The effects of differential rearing and abstinence period on post-synaptic glutamate receptors and amphetamine seeking

by

Erik Joseph Garcia

B.S., Colorado State University, 2010

M.S., Kansas State University, 2014

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

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Department of Psychological Sciences
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Abstract

Drug addiction is a chronic cyclical disease characterized by periods of drug use and abstinence. Drug craving increases as a function of abstinence period, such that longer periods of abstinence result in greater feelings of craving. Longer periods of abstinence may render cues to become more powerful motivators of drug seeking behavior because of the greater craving response. Neurobiological evidence suggests that changes in glutamatergic transmission in the nucleus accumbens (NAc) plays a pivotal role in the incubation of craving and drug seeking motivation. Specifically, the upregulation of Ca^{2+} permeable AMPA receptors may increase drug seeking following the presentation of a drug cue. Environmental housing manipulations also change the expression of metabotropic glutamate receptors (mGlur) and psychostimulant self-administration. In the current experiments, Sprague-Dawley rats were reared in enriched (EC) or isolated (IC) conditions from PND 21-51. Then rats were implanted with indwelling jugular catheters and allowed to self-administer amphetamine (0.1 mg/kg/infusion) or saline paired with a cue light for 16 days for 1h. Then rats went through a forced abstinence period of 1 day and were then tested in a cue-induced seeking test. Immediately after the seeking test, half the rats were sacrificed and the NAc was dissected and prepared for western blot analyses. The other half of rats rested for 40 days and were tested again in the cue-induced seeking test. Immediately following the seeking test, rats were sacrificed and their NAc was dissected. Factorial ANOVA results indicate that rearing in the IC environment increased drug seeking when compared to EC rats after 1 day of abstinence and after 40 days of abstinence, but drug seeking did not increase after 40 days. Rats in the saline groups showed an increase in seeking after 40 days of abstinence, providing evidence of increased responding. Saline responding was significantly lower when compared to rats that responded for amphetamine. When rats self-administered

saline, generally IC rats had more responding than EC rats. Western blot analyses indicated that expression of AMPA subunits GluA1, and GluA2, as well as metabotropic glutamate receptors 1 and 5 (mGlur1, and mGlur5) were not different across the experimental groups, suggesting another mechanism could be implicated in drug seeking after short and long abstinence periods. These results suggest that early life experience can have long lasting effects into adulthood and increase the vulnerability of drug abuse. Our results provide mixed results of incubated seeking. Positive early life experiences reduce drug seeking motivation after short and long abstinence periods, providing evidence for further research to examine how early life experience changes the reward seeking and subsequent structures in the mesocorticolimbic pathway.

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Approved by:

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Quote from Karen Moning

Dedication

This dissertation is dedicated to my mother. She struggled with so much, yet remarkably she always gave when others suggested she take. Thank you for your strength.

To Chloe': Iggy and I are lucky to have you. Care, love, and joy are the first things that come to mind.

Preface

Drug addiction is a cyclical process that involves periods of drug abstinence and periods of drug use. Often drug users struggle to maintain sobriety even though they desire to do so, suggesting that their motivation to seek drug rewards is greater than their motivation to maintain sobriety. The motivation to seek drug rewards is sometimes referred to as craving. In a linear fashion, drug craving progressively increases as period of abstinence gets longer, indicating that craving for drugs rewards does not subside, but rather, it can surmount the motivation to maintain sobriety. Drug craving can be modeled in rats, with a nearly identical linear increase in drug cue responding, i.e. craving. The progressive increase in drug cue responding is termed, 'the incubation of drug craving'.

Many of the neurobiological factors that influence the incubation of drug craving are largely unknown, but glutamate transmission has been largely studied. Specifically, calcium permeable AMPA (CP-AMPA) receptors mature and insert into post-synaptic neurons after a long abstinence period of 25-35 days and persist for greater than 70 days. The insertion of these receptors seems to be a critical neuronal moment that may underlie the inability to withstand craving and maintain sobriety. Therefore, understanding manipulations that may alter the development and insertion of these receptors may be a promising intervention to reduce drug craving after long periods of abstinence.

Differential rearing is a manipulation that alters a variety of behaviors, namely learning, memory, and reward seeking. When compared to rats reared in isolation (IC), rats reared in enrichment (EC) self-administer less psychomotor stimulants, and show less reinstatement of drug seeking behavior, indicating that the EC may strengthen protective factors. Alternatively, rearing in isolation likely increases vulnerability factors that may be important for maintaining

sobriety after long periods of abstinence. In addition to the behavioral differences observed between EC and IC rats, they also have marked differences in brain anatomy, including differences in glutamate receptor expression and function.

When compared to EC rats, IC rats have glutamatergic alterations that affect glutamate homeostasis; a hypothesized mechanism that contributes to addictive drug taking behavior. When glutamate homeostasis is in balance it acts to protect against drug taking behavior, and when homeostasis is not in balance drug seeking behavior is augmented. The factors that contribute to maintaining glutamate homeostasis are referred to generally as glutamatergic tone. Rearing in an EC influences glutamatergic tone in such a way that results in neuroprotection from drug seeking, while rearing in the IC leads to alterations that negatively affect glutamatergic tone and increase drug abuse vulnerability.

We hypothesize that rearing-induced changes will alter glutamate homeostasis and will lead to differences in drug seeking after short and long term abstinence. Further, we propose that these neuronal modifications that are induced by differential rearing will be measured in post-synaptic receptors, such that CP-AMPA, metabotropic glutamate receptors (mGlur) 1 and 5 will be altered differently in EC and IC rats after short and long abstinence periods.

The current experimental design will determine if differential rearing alters drug craving/seeking behavior in Sprague-Dawley rats after short and long abstinence, and will measure post-synaptic glutamate receptor expression using western blot protein analysis. This contribution is important because it will show how early development can alter protein expression that contributes to drug seeking after prolonged abstinence, and demonstrate how these receptors contribute to glutamate remodeling in the NAc.

Chapter 1 - Introduction

At the most basic level, learning can be described as acquiring new knowledge. Does learning and memory have the capability to physically change how the brain communicates or the connections within it? Freud was one of the first scientists to hypothesize that learning could directly affect the strength of neural communication. Similarly, Donald Hebb realized that psychological phenomena like motivation and attention influence the biology from which they arise, indicating a bidirectional relationship (Brown & Milner, 2003). To Hebb, Stimulus-Response relationships were oversimplified, but a unified approach of biology, psychology, and the dynamic interaction between them would lead to discovery and innovation in neuroscience. Hebb proposed that neural connections strengthened with repeated stimulation—neurons that fire together wire together. This type of strengthening is understood to be a biological correlate for learning and memory. At the time, Hebb developed research designs to test his ideas about neuroplasticity that helped shape the work of neuroscientists for decades. To his credit, he discovered that rats reared in enriching environments and outside of laboratory cages performed better during maze learning and memory tests, suggesting a potential enhancement in long term potentiation for enrichment-reared rats (Hebb, 1947). Further, this suggested that there was a critical period during development that promotes healthy learning and subsequently, synaptic strengthening that accompanies these learning enhancements in adulthood. However, learning, particularly learning about drug cues, can be damaging and lead to compulsive and maladaptive behavior. When drugs of abuse repeatedly stimulate neurons the natural strengthening is hijacked and the connections are strengthened beyond normal levels resulting in hyper-strengthened neural connections that are challenging to break. Within the nucleus accumbens (NAc), medium

spiny neuron's (MSN) baseline responsiveness to glutamatergic transmission has been enhanced and it is hypothesized these enhancements promote drug seeking (Purgianto et al., 2013).

Drug and alcohol addiction is a chronic cyclical disease characterized by recurring episodes of abstinence and relapse. High rates of relapse are present across a variety of drugs of abuse, and clinical observations show that drug craving is a critical factor that contributes to increased rates of relapse (Childress et al., 1999). Drug craving can be induced by a number of different factors including stress, cues associated with drug taking or drug receipt, and environments where drugs are administered. In this sense, craving is the conditioned response that results from presentation of drug associated cues. It is hypothesized that the conditioned response of craving motivates drug seeking or drug acquisition behavior (Childress et al., 1999; Gawin & Kleber, 1986; Ludwig & Wikler, 1974).

Many drugs of abuse activate the mesocorticolimbic pathway in the brain. This pathway is important for reward processing and learning (Everitt & Robbins, 2005; Everitt et al., 2008). More critically, this pathway is heavily involved in the switch from recreational drug taking to pathological or compulsive drug taking; from Action-Outcome to Stimulus-Response behavior (Everitt & Robbins, 2005). Early during acquisition, drug taking is hypothesized to be an Action-Outcome relationship, such that drug taking requires an action with the intention of a receiving an outcome or goal. In this case, the outcome reinforces the action, and results in a greater likelihood of occurring again in the future. However, that is not the entirety of the story. Stimuli or cues in the environment are also associated with drug reinforcement. Often through Pavlovian conditioning, cues become attractive and can motivate behavior through incentive salience (Flagel, Watson, Robinson, & Akil, 2007; Robinson & Berridge, 1993a; Robinson & Berridge, 2008). Once acquired, conditioned stimuli initiate an arousal state, which increases instrumental

responding or drug seeking/responding. Therefore, the associative learning that accompanies drug taking is a maladaptive learning process that motivates pursuit of drug reinforcers. In this sense, Everitt and Robbins (2005) argue that that the pursuit of drug reinforcement corresponds to a compulsive ("must do!") aspect of behavior, and reflects habit responding rather than conscious Action-Outcome behavior. Through this perspective, drug paired cues initiate drug-seeking through Stimulus-Response behaviors that are largely reflexive.

The stimuli that become associated with drug taking acquire value. After these stimuli have been conditioned (CS), they initiate an increase in phasic dopamine response (Fiorillo, Tobler, & Schultz, 2003). However, responses of dopamine neurons in the mesolimbic pathway generalize to other stimuli and show burst activity in response to surprising or novel stimuli (Anselme, 2009; Barto, 2013). Therefore, in addition to dopaminergic projections within midbrain dopamine neurons, it is likely that other neurotransmitters and brain regions are involved in mediating Stimulus-Response behavior. In other cases, conditioned stimuli themselves are reinforcing and become conditioned reinforcers. Conditioned reinforcement occurs when the stimuli associated with reinforcement acquire value and operant responding can be maintained for the conditioned reinforcer. Interestingly, conditioned reinforcement, but not conditioned stimuli maintain drug seeking (Di Ciano & Everitt, 2003), although both are mediated by midbrain dopamine activity (Everitt & Robbins, 2005), suggesting another mechanism may be important for how these learned associations motivate future drug seeking behavior compulsively.

Random presentations of drug associated conditioned stimuli do not maintain operant responding. Conversely, conditioned reinforcement does maintain operant responding, suggesting that the stimuli repeatedly paired with drug reinforcement acquire value and are

rewarding (Di Ciano & Everitt, 2003; Grimm, Kruzich, & See, 2000). Learning about drug associated stimuli is also dependent of dopamine activity, but learning can be augmented or mitigated by glutamatergic activity (Di Ciano, Cardinal, Cowell, Little, & Everitt, 2001; Ito, Dalley, Howes, Robbins, & Everitt, 2000), suggesting that both glutamate and dopamine are critical for conditioned responding and conditioned reinforcement.

In order to understand conditioned responding and conditioned reinforcement and their contribution to drug seeking behavior animal models have been developed. In the case of understanding how associative stimuli control drug seeking second order schedules of reinforcement are widely used (Di Ciano & Everitt, 2005), as these may better model the sequence of events completed to obtain drug reinforcement. Drug seeking has many components, namely when drug taking has not ceased and second, when drug seeking is commenced following a prolonged abstinence period. For example, second order schedules mimic the chain of behaviors addicts complete to obtain drug reinforcement, not necessarily drug seeking after a period of abstinence such as relapse. To understand drug seeking in the context of relapse a separate animal model of drug self-administration and relapse is utilized.

An Animal Model of Relapse

In order to study the behavioral and neurobiological mechanisms implicated in relapse behavior, an animal model of relapse was developed. In this model, animals are trained to make an operant response (e.g. nose poke or lever press) for drug reinforcement commonly paired with conditioned stimuli (cue light and tone). After animals reach stable responding, extinction training commences. During this phase, drug reinforcement and cue lights are removed and operant responding no longer results in a consequence. Importantly, animals are not forgetting about the Action-Outcome relationship, but rather are learning a new contingency, such that

operant responding no longer results in drug reinforcement. After animals have reduced responding they are tested in a relapse-like test. During the relapse-like test or reinstatement test, animals are presented with drug-paired cues, stress, or a small dose of drug. Then, drug seeking is measured by operant responding that once led to drug reinforcement, but these lever presses are not reinforced with drugs (Katz & Higgins, 2003; Shaham, Shalev, Lu, Wit, & Stewart, 2003). Drug-paired cues, stress, and small unit doses of drug all significantly increase operant responding, indicating a successful reinstatement manipulation, and a reliable model animal model of drug relapse.

The rodent model of relapse has high face validity but others have questioned the model's ability to capture construct or criterion validity (Katz & Higgins, 2003). Without construct and criterion validity, the results found in the model are equivocal. In this section, I present some of the cited shortcomings of the reinstatement model and discuss how other research has addressed them; validating the reinstatement model as a research tool to understand relapse and craving in human addicts. Currently, the reinstatement model is utilized to understand the behavioral and neurobiological mechanisms of craving and relapse, but as several papers suggest the validity of such broad generalizations should be interpreted cautiously.

One of the largest criticisms of the animal reinstatement model is the difference observed between conditioned stimuli and conditioned reinforcers. In humans, drug paired stimuli can induce craving (Childress et al., 1999) and lead to drug seeking behavior through a conditioned motivation state (Robinson & Berridge, 1993a). However, this relationship is not observed in a controlled laboratory setting. Instead, successful reinstatement behavior requires conditioned reinforcement (Grimm et al., 2000). Saunders et al. (2013) points out that the problem with current cue-reinstatement models is that the cue is not really inducing the behavior, but rather the

drug cue is the result of drug seeking behavior and is confounded with conditioned reinforcement.

Despite the concerns discussed above about the reinstatement model as a model of relapse, the reinstatement model has led to important advances in understanding relapse in human addicts (Kalivas & McFarland, 2003). Epstein et al. (2003) suggest that at the clinical level it is more important to test and develop medications that work, and the preclinical reinstatement model has been useful for testing medications prior to administration in humans. Importantly, reinstatement has been useful for eliminating medications that are true negatives; those that induce reinstatement in animals and relapse in humans (Epstein & Preston, 2003; Markou, Chiamulera, Geyer, Tricklebank, & Steckler, 2009). While Katz et al. (2003) suggests that the animal reinstatement model does not have predictive validity, Epstein et al. (2003) offers a different perspective, and suggests that in order to truly test the predictive validity of the reinstatement model clinical trials that resemble reinstatement need to be tested. In support of this idea, much of the clinical data surrounding relapse, craving, and propensity to relapse is anecdotal because of self-report bias or erroneous accounts of events prior to relapse (Shiffman et al., 1997). Taken together, until clinical data can better model relapse behavior in a controlled laboratory setting or more accurate accounts of relapse can be measured from addicts, the animal reinstatement model is a reliable model that has been shown to be a good model of human relapse behavior (Shaham et al., 2003).

Cue-induced craving and drug seeking

One missing aspect of the Stimulus-Response relationship is that there may be an intervening variable that accounts for individual differences in the propensity to relapse. The Stimulus-Response relationship predicts that once the relationship is formed, a ‘must do!’ action

is followed, suggesting that relapse rates are unequivocally high across all drugs, which is not supported (Ramo & Brown, 2008). Instead, I hypothesize that the stimulus results in an internal motivation that can motivate a behavior if the internalized state is great enough. I hypothesize that conditioned drug cues can result in craving to motivate behavior.

Empirical evidence suggests drug craving mediates relapse, such that when craving increases the probability of relapse also increases (Childress et al., 1999; Gawin & Kleber, 1986; Robinson & Berridge, 1993a; Saunders et al., 2013). Drug craving is currently modeled using the forced abstinence cue-induced drug seeking procedure. In this rodent model of craving, rats are subjected to the same intravenous self-administration procedures as discussed above. However, the similarities between these models of relapse diverge after self-administration. The main difference here is that many craving models (i.e. seeking tests) do not utilize a series of extinction days to diminish lever pressing (Loweth et al., 2014; Purgianto et al., 2013; Scheyer et al., 2016). In the craving model, after approximately 10-15 days of drug self-administration rats are rested for a single day. Then rats are tested in a cue-induced drug seeking test and active lever pressing/nose poking is measured. However, to model how craving changes the motivation to drug seek another set of animals rest for 30-90 days and are then tested in the same cue-induced drug seeking test (Conrad et al., 2008). Results indicate that longer durations of forced abstinence are positively correlated with greater levels of responding, such that the animals in the longer forced abstinence group respond more than animals in the short abstinence group. This time dependent increase in responding is termed the incubation of drug craving effect (Grimm, Hope, Wise, & Shaham, 2001).

Neurobiology of Craving and Cue-induced Drug Seeking

Craving is a psychological construct used to describe a motivational state of desire and wanting. Craving is a potentially rich data source for understanding drug addiction and relapse, because craving is hypothesized to increase with prolonged abstinence (Gawin & Kleber, 1986), and is evoked by contexts and stimuli associated with drug taking (Robinson & Berridge, 2001). The rodent incubation model of drug craving is suggested to be a valid model to understand human drug craving, because of the similarities in increased responding/drug seeking after prolonged abstinence in response to drug or natural reward paired cues (Conrad et al., 2008; Counotte, Schiefer, Shaham, & O'Donnell, 2014; Ferrario et al., 2011; Grimm et al., 2001).

The time-dependent increase in cocaine craving in human subjects follows an inverted U function, and suggests that craving peaks around one month and begins to decline after six months (Parvaz, Moeller, & Goldstein, 2016). Using the Late Positive Potential as an indicator of craving they determined that this brain correlated peaked around one month, but interestingly the subjective feelings of 'wanting' and 'liking' cocaine were highest around two days of abstinence. This study provides clear evidence that subjective ratings may not be the best measure to understand how cues motivate drug seeking behavior and scientists should rely on more objective measures.

The time-dependent increase in drug craving has been demonstrated most commonly with cocaine (Ferrario et al., 2011; Grimm et al., 2001; X. Li et al., 2013; Loweth, Tseng, & Wolf, 2014; L. Lu et al., 2005; Ma et al., 2014; Thiel et al., 2012), but heroin (Shalev, Morales, Hope, Yap, & Shaham, 2001), methamphetamine (Shepard, Bossert, Liu, & Shaham, 2004), alcohol (Bienkowski et al., 2004), and nicotine (Abdolahi, Acosta, Breslin, Hemby, & Lynch, 2010) also result in the increased drug craving. Interestingly, the time-dependent increase in drug craving is mediated by cues associated with drug taking being present, because general craving

decreases when drug paired cues are absent (X. Li, Caprioli, & Marchant, 2014; Thiel et al., 2012).

Neurobiological and pharmacological manipulations have identified the medial prefrontal cortex (mPFC), nucleus accumbens (NAc), ventral tegmental area (VTA), and the amygdala as important for incubation (Pickens et al., 2011). Koya and colleagues (2009) demonstrated that the ventral mPFC and not the dorsal mPFC is implicated in the incubation of drug craving. After cocaine self-administration rats were tested in a cue-induced reinstatement test after 1 or 30 days of abstinence. The increase in responding after the incubation period was attenuated by muscimol and baclofen (GABA agonist) administered to the ventral but not the dorsal medial prefrontal cortex after the 30 abstinence period. Also in support of the ventral mPFC in the incubation of craving effect is that phosphorylated extracellular signal-regulated kinases ERK activity was greater in the ventral mPFC when compared to the dorsal mPFC. The authors (Koya et al., 2009) suggest that phosphorylated ERK are an indicator of neuronal activation.

Other empirical evidence suggests a bidirectional relationship between the prelimbic (dorsal) and infralimbic (ventral) regions of the mPFC and the glutamatergic efferent projections to the NAc. Using a retrograde tracer Ma et al. (2014) revealed the majority of infralimbic mPFC efferents project to the NAc shell, while the majority of prelimbic mPFC efferents project to the NAc core. Interestingly, prelimbic mPFC to NAc core activation is required for cocaine reinstatement and inactivation of infralimbic mPFC to NAc shell also augments reinstatement (Kalivas & McFarland, 2003; Kalivas, 2009). This suggests that the circuits may have modulatory roles in the incubation of drug craving. Support of modulatory roles of the ventral and dorsal mPFC was observed by Ma et al. (2014). After cocaine self-administration, the NAc shell and core accumulate an abundance of silent synapses, which are thought to be immature,

inactive AMPA receptor synapses with the functional presence of NMDA receptors (Kerchner & Nicoll, 2008). After a 45 day abstinence period, in the NAc shell and NAc core, the augmented levels of silent synapses return to levels comparable to animals self-administering saline. Importantly, how these silent synapses became unsilenced illustrates the modulatory role of the shell and core. The NAc shell showed greater sensitivity to Naspm,—a CP-AMPA antagonist—and significant difference in inward rectification when compared to animals that self-administered saline. The NAc core AMPA receptors did not show a similar pattern. In the NAc core, the unsilenced synapses were not sensitive to Naspm and did not show a difference in inward rectification when compared to animals that self-administered saline. Taken together, these results provide evidence that glutamatergic transmission in the NAc is important for the incubation of drug craving and the infralimbic mPFC to NAc shell circuit and prelimbic mPFC to NAc core circuit have modulatory roles in the trafficking of AMPA receptors.

Glutamate mediated transmission in the amygdala has also been suggested to play a role in the incubation of drug craving. The amygdala is a limbic structure important for processing emotional stimuli, memory consolidation, and psychostimulant drug reward (Cain, Denehy, & Bardo, 2008; Knapp, Duncan, Crews, & Breese, 1998; Lee et al., 2013; L. Lu, Uejima, Gray, Bossert, & Shaham, 2007; Sharko, Kaigler, Fadel, & Wilson, 2013; Theberge, Milton, Belin, Lee, & Everitt, 2010). Evidence for a causal role of the central nucleus of the amygdala and not the basolateral amygdala has been suggested, because of the increase in ERK activity that coincides with the increase in responding after prolonged abstinence (L. Lu et al., 2007; Pickens et al., 2011). Recently, other evidence provides strong support that the basolateral amygdala to NAc shell circuit is critical the incubation of cocaine craving. In this circuit, after cocaine self-administration, the development of silent synapses are observed after 1 day of abstinence that

become unsilenced by CP-AMPA receptors insertion after prolonged abstinence and lead to increased cue-induced responding (Lee et al., 2013). At this time, both the basolateral and central nucleus of the amygdala require additional research, but it also suggests that the NAc may be the critical brain region where highly conductive CP-AMPA receptors are accumulating after prolonged abstinence.

Glutamatergic Transmission

Glutamate is an excitatory neurotransmitter. Glutamatergic transmission occurs through three types of ionotropic receptors and eight types of metabotropic receptors. Collectively, these receptors can account for upwards of 70% of central nervous system synaptic transmission (Pomierny Chamiolo et al., 2014). Ionotropic glutamate transmission is important for the development of psychostimulant locomotor sensitization and the acquisition of psychostimulant self-administration, suggesting glutamate transmission could mediate prolonged drug abuse. Further evidence for a causal role of glutamate in psychostimulant addiction is proposed by Kalivas (2009), which suggests that glutamate homeostasis is important for drug abuse resistance and reinstatement. A lack of glutamatergic tone results in greater extracellular glutamate, which leads to excitatory synapses that promote reinstatement of drug seeking behavior. Glutamatergic tone can be lost through presynaptic mGlur2/3 receptors and by loss of effective glutamate exchange. One important note about glutamate pharmacological interventions is that manipulations targeted at changing ionotropic transmission often induce considerable side effects. Agonism of these receptors can result in excitotoxicity, while antagonism can lead to deficits in cognitive function, and memory loss (Olive, 2009; Pomierny Chamiolo et al., 2014).

Ionotropic

Ionotropic glutamatergic transmission is fast and excitatory. Ionotropic transmission occurs at three different receptor subtypes: AMPA, kainate, and NMDA. AMPA and NMDA transmission is important for the development of amphetamine, and cocaine sensitization. AMPA receptors can broadly be classified into two different groups based on their calcium (Ca^{2+}) permeability. AMPA receptors with the GluA2 subunit do not pass Ca^{2+} through the ion channel meaning that those receptors are impermeable to Ca^{2+}(CI-AMPA). AMPA receptors that do not contain the GluA2 subunit are Ca^{2+} permeable (CP-AMPA). Differences between CP-AMPA and CI-AMPA receptors are observed in the kinetics with the Ca^{2+} permeable receptors having faster kinetics (Wolf & Tseng, 2012).

Calcium permeable AMPA receptors

Calcium permeable AMPA receptors (CP-AMPA) are a special AMPA receptor that does not contain the GluA2 subunit. CP-AMPA receptors can be homomeric and are composed of GluA1 or are heteromeric and are composed of GluA1 and GluA3 subunits. In either case, homomeric or heteromeric, CP-AMPA have unique qualities that result in special synaptic properties. Prior to being active and capable of synaptic transmission, CP-AMPA receptors are contained within the cytosol. However, synaptic activation via NMDA receptor and CaMKII activates AMPA receptors in the storage pool and rapid insertion into the postsynaptic membrane (Wolf & Ferrario, 2010).

Metabotropic

Metabotropic glutamate receptors (mGlur) are another type of glutamate receptor. MGlur are a transmembrane G-protein-coupled receptors and are implicated in slower neurotransmission and second messenger signaling. Currently, eight different mGlur receptors

have been identified, which generally fit into three different mGlur groups. Group I mGlurs encompass mGlur1 and mGlur5. Group II encompasses mGlur2, and mGlur3; finally, mGlur4, mGlur6, mGlur7, and mGlur8 are grouped into Group III (Olive, 2009). Through intracellular signaling, Group I mGlur receptor activation can indirectly activate protein kinase C and calcium/calmodulin-dependent kinase II (CaMKII) (Hermans & Challiss, 2001). Activation of these signaling pathways is especially important because protein kinase C and CaMKII mediate the release of dopamine following amphetamine or cocaine treatment (Hyman, 1996; Kantor & Gnegy, 1998). Therefore, identifying an upstream mechanism could be beneficial to reducing cocaine and amphetamine self-administration and relapse.

Although Group I and Group II mGlur have been implicated in addiction research, I am focusing only on Group I (mGlur1 and mGlur5). In a nicotine reinstatement procedure negative allosteric modulator (NAM), MPEP, significantly reduced cue and drug-induced reinstatement (Bespalov et al., 2005), suggesting that mGlur1 may play a significant role in relapse-like behavior. The same NAM also mitigates locomotor sensitization to cocaine (Dravolina, Danysz, & Bespalov, 2006) and morphine (Kotlinska & Bochenski, 2007) further supporting that mGlur1 has a significant role in addictive behavior. Certainly, further work is needed to understand the role of mGlur1 in addiction, especially because of the conflicting evidence suggesting that NAM of mGlur1 attenuates or has no effect on ethanol consumption and the expression of CPP (Hodge et al., 2006; Lominac et al., 2006; Schroeder, Overstreet, & Hodge, 2005), and that high doses of the potent NAM—JNJ16259685 resulted in reductions in locomotor activity. Nonetheless, evidence has shown that mGlur1 transmission reduces glutamate and dopamine release and is implicated in drug reinforcement and drug wanting in a CPP paradigm (Lominac et al., 2006).

Interestingly, and in slight opposition of the above mentioned research, blocking mGlur1 transmission with a non-competitive antagonist *increased* responding in an incubation of drug craving cue-induced reinstatement procedure (Halbout, Bernardi, Hansson, & Spanagel, 2014). This result and others support a negative coupling between of mGlur1 and CP-AMPA maturation and insertion in the NAc, such that increased mGlur1 stimulation results in less CP-AMPA receptor expression in the NAc (Loweth et al., 2014). Thus, this provides evidence that mGlur1 transmission may serve as a potential neural regulator of AMPA receptors and specifically CP-AMPA from being inserted in the post-synaptic membrane (Halbout et al., 2014).

The other Group I mGlur—mGlur5—is more widely researched in addiction. Similar to mGlur1, mGlur5 antagonism with MTEP, a NAM, also reduces the expression of morphine locomotor sensitization in a dose dependent manner, without affecting general locomotor activity (Kotlinska & Bochenski, 2007). Importantly, in Kotlinska and Bochenski's (2007) data suggest that mGlur1 or mGlur5 antagonism with NAM does not affect the increase in locomotor activity following acute morphine treatment, and suggests that there may be separate mechanisms that develop with repeated morphine exposure that mGlur1 and mGlur5 attenuate only following prolonged drug exposure. However, a causal role for mGlur5 was observed after acute cocaine treatment. When mice lacking mGlur5 were challenged with moderate and high doses of cocaine (10, 20, and 40 mg/kg) the mice failed to show an increase in locomotor activity (Chiamulera et al., 2001). The genetic alterations changed the reinforcing properties of cocaine as evidenced by mGlur5-lacking mice failing to acquire cocaine self-administration, but not food self-administration. Wild type and mGlur5-lacking mice were also not different extracellular dopamine release in the NAc following cocaine challenge or dopamine receptor or transporter expression.

Other research has determined mGlur5 antagonism with NAMs reduces ethanol (Backstrom, Bachteler, Koch, Hyytia, & Spanagel, 2004), heroin (Lou, Chen, Liu, Ruan, & Zhou, 2014), and nicotine (Paterson, Semenova, Gasparini, & Markou, 2003) self-administration, suggesting a promising role as a therapeutic in reducing drug reinforcement or drug seeking. Regardless of short or long access self-administration, MPEP significantly reduced cocaine self-administration (Kenny, Boutrel, Gasparini, Koob, & Markou, 2005). MGlur5 antagonism with MPEP significantly reduced nicotine-primed reinstatement (Tessari, Pilla, Andreoli, Hutcheson, & Heidbreder, 2004). Similarly, MPEP also dose dependently reduces ethanol-paired cue-induced reinstatement (Backstrom et al., 2004). MTEP, a slightly more potent and specific mGlur5 NAM, reduces methamphetamine self-administration, cue, and methamphetamine-induced reinstatement (Gass, Osborne, Watson, Brown, & Olive, 2009). Direct injections of MPEP into the NAc core reduced cue-induced reinstatement, but the suppression of reinstatement was likely dependent on an elevated anhedonic state or conditioned suppression as evidenced by elevations in intracranial self-stimulation (Backstrom et al., 2004; Kenny et al., 2005). MTEP, the more selective mGlur5 NAM, infused into the NAc core reduced both context-induced and cue-induced reinstatement following forced abstinence and extinction. These effects were not observed following MTEP infused into the dorsal striatum, indicating that glutamate signaling in the NAc core through mGlur5 is critical for cue-induced reinstatement (Kalivas & McFarland, 2003; Kalivas, 2009; Knackstedt, 2014).

In summary, the results clearly illustrate a relationship between Group I mGlur and drug reinforcement and drug seeking. However, given that mGlur1 antagonism results in increased responding after prolonged withdrawal and is negatively coupled with CP-AMPA insertion, while mGlur5 antagonism generally reduces psychostimulant reinstatement, it could suggest that

mGlur1 and mGlur5 have modulatory roles in drug seeking during reinstatement. To be specific, mGlur1 may protect against reinstatement and mGlur5 may promote drug reinstatement.

Synaptic strengthening

Long term potentiation is generally understood to reflect learning and memory at the neuronal level (Malinow, Schulman, & Tsien, 1989; Malinow & Malenka, 2002; Shi, Hayashi, Esteban, & Malinow, 2001; Shi et al., 1999). AMPA receptor movement into and out of the post-synaptic membrane is termed trafficking. AMPA receptor movement directly influences the synaptic strength of synapses (Malinow & Malenka, 2002).

Post-synaptic long term potentiation (LTP) within the hippocampus is largely dependent on AMPA receptor trafficking. AMPA receptor lability is not specific to the hippocampus. The NAc also shows AMPA receptor trafficking (Loweth et al., 2014; Wolf & Ferrario, 2010). The overwhelming majority of NAc neurons are medium spiny neurons. These medium spiny neurons are GABA neurons and are excited by AMPA receptor activation via glutamate (Hu & White, 1996; Pennartz, Boeijinga, & Lopes da Silva, 1990). AMPA receptor transmission has a bidirectional relationship with drug seeking, such that cue-induced reinstatement, drug-induced reinstatement, and drug seeking under second order schedules of reinforcement are all reduced when AMPA receptor transmission is blocked (Everitt & Robbins, 2005; Kalivas & McFarland, 2003; Kalivas, 2009). Alternatively, when AMPA receptor transmission is augmented prior to reinstatement testing, responding increases cocaine seeking (Cornish & Kalivas, 2000). This bidirectional modification to cocaine seeking is observed with intracranial infusions of AMPA antagonists and agonist directly into the NAc. It has been suggested that both the shell and the core of the NAc are integral in the increase and decrease in cocaine seeking, but the core is more important for cue-motivated drug seeking (Wolf & Ferrario, 2010).

Synaptic strengthening in the NAc is thought to mediate the long term alterations induced by drugs of abuse (Kalivas, 2009; Robinson & Berridge, 1993b; Robinson & Berridge, 2008; Wolf & Tseng, 2012; Wolf & Ferrario, 2010). In the drug naïve adult rat, the NAc contains few CP-AMPA receptors present in the core and the shell, suggesting that GluA1 receptors are coupled with GluA2 receptors and rendered Ca^{2+} impermeable (Boudreau, Reimers, Milovanovic, & Wolf, 2007). The small percentage of CP-AMPA receptors in the NAc are likely heteromeric GluA1/GluA3 with an even smaller proportion being homomeric GluA1 (Boudreau et al., 2007; Wolf & Ferrario, 2010). Further support of the idea that CP-AMPA receptors are in low abundance in drug naïve adult rats is that a selective CP-AMPA receptor antagonist has little effect in reducing excitatory post-synaptic currents (EPSC) (Conrad et al., 2008). These results provide strong evidence that in drug naïve animals, NAc AMPA transmission is mediated by GluA2-Containing (CI-AMPA receptors).

CP-AMPA receptors are highly conductive and even small changes in their post-synaptic expression can increase the strength of synaptic communication. Consistently, after long-access cocaine self-administration CP-AMPA receptors accumulate within the NAc core and shell (Wolf & Tseng, 2012; Wolf & Ferrario, 2010). Other research suggests that CP-AMPA maturation and insertion occurs primarily in the shell of the NAc (Kauer & Polter, 2014; Ma et al., 2014). In summary, the accumulation of CP-AMPA receptors in the NAc core and shell contribute to increased synaptic strength that lowers the threshold and invigorates drug seeking behavior following long abstinence periods.

Dysfunctional long-term depression

Recently, several research studies have shown that activation of long term depression (LTD) can reduce the strength of CP-AMPA receptor conductance (Loweth et al., 2014;

Mccutcheon et al., 2011). CP-AMPA receptors are highly labile and their maturation and insertion is dependent on neural activation. Using this idea it should be possible to remove these highly conductive receptors through synaptic depression. Because AMPA receptor transmission is prolific and governs the majority of excitatory synaptic transmission, systemic ionotropic manipulations in vivo are not without considerable side effects to learning, memory, and general cognition (Olive, 2009; Pomierny Chamiolo et al., 2014). To explore the possible therapeutic benefit of reducing CP-AMPA synaptic strength, metabotropic receptors (mGlur) have been explored. In further support of this idea, is that mGlur messaging moderates LTP in the NAc (Anwyl, 2009).

The vulnerability of future relapse episodes could be reduced if the highly conductive CP-AMPA receptor could be removed from the NAc. Removal of the CP-AMPA receptor would result in a net decrease in synaptic strength and could reduce the craving evoked by drug paired stimulation. Ideally, this form of long term depression would primarily remove CP-AMPA receptors and leave CI-AMPA receptors unaffected; or replace CP-AMPA receptors with CI-AMPA receptors. Importantly, group I mGlur (mGlur1 and mGlur5) are primarily located on post synaptic neurons and recent research indicates that mGlur1 stimulation results in a post-synaptic form of LTD. Previously however, LTD through mGlur transmission was understood to occur pre-synaptically through cannabinoid receptors (CB1) mediated depression (Robbe, Kopf, Remaury, Bockaert, & Manzoni, 2002). In this experiment, Robbe et al. (2002) hypothesized that activation of cortical glutamatergic afferents to the NAc would activate mGlur5, and further, that mGlur5 translates the signal to reach CB1 receptors that results in synaptic depression. Under control conditions, LTD is expressed following prolonged mGlur5 activation. After administration of MPEP a potent mGlur5 antagonist the effect of LTD was removed, and

administration of a mGlur5 agonist resulted in LTD. Taken together, these results suggest that in drug naïve NAc tissue mGlur5 is coupled with pre-synaptic CB1 receptors that together result in LTD.

Even a single exposure to cocaine eliminates the mGlur-CB1 receptor synaptic depression mechanism (Fourgeaud et al., 2004). Evidence from McCutcheon (2011) extends this finding and suggests that mGlur5's coupling with presynaptic CB1 may be altered after a prolonged abstinence period. Importantly, the CB1 synaptic depression mechanism remains intact after prolonged abstinence, and indicates that Group I mGlurs play an integral role in the accumulation of CP-AMPA receptors. Following DHPG (Group I mGlur agonist) administration, the rectification index is reduced resulting in LTD, which indicates that CP-AMPA receptors are either replaced by CI-AMPA or CI-AMPA function is enhanced. Interestingly, the synaptic depression effects of DHPG are modulated by cocaine exposure and by Group I mGlurs. In drug naïve rats, the effects of DHPG were blocked by MTEP (mGlur5 antagonist), however, in cocaine experienced rats the effects of DHPG were blocked by LY367385 (mGlur1 antagonist). These results clearly illustrate that in the NAc, the synaptic depression mechanisms change from mGlur5 to mGlur1 dependent following cocaine exposure and prolonged abstinence. Therefore, stimulating mGlur1 would theoretically reduce or remove CP-AMPA receptors and result in synaptic depression only in cocaine exposed animals after prolonged abstinence.

In support of this idea are data presented by Loweth et al. (2014). Their data suggested that CP-AMPA insertion resulted after the mGlur1 synaptic depression mechanism became dysfunctional. Interestingly, this showed that mGlur1 transmission is critical to preventing LTP from running rampant and synapses becoming over strengthened. When DHPG (Group I mGlur agonist) is injected directly into the NAc (single administration) it resulted in reduced drug

seeking after the long abstinence period, providing evidence that post-synaptic mGlur transmission can mitigate the effects of already accumulated CP-AMPA receptors. To provide more specific evidence a different PAM of mGlur1 (R067-7476) was administered to the NAc and it resulted in a similar reduction in drug seeking behavior. Systemic administration of mGlur1 PAMs also resulted in reduced drug seeking after prolonged abstinence, and importantly, provided evidence that the reduced drug seeking effect was the result of CP-AMPA removal. CP-AMPA removal is suspected because bath application of Naspm (CP-AMPA antagonist) did not have an effect after mGlur PAM stimulation, suggesting LTD resulted from removal. Taken together, these data provide strong evidence that within the NAc, mGlur1 transmission is directly linked to CP-AMPA maturation and insertion into the post-synaptic membrane. These data also support a therapeutic role for mGlur1 in reducing drug seeking behavior without affecting general locomotor/activity or natural reward responding.

The final piece of evidence that supports a dysfunctional control mechanism that promotes CP-AMPA accumulation is also evidence offered by Loweth et al. (2014). Using a cross-sectional design, rats' surface and total protein expression in the NAc was quantified at abstinence day 14, 25, or 48. Results indicated that immediately prior to CP-AMPA receptor insertion, mGlur1 surface expression was significantly decreased (abstinence day 25). It is hypothesized that this significant decrease in surface expression coincides with a significant reduction in mGlur1 transmission that allows CP-AMPA receptors to increase because there is no braking mechanism to control rampant CP-AMPA insertion. In this way, the LTD mechanism has become dysfunctional and the synapses in the NAc become over strengthened and hyper-responsive. Importantly, treatments of a mGlur1 PAM administered on intervening days from abstinence days 15-33 significantly reduced drug seeking after the prolonged abstinence and

suggests that enhancing the transmission of mGlur1 is a possible pharmacotherapy for reducing cocaine craving. However, 4-5 days after discontinuing mGlur PAM, increased levels of drug seeking and increased CP-AMPA receptors were measured, suggesting that continuous treatment with mGlur1 PAM is necessary to reduce drug craving.

Differential Rearing Alters Reward Response

The differential rearing model has not been widely researched with the incubation of drug craving. Differential rearing could induce the molecular changes in glutamate receptor expression that could result in protection from drug craving after prolonged abstinence (Chauvet, Goldberg, Jaber, & Solinas, 2012; Ma et al., 2016; Thiel, Sanabria, Pentkowski, & Neisewander, 2009; Thiel et al., 2012).

Rats reared in enrichment (EC) show an increase in learning, spatial memory, and novel object recognition (Duffy, Craddock, Abel, & Nguyen, 2001; Hullinger, O'Riordan, & Burger, 2015). Alternatively, rats reared in isolation (IC) show deficits in learning and spatial memory. Many of the behavioral tests that EC and IC rats perform differently rely on the hippocampus and prefrontal cortex, which suggests that the greatest difference between EC and IC rats lies within those regions. Neurobiological experiments have determined that EC rats have more dendritic branching on medium spiny neurons (MSN) (Comery, Stamoudis, Irwin, & Greenough, 1996), greater density of astrocytes, increased cortical thickness, and reduced glutamate release in response to amphetamine when compared to IC rats (Bowling, Rowlett, & Bardo, 1993; Diamond, Lindner, Johnson, Bennett, & Rosenzweig, 1975; Rahman & Bardo, 2008; Rosenzweig & Bennett, 1972). These results indicate the environmental housing manipulation is pervasive; capable of affecting change in many brain nuclei. Given the differences in brain weight and proliferation of dendritic spines between EC and IC rats, I hypothesize that there may

be differences between the groups in expression between glutamate receptors. Ultimately, these differences in glutamate receptor expression will contribute to protection (EC) or increased vulnerability (IC) during the drug seeking tests.

Differential rearing changes the responding for, and response to rewarding stimuli. Rats reared in enrichment respond less for novelty (Cain, Green, & Bardo, 2006) and demonstrate less locomotor activity in novel environments when compared to rats reared in isolation (Cain et al., 2006; Cain, Mersmann, Gill, & Pittenger, 2012). Differential rearing also changes the response to low unit doses of amphetamine, development of amphetamine sensitization, and self-administration of low unit doses of amphetamine (Arndt, Arnold, & Cain, 2014; Arndt, Johns, Dietz, & Cain, 2015; Bardo, Klebaur, Valone, & Deaton, 2001; Bardo et al., 1995; Bowling et al., 1993; Rahman & Bardo, 2008; Stairs, Klein, & Bardo, 2006; Stairs & Bardo, 2009). The protective effects of EC rearing are also observed across different drugs of abuse, providing more evidence of the protective effect induced by EC rearing. Rats and mice reared in the EC self-administer less amphetamine, cocaine, ethanol, and methamphetamine (Arndt et al., 2015; Chauvet, Lardeux, Goldberg, Jaber, & Solinas, 2009; Deehan, Cain, & Kiefer, 2007; Hofford, Darna, Wilmouth, Dwoskin, & Bardo, 2014). The most powerful effects of enrichment are observed at low-unit doses of psychostimulants, such that when higher doses are non-contingently or contingently administered the protective effects of enrichment are removed (Arndt et al., 2015; Bardo et al., 2001; Green, Gehrke, & Bardo, 2002; Solinas, Thiriet, Chauvet, & Jaber, 2010). This indicates that, while enrichment can be protective there is a limited range in its ability to protect against drug vulnerability. Maintaining an enrichment housing condition is also critical. When mice lose EC rearing, the rewarding effects of cocaine (assessed with CPP) are increased through a corticotropin releasing factor mechanism, which suggests that the loss of

enrichment could have induced an anxiety-like response. Notably, the mice switched from standard to enriched housing did not show a similar anxiety-like increase, suggesting the loss of enrichment led to the increase in corticotropin releasing factor, and not a general switch in housing condition (Nader et al., 2013). Taken together, continuous housing in the EC condition results in the greatest benefit to reducing the rewarding effects of cocaine. Secondly, intervention models of enrichment may induce non-specific alterations that result in negative affect and in turn, affect the rewarding properties of many psychomotor stimulants, especially because corticosterone is necessary to acquire amphetamine and cocaine self-administration (Marinelli, Rouge-Pont, De Jesus-Oliveira, Le Moal, & Piazza, 1997; Piazza et al., 1991). Therefore, the current proposal used a continuous EC or IC rearing environments beginning at postnatal day 21 through the final drug seeking test. This model of differential housing models the protective effects of enrichment and not the intervention effects cited in other differential housing experiments.

Enrichment affects reinstatement and incubation

In agreement with the idea that EC rearing is generally protective against drug vulnerability, EC rearing also reduces drug-induced reinstatement, but only when low unit doses are used (Stairs et al., 2006). In this experiment, EC and IC rats were allowed to self-administer amphetamine followed by extinction. During extinction, EC rats showed a faster rate of extinction learning when compared to IC rats. During the reinstatement tests rats were administered saline, a low (0.25 mg/kg), or high (1.0 mg/kg) dose of amphetamine and responding was measured. While both EC and IC rats increased responding on the active lever after administration of the low and high doses of amphetamine, EC rats did not increase to the same magnitude as IC rats at the low dose, again providing evidence of a dose dependent

protective effect of EC rearing. Differential rearing also changes reinstatement responding to a cue previously associated with methamphetamine reinforcement, such that EC rats continuously housed in enrichment did not respond as much as IC rats in the cue-induced reinstatement test (Hofford et al., 2014). Each of these differential rearing reinstatement tests followed extinction training, but determining if enrichment or isolation affects reinstatement responding after prolonged abstinence is less understood.

Recent research indicates that differential rearing can alter reinstatement responding after prolonged abstinence to both cocaine and to sucrose. However, the methodological differences between 'differential rearing' paradigms warrants discussion. After rats were housed in standard conditions for cocaine self-administration acquisition they were equally divided into EC or IC housing conditions during the abstinence periods. Rats remained in their new respective housing environment for 21 days and were then tested in extinction, cue-induced, and cocaine-induced (10 mg/kg) reinstatement (Thiel et al., 2009). Results clearly demonstrate a protective anti-craving effect of EC housing when compared to IC housing. Importantly, this model of enrichment is termed an intervention model, because the differential housing manipulation was introduced after stable cocaine (or any drug) self-administration. Using a similar intervention type model, EC housing resulted in protection from cue-induced drug seeking but not cocaine-induced reinstatement (Chauvet et al., 2009), suggesting that EC housing during the abstinence period could protect against the development of intensified craving induced by cues associated with drug reinforcement. Other evidence has also emerged that indicates that EC housing during the abstinence period does not attenuate the incubation effect (Thiel et al., 2012). When comparing EC and IC rats, EC rats responded less than IC rats during reinstatement tests, but EC rats' response rates increase from the short to long abstinence test. Despite previous evidence

that EC housing during the abstinence period mitigates the incubation effect (Chauvet et al., 2009; Thiel et al., 2009), it appears that further research is needed to truly determine if EC or IC housing affects the incubation of drug craving. Interestingly, these previously discussed studies all used differential housing as an intervention rather than a protective model. In a protective model, rats are reared from PND 21-51 in their respective housing condition and continue living in the housing condition throughout experimental testing. Our lab utilizes the differential rearing model that focuses on protection from compulsive drug taking and drug seeking. We hypothesize that the greatest effects of differential rearing occur during childhood, adolescence, and early adulthood. Second, our lab maintains the housing condition throughout all testing because continuous enrichment housing is most protective (Garcia, Haddon, Saucier, & Cain, 2017). Finally, our model of enrichment housing is different from the above mentioned studies in that our model houses 8-16 rats in a large cage, with daily handling and toy changes during the rearing period. After rearing (PND 51), novelty is maintained by daily toy changes. The abovementioned research studies only changed toys 3 times per week and did not include handling. Further, rats were in groups of 3-6 animals per cage, which does not result in robust enrichment effects (Renner & Rosenzweig, 2013). With all of this in consideration, a stronger enrichment environment may reduce responding during after the long abstinence period and mitigate the incubation effect.

Enrichment affects mGlurs and Long term potentiation

Rats reared in the EC housing have alterations in dopamine and glutamate systems. Neurobiological evidence clearly indicates that EC housing results in persistent changes within many brain areas, but importantly the mesolimbic and mesocortical dopamine pathways are also changed (Rahman & Bardo, 2008; Zhu, Apparsundaram, Bardo, & Dwoskin, 2005). It has been

suggested that multiple synaptic contacts in dendrites could help regulate synaptic strength and how neural circuits communicate (Harris, 1995). Within the dorsolateral striatum, EC rats display significantly more multiple-head spines on MSN when compared to IC rats (Comery et al., 1996). Presumably, the induction of multiple-head spines on dendrites enhances glutamatergic signaling and allows for modulated post-synaptic neural circuits, possibly allowing LTP and LTD to work synergistically to control synaptic strength.

In addition to the structural changes induced by EC or IC housing, there appear to be differences in dopamine and glutamate function. The *basal* levels of monoamines do not appear to be different between EC, IC and Standard (SC) rats, but differences in functional activity and turnover (reuptake and enzymatic breakdown) of monoamines are widely cited (Bardo et al., 1995; Bowling et al., 1993; Stairs & Bardo, 2009). When compared to IC rats, EC rats have less dopamine transporter expression in the mPFC (Zhu et al., 2005), and when challenged with amphetamine, EC rats show greater extracellular dopamine levels in the NAc when compared to IC rats. Glutamatergic tone is also altered by differential rearing. When challenged with amphetamine EC rats show a greater increase of glutamate release in the NAc when compared to IC rats (Rahman & Bardo, 2008). This glutamatergic increase has been suggested to be mediated by NMDA receptors but a definitive mechanism is not yet determined.

Further evidence of glutamatergic alterations induced by differential rearing have been identified in mGlur receptors. After rats are reared in EC or IC conditions, Group I and Group II mGlur receptors are expressed differently in the PFC. When compared to IC rats, EC rats have more expression of dimers mGlur1, and mGlur5. These alterations to the dimer form represent the functional mGlur1 and mGlur5, which suggests that IC rats have less expression of the functional Group I mGlurs, which may contribute to compromised glutamatergic tone

(Melendez, Gregory, Bardo, & Kalivas, 2004). To demonstrate differences in function DHPG (Group I agonist) or LY341495 (Group II/III antagonist) were administered followed by microdialysis measurement from the mPFC. As expected, the elevation in glutamate was greater in EC rats following both administrations, suggesting the alterations in Group I and Group II/III mGlurs occur at functional glutamate receptors. Furthermore, these alterations directly influence glutamatergic tone. Our lab has recently published results that indicate that altered Group I (Arndt et al., 2015) and Group II (Arndt et al., 2014) mGlurs that result from differential rearing. Briefly, our results indicate that IC rats have reduced function in mGlurs and it results in greater behavioral sensitization and reduced sensitivity to mGlur5 antagonism in an amphetamine self-administration paradigm. However, our lab has not quantified the expression of these different receptors following self-administration or after a forced abstinence reinstatement paradigm. Further, to my knowledge, Group I mGlurs have not been quantified in the NAc in differentially reared rats. Taken together, previous research suggests that differential rearing alters mGlur expression and cue-induced reinstatement following prolonged abstinence.

A new hypothesis about differential rearing and incubation of drug craving

Environmental enrichment protects against psychostimulant self-administration and reinstatement, and alters metabotropic glutamate function. With this in mind, and the abundance of evidence that EC housing alters learning when compared to IC or standard housed rats, I propose that the incubation of drug craving results from the loss of a cellular process important for LTD, which I hypothesize, signals through a mGlur1 mechanism. In accord with these behavioral changes, are differences in expression of glutamate receptors that control associative learning and synaptic strength. I hypothesize that EC housing will result in mGlur alterations that will control the accumulation of CP-AMPA receptors after prolonged abstinence. Alternatively,

IC housing will result in deleterious effects to mGlur expression rendering it dysfunctional and resulting in CP-AMPA accumulation. Together, these predictions suggest that housing conditions will directly influence the mechanism important for synaptic depression (LTD), and importantly will determine developmental factors that contribute to drug craving.

Hypotheses

The overarching hypothesis of this dissertation experiment is that EC rearing and IC rearing will result in different alterations to mGlur1 and mGlur5 expression. Collectively, these alterations will directly influence the amount of CP-AMPA that mature and insert into the post-synaptic membrane. The differences in CP-AMPA receptor expression will influence the magnitude of cue-induced drug seeking following long but not short abstinence.

Behavioral Hypotheses: Self-administration

The differences in amphetamine self-administration between EC and IC rats are greatest at low unit doses of amphetamine (Green et al., 2002). The current experiment used a high dose of amphetamine (0.1 mg/kg/infusion). We hypothesized that there would not be differences between EC and IC rats in the number of amphetamine infusions earned during the 1h self-administration testing. We are using a high dose to ensure equivalent amphetamine exposure between the rearing conditions (Arndt et al., 2015).

Hypothesis 1: EC and IC rats will self-administer equal amounts of amphetamine and saline during the operant self-administration phase.

Behavioral hypothesis: Cue-induced drug seeking after short abstinence

EC rats show faster extinction learning when compared to IC rats (Stairs et al., 2006). During the short abstinence seeking test, EC rats will show less drug seeking responding compared to IC rats. To measure drug seeking active lever presses were compared after the non-contingent cue presentation.

Hypothesis 2: EC and IC rats that self-administer amphetamine will have different rates of responding on the active lever during the cue-induced seeking test after the short abstinence period, such that IC rats will have higher response rates than EC rats. EC and IC rats self-administering saline will not be different.

Glutamate receptor expression hypotheses: Short abstinence

EC and IC rearing results in different mGlur1 and mGlur5 expression in the prelimbic (dorsal) and infralimbic (ventral) PFC (Melendez et al., 2004). However, mGlur1 and mGlur5 have not been measured in the NAc in EC and IC rats. After the short seeking test, EC and IC rats were sacrificed, the NAc dissected, and glutamate receptors were quantified. We hypothesize that EC rats will show elevated expression of mGlur1 and reduced expression of mGlur5 when compared to IC rats. Together these alterations will result in protection during the incubation period. IC rats will show the opposite effect. We hypothesize that IC rats will have decreased mGlur1 expression and increased mGlur5 expression when compared to the EC rats. As a result of these mGlur modifications, the IC rats will develop intensified craving during the incubation period. CP-AMPA insertion requires a prolonged abstinence period following stimulant self-administration, and therefore we hypothesize that GluA1 expression will not be different following the short abstinence seeking test.

Hypothesis 3: EC rearing will increase mGlur1 expression in the NAc when compared to IC rats. This increase will be evident in both amphetamine and saline groups.

Alternative 3: The hypothesized protective EC effect is reduced after high dose amphetamine self-administration. This results in similar mGlur1 expression in the NAc in EC and IC rats. However, in rats that self-administer saline, EC rats have more mGlur1 expression in the NAc when compared to IC rats.

Hypothesis 4: EC rearing will decrease mGlur5 expression in the NAc when compared to IC rats. The decrease will result in both amphetamine and saline groups.

Alternative 4: The protective effect of enrichment is lost after high dose amphetamine self-administration. EC and IC rats will express mGlur5 similarly in the NAc. In rats that self-administer saline, EC rats have less mGlur5 expression in the NAc when compared to IC rats.

Hypothesis 5: After the short abstinence seeking test, EC and IC rats will not express GluA1 differently in the NAc, and there will be no differences in GluA1 expression between the amphetamine and saline groups.

Hypothesis 6: After the short abstinence seeking test, EC and IC rats will not express GluA2 differently in the NAc, and there will be no differences in GluA2 expression between the amphetamine and saline groups.

Behavioral hypotheses: Drug seeking after prolonged abstinence

Drug craving increases across time. To model craving, rats were tested in a cue-induced seeking procedure after a prolonged abstinence period. We hypothesize that continuous rearing in the EC will result in protection from the incubation effect, such that EC rats will not show an increase in drug craving/drug seeking behavior; average responding during the short and long seeking tests will be similar in the EC rats. IC rats will show the progressive increase in responding after the prolonged withdrawal periods, such that responding during the seeking test will be significantly greater than the short abstinence seeking test and greater than EC responding during the long abstinence seeking test.

Hypothesis 7: For amphetamine rats, EC and IC rats will have different rates of responding on the active lever during the seeking test after the long abstinence period, such that IC rats will respond more when compared to IC responding during the short abstinence seeking test and when compared to the EC rats' responding during long abstinence seeking test. For saline rats, there will be no differences between the short and long response rates.

Glutamate receptor expression hypotheses: Prolonged abstinence

CP-AMPA receptor insertion requires a long 35-40 day abstinence period. Therefore, we hypothesize that CP-AMPA receptors will increase in expression following the long abstinence period, and CI-AMPA will be reduced or not changed. We hypothesize that expression of CP-AMPA receptors will be increased in IC rats only. MGlur1 and mGlur5 will also not be changed during the long incubation period. This result would indicate that mGlur's are hardwired during development and contribute protection from compulsive drug relapse. We hypothesized this

because rodents without mGlur5 do no acquire self-administration and blocking mGlur5 transmission reduces drug seeking (Chiamulera et al., 2001)

Hypothesis 8: For amphetamine and saline groups, after the long abstinence incubation test, EC and IC rats will express mGlur1 differently, such that EC rats will show greater expression in the NAc when compared to IC rats.

Hypothesis 9: For amphetamine and saline groups, after the long abstinence incubation test, EC and IC rats will express mGlur5 differently, such that EC rats will show decreased expression in the NAc when compared to IC rats.

Hypothesis 10: After the long abstinence incubation test, EC and IC rats will express GluA1 differently in the NAc, such that IC rats will have more expression when compared to EC rats. This effect will only be observed in the rats that self-administered amphetamine. There will be no change in GluA1 expression when comparing short vs. long or across housing group EC vs IC in the saline rats.

Hypothesis 11: After the long abstinence incubation test, EC and IC rats will express GluA2 differently, such that EC rats will have more expression in the NAc when compared to IC rats. This effect will only be observed in the rats that self-administered amphetamine. There will be no change in GluA2 expression when comparing short vs. long or across housing group EC vs. IC in the saline rats.

Chapter 2 - Methods

Animals

Male Sprague-Dawley rats (Charles River, Portage, MI, USA) arrived to the laboratory animal care facility at Kansas State University at exactly 21 days of age. Rats were housed in one of two environmental rearing conditions: enriched (EC), or isolated (IC). Rats were given ad libitum access to food and water throughout the experiment, with the exception of lever press training. During lever press training rats were maintained at 85% of free feeding weight until successful lever acquisition (see Lever press training). The colony room was set to a 12:12-h light:dark cycle and be maintained at approximately 22°C, with the humidity ranging from 30-45%. All behavioral testing was conducted during the light portion of the rats' cycle. All experimental procedures were conducted in accordance with the Institutional Animal Care and Use Committee at Kansas State University and the NIH guidelines (Council 2011).

Environmental Conditions

Upon arrival rats were randomly assigned to rear in the EC or IC for 30 days (PND 22-51). Enriched condition (EC) rats lived with 8-16 other cohorts and were housed in a large metal cage (60x120x45 cm) that was lined with pine wood chip bedding. Novelty, a component of the EC, was maintained by rotating 14 objects (small children's toys and PVC pipe) daily. Seven of the 14 objects were changed daily and all objects were changed two times weekly for cleaning and sanitation. EC rats were handled every day for approximately one minute. Isolated condition (IC) rats were housed individually in hanging metal wire cages (17x24x20 cm) during the 30 day rearing period. The IC rats were not handled during the rearing period and were not be exposed to novel objects or pine chip bedding.

Apparatus

Operant Chambers

Lever press training, sucrose self-administration, and amphetamine self-administration was conducted inside operant conditioning chambers (Med Associates, St. Albans, Vermont). Each operant chamber was enclosed in a sound attenuating box and controlled by a separate computer interface. The operant chamber was equipped with metal retractable levers. White cue lights (3 cm diameter) were centered above the levers. A white house light was on the opposite side of the levers and cue lights and illuminated the chamber during timeout periods. To deliver sucrose presentations a food magazine receptacle contained a liquid dipper capable of dispensing 0.1 ml of solution. Intravenous amphetamine solutions were delivered via a syringe pump (PHM-100, Med Associates) that was connected to a 10 ml syringe. From the syringe, catheter tubing was connected to a swivel and metal extension spring to protect the tubing from accidental tubing punctures. The extension spring leash was tethered to the rats' back mount to allow free movement throughout the operant chamber during all behavioral testing.

Drugs and Solutions

Cefazolin

The beta lactam antibiotic, cefazolin was dissolved in sterile water to yield a concentration of 50 mg/ml. Rats were administered 0.1 ml of cefazolin intravenously daily to reduce the probability of infection. Cefazolin was obtained from the Veterinary Pharmacy at Kansas State University.

D-amphetamine

D-amphetamine (Sigma Aldrich, MO, USA) was dissolved in 0.9% sterile saline to yield a concentration of 0.1 mg/kg/infusion. D-amphetamine was self-administered intravenously.

Diazepam

Diazepam was used for anesthesia before indwelling jugular catheter implantation. Diazepam was administered in a volume of 5 mg/kg/ml through intraperitoneal injection. Diazepam was obtained from the Veterinary Pharmacy at Kansas State University.

Flunixin (Banamine)

Flunixin was dissolved in sterile saline to yield a dose of 2.5 mg/kg/ml and injected subcutaneously to reduce inflammation and reduce discomfort or pain. Rats were administered flunixin injections for three days after surgery. Flunixin was obtained from the Veterinary Pharmacy at Kansas State University.

Ketamine

Ketamine was used for anesthesia before indwelling jugular catheter implantation. Ketamine was administered in a volume of 80 mg/kg/ml through intraperitoneal injection. Ketamine was obtained from the Veterinary Pharmacy at Kansas State University.

Heparinized Saline

Heparin (10-30 IU/ml) was dissolved in 0.9% sterile saline. Heparinized saline was used daily to help maintain catheter patency. Heparinized saline was administered in a volume of 0.1 ml intravenously. Heparin was obtained from the Veterinary Pharmacy at Kansas State University.

Sucrose

Sucrose was used to train rats to acquire lever pressing. Sucrose was dissolved in deionized water. To reach a concentration of 20% (w/v) sucrose concentration, 200g was dissolved in one liter of deionized water.

Procedures

Lever press training

At approximately 52 days of age, rats were food deprived to 85% of their free-feeding weight. Rat chow was restricted to 5-18 g per day until the target weight was reached. Rats were then trained to lever press on a single lever for 20% sucrose solution. First, rats were familiarized with the operant chamber and liquid dipper by presenting the rats with 20 non-contingent presentations of 20% sucrose over ~15 min. The next day rats were shaped to lever press on a fixed-ratio 1 (FR-1) schedule of reinforcement. Rats were allowed to earn up to 100 sucrose reinforcements. After rats acquired lever press training, the other lever was available and rats had access to both the active and inactive levers. Active lever presses resulted in sucrose presentation on a FR-1 schedule of reinforcement, and lever presses on the inactive lever had no programmed consequence. Data for the active and inactive levers was recorded and saved. Twenty percent (20%) sucrose self-administration sessions were 30 min. After three days of sucrose self-administration, rats were allowed to regain weight; rats were maintained on free-feeding weight throughout the duration of the subsequent experimental procedures. During all lever press training and the three (3) days of 20% sucrose self-administration the house light was illuminated and cue lights above the levers were not available.

Amphetamine self-administration

Surgical procedures

After lever press acquisition, rats regained weight for approximately four days prior to surgical procedures. Rats were deeply anesthetized with intraperitoneal injections of ketamine (80 mg/kg) and diazepam (5 mg/kg). Once anesthetized, the rats' dorsal area and ventral side of the neck were shaved, and scrubbed with chlorihexidine. A small 3cm horizontal incision was

made approximately 6-8cm below the shoulder blade. An additional incision (~.25cm) was made approximately 4cm above the initial incision. This smaller incision allowed the catheter port to exit. The final 1cm incision was made on the left ventral side of the neck, just above the clavicle, and perpendicular to the clavicle. The neck incision was superficial to the internal jugular vein. Once the catheter port was secure, the catheter tubing was tunneled subcutaneously up the dorsal side, over the rats' shoulder, and exited the neck incision. The internal jugular vein was located and a small incision was made across the vein. The catheter tubing was inserted into the left jugular vein. Once the catheter was inserted, the tubing was secured to the vein and connective tissue with suture thread and Vetbond adhesive (3M). The neck incision was closed with Vetbond adhesive (3M). The horizontal initial back wound was sutured with 3-4 suture knots and Vetbond secured each suture knot. Rats were administered flunixin (2.5 mg/kg/ml) to reduce post-surgical inflammation and 3ml of sterile saline to reduce dehydration. Catheter tubing was made from polyurethane plastic and measured 120mm with an internal diameter of 0.2mm (SAI Infusion Technologies). Catheter tubing was secured to a 22-gauge back-mounted cannula (Plastics One) with a suture knot. The back mount was secured to surgical mesh (Biomedical Structures). To protect the cannula from damage, it was covered with a stainless steel bolt. EC rats were housed in a standard shoebox cage with one other EC rat and one novel object for the night immediately after surgery. Following the post-surgical check the morning after surgery, EC rats were returned to the group cage for the remainder of the experiment. Rats were flushed daily with 0.1 ml of heparinized saline and 0.1 ml cefazolin to reduce the likelihood of infection and maintain catheter patency. Rats were allowed to recover from surgery for at least five days prior to experimentation.

Self-administration

After recovery rats were returned to the operant chambers and allowed to self-administer amphetamine (0.1 mg/kg/infusion) or saline. Responses on the active lever resulted in illumination of the cue light above the active lever, and 5.9s infusion of amphetamine or saline. After the 5.9s interval, the cue light turned off and the house light illuminated to signal a 20s timeout period. Responses on the active lever during the timeout were recorded but did not have a programmed consequence. Responses on the inactive lever had no programmed consequence throughout the experimental procedures. Rats self-administered amphetamine (0.1 mg/kg/infusion) for 1 hour for 10-15 sessions. Successful acquisition was defined as an average of 10 or more infusions across all self-administration sessions and a 2:1 ratio of active to inactive lever presses for amphetamine rats. Rats' catheter patency was checked with brevital (0.1 ml at 10mg/ml) before self-administration session and after the last self-administration session. Administration of brevital when the catheter is patent results in freezing behavior and sometimes, loss of the righting reflex. Rats with faulty catheters or that failed to acquire amphetamine self-administration were excluded from analyses.

Cue-induced drug seeking

Following the last day of amphetamine self-administration all rats rested for one day and were then tested in a cue-induced seeking procedure. Rats were tethered to the intravenous pump as if it were a normal self-administration session. During the first 10 min of the seeking test, all lever pressing had no programmed consequence, but active and inactive lever presses were counted. This was done to examine the invigoration by the context and to see the reduction in behavior prior to the cue presentation. After a Pre-CS interval of 10 min, rats were presented with a single 5.9s noncontingent presentation of the cue light associated with amphetamine or saline reinforcement. Subsequent responses on the active lever resulted in 5.9s illumination of

the cue light above the active lever and illumination of the house light for 20s. Responses on the inactive lever had no programmed consequence. The total duration of the cue-induced seeking test was 70 min in length: A Pre-CS interval of 10 min + 60 min seeking test. Immediately after the seeking test half of the rats were rapidly decapitated and their brains were harvested for tissue dissection. The remaining rats rested for 40 days in their home cage environmental condition (see Table 1). During the forced abstinence or long incubation period, rats were weighed daily to monitor health but were generally not handled unless a rat's health was a concern. The EC rats had daily toy rotations, and all toys were swapped two times per week. After 40 days, the rats were tested in the same cue-induced seeking experimental procedure, rapidly decapitated, and brain harvested for tissue extraction.

Protein analysis methods

This section outlines the methods used to extract, dissect, store, and analyze the brain tissue harvested after the rats' cue-induced seeking test.

Tissue collection

Immediately after the cue-induced seeking test, designated rats were transported to the surgery room, and deeply anesthetized using 4% isoflurane. Each rat had approximately 1-2 min of isoflurane exposure until effect. Rats were then rapidly decapitated and the brain tissue was harvested in ice cold saline and frozen on powdered dry ice. The brain was sealed in foil and kept at -80ºC until the brain was dissected. To dissect the NAc, the whole brain was sliced into 1mm coronal slices using a brain slicer (Brain Matrix Kent Scientific). The whole NAc was dissected on an ice block using biopsy punches (2mm). Tissue punches were immediately placed in 1ml of sucrose harvest buffer containing 10µl of protease inhibitors (ThermoScientific

Protease Cocktail) and homogenized mechanically using a tissue pedestal in a 1.5ml centrifuge tube. Tissue incubated on ice for 15-20 min and then was frozen at -80ºC.

Subcellular Fractional Protocol

Tissue samples removed from the freezer and thawed at room temperature. To remove the nuclear fraction samples were spun in a refrigerated 4ºC centrifuge for 5 min at 1,000g (P_1). The supernatant was transferred into a new aliquot (S_1). The S_1 tube contains the crude membrane and cytosolic fraction. The S_1 aliquot was returned to the refrigerated centrifuge and spun for 20 min at 10,000g. The supernatant was discarded and the resulting pellet (P_2) contained the cleared cellular membrane fraction. This cellular membrane fraction was used for quantification of all glutamate receptors and the loading control protein, calnexin. The pellet (P_2) was re-suspended in 200µl of NP-40 lysis buffer and 2µl of protease inhibitors and vortexed. The samples then incubated on ice for 15 min and stored at -80ºC (Lacrosse, Hill, & Knackstedt, 2016).

Protein Quantification

Unknown sample protein concentrations were determined using the Pierce BCA protein assay (ThermoScientific). Briefly, the Pierce BCA albumin protein standards were diluted according to the manufacturer's instructions and yielded a working range up to 2mg/ml. Once the known albumin standards were diluted, 25µl of each standard albumin sample was pipetted into a centrifuge tube. The working reagents (200µl) were applied to the standard dilutions and incubated for two hours at room temperature. The standard albumin samples were then tested for their absorbance of 562nm. Greater absorbance indicated more total protein. The computer was then calibrated to determine the total protein concentration in each albumin standard. Each albumin standard was tested three times for reliability. This process generated a standard curve

that could be used to interpolate to the unknown samples collected after the cue-induced seeking test. For each tissue sample collected, 25µl of unknown protein sample was added to 200µl of working reagent. Absorbance of 562nm was tested in the same way as the known albumin protein samples using the Nanodrop 8000. The unknown samples' absorbance was then compared to the standard curve and the protein concentration of the unknown samples was estimated. Each unknown sample was measured three times and the average of the three measurements was used for subsequent western blot analyses.

Western blot

Precast acrylamide gels were purchased (Bio Rad) in the gradient of 4-20% bis-acrylamide and were 1mm thick. Ten micrograms of total protein from rat NAc tissue from EC and IC housing conditions was added to Lamefili loading buffer under reducing conditions using βME. The total volume loaded into the SDS-PAGE gel for each well was 30µl. Sample was not pooled; allowing for individual quantification of each receptor of interest. Proteins were separated through the gel using 100v for 90 min. Gel was rinsed with deionized water and placed in transfer buffer solution until the transfer apparatus was ready. From negative to positive the order of the transfer sandwich was: a sponge, two pieces of filter paper, the gel, the PVDF membrane, two pieces of filter paper, and a sponge. The transfer sandwich was loaded in the protean transfer apparatus (Bio Rad mini) and ran at 75-90v for 120 min. After the transfer, the visible protein standards (Precision Plus Protein Standard Dual Color; Bio Rad) were marked with a pen on the back of the membrane. This helped ensure that the protein standard did not fade during the antibody application, or TBST (Tris buffered Saline + 0.1%Tween 20) washes.

The membrane was blocked in 3% non-fat dry milk+TBST for 30 min and gently rocked. The primary antibodies (GluA1: Rabbit polyclonal AB31232, 1:2,000, ~106 kDa; GluA2: Mouse

monoclonal AB106515, 1:1,000, ~100 kDa; mGlur1: Rabbit polyclonal AB82211, 1:1,000, ~300 kDa; mGlur5: Rabbit monoclonal AB76316, 1:5,000, ~300 kDa; all Abcam) were dissolved in 3% non-fat dry milk +TBST (Loweth et al., 2014; Melendez et al., 2004). The blocking solution was poured off and the primary antibody solution was applied and left to incubate overnight at 4ºC. The following morning, the membrane was washed in TBST for 60 min, exchanging the TBST with fresh TBST every 10 min. The secondary antibodies were diluted in 5% non-fat dry milk. The secondary antibodies were goat Anti-rabbit (AB97051; Abcam) or Anti-mouse (AB205719; Abcam) with a horseradish peroxidase conjugate. The secondary antibody was applied to the membrane and gently rocked for 60 min at room temperature. Then the membrane was washed a second time in TBST for 60 min, exchanging the TBST with fresh TBST every 10 min. The horseradish peroxidase conjugate was probed using a chemiluminescent (Clarity ECL; Bio Rad) solution. One and half milliliters of Clarity ECL was applied to the membrane for 30-60s and digital images were captured with a Kodak Image Station. Digital (.TIF) files were analyzed in ImageJ. The signal of each protein of interest was normalized to calnexin (Rabbit polyclonal AB22595, 1:10,000; Abcam). To make comparisons across EC and IC rats and drug conditions, protein values were normalized to the IC Sal rats.

Data analysis

Mixed factorial repeated measures ANOVA

A mixed factorial ANOVA was used to analyze group differences between EC and IC rats in amphetamine/saline self-administration. The between subjects factors were environmental group (EC or IC) and drug self-administered (amphetamine or saline). Typically, EC and IC rats do not differ in amphetamine self-administration at high unit doses. Therefore, we designed this study to use a dose of 0.1 mg/kg/infusion of amphetamine to help reduce baseline differences

between EC and IC rats. Preliminary analyses were completed to ensure that the average saline and amphetamine infusions were equal for the group assignments for the short and long incubation periods. Inactive lever pressing during self-administration were analyzed with a similar mixed factorial repeated measures ANOVA.

Prior to the seeking test, preliminary analyses were completed to ensure that there were not mean differences between rats assigned to the short or long incubation periods. There were no average differences in amphetamine exposure between EC and IC rats assigned to the short or long incubation periods. These preliminary analyses ensured that differences in drug seeking during the seeking tests were not an artifact of group assignment.

To determine differences in drug seeking behavior during the short and long seeking tests, two between subjects factorial ANOVAs were used to analyze potential group differences. The rationale for using separate ANOVAs is that the unequal cell sizes (behavioral data only) is a violation of the assumptions of ANOVA. For each ANOVA, the between subjects factors were environmental group (EC or IC), and drug self-administered (amphetamine or saline). Significant interactions observed in the omnibus ANOVA were probed with simple effects tests and corrected with Sidak post hoc comparisons. Sidak-corrected t-tests were used to compare average response rates for short and long incubation groups. Inactive lever pressing during the seeking test were analyzed with a similar factorial ANOVA model. Additional analyses (described below; Mixed effects model) were used to complement the ANOVA results to test continuous x categorical interactions (e.g. protein expression c rearing group) across each seeking test.

During the seeking tests, active and inactive lever presses were recorded every five min. This let us examine when the rats were making their active and inactive lever press responses

within the 70 min test session. In these analyses, group and drug were treated as between subjects variables and time bin was treated as a within subject variable. Separate factorial ANOVAs were used to analyze active lever presses for the short and long seeking tests. Inactive lever presses were analyzed using the same ANOVA model. Significant interactions observed in the omnibus ANOVA were probed with simple effects tests and corrected with Sidak post hoc comparisons.

To determine differences in receptor expression (mGlur1, mGlur5, and GluA1 & GluA2) between the experimental groups after the cue-induced drug seeking tests, a between-subjects factorial ANOVA was used for each glutamate receptor/subunit. Here, there were approximately equal cell sizes and incubation period could be used as a variable and separate ANOVAs were not required. The between subjects factors were environmental group (EC or IC), drug (amphetamine or saline), and abstinence period (short or long). The dependent variable was the average normalized expression value for each glutamate receptor. A separate factorial ANOVA was used for each receptor.

Chapter 3 - Results

Self-Administration Sessions

Infusions earned across self-administration sessions

The number of rats needed to reliably test the hypotheses exceeded what could be completed in one shipment of rats. Therefore, three shipments of rats arrived at Kansas State University spaced approximately eight weeks apart. Preliminary analyses were completed to ensure that cohort did not significantly affect infusions across the self-administration sessions. The repeated measures ANOVA confirmed that the rat's cohort did not affect amphetamine or saline self-administration. There was no main effect of cohort ($F(1, 77) = 0.70$, $p>.05$), and no significant interaction of cohort x session ($F(1, 75) = 1.43$, $p>.05$). For all subsequent analyses the rats across all cohorts were collapsed, because there were no differences between them in average amphetamine or saline self-administration.

The repeated measures factorial ANOVA revealed significant effects of rearing group ($F(1, 75) = 36.93$, $p<.001$), drug ($F(1, 75) = 126.80$, $p<.001$), and session ($F(15, 1{,}125) = 56.03$, $p<.001$). In addition there were significant two way interactions of session x drug ($F(15, 1{,}125) = 2.30$, $p<.005$), and rearing group x drug ($F(1, 75) = 33.37$, $p<.001$). The session x group interaction was not significant ($F(15, 1{,}125) = 1.28$, $p=.20$). However, this lack of session x group interaction was likely moved out of significance because of the significant three way interaction between rearing group, drug, and session ($F(15, 1{,}125) = 8.92$, $p<.001$). This result indicates that EC and IC rats took different number of infusions of amphetamine or saline across the 16 self-administration sessions. To further understand the average differences across these variables, the significant three way interaction was probed with simple effects analyses. Group differences in infusions earned between EC and IC rats were compared within each drug

condition for each session. Type I error rate was corrected with Sidak corrections. When rats self-administered amphetamine, the only significant difference between EC and IC rats in average infusions earned was during session one (Figure 1). EC rats had more infusions of amphetamine when compared to IC rats ($F(1, 1,125) = 10.01$, $p<.0016$). A summary table of the simple effect calculations can be found in Table 2.

To determine how EC and IC rats self-administered saline differently across the self-administration sessions, means were probed with simple effects analyses and corrected with Sidak corrections. For the EC and IC rats that self-administered saline, there were significant differences throughout the self-administration sessions. IC rats earned more saline infusions when compared to EC rats in sessions 1-15 (all $F's > 10.01$, $p<.0016$; Figure 1). EC and IC rats earned similar infusions of saline in the final self-administration session ($F(1, 1,125) = 9.17$, $p>.0016$). A summary table of the simple effect calculations can be found in Table 3.

Total active lever presses including timeout responding

A separate repeated measures factorial ANOVA was used to determine if rearing group, drug, session, or the interactions significantly altered total active lever pressing during the self-administration session. For these analyses the timeout responding is included in the analyses. The factorial ANOVA revealed that there were main effects of drug ($F(1, 75) = 49.70$, $p<.001$), session ($F(15, 1,125) = 39.27$, $p<.001$), but no main effect of group ($F(1, 75) = 0.96$, $p>.05$). This lack of effect of group was likely moved out of significance by significant interactions of group x drug ($F(1, 75) = 19.04$, $p<.001$), session x group ($F(15, 1,125) = 2.45$, $p<.005$), and session x drug ($F(15, 1,125) = 2.32$, $p<.005$). In addition there was a significant three-way interaction of group, drug, and session ($F(15, 1,125) = 7.69$, $p<.001$). Taken together, these results indicate that EC and IC rats had different average lever pressing across all sessions and

their responses depended on whether they self-administered amphetamine or saline. To determine when EC and IC rats differed in total lever pressing across sessions the significant three-way interaction was probed using simple effects with a Sidak correction.

For rats responding for amphetamine, the simple effects analysis revealed that EC rats had significantly more total active lever presses during session one ($F(1, 1{,}125) = 119.33$, $p<.0016$), and during session two ($F(1, 1{,}125) = 36.48$, $p<.0016$) than IC rats. During all remaining sessions EC and IC rats in the amphetamine group had similar total active lever pressing (Figure 2).

For the rats responding for saline, the opposite was observed. While there were significant differences between EC and IC rats in total active lever pressing, it was the IC rats responding more than the EC rats. This result was true for session one ($F(1, 1{,}125) = 21.13$, $p<.0016$) and for session two ($F(1, 1{,}125) = 13.15$, $p<.0016$). During the remaining sessions EC and IC rats in the saline group had similar rates of responding (Figure 2).

In summary, the significant three-way interaction indicated that EC and IC rats had different total active lever pressing across session and their rates of responding depended on amphetamine or saline assignment. Closer analyses revealed that these differences in responding were early in the self-administration sessions and were transient. However, this may also suggest differences in motivation that are prevalent in acquisition but dissipate in subsequent sessions.

Inactive lever presses across self-administration sessions

The repeated measures factorial ANOVA revealed that there were no main effects of rearing group ($F(1, 75) = 2.55$, $p>.05$), or drug ($F(1, 75) = 0.38$, $p>.05$), but there was a main effect of session ($F(15, 1{,}125) = 14.76$, $p<.001$). However, there was a significant interaction between group and drug ($F(1, 75) = 23.93$, $p<.001$) and a significant interaction between drug

and session ($F(15, 1{,}125) = 2.32$, $p<.005$). Probing the group and drug interaction indicated that EC and IC rats had different average response rates on the inactive lever for amphetamine and saline. On average, when responding for amphetamine the EC rats had significantly more inactive lever presses ($F(1, 75) = 5.50$, $p<.025$) than IC rats. The opposite effect was observed when testing the simple effects for saline, such that IC rats had significantly more responding on the inactive lever on average ($F(1, 75) = 20.78$, $p<.025$). To probe the simple effects of the drug and session the EC and IC rats were collapsed and the average response rates were compared between amphetamine or saline at each session. This simple effect resulted in a significant difference in average inactive lever presses for amphetamine and saline on session 1 with rats in the saline condition showing more inactive lever presses ($F(1, 1{,}125) = 25.69$, $p<.025$). It should also be mentioned that average differences between EC (M=2.49*)* and IC (M=6.76*)* rats in the saline group and in the amphetamine group EC (M=6.11) and IC (M=3.95) was small. Taken together these results clearly indicate that difference in inactive lever responding were early in the session and there were minimal difference between EC and IC rats across all sessions (see Figure 3).

Seeking after 1 day abstinence

Total active lever presses

Generally, I hypothesized that rearing in the EC would result in less drug seeking after a short 1day forced abstinence period in response to a drug associated cue. I hypothesized that this effect would be observed in the amphetamine rats but not the saline rats. To test this hypothesis a factorial ANOVA was completed and results indicated that there was main effect of group ($F(1, 77) = 10.55$, $p<.005$), a main effect of drug ($F(1, 77) = 52.73$, $p<.001$), but there was no significant interaction of group x drug ($F(1, 77) = 2.13$, $p>.05$). Examination of the group means

indicated that rats in the IC group responded more during the short seeking test when compared to EC rats. Similarly, rats that self-administered amphetamine had greater total active lever pressing during the short seeking test when compared with the saline group. (Figure 4).

Total inactive lever presses

With regards to the inactive responding during the seeking test after one day, I hypothesized that there would be no difference between EC and IC rats or across the drug groups. My hypothesis was not supported and the factorial ANOVA revealed that there was a main effect of group ($F(1, 77) = 9.35$, $p<.005$), main effect of drug ($F(1, 77) = 10.60$, $p<.005$), but no significant interaction of group x drug ($F(1, 77) = 0.71$, $p>.05$). Examination of the means suggest that IC rats had more inactive lever responses when compared to EC rats, and that amphetamine rats responded more when compared to IC rats (Figure 5).

Active lever pressing during the pre-cue interval

At the beginning of the seeking test there was a 10 min interval that had complete extinction contingencies. During this interval responses on the active and inactive lever were counted but presses resulted in no programmed consequence. This ANOVA compares responding across groups during that 10 min interval. The factorial ANOVA results revealed that there were a main effects of group ($F(1, 77) = 40.07$, $p<.001$), drug ($F(1, 77) = 51.30$, $p<.001$), and a significant interaction of group x drug ($F(1, 77) = 7.16$, $p<.01$). This interaction was probed with Sidak corrected simple effects. The simple effects analysis indicated that IC rats responded more than EC rats during the pre-cue interval for both amphetamine ($F(1, 77) = 40.06$, $p<.001$), and saline ($F(1, 77) = 6.76$, $p<.025$). Taken together, these results suggest that IC rats had significantly more lever presses during the pre-cue interval. The IC rats in the amphetamine condition showed the greatest rates of responding (Figure 6).

Inactive lever pressing during the pre-cue interval

The factorial ANOVA results indicated that there were significant main effects of group ($F(1, 77) = 15.74$, $p<.001$), drug ($F(1, 77) = 11.55$, $p<.001$), but there was no significant interaction of group x drug ($F(1, 77) = 2.39$, $p>.05$). Examination of the group means indicate that IC rats responded more than EC rats regardless of drug condition. Similarly, rats that previously self-administered amphetamine responded more when compared to saline rats (Figure 7).

Seeking after 40 day abstinence

Total active lever presses

I hypothesized that after the 40 day forced abstinence period drug seeking would increase in the IC rats but not the EC rats. My hypothesis was not supported because drug seeking motivation did not increase after the 40 day abstinence period in the IC or EC rats. This lack of result was confirmed in a series of paired samples t-tests (all p's >.05). Nonetheless, the factorial ANOVA revealed that EC and IC rats responded differently after 40 days of forced abstinence, such that IC rats responded more than EC rats ($F(1, 37) = 6.50$, $p<.05$). There was not an effect of drug ($F(1, 37) = 2.93$, $p>.05$), meaning that rats previously self-administering amphetamine and saline responded at similar rates following prolonged abstinence. Finally, there was not a significant interaction of group x drug ($F(1, 37) = 0.95$, $p>.05$). In summary, IC rats responded more than EC rats after 40 days of forced abstinence regardless of drug group (Figure 8).

Total inactive lever presses

The factorial ANOVA revealed that there was no main effects of group ($F(1, 37) = 0.48$, $p>.05$), drug ($F(1, 37) = 0.01$, $p>.05$), and no significant group x drug interaction ($F(1, 37) =$

3.61, p>.05). These results indicate no significant differences between EC and IC rats in the average inactive lever presses (Figure 9).

Active lever pressing during the pre-cue interval

An additional factorial ANOVA also revealed no main effects of group (F(1, 37) = 1.45, p>.05), drug (F(1, 37) = 0.38, p>.05), or group x drug interaction (F(1, 37) = 0.03, p>.05) for active lever presses during the 10 min pre-cue interval. This should not be confused for an absence of responding during this time. IC AMP rats had the greatest response rate (M = 18.42, SD = 9.96), followed by IC SAL rats (M = 16.40, SD = 6.50), EC AMP (M = 14.89, SD = 7.47), and finally EC SAL (M = 13.70, SD = 8.17) had the least active lever pressing during the pre-cue interval (Figure 10). I then used Sidak-corrected paired t-tests to determine if pre-cue interval responding increased within each experimental group. The t-test results indicate that within the saline group both EC and IC rats increased active lever presses during the pre-cue interval after the 40 day abstinence period (t(9) = 4.29, p=.002 and (t(9) = 3.57, p=.006). Within the amphetamine group, the responses of the EC and IC rats were the opposite. In EC rats, active lever pressing during the pre-cue interval increased after 40 days of abstinence, (t(9) = 3.02, p=.017). The IC rats active lever presses decreased after 40 days of abstinence, (t(9) = 2.46, p=.03), however, the Sidak-corrected adjustments results in a nonsignificant decrease between day 1 and day 40 responding in IC rats, suggesting that pre-cue response rates did not change in IC rats. These results suggest that the rats show augmented seeking behavior that was not specific to amphetamine reinforcement during the pre-cue interval. However, rats in the IC amphetamine condition decreased, offering the possibility of differences in seeking motivation invigorated by the context.

Inactive lever pressing during the pre-cue interval

The factorial ANOVA indicated that there were no main effects of group ($F(1, 37) = 0.66$, $p>.05$), drug ($F(1, 37) = 1.13$, $p>.05$), or a group x drug interaction($F(1, 37) = 0.79$, $p>.05$), suggesting that inactive lever presses across all groups were similar during the 10 min pre-cue interval. Although not statistically different from each other EC SAL rats had the most inactive lever presses (M =12.40, SD = 11.57), followed by IC SAL (M =12.20, SD = 7.66), IC AMP (M =11.75, SD = 7.74), and EC AMP (M =7.33, SD = 4.27). These results are summarized in Figure 11. As with the active lever press data from the pre-cue interval, Sidak-corrected t-tests were used to compare day 1 and day 40 inactive lever press responses. All of the analyses revealed no significant differences in inactive lever presses (all p's >.05), suggesting that the abstinence period's effects are specific to the active lever presses.

Time course analyses of the seeking tests

The 70 min seeking test was divided into 14-five min time bins. Breaking the seeking test into 5 min time bins enabled us to determine when the groups are responding on both the active and inactive levers. For these analyses, the between-subjects variables were group and drug, while time bin was treated as a within subjects variable. I hypothesized that EC would show less drug seeking when compared to IC rats. I hypothesized that rats that self-administered amphetamine would show greater lever pressing behavior throughout the seeking test.

After 1 day abstinence

Active lever presses

The repeated measures factorial ANOVA indicated that there were main effects of group($F(1, 77) = 20.29$, $p<.001$), drug ($F(1, 77) = 62.34$, $p<.001$), and time bin ($F(13, 1{,}001) = 44.00$, $p<.001$). There was also significant two-way interactions between group and time bin

($F(13, 1{,}001) = 12.00$, $p<.001$), and drug and time bin ($F(13, 1{,}001) = 13.75$, $p<.001$). The interaction of group x drug was not statistically significant but it was trending toward significance ($F(1, 77) = 3.89$, $p=.052$). Finally, there was a significant three-way interaction of group, drug, and time bin ($F(1, 1{,}001) = 3.29$, $p<.001$), indicating that across the seeking test EC and IC rats drug seeking depended on whether they had previously self-administered amphetamine or saline. To fully probe this significant three-way interaction simple effects with Sidak corrections were used. The simple effects analyses compared EC vs IC rats within a drug condition at every time bin. For example, EC rats' average responding was compared to IC rats' average responding within the amphetamine group for time bins one, two, three thru time bin 14.

The simple effects tests indicated that within the amphetamine condition EC and IC rats had different drug seeking rates early in the session and the differences between the groups became smaller and nonsignificant as the drug seeking test progressed. During the pre-cue interval where rats were in complete extinction (no cues presented after active lever presses), in the amphetamine group, IC rats had more drug seeking during time bin one ($F(1, 1{,}001) = 128.92$, $p<.001$), time bin two ($F(1, 1{,}001) = 30.74$, $p<.001$). After the second time bin elapsed a single presentation of the drug-paired cue was presented, and every active lever press resulted in the cue light and sound of the infusion pump. The cue invigorated drug seeking more in IC rats compared to EC rats for time bin three ($F(1, 1{,}001) = 73.58$, $p<.001$), and time bin five ($F(1, 1{,}001) = 22.65$, $p<.001$). In time bins six thru fourteen, there were no differences between EC and IC rats in amphetamine seeking all p's $>.05$.

For the rats that self-administered saline, the only significant difference between EC and IC rats occurred during the pre-cue interval in the first 5 min time bin. IC rats had significantly

more saline seeking during the beginning of the test ($F(1, 1{,}001) = 18.19$, $p<.001$). This effect was only transient and dissipated as the seeking test progressed (See Figure 12).

Inactive lever presses

An additional repeated measures factorial ANOVA was conducted on the inactive lever presses measured during the seeking test after one day of abstinence. This ANOVA revealed that there were main effects of group ($F(1, 77) = 16.83$, $p<.001$), drug ($F(1, 77) = 14.98$, $p<.001$), and time bin ($F(13, 1{,}001) = 17.09$, $p<.001$). Additionally, there were significant two-way interactions between group and time bin ($F(13, 1{,}001) = 4.57$, $p<.001$), and drug and time bin ($F(13, 1{,}001) = 2.33$, $p<.005$). The interaction of group x drug was not statistically significant ($F(1, 77) = 1.30$, $p>.05$). Similarly, the three-way interaction of group x drug x time bin was not statistically significant ($F(13, 1{,}001) = 1.35$, $p>.05$). The significant two-way interactions were probed with simple effects analyses and a Sidak correction.

The simple effects results from probing the group x time bin interaction indicated that during the pre-cue interval, IC rats had more inactive lever presses on time bin one ($F(1, 1{,}001) = 69.91$, $p<.001$), but there were no differences between EC and IC rats in the last 5 min of the pre-cue interval. Presentation of the cue light resulted in greater responding in IC rats for time bin three ($F(1, 1{,}001) = 12.54$, $p<.005$), and time bin four ($F(1, 1{,}001) = 22.76$, $p<.005$) when compared to EC rats. EC and IC rats were not different from time bins five thru fourteen. The other set of simple effects probed the interaction of drug x time bin. This set of simple effects determined that rats that previously self-administered amphetamine had significantly more inactive lever presses during the pre-cue interval at both time bins, one ($F(1, 1{,}001) = 34.13$, $p<.001$), time bin two ($F(1, 1{,}001) = 16.92$, $p<.005$). After the presentation of the cue, there was no immediate invigoration of inactive lever pressing, but amphetamine rats had more responses

during time bin four ($F(1, 1{,}001) = 18.11$, $p<.005$). Taken together, these results indicate that IC rats had more inactive lever presses but that was not drug dependent, and rats that previously self-administered amphetamine had more inactive lever presses. However, it should be mentioned that the differences across these groups was only transient and dissipated approximately 20 min into the 70 min seeking test (see Figure 13).

After 40 day abstinence

After the 40 day abstinence period, I hypothesized that rats that self-administered amphetamine would show increased drug seeking compared to the rats that self-administered saline. I hypothesized that EC housing during the 40 day abstinence periods would reduce drug seeking when compared to IC rats.

Active lever presses

The repeated measures factorial ANOVA determined that there were significant main effects of group ($F(1, 37) = 5.69$, $p<.05$), and time bin ($F(13, 481) = 39.14$, $p<.001$). The main effect of drug was not significant ($F(1, 37) = 2.64$, $p=.11$), indicating that amphetamine and saline rats—regardless of EC or IC group—responded similarly across the sessions. This interpretation was confirmed by a nonsignificant interactions of drug x time bin ($F(13, 481) = 0.57$, $p>.05$), and group x drug ($F(1, 37) = 0.78$, $p>.05$). There was, however, a significant two-way interaction between group x time bin ($F(13, 481) = 3.17$, $p<.001$), suggesting that EC and IC rats had different active lever pressing across the seeking test after 40 days of abstinence. This interaction was probed with simple effects and determined that EC and IC rats were not different during the pre-cue interval for time bin one ($F(1, 1{,}001) = 0.12$, $p>.002$), or during time bin two ($F(1, 1{,}001) = 6.95$, $p>.002$). However, after the presentation of the cue light associated with lever reinforcement, there was a significant difference in active lever presses between EC and IC

rats that persisted for 15 min. Specifically, IC rats—regardless of drug group—had more active lever presses at time bin three ($F(1, 1{,}001) = 32.11$, $p<.002$), time bin four ($F(1, 1{,}001) = 21.50$, $p<.002$), and time bin five ($F(1, 1{,}001) = 8.96$, $p<.002$). Importantly, these differences between EC and IC rats are across the amphetamine and saline conditions and are not specific to drug reinforcement (Figure 14).

Inactive lever presses

The repeated measures factorial ANOVA indicated that there was a main effect of time bin ($F(13, 481) = 21.29$, $p<.001$). There was no main effect of group ($F(1, 37) = 0.72$, $p>.05$), and no main effect of drug ($F(1, 37) = 0.50$, $p>.05$). There was also no interactions between group x drug ($F(1, 37) = 2.34$, $p>.05$), group x time bin ($F(13, 481) = 0.79$, $p>.05$), or drug x time bin ($F(13, 481) = 1.21$, $p>.05$). Finally, the three-way interaction between group x drug x time bin was not significant ($F(13, 481) = 0.20$, $p>.05$). Using Sidak corrected pairwise comparisons to compare the average inactive lever presses across the time bins indicated that responding during the pre-cue interval during the first 5 min (time bin one) had the greatest response rates. Time bin one had significantly higher inactive lever presses when compared to all other time bins. Time bins two and three were sporadically different from subsequent time bins. During the contingent cue presentation portion of the seeking test, there were no significant differences between time bins four thru fourteen, suggesting that all rats had similar inactive lever presses from minutes 20-70 during the seeking test (Figure 15).

Expression of AMPA receptor subunit 1 (GluA1)

A factorial ANOVA was used to analyze differences in AMPA subunit GluA1 across the different groups. The dependent variable being analyzed is the value that was normalized to the IC saline rats in the short test. I hypothesized that the GluA1 would increase in rats that self-

administered amphetamine after the long incubation period. However, I thought that EC rats would be 'protected' from this increase and the increase would only be observed in IC rats. The ANOVA results indicate that there was no main effects of group ($F(1, 71) = 0.96$, $p>.05$), drug ($F(1, 71) = 0.76$, $p>.05$), or incubation period ($F(1, 71) = 0.85$, $p>.05$). All two-way interactions were also not significant (p's >.05). The three-way interaction of group x drug x incubation period was also not significant ($F(1, 71) = 0.22$, $p>.05$). Taken together, these results suggest that AMPA subunit expression does not change differently across EC and IC rats, nor does the expression change as a function of amphetamine self-administration (Figure 16). My hypothesis about GluA1 expression increasing in IC rats that self-administered amphetamine after a prolonged abstinence period was not supported. While these results were surprising, it is not completely at odds with previous literature, because longer self-administration sessions may be required for the accumulation of Ca^{2+} permeable AMPA receptors (Purgianto et al., 2013).

Expression of AMPA receptor subunit 2 (GluA2)

Similarly to the previous analysis, the dependent variable being analyzed here is the GluA2 value that was normalized to the IC saline rats in the short test. I hypothesized that EC rats would have more GluA2 expression when compared to IC rats after the long abstinence period. The factorial ANOVA results did not provide support for my hypothesis and revealed no main effects of group ($F(1, 71) = 0.01$, $p>.05$), drug ($F(1, 71) = 0.01$, $p>.05$), or incubation period ($F(1, 71) = 0.00$, $p>.05$). All of the two-way interactions were not significant and the three-way interaction of group x drug x incubation period was also not significant ($F(1, 71) = 0.29$, $p>.05$). These results suggest that GluA2 expression was not changed by the incubation period, amphetamine exposure, or as a result of differential rearing (Figure 17).

Expression of metabotropic glutamate receptor 1 (mGlur1)

Rats reared in the EC show increases in the expression of mGlur1 in the prelimbic regions of the prefrontal cortex when compared to IC rats (Melendez et al., 2004). With this in mind, I hypothesized that mGlur1 expression would be greater in EC rats in the whole NAc (core + shell). In addition, I hypothesized that mGlur1 expression would decrease progressively during the abstinence period in the IC rats that self-administered amphetamine. I analyzed the mGlur1 dimer expression in each rat; normalized to IC rats that self-administered saline. The factorial ANOVA results did not provide support for my hypotheses. The results show that there was no effect of group ($F(1, 71) = 0.00$, $p>.05$), drug ($F(1, 71) = 0.87$, $p>.05$), or incubation period ($F(1, 71) = 0.59$, $p>.05$). The interaction of group x drug was trending but was not significant ($F(1, 71) = 2.00$, $p=.15$). The interactions of group x incubation period ($F(1, 71) = 0.18$, $p>.05$), and drug x incubation period ($F(1, 71) = 0.04$, $p>.05$), were also not significant. Finally, the three-way interaction of group x drug x incubation period was also not significant ($F(1, 71) = 0.44$, $p>.05$), providing evidence that mGlur1 expression was not changed as a result of the experimental manipulations (Figure 18).

Expression of metabotropic glutamate receptor 5 (mGlur5)

Melendez et al. (2004) also determined that mGlur5 expression is different in the prelimbic region of the prefrontal cortex between EC and IC rats, such that EC rats have more expression. I hypothesized that within the NAc there would be an increase in mGlur5 expression in IC rats compared to EC rats, because mGlur5 has been implicated in increasing drug abuse vulnerability and cue reactivity (Bespalov et al., 2005; Gass et al., 2009). For this analysis, I analyzed the mGlur5 dimer normalized expression. The factorial ANOVA indicated that there was no main effect of group ($F(1, 71) = 1.06$, $p>.05$), drug ($F(1, 71) = 1.07$, $p>.05$), or

incubation period ($F(1, 71) = 0.02$, $p>.05$). The group x drug interaction was not significant ($F(1, 71) = 2.38$, $p=.13$). The group x incubation period interaction was not significant ($F(1, 71) = 0.83$, $p>.05$). The last two-way interaction of drug x incubation period was also trending toward significance ($F(1, 71) = 2.84$, $p=.09$). These results possibly suggest that mGlur5 expression declines during the prolonged 40 day abstinence period in saline rats, but in amphetamine rats the expression may increase, specifically in IC rats. This potential interaction was probed with a Sidak-corrected planned comparison and revealed that at the short seeking test amphetamine and saline groups were not different ($F(1, 36) = 0.66$, $p>.05$). After the 40 day abstinence period amphetamine and saline groups were still not statistically different but it was close ($F(1, 39) = 3.94$, $p=.054$). These results were not significant but were trending in the described direction that mGlur5 may increase during prolonged abstinence in amphetamine exposed rats (Figure 19). Finally, the three-way interaction between group x drug x incubation period was not significant ($F(1, 71) = 0.47$, $p>.05$), suggesting that the expression of mGlur5 does not change differently in EC and IC rats regardless of drug condition across time. These results do not provide support for my hypotheses regarding mGlur5 expression and may actually provide evidence for the opposite to be supported, because EC rats tended to have more mGlur5 expression—albeit not statistically different—and I predicted IC rats would have more mGlur5 expression because of augmented amphetamine seeking.

Amphetamine intake is not correlated with glutamate receptor expression

There were no differences between EC and IC rats in protein expression so their data was collapsed and used to calculate correlations between average infusions of amphetamine per self-administration session and the protein expression for each glutamate receptors. These results indicated there was no correlation between amphetamine infusions and GluA1expression (r = -

0.01, $p > .05$). There was also not a significant correlation between amphetamine infusions and GluA2 expression ($r = 0.05$, $p > .05$). The correlation between amphetamine infusions and mGlur1 was small and nonsignificant ($r = 0.13$, $p > .05$). The final correlation between amphetamine infusions and mGlur5 expression was not significant, ($r = -0.09$, $p > .05$).

Chapter 4 - Discussion

Generally, I hypothesized that rearing in the EC would result in less drug seeking motivation when compared to IC rats at both the short and long seeking tests. This hypothesis was supported and provides further evidence for the protective effect environmental enrichment can have especially when introduced in early life. Second, I hypothesized that amphetamine seeking motivation would be augmented as a function of abstinence period, such that amphetamine seeking would progressively increase during the abstinence period. This hypothesis was not supported by the current data. This result was somewhat surprising given the amount of literature that shows augmented drug seeking after prolonged abstinence in cocaine (Grimm et al., 2001; L. Lu, Grimm, Dempsey, & Shaham, 2004) and methamphetamine (Krasnova et al., 2014; X. Li, Zeric, Kambhampati, Bossert, & Shaham, 2015; Scheyer et al., 2016) and, even more traditional thought of depressant drugs such as ethanol (Bienkowski et al., 2004) and heroin (Shalev et al., 2001). The current study did not show drug incubation and it would have been the first empirical study to show that *d*-amphetamine results in similar time-dependent increases in craving and amphetamine seeking using a short accessmodel. Here, the results suggest that the pharmacological action of stimulants is important to the development of the incubation of craving, because the development of intensified or persistent drug seeking did not develop. Alternately, amplified responding could have been observed after the short one day abstinence period and stayed elevated throughout the prolonged abstinence period. Nonetheless, these results suggest that the development of intensified drug craving is changed by drug or early life experience.

Enrichment reduces drug seeking after short and long abstinence

The current experiment determined that differential rearing changes amphetamine seeking after a short and long abstinence period, such that IC rats had greater drug seeking in amphetamine seeking. This result is in support of previous literature that indicates that early life experiences can have a profound influence on the abuse vulnerability (Bardo, Neisewander, & Kelly, 2013; Bardo, Donohew, & Harrington, 1996). The elevated drug response shown in the current study, and provides clear evidence that rearing in isolation is damaging and increases amphetamine self-administration and amphetamine seeking into adulthood.

Enrichment attenuates augmented drug seeking

The results from the current study indicate that EC housing beginning in early life and maintained throughout adulthood reduces amphetamine seeking when compared to IC rats. Our results are in agreement with other published studies examining the effects of enrichment to reduce drug seeking after prolonged abstinence in a cued seeking test (Chauvet et al., 2009; Thiel et al., 2009).

While our results do fit with current literature examining the effects of environmental enrichment there is one major difference between the previous studies and the current experiment. The previous studies used enrichment as an intervention to determine if it could reduce drug seeking, and our study design used a *prevention* model. In our study, our goal was to determine if early life experience could change adult drug seeking behavior after different abstinence periods. This study design explored the situational factors that lead to the development of augmented drug seeking and persistence in responding, while other studies examine if enrichment can be used as a therapeutic to reduce drug seeking behaviors (Solinas,

Chauvet, Thiriet, El Rawas, & Jaber, 2008; Solinas et al., 2010). Naturally, these methodological differences lead to different conclusions about how enrichment may be reducing drug seeking.

One hypothesis suggests that enrichment does not protect against the neurobiological alterations induced by cocaine self-administration but instead, enrichment protects against the expression of augmented cocaine seeking. This hypothesis is supported by evidence that losing enrichment, even after cessation of drug taking results in incubation of drug craving (Chauvet et al., 2012; Ma et al., 2016). Other results from our lab suggest that early life enrichment and maintaining enrichment in adulthood is critical for reducing behavioral responses that coincide with abuse criteria (Garcia et al., 2017). While the effects of EC housing are beneficial to reducing drug seeking behavior induced by drug cues and stress (Chauvet et al., 2009), their results also suggest that the effects of enrichment may deteriorate as quickly as seven days if enrichment is lost. Alternatively, exposing rodents to enrichment for as little as seven days can reduce drug seeking after prolonged abstinence (Chauvet et al., 2012). Thus, the positive effects of enrichment are induced and lost rapidly, suggesting that maintenance of enrichment throughout the withdrawal period is most beneficial to reducing drug seeking motivation.

The other hypothesis suggests that prolonged enrichment before drug exposure results in neurobiological adaptations that protect against the induction of drug-induced neuroadaptations that promote drug vulnerability. This hypothesis is supported by literature that suggests that enrichment beginning at postnatal day 21 prevents the development and expression of locomotor sensitization (Arndt et al., 2014; Cain et al., 2012; Gill, Arnold, & Cain, 2012; Green, Cain, Thompson, & Bardo, 2003). Importantly, however, these preventative effects are most robust at low-unit doses (Bardo et al., 2001; Cain et al., 2012; Garcia et al., 2017), suggesting that

enrichment can protect against adaptations that may accompany early drug experimentation before binge doses are self-administered.

Here, our data fit more with the former hypothesis in that similar neuroadaptations result in EC and IC rats after repeated amphetamine exposure but the expression of drug seeking is mitigated. EC and IC rats did not have differences in any of the glutamate receptors quantified in the NAc, but EC rats had significantly less drug seeking when compared to IC rats. It should also be mentioned that there were not any differences in the between the EC and IC saline rats, but the IC rats responded more during saline self-administration sessions and during each of the seeking tests. This result suggests that EC and IC rats may demonstrate a different glutamate plasticity pattern when compared to standard housed rats (W. Lu & Wolf, 1999; Mead & Stephens, 1998), because GluA1 and GluA2 show a decrease in the accumbens after repeated amphetamine. Interestingly, GluA1 expression was not different between amphetamine and saline rats after 3 days, but in amphetamine treated rats GluA1 and GluA2 decreased after 14 days. This study's (W. Lu & Wolf, 1999) experimental timeline and use of passive injections is different than the ones used in the current study, but it does suggest the onset of AMPA subunit changes could occur early (14 days) and normalize to baseline by 40 days. Alternatively, another explanation is that the timeline for increased craving and glutamate receptor adaptations is much longer for amphetamine than cocaine. For cocaine, intensified drug seeking peaks around abstinence day 35 and stays elevated through abstinence day 90 (Grimm et al., 2001; Wolf & Tseng, 2012). A similar timeline has not been established with amphetamine, meaning it could peak at 14 days and last until day 40, or intensified craving could peak at day 60 and last until day 90. Without knowing this timeline and given the lack of increased seeking, future studies researching amphetamine should utilize an experimental design that tests rats at various

abstinence periods. This would elucidate the critical time period when amphetamine craving intensifies and results in enhanced drug seeking. Additionally, it would result in a focused timeline to identify important neurobiological processes that accompany the increase in amphetamine seeking.

Additionally, one other explanation for no differences in protein expression is that we used a subcellular fractional protocol to get the crude membrane fraction. A more focused approach using biotinylation could have resulted in differences between surface protein expression and total expression. Further, looking specifically at different synaptic compartments could have yielded different results especially with the AMPA subunits because they are expressed more in the cytoplasm.

Isolated Housing Increases and Maintains Drug Seeking

The deficits that result in IC rearing and housing when compared to EC rearing are large and span many different behaviors. To my knowledge, the effects of IC rearing and housing have not been examined in the drug incubation model. The data indicate that early life experience enhances drug seeking motivation and IC rats resemble an 'incubated' drug seeking response in a standard housed rat on day1. I hypothesize that the reason that the current experiment did not observe an increase in amphetamine seeking after 40 days of abstinence was not because of a decrease in drug seeking motivation after 40 days. Instead, it is possible that amphetamine seeking motivation was elevated on day 1, and any subsequent increase in responding would be difficult to achieve. Comparing the IC rats responding in our current experiment with other published manuscripts examining the incubation effect, it appears that our IC responding was significantly higher than what is typically observed on day 1 responding (Conrad et al., 2008; Grimm et al., 2003; Scheyer et al., 2016). It is somewhat difficult to collapse across the many

incubation studies especially when considering drug, whether an experiment had extinction trials, and the length of the reinstatement/seeking tests. With all of those variables considered, it appears that the IC rats in our study showed elevated responding when compared to cocaine (Conrad et al., 2008; Grimm et al., 2003) and methamphetamine (Scheyer et al., 2016) seeking tests on day 1. Not including the pre-cue interval responding, in the first 30 min of the seeking (after the initial cue presentation) the IC rats had an average of approximately 37 drug seeking lever presses, while other published data typically observes an average of 15-20 drug seeking lever presses. This does not take away from the fact that IC rats did not elevate their responding after the prolonged abstinence period, but it does suggest that a possible ceiling effect occurred. Looking more closely at the time course data from the short and long seeking tests (Figures 12 and 14), the rate of extinction is faster in the short test IC amphetamine rats when compared to the IC s rats in the long test. This suggests that while the initial invigoration of drug seeking may be higher after a short abstinence period, the persistence is motivation is longer after the long abstinence period. Greater resistance to extinction is in accord with previously published studies examining the incubation of drug craving (Grimm et al., 2001; L. Lu et al., 2004). It should be mentioned however, these studies utilized a resistance to extinction protocol in which animals were repeatedly tested in six-1h extinction sessions. Nonetheless, our IC rats showed greater drug seeking motivation when compared to the EC rats and suggests that negative early life experience increases drug seeking motivation after short and long abstinence periods.

Short Access (1h) Does Not Result in Time-dependent Increases in Amphetamine Seeking

Another interpretation of the current data is that incubation did not develop as a result of the short access (1h) self-administration sessions. The incubation of drug craving has been

observed when using short access models, but only when rodents self-administered cocaine (Swinford-Jackson, Anastasio, Fox, Stutz, & Cunningham, 2016). To my knowledge, the time dependent increase in drug seeking has not been observed using amphetamine, suggesting that amphetamine self-administration does not result in an incubation effect or, more likely, that longer self-administrations sessions may be required. Furthermore, the accumulation of CP-AMPA receptors after the cessation of cocaine self-administration require that rodents undergo a long access (6h+) self-administration protocol; however, session length has not been manipulated using other stimulant drugs, meaning that the self-administration session length needed for the accumulation of CP-AMPA receptors could be different for different drugs of abuse. Thus, it is possible that the length of the self-administration session could represent a threshold that mediates the development of the incubation of drug craving. For example, the drug incubation effect is robustly observed when using long access self-administration models, while the incubation effect is less robust when using short access models. It is possible that if the length of the self-administration session is important for the development of the incubation effect, the threshold needed to observe the progressive increase in drug seeking can be moderated by the drug. In relation of the current data, maybe 1h *amphetamine* self-administration session will rarely result in rodents that show a progressive increase in amphetamine seeking motivation. This interpretation is reasonable to consider and suggests that future models examining amphetamine drug seeking motivation after prolonged periods of abstinence utilize a longer self-administration protocol, because the current data suggest a session length greater than 1h is required. What we do not know yet is what session length is needed that results in robust amphetamine seeking that increases as a function of abstinence period. Future experiments should examine this hypothesized threshold (session length and probability to 'incubate') to

determine if any biological, and developmental factors contribute to the time-dependent increase in drug seeking.

Exploring this idea further, Li and Frantz (2009) showed that the incubation of cocaine craving developed using a short access (2h) self-administration session, but the robustness of the incubation effect depended on when the rats began cocaine self-administration. Rats that acquired cocaine self-administration in adulthood were more likely to show the progressive increase in responding when compared to rats that acquired in adolescence after 30 and 60 days of abstinence. In another publication, Li and Frantz (2010) showed that the age effect was not a learning deficit by the adolescent rats. Rats that acquired sucrose self-administration in adolescence were just as likely to incubate after 30 days when compared to adults. Together, their results show that biological factors early in development can influence the incubation of drug craving when a short access self-administration model is used (C. Li & Frantz, 2009). It also suggests that if a researcher uses a manipulation that lowers the probability that 'drug incubation' will happen (because it is short access) that individual differences in the development of increased cocaine seeking emerge. These data support my hypothesis that other variables account for some variability in the development of an increased drug seeking response.

One other explanation of the lack of incubation effect is that it is possible that it could have been difficult to see an increase because the rats were trained on sucrose and then switched to amphetamine. After the switch and first day of amphetamine, the rats' active lever presses dropped significantly and remained lower throughout subsequent sessions. Therefore, if the rats were anticipating amphetamine then they would not show increased seeking because they were trained to maintain low levels of responding. Another explanation for the mixed incubation results is that the rats did not know what to anticipate prior to the seeking tests. They could be

anticipating sucrose or amphetamine/saline and the increase in responding is motivation to respond for sucrose and not visual stimulation. In either case, future studies examining the differences in reward seeking in differentially reared rats should explore other training methods to get the rats to acquire lever press training to reduce potential confounds in interpretation.

Enrichment Alters Glutamate Expression and Function

Environmental enrichment in rodents results in numerous behavioral and neurobiological changes including adaptations to glutamate receptor expression and transmission (Melendez et al., 2004). Recently, another study has determined that the glutamatergic alterations induced by EC housing affect AMPA receptors (Gauthier et al., 2015). While there were a number of differences between the current study and Gauthier et al. (2015), it does suggest that GluA1 expression increases as a function of extinction training with or without enrichment. Following cocaine self-administration that resulted in a net decrease of GluA1 expression, rats were trained under extinction conditions. Interestingly, reinstatement of cocaine seeking was only reduced in the groups that got extinction training, environmental enrichment, and showed elevations in GluA1. These data suggest that the expression of GluA1 increases as a result of extinction training but it appears that the changes in expression are only important if a behavioral intervention is also incorporated. These results fit nicely with the current data and with research in standard housed rats (Sutton et al., 2003). Keep in mind that while there were no statistically significant differences between the rearing groups, or drugs, our data for GluA1 expression were trending in this direction. After 40 days of forced abstinence, the EC amphetamine rats showed an increase in GluA1 expression that was not observed in the EC saline or IC amphetamine rats. This suggests that EC housing during the abstinence period may actually result in increases in GluA1 expression that are important for reducing drug seeking behavior. Alternatively, it is

possible that the trending increase in GluA1 expression is really the result of enhancements in general learning and memory that resulted from continuous EC housing during the abstinence period.

Glutamate receptor transmission in the context of extinction learning may be an important variable to consider. In the current experiment, the IC rats showed elevated response rates when compared to EC rats during saline self-administration and during the cued seeking test, even when they had previously responded for saline. This result was not entirely surprising given that IC rats after amphetamine self-administration did not show clear evidence of extinction learning until after 10 sessions (Stairs et al., 2006). The persistence in drug seeking was specific to amphetamine reinforcement because the IC rats in the sucrose self-administration study were not different from EC rats after four extinction sessions (Stairs et al., 2006), suggesting that amphetamine imparts neurobiological changes that increase drug seeking above and beyond natural reward reinforcement learning. These neurobiological deficits that result from amphetamine exposure may be exacerbated by IC housing and contribute to increased drug seeking even in the absence of drug-associated cues.

Amphetamine and enrichment condition do not affect NAc CP-AMPA

I hypothesized that amphetamine self-administration would result in significant differences between EC and IC rats after a prolonged incubation period, such that IC rats would show GluA1 increases and EC rats would not. My hypothesis was not supported by the current data because EC and IC amphetamine rats did not have different expression from saline rats. The same result was true for GluA2 expression.

I hypothesized that the accompanying changes would occur with the time-dependent increase in drug seeking because recent research examining the incubation of craving effect has

determined that within the nucleus NAc Ca^{2+} permeable AMPA (CP-AMPA) receptors accumulate after approximately 35-40 days of drug abstinence (Conrad et al., 2008). Importantly, the increase in CP-AMPA receptors only occurs when long access self-administration models are used (Purgianto et al., 2013), although see Ma et al. 2016. Even when the number of short access self-administration sessions are increased, and the number of cocaine infusions are equal, CP-AMPA receptors only increase using long access self-administration models (Purgianto et al., 2013). Given that the length of the session seems to be most important and not activation, these authors hypothesized that prolonged activation of monoamines could 'flip a switch' that results in increased CP-AMPA receptor trafficking. One way to test this hypothesis is to administer a monoamine oxidase inhibitor in the short access rats to determine if prolonged monoamine activation results in similar CP-AMPA receptor increases.

Amphetamine and enrichment condition do not affect NAc metabotropic receptors

Previous research has determined that IC rearing environment reduces mGlur1 and mGlur5 expression in the medial prefrontal cortex, but has no effect on mGlur2/3 receptors. Interestingly, when challenged with a Group I mGlur agonist (DHPG) EC and standard-housed rats show an elevation, supporting the western blot data. However, when rats are challenged with a mGlur2/3 antagonist—which would block the reuptake mechanism and result in more glutamate—IC rats show a similar deficit in glutamate release. These results indicate that despite showing no differences in mGlur2/3 expression, IC rats may have diminished function when compared to EC rats (Melendez et al., 2004).

The current experiment did not observe any differences in mGlur1 or mGlur5 expression across any of the experimental groups. However, with Melendez et al's (2004) data in mind, it is possible that despite not having differences in expression the function of these receptors is

different. The current experiment did not use any pharmacology to test this hypothesis but data from previous experiments in our lab supports this (Arndt et al., 2015; Gill et al., 2012). In previous work, we have determined that EC rats are more sensitive to pharmacological treatments targeting mGlur5 (Arndt et al., 2015), but IC rats may be more sensitive to treatments targeting mGlur2/3 (Arndt et al., 2014). Taken together, these data support the idea that when rats are reared in different environments it results in differences in mGlur function that may contribute to pre and post synaptic glutamatergic tone. To my knowledge, previous work has not specifically examined the expression of mGlurs in the NAc in EC and IC rats. Another way to explore the differences between EC and IC rats in NAc protein is by using biotinylation to determine if any differences arise in surface and total protein expression. The current data suggest that there are no alterations to mGlurs in the NAc, and differential rearing affect mGlurs in the medial prefrontal cortex (Melendez et al., 2004).

Brain-Derived-Nerve-Growth Factor, enrichment and incubation

One important aspect about the time-dependent increases is drug seeking and CP-AMPA receptor insertion is that the timelines do not match. The behavioral increases in drug seeking are observed after just one week of abstinence, while CP-AMPA receptor insertion does not accumulate in the NAc until abstinence day 25-35 (Wolf & Tseng, 2012), suggesting another neurobiological mechanism may is implicated in early increased drug seeking. Brain-derived-neurotrophic-factor could be that mechanism because it plays a significant role in AMPA receptor trafficking and the incubation of cocaine seeking. Exposure to enrichment has been shown to increase the gene expression of BDNF (Falkenberg et al., 1992) as well as nerve growth factor (Pham, Söderström, Henriksson, & Mohammed, 1997; Pham, Winblad, Granholm, & Mohammed, 2002) in the hippocampus. Similar results have not been published within the

NAc in EC rats. Nonetheless, in standard-housed rats, BDNF has been suggested to play an integral role in the time-dependent increase in cocaine seeking because BDNF protein levels also show a similar time-dependent increase that mirrors the increase in cocaine drug seeking (Grimm et al., 2003). These same time-dependent increases in BDNF levels are not observed when sucrose is self-administered, suggesting that the growth factor increase is specific to drug reward (Grimm et al., 2003). Specifically, Grimm et al. (2003) determined that BDNF increases in the ventral tegmental area, NAc, and amygdala are observed after 6h daily access to cocaine self-administration. A time-dependent increase was not observed in other growth factors including nerve growth factor suggesting that the increase was specific to BDNF and does not generalize to other growth factors. This is in support of other research examining the role of BDNF in conditioned reinforcement procedures (Horger et al., 1999). BDNF infused into the ventral tegmental area increased locomotor activity in response to a cocaine injection (15 mg/kg), and increased conditioned reinforcement responding when infused into the NAc, suggesting that BDNF enhances the stimulant and rewarding effects of cocaine when infused into the mesolimbic dopamine pathway (Horger et al., 1999). However, Pickens et al. (2011) offer another explanation for BDNF and its role in the incubation of drug craving. They suggest that BDNF levels in the ventral tegmental area are probably involved in long-term cellular processes implicated in the incubation of drug craving and not immediate synaptic activity because BDNF injections 2h before short abstinence cocaine seeking tests have no effect. Taken together with the data from Horger et al. (1999), it is possible that exogenous BDNF levels are elevated to levels above what are seen in endogenously, and that contributes to the increases in drug seeking. Furthermore, they (Pickens et al., 2011) also suggest that acute actions of BDNF may increase

general drug seeking and reinstatement or even enhance the acquisition, but BDNF's role in the development of *incubation* of drug seeking is less defined.

Given the role that BDNF level in the NAc has on the incubation of drug seeking, it is possible that increases in BDNF levels could result in other synaptic adaptations that are integral for the development of an increase drug seeking response after prolonged periods of abstinence. BDNF has also been suggested to play a role in AMPA receptor trafficking. In drug naïve rats, BDNF infusions into the core, but not the shell, of the NAc resulted in significant increases in GluA1 surface expression, while other AMPA receptor subunits were not affected (X. Li & Wolf, 2011). Results also determined that the increases in GluA1 surface expression were dependent on an extracellular signal-regulated kinase (ERK) mechanism. If an ERK activation blocker was administered before the BDNF NAc infusion, BDNF levels increased but the corresponding GluA1 surface expression was not observed. This study demonstrated that while BDNF is important for AMPA receptor modulation another mechanism may contribute to the progressive increase in AMPA receptor trafficking because these exogenous infusions of BDNF into the NAc core only transiently increased GluA1 receptor expression. The increase in GluA1 surface expression was lost 3h after the infusion and remained at baseline control levels when measured at 24h and 3 days later. These results clearly suggest that exogenous increases in BDNF may specifically regulate GluA1 while not affecting other AMPA receptor subunits in the accumbens, possibly suggesting that BDNF affect homomeric AMPA receptors. Further, it offers an explanation that another mechanism may be important for the maintenance of GluA1 after the insertion of them into the surface of the cellular membrane.

As I mentioned above, it is generally accepted that EC housing increases BDNF levels in the hippocampus and visual cortex (Falkenberg et al., 1992; Franklin, Murphy, Myers, Clarke, &

Currie, 2006), but these same results have not been published in the NAc. There is however some evidence that other mesocorticolimbic structures are affected by enrichment rearing and ethanol. Within the mPFC, BDNF levels decrease following enrichment and chronic ethanol exposure but BDNF levels are unaffected with acute ethanol exposure. These results suggest that other cellular mechanisms are implicated in how BDNF levels are expressed, and they may moderate synaptic changes differently. This idea is supported by other research in standard-housed rats that suggests a moderating role for BDNF signaling that is not only region specific but also time dependent too. BDNF within the NAc core may actually have a net suppressive effect at early withdrawal periods because reduced BDNF signaling increases cocaine seeking (X. Li et al., 2013). However, in the NAc shell, after 90 days of cocaine abstinence—a time when BDNF levels increase with incubated responding—reductions in BDNF signaling reduced cocaine seeking (X. Li et al., 2013). These results demonstrate that BDNF levels and its subsequent downstream signaling affect reward, and seeking differently within the NAc. While the effects of enrichment or isolation housing on BDNF levels in the NAc are not yet established it does suggest the possibility that the differences in early amphetamine seeking may be partially attributable to BDNF expression.

A Critical Developmental Period

One overarching research question in the current proposal was to determine when environmental housing was having its protective effect on drug abuse vulnerability. As mentioned earlier, our lab utilizes a prevention model to understand the critical adaptations that occur during development that exacerbate drug vulnerability. Although there are limitations, I hypothesized that mGlur1 expression could be compared at different withdrawal periods to help answer this research question.

After stimulant self-administration, mGlur1 signaling is thought to act as the LTD mechanism. Intact mGlur1 signaling is thought to maintain glutamatergic tone and help maintain homeostatic balance. Upon mGlur1 downregulation, CP-AMPA receptors quickly accumulate and increase synaptic strength, suggesting that mGlur1 may act as a regulator of CP-AMPA and CI-AMPA receptors (Loweth et al., 2014; Loweth, Tseng, & Wolf, 2013; Mccutcheon et al., 2011). I hypothesized that if IC rats had lower mGlur1expression after the short abstinence period that would provide evidence that EC rearing protected amphetamine-induced adaptations that would promote CP-AMPA insertion. Alternatively, the other hypothesis suggested that expression would be similar after short abstinence but would change after prolonged abstinence. This hypothesis suggested that enrichment would not have its protective effect until after amphetamine self-administration ceased. Our results suggested that mGlur1 in IC amphetamine rats had reduced mGlur1 expression at both short and prolonged abstinence periods, but it was not statistically different. This result, coupled with the lack of increased GluA1 expression in IC rats, provides no evidence of a deficiency in glutamatergic tone in the NAc that results in increased amphetamine seeking.

Given the lack of any mGlur1 or GluA1 expression differences, it is difficult to conclude that either early life enrichment or enrichment used as an intervention model has any impact on glutamatergic tone in the NAc, but this does not discount the behavioral effect enrichment has on drug seeking. During the pre-cue interval and after the cue presentation, EC animals show reduced drug seeking after the 1 day abstinence period. Similarly, after the 40 day abstinence period, EC rats also had less invigorated drug seeking following the cue presentation. Again, suggesting that while expression of these glutamate receptors in the NAc are not different, cue-induced drug seeking is increased in IC rats.

Chapter 5 - Exploratory Analyses

The nature of the experimental design did not fulfill all of the assumptions of the ANOVA. Therefore, to supplement the proposed repeated measures ANOVA results I am using a mixed effects model to determine how receptor expression and environmental condition interact to predict drug seeking patterns after short and long abstinence periods.

Mixed effects models can also provide estimates of model fit for comparison and help illustrate the meaningful relationships that may be unaccounted for with traditional ANOVA analyses, especially given the unequal cell sizes. For example, in addition to calculating simple correlations between lever pressing and receptor expression, mixed effects models allow for a complex (interactions) predictive relationship that will utilize all of the receptor expression values to understand if they uniquely or collectively predict lever pressing (drug seeking) during the seeking tests. Mixed effects analyses will allow powerful comparisons both across continuous and categorical groups (Cudeck, 1996; Laird & Ware, 1982; Young, Clark, Goffus, & Hoane, 2009). Another strength of using mixed effects analyses when examining individual differences in data is that this analysis is not dependent on experimenter determined divisions, and all rats are used in the analysis. A major problem common in many animal and human individual difference studies is the use of extreme comparisons. For example, ANOVAs require categories, which require researchers to compare the top third to the bottom third or they median split data. This not only can result in errors in statistical calculations or determining effects (Cohen, 1968; Cohen, 1983; De Coster, DeCoster, Gallucci, & Iselin, 2011; Irwin, Irwin, & McClelland, 2003; Maxwell, Maxwell, & Delaney, 1993; Owen & Froman, 2005), but it requires a large use of animals because upwards of a third of the sample is not used in analysis. For these reasons, mixed effects models were used to supplement the ANOVA analyses to more accurately

model the effects between rearing environment and receptor expression changes after short and prolonged abstinence.

Does the influence of receptor expression change depending on early life experience?

These analyses were completed to test the hypothesis that the environmental housing condition interacts with receptor expression to change the pattern of drug seeking during the short and long seeking tests. To test this hypothesis, a repeated measures regression model was constructed. To be clear, these data are preliminary and need to be followed up with experimental manipulations that directly alter these receptors to observe if the mathematically based conclusions are supported by behavioral or molecular data.

Each repeated measure regression model was constructed using the following guidelines. The dependent variable was the active lever presses during the seeking test, but the data specifically used was the data from the time bin analyses. This allows more flexibility because we have multiple time bins for each rat and not just the summed responses. Inactive lever presses were not analyzed in this preliminary data analysis because of the general lack of effect across the between subject variables (see Result inactive sections).

The independent variables for the preliminary mathematical model are: group (EC or IC), drug (AMP or SAL), and the expression of a given protein receptor (e.g .normalized GluA1). The time bin was treated as the repeated measured variable. Importantly, in the previous ANOVA analyses (see result sections for receptor expression) "the normalized expression" values were the dependent variables. Those analyses determined that the expression levels generally did not change across treatment groups. Here, these analyses explore if the expression of each of those glutamate receptors predicts drug seeking after 1 or 40 days of abstinence.

Importantly, these results also test the interaction that the expression of a given receptor and its influence on drug seeking may change depending on whether the rat was enriched or isolated.

General mathematical model

The models were constructed with the following effects:

- Group (Main effect)
- Drug (Main effect)
- Expression of normalized protein (Main effect)
- Time bin (Main effect)
- Expression of normalized protein x Drug
- Expression of normalized protein x Drug x Group
- Expression of normalized protein x Drug x Group x Time bin (Random Effect)

Each mathematical model had approximately 445 degrees of freedom. The dependent variable is the active lever presses during each five minute time bin. The following model is a linear mixed effect model. While all the time bins (14) can be included in the model, the model would not accurately represent the data because of the increase in responding after the cue presentation makes the function nonlinear. To fully account for these effects a nonlinear mixed effects model would need to be constructed. Therefore, the current models only model drug seeking behavior after the cue presentation (12 time bins). A model was constructed for the short test and a separate model using the same effects and interactions was fit for the seeking test after prolonged abstinence. Each model fit was assessed with Akaike information criterion (AIC). Lower values of the AIC suggest a better model fit and inclusion of too many predictors in the model result in 'overfitting' and the mathematical model is penalized by increasing the AIC value. For this reason the AIC has been suggested to be a better criterion to use when

constructing models over the more traditional R^2 model assessment because R^2 does not penalize for overfitting with too many predictors (Dixon, 2013). For the purposes of transparency, I included both model criterions (AIC and R^2) in the reported interpretations.

Increased GluA1 expression augments drug seeking in IC rats

For the seeking test after the one day abstinence period, the linear mixed effects model revealed that there were main effects of group, drug, and time bin (all F's <.05). There was not a main effect of GluA1 on active lever presses, suggesting that GluA1 expression did not affect drug seeking responses ($F(1, 445) = 0.31$, $p>.05$). The interaction of GluA1 x drug was trending toward significance, ($F(1, 445) = 3.27$, $p=.07$), suggesting that when rats self-administered saline the expression of GluA1 did not affect lever pressing. However, when rats self-administered amphetamine the influence of GluA1 expression changed drug seeking, such that higher levels of GluA1 resulted in more drug seeking. The three-way interaction of GluA1 x drug x group was not statistically significant, ($F(1, 445) = 1.28$, $p>.05$). Looking at the random effect predictions, the results suggest a predictive relationship for the interaction of GluA1 x drug x group x time bin (Random) only for IC rats that self-administered amphetamine ($t(14) = 2.48$, $p<.05$). This same effect was not observed in the IC saline rats or the EC amphetamine rats, suggesting that drug seeking was enhanced in IC rats in response to the cue light and higher levels of GluA1 augmented the invigorated response to the cue light. For the overall model a total of 28% of the variance in active lever responding was accounted for by the model used ($R^2 = .28$). The AIC for this model was 2,496.93 (Figure 20A).

This same mathematical model was used to account for drug seeking after the long abstinence period. The results indicate main effects of group, drug, and time bin (all F's <.05). Like in the short test, the effect of GluA1 expression was not significant, indicating that GluA1

expression does not account for drug seeking after prolonged abstinence ($F(1, 445) = 1.69$, $p>.05$). The interaction of GluA1 x drug did not interact to account for active lever presses ($F(1, 469) = 2.37$, $p>.05$). Similarly, the interaction of GluA1 x drug x group was also not predictive of active lever presses after prolonged abstinence, but it was trending ($F(1, 469) = 2.92$, $p=.08$). Further exploring this potential interaction, by examining the random effect predictions, it appears that all groups are not different from a slope of zero. The only group that shows any indication of a trending significant slope change across time are the IC amphetamine rats ($t(7) = 1.63$, $p=.14$). This likely suggests that the IC amphetamine rats showed greater reactivity to the cue light as GluA1 expression increases, but the IC saline rats also showed an increase in active lever presses mitigating any differences between drug groups or GluA1 expression (Figure 20B). The overall model accounted for slightly less variance that the short test's model ($R^2 = .24$), and the model fit was also slightly worse (AIC = 2,528).

GluA2 expression has no effect on drug seeking after 1 day but does after 40 days

A similar mathematical was used to examine how GluA2 expression interacts with drug and environmental condition to predict drug seeking after short and long abstinence periods. For the seeking test after the one day abstinence period , the mixed effects model indicated that there were main effects of group, drug, and time bin (all F's $<.05$). There was not an effect of GluA2 expression indicating that GluA2 does not account for any active lever pressing after one day of abstinence ($F(1, 445) = 0.79$, $p>.05$). There was also not evidence of a GluA2 x drug interaction ($F(1, 445) = 0.69$, $p>.05$) or a GluA2 x drug x group interaction ($F(1, 445) = 0.49$, $p>.05$). There were also no significant random effects or any random effects that were trending toward significant prediction. Together, these results provide mathematical evidence that GluA2 expression has no effect on amphetamine seeking behavior after 1 day of abstinence, the best

predictor in this model of active lever presses is whether the rat self-administered amphetamine. The overall model accounted for a large proportion of variance (R^2 = .27) and the AIC model fit was 2,503 (Figure 21A).

The same model was applied to predict the drug seeking after 40 days of abstinence. This model revealed main effects of group, drug, and time bin (all *F*'s <.05). There was no evidence that GluA2 expression after 40 days of abstinence predicted drug seeking, ($F(1, 470) = 1.28$, $p>.05$). There was no evidence of a GluA2 x drug interaction suggesting that GluA2 levels did not interact to predict drug seeking, ($F(1, 470) = 1.45$, $p>.05$). There was a significant three-way interaction of group x drug x GluA2 expression, ($F(1, 470) = 2.64$, $p<.01$). This result suggests the ability for GluA2 expression to predict drug seeking depends on both the solution the rats self-administered and the housing group, such that IC housing coupled with increased GluA2 expression resulted in greater drug seeking, but only after 40 days of abstinence (Figure 21B). Random effects estimates revealed that IC amphetamine rats had were trending toward a significant slope difference ($t(11) = 2.15$, $p=.054$), suggesting increased drug seeking throughout the test when GluA2 expression was higher. The model accounted for slightly less variance than the short seeking model (R^2 = .26) and the model fit was slightly worse too (AIC = 2,515).

Increased mGlur1 increases drug seeking after 1 day and loss of mGlur1 expression increases drug seeking after 40 days

The next model used was to examine the effects of the dimer form of mGlur1 on drug seeking after short and long abstinence periods. For the seeking test, after the one day abstinence period , the mixed effect model revealed main effects of group, drug, and time bin (all *F*'s <.05). MGlur1 expression had a positive relationship with drug seeking, but it was not significant, ($F(1, 446) = 2.83$, $p=.09$). There was a significant interaction of mGlur1 x drug, ($F(1, 446) = 5.80$,

p<.05), meaning that increases in mGlur1 expression did not affect active lever presses in saline animals. There was no statistical evidence for a group x drug x mGlur1 expression, suggesting that the increased responding as a result of mGlur1 expression and amphetamine exposure was not different between EC and IC rats (Figure 22A). Examination of the random effects confirmed this interpretation of the data and indicated the three-way interaction was not changing across time bin. This model accounted for 28% of the variance in active lever responding (R^2 =.28) and had an AIC model fit of 2,498.

The next mixed effects model revealed that after 40 days of abstinence the predictive relationship that mGlur1 has on active lever presses changes. There were main effects of group, drug, and time bin (all F's <.05). There was a strong negative relationship with mGlur1 that indicated active lever pressing was highest when mGlur1 expression was low (F(1, 470) = 3.37, p=.06). After one day of abstinence mGlur1 expression had nonsignificant positive relationship, but after 40 days of abstinence the predictive relationship became more negative, meaning that active lever presses were increased when mGlur1 expression was low. There was not a significant interaction between mGlur1 expression and drug when predicting active lever pressing, (F(1, 470) = 0.02, p>.05). However, the three-way interaction of group x drug x mGlur1 expression was nearly significant, (F(1, 470) = 3.72, p=.054), suggesting that decreases in mGlur1 expression result in increased amphetamine seeking only in IC rats (Figure 22B). The negative relationship that mGlur1 has with drug seeking is supported by previous literature (Loweth et al., 2014; Loweth et al., 2013). Despite these unique relationships that are supported by previous literature, this model only accounted for 23% of the variance in responses (R^2 = .23) and had a poorer model fit (AIC 2,533).

mGlur5 may increase drug seeking early, but not after prolonged abstinence

The next model tested was designed to test the interaction of mGlur5 expression with drug and group to predict active lever pressing during the seeking tests. The first model predicted active lever presses after one day of abstinence. This model determined that there were main effects of group x drug x time bin (all *F*'s <.05). There was not an effect of mGlur5 on active lever presses, ($F(1, 445) = 1.16$, $p>.05$). There was also no evidence of an interaction between mGlur5 expression x drug, ($F(1, 445) = 0.09$, $p>.05$), or between group x drug x mGlur5 expression, ($F(1, 445) = 0.74$, $p>.05$), providing no evidence that mGlur5 expression is accounting for any prediction in active lever presses during the seeking test. When examining the random effect predictions there was a significant slope increase in IC amphetamine rats across the time bins, but not IC saline rats, while the other groups were not different in their slopes. This effect was probably observed from the increased responding toward the end of the session in the higher mGlur5 expression rats (Figure 23A). Overall, the model accounted for 29% of the variance in active lever presses ($R^2 = .29$) and the AIC equaled 2,492.

Using the same predictors, I applied the same model to predict active lever presses after 40 days of abstinence. This mixed effects model revealed main effects of group, drug, and time bin (all *F*'s <.05). MGlur5 expression did not predict lever pressing, ($F(1, 469) = 0.04$, $p>.05$). There was also no evidence of a drug x mGlur5 expression interaction, ($F(1, 469) = 1.14$, $p>.05$), or a group x drug x mGlur5 expression interaction, ($F(1, 469) = 1.76$, $p>.05$). An examination of the random effect structures also provided no evidence of a predictive relationship across any of the groups. This result suggests that mGlur5 expression may not implicated in cue-induced drug seeking following a prolonged abstinence periods (Figure 23B).

Broad implications of the exploratory analyses

Using these mixed effects models I was able to use a repeated measures regression to test if protein expression and environmental condition interact to predict enhanced drug seeking. Our mathematical modeling data provide support that environmental housing condition plays a critical role in how these receptor changes motivate behavior. Authors from current literature hypothesize that the significance of CP-AMPA receptor insertion to increased drug seeking is that CP-AMPA receptors increase excitatory neural activity in response to cues (Loweth et al., 2014; Wolf & Tseng, 2012). While our data did support this conclusion, our results indicate environmental factors interact with GluA1 expression and could be indicative of CP-AMPA receptor expression. These changes moderate the ability to control drug seeking, especially after prolonged abstinence.

In the current study, IC rats showed increased drug seeking when GluA1 expression was higher after 1 day of abstinence, possibly suggesting that small increases in excitatory potentials in the NAc are forming as early as 1 day in IC rats. After 40 days, while IC amphetamine rats showed an increase in cue reactivity, the IC saline rats did too, suggesting that prolonged IC housing not only changed reward seeking but can enhance sensory reinforcement to visual cues. This result is in support of previous literature that shows that IC rearing results in augmented operant responding for visual cue lights (Cain et al., 2006). Importantly, EC amphetamine rats that showed similar GluA1 expression levels did not have the same invigoration to behavior, suggesting that another brain nucleus may have a braking effect that limits drug seeking. Cain et al. (2006) hypothesized that these differences between EC and IC rats in incentive salience could result from decreased dopamine transporter function in IC rats in the medial prefrontal cortex. Given the modulatory role of the prelimbic and infralimbic regions in the incubation of cocaine

seeking (Ma et al., 2014), further research should examine how enrichment is changing these cortical areas.

GluA2 expression had quite a different predictive outcome. Given the large differences in methodology between studies, GluA2 expression still warrants future examination especially if short access self-administration models are used. Mathematically, GluA2 expression showed no predictive relationship with drug seeking after 1 day of abstinence. Following a forced prolonged abstinence period in isolation, higher GluA2 expression was predictive of higher drug seeking, while housing in enrichment mitigated the augmented drug seeking response. This is in agreement with literature that hypothesizes that enriched animals actually show the same neural adaptations in response to drugs of abuse as standard housed rats, but for reasons not known, enriched animals do not express the addictive behavior (Chauvet et al., 2012). This conclusion likely means another brain region projecting to the NAc reduces drug seeking through an indirect circuit. IC rats likely have this circuit because recent data from our lab showed that enrichment was able to rescue IC rats (Garcia et al., 2017). Given the significant glutamatergic projections from the medial prefrontal cortex to the NAc, the medial prefrontal cortex is a candidate region of interest, and should be explored more following manipulations to environmental rearing and housing.

We explored mGlur1 because its activation results in the removal of CP-AMPA receptors (Loweth et al., 2014), suggesting that it has the ability to reduce synaptic strength and attenuate cue-induced drug seeking. The mathematical modeling data are in agreement with these prior conclusions and demonstrate that with short access amphetamine self-administration, mGlur1 increases may promote drug seeking in IC rats. After 40 days, decreased expression in IC rats was predictive of increased amphetamine seeking. While there was not a change in average

mGlur1 expression or GluA1, the individual animals with lower expression in the IC amphetamine group had increased drug seeking. Even minor changes in mGlur1 expression seem to be critical in promoting drug seeking, especially, when early life experiences are not supportive of healthy development.

MGlur5, which has been shown to reduce cue-induced drug seeking and mediate psychomotor stimulant reward responding (Kenny et al., 2005; Paterson et al., 2003) was not only unaffected by rearing condition, but in this experiment, mGlur5 was not predictive of drug seeking after short or long abstinence periods. The modeling data clearly showed that mGlur5 expression does not interaction with drug exposure or environmental housing condition to predict drug seeking. This result is in opposition of current literature examining cocaine (Kenny et al., 2005) and methamphetamine (Gass et al., 2009). Although these experiments used pharmacological interventions to block mGlur5 signaling while the current study did not.

Limitations of the models

The most obvious limitation to the mathematical models is that the temporal precedence assumption is not met. Temporal precedence was not met because the rats' NAc was harvested and dissected after the cue-induced seeking test, while the goal of the models was to determine if expression predicted drug seeking. In a more appropriate experimental design, we would have two groups of rats that self-administer amphetamine and go through the prolonged abstinence procedure. The first group would be sacrificed before the cue-induced seeking test and the second group would be sacrificed immediately after the seeking test. This design would allow more causal interpretations from the mathematical model, because we could directly test the effects of abstinence period and abstinence period + cue exposure on protein expression.

The next limitation from the mathematical model is that each receptor was tested independently. In a more appropriate model, all of the receptors could be entered in together or at least in a hierarchical manner that is theoretically based. The present data did not allow for this because of the limits to sample size/number of observations. This is the reason we did not test a full factorial mathematical model with random effects.

Despite these limitations these models show unique pattern in data that may not be observed by just examining the group means. It is important to keep in mind that mathematical models will never result in a statistically significant effect when experiments are poorly designed. They can however, shine light and present data in new, interesting ways that leads to better experimental design and interpretations; which is my focus here.

Future directions

The data here clearly show that glutamate receptor expression in the NAc does not fully account for differences in drug seeking. Instead, environmental factors moderate the influence that these receptors have to control drug seeking behavior. With this in mind, future research should fully explore how environmental rearing conditions early in life (preventative) and after drug taking (intervention) affect drug seeking behavior. There are a number of advanced genetic techniques to explore these research questions to determine how they may interact.

Recently, an experiment showed that an optogenetic protocol that results in LTD was able to reduce CP-AMPA receptors in the NAc. Interestingly, after a brief time the CP-AMPA receptors re-accumulated and neural strength in the NAc was again above normal levels, suggesting that these adaptations in AMPA receptors may be permanent without intervention (Ma et al., 2016). Enrichment without the LTD applied did not have any effect of CP-AMPA or basal levels of silent synapses. However, after optogenetic LTD induction, there is a therapeutic

window in which enrichment can successfully remodel silent synapses into CI-AMPA receptors and reduced incubated cocaine craving (Ma et al., 2016). These remodeling effects were in the basolateral amygdala to NAc circuit and provide evidence of a possible upstream brain area to target therapeutically. The infralimbic and prelimbic regions also project to the NAc and have been shown to have significant modulatory roles in the incubation of drug craving (Kauer & Polter, 2014; Ma et al., 2014). Using a similar ontogenetic strategy it would be possible to determine if these circuits communicate similarly between EC and IC rats.

Repeated systemic injections of a mGlur1 positive allosteric modulator also results in decreased CP-AMPA, but CP-AMPA receptors re-accumulate 2-3 days after injections are stopped (Loweth et al., 2014). Based on Ma et al.'s (2016) results, it is possible that mGlur1 activation opens up a therapeutic window where EC rearing could have a similar effect (Ma et al., 2016). I predict that after mGlur1 stimulation, CP-AMPA would recede back temporarily, opening an opportune time for an enrichment behavioral intervention. I hypothesize that enrichment would remodel the NAc with CI-AMPA receptor and reduce the incubation of craving effect.

Another interesting idea to pursue would be to determine if the same pharmacological mGlur1 stimulation has the same effect in EC and IC rats. I would hypothesize that IC rats' dose response would be shifted to the right, thus requiring a higher dose to see the same effect. Exploring the dose response relationships could further tell us how early life experience changes the efficacy of pharmacotherapies.

Finally, the western blot data and modeling analyses indicated significant interactions in how protein expression changes behavior during amphetamine seeking tests. While the western blot data are reliable, stronger biochemical measures can be used to separate surface and total

proteins. It is possible that there are large differences between groups in surface expression that would not have been observed in the subcellular fraction used in the current protocols. Another biological mechanism to explore would be to mRNA to determine if there are differences in translation and transcription of AMPA or mGlur proteins between EC and IC rats after amphetamine exposure. Once baseline measures for EC and IC rats have been determined, using genetic techniques to change receptor expression could result in empirical support for the mathematical analyses. These receptor overexpression and knockout studies could reveal how EC and IC rats differ from standard-housed rats in drug seeking after prolonged abstinence. In addition, these would determine how environmental condition changes the influence of glutamate receptors at different stages of drug seeking.

Chapter 6 - Concluding Statement

Early life experience can have a profound impact on behavior and neurobiology that persists into adulthood. While our data did not show differences in glutamate receptors across the environmental conditions, our data indicate that early life rearing condition may change the control that these receptors have on behavior. Enrichment does not protect against the long lasting effects of repeated drug abuse, but it does prevent the expression of persistent drug seeking in response to cues (Chauvet et al., 2012; Chauvet et al., 2009; Solinas et al., 2010). Our data add to current literature by determining glutamate receptor expression is not only changed by environmental factors, but importantly, its influence on behavior is in constant interaction. Furthermore, damaging early life conditions change reward that results in amplified drug seeking after early abstinence. This amplified seeking could result in persistent drug seeking motivation (i.e. craving) that persists for at least 40 days and perpetuates relapse.

When the synapses within the mesolimbic reward pathway are strengthened they become hypersensitive to drugs and cues associated with drug taking, motivating behavior in a reflexive or habitual fashion (Everitt et al., 2008; Robinson & Berridge, 1993a; Robinson & Berridge, 2001; Robinson & Berridge, 2008). Importantly, in the hypersensitive state, the 'wanting' and 'craving' becomes hypersensitive, while 'liking' decreases. Together, these biological and psychological adaptations contribute to compulsive drug seeking even after prolonged abstinence, which suggests that the adaptations induced by drugs of abuse are long lasting and contribute to future vulnerability.

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Figures

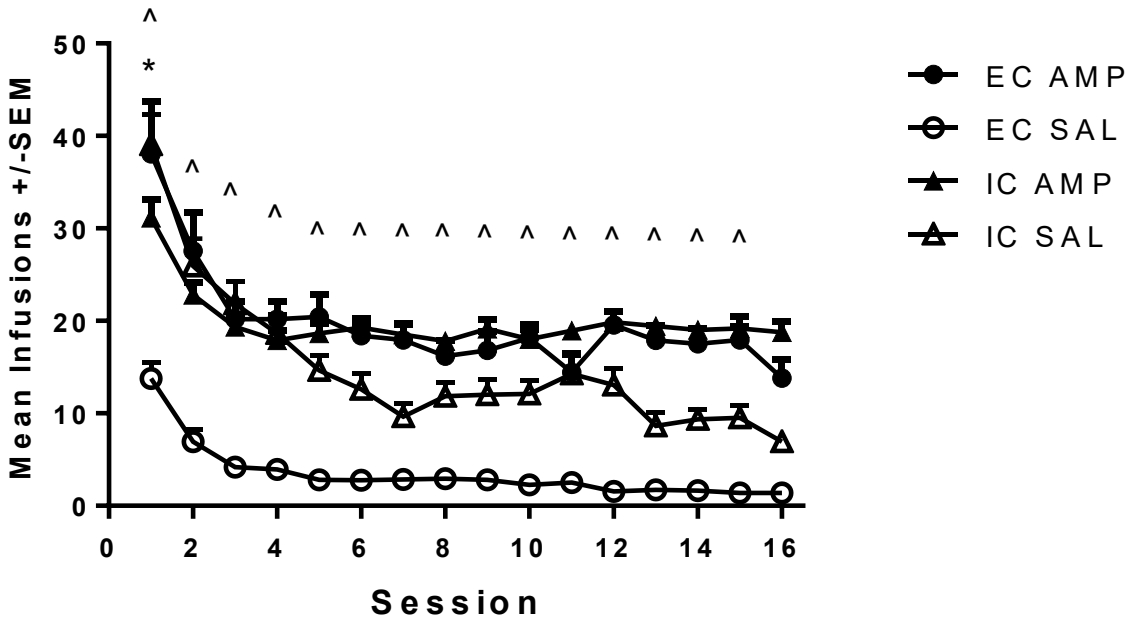

Figure 1. **The mean number of infusions of amphetamine (AMP) or saline (SAL) earned across the 60 min self-administration sessions for EC and IC rats. Rats responded on a FR-1 schedule of reinforcement throughout all self-administration sessions. Caret symbol (^) indicates a significant difference between EC SAL and IC SAL. Asterisk symbol (*) indicates a significant differences between EC AMP and IC AMP. The omnibus alpha is set at $p<.05$ and simple effects alpha is at $p<.0016$.**

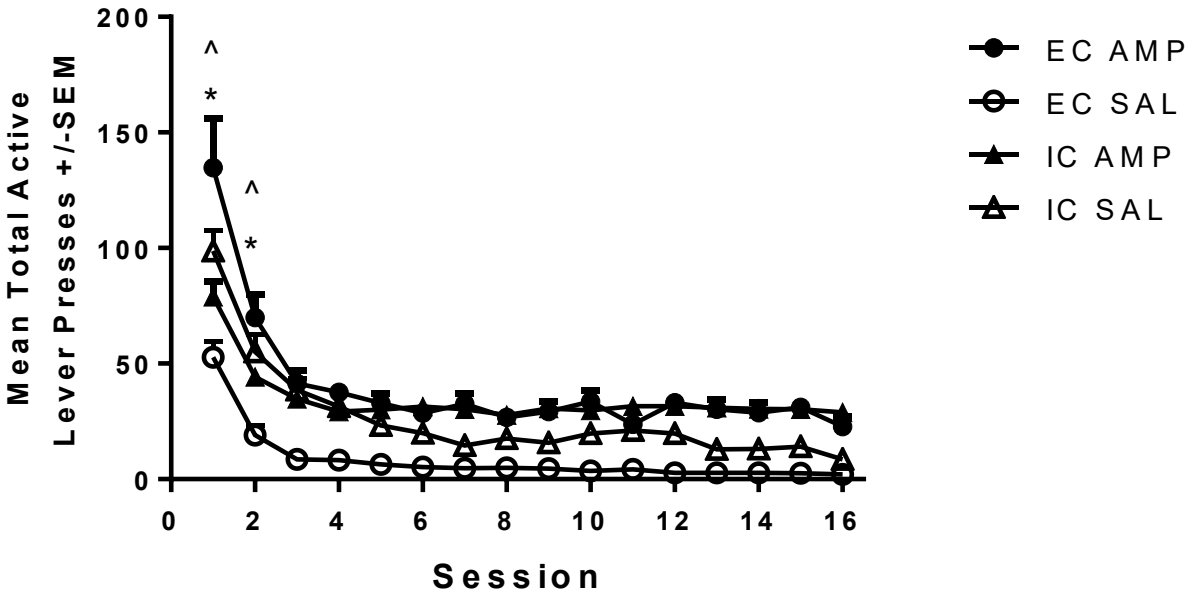

Figure 2. **The mean total active lever presses--including timeout responses--during the 60 min AMP or SAL self-administration sessions for EC and IC rats. Caret symbol (^) indicates a significant difference between EC SAL and IC SAL. Asterisk symbol (*) indicates a significant differences between EC AMP and IC AMP. The omnibus alpha is set at $p<.05$ and simple effects alpha is at $p<.0016$.**

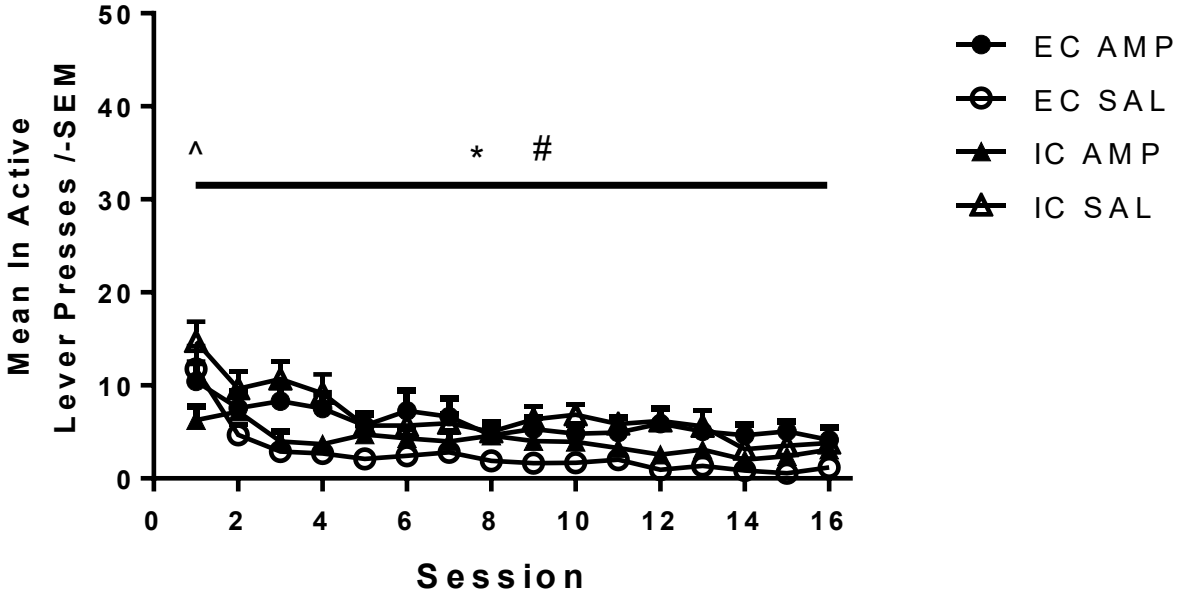

Figure 3. **The mean inactive lever presses during the 60 min AMP or SAL self-administration sessions for EC and IC rats. Caret (^) indicates SAL responding is higher than AMP on session one. Asterisk (*) indicates on average, EC rats responded more when in the AMP group, and # indicates IC responded more for SAL on average. The omnibus alpha is set at $p<.05$ and simple effects alpha is at $p<.0032$ or $p<.0253$.**

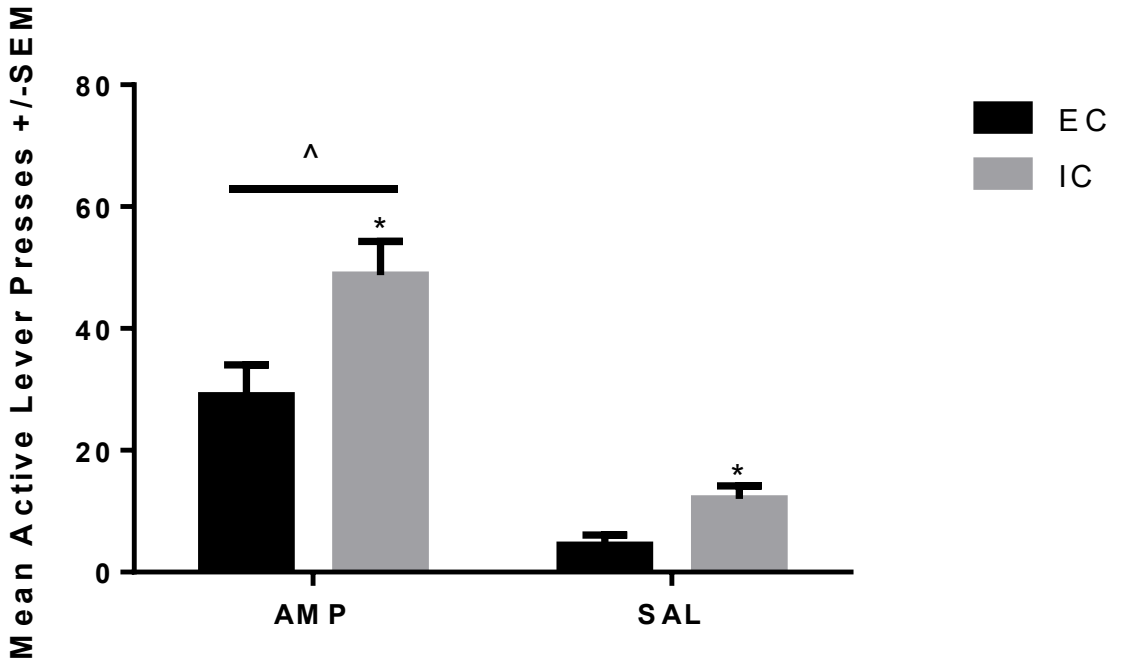

Figure 4. **Sixty min seeking test after 1 day of abstinence. Total active lever presses during the short seeking test. Asterisk (*) indicates IC rats pressed more than EC rats. Caret (^) indicates AMP pressed more than SAL rats. All p values were set at .05.**

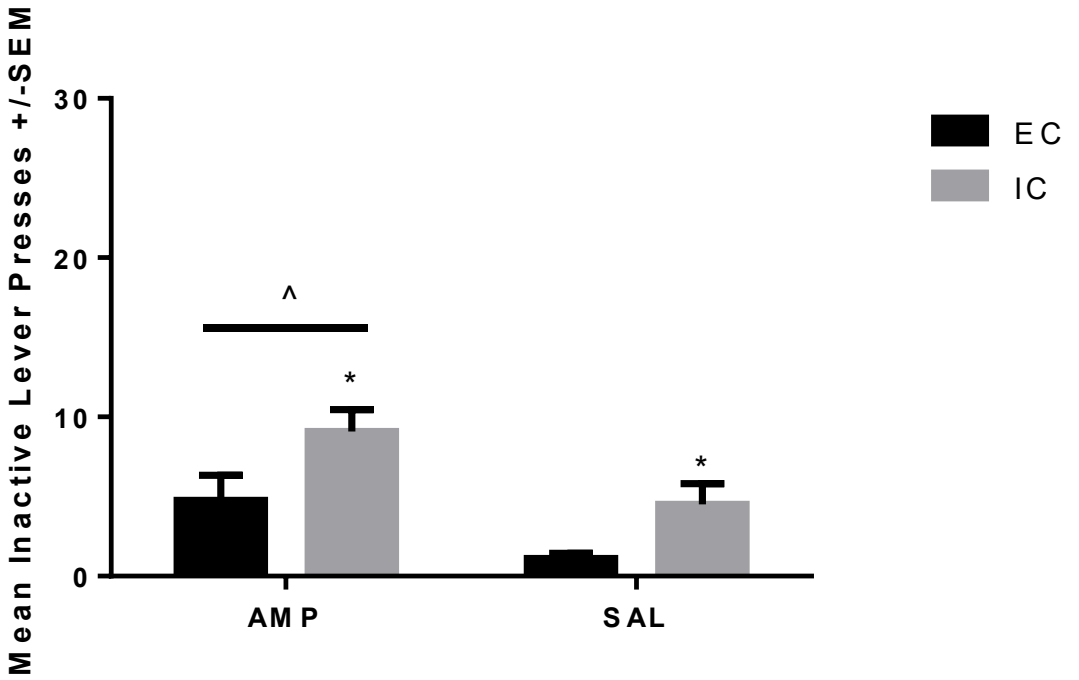

Figure 5. **Sixty min seeking test after 1 day of abstinence. Total inactive lever presses during the short seeking test. Asterisk (*) indicates IC rats pressed more than EC rats. Caret (^) indicates AMP pressed more than SAL rats. All p values were set at .05.**

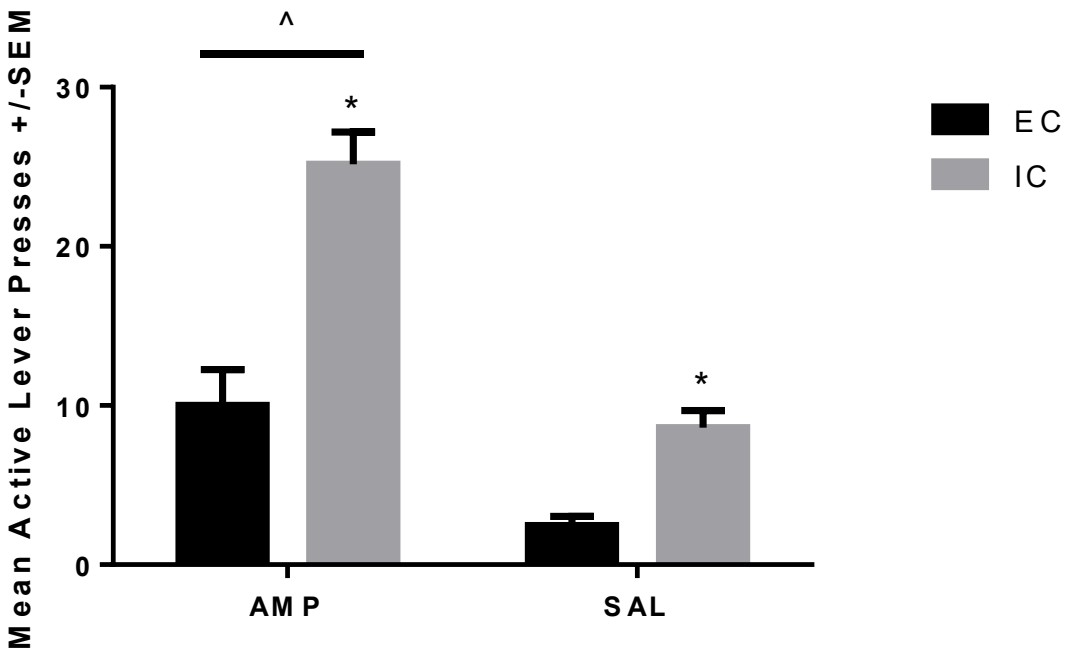

Figure 6. **Ten min pre-cue interval after 1 day of abstinence. Seeking test after 1 day of abstinence. Active lever presses during the 10 min pre-cue interval. Interaction was probed with Sidak correction. Asterisk (*) indicates p <.0253.**

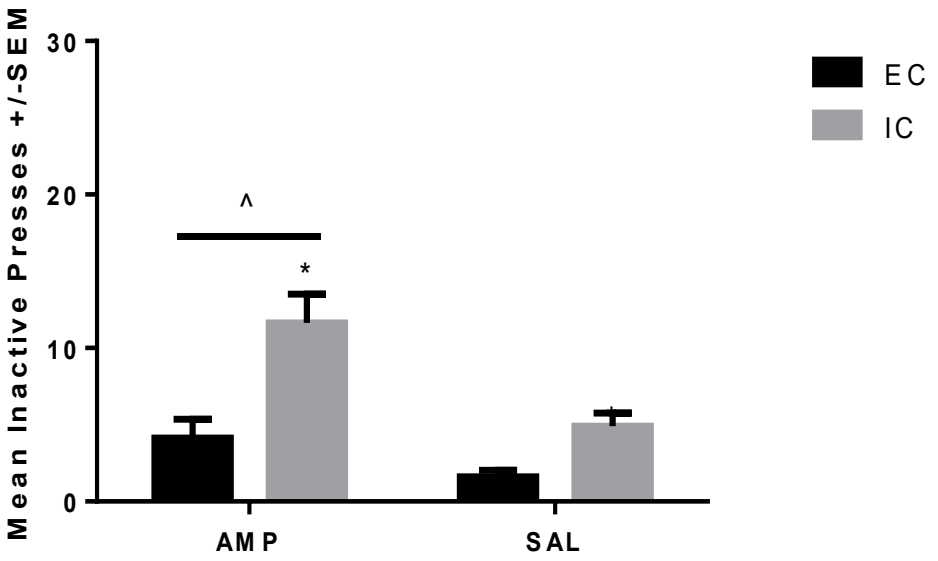

Figure 7. **Ten min pre-cue interval after 1 day of abstinence. Seeking test after 1 day of abstinence. Inactive lever presses during the 10 min pre-cue interval. Asterisk (*) indicates IC rats pressed more than EC rats. Caret (^) indicates AMP pressed more than SAL rats. All p values were set at .05.**

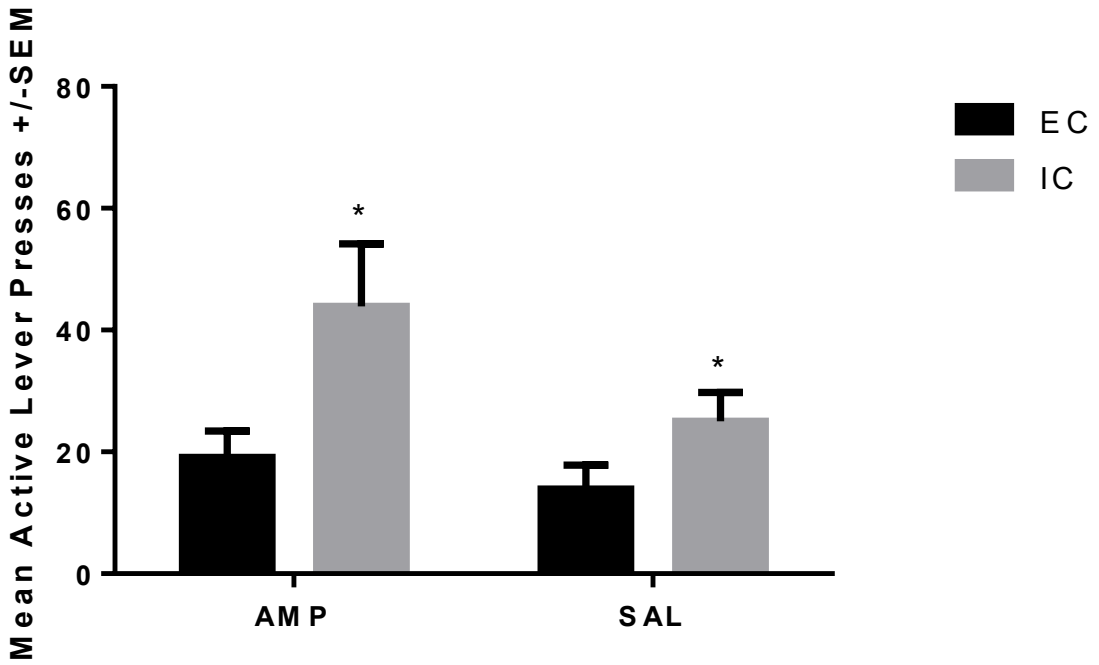

Figure 8. **Sixty min seeking test after 40 days of abstinence. Total active lever presses during the seeking test after 40 days. Asterisk (*) indicates IC rats had significantly more responding that EC rats regardless of drug group. All p values were set at .05.**

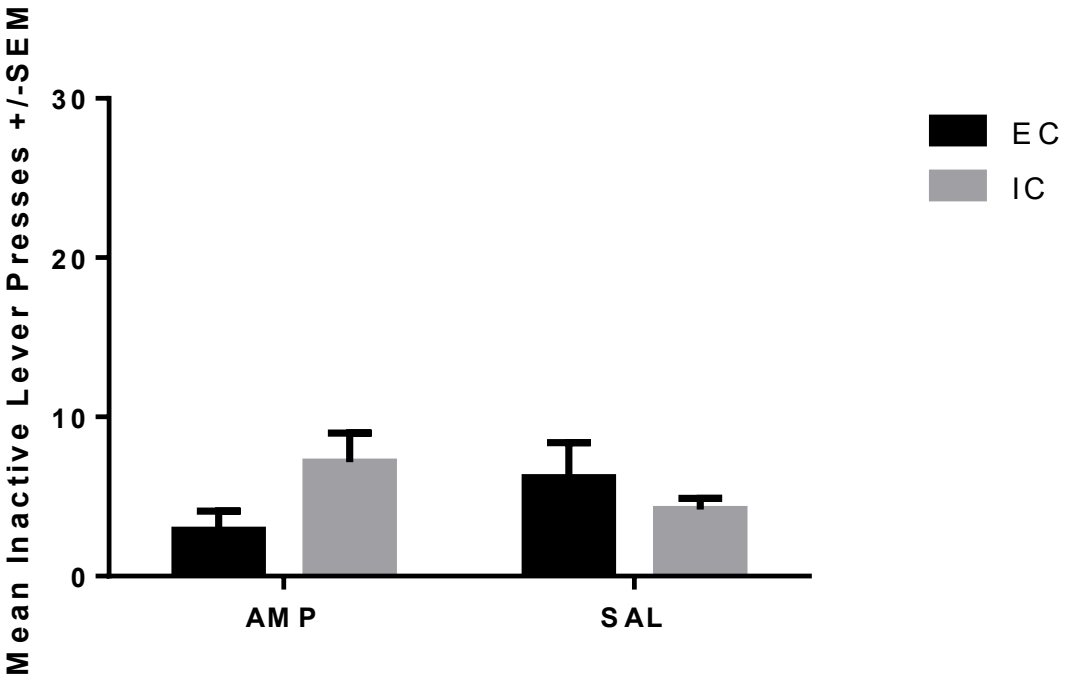

Figure 9. **Sixty min seeking test after 40 days of abstinence. Total inactive lever presses during the seeking test after 40 days. All p values were set at .05. There were no significant differences between groups or drug in average inactive lever presses.**

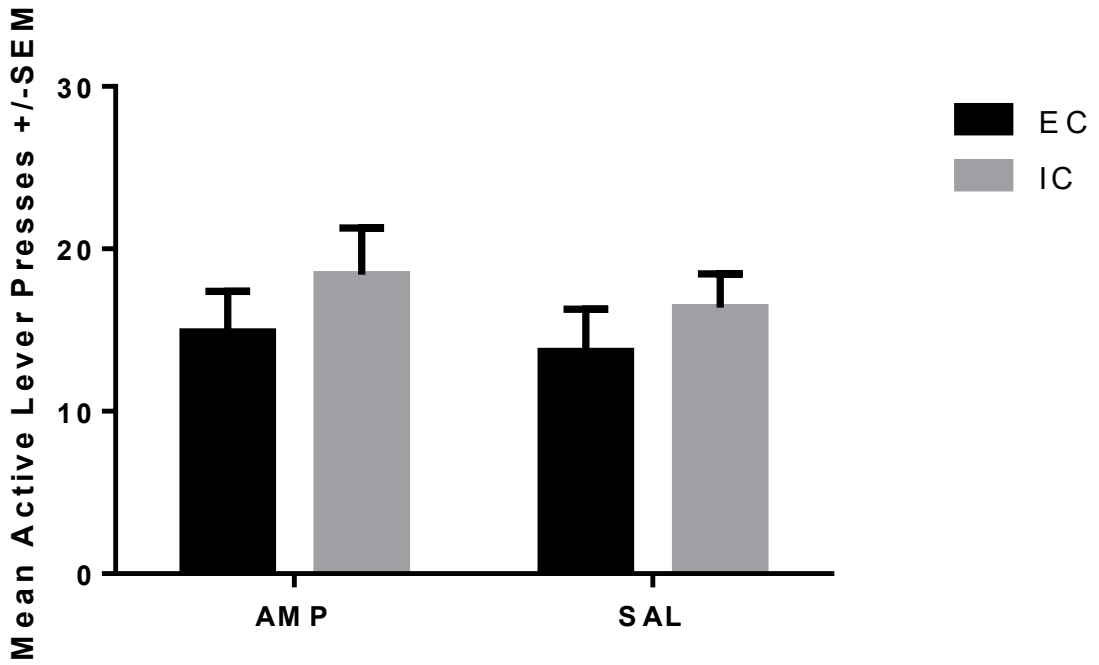

Figure 10. **Ten min pre-cue interval after 40 days of abstinence. Active lever presses during the 10 min pre-cue interval. There were no differences between groups. All p values set at .05.**

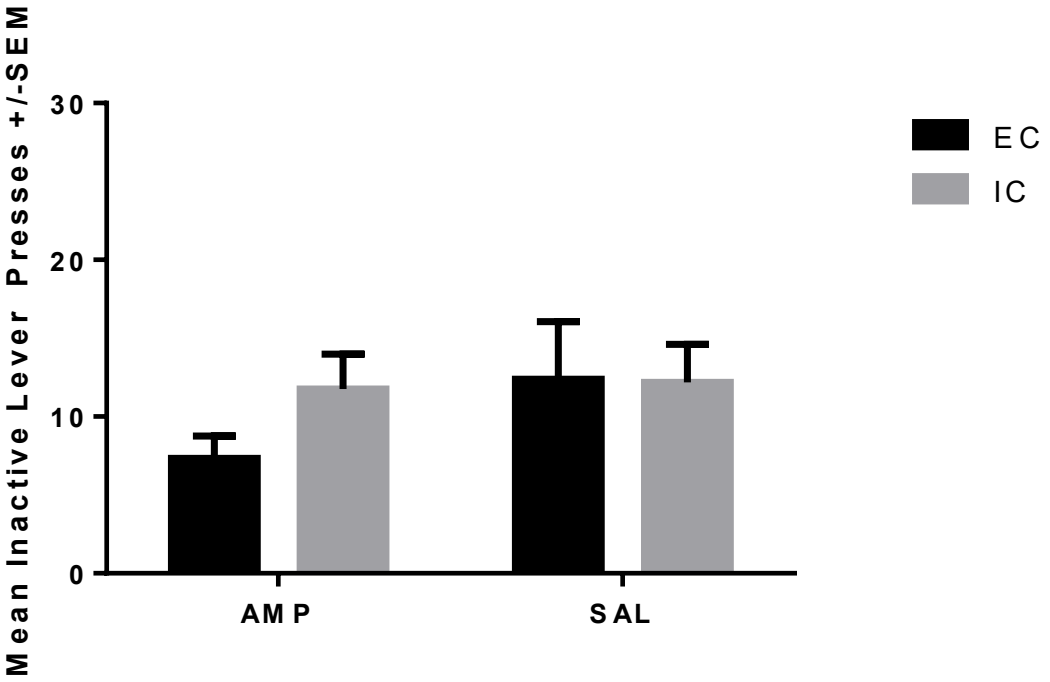

Figure 11. **Ten min pre-cue interval after 40 days of abstinence. Total inactive lever presses during the 10 min pre-cue interval. There were no significant differences between groups. All p values set at .05.**

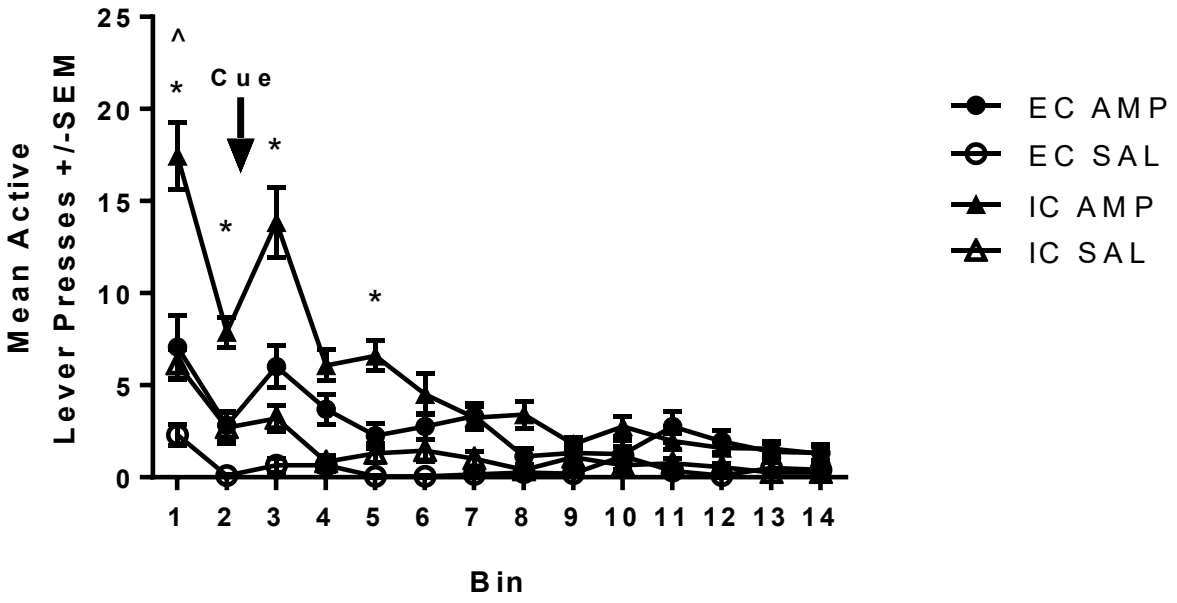

Figure 12. **Seeking test after 1 day of abstinence. Active lever presses during the seeking test after 1 day of abstinence. Cue arrow indicates the onset of the drug associated cue light. The cue was presented after the conclusion of the second (2nd) time bin. Caret symbol (^) indicates a significant difference between EC SAL and IC SAL. Asterisk symbol (*) indicates a significant differences between EC AMP and IC AMP. The omnibus alpha is set at p<.05 and simple effects alpha is at p<.0018.**

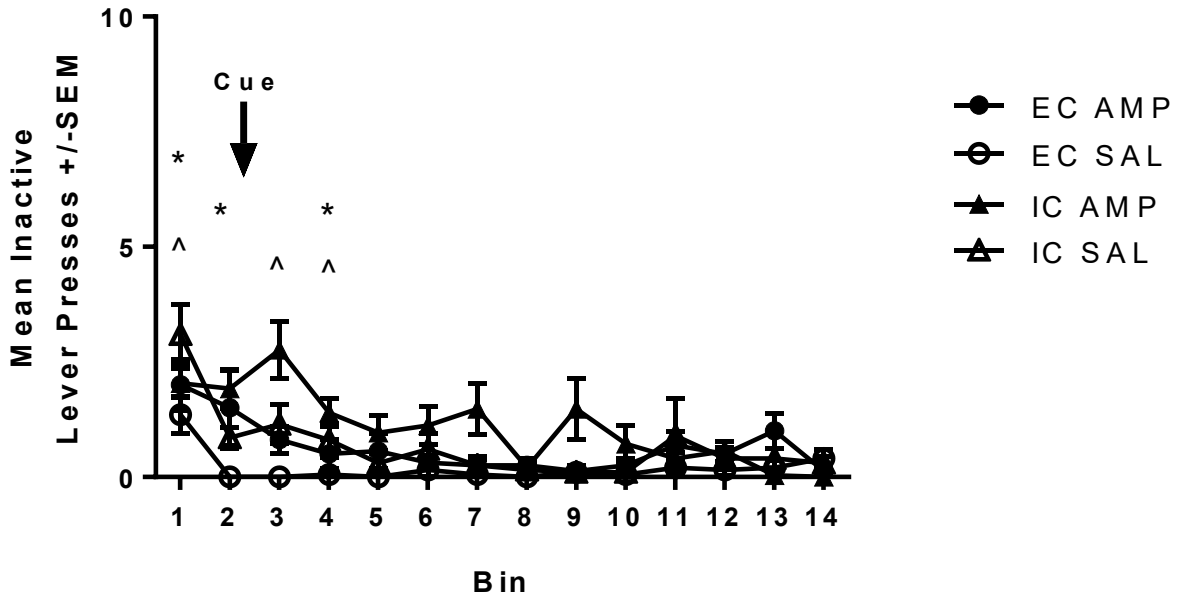

Figure 13. **Seeking test after 1 day of abstinence. Inactive lever presses during the seeking test after 1 day of abstinence. Cue arrow indicates the onset of the drug associated cue light. The cue was presented after the conclusion of the second (2nd) time bin. Caret symbol (^) indicates a significant difference between EC and IC with IC responding more. Asterisk symbol (*) indicates a significant differences between AMP and SAL with AMP rats responding more. The omnibus alpha is set at p<.05 and simple effects alpha is at p<.0037.**

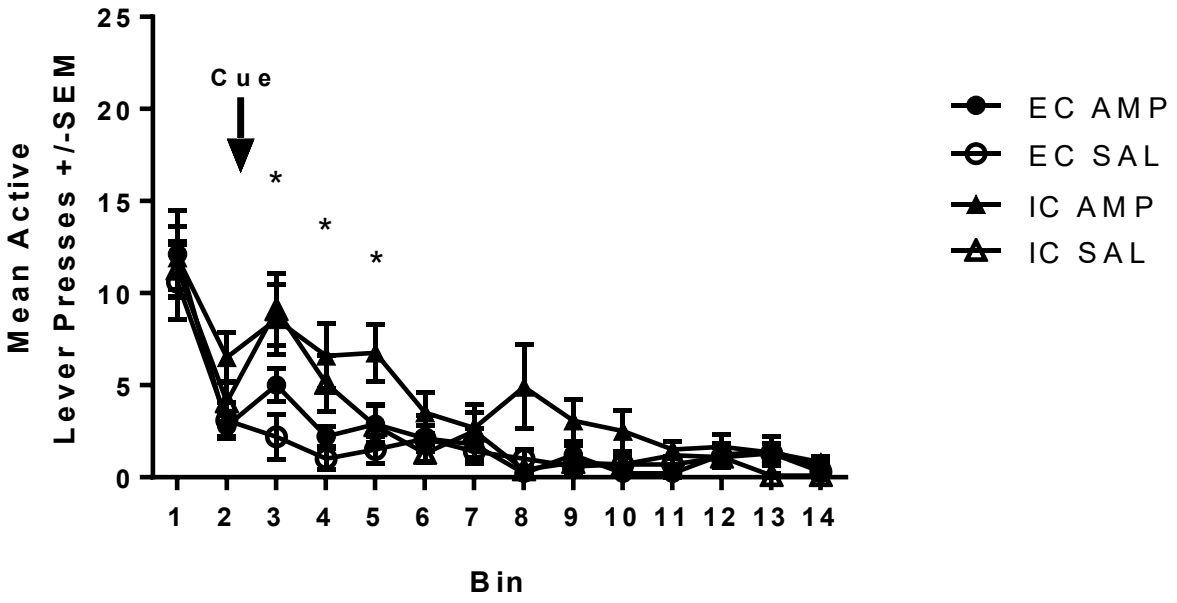

Figure 14. **Seeking test after 40 days of abstinence. Active lever presses during the seeking test after 40 days of abstinence. Cue arrow indicates the onset of the drug associated cue light. The cue was presented after the conclusion of the second (2nd) time bin. Asterisk symbol (*) indicates IC rats responded more after the cue presentation when compared to EC rats. The omnibus alpha is set at p<.05 and simple effects alpha is at p<.0016.**

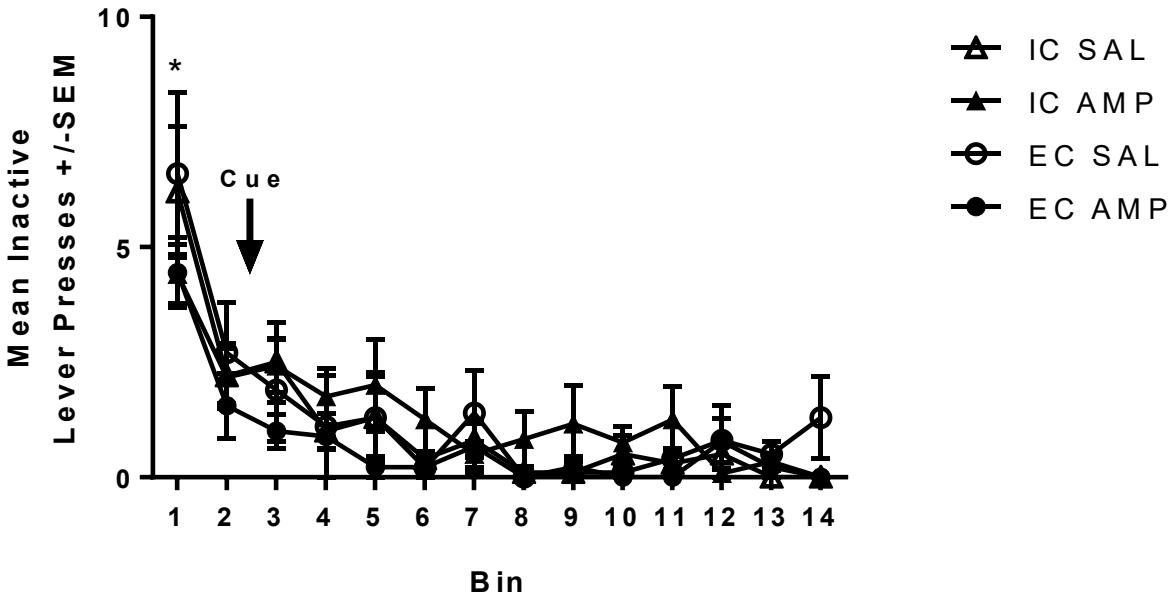

Figure 15. **Seeking test after 40 days of abstinence. Inactive lever presses during the seeking test after 40 days of abstinence. Cue arrow indicates the onset of the drug associated cue light. The cue was presented after the conclusion of the second (2nd) time bin. Asterisk symbol (*) indicates across all rats and groups responding on the inactive lever was higher during time bin 1 when compared to all other time bins. The omnibus alpha is set at $p<.05$ and simple effects alpha is at $p<.0016$.**

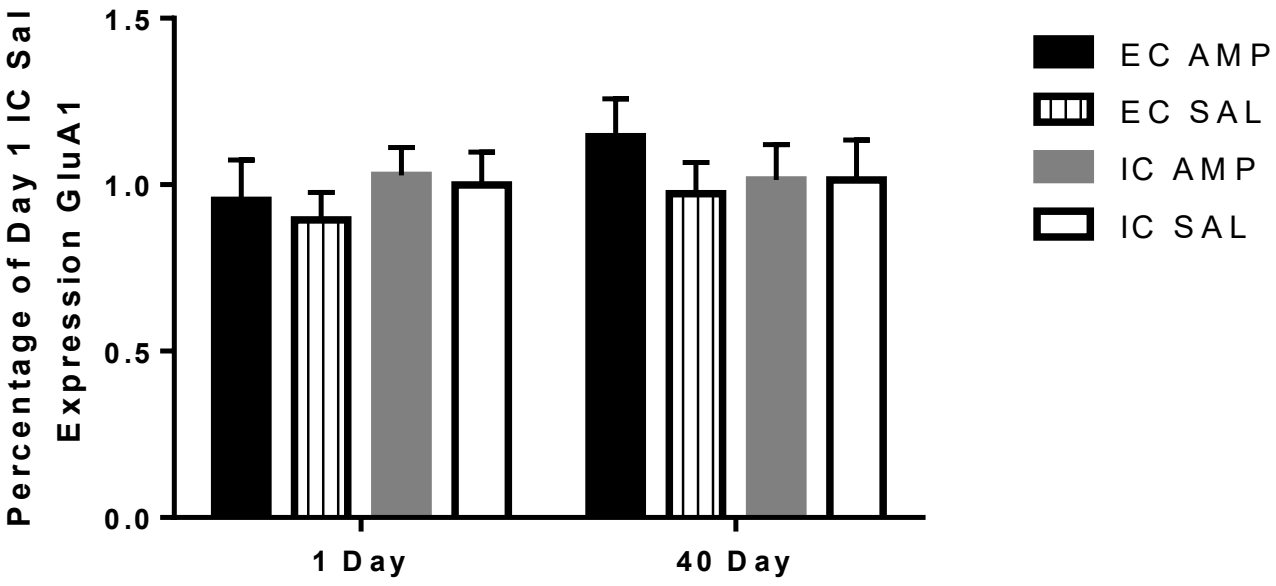

Figure 16. **The expression of AMPA subunit GluA1 was not different across any of the groups after 1 day of abstinence. After 40 days of abstinence, GluA1 expression was not different across any of the groups, indicating that differential housing and amphetamine exposure did not affect the expression of GluA1. There was trend that GluA1 increased in EC amphetamine rats, but it was not significant.**

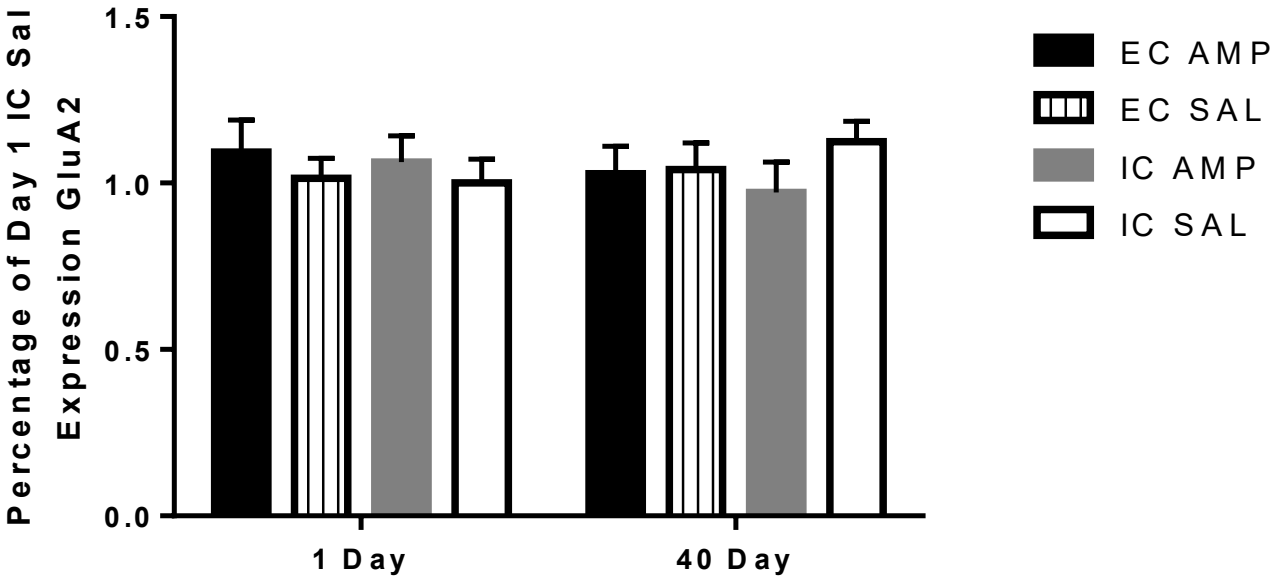

Figure 17. **The expression of AMPA subunit GluA2 was not different across any of the groups after 1 day of abstinence. After 40 days of abstinence, GluA2 expression was not different across any of the groups, indicating that differential housing and amphetamine exposure did not affect the expression of GluA2.**

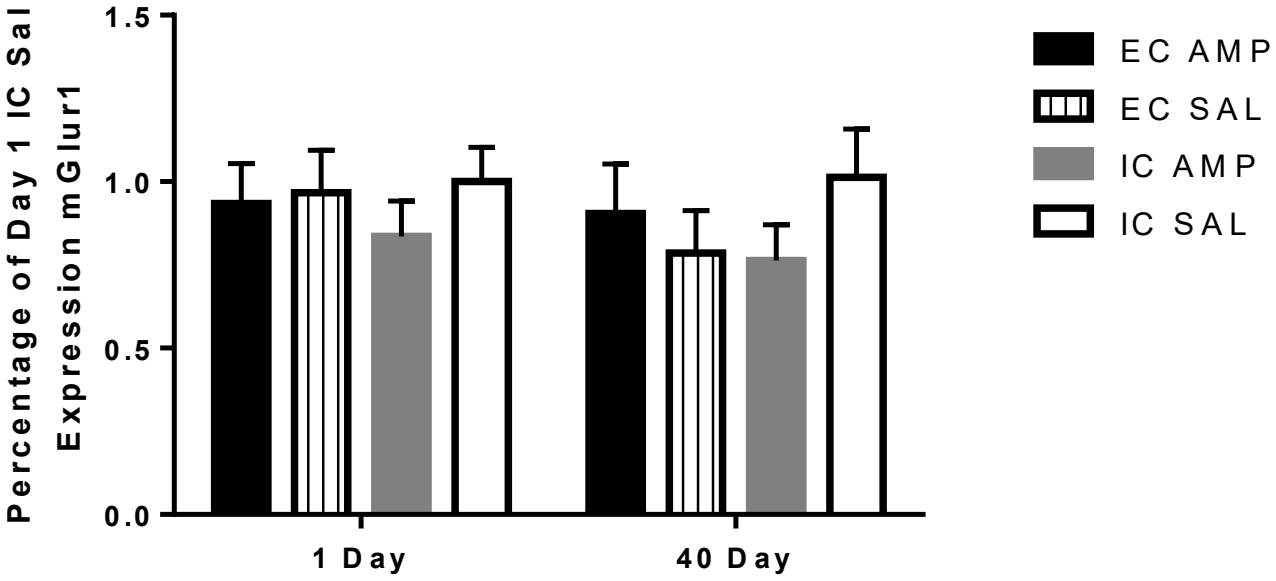

Figure 18. **The expression of the dimer of metabotropic glutamate receptor 1 (mGlur1) was not different across any of the groups after 1 day of abstinence. After 40 days of abstinence, mGlur1 dimer expression was not different across any of the groups, indicating that differential housing and amphetamine exposure did not affect the expression of the dimer form of mGlur1.**

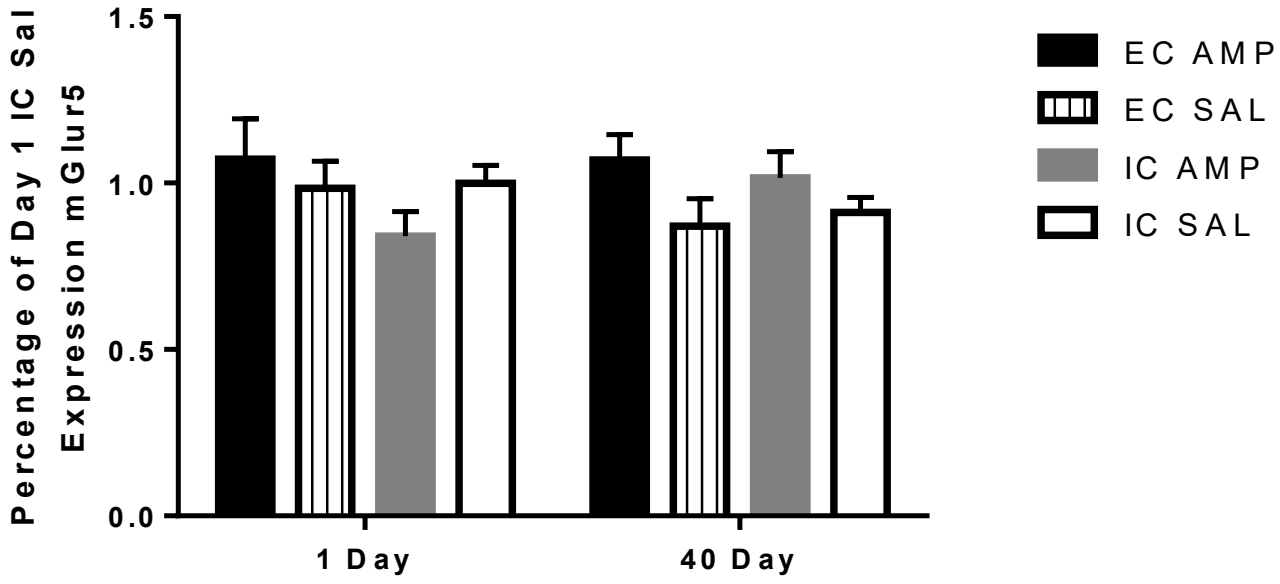

Figure 19. **The expression of metabotropic glutamate receptor 5 dimer (mGlur5) was not different across any of the groups after 1 day of abstinence. After 40 days of abstinence, mGlur5 dimer expression was not different across any of the groups, indicating that differential housing and amphetamine exposure did not affect the expression of the dimer form of mGlur5.**

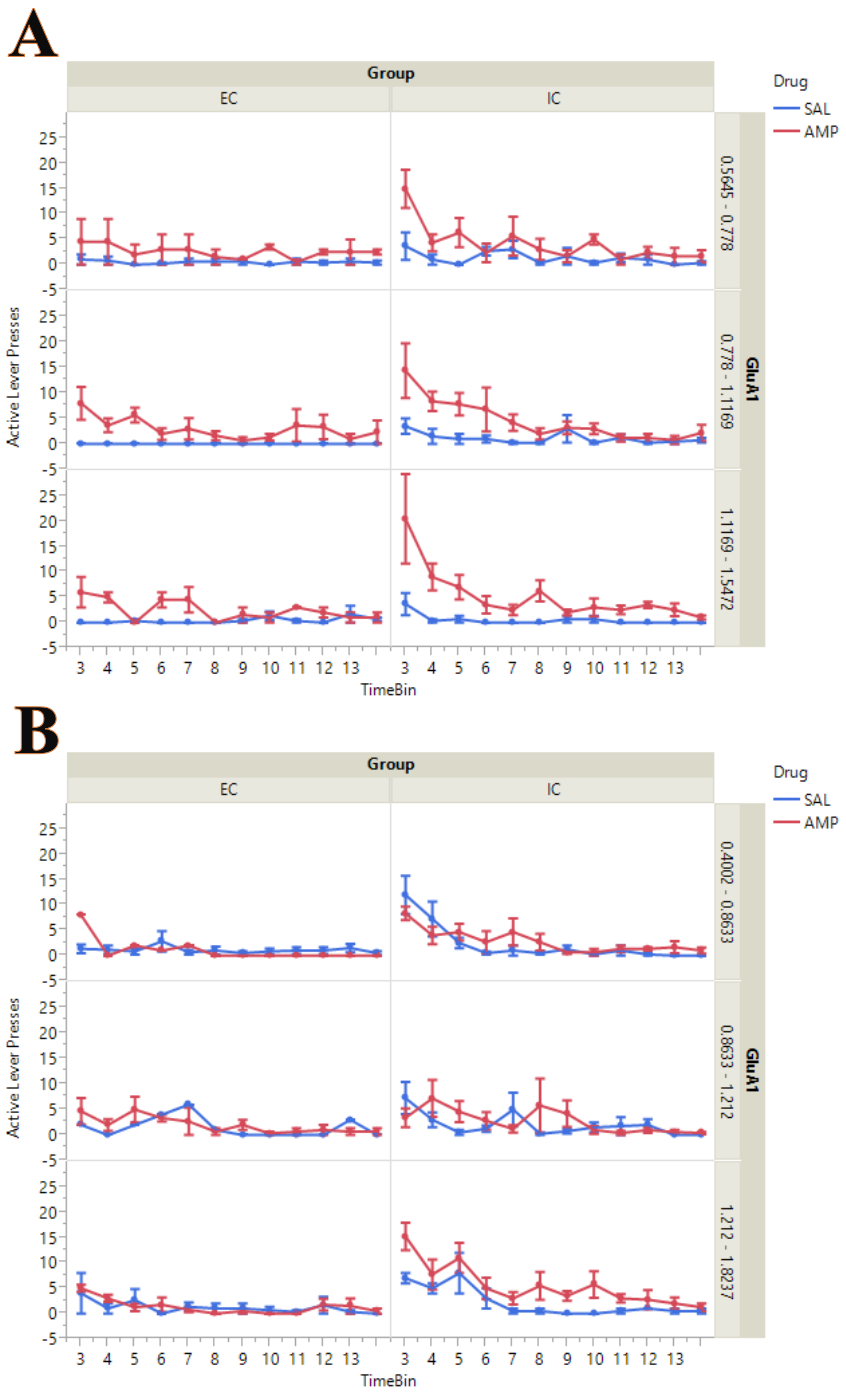

Figure 20. **Total active lever presses during the last hour of the seeking test as a function of environmental housing condition and GluA1 receptor expression. A) illustrates the responses during the seeking test after 1 day of abstinence and B) illustrates the responses after the 40 day abstinence period. IC amphetamine rats showed more drug seeking as receptor expression increase immediately following cue presentation.**

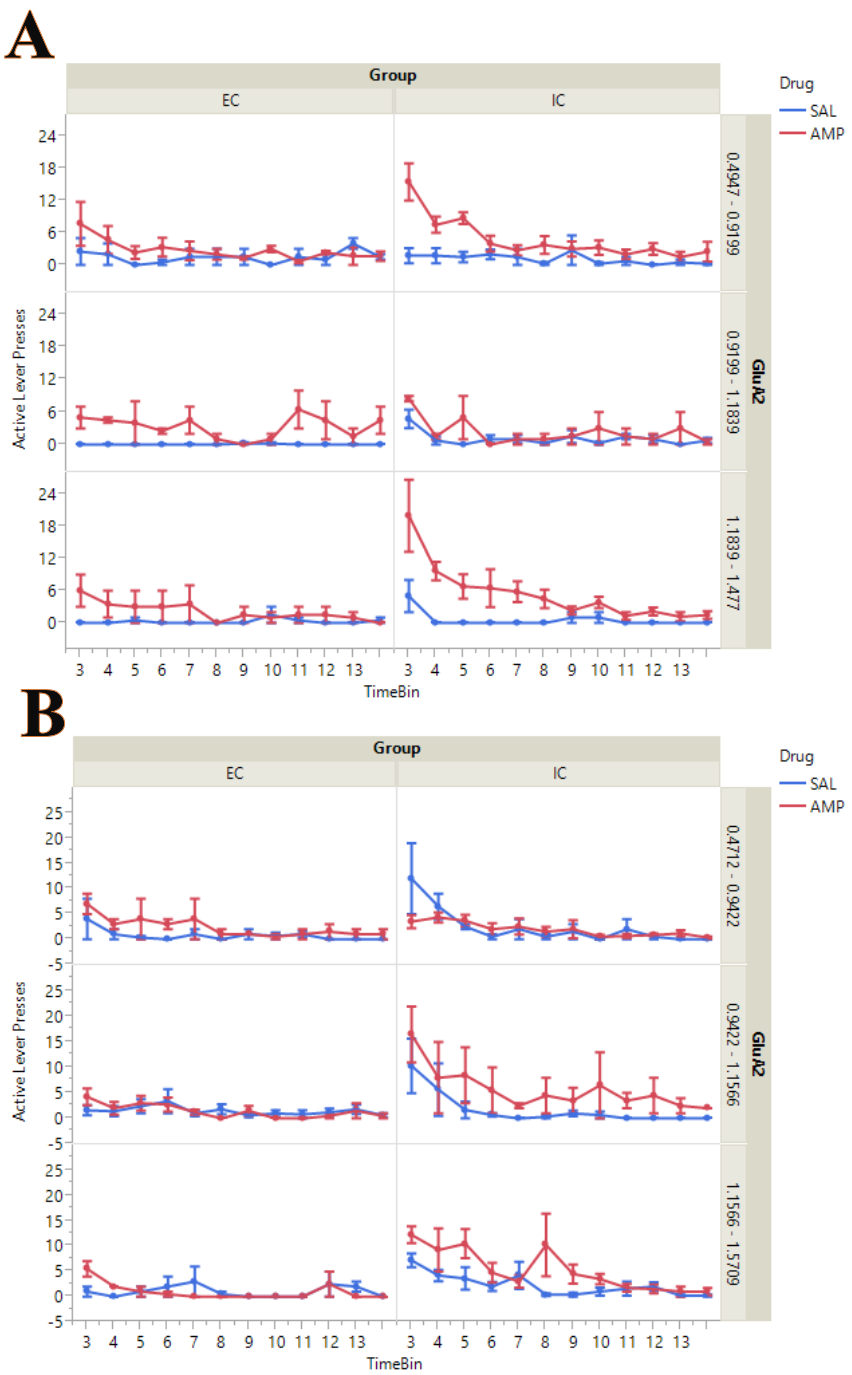

Figure 21. **Total active lever presses during the last hour of the seeking test as a function of environmental housing condition and GluA2 receptor expression. A) illustrates the responses during the seeking test after 1 day of abstinence and B) illustrates the responses after the 40 day abstinence period. GluA2 receptor expression did not interact with environmental condition or drug to predict drug seeking after short abstinence but did after long abstinence, resulting in increased drug seeking in IC amphetamine rats with higher expression.**

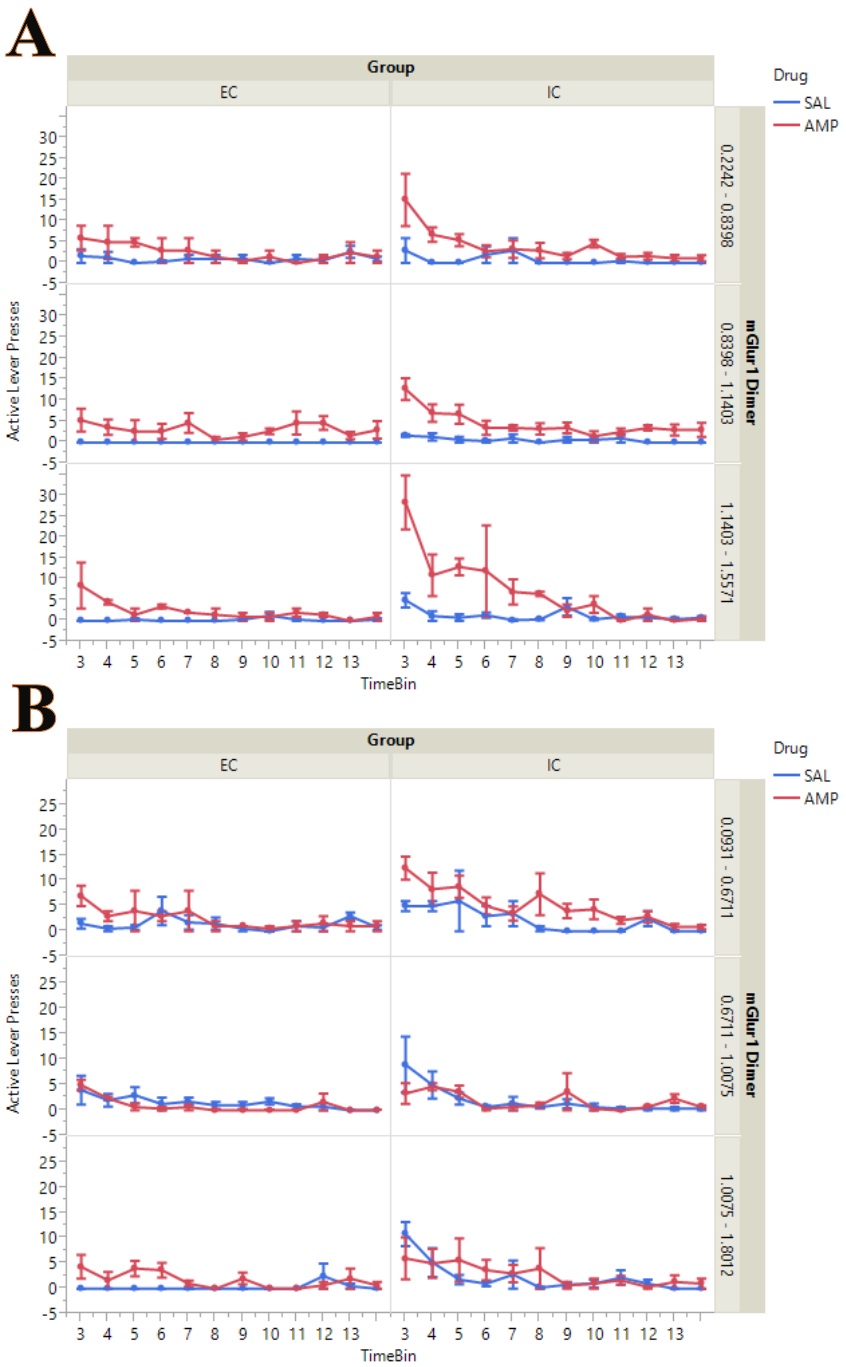

Figure 22. **Total active lever presses during the last hour of the seeking test as a function of environmental housing condition and mGlur1 dimer receptor expression. A) illustrates the responses during the seeking test after 1 day of abstinence and B) illustrates the responses after the 40 day abstinence period. The influence of mGlur1 expression on amphetamine seeking changed. After 1 day of abstinence, higher expression resulted in higher amphetamine seeking. After 40 days this relationship became negative, such that lower expression resulted in higher amphetamine seeking. These relationships were only observed in IC rats.**

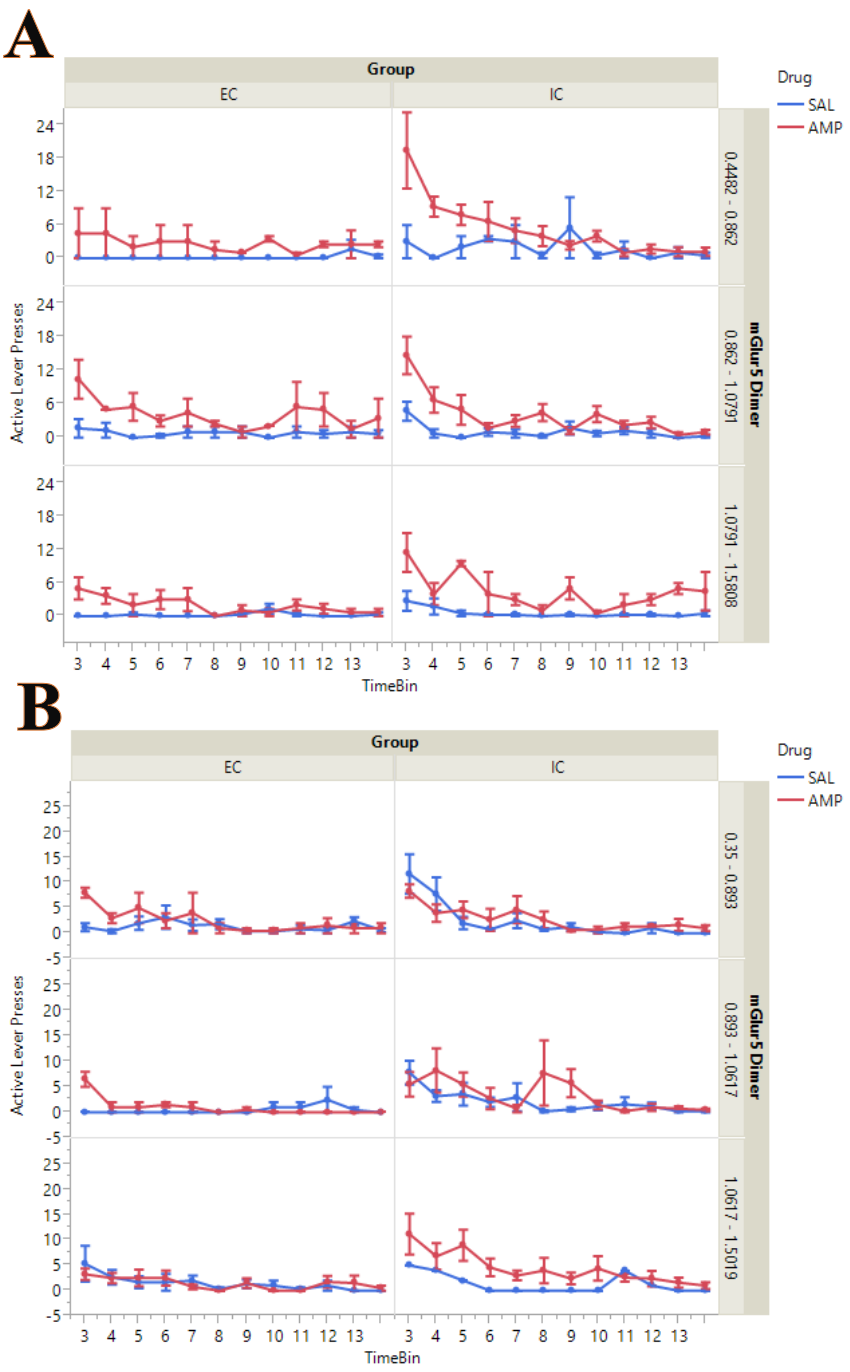

Figure 23. **Total active lever presses during the last hour of the seeking test as a function of environmental housing condition and mGlur5 dimer receptor expression. A) illustrates the responses during the seeking test after 1 day of abstinence and B) illustrates the responses after the 40 day abstinence period. MGlur5 did not predict or interact with other variable to predict amphetamine seeking after 1 day or 40 days of abstinence.**

Table 1: ***A total of 81 Sprague-Dawley rats were used in the experimental procedures. Below is the sample size of each experimental group. AMP indicates animals that self-administered amphetamine. SAL indicates animals that self-administered saline. Short refers to the cue-induced seeking test after 1 day. Long refers to the cue-induced seeking test after 40 days.***

	Seeking Test	
Rearing Group	Short	Long
EC-AMP	16	9
EC-SAL	20	10
IC-AMP	25	12
IC-SAL	20	10

Table 2*: Comparisons are between EC and IC rats for amphetamine infusions only. Calculated simple effects summary using the Sidak correction. The adjusted alpha is p<.0016 and the critical value is 10.008. Asterisk indicates a significant difference in infusions.*

Source	df	MS effect	MS error	*F*
Session 1	1	464.82	33.61	13.83*
Session 2	1	221.28	33.61	6.58
Session 3	1	6.68	33.61	0.19
Session 4	1	51.95	33.61	1.54
Session 5	1	31.52	33.61	0.93
Session 6	1	31.96	33.61	0.95
Session 7	1	3.31	33.61	0.09
Session 8	1	25.37	33.61	0.75
Session 9	1	53.76	33.61	1.6
Session 10	1	0.07	33.61	0.002
Session 11	1	196.03	33.61	5.83
Session 12	1	0.75	33.61	0.02
Session 13	1	23.90	33.61	0.71
Session 14	1	23.14	33.61	0.69
Session 15	1	15.55	33.61	0.46
Session 16	1	323.68	33.61	9.63
Error	1,125		33.61	

Table 3*: Comparisons are between EC and IC rats for saline infusions only. Calculated simple effects summary using the Sidak correction. The adjusted alpha is p<.0016 and the critical value is 10.008. Asterisk indicates a significant difference in infusions.*

Source	df	MS effect	MS error	*F*
Session 1	1	6,238.07	33.61	185.62*
Session 2	1	3,610.00	33.61	107.42*
Session 3	1	3,115.23	33.61	92.69*
Session 4	1	2160.90	33.61	64.30*
Session 5	1	1,404.23	33.61	41.78*
Session 6	1	970.23	33.61	28.87*
Session 7	1	462.40	33.61	13.76*
Session 8	1	792.10	33.61	23.57*
Session 9	1	846.40	33.61	25.19*
Session 10	1	970.23	33.61	28.87*
Session 11	1	1,380.63	33.61	41.08*
Session 12	1	1,322.50	33.61	39.35*
Session 13	1	465.66	33.61	13.85*
Session 14	1	592.90	33.61	17.64*
Session 15	1	656.10	33.61	19.52*
Session 16	1	308.03	33.61	9.16
Error	1,125		33.61	