REGULAR ARTICLE

The dark side of BrdU in neural stem cell biology: detrimental effects on cell cycle, differentiation and survival

Bernadette Lehner · Beatrice Sandner · Julia Marschallinger · Christine Lehner ·
Tanja Furtner · Sebastien Couillard-Despres · Francisco J. Rivera · Gero Brockhoff ·
Hans-Christian Bauer · Norbert Weidner · Ludwig Aigner

Abstract 5-Bromo-2′-deoxyuridin (BrdU) is frequently used in anaylsis of neural stem cell biology, in particular to label and to fate-map dividing cells. However, up to now, only a few studies have addressed the question as to whether BrdU labeling per se affects the cells to be investigated. Here, we focused on the potential impact of BrdU on neurosphere cultures derived from the adult rat brain and on proliferation of progenitors in vivo. In vitro, neurospheres were pulsed for 48 h with BrdU, and cell proliferation, cell cycle, differentiation, survival and adhesion properties were subsequently analyzed. BrdU inhibited the expansion of neural progenitors as assessed by MTS assay and increased the fraction of cells in the G0/G1-phase of the cell cycle. Moreover, BrdU increased cell death and dose-dependently induced adherence of NPCs. Cell adherence was accompanied by a reduced amount of active matrix-metalloproteinase-2 (MMP-2). Furthermore, BrdU repressed neuronal and oligodendroglial differentiation, whereas astroglial fate was not affected. In contrast to the in vitro situation, BrdU apparently did not influence endogenous proliferation of NPCs or neurogenesis in concentrations that are typically used for labeling of neural progenitors in vivo. Our results reveal so far uncharacterized effects of BrdU on adult NPCs. We conclude that, because of its ubiquitous use in stem cell biology, any potential effect of BrdU of NPCs has to be scrutinized prior to interpretation of data.

Keywords Neural progenitors · Neurogenesis · Cell culture · Cell labeling · Rat (Fischer 344)

B. Lehner · J. Marschallinger · T. Furtner · S. Couillard-Despres · F. J. Rivera · L. Aigner (✉)
Institute of Molecular Regenerative Medicine
Paracelsus Medical University Salzburg,
Strubergasse 21,
5020 Salzburg, Austria
e-mail: ludwig.aigner@pmu.ac.at

B. Lehner · B. Sandner · N. Weidner
Department of Neurology, University of Regensburg,
Regensburg, Germany

C. Lehner · H.-C. Bauer
Department of Organismic Biology, Division of Zoology and
Functional Anatomy, University of Salzburg,
Salzburg, Austria

C. Lehner · H.-C. Bauer
Applied Cell Biology
Paracelsus Medical University Salzburg,
Salzburg, Austria

G. Brockhoff
Clinic of Gynaecology and Obstetrics,
Caritas Hospital St. Josef, University of Regensburg,
Regensburg, Germany

N. Weidner
Clinic of Paraplegiology, University of Heidelberg,
Heidelberg, Germany

Introduction

5-Bromo-2′deoxyuridine (BrdU) is a chemically synthesized, bromine-tagged base analogue that incorporates instead of thymidine into newly synthesized DNA during the S-phase of the cell cycle (Nowakowski et al. 1989). The availability of specific antibodies makes BrdU a widely used reagent to label newly generated cells for birth dating and cell fate studies, for tracking of cells after transplantation, and also to

© Springer

determine the proliferative capacity of cells, e.g. neural stem and progenitor cells (for review see (Cooper-Kuhn and Kuhn 2002; Kuhn and Cooper-Kuhn 2007)). Currently, BrdU labelling is the most widely used technique for studying adult neurogenesis.

Although it has been known for a long time that the incorporation of brominated deoxyuridine into DNA provokes conformational changes in its structure (Goz 1977) and that the balanced nucleotide pool in cells might be influenced as a consequence of permanent BrdU exposure, the potential cytostatic implications that could lead to alterations in cell cycle progression (Poot et al. 1994) as well as to an increase in cell death after BrdU treatment were almost consistently ignored up to now. Hence, BrdU was in general considered to substitute relatively benignly for thymidine into replicating DNA chains, at least in the absence of secondary stressors. But, recently, some data arose indicating that BrdU could induce a wide variety of negative side effects–apart from the effects that have been described earlier. For example, recent work suggests that BrdU is selectively toxic to neuronal precursors in vitro (Caldwell et al. 2005) and could play an important role in the induction of premature senescence in many different cell types (Michishita et al. 1999; Suzuki et al. 2001). BrdU has also been shown to influence the structure and stability of the DNA, thereby increasing the risk of sister-chromatid exchanges, mutations and double-strand breaks (reviewed in Taupin 2007). This is particularly of relevance, since co-segregation of template DNA is a crucial prerequisite for stem cells (Shinin et al. 2006). Moreover, it has been reported that a single low-dose pulse of BrdU induces senescence in early postnatal neural progenitors (Ross et al. 2008) and that an individual brief exposure to BrdU affects different cancer cell lines in vitro, leading to a progressive and persistent impairment of cell cycle progression (Diermeier et al. 2004; Levkoff et al. 2008).

Hence, BrdU labeling for studying adult neurogenesis is not without pitfalls and limitations (Nowakowski and Hayes 2001; Gould and Gross, 2002; Rakic 2002), and there is a definite need to systematically investigate the consequences of BrdU incorporation in multipotent stem and progenitor cells to exclude any potential side effects due to DNA labeling, which in the end could lead to a misinterpretation of data. In the present study, we have evaluated the effects of various BrdU concentrations ranging between 0.2 and 20.0 μM on adult neural stem and progenitor cells. We performed MTS assay to determine the proliferative capacity of these cells after BrdU treatment in vitro. We also used PCNA immunocytochemistry to determine the effects of intraperitoneal injections of BrdU on proliferating cells in vivo. Additionally, single parametric DNA measurements with PI were done to calculate the amount of cells in the G0/G1-, S- and G2/M-phase of the cell cycle after administration of BrdU. Cytotox assays were performed to assess whether BrdU induces cell death in adult neural stem and progenitor cultures. Furthermore, we used zymography to analyze if a reduced metalloproteinase activity might be involved in the observed adherence of cells treated with concentrations higher than 2.5 μM BrdU. Finally, we treated cells with different BrdU concentrations under defined astroglial, oligodendroglial and neuronal differentiation conditions and investigated GFAP, GalC and Map2ab expressions, respectively.

Materials and methods

Adult neural progenitor cell cultures

Adult neural progenitor cells (NPCs) of the ventricle wall derived from 2- to 4-month-old female Fischer 344 rats (Charles River, Sulzfeld, Germany) were isolated and grown as described (Wachs et al. 2006). The tissue was dissociated mechanically using a scalpel, resuspended in PPD solution containing 0.01% Papain (Worthington Biochemicals, Lakewood, NJ, USA), 0.1% dispase II (Boehringer, Mannheim, Germany), 0.01 % DNase I (Worthington Biochemicals), and 12.4 mM $MgSO_4$ in HBSS (PAN, Aidenbach, Germany) without Mg^{++}/Ca^{++} (PAA, Pasching, Austria) and digested for 30–40 min at room temperature. The cell suspension was triturated every 10 min. The single cell suspension was resuspended in NB medium (Gibco, Karlsruhe, Germany) supplemented with B27 (Gibco) (NB/B27), 2 mM L-glutamine (PAN), 100 U/mL penicillin and 100 μg/mL streptomycin (PAN), 2 μg / mL heparin (Sigma, Munich, Germany), 20 ng/mL bFGF-2 (R & D Systems, Abingdon, UK), and 20 ng/mL EGF (R & D Systems). This medium composition is defined as proliferation conditions (PC) in the present study. Cells were seeded in T25 culture flasks and cultures were maintained at 37°C in an incubator with 5% CO_2. Single cells began to form spheres within 5–7 days of suspension culture and continued to grow in mass and number over the next weeks. For passaging of cells, the culture medium containing floating neurospheres was collected in a 15-mL centrifuge tube and centrifuged at 120g for 5 min. The pellet was resuspended in 200 μL of Accutase (Innovative Cell Technologies, San Diego, CA, USA) and triturated approximately 10 times using a pipette. Cells were incubated for 10 min at 37°C in a water bath. The dissociated cells were then resuspended, counted in a Neubauer counting chamber and seeded in defined concentrations of 5 x 10^5 cells/ml (for MTS-assay) or 5 x 10^4 cells/ml (for all other experiments) under PC or differentiation conditions (DC=PC without EGF, FGF, Heparin). Cells from passage numbers 4–7 were used for the experiments.

Treatment of NPCs with BrdU

Cells were treated with 0.2, 1.0, 2.5, 5.0, 10.0 or 20.0 µM BrdU (Sigma) or PBS (PAN) 48 h after seeding. Again 48 h, or alternatively in some experiments 6 h later, cells were either processed for FACS analysis, time lapse recording, zymography, cell death detection ELISA, LDH or MTT/MTS assay, or immunofluorescence stainings.

Immunofluorescence analysis or differentiation of NPCs after BrdU treatment for 48 h

For BrdU immunofluorescence stainings, BrdU-treated neurospheres were seeded overnight on polyornithine (250 µg/mL; Sigma) and laminin (5 µg/mL; Sigma)-coated 24-well glass coverslips (Menzel, Braunschweig, Germany) in 500 µl of proliferation media with BrdU, fixed and stained for BrdU immunoreactivity. Briefly, cells were fixed at room temperature in 4% paraformaldehyde (0.8 g paraformaldeyhe, 15 ml aqua dest., 50 µl 1 M NaOH, 8 µl 1 M CaCl$_2$, 1 ml 1 M Sucrose, 4 ml 0.5 M Na$_3$PO$_4$ pH 7.0) for 30 min. They were washed three times for 5 min in 1 ml PBS and blocked with 1 ml fish skin gelatine (FSG) buffer for 2 h at room temperature. Following this, the cells were incubated in 2 N HCl for 30 min at 37°C. After that, the cells were rinsed four times with TBS (100 mM Tris Base, 150 mM NaCl) and then incubated with the primary antibody (rat anti BrdU (1:500; ImmunologicalsDirect, USA) diluted in 500 µl FSG buffer overnight at 4°C. Next day, cells were washed 3 times for 10 min in 1 ml FSG buffer before they were incubated with the secondary antibodies and processed further (see below).

For differentiation, 5 x 10^4 cells/ml were seeded on polyornithine (250 µg/mL; Sigma) and laminin (5 µg/mL; Sigma)-coated 24-well glass coverslips (Menzel) in 500 µl proliferation media. After 2 days, either PBS (control), 0.2 µM BrdU or 20.0 µM BrdU were added. Additional 48 h later, medium was changed to astroglial (AC), oligodendroglial (OC) or neuronal (NC) differentiation conditions for 7 days [AC: PC+10 ng/ml BMP 2+10 ng/ml BMP 4; OC: mesenchymal stem cell conditioned medium (MSC-CM) (Rivera et al. 2006); NC: DC +5% fetal calf serum (FCS)+2 µM retinoic acid]. After 1 week, cells were fixed, washed three times for 5 min in 1 ml PBS and blocked with 1 ml fish skin gelatine (FSG) buffer (for later staining with GFAP and Map2ab, Triton was added, whereas for staining with GalC, no Triton was used) for 2 h at room temperature. Following this, the cells were incubated with the primary antibody (AC: rabbit anti-GFAP 1:1,000, Dako, Glostrup, Denmark; NC: mouse anti-Map 2ab 1:250; OC: rabbit anti-GalC 1:200, Millipore, Schwalbach, Germany) diluted in 500 µl FSG buffer (with or without triton) overnight at 4°C. Next day, cells were washed 3 times for

10 min in 1 ml FSG buffer (with or without Triton) before they were incubated with the secondary antibodies (donkey anti-mouse A488, 1:1,000, Invitrogen, Darmstadt, Germany; donkey anti rabbit A488, 1:1,000, Invitrogen) diluted in 500 µl FSG buffer (with or without triton) for at least 4 h in the dark. This was followed by one washing step in 1 ml FSG buffer containing Dapi 1:1,000 for 15 min. Subsequently, two more 10-min washing steps were performed with 1 ml FSG buffer and 1 ml PBS. Finally, coverslips were mounted on glass slides with Prolong (Invitrogen).

For mRNA expression analysis, NPCs were seeded in coated T75 culture flasks at a density of 2.5 x 10^4 cells/ml in 10 ml proliferation media. After 2 days. either PBS (control), 0.2 µM BrdU, 1.0 µM BrdU or 20.0 µM BrdU were added. An additional 48 h later, the medium was changed to AC, OC or NC as described above. One week later, RNA was extracted with the RNeasy Mini Kit (Qiagen, Hilden, Germany) and cDNA was synthesized using the Reverse TrNPCription System (Promega, Mannheim, Germany), both according to the manufacturers' instructions. Expression analysis was performed by real-time quantitative PCR with Stratagene Mx3005P (Agilent Technologies, Waldbronn, Germany) using SYBR Green JumpStart Taq ReadyMix (Sigma) and specific primers for GFAP (Quanti Tect® Primer-Assay; no sequence information available), DCX (forward: GGAAGGGGAAAGC-TATGTCTG; reverse: TTGCTGCTAGCCAAGGACTG) or MBP (forward: GCTTCTTTAGCGGTGACAGG; reverse: CCAGCTAAATCTGCTGAGGG). For quantification, standard curves for AC, OC and PC were established by amplification of serial dilutions of mixed cDNA obtained from cells that were treated with PBS or BrdU. Samples were assayed in triplicates and 18S rRNA as used as the internal reference. To exclude genomic DNA contamination, a control reaction, in which no reverse trNPCription took place, was included. The following temperature profile was used: 95°C, 10 min; 95°C; 30 s (45 cycles); annealing: 55°C for DCX, MBP, 18S; 58°C for GFAP, 1 min; elongation: 72°C, 30 s. The quality of the product was controlled by a melting curve.

FACS analysis

NPCs that were treated either with different concentrations of BrdU or PBS for 48 h were dissociated into single cells with accutase. After 10 min incubation, accutase was aspirated and cells were resuspended in 1 ml of ice-cold 70% ethanol. They were kept overnight at −20°C. One day later, cells were centrifuged at 120g for 5 min (4°C). The supernatant was removed and cells were resuspended in 470 µL of PBS, and then 5 µL of RNaseA (Roche Diagnostics, Mannheim, Germany) (stock 1 mg/ml) was added. After a 60-min incubation at 37°C,

25 μL of propidium-iodide (stock 1 mg/ml) (Sigma-Aldrich, Taufkirchen, Germany) were added and samples were analyzed on a FACSCalibur flow cytometer (Becton Dickinson, Heidelberg, Germany). Data were processed using WinMDI 2.8 software.

For BrdU/PI FACS analysis, cells were pulse-labeled 96 h after seeding with 0.2, 1.0, 2.5, 5.0, 10.0, 20.0 or 40.0 μM BrdU for 20 min. Then, they were washed once with PBS to remove BrdU-containing supernatant. Cells were centrifuged (120g for 5 min, 4°C) and fixed in 1 ml of ice-cold ethanol. At the earliest 24 h later, cells were centrifuged for 5 min (1,200 U/min) and washed three times with ice-cold PBS containing 2% BSA. The pellet was resuspended in 50 μl of PBS+2% BSA before 100 μl of RNase (1 mg/ml stock) were added. Afterwards, the cells were incubated for 12 min at 37°C (water bath) followed by a 40-min incubation step at room temperature. Cells were washed again and the pellet was resuspended in 50 μl of PBS+2% BSA and incubated for 3 min at 37°C. The cells were treated with 1 ml of a pepsin/HCl solution (5 mg enzyme in 15 ml 0.01 M HCl) for 4 min. The reaction was stopped with an excess (3 ml) of ice-cold PBS+2% BSA and cells centrifuged at 20,00 U/min. The supernatant was removed before DNA denaturation was completed with 400 μl of 2 M HCl within a 10-min incubation. Cell suspensions were adjusted to pH 7 by at least two wash steps. Then, the cells with incorporated BrdU were labeled with the FITC-conjugated mouse anti-BrdU antibody (BD Pharmingen, Heidelberg, Germany) at a concentration of 20 μl per test for 45 min, after they were resuspended in 100 μl PBS. Cells were washed and 500 μl PBS were added. The entire DNA was stained stoichiometrically by adding 1.25 μl of PI (stock: 1 mg/ml).

MTS and MTT assays

After 48 h of incubation with either BrdU or PBS, 20 μl of CellTiter 96® AQueous One Solution Reagent (Promega) were pipetted into each well of the 96-well assay plate containing the samples in 100 μl of culture medium. The plate was incubated for 4 h at 37°C in a humidified, 5% CO₂ atmosphere. To measure the amount of soluble formazan produced by cellular reduction of the MTS, the absorbance was recorded immediately at 490 nm using a 96-well plate reader. In some experiments, MTT assays were used: after 48 h of incubation with either BrdU or PBS, the supernatant (1 ml), which contained free-floating spheres, from each well was removed separately and transferred to a fresh 12-well plate so that the adherent cells remained in the original plate. The attached cells obtained 1 ml of fresh media per well. Afterwards, 100 μl of MTT solution (Merck, Darmstadt, Germany) were added to every well of the original plate as well as the new plate. Plates were incubated for 2 h in a humidified, 5% CO₂ atmosphere. Subsequently, the volume

of each well was transferred separately to a 1.5-ml tube and in addition the bottom of every well was rinsed with 200 μl of PBS. Cells were centrifuged for 5 min at 20,800g and most of the supernatant was removed with a pipette. Cells were centrifuged again for 5 min at 20,800g before the rest of the media was carefully removed. The remaining precipitate was solved in 200 μl of MTT solvent (0.1 M HCl in isopropanol) and was pipetted into a 96-well plate. Absorbance was recorded at 590–650 nm using a 96-well plate reader.

Time lapse assays

Cells were treated with 2.5 μM BrdU or PBS 48 h after seeding in T25 culture flasks (Peske, Aindlingen-Arnhofen, Germany). They were grown and maintained in a humidified chamber at 37°C and 5% CO₂. Pictures were taken every 10 min for the next 2 days and data were assembled using analySIS software (Olympus, Hamburg, Germany).

LDH and cell death assays

The supernatant of each well was transferred separately to a 1.5-ml tube after 48 h of incubation with either BrdU or PBS. Afterwards, cells were centrifuged at 240g for 5 min. Aliquots of 50 μl from all tubes were carried over to a fresh 96-well plate and 50 μl substrate mix of CytoTox 96®Non-Radioactive Cytotoxicity Assay (Promega) were added to each well of the plate. Cells were incubated at room temperature for 30 min under protection from light. Subsequently, 50 μl of stop solution were added to each well before the absorbance was recorded at 490 nm. Cell death (necrosis) was further detected by measuring cytoplasmic histone-associated DNA fragments (mono- and oligonucleosomes) in the supernatant of BrdU-treated cells using a photometric enzyme immunoassay (Cell Death Detection ELISA[PLUS]; Roche Diagnostics, Mannheim, Germany).

Zymography

Gelatine zymography was performed as described previously (Tempfer et al. 2009). Briefly, after 48 h of incubation with either BrdU or PBS cells were lysed in a buffer containing 20 mM TRIS-HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, and 0.1% SDS. Equal amounts of protein were loaded on 7.5% polyacrylamide gels containing gelatine (1 mg/mL). Following electrophoresis, the gels were incubated in renaturing buffer (ddH₂O containing 2.5% Triton X-100) with gentle agitation for 30 min. Then, gels were equilibrated in developing buffer (50 mM TRIS base, 50 mM TRIS-HCl, 0.2 M NaCl, 5 mM CaCl₂, 0.02 % Brij 35) for 30 min at room temperature and incubated in

the same buffer overnight at 37°C. The gels were then stained with 0.5 % Coomassie brilliant blue R-250 for 1 h and destained with a destaining solution (50% methanol, 10% acetic acid and 40% water) until clear bands against a dark blue background appeared.

Analysis of cell proliferation and neurogenesis in vivo

All experiments were carried out in accordance with the European Communities Council Directive (86/609/EEC) and institutional guidelines. Animals had ad libidium access to food and water throughout the study. All efforts were made to minimize the number of used animals and their suffering. Fifteen male Fischer-344 rats with a weight around 200 g were randomly divided into 3 groups (5 animals per group). On days 1 and 2, animals received intraperitoneal injections of either BrdU dissolved in NaCl solution (Braun, Melsungen, Germany) (group 2: 50 mg/kg BrdU; group 3: 200 mg/kg BrdU) or NaCl solution alone (group 1) as control. On either day 4 or day 12, animals were intracardially perfused with a 0.9 % saline solution followed by 4% paraformaldehyde in 0.1 M phosphate buffer. The brains were removed, post-fixed in the paraformaldehyde solution overnight at 4°C. Tissues were then cryoprotected in a 30% (w/v) sucrose, 0.1 M sodium phosphate solution (pH 7.4). Brain tissue was cut into 40-μM sagittal cryostat sections using a sliding microtome on dry ice. Sections were stored at –20°C in cryoprotectant solution (ethylene glycol, glycerol, 0.1 M phosphate buffer pH 7.4, 1:1:2 by volume). Every sixth section (240-μm interval) of one hemisphere was selected from each animal and processed for immunohistochemistry. The following antibodies were used: primary antibody was mouse anti-PCNA at 1:500 (Santa Cruz Biotechnology, Heidelberg, Germany), secondary antibody was donkey anti-mouse conjugated with biotin 1:500 (Dianova, Hamburg, Germany). Free-floating sections were rinsed in PBS 3 times for 5 min, incubated in 0.6% H_2O_2/PBS for 30 min and washed again 3 times in PBS. They were incubated for 2 h in 50 % formamide /2xSSC (0.3 M NaCl, 0.03 M sodium citrate) at 65°C. Afterwards, sections were rinsed in 2× SSC for 5 min, incubated for 30 min in 2 M HCl at 37°C, and rinsed for 10 min in 0.1 M borate buffer (pH 8.5) at room temperature. After washing in PBS 4 times for 5 min, sections were incubated in FSG buffer (TBS, 1.0 % BSA, 0.2 % FSG)+0.1% Triton-X (Sigma) for 2 h before they were incubated with mouse anti-PCNA antibody in FSG buffer+0.1% Triton-X overnight at 4°C. The following day, sections were rinsed 3 times in PBS for 5 min and incubated with a biotin conjugated secondary donkey anti-mouse antibody in FSG buffer+0.1% Triton-X for 1 h. During this incubation period, an avidin-biotin-peroxidase solution (Vector Elite Kit; Vector Laboratories, Wertheim,

Germany) was prepared for later use. After 1 h of incubation with the secondary antibody, 3 rinsing steps in PBS for 5 min were performed, before the sections were incubated for 1 h with the avidin-biotin-peroxidase solution. They were rinsed again 3 times for 5 min in PBS and were incubated for 5–10 min in DAB solution (5 ml PBS, 2 drops DAB, 2 drops H_2O_2, 2 drops $NiCl_2$). Sections were washed 4 times in tap water and were mounted onto gelatin-coated glass slides, air dried, dehydrated and coverslipped with Neo Mount (Merck). All morphological analyses were performed on blind-coded slides. To analyze cell proliferation in the DG, PCNA immunopositive cells were counted using a ×40 objective lens of a light microscope (Leica, Wetzlar, Germany) and multiplied by 6 to obtain an estimate of the total immunopositive cell numbers. The reference volume was determined by tracing the granule cell layer of the hippocampal DG using a semiautomatic stereology system (Stereoinvestigator, MicroBrightField, Colchester, USA). All extrapolations were calculated for one hippocampus and should be doubled to represent the total hippocampal values.

For immunofluorescence stainings, sections were pretreated with 2 M HCl for 30 min at 37°C, otherwise staining protocols were as described above. Primary antibodies were rat anti-BrdU 1:500 (AbD Serotec; MorphoSys, Oxford, UK) and goat anti-DCX (Doublecortin) (C-18) 1:250 (Santa Cruz Labs, Santa Cruz, USA), and secondary antibodies were donkey anti-goat, -rat conjugated with Alexa 488 (1:1,000; Molecular Probes, Eugene, USA), rhodamine X (Dianova). Sections were washed extensively and incubated with fluorochrome-conjugated species-specific secondary antibodies for overnight at 4°C. Sections were put on slides and mounted in Prolong Antifade kit (Molecular Probes). Photodocumentation was done using an Olympus microscope equipped with an optigrid system. For quantitative analysis of the DCX epressing cell popultions, 4 randomly selected visual field per animal were used to count DCX and/or BrdU positive cells within the dentate gyrus subgranular and granular zones, and the corresponding tissue area was measured and multiplied by 40 μm to obtain the tissue volume. To obtain the neuronal density, the total number of cells counted is divided by the sample volume and represented as cells/mm^3.

Preparation of respective cell types for transplantation

NPC were incubated with either 0.2, 1.0 or 2.5 μM BrdU in growth medium for 48 h just before transplantation. A sample of single cell suspension was stained with Trypan Blue (Sigma) and counted in a Neubauer hemocytometer. The remaining single cell suspension was washed twice and resuspended in PBS to yield a final concentration of 1.2 x 10^5 NPC/μl.

Cell transplantation into the intact spinal cord

Adult female Fischer 344 rats (Charles River) weighing 160–180 g (3–4 months old) were used for injection of respective cell types into the intact spinal cord. For all surgical procedures, animals were anesthetized using a cocktail of ketamine (62.5 mg/kg; WDT, Garbsen, Germany), xylazine (3.175 mg/kg; WDT) and acepromazine (0.625 mg/kg; Sanofi-Ceva, Düsseldorf, Germany) in 0.9% sterile saline. Animals received a partial laminectomy at cervical level C3. Then, a total volume of 2 µl cell suspension containing 1.2×10^5 NSCs/µl labeled with (1) 0.2 µM BrdU, $n=6$, (2) 1.0 µM BrdU, $n=5$) or (3) 2.5 µM BrdU, $n=6$) was injected stereotactically guided (medio-lateral midline, dorso-ventral–0.08) into the spinal cord through a pulled glass micropipette (100 µm internal diameter) using a Picospritzer II (General Valve, Fairfield, USA). The glass micropipette was raised slightly and left in place for at least 1 min to minimize cell diffusion up the needle track. The implantation site was covered with gelfoam (Gelita Tampon; Braun, Germany) before readapting muscular layers and stapling the skin above the lesion.

Spinal cord histological analysis

At 4 days postoperatively, animals were trNPCardially perfused as described above. The spinal cords were dissected, post-fixed overnight and cyroprotected in 30% sucrose. Cervical spinal cords were cut into sagittal 35-µm-thick cyrostat sections and processed for immunohistochemistry. Every seventh section was taken for Nissl staining to determine the injection site. Sections were stored in 25% glycerol, 25% ethylene glycol, 0.1 M sodium phosphate solution at 4°C. Double-labeling immunofluorescence techniques were performed with free-floating sections to assess the proliferation of the transplanted NSCs in vivo. The sections were washed in TBS, then blocked with a solution composed of TBS+3% donkey serum+0.1% Triton-X100 for 1 h, then transferred into the first primary antibody and incubated overnight at 4°C on a rotating platform. The following primary antibodies were used: rat anti-BrdU (1:500; AbDSerotec, UK) for grafted NSCs, mouse anti-PCNA (1:500; Santa Cruz Labs, Santa Cruz, USA). For detection of BrdU-prelabeled nuclei of adult NSCs, the following DNA denaturation steps preceded the incubation with rat anti-BrdU antibody: after rinsing in TBS, sections were incubated for 1 h in 50% formamide/2xSSC (0.3 M NaCl, 0.03 M sodium citrate) at 65°C. Sections were rinsed in 2× SSC, incubated for 30 min in 2 M HCl at 37°C and rinsed for 10 min on 0.1 M boric acid (pH 8.5). After rinsing in TBS, sections were incubated in TBS+3% donkey serum+0.1% Triton-X100 as described above together with rat anti-BrdU

(1:500; AbDSerotec). The following day, sections were rinsed and incubated with fluorophor rhodamine-X (1:500; Dianova) or fluorescine (1:1000; Molecular Probes) conjugated donkey secondary antibodies for 2 h. After a final rinsing step in TBS, sections were mounted onto glass slides and coverslipped with Prolong Antifade (Invitrogen). Diaminobenzidine (DAB) stainings were obtained using the Vectastain Elite ABC-kit (Vector Laboratories, Burlingame, CA, USA) and DAB-stained sections were dehydrated and mounted in NeoMount (Merck).

Immunhistochemical analysis was performed with a confocal fluorescence microscope (Leica TCS-NT). Co-localization of BrdU-prelabeled NSCs with PCNA was determined by analyzing between 30–35 optical sections through the z-axis of a 40-µm-thick coronal section at ×400 magnification. Co-localization was confirmed, once the differentiation marker was spatially associated to BrdU nuclear labeling through subsequent optical sections in the z-axis. In order to estimate the survival of the transplanted NSCs, the total number of BrdU-labeled cells within the section containing the most cells were counted.

Statistics

Statistical analyses was performed in general with Graph-Pad Prism 5 (GraphPad Software, La Jolla, USA) using one-way analysis of variance (ANOVA) with Tukey's multiple comparison test. Moreover, two-way ANOVA with Bonferroni post-test was used to compare cell cycle phases of BrdU- versus BrdU/DC-treated cells. For the in vivo experiments, Kruskal–Wallis test with Dunn's multiple comparison test was applied. Averages are expressed with their standard deviations reflecting the spread of individual data points around the mean value. In contrast, the standard error as an index of the variability of the sample mean would be less meaningful for the present data.

Results

BrdU inhibits proliferation of NPCs in vitro

To investigate on the effects of BrdU on neural progenitor cell biology, we used NPC cultures derived from adult rat SVZ (Wachs et al. 2006). These cultures grow in spheres (Fig. 1a) consisting of undifferentiated neural progenitor cells and, to a lesser extent, of already lineage-determined precursors (Wachs et al. 2006). Exposure of the cells with BrdU for 48 h in different concentrations from 1.0 to 20.0 µM significantly diminished the expansion rate of the cultures to about 60% of that observed under control conditions as shown by MTS assay (Fig. 1b). The maximal effect was evident already at a concentration of 1 µM BrdU; 0.2 µM BrdU, however, had

no inhibitory effect on proliferation of NPCs, even though a BrdU-immunofluorescence labeling of proliferating cells, which was qualitatively indistinguishable from a labeling intensity achieved with higher concentration, was clearly visible (Fig. 1b-j).

BrdU alters the cell cycle profile of NPCs

Since a reduced proliferation of NPCs should be reflected by changes in the cell cycle, we performed FACS-based cell cycle analysis with propidium-iodide to validate the observed inhibitory effects of BrdU on NPCs. Therefore, we treated cells with different BrdU concentrations ranging from 0 to 20.0 μM for 48 h and compared their cell cycle profile to that of control cells. A typical DNA histogram is shown in Fig. 2a. DNA content FACS analysis indicated a BrdU-induced shift from G2/M- and S-phase toward the G0/G1-phase (Fig. 2b–d). As expected, we observed a statistically significant reduction in the amount of BrdU-treated cells in S-phase from 13% (control) to approximately 8%, when BrdU was applied in concentrations over 0.2 μM. Again, the maximal effect was observed already with 1.0 μM BrdU, while 0.2 μM BrdU had no effect. FACS analysis revealed a significant increase in G0/G1-phase cells from 63% (control) to around 76%, and a significant decrease in G2/M-phase cells from 23% (control) to 15% at every BrdU concentration investigated (0.2–20.0 μM). These findings clearly demonstrate that exposure to BrdU leads to a pronounced alteration in cell cycle distribution.

Desoxycytidine (DC) is known to minimize potential BrdU treatment-associated disturbances in the nucleotide pathway. Thus, experiments were repeated and the medium was supplemented with DC at half-equimolar concentrations of BrdU, respectively. FACS analysis uncovered a significant decrease in S-phase cells from 19% (control) to around 9% and a significant increase in G0/G1-phase cells from 60% (control) to approximately 75% at every BrdU/DC concentration investigated (0.2–20.0 μM BrdU/10.0 μM DC) (Fig. 2e, f). In contrast to the BrdU-treated cells (without DC), which showed a significant reduction in G2/M phase cells (Fig. 2d) compared to control, the amount of G2/M-phase cells after BrdU/DC application remained unchanged at around 16% (Fig. 2g). However, comparing the BrdU- versus BrdU/DC-treated cells, we could not detect any significant differences regarding the alteration in cell cycle distribution after BrdU treatment, indicating that DC was not able to counteract the BrdU-induced effects.

BrdU induces cell death in vitro

To determine whether BrdU administration causes cell death in NPC, cells were treated for 48 h with different concentrations of BrdU from 0.2 to 20.0 μM and LDH assays were performed. A

concentration of only 1.0 μM BrdU significantly increased the amount of LDH in NPC cultures from 15% (control) to about 25% (Fig. 3a), whereas a BrdU concentration of 0.2 μM had no significant influence on cell death. Surprisingly, LDH in the 2.5 and 5.0 μM BrdU-treated groups was significantly reduced to 23 and 24%, respectively, compared to the 1.0 μM BrdU-treated group. Nevertheless, higher concentrations of 10.0 and 20.0 μM BrdU led to a significant elevated level of cell death (27 and 26%, respectively) in comparison with the 2.5 and 5.0 μM BrdU-treated cells, suggesting a dose-dependent effect of BrdU on NPC cell death.

To further strengthen the observation that BrdU induces cell death in NPCs, we performed cell death detection ELISA of BrdU treated cells to identify necrotic cell death in these cultures. Whereas the LDH assay did not detect any effect at 0.2 μM BrdU concentrations, the cell death detection ELISA revealed that the level of necrosis was already significantly increased in the 0.2 μM BrdU-treated cultures; 1.0 μM BrdU further increased the level of necrosis (Fig. 3b). Again, as in the LDH assay, using higher BrdU concentrations such as 2.5 and 5.0 μM BrdU, the absorbance value was significantly decreased compared to the 1.0 μM BrdU-treated cells. Concentrations of 10.0 and 20.0 μM BrdU, however, led to an elevated level of cell death compared to the 2.5 and 5.0 μM treated groups in accordance with the results of the LDH assay. In summary, our experiments clearly demonstrate that exposure to BrdU results in an increase of necrotic cells in NPC cultures.

Concentrations above 1,0 μM BrdU induce adhesion of neurospheres

Along the course of the study, we noticed to our surprise that during the 48 h incubation period with BrdU, neurospheres started to attach. However, the observed effect was dependent on the applied BrdU concentration. Low concentrations like 0.2 μM BrdU had no effect on the spheres, whereas a small amount of spheres that were treated with 1.0 μM of BrdU began to adhere. By contrast, concentrations between 2.5 and 20.0 μM BrdU led to adherence of almost all neurospheres in the culture (Fig. 4a–g). To validate these observations, we performed an MTT assay to measure the activity of living cells via mitochondrial dehydrogenase activity in BrdU-treated and control cells, and we compared the adherent versus the non-adherent cell fraction. Whereas treatment with 0.2 and 1.0 μM BrdU demonstrated no significant effect concerning the adhesion of neurospheres compared to control cells, results showed a significant increase in the amount of adherent cells when BrdU was applied in concentrations of 2.5–20.0 μM (Fig. 4i). Conversely, in comparison with the control, the quantity of free-floating spheres was significantly decreased when BrdU was added in concentrations between 2.5 and 20.0 μM (Fig. 4h). Within

Fig. 1 BrdU inhibits NPC proliferation in vitro. NPCs derived from SVZ were treated for 48 h with different concentrations of BrdU (0.2–20.0 μM). **a** Phase-contrast micrographs of proliferating neurospheres without BrdU treatment (×100 magnification). **b** MTS assay demonstrated an impaired expansion of NPC cultures treated with BrdU in concentrations between 1.0 and 20.0 μM. Data are presented as mean±standard deviation. Significant differences in respect to the control are marked with *asterisks*. ***$p < 0.001$. (**c**–**f**) Phase-contrast micrographs of BrdU labeled NPCs. (**g**–**j**) Fluorescent micrographs of labeled NPCs stained for BrdU. NPCs were untreated (**c**, **g**) or treated with different BrdU concentrations: 0.2 μM (**d**, **h**), 1.0 μM (**e**, **i**), and 20.0 μM (**f**, **j**). Note that already a 0.2-μM dose leads to a immunofluoresence label indistinguishable from higher doses. *Scale bars* 100 μm
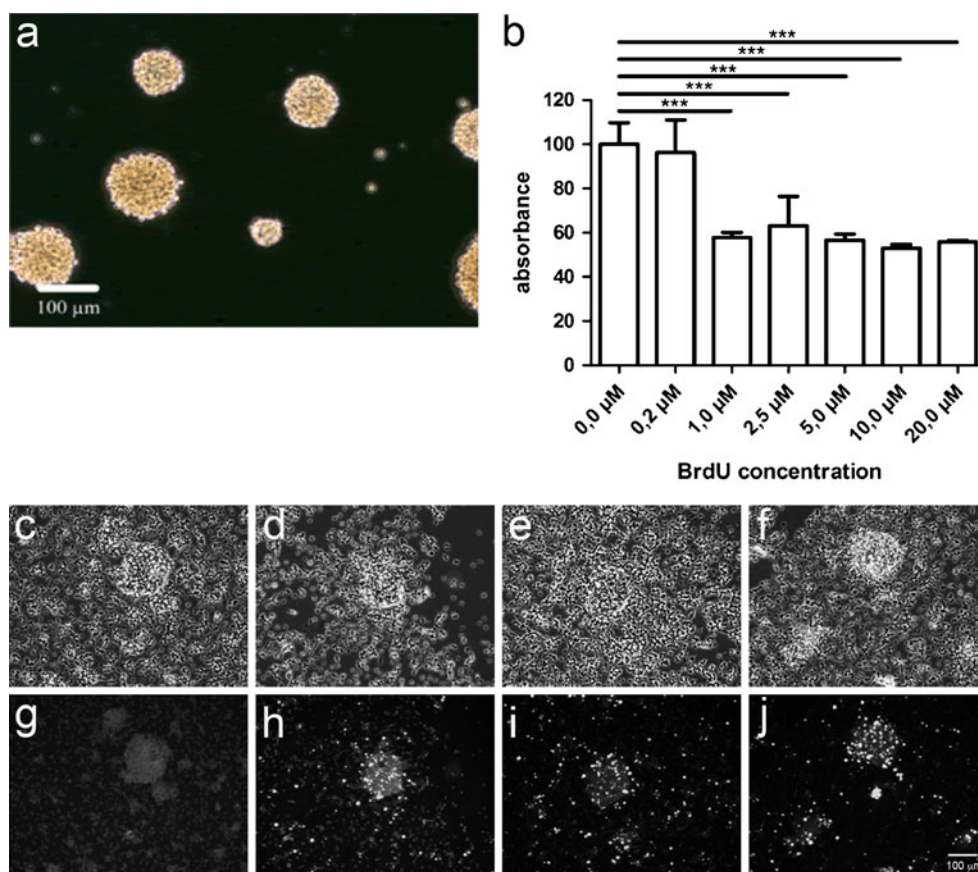
this concentration range, the adherence of neurospheres caused by BrdU as well as the amount of free-floating spheres in the supernatant were dose independent. Indeed, cells that were treated with 1.0 μM BrdU also revealed a significant reduction in the number of free-floating cells in the supernatant compared to control. Since this effect was even more pronounced in the 2.5 μM BrdU-treated cells than in the 1.0 μM BrdU-treated group, this suggests a dose-dependency within this concentration range. Taken together, these observations clearly demonstrate that BrdU induces adhesion of neurospheres when applied in concentrations of 2.5–20.0 μM BrdU.

Reduced levels of active MMP-2 are involved in the induction of neurosphere adherence after BrdU treatment

To identify potential molecules involved in the adhesion of neurospheres after BrdU treatment, we focused on proteins of the matrix metalloproteinase (MMP) family. MMPs are known to be capable of degrading extracellular matrix proteins in physiological and pathophysiological processes. Furthermore, they are thought to play a major role in cell behaviors such as cell proliferation, migration (adhesion/dispersion), differentiation, and apoptosis (Elkington et al. 2005). Therefore, we hypothesized that some of these

matrix metalloproteinases might participate in the induction of adherence of BrdU-treated neurospheres.

To corroborate these speculations, we applied gelatine zymography to analyze cell lysates of control and BrdU-treated cells for the presence of gelatinolytically active proenzyme, activation intermediate, and mature forms. We explored some of the key effectors of extracellular matrix remodeling, namely MMP-2 and MMP-9. In the case of MMP-2, all the functional forms of the enzyme that have been described to date (active MMP-2 with a size of 62 kD, pro-MMP-2 of about 68 kD, and the activation intermediate of 64 kD) were present in control and 1.0 μM BrdU-treated cells, with the control monitoring the highest gelatinase activity (Fig. 4j). Cells that were treated with 2.5 μM BrdU also demonstrated a high level of proMMP-2, whereas only a very low amount of active MMP-2 was observed. In agreement with lower BrdU concentrations, the 20.0 μM BrdU-treated cells displayed a high amount of proMMP-2. Indeed, the double band representing the active forms of MMP-2 was completely absent in this group. Concerning MMP-9, no enzyme was detected in the cell lysates of neither control nor BrdU-treated cells. In summary, our observations imply that a reduced level of active MMP-2 might be responsible for the adhesion of BrdU-treated neurospheres.
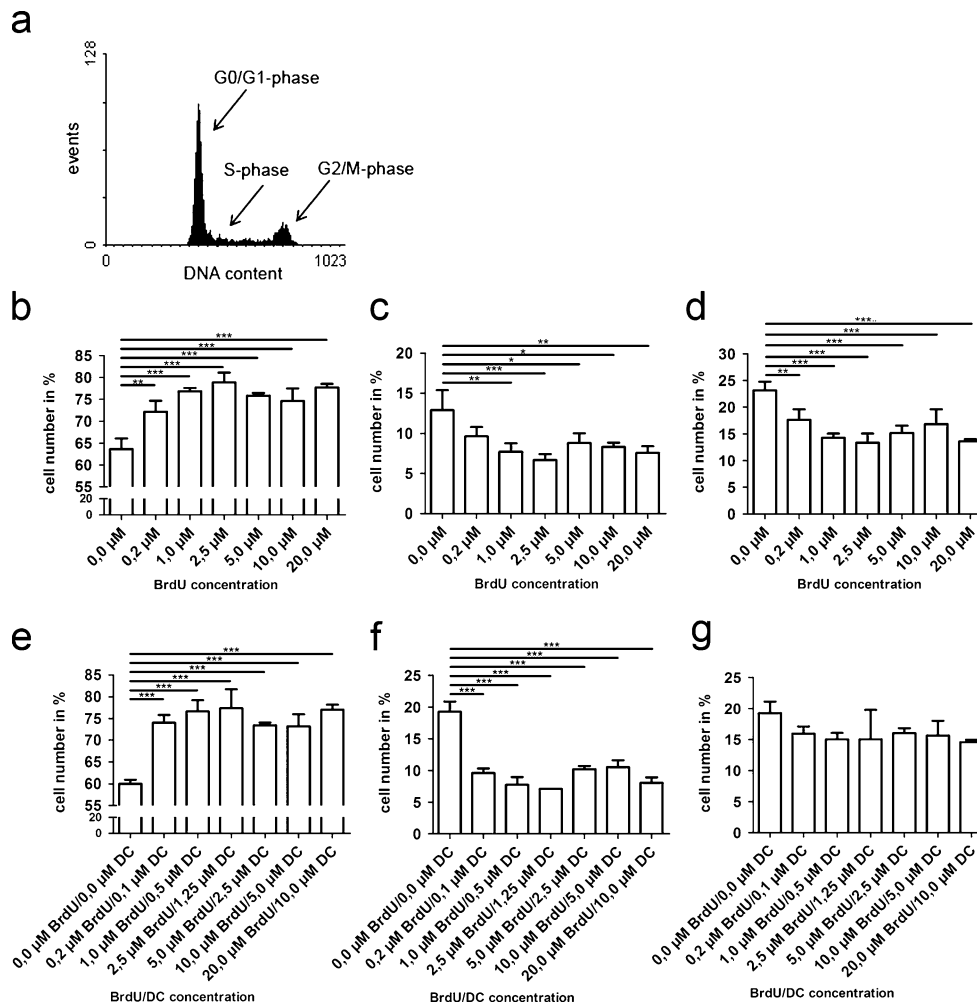
Fig. 2 BrdU affects cell cycle distribution in NPC cultures. NPCs derived from SVZ were treated for 48 h with different concentrations of BrdU (0.2–20.0 μM). a Representative cell cycle profile (*histogram*) of proliferating cells, displaying the different cell cycle phases. The DNA was stained with propidium iodide. b–d Quantification of cell cycles phases with Win MDI 2.8 showed an increase in the number of cells within the G0/G1-phase (b) of the cell cycle after BrdU treatment, whereas the fraction of S- and G2/M-phase (c and d, respectively) cells was decreased. Data are presented as mean±standard deviation (*n*=3). Significant differences in respect to the control are marked with asterisks. *$p<0.05$, **$p<0.01$, ***$p<0.001$. e–g Quantification of cell cycles phases after a combined BrdU/DC treatment again demonstrated an elevated G0/G1-phase (e) and a reduced S- and G2/M-phase (f and g, respectively), indicating that DC was not able to counteract the BrdU induced effects on cell cycle. Data are presented as mean±standard deviation (*n*=3). Significant differences in respect to the control are marked with *asterisks*. ***$p<0.001$

BrdU incorporation results in phenotypic changes in NPC cultures

Due to the fact that BrdU is widely used to label proliferating cells, we investigated potential effects of BrdU on the differentiation fate of NPCs, which are in general capable of generating neurons, astrocytes and oligodendrocytes. In order to assess multipotency, we used quantitative RT-PCR (qRT-PCR) to examine the expression level of doublecortin (DCX, neuronal precursor cells and young immature neurons), glial fibrillary acidic protein (GFAP, astrocytes) and myelin basic protein (MBP; oligodendrocytes).

After 7 days of incubation under neuronal differentiation conditions (NC), we found that 0.2 μM BrdU induced a highly significant reduction in relative expression of DCX mRNA from approximately 2.1 (control) to 1.6 (0.2 μM BrdU) (76.2% of control) (Fig. 5a). Cells that were treated with 1.0 μM BrdU displayed a relative mRNA expression level of 0.2, that was significantly decreased by 8-fold with respect to the 0.2 μM BrdU-treated group, whereas 20.0 μM BrdU-treated cells demonstrated no DCX expression at all. These data suggest a dose-dependent effect of BrdU on the expression of the neuronal precursor cell marker DCX in the concentration range between 0.0 and 1.0 μM BrdU. Higher BrdU concentrations like 20.0 μM had no further inhibitory effects on DCX expression, since they showed a non-significant decrease in DCX mRNA compared to the 1.0 μM BrdU-treated group. Cells that were exposed to oligodendroglial

Fig. 3 BrdU promotes cell death in NPC cultures. NPCs derived from SVZ were treated for 48 h with different concentrations of BrdU (0.2–20.0 μM). **a** LDH assay (LDH values are presented relative to MTS values) and **b** Cell death detection ELISA revealed a dose-dependent effect of BrdU on cell death in NPC cultures. Data are presented as mean±standard deviation. Significant differences in respect to the control are marked with *asterisks*. ***p< 0.001
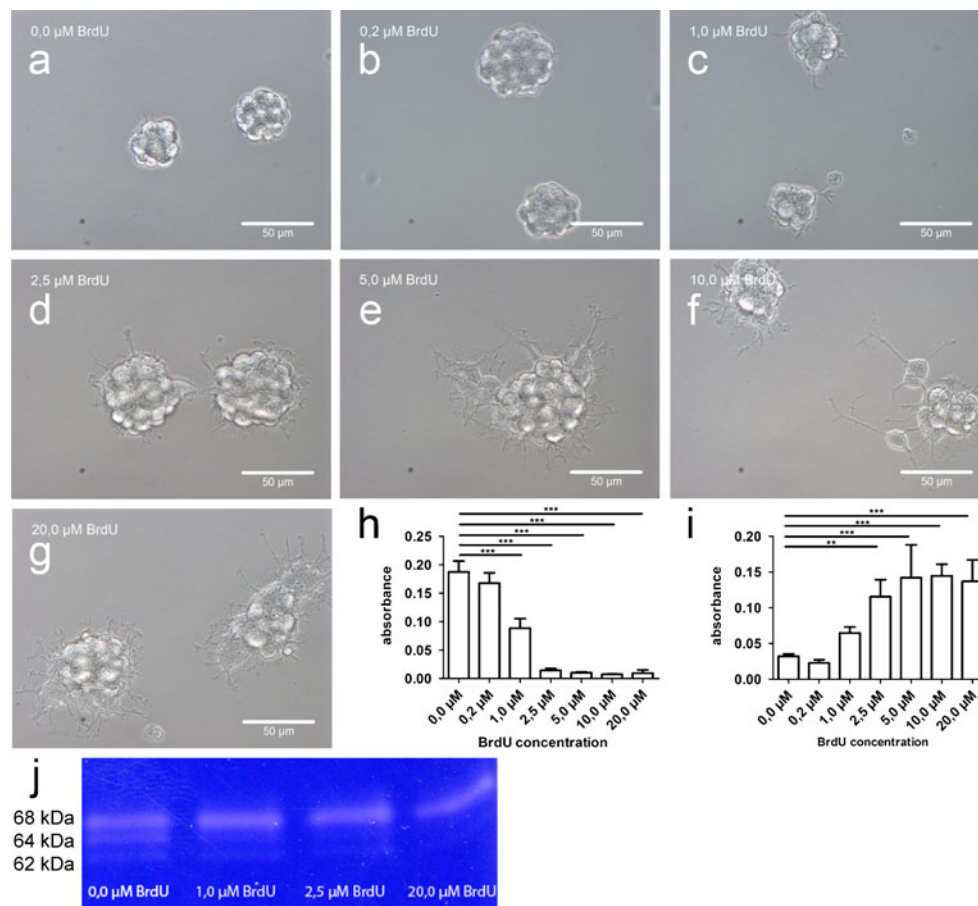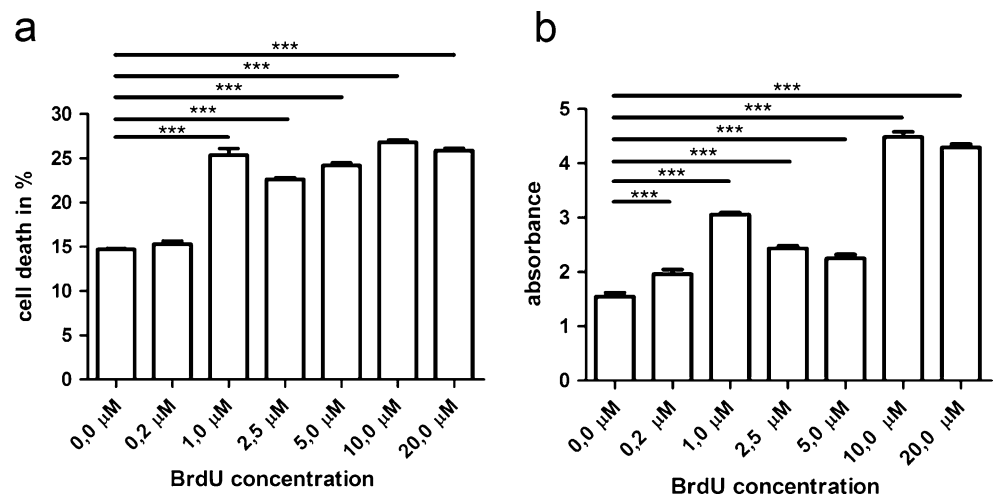
Fig. 4 BrdU induces adhesion of neurospheres when applied in concentrations higher than 1.0 μM. NPCs derived from SVZ were treated for 48 h with different concentrations of BrdU (0.2–20.0 μM). **a–g** Phase-contrast micrographs of BrdU-treated NPC cultures in a ×40 magnification. *Scale bars* 50 μm. NPCs were untreated (**a**) or treated with 0.2 μM (**b**); 1.0 μM (**c**); 2.5 μM (**d**); 5.0 μM (**e**); 10.0 μM (**f**) and 20.0 μM (**g**) BrdU. **h, i** MTT assay revealed a strong increase in the amount of adherent cells (**i**) accompanied by a robust decrease of cells in the cell culture supernatant (**h**) if cultures were treated with BrdU concentrations of 2.5 μM or higher. Data are presented as mean±standard deviation. Significant differences in respect to the control are marked with *asterisks*. **p<0.01, ***p<0.001. **j** Gelatin zymography demonstrated a reduced amount of active MMP-2 with increasing BrdU concentrations, which is supposed to be responsible for the induction of adherence in BrdU-treated cultures

differentiation conditions (OC) for 7 days demonstrated a significant impairment in MBP mRNA expression from 2.4 units (control) to 0.2 units (8,3% of the control) (1.0 μM BrdU-treated group) and 0.3 units (12,5% of the control) (20.0 μM BrdU-treated group) (Fig. 5c). On the other hand, administration of 0.2 μM BrdU had no significant effect concerning the expression of MBP with respect to control cells. In BrdU applications subsequent to astroglial (AC) differentiation conditions, there was a tendency towards elevated expression of GFAP mRNA, when BrdU was applied in concentrations of 1.0 and 20.0 μM (Fig. 5b). However, this observation was not statistically significant. To analyze if a shorter BrdU treatment would lead to similar changes in the differentiation phenotype, cells were treated with different BrdU concentrations for 6 h and analyzed as above. While there was no significant effect on DCX expression, the expression of MBP was similarly affected as in the 48 h treated groups (data not shown). Taken together, our data point to an altered expression of markers for neuronal precursor cells as well as mature oligodendrocytes as demonstrated by DCX and MBP expression, respectively, suggesting an aberrant progression of cell differentiation pathways when NPC are exposed to BrdU.

Owing to the massive changes in DCX and MBP mRNA expression, we analyzed the differentiation potential of the BrdU-treated cells by immunostainings for the lineage markers Map2ab (mature neurons), GFAP (astrocytes) and GalC (oligodendrocytes) after 7 days exposure with neuronal, astroglial and oligodendroglial differentiation conditions. Surprisingly, even cultures treated with 20.0 μM were showing MAP2ab, GalC and GFAP expression (Fig. 5d–f). This indicates that cells grown in the presence of BrdU, although they respond with changes in oligodendroglial and neuronal gene expression, retain their multipotent capacity.

BrdU has no immediate effect on endogenous NPC proliferation and neurogenesis in vivo

To investigate the effects of BrdU on endogenous cell proliferation and neurogenesis in vivo, animals received daily intraperitoneal injections of either 0, 50 or 200 mg/kg BrdU dissolved in NaCl solution or NaCl solution alone as a control for two consecutive days. After 48 h, animals were intracardially perfused. Cell proliferation was analyzed by quantification of PCNA-positive cells in the dentate gyrus of the hippocampus (Fig. 6a, b). After infusion of BrdU, no marked reduction in the number of PCNA-positive cells was observed in the dentate gyrus of neither the 50 mg/kg nor the 200 mg/kg BrdU-treated animals compared to control (Fig. 6b), suggesting that in vivo application of BrdU in doses that are typically used in neurogenesis studies does not interfere with progenitor proliferation in vivo, at least in the acute situation.

Unfortunately, the effects of in vivo BrdU labeling on the differentiation fate of proliferating cells is not approachable, since the cell fate analysis actually requires BrdU labeling. To circumvent this limitation, we analyzed the effects of BrdU treatments on neurogenesis by investigating the DCX populations of newly generated young immature neurons in the dentate gyrus (Fig. 6c, d). The quantitative analysis demonstrated that neither the total number of DCX expressing cells nor the DCX/BrdU double-labeled cells, nor the DCX-positive/BrdU-negative population differed between the high (200 mg/kg) and the low (50 mg/kg) doses (Fig. 6d). Also, the relative amount of DCX/BrdU double-labelled cells compared to the total population of DCX-positive cells was similar in the two groups (Fig. 6d).

Detection of BrdU prelabeled NPCs in vivo after transplantation

Finally, we aimed to analyze if the BrdU labeling of proliferating NPCs would affect their proliferative capacity after transplantation in vivo. NPCs were pre-labeled with either 0.2, 1.0 or 2.5 μM BrdU for 48 h before they were transplanted into the intact spinal cord of adult female Fischer 344 rats. At 4 days postoperatively, animals were transcardially perfused and double-labeling immunofluorescence techniques (BrdU/PCNA) were performed with free-floating sections to assess the proliferation of the transplanted cells in vivo. In addition, for immunohistological detection of the incorporated BrdU, a BrdU/DAB staining was employed to analyze the number of BrdU-labeled cells. The staining revealed a slight, but non-significant increase in the number of BrdU-positive cells labeled with 1.0 or 2.5 μM BrdU with respect to 0.2 μM BrdU-treated cells (Fig. 7a). This observation was in clear contrast to our in vitro data. Based on this, it seems that negative side effects associated with BrdU application are more pronounced in vitro than in vivo, at least in the observed concentration range. However, we cannot exclude the possibility that pre-labeled cells, which might have been harmed by BrdU application, died during the transplantation and are, therefore, no longer detectable. Alternatively, continuous proliferation of transplanted cells might have diluted the BrdU label in the low dose group. This assumption implicates that only part of the cells labeled with 0.2 μM BrdU are detected and this in turn might lead to an underestimation of BrdU-positive transplanted cells. In fact, immunfluorescence stainings exhibited a trend towards higher percentage of PCNA-positive cells that co-label with BrdU after treatment with 1.0 and 2.5 μM BrdU (Fig. 7b) compared to the 0.2 μM BrdU-treated cells. To evaluate our assumption, we used FACS analysis to determine the intensity of BrdU staining in 0.2, 1.0 and 2.5 μM BrdU-treated cells. As illustrated in Fig. 7c, d, BrdU staining intensities were considerably lower in 0.2 μM BrdU
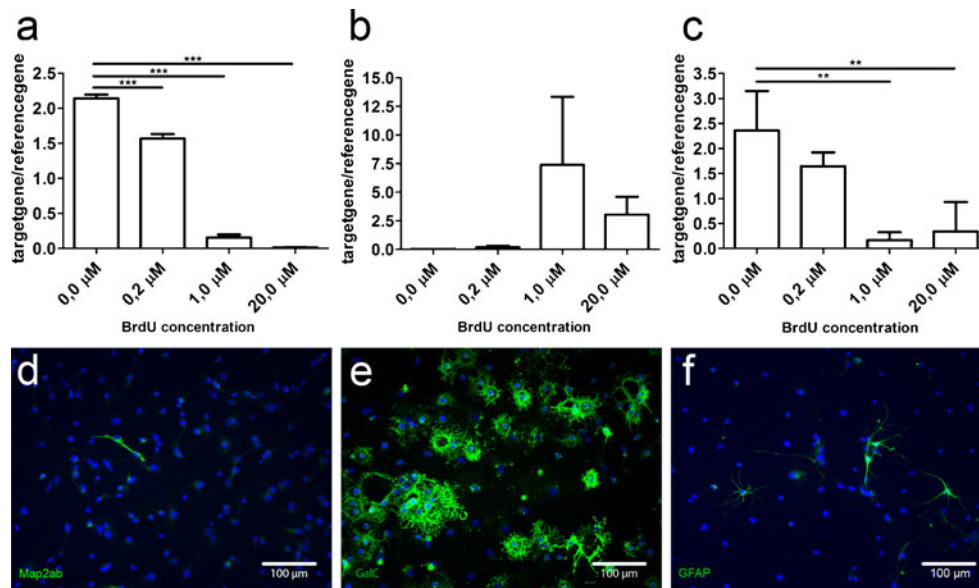
Fig. 5 BrdU affects the differentiation fate of NPCs, but does not interfere with their multipotency. NPCs derived from SVZ were treated for 48 h with different concentrations of BrdU (0.2–20.0 μM). Subsequently, media were changed to neuronal, oligodendroglial or astroglial differentiation conditions and cells were incubated therein for 7 days. Afterwards RNA was isolated. **a–c** Real-time PCR was performed to quantify relative expression of **a** Dcx, a marker for neuronal precursor cells, **b** GFAP, a marker for astrocytes, and **c** Mbp, a marker for oligodendrocytes. **d–f** Alternatively, immunofluorescent stainings was performed in differentiated NPCs, previously treated with 20.0 μM BrdU, to detect the expression of **d** Map2ab in neurons, **e** GalC in oligodendrocytes, and **f** GFAP in astrocytes. Altered phenotype of cells after BrdU treatment. Dcx and Mbp expression were found to be significantly reduced in cells treated with 1.0 μM BrdU. However, GFAP expression remained unchanged. Data are presented as mean±standard deviation. Significant differences in respect to the control are marked with *asterisks*. ***p<0.01, ***p<0.001.* Multipotency was not affected by BrdU treatment. Even cells treated with 20.0 μM were able to generate astrocytes, oligodendrocytes and neurons. *Scale bars* 100 μm
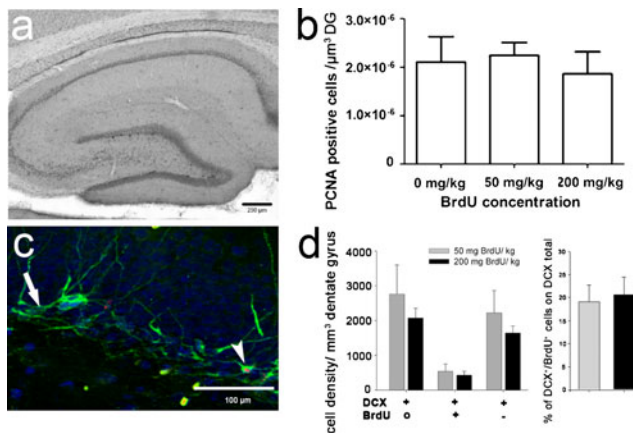
Fig. 6 BrdU labeling does not affect endogenous proliferation in vivo. Adult Fischer 344 rats received intraperitoneal injections of BrdU (50 or 200 mg/kg) or NaCl solution (control) on two consecutive days. After 48 h, animals were perfused and sections were processed for immunohistochemical analysis. **a** Representative PCNA staining in the hippocampal region. *Scale bar* 250 μm. **b** Quantification of PCNA density in the dentate gyrus (DG) of the hippocampus, showing no significant differences between control and BrdU-treated animals. **c** Immunohistological staining showing DCX-positive (*green*) and BrdU-labeled (*red*) cells in the dentate gyrus of a BrdU-treated (50 mg/kg) animal. *Arrow* points towards a DCX-positive/BrdU-negative cell. *Arrowhead* highlights a DCX/BrdU double-positive cells. *Scale bars* 100 μm. **d** Quantitative analysis of DCX populations in the dentate gyrus after BrdU labeling. Data are presented as mean±standard deviation (*n*=5)

compared to 1.0 μM BrdU-treated cells. This points toward an underestimation of transplanted cells in the low dose group. Thus, potentially higher levels of cell death in the 1.0 and 2.5 μM BrdU-treated cells (with respect to the low dose group) might have been obscured by the apparently depressed number of BrdU positive cells in the 0.2 μM BrdU-labeled group. Finally, we conclude that labeling of cells with 0.2 μM BrdU is not sufficient to track transplanted cells in vivo and may lead to misinterpretation of results.

Discussion

The use of BrdU in labeling newly generated neurons was a crucial and essential step in neurogenesis research such as in the first demonstration of neurogenesis in the adult human brain (Eriksson et al. 1998). The huge impact of this method on the scientific progress in the field and some initial data demonstrating the BrdU application during pregnancy in rodents did not have any obvious toxic effects on the progeny in terms of litter size, pup weight and neurological function (Miller and Nowakowski 1988), led to the neglection of any possible side effects of BrdU incorporation. This is surprising, since earlier studies had demonstrated quite unambiguously that BrdU has detrimental effects on fetal and

neonatal development (Garner 1974; Agnish and Kochhar 1976; Pollard et al. 1976; Barasch and Bressler 1977). Moreover, more recent work has demonstrated that BrdU impairs proliferation of mouse and human fibroblasts, of lymphocytes, and of carcinoma cell lines in vitro (Poot et al. 1994; Diermeier et al. 2004). The present work was aimed to comprehensively and systematically analyze the effects of BrdU on NPCs' proliferation, cell cycle, cell death and differentiation in vitro and partially also in vivo. We demonstrate that already a low concentration of 0.2 and 1.0 μM BrdU, which is at the lower end of what is typically used in BrdU-labeling experiments in vitro, inhibited cell proliferation of rat NPCs in cell culture. This is in agreement with recently published data demonstrating anti-proliferative effects of BrdU on mouse neural stem and progenitors in vitro (Ross et al. 2008). This study also demonstrated that a short pulse of BrdU in vivo reduces the proliferative capacity of neurosphere-forming cells derived from the subventricular zone (Ross et al. 2008). Despite this robust anti-mitotic effect of BrdU on NPCs in vitro, 50 or 200 mg/kg BrdU, a typical dose used in in vivo studies, did not affect cell proliferation in the hippocampal neurogenic niche in vivo, at least in the acute situation. This is consistent with findings showing that 40–480 mg/kg of BrdU did not affect NPCs' proliferation in the dentate gyrus (Hancock et al. 2009). Moreover, a daily dose of 50 mg/kg BrdU for a total period of 12 days or doses of up to 300 mg/kg BrdU in adult rats neither caused weight loss or behavioral abnormalities nor inhibited cell proliferation in the hippocampus (Cameron and McKay 2001; Cooper-Kuhn and Kuhn 2002). The present data suggest that the BrdU treatment did not affect neurogenesis at the level of the DCX-expressing population of young neurons. However, we cannot exclude a more protracted effect of BrdU on NPCs biology, for example during functional integration. Experiments using co-labeling of proliferating cells with, for example, viral labeling and BrdU incorporation could approach such issues.

At present, we can only speculate on the apparent discrepancy between the in vitro and in vivo effects of BrdU incorporation. An obvious reason might be the relatively short bio-availability of BrdU in vivo and the limited penetrance through the blood–brain barrier. The extracellular concentration of BrdU in the body has already decreased dramatically 2 h after BrdU injection (Packard et al. 1973; Hayes and Nowakowski 2000). Considering that the cell cycle's S-phase lasts typically for about 8 h, a significant proportion of cells in the S-phase might not be labeled within the 2 h of BrdU bio-availability (Phuphanich and Levin 1985). Indeed, only about 40% of all dividing cells can be detected by BrdU immunohistochehmistry after a single BrdU pulse (Kuhn and Cooper-Kuhn 2007). A further aspect might be the fact that BrdU is not necessarily stably integrated into DNA, since it is subjected to dehalogenation followed by an excision of the

uracil residue and degradation (Hume and Saffhill 1986). Differences in BrdU metabolism might contribute to the higher vulnerability of NPCs to BrdU in vitro compared to in vivo.

What are the underlying mechanisms of the anti-proliferative effect of BrdU on NPCs in culture? The present data on cell cycle analysis demonstrate that BrdU in concentrations of 1.0 μM and higher induce a shift from S- and G2/M-phase to the G0-phase of the cell cycle. Since DC, which is known to rescue cells from BrdU-induced effects on the nucleotide metabolism (Kaufman and Davidson 1978; Davidson and Kaufman 1979), did not save NPCs from BrdU-induced anti-proliferative effects, a mechanism related to nucleotide metabolism seems not to be involved. Alternatively, BrdU directly harms DNA, alters the binding properties of DNA regulatory proteins due to alterations in the DNA's tertiary structure, changes the DNA methylation pattern and destabilizes DNA, which might cause chromosomal aberrations and mutations (Goz 1977; Poot et al. 1994; Kuhn and Cooper-Kuhn 2007; Taupin 2007). In addition, BrdU might affect cell signaling pathways, which are crucially involved in cell proliferation. This, at least, was suggested by experiments of Caldwell et al. (2005), showing that an incubation of cells of the striatum—a region in close proximity to the SVZ—with 10.0 μM BrdU resulted in a decreased ERK phosphorylation and hence an increase in cell death compared to control cells. These findings raise the question, whether similar mechanisms cause the BrdU-induced cell death in adult NPC cultures and whether the observed cell death is attributed to an impaired phosphorylation or rather an inhibition of ERK at higher BrdU concentrations. Since a DNA array expression analysis performed with cells derived from the SVZ yielded—in contrast to the activation of ERK1/2 in hippocampal cell cultures (Zhu et al. 2002)—a reduced expression of ERK1 (=Mapk3) after transforming growth factor (TGF)-beta1 treatment. and further experiments have shown that treatment of adult NPCs with BrdU induces expression of TGF-beta1 (unpublished data), the involvement of ERK signaling in BrdU-mediated cell death of adult NPCs seems at least not implausible.

Besides its anti-proliferative effects on NPCs, BrdU inhibited neuronal and oligodendroglial differentiation as demonstrated by reduced DCX and MBP expression. BrdU-induced inhibition of neuronal differentiation was previously demonstrated using fetal neuronal precursors (Caldwell et al. 2005). In contrast to the study by Caldwell et al., which reported such effects only in BrdU concentrations of 1.0 μM and higher, we demonstrate a reduced DCX expression already with 0.2 μM BrdU. Moreover, the Caldwell study did not observe any effects on oligodendroglial differentiation. The reason for these differences might be found in the different nature and/or origin of the
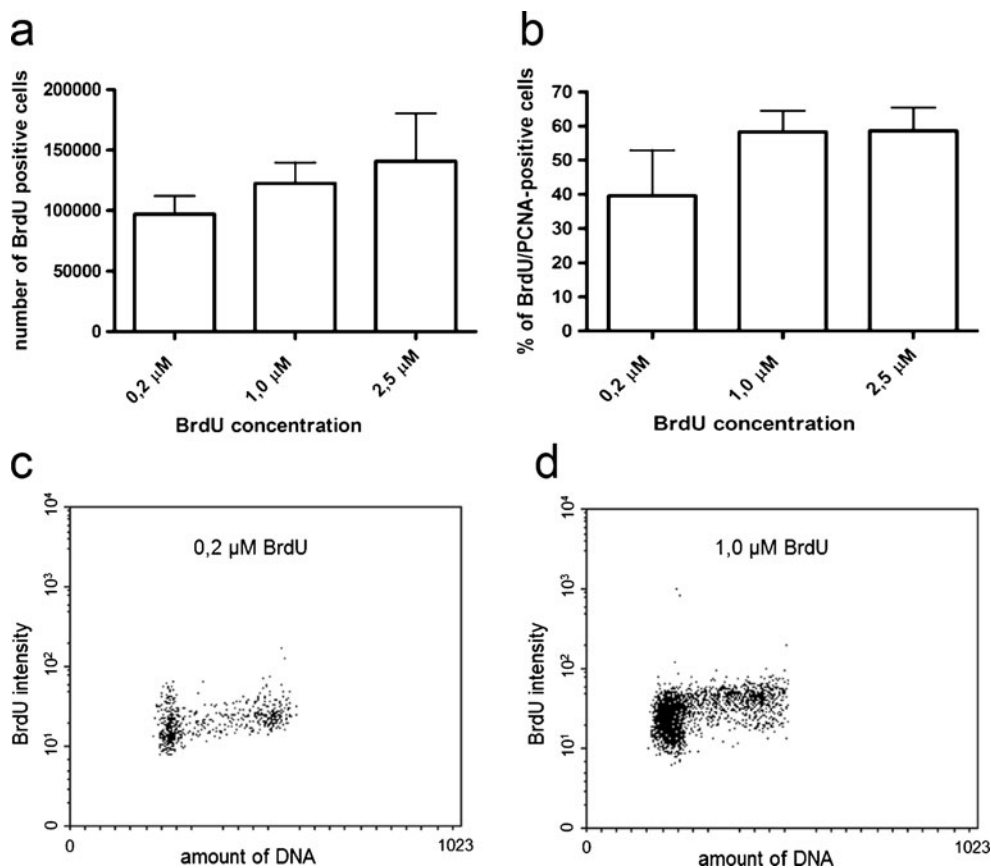
Fig. 7 Incorporated BrdU appears not to interfere with proliferation of transplanted cells in vivo. NPCs derived from the SVZ were treated for 48 h with different concentrations of BrdU prior to transplantation into the intact spinal cord of adult rats. After 4 days, animals were perfused and immunohistochemical stainings for **a** BrdU or **b** BrdU/PCNA were performed. **a** Quantification of BrdU staining, demonstrating no obvious effect of different BrdU concentrations on the number of BrdU-labeled cells. Data are presented as mean±standard deviation. **b** Quantification of BrdU/PCNA staining. No significant differences in proliferation of BrdU-labeled cells were observed. Data are presented as mean±standard deviation. **c, d** NPCs derived from the SVZ were incubated with BrdU in concentrations of **c** 0.2 or **d** 1.0 μM for 20 min and stained with an antibody against BrdU and propidium iodide (DNA staining). Afterwards, FACS analysis was performed to assess the intensity of BrdU staining at different BrdU concentrations. Representative dot plots of BrdU versus PI signal are shown. Note the considerably lower BrdU staining intensity after incubation with 0.2 μM BrdU compared to the 1.0 BrdU-treated group, suggesting that the number of BrdU-positive cells in the low doses group (0.2 μM BrdU) in Fig. 7a might have been underestimated due to the continuous proliferation of BrdU-labeled cells after transplantation

cells, meaning adult SVZ-derived neural progenitors versus fetal striatal precursors. In agreement with recently published data (Caldwell et al. 2005; Ross et al. 2008), we noticed that BrdU treatment of neural progenitors or precursors does not abolish the general ability to differentiate into the three neural lineages. However, a long-term 7-day exposure of neurosphere-forming cells to BrdU dramatically inhibited neuronal and astroglial differentiation (Ross et al. 2008) underscoring the negative side effects of the use of BrdU in neural stem cell biology in vitro. In vivo, however, BrdU did apparently not influence neuronal differentiation, even in doses of 240 and 480 mg/kg (Hancock et al. 2009).

In addition to the effects of BrdU on NPCs' proliferation and differentiation, we noticed that BrdU-induced cell death in NPC cultures. Similar observations were made in vivo

during fetal and neonatal development, where BrdU induced neuronal cell death (Bannigan 1985; Nagao et al. 1998), and in vitro, where high concentrations of BrdU (10.0 μM) caused a 30% reduction in neuronal precursor survival (Caldwell et al. 2005). In contrast to these findings, Ross et al. (2008) did not find any signs of cell death in mouse neural stem and progenitor cultures after a 24 h treatment with 50,0 μM BrdU. Moreover, the BrdU treatment induced a resistance in the mouse neural stem and progenitors to a chemically induced apoptosis (Ross et al. 2008). The reason for the apparent discrepancy between the Ross et al. study and other data including the present work is unclear, but species differences might contribute to it, as we recently demonstrated major differences between mouse and rat derived neural progenitors (Steffenhagen et al. 2011). While we used rat-derived neurospheres, the

Ross et al. study used mouse neural stem and progenitor cultures.

A surprising and to our knowledge novel finding of the present work is the dose-dependent BrdU-induced adherence of neurosphere cells. There is only one study in the literature, which relates BrdU treatment to alterations in cell adherence. But in contrast to our findings, BrdU-treated fibroblasts showed a reduced adherence to the cell culture plate (Berger 2001). The BrdU effect on NPC adherence apparently involved inhibition or lack of MMP-2 activation. While the expression of the pro-enzyme was not affected by the BrdU treatment, the MMP-2 activity was reduced in the presence of BrdU. Elevated levels of members of the tissue inhibitors of metalloproteinase proteins (TIMPS) might be responsible for this effect, since TIMPS are the main regulators/inhibitors of MMP activity (Gomez et al. 1997; Sternlicht et al. 1999). This, however, requires further investigation.

The versatile effects of BrdU on adult NPCs presented here urge for caution in the interpretation of proliferation and differentiation data on BrdU-treated NPCs. Besides the effects presented here, it has to be mentioned that BrdU gets incorporated in cells undergoing DNA-repair or re-entering the cell cycle (Taupin 2007). This is in particular of importance, since terminal differentiated post-mitotic neurons of the adult brain are able to activate cell cycle-associated proteins and may re-enter the cell cycle prior to be ultimately subjected to cell death (Katchanov et al. 2001; Herrup and Arendt 2002). For example, hypoxic and ischemic conditions induce neurons to re-initiate DNA synthesis as shown by expression of G1/S-phase transition markers and by detection of BrdU incorporation. Simultaneously, the majority of BrdU/NeuN-immunoreactive cells in this study showed a positive TUNEL-staining, indicating the apoptotic nature of these cells (Kuan et al. 2004). Thus, data showing elevated rates of BrdU uptake after brain injury should consider the possibility that the BrdU-labeled cells might be damaged rather than proliferating cells. A further issue that needs to be considered is the possibility of graft to host transfer of BrdU. Here, a recently published study suggests that BrdU may be transferred from transplanted cells into the genome of the recipient, e.g., into adult neuronal cells (Burns et al. 2006). This again, importantly, would contribute to a misinterpretation of gained data.

Despite the critical aspects listed above, there are currently only a few alternatives to BrdU labeling for the detection of proliferating cells available. Retroviral labeling has the advantage, that transgenes are passed to all progeny of the primarily infected cell, irrespective of how many divisions this cell runs through, whereas BrdU is diluted in the course of each cell cycle. The fundamental disadvantage of this method, however, is due to a potential inactivation of the promoter, that hence could result in an impaired or actually complete absent

transgene expression (Kuhn and Cooper-Kuhn 2007). In contrast, endogenous markers like PCNA and Ki67 indeed qualify excellently to analyze biopsies and human postmortem tissues, where utilization of BrdU–unless yet applied in one`s lifetime in the context of cancer-therapeutic reasons–is not arguable. However, in particular for the detection of neurogenesis, these markers are quite inappropriate, since their expression does not account for newly generated neurons, but endorses solely the proliferative activity of cells investigated. A stringent proof of neurogenesis by means of PCNA or Ki67 therefore absolutely requires a combination with additional phenotypic markers such as DCX (Eisch and Mandyam 2007).

In summary, in the present study, we aimed to investigate in detail the possible implications of a thymidine substitution by BrdU on different parameters of adult rat NPC cultures. The experiments showed that BrdU exerts a cytotoxic action on adult NPCs and negatively influences their proliferation, cell cycle and differentiation as well as their survival, and affects the cells' adhesion properties in vitro. These critical, but nevertheless often underestimated or rather unattended, aspects of BrdU labeling underscore the need to investigate the potential impact of this marker on the employed model system prior to the planned experiments. Collectively, labeling of mitotic cells with BrdU constitutes an adequate approach, given that one gets straight to the fact that its application can basically hold risks, and definitely calls for careful attention.

References

Agnish ND, Kochhar DM (1976) Direct exposure of postimplantation mouse embryos to 5-bromodeoxyuridine in vitro and its effect on subsequent chondrogenesis in the limbs. J Embryol Exp Morphol 36:623–638

Bannigan JG (1985) The effects of 5-bromodeoxyuridine on fusion of the cranial neural folds in the mouse embryo. Teratology 32:229–239

Barasch JM, Bressler RS (1977) The effect of 5-bromodeoxyuridine on the postnatal development of the rat testis. J Exp Zool 200:1–8

Berger S (2001) Untersuchung der Wirkung von Schwerionenstrahlen auf menschliche Hautfibroblasten unter besonderer Berücksichtigung chromosomaler Veränderungen. Dissertation, Universität Mainz

Burns TC, Ortiz-Gonzalez XR, Gutierrez-Perez M, Keene CD, Sharda R, Demorest ZL, Jiang Y, Nelson-Holte M, Soriano M, Nakagawa Y, Luquin MR, Garcia-Verdugo JM, Prosper F, Low WC, Verfaillie CM (2006) Thymidine analogs are transferred from prelabeled donor to host cells in the central nervous system after transplantation: a word of caution. Stem Cells 24:1121–1127

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

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

Cooper-Kuhn CM, Kuhn HG (2002) Is it all DNA repair? Methodological considerations for detecting neurogenesis in the adult brain. Brain Res Dev Brain Res 134:13–21

Davidson RL, Kaufman ER (1979) Resistance to bromodeoxyuridine mutagenesis and toxicity in mammalian cells selected for resistance to hydroxyurea. Somatic Cell Genet 5:873–885

Diermeier S, Schmidt-Bruecken E, Kubbies M, Kunz-Schughart LA, Brockhoff G (2004) Exposure to continuous bromodeoxyuridine (BrdU) differentially affects cell cycle progression of human breast and bladder cancer cell lines. Cell Prolif 37:195–206

Eisch AJ, Mandyam CD (2007) Adult neurogenesis: can analysis of cell cycle proteins move us "Beyond BrdU"? Curr Pharm Biotechnol 8:147–165

Elkington PT, O'Kane CM, Friedland JS (2005) The paradox of matrix metalloproteinases in infectious disease. Clin Exp Immunol 142:12–20

Eriksson PS, Perfilieva E, Bjork-Eriksson T, Alborn AM, Nordborg C, Peterson DA, Gage FH (1998) Neurogenesis in the adult human hippocampus. Nat Med 4:1313–1317

Garner W (1974) The effect of 5-bromodeoxyuridine on early mouse embryos in vitro. J Embryol Exp Morphol 32:849–855

Gomez DE, Alonso DF, Yoshiji H, Thorgeirsson UP (1997) Tissue inhibitors of metalloproteinases: structure, regulation and biological functions. Eur J Cell Biol 74:111–122

Gould E, Gross CG (2002) Neurogenesis in adult mammals: some progress and problems. J Neurosci 22:619–623

Goz B (1977) The effects of incorporation of 5-halogenated deoxyuridines into the DNA of eukaryotic cells. Pharmacol Rev 29:249–272

Hancock A, Priester C, Kidder E, Keith JR (2009) Does 5-bromo-2′-deoxyuridine (BrdU) disrupt cell proliferation and neuronal maturation in the adult rat hippocampus in vivo? Behav Brain Res 199:218–221

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

Herrup K, Arendt T (2002) Re-expression of cell cycle proteins induces neuronal cell death during Alzheimer's disease. J Alzheimers Dis 4:243–247

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

Katchanov J, Harms C, Gertz K, Hauck L, Waeber C, Hirt L, Priller J, von Harsdorf R, Bruck W, Hortnagl H, Dirnagl U, Bhide PG, Endres M (2001) Mild cerebral ischemia induces loss of cyclin-dependent kinase inhibitors and activation of cell cycle machinery before delayed neuronal cell death. J Neurosci 21:5045–5053

Kaufman ER, Davidson RL (1978) Biological and biochemical effects of bromodeoxyuridine and deoxycytidine on Syrian hamster melanoma cells. Somatic Cell Genet 4:587–601

Kuan CY, Schloemer AJ, Lu A, Burns KA, Weng WL, Williams MT, Strauss KI, Vorhees CV, Flavell RA, Davis RJ, Sharp FR, Rakic P (2004) Hypoxia-ischemia induces DNA synthesis without cell proliferation in dying neurons in adult rodent brain. J Neurosci 24:10763–10772

Kuhn HG, Cooper-Kuhn CM (2007) Bromodeoxyuridine and the detection of neurogenesis. Curr Pharm Biotechnol 8:127–131

Levkoff LH, Marshall GP 2nd, Ross HH, Caldeira M, Reynolds BA, Cakiroglu M, Mariani CL, Streit WJ, Laywell ED (2008) Bromodeoxyuridine inhibits cancer cell proliferation in vitro and in vivo. Neoplasia 10:804–816

Michishita E, Nakabayashi K, Suzuki T, Kaul SC, Ogino H, Fujii M, Mitsui Y, Ayusawa D (1999) 5-Bromodeoxyuridine induces senescence-like phenomena in mammalian cells regardless of cell type or species. J Biochem 126:1052–1059

Miller MW, Nowakowski RS (1988) Use of bromodeoxyuridine-immunohistochemistry to examine the proliferation, migration and time of origin of cells in the central nervous system. Brain Res 457:44–52

Nagao T, Kuwagata M, Saito Y (1998) Effects of prenatal exposure to 5-fluoro-2′-deoxyuridine on developing central nervous system and reproductive function in male offspring of mice. Teratog Carcinog Mutagen 18:73–92

Nowakowski RS, Hayes NL (2001) Stem cells: the promises and pitfalls. Neuropsychopharmacology 25:799–804

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

Packard DS Jr, Menzies RA, Skalko RG (1973) Incorporation of thymidine and its analogue, bromodeoxyuridine, into embryos and maternal tissues of the mouse. Differentiation 1:397–404

Phuphanich S, Levin VA (1985) Bioavailability of bromodeoxyuridine in dogs and toxicity in rats. Cancer Res 45:2387–2389

Pollard DR, Baran MM, Bachvarova R (1976) The effect of 5-bromodeoxyuridine on cell division and differentiation of preimplantation mouse embryos. J Embryol Exp Morphol 35:169–178

Poot M, Hoehn H, Kubbies M, Grossmann A, Chen Y, Rabinovitch PS (1994) Cell-cycle analysis using continuous bromodeoxyuridine labeling and Hoechst 33358-ethidium bromide bivariate flow cytometry. Methods Cell Biol 41:327–340

Rakic P (2002) Adult neurogenesis in mammals: an identity crisis. J Neurosci 22:614–618

Rivera FJ, Couillard-Despres S, Pedre X, Ploetz S, Caioni M, Lois C, Bogdahn U, Aigner L (2006) Mesenchymal stem cells instruct oligodendrogenic fate decision on adult neural stem cells. Stem Cells 24:2209–2219

Ross HH, Levkoff LH, Marshall GP 2nd, Caldeira M, Steindler DA, Reynolds BA, Laywell ED (2008) Bromodeoxyuridine induces senescence in neural stem and progenitor cells. Stem Cells 26:3218–3227

Shinin V, Gayraud-Morel B, Gomes D, Tajbakhsh S (2006) Asymmetric division and cosegregation of template DNA strands in adult muscle satellite cells. Nat Cell Biol 8:677–687

Steffenhagen C, Kraus S, Dechant FX, Kandasamy M, Lehner B, Poehler AM, Furtner T, Siebzehnrubl FA, Couillard-Despres S, Strauss O, Aigner L, Rivera FJ (2011) Identity, Fate and Potential of Cells Grown as Neurospheres: Species Matters. Stem Cell Rev: Mar 24. [Epub ahead of print]

Sternlicht MD, Lochter A, Sympson CJ, Huey B, Rougier JP, Gray JW, Pinkel D, Bissell MJ, Werb Z (1999) The stromal proteinase MMP3/stromelysin-1 promotes mammary carcinogenesis. Cell 98:137–146

Suzuki T, Minagawa S, Michishita E, Ogino H, Fujii M, Mitsui Y, Ayusawa D (2001) Induction of senescence-associated genes by 5-bromodeoxyuridine in HeLa cells. Exp Gerontol 36:465–474

Taupin P (2007) BrdU immunohistochemistry for studying adult neurogenesis: paradigms, pitfalls, limitations, and validation. Brain Res Rev 53:198–214

Tempfer H, Gehwolf R, Lehner C, Wagner A, Mtsariashvili M, Bauer HC, Resch H, Tauber M (2009) Effects of crystalline glucocorticoid triamcinolone acetonide on cultered human supraspinatus tendon cells. Acta Orthop 80:357–362

Wachs FP, Winner B, Couillard-Despres S, Schiller T, Aigner R, Winkler J, Bogdahn U, Aigner L (2006) Transforming growth factor-beta1 is a negative modulator of adult neurogenesis. J Neuropathol Exp Neurol 65:358–370

Zhu Y, Yang GY, Ahlemeyer B, Pang L, Che XM, Culmsee C, Klumpp S, Krieglstein J (2002) Transforming growth factor-beta 1 increases bad phosphorylation and protects neurons against damage. J Neurosci 22:3898–3909