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Research Article

Cholinergic, monoaminergic and glutamatergic changes following perinatal asphyxia in the rat

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Abstract. Perinatal asphyxia (PA) is considered to lead to a variety of brain disorders including spasticity, epilepsy, mental retardation, and minimal brain disorder syndromes and may form the basis for psychiatric and neurodegenerative diseases later in life. We examined markers for neuronal transmission involved in the pathomechanisms of PA and candidates as mediators for long-term sequelae. We tested tyrosine hydroxylase (TH) and the vesicular monoamine transporter (VMAT) representing the monoaminergic system, the vesicular acetylcholine transporter (VAChT), and the excitatory amino acid carrier 1 (EAAC1), a neuronal subtype of the glutamate transporter, using immunohistochemistry on brain sections of rats subjected to graded PA. Three months following the asphyxiant insult immunoreactive (IR)-TH was decreased in striatum, hippocampus, thalamus, frontal cortex, and cerebellum; IR-VMAT was increased, and IR-VAChT was decreased in striatum. IR-EAAC1 glutamate transporter was increased in frontal cortex. The cholinergic, monoaminergic, and glutamatergic changes, still observed 3 months after the asphyxiant insult, may reflect their involvement in the pathomechanisms of PA and indicate mechanisms leading to long-term complications of PA. The variable consequences on the individual markers in several brain regions may be explained by specific susceptibility of cholinergic, monoaminergic, and glutamatergic neurons to the asphyxiant insult.

Key words. Perinatal asphyxia; cholinergic; monoamine; glutamate; excitatory amino acid; brain; neuronal death.

Introduction

Perinatal asphyxia (PA) is considered to lead to a variety of brain disorders including spasticity, epilepsy, mental retardation, attention deficit disorders [1–3], and minimal brain disorder syndromes, and may form the basis for psychiatric and neurodegenerative diseases later in life [1–5]. It is therefore of the utmost importance to investigate tentative mediators as, e.g., neurotransmitters, involved in the pathogenesis of PA and its long-term complications.

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The monoaminergic system in PA has been examined by several groups and the sensitivity of this system to asphyxiant damage has been demonstrated in humans and in animal models [5–7] mainly revealing short- and long-term dopaminergic and tyrosine hydroxylase deficits [8–11 and references cited therein].

With the exception of recent studies on the effect of nicotine on the pathophysiology of PA, indicating an interaction between the cholinergic and the monoaminergic neurons [8], the only systematic study on cholinergic neurons following PA has been that of Burke and Karanas [12]: in a quantitative morphological analysis,

the authors described a 23% decrease in cholinergic neurons at the age of 4 weeks and a long-lasting cholinergic deficit in the striatum.

Information on the role of excitatory amino acids (EAAs) in PA is limited and only a few groups have been stressing the problem of glutamatergic functions: Otoya et al. [13] showed that exposure of newborn rats to hypoxia can generate acute and long-lasting effects on the N-methyl-D-aspartate (NMDA) receptor interfering with glutamatergic transmission. The effect of

Table 1. IR-VAChT in individual brain regions.

	Normoxia	10 min PA	20 min PA
Striatum (caudate-putamen)			
IR-perikarya in fiber bundles			
Mean ± SD	3.1 ± 1.24	2.4 ± 1.43	1.2 ± 1.41
n	10	8	10
P		n.s.	<0.01
Hippocampus (CA1)			
IR-perikarya per 1000 μm			
Mean ± SD	3.6 ± 1.84	3.4 ± 1.34	2.8 ± 1.75
n	9	8	10
P		n.s.	n.s.
Thalamus (ventrolateral/ventroanterior)			
IR-fibers per area			
Mean ± SD	9.1 ± 3.43	8.5 ± 1.98	8.7 ± 2.73
n	10	8	10
P		n.s.	n. s.
Cortex (frontal; outer pyramidal layer)			
IR-perikarya per area			
Mean ± SD	2.3 ± 0.32	2.0 ± 0.54	2.2 ± 0.72
n	9	8	10
P		n.s.	n.s.
Cerebellum (granular layer)			
Percent of all perikarya			
Mean ± SD	37.6 ± 5.6	39.9 ± 3.6	40.1 ± 5.2
n	10	10	10
P		n.s.	n.s.

Table 2. IR-VMAT in individual brain regions.

	Normoxia	10 min PA	20 min PA
Striatum (globus pallidus)			
Fibers			
Mean ± SD	13.7 ± 5.18	15.7 ± 4.55	21.0 ± 4.42
n	10	10	10
P		n.s.	<0.01
Hippocampus (n. dentatus)			
Fibers in white matter			
Mean ± SD	38.8 ± 18.0	42.3 ± 13.7	25.7 ± 8.52
n	9	10	10
P		n.s.	n.s.
Thalamus (ventrolateral/ventroanterior)			
IR-fibers per area			
Mean ± SD	9.9 ± 3.52	8.0 ± 4.12	6.8 ± 3.55
n	10	10	10
P		n.s.	n.s.
Cortex (frontal; outer pyramidal layer)			
IR-fibers			
Mean ± SD	16.5 ± 3.71	16.0 ± 3.97	16.4 ± 4.17
n	10	10	10
P	n.s.	n.s.	
Cerebellum			
IR-fibers in zona moleculare			
Mean ± SD	6.0 ± 3.24	4.8 ± 3.51	6.9 ± 3.48
n	10	8	10
P		n.s.	n.s.

Table 3. IR-TH in individual brain regions.

	Normoxia	10 min PA	20 min PA
Striatum (globus pallidus)			
IR-fibers			
Mean ± SD	38.5 ± 11.84	19.1 ± 8.78	11.4 ± 6.83
n	14	12	14
P		<0.001	<0.001
Hippocampus (n. dentatus)			
IR-fibers in white matter			
Mean ± SD	45.3 ± 34.86	19.2 ± 14.43	20.3 ± 11.99
n	10	10	10
P		<0.05	<0.05
Thalamus (ventrolateral/ventroanterior)			
IR-fibers			
Mean ± SD	10.7 ± 5.36	8.5 ± 2.65	5.2 ± 2.01
n	10	10	10
P		n.s.	<0.02
Cortex (frontal; outer pyramidal layer)			
IR-fibers			
Mean ± SD	12.0 ± 4.39	10.2 ± 4.83	6.2 ± 2.99
n	10	10	10
P		n.s.	<0.01
Cerebellum			
IR-fibers in white matter			
Mean ± SD	11.5 ± 5.15	5.0 ± 2.39	6.4 ± 3.87
n	10	15	12
P		<0.01	<0.02

Table 4. IR-EAAC1 in individual brain regions.

	Normoxia	10 min PA	20 min PA
Striatum (caudate-putamen)			
IR-perikarya in fiber bundles			
Mean ± SD	1.3 ± 1.02	0.9 ± 0.59	0.8 ± 0.32
n	10	10	10
P		n.s.	n.s.
Hippocampus (CA2 and CA3)			
IR-perikarya per 1000 μm			
Mean ± SD	52.8 ± 14.53	53.2 ± 12.28	47.2 ± 11.13
n	10	9	10
P		n.s.	n.s.
Thalamus (ventrolateral/ventroanterior)			
IR-perikarya per area			
Mean ± SD	0.6 ± 0.33	0.4 ± 0.23	0.6 ± 0.34
n	10	10	9
P		n.s.	n.s.
Cortex (frontal; outer pyramidal layer)			
IR-perikarya per area			
Mean ± SD	1.5 ± 0.47	1.5 ± 0.43	2.3 ± 0.55
n	10	9	10
P		n.s.	<0.001
Cerebellum (medial nucleus)			
IR-perikarya			
Mean ± SD	2.4 ± 0.5	2.0 ± 0.3	2.0 ± 0.7
n	10	10	9
P		n.s.	n.s.

cerebral hypoxia on NMDA-receptor-binding characteristics in newborn piglets was studied by Fritz et al. [14], who showed that hypoxia decreased glutamate binding to the NMDA receptor. Martin et al. [15] reported that hypoxia-ischemia caused abnormalities in glutamate transporters and death of glial cells and neurons in piglet newborn striatum. Glutamate accumulation in cultured rat cerebellar granule cells obtained

from intrauterine hypoxia-ischemia experiments was shown by Cai et al. [16]. Gilland et al. [17] provided indirect evidence for the intraischemic involvement of glutamate release: using a glutamate release inhibitor, the authors showed that pre- but not post-treatment with this inhibitor was neuroprotective. Nozaki and Beal [18] found that kynurenic acid, the only endogenous EAA receptor antagonist in the central nervous system, was neuroprotective in a neonatal model of hypoxia-ischemia. Sher [19] showed that competitive EAA antagonists in contrast to MK-801, a non-competitive EAA antagonist, were neuroprotective in fetal mouse cerebral cortical cell cultures. Silverstein et al. [20] used microdialysis to show that hypoxia-ischemia stimulated hippocampal glutamate efflux in perinatal rat brain, providing evidence for the hypothesis that synaptic concentrations of the endogenous EAA glutamate increase during the development of hippocampal ischemic injury. We showed increased glutamate and aspartate in the identical animal model at the same time, i.e., 3 months following PA, in hypothalamus but not in other brain areas as e.g., frontal cortex, striatum, hippocampus and cerebellum, although lesions were clearly identified in these regions [21].

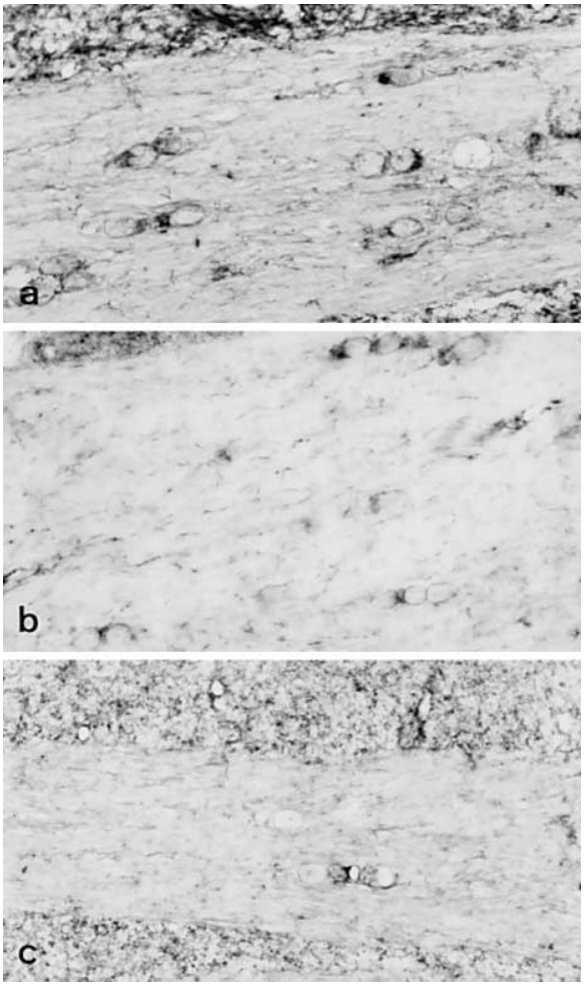

Figure 1. Striatum, immunohistochemical staining of VAChT after PA (left = rostral, right = dorsal). Under normoxic conditions (*a*), most of the perikarya within the fiber bundles are immunoreactive in the cytoplasm. After 10 min of PA (*b*), the density of stained cells is reduced. Following 20 min of PA (*c*), the overall density of neurons including immunoreactive ones is reduced. × 500.

Taken together, the limited information available does not allow an evaluation of the nature of neurotransmitter imbalances in PA. The involvement of the monoaminergic system in post-asphyxiant brain damage as reflected by reduced tyrosine hydroxylase (TH) and dopamine levels, however, is widely accepted. Cholinergic neuronal loss in PA has been documented by a single morphological study whereas overexcitation by EAAs leading to excitotoxic lesions has been published by several groups.

The aim of this study was to characterize the nature (monoaminergic, cholinergic, glutamatergic) of neuronal cell loss described in a previous publication on our model of PA, by studying TH and the vesicular monoamine transporter (VMAT), both markers for the monoaminergic system, the cholinergic marker vesicular acetylcholine transporter (VAChT) and the EAA carrier 1 (EAAC1), a marker for the glutamatergic system. The brain regions frontal cortex, striatum, thalamus, and cerebellum were selected as they are considered hypoxia sensitive and hippocampus was included, because neuronal cell loss in PA was prominent there, as shown recently [21].

Materials and methods

Animal experimental design. Asphyxia was induced in pups delivered by cesarean section on pregnant Sprague-Dawley rats [22–24]. Within the last day of gestation as evaluated by estabularium protocols, animals were sacrificed by neck dislocation and hysterectomized. The uterus horns, still containing the fetuses, were extirpated and placed in a water bath at 37 °C for various periods from 5 to 20 min. Cesarean-delivered control and asphyxiated pups were obtained from the same mother, since each rat delivered approximately 10–14 pups. Following the asphyxiant period, i.e., incubation at 37 °C, the uterus horns were rapidly opened and the pups removed. Pups were cleaned, the umbilical cord was ligated, and the animals were allowed to recover in a hood. Only litters with pups weighing more than 4.5 g at the time of delivery were used in the experiments. Ten pups per group (normoxia, 10 min and 20 min of asphyxia) were studied. In the group with

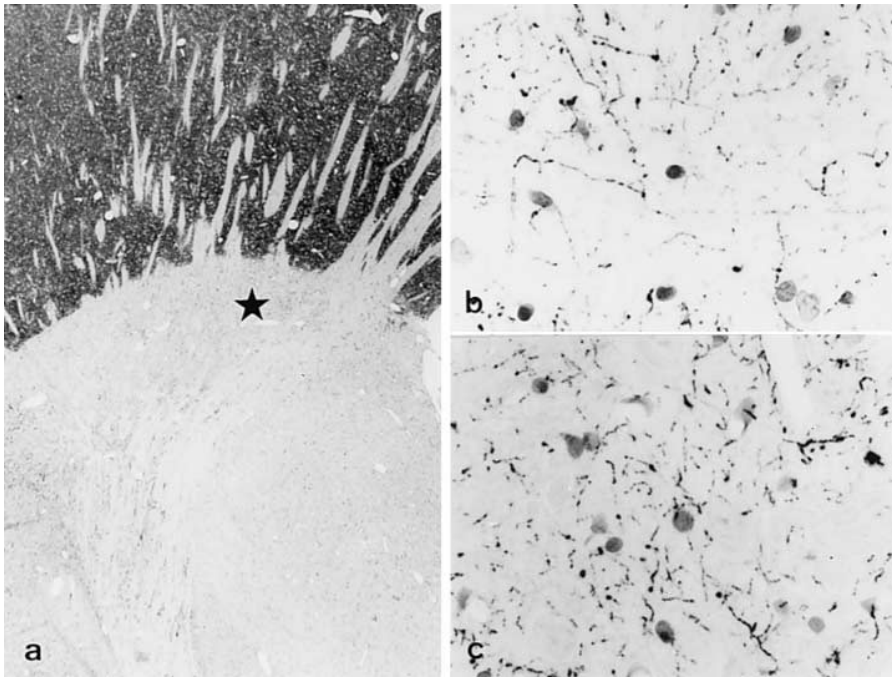

Figure 2. Striatum, immunohistochemical staining of VMAT and nuclear counterstaining in controls (*a*, *b*) and after 20 min PA (*c*) (left = rostral, right = dorsal). The asterisk indicates the region which was morphometrically analyzed for fiber density and is shown in detail in (*b*, *c*). Note the increase in immunoreactive fibers after 20 min PA. (*a*) × 32, (*b*, *c*) × 500.

normoxia, and 10 min asphyxia, 100% of pups survived; in the group with 20 min of asphyxia, 74% survived. At 21 min of asphyxia, only 10% of pups survived. After adaptation in a hood, rat pups were given to surrogate mothers until the end of the study.

Animal experiments were carried out according to the rules of the American Physiology Society.

Histological examinations. Animals used for histological studies at the age of 3 months were anesthetized (Membumal 50 mg/kg body weight, intraperitioneal) and perfused transcardially with 30–50 ml of 0.1 M phosphate-buffered saline (PBS) pH 7.4 containing 4% paraformaldehyde. Approximately 50–60 min after perfusion, the brain was removed from the skull, post-fixed in the same solution for 12–18 h and then kept in PBS containing 20% sucrose at 4 °C, pending sectioning. Paraffin embedding, preparation of 20-µm sections cut in the parasagittal plane, and dewaxing by xylene have been described elsewhere [25].

Immunostaining. The brain regions striatum, frontal cortex, hippocampus, thalamus and cerebellum, known to be hypoxia sensitive, were selected for evaluation of neuronal cell loss and/or morphological evaluations. In striatum, caudate-putamen was used for determination of immunoreactive (IR)-VAChT and IR-EAAC1 and globus pallidus for determination of IR-TH and IR-VMAT according to the regional distribution of neurotransmitters in control animals. In the hippocampus we used area CA1 for evaluation of cholinergic, dentate nucleus for monoaminergic (IR-VMAT and IR-TH) and CA2 and CA3 for glutamatergic (IR-EAAC1) innervation following the principle cited above. Ventrolateral/ventroanterior thalamus and the outer pyramidal layer of frontal cortex present all three types of innervation and therefore these structures were used for neurotransmitter immunohistochemistry. In cerebellum, the granular layer presents with cholinergic innervation, zona moleculare with VMAT immunoreactivity, white matter with IR-TH and medial nucleus with glutamatergic innervation, thus forming the rationale for selecting the individual cerebellar structures. Antibodies against VAChT and EAAC1 predominantly stained perikarya, whereas VMAT-, IR-TH preferably stained fibers.

Paraffin sections mounted on silanized glass slides were dewaxed in four changes of xylol for 15 min each, rehydrated in decreasing concentrations of ethanol, and rinsed in distilled water and two changes of PBS pH 7.4. Slides were subjected to an antigen retrieval procedure which consisted of autoclaving them in 1 mM EDTA pH 7.9 at 121 °C for 2 min and allowing to cool down overnight. Slides were rinsed in PBS twice, incubated

with protein blocking solution designed for automated immunostaining (Dako, Glostrup, Denmark) for 10 min at room temperature and incubated with the primary antibodies (all purchased from Chemicon International, Temecula, Calif.). For staining of the monoaminergic structures, we used rabbit anti-VMAT2 affinity-purified polyclonal antibody diluted 1:500 and rabbit anti-TH affinity-purified antibody diluted 1:100. For staining of cholinergic structures, we used goat anti-VAChT polyclonal antibodies diluted 1:100. For visualization of the glutamatergic system, we used goat anti-glutamate transporter (EAAC1) polyclonal antibody diluted 1:400. All primary antibodies were diluted in Dako antibody diluens and incubated for 2 h. Following the incubation with the first antibody, slides were rinsed in three changes of PBS. All secondary antibodies and chemicals were purchased for automated immunostaining applications (Dako ChemMate system). The secondary antibodies were species specific and biotinylated and were applied for 1 h. Biotin was de-

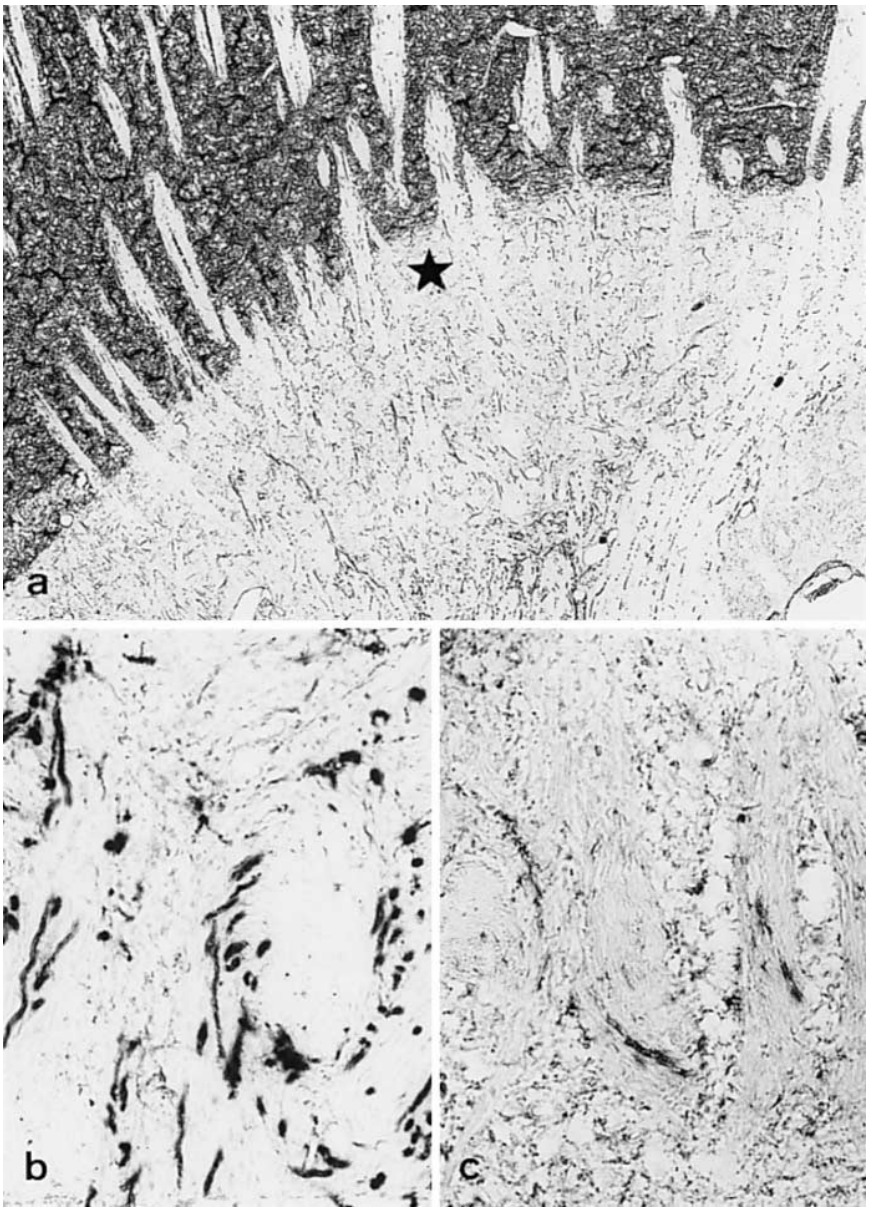

Figure 3. Striatum, immunohistochemical staining of TH in controls (*a*, *b*) and after 20 min PA (*c*) (left = rostral, right = dorsal). The asterisk indicates the region which was morphometrically analyzed for TH-positive fibers. Sections from this region are shown in detail in (*b*, *c*). After 20 min of PA there are evidently less TH-positive fibers (*a*) × 50, (*b*, *c*) × 500.

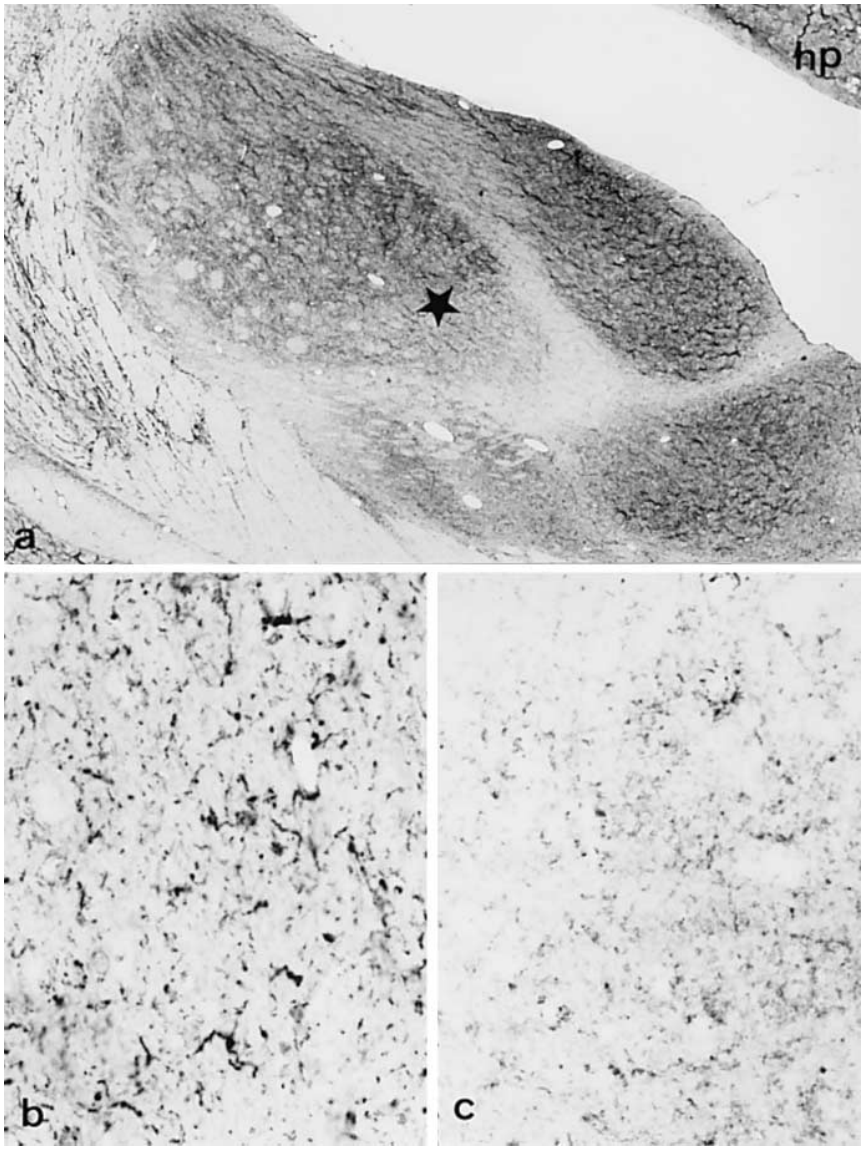

Figure 4. Thalamus and a small part of hippocampus (hp): immunohistochemical staining of TH in controls (*a*, *b*) and after 20 min PA (*c*) (left = rostral, right = dorsal). The asterisk indicates the region which was morphometrically analyzed for TH-positive fibers. Sections from this region are shown in detail in (*b*, *c*). After 20 min of PA, the density of TH-positive fibers is decreased compared to the normoxic control. (*a*) × 32, (*b*, *c*) × 500.

tected with the horseradish-peroxidase-conjugated streptavidin and diaminobenzidine reaction. When appropriate, slides were counterstained with hematoxylin (Dako), rinsed in water, dehydrated in an ethanol series and mounted with eukitt. Photographs were taken on a Nikon Microphot FMX light microscope. Kodak Technical Pan films 160 ISO were used and printed on Ilford photographic paper.

Morphometry. To determine differences in immunoreactivity between brain regions of controls and asphyxiated animals, we used morphometric procedures on histological sections based upon the principles of Burck [26]. IR neuronal fibers or perikarya were counted on micrographs or camera lucida drawings over a microscopic field corresponding to 5000–15,000 μm^2. The density values obtained were converted to immunoreac-

tivity units per 1000 μm^2, except for the neuronal cell counts in the hippocampus which were evaluated along the cell layer of 1000 μm.

Statistical calculations. Means and standard deviations were calculated. For the comparison of groups, morphometrical data were analyzed using ANOVA with a subsequent Kruskal-Wallis test.

Results

The results of the immunoreactivities of TH and transporters are summarized in tables 1–4 where number of samples, means and standard deviations, and the level of statistical significance are given.

Striatum. IR-VAChT perikarya were significantly reduced in striatal fiber bundles of the caudate-putamen region at 20 min of asphyxia (table 1, fig. 1). VMAT staining revealed a significant increase of VMAT-positive fibers in striatum of brains asphyxiated for 20 min.

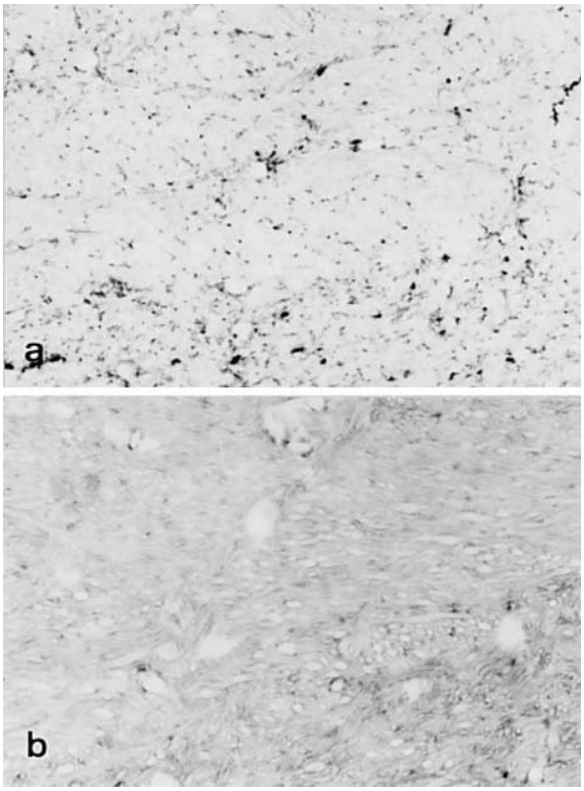

Figure 5. Frontal cortex, immunohistochemical stainig of EAAC1 glutamate transporter (left = rostral, right = dorsal). Outer pyramidal layer of normoxic controls (*a*) and 20 min after perinatal asphyxia (*b*) with increasing density of perikarya expressing glutamate transporter. × 250.

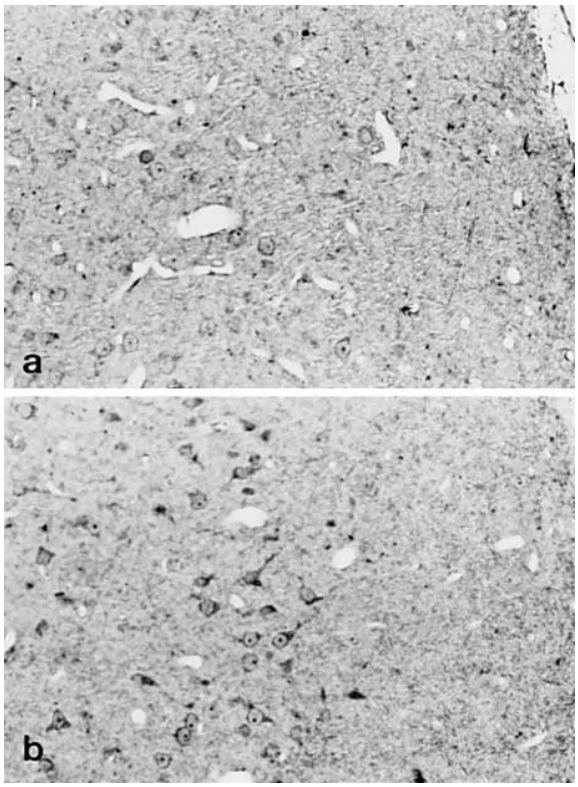

Figure 6. Cerebellum central white matter of a lobulus, immunohistochemical staining of TH in controls (*a*), and after 20 min PA (*b*) when there are less TH-positive fibers (left = rostral, right = dorsal). × 500.

The differences in IR-VMAT fiber density were perceivable throughout the globus pallidus (table 2, fig. 2). TH staining of the globus pallidus in striatum was significantly reduced from 10 min of PA (table 3, fig. 3). No significant changes in the glutamatergic system represented by IR-EAAC1 were observed (table 4).

Hippocampus. Immunoreactivity of VAChT, VAMT and EAAC1 showed no significant differences between normoxia and PA. Staining for IR-TH was significantly decreased from 10 min of PA (table 3).

Thalamus. In ventrolateral/ventroanterior thalamus, immunoreactivity of VAChT, VAMT and EAAC1 showed no significant differences between normoxia and PA. Staining for IR-TH was significantly decreased at 20 min PA (table 3, fig. 4).

Frontal cortex. In the outer pyramidal layer of frontal cortex, immunoreactivity of VAChT in perikarya and VMAT in fibers showed no significant difference between normoxia and PA. IR-TH in fibers was significantly decreased at 20 min of PA (table 3). A significant increase of EAAC1-positive perikarya following 20 min of PA was observed (table 4, fig. 5).

Cerebellum. In the granular layer of cerebellum, IR-VAChT of perikarya, IR-VMAT in zona moleculare,

and EAAC1 of perikarya in medial nucleus did not-show statistically significant differences between normoxia and PA. IR-TH of fibers in white matter showed decreased staining from 10 min of PA (table 3, fig. 6).

Discussion

A cascade of several mechanisms leading to the deterioration of brain function and neuronal death in perinatal asphyxia has been proposed and several neurotransmitter systems have been incriminated, but information on neuronal transmission is still incomplete.

Our results show deterioration of the monoaminergic system as reflected by decreased IR-TH in all brain regions investigated and increased IR-VMAT in striatum. A decrease in IR-TH, the rate-limiting enzyme in the biosynthetic pathway of the catecholamines, L-DOPA, dopamine, norepinephrine, and epinephrine, may therefore reflect deficient dopaminergic and noradrenergic innervation. Deficient dopaminergic/noradrenergic innervation may be compensated by the increase in IR-VMAT, the principal monoamine transporter pumping dopamine, norepinephrine, and others from neuronal cytoplasm into synaptic vesicles [27]. Although we provide evidence for decreased IR-TH in fibers, we cannot discriminate whether decreased IR resulted from neuronal loss or from decreased expression. Our findings are in agreement with previous observations. Chen et al. [8] have shown a reduction in IR-TH mesencephalic cell bodies in the 4-week-old rat using the same model and long asphyxiant periods of 20 min, as in our study. Ungethum et al. [9] revealed that dopamine levels were decreased in the substantia nigra/ventral tegmental area, in striatum, and in the accumbens nucleus/olfactory tubercle [9]. TH can be used as a marker for the dopaminergic system which is responsible for a series of psychomotor functions and, indeed, motor behavior was found to be impaired in a study using rats following 1 month of PA [10]. At the age of 3 months, using the same animal model, we were not able to find any deranged motor or behavioral functions, which may indicate that clinically, the TH dopaminergic deficit may be compensated [28].

No previous work is available on the role of VMAT2 in PA. It is localized in monoaminergic cell groups of the central nervous system (CNS) including brainstem, sensory relay nuclei of the CNS, limbic structures, basal ganglia, and hypothalamic nuclei [29]. VMAT2 is present along axons, soma, and dendrites [30], and this transporter is a major determinant regulating monoamine levels. Knockout of the VAMT2 gene resulted in neonatal death and sensitivity to cocaine and amphetamine [31]. Fon et al. [32], disrupting the VMAT2 gene, confirmed the pivotal role for this transporter. Evidence for the biological relevance of VMAT in human disease was provided by Thibaut et al. [33], who showed reduction of VMAT in Parkinson's disease.

The significant increase of IR-VMAT2 in asphyxiated animals, probably indicating compensation of decreased monoamines by increased monoamine transport, was limited to striatum. A possible explanation could be that striatum, abundantly innervated by the dopaminergic pathway, is particularly susceptible to asphyxiant neuronal damage. No data on brain monoamine levels at about 3 months following asphyxia are available to interpret with confidence the increase in VMAT2, but the interpretation given may be in agreement with biological consequences of decreased TH.

The cholinergic pathways are widespread in cortical and subcortical areas of the brain including the brain regions used in our experimental design [34]. In PA of 20 min, VAChT, which transports acetylcholine into synaptic vesicles, was significantly reduced in perikarya in fiber bundles of striatum. This reduction in IR-VAChT was not found in the other brain regions tested, possibly also indicating increased sensitivity to PA of the cholinergic system in this brain region. Expression of choline acetyl-transferase, VAChT, and the high-affinity membrane choline transporter define the cholinergic phenotype in the mammalian CNS, and the expression pattern of VAChT mRNA is consistent with anatomical, pharmacological, and histochemical information on the distribution of functional cholinergic neurons in the brain [35]. At near term, which is the case in our study, the distribution of VAChT already resembles the pattern found in the adult [36]. VAChT is detectable in the brain regions investigated in our study [37–39], and the cholinergic deficit in terms of decreased IR-VAChT in perikarya may indicate cholinergic neuronal loss, which would be in agreement with data from Burke and Karanas [12] demonstrating a significant loss of cholinergic neurons in striatum. The overall neuronal density, however, was only reduced in the hippocampal region CA1, not in striatum [21]. The cholinergic derangement suggested by our data may help in interpreting neurological sequelae following PA.

Glutamatergic neurotransmission is seen in abundance in structures such as cerebral cortex, hippocampus, and cerebellum [40], and indeed the EAAC1 glutamate transporter-immunoreactivity (IR-GLT) increased in the frontal cortex of animals with PA, which may well be compatible with overexcitation, described as occurring in PA of newborns and animal models [see above and refs. 41, 42]. Extracellular glutamate concentrations are regulated by glial and neuronal transporter proteins. Four glutamate transporter subtypes have been iden-

tified in rat brain: GLAST and GLT-1 are primarily astrocytic whereas EAAC1 and EAAT4 are neuronal. EAAC1, used in our study, seems to be an appropriate marker, since localization and distribution even in the newborn period are well documented [43]. The finding that in our model, 3 months following the asphyxiant insult, EAAs were only increased in hypothalamus [21] does not contradict the present study, because an increase in IR-GLT seems a more appropriate and functional test system than steady-state levels of EAAs. No long-term studies have reported on the excitotoxicity in PA and we therefore provide the first preliminary evidence for persistence of excitotoxicity. In a study using a nearly identical model, the early excitatory response to PA was described and the use of EAA antagonists showed some protective effect [44].

In conclusion, we detected changes in monoamine synthesis and transport, cholinergic deficit, and derangement of glutamate transport in individual brain regions following 3 months of graded perinatal asphyxia. We have furthermore shown the susceptibility of individual cholinergic, monoaminergic, and glutamatergic neurons to the asphyxiant insult. These findings may be relevant for understanding of the pathomechanisms of PA and form the basis for further studies on the nature of the deficits observed in PA.

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