$) doses of URB597 significantly increased CB_1 receptor expression when compared to the vehicle treatment. These results are shown in Figure 12 and Table 9.



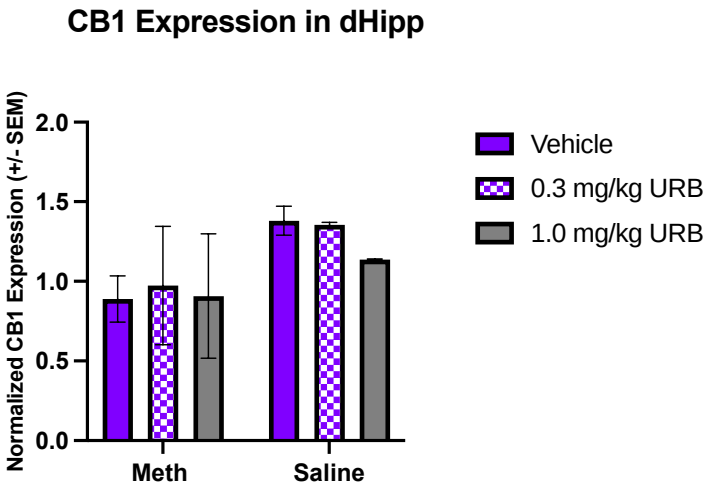


Figure 9. Mean normalized CB_1 expression (+/- SEM) in the dHipp. Neither methamphetamine self-administration or URB597 were found to significantly affect CB_1 expression in dHipp.

Table 6. *Results from Two-Way Analysis of Variance of CB_1 Expression in dHipp.*

	df	*SS*	*F*	*p*
Drug (Meth or Saline)	1	0.40	3.80	.09
URB Dose	2	0.05	0.21	.82
Drug*URB Dose	2	0.03	0.16	.85
Error	6	0.11		
Total	11	0.59		

Note: Significance is indicated by boldfaced font

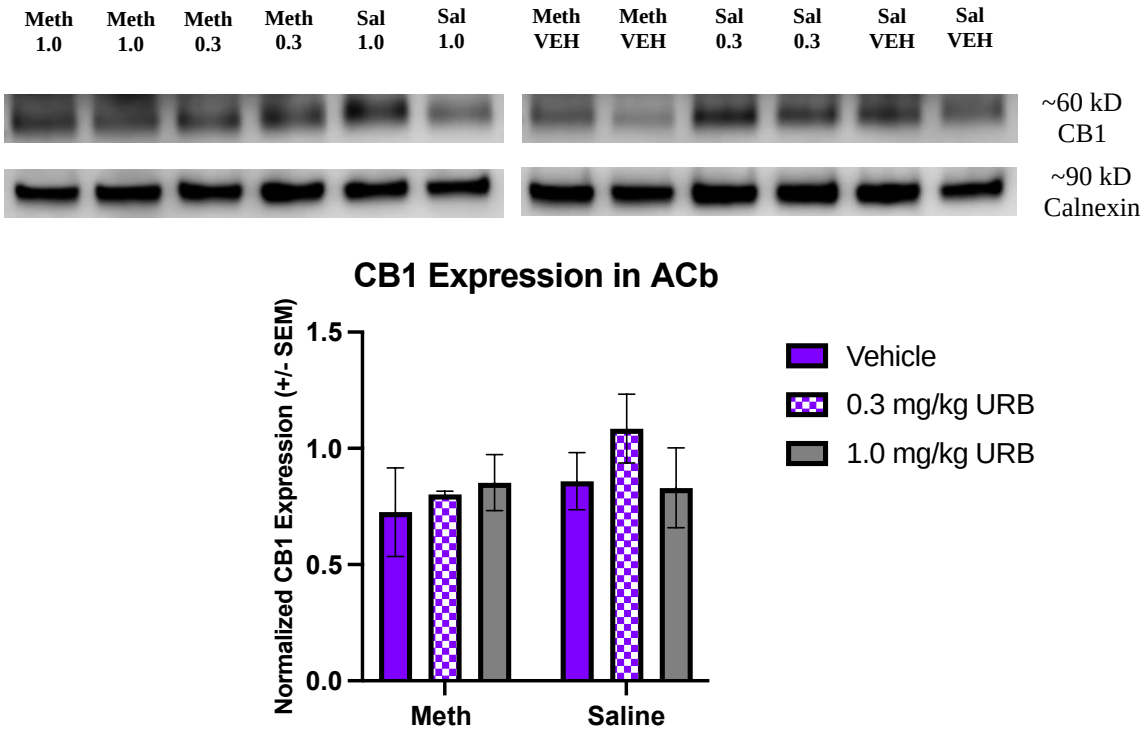


Figure 10. Mean normalized CB_1 expression (+/- SEM) in the ACb. Results did not indicate that methamphetamine self-administration and URB57 treatment did not affect normalized CB_1 expression.

Table 7. *Results from Two-Way Analysis of Variance of CB_1 Expression in ACb.*

	df	*SS*	*F*	*p*
Drug (Meth or Saline)	1	0.05	1.32	.29
URB Dose	2	0.05	0.60	.58
Drug*URB Dose	2	0.05	0.60	.58
Error	6	0.24		
Total	11	0.38		

Note: Significance is indicated by boldfaced font

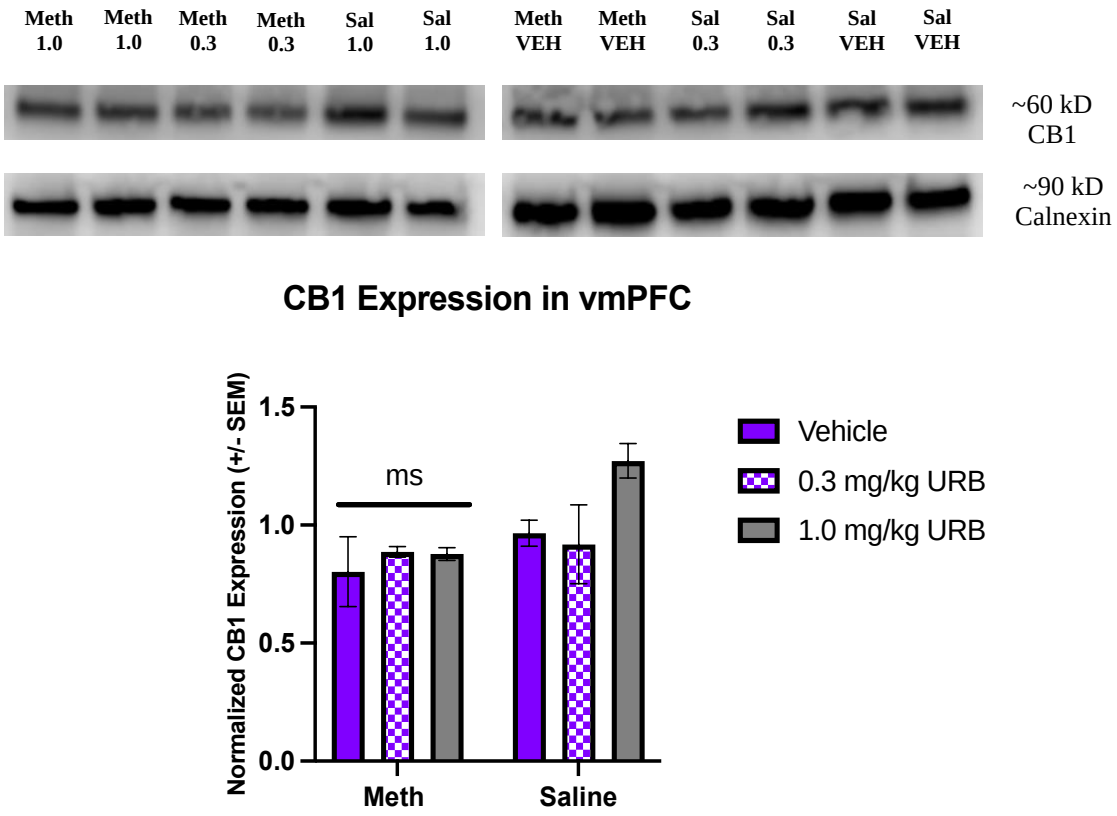
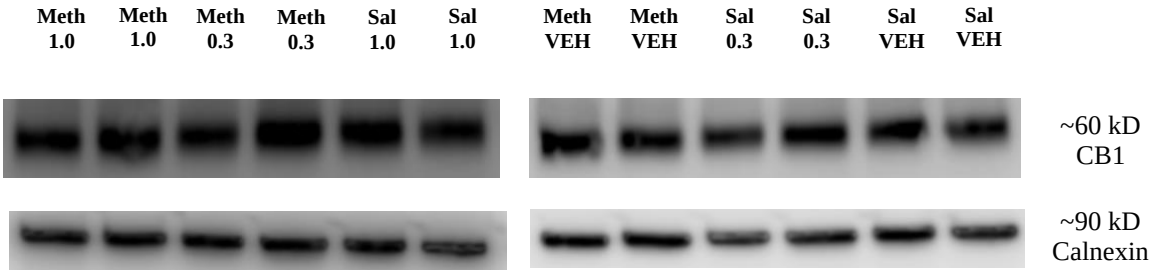


Figure 11. Mean normalized CB_1 expression (+/- SEM) in the vmPFC. Methamphetamine self-administration produced marginally significant decreases in normalized CB_1 expression (ms, $p = .051$).

Table 8. *Results from Two-Way Analysis of Variance of CB_1 Expression in vmPFC.*

	df	*SS*	*F*	*p*
Drug (Meth or Saline)	1	0.12	5.88	.051
URB Dose	2	0.09	2.25	.19
Drug*URB Dose	2	0.07	1.71	.26
Error	6	0.19		
Total	11	0.39		

Note: Significance is indicated by boldfaced font



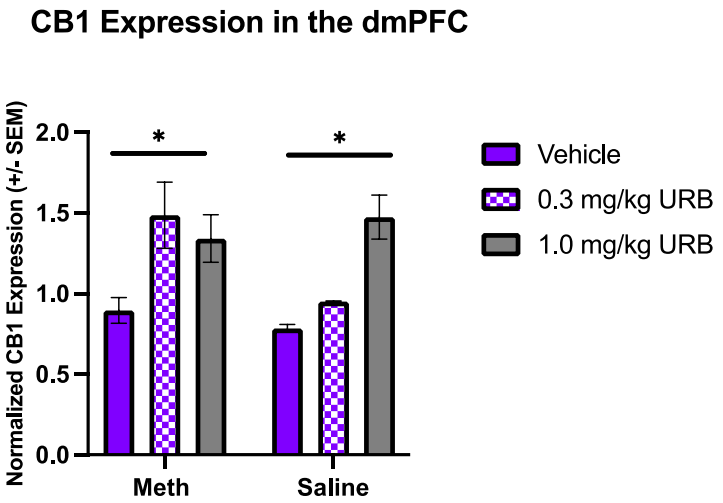


Figure 12. Mean normalized CB_1 expression (+/- SEM) in the dmPFC. Both doses of URB597 were shown to increase CB_1 expression compared to vehicle-treated animals, $p < .05$.

Table 9. *Results from Two-Way Analysis of Variance of CB_1 Expression in dmPFC.*

	Df	*SS*	*F*	*p*
Drug (Meth or Saline)	1	0.09	2.95	.14
URB Dose	2	0.67	11.24	**.01**
Drug*URB Dose	2	0.23	5.18	.08
Error	6	0.18		
Total	11	1.08		

Note: Significance is indicated by boldfaced font

Expression Changes in Metabotropic Glutamate Receptor-5 (mGluR5)

Separate two-way factorial ANOVAs were used to assess mGluR5 expression changes in the dHipp, ACb, dmPFC, and vmPFC. The dependent variable in each ANOVA was the amount of relative mGluR5 expression after being normalized to the loading control, Calnexin, on that given membrane.

In the dHipp, there was a significant main effect of URB597 treatment on normalized mGluR5 expression, such that the 0.3 mg/kg dose increased expression compared to the 1.0 mg/kg and vehicle doses, $F(2, 6) = 5.46$, $p = .04$. There was a significant two-way interaction between the self-administration condition and URB597 treatment, $F(2, 6) = 7.76$, $p = .02$, such that methamphetamine increased mGluR5 expression when compared to saline in vehicle-treated animals, $t(6) = -2.98$, $p = .03$ (see Figure 13 and Table 10). There was no significant difference in the mGluR5 expression of methamphetamine and saline self-administering rats at either dose of URB597 ($p > .05$).

In the ACb, the two-way ANOVA examining mGluR5 expression changes did not indicate that there were significant main effects of the drug self-administration condition (methamphetamine or saline) or URB597 treatment, or their two-way interaction (see Figure 14 and Table 11).

In the vmPFC, the ANOVA results showed a marginally significant main effect of the drug self-administration condition, such that normalized mGluR5 expression tended to be higher in animals that previously self-administered methamphetamine as compared to saline, $F(2, 6) = 4.51$, $p = .07$. There were no other significant main effects or interactions. These results are shown in Figure 15 and Table 12.

In the dmPFC, the two-way ANOVA examining mGluR5 expression changes did not indicate that there were significant main effects of the drug self-administration condition (methamphetamine or saline) or URB597 treatment, or their two-way interaction (see Figure 16).

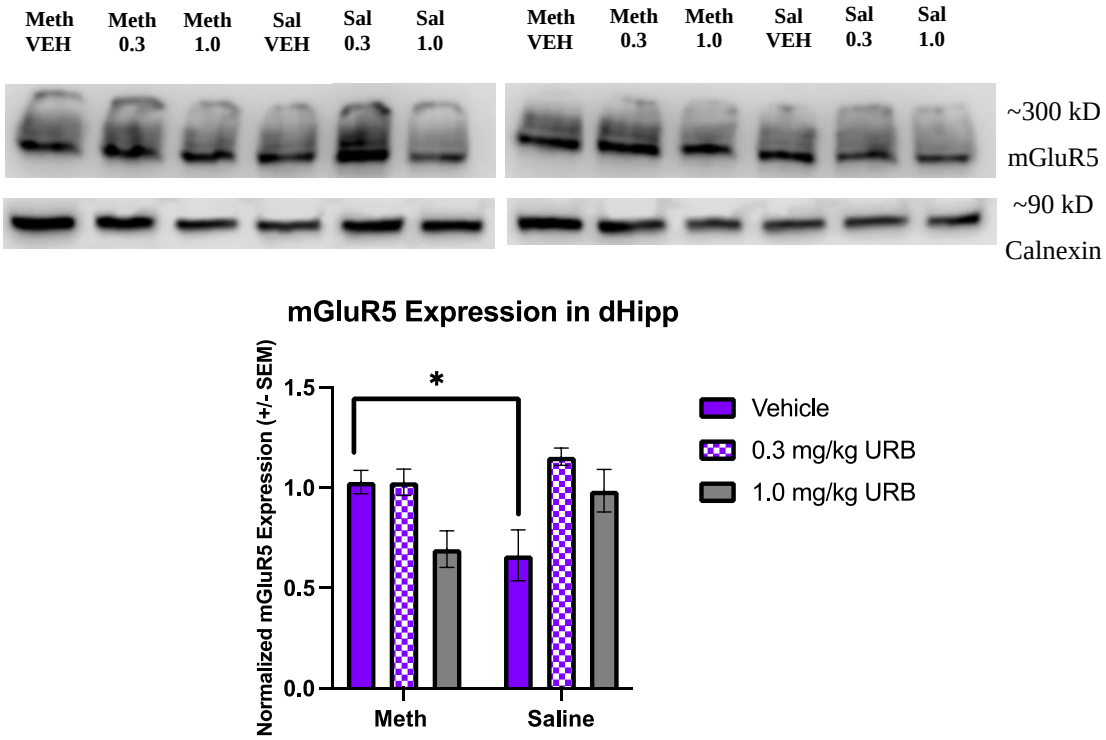


Figure 13. Mean normalized mGluR5 (+/- SEM) in the dHipp. Results indicated that methamphetamine exposure increased mGluR5 expression in vehicle-treated rats but not in URB597-treated rats, * $p < .05$.

Table 10. *Results from Two-Way Analysis of Variance of mGluR5 Expression in dHipp.*

	df	*SS*	*F*	*p*
Drug (Meth or Saline)	1	0.0009	0.06	.81
URB Dose	2	0.16	5.46	**.04**
Drug*URB Dose	2	0.23	7.76	**.02**
Error	6	0.09		
Total	11	0.49		

Note: Significance is indicated by boldfaced font

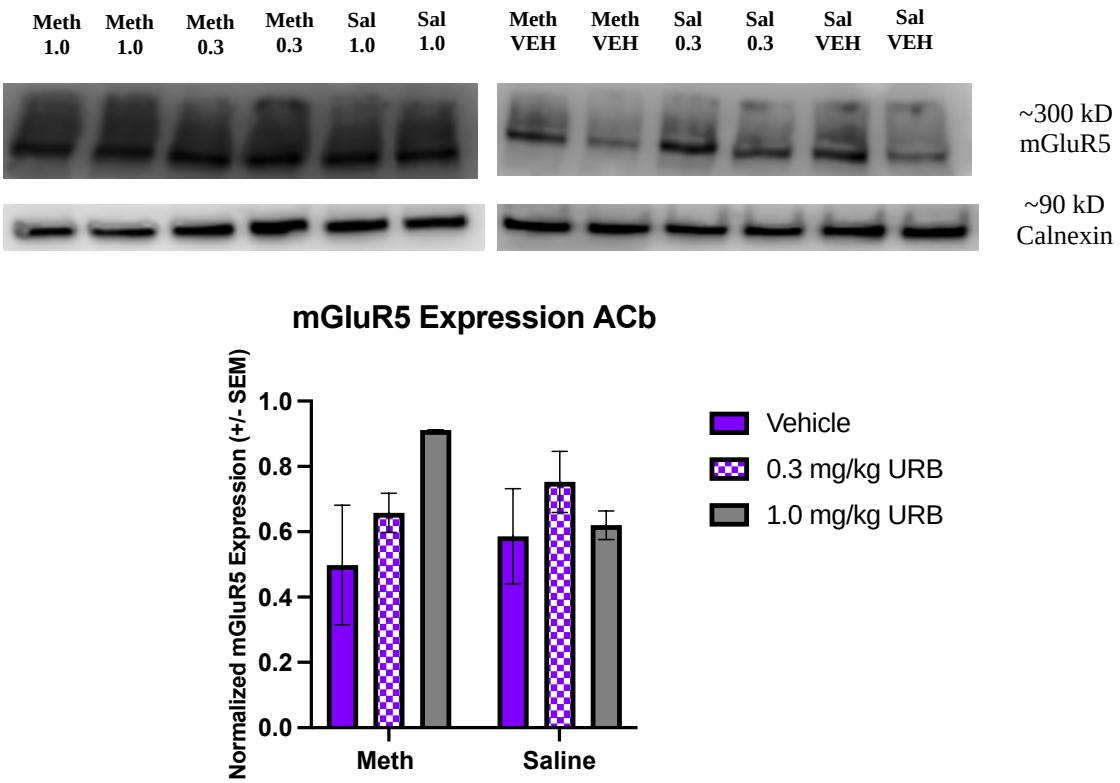


Figure 14. Mean normalized mGluR5 (+/- SEM) in the ACb. Results did not indicate that methamphetamine self-administration or URB597 treatment significantly affected mGluR5 expression in the ACb.

Table 11. *Results from Two-Way Analysis of Variance of mGluR5 Expression in ACb.*

	df	*SS*	*F*	*p*
Drug (Meth or Saline)	1	0.004	0.17	.69
URB Dose	2	0.11	2.33	.18
Drug*URB Dose	2	0.01	2.12	.20
Error	6	0.14		
Total	11	0.35		

Note: Significance is indicated by boldfaced font

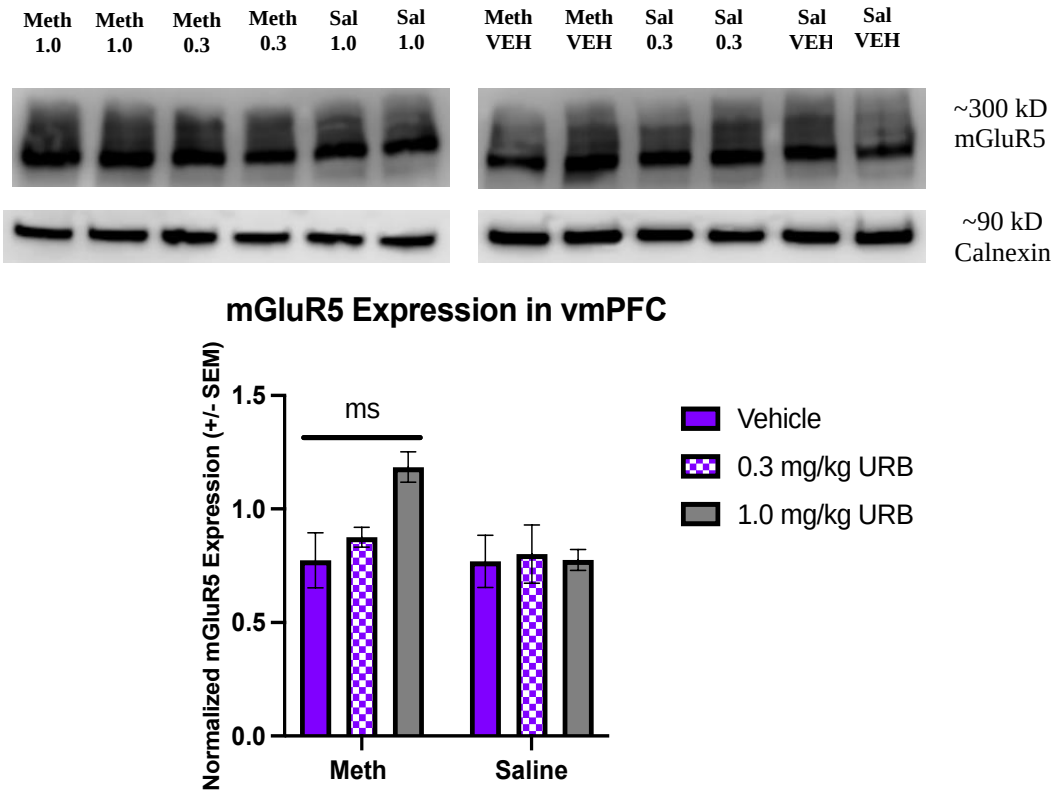


Figure 15. Mean normalized mGluR5 (+/- SEM) in the vmPFC. Results suggested a marginally significant increase in normalized mGluR5 expression for animals that previously self-administered methamphetamine as compared saline (ms, $p = .07$).

Table 12. *Results from Two-Way Analysis of Variance of mGluR5 Expression in vmPFC.*

	df	*SS*	*F*	*p*
Drug (Meth or Saline)	1	0.08	4.51	.07
URB Dose	2	0.09	2.58	.16
Drug*URB Dose	2	0.09	2.66	.15
Error	6	0.11		
Total	11	0.37		

Note: Significance is indicated by boldfaced font

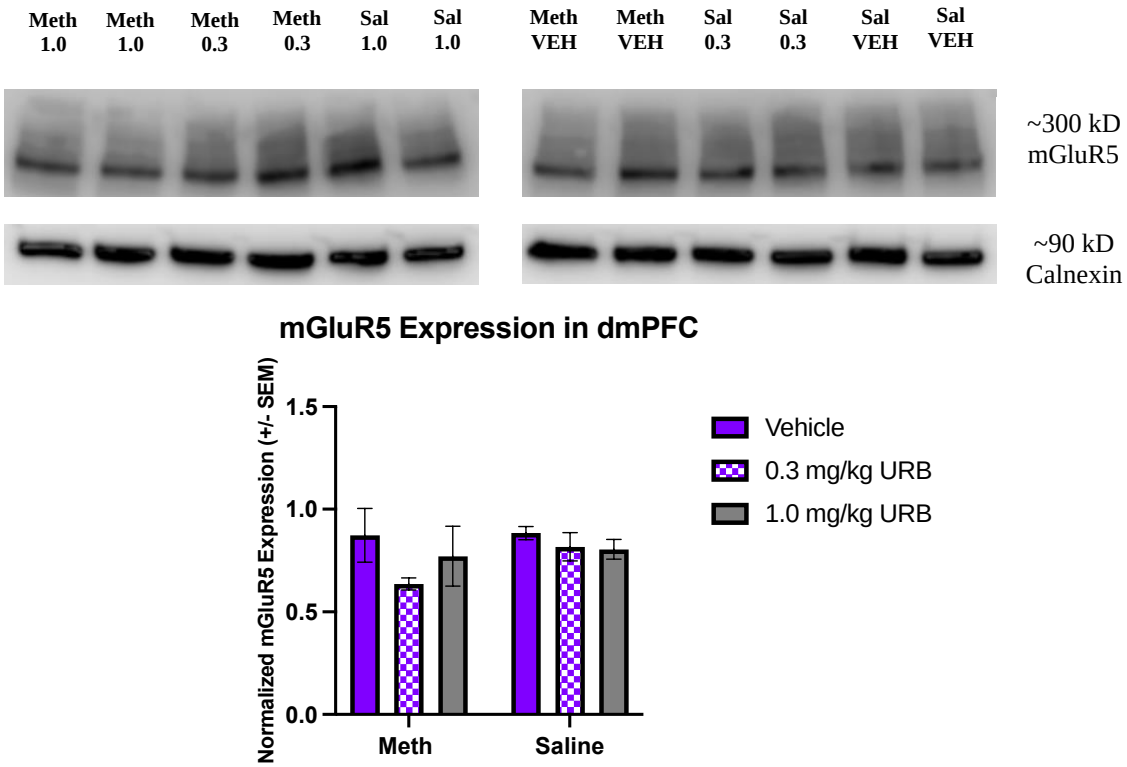


Figure 16. Mean normalized mGluR5 expression (+/- SEM) in the dmPFC. Analyses did not indicate significant effects of URB597 treatment or methamphetamine self-administration.

Table 13. *Results from Two-Way Analysis of Variance of mGluR5 Expression in dmPFC.*

	df	*SS*	*F*	*p*
Drug (Meth or Saline)	1	0.02	1.07	.34
URB Dose	2	0.05	1.48	.30
Drug*URB Dose	2	0.02	0.54	.61
Error	6	0.10		
Total	11	0.19		

Note: Significance is indicated by boldfaced font

Expression Changes in Fatty-Acid Amide Hydrolase (FAAH)

Separate two-way factorial ANOVAs were used to assess FAAH expression changes in the dHipp, ACb, vmPFC, and dmPFC. The dependent variable in each ANOVA was the amount of relative FAAH expression after being normalized to the loading control, Calnexin, on that given membrane.

Analysis of FAAH expression in the dHipp did not indicate significant effects of the self-administration condition, the URB597 treatment condition, or their two-way interaction. These results are shown in Figure 17 and Table 14.

Within the ACb, analysis of FAAH expression did not indicate significant expression changes resulting from the self-administration condition, the URB597 treatment condition, or their two-way interaction. These results are shown in Figure 18 and Table 15.

Results of analysis of FAAH expression in the vmPFC indicated that there was a significant main effect of URB597 treatment, $F(2, 6) = 11.08$, $p = .01$. Tukey's HSD post-hoc probing indicated that that both the 0.3mg/kg, $t(6) = -4.71$, $p = .003$, and 1.0 mg/kg, $t(6) = -2.49$, $p = .04$, doses of URB597 increased normalized FAAH expression when compared to the vehicle treatment (see Figure 19 and Table 16). No other main effects or interactions were observed.

Analysis of FAAH expression in the dmPFC was not conducted as the expression in this region was not quantifiable due to extremely low signal.

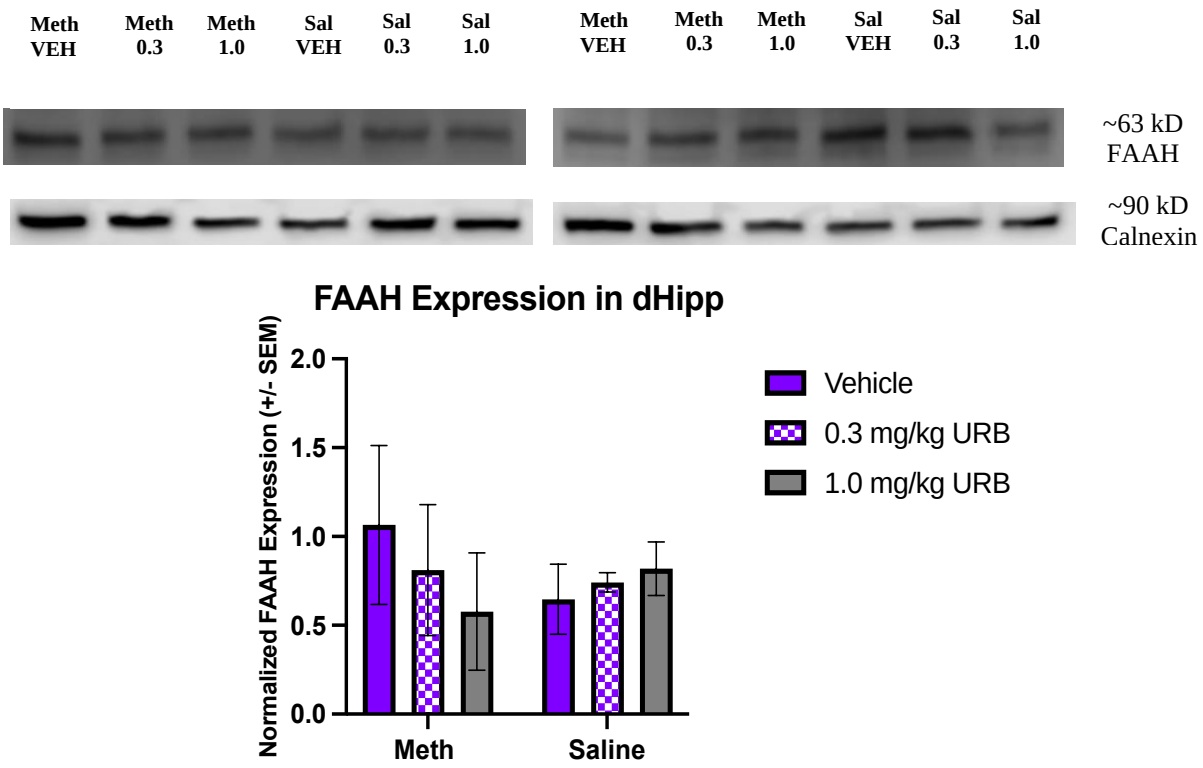
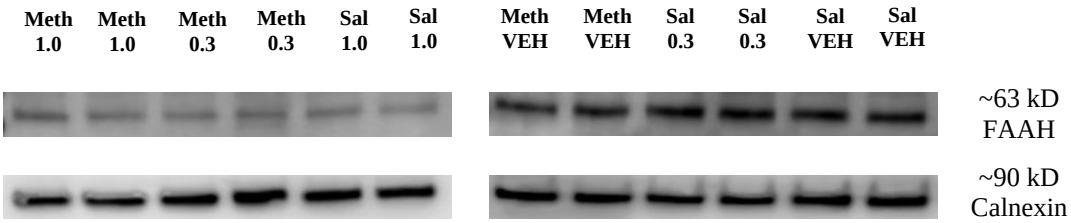


Figure 17. Mean normalized FAAH expression (+/- SEM) in the dHipp. Analysis did not indicate that the self-administration condition and URB597 treatment significantly impacted FAAH expression.

Table 14. *Results from Two-Way Analysis of Variance of FAAH Expression in dHipp.*

	df	*SS*	*F*	*p*
Drug (Meth or Saline)	1	0.02	0.12	.74
URB Dose	2	0.05	0.15	.87
Drug*URB Dose	2	0.22	0.64	.56
Error	6	1.02		
Total	11	1.31		

Note: Significance is indicated by boldfaced font



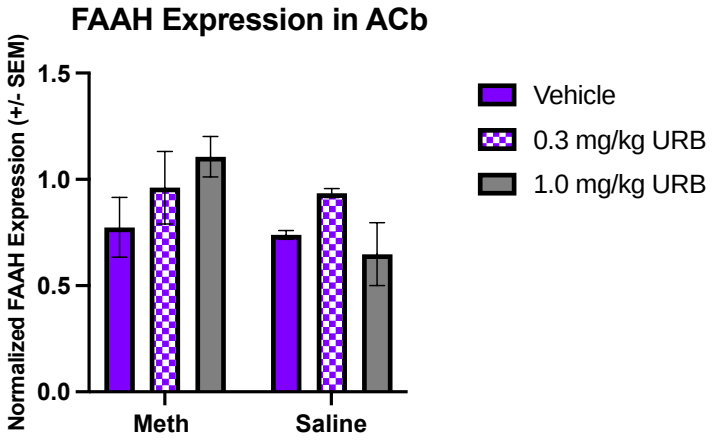


Figure 18. Mean normalized FAAH expression (+/- SEM) in the ACb. Analysis did not indicate that the self-administration condition and URB597 treatment significantly impacted FAAH expression.

Table 15. *Results from Two-Way Analysis of Variance of FAAH Expression in ACb.*

	df	*SS*	*F*	*p*
Drug (Meth or Saline)	1	0.09	3.35	.12
URB Dose	2	0.07	1.39	.32
Drug*URB Dose	2	0.12	2.26	.19
Error	6	0.16		
Total	11	0.45		

Note: Significance is indicated by boldfaced font

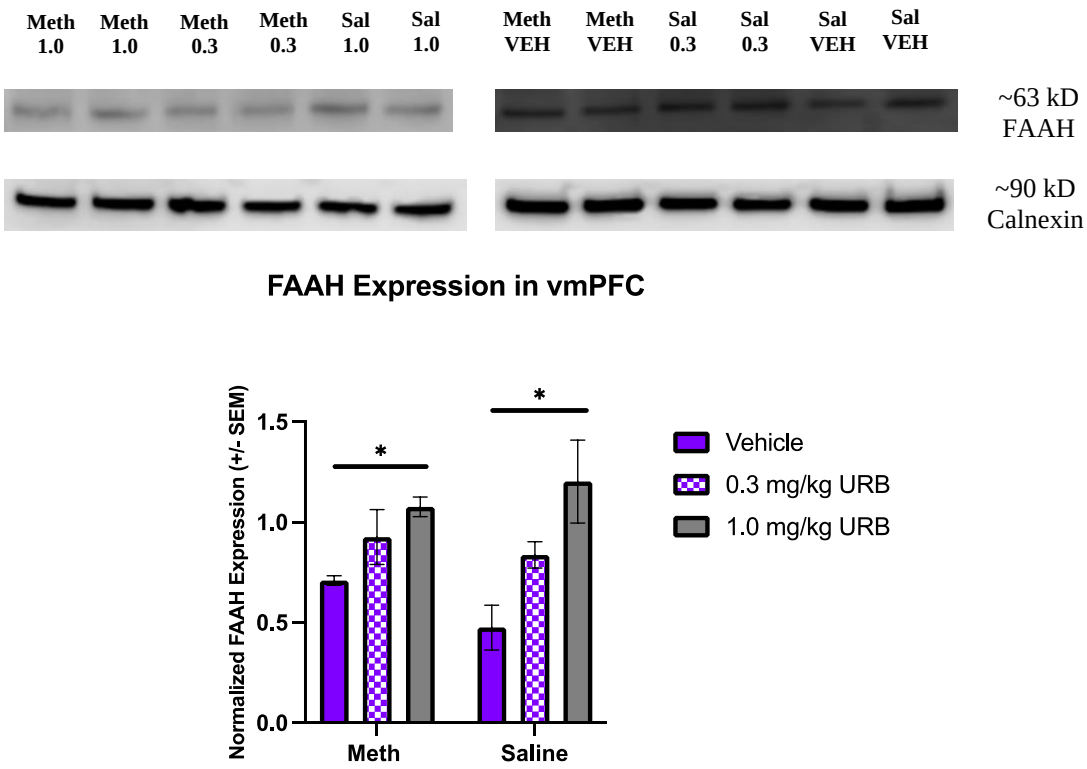


Figure 19. Mean normalized FAAH expression (+/- SEM) in the vmPFC. Analyses indicated that both the 0.3 mg/kg and 1.0 mg/kg doses of URB597 increased normalized FAAH expression when compared to vehicle-treated subjects, * $p < .05$.

Table 16. *Results from Two-Way Analysis of Variance of FAAH Expression in vmPFC.*

	df	*SS*	*F*	*p*
Drug (Meth or Saline)	1	0.01	0.47	.52
URB Dose	2	0.60	11.08	**.01**
Drug*URB Dose	2	0.07	1.21	.36
Error	6	0.16		
Total	11	0.84		

Note: Significance is indicated by boldfaced font

Anandamide (AEA) Concentrations in the Nucleus Accumbens

A two-way factorial ANOVA was used to analyze data from the AEA ELISA assay. The two-way ANOVA examining AEA in the ACb did not show significant main effects of the drug self-administration condition or URB597 treatment, and no significant two-way interaction. The ELISA data are shown in Figure 20 and Table 17.

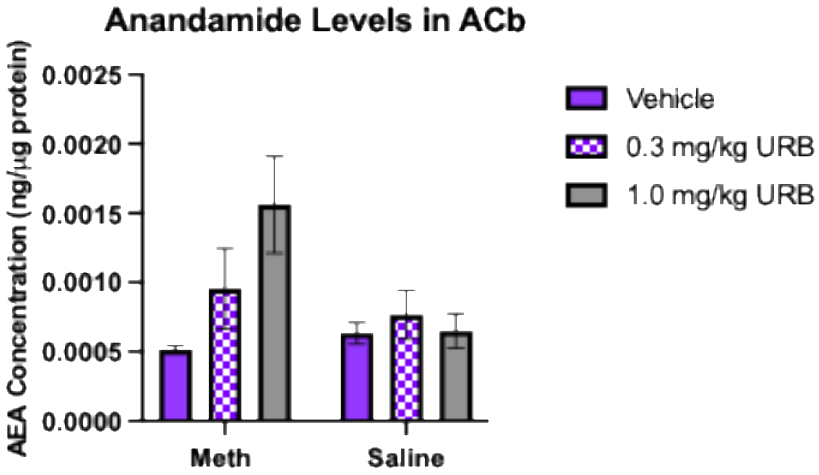


Figure 20. Mean AEA concentrations (+/- SEM) in the ACb. Analyses did not indicate significant effects of the drug self-administration condition, URB597 treatment, or their two-way interaction.

Table 17.*Results from Two-Way Analysis of Variance of AEA ELISA in ACb.*

	df	*SS*	*F*	*p*
Drug (Meth or Saline)	1	3.22e-7	3.70	.10
URB Dose	2	5.66e-7	3.25	.11
Drug*URB Dose	2	5.59e-7	3.21	.11
Error	6	5.22e-7		
Total	11	1.97e-6		

Note: Significance is indicated by boldfaced font

Chapter 5 - Discussion

Summary of Main Results

The current study sought to examine the potential efficacy of modulating eCB signaling in attenuating cue-induced methamphetamine craving following extended-access self-administration. Additionally, this study examined and further profiled molecular and neurochemical changes occurring during abstinence to methamphetamine and treatment with URB597. These data show a robust increase or escalation in methamphetamine-intake during the course of extended-access self-administration, consistent with a plethora of previous literature using similar methodologies (Murray et al., 2019; Funke et al., 2023; Scheyer et al., 2016). Second, we show that treatment with the FAAH-inhibitor, URB597, did not significantly reduce relapse responding following either acute or protracted methamphetamine abstinence, a finding not consistent with pre-experiment hypotheses. We demonstrate that methamphetamine abstinence significantly decreased the expression of CB_1 in the vmPFC. Methamphetamine abstinence also significantly decreased the expression of mGluR5 in the dHipp. Treatment with URB597 during abstinence increased CB_1 expression in the dmPFC while URB597 tended to increase FAAH in the vmPFC. Finally, ELISA measurements of AEA concentrations in the ACb did not indicate that methamphetamine significantly lowered AEA levels as predicted. These results are summarized in Figure 21 B.

Extended-Access Methamphetamine Self-Administration

Our self-administration data suggest several key points of note. First, these data suggest a robust preference for the active lever in methamphetamine self-administering animals when compared to saline self-administering animals. This finding is consistent with our pre-

experimental hypotheses that active-lever responding would be markedly higher in methamphetamine self-administering animals when compared to saline self-administering animals. Second, these data indicated a strong escalation in drug-intake for methamphetamine animals, again consistent with pre-experimental hypotheses. This finding further corroborates other reports of reliable increases in drug-intake using similar extended-access paradigms (Edwards & Koob, 2013; Murray et al., 2019; Funke et al., 2023; Scheyer et al., 2016).

Incubations in Cue-Induced Craving Do Not Occur Following Abstinence to Extended-Access Methamphetamine Self-Administration

Prior to the experiment, it was hypothesized that we would observe increased cue-induced methamphetamine craving as indicated by increase active-lever responding on WD28 when compared to WD2. Such a finding would support the *incubation of craving* phenomenon that has been previously observed following abstinence to a number of drugs of abuse (Pickens et al., 2011). However, the current data do not support this hypothesis as animals that previously self-administered methamphetamine did not show increased active-lever responding at WD28 compared to WD2.

Despite the absence of incubations in cue-induced methamphetamine craving in the current study, there is significant prior literature showing reliable and robust increases in craving for a number of drugs of abuse. Future discussion in this section will highlight differences in methodology and experimental design that may account for this difference. There is evidence illustrating incubation of craving for methamphetamine (Caprioli, Venniro, Zhang, Bosser, Warren, Hope, & Shaham, 2017; Li, et al., 2015;), cocaine (Grimm, et al., 2001; Mead, et al., 2007), heroin (Shalev, et al, 2001; Airavaara, et al., 2011), nicotine (Abdolahi, et al, 2010), and

alcohol (Bienkowski, et al., 2004). This previous literature suggests that extended- but not short-access cocaine and methamphetamine self-administration elicit CP-AMPA receptor accumulation in the ACb core (Purgianto, Scheyer, Loweth, Ford, Tseng, & Wolf, 2013; Loweth, Tseng, & Wolf, 2014; Murray et al., 2019). This increase in CP-AMPA receptor expression increases the long-term potentiation of glutamatergic synapses and drive incubations in craving during abstinence. However, there are molecular changes occurring during drug abstinence that could elicit an increase in CP-AMPA receptor expression. For instance, the expression of Group I mGluRs (mGlur1 and mGluR5), which predominate on the post-synaptic cell, and Group II mGluRs (mGluR2 and mGluR3), which predominate on the pre-synaptic cell, are inversely related CP-AMPA receptor expression (Loweth, Tseng, & Wolf, 2013; Loweth, Scheyer, et al., 2014). Given that both Group I and Group II mGluRs are a primary mechanism of long-term depression (LTD), a loss of glutamatergic tone on pre- or post-synaptic mGluRs, or a decrease in the basal expression mGluRs, decreases the efficiency of LTD mechanisms and a concomitant increase in CP-AMPA receptors. The mGluR5-eCB-CB_1 mediated synaptic depression mechanism was of particular interest to this project.

One viable answer as to why the observed incubation effects were not as robust as expected lie in the incubation timeline of methamphetamine. Specifically, as has been suggested in previous descriptions of the *incubation of craving* paradigm, drug-craving changes as a function of the abstinence period. However, this is not to say that increased drug craving continually increases during abstinence. Rather, the arc of drug craving during abstinence follow an inverted-U arc, with peak craving varying across different drugs of abuse, as well as depending on methodological factors such as extended- or short-access self-administration. For instance, peak cue-induced craving for cocaine occurs at Withdrawal Day 60 (Grimm, et al.,

2001; Lu, et al., 2004), while the incubation profile of opioids, such as heroin occur much faster with peak cue-induced craving occurring around Withdrawal Day 6 (Shalev, et al., 2001; Airavaara, et al., 2011). As for methamphetamine, previous work has demonstrated that peak cue-induced craving occurs somewhere between four and five weeks of abstinence (Murray, et al. 2022; Funke, et al., 2023; Scheyer, et al., 2016). The timepoint used in the current experiment to examine relapse under periods of protracted drug abstinence is consistent with this incubation timeline, as relapse was tested on Withdrawal Day 28. As such, it is unlikely that the lack of incubation effects in the current data are a result of testing at the wrong points in the incubation arc for methamphetamine.

Two additional points worthy of further discussion are the length of the cue-induced relapse tests, as well as the overall design of the relapse testing paradigm used in the current study. The overwhelming majority of previous research showing incubation of craving following methamphetamine self-administration has done so using between-subjects designs in which animals are randomly assigned to separate groups and undergo cue-induced relapse testing under acute or protracted abstinence timepoints (Li et al., 2015; Li et al., 2018; Murray et al., 2021; Rossi et al., 2020; Funke et al., 2023). With the between-subjects design, the studies have used various lengths of cue-test sessions, ranging anywhere from 30 minutes (Murray et al., 2021) to 3 hours in total test duration (Shepard et al., 2004). Contrasting this design, fewer reports have tested the incubation of methamphetamine craving using within-subject designs. Venniro et al. (2017) observed incubations in methamphetamine craving from Withdrawal Day 1 to Withdrawal Day 21 using a 30 minute cue-test. In another report, Krasnova and colleagues (2014) report incubations in methamphetamine craving from Withdrawal Day 1 to Withdrawal Day 21 using a 1 hour cue-seeking test using a within-subjects design. To control for the longer

cue-test then what has previously been used, we conducted exploratory analyses of active lever responding during the first 30 minutes of each relapse test session and observed that cue-induced responding was significantly higher on WD28 compared to WD2. However, given that this effect was not specific to methamphetamine self-administering animals, and that fact that this analysis does not account for the additional time spent in the operant chamber during the cue-induced relapse, we cannot rule out the potential for extinction that could have occurred during the remainder of WD2 cue session.

Another key difference in the experimental design between the current study and previous reports of incubation of methamphetamine craving is that acute craving was assessed on Withdrawal Day 2, rather than Withdrawal Day 1. The decision to test on Withdrawal Day 2 was made primarily made for two reasons. First, previous reports suggesting the efficacy of URB597 in attenuating cue-induced psychostimulant seeking (Chauvet et al., 2015) indicated that they did not administer URB597 on the day that cue-induced relapse test occurred. As such, we chose to administer URB597 on the first true day of methamphetamine abstinence (WD1) and then test relapse on Withdrawal 2 in the absence of URB597. Second, though we could have elected conduct the acute relapse test session on WD1 and not administered URB597, this decision would have confused the comparison across withdrawal days due to the absence of URB597 on WD1. With that being said, it is possible that there are behavioral differences in cue-induced responding that occur on WD1 versus those occurring on WD2. For instance, it is conceivable that given longer abstinence on WD2, animals could possibly respond more to the associated cues, thus decreasing the likelihood of observing potential incubation effects.

Systemic FAAH Inhibition Does Not Attenuate Cue-Induced Craving

Our present data did not indicate that inhibition of FAAH-breakdown of AEA, via systemic treatment with the FAAH-inhibitor URB597, was effective in attenuating cue-induced craving for methamphetamine. As discussed previously, the approach of using systemic intraperitoneal injections of URB597 was informed by previous research showing efficacy in attenuating cue-induced craving for nicotine (Justinova et al., 2015; Forget et al., 2009) and cocaine (Chauvet et al., 2015; Adamczyk et al., 2009), as well as its ability to attenuate DA-dependent neurotoxicity to methamphetamine (Nader et al., 2014). The doses of URB597 (0.3 and 1.0 mg/kg; ip) used in this experiment were consistent with prior literature. Though disappointing, this result has several suggestions which warrant future investigation, and the details of which will be detailed in this section.

An initial point warranting further discussion is the range of URB597 doses used in the current experiment. To summarize, the doses of 0.3 mg/kg and 1.0 mg/kg were chosen because these doses have previously been shown to be effective in mitigating either cue-induced drug-seeking or self-administration. Chauvet and colleagues (2015) found that URB597 (0.3mg/kg; ip) was effective in mitigating cue-induced reinstatement to cocaine. This same 0.3 mg/kg dose was effective in attenuating cue-induced nicotine-seeking (Forget et al., 2016). With that being said, other work has shown that more robust decreases in craving are evident with higher doses of URB597. Adamczyk et al. (2009) show that both 1 mg/kg and 3mg/kg URB597 are effective in attenuating cue-induced cocaine-seeking, with the 3 mg/kg dose being especially robust. Likewise, Nawata and colleagues showed that a 3.2 mg/kg dose of URB597 was effective in attenuating cue-induced methamphetamine-reinstatement. However, it was worth noting that this was the lowest dose of URB597 tested in this experiment. As such, it is possible that a higher

dose beyond those tested here, could have shown a significant reduction in methamphetamine craving on Withdrawal Day 28.

Circling back to the initial impetus of manipulating the eCB system to treat drug relapse, CB_1 receptors are ubiquitously expressed throughout the mesocortiolimbic circuitry and play a critical role in motivation and decision making (Cheer et al., 2007; Parsons & Hurd, 2015). Within this pathway, CB_1 receptors help to regulate dopamine (DA) release within the striatum and prefrontal cortex, two regions undergoing significant changes during drug exposure. In this way, CB receptors, in addition to glutamate receptors, are a critical player in regulating DA release, a system that is significantly dysregulated in the SUD disease state. The primary eCB of interest to this study, anandamide (AEA), is one endogenous ligand for the CB_1 receptor and is crucial in inhibiting cue-evoked DA release (Oleson et al., 2014). Given that attenuating cue-evoked DA release is central to many pharmacotherapies to treat SUD and cue-induced craving, AEA has become a central target for many addiction pharmacotherapies.

As previously stated, 2-AG and AEA show distinction anatomical localization with AEA predominately being produced and released from post-synaptic neurons while 2-AG is predominately produced and released from pre-synaptic neurons. In addition to this, the data on the receptor binding of 2-AG and AEA has become more complex than previously thought. Specifically, AEA and 2-AG both exert some agonist activity at both presynaptic and postsynaptic CB_1 receptors. This differs from what was previously understood as totally divergent routes of receptor binding with AEA binding to CB_1 receptors, and 2-AG primarily being responsible for binding to CB_2 receptors (Parsons & Hurd, 2015). Yet another finding that shows the complex nature of the eCB system is the finding that CB_1 receptor agonists have been found to increase nicotine and cocaine-seeking (Serrano & Parson, 2011). This contrasts with the

results that informed the current study showing that inhibition of eCB-degrading enzymes, such as FAAH, have been shown to decrease nicotine- and cocaine-seeking, and effect which is blocked by the administration of CB_1 receptor antagonists (Serrano & Parsons, 2011; Scherma et al., 2008).

The question of why such divergent results exist between CB agonists and eCB-enzyme inhibiting drugs likely exists in further downstream targets that are achieved by the different approaches. First, inhibiting eCB-degrading enzymes, such as FAAH, likely preferentially enhances eCB signaling at synapses activated by drug-related stimuli/cues (Parsons & Hurd, 2015). Such activation gives FAAH-inhibiting drugs greater specificity than exogenous CB_1 agonists and may lead to potential advantageous off-target effects. For instance, FAAH not only exerts effects on AEA, but also is responsible for regulating a host of other non-cannabinoid lipid signaling targets. Some research has recently suggested that therapeutic benefit of elevations in AEA tone via FAAH inhibition lie not necessarily in elevated CB_1 binding, but through binding of members of the "extended eCB system", such as peroxisome proliferator activated receptors (PPAR) (Lujan, Cheer, & Melis, 2021; Cristino, Bisogno, & Di Marzo, 2020).

PPARs function as ligand-gated transcription factors that are responsible for binding to promoter elements to regulate gene expression (Glass, 2006) and are essential in helping to regulate lipid homeostasis, inflammation, and immune response (Panlilio, Justinova, & Goldberg, 2013). The linkage between AEA and PPARs is illustrated by the finding that binding of AEA (and other eCB ligands) to PPARs changes target gene expression (Bouaboula et al., 2005; Sun et al., 2006; Panilio, Justinova, & Goldberg, 2013). As such, interest in the PPAR system has grown as a potential mechanism of action for substance-use therapeutics. This interest has shown a number of promising effects across a number of drugs of abuse and SUD-

related behaviors. The PPARα agonist, clofibrate, has been shown to decrease operant self-administration of nicotine, and effect which is reversed with the administration of the PPARα antagonist, MK866 (Panlilio et al., 2012; Mascia, et al., 2011). The administration of PPARα agonists have also been shown to attenuate cue-induced reinstatement to nicotine (Panlilio et al., 2012). Another PPAR isotype of significant interest is PPARγ as agonists of this receptor, such as pioglitazone and ciglitazone, have been shown to attenuate behavioral sensitization to methamphetamine (Maeda et al., 2007). In summary, the efficacy of FAAH inhibition is tied to PPAR activity.

The current study showed that AEA concentrations within the ACb were roughly equivalent in methamphetamine and saline animals that received the vehicle treatment during forced drug abstinence. Given this result, it could be deduced that AEA concentrations may be unaffected by abstinence to methamphetamine, at least within the ACb. However, future work should continue to characterize these results across other regions of the mesocorticolimbic pathway. In the event that AEA concentrations are only minorly affected by methamphetamine abstinence, future therapeutics, such as PPAR agonists, may prove more efficacious as these pharmacotherapies bypass the need for other mediating eCB ligands.

Changes in Cannabinoid Receptor-1 (CB_1) Expression Following Abstinence to Extended-Access Methamphetamine

In the current study, it was predicted that we would observe increased surface-level CB_1 receptor expression in animals that previously self-administered methamphetamine. This hypothesis was informed by previous findings showing that repeated psychostimulant administration produces increases in CB_1 receptor expression (Blanco et al., 2015). Further, we

also predicted that treatment with URB597 during abstinence would significantly decrease CB_1 expression, consistent with previous reports of such decreases in the ACb and Hipp (Marco et al., 2009).

Results of western blot analysis of CB_1 expression in the dorsal hippocampus (dHipp) was not altered by either methamphetamine abstinence or URB597 treatment. Thus, our pre-experimental hypotheses were not supported. This finding is consistent with prior research from Gonzalez et al. (2002) in which they demonstrated that chronic exposure to cocaine was not associated with changes in CB_1 mRNA in the hippocampus. However, this finding does conflict with work showing that neurotoxic doses of methamphetamine are associated with increased CB_1 expression within the hippocampus (Bortolato et al., 2010). This discrepancy in these findings and our own may be contributable to a difference in dosage as we used subtoxic doses of methamphetamine in the current study. The significance of the differences in the effects produced by subtoxic versus neurotoxic doses are illustrated by literature suggesting divergent effects on the glutamatergic system (Wolf, 2016). As such, it is conceivably possible that the effect that methamphetamine exposure, and methamphetamine abstinence, has on CB_1 expression may be dose-dependent.

Within the nucleus accumbens (ACb), CB_1 receptor expression was shown to be unaffected by methamphetamine abstinence or URB597 treatment, thus differing from our hypotheses. Though the ACb is of great importance to the mesocorticolimbic reward circuit, basal expression of CB_1 in the ACb is quite low (Winters et al., 2012). As such, this lack of effect within the ACb could possibly due to a floor effect resulting from low basal CB_1 expression. Interestingly, despite low CB_1 expression within the ACb, preferential manipulations of CB_1 receptors within the ACb produces significant changes in emotional and motivational

states. For instance, specific inhibition of CB_1-expressing neurons within the ACb has been shown to blunt cocaine reinforcement (Ramiro-Fuentes, et al., 2010). These results suggest that despite low overall expression of CB_1 in the ACb, specific targeting of CB_1 receptors within this brain region are crucial for behavioral changes occurring in response to psychostimulants. Such a suggestion is corroborated by findings that intracranial infusions of AEA into the ACb have been shown to mitigate increases in cue-induced cocaine craving (Zhang et al., 2021). Additionally, prior work has shown that treatment with URB597 significantly lowers the density of CB_1 receptors in the ACb and Hipp (Marco et al., 2009). However, this work administered only five doses of URB597 before assessing expression compared to the 26 days of URB597 administration used during the methamphetamine abstinence period here.

Within the medial prefrontal cortex (mPFC), we observed that methamphetamine did not significantly affect CB_1 receptor expression as predicted. Rather, CB_1 expression in the vmPFC showed a decreasing trend. However, though previous research has shown that methamphetamine administration decreases levels of CB_1 in the mPFC, this work has utilized neurotoxic doses of methamphetamine (Bortolato et al., 2010). Significantly less work has evaluated these effects in response to subtoxic doses of methamphetamine. As such, these results suggest that the effect of methamphetamine on CB_1 receptor expression likely vary as a result of the frequency and dose being administered.

Within the dorsomedial prefrontal cortex (dmPFC), we did not see a decrease in CB_1 expression in response to methamphetamine abstinence. However, we observed that URB597 treatment significantly increased expression when compared to vehicle-treated animals. These results contradict those predicted to occur following treatment with URB597 in methamphetamine animals. I predicted that increased CB_1 expression in response to

methamphetamine serves as a compensatory mechanism for overall decreases in AEA concentrations. As such, I predicted that the increase in AEA produced by URB597 treatment would significantly lower CB_1 expression. Though the finding that URB597 treatment increased CB_1 expression in the dmPFC is unexpected, there is evidence showing that eCB-metabolism inhibition (via FAAH) produces increases in CB_1 expression within the prelimbic cortex (Lisboa et al., 2015). The fact this effect was only present in the dmPFC, but not in any other brain area assessed, suggests neuroanatomical specificity with regard to the effects of URB597 on CB_1.

Firstly, it is worth noting the importance the delineation of dmPFC and vmPFC used here, as most reports do not split these up by subregion. In the context of the work from Lisboa and colleagues (2015), they observed increased CB_1 expression in the prelimbic cortex, a subregion located in the dmPFC. The functional role of CB_1 receptors in the PFC is to maintain a relative balance of glutamatergic excitatory postsynaptic currents (EPSCs). Specifically, CB_1 agonism decreases EPSCs in the mPFC while CB_1 antagonism increases the rate of EPSCs (Auclair et al., 2000). In the context of the paradigm used in the current study, these results could be compared to other results showing that pharmacological inactivations (i.e. muscimol, baclofen) of PL generally decrease cocaine-seeking, thus demonstrating that PL plays a pivotal role in drug-seeking (Di Pietro et al., 2006).

Metabotropic Glutamate Receptor-5 (mGluR5) Expression Changes in Response to Methamphetamine

The current study did not predict expression changes in response to either methamphetamine abstinence or treatment with URB597 for any of the four brain areas tested: the dHipp, ACb, vmPFC, or dmPFC. This hypothesis is consistent with prior literature showing

that surface mGluR5 expression in the ACb core is unaffected by protracted abstinence to methamphetamine (Murray et al., 2021).

In the dHipp, we found that mGluR5 expression was significantly and preferentially increased by methamphetamine in rats that received the vehicle treatment. Though this finding differs from those found in Murray et al. (2021), they share an important distinction, namely the brain area being evaluated. Increased mGluR5 expression in the dHipp is consistent with research from Liao and colleagues (2018) who have shown increased mGluR5 expression in dHipp following 10 days of methamphetamine injections in a behavioral sensitization procedure. Other work has shown similar effects in hippocampal neurons in response to amphetamine. Yu et al. (2000) used in situ hybridization to demonstrate that the administration of amphetamine to cultured hippocampal neurons resulted in a 50-62% increase in mGluR5 expression as compared to control.

Interestingly, we saw divergent patterns of effects for URB597 with respect to the impact that treatment had in methamphetamine and saline self-administering rats. URB597 treatment tended to increase mGluR5 expression in saline self-administering animals, in line with hypotheses. However, this effect showed the opposite pattern in methamphetamine self-administering animals, with URB597 producing a decreasing trend of mGluR5 expression. These divergent patterns are interesting and warrant further investigation. We hypothesized that, given the role of mGluR5 in inducing LTD at glutamatergic synapses, URB597 treatment would increase the expression of mGluR5. Though we predicted that this pattern would occur in methamphetamine animals and not saline, this result suggests a possible compensation mechanism. Namely, mGluR5 expression may increase in saline self-administering animals due to increases in AEA occurring in response to URB597 treatment. Referring back to Figure 1,

AEA serves as the primary ligand to bind to presynaptic CB_1 receptors and subsequently results in the LTD of glutamatergic synapses. Given that increased AEA to CB_1 binding could potentially bypass the need for mGluR5 expression in the typical mGluR5-eCB-CB_1 LTD signaling cascade, this could trigger compensatory increases in mGluR5 expression. As indicated, URB597 tended to have the opposite effect in methamphetamine self-administering animals. Here, mGluR5 expression was found to be highest in vehicle-treated rats. It is conceivable that the elevations in AEA concentration purportedly produced by URB597 treatment could induce the opposite pattern to those described in saline self-administering rats. Namely, the predicted decreases in AEA concentrations induced by abstinence to methamphetamine could increase mGluR5 expression, an effect which is supported by increased expression in methamphetamine-vehicle rats as compared to saline-vehicle rats. However, treatment with URB597 could likely be attenuating this increase, likely due to its purported ability to increase AEA concentrations, thus restoring the imbalance of AEA present in methamphetamine abstinence. Though not assessed here, future work should continue to examine AEA concentrations in the dHipp to explore the validity of this hypothesis.

We did not observe that either methamphetamine abstinence or treatment with URB597 during the abstinence period significantly impacted mGluR5 expression in the ACb. This result is well aligned with Murray et al. (2021) where the authors demonstrated that mGluR5 expression was not significantly altered by methamphetamine at either 3, 21, or 48 days of withdrawal from methamphetamine self-administration. However, one should not take this as evidence that the mGluR5 receptor is not implicated in cue-induced drug-seeking, at least as it is expressed in the ACb. In the same study, the authors found that intra-ACb infusions of the mGluR5 negative allosteric modulatory (NAM) drug, MTEP, significantly lowered cue-induced

nose poking at both 21 and 33 days of withdrawal (Murray et al., 2021). This adds to a litany of research showing that mGluR5 NAMs, such as MTEP, have a significant effect on reward and subsequent drug-seeking (Arndt, Johns, Dietz, & Cain, 2015; Brown et al., 2012).

Interestingly, we observed a slight, albeit non-statistically significant, increase in mGluR5 expression in the vmPFC in methamphetamine animals as compared to saline. These results are similar to those observed by Shaffer and colleagues (2010) in which the authors investigated the effects of acute amphetamine injections on mGluR5 expression in the mPFC. The authors observed that peak increases in mGluR5 expression occurred one hour after administration of the 5 mg/kg amphetamine injection. Perhaps more intriguing, the authors observed the opposite effect in the striatum with amphetamine significantly lowering mGluR5 expression one hour following the injection of amphetamine (Shaffer et al., 2010). These differential effects highlight important dichotomies in the patterns of effects produced by psychostimulant administration in the mPFC and ACb. Using in vivo microdialysates, Shoblock et al. (2003) determined that amphetamine and methamphetamine administration exert separable effects between the mPFC and ACb. Specifically, amphetamine was shown to increase glutamate concentrations in the ACb but failed to do so in the ACb. These effects were opposed to those obtained with methamphetamine administration where glutamate concentrations were unaffected in the ACb but were increased in the mPFC. These data, coupled with our own suggest distinct functional roles of mGluR5 between the ACb and mPFC in response to methamphetamine.

Changes in the Expression of Fatty-Acid Amide Hydrolase (FAAH)

We predicted that methamphetamine abstinence would result in significant increases in FAAH expression relative to the saline control animals. This prediction is tied to previous

research showing cocaine sensitization is associated with increases in FAAH expression (Blanco et al, 2015) and subsequent research showing decreased AEA concentrations in response to chronic amphetamine exposure (Thiemann et al., 2008). Additionally, we predicted that treatment with URB597 during the abstinence period would attenuate this increase in animals that previously self-administered methamphetamine.

In the dHipp, we did not observe significant changes in FAAH expression resulting from either methamphetamine abstinence or URB597 treatment. This result does differ from some prior research conducted by Jayanthi et al., (2022). In this study, the authors used a model of methamphetamine self-administration that helps to separate compulsive and non-compulsive phenotypes via the administration of footshocks following methamphetamine self-administration. The authors investigate how these phenotypes were associated with certain eCB synthesizing and metabolic enzymes. They found that FAAH mRNA levels in the Hipp were significantly increased in methamphetamine animals that showed sensitivity to the footstock; that is, animals that suppressed their methamphetamine-intake during the punishment phase when methamphetamine and footshocks were co-administered. Given the role of the eCB system in regulating stress and anxiety-states, it is possible that stressful states are required to elicit increases in FAAH expression. In the context of the current study, it is possible that increases in FAAH expression may occur earlier in the withdrawal period and taper as drug abstinence progresses.

We did not see changes in FAAH expression within the ACb resulting from either methamphetamine abstinence or URB597 treatment. This finding is somewhat surprising given that previous research has shown that single, high-unit doses of cocaine (20 mg/kg) are sufficient to eliminate eCB-mediated LTD in the ACb (Alegre-Zurano et al., 2023). This work served as

pretense for the increases in FAAH expression that were predicted to occur in response to methamphetamine in the current study. Namely, we theorized that increased FAAH levels decrease basal levels of AEA and the subsequent binding of CB_1 receptors, therefore decreasing eCB-mediated LTD that would serve to decrease cue-induced craving during methamphetamine abstinence. However, the current study differed from that of Alegre-Zurano and colleagues (2023) in several key facets. Namely, we measured eCB and glutamatergic receptor expression following abstinence to extended-access to a much lower-unit dose of methamphetamine (0.1 mg/kg/infusion). As such, it is likely that the duration of abstinence to the drug, the dosage used, and the drug administered (cocaine versus methamphetamine) may exert different effects on these mechanisms.

In the vmPFC, we did not see that methamphetamine abstinence significantly decreased FAAH expression as hypothesized. Previous research using cocaine-induced behavioral sensitization has shown that acute cocaine administration significantly increased FAAH expression relative to control-treated animals in the mPFC (Blanco et al., 2015). However, this work did not delineate whether this effect may differ across the ventromedial and dorsomedial regions of the mPFC. Interestingly, data from human clinical studies have shown linkages between FAAH expression and susceptibility to methamphetamine-use. Zhang and colleagues (2020) used a sample of methamphetamine-dependent and control subjects to examine FAAH expression differences by measuring the 385C/A polymorphism. Ultimately, they found that plasma FAAH mRNA levels were significantly lower in control subjects as compared to methamphetamine-dependent subjects. Further, the authors found that individuals carrying the A allele from the 385C/A polymorphism at significantly higher risk (1.65 times) of methamphetamine dependence. Though these results implicate FAAH expression in

psychostimulant expression, it is possible that such increases in FAAH occur more transiently. This is possible as the studies discussed above measured FAAH in animals immediately following cocaine administration, or in current methamphetamine-dependent individuals. Future studies should explore how FAAH expression is disrupted during abstinence to psychostimulant-use as it is possible that the degree of decrease varies as a function of the abstinence period. Additionally, the above studies are not as specific in the localization of the FAAH effects. For instance, Blanco et al. (2015) cites a difference in FAAH expression engendered by cocaine administration while our results suggest that the effect of methamphetamine abstinence and URB597 treatment may vary across subregions.

In the vmPFC, we did observe that systemic injections of either 0.3 mg/kg or 1.0 mg/kg URB597 during the abstinence period significantly increased FAAH expression relative to vehicle-treated rats. This result was contrary to those hypothesized as we predicted that supplementation of URB597 during abstinence would serve to attenuate the predicted increases in FAAH in response to methamphetamine. To our knowledge, very few studies have evaluated the effects of URB597 treatment on FAAH expression in the mPFC. Some previous work has shown that repeated treatment with URB597 produces modest decreases in FAAH expression in the Hipp (Rivera et al., 2015). However, it is worth noting that this study only administered URB597 (0.3 mg/kg; ip) for five consecutive days as compared to the 26 treatments that were given in the current experiment. It is possible that extended administration of URB597 may induce compensatory increases in the overall expression to counter the downregulation in FAAH that is present in acute treatment regimens.

This is a hypothesis that warrants future investigation as the impact of protracted URB597 treatment on FAAH expression may likely affect the efficacy of the treatment over

time. The question remains as to why this effect would be present in the vmPFC, while not evident in the ACb or dHipp. One plausible explanation for this pattern of effects could be due to differences in the density of glutamatergic projection neurons that emanate from the vmPFC. It is possible that the increased number of glutamatergic cells in this region necessitates increased levels of FAAH. As previously suggested, it is also possible that higher doses of URB597 are required to elicit similar increases in other brain areas beyond the vmPFC.

Anandamide (AEA) Concentrations in Response to Methamphetamine Abstinence and URB597 Treatment

In the current study, it was hypothesized that methamphetamine abstinence would significantly decrease AEA concentrations, and this would be mitigated by treatment with URB597. This hypothesis was informed by evidence suggesting that AEA concentrations are decreased in response to chronically administered methamphetamine (Nader et al., 2014) and that amphetamine-sensitization has been shown to decrease concentrations of AEA and 2-AG in the ACb (Thiemann et al., 2008). Overall, our current results do not suggest a decrease in AEA concentrations in the ACb resulting from methamphetamine abstinence. Such findings could suggest that the 28-day abstinence period used in the current study could possibly allow the the eCB system to recover to baseline levels. Such a suggestion aligns with work that shows that decreased AEA concentrations occur shortly following extended methamphetamine exposure (Nader et al., 2014).

Though the effect was not found to be statistically significant, we did observe a trend that URB597 treatment tended to increase AEA concentrations in methamphetamine-abstinent rats (see Figure 20). This finding is consistent with prior literature showing that URB597 treatment

increases brain and peripheral AEA concentrations (Kathuria et al., 2003). However, there are still methodological discrepancies between our own and Kathuria and colleagues (2003). First, we measured AEA concentrations specifically within the ACb. Given that Kathuria et al. (2003) did not take such a localized approach in their measurement, this could attribute to some discrepancies between these two sets of data. Second, Kathuria et al. (2003) did not assess the effect of FAAH in conjunction with psychostimulant exposure.

Integration of Western Effects: Implications for Glutamate Signaling in Mesocorticolimbic Circuit

In the current study, I demonstrate that methamphetamine abstinence significantly decreased the expression of CB_1 in the vmPFC. Likewise, methamphetamine abstinence decreased expression of mGluR5 in the dHipp. Treatment with URB597 during abstinence increased CB_1 expression in the dmPFC while URB597 tended to increase FAAH in the vmPFC. Finally, ELISA measurements of AEA concentrations in the ACb did not indicate that methamphetamine significantly lowered AEA levels as predicted. The patterns of effects illustrated here offer several possible changes in the mesocorticolimbic circuit that could result from either methamphetamine abstinence or treatment with URB597. The general mesocorticolimbic circuit, and its possible changes will be highlighted in subsequent sections.

As previously discussed, the dmPFC, vmPFC, ACb, Hipp, and VTA all play integral roles in mesocorticolimbic circuit and help to mediate motivation and reward-based processing. Figure 21A shows a simplified depiction of this circuit. Collectively, the dmPFC, vmPFC, and dHipp all send excitatory glutamatergic projections to the ACb. The functional consequence of these projections within the ACb is the activation of GABAergic projections stemming from the

ACb to the VTA and ultimately, the stimulation of dopamine projections extending from the VTA back to the ACb. This system is well-studied and understood to underscore the rewarding effects of drugs of abuse.

However, as I have laid out, the neurobiological changes shown by these data indicate several possible changes in this circuit that could provide insights into how this system might change in response to methamphetamine abstinence and URB597 treatment. Figure 21B shows a summary of the observed neurobiological effects as well as the putative changes that may occur in the mesorticolimbic neurocircuitry. First, we observed that methamphetamine abstinence significantly decreased CB_1 expression in the vmPFC. This could suggest a strengthening of glutamatergic projections to the dHipp and the ACb (more specifically the ACb shell) due to the role of CB_1 in mediating LTD at glutamatergic synapses (sort of like cutting the brakes on the glutamate system). The maladaptive consequence of such strengthening of these vmPFC-ACb projections could be increased disinhibition of VTA dopamine projections and increased dopamine release in the ACb, thus potentially increasing the incentive salience of drug-related cues (Berridge and Robinson, 1998). Within the dmPFC, we saw increases in CB_1 expression in response to URB597 treatment. This could, by the same logic, weaken glutamatergic afferents extending from the dmPFC to the ACb (specifically the ACb core). Finally, we observed that methamphetamine increased mGluR5 expression in the dHipp. Although we cannot pinpoint that this increase in mGluR5 specifically occurred in dHipp-ACb glutamatergic projection neurons, this is one idea that is explored in Figure 21B. We propose that the increase in mGluR5 expression in dHipp potentiates dHipp-ACb projections, thus increasing evoked dopamine in the ACb in such a way as that described by the vmPFC above. Such a suggestion is somewhat supported by previous reports showing that administration of the mGluR5 antagonist, MTEP,

tends to decrease cue-induced drug-seeking (Arndt, Johns, Dietz, & Cain, 2015; Brown et al., 2012) while administration of the mGluR5 agonist, CHPG, tends to promote cue-induced drug-seeking (Benneyworth et al., 2019). In total, we propose that many of the neurobiological changes induced by methamphetamine abstinence promote increased excitatory input to the ACb and subsequent potentiation of drug-seeking behaviors.

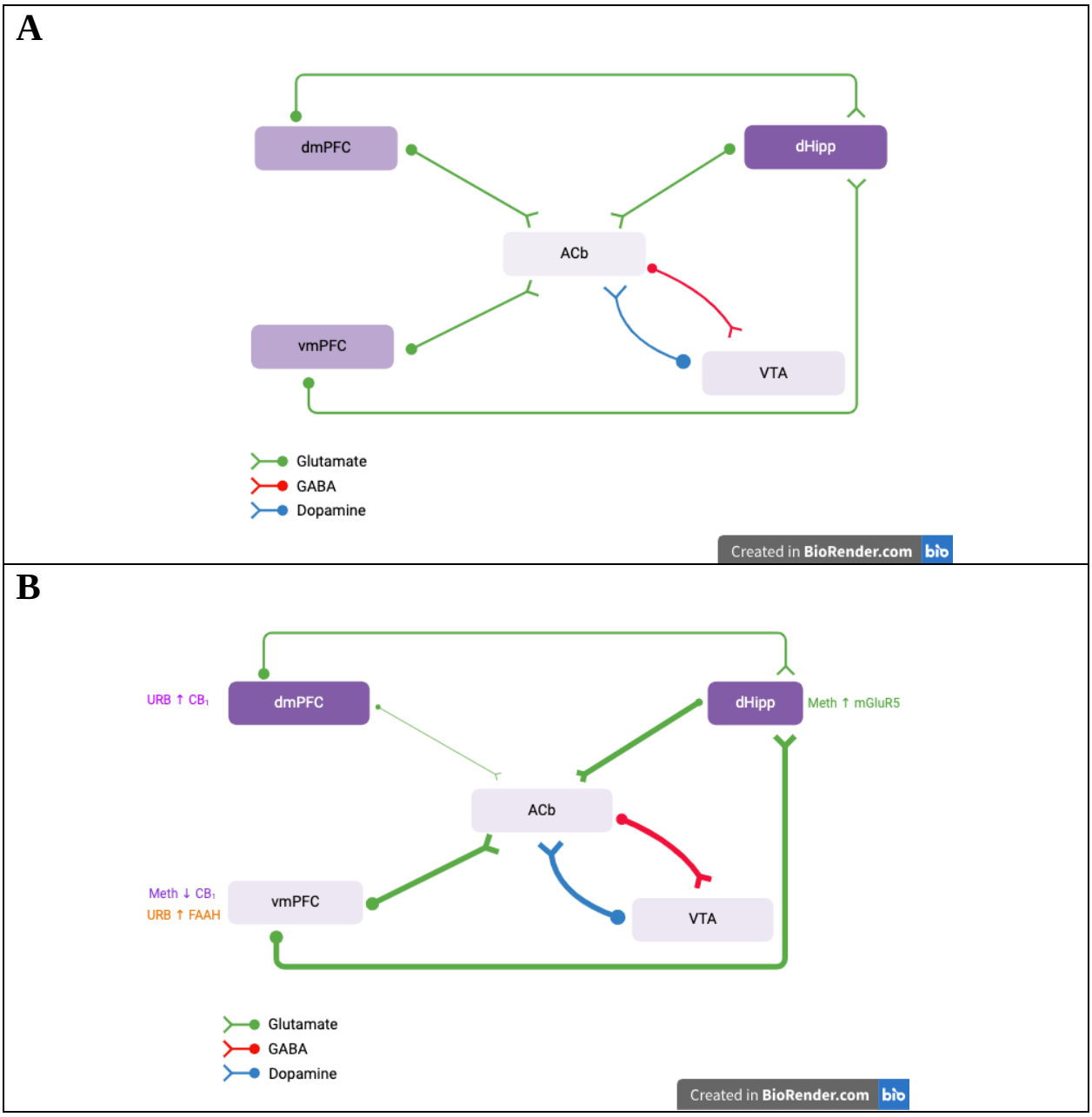


Figure 21. A) Simplified depiction of the mesocorticolimbic circuit, adapted from Parsons & Hurd (2015). B) Depiction of proposed changes in the mesocorticolimbic circuit in response to methamphetamine and URB597 treatment. An increase in the boldness of the line indicates a strengthening of that pathway relative to Panel A. The gradient of purple indicates the expression of CB_1 with darker purple indicating more expression. Darker or lighter purple in Panel B indicates a change in CB_1 in that brain area relative to Panel A.

Future Directions and Concluding Remarks

The results of the current study suggest that URB597 is likely ineffective for attenuating cue-induced craving for methamphetamine, despite previous demonstrations of its potential for attenuating cue-induced relapse (Chauvet et al., 2014; Forget, Coen, & Foll, 2009). More broadly, the results of the current study further demonstrate the complex nature of the eCB system as it pertains to the treatment of substance-use. Not only do the impacts of psychostimulants on the eCB and glutamate homeostasis systems vary across the psychostimulants, but these impacts also vary across brain region. Future research should continue to explore the functional role of eCB signaling within different brain regions as prior data, and those obtained from this dissertation, suggest that methamphetamine differentially affects eCB and glutamate receptor expression in a regionally specific manner.

Future work should also aim to evaluate how, or if, the effects of methamphetamine on the eCB and glutamatergic systems differ based on sex. A multitude of prior literature indicates sex differences in cue-induced craving and relapse, with females typically exhibiting greater cue-induced craving when compared to males (Robbins et al., 1999; Kennedy et al., 2013; Nicolas et al., 2019). Adding to this increased susceptibility to relapse in female subjects, there are marked sex differences in the eCB system. For instance, females tend to show higher resting concentrations of 11-hydroxy-tetrahyrocannabinol, thus suggesting increased sensitivity to tetrahydrocannabinol (THC) as compared to males (Wiley & Burston, 2014). Other evidence suggests sex differences in CB_1 and CB2 receptor expression across numerous brain areas including the Hipp (Liu et al., 2020), mPFC (Liu et al., 2020; Castelli et al., 2014), and striatum (Rodriguez de Fonesca, et al., 1999; Liu et al., 2020). In total, these results suggest that the efficacy of eCB-modulating therapeutics for SUD may likely vary based on sex.

Two additional fundamental questions on the topic of the eCB system as it pertains to psychostimulant-use should be addressed in future research. First, further examination of how eCB and glutamate receptor expression change as a function of drug abstinence should be addressed. Namely, how do the alterations of these receptors change in acute versus protracted abstinence? Such answers are critical when determining the mechanism of action for a particular intervention, as well as the timing of the intervention during the course of drug abstinence. Second, additional research using more translationally relevant dose ranges should occur. A number of prior studies examining the impact of psychostimulants on the eCB system have done so with extremely high doses of these psychostimulants. As discussed earlier, neurotoxic doses of methamphetamine have been shown to produce decreases in glutamate receptors not produced by subtoxic doses (Wolf, 2016). Such a dichotomy could potentially exist in the eCB system and should be explored in the future.

One final future direction that should be considered is the mechanism of action that is being targeted for methamphetamine relapse-preventative therapeutics. Though there is evidence to support the strategy of inhibiting FAAH-mediated metabolism of AEA to treat cue-induced methamphetamine-seeking, alternative mechanisms to the same end exist. The enzyme monoacylglycerol lipase (MAGL), metabolizes both AEA and 2-AG. The importance of 2-AG, in addition to AEA, may be illustrated by findings that show that neurotoxic doses of methamphetamine decrease 2-AG concentrations in the striatum (Nader et al., 2014). Future work should explore the efficacy of MAGL-inhibitors as the efficacy of eCB-targeting therapeutics may depend on the modulation of multiple eCBs.

To conclude, our results did not suggest that treatment with URB597 during abstinence from methamphetamine self-administration was sufficient to lower cue-induced relapse to

methamphetamine. However, our results do show multiple patterns of changes in CB and glutamate receptor expression occurring in a number of brain areas in response to methamphetamine. As such, though we did not show that inhibition of FAAH-mediated AEA metabolism was an effective treatment strategy, our results do demonstrate evidence for the potential validity of eCB-modulating therapeutics for the future treatment of methamphetamine relapse.

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