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Perinatal Asphyxia Induces Neurogenesis in Hippocampus: an Organotypic Culture Study

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There is clinical and experimental evidence indicating that neurocircuitries of the hippocampus are vulnerable to hypoxia/ischemia occurring at birth, inducing, upon re-oxygenation/re-circulation, delayed neuronal death, but also compensatory mechanisms, including neurogenesis. In the present report, perinatal asphyxia was induced by immersing foetuses-containing uterine horns removed from ready-to-deliver rats into a water bath at 37°C for 20 min. Some pups were delivered immediately after the hysterectomy to be used as non-asphyxiated caesarean-delivered controls. The pups were sacrificed after seven days for preparing organotypic hippocampal cultures. The cultures were grown on a coverslip in a medium-containing culture tube inserted in a hole of a roller device standing on the internal area of a cell incubator at 35°C, 10% CO_2. At days *in vitro* (DIV) 25-27, cultures were fixed for assaying cell proliferation and neuronal phenotype with antibodies against 5-bromo-2'deoxyuridine (BrdU) and microtubule associated protein-2 (MAP-2), respectively. Confocal microscopy revealed that there was a 2-fold increase of BrdU-positive, but a 40% decrease of MAP-2-positive cells/mm^3 in cultures from asphyxia-exposed, compared to that from control animals. Approximately 30% of BrdU-positive cells were also positive for MAP-2 (approximately 4800 cells), mainly seen in the dentate gyrus of the hippocampus, demonstrating a 3-fold increase of postnatal neurogenesis, when the total amount of double-labelled cells seen in cultures from asphyxia-exposed animals is compared to that from control animals.

Keywords: Perinatal asphyxia; Hippocampus; Neurogenesis; Organotypic cultures

Hypoxia/ischemia at birth induces long-term neurodevelopment impairments, resulting in spasticity, epilepsy and mental retardation when the insult is severe (see Hill, 1991; Johnston, 1997; Volpe, 2001), or attention-deficit hyperactivity syndrome and minimal brain disorders when it is mild (see Volpe, 2001; 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.*, 2002) and experimental (Pulsinelli *et al.*, 1982; Larsson *et al.*, 2001) evidence indicating that neurocircuitries of the hippocampus are also extremely vulnerable to that

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type of insult (see Pulsinelli, 1988; Squire and Zola, 1996; Harry and d'Hellencourt, 2003). Following global (Kirino *et al.*,1984; Kirino, 2000), 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 (Larsson *et al.*, 2001) and dentate gyrus (DG) (Johansen *et al.*, 1992), 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 (Lendhal *et al.*, 1998; Jin *et al.*, 2001; Kee *et al.*, 2001; Yagita *et al.*, 2001; Daval *et al.*, 2004) and the CA1 region (Nakatomi *et al.*, 2002; Daval *et al.*, 2004) of the hippocampus.

As previously reported (Morales *et al.*, 2005), we show here evidence for neurogenesis following perinatal asphyxia, assessed with hippocampus organotypic cultures grown *in vitro* for 25-27 days (DIV 25-27).

Perinatal asphyxia was induced by immersing foetuses-containing uterine horns removed from on-term rats into a water bath at 37°C for 20 min. One or two pups were delivered immediately to be used as non-asphyxiated caesarean-delivered controls (see Bjelke *et al.*, 1991; Andersson *et al.*, 1992; Herrera-Marschitz *et al.*, 1993). After delivery, control and asphyxia-exposed pups were stimulated to breathe and given to surrogate dams. The pups were sacrificed after seven days for preparing organotypic cultures of hippocampus. 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 coagulated by a bovine thrombin (20 μl of a 20 μl/450 μl DMEM (Sigma, St. Louis, MO, USA) 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 unbuffered 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/antimycotic (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. At DIV 3, the cultures were transferred to a serum-free medium [Neurobasal-A medium complemented with B27 (GIBCO BRL), 5 mM glucose, 2.5 mM L-glutamine (Sigma)]. The medium was changed every 3-4 days. At approximately DIV 24, 10 μM of 5-bromo-2'deoxyuridine (BrdU) (Megabase, Lincoln, NE, USA), was added to the culture medium for three days before the cultures were fixed in a cold formalin solution (4% paraformaldehyde, PF; Sigma, for 45 min). After rinsing cycles, the tissue was detached from the coverslip, mounted onto a gelatin-coated microscope slide for immunostaining. Neuronal phenotype was assayed with an antibody against microtubule associated protein-2 (MAP-2) (Sigma), while cellular proliferation was labelled with the mitotic marker BrdU (see Morales *et al.*, 2005).

For MAP-2 immunocytochemistry, cultures were post-fixed in methanol 100% (30 min), rinsed three times and pre-incubated in 0.1 M PBS, 0.1% Triton and 5% normal goat serum (NGS) (Calbiochem, CA, USA) for 1h. 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, Eugene, OR, USA), 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 2N 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.

For BrdU, a rabbit Polyclonal antibody (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 first with an epi-fluorescence inverted microscope, and then with a Zeiss LSM410 confocal laser-scanning microscope equipped with a 633 (1.4 N.A.) oil immersion objective lens. MAP-2 or BrdU-positive cells were counted in hippocampal cultures by an investigator blinded to the treatment, using the optical disector technique described in detail by Gundersen *et al.* (1988). A local National Committee for Ethics approved the experimental protocol for laboratory animals.

MAP-2 immunocytochemistry revealed that there were many cells with a neuronal phenotype, with cell bodies in regions identified as CA1, CA3 and dentate gyrus (DG). When inspected with confocal microscopy, cultures from asphyxia-exposed pups (n=5) showed a decreased number of MAP-2 positive cells, as compared to the controls (n=5). The effect was particularly prominent in DG (42 ± 14%). When treated with the mitotic marker BrdU, many positive cells were seen in all cultures, but the number of BrdU positive cells/mm3 was larger (~2-fold) in cultures from asphyxia-exposed (14976 ± 3176 cells) than that from control (7548 ± 996 cells) animals. Double BrdU/MAP-2 immunostaining revealed that ~20% (21 ± 6%) of BrdU positive cells of cultures from control animals (approximately 1500 cells) (n=5) were also positive to MAP-2, while ~30% (32 ± 11%) of BrdU positive cells of cultures from asphyxia-exposed animals (approximately 4800 cells (n=4) were also positive to MAP-2 (FIG.. 1).

The present results show that perinatal asphyxia induces a 2-fold increase in BrdU-positive cells, compared to that seen in controls, suggesting an enhanced postnatal mitotic activity. Thirty percent (30%) of BrdU-positive cells were also positive for MAP-2 (approximately 4800 cells), mainly seen in DG, demonstrating a 3-fold increase of postnatal neurogenesis, when the total amount of double-labelled cells seen in cultures from asphyxia-exposed animals is compared to that from control animals.

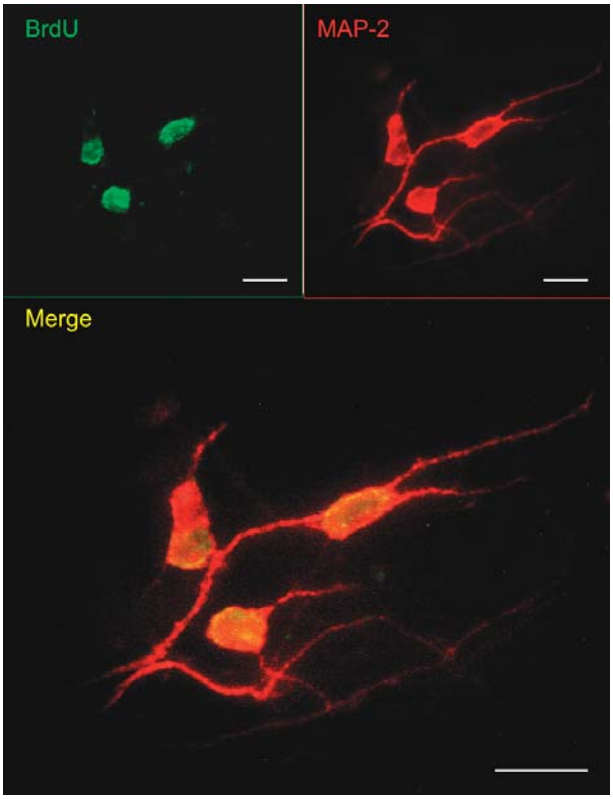

FIGURE 1 Perinatal asphyxia induces neurogenesis in hippocampus studied in organotypic cultures. 5-bromo-2'deoxyuridine (BrdU) positive (green) cells were found in the dentate gyrus (DG) of a culture from an asphyxia-exposed animal at days *in vitro* 27 (DIV 27). In the same region, there were microtubule associated protein (MAP)-2 positive (red) cells. When the images were merged, it became evident that the same cells were stained for the two markers, suggesting postnatal neurogenesis. Scale bar=20 μm.

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