THE ROLE OF ADULT NEUROGENESIS IN COCAINE ADDICTION

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DEDICATION

I dedicate this thesis to my husband Tim who has stood by me
through the joys and pains of research.

THE ROLE OF ADULT NEUROGENESIS IN COCAINE ADDICTION

by

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DISSERTATION

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The University of Texas Southwestern Medical Center at Dallas, 2008

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New neurons are born in the adult hippocampus in a region known as the subgranular zone (SGZ). This process is dynamically regulated and new neurons are thought to be important for certain types of spatial learning and memory. Proliferation of SGZ neural progenitors is decreased by drugs of abuse, yet it is not clear how the type and amount of drug as well as the pattern of administration changes long-term effects on neurogenesis. In addition, it is unclear what role if any SGZ neurogenesis plays in initiating drug-taking or relapse behaviors, or whether changes in neurogenesis are merely side effects of drug-taking.

I first examined effects of chronic cocaine self-administration and withdrawal on the different stages of neurogenesis. I found an early deficit in proliferation of neural progenitors, as well as a 4 week delayed increase in doublecortin-positive (DCX+) immature neurons which were common to both rats in withdrawal or those continuing to self-administer cocaine. I next asked the question of the functional consequence of

changes in adult hippocampal neurogenesis to the acquisition and maintenance of drug-taking, as well as relapse to drug-taking. I found that reduced adult neurogenesis via cranial irradiation prior to cocaine-taking was associated with increased acquisition of drug-taking and increased motivation for cocaine, but not sucrose, while reduced adult neurogenesis after rats have acquired cocaine self-administration was associated with increased resistance to extinction of drug-seeking behavior. Finally, I asked if formation of drug-context associations would be altered in rodents with reduced neurogenesis in a passive drug exposure paradigm. I found that a transgenic mouse with reduced adult neurogenesis has impaired long-term drug-context memory in the cocaine conditioned place preference paradigm (CPP).

Together these findings suggest that reduced adult hippocampal neurogenesis is a risk factor for drug addiction, that decreased proliferation after chronic drug intake likely contributes to drug-taking and drug-seeking behaviors, and that the delayed increase in immature neurons after drug-taking is likely protective against relapse. In sum, increases in adult hippocampal neurogenesis are beneficial both to the naïve and addicted brain, and therapeutics specifically increasing adult neurogenesis could aid in preventing initial addiction as well preventing future relapse.

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LIST OF ABBREVIATIONS

AC-3 – activated caspase-3

AraC – cytosine Arabinoside

ANOVA – analysis of variance

BDNF – brain-derived neurotrophic factor

BLA – basolateral amygdala

BrdU – 5-bromo-2’-deoxyuridine

CA1 – cornu ammonis region 1

CA3 – cornu ammonis region 3

Cre – cre recombinase

CMS – conditioned motor sensitization

CPP – conditioned place preference

CSA – cocaine self-administration

CONT – continuous

DAPI – 4',6-diamidino-2-phenylindole

DCX – doublecortin

DNA – deoxyribonucleic acid

DTA – diptheria toxin

EPM – elevated plus maze

FR – fixed ratio

FST – forced swim test

GABA – gamma-aminobutyric acid

GCL – granule cell layer

GFAP – glial fibrillary acidic protein

Hab – habenula

HPA – hypothalamic-pituitary-adrenal

IHC – immunohistochemistry

IRR – irradiation

I.p. – intraperitoneal

I.v. – intravenous

LTP – long-term potentiation

MAM – methylazoxymethanol

Mol – molecular layer

NAc – nucleus accumbens

Nestin-CreER^{T2} – nestin driving expression of cre recombinase fused to estrogen receptor

oGCL – outer granule cell layer

PBS – phosphate-buffered saline

PCR – polymerase chain reaction

PR – progressive ratio

RMS – rostral migratory stream

Sac – sacrifice

S.E.M – standard error of mean

SGZ – subgranular zone

SSA – sucrose self-administration

SVZ – subventricular zone

TAM – tamoxifen

Tg – transgenic

TrkB – tropomyosin related kinase B

VEGF – vascular endothelial growth factor

VTA – ventral tegmental area

WD – withdrawal

CHAPTER ONE
Introduction

COCAINE

History

Cocaine (benzoylmethyl ecgonine) is made from the erythoxylon coca plant, which is natively grown in South America (Karch, 2005). As early as 1500 years ago indigenous tribes continually chewed the coca leaves, which were 1% cocaine, as a mild stimulant (Bruckner and Peterson, 1977). Cocaine was isolated from coca leaves in 1859 by Dr. Albert Niemann, a graduate student of Dr. Friedrich Kohler at the University of Gottingen, (Karch, 2005) and it was soon touted by many, including Dr. Sigmund Freud, for it medicinal purposes. Cocaine was sold as an ingredient in wine tonics, cures of syphilis, whooping cough and morphine addiction, as well as in cough medicine (Haas, 1995). It was even used in Coca-Cola, developed by Dr. John Pemberton in 1886, although removed in 1906 once cocaine addiction became a well known side effect of use (Haas, 1995). Dr. Carl Koller in 1884 discovered cocaine had topical anesthetic properties and developed its use as eye anesthesia (Grzybowski, 2008), which is still used today. Medical usage of cocaine in modern times is still use sparingly when topically anesthesia is needed as well as vasoconstriction.

Modern day cocaine is rarely sold pure, and is often mixed with adulterants ranging from sugar to fertilizer (Bruckner and Peterson, 1977).

Cocaine can be self-administered in numerous ways: intranasally, intravenously alone or with heroin as “speedball”, smoke mixed with tobacco or marijuana cigarettes, smoked as crack cocaine, or even rubbed on mucus membranes when the nose has been damaged (Wesson and Smith, 1977). Cocaine is often used using while consuming alcohol, which leads to formation of the metabolite cocaethylene, which may produce both greater euphoria and toxicity than use of cocaine alone (Harris et al., 2003). Cocaine is broken down into norcocaine, which has many of the same properties as cocaine, and then is broken into inactive metabolites benzoylecgonine, as well as ecgonine methyl ester and ecgonine (Hawks, 1977).

Mechanism of cocaine’s effects

Cocaine mediates its pharmacological effects by binding to dopamine, serotonin, and norephinephrine reuptake transporters (Ritz et al., 1990), and causes an increase in these neurotransmitters at synapses between neurons. The “high” experienced by cocaine users is due to increased dopamine in particular between presynaptic terminals from the ventral tegmental area (VTA) and the postsynaptic receptors on neurons in the nucleus accumbens (NAc) (Wise, 1984), commonly known as the reward pathway. This dopaminergic projection, as well as glutamatergic projections from limbic regions including the prefrontal cortex,

hippocampus, and amygdala govern cocaine-taking and cocaine-seeking behaviors (Figure 1.1; (Everitt et al., 2008)).

Cocaine, due to binding multiple monoamine transporters both peripherally and in the brain, has many physiological effects. Acute effects of cocaine include euphoria, increased energy and alertness, decreased appetite, blood vessel constriction, increased body temperature, dilated pupils, and increased blood pressure (Byck and Van Dyke, 1977). Long term use of cocaine can result in addiction, irritability, paranoia, and auditory hallucinations (Siegel, 1977). Medical complications can include heart attack, heart rhythm alterations, respiratory failure, stroke, seizures, headaches, nausea, abdominal pain, and death due to overdose (Byck and Van Dyke, 1977; Siegel, 1977; NIDA, 2004).

Chronic cocaine use can result in both cognitive deficits, such as poor attention span, poor working memory, and impulsivity (Garavan and Hester, 2007), as well as drug addiction, which is defined as the inability to stop using a drug despite adverse consequences, including damage to health, finances, and family. There are currently no medications that cure cocaine addiction, leaving cocaine addicts with a high chance of relapse (Poling et al., 2007) after going “cold turkey” with or without psychotherapy or support groups, or substituting other drugs such as alcohol or nicotine. The high price tag of substance abuse to both society and family, coupled with the fact that 1 million Americans try a form of cocaine for the first time each year (NIDA, 2008), solidifies the necessity to

find therapeutics for cocaine addiction. This dissertation hopes to shed light on mechanisms of both cognitive deficits and addiction in a model of cocaine addiction by focusing on the impact of cocaine on a specific brain region, the hippocampus.

The hippocampus, a center for learning and memory

The hippocampus processes contextual information and is important for some forms of learning and memory (Eichenbaum et al., 1996) via its trisynaptic pathway. The first step in the pathway consists of input from entorhinal cortex via the medial and lateral perforant paths to the dentate gyrus. Excitatory granule cells of the dentate gyrus synapse onto neurons in layer CA3, making the mossy fiber pathway. CA3 neurons project to CA1, making the Schaffer-collateral pathway. CA1 then projects to the subiculum, which sends outputs to numerous brain regions, including important limbic regions such as the amygdala, prefrontal cortex, and nucleus accumbens, although CA1 itself can project to some of these regions (Figure 1.2; (Sahay and Hen, 2008)).

Effects of cocaine on the hippocampus

Long-term potentiation is a form of enhanced plasticity that occurs in the hippocampus and is altered by cocaine. Increased CA1 LTP occurs in hippocampal slices taken from rats that had self-administered cocaine 22 hours, 3 days, or 10 days prior (Thompson et al., 2004; del Olmo et al., 2006). However,

after 30 days of home cage withdrawal CA1, LTP was normal, and LTP was decreased after 100 days of withdrawal (Thompson et al., 2004). This suggests that the hippocampus may be primed to learn and remember things occurring during or shortly after drug taking, but may be less able to learn or remember things during long-term drug withdrawal. As cocaine use is associated with cognitive deficits (Garavan and Hester, 2007), these changes in hippocampal plasticity clearly impact function. A unique form of plasticity, addition of adult-generated neurons to the dentate gyrus, is also impacted by cocaine, as detailed below.

ADULT NEUROGENESIS

Adult neurogenesis has been discovered in many species, including humans (Eriksson et al., 1998), rats (Altman, 1962), mice (Messier et al., 1958), primates (Gould et al., 1999), lizards (Lopez-Garcia et al. 1988), and birds (Goldman and Nottebohm, 1983). There are two undisputed regions of adult neurogenesis in the rodent and human brain: the subgranular zone (SGZ) (Gould, 2007), which is in the inner layer of the dentate gyrus, and the subventricular zone (SVZ) (Gould, 2007), which occurs at the border of the lateral ventricle and the striatum. It is still controversial as to whether neurogenesis occurs brain regions besides the SGZ and SVZ, such as the hypothalamus, amygdala, striatum, and cortex (Gould, 2007), and which species this occurs in, and whether these regions

only have neurogenic potential after injury. Neural progenitors born in the SVZ migrate through the rostral migratory stream (RMS) and integrate into the olfactory bulb as interneurons in the granule cell layer. This occurs in both rodents (Doetsch et al., 1997) and humans (Curtis et al., 2007). It has been suggested that olfactory bulb neurogenesis may have some role in olfactory discrimination (Gheusi et al., 2000; Enwere et al., 2004), although that is controversial (Imayoshi et al., 2008).

The timecourse of adult neurogenesis and final type of neuron generated in the hippocampus differs from the olfactory bulb. Adult hippocampal neurogenesis is a tightly regulated multistep process. Neural stem cells expressing nestin (Type 1; Figure 1.3) asymmetrically divide to give rise to Type 2 neural progenitors (Figure 1.3; (Kempermann et al., 2004)). The rapidly dividing progenitor cells can be divided into two categories based on expression of markers: type 2a express nestin and type 2b no longer express nestin but express doublecortin (Figure 1.3; (Kempermann et al., 2004)). When type 2b cells become postmitotic and start growing dendrites and axons, they become type 3 cells (Figure 1.3; (Kempermann et al., 2004)). After about 4 weeks of age, these cells will have functional synapses onto CA3 cells, express mature neuronal markers, and become excitatory granule cells (Toni et al., 2008).

Stages of adult hippocampal neurogenesis

Newborn neurons with extended dendrites and axons have different physiological properties than developmentally generated neurons or younger neural progenitors. Neural progenitors less than 2 weeks old that lack developed axons and dendrites and are still excited by GABAergic inputs are unable to produce fast and large action potentials (Overstreet-Wadiche and Westbrook, 2006). Young neurons, approximately 2 weeks of age and older, can produce long-term potentiation (LTP) in the absence of GABA inhibitors (unlike older neurons) (Wang et al., 2000), and these neurons have a lower threshold for both LTP (Schmidt-Hieber et al., 2004) and long-term depression (LTD) (Schmidt-Hieber et al., 2004). Four to six week old neurons also have increased amplitude of LTP compared to older newborn neurons or developmentally generated neurons (Ge et al., 2007). This enhanced plasticity of young neurons suggests they may play a special role in learning and memory.

The proliferation, maturation, and survival of newborn neurons is complexly regulated. Basal levels of neurogenesis are different between species and strains of the same species (Kempermann et al., 1997), and neurogenesis decreases with age (Kuhn et al., 1996; Bizon and Gallagher, 2003). Circulating hormone levels (Cameron and Gould, 1994; Galea et al., 2006), as well as hippocampal levels of growth factors (Schmidt and Duman, 2007) and neurotransmitters (Powrozek et al., 2004) can dynamically influence

neurogenesis. Experiences such as stress (Dranovsky and Hen, 2006), treatment with drugs of abuse (Eisch and Harburg, 2006), learning (Dupret et al., 2007), exercise (van Praag et al., 1999), environmental enrichment, (Brown et al., 2003) and dietary restriction (Lee et al., 2000) also modulate neurogenesis. Long-term potentiation in the dentate gyrus increases neurogenesis (Bruel-Jungerman et al., 2006), as does injury such as seizures (Parent et al., 1997) or ischemia (Liu et al., 1998; Jin et al., 2001).

Effects of cocaine on adult hippocampal neurogenesis

In this thesis I will focus on the effects of one drug of abuse, the psychostimulant cocaine, on adult neurogenesis. Drugs of abuse, likely through direct toxicity, impair proliferation (Eisch et al., 2000; Nixon and Crews, 2002; Mandyam et al., 2008) or increase death of neural progenitors (Abrous et al., 2002). Cocaine, through oxidative stress, inhibits proliferation of neural progenitors by downregulating the cell cycle protein cyclin A2 and preventing the cell cycle transition from G1 to S (Lee et al., 2008). Interestingly, cocaine also inhibits proliferation of other dividing cell types, such as immune cells (Pacifici et al., 2003). Long-term effects of drugs of abuse on adult neurogenesis may be different between types of drugs due to different pharmacokinetics and pattern of administration, as well as effects on the hippocampal microenvironment such neurotransmitter and growth factor release (Powrozek et al., 2004).

Several studies have looked at effects of cocaine injections on neural progenitor proliferation, maturation, and survival. At least 8 days of chronic cocaine (20 mg/kg) given i.p. are necessary to reduce proliferation (Yamaguchi et al., 2005; Dominguez-Escriba et al., 2006). SGZ cells born immediately prior to or during the first week of a 24 day cocaine treatment are not affected by cocaine (Dominguez-Escriba et al., 2006), suggesting that cocaine impacts either type 1 or proliferating type 2 cells, and not more mature doublecortin+ (DCX+) cells (Figure 1.3). No changes were seen in dendrite morphology of DCX+ cells after chronic cocaine (Dominguez-Escriba et al., 2006), lending further support that immature neurons are not directly changed by cocaine. Mossy fiber synapse morphology was not changed after chronic cocaine (Dominguez-Escriba et al., 2006), suggesting signaling of mature dentate neurons to CA3 is normal after short-term chronic cocaine. Interestingly, glial fibrillary acidic protein-positive (GFAP)+ astrocytes in the dentate gyrus are increased in number, size, and complexity for at least 7 days, but not 14 days, after chronic cocaine (Fattore et al., 2002). As GFAP+ astrocytes are part of the neurogenic niche that support neurogenesis (Ma et al., 2005), and a proportion of those astrocytes are in fact type 1 stemlike cells (Garcia et al., 2004), determination of their contribution to regulation of neurogenesis after cocaine as beneficial or harmful will be important.

These previous studies suggest cocaine impairs hippocampal neurogenesis, but it is unclear whether this is simply a meaningless side effect of drug use or if this deficit causes cognitive deficits or promotes addictive behaviors. There is correlational evidence that there is an inverse relationship between drug-taking and drug-seeking and adult hippocampal neurogenesis. For example, manipulations known to increase neurogenesis, such as environmental enrichment (Brown et al., 2003), voluntary exercise (van Praag et al., 1999), and chronic treatment with antidepressants (Malberg et al., 2000) decrease drug-taking and drug-seeking (Kanarek et al., 1995; Baker et al., 2001; Green et al., 2002; Stairs et al., 2006; Smith et al., 2008). Conversely, manipulations that decrease neurogenesis, such as stress (Mirescu and Gould, 2006), increase drug-taking and drug-seeking (Tsien et al., 1996; Covington and Miczek, 2005). Functional studies determining that adult hippocampal neurogenesis has a role in learning, memory, or motivation would increase the clinical relevance of studies finding cocaine-altered proliferation in the dentate gyrus (Fattore et al., 2002; Yamaguchi et al., 2005; Dominguez-Escriba et al., 2006).

Methods of inhibiting adult neurogenesis

Study of the function of adult hippocampal neurogenesis require methods of ablating neurogenesis. Three available methods offer various benefits and drawbacks: mitotic inhibitors, cranial irradiation, and transgenic mouse models.

Mitotic inhibitors such as cytosine-b-D-arabinofuranoside (AraC) and methylazoxymethanol (MAM) have been used, however systemic administration causes illness and weight loss due to inhibition of proliferation in the gastrointestinal, immune, and circulatory systems (Dupret et al., 2005). Intracranial administration (Doetsch et al., 1999) can prevent these peripheral side effects, however this is more time-intensive and expensive. Regardless of route of administration, this method requires chronic administration, and neurogenesis resumes shortly after discontinuation of the drugs, due to rapidly dividing progenitors, and not stem cells, being the target of the drugs (Dupret et al., 2005). In addition, neurogenesis in the SGZ and SVZ, as well as any other area of proliferation, would be reduced, prohibiting regional specificity.

Cranial irradiation can induce permanent, irreversible suppression of neurogenesis depending on the paradigm used. It is the most effective method of neurogenesis suppression. Two methods have been recently used to protect proliferating cells in other parts of the body from irradiation exposure and subsequent death. Lead shielding can be used to protect regions of the head and body from irradiation while leaving a strip exposed over the hippocampus (Santarelli et al., 2003). Alternatively, irradiation can be focused in a small region over the hippocampus, such a 1 cm diameter circle (Snyder et al., 2005).

Variations in irradiation paradigms change the amount and endurance of neurogenesis suppression. Moderate doses of irradiation (7.5-10 Gy) provide

robust and permanent suppression of neurogenesis, but very high doses (25 Gy or higher) induce side effects months later including gliosis, vasculature damage (Chambers et al., 2005), demyelination (Panagiotakos et al., 2007) and necrosis (Yang et al., 2000). Paradigms delivering two consecutive days of irradiation provides more robust and permanent suppression of neurogenesis than one day of irradiation, likely due to targeting and killing quiescent stem cells on the second day of irradiation (Wojtowicz, 2006). The main drawbacks to the use of moderate doses of irradiation is inflammation, which lasts at least one month (Meshi et al., 2006; Airan et al., 2007), as well as reduced weight gain (Snyder et al., 2005). It is important to note that irradiation at moderate doses does not alter LTP between either CA3-CA1 synapses or dentate gyrus-CA3 synapses using a weak stimulus (4 trains of 1 sec 100 Hz stimulations every 15 sec; Saxe et al., 2006; 4 trains of 0.5 sec 100 Hz stimulations every 20 sec; Wang et al., 2005). In addition, neither synaptic release probability as measured by paired-pulse facilitation (Saxe et al., 2006) and paired-pulse depression (Wang et al., 2008), nor input-output curves (Wang et al., 2008; Saxe et al., 2006; Snyder et al., 2005; Wang et al., 2005) were different between irradiated and sham rodents, suggesting functional properties of adult neurons in the hippocampus are not impacted by irradiation. Benefits of irradiation compared to other methods of suppressing neurogenesis, such as AraC or transgenic mouse, include the ability to target hippocampal neurogenesis without ablating olfactory bulb neurogenesis, and the efficiency of the method.

There are several transgenic models of neurogenesis ablation available. Two involve using expression of diphtheria toxin, although one targets and kills neural stem cells (nestin-$CreER^{T2}$/floxedSTOP-DTA mice; laboratory of Amelia Eisch, unpublished) and the other targets and kills neural progenitors neurons once they mature into neurons (nestin-$CreER^{T2}$/floxedSTOP-NSE-DTA mice; (Imayoshi et al., 2008)) Other methods include gancyclovir-mediated cell death of stem cells (GFAP TK tg mice; (Saxe et al., 2006)) and overexpression of Bax, a protein involved in cell death (Bax tg mice; (Dupret et al., 2008)). These transgenic models have the caveats of being time-consuming, necessitating expensive minipumps or chronic i.p. injections, and also do not suppress neurogenesis to the same extent that irradiation does. Finally, these methods reduce neurogenesis both in the hippocampus and the olfactory bulb.

In all, functional studies of neurogenesis will require validation using at least two models, likely irradiation and a transgenic model. Future models transgenic mice that selectively increase neurogenesis to probe the therapeutic effects of increasing neurogenesis on behavioral tasks.

Function of adult hippocampal neurogenesis

Many studies have attempted to find a function for adult hippocampal neurogenesis; however the various combinations of methods of neurogenesis ablation, age and species used, and methods of behavior testing have produced

conflicting results. There are an equal number of studies using hippocampal-dependent learning and memory, such as the Morris water maze, Barnes maze, and passive avoidance that have found impaired or no change in learning and memory (Table 1.1). Interestingly, there is even a study that suggests some forms of complex hippocampal learning are improved when neurogenesis is suppressed (Saxe et al., 2007). The most consistent finding is that rodents with reduced hippocampus neurogenesis impairs learning of contextual fear conditioning (Saxe et al., 2006; Winocur et al., 2006; Imayoshi et al., 2008).

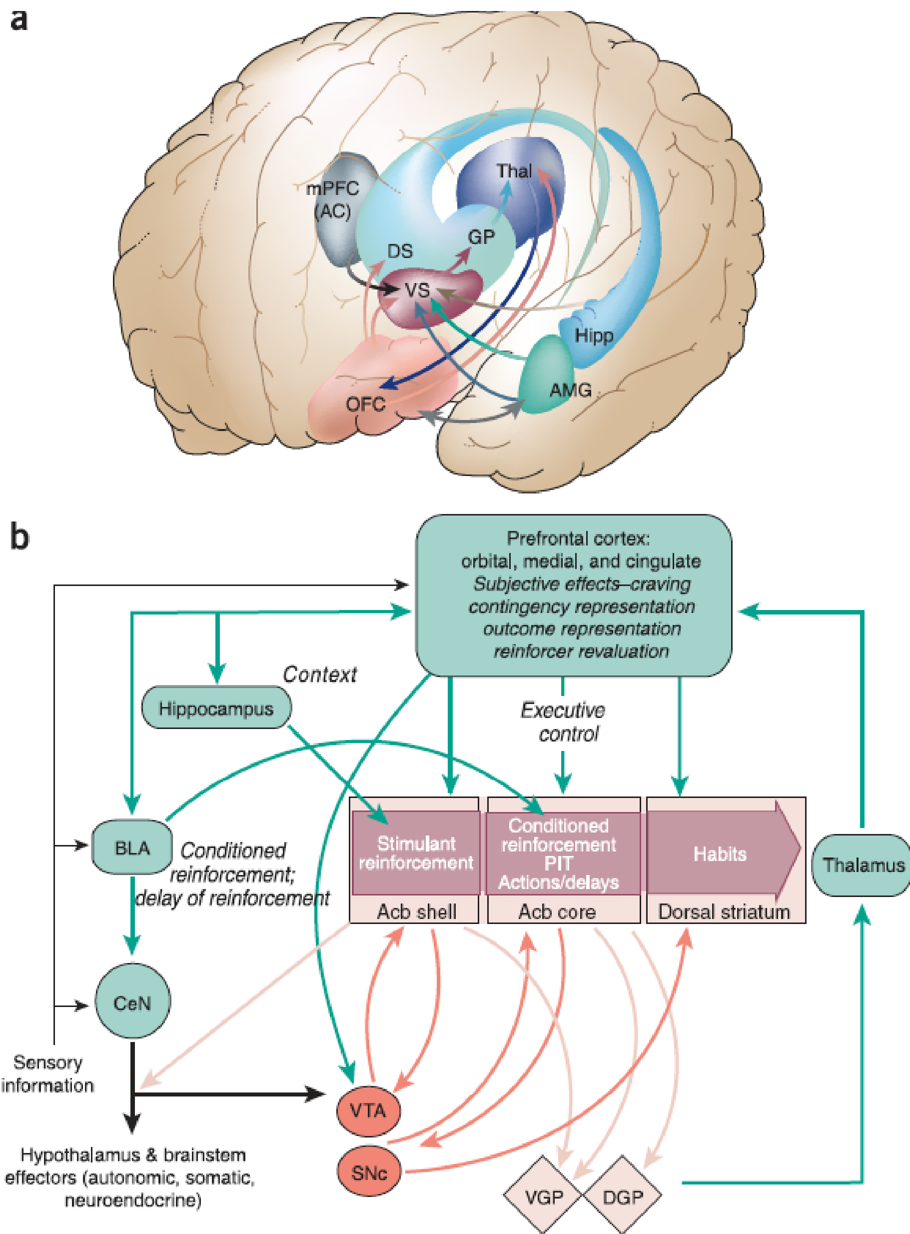
While most studies have looked at the role of neurogenesis in intrahippocampal processing, it is possible that altered neurogenesis could change neural circuitry influenced by the hippocampus's glutamatergic projections. For example, the hippocampus has glutamatergic projections to limbic regions such as the prefrontal cortex, amygdala, and nucleus accumbens (Figure 1.2; (Sahay and Hen, 2008)) that could influence mood and motivated behaviors. Indeed, it is known that activation of hippocampal output neurons increases drug-seeking behaviors (Vorel et al., 2001) as well as behavioral sensitization to stimulants (Lodge and Grace, 2008). Thus, alteration of neurogenesis could impact both intrahippocampal processing of the contextual stimuli as well as alter motivated behavior triggered by those stimuli. However, as of yet no study has found decreased adult hippocampal neurogenesis increases anxiety or depressive-like behaviors (Table 1.2).

ORGANIZING HYPOTHESIS

Adult neurogenesis is a young field, and it is still not clear how neurogenesis is impacted by external stimuli, and what the contribution of adult-generated neurons are to both hippocampal and systems level circuitry and behavior. The work presented in this dissertation will expand on these questions by investigating how neurogenesis is regulation by chronic cocaine self-administration and withdrawal, and testing the hypothesis that adult hippocampal neurogenesis has a role in cocaine self-administration and relapse, as well as drug-context memory in the cocaine conditioned place preference paradigm.

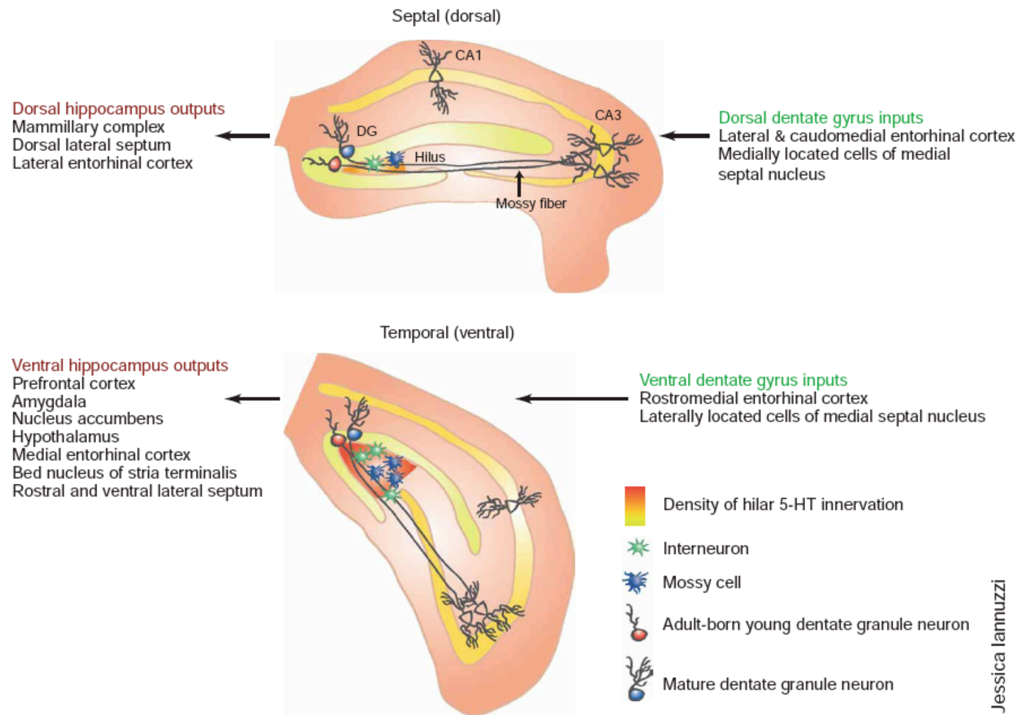
Chapter 1: Figures

Figure 1.1



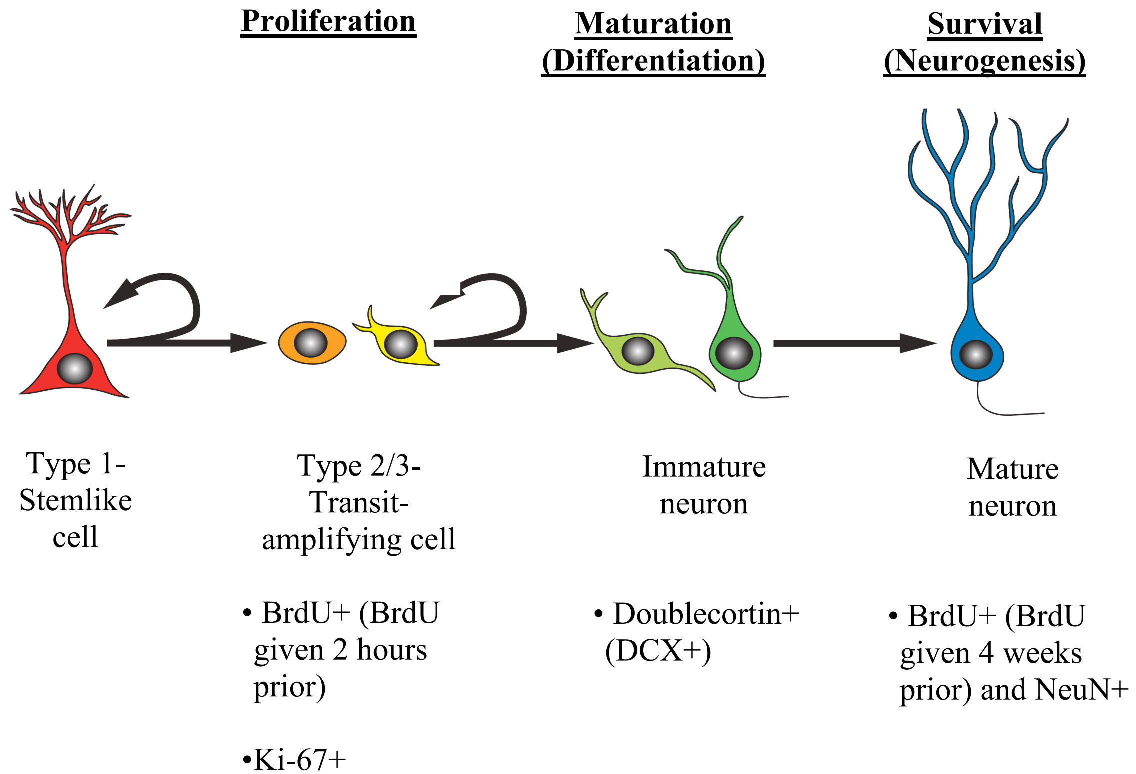
Neural circuitry of drug reinforcement. Glutamate from the prefrontal cortex, hippocampus, and amygdala, as well as dopamine from the ventral tegmental area, converge on the ventral striatum (nucleus accumbens) to control drug-taking and drug-seeking behavior. Green/blue arrows, glutamatergic projections; orange arrows, dopaminergic projections; pink arrows, GABAergic projections; Acb, nucleus accumbens; AMG = amygdala, BLA = basolateral amygdala; CeN = central nucleus of the amygdala; VTA = ventral tegmental area; SNc = substantia nigra pars compacta. GP = globus pallidus (D = dorsal, V = ventral); Hipp = hippocampus, mPFC = medial prefrontal cortex, AC = anterior cingulate cortex, OFC = orbitofrontal cortex, VS = ventral striatum, DS = dorsal striatum, Thal = thalamus. (Adapted from (Everitt et al., 2008)).

Figure 1.2



Hippocampal inputs and outputs. The hippocampus receives glutamatergic input from the entorhinal cortex, cholinergic and GABAergic input from the medial septal nucleus, serotonergic input from the raphe and dopaminergic input from the ventral tegmental area. Hippocampal outputs are numerous, but are distinct between the dorsal and ventral hippocampus. (Adapted from Sahay & Hen, 2008).

Figure 1.3



Stages of adult hippocampal neurogenesis. Type 1- stemlike cells divide infrequently and give rise to rapidly dividing type 2/3 transit-amplifying cells. Type 2/3 cells mature into postmitotic immature neurons. After growth of axons and dendrites and integration into hippocampal circuitry, newborn neurons become mature neurons at 4 weeks of age. BrdU = bromodeoxyuridine. (Adapted from Nathan DeCarolis).

Chapter 1: Tables

Table 1.1

Test	Results	Method	Species	Author
Barnes Maze	No recall 1 weeks later	Transgenic mouse	Nestin-CreERT2/floxed STOP-NSE-DTA mice	Imayoshi, 2008
Barnes Maze	Learning impaired	IRR	Mouse	Raber, 2004
Morris Water Maze	No recall 2 weeks later	IRR	Rat	Snyder, 2005
Morris Water Maze	Learning impaired	Transgenic mouse	Bax tg mice	Dupret, 2008
Morris Water Maze	No effect	IRR	Mouse	Raber, 2004
Novel object recognition	No effect	MAM	Rat	Bruel-Jungerman, 2005
Novel object recognition	No effect	IRR	Mouse	Raber, 2004
Y Maze	No effect	IRR	Mouse	Saxe, 2006
8-armed radial maze	Learning improved	IRR	Mouse	Saxe, 2007
Fear cond. (contextual)	Learning impaired	IRR	Mouse	Saxe, 2006
Fear cond. (contextual)	Learning impaired	IRR	Rat	Winocur, 2006
Fear cond. (contextual)	Learning impaired	Transgenic mouse	GFAP-TK tg mice	Saxe, 2006
Fear cond. (contextual)	Learning impaired	Transgenic mouse	DTA	Imayoshi, 2008
Fear cond. (contextual)	No effect	Transgenic mouse	Bax mice	Dupret, 2008
Fear cond. (contextual)	No effect	MAM	Rat	Shors, 2001

Fear cond. (trace)	Learning impaired	MAM	Rat	Shors, 2001
Eyeblink cond. (trace)	Learning impaired	MAM	Rat	Shors, 2001
Passive avoidance	No effect	IRR	Mouse	Raber, 2004
NMTS	No effect	IRR	Rat	Winocur, 2006
Delayed NMTS	Learning impaired	IRR	Rat	Winocur, 2006

Function of adult hippocampal neurogenesis in learning and memory. Studies of ablation of adult hippocampal neurogenesis have found mixed results on test of learning and memory. Only irradiation studies presented using doses less than 25 Gy which do not induce brain injury, irradiate at adulthood, and wait at least a month before behavioral testing to reduce inflammation are presented here.IRR = irradiation, MAM = methylazoxymethanol.

Table 1.2

Test	Results	Method	Species	Author
FST	No effect	IRR	Rat	Airan, 2007
EPM	No effect	IRR	Mouse	Raber, 2004
EPM	No effect	IRR	Mouse	Saxe, 2006
Light-dark box	No effect	IRR	Mouse	Saxe, 2006
Open field	No effect	IRR	Mouse	Raber, 2004
Latency to feed	No effect	IRR	Mouse	Santarelli, 2003
Grooming latency	No effect	IRR	Mouse	Santarelli, 2003
Coat score	No effect	IRR	Mouse	Santarelli, 2003

No function of adult neurogenesis in mood regulation. Irradiation studies using mice have not found any change in anxiety or depression. Other tests of mood regulation, as well as other methods of neurogenesis ablation and testing in rats are needed. FST = forced swim test, EPM = elevated plus maze.

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CHAPTER TWO
Impact of cocaine self-administration and withdrawal on adult neurogenesis

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INTRODUCTION

Cocaine addiction takes a devastating toll on society (Chychula and Okore, 1990). Cocaine-induced neuroadaptations are the focus of much research, as they may indicate novel treatment options for the persistent cognitive (Rogers and Robbins, 2001; Lawton-Craddock et al., 2003; Verdejo-Garcia et al., 2006) and olfactory deficits (Schwartz et al., 1989; Bauer and Mott, 1996; Podskarbi-Fayette et al., 2005) as well as the extraordinarily high incidence of relapse (Aharonovich et al., 2006) seen in addicts.

One neuroadaptation that may contribute to the constellation of deficits seen in cocaine addicts is altered adult neurogenesis. Two major sources of neurogenesis in the adult brain are the subgranular zone (SGZ), which generates hippocampal granule neurons, and the subventricular zone (SVZ), which generates olfactory bulb neurons (Gheusi et al., 2000; Petreanu and Alvarez-Buylla, 2002; Enwere et al., 2004). Neurogenesis is now appreciated as a process, not a time point (Abrous et al., 2005; Ming and Song, 2005). The modulation of SGZ and SVZ neurogenesis can impact hippocampal-based cognitive function

(Leuner et al., 2006) and olfaction (Gheusi et al., 2000; Shingo et al., 2003; Enwere et al., 2004) respectively. The hippocampus also plays a role in cocaine addiction and relapse (Canales, 2007). Interestingly, repeated experimenter-delivered intraperitoneal cocaine injections decrease SGZ proliferation (Yamaguchi et al., 2005; Dominguez-Escriba et al., 2006), yet no studies have examined whether the more clinically relevant paradigm of intravenous cocaine self-administration influences the process of SGZ neurogenesis.

Drug addiction is characterized by acquisition and maintenance of drug taking, followed by abstinence and subsequent relapse (Canales, 2007). A goal for drug addiction research is to identify pathways commonly used by all drugs of abuse, so as to generate more effective therapeutics for multisubstance abusers (Nestler, 2005). It is likely that the role of the hippocampus, and specifically adult neurogenesis, in memory (Bruel-Jungerman et al., 2007), as well as mood regulation (Becker and Wojtowicz, 2007), contribute to the acquisition of drug taking and relapse. Adult neurogenesis is modulated by heroin (Eisch et al., 2000), nicotine (Abrous et al., 2002), and alcohol self-administration (Crews et al., 2004), as well as passive administration of these and many other drugs of abuse (Eisch and Harburg, 2006). Thus, modulation of adult neurogenesis in the dentate gyrus may be an intriguing common pathway in drug addiction to which therapeutics should be targeted.

In this study, we examine the effects of cocaine self-administration (CSA) on the process of adult neurogenesis. We compare changes after 3 weeks of drug taking with changes that persist or arise after 4 weeks of either withdrawal (CSA-WD) or continued cocaine self-administration (CSA-CONT). By analyzing stages of adult neurogenesis – such as proliferation and survival of adult-generated neurons – we reveal enduring effects of cocaine self-administration that are only partially influenced by whether drug taking stops or continues. These data suggest that adult-generated neurons should be considered for their potential role in cocaine addiction and hippocampal-mediated relapse after cocaine withdrawal.

METHODS

Animals

Fifty-one male Sprague-Dawley rats (300-325 g, Charles River, Kingston, NY) were individually housed in a climate-controlled environment on a 12 hour light-dark cycle (lights on at 0700 hours). Rats were acclimated to vivarium conditions for at least one week prior to experimentation. Rats were allowed free access to water and lab chow, except during initial lever-press training as described below. All experiments were performed in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* in an Institutional Animal Care and Use Committee and Association for

Assessment and Accreditation of Laboratory Animal Care approved facility at UT Southwestern Medical Center.

Surgery

Rats were implanted with a chronic indwelling intravenous catheter, as described previously (Sutton et al., 2003; Edwards et al., 2007b). Briefly, rats were anesthetized with sodium pentobarbital (50 mg/kg, intraperitoneal (i.p.)) and atropine sulfate (0.10 mg/rat, subcutaneous) prior to surgical implantation of the catheter in the jugular vein. Following surgery, rats received a prophylactic injection of penicillin (60,000 IU/0.2 ml, intramuscular), and antibiotic ointment was applied daily to the surgical area. Catheters were flushed daily with 0.2 ml of heparinized (20 IU/ml) bacteriostatic saline containing gentamycin sulfate (0.33 mg/ml) to curb infection and preserve catheter patency.

Self-administration apparatus

The operant test chambers used for self-administration (Med Associates, Georgia, VT) were each enclosed in a ventilated, sound-attenuating box. Boxes were equipped with a Razel Model A injection pump (Stamford, CT) and a 10-ml glass syringe connected to a fluid swivel (Instech, Plymouth, PA) with Teflon tubing. Tygon tubing connected the swivel to the rat's catheter exit port and was enclosed by a metal spring secured to Teflon threads on the catheter assembly.

Each operant chamber contained two response levers located 2 cm off the floor. During self-administration, a response at the active lever delivered an intravenous cocaine infusion (or for control rats, a saline infusion), while a response at the inactive lever had no programmed consequence. Each cocaine or saline infusion was delivered over 5 s in a 0.1 mL volume. During the 5 s infusion, a cue light above the lever was illuminated and the house light was extinguished. This cue period was followed by an additional 10 s time-out interval when the house light remained off and responding at the active lever had no programmed consequences. The illumination of the house light signaled the end of the 15 s infusion time-out interval.

Cocaine self-administration

To facilitate acquisition of cocaine self-administration, rats were initially maintained on a restricted diet (~15 g of chow per day) for 3 successive days and trained to press the active lever for 45 mg sucrose pellets until they reached acquisition criterion (100 pellets for 3 consecutive days). After food training, rats were fed *ad libitum* at least 1 day prior to surgical catheterization and allowed to recover for 1 week before the onset of testing.

Cocaine hydrochloride was generously provided by the National Institute on Drug Abuse (Baltimore, MD). Rats were allowed to acquire cocaine self-administration (0.5 mg/kg infusion, intravenous (i.v.)) on a fixed-ratio

reinforcement schedule (e.g. one active lever press led to a single infusion followed by a 10 s time-out) in 4 hour daily sessions for 3 weeks (5 days/week). Reinforcements (delivery of drug or saline after active lever press) were capped at a maximum of 95 per 4 hour session.

Fifty-one rats were run for self-administration (Saline n = 24, Cocaine n = 27). The data presented here are replicates of two experiments. In the first experiment, rats were randomly assigned to either CSA (3 weeks cocaine self-administration; Figure 2.1A) or CSA-WD (3 weeks cocaine self-administration plus 4 weeks of withdrawal; Figure 2.1B). In the second experiment, rats were run in parallel and split into three complementary groups to ensure a similar average cocaine intake the last five days before BrdU injection: CSA, CSA-WD and CSA-CONT (continuous cocaine self-administration for 7 weeks; Figure 2.1C). As described in the results section, this allowed the CSA rats to serve as control for the initial BrdU labeling for the CSA-WD and CSA-CONT groups. The final number of rats in each group assessed for this study is CSA: n=7, Saline, n=8 Cocaine; CSA-WD: n=16 Saline-WD, n=12; and CSA-CONT, n=4, Saline-CONT, n=4. Catheter patency was verified for all rats after the final self-administration session via a 0.1 mL intravenous infusion of the short-acting barbiturate sodium methohexital (10 mg/mL), which induces a rapid loss of muscle tone.

BrdU injections and tissue preparation

As shown in Figure 2.1A-C, fifteen hours after the last self-administration session, all rats in the CSA, CSA-WD, and CSA-CONT groups received one 150 mg/kg i.p. injection of the S-phase marker bromodeoxyuridine (BrdU; Boehringer Mannheim, Mannheim, Germany). The CSA group was sacrificed 2 hours after BrdU injection to allow quantification of proliferating cells stained via BrdU immunohistochemistry (IHC; Figure 2.1A). In contrast, the CSA-WD and CSA-CONT groups were sacrificed 4 weeks after BrdU injection to allow quantification and assessment of surviving neurons stained via IHC for BrdU and the neuronal marker NeuN. The only difference between the CSA-WD and CSA-CONT groups was that after the BrdU injection and before sacrifice 4 weeks later, the CSA-WD rats were placed in their home cages (Figure 2.1B), whereas CSA-CONT rats continued self-administration of saline or cocaine (Figure 2.1C). Rats were sacrificed either via chloral hydrate anesthesia and intracardial perfusion with 0.1 M PBS (5 min with 10 ml/min flow rate) and 4% paraformaldehyde in 0.1 M PBS (15 min) or decapitation. After sacrifice, brains were removed and postfixed in 4% paraformaldehyde in 0.1 M PBS for at least 24 hours at 4 ºC. Brains were cryoprotected in 30% sucrose in 0.1 M PBS with 0.1% NaN_3 at 4ºC until coronal sectioning on a freezing microtome (Leica, Wetzlar, Germany) at 30 µm through the entire hippocampus (-1.80 to -7.64 mm from bregma; (Paxinos

and Watson, 1997)) and sections were stored in 0.1% NaN_3 in 0.1 M PBS at 4°C until processed for IHC.

Immunohistochemistry

IHC was performed as previously described (Eisch et al., 2000; Mandyam et al., 2004). Briefly, every ninth section of the hippocampus was mounted on glass slides (Fischer Superfrost/Plus, Hampton, NH) and allowed to dry overnight. Slides were coded prior to IHC and code was not broken until after microscopic analysis. For BrdU IHC, three pretreatment steps were used: antigen unmasking (0.01M citric acid, pH 6.0, 95°C, 15 min), membrane permeabilization (0.1% trypsin in 0.1M Tris and 0.1% $CaCl_2$, 10 min), and DNA denaturation (2M HCl in 1 X PBS, 30 min). For Ki-67 and doublecortin (DCX) IHC, pretreatment was limited to antigen unmasking. After pretreatment, sections were rinsed and immediately placed into blocking (3% serum) and then into primary antibody incubation overnight at room temperature. The primary antibodies used were rat anti-BrdU (Accurate, Westbury, NY; 1:100), mouse anti-BrdU (Becton-Dickinson, San Jose, CA; 1:100), rabbit anti-activated caspase 3 (AC-3; Cell Signaling Technology, Beverly, MA; 1:250), rabbit anti-Ki-67 (Vector Labs, Burlingame, CA; 1:500); mouse anti-NeuN (Chemicon, Temecula, CA; 1:50), and goat anti-doublecortin (Santa Cruz, Santa Cruz, CA; 1:3500). These antibodies were used to assess cells in stages of neurogenesis, including proliferating cells,

immature and mature neurons, and cells that had undergone cell death. For example, BrdU permanently labels cells in S phase at time of injection (Wojtowicz and Kee, 2006), and thus the BrdU antibody labels proliferating cells in the CSA group (injected 2 hours prior to sacrifice) and surviving cells in CSA-WD and CSA-CONT groups (injected 4 weeks prior to sacrifice). Ki-67 is an endogenous cell cycle protein, and thus the Ki-67 antibody labels proliferating cells in most phases of the cell cycle (Kee et al., 2002). When BrdU IHC is used to label surviving cells (e.g. BrdU given 4 weeks prior to sacrifice), Ki-67 IHC is often used as a reliable alternative and non-toxic measure of proliferation in the same tissue (Eisch and Mandyam, 2007). DCX is an endogenous cytoskeletal protein, and therefore the DCX antibody labels immature neurons (Brown et al., 2003). Finally, the NeuN antibody labels mature neurons (Mullen et al., 1992), and the AC-3 antibody labels apoptotic cells (Olney et al., 2002). Specificity of staining for each antibody was ensured by lack of signal after omission and/or dilution of primary antibody and by observation of expected subcellular localization and cellular populations stained for each antibody (Donovan et al., 2006; Lagace et al., 2006; Harburg et al., 2007; Lagace et al., 2007).

For BrdU or Ki-67 single labeling, primary incubation was followed by incubation in a biotinylated secondary (goat anti-mouse, Sigma-Aldrich, St. Louis, MO; or goat anti-rabbit, Vector Labs; 1:200), and visualization was accomplished with the avidin-biotin/diaminobenzidine method (Pierce, Rockford,

IL), followed by counterstaining with Fast Red (Vector Labs). For BrdU and NeuN double labeling, primary incubation was followed by incubation in fluorescent secondary antibody (Cy3 donkey anti-rat, and Cy5 donkey anti-mouse; Jackson Immunoresearch, Westgrove, PA; 1:200) and counterstaining with DAPI (Roche, Basel, Switzerland; 1:5,000) or green Nissl (Molecular Probes, Carlsbad, CA; 1:200). For DCX and AC-3 double labeling, primary incubation was followed by incubation in a biotinylated secondary for DCX (horse anti-goat, Vector Labs; 1:200), incubation with avidin-biotin and CY2 or CY3 tyramide signal amplification (Perkin-Elmer, Norton, OH), a fluorescent secondary antibody for AC-3 (Cy3 or Cy2 donkey anti-rabbit, Vector Labs; 1:200), and DAPI as a counterstain. Sections underwent rapid dehydration in ethanols and defatting in Citrosolv (Fischer) prior to coverslipping with DPX (Sigma-Aldrich).

Quantification of immunopositive (+) cells

Counts of BrdU+, Ki-67+, AC-3+, and DCX+ cells were performed at 400x magnification with an Olympus BX-51 microscope while continually adjusting the focal plane through the depth of the section. An observer blind to treatment group performed all cell counts.

BrdU+, Ki-67+, and AC-3+ cell counts were collected using the optical fractionator method. Briefly, exhaustive counts were collected from every ninth

hippocampal section throughout the anterior-posterior extent of the hippocampus (-1.80 to -7.64 mm from bregma; (Paxinos and Watson, 1997)). Resulting cell counts were multiplied by the fraction of the hippocampus examined (e.g. 9; (Eisch et al., 2000; Lagace et al., 2006)) and are reported as total number of cells in the brain region examined. Four discrete regions of the dentate gyrus (DG) were examined as previously described (Donovan et al., 2006): the SGZ, the outer granule cell layer (oGCL), the molecular layer (Mol), and the hilus (Figure 2A). Immunopositive cells were also counted in the habenula to control for bioavailability of BrdU and general levels of proliferation. BrdU+ cell densities were obtained by dividing the number of BrdU+ SGZ and oGCL cells by the volume of the anterior GCL (-1.80 to -4.52 mm from bregma) or posterior GCL (-4.80 to -7.64 mm from bregma), with anterior-posterior demarcation as previously described (Guzman-Marin et al., 2003).

DCX+ cell density quantification was restricted to two discrete points from bregma that represented the anterior (-2.80 mm from bregma) and posterior (-6.60 mm from bregma) dentate gyrus, respectively. These points were chosen in order to address specific hypotheses that emerged from our BrdU+ cell count data, allow exhaustive counting of cells at two discrete points from bregma, and to coincide with recent publications about rat DCX immunoreactivity (Driscoll et al., 2006) and anterior-posterior differences in the number of cells in stages of neurogenesis (Banasr et al., 2006; Lagace et al., 2006).

Stereological estimate of GCL and SVZ volume

Volumes of the GCL and SVZ were calculated by analyzing 30 µm sections stained with Fast Red (Donovan et al., 2006). Sections were coded so the experimenter was blind to treatment of the rat until completion of the analysis. All measurements were obtained using Stereoinvestigator software (MBF Bioscience, Williston, VT) and an Olympus BX51 microscope. Volumes were analyzed for the left hemisphere structure only due to previous work from our lab showing no statistical difference between the left and right hemispheres (Harburg et al., 2007).

For the GCL, the entire longitudinal axis of the hippocampus was analyzed: -1.80 to -7.64 mm from bregma (Paxinos and Watson, 1997). GCL volumes were measured according to the Cavaleri principle using a 10x objective (Gundersen and Jensen, 1987; West et al., 1991). For the anterior SVZ, analysis included four sections spanning 1.60 to -0.26 mm from bregma (Paxinos and Watson, 1997) (Figure 2.5A). Volumes were measured according to the Cavaleri principle using a 40x objective. The boundaries of the SVZ were based on definitions previously published (Gotts and Chesselet, 2005). Briefly, the SVZ contour included the lateral portion under the corpus callosum through the intersection of the lateral ventricle, as well as the SVZ 200 µm ventral and 200 µm medial to this intersection (Figure 2.5B).

Stereological estimate of SVZ Ki-67+ cell number

The optical fractionator method was used to obtain the absolute number of Ki-67+ cells in unilateral anterior SVZ (West et al., 1991; Gotts and Chesselet, 2005). A grid size of 50 x 50 μm^2 was superimposed over each section, and Ki-67+ cells within the SVZ were counted at 1000x in 15 x 15 x 6 um^3 sample volumes, with upper and lower guard volumes of 2 µm, resulting in an average of 140 sampling sites per rat. Gunderson coefficients were always under 0.1. The number of proliferating cells as measured by Ki-67 were similar to the numbers of proliferating cells as measured by multiple BrdU labelings in the adult rat (Gotts and Chesselet, 2005).

Phenotypic analysis

In order to determine the differentiation of newborn cells into neurons, CSA-WD and CSA-CONT sections were examined for the colocalization of BrdU with NeuN using confocal microscopy at 630x. On average more than 20 BrdU+ cells were analyzed in each rat (mean ± S.E.M.: Saline-WD 28.17 ± 3.87, CSA-WD 26.63 ± 2.90; Saline-CONT 23.00 ± 4.92, CSA-CONT 41.50 ± 6.81). BrdU+ nuclei were also analyzed for morphological characteristics such as nuclear pattern of BrdU staining (solid vs. punctate), shape (round vs. irregular), and orientation to the GCL (parallel vs. perpendicular) as previous publications have

linked these characteristics with maturation of the neuron (Cameron and McKay, 2001; Donovan et al., 2006).

The dendritic morphology of DCX+ cells after CSA, CSA-WD, and CSA-CONT was analyzed in sections -6.60 mm from bregma for characteristics previously linked to the maturity of DCX+ cells (Rao et al., 2005). Specifically, every DCX+ cell in the dorsal blade of the GCL (mean ± S.E.M.: Saline 82.75 ± 8.61, CSA 143.25 ± 27.33; Saline-WD 39.66 ± 9.66, CSA-WD 72.66 ± 22.56; Saline-CONT 81.50 ± 19.00, CSA-CONT 108.75 ± 5.65) was examined for the presence or absence of dendrites, vertical or horizontal orientation of dendrites, and whether or not the dendrites were branched (Rao et al., 2005).

Statistical analyses and presentation

Data are represented as mean ± S.E.M. Statistical analyses employed GraphPad Prism version 4.00 for Mac (GraphPad Software, San Diego, CA). A one-way ANOVA (t-test) was used for analyses with one variable, such as cocaine intake, effect of drug on the density of BrdU+ or DCX+ cells the posterior hippocampus, and effect of drug on the density of Ki-67+ cells in the SVZ. For analyses that had more than one variable (drug x dentate gyrus regions, drug x dendrite morphology), a two-way ANOVA was performed. If a main effect or interaction was found, a Bonferonni post-hoc test was performed to adjust the threshold of significance to guard against the type I error, which may occur with

multiple comparisons (Donovan et al., 2006). Statistical significance for main effects, interactions, and t-tests was defined as $p \leq 0.05$. Images were imported into Photoshop version 9.0.2 (Adobe Systems, Inc., San Jose, CA, USA) and the only adjustments made were via gamma in the Levels function.

RESULTS

Cocaine self-administration

Three groups of rats self-administered cocaine (CSA, CSA-WD, and CSA-CONT) with their respective control groups self-administering saline (Figure 2.1A-C). CSA, CSA-WD, and CSA-CONT rats took an average daily amount of 40 mg/kg of cocaine over the last five days before injection with the S-phase marker BrdU ($F_{2,24} = 0.05$, $p>0.05$; Figure 2.1D), indicating that cells labeled with BrdU were initially exposed to the same amount of cocaine in each group. CSA and CSA-WD rats took the same total amount of cocaine, while CSA-CONT rats took more than twice that amount ($F_{2,24} = 25.10$, $p<0.001$; Figure 2.1E), which was expected since the CSA-CONT rats self-administered for four additional weeks after the BrdU injection for a total of seven weeks of cocaine self-administration. Saline reinforcements for the last five days before BrdU injection were similar in controls across Saline, Saline-WD, and Saline-CONT groups (data not shown) and were minimal due to the lack of drug reinforcement. However, the fact that saline reinforcements in this study and many others were

still measurable is likely due to the stimulation provided by the cue light that was illuminated when the active lever was pressed. In support of this, responses on the inactive lever for Saline and Cocaine groups, which were not reinforced with drug or cue light, were virtually zero during the last five days before BrdU injection.

CSA decreases proliferation in the adult subgranular zone

To determine the effect of drug taking on SGZ proliferation, CSA rats were injected with BrdU 15 hours after the end of the last self-administration session and sacrificed 2 hours later (Figure 2.1A). BrdU IHC revealed a normal pattern of S-phase cells in the dentate gyrus, with BrdU+ cells most numerous in the SGZ, but also found in the outer granule cell layer, hilus, and molecular layer (Figure 2.2A) as previous reported (Donovan et al., 2006; Lagace et al., 2006; Harburg et al., 2007; Lagace et al., 2007). BrdU+ cells at this proliferation time point appeared small, irregular, and in clusters for both Saline and CSA groups (Figure 2.2B). However, rats that self-administered cocaine for 3 weeks had fewer BrdU+ cells relative to control rats ($F_{1,65} = 9.39$, $p < 0.01$; Figure 2.2C), with post-hoc analysis revealing a significant 47% decrease specifically in the SGZ ($p<0.001$; Figure 2.2C). BrdU+ cell density significantly decreased in the posterior SGZ ($p<0.05$; data not shown) but not in the anterior SGZ ($p>0.05$; data not shown), in keeping with previous reports on anterior-posterior differences in the number of cells in stages of neurogenesis (Banasr et al., 2006; Lagace et al.,

2006). While cocaine has potent vasoconstrictive effects (Kaufman et al., 1998; Herning et al., 1999), there was no significant effect of CSA on the number of BrdU+ cells in several control regions, including those within the dentate gyrus (p's>0.05 for the molecular layer, outer granule cell layer, and hilus; Figure 2.2C) and the habenula (p>0.05; Figure 2.2C). Therefore, the CSA-induced decrease in SGZ proliferation was not due to decreased BrdU passage across the blood brain barrier.

Quantification of AC-3+ cells showed no significant difference between CSA and Saline rats in the SGZ, indicating cell death was not changed after CSA (Saline = 16.88 ± 7.30, CSA = 25.71 ± 13.81; p>0.05). To address whether the CSA-induced decreased in proliferation (Figure 2.2C) led to a decrease in GCL volume, stereological estimates of the volume of the GCL were examined. GCL volumes were consistent with previous studies with adult rats (Isgor and Sengelaub, 1998). However, analysis revealed no significant change in volume of the GCL after CSA (Saline = 2.12 ± 0.21, CSA = 2.16 ± 0.17 mm^3; p>0.05). Therefore, 3 weeks of CSA decreased SGZ proliferation in a region-specific manner without influencing cell death or GCL volume.

CSA does not alter the number or dendritic morphology of immature neurons in the adult subgranular zone

As CSA decreased the number of proliferating SGZ cells, we next investigated if immature SGZ neurons, as labeled by DCX (Brown et al., 2003), were altered by CSA. DCX+ staining was present in both cell bodies and processes in both Saline and CSA rats (Figure 2.2D). We then quantified the density of DCX+ cells in the anterior SGZ (-2.80 mm from bregma), where proliferation as measured by BrdU+ cells was not significantly changed, and in the posterior SGZ (-6.60 mm from bregma) where proliferation was significantly decreased (Figure 2.2D). After CSA, the density of DCX+ cells in the SGZ showed a trend for an increase in the anterior hippocampal section, with no change in the posterior section (anterior: 38% increase, $p=0.06$; posterior: $p>0.05$; Figure 2.2E).

We examined the dendritic morphology of DCX+ cells in a posterior hippocampal section -6.60 mm from bregma. Previous work has shown that a DCX+ cell with a vertical, branched dendrite is more mature than a DCX+ cell that lacks a dendrite or whose dendrites are horizontal or unbranched (Rao and Shetty, 2004). Saline rats exhibited percentages of DCX+ cells with vertically oriented dendrites that were consistent with the literature (Rao et al., 2005). Analyses of Saline and CSA rats revealed a main effect of drug ($F_{1,39} = 6.86$; $p<0.05$; Figure 2.2F), but no post-hoc analyses were significant. Therefore, 3

weeks of CSA did not significantly influence the dendritic morphology of DCX+ SGZ neurons.

CSA-WD normalizes proliferation and survival of adult-generated neurons in the subgranular zone, but enhances maturity of adult-generated neurons

Since CSA decreased SGZ proliferation (Figure 2.2C), we then examined the CSA-WD group to see if cessation of cocaine self-administration for 4 weeks normalized SGZ proliferation. BrdU was given to the CSA-WD group 4 weeks prior to sacrifice (Figure 2.1B) thus labeling surviving, not proliferating, cells. Therefore, proliferating SGZ cells in the CSA-WD group were detected with an antibody against Ki-67, an endogenous marker of proliferation that has been used interchangeably with BrdU antibodies to detect cells labeled with BrdU 2 hours prior to sacrifice (Kee et al., 2002). Ki-67+ cells were small and often clustered (Figure 2.3A) and were qualitatively similar in the SGZ of CSA-WD rats and controls. There was no significant change in Ki-67+ cell number after CSA-WD, although there was a trend for an effect of drug ($F_{1,130} = 3.46$, $p = 0.06$; Figure 2.3B). As proliferation was decreased by 47% in CSA rats relative to controls (Figure 2.2C), the normal level of SGZ proliferation after CSA-WD suggested that proliferation rebounded at some point between the initial cessation of CSA and the 4-week withdrawal time point. Cell death was not changed after CSA-WD, as assessed via cell counts for AC-3+ cells (Saline-WD = 13.15 ± 8.26,

CSA-WD = 21.71 ± 7.60; $p > 0.05$). This suggests that the normalization of proliferation in the SGZ is not due to a compensatory decrease in cell death after 4 weeks of withdrawal, although compensation may have occurred at other time points not assessed.

While the number of proliferating SGZ cells was relatively normal after CSA-WD, we investigated if cells generated during CSA (e.g. labeled with BrdU 4 weeks earlier) were influenced by the 4-week withdrawal period after self-administration (Figure 2.1B). The CSA and CSA-WD groups both received BrdU injections 15 hours after 3 weeks of self-administration. Therefore we predicted CSA-induced decrease in the number of proliferating BrdU+ cells (Figure 2.2C) would equate with fewer surviving BrdU+ cells in the CSA-WD group 4 weeks later. BrdU IHC revealed cells in both Saline-WD and CSA-WD rats that were round and less clustered, and presented more punctate BrdU staining, typical of SGZ cells at 4 weeks after BrdU injection (Figure 2.3A; (Cameron and McKay, 2001; Donovan et al., 2006)). Interestingly, after CSA-WD there was no effect of drug on the total number of BrdU+ cells ($F_{1,130} = 0.51$; $p>0.05$; Figure 2.3C) or on the density of BrdU+ cells in the anterior or posterior SGZ (p’s>0.05; data not shown). Thus, despite the initial decrease in proliferating BrdU+ SGZ cells after CSA (Figure 2.2C), CSA-WD resulted in a surprising normalization of the number of surviving BrdU+ SGZ cells.

The vast majority of BrdU+ cells became neurons in both control and CSA-WD rats, as indicated by double labeling with BrdU and the neuronal marker NeuN ($p>0.05$; Figure 2.3D) and confocal analysis ($p>0.05$; Figure 2.3E). Therefore, CSA-WD did not grossly influence neuronal fate of cells labeled with BrdU 4 weeks earlier. The BrdU staining pattern of the surviving BrdU+ cells was also analyzed, as previous publications have linked this to maturity of the adult-generated neurons (Rietze et al., 2000; Cameron and McKay, 2001; Donovan et al., 2006). CSA-WD rats had a significantly greater percentage of cells with punctate BrdU staining (Saline-WD 48 ± 4.85%, CSA-WD 62 ± 2.79%; $p<0.05$; Figure 2.3E) as opposed to solid BrdU staining. While other characteristics of cells such as shape and orientation did not differ after CSA-WD (p's>0.05), the significant increase in the proportion of BrdU+ cells displaying punctate BrdU staining pattern after CSA-WD is consistent with accelerated maturation of adult-generated hippocampal granule cell neurons (Rietze et al., 2000; Cameron and McKay, 2001; Donovan et al., 2006). Thus, although CSA-WD normalized neurogenesis, as measured by the normal number of surviving BrdU+ cells and proportion of BrdU+/NeuN+ cells, more of the surviving BrdU+ cells presented this indicator of enhanced maturity.

Stereological methods were used to estimate the volume of the GCL after CSA-WD. There was no change in GCL volume after CSA-WD (Saline-WD = 2.58 ± 0.09, CSA-WD = 2.42 ± 0.17 mm^3; $p>0.05$).

CSA-WD increases the number of immature neurons in the posterior subgranular zone

Since CSA-WD normalized proliferation and resulted in the enhanced the maturity of surviving BrdU+ cells, we examined cells in an intermediate stage of neurogenesis using the immature neuronal marker DCX. After CSA-WD, a qualitative increase in DCX+ cell number was observed in the posterior SGZ (Figure 2.3F). Quantification of the density of DCX+ cells confirmed an increase in the posterior SGZ (-6.60 mm from bregma; $p<0.01$) but not in the anterior SGZ (-2.80 mm from bregma; $p>0.05$; Figure 2.3G). While there was a significant effect of drug on dendritic morphology ($F_{1,78} = 4.76$; $p<0.05$; Figure 2.3H), post-hoc analyses were not significant. Thus, consistent with the trend for an increase in proliferation after CSA-WD (Figure 2.3B) and the enhanced maturity of surviving BrdU+ cells (Figure 2.3E), the significant increase in posterior SGZ DCX+ cell number suggests that 4 weeks of withdrawal from self-administration is not sufficient to normalize the process of neurogenesis in the adult rat SGZ.

CSA-CONT normalizes progenitor proliferation as well as survival of adult-generated neurons in the subgranular zone without altering maturation

We hypothesized that the increased immature SGZ neuron number and enhanced maturity of surviving BrdU+ SGZ cells in CSA-WD rats was due to

compensation that occurred during the 4 weeks of withdrawal after cocaine self-administration. To test this, another group of rats was prepared similarly to the CSA-WD group – 3 weeks of saline or cocaine self-administration and BrdU injection 15 hours after last session – but instead of withdrawal in the home cage, these CSA-CONT rats continued to self-administer saline or cocaine for an additional 4 weeks (Figure 2.1C). CSA decreased SGZ proliferation (Figure 2.2C) while CSA-WD normalized it (Figure 2.3B); thus we expected 4 additional weeks of cocaine self-administration to sustain or further the decrease in proliferation seen after 3 weeks of cocaine self-administration. However, proliferation of Ki-67+ cells was unchanged ($F_{1,30} = 1.43$; $p<0.05$; Figure 2.4A). We also predicted the additional 4 weeks of cocaine self-administration in the CSA-CONT group would significantly decrease the number of surviving BrdU+ cells. Contrary to our hypothesis, 4 additional weeks of cocaine self-administration had no effect on the total number of BrdU+ cells (drug effect: $F_{1,30} = 2.47$; $p>0.05$; Figure 2.4B),although there was a trend for a decrease in the density of BrdU+ cells in the posterior SGZ ($p=0.07$; data not shown). Neuronal fate, as indicated by double labeling with BrdU, was also unaffected by continuous self-administration of cocaine ($p>0.05$; Figure 2.4C). Other characteristics of cells, such as BrdU staining pattern ($p>0.05$; Figure 2.4C), shape and orientation did not differ after CSA-CONT (p’s>0.05). Stereological methods were used to estimate the volume of the GCL after CSA-CONT. There was no significant change in GCL volume

after CSA-CONT (Saline-CONT = 2.34 ± 0.12, CSA-CONT = 2.81 ± 0.38 mm^3; $p>0.05$).

CSA-CONT increases the number of immature neurons in the posterior subgranular zone

Having discovered some unexpected similarities between the impact of 4 weeks of withdrawal or 4 additional weeks of cocaine self-administration on the number of proliferating cells and adult-generated BrdU+ neurons, we then explored if CSA-WD and CSA-CONT had similar effects on DCX+ immature neuron number. After CSA-CONT, the density of DCX+ cells was significantly greater in the posterior SGZ relative to controls (-6.60 mm from bregma; $p<0.05$; Figure 2.4D) but not in the anterior SGZ (-2.80 mm from bregma; $p>0.05$; Figure 2.4D). Additionally, CSA-CONT did not change the dendritic morphology of DCX+ cells in the posterior SGZ, as there was no main effect of drug ($F_{1,18}$ = 0.60; $p>0.05$; Figure 2.4E). Thus, similar to CSA-WD, CSA-CONT normalized the number of surviving BrdU+ cells and increased the number of immature neurons in the posterior SGZ without influencing their dendritic morphology. However, unlike CSA-WD, CSA-CONT had no effect on BrdU staining pattern of adult-generated neurons, an indicator of neuronal maturity.

CSA decreases proliferation in the subventricular zone

CSA produced such a striking decrease in hippocampal progenitor proliferation that an additional region of adult neurogenesis, the SVZ, was examined to see if proliferation in this region was altered in a similar fashion. Ki-67 IHC in the SVZ was used to assay proliferation (Figure 2.5A), and the density of Ki-67+ cells was obtained via the optical fractionator method (Figure 2.5B). CSA significantly decreased proliferation in the SVZ by 20% (Saline 9.76 ± 0.49 x 10^5; CSA = 8.23 ± 0.53 x 10^5 Ki-67+ cells/mm^3; p=0.05). While SVZ volumes of control rats were similar to previous studies of adult rats (Gotts and Chesselet, 2005), SVZ volumes of CSA rats were not significantly different from control (Saline-WD = 112.89 ± 9.00 x 10^6; CSA = 108.42 ± 3.56 x 10^6 μm^3; p>0.05), suggesting that the cocaine-induced decreased in SVZ proliferation was not sufficient in magnitude or duration to impact the volume of the SVZ.

CSA-WD normalizes proliferation in the subventricular zone

Ki-67+ cells in the SVZ of the CSA-WD group were quantified to see if cessation of drug taking reversed the CSA-induced decrease in SVZ proliferation. After CSA-WD there was no change in proliferation in the SVZ as measured by density of Ki-67+ cells (Saline-WD 5.92 ± 0.81 x 10^5; CSA-WD = 5.72 ± 0.72 x 10^5 Ki-67+ cells/mm^3; p>0.05). Additionally, after CSA-WD there was no change in volume in the SVZ as estimated by optical fractionator (Saline-WD = 119.34 ±

5.35×10^6, CSA-WD = $121.59 \pm 6.47 \times 10^6$ μm^3; $p>0.05$). Therefore, 4 weeks of withdrawal is sufficient to normalize the level of SVZ proliferation.

DISCUSSION

Our data highlight how cells in stages of adult hippocampal neurogenesis – proliferating cells, immature neurons, and surviving, adult-generated, mature neurons – are differentially influenced by intravenous cocaine self-administration and/or withdrawal. Proliferation in both the SGZ and SVZ is decreased after 3 weeks of cocaine self-administration, and 4 weeks of withdrawal reverses these changes. Neither the number or dendritic morphology of immature SGZ neurons are immediately influenced by cocaine self-administration, but surprisingly, the number of immature SGZ neurons is increased after 4 weeks of withdrawal or 4 additional weeks of cocaine self-administration. The number of surviving BrdU+ cells in the SGZ is normal after either 4 weeks of withdrawal or 4 additional weeks of cocaine self-administration, but the surviving BrdU+ neurons after 4 weeks of withdrawal unexpectedly display an indicator of enhanced maturity. These data add to the growing amount of evidence that cells in discrete stages of adult neurogenesis can be independently regulated (Garcia et al., 2004; Plumpe et al., 2006; Nacher et al., 2007). Importantly, given that adult-generated granule cells are important for aspects of hippocampal function (Leuner et al., 2006; Saxe et al., 2006; Kee et al., 2007) and that the hippocampus is important in addiction

and withdrawal (Goto and Grace, 2005; Rademacher et al., 2006; Rogers and See, 2007), our data urge consideration of how cocaine-induced alterations in adult neurogenesis arise, and how they may impact hippocampal function in general, and drug taking and relapse in particular.

Proliferating cells

We considerably extend previous results with non-contingent, intraperitoneal cocaine by showing that the more clinically relevant paradigm of reinforcement-related, intravenous cocaine self-administration decreases proliferation in the adult SGZ. We further show the CSA-induced decrease in proliferation is not due to decreased BrdU bioavailability, an important concern given cocaine's action on the vasculature. This decrease is likely not due to cell death because we found no change in AC-3+ cell number after CSA, an interpretation supported by previous cocaine work (Dominguez-Escriba et al., 2006). However, the trend in and high variability of the AC-3 data encourage future examination of additional time points after cocaine self-administration. An additional novel neuroadaptation we identify after cocaine self-administration is decreased SVZ proliferation. Finally, we show that both the decreased proliferation in the SGZ and SVZ are reversed by 3 weeks of withdrawal, indicating the homeostatic capabilities of the populations of dividing cells and/or of the neurogenic niche.

It is tempting to speculate that cocaine-induced dysregulation of adult hippocampal proliferation contributes to the cognitive deficits seen in cocaine addicts. However, this hypothesis requires in-depth clinical and translational research, and basic research to reveal the detailed time course of cocaine's impact on proliferation and to explore how proliferating SGZ cells might influence the hippocampal neurogenic niche. The functional relevance of cocaine-induced alterations in neurogenesis may be clearer in the SVZ, as the addition of new SVZ neurons to the olfactory bulb incontrovertibly enhances aspects of olfaction (Gheusi et al., 2000; Shingo et al., 2003; Enwere et al., 2004). In humans, cocaine use impairs olfactory function which improves with abstinence (Gordon et al., 1990; Bauer and Mott, 1996). Notably, both intravenous cocaine in humans and intraperitoneal cocaine in rats alter olfaction (Stripling and Ellinwood, 1977; Podskarbi-Fayette et al., 2005), thus addressing the apparent confound of nasal septum damage seen after intranasal cocaine use (Schwartz et al., 1989). An interesting, but as-yet untested, hypothesis is that cocaine-induced deficits in olfaction are secondary to decreased SVZ proliferation and the consequential decrease in adult-generated olfactory bulb neurons.

Immature neurons

One of our most surprising findings was that both CSA-WD and CSA-CONT result in a greater number of immature neurons specifically in the posterior

SGZ. As this occurred regardless of whether drug taking continued or stopped, this may reflect a delayed response to – and long-lasting, stable neuroadaptation resulting from – cocaine self-administration. This interpretation is supported by studies with non-contingent cocaine exposure, which find a transient increase in immature neuron number (Mackowiak et al., 2005) but no effect on immature neuron dendritic morphology (Dominguez-Escriba et al., 2006). While beyond the scope of this study, studies are required to identify whether immature neurons in particular, and neurogenesis in general, are differentially regulated during acquisition, maintenance, and long-term administration of cocaine and withdrawal, as has been shown with other neuroadaptations (Ferrario et al., 2005).

As immature neurons are important to hippocampal structure and function (Markakis and Gage, 1999; Esposito et al., 2005), it is intriguing to consider how a greater number of immature neurons in the posterior SGZ might impact hippocampal function. The posterior SGZ receives many limbic projections, and is more involved in emotion relative to the anterior SGZ, which plays a greater role in spatial processing (Sahay and Hen, 2007). Doublecortin regulates synaptic plasticity (Nacher et al., 2001; Brown et al., 2003), and adult-generated DCX+ neurons possess distinct properties from granule cells generated in the early postnatal period (Wang et al., 2000; Snyder et al., 2001; Couillard-Despres et al., 2006), including lowered threshold for induction of long-term potentiation (LTP). Therefore, it is possible that having more immature neurons would lead to

enhanced excitability of the hippocampus. This would fit well with the fact that chronic cocaine self-administration may increase hippocampal neurotransmitter release (Edwards et al., 2007a) and LTP at Schaffer collateral-CA1 synapses (Thompson et al., 2004; del Olmo et al., 2006). Behaviorally, a greater number of immature neurons may be related to the ability of cocaine to modulate hippocampal influence over reward circuitry and goal-directed behavior (Vorel et al., 2001; Sun and Rebec, 2003; Fuchs et al., 2005; Goto and Grace, 2005) and memory (Kilts et al., 2001; Hester et al., 2006; Del Olmo et al., 2007). Further study is needed to determine the functional role of the dentate gyrus and increased DCX+ cells in the posterior SGZ in the encoding of contextual associations with drug taking, as these associations are a major obstacle in addiction treatment.

Surviving, adult-generated, mature neurons

While 3 weeks of cocaine self-administration robustly decreased SGZ proliferation, it is striking that 4 weeks later, with or without continuation of cocaine self-administration, the number of surviving BrdU+ neurons in the SGZ is normal relative to saline self-administering controls. When analyzed in just the posterior SGZ, there was a trend to a decrease in CSA-CONT rats, encouraging additional analyses to pinpoint differences between CSA-WD and CSA-CONT. However, given the lack of change when the SGZ was considered as a whole, clearly some level of compensation must have occurred for the decreased

population of progenitors after 3 weeks of CSA to result in normal numbers of surviving BrdU+ neurons after 4 weeks of withdrawal and 4 additional weeks of cocaine. A decrease in cell death is unlikely, as we found no change in AC-3+ cells, although it is possible that we missed the narrow window in which cell death is detectable (Harburg et al., 2007) or that the low basal rate of apoptosis in the adult SGZ results in a floor effect for detecting further decreases in cell death (Heine et al., 2004). Alternatively, the decreased pool of progenitors could have divided more frequently or symmetrically, giving rise to two daughter cells more often than one. Both scenarios would result in the normal number of surviving BrdU+ neurons in the SGZ of CSA-WD and CSA-CONT rats reported here.

Aside from the many surprising similarities between the CSA-WD and CSA-CONT groups, an abstinence-specific neuroadaptation worth noting is the enhanced maturity of adult-generated BrdU+ neurons after withdrawal. This suggests a benefit of abstinence for the dentate gyrus, but clearly encourages functional exploration of this putative enhanced maturity as well as identification of other neuroadaptations during CSA-WD or CSA-CONT that may be compensatory, encouraging a return to homeostasis.

Conclusion

The present study defines the novel impact of cocaine self-administration and/or withdrawal on cells in various stages of adult neurogenesis. Self-

administration of cocaine, which exposed rats to more cocaine daily than passive administration of cocaine, resulted in a greater reduction of proliferation (50%) than passive administration (25%; Dominguez-Escriba et al., 2006; Yamaguchi et al., 2005) likely due to greater toxicity to neural progenitors (Lee et al., 2008). While detailed discussion about underlying mechanisms of long-term impact on adult neurogenesis is speculative, it is likely that cocaine self-administration and/or withdrawal alters the microenviroment of the neurogenic niche, (Abrous et al., 2005; Ming and Song, 2005), perhaps via alterations in levels of growth factors. A potential role for brain-derived neurotrophic factor (BDNF) is particularly intriguing, since BDNF signaling regulates cocaine self-administration (Graham et al., 2007), incubation of drug craving (Grimm et al., 2003), and specifically in the dentate gyrus, the acquisition of stimulant/context associations and conditioned locomotor sensitization to stimulants (Rademacher et al., 2006; Shen et al., 2006). While BDNF is one of many candidates, clearly understanding cocaine-induced alterations in the neurogenic microenvironment may point to a critical role for dentate gyrus neurogenesis in cocaine cravings and relapse, perhaps via facilitation of recall of contextual associations that stimulate cocaine cravings and relapse.

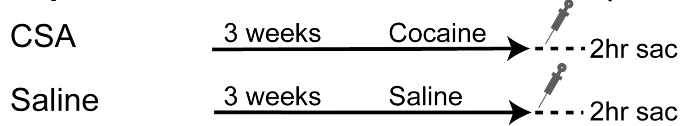
It will be interesting in the future to test whether manipulations of neurogenesis can alter the propensity for addictive behavior. Such studies await refinement in techniques to specifically alter adult neurogenesis with minimal side

effects. It is intriguing that conditions often comorbid with substance abuse, like stress, depression, and schizophrenia (Brady and Sinha, 2005) are marked by dysregulation of adult neurogenesis (Paizanis et al., 2007). The present study establishes neurogenesis as a potential target for therapeutic approaches to drug addiction treatment which may have broad implications for psychiatric disorders.

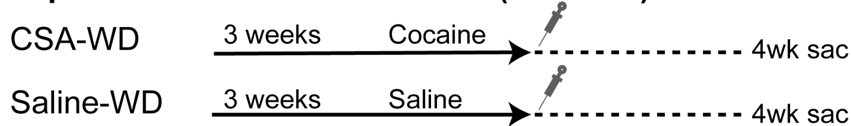
CHAPTER 2: Figures

Figure 2.1

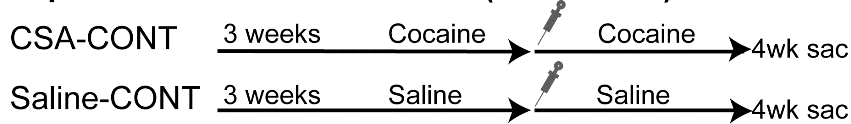
A Impact of Cocaine Self-Administration (CSA) on Proliferation



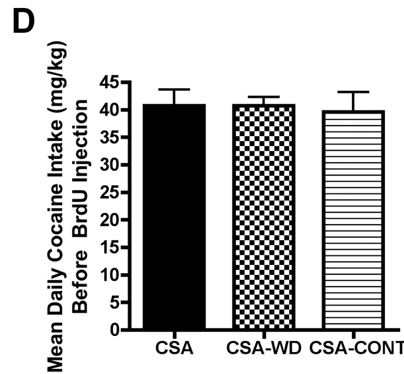
B Impact of Withdrawal from CSA (CSA-WD) on Survival



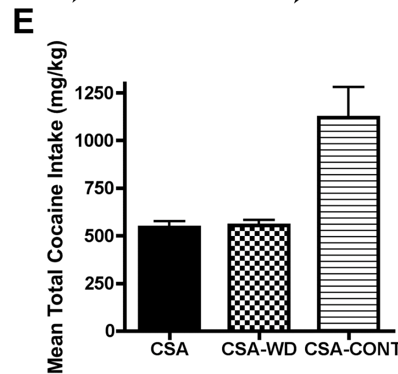
C Impact of Continuation of CSA (CSA-CONT) on Survival



D



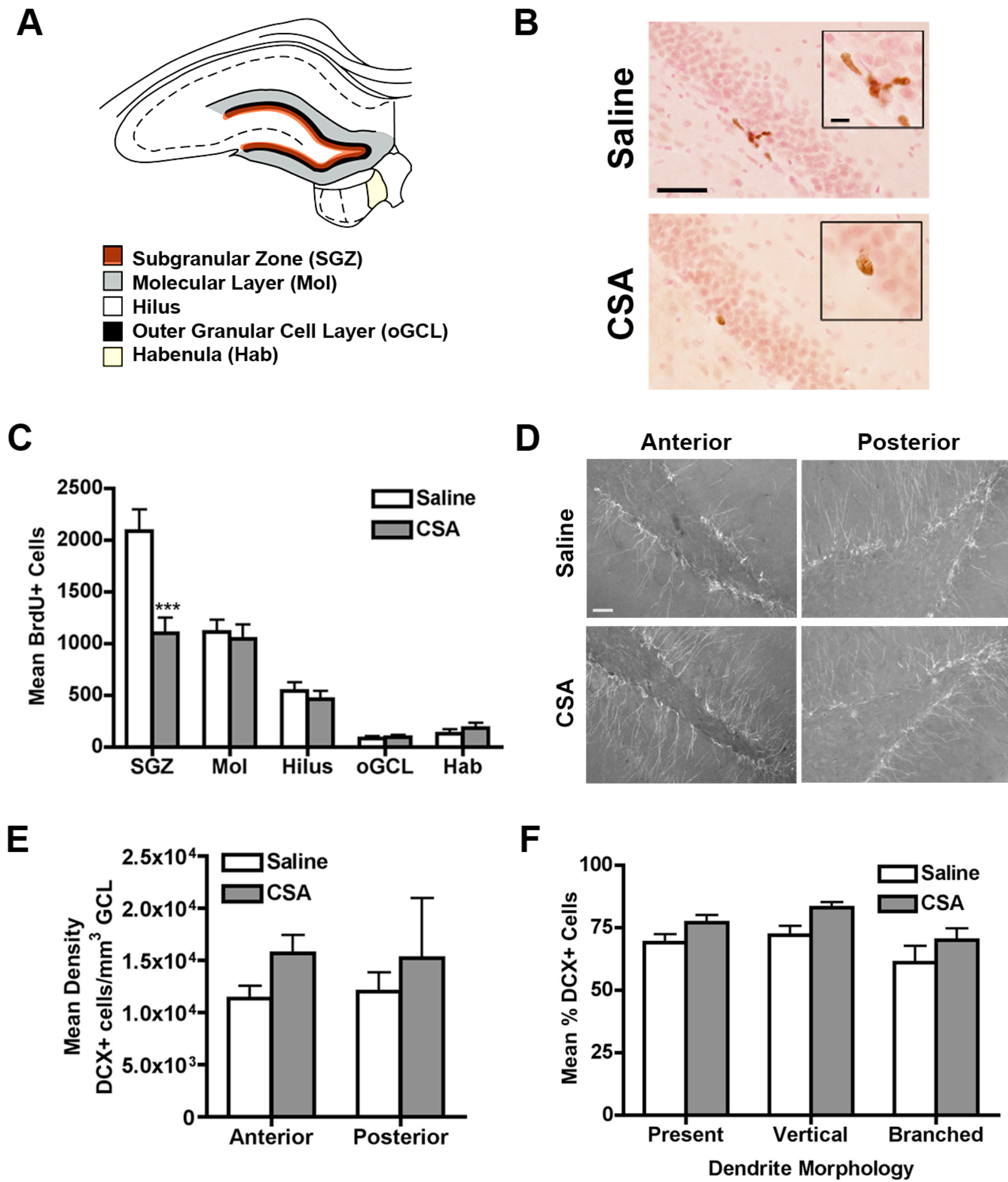
E



Experimental design. Animals were trained to lever press for i.v. saline or cocaine and then self-administered their respective solutions for 3 weeks. One BrdU injection (150 mg/kg, i.p., indicated by syringe) was administered 15 hours after the last self-administration session. (A) CSA and Saline rats were sacrificed 2 hours later to assess proliferation via BrdU+ cell counts. (B) CSA-WD and Saline-WD rats were returned to their home cage and then sacrificed 4 weeks later to assess the impact of withdrawal on survival or retention of BrdU+ cells labeled 4 weeks earlier. (C) CSA-CONT and Saline-CONT rats continued self-administration for 4 additional weeks before sacrifice to assess the influence of

cocaine on survival or retention of BrdU+ cells labeled 4 weeks earlier. (D) CSA, CSA-WD, and CSA-CONT rats all self-administered the same average amount of cocaine during the last five sessions before BrdU injection. (E) CSA and CSA-WD rats self-administered the same total amount of cocaine, but CSA-CONT rats self-administered more than twice as much total cocaine due to four additional weeks of sessions. Data for D-E presented as mean ± S.E.M. Sac = sacrifice.

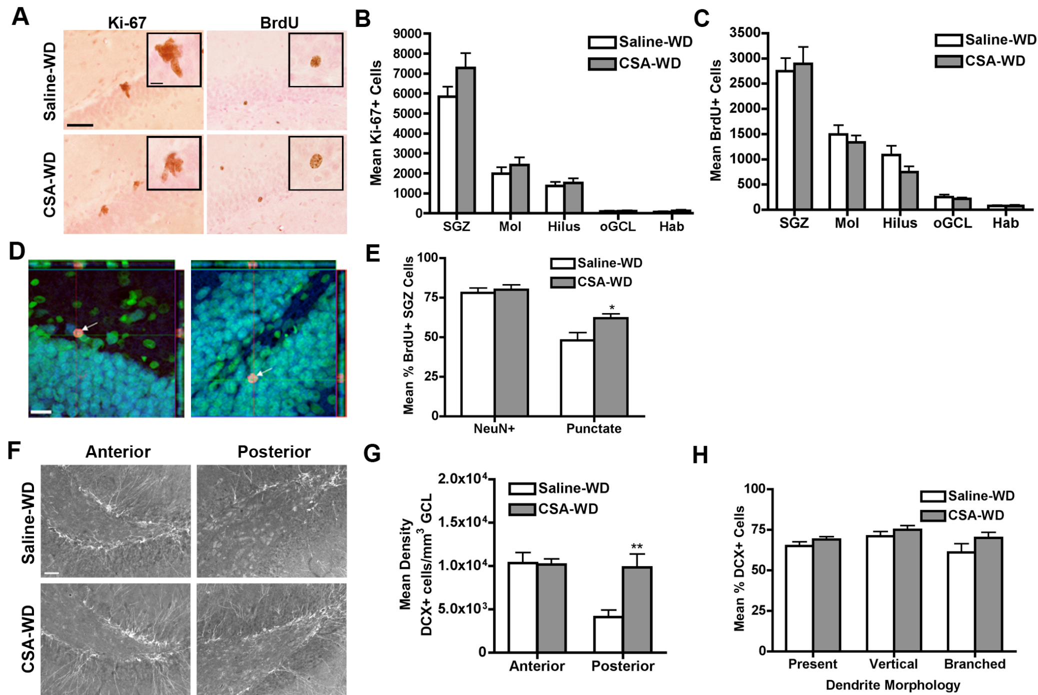
Figure 2.2



CSA decreases proliferation in the SGZ but does not alter DCX+ neuron number or dendritic morphology. (A) Schematic of the four regions of the dentate gyrus and medial habenula in which cell counts were collected for this

study. (B) Representative images at a proliferation time point (2 hours post BrdU) injection show the decreased number of BrdU+ cells in the SGZ after CSA. BrdU+ cells (brown, stained via DAB IHC) appear in the SGZ on the border of the granule cell layer labeled (pink, stained with Fast Red) and the hilus. Inset is a magnification of the BrdU+ cell clusters. Main scale bar = 50 µm. Inset bar = 10 µm. (C) Quantitative analysis of BrdU+ cells in the dentate gyrus after CSA found a decrease in proliferation specific to the SGZ. (D) Representative images of DCX+ cells in the SGZ at an anterior section (-2.80 mm from bregma) and posterior section (-6.60 mm from bregma) show staining of both the cell bodies and processes in both Saline and CSA rats. Scale bar = 50 µm. (E) Quantitative analysis of density of DCX+ cells in the SGZ after CSA found no change. (F) CSA did not change the dendritic morphology of DCX+ cells in the posterior SGZ. Data for C, E-F presented as mean ± S.E.M. Data in C multiplied by fraction of sections examined to result in BrdU+ cell number. *** = $p<0.001$.

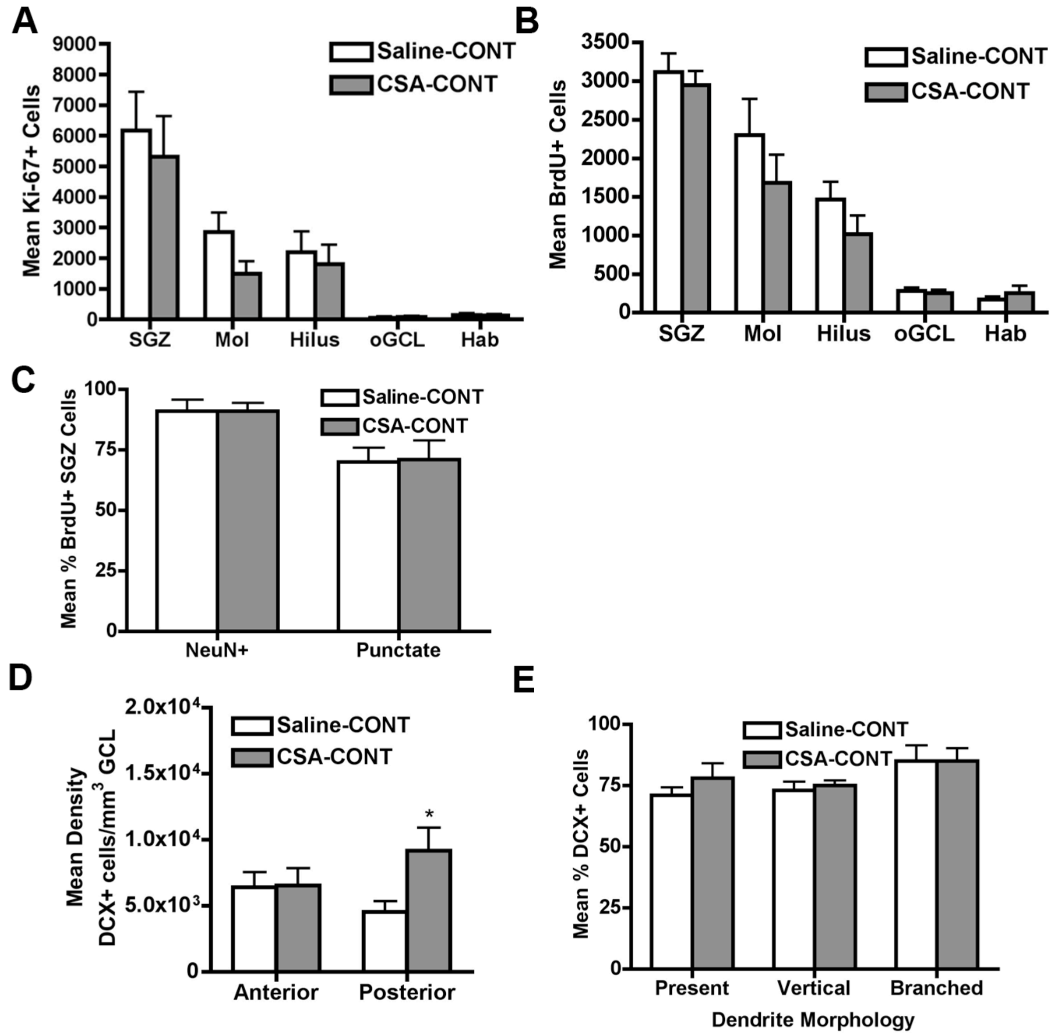
Figure 2.3



CSA-WD normalizes the number of proliferating cells and BrdU+ adult-generated neurons in the SGZ, but increases posterior DCX+ cells. (A) Representative images of cells immunopositive for Ki-67 (a marker of proliferation; left column) and BrdU (a marker of survival at this survival time point; right column) 4 weeks after CSA cessation. Note many BrdU+ cells at this 4 week survival time point present punctuate nuclear staining. Insets are magnification of immunopositive clusters. Main scale bar = 50 µm. Inset bar = 10 µm. (B) Quantitative analysis of Ki-67+ cells revealed no significant change in any region analyzed in the number of proliferating cells after CSA-WD. (C) Quantitative analysis of BrdU+ cells revealed no change in the number of surviving cells in any region analyzed. (D) White arrows indicate BrdU+ (red) cells in representative images of the SGZ. The left and right panels depict orthogonal confocal images with xy, xy, and yz planes shown. Note BrdU+ cells are also NeuN+ (neuronal marker; blue) as well as Nissl+ (cell body counterstain; green), indicating the majority of surviving cells had a neuronal phenotype. Scale bar = 10 µm. (E) CSA-WD did not alter the percentage of BrdU+ SGZ cells colocalizing with the neuronal marker NeuN, but the percentage of BrdU+ SGZ cells that had a punctuate nuclear staining pattern significantly

increased after CSA-WD. (F) Representative images of DCX+ cells in the anterior (-2.80 mm from bregma) and posterior hippocampus (-6.60 mm from bregma) after four weeks of CSA-WD. Scale bar = 50 µm. (G) Quantitative analysis of density of DCX+ cells after CSA-WD revealed a significant increase in the number of DCX+ cells in the posterior SGZ after CSA-WD. (H) CSA-WD did not change the dendritic morphology of DCX+ cells in the posterior SGZ. Data for B, C, E, G-H presented as mean ± S.E.M. * = $p<0.05$, ** = $p<0.01$. SGZ = subgranular zone; Mol = molecular layer; oGCL = outer granule cell layer; Hab = habenula.

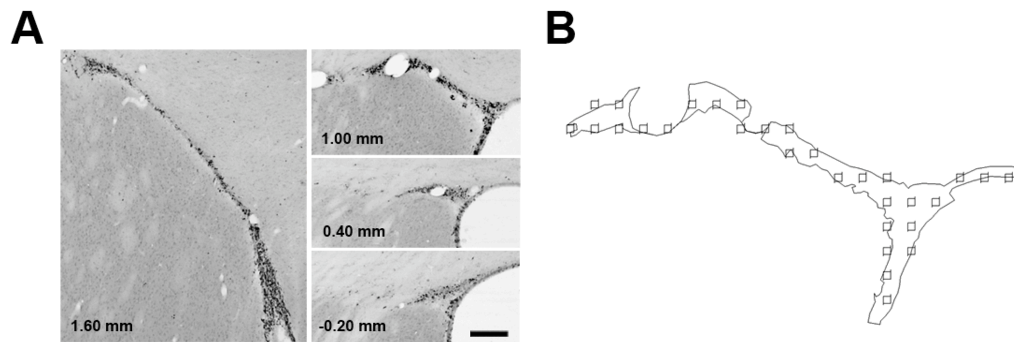
Figure 2.4



CSA-CONT normalizes the number of proliferating cells and BrdU+ adult-generated neurons in the SGZ but increases posterior DCX+ cells. (A) Quantitative analysis of Ki-67+ cells revealed no significant change in any region analyzed in the number of proliferating cells after CSA-CONT. (B) Quantitative analysis of BrdU+ cells revealed no change in the number of surviving cells in the SGZ after CSA-CONT. (C) CSA-CONT did not change the percentage of BrdU+ SGZ cells colocalizing with the neuronal marker NeuN or displaying a punctate BrdU staining pattern. (D) Quantitative analysis of the density of DCX+ cells in the SGZ after CSA-CONT revealed a significant increase in the number of DCX+

cells in the posterior SGZ after CSA-CONT. (E) CSA-CONT did not change the dendritic morphology of DCX+ cells in the posterior SGZ. Data for A-E presented as mean ± S.E.M. * = p=0.05. SGZ = subgranular zone; Mol = molecular layer; oGCL = outer granule cell layer; Hab = habenula.

Figure 2.5



Stereologic quantification of Ki-67+ cells in the SVZ. (A) Representative images of SVZ sections chosen for quantification of Ki-67+ cells with respective distances from bregma displayed in mm. Scale bar = 200 µm. (B) Representative outline from Stereoinvestigator of a SVZ section 1.00 mm from bregma shown in (A) depicting boundaries of SVZ as well as distribution of counting frames.

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CHAPTER THREE:
Suppression of adult neurogenesis via cranial irradiation increases drug-taking and drug-seeking behaviors

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INTRODUCTION

Drug addiction is an enormous problem that influences societies across the globe, with 4.9% of the world population abusing drugs in a year (UNODC, 2008). Addiction is exceptionally challenging to prevent and treat, as underscored by the high propensity of addicts to relapse to drug taking even after long periods of abstinence. For example, one study found 44% of cocaine users relapsed in an average of 72 days after rehab (Hubbard and Marsden, 1986). One promising approach for addiction prevention and improved treatment of drug users is the identification of vulnerability factors. For example, chronic stress (Miczek and Mutschler, 1996), high locomotor response to novelty (Piazza et al., 2000), and high levels of cocaine self-administration (Edwards et al., 2007) are addiction vulnerability factors, as each meets the criteria of producing a vertical shift in the dose-response curve and enhancing drug-seeking on a progressive ratio schedule (Piazza et al., 2000). Patients with substance use disorders are often comorbid for other psychiatric disorders (Buckley, 2006) and suffer from cognitive deficits

(Rogers and Robbins, 2001), but is it unclear whether drug use induced these disorders or whether they were pre-existing. Clearly more thorough identification of vulnerability factors for addiction has the potential to notably expand our understanding of addiction and improve treatment options.

The hippocampal formation has recently received attention for its potential role in addiction, due to its ability to impact contextual processing of drug-taking. The hippocampal formation is implicated in drug-context memory (Meyers et al., 2006; Shen et al., 2006; Hernandez-Rabaza et al., 2008) and relapse to drug-seeking (Vorel et al., 2001; Fuchs et al., 2005). A notable aspect of hippocampal plasticity that has not yet been thoroughly considered as a potential vulnerability factor for addiction is neurogenesis, or the ability of the subgranular zone (SGZ) or the hippocampus to give rise to new neurons throughout life (Eisch, 2002; Abrous et al., 2005). Clinically relevant self-administration studies have found dynamic regulation of adult neurogenesis in the hippocampus by diverse drugs of abuse such as cocaine (Noonan et al., 2008), methamphetamine (Mandyam et al., 2008), nicotine (Abrous et al., 2002), and heroin (Eisch et al., 2000). There appears to be negative correlation between levels of adult neurogenesis and drug-taking and seeking behaviors. Manipulations that increase adult hippocampal neurogenesis, such as environmental enrichment, chronic treatment with antidepressants, and exercise (van Praag et al., 1999; Malberg et al., 2000; Brown et al., 2003a), are also associated with decreased drug-taking and relapse

(Kanarek et al., 1995; Baker et al., 2001; Green et al., 2002; Stairs et al., 2006; Smith et al., 2008) and conversely, manipulations that decrease adult neurogenesis, such as stress and schizophrenia (Mirescu and Gould, 2006; Reif et al., 2006), are associated with increased drug-taking and relapse (Covington, 2005; Erb, 1996; Chambers, 2002}. Taken together with data that reduced adult neurogenesis can lead to cognitive deficits (Snyder et al., 2005; Saxe et al., 2006; Imayoshi et al., 2008), these correlative findings suggest that reduced hippocampal neurogenesis is a vulnerability factor for addiction.

Using published protocols to reduce hippocampal neurogenesis in the adult rat (Snyder et al., 2005; Winocur et al., 2006) and to assess vulnerability to addiction and propensity for relapse (Graham et al., 2007), we hypothesized that reduced adult hippocampal neurogenesis would lead to increased drug-taking and drug-seeking behaviors. We irradiated rats before cocaine self-administration to study the impact of irradiation on vulnerability to addiction and irradiated a separate group of rats after they had already become cocaine-experienced to study the impact of irradiation on relapse to drug-seeking. We irradiated rats before sucrose self-administration to test if effects of cranial irradiation were specific to drug-taking and drug-seeking, and not a generalized disruption of motivation, learning or memory. This is the first study to show that a reduction of adult hippocampal neurogenesis confers vulnerability to drug addiction.

METHODS

Animals

One hundred and two adult male Sprague-Dawley rats (Charles River, Kingston, NY) were individually housed in a climate-controlled environment on a 12 hour light-dark cycle (lights on at 0700 hours). Rats were acclimated to vivarium conditions for at least one week prior to experimentation. All experiments were performed in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* in an Institutional Animal Care and Use Committee and Association for Assessment and Accreditation of Laboratory Animal Care approved facility at UT Southwestern Medical Center. All steps were taken to minimize the number of rats used as well as the pain and suffering of the rats.

Experimental overview

As shown in Figure 3.1 and as described in detail below, rats used for this study were divided among three experiments. First, to examine the effect of cranial irradiation (IRR) on cocaine self-administration (CSA), rats received either IRR or sham irradiation prior to CSA (IRR-CSA n=21; Sham-CSA n=18; Figure 3.1A). Second, to examine the effect of IRR on a natural reward, rats received either IRR or sham irradiation prior to sucrose self-administration (IRR-SSA n=21; Sham-SSA n=18; Figure 3.1B). Finally, to examine the effect of

irradiation on reinstatement behaviors, rats received either IRR or sham irradiation after CSA and four weeks of withdrawal (CSA-WD-IRR n=15; CSA-WD/Sham n=9; Figure 3.1C).

Cranial irradiation

We used a cranial irradiation paradigm previously shown to be optimal for irreversibly suppressing adult hippocampal neurogenesis in the rat (Snyder et al., 2005; Winocur et al., 2006). For two consecutive days, rats were anesthetized with sodium pentobarbital (50 mg/kg, intraperitoneal (i.p.)) and exposed to either 0 Gy (Sham rats) or 10 Gy of irradiation (IRR rats; 1.08 Gy/min, 250 kV, 15 mA) via an X-RAD 320 self-contained irradiation system (Precision X-ray Inc, City, State). For IRR rats, irradiation was focused in a 1 cm diameter circle over the hippocampus (interaural 8.00 mm to -2.00 mm; (Paxinos and Watson, 1997)) to minimize irradiation of other brain regions (e.g. olfactory bulb, rostral migratory stream, cerebellum). As shown in Figure 3.1, rats were exposed to sham or cranial IRR either at 37 days of age (IRR-CSA, Sham-CSA; IRR-SSA, Sham-SSA) or 98 days of age (CSA-WD/IRR, CSA-WD/Sham).

Cocaine self-administration

To facilitate acquisition of cocaine self-administration for the four groups of CSA rats (IRR-CSA, Sham-CSA, CSA-WD/IRR, and CSA-WD/Sham; Figure

3.1A, 3.1C), these rats were placed on a restricted diet (~15 g of chow per day). For the next 3 days, rats were placed in operant chambers (Med Associates, Georgia, VT) where they learned to press the active left lever for 45 mg sucrose pellets (Bio-serv, Frenchtown, NJ). All rats used for the CSA studies reported here reached acquisition criterion, which was 100 pellets/day for 3 consecutive days. After food training, rats were fed *ad libitum* at least 1 day prior to implantation with a chronic indwelling intravenous (i.v.) catheter as described previously (Noonan et al., 2008). Cocaine self-administration via the i.v. catheter began after one week of recovery from the surgery.

Cocaine hydrochloride was provided by the National Institute on Drug Abuse (Baltimore, MD). IRR-CSA, Sham-CSA, CSA-WD/IRR, and CSA-WD/Sham rats self-administered cocaine i.v. in 4-hour daily sessions for 15 days (Figure 3.1A, 3.1C). During CSA, the i.v. infusions of cocaine were always a) paired with the active/left lever, b) accompanied by illumination of the cue light above the left lever, c) delivered in a 0.1 mL volume over 5 sec, and d) followed by a 15 second (sec) time-out during which the cue and house lights were off for the last 10 sec. The response requirement for cocaine infusions was steadily increased to facilitate drug-seeking during later reinstatement testing (Keiflin et al., 2008). For example, for days 1-6 of CSA (acquisition phase), rats self-administered cocaine on a fixed-ratio reinforcement schedule (FR1): one active/left lever press led to a 0.5 mg/kg i.v. infusion of cocaine. For days 7-8 of

CSA, the schedule increased to FR3, requiring three lever presses for an infusion. For days 9-15 of CSA, the schedule increased to FR5, requiring five lever presses for an infusion.

On the day 16, most IRR-CSA (n=14) and Sham-CSA (n=13) and all CSA-WD/IRR and CSA-WD/Sham rats underwent within-session dose-response testing. Four doses of cocaine (1, 0.33, 0.10, 0.03 mg/kg) were made available in descending order, as previously described (Graham et al., 2007).

The remaining rats from the IRR-CSA (n=7) and Sham-CSA (n=5) groups did not undergo dose-response testing but rather progressive-ratio testing on days 16-21 as previously described (Figure 3.1A). The rats that underwent progressive-ratio testing self-administered 1 of 3 doses of cocaine (0.1, 0.25, 0.75 mg/kg) in two consecutive daily sessions in counterbalanced dose order over a total of 6 days. The testing followed the progressive-ratio schedule previously described (Richardson and Roberts, 1996), where each successive infusion required progressively higher lever pressing according to the following series: 1, 2, 4, 6, 9, 12, 15, 20, 25, 32, 40, 50,62, etc. according to response requirement = [5*e*(injection # x 0.2)] - 5. The “break point”, or highest ratio of responses/infusions completed before a 1-hour lapse in earning infusions, was analyzed using data from the second test at each dose (Graham et al., 2007).

Immediately after the dose-response testing or the last day of progressive-ratio testing, all rats were tested for catheter patency with sodium methohexital

(0.1 mg in 0.1 mL). IRR-CSA and Sham-CSA rats that underwent progressive-ratio testing were sacrificed the following day for immunohistochemical analyses. However, to enable reinstatement testing in the remaining IRR-CSA and Sham-CSA rats and in all CSA-WD/IRR and CSA-WD/Sham rats, these rats received four weeks of withdrawal in their home cage after dose-response testing (Figure 3.1A, 3.1C). Rats were handled every three days during this period.

Reinstatement testing

After CSA and four weeks of withdrawal in the home cage, the remaining IRR-CSA and Sham-CSA and all the CSA-WD/IRR and CSA-WD/Sham rats went through 6 days of reinstatement testing (Graham et al., 2007) followed by 1 day of locomotor testing. Reinstatement testing involved exposing the rat to stimuli known to induce relapse to drug seeking (the context or cue light previously-paired with CSA, the drug itself, or a stressful stimuli). Lever presses were recorded during the context-, cue-, drug-, and stress-induced reinstatement testing, but presses on the formerly drug-paired left lever were not reinforced with cues or cocaine infusion. Left lever presses were used as a measure of drug seeking. On day 1, contextual reinstatement was tested in a 4-hour session. For the entire 4-hour session, rats were placed in the self-administration chambers where they had previously received cocaine. On day 2, cue-induced reinstatement was tested in a 4-hour session. After 3 hours in the self-administration chamber,

the cue light above the formerly drug-paired left lever was illuminated for 5 sec every minute over a span of 30 minutes. On days 3-5, rats were tested for cocaine-induced reinstatement in 2-hour daily sessions. After 1 hour in the self-administration chamber, rats received an intraperitoneal (i.p.) injection of 0 mg/kg cocaine on day 3, and 5 or 15 mg/kg cocaine on days 4 and 5 in counterbalanced order. On day 6, stress-induced reinstatement was tested in a 2-hour session. After 1 hour in the self-administration chamber, mild foot shocks were delivered intermittently for 30 minutes (1.0 mA in 0.5 s with random intervals averaging 30s). On day 7, locomotor activity was recorded 90 minutes prior to and after an injection of cocaine (15 mg/kg i.p.) in a circular test chamber as reported previously (Edwards et al., 2007). As described below, all rats were sacrificed the following day for immunohistochemical analyses.

Sucrose self-administration and reinstatement testing

To assess the impact of cranial irradiation on a non-drug reward, rats in the IRR-SSA and Sham-SSA groups self-administered sucrose pellets (Figure 3.1B) in a manner as similar as possible to cocaine administration in the IRR-CSA and Sham-CSA groups (Figure 3.1A). IRR-SSA and Sham-SSA rats received cranial or sham irradiation as described above, and left in home cage for 4 weeks. Rats were handled every 3 days during this period, and were place on food restriction to encourage sucrose pellet self-administration one day prior to food

training (the last day of the four weeks in home cage). Four weeks after irradiation, IRR-SSA and Sham-SSA rats underwent food training on an FR1 schedule to reach an acquisition criterion of 100 pellets/day for three consecutive days. Two days after acquisition, and almost 5 weeks after IRR, locomotor activity was measured for 90 minutes as previously described (Edwards et al., 2007) to determine if irradiated rats showed signs of sickness that might impair their ability or motivation to seek food. After food training and locomotor testing, rats self-administered sucrose pellets in 30-minute daily sessions for 15 days following the escalating FR schedule used for the CSA groups (days 1-6 FR1, days 7-8 FR3, Days 9-15 FR5; 15 sec timeout after each reinforcement).

After SSA, rats were run for one week on progressive-ratio testing. Rats were examined in both food-restricted and sated states (Schmelzeis and Mittleman, 1996), to assess potential alterations in the motivation for food or sensitivity to reward devaluation, respectively. On days 1-3 food-restricted progressive-ratio testing occurred. Rats were then fed *ad libitum* on day 4, and on days 5-7 sated progressive-ratio testing occurred. Rats were then food restricted on day 8, and placed back on the FR5 timeout 15 sec schedule of reinforcement for days 9-11 to restabilize lever pressing before reinstatement testing.

Reinstatement testing involved exposing the rat to stimuli known to induce relapse to sucrose-seeking (the context or cue light previously-paired with SSA, or sucrose pellets). Lever presses were recorded during the context-, cue-, and

sucrose-induced reinstatement testing, but presses on the formerly drug-paired left lever were not reinforced with cues or sucrose pellet administration. Left lever presses were used as a measure of sucrose-seeking. On day 1, contextual reinstatement of sucrose-seeking was tested during the 4-hour session. Rats were placed in the operant chamber, and left lever presses were recorded but the cue light did not illuminate and no sucrose pellet was delivered. On day 2, cue reinstatement was tested in a 4-hour session. After 3 hours in the self-administration chamber, the cue light above the formerly sucrose-paired left lever was illuminated for 5 sec every minute over a span of 30 minutes. On days 3-5, rats were placed in the self-administration chambers for one hour to extinguish lever pressing. Together with days 1, 2, and 6, this allowed a total of 6 within-session extinction in both the SSA and CSA reinstatement studies (compare Figure 3.1A and 3.1B). On day 6, sucrose reinstatement was tested in a 4-hour session. After 3 hours, non-contingent presentation of 2 sucrose pellets occurred, followed by 1 sucrose pellet released every minute for 30 minutes. Stress-induced reinstatement was not performed, as stress does not reinstate sucrose-seeking (Buczek et al., 1999).

Cocaine brain levels assay

To determine if irradiation altered the blood brain barrier and thus potentially bioavailability of cocaine, a subset of IRR-SSA (n=8) and Sham-SSA

(n=8) rats were injected with 20 mg/kg of cocaine i.p. two days after the sucrose reinstatement session (10 weeks after irradiation), and decapitated 15 minutes later. Brains were removed from the skull, the cerebellum was removed, and the forebrain frozen in ice-cold isopentane. The brains were stored at -80°C until assay of cocaine levels, as previously published (Edwards et al., 2007). 1ml of the 1:4 diluted homogenate (0.25 g tissue) from each brain was assayed as follows: 200 ng of deuterated cocaine (Cerilliant, Round Rock, TX) was added as an internal standard, and the pH was adjusted to 9.3 with ammonium chloride buffer. Cocaine was extracted into 4ml of n-butyl chloride (Fisher Scientific, Pittsburgh, PA), and subsequently back-extracted into 0.5 ml of 0.1N sulfuric acid (Fisher Scientific). The pH was adjusted back to 9.3 with ammonium chloride buffer and the cocaine was extracted into 2 ml of n-butyl chloride. The dried residue was reconstituted with 30 ml of n-butyl chloride. Extracts were quantified on an Agilent 5973N GC-MS (Agilent Technologies, Wilmington, DE) in selected ion mode, and results corrected for deuterated cocaine recovery.

Tissue preparation

All rats except those used in the assay of cocaine brain levels were sacrificed via chloral hydrate anesthesia and intracardial perfusion with 0.1 M PBS (5 min with 10 ml/min flow rate) and 4% paraformaldehyde in 0.1 M PBS (20 min) as previously described (Noonan et al., 2008). After sacrifice, brains

were removed and postfixed in 4% paraformaldehyde in 0.1 M PBS for 24 hours at 4ºC. Brains were cryoprotected in 30% sucrose in 0.1 M PBS with 0.1% NaN_3 at 4ºC until coronal sectioning on a freezing microtome (Leica, Wetzlar, Germany) at 30 µm through the entire hippocampus (-1.80 to -7.64 mm from bregma; (Paxinos and Watson, 1997)) and the olfactory bulb (Noonan et al., 2008). Sections were stored in 0.1% NaN_3 in 0.1 M PBS at 4°C until processed for immunohistochemistry (IHC).

Immunohistochemistry

IHC was performed as previously described (Noonan, 2008). Briefly, every ninth section of the hippocampus was mounted on glass slides (Superfrost/Plus; Fisher Scientific) and allowed to dry overnight. Slides were coded prior to IHC and the code was not broken until after microscopic and data analyses were complete.

To assess irradiation-induced alterations in neurogenesis, an entire series of hippocampal sections was stained with an antibody against the immature neuron marker, doublecortin (DCX; (Brown et al., 2003b)). For DCX IHC, antigen unmasking (0.01M citric acid, pH 6.0, 95°C, 15 min) and quenching of endogenous perixodases (0.3% H_2O_2, 30 min) were performed, and sections were placed into blocking solution for 1 hour (3% serum) followed by primary antibody incubation (goat anti-doublecortin, Santa Cruz, Santa Cruz, CA; 1:3000)

overnight at room temperature. Sections were then incubated with a biotinylated secondary antibody (horse anti-goat, Vector Labs, Burlingame, CA; 1:200), followed by incubation with avidin-biotin (ABC Elite, Vector Labs; 1:50). DCX-immunoreactive (DCX+) cells were visualized via CY2 tyramide signal amplification (Perkin-Elmer, Norton, OH) and DAPI was used as a nuclear counterstain (1:5000, Roche, Nutley, NJ). Sections underwent rapid dehydration in ethanols and defatting in Citrosolv (Fischer Scientific) prior to coverslipping with DPX (Sigma-Aldrich, St. Louis, MO) While this antibody is widely used and has been shown to lack staining in knockout animals and provide a single band via immunoblotting (Kappeler et al., 2006), specificity of DCX staining was also ensured by lack of signal after omission of primary antibody and by observation of expected subcellular localization and cellular populations.

Quantification of immunopositive (+) cells

Using the optical fractionator method, DCX+ cells in the SGZ were counted at 400x magnification with an Olympus BX-51 microscope while continually adjusting the focal plane through the depth of the section (Noonan et al., 2008). An observer blind to treatment group performed all cell counts. Exhaustive counts were collected from every ninth hippocampal section throughout the anterior-posterior extent of the hippocampus (-1.80 to -7.64 mm from Bregma; (Paxinos and Watson, 1997)). Resulting cell counts were multiplied

by the fraction of the hippocampus examined (e.g. 9; (Noonan et al., 2008)) and are reported as total number of cells in the dentate gyrus.

Statistical analyses and presentation

Data are presented as mean ± S.E.M. Statistical analyses employed SPSS version 11.0 for Mac (Chicago, IL). Self-administration and extinction data were analyzed using two factor ANOVA with repeated measures on test session. Reinstatement data were analyzed using two or three factor ANOVA with repeated measures on dose or test session and lever. DCX+ cell counts were analyzed with two factor ANOVA. Main effect analyses were followed with Bonferonni post hoc tests. For analyses with one variable, such as latency to extinction, a t-test was used. Statistical significance for main effects, interactions, and t-tests was defined as $p<0.05$. Images were imported into Photoshop version 9.0.2 (Adobe Systems, Inc., San Jose, CA, USA) and the only adjustments made were via gamma in the Levels function.

RESULTS

Cranial irradiation produced long-term ablation of adult hippocampal neurogenesis without changing brain levels of cocaine

We explored the role of adult hippocampal neurogenesis in cocaine self-administration by giving rats cranial or sham irradiation 5 weeks before self-administration (Figure 3.1A). As shown previously (Snyder et al., 2005), two consecutive sessions of 10 Gy of cranial irradiation directed over the hippocampus was sufficient to robustly decrease adult hippocampal neurogenesis, as measured by DCX+ cell number in the SGZ (Supplemental Figure 3.6A). The cranial irradiation-induced reduction in neurogenesis was long-lasting, as rats examined between 5 and 13 weeks after radiation all showed at least a 70% reduction in DCX+ SGZ cell number as compared to controls (main effect of treatment, $F_{1,33} = 213.05$, $p<0.001$; Supplemental Figure 3.6B).

To assess whether irradiation produced a nonspecific injury that might change brain bioavailability of cocaine, we used mass spectrometry to measure brain levels of cocaine after a single cocaine injection given 10 weeks after irradiation. Brain levels of cocaine (ng/gram of brain) were similar between control and irradiated rats (Sham = 5956.38 ± 1526.27, IRR = 7365.00 ± 974.87; $p=0.45$). These data suggest that the blood brain barrier and the bioavailability of cocaine is not grossly influenced by cranial irradiation, and that the cocaine self-administration experiments are not confounded by differences in uptake of cocaine into the brain.

Irradiation before cocaine self-administration increased motivation for cocaine

To address the hypothesis that reduced adult hippocampal neurogenesis is a vulnerability factor for addiction, rats were irradiated (IRR-CSA) or sham-irradiated (Sham-CSA) prior to cocaine self-administration (Figure 3.1A). IRR-CSA rats did not differ from Sham-CSA rats in food training, as the latency to acquire lever pressing at the active left lever for 100 sucrose pellets across sessions was similar (no effect of treatment, $F_{1, 111} = 0.038$, $p = 0.846$; Figure 3.2A). These data suggest irradiation did not disrupt basic operant learning. However, IRR-CSA rats self-administered more cocaine across 15 daily 4-hour sessions (main effect of treatment, $F_{1, 518} = 8.35$, $p<0.01$; Figure 3.2B). These data show that cranial irradiation, which is accompanied by a robust decrease in hippocampal neurogenesis (Supplemental Figure 3.6B), leads to increased self-administration of cocaine.

Since lesions of the hippocampus produce hyperactivity (Tani et al., 2001; Hernandez-Rabaza et al., 2008) as well as increase perseverative lever pressing lever for both water and cocaine self-administration (Rabe and Haddad, 1968; Chambers and Self, 2002), we addressed the possibility that, like a hippocampal lesion, reduced neurogenesis via cranial irradiation might lead to hyperactivity and perseverative responding. Indeed, IRR-CSA rats pressed significantly more on the drug-paired left lever during the 15 sec timeout in the first two self-

administration sessions (interaction of treatment and day, $F_{14,518} = 1.72$, $p<0.05$; Supplementary Figure 3.7A), suggesting greater cocaine intake during the first and second self-administration sessions is likely due to perseveration. However, as perseveration did not occur on the subsequent 13 daily self-administration sessions, and there was no difference between IRR-CSA and Sham-CSA rats in inactive right lever pressing on any of the 15 daily sessions (no effect of treatment, $F_{1,518} = 0.27$, $p=0.61$; Supplementary Figure 3.7B), these data suggest that the overall increase in cocaine intake was not due to hyperactivity or perseverative behavior. Taken together with the fact that irradiation did not alter bioavailability of cocaine in the brain, we then explored other hypotheses as to why irradiated rats self-administered more cocaine.

First, we tested the hypothesis that cranial irradiation led to greater cocaine self-administration because they were more sensitive to cocaine's reinforcing effects. To this end, rats underwent a within-session dose-response test the day after the 15th self-administration session. IRR-CSA rats had a vertical shift in the dose-response curve (interaction of treatment and dose, $F_{3, 75} = 3.14$, $p<0.05$; Figure 3.2C). This vertical shift is indicative of increased sensitivity to cocaine and is considered a standard measure of the "addicted" phenotype in laboratory animals (Piazza et al., 2000). When the number of infusions is multiplied by the amount of cocaine infused, IRR-CSA rats took more cocaine at

several doses (interaction of treatment and dose, $F_{3, 75} = 3.76$, $p<0.05$; Figure 3.2D).

Second, we extended our exploration of the impact of cranial irradiation on the reinforcing aspects of cocaine via progressive-ratio testing, which assesses how many lever presses a rat is willing to perform for a single infusion of cocaine (Richardson and Roberts, 1996). To this end, a subset of IRR-CSA and Sham-CSA rats received progressive-ratio testing instead of a dose-response testing. IRR-CSA rats worked harder than Sham-CSA rats to receive their last infusion of cocaine (main effect of treatment, $F_{1,20} = 5.51$, $p<0.05$; Figure 3.2E). These data indicate cocaine was more reinforcing for irradiated rats, and rule out the possibility that rats took more cocaine because they were less sensitive to cocaine.

Together the fixed-ratio, dose-response, and progressive-ratio data suggest that rats that have been cranially irradiated and have reduced neurogenesis are more motivated to take cocaine as well as seek cocaine, and thus fit the definition of more vulnerable to drug addiction (Piazza et al., 2000).

Irradiation before sucrose self-administration does not change motivation for sucrose

To address whether suppression of adult hippocampal neurogenesis increased motivation for all reinforcers or only for drugs of abuse, we used a natural reward: sucrose self-administration. Rats were irradiated (IRR-SSA) or

sham-irradiated (Sham-SSA) prior to sucrose self-administration (Figure 3.1B). Latency to acquire lever pressing for 100 sucrose pellets was not different between groups (no effect of treatment, $F_{1,111} = 0.38$, $p = 0.54$; Figure 3.3A), confirming data from the CSA group that irradiation does not interfere with learning of an operant task. Locomotor activity assessed 5 weeks after irradiation was not different between groups (no effect of treatment, $F_{1,629} = 0.05$, $p=0.82$; Figure 3.3B), suggesting rats were not ill from irradiation and subsequent studies were not confounded by locomotor impairments. Sucrose self-administration on an FR1, FR3, or FR5 schedule with a 15 sec timeout did not differ between groups (no main effect of treatment; $F_{1, 518} = 2.04$, $p=0.16$; Figure 3.3C). Both IRR-SSA and Sham-SSA rats were similar in left lever pressing during the 15 sec timeout (no effect of treatment, $F_{1,518} = 1.87$, $p=0.18$; Supplementary Figure 3.7C) and pressing on the right inactive lever (no effect of treatment, $F_{1. 518} = 2.61$, $p = 0.12$; Supplementary Figure 3.7D), suggesting their general activity was the same. These data suggest that self-administration of food, a natural reward, was not altered by irradiation, and irradiated rats do not suffer from lack of appetite or motor impairment.

To confirm our results that irradiation did not alter motivation for food, we used a more sensitive assay that would be less influenced by satiety, progressive-ratio testing, while on and off food restriction. IRR-SSA rats worked as hard as Sham-SSA rats to receive their last sucrose pellet reward (no effect of treatment,

$F_{1,74} = 0.10$, $p=0.75$; Figure 3.3D), suggesting sucrose was equally reinforcing for them. When food restriction was removed, both groups were less willing to work for their last sucrose pellet reward than when they were food restricted (no effect of treatment, $F_{1,74} = 0.10$, $p=0.75$; Figure, 3.3E), suggesting equal sensitivity to reward devaluation. Together with the fixed-ratio data, these data suggest that irradiated rats do not have altered motivation for sucrose.

We next tested the ability of irradiated rats to learn that pressing on the left lever no longer was reinforced with sucrose in a series of extinction tests, as well as their ability to relapse to sucrose-seeking when presented with sucrose-paired cues or sucrose itself. Both IRR-SSA and Sham-SSA rats extinguished pressing on the formerly sucrose-paired lever (no effect of treatment, $F_{1,185} = 0.15$, $p<0.70$; Figure 3.3F). There was no difference between groups in pressing on the formerly sucrose-paired lever in response to presentation of sucrose-paired cues (no effect of treatment, $F_{1,37} = 0.20$, $p=0.66$; Figure 3.3G) or sucrose pellets (no effect of treatment, $F_{1,37} = 0.22$, $p = 0.65$; Figure 3.3H).

In summary, irradiated rats with reduced hippocampal neurogenesis have normal motivation for natural reward, as rats show no difference in self-administration of sucrose, extinction of sucrose-seeking, or relapse to sucrose-seeking after re-exposure to sucrose-related cues or sucrose itself. In addition, these experiments addressed possible confounds of the previous irradiation before cocaine-self administration experiment. Irradiated rats did not show perseveration,

general increased activity, or general learning enhancement when response requirements are increased (i.e. an increase from FR3 to FR5 schedule or the progressive-ratio task), and were able to learn to extinguish responding when reinforcement is withheld, suggesting any changes seen in motivation for reward after irradiation are not confounded.

Irradiation after cocaine self-administration enhanced resistance to extinction

Having identified that rats with suppressed adult neurogenesis are more vulnerable to acquisition of drug addiction, we next addressed the hypothesis that suppression of adult neurogenesis during withdrawal increases vulnerability to relapse to drug-seeking. To this end, rats were irradiated (CSA-WD/IRR) or sham-irradiated (CSA-WD/Sham) after acquisition of cocaine self-administration (Figure 3.1C). Rats were assigned to groups by balanced cocaine self-administration (no main effect of treatment, $F_{1,308} = 0.05$, $p = 0.83$; Figure 3.4A). After one month of withdrawal, rats went through reinstatement testing to assess their vulnerability to relapse to drug-seeking. CSA-WD/IRR rats pressed more on the formerly drug-paired lever when they were re-exposed to the former drug-taking context each day (interaction of treatment and session, $F_{5,18} = 2.99$, $p<0.05$; Figure 3.4B), and took more trials to extinguish their drug-seeking ($p<0.01$; Figure 3.4C). Both groups were similar in lever pressing in response to formerly

drug-paired cues (no main effect of treatment; $F_{1, 22} = 3.79$, p=0.06; Figure 3.4D) and low dose cocaine injections (no main effect of treatment; $F_{1,44} = 0.28$, p=0.60; Figure 3.4E). CSA-WD/IRR rats did not differ from sham rats in lever pressing in response to footshock stress (no main effect of treatment; $F_{1, 22} =1.26$, p=0.27; Figure 3.4F), suggesting that irradiation does not alter the HPA axis and response to stress. Both groups had similar basal and cocaine-induced locomotion (no main effect of treatment, $F_{1,374} = 0.23$, p=0.88; Figure 3.4G), suggesting increased extinction responding was not due to hyperactivity. These data suggest decreased neurogenesis increases vulnerability to relapse after exposure to the former drug-taking environment.

Rats irradiated before cocaine self-administration did not have altered extinction or reinstatement responding

We next addressed the hypothesis that reduced hippocampal neurogenesis during both cocaine self-administration and withdrawal would increase relapse to drug-seeking. Rats were irradiated or sham-irradiated one month before initial cocaine self-administration and went through reinstatement testing after one month of abstinence (Figure 3.1A). Because rats that have increased cocaine intake also have greater relapse to drug-seeking (Sutton et al., 2000; Edwards et al., 2007), and rats irradiated after cocaine self-administration (CSA-WD/IRR) had increased resistance to extinction (Figure 3.4B), we expected IRR-CSA rats

to show increased extinction and reinstatement test responding. The subset of IRR-CSA rats that continued onto reinstatement testing had increased cocaine self-administration compared to sham rats (effect of treatment, $F_{1, 350} = 4.56$, $p<0.05$; Figure 3.5A). However, irradiated rats did not differ in extinction of pressing on the formerly drug-paired lever (no effect of treatment, $F_{1, 125} = 0.85$, $p=0.36$; Figure 3.5B). In addition, latency to extinguish lever pressing was not different between sham and irradiated rats ($p<0.05$; Figure 3.5C). Irradiated rats did not differ from sham rats in lever pressing in response to formerly drug-paired cues (no effect of treatment, $F_{1,25} = 0.33$, $p=0.57$; Figure 3.5D) or in response to low dose cocaine injections (no effect of treatment, $F_{1,50} = 0.63$, $p=0.43$; Figure 3.5E). Additionally, irradiated rats did not differ from sham rats in lever pressing in response to footshock stress (no effect of treatment, $F_{1,25} = 1.82$, $p=0.19$; Figure 3.5F). Finally, irradiated rats did not differ from sham rats in basal locomotion or cocaine-induced hyperlocomotion (no effect of treatment, $F_{1,425} = 0.01$, $p=0.89$; Figure 3.5G), suggesting that no change in drug-seeking in IRR-CSA rats is not due to possible nonspecific side effects of irradiation such as lethargy. These data suggest that reduced neurogenesis prior to cocaine self-administration does not increase later drug-seeking, despite increasing cocaine intake prior to withdrawal (Figure 3.2).

DISCUSSION

There are two main findings presented here. First, suppression of adult hippocampal neurogenesis prior to drug-taking led to enhanced drug-taking. Second, suppression of adult hippocampal neurogenesis led to enhanced drug-seeking. Before we discuss these key findings and their implications, it is important to discuss our use of cranial irradiation to permanently reduce adult neurogenesis specifically in the hippocampus. While alternative methods of reducing neurogenesis are available, there are three reasons we chose to use cranial irradiation. First, cranial irradiation provides a reduction of hippocampal neurogenesis that is as large or larger in magnitude, and in a shorter period of time and with equal or greater consistency, compared with alternative methods, such as treatment with mitotic inhibitors (Dupret et al., 2005) or utilization of transgenic mice (Saxe et al., 2006; Imayoshi et al., 2008). Second, cranial irradiation provides a relatively regional-restriction of the suppression of neurogenesis and does not influence olfactory bulb neurogenesis. Mitotic inhibitors and most transgenic approaches suppress neurogenesis in the olfactory bulb as well as the hippocampus, and this could independently increase the sensitivity to psychostimulants like cocaine (Holmes et al., 2002; Slattery et al., 2007). Finally, cranial irradiation does not produce the reduced locomotion and sickness from mitotic inhibitors (Dupret et al., 2005) and is not accompanied by the technical challenges of intravenous catheterization of mice with mini-pump surgeries, which are required in some transgenic models of neurogenesis suppression (Saxe

et al., 2006). While there are issues related to cranial irradiation which are discussed at length below, these three factors made cranial irradiation our preferred methods to launch this fundamental studies on the relationship between adult hippocampal neurogenesis and cocaine self-administration.

Function of adult neurogenesis in drug-taking

The first notable finding of this work is that cranial irradiation, which diminished adult hippocampal neurogenesis, led to an enhanced vulnerability to cocaine addiction. One alternative interpretation of increased cocaine self-administration by irradiated rats on the first several fixed-ratio sessions is that irradiated rats had an extinction deficit of their prior food training, as the lever was first paired with sucrose pellets during food training, and then changed to 0.5 mg/kg infusion of cocaine in the self-administration sessions. One way to distinguish whether irradiated rats had increased lever pressing because they were expecting food but no longer being reinforced or that they found cocaine more reinforcing is to have irradiated rats self-administer cocaine without prior food training, so that the lever is only paired with drug and extinction of food responding is not a factor. While this experiment was not performed, the lack of extinction deficits in sucrose-self administration rats suggests that irradiated rats are not perseverative and can learn to extinguish responding on a formerly food-paired lever appropriately, and likely any increases in cocaine self-administration

in irradiated rats are due to cocaine being a stronger reinforcer of lever pressing for them compared to sham rats.

Irradiated rats had increased cocaine self-administration under both fixed and progressive-ratio schedules compared to sham-irradiated rats. A general increase in locomotion was not the cause of increased cocaine intake, as inactive lever pressing was not different between sham and irradiated rats. Perseveration at the active lever was not responsible for increased cocaine intake during fixed-ratio, dose-response and progressive-ratio testing, as lever pressing during the timeout was enhanced only for the first two days of cocaine self-administration. Brain levels of cocaine were equal between sham and irradiated rats ruling out the possibility that a compromised blood brain barrier or altered metabolism of cocaine are responsible for increased drug intake. Finally, sucrose self-administration was not different between sham and irradiated rats, suggesting decreased adult neurogenesis selectively enhanced motivation for drug.

There were two reasons we were surprised that reduced adult neurogenesis during cocaine self-administration (IRR-CSA) did not increase drug-seeking in the former drug-taking context after abstinence. First, rats that self-administer more cocaine on fixed and progressive-ratio schedules have increased drug-seeking (Sutton et al., 2003; Edwards et al., 2007). Second, rats irradiated before self-administration also lacked neurogenesis during the abstinence period, and rats lacking neurogenesis only during the abstinence period (CSA-WD/IRR) had

greater drug-seeking than sham-irradiated rats. It is possible that IRR-CSA rats did not differ from sham-irradiated rats in drug-seeking because adult-generated neurons encode part of the long-term drug-context memory, similar to adult neurogenesis being important for long-term spatial memory (Snyder et al., 2005; Imayoshi et al., 2008). This, in combination with enhanced motivation for drug-seeking due to reduced neurogenesis during the withdrawal period, resulted in no change in drug-seeking.

Function of adult neurogenesis in drug-seeking

Our second notable finding was that cranial irradiation, which reduced adult hippocampal neurogenesis, increased the propensity for relapse after a period of forced abstinence. Rats irradiated after cocaine self-administration had more drug-craving in the former drug-taking environment, and took more sessions to extinguish responding on the non-reinforced lever. This resistance to extinction was not a general learning deficit or perseveration, as irradiated rats were able to reduce responding within sessions and latency to last lever press was not different (data not shown). Previous work from the Eisch Laboratory has shown that 4 weeks of withdrawal from cocaine-self administration increases the number of DCX+ immature neurons in the SGZ of rats (Noonan et al., 2008). It was hypothesized that these newly-generated neurons were contributing to drug-craving or relapse. However, the results of our CSA-WD/IRR study suggest that a

loss of neurogenesis or prevention of a withdrawal-induced increase in neurogenesis enhances relapse to drug-seeking, and that the increase in new neurons generated during the withdrawal period is actually beneficial for the hippocampus and not contributing to addiction.

Changes are mediated by decreased neurogenesis and not other side effects of irradiation

It is important to consider whether possible side effects of cranial irradiation, such as inflammation, death of mature neurons, and alterations of synaptic function and the HPA axis may be responsible for the increase in motivation for cocaine-taking and cocaine-seeking (Deroche et al., 1997). We suggest that our irradiation paradigm maintains the integrity of mature hippocampal neurons, while ablating only neural progenitors. Irradiation did not cause a general dentate gyrus lesion in our study, as gross morphology (as measured by DAPI staining) was normal (data not shown), and hyperactivity was not seen, as has been reported with dentate gyrus lesions (Tani et al., 2001; Hernandez-Rabaza et al., 2008). Finally, a study using the same irradiation paradigm found no change in the electrophysiological properties of mature hippocampal neurons in CA1 and dentate gyrus (Snyder et al., 2005), suggesting that any alterations in hippocampal function are due to only to the loss of adult-generated neurons.

It is possible that irradiation may have caused general side effects that influenced brain regions other than the hippocampus. We can rule out altered hypothalamic-pituitary-adrenal (HPA) axis function, which could increase vulnerability to drug addiction (Oswald et al., 2005) because irradiation does not change levels of the stress hormone corticosterone (Santarelli et al., 2003). Our study also supports no change in HPA axis function after irradiation, because irradiated rats did not have altered footshock stress-induced reinstatement, which is influenced by HPA function (Erb et al., 1998). A known side effect of irradiation, inflammation, may have played some role in mediating changes in motivation for cocaine. We waited 4 weeks after irradiation to begin testing, a period after which inflammation is minimal (Snyder et al., 2005; Airan et al., 2007). However, we cannot rule out the impact of residual inflammation on drug-taking and drug-seeking, as alleviation of inflammation also restores adult neurogenesis (Monje et al., 2003) Irradiation decreased the weight gain of rats in all groups but did not cause weight loss (data not shown), as previously reported (Snyder et al., 2005), suggesting rats were not ill and had an appetite for food. Finally, increased drug-taking and drug-seeking in our study was not secondary to the development of a mood disorder, as reduction of adult hippocampal neurogenesis does not induce symptoms of depression (Santarelli et al., 2003; Surget et al., 2008). In sum, published findings and our data suggest that irradiation-induced reduction of adult hippocampal neurogenesis, and not side

effects of irradiation, are responsible for increased drug-taking and drug-seeking in our study.

Possible mechanism of control over drug-taking and drug-seeking by adult hippocampal neurogenesis

It is interesting to consider the mechanism of increased drug-taking and drug-seeking in irradiated rats with reduced adult hippocampal neurogenesis. Dentate gyrus neurons are 10 times likelier to synapse onto inhibitory interneurons than an excitatory CA3 pyramidal neuron (Ascady et al., 1998), and bursting of dentate gyrus granule neurons results in long lasting inhibitory input onto CA3, which results in suppression of activity in both CA3 and CA1 (Mori et al., 2007). Fitting with this idea of dentate gyrus neurons as part of the inhibitory filter is that lesions of the dentate gyrus are associated with hippocampal disinhibition and increased locomotion (Emerich and Walsh, 1990; Tani et al., 2001; Hernandez-Rabaza et al., 2008). In addition, activating or inactivating the subiculum, which provides the glutamatergic projections from the hippocampus to the NAc and other limbic regions increases or decreases drug-taking and drug-seeking, respectively (Caine et al., 2001; Vorel et al., 2001; Fuchs et al., 2005). It is possible that decreasing the number of adult-generated neurons could also result in hippocampal dishinhibition, with increased glutamatergic signaling from the hippocampus to limbic projection areas such as the cortex, basolateral

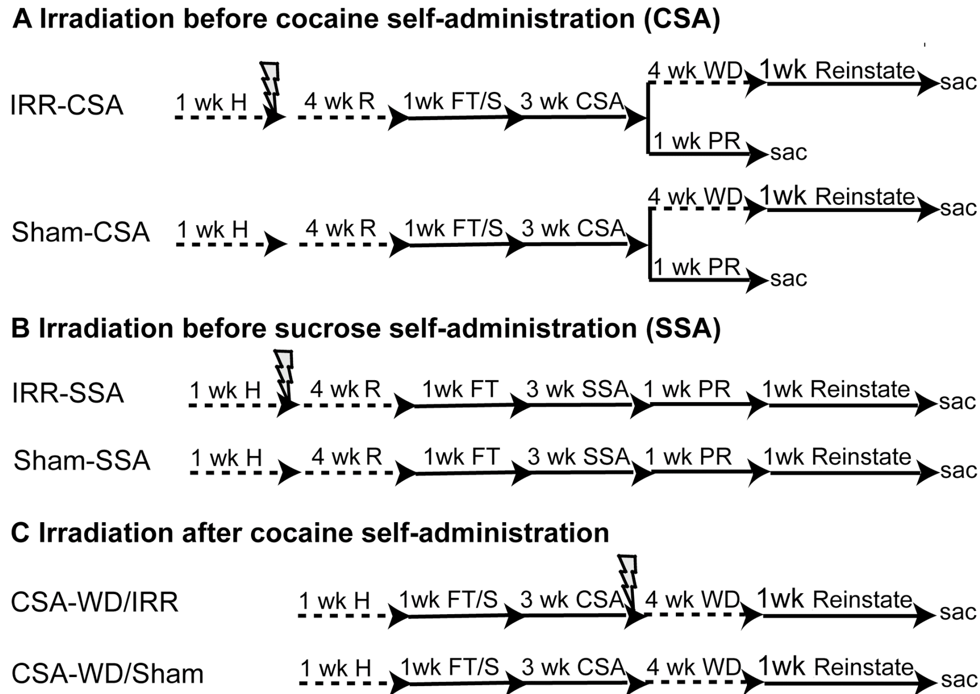
amygdala, and nucleus accumbens. In fact, other models of hippocampal disinhibition have found increased hippocampus-accumbens-ventral pallidum-ventral tegmental area circuitry activation (Floresco et al., 2001), which can increase dopamine release from the VTA in response to cocaine or the cocaine context, and may underlie behavioral sensitization to stimulants (Lodge and Grace, 2008). We suggest ablation of adult neurogenesis disinhibits neural circuitry responsible for initiating drug-taking and drug-seeking behavior, although clearly the mechanism of this disinhibition remains to be identified.

Possible clinical relevance

These studies suggest reduced adult hippocampal neurogenesis may enhance the transition from recreational user to addict as well as facilitate drug relapse, especially when faced with a former drug-taking environment. Treatments that increase adult neurogenesis may prevent addiction before it starts, which would be especially important for patients treated with potentially addictive prescription medication. Additionally, treatments that increase adult neurogenesis during abstinence may prevent relapse. Finally identification of cranial irradiation as a risk factor for addiction suggests cancer patients treated with irradiation should be monitored long-term for development of substance abuse disorders.

CHAPTER 3: Figures

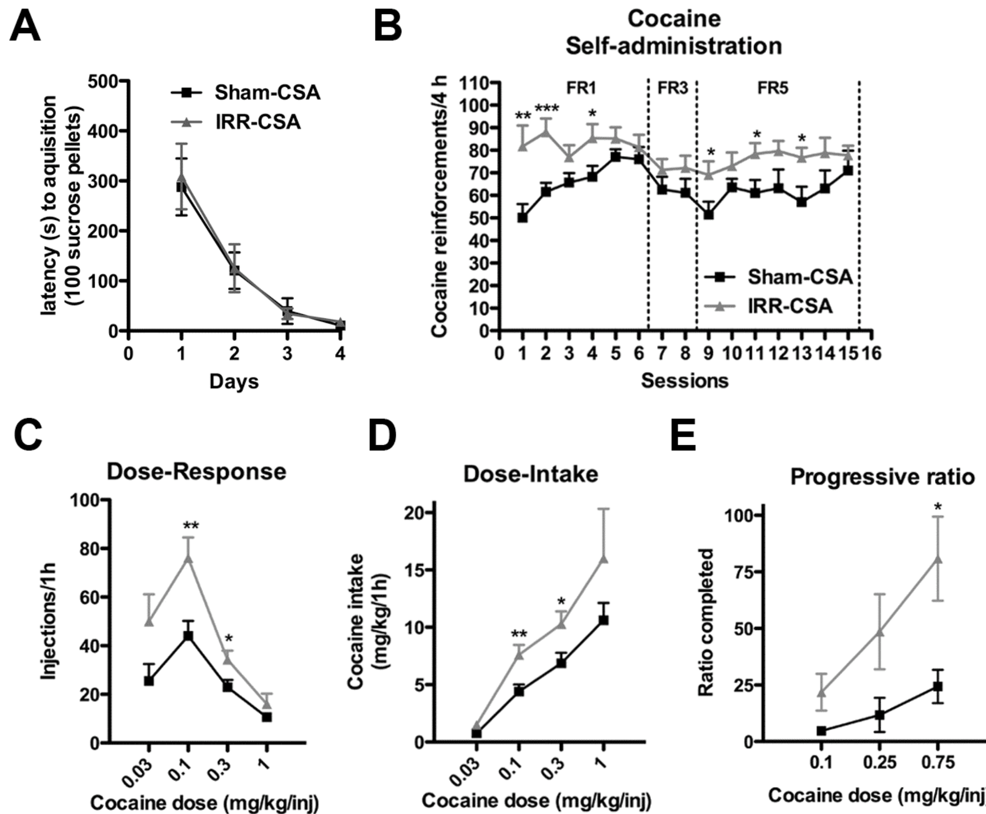
Figure 3.1



Cranial irradiation decreases adult neurogenesis. A. IRR-CSA (n=18) and Sham-CSA (n=21) rats were habituated to the animal facility for a week, anesthetized (50 mg/kg sodium pentobarbital, i.p.), irradiated, and left to recover for 4 weeks (with handling every 3 days). Rats completed food training, were implanted with an intravenous (i.v.) catheter, and allowed to recover for several days. 3 weeks of cocaine self-administration (CSA) followed. Rats either began progressive ratio testing and were sacrificed (IRR n=7, Sham n=5), or were given a dose-response test on the 16th day of CSA (IRR n=14, Sham n=13). 4 weeks of withdrawal in the home cage occurred next, followed by daily reinstatement testing, which consisted of an extinction session for at least one hour followed by reinstatement testing. The tests occurred in the following order: context, cue, saline, cocaine (15 mg/kg), cocaine (5 mg/kg), and footshock stress reinstatement. A locomotor test (basal and after a 15 mg/kg cocaine injection) was done on the

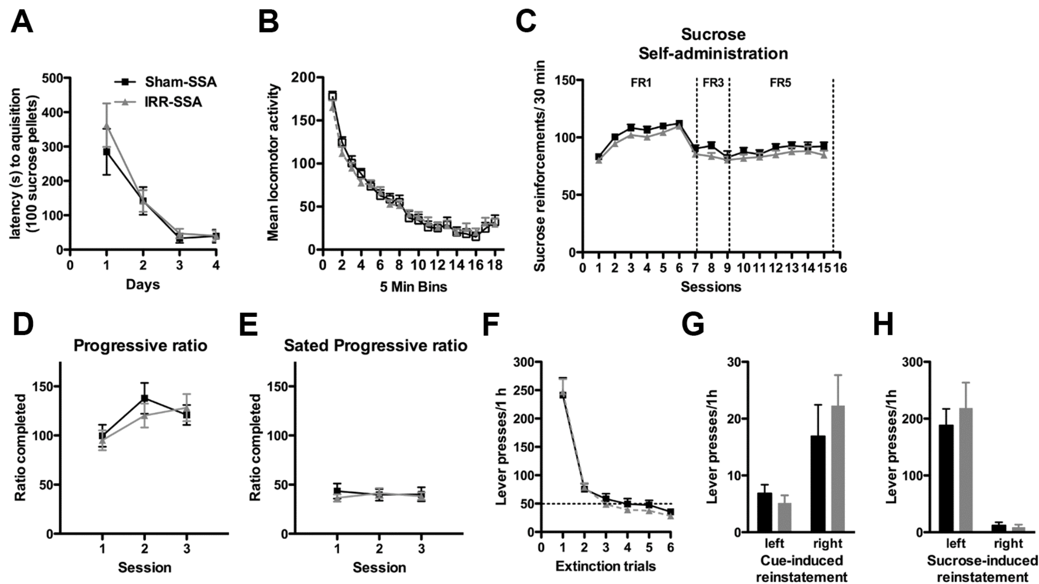
last day. Rats were sacrificed 24 hrs later. **B.** The experimental design for IRR-SSA (n=21) and Sham-SSA (n=18) rats was similar to Figure 2.1A, except that rats were not implanted with an i.v. catheter after food training and rats completed 3 weeks of sucrose pellet self-administration (SSA), followed by progressive ratio testing for sucrose pellets while food restricted for 3 day and while fed *ad libitum* for 3 days. Rats were then food restricted and restabilized on FR5 timeout 15 sec sucrose self-administration for 3 days. Reinstatement testing consisted of extinction of sucrose-seeking, cue-induced and sucrose-induced reinstatement testing. **C.** CSA-WD/IRR (n= 15) and CSA-WD/Sham (n=9) rats were irradiated after 3 weeks of CSA, and after 4 weeks of withdrawal that was coincident with the recovery period, were tested for reinstatement. All rats were age-matched to the beginning of CSA or SSA. Scale bar = 50 μm. Yellow bolt = 2 days of 10 Gy cranial irradiation, in a 1 cm diameter circle directed at the hippocampus. H = habituation, FT/S = food training, surgery, and recovery, CSA = cocaine self-administration, SSA = sucrose self-administration, WD = withdrawal, Reinstate = reinstatement testing, PR = progressive ratio, sac = sacrifice by intracardial perfusion.

Figure 3.2



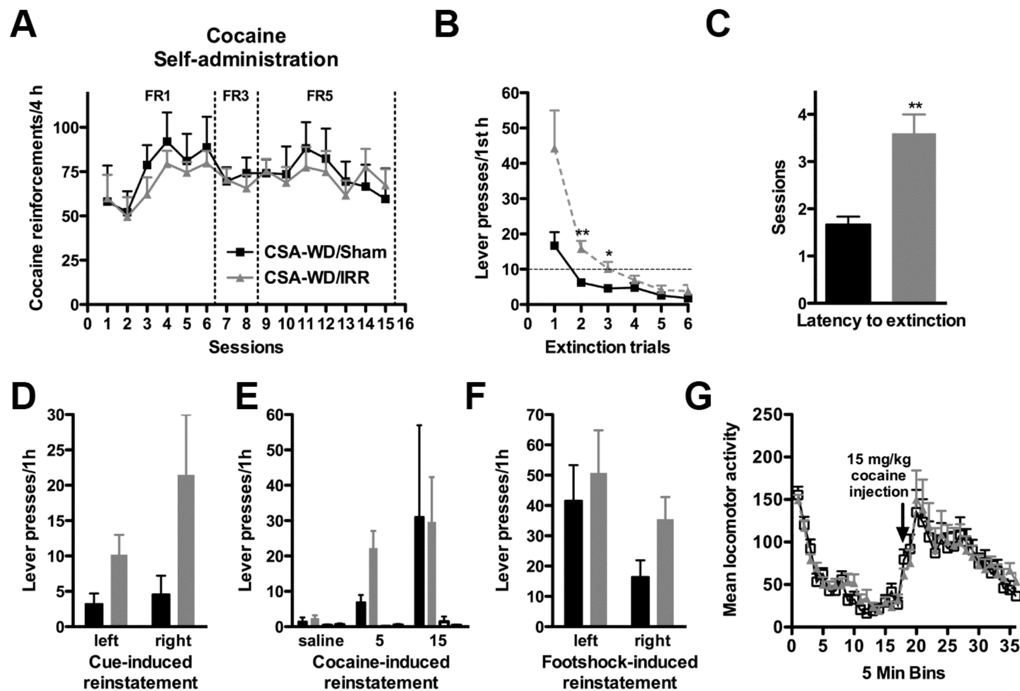
Cranial irradiation before cocaine self-administration increases cocaine reward. A. Irradiated rats did not differ from sham rats in the time it took to obtain 100 sucrose pellets by pressing the active left lever on a FR1 schedule during acquisition of food training. **B.** Cranial irradiation 4 weeks prior increased the amount of cocaine self-administered at a 0.5 mg/kg infusion dose. **C.** Irradiated rats self-administered more cocaine, causing a vertical shift in the dose-response curve. **D.** Dose-response data converted into dose-intake curves by multiplying infusions by dose show irradiated rats took more significantly more cocaine. **E.** Irradiated rats worked harder to get their last infusion of cocaine, suggesting they find cocaine more rewarding. Data for A-D presented as mean ± S.E.M.*p<0.05, **p<0.01, ***p<0.001.

Figure 3.3



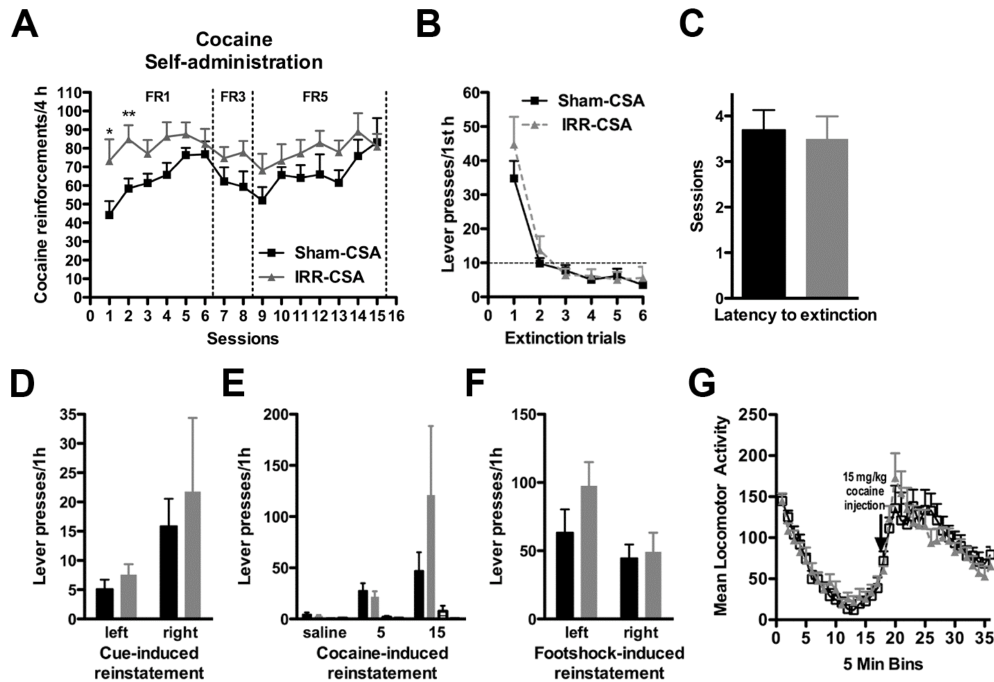
Cranial irradiation before sucrose self-administration does not change natural reward. A. Irradiated rats did not differ from sham rats in the time it took to obtain 100 sucrose pellets by pressing the left lever on a FR1 schedule during acquisition of food training. **B.** Basal locomotion was not different between irradiated and sham rats. **C.** Irradiated rats not differ from sham rats in sucrose pellet self-administration on a FR1, FR3, or FR5 schedule with timeout of 15 sec. **D.** Sucrose pellet self-administration on a progressive ratio was not different between irradiated and sham rats. **E.** Sucrose pellet self-administration on a progressive ratio when rats were removed from food restriction was not different between irradiated and sham rats. **F.** Irradiated rats did not differ from sham rats in their ability to extinguish responding on the formerly sucrose-paired lever. **G.** Irradiated rats did not differ from sham rats in responding to cues formerly paired with sucrose administration. **H.** Irradiated rats did not differ from sham rats in responding to presentation of sucrose pellets. Data for A-H presented as mean ± S.E.M.

Figure 3.4



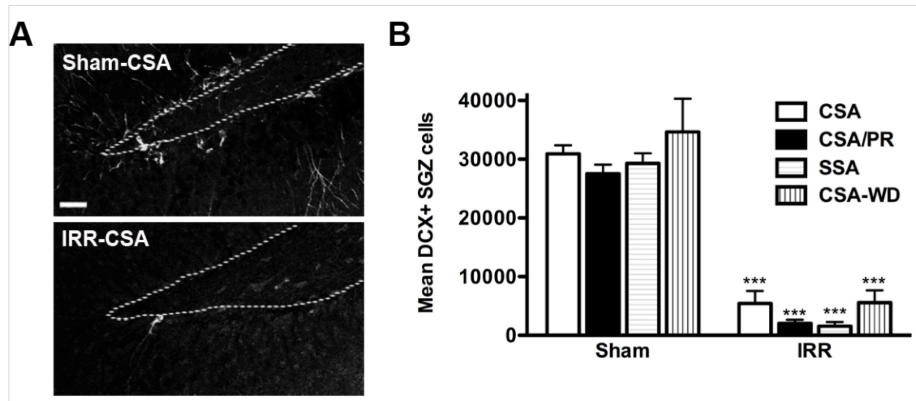
Cranial irradiation after cocaine self-administration increases context-induced relapse to drug-seeking. A. Rat were assigned to CSA-WD/IRR or CSA-WD/Sham groups based on balanced cocaine self-administration. **B.** Irradiated rats pressed more on the formerly drug-paired lever when re-exposed daily to the self-administration chamber after 4 weeks of abstinence. **C.** Irradiated rats took more sessions to extinguish lever pressing on the formerly drug-paired lever in the absence of reinforcement. **D.** Irradiated rats had a trend towards pressing both the formerly drug-paired lever and the inactive lever more than sham-irradiated rats in response to drug-cues. **E.** Irradiated rats had a trend towards pressing the formerly drug-paired lever more in response to low dose (5 mg/kg i.p.) cocaine exposure. **F.** Lever pressing between irradiated and sham-irradiated rats in response to footshock stress. **G.** Irradiated rats were not impaired in either basal locomotion or cocaine-induced hyperlocomotion (15 mg/kg i.p.). Data for A-G presented as mean ± S.E.M. *p<0.05, **p<0.01.

Figure 3.5



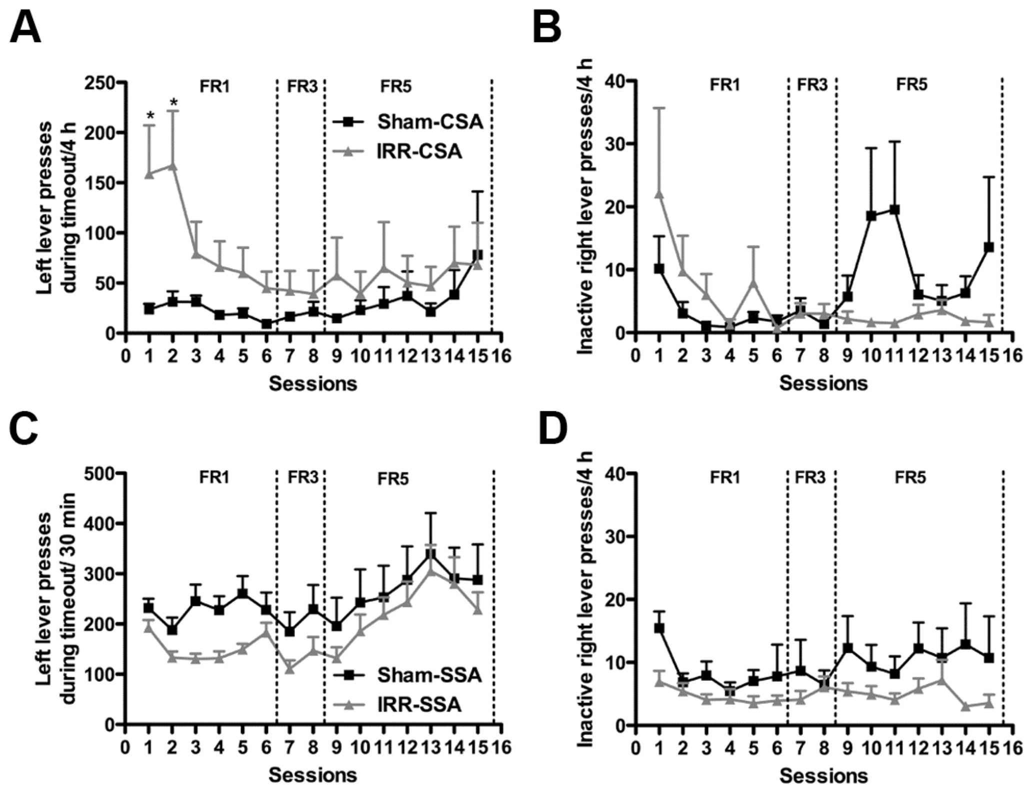
Cranial irradiation before cocaine self-administration does not change relapse to drug-seeking. A. Irradiated rats (subset of total IRR-CSA rats shown in Figure 3.2A) self-administered more cocaine than sham rats. **B.** Irradiated rats did not press more than sham rats on the formerly drug-paired lever when re-exposed to the self-administration chamber after 4 weeks of abstinence. **C.** Irradiated rats took the same number of days as sham rats to extinguish lever pressing at the formerly drug-paired left lever. **D.** Irradiated rats did not differ from sham rats in drug-seeking in response to drug cues. **E.** Irradiated rats did not differ from sham rats in drug-seeking in response to low dose cocaine injections. **F.** Lever pressing between irradiated and sham-irradiated rats in response to footshock stress was not significantly different. **G.** Irradiated rats were not impaired in either basal locomotion or cocaine-induced hyperlocomotion (15 mg/kg i.p.). Data for A-G presented as mean ± S.E.M.

Supplemental Figure 3.6



Cranial irradiation decreases adult hippocampal neurogenesis. A. Representative images at 20x of doublecortin+ (DCX+) immature neurons in the SGZ of Sham-CSA and IRR-CSA rats 13 weeks after irradiation. White dashed line is border between granule cell layer and hilus. Scale bar = 50 μm **B**. Quantitative analysis of DCX+ cells found a significant decrease in all irradiated groups at time of sacrifice compared to sham controls. Data for B presented as mean ± S.E.M. ***p<0.001.

Supplemental Figure 3.7



Cranial irradiation increases impulsive responding during the timeout period of cocaine self-administration, but not during sucrose self-administration. A. Irradiated rats pressed significantly press more on the drug-paired lever during the 15 second timeout on the first two self-administration sessions. **B.** Irradiated rats did not significantly press more on the inactive lever. **C.** Irradiated rats did not significantly press more on the food-paired lever during the 15 second timeout. **D.** Irradiated rats did not significantly press more on the inactive lever. Data for A-D presented as mean ± S.E.M.

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CHAPTER FOUR:
Suppression of adult neurogenesis via genetic ablation alters long-term drug-context memory

INTRODUCTION

The hippocampus is important for forming drug-context associations. Interestingly, passive or self-administration of stimulants in a novel environment (outside the homecage) increases locomotor and cellular responses to stimulants (Mattson et al., 2007; Mattson et al., 2008) and facilitates acquisition of cocaine self administration (Caprioli et al., 2007). The importance of drug administration environment is likely partly mediated by the hippocampus and studies suggest that this is likely specific to the dorsal region of the hippocampus. For example, lesions of the dorsal, but not ventral hippocampal subfields abolish cocaine CPP (Meyers et al., 2003; Meyers et al., 2006), and specifically, lesions of the dorsal dentate gyrus also abolish cocaine CPP (Hernandez-Rabaza et al., 2008). Additionally, inhibiting BDNF/TrkB signaling in the dorsal dentate gyrus is sufficient to block amphetamine CPP as well as conditioned motor sensitization (CMS) (Shen et al., 2006). These studies suggest that alterations in dorsal dentate gyrus plasticity could impact the learning or memory of drug-context associations.

The subgranular zone, which is at the border of the granule cell layer and the hilus of the dentate gyrus, is one of two regions in the adult brain that

continues to generate neurons throughout life (Gould, 2007). Adult-generated hippocampal neurons are thought to be important for learning and memory. Specifically, ablation of adult hippocampal neurogenesis via irradiation (Snyder et al., 2005) or transgenic mice (Imayoshi et al., 2008) both result in deficits in long-term spatial memory. It is controversial which types of learning and memory are impacted by loss of hippocampal neurogenesis, since for example, deficits associated with both contextual fear conditioning (Saxe et al., 2006; Winocur et al., 2006; Imayoshi et al., 2008), as well as Morris Water Maze (Snyder et al., 2005; Dupret et al., 2008) vary between different methods of ablation, behavioral protocols, and species. Contextual fear conditioning requires associating an aversive foot shock with the general context of the testing chamber, and requires both an intact hippocampus and amygdala (Phillips and LeDoux, 1992). Similarly, in behaviors paradigms such as drug conditioned place preference (CPP) there is an associative memory of the drug effect to the general context of the testing chamber, which also is dependent on the numerous connections between the hippocampus and limbic regions including the nucleus accumbens (Rademacher et al., 2006), but it remains to be determined whether adult-generated neurons may be required for this task. Investigation of whether CPP requires new neurons would address whether the new neurons may be important in drug-context associations and also help clarify the functional role for the new neurons.

Studies examining the function of adult neurogenesis often ablate neurogenesis with the confounding variable of unwanted side effects, such as inflammation after cranial irradiation (Monje et al., 2003) or illness after systemic administration of mitotic inhibitors such as MAM (Dupret et al., 2005). Other studies provide correlational evidence for the functional role of neurogenesis, such as reduced cognitive function in strains of mice with reduced neurogenesis, whether through age or gene deletion (Zhao et al., 2008). All of these methods include the caveat of incomplete temporal control, as neurogenesis is either suppressed irreversibly (irradiation; (Snyder et al., 2005)), for only 2 weeks (MAM; (Dupret et al., 2005)), or inefficiently (MAM; (Dupret et al., 2005), transgenic mouse; (Imayoshi et al., 2008). These studies also have the caveat of a lack of region specificity, since these methods often also inhibit neurogenesis in the subventricular zone/olfactory bulb (Imayoshi et al., 2008) or proliferation in the hypothalamus (Kokoeva et al., 2005). Currently lacking from the field of adult neurogenesis is a transgenic model that that selectively ablates adult neurogenesis in the hippocampus quickly and has the ability to restore neurogenesis with the same efficiency.

As the hippocampus, including the dentate gyrus, is important for drug-context associations, we hypothesize that adult-generated neurons are necessary for acquisition and recall of cocaine CPP. We have created a new transgenic

mouse model of neurogenesis ablation with enhanced temporal control to test this hypothesis.

METHODS

Mice

All mice were group housed in a climate-controlled environment on a 12 hour light-dark cycle (lights on at 0700 hours) with free access to water and lab chow. All experiments were performed in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* in an Institutional Animal Care and Use Committee and Association for Assessment and Accreditation of Laboratory Animal Care approved facility at UT Southwestern Medical Center.

Generation of nestin-CreER^{T2}/floxedSTOP-DTA mice.

Heterozygous nestin-CreER^{T2} mice (Lagace et al., 2007) were bred with homozygous floxed/STOP-DTA mice (Brockschnieder et al., 2006), resulting in all mice having the floxed/STOP-DTA gene but mice being positive or negative for the nestin-CreER^{T2} gene. Mice were genotyped by PCR using genomic DNA and primers previously published for Cre (Indra et al., 1999) and DTA (Brockschnieder et al., 2006).

Tamoxifen and BrdU administration

Nestin-CreER^{T2}/floxedSTOP-DTA mice (5 weeks old) were administered tamoxifen (TAM) at 180 mg/kg/d for 5 days as previously published (Lagace et al., 2007). Three days following TAM administration mice were administered BrdU (100 mg/kg 2x a day for 5 days) to permanently label cells dividing in the S-phase of the cell cycle (Figure 4.1B).

Cocaine CPP paradigm

A modified unbiased CPP paradigm was used (Renthal et al., 2007). Briefly, 29 days after the last TAM injection, nestin-CreER^{T2}/floxedSTOP-DTA mice (n=16 control, n=26 DTA) explored the 3 compartments of the CPP chambers (Med Associates, Georgia, VT) for 15 minutes on the pretest. For CPP training, mice were injected with saline (10 ml/kg, i.p.), and 5 minutes later confined to one side of CPP chamber. On the following day, mice were injected with cocaine (10 mg/kg) and confined to the opposite side of the CPP chamber 5 minutes later. The 5-minute delay between injection and conditioning enhances preference for the cocaine-paired side (Trantham et al., 2002). Training alternated for a total of 8 days. On posttest1, mice were allowed to explore the 3 compartments of the CPP chamber for 15 minutes to assess if the mice had a preference for the cocaine-paired side. Posttest14 was performed 2 weeks after posttest1 and the mice were again allowed to explore the 3 compartments of the

CPP chamber for 15 minutes to assess if the mice retained a preference for the cocaine-paired side Mice were sacrificed 90 minutes after posttest14 for immunohistochemistry (IHC). In a pilot study, adult male C57BLJ/6 mice went through the same CPP paradigm and were either conditioned with saline or cocaine (15 mg/kg). Mice were sacrificed immediately after posttest1 for IHC.

Olfactory habituation and discrimination testing

Nestin-CreER^{T2}/floxedSTOP-DTA mice that were previously used in a behavioral battery were tested for ability to smell and discriminate between odors (Tillerson et al., 2006) 50 (control n=7, DTA n=5) or 80 days (control n=26, DTA n=24) after the last TAM injection. Mice were single housed one week before experiments. Solution concentrations (1000 ng/ml) were freshly prepared before each experiment, and consisted of cinnamon (McCormick, Hunt Valley, MD), paprika (Private Selection, Cincinnati, OH), or MQ water (Millipore, Billerica, MA). The first day consisted of habituation to the cotton swabs, which hung upside down from the left and right side of the cage. On the second day olfactory testing in the form of five 3-minute olfactory habituation sessions began. 25 μl of odor solution was aliquoted onto one of the two cotton swabs, and 25 μl of water was aliquoted onto the other cotton swab. Sniffing was manually recorded and defined by the mouse's nose being within 1 cm of the cotton swab but did not include any time the mice spent biting the cotton swab. There was a 15-minute

interval between the five habituation sessions and between sessions the odor cotton swab position was randomized (left or right side of cage). Fifteen minutes after the 5th habituation session, discrimination testing was completed and consisted of a new odor replacing the odor the mice had habituated to, and water being placed on other cotton swab. Odor discrimination was defined as the time spent sniffing the new odor minus the time spent sniffing the old odor on the 5th session (Tillerson et al., 2006).

Tissue preparation

Animals were sacrificed via chloral hydrate anesthesia and intracardial perfusion with 0.1 M PBS (5 min with 7 ml/min flow rate) and 4% paraformaldehyde in 0.1 M PBS (15 min). After sacrifice, brains were removed and postfixed in 4% paraformaldehyde in 0.1 M PBS for 24 hours at 4 ºC. Brains were cryoprotected in 30% sucrose in 0.1 M PBS with 0.1% NaN_3 at 4ºC until coronal sectioning on a freezing microtome (Leica, Wetzlar, Germany) at 30 µm through the entire hippocampus and olfactory bulb. Sections were stored in 0.1% NaN_3 in 0.1 M PBS at 4°C until processed for IHC.

Immunohistochemistry

IHC was performed as previously described (Noonan, 2008). Briefly, every ninth section of the hippocampus was mounted on glass slides (Fischer

Superfrost/Plus, Hampton, NH) and allowed to dry overnight. Slides were coded prior to IHC and code was not broken until after microscopic analysis.

For DCX IHC, antigen unmasking (0.01M citric acid, pH 6.0, 95°C, 15 min), and quenching of endogenous peroxidases (0.3% H_2O_2, 30 min) was performed, and sections were placed into blocking (3% serum) and then into primary antibody incubation (goat anti-doublecortin (DCX), Santa Cruz, , CA; 1:3000) overnight at room temperature. Sections were then incubated in a biotinylated secondary (horse anti-goat, Vector Labs; 1:200), followed by incubation with avidin-biotin complex (ABC Elite, Vector Labs; 1:50) and then CY2 tyramide signal amplification (Perkin-Elmer, Norton, OH 1:50). DAPI was used as a nuclear counterstain.

For BrdU IHC, sections went through antigen unmasking, membrane permeabilzation, DNA denaturation, blocking, and primary antibody incubation (rat anti-BrdU, Accurate, Westbury, NY; 1:500) overnight. Sections were then incubated in a biotinylated secondary (goat anti-rat, Sigma; 1:200), followed by incubation with avidin-biotin complex, and then CY2 tyramide signal amplification, with DAPI counterstain.

For Ki-67 IHC, sections went through antigen unmasking, peroxidase quenching, blocking, and primary antibody incubation (rabbit anti-Ki-67, Vector Labs; 1:500) overnight. Sections were then incubated in a biotinylated secondary (goat anti-rabbit, Vector Labs; 1:200), followed by incubation with avidin-biotin

complex and then diaminobenzidine (DAB, Pierce, Rockford, IL 1:10). Fast Red was used as a counterstain (Vector Labs).

For all IHC experiments, sections underwent dehydration in ethanol and defatting in Citrosolv (Fischer) prior to coverslipping with DPX (Sigma-Aldrich). Specificity of staining was ensured by lack of signal after omission of primary antibody and by observation of expected subcellular localization and cellular populations stained for each antibody.

Quantification of immunopositive (+) cells

DCX+ cell counts were performed at 400x magnification with an Olympus BX-51 microscope while continually adjusting the focal plane through the depth of the section. An observer blind to treatment group performed all cell counts, using the optical fractionator method. Briefly, exhaustive counts were collected from every ninth hippocampal section throughout the anterior-posterior extent of the hippocampus (-1.80 to -7.64 mm from bregma; (Paxinos and Watson, 1997)). Resulting cell counts were multiplied by the fraction of the hippocampus examined and are reported as total number of cells in the dentate gyrus (Noonan et al., 2008).

Ki-67 and BrdU+ cell counts were performed similar to above, except cells were counted in four discrete regions of the dentate gyrus as previously described (Donovan et al., 2006): the SGZ, the outer granule cell layer (oGCL),

the molecular layer (Mol) and the hilus. BrdU cells were all counted in the medial habenula to control for bioavailability of BrdU and general levels of proliferation.

Statistical analyses and presentation

Data are presented as mean ± S.E.M. Statistical analyses employed SPSS version 11.0 for Mac (Chicago, IL) or GraphPad Prism version 5.00 for Mac (GraphPad Software, San Diego, CA). CPP data, olfactory habituation data and BrdU+ cell counts were analyzed using two factor ANOVA. Main effect analyses were followed with Bonferonni post hoc tests. For analyses with one variable, such Ki-67+ or DCX+ cell counts or olfactory discrimination testing, a t-test was used. Statistical significance for main effects, interactions, and t-tests was defined as $p<0.05$. Images were imported into Photoshop version 9.0.2 (Adobe Systems, Inc., San Jose, CA, USA) and the only adjustments made were via gamma in the Levels function.

RESULTS

Adult neurogenesis transiently suppressed in nestin-CreER^{T2}/floxedSTOP-DTA mice

To test the hypothesis that adult-generated neurons contribute to long-term drug-context memory, we used a novel transgenic mouse called nestin-CreER^{T2}/floxedSTOP-DTA that was created by crossing nestin-CreER^{T2} mice

crossed with floxedSTOP-DTA mice (Figure 4.1A). When nestin-CreER^{T2}/floxedSTOP-DTA mice are administered the estrogen receptor ligand TAM, cre is translocated to the nucleus allowing for the removal of the STOP codon and expression of diptheria toxin (DTA) in nestin-expressing neural stem and progenitor cells. Expression of DTA results in cell death and thus prevents subsequent birth of new neurons form the nestin-expressing cell. As depicted in Figure 4.1B, TAM was administered 4.5 weeks before mice began testing in the cocaine conditioned place preference (CPP) paradigm that assesses drug-context learning and memory .

To assess the number of adult-generated cells in the nestin-CreER^{T2}/floxedSTOP-DTA mice at multiple timepoints (12, 30, 52 days) after TAM we used IHC to label both proliferating cells that expressed the endogenous cell cycle protein Ki-67 and immature neurons that expressed DCX. Naive mice were used for the assessments at 12 and 30 days following TAM, however the mice that underwent CPP training were used for the 52 day time point in order to reduce animal numbers and since pilot studies in C57 mice demonstrated CPP training did not alter Ki-67+ or DCX+ cell number (Supplemental Figure 4.2). There was a significant reduction the number of proliferating Ki-67+ cells in the SGZ of Nestin-CreER^{T2}/floxedSTOP-DTA mice compared to control mice 12 days ($p<0.01$) and 30 days ($p<0.05$) after TAM (Figure 4.1C). However, there was no significant difference between DTA and control mice 52 days after TAM,

which was the timepoint of sacrifice after posttest 14, ($p>0.05$) (Figure 4.1C). Similarly, we found that the number of immature DCX+ neurons was significantly decreased 12 days ($p<0.05$), but not 30 days ($p>0.05$) or 52 days ($p>0.05$) after TAM in the nestin-CreER^{T2}/floxedSTOP-DTA mice (Figure 4.1C). Mice were injected with the thymidine analog BrdU to label cells born 4 weeks prior to the start of CPP training (Figure 4.1B). There was a significant and selective reduction of BrdU+ cells in the SGZ of nestin-CreER^{T2}/floxedSTOP-DTA mice, which were 7 weeks old at the time of sacrifice ($F_{4,50} = 2.62$, $p<0.05$; Figure 4.1D). This analysis of Ki-67+ and DCX+, and BrdU cell number suggests nestin-CreER^{T2}/floxedSTOP-DTA mice had reduced levels of proliferation between 3 and 30 days after TAM (prior to CPP training), but this normalized by 52 days of TAM when posttest14 was performed.

Nestin-CreER^{T2}/floxedSTOP-DTA mice learn cocaine conditioned place preference but lose preference 2 weeks later

Nestin-CreER^{T2}/floxedSTOP-DTA and control mice did not show a bias for either side of the box on the pretest, before CPP training ($p>0.05$; Figure 4.1E). In addition, locomotor activity was assessed in the cocaine and saline paired compartments during CPP training, and no difference was found between DTA and control mice ($p>0.05$; data not shown), suggesting there was no possible confound of locomotion on learning of CPP. After CPP training, on posttest1,

both the nestin-CreER^{T2}/floxedSTOP-DTA and control mice spent significantly more time in the drug-paired compartment than the saline-paired compartment and there was no difference between the two groups of mice ($p>0.05$; Figure 4.1E). In contrast, on a recall test of CPP two weeks following training, nestin-CreER^{T2}/floxedSTOP-DTA mice showed no preference for the cocaine paired side, while control mice still retained their preference (interaction of genotype and test session, $F_{2,39}= 3.646$, $p<0.05$; Figure 4.1E). This suggests adult-generated hippocampal neurons born prior to or during drug-context learning are necessary for long-term memory of drug-context associations.

Nestin-CreER^{T2}/floxedSTOP-DTA mice have no deficits in olfactory discrimination

We assessed primary olfactory function and olfactory discrimination at two timepoints after TAM administration, 50 and 80 days. Control mice at both timepoints spent more time sniffing an odored cotton swab than a water cotton swab in the first trial (Supplemental Figure 4.3A, 4.4A) and habituated to the odor by sniffing it less over trials. On the odor discrimination test, mice were presented with the habituated odor as well as a novel odor, and control mice correctly discriminated that there was a new odor and sniffed it more than the habituated odor (Supplemental Figure 4.3B, 4.4B). There was no difference between control mice and nestin-CreER^{T2}/floxedSTOP-DTA mice at either 50 or 80 days post

TAM, as nestin-CreER^{T2}/floxedSTOP-DTA mice smelled the first odor and habituated over sessions (Supplemental Figure 4.3C, 4.4C) and discriminated that there was a new odor (Supplemental Figure 4.3D, 4.4D).

DISCUSSION

This study characterized the dynamics of neurogenesis after multiple timepoints after diphtheria toxin expression in nestin+ neural stem and progenitor cells. The nestin-CreER^{T2}/floxedSTOP-DTA mouse provided a reversible method of neurogenesis suppression with temporal control over reduced neurogenesis. While this method of neurogenesis was surprisingly not as potent of an inhibitor of neurogenesis as we expected based on our previous reports of the high efficiency of this system (Lagace et al., 2007), there was a striking reduction in cocaine place preference at 2 weeks following cocaine place preference training in the nestin-CreER^{T2}/floxedSTOP-DTA mice, that was not present following cocaine place preference training. These findings suggest that the long-term drug-context memory on the cocaine place preference may require adult neurogenesis.

There are several explanations for why the nestin-CreER^{T2}/floxedSTOP-DTA mice had a reduction in neurogenesis that was transient. First, it is possible that nestin+ neural stem and progenitor cells are not the primary neural stem cell. It is possible that there are quiescent stem cells that become active when the pool of nestin+ stem cells is reduced, or that a non-nestin+ stem cell exists that could

give rise to nestin+ stem cells. While this is controversial, unpublished work from the laboratory of Amelia Eisch supports nestin+ cell may not be the only stem cell, and the recently published nestin-CreER^{T2}/floxedSTOP-NSE-DTA mice transgenic mouse model of neurogenesis ablation also reported an incomplete ablation (Imayoshi et al., 2008). New technologies, such as dual recombinase transgenic mice (Jensen et al., 2008) crossed to the floxedSTOP-DTA mice, could be used to target and ablate alternative pools of neural stem cells. Second, it is possible that nestin+ stem cells are resistant to diphtheria toxin induced death. Third, it is possible that the nestin+ stem cells are sensitive to diphtheria toxin induced death, that recombination occurred in 99% of nestin+ stem cells, and that there are no other stem cells besides nestin+ stem cells. However, even a population of 1% of stem cells remaining or downstream cell populations not impacted by diphtheria toxin, such as type 2 transit-amplifying cells or mitotic DCX+ cells could repopulate the SGZ, suggesting a mechanism for the normalization of neurogenesis we see with time. Finally, it is possible that cre recombinase was not efficient at excising the stop codon in front of the diphtheria toxin gene, and diphtheria toxin was not expressed in all nestin+ cells. While this may seem unlikely given that recombination occurred in almost 97% of radial glial stem-like SGZ cells in the nestin-CreER^{T2}/R26R-YFP mouse (Lagace et al., 2007), it is not unprecedented for different combinations of cre drivers and "floxed" genes to require different amount of tamoxifen to drive the most efficient

possible level of recombination. Thus, it is possible that our five day, 180 mg/kg treatment with tamoxifen was not ideal for inducing maximum recombination in the nestin-CreER^{T2}/floxedSTOP-DTA mouse. Alternative strategies to obtaining the highest amount of recombination include 1) using hydroxytamoxifen, which has greater affinity for the estrogen receptor than tamoxifen (Coward et al., 2001) using higher doses or longer treatment with tamoxifen, with the drawback being higher lethality, 3) changing the pattern of tamoxifen treatment and 4) delivering tamoxifen via an alternative route, such as orally in the drinking water (Imayoshi, et al., 2008) or via a minipump.

We found no difference between control and DTA mice in cocaine CPP at one day after training for CPP (posttest1). We were surprised by this finding, because in the previous chapter, we found that suppression of neurogenesis via cranial irradiation of the hippocampus resulted in increased motivation for cocaine (Figure 3.1). There are several explanations as to why DTA mice did not show increased cocaine CPP one day after training. First, cranial irradiation is a robust and irreversible method of neurogenesis ablation, resulting in at least 70% reduction of neurogenesis (Supplemental Figure 3.7), whereas neurogenesis reduction was modest and transient in the DTA mice (Figure 4.1C). Second, the irradiation study used rats, whereas the DTA study used mice, and it is known that mice have different dopamine receptor expression (Baker et al., 2005) and cellular and behavioral responses to drugs of abuse than rats (Zocchi et al., 2001). In fact,

all the studies that have found the dentate gyrus or hippocampus to be important for stimulant CPP have used rat and not mouse (Meyers et al., 2003; Shen et al., 2006; Hernandez-Rabaza et al., 2008). Third, cocaine was given in a chronic and voluntary manner in the previous chapter, whereas this study used passive injections of low-dose cocaine every other day for a total of 4 injections. DTA mice may not have been exposed to enough cocaine to show increased sensitivity to cocaine either via locomotion or CPP. It is possible that pre-exposing mice to cocaine injections, and then running CPP may have unmasked difference in cocaine reward (Renthal et al., 2007). Lastly, a combination of a hippocampal deficit in associating drug and context, as well as increased cocaine reward, could have resulted in no change in cocaine CPP.

Our second finding was that mice with reduced adult hippocampal neurogenesis prior to learning the cocaine CPP paradigm did not show a preference for the cocaine-paired side 2 weeks after learning the task. One explanation for lack of CPP memory is that olfactory bulb neurogenesis is also decreased in the nestin-CreER^{T2}/floxedSTOP-DTA mice. Olfactory bulbectomy leads to deficits in recall of cocaine CPP (Calcagnetti et al., 1996). It is possible that reduced OB neurogenesis may function similar to olfactory bulbectomy due to reduction of OB size. In the nestin-CreER^{T2}/floxedSTOP-DTA mice, we have not yet quantified neurogenesis, so it is not clear whether there is decreased neurogenesis in these mice. However, odor discrimination testing demonstrated

that nestin-CreER^{T2}/floxedSTOP-DTA they can both smell odors and discriminate between a new odor and an old odor, suggesting, unlike mice with olfactory bulbectomies, nestin-CreER^{T2}/floxedSTOP-DTA mice can smell. Together with qualitative data that the olfactory bulbs of DTA mice appear to be the same size as bulbs from control mice, this suggests it is unlikely that an olfactory bulb function in the nestin-CreER^{T2}/floxedSTOP-DTA mice could explain the lack of CPP memory.

There are several possible interpretations for why the nestin-CreER^{T2}/floxedSTOP-DTA mice did not show a preference for the cocaine-paired side two weeks after training. First, DTA mice may have not found cocaine as rewarding as control mice, and thus preference for the cocaine-paired side degraded faster. This could be tested by using lower, threshold doses of cocaine during training, such that DTA mice would not be able to show a place preference in the first test if they found cocaine less rewarding. Second, DTA mice could have had a problem with reconsolidation of the CPP memory, as the drug-context memory may be labile during posttest1 (Nader et al., 2000) While this could be tested by performing the CPP posttest 2 weeks after initial training without the first posttest 1 day after training, it has been found that a morphine CPP test does not cause memory to become labile (Milekic et al., 2006). Thus, we predict that loss of CPP memory in DTA mice is not due to reconsolidation problems,

although reduced cocaine reward is possible, but less likely due to work discussed in Chapter 3.

The third possibility is that adult-generated neurons are important for long-term memory of drug-context associations. Previous publications found a role for adult neurogenesis in long-term spatial memory (Snyder et al., 2005; Imayoshi et al., 2008). It has also been proposed that adult neurogenesis may be important for mood-related tasks that involve both the hippocampus and the amygdala (Saxe et al., 2006), such as contextual fear conditioning. Cocaine CPP requires both the hippocampus and the amygdala (Fuchs et al., 2002; Meyers et al., 2006) and as adult neurogenesis is necessary for long-term cocaine CPP memory, this study lends support to this theory. This is the first time adult hippocampal neurogenesis has been found to be necessary for appetitive learning and memory, as neurogenesis has been found to be important for either aversive learning tasks (fear conditioning) or purely spatial tasks (Morris Water Maze).

These studies lend some insight into the function of adult neurogenesis in hippocampal processing. One theory is that new neurons function to clear old memories out of the hippocampus and store them in new brain regions permanently (Feng et al., 2001). This study does not support this theory, as loss of new neurons in DTA mice did not result in stronger CPP memory. Another theory is that adult hippocampal neurogenesis prevents catastrophic interference (Wiskott et al.) or overloading of memory circuitry by too many new memories. It

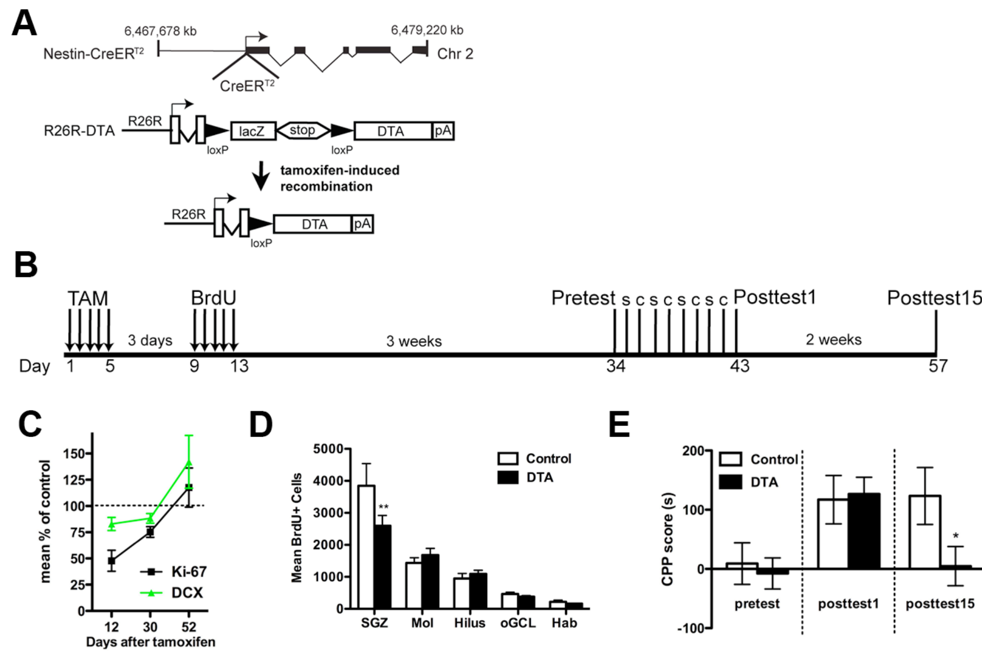
is possible that in the DTA mice, reduced neurogenesis lead to diminished memory storage capacity and results in a forgotten drug-context memory.

Conclusions

We have developed a new tool to study the function of adult neurogenesis, the nestin-CreER^{T2}/floxedSTOP-DTA mouse. Using this mouse, we have found no role for olfactory bulb neurogenesis in primary olfaction or olfactory discrimination. In addition, we found that long-term drug-context memory on the CPP task is likely disrupted in these mice, which is supported by other studies that have found deficits in long-term spatial memory on Morris water maze and Barnes maze (Snyder et al., 2005; Imayoshi et al., 2008).

CHAPTER 4: Figures

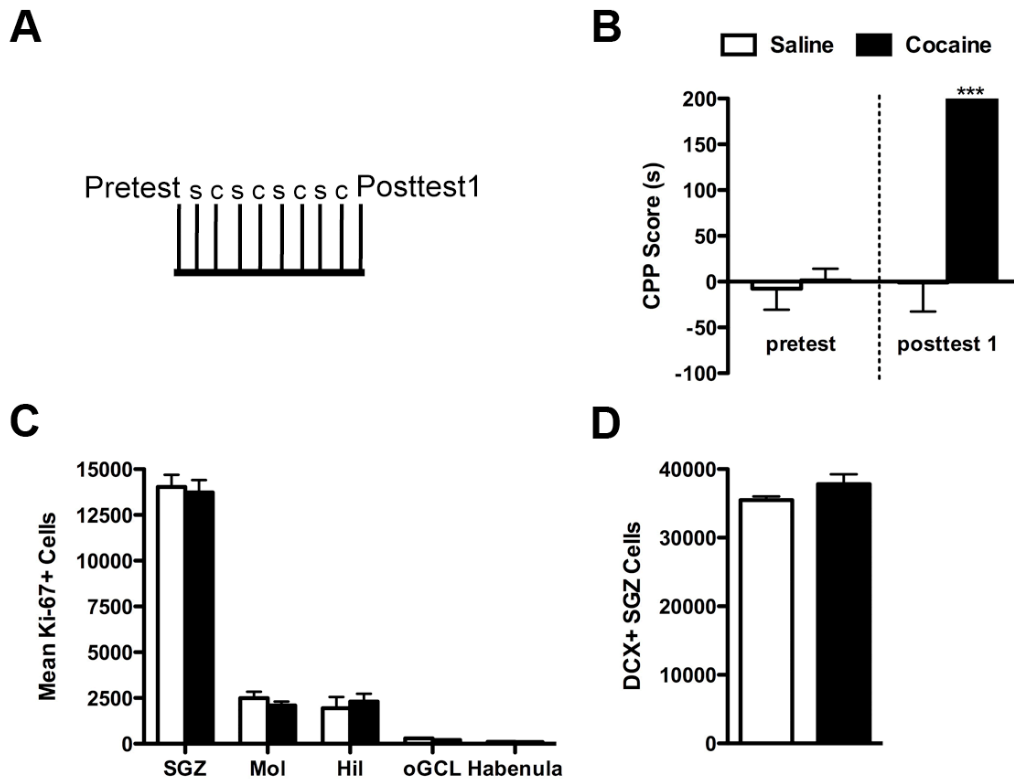
Figure 4.1



Suppression of adult neurogenesis in nestin-CreER^{T2}/floxedSTOP-DTA mice impairs recall of drug-context memory. A. Nestin-CreER^{T2} mice were crossed with floxed-STOP-DTA mice. **B.** Nestin-CreER^{T2}/floxed-STOP-DTA (DTA; n=26) and control (n=16) mice were injected with tamoxifen to suppress adult neurogenesis via cre-mediated expression of diphtheria toxin in nestin+ neural stem and progenitor cells. A permanent label of proliferating cells, BrdU, was injected 4 weeks prior to the start of cocaine conditioned place preference (CPP) training, in order to access whether these 4 week old neurons were decreased during training. CPP training consisted of a pretest to ensure there was no bias to either chamber side, and then alternating 4 daily saline injections and 4 cocaine injections that were paired with one side of the box for either saline or cocaine. A posttest was done after the last cocaine conditioning day to assess preference for the cocaine-paired side. Two weeks following posttest1, posttest 15 accessed recall of preference for the cocaine-paired side. **C.** DTA mice injected with tamoxifen 12 or 30 days earlier (equivalent to day 17 and 35 on the timeline) had

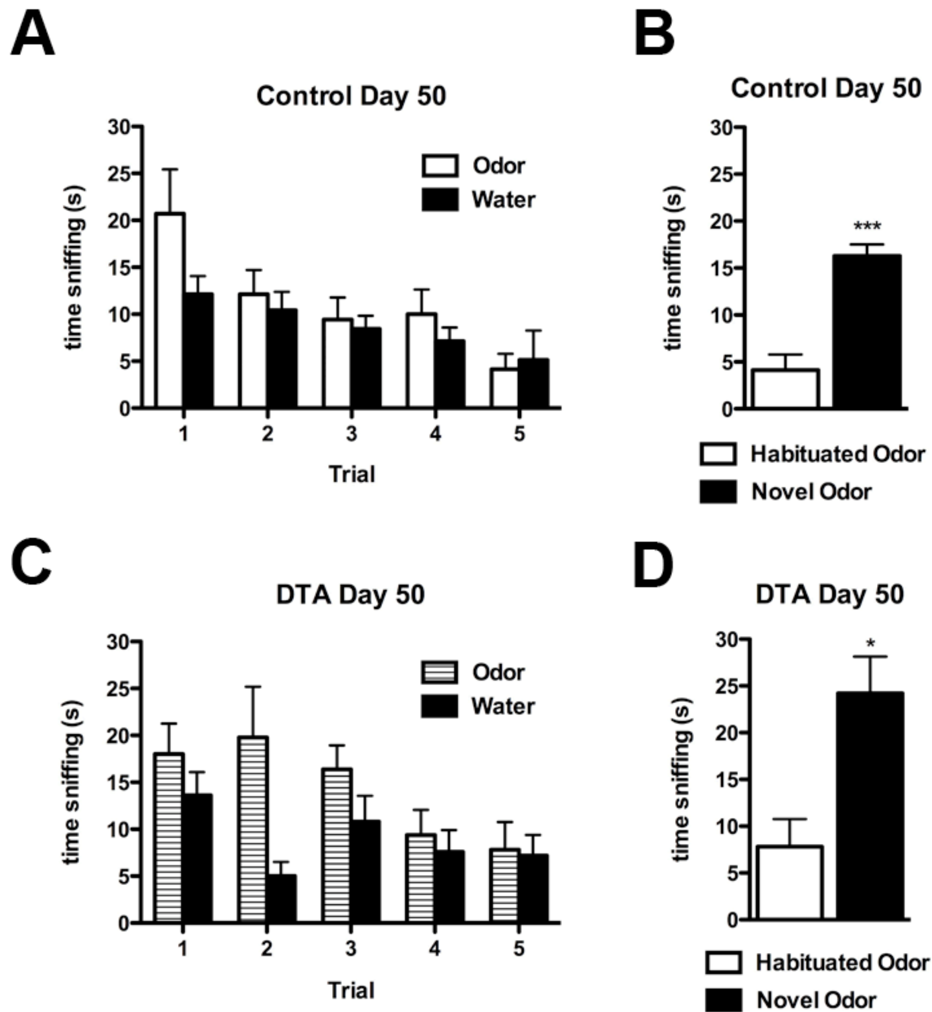
significantly less proliferating Ki-67+ cells, whereas DCX+ immature neurons were only significantly reduced 12 days after tamoxifen. **D.** DTA mice (n=6) have fewer 7-week old BrdU+ cells in the SGZ than control mice (n=6). **E.** DTA mice no longer show a preference for the cocaine-paired side at posttest14, whereas control mice do. Data for D-E presented as mean ± S.E.M. TAM = tamoxifen (180 mg/kg, 1x daily for 5 days), BrdU = bromodeoxyuridine (100 mg/kg, 2x daily for 5 days), S = saline pairing day, C = cocaine (10 mg/kg) pairing day.

Supplemental Figure 4.2



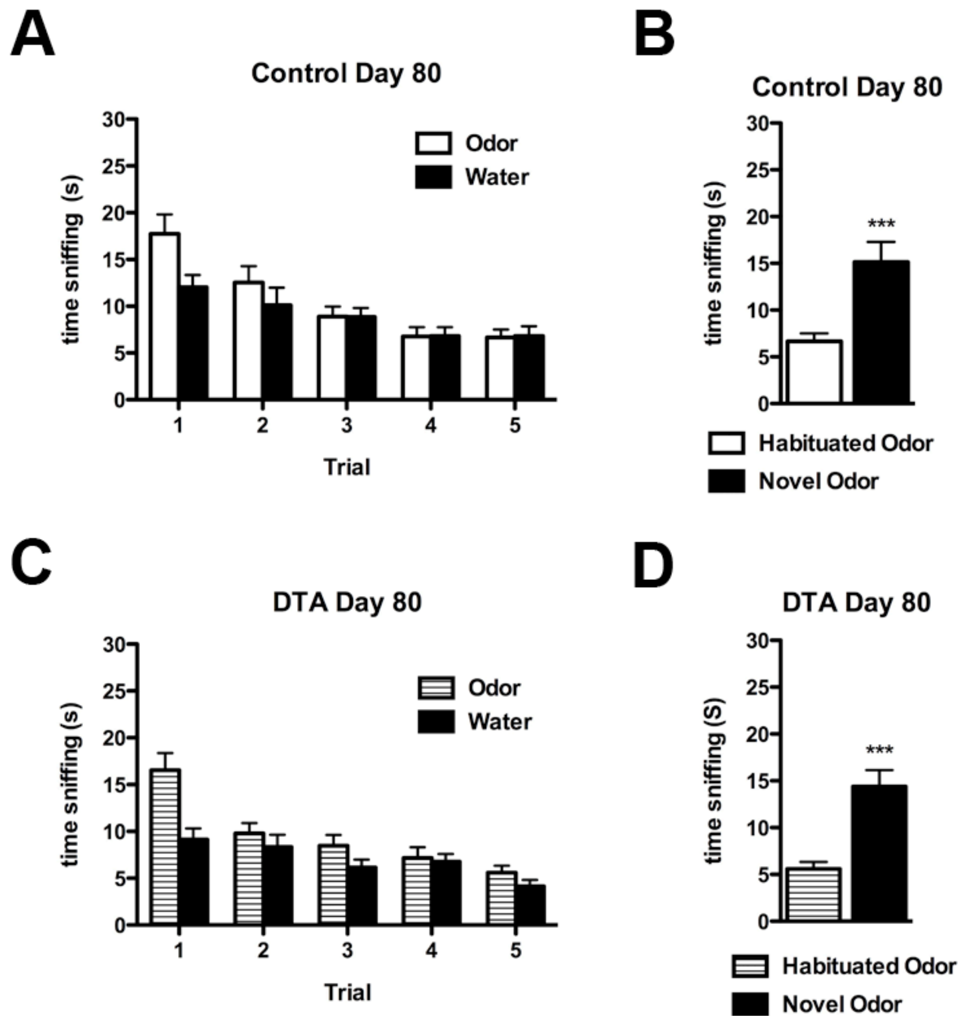
Acquisition and expression of cocaine CPP does not alter proliferation or maturation of neural progenitors. A. Mice were injected with saline or 15 mg/kg cocaine paired with a side of the CPP chamber in eight 15 minute daily trials. **B.** Mice conditioned with 15 mg/kg cocaine show a strong preference for the cocaine-paired side. **C.** There was no difference between saline and cocaine paired mice in SGZ Ki-67+ proliferating cells. **D.** There was no difference between saline and cocaine paired mice in SGZ DCX+ immature neurons. Data for B-D presented as mean ± S.E.M. s = saline, c = cocaine.

Supplemental Figure 4.3



Nestin-CreER^{T2}/floxedSTOP-DTA mice do not show any deficit in olfaction 50 days after recombination. A. Control mice show initial preference for the odor swab, and show proper habituation to the odor on repeated trials. **B.** Control mice discriminate between the novel odor and the habituated odor. **C.** 50 days after recombination, DTA mice also initial preference for the odor swab, and show proper habituation to the odor on repeated trials. **D.** 50 days after recombination, DTA mice discriminate between the novel odor and the habituated odor. Data for A-D presented as mean ± S.E.M.

Supplemental Figure 4.4



Nestin-CreER^{T2}/floxedSTOP-DTA mice do not show any deficit in olfaction 80 days after recombination. A. Control mice show initial preference for the odor swab, and show proper habituation to the odor on repeated trials. **B.** Control mice discriminate between the novel odor and the habituated odor. **C.** 80 days after recombination, DTA mice also initial preference for the odor swab, and show proper habituation to the odor on repeated trials. **D.** 80 days after recombination, DTA mice discriminate between the novel odor and the habituated odor. Data for A-D presented as mean ± S.E.M.

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CHAPTER FIVE:
Conclusions and future directions

Passive administration of cocaine has been previously shown to decrease proliferation, but not alter maturation of progenitors or neurogenesis in the hippocampus of rats. This dissertation expands on previous work to determine: 1) the impact of a clinically relevant model of drug addiction, cocaine self-administration and withdrawal, on multiple stages of adult neurogenesis, 2) the role of adult hippocampal neurogenesis in facilitating drug-taking and drug-seeking behaviors, and 3) the role of adult hippocampal neurogenesis in acquisition and recall of drug-context memory. In the previous three chapters, I provide evidence that decreased levels of adult hippocampal neurogenesis increase drug-taking and drug-seeking while impairing long-term memory of drug-context associations, and that drug-taking itself can complexly modulate adult neurogenesis both in the hippocampus and the olfactory bulb.

Chapter 2: Impact of cocaine self-administration and withdrawal on adult neurogenesis.

In order to understand how long-term voluntary drug self-administration and well as withdrawal impact adult neurogenesis, I examined the effects of cocaine self-administration versus saline self-administration on several stages of

neurogenesis at different timepoints after drug-taking. I found that proliferation of neural progenitors is decreased in both the subgranular zone and the subventricular zone after short-term cocaine self-administration and rescued by withdrawal, suggesting cocaine is toxic to neural progenitors in both neurogenic regions. Survival of the decreased pool of neural progenitors 4 weeks later was normal, suggesting compensatory mechanisms. This is supported by an increase in doublecortin-positive immature neurons 4 weeks after acquisition of cocaine self-administration. These findings suggest cocaine self-administration regulates different populations of neural progenitors at different stages of addiction, and is likely a more clinically relevant paradigm to study the effects of cocaine use on adult neurogenesis than passive administration.

Chapter 3: Suppression of adult neurogenesis via cranial irradiation increases drug-taking and drug-seeking behaviors.

In order to understand whether changes in adult hippocampal neurogenesis are neuroadaptations contributing to addiction or merely side effects of drug-taking, I used cranial irradiation to suppress adult hippocampal neurogenesis prior to or after cocaine self-administration. I found that decreased neurogenesis prior to cocaine self-administration increased acquisition of self-administration and total cocaine intake, likely through increased motivation for cocaine. Decreased neurogenesis after acquisition of cocaine self-administration increased resistance

to extinction, suggesting reduced neurogenesis increased motivation to seek drug. A control group irradiated prior to sucrose self-administration did not have any alteration in sucrose-taking or sucrose-seeking, suggesting reduced neurogenesis does not alter natural reward or basic operant learning. These findings suggest reduced adult neurogenesis disinhibits the hippocampus to promote drug-taking and drug-seeking behaviors.

Chapter 4: Suppression of adult neurogenesis via genetic ablation alters drug-context learning and drug reward.

In order to understand how adult hippocampal neurogenesis contributes to hippocampal processing of drug-context associations, I used a transgenic model of neurogenesis suppression, our nestin-CreER^{T2}/floxedSTOP-DTA mice in the cocaine conditioned place paradigm. I found that mice with decreased neurogenesis can learn drug-context associations and express a preference for the cocaine-paired side of the chamber, but long-term memory is impaired as the preference is lost two weeks later. These findings suggest adult neurogenesis is necessary for long-term recall of simple drug-context associations.

Future Directions

This research has suggested that modulation of adult hippocampal neurogenesis influences sensitivity to drugs of abuse. There are several pressing

questions raised by this research. First, does increased sensitivity to cocaine translate to the behavioral sensitization paradigm? Second, does reduced neurogenesis really disinhibit the hippocampus and increase activity of the VTA and NAc? Third, do increased levels of neurogenesis decrease drug-taking and drug-seeking, and does this apply to all drugs of abuse? Fourth, what long-term changes in the hippocampal microenvironment after heavy cocaine use mediate normalization of proliferation, upregulation of survival of progenitors, and upregulation of immature doublecortin+ immature neurons? Fifth, do human cocaine users have altered neurogenesis?

Currently I am confirming my results in irradiated rats that show increased sensitivity to cocaine via a vertical shift in the dose-response curve using another paradigm. Studies using a cocaine locomotor sensitization model (Benavides et al., 2007; Romieu et al., 2008) in both sham and irradiated rats, as well as control and nestin-CreER^{T2}/floxedSTOP-DTA (DTA) mice, are underway. I predict that irradiated rats and DTA mice will show increased locomotor sensitization to cocaine compared to control rats and mice, suggesting that reduced adult neurogenesis in fact alters hippocampal glutamatergic signaling to the nucleus accumbens that regulates behavioral sensitization (Lodge and Grace, 2008). No change in locomotion in DTA mice or irradiated rats compared to controls could happen if the cocaine dose was too high and a ceiling effect is seen. It is also possible that DTA mice could show no change in locomotion while irradiated rats

show an increase in locomotion. Two interpretations of this outcome are that increased sensitivity to cocaine in irradiated rats is due to side effects of irradiation rather than decreased neurogenesis, or that DTA mice may not have enough suppression of neurogenesis to enhance sensitivity to cocaine, while irradiated rats have robust neurogenesis suppression.

Studies suggest loss of dentate gyrus neurons may disinhibit the hippocampus (Tani et al., 2001; Won et al., 2003), perhaps via reduced activation of CA3 neurons that project back onto dentate gyrus GABAergic interneurons (Scharfman and Hen, 2007). Interestingly loss of only newborn dentate granule neurons may aid in learning some tasks (Saxe et al., 2007). Future studies in the Eisch laboratory may investigate system levels effects of reduced neurogenesis via counting c-fos labeled cells in regions downstream of glutamatergic signaling from the hippocampus, such as the NAc, BLA, and VTA, after injections with drugs of abuse or exposure to a former drug-taking context. I hypothesize that rodents with reduced neurogenesis would have more c-fos+ cells in the NAc, BLA, and VTA and injections with stimulants, indicating disinhibition of the hippocampus. If no change in c-fos+ cells is seen in any region, it may be necessary to use another marker of neuronal activation, such as zif268 (Saffen et al., 1988) or Arc (Kee et al., 2007) to make sure that lack of c-fos+ number changes is actually indicative of no change in neuronal activation. If we see a decrease in c-fos+ cells in any region, such as the BLA, it could suggest that

reduced neurogenesis leads to preferential activation of hippocampal-accumbens pathway as opposed to the BLA-accumbens pathway, and identify the neural circuitry underlying the behavior we are testing.

Another way to study how reduced hippocampal neurogenesis could alter reward pathway circuitry is by doing *in vivo* recordings from the NAc or VTA (Lodge and Grace, 2008). I hypothesize that rodents with reduced hippocampal neurogenesis would have increased firing in the NAc and VTA after injection with stimulants, similar to developmental hippocampal lesions (Lodge and Grace, 2007) or stimulation of the subiculum (Floresco et al., 2001). Alternatively, with cutting edge technology (Airan et al., 2007) it may be possible to stimulate or inhibit just adult-generated neurons of a certain age and determine if that changes drug-seeking behavior. I hypothesize that stimulating adult-generated neurons would inhibit drug-seeking behavior, because loss of newborn neurons increased drug-seeking and drug-taking behaviors (Chapter 3). It is also possible that activating a newborn neuron is not the opposite of removing it completely from the circuitry, and instead there could be increased or no change in drug-seeking behavior. Regardless of the outcome, these studies would yield a deeper understanding of the contribution of adult-generated neurons to intra- and extrahippocampal network processing, as well as behaviorally in the form of drug-taking and drug-seeking.

As all drugs of abuse initiate drug-taking and drug-seeking through the same neural circuitry (nucleus accumbens; (Bardo, 1998; Kalivas and McFarland, 2003)), it is likely that decreasing adult hippocampal neurogenesis will lead to increased drug-taking and drug-seeking for all drugs of abuse. Studies in the Eisch laboratory using self-administered morphine will be starting soon. In addition, increasing neurogenesis prior to self-administration may decrease acquisition and total drug intake, while increasing neurogenesis after self-administration may facilitate extinction of drug-seeking behaviors. Studies manipulating factors known to increase hippocampal neurogenesis, such as BDNF (Sairanen et al., 2005) and VEGF (Cao et al., 2004), will be pursued in the Eisch laboratory in the future.

Immunoblotting studies are ongoing using hippocampal tissue from CSA, CSA-WD, and CONT-CSA rats (and their Saline SA controls) from rat studies from Chapter 2. Due to relatively low abundance of neural progenitor proteins compared to mature hippocampal neurons proteins, it is unlikely that immunoblotting for neural progenitor proteins such as nestin or doublecortin would reveal changes between Saline SA and CSA groups. Thus, we will be looking at high abundance proteins in the hippocampal microenvironment, such as BDNF and VEGF, that are known to either influence neurogenesis (Cao et al., 2004; Sairanen et al., 2005) or addiction (Graham et al., 2007). These tissue samples have been sonicated, and antibody conditions are being optimized. I

hypothesize that either BDNF or VEGF will be increased in the hippocampus after CSA-WD, as compensation for initial proliferation deficits (Noonan et al., 2008). It is possible that we will see no change, and regulation of other proteins in the neurogenic niche are instead responsible for the pro-survival effect we see 4 weeks after CSA. If this is the case, a recent microarray study of hippocampi from cocaine addicts has yielded even more target proteins (Mash et al., 2007) that we could pursue.

Work from the Eisch laboratory suggests that proliferation, as measured by Ki-67+ cells, may be decreased or unchanged in the hippocampus of human heroin addicts (Harburg, 2007). It is likely that subgranular zone proliferation will be unchanged in cocaine addicts, as evidence from Chapter 2 suggests tolerance to proliferation deficits occur over long-periods of use or withdrawal. If proliferation in the human hippocampus is in fact increased or decreased in cocaine addicts, this could mean that neurogenesis in the human is regulated differently than in the rodent, or that human exposure to cocaine over a much longer timespan results in different changes in neurogenesis. More interesting to test is whether the number of immature neurons is altered in the hippocampi of human cocaine addicts, as immature neurons are increased in the hippocampi of heroin addicts (Weber et al., 2006). I hypothesize that DCX+ immature neurons cells would be increased in cocaine addicts as a compensation for reduced hippocampal volume (Bartzokis et al., 2000). It is feasible to explore these

questions of proliferation and neurogenesis in the hippocampi of human cocaine addicts because the Eisch laboratory has collaborated in the past with Dr. Yasmin Hurd from Mt. Sinai School of Medicine to receive brains of chronic heroin addicts, and is likely we would be to receive brain tissue from chronic cocaine addicts. Other sources of cocaine addict brain tissue would be the Stanley Foundation Brain Bank, and the tissue collection of Dr. Carol Tamminga at UT Southwestern Medical Center. Alternatively, new methods of magnetic imaging (Manganas et al., 2007) suggest that in the future we can image human addicts while they are alive to see if numbers of neural stem cells are altered during different stages of addiction and drug-craving. As we have collaborators such as Dr. Carol Tamminga at UT Southwestern's imaging facility, which can image both rats and humans, these studies would be feasible if the technology is developed.

Summary

This dissertation suggests that cognitive deficits in drug addicts may be both pre-existing due to decreased neurogenesis increasing vulnerability to addiction, as well as from drug-taking induced decreases in neurogenesis. In the future, new methods of manipulating adult neurogenesis, as well as assaying neurogenesis in live rodents and humans, will lead to greater understanding of

how drugs of abuse impact humans as well as how to treat addiction with therapeutics targeted at increasing neurogenesis.

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