

## STRIATAL CYTOMEGALIC NEURONS CONTAINING NITRIC OXIDE ARE ASSOCIATED WITH EXPERIMENTAL PERINATAL ASPHYXIA: IMPLICATION OF COLD TREATMENT

C. FABIAN LOIDL, FRANCISCO CAPANI, JUAN J. LOPEZ-COSTA, ESTER LOPEZ, ASIA SELVIN-TESTA, JORGE PECCI-SAAVEDRA

*Instituto de Biología Celular y Neurociencias Eduardo De Robertis, LANAIS-MIE, Facultad de Medicina, Universidad de Buenos Aires,*

**Summary** Neuropathological mechanisms triggered by excitatory aminoacids are known to involve nitric oxide (NO). Neurons containing NO are histochemically reactive to nicotinamide adenine dinucleotide phosphate diaphorase (NADPH-d), which labels NO synthase in CNS. Sprague-Dawley male rats subjected to perinatal asphyxia (PA) at 37°C, and PA plus 15°C hypothermia were evaluated when 6 months old by NADPH-d histochemical reaction. Computerized image analysis was used for quantification of stained sections. NADPH-d neurons in striatum from subsevere and severe PA showed a significant increment in soma size and dendritic process length versus control and hypothermic treated rats. Post-ischemic damage neurons are therefore involved in NO changes induced by PA that may be prevented by hypothermia treatment.

**Key words:** hypothermia, NADPH-diaforasa, perinatal asphyxia, striatum

Perinatal asphyxia (PA) remains a major complication in childbirth and its frequency is still high in spite of progress in health care. Affected newborns are prone to present neurological sequelae in the short- and long-term, their severity depending on the length of oxygen deprivation. Thus, PA may lead to attentional deficit, hyperactivity, epilepsy, mental retardation, motor disorders, cerebral palsy or even death<sup>1</sup>. The basic cause of cerebral hypoxia in PA is pre- and intrapartum ischemia that produces a global brain neurotransmission alteration involving the nigrostriatal pathway, developing in adult rats as a long-term increase in basal striatal dopamine (DA) levels<sup>2</sup>.

Hypothermia has been shown to prove critical for rat survival following PA induction. Indeed,

100% survival was achieved up to 15-16 min of asphyxia when PA was induced at body temperature (37°C), but dropped to 20% after 20-21 min, to fall to zero after 22 min of asphyxia. However, when PA was induced at a lower temperature (15°C), litter survival reached 100% even up to 100 min asphyxia<sup>2</sup>.

So far, the pathological mechanism triggered after 16 min of PA leading to chronic neurotransmission alterations evidenced in monoamines and GABA<sup>2,3</sup> is poorly understood but may well involve the participation of excitatory aminoacids, nitric oxide and free radicals. Nicotinamide adenine dinucleotide phosphate diaphorase (NADPH-d) is commonly employed as a histochemical marker of NO synthesis in neurons<sup>4</sup> and it was the fact that NO had been described to enhance DA release from striatum<sup>5</sup> that encouraged us to evaluate NADPH-d distribution.

As striatum is severely affected by PA, we attempted to delineate the pattern of NADPH-d labelling in 6-month-old rat sections after various PA periods, either with or without hypothermia treatment.

Received: 10-I-1996

Accepted: 21-II-1996

**Postal address:** Dr. Jorge Pecci Saavedra, Instituto de Biología Celular y Neurociencias, Eduardo De Robertis, Facultad de Medicina, Universidad de Buenos Aires, Paraguay 2155, 1121 Buenos Aires, Argentina.

Sprague-Dawley rats on the last day of gestation were anaesthetised with ether and hysterectomized, their gestational age being determined by palpation. The entire uterus containing the fetuses was taken out, the uterine horns were detached and placed in a water bath at 37°C for 5 or 10 min (both regarded as slight PA), 15 (moderate PA), 19 (subsevere PA) or over 20 (severe PA)<sup>2</sup>, or else in a bath at 15°C for 20 or 100 min.

Cesarean-delivered control and asphyctic pups were obtained from the same mother. Following induction of asphyxia, the uterine horns were rapidly opened and pups removed and stimulated to breathe on a heating pad by cleaning up the delivery fluid and by tactile stimulation with medical wipes. The umbilical cord was ligated and animals left to recover for around 1h before given to surrogate mothers which had delivered normally 24 hs before the experiment, mixing their normal litters with marked asphyctic and caesarean-delivered control pups.

On becoming adults at six months of age (N = 4-5 animals/group), they were anaesthetized with sodium pentobarbital (60 mg kg<sup>-1</sup>) and perfused through the aortic artery with 0.9% NaCl solution followed by 300ml of 4% paraformaldehyde in 0.1M phosphate buffer. Brains were removed and postfixed in the same solution during 2hs, then immersed overnight in a solution containing 20% sucrose in 0.1M phosphate buffer. Sections 40 µm thick were cut on an Oxford vibratome and mounted on gelatin-coated glass slides, then processed by the NADPH-d histochemical method. Briefly, sections were incubated for 1h at 37°C in a solution containing 0.1% β-NADPH and 0.02% nitroblue tetrazolium diluted in 0.1M phosphate buffer with 0.3% Triton X-100 (all reagents purchased from Sigma, U.S.A.). Sections were mounted in PBS/glycerol (1: 3), then observed and photographed with a Zeiss Axiophot microscope.

Mean number and cell perimeter of NADPH-d+ cells per random field from 10 striatal sections belonging to each group were calculated using a KONTRON/VIDAS image analyzer, quantifying a total of 10 cells per section. Sections were observed with a 40X objective, and images digitized using an Axiophot Zeiss microscope linked to the computer by a SONY video camera. Differences between groups were compared using analysis of

variance (ANOVA), taking p<0.05 as significant. Results are given as means ± SD.

Without exception, NADPH-d staining in striatum transverse sections from 6-month-old rats showed uniformly distributed medium-sized neurons. Subsevere and severe PA groups disclosed so-called neuronal cytomegaly<sup>6</sup>, together with an evident increase in tortuous dendritic arborizations compared to control, remaining PA groups at 37°C and those subjected to 20 and 100 minutes PA at 15°C.

In rats exposed to subsevere and severe PA, striatal cell perimeter disclosed highly significant cytomegaly (p<0.001) versus control, slight and moderate PA at 37°C and PA at 15°C groups during 20 or 100 minutes (Fig. 1 and Table 1). Mean striatal cell count per field with a 40X objective was 5 ± 1, lacking significant intergroup differences.

In agreement with reports describing NADPH-d+ striatal cells as interneurons containing somatostatine and neuropeptide Y<sup>7</sup>, in adult rat such neurons were of medium size and non-spiny type. Due to their lack of NMDA receptors<sup>7</sup> and high concentration of manganese superoxide dismutase<sup>8</sup>, these cells were regarded to be resistant to excitatory aminoacid toxicity and to free radicals toxicity respectively. Nevertheless, the broader implications of NO as a neurotoxic or neuroprotective modulator remain a subject of controversy.

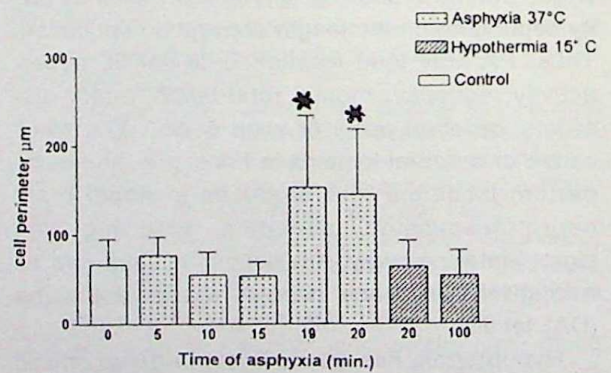


Fig. 1.— Measurement of striatal NADPH-d+ cell perimeter, in 6-month-old rats subjected to different periods of PA at 37°C or 15°C. Each value represents mean ± SD (vertical lines) of determinations made from n = 100 cells from each group. Asterisk indicates that NADPH-d+ in these two groups was significantly different (p<0.001) from the remainder. Statistical analysis was performed by ANOVA test. There were no significant differences between subsevere and severe PA.

TABLE 1.— *Cell perimeter of rat striatal neurons following various periods of newborn asphyxia*

| Time of asphyxia<br>(minutes) | Perimeter<br>( $\mu\text{m}$ )<br>Mean SD |
|-------------------------------|---|
| Zero (control) 37°C           | 67.10 $\pm$ 28.11                         |
| 5-6 37°C                      | 77.66 $\pm$ 20.55                         |
| 10-11 37°C                    | 55.30 $\pm$ 16.82                         |
| 15-16 37°C                    | 53.79 $\pm$ 16.82                         |
| 19-20 37°C                    | 153.28 $\pm$ 81.12*                       |
| 20-21 37°C                    | 145.56 $\pm$ 73.57*                       |
| 20-21 15°C                    | 64.47 $\pm$ 29.64                         |
| 100-101 15°C                  | 54.01 $\pm$ 28.36                         |

\* Significantly different from the remaining groups ( $P < 0.001$ ) but not between them.

Several neurological disorders are liable to induce changes in NADPH-d stained neurons. To illustrate, in Huntington's disease there is an increase in striatal NADPH-d+ cell measurements concomitantly with cell and fiber sparing, perhaps due to loss of striatal spiny neurons<sup>7</sup>. A recent report on human Parkinson's and Alzheimer's diseases has documented a relative NADPH-d+ cell sparing in striatal neurons displaying shrunken and foreshortened dendritic processes<sup>9</sup>. In the present study, an experimental model was specially designed to evaluate PA effects in adult animals<sup>2,3</sup>. NADPH-d technique disclosed positive cells of the same type, homogeneously distributed in asphyctic and control rat striatum. However, striatal NADPH-d+ neurons from rats subjected to subsevere or severe PA proved highly cytomegalic compared with control and hypothermia-treated animals, indicating that the pathological mechanism involved in PA is dissimilar to that in Alzheimer's, Parkinson's and Huntington's diseases.

Alterations found in rat striatal NADPH-d+ neurons closely resembled those reported by Mischel et al<sup>6</sup> who documented cortical neuronal cytomegaly in pediatric epilepsy.

As NO induces DA release, which may be blocked by the NO synthase inhibitor L-Me-Arg<sup>5</sup>, the higher basal level of striatal DA after an episode of severe PA<sup>2</sup> seems to agree with the number of NO-containing hypertrophic striatal neurons.

Besides, NO plays a major role in regulating blood flow by inducing relaxation of the vascular smooth muscle<sup>10</sup>, and most likely protects against ischemia by enhancing oxygen supply. The ability to stimulate DA release, considered toxic in striatum after ischemia<sup>11</sup>, together with its potent vasoconstrictor effect may worsen cell damage. However, since DA release is inhibited by hypothermia<sup>12</sup>, its protective mechanism is attributable to inhibition of the excessive release of striatal DA indirectly stimulated by NO.

In spite of the therapeutic application of hypothermia to reduce the severity of ischemic cerebral damage described in several studies in rats and gerbils<sup>22</sup>, data on PA is mostly limited to the 1950s and 1960s, when cold therapy plus positive pressure ventilation was introduced to resuscitate severely asphyxiated human neonates, previously regarded as incapable of recovery, with an excellent outcome evidenced by a rapid increase in Apgar scores<sup>13,14</sup>. Metabolic demands decrease when body temperature is lowered<sup>15</sup> so that toxic effects are weakened and survival to asphyxia ensured without permanent brain lesions.

However, the functional consequences arising from striatal NO-containing cytomegalic neurons after an episode of subsevere and severe PA are still obscure, so that further studies on cold therapy in the treatment of severely asphyctic babies are essential.

In conclusion, our findings demonstrate that NADPH-d reactivity is enhanced in striatal neurons containing NO secondary to chronic PA-induced ischemia due to a subsevere or severe lack of oxygen and that permanent brain damage may be prevented by hypothermia treatment.

## Resumen

*Neuronas estriatales citomegálicas que expresan óxido nítrico se asocian con asfíxia perinatal experimental: implicancias del tratamiento con frío*

El óxido nítrico (NO) se encuentra directa o indirectamente relacionado con mecanismos neuropatológicos iniciados con la liberación de aminoácidos excitatorios. Se pueden detectar histoquímicamente las neuronas NO+ por su reacción a la nicotinamida adenina dinucleótido

fosfato diaforasa (NADPH-d), cofactor específico para la enzima NO sintetasa en el SNC. Se estudiaron con NADPH-d secciones de núcleo estriado de ratas Sprague-Dawley de 6 meses de edad que fueron expuestas a un modelo de asfixia perinatal (PA) durante varios períodos de tiempo tanto a 37°C como a 15°C (tratamiento hipotérmico). Para comparar el patrón de tinción de los diferentes grupos experimentales se realizó un estudio de análisis de imágenes cuantitativo. Las neuronas estriatales NADPH-d+ de ratas de 6 meses que fueron expuestas a PA subsevera y severa mostraron un aumento significativo en sus prolongaciones dendríticas y del tamaño del soma en relación a los controles, a los animales tratados con hipotermia y a los restantes grupos de PA a 37°C. Estos datos indican que existen cambios crónicos en las neuronas que expresan NO del estriado post-isquémico y que estas alteraciones inducidas por PA pueden ser prevenidas con el tratamiento hipotérmico.

**Acknowledgements**

We would like to express our gratitude for the excellent technical assistance of Mrs Emérita Jorge Vilela de Banchieri. This work was supported by grants from the University of Buenos Aires and CONICET (Consejo Nacional de Investigaciones Científicas y Técnicas).

**References**

1. Volpe JJ. Neurology of the newborn. Philadelphia: WB Saunders, 1987.
2. Herrera-Marschitz M, Loidl CF, You ZB, et al. Neurocircuitry of the basal ganglia studied by monitoring neurotransmitter release. *Molec Neurobiol* 1994; 9: 171-82.

3. Loidl CF, Herrera-Marschitz M, Andersson K et al. Long-term effects of perinatal asphyxia on basal ganglia neurotransmitter systems studied with microdialysis in rat. *Neurosci Lett* 1994; 175: 9-12.
4. Hope B, Michael G, Knigge K et al. Neuronal NADPH-diaforase is a nitric oxide synthetase. *Proc Natl Acad Sci USA* 1991; 88: 2811-4.
5. Zhu X, Luo L. Effect of nitropusside (nitric oxide) on endogenous dopamine release from rat striatal slices. *J Neurochem* 1992; 59: 932-5.
6. Mischel P, Nguyen Land Vinters H. Cerebral cortical dysplasia associated with pediatric epilepsy. Review of neuropathologic features and proposal for a grading system. *J Neuropathol Exp Neurol* 1995; 54: 137-53.
7. Ferrante R, Kowall N, Beal M et al. Selective sparing of a class of striatal neurons in Huntington's disease. *Science* 1985; 230: 561-3.
8. Inagaki S, Suzuki K, Taniguchi N et al. Localization of Mn-superoxide dismutase (Mn-SOD) in cholinergic and somatostatin-containing neurons in the rat neostriatum. *Brain Res* 1991; 549: 174-7.
9. Mufson E, Brandabur M. Sparing of NADPH-diaforase striatal neurons in Parkinson's and Alzheimer's diseases. *NeuroReport* 1994; 5: 705-8.
10. Palmer R, Ferrige A, Moncada S. Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature* 1987; 327: 524-6.
11. Globus M, Ginsberg M, Dietrich W et al. Substantia nigra lesion protects against ischemic damage in the striatum. *Neurosci Lett* 1987; 80: 251-6.
12. Globus M, Busto R, Dietrich W et al. Effect of ischemia on the in vivo release of striatal dopamine, glutamate and GABA studied by intracerebral microdialysis. *J Neurochem* 1988; 1455-64.
13. Dunn JM, Miller JA Jr. Hypothermia combined with positive pressure ventilation in resuscitation of the asphyxiated neonate. *Amer J Obstet Gynec* 1969; 104: 58-67.
14. Westin B. Infant resuscitation and prevention of mental retardation. *Am J Obstet Gynecol* 1971; 110: 1134-8.
15. Miller JA Jr. New approaches to preventing brain damage during asphyxia. *Am J Obstet Gynecol* 1971; 110: 1125-33.

-----

*We cannot count on the medical solutions of the past to solve the problems of the future. Indeed, the re-emergence of old problems in new garments is programmed in the genetic machinery of evolution.*

No podemos contar con las soluciones médicas del pasado para los problemas del futuro. De hecho, la re-emergencia de viejos problemas en nuevas vestiduras está programada en la maquinaria genética de la evolución.

Richard Krause

In: *A dancing matrix. How science confronts emerging viruses.* Robin Marantz Henig. New York: Vintage Books, 1994, p 35