

Perinatal asphyxia impairs connectivity and dopamine neurite branching in organotypic triple culture from rat substantia nigra, neostriatum and neocortex

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Abstract

The effect of perinatal asphyxia on brain development was studied with organotypic cultures from substantia nigra, neostriatum and neocortex. Asphyxia was induced by immersing fetuses-containing uterine horns removed from ready-to-deliver rats into a water bath for 20 min. Following asphyxia, the pups were nursed by a surrogate dam and sacrificed after 3 days to prepare organotypic cultures. Non-asphyxiated caesarean-delivered pups were used as controls. Morphological features were recorded during *in vitro* development. At day *in vitro* (DIV) 24, the cultures were treated for histochemistry using fast red for cell nucleus labelling and antibodies against tyrosine hydroxylase for dopaminergic neurons. Compared to controls, cultures from asphyxiated pups revealed a diminished integration quantified during 21 DIV. After immunocytochemistry and camera lucida reconstruction, tyrosine hydroxylase-positive neurons showed a decreased number of neurites from secondary and higher level branching, demonstrating a vulnerability of the dopaminergic systems after perinatal asphyxia.

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While the clinical relevance of perinatal asphyxia is well established, the pre-clinical research is still at an exploratory phase, mainly because of a lack of consensus on a reliable and predictable experimental model. At the Karolinska Institutet, we have developed a model for investigating perinatal asphyxia in the rat [2,10], showing that the neurocircuitries of the basal ganglia are particularly vulnerable to perinatal asphyxia. Depending upon the severity of the insult, several effects have been reported on dopamine systems, affecting cell body [1–4] and terminal [5,6,11,13] regions differently. Severe asphyxia (> 20 min) has been shown to decrease tyrosine hydroxylase immunoreactivity (TH-IR) in the substantia nigra (SN)

[4]. In terminal regions, such as neostriatum (Str), TH-IR fibres have also been shown to be decreased following severe perinatal asphyxia [12], together with a decrease in dopamine levels [17] and release [5,13]. In agreement, it has been shown with a quantitative reverse transcription polymerase chain reaction method that striatal dopamine D1 and D2 receptor subtype levels are increased following severe asphyxia, suggesting a functional dopamine receptor supersensitivity related to a presynaptic deficit [9].

Thus, it has been interesting to establish at a cellular level the long-term effects of perinatal asphyxia. For that purpose, perinatal asphyxia was performed *in vivo*, and then, the interesting brain regions were used for organotypic cultures. This method allows the reconstruction of the basal ganglia neurocircuitries, preserving many of the electrophysiological [15,16] and neurochemical [8] features seen *in vivo*.

In the present paper, we studied the effects of perinatal asphyxia on basal ganglia neurocircuitries reconstructed

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with organotypic cultures, assaying (i) *in vitro* survival, (ii) connectivity, and (iii) neuronal phenotype, focusing on dopaminergic neurons.

Pregnant Wistar rats (UCHA, bred at a local colony) within the last day of gestation (G22) were anaesthetized, sacrificed by neck dislocation and hysterectomized. One or two pups were removed immediately and used as non-asphyxiated caesarean-delivered controls, and the remaining fetuses-containing uterine horns were immersed in a water bath at 37 °C for 20 min. Following asphyxia, the uterine horns were incised and the pups were removed, stimulated to breathe and after a 60 min observation period were given to surrogate dams for nursing, pending further experiments. Three days after birth (P3), the pups were used for preparing organotypic cultures using a modification of a protocol developed by Plenz and Kitai [15,16] (see Ref. [8]). Following decapitation, the brain was rapidly removed under sterile conditions and stored in a Petri dish containing Dulbecco's modified Eagle medium (DMEM; GIBCO AB, Täby, Sweden). Coronal sections were cut at mesencephalic (300 µm thick) and telencephalic (350 µm thick) levels and stored in cold DMEM. Samples from SN, Str and frontoparietal cortex (Cx) were dissected using the atlas by G.A. Foster as a reference (Chemical Neuroanatomy of the Prenatal Rat Brain, Oxford University Press, New York, 1998). The dissected tissue was placed on a coverslip (Nunc Thermanox Coverslips; Nunc, Naperville, IL) containing a spread layer of chicken plasma (25 µl), and further coagulated by bovine thrombin (20 µl, 1000 NIH units in 0.75 ml DMEM; Sigma-Aldrich Sweden AB, Stockholm, Sweden). Care was given to placing the tissue in a row, Cx, Str and SN, with a maximal possible distance between each pair of tissues. Then, the coverslips were transferred to sterile Nunc flat CT-tubes (Nunc, Naperville, IL), containing an un-buffered culture medium (50% Basal Medium Eagle, 25% Hanks Balanced Salt Solution and 25% horse serum (Gibco BRL; Life Technologies AB, Täby, Sweden), 0.5% glucose, 0.5 mM of L-glutamine (Sigma-Aldrich AB, Stockholm, Sweden), and 0.1% antibiotic/antimycotic (Gibco BRL)). The cultures were grown at 35 °C, 5% CO₂ in a Cell Incubator (Model TC2323, ShelLab, USA), with a roller device exposing the cultures to gaseous or water phases every minute. At day *in vitro* (DIV) 3, 10 µl of a mitosis inhibitor cocktail (4.4 mM cytosine-β-D-arabino-furanoside, 4.4 mM uridine and 4.4 mM 5-fluoro-2'-deoxyuridine; all from Sigma-Aldrich AB) was added for 24 h to decrease glial proliferation. The medium was changed every 3–4 days. The experimental protocol was approved by a local National Committee for Ethic Experiments with Laboratory Animals.

Four different rat series were used for preparing asphyxiated and control pups, and each pup provided approximately four triple cultures, depending upon the amount of dissected SN. Growth was periodically monitored with an inverted microscope equipped with Hoffmann optic (Nikon T100). Pictures were regularly taken and

transferred to a computer for storing and image-analysis. The regions were identified and the distance between them was successively measured during development (DIV 3, 4, 7, 14 and 21) by an investigator blinded to the treatment received by the pups from whom the tissue was obtained (i.e. asphyxiated versus non-asphyxiated controls), using as a reference the closest points between each pair of tissue. Selected cases (one from asphyxiated and one from control pups) were kept *alive* for 27 days to test cell viability, using ethidium homodimer and calcein-AM for labelling dead and alive cells, respectively (Molecular Probes L3224).

The distance between the regions was measured in millimetres (mm) (means ± SEM), and expressed as the percentage of the distance estimated at DIV 3 for each case. Differences between groups were analyzed with F-ANOVA. A level of $P < 0.05$ for the two-tailed test was considered as the limit for statistical significance.

At DIV 3, the distances were 3.03 ± 0.5 and 3.6 ± 1.5 mm between Cx and Str and 5.3 ± 1.2 and 3.5 ± 0.8 mm between Str and SN in control ($n = 6$) and asphyxiated ($n = 4$) pups, respectively. The distance among the regions diminished during development in both groups to an almost integrated feature as exemplified in Fig. 1A–D. At DIV 21, there was, however, an apparently larger gap between the structures in cultures from asphyxiated pups (Fig. 1C,D) than that seen in the controls (Fig. 1A,B). The effect is quantified in Fig. 2 (control ($n = 6$) versus asphyxiated ($n = 4$) cultures during DIV: F-ANOVA for the gap between Cx and Str = 4.698, d.f. 1,38, $P < 0.05$; and for the gap between Str and SN = 11, d.f. 1,38, $P < 0.05$). Nevertheless, both control and asphyxiated cultures survived well and viability tests revealed many alive and few dead cells in cultures investigated at DIV 27 (data not shown).

The cultures were fixed in a formalin solution at DIV 24. After rinsing cycles, the tissue was detached from the coverslip, mounted onto a gelatine-coated microscope slide and preincubated in 5% normal goat serum, NGS, 0.1% Triton X-100 in PBS. The slices were then incubated overnight at 4 °C with a mouse monoclonal antibody against TH (Sigma, St. Louis, MO) (1:250 diluted in PBS/5% NGS, 0.1% Triton X-100), rinsed and treated at room temperature with biotinylated anti-mouse IgG (Vector Laboratories, Burlingame, CA) for 1 h, followed by a further 1 h incubation with streptavidin-alkaline phosphatase complex. The reaction products were visualized with a 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT) substrate kit (Vector Laboratories), counterstained with fast red (Vector Laboratories) for cell nucleus labelling. Controls in which primary antibody were omitted resulted in no detectable blue staining for BCIP/NBT.

TH immunocytochemistry revealed several multipolar TH-positive neurons in SN and surrounding tissue, both in asphyxiated and control cultures. Control TH-positive neurons showed extensive branching, at primary and secondary neurites (Fig. 3A), while asphyxiated TH-

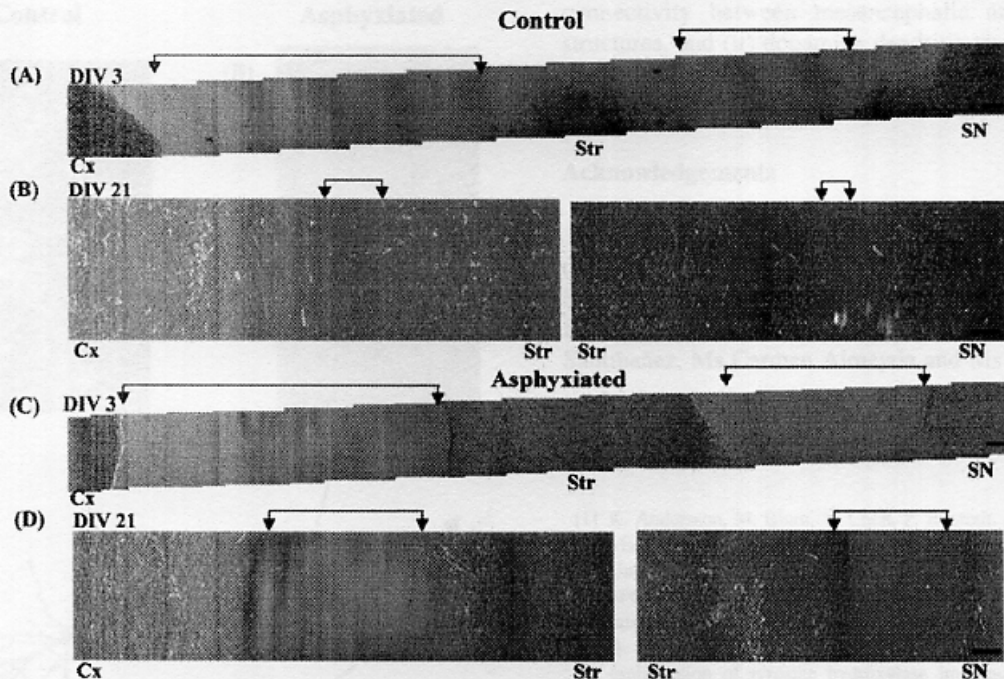
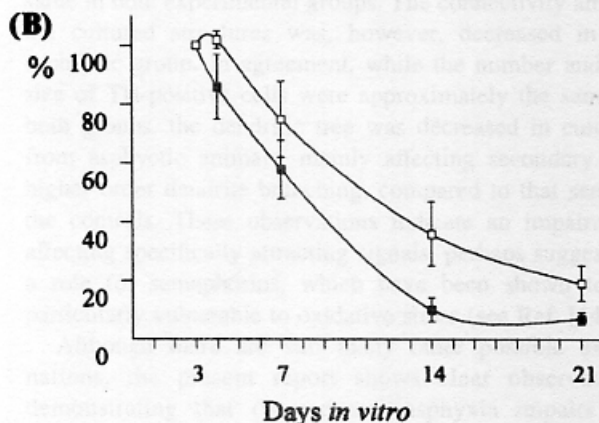
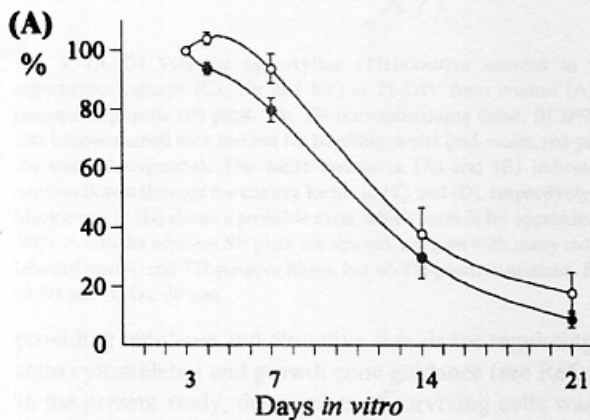


Fig. 1. (A–D) Overview of organotypic triple cultures (Cx, Str and SN) from control (A,B) and perinatal asphyxiated (C,D) pups at 3 (A,C) and 21 (B,D) days in vitro culture (DIV). The distance between Cx-Str and Str-SN at 3 DIV (A,C) and at 21 DIV (B,D) is indicated by a line connecting pairs of corresponding arrowheads. Bar: 1 mm.



positive neurons showed a rather sparse branching (Fig. 3B), as illustrated in camera lucida drawings (cf. Fig. 3C versus D). No differences in soma size and number of primary dendrites could be observed (cf. Fig. 3A versus B). When counterstained with fast red many well-shaped cells with red (red-pink and/or red-violet) labelled nuclei were evident in both groups, extending to regions where no TH-positive cell bodies could be seen (Fig. 3A,B).

Regarding the brain dopaminergic systems, the number of neurons is already established at P3 (see Ref. [5]), the time when the tissue was dissected to produce the organotypic cultures. However, it is well established that neuritogenesis is not fully completed at that period, since the final wiring of the central nervous system is largely a postnatal event. Neuritogenesis depends on forces generated from within the cells, although the task of locating an appropriate target is delegated to the growth cone. For navigation, however, guidance molecules presented by neighbouring cells are required, which instruct the growth cone to advance, retract or turn. Several guiding molecule families have been identified during evolution, i.e. (i) semaphorins, (ii) Slits, (iii) netrins, and (iv) ephrins,

Fig. 2. (A,B) The changes in connectivity during in vitro development are expressed as the percentage of the distance observed at DIV 3 between Cx and Str (A), and Str and SN (B) in control (full symbols) ($N = 6$) and asphyxiated (open symbols) pups ($N = 4$). While a decrease in the distance between Cx and Str, and Str and SN was evident in both control and asphyxiated cultures, that decrease was less evident in cultures from asphyxiated pups.

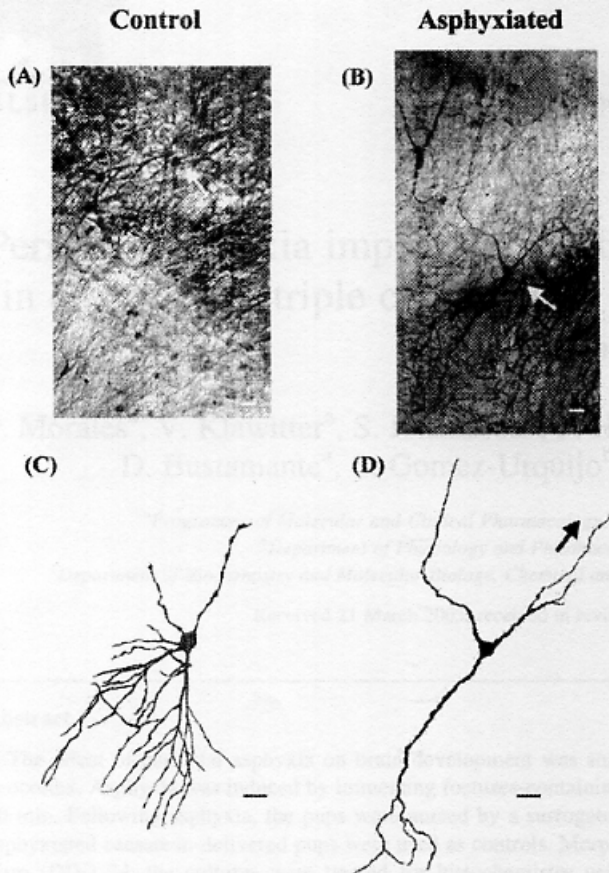


Fig. 3. (A–D) Tyrosine hydroxylase (TH)-positive neurons in triple organotypic cultures (Cx, Str and SN) at 21 DIV from control (A) and perinatal asphyctic (B) pups. The TH immunostaining (blue; BCIP/NBT) was counterstained with fast red for labelling nuclei (red-violet, red-pink in the microphotographs). The white arrows in (A) and (B) indicate the neurons drawn through the camera lucida in (C) and (D), respectively. The black arrow in (D) shows a probable axon, which extends for approximately 300 μm into the adjacent Str (data not shown), a region with many red-pink labelled nuclei, and TH-positive fibres, but no TH-positive neurons. Bar in (A,B) and (C,D): 20 μm .

providing repulsive and attractive signals for regulating the actin cytoskeleton and growth cone guidance (see Ref. [7]). In the present study, the number of surviving cells was the same in both experimental groups. The connectivity among the cultured structures was, however, decreased in the asphyctic group. In agreement, while the number and the size of TH-positive cells were approximately the same in both groups, the dendritic tree was decreased in cultures from asphyctic animals, mainly affecting secondary and higher order dendrite branching, compared to that seen in the controls. These observations indicate an impairment affecting specifically attracting signals, perhaps suggesting a role for semaphorins, which have been shown to be particularly vulnerable to oxidative stress (see Ref. [14]).

Although there are still many other possible explanations, the present report shows clear observations demonstrating that (i) perinatal asphyxia impairs the

connectivity between mesencephalic and telencephalic structures, and (ii) dopamine dendritic trees are vulnerable to perinatal asphyxia.

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