THE IMPACT OF OPIOIDS AND OPIATES ON ADULT HIPPOCAMPAL NEUROGENESIS

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DEDICATION

I dedicate this thesis to my family

for supporting me through all the ups and downs.

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I cannot possibly thank my mentor, Amelia Eisch, enough for giving me the opportunity to do a Ph.D. in her laboratory. From day one she's been extremely supportive and a constant source of energy and optimism. Under her guidance, I've renewed my love for science. I couldn't have a better role model.

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THE IMPACT OF OPIOIDS AND OPIATES ON ADULT HIPPOCAMPAL NEUROGENESIS

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Opiate addiction is a growing problem in today's society. Thus, it is of crucial importance that we understand the physiological basis for opiate addiction and the long-term consequences of opiate use in order to develop more effective means of treatment. Chronic morphine and heroin have previously been shown to decrease proliferation and survival of progenitor cells in the adult rat and mouse hippocampus. Here, I show that endogenous opioids may act through the mu opioid receptor (MOR) to similarly decrease survival of new hippocampal neurons. An exon 1 MOR knockout mouse showed increased survival of new neurons independent of effects on cell proliferation or cell death. In concordance with the

increased numbers of granule cells maturing into neurons, knockout mice also had larger hippocampal granule cells layers and increased numbers of granule cells.

Exploration of the impact of chronic morphine on different stages of neurogenesis showed that chronic morphine decreased numbers of Type 1 stem cells and proliferating progenitor cells. Progenitor cells exposed to chronic morphine during early maturation were not significantly decreased in number, but appeared to have retarded cell maturation since fewer had reached the immature neuron stage in chronic morphine mice. Chronic morphine also appeared to result in anterior hippocampus specific decreases in stem cells as well as maturation retardation. These findings show that morphine has distinct effects on different stages of neurogenesis, and that the anterior hippocampus may be more sensitive to some effects.

Cell proliferation levels in the brains of human heroin abusers and normal controls were assessed using the endogenous proliferation marker Ki67. Heroin abusers had decreased numbers, but larger clusters of proliferating cells in the dentate gyrus hilus as compared with controls. There was also a trend towards a decrease in number of proliferating cells in the granule cell layer of heroin abusers. Although these findings are preliminary, they suggest that chronic heroin use in humans, as in rodents, may negatively impact neurogenesis.

Together, these findings support a negative role for opioids and opiates in regulating adult hippocampal neurogenesis.

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LIST OF DEFINITIONS

5-HT – serotonin

AAALAC – Association for Assessment and Accreditation of Animal Care

AC3 – activated caspase-3

Ach – acetylcholine

AChE – acetylcholinesterase

ACTH – adrenocorticotropic hormone

AMPA – alpha-amino-3-hydroxy-5 methyl-4-isoxazolepropionic acid

ANOVA – analysis of variance

β-CNA – beta-chlornaltrexamine

BDNF – brain-derived neurotrophic factor

β-FNA – beta-funaltrexamine

BrdU – 5-bromo-2'-deoxyuridine

cAMP – 3'-5'-cyclic adenosine monophosphate

CNS – central nervous system

CREB – cAMP response element-binding

DNA – deoxyribonucleic acid

eGFP – enhanced green fluorescent protein

GABA – gamma-aminobutyric acid

GCL – granule cell layer

GFAP – glial fibrillary acidic protein

GFP – green fluorescent protein

h – hour

IHC – immunohistochemistry

IR – immunoreactive

KO – knockout

LC – locus coeruleus

LTP – long-term potentiation

MHPG – 3-methoxy-4-hydroxyphenylglycol

MOR – mu opioid receptor

NDS – normal donkey serum

NE – norepinephrine

NGF – nerve growth factor

NMDA – N-methyl-D-aspartic acid

NTX – naltrexone

PBS – phosphate-buffered saline

PCNA – proliferating cell nuclear antigen

PCR – polymerase chain reaction

PKA – cAMP-dependent protein kinase

POMC – proopiomelanocortin

PSA-NCAM – polysialic acid neural cell adhesion molecule

RT – room temperature

SEM – standard error of measurement

SGZ – subgranular zone

shRNA – short hairpin ribonucleic acid

SVZ – subventricular zone

TSA – tyramide signal amplification

TUNEL – terminal transferase mediated dUTP nick-end labeling

WT – wild-type

CHAPTER ONE
Introduction

History of Opiate Use and Abuse

Opiates have long been used for medicinal and recreational purposes. Opium, derived from the milky juice of the poppy, *Papaver somiferum*, has been produced since 4000 B.C. in Mesopotamia (Gold et al., 1982). It was initially used by Arabic physicians, due to its medicinal properties of analgesia, cough suppression, and the control of dysentery, and although not well documented, was probably used for similar purposes by the Greeks and Romans (van Ree et al., 1999). Beginning around 800 A.D., Arabic traders brought opium to the East, India, China, and Europe (van Ree et al., 1999), leading to the wide dispersal of a drug that has been of great importance to medicine, but has also had terrible social consequences.

Opium was lauded as one of the most effective drugs in the medicinal arsenal as early as the early 20th century. "If the entire material medica at our disposal were limited to the choice and use of only one drug, I am sure that a great many, if not the majority, of us would choose opium." (Macht, 1915) In the 17th century, laudanum, a mixture of opium, wine, saffron, and various spices became widely prescribed (London, 2005). Opiate use escalated during the 19th century, as it was included in a number of proprietary medicines easily obtainable from the local chemist (London, 2005), and was advertised to cure such maladies

as toothaches, ingrown toenails, bedwetting, and fretful babies. During this time period, opium demand increased greatly: in the United States, opium imports for 1840 were around 24,000 lbs; by 1867, 135,000 lbs, by the 1890's over 500,000 lbs. In line with increased opium use, opium addiction also became a large-scale problem. (Hodgson, 2001)

During the 1800's medical science had a great interest in finding and creating more potent opiates. Morphine, the principle alkaloid, or active ingredient in opium, was isolated in the first decade of the 1800's (Hamilton and Baskett, 2000). Still used today for short-term use and palliative care, morphine is effective in reducing pain, coughing, diarrhea, and vomiting. However, morphine, like opium, proved to be highly addicting and so medicinal chemists searched for non-addictive morphine substitutes. Out of this search came heroin (diacetylmorphine), discovered by Bayer company chemist Heinrich Dreser in 1898. Unfortunately, heroin was even more potent and addicting than morphine and thus its medicinal use for coughs and colds was discontinued in the early 1900's, although it was still used to control pain until relatively recently, especially in Britain and Australia (Hodgson, 2001).

Opiate distribution became highly regulated during the early 1900's and was no longer available in patent medicines. However, even in the absence of over-the-counter opiates, addiction remains a serious problem in today's society. According to the 2003 National Survey on Drug Use and Health, an estimated 3.7 million people in the United States had used heroin at some point in their lives, and over 119,000 of them reported using it within the

month preceding the survey. From 1995 through 2002, the annual number of new heroin users ranged from 121,000 to 164,000, with the majority of new users being males over the age of 18 (Abuse, 2005). In 2006, heroin use continued to escalate and was the primary drug of abuse for large portions of drug abuse treatment admission in Chicago, Denver, Los Angeles, Minneapolis/St. Paul, and St. Louis (Group, 2006). Abuse of other opiates, including morphine, also continued to rise or remain stable across the country. Opiate abuse has become of great concern recently in Dallas County, Texas, with a surge in the use of a heroin-cold medicine mixture termed "cheese". Containing 2-8% heroin, cheese is a cheap recreational drug that has gained popularity among adolescents and children as early as 9 years old. At least 21 young people have died in Dallas county from heroin overdose resulting from cheese use since 2005, and use continues to escalate (Ellis, 2007).

Opiate addiction is a pertinent and pressing problem in today's society. Thus, it is of crucial importance that we understand the physiological basis for opiate addiction and the long-term consequences of opiate use in order to develop more effective means of treatment.

Types of Opiates and Endogenous Opioids

There now exist a number of opiates, all possessing a similar chemical structure to morphine (Figure 1). Morphine and codeine are both alkaloids derived from the opium poppy and make up 10% and 0.5% of opium, respectively (Hardman et al., 2001). Other opiates are semisynthetic derivatives made by relatively simple modifications of morphine

(e.g heroin, oxycodone (Demerol), and buprenorphine), or another opium alkaloid, thebaine (e.g. etorphine).

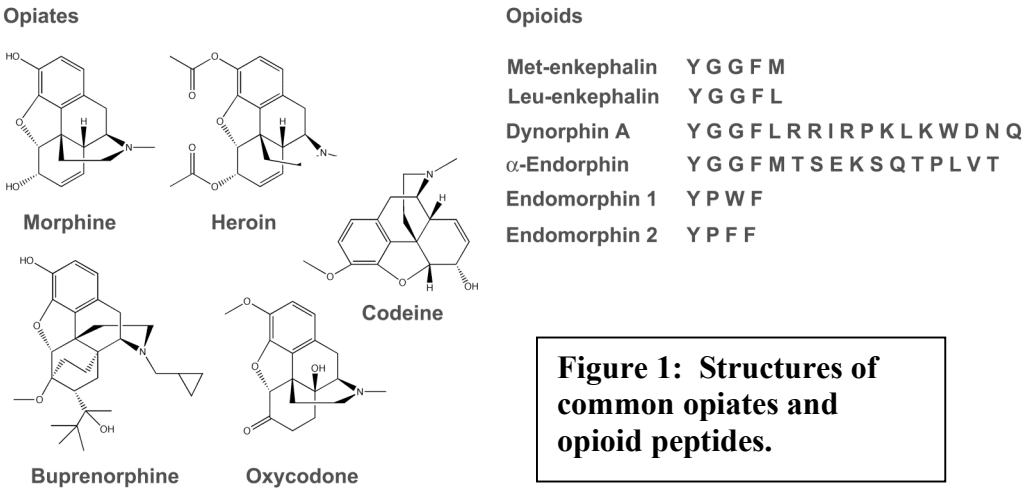

Figure 1: Structures of common opiates and opioid peptides.

While opiates are all drugs derived from opium, opioids refer to all compounds related to opium. These include endogenous opioids: peptides with opiate-like pharmacology that were identified in the brain during the mid-1970's (Hughes et al., 1975; Terenius and Wahlstrom, 1974). Indeed, the name for one endogenous opioid class, endorphins, is a conjunction of the phrase "endogenous morphine", emphasizing that the exogenous, but naturally occurring opiate morphine was discovered long before the endogenous compounds. The endogenous opioid peptides are products of three precursor proteins, each encoded by a separate gene: preproopiomelanocortin (POMC), preproenkephalin, and preprodynorphin. These proteins undergo proteolytic processing to give rise to the active peptides. POMC contains the opioid peptide β-endorphin, as well as several non-opioid peptides, including adrenocorticotropic hormone (ACTH), melanocyte-stimulating hormone, and β-lipotropin.

Preproenkephalin yields the active opioid peptides leu- and met-enkephalin, while preprodynorphin gives rise to dynorphins A and B and α- and β-neoendorphins. All of these opioid peptides contain the same four amino acid sequence Tyr-Gly-Gly-Phe at their N terminus, followed by either Met or Leu (Nestler et al., 2001).

Opioid Receptors

Opiates act by mimicking the endogenous opioid peptides, which are the naturally occurring ligands for opioid receptors. Although the existence of an opioid receptor had been hypothesized, it was not until 1973 that three independent groups presented evidence of stereospecific opiate receptor binding in the brain (Pert and Snyder, 1973; Simon et al., 1973; Terenius, 1974). There are three classes of opioid receptors: mu, delta, and kappa. These classes differ in their affinity for opiate and opioid ligands. The endogenous ligands for the mu, delta, and kappa opioid receptors are, respectively, endorphin, dynorphin A, and enkephalin. All of the opium-derived opiates and most of their derivatives are ligands for the mu opioid receptor (MOR). Thus, it is of particular importance the role of this receptor in regulating both physiological and molecular events in the brain is understood.

Opioid receptors are seven-transmembrane Gi or Go-coupled protein receptors that inhibit the cAMP pathway (Gold et al., 1982). The downstream mechanism of morphine action has been explored in neurons of the locus coeruleus (LC) and has been found to occur through a cascade of processes. Morphine acutely inhibits LC neurons via the opening of potassium inward rectifying channels, closing of sodium-dependent currents, and inhibition of adenyl

cyclase (Nestler and Aghajanian, 1997). Adenyl cyclase inhibition results in decreased activity of protein kinase A (PKA) and lowered levels of several phosphoproteins, including CREB. This decrease in phosphorylated CREB results in increased transcription, through a CRE site in its gene promoter, of CREB. This, in turn, results in increased transcription of adenyl cyclase (type VIII). Upregulation of adenyl cyclase (type 1) and PKA also occurs during chronic morphine administration through CREB-independent mechanisms. This upregulation of the cAMP pathway results in increased electrical excitability of the neurons by activating the sodium current. These adaptations to chronic opiates may be the mechanism for morphine tolerance, by serving to return neuron firing to normal rates in the continued presence of drug, and for opiate dependence and withdrawal, by resulting in higher than normal firing rates after removal of drug (Nestler and Aghajanian, 1997). Although these mechanisms have not yet been shown to occur in hippocampal neurons, it is likely that similar adaptions occur after chronic opiate exposure, resulting in drug tolerance and dependence.

Effect of Opiates and Opioids on the Hippocampus

Electrophysiology

Opiates and opiates also have a demonstrated role in regulating electrophysiological responses in the hippocampus. Activation of opioid receptors, and in particular the mu opioid receptor, is critical for the induction of long-term potentiation (LTP) at opioid containing synapses such as those in the lateral perforant pathway (Zieglgansberger et al., 1979). Early studies showed that activation of opioid receptors facilitated excitation of

pyramidal cells via disinhibition of inhibitory GABAergic interneurons. More recently, this has been shown to be true for dentate gyrus granule cells as well (Bramham and Sarvey, 1996). In addition, granule cells can be directly hyperpolarized by mu and delta agonists, distinguishing the dentate gyrus from CA1 and CA3, which are indirectly affected (Bramham and Sarvey, 1996). These findings point to opiates having a uniquely interesting role in dentate gyrus function, making it important to understand how opiates influence this important gateway to the hippocampus.

Anatomy

Electrophysiological changes in the hippocampus in response to opioids and opiates may be mediated in part by subtle alterations in hippocampal anatomy. Blockade of opioid receptors in early postnatal rats increases neuronal maturation in the CA1 and dentate gyrus regions of the hippocampus (Hauser et al., 1987; 1989). The length and concentration of basilar dendrites also significantly increases on CA1 pyramidal cells after chronic blockade (Hauser et al., 1987). In the dentate gyrus, the concentration of spines on granule cells and pyramidal neurons also increases significantly after chronic blockade both *in vitro* and *in vivo* (Hauser 1987, Liao 2005). Chronic morphine has the opposite effect, decreasing the complexity of dendrites on hippocampal granule cells and pyramidal neurons in rats exposed perinatally and decreasing spine density in self-administering adult rats (Robinson 2002). *In vivo* studies have shown that morphine causes collapse of preexisting spines via MOR-mediated activity (Liao 2005). These anatomical changes most likely reflect a reorganization

of synapses on to hippocampal neurons, which may in turn lead to long-term deficits in hippocampal function and behaviors.

Molecular Biology

Morphine and other MOR agonists also act to change the levels of many neurotransmitters in the hippocampus. Many of these changes are proposed to arise from morphine's effects on hippocampal glutamatergic and GABAergic neurotransmission. Some of the changes that have been observed after chronic, but not acute, morphine are decreased glutamine, increased glycine, and decreased GABA levels in the hippocampus (Gao et al., 2007). This may be due to changes in neurotransmitter release and uptake. (Capogna et al., 1993; Mao et al., 2002; Vaughan et al., 1997; Xu et al., 2003). Morphine also induces release of dopamine from the ventral tegmental area and other brain regions to the hippocampus (Gasbarri et al., 1994). Morphine and other MOR agonists have been reported to inhibit the release of acetylcholine (ACh), norepinephrine (NE), and serotonin (5-HT) in the hippocampus (Lapchak et al., 1989; Matsumoto et al., 1994; Yoshioka et al., 1993). These profound changes in hippocampal neurotransmitters alter hippocampal activity and impair its function, contributing to addiction and relapse in chronic opiate abusers (Nestler, 2001; O'Brien, 1997).

Behavior

Activation of MOR results in numerous physiological effects including: analgesia, depressed respiratory function, constipation, increased feeding, and sedation. Of interest

here, MOR agonists also have been shown to affect cognition. Both acute and chronic opiate administration results in impaired memory in rats and mice on a multitude of behavioral tasks (Hepner et al., 2002; McNay et al., 2006; Spain and Newsom, 1991). Acute administration of opiates causes deficits in spatial working memory (Hepner et al., 2002; McNay et al., 2006), while chronic opiate administration results in deficits in memory acquisition, but spares working memory (Spain and Newsom, 1991). Conversely, acute or chronic administration of MOR antagonists, naltrexone and naloxone, enhances memory retention in rats and mice (Canli et al., 1990; Castellano et al., 1989; Gallagher, 1982; Gallagher et al., 1983; Ilyutchenok and Dubrovina, 1995; Izquierdo, 1979; Spain and Newsom, 1989). This suggests that endogenous opioids, which are blocked by the antagonists, may act similarly to opiates in impairing memory.

Opiates and opioids may also negatively impact cognition in humans, where it has been found that opiate abusers and patients on methadone maintenance therapy perform more poorly than controls on tests of information processing, problem solving, verbal fluency, and other memory tasks (Darke et al., 2000; Hepner et al., 2002). These cognitive deficits are particularly troubling since they may contribute to difficulties in substance abuse counseling and patient adherence to therapies (Darke et al., 2000). Finding the mechanism by which these deficits occur is crucial for the development of new therapies for opiate addiction.

Effect of Opiates and Opioids on Brain Development

One possible mechanism of opiate's cognitive effects is that MOR is a regulator of cell survival in the central nervous system. Several reports have shown that prenatal exposure to the MOR antagonist naltrexone resulted in an increase in brain size (de Cabo and Paz Viveros, 1997; Zagon and McLaughlin, 1984; 1987). During early postnatal development, rats exposed to high doses of naltrexone had increased numbers of neurons in the hippocampus granule cell layer, a region of the brain notable for high levels of neurogenesis during this time period. Those regions not undergoing neurogenesis were not altered in neuron number (Zagon and McLaughlin, 1986c). These early studies supply a tempting correlation between endogenous opioids and neurogenesis. However, we have found that adult rats treated with naltrexone for five days show no changes in numbers of proliferating cells (Eisch, unpublished), suggesting that either a 5 day administration of naltrexone is not sufficient to induce changes in neurogenesis, or that Zagon's findings at earlier timepoints do not necessarily hold true in adult animals. In line with these findings, another study, using the mu specific antagonist, β-funaltrexamine, showed no increases in hippocampal granule cell numbers, suggesting that naltrexone's influence on neurogenesis is not through the mu opioid receptor (Zagon and McLaughlin, 1986a). In total, these studies suggest that endogenous opioids have a suppressive effect on prenatal and early postnatal neurogenesis, but that this effect is most likely not MOR mediated.

Effect of Opioids and Opiates on Cell Proliferation and Gliogenesis

In the adult, opiates tend to have a negative impact on proliferating cells, regardless of cell type. The greatest body of literature details the anti-proliferative effect of morphine on

the highly proliferative cells of the immune system - lymphocytes and thymocytes (Glasel, 2000). However, there is increasing evidence that opiates also impact more rare populations of proliferating cells in the brain. Morphine has been shown to decrease gliogenesis in the adult brain. Rats repeatedly injected with morphine have increased immunodensity of an astrocytic marker, GFAP, and decreased numbers of GFAP-IR cells in the spinal cord, posterior cingulated cortex, and hippocampus (Song and Zhao, 2001). This finding complements *in vitro* studies showing that morphine suppresses gliogenesis in glial cultures, including those derived from mouse hippocampus, by inhibiting cell proliferation and promoting cell differentiation and astrocyte hypertrophy (Stiene-Martin et al., 1991; Stiene-Martin and Hauser, 1993). This impact of morphine on astrocyte proliferation may also directly alter neurogenesis, as will be discussed in greater detail later. Neural progenitor cells may arise from radial glial stem cells (Figure 2) that share many characteristics with astrocytes (Seri et al., 2001). Although there is no direct evidence that morphine affects radial glial cells, mice treated with chronic morphine have decreased numbers of BrdU-labeled GFAP-IR cells within the subventricular zone (SVZ), a region rich in this cell type (Stiene-Martin et al., 2001). This indicates that morphine may both alter the hippocampal microenvironment through decreased numbers of astrocytes and impact neurogenesis via regulation of hippocampal stem cells.

The Impact of Opiates on Adult Hippocampal Neurogenesis

In the 1960's the birth of new neurons in the adult rat brain was discovered, however, it was not until the 1990's that the longstanding dogma that the adult brain was a static organ

was overturned and neurogenesis in the adult human brain was first demonstrated(Altman and Das, 1965; Eriksson et al., 1998; Gross, 2000). It is now accepted that adult neurogenesis occurs in two regions of the adult brain – the subventricular zone (SVZ) and the subgranular zone (SGZ) of the hippocampus dentate gyrus. New neurons in the hippocampus can functionally integrate into the hippocampal circuitry, and thereby impact hippocampal functions (Abrous et al., 2005).

Markers and Physiology of Neuron Maturation

In contrast to early studies that considered neurogenesis to consist of two time-points – proliferation (new neuron birth) and survival (mature neuron) – neurogenesis is now thought of as a continual process between these two time points (Kempermann et al., 2004a). New cells arise from slow-dividing radial glia stem cells (Type 1) through asymmetric division. The new cells, termed progenitor cells, can divide quickly in a series of symmetric divisions, giving rise to a large progenitor cell pool. Progenitor cells have the potential, in the adult hippocampus, to mature into neurons or glia, with the majority of cells taking on a neuronal phenotype. Utilizing a nestin-eGFP reporter mouse, we can detect these early steps via GFP (Yamaguchi et al., 2000). Radial glia cells and early progenitor cells (Type 2) both express nestin, and thus can be detected with GFP. These two cell types can be differentiated by their morphology. Radial glia cells possess a characteristic shape with a process extending through the granule cell layer of the dentate gyrus into the molecular layer. Type 2 cells are ovoid in shape with short non-oriented dendrites. If a Type 2 progenitor cell adopts a neuronal phenotype, it begins expressing a early neuronal marker – doublecortin. Using this

marker, Type 2 cells are split into two subtypes: Type 2a - early progenitor cells that only express GFP and Type 2b – later progenitor cells that also express doublecortin. As Type 2b progenitor cells mature, they no longer express GFP and they change morphology to more closely resemble that of neurons with a primary apical dendrite extending into the granule

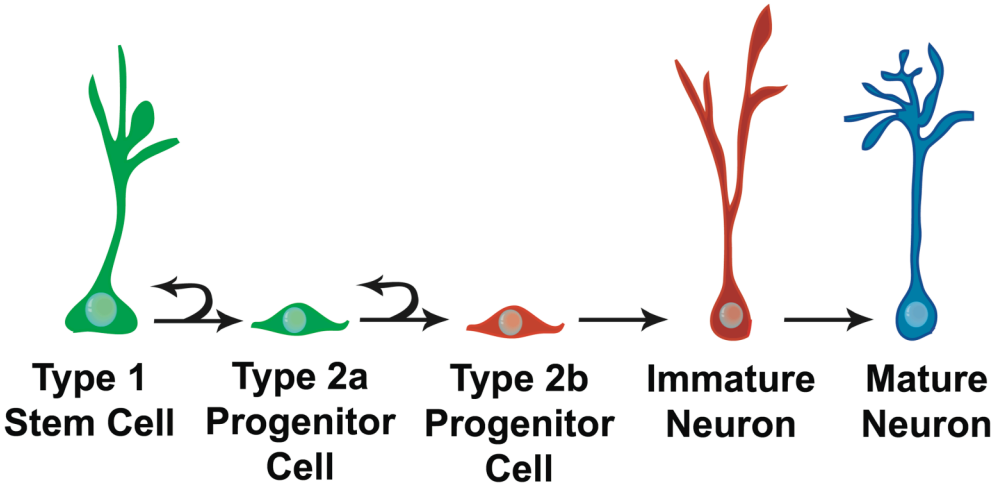

Figure 2: Stages of Neurogenesis

cell layer. These cells are termed immature neurons, and they are cells of approximately 1-3 weeks of age. Immature neurons eventually stop expressing doublecortin as they become new neurons. At 4 weeks of age, the new cells are morphologically virtually indistinguishable from mature granule cells (Kempermann et al., 2004a).

The different stages of neurogenesis also have distinct electrophysiological profiles reflecting their maturity (Overstreet-Wadiche and Westbrook, 2006). Type 1 stem cells have astrocytic properties, including low input resistance, A-type potassium currents, and a resting potential near the potassium equilibrium potential (Filippov et al., 2003; Fukuda et al., 2003). Type 2 progenitor cells have a high input resistance and rapidly inactivating voltage gated potassium currents. A small proportion of Type 2 cells, which are most likely the more mature Type 2b cells, also have small sodium and GABAergic synaptic currents and fire action potentials (Fukuda et al., 2003; Tozuka et al., 2005; Wang et al., 2005). Immature

neurons have a high input resistance and depolarized resting membrane potential. They fire small and broad action potentials mediated by sodium channels. They are notable for their lower threshold induction for long-term potentiation and long-term depression (Schmidt-Hieber et al., 2004). These distinct properties of the different stages of maturation make it likely that each stage will respond differently to hippocampal activity and microenvironment changes (Tashiro et al., 2007). It remains to be explored what the impact of opiates is on each stage of maturation.

Behavior and Neurogenesis

One of the most controversial topics in the neurogenesis field is the behavioral function of neurogenesis (Leuner et al., 2006). There are several studies supporting a link between learning and memory and neurogenesis. First, there is a positive correlation between increased neurogenesis produced by environmental enrichment and/or exercise and performance on hippocampal-dependent memory tests (Kempermann et al., 1997; van Praag et al., 1999). Conversely, ablating new neurons through the use of antimitotic drug treatment or localized x-ray irradiation correlates with impaired performance on hippocampal dependent-memory tasks (Shors et al., 2001; Snyder et al., 2005). Learning also increases the number of new neurons in a trace eyeblink conditioning paradigm (Gould et al., 1999a; Leuner et al., 2004). However, for each of these examples there are multiple studies using other manipulations or behavioral tasks that do not show a correlation between neurogenesis and memory (Shors et al., 2002; van Praag et al., 1999; Wood et al., 2001). Despite the inconclusive evidence for a linkage, it seems likely that neurogenesis is more than a vestigial

phenomenon since it has been observed in nearly every species, including humans (Gould, 2007). One possibility is that the behavioral tasks currently used to assess learning and memory are not appropriate to test the functional contribution of newly generated neurons. One new theory of neurogenesis, which has not yet been tested in animals, suggests that new neurons may actually contribute to temporal encoding of memories (Aimone et al., 2006). Although the function of new neurons remains to be proven, there is some evidence for a contribution of neurogenesis to hippocampal-dependent behaviors. Thus, alterations of neurogenesis in heroin abusers could negatively impact hippocampal function and lead to impaired cognition and mental inflexibility.

Opiates and Neurogenesis

In adult rats, chronic, but not acute, administration of morphine reduces the number of proliferating cells within the hippocampus by 28-54 percent (Eisch et al., 2000; Kahn et al., 2005). The decrease in proliferating cells after chronic morphine was not due to cell death, as there was no change in the number of pyknotic cells. These data support the hypothesis that chronic morphine affects the cell cycle, and thus the generation of new progenitor cells.

The mechanism by which morphine decreases cell proliferation has not yet been ascertained. *In vivo*, it has been suggested that chronic morphine results in premature mitosis, which might activate cell cycle checkpoint proteins, resulting in decreased levels of proliferating cells (Mandyam et al., 2004). While *in vitro* studies would be a simple means of dissecting apart the mechanism of morphine's affect on neurogenesis, there seems to be a

disconnect between *in vivo* and *in vitro* studies on neurogenesis. *In vitro*, neurogenesis is increased in isolated adult hippocampal progenitor cells by both mu and delta antagonists (Persson et al., 2003). While proliferation rates were decreased, there was an increase in the proportion of progenitor cells maturing into a neuronal fate, resulting in a net increase in neurogenesis. The disparity of these findings in comparison with *in vivo* antagonist effects underscores the importance of the hippocampal microenvironment in mediating opiate-induced changes in neurogenesis. Indeed, preliminary results show that chronic morphine does cause changes in growth factors and neurotransmitters in the adult hippocampus (Arguello et al., 2005; Mandyam and Eisch, 2004).

Additional experiments indicate that opiates may also have a negative impact on the maturation and survival of new neurons in the hippocampus. One week after chronic morphine administration, the number of immature PSA-NCAM-immunoreactive neurons in the hippocampus decreased by 68% (Kahn et al., 2005). Four weeks after chronic morphine, the number of new neurons reaching maturation was decreased by 42% (Eisch et al., 2000). These studies suggest that progenitor cells generated in an environment of chronic morphine continue to be negatively affected by the drug as they mature, with fewer cells reaching full maturation.

It is intriguing to consider if some of the cognitive deficits in the adult addicts is due to loss of new neurons, or if recovery is so difficult because new neurons are needed to learn extinction from a drug. It will be of great interest to determine if neurogenesis is altered in

the hippocampus of human opiate addicts. Future studies are needed to address this and the mechanisms by which morphine impacts neurogenesis.

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CHAPTER TWO
Impact of Chronic Morphine on Proliferation and Maturation of Adult Hippocampal Progenitor Cells

Introduction

The hippocampus is notable as being one of two anatomical regions in the brain that has the potential to generate new neurons during adulthood. These new neurons have been shown to functionally integrate into the hippocampus and may contribute to hippocampal function (Gould et al., 1999b; van Praag et al., 2002). Chronic opiate use has been shown to cause cognitive deficits on hippocampal-dependent tasks both in humans and rodents, a phenomenon that might be accounted for by correlative evidence of chronic opiate use decreasing adult hippocampal neurogenesis (Eisch et al., 2000; Kahn et al., 2005; Mandyam et al., 2004). Studies to date have focused primarily on chronic opiate's impact on proliferating cells in the hippocampus.

It is now appreciated that newly generated cells go through several distinct stages of maturation during neurogenesis (Kempermann et al., 2004a). Type 1 stem cells within the subgranular zone (SGZ) of the hippocampus give rise to Type 2 progenitor cells. These cells can take on a neuronal fate and mature into immature neurons, which then further mature into fully mature neurons (Kempermann et al., 2004a). A single publication showed that repeated morphine injection decreased numbers of putative immature neurons, suggesting that

morphine may negatively impact maturing cells as well (Kahn et al., 2005). However, a thorough analysis of how chronic morphine affects each stage of adult neurogenesis is lacking. Here we examine the impact of chronic morphine on early maturation of progenitor cells in the adult mouse hippocampus.

Experimental Procedures

Animals. The experiments were performed on adult male mice expressing green fluorescent protein (GFP) under the control of the nestin promoter on a full C57/BL6 background (Yamaguchi et al., 2000). The mice were housed in a facility approved by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) at the UT Southwestern Medical Center with a 12-h light/dark cycle and *ad libitum* access to food and water. All procedures were performed in accordance with guidelines of both the UT Southwestern Institutional Animal Care and Use Committee and AAALAC. Experiments were designed to minimize the number of animals used and their suffering.

Bromodeoxyuridine (BrdU) injections and tissue preparation. In order to assess cell differentiation in the dentate gyrus, mice were given one i.p. injection of BrdU (150 mg/kg; Boehringer Mannheim, Germany) dissolved in 0.9% saline and 0.007 N NaOH at 10 mg/ml as previously described (Mandyam and Eisch, 2004). A total of 15 male nestin-eGFPmice (sham=8, morphine=7) were used for this study (Yamaguchi et al., 2000). Five days after BrdU injection, all mice were anesthetized with chloral hydrate and perfused transcardially with cold 0.1 M phosphate-buffered saline (PBS) followed by 4% paraformaldehyde in 0.1

M PBS (pH 7.4) for 20 min at a rate of 7 ml/min. After perfusion, the brains were removed and postfixed overnight at room temperature (RT) with 4% paraformaldehyde in 0.1 M PBS. Brains were cryoprotected in 30% sucrose in 0.1 M PBS with 0.1% NaN_3 at 4^{o}C until sectioning. Thirty micrometer coronal sections were taken on a freezing microtome (Leica, Wetzlar, Germany). Nine serial sets of sections were collected through the entire hippocampus. Sections were stored in 0.1% NaN_3 in 0.1 M PBS at 4^{o}C until processed for immunohistochemistry (IHC).

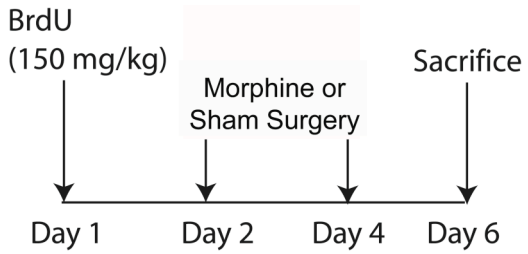

Figure 1: Experimental paradigm: Nestin-GFP transgenic mice were injected with 150 mg/kg BrdU on the first day of the experiment. Twenty-four (1 day) and seventy-two hours (3 days) later, the mice were either implanted with a 25 mg morphine pellet or underwent sham surgery. Forty-eight hours after the last surgery, all animals were sacrificed.

Morphine treatment. Mice were anesthetized via isoflurane (Henry Schein, Inc., Melville, NY, USA) and received a subcutaneous 25-mg morphine pellet (n=6) or sham surgery 24 hours and 72 hours after injection with BrdU (Mandyam et al., 2004; Shaw-Lutchman et al., 2002). All mice were perfused 48 hours after the last pellet implantation (Figure 1).

Immunohistochemistry. Every ninth section of the hippocampus was mounted on glass slides (Fisher Superfrost/Plus, Hampton, NH, USA) and left to dry overnight prior to IHC. Slides were coded so the experimenter was blind to the genotype of the animal until completion of analysis. Sections used for BrdU IHC were pretreated as follows: antigen unmasking (0.01 M citric acid, pH 6.0, 95^{o}C, 10 min), peroxidase quenching (0.3% hydrogen peroxide in 1xPBS, 30 min), membrane permeabilization (0.1% trypsin in 0.1 M Tris and 0.1% $CaCl_2$, 10 min), and acidification (2 M HCl in 1xPBS, 30 min). Primary antibody concentrations were as follows: rat anti-BrdU (Accurate, Westbury, NY, USA; 1:500), rabbit anti-Ki67 (Vector Laboratories, Burlingame, CA, USA; 1:2000), rabbit anti-GFP (Invitrogen, 1:500), goat anti-doublecortin (Santa Cruz Antibodies; 1:5000). Single-labeling IHC for BrdU was completed using the avidin-biotin/diaminobenzidine visualization method (Vector Laboratories, Pierce, Rockford, IL, USA) followed by counterstaining with Fast Red (Vector). Single-labeling for Ki67 was visualized using tyramide signal amplification (Cy3-TSA). Triple labeling for BrdU, GFP, and doublecortin was performed by pretreating sections with 0.01 citric acid and hydrogen peroxide as previously described then performing sequential IHC for doublecortin and GFP, which were visualized by CY5-TSA and CY3-conjugated secondary antibody respectively. The sections were then pretreated with trypsin and HCl and BrdU IHC carried out as previously described, but with visualization by Cy2-conjugated secondary antibody.

Microscopic Analysis and Quantification. BrdU, Ki67, and GFP-IR Type 1 stem cells were assessed within the subgranular zone (SGZ) in adjacent sets of serial sections. Using the optical fractionator method (Eisch et al., 2000), IR cells were quantified in the SGZ along the

longitudinal extent of the hippocampus [bregma -0.94 to -4.16;(Franklin and Paxinos, 1997)]. The SGZ was defined as a region straddling the border of the granule cell layer and the hilus: three granule cell widths into the hilus, and two-thirds of the GCL adjacent to the hilus. Sections were coded so the experimenter was blind to the treatment of the animal until completion of analysis. All cell counts were performed at 400x magnification with an Olympus BX-51 microscope while continually adjusting the focal plane through the Z plane. Since counting of cells was conducted on every ninth section of the hippocampus, the number of counted cells in the region was multiplied by nine to obtain an estimate of the total number of cells per region. To control for possible differences in bioavailability of BrdU between sham and morphine groups, BrdU-IR cells within the habenula were also quantified.

Phenotypic analysis. In order to determine the maturation stage of adult-generated cells into Types 2a, 2b, and immature neurons, triple-labeled sections were examined for colocalization of BrdU with GFP or doublecortin. An average of 91 BrdU-IR cells in either the anterior (89.23±8.22 cells, Bregma -1.48 to -1.78) or posterior (93.92±7.5 cells; Bregma -3.08 to -3.35) SGZ were optically sectioned in the Z plane using a confocal microscope (Zeiss Axiovert 200 and LSM510-META, Carl Zeiss, Oberkochen, Germany) with three laser lines (emission wavelengths 488, 543, and 633), multitrack scanning, and a section thickness of 0.45 mm. Orthogonal and rotational analysis in a 3D reconstruction program (Volocity, Improvision Inc., Lexington, VA, USA) confirmed colocalization.

Statistical analyses and presentation. Data are represented as mean±standard error of the mean. Statistical analyses were performed using a multiple variable analysis of variance (ANOVA) followed by a Bonferroni post hoc test. For repeated measure variables (bregma), a repeated measures ANOVA was used to assess the effect of drug treatment on the number of Ki67, BrdU, or Type 1 stem cells at specific bregma points, followed by a Bonferroni post hoc test. All statistical analysis was performed on a Macintosh computer using SPSS version 11.0.2 (SPSS Inc., Chicago, IL, USA). Statistical significance was defined as $P<0.05$. Images were imported into Photoshop version 7.0.1 (Adobe Systems Inc., San Jose, CA, USA) and the only adjustments made were via gamma in the Levels function.

Results

Ki67 and BrdU Quantification

Chronic morphine exposure has previously been shown to decrease the number of proliferating cells in the SGZ of both mice and rats (Eisch et al., 2000; Kahn et al., 2005; Mandyam et al., 2004). To determine if chronic morphine also decreased proliferation in nestin-GFP transgenic mice, we examined the expression of an endogenous marker of proliferation, Ki67 48 hours after the last sham surgery or morphine pellet implantation. In support of previous findings, we found the number of Ki67-IR cells in chronic morphine mice to be significantly decreased to 46% of control levels (Figure 2a; $F_{(2.12)}=9.455$, $P\leq0.01$). The distribution of Ki67-IR cells differed significantly across the longitudinal axis of the SGZ, with two peaks at bregma -1.48 and -3.62 (Figure 2b, $F_{(2,26)}=27.352$, $P\leq0.001$). Chronic morphine treatment also had a significant effect on the distribution of Ki67-IR cells

over the longitudinal axis of the hippocampus, with a significant interaction between bregma and treatment (Figure 2b, $F_{(2,26)}$=3.165, P≤0.001). Post hoc analysis showed that proliferation was uniformly decreased across the longitudinal axis of the SGZ (Figure 2b).

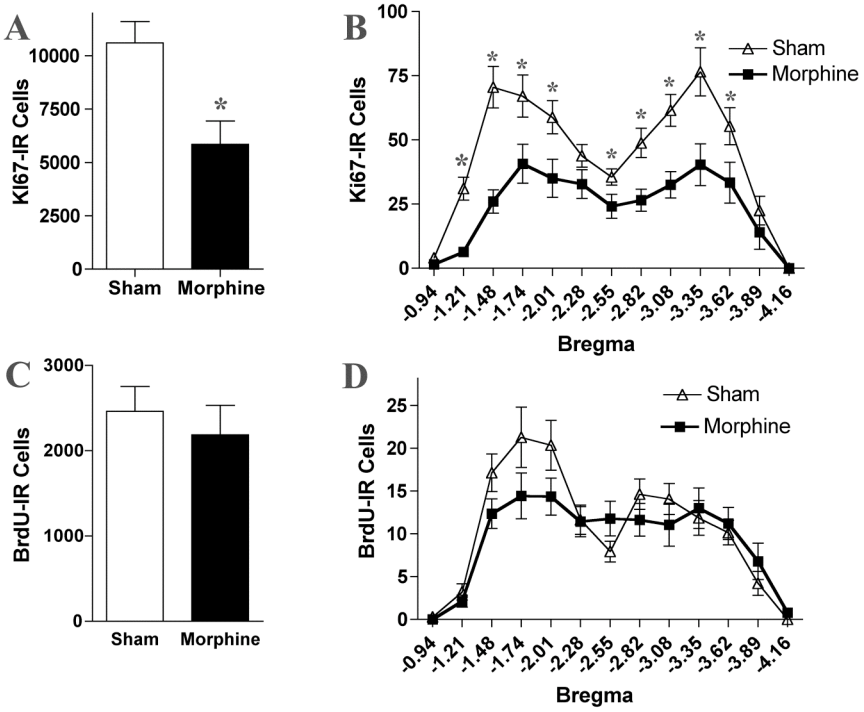

Figure 2: Progenitor cells in S phase 24 hours before morphine exposure are resistant to the drug's negative impact on proliferating cells. A. Cells proliferating during chronic morphine exposure, as identified by the endogenous proliferation marker Ki67, were greatly reduced in the SGZ (P<0.01). B. Distribution of Ki67-labelled cells along the anterior-posterior axis of the hippocampus. The number of Ki67-labeled cells was almost uniformly decreased across the anterior-posterior axis (P<0.05). C. Cells in the SGZ labeled with BrdU 24 hours before morphine exposure were assessed following chronic morphine. The number of BrdU-IR cells was not significantly changed by chronic morphine exposure. D. Distribution of BrdU-IR cells along the anterior-posterior axis of the hippocampus. There was not a significant interaction between bregma and treatment.

We next examined the impact of chronic morphine on cells labeled with BrdU 24 hours before drug exposure. In contrast to the large decrease in number of Ki67 cells, a population born during morphine exposure, there was no change in the number of BrdU-IR cells (Figure 2c, $F_{(2,13)}$=0.372, P≥0.05). The distribution of BrdU-IR cells differed significantly across the longitudinal axis of the SGZ, with a single large peak at bregma -1.74 (Figure 2d, $F_{(2,26)}$=25.827, P≤0.0001). There was also a significant interaction between bregma and drug treatment (Figure 2d, $F_{(2,26)}$=1.975, P≤0.05). Post hoc analysis showed that morphine treatment resulted in a slight trend to a decrease in BrdU-IR cells in the anterior SGZ, particularly at bregma -1.48 (P=0.10).

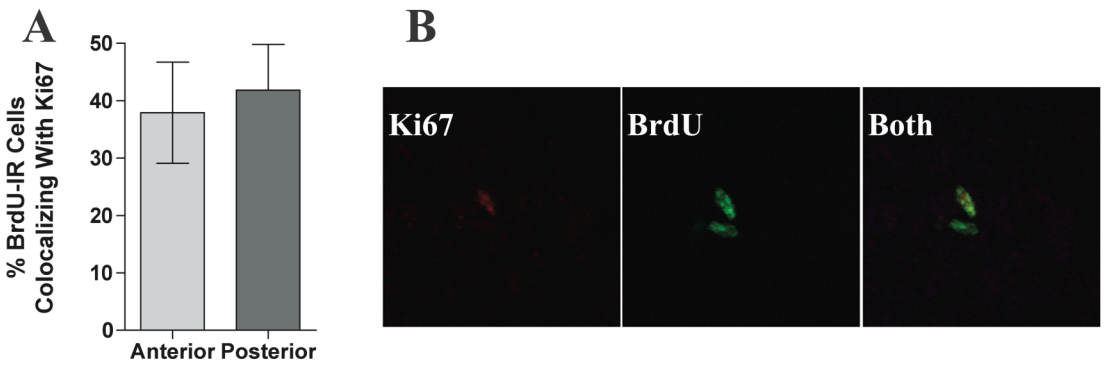

Figure 3: The proportion of BrdU-IR cells that continue to cycle 24 hours after labeling is equal in the anterior and posterior regions of the hippocampus. A. Proportions of BrdU-IR cells in either the anterior SGZ (Bregma -1.48 to -1.78) or posterior SGZ (Bregma -3.08 to -3.35) that colocalized with Ki67 24 hours after BrdU labeling. B. Example of BrdU-IR (green) cell clusters that colocalize with Ki67 (red; top cluster) or no longer express Ki67 (bottom cluster).

At 24 hours post-BrdU labeling, approximately 30% of labeled cells are still proliferating, as evidenced by colabeling with Ki67 (Mandyam et al., 2007). Since morphine has a strong

negative impact on proliferating Ki67 cells, the effect of morphine on cells labeled 24 hours prior to drug exposure could be due to the impact of morphine on those BrdU cells which continue to proliferate. Given morphine's trend to preferentially impact on BrdU-IR cells in the anterior SGZ, it was therefore hypothesized that a greater proportion of BrdU-IR cells in the anterior SGZ have re-entered the cell cycle at 24 hours post-labeling. To test this hypothesis, we examined the proportion of BrdU/Ki67 double-labeled cells in the anterior SGZ and posterior SGZ 24 hours after BrdU-labeling. The percent of BrdU-IR cells that co-labeled with Ki67 was equivalent in the anterior and posterior SGZ (Figure 3, $F_{(2,5)}=0.109$, $P>0.05$). This suggests that morphine's trend to preferentially impact BrdU-IR cells in the anterior SGZ is not due to the proliferative status of those cells at the time of morphine exposure.

Maturation Stage Analysis

As SGZ progenitor cells mature into granule cell neurons, they undergo a process of maturation that has been characterized by a number of markers (Kempermann et al., 2004a). In nestin-GFP mice, stem-like cells, characterized by a radial glia like morphology, (Type 1; Figure 4a) and ovoid-shaped early progenitor cells (Type 2a; Figure 4b) express GFP. As progenitor cells take on a neuronal fate, they begin to express an early neuronal marker, doublecortin (Type 2b; Figure 4c), and will also extend a process into the GCL to take on the distinctive morphology of an immature neuron (Figure 4d). After five days of morphine or sham treatment, the cells in S phase 6 days prior were assessed for colocalization of BrdU, GFP, and doublecortin to determine which stage of maturation the cells had reached. Equal

proportions of BrdU-IR cells located in the anterior SGZ (bregma -1.48 to -1.78) colocalized with only a single marker, GFP, and exhibited either a radial glial morphology (Type 1; $F_{(2,12)}$=0.272, P>0.05) or ovoid morphology (Type 2a; $F_{(2,12)}$=0.121, P>0.05) in both the sham and morphine treated mice. There was a strong trend to an increase of 29% in the proportion

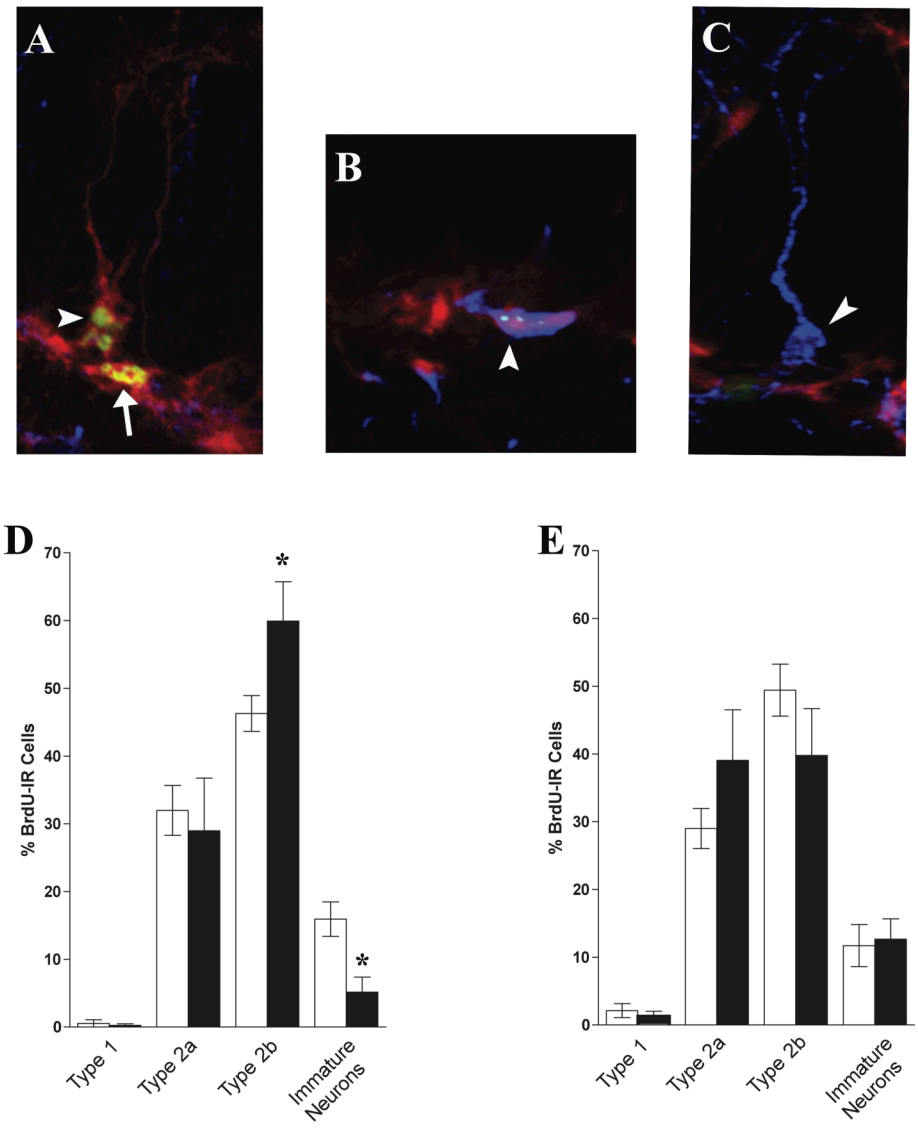

Figure 4: Chronic morphine retards the maturation of cells in the anterior hippocampus labeled 24 hours before morphine exposure. (A-C) Nestin-GFP and doublecortin, along with cell morphology, were used to determine the maturation stage of BrdU-IR cells. A. Cells expressing nestin-GFP alone were classified as either a Type 1 stem cell if they had a clear process extending into the GCL (arrowhead) or a Type 2a progenitor cell if they did not possess a process (arrow). B. Cells expressing both nestin-GFP and doublecortin were classified as Type 2b progenitor cells (arrowhead). C. Cells expressing doublecortin alone that extended processes into the GCL were classified as immature neurons (arrowhead). D. In the anterior hippocampus, chronic morphine mice had an increase in the proportion BrdU-IR cells with a Type 2b phenotype ($P<0.05$) and a decrease in the proportion of cells with an immature neuron phenotype ($P<0.05$). E. In the posterior hippocampus, chronic morphine mice did not show a significant difference in the proportions of BrdU-IR cells at any stage of maturation.

of BrdU-IR cells in the morphine treated mice that colocalized for both GFP and doublecortin (Type 2b; $F_{(2,12)}=4.558$, $P=0.059$), with the majority of these cells possessing an ovoid morphology. In contrast, there was a significant decrease of 32% of sham levels in the proportion of BrdU-IR cells colocalizing with doublecortin alone in the morphine treated mice ($F_{(2,12)}=10.123$, $P\leq0.01$). In the posterior hippocampus (bregma -3.08 to -3.35), morphine did not affect the proportion of BrdU-IR cells colocalizing with GFP (Type 1; $F_{(2,12)}=0.431$, $P>0.05$; Type 2a; $F_{(2,12)}=1.570$, $P>0.05$), doublecortin ($F_{(2,12)}=0.048$), $P>0.05$), or both (Type 2b; $F_{(2,12)}=1.490$, $P>0.05$). These findings suggest that maturation of the BrdU-IR cells is retarded by morphine specifically in the anterior hippocampus.

Stem Cell Analysis

It is clear that morphine has a negative impact on highly proliferating cell populations, however there have been no studies to date addressing the impact of morphine on slowly dividing stem cells *in vivo*. The number of BrdU-IR cells possessing a stem cell phenotype (GFP expression and a radial glial-like morphology) was extremely low (mean=1), emphasizing the slowly dividing nature of this cell population. We next assessed the overall number of Type 1 stem cells and found that chronic morphine treatment decreased the number of stem cells (Figure 5A, $F_{(2,26)}=10.432$, $P\leq0.01$). The distribution of BrdU-IR cells differed significantly across the longitudinal axis of the SGZ, with two peaks at the anterior and posterior regions, respectively (Figure 5B, $F_{(2,12)}=55.448$, $P\leq0.0001$). There was also a strong trend for an interaction between bregma and drug treatment (Figure 5B, $F_{(2,12)}=1.632$, $P=0.08$). In the anterior dentate gyrus (bregma -0.94 to -2.55), there was a significant interaction between bregma and drug treatment (Figure 5B, $F_{(2,7)}=2.319$, $P\leq0.05$). These findings suggest that chronic morphine treatment has a negative impact on numbers of Type 1 stem cells in the dentate gyrus, and that this effect is primarily seen in the anterior region of the dentate gyrus.

Discussion

These data suggest that morphine differentially affects hippocampal progenitor cells depending on the stage of cell maturation at the time of drug exposure. Cells that are proliferating during chronic morphine exposure are decreased in number, while those in S

phase 24 hours before, and thus are less likely to still be cycling at the time of exposure, do not significantly change in number. However, the cells that were born before chronic morphine treatment appear to have retarded cell maturation specifically in the anterior hippocampus. Morphine not only affects the proliferation and maturation of

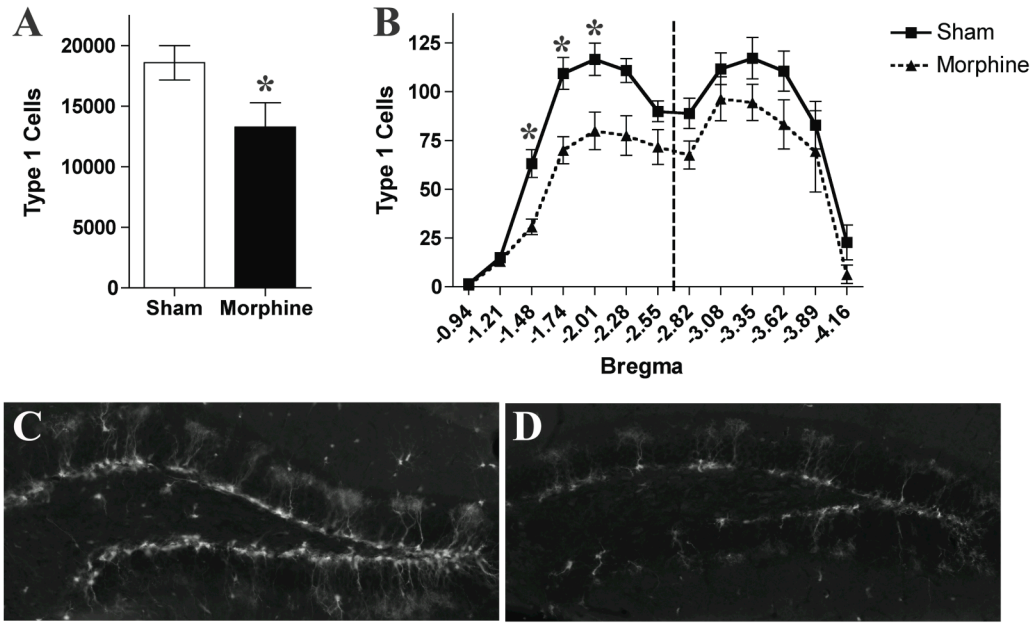

Figure 5: Hippocampal stem cells are decreased by chronic morphine exposure. A. The number of nestin-GFP Type 1 stem cells was significantly decreased in mice exposed to chronic morphine ($P<<0.05$). B. Distribution of Type 1 stem cells along the anterior-posterior axis of the hippocampus. Along the entire axis there was not a significant interaction between bregma and treatment. However, when the anterior and posterior regions were analyzed separately (as indicated by the dashed line), there was a significant interaction between bregma and treatment in the anterior region of the hippocampus. Type 1 stem cells were significantly decreased at bregmas -1.48 through -2.01 ($P<0.05$). C-D. Examples of nestin-GFP expression in the hippocampus of a sham (C) and chronic morphine exposed (D) mouse.

progenitor cells, but also decreases the number of Type 1 stem cells, which give rise to the progenitor cell pool. While previous studies have focused primarily on chronic morphine's

effects on proliferating cells in the hippocampus, our findings indicate that chronic morphine also negatively impacts slowly dividing or quiescent stem cells and maturing progenitor cells.

It is well established that chronic morphine decreases the number of proliferating cells in the hippocampus of both rats and mice (Eisch et al., 2000; Kahn et al., 2005; Mandyam et al., 2004). Here we extend the negative impact of morphine on proliferating cells to a nestin-eGFP transgenic mouse line. Furthermore, we show that proliferation is uniformly decreased throughout the entire hippocampus, suggesting that this effect is a global one. In contrast, we wanted to determine if cells that were born before morphine exposure would also be negatively impacted by the drug. Surprisingly, we did not find a decrease in the number of BrdU-IR cells subjected to chronic morphine 24 hours after BrdU-labeling. However, there was a non-significant decrease in the number of BrdU-IR cells in the anterior region of the hippocampus. One possible reason for the different effects of morphine on cells born before and after exposure is that morphine may decrease cell number by affecting cells undergoing mitosis, and thus would only impact actively proliferating cells (Mandyam and Eisch, 2004). However, twenty-four hours after BrdU labeling, approximately 60-70% of labeled cells have exited the cell cycle and would thus no longer be subject to morphine's effect on proliferating cells (Mandyam et al., 2007). The remainder of BrdU-IR cells continue to cycle and would thus, theoretically, be vulnerable to morphine exposure. Since we observe a slight, but non-significant decrease in the number of BrdU-IR cells in the anterior hippocampus, we hypothesized that more BrdU-IR cells in that region might re-enter the cell

cycle. However, when we analyzed the proportion of BrdU-IR cells colocalizing with Ki67 24 hours after BrdU labeling in untreated mice, the proportion in both the anterior and posterior regions of the hippocampus was 40%. This suggests that the non-significant decrease in the anterior hippocampus is not due to more cells re-entering the cell cycle in that region. It is interesting to note that despite the relatively large proportion of BrdU-IR cells still cycling at the initial time of morphine exposure, there was not a decrease in number of BrdU-IR cells. This finding is in line with previous studies showing that acute morphine exposure does not impact the number of proliferating cells in the hippocampus (Eisch et al., 2000). This suggests that chronic morphine exerts its effects on proliferating cells indirectly via changes in the hippocampal microenvironment (Arguello et al., 2005; Mandyam and Eisch, 2004). The reason we do not see a change in number of BrdU-IR cells may be because the hippocampal microenvironment remains permissive for proliferation for the time period during which this cell population remains cycling. Future experiments will address this by using later BrdU time points (e.g. concurrent or 24 hours after administration of the first morphine pellet) to determine exactly how long morphine exposure must be to induce a non-permissive hippocampal microenviroment for proliferating cells.

Although chronic morphine is known to decrease both proliferation and neurogenesis in rodents (Eisch et al., 2000), little is still known about its effects on the period of maturation that lies between these two points. One study found that rats treated with repeated morphine injections had decreased numbers of polysialated neural cell adhesion molecule (PSA-NCAM) expression cells in the SGZ (Kahn et al., 2005). This may be due to a decrease in

number of immature neurons, which have been shown to express PSA-NCAM (Seki, 2002). However, PSA-NCAM is also a marker of hippocampal structural plasticity (Bonfanti et al., 1992), so it is as yet unclear whether chronic morphine actually negatively impacts maturing cells. Our experimental paradigm allows us to examine the effects of chronic morphine on BrdU-IR cells undergoing the early stages of maturation. At the time of BrdU-labelling, the majority of cells are thought to express nestin-eGFP and are ovoid in shape (Figure 4A; (Kronenberg et al., 2003). Upon adoption of a neuronal fate, they continue to express eGFP, but also begin to express an early neuronal marker, doublecortin (Figure 4B; Kronenberg et al., 2003). As the cells continue to mature into immature neurons, only doublecortin is expressed and the cells take on a characteristic shape, extending a process into the granule cell layer (Figure 4C; Brandt et al., 2003; Kronenberg et al., 2003). 120 hours post-BrdU-labeling, after sacrifice, we found that BrdU-IR cells in sham mice were approximately 31% Type 2a cells, 48% Type 2b cells, and 14% immature neurons regardless of whether the anterior or posterior hippocampus was analyzed. While mice chronically exposed to morphine had similar proportions of BrdU-IR cells in the posterior region of the hippocampus, they had a higher proportion of BrdU-IR cells with a Type 2b phenotype and lower proportion of BrdU-IR cells with an immature neuron phenotype in the anterior hippocampus. This suggests that maturation of BrdU-IR cells is retarded by chronic morphine in the anterior hippocampus. An alternate interpretation is that immature neurons are being depleted in another manner, e.g. by apoptosis, which would also result in a similar change in distribution of proportions. To date, there have been no observed changes in apoptosis in the SGZ after chronic morphine treatment (Eisch et al., 2000; Kahn et al., 2005)

however *in vivo* and *in vitro* exposure of neurons to mu opioid receptor agonists has been shown to upregulate expression of pro-apoptotic proteins caspase-3, and Fas receptor with a concurrent downregulation of the antiapoptotic protein Bcl-2 (Boronat et al., 2001; Kugawa et al., 1998; Kugawa et al., 2000). This response does not necessarily cause apoptosis, but is thought to prime cells for death. Due to limitations in sensitivity with currently available techniques, we are not able to determine if more BrdU/doublecortin double-labeled cells are dying in chronic morphine mice. We may be able to differentiate between these two hypotheses (retarded maturation vs. immature neuron death) by assessing overall numbers of Type 2b and immature neurons in the anterior hippocampus after chronic morphine. If maturation is retarded, there should be an increase in the total number of Type 2b cells and decrease in the number of immature neurons. If immature neurons are dying, there would be no change in the overall number of Type 2b cells and a decrease in the number of immature neurons. Either of these outcomes suggest that there is most likely a decrease in the number of immature neurons in the anterior region of the hippocampus after chronic morphine, in line with the previous findings of decreased PSA-NCAM after repeated morphine in the rat (Kahn et al., 2005). In the future we will also be examining whether BrdU-IR cells at later stages of maturation (e.g. 72-120 hours after BrdU-labelling) are also negatively impacted by morphine.

Hippocampal progenitor cells are thought to be derived from a resident population of radial glia-like Type 1 stem cells (Alvarez-Buylla et al., 2001). This cell population is easily detectable in nestin-eGFP mice as GFP expressing cells with long radial glia-like processes

extending through the GCL (Figure 4A; Yamaguchi et al., 2000). We used this property of the nestin-eGFP mice to examine how chronic morphine affects hippocampal stem cells and found that Type 1 cells were significantly decreased in morphine treated mice (Figure 5A). Moreover, the decrease was significantly decreased specifically in the anterior hippocampus (Figure 5B). This is the first demonstration of opiates impacting stem cells in the adult brain. This finding is particularly interesting since the Type 1 cells are considered to be slowly-dividing or quiescent cells and thus, like maturing neurons, should also be refractory to morphine's anti-proliferative effects. It is unclear by what mechanism morphine decreases Type 1 cells. We are currently determining if this could be a direct effect by examining whether Type 1 cells express the mu opioid receptor. Alternatively, this effect could also be due to an indirect effect, such as changes in the microenvironment. We will also be assessing how quickly after chronic morphine stem cell numbers decrease. This will be important in interpreting our finding that there is a very strong correlation between Type 1 cell numbers and Ki67 cell numbers (data not shown). These data suggest that decreased proliferation in response to chronic morphine may be, at least in part, reflective of a decrease in the number of Type 1 cells giving rise to the highly proliferative progenitor cells. It will be of great interest to determine if the decrease in Type 1 cells preceded the decrease in proliferating progenitor cells after chronic morphine treatment.

Chronic morphine appears to differentially effect hippocampal cells depending on their stage of maturation at the time of exposure. One possible reason for this effect is that some key components, such as receptors, fluctuate in expression during cell maturation. The

NMDA receptor subunits NR1 and NR2B appear to be expressed by Type 1 stem cells, absent from Type 2 progenitor cells, and re-expressed by immature neurons (Nacher et al., 2007). Another receptor demonstrated to have stage-dependent expression is the glucocorticoid receptor. This receptor is expressed by 50% of Type 1 and Type 2a cells, is downregulated in Type 2b and immature neurons, and re-expressed in immature neurons (Garcia et al., 2004). Morphine could be acting directly through the mu opioid receptor (MOR), which has been shown *in vitro* to be expressed by adult hippocampal progenitor cells (Persson et al., 2004). We are currently examining the expression of MOR by cells at different stages of maturation *in vivo* to determine if receptor expression changes during the process of neurogenesis. Although expression of MOR in neurogenic cells is not yet known, constitutive knockout of MOR increases neurogenesis by conferring a survival advantage to cells in the later stages of maturation, but does not affect cells in the earlier stages of maturation (Harburg et al., 2007). This supports the idea that manipulations of the opioid system, either through deletion of MOR or activation by morphine, can influence one stage of neurogenesis over another. As progenitor cells mature, they also undergo changes in their electrophysiological properties. Type 1 stem cells do not respond physiologically to GABA, NMDA, AMPA, or glycine (Tozuka et al., 2005). Nestin-GFP/PSA-NCAM-IR cells, which are most likely what we define as Type 2b cells, respond to GABA through the $GABA_A$ receptor, but do not respond to NMDA, AMPA, or glycine (Tozuka et al., 2005). Immature neurons express $GABA_A$ and glutamate receptors and respond to NMDA, AMPA, and GABA (Overstreet Wadiche et al., 2005). In the dentate gyrus, opiates can, through MOR, directly inhibit GABAergic interneurons and excite mature granule cell neurons (Akaishi et

al., 2000; Bramham and Sarvey, 1996; Neumaier et al., 1988). Thus, the different effects of morphine we observe on Type 1 cells and maturing progenitor cells could be due to their different abilities to respond to alterations in glutamatergic and GABAergic stimulation in the hippocampus.

Another very interesting finding is that morphine seems to preferentially impact the anterior hippocampus, particularly in its effects on putatively non-dividing cells. The anterior, or rostral hippocampus, is anatomically and functionally distinct from the posterior, or caudal hippocampus (Moser and Moser, 1998). It receives projections primarily from the lateral entorhinal cortex, association cortices and perirhinal area (Moser and Moser, 1998). Through these anatomical connections, the anterior hippocampus receives information derived from the visual auditory, and somatosensory cortices (Moser and Moser, 1998). Functionally, the anterior hippocampus is necessary for spatial learning, as has been proven by numerous lesion studies (Moser and Moser, 1998). The anterior and posterior regions of the hippocampus may also be neurochemically distinct. In mice, the dorsal hippocampus has 25-30% higher BDNF and NGF levels than the ventral hippocampus (Zhu et al., 2006). In rats, the dorsal hippocampus has higher levels of taurine, serotonin, and a metabolite of norepinephrine (NE), 3-methoxy-4hydroxyphenylglycol (MHPG) (Hortnagl et al., 1991). Nothing is yet known about the effects of morphine on NGF and BDNF. Serotonin levels are not altered in the dorsal hippocampus by morphine (Tao and Auerbach, 1994). Hippocampal levels of NE and taurine are both decreased by morphine (Gao et al., 2007; Matsumoto et al., 1994). Depletion of NE is known to decrease proliferating cells in the hippocampus, but

does not affect differentiation or survival (Kulkarni et al., 2002). The role of taurine in hippocampal neurogenesis has not yet been explored, but it is known to induce proliferation in fetal neuron cells and is important for differentiation and survival of both fetal neuron cells and retinal progenitor cells (Chen et al., 1998; Levine et al., 2000). Whether taurine, NE, or other neurotrophins or transmitters are involved in chronic morphine's negative impact on various stages of neurogenesis remains to be explored.

In conclusion, we found that chronic morphine administration causes anterior hippocampus specific decreased in Type 1 stem cells and retarded maturation of progenitor cells into immature neurons. These findings show that morphine has distinct effects on several stages of neurogenesis, and suggest that these effects may have long-term consequences on adult hippocampal neurogenesis. Chronic morphine's anterior specific effects also point to differential regulation of neurogenesis by opiates along the anterior-posterior axis of the hippocampus. Since the anterior region of the hippocampus is necessary for spatial memory, the anterior specific impact of chronic morphine on stem cells and maturation maybe the mechanism by which memory is impaired after chronic morphine (Moser and Moser, 1998). Further studies are necessary to determine the behavioral consequences and the mechanisms by which morphine impacts neurogenesis.

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CHAPTER THREE
Knockout of the Mu Opioid Receptor Enhances Survival of Progenitor Cells in the Adult Hippocampus

(Adapted from: Harburg, G. C., Hall, F. S., Harrist, A. V., Sora, I., Uhl, G. R. and Eisch, A. J., 2007. Knockout of the mu opioid receptor enhances the survival of adult-generated hippocampal granule cell neurons. Neuroscience. 144, 77-87.)

Introduction

The mu opioid receptor (MOR) is highly expressed in brain monoaminergic pathways, brainstem, and spinal cord, and has long been implicated in processes related to these brain regions, such as addiction, alertness, and nociception (Arvidsson et al., 1995; Kreek et al., 2005; Mansour et al., 1995; Uhl et al., 1999). However, recent evidence points to a more novel role for MOR: regulating hippocampal structure and function (Guo et al., 2005; Harrison et al., 2002; Kearns et al., 2001; Martinez and Derrick, 1996; Morris and Johnston, 1995; Terman et al., 2000; Vigano et al., 2005a; Vigano et al., 2005b). MOR is expressed on interneurons and primary cell types in all hippocampal regions (Arvidsson et al., 1995; Drake and Milner, 2002; Mansour et al., 1995; Meibach and Maayani, 1980; Svoboda et al., 1999), including in the granule cell layer (GCL) of the dentate gyrus. Hippocampal structure, physiology, and biochemistry are potently influenced by exogenous MOR agonists, like morphine and heroin, and by perturbation of the levels of endogenous agonists, like enkephalin and dynorphin (Chavkin, 2000; Morris and Johnston, 1995; Simmons and Chavkin, 1996). For example, chronic exposure to MOR agonists negatively impacts hippocampal function, defined here as hippocampal-dependent behaviors, while blockade of MOR and other opiate receptors enhances hippocampal function (Spain and Newsom, 1989;

1991). Supporting the role for MOR in hippocampal function, transgenic mice that lack MOR or endogenous MOR ligands have altered behavior in hippocampal-dependent tasks (Jamot et al., 2003; Jang et al., 2003; Nguyen et al., 2005; Sanders et al., 2005). These studies encourage more detailed exploration of how MOR regulates hippocampal structure and function.

In this regard, an intriguing property of the adult hippocampal dentate gyrus is its capability to generate new neurons throughout life (Abrous et al., 2005; Eisch, 2002). Neural progenitors located in and adjacent to the GCL proliferate, differentiate, and mature to become functional granule cell neurons (Kempermann et al., 2004b; van Praag et al., 2002). All stages of hippocampal progenitor development – proliferation, differentiation, and survival – can be influenced by environmental and physiological stimuli (Abrous et al., 2005; Cameron et al., 1998; Eisch, 2002). For example, adult *in vivo* exposure to MOR agonists decreases the proliferation and survival of new neurons in the hippocampus (Eisch et al., 2000; Eisch and Harburg, 2006; Kahn et al., 2005; Mandyam et al., 2004). In order to examine the impact of loss of MOR on adult hippocampal neurogenesis, we examine the proliferation, differentiation, and survival of new neurons in the hippocampus of adult transgenic mice lacking exon 1 of MOR (Sora et al., 1997). We find that MOR knockout (KO) mice have normal proliferation and differentiation, but enhanced survival of new neurons and more granule cells. These results suggest a previously unappreciated role for MOR in the normal development of hippocampal granule cells.

Experimental procedures

Animals. Adult male mice with a deletion of exon 1 of MOR were created on a C57BL/6 and 129Sv mixed background as previously described (Sora et al., 1997). MOR KO mice were generated from heterozygote crosses to produce wild-type (WT), heterozygote, and homozygote littermates. WT, heterozygote KO and homozygote KO mice were genotyped by PCR using two internal primers, one targeted at the NEO insertion in the KO construct and one targeted at the WT gene, and one external primer, which generated two products identifying the WT and KO genes. PCR using Tsg DNA polymerase (Lamda Biotech, St. Louis, MO) was performed on tail DNA eluted after digestion overnight in Protease K. The forward primer (5' CTG GAT GAG CTG TAA GAA TAG G 3') and the WT primer (5' CAG CCA ACA CAA TAT CAC ATT C 3') produced a 550 bp band, while the forward primer and the NEO primer (5' CGG ACA GGT CGG TCT TGA C 3') produced an 800 bp band. PCR amplification products were separated by electrophoresis on 4% agarose gels and bands were visualized under UV illumination. The mice were housed at 24ºC with a 12:12 light/dark cycle and *ad libitum* access to food and water, and all procedures were performed according to AAALAC guidelines in a vivarium at NIDA-IRP in Baltimore, Maryland. Pharmacology experiments utilized 6-7 week old adult male C57BL/6J mice. These mice were housed in a facility approved by the AAALAC at the UT Southwestern Medical Center with a 12-h light/dark cycle and *ad libitum* access to food and water. All procedures were performed in accordance with guidelines of both the UT Southwestern Institutional Animal Care and Use Committee and AAALAC. Experiments were designed to minimize the number of animals used and their suffering.

Bromodeoxyuridine (BrdU) injections and tissue preparation. In order to assess levels of cell proliferation, differentiation, and survival of new cells in the dentate gyrus, mice were given one i.p. injection of BrdU (150 mg/kg; Boehringer Mannheim, Mannheim, Germany) dissolved in 0.9% saline and 0.007 N NaOH at 10 mg/ml as previously described (Mandyam et al., 2004). A total of forty-four male mice were used for this study. To assess proliferation, 22 mice (WT=10, heterozygote=5, homozygote=7) were perfused two hours after BrdU injection. To assess differentiation and survival 22 mice (WT=8, heterozygote=5, homozygote=9) were perfused 28 days after BrdU injection to allow time for BrdU cells born four weeks earlier to achieve their mature phenotype. All mice were anesthetized with chloral hydrate and perfused transcardially with cold 0.1 M phosphate-buffered saline (PBS) followed by 4% paraformaldehyde in 0.1 M PBS (pH 7.4) for 30 minutes at a rate of 7 ml/min. After perfusion, brains were removed and postfixed in 4% paraformaldehyde in 0.1 M PBS for at least 24 hours at 4ºC. Brains were cryoprotected in 30% sucrose in 0.1M PBS with 0.1% NaN_3 at 4ºC until sectioning. Thirty μm coronal sections were taken on a freezing microtome (Leica, Wetzlar, Germany). Nine serial sets of sections were collected through the entire hippocampus. Sections were stored in 0.1% NaN_3 in 1xPBS at 4ºC until processed for immunohistochemistry (IHC).

In vivo administration of MOR antagonist. Adult male C57/BL mice received subcutaneous implantation of Alzet osmotic pumps (Durect Corporation, Cupertino, CA) that delivered 50 mg/kg/day naltrexone (NTX; n=12; Sigma-Aldrich, St. Louis, MO). Sham mice (n=12) were subcutaneously implanted with an inert pellet to reduce experimental costs (Yoburn et al.,

1994). To address the impact of naltrexone on proliferation, mice (sham n=4; NTX n=4) were injected with BrdU (150 mg/kg) 4 days after implantation and sacrificed 2 hours later (Figure 5A). To address the impact of naltrexone on survival, two additional 28-day experiments were performed. Mice were given BrdU either 4 days after implantation (Figure 5B; sham n=4, NTX n=4) or prior to implantation (Figure 5C; sham n=4, NTX n=4). Both survival groups underwent pump re-implantation or sham surgery on day 14 of the experiment to allow replenishment of naltrexone and to avoid degradation of the compound.

IHC and histology. Every ninth section of the hippocampus was mounted on glass slides (Fisher Superfrost/Plus, Hampton, NH) and left to dry overnight prior to IHC. Slides were coded so the experimenter was blind to the genotype of the animal until completion of analysis. Sections used for IHC were pretreated as follows: DNA denaturation (0.01 M citric acid, pH 6.0, 95ºC, 10 min), membrane permeabilization (0.1% trypsin in 0.1 M Tris and 0.1% $CaCl_2$, 10 min), and acidification (2 M HCl in 1xPBS, 30 min). Primary antibody concentrations were as follows: rat anti-BrdU (Accurate, Westbury, NY; 1:100), rabbit anti-activated-caspase 3 (AC3; Cell Signaling Technology, Beverly, MA; 1:500), rabbit anti-glial fibrillary acidic protein (GFAP; Dako, Carpinteria, CA; 1:500), and mouse anti-NeuN (Chemicon, Temecula, CA; 1:50). Single-labeling IHC for BrdU or AC3 was completed using the avidin-biotin/diaminobenzidine visualization method (Vector Laboratories, Pierce, Rockford, IL) followed by counterstaining with Fast Red (Vector). Triple-labeling for BrdU, GFAP, and NeuN was performed by co-incubation in a primary cocktail overnight followed by co-incubation in a cocktail of the following fluorescent secondary antibodies (Jackson

Immunoresearch, West Grove, PA; anti-rat CY2 (1:200), anti-mouse CY3 (1:200), and anti-rabbit CY5, (1:500)), and completed by counterstaining with DAPI (Roche, Basel, Switzerland; 1:5000). Apoptotic nuclei were detected using AC3-immunoreactivity as described above, presence of pyknotic cells in Fast Red-stained sections, or terminal transferase-mediated dUTP nick-end labeling (TUNEL). Before beginning the TUNEL procedure (DeadEnd Colorimetric TUNEL System; Promega, Madison, WI), slide-mounted sections were pretreated with 0.1 M sodium citrate buffer, pH 6.0 at 95ºC for 5 min (Heine et al., 2004).

Quantification of immunoreactive (IR) and apoptotic cells. Cell birth, survival, and death were examined in the neurogenic region of the dentate gyrus. This region, referred to here as the GCL, was composed of the subgranular zone (SGZ; extending from the border of the GCL to three granule cell widths into the hilus) and the adjacent GCL (Donovan et al., 2006; Kempermann et al., 1997; Kuhn et al., 1996). Using modified stereology with the optical fractionator method (Eisch et al., 2000; Mouton, 2002), immunoreactive cells were quantified in the GCL along the longitudinal extent of the hippocampus (bregma -0.70 to -4.16,Franklin and Paxinos, 1997). Cell counts were performed at 400x and 1000x magnification with an Olympus BX-51 microscope while continually adjusting the focal plane through the depth of the section. Since counting of cells was conducted on every ninth section of the hippocampus, the number of counted cells in the region was multiplied by nine to obtain an estimate of the total number of cells per region. Cell birth and survival were determined by the number of BrdU-IR cells present in the GCL two hours or four weeks after

BrdU injection, respectively. To control for bioavailability of BrdU between genotypes, BrdU-IR cells within the medial habenula, putatively dividing mast cells (Zhuang et al., 1999), were also quantified (Donovan et al., 2006; Lagace et al., 2006; Mandyam et al., 2004). The habenula is non-neurogenic, unlike more commonly used control regions (e.g. subventricular zone (SVZ) (Ming and Song, 2005), corpus callosum (Dayer et al., 2005)), and its small volume and relative homogeneity allows appropriate stereologic analysis throughout its longitudinal axis. Cell death was assessed in GCL and hilus by quantification of AC3-IR, TUNEL-positive, or pyknotic cells (Donovan et al., 2006; Eisch et al., 2000) as previously described (Geloso et al., 2002; Gould et al., 1990; Heine et al., 2004).

Phenotypic analysis. In order to determine the differentiation of adult-generated GCL cells into either neurons or glial, triple labeled sections from the four-week group (n=4 animals of each genotype) were examined for co-localization of BrdU with NeuN or GFAP. At least 45 BrdU-IR cells in the GCL of each animal (WT 48±3, heterozygote 74±9, homozygote 66±6) were analyzed. All cells were optically sectioned in the Z plane using a confocal microscope (Zeiss Axiovert 200 and LSM510-META, Carl Zeiss, Oberkochen, Germany) with three laser lines (emission wavelengths 488, 543, and 633), multi-track scanning, and a section thickness of 0.45 μm. Orthogonal and rotational analysis in a 3D reconstruction program (Volocity, Improvision Inc, Lexington, MA) confirmed co-localization.

Stereological estimation of structural volume and granule cell number. GCL volume and granule cell number were determined in 30 μm sections stained with Fast Red (Donovan et

al., 2006). One section per 240 μm was analyzed spanning bregma -0.70 to -4.16 (Franklin and Paxinos, 1997) such that the entire longitudinal axis of the hippocampus was analyzed. Sections were coded so the experimenter was blind to the genotype of the animal until completion of analysis. All measurements were obtained using StereoInvestigator software (MBF Bioscience, Williston, VT) and an Olympus BX51 microscope. Volumes were measured according to the Cavalieri principle (Gundersen and Jensen, 1987; West and Gundersen, 1990) using a 40x objective. The total number of granule cells was determined using optical disectors along with the optical fractionator method (West et al., 1991) and a 100x/NA 1.30 oil objective. The right GCL of each animal was analyzed for neuron number since there was no statistical difference between the right and left GCL volumes. Data are presented as right GCL neuron numbers only. A grid size of 125 μm x 125 μm was superimposed over each section, and granule cells in fields within the GCL were counted in 15 x 15 x 6 μm sample volumes, with upper and lower guard volumes of 1 μm, resulting in an average of 160 sampling sites per GCL.

Statistical analyses and presentation. Data are represented as mean ± SEM. Statistical analyses were performed using a multiple variable analysis of variance (ANOVA) followed by a Bonferroni post-hoc test. For repeated measure variables (bregma), a repeated measures ANOVA was used to assess the effect of genotype on the number of BrdU-IR cells as specific bregma points, followed by a Bonferroni post-hoc test. All statistical analysis was performed on a Macintosh computer using SPSS version 11.0.2. Statistical significance was

defined as $p<0.05$. Images were imported into Photoshop version 7.0.1 (Adobe Systems Inc., San Jose, California) and the only adjustments made were via gamma in the Levels function.

Results

Proliferation

Two hours after BrdU injection, BrdU-IR cells were evident in the SGZ and GCL of the hippocampus. As previously shown, proliferating cells were small, clustered, and irregularly shaped (Fig. 1). No differences were evident in the size, clustering, or shape of BrdU-IR cells across genotypes. Quantification of the total number of BrdU-IR cells showed no significant effect of genotype in the GCL (Fig. 1B; $F_{(2,19)}=1.430$, $p>0.05$) or the habenula (Fig. 1B; $F_{(2,19)}=0.478$, $p>0.05$), a region we use to assess bioavailability of BrdU (Donovan et al., 2006; Mandyam et al., 2004). The distribution of BrdU-IR cells in all groups differed significantly across the longitudinal axis of the GCL (Fig. 1C; $F_{(14,266)}=31.116$, $p<0.001$), with two peaks of BrdU-IR cells in the GCL at bregma -1.66 and bregma -3.2. There was also a significant interaction of bregma and genotype (Fig. 1C, $F_{(28,266)}=1.843$, $p<0.01$). However, post hoc analysis revealed no significant difference in cell number at any bregma value among the genotypes. Therefore, while a trend exists (Fig. 1B, 1C), the number of proliferating cells in the adult mouse GCL is unchanged in MOR KO mice.

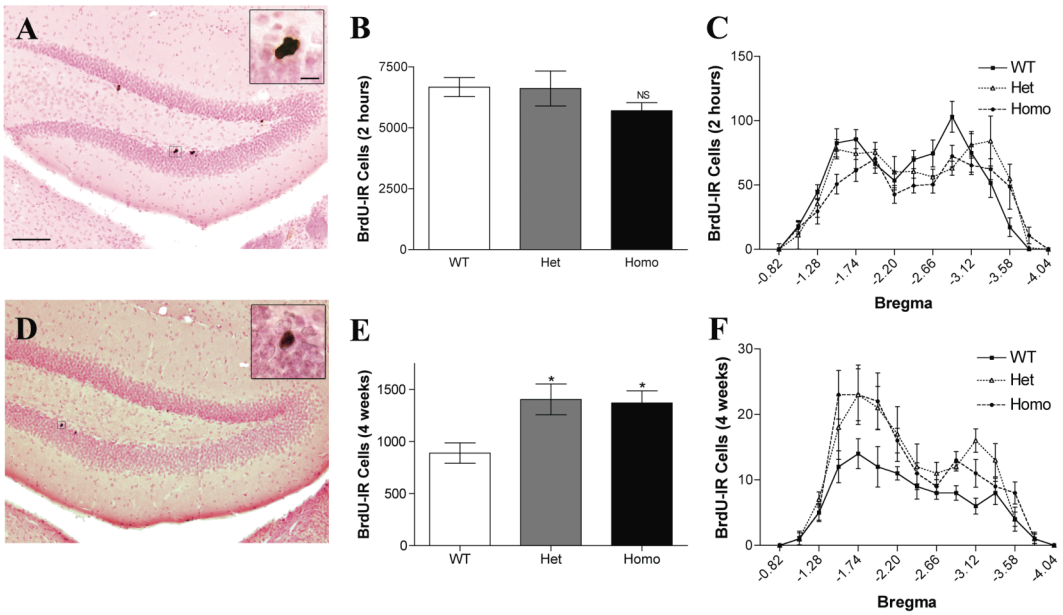

Figure 1: MOR knockout (KO) mice have increased cell survival without changes in proliferation.A-C: Cell proliferation in the adult hippocampal granule cell layer 2 hours after BrdU administration. A. BrdU-labeled cells in the subgranular zone of the dentate gyrus displayed a typical clustered appearance. Inset is a higher magnification image of a BrdU-IR cell cluster. Scale bar, 100 μm. Inset scale bar, 10 μm. B. Homozygote MOR KO mice show a non-significant trend to decrease in cell proliferation in comparison to wild-type (WT) mice (p=0.26). C. Distribution of BrdU-labeled cells along the anterior-posterior axis of the hippocampus. D-E: Survival of new cells in the adult hippocampal granule cell layer 4 weeks after BrdU administration. D. BrdU-labeled cells in the subgranular zone of the dentate gyrus displayed a mature neuron phenotype. Inset is a higher magnification of a BrdU-IR cell. E. Both heterozygote and homozygote animals show increased survival of newly born cells at 4 weeks in comparison to WT animals (* $p<0.05$). F. Distribution of BrdU-labeled cells along the anterior-posterior axis of the hippocampus. Data for B, C, E, and F are presented as mean ± SEM.

Survival

Surviving cells in the hippocampus were assessed in mice injected with BrdU and sacrificed four weeks later. Reflective of the maturation they undergo in the intervening four weeks, surviving BrdU-IR cells in the GCL were large, round, generally spotted or solid in

staining, and rarely clustered (Fig. 1D). Quantification of BrdU-IR cells at this survival time point revealed a significant effect of genotype (Fig. 1E; $F_{(2,19)}=6.042$; $p<0.01$). Heterozygous and homozygous mice had significantly more BrdU-IR GCL cells in comparison to wild-type mice, with increases of 57% and 54% respectively (p's<0.05). There was no effect of genotype on the number of BrdU-IR cells in the habenula ($F_{(2,19)}=0.378$, $p>0.05$), indicating that the increased BrdU-IR cell number was selective for the hippocampus. There was not an interaction of bregma and genotype (Fig. 1F; $F_{(24,228)}=1.281$, $p>0.05$), suggesting that the increase in homozygous and heterozygous mice was spread across the hippocampus. These data show that while proliferation is unchanged in MOR KO mice, constitutive loss of or reduction in levels of MOR increases the number of surviving cells in the adult GCL.

Differentiation

Hippocampal progenitor cells can differentiate into several fates, including astrocytes or neurons (Reynolds and Weiss, 1992). To determine if loss or depletion of MOR influenced the fate of adult-generated hippocampal cells, we triple-labeled sections from mice given BrdU four weeks earlier with antibodies against glia (GFAP), neurons (NeuN), and BrdU. Confocal analysis showed that the majority of BrdU-IR cells in all three genotypes became neurons (Fig. 2; Table 1). There was no effect of genotype on the proportion of cells that became neurons ($F_{(2,11)}=1.349$, $p>0.05$), glia ($F_{(2,11)}=0.919$, $p>0.05$), or had an indeterminate fate ($F_{(2,11)}=0.616$, $p>0.05$). These data show that the increase in surviving BrdU-IR cells seen after loss or reduction of MOR (Fig. 1D-F) reflects an increase in adult hippocampal neurogenesis.

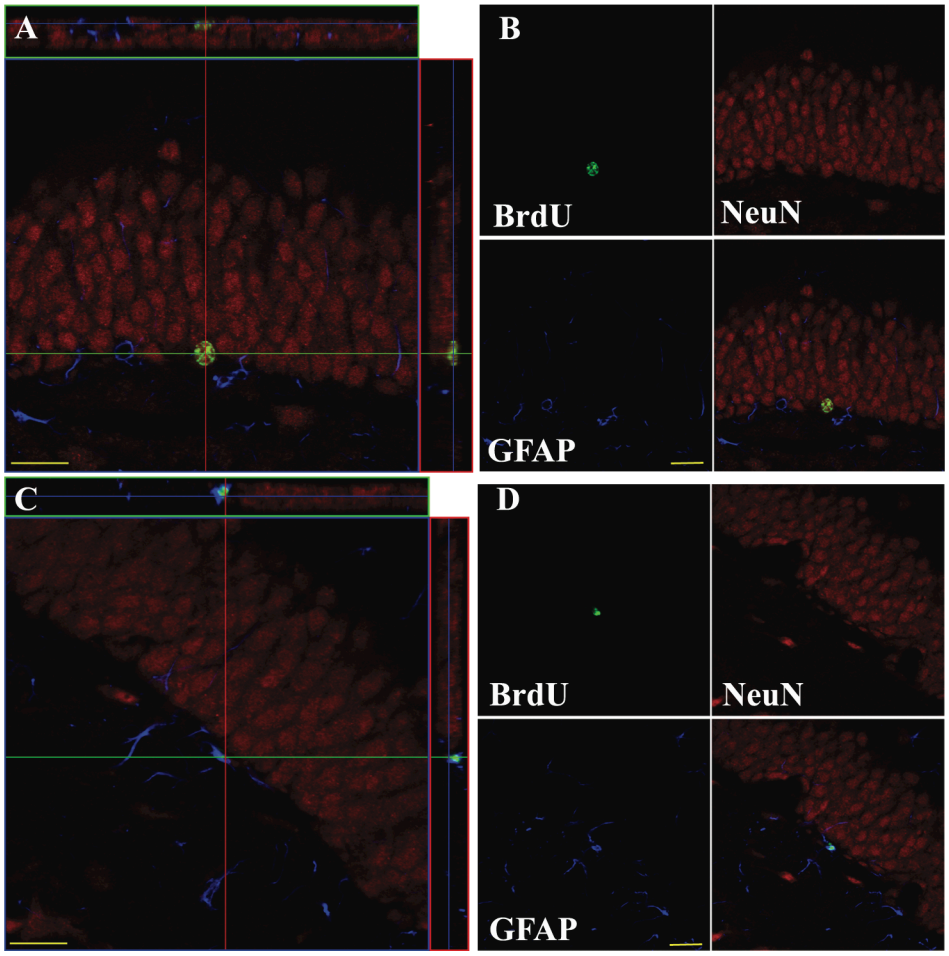

Figure 2: Knockout of the mu opioid receptor does not alter cell fate during maturation. A, B: Example of a BrdU-labeled cell with a neuronal phenotype. BrdU labeling (green) colocalizes with the neuronal marker, NeuN (red), but not a glial marker, GFAP (blue). C, D: Example of a BrdU-labeled cell with a glial phenotype. BrdU labeling colocalizes with GFAP and not NeuN. Scale bars, 20 μm.

Table 1: Phenotype of BrdU-IR GCL Cells

	% BrdU cells colocalizing with		
	NeuN	*GFAP*	*Neither*
Wild-Type	93.5(±2.4)	1.7(±1.2)	4.8(±2.7)
Heterozygote	96.3(±2.9)	0.6(±0.6)	3.1(±3.1)
Homozygote	98.6(±0.5)	0.3(±0.3)	1.1(±0.4)

Cell Death

Many adult-generated hippocampal neurons will die prior to incorporation into hippocampal circuitry (Dayer et al., 2003). Since we found that loss or reduction of MOR increases the survival of adult-generated neurons (Fig. 1D-F), we hypothesized that the increased survival was due to decreased cell death. To assess apoptosis, adjacent sets of sections were examined for the number of AC3-IR, TUNEL-positive, and pyknotic cells (Fig. 3). The majority of apoptotic cells were located within the SGZ region of the GCL, underscoring the high turnover of cells in this region (Dayer et al., 2003; Gould et al., 1999a; Heine et al., 2004; Thomaidou et al., 1997). As noted previously (Geloso et al., 2002), the majority of AC3-IR cells were large, round cells, and many extended processes through the GCL (Fig. 3D). This morphology reflects AC3's early role in the apoptotic cascade (Faherty et al., 1999). TUNEL-positive (Fig. 3E) and pyknotic cells (Fig. 3F) were small and round, and processes were rare, emphasizing that these two methods of cell death detection capture cells in later stages of apoptosis (Faherty et al., 1999). Regardless of the method used to detect apoptosis, there was no significant effect of genotype (AC3, $F_{(2,19)}=0.984$, $p>0.05$; TUNEL, $F_{(2,12)}=0.055$, $p>0.05$, pyknosis, $F_{(2,12)}=1.166$, $p>0.05$). These data show that GCL cell death is not different between WT and MOR KO mice.

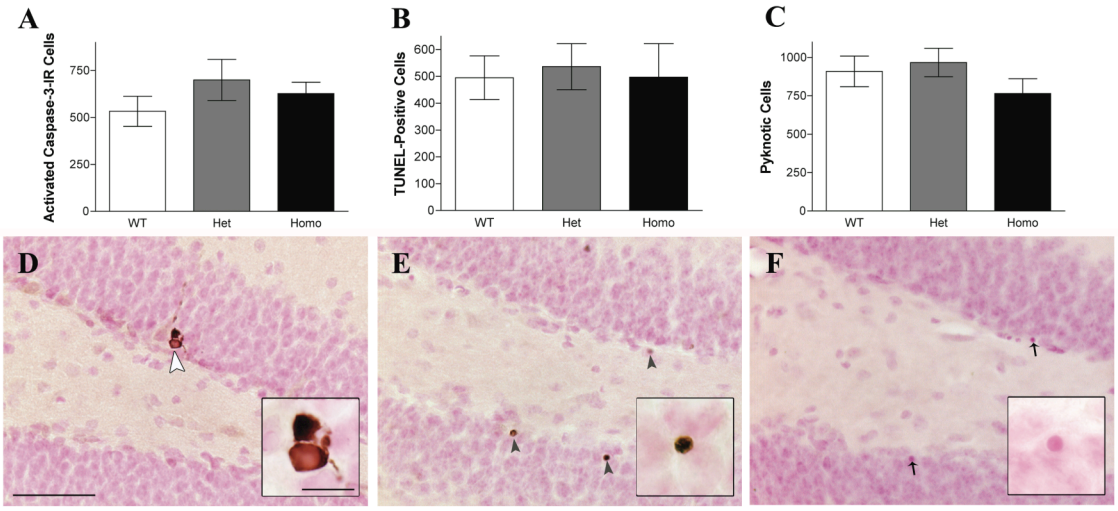

Figure 3: Increased survival of neurons in MOR KO mice is not due to a detectable decrease in levels of apoptosis. Quantitative analysis of (A) activated caspase-3-IR cells, (B) TUNEL-positive cells, and (C) pyknotic cells in the GCL. Representative images of (D) activated caspase-3 cells (indicated by white arrowhead), (E) TUNEL-positive cells (indicated by gray arrowheads), and (F) pyknotic cells (indicated by black arrows) in the GCL. Data for A-C are presented as mean ± SEM. Scale bar, 50 μm. Inset scale bar, 10 μm.

Stereological Estimation of Structural Volume and Granule Cell Number

Given that cell death was not different across genotypes (Fig. 3), the increased survival of newborn cells in the GCL of heterozygote and homozygote MOR KO mice (Fig. 1) would be expected to lead to an increased number of GCL cells. To address this possibility, we calculated the volume of the GCL and determined the number of granule cells using optical disectors along with the optical fractionator method (Gundersen and Jensen, 1987; West and Andersen, 1980; West and Gundersen, 1990). In regards to GCL volume, there was a significant effect of genotype ($F_{(2,18)}$=5.833; $p<0.05$; Table 2) with the GCL of MOR heterozygote and homozygote KO mice being 22% and 17% larger than WT littermates,

respectively ($p<0.05$ in each case). This increase in GCL volume was specific to the hippocampus since a main effect of genotype was also seen in the hilus ($F_{(2,18)}=5.071$; $p<0.05$; heterozygote and homozygote 20% and 23% larger than WT), but no main effect of genotype was seen on the volume of the habenula, a region rich in MOR ($F_{(2,17)}=0.107$; $p>0.05$; Fig. 4A, Table 2). There was also a main effect of genotype on the number of neurons in the GCL ($F_{(2,17)}=11.06$; $p<0.001$) with heterozygote and homozygote possessing 30% ($p<0.05$) and 44% ($p<0.001$) more GCL neurons than WT (Fig. 4B, Table 3). Taken together these data show that enhanced survival of adult-generated neurons results in increased numbers of neurons in the GCL of adult mice lacking MOR.

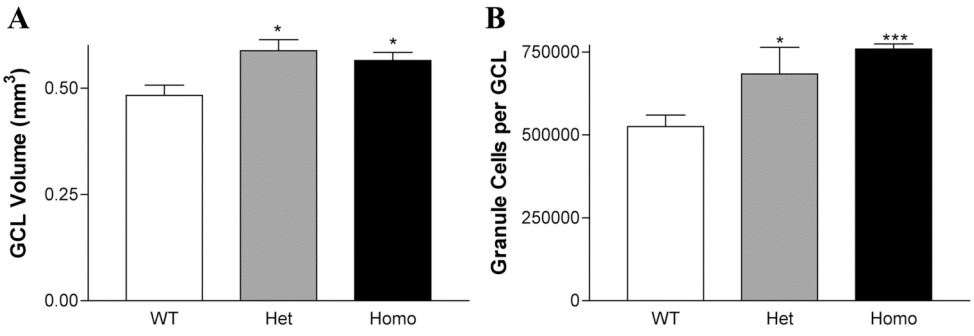

Figure 4: MOR KO mice have larger granule cell layers and increased numbers of granule cells. A. Granule cell layer volumes. B. Number of granule cells per GCL as estimated by optical fractionator. (* $p<0.05$, *** $p<0.001$) Data is presented as mean ± SEM.

Table 2: Effect of Genotype on Size of Brain Regions

	GCL Volume (mm^3)	*% WT*	*Hilus Volume (mm^3)*	*% WT*	*Habenula Volume(mm^3)*	*% WT*
Wild-Type	0.482(±0.024)	100	0.494(±0.032)	100	0.069(±0.002)	100
Heterozygote	0.588(±0.026)*	122	0.594(±0.021)*	120	0.069(±0.003)	100
Homozygote	0.565(±0.016)*	117	0.608(±0.003)*	123	0.067(±0.003)	97

* $p<0.05$

Table 3: Effect of Genotype on GCL Neuron Number

	GCL Volume (mm^3)	*% WT*	*GCL Neurons ($x10^5$)*	*% WT*	*Neuron Density ($x10^5/mm^3$)*
Wild-Type	0.482(±0.024)	100	5.254(±0.346)	100	10.90(±0.490)
Heterozygote	0.588(±0.026)*	122	6.840(±0.798)*	130	12.00(±1.222)
Homozygote	0.565(±0.016)*	117	7.590(±0.153)***	144	13.49(±0.386)*

* $p< 0.05$; *** $p<0.001$

In vivo Administration of an MOR Antagonist

We found that a four-day exposure to the opioid receptor antagonist naltrexone had no effect on the number of proliferating cells in the SGZ (Figure 5A). We next examined whether neurons born and maturing under the condition of opioid blockade had any alterations in cell survival. Mice exposed to naltrexone throughout cell birth and maturation, surprisingly, had no change in levels of cell survival (Figure 5B). We also examined the effects of opioid blockade specifically on cell maturation and survival by exposing mice to naltrexone 4 days after BrdU injection, this being a time-point where the majority of labeled cells have stopped proliferating (Dayer et al., 2003). We found that neurons maturing in an environment of opioid blockade had no alterations in cell survival (Figure 5C).

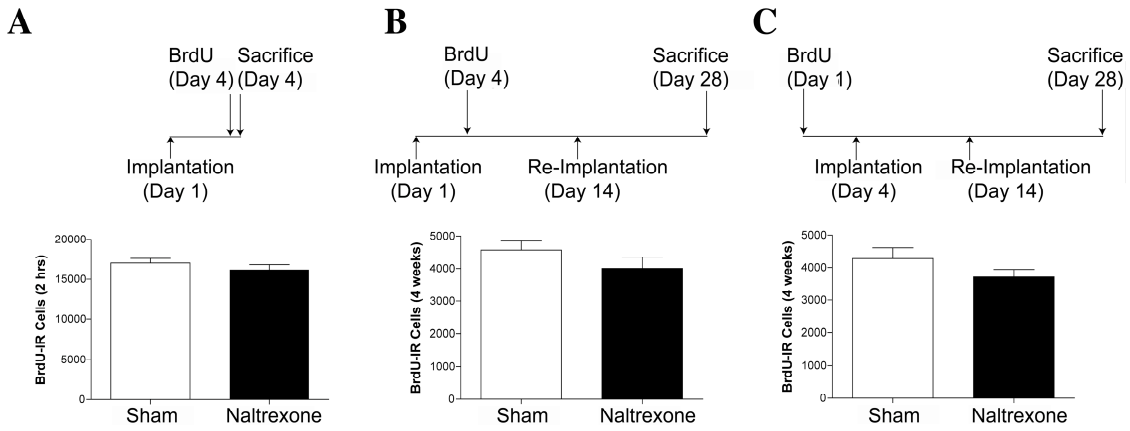

Figure 5: Naltrexone does not effect cell proliferation or survival in the adult mouse hippocampus. A) Mice that received naltrexone (NTX) subcutaneously for 4 days did not have altered numbers of proliferating cells in the SGZ. B) and C) Long term exposure to naltrexone did not alter the number of BrdU+ cells in the SGZ, regardless of whether naltrexone was in the system before (B) or after (C) labeling of the cells with BrdU.

Discussion

These data show that heterozygote and homozygote MOR KO mice have enhanced adult hippocampal neurogenesis. This effect is due to an increase in the number of progenitor cells that survive, not to alteration in the number of proliferating GCL cells, and is independent of detectable changes in cell death. There is no influence of the MOR KO on cell fate determination since WT, heterozygote and homozygote mice all had equivalent proportions of surviving cells maturing into neurons. The impact of increased neurogenesis on the hippocampus is emphasized by the increased volume and neuron number in the GCL of the dentate gyrus in heterozygote and homozygote mice. These results indicate that MOR plays a role in regulating the survival of maturing neurons in adult hippocampal neurogenesis.

The majority of factors currently known to influence neurogenesis do so via influence on proliferating progenitor cells. Thus, it is of particular interest that deletion of MOR appears to confer a survival advantage to maturing neurons without altering the numbers of proliferating progenitor cells. These data add MOR to the limited list of factors that have a stronger effect on survival versus proliferation of new hippocampal neurons (Ambrogini et al., 2005; Galea et al., 2006; Lichtenwalner et al., 2006; Rizk et al., 2005). The data presented here suggest that MOR is critical to normal maturation of adult-generated granule cells. Studies are in progress to explore whether other aspects of progenitor cell development, e.g. establishment of the granule cell dendritic tree, are altered in MOR KO mice.

The increase in survival of maturing neurons in MOR KO mice occurs without the expected concurrent decrease in cell death, as three measures (AC3, TUNEL, pyknosis) were similar between WT and KO mice. While it is difficult to reconcile increased cell survival in the face of no decrease in cell death, two things give us confidence that MOR KO mice indeed do have enhanced cell survival. First, despite the lack of detectable changes in cell death levels, our finding of increased cell survival (Fig. 1) is supported by a concomitant increase in the number of granule cell layer neurons (Fig. 4, Table 3). This emphasizes that more neurons appear to survive and likely integrate into the hippocampal circuitry in MOR KO mice. Second, available methods of cell death detection are hampered by the difficulty in catching the short window of time in which cells can be detected. Even those studies that show a correlation between cell birth and cell death show large differences between the number of proliferating cells and dying cells (Biebl et al., 2000; Cameron and Gould, 1996;

Cameron and McKay, 1999; Heine et al., 2004; Montaron et al., 1999). This commonly-reported discrepancy between the number of proliferating progenitors and dying cells underscores the need for global technical advances in detection of cell death, as well as encourages more specific research into the relationship between these events as it relates to adult hippocampal neurogenesis.

It is interesting to note that heterozygote and homozygote MOR KO mice both have similar increases in cell survival in the GCL. While it is difficult to pinpoint the reason for the lack of a gene dosage effect, it is interesting to speculate if receptor density plays a role. MOR is expressed at low levels in the dentate gyrus (Arvidsson et al., 1995; Drake and Milner, 2002; Mansour et al., 1995), so a 50% reduction in MOR would have a significant impact on receptor density. *In vivo* studies have shown that MOR activity is highly dependent on receptor density (Law et al., 2000; Pak et al., 1996). Decreasing the number of functional MOR receptors by treatment with irreversible MOR antagonists (β-CNA, β-FNA) results in a non-linear decrease in receptor function. This reduction in functional efficacy of the receptor/effector unit necessitates a greater percentage occupancy of receptors in order to generate a functional response (Pak et al., 1996). This work also suggests that secondary cascades initiated by MOR may be uniquely sensitive to MOR receptor density (Pak et al., 1996). Thus, loss of one copy of MOR could translate into greater than expected decrease in receptor function and downstream activity in the dentate gyrus, resulting in a phenotype similar to the homozygote. Future studies, beyond the scope of this paper, will explore the

mechanism behind our heterozygote phenotype by focusing on the kinetics of MOR specifically in dentate gyrus neurons.

Possible Mechanisms for MOR Regulation of Neurogenesis

Pharmacological evidence supports the hypothesis that MOR is a regulator of cell survival in the CNS. For example, pre- and postnatal exposure to the opioid antagonist naltrexone results in an increase in brain size (Zagon and McLaughlin, 1984; 1986b), while exposure to MOR agonists decreases brain size (Ford and Rhines, 1979; Zagon and McLaughlin, 1977a; b). Full exploration of the mechanism for MOR regulation of adult neurogenesis is outside the scope of the present study. However, several recent findings provide clues to how MOR regulates the survival of new neurons, and these deserve discussion. The most pressing question to be addressed is whether MOR impacts maturing neurons directly, indirectly, or both. Here we propose two different mechanisms – one direct, and one indirect – via which MOR could regulate neurogenesis.

Indirectly, MOR could regulate neurogenesis by altering the levels of pro-survival factors in the dentate gyrus. MOR activity has been shown to be involved in the regulation of a number of neurotransmitters in the hippocampus including acetylcholine (ACh, Kaplan et al., 2004; Lapchak et al., 1989), GABA (Akaishi et al., 2000; Drake and Milner, 1999; 2002), and norepinephrine (NE, Matsumoto et al., 1994). GABA modulates neurogenesis, but appears to do so by altering cell differentiation, not survival (Karten et al., 2006; Tozuka et al., 2005). Manipulations of ACh levels can alter both proliferation and survival (Cooper-

Kuhn et al., 2004; Mohapel et al., 2005). However, a recent paper showed that chronic acetylcholinesterase (AChE) antagonist treatment results in increased cell survival in the SGZ without affecting cell proliferation or fate (Kotani et al., 2006). While it is intriguing to consider whether perturbations in cholinergic signaling contribute to the enhanced survival reported here, cholinergic receptor binding as well as AChE activity are both normal in MOR KO mice (Tien et al., 2004). Thus, it seems unlikely that alterations in the cholinergic system are the cause of increased cell survival in MOR KO mice. NE is perhaps a more likely candidate. Antagonism of the alpha2-adrenoceptor, which increases NE levels, enhances neurogenesis by specifically increasing survival of new neurons in the hippocampus independent of changes in cell proliferation or fate (Rizk et al., 2005). MOR agonists inhibit release of NE (Matsumoto et al., 1994) and ACh (Lapchak et al., 1989) in the rat hippocampus. Perhaps deletion of MOR results in increased levels of these neurotransmitters in the hippocampus, leading to the increase in survival of new neurons reported here. The mechanisms by which these neurotransmitters promote cell survival have not yet been elucidated, however it has been proposed that NE, and possibly ACh, promote the survival of new neurons by increasing BDNF levels in the hippocampus (Rizk et al., 2005). Future experiments will address whether constitutive KO of MOR affects the levels of NE and BDNF in the hippocampus.

Alternatively, opioids could impact neurogenesis via direct activation of MOR. An *in vitro* study supports both the presence of MOR on hippocampal progenitors cells as well as the role of MOR in neuronal fate specification (Persson et al., 2003), and an *in vivo* study

shows the presence of MOR on progenitors in another neurogenic region of the brain, the SVZ (Stiene-Martin et al., 2001). Furthermore, MOR may be expressed on a small population of cells of unknown identity in the dentate gyrus (Drake and Milner, 1999); these cells deserve further analysis for the possibility that they are progenitor cells. Studies are currently ongoing in our laboratory to establish if and when hippocampal progenitor cells and immature granule cells *in vivo* express MOR, and can thus directly respond to MOR agonists and antagonists. If these progenitor cells do express MOR, future studies could specifically knockout MOR in progenitor cells or immature neurons (e.g. via viral knockdown (van Praag et al., 2002) or specific transgenic KO) to determine if direct activation of MOR in these cells is necessary for its regulatory effects on neurogenesis.

As with all constitutive KO mice, the possibility that the findings reported here are due to compensatory changes in the brain must also be considered. Potential adaptive alterations in the brains of MOR KO mice have previously been described (Hall and Uhl, 2006), including changes in the cholinergic system (discussed above), as well as changes in the dopaminergic and glutamatergic systems. In the hippocampus, MOR KO mice have been shown to have alterations in non-opioid receptor activities, including reduced dopamine D2 receptor binding (Matthies et al., 2000) and enhanced metabotropic glutamate receptor and somatostatin binding (Grecksch et al., 2004). One way to address whether the reported increase in cell survival is MOR-specific or due to compensatory changes is to examine the effects of chronic administration of an MOR antagonist. In line with our findings in the MOR KO mice, chronic naltrexone treatment in early postnatal rats increases hippocampal size and

granule cell number (Zagon and McLaughlin, 1984; 1986c). *In vivo*, chronic exposure of progenitor cells to another opioid antagonist, naloxone, results in increased neurogenesis (Persson et al., 2003). However, we have found that pharmacological blockade of MOR via chronic treatment with a high dose of the opioid antagonist naltrexone in adult mice does not alter cell proliferation or survival in the SGZ. This discrepancy with the MOR KO mice may be due to naltrexone's non-specificity since it acts on the delta and kappa opioid receptors as well (Raynor et al., 1994). This multiple opioid receptor antagonism could mask MOR-specific effects. Contradictory results have also been found with naltrexone's effects on cell proliferation in the SGZ (Galea et al., 2006; Persson et al., 2004), emphasizing the complexity of *in vivo* use of this compound, and the general challenges associated with attempts to mimic genetic manipulation with pharmacological blockade. Future studies will circumvent the natural confounds associated with constitutive KOs and the non-specificity of available MOR antagonists by studying the link between MOR and cell survival in a conditional MOR KO mouse.

Clearly, further studies are needed to elucidate whether the positive impact demonstrated here on adult neurogenesis is a result of the developmental expression of MOR on adult-generated neurons, an indirect effect such as via neurotransmitters, or perhaps a more global alteration in the hippocampal *milieu* that results in enhanced neurogenesis.

Implications of MOR Regulation of Neurogenesis on Behavior

Increased neurogenesis can positively impact hippocampal function, resulting in enhanced hippocampal-dependent learning. Neurons produced by adult hippocampal neurogenesis integrate into the structure of the hippocampus, extending axons along the mossy fiber tract to CA3 (Hastings and Gould, 1999; Markakis and Gage, 1999; Overstreet et al., 2004; Stanfield and Trice, 1988; Wadiche et al., 2005). The new neurons also gain the electrophysiological properties of mature granule cells and respond appropriately to stimulus of the perforant pathway (van Praag et al., 2002) strengthening the position that these cells are functionally integrated into the synaptic circuitry of the hippocampus. Correlative evidence suggests neurogenesis can enhance hippocampal-dependent learning in rodents (Drapeau et al., 2003; Shors et al., 2001; Shors et al., 2002; Snyder et al., 2005). In line with this theory, heterozygote and homozygote exon 1 MOR KO mice perform better than WT littermates in the Morris Water Maze, a task of hippocampal-dependent memory (Meilandt et al., 2004) and have enhanced adult hippocampal neurogenesis (present data). Further exploration of adult neurogenesis and hippocampal-dependent memory in MOR KO mice is warranted given differences in key physiological measures and behavior among distinct transgenic MOR KO mice (Hall and Uhl, 2006; Jamot et al., 2003; Jang et al., 2003; Kitanaka et al., 1998; Loh et al., 1998; Matthes et al., 1996; Matthies et al., 2000; Meilandt et al., 2004; Schuller et al., 1999; Sora et al., 1997). Identification of the similarities and differences among these mice will be central to determination of the mechanism that underlies the enhanced neurogenesis reported here in MOR KO mice.

In summary, these findings implicate MOR as necessary for the survival of newly mature neurons in the hippocampus. We extrapolate that endogenous opioids may act to regulate numbers of surviving newly born neurons by binding to MOR to cause this suppression. Thus, opioids and opiates have the potential to negatively impact hippocampal-dependent learning and memory by decreasing neurogenesis.

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CHAPTER FOUR
Effect of Heroin Abuse on Adult Hippocampal Neurogenesis

Introduction

Heroin abuse is a significant and expanding problem in today's society (Abuse, 2005; Group, 2006). Chronic opiate abusers show cognitive deficits and perform more poorly than controls on tests of information processing, problem solving, verbal fluency, and other memory tasks (Darke et al., 2000). Some of these deficits may be due to impaired hippocampal function (Guerra et al., 1987).

The hippocampus is notable as being one of only two regions of the adult brain that gives rise to new neurons during adulthood (Abrous et al., 2005). One of the most exciting findings of the last decade was that new neurons are generated in the adult human hippocampus (Eriksson et al., 1998). These new neurons integrate into the hippocampal circuitry and may contribute to hippocampal function (van Praag et al., 2002). Chronic opiate administration in mice and rats results in decreased progenitor cell proliferation and neurogenesis as well as impaired spatial memory (Eisch et al., 2000; Kahn et al., 2005; Mandyam et al., 2004; Spain and Newsom, 1991). Previous postmortem analysis has established that expression of polysialic acid neural cell adhesion molecule (PSA-NCAM), a protein linked to both neurogenesis and neural plasticity, is increased in the brains of heroin addicts (Kahn et al., 2005). However, the results of this study suggested that the increased

expression was not due to increased neurogenesis, but rather a compensatory response to neural insult. These findings do support the hypothesis that heroin abuse in humans results in significant changes in the hippocampus that might impact levels of neurogenesis. In this study, we wanted to address whether progenitor cell proliferation is negatively impacted in the hippocampus of human heroin abusers.

Methods

Subjects. Human brains from apparent heroin overdose and normal control Caucasian subjects without head trauma were collected as previously described from the Department of Forensic Medicine, Semmelweis University and the National Institute of Forensic Medicine, Karolinska Institutet (Drakenberg et al., 2006). All material was obtained under approved local ethical guidelines. The cause and manner of death were determined by a forensic pathologist after evaluating the circumstances of death, toxicology data, autopsy results, and police reports, as well as family interviews and medical records, when available. All cases were assessed for common drugs of abuse (including alcohol) and for therapeutic agents. The general characteristics of inclusion criteria for control and heroin subjects are described in Table 1. The heroin group represented a unique drug abuse population, because this group consisted of predominantly heroin users with no methadone treatment.

The total population of subjects consisted of 31 subjects (heroin users, n = 18; control, n = 13). Immediately after autopsy, brains were cut coronally in 1.5 cm slabs, frozen, and kept at –70°C. Blocks (5 x 7 cm) were obtained from the caudal region of the left hippocampus;

cryosections sectioned at Mount Sinai School of Medicine (20 μm, Microm HM560; Microm International, Walldorf, Germany) were quickly mounted on Superfrost Plus glass (BRL, Newton, MA) and kept at –80°C until analysis. All procedures were carried out blinded to the subject group.

Table 1:

Group	**Control**	**Heroin**
Number	n=4	n=6
Age (years)	33.50±5.06 (22-46)	25.83±1.72 (21-31)
Gender	M=3, F=1	M=6, F=0
PMI (hours)	19.5±4.50 (6-24)	22.67±1.33 (16-24)
Storage time (weeks)	419.5±52.80 (352-577)	458.33±66.71 (328-778)
pH	6.70±0.12 (6.33-6.85)	6.44±0.11 (6.07-6.74)
Ethanol (blood)	0 (0)	0.09±0.09 (0-0.52)
Cause of Death	Cardiac Failure (n=4)	Heroin overdose
Inclusion Criteria	Negative toxicology of opiates/other drugs No history of opiate abuse No history of abuse of other drugs No physical body-needle tracks No history of psychiatric disorder	Positive opiate toxicology History of opiate abuse No history of abuse of other drugs Physical body-needle tracks

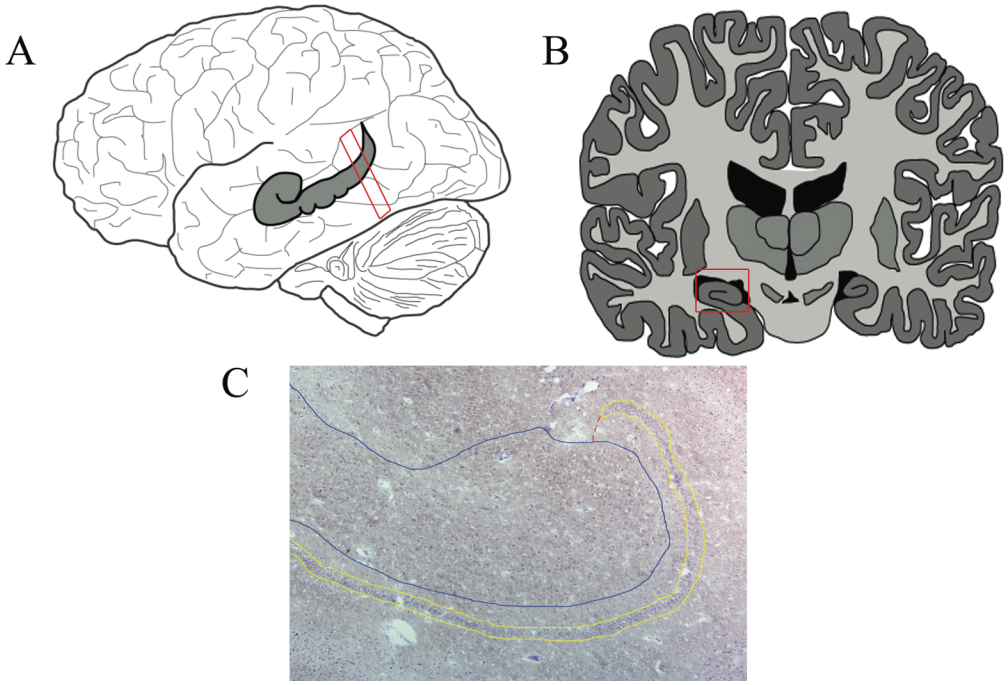

Figure 1: Hippocampal Region of Analysis. A. Hippocampal sections for this study were obtained from the caudal region of the hippocampus. B. Coronal view of the human brain. The red box shows the approximate region of the brain shown in the picture below. C. Example of the human dentate gyrus. The regions analyzed for this study are shown as follows: granule cell layer (yellow), hilus (blue). The subgranular zone is the region between these two structures enclosed by the dashed red line.

Immunohistochemistry. For detection of proliferating cells in the dentate gyrus of the hippocampus, 10 sections from all sectioned cases (n=31 subjects), spaced approximately 200 um apart, were immunohistochemically labeled using a Ki67 antibody. Slides were removed from -80^{o}C storage and dried overnight at room temperature. The sections were fixed with -20^{o}C acetone for 10 minutes, dried, and then rehydrated in phosphate-buffered

saline (PBS). They were blocked with 3% normal donkey serum (NDS) and 0.03% Triton X-100 for 1 hour, then incubated overnight in a room temperature humid chamber in mouse anti-Ki67 (MIB-1 clone) monoclonal antibody (1:250; DAKO, Carpenteria, CA, USA), 3% NDS and 0.03% Tween-20 in PBS. The following day the sections were incubated in biotinylated donkey-anti-mouse polyclonal antibody (1:250, Jackson ImmunoResearch, West Grove, PA, USA) in 1.5% NDS and PBS for 1 hour, followed by a 1.5 hour incubation in avidin-biotin (Vector Laboratories). Ki67 was detected with diaminobenzidine (Pierce Laboratories, Rockford, IL, USA), followed by light counterstaining with cresyl violet. Fresh frozen human tumor tissue was used as a positive-control for Ki67. No positively stained cells were observed when primary or secondary antibodies were omitted.

Quantification of Ki67-immunoreactive (IR) cells. Proliferating cells, indicated by Ki67, were assessed in a subset of cases (Table 1; control=5, heroin=7). Two subjects (control=1, heroin=1) were excluded from analysis as outliers by box-plot analysis. Several regions of the dentate gyrus were analyzed: the granule cell layer (GCL), putative subgranular layer (SGZ), and hilus (Figure 1). The GCL was easily identifiable as a dense band of cells in the hippocampus. The hilus was defined as a region mostly enclosed within the GCL, possessing obvious large hilar cells as detected by cresyl violet staining. The subgranular zone was defined as a largely cell-free region between the GCL and hilus (refs). Ki67-immunoreactive (IR) cells that were clearly located within the structure of blood vessels (e.g. colocalizing with the blood vessel wall) were excluded from counts. Cell counts were performed at 200x and 400x magnification with an Olympus BX-51. Region volumes were

obtained using StereoInvestigator software (MBF Bioscience, Williston, VT) and an Olympus BX51 microscope. Areas were measured according to the Cavalieri principle (Gundersen and Jensen, 1987; West and Gundersen, 1990) using a 20x objective.

Statistical analyses and presentation. Data are represented as mean ± SEM. Statistical analyses were performed using a paired sample two-tailed student's t-test followed by a Bonferroni post-hoc test analysis. All statistical analysis was performed on a Macintosh computer using Prism 4 for Macintosh version 4.0c (GraphPad Software, Inc., San Diego, CA, USA). Statistical significance was defined as $p<0.05$. Images were imported into Photoshop version 7.0.1 (Adobe Systems Inc., San Jose, California) and the only adjustments made were via gamma in the Levels function.

Results

Gross Morphology and Distribution of Ki67-IR Cells

Ki67 is well established as a marker to detect proliferating cells within the human hippocampus (Boekhoorn et al., 2006; Del Bigio, 1999). Although Ki67 has a relatively short half-life of 1 hour, and thus may reflect changes in proliferation occurring during the postmortem interval, it is not as susceptible to postmortem degradation as another commonly used endogenous proliferation marker, proliferating cell nuclear antigen (PCNA), that has a longer half-life of 20 hours, but degrades quickly in postmortem tissues (Eisch and Mandyam, 2007; Wolf and Dittrich, 1992). Ki67-IR cells were detected in all regions of the dentate gyrus analyzed. The majority of Ki67-IR cells presented in clusters of multiple

small, irregularly shaped cells similar to what has been reported in the rodent hippocampus (Figure 2;Mandyam et al., 2007). Cell clusters were dispersed throughout the hilus and SGZ regions without any discernable pattern. Within the GCL, the majority of Ki67-IR cells were observed within the inner half of the structure closest to the SGZ, but cells were also detected in the outer half of the structure as well. Although not quantified, Ki67-IR cells were also observed located in stratum moleculare.

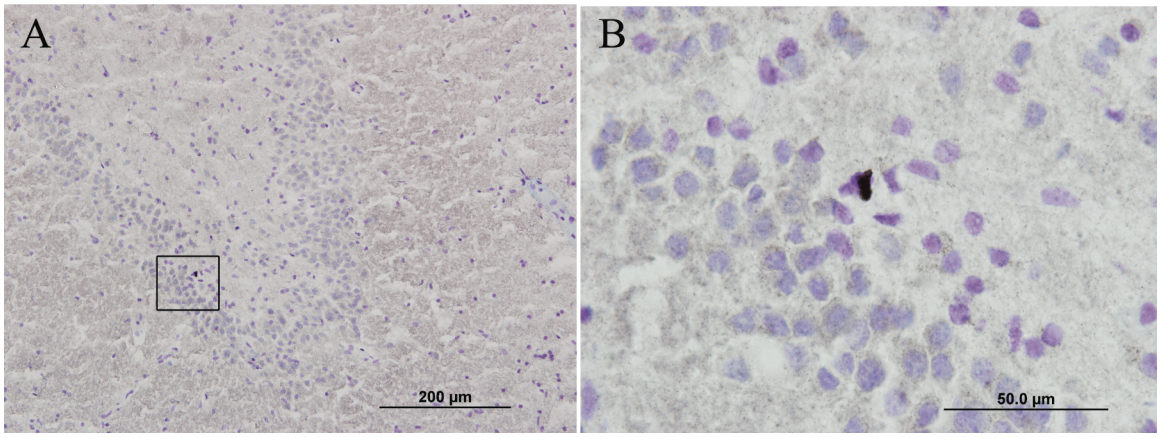

Figure 2: Ki67-immunoreactive (IR) cells localized to the granule cell layer. A. This is a single cluster of Ki67-IR cells located near the crest of the granule cells layer in the dentate gyrus. B. Higher magnification view of the same cluster shows that there are multiple cells present within a single cluster.

Quantification of Ki67 Cells

The number of Ki67-IR cells/mm^2 of each region did not vary significantly between the GCL, SGZ and the hilus by one way ANOVA (Control: $F_{(2,15)}$=0.7492, P>0.05; Heroin: $F_{(2,15)}$=0.1040, P>0.05). There was a trend towards a decrease in the number of Ki67-IR cells in the GCL of heroin abusers compared to matched controls by student's t-test (Figure 3A;

P=0.0997). There was no significant change in Ki67-IR cell numbers in either the SGZ or hilus (Figure 3A).

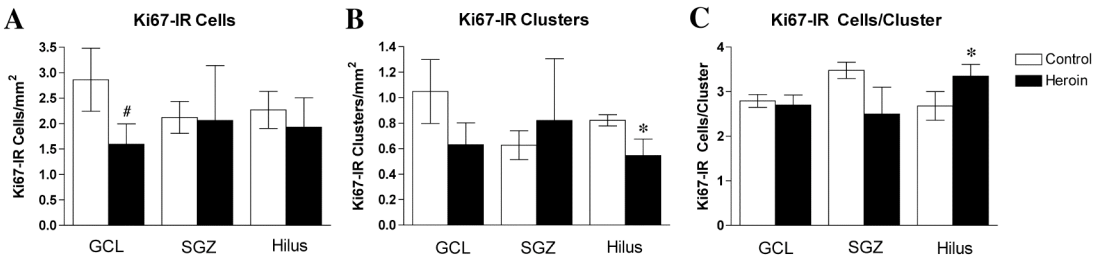

Figure 3: Heroin abusers show altered proliferation in the hilus region of the hippocampus. A. Quantification of Ki67-IR cells in the dentate gyrus. The total number of Ki67-IR cells in the granule cell layer trends towards a decrease (#, P=0.099), but does not reach significance. B. Quantification of Ki67-IR clusters in the dentate gyrus. C. Quantification of the number of Ki67-IR cells per cluster in the dentate gyrus. (*, P<0.05)

Quantification of Ki67 Clusters

The number of Ki67-IR clusters/mm^2 of each region did not vary significantly between the GCL, SGZ and the hilus by one way ANOVA (Control: $F_{(2,15)}$=1.719, P>0.05; Heroin: $F_{(2,15)}$=0.2104, P>0.05). There was no significant change in the number of Ki67-IR clusters between controls and heroin abusers in the GCL (Figure 3B) or the SGZ (Figure 3B). There was a significant decrease in the number of Ki67-IR clusters in the hilus in heroin abusers (Figure 3B; P<0.05).

Using the Ki67 cell and cluster counts, we determined the average number of Ki67-IR cells per cluster for each region in all subjects analyzed. We found that Ki67-IR cells per

cluster did not change between controls and heroin abusers in the GCL or SGZ (Figure 3C, P>0.05). However, there was an increase in the number of Ki67-IR cells per cluster in the hilus of heroin abusers (Figure 3C, P=0.02).

Discussion

Chronic opiate exposure in mice and rats has been shown to decrease proliferating cells in the adult hippocampus, leading to decreased neurogenesis (Eisch et al., 2000; Kahn et al., 2005; Mandyam et al., 2004). Opiate use is also known to cause cognitive deficits in both rats and human heroin abusers (Guerra et al., 1987; Ornstein et al., 2000; Spain and Newsom, 1991). Here we present our findings from a subset of a larger cohort of heroin abuser and control subjects. While the entire cohort remains to be analyzed in the future by a separate blinded experimenter, this smaller subset showed that, in comparison with matched controls, heroin abusers have significantly fewer clusters of Ki67-IR cells in the hilus and a trend towards a decrease in Ki67-IR cells in the GCL. While preliminary, these results suggest that heroin abuse results in decreased levels of proliferation in the adult human hippocampus.

For this study, we analyzed proliferation specifically in the caudal portion of the hippocampus. The homologous region in rodents, the anterior hippocampus (Sasaki et al., 2004), appears to particularly sensitive to chronic opiate administration, resulting in decreased numbers of hippocampal stem cells and retarded progenitor cell maturation (Chapter 3). Although proliferation is uniformly decreased along the anterior-posterior axis of the mouse hippocampus, these anterior specific effects could result in gross changes in

neurogenesis in the anterior hippocampus in response to chronic opiate administration. The anterior hippocampus in rodents is known to be necessary for spatial memory (Moser and Moser, 1998), a behavior impaired by chronic opiate administration (Spain and Newsom, 1991). In humans, the caudal hippocampus is associated with a number of cognitive functions, including semantic and spatial memory acquisition (Alvarez and Squire, 1994; Maguire et al., 2000) and episodic memory retrieval (Lepage et al., 1998). Hippocampal function, and in particular, caudal hippocampal function, seems to be impaired in heroin abusers, who perform more poorly on verbal fluency and memory tasks in comparison to controls (Guerra et al., 1987; Ornstein et al., 2000). Thus, we focused on the caudal hippocampus in this study as the most likely part of that hippocampus to show drug-induced alterations in heroin abusers.

Chronic heroin self-administration in rats results in decreased numbers of proliferating cells in both the hippocampal granule cell layer and the hilus (Eisch et al., 2000). We see a similar trend in this study towards decreased proliferation in the GCL (Figure 3A). This finding suggests that heroin may act to decrease proliferation, and perhaps neurogenesis, in humans as has been shown in rodent models (Eisch et al., 2000; Kahn et al., 2005; Mandyam et al., 2004). However, with the future analysis and inclusion of additional subjects, this trend towards a decrease may or may not become significant. If we ultimately find that GCL proliferation is unchanged in heroin abusers, this may be due to the much longer time-span and intermittency of drug use in contrast to animal studies. Preliminary results from our laboratory suggest that hippocampal proliferation may level out with acquisition of tolerance

during chronic opiate administration (Arguello et al., 2005). Increases in hippocampal proliferation have also been observed during withdrawal (Kahn et al., 2005). Although, all heroin subjects in this study died of heroin overdose, and thus were not in withdrawal at the time of death, any increases in proliferation observed might suggest that the subject was in withdrawal immediately preceding the lethal dose of heroin. Alternatively, withdrawal-induced seizures may also increase hippocampal proliferation (A. Arguello, personal communication). Thus, although our observed trend towards a decrease in GCL proliferation is interesting, at the completion of this study the outcome could change due to one of the reasons discussed above.

Chronic heroin self-administration in rats also results in decreased numbers of proliferating cells in the hilus of the hippocampus (Eisch et al., 2000). Although we did not observe a change in Ki67-IR cell number in the hilus, we did observe a significant decrease in the number of Ki67-IR clusters. Proliferating cells in the adult hippocampus divide in clusters (Kahn et al., 2005; Mandyam et al., 2004), so we calculated the number of Ki67-IR cells per cluster and found that there was a significant increase in the number of Ki67-IR cells per cluster in the hilus, but not the GCL or SGZ. This could be due to increased proliferation within a cluster to increase the number of cells within each individual cluster, or decreased numbers of one or two cell clusters. We may be able to distinguish these two possibilities by analyzing the distribution of individual cluster sizes within the hilus. In rats repeatedly administered with morphine, there was a trend towards a decrease in small cluster sizes (one to two cell clusters) in the SGZ. Conversely, rats in morphine withdrawal had

increased cluster sizes in the SGZ (Kahn et al., 2005). The finding supports the hypothesis that cluster size is regulated by chronic opiate administration. However the mechanism for cluster size regulation has not yet been investigated. One mechanism may be that pro-proliferative factors are increased within the hilus in response to chronic heroin use. Or lab has shown that BDNF levels increase in the mouse hippocampus after 5 days of morphine administration (Arguello et al., 2005). A similar phenomenon may occur in the human hippocampus, leading to increased proliferation within individual clusters (Sairanen et al., 2005). Alternatively, increased cluster size and decreased cluster numbers may reflect a response to hippocampal insult. Nerve cell loss has been reported as a consequence of toxic respiratory failure in chronic drug abusers. In 80% of the cases, the hippocampus had increased expression of GFAP, indicative of astrocytes, and often a concordant increase in microglia proliferation (Oehmichen et al., 1996). A recent study has also shown that polysialic acid neural cell adhesion molecule (PSA-NCAM) expression is increased in the hilus of human heroin abusers. They reported the upregulation in PSA-NCAM occurred in both neurons and glia and suggested that this might occur as a repair mechanism in response to either activated microglia or neurotoxicity (Weber et al., 2006). Since Ki67 does not distinguish between progenitor cells and proliferating glia, we would need to address this possibility by double-labeling for Ki67 and glial markers to determine if a proportion of the proliferating cells in the hippocampus are glia (Quinones-Hinojosa et al., 2006). Alterations in GFAP immunoreactivity could also be indicative of changes in the number of radial glia stem cells in the hippocampus (Seri et al., 2001). Future studies are examining this possibility by using a number of different stem cell markers (e.g. Prox1 and Musashi) to

estimate the number of radial glia stem cells in the dentate gyrus of controls and heroin abusers (Christie and Cameron, 2006). Further studies in animal models are still necessary to determine how cluster size is regulated.

In conclusion, we observed a significant decrease in the number of proliferating cell clusters and increased cluster size in the hilus as well as a trend towards decreased proliferating cells in the GCL of heroin abusers. Further studies are necessary to determine whether these changes reflect alterations in neurogenesis or gliogenesis.

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CHAPTER FIVE
Conclusions and Future Directions

Conclusion

Chronic morphine and heroin use has previously been shown to decrease proliferation and neurogenesis in rats and mice. This thesis has expanded previous research to determine: 1) the impact of chronic morphine on early stages of neurogenesis, 2) the role of MOR in adult neurogenesis, and 3) the effect of heroin abuse on proliferating cells in the human hippocampus. Using these several different approaches, opioids and opiates are demonstrated to negatively impact adult hippocampal neurogenesis.

In order to understand more about how morphine may decrease neurogenesis, I examined the impact of chronic morphine administration on different stages of neurogenesis (Chapter 2). I found that chronic morphine decreased the number of both Type 1 stem cells and proliferating progenitor cells. In contrast, progenitor cells exposed to chronic morphine during early maturation were not significantly decreased in number. However, exposure during early maturation appeared to retard cell maturation, since morphine decreased the number of cells that had an immature neuronal phenotype. Assessment of the effects of morphine along the septotemporal axis of the hippocampus revealed that the decrease in both stem cell number and maturation associated with morphine treatment was more pronounced

in the anterior hippocampus. These findings show that morphine has distinct effects on different stages of neurogenesis, and highlight the need for future work to determine why the anterior hippocampus may be more sensitive to the effects of morphine.

Endogenous opioids endorphin and enkephalin and the exogenous opiates heroin and morphine bind to MOR (Nestler et al., 2001). Thus I examined neurogenesis in a MOR knockout (KO) mouse model (Chapter 3) (Harburg et al., 2007). Interestingly, MOR KO mice had increased cell survival in the absence of a change in cell proliferation. In line with this finding, these mice also had larger hippocampal granule cell layers containing more granule cells. These findings suggested that opioids might act to suppress cell survival via MOR. In order to test this hypothesis, I also measured how pharmacological blockage of all three opioid receptor types (mu, delta, and kappa) by chronic administration of an opioid receptor antagonist, naltrexone. This antagonist has previously been shown to decrease neurogenesis when chronically administered pre- or postnatally. In contrast to these developmental effects, chronic blockade of all three opioid receptors by naltrexone did not have a significant effect on adult hippocampal neurogenesis. These convergent lines of data suggest that adult hippocampal neurogenesis is altered by blockade or knockout of mu opioid receptors during development rather than adulthood.

For the last aim of this thesis I wanted to determine whether the decrease in neurogenesis caused by drugs of abuse described in rodents is clinically relevant and occurs in human patients suffering from drug abuse. In order to achieve this goal, I assessed cell proliferation

levels in the brains of human heroin abusers and normal controls (Chapter 4). I found decreased clusters of proliferating cells, containing a greater number of cells, in the dentate gyrus hilus of heroin abusers, as compared with controls. There was also a trend towards a decrease in numbers of proliferating cells in the granule cell layer of heroin abusers. Although these findings are preliminary in nature, they suggest that chronic heroin use in humans, as in rodents, may negatively impact neurogenesis and highlight the feasibility for the continuation of this important line of study.

Together, these findings support a negative role for opioids and opiates in regulating adult hippocampal neurogenesis. They also dissect the role of MOR in the mechanism of morphine's effects, highlight how morphine alters cells in the different stages of maturation during neurogenesis, and make a clinical connection to human drug abusers.

Future Directions

This research has also provoked some very intriguing questions about the mechanism responsible for opioids' and opiates' negative impact on neurogenesis. The most pressing question is whether the effect of opiates is direct or indirect. To answer this, we need to know which cell types in the hippocampus express MOR. It has been shown that MOR is most highly expressed in the hippocampus by interneurons (Drake and Milner, 2002) and is also expressed at low levels in mature granule cell neurons (Stumm et al., 2004). It is as yet unknown whether MOR is expressed at different stages in development of new neurons. I have attempted to address this question using immunohistochemistry for MOR, but have

found that currently available antibodies show non-specific staining in the mouse hippocampus. In order to overcome this methodological issue, I am currently taking another approach by using *in situ* to detect MOR expression in a nestin-eGFP transgenic mouse (Yamaguchi et al., 2000). In conjunction with immunohistochemistry for GFP and doublecortin, this technique will allow me to determine what cells types in the different stages of maturation during neurogenesis express MOR. This study is currently underway and preliminary results look promising.

Identification of the neurogenic cell types expressing MOR, will allow for subsequent studies to determine whether opioids and opiates act directly on these cells to impact neurogenesis. Our laboratory has generated an inducible transgenic mouse that expresses Cre-ER^{T2} under control of the nestin promoter (Battiste et al., 2007). Administration of tamoxifen results in the translocation of Cre-ER^{T2} into the nucleus of nestin expressing cells (Type 1 and 2), where it can act on floxed genes. Although there are no known available floxed-MOR mice, generation of a floxed MOR mouse would allow for the specific knockout of MOR expression in Type 1 stem cells and Type 2 progenitor cells. Alternatively, targeted viral infection of progenitor cells with a short hairpin RNA (shRNA) for MOR could also allow for the specific deletion of MOR and study of its role in neurogenesis. Retroviral transduction has been shown to be effective at targeting proliferating cells (e.g. Type 2 progenitors) in the mouse hippocampus (Tashiro et al., 2007). In order to target not only the cells, but the cells at the other stages of maturation different virus could be utilized that transduces non-proliferating cells (e.g. adeno-associated virus; Baum et al., 2006) or the

MOR shRNA could to be expressed under the control of cell-type specific promoters (e.g. doublecortin). With the increasing rise in the use of viral and shRNA techniques, these studies should be both feasible, as well as highly informative in ascertaining how MOR influences neurogenesis.

A cell-specific conditional knockout of MOR may also be able to address how opioids affect neurogenesis more definitively than the currently available methods of constitutive MOR knockout and chronic receptor blockade (Chapter 3). Constitutive genetic manipulations of the opioid system either through knockout of MOR or its endogenous ligand β-endorphin, both result in increased neurogenesis, although this phenotype is only evident in β-endorphin KO mice engaged in voluntary running (Harburg et al., 2007; Koehl et al., 2006). While intriguing, neither of these studies can rule out possible confounds of compensatory changes. However, these findings are supported by pharmacological studies in which chronic administration of the opioid antagonist naltrexone to either prenatal or early postnatal rats results in increased neurogenesis (Zagon and McLaughlin, 1984; 1986b; c; 1987). In adult rats, subchronic administration of naltrexone also increases hippocampal neurogenesis (Holmes et al., 2004; Persson et al., 2004). In contrast, in adult mice acute administration of naltrexone results in decreased neurogenesis, while I have shown that subchronic or chronic administration does not affect neurogenesis (Galea et al., 2006; Harburg et al., 2007). These conflicting data reveal the challenges of *in vivo* use of naltrexone, which may give non-specific effects by binding to multiple receptor types and is also prone to secondary effects of administration paradigms. A temporal and cell-specific

MOR knockout will avoid the confounds associated with constitutive knockout and pharmacological blockades to determine if opiates and opioids truly act through MOR to regulate adult hippocampal neurogenesis by suppressing cell survival.

My work has discovered that morphine decreases the number of Type 1 stem cells and early Type 2 progenitor cells. A more difficult question to address is how morphine affects later stages of maturation. Recent studies have reported that both genetic and environmental manipulations can impact maturation and survival of immature neurons (Smrt et al., 2007; Tashiro et al., 2007). Thus, it would be of particular interest to determine if morphine affects the maturation and survival of postmitotic progenitor cells and immature neurons. To address this question, I have begun treating nestin-eGFP mice with chronic morphine at time points following the BrdU injection, when the cells have left the cell cycle or have reached the immature neuron stage of development. Specifically, these studies are examining the effects of morphine treatment at 1) 5 days after BrdU, since this is the earliest timepoint at which all BrdU-IR cells have left the cell cycle (Mandyam et al., 2007); and 2) 14 days after BrdU, since this is the earliest time point at which all BrdU-IR cells have reached the immature neuron stage (Harburg and Eisch, 2006). The findings from these experiments will further contribute to the current understanding of how morphine affects neurogenesis.

Our finding of altered proliferation in human heroin abuser hippocampuses opens up new avenues of research to unravel the complex study of neurogenesis in humans. First, continuation of the analysis of proliferation in the control and heroin abuser brains will help

determine the strength of our preliminary results. In order to determine the specificity for the alterations in proliferation in the hippocampus, examination of other non-neurogenic regions of the brain (e.g. CA1 and white matter) will be assessed for proliferation. Moreover, establishment of a collaboration with Carol Tamminga at UT Southwestern will allow for examination of proliferation in a cohort of control and schizophrenic subjects. This study will allow for the assessment of proliferation in another clinical population, provide the opportunity to assess two other avenues of research, and enlighten the basic study of human neurogenesis. The large number of cases and age ranges in this study will allow for a correlation between age and proliferation in control subjects. Although it is well established that neurogenesis in rodents decreases with age (Heine et al., 2004), surprisingly no human postmortem studies have had large enough sample sizes or a wide enough range of ages to show a similar correlation. This study will also allow for examination of proliferation in sections from both the anterior and posterior hippocampus, which will determine if basal proliferation in controls or patients differs between these two regions. Of note, preliminary results from this study suggest that GCL proliferation is significantly reduced in the caudal, but not the rostral hippocampus in schizophrenic, but not control brains. These data are interesting in light of our finding of hippocampus region effects in chronic morphine treated mice and suggest that humans may also have region-specific effects on neurogenesis.

General Conclusions and Speculations

It is clear that opioids and opiates negatively impact hippocampal neurogenesis, yet we can only speculate on the significance of this finding in the context of drug abuse. As

mentioned in the introduction, the holy grail of the study of neurogenesis is determining the contribution of new hippocampal neurons to function (Leuner et al., 2006). Since the functional role of neurogenesis remains unclear, I have interpreted my findings in the context of hippocampal neurogenesis being either significant or irrelevant to hippocampal-dependent behaviors, such as learning and memory.

If adult hippocampal neurogenesis does contribute to learning and memory, opiates and opioids may modulate hippocampal-dependent functions through their effects on neurogenesis. As mentioned previously, chronic opiate use impairs function on hippocampal-dependent tasks in both rodents (Spain and Newsom, 1991) and humans (Darke et al., 2000; Guerra et al., 1987; Ornstein et al., 2000). As shown in this thesis and in the literature, opiates and opioids also decrease neurogenesis by a decrease in stem and progenitor cell numbers, as well as impaired maturation of new neurons. This results in opiates and opioids having a net effect of decreasing the number of new neurons functionally integrating into the hippocampus and could lead to reduced learning and memory.

Alternatively, adult hippocampal neurogenesis may not be involved in learning and memory, but is, rather, vestigial, or possibly has an undetermined cognitive function. In this case, we cannot extrapolate our findings to hippocampal-dependent behavior, but have gained valuable insight into how opioids and opiates act on neural stem cells and their offspring. For example, this work may prove to be applicable to the study of brain development, or other types of stem and progenitor cells throughout the body. With the

increasing interest lately in the concept of cancer stem cells, it would be interesting for future studies to address the impact of opiates on tumor growth and survival in cancer patients, particularly those with neural tumors, undergoing morphine treatment for pain alleviation.

In conclusion, opiates and opioids negatively impact hippocampal neurogenesis in rodents and humans by as of yet unknown mechanisms. Ongoing studies are pursuing whether there is a direct effect of opiate and opioids on stem cells and their offspring. Although controversial, our findings suggest that impaired hippocampal function in heroin abusers and rodents exposed to chronic opiates may be due to decreases in adult hippocampal neurogenesis.

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VITAE

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