THE ROLE OF ADULT HIPPOCAMPAL NEUROGENESIS
IN MORPHINE ADDICTION

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DEDICATION

I would like to thank my mentor, Dr. Amelia Eisch, the members of my Graduate Committee, and my family who have supported me through the joys and hardships of this research.

THE ROLE OF ADULT HIPPOCAMPAL NEUROGENESIS IN MORPHINE ADDICTION

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THE ROLE OF ADULT DENTATE GYRUS NEUROGENESIS
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ABSTRACT

The hippocampus plays a large role in modulating the reward pathway, being especially important in craving and context-dependent relapse. One form a neuroplasticity within the hippocampus is adult neurogenesis, which occurs in the subgranular zone of the dentate gyrus. While a growing amount of literature has explored the effects of drugs of abuse on adult DG neurogenesis, the relationship between self-administered opiates and adult DG neurogenesis remains unexplored. This dissertation investigates both the role of adult DG neurogenesis in morphine-

related behaviors and the effects of self-administered opiates (morphine and heroin) on adult DG neurogenesis. I first explore the background literature important in the work completed within this dissertation (Chapter 1). Next, using a self-administration paradigm, I proceed to show that ablation of adult neurogenesis via cranial irradiation results in increased in morphine intake, decreased extinction, and decreased cognitive flexibility. Additionally, rats lacking adult DG neurogenesis exhibited increased morphine locomotor sensitization with increased DG activation in the infrablade after a low dose morphine challenge (Chapter 2). I will then go on to investigate the consequences of long-term self-administered opiates (morphine and heroin) on the different stages of maturation of adult-generated neurons. I demonstrate that morphine self-administration has no effect on proliferation, survival, or maturation immediately after exposure or after 28 days of withdrawal (Chapter 3). Additionally, I demonstrate that heroin self-administration does not alter DCX+ cell density or granule cell layer volume (Chapter 4). Taken together, my data suggests the adult DG neurogenesis is robust and normally unaffected by self-administered opiates. However, preexisting deficits in DG neurogenesis may lead to an increased vulnerability to addiction-related behaviors. In the final chapter (Chapter 5), I discuss potential implications of this work and future directions in which it may be taken.

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Palchik G*, Rivera PD*, Shih H-Y*, Amaral WZ, **Bulin SE,** Chen BPC, Eisch AJ. 28Si particle exposure decreases adult hippocampal neurogenesis in vivo. *In preparation for Winter 2015 submission.* *equal contribution

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Addiction – the condition of being addicted to a particular drug of abuse

Cognitive Flexibility – the ability to ignore previous information when circumstances change, and learn new information

Extinction – a variation of learning that requires the suppression of old knowledge

Opiate – as any drug that is naturally derived from the opium poppy plant

Opioid – a universal term for all drugs and endogenous peptides that bind to the opioid receptor

Self-administration – a behavioral task in which the animal choses to take a drug via pressing a lever or nosepoke

LIST OF ABBREVIATIONS

CSA – Cocaine self-administration

DG – Dentate gyrus

EC – Entorhinal cortex

GCL – Granule cell layer

HSA – Heroin self-administration

ip – Intraperitoneal

MLS – Morphine locomotor sensitization

MSA – Morphine self-administration

NAc – Nucleus accumbens

PFC – Prefrontal cortex

sc – subcutaneous

SGZ – subgranular zone

VTA – ventral tegmental area

CHAPTER ONE

Introduction

Portions of this chapter have been published (Bulin et al., 2011). However, they have been updated for content and references, and edited for clarity.

This introductory chapter is separated into four main sections that provide the background information necessary to understand the subsequent data chapters: 1. Hippocampus Fundamentals, 2. Addiction Overview, 3. Adult Neurogenesis and Drugs of Abuse, and 4. Unanswered Questions.

1. Hippocampus Fundamentals

The hippocampus is a complex brain region that is part of the limbic system and plays a vital role in several brain functions, including spatial learning and memory and mood regulation. Below, I provide an overview of the fundamental anatomy of the hippocampus and its function before narrowing down to emphasize one type of neuroplasticity seen within the hippocampus: adult dentate gyrus (DG) neurogenesis.

Hippocampal Circuitry

A simplified but still useful perspective of the hippocampus is that it is a unidirectional excitatory circuitry whose activity facilitates regulation of many brain regions (Teitelbaum and Milner, 1963, Amaral and Witter, 1989, Andersen et al., 2006).

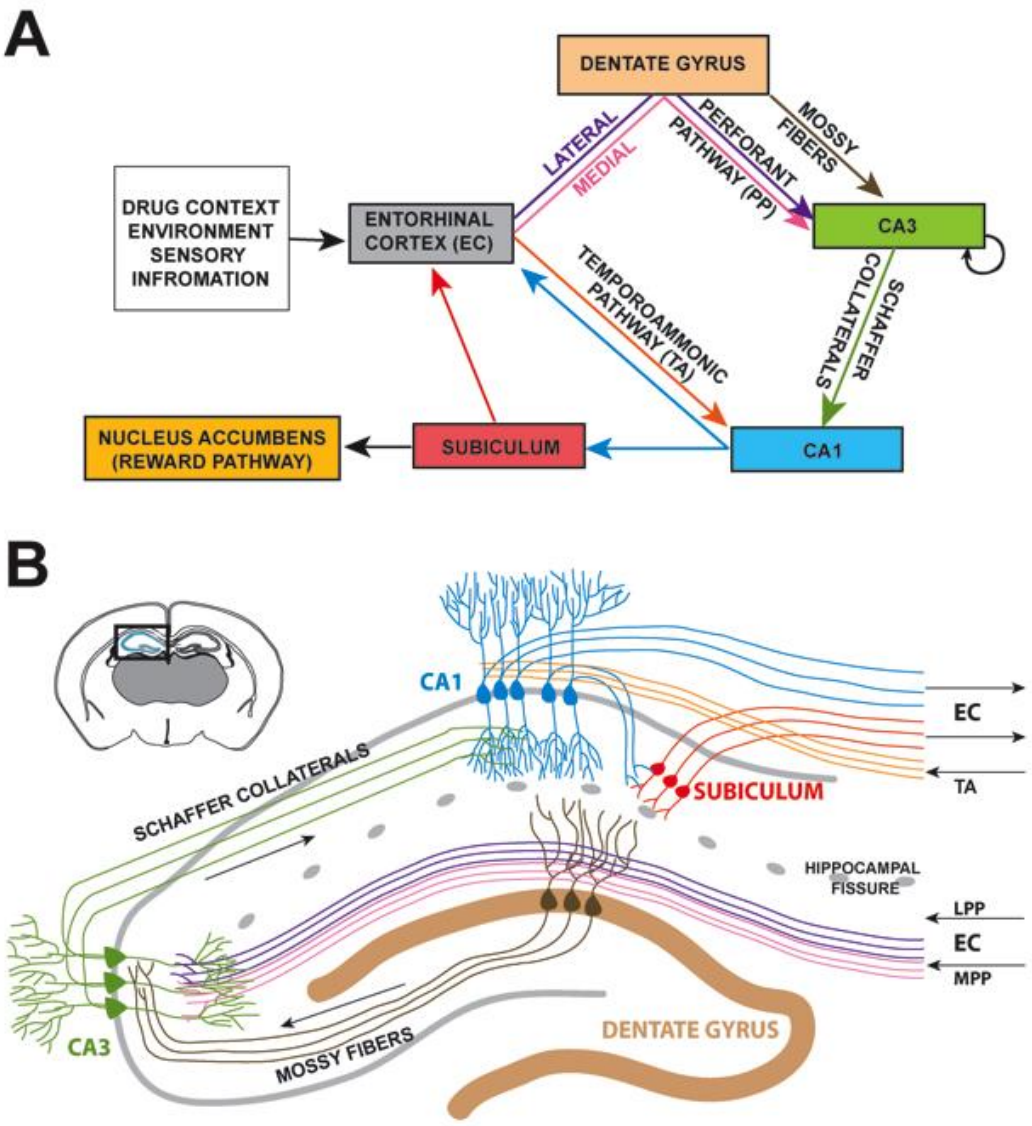

Figure 1-1. Hippocampal Circuitry. A) Simplified flowchart of hippocampal and dentate gyrus (DG) circuitry. One key target region of the hippocampal output is the nucleus accumbens (NAc), a main structure in the brain's reward pathway. **B)** Simplified diagram of hippocampal and DG circuitry. **B** shows the circuitry from **A** in more detail in the context of the hippocampal and DG anatomy. Briefly, axons from DG granule cells project onto the CA3 pyramidal cells (mossy fibers). CA3 receives projections from itself through recurrent collaterals, and then projects axons (Schaffer collaterals) to CA1 pyramidal neurons. The CA1 sends projection either directly to the Entorhinal Cortex (EC) or indirectly through the subiculum. The EC projects axons to the DG and CA3 through the lateral perforant pathway (LPP) and medial perforant pathway (MPP). The CA1 also receives projections from the EC through the Temporoammonic pathway (TA). Previously published in (Bulin et al., 2011).

A view of the essential anatomy of the hippocampus is shown in **Figure 1-1A.** The key flow of information through the hippocampus is fairly straightforward: cortical input from the entorhinal cortex (EC) stimulates the DG, CA3, and CA1 regions of Ammon's horn (Cornu Ammon's, or CA), and then exits the hippocampus via the EC again. This is a powerful, subcortical, excitatory loop that underlies spatial learning and memory and mood regulation, and participates in higher cognitive processes, such as pattern separation and pattern completion. As will be a central point in this thesis, and as is emphasized in **Figure 1-1A**, the hippocampus also participates in regulation of brain regions in the "reward" pathway, such as the nucleus accombens (NAc), which in turn can regulate the hippocampus.

It is useful to take an even closer look at the functional anatomy of the hippocampal circuitry, as it is critical to aspects of hippocampal neuroplasticity (**Figure 1-1B**). Hippocampal afferents, or inputs, come primarily through the perforant path into the DG, terminating on the dendrites of the granule neurons at varying distances from the cell soma (e.g. pink and purple in **Figure 1-1B** indicate medial and lateral perforant path, respectively). The excitatory EC signal, via the neurotransmitter glutamate, is then transmitted by the axons of the DG granule cells, also called mossy fibers, into the CA3 region of the hippocampus. This excitatory signal is detected by CA3 pyramidal neurons, and then passed to the CA1 region through the Schaffer collateral axons, again via glutamatergic transmission. The CA1 then projects either back to the EC to begin the excitatory loop again, or to the subiculum, which will then project to other areas of the brain, including other areas of the reward pathway such as the NAc and

PFC. While modern techniques have augmented this straightforward, relatively unidirectional view of hippocampal circuitry, the “predominantly unidirectional connectivity” seen in the hippocampus remains strikingly different from the reciprocal connectivity of other neocortical-related regions (Abbott et al., 1984).

Hippocampal Function

An overview of published literature suggest that the functions of the hippocampus can be divided into two major divisions: spatial learning and memory and mood regulation (Gould et al., 1999, Schinder and Gage, 2004, Warner-Schmidt and Duman, 2006, Eisch et al., 2008, Zhao et al., 2008, Petrik et al., 2012b). These two functions can be attributed to different parts of the hippocampus along the longitudinal axis. For example, lesion and inactivation studies indicate that the hippocampus, and more specifically, the dorsal hippocampus (also called anterior or septal), is required in order for animals to learn a spatial task (Morris et al., 1982, Moser et al., 1993, Meyers et al., 2003). In contrast, the ventral hippocampus (also called posterior or temporal) appears to be more involved in mood regulation, likely due to its connectivity with the reward system (Tseng et al., 2009, Britt et al., 2012). As discussed later in **Chapter 1-1**, the dorsal and ventral areas of the hippocampus have unique inputs and outputs to other brain areas that play vital roles in each of these functions. Additionally, plasticity within the entire hippocampus contributes to its ability to function. While there are multiple subregions of the hippocampus where plasticity is vital, the DG is unique in its ability to produce new neurons throughout adult life that incorporate into hippocampal circuity.

This adult DG neurogenesis is the emphasis of this dissertation, and will be discussed in detail below.

Adult DG Neurogenesis

History and Overview

Studies near the beginning of the 20th century suggested that new neurons were only added to the brain during embryonic development (Ramon y Cajal, 1913). Given the limitations of the time (such as the emergence of histological stains, and the early use of the Golgi stain) and the more prominent debates of the time (such as between the theories of Ramon y Cajal's "Neuron Doctrine" and Golgi's "Reticular Theory"), it was incredibly difficult to prove otherwise. The dogma that no new neurons were added to the brain after the embryonic or early postnatal development became engrained for many years.

Almost 50 years later, radioactive isotopes such as [H^3]-thymidine were first used to label dividing cells in the adult rodent brain, revealing new cells within multiple brain regions (Altman, 1962, Altman and Das, 1965, 1966). However, these studies gained little attention until the 1980s, when studies suggested that these new neurons were functional, received synaptic inputs, and extended processes into hippocampal circuitry (Kaplan 1983, Stanfield 1988). Thus, began the exciting field of adult neurogenesis.

Adult neurogenesis is the generation of new neurons throughout adult life. It is known to occur in at least two regions of the brain: the subventricular zone (SVZ)/rostral

migratory stream (RMS), which gives rise to olfactory interneurons, and the hippocampal subgranular zone (SGZ), which gives rise to granule cell neurons in the hippocampal dentate gyrus. Hippocampal SGZ neurogenesis is the focus of this dissertation. The SGZ is a region bordering the hippocampal dentate gyrus granule cell layer and the intervening hilus. When considered within hippocampal circuitry (**Figure 1-1B**), it becomes apparent that the SGZ of the dentate gyrus is positioned at one of the main entry points into the hippocampal circuitry.

The SGZ is the focus of this dissertation; however, SVZ is mentioned where appropriate. It is currently proposed that adult DG neurogenesis occurs in "stages" with stem-like cells giving rise to more rapidly dividing proliferating progenitors whose progeny then give rise to immature neurons which mature into granule cell neurons (Kempermann et al., 2004).

Stages of Adult Dentate Gyrus Neurogenesis

The process of adult DG neurogenesis has been divided into distinct stages based on the types of cells involved (Seri et al., 2001, Kronenberg et al., 2003, Kempermann et al., 2004). Each cell type can be characterized by the expression of certain key markers and cellular morphology.

For example, Type-1 cells, also called radial glia-like cells, are thought to be the putative stem cell, or the source of DG neurogenesis. Type-1 cells divide rarely, making it difficult to label them with mitotic markers (which label cells in the cell division cycle).

However, Type-1 cells express specific markers such as nestin, GFAP (glial fibrillary acidic protein), GLAST (glutamate/L-aspartate transporter), and BLBP (Brain Lipid Binding Protein) (Hartfuss et al., 2001, Lagace et al., 2007, DeCarolis et al., 2013), allowing for labeling using a combination of these markers. Type-1 cells also demonstrate astrocyte-like electrophysiological properties (Filippov et al., 2003). While much remains to be known about Type-1 cells, it appears that they can divide asymmetrically, giving rise to one Type-1 cell and an additional Type-2a daughter cell.

Type-2 cells, also known as Type-B cells (Seri et al., 2001) or quiescent neural precursors (Encinas et al., 2011) are considered to be progenitors or precursors, and they also express nestin. There are two subtypes of Type-2 cells that can be distinguished by their marker expression. For example, “younger” Type-2a cells are more likely to express Ki67, Ascl1 or BLBP (Steiner et al., 2006), while “older” Type-2b cells are more likely to express immature neuron markers such as doublecortin (DCX) and NeuroD (Miyata et al., 1999, Gao et al., 2009). These older cells can be identified by their morphology, as they tend to have a process extending either parallel or perpendicular to the granule cell layer, reflecting their morphological maturation into neurons (Plumpe et al., 2006). Type-2 cells are also called “transiently-amplifying cells” to reflect that they can rapidly increase their cell number via symmetric division.

A large number of these progenitors die by undergoing apoptosis (Biebl et al., 2000), but some Type-2 cells and their offspring will mature into Type-3 cells. Type-3 cells are also called immature granule cells since they are the first stage at which

functional integration into the hippocampal circuitry can be identified, and since in most cases they are post-mitotic (Kempermann et al., 2004). Type-3 cells lose nestin expression but still express DCX and NeuroD. Being “older” than Type-2 cells, as they also express neuronal markers, such as Calretinin, and have a characteristically larger and more complex dendritic tree which extends into the molecular layer to receive inputs from the EC. These immature Type-3 granule cells develop over time into mature granule cells (Brandt et al., 2010). Typically, adult-generated DG neurons take 4-6 weeks from the Type-2 stage (active cell division) to mature granule cells neurons when they are functionally incorporated into neural circuits (Stanfield and Trice, 1988, Hastings and Gould, 1999, Markakis and Gage, 1999, Kee et al., 2007).

While each stage has been presented here, there remains debate about the process of neurogenesis. For example, it is still unclear whether the sole source of new neurons is from nestin-expressing Type-1 cell division, or from where the Type-1 cells specifically originate (DeCarolis et al., 2013). This raises the important question of what precisely regulates the promotion of a cell from one cell stage to another, a question which many in the field and in the Eisch Lab have studied (Lagace et al., 2008, Gao et al., 2009, Ables et al., 2010, Guo et al., 2011). It is also important to acknowledge that while there are discrete stages of neurogenesis, neurogenesis is a process and not a timepoint (Kempermann et al., 2004).

Function of Adult Dentate Gyrus Neurogenesis

Spatial Learning and Memory (Pattern Separation)

For the past two decades, adult hippocampal neurogenesis has been believed to broadly impact hippocampal learning and memory (Fritzsch and Crapon de Caprona, 1990, Gould et al., 1999, Rola et al., 2004, Doetsch and Hen, 2005, Leuner et al., 2006, Saxe et al., 2006, Lagace et al., 2010). However, many studies often conflict with each other on how rodents lacking neurogenesis respond to different behavioral tests (Petrik et al., 2012b). Recent advances in the field point to newborn neurons in the hippocampus being more specifically important for pattern separation (Aimone et al., 2011, Sahay et al., 2011a, Sahay et al., 2011b). Pattern separation can be defined as the ability to distinguish between two fairly similar objects or contexts (Aimone et al., 2009). Computational studies suggest that the addition of new DG neurons during different experiences can result in a system that encodes new memories in familiar contexts differently than it would treat novel contexts. These studies also suggest that time is a another dimension of information that adult DG neurogenesis stores in relation to spatial contexts (Aimone et al., 2009). Though computational studies are informative, transgenic mouse work strengthens these claims. Using a conditional *Bax* knockout in a *nestin-ER*^{T2} mouse line, Sahay et al. was able to block apoptosis in proliferating cells in the SGZ, resulting in an overall increase in adult DG neurogenesis, with more functional cells (Sahay et al., 2011a). These mice with increased functional immature neurons have increased pattern separation but normal learning in a variety of other tasks that resulted in behavioral deficits with decreased DG neurogenesis,

suggesting that enhanced neurogenesis can increase ability in some spatial tasks but not others (Sahay et al., 2011a).

Depression and Anxiety

The role of adult DG neurogenesis in mood regulation was first suggested when it was discovered that DG neurogenesis was required for the efficacy of antidepressants (Santarelli et al., 2003). Since that landmark study, hundreds of additional studies have explored the interaction between adult DG neurogenesis and depression/anxiety. However, behavior data has been inconsistent, with animals lacking neurogenesis only exhibiting a depressive phenotype in certain tasks or sometimes not at all (Petrik et al., 2012b). Recent work using GFAP-TK transgenic rats has perhaps finally explained the mixed results (Snyder et al., 2011). Rats lacking adult DG neurogenesis exhibit similar anxiety levels to that of intact rats, but when both groups of rats were stressed prior to a novelty suppressed feeding task GFAP-TK rats exhibited higher anxiety (Snyder et al., 2011). Additionally, transgenic mice lacking adult neurogenesis also exhibited anhedonia in a sucrose preference test only after being stressed, suggesting that the role of adult DG neurogenesis in depression and anxiety is through buffering the stress response (Snyder et al., 2011). These data could explain earlier inconsistencies to most likely be caused by variations in stress levels between experimenters and tasks. Thus, a current hypothesis is that new neurons are important for mood regulation, particularly in times of stress.

Ablation of Adult Dentate Gyrus Neurogenesis

Another way to assess the function of adult-generated dentate gyrus neurons is via ablation of new neurons followed by behavioral and/or physiological analysis. Included in this dissertation is a curated table (non-exhaustive) of select publications that utilize ablation of new neurons to probe the function of these cells (**Table 1-1**). There are multiple ways ablate DG neurogenesis, including X-ray irradiation, mitotic inhibitors, and inducible transgenic mice and rats. Each of these techniques has their strengths, but also includes side effects and weaknesses.

For example, many early studies used mitotic inhibitors, such as methylazoxymethanol acetate (MAM), to ablate adult DG neurogenesis (Shors et al., 2002, Lledo et al., 2006, Ko et al., 2009). However, it was discovered that MAM did not result in a full ablation, and due to its peripheral effects on dividing cells was also detrimental to animal health leading to behavioral results that needed to be interpreted cautiously (Dupret et al., 2005). As mitotic inhibitors fell out of favor, many studies began to use X-ray irradiation or inducible transgenic ablation models, which are discussed in turn below.

X-ray irradiation is a quick technique that results in double-stranded DNA breaks in dividing cells, while sparing non-mitotic mature cells (Bradley and Kohn, 1979). It is a reliable technique for ablating mitotic cells, and can be performed on any wild-type rat or mouse. This can save time and money, since cohorts of transgenic animals do not need to be bred, and eliminates worries about background strain or littermate issues. It

Table 1-1. Curated List of Publications Assessing Behavior After Inducible Ablation or Increase of Adult Neurogenesis. Ordered by ascending year, and then alphabetical by first-author.

Reference	Animal Model	Method of Ablation	Behavioral Task	Outcome
(Burghardt et al., 2012)	Mice	X-ray/ Transgenic	Rotating Shock Zone	Impaired avoidance, Impaired cognitive flexibility
(Hernandez-Rabaza et al., 2009)	Rats	X-ray	T-maze, Contextual Fear conditioning	Working memory unchanged, Impaired retention of fear memory
(Jayatissa et al., 2009)	Rats	MAM/ Chronic Stress	Sucrose Preference	Chronic stress produced anhedonia, MAM no change
(Ko et al., 2009)	Mice	MAM/ g-ray irradiation	Contextual Fear conditioning	Decreased fear memory with high dose of g-rays, Extinction unchanged
(Lagace et al., 2010)	Mice	X-ray	Social Avoidance	Inhibited social avoidance after social defeat stress
(Luu et al., 2012)	Rats	X-ray	Olfactory Discrimination Task	Impaired learning of second odor after learning initial odor
(Meshi et al., 2006)	Mice	X-ray	Novelty Suppressed Feeding, Morris Water Maze	Increased latency to feed, Impaired learning of the water maze
(Noonan et al., 2010)	Rats	X-ray	Cocaine Self-administration	Increased cocaine intake, Higher lever pressing during extinction
(Sahay et al., 2011a)	Mice	Transgenic **Increase**	Contextual Fear Conditioning	Improved pattern separation
(Santarelli et al., 2003)	Mice	X-ray	Novelty Suppressed Feeding	Increased latency to feed with and without fluoxetine treatment
(Saxe et al., 2006)	Mice	X-ray/ Transgenic	Contextual and Cued Fear Conditioning	Impaired context fear memory, but cue unchanged
(Saxe et al., 2007)	Mice	X-ray/ Transgenic	Radial Arm Maze	Working memory improved in hippocampal-dependent tasks
(Shors et al., 2002)	Rats	MAM	Contextual Fear Conditioning, Morris Water Maze, Trace Fear Conditioning	Decreased Trace memory, Unchanged learning of the water maze and contextual fear memory
(Snyder et al., 2005)	Rats	X-ray	Morris Water Maze	Decreased retention of spatial memory
(Snyder et al., 2011)	Rats	Transgenic	Novelty Suppressed Feeding, Sucrose Preference	Increased latency to feed, anhedonia
(Winocur et al., 2006)	Rats	X-ray	Non-matching-to-sample task	Impaired memory when intervals were long, unchanged when short
(Wojtowicz et al., 2008)	Rats	X-ray	Contextual Fear Conditioning, Morris Water Maze	Impaired fear memory, Unchanged learning in the water maze

can be targeted to a small area, such as the hippocampus, while shielding the rest of the brain and body. However, there is the drawback that tissue and brain areas directly above and below the hippocampus would also receive irradiation (e.g. irradiated rats often exhibit hair loss in the targeted area). It is also a more "dirty" technique that can and does result in other changes to the brain. For example, inflammation is increased after irradiation for at least 30 days before returning to normal levels, as indicated by the number of activated microglia in the rat hippocampus (Monje et al., 2002). However, other work has shown that indices of inflammation are on the downturn after 30 days, and thus behavioral studies wait for at least this period of time before experimentation (Noonan et al., 2010). When performed in early life, X-ray irradiation – even when restricted to the head or hippocampus – can also decrease subsequent rate of weight gain (Winocur et al., 2006, Wojtowicz, 2006, Wojtowicz et al., 2008). In our hands, while we use a novel image-guided irradiation approach that restricts exposure to the hippocampal region (Song et al., 2010; expanded on in **Chapter 2**), we still see a slower weight gain post-irradiation, which cumulates in a 40-50g lower weight months later (**Figure 1-2**). In our experience, the divergence in weight gain usually begins at around 300g, regardless of weight at the time of irradiation. This weight gain phenomenon has been reported since X-ray irradiation began as an ablation technique for DG neurogenesis, but the neural basis for this has never been fully addressed.

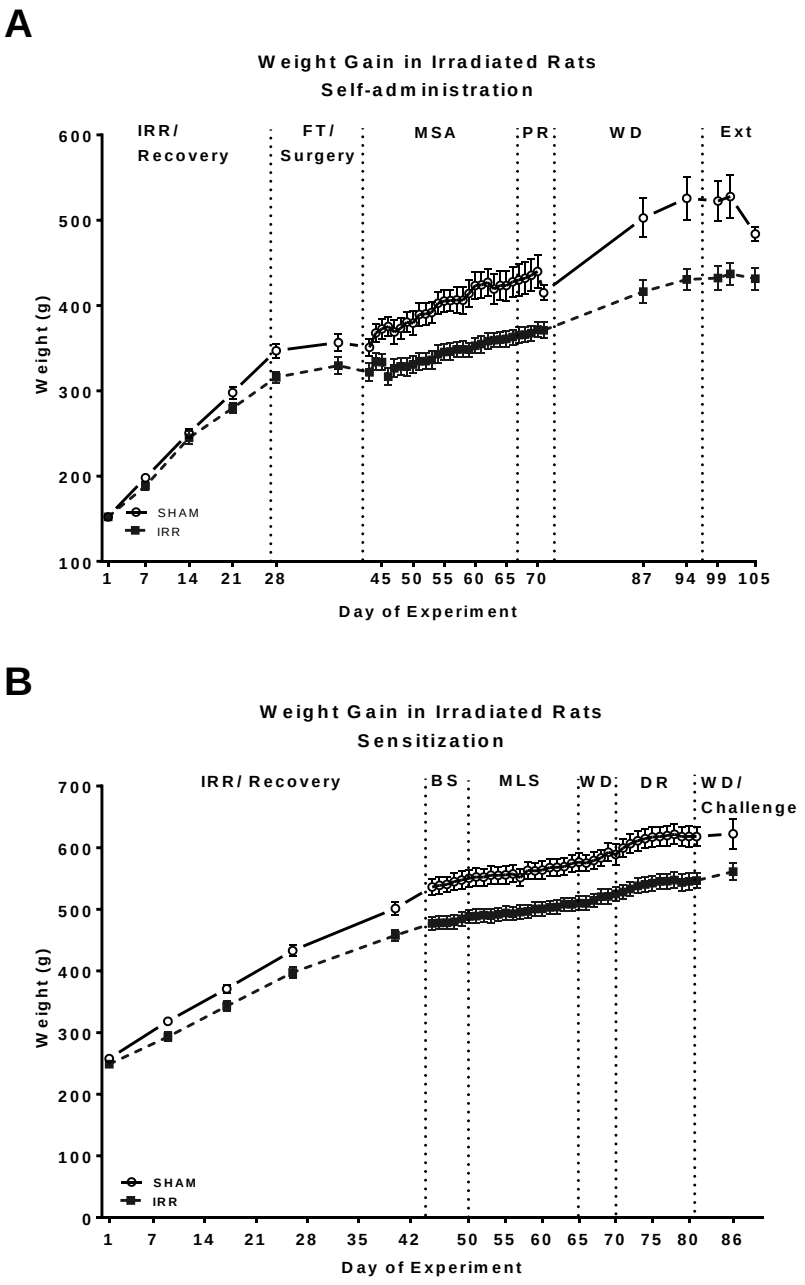

Figure 1-2. Weight gain in rats after X-ray irradiation. A) Body weight of Sham and IRR rats through morphine self-administration experiments shown in Chapter 2. After habituation, rats were divided into Sham or IRR groups and received the appropriate treatment over two days (see Methods). Rats then underwent 28D of home cage recovery (Rec) and were then separated into individual housing. Rats received 7D of food training (FT) and 7D of I.V. catheterization surgery (S). Rats then underwent 21D of morphine self-administration (MSA), followed by 7D of progressive ratio (PR; data not shown). Rats then had 28D of home cage withdrawal (WD) followed by 5D of extinction studies (Ext). Rats were killed 3D after the last extinction session. **B)** Body weight of Sham and IRR rats throughout morphine sensitization experiments shown in Chapter 2. After arrival in facility, rats received 14D of group-house habituation (H). After habituation, rats were divided into Sham or IRR groups and received the appropriate treatment over two days (see Methods). Rats then received six weeks of recovery (Rec) so that sensitization would start at approximately the same time as MSA. After 5D of baseline locomotor incurred by saline injections (BS), rats received 15D of 5 mg/kg i.p. to measure MLS. This was followed by 5D of withdrawal with daily locomotor sessions (WD), and 10D of an escalating dose response (DR). After DR, rats received 6D of withdrawal in the homecage (WD-HC). Finally, rats received a 1 mg/kg i.p. challenge dose (CHALLENGE), were placed into the locomotor chambers for 90 min, and then killed immediately afterwards

Another method of decreasing neurogenesis is through inducible transgenic models. Many different transgenic mouse lines have been made that allow selective ablation of neurogenesis. One popular mouse model is the Glial Fibrillary Acidic Protein-Thymidine Kinase (GFAP-TK) mouse. This model expresses the herpes virus TK under the GFAP promoter, causing its expression in all GFAP+ cells. When the drug ganciclovir is administered to the animal, only dividing cells expressing GFAP-TK will be killed by the antiviral drug, resulting in the ablation of adult DG neurogenesis, but sparing adult glia expressing GFAP (Saxe et al., 2006, Snyder et al., 2011, Burghardt et al., 2012, Lacefield et al., 2012). This model has recently been replicated within a transgenic rat (Snyder et al., 2011). A second popular transgenic approach of ablating adult neurogenesis requires the combination of a Nestin-CreER^{T2} line and a "killer" cre-sensitive line, such as a "floxed" Stop-Diphtheria Toxin (DT) line. In such a transgenic mouse, administration of tamoxifen leads to the deletion of the stop codon, resulting in the expression of DT and subsequent death of Nestin+ cells. This makes the deletion specific to cells fated for the neuronal lineage (Imayoshi et al., 2008). These inducible transgenic models have great benefits, as the animal is allowed to mature normally and only affects cells expressing the transgenes. Additionally, the side effects seen to other brain areas that occur in irradiation and the massive damage seen with mitotic inhibitors are no longer a concern when using transgenic models. However, there are drawbacks to inducible transgenics for ablation of neurogenesis. As they require breeding, they can be time- and labor-intensive. Also, there are multiple versions of most transgenic lines, and they can vary from line to line in regards to genome insertion location, efficacy of the ablation, approach to and toxicity of the ligand needed to induce cell

killing, and even the specificity of the driver line to target cells in the neurogenic lineage. Breeding and cost concerns are also warranted, as large cohorts may need to be bred and there is concern of genetic drift over time.

While this brief review above of the technical aspects of X-ray irradiation and inducible transgenic mouse models reveals that both approaches have pros and cons, review of the literature from these ablation approaches reveals multiple functions of adult-generated DG neurons. For example, hippocampal-dependent learning is decreased after ablation (Saxe et al., 2006, Guo et al., 2011) and irradiation studies targeting only the dorsal DG indicate that neurogenesis in this region is particularly important in spatial learning and memory (Wu and Hen, 2014). Ablation studies have also revealed a role of neurogenesis in pattern separation (Clelland et al., 2009) and cognitive flexibility in spatial tasks (Burghardt et al., 2012). In addition, different aspects of mood are also detrimentally affected by ablation of newborn neurons. Stressed animals lacking DG neurogenesis have heightened anxiety (Snyder et al., 2011) and these neurons are also important in stress-induced social avoidance (Lagace et al., 2010). Additionally, newborn neurons are required for the efficacy of antidepressants (Santarelli et al., 2003). Interestingly, some studies use X-ray irradiation and inducible transgenic models in parallel to doubly confirm that the results are because of the deletion of newborn neurons. For example, both methods of ablation were used to demonstrate increased synchronization of firing in the hippocampus after ablation (Lacefield et al., 2012, Nakashiba et al., 2012). In conclusion, these ablation techniques

are vital for understanding the role of adult neurogenesis in many aspects of hippocampal function.

Increasing the Number of New Dentate Gyrus Neurons

Just as the function of new neurons can be elucidated by ablation of adult DG neurogenesis, it can also be gauged by the approaches that inducibly increase new neurons and the functional changes that follow. Certainly there are many correlations between general stimuli and agents that increase new neurons and improved hippocampal function, and visa versa. For example, running increases neurogenesis, in part by increasing vasculature and growth factors to stimulate the neurogenic niche, and can improve hippocampal function and ameliorate or prevent depression- and anxiety-like behaviors (van Praag et al., 1999, Farmer et al., 2004, van Praag et al., 2005, Uda et al., 2006, Naylor et al., 2008, Brandt et al., 2010). For example, aerobic exercise or environmental enrichment were often used early in the study of adult DG neurogenesis, as both of these naturally increase the number of proliferating cells and immature neurons (Nilsson et al., 1999, van Praag et al., 1999, Young et al., 1999, van Praag et al., 2005, Uda et al., 2006, Clark et al., 2008, Trejo et al., 2008, Wojtowicz et al., 2008). However, both of these result in a great deal of changes throughout the brain and the body (Simpson and Kelly, 2011, van Praag et al., 2014), making the causative relationship between enhanced DG neurogenesis and enhanced ability to perform hippocampal-specific tasks difficult to make. As another example – but in the opposite direction – stress decreases neurogenesis, in part by acting on glucocorticoid receptors expressed on Type-1 and Type-2 cells, and can impede learning and memory and

exacerbate depression- and anxiety-like behaviors (Garcia et al., 2004). While there are many of these correlatives studies suggesting new neurons improve hippocampal function, causative studies have proven more challenging. However, inducible increase of DG neurogenesis has been accomplished through inducible transgenic mice, such as the *Bax* mouse (Sahay et al., 2011a), and also via novel neurogenic compounds. As the *Bax* mouse was mentioned above and again below, here I will only focus on novel neurogenic compounds.

Neurogenic compounds have been known for over 15 years, beginning with the publication that antidepressants increase neurogenesis (Malberg et al., 2000). However, with the advent of large scale screening of small molecule libraries, new compounds have been discovered that are putatively specific in their action at increasing neurogenesis. Two such compounds discovered at UT Southwestern are P7C3 or isoxazole-9 (Isx-9), which are thought to increase DG neurogenesis through distinct mechanisms. P7C3 is a compound that has been shown to rescue deficits in hippocampal neurogenesis and function caused by aging or disease. Notably, P7C3 does not elevate neurogenesis past basal levels in a healthy, young animal (Pieper et al., 2010, De Jesus-Cortes et al., 2012, Blaya et al., 2014, Latchney et al., 2015). In support of this, unpublished data from the Eisch Lab indicate that P7C3 does not increase immature neuron number or survival in young, healthy male rats. This suggests that while P7C3 may be a beneficial drug for when DG neurogenesis is already decreased, P7C3 cannot be used to increase neurogenesis when the animal is in a non-pathological state. A mechanism underlying the ability of P7C3 and related

compounds to increase neurogenesis has recently been proposed: prevention of apoptosis through prevention of oxidative toxicity (Pieper et al., 2010, Wang et al., 2014). Up until recently, the ability of P7C3 and related compounds to improve hippocampal function has never been shown to be specifically caused by increased neurogenesis. However, Walker et al., use X-ray ablation to show that prevention of P7C3-induced increase in neurogenesis blocks its behavioral effects in a depression-related task (Walker et al., 2014). It remains unknown whether the other published actions of P7C3 to improve hippocampal function are also reliant on P7C3-induced increases in hippocampal neurogenesis.

A second neurogenic small molecule discovered at UT Southwestern is Isx-9. First identified for its ability to drive neuronal differentiation *in vitro* (Schneider et al., 2008), more recently Isx-9 has been shown to increase proliferation, immature neuron number, dendritic arborization, and spatial memory *in vivo* (Petrik et al., 2012a). *In vitro* studies with Isx-9 first showed that it triggered differentiation via calcium influx and subsequent intracellular signaling via transcriptional regulators of the MEF2 family. Additional investigation *in vivo* showed that Isx-9 increases Mef2 expression in Type-1 stem-like cells and progenitors, leading to an increase in the number of dividing cells (Petrik et al., 2012a). Furthermore, use of transgenic mice where Mef2 could be inducibly deleted from Type-1 stem-like cells and their progeny showed the requirement of Mef2 for Isx-9's neurogenic influence (Petrik et al., 2012a).

So what can we learn about the function of adult neurogenesis from the studies that use these neurogenic small molecules? Minimally, it appears as if the selective increase in neurogenesis via P7C3 or Isx-9 can improve hippocampal function. This bodes very well for the ongoing efforts by pharmaceutical companies to develop these small molecules and related compounds for their eventual therapeutic human use. However, moving ahead it will be important to ensure that the global, non-neurogenesis effects of these and other small molecules are elucidated, and not to assume that their effects to improve brain function are all reliant on enhanced neurogenesis, particularly since neurogenesis is thought to occur at a much slower rate and lower amount in the primate and human brain (Knoth et al., 2010, Kohler et al., 2011, Sierra et al., 2011, Ernst and Frisen, 2015).

As potentially beneficial as P7C3 and Isx-9 appear to be, there are significant caveats. For example, both these small molecules (and many of their chemically-related compounds) are under patent, and therefore their availability is limited and their synthesis is cost restrictive. Both compounds would benefit from proof-of-principle studies with i.c.v. infusions in order to use smaller amounts, and even intrahippocampal infusions and to assess brain region-specificity and to clarify what brain effects are due to the compound vs. are indirect effects. Both P7C3 and Isx-9 represent an additional technique to increase in adult DG neurogenesis in wild-type animals, allowing for the study of large cohorts without breeding restraints and without the widespread changes that occur with aerobic exercise or environmental enrichment.

Adult Dentate Gyrus Neurogenesis in Humans

While almost all adult DG neurogenesis studies have focused on animal models, it was long debated as to whether humans also produced new neurons in the hippocampus throughout adult life. Studies have now used three different techniques to confirm the existence of DG neurogenesis in human populations. First, biomarkers for neuroprogenitor cells seen *in vitro* were detected within the hippocampus of young and adult humans using magnetic resonance spectroscopy (Manganas et al., 2007). Second, immunohistochemical work has shown adult DG neurogenesis in primate and human postmortem tissue (Knoth et al., 2010, Kohler et al., 2011, Sierra et al., 2011), and its regulation with age. Such post-mortem studies have also found an increased number of proliferating cells in humans that had been taking antidepressants prior to death, paralleling previous animal studies (Boldrini et al., 2009).

Perhaps some of the most interesting work supporting the existence of human adult DG neurogenesis involves the atomic bomb testing of the 1950s. After the blast, ^{14}C was released into the atmosphere, incorporated into the DNA of plants, which were then eaten by humans, resulting in ^{14}C incorporation into actively dividing cells (Ernst and Frisen, 2015). In adults, ^{14}C would only be incorporated into neurons that were dividing at the time, much like the BrdU used in animal studies. Studies on postmortem tissue found cells labeled with ^{14}C in the DG of individuals that were adults at the time of the atomic bomb (Spalding et al., 2013). While adult DG neurogenesis in humans has been long debated, the many techniques used in the past decade to address this

question that have all reached the same conclusion: DG neurogenesis does exist in the adult human.

In summary, correlative and causative studies suggest that adult DG neurogenesis plays a role in hippocampal-dependent functions. It is interesting that mood regulation appears to be a prime function of neurogenesis, particularly under stressful conditions. There remains great interest in the role of new neurons in depression and related disorders, as the advances in basic science may have implications for treating and perhaps even preventing depression (Santarelli et al., 2003, Warner-Schmidt and Duman, 2006). Given the strong connections between depression and addiction (Koob, 2000, Boden and Fergusson, 2011, Pettinati et al., 2013), it is curious that there is far less work done in regards to the role of adult neurogenesis in addiction. The sections below provide a brief overview of addiction – and its relationship to depression and mood disorders – and the work that has been done exploring the link between addiction and adult DG neurogenesis. This will set the background for my data in **Chapter 2,** which show that depletion of neurogenesis enhances vulnerability in an animal model of morphine addiction, and in **Chapter 3** and **Chapter 4**, which show that morphine and heroin, respectively, taken in a clinically-relevant paradigm have no gross influence on the generation of new neurons in the DG.

2. Addiction Overview

Definition of Addiction

Addiction, defined broadly under the umbrella of "substance abuse" in the DSM-IV (American Psychiatric Association, 2000), has now been divided into drug of abuse-specific sections in the DSM-V. Since opioids are the focus of this dissertation, the criteria for opioid abuse will be used to define addiction in humans. Opioid abuse is defined by the presence of at least two of the following criteria within the past year (quoted directly from the DSM-V; American Psychiatric Association, 2014):

"1. Opioids are often taken in larger amounts or over a longer period than was intended.
2. There is a persistent desire or unsuccessful efforts to cut down or control opioid use.
3. A great deal of time is spent in activities necessary to obtain the opioid, use the opioid, or recover from its effects.
4. Craving, or a strong desire or urge to use opioids.
5. Recurrent opioid use resulting in a failure to fulfill major role obligations at work, school, or home.
6. Continued opioid use despite having persistent or recurrent social or interpersonal problems caused or exacerbated by the effects of opioids.
7. Important social, occupational, or recreational activities are given up or reduced because of opioid use.
8. Recurrent opioid use in situations in which it is physically hazardous.
9. Continued opioid use despite knowledge of having a persistent or recurrent physical or psychological problem that is likely to have been caused or exacerbated by the substance.
10. Tolerance
11. Withdrawal"

As is central to this thesis, and particularly **Chapters 2-4**, many of these criteria for opioid addiction from the DSM-V can be replicated in animal models of addiction. For example, as expanded on below in the section "Animal Models of Addiction", criteria 1-4,8 and 10 can be replicated with self-administration, but certain aspects remain unable

to be tested in animals (i.e. criterion 7). Therefore, while addiction is a uniquely human disorder, key aspects of opioid addiction can be mimicked in a laboratory setting.

Societal /Economic Costs

According to the Centers for Disease Control and Prevention, substance abuse costs the economy of the United States over $600 billion annually, including costs related to crime, lost work productivity, and healthcare costs (NIDA, 2012). Of this, $428 billion is from legal substances, such as tobacco and alcohol. An additional $193 billion is spent each year because of illicit, or illegally obtained, narcotics. This is an immense pressure on the American economy, and our healthcare system, as the healthcare costs alone add up to more than $137 billion each year.

A 2012 study estimated 23.9 million Americans (9.2% of the population) had used an illicit drug or abused a medication within the last 30 days (NIDA, 2014). Additionally, this estimate was an 8.3% increase from the last study performed in 2002. Focusing on prescription opiates, such as oxycontin and hydrocodone, in 2012 6.8 million Americans (2.6% of the population) had used a psychotherapeutic medication either without a prescription or for a purpose for which it was not prescribed. Additionally, when examining first time drug users, 17% start with pain relievers, with 1.3% more beginning with sedatives. Opiates are increasingly becoming the leading cause of overdose deaths within the United States, with a 400% increase within the past ten years (NIDA, 2015). Therefore there is an urgent need to prevent and treat opioid addiction.

Current Treatment Options

A major problem within the addiction field is the “treatment gap”, in which individuals that need care to overcome their condition rarely receive the treatment they require. For example, in 2012 only 2.5 million people received in-facility treatment for problems related to drugs when a total of 23.1 million people needed care (NIDA, 2009). It is urgent that the treatment gap is filled so the remaining 89% of people can also receive proper treatment.

Many current treatments for addiction that involve medication treat the symptoms rather than the disease, primarily through preventing withdrawal symptoms with a similar compound that lacks the abuse potential (NIDA, 2009) in an approach called “replacement therapy”. For example, opiates, such as heroin and morphine, are commonly treated using methadone (Dole and Nyswander, 1965, Freedman and Senay, 1973). Methadone is a long-lasting, lower reward opiate that binds to the same receptor as heroin and morphine (the mu opioid receptor), thus preventing withdrawal symptoms and perhaps relieving cravings (Dole et al., 1991). Replacement therapy can be used for other drugs of abuse as well, and is often used by smokers attempting to quit. Smoking cessation often uses nicotine patches, gum, or newer e-cigarettes to slowly wean the user off of nicotine (Silagy et al., 2004).

In addition to replacement therapies, behavioral treatment is commonly used in parallel. Behavioral treatment often includes cognitive-behavioral therapy, which attempts to help users to “recognize, avoid, and cope with the situations in which they

are mostly likely to abuse drugs" (Tuten et al., 2012). However, even with behavioral and medicinal treatments used in tandem, relapse rates are fairly high, with 70% of opiate abusers relapsing within six months of treatment (Tuten et al., 2012).

Taken together, addiction is marked by high prevalence and high costs, and the current treatment options are limited, low in availability, and poor in their efficacy. Therefore, another approach to treating addiction is to understand its neural basis more effectively. As described below, human and laboratory animal studies have been effective in revealing the brain regions engaged in addictive processes, and suggesting novel approaches for preventing and treating addiction.

Brain Regions Implicated in Addiction

Reward Pathway

The reward pathway comprises a key set of brain structures on which addiction researchers have focused to figure out why people abuse drugs and why they commonly relapse. In other words, researchers believe that examining the neuroplasticity of reward pathway structures will probably give us clues to the mysterious process of addiction, help us design better treatments for addiction, and perhaps prevent it from occurring in the first place (Koob and Volkow, 2010).

The initial "liking" of a drug originates in the reward pathway, a series of interconnected brain structures that are acutely regulated by drugs of abuse. In general, the reward pathway begins with the dopamine-containing ("dopaminergic" or

dopamine (DA)) neurons of the ventral tegmental area (VTA) sending direct efferents, or projections, via the mesolimbic pathway to neurons in the nucleus accumbens (NAc), prefrontal cortex (PFC), ventral pallidum (VP), and the hippocampus and indirect connections to the basolateral amygdala (BLA) and extended amygdala (EA, which includes the central nucleus of the amygdala, the bed nucleus of the stria terminalis and a subregion of the NAc shell) (Swanson, 1982, Gasbarri et al., 1994b). A more inclusive version of the reward pathway is known as the limbic system (Koob and Volkow, 2010), and also includes multiple brain areas that also influence the NAc through glutamatergic projections. Regions that send glutamatergic efferents to the reward pathway and the NAc in particular include the hippocampus, the PFC, and the amygdala (Britt et al., 2012).

Brain Regions Involved in the Stress Response

The brain regions involved in the stress response have a strong role in addiction, as stress can often trigger a relapse in human patients (Breese et al., 2005) and in animal models (Shaham et al., 2000). The “stress axis” as it is known is an endocrine system consisting of three major areas – the paraventricular nucleus of the **h**ypothalamus, the **p**ituitary gland, and the **a**drenal glands – and thus is also known as the HPA axis. The HPA axis controls the body’s response to stress through the production and release of glucocorticoids. This process is tightly controlled through a series of hormones and negative feedback loops. Many areas of the brain involved in reward circuitry respond to elevated glucocorticoid levels, including the hippocampus

(Woolley et al., 1990, Atsak et al., 2012, Nasca et al., 2014) and the amygdala (Quirarte et al., 1997, Roozendaal et al., 2002).

Hippocampus

Although the reward pathway and the HPA axis are clearly important in addiction, another structure has more recently been appreciated for its potential role: the hippocampus. As mentioned above, the hippocampus has long been known to play a role in brain functions like spatial learning and memory and mood regulation. However, closer examination at how the hippocampus is connected to the reward pathway reveals why the hippocampus has gained more attention in recent years for its role in addiction (**Figure 1-1A, Figure 1-3**). As the hippocampus receives input from key structures in the brain reward pathway, such as the VTA, and sends output to reward structures like the NAc, PFC, EA, and BLA (Carr and Sesack, 1996, Pitkänen et al., 2000, Britt et al., 2012), the hippocampus is in an ideal position to influence both the acute and chronic effects of drugs of abuse. In fact, many drugs of abuse – including opioids – have direct action on hippocampal neurons, as well as on reward pathway neurons. As discussed in greater detail below, one example is that DG neurons express opioid receptors(Drake et al., 2007). Thus, this further supports a role for the hippocampus in addiction.

In addition to “being in the right place”, the hippocampus also appears to “be in the right place at the right time”, as additional research with animal models of drug intake and human imaging studies show that the hippocampus plays a vital role in many aspects of addiction, including craving, the anticipation of the drug, and the

establishment of drug/context associations (Koob and Volkow, 2010). For example, the hippocampus is required in order for animals to form a preference in conditioned place preference (CPP) (Ferbinteanu and McDonald, 2001, Meyers et al., 2003), and a novel context is required for locomotor sensitization to occur (Badiani et al., 2000).

The last point – that locomotor sensitization requires a novel context – is particularly interesting given the long-known role of the hippocampus in spatial learning and memory and the importance of context in learning and remembering during the process of addiction. In fact, the role of the hippocampus in addiction might be generally summarized as being keenly involved in the "memory of addiction." This view has gained support from addiction studies with laboratory animals that relapse, also known in the scientific field as reinstatement. For example, "addicted" laboratory animals that were in "recovery" from drug taking will relapse, or reinstate lever pressing for the drug, if the hippocampal outputs are stimulated (Martin-Fardon et al., 2008) and inactivation of the same outputs attenuate reinstatement (Rogers and See, 2007). This is strikingly similar to the clinical situation where the association drug addicts make between their drug intake and their environment is so powerful that re-exposure to the environment can result in relapse (Wikler, 1973, O'Brien et al., 1992). The importance of the hippocampus in relapse fits with the growing appreciation that the hippocampus may also be important for the craving of a drug during a drug-abstinent or withdrawal phase (Koob and Volkow, 2010).

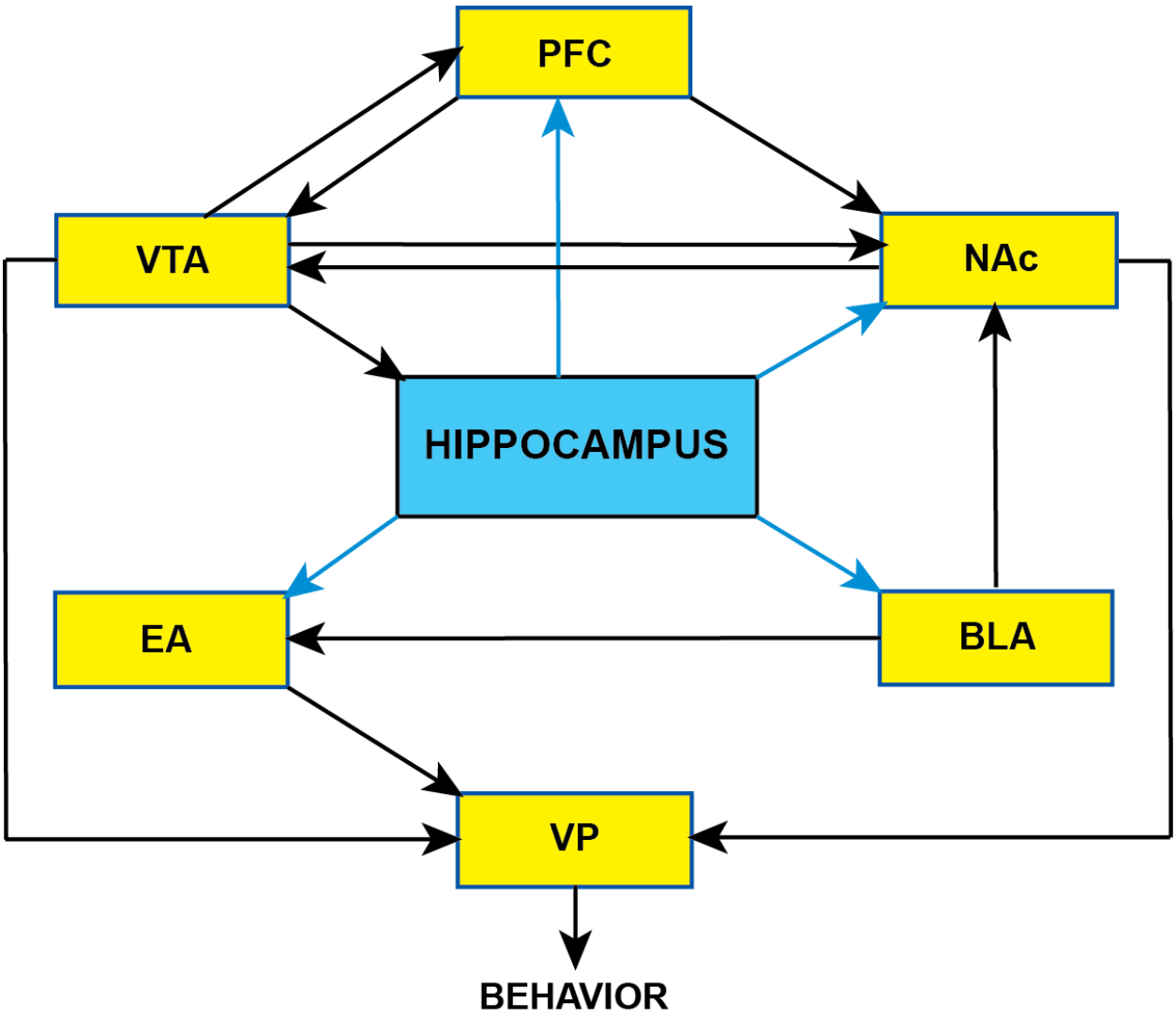

Figure 1-3. The hippocampus: a key player in the process of addiction. As elaborated in the text, addiction has long been known to involve the brain's "reward pathway", which includes dopamine-containing ("dopaminergic" or DA) cells in the ventral tegmental area (VTA) and their direct or indirect connections to the nucleus accumbens (NAc), prefrontal cortex (PFC) and ventral pallidum (VP), which is one of the main output pathways that mediates behavior (both in naïve and addicted states). More recent work has also implicated other limbic regions in addiction, like the basolateral amygdala (BLA) and extended amygdala (EA, which includes the central nucleus of the amygdala, the bed nucleus of the stria terminalis and a subregion of the NAc shell). More recently, researchers have come to appreciate that the hippocampus (blue) plays critical roles in the processes of drug addiction, drug craving, and processing context-dependent information. This role for the hippocampus could have been surmised from the afferent (input) and efferent (outputs) connections with structures in the brain reward pathway. Some of these connections are highlighted here: the hippocampus receives inputs from the VTA, and sends outputs to the NAc, EA and indirectly to the VP. Some connections from other monoaminergic and indolaminergic nuclei and to the thalamus have been omitted for clarity.

Networks Implicated Hippocampal-Reward Circuitry

As mentioned briefly above, the hippocampus is in a prime position to influence many parts of the reward pathway, as it sends efferents to, receives afferents from, or is bidirectionally connected to almost all the regions that play a role in reward (**Figure 1-3**). It is useful at this juncture to take a less brain region-specific view and instead take a more network level approach. Probably the most influential of these connections are the ventral hippocampal inputs through the ventral subiculum into the medial NAc shell (Zornoza et al., 2005, Britt et al., 2012). Glutamatergic inputs from the hippocampus can alter the proportion of tonic versus phasic neurons within the NAc and has its influence both DA neuron population activity and regulate the firing properties of these neurons, this strong influence suggests that dysregulation of hippocampal circuits can contribute to overexcitability of the dopamine system (Floresco et al., 2001). Interestingly, the hippocampus is not the only influence on these DA neurons, as PFC afferents also synapse on the same neurons (Carr and Sesack, 1996, Finch, 1996), causing integration of signals from multiple brain regions. The fact that the PFC, the amygdala, and the hippocampus all influence the dopamine system through glutamatergic inputs is even more interesting when you consider that both the PFC and amygdala receive strong inputs from the hippocampus itself. The connections from the hippocampus to the PFC occur through both the subiculum and directly from the CA1 (Swanson, 1981, Jay and Witter, 1991), allowing for influence from two parts of the hippocampal glutamatergic circuit. The amygdala also heavily influences the NAc through glutamatergic inputs and also receives afferents from the hippocampus (Pitkänen et al., 2000). In addition to the glutamatergic interconnectivity, the hippocampus also receives input from several brain regions important in reward,

including the VTA (Gasbarri et al., 1994a, Gasbarri et al., 1994b). Dopaminergic inputs from the VTA into the hippocampus occur at both the subiculum and the CA1, allowing for another level of control through the dopamine system (Gasbarri et al., 1994a).

In summary, the hippocampus – a brain region known for its role in memory and mood regulation – is itself a target of some addictive drugs and is well positioned to influence the main target of addictive drugs (the reward pathway). The hippocampus appears to be important particularly in mediating the associations addicts make between the drugs and the environment in which the drug is taken or acquired and in the craving for drugs. The effects that drugs have on the hippocampus may hold clues to why addiction presents clinically as an abnormally strong and focused memory. Understanding drug-induced neuroplasticity in the hippocampus may hold clues to why addiction is such an extremely difficult disorder to prevent and treat and may lead to novel strategies for addiction treatment and management.

Animal Models of Addiction

A reasonable question at this juncture in this Introduction would be “what does addiction do adult neurogenesis and hippocampal function?” However, the answer to this relies on the type of delivery of the drug. Therefore, a brief review of types of animal models of drug exposure is provided.

Typically, researchers use one of two approaches to expose subjects to a drug of abuse (usually laboratory animals, such as rodents or primates) when studying the

effect of drugs on adult DG neurogenesis: experimenter-delivered drug exposure and voluntary intake (often called “non-contingent” and “contingent”, respectively). These two very different paradigms come with advantages and disadvantages, and it is important to explore the results of both methods since changes seen in brain and behavior after drug exposure can be very different based on the method of delivery. The advantages of contingent and non-contingent drug delivery are further discussed below.

Experimenter-Delivered (Non-contingent) Drug Delivery

In studies using non-contingent drug delivery, the experimenter controls the amount of drug that the animal receives, and dosages remain consistent between subjects. The most common non-contingent drug administration method is injection, typically given either intraperitoneally (i.p.), subcutaneously (s.c.), or intramuscularly (i.m.). An advantage of using injections is that experimenters can closely mimic the blood levels of a drug experienced by a human addict in an experimental setting. In this method, the concentration of drug is highest following the injection, but decreases over time as the drug is metabolized or eliminated (e.g. Fischer et al., 2008). A secondary method of non-contingent drug delivery is subcutaneously pellets, which are implanted under the skin and allow for consistent, high delivery of drug through absorption. Noncontingent drug delivery through injection is used in two commonly-employed behavioral paradigms relevant to addiction – CPP and locomotor sensitization – which are discussed in turn below.

CPP typically is performed in a three-chambered box with one small middle chamber and two larger chambers on either side. The two larger chambers vary in several characteristics, with differently patterned walls and textured floors. The CPP box allows for either free access to all three chambers, or sequestration into a single chamber. Animals are then paired to one chamber/context with a drug, and given saline when sequestered in the opposite chamber/context. After multiple pairings to either side, animals are again allowed free access to the entire box while drug free and time spent in each chamber is recorded. Animals that exhibit a preference will spend more time in the drug-paired chamber, while animals that find the drug aversive will spend more time in the saline-paired chamber. This behavioral paradigm is often used to explore drug-context memory, or motivation effects of a drug (Meyers et al., 2006, Tzschentke, 2007), and has been used extensively by another Eisch Lab member, Phil Rivera, to explore the relationship between new neurons and place preference.

A second behavioral paradigm that uses non-contingent drug delivery is locomotor sensitization. Sensitization requires animals to be placed in a novel environment and given repeated injections of a drug over a series of days. On the first day, the animals demonstrate the locomotor effects of the drug. After a short period of withdrawal (usually a day), the animals receive the same dose of the drug again, yet the animals demonstrate a greater locomotor response, thus exhibiting a higher sensitivity to the same dose. For example, rats that receive 10 mg/kg doses of morphine exhibit higher locomotion on Day 5 than on the day of their first exposure (Mickiewicz et al., 2009). Sensitization is also defined as “the augmented motor-

stimulant response that occurs with repeated, intermittent exposure to a specific drug" (Steketee and Kalivas, 2011). This behavioral paradigm is often used to study the circuitry behind addiction and its activation (Robinson and Berridge, 1993), is a central behavioral paradigm in my thesis work, and the data using locomotor sensitization are shown and discussed in **Chapter 2**.

Voluntary Intake (Contingent)

Voluntary intake offers an advantage that experimenter-delivered methods do not: the animal is allowed to choose how much drug to take. This is considered to mirror dependence and some aspects of human addiction. Additionally, voluntary intake does not result in the same stress to the animal that experimenter-delivered drugs impose. For example, animals will not receive the stress of forced injections seen with experimenter-delivered drugs, which may introduce another factor along with the pharmacological effects of the drug.

A common model of voluntary intake is intravenous (i.v.) self-administration (Weeks, 1962, Roberts et al., 2007, Winstanley et al., 2010). For i.v. self-administration, surgical implantation of a catheter into the jugular vein of the animal. Animals are then trained to press a lever in order to receive an infusion of the chosen drug of abuse. After this training, animals receive daily access to the drug that they can then choose to self-administer. Voluntary i.v. delivery of the drug of choice is beneficial as it is a more clinically relevant model, as addicts voluntarily take drugs. Additionally, the drug almost instantaneously reaches the brain, allowing for immediate effect, in

contrast to injections which can take several minutes for full effectiveness (Pan et al., 1991). I.v. self-administration is a major component of this thesis, with i.v. morphine self-administration the focus of **Chapter 2** and **Chapter 3** and i.v. heroin self-administration the focus of **Chapter 4.**

Another common model of self-administration is oral administration, sometimes performed with a two-bottle paradigm. For the two-bottle paradigm, the drug of choice is placed within a second water bottle in the homecage, and the amount of liquid in both bottles (water vs. drug) can be measured. This allows for 24-hour access without the need for catheterization surgery. This model is commonly used for drugs that are consumed, such as ethanol (Middaugh and Bandy, 2000, Meisch, 2001).

In sum, non-contingent vs. contingent drug administration result in different drug metabolism, blood concentration, and serum kinetics/dynamics even if the same dose were used in each method. These considerations will be taken into account when interpreting the data in this thesis that involve non-contingent drug delivery (morphine locomotor sensitization).

Opiates

So far, I have detailed addiction in general, examining the circuitry involved, behavioral studies used, and methods of administration. However, while there are many drugs of abuse that can cause addiction, the emphasis of this dissertation is opiate addiction. Before exploring the relationship between adult DG neurogenesis and

opiate-related behaviors in rodents, it is important to understand how opiates affect the brain at multiple levels. Below, I will discuss opiates and opioids, their receptors, and their effects on the brain and subsequent behavior.

Opiates vs Opioids

Opiates and opioids can be confusing terms for those not used to the nomenclature. Opioids are a universal term for all drugs and endogenous peptides that bind to the opioid receptor (Saunders, 2007b). On the other hand, opiates are classified as any drug that is naturally derived from the opium poppy plant (Saunders, 2007a). Therefore all opiates are opioids, but not all opioids are opiates. Examples of opiates include morphine and heroin, while opioids include the synthetics hydrocodone and oxycodone, and the endogenous enkephalins and dynorphins.

Opioids in the Rodent Brain

The reason that opiates have any effect on the brain is that they mimic endogenous opioids, and bind to their receptors. Endogenous opioids can be divided into two major groups, the enkephalins and the dynorphins. Both of these groups of peptides are expressed widely throughout the central nervous system, including the hippocampus, the NAc, the VTA, and the hypothalamus (Watson et al., 1982, Drake and Milner, 2002, Drake et al., 2007).

Opioid Receptors

There are three major opioid receptors within the rodent brain, mu- (μ), delta- (δ), and kappa- (κ) receptors (MOR, DOR, and KOR, respectively). These receptors typically dimerize with themselves or with each other to become hetero- or homodimers (Gomes et al., 2000, Jordan et al., 2000). All opioid receptors are G-coupled protein receptors. MOR and DOR are coupled to the Gi/o subtype, while KORs are coupled to G_s proteins (Childers, 1991). The focus of this dissertation is on an agonist – morphine – with higher relative affinity to MOR, with a lower affinity for KOR and DOR, and thus the sections below will primarily address MOR's general distribution and second messenger signaling cascade.

General action of morphine on MOR and DOR

When morphine binds to the MOR, it begins a G-coupled protein receptor signaling cascade. Since MOR is a g-protein coupled receptor, binding of an agonist results in the activation of a $G_{i/o}$ protein and a $G_{\beta\gamma}$ protein. $G_{i/o}$ inhibits adenylate cyclase, preventing production of the second messenger cyclic-AMP (cAMP) and the PKA pathway. This results in a decrease in downstream mediators, such as cAMP response element-binding protein (CREB), resulting in the closure of Ca^{2+} channels. $G_{\beta\gamma}$, also activated by the binding of morphine, results in the opening of G protein-coupled inwardly-rectifying K^+ channels. Through the combination of these downstream signaling cascades, the overall result is the hyperpolarization of the cell. *In vitro* assays suggest that both MORs and DORs activate the same g-coupled proteins, resulting in similar downstream signaling (Alt et al., 2002). As MORs (and DORs) are typically

expressed on interneurons (Zheng and Khanna, 1999, Drake et al., 2002, Drake and Milner, 2002), ligand binding to MOR on interneurons lowers inhibitory tone and leads to greater activity of neurons that are downstream from the interneuron in question.

Distribution of Opioid Receptors in the Rodent Brain

Opioid receptors occur widely throughout the brain and spinal cord. For brevity, I will focus on brain areas of interest and the receptors expressed within each area.

Hippocampus. Most of the cells in the hippocampus that express DOR and/or MOR are interneurons (Stumm et al., 2004). However, opioid receptor distribution and density within the hippocampus varies greatly depending on the subregion examined. In the dentate gyrus, DORs are expressed highly within the hilus. DORs are also expressed within the granule cell layer, but to a lesser extent and with a scattered distribution (Commons and Milner, 1996). MORs are also expressed within the dentate gyrus in a sparse scattered pattern similar to DOR (Drake and Milner, 2002). The CA3 and CA1 show similar expression of opioid receptor subtypes. In both of these areas, a large number of interneurons express one, or both, MORs and DORs (Stumm et al., 2004). Interestingly, activation of MORs exclusively within the CA3 is rewarding, as rats will self-administer MOR agonists specifically to the CA3 (Stevens et al., 1991). KOR expression within the hippocampus is much more limited than the other two receptors subtypes. The dentate gyrus has very little KOR expression, with expression being limited to interneurons in the outer molecular layer, while interneurons in the CA3

and CA1 also have limited expression (Unterwald et al., 1991, Halasy et al., 2000, Racz and Halasy, 2002).

Nucleus Accumbens. Though it is thought that most of the rewarding properties of opiates occur through inhibiting interneurons in the VTA, the NAc also expresses opioid receptors. In particular, medium spiny neurons in the NAc shell, which also express DA receptors, express MORs (Mansour et al., 1995b). Further evidence the opioid receptors have direct actions within NAc have been revealed through direct injections of opioid agonists directly into the NAc. For example, MOR agonists infused directly into the NAc increase dopamine release and increase locomotor activity, while KOR agonists decrease dopamine release and decrease activity, indicating opposing action directly with the NAc (Di Chiara and Imperato, 1988).

Ventral Tegmental Area. The most commonly expressed opioid receptor within the VTA is the MOR. It is highly expressed within interneurons of the VTA, with no coexpression with markers of dopamine neurons (Garzon and Pickel, 2001). However, MORs have high expression presynaptically to dopamine neurons, putting them in a prime position to directly modulate dopamine neuron firing (Svingos et al., 2001). Stimulation of MORs expressed within the VTA results in increased dopamine release in downstream regions (Johnson and North, 1992, Jalabert et al., 2011), including the NAc and the hippocampus.

Hypothalamus-Pituitary-Adrenal Axis. Unlike the NAc and hippocampus, the hypothalamus and pituitary have high expression of KORs, mainly within the paraventricular nucleus, the arcuate nucleus, and the supraoptic nucleus (Unterwald et al., 1991, Mansour et al., 1995a, Mansour et al., 1995b). There is a small amount of MOR expression within the pituitary, but it is limited to very few cells. Opioid receptors in the hypothalamus and pituitary are thought to play a minor role in hormone release (Mansour et al., 1995a).

Morphine-induced Alterations in Behavior and Brain

In order to understand how laboratory animal work with morphine may or may not reflect humans exposed to morphine, it is important to gauge what is known and not known about the influence of opiates on the brain and behavior of laboratory animals. The vast majority of literature examining the influence of morphine on behavior and the brain results from studies that use experimenter-delivered (non-contingent) morphine, such as s.c. pellets or injections. These are discussed below. However, the more translationally relevant morphine self-administration is discussed in a within a separate section afterward, and is highlighted in **Table 1-2**.

Acute Behavioral Effects. As opioid receptors are expressed widely throughout the brain, it is understandable that morphine would cause alterations in behavior during and after acute exposure. For example, the hippocampus has high expression of opioid receptors throughout, and after an acute dose of morphine spatial learning is impaired, with high doses leading to complete amnesia of the previous day's training (Ma et al.,

2007). Another effect on behavior is that morphine can increase impulsivity during an acute challenge (Pattij et al., 2009). Acute morphine exposure also causes rapid changes in locomotor activity. Morphine is often thought of as a depressant, and indeed it will cause an initial depression in activity at high doses (10-40 mg/kg), but it will eventually lead to increased locomotion. However, more moderate doses (1- 5 mg/kg) are excitatory and increase activity without the initial depression effect (Babbini and Davis, 1972, Mucha et al., 1981). In fact, the morphine-induced increases locomotion forms the basis for the use of morphine locomotion sensitization in chronic exposure studies, as mentioned above. Acute morphine can also alter behavior after it has been metabolized, as a single dose of morphine increases anxiety even when the drug is no longer detectable in the serum (Harris and Gewirtz, 2004, Zhang and Schulteis, 2008). Taken together, acute exposure to morphine affects several types of behavior.

Long-term Behavioral Effects. One difference between studies that explore acute versus chronic effects of morphine is that many of the studies that explore behavior after chronic exposure are performed when morphine is no longer "on board" and the animal is in a withdrawal state, while acute experiments are performed immediately after the injection. Long-term behavioral effects of morphine can also be highly reliant on the scheduling of exposure. Therefore, I will mention the scheduling of morphine delivery and whether experiments occur during exposure or withdrawal.

As with acute morphine, chronic exposure can lead to deficits in learning and memory. Chronic morphine impairs spatial learning and memory when the drug is

onboard in several different tasks, including the radial arm maze, Y-maze, and the Morris water maze (Spain and Newsom, 1991, Pu et al., 2002). However, working memory may be spared (Spain and Newsom, 1991). Chronic morphine also decreases spatial learning and memory during the withdrawal phase. For example, chronic morphine exposure followed by seven days of withdrawal impaired cued fear extinction learning, but did not alter acquisition of the learned behavior (Gu et al., 2008). While this study indicates that contextually-dependent cognitive flexibility in fear memory is decreased long-term after chronic morphine exposure, prior to the work presented in my thesis it was unknown if there was decreased cognitive flexibility in a behavioral model of addiction after morphine exposure. This will be addressed in **Chapter 2.**

Interestingly, many long-term behavioral effects of morphine can be reversed via running in animal models. For example, rats that have access to voluntary running wheels for several weeks before chronic morphine exposure do not have morphine-induce deficits in spatial learning and memory (Miladi Gorji et al., 2008), but do have have improved conditioned place preference (Eisenstein and Holmes, 2007), improved passive-avoidance memory (Saadipour et al., 2009), and lower morphine-induced anxiety (Miladi-Gorji et al., 2012). While no one has directly looked at morphine exposure, wheel running, and adult DG neurogenesis, it is also well known that chronic wheel running increases DG neurogenesis (van Praag et al., 1999, Farmer et al., 2004, van Praag et al., 2005, Uda et al., 2006, Clark et al., 2008, Naylor et al., 2008, Wojtowicz et al., 2008). It would be interesting to explore the role of adult DG

neurogenesis as a mechanism behind how chronic aerobic exercise ameliorates the behavioral effects of chronic morphine exposure.

Neuroplastic Alterations From Acute Exposure. It makes sense that if behavioral differences are observed after morphine exposure, there must be cellular and molecular changes driving these changes in behavior. One way to examine these changes is through immediate early gene (IEG) expression. IEGs are transcription factors that respond rapidly to stimuli to the cell and are commonly used as markers of cellular activity. One of the most commonly-used IEGs is c-fos, or its upstream mediators, CREB or cAMP. An acute dose of morphine results in c-fos expression in the NAc core and shell (Grabus et al., 2004), dorsomedial caudate-putamen, paraventricular nucleus of the thalamus, and the hippocampus (Gutstein et al., 1998, Marie-Claire et al., 2003). In addition to changes in IEGs, long-term potentiation (LTP) in the perforant path of the hippocampus is significantly decreased after a single 1h exposure to morphine (Ito et al., 2001). Regarding other brain regions, acute morphine impairs LTP at inhibitory synapses within the VTA, but has no effect after chronic use (Niehaus et al., 2010) and acute morphine also induces LTP in synapses from the PFC to the NAc shell (Zheng et al., 2014).

Neuroplastic Alterations From Chronic Exposure. While acute exposure to morphine results in c-fos expression, longer exposure can result in the expression of deltafosB, a truncated form of fosB that is a proposed “marker” of addiction because of its stability, long half-life, and correlative and causative association with addictive

behaviors (Nestler, 2013). Morphine s.c. pellets result in high deltafosB expression in a variety of brain regions, including the PFC, the dorsal striatum, the NAc core, and several regions of the hippocampus (the DG, CA1, and CA3) (Perrotti et al., 2008). While acute exposure to morphine does not change NAc CREB, an upstream mediator of fos and related proteins, chronic morphine exposure decreases NAc CREB expression, indicating cAMP pathways are downregulated over time (Widnell et al., 1996). In regards to electrophysiological changes, the effects of chronic morphine on LTP have been well documented. LTP at the mossy fiber synapse is enhanced after chronic morphine (Harrison et al., 2002). Interestingly, opioid antagonists, which increase LTP in naïve animals, have no effect in animals treated with chronic morphine (Harrison et al., 2002). In conjunction, reintroduction of opiates after chronic exposure and withdrawal restores LTP to basal levels, indicating that the system has adapted to opiates over time (Pu et al., 2002). However, this is not necessarily a benefit, as a parallel group of animals showed deficits in spatial tasks at the time that LTP is unchanged (Pu et al., 2002). Chronic morphine also inhibits LTP within the CA1 with impairments in spatial memory, through altering the expression of various channels (Zhou et al., 2015). Recordings in the Schaffer-collaterals to the CA1 had opposite effects, revealing greater LTP after repeated morphine than the saline controls (Sadegh and Fathollahi, 2014). There are also changes in gene expression, as microarrays on chronically sensitized rats “suggests impairments in neurotransmitter release and the activation of neuroprotective pathways” within the hippocampus (Marie-Claire et al., 2007). Finally, it is important to examine how morphine affects MOR expression chronically, as most of its functions occur through this receptor. *In vitro* studies with

show morphine has little long-term effect on MOR expression, suggesting that chronic morphine is poor at causing MOR desensitization (rat brain slices; Blanchet and Luscher, 2002) or endocytosis (HEK cells; Keith et al., 1996), at least *in vitro*. Morphine's poor ability to alter the number of active MORs on the cell surface is one hypothesis as to why chronic exposure to morphine quickly leads to tolerance (Martini and Whistler, 2007). However, these *in vitro* studies have been challenging to reproduce *in vivo,* leaving the question open as to how exactly MOR is modified *in vivo* after chronic exposure.

Neuroplastic Alterations During Withdrawal. Withdrawal can be defined as any period of time after which morphine and its main metabolites are no longer detectable in the serum. As the half-life of morphine is 120 minutes in both rodents and humans (Catlin, 1977), withdrawal can be precipitated using MOR antagonists or occur spontaneously after the morphine has been metabolized (Blasig et al., 1973). This is a useful period of time in which to examine behavioral and brain changes since in both animal models of addiction and in humans withdrawal (or abstinence, in the case of humans) is when reports of craving or incidents of relapse to drug seeking and taking will occur (Bossert et al., 2005).

Morphine continues to have effects during withdrawal that vary from both the naïve state and from when morphine is in the system. During morphine withdrawal, brain regions begin to express c-fos or CREB, including the cortex, hippocampus, thalamus, and cerebellum, revealing a different pattern than seen after acute exposure

(Beckmann et al., 1995, Shaw-Lutchman et al., 2002). In one study, LTP in the CA1 of the hippocampus, which is unchanged after this particular chronic morphine exposure, became decreased during withdrawal (Pu et al., 2002). Likewise, within the amount of burst firing and the firing rate of dopamine neurons into the mesolimbic area is reduced when compared to saline treated controls and likely contributes to the dysphoria felt during withdrawal (Diana et al., 1995).

Morphine Self-administration (MSA). It is interesting to note that almost all studies examining the biological consequences of morphine exposure (acute, chronic, or withdrawal) have implemented experimenter-delivered morphine. Studies using MSA will likely reveal different and perhaps more clinically-relevant changes in brain and behavior than what is seen after experimenter-delivered morphine. It is not surprising that so few MSA papers have been published, given that i.v. morphine poses challenges (e.g. diminished immune system response, highly oral/chewing behavior). While still not widely used, the number of studies using MSA has grown in recent years (**Table 1-2**) and likely will continue to grow given the epidemic proportions of opiate addiction. From the studies that have thus far used MSA and examined behavior and neuroplasticity have found changes distinct from those seen after experimenter-delivered morphine. For example, MSA results in decreases spine density in the CA3, CA1, and DG of the hippocampus, but equivalently dosed injections have no effect (Robinson et al., 2002). In addition, the turnover rates for various neurotransmitters vary between contingent and noncontingent i.v. morphine (Smith et al., 1982). Surprisingly, little behavior beyond MSA has been explored. It is currently unknown how

MSA may alter behaviors that are known to be affected by experimenter-delivered morphine, including mood, learning and memory, or cognition in general. It is also unknown if ablating adult DG neurogenesis has any effect on MSA, or if MSA is detrimental to adult DG neurogenesis. Within this dissertation I will address these gaps within the literature.

In sum, there are a variety of changes in brain and behavior seen after different types and durations of morphine exposure. The most important messages from this section are: morphine locomotor sensitization is a useful way to probe the responsivity of the reward circuitry; MSA is a clinically-relevant approach to examine the influence of morphine on brain and behavior; MSA appears to negatively influence hippocampal structure and function, but its influence on DG is poorly understood. As self-administration allows for the exploration of drug related behaviors, such as intake patterns, escalation, or how hard an animal is willing to work for the drug, it is a strong tool for later clinically-relevant studies.

Table 1-2. Comprehensive List of Morphine and Heroin Self-administration Literature through March 18,

Reference	# of subject	Drug	Dose	Pre-Training	Test Ratio	Session Length	Timepoint	Days of SA	Studies/Biological Consequences	Sex/ Breed	Infusion Vol.
(Mierzejewski et al., 2007)	32	Cocaine Then Morphine	**C -** 0.3 mg/kg/inf **M** –0.56 mg/kg/inf	N/A	FR	2 HR	During MSA	20C 40M	Cocaine has higher response rate. Animals with cocaine experience respond to lower doses of Morphine	Male Sprague-Dawley	N/A
(Singer et al., 1982)	N/A	Heroin	0.1 mg/kg	Food Training	FR	N/A	During HSA	10	Food Training and Schedules promote more robust SA	Male	N/A
(Koob et al., 1984)	20	Heroin	0.06 mg/kg/inf	Food Training	FR	3 HR	During HSA	11 months	Naltrexone is more potent than naloxone in increasing HSA at the lower doses, supporting that the opiate receptors are located in the CNS	Male Wistar	N/A
(Dai et al., 1989)	N/A	Heroin	0.03, 0.1,0.3 and 0.6 mg/kg/inf	N/A	Fixed	0.5 HR	During HSA	9	0.03 mg/kg increased in dependent rats, while not higher doses.	Male Long - Evans	N/A
(Eisch et al., 2000)	9	Heroin	60 µg/kg	Food Training	Fixed	6 HR	Immediate	26	Decreased Proliferation	Male Sprague-Dawley	0.1 ml over 5s
(Zhou et al., 2005)	50	Heroin	50 µg/kg	N/A	PR	4 HR	Immediate or 3D Extinction	14	Relapse can occur through cues: heroin-predictive discriminative stimuli or conditioned stimuli after withdrawal.	Male Sprague-Dawley	N/A
(Steffensen et al., 2006)	23	Heroin	0.06 mg/kg	N/A	FR	Until 9 infusions received	Immediate	N/A	Heroin inhibits VTA GABA neurons by acting on dendritic µ Opioid Receptors	Male Sprague-Dawley	0.1 ml

(Edwards et al., 2009)	24	Heroin	60µg/kg per 100 µL	Food Training	FR	6 HR	12 HR or 24 HR WD	18	Increase in GluR1 phosphorylation in CA1 and CA3 during WD	Male Sprague -Dawley	N/A
(Cleva et al., 2011)	N/A	Heroin	N/A	N/A	FR	3 HR	3 HR	12	Extinction increased the number of proliferating cells in the SGZ	N/A	N/A
(Cicero et al., 2003)	48	Heroin Morphine	DR	Priming	FR and PR	4 HR	During MSA	4	Females take more, are reinforced at more doses than males.	Male/ Female Sprague-Dawley	N/A
(Werner et al., 1976)	40	Morphine	0.03, 0.1 or 0.3 mg/kg/ infusion	N/A	FR	10 HR	During MSA	6	The larger the dose, the less infusions the animals took	Male Sprague -Dawley	N/A
(Weeks and Collins, 1979)	80	Morphine	.0032 mg/kg/inf - 10 mg/kg DR Curve	N/A	FR	24 HR	During MSA	7	Largest difference was seen between 0.32 mg/kg (65% dependence) and 1.0 mg/kg (91% dependence)	Female Sprague -Dawley	N/A
(Beck and O'Brien, 1980)	N/A	Morphine	1.0, 2.0 mg/kg	Food Sprinkled on Lever	FR/ Variable	12 HR	During MSA	N/A	Animals receiving shock stress were more likely to overdose. ODs occurred more in the higher doses.	Female Sprague -Dawley	N/A
(Smith et al., 1982)	33	Morphine	10 mg/kg	Forced Infusion	FR10	24 HR	Immediate	>24	The passive infusion of morphine resulted changes turnover rates of the biogenic monoamine and ammo acid neurotransmitters The contingent morphine resulted in higher utilization rates than noncontingent morphine	Male Fischer 344	N/A

(Dworkin et al., 1988)	12	Morphine	0.41 mg/inf - 3.3 mg/ inf escalating	Food Training	Fixed	24 HR	During MSA	9	Rats with lesions taking out cholinergic and GABAergic neurons in the NAc took less morphine than controls.	Male Fisher	N/A
(Grasing and Szeto, 1993)	6	Morphine	30 µg/kg	N/A	Fixed	24 HR	During MSA	84	Morphine SA at medium and high levels disrupts circadian rhythms	Male Sprague -Dawley	30 µL over 0.5s
(Sahraei et al., 1999)	32	Morphine	Escalating from 0.3 mg/kg to 3.0 mg/kg	Priming	FR	2 HR	During MSA	12	Animals had greatest # of infusions at 0.75 mg/kg. Agonists for adenosine R2 decreased SA. Agonists for R1 had no effect.	Male Sprague -Dawley	0.1 mL over 5s
(Robinson et al., 2002)	N/A	Morphine	0.5 mg/kg/inf	Food Training	FR1	2 HR	4 WK WD	13-30	However, only MSA decreased spine density in the hippocampus and only MEA decreased spine density in Par1. In the orbital frontal cortex morphine significantly increased spine density in both Groups SA and EA, although to a much greater extent in SA	Male Sprague -Dawley	0.2µL over 5s
(Kruzich et al., 2003)	24	Morphine	0.3, 1.0, 3.0 mg/kg/inf	N/A	FR	4 HR; 18 HR	24H WD	7	More escalation at 0.3 mg/kg, but consumption higher at 3.0 mg/kg. Intake was more consistent at 18 HR than at 4 HR.	Male Sprague -Dawley	0.05 ml over 1.26s
(Rodriguez Parkitna et al., 2004)	40	Morphine	0.56 mg/kg	N/A	FR	2 HR	4 HR after or 30 D WD	40	Increased gene expression during SA in cellular stress genes, Decreased expression of GABA receptors	Male Sprague-Dawley	250 µL/kg over 2s

(Alaei et al., 2005)	30	Morphine	1 mg/ml/inf	Priming Injections	FR	2 HR	During MSA	10	Ascorbic acid decreases SA and withdrawal symptoms	Male Wistar	0.2 ml over 10s
(Glass et al., 2005)	3	Morphine	0.3 mg/kg to 2.4 mg/kg (doubling every other day)	N/A	FR	18 HR	Immediate	14	Increase a GluR1 subunit in dendrites in the amygdala	Male Sprague-Dawley	N/A
(He and Grasing, 2006)	59	Morphine	3.2 mg/kg/inf	Food Training/ Priming	FR PR	17 HR	During MSA	>7	SA was decreased by Type A MAO inhibitors and Type B MAO Inhibitors	Male Wistar	N/A
(Ballesteros-Yanez et al., 2007)	12	Morphine	1 mg/kg/inf	Food Training	FR1	12 HR	Immediate	15	Reduction in the size and branching complexity of the dendrites of pyramidal cells in the motor cortex.	Male Lewis	N/A
(Sanchez-Cardoso et al., 2007)	76	Morphine	1 mg/kg	Food Training	PR	60m after last infusion	Immediate or 18D Extinction	15	LEW rats had less binding to MORs in basal conditions than F344 rats; after morphine SA, both showed increased levels of binding to MORs as compared to basal groups; these binding levels came down in each extinction group.	Male Lewis/ Fisher344	N/A
(Sudakov et al., 2007)	20	Morphine	100 µL/infusion	N/A	FR	1 HR	Immediate	2	SA, but not forced, morphine increases 5-HT, in the anterior cingulate. Both forced and SA increased DA and NE in that same area.	Male Wistar	N/A

(Yoon et al., 2007)	45	Morphine	0.1 mg/kg	Food Training	FR1	1 HR	During MSA	6	GABA receptor antagonists reduced morphine maintenance response in a dose-dependent fashion	Male Sprague-Dawley	0.1 ml over 5s
(Ballesteros-Yanez et al., 2008)	8	Morphine	1 mg/kg	Food Training	FR1	16 HR	Immediate	15	Does not the dendritic arbors or in the spine density of pyramidal neurons in either the prelimbic or motor cortex of F344 rats.	Male Fisher 344	N/A
(Biscaia et al., 2008)	41	Morphine	1 mg/kg/inf	Food Training	FR; PR	3 HR – FR 12 HR-PR	Immediate	16	Males take more morphine after cannabinoids in PR, but not FR. Females have no change in either.	Male/ Female Wistar	90-115µL over 15s
(Doherty et al., 2009)	26	Morphine	0.375 mg/kg/inf	None	FR1	1HR or 8 HR	After Extinction	18	Adult animals escalate from taking approximately 75 infusions per session on 1D to 200-250 infusions on 18D during long access.	Male Sprague-Dawley	0.625 ml over 5s
(Hosseini et al., 2009)	32	Morphine	0.5 mg/ml/inf + sucrose pellet	Food Training	FR1	2 HR	During MSA	14	Exercise morphine animals looked similar to saline controls.	Male Wistar	0.1 ml over 10s
(Sanchez-Cardoso et al., 2009)	70	Morphine	1 mg/kg/inf	Food Training	PR	12 HR /Dark Cycle	0, 3, 7, or 14 D	15	Lewis rats had a larger amount of infusions than F344 rats	Male Lewis/ Fisher	N/A
(Su et al., 2009)	24	Morphine	1 mg/kg/inf	Food Training	FR1-5	4 HR	During MSA	40	Morphine only animals escalated from taking 6mg/kg/session to 12mg/kg/session at D18. After D18, declined in total intake back to around initial levels.	Male Sprague-Dawley	N/A

(Garcia-Lecumberri et al., 2011)	63	Morphine	0.25;0.5;1;2 mg/kg/inf DR	Food Training	PR	1.2HR	During MSA	15	LEW rats took more morphine at all doses tested had greater basal impulsivity. The F344 strain showed a preference for the dose of 0.5 mg/kg.	Male Lewis/ Fischer344	N/A
(Suto et al., 2011)	92	Morphine	0.5 mg/kg/inf And DR	None	FR2 PR	4 HR	During MSA	N/A	NAC lesions reduced MSA to a greater extent than did dCPu or vCPu lesions.	Male Long-Evans	0.07–0.13 ml rate of 1.6 ml/min
(Yoon et al., 2012)	23	Morphine	0.1 mg/kg/inf	Food Training	FR1	1 HR	During MSA	14-21	Saikosaponin A may effectively suppress morphine-reinforced behavior by activating GABAB receptors	Male Sprague-Dawley	0.1 ml over 5s
(Ramshini et al., 2013)	48	Morphine	1 mg/inf	Food Training	FR1	2 HR	During MSA	10	GABAB receptor agonists reduced MSA	Male Wistar	0.2 ml over 10s
(Kong et al., 2014)	42	Morphine	0.3 mg/kg/inf	N/A	FR1	3 HR	1 or 10D WD	18	Phosphorylation of GluR1 at Ser845, increases in the NAc and amygdala from 1 to 10 days of withdrawal, and no changes in GluR1 in the hippocampal CA1 from 1 to 10 days of withdrawal.	Male Sprague-Dawley	N/A
(Le et al., 2014)	40	Morphine	0.5 mg/kg/inf	Food Training + Drug	FR	4-6 HR	1D or 7D Extinction	15	Rats that took more morphine at the beginning each session had weight gain, reduced startle reflex, and enhanced drug seeking	Male Sprague-Dawley	N/A

(Talkhoonch eh et al., 2014)	30	Morphine	0.5 mg/inf		FR	2 HR		12	Pretreatment with ascorbic acid decreases MSA	Male Wistar	0.1 ml over 10s
(Yu et al., 2014)	20	Morphine	0.5 mg/kg/inf	Food Training	FR	4 HR	During MSA	7	Rats pretreated with intermittent morphine took more than rats pretreated with continuous morphine	Male Sprague -Dawley	N/A

3. Adult Dentate Gyrus Neurogenesis and Drugs of Abuse

More than 15 years ago, the first research was published showing that indeed drugs of abuse like s.c. morphine and i.v. heroin decreased adult hippocampal neurogenesis (Eisch et al., 2000). Since then, numerous additional publications have appeared that support the hypothesis that drugs of abuse decrease or in some way alter hippocampal neurogenesis (e.g., Abrous et al., 2002, Kahn et al., 2005, Noonan et al., 2008, Kochman et al., 2009). The past 15 years have also brought advances in our understanding of the function of adult-generated hippocampal neurons. Interestingly, the deficits in hippocampal-dependent tasks seen after intake of drugs of abuse (Spain and Newsom, 1991, Ornstein et al., 2000, Sudai et al., 2011) are very similar to the deficits in hippocampal-dependent tasks seen after ablation of neurogenesis (van Praag et al., 2002, Leuner et al., 2006, Winocur et al., 2006, Wojtowicz et al., 2008, Deng et al., 2009, Ko et al., 2009). This had led several researchers to hypothesize that the drug-induced deficits in hippocampal function are caused by drug-induced deficits in hippocampal neurogenesis (Canales, 2007, Eisch et al., 2008). Below I highlight the work previously completed in the field of adult DG neurogenesis and drugs of abuse. As this is an extensive field, I will focus on work dealing with opiates, but for contrast purposes will also review studies involving psychostimulants like cocaine. While all published primary literature exploring the relationship between adult DG neurogenesis and drugs of abuse is listed in **Table 1-3**, only the work on opiates and psychostimulants will be elaborated on in the text.

Opiates and the Effects on Adult Dentate Gyrus Neurogenesis

Morphine

Many studies have examined the effects of opiates on hippocampal neurogenesis. In general, opiates decrease proliferation and neurogenesis. For example, mice or rats that received high continuous doses of morphine (via s.c. pellet for 96h in mice or 5d in rats) had decreased SGZ proliferation (Eisch et al., 2000, Fischer et al., 2008, Arguello et al., 2009). The negative effect of morphine on proliferation appears to be dose- and "dependency"-dependent. For example, there are a variety of injection paradigms in mice that do not result in physical dependence and also do not change SGZ proliferation (Fischer et al., 2008). Morphine may also slow the process of maturation of adult-generated neurons. Mice that received s.c. pellet morphine had a greater proportion of actively dividing cells and a decreased proportion of older BrdU+/DCX+ immature neurons, indicating a backlog in the developmental process (Arguello et al., 2008). Additionally, mice that received s.c. pellet morphine had an increase in activated caspase-3 immunoreactive (AC3+) cells at 24hrs, but this increase had normalized after 96 hours of exposure to morphine (Arguello et al., 2008). This indicates that cell death was transiently increased during early morphine exposure, but then stabilized. Subcutaneous morphine pellets may also alter the cell cycle by slowing S-phase through interfering with DNA synthesis. The studies mentioned above support the hypothesis that morphine affects adult hippocampal neurogenesis through both increasing cell death and altering the cell cycle.

Morphine-induced alterations in adult hippocampal neurogenesis are highly dependent on method of delivery and animal model used. While much of the s.c. pellet work in mice described above have resulted in detrimental changes to neurogenesis, another experimenter-delivered method did not lead to the same results. Chronic injections of morphine (i.p., escalating doses) in mice every 6 hours for up to 256 hours resulted in no change in proliferation (Fischer et al., 2008). Alterations based on method of delivery are also dependent on species used, as chronic morphine injections in rats (20mg/kg, i.p., twice daily for 7 days) resulted in decreased proliferation (Kahn et al., 2005).

While many studies have explored the influence of non-contingent morphine in adult hippocampal neurogenesis, no experiments have explored the influence of voluntary intake via MSA on hippocampal neurogenesis prior to my data presented in **Chapter 3.** Since morphine's negative effects are highly dependent on dose, method of administration, and animal model, it is increasing important that morphine's effects are examined under the more clinically relevant model of self-administration.

While not specifically examining morphine and adult neurogenesis, there are relevant publications that warrant mention. For example, early postnatal administration of non-contingent chronic morphine disrupts early life neurogenesis and particularly interneuron development (Traudt et al., 2012). This is in keeping with the non-contingent chronic morphine studies performed in adults mentioned above, but also suggests that the studies with adults and morphine would benefit from analysis of

interneuron number and function. In addition, studies with MOR agonist support the conclusion that MOR-induced changes in neurogenesis are ligand- and dose-dependent. For example, chronic methadone – which is used for the replacement therapy mentioned above – has mild effects on behavior (Andersen et al., 2011), but has no influence on hippocampal neurogenesis (Sankararaman et al., 2012). In addition, tapentadol, a novel central analgesic combining μ-opioid receptor (MOR) agonism with norepinephrine reuptake inhibition, also fails to alter adult neurogenesis *in vivo* (Meneghini et al., 2014). However, buprenorphine – a common analgesic that is a MOR and opioid-like receptor-1 agonist with mixed KOR agonist and antagonist activities – does decrease aspects of neurogenesis (Pettit et al., 2012). Given the increasing problem of addiction to other opiates, it will be important to see how other opiates influence neurogenesis in future studies.

Heroin

The study of heroin's effects on adult hippocampal neurogenesis is very limited in comparison to the work that has been accomplished with other opiates. While no experiments have been published examining non-contingent heroin administration and neurogenesis, two heroin self-administration studies have been published. Rats that received six hours of heroin access each night had decreased proliferation when compared to controls that received saline self-administration (Eisch et al., 2000). No studies have explored possible alterations in survival of adult-generated cells in the hippocampus after heroin. However, experiments investigating heroin's effects on the hippocampal function may provide hints into what changes may be occurring in the

hippocampus. For example, mice receiving heroin injections for 34 days had deficits in performance in hippocampal-based tasks, such the Morris water maze (Tramullas et al., 2008). Deficits in the Morris water maze could also be rescued through transplanting embryonic neural stem cells into the hippocampus (Kazma et al., 2010). This indicates that mice given chronic heroin demonstrated decreased learning and memory, as well as decreased cognitive flexibility specifically through dysfunction of the hippocampus.

Psychostimulants and the Effects on Adult Dentate Gyrus Neurogenesis: Lessons for Opiates

While the focus of this thesis is on opiates and DG neurogenesis, there is far more literature on the influence of psychostimulants on adult neurogenesis. Therefore, it is useful to briefly review this literature on psychostimulants and adult neurogenesis, and to extract hypotheses that could be applied to the question of opiates and neurogenesis.

As defined by the American Heritage Stedman's Medical Dictionary, psychostimulants are a broad category of substances that are known for elevating mood and increasing locomotor activity (Stedman, 2004). Psychostimulants include illegal drugs of abuse, such as cocaine and methamphetamine, but also legal substances that can be a part of everyday life, such as caffeine, and medically useful prescription drugs, such as methylphenidate. The exact mechanism by which psychostimulants cause their stimulatory effects varies. However, all psychostimulants result in an elevation of the neurotransmitter dopamine within the synaptic cleft (Mach et al., 1997).

Many psychostimulants – including cocaine, the amphetamines, and methylphenidate – bind to and affect a family of membrane-bound proteins known as monoamine transporters, including transporters for norepinephrine, serotonin, and dopamine (Sora et al., Riddle et al., 2005, Howell and Kimmel, 2008). All of these transporters are expressed within the DG in the hippocampus and can have potent effects on adult neurogenesis. For example, serotonin 1A knockout mice are insensitive to the neurogenic effects of antidepressants (Santarelli et al., 2003) and depletion of dopamine neurons that occurs during Parkinson's disease can lead to decreased proliferation (Hoglinger et al., 2004). The transporter most commonly affected by psychostimulants is the dopamine reuptake transporter (DAT) (Giros et al., 1996, Gorentla and Vaughan, 2005, Kahlig et al., 2006, Tilley et al., 2007, Schmitt et al., 2008, Zhu and Reith, 2008). DAT normally acts to transport dopamine back into the cell after it has been released into the synaptic cleft (Hitri et al., 1994). Psychostimulant binding to DAT results in an elevation of synaptic dopamine levels in the reward pathway, including the NAc, the PFC, and the VTA (Bardo, 1998). This elevation of synaptic dopamine is thought to be critical to the reinforcing, or addicting, effects of psychostimulants (Di Ciano et al., 1995, Giros et al., 1996). Since dopamine neurons also project to the hippocampus (Gasbarri et al., 1991, Gasbarri et al., 1994a), it is interesting to note that DAT is also expressed in several hippocampal regions, including the DG, the SGZ, and hilus (Lewis et al., 2001). Therefore, it is likely that psychostimulants (and opiates) cause an increase in synaptic DA in the hippocampus directly as well as in the reward pathway.

It is instructive to take just one of these psychostimulants – for example, cocaine – and compare what we know about how it influences adult neurogenesis to what we know about opiates and adult neurogenesis. There is a clear trend from a multitude of studies examining cocaine's effects on neurogenesis: cocaine reduces SGZ proliferation. Rats given repeated injections of cocaine have fewer proliferating SGZ cells, regardless of dose administered (7 mg/kg, 15 mg/kg, or 20 mg/kg) (Yamaguchi et al., 2004, Dominguez-Escriba et al., 2006, Andersen et al., 2007, Garcia-Fuster et al., 2010). Two studies used rats that self-administer cocaine through an i.v. catheter both showed that three weeks of cocaine access decreased proliferation in the SGZ immediately after cocaine self-administration (CSA; 0 hours of withdrawal). However, there was a disagreement in the dose required for a decrease in proliferation. In one study, a dose of 0.5 mg/kg/infusion was sufficient to decrease proliferation (Noonan et al., 2008). Another study did not see a decrease in proliferation at 0.5 mg/kg/infusion, but proliferation was decreased after a self-administered dose of 1.5 mg/kg/infusion (Sudai et al., 2011). The disagreement in the dose required could be because of differences in the experimental methods between the two studies. For example, Noonan et al. 2008 provided cocaine access to rats during the light cycle and the rats in Sudai et al. received access during the dark cycle. Future studies should take into account this potential difference in the time of day of drug availability. Cocaine has very long-lasting effects in the human addict, with relapse occurring frequently during attempted abstinence, regardless of "desire for help" or "readiness for treatment" (Siegal et al., 2002). One question is if the long-lasting effects of cocaine craving correlate with long-lasting changes in adult neurogenesis. One study has shown that proliferation

normalizes regardless of if the rat goes into withdrawal or receives continued access to cocaine for another four weeks (Noonan et al., 2008). While this finding suggests that proliferation may be unaffected in long-term addicts, one study at one timepoint is not sufficient to conclude this, and this is an area that warrants additional research.

While cocaine's inhibition of proliferation is well documented in the adult hippocampus (Dominguez-Escriba et al., 2006, Andersen et al., 2007, Noonan et al., 2008, Garcia-Fuster et al., 2010, Sudai et al., 2011), one study also found decreased proliferation in another area of neurogenesis, the subventricular zone (SVZ) (Noonan et al., 2008). Decreased proliferation in the SVZ did not result in decreased olfaction, even though these neurons project down the rostral migratory stream into the olfactory bulb and are important in olfaction (Lazarini et al., 2009). Interestingly, cocaine addicts exhibit deficits in olfaction, regardless of method of cocaine administration (Stripling and Ellinwood, 1977, Podskarbi-Fayette et al., 2005). The decrease in the proliferation of new olfactory interneurons could explain the cocaine users' loss of smell. However, in the same study 4 weeks of withdrawal led to normalization of the number of SVZ dividing cells (Noonan et al., 2008). Future research should examine whether even a temporary decrease in SVZ neurogenesis has long-lasting functional implications, such as diminished olfaction or olfactory discrimination (Imayoshi et al., 2008, Lazarini et al., 2009).

Given the ability of cocaine to decrease proliferation, it is notable that no studies have shown an effect of experimenter-delivered cocaine on the number of cells in "later"

stages of neurogenesis. For example, Dominguez-Escriba et al. examined not only DCX+ cell number, but also dendritic morphology of DCX+ cells and their mossy fiber projections to the CA3. Noncontingent cocaine injections (8 or 24 days, 20mg/kg/day i.p.) did not alter DCX+ cell number, dendritic morphology or mossy fiber projections. It also did not alter the number of "surviving" BrdU+ cells. This lack of effect on immature neurons and BrdU+ cell survival is interesting since in the same study proliferation (as assessed by BrdU and Ki67) was decreased. It remains unknown whether an even longer injection paradigm would be effective in influencing the later stages of neurogenesis.

In contrast to the lack of effects on later stages of neurogenesis with non-contingent cocaine, cocaine self-administration (CSA) has produced some interesting data in terms of survival. While the proportion of DCX+ cells did not change immediately after CSA, there was – surprisingly - an increase in the proportion of DCX+ cells in the posterior hippocampus after either four additional weeks of cocaine access or withdrawal (Noonan et al., 2008). Since this effect occurred regardless of whether cocaine taking was continued or stopped, it may represent a long-lasting neuroadaptation resulting from the CSA. The fact that long-term CSA altered DCX+ cell number, but injections did not, could be due to the difference in length of drug-exposure or the route of administration (i.v. vs. i.p.). Alternatively, it could reflect more fundamental differences between contingent and non-contingent drug studies.

It is currently unknown how cocaine negatively influences proliferation and survival. One possibility though is suggested from embryonic studies: perhaps cocaine alters key aspects of the cell division cycle (e.g. Caviness et al., 2003, Lee et al., 2008). Another possibility is that cocaine is somehow toxic to proliferating cells, leading to cell death and thus fewer proliferating cells. This is currently not supported by the literature. For example, there was no difference in the number of activated caspase-3+ cells, a marker of cell death, after three weeks of self-administered cocaine (Noonan et al., 2008). Additionally, there was no change in the number of TUNEL+ cells, another marker for cell death, after experimenter-delivered cocaine (Dominguez-Escriba et al., 2006). However, as dead cells are often cleared rapidly from brain parenchyma (Sierra et al., 2010), it is likely more timepoints and more studies are needed prior to concluding that cell death is not contributing to cocaine-induced decrease in proliferation and survival.

Recently, scientists have begun trying to tease out the role of adult hippocampal neurogenesis in addictive behaviors, specifically with cocaine. Many studies have found correlative evidence to support that manipulations or stimuli that increase neurogenesis, such as running, typically decrease drug-taking and drug-seeking (van Praag et al., 1999, Chauvet et al., 2009, Hosseini et al., 2009). Additionally, manipulations or stimuli that typically decrease adult neurogenesis, such as stress, increase drug-taking and drug-seeking (Sutton et al., 2000, Pham et al., 2003, Greenwell et al., 2009). However, there was little causative evidence to support these correlative studies until recently. In a study from our laboratory, we showed that rats

that received hippocampal-directed, image-guided X-ray irradiation to suppress adult neurogenesis took more cocaine than rats with intact neurogenesis (Noonan et al., 2010). In addition, irradiated rats were willing to work harder for cocaine in a progressive ratio experiment and responded more in a dose-response curve. This behavior was restricted to the drug reward, as irradiated rats had no differences in sucrose self-administration, a natural reward (Noonan et al., 2010). With new neurogenic drugs that increase adult neurogenesis, it is also possible to now test how increasing, rather than decreasing, DG neurogenesis may affect CSA. For example, increasing neurogenesis after CSA can decrease reinstatement of drug-seeking behavior after a priming dose of cocaine (Deschaux et al., 2014). These studies indicate that rats with suppressed neurogenesis have a higher vulnerability to cocaine addiction and that elevated DG neurogenesis may be protective against relapse.

There is one additional study worth mentioning that examined the involvement of adult neurogenesis in the ability to learn and remember a context in which they received cocaine (Brown et al., 2010). In this paradigm, known as a conditioned place preference (CPP), irradiated rats were injected with cocaine or saline and placed in a specific context for several days. Irradiated and sham rats were then given a choice of spending time in the cocaine context or the saline context. Sham rats typically spend more time in the cocaine context, indicating a preference. Rats with suppressed neurogenesis exhibited the same preference as intact rats. These data suggest that cocaine-associated learning of CPP is regulated by hippocampal neurogenesis-independent mechanisms. However, much remains to be explored in animals with

suppressed neurogenesis using the CPP paradigm. For example, it remains unknown if testing at longer or shorter timepoints after CPP training would be influenced, or if the memory of the learned drug context or extinction of cocaine-associated learning is disrupted. In addition, alternative approaches to decrease or ablate DG neurogenesis (Shors et al., 2002, Snyder et al., 2011) will be useful to tease apart the functional role of neurogenesis in cocaine CPP and addiction in general. Selectively activating or silencing newborns, through DREADDS or optogenetics, would also allow for the analysis of the function of neurogenesis at critical timepoints during CPP.

The effects of drugs of abuse on adult DG neurogenesis have been the most extensively studied in regards to cocaine, but also have been investigated for many different drugs of abuse (**Table 1-3**). My own research explores a large gap that remains within the literature. Within this dissertation I will demonstrate the effects of self-administered morphine (**Chapter 3**) and heroin (**Chapter 4**) on stages of DG neurogenesis.

4. Unanswered Questions

While much study has gone into the relationship between addiction and adult hippocampal neurogenesis, much remains to be explored. Most of the research already completed is correlative, such as exposure to different drugs of abuse can alter the proliferation and survival of new neurons in the hippocampus. Further progress within the field will hopefully lead to new therapies and treatment, allowing for better lives for those who suffer from substance abuse. However, there are still large gaps in the

Table 1-3. Adult DG Neurogenesis and Drugs of Abuse Literature. Ordered by drug (alphabetical), and then by ascending year

Reference	Drug	Model	Strain	Sex	Age/ Weight	Dose	Method of Delivery	Length of Exposure	Length of Withdrawal	Behavioral Tests	Outcomes
(**Barr et al., 2010**)	Amphetamine	Rat	Sprague-Dawley	Male	60-75D	2.5 mg/kg	IP	14D	20HR/ 4WK	Elevated Plus Maze	No effect after Short Withdrawal Decreases Proliferation after Long Withdrawal
(**Yamaguchi et al., 2005**)	Cocaine	Rat	Sprague-Dawley	Male	260-300g	20 mg/kg	IP	14D	7D	Locomotion /Stereotypy Scoring	Chronic decreases Proliferation Withdrawal normalizes Proliferation. Acute no effect
(Dominguez-Escriba et al., 2006)		Rat	Wistar	Male	250-275g	20 mg/kg	IP	8D/ 24D	None	None	Decreases Proliferation No effect on Survival or Maturation
(Andersen et al., 2007)		Rat	Wistar	Male	21D	7 mg/kg	IP	12WK	None	None	Decreases Proliferation
(Noonan et al., 2008)		Rat	Sprague-Dawley	Male	300-325g	0.5 mg/kg/inf	IV	3WK/ 7WK	4WK	Self- Admin	Decreases Proliferation No effect on Survival or Maturation CSA-WD/CSA-CONT normalizes Proliferation
(Xie et al., 2009)		Mouse	AQP4 KO	Male	60-90D	20mg/kg	IP	14D	None	None	Decreases Proliferation Withdrawal normalizes Proliferation
(**Brown et al., 2010**)		Rat	Sprague-Dawley	Male	280-300g	12 mg/kg	IP	4D	None	CPP	Acquisition unchanged after ablation
(Garcia-Fuster et al., 2010)		Rat	Sprague-Dawley	Male	225-250g	15 mg/kg	IP	7D	3D/ 14D	Locomotion to Novelty	Decreases Proliferation Decreases Survival
(Noonan et al., 2010)		Rat	Sprague-Dawley	Male	N/A	0.5 mg/kg/inf	IV	15D	4WK	None	Increases CSA Decreases extinction learning after ablation

(Lloyd et al., 2010)	Cocaine	Mouse	C57BL/6J	Male	90-120D	10 mg/kg	SC	28D	1D - 5D	None	Increases Proliferation after short withdrawal No effect on Proliferation after longer withdrawal
(**Sudai et al., 2011)**		Rat	Sprague-Dawley	Male	250-280g	0.13 mg/kg/inf 0.5 mg/kg/inf 1.5 mg/kg/inf	IV	14D	None/ 27D	Water T-Maze	Decreases Proliferation
(Deschaux et al., 2014)		Rat	Wistar	Male	280-300g	0.5 mg/kg/inf	IV	29D	None	Extinction	Decreases Proliferation Extinction normalized Proliferation
(Barr and Unterwald, 2014)		Rat	Sprague-Dawley	Male	225-250g	20 mg/kg	i.p.	3D	None	CPP	Increased c-fos+ /DCX+ in the temporal DG after drug-context exposure
(Mustroph et al., 2015)		Mouse	Nestin-TK	Male	~65D	10 mg/kg	i.p.	4D	None	CPP after Running	DG neurogenesis is not necessary for wheel running to abolish CPP
(Blanco-Calvo et al., 2014)		Rat	Wistar	Male	250g	10-20 mg/kg	i.p.	Acute/ 5D	None	Sensiti-zation	Blocking CB1 or CB2 receptors prevents both sensitization and cocaine-induced decreases in proliferation
(Han et al., 2007)	Caffeine	Rat	Sprague-Dawley	Male	280-320g	0.3g/L	Oral	4WK	None	MWM	Decreases Proliferation at 2WK and 4WK Proliferation unchanged at 1WK
(Kochman et al., 2009)		Rat	Sprague-Dawley	Male	300g	20 mg/kg	IP	12HR	None	EEG, Locomotion	Light phase decreases Proliferation. No effect on dark phase on Proliferation.

(Wentz and Magavi, 2009)		Mouse	C57/Bl6	Female	60D	10-60 mg/kg	IP	Acute/ 7D/ 26D	None/ 28D	None	High, chronic doses decreases Proliferation No effect on Survival Acute Caffeine no effect
(Sahu et al., 2013)		Rat	Sprague-Dawley	Male	250-280g	60 mg/kg	Gavage	2D	None	Sleep Deprivation	Caffeine ameliorates deficits in proliferation caused by sleep deprivation
(Kochman et al., 2009)	Caffeine/ Methamphetamine / Modafinil	Rat	Sprague-Dawley	Male	65D	1.5/ 20/ 300 mg/kg	IP	12HR	2HR/ 21D	Sleep-wake Recordings	Decreases Proliferation only during Dark Phase
(Nixon and Crews, 2002)	Ethanol	Rat	Sprague-Dawley	Male	275-330g	5 g/kg	Gavage	1D/ 4D	26D	Intoxication Scoring	Acute and chronic decreases Proliferation Chronic decreases Survival Withdrawal decreases Survival
(Herrera et al., 2003)		Rat	Sprague-Dawley	N/A	300-325g	6.4% vol	In Diet	1WK/ 7WK	None	None	Decreases Proliferation Increases Cell Death
(Crews et al., 2004)		Mouse	C57/Bl6	Male	~25g	2%-10% vol	Oral SA	8D	None	LM	Decreases Proliferation Running+Ethanonl increases Proliferation
(Crews et al., 2006)		Rat	Sprague-Dawley	Male	250-300g	5 g/kg with escalation	Gavage	4D	None	None	Decreases Proliferation Decreases Survival
(Richardson et al., 2009)		Rat	Wistar	Male	180-200g	10% solution plus alcohol vapors	Oral SA	9 WK	4WK	None	Decreases Proliferation Decreases Differentiation Increases Apoptosis
(Alen et al., 2010)		Rat	Wistar	Male	150-175g	10% vol SA/ 5 g/kg gavage	Oral SA/ Gavage	45D	5D	None	Decreases Proliferation Decreases Survival

(Taffe et al., 2010)		Rhesus Monkey	Chinese	Male	4-5 yrs	6% vol	Oral SA	11MON	2-2.5 MON	Spatial and Working Memory Tasks	Decreases Proliferation
(Anderson et al., 2012)		Rat	Sprague-Dawley	Male	>60D	4% w/v	In Diet	14D	None	Locomotion and associative learning	Decreases Proliferation
(Maynard and Leasure, 2013)		Rat	Long-Evans	Female	175 - 200g	5 g/kg	Gavage	2D	28D	Locomotion	Decreases DG Volume Decreases Total Granule Cells Running+Ethanol Increases Proliferation
(Eisch et al., 2000)	Heroin/ Morphine	Rat	Sprague-Dawley	Male	275-300g	Morphine: 10 mg/kg Heroin: 60µg/kg/ inf	Pellet, IP, IV	6D/ 30D	None	None	Morphine Pellet Decreases Proliferation HSA Decreases Proliferation
(Hernandez-Rabaza et al., 2006)	MDMA	Rat	Wistar	Male	250-275g	5 mg/kg	IP	2D	14D	None	No effect on Proliferation or Maturation Decreases Survival
(Cho et al., 2007)		Mouse	C57BL/6	Both	22-25g	1.25 mg/kg 20 mg/kg	Gavage	30D	4D	None	Decreases Proliferation
(Cho et al., 2008)		Mouse	C57BL/6	Both	75-80D	1.25 mg/kg 20 mg/kg	Gavage	16D	None	None	Decreases Proliferation in adult after developmental exposure
(**Renoir et al., 2008**)		Mouse	C57BL/6J, 5-HTT KO	Male	60D	20 mg/kg	IP	4D	4WK	FST/ TST	Decreases Proliferation
(Catlow et al., 2010)		Rat	Sprague-Dawley	Male	28-54D	1.25, 2.5, 5.0 mg/kg	IP	4D	None	CPP	Increases Proliferation at 5.0mg/kg Decreases Survival at 5.0mg/kg
(Sankararaman et al., 2012)	Methadone	Rat	Wistar	Male	210-215g	2.5, 5, 10 mg/kg	SC	Acute/ 5D/ 15D	None	LM	No Effect

(Teuchert-Noodt et al., 2000)	Methamphetamine	Gerbil	N/A	Male	90D	25 mg/kg	IP	Acute	36HR	None	Decreases Proliferation
(Dawirs and Teuchert-Noodt, 2001)		Gerbil	N/A	N/A	14D	50 mg/kg	IP	Acute	76D	OFT	Decreases Proliferation
(Tian et al., 2009)		Rat	N/A	Male	E17	300uM	Culture	1D	None	None	Increases Apoptosis
(Goncalves et al., 2010)		Mouse	C57/Bl	Male	90D	30 mg/kg	IP	Acute	1HR/ 7D	None	Increases Inflammation in the Hippocampus
(Mandyam et al., 2008)		Rat	Wistar	Male	250-300g	0.05 mg/kg/infusion	IV	29D	Intermittent	Self-Admin	Daily SA Decreases Proliferation, Maturation, Survival No effect of Intermittent SA
(Lagace et al., 2006)	Methylphenidate	Rat	Sprague-Dawley	Male	20-35D	2 mg/kg	IP	16D	None	Locomotion	No effect on Proliferation Decreased Survival
(Kahn et al., 2005)	Morphine	Rat	Sprague-Dawley	Male	240-250g	20 mg/kg	IP	7D	None, 1WK, 2WK, 4WK	None	Decreases Proliferation Normalized By Withdrawal
(Arguello et al., 2008)		Mouse	C57/Bl6	Male	N/A	25 mg/pellet	Pellet	1D/ 3D/ 4D	None	None	Decreased Proliferation Increased Apoptosis
(Fischer et al., 2008)		Mouse	C57/Bl6/J	Male	45-50D	20 mg/kg	Pellet, IP	Pellet: 24, 72, 96HR IP: 6, 52, 124, 256HR	None	Precipitated Withdrawal assessment	Morphine Pellet Decreases Proliferation. No effect on Proliferation From IP Morphine
(Arguello et al., 2009)		Mouse	C57/Bl6/J	Male	N/A	25 mg/pellet	Pellet	1D/ 3D/ 4D	None	None	Decreases Proliferation No effect on Survival or Maturation CSA-WD/CSA-CONT normalizes Proliferation

knowledge of the field when it comes to drug addiction and adult DG neurogenesis. For example, while a large amount of study of study has example how psychostimulants, such as cocaine, affect DG neurogenesis after self-administration, little research has been conducted on opiates. It remains unknown how MSA affects the different stages DG of neurogenesis. Likewise, while proliferation after HSA has been examined, little is known about its effects on differentiation or survival of newborn neurons.

Another area that requires additional study is the role of DG neurogenesis and drug-induced behaviors. Ablation of DG neurogenesis leads to an increase in cocaine-seeking and cocaine-taking, but this increase in behavior has only been documented with a single psychostimulant. It remains unknown if the increases in drug-taking are universal to any type of drug, or if it is a specific relationship between cocaine and adult neurogenesis. If ablation of adult DG neurogenesis results in similar results with another class of drugs, such as opiates, it opens the possibility that DG neurogenesis plays a role in how the hippocampus modulates the reward pathway at the circuit level.

Overview of this Dissertation

The hippocampus plays a large role in modulating the reward pathway, being especially important in craving and context-dependent relapse. One form of neuroplasticity within the hippocampus is adult neurogenesis, which occurs in the subgranular zone of the dentate gyrus. While a growing amount of literature has explored the effects of drugs of abuse on adult DG neurogenesis, the relationship between self-administered opiates and adult DG neurogenesis remains unexplored.

Additionally, few studies have investigated the functional role of adult DG neurogenesis in addiction-related behaviors. This dissertation's goal is to explore two sides of the proverbial coin: 1) to further understand the functional role of adult DG neurogenesis and 2) to investigate the consequences of long-term self-administered opiates (morphine and heroin) on the different stages of maturation of adult-generated neurons. Better understanding of opiates effects on adult DG neurogenesis and how addiction-related behaviors change in animals lacking neurogenesis may lead to better therapeutic treatments for recovering opiate abusers and may prevent addiction in patients prescribed opiates for pain.

To explore these questions, the data that result from my dissertation research is presented in the three subsequent chapters, briefly described below. This is concluded with a chapter describing conclusions, comments, and potential future directions.

Chapter 2: Dentate Gyrus Neurogenesis Ablation via Cranial Irradiation Enhances Morphine Self-administration and Sensitization.

A previous publication, on which I was a co-author (Noonan et al., 2008), showed that rats with suppressed adult DG neurogenesis are more vulnerable in an animal model of cocaine addiction. I hypothesized that this vulnerability was due to the lack of adult-generated neurons, thus resulting in an absence of hippocampal circuitry influence on the downstream reward pathway. To test this hypothesis, I suppressed DG neurogenesis and then used a separate class of drugs of abuse, opiates, to examine if the vulnerability seen with cocaine after suppressed neurogenesis was universal. I

demonstrate that after image-guided, hippocampal-focused X-ray irradiation and subsequent loss of immature DG neurons, rats show greater morphine intake and slower extinction after morphine i.v. self-administration, and increased morphine locomotor sensitization relative to sham-irradiated rats. In addition, I demonstrate that after image-guided, hippocampal-focused X-ray irradiation and subsequent loss of immature DG neurons and morphine locomotor sensitization and morphine challenge, a subregion of the DG shows a greater proportion of neurons that are active relative to sham-irradiated rats. While this **Chapter 2** underscores that suppressed neurogenesis enhances vulnerability across distinct classes of drugs of abuse, it is also novel in that it explores the role of adult DG neurogenesis in cognitive flexibility from an addiction perspective.

Chapter 3: Morphine Self-administration and Subsequent Withdrawal Do Not Change Indices of Dentate Gyrus Neurogenesis in the Adult Rat.

Previous work within the Eisch lab has explored the effects of morphine on adult DG neurogenesis using multiple experimenter-delivered administration paradigms (Eisch et al., 2000, Arguello et al., 2008, Fischer et al., 2008, Arguello et al., 2009). However, it remains unknown how self-administered morphine, a more clinically relevant model, may affect the different stages of adult neurogenesis. In **Chapter 3**, I test the hypothesis that morphine self-administration decreases adult DG neurogenesis. Interestingly, I demonstrate that adult DG neurogenesis is robustly unaffected by MSA, either immediately after or after 28 days of withdrawal. I conclude that while some methods of non-contingent administration of morphine decrease proliferation, voluntary

intake under contingent conditions has no detrimental effect on adult DG neurogenesis.

Chapter 4: Immature Neuron Density in the Adult Rat Dentate Gyrus is Unchanged by Heroin Self-administration

In the first study exploring the effects of drugs of abuse on adult DG neurogenesis (Eisch et al., 2000), Dr. Eisch demonstrated the heroin self-administration resulted in a decreased number of proliferating cells. However, there has remained a gap in the literature as to the effects of self-administered heroin on later stages of adult DG neurogenesis. In **Chapter 4**, I demonstrate that HSA does not result in altered immature neuron density and does not change granule cell layer volume. I reconcile the difference between our prior work – heroin decreases proliferating cell number – and the data in **Chapter 4** – heroin does not change the density of immature neurons or granule cell layer volume and discuss the importance of the functionality of these immature neurons.

Taken together, the data in my thesis suggest the adult DG neurogenesis is robust and normally unaffected by self-administered opiates. However, preexisting deficits in DG neurogenesis may lead to an increased vulnerability to addiction-related behaviors. In the final chapter (**Chapter 5**), I discuss potential implications of this work and future directions in which it may be taken.

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CHAPTER TWO

Dentate Gyrus Neurogenesis Ablation via Cranial Irradiation Enhances Morphine Self-administration and Sensitization

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ABSTRACT

Background: Adult dentate gyrus (DG) neurogenesis is important for hippocampal-dependent learning and memory, but the role of new neurons in learning and memory processes that contribute to addiction is unclear. To test the hypothesis that neurogenesis is involved in the vulnerability to morphine addiction, we ablated adult DG neurogenesis and examined morphine self-administration (MSA), locomotor sensitization (MLS), and DG cellular activation.

Methods: Male Sprague-Dawley rats underwent hippocampal-focused, image-guided X-ray irradiation (IRR) to eliminate new DG neurons or sham treatment (Sham). Six weeks later, rats began either MSA (Sham=16, IRR=15) or MLS (Sham=12, IRR=12). Behavioral data were collected for MSA and MLS, and cellular data (c-fos) from MLS.

Results: Over 21 days of MSA, IRR rats self-administered ~70% more morphine than Sham rats. This was not a general enhancement of learning or locomotion, as operant learning and inactive lever presses were similar in both groups. After 28 days of

withdrawal, IRR rats pressed the active lever 40% more than Sham during extinction, suggesting cognitive inflexibiity. For MLS, both IRR and Sham rats sensitized, but IRR rats sensitized faster and to a greater extent. During dose response, IRR rats were more sensitive to a lower morphine dose. IRR rats also had greater cellular activation (more c-fos+ cells) in a DG subregion sensitive to stress.

Conclusions: Along with prior studies, these data show reduced hippocampal neurogenesis confers vulnerability for multiple classes of drugs. Thus, therapeutics to specifically increase or stabilize hippocampal neurogenesis could aid in preventing initial addiction as well as future relapse.

INTRODUCTION

Opiate abuse is a major problem, with over 2 million people diagnosed with prescription opioid abuse in 2013 alone (NIDA, 2014). The study of the addicted brain has recently benefitted from a broadened focus that includes study not only of classical "reward pathway" brain regions (e.g. ventral tegmental area [VTA] to nucleus accumbens [NAc] and prefrontal cortex [PFC]) but also synaptically connected brain regions, such as the amygdala and hippocampus (Wolf, 1998, Lodge and Grace, 2006, Britt et al., 2012). The hippocampus in particular is detrimentally affected by long-term drug exposure (Pu et al., 2002, Kahn et al., 2005, Bao et al., 2007, Marie-Claire et al., 2007, Niu et al., 2009). For example, heroin users have smaller hippocampi and reduced blood flow when compared to healthy controls (Pezawas et al., 2002). Such hippocampal drug-induced plasticity may also play a causative role, as the hippocampus of abstinent drug users is active during stimuli, like cue presentation, that can induce craving and relapse (Sell et al., 2000). As the hippocampus has a potent glutamatergic influence on NAc cell firing (Totterdell and Smith, 1989, Blaha et al., 1997, Lodge and Grace, 2006, Britt et al., 2012), drug- or even experience-induced hippocampal neuroplasticity may have long-term consequences for the function of the reward pathway. Indeed, of the glutamatergic NAc afferents, projections from the hippocampus uniquely synapse in the medial NAc shell where they regulate drug-induced locomotion (Britt et al., 2012). Therefore, it is important to understand hippocampal drug-induced neuroplasticity and its subsequent influence on the reward pathway.

Adult hippocampal neurogenesis is the ongoing process of adding new glutamatergic neurons to the dentate gyrus granule cell layer (DG GCL). Interestingly, many drugs of abuse, including opiates, decrease adult neurogenesis (Eisch et al., 2000, Kahn et al., 2005, Arguello et al., 2008, Fischer et al., 2008, Arguello et al., 2009). Drug-induced alterations in DG neurogenesis may contribute to addiction and relapse (Canales, 2013). Indeed, ablation of new DG neurons decreases cognitive flexibility and extinction in spatial tasks (Snyder et al., 2005, Winocur et al., 2006, Burghardt et al., 2012, Pan et al., 2012). Directly relevant to a functional role for new neurons in addiction, reduction of new DG neurons increases vulnerability in a rat model of cocaine addiction (Noonan et al., 2010). However, it is unknown if this vulnerability generalizes from psychostimulants to other classes of drugs of abuse, such as opiates. In addition, it is unknown if this vulnerability occurs through altering the hippocampal influence on the reward pathway+ by altering drug-induced behavior or DG cellular activation.

Using a new image-guided cranial X-ray technique (Song et al., 2010), here we completely ablate DG neurogenesis to test the hypothesis that decreased neurogenesis leads to greater morphine self-administration (MSA). We also hypothesized that ablation of neurogenesis would increase morphine locomotor sensitization (MLS). Locomotor sensitization reflects enhanced sensitivity within the NAc to glutamate release (Robinson and Berridge, 1993, Kalivas et al., 2009), and thus MLS allows for a behavioral exploration of hippocampal-NAc circuit changes. As a related cellular index of circuit changes, we finally hypothesized that ablated neurogenesis would lead to greater morphine-induced DG cellular activation.

METHODS AND MATERIALS

Animals

Male Sprague-Dawley rats were ordered from Charles River (Kingston, NY) at 100g. Rats were housed in a climate-controlled environment on a 12 hours (hr) light/dark cycle (lights on 7:00 A.M.) at the University of Texas Southwestern Medical Center (UTSW). The vivarium was maintained at 72ºF with a 30%-40% humidity range. All rats were group-housed and habituated (H; **Figures 2-1A, 2-2A, 2-3A**) to vivarium conditions for at least 14 days (D) prior to irradiation. For MSA experiments, rats (n=31) were individually housed prior to food training. For MLS, rats (n=24) remained group-housed (2/cage) for the entirety of the experiment. 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 UTSW.

Drugs

Morphine sulfate was provided by NIDA (Baltimore, MD). For MSA, morphine sulfate was dissolved in 0.9% NaCl and administered as an intravenous (i.v.) 0.5 mg/kg/infusion. For MLS, morphine sulfate was dissolved in 0.9% NaCl and administered as an intraperitoneal (i.p.) 5 mg/kg injection, with doses ranging from 0.5-10mg/kg used for dose response and 5 and 10 mg/kg used for pilot data (data not shown). Sodium pentobarbital (50 mg/ml; Lundbeck, Deerfield, IL) was given as an i.p. 60 mg/kg injection. Atropine (Med-Pharmex Inc.; Pomona, CA) was given as a s.c. 1ml/kg injection. Chloral hydrate (Sigma) was given as an i.p. 400 mg/ml injection.

Cranial irradiation (IRR)

When rats (n=64) were ~45-55D old (~200g), they were either irradiated (IRR, n=28) using an image-guided cranial-directed X-ray irradiation or received sham irradiation (Sham, n=28; Song et al., 2010). IRR and Sham rats were anesthetized with sodium pentobarbital and placed in an X-RAD 320 self-contained irradiation system (Precision X-Ray; North Branford, CT). For 2 consecutive days, IRR rats were exposed to 10.63 Gy of X-ray radiation (total 21.26 Gy) focused in a 1-cm diameter circle over the hippocampus effectively sparing tissues outside the field (**Figure 2-1B-C**). Sham rats were placed in the X-RAD machine, but radiation was not delivered. After IRR or Sham treatment, rats recovered from anesthesia in a temperature-controlled environment and were monitored until fully ambulatory. A subset of rats (IRR=4; Sham=4) was sacrificed 5 weeks post-IRR to confirm the loss of adult-generated neural progenitors/immature neurons (via doublecortin-immunoreactive [DCX+] cell number, **Figure 2-1A**). The remaining rats began behavioral studies 4-5 weeks after IRR, a time when IRR-induced indices of inflammation have returned to basal levels (Wojtowicz, 2006, Noonan et al., 2010, and data not shown). MSA rats started food training 28D post-IRR (**Figure 2-2A**), and MLS rats started locomotion testing 42D post-IRR (**Figure 2-3A**), thus synchronizing the time post-IRR when MSA and MLS rats were exposed to morphine (compare **Figure 2-2A** and **Figure 2-3A**).

Food training and i.v. catheter surgery

A total of n=31 rats (IRR n=15, Sham n=16) were used for MSA experiments (**Figure 2-2A**). Twenty-eight days post-IRR or Sham, MSA rats were food restricted

(~15g of chow/D) to facilitate acquisition of operant lever pressing. Rats were then placed in a 2-lever operant chamber (Med Associates; St. Albans City, VT) where pressing of the active (left) lever delivered a sucrose pellet to a central trough (45 grams, Bio-Serv; Flemington, NJ) while pressing of the inactive (right) lever resulted in no consequence. Rats received one food training session a day for at least 3 consecutive days. All IRR and Sham rats used for MSA reached food training acquisition criterion (100 pellets/D for 3 consecutive D), and 95-100% of rats reached criterion for the first time by day 3 of training (data not shown). After food training, rats were fed *ad libitum* at least 2D prior to surgical implantation with a chronic indwelling i.v. jugular catheter (Tubing: Cole-Palmer, Vernon Hills, IL, Catalog #EW-96115-02; Cannula: Plastic One, Roanoke, VA, Catalog # C313G-5UP/SPC) as described previously (Noonan et al., 2008, Noonan et al., 2010).

Morphine self-administration (MSA)

Fourteen days after the start of food training and 7D after i.v. jugular catheter surgery (FT/S), MSA rats were placed in operant chambers for 4hr/D for 21D (**Figure 2-2A**), as previously described (Noonan et al., 2010). During MSA, rats acquired lever pressing for morphine over at least 12 consecutive days. Between acquisition and the conclusion of MSA, rats received 2 interspersed days of withdrawal to encourage heightened lever pressing. Specifically, two separate cohorts of rats were used for the experiment and data shown in **Figure 2-2**. The first cohort experienced D1-D11 of MSA, then 1D of withdrawal (WD), D12-D17 of MSA, 1D of WD, and finally D18-D21 of MSA. The second cohort experienced D1-D9 of MSA, then 1D of WD, D10-D15 of

MSA, 1D of WD, and finally D16-D21 MSA. No significant difference was found in the MSA between the 2 cohorts, so the self-administration data are combined for **Figure 2-2.** During MSA, i.v. infusions of morphine were always paired with the active/left lever, accompanied by illumination of the cue light above the left lever, delivered in a 0.1 ml volume over 5seconds (s), and followed by a 15s timeout during which the cue and house lights were off for the last 10s. With the exception of this 15s timeout period, the house lights remained on for the entire 4hr session. Rats self-administered at a fixed ratio schedule of 1. Rats were omitted from the study if catheter patency failed, and individual sessions for individual rats were discarded if technical issues occurred (e.g. clogged tubing). Subsequent to MSA, rats underwent progressive ratio (PR) as previously described (Noonan et al., 2010) using a PR9-4 schedule (1,1,1,2,2,2,3,3,4,4,5,5….) described in (Grasing et al., 2003). However, PR data are not presented here due to rats reaching satiety too swiftly on the FR1 schedule. Data are presented as reinforcements (successful lever pressed-induced infusions) per session, total morphine intake (as mg/kg) per 21D of MSA, and number of inactive lever presses per session.

Extinction testing (Ext)

After 28D of WD in the home cage, rats underwent 5D of extinction testing (Ext; **Figure 2-2A**). Rats were placed back into the self-administration chamber for 4hr/D during which no drug was available. The lever presses during the 1st hour of each session is reported here as extinction. As previously published (Noonan et al., 2010), the 2nd-4th hours of each session/D provided a distinct reinstatement environment (D1

context, D2 cue, D3-5 drug), and those data are not presented as they are the focus of another study. Half of the IRR and Sham rats underwent one 120-minute (min) locomotor session after extinction testing. Rats were killed 7D after the last extinction test.

Morphine locomotor sensitization (MLS)

A total of n=24 rats (IRR n=12, Sham n=12) were used for MLS experiments (**Figure 2-3A**). To coordinate the beginning of MLS and MSA to occur at the same time post-IRR, MLS rats recovered for 42D post-IRR or Sham treatment. For behavioral assessment in locomotor chambers (**Figure 2-3A**), rats were transported to the locomotor test room at 11am-12pm each day, and habituated to red light for 1hr before the start of the daily session. Locomotor activity was assessed in circular chambers (diameter 38", height 12", track width 10"**,** four movement detection beams placed at 90° angles). The daily 90-min locomotor sensitization session was divided into eighteen 5-min time bins. The number of beam breaks was automatically recorded by Med Associates software and totaled for each bin. Beam breaks are reported as total breaks/session. Rats were returned to homecage after the end of the daily locomotor session.

The 5 phases of MLS are described below. The dose of morphine used for MLS (5 mg/kg) was based on pilot data with 10 mg/kg, a typical dose used for rat morphine sensitization (Wolf and Jeziorski, 1993, Jeziorski and White, 1995). Indeed, while with 10 mg/kg our pilot IRR and Sham rats sensitized (showing greater number of beam

breaks after repeated daily injections of morphine), there appeared to be a ceiling effect, and IRR-MLS rats showed stereotypy sooner than Sham-MLS animals (qualitative data not known), while a differential sensitization was seen with 5 mg/kg. Therefore, all additional experiments with Sham and IRR MLS rats utilized the lower dose of 5 mg/kg in the MLS phase.

Based on previous work (Johnson and Napier, 2000), the MLS paradigm consisted of 5 phases conducted over 42D (**Figure 2-3A**). First, in the baseline phase (BL-Sal, 5D); all rats received a daily i.p. injection of 1.0 ml/kg of saline and were immediately placed in the locomotor chamber where beam breaks recorded activity for 90-min. Second, in the morphine locomotor sensitization phase (MLS, 15D); all rats received a daily i.p. injection of 5 mg/kg of morphine and were immediately placed in the locomotor chamber where beam break recorded activity for 90-min (see also SI). Third, potential conditioned-locomotor activity (Hinson and Poulos, 1981, Weiss et al., 1989) was assessed during a withdrawal phase (WD-LM, 5D). Locomotion was recorded for 90-min in the locomotor chamber, but without the stimulus of a morphine or saline injection. Fourth, rats then underwent dose-response (DR, 10D) testing. Rats were given 2D of escalating doses of i.p. morphine: 0.5, 1, 2.5, 5, and 10 mg/kg. Data were taken from the first day of each dose. The DR phase was followed by 6D of WD in the homecage (WD-HC). The fifth and final phase was the challenge phase (1D), when all rats received one morphine injection (1 mg/kg i.p.) followed by 90-min in the locomotor chamber. The challenge dose of 1 mg/kg was chosen based on the greatest change in response during DR testing. Rats were killed immediately after the challenge locomotor

session.

Tissue preparation

At the end of both the MSA and MLS experiments, rats received chloral hydrate anesthesia and subsequent intracardial perfusion with 0.1M PBS (8-min, 8 ml/min flow rate) and 4% paraformaldehyde in 0.1M PBS (20-min) as previously described (Noonan et al., 2010). After perfusion, brains were removed and postfixed in 4% paraformaldehyde in 0.1M PBS for 24hr. Brains were cryoprotected in 30% sucrose in 0.1M PBS with 0.1% NaN_3 at 4°C until coronal sectioning on a freezing microtome (Leica; Buffalo Grove, IL) at 30µm through the NAc and the entire hippocampus (4.20mm to -7.64mm from Bregma). Sections were stored in 0.1% NaN_3 in 0.1M PBS at 4°C until processed for immunohistochemistry (IHC).

IHC, histology, and quantification of immunopositive cells

IHC was performed as previously described (Noonan et al., 2010). For slide-mounted doublecortin (DCX) IHC, every 10^{th} serial section of the hippocampus was slide-mounted (Superfrost/Plus; Fisher Scientific; Waltham, MA). Slides were coded prior to IHC and the code was not broken until after microscopic and data analyses were complete. 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) were performed, and sections were placed into blocking solution for 1hr (3% serum) followed by primary antibody incubation (goat anti-DCX, Santa Cruz, 1:500) overnight at room temperature.

Sections were then incubated for 60-min with a biotinylated secondary antibody (donkey anti-goat DCX, 1:200, Santa Cruz Biotechnology; Dallas, TX) followed by incubation a 90-min with avidin-biotin (ABC Elite, 1:50; Vector Labs, Burlingame, CA). Immunoreactive (+) cells were visualized via DAB and sections stained with a counter stain (FastRed, Vector Labs).

For free-floating c-fos IHC, every 30th section was placed in a tissue culture well by an experimenter blind to treatment. Sections were quenched of endogenous peroxidases (0.1% H202, 30-min), blocked for 1hr (3% serum), and incubated with primary antibody (rabbit anti-cfos Santa Cruz, 1:2500) for 72hr at 4°C. Sections were then incubated with a biotinylated secondary antibody (donkey anti-rabbit, 1:200) followed by avidin biotin. C-fos+ cells were then visualized with DAB and then mounted onto charged glass slides prior to counterstain with FastRed.

After staining, all sections underwent rapid dehydration in ethanols and defatting in Citrosolv (Fischer Scientific) prior to coverslipping with DPX (Sigma-Aldrich; St. Louis, MO). DCX+ subgranular zone (SGZ) cells and c-fos GCL cells were counted at 400X magnification (40x/NA 0.90 lens) with an Olympus BX-51 microscope by an observer blind to experimental treatment. Using stereological principles for the quantification of rare populations of cells (Lagace et al., 2010), exhaustive counts were collected from every 10th section throughout the hippocampus for DCX+ SGZ cell counts and every 30th section for c-fos+ GCL cell counts. For DCX, cell counts were then multiplied by the fraction of the hippocampus examined and reported as total

number. All rats in this in MLS, and a subset from MSA, (from Figures 1-4) were examined for DCX IHC, although only the data from Figure 1 rats are shown. For c-fos, cell counts were multiplied by the fraction of the hippocampus examined, and then divided by the volume of the GCL. For GCL volume determination, a set of sections adjacent to those used for c-fos was processed for histological staining for cresyl violet. GCL volume was determined using the Cavalieri probe with the Stereo Investigator program (Microbrightfield; Williston, VT, Latchney et al., 2014) via an Olympus BX51 microscope with a 10X/NA 0.30 lens. Volume data are reported as volume in cubic micrometers. Density of c-fos+ cells is reported as the total number of cells divided by volume.

Statistical analysis

Data are presented as mean and standard error of the mean. Analyses were performed with GraphPad Prism (version 6.05). For DCX+ cell counts (**Figure 2-1F**), MSA daily session data (**Figure 2-2B-D, F, G**), MLS daily session data (**Figure 2-3B, E-G**), and Food training (Data not shown) analyses were performed via two-way analysis of variance (ANOVA). For MSA total intake of IRR vs. Sham, data were analyzed via two-tailed unpaired Student's t-test. Data from the MLS morphine sensitization days were also analyzed within Treatment, by Individual, and across Session via one-way ANOVA with repeated measures and a Greenhouse-Geisser correction (reported in Results text). Posthoc analyses were performed via Bonferroni (DCX, **Figure 2-1F**; MSA extinction, **Figure 2-2G**), two-tailed unpaired Student's t-test with Holm-Sidak multiple comparison correction (MSA daily sessions, **Figure 2-2B-D,F**; MLS daily

sessions, **Figure 2-3E**; MLS dose response, **Figure 2-3G**), or Dunnett's multiple comparison test with individual variance computed for each comparison of a session to the first morphine session (MLS morphine sensitization days within Treatment, by Individual, and across Session, reported in Results text). For DG GCL volume and c-fos+ cell density, data were analyzed with two-tailed Student's t-test (**Figure 2-4**). For certain data, such as the MSA daily session intake data, the rare instance of missing values due to experimental obstacles (e.g. power outage, blocked infusion syringe) led to variable degrees of freedom in ANOVA analyses. Therefore, the precise degrees of freedom for each analysis are reported in the Results, and the full table of statistics is provided in the Supplemental Information (below). For all analyses, significance was set at $p<0.05$. In figures, significance is indicated by ***$p<0.001$, **$p<0.01$, and *$p<0.05$, and trends are indicated by #<0.11.

RESULTS

Image-guided cranial irradiation produces a long-term ablation of adult DG neurogenesis

In order to explore the relationship between adult DG neurogenesis and morphine-related behaviors, we exposed rats to either IRR or Sham treatments six weeks prior to MSA or MLS (**Figure 2-2A, 2-3A**). Prior work indicated 2 consecutive days of 10 Gy irradiation leads to a complete ablation of neurogenesis in the dorsal hippocampus, with 50% suppression in the ventral hippocampus (Wojtowicz et al., 2008, Noonan et al., 2010). Here we used a novel diagnostic image-guided approach to accurately deliver the planned radiation dose to the hippocampus (Figure 2-1A-B;

Song et al., 2010). For IRR rats, a planar X-ray image was first taken to ensure the head was properly aligned and to target the specific Bregma (**Figure 2-1B**). A collimator defining the radiation field was inserted, and then a second planar X-ray image was taken to confirm the target area (**Figure 2-1C**). These diagnostic X-ray images were extremely low in dose, resulting in less than 0.03 Gy (3 cGy) in addition to their daily 10.57 Gy dose, for a total of 10.63 Gy daily. When examined 5 weeks later, IRR and Sham rats were indistinguishable based on qualitative examination of DG GCL histology, emphasizing this new X-ray irradiation technique does not lead to the gross hippocampal damage as seen in lesions studies (Hernandez-Rabaza et al., 2008). While qualitatively similar based on general histology, IRR differed from Sham rats in one notable aspect: there was a complete ablation of DCX+ immature neurons along the entire longitudinal axis of the hippocampus (**Figure 2-1D-H**) in IRR rats five weeks post-IRR (main effect of Bregma [$F_{18,108}$=6.055, p<0.0001] and Treatment [$F_{1,108}$=625.2, p<0.0001] and significant interaction [$F_{18,108}$=5.578 p<0.0001]; all post-hoc p's<0.001). Thus, this new X-Ray irradiation technique produced a more thorough ablation than previously-used X-ray irradiation techniques employed in rat. Additionally, the ablation was long lasting, as DCX+ cells were still absent 4-months post-IRR (data not shown).

Rats irradiated prior to MSA take more i.v. morphine and press the active lever more during extinction

To test the hypothesis that ablation of adult neurogenesis leads to a greater vulnerability to an animal model of morphine addiction, rats received either IRR or Sham prior to operant training and subsequent MSA (**Figure 2-2A**). All IRR and Sham rats

used for MSA reached food training acquisition criterion (100 pellets/D for 3 consecutive D), and 95-100% of rats reached criterion for the first time by day 3 of training (see SI). However during MSA, IRR rats pressed the active lever for morphine significantly more than Sham rats (**Figure 2-2B, E**). Morphine intake analyzed across sessions revealed a significant main effect of Treatment ($F_{1,586}$ =54.59, p<0.0001) and Session ($F_{22,586}$=2.381, p<0.005). This increase was particularly evident during two phases. First, IRR rats took more morphine than Sham in the first 4D of MSA when the rats were switching from self-administration of sucrose pellets to self-administration of morphine (**Figure 2-2C;** main effect of Treatment [$F_{1,117}$=15.12, p<0.0005] and Session [$F_{3,117}$=4.982, p<0.005]). Second, IRR rats took more daily morphine during the maintenance phase of self-administration when both groups began stable daily intake (**Figure 2-2D**; main effect of Treatment [$F_{1,102}$ =25.97, p<0.0001]). IRR rats also took more morphine over the length of all MSA sessions, having an almost 100 mg/kg increase in total morphine intake (**Figure 2-2E;** p<0.05). As hippocampal lesions may increase locomotor activity (Teitelbaum and Milner, 1963, Tani et al., 2001, Won et al., 2003), one explanation for greater morphine intake in IRR rats is that they may exhibit higher locomotion due to hippocampal damage. However, IRR and Sham rats had similar indices of general locomotor activity, such as inactive lever presses (**Figure 2-2F;** $F_{1,229}$=1.292, p>0.05), suggesting again that this X-Ray IRR approach does not produce gross hippocampal damage.

After 28D of WD, a subset of rats (Sham=8; IRR=8) was placed back into the self-administration chamber for extinction trials. IRR rats pressed more on their first day

of extinction vs. Sham rats (**Figure 2-2G,** main effect of Treatment [$F_{1,69}$=8.978, p<0.005] and Session [$F_{4,69}$=74.00, p<0.0001], and significant interaction [$F_{4,69}$=4.784 p<0.005]; post-hoc p<0.001). These data suggest that IRR rats – which lack DG neurogenesis – take longer to learn that the morphine is no longer available in this operant task.

Rats irradiated prior to MLS are more sensitive to the locomotor effects of repeated, daily morphine, particularly at lower doses

Locomotor sensitization is used to measure the sensitivity of the reward pathway to repeated drug exposure (Robinson and Berridge, 1993). Given that during self-administration IRR rats took more morphine and pressed the active lever more during extinction vs. Sham rats (**Figure 2-2**), we used MLS to measure IRR-induced behavioral activation of the reward pathway. Rats underwent MLS (**Figure 2-3A**), beginning with BL-Sal. Both IRR and Sham rats showed similar baseline activity, including during the first day when the locomotor context was novel (**Figure 2-3B**; $F_{1,108}$ =0.3470, p>0.05)**.** During the MLS phase, IRR and Sham rats also showed similar activity levels on their first exposure to morphine (acute exposure; **Figure 2-3C**). In addition, over the 15D of MLS, IRR and Sham rats both sensitized to 5 mg/kg morphine, having the greatest number of beam breaks on D10 (**Figure 2-3D**), and having significantly higher number of beam breaks on D15 when compared to D1 (data not shown, Sham, p<0.05; IRR, p<0.001). Qualitative observational analysis during the last 5D of the MLS phase suggested both IRR and Sham rats were sensitizing to the point of displaying stereotypical behavior. However, closer examination of the daily MLS data

revealed that IRR rats sensitized significantly more than Sham rats on the day of peak morphine-induced locomotor activity (**Figure 2-3D**), with IRR rats showing 25% more locomotion than Sham rats on D10 of exposure ($p<0.05$). Additionally, IRR rats had shorter latency to sensitization, showing significantly more morphine-induced beam breaks on D7 compared to their D1 locomotion (main effect of Session [$F_{3.912,43.04}=8.846$] and Individual [$F_{11,154}=27.71$, $p<0.0001$]; first $p<0.05$ is D7), while Sham rats took one more day to reach significance (main effect of Session [$F_{3.924,43.16}=6.217$] and Individual [$F_{11,154}=33.44$, $p<0.0001$]; first $p<0.05$ is D8). Overall, as shown in the highlighted MLS session days in **Figure 2-3E**, IRR rats have greater sensitization than Sham rats (main effect of Treatment [$F_{1,110}=6.212$, $p<0.05$] and Session [$F_{4,110}=9.073$, $p<0.0001$]).

To assess if the differences in locomotion were the result of changes in Pavlovian conditioning rather than a locomotor response to morphine (Hinson and Poulos, 1981), rats were placed back into locomotor chambers during the withdrawal phase (WD-LM). Notably, the activity of IRR rats was no longer higher than Sham rats, and both IRR and Sham rats showed activity levels similar to baseline (**Figure 2-3F**; no effect of Treatment [$F_{1,109}=0.01278$, $p>0.05$] or Session [$F_{4,109}=0.9208$, $p>0.05$]). This indicated that the increased locomotion in both IRR and Sham rats during the MLS phase relied on morphine administration, and was not contextually-conditioned locomotion. After WD-LM, a dose-response (DR) experiment was used to identify whether sensitization was enhanced in IRR rats relative to Sham rats. IRR rats showed significantly more locomotion at 1.0 mg/kg and a trend at 0.5 mg/kg vs. Sham rats

(**Figure 2-3G;** main effect of Treatment [$F_{1,109}$=5.194, p<0.05] and Dose [$F_{4,109}$ =17.69, p<0.0001])**.** IRR and Sham rats both showed a maximum locomotor response at 3.0 mg/kg, with decreasing activity at doses higher than 3.0 mg/kg, most likely due to a shift from locomotion into stereotypic behavior. As the 1.0 mg/kg dose resulted in the largest difference in locomotion during DR, we opted to this dose for a morphine challenge prior to sacrifice. However, both IRR and Sham rats showed similar locomotor response to 1.0 mg/kg on challenge day (**Figure 2-3H**; p>0.05).

Rats irradiated prior to MLS have greater density of morphine challenge-induced c-fos+ cells in the infrapyramidal blade of the DG GCL

While IRR and Sham rats showed similar locomotor response from 1.0 mg/kg during the challenge phase (**Figure 2-3H**), the heightened sensitivity of IRR rats to MLS (**Figure 2-3D,E,G**) encouraged us to hypothesize that there would be lasting anatomical and cellular imprints of irradiation and its influence on hippocampal and reward circuitry. We also hypothesized that these imprints would be only evident in either the suprapyramidal or infrapyramidal blades of the DG GCL (**Figure 2-4A**), as these DG GCL subregions are differentially sensitive to locomotion and stress, respectively (Lee et al., 2003, Snyder et al., 2011a, Hoffman et al., 2013). We first examined volume of the DG GCL and GCL subregions in IRR and Sham rats after the 1 mg/kg sensitization challenge. There was no difference in volume of the total DG GCL or the suprapyramidal blade between IRR and Sham rats (**Figure 2-4B,** p>0.05). However, the volume of the infrapyramidal blade was 13% smaller in IRR rats relative to Sham rats (**Figure 2-4B**, p<0.05). Then, as an index of cellular activation we quantified the

density of cells immunoreactive for the immediate early gene, c-fos in the total DG GCL and GCL subregions, (Figure 4C; Jessberger and Kempermann, 2003, Lee et al., 2003, Rademacher et al., 2007). C-fos+ cells density was similar between IRR and Sham rats in both the total DG and in the suprapyramidal blade subregion. However, IRR rats had 36% greater density of c-fos+ cells in the infrapyramidal blade vs. Sham rats (**Figure 2-4D,** $p<0.05$). The IRR-induced increase in c-fos+ density in the infrapyramidal blade was not due to differences in locomotion, as IRR and Sham exhibited similar number of beam breaks after morphine challenge (**Figure 2-3H**). These data show that after MLS and subsequent morphine challenge, a greater proportion of DG GCL neurons in the infrapyramidal blade are active after morphine challenge in IRR rats relative to Sham rats.

DISCUSSION

The first notable finding presented here is that image-guided, hippocampal-focused X-ray irradiation and subsequent ablation of DG neurogenesis increased vulnerability in an animal model of morphine addiction 6 weeks later. IRR rats took more morphine in MSA, and this increase was not the result of IRR-induced changes in locomotion or operant learning. Given that cranial IRR increased drug taking for both a psychostimulant (Noonan et al., 2010) and an opiate (present work) – drug classes with distinct mechanisms of action – it suggests IRR-induced ablation of DG neurogenesis may generally disrupt hippocampal contribution to reward-based behavior. This disruption may be more easily observed with drug-induced activation than with natural reward-induced activation, as IRR rats self-administer sucrose similarly to controls

(Noonan et al., 2010), but stressed mice lacking neurogenesis exhibit anhedonia in sucrose preference tests (Snyder et al., 2011b). It is logical that DG neurogenesis would influence reward circuitry activation with all types of reward, but the disruption is more obvious with drugs of abuse that activate the reward pathway beyond its normal scope.

Our second notable finding is that IRR rats took longer than Sham rats to suppress old knowledge in order to learn something new. There were two examples of this presented here, both in the MSA experiment. First, in the first 4D of MSA, IRR rats pressed the morphine-paired lever 25% more than Sham rats, presumably taking longer to learn that the previously sucrose-paired lever was now paired with morphine. Second, during extinction, IRR rats pressed the formerly morphine-paired lever 40% more than Sham rats, again presumably taking longer to learn that this lever no longer delivered morphine. One explanation for the increased lever pressing is that IRR rats have deficits in extinction learning and/or in cognitive flexibility, both hallmarks of addiction (Baldacchino et al., 2012). In support of IRR-induced extinction deficits, fear- and contextual-learning studies suggest DG neurogenesis is important in extinction (Dupret et al., 2005, Ko et al., 2009, Sahay et al., 2011), where old knowledge must be suppressed in order to learn something new, and also in cognitive flexibility, which is the ability to ignore previous information when circumstances change, and learn new information (Wiskott et al., 2006, Burghardt et al., 2012). In this regard, our study is the first to illustrate IRR-induced cognitive flexibility deficits in an animal model of addiction. It has been argued that ablation of adult neurogenesis leads to an overall deficit in

learning rather than in extinction, as some studies have seen deficits in spatial and contextual learning (Shors et al., 2002, Drapeau et al., 2007). However, we found no deficits in the initial learning of the operant task, but only deficits in extinction/cognitive flexibility. If, as our study suggests, IRR rats indeed have deficits in extinction/cognitive flexibility, this would suggest that strategies to bolster neurogenesis might accelerate extinction learning or prevent relapse. In support of this, DG neurogenesis protects against relapse in an animal model of cocaine addiction (Deschaux et al., 2014). While cognitive flexibility has been indirectly linked to hippocampal neurogenesis (Burghardt et al., 2012), the vast majority of work indicates a PFC mechanism for cognitive flexibility (Gruber et al., 2010, Blot et al., 2013, Rubin et al., 2014). Thus altered DG neurogenesis may robustly influence PFC structure and function, and future studies are warranted to explore this as-yet unknown link.

Our third notable finding presented here is that IRR rats lacking DG neurogenesis sensitize more to morphine relative to Sham rats. Of particular note, IRR and Sham rats had similar locomotion at baseline and on the first day of morphine injection (acute exposure). Locomotor sensitization is a well-known behavioral paradigm that provides insight into the long-term, drug-induced, cellular – and ultimately functional – changes in the reward pathway and related circuitry (Steketee and Kalivas, 2011). In fact, locomotor sensitization is considered to be a behavioral reflection of a hypersensitive reward pathway (Robinson and Berridge, 1993, Rademacher et al., 2007, Vanderschuren and Pierce, 2010). Additionally, the hippocampus plays a central role in locomotor sensitization. For example, sensitization will only develop in a novel

context, and exposure to the sensitized context results in more c-fos expression in the hippocampus and NAc (Badiani et al., 2000, Badiani and Robinson, 2004, Rademacher et al., 2007). Interestingly, sensitization results in more morphine-induced glutamate release in the hippocampus (Farahmandfar et al., 2011), and it is reasonable to hypothesize that this is even greater in MLS IRR rats than in MLS Sham rats. A greater IRR-induced increase in hippocampal glutamate might ultimately result in altered glutamate transmission downstream in the NAc, though this remains untested. Indeed, sensitization and subsequent WD results in less basal glutamate in the NAc (Xi et al., 2002), setting the stage for a stronger response to drug-induced hippocampal-NAc glutamate release (Reid and Berger, 1996). Alternatively, it will be also productive to explore potential alterations in hippocampal inhibitory tone that may influence the NAc. For example, recent work shows decreasing hippocampal interneuron number can strongly increase spontaneous dopaminergic firing (Perez and Lodge, 2013, Boley et al., 2014). Also, as interneurons are increasingly appreciated as potent regulators DG neurogenesis (Li et al., 2013, Song et al., 2013), more work is needed to understand the relationship between hippocampal interneurons and neurogenesis as a potential mechanism behind ablation-induced alteration of hippocampal output. In sum, while it is unclear what drives the increased and faster onset of hypersensitivity to morphine after X-ray-induced ablation of DG neurogenesis, we hypothesize it may be due to less inhibitory tone in the DG, which results in greater subsequent activation of the reward pathway. This hypothesis is ripe for testing, particularly with the development of approaches to inducibly delete or silence adult DG neurons (Saxe et al., 2006, Dhaliwal and Lagace, 2011, Snyder et al., 2011b) and inducibly activate select cell populations

the hippocampal and reward circuitry (Deisseroth, 2012, Urban and Roth, 2015).

It has been difficult to predict changes in hippocampal activity after the ablation of adult neurogenesis. While no one has assessed hippocampal output to the NAc after image-guided cranial irradiation or transgenic inducible deletion of DG neurons, ablation studies of adult neurogenesis through other methods suggest that removing these newborn neurons increases spontaneous activity within the hippocampus (Lacefield et al., 2012, Ikrar et al., 2013). Here we quantified DG GCL c-fos+ cells after MLS and morphine challenge (rather than after MSA) as Sham and IRR rats were exposed to equal amounts of morphine. Notably, Sham and IRR rats had no difference in morphine challenge-induced locomotion, which allowed us to probe for a cellular imprint of the prior enhancement in sensitization. Our key finding in this regard – a greater density of DG GCL c-fos+ cells in the infrapyramidal blade in IRR rats vs. Sham rats – is very interesting given the sensitivity of the infra- and suprapyramidal blades to distinct experiences. For example, the suprapyramidal blade shows higher c-fos expression after a spatial task (Snyder et al., 2011a), while the infrapyramidal blade shows selective c-fos activation after a stressful experience (Hoffman et al., 2013). The neuroanatomical and physiological underpinnings of the differential response of the DG GCL subregions are only partially understood (Scharfman et al., 2002, Cosgrove et al., 2010, Jinno and Kosaka, 2010). However, current literature suggests the two blades have different circuit functions, and that the infrapyramidal blade may play a greater role in activating the hippocampus, particularly under stressful conditions. In fact, a recent theory of DG neurogenesis is that new neurons buffer vulnerability to stress. For

example, rats lacking neurogenesis only exhibit increased anxiety in certain behavioral tests after chronic stress (Snyder et al., 2011b). Taking this into account, one explanation for our data is that IRR rats are more vulnerable to stress, which results in increased MSA and MLS. In support of this, morphine sensitization is increased by stress, but is prevented when blocking glutamatergic receptors (del Rosario et al., 2002). It could be that the loss of DG neurogenesis influences the reward circuitry no only via hippocampal-NAc inputs, but also through hippocampal-hypothalamic inputs. The relationship between stress and addiction is well-studied (Deroche et al., 1992, Deroche et al., 1997, Ribeiro Do Couto et al., 2006, Sinha, 2008), as is the relationship between stress and DG neurogenesis (Gass et al., 2000, Herrera et al., 2003, Warner-Schmidt and Duman, 2006, Earnheart et al., 2007, Lagace et al., 2010, Petrik et al., 2012b, Lucassen et al., 2013). However, the relationship among stress, DG neurogenesis, and addiction is unknown. While our work was not designed to specifically address this relationship, this explanation of our data is reasonable, and warrants additional testing.

A reasonable methodological concern of this study – and of all studies that utilize irradiation – is the nonspecific nature of cranial irradiation. X-ray radiation kills dividing cells and suppresses DG neurogenesis, but it also induces inflammation and can compromise neuronal synapses and dendrites, vasculature integrity, and the neurogenic niche (Wojtowicz, 2006, Raber, 2010). Certainly, our hypothesis of the influence of DG neurogenesis on the reward pathway merits testing in future studies via methods of temporary ablation (optogenetics) or silencing (DREADDS) of newborn

neurons at key points during the addiction process. However, there are three reasons why we still favor the hypothesis that the enhanced morphine reward shown here is due to suppressed DG neurogenesis. First, these experiments were performed at a time post-IRR when indices of inflammation were low or nonexistent (Noonan et al., 2010, and data not shown). Second, the dose of irradiation used here had no obvious influence on operant learning, locomotion, or other behaviors that would be sensitive to damage of hippocampal neurons or gross interference with the neurogenic niche (Present data; Wojtowicz et al., 2008). Finally, the IRR-induced enhanced MSA and MLS shown here was achieved with complete ablation of DG via image-guided irradiation, yet our prior work with IRR-induced enhanced cocaine SA was achieved with complete ablation in the dorsal but only 50% ablation in the ventral DG (Noonan et al., 2010). The similar findings with different drugs and different levels of neurogenesis minimally do not contradict the idea that neurogenesis suppression is a common denominator of these studies. Given the more selective targeting available with the image-guided cranial irradiation used here, it would be valuable to specifically assess hippocampal physiology, microanatomy, and a broader spectrum of behaviors after such directed ablation.

While limited comparison can be made between our work and those performed with other drugs of abuse and approaches to ablate neurogenesis, it remains notable that both 50% reduction (Noonan et al., 2010) and 100% ablation of ventral DG neurogenesis (present work) enhance drug reward. As the ventral hippocampus both sends afferents to the NAc and is contributes to functions closely linked to addiction –

like anxiety and response to stress – (Siegel and Tassoni, 1971, Swanson et al., 1978, Moser and Moser, 1998, Bannerman et al., 2004, O'Leary and Cryan, 2014), one might expect a more robust effect in morphine-related behaviors when more of the ventral DG neurogenesis is ablated. It is possible that there is an under-appreciated role of the dorsal DG in regulation of the reward pathway, particularly through indirect connections (e.g. via the stress axis or PFC). Indeed, recent work supports a role for dorsal hippocampal neurogenesis in some behavioral responses to antidepressants (Wu and Hen, 2014). While we hypothesize that it is the IRR-induced ablation of new neurons in the ventral DG that primarily contributes to the enhanced morphine reward shown here, this warrants testing with discrete ablation of dorsal vs. ventral DG neurogenesis (Wu and Hen, 2014).

In sum, we report that image-guided cranial X-ray IRR and subsequent ablation of DG neurogenesis increases drug intake and results in extinction/cognitive flexibility deficits in an animal model of morphine addiction (MSA), increases locomotor sensitivity to repeated morphine (MLS), and increases morphine-induced cellular activation in the infrapyramidal blade of the DG, a subregion linked to stress. These data underscore a role for DG neurogenesis in the regulation of the reward pathway and behaviors linked to drugs of abuse. While these data show reduced hippocampal neurogenesis confers vulnerability for multiple classes of drugs, therapeutics to specifically increase or stabilize hippocampal neurogenesis (Pieper et al., 2010, Petrik et al., 2012a) could aid in preventing initial addiction as well as future relapse.

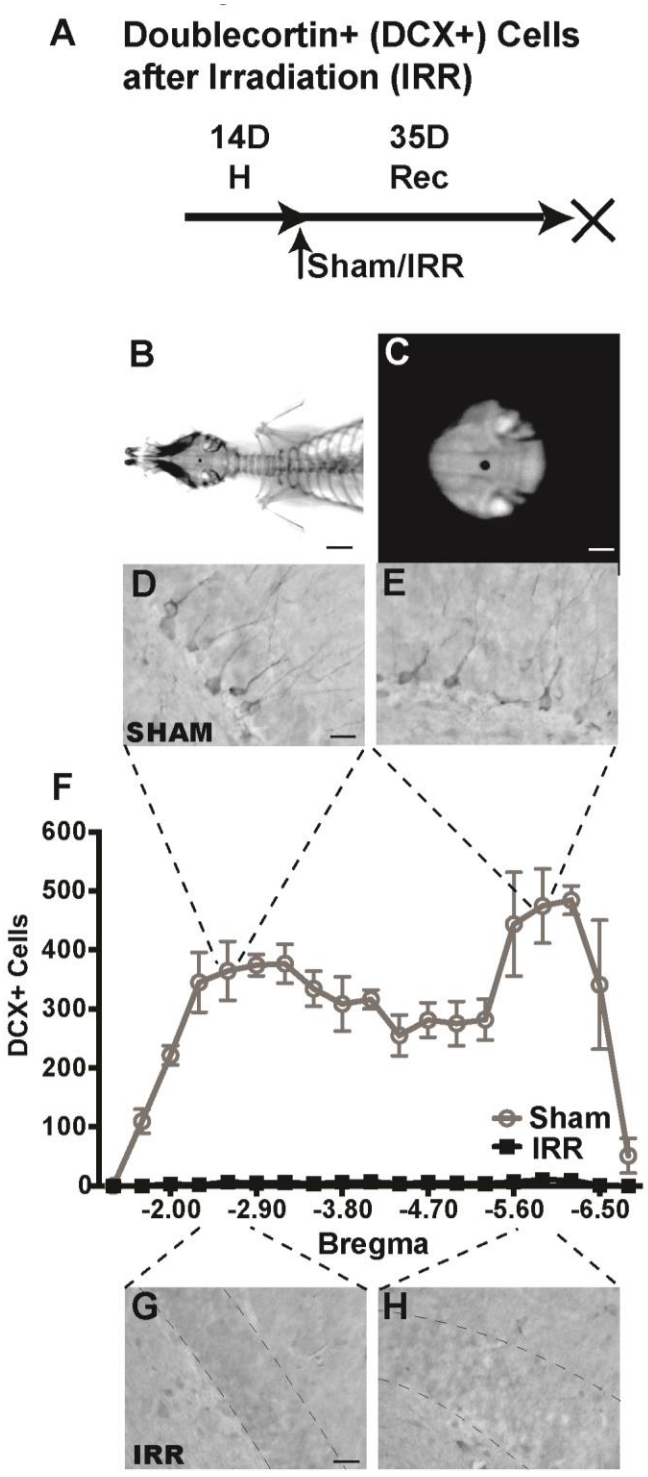

Figure 2-1. Image-guided cranial irradiation ablates adult dentate gyrus (DG) neurogenesis. **A.** Timeline for experiment. After arrival in facility, rats received 14D of group-house habituation (H). After habituation, rats were divided into Sham or IRR groups and received the appropriate treatment over 2D (see Methods). Rats were killed (X) 5 weeks later. **B.** A planar X-ray image without collimator in for proper radiation beam placement to the hippocampus. Scale bar=10mm. **C.** Higher magnification of 2nd planar X-ray image with a 10-mm circular collimator in place. Black dot indicates the center of the radiation field. Scale bar=2.5mm. **D-E.** Image of DCX+ immature neurons in the subgranular zone of the dorsal (**D**) and ventral (**E**) DG in a Sham rat. **F.** Quantification of DCX+ immature neurons along the longitudinal axis of the DG (distance from Bregma in mm) in Sham and IRR rats 5 weeks post-IRR. **G-H.** Image of DCX+ immature neurons in the subgranular zone of the dorsal (**G**) and ventral (**H**) DG in an IRR rat. Dotted outline=granule cell layer (GCL). Scale bar=10um in (**D**) applies **D-E, G-H**.

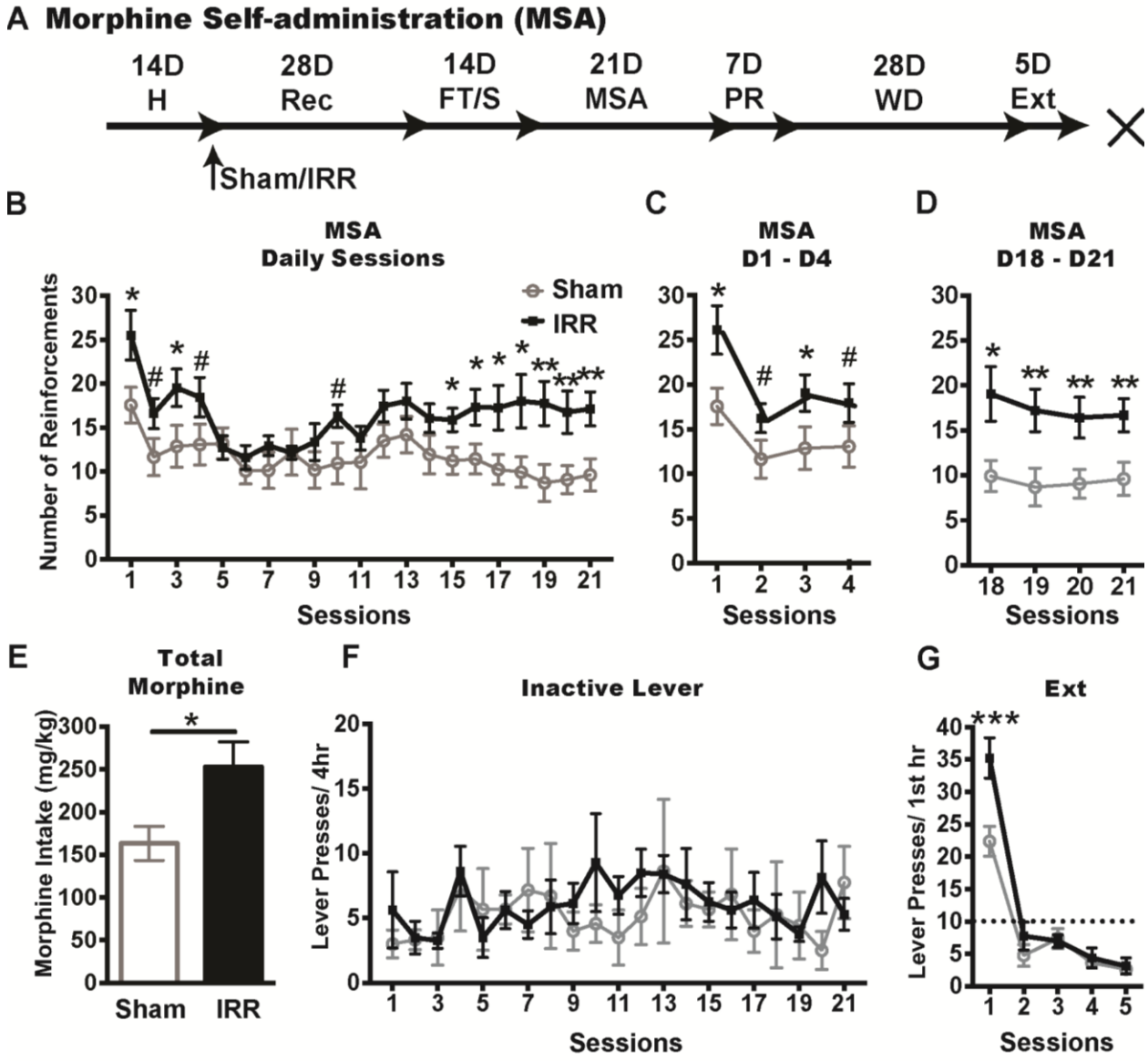

Figure 2-2. Morphine self-administration (MSA) is increased in irradiated rats.
A. Timeline for MSA. After arrival in facility, rats received 14D of group-house habituation (H). After habituation, rats were divided into Sham or IRR groups and received the appropriate treatment over two days (see Methods). Rats then underwent 28D of home cage recovery (Rec) and were then separated into individual housing. Rats received 7D of food training (FT) and 7D of I.V. catheterization surgery (S). Rats then underwent 21D of morphine self-administration (MSA), followed by 7D of progressive ratio (PR; data not shown). Rats then had 28D of home cage withdrawal (WD) followed by 5D of extinction studies (Ext). Rats were killed (X) 3D after the last extinction session. **B.** Rats that received cranial irradiation six weeks prior to MSA took more morphine. **C.** IRR rats self-administered more morphine during the first four days of self-administration (acquisition phase, D1-D4) than Sham rats. **D.** IRR rats administered more morphine during the last four days of the self-administration (maintenance phase, D18-D21) than Sham rats. **E.** Sum of all morphine taken during 21D of MSA. **F.** No changes in inactive lever presses after irradiation indicates no alterations in locomotor activity during MSA. **G.** Irradiated rats pressed more on D1 of extinction training. *p<0.05, ***p<0.001

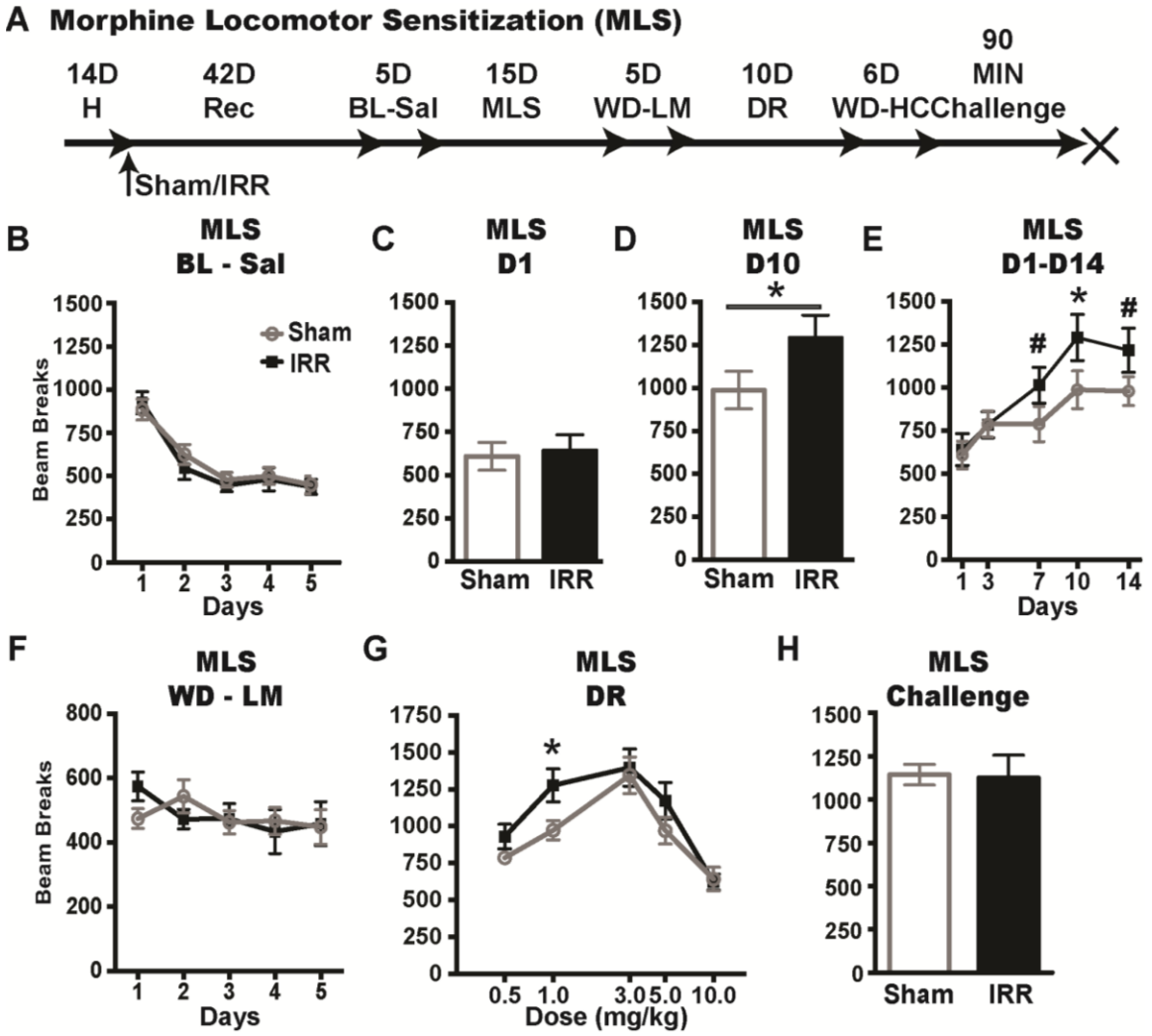

Figure 2-3. Morphine locomotor sensitization (MLS) is increased in irradiated rats. A. Timeline for MLS. After arrival in facility, rats received 14D of group-house habituation (H). After habituation, rats were divided into Sham or IRR groups and received the appropriate treatment over two days (see Methods). Rats then received six weeks of recovery (Rec) so that sensitization would start at approximately the same time as self-administration (Figure 2). After 5D of baseline locomotor incurred by saline injections (BL-Sal), rats received 15D of 5 mg/kg i.p. to measure MLS. This was followed by 5D of withdrawal with daily locomotor sessions (WD-LM), and 10D of an escalating dose response (DR). After DR, rats received 6D of withdrawal in the homecage (WD-HC). Finally, rats received a 1 mg/kg i.p. challenge dose (CHALLENGE), were placed into the locomotor chambers for 90 min, and then killed immediately afterwards (X). **B.** BL-Sal locomotion was not different between Sham and IRR rats. **C.** Morphine-induced locomotion was not different between Sham and IRR rats on D1 of morphine exposure. **D.** Morphine-induced locomotion was greater in IRR vs. Sham on D10 of morphine exposure. **E.** Both Sham and IRR rats sensitized to 5 mg/kg morphine on D10, but highlighted days of MLS presented here show that IRR rats exhibited a strong trend to more locomotion on D4 and D14 as well. **F.** Locomotor responses returned to baseline levels during WD-LM. **G.** Locomotor activity of rats during DR when they received escalating doses of morphine after sensitization. D1 of each dose is shown. **H.** No difference between IRR and Sham locomotor during the 1 mg/kg challenge. #p=0.013, *p<0.05

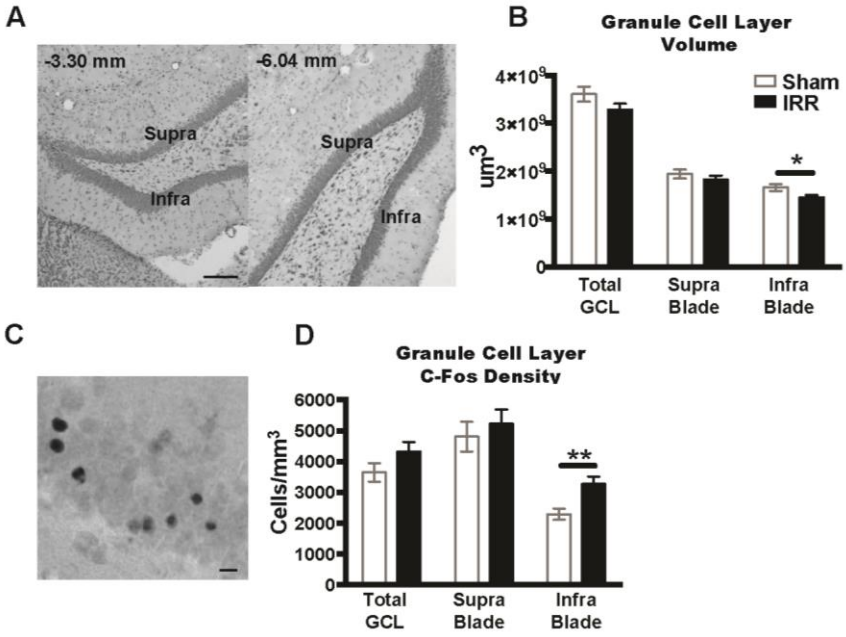

Figure 2-4. After MLS and morphine challenge, irradiated rats have greater c-fos+ cell density in the infrapyramidal blade of the dentate gyrus granule cell layer (GCL). **A.** Representative photomicrographs depicting the suprapyramidal (Supra) and infrapyramidal (Infra) blade subregions of the dentate gyrus in the anterior (-3.30mm from Bregma) and posterior (-6.04mm from Bregma) hippocampus. Scale bar=100um **B.** Cavalieri analysis reveals Sham and IRR rats have similar volume of the total GCL, and in the suprapyramidal blade subregion. The infrapyramidal blade is significantly decreased in volume in IRR rats when compared to Sham. **C.** Representative photomicrograph depicting the c-fos+ cells in the dentate gyrus GCL. Scale bar=10um **D.** Stereological quantification reveals Sham and IRR rats have similar c-fos+ cell density in the dentate gyrus GCL and suprapyramidal blade, but that IRR rats have significantly greater c-fos+ cell density in the infrapyramidal blade subregion. **p<0.01

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CHAPTER THREE

Morphine Self-administration and Subsequent Withdrawal Do Not Change Indices of Dentate Gyrus Neurogenesis in the Adult Rat

Bulin SE, Richardson DR, Latchney SE, Eisch AJ. "Dentate Gyrus Neurogenesis Ablation via Cranial Irradiation Enhances Morphine Self-administration and Sensitization". In preparation.

ABSTRACT

Opiates – including morphine – are powerful analgesics but have high abuse potential, underscoring the importance of understanding the neurobiological consequences of their extended use. Clinical and preclinical research suggests negative effects of extended opiate use, particularly for hippocampal-dependent measures, but these effects are highly dependent on the dose, duration, and route of administration. Surprisingly, it is unknown how morphine self-administration (MSA) influences a key aspect of hippocampal plasticity, adult dentate gyrus (DG) neurogenesis. Here we examined how adult DG neurogenesis was regulated by MSA relative to saline self-administration (SSA). Based on prior work with non-contingent morphine and other drugs of abuse, we hypothesized that intravenous (i.v.) self-administered morphine (three weeks, 4 hour daily sessions, average dose 8 mg/kg/day) would decrease indices of adult DG neurogenesis. Rats were injected with the S-phase marker bromodeoxyuridine (BrdU) 24 hours prior to the first day of self-administration, and were divided into either MSA or SSA for 21 days. Brains were collected either immediately after SA (MSA zero day withdrawal [MSA-0D], SSA-0D) or after SA and 28

days of withdrawal (MSA-28D, SSA-28D). Hippocampal tissue was processed for quantification of proliferating precursors (Ki67+ nuclei), neuroblast/immature neurons (DCX+ cells), and surviving granule cells (BrdU+ nuclei) in the DG. Surprisingly, MSA-0D and MSA-28D rats had similar number of proliferating precursors, neuroblast/immature neurons, and surviving granule cells relative to SSA-0D and SSA-28D, respectively. Thus, in a clinically-relevant model of morphine administration, key indices of adult DG neurogenesis are unaffected. Taken together with prior work, these data suggest that while some methods of non-contingent administration of morphine decrease proliferation, voluntary intake under contingent conditions has no overt detrimental effect on the number of cells proceeding through the process of adult DG neurogenesis.

INTRODUCTION

Opiates – including morphine – represent a double-edged sword. On one hand, they are effective and widely used for acute pain relief. In 2013 alone, over 200 million people received prescription pain relief from opioids (United Nations, 2009). On the other hand, their rewarding properties can lead to their overuse and to subsequent addiction, which is supported by the over 30 million people worldwide who abuse opiates (UNODC, World Drug Report 2012). In the effort to understand both the analgesic properties and abuse potential of opioids, clinical and basic researchers have uncovered a range of neurobiological and behavioral alterations induced by opiate exposure and subsequent abstinence or withdrawal. From these studies, the hippocampus has emerged as a brain region dynamically responsive to opiate exposure and withdrawal. For example, extended opiate exposure is marked by decreased hippocampal blood flow and size, hippocampal-dependent learning, memory, and neuroplasticity, and birth of new neurons in the dentate gyrus (DG) (Eisch et al., 2000, Pezawas et al., 2002, Kahn et al., 2005, Drake et al., 2007, Ma et al., 2007, Gu et al., 2008, Arguello et al., 2009, Lu et al., 2010). In contrast, opiate withdrawal is marked exacerbated impulsivity, withdrawal-induced anxiety, and elevated levels of corticosterone (Grasing et al., 1996, Rabbani et al., 2009, Harvey-Lewis et al., 2012, Miladi-Gorji et al., 2012), traits that involve several brain regions including the hippocampus. In fact, identification and subsequent prevention or reversal of opiate-induced hippocampal plasticity may help develop novel approaches to addiction treatment or to prevent relapse (Feltenstein and See, 2008).

While the published literature raises the possibility of long-term opiate-induced changes in cognition and hippocampal function in humans (Mintzer et al., 2010, Rapeli et al., 2011, Darke et al., 2012), closer consideration of specific studies reveals significant knowledge gaps. For example, many studies exploring the neurobiological influence of morphine reveal dose- and time-dependent effects in measures of hippocampal plasticity (Babbini and Davis, 1972, Lamb et al., 1991, Ito et al., 2001, Fischer et al., 2008). There is also a clear dependency on route of administration, as delivery via the clinically-relevant intravenous (i.v.) morphine self-administration (MSA) (Weeks, 1962, Weeks and Collins, 1979, Haney and Spealman, 2008) can result in hippocampal changes not seen after non-contingent administration of the same morphine dose (Smith et al., 1982, Robinson et al., 2002). Even within MSA studies there are time-dependent effects, with distinct hippocampal neuroadaptations seen soon after cessation of MSA vs. after withdrawal (Kong et al., 2014). This dose- and time-dependency is particularly evident in regards to the influence of morphine on adult DG neurogenesis, or the proliferation, maturation, and survival of adult-generated DG neurons. For example, subcutaneous (s.c.) morphine pellets decrease DG proliferation in both rats and mice (Eisch et al., 2000, Arguello et al., 2009), but repeated intraperitoneal (i.p.) morphine injections can either decrease neuroblast/immature neuron number (Kahn et al., 2005) but still have no effect on proliferation (Fischer et al., 2008). This difference is likely due to route of administration in these non-contingent experimental paradigms (s.c. vs. i.p.), the species studies, and/or the morphine dose employed, as these and other factors influence morphine serum levels, kinetics, and metabolism which in turn influence aspects of DG neurogenesis (Fischer et al., 2008).

These varying results raise this possibility: perhaps published opiate-induced changes in hippocampal plasticity in general and DG neurogenesis in particular are not relevant to humans since the effects in laboratory animals occur at much higher doses than those typically given to or taken by humans. However, very few studies have examined hippocampal plasticity – and none have examined DG neurogenesis – after clinically-relevant paradigms like MSA. This is important work to perform for three reasons. First, diverse drugs of abuse dysregulate DG neurogenesis (Eisch et al., 2000, Abrous et al., 2002, Nixon and Crews, 2002, Mandyam et al., 2008, Noonan et al., 2008, Arguello et al., 2009, Richardson et al., 2009), suggesting altered neurogenesis may be a common maladaptation contributing to the addicted state. Second, as ablation of new neurons decreases cognitive flexibility and extinction in spatial tasks (Snyder et al., 2005, Winocur et al., 2006, Burghardt et al., 2012, Pan et al., 2012), and as cognitive inflexibility and diminished extinction are hallmarks of addiction (Stalnaker et al., 2009, Baldacchino et al., 2012), drug-induced alterations in DG neurogenesis may contribute to addiction and relapse (Cleva et al., 2011, Mandyam and Koob, 2012, Canales, 2013). Third, drug-induced alterations in neurogenesis may be functionally important, as DG neurogenesis may be protective against relapse (Dupret et al., 2005, Noonan et al., 2010) and thus normalization of neurogenesis may represent a novel therapeutic avenue. This emphasizes that it is important to explore if morphine delivered in a clinically-relevant model like MSA can dysregulate DG neurogenesis.

Here we asked the question of how MSA influences key indices of DG neurogenesis in the adult male rat. Knowing the results would rely on morphine dose, route of administration, and withdrawal interval, we selected a moderate dose common in the MSA literature (0.5 mg/kg/infusion, i.v.; Yoon et al., 2007, Garcia-Lecumberri et al., 2011, Kong et al., 2014, Le et al., 2014, Yu et al., 2014), a 21 day self-administration period to allow acquisition and maintenance, and two timepoints of 0 and 28 day (D) withdrawal after MSA or control infusions (saline, SSA). Knowing the dynamic nature of adult DG neurogenesis, we quantified DG cells immunoreactive (+) for proliferating precursors (Ki67+ nuclei), neuroblast/immature neuron number (doublecortin [DCX+] cells), and surviving granule cells (BrdU+ nuclei, BrdU given prior to SA). Given work with other drugs of abuse and from morphine delivered in non-contingent paradigms, we hypothesized that MSA-0D would have decreased neurogenesis relative to control rats. Given the long lasting nature of addiction, the exacerbated impulsivity, anxiety, and stress that can occur after withdrawal, and the contribution of new neurons to these behaviors (Grasing et al., 1996, Snyder et al., 2011), we further hypothesized MSA-28D neurogenesis would still be decreased relative to control rats.

METHODS

Animals

32 male Sprague Dawley rats (300–325 g; Charles River Laboratories, Kingston, NY) were individually housed in a climate-controlled environment on a 12 hour (h) light/dark cycle (lights on 7:00 A.M.). Rats were habituated to vivarium conditions for at least 1 week before experimentation **(Figure 3-1A, 3-1B)**. Rats were allowed *ad libitum*

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 University of Texas Southwestern Medical Center.

Food Training

After habituation, rats were maintained on a restricted diet (~15 g of chow per day) for 3 successive days and trained to press the active (left) lever for 45 mg sucrose pellets until they reached acquisition criterion (100 pellets for 3 consecutive days). This training method is used to facilitate the SA of drug by forming the association of the left lever for a reward. Rats were weighed daily. After food training, rats were fed *ad libitum* at least 2 days before surgical catheterization **(Figure 3-1A, 3-1B)**.

Surgery

Rats were implanted with a chronic indwelling intravenous catheter (Sutton et al., 2003; Edwards et al., 2007b). Briefly, rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and atropine sulfate (0.10 mg/rat, s.c.) before surgical implantation of the catheter in the jugular vein. After surgery, rats received a prophylactic injection of gentamyicin 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. Rats were allowed to recover from surgery for 7 days before the onset of testing **(Figure 3-**

1A, 3-1B).

Self-administration apparatus

Each operant test chamber used for self-administration (Med Associates, Georgia, VT) was enclosed in a ventilated, sound-attenuating box. Boxes were equipped with a Razel (Stamford, CT) model A injection pump 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), whereas a response at the inactive lever had no programmed consequence. One square inch cubes of soft wood were placed within the chamber to alleviate opiate-induced chewing stereotypy (Bio-Serv, Flemington, NJ). Each morphine 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.

BrdU injections

Twenty-four h prior to the first SA session for morphine or saline **(Figure 2-1A, 2-1B)**, all rats received one 150 mg/kg i.p. injection of the S-phase marker BrdU

(Boehringer Mannheim, Mannheim, Germany). This dose allowed labeling of the nuclei of DG cells in S-phase, and this timing prior to SA allowed dividing cells to undergo at least one cell cycle (24h; Cameron and McKay, 2001) prior to MSA or SSA. Rats were left undisturbed after BrdU injection until SA began the following day.

Self-administration

After food training, rats were randomly divided into either the control group (SSA, 0.1ml/kg infusion) or MSA (0.5 mg/kg infusion, i.v.) **(Figure 3-1A, 3-1B)**. This moderate dose for MSA was selected based on its wide use (Yoon et al., 2007, Garcia-Lecumberri et al., 2011, Kong et al., 2014, Le et al., 2014, Yu et al., 2014) and its ability to induce the greatest intake in a dose-response curve for several rat strains (Garcia-Lecumberri et al., 2011). Morphine sulfide was generously provided by the National Institute on Drug Abuse (Baltimore, MD). 0.9% Saline was generated via mixing NaCl in mqH_2O. Saline was then filtered and autoclaved. For 20-21 days (with 2 interspersed days of withdrawal to promote escalation), rats were allowed one 4 h session per day in the operant chamber. I.v. injections were delivered on a fixed-ratio reinforcement schedule of 1 (e.g. one active lever press led to a single infusion followed by a 10 s time out). The data presented here are replicates of two experiments (SSA total n=16, MSA total n=16).

Tissue preparation

Immediately after the last session (MSA-0D and SSA-0D) or 28 days later (MSA-28D and SSA-28D; **Figure 3-1A, 3-1B**), rats were anesthetized via chloral hydrate and

killed via intracardial perfusion with 0.1 M PBS (8 min with 8 ml/min flow rate) and 4% paraformaldehyde in 0.1 M PBS (20 min). Brains were removed and postfixed in 4% paraformaldehyde in 0.1 M PBS for at least 24 h. Brains were cryoprotected in 30% sucrose in 0.1 M PBS with 0.1% NaN3 at 4°C until coronal sectioning at 30 μm through the entire hippocampus (-1.80 to -7.64 mm from Bregma; Paxinos and Watson, 1997). Sections were stored in 0.1% NaN3 in 0.1 M PBS at 4°C until processed for immunohistochemistry (IHC).

IHC

IHC was performed as described previously (Eisch et al., 2000, Mandyam et al., 2004). Every tenth section of the hippocampus was mounted on glass slides (Superfrost/Plus; Fisher Scientific, Hampton, NH). Slides were coded before IHC and code was not broken until after microscopic analysis. For BrdU IHC, three pretreatment steps were used: antigen unmasking (0.01 M citric acid, pH 6.0, 95°C, 15 min), membrane permeabilization (0.1% trypsin in 0.1 M Tris and 0.1% CaCl2, 10 min), and DNA denaturation (2 M HCl in PBS, 30 min). Pretreatment was limited to antigen unmasking for Ki-67 and DCX IHC. 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 mouse anti-BrdU (BD Biosciences, San Jose, CA; 1:200), rabbit anti-Ki-67 (Vector Laboratories, Burlingame, CA; 1:500), and goat anti-doublecortin (Santa Cruz Biotechnology, Santa Cruz, CA; 1:500). These antibodies were used to assess cells in stages of neurogenesis, including proliferating cells (Ki67+ nuclei), neuroblasts/immature neurons

(DCX+ cells), and cells that matured during the self-administration (BrdU+ nuclei).

For BrdU or Ki-67 single labeling, primary incubation was followed by incubation in a biotinylated secondary (goat anti-mouse [Sigma, St. Louis, MO] or goat anti-rabbit [Vector Laboratories], 1:200), followed by amplification using avidin-biotin, 10-15 minutes of diaminobenzidine for visualization (Pierce, Rockford, IL), and counterstaining with Fast red (Vector Laboratories). Sections underwent dehydration in ethanols and defatting in Citrosolv (Fisher Scientific) before coverslipping with DPX (Sigma).

Quantification of immunoreactive (+) cells

BrdU+ and Ki-67+ nuclei and DCX+ cell counts were performed at 400x magnification with an Olympus (Tokyo, Japan) BX-51 microscope while continually adjusting the focal plane through the depth of the section. An observer blind to treatment group performed quantification. All counts were collected using the optical fractionator method. Briefly, exhaustive counts were collected from every tenth hippocampal section throughout the anterior–posterior extent of the DG (-1.80 to -7.64 mm from Bregma; Paxinos and Watson, 1997). Resulting counts were multiplied by the fraction of the hippocampus examined (10) (Eisch et al., 2000, Lagace et al., 2006) and are reported as either as total number of cells in the subgranular zone (SGZ) of the DG for Ki67 and DCX, or as total number of cells in the granule cell layer (GCL) of the DG for BrdU.

Statistics

All analyses were performed with GraphPad Prism (version 6.0). Data are presented as mean and standard error of the mean. MSA behavioral data and comparison of immunoreactive nuclei or cells across age groups (0D vs. 28D) was analyzed via two-way analysis of variance (ANOVA). Posthoc analyses were performed using the Sidak method. Nuclear/cellular counts within age group (0D or 28D) were analyzed via a Student's t-test. For all analyses, significance was set at $p<0.05$.

RESULTS

Rats self-administer i.v. morphine, but not i.v. saline, and will do so consistently for up to 3 weeks.

To explore the effects of MSA on adult DG neurogenesis, adult rats underwent MSA or SSA for 21 days **(Figure 3-1A, 3-1B)**. Prior to jugular catheterization and SA, all rats were trained in the use of the operant chamber through using sucrose pellets as the reward for pressing the active (left) lever. As there were no differences among rats in the speed of learning this operant task of pressing for sucrose pellets, rats were then randomly assigned to either the SSA or MSA group.

Both MSA-0D and MSA-28D rats self-administered and established stable intake of morphine, as indicated by number of reinforcements over the daily SA sessions (**Figure 3-2A, 3-2B**). Morphine intake for MSA-0D analyzed across daily sessions revealed a significant main effect of Treatment ($F_{1,235}=254.7$, $p<0.0001$) and Session ($F_{21,235}=1.802$, $p<0.05$), with no interaction. MSA-28D analyzed across sessions also

revealed a significant main effect of Treatment ($F_{1,285}$ =131.5, p<0.0001) and Session ($F_{19,258}$ = 10.20, p<0.0001), with no interaction. In contrast, after the first day, SSA-0D and SSA-28D rats pressed very little for saline (<5 daily reinforcements) compared to MSA rats (10-25 daily reinforcements). This indicates that morphine groups for both time points were self-administering over a non-rewarding baseline. While there was a visual trend for the MSA-28D rats to take less morphine than MSA-0D (compare **Figure 3-2B with 3-2A)**, there was no significant difference in the amount of total morphine intake over 20 days between MSA-0D and MSA-28D (**Figure 3-2C**, p>0.05). Since MSA-0D is 21 and MSA-28-D is 20 days long, is Figure 3-2D just comparing the first 20 days of each? From eyeballing the area under the curve in a and B I would have expected C to look more different, so just want to make sure.

Increased activity levels can increase the number of new neurons (van Praag et al., 1999, Naylor et al., 2008, Wojtowicz et al., 2008). Since MSA-0D and MSA-28D press the active lever more than their SSA-0D and SSA-28D counterparts, we also recorded number of inactive lever presses during each session. The inactive (right) lever is not paired to any reward and results in no consequence, and thus can be used as an indicator of activity during an SA session. Analysis across sessions revealed more inactive lever presses in MSA-0D vs. SSA-0D. As morphine can induce a locomotor response at some doses, this was not surprising (Johnson and Napier, 2000, Rademacher et al., 2007). However, post-hoc analysis did not reveal any specific day that was different between MSA-0D and SSA-0D (**Figure 3-2D**, two-way ANOVA, Treatment [$F_{1,213}$=9.049 p<0.05], Session [$F_{21,213}$=0.5147, p>0.05]). Inactive lever

presses were not different between MSA-28D and SSA-28D (**Figure 3-2E**, two-way ANOVA, p>0.05).

Number of proliferating precursors, neuroblasts/immature neurons, surviving granule cells are unchanged after morphine self-administration and withdrawal when compared to controls.

To assess cells in stages of DG neurogenesis after MSA and withdrawal, we quantified the number of proliferating precursors (Ki67+ nuclei), neuroblast/immature neurons (DCX+ cells), and surviving granule cells (BrdU+ nuclei) immediately after MSA or SSA, or after 28 days of withdrawal. Proliferating precursors (Ki67+ cells) were clearly evident in the SGZ of all rats, presenting the typical clusters of densely labeled nuclei slight less than 10um in diameter (**Figure 3-3A**). Stereological quantification showed Ki67+ nuclei number was similar between SSA-0D and MSA-0D, and between SSA-28D and MSA-28D both when graphed as total DG number (**Figure 3-3B, 3-3C**) and as distance from Bregma (data not shown, p's>0.05). Ki67+ nuclei cluster number was also unchanged (data not shown). DCX+ neuroblasts/immature neurons were also evident in the SGZ of all rats (**Figure 3-4A**), presenting their characteristic soma size and morphology (which ranged from 8-10um, and from irregular to tear-drop shaped), with many cells extending processes and dendrites into and even through the DG granule cell layer (GCL). Stereological quantification showed DCX+ cell number was similar between SSA-0D and MSA-0D, and between SSA-28D and MSA-28D both when graphed as total DG number (**Figure 3-4B, 3-4C**) and as distance from Bregma (data not shown, p's>0.05). Finally, BrdU+ nuclei were evident in the GCL of all rats (**Figure 3-5A**). As BrdU was injected at baseline 24 h prior to MSA or SSA, BrdU+

nuclei number reflect the influence of MSA or SSA on the differentiation and maturation of cells “born” prior to the start of SA. Indeed, the BrdU+ nuclei in both 0D and 28D groups presented characteristics of surviving cells: nuclei with round morphology and a range of labeling (punctate to solid) putatively indicative of maturational state (Donovan et al., 2006). Stereological quantification showed BrdU+ nuclei number was similar between SSA-0D and MSA-0D, and between SSA-28D and MSA-28D (**Figure 3-5B, 3-5C**) and as distance from Bregma (data not shown, p’s>0.05).

DISCUSSION

The major finding in this study is that MSA – a clinically relevant model of morphine administration – does not alter key indices of adult DG neurogenesis. After 3 weeks of MSA, the number of proliferating cells (Ki67+ nuclei), neuroblasts/immature neurons (DCX+ cells), and surviving GCL cells (BrdU+ nuclei) are the same as control rats. These findings are consistent at two separate timepoints: both immediately after 3 weeks of MSA and after 3 weeks of MSA and 28 days of withdrawal.

It is notable that MSA does not decrease the number of cells in either the early (proliferating) or intermediate (differentiating) stages of neurogenesis, as this is in contrast to work in non-contingent morphine delivery. For example, s.c. pellets in mice and rats (Eisch et al., 2000, Arguello et al., 2008, Arguello et al., 2009) or chronic, high dose injections in rats (Kahn et al., 2005) decrease cell number in these stages of neurogenesis. However, it is proposed that the opiate-induced decrease in these stages of neurogenesis requires consistently high levels of morphine within the blood

(Arguello et al., 2008, Fischer et al., 2008), which may be achieved with s.c. pellets, minipumps, or high dose injections but not with lower dose injections or with the MSA paradigm employed here (4h/day, moderate dose, FR1). Thus, our negative data presented here support the hypothesis that opiate-induced deficits in proliferation requires higher doses than rats are willing to self-administer in this paradigm. On a related note, the opiate-withdrawal induced elevation of proliferation seen after non-contingent morphine administration (Kahn et al., 2005) is not seen in our MSA-28D withdrawal group. This further fuels our hypothesis that non-contingent chronic morphine results in a more dependent, tolerant state with greater withdrawal-induced compensation than can be achieved by voluntary rat MSA. However, we cannot exclude an alternative hypothesis that a shorter withdrawal interval – perhaps 7D instead of 28D of withdrawal– would have revealed a similar withdrawal-induced elevation in proliferation ere would be increased proliferation (Kahn et al., 2005). This hypothesis could be tested by examination of additional withdrawal timepoints, but also could be tested via analysis of phenotypic and morphological characteristics of cells in the current study. For example, morphological and phenotypica analysis of DCX+ cells (Plumpe et al., 2006) or BrdU+ nuclei (Donovan et al., 2006) would provide finer temporal detail on the influence of MSA on the complex kinetics of DG neurogenesis.

In is interesting that in rats heroin self-administration (HSA) decreases proliferation (Eisch et al., 2000), but MSA does not (present study). There are several possible explanations for the distinct results these two studies. First, HSA was performed during the rat's dark cycle (or active phase), while MSA was completed

during the light cycle (or inactive phase). Rats commonly take more drug during the dark cycle, as this is the natural active phase of a nocturnal animal. This may also be the reason that escalation was seen in this previous HSA study (Eisch et al., 2000), while the MSA rats demonstrated stable daily intake with no signs of escalation. Escalation is also an indication of becoming "addicted" (Deroche-Gamonet et al., 2004), and thus it is possible that adult DG neurogenesis is unaltered in "non-addicted" animals, but results only in deficits when animals reach an "addictive" state or demonstrate tolerance and dependence (Fischer et al., 2008). Future work may attempt active phase MSA with a long-access paradigm, promoting higher drug intake and escalation (Ahmed et al., 2000).

A second consideration of the differences seen between HSA and MSA is that the kinetics of heroin and morphine are different. Heroin has an initial short half-life of less than five minutes before it is metabolized, compared to 120 minutes for morphine (Catlin, 1977, Inturrisi et al., 1984). However, heroin then metabolizes into morphine, resulting in a "double hit" to mu opioid receptors, resulting in a different response from the receptor. For example, HSA can actually increase mu receptor binding in the hippocampus, while desensitizing receptors in other brain regions (Sim-Selley et al., 2000). In contrast, morphine is very poor at causing mu receptor desensitization (Blanchet and Luscher, 2002) or endocytosis (Keith et al., 1996). Taken together, while it is tempting to compare results from HSA and MSA studies – since they are both classified as opiates – differences in their pharmacological actions can result in very different effects throughout the brain and the hippocampus.

Other major and quite novel findings of this study are that the number of neuroblasts/immature neuron and surviving neurons (labeled prior to MSA, but matured during MSA) were unaffected by MSA either immediately or after 28 days of withdrawal. This is the first study to track the survival of proliferating cells throughout chronic morphine exposure. There have been mixed results about whether non-contingent morphine alters the number of immature or surviving neurons immediately after or after withdrawal (Eisch et al., 2000, Kahn et al., 2005, Arguello et al., 2008). Interestingly, previous studies using s.c. pellets suggest that morphine slows the maturation process, leading to no change in the number of immature neurons, but a greater proportion of younger DCX+ cells (Arguello et al., 2008), and that the survival of adult-generated neurons in the SGZ – but not in the hilus – may be diminished by chronic exposure to morphine (Eisch et al., 2000). Our negative DCX and BrdU findings support the concept that opiate-induced influence on cell differentiation/maturation is dose-dependent. One possibility is that MSA slowed the process of maturation as well, leading to immature neurons expressing DCX for longer. While this is unlikely (as both proliferation and survival are unchanged in the present study, and thus delayed maturation would have resulted in an increase in DCX+ number), this possibility can be assessed with morphological/phenotypic analysis of DCX+ cells in the current study.

Since proliferation and survival are unchanged at MSA-0D, it is logical to assume that DCX+ number would be unchanged after 28D of withdrawal. However, it is interesting to compare the withdrawal from MSA to other previous studies. For example, previous self-administration studies involving another drug of abuse, cocaine,

found increases in DCX+ cell density in the ventral hippocampus after 28D of withdrawal even with decreases in proliferation immediately after exposure (Noonan et al., 2008). Also, cessation of non-contingent morphine administration also led to enhanced proliferation and normalized immature neuron number in rats (Kahn et al., 2005). Our findings suggest that adult DG immature neuron number is robust enough to be unaffected by this paradigm of MSA.

While we found no deficits in adult DG neurogenesis after MSA, it is interesting to consider the functional role of DG neurogenesis. Increasing evidence suggests DG neurogenesis is protective against addiction-related behaviors (Noonan et al., 2010, Mandyam and Koob, 2012, Deschaux et al., 2014). No studies have yet to inducibly increase DG neurogenesis and examine deficits caused by addiction. However, it is also well known that global stimulation of activity and brain function, as seen after chronic wheel running, increases DG neurogenesis (van Praag et al., 1999, Farmer et al., 2004, van Praag et al., 2005, Uda et al., 2006, Clark et al., 2008, Naylor et al., 2008, Wojtowicz et al., 2008), and that running is proposed to be an effective adjunct treatment for addiction (need ref here). Interestingly, many long-term behavioral effects of morphine can be reversed via running in animal models. For example, rats that have access to voluntary running wheels for several weeks before chronic morphine exposure do not have spatial learning and memory deficits (Miladi Gorji et al., 2008), have improved conditioned place preference (Eisenstein and Holmes, 2007), have improved passive-avoidance memory (Saadipour et al., 2009), and lower morphine-induced anxiety (Miladi-Gorji et al., 2012). While no one has directly looked at morphine

exposure, wheel running, and adult DG neurogenesis, it would be interesting to explore the role of adult DG neurogenesis as a mechanism behind how chronic aerobic exercise ameliorates the behavioral effects of chronic morphine exposure.

When designing this experiment, we spent significant time considering which morphine dose, paradigm, and duration of SA to use. Ideally, we would have utilized a paradigm and dose known to produce cognitive dysfunction or other lasting neurobiological or behavioral consequences. However, the MSA literature has not identified such paradigms or doses yet. Certainly, MSA results in neuroplastic changes within the hippocampus and other brains areas by altering GluR1 subunit number and phosphorylation (Glass et al., 2005, Kong et al., 2014). MSA also alters circadian rhythms and decreased weight gain, indicating disruption of other brain functions (Grasing et al., 2003, Le et al., 2014). Certainly many studies with non-contingent morphine have identified negative effects on learning and memory (Spain and Newsom, 1991, Gu et al., 2008, Miladi Gorji et al., 2008) and anxiety (Grasing et al., 1996), but it is unknown how relevant these studies are towards MSA. Clearly, data are lacking on the influence of voluntary, long-term intake of morphine on cognition and behavior. This underscores a major knowledge gap on the effects of MSA on cognition, learning and memory, and anxiety that warrants intense exploration in the near future.

In conclusion, the MSA paradigm used here did not alter the number of cells in key stages of adult DG neurogenesis. This is a promising result, suggesting that short-term chronic i.v. morphine (<3 weeks) will grossly influence neurogenesis in mammalian

and human subjects. While the present work are a notable start, neurogenesis is far more than just the number of cells in each stage of maturation, and future work is needed to assess functional characteristics of these cells (such as synaptic integration, morphological maturation). This angle of analysis may be particularly useful, given that new neurons mature over time and become integrated into hippocampal circuitry, and that addiction is marked by persistence of drug-seeking even after long period of withdrawal (Grimm et al., 2001, Madsen et al., 2012). Thus, if we can deepen our understanding if and how new neurons are altered by MSA, we may shed light both on the process of adult neurogenesis but also on new therapeutics to treat addiction and prevent relapse.

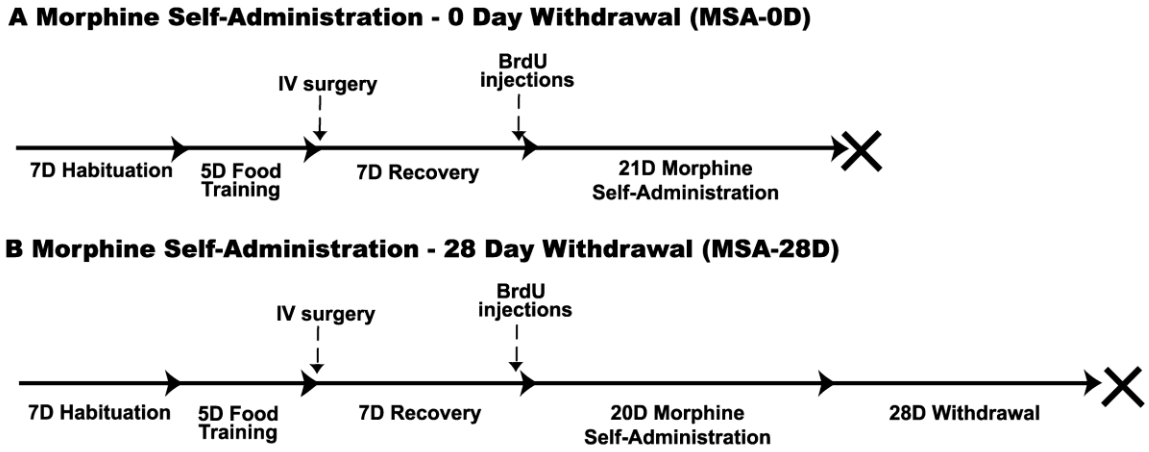

Figure 3-1. Morphine self-administration (MSA) timelines. After arrival in the facility, rats received 7 days (D) of habituation. After habituation, all rats 3-5D of food training, followed by I.V. catheterization surgery. Rats were allowed to recover for 7D. 24 h prior to the first self-administration session, they received one 150 mg/kg BrdU injection. Rats were then divided into two groups: morphine self-administration (MSA) or saline self-administration (SSA) and underwent 20-21 sessions. Rats were either sacrificed immediately after the last session (**A**) or after 28D of withdrawal (**B**).

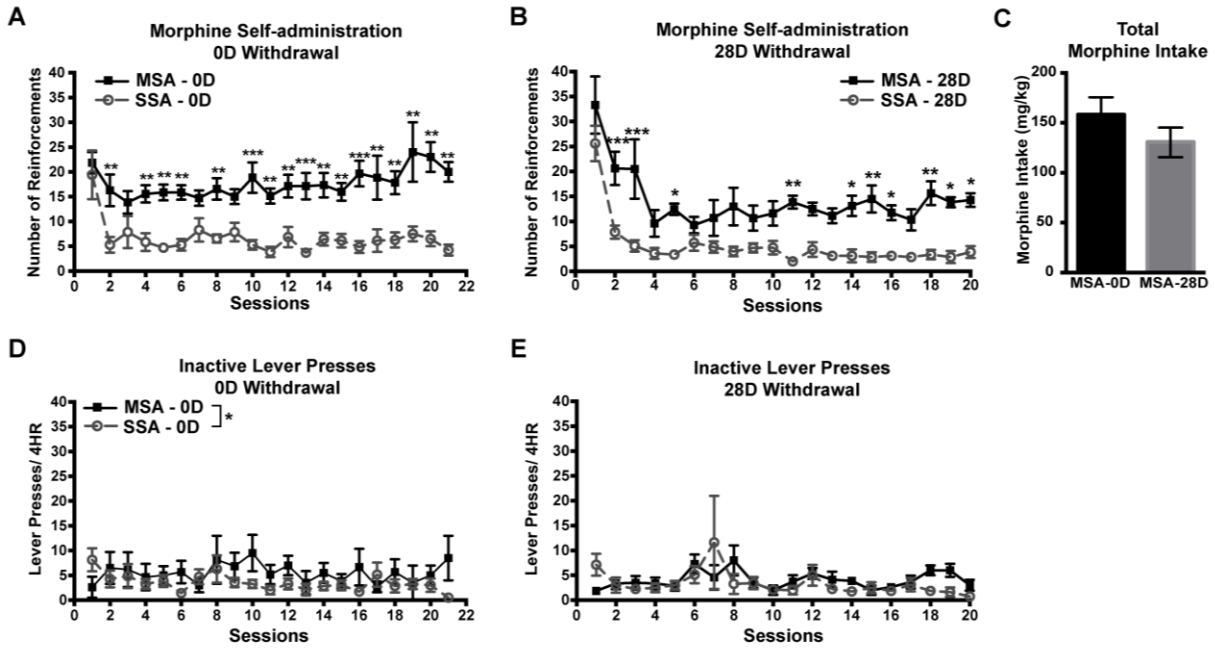

Figure 3-2. Rats self-administer i.v. morphine, but not i.v. saline, and will do so consistently for up to 3 weeks. A, B. Number of reinforcements (active lever presses that resulted in infusion of morphine or saline) per daily 4 h self-administration session in **(A)** MSA-0D and SSA-0D rats and **(B)** MSA-28D and SSA-28D rats. **C.** Total morphine intake for 20 days of MSA-0D and MSA-28D rats. **D, E.** Number of inactive lever presses per daily 4 h self-administration session in **(D)** MSA-0D and SSA-0D rats and **(E)** MSA-28D and SSA-28D rats.

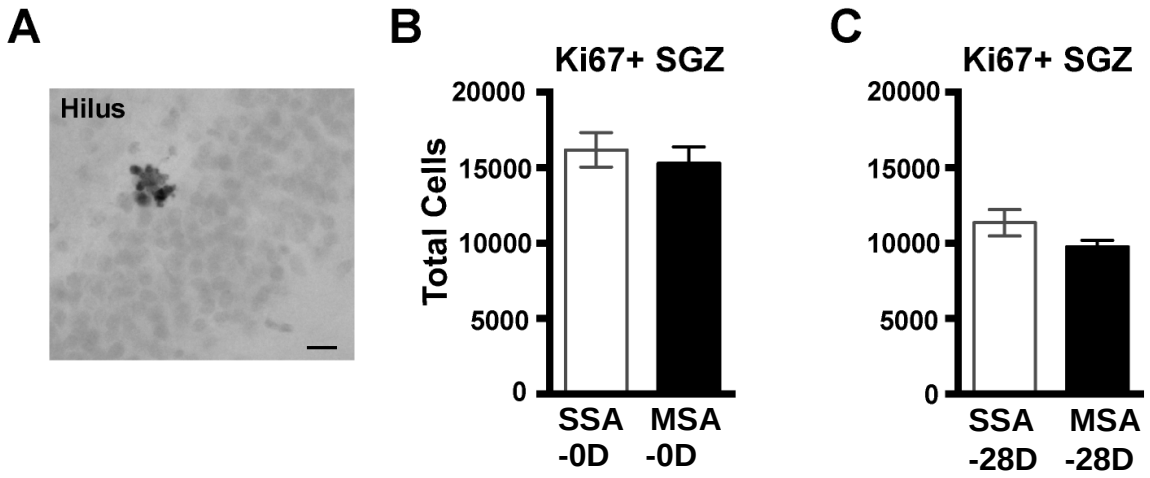

Figure 3-3. The number of proliferating precursors in the dentate gyrus subgranular zone (DG SGZ) is unchanged after morphine self-administration and withdrawal compared to controls. A. Representative photomicrograph of a cluster of Ki67+ nuclei from a control rat. Scale bar=50um **B-C.** Quantification of proliferating nuclei (Ki67+) in the DG SGZ either immediately after MSA **(B)** or after 28 days of withdrawal **(C).**

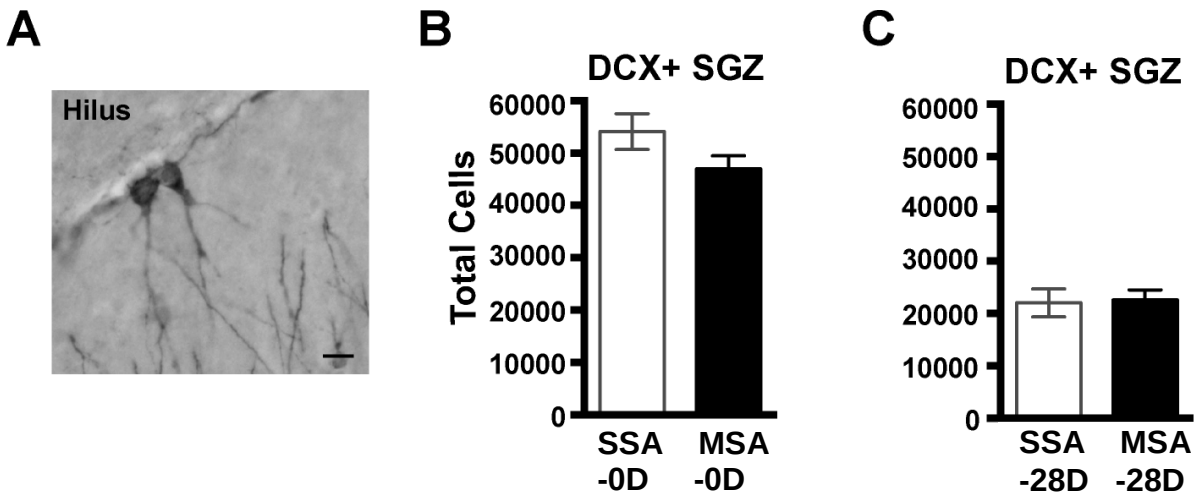

Figure 3-4. The number of DCX+ neuroblasts/immature neurons in the DG SGZ is unchanged after morphine self-administration and withdrawal compared to controls. Scale bar=10um **A.** Representative photomicrograph of a DCX+ cell from a control rat. **B-C.** Quantification of neuroblast/immature neurons (DCX+ cells) within the subgranular zone (SGZ) of the dentate gyrus either immediately after MSA **(B)** or after 28 days of withdrawal **(C).**

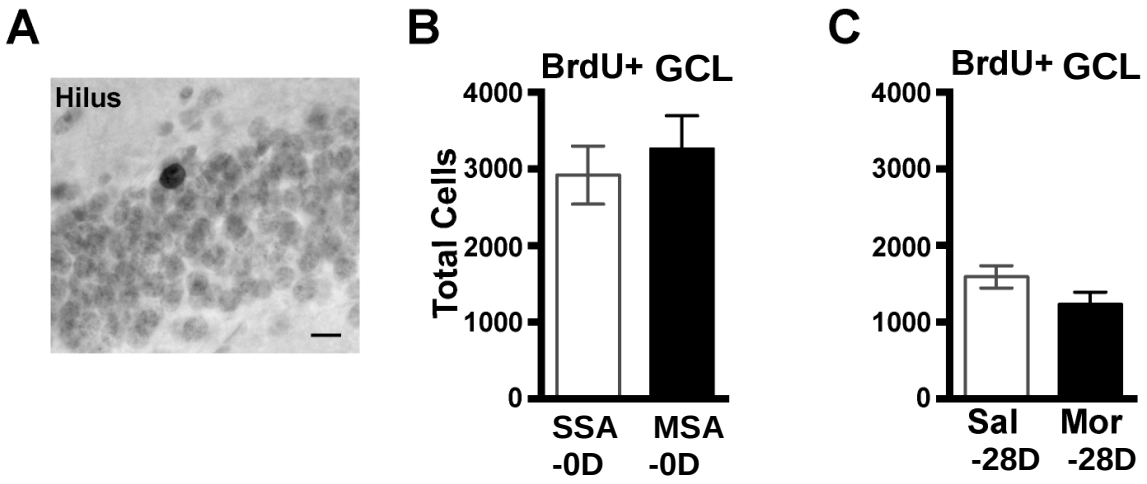

Figure 3-5. The number of surviving BrdU+ nuclei in the DG GCL is unchanged after morphine self-administration and withdrawal compared to controls. Scale bar=10um **A.** Representative photomicrograph of a BrdU+ nuclei from a control rat. **B-C.** Quantification of surviving granule cells (BrdU+ nuclei) in DG either immediately after MSA **(B)** or after 28 days of withdrawal **(C).**

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CHAPTER FOUR

Immature Neuron Density in the Adult Rat Dentate Gyrus is Unchanged by Heroin Self-administration

Bulin SE, Carlton AF, Eisch AJ. "Immature Neuron Density in the Adult Rat Dentate Gyrus is Unchanged by Heroin Self-administration". In preparation.

ABSTRACT

Studies in addicted humans and animal models of addiction, such as i.v. self-administration, show that extended exposure to opiates, including heroin, decrease cognitive function. While the underlying neuroplasticity of this cognitive dysfunction is unclear, prior work (Eisch et al., 2000) found that rats that self-administered heroin had fewer dividing neural precursors in the adult rat dentate gyrus (DG) relative to control rats. Given that reduction of neurogenesis can result in increased vulnerability to addictive behaviors (Noonan et al., 2010), it is notable that it is not known if heroin self-administration (HSA) decreases the number of cells in later stages of DG neurogenesis or alters DG granule cell layer (GCL) volume. Here we quantified both GCL volume and the density of immature neurons in the DG of rats that self-administered heroin (N=8, 2.1 µm/kg/day) or saline (N=7) for 6 hours/day for 26 days (Eisch et al., 2000). HSA did not significantly alter GCL volume. In addition, analysis of immature neuron density (via doublecortin-immunoreactive cell density) showed no difference between saline and HSA rats. This lack of change in neuroblast/immature neuron density or GCL volume suggests HSA does not robustly influence cells in later stages of neurogenesis after 26 days, or that cells in later stages of neurogenesis have compensated by the time of

tissue analysis. Additional work is needed to clarify whether immature neurons that develop during HSA have intact function and morphology, and what if any cognitive impact emerges from 21 days of HSA.

INTRODUCTION

Heroin use and abuse peaked in the USA in the 1970's (SAMHSA, 2013), but unfortunately it is again on the rise, likely from drug users shifting to heroin from prescription opiates in the search for a greater "high" or a more accessible alternative when prescriptions become difficult to acquire (Pollini et al., 2011, Lankenau et al., 2012, SAMHSA, 2013). In humans and in animal models, chronic heroin exposure decreases cognition, for example leading to spatial learning and memory deficits (Ornstein et al., 2000, Fu et al., 2008, Tramullas et al., 2008). A key brain region in these behavioral tasks learning and memory is the hippocampus (Bannerman et al., 1999, Strange et al., 1999), a region particularly vulnerable to the negative consequences of long-term opiate use (Sell et al., 2000, Bao et al., 2007). For example, heroin users have reduced blood flow to the hippocampus and smaller hippocampi overall when compared to normal, healthy controls (Pezawas et al., 2002). Additionally, the hippocampus becomes activated in heroin users that are presented with drug cues, indicating that hippocampal drug-induced plasticity may also play a causative role in craving and relapse (Sell et al., 2000). Therefore, there is great interest in delineating if and how opiate like heroin influence hippocampal structure and function.

One form of hippocampal neuroplasticity that has been under-examined in regard to heroin exposure and use is adult neurogenesis, the ongoing addition of new glutamatergic dentate gyrus (DG) granule cell layer (GCL) neurons to the existing hippocampal circuitry. Adult DG neurogenesis is sensitive to a variety of stimuli,

including chronic exposure to drugs of abuse. Indeed, proliferation of neural precursors is decreased after either chronic morphine (Eisch et al., 2000, Arguello et al., 2008) or heroin self-administration (HSA) (Eisch et al., 2000), and this may suggest that heroin decreases neurogenesis. However, adult DG neurogenesis is a dynamic, complex process that consists of many stages of cells, each with characteristic morphology and marker presentation (Kempermann et al., 2004, Ming and Song, 2005). While decreased proliferation often indicates decreased number or density of neurons – as is indicated by GCL volume and doublecortin-immunoreactive (DCX+) neuroblast/immature neurons – some stimuli influence proliferation without altering these later stages. Here we asked the question: does an HSA regimen that deceases DG proliferation change GCL volume and/or DCX+ cell density? Based on the decreased proliferation reported in this same tissue (Eisch et al., 2000), we hypothesized GCL volume, DCX+ cell density, or both would be decreased in HSA tissue relative to control rats.

METHODS AND MATERIALS

Animals

Adult, male Sprague–Dawley rats (initial weight 275–300 g; Charles River Breeding Laboratories) were used for all experiments. Animals were singling housed during self-administration studies. Rats were housed in a climate-controlled environment on a 12 hours (h) light/dark cycle (lights on 7:00 A.M.) at the University of Texas Southwestern Medical Center (UTSW). 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 UTSW.

Self-Administration Paradigm

While the self-administration data for this cohort of rats has been previously published (Eisch et al., 2000), the paradigm is described here for reader convenience. To facilitate acquisition of i.v. drug self-administration, rats were first food-restricted and trained to press one of two levers for food pellets. Once correct active lever-press behavior had been established, rats were fed *ad libitum* for 2d before surgical implantation of a jugular catheter. After 5d of recovery, rats were returned to the operant chamber and allowed to self-administer saline or heroin on a FR1 schedule of reinforcement in a daily 6-h session. Heroin self-administering rats (n=8) received 60 mg/kg per injection in 0.1 ml volume per active-lever press. Saline self-administering rats (n=7) received 0.1 ml saline per active-lever press. Active-lever presses resulted in infusion of heroin or saline over a 5-sec period, followed by a 10-sec timeout. Inactive-lever presses were recorded as a measure of general activity. The two groups were tested simultaneously for 6 h/d during their dark cycle (10pm-4am), 7 d/wk for 26 days. During the three sessions before the final session (d 23–25), rats received 0.9% NaCl (10 ml/kg, i.p.) at the fourth h of the 6-h session to habituate them to injection. During the final session (d 26), rats were perfused at the conclusion of the 6-h session.

Immunocytochemistry (IHC)

All rats were killed via terminal anesthesia followed by intracardial perfusion with

4% paraformaldehyde. Brains were removed and postfixed in 4% paraformaldehyde in 0.1 M PBS for at least 24 h at 4°C. Brains were cryoprotected in 30% sucrose in 0.1 M PBS with 0.1% NaN3 at 4°C until sectioning. A freezing microtome was used to collect serial coronal 30-μm sections through striatum and hippocampus. Sections were stored in 0.1% NaN3 in 0.1 M PBS at 4°C until processed for IHC. Every ninth section was slide-mounted and coded before processing for immunocytochemistry to ensure objectivity.

IHC was performed as previously described (Noonan et al., 2008). Briefly, for doublecortin (DCX) IHC, sections were pretreated for antigen unmasking. After pretreatment, sections were rinsed and immediately placed into blocking (3% serum) and then into primary DCX antibody (goat anti-DCX, Santa Cruz Biotech, 1:500) for incubation overnight at room temperature. After rinsing, sections were incubated for 1h in a biotinylated anti-goat secondary antibody (Jackson Labs, 1:200) followed by visualization via ABC/DAB (Vector Laboratories). Sections were counterstained with Fast Red, dehydrated, and coverslipped with DPX.

Quantification of DG GCL volume and DCX+ cell density

For all quantitative measures, an observer blind to treatment group performed the analysis. The volume of the dentate gyrus GCL was assessed using Cavalieri as previously described (Latchney et al., 2014). Data are presented as volume at points across Bregma. Using the optical fractionator method, DCX+ cells in the GCL 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). Exhaustive counts were collected from every ninth hippocampal section throughout the anteroposterior axis. Resulting cell counts were divided by the volume of the GCL at the corresponding Bregma position. DCX+ cell density is reported as DCX+ cells/mm^3 and as density of DCX+ cells at each Bregma level in the dentate gyrus.

Statistical analyses and presentation

Data are presented as mean-SEM. Statistical analyses used GraphPad Prism. GCL volume across Bregma and DCX+ cell density across Bregma were analyzed with two-factor ANOVA, while DCX+ cells/mm^3 were analyzed via Student's t-test.

RESULTS

As previously published, rats took daily i.v. heroin with a daily average dose of 2.1 mg/kg (Eisch et al., 2000). Rats self-administered heroin for 6h/ d for 26 consecutive days during the dark cycle and were sacrificed immediately after the last session and 2h after an i.p BrdU injection. Saline injections were administered for the 3d prior to the day of sacrifice to acclimate the rats to the mid-session injection. This previous study found a 27% decrease in BrdU-labelled cells after HSA (Eisch et al., 2000). This was the first finding to show opiate-induced deficits in adult DG neurogenesis, but only explored proliferation through BrdU labeling. We wanted to test the hypothesis that HSA resulted in deficits in dentate granule cell layer volume and DCX+ cell density within the hippocampus.

GCL volume was unchanged after heroin self-administration

The dorsal and ventral hippocampi vary in their function and neuroanatomical connectivity (Kelley and Domesick, 1982, Thierry et al., 2000, Britt et al., 2012, Wu and Hen, 2014). Therefore, it is important to examine the effect of HSA on immature neurons throughout the hippocampus. In order to explore the effect of HSA on the volume of the GCL volume we performed a Cavaleri analysis of volume by Bregma (Kempermann et al., 1997). This allowed for insight into the effect of HSA for both the dorsal and ventral GCL independently. The dorsal and ventral regions of the hippocampus vary greatly in both function (Snyder et al., 2011, Wu and Hen, 2014) and anatomical connections (Moser and Moser, 1998). However, there was no change in GCL volume along the antero-posterior axis between control and HSA rats (**Figure 4-1A**, Two-way ANOVA, $p>0.05$).

Doublecortin+ cell density was unchanged after HSA

In control rats, stereological quantification of DCX+ cell density found an average of ~1000 DCX+/mm^3 per GCL, similar to what has been presented in the literature in rats of a similar age (Epp et al., 2009). Notably, total DCX+ cell density was not changed by HSA (**Figure 4-1B**, $p>0.05$). In addition, DCX+ cell density along the anteroposterior axis was not different between control and HSA rats (**Figure 4-1C,** Two-way ANOVA, $p>0.05$).

DISCUSSION

The first notable finding presented here is that the GCL is unchanged in volume after 21d of HSA. While this is surprising, as chronic human users have smaller hippocampi than healthy controls (Pezawas et al., 2002), there are several possible explanations, two of which are mentioned here. First, human studies incorporate the entire hippocampal structure, while this study concentrated on the DG GCL. We chose to restrict our focus to the GCL, the site of adult DG neurogenesis, as changes in volume would likely be from the addition of new neurons. Second, in humans chronic heroin use is reported as 10-240 months of daily use (Pezawas et al., 2002), while rats were exposed for the much shorter time frame of three weeks. It is likely that the decreased volume seen in humans is the result of a much longer exposure that would require an extended time frame of several months of HSA in rodents. Another possibility is that this extended time frame resulted in large differences in dose between human heroin abusers and the animal model of HSA. As abusers become more tolerant to the rewarding effects of heroin, they will begin to consume much larger doses to feel the effects of the drug (Best et al., 2008). It is possible that no change is seen in our study because the animals were not taking equivalent doses.

Our second notable finding presented here is that DCX+ density is unchanged throughout the hippocampus after HSA. This was surprising, there was decreased proliferation reported in this same tissue (Eisch et al., 2000). The lack of a decrease in DCX+ density suggests that some compensation is taking place during the maturation of these cells. One characteristic of adult DG neurogenesis is that many proliferating

cells undergo apoptosis and only a subset go on to mature and integrate with the circuitry (Young et al., 1999, Biebl et al., 2000, Kempermann et al., 2003). It is possible that a greater proportion of progenitor cells progress through maturation when there is a smaller pool generated from stunted proliferation. Another possibility is that maturation is slowed in HSA rats, resulting in cells remaining within the immature neuron stage for longer than normal. For example, decreases in proliferation with no change in immature neuron number also occur after subcutaneous morphine pellets (Arguello et al., 2008). However, this study also found that maturation was stunted by morphine, resulting in a backlog of younger immature neurons. Slowed maturation also may have repercussions on that circuitry, regardless of the density of the neurons. One could speculate that DCX+ cells that take longer to integrate into circuitry may not be as functional as those that integrate normally, resulting in changes to downstream circuitry. Future studies examining HSA and adult DG neurogenesis should explore the timing of maturation to test the hypothesis that maturation is slowed by opiates, resulting in no net change in immature neuron density. Additionally, it would be informative to explore the morphology, synaptic integrity, and even electrophysiological properties of immature neurons after chronic opiate exposure, as aberrant neurogenesis may detrimentally affect the circuit (Parent et al., 1997, Jessberger et al., 2007).

Another possible explanation for the apparent dissociation of HSA-induced decrease in DG proliferation from a lack of an HSA effect on neuroblast/immature neuron density and GCL volume is that the prior data showing decreased proliferation is not valid. However, there are at least two reasons why we think indeed HSA decreases

DG proliferation. First, s.c. morphine causes a decrease in proliferation but no change in DCX+ cell number (Arguello et al., 2008). As one of heroin's metabolites is morphine, HSA may indeed result in influence of both heroin and morphine on proliferating cells. Second, opiates in vitro decrease proliferation through altering the length of the cell cycle (Arguello et al., 2009), but do not appear to influence the number of cells in later stages of maturation (Kahn et al., 2005, Arguello et al., 2008, Fischer et al., 2008). Notably, while DCX+ cells are nearly impossible to quantify in postmortem human tissue (e.g. Knoth et al., 2010), indices of proliferative are quite amenable to quantification (e.g. Boldrini et al., 2009). If human heroin addicts have decreased DG proliferation, it is possible that the loss of those cells could be a contributing factor in heroin-induced cognitive dysfunction. Future studies with post mortem tissue from heroin users that received cognitive assessment pre mortem may be valuable in addressing the potential role of DG proliferating cells in hippocampal function.

In conclusion, we reported HSA does not result in changes in immature neuron density, indicating that while proliferation is decreased, later stages remain unaffected. Additionally, we have shown that GCL volume remains stable after HSA, suggesting that the decreased volume indicated in human studies require long-term heroin exposure to take effect. Taken together with prior work, we show that HSA decreases proliferation without influencing the density of neuroblast/immature neurons or GCL volume, thus adding this HSA paradigm to the growing list of drugs of abuse that differentially influence cells in distinct stages of neurogenesis (Teuchert-Noodt et al., 2000, Dominguez-Escriba et al., 2006, Schaefers et al., 2009).

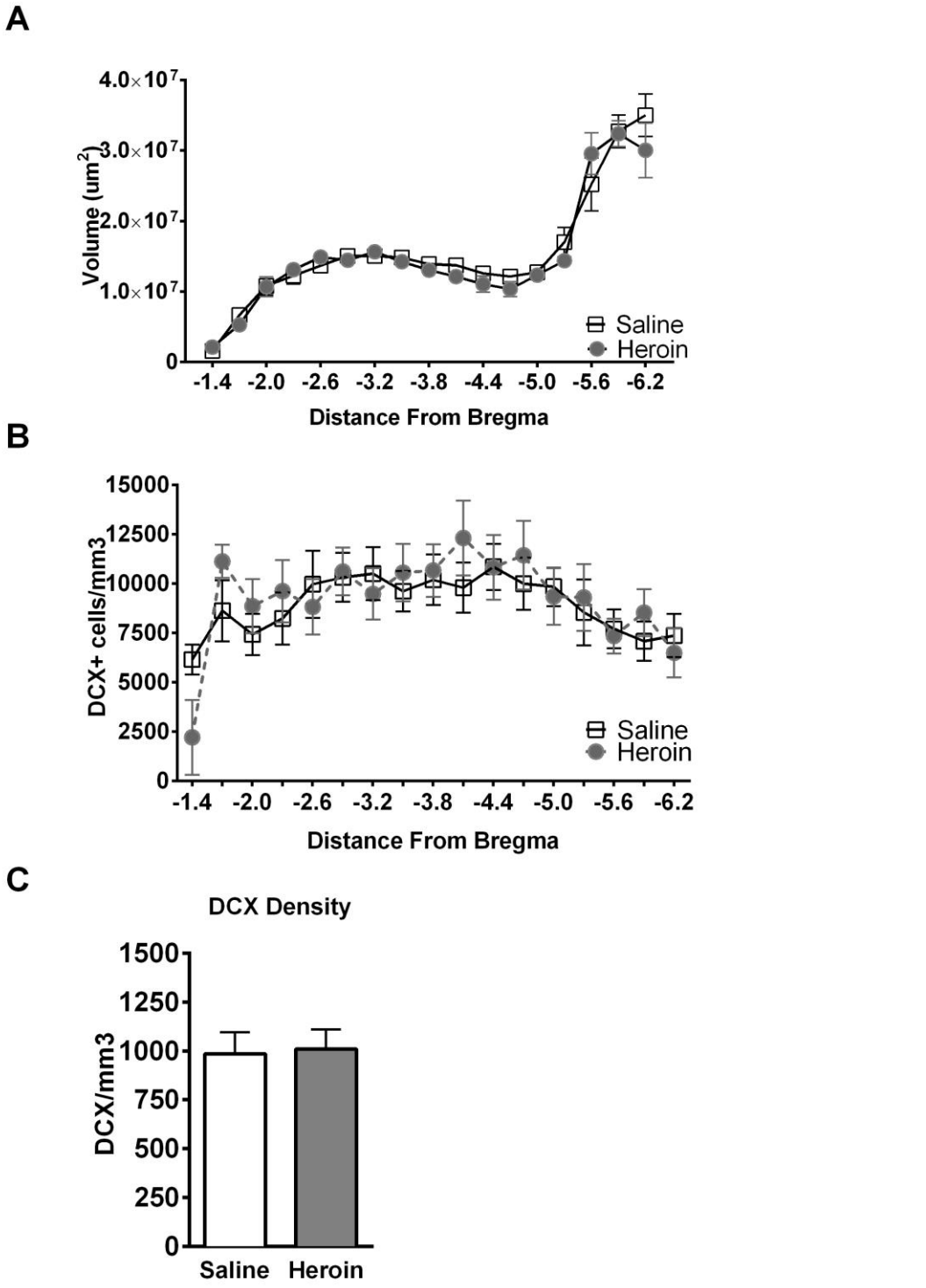

Figure 4-1. Effect of HSA on GCL Volume and DCX+ Cell Density. A) Volume of the GCL along the anteroposterior axis of the hippocampus (Two-way ANOVA; p>0.05) **B)** Density of DCX+ cells along the anteroposterior axis of the hippocampus (Two-way ANOVA, p>0.05 **C)** Total density of DCX+ cells within the GCL (p>0.05).

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CHAPTER FIVE

Conclusions and Future Directions

Overview

The work of this thesis has primarily focused on the two-way relationship between morphine addiction and adult dentate gyrus (DG) neurogenesis. Understanding the role of DG neurogenesis in addiction may reveal new methods of preventing relapse, as well as preventing addiction after initial drug exposure. It would also reveal which individuals may be at more risk to addiction when exposed to morphine in a clinical setting, as decreased neurogenesis via stress or irradiation could be a risk factor for developing a substance abuse problem. Additionally, understanding if DG neurogenesis is detrimentally affected by chronic exposure to morphine in a clinically-relevant model is critical, as the drug itself could be increasing vulnerability to addictive behavior through suppressing adult DG neurogenesis.

In regards to the field of addiction, this dissertation has added multiple pieces to the puzzle of the role of adult DG neurogenesis in addiction behaviors and reward circuitry. This work is the first to show that ablation of adult DG neurogenesis leads to increases in morphine intake, deficits in cognitive flexibility, increased sensitivity to

morphine, and increased activation in the infrapyramidal blade of the DG. Additionally, I demonstrate that morphine self-administration (MSA), the most clinically-relevant animal model available, does not detrimentally affect any stage of adult DG neurogenesis examined, including proliferation, survival, or immature neuron cell number. I also contributed to previous heroin self-administration (HSA) work; demonstrating that intravenous heroin does not result in changes to the volume of the dentate gyrus and does not alter immature neuron density. Taken together, these data suggests that adult DG neurogenesis is robust enough to be unaffected by chronic voluntary morphine intake. However, if DG neurogenesis is disrupted this can lead increased vulnerability to addictive behaviors and relapse.

Below, I will explore the major conclusions of each chapter, address key points and considerations, and discuss future experiments that would further the contributions of this work.

Chapter 2: Dentate Gyrus Neurogenesis Ablation via Cranial Irradiation Enhances Morphine Self-administration and Sensitization

Within this chapter, I hypothesized that ablation of adult DG neurogenesis would result in an increased vulnerability to addiction-related behaviors through altering hippocampal circuitry, resulting in changes downstream in the reward pathway. In order to understand the role of adult DG neurogenesis in a clinically-relevant model of morphine addiction, I used image-guided, hippocampal-directed X-ray irradiation (IRR)

to ablate neurogenesis and explored several morphine related behaviors, including MSA and MLS after ablation. I reported that IRR and subsequent ablation of DG neurogenesis increased drug intake and resulted in extinction/cognitive flexibility deficits in MSA, increased sensitivity to MLS, and increased morphine-induced cellular activation in the infrapyramidal blade of the DG. These data support my hypothesis, suggest DG neurogenesis plays a role in the regulation of the reward pathway, and indicate novel approaches to the prevention and treatment of addiction.

Chapter 2, Considerations and Future Directions

This research suggests that adult DG neurogenesis plays an important role in addiction, regardless of the type of drug of abuse (opiates vs. psychostimulants). Not only do rats lacking DG neurogenesis take more drug than their normal counterparts, IRR rats also struggle with cognitive inflexibility and hypersensitivity to repeated dosages. These findings open up a new series of questions that could be addressed in future work.

First, there is an elevation in cellular activation within the infrapyramidal blade of the dentate gyrus and a trend towards an increase within the nucleus accumbens (NAc) shell where hippocampal inputs have been observed (**Figure 5-1** and other **Chapter 2** data not shown). This begs the question of identifying other areas of the reward pathway that may show increased cellular activation after morphine sensitization. Ideal areas to explore would include not only other subregions of the hippocampus, such as

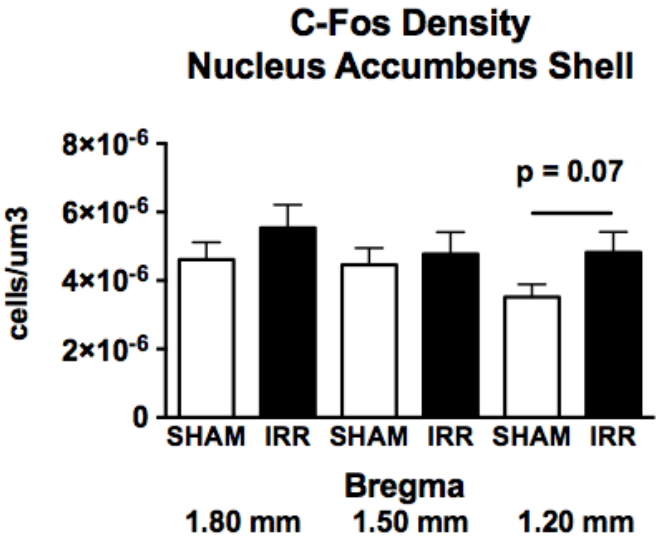

Figure 5-1. Nucleus Accumbens Shell C-fos Density. Rats underwent morphine locomotor sensitization (15D), withdrawal (5D), a dose-response curve within the locomotor chamber (10D), and a second withdrawal phases (6D; See Figure 2-3 for timeline). Rats were then sacrificed 90 minutes after a 1 mg/kg challenge in the locomotor chamber via pericardial perfusion. A strong trend towards an increase in c-fos density was observed in portions of the NAc that receive hippocampal input.

the CA1 or the subiculum, but also other brain regions that receive inputs from the hippocampus, such as the prefrontal cortex. This experiment would clarify the role of DG neurogenesis even more, as the hippocampus may be over activating several brain regions, not just the NAc. In parallel with this work, using electrophysiology to study changes in the pathway between the ventral hippocampus and the nucleus accumbens would be very informative. However, this would have to be done in irradiated rats after chronic morphine exposure (vs. sham rats with chronic morphine vs. irradiated rats with no exposure), which most likely would need to be done *in vivo* because of the slice recordings between the hippocampus and accumbens being difficult to isolate. Additionally, attempting electrophysiology in older adult rats would also prove difficult. Alternatively, another future experiment exploring circuit wide activity changes would look at brain activity using a small animal MRI machine after Sham/IRR both before and after morphine exposure. This would be an interesting approach, as the same animals could be examined over time, allowing for comparison of regional activity throughout the process of addiction (i.e. acute or chronic exposure, withdrawal).

As adult-generated neurons are glutamatergic, it seems counterintuitive that ablating these cells would result in greater excitation both in the hippocampus and downstream. Some studies suggest increased hippocampal output (Snyder et al., 2011b) or increased synchronization of the hippocampus after ablation (Lacefield et al., 2012). Another study found decreased excitability in the DG of mice with increased neurogenesis, and increased excitability after ablation, indicating that newborn neuron modulate excitability of mature granule cells (Ikrar et al., 2013). Interestingly, when older granule cells are silenced in mice, these animals exhibited higher amounts of

pattern separation (Nakashiba et al., 2012). This effect was then reversed with irradiation, suggesting that the newborn neurons are responsible for this increase in function and that the function of these cells change as they mature. One hypothesis for increased excitability of the hippocampus is that immature neurons synapse onto nearby hilar mossy cells and other inhibitory interneurons during their maturation process (Toni et al., 2008). The glutamatergic input from immature neurons, along with their unique excitability, may result in more interneuron firing, ultimately quieting the hippocampus. Further experiments examining the activity of hilar mossy cells after ablation of DG neurogenesis may provide additional support for the role of immature neurons in hippocampal excitability.

As mentioned above, c-fos density was elevated specifically in the infrapyramdinal blade of the DG after morphine sensitization, but was unchanged in the suprapyramidal blade. This finding adds to a growing amount of literature that there may be functional and anatomical differences between the two blades of the dentate gyrus. As the supra- and infrapyramdal blades preferentially activate to particular stimuli (Snyder et al., 2011a, Hoffman et al., 2013), there is a case that inputs and outputs of the two blades are different. For example, granular cells from both blades feed into the mossy fiber pathway and synapse on pyramidal cells in the CA3 (Andersen et al., 2006), but these synapses may not be on the same subpopulation of CA3 cells or may be synapsing onto different areas of the same cells (Cosgrove et al., 2010). Likewise, inputs into the molecular layer may vary slightly between the two blades. Tracing studies may reveal differences in the circuitry of the supra- and infrapyramidal

blades that be informative in not only the preferential roles of the two subregions of the dentate gyrus, but how adult DG neurogenesis may play a role in those functions.

Interestingly, almost all work in regarding to the function of adult DG neurogenesis and drug addiction has examined drug-induced behaviors after disruption or ablation (Noonan et al., 2010, Deschaux et al., 2014). Ablation studies, such as my work and that previously performed, are important to understand the functionality of newborn neurons in drug addiction and have resulted in the knowledge that less adult DG neurogenesis results in more vulnerability to addiction-related behaviors. However, it is still unknown if increasing DG neurogenesis results in less vulnerability to these same behaviors. One potential future experiment to build on this work would use neurogenic compounds to increase neurogenesis (such as i.c.v. or intra-brain region infusion of Isoxazole-9 or P7C3) and then examine drug intake, extinction, and sensitization. One hypothesis for this work would be that elevated DG neurogenesis is protective against drug-induced behaviors and results in enhanced cognitive flexibility, leading to lowered drug intake and faster extinction rates. Exploring these future directions, and taking them together with my own work, may provide the first steps in creating therapeutic medications for drug abusers that aid in suppressing craving and relapse.

My work has revealed that adult DG neurogenesis contributes to addiction-related behaviors, regardless of the type of drug of abuse. However, due to the permanent nature of irradiation-induced ablation, it is difficult to isolate each stage of

addiction and explore if adult DG neurogenesis contributes to each part of the addictive process. It could be that adult DG neurogenesis is important in the initial stages, and once the animals have taken more morphine it could alter their behavior in later parts of MSA. One way around this is through the use of a new tool in the field: DREADDs. DREADDs (Designer Receptors Exclusively Activated by Designer Drugs) allow for short-term activation (hM3Dq) or silencing of neurons (hM4Di) (Urban and Roth, 2015). This occurs through virally expressing a designer receptor that does not normally exist in the mammalian genome within the neurons of interest. These receptors can be activated through an injection of a designer ligand (clozapine-N-oxide [CNO]) and can last for a short period of time (2hr) (Guettier et al., 2009, Urban and Roth, 2015). This tool would allow for silencing of newborn neurons during different stages of addiction to further pinpoint the role of DG neurogenesis in these individual stages. This can include allowing rats to self-administer until they all have reached the maintenance phase, then silencing newborn neurons, or have the neurons silenced during the initial few days, then have them activated once the maintenance phase begins. These proposed experiments would further the works already completed within this thesis are providing greater insight into the specific role of DG neurogenesis in addiction.

Besides these future experiments, there are also some confounds that should be addressed. One confound of the irradiation work is the weight difference between Sham and IRR rats. Irradiated rats typically gain weight slower than their sham counterparts for a few weeks post-irradiation, this slowed weight gain is then normalized later on (i.e. they gain weight at the same rate). However, this lost weight is never fully

recovered, leading to IRR rats weighing 40-50g less than their Sham counterparts throughout the experiment. One reason that this may be a confound is that all dosages were calculated by weight, and while IRR rats weighed less they also likely had a smaller percentage of body fat, which may skew dosing. However, even when weight is taken into account, irradiated rats consume more total morphine (**Figure 5-2**). This slowed weight gain caused by focal irradiation over the hippocampus is well documented within the literature (Winocur et al., 2006, Wojtowicz, 2006, Wojtowicz et al., 2008). While it is a well-known feature of cranial X-ray irradiation, there is no clear mechanism behind the phenomenon. One potential region of interest for examining the change in weight gain is the hypothalamus. The hypothalamus is incredibly important for different parts of homeostasis (Williams et al., 2000). Additionally, small amounts of neurogenesis are thought to occur in the arcuate nucleus, a subregion responsible for food intake and hunger (Kokoeva et al., 2005, 2007, Pierce and Xu, 2010). It is likely that the irradiation would affect the proliferating cells in this brain region as well, since the hypothalamus would be in the path of the restricted beam. However, since the beam must go through a larger amount of tissue, there is the possibility that the dose received by the hypothalamus is much lower than that received by the hippocampus. Experiments exploring this potential additional ablation of new neurons in the arcuate nucleus would be very informative, potentially finding the mechanism behind the often overlooked side effect of lowered weight gain after irradiation. Pilot data in our lab (Walker, Bulin, and Eisch, in progress), though, confirm what is shown in the literature: the new cells in the arcuate do not label with convention “immature neuron” markers like DCX, but rather only with proliferation markers, making it difficult to address this

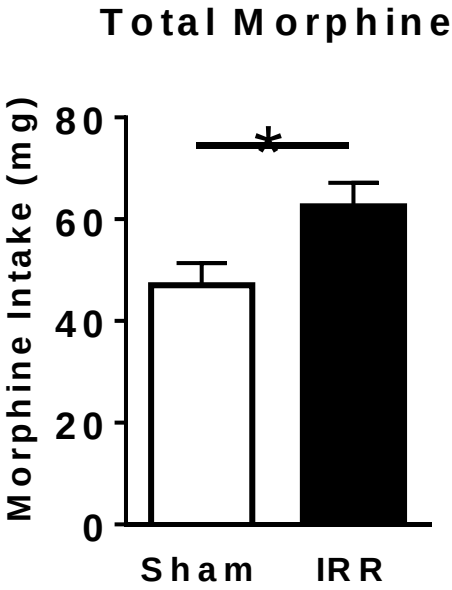

Figure 5-2 Total morphine intake during morphine self-administration. Total milligrams of morphine consumed were calculated by multiplying total mg/kg consumed by each individual animal's weight in kilograms (~0.50 kg). Irradiated rats consumed approximately 20% more total morphine than their sham counterparts.

question without BrdU or another way to lineage trace the cells. Additionally, using metabolic chambers and/or measuring food intake of rats after irradiation may uncover if the weight loss is due to alternations in metabolic processes that would ultimately by responsible for the difference in weight gain.

Another confound of these irradiation studies is the age of the rats when the irradiation is completed. Irradiation is completed when the rats are approximately six weeks of age (or 45D). In adult DG neurogenesis literature, rats are not considered young adult until eight weeks of age, so some developmental processes may be disrupted by the irradiation treatment. However, the rats in this study are actually slightly older than when young rats are often irradiated for neurogenesis. For example, a previous irradiation study in which the Eisch lab irradiated young rats was at approximately 100g, which is between four and five weeks of age (Noonan et al., 2010). This timing of irradiation is common throughout the literature (Winocur et al., 2006, Wojtowicz, 2006, Wojtowicz et al., 2008). Gross damage to the developing hippocampus is also not an issue, as postnatal development of the hippocampus is complete by four weeks of age (Andersen et al., 2006). To help prevent any additional damage that might have occurred from post-irradiation inflammation, the anesthetic pentobarbital was used during the irradiation. This anesthetic was chosen specifically for its neuroprotective properties that might aid against damage caused through inflammation or to mature cells are may receive small amounts of DNA damage that would need to be repaired (Araki et al., 1990). In conclusion, irradiation is a "dirty" method of ablation that can affect other brain regions besides the hippocampus and

may affect the hippocampus differently based on age, but we took as many precautions as possible to prevent off target effects.

One alternative interpretation of the increased morphine intake by irradiated rats is that rats lacking adult DG neurogenesis have increased impulsivity. If this were the case, irradiated rats would press the lever more not only during when drug in available, but also when the drug in not available, such as during the timeout periods (i.e. immediately after an infusion of morphine). Within my data, a two-way ANOVA analysis revealed that irradiated rats pressed more across all sessions, but Sidak post-hoc analysis found no differences between groups during individual sessions (**Figure 5-3**). This indicates that irradiated rats did not take more because of increased impulsivity (i.e. continuing to press the lever after the drug had been administered). Additionally, this interpretation can be extended to the extinction experiment. Irradiated animals may have higher lever presses during extinction because they pressed more during previous self-administration, therefore having a higher baseline. However, during the first extinction session irradiated rats and sham rats exhibited similar amounts of lever presses during the first 30 minutes (**Figure 5-4A**). IRR rats continued to press during the next 30 minutes, while Sham rats had ceased lever pressing. There was no difference in additional days of extinction training (**Figure 5-4B-D**).

Chapter 3: Morphine Self-administration and Subsequent Withdrawal Do Not Change Indices of Dentate Gyrus Neurogenesis in the Adult Rat

Within this chapter, I hypothesize that MSA decreases adult DG neurogenesis.

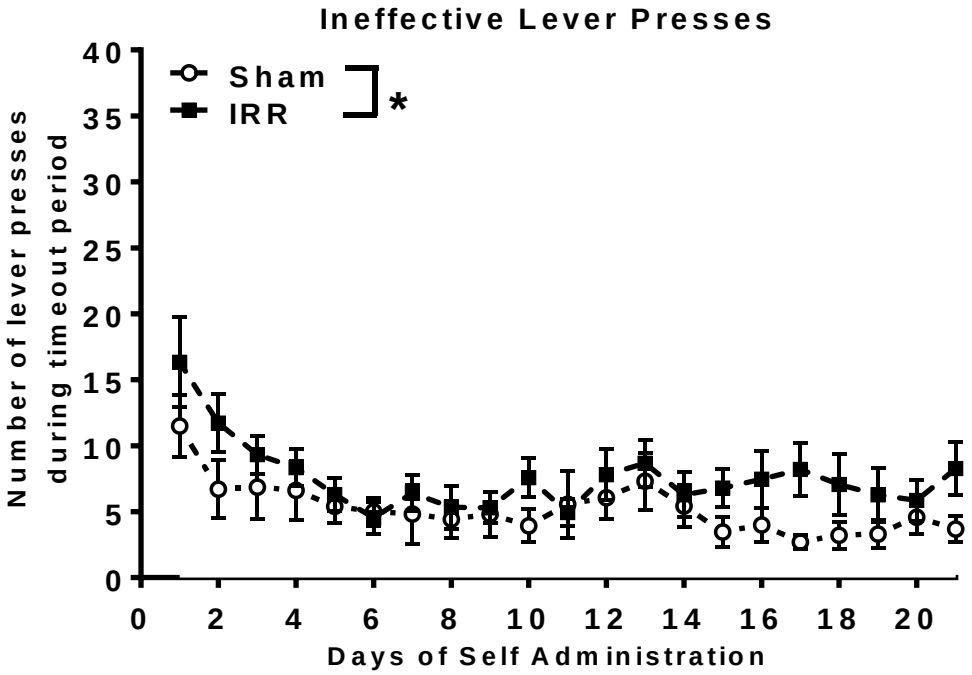

Figure 5-3. Ineffective Lever Presses during Timeout Periods. The number of level presses was recorded during periods in which rats could not receive morphine. Two-way ANOVA analysis revealed a main effect of Treatment ($F_{1,553}$=17.84, p<0.0001) and Session ($F_{20,553}$=2.939, p<0.0001) with no interaction. Holm-Sidak post-hoc analysis found no differences in individual sessions.

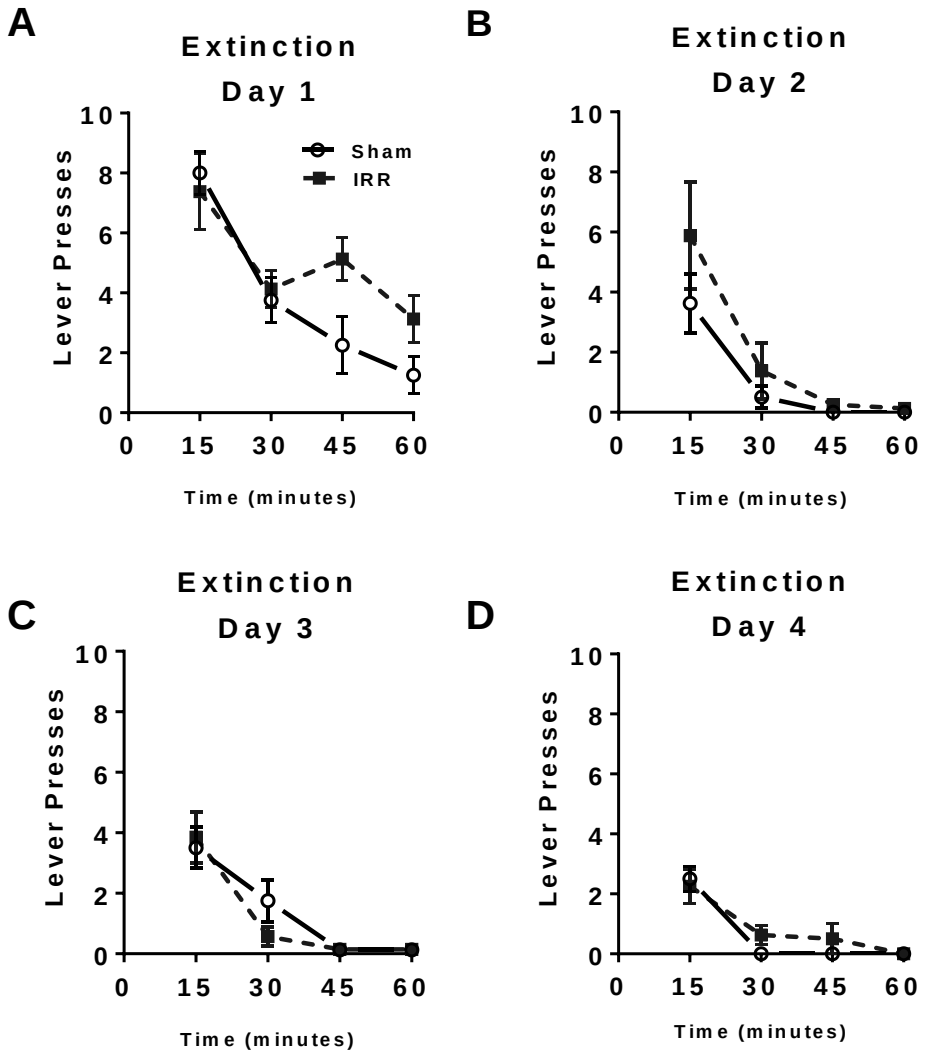

Figure 5-4. Fifteen-minute Bins of Extinction Sessions (Day 1- Day 4). A-D) The number of lever presses during each fifteen minutes of the first four days of extinction. **A**) Both IRR and Sham rats began extinction with similar lever presses during the first 30 minutes. IRR rats continued to press during the 30-45 and 45-60 minute bins while Sham rats extinguished fairly quickly. Two-way ANOVA analysis found an effect of time ($F_{3,56}$=15.77, $p<0.0001$), with a trend towards significance in treatment ($F_{1,56}$ =3.646, $p=0.0613$) with no interaction ($F_{3,56}$=1.741, $p=0.1691$).

In order to understand the effects of MSA and subsequent withdrawal on adult DG neurogenesis, rats underwent 21 days of MSA and then were sacrificed immediately after the last session, or were given 28 days of forced abstinence in their home cage. I reported that proliferation, survival, and immature neuron number remained unchanged immediately after MSA and after the withdrawal period, disproving my original hypothesis. This research suggests that MSA under these conditions does not have detrimental effects on adult DG neurogenesis.

Chapter 3, Considerations and Future Directions

While this research shows that rats that undergo 21 days of MSA have no change in proliferation, survival, or in immature neuron number, it is still unknown if there are any alterations to adult DG neurogenesis that is not limited by the markers used or smaller differences that would not be revealed through cell counts. For example, previous morphine pellet work revealed that maturation of new DG neurons was slowed by morphine, resulting in a backlog of younger DCX+ cells versus older DCX+ cells while not affecting the overall number of cells (Arguello et al., 2008). These subpopulations were separated using dual-labelling of DCX+ and GFP in a nestin-GFP mouse line. This would not be possible in the outbred rat strain used in this study, but other methods are classifying DCX+ cells by their processes may be used, such as classification of dendritic maturity (Plumpe et al., 2006).

One consideration is that MSA does not affect adult DG neurogenesis because rats do not voluntarily take morphine at high enough levels to cause the deficits seen in

proliferation after subcutaneous morphine pellets. My research was always performed during the light cycle, or when rats are less active. It would be interesting to investigate if adult DG neurogenesis is also unchanged during dark cycle MSA, as rats are more active during this time and often will exhibit higher amounts of drug-taking (Fitch and Roberts, 1993, O'Dell et al., 2007). Because of shared housing and limited space in the NC4 and NG2 housing facilities, reversed light cycles were not attempted for my studies. However, other methods were attempted in order to promote higher morphine intake rather than performing dark cycle MSA. For example, longer term access was attempted early on, with the rats having 8hr daily morphine sessions rather than the 4hr session data reported in **Chapter 3**. However, no differences were seen in adult DG neurogenesis were seen at this length of access, so the short access was chosen (**Figure 5-5**). Long daily access is often associated with tolerance and escalation, markers of rats becoming “addicted” (Ahmed and Koob, 1998, Deroche-Gamonet et al., 2004), as it would be interesting to compare DG neurogenesis in “addicted” and “nonaddicted” rats. However, many issues arose during the experiment that made completing long daily access difficult. First, chronic morphine suppresses the immune system (Bryant and Roudebush, 1990, Roy et al., 2006), making MSA rats much more susceptible to infection. Second, the combination of morphine-related anesthesia and morphine-induced itching could result in self harm of the animals, which could be compounded by a suppressed immune response. Third, morphine induced stereotypic behaviors observed during 8hr access including paw-chewing and chewing of the equipment. Some of this chewing behavior was alleviated in the 4hr access group was alleviated through the use of soft wooden blocks placed within the operant chamber with

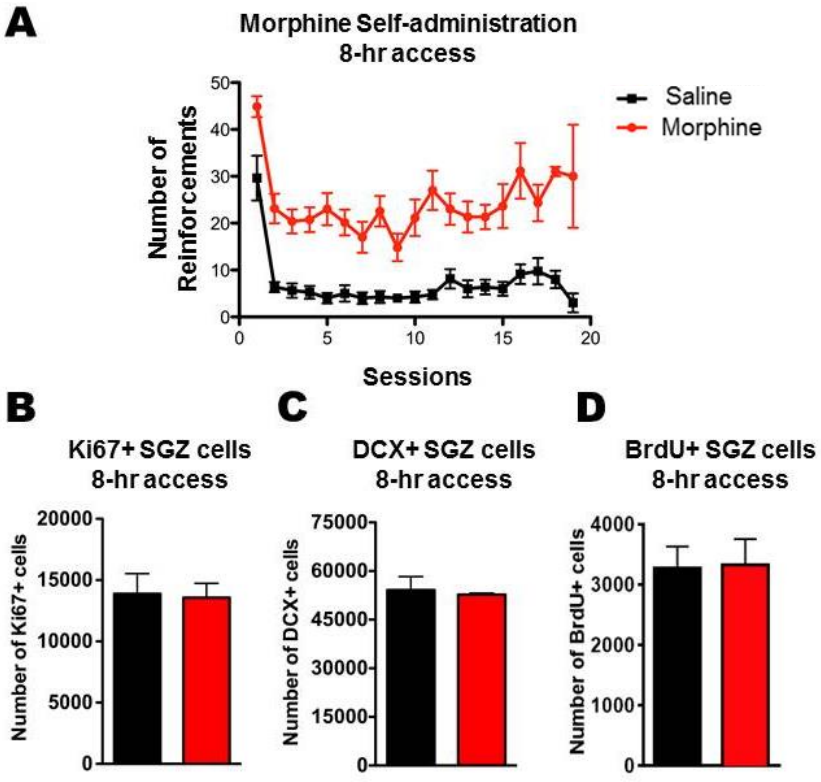

Figure 5-5. 8 Hour Morphine Self-administration Access. A) Sessions of morphine self-administrations **B)** Proliferation (Ki67+) is unchanged after 21 days of 8hr MSA **C)** Immature neuron number (doublecortrin +) is unchanged after 21 days of 8 hr MSA **D)** Survival (BrdU+) is unchanged after 21 days of MSA.

the animals. The blocks allowed for the rats to chew on something other than the equipment, sparing tethers and any plastic piece they could reach. These blocks were replaced every few sessions, as the animals would not chew on them once the corners were removed.

It is interesting to note that morphine results in deficits in many hippocampal tasks while not affecting adult DG neurogenesis. One thing to consider is that all behavioral studies performed were completed after experimenter-delivered morphine. It would be interesting to explore if animals exposed to MSA also exhibit the same decreased learning and memory seen after injections (Ma et al., 2007, Gu et al., 2008). If MSA rats exhibit deficits in learning and memory, or spatial tasks, it is important to consider other subregions of the hippocampus. Alternative hypotheses behind these hippocampal-dependent deficits may explore mechanisms focusing on changes in LTP (Dong et al., 2006, Bao et al., 2007, Lu et al., 2010, Portugal et al., 2014) or changes in the expression of various receptors (Le Greves et al., 1998, Zarrindast et al., 2006).

When comparing this work to previous studies, it is interesting that HSA resulted in changes to proliferation (Eisch et al., 2000) while MSA did not. There could be several different reasons for discrepancy. First, heroin metabolizes into morphine (Goldberger et al., 1993), resulting in a “one-two punch” to the system and heroin results in different modulations of hippocampal circuitry (Bao et al., 2007). Second, proliferation was examined via BrdU injections 2hr prior to sacrifice in HSA rats, while proliferation seen in both experiments. Additional experiments suggested in the

Chapter 4 section may reveal a better comparison.

Chapter 4: Immature Neuron Density in the Adult Rat Dentate Gyrus is Unchanged by Heroin Self-administration

Based on the large deficit in proliferation already reported (Eisch et al., 2000) and hippocampal alterations seen in human studies (Pezawas et al., 2002, Weber et al., 2006), I hypothesized that granule cell layer volume (GCL) would be reduced, along with the density of immature neurons, in rats that underwent 21D of HSA. In order to explore the effect of heroin self-administration on immature neuron density and granule cell layer volume, rats underwent 26 days of HSA and were sacrificed immediately after the last session. I found that HSA did not alter the volume of the GCL of the hippocampus and did not alter the density of DCX+ cells within the GCL, disproving my original hypothesis.

Chapter 4, Considerations and Future Directions

While the relationship between HSA and adult DG neurogenesis was initially explored over 15 years ago (Eisch et al., 2000), the effects of HSA on later stages of DG neurogenesis were never investigated until now. The first finding of this chapter, that DCX+ cell density was unchanged after HSA, still leaves several questions unanswered. This analysis of DCX+ cell density did not include examining the dendritic processes of the DCX+ cells. Qualitative observation of the stained tissue suggested that the processes might be smaller in heroin self-administering animals than saline self-administering controls, but the age of the tissue made it difficult to distinguish between staining issues and actual lack of dendritic processes. Either new staining

conditioning (optimized for this older tissue) or a new cohort would be needed to complete HSA under similar conditions to conclusively study the effect of HSA on immature neuron maturity and processes.

As mentioned briefly in the previous section, prior HSA publications indicate that HSA decreases proliferation through labelling dividing cells with BrdU on the last day of self-administration, resulting in a ~30% decrease in BrdU labelling (Eisch et al., 2000). While BrdU is a valuable tool is studying proliferation within the DG, it has its limitations. First, it only labels a subset of dividing cells, particularly those that are in the S phase of division, since this is when the BrdU is incorporated into the DNA (Kee et al., 2002, Wojtowicz and Kee, 2006). Second, BrdU does not label every cell in S phase, but only a subset (Cameron and McKay, 2001, Taupin, 2007). Third, stress can increase the permeability of the blood brain barrier, which may alter the amount of BrdU that can enter the brain (Esposito et al., 2001). It would be useful to examine proliferation in another manner. Future studies should include the use of an endogenous marker, such as Ki67, to analyze proliferation after HSA. Use of Ki67 would label all dividing cells, not just a subset, allowing for a more thorough analysis. While it is unknown if heroin has any direct action on dividing cells, morphine pellets can directly alter the length of the cell cycle (Arguello et al., 2009). When staining the DG for Ki67, cells are often labelled in clusters, or groupings of dividing cells. Since opiates affect proliferation, it would be interesting to explore if the total number of clusters is also decreased or if the number of cells per cluster is determinately affected.

Overall Conclusions

The work detailed within this dissertation has laid a strong foundation for future work regarding adult DG neurogenesis and addiction. First, I have shown that ablation of newborn neurons within the DG of the hippocampus increases in morphine intake, decreases cognitive flexibility in an addiction model, increases the rate and amount sensitivity to repeated doses of morphine, and increases cellular activation in the infrapyramidal blade of the DG after an exposure to morphine within the drug context. These results highlight the contribution of newborn DG neurons in modulating addiction-related behaviors and give further understanding as to how adult DG neurogenesis may contribute to hippocampal influence on the reward pathway.

Second, I have shown that adult DG neurogenesis is so robust that it is unaltered by MSA. This finding is positive for the field. Morphine is widely prescribed for pain, and I have shown that decreased DG neurogenesis can increase morphine intake and extinction. Since MSA does not decrease DG neurogenesis, morphine itself does not increase susceptibility through affecting newborn neuron number. These first two findings are the first two steps in understanding the importance of examining neurogenic compounds as potential therapeutic drugs to aid in the prevention increased intake or relapse. Increasing adult DG neurogenesis may prove to be protective against addiction-related behaviors and may, in the future, be administered alongside addictive prescriptions to aid in preventing the development of addiction.

Lastly, I have provided additional insight to the effects of HSA on immature neuron density and GCL volume. However, more work is necessary to truly understand the role of neurogenesis in addiction, and how newborn neurons affect the brain at the circuit level. Overall, adult DG neurogenesis contributes to addiction-related behaviors and cognitive flexibility. Additional work within the field is required for the development of therapeutics that may improve the lives and recovery of human patients suffering from addiction.

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