ADMINISTRATION OF 5-BROMO-2´-DEOXYURIDINE INTERFERES WITH NEUROBLAST PROLIFERATION AND PROMOTES APOPTOTIC CELL DEATH IN THE RAT CEREBELLAR NEUROEPITHELIUM

Running title: BrdU alters the early cerebellar development

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ABSTRACT

The current study was conducted to assess whether a single administration of 5-bromo-2'-deoxyuridine (BrdU) interferes with cell proliferation and leads to the activation of apoptotic cellular events in the prenatal cerebellum. BrdU effects across a wide range of doses (25 to 300 µg/g b.w.) were analyzed using immunohistochemical and ultrastructural procedures. The pregnant rats were injected with BrdU at embryonic day 13, and their fetuses were sacrificed from 5 to 35 h after exposure. The quantification of several parameters such as the density of mitotic figures, and BrdU and proliferating cell nuclear antigen (PCNA)-reactive cells showed that, in comparison with the saline injected rats, the administration of BrdU impairs the proliferative behavior of neuroepithelial cells. The above mentioned parameters were significantly reduced in rats injected with 100 µg/g b.w. of BrdU. The reduction was more evident using 200 µg/g b.w. The most severe effects were found with 300 µg/g b.w. of BrdU. The present findings also revealed that high doses of BrdU lead to the activation of apoptotic cellular events as evidenced by both terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay and immunohistochemistry for active caspase-3. In comparison with saline rats, many apoptotic cells were found in rats injected with 100 µg/g b.w. of BrdU. The number of dying cells increased with 200 µg/g b.w. The most important number of apoptotic cells were observed in animals injected with 300 µg/g b.w. of BrdU. Ultrastructural studies confirmed the presence of neuroblasts at different stages of apoptosis.

Keywords: embryonic life; 5-bromo-2'-deoxyuridine; cerebellar neuroepithelium; apoptosis; immunohistochemistry; RRID: AB_10013660; RRID: AB_628110; RRID: AB_476884; RRID: AB_258588; RRID: AB_2313609; transmission electron microscopy.

1. INTRODUCTION

The cerebellum is involved not only in motor coordination, but also in the processing of signals for cognitive, sensory discriminative and affective functions (D'Angelo, 2018; Mariën & Borgatti, 2018; Schmahmann, 2019). This region presents a laminated outer area, the cerebellar cortex, and a set of cerebellar nuclei neurons buried within the white matter (Altman and Bayer, 1997). This regular neuroarchitecture has allowed to analyze the cellular mechanisms involved in the development of the cerebellum (Butts, Green & Wingate, 2014; Cerminara et al., 2015). Many lines of evidence indicate that the generation of cerebellar neurons is compartmentalized, with ventricular zone progenitors giving rise to GABAergic neurons, and rhombic lip precursors producing glutamatergic cells (Leto et al., 2016).

The halopyrimidine 5-bromo-2´-deoxyuridine (BrdU) is a chemically synthesized bromine-tagged base analogue that is incorporated instead of thymidine into newly synthesized DNA during the S-phase of the cell cycle (Lehner et al., 2011). Since the introduction of monoclonal antibodies against BrdU (Graztner 1982), an increasing number of methodologies have been used for the immunodetection of this nucleoside in the replicating DNA (Molina et al., 2017). One of the advantages of the use of BrdU is that it has allowed to microscopically analyze the developmental timetables, fate and survival of different neuron types in the central nervous system (Taupin 2007; Marti et al. 2015; 2016; Lanctot et al., 2017).

Previous reports have indicated that the injection of BrdU to gestating dams is well tolerated and produces neither cytotoxic effects nor alters the cell cycle progression in the cerebral wall of prenatal mice (Miller and Nowakowski, 1988; Takahashi, Nowakowski & Caviness, 1992; Takahashi et al., 1999). Despite that, several studies have revealed that the incorporation of this thymidine analogue into replicating DNA

induces sister-chromatid exchanges and double-strand breaks (Taupin 2007). Other authors have indicated that the exposure of rodent neural stem cells to BrdU causes a decrease in DNA methylation and a reduced marker's expression in neural stem cells (Schneider & di Fagagna, 2012). It has also been shown that BrdU is toxic to neuronal precursors *in vitro* (Caldwell, He & Svendsen, 2005; Lehner et al., 2011). Moreover, there are evidences revealing that the injection of BrdU to mammalian embryos has harmful effects on the development of several regions of the central nervous system (Kolb et al. 1999; Kuwagata et al. 2007; Duque and Rakic, 2011; Lehner et al., 2011), including the cerebellum (Sekerkova, Ilijic & Mugnani, 2004; Martí et al., 2015). Deleterious effects on neuron fate and function (Duque and Rakic, 2011; 2015), as well as senescence in several cell types (Michishita et al., 1999; Suzuki et al., 2001) including neural progenitors (Ross et al., 2008), have also been reported. Despite these evidences indicating the toxicity of BrdU, the effect of the incorporation of this molecule into DNA on cell proliferation is frequently neglected (Duque and Rakic, 2011).

In light of the above, we began a set of experiments. The major goal of this study is to analyze whether a single injection of BrdU, doses ranging from 25 to 300 µg/g b.w., alters the development of the cerebellar neuroepithelium. Dams were injected on embryonic day (E) 13, as it corresponds to the onset of Purkinje cells and deep cerebellar nuclei neurons neurogenesis in wild type rats (Altman and Bayer, 1997; Martí et al., 2015; 2016). The selection of the cerebellum was supported by the evidence that this region is highly vulnerable to poisoning (Samson and Claassen 2017).

Specifically, the following aspects were addressed. (I) We determined the proliferative behavior of cerebellar neuroepithelial cells after a single injection of BrdU. This was carried out by quantifying several parameters, such as the density of mitotic

figures, BrdU and PCNA-positive cells. (II) We used TUNEL, immunohistochemistry for active caspase-3 and transmission electron microscopy to determine whether BrdU-exposure leads to the activation of apoptotic cellular events in the growing cerebellar neuroepithelium, and if so, to study their ultrastructural features.

2. MATERIALS AND METHODS

2.1 Animals and experimental design

All the experiments were approved by the Animal Research Committee of the Universitat Autònoma de Barcelona (UAB) and the Government of Catalonia, and conducted in accordance with the legislation for the protection of animals used for scientific purposes (directive 2010/63/EU, RD 53/2013). The number of animals was kept to a minimum and all efforts were made to minimize their suffering. Adult female Sprague-Dawley OFA rats were put into the home cages of individual housed male rats at 5:00 pm and were removed at 8:00 am the next morning. At that time, vaginal smears were analyzed from each female and those that had sperm were placed into separate cages. This day was considered E1. Pregnant dams were injected, at E13, with a single intraperitoneal injection of saline (0.9% NaCl) or BrdU (Sigma, St. Louis, MO, USA) solution (10mg/ml in sterile saline with 0.007N sodium hydroxide) at the doses of 25, 50, 75, 100, 200 or 300 µg/g b.w.

The pregnant dams were sacrificed and the embryos were harvested at regular, 5 h intervals from 5 to 35 h after saline or BrdU-exposure. A total of 245 pregnant dams were used; 35 were injected with saline and 210 with BrdU. A total of 490 pups were utilized. For example, in animals sacrificed 5 h after saline or BrdU-exposure, 35 different pregnant dams were used; 5 were injected with saline; 5 with BrdU (25 µg/g b.w); 5 with BrdU (50 µg/g b.w); 5 with BrdU (75 µg/g b.w); 5 with BrdU (100 µg/g b.w); 5 with BrdU (200 µg/g b.w) and 5 with BrdU (300 µg/g b.w). To prevent all the pups from a dam accumulating in the same experimental group, from each dam, 2 embryos were analyzed. Data from both embryos were combined to obtain a mean. The same was carried out in the remaining survival times. The dams were maintained under standard laboratory conditions (temperature of 22 ± 2ºC and relative humidity of 55 ±

5%, lights on from 08:00h to 20:00h) throughout gestation. Food and water were provided *ad libitum*.

2.2 Tissue processing

Dams were anesthetized with a ketamine-xylazine mixture (90:10 mg/ml; 1 ml/kg, intraperitoneal). Embryos were removed by caesarian. They were decapitated and their heads were immediately immersed in 10% neutral buffered formalin for 24h at 4°C. These were dehydrated, paraffin-embedded and serially cut in the sagittal plane at 10 µm using a rotary microtome. Only one tissue section of every fifth was placed on poly-(L-lysine)-coated slides in order to avoid overestimation of cell counts. Figure 1 shows the level chosen for analysis, which corresponds to the figure number 2 (page number 100) and plate 2 (page number 101) from the Altman and Bayer atlas (Altman and Bayer, 1995).

2.3 Feulgen Method

The Feulgen staining was performed according to previously published procedures (Rodríguez-Vázquez and Martí 2017; Molina, Rodríguez-Vázquez & Marti, 2020). Sections were deparaffinized in xylene and rehydrated through a series of graded ethanols. Partial denaturation of DNA was carried out with 3N HCl at 40°C for 15 min. Hydrolysis was halted with two washes in distilled water at room temperature (RT) and then the sections were treated with Schiff's reagent (prepared from basic fuchsin; Fluka Chemie, Buchs, Switzerland) at RT for 1h in darkness. After washing them in a fresh sulphurous acid solution, the stained sections were rinsed in distilled water, dehydrated and coverslipped.

2.4 TUNEL staining

TUNEL staining was carried out with an *in situ* cell death detection kit (POD Roche Diagnostics, cat 11684817910). The manufacturer's protocol was followed. In brief, after removing paraffin, sections were incubated with proteinase K, nuclease free (20 µg/ml in 10 mM Tris-HCl, pH 7.5) during 30 min at RT. They were then cleaned with PBS and incubated for 10 min with 3 %H_2O_2 in methanol. Slices were soaked in the TUNEL reaction mixture for 60 min at 37ºC and then cleaned with PBS. Sections were then incubated with converted-POD for 30 min at 37ºC. Apoptotic cells were visualized with a peroxidase-diaminobenzidine reaction and sections were then counterstained with haematoxylin. For positive control of TUNEL labeling, sections were incubated with DNase (5 µg/ml) at 37 °C during 10 min to induce DNA strand breaks. For negative control, terminal deoxynucleotidyl transferase was replaced with PBS.

2.5 Immunohistochemical staining of cerebellar neuroepithelium

Immunohistochemistry for BrdU, PCNA and active caspase-3 were performed according to previous procedures (Rodríguez-Vázquez and Martí 2017; Rodríguez-Vázquez et al., 2019; Molina et al., 2020). Sections were deparaffinized in xylene, hydrated in decreasing concentrations of ethanol solutions, and extensively washed in distilled water. Pretreatment with 3N HCl at 40ºC during 15 min was performed for BrdU immunostaining. On the other hand, for PCNA and active caspase-3 immunodetection, sections were immersed in Coplin jars containing 15 mM of heated (96-99°C) sodium citrate buffer (pH 6.0) for 30 min. After that, the slices were allowed to cool for 30 min. Endogenous peroxidase activity was blocked with 3 % H_2O_2 for 10 min at RT. Non-binding sites were blocked using 5% bovine serum albumin in PBS

containing 0.1% Tween-20 and 5% normal goat serum for 1h at RT. Following this, the slides were incubated overnight at 4°C with one of the following primary antibodies: anti-BrdU (mouse monoclonal; 1:150; Dako, Glostrup, Demark. RRID: AB_10013660), anti-PCNA (mouse monoclonal; 1:1500; Santa Cruz Biotechnology, CA. RRID: AB_628110) or anti-active caspase-3 (rabbit polyclonal; 1:250; Sigma-Aldrich, St. Louis, MO. RRID: AB_476884). Primary antibodies were diluted in PBS (Boheringer Mannhein, Germany) supplemented with 1% bovine seroalbumine. After this, sections were rinsed with PBS and incubated at room temperature for 30 min with a biotin conjugated goat anti-mouse IgG antibody (Sigma, St. Louis, MO; Cat# B6649; RRID: AB_258588) 1:50 for 30 min and then exposed to ExtrAvidin-peroxidase (Sigma, St. Louis, MO) 1:50 for 30 min (for anti-BrdU and anti-PCNA) or incubated with a biotin-conjugated goat anti-rabbit antibody (Dako, Glostrup, Demark; Cat# E0432; RRID: AB_2313609) 1:100 for 60 min and then exposed to ExtrAvidin-peroxidase (Sigma, St. Louis, MO; E2886) 1:20 for 30 min (for anti active caspase-3). The peroxidase activity was developed by subjecting the slides to 0.7 mg ml^{-1} 3,3'-diaminobenzidine (Sigma, St. Louis, MO) plus 3% H_2O_2 in tris-phosphate buffered saline 0.05 M (pH 7.6) for 5 min, and were then rinsed in distilled water. Sections were counterstained with hematoxilin, dehydrated with ethanol and xylene, and coverslipped with Merckoglass. Control sections were prepared by replacing the primary antibody with PBS. These showed no immunolabeling.

2.6 Antibody characterization

1. Mouse monoclonal antibody against BrdU (bromodeoxyuridine; clone Bu20a; Dako; #M 0744; RRID: AB_10013660) was used to detect BrdU, a synthetic halogenated

pyrimidine analogue, that is incorporated into DNA in place of thymidine during the S phase of the cell cycle. This antibody reacts with BrdU in single stranded DNA (produced by partial denaturation of double stranded DNA). Isotype: IgG1, kappa (DAKO antibody datasheet). Using immunohistochemical techniques with this antibody, BrdU has been detected in several mammalian species including rat (Rodríguez-Vázquez et al., 2019), mouse (Marti et al., 2015; Hu et al., 2017) and primates (Block et al., 2011).

2. The mouse monoclonal antibody against PCNA (proliferating cell nuclear antigen; clone PC10; Santa Cruz Biotechnology: Cat# sc-56, RRID: AB_628110) is made in the protein A expression vector pR1T2T of rat origin. The PCNA, a nuclear protein synthesized in early G1 and S phases of the cell cycle, functions in cell cycle progression, DNA replication and DNA repair. Near-infrared western blot analysis of PCNA expression in Raji, HCT-116, MOLT-4, HeLa, NIH/3T3 and C6 whole cell lysates show a single, strong band at ~35kDa (manufacturer's information). Using immunohistochemical techniques with this antibody, PCNA has been detected in several mammalian species including rat (Rodríguez-Vázquez and Martí, 2017), mouse (Jeong et al., 2019), gerbil (Campos et al., 2018) and human (Shan et al., 2019).

3. Rabbit policlonal anti-caspase-3, active (Sigma: Cat# C8487, RRID: AB_476884) is produced in rabbit using as immunogen a synthetic peptide corresponding to the cleavage site of human caspase 3. This sequence is identical in many species including mouse, rat, dog, pig and bovine caspase 3. Whole antiserum is purified to provide an IgG fraction of antiserum. The resulting IgG fraction is further purified by absorption on the caspase 3 peptide (human) corresponding to the uncleaved caspase 3 site sequence (Sigma antibody datasheet). Using immunohistochemical techniques with this

antibody, active caspase 3 has been detected in several mammalian species including rat (Benitez et al., 2014; Molina et al., 2020) and mouse (Zhu et al., 2020).

2.7 Quantitative analyses

Light microscopic observations were made with a Zeiss Axiophot microscope using a 63X oil immersion objective and equipped with a 10 x 10 reticule in one of the eyepieces. Counts of mitotic figures, BrdU, PCNA, TUNEL and active caspase-3 positive cells were made in the pertinent sections, i.e., each section had the whole profile of the cerebellar neuroepithelium. The density of mitotic figures, BrdU, PCNA, TUNEL and active caspase-3 positive cells were calculated as the percentage of the respective cell type over the area of the cerebellar neuroepithelium. This parameter was determined using the Image J software (v. 1.43 u, NIH, Bethesda, MD, USA). The density of each parameter was determined in two sections of each experimental embryo. Data from both sections were added to obtain a mean per embryo.

2.8 Transmission Electron microscopy

For ultrastructural analysis, 2 pups per experimental condition were used. For example, in animals sacrificed 10 h after saline or BrdU-exposure, 14 different pregnant dams were used; 2 were injected with saline; 2 with BrdU (25 µg/g b.w); 2 with BrdU (50 µg/g b.w); 2 with BrdU (75 µg/g b.w); 2 with BrdU (100 µg/g b.w); 2 with BrdU (200 µg/g b.w) and 2 with BrdU (300 µg/g b.w). From each dam, one pup was used to electron microscopy study. From each pup, two ultrathin sections were studied. The same was carried out in the remaining survival times. Electron microscopy samples were prepared following a method previously described (Martí et al., 2017; Rodríguez-Vázquez et al., 2019). In brief, embryo heads were immersed in 2% glutaraldehyde, 2%

paraformaldehyde in 0.1M cacodylate buffer, pH 7.2. Tissue cubes (~1mm^3) were rinsed in PBS, postfixed in 1% OsO_4 for 2h, dehydrated in a graded ethanol series, infiltrated with propylene oxide, and embedded in Epon. Ultrathin sections (60nm) were generated with an ultramicrotome (Leica AG, Reichert ultracut S) and then analyzed with a transmission electron microscope (JEM-1400). Digital images were obtained with a CCD GATAN 794 MSC 600HP digital camera system.

2.9 Data analysis

Data were analyzed with the one-way ANOVA, followed by individual comparison of means with the Student-Newman-Keuls (SNK) test using the SPSS (version 18) statistical software. The results are expressed as mean ± SEM. Differences are considered to be significant when the '*P*' value is < 0.05.

2.10 Photographic Material

The digitized images were prepared for publication with the Adobe Photoshop (v. 7.0, Adobe Inc., San Jose, CA, USA) software.

3. RESULTS

3.1 Experiment 1

During the embryonic life, the cerebellar neuroepithelium contains neuroblasts undergoing DNA synthesis. They are the precursors of several neuronal types including Purkinje cells and deep cerebellar nuclei neurons (Altman and Bayer 1997; Leto et al., 2016). There are evidences showing that the administration of BrdU affects the cell cycle progression of neural stem cells (Kuwagata et al., 2007). This experiment was carried out to determine whether a single-pulse of BrdU exposure, the doses ranging from 25 to 300 µg/g, modifies the proliferative behavior of neuroepithelial cells. In order to assess this, the number of mitotic figures, and BrdU and PCNA-immunoreactive cells per area of the cerebellar neuroepithelium were quantified.

Figure 2 shows examples of Feulgen-stained cells in the cerebellar neuroepithelium of rats injected with saline. This histochemical technique has been accepted as a stoichiometric procedure for exclusive staining of nuclear DNA in a reproducible and standardized manner (Biesterfeld et al., 2011). The Feulgen staining provides excellent detail and morphology of mitotic cells. With this procedure, they are magenta-stained.

Figure 3 exhibits examples of BrdU-positive cells in animals injected with 50 µg/g of BrdU and sacrificed 5 h later. The immunolabeled nuclei show a granular staining texture and the intensity of the nuclear label appears to vary in the same histological preparation. Figure 4 epitomizes the decrease in the density of BrdU-reactive cells between rats injected with 50 µg/g or 200µg/g of BrdU and sacrificed 15 h later. The data of the density of mitotic figures, and BrdU and PCNA-immunoreactive cells, in addition to the statistical analysis of the former parameters, are given in Tables 1-3. The current results reveal that, irrespective of the studied survival times, the density of mitotic cells was similar in rats exposed to doses of BrdU ranging from 25 to 75 µg/g

(Table 1). No statistical differences were found in comparison to the saline group. When doses of 100 to 300 µg/g of BrdU were analyzed, significant effects on the density of mitotic figures were observed. For each dose, the density decreased from 5 to 35 h after the exposure to the marker. Additionally, with the exception of rats sacrificed 5 h after BrdU-administration, the density of mitotic cells was lower using doses of 100 to 300 µg/g of BrdU in comparison to the saline group. The lowest values of this parameter were observed in rats exposed to 300 µg/g of BrdU and sacrificed from 25 to 35 h after treatment. Similar results were found when the density of BrdU (Table 2) and PCNA-reactive cells (Table 3) were studied. To illustrate the findings, a visual rendering of our current data is presented in Figure 5.

3.2 Experiment 2

The brominated thymidine analog BrdU has provided new advances in the study of cell type-specific birth dating, migration and fate in the developing brain (Takahashi, Nowakowski & Caviness, 1992; Duque and Rakic, 2011; Martí et al., 2015; 2016). Despite that, the use of the BrdU has risks and limitations. In this context, there are evidences indicating that BrdU-injection leads to the activation of apoptotic cellular events during the development of the central nervous system (Kuwagata et al., 2007). In this section, we used the TUNEL reaction, immunohistochemitry for active caspase-3 and transmission electron microscopy to determine whether the prenatal administration of the BrdU triggers apoptotic cell death in the cerebellar neuroepithelium.

Figure 6 epitomizes the increase in the density of TUNEL-stained cells between rats injected with saline or 200µg/g of BrdU and sacrificed 20 h later. The data of the density of TUNEL-stained neuroblasts, in addition to the statistical analysis of the former parameters, are depicted in Table 4. Our results show that, in comparison to

saline injected rats and irrespective of the analyzed survival times, the density of TUNEL-positive cells was similar in animals exposed to doses of BrdU ranging from 25 to 75 µg/g of BrdU. No statistical differences were found in comparison to the saline group. When doses of 100 to 300 µg/g of BrdU were studied, significant effects on the density of TUNEL-stained cells were found. For each dose, the density of this parameter increased from 5 to 35 h after marker exposure. Additionally, with the exception of rats sacrificed 5 h after BrdU-administration, the density of TUNEL-positive cells was higher using doses from 100 to 300 µg/g of BrdU in comparison to the saline group. The highest values were found in rats exposed to 200 and 300 µg/g of BrdU and sacrificed from 30 to 35 h after the exposure to the agent. Similar results were found when the density of active caspase-3 was studied (Table 5). To illustrate the findings, a visual rendering of the our current data is presented in Figure 7.

Light and electron microscopy were used to verify morphological features of cell death, in neuroepithelial neuroblasts treated with several doses of BrdU. Optical microscopical studies based on plastic sections indicated that, in saline rats, the neuroepithelium is formed by a homogenous population of small and packed neuroblasts, most of which were round-like in shape. They present large nuclei, which occupy much of the space within the soma. On the other hand, rats injected with BrdU at doses ranging from 100 to 300 µg/g, showed degenerating cells with no preferential location within the neuroepithelium. The microscopic analysis indicated conspicuous alterations of the neuroblast morphology consistent with apoptotic processes, including the presence of spherical chromatin balls (Figure 8a-b). Very few dying cells were observed in rats administered with saline or with BrdU at doses ranging from 25 to 75 µg/g of BrdU.

Under the electron microscope, we examined neuroepithelial cells treated with saline or BrdU. In the first of these, our current observations revealed that cell somas are either ovoid or have the shape of a polyhedron, and the nuclei exhibit one nucleolus surrounded by heterochromatin (Figure 8c). In rats injected with BrdU at doses ranging from 100 to 300 µg/g, dying cells with typical apoptotic features were found (Figure 8d-i). Damaged neuroblasts showed clumps of nuclear chromatin of high electron density, in close contact with the inner nuclear envelope (Figure 8d). Another important feature was the presence of several round electron-dense apoptotic bodies containing condensed dark chromatin masses (Figure 8e-i). In some cases, the apoptotic bodies were broken and their content extruded into the cytoplasm (Figure 8f-h). This occurs while the cytoplasmic organelles are destroyed (Figure 8i). On the contrary, only a few neuroblasts displaying apoptotic morphology were detected in the rats administered with saline or with BrdU at doses ranging from 25 to 75 µg/g of BrdU.

4.- DISCUSSION

As a marker of DNA synthesis (Duque and Rakic, 2011), BrdU has provided important insights into the cellular mechanisms of the brain development (Miller and Nowakowski 1988; Taupin, 2007; Marti et al., 2016). BrdU is generally thought to be a relatively benign substitute for the endogenous thymidine. However, several studies have revealed that the incorporation of this thymidine analogue into newly synthesized DNA can cause unforeseen problems (Costandi, 2011). In this context, it has been shown that BrdU induces alterations during the growth of the chick dorsal telencephalon (Rowell and Ragsdale, 2012). In mammalian embryos, this agent is toxic to cultured neuroblasts obtained from the striatum (Calswell et al., 2005). In addition to this, BrdU has a harmful effect on the embryonic development of the neocortex, the striatum (Kuwagata et al., 2007) and the cerebral cortex (Duque and Rakic, 2011). Moreover, it causes a decrease in cell proliferation, migration and patterning of the cerebellum (Sekerkova, Ilijic & Mugnani, 2004). In this current paper, we investigated the effect of a genotoxic agent, BrdU, on the development of the rat cerebellar neuroepithelium.

The present study revealed no apparent harmful effects when low doses of BrdU (25 to 75 µg/g) were administered. No prominent cytotoxic effects on the cerebellar neuroepithelium were found, which suggests that these doses are appropriate, at least in the acute situation, for tagging proliferative neuroblasts during the development of the central nervous system. These results are in line with previous reports, in which the cell cycle kinetics of neural progenitors in the embryonic cerebral wall (Takahashi, Nowakowski & Caviness, 1995) and cerebellar external granular layer (Molina et al., 2017) of rodents were not altered by the administration of 50 µg/g of BrdU. Despite this, we cannot exclude a more protracted effect of BrdU on neuroepithelial cell

biology, i.e., cell differentiation and final fate. It is important to indicate that no difference in BrdU-signal was observed between 50 and 75 µg/g, which allows a confident identification of those neuron precursors engaged in DNA synthesis. On the premise that low doses are preferable to higher ones, we propose that the dose of 50 µg/g of BrdU should be used in those experiments involving the prenatal period of life. In our experimental conditions (a BrdU/ diaminobenzidine staining was employed to detect BrdU-reactive cells), lower concentrations of BrdU (25 µg/g) proved to be insufficient to detect DNA synthesis with light microscopy. Several factors may explain these current results: (I) the limited penetrance through the blood-brain barrier and the short bio-availability of BrdU. In this context, there are evidences indicating that the concentration of this marker in the body has already decreased 2 h after BrdU injection (Packard, Menzies & Skalko, 2014; Hayes & Nowakowski, 2000; Lehner et al., 2011), (II) BrdU is not necessary stably integrated into the DNA, since it is subjected to dehalogenation followed by an excision of the uracil residue and degradation (Hume & Saffhill, 1986; Lehner et al., 2011) and (III) it cannot be excluded the possibility that the proliferation of neuroepithelial cells might have diluted the BrdU label and, therefore, they are no longer detectable.

Our analysis also denotes that a single administration of BrdU at doses ranging from 100 to 300 µg/g is able to alter neuroblast proliferation and leads to the activation of apoptotic cellular events in the rat embryonic cerebellum. The results also indicate that, for the cerebellum, E13 is a developmental stage particularly susceptible to high doses of BrdU. The current findings may be also true for any other structure that happen to be highly proliferative at this embryonic age. As the onset of Purkinje cells and deep nuclei neurons production is on E13 (Altman and Bayer 1997) and the day of BrdU administration corresponds with the times of neuron origin of these macroneurons, we

propose that several of these cells which were supposed to originate from the neuroblasts were never born. The extension of damage induced by BrdU would determine the level of abnormalities in this region of the central nervous system.

The thymidine analog BrdU is an anticancer drug, and it is known that in combination with secondary stressors, such as ionizing radiation, BrdU has lethal consequences for cancer cells (Levkoff et al., 2008). The current findings indicate that in the absence of secondary insults that would stress the cerebellar neuroblasts, a single administration of a high dose of BrdU damages the cell cycle progression. Our results show that the cerebellar neuroblasts seem to be especially vulnerable to the antiproliferative effects of BrdU. In line with this scenario, several reports have denoted negative effects of BrdU incorporation on the proliferative dynamics of mouse and human fibroblasts, and adult neural progenitor cells *in vitro* (Michishita et al., 1999; Ross et al., 2008; Caldwell, He & Svendsen, 2005; Lehner et al., 2011). We do not know the underlying mechanisms of the antiproliferative effect of BrdU on neuroepithelial cells. A previous report has demonstrated that, *in vitro*, this agent induces a shift from S and G2/M-phase to the G0-phase of the cell cycle (Lehner et al., 2011). Additionally, BrdU might be altering cell signaling pathways involved in cell proliferation. Future studies should address this and examine the effect of BrdU exposure on other germinal sources of neurons.

The results of our light microscopy study indicate that exposure of the embryonic cerebellum to BrdU at doses ranging from 100 to 300 μg/g depletes the proliferating precursor cells. TUNEL staining and immunohistochemistry for active caspase-3 reveal that neuroepithelial cells died by apoptosis. In order to verify it, an ultrastructural analysis was carried out. With electron microscopy, it was possible to see features that are characteristic in apoptosis, such as chromatin condensation, nuclear fragmentation

and apoptotic bodies. Similar results were observed in electron micrographs after treatment with 5-azacytidine (Ueno et al., 2002) and hydroxyurea (Rodríguez-Vázquez and Martí 2017).

At this point of the discussion, we are not able to provide a clear answer regarding to the underlying mechanisms through which BrdU alters the proliferative activity of neuroepithelium cells and induces apoptotic events. There is no accepted consensual mechanism of action for BrdU. Here, we propose that both detrimental consequences may be mediated by the toxicity of this agent. The reason is the BrdU structure. It is known that the molecular structure of BrdU differs considerably from the natural structure of the endogenous thymidine (Costandi, 2011; Duque and Rakic, 2011). Moreover, the incorporation of BrdU into replicating DNA produces an abnormal base pairing of the guanine and adenine with the bromouracil. This structural change in the DNA helixes may have a detrimental effect on RNA transcription, and eventually the protein (Duque and Rakic, 2011; 2015; Rowell and Ragsdale 2012). These differences may result in mutations leading to the aforementioned effects on the neuroblasts analyzed in this current study. Given that the current work only assesses BrdU effects, additional experiments are needed to determine to the extent to which these findings apply to other halogenated deoxyuridines such as chlorodeoxyuridine and iododeoxyuridine.

CONCLUSIONS

We show here that high doses of BrdU have a cytotoxic action on the prenatal cerebellum. Our current findings indicate that the administration of this marker to embryos may lead to false results and misinterpretation in the identification of proliferative neuroblasts. Based on our results, we strongly advise to take into account the adverse effects of BrdU in future studies. Moreover, data obtained with this thymidine analogue should be prudently interpreted and suitable controls should be set up in order to ensure that BrdU-labeling reflects the neural precursor fraction engaged in DNA synthesis during the development of the central nervous system.

Conflict of interest statement

The authors declare that they have no conflicts of interest.

Author's contribution

L:R-V.: performed experiments, data analysis, approved final manuscript; J.M.: designed the study, performed experiments, wrote the manuscript, generated all figures, approved final manuscript.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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FIGURE LEGEND

Figure 1. (a) Schematic illustration at embryonic day 13 of the level used for quantitative evaluation in sagittal section. Red area and lines delimit the cerebellar neuroepithelium. Modified from Altman and Bayer, 1995. (b) Sagittal low-power photomicrograph of a rat injected with saline at embryonic day 13 and sacrificed 5 h later. Arrowhead outlines the rostral boundary of the cerebellar neuroepithelium. The caudal one is traced by the vellum medullare. vm, vellum medullare; v4, fourth ventricle; in, isthmal neuroepithelium. Scale bar = 200 µm.

Figure 2. (a-b) Examples of Feulgen stained cells in the cerebellar neuroepithelium from a rat injected with saline and examined 10 hours later. Tissue is stained in magenta. In both images, arrow heads point to interphasic nuclei, while black arrows indicate mitotic figures. Calibration bar for (a) and (b) represent 20 and 5 µm, respectively.

Figure 3. High magnification of BrdU-reactive cells in the cerebellar neuroepithelium of rats allowed to survive for 5 h after a single injection of 50 µg/g of BrdU. Note that BrdU-immunopositive nuclei present different labeling patterns. Insets in (a) and (b) show examples of immunolabeled cells in metaphase and anaphase, respectively. Scale bars = 10 µm (a-b), 5 µm in insets (a) and (b).

Figure 4. BrdU-labeling in the cerebellar neuroepithelium of rats allowed to survive for 15 h after a single injection of 50 µg/g (a) or 200µg/g (b) of BrdU. Note that the proportion of nuclei BrdU-reactive decreased in animals injected with 200µg/g of BrdU. Short arrows in (a) show immunolabeled mitotic figures. Long arrows indicated BrdU-positive interphasic nuclei. They stand out with respect to other nuclei that are immunonegative (arrow heads). Scale bars = 30 µm.

Figure 5. Mean ± SEM density of mitotic figures (a), BrdU (b) and PCNA (c) positive cells in the cerebellar neuroepithelium of rats injected, at embryonic day 13, with saline (closed diamonds, and double-dotted and dashed lines) or 25 µg/g (closed squares and solid black line), 50 µg/g (open triangles and short broken lines), 75 µg/g (open squares and long broken lines), 100 µg/g (closed diamonds, and dotted and dashed lines), 200 µg/g (closed circles and long broken lines) and 300 µg/g of BrdU (plus signs and long broken lines). Animals were sacrificed at regular, 5 h intervals from 5 to 35 h after saline or BrdU-injection.

Figure 6. Microscopic detection of TUNEL-positive cells in rats injected with saline or BrdU (200µg/g) at postnatal day 13 and sacrificed 20 h later. TUNEL-stained cells are those presenting a brown reaction product in their nuclei. (a) Very few TUNEL-positive cells are detected in rats injected with saline. (b) After BrdU injection, numerous TUNEL-stained cells are found in the cerebellar neuroepithelium. Scale bars = 100 µm.

Figure 7. Mean ± SEM density of TUNEL (a) and active caspase-3 (b) positive cells in the cerebellar neuroepithelium of rats injected, at embryonic day 13, with saline (closed diamonds, and double-dotted and dashed lines) or 25 µg/g (closed squares and solid black line), 50 µg/g (open triangles and short broken lines), 75 µg/g (open squares and long broken lines), 100 µg/g (closed diamonds, and dotted and dashed lines), 200 µg/g (closed circles and long broken lines) and 300 µg/g of BrdU (plus signs and long broken lines). Animals were sacrificed at regular, 5 h intervals from 5 to 35 h after saline or BrdU-injection.

Figure 8. (a-b) Light microscopic view of toluidine blue stained semi-thin sections of healthy (white arrows) and degenerating neuroblasts (black arrows) in the cerebellar

neuroepithelium of rats exposed to 100 µg/g of BrdU and sacrificed 15 hours later. (c-i) Electron micrograph of a healthy cell (c) and apoptotic cellular profiles (d-i) from the neuroepithelium 10 hours following BrdU treatment (100 µg/g). (d) Ultrastructural morphology of an apoptotic cell. The masses of compact chromatin display a high electron density and a homogeneous texture. They are associated with an intact nuclear envelope. (e-i) Typical electron-dense apoptotic bodies. (f-h) Breaking of apoptotic bodies and release of their contents into the cytoplasm. (i) One dying cell exhibiting a round and very electron-dense apoptotic body. The membranes and organelles are destroyed. Scale bar = 20 µm (a-b), 2 µm (c), 1 µm (d, f), 2.5 µm (e), 0.5 µm (g-i).

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