UNIVERSITY OF CALIFORNIA, SAN DIEGO

Methamphetamine and Neurogenesis: The Role of Adult Hippocampal Neurogenesis in Drug Seeking Behavior

A Dissertation submitted in partial satisfaction of the requirement for the degree Doctor of Philosophy

in

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by

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DEDICATION

I dedicate this dissertation to Alexandre Galinato Colavin and my family.

EPIGRAPH

The brain is a world consisting of a number of unexplored continents and great stretches of unknown territory.

- Santiago Ramón Cajal

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ABSTRACT OF THE DISSERTATION

Methamphetamine and Neurogenesis: The Role of Adult Hippocampal Neurogenesis in Drug Seeking Behavior

by

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The research field of drug addiction is vast with multiple drugs, models, techniques, and brain regions to explore with hope of improving therapeutic treatments. Much of the work has focused on dopamine systems and brain structures important for reward related behavior. More recently the role of the hippocampus in drug addiction has been studied for its importance in learning and memory. One unique feature of the hippocampus is that it produces newborn

neurons that become mature granule cell neurons in the dentate gyrus. This process, adult hippocampal neurogenesis, is important for specific memory functions involving discrimination; however, the functional role of neurogenesis has often been disputed.

Adult hippocampal neurogenesis is impacted by several environmental factors including drugs of abuse. The studies presented in this dissertation aim to understand the nature of altered neurogenesis caused by methamphetamine, which is a strongly reinforcing psychostimulant drug of abuse. The overall goal of the dissertation was to investigate changes occurring in the dentate gyrus during abstinence that could contribute to drug seeking behavior. In chapter 2, we investigate changes in hippocampal biochemistry during acute withdrawal, measuring proteins involved in cell growth, cell death, and synaptic plasticity needed for memory. We found significant changes primarily in animals that exhibited escalating drug intake patterns demonstrating compulsive-like drug seeking, which later results in enhanced neurogenesis during abstinence.

The following two studies tested the hypothesis that increased hippocampal neurogenesis induced by abstinence from meth plays a functional role in promoting drug seeking behavior during abstinence from meth. We used two different approaches for manipulating neurogenesis in order to test its effects on drug seeking. In chapter 3, the study used a synthetic compound, isoxazole-9, which reduced the abstinence induced neurogenesis back down to control levels. Normalized neurogenesis was associated with reductions in drug seeking behavior

in response to the drug-related context. In chapter 4, our study used a transgenic rat that allowed us to completely ablate neurogenesis during abstinence. Animals with inhibited neurogenesis showed impairments in drug seeking behavior during extinction and context reinstatement. Our findings from the studies in this dissertation supported the hypothesis that abstinence induced neurogenesis plays a direct role in reinstatement of drug seeking behavior.

CHAPTER 1:

INTRODUCTION

Methamphetamine and public health

Methamphetamine (meth) addiction is a global health issue and a rising public health issue especially in San Diego, California. Meth is a highly addictive psychostimulant that is used recreationally for its euphoric effects and meth can be used medically in rare cases to treat attention-deficit/hyperactivity disorder and obesity (Castle et al. 2007). In large doses, meth can be fatally toxic, and in San Diego, overdose deaths due to meth have been rising since 2010, numbering above overdose deaths due to cocaine and heroin (Goldberg 2016). The number of meth related visits to hospital emergency rooms (ERs) has also risen from over three thousand meth-related ER visits in 2011 to over ten thousand meth-related ER visits in 2014 (Goldberg 2016). Currently meth addiction treatment focuses on cognitive and behavioral therapies to shape behavior around reducing craving and relapse (Lee and Rawson 2008); however, there are no FDA approved pharmacological treatments for methamphetamine addiction. There is a clear need for better therapies to treat meth addiction, and there is a need to find effective therapeutic targets. In order to find those targets, we need to better understand the psychobiology and neurobiology of meth addiction and meth seeking behavior.

The psychobiology of addiction

Researchers define drug addiction as a chronic relapsing disorder with three distinct phases; namely, intoxication -when the individual experiences the drug, withdrawal -when the individual is not consuming the drug and anticipation or craving -when the individual seeks the drug to gain relief from physical withdrawal symptoms (Koob and Volkow 2010). The three phases form the cycle of addiction where they repeat over and over again. In addition, the individual builds extensive association between the drug and the physiological and pharmacological effects produced by the drug (e.g. euphoria, alertness, sedation, psychedelic effects) (Solomon and Corbit 1974). Therefore, addiction also involves learning and memory processes that become dysfunctional over time due to acquiring and maintaining new drug-related behaviors as well as losing control over drug taking behavior. For example, during the binge/intoxication phase, the brain is making associations between drug taking, the environment, the context, the cues, and pleasurable feelings (Koob and Volkow 2010; Everitt and Robbins 2005). Initially, drug may be taken for its pleasurable effects; however, as the drug addiction cycle continues, escalating drug intake occurs due to changes in drug effects, and tolerance to the drug, despite negative effects (Solomon and Corbit 1974; Koob and Le Moal 2001). The second phase is withdrawal from the drug during which physical and psychological symptoms create negative affect when the drug is no longer being taken. Withdrawal symptoms in people who chronically use meth include impaired sleep, dysphoric mood, lack of motivation, and increased anxiety, and these symptoms can last several weeks (McGregor et al. 2005). The third phase is the

anticipation/preoccupation stage when craving is experienced and drives thoughts about when and where to seek the drug. These thoughts and cravings can arise in response to drug associated contexts and cues or other drug-related memories that were learned during drug experience (Everitt et al. 2008). Moreover, these learned associations may be generalized to cues and environments that are similar to the drug-related objects, places, and feelings, which make it even harder to avoid craving.

Animal model of addiction and craving, and the neurobiology of addiction

Researchers have begun to define addiction as a brain disease. In order to measure changes in the brain associated with the stages of drug addiction, researchers developed rodent models of addiction. Clinically defined, addiction or substance dependence involves the loss of behavioral control over drug taking and drug seeking. The limited use of drugs with the potential for abuse is distinct from escalated drug use and the materialization of a chronic drug-dependent state. Such addiction-like behavior has been demonstrated in rodent models of intravenous drug self-administration (Hyman, Malenka, and Nestler 2006; Everitt et al. 2008). Rodents can learn arbitrary instrumental actions, such as lever pressing, to gain access to positive reinforcement, such as food or drugs of abuse. Therefore, there is much clinical relevance in intravenous drug self-administration, in which rodents are trained to self-administer drugs by pressing a lever for an intravenous drug infusion in an operant conditioning chamber (Collins et al. 1983), with intermittent

(1 h twice a week access mimicking recreational intake in humans), limited (1 h daily access mimicking frequent, but non-dependent intake in humans), or long (extended) access (> 4 h daily access mimicking compulsive intake and thus dependence in humans). An increase in drug availability or a history of drug intake has been shown to accelerate the development of dependence in humans. In rats, extended access to drugs of abuse, including cocaine, methamphetamine, nicotine, heroin, and alcohol, produce an escalation of drug self-administration, suggesting compulsive drug intake and therefore reflecting dependence-like behavior (Gilpin and Koob 2008; Koob 2009). Animal models of escalation, therefore, may provide a useful approach to understanding the neurobiological mechanisms responsible for the transition from limited drug use to compulsive intake and may represent a particularly suitable model for testing the hypothesis that alterations in adult brain plasticity by the drug is partially responsible for the addictive behavior.

Animal Models of Craving

The reinstatement of drug-seeking behavior in rats is a widely used model of craving that mimics the relapse stage of addiction in human addicts. Two models have been extensively used to uncover the key brain regions, brain circuitry, neurotransmitters, and neuromodulators associated with reinstatement behavior. The first is the drug self-administration paradigm. After the self-administration behavior is learned, it is extinguished by explicit nonreward; reinstatement drug-seeking behavior (e.g., lever pressing in the operant chamber) in response to a

priming stimulus is then measured following a specific period of extinction (Sanchis-Segura and Spanagel 2006; Collins et al. 1983). Priming stimuli include cues previously paired with drug self-administration (cue priming), acute noncontingent exposure to the drug (which is usually delivered to the rat by the experimenter; i.e., drug priming) or context (spatial location) where the drug was self-administered (context) (Sanchis-Segura and Spanagel 2006). The second is the conditioned place preference model of reinstatement, in which rats are administered the drug by the experimenter (passive exposure during training) and are tested (cue/context) in a drug-free state (Sanchis-Segura and Spanagel 2006). Although both models have face validity, the self-administration model can be used to produce distinct drug intake patterns (limited *vs*. compulsive intake of the drug) that mimic recreational use *vs*. dependent use in human addicts, measure repeated operant behavior (during drug taking and seeking) that mimic an addict's drug-response pattern, and produce high rates of relapse. Thus, the intravenous self-administration model of drug exposure appears to be best suited for studying the neural mechanisms of relapse.

Reward and Relapse Circuitry in the Adult Brain

Addiction research indicates that dysregulation of the "hedonic set point" and the allostatic processes of the brain reward system that support it may underlie relapse to drug seeking and promote addiction to the drug of abuse (Ahmed and Koob 1998; Solomon and Corbit 1974; Koob and Le Moal 2001). The reward and

relapse circuitry in the adult mammalian brain has been delineated based on multiple groundbreaking studies performed in rodent models of acute and chronic reinforcement schedules and reinstatement to drug-seeking behavior (Kalivas and Volkow 2005).

The focus of this thesis is on the neurobiological changes occurring during withdrawal and protracted abstinence and the anticipation/preoccupation stage of drug addiction. Based on several preclinical and recent clinical studies, it has been demonstrated that the brain regions involved in the relapse stage of addiction include, but are not limited to, the prefrontal cortex (Clark et al. 2008), basolateral amygdala (Murray 2007), central nucleus of the amygdala, nucleus accumbens (Kalivas and Volkow 2005), ventral tegmental area, and the hippocampus (Ramirez et al. 2009; Xie et al. 2009; Fuchs et al. 2005). The nucleus accumbens is considered a focal point for reward and reinstatement of drug-seeking behaviors (Kalivas and Volkow 2005). Most importantly, it is believed that the release of the neurotransmitters dopamine, glutamate, and corticotropin-releasing factor in these key brain regions is essential for the behavioral outcomes of the drug (Koob 2009; Martin et al. 2012; McFadden et al. 2012).

This thesis specifically focuses on the role of the hippocampus in relapse to meth seeking behaviors. Previous studies conducted in animals show that the hippocampus is involved in relapse to meth seeking behaviors (Fuchs et al. 2005). For example, researchers demonstrate that when the hippocampus is functionally inactivated in an animal model of meth addiction, the animals were unable to

reinstate meth seeking triggered by drug context, drug cues and the drug itself. From these pioneering experiments we have learned that the hippocampus is involved in meth seeking behavior in response to meth-related contexts.

Meth experience affects the structure and function of the hippocampus

Several studies have shown how meth addiction impacts the hippocampus in both humans and rodents. Human imaging studies show reduced hippocampal volume, particularly gray matter volume, and decreased hippocampal responsiveness in chronic meth users (Kim et al. 2010; Thompson et al. 2004; Nakama et al. 2011), indicating maladaptive hippocampal networking in meth-exposed individuals. Postmortem analyses in human brain tissue confirms that chronic methamphetamine use produces neurotoxicity in the hippocampus (Kitamura 2009; Kitamura et al. 2010), which suggests an association between hippocampal dysfunction and toxicity in individuals addicted to meth. In rodent studies, researchers found reductions in hippocampal volume due to apoptosis or cell death in meth exposed animals (Schmued and Bowyer 1997). In studies looking at electrophysiology of neurons in the hippocampus, disruptions in normal forms of plasticity (long-term potentiation) were found in rats exposed to meth (Swant et al. 2010). In the dentate gyrus, acute meth exposure reduced neuronal excitability, whereas repeated exposure to meth increased excitability of these neurons (Criado et al. 2000). In terms of morphology, self-administration of methamphetamine produces robust and persistent changes in CA1 neurons (Onaivi et al. 2002;

Crombag et al. 2005; Swant et al. 2010). These studies emphasize that the hippocampus is sensitive to meth and the meth-induced changes in the hippocampus could possibly contribute to enhanced propensity for relapse.

Meth addiction effects learning and memory dependent on the hippocampus

There is an enormous body of literature to support the role of the hippocampus in learning and memory. When we discuss drug addiction as a learning process, it is easier to understand how the hippocampus can be involved in learned associations that contribute to drug seeking behavior. The hippocampus contains distinct subregions connected and organized with specific cell types to perform the multitude of memory and learning tasks that involve this brain region. The hippocampus is famously important for spatial memory with the discovery of place cells and grid cells that are active in response to specific locations (O'Keefe and Dostrovsky 1971; Hafting et al. 2005), which led to the 2014 Nobel Prize in Physiology or Medicine awarded to John O'Keefe, May-Britt Moser, and Edvard Moser. Because the hippocampal circuitry has been well studied, we are able to understand the different functional roles of the hippocampal subregions. For example, the CA3 and dentate gyrus subregions of the hippocampus are specifically involved in memory tasks that use spatial discrimination or pattern separation (Leutgeb et al. 2007), which is the ability to remember a context or environment as different from one that is similar. The focus of this thesis is the dentate gyrus

subregion of the hippocampus for its unique ability to produce newborn neurons in the adult brain in a process called adult neurogenesis.

Adult neurogenesis in the hippocampus

Neurogenesis is the process by which early neural precursor cells divide, multiply, and develop into immature neurons, and then become mature granule cell neurons in the dentate gyrus of the hippocampus (Zhao, Deng, and Gage 2008). There are multiple stages to neurogenesis and so it is important to specify which stages or cell types are being studied. For example, simply stating that neurogenesis has increased is a rather vague statement, which can be clarified by specifying whether cell proliferation (early stages) or cell survival (late stages) has increased. At each stage, the cells express different proteins or factors that can be used as markers for that particular stage of neurogenesis. Researchers can use immunohistochemistry techniques to label the cells and quantify how much the cells are dividing or how much the cells survive in a given time period. Using those techniques, adult hippocampal neurogenesis has been identified in rodents, in nonhuman primates, and in humans (Eriksson et al. 1998; Gage 2000; Zhao, Deng, and Gage 2008). The existence of adult neurogenesis was discovered relatively recently, and so the exact function of adult neurogenesis in the dentate gyrus is still being explored.

In order to understand the function of hippocampal neurogenesis, we need ways to either enhance or inhibit neurogenesis followed by measures of behavior that depend on the hippocampus, specifically the dentate gyrus. Several external and internal factors that regulate neurogenesis have been identified. Reduced neurogenesis has been observed in animal models of stress, depression, aging, and drug addiction (Mandyam and Koob 2012; Mandyam et al. 2008; Chetty et al. 2014; Sahay and Hen 2007), which contributes to the growing interest in neurogenesis as a potential therapeutic target in the field of psychiatry. To enhance neurogenesis one can use physical activity, enriched environment, antidepressants, deep brain stimulation, and ischemia (Zhao, Deng, and Gage 2008; Deng, Aimone, and Gage 2010; Aimone et al. 2014). Some of these neurogenic methods induce gene expression and biochemical pathways that promote neuronal proliferation and cell survival, which increases neurogenesis. To reduce or inhibit neurogenesis in experiments, most researchers have used x-irradiation, low frequency stimulation, or genetic approaches targeting genes needed for neuronal growth (Deschaux et al. 2014; Clelland et al. 2009; Sahay, Wilson, and Hen 2011). While all these methods of manipulating neurogenesis have been effective, none of them are perfect methods of exclusively targeting hippocampal neurogenesis. For example, wheel running as a form of physical activity in rodents has multiple effects on the brain and body in addition to upregulation of neurogenesis, and so behavioral effects of wheel running may be attributed to changes in heart or lung health rather than neurogenesis alone. In chapters 3 and 4, the pharmacologic and genetic tools employed to reduce

neurogenesis are more selective than previously used methods because they specifically target mechanisms of newborn cell growth, and these tools enable us to better understand the function of neurogenesis in the dentate gyrus during drug addiction.

Neurogenesis and context discrimination

Regarding drug addiction, functional roles of the dentate gyrus may be involved in remembering drug-associated contexts. As mentioned earlier, one function of the dentate gyrus is called pattern separation or context discrimination, which is the ability to discern between two similar objects or contexts (Leutgeb et al. 2007). For example, the dentate gyrus is involved when a person is able to tell the difference between a visit to the conference room in the morning for a talk and another visit to the same conference room in the afternoon for a different meeting. The room is the same, and there may be some of the same people in both situations, but there are a few differences recognized by the dentate gyrus that help distinguish the two memories. In rodent studies, reducing neurogenesis using x-irradiation and genetic approaches can impair performance on memory tasks that involve pattern separation or context discrimination (Deng, Aimone, and Gage 2010; Clelland et al. 2009). One of these tasks is a two-choice spatial discrimination task in which rodents must learn to respond to the correct location on a touch screen given the choice between two colored boxes on the screen. Performance in this task is impaired in irradiated animals only when the boxes are close to each other rather

than far apart on the screen, which indicates that neurogenesis is needed to tell the difference between spatially similar locations (Clelland et al. 2009). This role of neurogenesis can come into play during drug addiction when encountering environments that are similar to drug-associated contexts.

Drugs of abuse and neurogenesis

As previously mentioned, drugs of abuse are known to alter hippocampal neurogenesis during the various stages of drug addiction. Studies have shown how cell proliferation is reduced by self-administration of cocaine, heroin, nicotine, alcohol, and meth. For review see (Mandyam and Koob 2012). There are limited studies on the nature of altered neurogenesis during drug addiction.

Previous studies from the Mandyam lab have shown how different patterns of drug taking behavior can differentially affect neurogenesis in the hippocampus as well as drug seeking behavior after abstinence. Extended-access to meth self-administration reduced both cell proliferation and cell survival of neurons born during drug acquisition (Recinto et al. 2012). In contrast, forced abstinence increased cell proliferation and rate of cell survival, suggesting a rebound effect or maybe a form of recovery. Positive correlations were found between meth intake and cell survival during abstinence as well as drug seeking during abstinence (Recinto et al. 2012). Together these associations suggest a relationship between abstinence-induced hippocampal neurogenesis and drug seeking behavior after abstinence. However, studies have failed to show the exact contributions of

hippocampal neurogenesis in drug seeking behavior using a model of compulsive-like drug seeking. These studies showing impairments to neurogenesis during and after meth self-administration demonstrate the need to study the role of hippocampal neurogenesis in drug addiction.

We hypothesize that Meth withdrawal-induced maladaptive changes in neurogenesis is involved in the enhancement of reinstatement of drug seeking following abstinence, and that normalizing neurogenesis during abstinence will contribute to repair after Meth self-administration via neuronal turnover and will reduce relapse. This hypothesis is addressed by three specific aims:

Specific Aims

1. Determine the effects of Meth self-administration on cellular and biochemical events in the dentate gyrus.
2. Determine the cellular mechanisms in the dentate gyrus underlying enhanced reinstatement of Meth seeking during abstinence.
3. Determine whether aberrant neurogenesis during abstinence plays a mechanistic role in reinstatement of Meth seeking.

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CHAPTER 2:

METHAMPHETAMINE DIFFERENTIALLY AFFECTS BDNF AND CELL DEATH FACTORS IN ANATOMICALLY DEFINED REGIONS OF THE HIPPOCAMPUS

Abstract

Methamphetamine exposure reduces hippocampal long-term potentiation (LTP) and neurogenesis and these alterations partially contribute to hippocampal maladaptive plasticity. The potential mechanisms underlying methamphetamine-induced maladaptive plasticity were identified in the present study. Expression of brain-derived neurotrophic factor (BDNF; a regulator of LTP and neurogenesis), and its receptor tropomyosin-related kinase B (TrkB) were studied in the dorsal and ventral hippocampal tissue lysates in rats that intravenously self-administered methamphetamine in a limited access (1 h/day) or extended access (6 h/day) paradigm for 17 days post baseline sessions. Extended access methamphetamine enhanced expression of BDNF with significant effects observed in the dorsal and ventral hippocampus. Methamphetamine-induced enhancements in BDNF expression were not associated with TrkB receptor activation as indicated by phospho (p)-TrkB-706 levels. Conversely, methamphetamine produced hypophosphorylation of N-methyl-d-aspartate (NMDA) receptor subunit 2B (GluN2B) at Tyr-1472 in the ventral hippocampus, indicating reduced receptor activation. In addition, methamphetamine enhanced expression of anti-apoptotic

protein Bcl-2 and reduced pro-apoptotic protein Bax levels in the ventral hippocampus, suggesting a mechanism for reducing cell death. Analysis of Akt, a pro-survival kinase that suppresses apoptotic pathways and pAkt at Ser-473 demonstrated that extended access methamphetamine reduces Akt expression in the ventral hippocampus. These data reveal that alterations in Bcl-2 and Bax levels by methamphetamine were not associated with enhanced Akt expression. Given that hippocampal function and neurogenesis vary in a subregion-specific fashion, where dorsal hippocampus regulates spatial processing and has higher levels of neurogenesis, whereas ventral hippocampus regulates anxiety-related behaviors, these data suggest that methamphetamine self-administration initiates distinct allostatic changes in hippocampal subregions that may contribute to the altered synaptic activity in the hippocampus, which may underlie enhanced negative affective symptoms and perpetuation of the addiction cycle.

Introduction

Methamphetamine abuse takes severe emotional and financial tolls on society, cutting across ages, races, ethnicities, and genders, and causes significant damage to self by producing cognitive impairment (Thompson et al., 2004, SAMHSA, 2008, Price et al., 2011 and Weber et al., 2012). Hippocampus-dependent cognitive impairments in methamphetamine-abusing subjects can be attributed to

methamphetamine-induced alterations in structural and functional plasticity of hippocampal neurons. For example, human imaging studies show reduced hippocampal volume, particularly gray matter volume, and decreased hippocampal responsiveness in chronic methamphetamine users (Thompson et al., 2004, Kim et al., 2010, Schwartz et al., 2010, Daumann et al., 2011, Nakama et al., 2011, Orikabe et al., 2011 and Morales et al., 2012), indicating maladaptive hippocampal networking in methamphetamine-exposed individuals. Postmortem analyses in human brain tissue confirms that chronic methamphetamine use produces neurotoxicity in the hippocampus (Kitamura, 2009 and Kitamura et al., 2010), which suggests an association between hippocampal dysfunction and toxicity in methamphetamine addicts.

The behavioral deficits observed in methamphetamine addicts have been demonstrated in preclinical models of binge methamphetamine exposure and methamphetamine self-administration with extended access to the drug. For example, these experimental paradigms produce cognitive dysfunction and memory impairments dependent on the hippocampus (Itoh et al., 1984, Yoshikawa et al., 1991, Yamamoto, 1997, Friedman et al., 1998, Rogers et al., 2008 and Recinto et al., 2012). Neurodegeneration and neurotoxicity in the hippocampus is also observed in animal models of binge methamphetamine exposure and methamphetamine self-administration, suggesting a positive correlation between methamphetamine-induced toxicity and methamphetamine-induced behavioral deficits (Commins and Seiden, 1986, Eisch et al., 1996, Schmued and Bowyer, 1997 and Mandyam et al.,

2008). Additional mechanistic studies show that methamphetamine exposure alters the functional and structural plasticity of hippocampal neurons. For example, acute and systemic methamphetamine treatment reduces long-term potentiation (LTP) of CA1 pyramidal neurons through activation of D1 receptors and increases baseline excitatory synaptic transmission (Swant et al., 2010). Alternatively, self-administration of methamphetamine produces robust and long-lasting morphological changes in CA1 neurons (Onaivi et al., 2002, Crombag et al., 2005 and Swant et al., 2010). Acute methamphetamine exposure reduces excitability of dentate gyrus neurons, whereas repeated exposure to methamphetamine increases excitability of these neurons (Criado et al., 2000). Furthermore, emerging studies in adult rats self-administering methamphetamine under extended access conditions (6 h of drug access per day) demonstrate that methamphetamine self-administration reduces dentate gyrus neurogenesis, and these effects were relative to the amount of methamphetamine consumed (Mandyam et al., 2008 and Yuan et al., 2011). Withdrawal from methamphetamine self-administration produces compensatory changes in dentate gyrus neurogenesis (i.e., the enhanced survival of newly born neurons born after methamphetamine self-administration), and the hypothesis is that these changes in the hippocampus during withdrawal may regulate relapse to methamphetamine seeking (Recinto et al., 2012). These studies demonstrate that methamphetamine exposure (via either experimenter-delivered or self-administration paradigms) produces synaptic maladaptation in the

hippocampus that may mediate some of the addiction behaviors dependent on the hippocampus.

In this context, brain-derived neurotrophic factor (BDNF), via interactions with its receptor tropomyosin-related kinase B (TrkB), regulates the function of neurons within the mesolimbic dopamine system and other reward regions to modulate the motivation to take drugs, while BDNF also independently affects hippocampal neurogenesis (Lee et al., 2000, Pierce and Bari, 2001, Katoh-Semba et al., 2002, Bolanos and Nestler, 2004, Scharfman et al., 2005, Donovan et al., 2008 and Russo et al., 2010). Enhanced BDNF levels in the dentate gyrus positively correlate with increased number of newly born neurons in the dentate gyrus in non-pathological conditions; however, enhancing BDNF in an ischemic brain reduces ischemia-induced neurogenesis (Lee et al., 2000, Katoh-Semba et al., 2002, Larsson et al., 2002 and Scharfman et al., 2005), suggesting a critical relationship between the neurogenic niche, BDNF, and neurogenesis in the hippocampus. In the context of addiction, cocaine exposure produces a widespread, but transient, induction of BDNF protein in the nucleus accumbens, prefrontal cortex, ventral tegmental area, and the central and basolateral nuclei of the amygdala (Grimm et al., 2003, Le Foll et al., 2005 and Graham et al., 2007), whereas fewer studies have been conducted to examine BDNF protein levels after exposure to amphetamines. For example, BDNF protein expression in the hippocampus is enhanced in juvenile animals after forced administration of neurotoxic doses of amphetamines and the findings demonstrate that these changes in the neurotrophin may relate to the cognitive deficits in

methamphetamine experienced animals (Skelton et al., 2007 and Grace et al., 2008). In contrast to these studies using noncontingent methamphetamine injections, there are no published studies on the effect of methamphetamine self-administration on hippocampal BDNF levels, and therefore, was the focus of the current study. We also investigated the receptor systems (TrkB, N-methyl-d-aspartate (NMDA) receptor subunit 2B (GluN2B)), cell death factors (Bcl2 and Bax) and downstream signaling proteins (Akt, a pro-survival kinase) modulated by BDNF (Lin et al., 1998, Chao, 2003 and Almeida et al., 2005).

Experimental procedures

Animals

Surgical and experimental procedures were carried out in strict adherence to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH publication number 85-23, revised 1996) and approved by the Institutional Animal Care and Use Committee of The Scripps Research Institute. Nineteen adult, male Wistar rats (Charles River), weighing 200–250 g at the start of the experiment, were housed two per cage in a temperature-controlled vivarium under a reverse light/dark cycle (lights off 8:00 AM–8:00 PM) for at least 1 week.

Surgery

Twenty-two rats underwent surgery for catheter implantation for intravenous self-administration. Rats were anesthetized with 2–3% of isoflurane mixed in oxygen and implanted with a sterilized silastic catheter (0.64 ID × 1.19 OD mm; Dow Corning Co., Midland, MI, USA) into the right jugular vein under aseptic conditions. The distal end of the catheter was threaded under the skin to the back of the rat and exited the skin via a metal guide cannula (22G, Plastic One, Inc., Roanoke, VA, USA). Immediately after surgery, Flunixin® (2.5 mg/kg, s.c.; Bimeda – MTC Animal Health Inc., Oakbrook Terrace, IL, USA) was given as analgesic. The rats were subjected to antibiotic therapy with Timentin® (20 mg, i.v, once daily; SmithKline Beecham, Brentford, London, UK) during 10 days after the surgeries, and sulfamethoxazoletrimethoprim oral suspension (TMS, Hi-Tech Pharmacal Co., Inc., Amityville, NY, USA) in the drinking water (0.48 mg/ml) when health complications appeared during the study. Catheters were flushed daily with heparinized saline (30 USP units/ml) and tested eventually for patency using methohexital sodium (Brevital®, 10 mg/ml, 2 mg/rat; King Pharmaceutical Inc., Bristol, TN, USA). Eight rats did not experience methamphetamine. Three of the eight rats were trained to self-administer saline (under FR1 schedule with each active lever delivering 90–100 µl of sterile saline) and experienced saline self-administration for 1 h for the same number of sessions as methamphetamine rats. Fourteen rats experienced methamphetamine self-administration.

Baseline training sessions and maintenance on an extended access schedule

Four to five days after surgery rats (n = 14) were trained to press a lever according to an FR1 schedule of methamphetamine reinforcement (0.05 mg/kg/injection of methamphetamine hydrochloride, generously provided by the National Institute on Drug Abuse) in operant boxes (Med Associates, St Albans, VT, USA) under baseline (acquisition) conditions (1 h access per day for 6–7 days). During daily sessions, a response on the active lever resulted in a 4-s infusion (90–100 µl volume), followed by a 20-s time-out period to prevent overdose. Each infusion was paired for 4 s with white stimulus light over the active lever (conditioned stimulus [CS]). Response during the time-out or on the inactive lever was recorded but resulted in no programed consequences. All animals were housed on a reverse cycle (lights off at 8 am) and were transferred from their home cages to their operant chambers between 9 and 10 am. Training on the first and second days was initiated with two–three priming (non-contingent) infusions of methamphetamine during the first 10 min. Rats were allowed to respond for the remaining 50 min without any additional priming. Acquiring methamphetamine self-administration was defined as maintenance of a similar number of infusions over three consecutive days during baseline training sessions. All animals acquired methamphetamine self-administration (data not shown). After baseline (acquisition) training, the rats were subjected to a short (ShA, n = 8, 1 h per day under an FR1 schedule) or long (LgA, n = 6, 6 h per day under an FR1 schedule) access schedule of methamphetamine reinforcement. Methamphetamine self-administration was performed for 17 days.

Western blotting

Procedures optimized for measuring levels of both phosphoproteins and total proteins were employed (Graham et al., 2007, Orio et al., 2009 and Edwards et al., 2013). Methamphetamine-naive rats (n = 8), ShA (n = 8) and LgA (n = 6) rats were killed via rapid decapitation under light isoflurane anesthesia 16–20 h after the last self-administration session. Brains were quickly removed and flash-frozen. Tissue punches enriched in the dorsal hippocampus (−3.12 to −4.44 mm from bregma) or ventral hippocampus (−5.40 to −6.12 mm from bregma; Fig. 1b) from 500-μm-thick sections were homogenized on ice by sonication in buffer (320 mM sucrose, 5 mM HEPES, 1 mM EGTA, 1 mM EDTA, 1% sodium dodecyl sulfate (SDS), with Protease Inhibitor Cocktail and Phosphatase Inhibitor Cocktails II and III diluted 1:100; Sigma, St. Louis, MO, USA), heated at 100 °C for 5 min, and stored at −80 °C until determination of protein concentration by a detergent-compatible Lowry method (Bio-Rad, Hercules, CA, USA).

Mature BDNF protein levels were determined in 20-μg protein samples (mixed (1:1) with a Tricine sample buffer containing β-mercaptoethanol) subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) (15% acrylamide) using a Tris–Tricine–SDS buffer (Bio-Rad, Hercules, CA, USA), followed by electrophoretic transfer to polyvinylidene fluoride membranes (PVDF pore size 0.2 μm). TrkB, pTrkB, GluN2B, pGluN2B, Bcl-2 and Bax protein levels were determined in 20–30-μg protein samples (mixed (1:1) with a Laemmli sample buffer

containing β-mercaptoethanol) subjected to SDS–PAGE (8–12% acrylamide) using a Tris–Glycine–SDS buffer (Bio-Rad, Hercules, CA, USA), followed by electrophoretic transfer to PVDF membranes. Blots were blocked with 5% milk (w/v) in Tris-buffered saline and Tween 20 (TBST) (25 mM Tris–HCl (pH 7.4), 150 mM NaCl and 0.1% Tween 20 (v/v)) for 1 h at room temperature and were incubated with the primary antibody for 16–20 h at 4 °C: antibody to BDNF (1:200, Santa Cruz cat. no. sc-546, predicted molecular weight 14 kDa, observed band between 15 and 20 kDa), TrkB (1:200, Santa Cruz cat. no. sc-8316, predicted molecular weight 95–145 kDa, observed band ~130 kDa), pTrkB Tyr-706 (1:200, Santa Cruz cat. no. sc-8316, predicted molecular weight 95–145 kDa, observed band ~95 kDa), GluN2B (1:200, Santa Cruz cat. no. sc-9057, predicted molecular weight 178 kDa, observed band ~180 kDa), antibody to pGluN2B Tyr-1472 (1:200, Cell Signaling cat. no. 4208S, predicted molecular weight 190 kDa, observed band ~180 kDa), Bcl-2 (1:500, R&D Systems cat. no. MAB8272, predicted molecular weight 24 kDa, observed band ~25 kDa), Bax (1:500, Santa Cruz cat. no. sc-493, predicted molecular weight 23 kDa, observed band ~20 kDa), antibody to Akt (1:500, Cell Signaling cat. no. 4691S, predicted molecular weight 60 kDa, observed band ~60 kDa), antibody to pAkt Ser-473 (1:500, Cell Signaling cat. no. 4060S, predicted molecular weight 60 kDa, observed band ~60 kDa). Blots were then washed three times for 15 min in TBST, and then incubated for 1 h at room temperature (24 °C), with horseradish peroxide-conjugated goat antibody to rabbit (1:2000, Bio-Rad, Hercules, CA, USA) in TBST. After another three washes for 15 min with TBST, immunoreactivity was detected

using SuperSignal West Dura chemiluminescence detection reagent (Thermo Scientific, Waltham, MA, USA) and collected using HyBlot CL Autoradiography film (Denville Scientific, South Plainfield, NJ, USA) and a Kodak film processor. Net intensity values were determined using the Image Studio Lite (version 3.1). For normalization purposes, membranes were incubated with 0.125% coomassie stain for 5 min and washed three times for 5–10 min in destain solution.

Statistical analysis

The methamphetamine and saline self-administration data are expressed as active lever presses and methamphetamine data are also expressed as mean mg/kg methamphetamine self-administered per session. The effect of session duration on methamphetamine self-administration during the 1-h session, 6-h session and during the first hour of the 6-h session was examined over the 17 escalation sessions using a two-way repeated-measures analysis of variance (ANOVA; session duration × daily session) followed by Fisher's Least Significant Difference (LSD) post hoc test or Student–Newman–Keuls post hoc test. The pattern of responding for methamphetamine is expressed as the mean mg/kg per hour over 6-h sessions in LgA rats and were compared between the first and >10th escalation sessions. Differences in the rate of responding between the first and other escalation sessions were evaluated using the paired t-test. Differences in the rate of responding between the first and other saline sessions were evaluated using a one-way repeated-measures ANOVA. Western blotting analysis was conducted separately for

four groups, nonsaline controls, saline controls, limited access and extended access methamphetamine. Because protein levels were not significantly different between nonsaline and saline controls they were combined and were analyzed as methamphetamine naïve controls. Differences in density of proteins were analyzed by split-plot ANOVAs with methamphetamine access as a between-subject factor and hippocampal subregion as a within-subject factor. Following significant omnibus effects ($p < 0.05$), pairwise comparisons used Student's t-tests for split-plot ANOVAs. Data are expressed as mean ± SEM and were analyzed using SPSS or GraphPad Prism. Values of $p < 0.05$ were considered statistically significant. Graphs were generated using GraphPad Prism 5.0 software.

Results

Extended access to methamphetamine self-administration resulted in escalation of methamphetamine intake

Rats experienced saline or methamphetamine for 17 days (Fig. 1a). A repeated measures ANOVA did not detect a change in saline intake in control animals (Fig. 1c). A repeated measures two-way ANOVA detected a significant Days of Self-Administration × Methamphetamine Access interaction (Fig. 1c, d, $F_{16,192} = 6.323$, $p < 0.0001$) and an increase in methamphetamine intake across 17 sessions when analyzing both the first hour of the session (Fig. 1d, $F_{16,80} = 2.684$, $p < 0.01$)

and the total 6-h session ($F_{16,80} = 4.136$, $p < 0.0001$) in LgA animals. Post hoc analysis revealed a significant increase in methamphetamine intake during the 6 h session in LgA animals during Days 13–17 compared with Days 1–2. A repeated measures ANOVA did not detect a change in methamphetamine intake for ShA animals (Fig. 1c, d).

Limited and extended access methamphetamine self-administration increase BDNF expression but do not alter expression of TrkB or the phosphorylation of TrkB at Tyr-706

To determine whether limited access and extended access methamphetamine self-administration alters BDNF expression in the dorsal and ventral hippocampus, Western blot analyses were conducted on hippocampal protein lysates for methamphetamine naïve control animals, LgA, and ShA animals sacrificed 16–20 h after the last methamphetamine self-administration session. Protein levels of BDNF (mature form) were analyzed in the dorsal and ventral hippocampus-enriched regions in control, LgA and ShA animals (Fig. 1b). Data were subjected to an ANOVA to examine the effects of treatment (control vs. methamphetamine groups) across hippocampal subregion (dorsal vs. ventral). An ANOVA detected a significant main effect of methamphetamine access on BDNF expression ($F_{2,38} = 3.7$, $p = 0.03$), indicating that methamphetamine experience enhanced BDNF protein expression in the hippocampus. Unpaired-t-test demonstrated significant increases in BDNF protein levels in the LgA animals in the

dorsal and ventral hippocampus compared with drug naïve controls (p's = 0.02; Fig. 2).

Additionally, we determined whether methamphetamine self-administration altered total TrkB receptor expression and pTrkB expression in the dorsal and ventral hippocampus. Protein levels of TrkB (total and pTrkB at Tyr-706) were analyzed in the dorsal and ventral hippocampus-enriched regions in drug naïve controls, LgA and ShA animals. Data were subjected to ANOVAs to examine the effects of treatment (control vs. methamphetamine groups) across hippocampal subregion (dorsal vs. ventral) on total TrkB and pTrkB/TrkB expression. An ANOVA did not detect a significant main effect of methamphetamine or hippocampal region on total TrkB and pTrkB/TrkB expression (Fig. 3).

Extended access methamphetamine self-administration results in hypophosphorylation of GluN2B at Tyr-1472 in the ventral hippocampus

To determine whether methamphetamine self-administration altered total GluN2B receptor expression and pGluN2B expression in the dorsal and ventral hippocampus, protein levels of GluN2B (total and pGluN2B at Tyr-1472) were analyzed in the dorsal and ventral hippocampus-enriched regions in drug naïve control, LgA and ShA animals. Data were subjected to ANOVAs to examine the effects of treatment (control vs. methamphetamine groups) across hippocampal subregion (dorsal vs. ventral) on total and pGluN2B/GluN2B expression. An ANOVA did not detect a significant main effect of methamphetamine or hippocampal region

on total GluN2B expression. An ANOVA showed a significant main effect of methamphetamine access on pGluN2B/GluN2B ratio ($F_{2,38} = 3.2$, $p = 0.05$). Unpaired-t-test demonstrated a significant reduction in pGluN2B/GluN2B ratio in the ventral hippocampus in LgA animals compared with drug naïve controls ($p = 0.008$; Fig. 4).

Both limited and extended access methamphetamine self-administration increased anti-apoptotic protein levels and reduced pro-apoptotic protein levels in the ventral hippocampus.

We next determined whether methamphetamine self-administration alters expression of pro-apoptotic and anti-apoptotic factors in the dorsal and ventral hippocampus. Protein levels of Bcl-2 (anti-apoptotic) and Bax (pro-apoptotic) were analyzed in the dorsal and ventral hippocampus-enriched regions in controls, LgA and ShA animals. Data were subjected to two-way ANOVAs to examine the effects of treatment (control vs. methamphetamine groups) across hippocampal subregion (dorsal vs. ventral) on pro- and anti-cell death factor expression. An ANOVA detected a significant effect of hippocampal subregion on Bcl-2 expression ($F_{1,34} = 4.8$, $p = 0.03$; Fig. 5). Unpaired-t-test detected an increase in Bcl-2 protein levels in the ventral hippocampus of LgA rats ($p = 0.04$) compared with controls. An ANOVA detected a significant access × hippocampal subregion interaction ($F_{2,34} = 6.1$, $p = 0.001$), effect of hippocampal subregion ($F_{1,34} = 24.7$, $p < 0.001$), and effect of methamphetamine ($F_{2,34} = 4.4$, $p = 0.02$; Fig. 5) on Bax expression. Post hoc

analysis revealed a significant decrease in Bax protein levels in the ventral hippocampus of LgA animals ($p < 0.0001$) and ShA animals ($p = 0.01$) compared with controls.

Extended access methamphetamine self-administration reduces the expression of Akt in the ventral hippocampus

To determine whether methamphetamine self-administration altered total Akt expression and pAkt expression in the ventral hippocampus, protein levels of Akt (total and pAkt at Ser-473) were analyzed in the ventral hippocampus-enriched regions in drug naïve control, LgA and ShA animals. Data were subjected to one-way ANOVAs to examine the effects of treatment (control vs. methamphetamine groups) on total and pAkt/tAkt expression. An ANOVA showed a significant main effect of methamphetamine access on tAkt ($F_{2,21} = 4.29$, $p = 0.02$). Dunnett's multiple comparison test demonstrated a significant reduction in tAkt in the ventral hippocampus in LgA animals compared with drug naïve controls ($p < 0.05$; Fig. 6). An ANOVA did not detect a significant main effect of methamphetamine on pAkt or p/tAkt expression.

Discussion

Functional dissociation exists along the dorsal–ventral gradient in the rat hippocampus. For example, the ventral hippocampus when compared with the dorsal hippocampus has greater output connections with the prefrontal cortex and bed nucleus of the stria terminalis, and also has extensive reciprocal connections with the amygdala (Henke, 1990, Pitkanen et al., 2000 and Ishikawa and Nakamura, 2006), suggesting that neuroadaptations in the ventral hippocampus may be strongly associated with negative affective symptoms that promote drug dependence (Koob, 2003). Indeed, partial lesion and regional inactivation studies have proposed that the dorsal hippocampus is vital for spatial learning, whereas the ventral hippocampus is involved in regulating fear and anxiety (Moser et al., 1995, Kjelstrup et al., 2002, Bannerman et al., 2004, Pothuizen et al., 2004 and Pentkowski et al., 2006). In the context of drug-seeking behaviors, the dorsal hippocampus is particularly critical for acquisition of drug memories and reconsolidation of reactivated drug-related associative memories (Meyers et al., 2006 and Wells et al., 2011), suggesting that the act of drug taking and seeking produces neuroadaptive changes in the dorsal hippocampus that allow for long-term memory storage of drug-context memories, and allow the recall of associative memories during relapse. The ventral hippocampus, however, is important for reinstatement of drug-seeking behavior triggered by drug contexts, drug cues, or the drug itself (Rogers and See, 2007, Lasseter et al., 2010 and Deschaux et al., 2012), suggesting that neuroadaptive changes in the ventral hippocampus contribute to the motivational state that drives drug-seeking behavior during abstinence. Given the distinct, yet supporting roles of

the dorsal and ventral hippocampal regions in addiction, we examined the specific proteins implicated in hippocampal neuroplasticity in dorsal and ventral hippocampus-enriched tissue lysates.

Synaptic plasticity, and consequently memory formation, requires new protein synthesis (Kandel, 2001, Bruel-Jungerman et al., 2007 and Costa-Mattioli et al., 2009). Endogenous pro-BDNF is the precursor for mature BDNF and undergoes intracellular processing to be rapidly converted to the mature form, indicating that pro-BDNF is a transient biosynthetic intermediate (Matsumoto et al., 2008). BDNF, in its mature form is a prominent neurotrophin, mediating many enduring changes in neuroplasticity and synaptogenesis; it has been shown to play a key role as mediator of activity-induced LTP in the hippocampus (Bramham and Messaoudi, 2005, Lu et al., 2008, Minichiello, 2009, Park and Poo, 2013 and Leal et al., 2014). Upon high-frequency stimulation, endogenous hippocampal BDNF (is generated and) is secreted from axon terminals (presynaptic site) and dendrites (postsynaptic site) of glutamatergic principal neurons (hippocampal granule cells and pyramidal cells; (Lu, 2003)), in a calcium-dependent and GluN2B-dependent manner (Hartmann et al., 2001, Balkowiec and Katz, 2002, Gartner and Staiger, 2002 and Aicardi et al., 2004). Emerging evidence demonstrates that elevated endogenous BDNF is also required for the maintenance of high frequency stimulation-associated late-phase hippocampal LTP (Lu et al., 2008), suggesting that BDNF levels are low under normal conditions and are elevated by stimuli that enhance hippocampal activity. In this context, chronic methamphetamine exposure enhances baseline

hippocampal CA1 activity, increases excitability of dentate gyrus neurons, and increases paired pulse facilitation, and these alterations result in occlusion of hippocampal LTP development (Criado et al., 2000, Onaivi et al., 2002, Ishikawa et al., 2005, Hori et al., 2010, Swant et al., 2010 and North et al., 2013). Therefore, it can be hypothesized that methamphetamine induces the same neurobiological changes in the hippocampus that are required for generation and maintenance of hippocampal synaptic activity and additional activation as a consequence of tetanic stimulations in the hippocampus of methamphetamine experienced animals may not produce LTP (Hope, 1998, Schulz et al., 1999 and Onaivi et al., 2002).

In the context of the above hypothesis, our findings demonstrate that extended access methamphetamine self-administration enhances BDNF expression in the dorsal and ventral hippocampus that may result in a modification of the characteristics of synaptic transmission in the dorsal and ventral hippocampus (Scharfman et al., 2005). This finding is consistent with previous studies in amphetamine-experienced animals, in which drug exposure (experimenter-delivered) significantly enhanced BDNF protein levels in the hippocampus (Skelton et al., 2007 and Grace et al., 2008), but significantly extend the findings with methamphetamine self-administration, a paradigm that produces much lower levels of drug intake compared with high-dose experimenter-delivered paradigms. Upon release into the synaptic cleft, BDNF can bind to its receptor TrkB, localized at both pre- and postsynaptic sites of glutamatergic synapses in the hippocampus, and BDNF binding to TrkB induces receptor dimerization and phosphorylation at the

autophosphorylation site Tyr-706 (Drake et al., 1999 and Chao, 2003). Further analysis revealed that enhanced BDNF expression in the hippocampus in extended access animals did not concurrently alter expression of total TrkB or pTrkB-706. While the lack of effect of methamphetamine on total TrkB is consistent with previous studies using amphetamine treatments (Meredith et al., 2002 and McGinty et al., 2011), a possible explanation for no effect on pTrkB-706 could be that BDNF-mediated TrkB activation occurs in a time-dependent manner following BDNF release. For example, TrkB phosphorylation was not evident 15 min after a single amphetamine injection but was detected 2 h later, suggesting that there is a delicate relationship between BDNF release and TrkB activation (McGinty et al., 2011). Thus, it is possible that TrkB activation occurs at a different time point compared with the one used for the current study and should be further investigated. It is also possible that neuroadaptations have occurred in the hippocampus following methamphetamine self-administration, such that the typical neuronal activity-induced secretion of pro-BDNF and conversion of pro-BDNF to mature BDNF, and TrkB phosphorylation via BDNF release is hampered and therefore hinders hippocampal synaptic activity (LTP) that is dependent on TrkB activation (Aloyz et al., 1999, Binder et al., 1999, Pang et al., 2004 and Nagappan and Lu, 2005).

BDNF release in the hippocampus also enhances tyrosine phosphorylation of GluN2B subunits at Tyr-1472 of the NMDA receptor via TrkB-mediated phosphorylation, and this constitutes a critical step in mediating BDNF stimulation of synaptic transmission (Lin et al., 1998 and Caldeira et al., 2007). Furthermore, the

increase in GluN2B tyrosine phosphorylation elicited by BDNF is comparable to that seen after hippocampal LTP induction (Rosenblum et al., 1996 and Rostas et al., 1996), suggesting that BDNF-stimulated phosphorylation of GluN2B may provide a mechanism for trophin modulation of hippocampal LTP. The present results demonstrate that extended access methamphetamine self-administration reduces phosphorylation of GluN2B at Tyr-1472 in the ventral hippocampus. Functional changes in NMDARs are significantly linked with phosphorylation and dephosphorylation of GluN2B subunits (Ali and Salter, 2001, Nakazawa et al., 2001 and Nakazawa et al., 2006), with phosphorylation of GluN2B at Tyr-1472 regulating internalization of NMDARs via clathrin-mediated endocytosis, such that phosphorylation suppresses NMDAR endocytosis and dephosphorylation enhances NMDAR endocytosis (Lavezzari et al., 2003 and Prybylowski et al., 2005). Dephosphorylation of GluN2B by methamphetamine may thus prevent neuronal activity-induced potentiation of LTP in the hippocampus. Our observations add to the growing body of evidence indicating that modification of neuronal networks in the hippocampus by methamphetamine may play a role in altering hippocampal plasticity, which may partly underlie dysfunctional hippocampus-dependent behaviors.

Emerging evidence supports the beneficial role of BDNF against excitotoxicity-induced cell death in the hippocampus (Almeida et al., 2005). For example, BDNF prevents nitrous oxide (NO)-induced cell death in hippocampal neurons (Mattson et al., 1995 and Kume et al., 1997), and these effects are mediated

via BDNF-induced activation of extracellular signal-regulated kinase (ERK) 1/2 and phosphatidylinositol-3-kinase (PI3K)/Akt pathways (Skaper et al., 1998, Hetman et al., 1999, Han and Holtzman, 2000 and Almeida et al., 2005). Notably, hypophosphorylation of GluN2B at Tyr-1472 also reduces excitotoxicity-induced cell death in cortical neurons and these effects could be mediated via Akt activation (Chalecka-Franaszek and Chuang, 1999 and Hashimoto et al., 2002). Furthermore, Akt activation inhibits neuronal apoptosis by reducing the expression of pro-apoptotic protein Bax and enhancing the expression of anti-apoptotic protein Bcl-2 (Datta et al., 1997 and del Peso et al., 1997), suggesting that Akt activation by BDNF is critical for BDNF-induced increases in Bcl-2 and reduction in Bax (Almeida et al., 2005). In this context, methamphetamine has been reported to increase Akt activity in cultured hippocampal neurons and reduce apoptosis in these neurons (Rau et al., 2011), suggesting enhancement of neuronal survival in the hippocampus. Our findings extend this in vitro study to demonstrate that limited and extended access methamphetamine self-administration increases Bcl-2 and reduces Bax expression in the ventral hippocampus, suggesting enhancement of survival of hippocampal neurons in the ventral hippocampus. These data also suggest that alterations in cell death proteins by methamphetamine are not predicted by the amount of methamphetamine consumed. Analysis of Akt expression and Akt activity via pAkt expression suggests that methamphetamine self-administration induced increases in Bcl-2 and reduction in Bax expression in the ventral hippocampus did not correlate with increases in Akt expression, whereas, these changes in the extended

access animals were associated with reduced Akt expression. One of the most compelling explanations for the differences observed in our study versus the in vitro study is that we examined the effects of methamphetamine under self-administration conditions. It is intriguing to consider the possibility that the effects of methamphetamine on Akt expression observed here are neurotoxic and are not associated with neuroprotection. Indeed, the Akt signaling cascade via phosphorylation at Ser-473 has been identified as being crucial to the induction of protein synthesis-dependent synaptic plasticity required for hippocampus-dependent learning and memory processes (Opazo et al., 2003, Shehadah et al., 2010 and Zhang et al., 2010), and a reduction in Akt expression in extended access animals suggests reduction in synaptic plasticity (Swant et al., 2010). Therefore, the ability of methamphetamine to enhance BDNF expression, produce hypophosphorylation of GluN2B, together with increases in Bcl-2 and reductions in Bax and Akt, suggests that methamphetamine-induced activation of signaling in the hippocampus may function to transduce the BDNF-initiated survival signal in the ventral hippocampal neurons to assist with maladaptive hippocampal plasticity that contributes to the cognitive and motivational states that drive methamphetamine addiction. Future mechanistic studies with these candidate proteins will determine their involvement in the behavioral deficits that are associated with extended access methamphetamine self-administration.

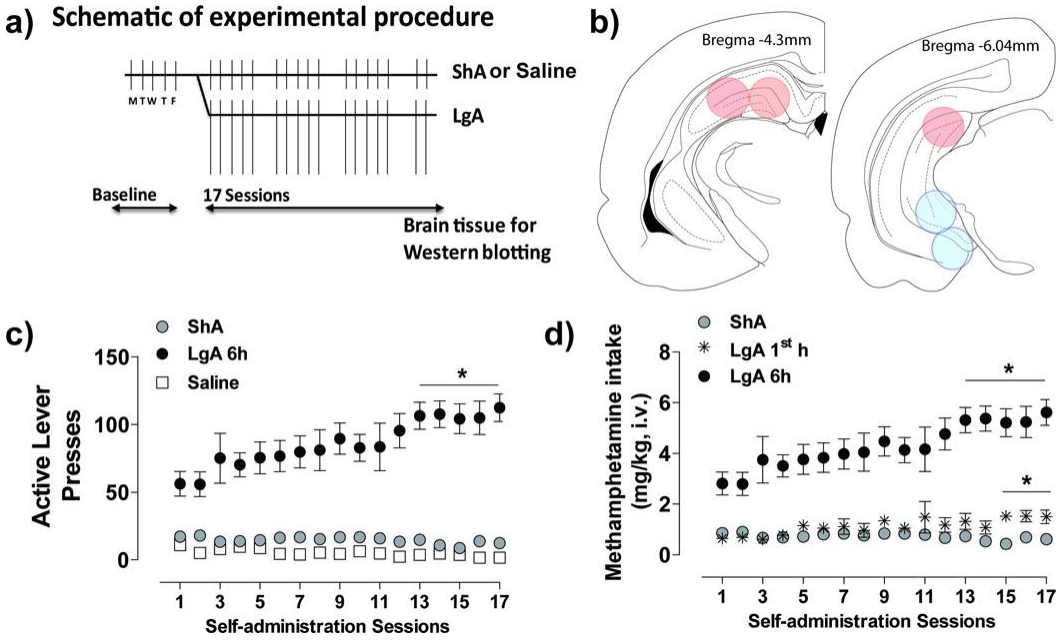

Figure 2.1: Rats that underwent extended access to methamphetamine self-administration escalated methamphetamine intake. (**a**) Schematic showing baseline training followed by 17 sessions of either 1 hr/day saline (n = 3), 1hr/day (ShA, n = 8; short vertical lines) or 6 hr/day (LgA, n = 6; long vertical lines) methamphetamine self-administration. (**b**) Schematic of brain regions indicating area of tissue punches used for Western blot analysis. Red circles, dorsal hippocampus; blue circles, ventral hippocampus. (**c–d**) Active lever presses for saline and methamphetamine (c) and methamphetamine intake in mg/kg per session from ShA and LgA animals (d). The first hour intake is also indicated for LgA animals. *p< 0.01 compared with initial sessions in the same animals by repeated measures two-way ANOVA followed by followed by Student-Newman-Keuls *post hoc* test. Data are represented as mean ± SEM.

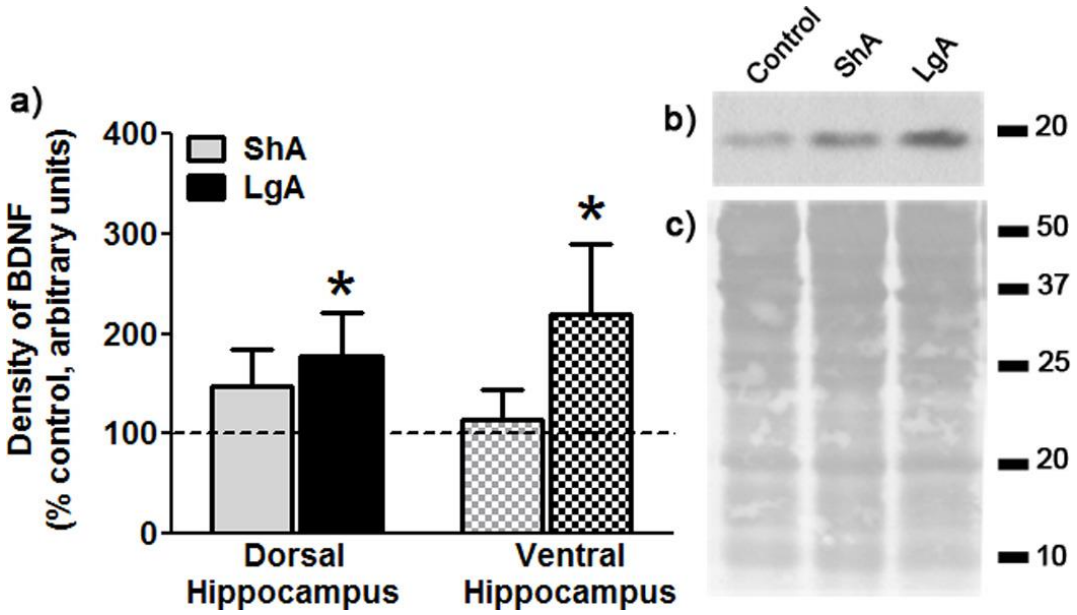

Figure 2.2: Methamphetamine self-administration enhances BDNF expression in the dorsal and ventral hippocampus. (**a**): Quantitative analysis of BDNF expression (mature form) by Western blot analysis of dorsal and ventral hippocampus-enriched protein lysates. (**b**): Qualitative representation of BDNF immunoreactive bands used for quantitative data analysis from dorsal hippocampus; (**c**) corresponding Coomassie staining of the membrane is shown as loading control. Molecular weights of proteins are indicated in kDa. *p<0.05 compared to naïve controls. Data are represented as mean + SEM.

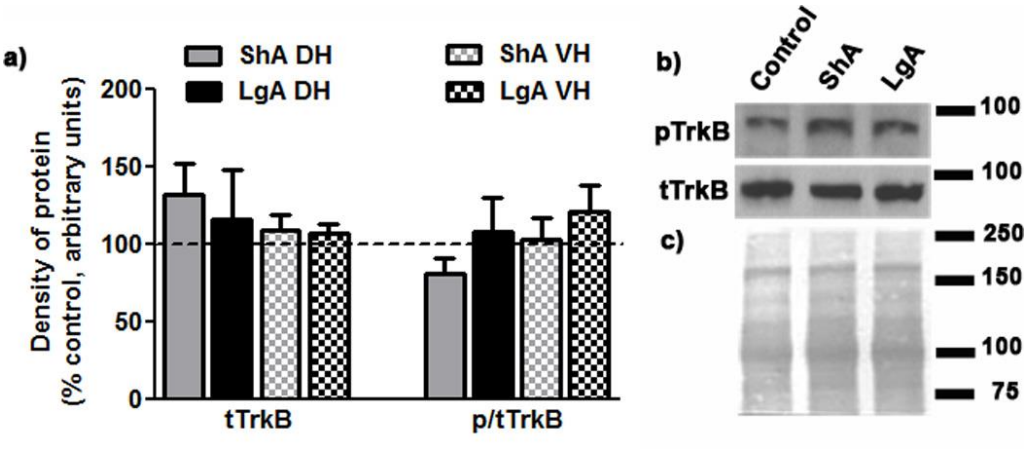

Figure 2.3: Methamphetamine self-administration does not alter TrkB expression or pTrkB levels in the hippocampus. (**a**): Quantitative analysis of TrkB and pTrkB/TrkB expression by Western blot analysis of dorsal and ventral hippocampus-enriched protein lysates. (**b**): Qualitative representation of phospho (p)TrkB and total (t)TrkB immunoreactive bands used for quantitative data analysis from dorsal hippocampus; (**c**) corresponding Coomassie staining of the membrane is shown as loading control. Molecular weights of proteins are indicated in kDa.

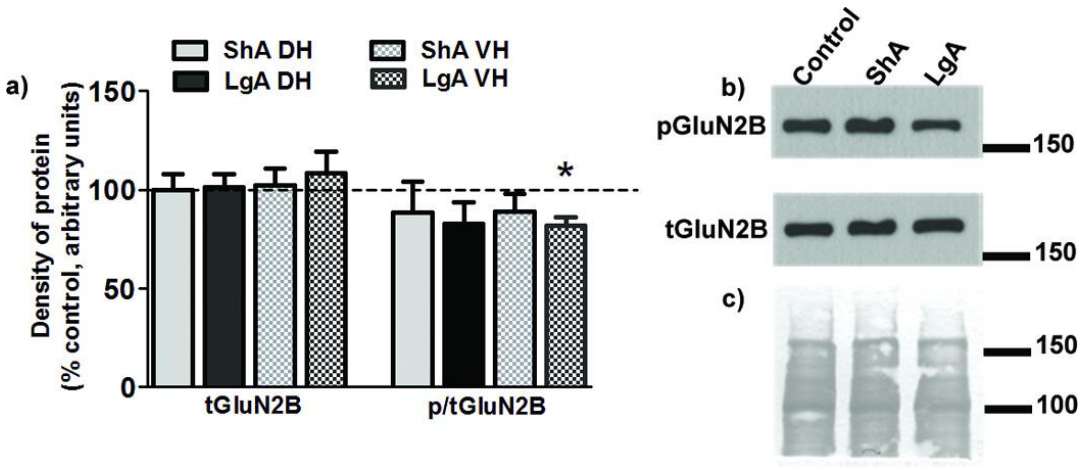

Figure 2.4: Methamphetamine self-administration produces hypophosphorylation of GluN2B receptors at Tyr-1472 in the ventral hippocampus. (**a**): Quantitative analysis of total (t)GluN2B and phospho (p)GluN2B/tGluN2B expression by Western blot analysis of dorsal and ventral hippocampus-enriched protein lysates. (**b**): Qualitative representation of pGluN2B and tGluN2B immunoreactive bands used for quantitative data analysis. (**c**) corresponding Coomassie staining of the membrane is shown as loading control. Molecular weights of proteins are indicated in kDa. *$p<0.05$ compared to naïve controls. Data are represented as mean + SEM.

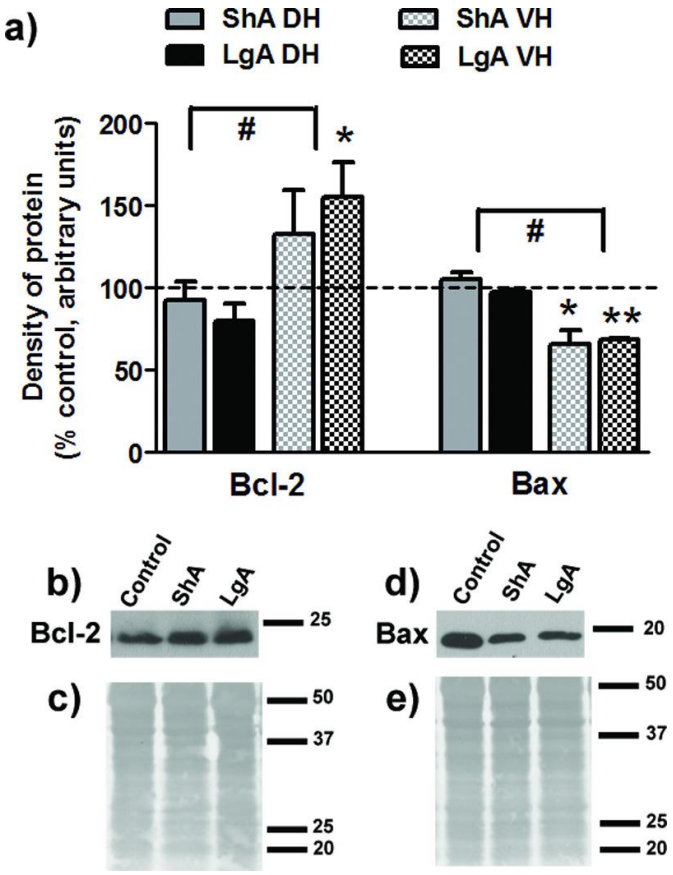

Figure 2.5: Methamphetamine self-administration regulates cell death factor expression in the ventral hippocampus. (**a**): Quantitative analysis of Bcl-2 and Bax expression by Western blot analysis of dorsal and ventral hippocampus-enriched protein lysates. (**b–c**): Qualitative representation of Bcl-2 immunoreactive bands used for quantitative data analysis (**b**); corresponding Coomassie staining of the membrane used as loading control (**c**). (**d–e**): Qualitative representation of Bax immunoreactive bands used for quantitative data analysis (**d**); corresponding Coomassie staining of the membrane used as loading control (**e**). Molecular weights of proteins are indicated in kDa. *p<0.05 compared to naïve controls; **p<0.01 compared to naïve controls; #p<0.01 main effect of hippocampal subregion. Data are represented as mean + SEM.

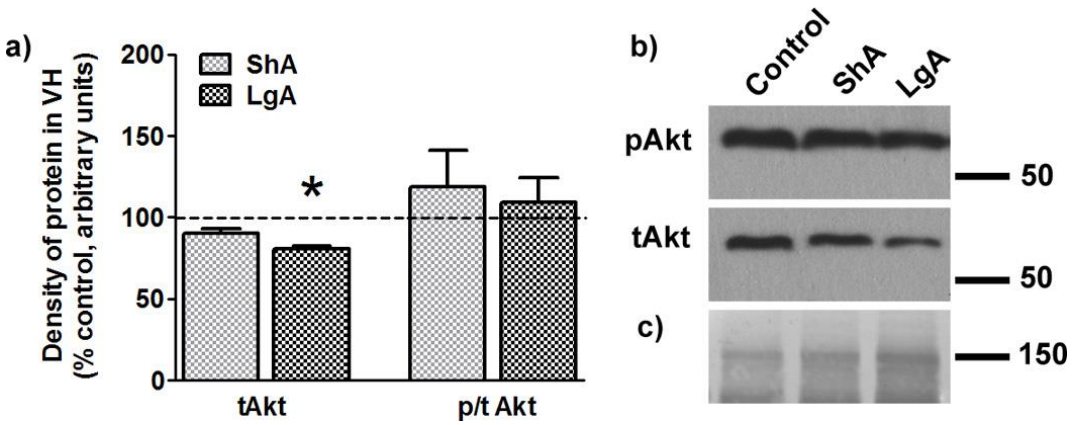

Figure 2.6: Methamphetamine self-administration reduces Akt levels in the ventral hippocampus. (**a**): Quantitative analysis of total (t)Akt and phospho (p)Akt/tAkt expression by Western blot analysis of ventral hippocampus-enriched protein lysates. (**b**): Qualitative representation of pAkt and tAkt immunoreactive bands used for quantitative data analysis. (**c**) corresponding Coomassie staining of the membrane is shown as loading control. Molecular weights of proteins are indicated in kDa. *p<0.05 compared to naïve controls. Data are represented as mean + SEM.

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CHAPTER 3:

INHIBITION OF WITHDRAWAL-INDUCED NEUROGENESIS IN THE DENTATE GYRUS BLOCKS METHAMPHETAMINE RELAPSE

Abstract

Adult neurogenesis in the dentate gyrus (DG) is strongly influenced by drug taking behavior and may have a role in the etiology of drug seeking behavior. However, mechanistic studies on the relationship of neurogenesis on drug seeking are limited. Outbred Wistar rats experienced extended access methamphetamine self-administration and individual differences in drug taking defined animals with higher preferred and lower preferred levels of drug intake. Forced abstinence from higher preferred levels of drug taking enhanced neurogenesis and neuronal activation of granule cell neurons (GCNs) in the DG and produced compulsive-like drug reinstatement. Systemic treatment with the drug Isoxazole-9 (a synthetic small molecule known to modulate neurogenesis in adult rodent brain) during abstinence blocked compulsive-like context-driven methamphetamine reinstatement. Isoxazole-9 modulated neurogenesis, neuronal activation and structural plasticity of GCNs, and expression of synaptic proteins associated with learning and memory in the DG. These findings identify a subset of newly born GCNs within the DG that could directly contribute to drug-seeking behavior. Taken together, these results support

a direct role for the importance of adult neurogenesis during abstinence in compulsive-like drug reinstatement.

Introduction

Methamphetamine addiction is a serious public health problem and the rate of recovery from methamphetamine addiction is extremely low. One aspect of addiction, compulsive drug seeking, can be modeled in rodents with extended access to methamphetamine [1]. One distinct advantage of these models is that the neuroplasticity in neurobiological function can be delineated in rodents based on individual differences in drug seeking animals [2-4]. These findings demonstrate that animals with preferentially higher levels of drug intake are powerful models for identifying neurobiological factors involved in the acquisition, maintenance and risk of relapse and provide a means to increase our understanding of addiction-like behaviors using rodent models.

Functional granule cell neurons (GCNs) are generated in the granule cell layer (GCL) of the dentate gyrus (DG) of the hippocampus throughout life by a multistep process called neurogenesis [5, 6]. The process of neurogenesis involves stem-like precursor cells that proliferate into preneuronal progenitors, which in turn differentiate into immature neurons and eventually mature into GCNs [7]. GCNs generated during adulthood assist with neuronal turnover [8]. Computational and behavioral models combined with electrophysiological findings indicate that the DG

participates in an array of behaviors to assist with hippocampal dependent spatial memory [9]. For example, GCNs in the DG communicate with CA3 neurons, mossy cells and hilar interneurons to modulate interference between similar spatial inputs via cognitive discrimination. Furthermore, newly born GCNs modulate sparseness of activity of pre-existing GCNs through recruitment of feedback inhibition, and via adaptive changes to DG network excitability affect and strengthen cognitive discrimination [10, 11]. Neurogenesis may also enable animals to distinguish related stimuli and events rapidly and support contextual discrimination. In addition to their role in discrimination, new evidence supports the functional significance of neurogenesis in hippocampal memory clearance [12, 13], suggesting that endogenous alterations in neurogenesis and DG excitability could contribute to memory-related disorders [14].

Neuroanatomical studies in the hippocampus support segregation of neuronal outputs along the dorso-ventral axis whose connectivity may influence the expression of neurogenesis in the DG and behavior dependent on the hippocampus [15, 16]. For example, the dorsal hippocampus has higher levels of neurogenesis and is vital for spatial learning, and is particularly critical in mediating contextual discrimination [17]. In contrast, the ventral hippocampus has lower levels of neurogenesis and is strongly associated with negative affective symptoms that promote propensity for reinstatement of drug seeking [18, 19]. Similar functional differences have been noted along the septo-temporal axis of the hippocampus in humans, with ventral hippocampus demonstrating greater activity in response to

negative affective symptoms [20]. Given the functional distinction between the dorsal and ventral hippocampal regions, the role of neurogenesis and hippocampal synaptic events along the dorsal-ventral gradient in regulating contextual discrimination should be investigated.

In the context of substance and alcohol use disorders, it is predicted that hippocampal neurogenesis protects the neural and behavioral plasticity suppressed by drugs of abuse and alcohol. For example, reinforcing doses of stimulants, opiates and alcohol during drug taking suppress proliferation, differentiation and survival of neural progenitors. Forced abstinence from stimulants and alcohol stimulate proliferation and enhance survival of neural progenitors, suggesting a rebound effect [21]. These findings demonstrate that suppression of, and stimulation of neurogenesis are being observed at various stages of substance abuse disorders, and it has been hypothesized that spontaneous neurogenesis during forced abstinence may block memories associated with the contextual reinstatement of drug seeking or promote extinction learning [22]. However, the reduction in spontaneous neurogenesis during self-administration and robust rebound in neural progenitors and neurogenesis during abstinence are associated with enhanced propensity for reinstatement in methamphetamine experienced animals [23], suggesting reinforcement of drug memories by enhanced neurogenesis during abstinence. This led us to hypothesize that spontaneous neurogenesis during abstinence may produce productive effects on reinstatement of drug seeking by strengthening drug associated memories (enhance contextual discrimination

between drug-paired context and drug-unpaired context and prevent memory clearance), and inhibiting or preventing neurogenesis during abstinence would clear drug associated memories and reduce the efficacy of context-driven reinstatement. To test this hypothesis, a synthetic small molecule isoxazole-9 (Isx-9; [N-cyclopropyl-5-(thiophen-2-yl)isoxazole-3-carboxamide]; [24-26]) was administered during forced abstinence to evaluate the efficacy of the molecule in modulating the neurogenesis response during abstinence, and in reducing context-driven reinstatement of drug seeking.

Materials and Methods

Detailed methods are provided for all behaviors and procedures conducted in the supplementary methods section. One hundred-fifty two, adult male Wistar rats (Charles River), weighing 200-250 g (8 weeks old) at the start of the experiment were used for the study. All procedures were approved by the IACUC at The Scripps Research Institute. One hundred and nine rats underwent surgery for catheter implantation for intravenous self-administration (Supplementary methods). Following 4 days of recovery after surgery, ninety-nine animals were trained to lever press for i.v. infusions of methamphetamine (0.05 mg/kg per infusion) and ten animals were trained to lever press for i.v. infusions of saline (0.9%) in an operant chamber (context A) on an FR1 schedule for 6 hours per

session for 17 sessions. Some animals were trained to self-administer sucrose (context A, oral, 10%w/v). After 17 sessions of methamphetamine or sucrose self-administration animals experienced forced abstinence for 24 days. During abstinence animals received one i.p. injection of isoxazole-9 or vehicle (25% HBC; Supplementary methods) each day, starting on day 1 of abstinence and continued injections for 12 days into abstinence [26-28] (treatment was based on an in vivo study [27]). The day after last isoxazole-9 injection, a subset of animals received one i.p. injection of BrdU (150 mg/kg) or i.c. injection of mCherry retrovirus (Supplementary methods). Animals then experienced extinction (context B) and reinstatement (context A) sessions (Supplementary methods). One hour after reinstatement session animals were euthanized and brain tissue was processed for histology, Western blotting analysis, and cellular quantification (Supplementary methods). Statistical analysis were conducted using one-way, two-way ANOVAs followed by Student-Newman-Keuls post hoc test (Supplementary methods).

Results

Extended access methamphetamine self-administration in 99 outbred adult Wistar rats demonstrates high and low preferred intake in methamphetamine.

Animals were separated into compulsive-like responders (high responders, HR) and noncompulsive-like responders (low responders, LR) based on escalation

criteria (defined as greater than 150 % change in active lever (reinforced) responses during sessions 13-17 compared with sessions 1-5 after a median split analysis on their reinforced lever responses during sessions 13-17. HR have higher responding on reinforced (active) levers indicated by a significant methamphetamine group x active lever responses interaction $F(16,1581)=8.2$, main effect of methamphetamine group $F(1,1581)=608.4$ and number of active lever presses $F(16,1581)=10.2$ by two-way ANOVA, $P<0.01$ (Figure 1A-B). HR have higher responding on the non-reinforced (inactive) levers compare to LR (no interaction, no effect of days, significant effect of methamphetamine group $F(1,1585)=88.3$, $P<0.01$; Figure 1C; Supplementary Figure 1A-B). HR have higher responding on the active levers during the 1st hour of the 6 hour session, indicated by a significant methamphetamine group x active lever responses interaction $F(16,1456)=3.3$, main effect of methamphetamine group $F(1,1456)=289.9$ and number of active lever presses $F(16,1456)=4.296$ $P<0.01$ by two-way ANOVA (Figure 1D). HR exhibit an upward shift in peak self-administration rates, and a rightward shift in the descending limb of the self-administration dose–response curve compared to LR indicated by a significant methamphetamine group x dose interaction $F(3,42)=4.018$, main effect of methamphetamine group $F(1,14)=11.62$ and number of active lever presses $F(3,42)=12.96$ $P<0.01$ by two-way ANOVA (Figure 1E). Methamphetamine self-administration data converted to dose-intake curves shows that HR take greater daily amounts of methamphetamine at doses on the descending limb of the dose–response curve compared to LR, $P<0.01$ (Figure 1F). HR

demonstrate higher escalation in drug self-administration ($t=5.46$, $df=61.08$, $P<0.0001$ by unpaired t test; Figure 1G). HR demonstrate uncontrolled responding during time-out indicated by main effect of methamphetamine group $F(1,1536)=250.4$, $P<0.0001$ by two-way ANOVA, indicating inability to suppress unrewarded behavior, while maintaining the ability to discriminate reinforced active lever responses from non-reinforced inactive lever responses (Supplementary Figure 1C-D). HR demonstrate higher motivation to seek methamphetamine as measured by infusions earned during self-administration on a progressive ratio schedule ($t=2.4$, $df=13$, $P<0.05$ by unpaired t test; Figure 1H). Amount of meth measured by mass spectroscopy in the hippocampus following meth challenge (0.4 mg/kg, i.v.) showed no significant differences between HR and LR (Figure 1I). Plasma corticosterone levels collected at two time points during animals' dark cycle before initiation of self-administration and after completion of the session on the same day shows no difference between HR and LR, however, demonstrate a significant increase in basal corticosterone levels in HR and LR compared to saline self-administering animals ($F=13.1$, $P<0.01$; Figure 1J).

Synthetic small molecule Isoxazole-9 reduces drug seeking during context- and cue-induced reinstatement in abstinent HR.

Isx-9 was synthesized according to [26], and pharmacokinetic studies indicate that systemic injections of the compound crosses the blood-brain barrier

and into the hippocampus with a half-life of 29.2 minutes (Supplementary Figure 2A-C). To determine whether Isx-9 itself produced any confounding behavioral responses we treated a separate set of drug and behavior naïve rats with Isx-9 (Supplementary Figure 3A) and investigated the potential effects of Isx-9 in functional observational battery tests. Isx-9 treatment did not alter body weight, locomotor activity and sensory/motor reflex responses (Supplementary Figure 3B-D; all Ps>0.05).

Prior to initiation of Isx-9 administration (vehicle [HBC; (2-hydroxypropyl)-β-cyclodextrin] and Isx-9 rats were matched for self-administration behavior (Figure 2B). HBC-HR responded higher than HBC-LR during days 1-3 of extinction (significant methamphetamine group x extinction days interaction $F(5,210)=3.027$, main effect of extinction days $F(5,210)=29.61$ and methamphetamine group $F(1,42)=7.339$, $P<0.001$ by two-way ANOVA; Figure 2E,F). Isx-9-HR responded higher than Isx-9-LR during days 1-3 of extinction (no significant interaction, main effect of extinction days $F(5,175)=24.05$ and methamphetamine group $F(1,35)=7.165$, $P<0.001$ by two-way ANOVA). Lever responses on the previously paired active levers were higher than inactive levers in HBC-HR, HBC-LR and Isx-9-LR animals ($P<0.05$). HBC-HR responded higher than HBC-LR and Isx-9-HR during context-driven reinstatement (significant methamphetamine group x lever responses interaction $F(6,154)=6.215$, $P<0.001$; main effect of reinstatement $F(2,154)=30.24$, $P<0.01$; and methamphetamine group $F(3,77)=4.710$, $P<0.001$ by repeated measures two-way ANOVA; Figure 2G). Lever responses on the previously

paired active levers were higher than inactive levers in HBC-HR ($P<0.05$). HBC-HR responded higher than HBC-LR and Isx-9-HR during contextual cued reinstatement (significant meth group x lever responses interaction $F(6,154)=4.911$, $P<0.001$; main effect of reinstatement $F(2,154)=20.88$, $P<0.01$; and meth group $F(3,77)=7.798$, $P<0.001$ by repeated measures two-way ANOVA; Figure 2H).

Isoxazole-9 produced distinct alterations in neurogenesis and neuronal activation of GCNs in the dorsal and ventral DG in abstinent HR.

17-day-old 5-bromo-2'-deoxyuridine (BrdU) cells in HBC and Isx-9 HR and LR were examined in the GCL immediately after the reinstatement session to quantify the number of newly born GCNs (BrdU colabeled with neuronal marker neuronal nuclease, NeuN), and activation of newly born GCNs (BrdU colabeled with NeuN and Fos). Brain tissue was also processed for Ki-67 to quantify proliferation of newly born progenitors and Fos to quantify activation of GCNs that were preexisting relative to newly born (BrdU) GCNs. Isx-9 controls had higher number of BrdU and Ki-67 cells compared to HBC controls; Isx-9-HR had reduced number of BrdU and Ki-67 cells compared with HBC-HR and similar number of cells compared to HBC controls. Two-way ANOVA demonstrated a significant methamphetamine group x Isx-9 interaction $F(3,35)=4.111$, main effect of methamphetamine $F(3,35)=3.437$ and main effect of Isx-9 $F(1,35)=5.694$ on BrdU (Figure 3C; $Ps<0.01$), and a significant methamphetamine group x Isx-9 interaction $F(2,31)=6.092$ on Ki-67 cells

(Figure 3D; P<0.01). Isx-9-HR and -LR had similar number of activated caspase-3 cells compared with HBC-HR and -LR and controls (Supplementary Figure 4A). Isx-9 controls had similar ratio of the phenotype of BrdU cells compared to HBC controls. Isx-9-HR and -LR had similar ratio of the phenotype of BrdU cells compared to HBC-HR and -LR and controls. Two-way ANOVA demonstrated a main effect of methamphetamine group $F(5,72)=5.69$ and main effect of phenotype $F(2,72)=62.9$ in dorsal BrdU cells (Figure 3G; P<0.001), and a significant methamphetamine group x Isx-9 interaction $F(10,69)=2.1$, main effect of methamphetamine group $F(5,69)=5.4$ and main effect of phenotype $F(2,69)=52.04$ in ventral BrdU cells (Figure 3H; P<0.001). Quantitative analysis showed higher number of nonneuronal BrdU cells in dorsal GCL and higher number of neuronal BrdU cells in dorsal and ventral GCL in Isx-9 controls compared to HBC controls (Figure 3G, H). HBC-HR had higher number of neuronal BrdU cells compared to HBC controls in dorsal and ventral GCL (Ps<0.05). Isx-9 controls did not have higher number of activated BrdU cells in dorsal and ventral GCL compared to HBC controls. Isx-9-HR had reduced number of activated BrdU cells in dorsal GCL compared to HBC-HR (P<0.05; Figure 3G).

Quantitative analysis of Fos expressing GCNs demonstrated an increase in the number of activated cells in the dorsal and ventral GCL in HBC-HR and -LR compared to controls and a reduction in the number of activated cells in Isx-9-HR in the ventral GCL compared to HBC-HR (main effect of methamphetamine in the dorsal hippocampus $F(2,24)=10.96$, P<0.01; a significant interaction $F(2,22)=3.863$

and main effect of methamphetamine in the ventral hippocampus $F(2,22)=7.759$, $P<0.01$ by two-way ANOVA; Figure 3K).

The number of BrdU cells were quantified in the prefrontal cortex, a brain region where newly born progenitors mostly differentiate into oligodendrocyte progenitors [29]. We demonstrate that Isx-9's effects were attributable to specific effects in the GCNs as no alterations were found in gliogenic progenitors in the medial prefrontal cortex (Supplementary Figure 7).

Isoxazole-9 produced alterations in structural plasticity of pre-existing and newly born GCNs in the dorsal DG.

17-day-old retrovirus expressing mCherry (mCherry) cells in HBC and Isx-9 HR and LR were examined in the GCL immediately after the reinstatement session to quantify the dendritic structure of newly born GCNs. Brain tissue was also processed for Golgi-Cox analysis to examine structural alterations in GCNs that were preexisting relative to newly born (mCherry) GCNs. Isx-9-HR showed enhanced structural plasticity of mCherry and Golgi-Cox GCNs compared to HBC-HR, indicated by enhanced spine density and dendritic extent (main effect of Isx-9 by two-way ANOVA $F(1,103)=7.543$, $P<0.01$, Figure 4G), dendritic extent of Golgi-Cox GCNs (main effect of Isx-9 by two-way ANOVA $F(1,127)=7.249$; $P<0.01$, Figure1H) and mCherry GCNs (main effect of methamphetamine by two-way ANOVA $F(2,125)=7.138$, $P<0.01$, Figure 1I). 3D Sholl analyses of Golgi-Cox and mCherry

GCNs demonstrated a significant neuron type x distance from soma interaction ($F(15,690)=7.166$, $P<0.01$, Figure 4J). 3D Sholl analyses of Golgi-Cox GCNs demonstrated main effect of methamphetamine ($F(5,386)=6.506$, $P<0.05$) and distance from soma ($F(15,386)=43.40$, $P<0.01$, Figure 4K). 3D sholl analyses of mCherry GCNs demonstrated main effect of methamphetamine ($F(5,559)=10.97$, $P<0.01$) and distance from soma ($F(15,559)=34.83$, $P<0.01$, Figure 4L).

Isoxazole-9 alters the expression of synaptic plasticity proteins in the dorsal and ventral DG.

Brain tissue was snap frozen one hour after the reinstatement session and micropunches enriched in the dorsal and ventral DG GCNs were separated and homogenized and cytoplasm enriched fractions were processed for immunoblotting. Isx-9-HR showed reduced density of total N-methyl-D-aspartate (NMDA) glutamate receptor (GluN), GluN2B, and enhanced expression of phosphorylated GluN2B in dorsal DG without producing any changes in the ventral DG (Dorsal dentate gyrus: tGluN2B- main effect of Isx-9: $F(1,41)=4.741$, $P<0.05$; pGluN2B- main effect of methamphetamine $F(2,41)=4.218$, $P<0.05$; main effect of Isx-9 $F(1,41)=9.845$, $P<0.01$, Figure 5E). Isx-9 controls showed higher levels of phosphorylated Ca2+/calmodulin (CaM)-dependent protein kinase (CaMK), pCaMKII, in the dorsal and ventral DG compared to HBC controls. Isx-9-HR and -LR showed higher levels of pCaMKII in dorsal DG compared to HBC-HR and -LR. Isx-9-HR showed lower levels of pCaMKII in ventral DG compared to HBC-HR (Dorsal DG: pCaMKII- main effect of

Isx-9 $F(1,43)=13.10$, $P<0.001$, Figure 5G; Ventral DG: pCaMKII- methamphetamine group x Isx-9 treatment interaction $F(2,38)=6.561$, $P<0.01$; main effect of methamphetamine $F(2,38)=6.273$, $P<0.01$, Figure 5H). Isx-9-HR showed higher levels of phosphorylated class IIa histone deacetylase (HDAC5) in dorsal DG compared to HBC-HR (pHDAC5- methamphetamine group x Isx-9 treatment interaction $F(2,40)=3.820$, $P<0.05$, Figure 5I).

Discussion

Compulsive-like Methamphetamine Intake Predicts Higher Reinstatement of Methamphetamine Seeking

We demonstrate compulsive-like behavior in HR self-administering intravenous infusions of methamphetamine in an extended access schedule of reinforcement. This was evident as increased responding during self-administration sessions and during time-out periods, increased peak self-administration rates and responding during progressive-ratio schedules reflecting enhanced motivation for methamphetamine [2]. These behavioral differences between HR and LR cannot be explained by differential metabolism or bioavailability of methamphetamine, or differences in circadian dependent corticosterone release by methamphetamine. These findings demonstrate that HR are powerful models for identifying neurobiological factors involved in determining risk for relapse and will improve

our understanding of addiction-like behavior with regard to its translational value to human addiction.

To determine differences in propensity for relapse, HR and LR were withdrawn from methamphetamine and after a period of protracted abstinence (22 days; a timeframe required for preneuronal progenitor cells to become GCNs), all animals were tested for reinstatement of drug seeking in an A-B-A self-administration-extinction-reinstatement paradigm [30]. HR demonstrated significantly greater drug seeking behavior (as defined by lever pressing) during extinction compared to LR. However, responding was significantly reduced in both HR and LR rats, reaching equivalent levels of performance after 6 days of extinction training. Following extinction, reinstatement of drug-seeking behavior was tested by re-exposing the animal to the training drug context (context A) without cues (context only) or with cues (context A + cues). HR exhibited greater methamphetamine-seeking behavior on the previously associated drug-paired lever compared to LR despite the continued absence of the drug reinforcer. These findings provide further validity to the enhanced compulsive-like drug seeking observed in HR, as they demonstrated enhanced propensity for reinstatement after protracted abstinence via enhanced contextual discrimination.

Isoxazole-9 Reduces Reinstatement of Methamphetamine Seeking in HR

Robust modulation of neurogenesis is achieved by pharmacological agents (e.g., antidepressants, anticonvulsants, synthetic small molecules [31]). In vitro

studies demonstrate that the synthetic small molecule Isx-9 triggers release of intracellular calcium (Ca2+), specifically in neuronal progenitor cells via high-voltage Ca2+ channels and GluNs, suggesting neurotransmitter-like properties selective to this cellular population [24, 26, 28]. Increases in intracellular Ca2+ by Isx-9 produce cellular excitation in progenitor cells that drives expression of genes and epigenetic factors such as HDAC5 via CaMK activity to direct the phenotype of progenitors into neurons [26, 32]. Isx-9 also increases neurogenesis in vivo [25, 27]. These findings suggest that Isx-9 can modulate excitatory neurotransmission and synaptic plasticity, particularly in the hippocampus via GluNs [26], and mediate synaptic events in the hippocampus and long-term memory storage dependent on the hippocampus via CaMKII [33]. Notably, Isx-9 produces growth arrest by inhibiting cell differentiation in cells selectively sensitive to disturbances in Ca2+ homeostasis (e.g., cells born during hyperglutamatergic state or cells expressing altered GluNs), providing a potential target mechanism for reducing cells of an immature progenitor status with compromised function [28]. Therefore, Isx-9 could be used to modulate neurogenesis and thereby influence contextual discrimination by either increasing or reducing the available pool of adult generated neurons to alter their capacity for information processing.

To investigate the role of Isx-9 in context-driven reinstatement, we injected HR and LR with Isx-9. Following extinction, Isx-9 treated HR failed to reinstate drug-seeking responding compared to vehicle treated HR. These findings demonstrate

that Isx-9 reduces propensity for reinstatement, possibly by promoting methamphetamine context-specific extinction learning mechanisms.

Isx-9 reduces the neuronal activation of newly born GCNs in HR

We studied Isx-9-induced alterations in the number of newly born GCNs and activation of GCNs that could be associated with reduced methamphetamine seeking. Quantification of BrdU cells revealed that Isx-9 increased the number of cells in methamphetamine naïve and sucrose self-administering animals. In methamphetamine treated animals, forced abstinence increased the number of BrdU cells by 70% and 52% in vehicle treated HR, relative to both methamphetamine naïve controls and LR. Isx-9 treatment in methamphetamine treated animals did not increase the number of BrdU cells during forced abstinence compared to vehicle treated controls, and this effect could be due to a hostile cellular environment in the progenitor pool created during methamphetamine experience [34]. For example, methamphetamine experience reduces net proliferation of progenitors and immature neurons by reducing the number of proliferating preneuronal neuroblasts and increasing the number of proliferating preneuronal progenitor cells [34], suggesting that a decrease in the number of progenitors and immature neurons, to a large degree, is attributable to the decrease in the ability of neuroblasts to divide and produce stable progenitor cells that survive as immature neurons [34, 35]. However, abstinence from methamphetamine experience increases net proliferation of progenitors and

survival of newly born GCNs, suggesting that cell intrinsic signals that maintain cell proliferation are differentially regulated during abstinence from the drug [23]. The increases in net proliferation and survival during abstinence observed in vehicle treated HR were not evident in Isx-9 treated HR when compared to drug naïve controls. Taken together, while the effects of Isx-9 on proliferation and survival in methamphetamine naïve and methamphetamine treated animals are quantitatively different, further investigation is required to determine the cellular mechanism underlying the difference. Labeling for activated caspase-3 and analyses of pro- and anti-cell death factors indicated that the higher number of BrdU cells in vehicle treated HR and similar number of BrdU cells in Isx-9 treated HR compared to drug naïve controls was not correlated with alterations in apoptosis.

Isx-9 increased neurogenesis in the dorsal and ventral GCL, and enhanced generation of nonneuronal cells in the dorsal GCL compared to vehicle treated controls. Colabeling analysis of BrdU with Fos demonstrated neuronal activation of BrdU GCNs, however, context-driven reinstatement did not alter the activity of BrdU GCNs in vehicle treated HR and LR compared to controls. Notably, Isx-9 treatment reduced the activity of dorsal BrdU GCNs in HR compared to vehicle treated HR. These findings demonstrate that the behavioral effects of reduced context-driven reinstatement produced by Isx-9 in HR is associated with reduced activation of newly born GCNs in the dorsal GCL. Examination of Fos activation of preexisting GCNs demonstrated that significant number of GCNs were activated during context-driven reinstatement in dorsal and ventral GCL in HR (450%) and LR (300%)

relative to methamphetamine naïve controls. Isx-9 treatment did not alter reinstatement-induced Fos activation in the dorsal GCL. In contrast, Isx-9 abolished Fos activation in the ventral GCL in HR compared to vehicle treated HR. Therefore, the findings with Fos demonstrate that dorsal and ventral GCNs respond similarly to context-driven reinstatement-elicited brain activity, and reduced reinstatement is associated with reduced activation of newly born GCNs in the dorsal GCL and preexisting GCNs in the ventral GCL in Isx-9 treated HR. Taken together, Isx-9 treatment may have reduced the incentive motivational effects of the drug context to some degree, resulting in the reduced Fos expression in GCNs.

To examine the specificity of these findings, separate groups of animals were trained to self-administer sucrose or saline and tested using an identical extinction-reinstatement procedure (Supplementary Figure 5-6). The results of these studies show that neurogenesis and Fos responses were not altered in sucrose-trained or saline-trained animals suggesting that Isx-9's effects on alterations in neurogenesis, neuronal activation and the behavioral responses are attributable to drug specific effects and not to general nondrug reward seeking or other procedural influences. Therefore, while these experimental groups served to demonstrate specificity of Isx-9 on drug reward, additional studies are needed to determine the mechanisms underlying Isx-9's effects on neurogenesis and neuronal activation of GCNs and reduced methamphetamine seeking in HR.

The neuronal phenotype of newly born GCNs was further confirmed by retroviral labeling studies, where 17-day-old mCherry-labeled cells exhibited

neuron-like morphology with apical dendrites arborizing in the molecular layer of the DG. Because Isx-9 reduced activation of BrdU GCNs in the dorsal GCL, we examined alterations in structural plasticity in newly born and preexsisting Golgi-Cox labeled dorsal GCNs. Three dimensional Sholl analysis demonstrated that newly born GCNs have distinct arborization profiles relative to older preexisting GCNs. Further analysis revealed that HR exhibited reduced arborization of newly born and preexisting GCNs, and reduced dendritic extent in distal dendrites and reduced spine density relative to methamphetamine naïve controls, and this effect was inhibited by Isx-9 treatment. The effects in structural changes were not evident in LR. These findings demonstrate that forced abstinence from methamphetamine increases neurogenesis with compromised structural arborization of newly born GCNs in the dorsal GCL and the effect was specifically seen in animals that demonstrated compulsive-like behavior and higher propensity for reinstatement. Isx-9 treatment reduced context-driven reinstatement of drug seeking in HR and reduced activation of, and modified the structure of newly born GCNs. Taken together, these results demonstrate a novel relationship between abstinence-induced alterations in newly born GCNs and enhanced propensity for reinstatement.

We also studied possible synaptic and epigenetic mechanisms underlying the changes in neuronal activation and structural plasticity, using standard immunoblotting techniques. Density of total and phosphorylated proteins was evaluated for GluN receptors, CaMKII, and HDAC5 (Figure 5; Supplementary Table 1). Density of total GluN2B receptors were reduced and phosphorylated GluN2B at

Tyr1472 and phosphorylated CaMKII at Thr286 were enhanced by Isx-9 treatment in the dorsal DG, indicating enhanced activity of synaptic plasticity proteins [33, 36], 37; whereas an opposite effect of Isx-9 treatment on these phosphorylated subunits were observed in ventral DG (GluN2B no change; CaMKII reduced phosphorylation). Enhanced CaMKII activity in the dorsal DG correlated with enhanced phosphorylation of HDAC5 at Ser259, supporting epigenetic alterations and derepression of target gene expression 26. The opposing effects of Isx-9 on the activity of GluN2B, CaMKII and HDAC5 in the dorsal and ventral DG support the functional dissociation that exists along the dorsal-ventral gradient in the rat hippocampus [15, 16]. In summary, the most parsimonious interpretation of our results is that Isx-9 treatment during abstinence protects against context-driven reinstatement and these behavioral benefits were associated with reduced activation and enhanced structural plasticity of newly born GCNs in the dorsal GCL and reduced activation of preexisting GCNs in the ventral GCL. Mechanisms underlying this protection include enhanced expression of synaptic proteins that are known to promote long-term memory storage of extinguished memory in the dorsal DG. Further studies are now needed to explore the circuit-level consequences of the small yet significant number of newly born GCNs during abstinence and how they influence other cognitive behaviors that are context-driven, and pathologies associated with other types of drugs of abuse.

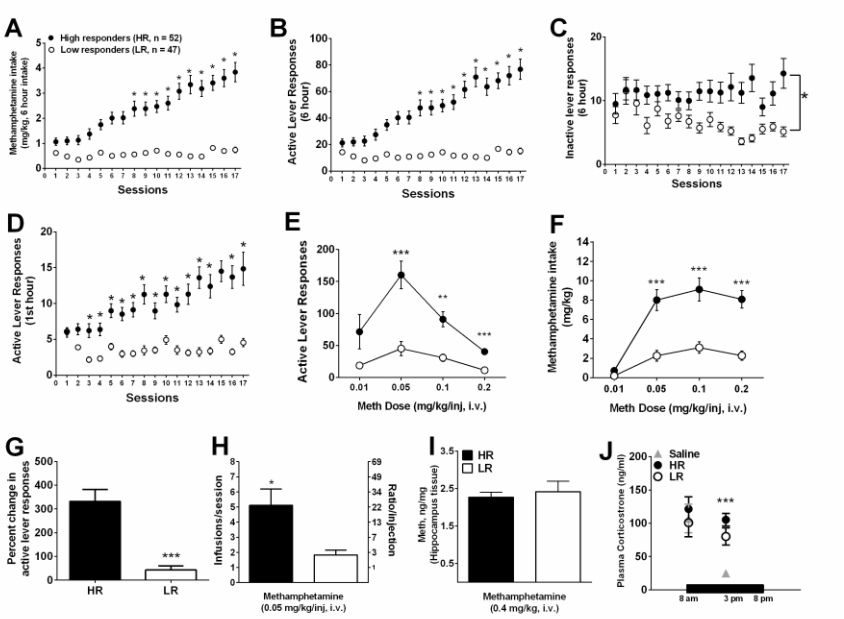

Figure 3.1: Extended access methamphetamine self-administration in 99 outbred adult Wistar rats demonstrates high and low preferred intake in methamphetamine. (A) Methamphetamine intake during six hour sessions, (B) active lever responses during six hour sessions (C) inactive lever responses during six hour sessions and (D) active lever responses during the first hour of the six hour session in High Responders (HR, n=52) and Low Responders (LR, n=47). HR and LR phenotype during methamphetamine self-administration were determined by the median of average daily methamphetamine self-administration (0.05 mg/kg/injection, FR1 schedule of reinforcement) during the 14 of 17 six hour self-administration sessions. (E) HR animals (n=10) exhibit an upward shift in peak self-administration rates, and a rightward shift in the descending limb of the self-administration dose–response curve compared to LR animals (n=6). (F) Methamphetamine self-administration lever responses shown in (E) converted to dose-intake curves shows that HR animals take greater daily amounts of methamphetamine compared to LR animals at doses on the descending limb of the dose–response curve shown in panel (E). (G) Percent changes in active lever responses in HR (n=52) and LR (n=47) animals when lever responses during sessions 13 to 17 were compared with sessions 1 to 5. (H) Methamphetamine-maintained breakpoints in HR (n=10) and LR (n=6) rats when tested in on a progressive ratio schedule of reinforcement. Left axis in (H) depicts the number of infusions obtained; right axis in (H) depicts the final ratio value completed. (I) Brain methamphetamine levels are similar in HR (n=10) and LR (n=6) animals. Brain tissue was collected 45 min after a 0.4 mg/kg intravenous methamphetamine injection and methamphetamine levels were analyzed by gas chromatography/mass spectrophotometry methods. (J) Plasma corticosterone (CORT) levels evaluated by ELISA demonstrates that HR (n=10) and LR (n=6) have similar peak CORT levels compared to animals that self-administered saline (n=8). However, nadir CORT was higher in HR and LR rats compared to saline rats. Saline, n=8; HR, n=10-52; LR, n=6-47 each group). Data shown are represented as mean +/- SEM. * P <0.05, compared to session 1 (A); * P <0.05, ** P <0.01, *** P <0.001 vs.LR (C-E), or saline (G) by repeated measures or standard two way analysis of variance (ANOVA) and Student-Newman-Keuls posttests.

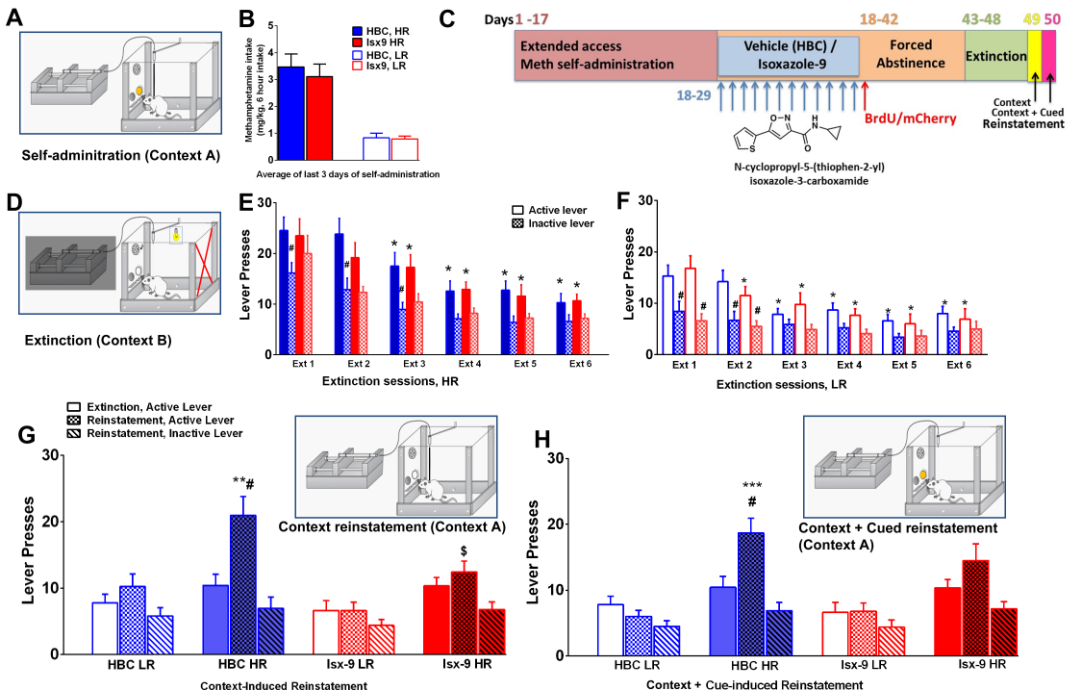

Figure 3.2: Neurogenic small molecule Isoxazole-9 reduces drug seeking during context- and cue-induced reinstatement in methamphetamine addicted animals. (A) Cartoon representation of operant box 'context A' for methamphetamine (meth) self-administration modified from Watterson and Olive 2014. (B) Average meth intake during last three sessions of self-administration in HR and LR animals injected with HBC control or Isx-9 during abstinence. (C) Schematic of experimental design indicating self-administration, Isx-9 injections (20 mg/kg, i.p.), BrdU injection (150 mg/kb, i.p.), abstinent days, extinction days and reinstatement days. (D-F) Cartoon representation of operant box 'context B' for extinction sessions (D), and active and inactive lever responses during extinction sessions in HR (E, control and Isx-9) and LR (F, control and Isx-9) animals. (G-H) Cartoon representation of operant box for contexual and cued reinstatement (insets in G-H), and active and inactive lever responses during contextual (G) and contextual-cued (H) reinstatement sessions in HR and LR, control and Isx-9 animals. HBC-LR, n=20; HBC-HR, n=24; Isx9 LR, n=19; Isx9-HR, n=18. Data shown are represented as mean +/- SEM. # P<0.05, vs. active lever presses and * P <0.05 vs. day 1 active lever presses in E-F by repeated measures two way ANOVA and Bonferroni posttests. ** P <0.01, *** P <0.001 vs. extinction session 6, # P<0.05, vs. inactive lever presses, $ P<0.01 vs. HBC-HR active lever presses in G-H by repeated measures two way ANOVA and Student-Newman-Keuls posttests.

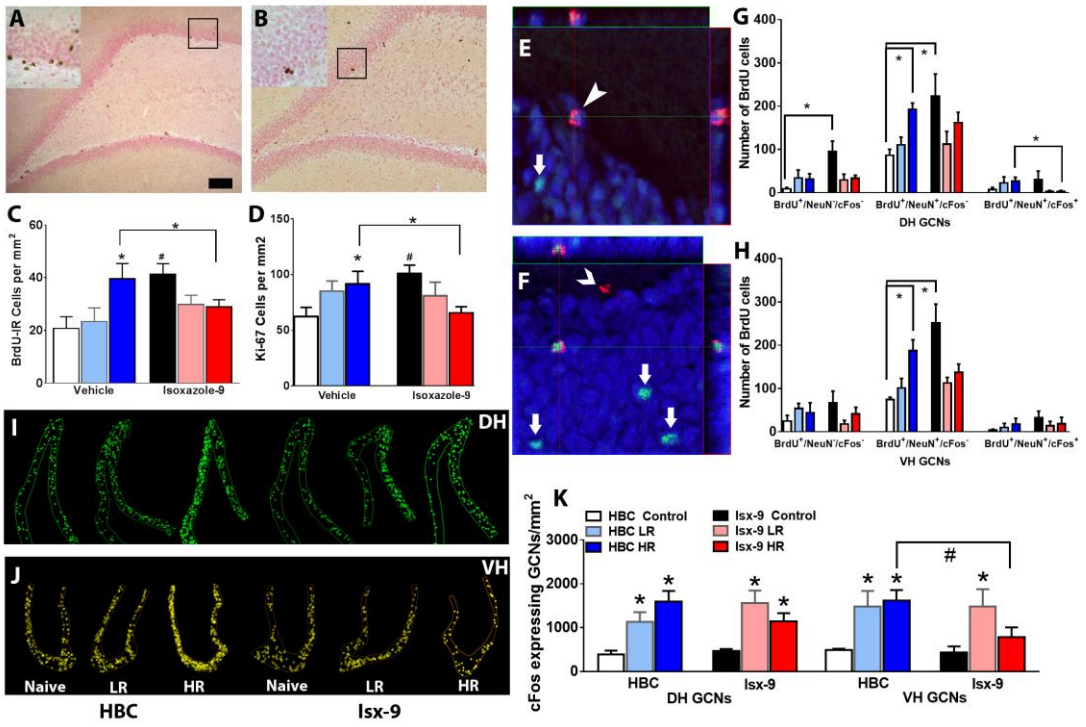

Figure 3.3: Isoxazole-9 prevented methamphetamine abstinence-induced alterations in neurogenesis and neuronal activation of GCNs in the dentate gyrus. (A-B) Photomicrographs of 17-day-old BrdU (A) and Ki-67 (B) labelled cells in the dorsal dentate gyrus granule cell layer. (C-D) Quantitative analysis of BrdU (C) and Ki-67 (D) labelled cells. (E-F) Orthogonal z stack confocal images of BrdU (red, CY3) cells expressing NeuN (blue, CY5), and/or cFos (green, FITC) in dorsal (E) and ventral (F) GCNs. Open arrowhead, BrdU alone; filled arrowhead, BrdU/NeuN; thin arrow, BrdU/NeuN/cFos; thick arrows, cFos/NeuN. (G-H) Phenotypic analysis of BrdU labelled cells in dorsal (G) and ventral (H) GCNs. (I-J) Schematic representation of expression of cFos in dorsal (I) and ventral (J) GCNs as depicted in StereoInvestigator that was used for cell quantification. (K) Quantitative analysis of cFos expressing GCNs. HBC control, n=4; HBC-LR, n=5; HBC-HR, n=7; Isx-9 control, n=6; Isx-9 LR, n=5; Isx-9 HR, n=7. Scale bar in A, main panel is 100 um (applies A-B), is 15 um (applies E-F). Data shown are represented as mean +/- SEM. *P<0.05 vs. vehicle control, #P<0.05 vs. Isx-HR in C-D, *P<0.05 vs. vehicle control in (K) by two way ANOVA and Student-Newman-Keuls posttests.

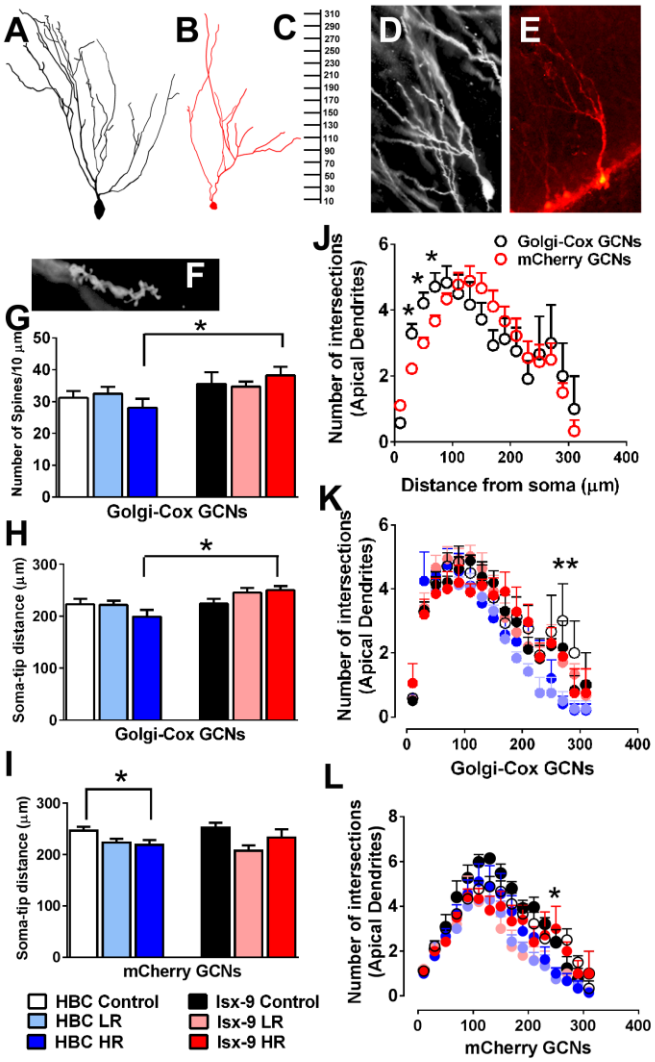

Figure 3.4: Isoxazole-9 prevented methamphetamine abstinence-induced altered structural plasticity of pre-existing and newly born granule cell neurons (GCNs). (A-F) 3D tracings of preexisting (A) and newly born (B) GCNs with Sholl ring distance indicated in (C), and images of Golgi-Cox (D, inverted image of black cells over white background) and 17-day-old mCherry (E, red cells over black background) labeled GCNs used for Sholl analysis. (F) Illustration of dendritic spines on Golgi-Cox labeled cells. (G-I) Structural plasticity of Golgi-Cox-labeled and mCherry labeled neurons shown as alterations in the number of dendritic spines on apical dendrites of Golgi-Cox-labeled cells (G), soma-to-tip distance of Golgi-Cox-labeled cells (H) and mCherry-labelled cells (I). (J-L) 3D Sholl analyses of preexisting and newly born GCNs (J), and newly born GCNs (L). HBC controls (n=6-9), Isx-9 controls (n=6-9), HBC-LR (n=8-9), HBC-HR (n=9-12), Isx-9 LR (n=5-8), Isx-9 HR (n=9-12). Data shown are represented as mean +/- SEM. *P<0.05 in G-I; * P<0.05 vs mCherry GCNs in J; **P<0.01 and *P<0.05 vs. HBC control in K-L compared to groups indicated by lines or to vehicle control by two way repeated measures ANOVA and Student-Newman-Keuls posttests.

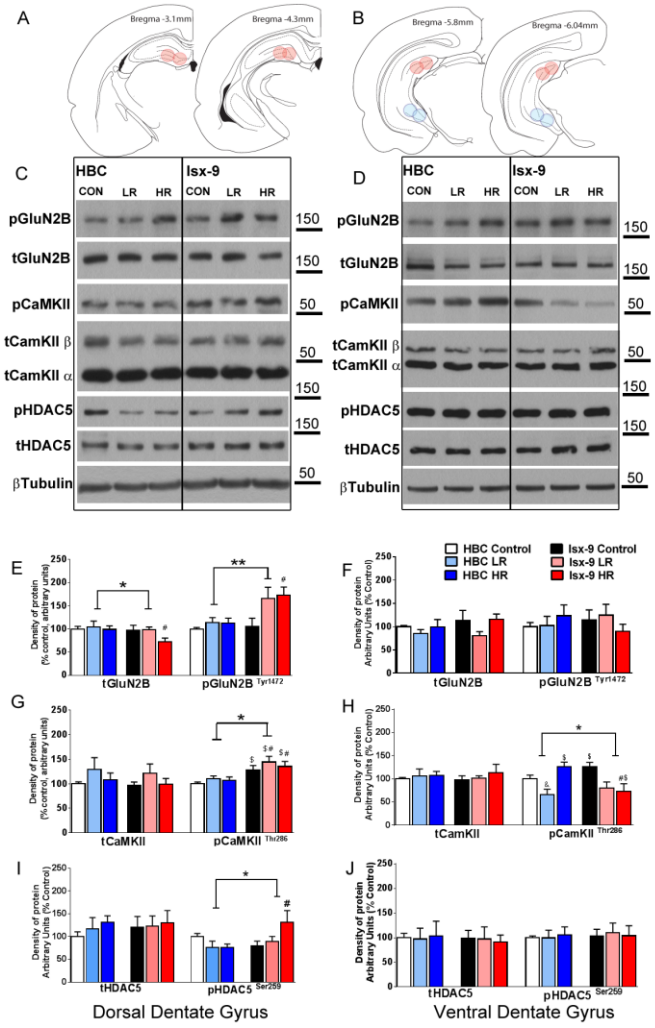

Figure 3.5: Isoxazole-9 alters the expression of synaptic plasticity proteins in the dorsal and ventral dentate gyrus. (A-B) Schematic showing location of tissue punches taken in the dorsal (A) and ventral (B) dentate gyrus of the hippocampus. (C-D) Representative Western blots for protein expression in dorsal (C) and ventral (D) dentate gyrus enriched tissue of total and phosphorylated GluN2B, CaMKII and HDAC5. (E-F) Density of protein expression for total and phosphorylated GluN2B in dorsal (E) and ventral (F) dentate gyrus (Dorsal dentate gyrus: tGluN2B- main effect of Isx-9: $F(1,41) = 4.741$, $P<0.05$; pGluN2B- main effect of meth $F(2,41) = 4.218$, $P<0.05$; main effect of Isx-9 $F(1,41) = 9.845$, $P<0.01$). (G-H) Density of protein expression for total and phosphorylated CaMKII in dorsal (G) and ventral (H) dentate gyrus (Dorsal dentate gyrus: pCaMKII- main effect of Isx-9 $F(1,43) = 13.10$, $P<0.001$; Ventral dentate gyrus: pCaMKII- meth group x Isx-9 treatment interaction $F(2,38) = 6.561$, $P<0.01$; main effect of meth $F(2,38) = 6.273$, $P<0.01$). (I-J) Density of protein expression for total and phosphorylated HDAC5 in dorsal (I) and ventral (J) dentate gyrus (Dorsal dentate gyrus: pHDAC5- meth group x Isx-9 treatment interaction $F(2,40) = 3.820$, $P<0.05$). HBC control, n=8; HBC-LR, n=8; HBC-HR, n=8; Isx-9 control, n=8; Isx-9 LR, n=9; Isx-9 HR, n=8. *$P<0.05$, **$P<0.01$ by two way ANOVA and Student-Newman-Keuls posttests.

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Supporting Materials and Methods

Animals: Surgical and experimental procedures were carried out in strict adherence to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH publication number 85–23, revised 1996) and approved by the Institutional Animal Care and Use Committee of The Scripps Research Institute. One hundred-fifty two, male Wistar rats (Charles River), weighing 200-250 g (8 weeks old) at the start of the experiment, were housed two per cage in a temperature-controlled vivarium under a reverse light/dark cycle (lights off 8:00 AM–8:00 PM) for at least one week prior to surgery. The number of animals for each experiment is indicated in the figure legends, and the sample size for each experiment indicated adequate power (>80%) when analyzed with GraphPad StatMate software. All experimental analysis were conducted as a double blinded study where the investigator performing and examining postmortem tissue analysis did not have the code for the animal group or treatment.

Intravenous Catheter Surgery: One hundred and nine rats underwent surgery for catheter implantation for intravenous self-administration. Rats were anesthetized with 2–3% of isofluorane mixed in oxygen and implanted with a sterilized silastic catheter (0.64 ID x 1.19 OD mm; Dow Corning Co.) into the right jugular vein under aseptic conditions. The distal end of the catheter was threaded under the skin to the back of the rat and exited the skin via a metal guide cannula (22G, Plastic One, Inc.).

Immediately after surgery, Flunixin® (2.5 mg/kg, s.c.; Bimeda – MTC Animal Health Inc) was given as analgesic. The rats were subjected to antibiotic therapy with Cefoxitin or Cefazolin during 10 days after the surgeries. Catheters were flushed daily with antibiotic in heparinized saline (30USPunits/ml) and tested for patency using methohexital sodium (Brevital®, 10mg/ ml, 2 mg/rat; King Pharmaceutical Inc.).

Methamphetamine Self-Administration: Following 4 days of recovery after surgery, ninety-nine animals were trained to lever press for i.v. infusions of methamphetamine (0.05 mg/kg per infusion) in an operant chamber (context A) on an FR1 schedule for 6 hours per session for 17 sessions. Animals were primed for the first hour of the session for the first two sessions. Animals had 17 sessions of methamphetamine self-administration followed by 12 days of abstinence during which animals received one i.p. injection of isoxazole-9 or vehicle (25% HBC) each day, starting on day 1 of abstinence and continued injections for 12 days into abstinence 1-3 (treatment was based on an in vivo study 1). The day after last isoxazole-9 injection, a subset of animals received one i.p. injection of BrdU (150 mg/kg). Animals remained in their home cages for an additional 13 days followed by extinction and reinstatement sessions.

Saline self-administration: Following 4 days of recovery after surgery, ten animals were trained to lever press for i.v. saline (0.9% sterile) in an operant chamber

(context A) similar to the paradigm used for methamphetamine self-administration (FR1; 6 hours per session for 17 sessions). Animals had 17 sessions of saline self-administration followed by 12 days of withdrawal. On day thirteen, five animals received one i.p. injection of BrdU (150 mg/kg). Animals remained in their home cages for an additional 13 days followed by extinction and reinstatement sessions.

Extinction: Extinction sessions for methamphetamine and saline animals were performed in a new context (context B) for 1h for six sessions. In this context, animals were not attached to the drug infusion apparatus, white noise was added during the entire session, a house light was turned on for the entire session, and black colored tape was pasted on the operant door. Responses on either the active or inactive lever were recorded and did not result in programmed consequences (i.e., no infusions and no conditioned stimulus presentations). Extinction was defined as reduced (<50%) active lever pressing on days 4-6 vs. day 1 of extinction.

Reinstatement: Twenty-four hours after the final extinction session, animals underwent context-induced reinstatement in which they were placed into the methamphetamine- or saline- paired context (context A) for 1 h, during which they were connected to the infusion apparatus to allow for a similar interaction with the spatial elements of the context as during methamphetamine self-administration training. Lever presses were used as a measure of drug seeking, and responses on either the active or inactive lever were recorded and did not result in an infusion of

fluids through the catheter or other programmed consequences (i.e., conditioned stimulus presentations). The next day, animals underwent context-plus-cue-induced reinstatement in which conditions were the same as context-induced reinstatement, and responses on the active lever resulted in the conditioned stimulus light presentation. One hour after the end of the session, animals were euthanized by rapid decapitation or transcardial perfusions.

Dose-response: Sixteen animals experienced 14 days of 6h methamphetamine self-administration sessions as described above. The four following days, animals underwent dose-response studies in which the methamphetamine dose administered was changed each day: 0.01, 0.05, 0.1, and 0.2 mg/kg/injection. Dose-response sessions were maintained at 6h and FR1 schedule.

Progressive ratio: The day following the last dose-response session, responding was reinforced on a progressive ratio (PR) schedule of reinforcement 4. On this schedule, the number of responses required for reinforcement incremented progressively, and each session continued until a breakpoint (defined as the number of infusions obtained before 1h elapsed with no infusions) was reached. Breakpoint was determined for 0.05 mg/kg/infusion methamphetamine.

Functional observational battery tests: Animals either received 12 injections of Isx-9 (1 injection per day; 20 mg/kg in 25% HBC, i.p.; n=15) or none (controls, n=12) and

were weighed after the last day of injections. Following body weight measures, animals were allowed to freely move in an enclosed chamber fitted with a video tracking system for 3 minutes and locomoter activity was measured during the 3 minute duration. Following activity measures, animals were trained on a trace fear conditioning paradigm to determine any effects of Isx-9 injections on motor and sensory reflexes. Animals received 5 CS-US pairings of a 30 second (s) tone (80 dB, CS) that was followed by 45s trace period which terminated with a 2 s footshock (0.5 mA, US) presented on a 20-90 s variable ITI. All animals remained in the chamber for 4 minutes following the final footshock. After conditioning, animals were immediately returned to the colony room.

Brain methamphetamine quantification: Following the completion of the last self-administration session (progressive ratio schedule), methamphetamine (0.4mg/kg, i.v.) was injected into the rats (n=16) via the indwelling jugular catheter. Within an hour, the rats were deeply anesthetized (isoflurane) and killed by decapitation. The striatum and the hippocampus from the left hemisphere of the brain were rapidly dissected, and weighed in microcentrifuge tubes. Tissue methamphetamine concentrations were measured using a previously published liquid chromatography-tandem mass spectrometry assay 5, 6 with minor modifications. Briefly, striatal and hippocampal tissue samples were homogenized by sonication in 500 µl deionized sterile water, and stored at -80 °C. The entire sample homogenate was used for analysis.

Preparation of Samples and Standards: On the day of the assay, the homogenates were equilibrated to room temperature. Deuterated methamphetamine (250 ng; METH-d8) was added as internal standard to 500 ul of homogenate. Samples were vortexed for 5 s and then made strongly basic by adding alkaline (pH > 12) with 100 µl of concentrated ammonium hydroxide. Homogenates were extracted into 6 ml of a 4:1 v/v mixture of n-butyl chloride and chloroform for 30 min with gentle shaking. Samples were centrifuged for 10 min at 1200g. The organic phase containing the analyte of interest was transferred to a 16 x 100-mm silanized glass screw-capped test tube and evaporated to dryness at 20°C. Residues were reconstituted with 100 µl of 95:5 formic acid (0.1%) and 5% acetonitrile (v/v) prior to analysis by liquid chromatography/tandem mass spectrometry. A multipoint calibration curve ranging from 1 to 1500 ng/ml homogenate was prepared with drug-free rat plasma and extracted as described above. Analytical intra-assay accuracy and the lack of matrix effect were verified by concurrent analysis of quality control (known standard) samples that were prepared in drug-free rat brain tissue homogenate.

Determination of Methamphetamine Concentration: Concentrations of methamphetamine were determined with Agilent 6490 triple quadrupole mass spectrometer with an electrospray ionization source operated in MRM/positive ion mode (Agilent Technologies, Santa Clara, CA). Separation by liquid chromatography was carried out using a 2.1x50mm Symmetry ® LC C18 column (Waters, Milford, MA). Water (with 0.1% formic acid) and acetonitrile (with 0.1% formic acid) were used for the gradient mobile phase; gradient used was as follows – 100/0 at T=0

min, 100/0 at T=1 min, 65/35 at T=5 min, 0/100 at T=6, Off at T=9 min. A 4-min re-equilibration step was included between two consecutive sample injections. Transition states monitored were m/z 150.1 -> 91.0 for methamphetamine and m/z 158.2 -> 93.1 for METH-d8. The limit of detection in these experiments was 100 fg/µl. Accuracy was within 10% of the methamphetamine concentrations from brain homogenate quality control (known standard) samples.

Corticosterone assay: Plasma corticosterone was measure in rats self-administering methamphetamine and in rats self-administering saline using the DetectX ® Corticosterone Enzyme Immunoassay Kit (Arbor Assays, Ann Arbor, MI) following manufacturer instructions. Briefly, 100-150 ul tail blood was collected from the rats before and/or after the self-administration session in specialized heparin coated microcentrifuge tubes. Plasma was separated by centrifugation of the blood samples at 3000 rpm for 15 min at 4°C, and stored at – 80°C. On the day of the assay, the plasma were allowed to thaw on ice and the reagents of the Enzyme Immunoassay Kit were allowed to warm to room temperature prior to use. Samples were prepared by mixing plasma with the Dissociation Reagent in a 1:1 ratio, and then diluting the mixture with Assay Buffer to get a final dilution of 1:100 for the plasma. The manufacturer provided corticosterone standard (100 ng/ml) was serially diluted to generate an 8-point standard curve ranging from 78.125 pg/ml to 10,000 pg/ml. Standards and the diluted samples were added to microtiter plate coated with secondary antibody against sheep. A sheep polyclonal antibody against

corticosterone and corticosterone-peroxidase conjugate were added to sample and standard wells. Following an hour of incubation, binding of the corticosterone and corticosterone-peroxidase conjugate to the plate was stopped by washing the wells. Then a peroxidase substrate was added to the wells that produced a colorimetric reaction with the bound corticosterone-peroxidase conjugate. This reaction was stopped and the intensity of the generated color (or optical density) was measured at 450nm using a microtiter plate reader. The concentration of corticosterone in the samples was calculated from the 4-parameter logistic non-linear regression obtained from concentration-optical density plot generated by the 8 known standard dilutions using Graphpad Prism.

Sucrose Self-administration: Twelve rats were trained to orally self-administer sucrose solution (10% sucrose on an FR1 schedule; context A) for seventeen sessions. The first four sessions served as training for the animal to learn to 1) press the correct lever for a fluid reward and 2) drink the administered fluid from the delivery cup. The first two training sessions were conducted overnight in the operant chambers and active lever presses were rewarded with tap water while the following four sessions were two hours in duration and 10% sucrose was delivered following an active lever press. Subsequent sessions were 30 minutes in duration, and each active lever press activated delivery of the 10% sucrose solution into the delivery cup. Operant sessions were conducted for 5 days followed by 2 days of abstinence for a total of 17 30-minute sessions. Following the last operant session,

animals were injected i.p. with either 20mg/kg body weight Isoxazole-9 or vehicle (25% HBC) once per day for 12 days. Subsequently, animals received one i.p. injection of BrdU (150 mg/kg). 13 days following BrdU administration, animals were trained to extinguish their previous operant training. For this, the session duration was the same (30 minutes) but the extinction sessions occurred in a novel context B and active lever presses were not rewarded. Following six extinction sessions, animals were then returned to their original operant context for a single reinstatement session (30 min); however, responses on the active lever did not result in programmed consequences. One hour after the end of the session, animals were euthanized by transcardial perfusions.

Synthesis and MS of Isoxazole-9: Isx-9 was prepared according to cyclodehydration route outlined by Schneider et al 2007 2. Supplementary Figure 2 demonstrates the 1H NMR and HPLC of Isx-9 to confirm identity and purity. The concentration of Isx-9 in plasma or hippocampus tissue was measured via MS using a previously reported method 1, 7. Rats were injected with Isx-9 (20 mg/kg, i.p.) and were decapitated 10, 30 or 60 minutes after the injection (n = 3 each time point) and trunk blood was collected in heparin coated microfuge tubes and hippocampal tissue was dissected, weighed and snap frozen on dry ice. Plasma was isolated and 500 ul of plasma was used for MS. Brain tissue was homogenized in 500 ul of acetonitrile and used for MS. Samples were run on Agilent 6490 triple quadrupole mass spectrometer by the core

facility at Scripps Research Institute to estimate amount of Isx-9 (ng) per weight unit (g tissue or ml plasma) (supplementary figure 2).

Retroviral Constructs and Virus Preparations. The mCherry plasmid and retrovirus was generated as previously reported in Magill et al 2010 8.

Intracranial Surgery: Intracranial surgery was performed the day following the last isoxazole-9 injection or 13 days after the last methamphetamine self-administration session. Animals were anesthetized with isofluorane (2-3%) mixed with oxygen and received stereotaxic bilateral infusions of mCherry retrovirus in the dorsal hippocampus (AP, -4.3mm from bregma; ML, -/+ 2.6mm from bregma ; DV, -3.4, -3.25, and -3.0 from dura; 9) with a stainless steel cannula attached to a syringe pump connected by plastic tubing. Animals were infused at a flow rate of 1 ul/min with a total volume of 4.5 ul (1.5ul infused at -3.4mm, 2ul infused at -3.25mm, and 1ul infused at -3.0mm). Immediately after surgery, Flunixin® (2.5 mg/kg, s.c.; Bimeda – MTC Animal Health Inc) was given as analgesic, and Cefoxitin was administered as antibiotic.

Perfusions and brain tissue collection: One hour after behavioral experiments animals were fully anaesthetized using chloral hydrate (240 mg/kg, i.p.). Rats were then transcardially perfused with phosphate-buffered saline (2 min at 15 ml/min and 4 % paraformaldehyde (20 min at 15 ml/min). The brains were dissected out

and postfixed in 4 % paraformaldehyde at 4ºC for 16–20 h and sectioned in the coronal plane at a thickness of 40 μm on a freezing microtome. The sections through the brain were collected in nine vials [containing 0.1 % NaN3 in 1X phosphate-buffered saline (PBS)] and stored at 4 ºC. One ninth of the brain region through the hippocampus and the prefrontal cortex was used for immunohistochemical analysis.

Immunohistochemistry: The following primary antibodies were used for immunohistochemistry (IHC): Ki-67 (1 : 1000), BrdU (1 : 400), activated caspase-3 (AC-3, 1:500) and c-Fos (1 : 1000). For Ki-67, BrdU and AC-3 IHC, left and right hemispheres of every ninth section through the hippocampus and mPFC (including the anterior cingulate, prelimbic, and infralimbic cortices) were slide-mounted, coded, and dried overnight before IHC. For Fos IHC, two bilateral sections that contained the dentate gyrus, two bilateral sections that contained the mPFC were used for cell quantification from each rat from each group. The sections were pretreated 10, blocked, and incubated with the primary antibodies (BrdU, Ki-67, AC-3, Fos) followed by biotin-tagged secondary antibodies. Immunoreactive cells in the SGZ (ie, cells that touched and were within three cell widths inside and outside the hippocampal granule cell-hilus border for BrdU, Ki-67 and AC-3) or granule cell layer (GCL; Fos) were quantified with a Zeiss AxioImagerA2 (×400 magnification) using the optical fractionator method, in which sections through the dentate gyrus (−1.4 to −6.7 mm from bregma; 9) were examined. Cells in the SGZ and GCL were summed and divided by the area of the granule cell layer to give the total number of

cells per mm2. BrdU and Fos cells in the mPFC (3.7–2.2 mm from bregma) were examined, and cells from the left and right hemispheres that were localized in the counting frame (mPFC, 0.09 mm2) were visually quantified. The total number of immunoreactive cells both in the left and right hemispheres was counted and are expressed as the total number of immunoreactive cells per mm2 in each rat averaged across rats within a group.

Western Blotting: Procedures optimized for measuring both phosphoproteins and total proteins were employed 11. Animals were euthanized via rapid decapitation under light isoflurane anesthesia 1 hour after end of reinstatement session. Brains were quickly removed and flash-frozen. Brain tissue was cut at the mid-sagittal axis and the right hemisphere was processed for Western blotting. Tissue punches from dorsal and ventral hippocampal formation from 500 μm thick sections were homogenized on ice by sonication in buffer (320 mM sucrose, 5 mM HEPES, 1 mM EGTA, 1mMEDTA, 1% SDS, with Protease Inhibitor Cocktail and Phosphatase Inhibitor Cocktails II and III diluted 1:100; Sigma), heated at 100 °C for five minutes, and stored at−80 °C until determination of protein concentration by a detergent-compatible Lowry method (Bio-Rad, Hercules, CA). Samples were mixed (1:1) with a Laemmli sample buffer containing β-mercaptoethanol. Each sample containing protein from one animal was run (20 μg per lane) on 8–12% SDS-PAGE gels (Bio-Rad) and transferred to polyvinylidene fluoride membranes (PVDF pore size 0.2 μm). Blots were blocked with 2.5% bovine serum albumin (for phosphoproteins) or

5% milk (w/v) in TBST (25 mM Tris–HCl (pH 7.4), 150 mM NaCl and 0.1% Tween 20 (v/v)) for 16–20 h at 4 °C and were incubated with the primary antibody for 16–20 h at 4 °C: antibody to NR1 (1:200, Santa Cruz Biotechnology cat. no. sc-1467, predicted molecular weight 115 kDa, observed band ~115 kDa), antibody to NR2A (1:200, Santa Cruz Biotechnology cat. no. sc-9056, predicted molecular weight 177 kDa, observed band ~170 kDa), antibody to pNR2A Tyr-1325 (1:200, PhosphoSolutions cat. no. p1514-1325, predicted molecular weight 180 kDa, observed band ~180 kDa), antibody to NR2B (1:200, Santa Cruz cat. no. sc-9057, predicted molecular weight 178 kDa, observed band ~180 kDa), antibody to pNR2B Tyr-1472 (1:200, Cell Signaling cat. no. 4208S, predicted molecular weight 190 kDa, observed band ~180 kDa), antibody to pNR2B Ser-1480 (1:200, Thermo Scientific cat. no. PA14733, predicted molecular weight 190 kDa, observed band ~180 kDa), antibody to pNR2B Ser-1480 (1:200, Thermo Scientific cat. no. PA14733, predicted molecular weight 190 kDa, observed band ~180 kDa), antibody to CamKII (1:200, Abcam cat. no. ab52476, predicted molecular weight 47 and 60 kDa, observed band ~47 and 60 kDa), antibody to pCamKII Tyr-286 (1:200, Abcam cat. no. ab5683, predicted molecular weight 50 kDa, observed band ~50 kDa), antibody to HDAC5 (1:200, Abcam cat. no. ab50001, predicted molecular weight 122 kDa, observed band ~130 kDa), antibody to pHDAC5 Ser-259 (1:200, Abcam cat. no. ab192339, predicted molecular weight 122 kDa, observed band ~130 kDa), antibody to pHDAC5 Ser-498 (1:200, Abcam cat. no. ab47283, predicted molecular weight 122 kDa, observed band ~130 kDa), Bcl-2 (1:500, R&D Systems cat. no. MAB8272,

predicted molecular weight 24 kDa, observed band ~25 kDa), Bax (1:500, Santa Cruz cat. no. sc-493, predicted molecular weight 23 kDa, observed band ~20 kDa), and antibody to PSD-95 (1:500, Millipore, cat. no. 04-1066, predicted band size 95 kDa, observed band ~95 kDa). Blots were then washed three times for 15min in TBST, and then incubated for 1 h at room temperature (24 °C), appropriately with horseradish peroxide-conjugated goat antibody to rabbit or horseradish peroxide–conjugated goat antibody to mouse (1:10,000, BioRad) in TBST. After another three washes for 15 min with TBST, immunoreactivity was detected using SuperSignal West Dura chemiluminescence detection reagent (Thermo Scientific) and collected using HyBlot CL Autoradiography film (Denville Scientific) and a Kodak film processor. Following chemiluminescence detection, blots were stripped for 20 minutes at room temperature (Restore, Thermo Scientific) and reprobed for total protein levels of β-Tubulin (1:4000, Santa Cruz cat. no.sc-53140, predicted molecular weight 50 kDa, observed band ~50 kDa), for normalization purposes. Densitometry was performed using ImageStudio software (Li-Cor Biosciences). X-ray films were digitally scanned at 600 dpi resolution, then bands of interest were selected in identically sized selection boxes within the imaging program which included a 3 pixel extended rectangle for assessment of the background signal. The average signal of the pixels in the 'background' region (between the exterior border of the region of interest selection box and the additional 3 pixel border) was then subtracted from the signal value calculated for the band of interest. This was repeated for β-Tubulin, and the signal value of the band of interest following

subtraction of the background calculation was then expressed as a ratio of the corresponding β-Tubulin signal (following background subtraction). This ratio of expression for each band was then expressed as a percent of the drug naïve control animals included on the same blot.

Golgi-cox staining and neuron morphology analysis: For determination of the effect of methamphetamine on hippocampal neuronal architecture, the left hemisphere was processed for Golgi–Cox staining. The other half (right hemisphere) was used for Western blotting analysis (See above). For Golgi–Cox staining, the brain was submerged in Golgi–Cox solution A+B (FD Neurotechnologies Inc.) for 8 days at room temperature, followed by solution C for 4 days at room temperature and stored at–80 °C until processed for staining. Frozen brain tissue was coronally cut on a cryostat at 100 um-thick sections and stained with solution D + E and dehydrated according to manufacturer's instructions. Brains were coded before sectioning to ensure that experimenters were blind to treatments. To evaluate hippocampal neuron morphology, a Zeiss Axiophotmicroscope and a computer-based system (Neurolucida; Micro Bright Field) was used to generate three-dimensional neuron tracings that were subsequently visualized and analyzed using NeuroExplorer (Micro Bright Field). In order for a neuron to be selected the following four criteria were met: (1) the neuron was in the region of interest (DG [outer granule cell layer of the superior or inferior blade] of the hippocampus,–2.56 to –3.8mm to bregma), (2) the neuron was distinct from other pyramidal and

interneurons to allow for identification of dendrites, (3) the neuron was not truncated or broken, and (4) the neuron exhibited dark, well filled staining throughout including spines. For each animal, 4 granule cell neurons in the DG were traced at 40× magnification. No more than one neuron per region per section was used in the structural analysis. Both the apical and basal trees were traced, and morphological measurements were analyzed separately. For each reconstructed neuron, an estimate of dendritic complexity was obtained using the Sholl ring method. A 3D Sholl analysis was performed in which concentric spheres of increasing radius (starting sphere 10 μm and increasing in 20 μm increments) were layered around the cell body until dendrites were completely encompassed. The number of dendritic intersections at each increment was counted, and results were expressed as total intersections and the number of intersections per radial distance from the soma. Additionally, total dendritic length and longest soma-to-tip length were calculated for each tree of each reconstructed neuron. For all neurons, spine density was measured at 60×–100× magnification with an oil immersion lens (equipped with a 10× eye piece) on the same dendritic branches used for Sholl analysis. Dendritic spine density was measured on three dendritic segments from both the apical tree. For the apical tree, spines were counted along 10 μm segments of primary apical branches located at least 50 μm away from the cell body. All measurements of spine density were taken from 1–2-μm-thick dendritic segments to minimize the number of spines hidden by the dendritic shaft and ensure that the number of hidden spines was proportional across all segments counted and among

treatment groups. The total number of dendritic spines visible along both sides of the segment were counted and expressed as number of spines per total length of dendrite.

mCherry staining and neuron morphology analysis: For detecting retrovirus labeled newly born neurons, animals were perfused as previous indicated and brain tissue was sectioned on a cryostat at 100um thickness. Sections were stained with primary antibody against mCherry (1:500, Clontech Laboratories Inc., cat. No. 632543) and stained neurons were traced to evaluate hippocampal neuron morphology as described for Golgi-Cox analysis.

Statistical analyses: The methamphetamine self-administration data is expressed as the mean mg/kg per session of methamphetamine self-administration. The effect of session duration on methamphetamine self-administration during the 6h session and during the first hour of the 6h session was examined over the 17 escalation sessions using a two-way repeated-measures analysis of variance (ANOVA; session duration □ daily session) followed by the Student-Newman-Keuls post hoc test. The pattern of responding for methamphetamine is expressed as the mean mg/kg per hour over 6 h sessions and active and inactive lever responses in HR and LR rats and were compared between the first and > 10th escalation sessions. Differences in the rate of responding between the first and other escalation sessions were evaluated using the paired t-test. Running data is expressed as revolutions per day or per

hour. The effect of methamphetamine withdrawal on extinction and reinstatement in vehicle treated and Isx-9 treated animals was examined using a two-way repeated-measures (ANOVA; meth group x session) followed by the Student-Newman-Keuls post hoc test. Similar analyses were performed for sucrose and saline self-administering animals. For the Ki-67, BrdU, AC-3, Fos Golgi-Cox, mCherry and Western blotting analyses, two-way ANOVA was used. The data are expressed as mean ± SEM in all graphs.

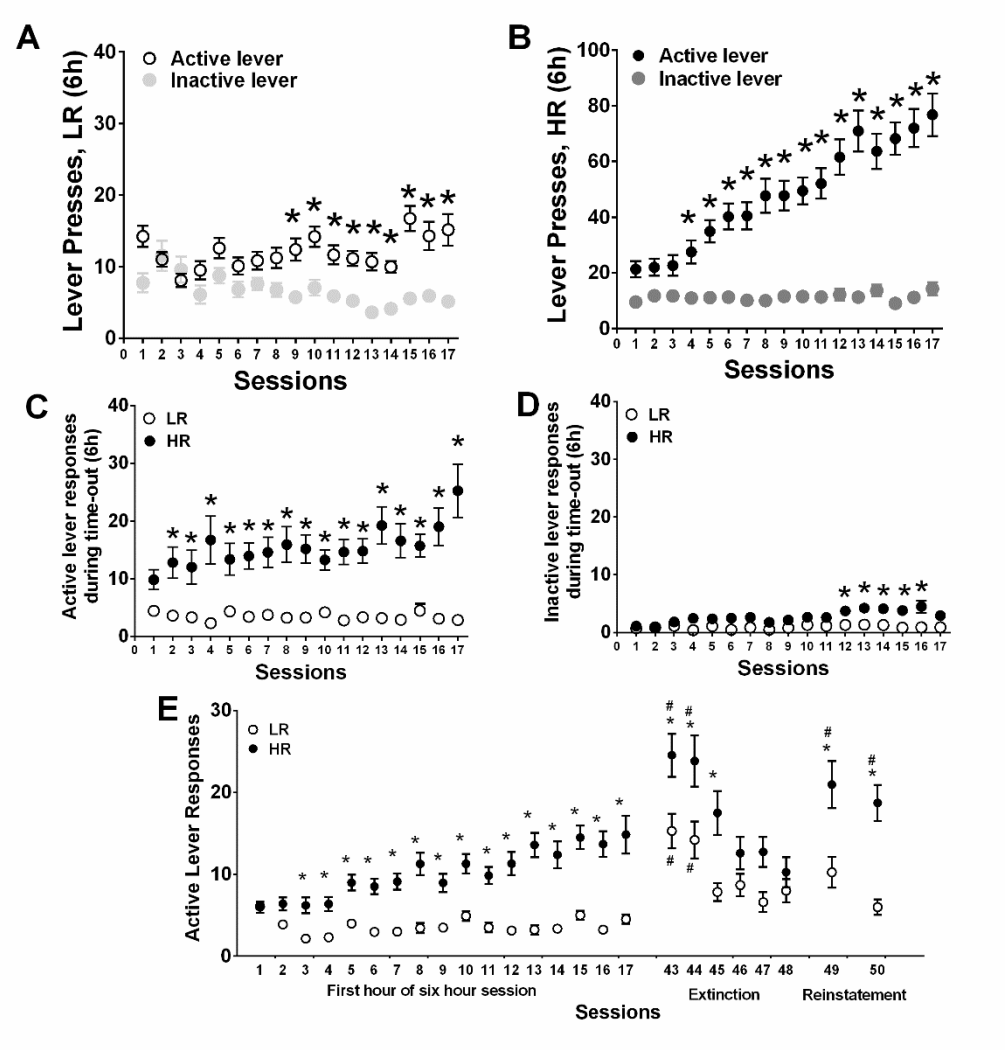


Figure S3.1: Extended access methamphetamine self-administration in 99 outbred adult Wistar rats demonstrates enhanced compulsive-like responses in HR animals. (A-B) Active and inactive lever responses in LR (A) and HR (B). LR animals have higher responding on reinforced (active) levers indicated by a significant number of sessions x lever responses interaction $F(16, 1498) = 3.247$, main effect of number of sessions $F(16, 1498) = 2.258$ and lever presses $F(1, 1498) = 148.5$ by repeated measures two-way ANOVA, $P<0.01$. HR animals have higher responding on reinforced (active) levers indicated by a significant effect of lever presses $F(1, 1585) = 88.31$ by repeated measures two-way ANOVA, $P<0.01$. (C) HR animals have higher responding on the active levers during time-out, no interaction, no effect of days, significant effect of session length $F(1, 1536) = 250.4$, $P<0.01$. (D) HR animals have higher responding on the inactive levers during time-out, significant session length x inactive lever responses interaction $F(16, 1480) = 2.028$, main effect of meth group $F(1, 1480) = 118.9$ and number of sessions $F(16, 1480) = 3.249$, $P<0.01$ by two-way ANOVA. (E) Active lever presses during the first hour of the six hour session during self-administration sessions, followed by lever presses during extinction sessions and reinstatement sessions. *$P<0.05$ vs. LR; #$P<0.05$ vs. day 17 compared to days 43 and 44; #$P<0.05$ vs. day 48 compared to days 49 and 50.

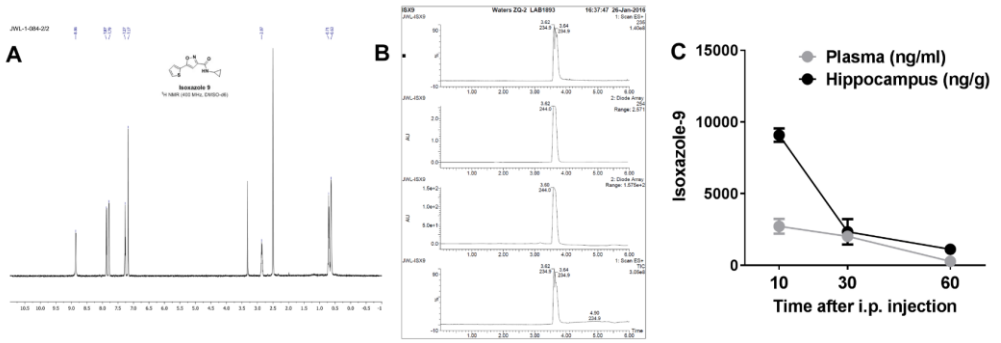


Figure S3.2: Synthesis of Isx-9 and pharmacokinetics of Isx-9 in adult Wistar rats. HPLC (A) and MS (B) data for Isx-9. Isx-9 was synthesized at the Scripps Research Institute according to 2. (c) MS analysis of Isx-9 in plasma and hippocampus tissue. Isx-9 crosses the BBB, as shown by the concentration of Isx-9 (ng/g) in hippocampus tissue or plasma (ng/ml) 10, 30, and 60 min after 1 i.p. injection of Isx-9 (n=3 per time point). Two-way ANOVA demonstrated a significant Isx-9 amount x time after injection interaction $F(2, 9) = 17.35$, significant increases in brain Isx-9 vs. plasma $F(1, 9) = 32.31$, and significant effect of time after injection $F(2, 9) = 47.30$, $P<0.01$. Posthoc analysis revealed higher levels of Isx-9 10 min after injection compared with other time points in the adult rat hippocampus tissue, supporting previously reported pharmacokinetic data in the adult mouse brain tissue 1.

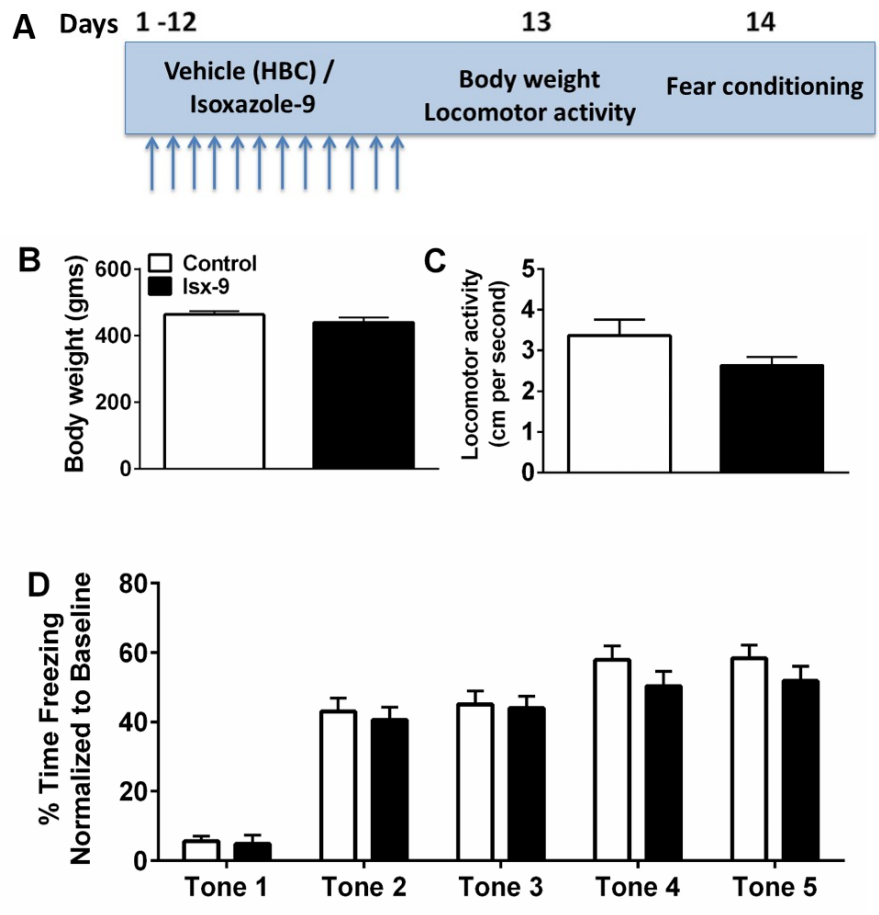
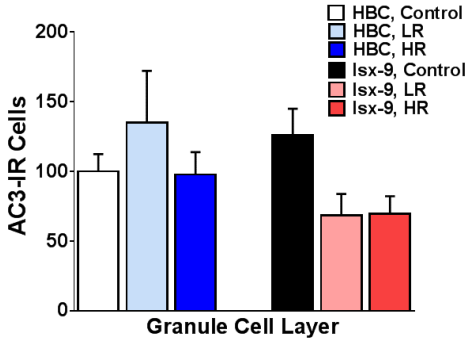


Figure S3.3: Functional observational battery tests were performed on animals treated with Isx-9 to determine the effects of the small molecule on general behavioral endpoints. (A) Schematic of experimental design and behavioral tests. (B) Body weight measured in grams. (C) Locomotor activity indicated as distance travelled in cm in seconds. (D) Freezing behavior in response to CS-US pairings indicated as percent time freezing normalized to baseline freezing. No significant differences were observed between controls and Isx-9 animals in any of measures indicated.

A



B

DORSAL Dentate gyrus	Density of protein (% control)				
Protein markers	LR, HBC	HR, HBC	drug naïve, Isx-9	LR, Isx-9	HR, Isx-9
Bax	81.90 ± 6.90	94.20 ± 15.39	101.10 ± 12.78	88.99 ± 6.89	88.12 ± 12.12
Bcl-2	108.30 ± 7.40	117.40 ± 8.17	116.05 ± 16.34	118.60 ± 9.97	130.30 ± 17.94

VENTRAL Dentate gyrus	Density of protein (% control)				
Protein Markers	LR, HBC	HR, HBC	drug naïve, Isx-9	LR, Isx-9	HR, Isx-9
Bax	103.20 ± 9.96	103.10 ± 5.60	101.40 ± 7.31	100.60 ± 6.54	99.69 ± 9.42
Bcl-2	130.60 ± 33.10	139.7 ± 32.15	107.70 ± 12.98	122.80 ± 39.04	139.60 ± 45.97

Figure S3.4: Cell death measured by activate caspase-3 is not altered by Isx-9 treatment in the granule cell layer of the hippocampus.
(A) Quantitative analysis of the number of activated caspase-3 cells in the granule cell layer of the hippocampus does not demonstrate an effect of methamphetamine or Isx-9. (B) Density of cell death factors in the dorsal and ventral dentate gyrus enriched regions demonstrates no significant effect in any groups.

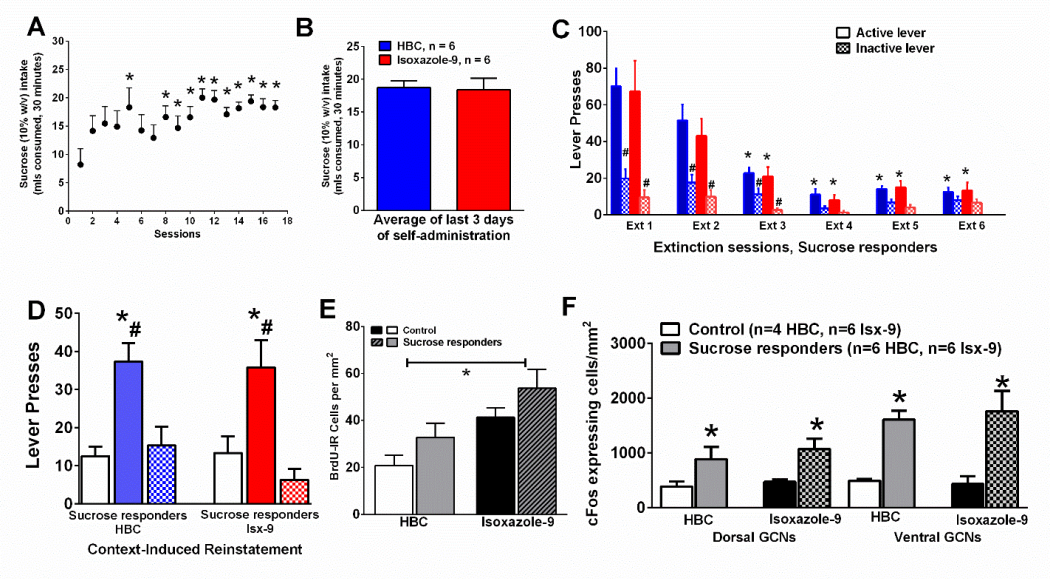


Figure S3.5: Synthetic small molecule Isx-9 does not alter reinstatement of sucrose seeking behaviors. (A) Sucrose consumption increases over days of self-administration. Repeated measures one-way ANOVA demonstrates significant increase in sucrose consumption ($F(16, 176) = 4.03$, $P=0.005$). Post hoc analysis indicated sustained increase in consumption during days 9-17 when compared to the first day of sucrose experience (Ps <0.05 by Fishers LSD). (B) Animals injected with Isx-9 did not differ in self-administration behavior compared with animals injected with vehicle control. (c) Vehicle and Isx-9 injected animals do not differ in their latency to extinguish sucrose-seeking behaviors, all animals extinguished before reinstatement session. (D) Vehicle and Isx-9 animals do not differ in their reinstatement to sucrose seeking triggered by sucrose context. (E) Withdrawal and protracted abstinence from sucrose self-administration does not enhance survival of BrdU cells in the DG. Isx-9 increased survival of BrdU cells in controls and sucrose experienced animals, two-way ANOVA demonstrated a significant main effect of Isx-9, $F(1, 16) = 9.598$, $P<0.05$. (F) Reinstatement of sucrose seeking increased neuronal activation of GCNs in the dorsal and ventral DG and Isx-9 treatment did not affect these increases, main effect of sucrose $F(3, 32) = 10.40$, $P <0.01$.

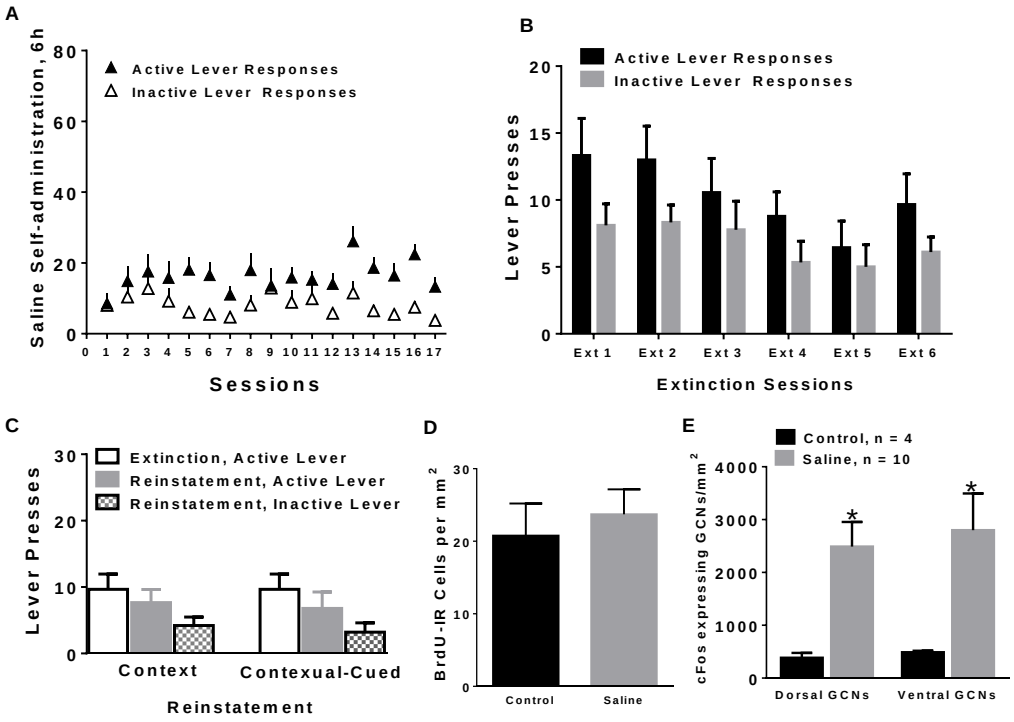


Figure S3.6: Neurogenesis is not altered by drug seeking behaviors in saline self-administering animals. (A) Animals trained to self-administer saline do not increase lever responses over sessions and do not demonstrate lever discrimination between active (reinforcing) and inactive lever responses. (B) Animals trained to self-administer saline do not seek saline and do not demonstrate extinction behavior. (C) Animals trained to self-administer saline do not reinstate saline seeking triggered by context. (D) Withdrawal and protracted abstinence from saline self-administration does not alter survival of BrdU cells in the DG. (E) Neuronal activation is enhanced in the GCNs in saline seeking animals that did not demonstrate reinstatement triggered by saline context. Two-way ANOVA demonstrated significant effect of saline context on the number of cFos cells $F(1, 14) = 21.55$, $P<0.05$.

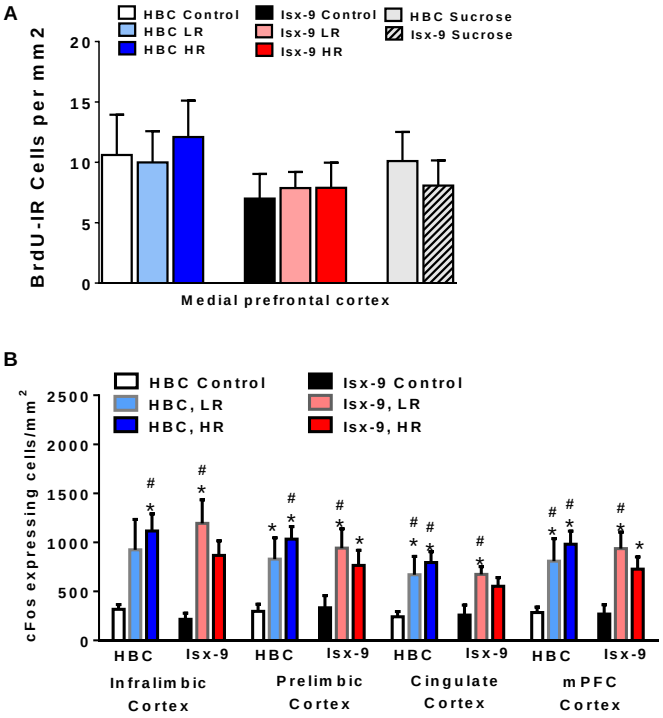


Figure S3.7: Isx-9 does not alter the number of glial progenitors and gliogenesis in the medial prefrontal cortex of methamphetamine seeking rats. (A) Withdrawal and protracted abstinence from methamphetamine self-administration does not alter the number of glial progenitors in the medial prefrontal cortex. (B) Isx-9 treatment did not alter the number of glial progenitors in the prefrontal cortex. (C) Reinstatement of methamphetamine seeking triggered by drug context and cues enhanced neuronal activation in the medial prefrontal cortex, and Isx-9 did not modify these effects. Main effect of treatment, $F(5, 22) = 4.015$, $P<0.05$.

Table S3.1: Isx-9 does not alter GluN1, GluN2A receptor and PSD-95 expression in the dentate gyrus. Withdrawal and protracted abstinence does not alter the expression of other proteins implicated in synaptic activity.

DORSAL Dentate gyrus	Density of protein (% control)				
Protein markers	LR, HBC	HR, HBC	drug naïve, Isx-9	LR, Isx-9	HR, Isx-9
tGluN1	112.02 ± 9.69	114.8 ± 13.32	95.99 ± 6.40	109.80 ± 8.97	104.40 ± 12.44
tGluN2A	113.30 ± 16.40	101.30 ± 9.87	90.05 ± 12.34	108.40 ± 12.89	87.57 ± 8.86
pGluN2A Tyr 1246	107.2 ± 9.59	121.8 ± 17.39	121.3 ± 20.10	118.20 ± 11.27	132.60 ± 17.44
pGluN2B Ser1480	108.3 ± 7.4	117.4 ± 8.1	116 ± 16.2	118.6 ± 9.9	130.3 ± 17.9
pHDAC5 Ser498	88.1 ± 16.2	93.4 ± 10.4	101.5 ± 6.1	96.1 ± 21.9	90.2 ± 15.6
PSD-95	80.84 ± 16.18	99.43 ± 9.17	100.60 ± 13.14	122.10 ± 20.64	73.39 ± 7.32

VENTRAL Dentate gyrus	Density of protein (% control)				
Protein Markers	LR, HBC	HR, HBC	drug naïve, Isx-9	LR, Isx-9	HR, Isx-9
PSD-95	120.05 ± 12.71	122.89 ± 13.95	99.83 ± 4.57	108.00 ± 7.89	130.29 ± 15.69

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CHAPTER 4:

CONTRIBUTION OF WITHDRAWAL-INDUCED NEUROGENESIS TO DRUG CONTEXT-INDUCED REINSTATEMENT OF METHAMPHETAMINE SEEKING BEHAVIOR IN RATS

Abstract

Previous studies have demonstrated that adult hippocampal neurogenesis is impacted by drugs of abuse. However, there are limited studies testing causation between neurogenesis and drug seeking behavior. In this study, we tested the hypothesis that increased adult hippocampal neurogenesis induced by forced abstinence from methamphetamine (meth) facilitates drug seeking in response to drug-associated contexts and cues. Male adult GFAP-TK transgenic rats (express herpes simplex virus thymidine kinase specifically in type-1 GFAP stem cells) experienced extended-access to intravenous meth or oral sucrose self-administration in an operant chamber where they learned to associate the context, a cue light, and active lever presses with a reward. After several sessions of self-administration, animals experienced forced abstinence from meth and sucrose. During abstinence animals received either Valcyte (valganciclovir, TK+Valcyte) or vehicle (TK-Valcyte) and were tested for extinction in a novel non-drug context, followed by context reinstatement, and context+cued reinstatement in the drug context. Valcyte treatment completely ablated generation of immature neurons and

produced significant reduction in the number of neural progenitor cells in meth and sucrose animals. Valcyte treatment in meth animals produced impairments in lever discrimination during extinction and context reinstatement, such that TK+Valcyte meth animals did not show spontaneous reinstatement during extinction and context-induced reinstatement compared to TK-Valcyte controls. Both meth groups showed robust cued reinstatement. No such behavioral effects were observed in sucrose ±Valcyte animals. These behavioral effects of Valcyte in meth animals were associated with reduced CaMKII activity in the dorsal dentate gyrus compared to TK–Valcyte controls. These findings support the role of neurogenesis during abstinence following escalating meth intake to drug-seeking behavior. These findings also demonstrate that neurogenesis during abstinence contributes to alterations in synaptic proteins associated with learning and memory consolidation dependent on the hippocampus which could support drug seeking behaviors triggered by drug context and not drug cues.

Introduction

Methamphetamine addiction is a national and global public health problem with treatment focusing on cognitive behavioral therapies aimed to reduce craving and relapse behavior (Lee and Rawson 2008). There is a need to identify effective

treatments that can enhance currently available therapy as there are no approved drugs to treat methamphetamine addiction.

In order to study the role of neurobiological processes underlying drug addiction, we are able to employ robust animal models of drug addiction that produce compulsive-like drug seeking. Kitamura et al. demonstrated how extended access to methamphetamine self-administration leads to an escalation in drug intake as well as reinstatement behavior following extinction (Kitamura et al. 2006). Compared to other schedules of drug administration, this protocol provides a means to ask questions regarding changes during abstinence that lead to drug seeking behavior after halting drug self-administration. Extended access to methamphetamine self-administration produces hallmark behaviors that define drug addiction in a robust manner.

Additional studies have shown the phenomenon of incubation of craving during abstinence from drugs of abuse (Shaham et al. 2003; Pickens et al. 2011). These studies demonstrated how animals that undergo longer periods of forced abstinence are more motivated to seek drug during reinstatement triggered by drug context, drug cues and the drug itself. Studies from our lab have also shown how animals that take more drug initially, are more likely to show higher levels of drug seeking during reinstatement triggered by drug itself (Recinto et al. 2012). This positive relationship between drug taking and reinstatement behavior highlights the role of meth-dependent plasticity in the brain during abstinence.

To model relapse behavior, reinstatement testing for drug-seeking behavior is used to assess motivation to seek drug, which is induced by exposing the animal to drug-associated context, cues, and/or the drug itself (Shaham et al. 2003; Rogers, De Santis, and See 2008; Crombag et al. 2008). Animals will continue to lever press for drug despite receiving no reward when exposed to the drug-associated context. This reinstatement behavior is mediated by the hippocampus, and if the hippocampus is functionally inactivated, drug-seeking behavior in rats is inhibited (Ramirez et al. 2009; Xie et al. 2013). The role of the hippocampus in context-induced reinstatement is not surprising given the importance of the hippocampus in numerous learning and memory functions (Gonçalves, Schafer, and Gage 2016; Goodman and Packard 2016) . Notably adult hippocampal neurogenesis has been identified as a process involved in discerning contexts (Kheirbek, Tannenholz, and Hen 2012; Clelland et al. 2009; Sahay, Wilson, and Hen 2011), which may be a contributor in the role of the hippocampus in reinstatement behavior in a drug-associated context.

Neurogenesis is the process by which neural precursor cells divide, proliferate and mature into dentate granule cell neurons. These newborn neurons integrate into the hippocampal circuitry and play functional roles in learning and memory (Zhao, Deng, and Gage 2008; Aimone et al. 2014). Specifically newborn granule cells can destabilize network activity in the hippocampus by extending dendrites to compete for the same synapses as mature granule cell neurons as well as by activating feedback inhibition via hilar interneurons (Lacefield et al. 2012).

The resulting destabilized network activity can function as a form of plasticity important for learning and memory.

Several studies found that adult hippocampal neurogenesis was impacted by various drugs of abuse, including alcohol, methamphetamine, cocaine, and heroin (Mandyam and Koob 2012). The manner in which neurogenesis is changed depends on the drug and the timing. Previous studies from our lab have shown that extended-access to methamphetamine self-administration reduces both cell proliferation and cell survival of neurons born during drug acquisition (Recinto et al. 2012). Forced abstinence, however, produces an increase in cell proliferation and rate of cell survival, suggesting a rebound effect 28 days after the last exposure to meth. There was a positive correlation between the number of newborn cells and the amount of meth intake (Recinto et al. 2012). Concurrently, there was a positive correlation between meth intake and active lever presses during reinstatement. Together these associations suggest a relationship between abstinence-induced hippocampal neurogenesis and drug seeking behavior after abstinence. However, studies have failed to show the exact contributions of hippocampal neurogenesis in drug seeking behavior using a model of compulsive-like drug seeking. These studies showing impairments to neurogenesis and hippocampal function support the need to study the role of hippocampal neurogenesis in drug addiction.

Recent state-of-the-art genetic tools have been developed in rats where we can selectively produce apoptosis in actively dividing neural precursor cells to conditionally ablate progenitor pool and neurogenesis in the adult hippocampus

(Snyder et al. 2016). The human GFAP promoter drives expression of a modified herpes simplex virus thymidine kinase, and only when we administer a drug valganciclovir (Valcyte) does the suicide gene kill proliferating progenitor cells. This method is selective for neural precursor cells undergoing mitosis, and does not inhibit growth of post mitotic astrocytes that also express GFAP (Snyder et al. 2016). Thus we have a novel, inducible method with which we can inhibit neurogenesis with temporal control with more specificity compared to x-irradiation.

Given that we know changes in neurogenesis are occurring during abstinence from meth, and that incubation of meth craving during abstinence drives reinstatement of drug seeking behavior, we hypothesize that the increase in neurogenesis during forced abstinence is maladaptive and contributes to context-induced reinstatement. To test this hypothesis we will use the extended access model of drug self-administration and a transgenic line of rats in which we have temporal control over inhibition of neurogenesis so that we can specifically inhibit neurogenesis during abstinence and measure differences in reinstatement of drug seeking following abstinence.

Materials and Methods

Animals

Surgical and experimental procedures were carried out in strict adherence to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH publication number 85-23, revised 1996) and approved by the Institutional Animal Care and Use Committee of The Scripps Research Institute. Adult male GFAP-TK rats (Long-Evans, weighing 450-500 g, 16-17 weeks old at the end of experiment) were provided by Dr. Heather Cameron via a MTA to the Scripps Research Institute. Breeders were provided with additional animals that were used for studies. Additional rats were bred at the Scripps Research Institute. Rats were weaned at 21–24 d of age, pair-housed, and genotyped by PCR (TransnetYX). Alls rats used for the study were housed two per cage and habituated in a temperature-controlled vivarium under a reverse light/ dark cycle (lights off 8:00 AM–8:00 PM) for at least 1 week prior to surgery.

Surgery

Procedures for catheter implantation for intravenous self-administration are described in chapters 2 and 3 [ref]. The rats were subjected to antibiotic therapy with Cefoxitin or Cefazolin (10.0 mg/mL heparinized saline, 2.5 mg/animal, s.c. SavMart Pharmaceuticals) during 10 days after the surgeries. Catheters were flushed daily with antibiotic in heparinized saline (30 USP units/ml) and tested for patency using methohexital sodium (Brevital®, 10mg/ ml, 2 mg/rat; King Pharmaceutical Inc.).

Animal Behavior Experiments

Procedures for animal experiments can be found in more detail in chapter 3.

Meth self-administration: Following 2-5 days of recovery after surgery, animals were trained to lever press for IV infusions of methamphetamine (0.05 mg/Kg per infusion) in an operant chamber on an FR1 schedule with a cue light (conditioned stimulus) for 6 hours per session for 17 sessions. Animals were primed for the first hour of the session for the first two sessions. Animals had 17 sessions of methamphetamine self-administration followed by 9 days of withdrawal during which animals received daily peanut butter balls or sucrose pellets (vehicle for TK-Valcyte and mixed with Valcyte (7.5 mg/kg p.o.; Roche) for TK+valcyte). Animals remained in their home cages for an additional 12 days, followed by six extinction sessions, during which animals received peanut butter balls twice a week.

Extinction: During extinction animals were exposed to a new operant chamber (context B) in which they were not attached to the drug infusion apparatus, and the operant box had white noise, a house light, and colored tape on the door. Responses on either the active or inactive lever were recorded and did not result in programmed consequences (i.e., no infusions and no conditioned stimulus presentations).

Reinstatement: Twenty-four hours after the final extinction session, animals underwent context-induced reinstatement in which they were placed into the methamphetamine-paired context (i.e., the same operant box used for self-administration sessions) for 1 h, during which they were connected to the infusion apparatus to allow for a similar interaction with the spatial elements of the context as during methamphetamine self-administration training. Lever presses were used as a measure of drug seeking, and responses on either the active or inactive lever were recorded and did not result in an infusion of fluids through the catheter or other programmed consequences (i.e., conditioned stimulus presentations). The next day, animals underwent context-plus-cue-induced reinstatement in which conditions were the same as context-induced reinstatement, and responses on the active lever resulted in the conditioned stimulus light presentation. One hour after the end of the session, animals were sacrificed.

Sucrose self-administration: Rats were trained to self-administer 10% sucrose on an FR1 schedule in an operant chamber with a cue light (conditioned stimulus). Animals were primed for one hour during the training session. Subsequent sessions were 30 minutes in duration, and each active lever press activated delivery of the 10% sucrose solution into the delivery cup. Operant sessions were conducted for 5 days followed by 2 days of abstinence for a total of 17 30-minute sessions. Animals were administered peanut butter balls on the same schedule as the meth animals.

Sucrose extinction: The session duration was the same (30 minutes) and the extinction sessions occurred in a novel context B and active lever presses were not rewarded.

Sucrose reinstatement: Following six extinction sessions, animals were then returned to their original operant box, context A, for context reinstatement (30 min). The following day animals were tested for context plus cued reinstatement session (30 min). Presses of the active lever were not rewarded.

Tissue collection: Animals were sacrificed one hour after the end of the last reinstatement session. Animals were anesthetized with isoflurane and rapidly decapitated using a guillotine. Brains were removed, and cut along the mid sagittal plane into left and right hemisphere. The left hemisphere of the brain was post fixed in 4% PFA and cryoprotected in 30% sucrose until histological sectioning and analysis. The right hemisphere of the brain was frozen and stored at -80°C and was used for Western blotting.

Immunohistochemistry and cell quantification

Procedures can be found in more detail in chapters 2 and 3. Also see references (Mandyam, Norris, and Eisch 2004).

Frozen brain tissue was sectioned (40um) using a sliding microtome. The following primary antibodies were used for immunohistochemistry (IHC): anti-Ki-67 (1 : 500), anti-NeuroD (1 : 500), anti-c-Fos (1:1000). For each animal, two dorsal

and two ventral regions of the rat hippocampus (−1.4 to −6.7 mm from bregma; Paxinos and Watson, 1997) were slide-mounted, coded, and dried overnight before IHC. The sections were pretreated (Mandyam et al, 2004), blocked, and incubated with the primary antibodies (Ki-67 and NeuroD) followed by biotin-tagged secondary antibodies. Immunoreactive cells in the SGZ (ie, cells that touched and were within three cell widths inside and outside the hippocampal granule cell-hilus border for Ki-67) or granule cell layer (GCL) were quantified with a Zeiss Axiophot photomicroscope (× 400 magnification) using the optical fractionator method, in which sections through the dentate gyrus were examined. Cells in the SGZ and GCL were summed and divided by the area of the granule cell layer to give the total number of cells per mm2.

Western Blot Analysis

General procedures for western blots were performed as described in Staples et al., 2015. Procedures optimized for measuring neuronal levels of both phosphoproteins and total proteins were employed (Kim et al., 2014). Procedures can also be found in chapter 3.

Blots were blocked with 1.5% bovine serum albumin (for phosphoproteins) or 5% milk (w/v) in TBST (25 mM Tris–HCl (pH 7.4), 150 mM NaCl and 0.1% Tween 20 (v/v)) for 1 h at room temperature and were incubated with the primary antibody for 16–20 h at 4 °C: antibody to phospho CaMKII Tyr 286 (1:2000, Abcam cat. no. 5683, predicted molecular weight 50 kDa, observed bands 50 and 55 kDa),

antibody to total CaMKII (1:1000, Abcam cat. no. 52476, predicted molecular weight 54 (45-70) kDa, observed bands 50 and 55 kDa), antibody to phospho HDAC5 Ser 259 (1:200, Abcam cat. no. 192339, predicted molecular weight 124 kDa, observed band ~125 kDa), antibody to total HDAC5 (1:200, Abcam cat. no. 50001, predicted molecular weight 122 kDa, observed band ~125 kDa), antibody to total GluN2B (1:200, Santa Cruz Biotechnology, Inc. cat. no. sc-9057, predicted molecular weight 178 kDa, observed band ~180 kDa), antibody to phospho GluN2B Tyr-1472 (1:200, Cell Signaling cat. no. 4208S, predicted molecular weight 190 kDa, observed band ~180 kDa), antibody to β tubulin (1:8000, Santa Cruz Biotechnology, Inc. cat. no. sc-53140, predicted band size 55 kDa, observed band ~50 kDa). Blots were then washed three times for 15min in TBST, and then incubated for 1 h at room temperature (24 °C), appropriately with horseradish peroxide-conjugated goat antibody to rabbit or mouse (1:2000 BioRad) in TBST. After another three washes for 15 min with TBST, immunoreactivity was detected using SuperSignal West Dura chemiluminescence detection reagent (Thermo Scientific) and collected using HyBlot CL Autoradiography film (Denville Scientific) and a Kodak film processor. Net intensity values were determined using the Image Studio Lite (version 3.1). For normalization purposes, membranes were incubated with 0.125% coomassie stain for 5 min and washed three times for 5–10 min in destain solution.

Statistical Analysis

Depending on the experiment, we analyzed data using one of the following statistical tests (see figure legends): two-tailed paired or unpaired t-test, two-way analysis of variance (ANOVA) or one- or two-way repeated measures ANOVA. If a significant main effect and interaction was found, a Student–Newman–Keuls post hoc test was used. Data are expressed as mean±s.e.m. Values of $p<0.05$ were considered statistically significant. Statistical analysis was performed and graphs were generated using GraphPad Prism 5.0 software.

Results

Abstinence from extended access to meth self-administration and sucrose self-administration enhances neuronal differentiation in the dentate gyrus.

Our lab had previously shown that extended-access to meth self-administration as well as forced abstinence from meth led to changes in cell proliferation and cell survival of newborn dentate granule cell neurons in male adult Wister rats. For this study we use the same schedule and timeline of self-administration (figure 1a) in which we saw abnormalities in neurogenesis in order to investigate the effects of inhibiting neurogenesis during forced abstinence.

We first wanted to confirm that GFAP-TK rats (Long Evans background) also showed similar changes in neurogenesis following abstinence from meth self-administration, and that they were associated with previous escalating meth intake.

We confirm that GFAP-TK rats are able to acquire operant meth-self administration and they both escalate in meth intake across 17 sessions of extended-access to meth-self administration (repeated measures two-way ANOVA indicates significant effect of sessions in both groups: $F(16, 368) = 12.34$, $p<0.001$; figure 1c). All rats reached escalation criteria by day 12. To test whether the effects of neurogenesis inhibition would be specific to meth, we also trained GFAP-TK rats to self-administer sucrose. Rats that experienced sucrose self-administration also escalated sucrose intake by day 6 (repeated measures two-way ANOVA indicates significant effect of sessions in both groups: $F(16, 208) = 42.65$, $p<0.0001$; figure 1d).

Neurogenesis was inhibited during abstinence, and differences in the number of progenitor cells and neurogenesis between rats that received Valcyte (TK+Valcyte) or vehicle (TK-Valcyte) were determined by postmortem analysis. To measure changes in cell number, brain tissue extracted 28 days after the last self-administration session was treated to label cells expressing Ki-67, as a marker for cell proliferation, and NeuroD, as a marker for cell differentiation (figure 2a-d). Neurogenesis was inhibited in meth and sucrose TK+valcyte animals as shown by significant reductions in cells expressing Ki-67 (one-way ANOVA indicates a significant effect of treatment: $F(4, 45) = 12.47$, $p<0.001$; figure 2e) and NeuroD (one-way ANOVA indicates a significant effect of treatment: $F(4, 45) = 41.09$, $p<0.001$; figure 2f) compared to age matched controls. TK-valcyte animals that experienced meth and sucrose self-administration showed an increase in cells expressing NeuroD ($ps<0.05$; figure 2f) compared to age matched controls, and

there was no increase in cells expressing Ki-67 (figure 2e). These results indicate an increase in neuronal differentiation, but not cell proliferation, in the dentate gyrus during abstinence for animals that experienced self-administration.

Inhibition of hippocampal neurogenesis during abstinence resulted in reduction in spontaneous reinstatement during extinction and impairments in lever discrimination during context reinstatement for Meth TK+valcyte rats.

Following 21 days after the last self-administration session, all animals experienced extinction training (6 one-hour sessions, 1/day) in a new operant chamber with a context different from the one in which they learned to self-administer meth or sucrose. Meth TK-valcyte rats showed normal extinction training, with spontaneous reinstatement on day 1 of extinction followed by reduction in active lever presses, reaching extinction criteria by day 4 . Meth TK+valcyte rats did not exhibit spontaneous reinstatement as active lever presses on day 1 of extinction were significantly reduced compared to the last day of self-administration. Meth TK+valcyte rats also did not reduce their active lever presses during extinction session, and active lever presses were not statistically different from inactive lever presses (two-way ANOVA demonstrates a significant treatment x session interaction $F(24, 436) = 1.535$, $p=0.05$; significant effect of session $F(8, 436) = 6.309$, $p=0.001$; and significant effect of treatment $F(3, 436) = 33.99$, $p<0.001$; Figure 3a). Post hoc analysis demonstrated a significant difference between –Valcyte and +Valcyte groups on first day of extinction ($p<0.05$). These

results indicate that inhibition of neurogenesis during abstinence led to impairments in lever discrimination during extinction of drug-seeking behavior in a non-drug related context. There were no significant differences between sucrose TK-valcyte and TK+valcyte rats during extinction (Main effect of session, $F(8, 233) = 32.59$, p ,0.001; figure 3b). Notably, sucrose animals did not exhibit spontaneous reinstatement on the first day of extinction. These results indicate that effects of neurogenesis inhibition are specific to animals exposed to meth self-administration.

Reinstatement is used to measure drug-seeking behavior in response to drug-related environmental cues without the reinforcement of drug on board. In this study we employed two separate reinstatement sessions, one with context alone, and the next day with context plus cue light to induce reinstatement of drug-seeking behavior. Meth TK-valcyte rats showed robust reinstatement behavior during context reinstatement and context-plus-cue-induced reinstatement ($p<0.05$; figure 3a). During context and cued reinstatement, Meth TK-valcyte active lever presses were significantly higher than the last extinction session, and active lever presses were higher than inactive lever presses for each reinstatement session. Meth TK+valcyte rats had slightly elevated active lever presses in response to context compared to last day of extinction, however the difference was not statistically significant ($p=0.08$, figure 3a). During context reinstatement, Meth TK+valcyte rats also exhibited impairments in lever discrimination as there was no significant difference between active and inactive levers. However during cued

reinstatement, TK+valcyte rats showed increased active lever responses compared to last day of extinction and active lever responses were significantly higher than inactive lever presses ($p<0.05$). These results indicate that neurogenesis is needed for context reinstatement and lever discrimination in response to context; however, neurogenesis is not necessary for cued reinstatement.

Changes in CaMKII activity are associated with changes in drug seeking behavior in TK+valcyte rats.

To investigate molecular mechanisms underlying changes in behavior with neurogenesis inhibition, we homogenized tissue from the dorsal and ventral hippocampus (figure 4a-b) and analyzed the tissue for proteins important for synaptic plasticity. In the dorsal dentate gyrus enriched tissue, Meth TK+valcyte had reduced pCaMKII compared to Meth TK-valcyte, indicating a reduction in CaMKII activity in meth animals with inhibited neurogenesis ($p<0.05$ by one-way ANOVA; figure 4g). In the ventral dentate gyrus enriched tissue, Meth TK+valcyte had reduced total CaMKII compared to Meth TK-valcyte, whereas there was no significant difference for pCaMKII ($p<0.05$ by one-way ANOVA; figure 4h). There were no significant differences in protein expression for p/t GluN2B and p/t HDAC5 between Meth TK+valcyte and Meth TK-valcyte. There were also were no significant differences in protein expression between sucrose TK+valcyte and sucrose TK-valcyte animals (figure 4g-h).

To further investigate whether changes in neuronal activity could underlie changes in behavior with neurogenesis inhibition, we analyzed the BLA, ventral DG, and dorsal DG for neuronal activation during context-plus-cue induced reinstatement. There were no significant differences in cFos expression between TK+valcyte and TK-valcyte animals for BLA, ventral DG and dorsal DG (figure 5).

Discussion

To the best of our knowledge, this study is the first to demonstrate that hippocampal neurogenesis during abstinence contributes towards context-induced reinstatement. Using novel GFAP-TK rats exposed to the well-established extended-access methamphetamine self-administration, we show new evidence to support the involvement of neurogenesis specific to context driven drug seeking rather than cue-induced reinstatement. This study also provides evidence of neurogenesis being involved in drug seeking in a non-drug-related context during extinction. These findings are consistent with previous findings in the lab that showed how reducing abstinence-induced neurogenesis using a synthetic compound isoxazole-9 also reduced context-induced reinstatement (chapter 3). Taken together these studies highlight the aberrant effects of newborn granule cells born during abstinence and their contribution toward reinstatement of drug seeking behavior.

Several studies support the role of adult hippocampal neurogenesis in context discrimination (Clelland et al. 2009; Sahay, Wilson, and Hen 2011; Sahay et al. 2011), which is the ability to detect the difference between two or more similar environments or locations. For example, performance in a two-choice spatial discrimination task (choosing the correct location between two colored boxes on a screen) is impaired in animals with reduced neurogenesis (x-irradiation) only when the boxes are spatially close to each other rather than far apart on the screen (Sahay et al. 2011), which indicates that neurogenesis is needed to tell the difference between spatially similar locations. This role of neurogenesis can come into play during relapse to drug seeking when encountering environments that are similar to drug-associated contexts and, in the rodent case, choosing between identical levers that are spatially close to each other.

Indeed our findings support the importance of neurogenesis in context discrimination by showing how inhibition of neurogenesis disrupts lever discrimination during extinction and context-induced reinstatement. One could interpret that when neurogenesis was blocked during abstinence, the rats had difficulty remembering the previously rewarding lever in both the non-drug and drug-related contexts. Whereas, the rats with abstinence-induced neurogenesis exhibited spontaneous reinstatement on day one of extinction and they consistently pressed the previously rewarding lever more than the inactive lever until the last day of extinction, meaning the similar but non-drug related context was enough to produce drug seeking behavior and recall drug-related memory needed for lever

discrimination. Together with our findings, these studies show how aberrant adult hippocampal neurogenesis can play a role in drug seeking behavior in response to environments that are similar to drug-related contexts.

However, our study shows that neurogenesis, is not needed for cue-induced reinstatement. Previous studies have identified the basolateral amygdala and the nucleus accumbens as brain regions that are involved in drug seeking behavior in response to drug-related cues (Fuchs et al. 2005). Our findings from the cue-induced reinstatement in rats with inhibited neurogenesis further confirm that drug-related memory for lever discrimination is not completely reliant on neurogenesis, and so inhibiting neurogenesis does not abolish the memory for the rewarding lever altogether. The neuronal activation results are consistent with the involvement of the amygdala during cue-induced reinstatement such that both groups, with or without intact hippocampal neurogenesis, would have similar neuronal activation in the amygdala in response to the drug-related cue and thus similar drug seeking behavior. These results together confirm the specificity of hippocampal neurogenesis in driving context-driven drug-seeking behavior rather than cue-driven, and demonstrate that Valcyte treatment did not alter neuronal activation of preexisting granule cell neurons or neurons in the BLA.

We further investigated biochemical mechanisms underlying changes in behavior when neurogenesis was inhibited. We targeted proteins that drive neuronal differentiation as well as pathways known to facilitate synaptic plasticity needed for learning and memory (Kauer and Malenka 2007; Prybylowski et al.

2005). Differences in drug seeking behavior were not associated with differences in protein expression of the epigenetic factor HDAC5, total GluN2B or phospho GluN2B (tyr1472). However, the reductions we observed in phospho CaMKII (tyr286) in the dorsal dentate gyrus reflect the changes in drug seeking behavior when neurogenesis was inhibited. CaMKII autophosphorylation is needed for long term memory formation by activating transcription factors needed for memory consolidation (Zhou et al. 2007; Katano et al. 2011). Reduced CaMKII activity in the dorsal dentate gyrus is one potential mechanism by which inhibiting neurogenesis could disrupt drug-related memory in response to the drug-related context. The differences between dorsal and ventral dentate gyrus in the protein expression of phospho and total CaMKII highlights the disassociated functional roles of the subregions with dorsal hippocampus being more involved in spatial memory, which would be needed for lever discrimination (Wu and Hen 2014; Esclassan et al. 2009). With respect to cue-induced reinstatement, the involvement of the basolateral amygdala and the nucleus accumbens are likely to overcome the deficits induced by changes in CaMKII activity in the hippocampus (Fuchs et al. 2005).

There are several factors that limit our ability to interpret the results of this study. While the A-B-A experimental paradigm allows us to test the involvement of neurogenesis in a previously drug-associated context, an additional A-B-C experiment would allow us to interpret whether the reduced drug-seeking results are exclusive to the drug-associated context or whether such behavior would be observed in a novel environment as well. We were also limited in our analysis of

protein levels due to sourcing whole tissue homogenates from tissue punches rather than taking synaptic fractions or specifying cytosolic versus nucleic expression. However, the findings for changes in CaMKII activity should be robust given existing evidence of autophosphorylated CaMKII being specifically localized to mediate activation of transcription factors and effects on learning and memory.

Conclusion

Together our findings support the hypothesis that abstinence following extended access to methamphetamine self-administration produces increased neurogenesis which contributes to drug seeking behavior during abstinence. More specifically, this study provides the first evidence for the role of neurogenesis in lever discrimination and drug seeking during context-induced reinstatement, but not cue-induced reinstatement. We further explored potential mechanisms underlying changes in behavior and found reductions in CaMKII activity in the dorsal dentate gyrus that could possibly explain memory deficits in rats with inhibited neurogenesis during context-induced reinstatement. Future work will address the alterations in the neuronal functioning of the granule cell neurons during abstinence and whether disruption of neurogenesis during abstinence will affect neuronal functioning of granule cell neurons to protect against propensity for relapse.

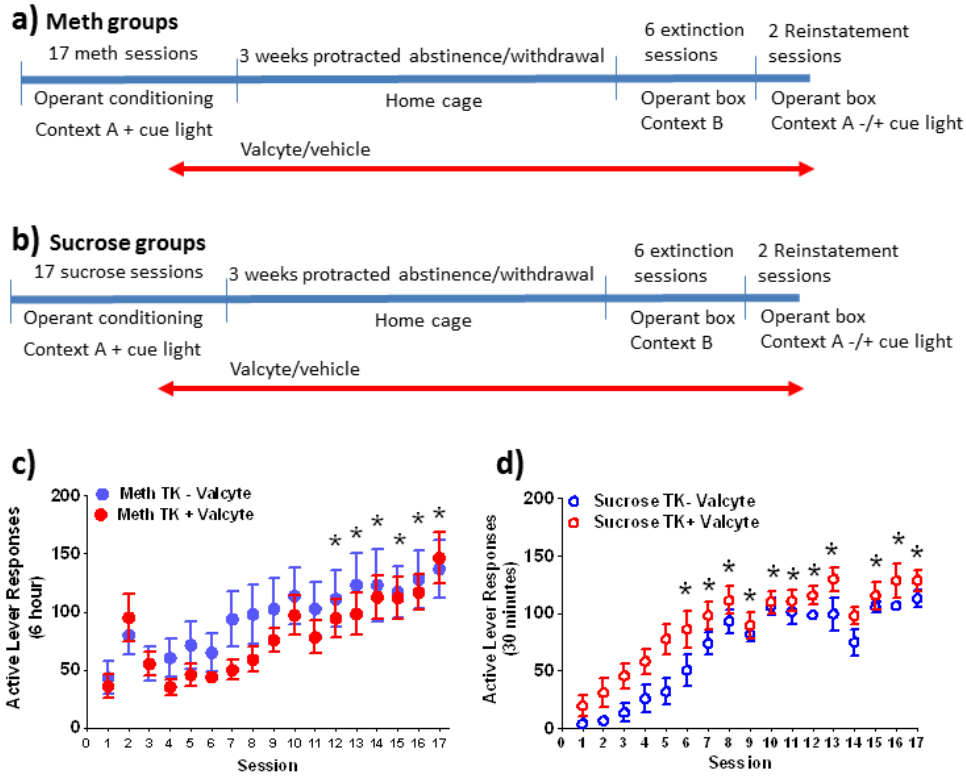

Figure 4.1: Rats that underwent extended access to methamphetamine self-administration escalated methamphetamine intake. (**a**) Schematic of experimental design for meth self-administration and (**b**) sucrose self-administration. (**c–d**) Active lever presses for meth and sucrose self-administration sessions. Meth TK-valcyte, n=16; Meth TK+valcyte, n=14; sucrose TK-valcyte, n=7; sucrose TK+valcyte, n=9. *p< 0.05 compared with initial sessions in the same animals by repeated measures two-way ANOVA followed by followed by Student-Newman-Keuls *post hoc* test. Data are represented as mean ± SEM.

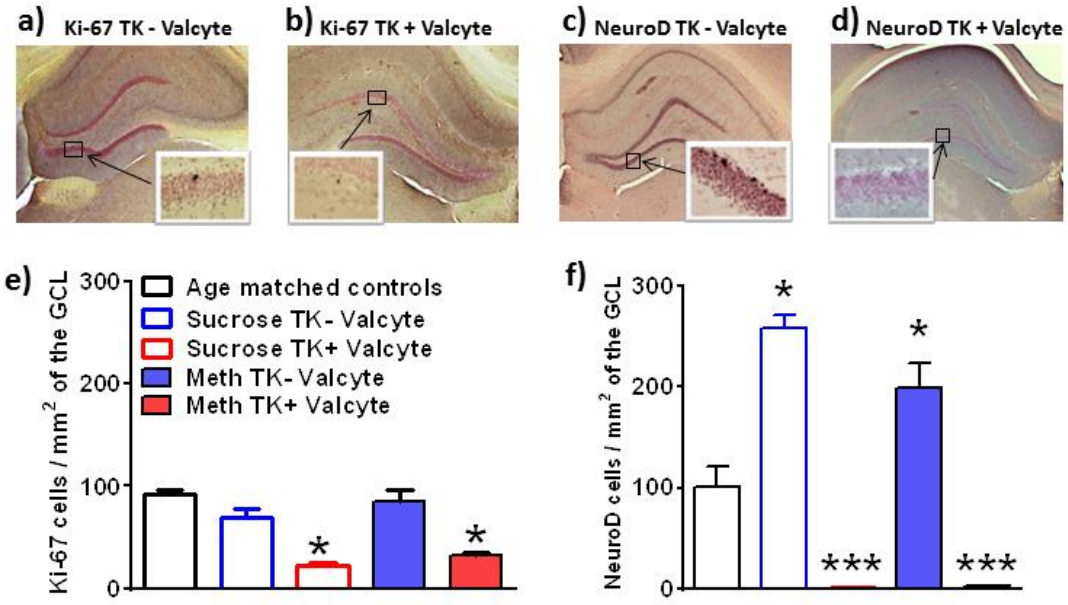

Figure 4.2: GFAP-TK rats that received valcyte had reduced proliferation and differentiation of dentate granule cells during abstinence compared to age matched naïve controls. (**a-d**) Example light microscopy images of immunohistochemically treated coronal hippocampal sections from animals exposed to meth self-administration used for quantification of cells expressing Ki-67 (**a-b**) and NeuroD (**e**) Quantification of dentate granule cells expressing Ki-67, showing a reduction in TK + valcyte animals. (**f**) Quantification of dentate granule cells expressing NeuroD, showing an increase in TK - valcyte animals and a significant reduction in TK + valcyte animals. Meth TK-valcyte, n=16; Meth TK+valcyte, n=14; sucrose TK-valcyte, n=7; sucrose TK+valcyte, n=9; controls, n=5. *p< 0.05, ***p< 0.001 compared with age matched naïve controls by unpaired t test. Data are represented as mean ± SEM.

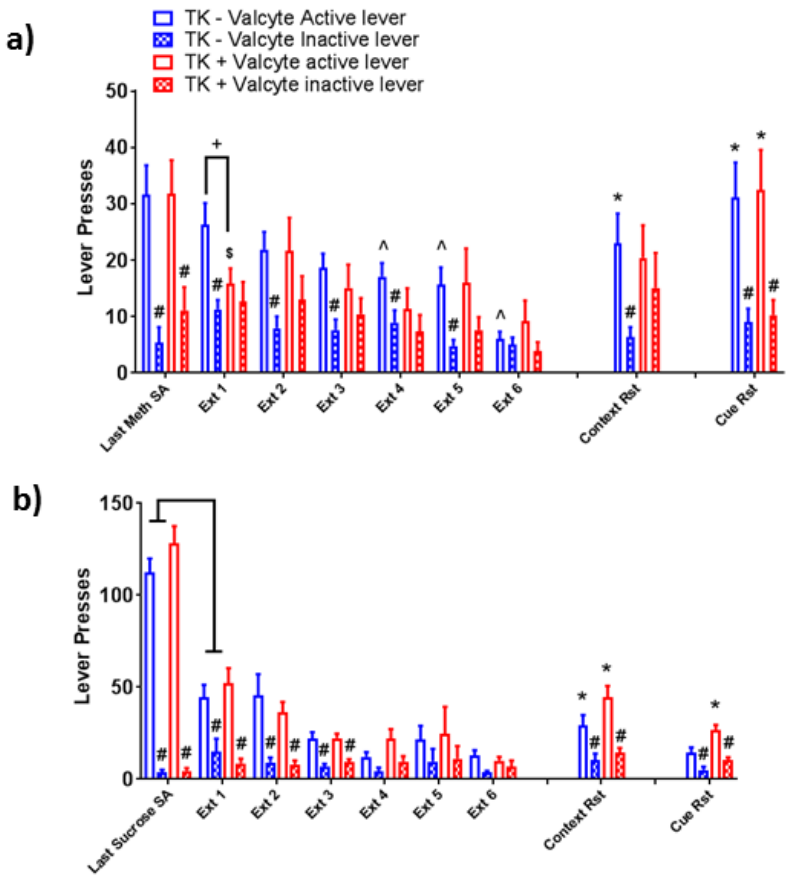

Figure 4.3: Inhibition of neurogenesis during abstinence led to changes in lever responses during extinction and reinstatement sessions in animals exposed to meth self-administration, but not sucrose self-administration. (**a**) TK-valcyte rats (blue) exposed to meth self-administration demonstrated extinction (reduction in active lever presses) and reinstatement (increase in active lever presses) of drug seeking in response to drug context and drug cued sessions. TK + valcyte rats (red) exposed to meth self-administration showed impaired lever discrimination behavior (no significant difference between active and inactive lever presses) during extinction sessions and drug context-induced reinstatement, but showed robust reinstatement to drug cues. (**b**) There were no significant differences in drug seeking behavior between TK - valcyte and TK + valcyte animals exposed to sucrose self-administration. Meth TK-valcyte, n=16; Meth TK+valcyte, n=14; sucrose TK-valcyte, n=7; sucrose TK+valcyte, n=9.*$p< 0.05$ vs. extinction day 6, #$p< 0.05$ vs. active lever presses, ^$p< 0.05$ vs. extinction day 1, +p< 0.05 vs. TK-valcyte active lever presses on extinction day 1, $p< 0.05 vs. last Meth SA session by repeated measures two way ANOVA and Student-Newman-Keuls posttests. Data are represented as mean ± SEM.

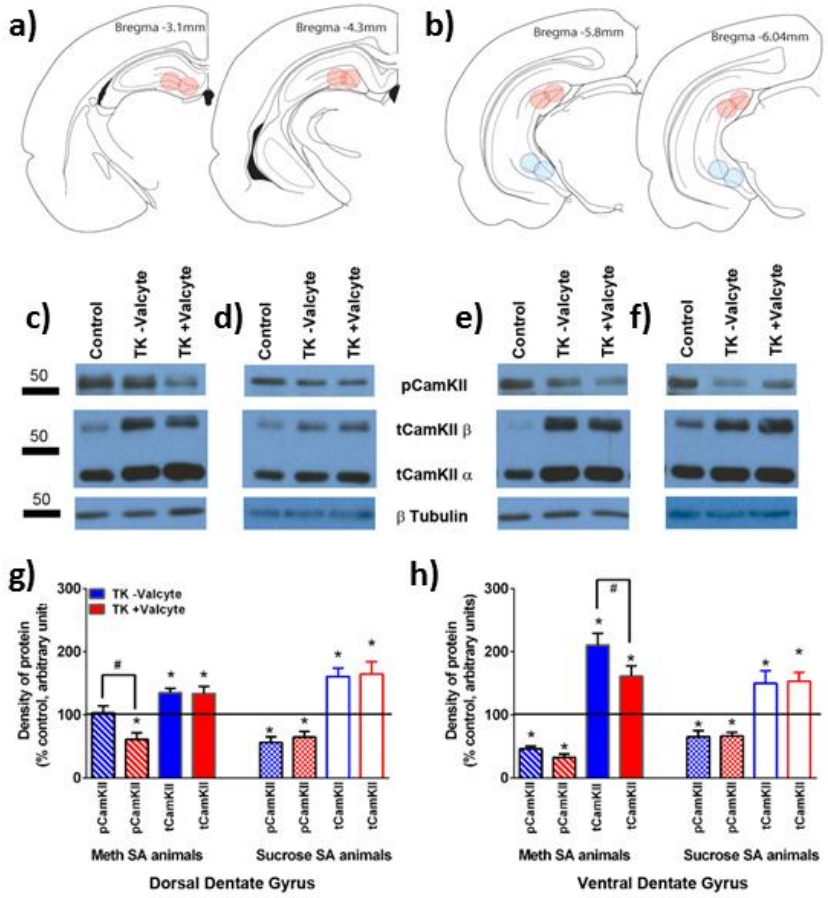

Figure 4.4: Potential molecular and cellular mechanisms underlying drug seeking behavior during context and cued reinstatement. (**a-b**) Schematic showing location of tissue punches taken in the dorsal (**a**) and ventral (**b**) dentate gyrus of the hippocampus. (**c-f**) Representative Western blots for protein expression in dorsal (**c-d**) and ventral (**e-f**) dentate gyrus enriched tissue of total and phosphorylated CaMKII (Thr-286) and β Tubulin in rats exposed to meth (**c, e**) and sucrose (**d, f**) self-administration. (**g-h**) Density of protein expression for total and phosphorylated CaMKII in dorsal (**g**) and ventral (**h**) dentate gyrus. Meth TK-valcyte, n=16; Meth TK+valcyte, n=14; sucrose TK-valcyte, n=7; sucrose TK+valcyte, n=9; control, n=5. *p< 0.05 vs. control, #p< 0.05 vs. TK-valcyte by repeated measures two way ANOVA and Student-Newman-Keuls posttests. Data are represented as mean ± SEM.

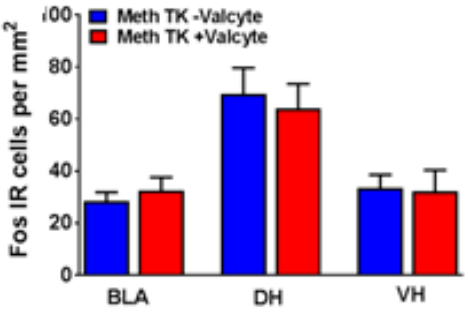

Figure 4.5: Quantification of cells in the basolateral amygdala (BLA), dorsal dentate gyrus (DH) and ventral dentate gyrus (VH) expressing cFos in brain tissue extracted one hour after the end of the cued reinstatement session. There were no significant differences in cFos expression between TK-valcyte and TK+valcyte groups. Meth TK-valcyte, n=16; Meth TK+valcyte, n=14; sucrose TK-valcyte, n=7; sucrose TK+valcyte, n=9; control, n=5. Data are represented as mean ± SEM.

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CHAPTER 5:

CONCLUSIONS

All the studies described in this dissertation investigated changes in neurogenesis and related cellular and molecular mechanisms during acute and protracted abstinence from methamphetamine. The goal was to find evidence in support of the hypothesis that adult hippocampal neurogenesis induced by abstinence from methamphetamine contributed to drug seeking behavior. The studies indeed support this hypothesis by showing how inhibiting or reducing neurogenesis during abstinence from meth can alter drug seeking during extinction and reinstatement. Neurogenesis was needed to exhibit normal extinction and reinstatement behavior. The changes in behavior were associated with changes in proteins important for synaptic plasticity and memory. The studies identify newborn dentate granule cells born during abstinence as aberrant contributors to drug seeking, specifically in response to drug-associated contexts.

Chapter 2 presented the investigation of acute abstinence from meth self-administration looking at mechanisms of maladaptive plasticity that could explain changes in neurogenesis during abstinence from meth. This study used both limited/short access (ShA) and extended/long access (LgA) to meth self-administration and showed that only LgA rats showed significantly enhanced brain derived neurotrophic factor (BDNF) in dorsal and ventral hippocampus. There was

also a reduction in activated GluN2B receptors only in the ventral hippocampus, which may be due to the different functional roles between the dorsal and ventral hippocampus. The dorsal hippocampus is more involved in spatial memory while the ventral hippocampus regulates anxiety and emotional memory. This subregion specificity is further highlighted by differences in expression of cell death factors. Both ShA and LgA animals showed an increase in anti-apoptotic protein Bcl-2 and concurrent decrease in pro-apoptotic protein Bax in the ventral hippocampus, which indicate a mechanism preventing cell death. We concluded that these data suggest methamphetamine self-administration initiates distinct allostatic changes in hippocampal subregions that may contribute to the altered synaptic activity in the hippocampus, which may facilitate negative affect and perpetuate the addiction cycle.

Chapter 3 presented the first study on the role of hippocampal neurogenesis during protracted abstinence from extended access to meth self-administration. We used a synthetic compound, isoxazole-9, to manipulate neurogenesis during abstinence from meth, and found that compared to vehicle, isx-9 reduced hippocampal neurogenesis down to control levels. This normalization of neurogenesis was associated with reduced drug seeking behavior during reinstatement as well as alterations in neuronal activity, structural plasticity, and expression of synaptic proteins important for learning and memory in the dentate gyrus. Furthermore, the behavioral effects and most of the cellular and molecular

effects of isx-9 were exclusive to high responders, rats that escalated meth intake demonstrating compulsive-like drug seeking, and not in low responders that maintained a baseline level of meth intake during self-administration sessions. We concluded that abstinence from escalating meth intake enhances neurogenesis, which produces a subset of neurons that could directly contribute to drug seeking behavior during abstinence in compulsive-like drug reinstatement.

Chapter 4 presented the second study investigating the role of enhanced hippocampal neurogenesis during protracted abstinence from extended access to meth self-administration. Using GFAP-TK transgenic rats we ablated neurogenesis with temporal control during abstinence from meth in order to test the functional role of neurogenesis in drug seeking behavior. Inhibition of neurogenesis caused impairments in lever discrimination during extinction in a novel non-drug associated context and during context reinstatement, but not during cued reinstatement in a drug-associated context. These behavioral effects in meth animals with inhibited neurogenesis were associated with reduced CaMKII activity in the dorsal dentate gyrus. CaMKII activity promotes synaptic plasticity which is important for hippocampal dependent learning and memory consolidation and could thus support drug seeking behavior in response to drug associated environments but not drug related cues. This is the first evidence supporting the role of abstinence induced neurogenesis in lever discrimination and drug seeking during context-induced reinstatement, and not cue-induced reinstatement.

These studies call for a shift in how the field views neurogenesis as a generally positive phenomenon promoting cognitive and emotional well-being. In contrast to previous neurogenesis research, these studies identify an aberrant form of enhanced neurogenesis during abstinence from methamphetamine which facilitates drug seeking in response to drug-associated contexts. Previously identified roles of neurogenesis in spatial discrimination may come into play given the result of impaired lever discrimination when neurogenesis was ablated. In addition, these studies also show contributions of neurogenesis to drug seeking when neurogenesis was normalized to control levels rather than completely inhibited. The role of neurogenesis in drug seeking, seems to be specific to responses to drug associated contexts rather than cues. Going forward, future studies may address biophysical properties of newborn cells borns during abstinence from meth and how they affect hippocampal circuitry. The studies presented in this dissertation open new avenues of research in the fields of neurogenesis and drug addiction.