EFFECTS OF PERINATAL ASPHYXIA ON CELL PROLIFERATION AND NEURONAL PHENOTYPE EVALUATED WITH ORGANOTYPIC HIPPOCAMPAL CULTURES

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Abstract—The present report summarizes studies combining an *in vivo* and *in vitro* approach, where asphyxia is induced *in vivo* at delivery time of Wistar rats, and the long term effects on hippocampus neurocircuitry are investigated *in vitro* with organotypic cultures plated at postnatal day seven.

The cultures preserved hippocampus layering and regional subdivisions shown *in vivo*, and only few dying cells were observed when assayed with a viability test at day *in vitro* 27. When properly fixed, cultures from asphyxia-exposed animals showed a decreased amount of microtubule-associated protein-2 immunocytochemically positive cells (~30%), as compared with that from controls. The decrease in microtubule-associated protein-2 immunocytochemistry was particularly prominent in Ammon's horn 1 and dentate gyrus regions (~40%). 5-Bromo-2′deoxyuridine labeling revealed a two-fold increase in cellular proliferation in cultures from asphyxia-exposed, compared with that from control animals. Furthermore, confocal microscopy and quantification using the optical disector technique demonstrated that in cultures from asphyxia-exposed animals ~30% of 5-bromo-2′deoxyuridine-positive cells were also positive to microtubule-associated protein-2, a marker for neuronal phenotype. That proportion was ~20% in cultures from control animals. Glial fibrillary acidic protein-immunocytochemistry and Fast Red nuclear staining revealed that the core of the hippocampus culture was surrounded by a well-developed network of glial fibrillary acidic protein-positive cells and glial fibrillary acidic protein-processes providing an apparent protective shield around the hippocampus. That shield was less developed in cultures from asphyxia-exposed animals.

The increased mitotic activity observed in this study suggests a compensatory mechanism for the long-term impairment induced by perinatal asphyxia, although it is not clear yet if that mechanism leads to neurogenesis, astrogliogenesis, or to further apoptosis.

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Abbreviations: AM, Acetomethoxy; BrdU, 5-bromo-2′deoxyuridine; CA1, CA2, CA3, Ammon's horn 1, 2, 3; DG, dentate gyrus; DIV, days *in vitro*; DMEM, Dulbecco's modified Eagle medium; FGF2, fibroblast growth factor; GFAP, glial fibrillary acidic protein; MAP, microtubule-associated protein; NGS, normal goat serum; P, days after birth; PBS, phosphate-buffered saline; PF, paraformaldehyde.

Key words: neonatal, anoxia, hippocampus, neurocircuitry, neurogenesis, rat.

Hypoxia/ischemia at birth induces severe long-term neurodevelopment impairments, resulting in spasticity, epilepsy and mental retardation when the insult is severe, or attention-deficit hyperactivity syndrome and minimal brain disorder when it is mild (Boksa and El-Khodor, 2003). The neurocircuitries of the basal ganglia have been shown to be particularly vulnerable to hypoxia/ischemia (Pasternak et al., 1991), but there is clinical (van Erp et al., 2001) and experimental (Pulsinelli et al., 1982) evidence indicating that circuitries of the hippocampus are also extremely vulnerable to that type of insult (Harry and d'Hellencourt, 2003).

Perinatal asphyxia is a major cause of death and neurological injury in newborn babies, frequently associated to difficult or elongated birth processes (Berger and Garnier, 1999; Volpe, 2001). At the Karolinska Institutet, Stockholm, Sweden, a model for investigating perinatal asphyxia in the rat was proposed (Bjelke et al., 1991; Herrera-Marschitz et al., 1993), demonstrating the effects on dopamine (Chen et al., 1997a,b,c; Kohlhauser et al., 1999), and amino acid (Chen et al., 1997b; Kohlhauser et al., 1999; Engidawork et al., 2001) neurocircuitries of the basal ganglia. While the hippocampus is perhaps the most plastic structure of the CNS, playing a key role in memory and learning, little attention has been given in that model to the effect of perinatal asphyxia on the hippocampus, although in the original paper by Bjelke et al. (1991), it was shown that severe perinatal asphyxia induced a reduction in the number of neural cell bodies in the Ammon's horn (CA) 1 and CA3 regions, reflecting neuronal death.

Following global (Kirino, 1982; Kirino et al., 1984), or focal (Nakano et al., 1990) anoxia/ischemia, neurons of the hippocampus show a delayed death that can occur days after the insult, involving neuronal cells in CA1 (Johansen et al., 1992) and dentate gyrus (DG) (Wang et al., 1999), suggesting an apoptotic mechanism (Nakajima et al., 2000). It has also been shown that anoxic/ischemic insults can trigger several compensatory mechanisms to neuronal death including neurogenesis (Gould and Tanapat, 1997). Indeed, neurogenesis has been observed in several regions of the brain (Gage, 2000), including DG (Liu et al., 1998; Jin et al., 2001; Kee et al., 2001; Daval et al., 2004) and the CA1 region (Nakatomi et al., 2002; Daval et al., 2004) of the hippocampus.

Thus, the present study investigated the long-term consequences of perinatal asphyxia performed *in vivo* on hippocampus organotypic cultures (Gähwiler, 1981), focusing on: (i) *in vitro* cell survival, by direct monitoring with Hoffman's microscopy, and labeling alive and dead cells with a viability test; (ii) neuronal phenotype, by labeling microtubule-associated protein (MAP)-2 positive cells in fixed material, and (iii) postnatal neurogenesis, by treating the cultures with 5-bromo-2′-deoxyuridine (BrdU) and ulterior immunocytochemistry. (iv) Astrocyte proliferation was also examined using an antibody against glial fibrillary acidic protein (GFAP), together with Fast Red nuclear staining.

EXPERIMENTAL PROCEDURES

Perinatal asphyxia

Pregnant Wistar rats within the last day of gestation (G22) were killed by neck dislocation and hysterectomized. One or two pups were removed immediately from a uterine horn and stimulated to breathe to be used as non-asphyxiated caesarean-delivered controls. The remaining fetus-containing uterine horns were immersed in a water bath at 37 °C for 20 min, and then the fetuses were removed from the uterine horns, stimulated to breathe and after a 60 min observation period given to surrogate dams for nursing, pending further experiments. Seven days after birth (P7), the pups were used for preparing organotypic cultures using a modification of a protocol developed by Gähwiler (1981).

Organotypic cultures

Different rat series (>10) were used for preparing cultures. Following decapitation, the brain was rapidly removed under sterile conditions and stored in a Petri dish containing Dulbecco's modified Eagle medium (DMEM; GIBCO BRL, Life Technologies AB, Täby, Sweden). Coronal sections were cut with a microslicer (DTK-2000, Dosaka, CO, Japan) at 350 μm thick and stored in cold DMEM. Sections from the hippocampus were dissected and placed on a coverslip (Nunc Thermanox Coverslips; Nunc, Naperville, IL, USA), containing a spread layer of chicken plasma (25 μl), and further coagulated by a bovine thrombin ([Sigma, St. Louis, MO, USA]; 20 μl of a 20 μl/450 μl DMEM solution, freshly prepared from frozen aliquots containing 1000 NIH units in 0.75 ml H_2O). The coverslips were then transferred to sterile Nunc flat CT-tubes containing an un-buffered culture medium [50% Basal Medium Eagle, 25% Hanks' Balanced Salt Solution and 25% horse serum (GIBCO BRL), 0.5% glucose, 0.5 mM of L-glutamine (Sigma), and 0.1% antibiotic/anti-mycotic (GIBCO BRL)]. The cultures were grown at 35 °C, 10% CO_2 in a Cell Incubator (Model TC2323, ShelLab, USA), with a roller device exposing the cultures to gaseous or water phases every minute. After 3 days, the cultures were transferred to a serum-free medium (Neurobasal-A medium with B27 complement [GIBCO BRL], glucose 5 mM, L-glutamine 2.5 mM [Sigma]). The medium was changed every 3–4 days.

In vitro and ex vivo monitoring

Growth was periodically monitored with an inverted microscope equipped with Hoffmann optic (Nikon T100). Pictures were regularly taken (4, 7, 14 and 21 days *in vitro*, DIV), and then the cultures were treated with 10 μM of BrdU (Sigma), added to the medium for three days, and fixed with a formalin solution. Alternatively, the cultures were analyzed for cell viability at DIV 27, using ethidium-homodimer-1 and calcein-Acetomethoxy (AM) for labeling dead and alive cells, respectively (Molecular Probes L3224, Eugene, OR, USA). For quantification two samples were taken from the body and border of the tissue, focusing on areas presenting the majority of ethidium-homodimer positive cells.

Immunocytochemistry

For immunocytochemistry, the cultures were fixed in a formalin solution (4% paraformaldehyde, PF; Sigma) for 45 min at 4 °C. After rinsing cycles, the tissue was detached from the coverslip, mounted onto a gelatin-coated microscope slide for immunostaining. Cellular proliferation was labeled with the mitotic marker BrdU (Megabase, Lincoln, NB, USA), and neuronal phenotype with an antibody against MAP-2 (Sigma).

For MAP-2 immunocytochemistry, cultures were post-fixed in methanol 100% (30 min), rinsed three times and pre-incubated in 0.1 M phosphate-buffered saline (PBS), 0.1% Triton and 5% normal goat serum (NGS) (Calbiochem, CA, USA) for 1 h. A mouse monoclonal antibody against MAP-2, immunospecific for all forms of mature and immature neurons (1:2000, Sigma), was applied overnight at 4 °C in 0.1 M PBS, 0.1% Triton and 5% NGS. Following extensively washings, cultures were incubated in a Tyramide Amplification Kit #3 (TSA™, Molecular Probes), according to the instructions of the supplier. After that, the cultures were post-fixed in 4% PF for 15 min at 4 °C. The cultures were then washed extensively. DNA denaturation was achieved by treating the slices with 2 N HCL for 30 min at 37 °C. They were extensively washed in 0.1 M PBS before pre-incubation for 1 h at room temperature in 0.1 M PBS, 0.1% Triton and 5% NGS. A rabbit polyclonal antibody against anti-BrdU (1:4000, Megabase) was applied overnight at 4 °C in 0.1 M PBS, 0.1% Triton and 5% NGS. Following extensively washings, cultures were incubated in the TSA™ kit #12. The sections were washed again, coverslipped with DAKO fluorescent mounting medium (DAKO Corp, Carpinteria, CA, USA) and examined in an epi-fluorescence inverted microscope.

For GFAP, PF fixed tissue was washed in PBS, pre-incubated with 5% of NGS, 0.1% Triton X-100, in PBS, for 1 h at 37 °C, and incubated overnight with a mouse monoclonal antibody against GFAP (Sigma) (1:2000 diluted in 5% NGS, 0.1% Triton X-100, in PBS). Following extensively washings, the slices were treated with a biotinylated anti-mouse IgG (1:500 in PBS) for 1 h, followed by a further incubation with a streptavidin phosphatase complex for 1 h, rinsed and incubated with a levamisole solution (Vector Laboratories, Burlingame, CA, USA) for 15 min, to inhibit the endogenous alkaline phosphatases. The reaction was visualized with a 5-bromo-4-chloro-3-indoyl-phosphate/nitroblue tetrazolium (BCIP/NBT) substrate kit (Vector Laboratories). Then, the slices were counterstained with Fast Red (Sigma) for labeling cell nucleus. Sections were dehydrated through graded alcohols, cleared in xylene and coverslipped in entellan mounting medium (Merck, Darmstadt, Germany).

Confocal microscopy–optical disector quantification

Confocal microscopy was performed using a Zeiss LSM410 confocal laser-scanning microscope with a 633 (1.4 N.A.) oil immersion objective lens. MAP-2 or BrdU-positive cells in hippocampal cultures were counted by an investigator blinded to the treatment, using the optical disector technique described in detail by Gundersen et al. (1988). Briefly, MAP-2 or BrdU-positive nuclei were counted as they came into focus while scanning through the section. The disector height (h) was set at 10 μm and nuclei within the first 3 μm of the section were not counted. The area of dissector (a_{dis}) was set at 4.5×10^4 μm^2. The area of culture (a) was measured through an image J 1.32 software. The total number of MAP-2 or BrdU-positive nuclei in each hippocampus culture was then estimated as $N=\Sigma Q^{-}\times t/h\times a/a_{dis}$; where ΣQ^{-} is the total number of counted MAP-2 or BrdU positive nuclei in each culture; *t,* the average slice thickness; a, the area of culture; a_{dis},

the area of dissector; *h*, the dissector height. Cells were considered double-labeled when MAP-2 and BrdU immunoreactivity overlapped at four levels through a section (z-step 1 μm).

All data are presented as mean±standard error of the means (S.E.M.); comparisons were analyzed with a Student's *t*-test for unpaired data.

A local Committee for Ethics approved the experimental protocol for laboratory animals, according to international guidelines on the ethical use of animals, minimizing the number of animals used and their suffering.

RESULTS

In vitro monitoring

The hippocampus sections developed well *in vitro*, preserving the main layering features observed *in vivo*, making possible to distinguish the CA1, CA2 and CA3 regions, as well as the DG when observed with an inverted microscope along *in vitro* monitoring (4, 7, 14 and 21 DIV), without requiring any particular labeling. The Hoffman resolution was useful for identifying individual cell bodies, as well as processes, which was even easier along with *in vitro* development, because the tissue became thinner and presented a quasi monolayer appearance, as previously reported (Gähwiler et al., 1997; Plenz and Kitai, 1996a,b; Gomez-Urquijo et al., 1999). At the *in vitro* monitoring stage, no evident differences were observed between cultures from asphyxia-exposed or control pups. The quality of the tissue was, however, variable, because there were sometimes holes that appeared to tense the tissue. Despite these holes, the tissue and its stratification were well preserved, which was later confirmed by an *in vitro* viability test and by the immunocytochemical analysis, when the tissue was properly fixed.

In the *in vitro* viability test, developed by Novelli et al. (Fernandez et al., 1991; Novelli et al., 1988) and improved by Papadopoulos et al. (1994), alive cells are identified with calcein-AM, which is taken up by alive cells and split to calcein and AM by intracellular esterase. The calcein dye is retained within alive cells producing a green fluorescence. Dying and dead cells are identified with ethidium homodimer-1, entering to cells with damaged membranes,

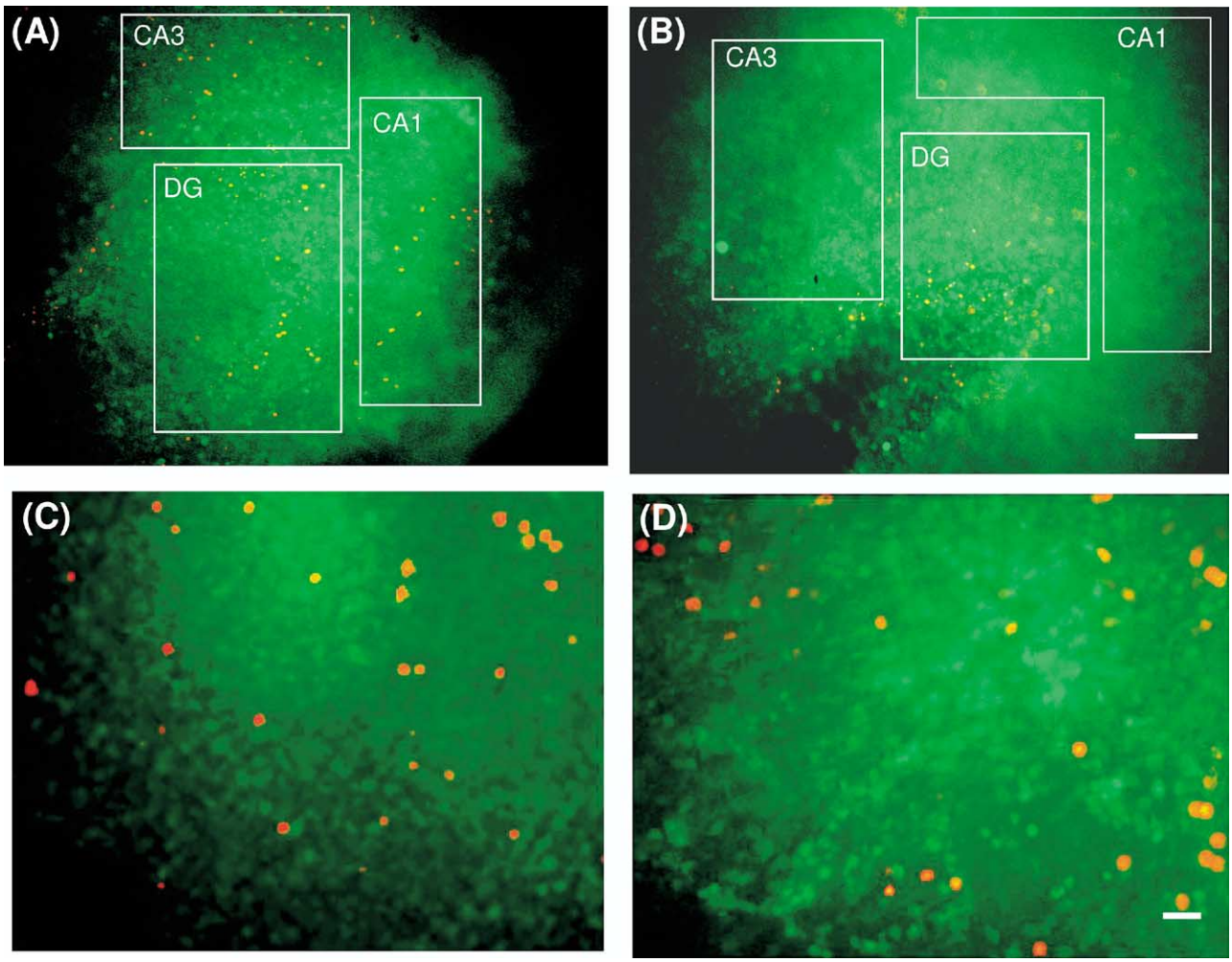

Fig. 1. Cell viability test applied to hippocampal organotypic cultures from control (A, C) and asphyxia-exposed (B, D) rats at DIV 27. The hippocampal regions CA1, CA3 and DG are demarked by filled lines. (C) and (D) correspond to a magnification from the area demarked as DG in (A, B). Many alive (green fluorescence), but few dying (red fluorescence) cells are seen in both groups. Scale bars=250 μm in A and B; 25 μm in C and D.

Table 1. Number of ethidium-homodimer positive cells (red) in hippocampal organotypic cultures from control and asphyxia-exposed animals at DIV 27, measured in 1 mm^2 samples from body and border regions of the tissue

Sampled region	Cultures from control animals (n=2)	Cultures from asphyxia-exposed animals (n=3)
Body	55±37	76±46
Border	99±26	92±24

binding to nucleic acids to produce a red fluorescence. In the present study, the majority of the cells showed an intensively green fluorescence surrounded by a greenish background (Fig. 1). Some migrating cells could be observed, surrounding the body of the tissue or in the holes, which stretched strips of well-preserved greenish tissue. Few ethidium-homodimer positive cells (red) were observed in cultures from both asphyxia-exposed (n=3) and control (n=2) pups, often on the top of the tissue, but also in the holes (Fig. 1). For quantification, ethidium-homodimer positive cells were counted in 1-mm^2 samples of organotypic cultures at DIV 27, from the body and border of the tissue. Table 1 shows that there was an apparent increase in the number of red cells in the body of the tissue of cultures from asphyxia-exposed animals compared with that seen in controls, but the difference did not reach the statistic level.

MAP-2 immunostaining

When PF-fixed (24–27 DIV), and treated with antibody against MAP-2, it was evident that many of the cells observed in the cultures presented a neuronal phenotype, with cell bodies in regions identified as CA1, CA3 and DG (Fig. 2A, B). When inspected with confocal microscopy, cultures from asphyxia-exposed pups showed a decreased number of MAP-2 positive cells (n=4), as compared with that seen in cultures from control animals (n=5) (c.f. Fig. 2C versus 2D). Quantification, by using the optical disector technique (Gundersen et al., 1988), revealed that the decrease was significant, whether considering the number of MAP-2 positive cells in the whole hippocampus tissue (32±16%) (Fig. 3A), or cells counted in CA1 (40±8%) and DG (42±14%) regions, respectively (Fig. 3B).

BrdU immunostaining

After *in vitro* treatment with the mitotic marker BrdU (10 μM BrdU to the culture medium for three days), fixation and immunocytochemistry, many BrdU positive cells were seen in all cultures, but the total amount of BrdU positive cells was larger in cultures from asphyxia-exposed (n=5) than that from control animals (n=5) (Fig. 4). That difference was even larger when expressed as the number of BrdU positive cells/mm^3 (~two-fold).

Double BrdU/MAP-2 immunostaining: confocal microscopy

Double staining revealed that approximately 25% of BrdU positive cells were also positive for MAP-2, in cultures from both control (21±6%; n=5) and asphyxia-exposed (32±11%, n=4) animals, respectively (Fig. 4). The majority of doubled-stained cells were observed in DG.

Fig. 5 shows confocal cases observed in DG of cultures from control (Fig. 5A–C), and asphyxia-exposed (Fig. 5D–F) animals. Several BrdU positive nuclei and MAP-2 perikarya can be seen in panels A, D and B, E, respectively, showing double staining when the pictures are merged (C, F). The double staining was confirmed when observed at four levels through a section. Double staining revealed that BrdU/MAP-2-positive cells in cultures from asphyxia-exposed animals (Fig. 5F) formed clusters of two or more cells. When compared with the controls, BrdU/MAP-2 positive cells showed a reduced dendritic development, indicating, perhaps, that cells in cultures from asphyxia-exposed animals are immature (c.f. Fig. 5C versus 5F).

GFAP- immunostaining

In both groups, GFAP immunostaining (DIV 27) revealed abundant cell bodies and processes at the border of the cultures, with few GFAP-positive cells in the core of the cultures, core that was intensively labeled with the nuclear marker Fast Red (Fig. 6). GFAP-positive cells showed a multipolar feature, with processes extending through a wide area of the tissue. The GFAP-positive network surrounded the core of the cultures, appearing to provide a protective shield to the cultures. No apparent differences were observed between cultures from control and asphyxia-exposed animals regarding the amount of GFAP-positive cells, but in cultures from control animals (Fig. 6A), the GFAP network was well organized, while in cultures from asphyxia-exposed animals (Fig. 6B) that network was found to be less tight and the border less outlined.

DISCUSSION

The experimental model used in the present study was originally developed by Bjelke et al. (1991), proposing a useful model for studying the short- and long-term consequences of perinatal asphyxia, still a main pediatric issue, despite the improvements in neonatal care (Hill, 1991; Volpe, 2001). A main feature of the model is that it is largely non-invasive, allowing to use the asphyxia-exposed animal for long-term studies (Loidl et al., 1994; Chen et al., 1997a,b,c; Kohlhauser et al., 1999; Gross et al., 2000, 2005; Bernert et al., 2003), or for preparing organotypic cultures (Morales et al., 2003; Klawitter et al., 2005), as shown in the present study.

We show here that hippocampus tissue from control and asphyxia-exposed animals survived and developed well when explanted at P7 and kept alive for approximately one month as organotypic cultures following a protocol proposed by Gähwiler and collaborators (Raineteau et al., 2004). The cultures preserved the typical layering and

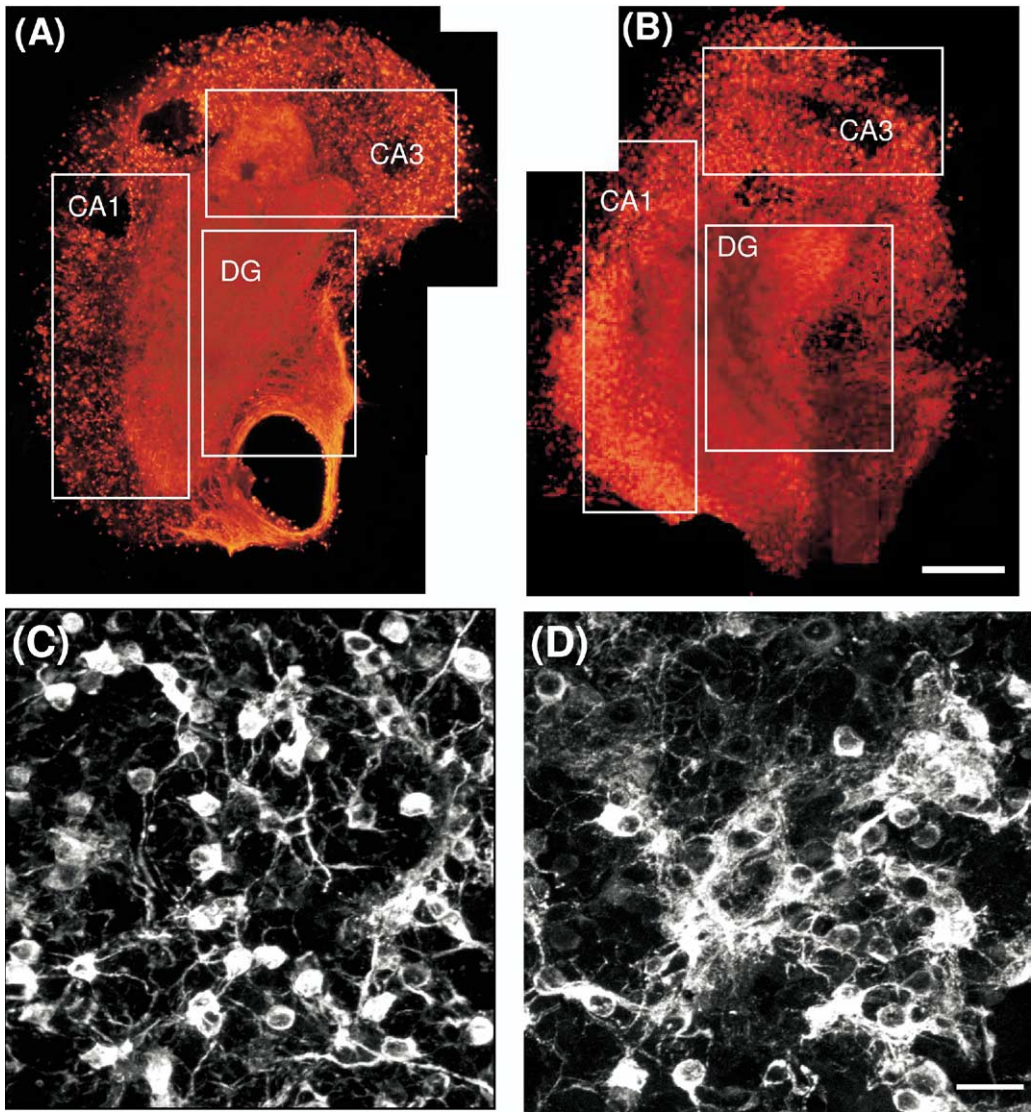

Fig. 2. MAP-2 positive cells in organotypic cultures from control (A, C) and asphyxia-exposed (B, D) rats at DIV 24–27. (C) and (D) are microphotographs taken with confocal microscopy from the same cultures shown in (A) and (B) respectively. Observe that in the culture from an asphyxia-exposed animal (D) the number of MAP-2 positive cells is decreased compared with that in the control (C). Scale bars=500 μm in A and B; 25 μm in C and D.

regional subdivisions shown *in vivo*, and only few dying cells were observed when assayed with a viability test at DIV 27.

No differences were observed regarding *in vitro* viability between cultures from control and asphyxia-exposed animals, although the number of red-positive dying cells/mm^2 in the body of the tissue of cultures from asphyxia-exposed animals appeared to be elevated compared with that seen in controls. In agreement, immunocytochemistry revealed a decrease in the total amount of MAP-2 positive cells in organotypic cultures prepared from hippocampus of asphyxia-exposed, compared with that from control pups. Furthermore, organotypic cultures from asphyxia-exposed animals showed a two-fold increase in BrdU-positive cells, compared with that seen in controls, suggesting an enhanced postnatal mitotic activity. BrdU positive cells were seen in all regions of hippocampus. Double labeling, further evaluated with confocal microscopy, showed that approximately 25% of the cells were positive for both MAP-2 and BrdU. The majority of double labeled cells was seen in DG of cultures from both asphyxia-exposed and control animals, suggesting postnatal neurogenesis. Quantitative analysis showed that the proportion of BrdU/MAP-2 double staining was, however, similar in both groups. The two-fold increase of total BrdU-positive cells/mm^3 observed in cultures from asphyxia-exposed animals suggests instead an increase of other types of cells (Raineteau et al., 2004; Kuan et al., 2004).

The GFAP-positive cells formed a network surrounding the core of the cultures, appearing to provide a protective shield to the cultures as previously reported (Raineteau et al., 2004). Interestingly, that network was well organized in

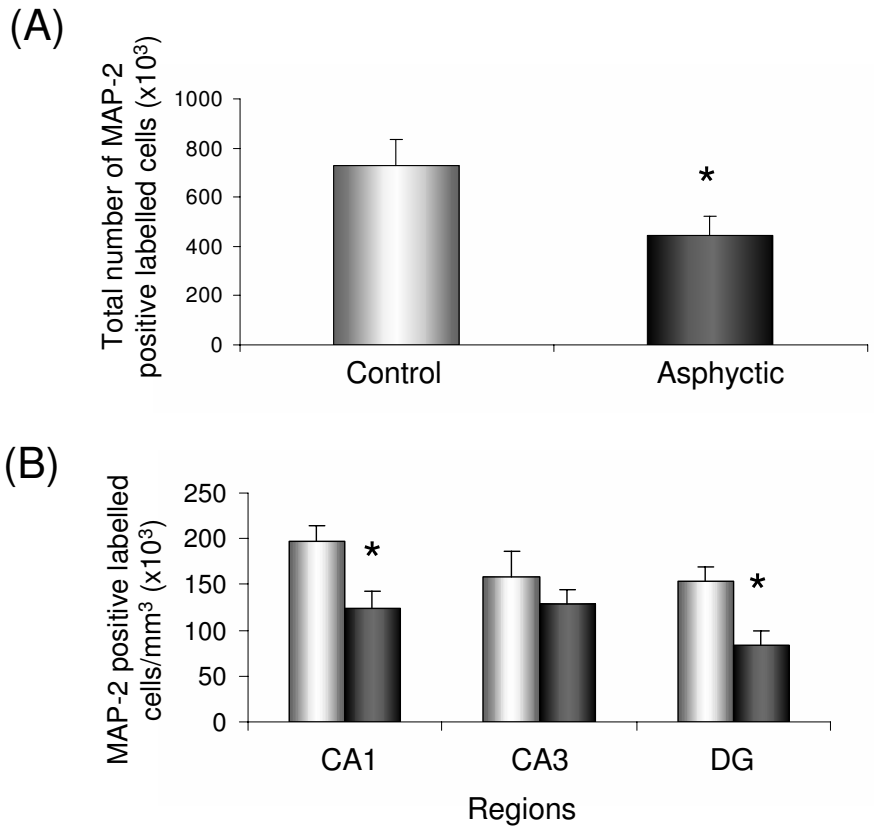

Fig. 3. Quantification of MAP-2 cells seen in hippocampal cultures from control (light bars) ($n=5$) and asphyxia-exposed (dark bars) ($n=4$) rats at DIV 24–27. In (A) the sampling is from the whole hippocampal tissue, showing a significant decrease (32±16%) of MAP-2 labeled cells in cultures from asphyctic rats. In (B), MAP-2 cells were seen in CA1 and CA3 and DG of hippocampus. There is a significant decrease of MAP-2 positive cells/mm^3 in asphyxia-exposed CA1 (40±8%) and DG (42±14%) regions compared with that in controls. * $P<0.05$ (Student's *t*-test).

cultures from control animals, but appeared to be less tight and less outlined in cultures from asphyxia-exposed animals, suggesting perhaps an increased vulnerability to noxious substances present in the surrounding environment. As previously reported (Strasser and Fischer, 1995; Scheepens et al., 2003; Bartley et al., 2005), no apparent differences were observed regarding the total amount of GFAP-positive cells, between cultures from control and asphyxia-exposed animals.

The susceptibility of hippocampus to anoxia, ischemia and other metabolic insults is well documented both in human (Squire and Zola, 1996; Johnston, 1997) and animal (Pulsinelli, 1988; Kirino, 2000; Harry and d'Hellencourt, 2003) models. Following global (Kirino, 1982; Pulsinelli et al.,

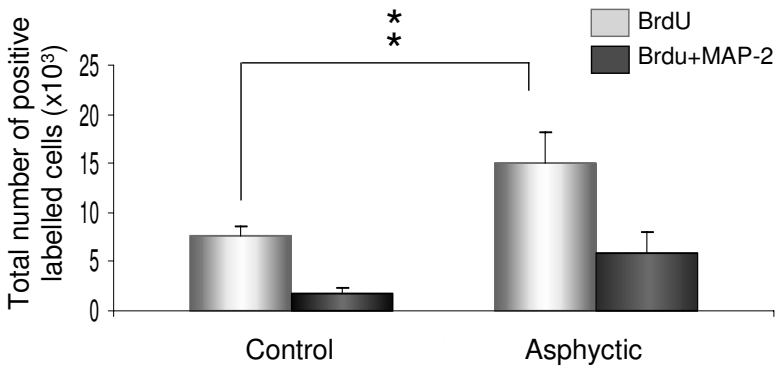

Fig. 4. Quantitative analysis of total BrdU (light bars) and BrdU/MAP-2 double labeled (dark bars) cells observed in cultures from asphyxia-exposed and control rats at DIV 24. There is a significant increase of BrdU-labeled cells in cultures from asphyxia-exposed rats (14,976±3176 BrdU-positive cells, $n=5$, versus 7548±996 cells in cultures from control animals, $n=5$; $P<0.01$; Student's *t*-test). In cultures from asphyxia-exposed rats, 32±11% of BrdU positive cells were also positive for MAP-2 (5790±2666 cells; $n=4$), while in cultures from control rats, 21±6% of BrdU positive cells were also positive for MAP-2 (1703±548, $n=5$), but that difference did not reach a statistically significant level.

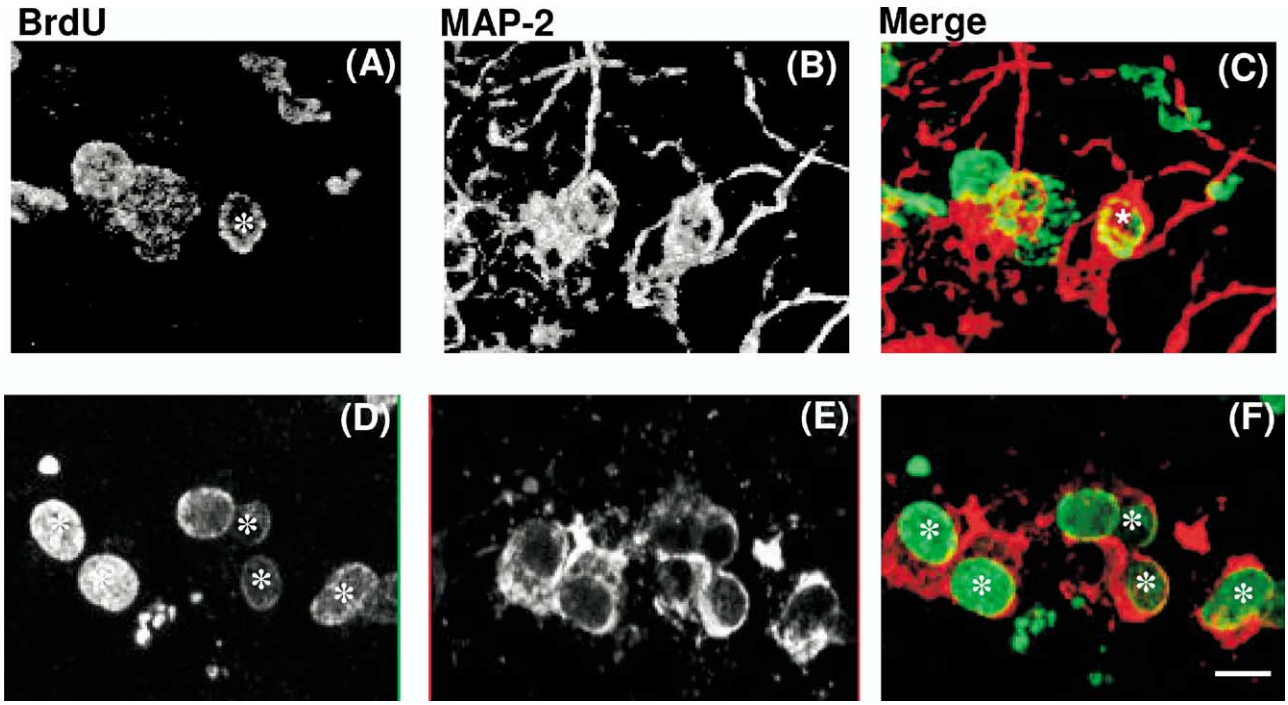

Fig. 5. Cell proliferation and neuronal phenotype evaluated at DIV 24 with confocal microscopy in DG of hippocampal cultures from control (A–C) and asphyxia-exposed (D–F) rats. (A) and (D) show BrdU positive cells only. (B) and (E) show MAP-2 positive cells only. (C) and (F) are the merging of the pictures. Scale bar=10 μm.

1982; Kirino et al., 1984), or focal ischemia (Nakano et al., 1990) in adult animals, neurons of the hippocampus show a delayed death that can be evident days after the insult, mainly seen in CA1 (Johansen et al., 1992) and DG (Wang et al., 1999), suggesting an apoptotic mechanism (Johnston, 2000; Nakajima et al., 2000). It has also been shown that anoxia/ischemia can trigger several compensatory mechanisms to neuronal death, including neurogenesis (Gould and Tanapat, 1997; Liu et al., 1998; Kee et al., 2001; Daval et al., 2004). These changes have also been observed following perinatal or neonatal asphyxia (Johnston et al., 2001; Volpe, 2001). Furthermore, using the present model for inducing perinatal asphyxia, it was demonstrated that the hippocampus suffers a significant amount of neuronal loss following severe perinatal asphyxia without changes in hippocampal volume at P21 (van de Berg et al., 2001). A similar result was reported when analyzing asphyxia-exposed guinea-pig three months after the perinatal insult (Bernert et al., 2003).

The effect of global hypoxia/ischemia has been further investigated with a stereological method, demonstrating the peculiar vulnerability of CA1 pyramidal neurons and dentate hilar interneurons (Larsson et al., 2001). Cell loss probably relates to (i) energy failure; (ii) free radical damage; (iii) cytokines and excitotoxicity and/or (iv) caspase-dependent cell death, occurring immediately after the insult, or progressing with time upon reperfusion/reoxygenation. It has been suggested that the immediate cell loss is by necrosis, while delayed neuronal death is by apoptosis (Northington et al., 2001), and that neuronal loss can further lead to damage by target deprivation of trophic support (Miller and Kuhn, 1997).

Apoptosis has been shown to play a prominent role in the neurodegeneration observed following hypoxia/ischemia in newborn rats (Johnston, 2000; Nakajima et al., 2000). Thus, a significant increase in the activity of caspase-3, a cysteine protease involved in the execution phase of apoptosis (Yuan et al., 1993; Chen et al., 1998; Endres et al., 1998; Namura et al., 1998; Ni et al., 1998) was reported to occur in hippocampus one week after perinatal asphyxia (Daval et al., 2004; van de Berg et al., in preparation). In our own *in vivo* studies (Morales et al., in preparation), using Hoechst DNA and terminal deoxynucleotidyl transferase-mediated UTP nick labeling, we have also found an enhanced amount of apoptotic nuclei in CA1, CA3 and GD regions, seven days after perinatal asphyxia. This peak in apoptotic cell loss observed one week after a hypoxic/ischemic insult appears to reflect a strict molecular mechanism, associated with a spontaneous anatomical recovery reported by several authors (Sharp et al., 2002; Nakatomi et al., 2002) and with a shift in the expression of pro-apoptotic (BAX and caspase-3), to the anti-apoptotic protein Bcl-2, recorded at 20 days post-hypoxia (Daval et al., 2004).

In the present study, we found a two-fold increase in BrdU-positive cells in organotypic cultures from asphyxia-exposed compared with that seen in control animals, suggesting an enhanced postnatal neurogenesis, although MAP-2 positive cells corresponded only to a ¼ of those labeled for BrdU. However, we used here a Sigma mono-

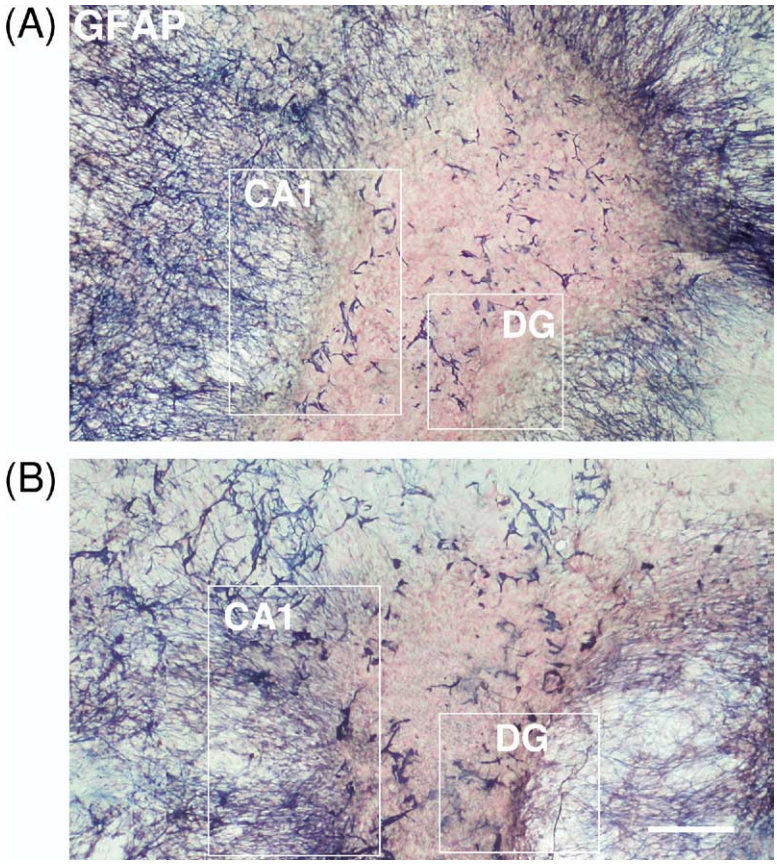

Fig. 6. GFAP-immunostaining (blue) and Fast Red nuclear staining (red) observed at 27 DIV in hippocampal cultures from control (A) and asphyxia-exposed (B) rats. Observe that the nuclear staining (red) is concentrated in the core of the cultures. Individual GFAP positive (blue) cells in that core are clearly distinguished, showing a multipolar feature, with processes extending for ~50 μm. No apparent differences were observed between cultures from control and asphyxia-exposed animals. In the periphery of the cultures a strong GFAP-positive network can be seen, surrounding the core of the cultures, providing an apparent barrier protecting the border of the cultures. In cultures from control animals (A) that barrier is well organized, while in cultures from asphyxia-exposed animals (B) that network is less tight and the border is less outlined. Scale bar=250 μm.

clonal antibody that reacts with all forms of MAP-2, a to c, not being able to differentiate among juvenile and adult isoforms of MAP-2. It is possible that with a more specific antibody we could demonstrate that some of the BrdU-positive cells observed here represent neurons in a more immature developmental stage, or even immature astrocytes or other types of glial cells (Raineteau et al., 2004).

Several studies have shown that experimental anoxia/ischemia can lead to neurogenesis as a compensatory mechanism for neuronal cell death in hippocampus (Liu et al., 1998; Kee et al., 2001). New neurons can generate from the subgranular cell layer of the DG, perhaps even migrating to CA1 and CA3 regions (Nakatomi et al., 2002; Daval et al., 2004). While less possible in the present preparation, cells migrating from the periventricular zone can also contribute to re-placement of neurons of the CA1 region (Daval et al., 2004). In adult animals, global ischemia produces a several-fold increase in the birth rate of new cells in the subgranular zone of the DG, beginning at 7 days after the insult, peaking at 11 days and decreasing thereafter (Sharp et al., 2002), news cells becoming NeuN-, calbindin- and MAP-2-immunostained neurons, or GFAP-labeled astrocytes (Kee et al., 2001).

It has been shown that there is an increase in the levels of basic fibroblast growth factor (FGF2) following hypoxia (Andersson et al., 1995), a protein expressed by astroglia throughout the forebrain (Ganat et al., 2002). However, FGF2-positive astrocytes do not show any detectable GFAP staining, suggesting a reversion to an immature cell stage, perhaps re-entering to the cell cycle and leading to neuronal regeneration, or resuming apoptosis-associated DNA synthesis (Kuan et al., 2004). In the present study, there was a wide amount of BrdU-positive cells in cultures from both asphyxia-exposed and control animals, not restricted to DG, but also comprising regions of the Ammon's horn, in agreement with that reported by Rietze et al. (2000). Since only ¼ of BrdU-positive cells were also positive to MAP-2, a significant amount of BrdU-positive cells shown in the present study may represent cells at an immature stage, potentially leading to neurogenesis via a radial glia pathway (Ganat et al., 2002; Kuan et al., 2004), or just gliogenesis.

Perinatal asphyxia may trigger gliogenesis, but no apparent increase in the number of GFAP-positive cells could be demonstrated in cultures from asphyxia-exposed animals, compared with that from controls. Using the same

model, Scheepens et al. (2003) did not find any change in hippocampus GFAP content assayed *in vivo* with an ELISA method (O'Callaghan, 1991), within a 15 days' post-asphyxia period. A lack of effect on GFAP-stained astrocytes was also reported by Strasser and Fischer (1995), studying the effect of combined oxygen/glucose deprivation in organotypic hippocampal cultures.

Interestingly, however, using a chronic neonatal hypoxia model, Ganat et al. (2002) observed a decreased number of GFAP-immunoreactive cells, but an increased density of vimentin-positive cells, a marker for radial glial cells and immature astrocytes (Pixley and de Villis, 1984). In the same line, it was reported that transient forebrain ischemia in adult rats can induce the expression of Musashi1 (Msi1) and nestin proteins by reactive astrocytes of the hippocampus (Yagita et al., 2002). Msi1 is a protein present in ependymal and subependymal cells and astrocytes, but not in microglia, oligodendrocyte or mature neurons (Sakakibara et al., 1996), while nestin is a protein used as a neural stem/progenitor cell marker (Lendhal et al., 1990). While almost all Msi1 and nestin immunoreactivity was co-localized with GFAP in the CA1 region after ischemia, Msi1-, but not nestin-positive cells were shown to form clusters after ischemia, co-expressing BrdU but not GFAP, in the subgranular zone of the hippocampal DG, whereas almost all nestin-positive cells in that region also expressed GFAP (Yagita et al., 2002). The increased expression of nestin in subgranular zone of the DG following brain injury has led to suggest that reactive astrocytes have the potential ability to revert to an undifferentiated stage (Doetsch et al., 1999; Seri et al., 2001; Yagita et al., 2001, 2002). Raineteau et al. (2004) have monitored cellular proliferation in hippocampal organotypic cultures, finding numerous BrdU-positive cells at the surface of all regions of the slices, the larger proportion in DG ($\sim$22 cells/250 μm^2), many positive to GFAP, showing type-I (differentiated during embryonic development) and type-2 (reactive astrocytes) morphology, co-expressing nestin, as seen in organotypic cultures from rat cortex (Schmidt-Kastner and Humpel, 2002). Raineteau et al. (2004) also demonstrated, as shown here, that astrocytes could form a protective shield around the hippocampal tissue. Interestingly, that shield appears to be less developed in cultures from asphyxia-exposed animals, suggesting that there is an increased vulnerability for noxious substances from the surrounding environment, as well as an impairment in the homeostasis of the extracellular compartment required for neurotransmission.

CONCLUSIONS

The present report summarizes studies combining an *in vivo* and *in vitro* approach, where asphyxia is induced *in vivo* at the time of delivery and the long-term effects on hippocampus neurocircuitry are investigated *in vitro* with organotypic cultures plated at P7. A major effect of perinatal asphyxia was a decrease in MAP-2 positive cells, observed in cultures prepared from asphyxia-exposed, compared with that from control animals, both in CA1 and DG regions of the hippocampus, indicating a regionally specific neuronal death. That effect was accompanied by a two-fold increase in mitotic activity, as evaluated with BrdU-staining in cultures from asphyxia-exposed animals, although it was not evident that that increase led to an enhanced number of MAP-2-labeled neurons. The increase in mitotic activity observed in this study can represent a compensatory attempt to revert a long-term impairment induced by perinatal asphyxia, although that compensation can equally lead to neurogenesis, astrogliagenesis, or to a final tagging for fulfilling an apoptotic program. The two-fold increase in the expression of BrdU can provide, however, a therapeutic opportunity, used for interventions preventing, or even reverting, the long-term neurological effects induced by a severe metabolic insult like anoxia occurring at birth time.

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