

Asphyxia during birth : biochemical and morphological study in basal ganglia : implication of hypothermia

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**ASPHYXIA DURING BIRTH:
BIOCHEMICAL AND MORPHOLOGICAL STUDY IN BASAL GANGLIA.
IMPLICATION OF HYPOTHERMIA.**

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ter verkrijging van de graad van doctor aan
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**Asphyxia during Birth: Biochemical and Morphological Study in Basal Ganglia.
Implication of Hypothermia.**

César Fablán Loidl.

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To Nora, Emilia and Eugenia

and in memory of my dear friend Mario Gullé

ASPHYXIA DURING BIRTH

Biochemical and morphological study on basal ganglia. Implication of hypothermia

	<i>Contents</i>	<i>page</i>
	Abbreviations	1
	Chapter 1	3
	Purpose of research	4
	Chapter 2	5
	General Introduction: review of the literature	6
2.1	Acute perinatal asphyxia	6
2.1.1	General introduction	6
2.1.2	Definition of perinatal asphyxia In the clinical practice	7
2.1.3	Brain and perinatal asphyxia	8
2.2	Brief description of basal ganglia anatomy	8
2.3	Possible neurotoxic mechanisms induced by asphyxia	11
2.3.1	Excessive excitatory aminoacids release	11
2.3.2	Free radicals production	13
2.3.3	Nitric oxide release	13
2.3.4	Lactic acid accumulation	14
2.3.5	Role of calcium	15
2.3.6	Excessive release of neurotransmitters	15
2.3.7	Membrane degradation	16
2.4	Asphyxia and intermediate filaments In neurons and gllia	16

Chapter 3	17
3	Material & methods 18
3.1	Subjects 18
3.2	Surgical technique to induce perinatal asphyxia 18
3.3	Behavioural studies 20
3.4	Microdialysis technique 20
3.4.1	Brief description 20
3.4.2	Subcutaneous microdialysis in newborn rats 22
3.4.3	Intracerebral microdialysis. Study of the nigrostriatal system 22
3.5	Possible therapeutic agents tested during experimental perinatal asphyxia 23
3.6	Cresyl violet histology 23
3.7	Immunocytochemistry 24
3.8	NADPH-diaphorase histochemical technique 24
3.9	Computerized Image analysis 25
3.10	Statistical analysis 25

Chapter 4	27
4	Results 28
4.1	The experimental model. Protection from mortality in PA by Hypothermia 28
4.2	SHORT-TERM EFFECTS OF PERINATAL ASPHYXIA 28
4.2.1	Survival, behaviour and subcutaneous microdialysis in asphyctic, controls and hypothermic-treated pups 28
	a. Effects of delivery through hysterectomy (caesarea) and normal vaginal delivery 28
	b. Slight and moderate asphyctic exposure 29
	c. Subsevere asphyctic exposure 29
	d. Severe asphyctic exposure 29

4.2.2	Effects of the antagonists of glutamate receptors	30
4.2.3	NADPH-d histochemical staining in asphyctic, control and hypothermic-treated pups	30
4.3	LONG-TERM EFFECTS OF PERINATAL ASPHYXIA	48
4.3.1	Intracerebral microdialysis	48
4.3.2	Immunocytochemistry and basal ganglia in asphyctic and control adult rats	48
4.3.3	NADPH-histochemical staining in 6 months old asphyctic, control and hypothermic-treated adult rats	48
4.3.4	Cresyl violet staining in striatum and cerebral cortex	49

Chapter 5		61
5	General discussion	62
5.1	Utility of the model	62
5.2	Behavioural evaluation	62
5.3	Why are neonates more resistant to asphyxia?	63
5.4	Hypothermia and survival protection to asphyxia	63
5.5	Glutamate antagonists and perinatal asphyxia	64
5.6	Subcutaneous microdialysis. Indicator of long-term damage as a prognosis tool?	65
5.7	Do long-term dopaminergic changes represent a sign of accelerated ageing?	66
5.8	Asphyxia and reactive astrocytosis. GFAP immunostaining	67
5.9	Perinatal asphyxia and hypertrophy of NF. A sign of neurodegenerative and accelerated ageing?	68
5.10	Cytomegalic NOS-containing neurons associated with PA: A sign of neuroprotection or neurotoxicity?	68
	a. Acute changes in NOS after PA (newborn rats)	68
	b. Chronic changes in NOS after PA (6-months old rats)	69

Chapter 6 71

Conclusions 72

Chapter 7 75

7.1 Summary 76

7.2 Samenvatting 78

References 80

Curriculum Vitae 91

Acknowledgements 93

Publicatlonns 95

ABBREVIATIONS

Ach	Acetylcholine
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
AMPA	Alpha-amino-3-hydroxy-5-methyl-4-isoxalepropionic acid
ANOVA	Analysis of variance
ATP	Adenosine 5'-triphosphate
Asc	Ascorbate
Asp	Aspartate
CBF	Cerebral blood flow
CSF	Cerebrospinal fluid
cGMP	cyclic Guanosyn monophosphate
CNS	Central nervous system
DA	Dopamine
DAB	Diaminobenzidine
GABA	Gamma-amino butyric acid
GFAP	Glial fibrillary acidic protein
Glu	Glutamate
HPLC	High performance liquid chromatography
Lac	Lactic acid
MK-801	(+)-5methyl-10,11-dihydro-5H-dibenzo(a,d)cyclohepten-5,10-imino maleate (Dizocilpine)
NADPH-d	Nicotin amide adenine dinucleotide phosphate diaphorase
NBQX	[2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(F)quinoxaline
NF	Neurofilament
NGS	Normal goat serum
NMDA	N-methyl-D-aspartate
NO	Nitric oxide
NOS	Nitric oxide synthase
NY	Neuropeptide Y
PA	Perinatal asphyxia
PAP	Peroxidase-antiperoxidase method
Pyr	Pyruvate
SD	Standard deviation
SOD	Superoxyde dismutase
SEM	Standard error of the mean
SOM	Somatostatine

CHAPTER 1

1. PURPOSE OF RESEARCH

Perinatal asphyxia (PA) is a major worldwide problem in perinatology. Per year, 4 million infants are affected by this problem, with 800.000 dying and another 800.000 suffering brain damage (WHO Report, 1991). PA is a serious problem in less developed countries (Costello, 1994), where care and precautions are neither always available nor properly applied.

The aim of this thesis is focused on:

1. The development of an original experimental model in order to study short and long-term consequences produced by asphyxia during birth.
2. The study of new therapeutical approaches in order to control asphyctic damage.
3. A biochemical and morphological study of basal ganglia in rats exposed to PA, using microdialysis, histochemistry and immunocytochemistry respectively.

CHAPTER 2

GENERAL INTRODUCTION: REVIEW OF THE LITERATURE

The normal growth and development of a fetus depends on a normal interchange of gases, nutrients and metabolic waste with the placenta. If an abnormality produces a breakdown in these homeostatic mechanisms, then a rapid or slow insufficiency in the fetal requirements develops, leading to an acute, subacute or chronic asphyxia.

2.1. ACUTE PERINATAL ASPHYXIA

2.1.1 General Introduction: Acute PA is the principal cause of death and neurological injury in newborn infants. The incidence of this problem has been estimated at 2-4 cases per each 1000 births at term, and this percentage has not decreased despite widespread medical advances in perinatal and obstetric care (Hill, 1991; Younkin, 1992). Most asphyctic infants die during the perinatal period and 20-30% of survivors present short or long-term evident sequelae, amongst which hyperactivity and attention deficit disorders (DSM III R, American Psychiatric Association), epilepsy, motor disorders, mental retardation and cerebral palsy or spasticity are included (Volpe, 1987; Younkin, 1992).

The word *asphyxia* derives from the words *a*, which means *lack or absence* and *sphyzo*, which means *palpitation or heart movements*. Also, it is used to describe a lack of body movement, specifically, those that refer to breathing (Bonnet, 1982). Asphyxia is a state of hypoxia and ischemia that if sustained can lead to death.

Hypoxia is a decrease in oxygen supply and *ischemia* is a decrease in blood supply to the tissues (Percy, 1986; Volpe, 1987).

Most cases of PA remain unexplained, but amongst the multiple aetiologies known to induce lack of oxygen the most common are: cord compression, true knot in cord, abruptio placenta, placenta previa, infarct of the placenta, haemorrhage or anaemia of the mother, toxemia and obstetric complications during delivery.

The combination of hypoxia and ischemia is called *hypoxia-ischemia*. If an organism is subjected to this state for a certain period of time, cells trigger self-defense mechanisms that lead to a degeneration, if the lack of oxygen persists. Some tissues suffer the harmful effects of a diminished blood flow and a reduced availability of oxygen and glucose more than others - the mammalian brain is an example of the rapidity with which a state of cerebral hypoxia-ischemia causes damage.

The main cause for cerebral hypoxia during PA is ischemia and in 90% of the cases this occurs during ante-and/or -intra partum. Asphyctic babies suffer a global decrease - or interruption - in oxygenation that in severe cases although also affecting other organs, mainly leads to neurological injuries.

According to its severity, cerebral hypoxia-ischemia can be divided in two categories: focal and global. *Focal* ischemia occurs when a specific region of the brain is affected, e.g. an occlusion or vascular interruption such as an acute thrombotic or embolic stroke. Its neuropathology is characterized by a pan-necrosis or infarction, where all cell types are affected - and this is well evident after 6 hours. If a decrease in cerebral blood flow (CBF) below 15-20% is sustained for 3-4 hours, then infarction of the tissue occurs. The severity of the damage depends on where the occlusion of the vessel occurs. The damage produced by an infarct has two zones: *core*, place of irreversible injury, and *penumbra*, the place of electrical and functional quiescence. Therefore, achieving reperfusion is extremely important (Ginsberg, 1995). *Global* ischemia occurs when all blood supply to the brain is interrupted, as in a cardiac arrest with resuscitation. A lowering in CBF towards zero, lasting at least 5-10 minutes, leads to abnormalities. In this case, there is a general involvement, but damage occurs mainly in the so-called "vulnerable" areas of the brain: hippocampus, striatum, cerebral cortex and cerebellum (Ginsberg, 1995). Since after an acute global oxygen arrest, the injury suffered by neurons continues to develop for hours - or even days - the possibility of using treatment to stop damage from expanding is potentially present (Pedto, 1987; Garcia, 1992). PA is a type of global hypoxia-ischemia, since all oxygen supply to the fetus is temporarily interrupted.

A severe state of hypoxia-ischemia depresses the respiratory centers, leading to anoxemia. In anoxia-ischemia, a lack of oxygen and nutrients added to an accumulation of metabolic products lead through different mechanisms to cell death if the problem is not rapidly solved (Cervós-Navarro, 1991).

2.1.2 Definition of Perinatal Asphyxia in the Clinical Practice:

Unfortunately, different medical specialisms define PA in different ways. To the obstetricians, the appearance of meconium, fetal heart rate alterations and acidosis signals asphyxia; to the neonatologists, it is a low Apgar score, a delay in the establishing of breathing accompanied by signs of behavioural alterations or encephalopathy that signals it. The most widely accepted clinical PA definition includes the following parameters: acidosis, a low Apgar score after birth, delay in breathing and a need for an active resuscitation (Hull & Dodd, 1991).

A major clinical problem is that when PA is not severe, neither mother nor infant present any sign or symptom; the infant's problem will become evident only in the long-term. At present, if PA is suspected, help through computerized tomography can be extremely useful to detect damage, showing hemorrhagic and low density areas in the brain - similar to typical infarct images. Also, an electroencephalographic study can help define the presence - or absence - of epileptic form activity.

In PA, prognosis is extremely difficult to establish: one same Apgar score lower than 3 at 10 minutes may lead one third of the cases to severe neurological sequelae whereas another third will be normal. If hypotonia - a sign that accompanies severe PA with a low Apgar score - disappears within a few hours then, usually, the infant will not have neurological sequelae. However, if within 24 hours hypotonia rapidly progresses to hypertonia, they will. (Percy, 1986). Also, cerebral creatin kinase isoenzyme in blood is measured and studied in order to assess neurological outcome sequelae in term infants (Walsh, 1982).

Evidently, the best solution to PA lies in its prevention. At present, new pharmacological approaches such as calcium blockers, glutamate antagonists, antagonism of oxygen free radicals, corticosteroids and others are being tried out on animals and promise to be useful for humans in the future.

2.1.3 Brain and perinatal asphyxia: A deficit of oxygen supply affects the carbohydrate metabolism and therefore, the energy supply: this is the first alteration produced on the tissues by asphyxia. The central nervous system (CNS) is highly vulnerable to lack of oxygen due to (1) its need for great amounts of energy in order to maintain its ionic cell homeostasis and (2) to the fact that its available substrate storage for the anaerobic metabolism is low (Hansen, 1985; Erecinska, 1989; Siesjö, 1988).

Numerous studies show that hypoxia does not affect all areas of the brain in one same way. The most vulnerable neurons are those in the following areas: (i) CA1, CA3 and CA4 of the hippocampus, with pyramidal cells in CA1 being the most vulnerable to global ischemia in both humans and animals, (ii) small-to-medium neurons of the basal ganglia, in particular those in the caudate-putamen nuclei, (iii) Purkinje cells in the cerebellum and (iv) neurons of layers 3, 5 and 6 in the cerebral cortex. The mechanisms responsible for making these areas more vulnerable than others are still unclear (Pasternak, 1991; Paschen, 1989; Cervós-Navarro, 1991; Ginsberg, 1995).

2.2 BRIEF DESCRIPTION OF BASAL GANGLIA ANATOMY

The basal ganglia is a complex neuronal net that transports and connects signals emitted by the cerebral cortex. In terms of structure, the basal ganglia is formed by a deep and voluminous nuclei on each side of the cerebral hemispheres (striatum, formed by the caudate-putamen and pallidum). In terms of function, this group of nuclei acts in connection with other mesencephalic nuclei (substantia nigra and subthalamic nuclei). There is a great diversity of neurotransmitters and neuromodulators in these cerebral pathways. Their functions include memory aspects, programming, selection and execution of motor movements.

In the striatum, dopamine (DA) terminals - mostly originating in substantia nigra neurons - are directly linked with medium-sized spiny neurons that utilize *gamma-amino butyric acid* (GABA) as principal neurotransmitter. The striatum also has intrinsic large-sized neurons containing acetylcholine (ACh) (Bolam, 1984). It has been suggested that striatal cholinergic neurons are not synaptically linked with DA terminals (Lehmann, 1983). However, electron microscopy studies provided evidence that striatal ACh neurons may indeed receive direct inputs from dopaminergic axons (Kubota, 1987), and *in situ* hybridization studies have demonstrated that 95% of large-sized neurons in the striatum express mRNA for DA D-2 receptors (Brené, 1990). The medium-sized spiny neurons emit axons both to the globus pallidus and the substantia nigra. Neurons emitting to the globus pallidus contain both GABA and enkephalin whereas striato-nigral fibers contain GABA, substance P and dynorphin (Graybiel, 1990). Adding a description of different types of dopamine receptors, e.g. DA acts on striato-pallidal neurons via D-2 type of receptors and on striato-nigral neurons via D-1 receptors, the net becomes even more complex (Gerfen, 1990). On the other hand, there is evidence that striatal DA release is presynaptically modulated by glutamatergic cortical inputs. It has been suggested that DA stimulation produced by glutamate reflects the direct axonal interactions between glutamatergic and dopaminergic terminals in the striatum.

Furthermore, the striatum contains the highest cerebral activity of cGMP and soluble guanylate cyclase, which is the target of nitric oxide (NO). The histochemical technique for NADPH-d and immunocytochemical technique for NO synthase (NOS) have revealed the presence of medium-sized non-spinal NOS-containing interneurons, which are the same contained by somatostatin (SOM) and neuropeptide Y (NY). Not only NOS but also cGMP appear to be absent in the giant cholinergic-interneurons. ACh and muscarinic agonists produce a striatal increase in Ca^{2+} -dependent cGMP levels. The last data is consistent with the presence of muscarinic receptors in neurons that express NOS and moreover, there is structural evidence suggesting that these cells are innervated by cholinergic terminals (Phelps, 1985). Glu, DA and opioid-receptors agonists also produce an increase in striatal cGMP levels (Minneman & Iversen, 1976; Foster & Roberts, 1980). This data suggests that NOS-interneurons may regulate the efferent response of the gabaergic medium-sized spiny neurons, which receive afferent stimulus from cortex, substantia nigra and local circuits (Vincent & Hope, 1992). Fig. 1 presents a diagram of the structures and connections studied in the present thesis.

Some neurodegenerative diseases have been associated with the neuronal circuitry in basal ganglia. It was clearly established that the most critical abnormality in Parkinson's disease is the degeneration of the nigrostriatal dopaminergic system, and that the principal characteristic of the chorea in Huntington's disease is a marked loss of striatal neurons. However, although Alzheimer's disease and dementia of Alzheimer's type are primarily associated with deficits of cerebral cortex and hippocampal formation, some of the symptoms presented by these

diseases are also linked to dysfunctions of the basal ganglia (Herrera-Marschitz & Ungerstedt, 1990). Furthermore, deficits of mesolimbic and mesocortical dopaminergic transmission have been associated with several functional syndromes, such as psychosis (Carlsson, 1990), drug addiction (Koob, 1992), schizophrenia and obsessive-compulsive diseases (Swerdlow, 1987)

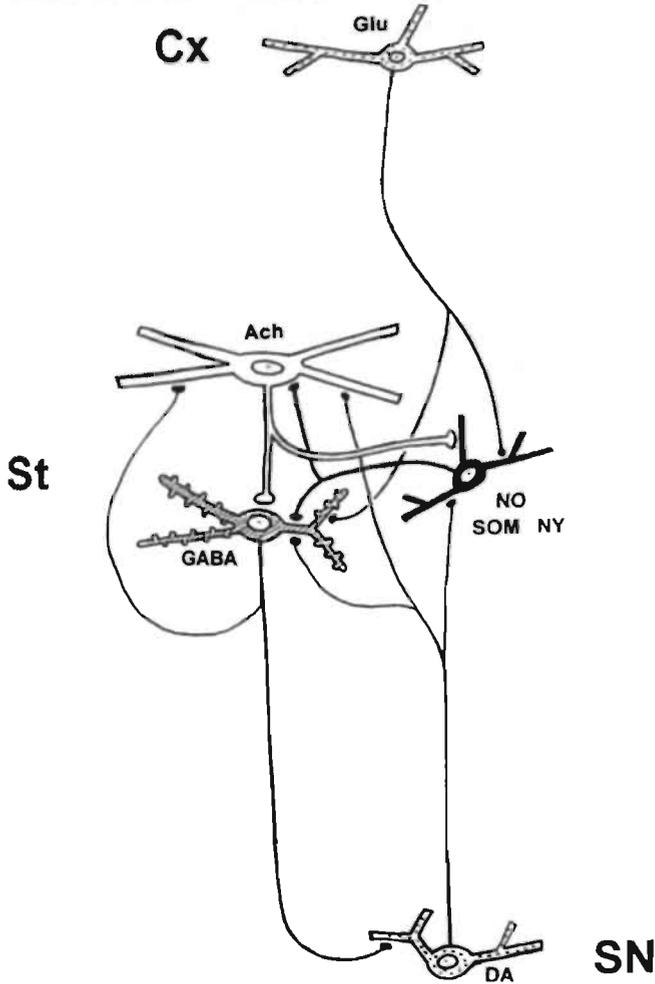


Fig 1: Diagram shows the most established cell nerve connections, their classical and putative neurotransmitters, in transverse sections at cortico-striato-nigral level. Large non-spiny interneuron-containing Ach is represented in white; medium-sized non-spiny interneuron-containing NO, SOM and NY is in black; two types of spiny projective neurons-containing GABA projecting their axons to SN and GP are represented together in one neuron in stripes. Nigrostriatal and cortical neurons are represented with spots. Abbreviations: St, striatum; SN, substantia nigra; Cx, cerebral cortex; Glu, glutamate; Ach, acetylcholine; NO, nitric oxide; SOM, somatostatine; NY, neuropeptide Y; GABA, gamma-aminobutyric acid; DA, dopamine.

2.3 POSSIBLE NEUROTOXIC MECHANISMS INDUCED BY ASPHYXIA

Under normal conditions, the brain uses oxygen and glucose in the form of ATP for its required energy. In seconds, ischemia produces a rapid decrease - or cessation - in oxidative phosphorylation and when this occurs, the only source of energy left is that of anaerobic glycolysis, which is not sufficient to maintain a normal cerebral function. Consequently, the brain is forced to utilize its stored glucose and glycogen, which only lasts for 2-3 minutes. The brain also contains a creatin-kinase enzyme that functions as a catalyzer to convert phosphocreatine into ATP, but this only helps for one minute longer. It is at this point that a last cerebral defensive mechanism is triggered since the increase in cytosol of ADP molecules stimulates another source of energy: the adenyl-kinase enzyme, which converts two ADP molecules in ATP plus AMP. In asphyctic conditions and with the help of this enzyme, the brain has approximately 5-10 minutes longer before consuming its available ATP.

If this energy failure is sustained in time, different cellular degenerative mechanisms such as (1) excitatory aminoacids release, (2) free radicals generation, (3) nitric oxide and other neurotransmitters release, (4) lactic acid accumulation, (5) massive entrance of calcium and (6) degradation of the membrane phospholipids amongst others will be triggered (Farooqui, 1994). In this condition, neurological damage or death may follow. The principal neurotoxic mechanisms involved in hypoxia are depicted in Fig. 2.

2.3.1 Excessive excitatory aminoacids release: In mammals, glutamate (Glu) is the principal endogenous excitatory aminoacid in the CNS. It couples with at least 5 subtypes of receptors, amongst which NMDA, kainate, AMPA and metabotropic trans-ACPD are the most important. There is strong evidence that in a state of hypoxia-ischemia an overstimulation of excitatory aminoacids release (glutamate and aspartate) plays an important role in the pathogenesis of cerebral damage and therefore, the competitive and non-competitive antagonists of N-methyl-D-aspartate (NMDA) receptors and those of alpha-amino-3-hydroxy-5-methyl-4-isoxalepropionic acid (AMPA) have been suggested as neuroprotective agents (Scatton, 1991; Barks & Silverstein, 1992; Sheardon, 1990).

The idea that these aminoacids play a neurotoxic role emerges from the following facts: (1) the most vulnerable areas of the brain (hippocampus, cerebral cortex, striatum and cerebellum) receive strong glutamatergic inputs, (2) lesions in these pathways prevent the neuronal damage induced by ischemia, (3) high concentrations of extracellular glutamic acid have been registered in these regions after ischemia and (4) a local micro injection of NMDA antagonists prevents the neuronal damage induced by ischemia (Cervos-Navarro, 1994).

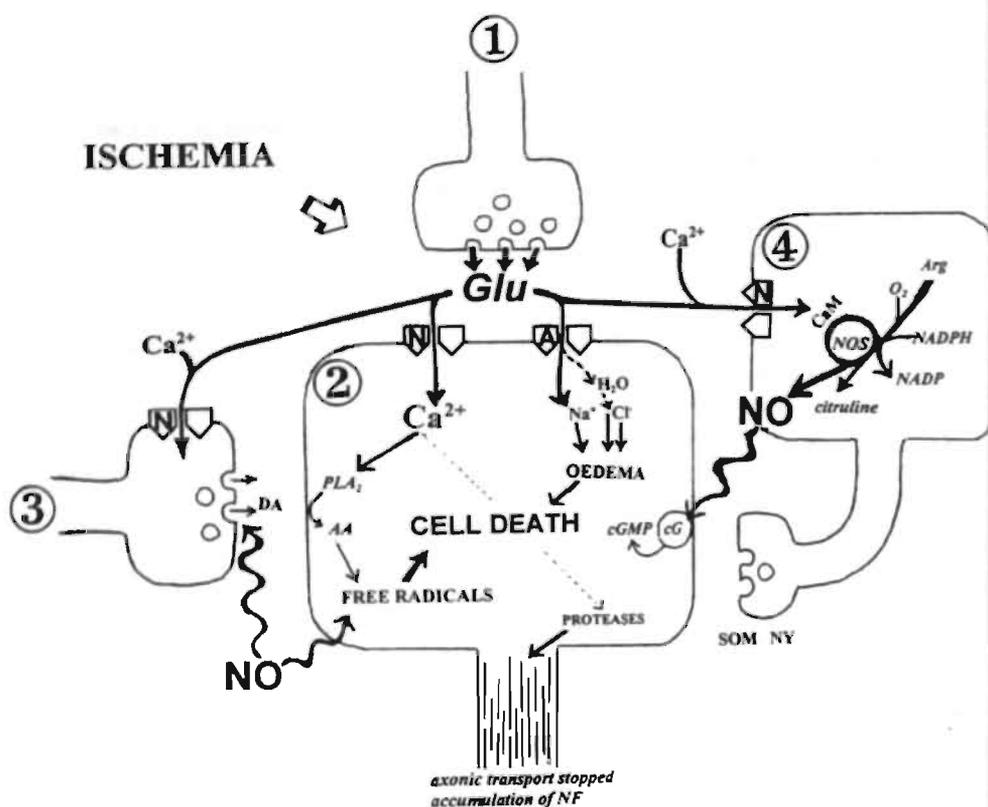


Fig 2: Possible mechanisms involved in striatal neurotoxicity. 1. Corticostriatal nerve ending, 2. Striatal spiny projective neuron-containing GABA, 3. Nigrostriatal nerve ending, 4. Medium-sized non-spiny interneuron-containing NOS, SOM and NY. Abbreviations: N: NMDA receptor; A: AMPA receptor; PLA₂: phospholipase A₂; AA: araquidonic acid; Gc: soluble guanylate cyclase; NY: neuropeptide Y; SOM: somatostatine; NOS: nitric oxide synthase; CaM: calmoduline; NF: neurofilaments.

When neurons are exposed to an excess of glutamate, an acute neuronal oedema occurs as a result of an active depolarization with cellular entrance of Na^+ , Cl^- and H_2O together with Na^+ , K^+ -ATPase channel inhibition. This process can be reversed if Glu is rapidly removed from the zone; but if the exposure is sustained, the entrance of water into the cells produces an osmotic swelling that alters the neuronal function. Moreover, a NMDA receptor stimulation speeds up channels, permitting an excessive entrance of Ca^{2+} and it is known that an intracellular increase in this ion activates lipases, phospholipases, proteases and protein kinases, which can produce considerable cell damage if not properly regulated (see Fig. 2).

The hypothesis that neuronal degeneration is the result of an excessive release of excitatory amino acids presents some weak points: (1) the hippocampal CA4 region has a low concentration of NMDA and yet is highly vulnerable to an ischemic insult, and (2) hippocampal neurons of the gyrus dentatus, a resistant zone to ischemia, has a high density of NMDA receptors.

2.3.2 Free radicals production: It is accepted that reperfusion after ischemia stimulates the production of free radicals. An indirect biochemical fact that demonstrates this production of free radicals and lipid peroxidation in ischemia is the reduction of endogenous antioxidants such as ascorbate, glutathione, ubiquinone, α -tocopherol and cholesterol in the cerebral tissue. Some of the most common radicals formed are: superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($\text{OH}\cdot$), catalyzed by cyclooxygenase and lipoxygenase enzyme activities and the Fenton reaction. Radicals are also produced by catecholamine autoxidation and xanthinoxidase. The significance of each of these processes is still not clear, but all of them are capable of producing free radicals during reperfusion. Recently, another oxygen radical, peroxynitrite anion (ONOO^-), has been proposed as a toxic agent in ischemia (Farooqui, 1994).

There is a type of enzymes called superoxide dismutase (SOD), catalase and glutathione peroxidase that is involved in the cerebral defense from free radicals formation. SOD mutations are associated with amyotrophic lateral sclerosis (ALS), a degenerative disease of the motor neurons in cerebral cortex, medulla and spinal cord (Rosen, 1993). This is a clear example where damage produced by free radicals is associated with a defect in the genetic code involved in defensive protein formation against oxidation.

2.3.3. Nitric oxide release: Nitric oxide (NO) is a very important and unusual messenger. Because it is a highly reactive and unstable gaseous free radical, it is capable of reacting and changing as soon as it is in a biological environment. NO is naturally generated from the amino acid L-arginine by nitric oxide synthase (NOS), a NADPH and calcium/calmodulin-dependant enzyme. Two types of NOS have been identified: 1) a constitutive form of NOS (cNOS), dependant upon Ca^{2+} /calmodulin that releases NO stimulated by increase in intracellular Ca^{2+} for only short periods of time and 2) a second form of NOS, inducible nitric oxide synthase (iNOS) that is expressed under cytokine activation

of the cells, is not dependant on Ca^{2+} and it produce NO for long time periods. iNOS is primarily found in macrophages, smooth muscle cells, endothelial cells and hepatocytes.

The observation that neuronal cNOS is identical to neuronal nicotin-amide-adenine-dinucleotide-phosphate diaphorase (NADPH-d) (Dawson, 1991; Hope, 1991, Vincent & Hope, 1992) has allowed the localization of NOS through a histochemical method first described by Thomas & Pearse (1964). The fact that cNOS is a calcium-calmoduline dependant enzyme has led to the hypothesis of a linkage between NOS and NMDA receptors, which are coupled to calcium ionic channels. In the last years, it was established that NO increases after stimulation of glutamatergic NMDA (Garthwaite, 1988) due to a NOS activation by calcium influx through NMDA (Bredt, 1990). The final result is an increase in NO, that activates a soluble guanylate cyclase with cGMP production (Garthwaite, 1988).

It was established that NO is involved in neurotransmission, vasodilation, neurotoxicity, inhibition of platelet aggregation, and the antiproliferative action of cytokines. As a neurotransmitter, the role of this messenger still remains unclear. The interaction between NO and NMDA is particularly interesting because of the role played by these receptors in neuronal plasticity, including sprouting of neurites, synaptic transmission and long-term potentiation (LTP) (Bohme, 1991; O'Dell, 1991; Shuman & Madson, 1991, 1994; Hess, 1993; Zhuo, 1993; Montaghe, 1994).

Under ischemic conditions, NO, generated in the brain by neurons and endothelial cells, reacts with oxygen radicals forming peroxynitrite, which rapidly decreases producing highly reactive radicals such as hydroxyl radicals ($\text{OH}\cdot$) and nitrogen dioxide ($\text{NO}_2\cdot$). Furthermore, a high concentration of NO can be toxic to cells, stimulating the ADP-ribosyltransferase and through its association with enzymatic centers containing iron-sulphur, interfering with the mitochondrial electron transport chain, tricarboxylic acid cycle and DNA synthesis (Garthwaite, 1991). According to different authors, NO could have either neuroprotective or neurotoxic effects. Recently, Lipton & Stamler (1994) have proposed that NO could have both effects, depending on the redox state of the medium. However, recent results of Terwel et al. do not lend support to this notion (unpublished data).

2.3.4 Lactic acid accumulation: Cerebral hypoxia-ischemia induces an increase in lactic acid (Lac) due to a deviation of glycolysis to the anaerobic pathway. An increase in Lac, added to an increase in CO_2 and intracellular protons such as ATP hydrolysis, produces an acidotic state. A decrease in intracellular pH is an optimal medium for the activity of the hydrolytic enzymes of lysosomes in cerebral tissue, producing a rapid destruction of Nissl granules and neuronal and glial swelling that leads to cellular death (Nedegaard, 1991). It has been demonstrated that the highest glucose consumption with Lac accumulation in brain after carotid ligatures is produced in the striatum (caudate/putamen), medial thalamus, Ammon's horn and substantia nigra pars reticularis.

It is interesting to point out that a mild exposure to an acidic medium (pH 6.6) might be neuroprotective because it induces a decrease in NMDA activity (Giffard, 1990; Tombaugh, 1990). However, an important decrease in pH amplifies the neurodegenerative processes triggered by glutamate excitotoxicity (Chleide, 1991). Studies performed with cell cultures have shown death of neurons and glial cells after a 10 minute exposure to an acidic medium with Lac at pH of 4.8. (Goldman, 1989). Other reports show that local sodium lactate injections decreasing cerebral pH to 5 induces cellular swelling, proteins and nucleic acid denaturation, free radicals formation, inhibition of neurotransmitter reuptake and an alteration in endothelial cells, leading to a secondary vascular insufficiency. It has also been suggested that the decrease in pH is partly due to an accumulation of excitatory aminoacids, which opens Na^+ channels inducing oedema, and eventually osmolytic.

2.3.5 Role of calcium: Neuronal calcium is a cellular messenger. It plays an extremely important role in regulating most enzymatic reactions and activating various phospholipases, proteases, protein kinases and phosphatases. The cytosolic calcium concentration in neurons is regulated by a great variety of membrane pumps and exchange systems. Most researchers have concluded that the homeostatic alteration produced by a Ca^{2+} release induced by ischemia is what ultimately leads to degeneration, since there always is an abnormal increase in this ion prior to any kind of detectable damage. A decrease in extracellular levels of calcium can also be observed and there are reports indicating that both the removal of calcium from the medium prior to ischemia and the utilization of NMDA antagonists prevent neuronal damage.

2.3.6 Excessive release of neurotransmitters: Following ischemia, the metabolism of most neurotransmitters is altered indicating neuronal failure. This is described for Ach, DA, noradrenaline and serotonin, besides GABA and the excitatory aminoacids previously mentioned (Volpe, 1987; Allain, 1991; Richards, 1993). With the exception of glutamate and aspartate, the idea that an excessive release of some neurotransmitters after ischemia, DA in particular, can be cause or effect for neurotoxicity is extremely debatable. Furthermore, NMDA receptors facilitate DA release in striatum (Roberts & Anderson, 1979; Chermamy, 1986; Clow & Jhamandas, 1989; Moghaddam & Gruen, 1991), an area that is rich in NOS (Bredt, 1990, 1991). Hypothetically, from a functional point of view, NMDA receptors, NO production and DA release might be related. According to this hypothesis, NOS inhibitors might block striatal release of DA mediated by NMDA (Hanbauer, 1992). Current bibliography concerning the interaction of NO and DA is contradictory, with some authors reporting a stimulant effect in DA release (Zhu & Luo, 1992; Lonart, 1993, Tokutake, 1993; Black & Humprey, 1994a, 1994b) and others an inhibitory effect (Rose, 1994; Guevera-Guzman, 1994).

2.3.7 Membrane degradation: During preparations including cultures, slices and synaptosomes performed with samples of hippocampus, striatum and hypothalamus, it was observed that agonists of excitatory aminoacids via NMDA receptors activate release of arachidonic acid (AA) from phospholipids of the membrane. The release of AA is mediated by phospholipase A₂, which also generates free radicals. Moreover, the activation of metabotropic-transACPD receptors seems to be the principal cause for hydrolysis of phosphoinositol by activating a phospholipase C.

2.4 ASPHYXIA AND INTERMEDIATE FILAMENTS IN NEURONS AND GLIA

In order to resist different types of insults, CNS reacts in a stereotyped manner; and changes observed in the cytoskeleton of glial cells and neurons are an example of this. The enhancement of glial fibrillary acidic protein (GFAP) reactivity, a feature of a damaged brain (Eng, 1987, Nathaniel, 1981), reflects an astrocytic response to brain insult (Eng, 1988; Vijayan, 1990). Following an insult, the so-called "reactive astrocytes" increase in number and size, with their specific intermediate filaments (GFAP) increased (Latov, 1979; Barret, 1981). After damage, changes in neuronal intermediate filaments can also be observed. The most common are called neurofilaments (NF). According to molecular weight given by their carboxy-terminal portion, 3 types have been described: light, NF-s (68kD), medium, NF-m (150kD) and heavy, NF-h (230kD) (Gelsler, 1983).

Information on subcellular changes associated with astroglial and neuronal intermediate filaments following ischemia can help to understand the role of reactive astrocytes in repairing damaged CNS. It can also provide information on the degree of neuronal damage, reflected in the amount of NF. Based on this information, astroglial reaction and NF content were studied in rats subjected to perinatal asphyxia.

CHAPTER 3

3. MATERIAL & METHODS

3.1 Subjects: Sprague-Dawley albino rats were used for all studies. Rats, in compliance with principles of *animal care and use of laboratory animals*, were kept in individual stainless-steel cages, fed purine chow and given tap water ad libitum. Room temperature was controlled and had a light/dark cycle of 12 hours. Experiments were performed: (1) immediately after birth and (2) at 6 months age.

3.2 Surgical technique to induce perinatal asphyxia: Asphyxia was induced in caesarean-section delivered pups from pregnant rats. Gestational age of the rat was determined by: estabularium protocols, vaginal smear and clinical palpation performed as of 3 days previous to the expected delivery day. In a fetus at term, the head can clearly be distinguished from the body. Furthermore, the mother is in a hypoactive state, frequently licking vaginal blood and instinctively building a nest when paper is placed in its cage. In the last gestational day, rats were anaesthetized with ether, hysterectomized and killed. The isolated uterus horns containing the fetuses were extracted, detached, and placed in a water bath at 37°C for several periods of time before the delivery of the pups. Since each rat delivered 12 to 15 pups, controls delivered by caesarian-section and asphyctic pups were obtained from one same mother. Following asphyxia, uterus horns were rapidly opened and pups removed. Pups were stimulated to breathe by cleaning delivery fluid and performing tactile stimulation on oral region with medical wipes. The umbilical cord was ligated, and pups were left to recover for 40-80 minutes on a heating pad before giving them to surrogate mothers. Pups delivered spontaneously by rats in the course of the experiments were used as spontaneously vaginal delivered controls. Time of asphyxia was measured as the time elapsed from when blood circulation to the uterus was cut off to the moment when pups started to breathe. Only male pups within the following parameters (1) occipitocaudal length > 41 mm and (2) body weight > 5.0g were included in the experiments. Rats that delivered normally within 48 hours before experiments were utilized as surrogate mothers. Each surrogate mother rat received 4 asphyctic male pups, 2 male control pups delivered by rapid caesarea and retained 2 of its own male pups, as normally delivered control animals. Each mother had a final litter of 8 pups to care for until weaning. Pups selected for the study of PA long-term effects were marked by cutting of different nails and left to grow until 6 months of age. Steps of the model used are shown in Fig. 3.

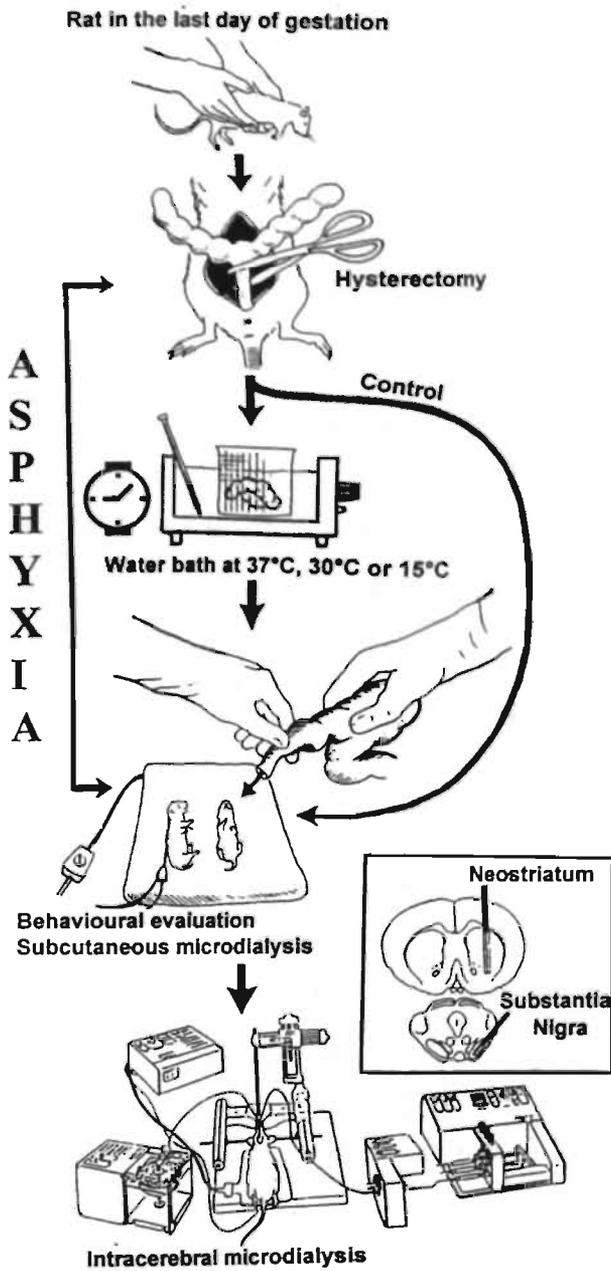


Fig 3: Diagram of the methodology to induce PA

3.3 Behavioural studies: Through direct observation, and in pends of 40-80 minutes after delivery, several parameters were recorded : (i) body weight, (ii) survival, (iii) respiratory frequency, (iv) gasping, (v) vocalization, (vi) skin colour, and (vii) spontaneous movements. These were scored according to the following scale: (0) akinesia and rigidity (mainly of hind legs); (1) movement of one of the following body structures: front legs, hind legs or head alone; (2) movement of two of the body structures; (3) movement of all body structures; (4) intensive movements shown by wriggling (Loidl, 1994). The term "gaping" refers to the effort to maintain respiration expressed by the opening of the mouth and movements of the diaphragm.

3.4 Microdialysis technique

3.4.1 Brief description: Microdialysis is a recent neurochemical technique that permits to study *in vivo* mechanisms of neurotransmitter release (Ungerstedt, 1982). It is a simple technique that to a certain extent imitates the physiology of capillaries. It is composed of a microdialysis fiber that consists of a steepil with an approximate diameter of 0.25 mm, and that in its extremity has a semipermeable membrane capable of obtaining, by simple diffusion, chemical components with low molecular weight from the extracellular space (see Fig. 3 and 4). Then, through a known liquid e.g. artificial cerebro spinal fluid (CSF), different substances can enter or leave the fiber through the membrane (see Fig. 3). Therefore, a later measuring of substances in the dialyzed liquid of perfusion is possible. Generally, the most widely used method to analyze collected samples is the high performance liquid chromatography (HPLC), coupled to electrochemical and fluorometric detectors (Ungerstedt, 1982). Introduction of drugs together with the perfusion liquid is also possible. This allows to measure chemical changes in the extracellular space.

In order to study PA short-term effects, a microdialysis fiber was implanted in the subcutaneous tissue of asphyctic and normal pups, thus allowing changes in glutamate (Glu), aspartate (Asp), lactate (Lac), pyruvate (Pyr) and ascorbate (Asc) to be measured (Fig. 4).

Long-term effects in 6 months old rats were studied with intracerebral microdialysis. The nucleus studied were striatum and substantia nigra. Extracellular levels of DA, GABA, Ach, Glu, Asp and metabolic products Lac, Pyr and Asc were evaluated in asphyctic and normal rats (Fig. 4)

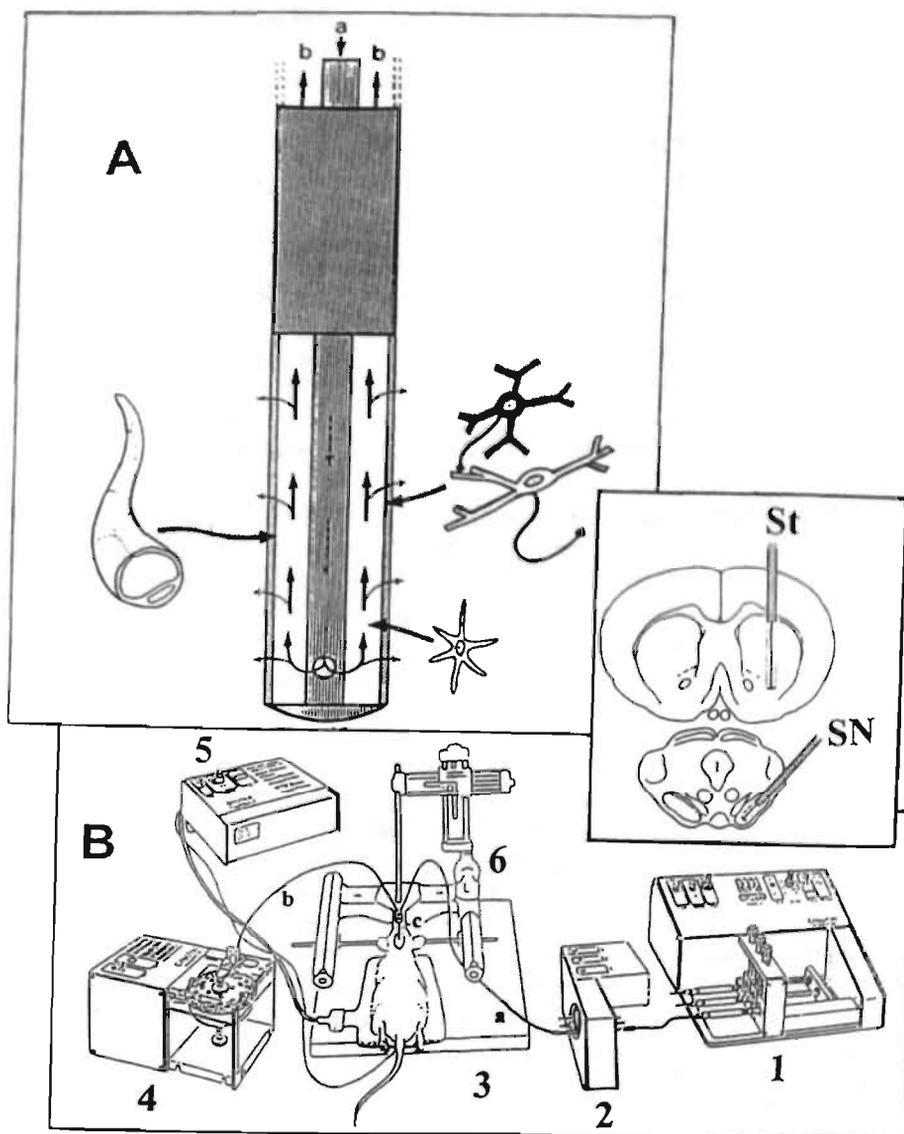


Fig 4: A: Transverse section of the microdialysis probe. Arrows indicate the path of perfused liquid, (a) inner canulae to perfusion liquid, (b) outer way to dialyzed liquid. B: Diagram of set employed for microdialysis technique, 1- perfusion pump coupled to microsyringes, 2- automatic switcher to microsyringes, 3- (a) inner canulae, (b) outer canulae, (c) microdialysis probe (see direction and size of intracerebral probes in striatum [St] and substantia nigra [SN]); 4-automatic collector of samples, 5- automatic body temperature controller, and 6- stereotaxic frame.

3.4.2 Subcutaneous microdialysis in newborn rats: Approximately 40 minutes after delivery both spontaneous vaginal and caesarean-section controls, as well as asphyctic pups, were implanted with a flexible microdialysis probe in the subcutaneous tissue of the dorsal region, in a caudo-rostral orientation, parallel to the spinal cord. Microdialysis probes (dialyzing length = 4mm; diameter = 0.5mm) were perfused with a modified CSF solution (148mM NaCl, 2.7mM KCl, 1.2mM CaCl₂, 0.85mM MgCl₂; pH 7) at a constant flow of 2 μ l/min that was maintained with a microdialysis pump. After two 20 minute samples, the microdialysis probe was carefully removed, the wound sealed with a drop of acrylic blue (Histoacryl, B. Braun Melsungen AG, Germany) and the pups were given to surrogate mothers. Samples were split in 10 μ l aliquots and were directly injected into high-performance liquid chromatography (HPLC) systems, coupled to a fluorometric or an ultraviolet detector to analyze Glu and Asp or Lac, Pyr and Asc respectively. Means and standard errors of means (SEM) were calculated and differences were tested with Fisher (F) ANOVA tests. A level of P < 0.05 for the two-tailed test was considered critical for statistical significance.

3.4.3 Intracerebral microdialysis. Study of the nigrostriatal system: After 6 months (~500g wt.), asphyctic and control rats were anaesthetized with halothane, placed in a David-Kopf stereotaxic frame and two microdialysis probes were implanted, one in the left striatum (coordinates: B 0.5, L -3.2, V -7.2)(dialysis length = 4mm; diameter = 0.5 mm) (CMA/ microdialysis AB, Stockholm, Sweden) and another in the left substantia nigra (B -6.2, L -7.6, V -8.6; Inserted at a 40° angle from vertical in the coronal plane) (dialyzing length = 2mm; diameter = 0.5 mm), according to the Atlas of Paxinos & Watson (1986) (see Fig. 4). Microdialysis probes were perfused with a modified CSF solution (148mM NaCl, 2.7mM KCl, 1.2mM CaCl₂, 0.85mM MgCl₂; pH 7). A constant flow of 2 μ l/min was maintained throughout the microdialysis experiment. Anaesthesia was performed by free breathing into a mask fitted over the nose of the rat (1% halothane in air flow of 1.5 l/min). Body temperature was kept at 37°C using a temperature control system (CMA/150, CMA/ μ dialysis AB). Samples (each of 80 μ l every 40 min) were collected automatically (CMA/140, μ dialysis AB) and directly injected into high performance liquid chromatography systems, coupled to electrochemical detection (HPLC-EC) systems for DA and its metabolites (Herrera-Marschitz, 1992) and γ -amino butyric acid (GABA)(Kehr & Ungerstedt, 1987), acetylcholine and choline (Maysinger, 1988) or to a fluorometric detector system for glutamate (Glu) and aspartate (Asp) (Herrera-Marschitz, 1992). Basal values were referred to those obtained before the administration of 2mg/kg s.c. of D-amphetamine (Sigma, St. Louis, MO, USA) (200 minutes after microdialysis implantation). Following every microdialysis experiment, animals were sacrificed, their brain removed and conserved in formaline for its ulterior histological study. Means and standard errors of the means (SEM) were calculated and differences were tested with Fisher (F)-ANOVA test. A level of P < 0.05 for the two tailed test was considered critical for statistical significance.

3.5 Possible therapeutic agents tested during experimental perinatal asphyxia

* **Hypothermia:** The model to induce PA was also performed under hypothermic conditions at 30°C and 15°C in order to study the possible protective effect of cold-treatment. Once PA was started, shifts in temperature from 37°C to 15°C were performed.

* **Drugs:** In a series of experiments, the non-competitive NMDA antagonist Dizocilpine, (+)-MK 801 hydrogen maleate [(+)-5-methyl-10,11-dihydro-5H-dibenzo(a,d)cyclohepten-5,10-imine maleate]; RBI, Natick, MA, USA (dissolved in saline) (0.2 mg/kg s.c.) or the AMPA antagonist NBQX [2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(F)quinoxaline], Novo Nordisk A/S, Måløv, Denmark (dissolved with a drop of NaOH in 5% glucose) was administered in a single dose to mothers, one hour before delivery. Comparisons on rate of survival were established with non-parametric Cochran's test (Q). A level of $P < 0.05$ was considered critical for statistical significance.

3.6 Cresyl violet histology:

At 6 months of age, control, asphyctic and treated rats were sacrificed with 28g % chloral hydrate, 0.1 ml/100g of body weight. Brains were removed, fixated in formalin, and coronal sections including striatum and neocortex were collected using an Oxford vibratome (40µm thick) and stained with Cresyl violet. Bilateral counts of viable looking neurons (well-defined nucleus and nucleoli, not shrunken) were performed by an observer blind to treatment conditions. Neurons were counted in the lateral, medial, dorsal and ventral striatum at bregma -0.40- interaural 8.6 and neurons in the medial and lateral sectors of neocortex were counted at bregma -0.40- interaural 8.6 (Paxinos & Watson, 1986).

3.7 Immunocytochemistry. Sternberger's peroxidase anti-peroxidase (PAP) method (1986):

The following primary antibodies were used: *anti-gial fibrillary acidic protein (GFAP)*, as a marker of astroglial response to PA, and *anti-neurofilaments protein (NF)*, with 68, 160 and 230 kD molecular weight, as neurodegenerative markers.

Adult animals were i.p. anaesthetized with 28g % chloral hydrate, 0.1ml/100g body weight, and perfused through the abdominal aorta (Gonzalez Aguilar & De Robertis, 1963). Rats were perfused with 4% paraformaldehyde, 0.25% glutaraldehyde in 0.1M phosphate buffer, pH 7.4. Coronal sections from the brain containing striatum, and others containing substantia nigra (40 μ m thick) were cut in an Oxford vibratome. Sections from treated and control animals were simultaneously processed. For each step of the immunohistochemical procedures, according to Sternberger's peroxidase-antiperoxidase (PAP) method (1986), the same protocol was used. Free floating sections were incubated either for GFAP or NF in the following sequence: (1) Incubation in 3% normal goat serum (NGS) for 30 minutes; (2) Incubation with the primary antibody, polyclonal anti-GFAP, monoclonal anti-NF (of the three types) in 1% NGS (v/v) at 4°C for 48 hours; (3) Incubation for 60 minutes in the secondary antibody (sheep polyclonal anti-rabbit) in 1% NGS (v/v) for GFAP; goat-monoclonal anti-mouse in 1% NGS (v/v) for NF; (4) Incubation for 45 minutes in PAP diluted 1:100 in 1% NGS (v/v); (5) development for 15-30 minutes in 0.035% DAB containing 2,5% (w/v) nickel ammonium sulphate, and 0.01% H₂O₂ in 0.1M acetate buffer (v/v) pH 6.0 (Hancock, 1984); (6) sections were mounted on glass slides, dehydrated, and examined in a Zeiss Axioptot photo microscope.

3.8 NADPH-diaphorase histochemical technique:

Technique in pups: Pups surviving asphyxia, caesarean delivered controls and hypothermic-treated pups (5 in each group) were immediately decapitated following the first 30 minutes of recovery after birth. Brains were directly fixated for 24 hours in 4% paraformaldehyde in 0.1M phosphate buffer, and then immersed overnight in a solution containing 5% sucrose in 0.1M phosphate buffer pH 7.4. After being fixated, the brains of the pups were cut on a Leltz cryostat in sections of 20 μ m and placed on gelatin-coated glass slides.

Technique in adult rats (6 months): Animals were perfused following the method described above and post-fixated in the same solution for 2 hours. Brains were cut in coronal sections (40 μ m thick) containing the striatum in an Oxford vibratome and placed on gelatin-coated glass slides.

Then, for both pups and adult rats the same protocol for each step of NADPH-d histochemical method was used (Vincent & Kimura, 1992). Cerebral sections from treated and control animals were simultaneously processed. In short, sections were incubated for 1 hour 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, St. Louis, MO, USA). Sections were placed in PBS/glycerol (1:3) and observed and photographed with a Zeiss Axophot microscope.

3.9 Computerized Image Analysis:

NADPH-d and Cresyl violet stained neurons were quantitatively measured using a computerized image analyzer (Kontron-ZEISS VIDAS). The selected fields were located in the lateral, medial, dorsal and ventral areas of the striatum and the medial and lateral sectors of the neocortex. Adequately labelled sections, 40 μ m thick, from striatum and surrounding neocortex were randomly selected. Ten cells per section were analyzed with a total of 100 per group. Observations and measurements obtained in the light microscope were transferred to a video camera attached and connected to an Interactive image analysis system on line, Kontron-ZEISS VIDAS. Images were digitized into an array of 512 x 512 pixels corresponding to 140 x 140 μ m (40X primary magnification). Resolution of each pixel was 256 (gray levels). After automatic normalization of the gray-scale, interactive delineation and contrast enhancement of images were performed, removing all interfering non-specific images. The projected surface of NADPH-d and Cresyl violet neurons was measured using morphometry: area, perimeter and maximum-minimum diameter form factor (F-shape). Data count in the measured field was stored and represented the number of positive structures in each field. Mean and SD were calculated for the different fields studied in asphyctic, treated and control groups.

3.10 Statistical analysis:

Differences between means and S.D. of experimental and control groups were statistically analyzed using one-way analysis of variance (ANOVA) and later the Newman-Keuls test, a $P < 0.05$ being considered significant. ANOVA and Newman-Keuls tests were routinely performed with an IBM compatible PC AT 486 package software (Primer, Mc Graw Hill, Inc.).

CHAPTER 4

4. RESULTS

4.1 The experimental model. Protection from mortality in PA by hypothermia: A non-invasive animal model to study short- and long-term consequences of hypoxic-ischemic lesions, similar to those produced under labour in clinical situations, was developed in rats. The findings were: 1) in a water bath at 37°C, a PA lasting longer than 22 minutes led to 100% mortality within the first 20 minute period after delivery; 2) when the uterus containing the fetuses was kept for 22 minutes in a 30°C water bath, 100% of the pups recovered respiratory function following tactile oral stimulation and were accepted by surrogate mother; 3) the protective effect of hypothermia at 30°C allows for a 47-48 minute asphyctic period and, 4) when asphyxia was induced in a water bath at 15°C, the 100% survival rate was extended to 101 minutes.

Fig. 5 shows survival percentage in animals exposed to different asphyctic periods of time at 37°C and Fig. 6 compares survival % at 37°C, 30°C and 15°C. According to survival % at 37°C, PA was classified into *slight* (5-6 minutes and 10-11 minutes), *moderate* (15-16 minutes), *subsevere* (19-20 minutes) and *severe* (≥ 20 minutes).

4.2 SHORT-TERM EFFECTS OF PERINATAL ASPHYXIA

4.2.1 Survival, behaviour and subcutaneous microdialysis in asphyctic, control and hypothermic-treated pups: Several parameters were acutely recorded by direct observation (see Tables I-IV) or in vivo microdialysis. After asphyxia, pups were subcutaneously implanted with 4mm flexible microdialysis probes in the dorsal region, while kept on a heating pad. In this way, subcutaneous levels of aminoacids (glutamate, aspartate), and metabolic products (lactate, pyruvate, and ascorbate) were monitored for 40-60 and 60-80 minute periods after removal of asphyctic and control pups from the uterus (see Figs. 7-11)

a) Effects of delivery through hysterectomy (caesarea) and normal vaginal delivery: Pups delivered from uterus horns by hysterectomy on their final day of gestation started regular breathing (respiratory frequency 60/min) almost immediately after delivery was completed. These control pups presented pink-colored skin and intensive vocalization and motility. They were accepted by surrogate mothers after an 80 minute observation period. When pups were accepted, their posterior development was similar to that of normally delivered rats (this was seen in the one month period of observation). Measurement of subcutaneous levels showed no difference between the two groups and were approximately: glutamate = $2\mu\text{M}$; aspartate = $0.4\mu\text{M}$; lactate 1mM ; pyruvate = $60\mu\text{M}$ and ascorbate $20\mu\text{M}$

b) Slight and moderate asphyctic exposure: Following a 2-3 and 5-6 minute asphyctic period induced in a water bath at 37°C (slight PA) or 30°C, all pups started breathing shortly after delivery. Their behaviour was similar to that observed in the control animals (see Tables I and II). Following a 5-6 minute asphyctic period at 37°C, glutamate levels were =7.5 μ M; aspartate=1.2 μ M; lactate=1.5mM; pyruvate=150 μ M and ascorbate=15 μ M. At 30°C, glutamate levels were=3.5 μ M; aspartate=1.6 μ M; lactate=1.3mM; pyruvate=150 μ M and ascorbate=10 μ M. Following a 15-16 minute asphyctic period induced in a water bath at 37°C (moderate PA) or 30°C, all pups survived, presenting no difference in skin colour or respiratory frequency when compared with control animals. Although slight decrease in spontaneous motility was observed, all pups were accepted by surrogate mothers. At 37°C, glutamate levels were=7 μ M; aspartate=1 μ M; lactate 2mM; pyruvate=60 μ M and ascorbate=15 μ M. At 30°C glutamate levels were=3 μ M; aspartate=1 μ M; lactate 1.5mM; pyruvate=60 μ M and ascorbate=5 μ M.

c) Subsevere asphyctic exposure: After a 19-20 minute asphyctic period at 37°C, pups had to be intensively stimulated in order to start breathing. Surviving pups remained aknetic for a long period after delivery and showed a significant decrease in respiratory frequency (=20/mln), accompanied by gasping and a pink/pale skin coloration. Approximately 30% of the pups died shortly after delivery. In contrast, at 30°C all pups survived a 19-20 minute asphyctic period. However, Initial gasping, a slight decrease in respiratory frequency (=40/min) and motility was observed. Skin colour was similar to that of control pups. All surviving pups were accepted by surrogate mothers. At 37°C, glutamate levels were =5 μ M; aspartate=0.5 μ M; lactate 2mM; pyruvate=60 μ M and ascorbate=4 μ M. At 30°C, glutamate levels were=5 μ M; aspartate=1 μ M; lactate 2mM; and pyruvate=40 μ M and ascorbate=12 μ M.

d) Severe asphyctic exposure: After a prolonged asphyctic period at 37°C (\geq 20 min), rate of survival rapidly decreased and, in general, the physiological condition of the surviving pups deteriorated (increased gasping, decreased respiratory frequency, lack of vocalization, akinesia and pale skin). No pups survived asphyctic periods longer than 22 minutes. In contrast, at 30°C, all pups survived up to a 30-31 minute asphyctic period, although some signs of physiological impairment (presence of gasping, decrease in respiratory frequency and motility and pale skin) were observed. At this temperature, 40% survival was observed after a 47-48 minute asphyctic period. All surviving pups showed gasping, decrease in respiratory frequency (\approx 10/mln), akinesia and pale skin. No survival was observed after asphyctic periods longer than 48 minutes. Following a 21-22 minute asphyctic period at 37°C, glutamate levels were=4 μ M; aspartate=0.4 μ M; lactate 2mM; pyruvate=90 μ M and ascorbate=10 μ M. At 30°C, glutamate levels were=9 μ M; aspartate=1 μ M; lactate 2mM; pyruvate=40 μ M and ascorbate=2.5 μ M.

At 15°C, 100% survival was observed up to 101 minutes of asphyxia (see Fig. 6). Following a 50-51 minute asphyctic period, gasping was observed in 50% of pups, respiratory frequency was =40/min, vocalization and motility decreased and skin was pink/pale. Following a 100-101 minute asphyctic period at 15°C, gasping was observed in all pups and respiratory frequency was 10/min. Pups were akinetic and pale, and no vocalization was observed (Table II). All substances measured by microdialysis in subcutaneous tissue were similar to those observed in control group.

In a series of experiments the water temperature was rapidly shifted from 37°C to 15°C in different asphyctic periods. High prevention of mortality was obtained when shifting was done before the first 15 minutes of PA. The sooner the shifting was performed, the higher the prevention of mortality was. (Fig 12).

4.2.2 Effects of the antagonists of glutamate receptors: Table IV shows the effect of pretreatment with saline, dizocilpine (MK-801) (0.2 mg/kg s.c.) or NBQX (10-30mg/kg s.c.) (no effects were observed after NBQX 3 mg/kg s.c.) administered to mothers one hour before hysterectomy (control group) or hysterectomy followed by 21-22 and 22-23 minute asphyctic periods at 37°C. Survival after a 21-22 minute asphyctic period at 37°C was slightly increased by pretreatment with dizocilpine or NBQX. An increase in survival following a 22-23 minute asphyctic period at 37°C was observed only when the highest dose of NBQX (30 mg/kg s.c.) in association with MK-801 was administered (see Fig. 13)

4.2.3 NADPH-d histochemical staining in asphyctic, control and hypothermic-treated pups: In control pups born through caesarea or natural vaginal delivery, NADPH-d(+) cells were present only in the lateral zone of the neostriatum, near the corpus callosum and at the end of corpus callosum (see Fig. 14 and Fig 23a). Few stained cells (6 ± 2) were found in control striatal sections and these were either non-polar type or presented short monopolar processes (Fig. 15a). No stained cells were seen in the cortex of control rats (Fig. 16a).

Rats exposed to severe PA showed NADPH-d(+) cells in the same areas as controls did; also, some, were detected in the cortex (Fig. 16c-d). Neurons in striatum presented a greater affinity to staining, and larger and longer processes (Fig. 15c-d). Animals exposed to 100 minutes of PA in hypothermic conditions (15°C) showed the same pattern of staining as controls (Fig 15b and 16b). All groups presented NADPH-d+ rounded cells at the end of the corpus callosum, with no morphological difference between them.

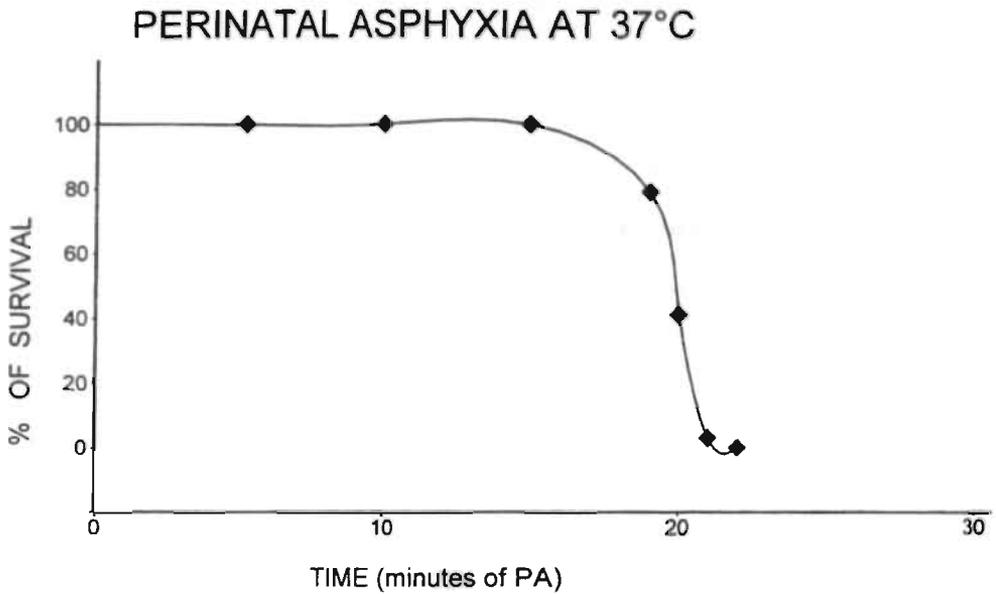


Fig 5: Percentage of survival at different PA periods of time at 37°C

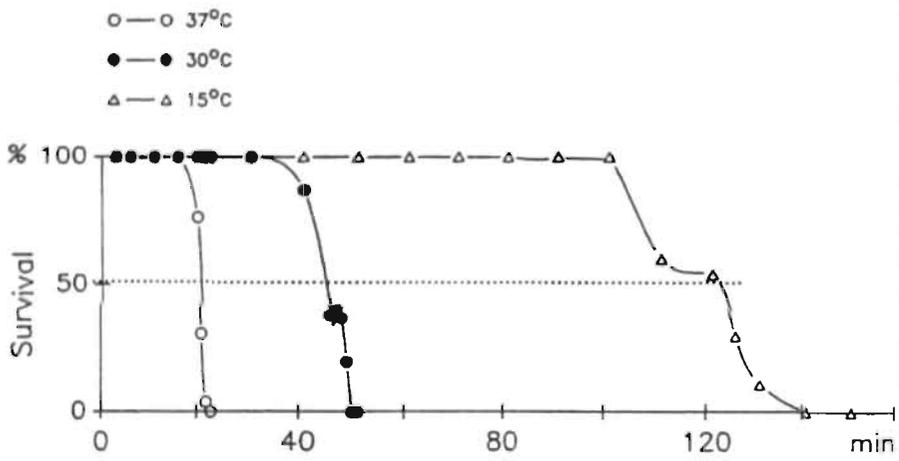


Fig 6 : Percentage of survival at different PA periods of time at 37°C, 30°C and 15°C

Table 1: Short-term effects of PA at 37°C monitored by direct observation at 40-60 min following delivery. Spontaneous movements were scored according to the following scale: (0) akinesia and rigidity (mainly of hind limbs); (1) movement of one of the following body structures: front limbs, hind limbs or head alone; (2) movement of two of the body structures; (3) movement of all body structures; (4) intensive movements shown by wriggling. Gasping refers to the effort to maintain respiration expressed by the opening of the mouth and movements in the diaphragm.

Experimental condition	Body weight (g)	Survival	Respiratory frequency	Gasping	Vocalization	Colour of the skin	Spontaneous movements
[1] Vaginal delivery (n=18)	6.1 ± 0.5	100%	79 ± 2	0%	100%	Pink	3.9 ± 0.1
[2] Caesarean delivery (n=42)	5.8 ± 0.5	100%	74 ± 2	0%	100%	Pink	3.8 ± 0.1
[3] PA at 2-3 min (n=4)	5.8 ± 0.5	100%	77 ± 7	0%	100%	Pink	3.9 ± 0.1
[4] PA at 5-6 min (n=14)	5.7 ± 0.4	100%	76 ± 3	0%	100%	Pink	3.7 ± 0.2
[5] PA at 10-11 min (n=12)	5.7 ± 0.3	100%	77 ± 2	0%	100%	Pink	3.3 ± 0.2
[6] PA at 15-16 min (n=12)	6.0 ± 0.5	100%	61 ± 3.7	0%	83%	Pink	2.9 ± 0.23
[7] PA at 19-20 min (n=33)	5.8 ± 0.2	79%	32 ± 3.4	55%	6%	Pink/pale	0.7 ± 0.08
[8] PA at 20-21 min (n=23)	5.8 ± 0.3	41%	24 ± 2.6	95.5%	0%	Pale	0
[9] PA at 21-22 min (n=13)	5.9 ± 0.6	3%	18 ± 3	100%	0%	Pale	0
[10] PA at 22-23 min (n=132)	5.8 ± 0.2	0%	-	-	-	-	-

Table II: Short-term effects of PA at 30°C monitored by direct observation at 40-60 min fo delivery.

Experimental condition	Body weight (g)	Survival	Respiratory frequency	Gasping	Vocalization	Colour of the skin	Spontaneous movements
[1] PA at 2-3 min (n=6)	5.8 ± 0.4	100%	69 ± 10	0%	100%	Pink	3.7 ±
[2] PA at 5-6 min (n=17)	5.8 ± 0.2	100%	56 ± 4	0%	100%	Pink	2.8 ±
[3] PA at 10-11 min (n=11)	5.7 ± 0.4	100%	56 ± 5	0%	100%	Pink	2.6 ±
[4] PA at 15-16 min (n=9)	5.8 ± 0.4	100%	54 ± 2.6	0%	89%	Pink	2.7 ±
[5] PA at 19-20 min (n=12)	6.0 ± 0.3	100%	39 ± 10	11%	22%	Pink	2.4 ±
[6] PA at 20-21 min (n=10)	6.0 ± 0.3	100%	47 ± 5	10%	50%	Pink	2.7 ±
[7] PA at 21-22 min (n=19)	6.0 ± 0.3	100%	47 ± 2	14%	57%	Pink/pale	2.6 ±
[8] PA at 22-23 min (n=23)	6.0 ± 0.3	100%	38 ± 9	14%	57%	Pink/pale	1.5 ±
[9] PA at 30-31 min (n=17)	6.0 ± 0.4	100%	45 ± 5	27%	40%	Pink/pale	1.8 ±
[10] PA at 40-41 min (n=15)	5.9 ± 0.3	87%	40 ± 7	44%	22%	Pale	1.1 ±
[11] PA at 45-46 min (n=18)	5.8 ± 0.3	44%	34 ± 7	88%	13%	Pale	0.8 ±
[12] PA at 46-47 min (n=14)	5.8 ± 0.3	43%	30 ± 7	83%	0%	Pale	0.2 ±
[13] PA at 47-48 min (n=22)	5.9 ± 0.6	36%	17 ± 5	100%	0%	Pale	0
[14] PA at 48-49 min (n=12)	5.9 ± 0.6	25%	25 ± 6	100%	0%	Pale	0
[15] PA at 50-51 min (n=15)	6.0 ± 0.6	0%	-	-	-	-	-

Table III: Short-term effects of PA at 15°C monitored by direct observation at 40-60 min following delivery.

Experimental condition	Body weight (g)	Survival	Respiratory frequency	Gasping	Vocalization	Colour of the skin	Spontaneous movements
[1] PA at 2-3 min (n=7)	6.0 ± 0.2	100%	83 ± 2	0%	100%	Pink	3.9 ± 0.3
[2] PA at 5-6 min (n=8)	5.7 ± 0.3	100%	67 ± 5	0%	100%	Pink	3.3 ± 0.3
[3] PA at 10-11 min (n=7)	5.8 ± 0.3	100%	77 ± 4	0%	100%	Pink	3.5 ± 0.2
[4] PA at 15-16 min (n=6)	5.8 ± 0.4	100%	80 ± 0	0%	100%	Pink	2.7 ± 0.16
[5] PA at 20-21 min (n=5)	5.8 ± 0.3	100%	68 ± 9	0%	100%	Pink	3.0 ± 0.57
[6] PA at 30-31 min (n=7)	5.9 ± 0.5	100%	79 ± 1	0%	50%	Pink/pale	2.8 ± 0.3
[7] PA at 40-41 min (n=7)	6.0 ± 0.3	100%	61 ± 6	0%	33%	Pink/pale	2.2 ± 0.16
[8] PA at 50-51 min (n=12)	5.8 ± 0.3	100%	55 ± 6	33%	8%	Pale	1.1 ± 0.33
[9] PA at 60-61 min (n=10)	5.7 ± 0.3	100%	48 ± 4	50%	0%	Pale	0.66 ± 0.33
[10] PA at 70-71 min (n=11)	5.8 ± 0.2	100%	50 ± 4	55%	0%	Pale	0.27 ± 0.14
[11] PA at 80-81 min (n=7)	6.0 ± 0.1	100%	41 ± 5	67%	0%	Pale	0.2 ± 0.17
[12] PA at 90-91 min (n=5)	5.9 ± 0.3	100%	23 ± 5	75%	0%	Pale	0
[13] PA at 100-101 min (n=8)	5.9 ± 0.3	100%	25 ± 6	88%	0%	Pale	0
[14] PA at 110-111 min (n=5)	5.9 ± 0.4	60%	24 ± 1	100%	0%	Pale	0
[15] PA at 120-121 min (n=12)	6.0 ± 0.1	54%	14 ± 4	100%	4%	Pale	0
[16] PA at 125-126 min (n=10)	5.6 ± 0.2	30%	23 ± 3	100%	0%	Pale	0
[17] PA at 130-131 min (n=9)	5.5 ± 0.0	11%	8 ± 0.0	100%	0%	Pale	0
[18] PA at 140-141 min (n=7)	6.0 ± 0.6	0%	-	-	-	-	-

Table IV: Effect of glutamate antagonists, administered to the mother one hour before hysterectomy, on the short-term effects of PA at 37°C monitored by direct observation at 40-60 min following delivery. Controls were taken from the same mother, but were not exposed to asphyxia. * $P < 0.05$, compared to the saline group.

Experimental condition	Body weight (g)	Survival	Respiratory frequency	Gasping	Vocalization	Colour of the skin	Spontaneous movements
SALINE							
Control (n=5)	6.0 ± 0.2	100%	81 ± 2	0%	100%	Pink	3.9 ± 0.1
PA at 21-22 min (n=78)	5.9 ± 0.3	3%	16 ± 5	100%	0%	Pale	0
PA at 22-23 min (n=10)	5.9 ± 0.2	0%	-	-	-	-	-
MK-801 (DIZOCLIPINE) 0.2 mg/kg							
Control (n=18)	5.8 ± 0.3	100%	62 ± 3	7%	40%	Pink/pale	2.2 ± 0.2
PA at 21-22 min (n=42)	5.7 ± 0.3	17%*	39 ± 7	60%	20%	Pink/pale	0.4 ± 0.2
PA at 22-23 min (n=11)	5.9 ± 0.3	0%	-	-	-	-	-
NBQX 10 mg/kg							
Control (n=12)	5.7 ± 0.4	100%	62 ± 5	0%	100%	Pink/pale	3.1 ± 0.3
PA at 21-22 min (n=31)	5.8 ± 0.1	13%	17 ± 7	100%	0%	Pale	0
PA at 22-23 min (n=28)	5.7 ± 0.2	0%	-	-	-	-	-
PA at 23-24 min (n=5)	5.9 ± 0.2	0%	-	-	-	-	-
NBQX 20 mg/kg							
Control (n=7)	5.7 ± 0.3	100%	73 ± 7	0%	100%	Pink/pale	3.5 ± 0.4
PA at 19-20 min (n=9)	5.8 ± 0.2	55%	52 ± 8	40%	60%	Pink/pale	2.1 ± 0.3
PA at 20-21 min (n=17)	5.8 ± 0.2	24%	32 ± 7	100%	0%	Pale	0
PA at 21-22 min (n=10)	5.7 ± 0.4	30%*	21 ± 8	100%	0%	Pale	0
PA at 22-23 min (n=10)	5.9 ± 0.2	9%*	32 ± 0.0	100%	0%	Pale	0
PA at 23-24 min (n=4)	5.9 ± 0.3	0%	-	-	-	-	-

Table IV (cont.)

Experimental condition	Body weight (g)	Survival	Respiratory frequency	Gasping	Vocalization	Colour of the skin	Spontaneous movements
NBQX 30 mg/kg							
Control (n=7)	5.9 ± 0.3	100%	73 ± 7	0%	71%	Pink	3.6 ± 0.2
PA at 19-20 min (n=13)	5.7 ± 0.2	100%	51 ± 3	8%	23%	Pink/pale	1.7 ± 0.3
PA at 20-21 min (n=18)	5.7 ± 0.1	61%	42 ± 7	41%	35%	Pink/pale	1.1 ± 0.2
PA at 21-22 min (n=20)	5.9 ± 0.2	20%*	60 ± 10	75%	0%	Pale	0
PA at 22-23 min (n=23)	5.7 ± 0.1	17%*	51 ± 5	100%	0%	Pale	0
PA at 23-24 min (n=5)	5.8 ± 0.2	0%	-	-	-	-	-
PA at 30-31 min (n=3)	5.9 ± 0.2	0%	-	-	-	-	-
NBQX 30 mg/kg + MK-801 0.2 mg/kg							
Control (n=10)	6.0 ± 0.5	100%	63 ± 3	0%	90%	Pink	4.0 ± 0.4
PA at 19-20 min (n=3)	5.8 ± 0.1	100%	36 ± 3	67%	0%	Pink	3.2 ± 0.4
PA at 20-21 min (n=22)	5.8 ± 0.3	41%	22 ± 2	94%	0%	Pale	0.5 ± 1.0
PA at 21-22 min (n=20)	5.9 ± 0.26	36%*	12 ± 2	100%	17%	Pink/pale	0
PA at 22-23 min (n=23)	5.7 ± 0.1	17%*	9 ± 2	100%	0%	Pale	0
PA at 23-24 min (n=5)	5.8 ± 0.5	20%*	8 ± 0.0	100%	0%	Pale	0

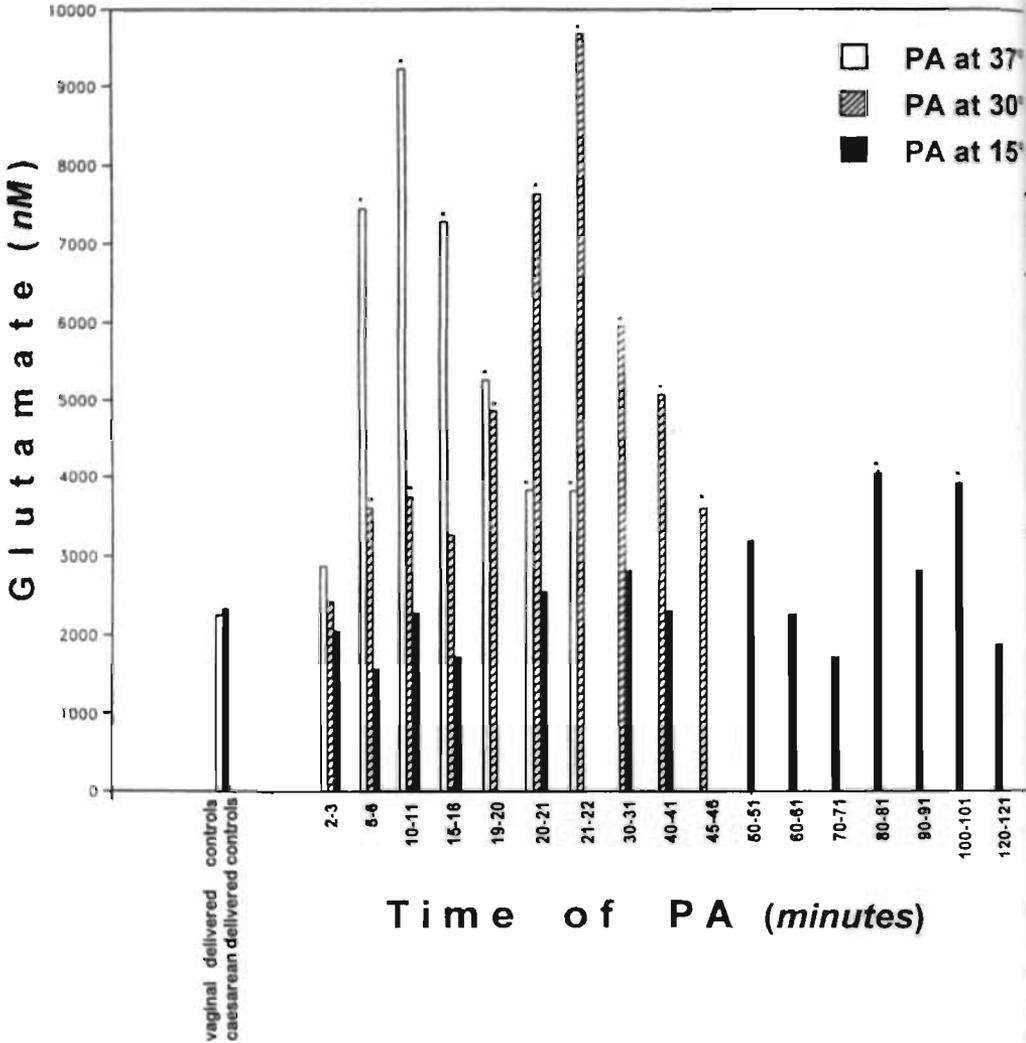


Fig 7: Short-term effects of PA at 37°C, 30°C and 15°C on subcutaneous levels of glutamate (Glu) measured at 60-80 min following delivery of vaginal and caesarean-delivered controls at different asphyctic periods of time in pups. (*) P < 0.05 for the two tailed test, compared with the caesarean-delivered group (N=4-26)

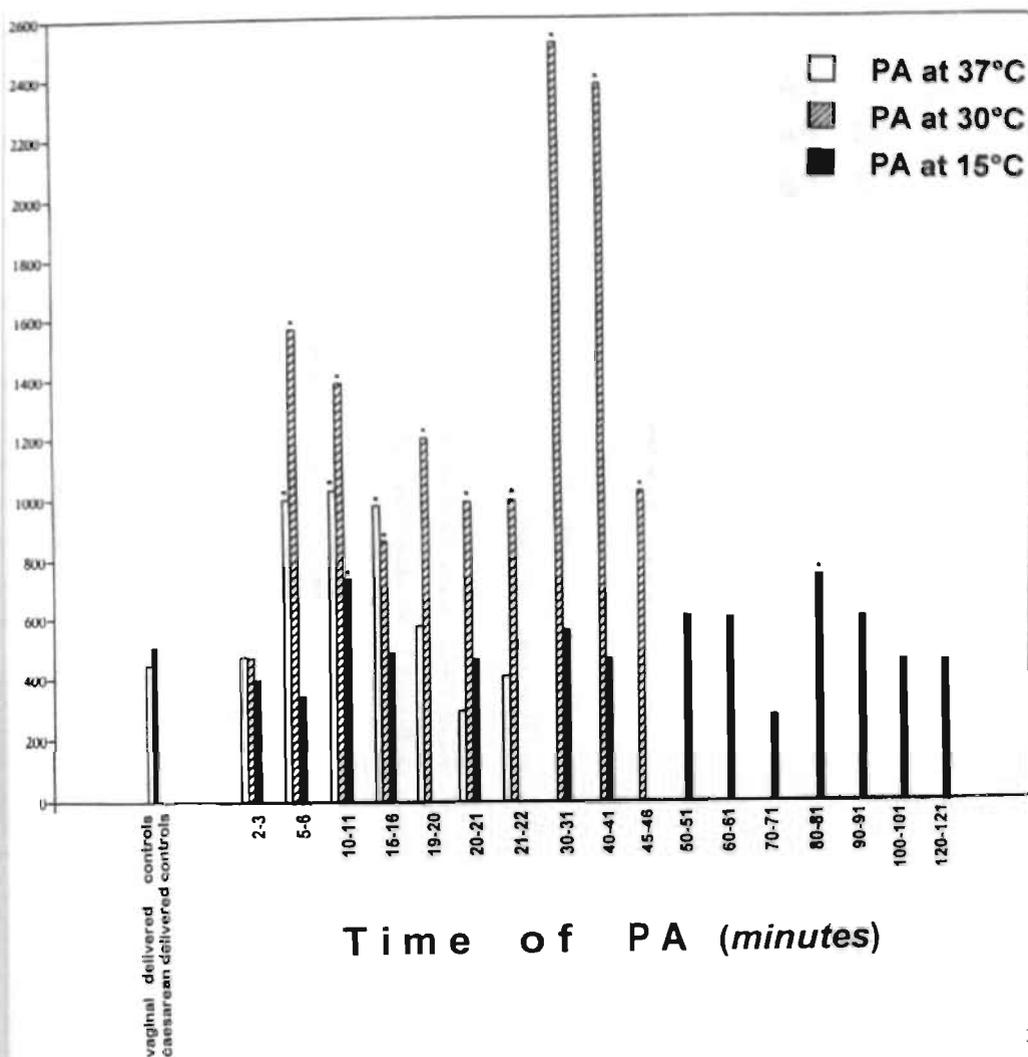


Fig 8: Short-term effects of PA at 37°C, 30°C and 15°C on subcutaneous levels of aspartate (Asp) measured at 60-80 min following delivery of vaginal and caesarean-delivered controls at different asphyctic periods of time in pups. (*) $P < 0.05$ for the two tailed test, compared with the caesarean-delivered group ($N = 4-26$)

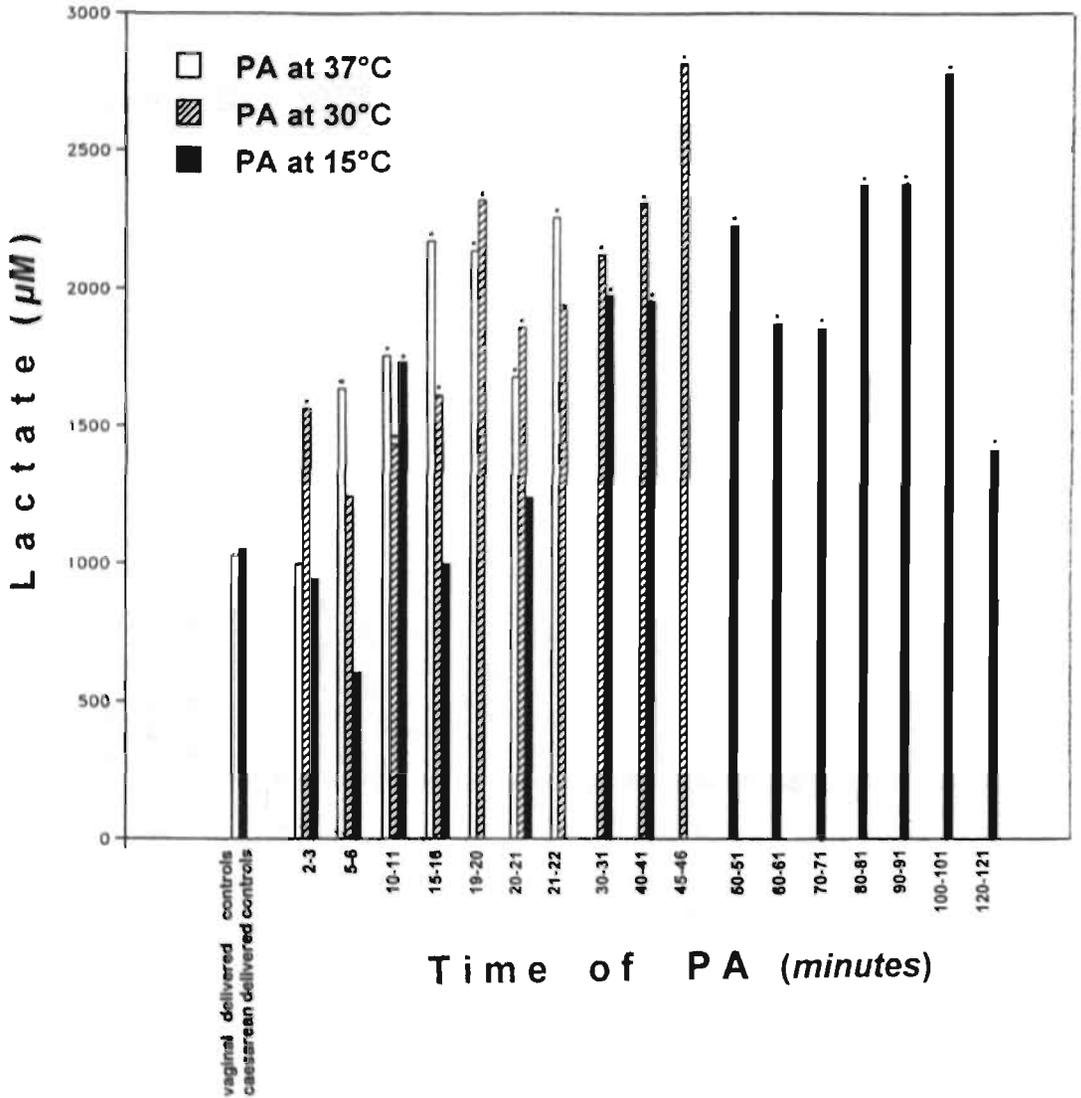


Fig 9 : Short-term effects of PA at 37°C, 30°C and 15°C on subcutaneous levels of lactate (Lac) measured at 60-80 min following delivery of vaginal and caesarean-delivered controls at different asphyctic periods of time in pups. (*) P < 0.05 for the two tailed test, compared with the caesarean-delivered group (N=4-26)

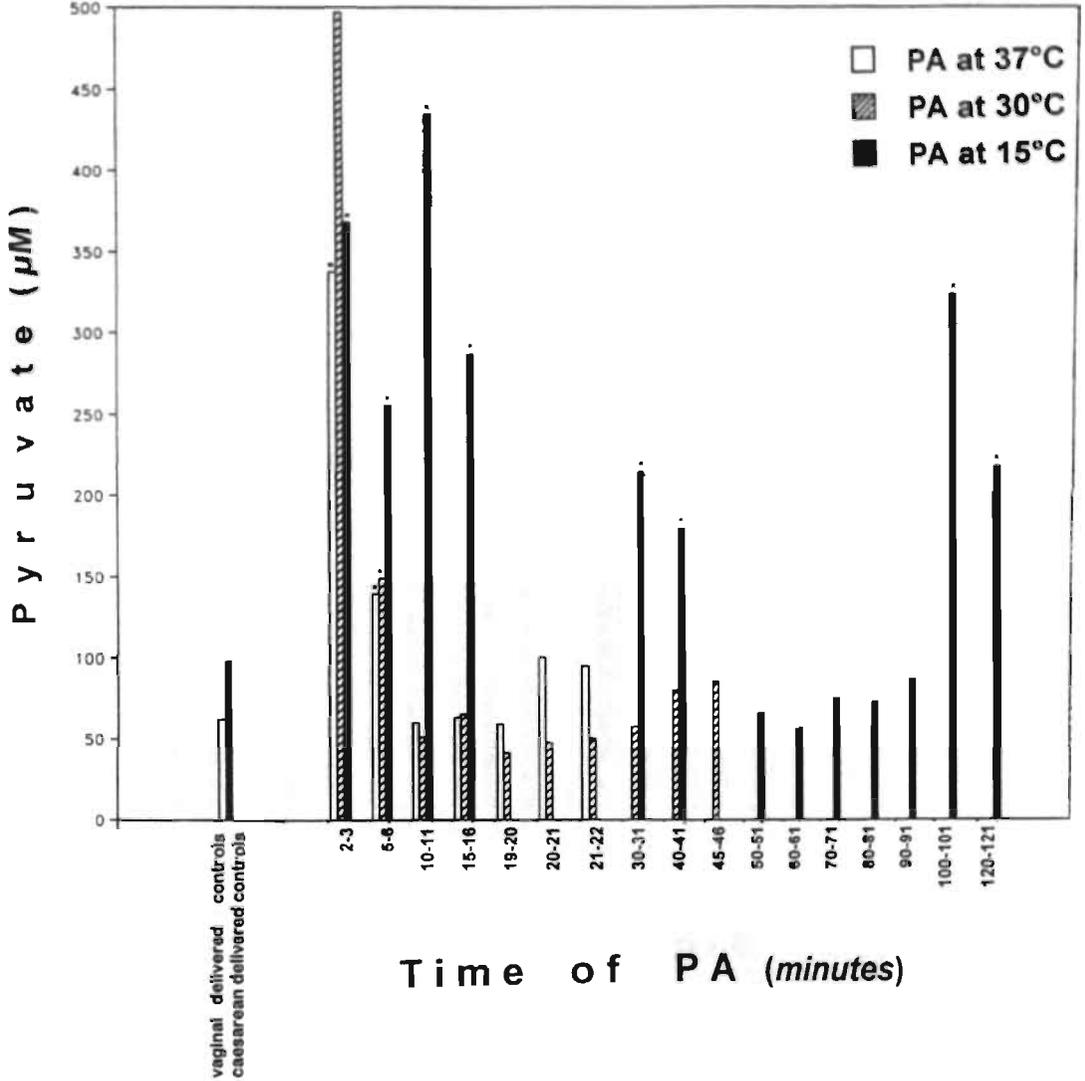


Fig 10: Short-term effects of PA at 37°C, 30°C and 15°C on subcutaneous levels of pyruvate (Pyr) measured at 60-80 min following delivery of vaginal and caesarean-delivered controls at different asphyctic periods of time in pups. (.) P < 0.05 for the two tailed test, compared with the caesarean-delivered group (N=4-26)

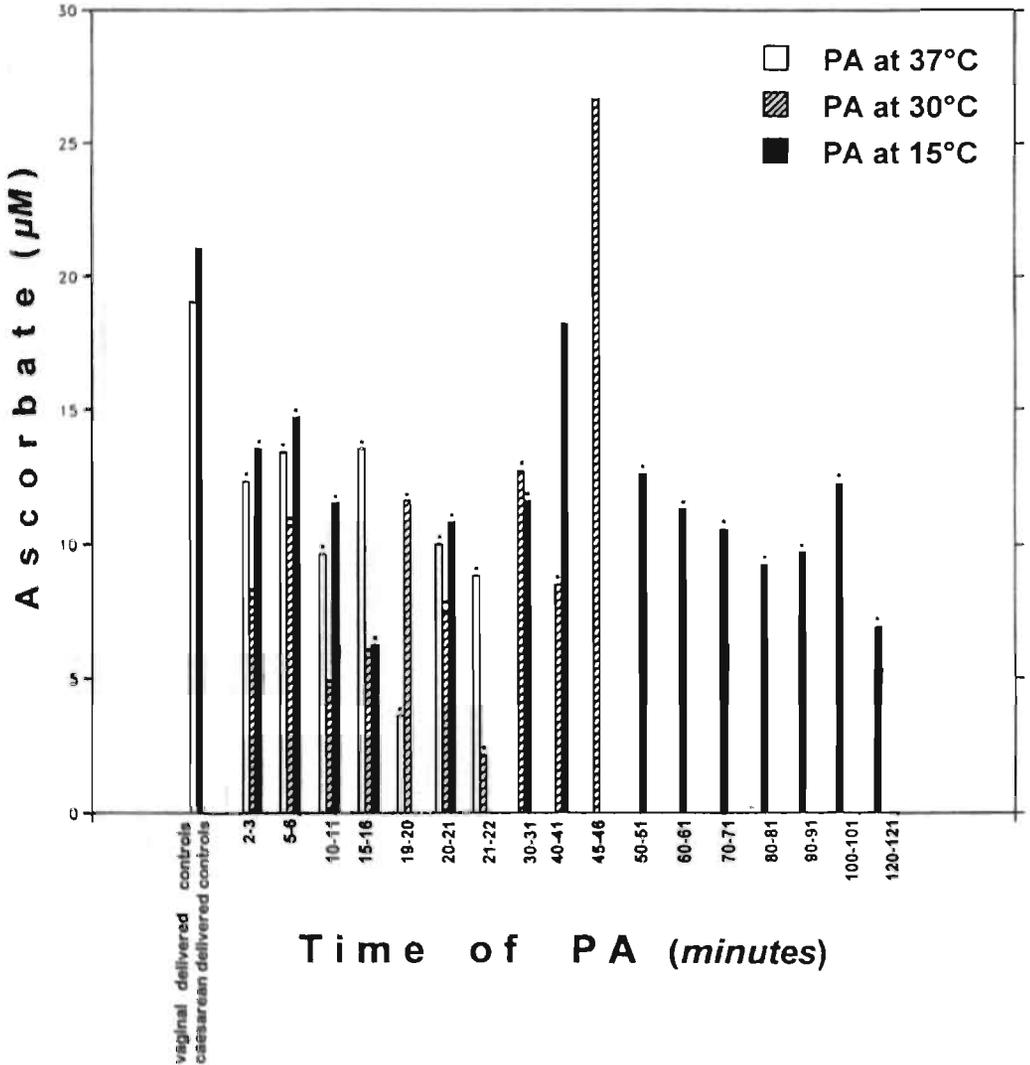


Fig 11: Short-term effects of PA at 37°C, 30°C and 15°C on subcutaneous levels of ascorbate (Asc) measured at 60-80 min following delivery of vaginal and caesarean-delivered controls at different asphyctic periods of time in pups. (*) P < 0.05 for the two tailed test, compared with the caesarean-delivered group (N=4-26)

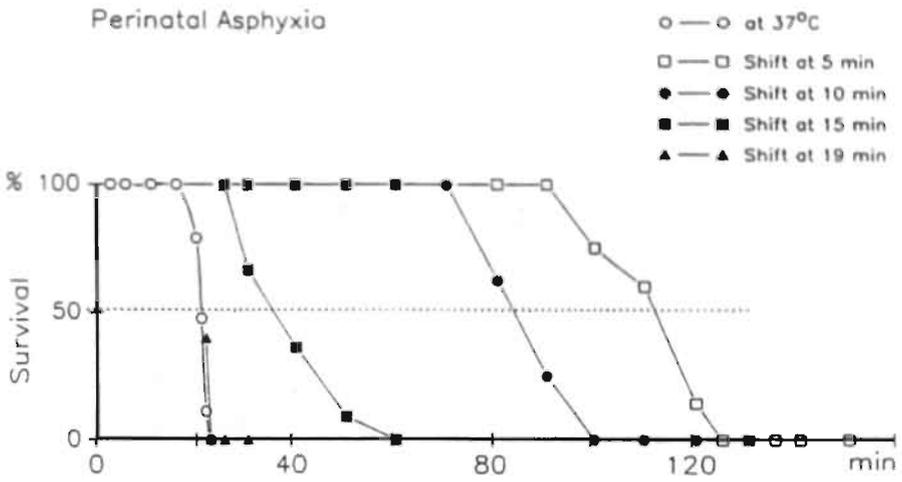


Fig 12 : Percentage of survival at different PA periods of time when shifting temperature from 37°C to 15°C was performed at 5, 10, 15 and 19 min from the starting of asphyxia.

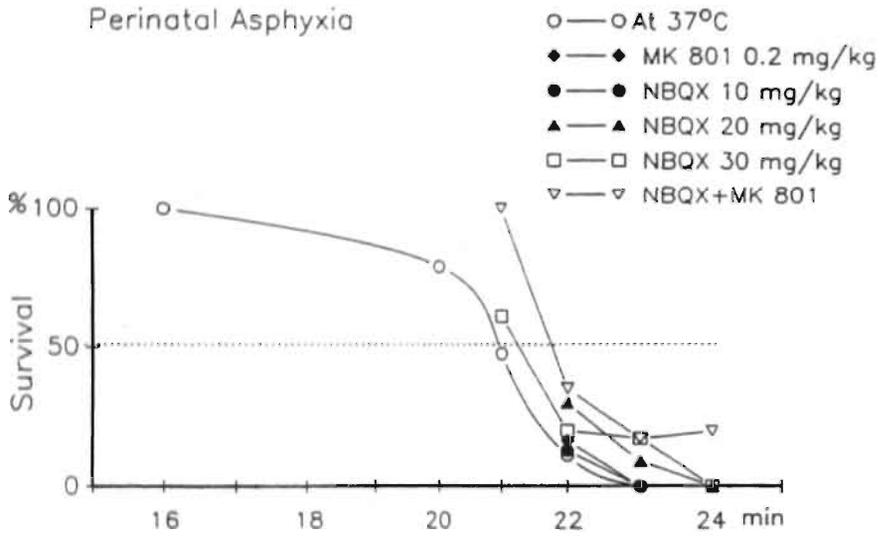


Fig 13: Percentage of pups that survived the different PA periods of time at 37°C when glutamate antagonists were administered intraperitoneally to the mother one hour prior to hysterectomy.

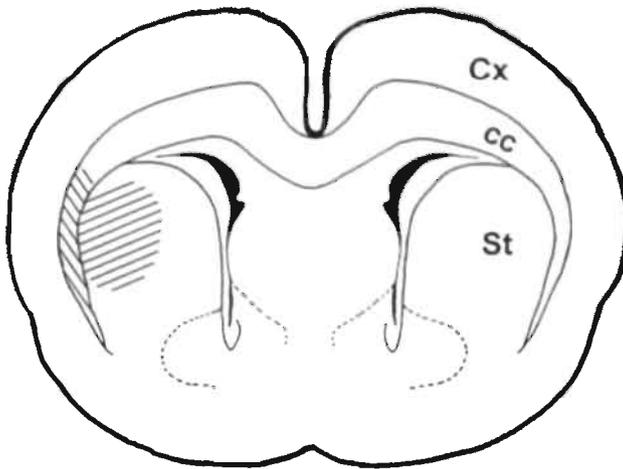


Fig 14 : NADPH-d positive areas (shadow) in control and in asphyctic newborn rats. Abbreviations: Cx: cerebral cortex; St: striatum; CC: corpus callosum

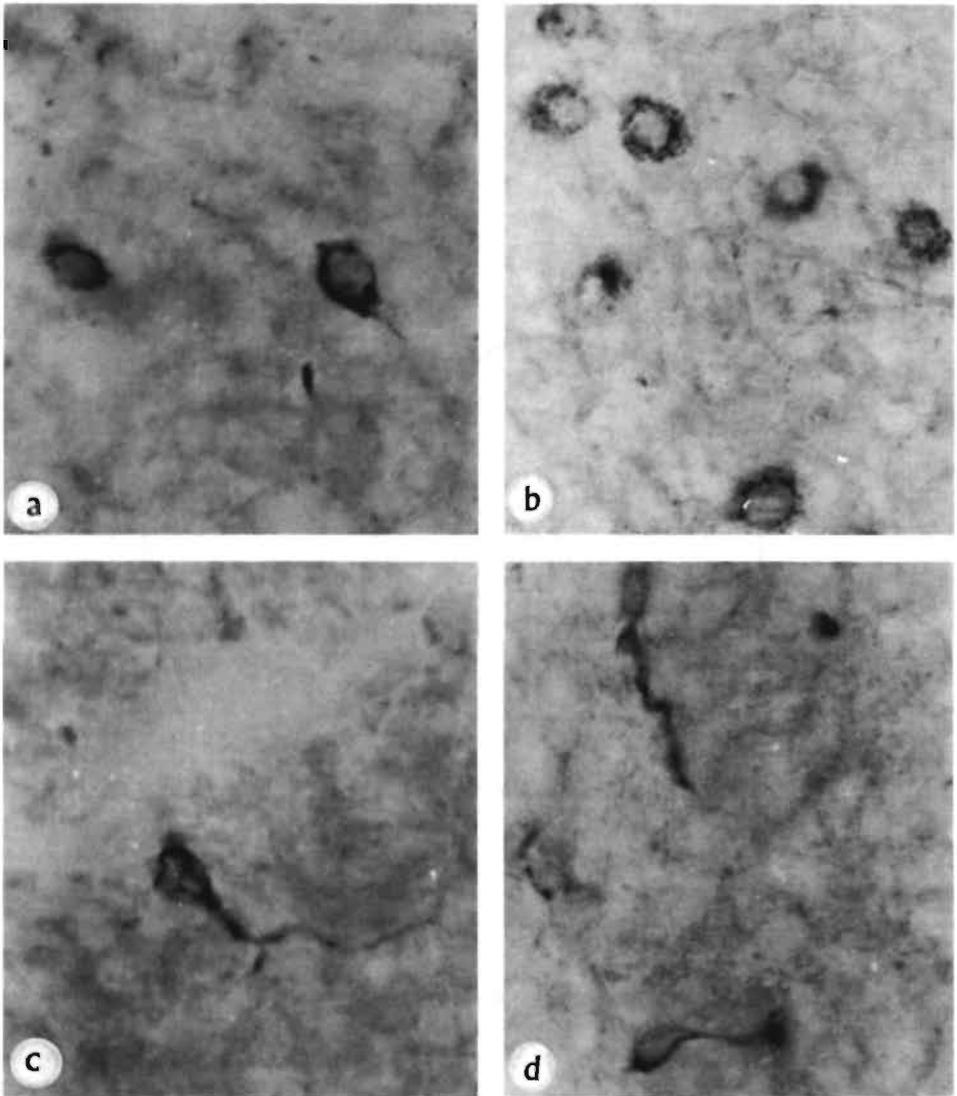


Fig 15 : Photomicrographs of NADPH-d positive neurons located in the lateral zone of the rostral striatum (900x). **a :** control; **b :** PA (100 min) + hypothermia (15°C), **c and d :** severe PA (≥ 20 min/37°C). Note the cell processes in severe PA.

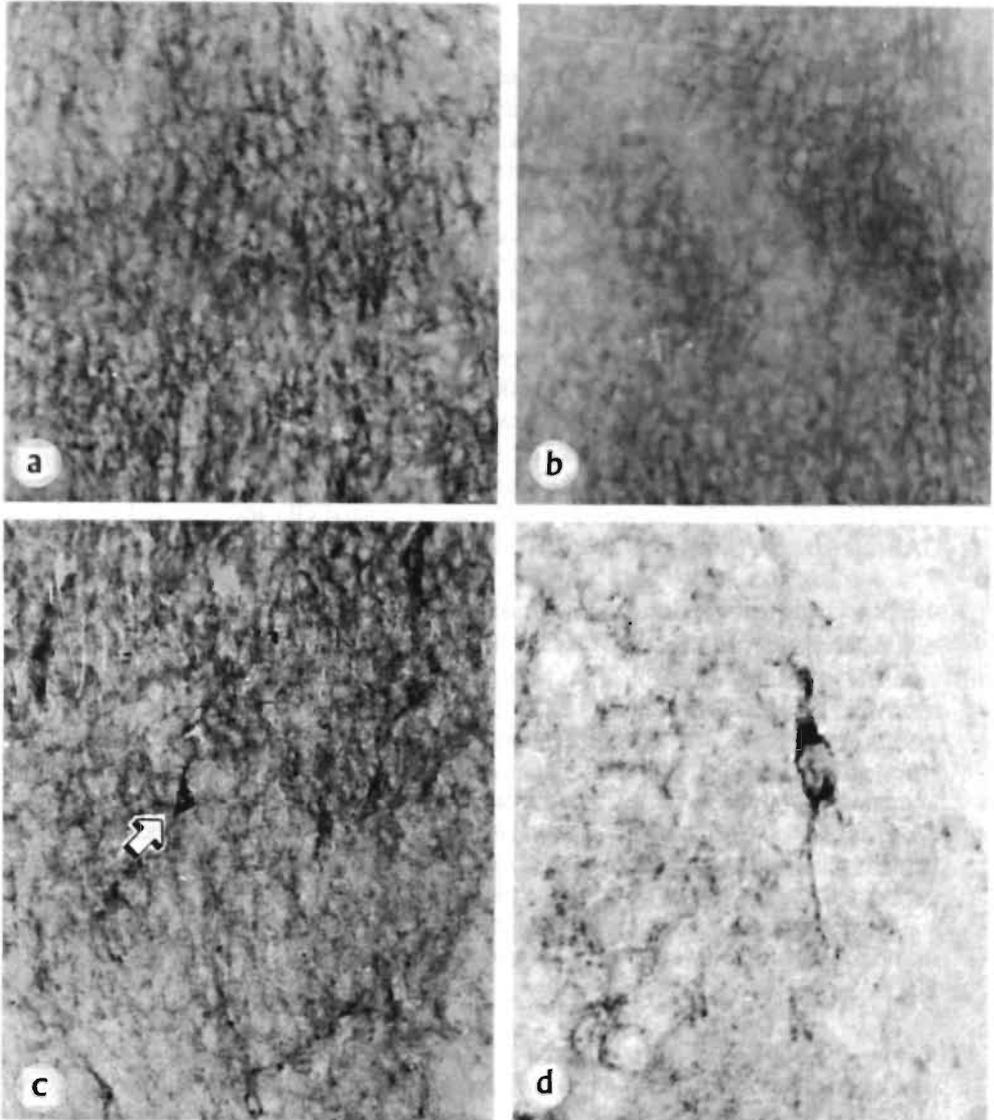


Fig 16 : Photomicrographs of NADPH-d positive neurons located in the cerebral cortex (150x). a : control; b : PA (100 min) + hypothermia (15°C), c and d : severe PA (≥ 20 min/37°C). Note the call processes in severe PA (900x). Note positive staining in some cortical neurons of animals subjected to severe PA.

4.3 LONG-TERM EFFECTS OF PERINATAL ASPHYXIA

4.3.1 Intracerebral microdialysis: After 6 months, rats that had suffered PA and controls, were implanted with two microdialysis probes, one in striatum and the other in substantia nigra. Monoamines and aminoacids were monitored under basal and D-amphetamine-stimulated conditions (see Tables V-VII).

Significant changes in monoamines, GABA and Glu were observed in rats exposed to subsevere and severe asphyxia. Striatal extracellular DA levels were significantly increased in animals exposed to subsevere (19-20 min, at 37°C) asphyxia (approximately twofold), although the effect of D-amphetamine on extracellular DA levels was significantly decreased (Fig. 17; Table VI). Striatal basal levels of Glu decreased. No significant effects were observed in Asp levels. Extracellular GABA levels in substantia nigra were 50% decreased (Table VI). In animals with severe asphyxia (≥ 20 min, at 37°C), a decrease in striatal DA levels under basal ($\approx 70\%$) and D-amphetamine stimulation ($\approx 50\%$ compared to controls) (Fig. 17; Table VII) as well as a decrease in striatal Glu and Asp and nigral GABA levels were observed (Table VII).

4.3.2 Immunocytochemistry and basal ganglia in 6 months old asphyctic and control rats: In 6 months old rats that suffered subsevere and severe PA, an astroglial hypertrophy in striatum (Fig 18c), substantia nigra (Fig 19b) and cortex illustrated by the enlargement and tortuosity of astrocytes was observed. These findings were compatible with the typical "astroglial reaction" also evident in the perivascular and limitant glia. The astroglial reaction immunostained with GFAP was more intense in those animals exposed to severe PA. The staining to GFAP in substantia nigra and striatum of the hypothermic-treated animals (exposed to PA at 15°C) for 20-21 and 100-101 minute periods were similar to that of the controls (Figs. 18b and 19c).

A great increase in immunostaining to NF was found in those animals that suffered severe PA. However, this was the case only in those of 230kD (see Fig. 20).

4.3.3 NADPH-d histochemical staining in 6 months old asphyctic, control and hypothermic-treated rats: Throughout, NADPH-d staining in striatum transverse sections of 6 months old rats showed uniformly distributed medium-sized neurons. Subsevere and severe PA groups presented an increase in soma size accompanied by an enlargement of processes, with evident tortuous dendritic arborization typical of the so-called neuronal cytomegaly (Mischel, 1995), when compared with control group and remaining PA groups at 37°C as well as 20 and 100 minutes PA at 15°C (compare Figs. 21a-c). Labelled cell area and cell perimeter were measured by image analysis in each experimental group. Both striatal cell area and cell perimeter of rats exposed to subsevere and severe PA showed a highly significant cytomegaly ($p < 0.001$) in

comparison with control, slight, moderate PA and PA at 15°C groups for 20 and 100 minutes (see Figs. 22a-b).

F-shape measurement of the neurons failed to disclose statistical differences between striatal neurons in all studied groups (see Figs. 22c and 22f).

Mean cell count per field with a 40X objective was 5 ± 1 in striatum and 4 ± 1 in cerebral cortex, lacking significant intergroup differences.

Likewise, in rats subjected to subsevere and severe PA, NADPH-d+ neurons in cerebral cortex sections, including medium-sized ones belonging to local circuit neurons, displayed morphological changes that presented similar characteristics to those of cytomegalic cells observed in striatum (compare Figs. 21d-e). Animals suffering 20 and 100 minutes of PA at 15°C showed a similar staining pattern to that of controls, slight and moderate PA groups (compare Figs. 21f and 21d).

Cortical cell area and perimeter increased significantly in rats exposed to subsevere and severe PA ($p < 0.001$) compared with control, slight, moderate PA and PA at 15°C-treated animals for 20 and 100 minutes (see Figs 22d-e).

After 6 months, those animals subjected to 20 or 100 minutes PA at 15°C disclosed typical NADPH-d(+) neurons in the corpus callosum (see Figs 23b-c).

4.3.4 Cresyl violet staining in the striatum and cortex

Subsevere and severe PA (normothermic ischemia) resulted in a loss of neurons in medial, posterior, ventral and dorsal sector of the striatum ($P < 0.001$). Hypothermia (20 min and 100 min at 15°C) reduced neuronal loss ($P < 0.001$). In the other groups (5, 10 and 15 min), no differences with controls were observed. When comparing different regions of the striatum (ventral, dorsal, lateral, medial) no significant differences were found ($P < 0.001$).

Subsevere and severe PA also resulted in a severe loss of neurons in medial and lateral neocortex with statistical difference ($P < 0.001$). Hypothermia (20 min and 100 min at 15°C) attenuated neuronal loss significantly ($P < 0.001$). When comparing different sectors of the neocortex (lateral and medial) no significant differences were found ($P < 0.001$).

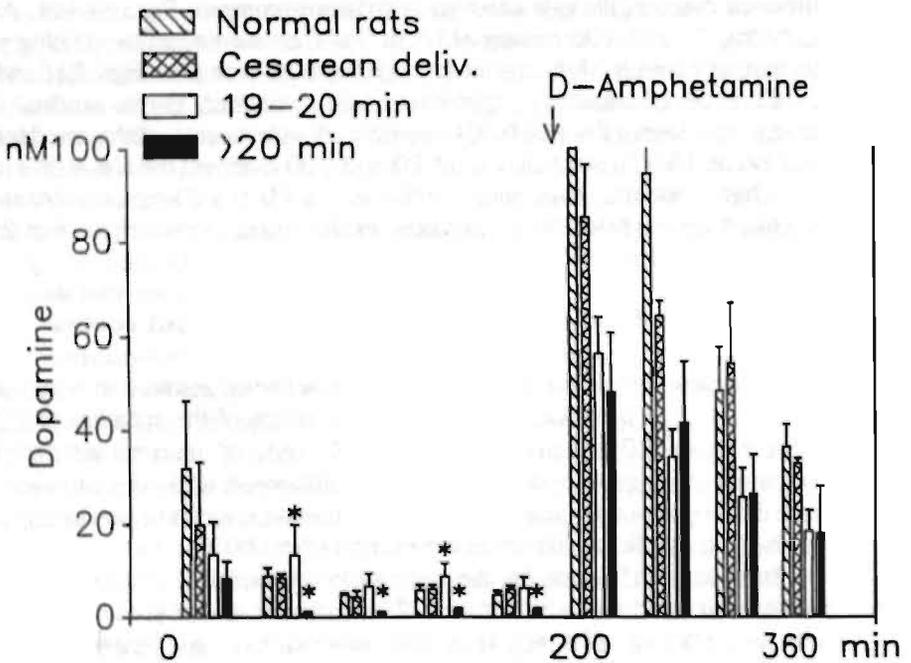


Fig 17: Effect of subsevere (19-20 min/37°C) and severe (≥ 20 min/37°C) PA on extracellular striatal dopamine levels measured 6 months after delivery. Microdialysis probes (4-mm length) perfused with a modified CSF solution were implanted in the striatum of: (1) vaginal-delivered controls (hatched columns, $n=8$), (2) caesarean-delivered controls (cross-hatched columns, $n=8$), (3) subsevere PA (open columns, $n=9$) and (4) severe PA (filled columns, $n=8$) rats. At a 200 min period of time following microdialysis implantation, a dose of D-amphetamine (2 mg/kg s.c.) was administered. Dopamine was assayed in 20 μ l samples using HPLC-EC. Vertical lines show SEM * $P < 0.05$ for the two tailed test (Mann-Whitney U-test, corrected by the Bonferroni's procedure).

Table V: Long-term effects (6 months after delivery) on monoamine, aminoacids and metabolic products levels monitored under basal (160-200 min period of time following microdialysis implantation) and D-amphetamine-stimulated (2 mg/kg s.c.) (200-240 min period) conditions in striatum and substantia nigra of caesarean delivered control rats.

	Basal (nM)	D-Amphetamine (nM)	%
Striatum			
<i>Control (n=15-22)</i>			
Dopamine	4.2 ± 1	72.4 ± 9	(1736%)
DOPAC	1175 ± 115	779 ± 95	(66%)
HVA	1052 ± 154	923 ± 133	(88%)
5-HIAA	3022 ± 321	2694 ± 266	(89%)
GABA	22 ± 3	27 ± 5	(121%)
Glutamate	1203 ± 281	1322 ± 321	(110%)
Aspartate	133 ± 30	101 ± 33	(77%)
Lactate	122 ± 41	152 ± 38	(124%)
Pyruvate	12 ± 2	15 ± 2	(134%)
Ascorbate	4 ± 0.7	4 ± 0.6	(106%)
Substantia nigra			
<i>Control (n=16-24)</i>			
Dopamine	0.9 ± 0.1	1.7 ± 0.3	(184%)
DOPAC	14 ± 2	4 ± 3	(29%)
HVA	38 ± 8	30 ± 7	(77%)
5-HIAA	496 ± 131	471 ± 137	(95%)
GABA	19 ± 3	34 ± 11	(178%)
Glutamate	788 ± 201	781 ± 232	(100%)
Aspartate	91 ± 20	80 ± 10	(89%)
Lactate	88 ± 9	95 ± 14	(108%)
Pyruvate	7 ± 0.6	8 ± 1	(118%)
Ascorbate	3.7 ± 0.7	3.6 ± 0.8	(97%)

Table VI: Long-term effects (6 months after delivery) on monoamines and aminoacids levels monitored under basal (160-200 min period of time following microdialysis implantation) and D-amphetamine-stimulated (2 mg/kg s.c.) (200-240 min period of time) conditions in striatum and substantia nigra of subsevere asphyctic (19-20 min of PA/37°C) rats.

	Basal (nM)	D-Amphetamine (nM)	%
(A) Striatum			
<i>Subsevere PA (n=7)</i>			
Dopamine	6.1 ± 1.7*	65.3 ± 8*	(1066%)
DOPAC	1047 ± 177	436 ± 115	(42%)
HVA	1417 ± 186	1086 ± 99	(77%)
5-HIAA	406 ± 25	395 ± 36	(97%)
GABA	28 ± 3	66 ± 18	(234%)
Glutamate	575 ± 161*	1360 ± 500	(237%)
Aspartate	100 ± 20	140 ± 50	(140%)
Acetylcholine	255 ± 56	467 ± 110	(183%)
Choline	799 ± 194	722 ± 172	(91%)
(A) Substantia nigra			
<i>Subsevere PA (n=7)</i>			
Dopamine	0.74 ± 0.1	1.8 ± 0.3	(234%)
DOPAC	14 ± 4.7	12 ± 4.4	(86%)
HVA	36 ± 5.4	40 ± 6	(110%)
5-HIAA	175 ± 24	629 ± 25	(93%)
GABA	11.5 ± 2.5*	12 ± 3.4*	(105%)
Glutamate	830 ± 370	720 ± 240	(87%)
Aspartate	100 ± 20	70 ± 120	(70%)
Acetylcholine	11 ± 2.5	20 ± 6	(176%)
Choline	389 ± 56	409 ± 184	(105%)

* =P <0.05 compared to the corresponding values observed in the controls.

Table VII: Long-term effects (6 months after delivery) on monoamine, aminoacids and metabolic products levels monitored under basal (160-200 min period of time following microdialysis implantation) and D-amphetamine-stimulated (2 mg/kg s.c.) (200-240 min period of time) conditions in the striatum and substantia nigra of severe asphyctic (≥ 20 min of PA at 37°C) rats.

	Basal (nM)	D-Amphetamine (nM)	%
Striatum			
<i>Severe PA (n=4-8)</i>			
Dopamine	1.6 \pm 0.44*	48 \pm 13*	(2951%)
DOPAC	1239 \pm 175	819 \pm 129	(66%)
HVA	773 \pm 88	694 \pm 77.4	(90%)
5-HIAA	926 \pm 67*	880 \pm 67*	(95%)
GABA	21 \pm 3	26 \pm 1.75	(123%)
Glutamate	230 \pm 40*	222 \pm 50*	(96%)
Aspartate	61 \pm 10*	240 \pm 130*	(400%)
Lactate	120 \pm 11.5	185 \pm 19	(154%)
Pyruvate	7 \pm 0.2	9 \pm 0.4	(126%)
Ascorbate	8 \pm 0.8	7.3 \pm 0.6	(95%)
Substantia nigra			
<i>Severe PA (n=4-8)</i>			
Dopamine	0.6 \pm 0.2	1.5 \pm 0.3	(237%)
DOPAC	20 \pm 7	17 \pm 7*	(87%)
HVA	65 \pm 5.2*	64 \pm 5.5*	(99%)
5-HIAA	453 \pm 105	457 \pm 96	(101%)
GABA	9 \pm 1.45*	11 \pm 2.4*	(122%)
Glutamate	991 \pm 150	940 \pm 160	(95%)
Aspartate	11 \pm 2.4*	10.5 \pm 2.2*	(91%)
Lactate	126 \pm 18	122 \pm 21	(97%)
Pyruvate	6.5 \pm 0.5	7.7 \pm 0.6	(119%)
Ascorbate	5.8 \pm 1.24	5.3 \pm 1.3	(91%)

* = $P < 0.05$ compared to the corresponding values observed in the controls.

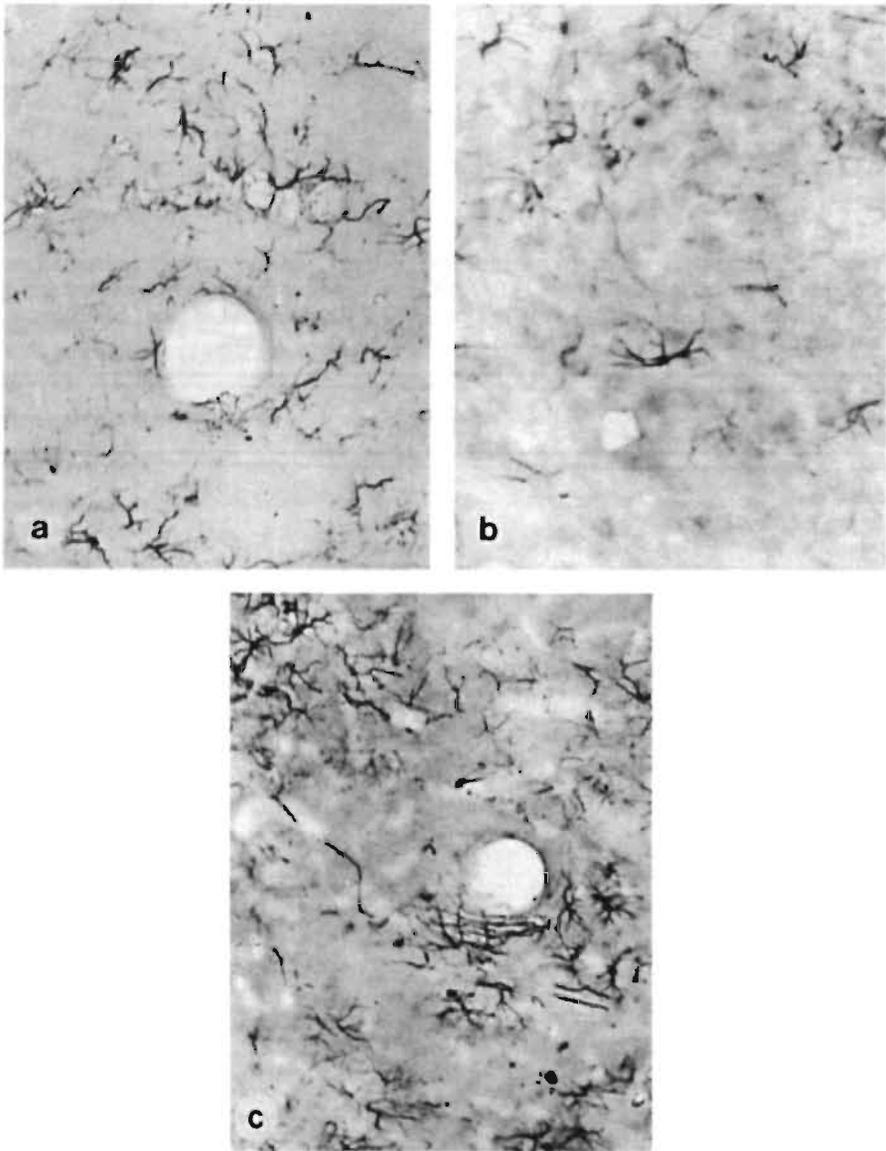


Fig 18 : Photomicrographs of striatum immunstained against GFAP 6 months after delivery (250x). **a :** control, **b :** 100 min of PA + hypothermia (15°C), **c :** severe PA (≥ 20 min/37°C).

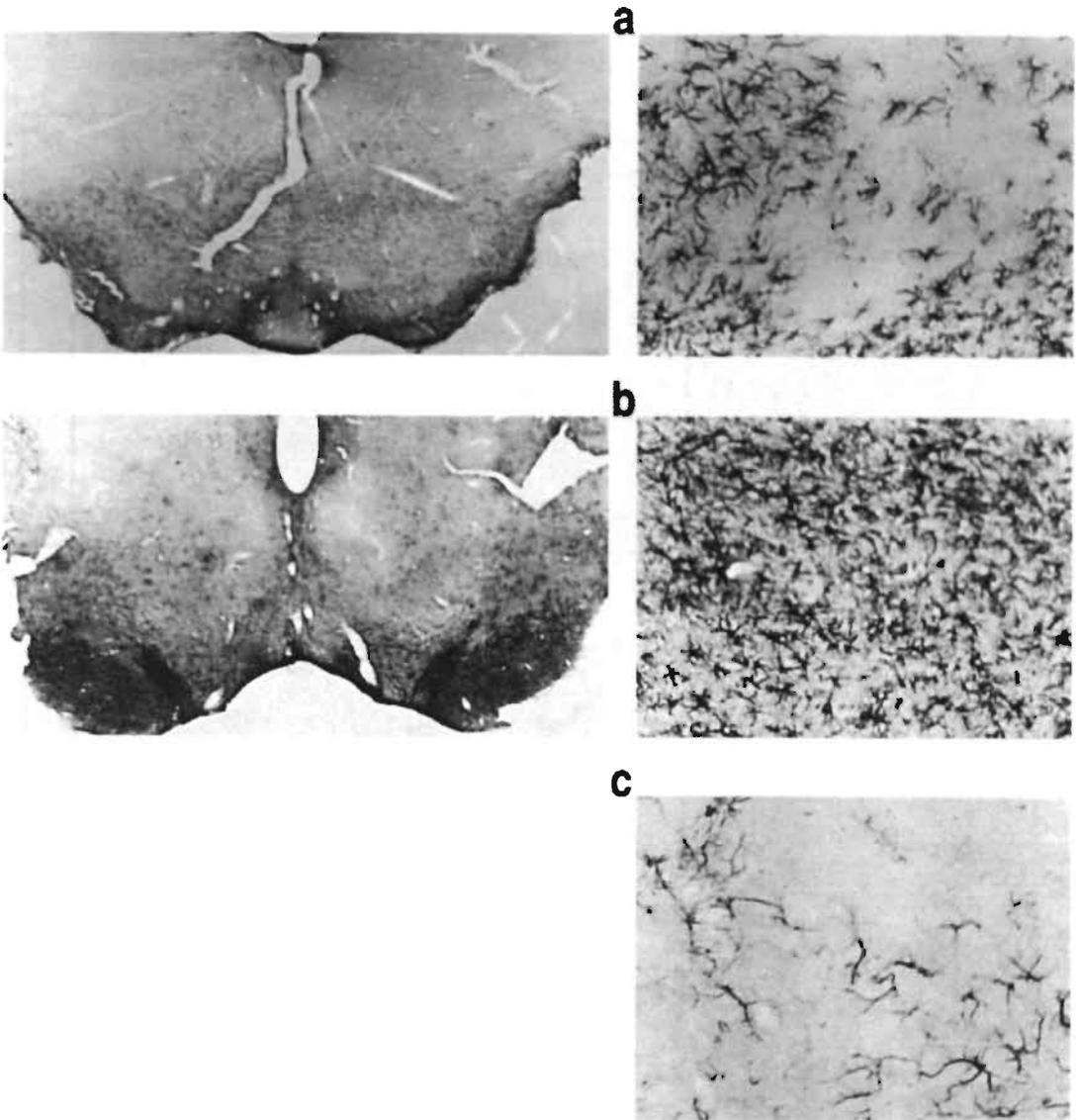


Fig 19 : Photomicrographs of substantia nigra immunstained against GFAP 6 months after delivery (250x). a : control, b : severe PA (≥ 20 min/ 37°C), and c: 100 min of PA + hypothermia (15°C) (250x).

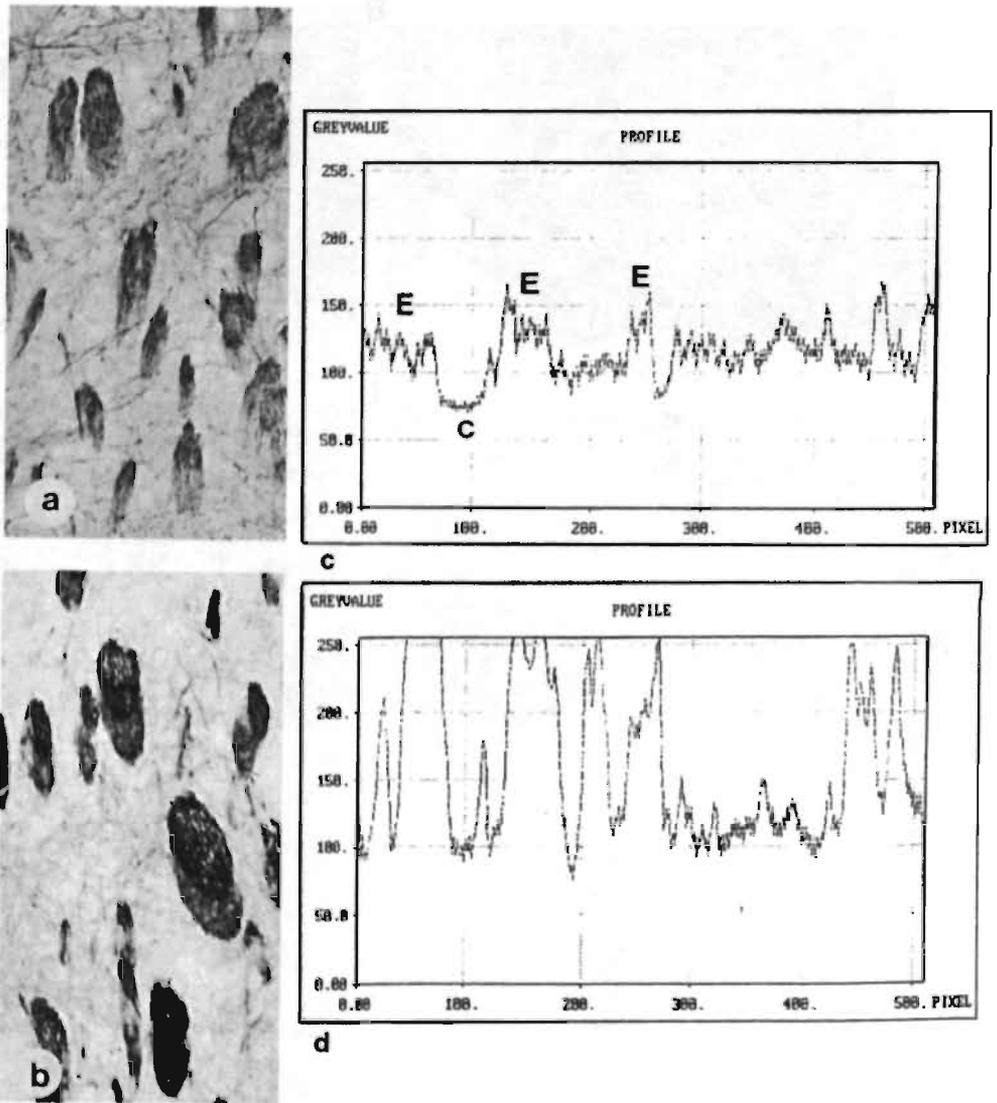


Fig 20 : Photomicrographs of 230 kD NF-immunostaining in striatum of (a) control (300x), and (b) severe PA (300x) and their correspondent greyvalue profile obtained when a computerized horizontal line crosses several striosomes (E) in (c) control striatum stained to 230 kD and (d) severe PA striatum stained to 230 kD. C=capilar. Note the increase in immunostaining showed by increase in greyvalue profile in (d).

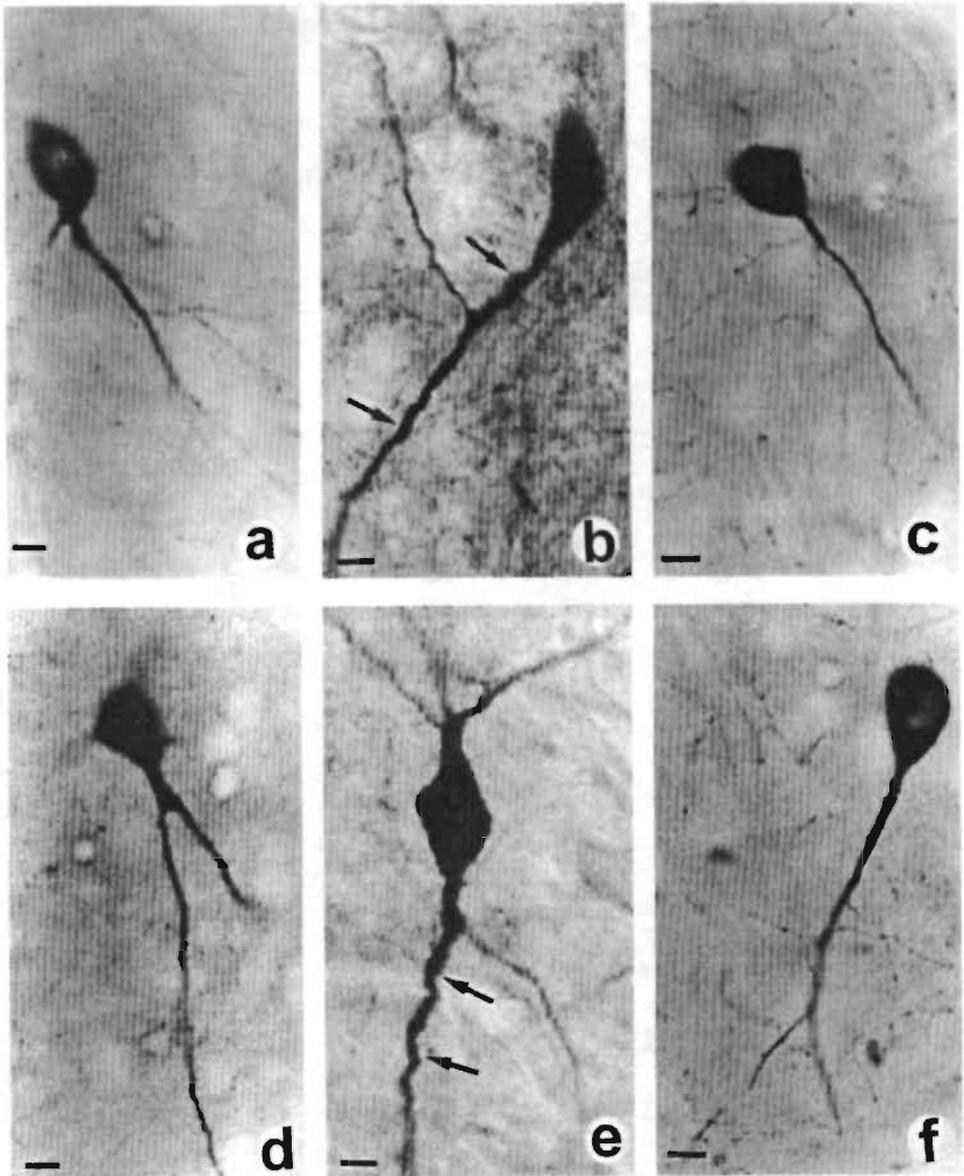


Fig 21 : Photomicrographs of striatal (a-c) and cortical (d-f) NADPH-d (+) neurons of 6 months old rats in sections from (a and d) control, (b and e) severe PA and (c and f) 100 min of PA at 15°C. Note cytomegalic soma and tortuous dendritic branches in a severe PA striatal (b) and cortical (e) neurons (arrows) compared with control and PA/15°C cells. All cells at same magnification. Scale bar: 10 μ m.

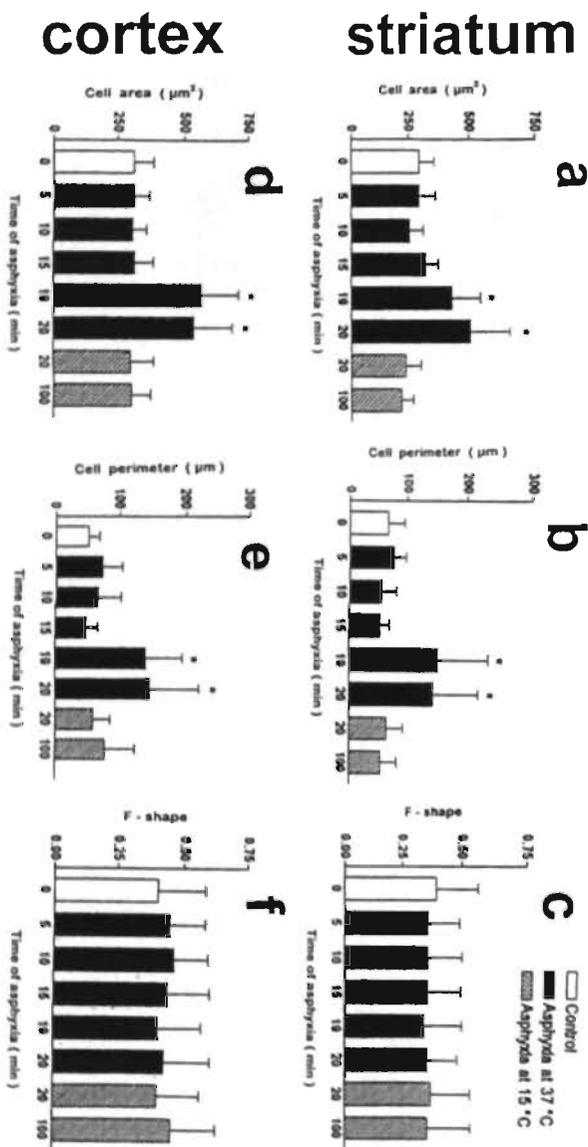


Fig 22 : Measurement of striatal NADPH-d (+) cell area (a), cell perimeter (b) and F-shape (c), in 6 months old rats subjected to different PA periods of time at 37°C or 15°C. Each value presents mean \pm SD (vertical lines) of determinations made from $n=100$ cells of each group. Asterisks indicates that differences in NADPH-d (+) cells in these two groups were highly significant ($p < 0.001$) compared with the remainder. Statistical analysis was performed by ANOVA test. There were no significant differences between subsevere and severe PA. No significant differences in F-shape (c) were found for any group inter se.

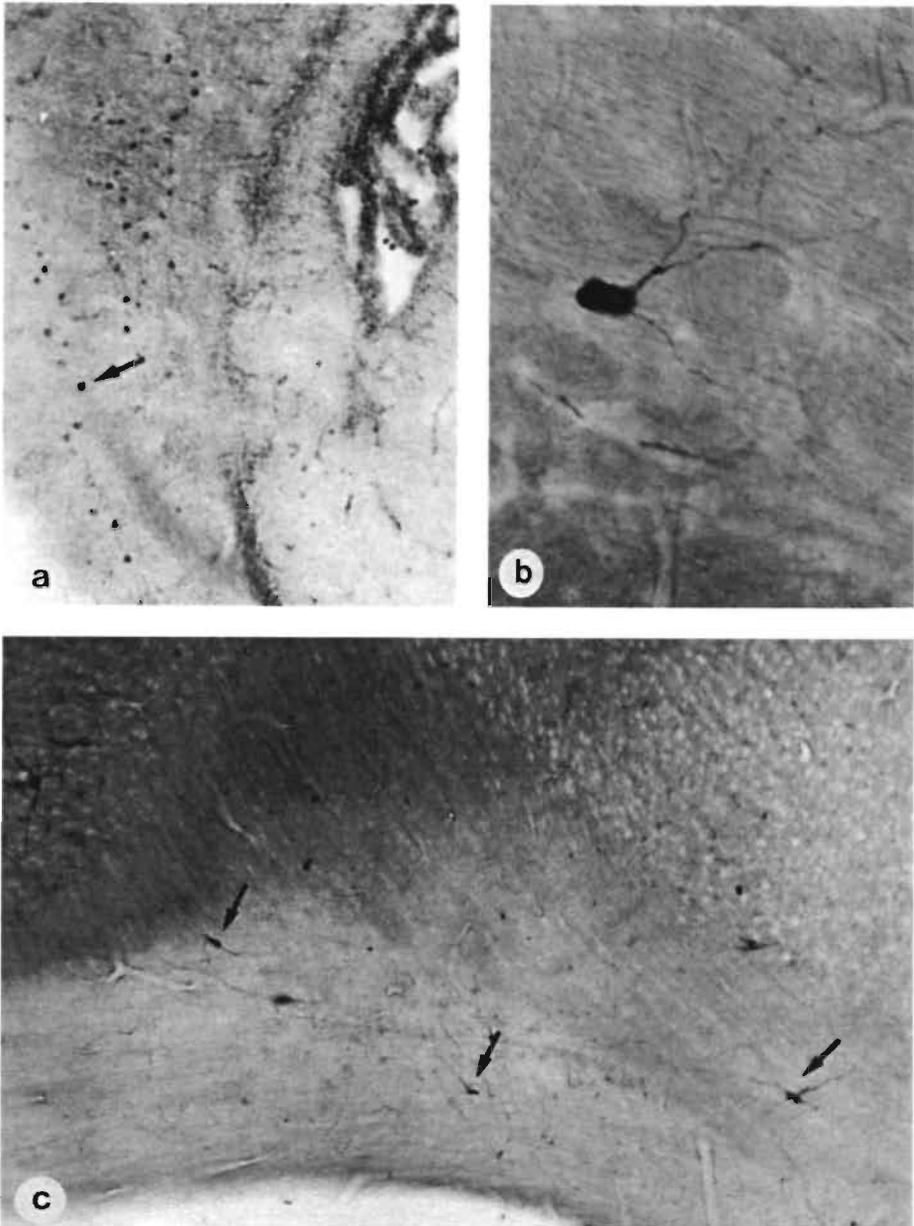


Fig 23 : Photomicrographs of the corpus callosum using NADPH-d staining in rats subjected to PA. **a :** tail of the corpus callosum in newborn rat after severe PA (100x), **b** (500x) and **c** (200x) shows the corpus callosum of 6 months old rats subjected to PA (100 min) and treated with hypothermia (15°C) during insult.

CHAPTER 5

5 GENERAL DISCUSSION

5.1 Utility of the model: The non-invasive experimental model used and presented in this thesis was developed in order to obtain global asphyxia without a need for invasive procedures such as carotid ligation. Precisely at delivery, it produces global asphyxia with the aim to mimic pathological situations occurring during human labour. This model presents the advantage of being relatively economic and easily reproducible. It allows to observe and evaluate behaviour and physical characteristics in newborn pups. Therefore, different parameters similar to those of the Apgar's test can be measured after birth. The present model also allows to study the long-term effects of PA.

In the study of severe neonatal ischemia, several models have been used. However, the majority are invasive (e.g. carried out with carotid ligation) and performed on one-week-old or older pups (Hossmann, 1991; Raju 1992). However, it must be pointed out that the use of one-week-old or older pups is preferred by many scientists (Vannucci, 1992) because there is evidence showing that, at one week of age, the maturation of the cerebral cortex of a rat best matches that of the full-term newborn human infant (Romijn, 1991). Nevertheless, whether this period is also optimal to study other brain regions is not clear.

5.2 Behavioural evaluation: As shown in Tables I-III and Figs. 5-6, the percentage of survival of pups is drastically decreased in the period >19min at 37°C, >30 min at 30°C and >100 min at 15°C. Pups surviving prolonged asphyctic periods showed several signs of physiological impairment; they were, however, accepted by surrogate mothers. An interesting observation was that animals suffering severe PA showed hypotony at the beginning, becoming hypertonic a few minutes later and presented an evident rigidity, mainly in their hind legs (Loidl, 1994). This is comparable to what is seen in human infants suffering PA with an Apgar score <3 after 10 minutes who change from hypotony to hypertony, something taken as a bad prognosis (Percy, 1986). Brann & Dykes (1977) suggest that the development of muscle tone implies a good prognosis for survival and posterior neurologic function. Hypotonic infants who rapidly recover a normal muscle tone within the first 2 hours survive without sequelae. Infants with a sustained hypotony lasting 4-5 days will either die or present sequelae; and babies born hypotonic, but who within their first 24 hours rapidly change to hypertony have both a higher chance of survival but also of presenting neurologic sequelae. When constant convulsions appear, or the EEG shows persistent abnormalities, the probability of sequelae increases. These short-term alterations in tone could indicate an early dysfunction in the basal ganglia.

5.3 Why are neonates more resistant to asphyxia? High resistance to lack of oxygen in the newborn is due to specific metabolic characteristics as well as differences in the structural organization of the cerebral and myocardial neonatal tissue.

During hypoxia, besides the cerebral defensive mechanisms, a neonate's brain consumes less oxygen than that of an adult, acidosis is milder, body temperature decreases and the still present fetal haemoglobin optimally carries oxygen. Moreover, the neonatal myocardium and cardiovascular system are more resistant to hypoxia than those in adults (Volpe, 1987; Drahota, 1991).

5.4 Hypothermia and survival protection to asphyxia: A temperature decrease from 37°C to 30°C or 15°C during asphyxia results in a significant increase in animal survival.

The protective effect of hypothermia has been used worldwide amongst primitive peoples (Sir J. Floyer, 1697; Floyer & Baynard, 1706) and its effect on survival following asphyxia was first studied by J.A. Miller et al. in the fifties and sixties. It was suggested that man may have discovered the advantages of low temperature for asphyctic neonates in the glacial period. Then, infants who seemed to be dead were taken outside the caves, left in the cold, and began breathing (see Miller, 1971). Miller et al. have demonstrated that deep hypothermia is an effective treatment in induced asphyxia in a number of species of newborn animals. Based on their experimental evidence, the use of hypothermia in the treatment for severe PA was recommended (Miller, 1964, Westin, 1959, 1963). Some clinical applications were carried out combining hypothermia with positive pressure ventilation in order to resuscitate asphyctic neonates that, according to clinical experience, were otherwise absolutely certain not to recover. Thus, if the infants died, their conscience would be clear (Dunn & Miller, 1969; Westin, 1971, Cordey, 1964). In one of these studies (Dunn & Miller, 1969), 28 infants were selected for cooling when after 5 minutes Apgar scores had neither changed nor increased. The Apgar of some of these infants was 0. It is important to remember that an Apgar score between 0 and 2 at 5 minutes results in mortality in almost 50% of the cases (Drage, 1964). Hypothermia was induced by immersing the infants - with the exception of eyes, nose, and mouth - into a watertub filled with cold water and ice cubes, maintaining temperature between 8 and 14°C. Infants were kept in water until spontaneous, unassisted respiration was established. After immersion, positive pressure ventilation was performed in order to stimulate stretch receptors of the lungs. Then, they were removed from the tub, dried and placed in the nursery without active rewarming. During this process, there was a mean increase in Apgar score of 6.4 units in 7.6 minutes, and all infants, but three, began to breathe. Three infants died: the death of two of them was explained as due to prematurity presenting respiratory distress and the third infant presented congenital cardiac abnormalities incompatible with life. No death was attributed to cold exposure. Surviving infants were

followed-up for two years, and their pediatric examination showed normal development (Dunn & Miller, 1969).

The term *hypothermia* is used rather loosely by clinicians. It refers to a central body temperature of less than 36°C. In young infants, this is best determined by inserting a low-reading thermometer into the rectum to a depth of not less than 2.5 centimeters, the reading being taken after a minimum period of two minutes (Mann, 1963). The high resistance to applying cold treatment, and the general belief that under no circumstance may an infant's temperature be allowed to fall, comes from the knowledge that extended exposure of premature infants to cold (Silverman, 1958, Buetow & Klein, 1964, Day, 1964) and accidental long-term exposure to cold (Mann & Elliot, 1957) increase mortality in term infants. Although it is accepted that long-time exposure to cold is dangerous, there is no evidence that a short-time decrease in body temperature is dangerous neither immediately after birth nor during asphyxia (Dunn & Miller, 1969).

Lately, the protective effect of hypothermia has also been widely studied in experimental transient forebrain ischemia (Globus, 1988; Coimbra & Wieloch, 1992, Ginsberg, 1992), where low intraschemic brain temperature can protect brain neurons. This effect is probably due to a reduction in the brain's energy demands and consequently, a decrease in the rate of ATP depletion (Young, 1983).

5.5 Glutamate antagonists and perinatal asphyxia: Competitive and non-competitive NMDA and AMPA antagonists have been suggested as useful neuroprotective agents in both focal and global ischemia (Sheardown, 1990; Scatton, 1991, Barks & Silverstein, 1992). Therefore, establishing a comparison between the protective effect of hypothermia with that of glutamate antagonists is interesting. A series of experiments yielded the following information: 1) if the dizocilpine dose was above 0.5 mg/kg s.c., it was lethal to pups delivered by hysterectomy, 2) if the dizocilpine dose was 0.3 mg/kg s.c., mortality was not increased - but pups were still not accepted by surrogate mothers, 3) if the dizocilpine dose was 0.2 mg/kg s.c., pups were accepted by surrogate mothers. It was also established that dizocilpine (0.2 mg/kg s.c.) could slightly increase survival (from 3% to 19%) in an induced 21-22 minute asphyctic period at 37°C (Herrera-Marschitz, 1993). However, when asphyctic periods were longer, no protective effect was observed. The observation that MK 801 induces a prolonged hypothermia in treated animals has led to controversy dizocilpine's protective effect (Bunchan, 1990, 1991).

Because Judge et al. (1991) reported a protective effect of the AMPA antagonist NBQX, this drug was also tested to increase survival rate following asphyxia. NBQX, in a 30 mg/kg s.c. dose, increased survival after asphyctic periods of 21-22 minutes (from 3% to 21%) and 22-23 (from 0 to 18%) (Table IV; Fig 13). No signs of protection were observed in pups subjected to asphyctic periods longer than 23 minutes. This data suggests a preventive effect on glutamate antagonists. However, its effect is less significant than that of hypothermia.

5.6 Subcutaneous microdialysis. Indicator of long-term damage as a prognosis tool? As shown in Figs. 7-11, in slight PA, there is a slight increase in the subcutaneous levels of Glu, Asp and Lac in comparison with control group, and Pyr is clearly higher and Asc, lower. In moderate asphyxia, s.c levels of Glu and Asp are peaking, Lac is still increasing, Pyr is decreasing towards normal levels and Asc remains under the control group's levels. In severe PA, Glu and Asp levels are decreasing, Lac is peaking, and no difference in Pyr and Asc was observed when compared with moderate PA. A subcutaneous measurement of these substances in infants who suffered PA, or those suspected to have suffered it, might be a useful tool in prognosis of possible damage. It was interesting to observe that when PA was induced at about 30°C, s.c. levels of excitatory aminoacids and metabolic products were similar to those observed at 37°C whereas when induced at about 15°C, levels were almost normal compared with those of control group, with the exception of Lac, which always increases while asphyxia is sustained.

Pyr is the main product of glycolysis which, under aerobic conditions, enters the mitochondria where it is completely oxidized to CO₂ and H₂O. Consequently, extracellular Pyr levels should increase whenever there is an increase in energy demand. If the supply of oxygen is insufficient, Pyr is converted into Lac (Hansen, 1985; Siesjö, 1978). Accumulation of Lac is an indication of a shift from aerobic to anaerobic metabolism. Therefore, an increase in Pyr levels after a short period of PA might indicate an increase in glycolysis, probably via an increase in phosphofructokinase and pyruvate kinase activity (Siesjö, 1978; Hansen, 1985; Volpe, 1987; Luz, 1992). After asphyctic periods longer than 10 minutes, glycolysis decreases, probably due to the production of H⁺ ions derived from NAD⁺ and inhibition of phosphofructokinase (Vanucci, 1991; Luz, 1992), resulting in a decrease in Pyr levels, while Lac accumulation is still sustained.

The origin of subcutaneous Glu and Asp is probably both metabolic and neuronal. A microdialysis probe was implanted into a region showing strong Glu- and Asp-IR, partly in fiber-like structures, which may represent sensory nerves (Nordlin, 1993). Both Glu and Asp levels increased significantly following PA, reaching the maximum at 10-11 minutes and 15-16 minutes asphyctic periods. This delayed increase in Glu probably reflects a shift in the aspartate-aminotransferase reaction, concomitant with the shift from aerobic to anaerobic metabolism (Hansen, 1985; Siesjö, 1978), as indicated by the Pyr to Lac conversion. However, the increase in Glu and Asp levels may also be due to inhibition of the ATP-dependent re-uptake pump for the aminoacids (Naito, 1983, 1985; Robinson, 1993). Increases in aminoacid levels have been observed in several experimental models of PA (Johnston, 1983), and are considered to be the cause of cytotoxic cascades with increased excitability, increased energy demands and intracellular calcium accumulation (Choi, 1990). However, it was found that subcutaneous Glu and Asp progressively decreased to control levels in longer asphyctic periods. This decrease in aminoacids probably reflects an inhibition of Glu synthesis in extreme anaerobic conditions (Luz, 1992).

Independently from the temperature used, the group exposed to PA had a lower level of Asc when compared with the control group. Furthermore, a decrease in ascorbate was found in the amniotic fluid of pregnant women who were smokers and in pregnancies complicated with premature rupture of membranes. This observation led to suggest that the use of Asc as an antioxidant might have a protective effect (Barret, 1991)

5.7 Do long-term dopaminergic changes represent a sign of accelerated ageing? The effect of PA on DA levels appears to depend on the length of an asphyctic period: In moderate PA an increase in DA was observed whereas in severe PA a decrease in DA was observed. The increase in basal DA levels in moderate asphyxia (19-20 min) might agree with a histochemical study (Bjelke, 1991) showing that given the same conditions, asphyxia produced an increase in number of tyrosine hydroxylase-immunoreactive (TH-IR) nerve cell bodies: a sign of proliferation of dopaminergic neurons. It has been suggested that the cause for this increase in nigral DA cell bodies is a deficit in GABAergic striato-nigral feedback, which would set the nigrostriatal DA neurons in hyperactive state (Herrera-Marschitz, 1994).

Some reports suggest that an excessive release of striatal DA might play a neurotoxic effect, and this cerebral area is particularly sensitive to ischemic injury (Smith, 1984). In agreement with this hypothesis, a lesion in the nigrostriatal dopaminergic neurons would protect from ischemic damage in the striatum (Globus, 1987), and D1 dopaminergic antagonist Sch 23390 would increase the protective effect of MK-801 (Globus, 1989). The toxic effect induced by DA might be due to oxidative products that lead to the production of hydrogen peroxide and hydroxyl radicals. Furthermore, the strong vasoconstrictor effect of DA might contribute to damage. It is interesting to add that DA release seems to be temperature-dependant, and can be decreased with hypothermia (Globus, 1988).

Rats surviving asphyctic periods longer than 20 minutes (severe PA) showed chronic deficits in the release of several putative neurotransmitters monitored with microdialysis in the basal ganglia. In comparison with the control group (vaginally or caesarean delivered rats), the main change observed in six-month-old rats after severe PA was a marked decrease in DA release monitored under basal and D-amphetamine stimulated conditions (Loidl, 1994). In agreement with the present observation of a reduced DA release in the neostriatum and a reduced GABA release in the substantia nigra, other experiments showed that following severe PA, rats presented a decrease in the rate of spontaneous locomotor activity (Andersson, 1992). Whether the chronic effect on DA release is primarily due to PA or secondary to other neurotransmitter systems is not yet known. However, in a recent study (Andersson, 1995), changes in the number of TH immunoreactive nerve cell bodies and also in the basic Fibroblast Growth Factor (bFGF) gene expression were observed in the substantia nigra of asphyctic rats, four weeks after birth.

In the case of severe PA, the decrease in DA levels might be correlated to some mechanisms of retrograde degeneration of the nigrostriatal pathway. Although it is accepted that dopaminergic transmission decreases with age, and that this decrease is enhanced in several neurodegenerative disorders such as Parkinson's and Alzheimer's diseases (Giaquinto, 1988), it is not known whether PA is related to biochemical features observed in these accelerated ageing processes. It is our hypothesis that e.g. decreases in DA levels observed in adult stages might be related to perinatal asphyctic lesions (Loidl, 1994).

5.8 Asphyxia and reactive astrocytosis. GFAP immunostaining:

For many years, the idea that gliosis was only a passive scar and therefore interfered with repairing the damaged brain was accepted. In recent years, this concept has been completely reviewed as a consequence of having established that astrocyte cells are capable of releasing certain factors that induce axonal growth and stimulates neuronal reparation. CNS responds to all types of noxae with astroglial hyperplasia and hypertrophia. Its extent can be determined by specific immunomarkers in these cells. Numerous reports have demonstrated the high specificity of glial fibrillary acidic proteins (GFAP) -which have a structural role - since they are the main intermediate filaments that form the cytoskeletal frame in these cells.

A perinatal hypoxic-ischemic lesion produces characteristic changes in the CNS, typically seen in children affected by severe PA and who, subsequently, present intellectual difficulties, epilepsy or motor alterations. One of these morphological alterations is the "marmoratus status", which is generally restricted to the basal ganglia and thalamus. This marmoratus status, so-called due to the typical marbled aspect of these cerebral structures, was attributed to a gliosis and hypermyelination (Percy, 1986).

The astroglial reaction observed with GFAP immunostaining in animals subjected to severe PA could on the one hand be correlated to the status marmoratus observed in humans and on the other explain the low levels in striatal extracellular Glu and Asp registered by *in vivo* microdialysis (Loidl, 1994) since their capacity as "buffers" to reuptake excessive excitatory aminoacids from the medium -something potentially toxic- is known (Drejer, 1985).

It is widely accepted that in ischemia there is an important decrease in protein syntheses, GFAP together with heat shock proteins (HSP) (Capani, 1996) and a neuronal c-Fos (Dell'Anna, 1995) and c-Jun increase. Although the role and significance of these proteins is still being discussed, the possibility of their playing an important role in resisting ischemia, cellular activation, differentiation and regeneration is present.

5.9 Perinatal asphyxia and hypertrophy of NF. A sign of neurodegenerative and accelerated ageing?

An increase in 230 kD neurofilaments (NF) immunoreactivity in severe asphyctic animals was clearly observed in the neostriatum, and this agrees with other authors' findings that postulate this NF as neurodegenerative markers (Nixon, 1991; Klossen, 1994). Immunostaining in NF is well evident in the "striatal patches", the place where axons - mostly originated in cortical neurons - cross this nuclei. Axons are more stained than somas because they have a higher amount of NF and because antibodies have a greater affinity with the phosphorylated epitopes, mainly present in axons (Drake, 1984). A great amount of neurodegenerative pathologies are characterized by a morphological reorganization of their cytoskeleton. Examples of this are: Alzheimer's disease, idiopathic and post-encephalic Parkinson's disease (with the Lewis's inclusion of filamentous nature), amyotrophic lateral sclerosis, Down's syndrome, Pick's disease (all with intra-neuronal inclusions) and in infantile spasms, where the presence of argyrophillic neurofibrillary-like tangles and cytoplasmic vacuoles is associated with cytomegalic neurons (Vinters, 1992). Tangles of neurofilaments, present both in Alzheimer's disease and normal ageing, reinforce the theory of a possible accelerated ageing process stimulated by severe PA. The massive and rapid accumulation of axonal aberrant NF might be associated to an increase in their production with hyperphosphorylation (Nixon, 1991) or to an increase in their degradation, performed by a kind of proteases called calpains (Roberts-Lewis, 1994).

Axonial transport associated with this type of NF could also be involved in these pathologies, but the mechanisms responsible for producing NF accumulation and the significance of these changes remain unclear (Gajdusek, 1985).

5.10 Cytomegalic NOS-containing neurons associated with PA: A sign of neuroprotection or neurotoxicity?

a. Acute changes in NOS after PA (newborn rats): Immediately after inducing asphyxia, the expression of NADPH-d changes in the lateral zone of the striatum and in some cortical neurons was observed. Striatal NADPH-d(+) neurons increased the thickness of their processes, but always kept a lateral localization in the striatum, suggesting that PA might induce some kind of stimulation in NOS expression. This phenomenon produced the appearance of NOS in places where normally, at this stage, it is not present.

The entire cell, including the soma and processes, was intensely stained. An increase in NADPH-d(+) staining of the blood vessels in striatum and cortex was also observed in severe asphyctic animals, indicating that at this early post-ischemic period, vasodilation takes place in an effort to enhance oxygen supply to the tissues.

Negative staining of NADPH-d in cortical neurons in the newborn control group agreed with a recent report showing that in rats this enzyme is present up until 19 days prior to birth, and then begins to decrease up until day 0 postnatal (Bredt, 1994). In pups with induced PA, some cortical granular type neurons expressed NADPH-d(+) staining.

It was striking to observe that under hypothermia at 15°C for 20 and 100 minutes, although PA survival increased around 5 times, it prevented changes in neurons containing NADPH-d. This lower metabolism induced by hypothermia might lead cells to save energy consumption, something that could be important for survival by inhibiting cellular neurotoxicants triggered by ischemia. Corpus callosum presented NADPH-d(+) rounded cells in all newborn group. The nature of these cells is controversial, and further experiments should be carried out so as to determine whether they are glial type cells, macrophages (Peng, 1994) or neurons in a migration process. NADPH-d+ cells with the typical neuronal morphology in corpus callosum of adult rats were observed only in the hypothermic treated group. A recent report attributed these NADPH+ cells in corpus callosum to Martinotti's neurons, present in the deeper layer of the cortex (Luth, 1994).

b. Chronic changes in NOS after PA (6 month-old rats): In agreement with authors who described NADPH-d(+) striatal and cortical cells as interneurons containing somatostatin and neuropeptide Y, these neurons in adult rats were medium-sized and non-spiny in type (Ferrante, 1985; Vincent & Hope, 1992). Given their lack of NMDA receptors (Vincent & Kimura, 1992) and their high concentration of manganese superoxide dismutase, (Inagaki, 1991) these cells were seen as resistant to excitatory amino acid toxicity and free radical toxicity respectively. However, wider implications of NO as a neurotoxic or neuroprotective modulator are still subject to controversy (Schuman & Madison, 1994). Certain neurological disorders have been reported to induce changes in NADPH-d stained neurons. Some studies on patients with Huntington's disease have disclosed an increase in the striatal NADPH-d(+) cell measurements associated with cell and fiber sparing, which can be attributed to the loss of striatal spiny neurons (Vincent & Hope, 1992). Recently, a report on human Parkinson's and Alzheimer's diseases has documented a relative NADPH-d(+) cell sparing in striatal neurons with shrunken and foreshortened dendritic processes (Mufson & Brandabur, 1994). In our findings, NADPH-d technique disclosed positive cells of the same type, homogeneously distributed in striatum and cortex of asphyctic and control animals, shown by F-shape analysis. However, striatal and cortical NADPH-d(+) neurons of rats subjected to moderate or severe PA were highly cytomegalic compared with control and hypothermia-treated animals, suggesting that the pathological mechanism related to NO involved in PA is different from that in Alzheimer's, Parkinson's, and Huntington's diseases.

In agreement with Mischel et al. (Mischel, 1995) who documented cortical neuronal cytomegaly in pediatric epilepsy (data from 77 patients who underwent surgical cortical resections for intractable seizures), our study found similar altera-

tions in rat striatal and cortical NADPH-d(+) neurons in the striatum and cerebral cortex of 6 month-old rats exposed to moderate or severe PA.

Since NO induces DA release, which can be blocked by administering the NO synthase inhibitor, L-Me-Arg (Zhu & Luo, 1992), the highest basal level of striatal DA chronically maintained following subsevere PA (Herrera-Marschitz, 1994) appears to correlate closely with the number of NOS-containing cytomegalic striatal neurons.

Amongst other functions, NO plays a relevant role in regulating blood flow by inducing relaxation of the vascular smooth muscle (Palmer, 1987) providing, perhaps, protection against ischemia by increasing oxygen supply. Since NO stimulates DA release, reportedly toxic in striatum following ischemia, its potent vasoconstrictor effect may well worsen cell damage. However, since DA release is inhibited by hypothermia (Globus, 1987, 1988), its protective mechanism might be the inhibition of an excessive release of striatal DA indirectly stimulated by NO. The functional consequences resulting from striatal and NO-containing cytomegalic neurons after subsevere or severe PA are yet to be determined.

Despite the therapeutic application of hypothermia in controlling severity of ischemic cerebral damage described in rats (Busto, 1987) and gerbils (Busto, 1989), only a few studies on PA were performed in the fifties and sixties, when cold therapy plus positive pressure ventilation was introduced in order to resuscitate severely asphyctic human neonates previously considered incapable of recovery (Westin, 1971). Mechanisms underlying the protective effect of hypothermia are not clear. A decrease in metabolic demands occurs when body temperature is lowered. Subsequent toxic effects are weakened and survival to prolonged exposure to asphyxia without permanent brain lesions is ensured. A prolonged duration of hypothermia might also help in the recovery of normal cellular Ca^{++} homeostasis (Colbourne, 1994).

Neuronal heterotopia, islands of disorganized neurons within the subcortical white matter, was found in some cases of pediatric epilepsy (Mischel, 1995) and it has been suggested that injury to radial fibers (e.g. in a vascular injury) might stop the migration of neuroblasts, later leading to a single heterotopic white matter neurons (Sarnat, 1992). Interstitial neurons in white matter are the oldest cells of the cortex/white matter and most are derived from subplate neurons; as an hypothesized result of apoptosis these neurons decrease and disappear during development and maturation. Therefore, heterotopic neurons in white matter may be due to a failure of programmed cell death (Chun & Schatz, 1989). In our study, heterotopic neurons in corpus callosum were only observed in 6 month old rats exposed to hypothermia. Either a halt to the neuroblasts migration process or a failure of the apoptotic mechanism - or both - might occur with hypothermia.

Further experimental studies on cold therapy for PA treatment are necessary in order to clarify the functional implication of this observation.

CHAPTER 6

6. CONCLUSIONS

- 1) A non-invasive experimental model that allows to study short- and long-term consequences of perinatal asphyxia is presented.

- 2) Survival rate after PA depends on both duration of asphyxia and temperature at which it is performed. *At 37°C body temperature, an asphyctic period longer than 22 minutes is associated with 100% mortality, 100% survival is only observed up to a 16 minute period of PA. At 30°, a 100% survival is observed up to a 30 minute period of PA. At 15°C, 100% survival is still observed up to a 100 minute period of PA.*

- 3) PA mortality at 37°C can be decreased by the shifting of temperature from 37°C to 15°C during the period when asphyxia is induced. *Prevention of mortality is obtained by the shifting of temperature from 37°C to 15°C during the period up to 15 minutes after starting induction of PA. After this period of time, a shift in temperature does not prevent mortality.*

- 4) A slight reduction in mortality rate is obtained when treating mothers with glutamate antagonists one hour before delivery. *NMDA and AMPA antagonists (+JMK 801 and NBQX) produced only a slight increase in survival at maximal doses; their effect was improved when both were simultaneously administered.*

- 5) Several metabolic products and excitatory aminoacids were monitored using *in vivo* microdialysis in subcutaneous tissue for 40-80 minutes after delivery. *The most important changes in PA at 37°C were seen in Glu and Asp levels with a maximal increase following a 10-11 minute asphyctic period. PA at 15°C proved to prevent changes in Glu and Asp.*

- 6) Rats surviving asphyctic periods longer than 19 minutes at 37°C showed chronic alterations in the release of neurotransmitters monitored with *in vivo* microdialysis in the basal ganglia. *The main change observed after 6 months in rats subjected to subsevere PA (19-20 min) was an increase in DA levels whereas those subjected to severe PA (> 20 min) presented a decrease in DA levels compared with controls. However, both groups presented a decrease in DA release after stimulation with D-amphetamine.*

7) Present findings show that NADPH-d reactivity is enhanced in striatal and cortical neurons containing-NOS secondary to subsevere or severe PA.

An increase in neuronal NADPH-d staining in pups, and chronic changes in adult animals revealed by cytomegalic neurons containing-NOS was observed after subsevere and severe PA. It was also demonstrated that these changes may be prevented with hypothermic treatment.

8) Immunostaining to glial fibrillary acidic protein (GFAP) revealed that subsevere and severe PA induces chronic changes in the expression of these intermediate filaments in striatum, substantia nigra and cortex. *PA induced under hypothermic condition (15°C) prevents reactive astrocytosis.*

9) Immunostaining to 230 kD neurofilaments (NF) revealed that subsevere and severe PA induces chronic changes in the expression of these intermediate filaments in the striatum. *Changes observed in this type of NF indicate that PA induces neuronal filamentous accumulation compatible with several diseases.*

10) Therefore, this experimental model appears to be useful to study short- and long-term consequences of hypoxic-ischemic lesions in rats, induced under conditions similar to those found in labour in clinical situations. *Further studies on cold therapy for the treatment of severe PA should be re-examined and carried out without delay.*

11) Asphyxia during birth might be a factor contributing to the development of neurodegenerative diseases such as accelerated ageing processes, Parkinson's and Alzheimer's diseases.

12) An irreversible cerebral lesion, resulting from PA, is a tragedy for a child and its family. Also, it increases medical, social and economic demands on society. Therefore, one of the objectives of obstetric and neonatal care that also is a challenge to science is to decrease the number of children damaged by PA.

CHAPTER 7

7.1. SUMMARY

Acute perinatal asphyxia (PA) is still the major cause of death and neurological injury in newborn infants at term. A non-invasive experimental model that allows to study short- and long-term consequences of PA is described.

The percentage of survival after PA depends on: a) duration of PA and b) temperature at which asphyxia is induced. At a body temperature of 37°C, PA lasting over 22 minutes results in 100% mortality whereas if PA ends before 16 minutes, there is 100% survival. At 30°C, there is 100% survival if the duration of PA is up to a period of 30 minutes. At 15°C, PA lasting up to 100 minutes still results in 100% survival. At 37°C, PA mortality can be decreased by shifting temperature from 37°C to 15°C during the asphyctic period, if this shifting is performed within the first 15 minutes of the beginning of asphyxia. Later than this 15 minute period, shifting temperature no longer prevents mortality.

A slight decrease in mortality rate can be achieved by administering glutamate antagonists to the mother, one hour before delivery. NMDA and AMPA antagonists (+)MK 801 and NBQX produced only a slight increase in survival at maximal doses; their effect was improved when both were simultaneously administered.

Several metabolic products and excitatory aminoacids were monitored using in vivo microdialysis in subcutaneous tissue for 40-80 minutes following delivery. The most important changes produced by PA at 37°C were seen in Glu and Asp levels, which presented a maximal increase after a 10-11 minute asphyctic period. At 15°C, PA produced no change in Glu and Asp.

Adult rats surviving asphyctic periods longer than 19 minutes at 37°C showed chronic alterations in the release of neurotransmitters, this was monitored with in vivo microdialysis in the basal ganglia. After 6 months, the main change observed in rats subjected to subsevere PA (19-20 minutes) was an increase in DA levels; those subjected to severe PA (≥ 20 min) presented a decrease in DA levels compared with controls. However, both groups presented a decreased DA release following stimulation with D-amphetamine.

Morphological studies demonstrated that NADPH-d reactivity is enhanced in striatal and cortical neurons containing-NO secondary to subsevere or severe PA. After subsevere and severe PA, an increase in neuronal NADPH-d staining in pups, and chronic changes in the adult animals, revealed by cytomegalic neurons containing-NO, was observed. Another observation was that these changes may be prevented by cold treatment. Immunostaining to glial fibrillary acidic protein (GFAP) revealed that subsevere and severe PA induce chronic changes in the expression of these intermediate filaments in striatum, substantia nigra and cortex. PA induced under hypothermic conditions (15°C) prevents reactive astrogliosis. Immunostaining to 230 Kd neurofilaments (NF) revealed that subsevere and severe PA induce chronic changes in the expression of these intermediate filaments

in the striatum. Changes observed in this type of NF indicate that PA induces neuronal filamentous accumulation compatible with several diseases.

Therefore, this experimental model appears to be useful to study short- and long-term consequences of hypoxic-ischemic lesions in rats, induced under conditions similar to those found in labour in clinical situations.

Further studies on cold therapy for the treatment of severe PA should be re-examined and carried out without delay. Asphyxia during birth may be a factor contributing to the development of neurodegenerative diseases, such as accelerated ageing processes, Parkinson's and Alzheimer's diseases. An irreversible cerebral lesion, resulting from PA, is a tragedy for a child and its family. Also, it increases medical, social and economic demands on society. Therefore, one of the objectives of obstetric and neonatal care - that also is a challenge to science - is to decrease the number of children damaged by PA.

7.2. SAMENVATTING

Acute perinatale asfyxie (PA) is nog steeds de belangrijkste oorzaak van sterfte en neurologische beschadiging bij a terme pasgeborenen. Een non-invasief experimenteel model dat het mogelijk maakt de vroege en late gevolgen van perinatale asfyxie te bestuderen, wordt beschreven.

Het overlevingspercentage na PA hangt af van de duur, zowel als van de temperatuur waarbij de asfyxie werd geïnduceerd. Bij een lichaamstemperatuur van 37°C, gaat een asfyctische periode van langer dan 22 minuten gepaard met 100% mortaliteit, terwijl 100% overleving optreedt bij een asfyctische periode korter dan 16 minuten. Bij een lichaamstemperatuur van 30°C echter, wordt 100% overleving gezien tot 30 minuten PA, terwijl bij een temperatuur van 15°C, 100% overleving wordt gezien bij 100 minuten PA. De mortaliteit ten gevolge van PA kan gereduceerd worden door de temperatuur te veranderen van 37°C naar 15°C gedurende de periode dat asfyxie wordt geïnduceerd, maar alleen als dat gebeurt binnen 15 minuten na de start van de asfyxie. Na deze tijd, voorkomt verandering in temperatuur geen mortaliteit.

Een kleine reductie van mortaliteit wordt ook verkregen door 1 uur voor de bevalling glutamine antagonisten aan de moeder toe te dienen. De NMDA en AMPA antagonisten (+)MK 801 en NBQX gaven maar een kleine stijging te zien in overleving bij een maximale dosis maar het effect werd verhoogd als beide tegelijkertijd werden toegediend.

Verschillende metabole producten en excitatoire aminozuren werden gemonitord middels microdialyse in het onderhuidse weefsel gedurende 40-80 minuten na de bevalling. De meest opvallende veranderingen tijdens PA bij 37°C traden op in glutamaat (Glu) en aspartaat (Asp) spiegels met een maximale toename na een 10-11 minuten durende asfyctische periode. PA bij 15°C bleek veranderingen in Glu and Asp spiegels te voorkomen.

Volwassen ratten die periodes van asfyxie langer dan 19 minuten bij 37°C overleven, vertonen chronische verhoging van het vrijkomen van neurotransmitters gemonitord met in vivo microdialyse in de basale ganglia. De belangrijkste verandering die gezien wordt in ratten 6 maanden na matig-ernstige PA (19-20 minuten) was een toename van Dopamine (DA) spiegels, terwijl de ratten die blootgesteld zijn aan extreme perinatale asfyxie (≥ 20 min) een daling in DA-spiegels laten zien, in vergelijking met de controles. Echter na stimulatie met D-amp hetamine is in beide groepen een daling in DA uitscheiding te zien.

Morphologische studies toonden aan dat NADPH-d reactiviteit verhoogd is in de NO bevattende striatale en corticale neuronon, optredend na matige of ernstige PA. Een toename in neuronale NADPH-d kleuring bij pups, zowel als chronische veranderingen in volwassen dieren, aangetoond door corticale neuronon die NOS bevatten, kon gezien worden na matige en ernstige PA. Tevens werd gezien dat deze veranderingen voorkomen kunnen worden door "koude behandeling".

Immunokleuring op gliale fibrillaire eiwitten (GFAP) toonde aan dat matige en ernstige PA chronische veranderingen in de expressie van deze vezels in het striatum, de substantia nigra en de cortex teweegbrengt. PA bij onderkoeling (15°C) voorkomt reactieve astrocytose. Immunocytochemische aankleuring van 230 kD neurofilamenten (NF) toonde aan dat matige en ernstige PA chronische veranderingen in de hoeveelheid van deze intermediaire filamenten in het striatum teweegbracht. Veranderingen die gezien werden in dit type neurofilamenten geven aan dat PA een toename in het aantal neuronale vezels induceert, compatibel met verschillende ziektes.

Daarom schijnt dit experimentele model waardevol te zijn om bij ratten de korte en langdurige gevolgen van hypoxie-ischemie, toegebracht onder identieke omstandigheden zoals die plaatsvinden tijdens de bevalling, te bestuderen.

Verdere studies naar "koude therapie" voor de behandeling van ernstige PA zouden op korte termijn opnieuw geëvalueerd en uitgevoerd moeten worden. Asfyxie gedurende de geboorte kan een factor zijn die bijdraagt aan de ontwikkeling van neurodegeneratieve ziekten zoals versnelde ouderdomsprocessen, en de ziekte van Parkinson en Alzheimer.

Een onherstelbaar hersenletsel als gevolg van PA is een tragedie voor het kind en zijn familie, en verhoogt de benodigde medische, sociale en economische middelen. Bovendien is het verkleinen van het aantal op deze manier beschadigde kinderen één van de doelstellingen van de obstetrische en neonatale zorg, en een uitdaging voor de wetenschap.

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CURRICULUM VITAE

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Abstracts:

Short-and-long term effects of perinatal asphyxia studied with microdialysis in rat: Protective effects of hypothermia and glutamate antagonism. Loidl CF, Herrera-Marschitz M, Andersson K, Gojny M, O'Connor WT and Ungerstedt U. *16th Annual Meeting of the European Neuroscience Association. Madrid. Spain. September 1993.*

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Influence of hypothermia in the distribution of nitric oxide synthase in the striatum of adult rats subjected to perinatal asphyxia. Capani F, Loidl CF, López EM, Goldstein J, López Costa JJ and Pecci Saavedra J. *XV Congreso de la Sociedad Brasileña de Microscopía Electrónica. Caxambú Brasil, Septiembre 1995*

Free radicals are released in the striatum after reoxygenation in a model of mild and severe perinatal asphyxia. Loidl C.F., Capani F, Aguilre F, Piehl L, Faccorro G, De Paoli T, Hager A. and Pecci Saavedra J. *Soc. for Neurosci. Abstr. Washington, USA 1996*

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**Short- and long-term effects of perinatal asphyxia in rats
monitored with peripheral and intracerebral microdialysis**

We are developing a novel non-invasive animal model for studying hypoxic-ischemic lesions, similar to those produced under labor in clinical situations. Perinatal asphyxia is induced by maintaining pups-containing uterus horns removed by hysterectomy in a water bath (at 37°C, 30°C or 15°C) for various periods of time (0–101 min). Control and asphyctic pups are obtained from the same mother. Following various asphyctic periods, the uterus horns are rapidly opened and the pups are removed and stimulated to breathe by cleaning of the delivery fluid and by tactile stimulation of the oral region. The time of asphyxia is measured from the time when the blood circulation to the uterus is cut off until the pups start to breathe. The umbilical cord is ligated and the animals are allowed to recover on a heating pad. Several parameters are acutely or chronically recorded by direct observation or by *in vivo* microdialysis. Following asphyxia, pups are implanted subcutaneously with microdialysis probes in the dorsal region, before they are presented to surrogate mothers. Thus, levels of aminoacids (glutamate, Glu; aspartate, Asp), and metabolic products (lactate, Lact; pyruvate, Pyr) are monitored after delivery in both asphyctic and controls pups.

It was found that, when the pups were kept in a water bath at 37°C, neonatal asphyxia led to 100% mortality within the first 20 min period following delivery, whenever the asphyctic period was longer than 22 min. However, when the pups were kept in a water bath at 30°C, 100% of the pups recovered respiratory function following tactile stimulation and were accepted by the surrogate mothers, even when the pups were exposed to an asphyctic period as long as 41 min. All pups died when the asphyctic period was extended to 50–51 min. At 15°C, 100% survival was observed following a 101 min asphyctic period.

Subcutaneous Glu, Asp and Lact levels were increased in all asphyctic groups, while no significant increases could be seen in Pyr levels. Glu was increased >4 fold in all groups, with a maximum increase seen after a 5–6 min at 37°C (> 10 fold).

We have completed series of experiments in which the pups were exposed to asphyxia and then, approximately one or six month later, the same rats were implanted with two microdialysis probes, one into the striatum and another into the substantia nigra. Monoamines and amino acids were monitored under basal and D-amphetamine-stimulated conditions. Significant changes in monoamines and GABA were observed in rats exposed to mild and severe asphyxia, while Glu and Asp levels were unchanged. Studies combining microdialysis and immunohistochemistry are

**Prevention of mortality induced by perinatal asphyxia:
Hypothermia or glutamate antagonism?**

Short Communication

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Summary. Perinatal asphyxia was induced by keeping pups-containing uterus horns, removed by hysterectomy, in a 37°C or a 30°C water bath. Asphyxia for a period of 21–22 min at 37°C led to a 97% mortality within the first 20 min period following delivery. When the asphyctic period was extended to more than 22 min all the pups died following delivery. When the asphyxia was induced at 30°C, 100% of the delivered pups survived and were accepted by surrogate mothers. The protective effect of hypothermia could be observed even when the pups-containing uterus horns were exposed to a 45–46 min asphyctic period. Pretreatment with dizocilpine (0.2 mg/kg s.c.), or 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(f)quinoxaline (NBQX) (3–30 mg/kg s.c.), administered to the mothers one hour before hysterectomy, reduced slightly the mortality induced by a 21–22 min asphyctic period at 37°C. An increase in survival following a 22–23 min asphyctic period could only be observed after the highest dose of NBQX.

Keywords: Amino acids – Asphyxia – Hypothermia – Glutamate receptors – Rat

Introduction

Acute perinatal asphyxia is a major cause of death and neurological injury in newborn babies. The incidence of asphyxia has been estimated as 2–4 per 1000 live term births, and has not decreased despite advances in perinatal and obstetric care (Hill, 1991; Younkin, 1992). Many babies die during the newborn period, and 20–30% of the survivors present long-term neurological deficits (Younkin, 1992). There is evidence that over-activation of excitatory amino acid receptors plays a role in the pathogenesis of perinatal hypoxic-ischemic brain injury, and therefore, it has been suggested that competitive and non-competitive

N-Methyl-D-Aspartate (NMDA), as well as α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) antagonists are useful as neuroprotective agents (Sheardown et al., 1990; Scatton et al., 1991; Barks and Silverstein, 1992).

We have developed a non-invasive animal model for studying the short- and long-term consequences of perinatal asphyctic lesions in rats, in conditions similar to those produced under labor in clinical situations (Bjelke et al., 1991). In this study we have analysed the short-term effects produced by asphyxia at 37°C, and the survival rate following hypothermia or glutamate antagonism.

Material and methods

Asphyxia was induced in pups-containing uterus horns obtained by cesarean section on pregnant Sprague-Dawley rats. Female rats at the final day of gestation (b.w. 400–500g) were anaesthetised with halothane and hysterectomised. The entire uterus was taken out, the uterus horns containing the foetuses were detached and placed in a 37°C or a 30°C water bath for various periods of time (0–51 min) (control and asphyctic pups could be obtained from the same mother, since each mother produced approximately 15 pups). Following hysterectomy alone or asphyxia, the uterus horns were rapidly opened, the pups were removed and stimulated to breathe on a heating pad by tactile stimulation of the oral region with pieces of medical wipes. The umbilical cord was ligated and after a 60 min period the pups were given to the surrogate mothers. The time of asphyxia was measured from the time when the blood circulation to the uterus was cut off until the pups started to breathe.

In a series of experiments the non-competitive NMDA antagonist, dizocilpine ((+)-MK-801 hydrogen maleate; RBI, Natick, MA, USA) (dissolved in saline) (0.2 mg/kg s.c.) or the AMPA antagonist NBQX (Novo Nordisk A/S, Måløv, Denmark, generously supplied by Dr L. Nordholm) (dissolved with a drop of NaOH in 5% glucose) (3–30 mg/kg s.c.) was administered to the mothers, in a single dose, 1 h before delivery.

Several parameters were acutely recorded by direct observation at a 40–60 min period following delivery: (1) survival, (2) gasping, and (3) colour of the skin. The reception by surrogate mothers and the survival for at least one month period were also recorded.

Comparisons on the rate of survival was tested by the non-parametric Cochran's test (Q). A level of $P < 0.05$ was considered critical for statistical significance.

Results

Pups delivered from uterus horns removed by hysterectomy from rats at the final day of gestation, started regular breathing (respiratory frequency ≈ 60 per min) almost immediately after delivery was completed. These control pups showed a pink coloured skin and intensive vocalization and motility. They were accepted by surrogate mothers after a 60 min observation period. When the pups were accepted, they grew up in a manner similar to that shown by normally delivered rats (all the surviving rats reported in this studied were followed up for at least one month period).

Following a 15–16 min asphyctic period, induced in a water bath at 37°C (Table 1A) or 30°C (Table 1B), 100% of the pups started breathing short after delivery, they survived and were accepted by the surrogate mothers. Following a 19–20 min asphyctic period at 37°C (Table 1A), the pups had to be intensively stimulated to start to breathe. The surviving pups remained akinetic for a long period after delivery, showed a significant decrease in respiratory frequency (≈ 20 per min), which was accompanied of gasping, and showed a pink/pale skin. Approximately 30% of the pups died shortly after delivery. In contrast all the

Table 1A,B. Short-term effects of neonatal asphyxia performed under 37°C (A) and under 30°C (B)

Treatment Time of asphyxia	S	Parameters G	CS
Controls (M = 30; N = 30)	100%	No	Pink
A.			
Asphyxia at 37°C			
15–16 min (M = 10; N = 96)	100%	No	Pink
19–20 min (M = 10; N = 100)	78 ± 9% *	83%	Pink/Pale
20–21 min (M = 10; N = 21)	40 ± 16% *	88%	Pale
21–22 min (M = 12; N = 100)	3 ± 2% *	100%	Pale
22–23 min (M = 10; N = 89)	0%	0	0
30–31 min (M = 6; N = 15)	0%	0	0
40–41 min (M = 4; N = 4)	0%	0	0
45–46 min (M = 5; N = 8)	0%	0	0
50–51 min (M = 4; N = 4)	0%	0	0
B.			
Asphyxia at 30°C			
15–16 min (M = 3; N = 6)	100%	No	Pink
19–20 min (M = 10; N = 50)	100%	10%	Pink
20–21 min (M = 10; N = 40)	100%	10%	Pink/Pale
21–22 min (M = 10; N = 40)	100%	14%	Pink/Pale
22–23 min (M = 5; N = 25)	100%	20%	Pale
30–31 min (M = 6; N = 15)	100%	27%	Pale
40–41 min (M = 6; N = 15)	83 ± 12%	44%	Pale
45–46 min (M = 5; N = 15)	46 ± 5% *	100%	Pale
50–51 min (M = 4; N = 15)	0%	0	0

S Survival (Means ± S.E.M.); G gasping, and CS colour of the skin. M number of mothers; N number of pups.

* P < 0.05, compared with controls

pups survived following a 19–20 min asphyctic period at 30°C (Table 1B). The pups started rapidly to breathe in a regular manner, although some initial gasping could be observed. The colour of the skin was similar to that in control pups.

The rate of survival rapidly decreased following prolonged asphyctic periods at 37°C (Table 1A), and as a whole, their physiological condition deteriorated. No pup survived following asphyctic periods longer than 22 min. In contrast, at 30°C (Table 1B), all the pups survived up to a 30–31 min asphyctic period, although some signs of physiological impairment (presence of gasping and pale skin) could be observed. Survival could still be observed following a 45–46 min asphyctic period. No survival was observed following asphyctic periods longer than 46 min.

Table 2. Effect of dizocilpine (0.2 mg/kg s.c.) and NBQX (10–30 mg/kg s.c.) (administered to the mother one hour before hysterectomy) on the short-term effects of neonatal asphyxia at 37°C. Controls are taken from the respective mother, but without undergoing asphyxia (see legend in Table 1).

* $P < 0.05$, compared with the saline group

Treatment Time of asphyxia	Parameters		
	S	G	CS
SALINE			
Control (M = 5; N = 5)	100%	No	Pink
21–22 min (M = 12; N = 78)	3 ± 2%	100%	Pale
22–23 min (M = 5; N = 10)	0	0	0
DIZOCILPINE			
Control (M = 9; N = 9)	100%	No	Pink
21–22 min (M = 9; N = 42)	19 ± 8%	86%	Pale
22–23 min (M = 9; N = 30)	0%	0	0
NBQX 10 mg/kg			
Control (M = 5; N = 5)	100%	No	Pink
21–22 min (M = 5; N = 31)	16 ± 12%	100%	Pale
22–23 min (M = 5; N = 28)	0%	0	0
NBQX 30 mg/kg			
Control (M = 6; N = 9)	100%	No	Pink
21–22 min (M = 6; N = 20)	21 ± 4%	* 50%	Pale
22–23 min (M = 6; N = 23)	18 ± 6%	* 25%	Pale

Table 2 shows the effect of pretreatment with saline, dizocilpine (0.2 mg/kg s.c.) or NBQX (10–30 mg/kg s.c.) (no effects were observed after NBQX 3 mg/kg s.c.), administered to the mothers, one hour before hysterectomy alone (controls) or hysterectomy followed by 21–22 or 22–23 min asphyctic periods at 37°C. Survival after a 21–22 min asphyctic period at 37°C was slightly increased by pretreatment with dizocilpine or NBQX. An increase in survival following a 22–23 min asphyctic period, at 37°C, could only be observed after the highest dose of NBQX (30 mg/kg s.c.).

Discussion

A novel animal model for studying the consequences of perinatal asphyxia in rats is presented. The model is largely non-invasive and it mimicks conditions similar to those produced under labor in clinical situations (Bjelke et al., 1991). In this report we show the short consequences of various asphyctic periods, focusing on survival, which remains the major goal in any medical intervention.

We found that perinatal asphyxia, induced by immersing pups-containing uterus horns into a water bath at 37°C for a period longer than 22 min, led to a 100% mortality within the first 20 min period following delivery. When the uterus horns were kept in a 30°C water bath, 100% of the delivered pups started respiratory functions following tactile stimulation and were accepted by the surrogate mothers, even when the pups were exposed to a 30–31 min asphyctic period. All pups, however, died when the asphyctic period was extended to 50–51 min. The pups surviving prolonged asphyctic periods showed several signs of physiological impairment (e.g. decrease in respiratory frequency, motility and vocalization, and changes in the colour of the skin), however, they were accepted by surrogate mothers and survived for at least one month.

The striking protective effect of hypothermia shown in the present study is in agreement with the report by Ginsberg et al. (1992) demonstrating that low intraschemic brain temperature can protect brain neurons in rats subjected to transient forebrain ischemia, an effect probably due to reduction in brain energy demands and to a consequent decrease in the rate of ATP depletion (Young et al., 1983). The protective effect of hypothermia has been utilized world wide among primitive peoples and its effect on survival following asphyxia was pionerly studied by J.A. Miller and collaborators in the fifties (see Miller, 1971).

It has been suggested that competitive and non-competitive NMDA, as well as AMPA antagonists are useful as neuroprotective agents in focal and global ischemia (Sheardown et al., 1990; Scatton et al., 1991; Barks and Silverstein, 1992). Thus, it was interesting to compare the protective effect of hypothermia with that of glutamate antagonists. In a series of experiments (Loidl et al. in preparation) it was found that dizocilpine given in doses above 0.5 mg/kg s.c., was lethal to the pups delivered by hysterectomy. A 100% survival was observed after 0.3 mg/kg s.c. of dizocilpine, however, they were not accepted by the surrogate mothers. However, following dizocilpine 0.2 mg/kg s.c. the pups were also accepted by the surrogate mothers. In the present study it was found that dizocilpine (0.2 mg/kg s.c.) could slightly increase survival induced by a 21–22 min asphyctic period at 37°C (from 3% to 19%). However, no protective effect

could be observed against longer asphyctic periods. In view of a report by Judge et al. (1991), demonstrating a protective effect of the AMPA antagonist NBQX, we also tested this drug in order to increase survival following asphyxia. NBQX, at the dose of 30 mg/kg s.c., increased survival following 21–22 min (from 3% to 21%) or 22–23 (from 0 to 18%) asphyctic periods (Table 2). No signs of protection were observed on pups suffering from longer than 23 min asphyctic periods (data not shown).

Thus, the present results suggest a preventive effect by the glutamate antagonists. The effect is, however, minor as compared with the prominent effect produced by hypothermia. Higher doses of dizocilpine are clearly toxic to the pups, and therefore cannot be tested. Higher doses of NBQX, as well as more specific and selective glutamate antagonists should be studied. However, whatever the results might be, the effects of the drugs should be compared with those produced by hypothermia.

In conclusion, a new non-invasive model for studying perinatal asphyxia is presented, which allows to study the short- and long-term consequences of hypoxic-ischemic lesions in rats, induced under conditions similar to those found under labor in clinical situations. Hypothermia appears to be superior to glutamate antagonism as a therapeutical intervention for increasing survival following perinatal asphyxia. Survival remains the most important goal of medicine, and therefore it should be considered when different therapeutical approaches are studied and should be compared with the effects of hypothermia.

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Long-term effects of perinatal asphyxia on basal ganglia neurotransmitter systems studied with microdialysis in rat

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Abstract

Asphyxia was induced in pups delivered by caesarean section on pregnant Sprague–Dawley rats. Rats within the last day of gestation were anaesthetised and hysterectomized. The uterus horns including the fetuses were placed in a water bath for various periods of time. Following asphyxia the uterus horns were opened. The pups were removed, stimulated to breathe, left to recover and given to surrogate mothers. Control and asphyctic pups were obtained from each mother. Rats surviving asphyctic periods longer than 20 min at 37°C showed chronic deficits in the release of neurotransmitters monitored with microdialysis in the basal ganglia. The main change observed in 6-month-old male rats that underwent severe perinatal asphyxia was a marked decrease in striatal dopamine release, monitored under basal and D-amphetamine stimulated conditions, as compared with control (normal- or caesarean-delivered) rats. Striatal glutamate and aspartate levels were also decreased following asphyxia. In the substantia nigra, the main effect of asphyxia was a decrease of both γ -aminobutyric acid (GABA) and aspartate levels. Thus, this study provides evidence that perinatal asphyxia leads to chronic deficits in neurotransmission in the basal ganglia.

Key words: Neonatal asphyxia; Basal ganglia; Dopamine; Parkinson's disease; Microdialysis; Ageing; Rat

Acute perinatal asphyxia can be a cause of death and neurological injury in newborn babies [8]. Many asphyctic babies die during the newborn period, and 20–30% of the survivors present long-term neurological sequelae, including Attention Deficit Hyperactivity Disorder, Cerebral Palsy, Seizures and Mental Retardation [15]. Although there is agreement that dopaminergic transmission decreases with age, and that this decrease is enhanced in several neurodegenerative disorders, such as Parkinson's and Alzheimer's diseases [4], it is not known whether perinatal asphyxia is related to the biochemical features observed in such accelerated ageing processes. It is our hypothesis that, e.g. decreases in dopamine (DA) levels observed at the adult stages might be related back to perinatal asphyctic lesions.

Several models have been used to study severe ischemia, but the majority of them are invasive and per-

formed in one-week-old or older pups [9,11]. At the Karolinska Institute, a novel non-invasive model for studying the short- and long-term consequences of hypoxic-ischemic lesions in rats, has been developed. Asphyxia is induced during delivery, mimicking the conditions resulting in asphyxia in human labor [3,6]. However, it should be kept in mind that the use of one-week-old or older pups is preferred by many scientists [14], since there is evidence showing that, at that age, the maturation grade of the rat cerebral cortex best matches that of the full-term newborn human baby [12]. It is not clear, however, whether this period is also optimal for studying other brain regions. Nevertheless, in the present model asphyxia is induced at the time when the rats do normally deliver.

The obvious and most serious short-term consequence of perinatal asphyxia observed in the present study was mortality. At 37°C, an asphyctic period longer than 22 min was inevitably associated with death. Rats surviving asphyctic periods longer than 20 min at 37°C showed

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chronic deficits in several putative neurotransmitter systems monitored with microdialysis [13] in the basal ganglia.

Asphyxia was induced in pups delivered by caesarean section on pregnant Sprague-Dawley rats. Rats within the last day of gestation were anaesthetised with ether and hysterectomized. The gestational age of the rat was determined by estabularium protocols and by clinical palpation. The entire uterus, still containing the foetuses, was taken out, the uterus horns were detached and placed in a water bath at 37°C for various periods of time. Caesarean-delivered control and asphyctic pups were obtained from the same mother, since each mother delivered approximately 12-15 pups. Following asphyxia, the uterus horns were rapidly opened and the pups were removed and stimulated to breathe on a heating pad by cleaning of the delivery fluid and by tactile stimulation of the oral region with pieces of medical wipes. The umbilical cord was ligated and the animals were left to recover for 40-80 min before they were given to surrogate mothers. Several parameters were recorded by direct observation at a 40-80 min period following delivery: (i) body weight, (ii) survival, (iii) respiratory frequency, (iv) gasping, (v) vocalisation, (vi) colour of the skin, and (viii) spontaneous movements (Table 1). Rats which had normally delivered 24 h before the experiments were utilised as surrogate mothers. Each surrogate mother conserved two of its own male pups and received four asphyctic and two caesarean delivered control male pups. After six months (=500 g b.wt.), asphyctic and control rats were anaesthetised with halothane, placed in a Kopf stereotaxic frame and implanted with two microdialysis probes, one into the left striatum (coordinates: B 0.5, L -3.2, V -7.2, according to the atlas of Paxinos and Watson [10] (dialysing length = 4 mm; diameter = 0.5 mm) (CMA/microdialysis AB, Stockholm, Sweden) and another into the left substantia nigra (B -6.2, L -7.6, V -8.6; inserted with a 40° angle from vertical in the coronal plane) (dialysing length = 2 mm; diameter = 0.5 mm). The microdialysis probes were perfused with a modified CSF solution (148 mM NaCl, 2.7 mM KCl, 1.2 mM CaCl₂, 0.85 mM MgCl₂; pH 7). A constant flow of 2 µl/min was maintained with a microdialysis pump. Halothane anaesthesia was maintained throughout the microdialysis experi-

ment by free breathing into a mask fitted over the nose of the rat (1% halothane in air flow of 1.5 l/min). Body temperature was kept at 37°C using a temperature control system. Samples (collected every 40 min) were directly injected onto high performance liquid chromatography systems, coupled to electrochemical detection (HPLC-EC) systems for DA and its metabolites and γ -aminobutyric acid (GABA), or to a fluorometric detection system for glutamate (Glu) and aspartate (Asp) (see [5]). The microdialysis probes used in this study showed approximately 20% and 14% in vitro recovery in 4 mm and 2 mm long probes, respectively, for DA, dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), GABA, Glu and Asp (expressed as the concentrations found in the perfusates; nM). Basal values are referred to those obtained before the administration of 2 mg/kg s.c. of D-amphetamine (200 min after microdialysis implantation). Means and standard errors of the means (S.E.M) were calculated and differences were tested with Fisher (*F*)-ANOVA test. A level of *P* < 0.05 for the two-tailed test was considered critical for statistical significance.

As shown in Table 1, 14% of the pups survived a 20-22 min asphyctic period. The surviving pups showed deficits in respiration and were largely akinetic. However, all the pups were accepted by the surrogate mothers. Following weaning, the animals were housed in groups of three or four per cage; at that stage no obvious estabularium differences were observed between control and asphyctic rats.

Table 2 shows DA, metabolites and amino acid levels found in the striatum (A) and substantia nigra (B) of control (caesarean-delivered) and asphyctic rats 6 months following delivery. No significant differences were observed between normal and caesarean-delivered rats, and therefore values obtained in normal rats are not shown. A significant decrease in striatal DA (=60%), Glu (=80%) and Asp (=50%) levels was observed in the asphyctic rats (Table 2A). In the substantia nigra, no significant changes were observed in DA levels, although HVA were significantly increased (=70%) in the asphyctic rats. In this region, the most marked effects were seen on GABA and Asp levels, which were decreased by =50% and =80%, respectively (Table 2B). The time course of the effect of D-amphetamine on striatal DA

Table 1
Short-term effects of Severe perinatal asphyxia, monitored by direct observation at the 40-80 min period following delivery

Experimental condition	Body weight (g)	Survival	Respiratory frequency	Gasping	Vocalization	Colour of the skin	Spontaneous movements
[1] Normal delivery (n = 18)	6.1 ± 0.5	100%	79 ± 2	0%	100%	Pink	3.9 ± 0.1
[2] Caesarean delivery (n = 42)	5.8 ± 0.5	100%	74 ± 2	0%	100%	Pink	3.8 ± 0.1
[3] 20-22 min asphyxia (n = 36)	5.8 ± 0.5	14%	21 ± 3	98%	0%	Pale	0

Spontaneous movements were scored by the following scale: (0) akinesia and rigidity (mainly of hind legs); (1) movement of one of the following body structures: front legs, hind legs or head alone; (2) movement of two of the body structures; (3) movement of all body structures; (4) intensive movements shown by wriggling. Gasping refers to an effort to maintain respiration shown by opening of the mouth and movements of the diaphragm.

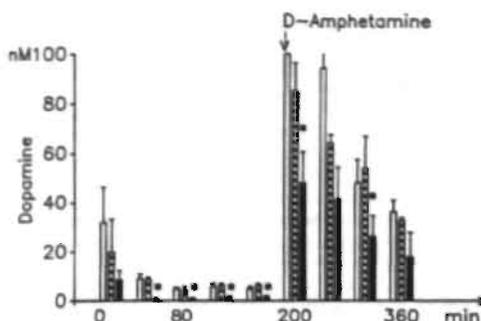


Fig. 1. Effect of severe perinatal asphyxia on striatal extracellular dopamine levels measured approximately six months after delivery. Microdialysis probes were implanted into the striatum of: (1) normal (open columns, $n = 8$), and (2) caesarean-delivered control (cross-hatched columns, $n = 8$) rats, as well as of (3) asphyctic (filled columns; $n = 8$) rats. Two-hundred minutes after the microdialysis implantation, a dose of *D*-amphetamine (2 mg/kg s.c.) was administered. Dopamine was assayed in 20 μ l samples using HPLC-EC. Vertical lines show S.E.M. * $P < 0.05$ for the two-tailed test.

levels in control and asphyctic rats is shown in Fig. 1. For comparison, striatal DA levels found in normal rats are also presented. Compared to levels in caesarean-delivered rats, the *D*-amphetamine effect on DA levels was decreased by $\approx 40\%$ in asphyctic rats.

The obvious and most serious short-term consequence of perinatal asphyxia is death. We have recently reported [6] that the rate of survival after perinatal asphyxia is dependent upon the duration of the asphyxia, as well as upon the temperature at which it occurs. At 37°C, the normal condition, 100% survival is observed up to a 16 min asphyctic period, whilst asphyctic periods longer than 22 min are inevitably associated with 100% mortality. However, a 100% survival is still observed up to a 30 min asphyctic period at 30°C, and even up to a 100 min asphyctic period at 15°C.

Rats surviving asphyctic periods longer than 20 min showed chronic deficits in the release of several putative neurotransmitters monitored with microdialysis in the basal ganglia. The main change observed in six month old rats that underwent severe perinatal asphyxia was a marked decrease in dopamine release, monitored under basal and *D*-amphetamine stimulated conditions, as compared with control (normally- or caesarean-delivered) rats. In agreement with the present observation of a reduced dopamine release in the neostriatum, and reduced GABA release in the substantia nigra, parallel experiments showed that following severe perinatal asphyxia, rats presented a decrease in the rate of spontaneous locomotor activity [1]. Whether the chronic effect on DA release is primarily due to perinatal asphyxia or secondary to other neurotransmitter systems is not yet known. However, in a recent study [2], changes in the number of tyrosine-hydroxylase (TH) immunoreactive

nerve cell bodies, as well as in basic Fibroblast Growth Factor (FGF) gene expression were observed in the substantia nigra of asphyctic rats, four weeks after birth. Furthermore, we have recently found that severe perinatal asphyxia produced a decrease in DA levels and DA turnover in the striatum, but not in the substantia nigra, measured postmortem at the adult stage (Ungethüm et al., in preparation). The effect of perinatal asphyxia on DA levels appears to depend upon the length of the asphyctic period, since we have found that following sub-severe asphyxia (19–20 min, at 37°C), basal extracellular DA levels were significantly increased (≈ 2 fold), although the effect of *D*-amphetamine was significantly decreased ($\approx 50\%$) [7]. Furthermore, a decrease in the total number of neurons in hippocampal (CA1, CA3) regions, which may contribute to the general behavioural syndrome observed after perinatal asphyctic lesions, has

Table 2

Long-term effects of Severe perinatal asphyxia (six months after delivery) on monoamine and amino acid levels monitored under basal (160–200 min period following microdialysis implantation) and *D*-amphetamine-stimulated (2 mg/kg s.c.) (200–240 min period) conditions in the striatum (A) and substantia nigra (B) of caesarean delivered (control) and asphyctic (>20 min asphyxia before delivery) rats.

	Basal (nM)	<i>D</i> -Amphetamine (nM)
(A) Striatum		
<i>Control (n = 15–22)</i>		
Dopamine	4.2 \pm 1	72.4 \pm 9
DOPAC	1175 \pm 115	779 \pm 95
HVA	1052 \pm 154	923 \pm 133
GABA	22 \pm 3	27 \pm 5
Glutamate	1203 \pm 281	1322 \pm 321
Aspartate	133 \pm 30	101 \pm 33
<i>Asphyctic (n = 4–8)</i>		
Dopamine	1.6 \pm 0.4 ^b	48.1 \pm 13 ^b
DOPAC	1239 \pm 175	818 \pm 129
HVA	773 \pm 88	694 \pm 77
GABA	21 \pm 3	25 \pm 2
Glutamate	230 \pm 40 ^b	222 \pm 51 ^b
Aspartate	61 \pm 10 ^b	120 \pm 60
(B) Substantia nigra		
<i>Control (n = 16–24)</i>		
Dopamine	0.9 \pm 0.1	1.7 \pm 0.3
DOPAC	14 \pm 2	4 \pm 3
HVA	38 \pm 8	30 \pm 7
GABA	19 \pm 3	34 \pm 11
Glutamate	788 \pm 201	781 \pm 232
Aspartate	91 \pm 20	80 \pm 10
<i>Asphyctic (n = 4–8)</i>		
Dopamine	0.6 \pm 0.2	1.5 \pm 0.3
DOPAC	20 \pm 7	17 \pm 6 ^b
HVA	65 \pm 5 ^b	64 \pm 6 ^b
GABA	9 \pm 2 ^b	11 \pm 2 ^b
Glutamate	991 \pm 150	940 \pm 160
Aspartate	11 \pm 2 ^b	11 \pm 2 ^b

^b = $P < 0.05$ compared to the corresponding values observed in the controls.

also been reported [3]. Further studies combining microdialysis, immunohistochemistry and quantitative histo-fluorometry are now in progress.

Thus, the present experimental model appears to be useful to study the short- and long-term consequences of hypoxic-ischemic lesions in rats, induced under conditions similar to those found during a human labor resulting in an asphyctic state. Asphyxia during birth may be a factor contributing to the development of neurodegenerative diseases such as Parkinson's and Alzheimer's diseases.

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Neurocircuitry of the Basal Ganglia Studied by Monitoring Neurotransmitter Release

Effects of Intracerebral and Perinatal Asphyctic Lesions

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Abstract

The neurocircuitries of the basal ganglia are studied with *in vivo* microdialysis, with special consideration to dopamine transmission and its interaction with other neurotransmitter systems. The aim is to develop experimental models to study the pathophysiology and therapy of neurodegenerative disorders of the basal ganglia, as well as to develop models to study the short- and long-term consequences of perinatal asphyctic lesions. A main goal of these studies is to find and to characterize new treatments for these disorders.

Index Entries: Basal ganglia; neurotransmitter release; microdialysis; chemical lesions; asphyxia; hypothermia; rats.

Introduction

In this article, we present our recent investigations about the neurocircuitry of the basal ganglia. The aims of these investigations have been:

1. To study the neurocircuitry and neuropharmacology of the basal ganglia, with special consideration to dopamine (DA) transmission and its interaction with other neuronal systems;
2. To develop experimental models to study the pathophysiology of neurodegenerative disorders of the basal ganglia;
3. To study new pharmacological approaches for

- the treatment of these disorders; and
4. To develop a novel model to study the short- and long-term consequences of perinatal asphyctic lesions.

The basal ganglia provide a complex neuronal network that conveys and integrates signals from and to the cerebral cortex. Neurocircuitries of the basal ganglia have been associated with several neurodegenerative diseases. Thus, it is well established that in Parkinson's disease degeneration of the nigrostriatal DA system constitutes the most critical abnormality. In Huntington's chorea the most prominent feature is a marked loss of

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striatal neurons. However, although Alzheimer's disease and dementia of Alzheimer's type are primarily associated with deficits of cerebral cortex and hippocampal formation, some of the symptoms presented by these diseases are also linked to dysfunctions of the basal ganglia (1). Furthermore, deficits of mesolimbic and mesocortical DA transmission have been associated with several functional syndromes, such as psychosis (2) and drug addiction (3).

Whereas DA is an essential neurotransmitter of the basal ganglia, it exerts modulatory actions on neuronal circuitries that utilize amino acids and/or neuropeptides as chemical messengers. In the striatum, DA terminals directly link with medium-size spiny neurons utilizing γ -amino butyric acid (GABA) as a principal neurotransmitter, but also with neurons utilizing opioid or tachykinin peptides (4). In the striatum, acetylcholine (ACh) is found in intrinsic large size neurons (5). It has been suggested that striatal ACh neurons are not synaptically linked with DA terminals (6). However, in an electron microscopy study, Kubota et al. (7) presented evidence that striatal cholinergic neurons may indeed receive direct inputs from dopaminergic axons. In agreement, we have recently shown, in an *in situ* hybridization study, that approx 95% of large size neurons in the striatum express mRNA for DA D-2 receptors (8).

The medium-size spiny neurons project axons to the globus pallidus and to the substantia nigra. Neurons projecting to the globus pallidus contain both GABA and enkephalin, whereas striato-nigral fibers contain GABA, substance P, and dynorphin (9). There are studies showing that in the striatum, DA exerts, via different receptors, selective actions on neuropeptides associated to different projection systems (10). Dopamine acts on striato-pallidal neurons via D-2 type of receptors and on striato-nigral neurons via D-1 receptors. Thus, DA receptor multiplicity provides a gating system funneling striatal activity via different efferent pathways, a hypothesis largely developed in our own laboratory (11-19).

The interaction between DA and the neuropeptide cholecystokinin (CCK) has been an important issue for describing the functioning of the basal ganglia. The issue has been discussed in rather oversimplified terms and without morphological background. In the striatum, several different CCK systems exist, with both intrinsic and extrinsic origin (20). A proportion of mesencephalic DA neurons projecting to the telencephalon utilizes CCK as

a cotransmitter (21), and this neuropeptide is also present in cortico-striatal projections, probably colocalized with glutamate. We have recently reported evidence for a partly crossed CCK cortico-striatal pathway in the rat (22-24). Little is, however, known about the functional interactions between CCK and DA and the contributions from each particular system to the final output of the basal ganglia.

Several methods have been applied to study interactions between monoamines and neuropeptides in the brain, the majority of them utilizing indirect neuroanatomical or behavioral techniques. We have chosen to utilize the novel technique, largely developed in our laboratory, of *in vivo* microdialysis (25), which allows the simultaneous monitoring of the release of monoamines, ACh, amino acids, and peptides in restricted regions of the brain. Thus, the interactions among different nuclei of the basal ganglia have been studied with *in vivo* microdialysis, in normal and lesioned rats. DA, ACh, glutamate, aspartate, GABA, purines, lactate, and pyruvate are assayed with high-performance liquid chromatography (HPLC), coupled to electrochemical (EC), fluorescence (F), or ultraviolet (UV) detection systems. Neuropeptides are measured by sensitive radioimmunoassays (RIA) utilizing selective antibodies. Rotational behavior (11,12,26), simultaneously with microdialysis or in parallel experiments has also been recorded. Histochemistry and *in situ* hybridization studies have also been performed.

The main aim of these studies is, however, to characterize pharmacological treatments. Drugs have been analyzed in experimental models mimicking Parkinson's or Alzheimer's diseases. Attention has been given to the effects of treatments with endogenous and exogenous trophic factors, such as nerve growth factor (NGF), the monosialoganglioside GM1, and nicotine.

Functional Neuroanatomical Studies

Modulation of Striatal DA Release by Striato-Nigral Pathways

Striatal DA release is differently modulated by striato-nigral GABA, dynorphin, substance P, and neurokinin A pathways. GABA and dynorphin exert a negative feedback on striatal DA, whereas substance P and neurokinin A provide a positive

feedback (27–29). The effects produced by substance P and neurokinin A are conveyed via different receptors (30,31), and via different neuronal (32,33) and metabolic (34,35) pathways (36). We have found that exogenously administered substance P may be cleaved to a shorter active fragment (substance P [1–7]), which can then have antagonistic properties against substance P (34), in contrast to the C-terminal substance P fragment (substance P [6–11]), which does not exert any significant modulation on the effects of substance P (35).

In order to monitor closely the neurotransmitter cascade connecting the substantia nigra and the striatum, the sensitivity of the analytical assays had to be in the lower pico- and femtomole levels for monoamines and amino acids, and peptides, respectively. Thus, following implantation of microdialysis probes into the striatum and into the substantia nigra, we can now simultaneously monitor monoamines, amino acids, and neuropeptides in both regions. Figure 1 shows dynorphin B and GABA levels simultaneously monitored in left striatum and left substantia nigra under basal and K⁺-depolarizing conditions. The GABA antagonist bicuculline has been included together with the KCl, in order to block the effect produced by a massive release of GABA.

The effects of striatal or intranigral administration of the dopamine D-1 agonist SKF 38393, and the D-2 agonist quinpirole on GABA and dynorphin release have been studied. We have found that 100 μ M of SKF 38393 included in the striatal perfusion medium produces a greater than twofold increase in nigral GABA and dynorphin levels, whereas no such effect has been observed after quinpirole. In contrast, nigral quinpirole, but not SKF-38393, produces a concentration-dependent decrease in striatal dopamine levels. In the nigra, SKF-38393 induces an increase of nigral GABA and dynorphin levels (85).

Modulation of Striatal DA Release by Cortico-Striatal Pathways

There is evidence that striatal DA release is presynaptically modulated by glutamatergic cortical inputs. It was proposed that the DA stimulation produced by glutamate reflected direct axonal interactions between glutamatergic and dopaminergic terminals in the striatum. This hypothesis received some support from biochemical and histochemical studies showing direct intrastriatal axonal interactions. However, the majority of the striatal afferents from the cortex and substantia

nigra make axodendritic synaptic contacts with striatal neurons (37), giving a basis for polysynaptic loops, including GABA and/or ACh neurons, by which cortical glutamate neurons can also modulate striatal DA release (38,39).

We have found that cortical stimulation produces an increase in striatal ACh and DA release (39). These effects, however, seem to be mediated by different glutamate receptors; kainate agonists produced an increase in striatal DA release, whereas NMDA agonists produced an increase in striatal ACh release (38,40). The development of this model will enable us to follow the changes in extracellular striatal monoamine, amino acid, and neuropeptide levels during cortical stimulations. We have found, however, that the study of the interactions between cortex and striatum is complicated by the fact that inputs onto the striatum are partly originating in the contralateral side. Thus, we have reported that glutamate and CCK are released from a partly crossed cortico-striatal pathway (41,22–24).

Evidence for the presence of DA nerve terminals in the deep layers of the fronto-parietal cortex of the rat has been presented (42). In this cortical region, extracellular DA is found in a 1-nM range and can be increased by K⁺-depolarization or amphetamine stimulation, and suppressed by mesencephalic 6OHDA lesions (42,43). Nigral administration of substance P produces an increase in both cortical and striatal DA release, whereas neurokinin A stimulates striatal DA release only (43). Differences in striatal and cortical DA functions have also been found in studies measuring the mRNA expression of several putative neurotransmitters with *in situ* hybridization and RNA blots. We have found that glutamic acid decarboxylase (GAD, a marker for GABA neurons), somatostatin, and NPY mRNA gene expression were increased in the striatum, but decreased in the cortex following DA deafferentation (44,45), suggesting therefore that DA has different functional roles in the striatum and frontoparietal cortex.

Extracellular levels of ACh can be simultaneously measured in the cortex and striatum of rats (46). These levels could be selectively stimulated by several pharmacological treatments and inhibited by specific lesions. A unilateral ibotenic acid lesion into the nucleus basalis, but not into the striatum, produced a strong decrease in extracellular ACh levels in the ipsilateral cortex (47). Unilateral decortication with the excitotoxin kainic acid, which selectively damages local neurons while sparing

nicotine administered by subcutaneously implanted Alzet minipumps counteracts the decrease in extracellular neostriatal DA induced by the transection, supporting the idea that chronic nicotine may protect against degeneration of central DA neurons (58,59).

Models for Dementia of Alzheimer Type

We have proposed two models to study dementia of Alzheimer type, one based on cortical infarcts induced by devascularizing lesions (46,60,61; see also ref. 1) producing retrograde degeneration of neurons in the nucleus basalis, and another where lesions are directly performed into the nucleus basalis (47). The effects of NGF and GM1 treatments on neural repairing have been studied in these models. We have found that chronic administration of GM1 or NGF could reverse biochemical and morphological changes induced by these lesions (46,60,61).

In vivo microdialysis has been used to study the effects of the aziridinium ion of ethylcholine mustard (AF64A), a powerful alkylating agent that binds to high-affinity choline uptake sites, on intrinsic and extrinsic neuronal systems of rat neostriatum. This neurotoxin has been proposed as a selective toxin for cholinergic neurons, and thus, perhaps, a useful tool in the development of animal models of Alzheimer's disease and senile dementia of the Alzheimer type (62). We compared the effect of AF64A to that of ibotenic acid on striatal ACh, GABA, DA, glutamate, and aspartate levels (63). We concluded that, although AF64A is a potent neurotoxin for intrinsic neuronal systems, it appears, like ibotenic acid, to have similar effects on local cholinergic and GABAergic neuronal systems in the striatum.

Pharmacological Treatments

Mechanisms of Actions of Antiparkinsonian Drugs

Most of the antiparkinsonian drugs are DA agonists. Therefore, we have studied the selectivity of several DA agonists on different receptor populations and proposed the idea that receptor multiplicity may constitute a mechanism by which the actions of DA are gated by different neuronal pathways (15). Studies with rotational behavior (11-14), microdialysis (14-19,40,64), and recently, studies with *in situ* hybridization histochemistry combined

with fluorogold tracing technique (10) support this hypothesis (65,66). We have found that, in the striatum, DA exerts an inhibitory modulation on GABA neurons via D-2 receptors, whereas D-1 stimulation exerts a stimulatory modulation on GABA neurons (19).

Looking for new antiparkinsonian therapies, we have found that caffeine shares some of the properties of DA agonists. Thus, the methylxanthines, caffeine, theophylline, and theobromide produce rotational behavior in 60HDA lesioned rats, a behavior partially inhibited by DA antagonists (67,68). Although we have studied several hypotheses, we still lack conclusive results to support a single mechanism for explaining the effects produced by methylxanthines in 60HDA lesioned rats (67-73).

Treatment with Endogenous and Exogenous Trophic Factors

The idea that degenerative processes may be delayed or even reversed by exogenously administered trophic factors, such as NGF and GM1, is now accepted. NGF is synthesized within target tissues of some peripheral and central neurons and can act on specific receptors (74). NGF, in turn, is internalized and retrogradely transported to cell bodies (75). Thus, NGF can be used as a pharmacological tool to induce nerve growth and repair.

At the beginning of the 1980s, we studied the promotion of phenotypical transformation of chromaffin cell grafts by NGF. We were the first to report *in vivo* studies (50,76,77) showing that NGF could induce changes in chromaffin cells grafted into a DA deafferented striatum, transforming their endocrine-like into a neuron-like feature. It was found that the changes were associated with the reversing of symptoms of experimentally induced parkinsonism. We also showed the effects of NGF treatments on extracellular ACh, DA, and adenosine levels in the cortex and striatum of rats with unilateral devascularizing cortical lesions (61).

Another neurotrophic factor, the monosialoganglioside GM1, has also been tested for the promotion of nerve growth and repair (78). GM1 can prevent retrograde changes in nucleus basalis produced by cortical lesions (79) and might also stimulate the activity of cortical cholineacetyltransferase in regions adjacent to the lesions. We have extended these studies by analyzing the effects of decortication and treatments with GM1 on cortical and striatal ACh, catecholamines, and adenosine lev-

els measured with microdialysis (46). A novel administration route for neurotrophic factors, i.e., microencapsulation into human serum albumin microspheres, which can then be topically applied onto damage regions, has also been studied (60). Such treatments with GM1 promote (1) recovery of retrograde morphological changes produced by devascularization and (2) a parallel increase in cortical ACh release. Although these results appear to be promising, the possibility that excessive trophic stimulation might lead to aberrant connections in addition to, or instead of functionally reparative ones has to be carefully investigated.

Perinatal Asphyctic Lesions

The Experimental Model

We have developed a noninvasive animal model for studying the short- and long-term consequences of hypoxic-ischemic lesions in rats, similar to those produced under labor in clinical situations (80). We found that perinatal asphyxia for a period longer than 22 min, in a water bath at 37°C, led to 100% mortality within the first 20-min period following delivery. However, when the uterus containing the pups was kept 22 min in a 30°C water bath, 100% of the pups recovered respiratory function following tactile oral stimulation and were accepted by the surrogate mothers (81, Loidl et al., in preparation). The protective effect of hypothermia at 30°C even allows for a 47–48-min asphyctic period. When asphyxia was induced in a water bath at 15°C, 100% survival could be extended to 101 min.

Short-Term Effects of Perinatal Asphyxia

Several parameters are acutely or chronically recorded by direct observation or by *in vivo* microdialysis. Following asphyxia, pups are subcutaneously implanted with 4-mm microdialysis probes in the dorsal region, while kept on a heating pad (Fig. 2). Thus, subcutaneous levels of amino acids (glutamate, aspartate), and metabolism products (lactate, pyruvate, and ascorbate) are monitored during the 40–60- and 60–80-min periods after removal from the uterus in asphyctic and controls pups.

Effects of Delivering by Hysterectomy

Pups delivered from uterus horns removed by hysterectomy from rats at the final day of gestation started regular breathing (respiratory frequency 60/min) almost immediately after the delivering

was completed. These control pups showed a pink-colored skin and intensive vocalization and motility. They were accepted by surrogate mothers after an 80-min observation period. When the pups were accepted, they grew up in a similar manner to that of normally delivered rats (at least during a 1-mo period of observation). Subcutaneous glutamate levels were =2 μ M; aspartate =0.4 μ M; lactate =1 mM; and pyruvate =60 μ M.

Short Asphyctic Exposure

Following a 5–6-min asphyctic period, induced in a water bath at 37 or 30°C, all the pups started breathing shortly after delivery. Their behavior was similar to that observed in the control animals. Following 5–6 min of asphyxia at 37°C, glutamate levels were =10 μ M; aspartate =1.2 μ M; lactate =2 mM; and pyruvate =60 μ M. At 30°C glutamate levels were =6 μ M; aspartate =2 μ M; lactate =1 mM; and pyruvate =40 μ M. Following 15–16 min asphyctic periods, induced in a water bath at 37 or 30°C, all the pups survived, without differences in color of the skin and respiratory frequency, as compared to the control animals. A slight decrease in spontaneous motility was observed, but all the pups were, however, accepted by the surrogate mothers. At 37°C, glutamate levels were =7 μ M; aspartate =1 μ M; lactate =2 mM; and pyruvate =60 μ M. At 30°C glutamate levels were =3 μ M; aspartate =0.5 μ M; lactate =2 mM; and pyruvate =60 μ M.

Intermediate Asphyctic Exposure

Following a 19–20-min asphyctic period at 37°C, the pups had to be intensively stimulated to start breathing. The surviving pups remained akinetic for a long period after delivery, showed a significant decrease in respiratory frequency (=20/min), which was accompanied by gasping and showed a pink/pale skin coloration. Approx 30% of the pups died shortly after delivery. In contrast, all the pups survived following a 19–20-min asphyctic period at 30°C. Initial gasping, a slight decrease in respiratory frequency (=40/min), and in motility could be observed. The color of the skin was similar to that in control pups. All the surviving pups were accepted by the surrogate mothers. At 37°C, glutamate levels were =5 μ M; aspartate =0.5 μ M; lactate =2 mM; and pyruvate =60 μ M. At 30°C glutamate levels were =6 μ M; aspartate =1 μ M; lactate =2 mM; and pyruvate =40 μ M.

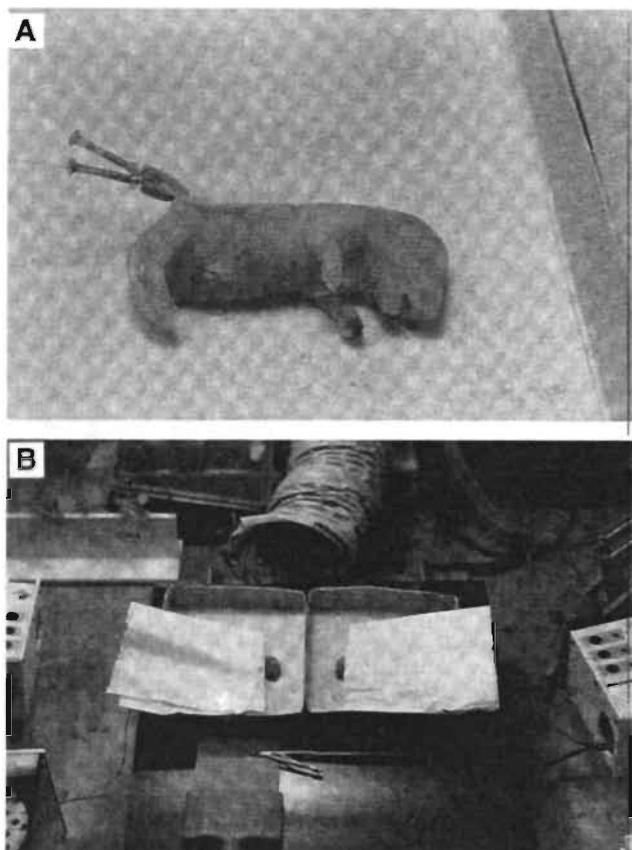


Fig. 2. (A) Pup recovering from a 19–20-min asphyctic period, at 37°C. A 4-mm microdialysis probe was subcutaneously implanted into the dorsal region in order to monitor peripheral glutamate, aspartate, lactate, and pyruvate in 20-min (40 μ L) perfusion samples. Two fraction samples were taken before the pup was given to surrogate mothers. The experimental conditions under which the pups are normally microdialysed are shown in (B).

Long Asphyctic Exposure

The rate of survival rapidly decreased following prolonged asphyctic periods at 37°C (>20 min), and as a whole, the physiological condition of the surviving pups deteriorated (increased gasping, decreased respiratory frequency, lack of vocalization, akinesia, and pale skin). No pups survived following asphyctic periods longer than 22 min. In contrast, at 30°C, all the pups survived up to a 30–31-min asphyctic period, although some signs of physiological impairment (presence of gasping, decrease in respiratory frequency and motility, and

pale skin) could be observed. At this temperature, 40% survival could be observed following a 47–48-min asphyctic period. All the surviving pups showed gasping, a decrease in respiratory frequency (\approx 10/min), akinesia, and pale skin. No survival was observed following asphyctic periods longer than 48 min. Following a 21–22-min asphyctic period at 37°C, glutamate levels were \approx 4 μ M; aspartate \approx 0.4 μ M; lactate \approx 2 mM; and pyruvate \approx 90 μ M. At 30°C glutamate levels were \approx 9 μ M; aspartate \approx 1 μ M; lactate \approx 2 mM; and pyruvate \approx 40 μ M.

At 15°C, 100% survival was observed up to 101 min of asphyxia. Following a 50–51-min asphyctic

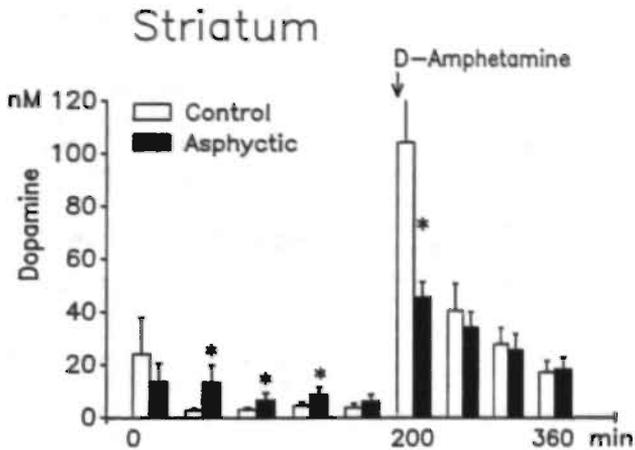


Fig. 3. Effect of perinatal 19–20-min asphyxia on extracellular striatal dopamine levels measured in samples collected by *in vivo* microdialysis 1 mo after delivery. A 4-mm microdialysis probe was implanted into the left striatum of halothane-anesthetized asphyctic ($n = 7$) and control ($n = 6$) rats. Microdialysis probes were perfused with a modified CSF solution. At the 200-min period following microdialysis implantation, a dose of *D*-amphetamine (2 mg/kg sc) was administered. Dopamine was detected in 20 μ L samples using a highly sensitive HPLC-EC. Vertical lines show SEM. * = $p < 0.05$ for the two-tailed test (Mann-Whitney U-test, corrected by the Bonferroni's procedure).

period, gasping was observed in 50% of the pups, the respiratory frequency was ≈ 40 /min, vocalization and motility were decreased, and the skin was pink/pale. Following a 100–101-min asphyctic period at 15°C, gasping was observed in all the pups, and the respiratory frequency was 10/min. The pups were akinetic and pale, and no vocalization was observed.

Long-Term Effects of Perinatal Asphyxia

We have completed a series of experiments in which the pups were exposed to asphyxia and, approx 1–6 mo later, implanted with two microdialysis probes, one into the striatum and another one into the substantia nigra. Monoamines and amino acids were monitored under basal and *D*-amphetamine-stimulated conditions.

Significant changes in monoamines and GABA were observed in rats exposed to subsevere and severe asphyxia, but nonsignificant effects were observed in glutamate and aspartate levels. Striatal extracellular DA levels were significantly increased in animals exposed to subsevere (19–20 min, at 37°C) asphyxia (approximately twofold), although the effect of *D*-amphetamine on extracellular DA levels was significantly decreased ($\approx 50\%$ compared

to controls) (Fig. 3). Extracellular GABA levels in the substantia nigra were decreased by $\approx 50\%$. In animals with severe asphyxia (>20 min, at 37°C), a decrease in striatal DA levels was observed under basal ($\approx 70\%$) and under *D*-amphetamine stimulation ($\approx 50\%$ compared to controls) (86). The increase in basal DA levels in subsevere asphyxia could be in agreement with a histochemical study (80) showing that under the same conditions, asphyxia produced an increase in the number of tyrosine hydroxylase-immunoreactive (TH-IR) nerve cell bodies, which was considered to be sign of proliferation of dopaminergic neurons. A cause for this increased number of nigral DA cell bodies was suggested to be a deficit in the GABAergic striato-nigral feedback, which would set the nigrostriatal DA neurons in a hyperactive state. In agreement with this is the present finding that there is a decrease in nigral GABA levels and a decrease in the effect of *D*-amphetamine. Further studies combining microdialysis, immunohistochemistry, and quantitative histofluorometry are now in progress.

The present results show that perinatal asphyxia leads to death or to long-term neuronal deficits, affecting such systems as the nigrostriatal dopamine pathway. The extent of the damage appears to

depend directly on the length of the asphyctic period, as well as on the general metabolic condition under which asphyxia is induced. Thus, it is striking that decreasing the temperature from 37 to 30 or 15°C resulted in a significant increase in survival. This finding is in agreement with experimental studies demonstrating that low brain temperature protects brain neurons in rats subjected to transient forebrain ischemia (82,83), an effect probably resulting from reduction in brain energy demands and a consequent decrease in the rate of ATP depletion (84).

Summary

The interactions among different nuclei of the basal ganglia have been studied with *in vivo* microdialysis, in normal and lesioned rats. DA, ACh, glutamate, aspartate, GABA, adenosine, and neuropeptides have been simultaneously monitored and assayed with highly sensitive HPLC and RIA methods. Rotational behavior has also been recorded, together or in parallel with microdialysis.

The modulation of striatal DA release by striato-nigral and cortico-striatal pathways has been studied. It has been found that striato-nigral GABAergic and dynorphinergic pathways exert a negative feedback on striatal DA, whereas tachykininergic pathways exert a positive feedback on striatal DA. Cortical stimulation produces an increase in striatal DA release, probably via glutamatergic receptors of the kainate type. The interaction between cortex and striatum is, however, complicated by the fact that there are ipsilateral and contralateral cortical inputs, utilizing glutamate and CCK as transmitter signals.

Several experimental models have been developed to study the pathophysiology and therapy of neurodegenerative disorders of the basal ganglia. Different pathways of the basal ganglia are destroyed by intracerebral injections of selective toxins or surgical knife cuts. Thereafter, various drugs, including endogenous and exogenous trophic factors, are tested to reverse the effects induced by lesions.

A novel animal model to study the short- and long-term consequences of perinatal asphyctic lesions is presented. It has been found that perinatal asphyxia leads to death or to long-term neuronal deficits. The extent of the damage appears to depend on the length of the asphyctic period, as well as on the general metabolic condition under

which asphyxia is induced. Hypothermia appears to be a powerful treatment to increase survival following severe asphyxia. The presented model is largely noninvasive on the pups, and it mimics the condition produced under labor in clinical situations. Therefore, it appears to be useful as a model for studying treatments to ameliorate the deleterious effects induced by hypoxic-ischemic lesions.

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

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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 death¹. 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) levels².

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 asphyxia³.

So far, the pathological mechanism triggered after 16 min of PA leading to chronic neurotransmission alterations evidenced in monoamines and GABA^{2,3} 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 neurons⁴ and it was the fact that NO had been described to enhance DA release from striatum⁵ 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.

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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)², 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⁻¹) 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 μ m 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 \pm 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 cytomegaly⁴, 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 somatostatin and neuropeptide Y⁷, in adult rat such neurons were of medium size and non-spiny type. Due to their lack of NMDA receptors⁷ and high concentration of manganese superoxide dismutase⁸, 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.

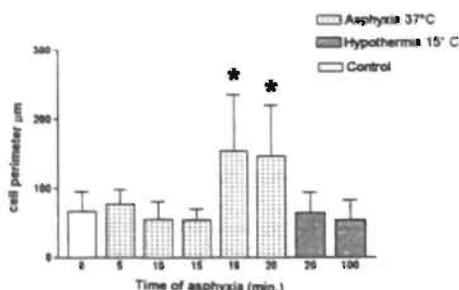


Fig. 1.— Measurement of striatal NADPH-d+ cell perimeter, in 6-month-old rats subjected to different periods of PA at 37°C or 15°C. Each value represents mean \pm SD (vertical lines) of determinations made from $n = 100$ cells from each group. Asterisk indicates that NADPH-d+ in these two groups was 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 (minutes)	Perimeter (μm) 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 neurons⁷. 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 processes⁸. In the present study, an experimental model was specially designed to evaluate PA effects in adult animals^{2,3}. 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 al⁶ who documented cortical neuronal cytomegaly in pediatric epilepsy.

As NO induces DA release, which may be blocked by the NO synthase inhibitor L-Me-Arg⁹, the higher basal level of striatal DA after an episode of severe PA² 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 muscle¹⁰, and most likely protects against ischemia by enhancing oxygen supply. The ability to stimulate DA release, considered toxic in striatum after ischemia¹¹, together with its potent vasoconstrictor effect may worsen cell damage. However, since DA release is inhibited by hypothermia¹², 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 gerbils¹³, 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^{13, 14}. Metabolic demands decrease when body temperature is lowered¹⁵ 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 asfisia 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.

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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

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LONG - TERM MORPHOLOGICAL CHANGES IN NADPH-DIAPHORASE
REACTIVITY IN STRIATAL AND CORTICAL NEURONS FOLLOWING
EXPERIMENTAL PERINATAL ASPHYXIA: NEUROPROTECTIVE EFFECTS
OF HYPOTHERMIA

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SUMMARY: Nitric oxide (NO) is known to be directly or indirectly involved in neuropathological mechanisms triggered by excitatory aminoacids. NO(+) neurons in brain may be detected histochemically by nicotinamide adenine dinucleotide phosphate diaphorase (NADPH-d) histochemical technique, as the latter readily labels NO synthase in central nervous system (CNS). NADPH-d stained striatal and cortical sections were studied in 6-month-old male Sprague-Dawley rats exposed to perinatal asphyxia (PA) at 37°C, as well as in animals subjected to PA plus hypothermia treatment at 15°C. Quantitative image analysis was performed to compare the staining pattern in the various groups. NADPH-d(+) neurons in striatum and cortex from subsevere and severe PA animals showed a significant increase in soma size and in dendritic processes versus controls and hypothermia-treated rats. These findings indicate that chronic NO changes are involved in post-ischemic striatal and cortical alterations induced by PA that may be prevented by hypothermia treatment.

RUNNING TITLE: NADPH Diaphorase changes in perinatal asphyxia.

KEY WORDS: *Perinatal asphyxia - Hypothermia - NADPH Diaphorase - Striatum - Cortex - Rats*

INTRODUCTION

Perinatal asphyxia (PA) is still a major complication in childbirth and its frequency has not been reduced despite advances in health care. Affected newborn are liable to present short- and long-term neurological sequelae whose severity depends on the period of oxygen deprivation. Thus, PA may lead to hyperactivity, attentional deficit, epilepsy, mental retardation, motor disorders, cerebral palsy or even death.(Volpe, 1987). Some regions of the brain are particularly affected than others being the most sensitive and selectively vulnerable neurones located in the CA1, CA3 and CA4 regions of the hippocampus, cerebellum, neocortex in layers 3, 5 and 6 and neostriatum.(Percy 1986). Although the mechanism responsible for this vulnerability are not clear (Zhu & Luo, 1992), a long-term increase in basal striatal dopamine (DA) levels has been demonstrated in the nigrostriatal pathway using microdialysis (Herrera-Marschitz, et al., 1994).

Fortunately , after ischemia, cell death does not occurs immediately and delayed for hours to days. This process, called delayed neuronal death (Paschen, 1991, Kirino, 1982), provides an opportunistic time window to reduce cell loss and associated learning and memory deficits. (Colbourne, F. & Corbett D., 1994).

At present the most effective treatment is intraischemic hypothermia, which has been repeatedly shown to confer remarkable histological and behavioral protection (Green, E.J. et. Al., 1992, Vicent, S., & Kimura, 1992). Post-ischemic hypothermia has also been found to be beneficial when initiated within 30 min., of reperfusion (Busto, R., et.al. 1987). It has already been demonstrated that hypothermia is critical for increases animal survival following PA induction. When PA was induced at body temperature

(37°C), 100% survival was achieved up to 15-16 min of asphyxia, whereas for 19-20 and 20-21 min periods, survival reached 70% and 20% respectively, with no survival after 22 min of asphyxia. In contrast, when PA was induced at lower temperature (15°C), litter survival reached 100% even up to 100 minutes of asphyxia (Herrera-Marschitz, 1994; Loidl, 1993).

The pathological mechanism triggered after 16 min of PA leading to chronic neurotransmission alterations evidenced in monoamines and GABA (Herrera-Marschitz, 1994; Loidl, 1993) is still poorly understood but may involve the participation of the sequence: excitatory aminoacids, nitric oxide and free radicals. As regards nitric oxide (NO), nicotinamide adenine dinucleotide phosphate diaphorase (NADPH-d) is employed as a histochemical marker of NO synthesis in neurons (Hope, 1991; Uemura, 1990). Indeed, NO has been reported to stimulate DA release from striatum (Hanbauer, 1990; Zhu, 1992).

Since both striatum and brain cortex are severely affected by PA (Pasternak, 1991) the aim of this work was to analyse the pattern of NADPH-d labelling in 6-month-old rat sections following a range of PA periods with or without hypothermia treatment.

MATERIALS AND METHODS

Subjects

Forty-two pregnant rats and fifty post-ischemic female rats, were included in this study. The dams were bred by housing three of them with one male until they were sperm positive as determined by vaginal smear. Pregnant rats were kept in individual stainless-steel cages and fed with Purine chow and tap water ad libitum. The 21st day following the original mating, individual females were placed in separate cages. Pregnancy was indicated by marked increase in weight, 25 to 30 grams, and by an enlargement of the abdomen which was evident at about the 13th day of gestation. Asphyxia was induced in pups delivered by caesarean operation.

Induction of asphyxia

Rats on the last day of gestation were anaesthetised with ether and hysterectomized. Gestational age was determined by vaginal smear. The entire uterus containing the foetuses, was taken out, the uterine horns were detached in a water bath at 37 ° C during the following periods : 5 min., 10 min., (both considered slight PA), 15 moderate PA), 19 min., (subsevere PA) and over 20 min. (severe PA)

Asphyxia was induced in pups delivered by caesarean operation on pregnant Sprague-Dawley rats. Rats on the last day of gestation were anaesthetised with ether and hysterectomized. Gestational age was determined by palpation. The entire uterus, containing the foetuses, was taken out, the uterine horns were detached and placed in a water bath at 37°C during the following periods in min: 5, 10 (both considered slight

PA), 15 (moderate PA), 19 (subsevere PA) and over 20 (severe PA) or at 15°C during 20 and 100 min.. (Herrera-Marschitz M., et. al., 1994) .

Caesarean-delivered control and asphyctic pups were obtained from the same mother. Following asphyxia, the uterine horns were rapidly opened and pups removed and stimulated to breathe on a heating pad by cleaning the delivery fluid and by tactile stimulation with pieces of medical wipes. The umbilical cord was ligated and animals left to recover for around 1h before given to surrogate mothers which delivered normally 24 hs. before the experiment, mixing their normal litters with previously marked asphyctic and control pups delivered by caesarea. Experimental and control pups were weaned at 24 days, and were transferred to stainless-steel cages, with free access to food and water, in compliance with the principles of animals care and Use of Laboratory Animals.

Once the animals became adults at an age of six months (N=4-5 animals/group), they were anaesthetised with 28% (w/v) chloral hydrate, 0.1 ml./100 g. of body weight, and perfused with mixture of aldehydes through the abdominal aorta (González-Aguilar, F., & De Robertis E., 1963)

Histochemistry

Rats were perfused 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 . Brains were removed and postfixed in the same solution during 2h., and then immersed overnight in solution containing 5% sucrose in 0.1 M phosphate buffer pH 7.4. Coronal sections from the brain containing the striatum (40µm thick) were cut on an Oxford vibratome and mounted on gelatine-coated glass slides. Sections from treated (PA, PA + hypothermia) and control animals were simultaneously processed using the same react mixture NADPH-d histochemical method. (Vicent, S., & Kimura, H., 1992).

Briefly, sections were incubated for 1h. at 37°C in a solution containing 0.1% β -NADPH and 0.02 % nitrobluetetrazolium diluted in 0.1M phosphate buffer with 0.3% Triton X-100 (all reagents were purchased from Sigma, St. Louis, MO, USA). Sections were mounted in PBS/ glycerol (1:3), and observed and photographed with a Zeus Axiophot microscope. In absence of NADPH, cells were not stained; substituting NADH for NADPH resulted in nonspecific staining of the entire section. Nitroblue tetrazolium contains a monoformazan impurity that gives a diffuse purple background (Valtschanoff, J.G., et. al., 1993); to remove this some sections were incubated in acetone dimethyl sulfoxide for 10 minutes before mounting.

Histology

Rats were sacrificed either 10 or 45 days after hypoxia with 28% (w/v) chloral hydrate. 0.1 ml/100 g. Of body weight. Adjacent coronal sections (40 μ m thick) were collected and stained with Cresyl violet. Bilateral counts of neurones (well defined nucleus, distinct cellular membrane and not shrunken) were performed by an observer blind to treatment conditions. Neurones were counted in the lateral, medial, dorsal and ventral striatum at bregma -0.40-interaural 8.6 and neurons in the medial and lateral sectors of neocortex at bregma -0.40 - interaural 8.6 (Paxinos and Watson, 1986).

Computerised Image Analysis

The NADPHd and cresyl violet stained neurons in sections of brains of different groups, were measured by using a computerised image analyser (Kontron-ZEISS VIDAS). The selected fields were located in the lateral, medial, dorsal and ventral of striatum and medial and lateral sectors of neocortex. Sections of 40 μm thick with adequate labelling of NADPH diaphorase were randomly selected from coded slides (6 for each time of considered groups). Data from 50 selected striatum and surrounding neocortex were averaged for each animal. Ten cells per sections were analysed with a total of 100 for each group. Images from striatum and neocortex were obtained with a the light microscope and were transferred to a video camera attached and connected to an interactive analysis system on line (KONTRON- ZEUS VIDAS). The images were digitised into an array of 512 X 512 pixels corresponding to 140 x 140 μm (40x primary magnification). The resolution of each pixel was 256 grey levels (8 grey bits level resolution). After automatic normalization of grey-scale, interactive delineation and contrast enhancement of the neuronal images were performed, following the removal of interfering non specify images. The projected surface of cresol violet and NADPH neurons were measured using morphometric parameters: area, perimeter, maximum and minimum diameter, shape factor in sections of experimental and control groups. The means and SD were calculated intreated and control groups.

The spatial distributions of NADPH and cresol violet staining neurones were counted using VIDEOPLAN image analysis system. They were digitised into 256 grey levels in measuring fields, 215 x 180 μm in size (20 x primary magnification). NADPH

positive cells count in per measuring field were performed . The mean and SD were calculated for the different fields studied in asphyctic and control groups.

Statistical Analysis

Differences between means and SAD. of the experimental and control groups were analysed statistically using one- way analysis of variance (ANOVA) and subsequently the Newman-Keuls test, with a p value of less than 0.05 being considered significant. ANOVA and Newman-Keuls test were routinely performed on an IBM compatible PC AT 486 package software (Primer, Mc Graw Hill, Inc.).

RESULTS

Cresol violet staining in striatum

Nineteen and twenty minutes of normothermic ischemia resulted in loss of neurones in medial, posterior, ventral and dorsal sector of the striatum (P 0.001). Hypothermia (20 minutes and 100 minutes at 15 °C), reduced neuronal loss (P: 0.001). In the others groups (5, 10 and 15 minutes) there were not differences with the control (Table 1).

NADPHd staining in the striatum

NADPH-d staining in striatum transverse sections from 6-month-old rats showed uniformly distributed medium -sized neurones. Subsevere and severe PA groups disclosed an increase of the soma size accompanied by an enlargement of processes which showed an evident tortuous dendritic arborizations, distinctive of the so-called neuronal cytomegaly (Mischel, 1995), compared to controls and remaining PA groups at 37 °C, as well as 20 and 100 min.. PA at 15 °C (compare Figs. 1a, 1b and 1c). Labelled cell area and cell perimeter were measured by image analysis in each experimental group. Both striatal cell area and cell perimeter of rats exposed to subsevere and severe PA showed a highly significant cytomegaly ($p < 0.001$) in comparison to control, slight , moderate PA and PA at 15 °C group (Loidl F., 1995, 1996), during 20 min.. and 100 min.. (see Fig. 3a and 3b).

Shape factor measurement of neurones failed to disclose statistical differences between striatal neurons in any studied group (see figs. 3c and 4c).

There were not significant intergroup differences ($P= 0.0001$) in numbers of cells (Table 1).

Cresol violet staining in the neocortex

Nineteen and twenty minutes of ischemia also resulted in severe loss of neurons in medial and lateral neocortex with statistically difference ($P= 0.0001$). Hypothermia (15°C, 20 min., and 100 min..) greatly attenuated neuronal loss ($P 0.0001$) (Table 2).

NADPHd staining in the neocortex

Likewise, in rats subjected to subsevere and severe PA, medium sized NADPH-d+ in cerebral cortex, displayed morphological changes with the same characteristics of cytomegalic cells observed in striatum. Meanwhile, animals undergoing 20 and 100 min. of PA at 15°C (Loidl et al, 1995) showed a staining pattern similar to controls and slight and moderate PA groups (compare Figs. 2a, 2b and 2c).

Cortical cell area and perimeter increased significantly in rats exposed to subsevere and severe PA ($p< 0.001$) compared with control, slight, moderate PA and PA at 15 ° treated animals during 20 min.. and 100min.., (see Figs. 4a and 4b).

There were no significant intergroup differences ($P= 0.0001$) in the number of cells (Table 4).

DISCUSSION

In agreement with authors who described NADPH-d(+) striatal and cortical cells as interneurons containing somatostatin and neuropeptide Y, such neurons in adult rat proved to be of medium size and non-spiny in type (Ferrante R. et al., 1985, Vicent S., & Hope, 1992). Given their lack of NMDA receptors, (Vicent S., and Kimura H., 1992) as well as their high concentration of manganese superoxide dismutase, (Inagaki et al. 1991) these cells were regarded to be resistant to excitatory amino acid toxicity and to free radical toxicity, respectively. However, the wider implications of NO as a neurotoxic or neuroprotective modulator are still a subject of controversy. (Schuman, E. & Madison D., 1994)

Certain neurological disorders have been reported to induce changes in NADPH-d stained neurons. Thus, studies in patients with Huntington's disease have disclosed an increase in striatal NADPH-d(+) cell measurements associated with cell and fibre sparing, attributable to loss of striatal spiny neurons. (Vicent S. & Hope B., 1992). Quite recently, a report on human Parkinson's and Alzheimer's diseases has documented a relative NADPH-d(+) cell sparing in striatal neurons with shrunken and foreshortened dendritic processes (Mufson E., & Branabur M., 1994). In the present study, an expressly developed experimental model was employed to evaluate the effects of PA in adult animals. (Clifton, et al., 1989, Ferrante, R., et al., 1985, Bjelke, B., et al. 1991). NADPH-d technique disclosed positive cells of the same type, homogeneously distributed in rat striatum and cortex in asphyctic and control animals as shown by shape factor analysis. However, striatal and cortical NADPH-d(+) neurons from rats subjected

to subsevere or severe PA proved highly cytomegalic compared with control and hypothermia-treated animals, suggesting that the pathological mechanism involved in PA is different from that in Alzheimer's, Parkinson's, and Huntington's diseases.

Cortical neuronal cytomegaly has been documented in pediatric epilepsy (Mischel, 1995). These findings are in agreement with similar alterations in rat striatal (Loidl, 1995, 1996) and cortical NADPH-d(+) neurons of the 6 month-old rats exposed to subsevere or severe PA.

Since NO induces DA release, which may be blocked the NO synthase inhibitor L-Me-Arg. (Zhu, X. & Luo, L., 1992) the highest basal level of striatal DA chronically maintained following an episode of severe PA (Herrera-Marchitz, M. et al., 1994) seems to correlate closely with the number of NO-containing cytomegalic striatal neurons.

Among other functions, NO plays a relevant role in regulating blood flow by inducing relaxation of the vascular smooth muscle (Palmer, R. et al., 1987) perhaps providing protection against ischemia by increasing oxygen supply. As NO stimulates DA release, which was reportedly to be toxic in striatum following ischemia, its potent vasoconstrictor effect may well worsen cell damage. However, as DA release is inhibited by hypothermia (Globus, M., et al., 1987, Globus, M. et al., 1988) its protective mechanism may be explained by inhibition of the excessive release of striatal DA.

Despite the importance of hypothermia to reduce the severity of ischemic cerebral damage described in rats (Busto, R. et al, 1987) and gerbils (Busto, R. et al, 1989) only a few studies were performed in PA, except during the fifties and sixties, when cold therapy plus positive pressure ventilation was introduced in order to resuscitate severely asphyctic human neonates, previously considered incapable of recovery. Results were excellent showing a rapid increase in Apgar scores. (Westin B., 1971, Busto, R., 1989).

The mechanisms underlying the protective effect of hypothermia are not clear. It is possible that one of the protective mechanisms in the hippocampus is a reduction of the normal synaptic drive on CA1 from the perforant path and CA3 (resulting in less glutamate release) at a time when these neurones are a reduction of the normal synaptic drive on CA1 from the perforant path and CA3 (resulting in less glutamate release) at a time when these neurones are in a highly vulnerable states. Perhaps, the prolonged duration of hypothermia also helped the cell to recover normal Ca^{2+} homeostasis. (Colbourne F., 1994). A decrease in metabolic demands takes place when body temperature is lowered, so that subsequent toxic effects are weakened and survival to prolonged exposure to asphyxia is ensured without permanent brain lesions.

The functional consequences that result from striatal and cortical NO-containing cytomegalic neurons after an episode of subsevere or severe PA remain to be determined. Further studies on cold therapy in the treatment of severe asphyxia in newborn should be carried out without delay.

In summary, the present findings demonstrate that an inappropriate activation of NOS enhanced in striatal and cortical neurons secondary to chronic PA in the presence of subsevere severe lack of oxygen is closely associated with ischemic neuronal injury. Moreover this study shows that hypothermia reduced neuronal damage suggesting that it may be a useful therapeutic following ischemic injury.

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LEGENDS TO FIGURES

Fig. 1: Photomicrographs of striatal NADPH-d(+) neurons from 6-month-old rats in sections from (a) control, (b) severe perinatal asphyxia (PA) and (c) 100min of PA at 15°C. Note cytomegalic soma and dendritic branches of a severe PA neuron (arrows) compared to control and PA-H cells. All cells at same magnification. Scale bar: 10µm.

Fig. 2: Photomicrographs of cortical NADPH-d(+) neurons from 6-month-old rats in sections from (a) control, (b) severe PA and (c) 100min of PA at 15°C. Note cytomegalic soma and tortuous dendritic branches of a severe PA neuron (arrows) compared to control and PA-H cells. All cells at same magnification. Scale bar: 10µm.

Fig. 3: Measurement of striatal NADPH-d(+) cell area (a), cell perimeter (b) and F-shape (c), in 6-month-old rats subjected to different periods of PA at 37°C or 15°C. Each value presents mean \pm SD (vertical lines) of determinations made from n=100 cells from each group. Asterisk indicates that differences in NADPH-d(+) cells in these two groups were highly significant ($p < 0.001$) compared with the remainder. Statistical analysis was performed by ANOVA test. There were no significant differences between subsevere and severe PA. No significant differences in F-shape (c) were found for any groups *inter se*.

Fig. 4: Measurement of cortical NADPH-d(+) cell area (a), cell perimeter (b) and F-shape (c), in 6-month-old rats subjected to different periods of PA at 37°C or 15°C. Data are means \pm SD (in vertical lines) of determinations made from n=100 cells from each group. Asterisk indicates that differences in NADPH-d(+) cells in these two groups were highly significant ($p < 0.001$) compared with the remainder (ANOVA test). There were no significant differences between subsevere and severe PA groups. No significant differences in F-shape (c) were found in any groups *inter se*.

Table 1. Number of neurones stained in the Striatum with NADPH-diaphorase techniques and asphyctic groups.

Region	Asphyctic time	Cell Number per field
Striatum Dorsal	Control	3.9 ± 1.19
	5' 37°	4.1 ± 1.19
	10' 37°	4.2 ± 1.03
	15' 37°	3.9 ± 1.07
	19' 37°	4.1 ± 1.19
	20' 37°	3.9 ± 1.07
	Hypothermia 20' 15°	3.8 ± 1.03
	Hypothermia 100' 15°	3.9 ± 1.19
Ventral Striatum	Control	4.1 ± 1.08
	5' 37°	3.9 ± 1.10
	10' 37°	4.1 ± 1.09
	15' 37°	3.9 ± 1.10
	19' 37°	3.8 ± 1.03
	20' 37°	4.5 ± 1.08
	Hypothermia 20' 15°	4.2 ± 1.09
	Hypothermia 100' 15°	4.1 ± 1.12
Medial Striatum	Control	3.9 ± 1.07
	5' 37°	3.8 ± 1.08
	10' 37°	3.9 ± 1.07
	15' 37°	3.9 ± 1.14
	19' 37°	3.9 ± 1.08
	20' 37°	3.6 ± 1.07
	Hypothermia 20' 15°	4.1 ± 1.07
	Hypothermia 100' 15°	4.1 ± 1.04
Striatum Lateral	Control	3.8 ± 1.09
	5' 37°	3.9 ± 0.97
	10' 37°	4.1 ± 1.05
	15' 37°	3.8 ± 1.07
	19' 37°	4.1 ± 1.05
	20' 37°	4.3 ± 0.94
	Hypothermia 20' 15°	3.9 ± 0.94
	Hypothermia 100' 15°	3.9 ± 1.17

Table 2. Number of neurons stained in the neocortex with NADPH-diaphorase techniques and aphyotic groups

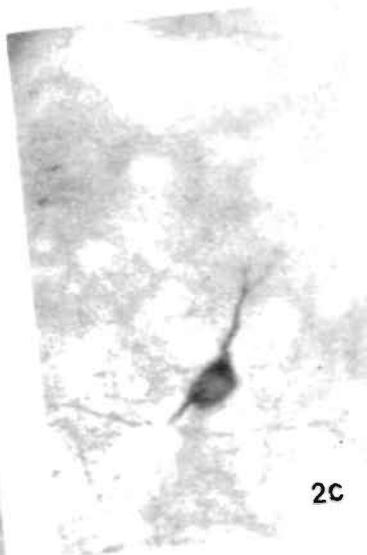
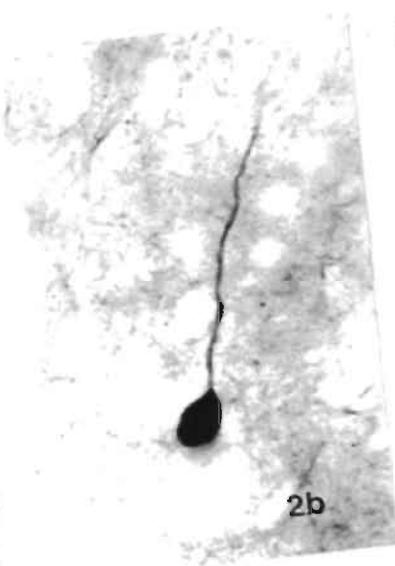
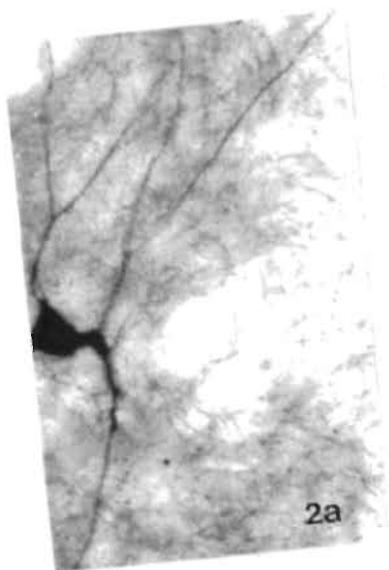
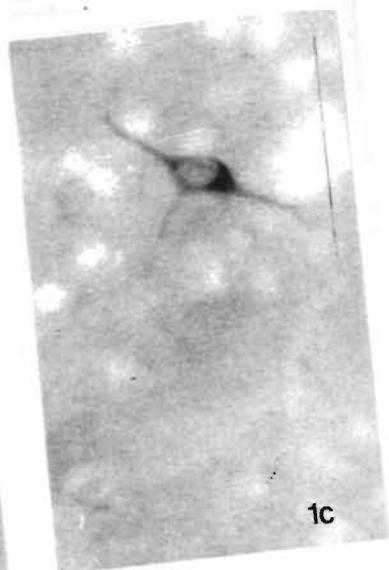
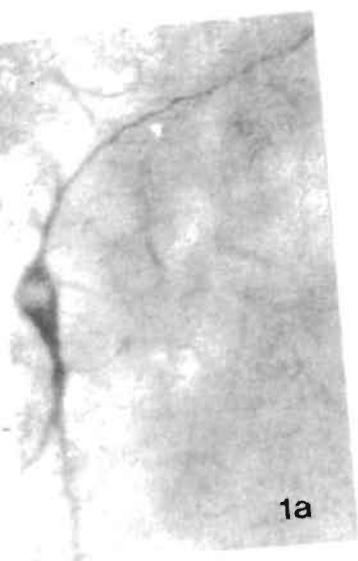
Region	Aphyotic time	Cell number per field
Lateral Neocortex	Control	3.7 ± 0.94
	5' 37*	4.3 ± 0.98
	10' 37*	3.6 ± 1.06
	15' 37*	3.5 ± 0.89
	19' 37*	4.2 ± 0.98
	20' 37*	3.7 ± 0.94
	Hypothermia 20' 15*	4.1 ± 0.97
	Hypothermia 100' 15*	4.3 ± 0.98
	Control	4.1 ± 0.81
	5' 37*	3.8 ± 0.91
	10' 37*	3.7 ± 0.97
	15' 37*	4.1 ± 0.99
	Medial Neocortex	19' 37*
20' 37*		3.6 ± 0.84
Hypothermia 20' 15*		4.3 ± 0.87
Hypothermia 100' 15*		4.1 ± 0.99

Table. 3 Striatum cells counts at 8.6 mm and 0.40 mm to bregma.

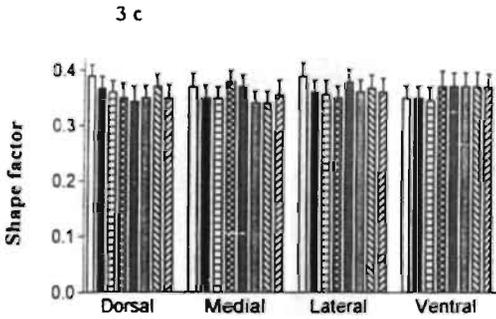
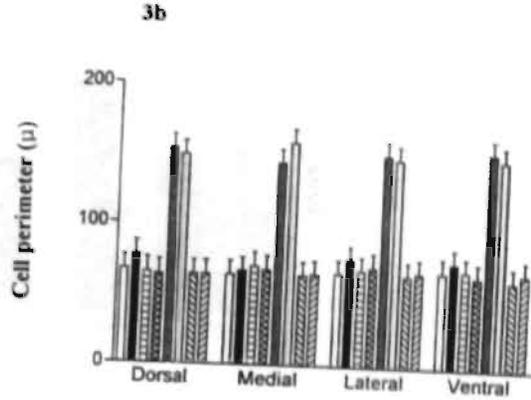
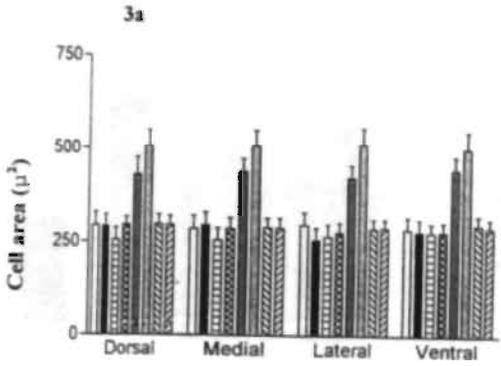
Region	Asphyctic time	Cell Number per field	
Medial striatum	Control	26.4 ± 3.92	
	5' 37°	26.9 ± 4.12	
	10' 37°	27.7 ± 4.27	
	15' 37°	28.2 ± 4.49	
	19' 37°	17.9 ± 3.68	
	20' 37°	17.3 ± 3.28	
	Hypothermia 20' 15°	28.6 ± 4.16	
	Hypothermia 100' 15°	28.1 ± 3.47	
	Control	27.1 ± 4.18	
	5' 37°	27.2 ± 3.85	
Dorsal Striatum	10' 37°	27.2 ± 3.61	
	15' 37°	27.3 ± 3.91	
	19' 37°	18.3 ± 3.71	
	20' 37°	18.3 ± 3.71	
	Hypothermia 20' 15°	27.6 ± 3.45	
	Hypothermia 100' 15°	26.1 ± 3.77	
	Control	25.8 ± 3.73	
	5' 37°	27.6 ± 4.53	
	10' 37°	27.9 ± 3.73	
	15' 37°	27.9 ± 3.73	
Lateral Striatum	19' 37°	18.2 ± 3.82	
	20' 37°	18.2 ± 3.92	
	Hypothermia 20' 15°	27.4 ± 3.79	
	Hypothermia 100' 15°	27.7 ± 3.90	
	Control	26.8 ± 3.91	
	5' 37°	26.2 ± 3.96	
	10' 37°	29.1 ± 3.94	
	15' 37°	26.5 ± 3.94	
	Ventral Striatum	19' 37°	17.8 ± 3.90
		20' 37°	17.1 ± 3.81
Hypothermia 20' 15°		29.3 ± 3.97	
Hypothermia 100' 15°		29.1 ± 3.96	

Table 4. Stratum cells counts at 8.6 mm and 0.40 to bregma.

Region	Anesthetic Time	Cell number per field
Lateral neocortex	Control	35.2 ± 4.19
	5' 37°	35.7 ± 4.43
	10' 37°	35.2 ± 4.07
	15' 37°	35.7 ± 3.92
	19' 37°	27.5 ± 3.56
	20' 37°	26.9 ± 3.67
	Hypothermia 20' 15°	35.2 ± 3.98
	Hypothermia 100' 15°	36.3 ± 3.76
	Medial neocortex	Control
5' 37°		37.4 ± 3.06
10' 37°		37.3 ± 3.19
15' 37°		37.1 ± 3.95
19' 37°		31.1 ± 3.78
20' 37°		31.7 ± 3.12
Hypothermia 20' 15°		39.6 ± 3.71
Hypothermia 100' 15°		39.9 ± 3.77

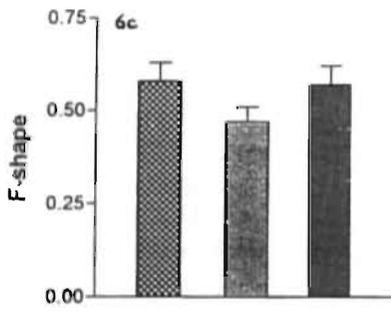
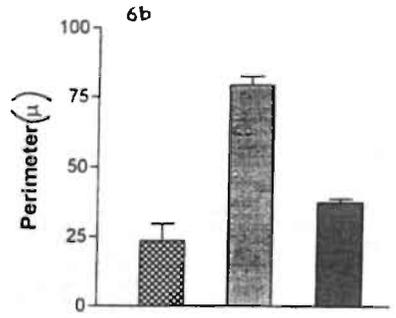
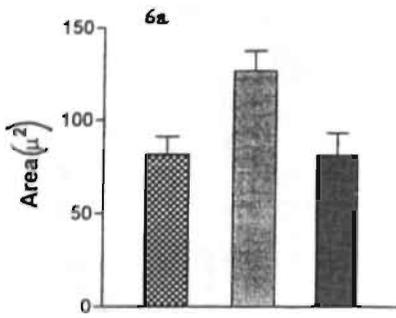


STRIATUM



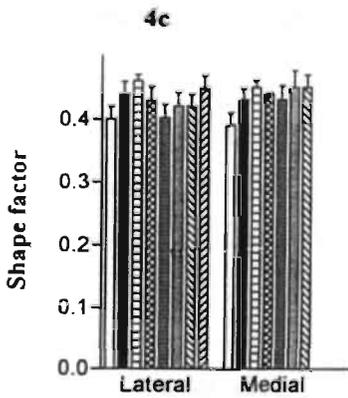
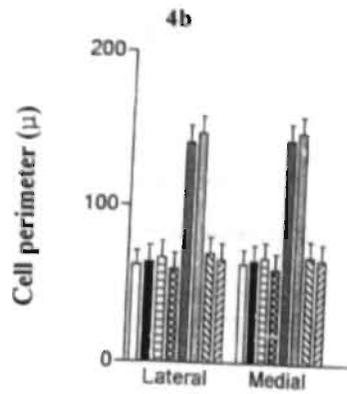
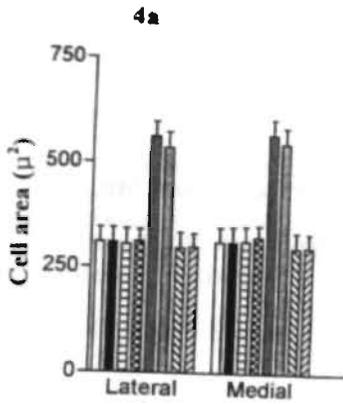
- Control
- 5' 37°C
- ▨ 10' 37°C
- ▩ 15' 37°C
- ▧ 19' 37°C
- ▦ 20' 37°C
- ▤ Hypothermia 20' 15°C
- ▣ Hypothermia 100' 15°C

STRIATUM



Control
19' 37°C
Hypothermia 100' 15°C

NEOCORTEX



- Control
- 5' 37°C
- 10' 37°C
- 15' 37°C
- 19' 37°C
- 20' 37°C
- Hypothermia 20' 15°C
- Hypothermia 100' 15°C

SHORT TERM CHANGES IN NADPH-DIAPHORASE REACTIVITY IN RAT BRAIN
FOLLOWING PERINATAL ASPHYXIA: NEUROPROTECTIVE EFFECTS OF COLD
TREATMENT.

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SUMMARY: Perinatal asphyxia (PA) produces changes in nitric oxide synthase (NOS) activity in neuronal and endothelial cells of the striatum and neocortex. The changes were examined using histochemical NADPH-diaphorase staining method. New born rats were exposed to severe PA at 37°C and another groups was subjected to severe PA under hypothermic condition (15°C) for 20 or 100 min. Quantitative image analysis was performed on striatal and neocortex in order to count cell number of reactive neurons and to compare the pattern of staining between the different groups of animals. Severe asphyctic pups showed an important neuronal loss in striatum and neocortex, that was reduced by hypothermia. NADPHd(+) neurons with reactive processes were found in the lateral zone of the striatum and neocortex in asphyctic pups. Controls and hypothermic striatum showed rounded cells without reactive process while no cells were stained in cortex. There was also an increase in NADPHd activity in endothelial cells in severe asphyctic pups in striatum and neocortex versus control and hypothermic treated animals. Our data evidenced that an inappropriate activation of NOS in neuronal and endothelial cells induced by PA is related with neuronal injury. Hypothermia inhibits neuronal injury and may be a valuable neuroprotective agent.

KEY WORDS: *Hypothermia - NADPH diaphorase - Perinatal asphyxia - Striatum - Cortex - Newborn rats*

1- INTRODUCTION

Asphyxia during birth is an important cause of perinatal mortality and neurology morbidity. Several organs may be affected during ischemia renal and cerebral injuries being the most common. (Williams, et al, 1992). Perinatal asphyxia (PA) is the main cause of cerebral hypoxia during ante and intra-partum. Although some infants recover completely from an asphyctic episode, others can develop permanent deficits such as hyperactivity syndrome, epilepsy, mental retardation or cerebral palsy (Volpe, 1987). The regions that are particularly sensitive to injury include layer 3-5 of the cortex, hippocampus (CA1 and CA4), striatum, thalamus, medial geniculate nucleus (MGN) and substantia nigra reticulata (Snr) (García et al, 1989, Meyer, 1989, Hossman, 1991). Although the mechanism responsible for this selective vulnerability is not well known (Pastchen, 1992) a brief event of global ischemia produce neurotransmitter alterations, e.g. in striatum and neocortex. (Bjelke et al, 1991, Herrera-Marschitz et al, 1993, Loidl et al, 1994)

It has been shown using animal experimental models of cerebral ischemia that decreases in brain temperature confer striking protection against ischemic neuronal injury (Grinsberg, et al 1992, Green, et al, 1992, Loidl et al, 1996). In previous reports we have demonstrated the important role of hypothermia in animal survival after the induction of PA. While 100% of survival was observed up to 16 min. of asphyxia when PA was induced at normal temperature (37°C) and no survival was seen after 22 min of asphyxia at the same temperature, on the opposite 100% survival was observed after 100 min. of asphyxia when PA was performed at low temperature (15°C) (Herrera Marschitz et al 1993, Loidl et al, 1995).

The precise cascade of events leading to secondary damage is unclear, although multiple mechanisms have been implicated. At the biochemical level, excitatory amino acids, (Choi, 1990) intracellular calcium accumulation, (Siesjo & Bengtsson, 1989) and increased free radical production are involved (Pellegrini-Giampietro, et al 1990). At the cellular level, apoptosis (programmed cell death) due to deprivation of growth factors and activity of inflammatory cells may play important roles. (Edwards, et al, 1995). Nitric oxide, initially identified as an endothelial-derived relaxing factor (Palmer et al., 1987), has been involved in the pathogenesis of brain injury (Moncada et al, 1990). NO reacts with superoxide anion to originate peroxynitrite anion (ONOO⁻), which in a protonate form, decays rapidly to form highly reactive radicals including hydroxyl radical (OH[•]) and nitrogen dioxide (NO₂) (Beckman et al, 1990). Furthermore, the excess of nitric oxide per se can be toxic to neurons (Garthwaite, J. 1991). The NOS possesses a NADPH binding site responsible of the diaphorase activity (Dawson and Snyder, 1994) making it possible to be detected by a histochemical reaction.

Therefore the relationship between the development of ischemic neuronal injury and NO production was examined following perinatal asphyxia in an attempt to clarify the role of NO in the generation of ischemic brain injury. In the present study NOS activity was investigated in neuronal cells of the striatum and neocortex, know to be the most vulnerable to a PA (Pasternak et al, 1991). In addition, immediately delivered pups that suffered severe PA were compared with asphyctic animals treated with hypothermia.

2-MATERIAL AND METHODS

Subjects

Fifty-two Sprague-Dawley pregnant rats (Certified Colony) and thirty post-ischemic male rats were purchased from the Breeding Animal Laboratory of the School of Biochemistry, and included in this study. The dams were bred by housing, three of them with one male until they were sperm positive as determined by vaginal smear. Pregnant rats were kept in individual stain-less steel cages and fed with Purine chow and tap water ad libitum. The 21st day following the original mating, individual female were placed in separate cages.

Induction of asphyxia

Asphyxia was induced in pups delivered by caesarean operations on pregnant rats. Gestational age was determined by vaginal smear. Rats within the last day of gestation were anaesthetised by i.p. injection of 28% (w/v) chloral hydrate (Merck) 0.1 ml/100g of body weight. Their entire uteri containing the foetuses were taken out by hysterectomy, and placed in a bath water at 37°C during 20 min. (severe asphyxia) (Herrera-Marschitz et. al., 1993) or at 15°C for 20 and 100 min. Caesarean-delivered control and asphyctic pups were obtained from the same mother. Following asphyxia, the uterus horns were rapidly opened and the pups removed and stimulated to breathe on a heating pad by cleaning off the delivery fluid and by tactile stimulation with small pieces of medical wipes.

Some survival asphyctic and caesarean delivered control pups (10 in each group) were decapitated following 30 min of recovery after birth, period during which survival, gasping, respiratory frequency, vocalisation, colour of the skin and spontaneous

movements were recorded. Animals used for morphological studies were killed and brains were fixed by immersion during 2 hs. with 4% paraformaldehyde in 0.1M phosphate buffer, pH 7.4. After fixation, brains were cut on a Leitz cryostat and 20 μ m sections mounted on gelatine-coated glass slides.

Histochemistry

Sections from treated (PA, PA + hypothermia) and control animals were processed using the NADPH-d histochemical method (Vincent and Kimura, 1992). Briefly, sections were incubated for 1h at 37°C in the same reaction mixture containing 0.1% NADPH and 0.02% nitroblue tetrazolium diluted in 0.1M phosphate buffer with 0.3% Triton X-100 (all reagents were purchased from Sigma). Sections were mounted in PBS/glycerol (1:3) observed and photographed with a Zeiss Axiophot microscope. In absence of β -NADPH, cells were not stained. When it were substituted NADPH for NADP nonspecific staining resulted in the entire section. Nitroblue tetrazolium contains a monoformazan impurity that gives a diffuse purple background (Valtschanoff, et. al., 1993); to remove this background, some sections were incubated in acetone dimethyl sulfoxide for 10 minutes before mounting.

Histology

Rats subjected to 30 min. of PA or PA + hypothermia were anaesthetised with 28 % (w/v) chloral hydrate, 0.1 ml/100g of body weight and decapitated. Adjacent coronal sections (40 μ m thick) were collected and stained with cresyl violet. Bilateral counts of neurons (well defined nucleus, distinct cellular membrane and not shrunken) were performed by an observer blind to treatment conditions.

Neurons were counted in the lateral, medial, dorsal and ventral striatum neurons and in the medial and lateral sectors of neocortex following the Paxinos Atlas. (Paxinos 1993).

Computerized Image Analysis

Selected brain sections of the NADPHd (+) and cresyl violet stained neurons of different groups, were measured by using a computerised image analyser (KONTRON-ZEISS VIDAS). Selected fields were located in the lateral sectors of neocortex. Sections of 40 μm thick with adequate labelling of NADPHd were randomly selected from coded slides (6 for each of considered groups). Data from 50 selected striatum and surrounding neocortex were averaged for each animal. Ten cells per sections were analysed (total: 100 cells per group). Light microscope images were transferred by a video camera connected to an interactive image analysis system on line. (KONTRON ZEISS VIDAS) . The images were digitised into an array of 512x 512 pixels corresponding to 140 x140 μm (40X primary magnification). The resolution of each pixel was 256 grey levels (8 grey bits level resolution). After automatic normalization of grey-scale, interactive delineation and contrast enhancement of the neuronal image were performed, following the removal of interfering non specific images. Morphometric parameters (area, perimeter, maximum and minimum diameter, shape factor) from NADPHd (+) neurons in sections of experimental and control groups were measured. The mean and SD were calculated in treated and control groups.

The spatial distribution of NADPHd(+) and cresyl violet staining neurons were counted using VIDEOPLAN image analysis system. They were digitised into 256 grey levels in measuring fields, 215 x 180 μm in size (20X primary magnification). NADPH

(+) cells count per measuring field were performed. The mean and SD were calculated in the different fields from asphyctic and control groups.

Statistical Analysis

Differences between means and SD of the experimental and control groups were analysed statistically using one-way analysis of variance (ANOVA) and subsequently the Newman-Keuls test, with a p value of less than 0.05 being considered significant. ANOVA and Newman-Keuls test were routinely performed on an IBM compatible PC AT486 package software (primer, Mc Graw Hill, Inc.)

RESULTS

Survival and early behaviour:

Rats subjected to > 20 min of PA were considered severe asphyctic animals. As showed in previous reports (Herrera-Marschitz et al, 1993, Loidl et al, 1993, 1994) a drastic decrease of survival (about 80%) and deteriorated physiological conditions were observed in this group of animals in comparison with the controls (presence of gasping, decrease in respiratory frequency and vocalization, akinesia and pale - skin). No pups survived more than 22 min of asphyxia, but at 15°C, 100% survived without behavioural differences with the control group. After 20 min. and 100 min of PA at 15°C all pups survived after intense manual stimulation but showed gasping, decrease in respiratory frequency, no vocalisation, pale skin and akinesia.

Cresyl Violet staining in the striatum

Normothermic (37°) severe asphyctic animals exhibited nearly 50% neuronal loss in striatum. Hypothermia (15°C) resulted in virtually no striatum neuronal injury as was also the case in the control animals. Table 1 summarizes the values of striatum neuronal counting and the estimate of neuronal loss in striatum of normothermic and hypothermic ischemic group. Normothermic animals exhibited significantly greater neuronal loss in striatum than hypothermic ischemic animals ($P>0.001$). When compared different regions of the striatum (ventral, dorsal, lateral, medial) no significant differences were found. (Table 1)

NADPH histochemical staining in striatum asphyctic new-born rats:

Pups exposed to severe PA, control PA + hypothermic conditions showed NADPHd (+) cells in the lateral zone of the striatum (Fig. 1). In control pups, obtained by caesarea or by natural delivery, the NADPH-d (+) cells stained only the soma but not the processes (Fig. 2a). Animals exposed to severe asphyxia exhibited NADPH-d (+) neurons with highly reactive processes (Fig 2b). Animals exposed to PA for 20 min. and 100 min in hypothermic at 15°C (Fig. 2c) showed the same pattern of staining than controls. Striatum cell area and perimeter increased significantly in rats exposed to severe asphyxia in comparison with control and PA + hypothermia 15°C treated animals during 20 min. and 100 min. (see Fig. 6a, 6b, 6c). There were not significant differences ($P < 0.001$) in number of cells (data not shown). When different regions of the striatum (dorsal, ventral, lateral and medial) were compared no significant differences (neither in any morphologic parameter nor in the number of cells) were detected. The NADPH diaphorase activities in endothelial cells of the medium-diameter vessels were more intense than in controls.(4a, 4b) Hypothermia during 20' and 100' reduced the NADPH diaphorase reactivity in endothelial cells, reaching similar levels to those observed in controls. (Fig 4c).

Cresyl violet in the neocortex

Severe normothermic (37°C) asphyctic animals showed a 45% neuronal loss. Hypothermia (15°C) during 20 min. and 100 min. reduced significantly neocortical neuronal loss ($P > 0.001$). Table 2 showed the neocortical neuronal counting and the estimate of the neuronal loss in normothermic and hypothermic groups. Normothermic

asphyctic animals exhibited significantly greater neuronal loss than hypothermic animals. When compared different sectors of the neocortex (lateral and medial) no significant differences were found ($P>0.001$) (Table 2)

NADPH histochemical staining in neocortex asphyctic newborn

Rats subjected to severe PA in parietal neocortex showed the same morphological neuronal changes as those observed in the striatum.(Fig 3b) Non- stained cells were also seen in the cortex of the control and hypothermic control rats (Fig 3a, 3c). The NADPH diaphorase staining in endothelial cells of medium-diameter vessels became more intense during severe asphyxia in comparison with controls.(5a, 5b) Hypothermia at 15°C during 20 min. and 100 min. reduces the NADPH-d(+) reactivity in endothelial cells reaching similar levels to those observed in controls.

DISCUSSION

Immediately after inducing asphyxia NADPH-d changes in the lateral zone of striatum as well as in some cortical neurons are observed. The entire cell including the soma and processes are intensely stained. Striatal NADPH-d (+) neurons increase the reactivity of their processes, but were always localized in the lateral zone of the striatum, suggesting that severe PA could stimulate some kind of NADPH reactivity. An increase in NADPH-d (+) staining of the blood vessels in striatum and cortex was also observed in the severe asphyctic animals, indicating that at this early post-ischemic period, vasodilatation takes place in an effort to enhance the oxygen supply to the tissues.

The negative staining to NADPH-d in cortical neurons of control rats is in agreement with a recent report showing that this enzyme is present up to the 19th prenatal day in the rat, and decrease until day 0 postnatal (Bredt et al, 1994). In those rats in which PA was induced, some cortical granular type neurons expressed NADPH-d

Although NO synthase (NOS) has several isoforms, they may be divided into two categories with different regulatory mechanisms and activities: The constitutive Ca^{2+} regulated NOS (Bredt et al, 1991) and the inducible iNOS, Ca^{2+} -independent (Xie, et al, 1992). Endothelial cells express both types of this enzyme. (Radomski et al., 1990). The cNOS in endothelial cells regulates blood vessel tone by continuously releasing NO but when there is an excess amount of NO produces an activation of endothelial iNOS, (Moncada et al., 1991) leading to an inappropriate vasodilatation. This process induces an increase in capillary permeability and the efflux of blood component decreasing blood

flow (Kubes and Granger, 1992; Chi et al., 1994). These alterations in endothelial permeability may mediate edema formation and subsequently accelerate a decrease in blood flow in the area with overactivation of endothelial NOS. In agreement with a recent report, (Nakashima, 1995) the observation that endothelial cells in the damaged striatum and neocortex exhibited a high level of NADPH-d reactivity after period of ischemia suggests a high correlation of endothelial NOS activity with the progression of neuronal loss in response to cerebral ischemia. Then, an excessive amount of endothelial NO response to a severe perinatal asphyxia seems to play a role in triggering ischemic neuronal injury.

The protective effect of hypothermia on the brain ischemia in the rat has been extensively investigated (Coimbra and Wieloch et al, 1992, Edwards et al, 1995, Loidl et al, 1996). The mechanisms of protection by hypothermia are multifactorial. (Ginsberg et al, 1992) The most important mechanism is a decrease in the basal metabolic rate, allowing the brain to preserve the energy (including ATP). The second probable mechanism is the reduction of the ischemia induced increase in glutamate and dopamine levels (Busto et al, 1987). Hypothermia may attenuate the free radical increments detected in the reperfusion period after cerebral ischemia (Baiping, L., 1994). In agreement with other studies >20 min. (Minasawa, et al 1990, Williams C.E., et al 1991) of brain asphyxia caused severe neuronal damage in hippocampus cortex and striatum. However, under hypothermic conditions (35° C, 33°C) the neuronal injury was reduced, with significant protection. Our data are consistent with the previous findings, and we now demonstrate that whole body hypothermia 15°C during 20 min. and 100 min. prevents asphyctic neuronal damage induced by 20 min. of asphyctic ischemia in the rat.

Hypothermia also reduces the NADPH-d reactivity of endothelial blood vessels cell protecting against neuronal damage.

Therefore, these findings demonstrate that an alteration in activation of NOS in striatum and neocortex neurons and endothelial cells is associated with PA neuronal cell damage in this regions of the brain. Moreover our data demonstrate that 15°C hypothermia during ischemia is remarkably beneficial and suggest that it may be of clinical use for cases of ischemic injury.

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LEGEND OF FIGURES

- Fig.1 NADPH-d positive areas (shadow) in control and asphyctic newborn rats. Cx cerebral cortex; St: striatum; CC: corpus callosum.
- Fig.2 Photomicrographs of striatal NADPH-d(+) neurons in sections from (a) control, (b) severe perinatal asphyxia (PA) and (c) after 100min of PA at 15°C. Note a unipolar process in PA severe asphyctic neurons (arrows) in comparison with control and hypothermia. All cells at the same magnification. Scale bar: 10µm.
- Fig. 3: Photomicrographs of cortical NADPH-d(+) neurons in sections from (a) control, (b) severe PA and (c) 100 min of PA at 15°C. Note a unipolar process in a severe PA neuron (arrows) and no stained cell in control and hypothermia sections. All cells at the same magnification. Scale bar: 10µm.
- Fig.4: Photomicrographs of striatal NADPHd (+) blood vessels in sections from (a) control, (b) severe PA and (c) 100 min. of PA at 15°C. Note an increase in endothelial NADPHd reactivity in severe PA compared with control and hypothermia.
- Fig.5 Photomicrographs of cortical NADPHd (+) blood vessels in sections from (a) control, (b) severe PA and (c) 100 min. of PA at 15°C. Note an increase in endothelial NADPHd reactivity in severe PA, compared with control and hypothermia.

Fig. 6 Measurement of striatal NADPH-d(+) cell area (a), cell perimeter (b) and F-shape (c), in rats subjected to different periods of PA at 37°C or 15°C. Each value presents mean \pm SD (vertical lines) of determinations made from n=100 cells from each group. Statistical analysis was performed by ANOVA test. No significant differences in shape factor (c) were found for any groups *inter se*.

Table 1. Striatum cells counts

Region	Asphyctic Time	Cell Number per field
Medial Striatum	Control	19.9 \pm 1.85
	20' 37°	11.5 \pm 1.78
	Hypothermia 20' 15°	19.4 \pm 1.92
	Hypothermia 100' 15°	19.3 \pm 1.84
Dorsal Striatum	Control	20.2 \pm 1.92
	20' 37°	12.2 \pm 1.96
	Hypothermia 20' 15°	19.6 \pm 1.95
	Hypothermia 100' 15°	19.5 \pm 1.87
Lateral Striatum	Control	20.4 \pm 1.95
	20' 37°	11.4 \pm 1.87
	Hypothermia 20' 15°	19.9 \pm 1.97

Table 2. Neocortex cells counts

Region	Asphyctic Time	Cell Number per field
Lateral NeoCortex	Control	25.4 ± 1.71
	19'37°	14.6 ± 1.75
	Hypothermia 20'15°	24.6 ± 1.76
	Hvnothermia 100'15°	25.6 ± 1.73

