THE IMPACT OF CHRONIC MORPHINE ON ADULT HIPPOCAMPAL PROGENITOR CELLS AND THE NEUROGENIC NICHE

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DEDICATION

I dedicate this thesis to my family
for their encouragement and support.

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by

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The birth of new neurons persists in the adult hippocampal subgranular zone (SGZ). Adult neurogenesis is dynamically regulated and thought to be important for certain types of spatial learning and memory. SGZ proliferation and neurogenesis are decreased by chronic morphine, yet how this alteration occurs is unknown. It is unclear if morphine causes alterations in cell cycle progression, progenitor cell maturation, or indirectly inhibits progenitor cells by altering the hippocampal neurogenic niche.

I first examined a time course of morphine's effect on the progenitor cell cycle, cell death and immature SGZ neurons. I found that S phase cycling cells were vulnerable to morphine at early time points with a concurrent increase in cell death. I found that although the total population of SGZ immature neurons remained unchanged, the proportion of progenitor cells that progressed to a more mature stage decreased. I next asked whether decreased levels of proliferation resulted from shortened S phase length. Using a modified double injection paradigm of halogenated thymidine analogs, I found that chronic morphine did not alter the length of S phase of progenitor cells. Next, I asked if chronic morphine could have an indirect inhibitory effect on progenitor cells by altering growth factors and neurovasculature within the hippocampal neurogenic niche. I found that protein levels of factors within the niche were maintained or upregulated (e.g. vascular endothelial growth factor) to compensate for the morphine-induced decrease in proliferation. Lastly, I asked whether chronic morphine would decrease proliferation in an inducible nestin-CreER^{T2}/R26R-yellow fluorescent protein transgenic mouse. I found that proliferation in this transgenic mouse was not altered after a particular paradigm of morphine exposure.

Together these findings suggest that morphine alters adult hippocampal proliferation through multiple effects: both on the progenitor cells themselves (cell cycle, maturation) and indirectly by alteration of the neurogenic niche. Additional work is needed to understand the mechanism of the morphine-induced changes

in progenitor cell cycle and the neurogenic niche. The present findings will benefit both the addiction field by offering new avenues for treatment and neural stem cell biology by demonstrating stages of neurogenesis that are more vulnerable to exogenous stimuli.

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PRIOR PUBLICATIONS

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Chapter 1: Introduction

LIST OF ABBREVIATIONS

^{3}H – tritiated thymidine

AC3 – activated caspase-3

BDNF – brain-derived neurotrophic factor

BrdU – bromodeoxyuridine

CFP – cyan fluorescent protein

CldU – chlorodeoxyuridine

CNS – central nervous system

DCX – doublecortin

DG – dentate gyrus

G1 – gap1

G2 – gap2

GCL – granule cell layer

GFAP – glial fibrillary acidic protein

GFP – green fluorescent protein

IdU – iododeoxyuridine

IHC – immunohistochemistry

IL1β – interleukin-1β

IL1R1– interleukin-1β receptor 1

i.p. – intraperitoneal

IR – immunoreactive

M – mitosis

MOR – mu opioid receptor

Nestin-GFP – nestin driven GFP transgenic mouse

NeuN – neuronal nuclei

OB – olfactory bulb

PBS – phosphate buffered saline

PCNA – proliferating cell nuclear antigen

pHH3 – phosphorylated histone H-3

PSA-NCAM – polysialic-neural cell adhesion molecule

s.c. – subcutaneous

SEM – standard error of the mean

SGZ – subgranular zone

SVZ – subventricular zone

TAM – tamoxifen

TrkB – tropomyosin related kinase B

VEGF – vascular endothelial growth factor

VEGFR2 – vascular endothelial growth factor receptor 2

VEH – vehicle

YFP – yellow fluorescent protein

CHAPTER ONE

Introduction

Adult Neurogenesis

The adult mammalian brain was once thought to be in a fixed state in which neurons could not be regenerated (Ramon y Cajal, 1913). However during the last few decades it has become clear that specific processes, such as neurogenesis, that occur in the developing brain also occur in the adult brain (Altman and Das, 1965; Gross, 2000). While controversial at its first discovery in the 1960's (Altman and Das, 1965), it has been well established that neurogenesis occurs in two regions of the mammalian brain throughout life: the subventricular zone (SVZ) and the hippocampal subgranular zone (SGZ) (Ming and Song, 2005). Several studies, which will be discussed below, have determined that adult neurogenesis can be altered, both positively or negatively, by various stimuli such as exercise or conversely drugs of abuse (Canales, 2007; Eisch and Harburg, 2006; Kempermann et al., 2004). The function of neurogenesis, however, is still controversial. Some studies have observed that neurogenesis is essential for memory while others have observed the opposite (Madsen et al., 2003; Shors et al., 2001; Shors et al., 2002). These seemingly contradictory studies are likely due to the complexity of the process of neurogenesis. This introduction will focus on adult neurogenesis in the hippocampal SGZ, with discussion of the SVZ only when relevant to discoveries made in the SGZ. However, a more broad discussion of SVZ neurogenesis in particular can be found in several excellent reviews (Alvarez-Buylla and

Lim, 2004; Ming and Song, 2005). This introduction will review the field of neurogenesis and concentrate on its regulation by morphine to highlight questions that remain to be answered.

Tools used to study adult neurogenesis

The complexity of experimental tools used to study neurogenesis has increased in the last forty years. While some of the first studies detected new neurons in the postnatal brain by tritiated thymidine (^{3}H) autoradiography (Altman and Das, 1965), neurogenesis is now more commonly visualized with the exogenous S phase marker bromodeoxyuridine (BrdU), which can be visualized by immunohistochemistry (Kee et al., 2002; Wojtowicz and Kee, 2006). Furthermore, combinations of endogenous cell cycle markers such as Ki67 and phosphorylated histone H-3 (pHH3), together with BrdU allow for analysis of specific phases of the progenitor cell cycle (Eisch and Mandyam, 2007; Mandyam et al., 2007). For example, a cell that is immunoreactive (IR) for BrdU and Ki67 would be considered an S phase cycling cell, whereas a cell that is IR for Ki67 but not BrdU would be considered a non-S phase cycling cell. Lastly, neurogenesis is now commonly detected with fluorescently labeled progenitor cells either by viral induction (van Praag et al., 2002; Zhao et al., 2006) or via transgenic reporter lines (Imayoshi et al., 2008; Lagace et al., 2007; Yamaguchi, 2005; Yamaguchi et al., 2000). Typical transgenic reporter lines used to label adult neural progenitor cells, including those in the hippocampus, use specific regions of the nestin promoter or gene to drive expression of green or yellow

fluorescent protein (GFP or YFP) in neuronal cell populations. For example, nestin-GFP mice express GFP only during nestin expression (Mignone et al., 2004; Yamaguchi et al., 2000) which makes it easier to track and study neural progenitor cells by morphological or electrophysiological analysis. However, more sophisticated transgenic lines have used the nestin promoter or wider areas of the nestin gene to inducibly label progenitor cells and their progeny (Carlen et al., 2006; Imayoshi et al., 2008; Lagace et al., 2007; Yu et al., 2005). As described below, both reporter and inducible nestin-driven transgenic lines have been useful in delineating discrete stages of neurogenesis (Kempermann et al., 2004). A key focus of this thesis is to further explore the regulation of neurogenesis in both the nestin-GFP and inducible nestin transgenic mouse lines.

Stages of adult hippocampal neurogenesis

Adult neurogenesis persists in the hippocampus of the brain of rodents, primates and humans among other mammalian species (Altman and Das, 1965; Eriksson et al., 1998; Kornack and Rakic, 1999). Specifically, within the hippocampus new neurons are born in a discrete region of the dentate gyrus (DG) termed the SGZ. This region is defined as an area three cell widths into the hilus and half of the granule cell layer (Figure 1.1). Cells that are born in the SGZ are not simply a single type of new cell, but rather a heterogeneous population of newly born, differentiating and mature cells (Kempermann et al., 2004; Kronenberg et al., 2003). Type 1 stem like cells, also referred to as radial glial-like cells, can give

rise to a rapidly amplifying cell, or the traditional proliferating progenitor cell. Rapidly amplifying cells divide and then exit the cell cycle or become post-mitotic, and pass through a series of stages where they become morphologically mature, start to express mature neuronal markers and eventually become incorporated into the existing hippocampal circuitry (Kempermann et al., 2004; Mignone et al., 2004; Ming and Song, 2005). This process can occur within the timespan of 1 to 4 weeks (Dayer et al., 2003). Figure 1.2 depicts the different stages of neurogenesis in more detail, as defined by marker expression and morphological features, as determined with a nestin-GFP transgenic mouse (Yamaguchi et al., 2000). Type 1 cells have a triangular cell soma, extend their dendrites into the molecular layer, and express the characteristic markers glial fibrillary acidic protein (GFAP) and GFP. Type 2 cells have a small, irregular shaped soma with no dendrites and can be further delineated into type 2a and type 2b depending on the presence (type 2b) or absence (type 2a) of doublecortin (DCX) expression (Kronenberg et al., 2003). Immature neuronal progenitor cells express DCX but lose expression of GFP and are defined by their round cell soma and process extending into the molecular layer. Progenitor cell expression of neuronal nuclei (NeuN) and extension of an elaborate dendritic process are typical indications that a progenitor cell has become a mature neuron. Lineage analysis studies support this chronological sequence of events (Suh et al., 2007). It has also been shown that mature neurons make functional synaptic contacts with the existing hippocampal circuitry and can form dendritic spines (Hastings et al., 1999; Markakis and Gage, 1999; Stanfield and Trice, 1988; Toni et al., 2008; Zhao et

al., 2006). The postnatal-generation of DG granule cell neurons and their clear contribution to the circuitry of the hippocampus is even more intriguing in that the process of adult neurogenesis is highly regulated, as described below.

Regulation of adult hippocampal neurogenesis

The process of neurogenesis has been shown to be very sensitive to exogenous and endogenous stimuli. Levels of adult hippocampal neurogenesis vary depending on mouse strain (Hayes and Nowakowski, 2002; Kempermann and Gage, 2002; Schauwecker, 2006) as well as age, with adolescent animals exhibiting more neurogenesis than aged animals (Maslov et al., 2004; Olariu et al., 2007). Although it is unknown why levels of adult neurogenesis vary with strain or age, it has been established that this is not due to differences in cell cycle length (Hayes and Nowakowski, 2002; Olariu et al., 2007). Many exogenous stimuli have been shown to alter adult neurogenesis. Some examples include exercise (Kempermann et al., 2003), environmental enrichment (Komitova et al., 2005), stress (Gould et al., 1997; Pham et al., 2003), application or inhibition of growth factors (Jin et al., 2002; Koo and Duman, 2008; Scharfman et al., 2005), irradiation (Monje et al., 2002) and antidepressants (Encinas et al., 2006; Wang et al., 2008). The mechanisms for how stimuli such as stress, growth factors or exercise alter neurogenesis have not been determined. Interestingly, although it has been suggested that neurogenesis is important for learning, the process of learning itself can also alter proliferation (Epp et al., 2007; Gould et al., 1999). Lastly, it has recently been shown that the maturational

stage of a progenitor cell can determine its response to certain stimuli (Dupret et al., 2007; Ge et al., 2007; Kee et al., 2007; Overstreet-Wadiche and Westbrook, 2006; Tashiro et al., 2007), increasing the complexity of the study of adult neurogenesis.

Most drugs of abuse have also been shown to alter neurogenesis. These include opiates, such as morphine and heroin (Eisch et al., 2000; Kahn et al., 2005), nicotine (Abrous et al., 2002), cocaine (Dominguez-Escriba et al., 2006; Noonan et al., 2008), methamphetamine (Mandyam et al., 2008), alcohol (Crews et al., 2006; Nixon and Crews, 2002) and 3, 4-methylenedioxymethamphetamine or ecstacy (Hernandez-Rabaza et al., 2006). The effects of other drugs of abuse on proliferation and neurogenesis, such as cannabis and its active ingredient Δ9-tetrahydrocannabinol, are still controversial (Kim et al., 2006; Kochman et al., 2006). It is also unclear how a more commonly studied drug such as morphine, alters neurogenesis. This will be a major focus of this thesis.

Function of adult hippocampal neurogenesis

While it is hypothesized that adult neurogenesis is important for learning and memory, this hypothesis is not yet confirmed (Aimone et al., 2006; Kempermann, 2002; Wiskott et al., 2006). For example, several studies have shown that adult neurogenesis is essential for the formation of a spatial or hipppocampal dependent memory (Shors et al., 2001; Snyder et al., 2005; Winocur et al., 2006). However, other studies have shown that neurogenesis is not essential for

certain types of spatial memory formation (Madsen et al., 2003; Shors et al., 2002). Many recent studies that have tried to unravel this inconsistency have revealed that the role of adult neurogenesis in learning and memory is quite complex, depending on both the task used to assess spatial memory (Saxe et al., 2007) as well as the removal and addition of adult hippocampal neurons (Dupret et al., 2007). A better understanding of both the process and regulation of neurogenesis will help to understand these conflicting results.

Morphine

History

Morphine has a long history of use in humans with the Greeks and Romans purportedly using morphine for medicinal use as far back as 300 BC (van Ree et al., 1999). In the 19th century, morphine was used to prepare patients for surgery as well as for treatment of pain, both postoperative and chronic (van Ree et al., 1999). Interstingly, morphine was also used as a cough suppressant, sleep aid, calmer of nerves (Aurin, 2000), and was used by several artists and writers of the 19th century for its psychological effects in enhancing creativity (Miller and Tran, 2000). The isolation of the active component of opium, morphine, synthesis of its derivative heroin, and doctor-prescribed use of morphine and heroin via injection coincided with increased risk for patients to become dependent on the drug (Aurin, 2000). The use of opiates was banned in the early 20th century in the United States, but was legalized for medical purposes, when prescribed (Aurin, 2000). Although morphine, heroin, and other opiates (such as oxycontin) are

used in the present day for treatment of chronic pain, they have an enormous potential for abuse. This is due to tolerance that develops after chronic use, negative effects of withdrawal (van Ree et al., 1999) as well as the growing availability of prescription opiates. Heroin abusers have impaired cognition (Guerra et al., 1987), hippocampal hyperactivity during drug craving (Daglish et al., 2003), and altered levels of immature neurons in the hippocampus (Weber et al., 2006). Determining the neuromechanism of morphine's effects on adult neurogenesis will help to advance treatment for opiate addiction.

Signaling and opiate receptor distribution in the brain

Morphine preferentially binds to the mu opioid receptor (MOR), a $G_{i/o}$ protein coupled receptor, and has lower affinity for the kappa and delta opioid receptors. The interaction between morphine and its receptor initiates a series of downstream cascade events. Acute exposure to morphine causes inhibition of adenyl cyclases and the cAMP pathway, activation of inwardly rectifying K+ channels and inhibition of voltage-gated Ca^{2+} channels, which together result in reduced neuronal excitability and neurotransmitter release (Nestler, 2004). However, after chronic morphine exposure, it is postulated that cells undergo compensatory action. Following chronic morphine exposure, there is increased activation of the adenyl cyclases and the cAMP pathway resulting in increased excitability of neurons, leading to tolerance and dependence (Nestler, 2004).

Opiate receptors are dense in the rat and the mouse brain, and are present on a variety of cell types. In rat, qualitative studies suggest that the relative density of MORs in the hippocampus is greater than that of the delta receptor, with a very low density of kappa receptor both via qualitative (Temple and Zukin, 1987) and quantitative autoradiography (Mansour et al., 1987). Studies that employed quantitative autoradiography of mu, delta and kappa receptor expression show that MOR expression is highest in the hippocampus, with half the expression of delta and very little to no expression of kappa receptor, though relative expression of receptors may not reflect activity of the receptor (Sharif and Hughes 1989). In mice, however, it seems that mu and kappa receptor densities are similar, with very low expression of kappa (Moscowitz and Goodman, 1984; Kitchen et al., 1997; Goody et al., 2002). However, it must be taken into account that these studies all have the similar problem of specificity of radioligand, as outlined in Tables 1.1 and 1.2. MORs are present in the hippocampus, a brain region that has been shown to be important for spatial memory, much like adult neurogenesis itself, and this is supported by a wide variety of studies that explore the effect of exogenous opiates on memory processes (Ben-Shaanan et al., 2008; Daglish et al., 2003; Guerra et al., 1987; Miladi Gorji et al., 2008; Spain and Newsom, 1991). Within the hippocampus, the MORs are on both primary cells (CA1-3 pyramidal and DG granule cell neurons) as well as interneurons (Drake et al., 2007). Of course, presence of opiate receptors in the hippocampus does not restrict opiate action to the hippocampus. In fact, the wide CNS distribution of opiate receptors makes it challenging to narrow the brain region of

action of morphine, including its action on adult hippocampal neurogenesis. It is thought that opiate-induced inhibition of hippocampal neurogenesis occurs via actions of opiates at MOR: systemic administration of the MOR antagonist naloxone attenuates the morphine-induced inhibition of hippocampal progenitor cell proliferation (Eisch et al., 2000).

Given that the focus of this thesis is on the effect of opiates on hippocampal progenitor cells, it is reasonable to ask whether MORs are on hippocampal progenitors. MORs are present on SVZ progenitor cells (Sargeant et al., 2007), cerebellar progenitor cells (Hauser et al., 2000) and on dendrites of mature hippocampal granule cells (Drake et al., 2007). However, lack of specific MOR antibodies and multiple splice variants of MOR have made it challenging to extend these studies to hippocampal progenitor cells (Eisch and Harburg, 2006). While work is ongoing in the Eisch Laboratory to identify whether or not MOR are on progenitor cells, this thesis contributes to the understanding of this complex issue by addressing the possibility that morphine can also alter progenitor cells indirectly through alteration of the progenitor cell neurogenic niche (Eisch and Harburg, 2006), as described in Chapter 4.

Morphine's effect on memory

Several studies in humans and in rodents have examined the impact of extended exposure to morphine on spatial memory. Rats exposed to chronic morphine, via subcutaneous (s. c.) pellet implantation have impairment in acquisition of a

reference memory as assessed by the radial arm and Y-maze tasks (Spain and Newsom, 1991). A subsequent study, in which the morris water maze was used to assess spatial memory, found that acquisition and retention of a short-term spatial memory was not altered with chronic exposure to morphine, but that retention of a long-term spatial memory was impaired (Miladi Gorji et al., 2008). These studies are particularly interesting given the hypothesis of the importance of adult hippocampal neurogenesis to spatial memory processes (Canales, 2007). A recent study indeed has shown that a morphine-induced decrease in neurogenesis (via prenatal morphine expousure) correlated with impared performance in the morris water maze (Ben-Shaanan et al., 2008). Strikingly, when proliferation was normalized by neural stem implantation in adulthood, the deficits in neurogenesis and spatial memory were restored.

Given the intriguing effect of morphine's inhibition on spatial memory, a process dependent on the hippocampus, and morphine's inhibition of hippocampal progenitor cell proliferation, it is of importance to elucidate exactly how this drug of abuse exerts its effect on adult hippocampal proliferation and neurogenesis (Canales, 2007; Eisch and Harburg, 2006). The relevance of studying morphine's effects on proliferation are also particularly important given that heroin abusers have cognitive deficits (Guerra et al., 1987), hippocampal hyperactivity during drug craving (Daglish et al., 2003) and altered levels of immature hippocampal neurons (Weber et al., 2006).

Effect on hippocampal progenitor cells

It was first demonstrated that SGZ progenitor cell proliferation and adult neurogenesis was decreased in rats exposed to chronic morphine, via s.c. pellet implantation or by self-administration of heroin (Eisch et al., 2000). Though the self-administration paradigm is more clinically relevant than s.c. pellet administration,(Eisch et al., 2000; Noonan et al., 2008), it is important to note that the magnitude of decreased neurogenesis was similar with both self-administration and s.c. pellet administration (Eisch et al., 2000). It was further demonstrated that SGZ progenitor cell proliferation was decreased in mice that received chronic morphine via s. c. morphine pellets (Mandyam et al., 2004), as well as in rats that received morphine via intraperitoneal (i.p.) injections (Kahn et al., 2005). Furthermore, a time course of chronic morphine's effects determined that SGZ progenitor cell proliferation was decreased after 24, 72 and 96 hrs of morphine exposure (Fischer et al., 2008). Although an escalating i.p. morphine paradigm did not result in decreased SGZ progenitor cell proliferation in mice, this likely was due to the difference in blood levels of morphine achieved with pellet vs. injections (Fischer et al., 2008). With the exception of escalating i.p. injections of morphine in mice, the strength of morphine administration is its consist inhibitory effect on SGZ neurogenesis, making it a useful paradigm to study morphine effects on neurogenesis as well as learn more about the progenitor cell response to exogenous stimuli.

After it was established that opiates decrease hippocampal progenitor cell proliferation, a possible mechanism of morphine's effects on adult hippocampal progenitor cells was determined: morphine causes premature mitosis of S phase cycling cells (Mandyam et al., 2004). However, several questions remain to be answered, including determination of which portions of the SGZ progenitor cell cycle are most vulnerable to morphine's effect and what stages of neurogenesis are more susceptible to morphine. By comparing and contrasting the effects of morphine on particular aspects of SGZ neurogenesis with other stimuli in the field (such as antidepressants, exercise and stress) it can be determined which stages of neurogenesis are more sensitive to alteration.

Effect on neurogenic niche

Progenitor cells in both the SGZ and SVZ reside in neurogenic niches rich in vasculature and key factors, such as vascular endothelial growth factor (VEGF), interleukin-1β (IL1β), and brain-derived neurotrophic factor (BDNF) (Riquelme et al., 2008), and often proliferate in close proximity to vasculature elements (Heine et al., 2005; Mercier et al., 2002; Palmer et al., 2000). Many studies show a correlative relationship between manipulation of factors in the neurogenic niche and hippocampal neurogenesis (Jin et al., 2002; Koo and Duman, 2008; Scharfman et al., 2005). Interstingly, heroin abusers also have altered levels of circulating growth factors (Angelucci et al., 2007) suggesting the possibility that neurotrophic factors may indirectly mediate the morphine-induced cognitive deficit in both humans and rodents (Spain and Newsom, 1991). However, there

are very few studies that have looked at changes in the neurogenic niche following morphine exposure. While a few studies have looked at changes in neurotrophic factors after morphine administration, these studies primarily observed changes in the ventral tegmental area (Akbarian et al., 2002). Although observing changes in the neurogenic niche would provide for an indirect mechanism of how morphine exerts its effect on progenitor cell proliferation, it is nonetheless important since morphine is likely to exert its effect not only through a direct mechanism. A recent study demonstrated that mice exposed to morphine prenatally had diminished levels of the unprocessed growth factor BDNF in the hippocampus (Schrott et al., 2008). *In vitro* data has also implicated morphine's impact on growth factors: morphine inhibits an increase in VEGF that is usually observed after myocardial infarction (Balasubramanian et al., 2001; Roy et al., 2003). Furthermore the clinical relevance of morphine actions on neurotrophins are important in that it is noted that serum levels of BDNF are altered in heroin abusers (Angelucci et al., 2007).

The above introduction has given facts about morphine's relationship to adult neurogenesis, however it is apparent that there are many questions that need to be addressed. These questions are discussed below.

Organizing hypothesis

Most drugs of abuse alter adult hippocampal neurogenesis. While the mechanism of how each each drug alters neurogenesis remains to be answered,

drugs of abuse can have very similar effects on progenitor proliferation but very different effects on progenitor cell maturation (Noonan et al, 2008). Knowing the pathways by which each drug diminishes adult neurogenesis will be essential to determine the detrimental effect of drugs on neural processes, as well as elucidate the function of adult neurogenesis. Therefore this thesis addresses questions that are related to morphine's effect on adult hippocampal progenitor cells. Specifically, I determine a time course of morphine's effect on progenitor cell proliferation and death. Furthermore I also address whether morphine alters progenitor cell cycle dynamics, length of S phase and the hippocampal neurogenic niche. Lastly, I address whether morphine alters proliferation in an inducible transgenic mouse model.

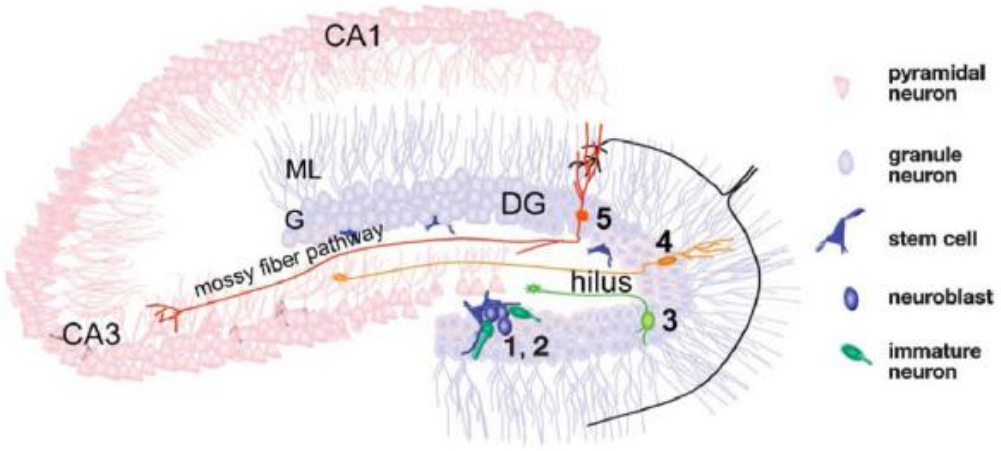

Figure 1.1. **Adult neurogenesis occurs in the hippocampal SGZ.** New neurons are born in the DG of the hippocampus, in a discrete region termed the SGZ. This SGZ region is defined as an area three cell widths into the hilus and half of the granule cell layer **(1)** Cells that are born in the SGZ start off as type 1 stem like cells, also referred to as radial glial, which give rise to **(2)** a rapidly amplifying cell, also called a neuroblast or the traditional proliferating progenitor cell. Rapidly amplifying cells divide, exit the cell cycle, and go through a series of stages **(3-5)** where they become morphologically mature, start to express mature neuronal markers, move into the inner portion of the granule cell layer. The newly born cells eventually become incorporated into the existing hippocampal circuitry as DG granule cell neurons and extend their axons into the mossy fiber pathway where they contact the dendrites of pyramidal neurons of CA3. CA1, cornu ammons area 1; CA3, cornu ammons area 3; DG, dentate gyrus; G, granule cell layer; ML, molecular cell layer. Figure from Ming and Song, 2005.

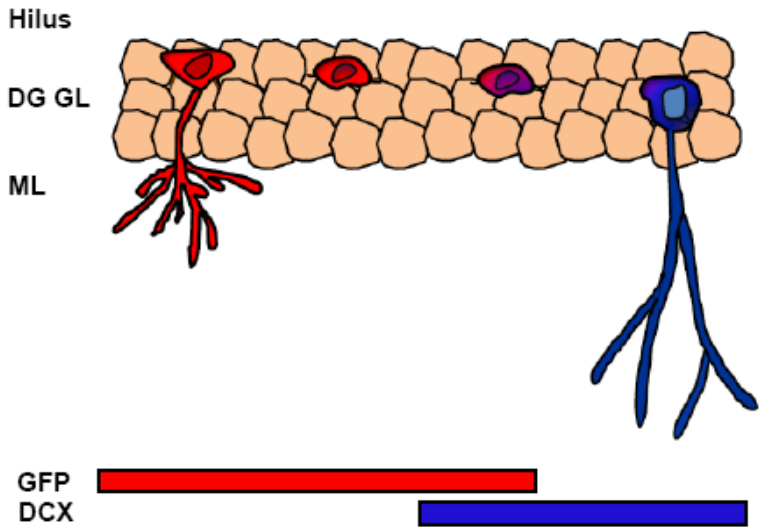

Figure 1.2. **Stages of adult hippocampal neurogenesis**. This process of neurogenesis occurs over a timespan of 1 to 4 weeks. The discrete stages of neurogenesis are defined by marker expression and morphological features. Type 1 cells have a triangular cell soma, extend their dendrites into the molecular layer, and express the characteristic marker GFP in the nestin-GFP reporter mouse (Yamaguchi et al., 2000). Type 2a cells have a small, irregular shaped soma with no dendrites and express GFP. Type 2b cells also have a small, irregular shaped soma but express GFP and the immature neuronal protein DCX (Kempermann et al., 2004). Immature neuronal progenitor cells express and DCX and are defined by their round cell soma and process. Mature neurons (not pictured) will gain expression of NeuN in addition to a more extensive dendritic process. Figure adapted from Arguello et al., 2008.

In Mouse						
Paper	**Method**	**Ligands Used**	**Region**	**Mu**	**Delta**	**Kappa**
Moskowitz, 1984	Quantitative autoradiography	mu=^{3}[H]dihydromorphine del=^{3}[H] enkephalin	Hpp stratum mol	(fmol/mg tissue)		
			CA1	11.3	19.7	N/A
			CA2	8.4	21.7	
			CA3	22.7	31.7	
			Hpp pyramidal			
			CA1	9.8	0.37	
			CA2	12.7	0.38	
			CA3	9.8	0.34	
Yasuda, 1993	In situ hybrid.	N/A	CA3, Dentate GCL	No units		
				N/A	N/A	High
Kitchen, 1997	Quantitative autoradiography	mu=^{3}[H] DAMGO del=^{3}[H] DELTI kap=^{3}[H] bemazocine +unlab. DAMGO,DPDPE		(fmol/mg tissue)		
			HPP	14.2	25.5	10.8
			DG	51.8	N/A	N/A
Goody, 2002	Quantitative autoradiography	mu=^{3}[H] DAMGO del=^{3}[H] DELTI kap=^{3}[H] bemazocine +unlab. DAMGO,DPDPE	HPP	(fmol/mg tissue)		
				20.4	38.4	2.4

***Table 1.1*. Expression of opioid recetors in mouse brain.**
Comparative expression of the opioid receptors, mu, delta and kappa, in the mouse brain as determined by quantitative autoradiography or in situ hybridization from various studies (Goody et al., 2002; Kitchen et al., 1997; Moskowitz and Goodman, 1984; Yasuda et al., 1993)

In Rat						
Paper	**Method**	**Ligands Used**	**Regions**	**Mu**	**Delta**	**Kappa**
Temple, 1987	Quantitative autoradiography	mu=^{3}[H] mu specific Enk del=^{3}[H] del specific Enk kap= ^{3}[H] EKC		(fmol/mg protein)		
			Stratum oriens	ND	ND	28.4
			Stratum molecular	ND	ND	28.6
			CA2	55.7	ND	41.9
			CA3	28.7	ND	41.9
			GCL	ND	ND	51
			Pyramidal cell layer	32.2	ND	41.9
Mansour, 1987	Qualitative autoradiography	mu=^{3}[H] DAGO del= ^{3}[H]DPDPE kap=^{3}[H] bemazocine +unlab. DAMGO,DPDPE		No units		
			Pyramidal CL	+++	++	+
			Dentate GCLventral	++++	+	+
Sharif, 1989	Quantitative autoradiography	mu=^{3}[H] DAGO del=^{3}[H] DPDPE kap=^{125}[I] dynorphin		(amol/mm^{3} tissue)		
			HPP mol lay	176	N/A	0.4
			HPP pyramidal lay	211	83	N/A
			HPP DG- ventral	265	N/A	N/A
			HPP granule	N/A	N/A	0.3

***Table 1.2*. Expression of opioid recetors in mouse brain.**
Comparative expression of the opioid receptors, mu, delta and kappa, in the rat brain as determined by quantitative and qualitative autoradiography from various studies (Mansour et al al., 1997; Sharif and Hughes, 1989; Tempel and Zukin, 1987).

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CHAPTER TWO

Time course of chronic morphine-induced inhibition of hippocampal cytogenesis

Previously published: **Arguello AA**, Harburg GC, Schonborn JR, Mandyam CD, Yamaguchi M, Eisch AJ. 2008. Time course of morphine's effects on adult hippocampal subgranular zone reveals preferential inhibition of cells in S phase of the cell cycle and a subpopulation of immature neurons. Neuroscience 157(1):70-79.

Introduction

Almost forty years after the initial observation that hippocampal neurogenesis persists into adulthood (Altman and Das, 1965), intriguing links have arisen between the dynamic regulation of adult neurogenesis and psychiatric disorders, such as addiction and depression (Eisch, 2002; Jacobs et al., 2000). Opiates were the first drug of abuse shown to negatively impact proliferation and neurogenesis in the adult mammalian hippocampus (Eisch et al., 2000), and other drugs of abuse have since been shown to also decrease neurogenesis (Crews et al., 2006; Dominguez-Escriba et al., 2006; Mandyam et al., 2008b; Nixon and Crews, 2002; Noonan et al., 2008; Yamaguchi et al., 2004). The reduction in neurogenesis caused by drugs of abuse is hypothesized to contribute to the cognitive deficits observed in addicts (Canales, 2007; Eisch and Harburg, 2006). For example, chronic morphine interferes with memory and

hippocampal function in rodents (Miladi Gorji et al., 2008; Spain and Newsom, 1991), and human heroin addicts have altered neurogenesis and hippocampal hyperactivity during drug craving (Daglish et al., 2003; Weber et al., 2006). While additional work is needed to understand the functional importance of opiate-induced alterations in adult hippocampal neurogenesis, another critical step is to clarify the cellular mechanisms underlying opiate-induced regulation of neurogenesis. Given the obvious interest in how neurogenesis is regulated by physiological and environmental stimuli (Kee et al., 2007; Overstreet-Wadiche and Westbrook, 2006), such mechanistic research on opiates would benefit not only the field of addiction, but neural stem cell biology as a whole.

Some key advances have been made in understanding how opiates inhibit neurogenesis in the adult subgranular zone (SGZ). For example, chronic, but not acute, opiates act via the mu opiate receptor (MOR) to decrease SGZ proliferation (Eisch et al., 2000). Opiate-induced reduction in SGZ proliferation occurs after myriad administration paradigms, including self-administration, subcutaneous (s.c.) pellet implantation, or intraperitoneal (i.p.) injection (Fischer et al., 2008; Kahn et al., 2005; Mandyam et al., 2004). Interestingly, the decreased proliferation is not due to decreased physical inactivity or enhanced levels of stress hormones (Eisch et al., 2000). While these studies provide essential information about opiate-induced reduction of SGZ proliferation, it is still not clear how the decrease in neurogenesis occurs. One possibility is that an altered cell cycle of progenitor cells contributes to decreased SGZ proliferation in

mice (Eisch and Mandyam, 2007). Indeed, chronic morphine results in premature mitosis of proliferating cells (Mandyam et al., 2004). However, cell cycle dynamics have only been evaluated at a single time point after chronic morphine exposure (96 hrs), and therefore a time course analysis is warranted. Increased cell death may also contribute to decreased SGZ proliferation, a theory supported by non-neurogenesis studies in which morphine increases hippocampal levels of activated caspase-3 (AC3) measured via immunoblotting (Emeterio et al., 2006). However, since neurogenesis studies find no increase in the number of dying SGZ cells following 96 hrs of morphine exposure (Eisch et al., 2000), additional time points should be examined. Finally, while neurogenesis is now appreciated to be a multi-staged process (Kempermann et al., 2004a) and specific stages of neurogenesis are preferentially altered by stimuli such as antidepressants and running (Encinas et al., 2006; Fukuda et al., 2003; Kronenberg et al., 2003; Overstreet-Wadiche and Westbrook, 2006), it is not clear how the multi-stage process of neurogenesis is affected by opiates. Immature neuron number, as determined by polysialic-neural cell adhesion molecule (PSA-NCAM) expression, is decreased by chronic morphine injections (Kahn et al., 2005), but a detailed analysis of morphine's impact on the progression from Type 1 stem cell to immature neurons has yet to be examined.

To gain insight into whether chronic morphine causes a decrease in hippocampal SGZ neurogenesis via alterations in cell cycle, cell death, or progression through stages of neurogenesis, an s.c. morphine pellet administration paradigm that

decreases proliferation in the adult mouse SGZ was utilized (Fischer et al., 2008; Mandyam et al., 2004). After 24 or 96 hrs of morphine exposure, changes in cell cycle, cell death and immature neurons were examined by quantifying the number of SGZ cells immunoreactive (IR) for the exogenous S phase marker bromodeoxyuridine (BrdU), the endogenous cell cycle marker Ki67, the cell death marker AC3, and the immature neuron marker doublecortin (DCX). Furthermore, to examine the effect of morphine on maturing progenitor cells, nestin-green fluorescent (GFP) mice were utilized to examine the impact of morphine on the progression of GFP-IR progenitors through discrete stages of maturation. These data demonstrate that cells in specific phases of the cell cycle as well as stages of progenitor cell maturation are highly sensitive to the effects of morphine, and thus provide novel insight into morphine's effects on proliferation and maturation.

Materials and Methods

Animals

Two experiments were performed using two strains of adult male C57BL/6J mice. For Experiment 1, C57BL/6J mice were received from Jackson laboratories (Bar Harbor, ME, USA, stock #: 000664) at least one week prior to experimentation. For Experiment 2, homozygous nestin-GFP mice expressing green fluorescent protein under the control of the nestin promoter were bred at UT Southwestern Medical Center. Nestin-GFP mice were initially generated and since maintained on a C57BL/6J background (Yamaguchi et al., 2000). All mice were group-housed four to a cage with free access to food and water in a facility at UT

Southwestern Medical Center run on a 12 hr light/dark cycle. The Institutional Animal Care and Use Committee (IACUC) approved all procedures. All measures were taken to minimize animal suffering and the number of animals used. All animal use procedures were in accordance with the U.S. National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Drug Treatment and BrdU Injections

For both Experiments 1 and 2, mice were given one 25 mg morphine pellet (generously provided by the National Institute on Drug Abuse, Bethesda, MD) or sham pellet/surgery s.c. under isoflurane anesthesia (2% in compressed oxygen for 1-2 minutes) at 0 and 48 hrs (Mandyam et al., 2004; Shaw-Lutchman et al., 2002). Morphine pellet administration was chosen due to the stable blood levels of morphine, high level of physical dependence, and decreased SGZ proliferation achieved in mice as compared with an escalating paradigm of morphine i.p. injections (Fischer et al., 2008). For Experiment 1, mice were given one 150 mg/kg injection of BrdU (i.p., Roche Diagnostics, Indianapolis, IN, USA, cat. #: 280-879, dissolved in 0.9% saline, 0.007N NaOH at 10 mg/mL) 2 hrs prior to sacrifice via live decapitation at 24, 72 or 96 hrs (sham n=4-8; morphine n=3-8). One hemisphere of the brain was post fixed in 4% paraformaldehyde for three days, followed by cryoprotection in 30% sucrose as previously described (Lagace et al., 2007c). For Experiment 2, one 150 mg/kg injection of BrdU was given 1 day prior to drug or sham administration so that the effect of morphine on maturing BrdU-IR cells could be examined. At 96 hrs, mice were anesthetized

with chloral hydrate (120 mg/kg dissolved in 0.9% saline) and perfused transcardially with 0.1M PBS (sham n=7-8; morphine n=6-7) followed by 4% paraformaldehyde, as previously described (Harburg et al., 2007).

Antibodies and Immunohistochemistry (IHC)

Tissue Sectioning and Preparation. Serial sets of 30μm coronal sections from brains were collected on a freezing microtome (Leica, SM2000R, Bannockburn, IL, USA) into nine wells containing 0.1% NaN_3 in 1X PBS and stored at 4°C until processing. For each set of antibodies examined, one well containing the entire mouse hippocampus, -0.82 to -4.24 from bregma (Franklin and Paxinos, 1997), was slide mounted prior to IHC. In preparation for subsequent analysis via stereological cell counting via the modified version of the optical fractionator method (Cameron and McKay, 2001; Ngwenya et al., 2005) sections anterior and posterior to the hippocampus were also mounted, resulting in a total of about 13 sections mounted per mouse and making additional volumetric estimations of the hippocampus unnecessary. Slides were coded before IHC and the code was not broken until data analysis was complete (Mandyam et al., 2004).

Antibodies. The following primary antibodies were used: rat anti-BrdU (Accurate, Westbury, NY, Cat #: OBT0030, Lot #: H7786, 1:300 for single labeling, 1:500 for double or triple labeling); rabbit anti-Ki67 (Vector laboratories, Burlingame, CA, Cat #: VP-K451, Lot#: 301108, 1:500); goat anti-DCX (C-18; Santa Cruz Biotechnology, Santa Cruz, CA, Cat#: sc-8066, Lot#: 12305, 1:3000 for single

labeling, 1:5000 for triple labeling); rabbit anti-cleaved caspase-3 (Asp 175; Cell Signaling Technology Inc., Danvers, MA, 01923, Cat#: 9661, Lot#: 18, 1:500); rabbit anti-GFP (Invitrogen, Eugene, OR, Cat #: A11122, Lot #: 41279A, 1:500). For each antibody, the specificity of staining was determined by lack of signal after omission and/or dilution of the primary antibody as well as observation of a similar pattern of staining, as previously reported: AC3 (Cooper-Kuhn and Kuhn, 2002; Donovan et al., 2006), GFP (Donovan et al., 2008a), Ki67 (Dayer et al., 2003; Mandyam et al., 2007), DCX (Donovan et al., 2006; Lagace et al., 2007c).

Single Label IHC. Single label IHC was performed for antibodies against BrdU, Ki67, DCX, and AC3. For BrdU and AC3 IHC, the following pretreatment steps were used: antigen unmasking (0.01M citric acid, pH 6.0, 100°C), permeabilization with trypsin (trypsin in 0.1M Tris, 0.1% $CaCl_2$), DNA denaturation (2N HCl in 1X PBS), and quenching of endogenous peroxidases (0.3% H_2O_2), as previously described (Donovan et al., 2006). For Ki67 and DCX IHC, only antigen unmasking and endogenous peroxidase quenching steps were used. Tissue sections underwent blocking (3% serum, 0.3% Triton-X) before incubation in primary antibody overnight (in 3% serum, 0.3% Tween-20). IHC was completed using the avidin-biotin diaminobenzidine visualization method, followed by counterstain with nuclear fast red (Vector laboratories, Burlingham CA, Cat# H-3403, Lot#: N0507). Single labeling for AC3 was visualized with CY2-fluorophore conjugated secondary.

Double and Triple Label IHC. For double labeling for Ki67/BrdU pretreatment steps for Ki67 were performed (antigen unmasking, peroxidase quenching) followed by staining for Ki67 (visualized with FITC-TSA). Sections then underwent remaining pretreatment steps for BrdU (permeabilization and denaturation), blocking and incubation in primary antibody overnight, followed by visualization with CY2-flourophore conjugated secondary as described previously (Lagace et al., 2007c). For triple labeling with BrdU/DCX/GFP, antigen unmasking and peroxidase quenching steps were performed, followed by sequential staining for DCX (visualized with CY5-TSA) then GFP (visualized with CY3 conjugated secondary). Sections were then processed through remaining pretreatment steps for BrdU followed by visualization with CY2-conjugated secondary.

Microscopic analysis and quantification

BrdU-IR, Ki67-IR, and DCX-IR cell numbers were quantified with stereology via the modified optical fractionator method throughout the rostral-caudal axis of the hippocampus, -0.82 to -4.24 from bregma (Franklin and Paxinos, 1997) as previously described (Harburg et al., 2007; Mandyam et al., 2007). Briefly, immunoreactive cell and cluster SGZ counts were performed at 400X magnification on an Olympus BX-51 microscope (Tokyo, Japan) in brightfield or fluorescence with continuous adjustment of the focal plane. Resulting counts were multiplied by the number of sections analyzed (e.g. 9) to reveal total cell or cluster counts (Cameron and McKay, 2001; Ngwenya et al., 2005).The SGZ was

defined as the region bordering the granule cell layer and hilus: three cell widths into the hilus and the inner half of the granule cell layer (Donovan et al., 2006; Mandyam et al., 2007).

For double label Ki67/BrdU analysis, the number of Ki67-IR cells that were BrdU-IR was counted and vice versa. To determine percentages of Ki67-IR cells that were BrdU-IR, the ratio of BrdU-IR cells/(BrdU-IR and Ki67-IR cells) was calculated (Siegenthaler and Miller, 2005). Ki67, rather than proliferating cell nuclear antigen (PCNA), was utilized as an endogenous marker of cycling cells as it provides for more accurate cell cycle analysis due to its shorter in vivo half life (Mandyam et al., 2007).

For phenotypic BrdU-IR cell analysis, a confocal microscope was used (Zeiss Axiovert 200M and LSM510-META, emission wavelengths 488, 543, and 633, magnification 630X). An average of 95 ± 9 and 76 ± 14 BrdU-IR cells were analyzed in nestin-GFP reporter mice given sham surgery or morphine administration, respectively. BrdU-IR cells in the anterior hippocampus, -1.48 to -1.78 from bregma (Franklin and Paxinos, 1997), were categorized into discrete maturational stages via their morphological characteristics and co-localization with GFP and DCX as follows: Type 1 cells: triangular soma, dendrite extending into molecular layer, GFP+/DCX-; Type 2a transiently amplifying: ovoid cell body, no dendrite, GFP+/DCX-; Type 2b, ovoid cell body, GFP+/DCX+; immature neurons: round cell body, dendrite extending into molecular layer, GFP-/DCX+.

Scanning and optical sectioning in the *Z* plane was performed as described previously (Donovan et al., 2006) and fluorescently labeled confocal images presented here were taken from one optical slice and imported into Photoshop (Adobe Systems, San Jose, CA) for composition purposes. Adjustments to images were only made via gamma levels.

Statistical analyses

Data are reported as mean ± SEM. Statistical analyses were performed using student's t-test for comparison of two groups, or 1 way ANOVA for multiple groups followed by a Bonferroni *post hoc* test for selected groups. For repeated measure variables, a 2 way ANOVA was used to determine the effect of drug treatment (sham or morphine) on the number of Ki67-IR cells in S phase (BrdU+) or not in S phase (BrdU-) followed by a Bonferroni *post hoc* test to take into account the multiple comparisons made. All statistical analyses were performed using Prism (version 5.0) software. Statistical significance was defined as $p \leq 0.05$ with $*p \leq 0.05$, $**p<0.01$, $***p<0.001$.

Results

Chronic morphine decreases SGZ proliferation: analysis of exogenous and endogenous cell cycle markers

C57BL/6J mice were given morphine or sham pellets s.c. at 0 and 48 hrs. Two hrs before sacrifice (24, 72, or 96 hrs), BrdU was given to exogenously label proliferating cells (Figure 2.1A). There was a qualitative decrease in the number

of BrdU-IR cells in the hippocampal SGZ (Figure 2.1Bi, ii). There was no obvious morphological difference in proliferating cells, as BrdU-IR cells were typically found in the SGZ in clusters of at least two cells with condensed or punctate heterochromatin (Figure 2.1Biii) (Mandyam et al., 2004). Quantitatively, mice exposed to morphine had fewer BrdU-IR cells compared to sham mice ($F_{5,30}$=7.306, p=0.0001). Post hoc analyses between morphine and sham exposed mice were significantly different at each time point (Figure 2.1C; 24 hr: p<0.05, 72 hr: p<0.01, 96 hr: p<0.001). The number of Ki67-IR cells was also decreased after chronic morphine ($F_{5,30}$=7.683, p<0.0001). Interestingly, post hoc analyses revealed that the number of Ki67-IR cells was decreased only at 72 and 96 hrs (Figure 2.1D; 72 hr: p<0.001, 96 hr: p<0.01). There was not a quantitative difference in the number of BrdU-IR or Ki67-IR cells per cluster between sham and morphine groups at any time point (Figure 2.1E-F). These data suggest that the pattern of decreased proliferation is similar with assessment of an exogenous or endogenous marker with the exception of early exposure (24 hrs) to morphine.

Chronic morphine alters the ratio of cells cycling in S phase versus non-S phase

To examine for possible alterations in S phase versus total cycling cells, the number of Ki67-IR cells that were BrdU- or BrdU+ was determined after 24 or 96 hrs of morphine exposure. Ki67+/BrdU- cells represent cycling cells in non-S phases of the cell cycle (Figure 2.2A, dashed red line only; Figure 2.2B, open arrows) whereas Ki67+/BrdU+ cells represent cycling cells in S phase (Figure

2.2A, dashed red and green lines; Figure 2.2B, closed arrows). After 24 hrs of morphine exposure, there was only a significant main effect for Ki67 cell type ($F_{1, 6}$=97.04; p<0.0001; Figure 2.2C). After 96 hrs of morphine exposure, there was a significant main effect for Ki67 cell type ($F_{1, 11}$=84.07; p<0.0001) and a significant main effect for drug treatment ($F_{1, 11}$=33.84; p<0.0001) with no significant interaction between Ki67 cell type and drug treatment. Bonferroni post hoc tests revealed significant differences between sham and morphine groups within BrdU- (p<0.001) and BrdU+ (p<0.01) cell type (Figure 2.2D). This suggests that morphine does not differentially affect S phase versus total cycling cells, as there is no interaction between drug treatment and Ki67 cell type at 24 and 96 hrs with raw numbers of cells.

However, the effect of morphine on cells in S phase relative to non-S phase cycling cells can also be assessed by calculating the percentage of Ki67-IR cells that were BrdU-IR. There was a significant decrease in the percentage of Ki67-IR cells that were BrdU-IR after 24 hrs (t_6=4.39, p=0.0046), but no change after 96 hrs (Figure 2.2E, F). Thus after 24 hrs of morphine exposure, the number of S phase cells was disproportionately decreased relative to SGZ cells in the other cell cycle phases. This suggests that cells cycling in S phase are initially more sensitive to morphine's inhibitory effects than SGZ cells cycling in other phases of the cell cycle.

Chronic morphine transiently increases cell death in the SGZ

The results in Figure 2.2E suggest that morphine may induce cell death after 24 hrs of exposure to morphine. Therefore, the numbers of AC3-IR SGZ cells was quantified after 24 and 96 hrs of morphine exposure. As previously reported, AC3-IR cells were easily identified by their discrete cell morphology (Figure 2.3, inset) (Harburg et al., 2007). Mice exposed to morphine had more AC3-IR cells compared to sham mice ($F_{3, 20} = 3.473$, $p=0.035$). Post-hoc analyses between sham and morphine exposed mice revealed that numbers of AC3-IR cells were increased by almost 100% after 24 hrs, but returned to baseline values by 96 hrs (Figure 2.3A), augmenting previous results (Eisch et al., 2000). This suggests that cell death is transiently increased after early exposure to morphine, and therefore may contribute to the morphine-induced decrease in S phase cells seen at 24 hrs (Figures 2.1C, 2.2E).

Chronic morphine alters maturation of progenitor cells

Thus far the present results extend previous observations of morphine-induced decrease in progenitor cell proliferation (Fischer et al., 2008) by revealing a disproportionate inhibition of S phase cells relative to other phases. However, the effect of morphine on progenitor cell maturation has not been examined; such analysis has previously been useful in identifying the stage of neurogenesis susceptible to neurogenic stimuli (Encinas et al., 2006; Mandyam et al., 2008b; Wang et al., 2008). As a first step, the number of DCX-IR SGZ cells was quantified after 96 hrs of morphine exposure. The gross morphology and number

of DCX-IR cells between sham and morphine exposed mice was not significantly different, suggesting that the total population of DCX-IR cells was not decreased by morphine (96 hrs: sham = 1112.75 ± 70.4; morphine = 1236.67 ± 41.3). DCX is expressed by a heterogeneous population of progenitor and maturing cells (Dominguez-Escriba et al., 2006; Plumpe et al., 2006) and therefore insensitivity of this total population to morphine is not surprising. However, knowing the number of DCX-IR cells remained unchanged after morphine made the subsequent analysis on stages of maturation more straightforward to interpret (Kempermann et al., 2004a).

To address whether morphine alters specific stages of progenitor cell and immature neuron maturation, nestin-GFP reporter mice (Yamaguchi et al., 2000) were given a single 150mg/kg injection of BrdU 24 hrs before morphine pellet implantation (Figure 2.5A). As in Experiment 1, the number of Ki67-IR proliferating cells was decreased at 96 hrs (Figure 2.5B; t_{13}=3.223, p=0.0067), verifying that morphine decreases SGZ proliferation. However, there was not a significant decrease in the number of BrdU-IR cells at 96 hrs after the first morphine pellet (Figure 2.5C; t_{13}=1.065, p=0.31). This shows that cells labeled with BrdU 5 days earlier are insensitive to morphine's inhibitory effect, and is in line with the lack of change in total DCX-IR cell number.

In Experiment 2, progenitor cells were first labeled with BrdU and then matured in the presence of morphine, which allowed for examination of the maturational

stage of BrdU-IR cells via triple IHC for BrdU, DCX and GFP (Donovan et al., 2008a; Kempermann et al., 2003; Kempermann et al., 2004a). As previously shown, GFP protein was robustly expressed in the SGZ (Figure 2.5Di). The majority of BrdU-IR cells were either Type 2a transiently amplifying (Figure 2.5Dii, iii, open arrows; ovoid cell body, no dendrite, GFP+/DCX-) or Type 2b (Figure 2.5Div, closed arrow; ovoid cell body, GFP+/DCX+). A smaller percentage of BrdU-IR cells were immature neurons (Figure 2.5Diii, closed arrow; round cell body, dendrite extending into molecular layer, GFP-/DCX+). As expected, very few BrdU-IR cells were Type 1, stem-like cells (Figure 2.5Div, unlabeled with cell body out of focal plane, open arrow; triangular cell body with process extending into molecular layer, GFP+/DCX-) (Donovan et al., 2008a).

The percentage of BrdU-IR cells that were Type 1 or Type 2a was unchanged after chronic morphine. However, there was a significant increase in the percent of BrdU-IR cells that were Type 2b with a corresponding decrease in the percent of BrdU-IR cells that were immature neurons (Figure 2.5E; Type 2b: t_{11}=2.197, p=0.050; immature: t_{11}=2.504, p=0.029). Thus while there was no change in overall DCX-IR cell number or BrdU cell number when labeled 5 days prior, these data suggest that morphine inhibits neurogenesis in part by inhibiting maturation of progenitor cells to an immature stage.

Discussion

The current results show that chronic morphine alters specific aspects of progenitor cell proliferation and maturation in the adult mouse SGZ. S phase cycling cells were particularly vulnerable after shorter exposure to morphine, but the decrease in proliferation extended to all phases of the cell cycle after longer exposure. Consistent with previous results (Eisch et al., 2000), cell death was not changed after 96 hrs of morphine, but shorter exposure to morphine revealed a robust increase in SGZ cell death. This effect was specific to proliferating cells, as the total number of immature neurons was not changed by morphine. Furthermore, although the total number of immature neurons remained unchanged, the proportion of progenitor cells that progressed to a more mature stage decreased. The current data point to increased sensitivity of dividing SGZ cells and subpopulations of immature SGZ neurons in response to morphine.

Chronic morphine inhibits cycling progenitor cells

Several administration paradigms have revealed that chronic morphine decreases proliferation as measured by fewer BrdU-IR cells (Eisch et al., 2000; Kahn et al., 2005). Recently, a time course of proliferation after morphine pellet administration determined that the number of BrdU-IR cells was decreased after 24, 72 and 96 hrs of morphine exposure (Fischer et al., 2008). Here, these previous findings were extended by determining the time course of morphine's inhibition on the number of cells immunoreactive for the endogenous cell cycle marker Ki67. In many cases, BrdU (given 2 hr prior to sacrifice) and Ki67 are

interchangeable as proliferation markers (Komitova et al., 2005; Lagace et al., 2007a; Wojtowicz and Kee, 2006). In line with this idea, the present results reveal that after longer exposure to morphine (72 and 96 hrs) BrdU and Ki67 labeled cells were similarly decreased, emphasizing their interchangeable use as general proliferation markers. However after a shorter exposure to morphine (24 hrs), only BrdU labeled cells were decreased. Thus these data presented here emphasize that BrdU and Ki67 label distinct aspects of the cell cycle (S phase vs. entire cell cycle), and therefore should not always be used interchangeably to measure proliferation.

The results shown in Figure 2.2 take advantage of the fact that BrdU given 2 hrs before sacrifice results in S phase cycling cells labeled with Ki67/BrdU and non-S phase cycling cells labeled only with Ki67 (Mandyam et al., 2007). This combinatorial analysis of two cell cycle markers revealed a detailed picture of how the progenitor cell cycle is influenced by morphine over time: S phase cycling cells are preferentially decreased in number after 24 hrs relative to non-S phase cycling cells, but all aspects of the cycle are influenced after 96 hrs. These data suggest that initially morphine interferes with DNA synthesis, perhaps by targeting cell cycle proteins essential for entrance into or movement through S phase. Previous work has shown that after 96 hrs, morphine induces premature mitosis of SGZ cells (Mandyam et al., 2004). Furthermore, non-neuronal cells exposed to morphine *in vitro* have increased levels of phosphorylated p53 and p21 with a concurrent decrease in proliferation (Tegeder et al., 2003), and loss of

the cell cycle related proteins p27Kip1 or p21cip1 can increase proliferation in the SVZ and hippocampal dentate gyrus (Doetsch et al., 2002; Pechnick et al., 2008). Examination of whether these and other S phase and cell cycle proteins are altered by morphine *in vivo* will be critical to understanding how morphine acts to alter adult hippocampal neurogenesis.

Chronic morphine increases progenitor cell death

A notable finding from the present work is that morphine exposure produced a robust increase in cell death in the SGZ after 24 hrs, but not 96 hrs, extending previous results on levels of cell death proteins in total hippocampus (Emeterio et al., 2006). Thus it will be important to further assess the relationship between the transient but robust increase in cell death reported here on S phase cycling cells at 24 hrs. For example, perhaps the decrease in S phase cells after 24 hrs is due to cell death, but the decrease in total number of cycling cells after 96 hrs reflects a new homeostatic number of proliferating cells. Phenotypic analysis of AC3-IR cells would be necessary to further explore this hypothesis. As AC3-IR cells are difficult to co-label with BrdU or non-death associated proteins (Bauer and Patterson, 2005; Cooper-Kuhn and Kuhn, 2002), testing of this hypothesis awaits additional technical advances in detection of cell death. However, since cell cycle and cell death are closely linked (Iaquinta and Lees, 2007), elaboration of the relationship between morphine-induced cell death and inhibition of proliferation is likely to be fruitful in explaining how morphine alters adult hippocampal neurogenesis.

Chronic morphine inhibits maturation of progenitor cells

Numerous neurogenesis studies have demonstrated that SGZ cells in discrete stages of progenitor cell maturation are differentially sensitive to physiological and pharmacological stimuli (Encinas et al., 2006; Kronenberg et al., 2003; Mandyam et al., 2008a; Mandyam et al., 2008b). Although the effect of stimuli such as running, antidepressants, and environmental enrichment on the maturation of progenitor cells has been well characterized (Ming and Song, 2005; Plumpe et al., 2006; Wang et al., 2008), the effect of chronic morphine on maturation of SGZ progenitors had not been assessed. In light of previous work showing that Type 2a cells are preferentially influenced by antidepressants and running, one of the most interesting findings from the present study is that morphine increased the percentage of BrdU-IR cells that were Type 2b and decreased the percentage that were DCX-IR. The present data add to the growing literature showing that the maturational stage of a progenitor cell influences its response to stimuli (Ge et al., 2007; Kee et al., 2007; Overstreet-Wadiche and Westbrook, 2006; Tashiro et al., 2007). Taken together with the finding that total number of immature neurons (identified by DCX or BrdU given 5 days prior) does not change after 96 hrs of morphine, these data emphasize that only a subpopulation of DCX-IR cells are sensitive to morphine. Since immature neurons appear to be functionally significant after integration into hippocampal circuitry (Doetsch and Hen, 2005), disruption of their maturation by morphine may be detrimental to hippocampal function. Future work could address this hypothesis by evaluating whether the dendritic morphology of immature neurons

is altered after morphine exposure, as has been assessed after antidepressants, seizures, or drugs of abuse (Hernandez-Rabaza et al., 2006; Jessberger et al., 2005; Wang et al., 2008).

Receptor expression of progenitor cells

Why are the percentages of BrdU-IR cells that are in the Type 2b and immature neuron stages preferentially affected by morphine? One possibility is that these subpopulations of progenitor cells express specific receptors that enable them to differentially respond to morphine directly or, more likely, to alterations in the neurogenic microenvironment (Duan et al., 2008; Encinas et al., 2006; Plumpe et al., 2006; Wang et al., 2008). In regards to responding to morphine directly, it remains unclear when the MOR is expressed on progenitor cells (Drake et al., 2007; Eisch and Harburg, 2006), but several lines of indirect evidence suggest that morphine and opioid receptors are important in neurogenesis (Drake et al., 2007; Eisch et al., 2000; Harburg et al., 2007; Hauser et al., 2000; Koehl et al., 2008). In light of the present data that morphine decreases the percent of BrdU-IR cells that reach an immature stage, it is interesting that mice that lack the MOR from birth have increased survival of dentate gyrus granule cells (Harburg et al., 2007). However, more work is needed to clarify if morphine directly inhibits particular progenitor cell types. In regards to responding to alterations in the neurogenic microenvironment, it is known that cells in discrete stages of neurogenesis are more likely to express tropomyosin related kinase B (TrkB), a key receptor for the neurotrophin brain-derived neurotrphic factor (BDNF),

NMDA, or subtypes of glucocorticoid receptors, all of which have been implicated in regulating neurogenesis (Donovan et al., 2008a; Garcia et al., 2004; Nacher et al., 2007). Perhaps morphine inhibits actively dividing progenitor cells preferentially due to altered expression of these receptors, such that the progenitor cells cannot compensate or respond to morphine-induced changes in the hippocampal microenvironment. Identification of vulnerable subpopulations after discrete stimuli (Encinas et al., 2006; Mandyam et al., 2008b; Wang et al., 2008) and their receptor expression patterns (Chumley et al., 2007; Suslov et al., 2000) will notably enhance our ability to independently manipulate adult neurogenesis in the future.

Functional relevance of morphine-induced inhibition of maturation

While the functional implications of the present data are merely speculative, it is important to consider how they might influence our understanding of addiction. It is notable that adult hippocampal neurogenesis is functionally implicated in spatial memory, for example, newborn neurons are incorporated into hippocampal circuits and activated during spatial memory tasks (Epp et al., 2007; Kee et al., 2007; Winocur et al., 2006). Interestingly, chronic morphine alters long-term spatial memories in rodent (Miladi Gorji et al., 2008; Spain and Newsom, 1991) and heroin addicts have altered levels of PSA-NCAM, which besides being a marker of immature neurons, appears important for long term memory formation (Lopez-Fernandez et al., 2007). It is also intriguing that blockade of NR2b receptor expression, one of the subtypes present on

progenitor cells, ablates morphine-induced conditioned place preference (Suzuki et al., 2000). Thus, future studies could take advantage of the fact that specific stages of drug taking represent very specific forms of learning, and use both conditioned place preference and drug self-administration to more closely examine the link between hippocampal neurogenesis, learning, and addiction.

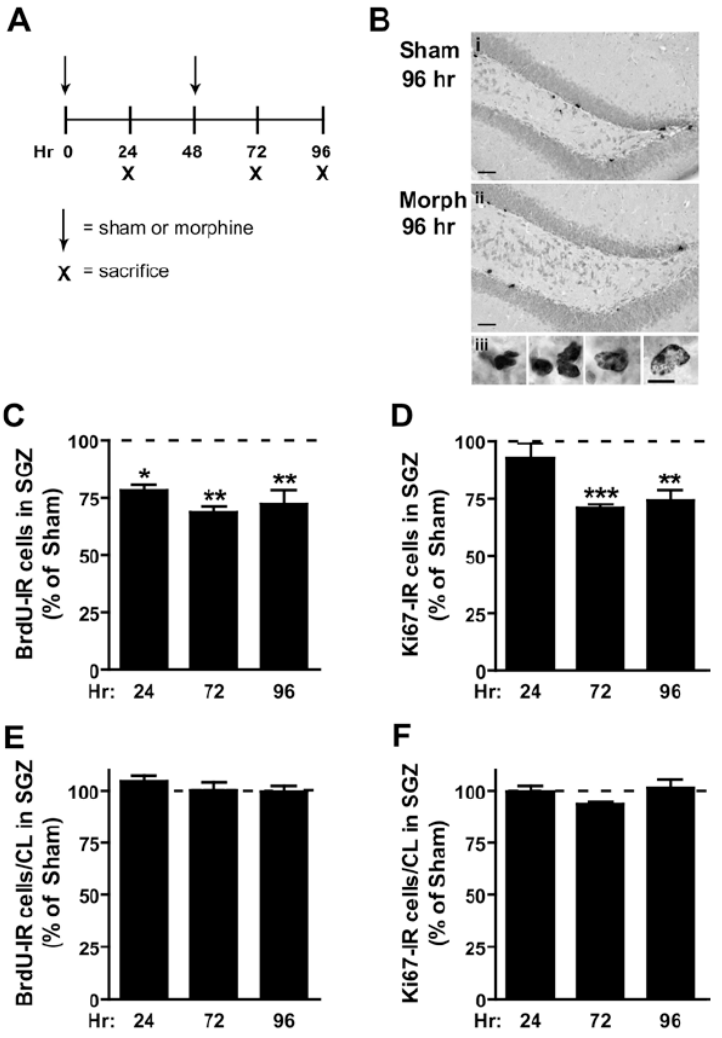

Figure 2.1. **Chronic morphine decreases SGZ proliferation. (A**) For Experiment 1, morphine or sham pellets were implanted subcutaneously at 0 and 48 hrs. Two hrs prior to sacrifice (24, 72 and 96 hrs) mice were given an injection of 150 mg/kg BrdU. **(B)** Qualitative IHC shows decreased numbers of BrdU-IR cells in the SGZ of **(ii)** morphine exposed mice compared to **(i)** sham exposed mice. Scale bar = 50 um. **(iii)** BrdU-IR cells, from a representative sham exposed mouse, divide in clusters and have dark or punctate heterochromatin. Scale bar = 10 um. **(C)** The number of BrdU-IR cells expressed as percent of sham is decreased at all time points. **(D)** The number of Ki67-IR cells expressed as percent of sham is decreased at 72 and 96 hr. **(E-F)** The numbers of BrdU-IR or Ki67-IR cells in a cluster are not changed after 24, 72, or 96 hrs of exposure to morphine. At all time points sham: n=6, morphine: n=6. *p≤0.05, **p<0.01, ***p<0.001.

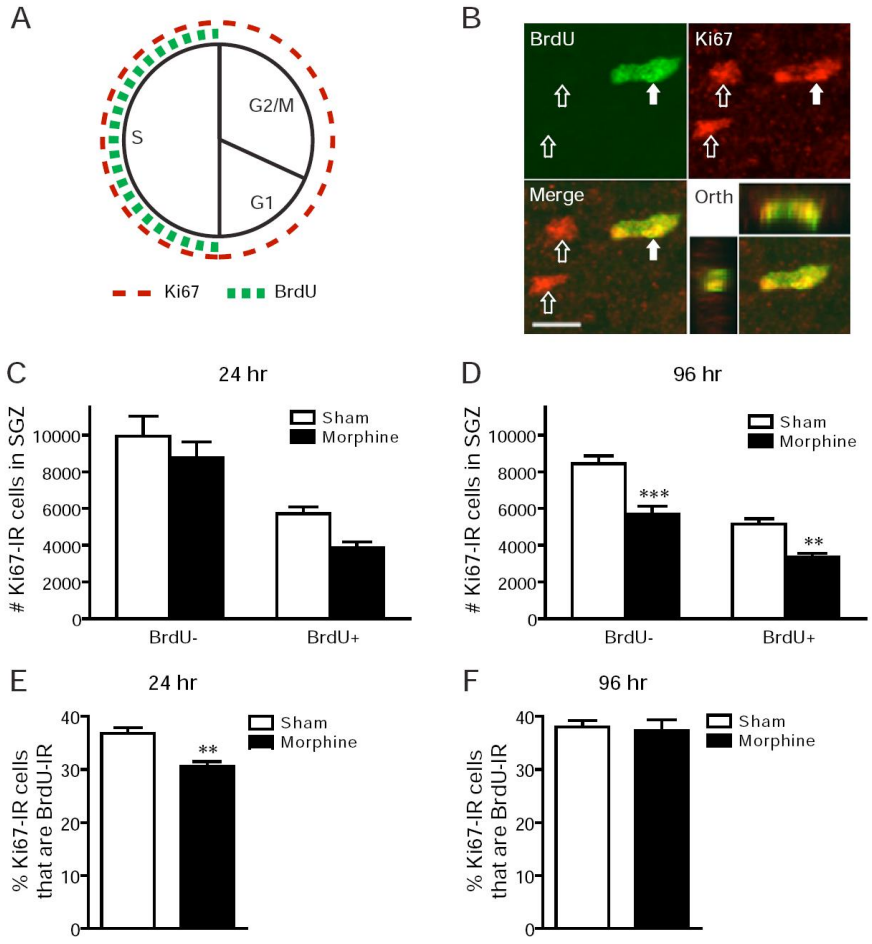

Figure 2.2. **Chronic morphine alters cell cycle dynamics. (A)** Schematic depicting putative expression of Ki67 and BrdU within the cell cycle. Cycling cells in non-S phases do not co-label with Ki67 and BrdU (Ki67+/BrdU-; dashed red line only), whereas S phase cycling cells co-label with Ki67 and BrdU (Ki67+/BrdU+; dashed red and green lines). **(B)** Confocal image of cells that are Ki67+/BrdU- (open arrows) and Ki67+/BrdU+ (closed arrows). Orthogonal view shows co-localization of Ki67 (red) and BrdU (green). Scale bar=10 um. **(C-D)** Quantification of the number of Ki67-IR cells that are BrdU- or BrdU+ reveals significant main effect for Ki67 cell type at 24 hrs. In contrast there was a significant main effect for Ki67 cell type and drug treatment at 96 hrs with no significant interaction. Bonferroni post hoc tests revealed significant differences between sham and morphine groups within Ki67 (BrdU- and BrdU+) cell type. **(E)** The percentage of Ki67-IR cells that were BrdU-IR was decreased after 24 hrs exposure to morphine **(F)** but not after 96 hrs. **(C, E 24 hr)** sham: n=4, morphine: n=4. **(D, F 96 hr)** sham: n=6, morphine: n=7. **p<0.01, ***p<0.001.

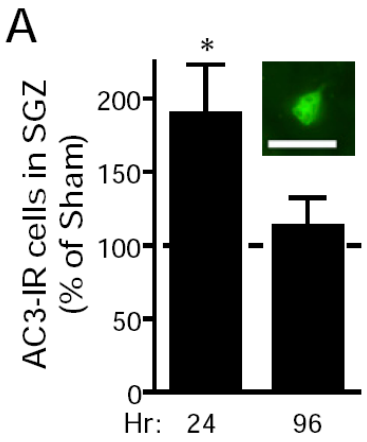

Figure 2.3. Chronic morphine transiently increases cell death. (A) The number of AC3-IR cells, expressed as percent of sham, was increased after 24 hrs but not after 96 hrs morphine exposure. **(Inset)** AC3-IR cells were identified by their distinct cell morphology. For both time points sham: n=6, morphine: n=6. Scale bar = 10 um. *p≤0.05.

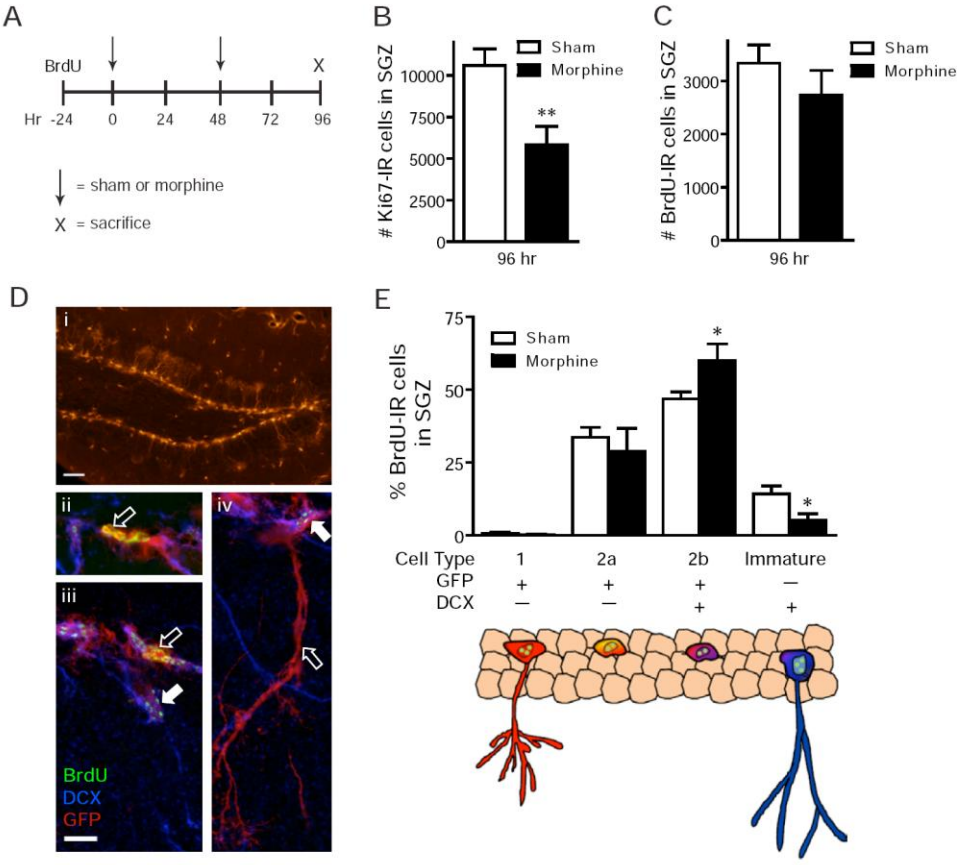

Figure 2.4. **Chronic morphine alters specific stages of progenitor cell maturation. (A)** A single 150 mg/kg BrdU injection was given to nestin-GFP mice 24 hrs before sham surgery or implantation of morphine pellet at 0 and 48. Mice were sacrificed after 96 hrs of exposure. **(B)** The number of Ki67-IR proliferating cells was decreased as previously shown (Figure 2.1D). **(C)** The number of BrdU-IR cells was not significantly decreased. **(D)** Phenotypic analysis of BrdU-IR cells was assessed by cell morphology and co-localization with GFP and DCX with confocal microscopy. **(i)** GFP was robustly expressed in the SGZ. Scale bar = 50 um. **(ii-iv)** BrdU-IR cells were classified as Type 1 (iv, open arrow; GFP+/DCX-, triangular cell body, dendrite extending into molecular layer), Type 2a (ii, iii, open arrows; GFP+/DCX-, ovoid cell body, no dendrites), Type 2b (iv, closed arrow; GFP+/DCX+, ovoid cell body, no dendrites;) or immature neuron (iii, closed arrow; GFP-/DCX+, mature morphology). Scale bar= 10 um in iii. **(E)** The percent of BrdU-IR cells that were Type 2b increased whereas those that were immature neurons decreased. Schematic diagram depicts cell morphology and marker expression of cell type affected by morphine. Green dots represent BrdU (as BrdU-IR cells were phenotyped). **(B,C)** sham: n=8, morphine: n=7. **(E)** sham: n=7, morphine: n=6. *p≤0.05, **p<0.01.

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CHAPTER THREE

Chronic morphine alters specific aspects of the progenitor cell cycle

Introduction

Adult neurogenesis occurs in two brain regions: the hippocampal subgranular zone (SGZ) and the subventricular zone (SVZ). In the hippocampal SGZ, progenitor cells proliferate and eventually differentiate into mature dentate gyrus (DG) granule neurons. Much research has focused on the regulation of adult neurogenesis, showing that age and drugs of abuse, like opiates, decrease the number of adult generated SGZ neurons (Eisch et al., 2000; Olariu et al., 2007). Regulation of the cell cycle and its individual phases (G1, gap1, preparation for DNA duplication; S, DNA replication; G2, gap2, preparation for cell division; M, mitosis) has been postulated as a possible mechanism for altered neurogenesis (Eisch and Mandyam, 2007). For example, alterations in the cell cycle are thought to regulate overall neuron number during evolution (Fish et al., 2008; Kriegstein et al., 2006) and contribute to alterations in SGZ neurogenesis as seen with neurodegenerative diseases (Okamoto et al., 2007; Nagy et al., 1997). However, less information is known about how alterations in the progenitor cell cycle contribute to increases or decreases in opiate-induced alterations in adult neurogenesis. Identifying this information has much potential to help understand opiate-induced regulation in particular and adult neurogenesis regulation in general.

Chronic morphine decreases hippocampal neurogenesis by impairing proliferation and maturation (Thesis Ch. 2; Arguello et al., 2008; Fischer et al., 2008). In regards to the cell cycle, morphine has a greater impact on cells that are proliferating in S phase of the cell cycle (Arguello et al., 2008). Furthermore, after morphine exposure S phase proliferating cells reach G2/M prematurely (Mandyam et al., 2004). These data suggest that morphine may cause S phase length to be shortened. However it has not been determined if a shortened S phase actually contributes to decreased SGZ proliferation. Distinguishing whether morphine-induced decrease in proliferation is a result of altered S phase length, diminished number of S phase cycling cells, or both will help elucidate how morphine exerts its effects on the progenitor cell cycle.

In the adult mouse SGZ, S phase is approximately 6-8 hours long and occupies half the cell cycle length of 12-14 hrs (Burns and Kuan, 2005; Hayes and Nowakowski, 2002; Mandyam et al., 2007). Traditionally, to determine the length of S phase of the cell cycle, two markers of S phase are used. In a traditional double injection paradigm, tritiated thymidine (^{3}H) is injected intraoeritoneally (i.p.) followed by time interval “t”, followed by a second injection of a different thymidine analog, bromodeoxyuridine (BrdU) (Cameron and McKay, 2001; Hayes and Nowakowski, 2002). A certain population of cells that are labeled with ^{3}H will move forward during the interinjection interval, thereby resulting in three distinct populations: ^{3}H, BrdU-immunoreactive (IR), and ^{3}H/BrdU-IR cells. A double injection protocol provides information about the number of proliferating

cells in S phase as well the length of S phase itself. However, since ^{3}H radioactivity can only be detected through the top 3-7 μm of tissue, it can be challenging to determine if a cell is truly co-labeled with ^{3}H and BrdU. In addition, the developing process of ^{3}H via radiolabeling can take weeks (Cameron and McKay, 2001). To overcome these problems with the traditional double-S phase labeling paradigm, an alternative approach to measure S phase has been proposed which uses the two halogenated thymidine analogs iododeoxyuridine (IdU) and chlorodeoxyuridine (CldU) (Aten et al., 1992; Vega and Peterson, 2005). IdU and CldU are incorporated into the DNA of dividing cells during S phase, but are differentially and specifically recognized by two different BrdU antibodies.

Here, I used this new approach to determine if morphine alters the length of S phase of the progenitor cell cycle. It was first necessary to validate the use of halogenated analogs for S phase length determination (Experiments 1 and 2) before combining morphine administration and a double thymdine analog injection paradigm (Experiment 3). For Experiment 1, the specific detection of IdU and CldU was validated by giving C57BL/6J mice 50 mg/kg equimolar equivalent (referred to as 50 mg/kg for simplicity) of IdU or CldU i.p., then quantifying the number of IdU and CldU-IR cells via immunohistochemistry. For Experiment 2, C57BL/6J mice received a double injection of both halogenated analogs: 50 mg/kg IdU followed 2 hrs later by a 50 mg/kg injection of CldU. Mice were perfused 30 minutes after the second injection to determine if three

populations of proliferating cells were labeled. For Experiment 3, C57Bl/6J mice were implanted with sham or morphine pellets subcutaneously (s.c.; 0 and 48 hrs) and perfused at 24 and 96 hrs. Two hrs and 30 min before the 24 and 96 hr sacrifice time points, mice received a double injection of IdU and CldU (as in Experiment 2). Based on previous work showing that the length of S phase is not altered after age or in different mouse strains (Hayes and Nowakowski, 2002; Olariu et al., 2007), it was hypothesized that chronic morphine would decrease the number of proliferating cells in S phase but not alter the length of S phase itself.

Material and Methods

Animals

For all experiments, male C57BL/6J mice from Jackson Laboratories (stock #000664) were used. Mice were housed four to a cage with free access to food and water in a UT Southwestern Medical Center facility on a 12 hr light/dark cycle. Mice were allowed to habituate for one week prior to start of experiments. All experimental procedures were approved by The Institutional Animal Care and Use Committee and were in accordance with the U.S. National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Drug treatment and halogenated thymidine injections

For Experiment 1, which provided the initial characterization of halogenated thymidine analogs, mice were given an injection of 50 mg/kg IdU (Sigma, Cat#

C6891, dissolved in 0.9% saline, 0.2N NaOH at 2.5 mg/mL, then normalized to pH 7 with 10N HCl) or a 50 mg/kg injection of CldU (MP Biomedicals, Cat# 100357, dissolved in 0.9% Saline, 0.007N NaOH at 10 mg/mL) 2 hrs before sacrifice. Mice were anesthetized with chloral hydrate (120 mg/kg dissolved in 0.9% saline), and perfused transcardially with 0.1M PBS followed by 4% paraformaldehyde in 0.1M PBS (pH 7.4), as previously described (Arguello et al., 2008; Harburg et al., 2007). During the initial stages of validation, it was noticed that rat anti-BrdU also non-specifically recognized IdU, though the staining was on the order of background level staining (data not shown). However, non-specific recognition of IdU by rat anti-BrdU antibody was eliminated by dilution of the primary antibody to the concentrations reported here.

For Experiment 2, which validated the use of double injection of halogenated thymidine analogs in the adult mouse SGZ to determine S phase length, mice received a single 50 mg/kg i.p. injection of IdU followed 2 hrs later by a 50 mg/kg injection of CldU. Mice were perfused 30 minutes later, as described above.

For Experiment 3, the double injection of halogenated thymidine analogs was combined with a morphine time course in order to examine how morphine alters S phase of SGZ progenitor cells. Mice were implanted with one 25 mg morphine pellet (generously provided by the National Institute on Drug Abuse, Bethesda, MD) or sham pellet s.c. under isoflurane anesthesia (2% in compressed oxygen for 1-2 minutes) at 0 and 48 hrs. Two hours and thirty minutes before the 24 hr or

96 hr sacrifice time point, mice were given an injection of IdU then CldU, as described for Experiment 2.

Tissue sectioning

Serial sets of 30μm coronal sections from brains were collected on a freezing microtome (Leica, SM2000R). Every ninth section through the entire mouse hippocampus was slide mounted prior to immunohistochemistry (IHC) (-0.82 to -4.24 from bregma) (Franklin and Paxinos, 1997). Slides were coded before IHC and the code was not broken until data analysis was complete (Arguello et al., 2008; Mandyam et al., 2004).

IHC

Antibodies. The following antibodies were used for IHC: rat anti-BrdU (Accurate, Westbury, NY, Cat #: OBT0030, Lot #: H7786, 1:5000); mouse anti-BrdU (BD Biosciences, San Jose, CA, Cat#: 347580, Lot #: 82737, 1:300). For each antibody, the specificity of staining was determined by lack of signal after omission and/or dilution of the primary antibody, as previously reported (Eisch et al., 2000; Mandyam et al., 2004).

Double label IHC. The following pretreatment steps were used: antigen unmasking (0.01M citric acid, pH 6.0, 100°C, 10 min), membrane permeabilization (trypsin in 0.1M Tris, 0.1% $CaCl_2$, 10 min), DNA denaturation (2M HCl in 1X PBS, 30 min) and quenching of endogenous peroxidases (0.3%

H_2O_2, 30 min), as described previously (Arguello et al., 2008). Nonspecific binding was blocked (3% serum, 0.3% Triton-X, 30-60 min) followed by co-incubation with mouse anti-BrdU and rat anti-BrdU primary antibodies overnight (3% serum, 0.3% Tween-20). Tissue from all experiments was co-incubated with both antibodies. IdU and CldU were visualized by co-incubation with fluorophore conjugated secondary antibodies: donkey anti-mouse Cy2 for IdU (Jackson, West Grove, PA, Cat#: 715-065-150,1:200, 4 hrs) and donkey anti-rat CY3 (Jackson Immunoresearch, West Grove, PA, Cat#: 712-165-153, 1:200, 4 hrs).

Microscopic analysis and quantification

IdU-IR and CldU-IR cell quantification. IdU and CldU-IR cell numbers were quantified at 400X magnification on an Olympus BX-51 microscope (Tokyo, Japan). Cell numbers were quantified with modified stereology via the optical fractionator method throughout the rostral-caudal axis of the hippocampus (-0.82 to -4.24 from bregma) (Franklin and Paxinos, 1997). Cell counts were collected from the SGZ (defined as the region bordering the granule cell layer and hilus: three cell widths into the hilus and half of the granule cell layer), with continuous adjustment of the focal plane, as described previously (Arguello et al., 2008; Donovan et al., 2006).

S phase length analysis

Data collected from IdU-IR and CldU-IR quantification above was used to determine the length of S phase, as previously described (Cameron and McKay,

2001; Olariu et al., 2007). The following equation was used: $(N_i/(N_c + N_{ic})) = (i/T_s)$, where N_i = number of IdU-IR cells, N_c = number of CldU-IR cells, N_{ic} = number of IdU/CldU-IR cells, i = interinjection interval (2 hrs), and T_s = length of S phase. T_s was calculated for each individual animal and the average T_s was calculated for each experimental group (24 hr sham, 24 hr morphine, 96 hr sham, 96 hr morphine).

Statistical analysis

Data are reported as mean ± SEM. Statistical analyses were performed using student's t-test for comparison of two groups. All statistical analyses were performed using Prism (version 5.0) software. Statistical significance was defined as $p<0.05$ with $*p<0.05$, $**p<0.01$.

Results

Halogenated thymidine analogs can be detected in mouse SGZ

The two halogenated thymidine analogs, IdU and CldU, have been shown *in vivo* to be differentially detected by two distinct BrdU antibodies raised in mouse or rat, respectively (Maslov et al., 2004; Vega and Peterson, 2005; Breunig et al., 2007). The halogenated analogs have been used to determine cell cycle exit or cell cycle re-entry in the adult rat SVZ by i.p. administration, or in the early post-natal SGZ by i.p. and oral administration. Therefore validation of specific detection of each analog in the adult mouse SGZ with sequential i.p. injections was necessary.

C57BL/6 mice were injected with a single 50 mg/kg i.p. injection of IdU or CldU and were perfused 2 hrs later (Experiment 1). As expected from published reports, IdU was only detected by mouse anti-BrdU antibody whereas CldU was only detected by rat anti-BrdU antibody (Figure 3.2A). To determine if both analogs labeled an equal population of proliferating cells, the number of IdU-IR and CldU-IR cells was quantified in the mouse SGZ. Although IdU-IR cells appeared more intensely stained than CldU-IR cells, both halogenated analogs labeled an equal number of proliferating cells in the SGZ when examined 2 hrs after injection (Figure 3.2B). Therefore in the mouse SGZ, IdU and CldU were specifically detected by mouse anti-BrdU and rat anti-BrdU antibodies, respectively. Furthermore, both analogs were incorporated equally into proliferating cells.

Double injection of two halogenated analogs labels three populations of proliferating cells

To determine if co-injection of IdU and CldU labeled three populations of proliferating cells, C57BL/6J mice were administered a single 50 mg/kg i.p. injection of IdU, followed 2 hrs later with a single 50 mg/kg i.p. injection of CldU (Figure 3.3A). In theory, the first injection of IdU would label a population of cells in S phase. During the 2 hr interinjection interval, some IdU-IR cells would move forward in the cell cycle. After the second injection of CldU is given 2 hrs later, there should be three distinct populations of labeled cells: IdU-IR cells (Figure

3.3B; purple hatched area), CldU-IR cells (Figure 3.3B; green hatched area) or IdU/CldU-IR double-labeled cells (Figure 3.3B; purple and green hatched area). A representative IHC image from tissue co-stained with mouse anti-BrdU and rat anti-BrdU antibodies depicts each of the three populations of proliferating cells: IdU-IR cells (Figure 3.3C, closed arrows), CldU-IR cells (Figure 3.3C, open arrows) and IdU/CldU-IR double-labeled cells (Figure 3.2C, no arrows). Interestingly, not all cell clusters were exlusively IR for IdU or CldU. There were several instances in which a cell cluster was heterogenous, with both IdU and CldU cells within a cluster (images not pictured). Having determined that the double injection of IdU and CldU labels three cell populations, the double analog injection paradigm could now be combined with a morphine administration paradigm to determine if morphine alters the length of S phase.

Chronic morphine decreases the number of cells dividing in S phase but does not alter length of S phase

It has previously been shown that either 24 hrs or 96 hrs of morphine exposure results in a decrease in the number of SGZ progenitor cells dividing in S phase (Arguello et al., 2008; Fischer et al., 2008). To determine if chronic morphine alters the length of S phase, the double injection of IdU and CldU was performed at these time points. C57BL/6J mice were given morphine or sham pellets (s.c. at 0 and 48 hrs). Two hrs and 30 minutes before perfusion (at 24 or 96 hrs), mice were given a 50 mg/kg i.p injection of IdU, followed by a 2 hr interinjection interval, then a 50 mg/kg i.p. injection of CldU (Figure 3.4A). At both the 24 hr

and 96 hr time point, the number of IdU-IR and CldU-IR cells was not changed after morphine exposure (Figure 3.4B, C). However at both time points, the number of IdU/CldU-IR cells was decreased (Figure 3.4A, B). These data are consistent with previous reports that 24 and 96 hrs of morphine exposure decreases the number of proliferating cells, as determined by BrdU-IR counts (Arguello et al., 2008; Fischer et al., 2008). However, by using the raw number of IdU-IR, CldU-IR and IdU/CldU-IR cells, the length of S phase could be determined, where $Ts = (i*(N_c + N_{ic}))/N_i$ (Cameron and McKay, 2001; Hayes and Nowakowski, 2002) (Figure 3.4D). The length of S phase (Ts) was calculated for each individual animal, and an average length of S phase per treatment groups was determined. The length of S phase was not different between sham and morphine treated groups after 24 or 96 hrs of morphine exposure (Figure 3.4E).

Discussion

These data show that the chronic morphine-induced decrease in the number of divding cells does not result from an alteration in the length of S phase. Furthermore, these data validate the use of IdU and CldU for a modified double injection paradigm of halogenated thymidine analogs with a short interinjection interval to determine length of S phase in adult mouse SGZ.

IdU and CldU are specifically recognized in the mouse SGZ

Previous work has used the halogenated thymidine analogs IdU and CldU (Breunig et al., 2007; Maslov et al., 2004; Thomas et al., 2007; Vega and

Peterson, 2005), but the data presented here are the first to use these analogs in a double injection protocol for the purpose of determining the length of S phase in the adult mouse SGZ. While halogenated thymidine analogs have been used for cell cycle analysis and pulse chase experiments *in vitro*, the first demonstrated used of both IdU and CldU to determine cell cycle re-entry was reported in 2004 in the mouse SVZ in which IdU was administered in the drinking water and CldU in water or by i.p. injection (Maslov et al., 2004). Therefore validation of the use of injected halogenated analogs was necessary before application to the double injection paradigm. IdU was specifically recognized by mouse anti-BrdU and CldU by rat anti-BrdU antibodies.

Double injection of IdU and CldU labels three proliferative cell populations

Double injection of the two halogenated analogs allowed for detection of three distinct proliferating cell populations: IdU-IR, CldU-IR and IdU/CldU-IR. An interesting finding from the present study was the heterogeneity of the cell populations labeled with IdU and CldU. For example, although most cell clusters were exclusively IdU-IR, or CldU-IR, approximately 20% of cell clusters were heterogenous such that an IdU-IR cell cluster contained one to two CldU-IR cells and vice versa. There are at least two possible explanations for the observation of heterogeneous clusters. One possibility is that an IdU-IR cluster is observed which is in close proximity to a smaller cluster of CldU-IR cells. A second possibility is that the first injection of IdU labeled a certain population of proliferating cells in S phase and when the second injection of CldU was given, it

labeled cells that were within the IdU-IR cluster but were in G1, or in G0 at the time of the first injection. Although difficult to test, this second possibility is very interesting in that it would suggest that proliferating cells within a cluster are positioned in close proximity to cells that are in G1 preparing to divide or G0 quiescent, post-mitotic cells. Could these G1 phase cells or G0 quiescent cells be providing some support to actively dividing cells? This is possible, as it has been shown that proliferating cells are clustered within immature neurons (Seki, 2002) and a niche rich in cell-adhesion molecules (Seki et al., 2007).

Chronic morphine does not alter the length of S phase

An important point to note is that the present data confirm previous reports showing that the number of cells dividing in S phase is decreased after both 24 and 96 hrs of morphine exposure (Arguello et al., 2008; Fischer et al., 2008). The present study validates that the decreased proliferation observed after 24 or 96 hrs of morphine exposure can be detected with BrdU, IdU or CldU. However, the highlight of the present study was the result that the length of S phase was not altered after chronic morphine administration. The fact that the length of S phase was not altered after 24 or 96 hrs of morphine exposure was initially surprising. Previous studies had determined that SGZ proliferating cells exposed to morphine reached G2/M prematurely when compared to proliferating cells not exposed to drug (Mandyam et al., 2004). Another study observed that mice exposed to morphine *in utero* had a lengthened G2/M phase in SVZ cycling cells (Sargeant et al., 2008) which suggested the possibility of altered S phase length.

However, it has also been noted that S phase is the least mutable of the phases of the cell cycle *in vivo* (Eisch and Mandyam, 2007), though this has not been observed in other areas of the CNS (Alexiades and Cepko, 1996). A previous study also found that an age-induced decrease in proliferation is not due to a change in the length of S phase or the entire cell cycle (Olariu et al., 2007). There are various studies that have determined that the duration of G1 can be lengthened or shortened after stroke (Zhang et al., 2006; Zhang et al., 2008). Perhaps future studies may determine if the length of another cell cycle phase is altered following chronic morphine.

A final discussion point concerns the absolute length of S phase. From calculations, the relative length of S phase was similar between sham and morphine treated groups, however the absolute length was much longer than expected. Several studies have determined that the length of S phase in the adult mouse SGZ is between 6-8 hrs long (Burns and Kuan, 2005; Hayes and Nowakowski, 2002; Mandyam et al., 2007). Importantly, each approximation of the length of S phase was determined with a distinct labeling method: combination of exogenous and endogenous cell cycle markers (Mandyam et al., 2007), tradition double injection paradigm (Hayes and Nowakowski, 2002), or combination of IdU and BrdU double injection (Burns and Kuan, 2005), suggesting that the longer estimation of S phase length reported here is not simply due to our experimental design. A more plausible explanation for the variability in calculated S phase length concerns the raw numbers of IdU-IR and

CldU-IR labeled cells. When data from IdU-IR, CldU-IR and IdU/CldU-IR were collapsed into total IdU-IR or total CldU-IR cells from Experiment 3 within control conditions, the number of total IdU-IR and total CldU-IR cells was equal. These data match well with the initial characterization data (Figure 3.2B) which determined that the number of IdU-IR and CldU-IR cells in the SGZ were equal. However, when the number of cells is narrowed to only IdU-IR and CldU-IR, there are significantly more IdU-IR cells than CldU-IR cells. Furthermore, the raw number of IdU-IR and CldU-IR cells is very few (e.g. 50-100 cells). Since raw numbers of single-labeled cells are used for the calculation of S phase length, low cell number can lead to increased variability particularly when a difference of 50 cells in the numerator or denominator can lead to a magnitude of difference. Other research groups have also noted variability in the calculation of length of the entire cell cycle of up to 9 hours (Olariu et al., 2007). This variability time is in line with the present results, in that S phase is expected to be approximately 6-8 hrs long, but it was actually between 12-17 hrs (an error of about 7-9 hrs).

How can the issue of variability in calculated S phase length be alleviated for future studies? It seems that the crux of the problem relates to the small number of proliferating cells that are single-labeled. So an obvious answer is to increase the number of cells that are labeled with the halogenated analogs. This could be accomplished with free-floating staining of tissue sections as opposed to the present slide mounted staining, by sectioning tissue at a reduced thickness to sample more of the hippocampus, or by increasing the length of secondary

antibody incubation, which has been shown to increase the total number of cells that are labeled (Neal Melvin, personal communication). Future studies might try these new alternate avenues of staining protocol to increase the raw number of cells labeled, but likely will also require re-working of antibody dilution concentration to avoid our initial problem of non-specific staining of IdU.
Future studies could also address why there are more IdU-IR cells than CldU-IR cells by determining the half-life of IdU and CldU. Perhaps there are more IdU-IR cells because this analog does not have the same half-life as CldU. To determine this, a time course of both analogs would need to be performed, as with a previous BrdU time course study (Mandyam et al., 2007). It is also possible that by the second injection of CldU, the nucleoside transporter has become saturated, which would lead to less cells incorporating CldU (Cameron and McKay, 2000).

The present study determined that length of S phase is not altered by chronic morphine, but what could be an explanation for cells prematurely entering G2/M phase (Mandyam et al., 2004)? Future studies should be geared to examine possible alterations in cell cycle check point proteins, especially those related to G2/M. For example, several proteins have been shown to regulate the transition from S to G2/M including Cdk1 and cyclin B1 (Sanchez and Dynlacht, 2005). These proteins could be analyzed by western blotting of DG-enriched hippocampal samples, to obtain a rough estimate of potential protein changes. Furthermore, since the present results suggest that S phase length is not altered,

future experiments could also examine whether the G1-S phase transition is altered, as there is ample evidence that G1 length, *in vivo*, can be altered by various stimuli (Heine et al., 2004; Zhang et al., 2006; Zhang et al., 2008). This could be examined with a modified version of the IdU/CldU double injection paradigm in which the interinjection interval is increased to 10-12 hrs, to determine if cell cycle re-entry is altered by chronic morphine. It is also possible that S phase progenitor cells get to G2/M prematurely because they are exiting S phase at a faster rate. This is very challenging to examine *in vivo*. The calculations that are currently used to determine if length of S phase or the cell cycle are altered, are all dependent on administration of an analog which gets incorporated during S phase of the cell cycle. Therefore future studies need to determine an alternate method to calculate length of phases of the cell cycle that are not dependent on S phase. A possible avenue for future studies could focus on FACS sorting of progenitor cells from sham and morphine treated animals. The sorted cells could be synchronized and then it could be determined whether the morphine-treated sorted cells enter G2/M prematurely and continue to cycle normally.

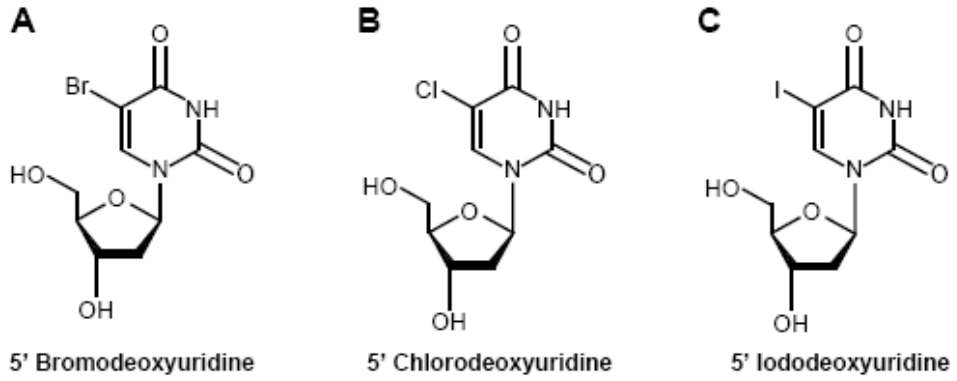

Figure 3.1. **Structure of halogenated thymidine analogs.**
(A) 5-Bromodeoxyuridine is typically administered exogenously via i.p. injection to label progenitor cells dividing in S phase of the cell cycle. **(B)** 5-Chlorodeoxyuridine and **(C)** 5-Iododeoxyuridine canalso be administered i.p. to exogenously label S phase dividing cells.

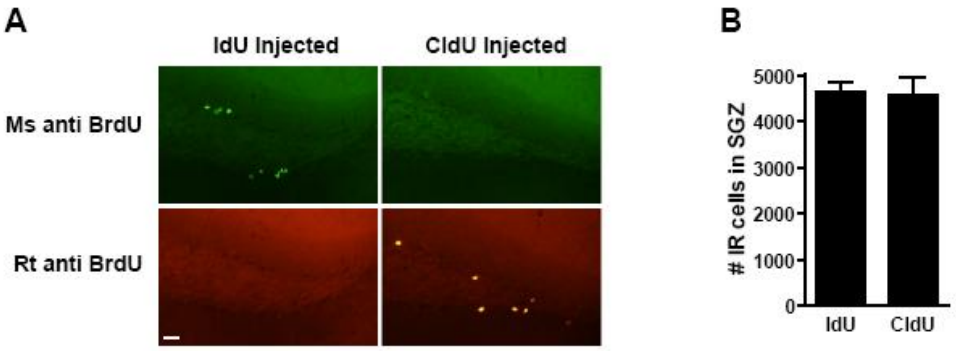

Figure 3.2. **CldU and IdU are specifically detected with BrdU Antibodies raised in two different species.** C57BL/6J mice were implanted were injected with 50 mg/kg IdU or CldU and prefused two hours after injection. **(A)** IdU is detected only by BrdU antibody raised in mouse (ms anti-BrdU) and CldU is detected only by BrdU antibody raised in rat (rt anti-BrdU). Scale bar=50 μm **(B)** The number of IdU-IR cells and CldU-IR cells in the SGZ are equal therefore both analogs label a similar population of proliferating cells. IdU: n=4, CldU: n=4.

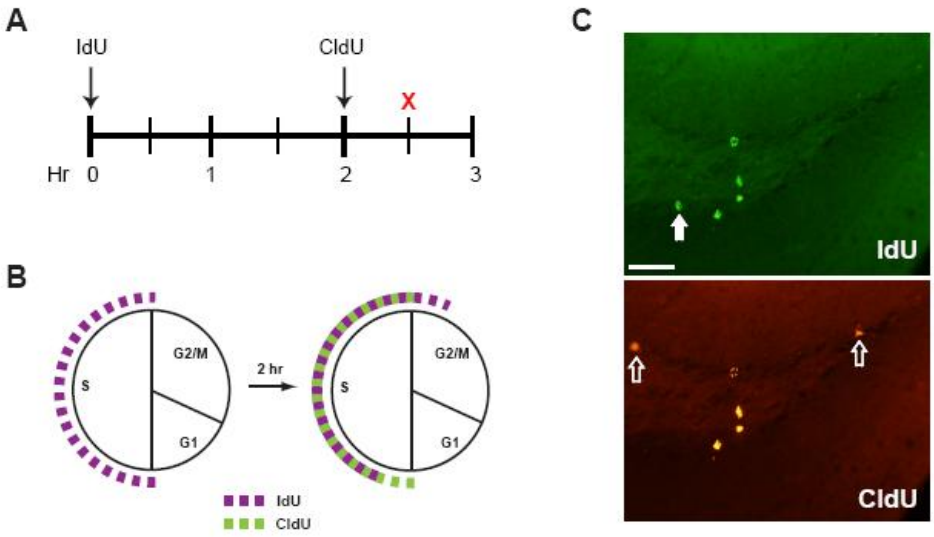

Figure 3.3. **Double injection of IdU and CldU label three populations of proliferating cells. (A)** C57BL6/J mice were given one 50 mg/kg i.p. injection of IdU followed 2 hrs later by another 50 mg/kg i.p. injection of CldU. Mice were perfused 30 minutes after the CldU injection. **(B)** Schematic depicting three populations that should be labeled after double injection of IdU and CldU: IdU only (purple hatched area), CldU only (green hatched area) and IdU/CldU double labeled (purple and green hatches). **(C)** IHC for IdU and CldU showing three populations of dividing cells in SGZ of hippocampus. IdU only labeled population (solid arrow), CldU only labeled population (open arrows), and double-labeled population (no arrows). Scale bar=100 μm.

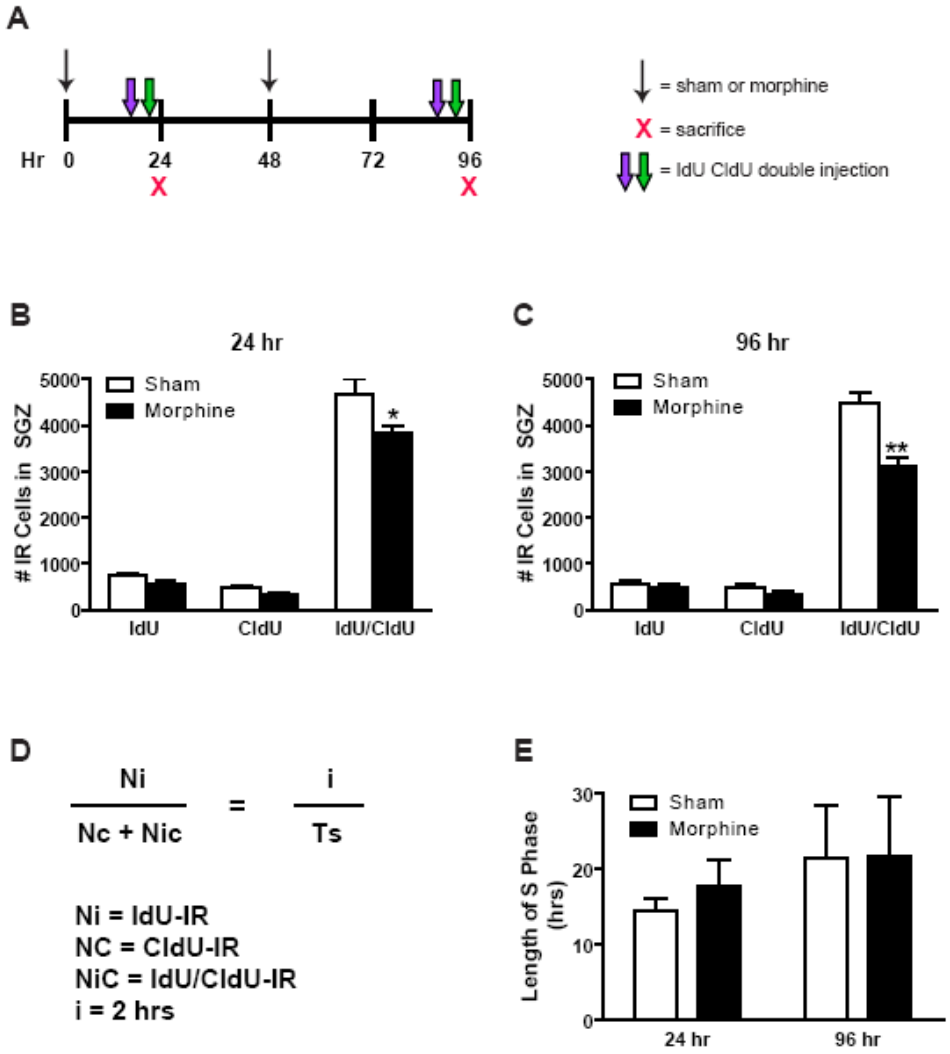

Figure 3.4. **Chronic morphine does not alter length of S phase.**
(A) Schematic of combined morphine time course and double injection paradigm. C57BL/6J mice were implanted with s.c. sham or morphine pellets at 0 and 48 hrs. Two hours and 30 minutes before sacrifice at 24 and 96 hrs, mice were given double injection of IdU (purple arrow) and CldU (green arrow) as in Figure 3A. **(B, C)** The number of IdU/CldU-IR cells in the SGZ was decreased at 24 and 96 hrs. Sham: n=5-6, morphine: n=5-7, *p<0.05, **p<0.01. **(D)** To determine the length of S phase, the following formula was used, where Ni= IdU–IR cells, Nc=CldU-IR cells, Nic=IdU/CldU-IR cells, i= interinjection interval and Ts= length of S phase. **(E)** The length of S phase is not different between sham and morphine exposed mice at either 24 or 96 hrs. Sham: n=6-7, morphine: n=5-7.

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CHAPTER FOUR

Chronic morphine's effect on the hippocampal neurogenic niche

Previously published: **Arguello AA**, Fischer SJ, Schonborn JR, Markus RW, Brekken RA, Eisch AJ. 2008. Effect of chronic morphine on the dentate gyrus neurogenic microenvironment. Neuroscience, *in press*.

Introduction

Neurogenesis occurs in two primary brain areas in the postnatal mammalian brain: the subgranular zone (SGZ) of the hippocampal dentate gyrus (DG) and the subventricular zone (SVZ) (Altman and Das, 1965; Ming and Song, 2005; Monje et al., 2002). Progenitor cells in both the SGZ and SVZ reside in neurogenic niches rich in vasculature and key factors, such as vascular endothelial growth factor (VEGF), interleukin-1β (IL1β), and brain-derived neurotrophic factor (BDNF) (Riquelme et al., 2008), and often proliferate in close proximity to vasculature elements (Heine et al., 2005; Mercier et al., 2002; Palmer et al., 2000). Many studies show a correlative relationship between manipulation of factors in the neurogenic niche and hippocampal neurogenesis. For example, exogenous application of growth factors and cytokines alters SGZ proliferation (Jin et al., 2002; Koo and Duman, 2008; Scharfman et al., 2005) and even influences hippocampal functions such as learning and memory (Cao et al., 2004; Mustafa et al., 2008; Warner-Schmidt et al., 2008). However, fewer studies have examined whether stimuli known to impact adult SGZ neurogenesis lead to

alterations in hippocampal levels of factors such as BDNF, IL1β, or VEGF or changes in the vasculature.

Chronic opiate exposure negatively impacts SGZ proliferation and thus can be useful to elucidate the relationship between the neurogenic niche and decreased SGZ neurogenesis. Opiates decrease SGZ proliferation by inhibition of progenitor proliferation, maturation (Arguello et al., 2008; Eisch et al., 2000; Kahn et al., 2005) and alteration of the progenitor cell cycle (Arguello et al., 2008; Mandyam et al., 2004). Notably, cognitive defects are evident in both laboratory animals chronically exposed to opiates (Spain and Newsom, 1991) as well as heroin abusers (Guerra et al., 1987). Heroin abusers also have altered levels of circulating growth factors (Angelucci et al., 2007) suggesting the possibility that neurotrophic factors may mediate the morphine-induced cognitive deficit in both humans and rodents (Spain and Newsom, 1991). However morphine's influence on the neurogenic niche *in vivo*, including levels of BDNF, IL1β, VEGF, and vasculature elements, has not been examined. Therefore, the present study addressed whether chronic morphine altered the neurogenic microenvironment of the DG SGZ. We hypothesized that chronic morphine would decrease the pro-proliferative factors VEGF and BDNF but increase the anti-proliferative factor IL1β, correlating with decreased SGZ proliferation.

To gain insight into whether the chronic morphine-induced decrease in SGZ proliferation correlates with changes in factors in the neurogenic niche, a

subcutaneous (s.c.) morphine pellet administration paradigm was utilized where C57BL/6J mice were exposed to morphine for 24 or 96 hrs (Arguello et al., 2008; Fischer et al., 2008). To correlate changes in proliferation with changes in the microenvironment, mice were killed via decapitation, one hemisphere was post fixed to observe changes in proliferation via immunohistochemistry (IHC), and the DG/CA1 region was dissected from the other hemisphere to observe changes in the neurogenic niche by immunoblotting.

Materials and Methods

Animals

For all experiments, C57BL/6J mice from Jackson Laboratories (stock #000664) were used. Mice were housed four to a cage with free access to food and water in a UT Southwestern Medical Center facility on a 12 hr light/dark cycle. Mice were allowed to habituate for one week prior to start of experiments. All experimental procedures were approved by The Institutional Animal Care and Use Committee and were in accordance with the U.S. National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Drug treatment and Bromodeoxyuridine (BrdU) injections

For Experiment 1, mice were implanted with one 25 mg morphine pellet (generously provided by the National Institute on Drug Abuse, Bethesda, MD) or sham pellet s.c. under isoflurane anesthesia (2% in compressed oxygen for 1-2 minutes) at 0 and 48 hrs and were killed via decapitation after 24 or 96 hrs of

morphine exposure. One hemisphere of the brain was post fixed in 4% paraformaldehyde in 0.1M phosphate buffered saline (PBS; pH 7.4) for three days, followed by cryoprotection in 30% sucrose as previously described (Lagace et al., 2007). The other hemisphere was regionally dissected (DG/CA1 and CA3) and snap frozen for future immunoblotting use. For Experiment 2, mice were also implanted with s.c. morphine or sham pellets (0 and 48 hrs). Two hours before perfusion (at 24 or 96 hrs) mice were given a single 150 mg/kg injection of BrdU intraperitoneally (i.p., Roche Diagnostics, dissolved in 0.9% saline, 0.007N NaOH at 10 mg/mL) and anesthetized with chloral hydrate (120 mg/kg dissolved in 0.9% saline). Mice were then transcardially exsanguinated with 0.1M PBS followed by perfusion of the fixative (4% paraformaldehyde in 0.1M PBS, pH 7.4), each provided in a 7 ml/min flow rate as previously described (Harburg et al., 2007).

Tissue sectioning

Serial sets of 30μm coronal sections from brains were collected on a freezing microtome (Leica, SM2000R). Every ninth section through the entire mouse hippocampus was slide mounted prior to IHC (-0.82 to -4.24 from bregma) (Franklin and Paxinos, 1997). Slides were coded before IHC and the code was not broken until data analysis was complete (Mandyam et al., 2004).

IHC

Antibodies. The following antibodies were used for IHC: rat anti-BrdU (Accurate, Westbury, NY, Cat #: OBT0030, Lot #: H8365, 1:300); rabbit anti-Ki67 (SP6, LabVision Corporation NeoMarkers, Fremont, CA, Cat#: RM-9128-R1, Lot#: 9106S603C, 1:400); rat anti-mouse endoglin (MJ7/18, Developmental Studies Hybridoma Bank, University of Iowa, Illinois, 1:500). For each antibody, the specificity of staining was determined by lack of signal after omission and/or dilution of the primary antibody, as previously reported: endoglin (Ge and Butcher, 1994; Korpanty et al., 2007) present study, BrdU (Mandyam et al., 2004), Ki67 (Dayer et al., 2003; Mandyam et al., 2007).

Single label IHC. Tissue from Experiment 1 was used for Ki67 single label IHC. The following pretreatment steps were used: antigen unmasking (0.01M citric acid, pH 6.0, 100°C, 10 min) and quenching of endogenous peroxidases (0.3% H_2O_2, 30 min). Nonspecific binding was blocked (3% serum, 0.3% Triton-X, 30-60 min) followed by primary antibody incubation overnight (3% serum, 0.3% Tween-20). Sections were then incubated with biotin-tagged secondary antibody (1:200, 60 min) and IHC was completed with the avidin-biotin/CY3 tyramide signal amplification method (ABC Elite, Vector Laboratories, Burlingame, CA and CY3-TSA, Perkin Elmer Life Sciences, Inc., Boston, MA, Cat#: SAT704A001EA, 1:50) as previously described (Lagace et al., 2007) followed by counterstain with DAPI and quenching of autofluorescence with 1X cupric sulfate (Fischer et al., 2008).

Double label IHC. Tissue from Experiment 2 was used for endoglin and BrdU double label IHC. Staining for endoglin was completed followed by staining for BrdU. Endogenous peroxidase quenching and blocking steps were performed for endoglin followed by incubation with endoglin primary antibody overnight. IHC for endoglin was completed via avidin-biotin/CY3 tyramide signal amplification as for Ki67. Sections then underwent the following pretreatment steps for BrdU: antigen unmasking, membrane permeabilization (trypsin in 0.1M Tris, 0.1% $CaCl_2$, 10 min) and DNA denaturation (2M HCl in 1X PBS, 30 min), as previously described (Donovan et al., 2006). Sections were then incubated in blocking serum followed by BrdU primary antibody incubation overnight. BrdU was visualized with a CY2-conjugated fluorophore (1:200, 4 hrs) and counterstained as described above.

Immunoblotting

Snap frozen DG/CA1 regionally dissected tissues from Experiment 1 were lysed with 50 mM NaF, 1% SDS buffer (for BDNF, TrkB, IL1β, IL1R1) or 50mM Tris, 50 mM NaF, 32 mM sucrose, 1% SDS (with 1X protease inhibitor cocktail (Roche Diagnostics, Cat# 11697498001) 1X phosphatase inhibitor 1 and 1X phosphatase inhibitor 2 (Sigma, Cat# P2850 and P5726, respectively)). Samples were sonicated for 10 seconds with 100°C lysis buffer, followed by 10 minutes of boiling. Following lysis, protein concentration was determined via BCA Protein Assay Kit (Pierce, Rockford, IL, Cat#: 23225). Forty μg of protein was loaded on a 4-20% gradient gel. Protein was transferred to nitrocellulose membranes for 1

hour at 100 V. Membranes were blocked in 5% milk for one hour, followed by primary antibody overnight at 4°C. The following primary antibodies from Santa Cruz Biotechnology (Santa Cruz, CA) were used: rabbit anti-BDNF(N-20, Cat#: sc-546, Lot#: C1804, 1:500); rabbit anti-IL1β (H-153, Cat#: sc-7884, Lot#: C1904, 1:250); rabbit anti- IL1R1(M-20, Cat#: sc-680, Lot#: E102, 1:1000). Additional antibodies used were: rabbit anti-TrkB (Millipore, Billerica, MA 01821, Cat#: 07-225, Lot#: 28846, 1:5000); rabbit anti-VEGF (SP28, LabVision Corporation NeoMarkers, Fremont, CA, Cat#: RM-9128-R1, Lot#: 9128R609E, 1:300); rabbit anti-VEGFR2 (T014, (Feng et al., 2000), 1:300); mouse anti-GAPDH (Research Diagnostic Inc., Flander, NJ, 07836, Cat#: RDI-TRK5G4-6C5, 1:200,000). Membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies (1:10,000 for BDNF, IL1β, IL1R1, TrkB, VEGF, VEGFR2; 1:100K for GAPDH) and developed with ECL Plus Detection Reagent (GE Healthcare, Buckinghamshire, UK). Membranes were washed with stripping buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 100 mM βME in 1X Tris-Buffered Saline Tween-20) before being reprobed with GAPDH. Proteins of interest were normalized to GAPDH and bands and were quantified with NIH Image J 1.63. Data are expressed as percent of sham (mean ± SEM).

Microscopic analysis and quantification

Ki67-IR cell quantification. Ki67-IR cell number was quantified at 400X magnification on an Olympus BX-51 microscope (Tokyo, Japan). Cell numbers were quantified with modified stereology via the optical fractionator method

throughout the rostral-caudal axis of the hippocampus (-0.82 to -4.24 from bregma) (Franklin and Paxinos, 1997). Cell counts were collected from the SGZ (defined as the region bordering the granule cell layer and hilus: three cell widths into the hilus and half of the granule cell layer), with continuous adjustment of the focal plane, as described previously (Arguello et al., 2008; Donovan et al., 2006).

Vessel and BrdU proximity analysis. Tissue from Experiment 2 was used for vessel analysis. We used a modified protocol to quantify endoglin staining: number of vessels, vessel area, and vessel perimeter (Heine et al., 2005). Sections of the DG were examined on a Nikon Eclipse E600 microscope at 100X (Nikon, Lewisville, TX) and images were captured with a Photometric Coolsnap HQ camera (Nikon) and imported into Metamorph software (Universal Imagining Corporation) for analysis. Photos of DG sections (-1.3 to -3.1μm from bregma) were captured for each stain (endoglin or BrdU) under identical conditions (exposure time, high and low limits, and scaling), and background levels were thresholded from control sections stained with secondary antibody alone. Integrated optical density and optical intensity were measured using MetaMorph's Integrated Morphometry Analysis for number of endoglin-IR vessels, vessel area and vessel perimeter, as previously described (Arnold et al., 2008). Additionally, proximity of BrdU-IR cells to endoglin-IR vessels was categorized by a blinded observer. Similar to previous studies (Heine et al., 2005), BrdU-IR cells that were vascular-associated were categorized as: “touch” if touching or overlapping a vessel. Non vascular-associated BrdU-IR cells were

further distinguished as either “near”, within 10 μm of vessel, or “far”, beyond 10 μm of vessel (Heine et al., 2005).

Statistical analyses

Data are reported as mean ± SEM. Statistical analyses were performed using student’s t-test for comparison of two groups. All statistical analyses were performed using Prism (version 5.0) software. Statistical significance was defined as $p < 0.05$ with $*p \leq 0.05$, $**p < 0.01$.

Results

Chronic morphine inhibits the total population of proliferating cells in the SGZ

Previous work has determined that chronic morphine decreases SGZ progenitor cell proliferation (Fischer et al., 2008). To correlate changes in the neurogenic SGZ microenvironment with a change in proliferation, C57BL/6J mice were implanted with morphine or sham pellets s.c. at 0 and 48 hrs and were killed at 24 or 96 hrs (Figure 4.1A). Progenitor cell proliferation was assessed with the endogenous cell cycle marker Ki67 in the SGZ as previously described (Arguello et al., 2008). The number of Ki67-IR cells, or total population of proliferating cells, was not decreased after 24 hrs of morphine exposure (Figure 4.1B) as previously shown (Fischer et al., 2008). However, after 96 hrs of morphine exposure, the number of Ki67-IR cells was significantly decreased from sham mice (Figure 4.1C; $t_6=4.74$, $p=0.0032$). A representative IHC image depicts this decrease

qualitatively (Figure 4.1D). This confirms earlier work that the total population of proliferating cells is decreased after 96 hrs but not 24 hrs of morphine exposure.

Chronic morphine does not alter certain pro- or anti-proliferative factors in the DG

To assess changes in pro- or anti-proliferative factors in the DG after chronic morphine, DG-enriched protein samples were immunoblotted for BDNF and IL1β and their corresponding receptors tropomyosin realted kinase B (TrkB) and interleukin-1β receptor (1IL1R1). DG levels of IL1β and IL1R1 were unchanged after 24 hrs of morphine exposure (Figure 4.2A, B). BDNF and TrkB levels also remained unchanged after 24 hrs of morphine exposure (Figure 4.2C, D). The same result was observed for IL1β, IL1R1, BDNF and TrkB at the 96 hr time point (Figure 4.2E-H). Therefore at a time point in which proliferation was decreased by morphine (96 hrs), DG levels of the pro- and anti-proliferative factors BDNF and IL1β remained unchanged.

Chronic morphine dynamically alters DG levels of VEGF

Given previous work linking VEGF to regulation of SGZ neurogenesis (Jin et al., 2002), protein levels of VEGF and vascular endothelial growth factor receptor 2 (VEGFR2) were also assessed after chronic morphine. After 24 hrs of morphine exposure, DG levels of VEGF and its primary receptor VEGFR2 were unchanged (Figure 4.3 A,B). Interestingly, after 96 hrs of exposure (a time point in which numbers of Ki67-IR cells were decreased) levels of VEGF were significantly

increased (Figure 4.3C; t_8=3.375, p=0.0097). Levels of VEGFR2 remained unchanged at 96 hrs (Figure 4.3D). These data suggest that rather than a parallel change, VEGF levels negatively correlate with the change in SGZ proliferation.

Endoglin expression in the hippocampal DG

VEGF is a unique pro-proliferative factor in that it also can function as an angiogenic factor (Palmer et al., 2000). To determine if the morphine-induced increase of VEGF levels correlated with altered neurovasculature, it was first necessary to stain for an appropriate vasculature marker in the SGZ. C57BL/6J mice were given morphine or sham pellets s.c. at 0 and 48 hrs and two hrs before perfusion (24 or 96 hrs) received a single 150mg/kg i.p. injection of BrdU. Co-labeling of endoglin, an endothelial cell marker, and BrdU demonstrated that endoglin-IR vessels are present throughout the DG (Figure 4.4A). Confocal images of endoglin-IR vessels revealed that BrdU-IR cells were closely associated with vasculature (Figure 4.4 B,C). This allowed examination of properties of the vessel themselves, as well as association of proliferating cells with the neurovasculature.

Chronic morphine-induced increase in VEGF correlates with altered vasculature in dentate SGZ

To determine if the morphine-induced increase in VEGF correlated with vasculature changes in the DG, several measures of vasculature remodeling

were examined: endoglin-IR vessel number, area and perimeter (Heine et al., 2005). First, to assess if increased VEGF correlated with increased angiogenesis, the number of endoglin-IR vessels was compared between sham and morphine exposed mice. The numbers of endoglin-IR vessels, vessel area and perimeter in the DG SGZ were unchanged after 24 hrs of morphine exposure (Figure 4.5A), a time point in which proliferation and VEGF levels were unchanged. This suggests that there was no change in angiogenesis or the neurovasculature after 24 hrs of morphine exposure. Similarly, after 96 hrs of morphine exposure the number of endoglin-IR vessels was the same between sham and morphine groups. However, the area and perimeter of endoglin-IR vessels was significantly increased (Figure 4.5B; area: t_8=4.803, p=0.0014; perimeter: t_8=3.890, p=0.0046). This suggests that although angiogenesis was unchanged, the neurovasculature was altered after 96 hrs of morphine exposure. To determine if morphine exposed mice had diminished association between proliferating cells and the neurovasculature, the proximity of BrdU-IR cells to endoglin-IR vessels was examined (Heine et al., 2005). No changes in progenitor cell proximity to endoglin-IR vessels was observed after 96 hrs of morphine exposure (Figure 4.5C).

Discussion

The current results suggest that following chronic morphine exposure, DG levels of factors within the neurogenic microenvironment are either maintained or up-regulated. At a time point in which progenitor cell proliferation was decreased,

levels of both pro and anti-proliferative factors (BDNF and IL1β, respectively) were maintained. Interestingly, VEGF, known as both a pro-proliferative and angiogenic factor, was increased when proliferation was decreased. Furthermore, although the morphine-induced increase in VEGF did not correlate with altered angiogenesis of endoglin-IR vessels within the DG, properties of the vessels themselves such as area and perimeter were also increased.

Chronic morphine inhibits proliferating cells in the hippocampal SGZ

The effect of chronic morphine on SGZ progenitor cells has been well characterized (Arguello et al., 2008; Fischer et al., 2008). However it was necessary to confirm changes in proliferation so that they could be correlated with possible alterations in the neurogenic microenvironment within a single subject. As expected from previous data, the current experiments demonstrate that the total population of proliferating cells was only decreased after 96 hrs of morphine exposure.

DG levels of BDNF and IL1β are not altered by chronic morphine

An interesting and unexpected finding from the present study was that DG levels of BDNF and IL1β, as well as their respective receptors TrkB and IL1R1, were not decreased after 24 or 96 hrs of morphine exposure. This is of interest as other studies show that exogenously applied BDNF or IL1β are pro-proliferative or anti-proliferative, respectively (Koo and Duman, 2008; Scharfman et al., 2005). However the present findings are not unprecedented, as another report also

observed a disconnect between levels of proliferation and growth factor mRNA levels (Warner-Schmidt et al., 2008). Perhaps these differences arise from different experimental designs: examination of the effect of exogenously applied growth factors versus examination of the effect of drug administration on endogenous growth factors. It is also possible that immunoblotting from a DG-enriched sample is not sensitive enough to detect changes in protein levels that may occur in discrete cellular populations. For example, levels of BDNF are increased in one cell population and decreased in another, resulting in the lack of change reported here. However, several studies have detected changes in cell death proteins in the hippocampus via immunoblotting following morphine exposure (Emeterio et al., 2006), as well as proBDNF after prenatal exposure to opiates (Schrott et al., 2008). Given that in the present work a robust change in VEGF levels from DG-enriched samples can be detected, it can minimally be concluded that the immunoblotting approach used here is sensitive enough to detect large changes in hippocampal levels of growth factors. A third explanation for the present results could center on temporal specificity. A previous study determined that an increase in VEGF precedes an increase in BDNF levels (Louissaint et al., 2002). Thus it is possible that BDNF protein levels would not be altered at 24 and 96 hr time points, but would only be revealed at later time points. However, examination of factors within the neurogenic niche would be challenging with the present morphine paradigm since mice may experience somatic withdrawal due to the decline of morphine blood levels when time points beyond 96 hrs are examined (Fischer et al., 2008; unpublished observations).

Since i.p. injections of morphine decrease SGZ proliferation in the rat (Kahn et al., 2005), a reasonable alternative to s.c. pellet administration is a paradigm using i.p. injections of escalating morphine doses (Shaw-Lutchman et al., 2002). However, the Eisch laboratory has recently showed that escalating injection paradigms in the mouse do not decrease SGZ proliferation (Fischer et al., 2008). Thus, examination of longer time points awaits development of alternative opiate administration paradigms that result in decreased SGZ proliferation in the mouse without somatic withdrawal symptoms (e.g. self-administration or alternative injection paradigms). Taken together the present data suggest that factors in the neurogenic niche are differentially sensitive to exogenous stimuli.

DG levels of VEGF are dynamically regulated

Another significant finding of the present study was that at a time point in which progenitor proliferation was decreased, DG levels of VEGF were increased. This was unexpected as levels of VEGF are usually positively correlated with changes in proliferation (Cao et al., 2004; Jin et al., 2002; van Praag et al., 2005; Warner-Schmidt et al., 2008). Furthermore, VEGF levels increase after myocardial infarction and morphine treatment prevents this increase (Balasubramanian et al., 2001; Roy et al., 2003). One potential hypothesis in the case of chronic morphine administration is that an increase in VEGF in the DG may reflect a compensatory response of the DG to prevent a further decrease in proliferation. This is very interesting in that although 30% of proliferating cells are inhibited by morphine, 70% of proliferating cells continue to divide, which suggests that

morphine alters a subpopulation of proliferating cells that are not be able to respond to the factors that are maintained in the neurogenic niche.

Chronic morphine-induced increase in VEGF correlates with altered SGZ neurovasculature

Although increased DG levels of VEGF were observed after 96 hr of morphine exposure, it was unclear if this increase was functional. Therefore it was examined whether the increased VEGF levels correlated with a measureable change in the vasculature. Several studies have observed that increased VEGF levels are associated with altered properties of neurovasculature such as increased area and perimeter (Louissaint et al., 2002; van Praag et al., 2005) as well as number of vessels (Cao et al., 2004). In the present study, increased VEGF levels did not correlate with the formation of new vessels, but did correlate with increased area and perimeter of endoglin-IR vessels, suggesting potential remodeling of the neurovasculature. It is possible that the morphine-induced increase in VEGF levels was not robust enough to lead to angiogenesis, as observed with previous overexpression studies (Cao et al., 2004). Given the discovery that progenitor cells are closely associated with the vasculature, (Palmer et al., 2000) the increase in vessel size after morphine treatment supports the hypothesis that the neurogenic niche may be providing more vascular support to compensate for decreased SGZ proliferation. To further explore the relationship between the morphine-induced alterations in VEGF and vasculature, future experiments should take advantage of the documented lack

of morphine's effect on SVZ proliferation (Eisch et al., 2000). Comparison between the potential changes in morphine-induced alterations in neurovasculature between the SVZ and the SGZ would shed light on the similarities and differences between these two neurogenic niches.

While this correlative study highlights several interesting findings, key questions remain to be answered. What cell populations are secreting VEGF? More importantly, which progenitor cell populations express VEGFR2 and might therefore be able to respond to increased VEGF levels? Several studies have shown that progenitor cells and the surrounding granule cells express receptors to respond to growth factors, such as TrkB (Donovan et al., 2008; Li et al., 2008) and VEGFR2 (Cao et al., 2004; Jin et al., 2002), suggesting that the mature neurons themselves might provide trophic support for dividing cells. Similar to TrkB and VEGFR2 expression, IL1R1 is highly expressed in the DG and has been observed on proliferating progenitor cells (Koo and Duman, 2008). Immunohistochemical staining has shown expression of the primary VEGF receptor VEGFR2 (Flk1) on progenitor cells in the DG. Specifically, VEGFR2 expression is present on immature doublecortin (DCX)-IR cells as well as mature BrdU-IR cells (Jin et al., 2002; Palmer et al., 2000). A previous study by the Eisch laboratory determined that the total population of DCX-IR cells was not altered after chronic morphine (Arguello et al., 2008), suggesting that a subpopulation of DCX-IR cells that are also VEGFR2-IR are responsive to the morphine-induced increase in VEGF. However, VEGFR2 presence is not restricted to progenitor

cells, as colocalization of VEGFR2 has also been reported on both astrocytes and neurons in the hilus, and granule neurons of the DG (Heine et al., 2005; Palmer et al., 2000). Future studies should focus on determining if the expression pattern of VEGFR2 on specific progenitor cell types is altered after chronic morphine and whether over-expression of VEGF could attenuate the morphine-induced decrease in proliferation.

Conclusion

In sum, the present study shows that chronic morphine results in decreased SGZ proliferation, no change in DG levels of the cytokine IL1β, growth factor BDNF, or their respective receptors, but does enhance DG levels of VEGF and certain characteristics of the vasculature. The present results underscore the complex relationship between adult hippocampal neurogenesis and pro-and anti-proliferative factors within the neurogenic niche. Future work is warranted to more fully explore the functional consequence of treatments that enhance proliferation on neurovasculature (Newton and Duman, 2004). Interestingly, VEGF is important for spatial learning and memory formation (Cao et al., 2004) and human heroin abusers and rodents exposed to morphine have altered neurogenesis and impaired memory (Eisch et al., 2000; Guerra et al., 1987; Spain and Newsom, 1991; Weber et al., 2006). Therefore, future use of an opiate-self administration paradigm in rodents would be extremely useful in testing whether knockdown or overexpression of certain factors in the neurogenic

niche, such as VEGF, would impair or enhance learning of drug-context memories, indicating novel treatment avenues for addiction.

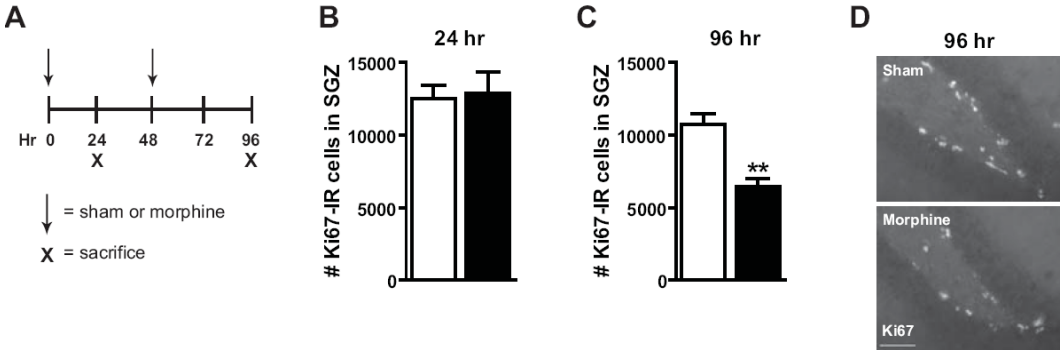

Figure 4.1. **Chronic morphine inhibits the total population of SGZ proliferating cells. (A)** C57BL/6J mice were implanted with s.c. sham or morphine pellets at 0 and 48 hrs and killed at 24 or 96 hrs via decapitation. **(B)** The number of Ki67-IR cells in the SGZ was not changed after 24 hrs. **(C)** The number of Ki67-IR cells was significantly decreased after 96 hrs of morphine exposure. **(D)** Representative IHC demonstrated decreased numbers of Ki67-IR cells in the SGZ. At all time points sham: n=4, morphine: n=4. Scale bar=100 μm, **p<0.01.

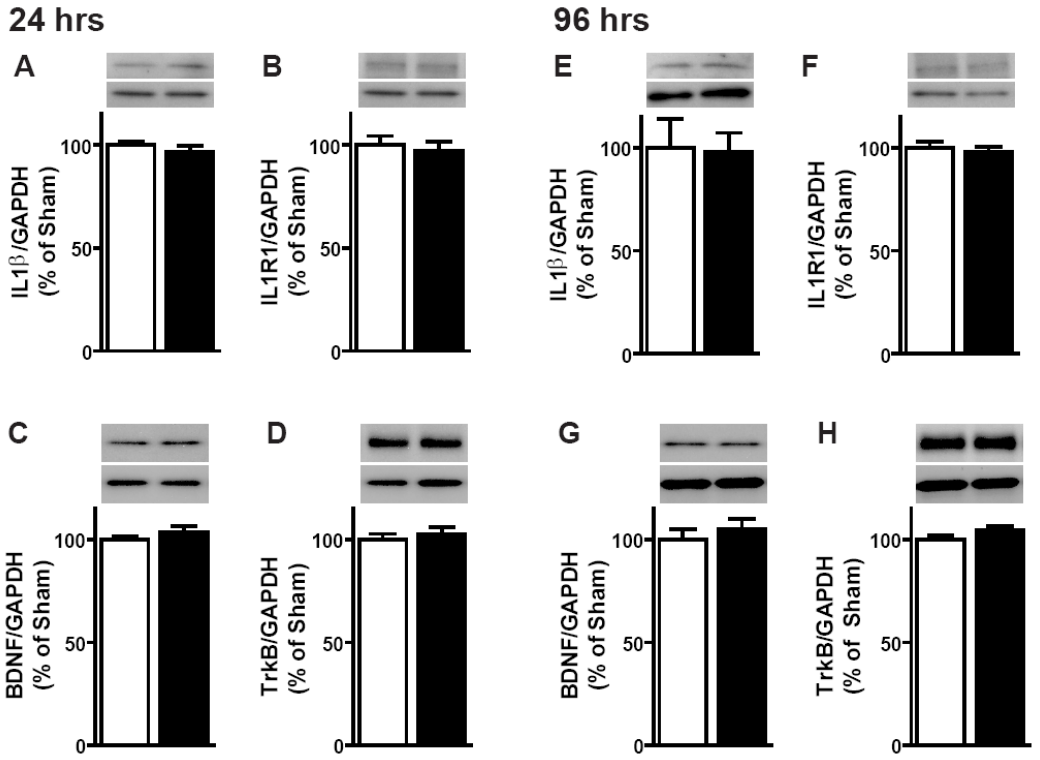

Figure 4.2. **Chronic morphine does not alter DG protein levels of BDNF or IL1β.** To determine if the microenvironment of the hippocampus was altered by chronic morphine, DG-enriched extracts were used to look for changes in protein levels via immunoblotting. **(A-D)** After 24 hrs of morphine exposure, dentate levels of IL1β, BDNF and their respective receptors, IL1R1 and TrkB remained unchanged. **(E-H)** After 96 hrs of morphine exposure, dentate levels of IL1β and BDNF and their respective receptors, IL1R1 and TrkB remained unchanged. At all time points sham: n=4-8, morphine: n=5-8.

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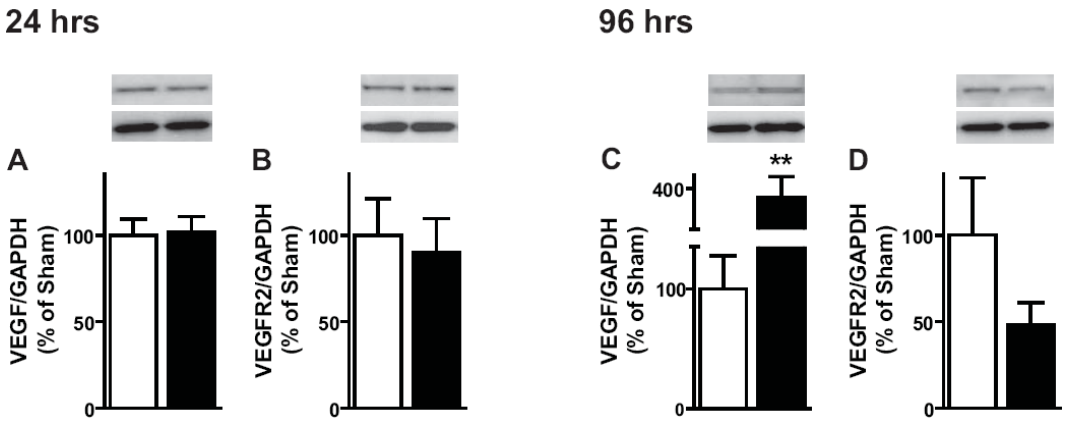

Figure 4.3. **Chronic morphine dynamically alters protein levels of VEGF in the DG. (A, B)** After 24 hrs of morphine, DG protein levels of VEGF and VEGFR2 are unchanged as measured via immunoblotting. **(C, D)** After 96 hrs, VEGF protein levels are increased whereas receptor levels remain unchanged. At all time points sham: n=5-6, morphine: n=4-8, **p<0.01.
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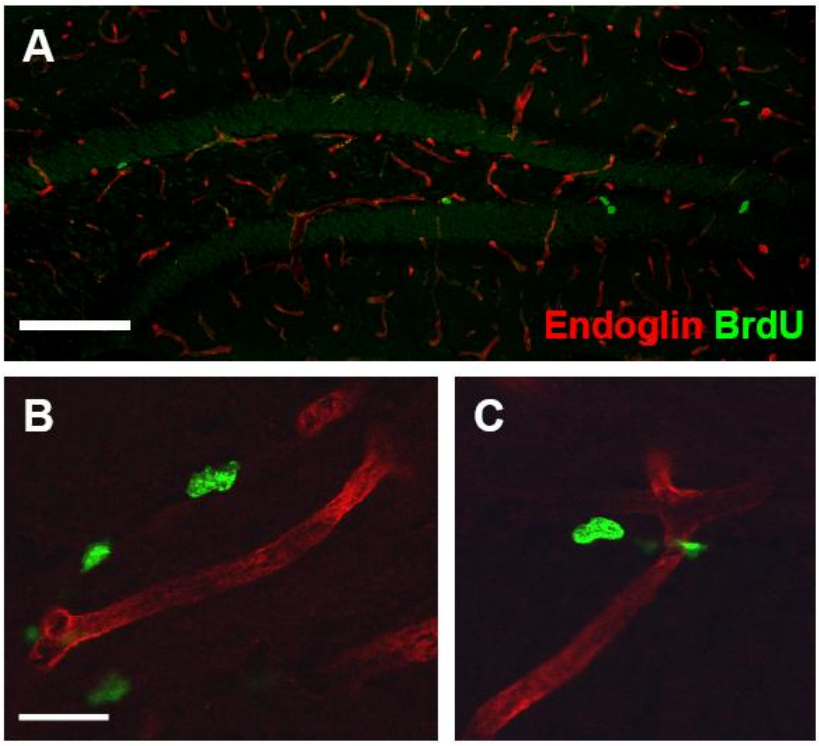

Figure 4.4. **Endoglin protein expression in the hippocampal DG.**
(A) Low-power image shows that endoglin staining is present throughout the hippocampus and in the SGZ where the majority of proliferating BrdU-IR cells reside. Scale bar=100 um. **(B-C)** Confocal images show close proximity of BrdU-IR cells to endoglin-IR vessels. Scale bar=10 um.

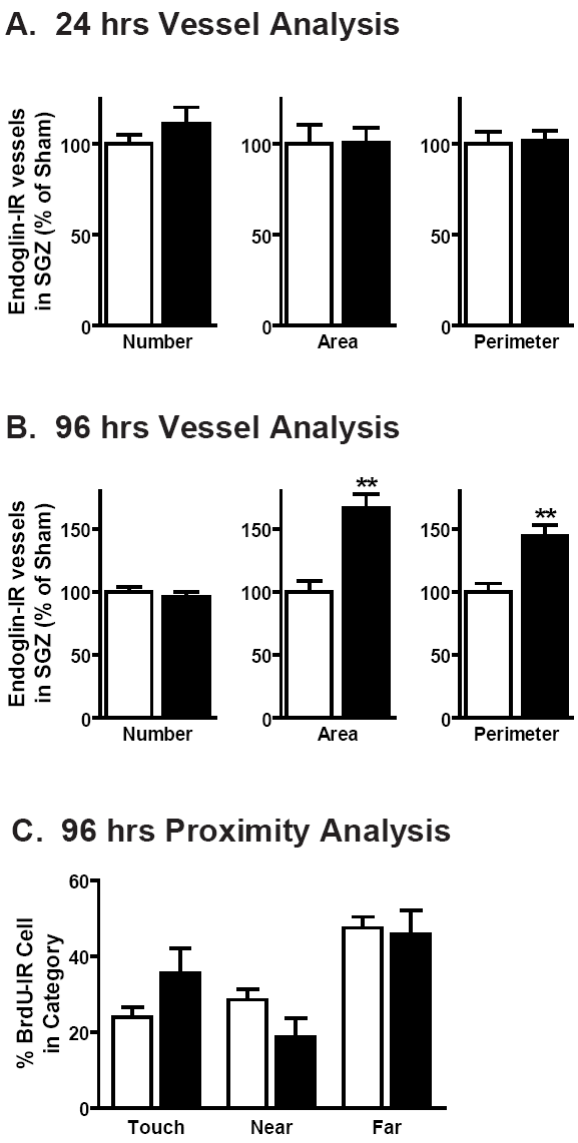

Figure 4.5. **Chronic morphine-induced increase in VEGF protein correlates with altered DG neurovasculature. (A)** After 24 hrs of morphine exposure, various properties of endoglin-IR cells including vessel number, area and perimeter remain unchanged. **(B)** After 96 hrs of morphine exposure, the number of endoglin-IR vessels between sham and morphine groups did not differ. However the area and perimeter of endoglin-IR vessels are increased. **(C)** The proximity of BrdU-IR cells to endoglin-IR vessels, does not differ between sham and morphine treated groups. Proximity was measured as "touch", touching or overlapping the vessel; "near", within 10 μm of vessel, or "far", beyond 10μm of vessel. For all time points sham: n=4-5, morphine: n=4-5, **p<0.01.

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CHAPTER FIVE

Chronic morphine's effect on neural progenitor cells labeled by an inducible stem-cell specific transgenic mouse

Introduction

Since the initial discovery of adult neurogenesis in rodent, molecular techniques to study the birth of new neurons have become more sophisticated (Ming and Song, 2005). The gold standard of observing proliferation is immunohistochemical detection of bromodeoxyuridine (BrdU), a thymidine analog that when given intraperitoneally (i.p.) gets incorporated into the DNA of dividing cells (Christie and Cameron, 2006; Kee et al., 2002). BrdU has an advantage over endogenous markers such as Ki67 (which labels proliferating cell in all cell cycle phases) in that it can provide temporal specificity by labeling only S phase cycling cells when given as a single pulse (Eisch and Mandyam, 2007; Mandyam et al., 2007). However, the use of an exogenous marker to label proliferating cells has several disadvantages. First, although minimal, even a single injection of BrdU can be stressful to mice. Second, an essential problem of BrdU labeling is the issue of dilution. A cell that is labeled with BrdU can undergo a single round of division and then exit the cell cycle; this cell can be detectable at survival time points of up to 4 weeks. However, a cell that re-enters S phase and cycles more than once will have dilution of BrdU, especially if only a single saturating dose of BrdU is administered (Cameron and McKay, 2001; Hayes and Nowakowski, 2002; Mandyam et al., 2007). Dilution of BrdU can also minimize the number of surviving cells that are observed at time points such as 4 weeks.

However, a transgenic model could potentially label a larger progenitor cell population and their progeny, so that a larger population of surviving cells could be examined at a 4 week survival time point. Therefore a more efficient, long-lasting method of labeling progenitor cells is needed.

To overcome these disadvantages of BrdU labeling, several labs have developed transgenic mice that express green or cyan fluorescent protein (GFP or CFP) under the control of the nestin promoter so that neural progenitor cells are labeled with GFP or CFP (Mignone et al., 2004; Yamaguchi et al., 2000; Encinas et al., 2006) and are typically referred to as nestin-GFP mice. These reporter lines have allowed for endogenous visualization and characterization of progenitor cells via electrophysiology and morphology, and were essential for delineating discrete stages of progenitor cell differentiation to mature neurons (Fukuda et al., 2003; Kempermann et al., 2004; Encinas et al., 2006). However these transgenic mice are limited by the fact that GFP is only expressed for as long as nestin is expressed in the neurogenic regions, making long-term analysis of progenitor cells and their progeny impossible.

A further advancement in transgenic approaches is that of the inducible-transgenic mouse. Several laboratories have generated mice in which, like the nestin-GFP mice, the nestin promoter is used but to drive expression of the CreER^{T2} fusion protein (Carlen et al., 2006; Imayoshi et al., 2008; Yu et al., 2005). Nestin-CreER^{T2} mice can then be bred with R26R-yellow fluorescent

protein (YFP) reporter mice, resulting in bitransgenic nestin-CreER^{T2}/R26R-YFP mice (Soriano, 1999). The CreER^{T2} protein remains in the cytoplasm, but when nestin-CreER^{T2}/R26R-YFP mice are given an injection of the synthetic estrogen receptor ligand tamoxifen (TAM), the CreER^{T2} fusion protein gets shuttled into the nucleus. There it can excise a floxed-stop portion of the R26R-YFP gene, leading to expression of YFP in nestin-expressing cells (Indra et al., 1999; Sauer, 1998). The advantage of this system is that once a cell is inducibly labeled, it and its progeny will be permanently labeled with YFP (Lagace et al., 2007b). This permanent labeling of progenitor cells overcomes the problems associated with dilution of BrdU and the loss of GFP expression when nestin expression declines in the nestin-GFP mice.

The Eisch laboratory has previously shown that chronic morphine decreases proliferation and maturation of progenitor cells in the mouse SGZ, via use of exogenous BrdU labeling and progenitor stage analysis with nestin-GFP mice (Arguello et al., 2008). However, to extend these studies to morphological analysis of dendrites and possibly electrophysiological analyses, a more sophisticated transgenic mouse system is needed. The Eisch Laboratory has also published a characterization the nestin-CreER^{T2}/R26R-YFP mouse, and shown that inducibly-labeled YFP cells can respond to exogenous stimuli such as running (Lagace et al., 2007b). Therefore, it was hypothesized that chronic morphine exposure would result in fewer inducibly-labeled YFP-immunoreactive (IR) cells in the nestin-CreER^{T2} mice.

Material and Methods

Animals

For all experiments, nestin-CreER^{T2}/R26R-YFP mice were used. Nestin-CreER^{T2} mice were bred with R26R-YFP reporter mice, resulting in bitransgenic mice heterozygous for both transgenes, as previously described (Lagace et al., 2007b). Mice were housed three or four to a cage with free access to food and water in a UT Southwestern Medical Center facility on a 12 hr light/dark cycle. Mice were allowed to habituate for one week, after weaning, prior to start of experiments. All experimental procedures were approved by The Institutional Animal Care and Use Committee and were in accordance with the U.S. National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Drug treatment, TAM and BrdU administration

The goal of Experiment 1 was to determine TAM's potential effect on progenitor cell proliferation and survival. As shown in Figure 5.1, nestin-CreER^{T2}/R26R-YFP mice received TAM (i.p., dissolved in 10% EtOH/90% sunflower oil) at 180 mg/kg/d for 5 days or vehicle (VEH; 10% EtOH/90% sunflower oil) starting at 5 weeks of age, as previously described (Lagace et al., 2007b). To determine TAM's potential impact on progenitor cell proliferation, 12 days post-TAM, mice received a single i.p. injection of 150 mg/kg BrdU (Roche Diagnostics, dissolved in 0.9% saline, 0.007N NaOH at 10 mg/mL) 2 hrs before sacrifice. To determine TAM's potential impact on progenitor cell survival, mice received a single

injection of BrdU 1 day prior to start of TAM treatment. 28 days post-TAM, mice were sacrificed.

The goal of Experiment 2 was to determine if the number of inducibly-labeled YFP cells in the nestin-CreER^{T2}/R26R-YFP mice was decreased after morphine exposure. As shown in Figures 5.2 and 5.3, mice received 5 daily injections of TAM (180 mg/kg/d). Twelve days or 30 days post-TAM mice were implanted with a 25 mg morphine pellet (generously provided by the National Institute on Drug Abuse, Bethesda, MD) or sham pellet subcutaneously (s.c.) under isoflurane anesthesia (2% in compressed oxygen for 1-2 minutes) at 0 and 48 hrs. 2 hrs before sacrifice (at 24 or 72 hrs) mice were administered a single injection of 150 mg/kg BrdU.

The goal of Experiment 3 was to determine TAM's possible interaction with morphine. As shown in Figure 5.4, mice received 5 daily injections of TAM (180 mg/kg/d) or VEH. Thirty days post-TAM, all mice received a 25 mg morphine pellet s.c. under isoflurane anesthesia. Two hrs before sacrifice (24 hrs) mice received a single injection of 150 mg/kg BrdU. For all experiments, mice were anesthetized with chloral hydrate (120 mg/kg dissolved in 0.9% saline), and perfused transcardially with 0.1M PBS followed by 4% paraformaldehyde in 0.1M PBS (pH 7.4), as previously described (Arguello et al., 2008; Harburg et al., 2007).

Tissue sectioning

Serial sets of 30μm coronal sections from brains were collected on a freezing microtome (Leica, SM2000R). Every ninth section through the entire mouse hippocampus was slide mounted prior to immunohistochemistry (IHC) (-0.82 to -4.24 from bregma) (Franklin and Paxinos, 1997). Slides were coded before IHC and the code was not broken until data analysis was complete (Arguello et al., 2008; Mandyam et al., 2004).

IHC

Antibodies. The following antibodies were used for IHC: rat anti-BrdU (Accurate, Westbury, NY, Cat #: OBT0030, Lot #: H7786, 1:300); rabbit anti-Ki67 (SP6, LabVision Corporation NeoMarkers, Fremont, CA, Cat#: RM-9128-R1, Lot#: 9106S603C, 1:500), rabbit anti-GFP (used to detect YFP; Invitrogen, Eugene, OR, Cat#: A11122, Lot#: 41724A, 1:3000). For each antibody, the specificity of staining was determined by lack of signal after omission and/or dilution of the primary antibody, as previously reported: BrdU (Mandyam et al., 2004), Ki67 (Dayer et al., 2003; Mandyam et al., 2007), YFP (Lagace et al., 2007b).

Single label IHC. For BrdU IHC, the following pretreatment steps were used: antigen unmasking (0.01M citric acid, pH 6.0, 100°C, 10 min), membrane permeabilization (trypsin in 0.1M Tris, 0.1% $CaCl_2$, 10 min), DNA denaturation (2M HCl in 1X PBS, 30 min) and quenching of endogenous peroxidases (0.3% H_2O_2, 30 min), as described previously (Arguello et al., 2008). Nonspecific

binding was blocked (3% serum, 0.3% Triton-X, 30-60 min) followed by incubation with rt anti BrdU primary antibody overnight (3% serum, 0.3% Tween-20). BrdU IHC was completed using the avidin-biotin diaminobenzidine visualization method, followed with counterstain by Nuclear Fast Red (Vector Laboratories, Cat#: H-3403, Lot#: N0507), as previously described (Arguello et al., 2008). For Ki67 IHC, pretreatment steps of antigen unmasking and quenching of endogenous peroxidases were used. Primary, secondary antibody incubations and visualization were the same as for BrdU IHC. For GFP IHC to detect YFP-expressing cells, pretreatment steps of antigen unmasking and quenching of endogenous peroxidases were used. Nonspecific binding was blocked, followed by incubation in rabbit anti-GFP primary antibody overnight. Sections were then incubated with biotin-tagged secondary antibody (1:300, 60 min) and IHC was completed with the avidin-biotin/FITC tyramide signal amplification method (FITC TSA, Perkin Elmer Life Sciences, Inc., Boston, MA, Lot#: 328163, 1:50), followed by counterstain with DAPI, as previously described (Lagace et al., 2007b).

Microscopic analysis and quantification

BrdU-IR, Ki67-IR and YFP-IR cell numbers were at 400X magnification on an Olympus BX-51 microscope (Tokyo, Japan). Cell numbers were quantified with modified stereology via the optical fractionator method throughout the rostral-caudal axis of the hippocampus (-0.82 to -4.24 from bregma) (Franklin and Paxinos, 1997). Cell counts were collected from the SGZ (defined as the region

bordering the granule cell layer and hilus: three cell widths into the hilus and half of the granule cell layer), with continuous adjustment of the focal plane, as described previously (Arguello et al., 2008; Donovan et al., 2006).

Statistical analysis

Data are reported as mean ± SEM. Statistical analyses were performed using student's t-test for comparison of two groups. All statistical analyses were performed using Prism (version 5.0) software. Statistical significance was defined as $p<0.05$ with *$p<0.05$, **$p<0.01$.

Results

Progenitor cells in the SGZ of nestin-CreER^{T2}/R26R-YFP mice are inducibly labeled with YFP

Nestin-CreER^{T2}/R26R-YFP mice express transgenes for both nestin-CreER^{T2}, driven by 5.8 kB of the nestin promoter, and R26R-YFP reporter (Lagace et al., 2007b). When given TAM (180 mg/kg/d for 5 days), a ligand for the modified estrogen receptor, ER^{T2}, the CreER^{T2} fusion protein in nestin-expressing cells translocates to the nucleus and excises a stop codon flanked by loxP sites, allowing for expression of YFP (Lagace et al., 2007b). Nestin-CreER^{T2}/R26R-YFP mice given TAM have robust expression of YFP in the SGZ and other neurogenic regions in the postnatal brain, whereas mice given VEH do not (Figure 5.1). Furthermore it has been determined that YFP-labeled cells give rise to immature and mature neuronal cell types (Lagace et al., 2007b).

TAM administration does not result in a long-lasting enhancement of progenitor cell survival

TAM can have both negative and positive effects on progenitor cell proliferation (Barker and Galea, 2008; Galea, 2008; Lagace et al., 2007a). It was important to evaluate the impact of TAM on proliferation and survival in nestin-CreER^{T2}/R26R-YFP mice in order to appropriately interpret results from later studies on the effect of morphine on YFP-expressing cells in this mouse. To address the impact of TAM on progenitor cell proliferation, nestin-CreER^{T2}/R26R-YFP mice were given TAM for 5 days. Twelve days post-TAM, they received an injection of BrdU 2 hrs prior to sacrifice (Figure 5.1B). Mice given TAM had significantly more BrdU-IR cells in the SGZ when compare to VEH treated mice (Figure 5.1C). To address the impact of TAM on progenitor cell survival, nestin-CreER^{T2}/R26R-YFP mice received an injection of BrdU one day prior to TAM. Twenty-eight days post-TAM, mice were sacrificed (Figure 5.1D). Mice treated with TAM had similar numbers of BrdU-IR cells in the SGZ when compared to VEH treated mice (Figure 5.1E). These data suggest that although TAM has a pro-proliferative effect 12 days post-TAM, the pro-proliferative effect does not result in increased progenitor cell survival.

Chronic morphine does not alter the number of inducibly-labeled YFP cells in the SGZ of nestin-CreER^{T2}/R26R-YFP mice

Although TAM had a transient effect on progenitor cell proliferation, all nestin-CreER^{T2}/R26R-YFP mice would be given TAM and therefore would have the

same baseline levels of proliferation prior to chronic morphine treatment. To determine the effect of chronic morphine on proliferation in the nestin-CreER^{T2}/R26R-YFP mice, a well-characterized morphine administration paradigm was employed (Arguello et al., 2008; Fischer et al., 2008; Mandyam et al., 2004). Nestin-CreER^{T2}/R26R-YFP mice were given TAM (Figure 5.2), and 12 days post-TAM they received either sham or morphine pellets s.c. (at 0 and 48 hrs). Two hrs before sacrifice (at 96 hrs), mice were given an injection of BrdU (Figure 5.2A). It has been shown previously that the number of BrdU-IR cells is decreased after 96 hrs of morphine exposure in C57BL/6J mice (Arguello et al., 2008; Fischer et al., 2008; Mandyam et al., 2004). Surprisingly, the number of inducibly-labeled YFP cells in the SGZ was not different between sham and morphine treated mice (Figure 5.2B). When proliferation was assessed with the traditional S phase marker BrdU, it was found that the number of BrdU-IR cells in the SGZ was not different between sham and morphine treated groups (Figure 5.2C), suggesting that nestin-CreER^{T2}/R26R-YFP mice did not respond similarly to morphine as C57BL/6J mice. It was of note that a large percent of nestin-CreER^{T2}/R26R-YFP mice died before the 96 hr sacrifice time point (50% lethality rate as opposed to 2% in C57BL/6J mice), suggesting an interaction between TAM and morphine. To address whether an interation between TAM and morphine could account for the increased lethality rate, the time interval between TAM and morphine administration was lengthened.

Chronic morphine does not alter SGZ proliferation in the nestin-CreER^{T2}/R26R-YFP mice with longer interval between TAM and morphine administration

Previous studies have shown that TAM *in vitro* can inhibit an enzyme required for metabolism and clearance of morphine, suggesting the potential for side effects when both drugs are co-adminsitered (Hara et al., 2007). To overcome a possible interaction between between TAM and morphine, the interval between TAM and morphine administration was increased. Nestin-CreER^{T2}/R26R-YFP mice were given TAM (Figure 5.3) and 30 days post-TAM received either sham or morphine pellets s.c. (at 0 and 48 hrs). Two hrs before sacrifice (at 24 or 96 hrs), mice were given an injection of BrdU (Figure 5.3A). Again, the number of BrdU-IR cells in the SGZ after 24 hrs of morphine exposure was not different from sham treated nestin-CreER^{T2}/R26R-YFP mice (Figure 5.3B). Proliferation was also assessed with the endogenous proliferation marker Ki67, as previous results from the Eisch laboratory have shown that Ki67 and BrdU do no always serve as equivalent proliferation markers (Arguello et al., 2008). However, the number of Ki67-IR cells in the SGZ was not different between sham and morphine treated nestin-CreER^{T2}/R26R-YFP mice after 24 hrs (Figure 5.3C) or 72 hrs of morphine exposure (Figure 5.3D). These data suggest that nestin-CreER^{T2}/R26R-YFP mice do not have decreased levels of proliferation after morphine exposure, perhaps suggesting a resistance to the morphine-induced decrease in SGZ proliferation. However, as it was determined that TAM led to a transient increase in proliferation (Figure 5.1C), it was necessary to determine if

proliferation was different between TAM /morphine and VEH/morphine treated mice.

TAM treatment does not alter proliferation 30 days after the last day of treatment

To determine if TAM resulted in an increased baseline of proliferation 30 days post-TAM, mice were given TAM or VEH as usual (Figure 5.4). Thirty days post-TAM or VEH, all mice received an s.c. morphine pellet at 0 hrs. Two hrs before sacrifice (at 24 hrs) mice received a single injection of BrdU (Figure 5.4A). Interestingly, whether mice were given VEH or TAM did not influence morphine's effect on SGZ proliferation. Both the number of BrdU-IR cells in the SGZ and the number of Ki67-IR cells in the SGZ were similar (Figures 5.4B, C). This suggests that TAM does not account for the lack of a morphine-induced decrease of SGZ proliferation in the nestin-CreER^{T2}/R26R-YFP mice.

Discussion

Nestin-CreER^{T2}/R26R-YFP mice can be used to permanently label progenitor cells

The nestin-CreER^{T2}/R26R-YFP mice were generated previously by the Eisch laboratory. It has been shown previously that TAM inducibly and reliably labels progenitor cells and their subsequent progeny in neurogenic regions of this transgenic mouse. This transgenic mouse system has the added advantage that it is not "leaky"; no recombination is observed when mice are treated with vehicle.

Furthermore, recombination is induced in a stem-like progenitor cell so that subsequent progeny are also YFP-IR, with an end result of a population of recombined cells that include proliferating, immature and mature progenitor cells (Lagace et al., 2007b). Finally, the TAM needed to induce recombination does not result in any long-term effects on progenitor cell survival. Therefore, progenitor cells were indubily labeled in this transgenic mouse with no long-term TAM-induced effects on cell survival.

Nestin-CreER^{T2}/R26R-YFP mice do not have decreased proliferation with chronic morphine exposure

The most challenging but also interesting finding of this study was the lack of decrease in the number of inducibly-labeled YFP-IR, BrdU-IR and Ki67-IR cells in the SGZ after morphine exposure. The lack of a decrease in proliferation as assessed by BrdU-IR and Ki67-IR counts is in striking contrast to previously published reports (Arguello et al., 2008; Fischer et al., 2008; Mandyam et al., 2004). Why do nestin-CreER^{T2}/R26R-YFP mice not show a morphine-induced decrease in SGZ proliferation? The initial observation of increased lethality in the nestin-CreER^{T2}/R26R-YFP mice suggested a potential interaction between TAM and morphine with a 12-day interval between TAM and morphine (50% lethality in nestin-CreER^{T2}/R26R-YFP mice compared to 2% lethality in C57BL/6J mice). Indeed, *in vitro* literature suggested that TAM and morphine could interact with each other (Gutierrez et al., 1998; Hara et al., 2007). Specifically, TAM can inhibit the activity of an enzyme required for metabolism and clearance of morphine 3

and 6-glucuronides (Hara et al., 2007). Morphine 6-glucuronide can be 20 times more potent as an analgesic when compared to morphine, therefore TAM's possible inhibition of morphine 6-glucuronide's clearance could result in side effects when both of these drugs are co-adminsitered (Hara et al., 2007).

Though these *in vitro* data do not provide a definitive answer for the increased lethality in nestin-CreER^{T2}/R26R-YFP transgenic mice, the possibility that TAM could interact with morphine *in vivo* provided a rationale for increasing the interval between TAM and morphine administration to 30 days. With the increased interval between TAM and morphine administration, the lethality rate of nestin-CreER^{T2}/R26R-YFP mice was diminished (from 50% to 14%), however the morphine-induced decrease in SGZ proliferation was still not observed. Given the data that TAM can increase proliferation, it is possible that the lack of decreased proliferation after morphine is due to a transient increase in proliferation after TAM. Although both sham and morphine treated mice received TAM injections, suggesting that both groups would have an enhanced baseline of proliferation, as possible transient increase in proliferation was an important issue to address. In our initial experiment testing the effect of TAM on proliferation, there was a 12-day interval between TAM and morphine administration. Since the morphine studies were conducted after a 30-day interval, we tested the potential effect of TAM on proliferation 30 days post-TAM. Interestingly, after a 30-day interval between TAM and morphine administration, the number of proliferating cells between VEH/morphine and TAM/morphine treated mice was not different. This

suggested that in fact the lack of a decrease in proliferation that was observed was due to the transgenic mouse itself.

Could mouse strain contribute to the lack of a decrease in proliferation in the nestin-CreER^{T2}/R26R-YFP mice? Although it has been shown that certain non-transgenic mouse strains can have an increased response to exogenous stimuli (Kempermann et al., 1998), the contrary has not been examined. This is unlikely since the nestin-CreER^{T2}/R26R-YFP mice are on a C57BL/6J background, which is similar to that of previous studies (Arguello et al., 2008; Fischer et al., 2008). It's also possible that the nestin-CreER^{T2} mice have a shifted dose response to morphine. This is also unlikely in that nestin-CreER^{T2} mice treated with morphine presented with similar behavioral response to morphine, as C57BL/6J mice (Fischer et al., 2008). However, to test this hypothesis two simple experiments could be conducted. One is to administer morphine to C57BL/6J mice and nestin-CreER^{T2}/R26R-YFP that are not treated with TAM. Another is to treat C57BL/6J mice with the usual TAM treatment and then administer morphine. Furthermore, more detailed behavioral assessments could be conducted after morphine exposure, as detailed previously (Fischer et al., 2008). These experiments would definitively address whether the lack of decreased proliferation is due to an effect of transgene insertion or due to the protective effect of TAM (though the data presented in Figure 5.4 does not support this possibility).

The data gained from these future experiments might afford a future alternative to block the observed morphine-induced decrease in proliferation in C57BL/6J mice. It is possible that this data could be translated to patients who are receiving TAM for cancer treatment, concurring with morphine for treatment of pain.

Figure 1

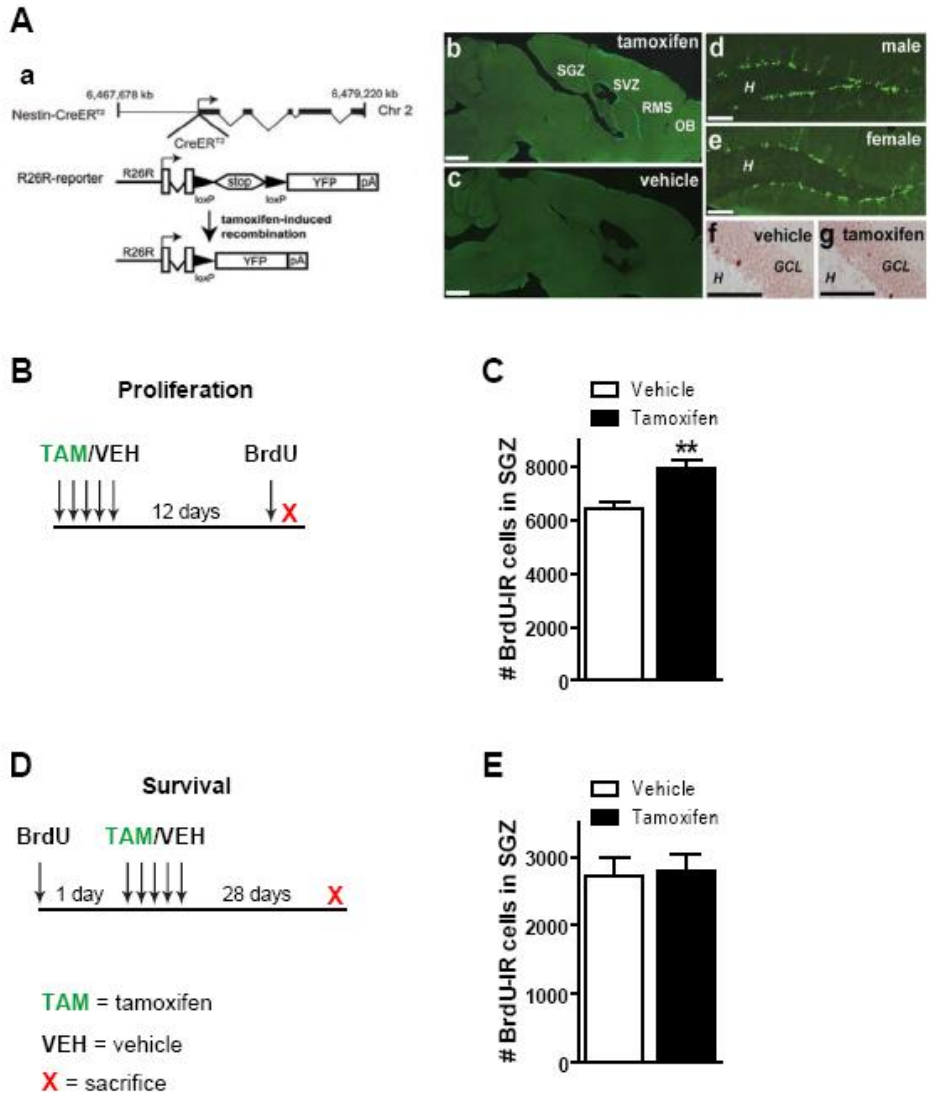

Figure 5.1. **Recombination in nestin-CreER^{T2}/R26R-YFP mice is induced by TAM and is specific to neurogenic regions. (A)** ***a***, The nestin-CreER^{T2}/R26R-YFP construct has 5.8 kb of the *nestin* promoter and exons 1–3, including the second intronic enhancer. ***b, c***, YFP-IR cells are evident 12 d post-TAM in the SVZ, Rostral Migratory Stream, Olfactory Bulb, Granule Cell Layer, and SGZ of nestin-CreER^{T2}/R26R-YFP mice given TAM but not VEH. ***d, e***, Recombination efficiency in the SGZ is similar in male and female mice 12 d after TAM. ***f***, ***g***,

Mice given BrdU 1 d before TAM or VEH and killed 28 d later have similar numbers of SGZ BrdU cells. Scale bars: ***b***, ***c***, 1 mm; ***d– g***, 100μm. **(B)** To assess TAM's possible effect on progenitor cell proliferation, nestin-CreER^{T2}/R26R-YFP mice were treated for 5 days with TAM or VEH. 12 days post-TAM or VEH, mice received a single 150 mg/kg i.p. injection of BrdU and 2 hrs later, mice were sacrificed. **(C)** The number of BrdU-IR cells was significantly increased after the typical TAM paradigm. Sham: n=7, morphine: n=8, ** $p<0.01$. **(D)** To assess TAM's possible effect on progenitor cell survival, nestin-CreER^{T2}/R26R-YFP mice were given a 150 mg/kg injection of BrdU one day before TAM treatment. Mice were sacrificed 28 days post-TAM. **(E)** The number of BrdU-IR cells in the SGZ after a 28 day survival time point is similar between VEH and TAM mice. Sham: n=5, morphine: n=6. Data from Figure 5.1A and E were previously published in Lagace et al, 2007b.

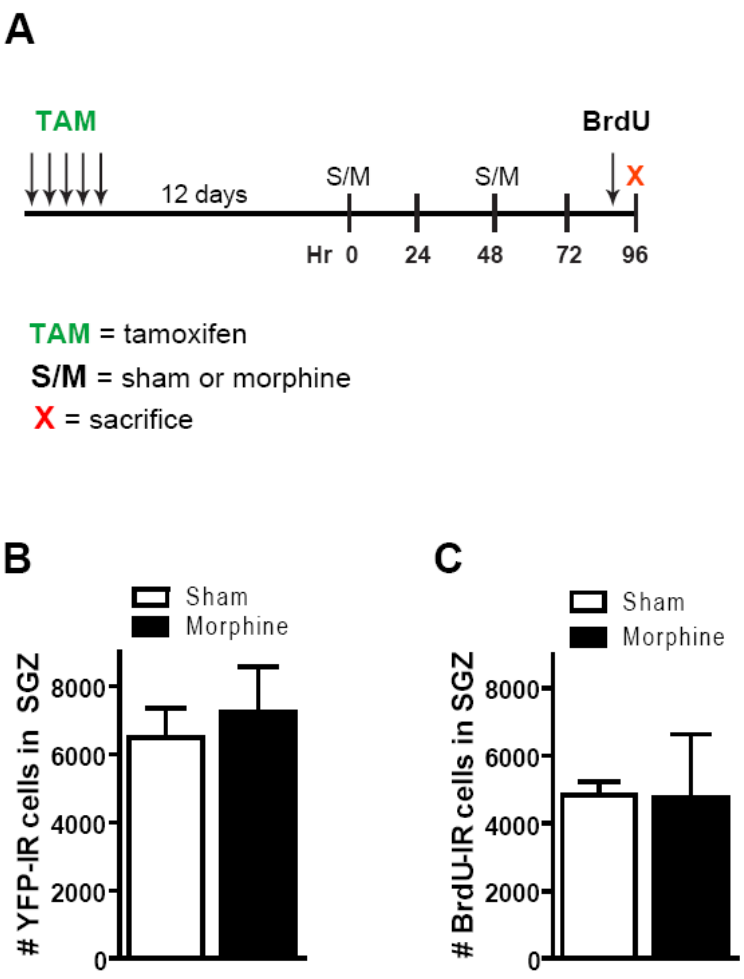

Figure 5.2. **Chronic morphine does not alter the number of inducibly-labeled YFP-IR cells in the SGZ. (A)** To determine if 96 hrs of morphine exposure alters the number of YFP-IR cells, nestin-CreER^{T2}/R26R-YFP mice received TAM injections for 5 days. Twelve days post-TAM, mice were implanted with s.c. sham or morphine pellets (0, 48 hrs) and sacrificed at 96 hrs. Two hrs before sacrifice, mice received a 150 mg/kg injection of BrdU. **(B)** Nestin-CreER^{T2}/R26R-YFP mice exposed to morphine for 96 hrs have an equal number of inducibly-labeled YFP-IR cells as compared to sham exposed mice. Sham: n=3, morphine: n=2. **(C)** nestin-CreER^{T2}/R26R-YFP mice exposed to morphine for 96 hrs do not have decreased numbers of BrdU-IR cells in the SGZ when compared to sham. Sham: n=3, morphine: n=2.

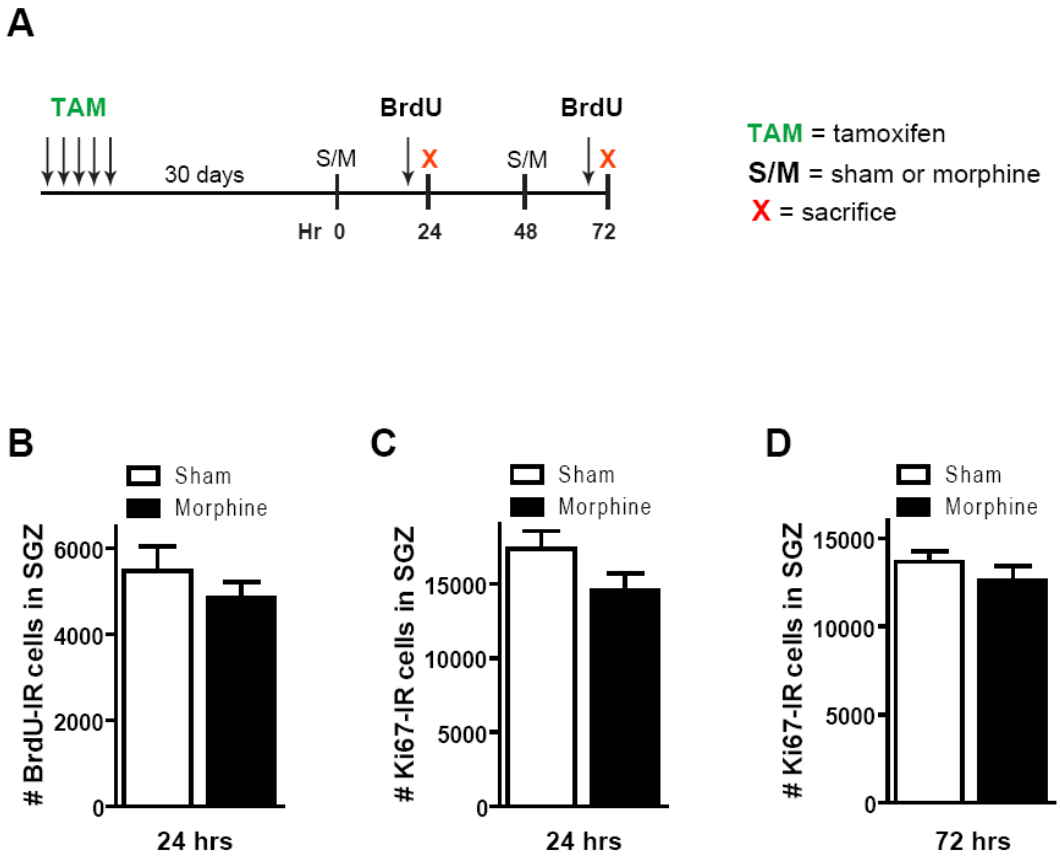

Figure 5.3. **Nestin-CreER^{T2}/R26R-YFP mice exposed to chronic morphine do not have decreased levels of SGZ proliferation. (A)** Nestin-CreER^{T2}/R26R-YFP mice received TAM injections for 5 days. Twelve days post-TAM mice were implanted with s.c. sham or morphine pellets (0, 48 hrs) and sacrificed at 24 or 72 hrs. Two hrs before sacrifice, mice received a 150 mg/kg injection of BrdU. **(B)** The number of BrdU-IR cells in the SGZ does not differ between sham and morphine treated groups after 24 hrs of morphine exposure. Sham: n=4, morphine: n=6. **(C)** The number of Ki67-IR cells in the SGZ does not differ between sham and morphine treated groups after 24 hrs of morphine exposure. Sham: n=4, morphine: n=6. **(D)** The number of Ki67-IR cells in the SGZ does not differ between sham and morphine treated groups after 72 hrs of morphine exposure. Sham: n=7, morphine: n=7.

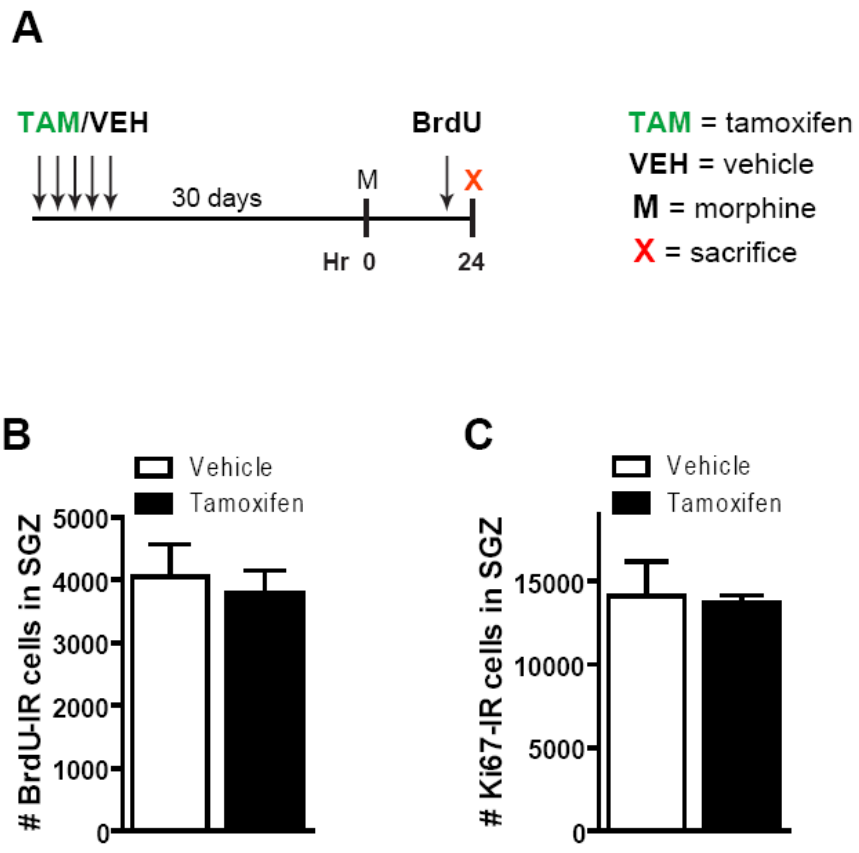

Figure 5.4. **TAM does not alter proliferation 30 days post-TAM (A**) Nestin-CreER^{T2}/R26R-YFP mice received TAM or VEH injections for 5 days and 30 days later all mice received a single s.c. morphine pellet and were sacrificed at 24 hrs. Two hrs before sacrifice, mice receive a single 150 mg/kg injection of BrdU. **(B)** The number of BrdU-IR cells does not differ between VEH or TAM mice that were all exposed to 24 hrs of morphine. Sham: n=5, morphine: n=6. **(C)** The number of Ki67-IR cells does not differ between VEH or TAM mice that were all exposed to 24 hrs of morphine. Sham: n=5, morphine: n=5.

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CHAPTER SIX
Conclusions and future directions

Conclusions

Chronic morphine has been shown previously to decrease adult hippocampal proliferation and neurogenesis in both mice and rats. This dissertation expands on previous work to determine: 1) a time course of morphine's effect on the progenitor cell cycle, cell death and immature neurons as well as maturation of immature neurons; 2) morphine's potential effect on alteration of S phase length; 3) morphine's indirect effect on the hippocampal neurogenic niche; 4) morphine's effect on proliferation in an inducible transgenic nestin-CreER^{T2}/R26R-yellow fluorescent protein (YFP) mouse. I provide evidence that morphine alters the cell cycle of SGZ progenitor cells and maturation of progenitor cells. Furthermore, I provide evidence that morphine up-regulates DG levels of the growth factor VEGF and enhances area and perimeter of neurovasculature within the neurogenic niche.

Chapter 2: *Time course of chronic morphine-induced inhibition of hippocampal cytogenesis*

In order to understand whether morphine exerts its effects on progenitor cell proliferation and neurogenesis by altering cell cycle dynamics, cell death and progenitor cell maturation, I used a time course of subcutaneous (s.c.) morphine pellet administration. I found that adult hippocampal proliferation was inhibited at all timepoints of morphine administration, S phase cycling cells were potently

inhibited by morphine at earlier exposure times, cell death was increased at early time points of exposure, and that morphine also inhibited progenitor cell maturation. These findings suggest that morphine inhibits adult neurogenesis by altering S phase proliferating cells as well as inhibiting progenitor cell maturation. These findings also raise new questions. Why are progenitor cells paused at the type 2b stage? Why doesn't the number of S phase progenitor cells decrease further with longer exposure to morphine?

One possibility is that these subpopulations of progenitor cells express specific receptors that enable them to differentially respond to morphine directly or, more likely, to alterations in the neurogenic microenvironment (Duan et al., 2008; Encinas et al., 2006; Plumpe et al., 2006; Wang et al., 2008). In regards to responding to morphine directly, it remains unclear when the mu opioid receptor (MOR) is expressed on progenitors cells (Drake et al., 2007; Eisch and Harburg, 2006), but several lines of indirect evidence suggest that morphine and opioid receptors are important in neurogenesis (Drake et al., 2007; Eisch et al., 2000; Harburg et al., 2007; Hauser et al., 2000; Koehl et al., 2008). In light of the present data that morphine decreases the percent of bromodeoxyuridine-immunoreactive (BrdU-IR) cells that reach an immature stage, it is interesting that mice that lack the MOR from birth have increased survival of dentate gyrus (DG) granule cells (Harburg et al., 2007). However, more work is needed to clarify if morphine directly inhibits particular progenitor cell types. In regards to responding to alterations in the neurogenic microenvironment, it is known that

cells in discrete stages of neurogenesis are more likely to express tropomyosin related kinase B (TrkB), a key receptor for the neurotrophin brain-derived neurotrophic factor (BDNF), NMDA, or subtypes of glucocorticoid receptors, all of which have been implicated in regulating neurogenesis (Donovan et al., 2008; Garcia et al., 2004; Nacher et al., 2007). Perhaps morphine inhibits actively dividing progenitor cells preferentially due to altered expression of these receptors, such that the progenitor cells cannot compensate or respond to morphine-induced changes in the hippocampal microenvironment.

Future Directions, Chapter 2

A future direction that the Eisch laboratory will take is to look at expression of MORs in these discrete subpopulations of cells via electron microscopy. Perhaps one or more populations will express less or more MOR and it's tempting to postulate the possibility that the type 2b cells will express more MORs or be more likely to express MORs, explaining their preferential inhibition by morphine. To determine why the number of S phase progenitor cells is decreased by 30% at all time points, future studies could quantify the number of type 1 stem cells, or the source of progenitor cells, in the SGZ. Perhaps morphine does not affect this stem cell population, allowing for stabilization of the S phase progenitor cell population after the initial decrease at 24 hrs.

Chapter 3: Chronic morphine alters specific aspects of the progenitor cell cycle

In order to understand whether a decrease in proliferation is due to a shortening of S phase length I used a modified double injection paradigm, using the two halogenated thymidine analogs iododeoxyuridine (IdU) and chlorodexoyuridine (CldU). I found that although IdU and CldU could be used to determine if S phase cells were inhibited after chronic morphine exposure, the relative length of S phase remained unchanged. These findings suggest that chronic morphine exposure simply decreases the number of cells dividing in S phase. If morphine inhibits only the number of dividing cells in S phase and doesn't alter the length of S phase, then how do progenitor cells reach G2/M prematurely?

Future Directions, Chapter 3

To examine how hippocampal progenitor cells reach G2/M prematurely, future studies should focus on determining whether specific cell cycle checkpoint proteins are altered after chronic morphine. Several proteins have been shown to regulate the transition from S to G2/M including Cdk1 and cyclin B1 (Sanchez and Dynlacht, 2005). These proteins could be analyzed by western blotting of DG-enriched hippocampal samples, to obtain a rough estimate of potential protein changes. However, if candidate checkpoint proteins are altered after chronic morphine they will need to be validated via immunohistochemistry (IHC). IHC will provide essential information as to the expression of these candidate proteins with regards to the cell cycle. For example, if Cdk1 protein doesn't co-localize with the mitotic marker phosphorylated histone H-3 (pHH3), it would be questionable if it really is expressed during the G2/M checkpoint *in vivo* in the

subgranular zone (SGZ). If the G2/M checkpoint proteins are not altered after chronic morphine, a second potential experiment could test to see if the G1-S phase transition is altered. Perhaps fewer cell are re-entering the S phase. This could be examined with a modified version of the IdU/CldU double injection paradigm in which the interinjection interval is increased to 10-12 hrs. In this case double labeled cells would represent those cells that were labeled with the first analog and labeled with the second analog because they re-entered S phase of the cell cycle. Future studies could also address why there are more IdU-IR cells than CldU-IR cells by determining the half-life of IdU and CldU. Perhaps there are more IdU-IR cells because this analog does not have the same half-life as CldU. To determine this, a time course of both analogs would need to be performed, as with a previous BrdU time course study (Mandyam et al., 2007). It is also possible that by the second injection of CldU, the nucleoside transporter has become saturated, which would lead to less cells incorporating CldU (Cameron and McKay, 2000).

Chapter 4: Chronic morphine's effect on the hippocampal neurogenic niche

In order to understand how morphine might indirectly affect progenitor cells, I observed various aspects of the hippocampal neurogenic niche, including growth factors and neurovasculature. I found that many factors in the neurogenic niche such as BDNF, Interleukin-1β (IL1β) and their receptors were maintained after chronic morphine. Furthermore I determined that other factors such as vascular endothelial growth factor (VEGF) and the dentate neurovasculature were

upregulated after morphine exposure (other factors such as TNF and TGFβ, were not detectable via western blotting). These findings suggest that factors within the neurogenic niche are either maintained or upregulated to compensate for the morphine-induced decrease in SGZ proliferation. The fact that elements of the neurogenic niche were upregulated was particularly striking. Why are certain elements upregulated? In regards to the compensatory response of VEGF and the neurovasculature, are these changes a result of decreased proliferation and how do progenitor cells signal to the neurogenic niche around them?

Several studies have shown that progenitor cells and the surrounding granule cells express receptors to respond to growth factors, such as TrkB (Donovan et al., 2008; Li et al., 2008) and vascular endothelial growth factor receptor 2 (VEGFR2) (Cao et al., 2004; Jin et al., 2002), suggesting that the mature neurons themselves might provide trophic support for dividing cells. Similar to TrkB and VEGFR2 expression, Interleukin-1β receptor 1 (IL1R1) is highly expressed in the DG and has been observed on proliferating progenitor cells (Koo and Duman, 2008). IHC staining has shown expression of the primary VEGF receptor VEGFR2 (Flk1) on progenitor cells in the DG. Specifically, VEGFR2 expression is present on immature doublecortin (DCX)-IR cells as well as mature BrdU-IR cells (Jin et al., 2002; Palmer et al., 2000). A previous study by the Eisch laboratory determined that the total population of DCX-IR cells was not altered after chronic morphine (Arguello et al., 2008), suggesting that a subpopulation of DCX-IR cells that are also VEGFR2-IR are responsive to the

morphine-induced increase in VEGF. However, VEGFR2 presence is not restricted to progenitor cells, as colocalization of VEGFR2 has also been reported on both astrocytes and neurons in the hilus, and granule neurons of the DG (Heine et al., 2005; Palmer et al., 2000).

Future Directions, Chapter 4

Future studies should focus on determining if the expression pattern of VEGFR2 on specific progenitor cell types is altered after chronic morphine and whether over-expression of VEGF could attenuate the morphine-induced decrease in proliferation. Furthermore to examine whether the increase in VEGF and neurovasculature is a consequence of morphine treatment, future experiments should focus on overexpressing or inhibiting VEGF expression during morphine administration to determine if this will block or exacerbate morphine's effect on progenitor cell proliferation. However, these studies will need to be conducted in a heroin self-adminsitration paradigm due to the limitations of the present s.c. morphine pellet paradigm (Chapter 4 discussion). Use of the heroin self-administreation paradigm will help to more fully explore the functional consequence of treatments that enhance proliferation on neurovasculature (Newton and Duman, 2004). Interestingly, VEGF is important for spatial learning and memory formation (Cao et al., 2004) and human heroin abusers and rodents exposed to morphine have altered neurogenesis and impaired memory (Eisch et al., 2000; Guerra et al., 1987; Spain and Newsom, 1991; Weber et al., 2006). Therefore, future use of an opiate-self administration paradigm in rodents would

be extremely useful in testing whether knockdown or overexpression of certain factors in the neurogenic niche, such as VEGF, would impair or enhance learning of drug-context memories, indicating novel treatment avenues for addiction.

Chapter 5: Chronic morphine's effect on neural progenitor cells labeled by an inducible stem-cell specific transgenic mouse

In order to facilitate potential future studies on morphine's effect on dendritic morphology or altered electrophysiological effects of progenitor cells using an inducible transgenic mouse, I assessed whether proliferation in these mice was altered. I found that the transgenic nestin-CreER^{T2}/R26R-YFP mice, in which progenitor cells and their progeny are permanently labeled, did not have altered levels of proliferation as assessed by YFP-IR, BrdU-IR, and Ki67-IR cell counts at various timepoints of morphine exposure. These findings suggest that nestin-CreER^{T2}/R26R-YFP mice do not respond similarly to morphine as C57BL/6J mice. Why do nestin-CreER^{T2}/R26R-YFP mice respond differently to morphine than C57BL/6J mice? Is this due to insertion of transgenes in the mouse genome or due to an interaction of tamoxifen (TAM) and morphine or due to a shifted dose response to morphine?

Future Directions, Chapter 5

To determine if the nestin-CreER^{T2}/R26R-YFP mice do not have the expected morphine induced-decrease in progenitor cell proliferation due to transgene insertion or due to a shifted dose-response to morphine, two potential

experiments should be conducted in the future. One is to administer morphine to three groups of mice that are not given TAM: C57BL/6J mice, nestin-CreER^{T2}/R26R-YFP, and mice that only contain a copy of the nestin-CreER^{T2} transgene. A second is to treat C57BL/6J mice with the usual TAM treatment and then administer morphine. These experiments would definitively address whether the lack of decreased proliferation is due to an effect of transgene insertion or due to the protective effect of TAM. To determine if the nestin-CreER^{T2} mice have a shifted dose response to morphine, detailed behavioral assessments could be conducted after morphine exposure in parallel with the two above experiments as detailed previously (Fischer et al., 2008).

Summary

This dissertation has determined various aspects of the progenitor cell cycle and niche that are altered with morphine treatment, and has provided the framework for future studies using the rodent model of heroin self-administration. Future work manipulating various factors such as VEGF or progenitor cell maturation in combination with heroin self-administration will help to shed light on the relationship between altered neurogenesis and its possible role in addiction.

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