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Does 5-Bromo-2′-deoxyuridine (BrdU) Disrupt Cell Proliferation and Neuronal Maturation in the Adult Rat Hippocampus *In Vivo*?

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Abstract

5-Bromo-2′-deoxyuridine (BrdU) is frequently used as a mitotic marker in studies of cell proliferation. Recent studies have reported cytotoxic effects of BrdU on neural progenitor cells in embryonic and neonatal brains *in vivo* and in adult tissue studied *in vitro*. The present study was conducted to assess whether BrdU interferes with cell proliferation and neuronal maturation in the rat adult hippocampus *in vivo*. BrdU effects across a wide range of doses (40 – 480 mg/kg) on cell proliferation and the population of immature neurons in the adult hippocampus were investigated using immunohistochemical labeling methods for the cell cycle marker Ki67 and a marker for immature neurons, doublecortin. BrdU did not influence cell proliferation in the dentate gyrus or the population of immature neurons observed in the adult hippocampus relative to those observed in saline treated controls. Thus, in contrast with reports of deleterious effects of BrdU in embryonic and neonatal tissue and adult tissue studied *in vitro*, BrdU does not appear to have cytotoxic effects on proliferating hippocampal cells or immature neurons *in vivo* in rats.

Keywords

BrdU; Neurogenesis; Doublecortin; Ki67; toxicity

Introduction

Many studies of cell proliferation in the adult hippocampus have been based on immunofluorescence cell labeling methods that use 5-Bromo-2′-deoxyuridine (BrdU) as a mitotic marker (for a review see Taupin, 2007b). BrdU is a thymidine analog that is incorporated into cells during the S phase of mitosis, and can be used in combination with neuron-specific markers, such as NeuN, to identify newly formed neurons (Cameron and McKay, 2001). Although BrdU has played an important role in definitively establishing that the neurons are added to the hippocampus during adulthood (Cameron et al., 1993), it is also clear that BrdU can be toxic to newborn neurons. For example, a recent *in vitro* study reported that BrdU doses in the concentration range that is recommended for cell

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proliferation studies (1–10 μM) interfered with the survival of newborn neurons (BrdU/TuJ1$^+$ cells) and that high doses of BrdU activated classical apoptosis pathways (Caldwell et al., 2005). Additionally, when administered to pregnant mice and rats, BrdU can interfere with embryonic brain development and cause body defects in embryos and postnatal behavioral abnormalities (Biggers et al., 1987; Nagao et al., 1997; Kolb et al., 1999; Sekerkova et al., 2004; Kuwagata et al., 2007).

In light of the findings mentioned above, that BrdU can be trigger apoptosis and genotoxic to embryonic neurons, we wondered whether BrdU administration regimens that are used to study adult hippocampal neurogenesis *in vivo* might interfere with cell proliferation in the hippocampus and neuronal maturation. To evaluate the effects of BrdU on neuronal maturation and cell proliferation in the hippocampus we used immunohistochemical markers for doublecortin (DCX) and Ki67. In the dentate gyrus, DCX is only expressed in cells that contribute to neurogenesis with the majority of DCX expressing cells being immature neurons that undergoing neurite elongation (>70%) while 20 percent are transiently amplifying progenitor cells (Plumpe et al., 2006). Ki67 is a marker for the cell cycle-associated protein mKi67 that marks proliferating cells that are in late G1, S, G2, and M phases of the cell cycle (Scholzen and Gerdes, 2000; Namba et al., 2005). Ki67 was used in the present study to determine if BrdU generally decreased cell proliferation in the dentate gyrus.

Materials and Methods

Sixty-eight, male, Long-Evans hooded rats weighing approximately 250 g, were housed in groups of two in clear Plexiglas® tub cages. Rats had continuous access to food and water and were treated in accordance with the University of North Carolina Wilmington Institutional Animal Care and Use Committee regulations. A 12 h light/dark cycle was in effect and all BrdU injections occurred during the light phase of the cycle. BrdU (Sigma, St. Louis, MO) was dissolved in 0.9% NaCl, and prepared in concentrations of 10, 60, or 120 mg/ml. Each animal received four separate injections of their assigned dose (saline, 10, 60, or 120 mg/kg BrdU) and injections were administered at approximately 07:00, 09:00, 11:00, and 13:00 h. Solutions were slowly warmed and stirred on a hotplate until BrdU dissolved, loaded in a syringe (in volumes of 1 ml/kg body weight) and injected i.p. when the syringe felt comfortable to the touch. The final cumulative BrdU doses for the four groups were 0, 40, 240, and 460 mg/kg.

In order to examine BrdU effects on cell proliferation and survival at two different time points, one subset of rats (N=36; n=9 per dose) was perfused two hours after the final BrdU dose and processed for BrdU and Ki67 immunohistochemistry and another subset (N=32; n=8 per dose) was perfused four days after the final BrdU dose and processed for BrdU, NeuN (neuronal nuclei; a neuronal marker), and DCX immunohistochemistry. If BrdU is toxic to immature neurons or transiently amplifying progenitor cells, fewer DCX-positive cells should be observed in groups exposed to BrdU than in the saline control group.

Rats were deeply anesthetized with sodium pentobarbital (100mg/kg), perfused with 5% sucrose in de-ionized (DI) water, followed by 4% paraformaldehyde in DI water, using a

Perfusion One (Coretech Holdings, St. Louis, MO) pump. Brains remained overnight in fixative, then were rinsed twice in 5% sucrose and placed in 30% sucrose for cryoprotection. Brains were sectioned at 40μm and every sixth section was collected for immunofluorescent labeling for BrdU, doublecortin (DCX), and Ki67. For BrdU and NeuN staining, the tissue was washed, treated with 50% Formamide/2X SSC buffer for 2 hours at 65 °C, washed in 2X SSC, incubated with 2M HCl at 37 °C for 30 minutes, and washed over 2hrs. Tissue was then placed in primary antibody overnight: 0.3% triton × (Sigma, St. Louis, MO), 2% Normal Goat Serum (Jackson Immunoresearch, West Grove, PA), 1:200 rat anti-BrdU (Accurate Chemical & Scientific Corp., Westbury, NY), and 1:1000 mouse anti-NeuN (Chemicon, Temecula, CA) in 0.1M PBS. Tissue was washed in 0.1M PBS and placed overnight in secondary antibody: 1:500 biotinylated Goat anti-rat IgG (Chemicon, Temecula, CA) in 0.1M PBS. Tissue was washed and placed in streptavidin-Alexa 568 (1:500) in 0.1M PBS for 45 minutes. Tissue was washed then mounted onto subbed slides (1% gel, 0.2% chromalum) and cover slipped using glycerol mounting medium (1–3% n-propyl galate in 1 part 0.1M phosphate buffer and 9 parts glycerol).

For DCX staining, tissue was washed and incubated in 0.3% H_2O_2 in 0.1M PBS for 25 min at room temperature. Tissue was washed then placed overnight in primary antibody solution: 1:1000 anti-DCX goat polyclonal (Santa Cruz Biotechnology, Santa Cruz, CA), 0.5% Triton-X, and 2% normal rabbit serum (Vectastain Elite ABC Kit Goat IgG – Vector Laboratories, Burlingame, CA), in 0.1M PBS. Tissue was washed and placed in secondary antibody solution: 1:1000 Biotinylated anti-goat IgG (Vectastain Elite ABC Kit Goat IgG, Vector Laboratories), in 0.1m PBS for 1 hour at room temperature. Tissue was washed and incubated in ABC solution (Vectastain Elite ABC Kit Goat IgG – Vector Laboratories) for 45 minutes at room temperature. Tissue was washed and incubated in DAB with Nickel solution (Vector Laboratories) until color changed (2–10 min). Tissue was washed and mounted onto subbed slides (1% gel, 0.2% chromalum). Slides were dehydrated with an ascending series of ethanol, cleared in Citrisol (Fisher Scientific), and coverslipped using Permount (Fisher Scientific) mounting medium.

For Ki67, staining tissue was washed and incubated overnight in primary antibody solution: 1:500 Ki67 rabbit polyclonal (Novocastra, Newcastle upon Tyne, UK), 0.3% Triton-X, 1% normal goat serum (Jackson ImmunoResearch, West Grove, PA) in 0.1M PBS. Tissue was washed and incubated for one hour at room temperature in secondary antibody solution: 1:1000 Anti-rabbit IgG biotinylated antibody (Vector Laboratories) in PBS. Tissue was washed and placed in 1:500 Streptavidin Alexa Fluor 568 conjugate (Molecular Probes) for 45 minutes at room temperature. Tissue was washed, mounted onto subbed slides (1% gel, 0.2% chromalum) and coverslipped using glycerol mounting medium (1–3% n-propyl galate in 1 part 0.1M phosphate buffer and 9 parts glycerol).

BrdU-, DCX-, and Ki67-positive cells were counted in brain sections that corresponded to plates 31, 35 and 44 of Paxinos and Watson's atlas of the rat brain (Paxinos and Watson, 1998). BrdU-, Ki67-, and DCX-labeled cells in the granule cell layer and subgranular zone of the dentate gyrus were counted in both hemispheres from each section. BrdU- and Ki67-labeled cells were observed with an Olympus BH-2 epifluorescence microscope with green excitation achieved by using a DM570 dichroic mirror with an IF545 excitation filter and a

590-nm barrier filter through a 40 × objective. DCX-labeled cells were observed with the light microscopy mode of the Olympus BH-2 microscope through a 20 × objective. An experimenter not involved in cell counting coded the slides such the experimental conditions of the samples were unknown to the experimenters who performed the cell counts. Two blind raters independently provided counts for each section. Inter-rater reliabilities were consistently high (all r's > 0.95). Composite means of the two experimenters' counts were used as dependent variables.

For BrdU- and Ki67-labeled cells image collection we used an Olympus LSM FluoView 1000 laser scanning confocal microscope. Cells were imaged through a 40 × or a 60 × objective, and Z-series of 1μm optical sections were merged to compose a two-dimensional micrograph representation of cells counted. For DCX-labeled cells image collection we used the light microscopy mode of an Olympus BX60 microscope with a 40 × objective lens.

Statistical analyses

All data analyses were based on ANOVA models and used Fisher's planned comparisons for post hoc tests (SPSS version 11.0.4 for Macintosh; Chicago, Il).

Results

Figure 1 shows representative photomicrographs of BrdU+ cells (top left). Nuclei of cells containing BrdU fluoresced in the red wavelength. Of the fluorescence observed, only the cells distinctively brighter than the background were counted as BrdU positive. Consistent with previous reports, clear dose-effect function was apparent such that the fewest BrdU labeled cells were observed at the lowest BrdU dose (40 mg/kg) whereas the intermediate (240 mg/kg) and largest (480 mg/kg) BrdU doses produced significantly more BrdU labeling (Fig. 1, bottom left) (Cameron and McKay, 2001; Hayes and Nowakowski, 2002). The 480 mg/kg dose, however, did not produce significantly more BrdU+ cells than were observed at the 240 mg/kg dose. Survival time after BrdU administration significantly affected the number of BrdU+ cells observed. Rats that survived for four days after BrdU administration had significantly more BrdU labeling than the rats that survived for two hours after the final BrdU injection. The observations reported above were confirmed by a two-way analysis of variance that detected main effects of dose, $F(3, 57)=43.06$, $p<0.001$, and survival time, $F(1, 57)=22.60$, $p<0.001$. A significant interaction between dose and survival time was not observed ($p>0.05$).

The top middle panel of Figure 1 shows representative photomicrographs of Ki67 positive cells. Ki67 positive cells fluoresced in the red wavelength. Of the fluorescence observed, only the cells distinctively brighter than the background were counted as Ki67 positive. Any fluorescing objects that did not match neuronal cell morphology were not counted as positive. BrdU did not influence cell proliferation, as measured by Ki67 labeling, at any of the doses studied (Fig. 1; bottom middle): $F(3, 32)=0.78$, $p=.97$.

The top right panel of Figure 1 is a representative photomicrograph of DCX positive cells. The cytoplasm and the axonal and dendritic extensions of DCX positive cells appeared dark purple/black in coloration. Cell bodies that appeared dark were counted as DCX positive

cells regardless of whether the dendrites and axons were visible. Axons and dendrites that were not connecting to any cell body visible in the section were not counted as positive cells. The cell body had to be clearly discernable in order for the cell to be counted as positive for DCX. We observed no evidence that any dose of BrdU decreased the population of neurons that were in the DCX phase of development in rats treated exposed to BrdU four days prior to perfusion: $F(3, 28)=.166$, $p=.92$ (Figure 1; bottom right).

Discussion

This study demonstrated that BrdU administered to adult rats by cumulative labeling does not decrease cell proliferation or the population of immature neurons in the dentate gyrus at any dose tested (40, 240 or 460 mg/kg) or at either survival time (2 hours or 4 days) as measured by Ki67 and DCX labeling. Thus, cumulative BrdU doses up to 480 mg/kg, administered over a 6-h interval, do not appear to disrupt cell proliferation or the population of immature neurons *in vivo*.

As reported by previous studies (Cameron and McKay, 2001), a clear dose-effect curve was observed in this study, such that the fewest BrdU positive cells were detected at the 40 mg/kg BrdU cumulative dose and significantly more BrdU positive cells were seen at the 240 and 480 mg/kg cumulative doses. Additionally, the amount of BrdU labeling produced by 240 and 480 mg/kg cumulative doses did not differ significantly from one another. This finding suggests that a plateau, perhaps due to BrdU saturation, is reached somewhere between these two doses.

If BrdU was toxic to either transiently amplifying neural progenitor cells or immature neurons, then a dose-related decrease in the population of Ki67- and/or DCX-labeled cells would have been observed. On the contrary, BrdU administration had no effect on the number of Ki67- or DCX- labeled cells in the 2-hour or 4-day survival group, respectively. Longer survival time after BrdU administration (4-days) was associated with a significant increase in the number of BrdU positive cells with the number of BrdU cells approximately doubling between 2 hours and 4 days after BrdU administration. In that BrdU is only available for incorporation into replicating DNA only for about 15 min after injection the observation that BrdU labeling doubled between the day of injections and four days later suggests that many BrdU labeled cells underwent mitosis at least once after incorporating BrdU into their DNA (Mandyam et al., 2007).

In *in vitro* experiments using adult brain tissue, BrdU activates apoptosis pathways in neurons, but not glia, at concentrations above 0.2 micromolar (Caldwell et al., 2005). This raises the question of why adult hippocampal neurogenesis was unaffected by BrdU in the present study. Several factors should be considered. In previous *in vitro* studies, a constant extracellular concentration of BrdU was maintained for 24 h, whereas under *in vivo* conditions such as that used the present study, the extracellular concentration of BrdU following systemic administration declines rapidly during the first 2 h after administration (Packard et al., 1973; Hayes and Nowakowski, 2000). Under *in vitro* conditions, however, the processes that normally support BrdU metabolism are disrupted. BrdU is metabolized through dehalogenation and then the uracil residue is excised from the DNA by the uracil

glycosylase repair system (Hume and Saffhill, 1986; Saffhill and Hume, 1986). In plasma, BrdU is also metabolized rapidly through dehalogenation (the half-life of BrdU in plasma is 8–11 min;(Kriss et al., 1963)). Thus, the concentration of BrdU that reaches the brain after systemic administration is only a fraction of the administered dose (Taupin, 2007a, b). Therefore, neuronal cells studied *in vitro* are exposed to a higher concentration of BrdU than are neuronal cells studied *in vivo*.

Data from the present study does not permit us to directly address the question of why cell proliferation neuronal maturation in the adult hippocampus do not display the same vulnerability to BrdU toxicity as do embryonic neurons (Kuwagata and Nagao, 1998; Kuwagata et al., 2004; Sekerkova et al., 2004; Ogawa et al., 2005; Muneoka et al., 2006; Kuwagata et al., 2007). One possible explanation is that relatively more BrdU permeates the blood-brain barrier in embryos than in adults because the blood-brain barrier is completed postnatally (Xu and Ling, 1994). Additional clues, however, come from the observation that BrdU's toxicity varies across brain areas, with each area having a critical period during embryonic development (Kuwagata et al., 2007). For example, cell death increased and neural progenitor cell proliferation decreased in the neocortex, mammillary body, and cerebellum, when BrdU was administered to to mice at embryonic day 11 (via ip injection to pregnant mothers) but not at embryonic day 9. If the vulnerability of embryonic brain cells was due to the relative immaturity of the embryonic blood-brain barrier, one would expect to observe that relatively uniform effects on levels of cell death and progenitor proliferation would have been observed across different brain areas and the neocortex, mammillary body, and cerebellum would not have been more vulnerable on embryonic day 11 than they were on embryonic day 9. Several studies show that the integration of a bromine atom into the DNA increases the risk of sister-chromatid exchanges, mutations and DNA double-strand breaks, and it lengthens the cell cycle of cells that incorporate it (Bannigan and Langman, 1979; Ockey et al., 1984; Saffhill and Ockey, 1985; Taupin, 2007b). Such effects of BrdU may be more influential during certain developmental periods than they are at others.

In summary, in the present study BrdU labeling was maximal at the 240mg/kg dose, and even cumulative dosing up to 480 mg/kg did not decrease proliferating (KI67 labeled) or differentiating cells (DCX labeled) hippocampal cells relative to saline treated controls. Thus, BrdU does not appear to have toxic effects on adult hippocampal neurogenesis when administered *in vivo* and at doses sufficient to achieve maximal labeling of newly born neurons.

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References

Bannigan J, Langman J. The cellular effect of 5-bromodeoxyuridine on the mammalian embryo. J Embryol Exp Morphol. 1979; 50:123–135. [PubMed: 458350]

Biggers WJ, Barnea ER, Sanyal MK. Anomalous neural differentiation induced by 5-bromo-2′-deoxyuridine during organogenesis in the rat. Teratology. 1987; 35:63–75. [PubMed: 3563938]

Caldwell MA, He X, Svendsen CN. 5-Bromo-2′-deoxyuridine is selectively toxic to neuronal precursors in vitro. Eur J Neurosci. 2005; 22:2965–2970. [PubMed: 16324131]

Cameron HA, McKay RD. Adult neurogenesis produces a large pool of new granule cells in the dentate gyrus. J Comp Neurol. 2001; 435:406–417. [PubMed: 11406822]

Cameron HA, Woolley CS, McEwen BS, Gould E. Differentiation of newly born neurons and glia in the dentate gyrus of the adult rat. Neuroscience. 1993; 56:337–344. [PubMed: 8247264]

Hayes NL, Nowakowski RS. Exploiting the dynamics of S-phase tracers in developing brain: interkinetic nuclear migration for cells entering versus leaving the S-phase. Dev Neurosci. 2000; 22:44–55. [PubMed: 10657697]

Hayes NL, Nowakowski RS. Dynamics of cell proliferation in the adult dentate gyrus of two inbred strains of mice. Brain Res Dev Brain Res. 2002; 134:77–85.

Hume WJ, Saffhill R. Iodo- and bromodeoxyuridine are excised at different rates from DNA of mouse tongue keratinocytes in vitro. Chem Biol Interact. 1986; 60:227–232. [PubMed: 2431799]

Kolb B, Pedersen B, Ballermann M, Gibb R, Whishaw IQ. Embryonic and postnatal injections of bromodeoxyuridine produce age-dependent morphological and behavioral abnormalities. J Neurosci. 1999; 19:2337–2346. [PubMed: 10066283]

Kriss JP, Maruyama Y, Tung LA, Bond SB, Revesz L. The fate of 5-bromodeoxyuridine, 5-bromodeoxycytidine, and 5-iododeoxycytidine in man. Cancer Res. 1963; 23:260–268. [PubMed: 14035826]

Kuwagata M, Nagao T. Behavior and reproductive function of rat male offspring treated prenatally with 5-bromo-2′-deoxyuridine. Reprod Toxicol. 1998; 12:541–549. [PubMed: 9763246]

Kuwagata M, Ogawa T, Nagata T, Shioda S. The evaluation of early embryonic neurogenesis after exposure to the genotoxic agent 5-bromo-2′-deoxyuridine in mice. Neurotoxicology. 2007; 28:780–789. [PubMed: 16956662]

Kuwagata M, Muneoka KT, Ogawa T, Takigawa M, Nagao T. Locomotor hyperactivity following prenatal exposure to 5-bromo-2′-deoxyuridine: neurochemical and behavioral evidence of dopaminergic and serotonergic alterations. Toxicol Lett. 2004; 152:63–71. [PubMed: 15294348]

Mandyam CD, Harburg GC, Eisch AJ. Determination of key aspects of precursor cell proliferation, cell cycle length and kinetics in the adult mouse subgranular zone. Neuroscience. 2007

Muneoka K, Kuwagata M, Iwata M, Shirayama Y, Ogawa T, Takigawa M. Dopamine transporter density and behavioral response to methylphenidate in a hyperlocomotor rat model. Congenit Anom (Kyoto). 2006; 46:155–159. [PubMed: 16922923]

Nagao T, Saito Y, Watanabe C, Imai K. Reproductive effects of prenatal exposure to 5-bromo-2′-deoxyuridine on male rats. Reprod Toxicol. 1997; 11:37–45. [PubMed: 9138632]

Namba T, Mochizuki H, Onodera M, Mizuno Y, Namiki H, Seki T. The fate of neural progenitor cells expressing astrocytic and radial glial markers in the postnatal rat dentate gyrus. Eur J Neurosci. 2005; 22:1928–1941. [PubMed: 16262632]

Ockey CH, Saffhill R, Booth JA. The effect of cell proliferation, bromodeoxyuridine concentration, and deoxynucleoside triphosphate pools on sister chromatid exchange induction. Basic Life Sci. 1984; 29(Pt A):267–279. [PubMed: 6532420]

Ogawa T, Kuwagata M, Muneoka KT, Shioda S. Neuropathological examination of fetal rat brain in the 5-bromo-2′-deoxyuridine-induced neurodevelopmental disorder model. Congenit Anom (Kyoto). 2005; 45:14–20. [PubMed: 15737126]

Packard DS Jr, Menzies RA, Skalko RG. Incorportaiton of thymidine and its analogue, bromodeoxyuridine, into embryos and maternal tissues of the mouse. Differentiation. 1973; 1:397–404. [PubMed: 4802502]

Paxinos, G.; Watson, C. The rat brain in stereotaxic coordinates. 4. San Diego: Academic Press; 1998.

Plumpe T, Ehninger D, Steiner B, Klempin F, Jessberger S, Brandt M, Romer B, Rodriguez GR, Kronenberg G, Kempermann G. Variability of doublecortin-associated dendrite maturation in adult hippocampal neurogenesis is independent of the regulation of precursor cell proliferation. BMC Neurosci. 2006; 7:77. [PubMed: 17105671]

Saffhill R, Ockey CH. Strand breaks arising from the repair of the 5-bromodeoxyuridine-substituted template and methyl methanesulphonate-induced lesions can explain the formation of sister chromatid exchanges. Chromosoma. 1985; 92:218–224. [PubMed: 4017746]

Saffhill R, Hume WJ. The degradation of 5-iododeoxyuridine and 5-bromodeoxyuridine by serum from different sources and its consequences for the use of the compounds for incorporation into DNA. Chem Biol Interact. 1986; 57:347–355. [PubMed: 3698122]

Scholzen T, Gerdes J. The Ki-67 protein: from the known and the unknown. J Cell Physiol. 2000; 182:311–322. [PubMed: 10653597]

Sekerkova G, Ilijic E, Mugnaini E. Bromodeoxyuridine administered during neurogenesis of the projection neurons causes cerebellar defects in rat. J Comp Neurol. 2004; 470:221–239. [PubMed: 14755513]

Taupin P. Protocols for studying adult neurogenesis: insights and recent developments. Regen Med. 2007a; 2:51–62. [PubMed: 17465775]

Taupin P. BrdU immunohistochemistry for studying adult neurogenesis: paradigms, pitfalls, limitations, and validation. Brain Res Rev. 2007b; 53:198–214. [PubMed: 17020783]

Xu J, Ling EA. Studies of the ultrastructure and permeability of the blood-brain barrier in the developing corpus callosum in postnatal rat brain using electron dense tracers. J Anat. 1994; 184(Pt 2):227–237. [PubMed: 8014116]

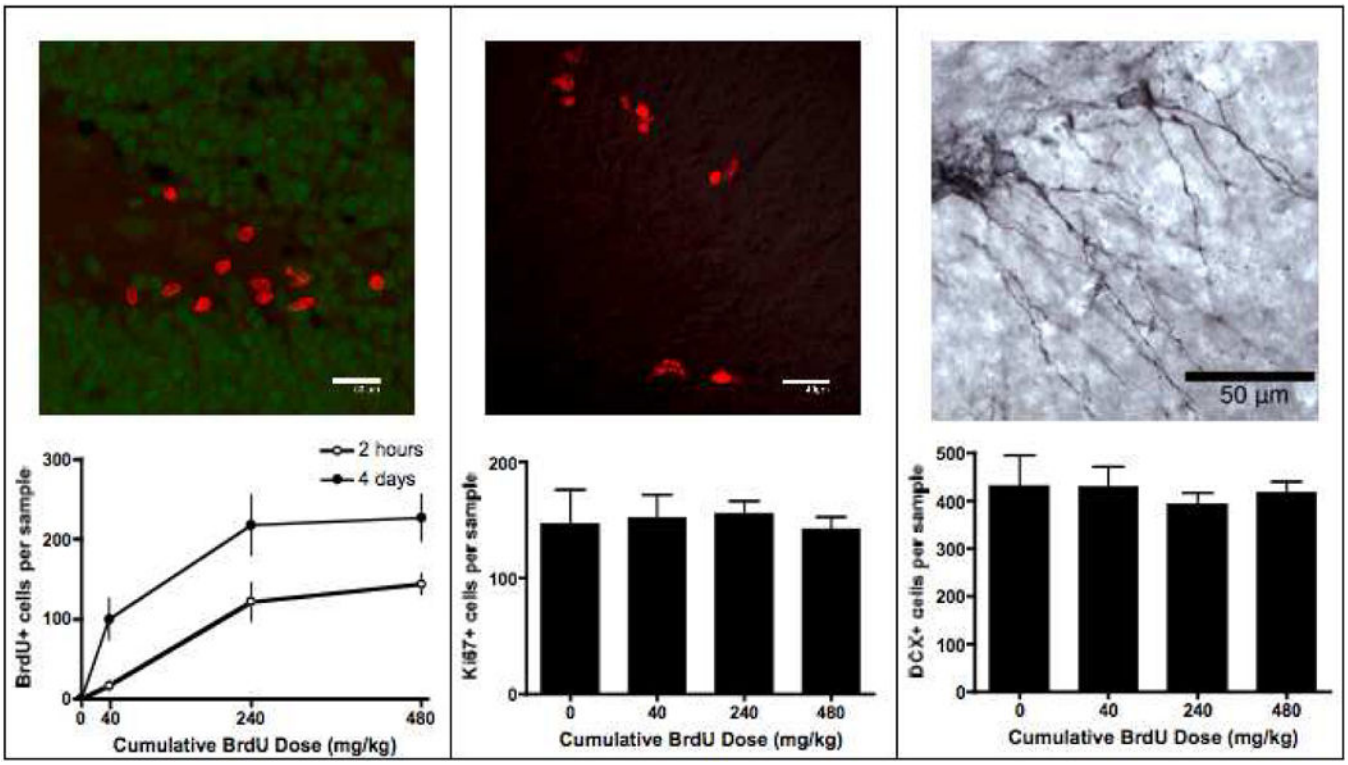

Fig 1.

LEFT TOP: Photomicrograph of representative examples of BrdU-positive cells located in the dentate gyrus of the hippocampus observed by laser scanning confocal microscopy at 40 × magnification. Nuclei of cells containing BrdU fluoresced in the red wavelength. Green fluorescing objects neuronal nuclei labeled with NeuN. LEFT BOTTOM. Mean BrdU-labeled cell counts in six regions of the dentate gyrus (3 per hemisphere) as a function of dose and time between the final BrdU or saline administration and perfusion. MIDDLE TOP. Photomicrograph of representative examples of Ki67-positive cells located in the dentate gyrus of the hippocampus observed by laser scanning confocal microscopy at 40 × magnification. Nuclei of cells containing Ki67 fluoresced in the red wavelength. MIDDLE BOTTOM. Mean Ki67-labeled cell counts in six regions of the dentate gyrus (3 per hemisphere) in rats perfused two hours after the final BrdU or saline administration. RIGHT TOP. Photomicrograph of representative examples of doublecortin-positive cells located in the dentate gyrus of the hippocampus observed with the light microscopy on an Olympus BH-2 microscope through a 40 × objective. Cells containing the doublecortin protein appear darker. RIGHT BOTTOM. Mean doublecortin-labeled cell counts in six regions of the dentate gyrus (3 per hemisphere) in rats perfused four days after the final BrdU or saline administration. Error bars represent standard error of the mean.