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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. © 2005 Published by Elsevier Ltd on behalf of IBRO.

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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.

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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},