Amino Acids (2006) 31: 385–396
DOI 10.1007/s00726-006-0393-z

Amino Acids
Printed in Austria

Long-term effect of moderate and profound hypothermia on morphology, neurological, cognitive and behavioural functions in a rat model of perinatal asphyxia*

H. Hoeger[1,**], **E. Engidawork**[2,3], **D. Stolzlechner**[3,**], **H. Bubna-Littitz**[4], and **B. Lubec**[3]

[1] Institute for Animal Breeding, Medical University of Vienna, Vienna, Austria
[2] Department of Pharmacology, School of Pharmacy, Addis Ababa University, Addis Ababa, Ethiopia
[3] Department of Neonatology, Medical University of Vienna, Vienna, Austria
[4] Institute for Physiology, Veterinary University, Vienna, Austria

Received May 15, 2005
Accepted January 26, 2006
Published online August 31, 2006;

Summary. *Background*. Perinatal asphyxia is a frequent cause of neurological handicap with no known therapy. However, hypothermic therapy has recently attracted attention owing to its neuroprotective property in brain of immature organisms.

Objectives. Hypothermia appears to be promising in reversing the immediate effect of perinatal asphyxia, but data on long-term neuroprotection is still lacking. We therefore intended to test the long-term effect of moderate and profound hypothermia on brain morphology and functions using a well established rat model of perinatal asphyxia.

Methods. Rat pups delivered by caesarean section were placed into a water bath, still in patent membranes, at 37 °C and variable hypothermic conditions to induce asphyxia and thereafter given to surrogate mothers. Examinations were performed at the age of three months, consisting of a battery of motor, behavioural, cognition and reflex tests including rota-rod, Morris water maze, multiple T-maze, elevated plus maze and open field studies. Morphological alterations were evaluated by Nissl staining of brain areas known to be hypoxia sensitive. Neurotransmission system markers, including tyrosine hydroxylase, vesicular monoamine transporter, vesicular acetylcholine transporter and excitatory amino acid carrier1 were analyzed by immunohistochemistry.

Results. Survival increased with hypothermia. The Nissl stain revealed neuronal loss in hippocampus and hypothalamus of normothermic asphyxiated group (20/37) compared to controls (0/37), but no neuroprotective patterns emerged from hypothermia. An overall inconsistent protection of the neural systems was noted by variable periods of hypothermia. Motor function was significantly impaired in 20/37 as compared to 0/37. In the Morris water maze and multiple T-maze, results were comparable between the groups. In the elevated plus maze, time spent in the closed arm was reduced and in the open field, vertical behaviour was altered in the 20/37 group with horizontal motor behaviour being unaffected. Hypothermia reversed all abnormalities seen in 20/37, with short-term moderate and profound hypothermia being superior to long-term hypothermia.

Conclusion. Hypothermia not only significantly increased survival, but also resulted in unimpaired motor as well as improved cognitive functions. Those findings are in contrast to altered brain morphology. As neuronal loss was present in various brain regions, we conclude that deficits may be compensated in the maturing animal. Intrahypoxic hypothermia was able to protect the rat from the devastating effect of perinatal asphyxia not in morphological, but in functional terms.

Keywords: Hypothermia – Perinatal asphyxia – Neurotransmitter markers

Abbreviations: EAAC, exitatory amino acid carrier; PBS, phosphate buffered saline; TH, tyrosine hydroxylase; VAChT, vesicular acetylcholine transporter; VMAT, vesicular monoamine transmitter

Introduction

Birth asphyxia has long been recognized as potentially damaging to normal neurological development. However, asphyxia ranges widely in severity from a degree that might be considered physiological to initiate respiration to that causing fetal or neonatal death. Acute perinatal asphyxia is a major cause of death and neurological injury in newborn infants. The incidence has been estimated as 2–4 per 1000 live births and has not decreased despite advances in perinatal and obstetric care. Many asphyxiated babies die during the newborn period, and 20–30% of the survivors present with long term neurological sequelae, including spasticity, epilepsy, and mental retardation. Milder forms of asphyxiating insults can be associated with attention deficit disorders, such as the minimal brain disorder syndrome (Volpe, 1987; Hill, 1991; Carter et al., 1993).

* This paper is dedicated to Professor Arnold Pollak for his continuous stimulation and support of neonatal research.
** These authors have contributed equally to this work.

Although the understanding of perinatal asphyxia-related pathophysiology is gradually increasing, limited therapeutic options are available to prevent or even mitigate the devastating process that unfolds after injury. A potential solution lies in the application of therapeutic hypothermia, which is thought to prevent or lessen neuronal injury probably by decreasing energy expenditure (Ginsberg et al., 1992). The protective effect of hypothermia following an asphyxiating insult was first demonstrated by the pioneering work of Miller (1971). Following this, several studies have confirmed the effectiveness of both intra- as well as post-asphyxic hypothermia. Post-asphyxic hypothermia ameliorates brain damage in asphyxiated neonates (Miller and Miller, 1972; Dunn and Miller, 1969) and in animal models of focal as well as global cerebral hypoxia/ischemia (Loidl et al., 1998; Sirimane et al., 1996; Thoresen et al., 1996; Edwars et al., 1995; Haaland et al., 1997; Laptook et al., 1994). However, delay in initiation is also shown to abolish its neuroprotective effect (Gunn et al., 1999). Intra-asphyxic hypothermia has also been shown to provide a robust neuroprotective effect in animal models of perinatal asphyxia (Engidawork et al., 2001) as well as hypoxia/ischemia (Loidl et al., 1998; Edwars et al., 1995). The majority of published reports focussed mainly on the short-term neuroprotective effects of hypothermia and only a handful of studies investigated its long-term benefits (Capani et al., 1997; Trescher et al., 1997; Yager et al., 1993). These later studies also assessed neuropathological changes associated with asphyxia and comprehensive information on the long-term beneficial effects of hypothermia is lacking. In the present study, an attempt was therefore made to evaluate the effect of moderate and profound hypothermia on emotion, motor and cognitive functions using a non-invasive rat model of perinatal asphyxia that mimics intrapartum asphyxia (Lubec et al., 1997a). To this end, appropriate tests were performed at three months of age following the asphyxiating insult. While these findings are interesting, they do not, however, have any therapeutic value unless otherwise it is demonstrated that hypothermia can be applied during intrapartal-asphyxia, which is currently not feasible in humans in the context of birth asphyxia. Nevertheless, the data still attest to the fact that hypothermia is a promising treatment for hypoxic/ischemic insults.

Materials and methods

Induction of perinatal asphyxia

Perinatal asphyxia was induced as described elsewhere (Lubec et al., 1997a, b; Klawitter et al., 2005). Briefly, pregnant Sprague–Dawley rats were sacrificed by neck dislocation and hysterectomized. The uterine horns containing the fetuses were placed in a water bath at 37 °C for twenty minutes (normothermic asphyxiated group, 20/37) following removal of pups that served as normoxic controls (0/37). In a similar experiment, other pups were subjected to different duration and magnitude of hypothermia (30 °C for 20 min, moderate hypothermic group, designated as 20/30; 15 °C for 20, 50 and 100 min, profound hypothermic asphyxiated group, designated as 20/15, 50/15 and 100/15, respectively) to obtain different hypothermic-asphyxiated groups.

Only litters with pups weighing more than 4.5 g were used for the experiments. All tests were carried out at three months of age only on female rats. Female rats were chosen based on the observation of sex-related differences in hypothermia-induced improvement of sensorymotor functions (Bona et al., 1998). The animal studies were performed according to the rules of the American Physiology Society and the experimenter was blind to grouping of the rats.

Histological examination

Rats were anesthetized (Membutal 50 mg/kg body weight, i.p.) and perfused intracardially 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, postfixed in the same solution for 12–18 h and then kept in PBS containing 20% sucrose at 4 °C pending sectioning. Paraffin embedding, sectioning (10 μm) and dewaxing by xylene were performed as described elsewhere (Kohlhauser et al., 1999a).

Nissl staining

Frontal cortex, striatum, hippocampus (area CA1, CA3), hypothalamus (ventral anterior thalamic nucleus, ventrolateral/ventromedial thalamic nucleus and anterior hypothalamic area central) and cerebellum (lobule L8) were selected for evaluation of neuronal cell-loss and morphological evaluations. The brain regions were selected because they are involved in cognitive, behavioural and motor functions as well as are known to be hypoxia sensitive. Dewaxed sections were incubated three times in 96% ethanol for five min and subsequently incubated in 0.1% luxolfastblue overnight at 60 °C. Sections were then rinsed briefly in 96% ethanol and differentiated in 0.05% lithiumcarbonate. Nissl-staining for the demonstration of nuclear and cytoplasmic RNA (Nissl-substance) was performed using cresylfastviolet (0.1% w/v, pH adjusted to 3.9 with acetic acid) staining for three minutes. This was followed by destaining with distilled water (2×) then with 96% ethanol (2×), and differentiation in dried ethanol for six minutes. After incubation in xylene, slides were mounted with a coverslip in Eukitt.

Morphometry: Determination of cell density was carried out in the brain regions/areas given above by taking digital micrographs using a Nikon Optiphot-2-microscope. Micrographs of each animal and of each brain region/area (10 per group) were subjected to analysis of cell density using standard procedures (Burck, 1981). Neuronal cells were positively identified by the Nissl stain and the number of positively stained nuclei were counted per defined field (= photographic frame). In cerebellum, L8, morphometry was applied to the granular layer evaluating the number of nuclei with chromatin changes.

Immunostaining

The same brain regions were used for determination of immunoreactivity of putative markers of neurotransmission systems. Markers selected were based on regional distribution of neurotransmitters. Accordingly, the caudate-putamen was used for determination of tyrosine hydroxylase (TH), vesicular monoamine transporter (VMAT), vesicular acetylcholine transporter (VAChT) and excitatory amino acid carrier-1 (EAAC1)-immunoreactivity. In hippocampus and hypothalamus, whilst the polymorph layer

of the dentate gyrus and ventrolateral/ventromedial thalamic nuclei, respectively were used for evaluation of VMAT and TH; area CA1 & CA3 and ventral anterior thalamic nucleus/anterior hypothalamic region central were used for evaluation of VAChT and EAAC1. The outer pyramidal layer of frontal cortex presents with all three types of innervation so used for determination of immunohistochemistry of cholinergic, monoaminergic and glutamatergic neurotransmission. In cerebellum, the granular layer was used for VAChT, zona moleculare for VMAT, white matter for TH and medial nucleus for EACC1 immunoreactivity.

For immunohistochemistry, paraffin sections mounted on silanized glass slides were dewaxed with xylol (4×15 min), rehydrated in decreasing concentrations of ethanol and rinsed with water followed by PBS, pH 7.4 ($2\times$). Slides were then subjected to an antigen retrieval procedure, which consisted of autoclaving slides in 1 mM EDTA, pH 7.9, at 121 °C for 2 min and cooling overnight. Slides were again rinsed in PBS twice, blocked with protein blocking solution designed for automated immunostaining (DAKO, Glostrup, Denmark) for 10 min at room temperature followed by incubation for 2 h with corresponding primary antibodies (polyclonal rabbit-anti-VMAT2, 1:500; rabbit anti-TH, 1:100; goat-anti-VAChT, 1:100 and goat-anti-EAAC1, 1:400, Chemicon International, Temecula, CA, USA) diluted in Dako antibody diluens. Following incubation with primary antibody, slides were rinsed three times with PBS and incubated for one hour with the corresponding secondary antibody. All secondary antibodies were biotinilated and species specific, and purchased from Dako ChemMate system, Glostrup, Denmark. Slides were also counterstained with hematoxylin (Dako) rinsed in water, dehydrated in ethanol 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. Morphometric procedures were applied on histological sections to determine differences in immunoreactivity between brain regions of different groups. To this end, immunoreactive-neuronal fibers or perikarya were counted on micrographs or camera lucida drawings over a microscopical field corresponding to 5000 to 15,000 μm. Values obtained for density were converted to immunoreactivity units/1000 μm^2, except for the neuronal cell counts in the hippocampus, which were evaluated along the cell layer of 1000 μm. Antibodies against VAChT and EAAC1 predominantly stained perikarya, whereas those of VMAT and TH preferably stained fibers.

Behavioural and cognitive function analyses

Neurological evaluation and rota-rod test

Rats were tested for postural and locomotor behaviour as well as triggered movements. Neurological assessment was made based on a battery of tests as described earlier (Irwin, 1968). The rota-rod test is a well-established procedure for testing balance and coordination effects of motor performance in rats and mice (Jones and Roberts, 1968). Particularly, the accelerating rota-rod task is shown to be a more sensitive index for the assessment of motor impairment (Hamm et al., 1994). For the rota-rod test, rats were conditioned to an accelerating rota-rod prior to the test. Each rat received a training session on the rota-rod set at a constant speed of 8 rpm with accelerating option until it was able to stay on the rotating spindle for 60 seconds. Each rat was then subjected to a single baseline trial on the accelerating rota-rod at which the speed of the spindle was increased from 4 to 70 rpm over a period of 5 min and time spent on the rod was recorded (Rogers et al., 1997).

Morris water maze

The test arena was a circular pool with diameter of 140 cm and height of 80 cm. The pool was filled with water (21 °C) up to 65 cm and a small hidden platform (10 cm diameter) made of plexiglass was placed in one of the imaginary quadrants such that its top was 1 cm beneath the surface of the water. The tests took place between 5 and 10 p.m. and were performed by a trained experimenter. The rats were subjected to a short series of training trials conducted four times per day for three days with a hidden platform in a fixed position. One week later, the animals were tested for memory (reference memory) with the hidden platform in the same position as that of the training session. After a lapse of one more week, the animals were subjected to another test (working memory) with a hidden platform in a new position (to the opposite quadrant). This was the recall or relearning test. There were four fixed starting positions and rats were placed into the water from each possible start positions during each trial. Rats were allowed to swim until they locate the escape platform by climbing onto it for 5 sec or until 60 sec had elapsed. Time of each trial was measured using a computerized tracking system. The inter-trial interval during which the animals rested on the platform was 20–30 seconds.

Multiple T-maze

Animals were tested for learning and memory (reference memory) in a Multiple T-Maze with 7 choice points and dimensions of 150×130 cm. The alley through which the animals traversed had a height of 15 cm and width of 8 cm. It is assumed that food deprivation motivates animals to reach the goal box where they would be rewarded with food. Rats deprived of food for three days were trained three times per day for four consecutive days and a fortnight later, memory was tested consisting of three runs on that day. Time (in seconds) to reach the goal box and correct decision (n) were monitored (Hoeger et al., 2000). Tests were performed between 8 and 10 p.m.

Elevated plus maze

The elevated plus maze was made out of dark-gray PVC consisting of a central area (10×10 cm) from which four arms (each 50 cm long and 10 cm wide) radiated outwards, rendering the shape of a plus sign. Three of the arms were open and the other closed, with a black box of 20×20 cm. It was elevated 73-cm above the floor and placed in a brightly-lit room. It is thought that the elevated plus maze combines fear of a novel brightly-lit open space and of height, thereby enabling to assess the emotional state of the animal. The behavioural patterns, such as entries, time spent in the closed arm and latency were scored. An entry was defined as having the animal placed all limbs into a defined area of the maze. The maze was cleaned thoroughly with water containing a detergent before a new rat was exposed to it. Exposure started with placing the animal on the central area facing the closed arm (Hoeger et al., 2000).

Open field studies

Animals were tested in an open field design as published previously (Hoeger et al., 2000). Briefly, the behaviour of animals placed in an open field (dimension, 80×80 cm) was monitored by an infrared tracking system (Viptronic, Vipdula 810 IR). The parameters for horizontal motor behaviour i.e. path length, number of entries, resting time and number of traversions, and vertical behaviour, such as sniffing and rearing were evaluated.

Statistical analyses

Data are expressed as mean ± standard deviation of the measured parameter. Morris water maze and multiple T-maze data were analyzed using two-way ANOVA (treatment × blocks of trial) with repeated measures on the second factor. Measurements obtained from the elevated plus maze were analyzed using completely randomized ANOVA. Post-hoc Tuckey's HSD test was applied, when required. For open field, the Kruskal–Wallis and Wilcoxon test were used to compare the parameters between groups. Histological data were compared using ANOVA with subsequent Kruskal–Wallis test or Mann–Whitney *U*-test when appropriate. The significance was set at $P < 0.05$ for a two-tailed test.

Results

Survival pattern

As shown in Table 1, survival varied with temperature and duration at which asphyxia was induced. At 0/37 survival was 100% and was decreased by 16.9% with 20/37. However, this survival rate was progressively increased with a decrease in temperature returning to normoxic level at 20/15. Although hypothermia increased survival rate, this rate was influenced by the duration of asphyxia. Moderate hypothermia increased survival rate by about 12% and a further increase was obtained with deepening of hypothermia i.e., profound hypothermia (15 °C). The normoxic survival rate attained at 20 min with profound hypothermia, however, was decreased with time and reached that of moderate hypothermia at 50 min of asphyxia, and further decreased with time.

Neuropathology

Nissl staining

Neuronal cells were detected in various regions using Nissl staining. Subsequent quantitative analysis was performed to evaluate neuronal loss in regions known to be sensitive to hypoxia and the outcome is presented in Table 2. Significant neuronal loss ($P < 0.05$) was observed in CA1 and CA3 regions of the hippocampus as well as in the ventral anterior thalamic nucleus and central anterior hypothalamic area of the diencephalon of the 20/37 group compared to the 0/37 group. However, no apparent loss was observed in cerebellum, striatum and frontal cortex. Moderate hypothermia failed to ameliorate this neuronal loss, as there was no significant difference in neuronal density between the 20/37 and 20/30 groups. The same was true for profound hypothermia, although there appeared to be a tendency of increasing neuronal density of the 20/15 group in area CA1 that failed reaching statistical significance. Unlike moderate hypothermia, profound hypothermia was shown to be associated with accentuation of neuronal loss in areas that had already been identified as target areas for an asphyxiating insult in the 20/37 group. In other regions, such as striatum, cerebellum and frontal cortex, which had not displayed neuronal loss in the 20/37 group, profound hypothermia caused significant reduction ($P < 0.05$) in neuronal density (Table 2).

Table 1. Survival data following asphyxia under normothermic and hypothermic conditions

Group	No.	Survival in %
0/37 (controls)	39	100
20/37	65	83.1
20/30	42	95.2
20/15	30	100
50/15	52	94.2
100/15	73	64.4

The data show survival pattern of rats 24 h following asphyxia and indicate the acute protective effect of hypothermia. Numerator represents time and denominator temperature at which asphyxia was induced

Immunostaining

Immunostaining patterns of the different neurotransmission markers investigated. Table 3A–D summarizes the results of morphometric analyses. 20/37 produced a significant decrease ($P < 0.05$) in immunoreactivity of TH in frontal cortex, striatum and thalamic regions, but that of hippocampus and cerebellum was unaffected. Immunoreactivity of VMAT, VAChT and EAAC1 more or less showed the same trend with little differences. The unaffected regions were striatum for VMAT, striatum and cerebellum for VAChT, and frontal cortex for EAAC1. It was noted that whilst VAChT was decreased in both CA1 and CA3 regions of the hippocampus, EAAC1 was decreased

Table 2. Histological outcome expressed as neuronal loss

Group	Frontal cortex	Caudate putamen	CA1	CA3	Ventral anterior thalamic nucleus	Anterior hypothalamic area central	Lobulus 8 (L8) cerebellum
0/37	669.1 ± 39.3	714.8 ± 166.4	95.7 ± 6.3	91.3 ± 14.1	755.5 ± 78.7	618.1 ± 228.5	27.8 ± 1.8
20/37	642.0 ± 51.0	725.0 ± 109.4	83.7 ± 6.9*	70.0 ± 10.3*	674.1 ± 74.2*	572.3 ± 57.8*	26.6 ± 2.3
20/30	569.2 ± 210.3	610.5 ± 226.3	78.0 ± 27.8	65.0 ± 24.7	567.3 ± 205.3	450.2 ± 170.9	23.0 ± 8.7
20/15	607.7 ± 87.7	620.7 ± 107.4*	91.5 ± 6.3	65.9 ± 11.1	633.4 ± 103.5	473.1 ± 64.8*	24.6 ± 2.5
50/15	626.3 ± 67.1	603.6 ± 96.6*	78.1 ± 7.3	54.3 ± 4.7*	679.9 ± 104.9	559.6 ± 83.6	22.2 ± 2.5*
100/15	542.2 ± 11.3*	615.6 ± 3.6*	68.0 ± 4.5*	48.5 ± 2.6*	681.7 ± 3.4	541.8 ± 2.8	24.0 ± 2.4*

Values represent mean ± standard deviation. Neuronal densities are described either along a cell border of 1 mm (CA1, CA3 and L8) or per mm^2 (frontal cortex, caudate putamen and thalamic/hypothalmic regions). Comparison was made between 0/37 and 20/37 as well as 20/37 and hypothermic-asphyxiated groups. An asterisk indicates statistical significance ($P < 0.05$) and $n = 10$ in all cases

Table 3. Results of immunoreactivity of TH (**A**), VMAT (**B**), VAChT (**C**) and EAAC1 (**D**) in different brain regions at different asphyxiated periods

Group	Frontal cortex (per mm^2)	Caudate putamen (per mm^2)	PoDG (per mm^2)	VL/VM (per mm^2)	L8 (per mm^2)
(A)					
0/37	3125.0 ± 650.4, $n = 10$	2976.2 ± 300.9, $n = 10$	4213.0 ± 1371.2, $n = 9$	2750.0 ± 381.4, $n = 10$	1117.7 ± 458.9, $n = 9$
20/37	1922.6 ± 358.3*, $n = 10$	2571.4 ± 313.5*, $n = 10$	4148.8 ± 1425.6, $n = 10$	1023.8 ± 383.5*, $n = 10$	870.5 ± 348.4, $n = 8$
20/30	2339.3 ± 438.4, $n = 10$	2595.2 ± 481.3, $n = 10$	4779.8 ± 1689.4, $n = 10$	1982.1 ± 419.1*, $n = 10$	1177.2 ± 377.4, $n = 9$
20/15	3174 ± 669.5*, $n = 9$	2602.0 ± 437.0, $n = 7$	5869.0 ± 1079.9*, $n = 10$	1744.0 ± 546.3*, $n = 10$	1107.1 ± 358.5, $n = 10$
50/15	2305.2 ± 401.1, $n = 11$	2305.2 ± 265.0, $n = 11$	4312.8 ± 1142.6, $n = 11$	1655.8 ± 314.8*, $n = 11$	1163.4 ± 258.6, $n = 11$
100/15	2196.4 ± 306.7, $n = 10$	2178.6 ± 341.6*, $n = 10$	3327.4 ± 1161.2, $n = 10$	1144.2 ± 553.4, $n = 9$	1035.7 ± 268.0, $n = 10$
(B)					
0/37	3698.9 ± 768.4, $n = 10$	1960.0 ± 454.8, $n = 9$	6426.9 ± 1182.3, $n = 9$	3183.1 ± 926.9, $n = 9$	1359.8 ± 300.8, $n = 9$
20/37	2064.8 ± 569.1*, $n = 10$	1808.0 ± 481.2, $n = 9$	4277.0 ± 1319.6*, $n = 10$	1800.4 ± 508.2.5*, $n = 9$	995.2 ± 342.0*, $n = 9$
20/30	3582.7 ± 849.2*, $n = 10$	2146.9 ± 460.6, $n = 10$	4963.8 ± 895.1, $n = 10$	2454.5 ± 479.6*, $n = 10$	957.2 ± 277.7, $n = 9$
20/15	3198.3 ± 853.8*, $n = 9$	2037.5 ± 315.5, $n = 10$	6276.5 ± 1587.1*, $n = 10$	2352.0 ± 904.3, $n = 10$	1010.4 ± 226.2, $n = 10$
50/15	2772.1 ± 1077.7, $n = 11$	2480.0 ± 269.6*, $n = 11$	4935.2 ± 1132.9, $n = 11$	1783.9 ± 392.1, $n = 11$	997.7 ± 342.7, $n = 10$
100/15	1353.8 ± 834.1, $n = 10$	1752.0 ± 559.0, $n = 8$	3449.0 ± 925.0, $n = 10$	1346.9 ± 525.7, $n = 10$	847.8 ± 239.5, $n = 10$

Group	Frontal cortex (per mm^2)	Caudate putamen (per mm^2)	CA1 (along 1 mm)	CA3 (along 1 mm)	VA (per mm^2)	AHC (per mm^2)	L8 (along 1 mm)
(C)							
0/37	78.3 ± 11.4, $n = 10$	72.4 ± 13.5, $n = 10$	74.9 ± 5.3, $n = 10$	62.2 ± 5.4, $n = 10$	54.4 ± 10.7, $n = 10$	85.4 ± 19.2, $n = 9$	15.3 ± 4.0, $n = 9$
20/37	64.0 ± 6.0*, $n = 10$	67.8 ± 7.1, $n = 10$	59.1 ± 8.8*, $n = 8$	54.7 ± 4.0*, $n = 10$	52.9 ± 11.0, $n = 10$	67.4 ± 9.4*, $n = 9$	15.0 ± 4.2, $n = 9$
20/30	67.1 ± 9.1, $n = 10$	64.9 ± 11.6, $n = 9$	64.3 ± 4.7, $n = 9$	56.0 ± 8.1, $n = 8$	56.1 ± 10.7, $n = 10$	78.9 ± 14.4, $n = 8$	15.2 ± 3.0, $n = 10$
20/15	71.6 ± 8.7, $n = 10$	72.5 ± 12.6, $n = 8$	73.3 ± 8.8*, $n = 8$	61.2 ± 3.0*, $n = 8$	47.6 ± 8.6, $n = 10$	82.0 ± 10.9*, $n = 9$	14.7 ± 3.2, $n = 8$
50/15	71.7 ± 9.2, $n = 11$	66.8 ± 11.4, $n = 10$	61.9 ± 6.8, $n = 10$	49.3 ± 6.8, $n = 11$	54.8 ± 9.4, $n = 11$	70.8 ± 14.8, $n = 11$	13.6 ± 3.4, $n = 9$
100/15	75.3 ± 9.3, $n = 10$	69.8 ± 10.4, $n = 10$	61.3 ± 6.5, $n = 9$	54.4 ± 6.8, $n = 9$	53.3 ± 9.6, $n = 10$	66.8 ± 13.4, $n = 10$	14.6 ± 2.2, $n = 9$
(D)							
0/37	928.7 ± 161.8, $n = 10$	1219.7 ± 93.7, $n = 10$	146.4 ± 10.2, $n = 9$	94.6 ± 11.9, $n = 9$	254.2 ± 43.8, $n = 10$	695.5 ± 133.8, $n = 10$	30.4 ± 4.2, $n = 10$
20/37	872.9 ± 101.4, $n = 10$	751.1 ± 69.9*, $n = 9$	119.1 ± 13.2*, $n = 10$	88.9 ± 6.3, $n = 10$	155.8 ± 47.1*, $n = 10$	586.3 ± 87.7, $n = 10$	18.6 ± 3.3*, $n = 9$
20/30	822.9 ± 171.9, $n = 10$	781.8 ± 87.6, $n = 10$	130.4 ± 13.5, $n = 10$	84.0 ± 5.6, $n = 10$	189.6 ± 78.5, $n = 10$	561.3 ± 121.9, $n = 10$	22.3 ± 2.2, $n = 9$
20/15	839.1 ± 107.0, $n = 10$	757.6 ± 90.0, $n = 9$	138.3 ± 12.6*, $n = 10$	85.2 ± 11.0, $n = 10$	220.4 ± 80.8, $n = 10$	674.5 ± 161.2, $n = 10$	25.2 ± 6.6*, $n = 10$
50/15	786.8 ± 165.4, $n = 11$	756.1 ± 85.5, $n = 11$	88.8 ± 8.9, $n = 11$	88.8 ± 8.9, $n = 11$	215.1 ± 62.1*, $n = 11$	565.7 ± 99.2, $n = 10$	22.3 ± 3.1, $n = 10$
100/15	833.2 ± 105.7, $n = 10$	677.6 ± 91.0, $n = 9$	88.4 ± 10.9, $n = 9$	88.4 ± 10.9, $n = 9$	215.5 ± 72.4, $n = 9$	580.4 ± 77.5, $n = 10$	22.5 ± 1.3*, $n = 10$

Values refer to mean ± standard deviation and immunoreactivity is described either along a cell border of 1 mm or per mm^2 as shown in the body of the table. Comparison was made between 0/37 and 20/37 as well as 20/37 and hypothermic-asphyxiated groups. An asterisk indicates statistical significance ($P < 0.05$); *PoDG* polymorph layer of the dentate gyrus; *VA* ventral anterior thalamic nucleus; *VL/VM* ventrolateral/ventromedial thalamic nucleus; *AHC* anterior hypothalamus central region

only in CA1 region. Likewise, diencephalon VAChT was shown to decrease in anterior hypothalamic region central, whereas EAAC1 in ventral anterior thalamic nucleus ($P<0.05$ in all cases).

Hypothermia produced variable effects in asphyxia-induced markers expression in different brain regions. In the frontal cortex, where expression of all but EAAC1 was shown to be reduced in the 20/37 group, 20/30 abrogated the abnormality as it significantly increased ($P<0.05$) expression of VMAT by ~74% (Table 3B). An increasing tendency (~21%) in TH expression by 20/30 was also observed in this region (Table 3A). Better protective effect was provided by profound hypothermia, although at times it led to recurrent loss when prolonged. 20/15 reversed 20/37-induced loss of TH and VMAT expression by ~65% ($P<0.05$) and 55% ($P<0.05$), respectively. Likewise, 100/15 increased expression of VAChT by ~17% ($P<0.05$), although it also led to decreased expression of VMAT by ~35% ($P<0.05$) (Table 3B).

In the striatum, where 20/37-mediated loss of TH and EAAC1 was observed, hypothermia failed to revert this loss regardless of magnitude and duration. Rather, 100/15 significantly intensified ($P<0.05$) loss of striatal TH (Table 3A). Striatal VMAT was also shown to be significantly increased by ~37% ($P<0.05$), which was originally unaffected by 20/37 (Table 3B). Hypothermia caused sub-field-dependent attenuation of 20/37-induced alterations of hippocampal VMAT, VAChT and EAAC1 expression. Dentate gyrus VMAT (26.4%), CA3 VAChT (12%) and CA1 VAChT (24%) as well as EAAC1 (16%) expression were significantly increased ($P<0.05$) and tended to return to normal levels only with 20/15. However, 20/15 also induced a significant increase in dentate gyrus TH expression by ~40% ($P<0.05$), which was unaffected by 20/37. Diencephalon is the only region where 20/37-induced reduction in all the markers was demonstrated. Ventrolateral/ventromedial thalamic nuclei expression of TH and VMAT appeared to be increased with all duration/magnitude of hypothermia, except for 100/15. The increase was significant ($P<0.05$) with TH, the rank order being 20/30 (94.2%) > 20/15 (70.3%) > 50/15 (62%). With VMAT, a significant increase by ~36.4% ($P<0.05$) was achieved only with 20/30 (Table 3B). Decreased VAChT expression caused by 20/37 in the anterior hypothalamic region central was reverted by 20/15, which caused a significant increase by ~22.3% ($P<0.05$) (Table 3C). Unlike VAChT, decreased expression of EAAC1 in the diencephalon was detected in the ventral anterior thalamic nucleus and although all levels of hypothermia caused an increased expression of EAAC1, significant reversal was achieved only with 50/15 (~39%, $P<0.05$). A similar effect by all levels of hypothermia, excluding 100/15 was observed on expression levels of anterior hypothalamic region central VAChT levels. VMAT, which was shown to be aberrant in cerebellum by 20/37 was refractory to the action of all levels of hypothermia. By contrast, cerebellar levels (lobular 8) of EAAC1 tended to increase and approached normal values with hypothermia, though significance ($P<0.05$) was achieved only with 20/15 and 100/15.

Table 4. Tabular presentation of the neurological observational battery

Group	0/37, $n=26$	20/37, $n=16$	20/30, $n=21$	20/15, $n=15$	50/15, $n=20$	100/15, $n=30$
Body position	2.19 ± 1.8	1.25 ± 1.6	1.81 ± 0.9	2.27 ± 1.9	2.05 ± 1.8	1.33 ± 1.2
Locomotor activity	1.46 ± 1.5	$0.38 \pm 1.1^*$	1.05 ± 1.5	1.2 ± 2.0	0.8 ± 1.0	1.27 ± 1.4
Negative geotaxis	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0
Cliff avoidance	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0
Transfer arousal	4.12 ± 0.3	$4.38 \pm 0.5^*$	4.09 ± 0.3	4.13 ± 0.4	4.0 ± 0.0	4.8 ± 0.7
Spatial locomotion	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0	6.33 ± 0.8
Startle response	6.08 ± 1.5	$8.0 \pm 0.0^*$	$5.9 \pm 2.0^*$	7.47 ± 1.4	$6.2 \pm 1.6^*$	7.13 ± 1.5
Crossing a narrow path	22.69 ± 11.3	$15.63 \pm 9.9^*$	16.9 ± 12.0	23.67 ± 10.9	17.0 ± 12.0	20.17 ± 13.0
Tail elevation	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
Pelvic elevation	6.54 ± 0.9	$6.0 \pm 0.0^*$	6.67 ± 1.0	6.27 ± 0.7	6.5 ± 0.9	6.47 ± 1.3
Finger approach	4.62 ± 1.2	$5.76 \pm 1.6^*$	4.48 ± 1.5	4.93 ± 1.3	6.2 ± 0.9	4.62 ± 1.4
Visual placing	6.23 ± 1.3	$5.25 \pm 1.0^*$	$6.57 \pm 1.1^*$	6.13 ± 0.9	$6.5 \pm 0.9^*$	5.87 ± 1.7
Vestibular drop	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0
Grip strength	5.92 ± 0.4	5.7 ± 1.0	5.62 ± 0.8	5.73 ± 0.7	6.1 ± 0.4	6.0 ± 0.0
Wire manoeuvre	0.00 ± 0.0	0.13 ± 0.5	0.57 ± 1.3	0.13 ± 0.5	0.4 ± 0.8	0.2 ± 0.6
Righting reflex	0.00 ± 0.0	0.00 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

Comparison was made between 0/37 and 20/37 as well as 20/37 and hypothermic-asphyxiated groups. An asterisk indicates statistical significance ($P<0.05$)

Table 5. Tabular presentation of rota-rod, elevated plus maze and open field studies

Group	0/37	20/37	20/30	20/15	50/15	100/15
Rota-rod test (sec)	113.8 ± 10.4	71.7 ± 9.6***	89.6 ± 7.2*	92.8 ± 9.6*	104.00 ± 11.2*	105.6 ± 12.8
Elevated plus maze test						
Motivation (sec)	20.37 ± 5.27	18.8 ± 6.1	21.4 ± 6.4	20.5 ± 6.1	20.0 ± 5.83	14.4 ± 4.72
Latencies (sec)	77.9 ± 25.5	74.5 ± 25.5	80.0 ± 20.7	81.4 ± 15.9	105.5 ± 11.7	107.6 ± 12.4
Time spent in closed arm (sec)	236.0 ± 60.0	164.0 ± 24.0*	216.0 ± 36.0	214.0 ± 48.0	196.0 ± 36.0	148.0 ± 20.0
Entries to the closed arm (n)	23.9 ± 5.5	25.5 ± 5.0	25.3 ± 6.6	22.7 ± 7.2	21.1 ± 7.5	23.3 ± 5.27
Open field test						
Path length (m)	61.1 ± 9.4	61.1 ± 11.4	61.6 ± 15.5	63.3 ± 10.0	57.2 ± 15.0	58.8 ± 9.4
Traversions (n)	14.0 ± 5.83	21.8 ± 5.3	17.5 ± 5.6	14.0 ± 5.6	12.6 ± 5.6	12.3 ± 3.6
Entries to the center (n)	98.8 ± 2.8	99.0 ± 2.4	99.0 ± 0.2	99.0 ± 0.2	98.9 ± 0.2	99.0 ± 0.2
Time spent at the wall (%)	58.6 ± 10.0	61.3 ± 1.3	64.0 ± 11.3	65.3 ± 8.0	64.0 ± 8.0	65.3 ± 8.0
Sniffing at the wall (n)	33.8 ± 5.1	23.8 ± 5.0*	31.1 ± 6.1	33.3 ± 4.4*	30.0 ± 6.1	32.2 ± 6.6
Grooming (n)	7.3 ± 3.4	12.7 ± 2.0*	7.7 ± 1.4*	5.9 ± 1.8*	6.8 ± 2.5*	8.0 ± 2.3*
Rearing (n)	6.6 ± 2.5	9.4 ± 2.0	6.6 ± 2.8	6.0 ± 2.4	6.8 ± 2.7	7.3 ± 3.0

Values refer to mean ± standard deviation and comparison was made between 0/37 and 20/37 as well as 20/37 and hypothermic-asphyxiated groups. An asterisk indicates statistical significance $^{***}P<0.001$, $^{*}P<0.05$

Behavioural and cognitive responses

Neurological examination and rota-rod test

Table 4 summarizes the findings obtained from a battery of neurological tests. The 20/37 group differed from the 0/37 controls in some of the motor function as well as reflex tests. Abnormal motor and/or affective responses were apparent in the 20/37 group, as there was a significant decrease in locomotor activity, pelvic elevation and alley traversions, and significant increase in transfer arousal. Although righting reflex, negative geotaxis, cliff avoidance, free fall acceleration and vestibular drop failed to show significant differences; visual placing and finger approach were significantly decreased, whereas the acoustic startle response was significantly increased in the 20/37 compared to 0/37. Hypothermia appeared to normalize some changes seen with 20/37. For example, both 20/30 and 50/15 had significantly decreased and increased ($P<0.05$) startle responses and visual placing, respectively, compared to 20/37. In the rota-rod test system, 20/37 rats remained on the rotating rod for a highly significantly shorter period of time ($P<0.001$) compared to 0/37 control rats. This abnormality was attenuated by hypothermia, as all hypothermic-asphyxiated groups remained on the rod for a significantly longer ($P<0.05$) time than 20/37 rats (Table 5).

Maze studies

Morris water maze: To study the effect of perinatal asphyxia with and without hypothermia on memory acquisition and consolidation, control and asphyxiated rats were subjected to the Morris water maze test as described in Materials and methods. The mean latencies before escape onto the hidden platform across the three days training sessions and memory and recall tests are shown in Fig. 1. For 0/37 rats, the latency decreased across training days, providing evidence of learning in normal controls. The latency was even dramatically decreased during the memory test, indicating acquisition and consolidation of the spatial memory. This was further corroborated by the recall test, in which the latency tended to decrease during

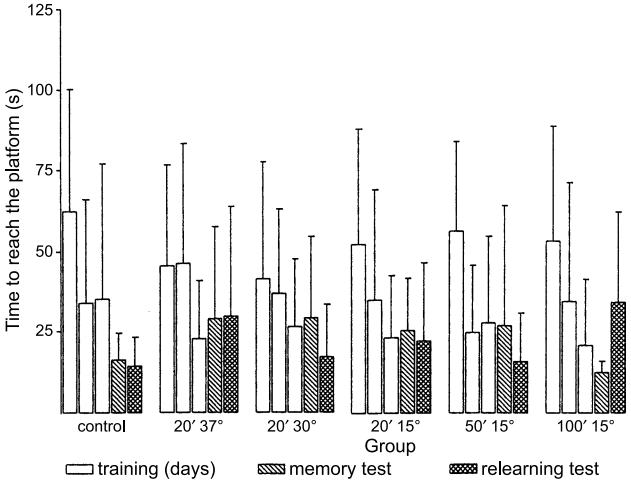

Fig. 1. In the Morris water maze the normoxic group was comparable to the asphyxiated groups i.e. the time to reach the platform was not different between panels. Three training days were followed by a memory test, which in turn was followed by the relearning test (different localization of the platform)

the recall test. 20/37 rats performed similarly to control rats across training days, but latency tended to increase in the memory and recall tests, though failed to reach significance level. A similar trend was observed with hypothermic-asphyxial rats in the test as well.

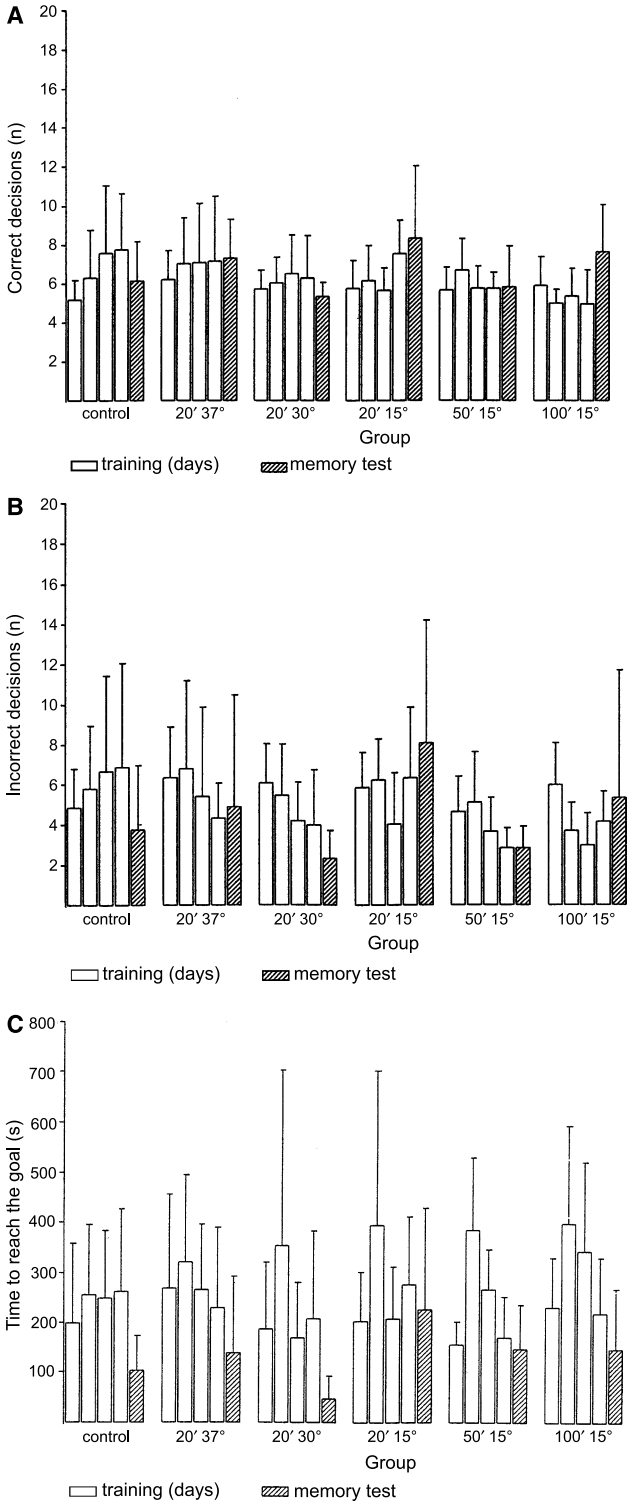

Fig. 2. Results from the multiple T-maze: The four training days in the MTM were followed by a memory test (**A–C**); no significant differences, however, were observed between cohorts

Multiple T-maze: Cognition was also evaluated by using a method studying memory elicitation in response to appetitive motivation in a multiple T-maze. Statistically significant deficiency in the T-maze performance was not observed in 20/37 rats at the day memory was evaluated, as measured by running time to reach the goal box and errors in the maze (Fig. 2A–C). Although hypothermic-asphyxiated rats performed equally well by and large to 20/37 rats in this task, 20/30 rats had shown a significantly lower time ($P<0.05$) than 20/37 rats in reaching the goal box.

Elevated plus maze: As shown in Table 5, latency to enter the closed arm, motivation/time to start moving and number of entries into the closed arm did not differ between the 0/37 and the 20/37 groups. However, time spent in the closed arm was significantly reduced ($P<0.05$) in the 20/37 group compared to the 0/37 ones (Table 5). Hypothermia did not affect significantly any of the parameters recorded in the 20/37 group, although there was a tendency of increasing anxiety-related behaviour by slightly increasing time spent in the closed arm.

Open field studies: Evaluation of spontaneous activity in an open field arena (Table 5) revealed that perinatal asphyxia alters most of the vertical but not horizontal motor behaviours. Horizontal motor behaviours, such as entry into the center, traversions, path length and time spent at the wall were comparable between the 0/37 and 20/37 groups. Whereas some vertical motor behaviours, such as grooming and sniffing at the wall showed a statistically significant increase and decrease ($P<0.05$), respectively in 20/37 rats; others, like rearing were comparable. General observations made in the open field arena also showed that freezing was seen only in the 20/37 group. Voiding and defecation, manifestations of autonomic stimulation, were noted in both the 0/37 and 20/37 groups. Hypothermia was shown to abrogate the abnormalities detected in 20/37 rats in open field studies. Sniffing at the wall was increased by all levels of hypothermia, the increase being significant ($P<0.05$) with 20/15 (Table 5). Grooming was also significantly decreased ($P<0.05$) in all the hypothermic-asphyxiated groups (Table 5). Not unlike the 20/37 group, defecation/voiding was also observed in the hypothermic asphyxiated groups as well.

Discussion

This is the first long-term study performed to test the effect of moderate and profound hypothermia on morphological and functional changes of the brain following

three months of an asphyxial insult. The study evaluated morphological alterations of the brain and its three neurotransmitter systems, and analyzed the impact of these alterations on neurological, behavioural, and cognitive functions as well as mood.

Survival decreased with 20/37 and the decrease was ablated with increasing magnitude of hypothermia. This prolonged survival may well be related to reduction of oxygen consumption/decreased metabolic rate under hypothermic conditions (Wagner et al., 1999). Hypothermia is also implicated in attenuating excitatory amino acid neurotoxicity and in prolongation of extracellular accumulation of the inhibitory neurotransmitter glycine during hypoxia-ischemia (Baker et al., 1991; Busto et al., 1989; Simpson et al., 1990). Hypothermia produces graded reduction in cerebral metabolism of about 5% for every degree of temperature reduction (Laptook et al., 1995), suggesting increased protection at lower temperatures. This is in line with the present study, as maximum survival was achieved at 15 °C. However, duration of hypothermia was shown to be a concern, since increasing duration beyond 20 min was associated with reduced survival. This shows that there is a cut-off time point where hypothermia could be protective. Once this limit is passed it can lead to death because very low temperature is associated with reduced oxygen availability, metabolic acidosis and increased blood viscosity (Gunn et al., 1998).

Evaluation of neuronal loss in five different brain regions revealed that 20/37-mediated neuronal loss is pronounced in the hippocampus and hypothalamus, which is consistent with our previous report (Kohlhauser et al., 1999b). Although moderate as well as profound hypothermia considerably extended survival of an asphyxiated pup, it failed to offer neuroprotective effect when evaluated at three months of age, at least, with respect to morphology. Surprisingly, hypothermia rather demonstrated dose- and time-dependent neurotoxic property. Morphological findings in the hippocampus are discordant with unimpaired cognitive functions in the spatial memory test. One could argue that the unaffected frontal cortex might have a role in this regard, since there is sufficient evidence that it is involved in aquisition of spatial/temporal learning and memory (Mitchel and Laiacona, 1998; Fritts et al., 1998; Solbrig et al., 1996). The results of Morris water maze and multiple T-maze in 100/15, however, speak against this notion, showing once again the paralleled relation of histological findings and function. This enigma may be explained by compensatory mechanisms that were operative in the perinatal brain, owing to its plasticity, such as taking over function of lost neurons by surviving neurons. This compensation might have thus enabled the asphyxiated adolescent animal to perform like its normal counterpart in memory tests. The observation of comparable cognitive functions among 0/37, 20/37, 20/30, 20/15, 50/15 and 100/15 rats as evaluated by the Morris water maze and multiple T-maze is a rather unexpected finding as profound hypothermia was supposed to have adverse effects. And it also presents challenging data to previous results of experiments describing anoxia induced impairment of cognitive functions in animal models of perinatal hypoxia without hypothermia (Hershkowitz et al., 1983; Speiser et al., 1983; Dell'Anna et al., 1991).

Several neurotransmitter systems have been incriminated in mechanisms leading to neuronal death and brain dysfunction following perinatal asphyxia. This study also revealed deterioration of all the transmitter systems in various brain regions, out of which some could be reversed by hypothermia. Decreased expression of TH, a rate-limiting enzyme in the biosynthetic pathway of catecholamines, may reflect deficient dopaminergic and noradrenergic innervation. TH can be used as a marker for the dopaminergic system, which in turn is responsible for a series of psychomotor functions. Motor behaviour was impaired in 4 week old rats following perinatal asphyxia and these rats demonstrated reduction of immunoreactive TH mesencephalic cell bodies (Chen et al., 1995). In the present study, deranged motor functions were noted in 20/37 rats following three months of asphyxia, which happened to display TH-deficit. Inability of 20/37 rats to remain longer on the rota-rod reflects impaired motor function and these abnormalities were virtually reversed by hypothermia. It is thus conceivable to assume that reversion of motor abnormalities may have to do with overcoming of TH-deficit by hypothermia. VMAT, the principal monoamine transporter, is evidenced to be of biological importance in human disease. Thiabut and coworkers showed that reduction in VMAT is associated with Parkinson's disease (Thiabut et al., 1995). Deficit of VMAT in 20/37 was found in various brain regions, showing its potential to cause brain disease in the long-term. Returning to normal level of VMAT by hypothermia also reflects how hypothermia could be of use in reducing the likelihood of developing long-term brain sequelae following perinatal insults. The cholinergic pathways are widespread in cortical and subcortical areas of the brain, including regions used in our experiment (Kassa, 1986). Expression of choline acetyltransferase, VAChT, which transports acetylcholine into synaptic vesicles, and the high affinity membrane choline transporter are defining markers of the cholinergic phenotype in the mammalian central nervous system (Schafer et al., 1994). At near term, the time at which asphyxia was induced in the

present work, VAChT is already present in a distributional pattern resembling the adult (Aubert et al., 1996). Reduced VAChT observed in 20/37 rats could thus reflect cholinergic neuronal loss in the perikarya of fiber bundles of frontal cortex, CA1 and CA3 of the hippocampus and the anterior hypothalamic region central. These findings were paralleled by a decreased number of Nissl stained cells in the same areas with the exception of frontal cortex. As the cholinergic neurons are involved in complex memory formation, cholinergic derangement suggested by our data and its reversal by profound hypothermia may help to interpret neurological sequelae and its prevention following perinatal asphyxia. Glutamatergic neurotransmission is seen in abundance in brain regions, such as cerebral cortex, hippocampus and cerebellum (Tohyama and Takatsuji, 1998). Extracellular glutamate concentrations are regulated by glial and neuronal transporter proteins. Four glutamate transporter subtypes have been identified in rat brain; GLAST and GLT-1 are primarily astrocytic, whereas EAAC1 and EAAT4 are neuronal. EAAC1 seems to be an appropriate marker for our purpose as its localization and distribution is well-documented even in newborns (Furuta et al., 1997). Excitotoxicity is described to be one of the mechanisms leading to neuronal death in newborn animal models of perinatal asphyxia and use of excitatory amino acid antagonists has been shown to provide some protective effect (Engidawork et al., 2001; Hagberg et al., 1987; Espinoza et al., 1991). Increased exitatory amino acid levels were also found in three months old asphyxiated rats (Kohlhauser et al., 1999b). Given this, lower levels of EAAC1 in 20/37 rats could therefore provide a tentative explanation for persistence of excitotoxicity. Moreover, returning EAAC1 to normal level by profound hypothermia (20/15 and 50/15) could indicate one possible mechanism by which the protective effect of hypothermia is effected.

In an observational battery for neurological evaluation, we found unimpaired reflexes with exception of increased acoustic startle response, probably pointing to increased irritability, and an impaired visual placing test reflecting positional passivity in 20/37 rats. Reduced scores in the alley traversion may reflect deteriorated exploratory behaviour activity that could be explained by freezing/immobilization seen in the group, since locomotor activity in the open field was unaffected and elevated plus maze findings ruled out that decreased activity was anxiety-related. Our data point to the fact that both moderate and profound hypothermia during a period of anoxia as long as 50 and 100 min are neuroprotective in terms of motor function.

Studies on long-term effects of perinatal asphyxia/hypoxia/anoxia have shown hyperactivity in the open field, including motor activity in ambulation, sniffing and rearing activities in early adulthood that returns to normal with age (Hershkowitz et al., 1983; Speiser et al., 1983; Dell'Anna et al., 1991). In the present study, we found increased grooming and decreased sniffing at the wall in the 20/37 group. Increased grooming could be explained by hypoxic damage of the hypothalamus. It is well known that electrical as well as chemical stimulation of the paraventricular nucleus of the hypothalamus and the adjacent thalamus induce self grooming responses in the rat, a behaviour which also can be influenced by certain neuropeptides, the administration of ACTH or dopamine antagonists for instance (van Erp et al., 1995; Poggioli et al., 1995; Bressers et al., 1995; Moøy, 1995). Increased grooming was totally abolished by hypothermia, probably an effect attributed to protection of the hypothalamus. Decrease of the parameter sniffing at the wall may reflect reduced exploratory behaviour paralleling the alley traversion test, though, as mentioned above the elevated plus maze results in this group ruled out anxiety-related behaviour and scores for the finger approach test were increased at the same time. Buwalda and coworkers (1995), studying the effects of early postnatal anoxia on adult learning and emotions in rats, reported normal outcome in rats with neonatal anoxia when tested in the elevated plus maze as compared to normoxic controls. This is in contrast to our finding that 20/37 rats spent significantly more time in the open arms of the maze. Reduced anxiety-related behaviour shown by these animals might be explained by hypothalamic neuronal loss observed in the present study and by altered concentrations of excitatory amino acids in the hypothalamus described in the identical animal model of perinatal asphyxia (Kohlhauser et al., 1999a). This notion appears to be valid, as hypothermic animals stayed relatively shorter in the open arm than 20/37 rats. Thus, impaired cognitive function or hyperactivity was not responsible for loss of anxiety related behaviour as it was ruled out by mazes and open field studies.

In conclusion, the present study confirmed and extended a previous study revealing the neuropathological, neurological and behavioural long-term outcome of perinatal asphyxia in the rat. The study clearly showed the inconsistent cause-and-effect relationships between morphological findings and functional changes. Morphological changes seen in hippocampal areas CA1 and CA3 of 20/37 rats were not accompanied by deficits in cognitive functions, as reflected by normal performance in the maze

studies. Conversely, absence of morphological pathology in motor regions, such as striatum and cerebellum was greeted by neurological findings unambiguously diagnosed by rota-rod tests. The absence of neuropathology in frontal cortex was reflected by unimpaired cognitive functions (spatial memory), but this was not in agreement with findings of decreased sniffing at the wall, representing exploratory behaviour in the open field, a function which can be assigned (although not exclusively) to frontal cortex. However, hypothalamic neuronal loss might have been responsible for changes in mood i.e. reduced anxiety-related behaviour and increased grooming. These observations as well as our previous report in guinea-pigs (Hoeger et al., 2003) strongly indicate that using only histological methods for evaluation of brain damage or neuroprotective strategies is insufficient and performing morphological studies, along with tests for neurological, behavioural and cognitive functions is therefore mandatory. We also strongly recommend the concomitant use of maze studies, open field and rota-rod in order to see findings in different context, as cognitive functions, mood and motor functions are closely related and interactive. The performance of either test alone would inevitably lead to misinterpretations. We also question the importance of the neurological observational battery as a reliable set of parameters for motor impairment as the unequivocal motor deficits found by rota-rod examination were not detected by this screening system in our study.

Hypothermia failed to attenuate total neuronal loss caused by 20/37. At times, it accentuated neuronal loss in duration and magnitude-dependent manner even in areas not affected by 20/37. On the other hand, short-term moderate (20/30) and profound (20/15) hypothermia provided inconsistent protective effect to different neurotransmitter systems, an effect known to depend on regiospecific sensitivity to hypoxia/ischemia. Hypothermia was, however, effective in preserving survival, motor functions, mood and behaviour. Based upon our current findings the hypothermic principle may be valid, as long as the optimal magnitude and duration is employed, we are thus now using pharmacological approaches to reduce body temperature in an identical model.

Acknowledgements

We are highly indebted to Prof. Dr. Gert Lubec, FRSC (UK) for scientific advice. EE is supported by the Austrian Embassy Development Cooperation, Addis Ababa, Ethiopia. We appreciate the supervision of morphological work by associate Prof. Dr. W. Mosgoeller, Institute for Tumor Biology, Medical University of Vienna. We highly appreciate the contribution of the Verein zur Durchführung der wissenschaftlichen Forschung auf dem Gebiet der Neonatologie und Kinderintensivmedizin ("Unser Kind").

References

Aubert I, Cecyre D, Gauthier S, Quirion R (1996) Comparative ontogenic profile of cholinergic markers, including nicotinic and muscarinic receptors in the brain. J Comp Neurol 369: 31–55

Baker AJ, Zornow MH, Grafe MR, Schiller MS, Skilling SR, Smullin DH, Larson AA (1991) Hypothermia prevents ischemia-induced increases in hippocampal glycine concentrations in rabbits. Stroke 22: 666–673

Bona E, Hagberg H, Loberg EM, Bagenholm R, Thoresen M (1998) Protective effects of moderate hypothermia after neonatal hypoxia-ischemia: short- and long-term outcome. Pediatr Res 43: 738–745

Bressers WM, Kruk MR, Van Erp AM, Willekens-Bramer DC, Haccou P, Meelis E (1995) Time structure of self grooming in the rat: self-faciliation and effects of hypothalamic stimulation and neuropeptides. Behav Neurosci 109: 955–964

Burck HC (1981) Histologische Technik. Thieme, Stuttgart, New York

Busto R, Globus MY-T, Dietrich D, Martinez E, Valdes I, Ginsberg MD (1989) Effect of mild hypothermia on ischemia-induced release of neurotransmitters and free fatty acids in rat brain. Stroke 20: 904–910

Buwalda B, Nyakas C, Vosselman HJ, Luiten PGM (1995) Effects of early postnatal anoxia on adult learning and emotion in rats. Behav Brain Res 67: 85–90

Capani F, Loidl F, Lopez-Costa JJ, Selvin-Testa A, Saavedra JP (1997) Ultrastructural changes in nitric oxide synthase immunoreactivity in the brain of rats subjected to perinatal asphyxia: neuroprotective effects of cold treatment. Brain Res 775: 11–23

Carter BS, Havercamp AD, Merenstein GB (1993) The definition of acute perinatal asphyxia. Clin Perinatol 20: 287–303

Chen Y, Orgen SO, Bielke B, Bolme P, Eneroth P, Gross J, Loidl F, Herrera-Marschitz M (1995) Nicotine treatment counteracts perinatal asphyxia induced changes in the mesostriatal/limbic dopamine system and in motor behavior in the four week old male rat. Neuroscience 68: 531–538

Dell'Anna ME, Calzolari S, Molinari M, Iuvone L, Calimici R (1991) Neonatal anoxia induces transitory hyperactivity, permanent spatial memory deficits and CA1 cell density reduction in developing rats. Behav Brain Res 45: 125–134

Dunn JM, Miller JA (1969) Hypothermia combined with positive pressure ventilation in the resuscitation of the asphyxiated neonate. Am J Obstet Gynecol 104: 58–67

Edwars AD, Yue X, Squier MV, Thoresen M, Cady EB, Penrice J, Cooper CE, Wyatt JS, Reynolds EOR, Mehmet H (1995) Specific inhibition of apoptosis after cerebral hypoxia-ischemia by moderate post-insult hypothermia. Biochem Biophys Res Commun 3: 1193–1199

Engidawork E, Loidl F, Chen Y, Kohlhauser C, Stoeckler S, Dell'Anna E, Lubec B, Lubec G, Goiny M, Gross J, Andersson K, Herrera-Marschitz M (2001) Comparison between hypothermia and glutamate antagonism treatments on the immediate outcome of perinatal aspyxia. Exp Brain Res 138: 375–383

Espinoza MI, Parer JT (1991) Mechanisms of asphyxial brain damage and possible pharmacologic interventions in the fetus. Am J Obstet Gynecol 164: 1582–1589

Fritts ME, Asbury ET, Horton JE, Isaac WL (1998) Medial prefrontal lesion deficits involving or sparing the prelimbic area in the rat. Physiol Behav 64: 373–380

Furuta A, Rothstein JD, Martin LJ (1997) Glutamate transporter protein subtypes are expressed differentially during rat CNS development. J Neurosci 17: 8363–8375

Ginsberg MD, Sternau LL, Globus MY, Dietrich WD, Busto R (1992) Therapeutic modulation of brain temperature: relevance to ischemic brain injury. Cerebrovasc Brain Metab Rev 4: 189–225

Gunn AJ, Gluckman PD, Gunn TR (1998) Selective head cooling in newborn infants after perinatal asphyxia: a saftey study. Pediatrics 102: 885–892

Gunn AJ, Bennet L, Gunning MI, Gluckman PD, Gunn TR (1999) Cerebral hypothermia is not neuroprotective when started after post-ischemic seizures in fetal sheep. Pediatr Res 46: 274–280

Haaland K, Loberg EM, Steen PA, Thoresen M (1997) Posthypoxic hypothermia in newborn piglets. Pediatr Res 41: 505–512

Hagberg H, Andersson P, Kjellmer I, Thiringer K, Thordstein M (1987) Extracellular overflow of glutamate, aspartate, GABA, and taurine in the cortex and basal ganglia of fetal lambs during hypoxia-ischemia. Neurosci Lett 78: 311–317

Hamm RJ, Pike BR, O'Dell DM, Lyeth BG, Jenkins LW (1994) The rota-rod test: an evaluation of its effectiveness in assessing motor deficits following traumatic brain injury. J Neurotrauma 11: 187–196

Hershkowitz M, Grimm VE, Speiser Z (1983) The effects of postnatal anoxia on behaviour and on the muscarinic and beta-adrenergic receptors in the hippocampus of the developing rat. Dev Brain Res 7: 379–382

Hill A (1991) Current concepts of hypoxic-ischemic cerebral injury in the term newborn. Pediatr Neurol 7: 317–325

Hoeger H, Engelmann M, Bernert G, Seidl R, Bubna-Littitz H, Mosgoeller W, Lubec B, Lubec G (2000) Long term neurological and behavioral effects of graded perinatal asphyxia in the rat. Life Sci 66: 944–962

Hoeger H, Bubna-Littitz H, Engelmann M, Schwerdtner I, Schmid D, Lahoda R, Seidl R, Lubec G, Lubec B (2003) Perinatal asphyxia in the guinea-pig leads to morphologic but not neurologic, cognitive or behavioral changes. J Invest Med 51: 233–239

Irwin S (1968) Comprehensive observational assessment: a systematic, quantitative procedure for assessing the behavioral and physiologic state of the mouse. Psychopharmacologia 13: 222–257

Jones BJ, Roberts DJ (1968) The quantitative measurement of motor incoordination in naive mice using an accelerating rota-rod. J Pharm Pharmacol 20: 302–304

Kassa P (1986) The cholinergic system in brain and spinal cord. Progr Neurobiol 26: 211–272

Klawitter V, Morales P, Johansson S, Bustamante D, Goiny M, Gross J, Luthman J, Herrera-Marschitz M (2005) Effects of perinatal asphyxia on cell survival, neuronal phenotype and neurite growth evaluated with organotypic triple cultures. Amino Acids 28: 149–155

Kohlhauser CH, Mosgoeller W, Hoeger H, Lubec G, Lubec B (1999a) Cholinergic, monoaminergic and glutamatergic changes following perinatal asphyxia in the rat. Cell Mol Life Sci 55: 1491–1501

Kohlhauser C, Kaehler S, Mosgoeller W, Singewald N, Kouvelas D, Prast H, Hoeger H, Lubec B (1999b) Histological changes and neurotransmitter levels three months following perinatal asphyxia in the rat. Life Sci 64: 2109–2124

Laptook AR, Corbett RJT, Sterett R, Burns DK, Tollefsbol G, Garcia D (1994) Modest hypothermia provides partial neuroprotection for ischemic neonatal brain. Pediatr Res 35: 436–442

Laptook AR, Corbett RJT, Sterett R, Garcia D, Tollsfbol G (1995) Quantitative relationship between brain temperature and energy utilisation rate measured in vivo using 31P and 1H magnetic resonance spectroscopy. Pediatr Res 38: 919–925

Loidl CF, De Vente J, Markerink van Ittersum M, van Dijk EHJ, Vles JSH, Steinbusch HWM, Blanco CE (1998) Hypothermia during or after severe perinatal asphyxia prevents increase in cyclic GMP-related nitric oxide levels in the newborn rat striatum. Brain Res 791: 303–307

Lubec B, Dell'Anna E, Fang-Kircher S, Marx M, Herrera-Marschitz M, Lubec G (1997a) Decrease of brain protein kinase C, protein kinase A, and cyclin-dependent kinase correlating with pH precedes neuronal death in neonatal asphyxia. J Invest Med 45: 284–294

Lubec B, Marx M, Herrera-Marschitz M, Labudova O, Hoeger H, Gille L, Nohl H, Mosgoeller W, Lubec G (1997b) Decrease of protein kinase C and cyclin-dependent kinase precedes death in perinatal asphyxia. FASEB J 11: 482–492

Miller J (1971) New approaches to preventing brain damage during asphyxia. Am J Obstet Gynecol 110: 1125–1133

Miller JA, Miller FS (1972) Mechanisms of hypothermic protection against anoxia. Adv Exp Med Biol 33: 571–586

Mitchel JB, Laiacona J (1998) The medial frontal cortex and temporal memory: tests using spontaneous exploratory behaviour in the rat. Behav Brain Res 97: 107–113

Moøy SS (1995) Impaired acquisition and operate responding after neonatal dopamine depletion in rats. Pharmacol Biochem Behav 52: 433–441

Poggioli R, Benelli A, Arletti R, Cavazzuti E, Bertolini A (1995) Nitric oxide is involved in the ACTH-induced behavioral syndrome. Peptides 16: 1263–1268

Rogers CD, Campbell CA, Stretton JL, Mackay KB (1997) Correlation between motor impairment and infarct volume after permanent and transient middle cerebral artery occlusion in the rat. Stroke 28: 2060–2066

Schafer MK, Weihe E, Varoqui H, Eiden LE, Erickson JD (1994) Distribution of the vesicular acetylcholine transporter (VAChT) in the central and peripheral nervous system of the rat. J Mol Neurosci 5: 1–26

Simpson RE, Walter GA, Phillis JW (1990) The effects of hypothermia on amino acid neurotransmitter release from the cerebral cortex. Neurosci Lett 124: 83–86

Sirimane ES, Blumberg RM, Bossano D, Guning M, Edwards AD, Gluckman PD, Williams CE (1996) The effect of prolonged modification of cerebral temperature on outcome after hypoxic-ischemic brain injury in the infant rat. Pediatr Res 39: 591–597

Solbrig MV, Koob GF, Reid S, Lipkin WI (1996) Prefrontal cortex dysfunction in Borna disease virus (BVD)-infected rats. Biol Psychiatry 40: 629–636

Speiser Z, Amitzi-Sonder J, Gitter S, Cohen S (1983) Behavioral differences in the developing rat following postnatal anoxia or post-natally injected AF-64, a cholinergic neurotoxin. Behav Brain Res 7: 379–382

Thiabut F, Faucheux BA, Marquez J, Villares J, Menard JF, Agid Y, Hirsch EC (1995) Regional distribution of monoamine vesicular uptake sites in the mesencephalon of control subjects and patients with Parkinson's disease: a postmortem study using tritiated tetrabenazine. Brain Res 692: 233–243

Thoresen M, Bagenholm R, Loberg EM, Apricena F, Kjellmer I (1996) Posthypoxic cooling of neonatal rats provides protection against brain injury. Arch Dis Childhood 74: F3–F9

Tohyama M, Takatsuji K (1998) The glutamatergic system. In: Tohyama M, Takatsuji K (eds) Atlas of neuroactive substances and their receptors in the rat. Oxford University Press, New York, pp 134–143

Trescher WH, Ishiwa S, Johnston MV (1997) Brief post-hypoxic-ischemic hypothermia markedly delays neonatal brain injury. Brain Dev 19: 326–338

van Erp AM, Kruk MR, Veening JG, Roeling TA, Meelis W (1995) Neuronal substrate of electrically induced grooming in the PVH of the rat: involvment of oxytocinergic systems? Physiol Behav 57: 881–885

Volpe JJ (1987) Neurology of the newborn. In: Volpe JJ (ed) Hypoxic-ischemic encephalopathy: biochemical and physiological aspects. WB Saunders, Philadelphia, p 22

Wagner CL, Eicher DJ, Katikaneni LD, Barbosa E, Holden KR (1999) The use of hypothermia: a role in treatment of neonatal asphyxia? Pediatr Neurol 21: 429–443

Yager J, Towfighi J, Vanucci RC (1993) Influence of mild hypothermia on hypoxic-ischemic brain damage in the immature rat. Pediatr Res 34: 525–529

Authors' address: Prof. Dr. Barbara Lubec, Department of Neonatology, Medical University of Vienna, Währinger Gürtel 18, A- 1090 Vienna, Austria,

Fax: +43-1-40400-3194, E-mail: gert.lubec@meduniwien.ac.at