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THE OPIATE ANALGESIC BUPRENORPHINE DECREASES PROLIFERATION OF ADULT HIPPOCAMPAL NEUROBLASTS AND INCREASES SURVIVAL OF THEIR PROGENY

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Abstract—Although opiate drugs of abuse have been shown to decrease adult hippocampal neurogenesis, the impact of opiate analgesics has not been tested. North American regulatory boards governing the ethical treatment of experimental animals require the administration of analgesics, such as buprenorphine, following minor surgical interventions. Here, we show that two commonly used post-operative buprenorphine dosing regimes significantly inhibit the proliferation of doublecortin-positive neuroblasts but not other hippocampal stem and progenitor cell populations in adult mice. Buprenorphine, administered in schedules of three 0.05 mg/kg subcutaneous injections over a single day or seven 0.05 mg/kg injections over a 3-day period decreased the number of actively proliferating 5-iodo-2′-deoxyuridine-labeled doublecortin-positive cells for up to 6 days after opiate withdrawal. The minimal (three injection), but not standard (seven injection), analgesic paradigm also reduced basal indices of hippocampal progenitor cell apoptosis and enhanced survival of newly born cells for up to 28 days. Taken together, these data provide the first evidence that the routine administration of opiate analgesics has transient but long-lasting effects on neurogenesis and further emphasize that analgesic dosage and schedule should be reported and considered when interpreting the magnitude of neural stem and progenitor cell activation in response to *in vivo* intervention.

Key words: buprenorphine, opiate, neurogenesis, analgesia, hippocampus, neuroblasts.

Adult neurogenesis is primarily restricted to two regions of the adult mammalian brain: the subgranular zone (SGZ) of the dentate gyrus of the hippocampus and the subventricular zone of the lateral ventricles (Gage, 2000). The production of new neurons in these regions occurs through the stepwise expansion, specification, migration, and terminal differentiation of increasingly committed neural stem and progenitor cell populations. In the SGZ, resident neural stem cells (NSCs) identified, in part, by their expression of both nestin and glial fibrillary acidic protein (GFAP), can self-renew or give rise to nestin-positive transient amplifying progenitor cells (TAPs). TAPs, in turn, can generate doublecortin-positive (DCX) neuroblasts capable of terminal neuronal differentiation (Doetsch, 2003; Garcia et al., 2004; Kempermann et al., 2004; Bérubé et al., 2005; Ables et al., 2010). Their post-mitotic neuronal progeny can either mature and integrate into adult hippocampal circuitry or be deleted by apoptosis (Deng et al., 2010). Each step of this process is discretely regulated and is postulated to affect hippocampal function in the uninjured brain (Zhao et al., 2008).

Here, we asked whether the routine prescription of opiate analgesics alters hippocampal neurogenesis. Although chronic administration of opiate drugs of abuse (i.e., morphine and heroin) is known to inhibit proliferation of neural progenitor cell populations in the SGZ (Eisch et al., 2000; Kahn et al., 2005; Arguello, 2008, 2009), the effects of opiate analgesics have yet to be assessed. We tested buprenorphine (Chiron Compounding Pharmacy Inc., Guelph, ON, Canada), a μ-opioid receptor (MOR) and opioid-like receptor-1 (ORL-1) agonist with mixed κ-opioid receptor (KOR) agonist and antagonist activities. Clinically buprenorphine is routinely prescribed for pain management and is increasingly administered "off-label" to treat addiction and mood disorders (Davis, 2007; Likar et al., 2007; Howland, 2010). Buprenorphine is also recommended by both American and Canadian regulatory boards as an analgesic following painful experimental interventions such as the minor surgeries commonly used to manipulate neurogenesis in laboratory animals (Olferts et al., 1993; Gebhart et al., 2009; Garber et al., 2011).

Certainly, the use of appropriate analgesia is one of the predominant refinements associated with the 3R principles of replacement, reduction, and refinement practiced to ensure the ethical use of experimental animals (Russell et al., 1959; Smythe, 1978). To obtain consistent plasma levels, reduce handling stress and target drug delivery, test compounds are frequently administered via osmotic minipumps. Basic subcutaneous (s.c.) implantation of these pumps involves a minor skin incision. North American regulatory boards mandate the administration of analgesia as a standard post-operative procedure in such cases (Olferts et al., 1993; Gebhart et al., 2009; Garber et al., 2011). Although best practice protocols vary between institutions, 0.05 mg/kg, s.c. buprenorphine administered at 3–12-h intervals is considered the "gold-standard" gener-

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Abbreviations: ANOVA, analysis of variance; BrdU, 5-bromo-2'-deoxyuridine; CldU, 5-chloro-2'-deoxyuridine; DCX, doublecortin; GCL, granule cell layer; GFAP, glial fibrillary acidic protein; HIL, hilus; HPA, hypothalamic-pituitary-adrenal; IdU, 5-iodo-2'-deoxyuridine; i.p., intraperitoneal; KOR, κ-opioid receptor; MOL, molecular layer of the dentate gyrus; MOR, μ-opioid receptor; NSCs, neural stem cells; OPCs, oligodendrocyte progenitor cells; ORL-1, opioid-like receptor-1; s.c., subcutaneous; SGZ, subgranular zone; TAPS, transient amplifying cells; TUNEL, terminal deoxynucleotidyl nick-end labeling.

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ating long-lasting analgesia with minimal side effects (Dobromylskyi et al., 2000; Martin et al., 2001; Hawk et al., 2005; Curtin et al., 2009). Injections are commonly performed as a standard post-operative procedure by veterinary staff often without the full awareness of the primary research team. Consequently, details of exposure, dosage, specific schedule, and type of opiate analgesic are rarely reported (Stokes et al., 2009). Such variations in best practice protocols are also characteristic of clinical practice (Likar et al., 2007).

Here, we assessed the effects of two common dosing schedules of buprenorphine on adult hippocampal progenitor cell proliferation, specification, and survival. Our data indicate that buprenorphine inhibits the proliferation of DCX-positive neuroblasts, regardless of the schedule of administration, and enhances survival of progeny born at the time of analgesic treatment. Taken together, these data provide the first evidence that the routine administration of opiate analgesics has transient but long-lasting effects on neurogenesis and further emphasize that analgesic dosage and schedule should be both reported and considered when interpreting the magnitude of neural stem and progenitor cell activation in response to pharmacological or molecular intervention *in vivo*.

EXPERIMENTAL PROCEDURES

Animals

Male C57BL/6×FVB/N×129/SV hybrid mice were 8 weeks of age at time of treatment. All experimental animals were compared with age-matched drug-naïve controls. With upward of 50% of mice used in research being genetically manipulated, this strain of mice was selected to assess impact of buprenorphine on one of the common genetic backgrounds used in neurogenic studies (Yoshiki and Moriwaki, 2006; Blunt, 2009). Specifically, the C57BL/6×FVB/N×129/SV strain is representative of the hybrid crosses of nestin-specific cre-recombinase FVB/N mice mated to floxed lines generated on a C57BL/6×129/SV background (Bérubé et al., 2005; Dubois et al., 2006; Ackermann et al., 2011; Schultz et al., 2011). Animals were kept on a 12-h light-dark cycle (light phase 600 h—1800 h) with access to food and water *ad libitum*. Five to ten mice were used per experimental group (Fig. 1A–C). All protocols were approved by the Animal Care Committee of the University of Ottawa according to guidelines set forth by the Canadian Council on Animal Care. Modifications to institutional standard operating procedures for the purposes of testing the effect of the analgesic schedule are indicated below and were approved by the University of Ottawa's Animal Care Committee. All experiments were designed to minimize the number of animals used and prevent their suffering.

Opiate analgesia

Buprenorphine (Chiron Compounding Pharmacy Inc) was prepared in sterile water to a final concentration of 0.03 mg/ml. Delivery schedules, defined as either as "Standard," representing the routine practice of veterinary staff at the University of Ottawa, or "Minimal," representing a reduced number of opiate analgesic injections that still provided effective pain management according to the Canadian Council for Animal Care guidelines, are depicted in Fig. 1A–C. Under the standard protocol, mice received three injections (0.05 mg/kg, s.c.) of analgesic every 6 h on the day of surgery and two injections (0.05 mg/kg, s.c.) separated by 8 h for two subsequent days for a total of seven injections over a 72-h period. Under the minimal protocol, mice received three injections (0.05 mg/kg, s.c.) separated by 6 h over a 24-h period. Delivery schedules were designed to model variations in pain management strategies observed across institutions (Dobromylskyi et al., 2000; Martin et al., 2001; Hawk et al., 2005; Curtin et al., 2009). All subjects were compared with age-matched drug-naïve control mice.

Surgery

To assess the effects of opiate analgesia with and without the need for pain management, uninjured mice were compared with mice that underwent minor surgery. The surgical group was anesthetized with an inhalant mixture of oxygen and isoflurane. Alzet osmotic minipumps (model 2001 (7-day implantation) or model 2004 (28-day implantation), DURECT Corporation, Cupertino, CA, USA) pre-filled with 20% (2-hydroxypropyl)-β-cyclodextrin (catalog #H107, Sigma, Saint Louis, MO, USA) in sterile normal saline (0.9% NaCl) were subcutaneously implanted on the back of each mouse. Both standard and minimal analgesic schedules were employed as pain management strategies (Fig. 1A, C).

In vivo labeling of proliferating cells

Mice received five injections over 3 days of 5-chloro-2′-deoxyuridine (CldU; 42.5 mg/kg) (prepared to 17 mg/ml in 0.625 M Tris base in sterile water; Sigma, catalog #C6891, Saint Louis, MO, USA) and/or 5-iodo-2′-deoxyuridine (IdU; 57.5 mg/kg) (prepared to 23 mg/ml in 0.895 M Tris base in sterile water; MP Biomedicals, catalog #100357, Solon, OH, USA) intraperitoneally (i.p.). Concentrations were as described in Vega and Peterson (2005) with the exception that Tris base was used to enhance solubility in place of sterile saline and 5 N NaOH. The final pH of the solutions was pH 8.5 as recommended by Vega and Peterson (Vega and Peterson, 2005). Delivery schedules for CldU and IdU (Fig. 1A–C) for each experiment were optimized to label both rapidly dividing TAPS and slowly dividing NSC population (Nowakowski et al., 1989; Taupin, 2007).

Tissue preparation

Mice were euthanized at the time points outlined in Fig. 1A–C by injection with euthanyl (Bimeda-MTC Animal Health Ins., Cambridge, ON, Canada) prepared in sterile water to a final concentration of 65 mg/ml and transcardial perfusion with 10 mM phosphate buffered saline (PBS; 10 mM sodium phosphate and 154 mM NaCl) followed by 3.7% paraformaldehyde in 10 mM PBS. Brains were removed, post-fixed for 24 h, and cryoprotected in 20% sucrose. Serial 10-μm coronal cryosections were collected using a Leica CM1900 cryostat (Leica Microsystems, Wetzlar, Germany), throughout the dorsal domain of hippocampus from bregma −1.0 to −3.4 using the coordinates of Hof et al. (Hof et al., 2001).

Immunofluorescence

Sections were incubated in 2 N HCl for 30 min at 37 °C and neutralized in 0.1 M borate buffer (pH 8.5). IdU and CldU incorporation was detected by immunofluorescence using mouse anti-5-bromo-2′-deoxyuridine (BrdU) clone 44 (1:500; Becton Dickinson Immunocytochemistry Systems, catalog #347580, San Jose, CA, USA) and rat anti-BrdU-FITC (1:100, AbD Serotec, catalog #OBT0030F, Raleigh, NC) respectively. We used double and triple immunofluorescence for antigenic lineage markers to determine (a) the identity of actively proliferating (IdU-labeled) cells and (b) fate of cells actively proliferating at the time of opiate analgesic exposure (CldU-labeled). The primary antibodies employed were rabbit anti-GFAP (1:100; Sigma, catalog #G9269, Saint Louis, MO, USA), rabbit anti-nestin (1:1000; Covance, catalog #PRB-

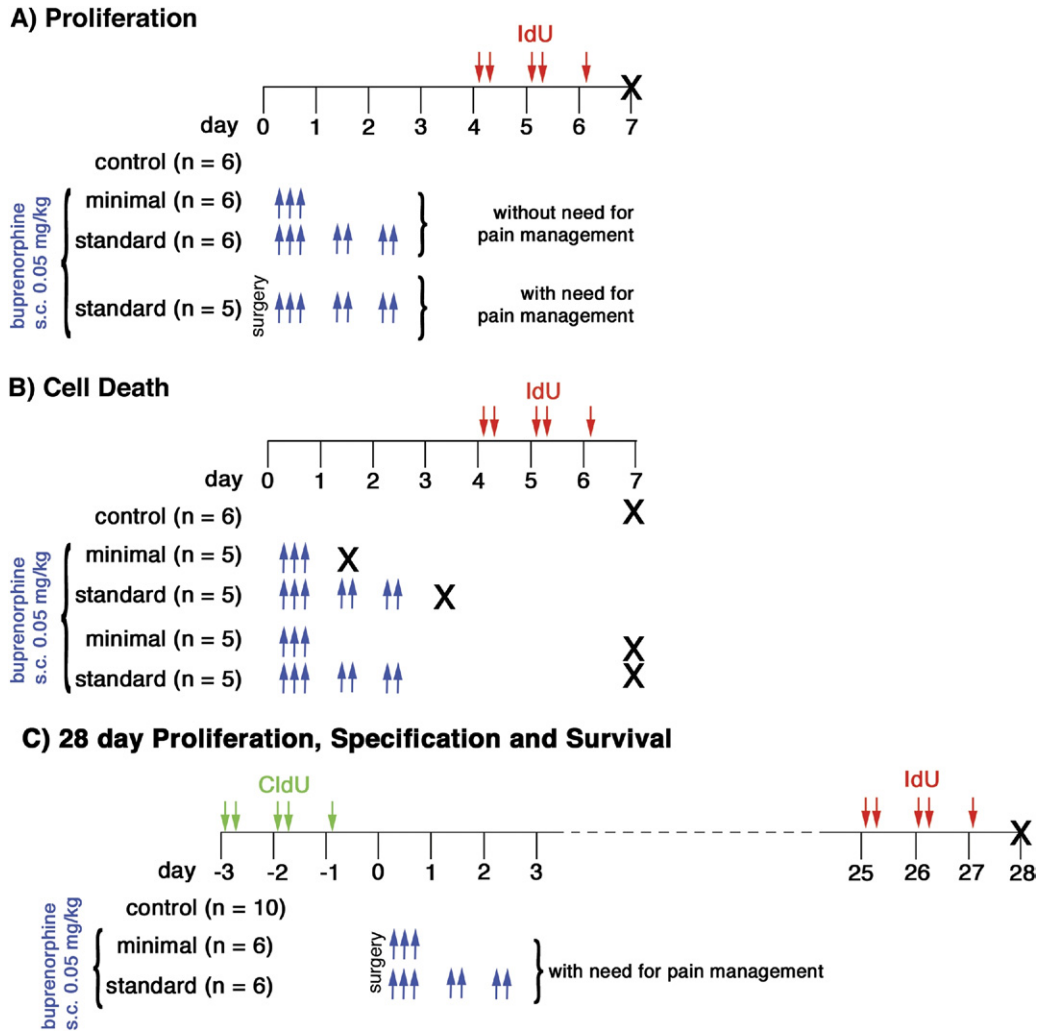

Fig. 1. Treatment paradigms. Buprenorphine injections (0.05 mg/kg, s.c.) are indicated by blue arrows, IdU injections (57.5 mg/kg, i.p.) are indicated by red arrows, and CldU injections (42.5 mg/kg, i.p.) are indicated by green arrows. Mice subjected to surgical intervention were implanted with osmotic minipumps subcutaneously on day 0, delivering 20% (2-hydroxypropyl)-β-cyclodextrin, as indicated by "surgery." Time of sacrifice is indicated by a black X. (A) Assessment of cell proliferation following minimal or standard schedules of analgesia with or without the need for pain management. Proliferating cells were labeled on days 4, 5 and 6 after the initiation of opiate analgesia. Animals were sacrificed 1 wk after the onset of analgesic treatment. (B) Apoptotic-like cell death was assessed 24 h, 72 h, and 6 d after the final buprenorphine injection for both the minimal and standard analgesia regimen. (C) To establish whether acute exposure to buprenorphine under either the minimal or standard schedule chronically alters proliferative capacity, IdU was administered on days 25, 26, and 27 following surgery, and IdU-positive proliferating cell number was assessed 28 d after the onset of opiate analgesia. To assess specification and survival of stem and progenitor cells following surgery and buprenorphine treatment, proliferating cells were labeled with CldU over the 3 d before surgery. Survival and fate specification were assessed 28 d later.

315C, Emeryville, CA, USA), guinea pig anti-DCX (1:400; Millipore, catalog #AB5910, Temecula, CA, USA), rabbit anti-NG2 (1:100; Millipore, catalog #AB5320), and Alexa488-conjugated and Alexa488-unconjugated mouse anti-NeuN (1:100; Millipore, catalog #MAB377X and MAB377, respectively). Secondary antibodies were FITC- or Cy3-conjugated anti-mouse IgG (1:100, 1:800; Jackson ImmunoResearch, catalog #715-095-150 and 715-165-150, respectively), Cy3-conjugated anti-rabbit IgG (1:600; Jackson ImmunoResearch, catalog #711-166-152), and Cy3-conjugated anti-guinea pig IgG (1:800; Jackson ImmunoResearch, catalog #706-165-147). Sections were incubated with primary antibodies overnight at 4 °C, washed with 10 mM PBS, incubated with secondary antibodies for 1 h at room temperature, and washed with PBS before being coverslipped in ProLong Gold antifade reagent (Invitrogen, catalog #P-36930, Eugene, OR, USA). All antibodies were diluted in antibody buffer (3% bovine serum albumin, 0.3% Triton X-100 in 10 mM PBS).

Cell death

Dying cells were labeled by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL, catalog #11684795910, Montreal, Canada) as we have previously described (Melanson-Drapeau et al., 2003).

Microscopic analysis and quantification

Immunofluorescence was assessed usinga DMXRA2 epifluorescent microscope (Leica Microsystems). Additional confocal imaging was performed on a Leica TCS SP5 (Leica Microsystems). Quantification of the total number of IdU- and CldU-labeled cells

was as described (Thallmair et al., 2006; Guo et al., 2010) with the following minor modification: counts were restricted to the dorsal domain of the hippocampus as defined by Fanselow and Dong, 2010. This focus facilitated a clear anatomical distinction between the dorsal zone of the hippocampus and the intermediate/ventral zones that would otherwise require the use of marker genes to distinguish boundaries in more posterior sections (Dong et al., 2009). An average of 10 serially sampled sections along the entire anterior–posterior axis of the dorsal hippocampus, bregma-matched between −1.0 and −3.4, were analyzed per animal. Five to ten animals were counted per condition (Fig. 1). The area of (a) the SGZ, defined as the region bordering the granule cell layer and the hilus (three cell widths into each), (b) the hilus, and (c) the entire granule cell layer (GCL) was established for each section using the Advanced Measurement Module of OpenLab 5.0.2 (PerkinElmer, Waltham, USA) software. The number of labeled cells was assessed in both the left and right hippocampi by two independent observers blinded as to the identity of the sections. These values were averaged to yield a single number per region, section, and animal. Because labeled cells were not evenly distributed and are relatively rare, we did not use counting frames but rather counted all labeled cells within defined substructures of the hippocampus in accordance with previous studies (Thallmair et al., 2006; Guo et al., 2010). In proliferation studies, IdU-labeled cells were counted in the SGZ and hilus using a 10× objective. In survival studies, CldU-labeled cells were counted throughout both the GCL and hilus as new cells, born in the SGZ, will migrate into the granule cell layer of the DG. The hilus was included in all analyses to include $NG2^+$ gliogenic progenitor cells as well as to acknowledge the possibility of ectopic localization of SGZ progenitor progeny following treatment. The total number of labeled cells in a given region per animal was calculated as the sum of the cell counts in all sampled sections multiplied by the total number of serial sections collected between bregma −1.0 and −3.4 and divided by the number of sections sampled (Thallmair et al., 2006; Guo et al., 2010). Means and standard errors of the mean (SEM) are reported for each experimental group.

Identification of IdU- or CldU-positive cells, co-labeled with antigenic lineage markers, were completed using a 40× objective for epifluorescence and confirmed by confocal laser scanning microscopy using a 63× objective. Total IdU- or CldU-positive nuclei in each area of interest were counted and then assessed for the presence of the lineage marker of interest in merged epifluorescent images. To provide further confirmation, confocal images were captured with Leica LAS AF software (version 2.41) using an HCX PL APO CS 63×1.4 oil objective with a pinhole size of 1 Airy unit (or equal optical slices for multiple fluorophores) through *z*-stacks. We considered antigenic lineage markers co-localized with IdU- or CldU-labeled nuclei only if the lineage label extended from top to bottom of the *z*-stack. An average of 350 IdU-positive cells per mouse were analyzed to identify proliferating cells, and 45 CldU-positive cells per mouse were analyzed to establish fate specification. The total number of cells positive for each proliferative phenotype was calculated by multiplying the total number of IdU-positive cells by the proportion positive for the appropriate lineage makers yielding a single value per mouse. Five to ten mice were assessed per condition as indicated (Fig. 1A–C). Data are presented as mean and SEM of all animals counted.

TUNEL counts were completed in the GCL, SGZ, and hilus of both hemispheres using a 10× objective in two serial sections between bregma −1.7 and −2.7 matched between mice (n=4 counts per mouse). Details are as we have previously published (Melanson-Drapeau et al., 2003). Briefly, counts were performed by two independent investigators blinded as to the identity of the sections. The area of the GCL, SGZ, and hilus was established for each hemisphere/section using the Advanced Measurement Module of OpenLab 5.0.2 (PerkinElmer) software. Area measurements allowed us to confirm anatomical bregma matching and to express final data as the number of positive cells per 0.1 mm^2 in the combined areas of interest (GCL, SGZ, and hilus).

Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) followed by post hoc Tukey's test or Bonferroni's test as indicated.

RESULTS

Buprenorphine reduces proliferating cell number in the adult hippocampus

The standard post-operative analgesic protocol following minor surgery (i.e., s.c. implantation of osmotic minipumps) at the University of Ottawa is seven buprenorphine injections (0.05 mg/kg, s.c.) administered over 3 days (Fig. 1A). We found that this schedule, in the absence of any other intervention, reduced the number of actively proliferating IdU-positive cells in the SGZ and hilus of the hippocampal dentate gyrus assessed 1 week after analgesic treatment (Fig. 2A, B; ANOVA, post hoc Tukey's test, ** $P<0.01$ relative to control). The effect of buprenorphine on hippocampal cell proliferation was not dependent on the schedule of opiate administration. Mice that received an abbreviated (minimal) experimental protocol of three injections over the course of a single day (Fig. 1A) also showed a significant reduction in proliferating IdU-positive cell number (Fig. 2A, B; ANOVA, post hoc Tukey's test, * $P<0.05$ relative to control). There was no difference in IdU incorporation in the CA1 region of the hippocampus between any of the treatment groups indicating that IdU bioavailability was equivalent across experimental manipulations.

To test whether buprenorphine inhibits hippocampal cell proliferation in the context in which it is routinely used (i.e., as a pain management strategy), mice were subcutaneously implanted with osmotic minipumps containing 20% (2-hydroxypropyl)-β-cyclodextrin, a common drug delivery vehicle (Loftsson et al., 2005), and treated under the standard schedule of buprenorphine administration (Fig. 1A). The number of IdU-positive cells in the SGZ and hilus of the adult hippocampal dentate gyrus was significantly reduced 7 days following the initiation of treatment both with and without need for pain management compared with drug-naïve uninjured animals (Fig. 3; ANOVA, post hoc Tukey's test, *$P<0.05$ and **$P<0.01$ vs. control). Moreover, proliferating cell number was found to be further suppressed in mice that had undergone minor surgery compared with pain-free animals (Fig. 3; ANOVA, post hoc Tukey's test, ## $P<0.01$ without vs. with the need for pain management). As it is required by the Canadian Council for Animal Care that mice receive opiate analgesia after minor surgery, we could not ethically include a surgical control group that did not receive buprenorphine to assess impact of pain alone on hippocampal cell proliferation. An isoflurane anesthesia alone control group also was not included, as recent reports indicate that isoflurane does not affect the proliferation or survival of adult hippocampal progenitors (Zhu et al., 2010).

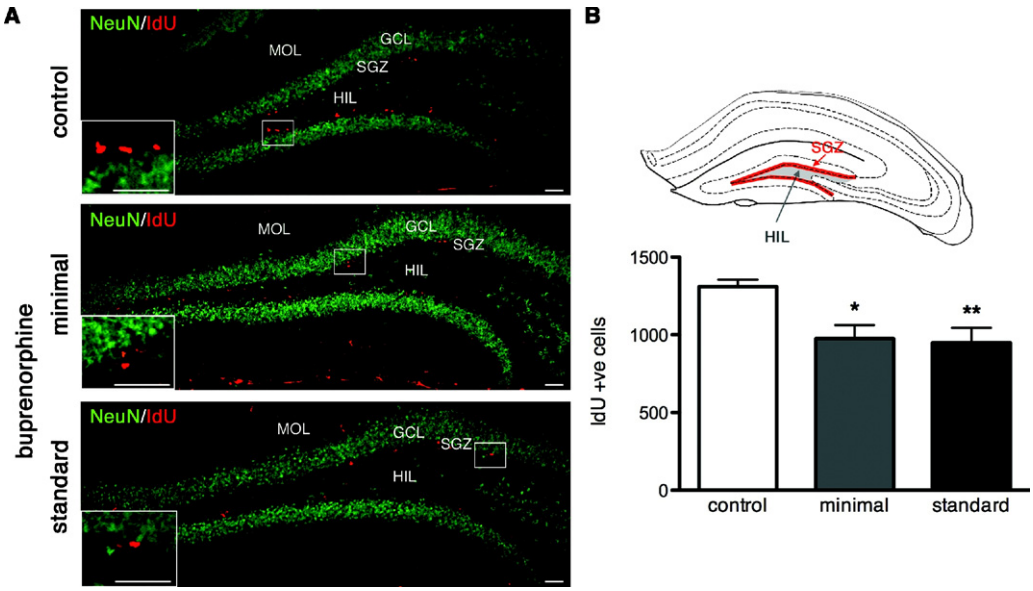

Fig. 2. Proliferating cell number is decreased in the SGZ and hilus of the hippocampal dentate gyrus 7 d after the initiation of either standard or minimal buprenorphine treatment schedules. Abbreviations: GCL, granule cell layer of the dentate gyrus; HIL, hilus; MOL, molecular layer of the dentate gyrus; SGZ, subgranular zone. (A) Double immunofluorescence for NeuN (green) and IdU (red) in the dentate gyrus of mice who did not receive buprenorphine injections (control) or following minimal or standard buprenorphine injection paradigms. Insets depict IdU labeling at higher magnification. Scale bar 50 μm. (B) Schematic of the hippocampal dentate gyrus highlighting the SGZ (red) and hilus (gray), the areas used for quantification of IdU-positive cells. Quantification of IdU immunoreactive cells in the SGZ and hilus of controls (n=6) and of mice receiving a minimal (n=6) or standard (n=6) buprenorphine treatment regimen. Statistics were ANOVA followed by post hoc Tukey's test. * P<0.05, ** P<0.01 relative to control.

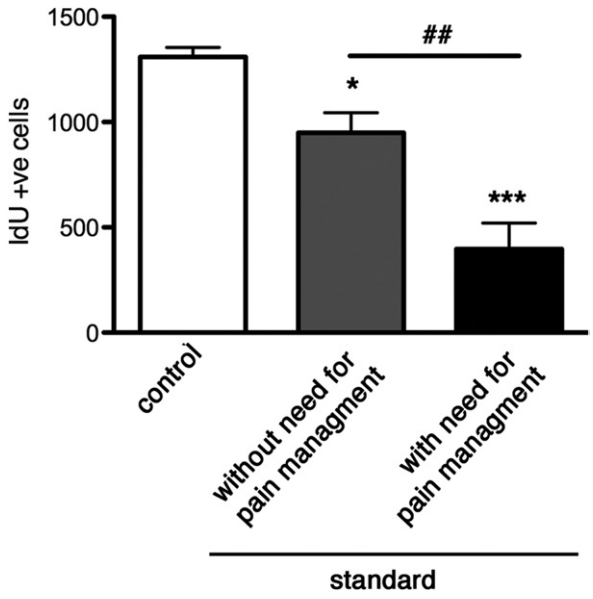

Fig. 3. Proliferating cell number is further suppressed in SGZ and hilus of the hippocampal dentate gyrus 7 d following surgery after application of the standard pain management strategy. Quantification of IdU-positive cells in the SGZ and hilus of drug-naïve littermates (control, n=6) and uninjured mice (n=6, without need for pain management) or mice implanted with osmotic mini-pumps (n=5, with need for pain management) administered the standard schedule of buprenorphine. Proliferating cell number was assessed 1 wk after the initiation of treatment. Statistics were ANOVA, post hoc Tukey's test. * P<0.05, ** P<0.01 relative to control, ## P<0.01 without vs. with the need for pain management.

The buprenorphine-mediated reduction in hippocampal cell proliferation is transient following surgery

To assess the duration of buprenorphine's effects on proliferation, we quantified the number of IdU-positive cells in the SGZ and hilus 28 days following standard or minimal buprenorphine treatment and minor surgery (Fig. 1C). Proliferating cell number was not permanently changed by opiate analgesia (Fig. 4; ANOVA, post hoc Tukey's test, P>0.05). We did, however, detect a difference in prolifer-

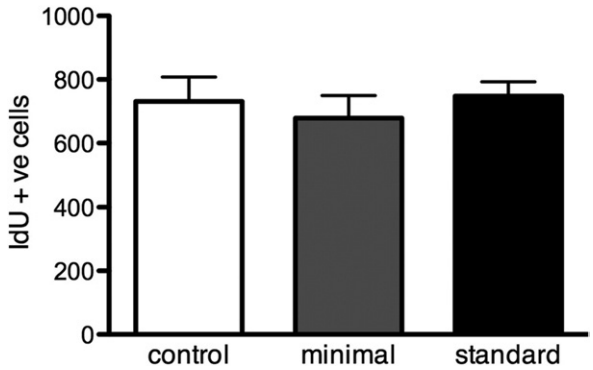

Fig. 4. Proliferating cell number is comparable to control levels 28 d after buprenorphine treatment. Quantification of IdU-positive cells in the SGZ and hilus of controls (n=10) and mice implanted with osmotic mini-pumps delivering 20% (2-hydroxypropyl)-β-cyclodextrin and administered either a minimal (n=6) or standard (n=6) schedule of buprenorphine. The control group represents uninjured drug-naïve littermates. Proliferating cell number was assessed 28 d after the initiation of treatment. Statistics were ANOVA, post hoc Tukey's test. P>0.05.

ating cell number between control animals aged 8 weeks of age (Fig. 3) and 12 weeks of age (Fig. 4) consistent with previously published data (Ben Abdallah et al., 2010).

Buprenorphine inhibits proliferation of DCX-positive neuroblasts

Hippocampal stem and progenitor cells exhibit differential sensitivity to various pharmacological manipulations including opiate drugs of abuse (Kronenberg et al., 2003; Sargeant et al., 2008). To identify the proliferating cell populations affected by buprenorphine, cells were labeled *in vivo* with IdU and identified 24 h after the last IdU injection (Fig. 5A). NSCs in the SGZ were identified by their co-expression of IdU and the glial antigen GFAP along with morphological criteria that included a distinctive triangular soma and single process projecting through the GCL (Fig. 5B–i, open arrow). All GFAP-positive cells with ovoid cell bodies lacking a single projection into the GCL were classified as astrocytes (Fig. 5B–i, filled arrow). Confocal imaging of IdU/Nestin/GFAP triple-labeled sections confirmed these results (Fig. 5B–ii). Proliferating TAPs were IdU-positive/nestin-positive but did not express GFAP (Fig. 5B–iii). Proliferating neuroblasts were identified by their DCX immunoreactivity and IdU labeling (Fig. 5B–iv). Proliferating early oligodendrocyte precursor cells (OPCs) were identified by their NG2 immunoreactivity (Fig. 5B–v) and were almost exclusively found in the hilus. All IdU-positive cells that did not co-label with these lineage markers were classified as "other" and were most likely immature neurons or possibly more committed gliogenic progenitor cells (Fig. 5A). Buprenorphine reduced the number of both IdU-labeled NSCs and neuroblasts, but not IdU-labeled TAPS, OPCs, astrocytes, or other cell types following either minimal or standard treatment protocols; however, only the reduction in IdU-labeled neuroblasts reached statistical significance (Fig. 5C; ANOVA, post hoc Bonferroni's test, $*P<0.05$, $**P<0.01$).

Buprenorphine enhances cell survival in adult hippocampus

The transient reduction in DCX-positive neuroblast cell proliferation following buprenorphine treatment could be due to three possibilities: a decrease in cell proliferation, as has been observed following chronic exposure to opiate drugs of abuse (Eisch et al., 2000), an increase in the apoptotic index of actively dividing cells, as has been observed in NG108-15 neuroblastoma/glioma cells treated *in vitro* with buprenorphine (Kugawa et al., 2000), and/or an increase in the maturation rate of DCX-positive neuroblasts. Because the percentage of IdU-positive cells identified as "other" (i.e., possible neuroblasts that had stepped out of the cell cycle over the course of labeling and developed into DCX-negative immature neurons) was lower than that of controls (Fig. 5C), it is unlikely that an increase in the rate of neuroblast maturation underlies the decrease in IdU labeling. To distinguish between the two remaining hypotheses, dying cells were labeled by TUNEL. Because cell loss in adult hippocampus attributed to opiate drugs of abuse is restricted to cells in S-phase and newly born immature neurons (Arguello et al., 2008), we assessed cell death in both actively proliferating cells, 24 h after IdU labeling, and immature neurons that had matured 7 days following the initiation of analgesic treatment (Fig. 1B). No significant difference in the total number of TUNEL-positive cells was observed 24 h after the last buprenorphine injection, suggesting that analgesic treatment does not compromise the viability of actively proliferating cells in the hippocampus whether administered through a standard or minimal schedule (Fig. 6A). In contrast, a reduction in the hippocampal apoptotic index was evident following both treatment paradigms 7 days after initiating buprenorphine treatment. Two-fold less TUNEL-positive cells were detected in the GCL, SGZ, and hilus compared with controls under both administration paradigms; however, this decrease only reached statistical significance in the group receiving the minimal analgesic schedule (Fig. 6B; ANOVA, post hoc Tukey's test, $*P<0.05$).

An abbreviated schedule of buprenorphine analgesia enhances the survival of newly born cells

To directly assess the impact of buprenorphine on hippocampal stem and progenitor cell survival, we tracked the number and fate of cells labeled with CldU before manipulation 28 days after minor surgery and analgesic treatment (Fig. 1C). Consistent with the decrease in cell death observed 7 days after minimal buprenorphine treatment in mice without need for pain management (Fig. 6B), we found that the survival of CldU-labeled cells in the hippocampal dentate gyrus of mice that received a minimal buprenorphine treatment regimen following surgery significantly increased relative to both control and standard treatment (Fig. 7A; ANOVA, post hoc Tukey's test, $*P<0.05$ relative to control, $\#\# P<0.01$ relative to standard treatment). We next identified these CldU-positive cells using markers for mature neurons (NeuN) or astrocytes (GFAP) (Fig. 7B, C). Double-positive cells were quantified and expressed as a percentage of the total number of CldU-positive cells. No difference in the percentage of terminally differentiated cells was detected between groups (Fig. 7D), indicating that although the minimal protocol of opiate analgesic intervention enhanced the survival of newly born cells, it did not alter their specification.

DISCUSSION

Here, we report that buprenorphine, a routinely administered opiate analgesic, transiently inhibits the proliferation of DCX-positive neuroblasts and, under an acute schedule of administration, enhances survival of newly born cells. Using two different administration paradigms (standard, seven injections over 72 h and minimal, three injections over 24 h), we show that buprenorphine decreases the number of proliferating DCX-positive cells in the adult hippocampus for up to 1 week after the last buprenorphine injection. These effects are further exacerbated by postoperative pain with animals subjected to both surgery and buprenorphine showing larger reductions in proliferating cell number than pain-free animals administered buprenor-

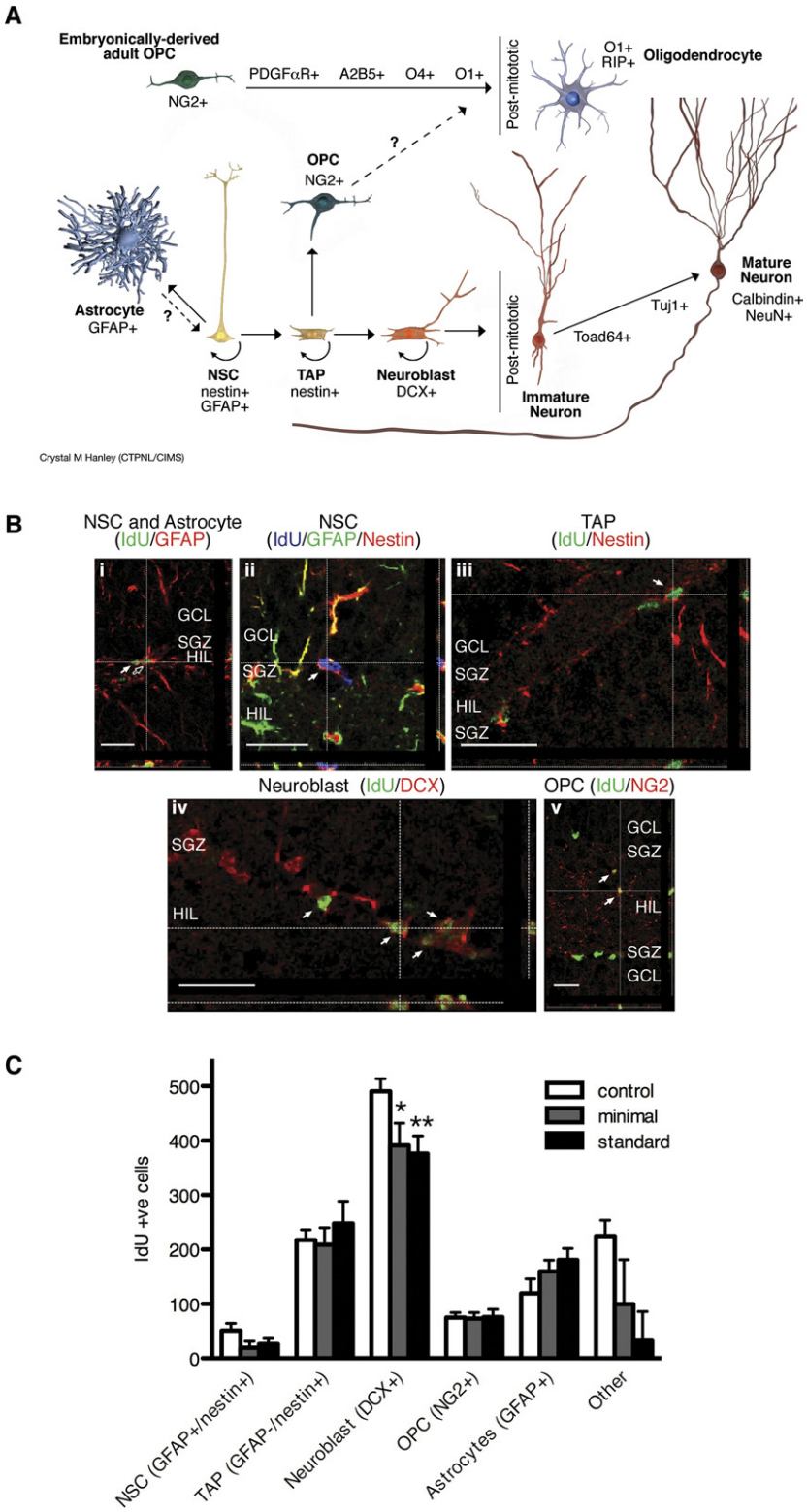

Fig. 5. Buprenorphine inhibits the proliferation of DCX-positive neuroblasts. Abbreviations are as defined in Fig. 2 (A) Schematic of the process of adult hippocampal neurogenesis depicting the morphological characteristics of the individual cell types and the antigenic lineage markers used to follow the process. (B) Representative confocal micrographs used for lineage analysis of proliferating stem and progenitor cells. (i) IdU/GFAP (NSC, open arrow and astrocyte, filled arrow), (ii) IdU/Nestin/GFAP (NSC, filled arrow), (iii) IdU/Nestin (TAP, filled arrow), (iv) IdU/DCX (neuroblast, filled arrows), and (v) IdU/NG2 (OPC, filled arrows) Scale bars 25 μm. (C) Quantification of IdU-labeled NSCs, TAPS, neuroblasts, OPCs, astrocytes, and others in drug-naïve mice (control, n=9) or following minimal (n=6) or standard (n=6) buprenorphine injection paradigms without requirement for pain management. The number of IdU-positive/DCX-positive neuroblasts were significantly reduced following treatment with either the minimal or standard schedule of buprenorphine treatment. Statistics for each lineage marker were ANOVA followed by post hoc Bonferroni's test. * $P<0.05$, ** $P<0.01$ relative to control.

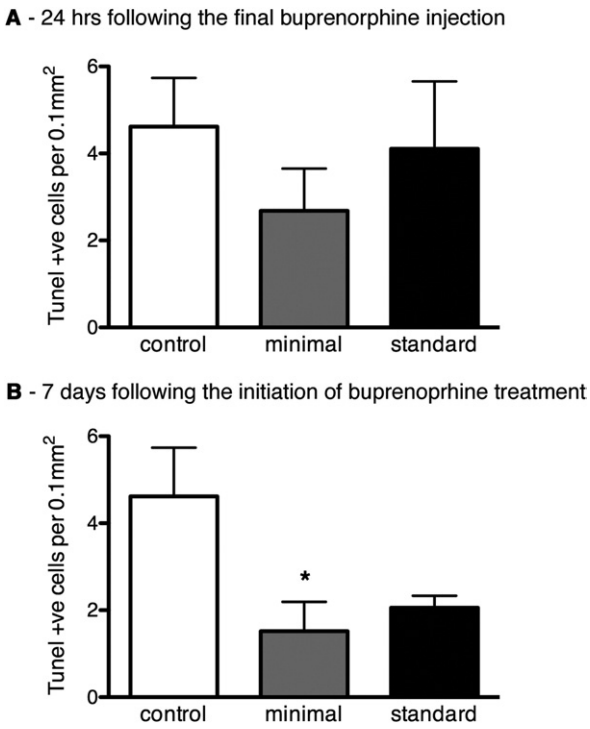

Fig. 6. Apoptotic cell death is decreased in the hippocampal dentate gyrus 7 d but not 24 h following the initiation of buprenorphine treatment. Quantification of TUNEL-positive cells per 0.1 mm^2 of control, minimal and standard buprenorphine-treated mice without need for pain management. (A) 24 h after the final buprenorphine injection (control $n=6$, minimal $n=5$, standard $n=5$) or (B) 7 days following the first buprenorphine injection (control $n=6$, minimal $n=5$, standard $n=5$). Statistics were ANOVA followed by post hoc Tukey's test. * $P<0.05$ relative to control.

phine alone. Surprisingly, our data also indicate that acute exposure to opiate analgesics enhances the survival of newly born cells for up to 28 days. Following a minimal schedule of buprenorphine administration, basal apoptotic indices are reduced, and long-term survival of newly born cells is enhanced.

Although this is the first study to demonstrate that opiate analgesics alter hippocampal neurogenesis, our data are consistent with the well-documented negative impact of opiate drugs of abuse on the proliferation of hippocampal neural progenitor cells and specifically DCX-positive/nestin-negative TAP populations (Eisch et al., 2000; Kahn et al., 2005; Arguello et al., 2009). We did not test whether the return to basal proliferative indices observed in this study is gradual or involves a rebound increase in proliferating cell number as seen after withdrawal from chronic morphine treatment (Kahn et al., 2005). The acute nature, however, of the buprenorphine-mediated phenotype is novel. Measurable decreases in hippocampal progenitor cell proliferation depend on chronic exposure to opiate drugs of abuse whether through s.c. pellet implantation (morphine) or i.v. self-administration (heroin) (Eisch et al., 2000). A single i.p. injection of morphine does not alter proliferating hippocampal progenitor cell number (Eisch et al., 2000). The threshold of opiate exposure required to elicit these effects has yet to be determined, and it may be that our acute injection paradigm of three s.c. injections in a single day represents the minimal threshold of opiate exposure necessary to inhibit neuroblast proliferation.

Perhaps even more intriguingly, the viability and maturation of hippocampal progenitor cells are known to be impaired by chronic exposure to opiate drugs of abuse

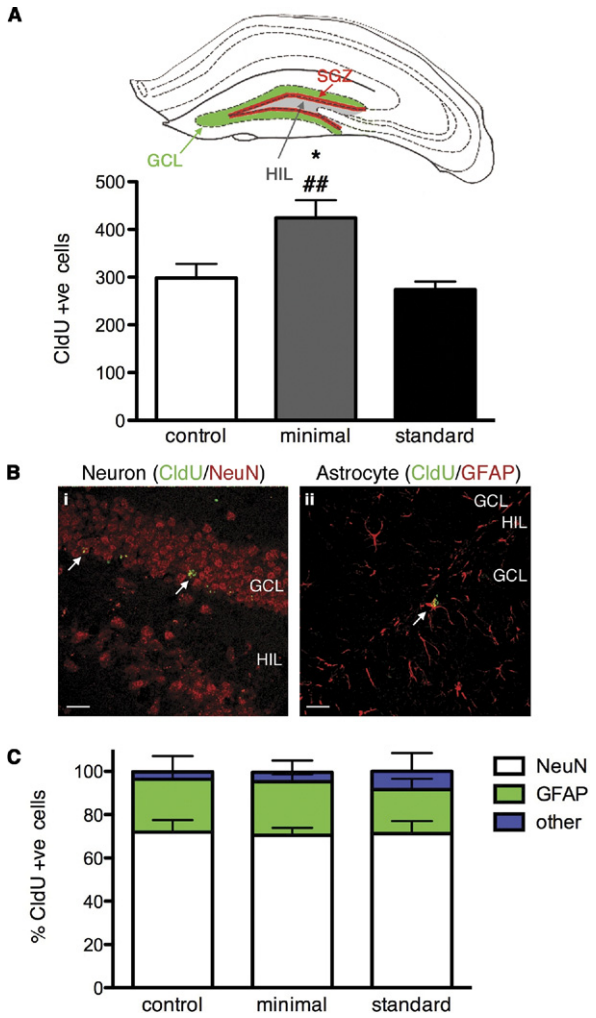

Fig. 7. The survival of newly born cells is increased with minimal buprenorphine treatment following surgery. Abbreviations are as defined in Fig. 2. (A) Schematic of the hippocampal dentate gyrus highlighting the GCL (green), SGZ (red), and HIL (gray), the areas used for quantification of CldU-positive cells and quantification of surviving CldU-positive cells in the dentate gyrus of controls ($n=10$) and mice implanted with osmotic mini-pumps administered a minimal ($n=6$) or standard ($n=6$) schedule of buprenorphine 28 d following surgery. Statistics were ANOVA followed by post hoc Tukey's test. * $P<0.05$ relative to control, ## $P<0.01$ relative to standard buprenorphine treatment. (B) Representative confocal micrographs of double immunofluorescence for (i) CldU/NeuN and (ii) CldU/GFAP used to assess fate specification in the dentate gyrus. Double-positive cells are indicated by white arrows. Scale bars 25 μm. (C) Percentage of total CldU-positive cells double-positive for the lineage markers NeuN (white), GFAP (green), or other (blue) in the dentate gyrus. Note: there is no difference in the percentage of terminally differentiated neurons or glia generated by NSC and progenitor cells in the presence or absence of opiate analgesia and surgery.

(Eisch et al., 2000; Arguello et al., 2009), whereas here we show here that the shortest buprenorphine analgesic paradigm improves survival of newly born cells exposed to opiate analgesia. Again, this may be a compensatory effect of analgesic withdrawal as extended exposure to buprenorphine *in vitro* has been shown to elicit apoptosis (Kugawa et al., 2000); thus, limiting the duration of exposure to an apoptogenic stimuli *in vivo* may be sufficient to trigger compensatory anti-apoptotic pathways that enhance cell survival in the absence of cell death. Alternatively, the differential effects of buprenorphine and opiate drugs of abuse on cell survival may reflect different mechanisms of action. Certainly stress and opiate treatment can activate the hypothalamic-pituitary-adrenal (HPA) axis causing an increase in the production and secretion of stress hormones, notably corticosterone, that negatively regulate neurogenesis (Urquhart et al., 1984; Bryant et al., 1991; Coe et al., 2003; Olfe et al., 2010). Buprenorphine is unique among opiates, as it does not activate the HPA axis (Gomez-Flores and Weber, 2000). We cannot rule out the possibility that the behavioral impact of our s.c. injection paradigm, independent of the drug administered, activated the HPA axis. However, while restraint stress is associated with activation of the HPA axis and impaired neurogenesis, this effect is variable and dependent on numerous factors including age, sex, duration and frequency of restraint, and habituation to handling (Barha et al., 2011; Hanson et al., 2011). Both of our treatment paradigms fall within the mild end of the stress spectrum suggesting that the reduced proliferation and enhanced survival observed is primarily directly attributable to buprenorphine.

Buprenorphine targets the MOR, KOR, and ORL-1 opiate receptors, all of which are expressed by hippocampal neurons (Yasuda et al., 1993; Mollereau et al., 1994; Tallent et al., 2001; Skyers et al., 2003). NSC and progenitor populations have been shown to express MORs and respond to MOR ligands but appear not to express KOR or respond to KOR-specific ligands, suggesting a direct role for MOR signaling in the phenotypes observed in this study (Hauser et al., 1996; Gutstein et al., 1997; Learish et al., 2000; Persson et al., 2003; Berglund et al., 2004; Zheng et al., 2010). Although not extensively studied, buprenorphine has also been reported to be neuroprotective following retinal optic nerve transection *in vivo* (Ozden and Isenmann, 2004), and this protection is thought to be mediated by its direct action on MORs (Faden, 1996). It is not yet known whether hippocampal neuroblasts express ORL-1, but given its robust expression by granule neurons of the dentate gyrus (Tallent et al., 2001), it may be that buprenorphine modulates the “instructive” signaling associated with neuron-progenitor cell interactions influencing their survival and specification (Song et al., 2002). *In vivo*, ORL-1 expression increases in neurons that survive traumatic brain injury, thus suggesting that activation of these receptors could be implicated in the increased survival of newly born cells observed in this study (Witta et al., 2003). Clearly, the expression of ORL-1 by hippocampal stem and progenitor cells must be determined before these hypotheses can be further explored mechanistically.

Taken together, this study identifies a hitherto unreported source of variability in neurogenic studies and a novel potential mechanism that could impact upon clinical efficacy. Here, we demonstrated the impact of buprenorphine on neurogenesis in the dorsal hippocampus. Adult hippocampal neurogenesis has been implicated in learning and memory, with newborn neurons capable of being functionally integrated into the existing hippocampal circuitry and activated by specific memory tasks, most often spatial in nature (Winocur et al., 2006; Kee et al., 2007; Zhao et al., 2008). Accordingly, we and others have focused our studies of neurogenesis on the dorsal domain of the hippocampus that is responsible for these cognitive functions (Fanselow and Dong, 2010). There is, however, an increasing awareness of the functional heterogeneity of the hippocampus along its longitudinal axis and a growing body of research investigating the role of neurogenesis within the ventral hippocampus with respect to the regulation of emotion and the outcome of motivated behaviors (Fanselow and Dong, 2010; Kheirbek and Hen, 2011). This is particularly interesting in the context of opiate treatment in general, and buprenorphine more specifically, as even acute treatment may lead to cognitive and psychomotor impairment as well as modulate the magnitude of emotional outcomes in healthy subjects (Panksepp et al., 1978; Dolcos et al., 1998; Jensen et al., 2008). Although such potential functional implications are important in the context of experimental design when examining neurogenesis and/or hippocampal dependent behaviors, they are of even greater importance in the clinic where the acute effect of buprenorphine on neurogenesis may negatively impact the already heightened negative emotions and cognitive impairments seen in patients with chronic pain, anxiety and/or depression, whereas timely removal of opiate treatment following amelioration of pain could facilitate recovery through neurogenic impact not only in dorsal but also in ventral hippocampus (Wade et al., 1990; Hart et al., 2000, 2003; Beaudreau and O’Hara, 2008; McDermott and Ebmeier, 2009). Future studies dissecting the impact of various opiates on neurogenesis in the dorsal versus ventral hippocampal domains and separating the functional impact on each region using appropriate behavioral paradigms will be of critical importance, notably when exploring the off-label use of buprenorphine to treat addiction and mood disorders (Davis, 2007; Likar et al., 2007; Howland, 2010).

In no way do our data support curtailing the use of analgesics in neurogenic studies. Rather, our findings are presented to highlight an important source of variability affecting the interpretation of changes in acute cell proliferation following neurogenic intervention strategies. It is widely accepted that surgery, even minor surgery, is stressful, both physically and psychologically to both humans and experimental animals, and that opiate analgesics can attenuate many of the immune modulatory events underlying the stress response in addition to pain management, thereby improving post-operative recovery (reviewed in (Shavit et al., 2006)). Here, we show that the inhibition of cell proliferation in animals treated with bu-

prenorphine is significantly greater in animals experiencing post-operative pain. Although ethical considerations precluded the direct examination of the effect of surgery alone on neurogenesis in our study, these data are evidence of a profound modulatory role of pain on hippocampal neurogenesis. Given the need to minimize the impact of pain in studies designed to test neurogenic interventions that require surgical manipulation of experimental animals, we encourage researchers to carefully consider the potential impact of analgesic treatments on experimental outcomes and to include these details in reported methodologies as a potential explanation for a transient decrease in neuroblast proliferation following surgical intervention. Only 20% of current peer-reviewed publications detail the post-operative care of animals undergoing minor experimental surgery (Piersma et al., 1999; Stokes et al., 2009; Coulter et al., 2011). Failure to report analgesic use is not surprising; these procedures are commonly performed as standard operating procedures by veterinary staff monitoring the post-operative recovery of experimental animals, often without the involvement (or direct knowledge) of the primary research team. Given these necessary but "silent" interventions, our data suggest that differences in practice between institutions likely affect the acute assessment of hippocampal progenitor cell proliferation following surgical interventions and, potentially, the long-term survival of progenitor cell progeny without research teams being fully aware of these critical procedural differences.

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REFERENCES

Ables JL, Decarolis NA, Johnson MA, Rivera PD, Gao Z, Cooper DC, Radtke F, Hsieh J, Eisch AJ (2010) Notch1 is required for maintenance of the reservoir of adult hippocampal stem cells. J Neurosci 30:10484–10492.

Ackermann J, Ashton G, Lyons S, James D, Hornung JP, Jones N, Breitwieser W (2011) Loss of ATF2 function leads to cranial motoneuron degeneration during embryonic mouse development. PLoS One 6:e19090.

Arguello AA, Fischer SJ, Schonborn JR, Markus RW, Brekken RA, Eisch AJ (2009) Effect of chronic morphine on the dentate gyrus neurogenic microenvironment. Neuroscience 159:1003–1010.

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

Barha CK, Brummelte S, Lieblich SE, Galea LA (2011) Chronic restraint stress in adolescence differentially influences hypothalamic-pituitary-adrenal axis function and adult hippocampal neurogenesis in male and female rats. Hippocampus 21:1216–1227.

Beaudreau SA, O'Hara R (2008) Late-life anxiety and cognitive impairment: a review. Am J Geriatr Psychiatry 16:790–803.

Ben Abdallah NM, Slomianka L, Vyssotski AL, Lipp HP (2010) Early age-related changes in adult hippocampal neurogenesis in C57 mice. Neurobiol Aging 31:151–161.

Berglund CM, Aarum J, Haeberlein SL, Nyengaard JR, Hökfelt T, Sandberg K, Näslund J, Persson MA (2004) Characterization of long-term mouse brain aggregating cultures: evidence for maintenance of neural precursor cells. J Comp Neurol 474:246–260.

Bérubé NG, Mangelsdorf M, Jagla M, Vanderluit J, Garrick D, Gibbons RJ, Higgs DR, Slack RS, Picketts DJ (2005) The chromatin-remodeling protein ATRX is critical for neuronal survival during corticogenesis. J Clin Invest 115:258–267.

Blunt D (2009) Statistics of Scientific Procedures on Living Animals. (HMSO, ed). London: The Stationary Office. Available at: http://webarchive.nationalarchives.gov.uk/+/http://www.homeoffice.gov.uk/publications/science-research-statistics/research-statistics/science-research/spanimals09/.

Bryant HU, Bernton EW, Kenner JR, Holaday JW (1991) Role of adrenal cortical activation in the immunosuppressive effects of chronic morphine treatment. Endocrinology 128:3253–3258.

Coe CL, Kramer M, Czéh B, Gould E, Reeves AJ, Kirschbaum C, Fuchs E (2003) Prenatal stress diminishes neurogenesis in the dentate gyrus of juvenile rhesus monkeys. Biol Psychiatry 54:1025–1034.

Coulter CA, Flecknell PA, Leach MC, Richardson CA (2011) Reported analgesic administration to rabbits undergoing experimental surgical procedures. BMC Vet Res 7:12.

Curtin LI, Grakowsky JA, Suarez M, Thompson AC, DiPirro JM, Martin LB, Kristal MB (2009) Evaluation of buprenorphine in a postoperative pain model in rats. Comp Med 59:60–71.

Davis MP (2007) What is new in neuropathic pain? Support Care Cancer 15:363–372.

Deng W, Aimone JB, Gage FH (2010) New neurons and new memories: how does adult hippocampal neurogenesis affect learning and memory? Nat Rev Neurosci 11:339–350.

Dobromylskyi P, Flecknell P, Lascelles B, Pascoe P, Taylor P, Waterman-Pearson A (2000) Management of postoperative and other acute pain. In: Pain management in animals (Flecknell PA, Waterman-Pearson A, eds), pp 81–147. London: WB Saunders.

Doetsch F (2003) The glial identity of neural stem cells. Nat Neurosci 6:1127–1134.

Dolcos F, Strungaru C, Dolcos SM, Turcu E (1998) The involvement of endogenous opiates in emotional-cognitive interaction in stress. Rom. J Physiol 35:259–274.

Dong HW, Swanson LW, Chen L, Fanselow MS, Toga AW (2009) Genomic-anatomic evidence for distinct functional domains in hippocampal field CA1. Proc Natl Acad Sci U S A 106:11794–11799.

Dubois NC, Hofmann D, Kaloulis K, Bishop JM, Trumpp A (2006) Nestin-Cre transgenic mouse line Nes-Cre1 mediates highly efficient Cre/loxP mediated recombination in the nervous system, kidney, and somite-derived tissues. Genesis 44:355–360.

Eisch AJ, Barrot M, Schad CA, Self DW, Nestler EJ (2000) Opiates inhibit neurogenesis in the adult rat hippocampus. Proc Natl Acad Sci U S A 97:7579–7584.

Faden AI (1996) Neurotoxic versus neuroprotective actions of endogenous opioid peptides: implications for treatment of CNS injury. NIDA Res Monogr 163:318–330.

Fanselow MS, Dong HW (2010) Are the dorsal and ventral hippocampus functionally distinct structures? Neuron 65:7–19.

Gage FH (2000) Mammalian neural stem cells. Science 287: 1433–1438.

Garber JC, Barbee RW, Bielitzki JT, Clayton LA, Hendriksen CFM, Kohn DF, Lipman NS, Locke PA, Melcher J, Quimby FW, Turner PV, Wood GA, Würbel H (2011) Guide for the Care and Use of Laboratory Animals, 8th ed. National Academies Press. Available at: http://wwwaaalacorg/resources/Guide_2010pdf.

Garcia AD, Doan NB, Imura T, Bush TG, Sofroniew MV (2004) GFAP-expressing progenitors are the principal source of constitutive neurogenesis in adult mouse forebrain. Nat Neurosci 7:1233–1241.

Gebhart GF, B AI, Flecknell P, Goodly L, Karas AZ, Kelley ST, Lacher K, Mason G, Sneddon LU, Soriano SG (2009) Recognition and alleviation of pain in laboratory animals. The National Academies Press. Available at: http://wwwnapedu/catalogphp?record_id=12526.

Gomez-Flores R, Weber RJ (2000) Differential effects of buprenorphine and morphine on immune and neuroendocrine functions following acute administration in the rat mesencephalon periaqueductal gray. Immunopharmacology 48:145–156.

Guo F, Maeda Y, Ma J, Xu J, Horiuchi M, Miers L, Vaccarino F, Pleasure D (2010) Pyramidal neurons are generated from oligodendroglial progenitor cells in adult piriform cortex. J Neurosci 30:12036–12049.

Gutstein HB, Rubie EA, Mansour A, Akil H, Woodgett JR (1997) Opioid effects on mitogen-activated protein kinase signaling cascades. Anesthesiology 87:1118–1126.

Hanson ND, Owens MJ, Boss-Williams KA, Weiss JM, Nemeroff CB (2011) Several stressors fail to reduce adult hippocampal neurogenesis. Psychoneuroendocrinology 36:1520–1529.

Hart RP, Martelli MF, Zasler ND (2000) Chronic pain and neuropsychological functioning. Neuropsychol Rev 10:131–149.

Hart RP, Wade JB, Martelli MF (2003) Cognitive impairment in patients with chronic pain: the significance of stress. Curr Pain Headache Rep 7:116–126.

Hauser KF, Stiene-Martin A, Mattson MP, Elde RP, Ryan SE, Godleske CC (1996) mu-Opioid receptor-induced Ca2+ mobilization and astroglial development: morphine inhibits DNA synthesis and stimulates cellular hypertrophy through a Ca (2+)-dependent mechanism. Brain Res 720:191–203.

Hawk T, Leary SL, Moris TH (2005) Formulary for Laboratory Animals 3rd ed. New York: Wiley-Blackwell.

Hof PY, Bloom FE, Belichenko PV, Celio MR (2001) Comparative cytoarchitectonic Atlas of the C57BL/6 and 129/Sv mouse brains. London: Elsevier Science.

Howland RH (2010) The diverse clinical uses of opioid receptor drugs. J Psychosoc Nurs Ment Health Serv 48:11–14.

Jensen ML, Sjøgren P, Upton RN, Foster DJ, Bonde P, Graae C, Skram U, Stevner L, Christrup LL (2008) Pharmacokinetic-pharmacodynamic relationships of cognitive and psychomotor effects of intravenous buprenorphine infusion in human volunteers. Basic Clin Pharmacol Toxicol 103:94–101.

Kahn L, Alonso G, Normand E, Manzoni OJ (2005) Repeated morphine treatment alters polysialylated neural cell adhesion molecule, glutamate decarboxylase-67 expression and cell proliferation in the adult rat hippocampus. Eur J Neurosci 21:493–500.

Kee N, Teixeira CM, Wang AH, Frankland PW (2007) Preferential incorporation of adult-generated granule cells into spatial memory networks in the dentate gyrus. Nat Neurosci 10:355–362.

Kempermann G, Jessberger S, Steiner B, Kronenberg G (2004) Milestones of neuronal development in the adult hippocampus. Trends Neurosci 27:447–452.

Kheirbek MA, Hen R (2011) Dorsal vs ventral hippocampal neurogenesis: implications for cognition and mood. Neuropsychopharmacology 36:373–374.

Kronenberg G, Reuter K, Steiner B, Brandt MD, Jessberger S, Yamaguchi M, Kempermann G (2003) Subpopulations of proliferating cells of the adult hippocampus respond differently to physiologic neurogenic stimuli. J Comp Neurol 467:455–463.

Kugawa F, Ueno A, Aoki M (2000) Apoptosis of NG108-15 cells induced by buprenorphine hydrochloride occurs via the caspase-3 pathway. Biol Pharm Bull 23:930–935.

Learish RD, Bruss MD, Haak-Frendscho M (2000) Inhibition of mitogen-activated protein kinase kinase blocks proliferation of neural progenitor cells. Brain Res Dev Brain Res 122:97–109.

Likar R, Lorenz V, Korak-Leiter M, Kager I, Sittl R (2007) Transdermal buprenorphine patches applied in a 4-day regimen versus a 3-day regimen: a single-site, Phase III, randomized, open-label, crossover comparison. Clin Ther 29:1591–1606.

Loftsson T, Jarho P, Másson M, Järvinen T (2005) Cyclodextrins in drug delivery. Expert Opin Drug Deliv 2:335–351.

Martin LB, Thompson AC, Martin T, Kristal MB (2001) Analgesic efficacy of orally administered buprenorphine in rats. Comp Med 51:43–48.

McDermott LM, Ebmeier KP (2009) A meta-analysis of depression severity and cognitive function. J Affect Disord 119:1–8.

Melanson-Drapeau L, Beyko S, Davé S, Hebb AL, Franks DJ, Sellitto C, Paul DL, Bennett SA (2003) Oligodendrocyte progenitor enrichment in the connexin32 null-mutant mouse. J Neurosci 23:1759–1768.

Mollereau C, Parmentier M, Mailleux P, Butour JL, Moisand C, Chalon P, Caput D, Vassart G, Meunier JC (1994) ORL1, a novel member of the opioid receptor family. Cloning, functional expression and localization. FEBS Lett 341:33–38.

Nowakowski RS, Lewin SB, Miller MW (1989) Bromodeoxyuridine immunohistochemical determination of the lengths of the cell cycle and the DNA-synthetic phase for an anatomically defined population. J Neurocytol 18:311–318.

Olfe J, Domanska G, Schuett C, Kiank C (2010) Different stress-related phenotypes of BALB/c mice from in-house or vendor: alterations of the sympathetic and HPA axis responsiveness. BMC Physiol 10:2.

Olferts EB, Cross BM, McWilliam AAE (1993) Control of animal pain in research, testing and teaching. In: Guide to the care and use of experimental animals, Vol. 1 Canadian Council on Animal Care. Available at: http://wwwccacca/en/CCAC_Programs/Guidelines_Policies/GUIDES/ENGLISH/V1_93/CHAP/CHXHTM.

Ozden S, Isenmann S (2004) Neuroprotective properties of different anesthetics on axotomized rat retinal ganglion cells in vivo. J Neurotrauma 21:73–82.

Panksepp J, Herman B, Conner R, Bishop P, Scott JP (1978) The biology of social attachments: opiates alleviate separation distress. Biol Psychiatry 13:607–618.

Persson AI, Thorlin T, Bull C, Eriksson PS (2003) Opioid-induced proliferation through the MAPK pathway in cultures of adult hippocampal progenitors. Mol Cell Neurosci 23:360–372.

Piersma FE, Daemen MA, Bogaard AE, Buurman WA (1999) Interference of pain control employing opioids in in vivo immunological experiments. Lab Anim 33:328–333.

Russell WMS, Birch RL (1959) The principles of humane experimental technique. Springfield: Charles S Thomas.

Sargeant TJ, Miller JH, Day DJ (2008) Opioidergic regulation of astroglial/neuronal proliferation: where are we now? J Neurochem 107:883–897.

Schultz KM, Banisadr G, Lastra RO, McGuire T, Kessler JA, Miller RJ, McGarry TJ (2011) Geminin-deficient neural stem cells exhibit normal cell division and normal neurogenesis. PLoS One 6:e17736.

Shavit Y, Fridel K, Beilin B (2006) Postoperative pain management and proinflammatory cytokines: animal and human studies. J Neuroimmune Pharmacol 1:443–451.

Skyers PS, Einheber S, Pierce JP, Milner TA (2003) Increased mu-opioid receptor labeling is found on inner molecular layer terminals of the dentate gyrus following seizures. Exp Neurol 179:200–209.

Smythe DH (1978) Alternatives to animal experiments. London: Scholar Press-Royal Defence Society.
Song H, Stevens CF, Gage FH (2002) Astroglia induce neurogenesis from adult neural stem cells. Nature 417:39–44.
Stokes EL, Flecknell PA, Richardson CA (2009) Reported analgesic and anaesthetic administration to rodents undergoing experimental surgical procedures. Lab Anim 43:149–154.
Tallent MK, Madamba SG, Siggins GR (2001) Nociceptin reduces epileptiform events in CA3 hippocampus via presynaptic and postsynaptic mechanisms. J Neurosci 21:6940–6948.
Taupin P (2007) BrdU immunohistochemistry for studying adult neurogenesis: paradigms, pitfalls, limitations, and validation. Brain Res Rev 53:198–214.
Thallmair M, Ray J, Stallcup WB, Gage FH (2006) Functional and morphological effects of NG2 proteoglycan deletion on hippocampal neurogenesis. Exp Neurol 202:167–178.
Urquhart J, Fara JW, Willis KL (1984) Rate-controlled delivery systems in drug and hormone research. Annu Rev Pharmacol Toxicol 24:199–236.
Vega CJ, Peterson DA (2005) Stem cell proliferative history in tissue revealed by temporal halogenated thymidine analog discrimination. Nat Methods 2:167–169.
Wade JB, Price DD, Hamer RM, Schwartz SM, Hart RP (1990) An emotional component analysis of chronic pain. Pain 40:303–310.
Winocur G, Wojtowicz JM, Sekeres M, Snyder JS, Wang S (2006) Inhibition of neurogenesis interferes with hippocampus-dependent memory function. Hippocampus 16:296–304.
Witta J, Buzas B, Cox BM (2003) Traumatic brain injury induces nociceptin/orphanin FQ expression in neurons of the rat cerebral cortex. J Neurotrauma 20:523–532.
Yasuda K, Raynor K, Kong H, Breder CD, Takeda J, Reisine T, Bell GI (1993) Cloning and functional comparison of kappa and delta opioid receptors from mouse brain. Proc Natl Acad Sci U S A 90:6736–6740.
Yoshiki A, Moriwaki K (2006) Mouse phenome research: implications of genetic background. ILAR J 47:94–102.
Zhao C, Deng W, Gage FH (2008) Mechanisms and functional implications of adult neurogenesis. Cell 132:645–660.
Zheng H, Zeng Y, Zhang X, Chu J, Loh HH, Law PY (2010) mu-Opioid receptor agonists differentially regulate the expression of miR-190 and NeuroD. Mol Pharmacol 77:102–109.
Zhu C, Gao J, Karlsson N, Li Q, Zhang Y, Huang Z, Li H, Kuhn HG, Blomgren K (2010) Isoflurane anesthesia induced persistent, progressive memory impairment, caused a loss of neural stem cells, and reduced neurogenesis in young, but not adult, rodents. J Cereb Blood Flow Metab 30:1017–1030.

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