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Time course of morphine's effects on adult hippocampal subgranular zone reveals preferential inhibition of cells in S phase of the cell cycle and a subpopulation of immature neurons

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Abstract

Opiates, such as morphine, decrease neurogenesis in the adult hippocampal subgranular zone (SGZ), raising the possibility that decreased neurogenesis contributes to opiate-induced cognitive deficits. However, there is an incomplete understanding of how alterations in cell cycle progression and progenitor maturation contribute to this decrease. The present study examined how morphine regulates progenitor cell cycle, cell death and immature SGZ neurons (Experiment 1) as well as the progression of SGZ progenitors through key stages of maturation (Experiment 2). In Experiment 1, mice received sham or morphine pellets (subcutaneous, 0 and 48 hrs) and bromodeoxyuridine (BrdU) 2 hrs prior to sacrifice (24, 72 or 96 hrs). Morphine decreased both the number of S phase and total cycling cells, as there were fewer cells immunoreactive (IR) for the S phase marker BrdU and the cell cycle marker Ki67. The percentage of Ki67-IR cells that were BrdU-IR was decreased after 24 but not 96 hrs of morphine, suggesting a disproportionate effect on S phase cells relative to all cycling cells at this time point. Cell death (activated caspase-3 counts) was increased after 24 but not 96 hrs. In Experiment 2, nestin-GFP mice given BrdU 1 day prior to morphine or sham surgery (0 and 48 hrs, sacrifice 96 hrs) had fewer Ki67-IR cells, but no change in BrdU-IR cell number, suggesting that this population of BrdU-IR cells were less sensitive to morphine. Interestingly, examination of key stages of progenitor cell maturation revealed that morphine increased the percent of BrdU-IR cells that were Type 2b and decreased the percent that were immature neurons. These data suggest that chronic morphine decreases SGZ neurogenesis by inhibiting dividing cells, particularly those in S phase, and progenitor cell progression to a more mature neuronal stage.

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Keywords

morphine; proliferation; maturation; nestin-GFP; cell cycle; cell death

Introduction

Almost forty years after the initial observation that hippocampal neurogenesis persists into adulthood (Altman and Das, 1965), intriguing links have arisen between the dynamic regulation of adult neurogenesis and psychiatric disorders, such as addiction and depression (Eisch, 2002; Jacobs et al., 2000). Opiates were the first drug of abuse shown to negatively impact proliferation and neurogenesis in the adult mammalian hippocampus (Eisch et al., 2000), and other drugs of abuse have since been shown to also decrease neurogenesis (Crews et al., 2006; Dominguez-Escriba et al., 2006; Mandyam et al., 2008b; Nixon and Crews, 2002; Noonan et al., 2008; Yamaguchi et al., 2004). The reduction in neurogenesis caused by drugs of abuse is hypothesized to contribute to the cognitive deficits observed in addicts (Canales, 2007; Eisch and Harburg, 2006). For example, chronic morphine interferes with memory and hippocampal function in rodents (Miladi Gorji et al., 2008; Spain and Newsom, 1991), and human heroin addicts have altered neurogenesis and hippocampal hyperactivity during drug craving (Daglish et al., 2003; Weber et al., 2006). While additional work is needed to understand the functional importance of opiate-induced alterations in adult hippocampal neurogenesis, another critical step is to clarify the cellular mechanisms underlying opiate-induced regulation of neurogenesis. Given the obvious interest in how neurogenesis is regulated by physiological and environmental stimuli (Kee et al., 2007; Overstreet-Wadiche and Westbrook, 2006), such mechanistic research on opiates would benefit not only the field of addiction, but neural stem cell biology as a whole.

Some key advances have been made in understanding how opiates inhibit neurogenesis in the adult subgranular zone (SGZ). For example, chronic, but not acute, opiates act via the mu opiate receptor to decrease SGZ proliferation (Eisch et al., 2000). Opiate-induced reduction in SGZ proliferation occurs after myriad administration paradigms, including self-administration, subcutaneous (s.c.) pellet implantation, or intraperitoneal (i.p.) injection (Fischer et al., 2008; Kahn et al., 2005; Mandyam et al., 2004). Interestingly, the decreased proliferation is not due to decreased physical inactivity or enhanced levels of stress hormones (Eisch et al., 2000). While these studies provide essential information about opiate-induced reduction of SGZ proliferation, it is still not clear how the decrease in neurogenesis occurs. One possibility is that an altered cell cycle of progenitor cells contributes to decreased SGZ proliferation in mice (Eisch and Mandyam, 2007). Indeed, chronic morphine results in premature mitosis of proliferating cells (Mandyam et al., 2004). However, cell cycle dynamics have only been evaluated at a single time point after chronic morphine exposure (96 hrs), and therefore a time course analysis is warranted. Increased cell death may also contribute to decreased SGZ proliferation, a theory supported by non-neurogenesis studies in which morphine increases hippocampal levels of activated caspase-3 (AC3) measured via immunoblotting (Emeterio et al., 2006). However, since neurogenesis studies find no increase in the number of dying SGZ cells following 96 hrs of morphine exposure (Eisch et al., 2000), additional time points should be examined. Finally, while neurogenesis is now appreciated to be a multi-staged process (Kempermann et al., 2004) and specific stages of neurogenesis are preferentially altered by stimuli such as antidepressants and running (Encinas et al., 2006; Fukuda et al., 2003; Kronenberg et al., 2003; Overstreet-Wadiche and Westbrook, 2006), it is not clear how the multi-stage process of neurogenesis is affected by opiates. Immature neuron number, as determined by polysialic-neural cell adhesion molecule (PSA-NCAM) expression, is decreased by chronic morphine injections

(Kahn et al., 2005), but a detailed analysis of morphine's impact on the progression from Type 1 stem cell to immature neurons has yet to be examined.

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

Materials and Methods

Animals

Two experiments were performed using two strains of adult male C57BL/6J mice. For Experiment 1, C57BL/6J mice were received from Jackson laboratories (stock # 000664) at least one week prior to experimentation. For Experiment 2, homozygous nestin-GFP mice expressing green fluorescent protein under the control of the nestin promoter were bred at UT Southwestern Medical Center. Nestin-GFP mice were initially generated and since maintained on a C57BL/6J background (Yamaguchi et al., 2000). All mice were group-housed four to a cage with free access to food and water in a facility at UT Southwestern Medical Center run on a 12 hr light/dark cycle. The Institutional Animal Care and Use Committee (IACUC) approved all procedures. All measures were taken to minimize animal suffering and the number of animals used.

Drug Treatment and BrdU Injections

For both Experiments 1 and 2, mice were given one 25 mg morphine pellet (generously provided by the National Institute on Drug Abuse, Bethesda, MD) or sham pellet/surgery s.c. under isoflurane anesthesia (2% in compressed oxygen for 1–2 minutes) at 0 and 48 hrs (Mandyam et al., 2004; Shaw-Lutchman et al., 2002). Morphine pellet administration was chosen due to the stable blood levels of morphine, high level of physical dependence, and decreased SGZ proliferation achieved in mice as compared with an escalating paradigm of morphine i.p. injections (Fischer et al., 2008). For Experiment 1, mice were given one 150 mg/kg injection of BrdU (i.p., Roche Diagnostics, dissolved in 0.9% saline, 0.007N NaOH at 10 mg/mL) 2 hrs prior to sacrifice via live decapitation at 24, 72 or 96 hrs (sham n=4–8; morphine n=3–8). One hemisphere of the brain was post fixed in 4% paraformaldehyde for three days, followed by cryoprotection in 30% sucrose as previously described (Lagace et al., 2007b). For Experiment 2, one 150 mg/kg injection of BrdU was given 1 day prior to drug or sham administration so that the effect of morphine on maturing BrdU-IR cells could be examined. At 96 hrs, mice were anesthetized with chloral hydrate (120 mg/kg dissolved in 0.9% saline) and perfused transcardially with 0.1M PBS (sham n=7–8; morphine n=6–7) followed by 4% paraformaldehyde, as previously described (Harburg et al., 2007).

Antibodies and Immunohistochemistry (IHC)

Tissue Sectioning and Preparation—Serial sets of 30µm coronal sections from brains were collected on a freezing microtome (Leica, SM2000R) into nine wells containing 0.1% NaN₃ in 1X PBS and stored at 4°C until processing. For each set of antibodies examined, one well containing the entire mouse hippocampus, −0.82 to −4.24 from bregma (Franklin and Paxinos, 1997), was slide mounted prior to IHC. In preparation for subsequent analysis via stereological cell counting via the modified version of the optical fractionator method (Cameron and McKay, 2001; Ngwenya et al., 2005) sections anterior and posterior to the hippocampus were also mounted, resulting in a total of about 13 sections mounted per mouse and making additional volumetric estimations of the hippocampus unnecessary. Slides were coded before IHC and the code was not broken until data analysis was complete (Mandyam et al., 2004).

Antibodies—The following primary antibodies were used: rat anti-BrdU (Accurate, Westbury, NY, Cat #: OBT0030, Lot #: H7786, 1:300 for single labeling, 1:500 for double or triple labeling); rabbit anti-Ki67 (Vector laboratories, Burlingame, CA, Cat #: VP-K451, Lot#: 301108, 1:500); goat anti-DCX (C-18; Santa Cruz Biotechnology, Santa Cruz, CA, Cat#: sc-8066, Lot#: 12305, 1:3000 for single labeling, 1:5000 for triple labeling); rabbit anti-cleaved caspase-3 (Asp 175; Cell Signaling Technology Inc., Danvers, MA, 01923, Cat#: 9661, Lot#: 18, 1:500); rabbit anti-GFP (Invitrogen, Eugene, OR, Cat #: A11122, Lot #: 41279A, 1:500). For each antibody, the specificity of staining was determined by lack of signal after omission and/or dilution of the primary antibody as well as observation of a similar pattern of staining, as previously reported: AC3 (Cooper-Kuhn and Kuhn, 2002; Donovan et al., 2006), GFP (Donovan et al., 2008), Ki67 (Dayer et al., 2003; Mandyam et al., 2007), DCX (Donovan et al., 2006; Lagace et al., 2007b).

Single Label IHC—Single label IHC was performed for antibodies against BrdU, Ki67, DCX, and AC3. For BrdU and AC3 IHC, the following pretreatment steps were used: antigen unmasking (0.01M citric acid, pH 6.0, 100°C), permeabilization with trypsin (trypsin in 0.1M Tris, 0.1% CaCl₂), DNA denaturation (2N HCl in 1X PBS), and quenching of endogenous peroxidases (0.3% H₂O₂), as previously described (Donovan et al., 2006). For Ki67 and DCX IHC, only antigen unmasking and endogenous peroxidase quenching steps were used. Tissue sections underwent blocking (3% serum, 0.3% Triton-X) before incubation in primary antibody overnight (in 3% serum, 0.3% Tween-20). IHC was completed using the avidin-biotin diaminobenzidine visualization method, followed by counterstain with nuclear fast red (Vector laboratories, Burlingame CA, Cat# H-3403, Lot#: N0507). Single labeling for AC3 was visualized with CY2-fluorophore conjugated secondary.

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

Microscopic analysis and quantification

BrdU-IR, Ki67-IR, and DCX-IR cell numbers were quantified with stereology via the modified optical fractionator method throughout the rostral-caudal axis of the hippocampus, -0.82 to -4.24 from bregma (Franklin and Paxinos, 1997) as previously described (Harburg et al., 2007; Mandyam et al., 2007). Briefly, immunoreactive cell and cluster SGZ counts were performed at 400X magnification on an Olympus BX-51 microscope (Tokyo, Japan) in brightfield or fluorescence with continuous adjustment of the focal plane. Resulting counts were multiplied by the number of sections analyzed (e.g. 9) to reveal total cell or cluster counts (Cameron and McKay, 2001; Ngwenya et al., 2005). The SGZ was defined as the region bordering the granule cell layer and hilus: three cell widths into the hilus and the inner half of the granule cell layer (Donovan et al., 2006; Mandyam et al., 2007).

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

For phenotypic BrdU-IR cell analysis, a confocal microscope was used (Zeiss Axiovert 200M and LSM510-META, emission wavelengths 488, 543, and 633, magnification 630X). An average of 95 ± 9 and 76 ± 14 BrdU-IR cells were analyzed in nestin-GFP reporter mice given sham surgery or morphine administration, respectively. BrdU-IR cells in the anterior hippocampus, -1.48 to -1.78 from bregma (Franklin and Paxinos, 1997), were categorized into discrete maturational stages via their morphological characteristics and co-localization with GFP and DCX as follows: Type 1 cells: triangular soma, dendrite extending into molecular layer, GFP+/DCX-; Type 2a transiently amplifying: ovoid cell body, no dendrite, GFP+/DCX-; Type 2b, ovoid cell body, GFP+/DCX+; immature neurons: round cell body, dendrite extending into molecular layer, GFP-/DCX+. Scanning and optical sectioning in the Z plane was performed as described previously (Donovan et al., 2006) and fluorescently labeled confocal images presented here were taken from one optical slice and imported into Photoshop (Adobe Systems, San Jose, CA) for composition purposes. Adjustments to images were only made via gamma levels.

Statistical analyses

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

Results

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

C57BL/6J mice were given morphine or sham pellets s.c. at 0 and 48 hrs. Two hrs before sacrifice (24, 72, or 96 hrs), BrdU was given to exogenously label proliferating cells (Figure 1A). There was a qualitative decrease in the number of BrdU-IR cells in the hippocampal SGZ (Figure 1Bi, ii). There was no obvious morphological difference in proliferating cells,

as BrdU-IR cells were typically found in the SGZ in clusters of at least two cells with condensed or punctate heterochromatin (Figure 1Biii) (Mandyam et al., 2004). Quantitatively, mice exposed to morphine had fewer BrdU-IR cells compared to sham mice ($F_{5,30}=7.306$, $p=0.0001$). Post hoc analyses between morphine and sham exposed mice were significantly different at each time point (Figure 1C; 24 hr: $p<0.05$, 72 hr: $p<0.01$, 96 hr: $p<0.001$). The number of Ki67-IR cells was also decreased after chronic morphine ($F_{5,30}=7.683$, $p<0.0001$). Interestingly, post hoc analyses revealed that the number of Ki67-IR cells was decreased only at 72 and 96 hrs (Figure 1D; 72 hr: $p<0.001$, 96 hr: $p<0.01$). There was not a quantitative difference in the number of BrdU-IR or Ki67-IR cells per cluster between sham and morphine groups at any time point (Figure 1E–F). These data suggest that the pattern of decreased proliferation is similar with assessment of an exogenous or endogenous marker with the exception of early exposure (24 hrs) to morphine.

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

To examine for possible alterations in S phase versus total cycling cells, the number of Ki67-IR cells that were BrdU– or BrdU+ was determined after 24 or 96 hrs of morphine exposure. Ki67+/BrdU– cells represent cycling cells in non-S phases of the cell cycle (Figure 2A, dashed red line only; Figure 2B, open arrows) whereas Ki67+/BrdU+ cells represent cycling cells in S phase (Figure 2A, dashed red and green lines; Figure 2B, closed arrows). After 24 hrs of morphine exposure, there was only a significant main effect for Ki67 cell type ($F_{1,6}=97.04$; $p<0.0001$; Figure 2C). After 96 hrs of morphine exposure, there was a significant main effect for Ki67 cell type ($F_{1,11}=84.07$; $p<0.0001$) and a significant main effect for drug treatment ($F_{1,11}=33.84$; $p<0.0001$) with no significant interaction between Ki67 cell type and drug treatment. Bonferroni post hoc tests revealed significant differences between sham and morphine groups within BrdU– ($p<0.001$) and BrdU+ ($p<0.01$) cell type (Figure 2D). This suggests that morphine does not differentially affect S phase versus total cycling cells, as there is no interaction between drug treatment and Ki67 cell type at 24 and 96 hrs with raw numbers of cells.

However, the effect of morphine on cells in S phase relative to non-S phase cycling cells can also be assessed by calculating the percentage of Ki67-IR cells that were BrdU-IR. There was a significant decrease in the percentage of Ki67-IR cells that were BrdU-IR after 24 hrs ($t_6=4.39$, $p=0.0046$), but no change after 96 hrs (Figure 2E, F). Thus after 24 hrs of morphine exposure, the number of S phase cells was disproportionately decreased relative to SGZ cells in the other cell cycle phases. This suggests that cells cycling in S phase are initially more sensitive to morphine's inhibitory effects than SGZ cells cycling in other phases of the cell cycle.

Chronic morphine transiently increases cell death in the SGZ

The results in Figure 2E suggest that morphine may induce cell death after 24 hrs of exposure to morphine. Therefore, the numbers of AC3-IR SGZ cells were quantified after 24 and 96 hrs of morphine exposure. As previously reported, AC3-IR cells were easily identified by their discrete cell morphology (Figure 3, inset) (Harburg et al., 2007). Mice exposed to morphine had more AC3-IR cells compared to control mice ($F_{3,20}=3.473$, $p=0.035$). Post-hoc analyses between sham and morphine exposed mice revealed that numbers of AC3-IR cells were increased by almost 100% after 24 hrs, but returned to baseline values by 96 hrs (Figure 3A), augmenting previous results (Eisch et al., 2000). This suggests that cell death is transiently increased after early exposure to morphine, and therefore may contribute to the morphine-induced decrease in S phase cells seen at 24 hrs (Figures 1C, 2E).

Chronic morphine alters maturation of progenitor cells

Thus far the present results extend previous observations of morphine-induced decrease in progenitor cell proliferation (Fischer et al., 2008) by revealing a disproportionate inhibition of S phase cells relative to other phases. However, the effect of morphine on progenitor cell maturation has not been examined; such analysis has previously been useful in identifying the stage of neurogenesis susceptible to neurogenic stimuli (Encinas et al., 2006; Mandyam et al., 2008b; Wang et al., 2008). As a first step, the number of DCX-IR SGZ cells was quantified after 96 hrs of morphine exposure. The gross morphology and number of DCX-IR cells between sham and morphine exposed mice was not significantly different, suggesting that the total population of DCX-IR cells was not decreased by morphine (96 hrs: sham = 1112.75 ± 70.4 ; morphine = 1236.67 ± 41.3). DCX is expressed by a heterogeneous population of progenitor and maturing cells (Dominguez-Escriba et al., 2006; Plumpe et al., 2006) and therefore insensitivity of this total population to morphine is not surprising. However, knowing the number of DCX-IR cells remained unchanged after morphine made the subsequent analysis on stages of maturation more straightforward to interpret (Kempermann et al., 2004).

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

In Experiment 2, progenitor cells were first labeled with BrdU and then matured in the presence of morphine, which allowed for examination of the maturational stage of BrdU-IR cells via triple IHC for BrdU, DCX and GFP (Donovan et al., 2008; Kempermann et al., 2003; Kempermann et al., 2004). As previously shown, GFP protein was robustly expressed in the SGZ (Figure 5Di). The majority of BrdU-IR cells were either Type 2a transiently amplifying (Figure 5Dii, iii, open arrows; ovoid cell body, no dendrite, GFP+/DCX-) or Type 2b (Figure 5Div, closed arrow; ovoid cell body, GFP+/DCX+). A smaller percentage of BrdU-IR cells were immature neurons (Figure 5Diii, closed arrow; round cell body, dendrite extending into molecular layer, GFP-/DCX+). As expected, very few BrdU-IR cells were Type 1, stem-like cells (Figure 5Div, unlabeled with cell body out of focal plane, open arrow; triangular cell body with process extending into molecular layer, GFP+/DCX-) (Donovan et al., 2008).

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

Discussion

The current results show that chronic morphine alters specific aspects of progenitor cell proliferation and maturation in the adult mouse SGZ. S phase cycling cells were particularly vulnerable after shorter exposure to morphine, but the decrease in proliferation extended to

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

Chronic morphine inhibits cycling progenitor cells

Several administration paradigms have revealed that chronic morphine decreases proliferation as measured by fewer BrdU-IR cells (Eisch et al., 2000; Kahn et al., 2005). Recently, a time course of proliferation after morphine pellet administration determined that the number of BrdU-IR cells was decreased after 24, 72 and 96 hrs of morphine exposure (Fischer et al., 2008). Here, these previous findings were extended by determining the time course of morphine's inhibition on the number of cells immunoreactive for the endogenous cell cycle marker Ki67. In many cases, BrdU (given 2 hr prior to sacrifice) and Ki67 are interchangeable as proliferation markers (Komitova et al., 2005; Lagace et al., 2007a; Wojtowicz and Kee, 2006). In line with this idea, the present results reveal that after longer exposure to morphine (72 and 96 hrs) BrdU and Ki67 labeled cells were similarly decreased, emphasizing their interchangeable use as general proliferation markers. However after a shorter exposure to morphine (24 hrs), only BrdU labeled cells were decreased. Thus these data presented here emphasize that BrdU and Ki67 label distinct aspects of the cell cycle (S phase vs. entire cell cycle), and therefore should not always be used interchangeably to measure proliferation.

The results shown in Figure 2 take advantage of the fact that BrdU given 2 hrs before sacrifice results in S phase cycling cells labeled with Ki67/BrdU and non-S phase cycling cells labeled only with Ki67 (Mandyam et al., 2007). This combinatorial analysis of two cell cycle markers revealed a detailed picture of how the progenitor cell cycle is influenced by morphine over time: S phase cycling cells are preferentially decreased in number after 24 hrs relative to non-S phase cycling cells, but all aspects of the cycle are influenced after 96 hrs. These data suggest that initially morphine interferes with DNA synthesis, perhaps by targeting cell cycle proteins essential for entrance into or movement through S phase. Previous work has shown that after 96 hrs, morphine induces premature mitosis of SGZ cells (Mandyam et al., 2004). Furthermore, non-neuronal cells exposed to morphine *in vitro* have increased levels of phosphorylated p53 and p21 with a concurrent decrease in proliferation (Tegeder et al., 2003), and loss of the cell cycle related proteins p27Kip1 or p21cip1 can increase proliferation in the SVZ and hippocampal dentate gyrus (Doetsch et al., 2002; Pechnick et al., 2008). Examination of whether these and other S phase and cell cycle proteins are altered by morphine *in vivo* will be critical to understanding how morphine acts to alter adult hippocampal neurogenesis.

Chronic morphine increases progenitor cell death

A notable finding from the present work is that morphine exposure produced a robust increase in cell death in the SGZ after 24 hrs, but not 96 hrs, extending previous results on levels of cell death proteins in total hippocampus (Emeterio et al., 2006). Thus it will be important to further assess the relationship between the transient but robust increase in cell death reported here on S phase cycling cells at 24 hrs. For example, perhaps the decrease in S phase cells after 24 hrs is due to cell death, but the decrease in total number of cycling cells after 96 hrs reflects a new homeostatic number of proliferating cells. Phenotypic analysis of AC3-IR cells would be necessary to further explore this hypothesis. As AC3-IR

cells are difficult to co-label with BrdU or non-death associated proteins (Bauer and Patterson, 2005; Cooper-Kuhn and Kuhn, 2002), testing of this hypothesis awaits additional technical advances in detection of cell death. However, since cell cycle and cell death are closely linked (Jaquinta and Lees, 2007), elaboration of the relationship between morphine-induced cell death and inhibition of proliferation is likely to be fruitful in explaining how morphine alters adult hippocampal neurogenesis.

Chronic morphine inhibits maturation of progenitor cells

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

Receptor expression of progenitor cells

Why are the percentages of BrdU-IR cells that are in the Type 2b and immature neuron stages preferentially affected by morphine? One possibility is that these subpopulations of progenitor cells express specific receptors that enable them to differentially respond to morphine directly or, more likely, to alterations in the neurogenic microenvironment (Duan et al., 2008; Encinas et al., 2006; Plumpe et al., 2006; Wang et al., 2008). In regards to responding to morphine directly, it remains unclear when the mu opioid receptor is expressed on progenitor cells (Drake et al., 2007; Eisch and Harburg, 2006), but several lines of indirect evidence suggest that morphine and opioid receptors are important in neurogenesis (Drake et al., 2007; Eisch et al., 2000; Harburg et al., 2007; Hauser et al., 2000; Koehl et al., 2008). In light of the present data that morphine decreases the percent of BrdU-IR cells that reach an immature stage, it is interesting that mice that lack the mu opioid receptor from birth have increased survival of dentate gyrus granule cells (Harburg et al., 2007). However, more work is needed to clarify if morphine directly inhibits particular progenitor cell types. In regards to responding to alterations in the neurogenic microenvironment, it is known that cells in discrete stages of neurogenesis are more likely to express TrkB, a key receptor for the neurotrophin BDNF, NMDA, or subtypes of glucocorticoid receptors, all of which have been implicated in regulating neurogenesis (Donovan et al., 2008; Garcia et al., 2004; Nacher et al., 2007). Perhaps morphine inhibits actively dividing progenitor cells preferentially due to altered expression of these receptors, such that the progenitor cells cannot compensate or respond to morphine-induced changes in

the hippocampal microenvironment. Identification of vulnerable subpopulations after discrete stimuli (Encinas et al., 2006; Mandyam et al., 2008b; Wang et al., 2008) and their receptor expression patterns (Chumley et al., 2007; Suslov et al., 2000) will notably enhance our ability to independently manipulate adult neurogenesis in the future.

Functional relevance of morphine-induced inhibition of maturation

While the functional implications of the present data are merely speculative, it is important to consider how they might influence our understanding of addiction. It is notable that adult hippocampal neurogenesis is functionally implicated in spatial memory, for example, newborn neurons are incorporated into hippocampal circuits and activated during spatial memory tasks (Epp et al., 2007; Kee et al., 2007; Winocur et al., 2006). Interestingly, chronic morphine alters long-term spatial memories in rodent (Miladi Gorji et al., 2008; Spain and Newsom, 1991) and heroin addicts have altered levels of PSA-NCAM, which besides being a marker of immature neurons, appears important for long term memory formation (Lopez-Fernandez et al., 2007). It is also intriguing that blockade of NR2b receptor expression, one of the subtypes present on progenitor cells, ablates morphine-induced conditioned place preference (Suzuki et al., 2000). Thus, future studies could take advantage of the fact that specific stages of drug taking represent very specific forms of learning, and use both conditioned place preference and drug self-administration to more closely examine the link between hippocampal neurogenesis, learning, and addiction.

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Abbreviations

AC3	activated caspase-3
BrdU	bromodeoxyuridine
DCX	doublecortin
IHC	immunohistochemistry
i.p.	intraperitoneal
PCNA	proliferating cell nuclear antigen
PSA-NCAM	polysialic-neural cell adhesion molecule
s.c.	subcutaneous
SGZ	subgranular zone

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Figure 1. Chronic morphine decreases SGZ proliferation

For Experiment 1, morphine or sham pellets were implanted subcutaneously at 0 and 48 hrs. Two hrs prior to sacrifice (24, 72 and 96 hrs) mice were given an injection of 150 mg/kg BrdU. **(B)** Qualitative IHC shows decreased numbers of BrdU-IR cells in the SGZ of **(ii)** morphine exposed mice compared to **(i)** sham exposed mice. Scale bar = 50 μ m. **(iii)** BrdU-IR cells, from a representative sham exposed mouse, divide in clusters and have dark or punctate heterochromatin. Scale bar = 10 μ m. **(C)** The number of BrdU-IR cells expressed as percent of sham is decreased at all time points. **(D)** The number of Ki67-IR cells expressed as percent of sham is decreased at 72 and 96 hr. **(E-F)** The numbers of BrdU-IR

or Ki67-IR cells in a cluster are not changed after 24, 72, or 96 hrs of exposure to morphine. At all time points sham: n=6, morphine: n=6. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Figure 2. Chronic morphine alters cell cycle dynamics

(A) Schematic depicting putative expression of Ki67 and BrdU within the cell cycle. Cycling cells in non-S phases do not co-label with Ki67 and BrdU (Ki67+/BrdU-; dashed red line only), whereas S phase cycling cells co-label with Ki67 and BrdU (Ki67+/BrdU+; dashed red and green lines). (B) Confocal image of cells that are Ki67+/BrdU- (open arrows) and Ki67+/BrdU+ (closed arrows). Orthogonal view shows co-localization of Ki67 (red) and BrdU (green). Scale bar=10 μm. (C–D) Quantification of the number of Ki67-IR cells that are BrdU- or BrdU+ reveals significant main effect for Ki67 cell type at 24 hrs. In contrast there was a significant main effect for Ki67 cell type and drug treatment at 96 hrs

with no significant interaction. Bonferroni post hoc tests revealed significant differences between sham and morphine groups within Ki67 (BrdU⁻ and BrdU⁺) cell type. **(E)** The percentage of Ki67-IR cells that were BrdU-IR was decreased after 24 hrs exposure to morphine **(F)** but not after 96 hrs. **(C, E 24 hr)** sham: n=4, morphine: n=4. **(D, E 96 hr)** sham: n=6, morphine: n=7. **p<0.01, ***p<0.001.

Figure 3. Chronic morphine transiently increases cell death

The number of AC3-IR cells, expressed as percent of sham, was increased after 24 hrs but not after 96 hrs morphine exposure. (**Inset**) AC3-IR cells were identified by their distinct cell morphology. For both time points sham: n=6, morphine: n=6. Scale bar = 10 μm.

*p<0.05.

Figure 4. Chronic morphine alters specific stages of progenitor cell maturation

(A) A single 150 mg/kg BrdU injection was given to nestin-GFP mice 24 hrs before sham surgery or implantation of morphine pellet at 0 and 48. Mice were killed after 96 hrs of exposure. (B) The number of Ki67-IR proliferating cells was decreased as previously shown (Figure 1D). (C) The number of BrdU-IR cells was not significantly decreased. (D) Phenotypic analysis of BrdU-IR cells was assessed by cell morphology and co-localization with GFP and DCX with confocal microscopy. (i) GFP was robustly expressed in the SGZ. Scale bar = 50 μ m. (ii–iv) BrdU-IR cells were classified as Type 1 (iv, open arrow; GFP+/DCX–, triangular cell body, dendrite extending into molecular layer), Type 2a (ii, iii, open arrows; GFP+/DCX–, ovoid cell body, no dendrites), Type 2b (iv, closed arrow; GFP+/DCX+, ovoid cell body, no dendrites) or immature neuron (iii, closed arrow; GFP–/DCX+, mature morphology). Scale bar= 10 μ m in (iii). (E) The percent of BrdU-IR cells that were Type 2b increased whereas those that were immature neurons decreased. Schematic diagram

depicts cell morphology and marker expression of cell type affected by morphine. Green dots represent BrdU (as BrdU-IR cells were phenotyped). **(B,C)** sham: n=8, morphine: n=7. **(E)** sham: n=7, morphine: n=6. *p 0.05, **p<0.01.