ASPHYXIA-INDUCED BRAIN OEDEMA FORMATION IN NEWBORN PIGS: A PARTICULAR ROLE FOR HISTAMINE

Ph. D. Thesis

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Contents

***In extenso* publications in the topic of the thesis**

I. Dux E, Temesvári P, Szerdahelyi P, Nagy Á, **Kovács J**, Joó F: Protective effect of antihistamines on cerebral oedema induced by experimental pneumothorax in newborn piglets. *Neuroscience* 1987; **22:** 317-321. (IF_{1987}: 4.547)

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SUMMARY

Prevention of brain damages caused by perinatal asphyxia and hypoxic-ischaemic insults has great clinical importance in neonatal intensive care. Cerebral oedema is an acute, life-threatening complication of hypoxic brain damages. The aim of the present Ph.D. thesis was to investigate the pathophysiology and therapeutic prevention of neonatal brain oedema using animal models. The study was particularly focused on the role of histamine, as a vasoactive mediator, in the pathogenesis of the disease.

The main new results of the thesis are summarized as follows:

It has been established that neonatal asphyxia-reperfusion increases the concentration of histamine in serum from the jugular vein, in cerebrospinal fluid, and in isolated cerebral microvessels. The investigations have revealed the role of cerebral microvessels in the elimination of excess histamine released from brain compartments during postasphyxial reperfusion in newborn piglets.

During asphyxia-reperfusion an increase in serum α-melanocyte-stimulating hormone concentration and a decrease in cerebrospinal fluid hormone level were observed in newborn pigs. It is known that the release of α-melanocyte-stimulating hormone from the intermediate lobe of the pituitary gland is regulated, among others, by hypothalamic histaminergic neurons in perinatal stress.

It has been proved that temporal and regional distribution of asphyxia-induced cytotoxic and vasogenic oedema in newborn pigs resembles to the characteristics of brain damages in human neonates. This observation further supports that porcine bilateral experimental pneumothorax is an appropriate animal model for studying the pathomechanism and therapy of brain damages evoked by neonatal asphyxia.

Intracarotid administration of exogenous histamine could induce vasogenic brain oedema formation in healthy newborn pigs. These data may serve as an indirect proof for the involvement of excess endogenous histamine in the blood-brain barrier permeability changes.

Moreover, the contribution of histamine to the development of cerebral injury is further supported by the efficacy of H_1 and H_2 histamine receptor antagonists in reduction of brain oedema formation in porcine model of neonatal asphyxia.

It has been also demonstrated that development of brain oedema in asphyxiated newborn pigs could be attenuated by treatment with magnesium sulphate or D-2-amino-5-phospho-valeric acid, *N*-methyl-D-aspartate type glutamate receptor antagonists. The crucial role for glutamic acid in the pathogenesis of neonatal encephalopathies has been previously established in series of experimental and clinical studies.

The present investigations on the pathophysiology and therapy of neonatal brain oedema may contribute to the better understanding of hypoxic-ischaemic neonatal brain damages, to the treatment of acute life-threatening complications, and to the prevention of neurological sequelae.

Abbreviations:

ACTH:	adrenocorticotropin
ADH:	antidiuretic hormone
ANOVA:	analysis of variance
APV:	D-2-amino-5-phospho-valeric acid
ATP:	adenosine triphosphate
BBB:	blood-brain barrier
b.w.:	body weight
cAMP:	cyclic adenosine 3'5'-monophosphate
CBF:	cerebral blood flow
CVP:	central venous pressure
cGMP:	cyclic guanosine 3'5'-monophosphate
CNS:	central nervous system
CSF:	cerebrospinal fluid
DPPE:	*N,N*-diethyl-2-(4-(phenyl-methyl)phenoxy)ethanamine
EBA:	Evan's blue-labelled albumin
H_1:	histamine type 1 receptor
H_2:	histamine type 2 receptor
H_3:	histamine type 3 receptor
HDC:	L-histidine decarboxylase
Hic:	intracellular histamine binding site
HIV-1:	human immunodeficiency virus-1
HMT:	histamine-*N*-methyltransferase
HPLC:	high pressure liquid chromatography
HR:	heart rate
i.c.v.:	intracerebroventricularly
IL:	interleukin
i.m.:	intramuscularly
i.p.:	intraperitoneally
i.v.:	intravenously
i.th.:	intrathecally
LTC:	lung-thoracic compliance
MABP:	mean arterial blood pressure
α-MSH:	α-melanocyte stimulating hormone
mw:	molecular weight
*N*MDA:	*N*-methyl-D-aspartate
P-creatine:	phosphocreatine
POMC:	proopiomelanocortin
PTX:	pneumothorax
RIA:	radioimmunoassay
s.c.:	subcutaneously
SF:	sodium fluorescein
TNF-α:	tumor necrosis factor-α
ZO:	zonula occludens

1. INTRODUCTION

1.1 Neonatal asphyxia

Perinatal asphyxia, *i.e.* failure to initiate and sustain breathing at birth, is one of the most frequent and serious clinical problems in neonatology [181]. According to World Health Organization estimates, around 3% of approximately 120 million infants born every year in developing countries present birth asphyxia requiring resuscitation, and about 900,000 of these infant die each year [181]. The incidence of perinatal asphyxia is higher in less developed countries, because of a higher prevalence of risk factors, such as (i) poor maternal health, (ii) high incidence of pregnancy and delivery complications, (iii) inadequate care during labour and delivery, and (iv) prematurity [181].

Despite its common occurrence, there is no agreed definition or specific accurate biological marker for ascertaining the diagnosis of perinatal asphyxia [149]. In clinical terms perinatal asphyxia is an insult to the foetus or newborn due to lack of oxygen or lack of perfusion to various organs. These are associated with tissue hypoxia and acidosis which provide the biochemical definition of the disorder [50].

The aetiopathology of asphyxia can be divided either to maternal, placental and foetal causes or to *ante-partum*, *intrapartum* and *postpartum* origin [66]. Approximately 90% of asphyxial insults occur during the first two time-periods (see Table 1).

Table 1 **Causes of perinatal asphyxia**

Maternal	• toxaemia of pregnancy, hypotension, vascular disease, diabetes mellitus, pulmonary or cardiac disease, infection, uterine tetany • narcotics, sedatives, anaesthetics • difficult delivery (forceps or vacuum extraction, caesarean section)
Placental	• abnormal anatomy of placenta, placental insufficiency, • premature separation or abruption of the placenta, infarction • umbilical cord accidents (prolapse, compression, knotting, entanglement) • abnormality of umbilical vessels
Foetal	• anaemia, intrauterine growth retardation, postmaturity, hydrops • immaturity of the lungs, intrauterine infections, meconium aspiration, diaphragmatic hernia, congenital cardiac or lung diseases, intracranial haemorrhage

This classification, however, seems to be rather artificial, since the etiologic factors above may belong to more than one category. Some of these problems may be either the cause of asphyxia or may be associated with it. In most cases of perinatal asphyxia a combination of these factors is present, with a resultant inability to provide oxygen to and to remove carbon dioxide and hydrogen from the foetal or neonatal organs.

During normal labour the blood flow to, and the gas exchange across the placenta are both reduced causing decreased oxygen delivery to the foetus. At the same time both the mother and the foetus have increased oxygen consumption. In consequence, most babies are born with little oxygen reserves. In addition to these normal events, any maternal, placental or cord factors that interfere with gas exchange across the placenta could result in perinatal asphyxia (Table 1). The following alterations could be seen along the pathogenetic process: early congestion, fluid leak from increased capillary permeability and endothelial cell swelling may lead to coagulation necrosis and cell death [66]. Vasoactive mediators and free oxygen radicals are known to be generated in abundance during perinatal ischaemia-reperfusion [178]. If foetal distress is prolonged, it may result in severe tissue hypoxia and acidosis (partially due to lactic acid production in anaerobic metabolism) which may be worsened when organ perfusion is deteriorated.

The clinical signs of perinatal hypoxia/ischaemia can also be differentiated according to their time-dependent appearance along the pregnancy and delivery:

- *antenatal*: decreased foetal movements, intrauterine growth retardation, increased vascular resistance, non-reactive stress testing, abnormal cardiotocogram (variable or late deceleration);
- *intrapartum*: meconium stained amniotic fluid, foetal heart deceleration, depressed Apgar scores, abnormally low pH and excessive base deficit in cord blood;
- *postpartum* effects of asphyxia depend on the severity of the insult and on the affected organ:
    - Central nervous system (CNS): hypoxic-ischaemic encephalopathy (periventricular leukomalacia), infarction, intracranial haemorrhage, cerebral oedema, seizures, abnormal muscle tone, feeding difficulties;
    - Circulation: myocardial ischemia, hypotension, bradycardia, pallor, cyanosis;
    - Adrenal gland: haemorrhage;
    - Lung: persistent pulmonary hypertension, haemorrhage, respiratory distress syndrome;

- Kidney: acute cortical and tubular necrosis;
- Gastrointestinal tract: haemorrhage, ulceration, necrosis, perforation

This multi-organ failure is accompanied also with metabolic and endocrine disturbances, such as hyponatraemia, hypoglycaemia, hypocalcaemia, inappropriate antidiuretic hormone (ADH) syndrome further increasing the water retention in body fluids. In the presence of hypoxic-ischaemic insult to the foetus, reflexes are initiated, shunting the blood to maintain the perfusion of brain, heart and adrenals in preference to lungs, gut, liver, kidneys and muscles.

The condition of the newborn after birth can be described by the Apgar scoring system, and low (3 or less at 5 min) Apgar score defines severe perinatal insult.

The outcome of perinatal asphyxia depends on the severity of metabolic, cardiovascular and cerebral complications. Besides the high mortality rate, the incidence of neurological sequelae in survivors is 20-45%. Morbidity and mortality are even higher in preterm infants, possibly because of the higher rate of brain damages. Good perinatal care can prevent or minimize asphyxia, but inadequate treatment may lead to death or major long-term neurological complications. Among all problems in neonatal medicine brain injury, and especially its prevention, is of particular importance.

1.2. Hypoxic-ischaemic encephalopathy in newborns

Perinatal asphyxia, with its attendant bradycardia and hypotension, results in ischaemia-reperfusion injury in several organs including brain [50]. Cerebral hypoxia-ischaemia may produce severe acute (intraventricular haemorrhage, brain oedema) and chronic (periventricular leukomalacia, cerebral palsy, neurological sequelae manifested in cognitive and behavioural deficits, serious school disturbances) consequences in preterm and term newborns [66]. Neonatal encephalopathy is characterized as mild, moderate, or severe by variable alterations in consciousness, reflex patterns, and muscle tone and possible brainstem and autonomic dysfunction [149].

Magnetic resonance imaging studies provided data on the detection of brain injury during the first hours of postasphyxial reperfusion [10,13,63,127,161]. Impairment in cerebral energy metabolism, such as elevated tissue lactate level or a decline in phosphocreatine/inorganic phosphate ratio, may allow prediction of neurodevelopmental outcome of the affected infants

during the early postasphyxial hours [63,126,127,161]. The regional patterns of brain injury of asphyxiated term newborns in the first days of life, however, remain to be revealed in details [13,161].

Brain oedema formation contributes to asphyxia or hypoxia-ischaemia induced brain damages in newborn animals [106,110,163,165,170]. In a porcine model of bilateral pneumothorax (PTX) originally described by Temesvári *et al.* [163] asphyxia and reperfusion resulted in deterioration of cerebral circulation, cerebral oedema formation, and neurological damage [2,3,162-165]. According to the classical neuropathological views [18,75,179], brain oedema has cytotoxic and vasogenic components. Water and electrolyte disturbances caused by cellular energy failure result in cytotoxic oedema formation, hence extravasation of serum constituents through the blood-brain barrier (BBB) produces vasogenic type of cerebral oedema. A correlation was reported between the duration of severe oedema, and the degree of ultimate brain damage in immature rats [170]. Neuronal susceptibility to hypoxia-ischaemia was also correlated with the extent of BBB permeability that was closely related to maturation of the animal [110]. Albumin, similarly to other serum factors, extravasating into the cerebral interstitium may exert neurotoxic effect *in vivo* [64].

1.3. Molecular mechanisms involved in the pathogenesis of neonatal brain injuries

Cellular and molecular mechanisms contributing to the development of brain damages in asphyxiated neonates include neuronal excitotoxicity, the receptor-mediated increase in intracellular Ca^{2+}, generation of reactive oxygen species and nitric oxide, release of vasoactive mediators and cytokines, *etc.* [37,50,91,161]. Ischaemic neuronal cell death involves various critical functional and structural changes, such as increased membrane permeability, decreased Na^{+}/K^{+}-ATPase activity, mitochondrial dysfunction, decreased protein synthesis, cytoskeletal damage, and prolonged changes in kinases or phosphatases [97]. The mechanisms relevant to the topic of thesis are summarized in the following paragraphs.

1.3.1. Excitatory amino acids

Neonatal asphyxia results in high brain tissue concentration of endogenous excitatory amino acids including aspartate and glutamate [134]. The action of these excitatory amino acids is

mediated by various receptor subtypes named after preferential agonists, such as *N*-methyl-D-aspartate (*N*MDA), α-amino-hydroxy-5-methyl-4-isoxazole proprionic acid (quisqualate), and kainate [37]. Excessive receptor activation leads to excitotoxicity to brain cells, and *N*MDA receptor-mediated neurotoxicity is stronger in the developing brain than in the adult one [37]. Glutamate-receptor-mediated neurotoxicity can be ameliorated through mechanisms (i) upstream (agents decreasing glutamate release, *e.g.* adenosine derivatives, sodium-channel blockers, voltage-dependent–calcium channel antagonists); or (ii) downstream (xanthine oxidase inhibitors, free radical scavengers, protein kinase C inhibitors, protease inhibitors, nitric oxide synthase inhibitors *etc.*) to glutamate receptors; and (iii) through glutamate receptor antagonists [98]. The *N*MDA receptor is an essential component of learning and memory and of mechanisms responsible for hypoxic-ischaemic injury [37]. The activity of *N*MDA receptor-ion channel complex can be modulated through pharmacologically distinct sites by various receptor antagonists [32,40,97,98,117], as it is shown in Table 2.

Table 2 **Antagonists of the *N*MDA receptor-ion channel complex**

	Binding sites	***N*MDA-receptor antagonists**
1	glutamate-binding site	CG19755
2	glycine-binding site	felbamate
3	polyamine site	ifenprodil, eliprodil, CP101606, Ro 25-6981, Ro 8-4304
4	receptor-associated channel	Mg^{2+}, Zn^{2+}, ketamine, dextrorphan, dextromethorphan, D-2-amino-5-phosphovaleric acid, memantine, remacimide, CNS1102,
5	redox modulatory site(s)	nitroglycerin, nitroprusside

Previous studies have shown that the *N*MDA receptor is modified during hypoxia in the cerebral cortex of newborn piglets [35]. The effect of receptor antagonists on brain oedema formation, however, was not previously demonstrated.

1.3.2. Histamine

Sensitivity of excitatory amino acid receptors is affected by a number of biological modulators and drugs. Accumulating data provide experimental evidence that histamine plays a role not only in the *N*MDA-mediated synaptic transmission and long-term potentiation [16], but also in the *N*MDA receptor-mediated excitotoxicity during cerebral ischaemia and epilepsy [150]. Histamine modulates the glutamate *N*MDA receptor *via* an action at the polyamine binding site [25].

Specific *N*MDA receptor subunits including NR1a/NR2B are responsible for the potentiation of *N*MDA receptor by histamine [180].

The presence of histamine in the CNS has long been revealed and its role in the neurohumoral transmission has been evidenced [146]. The brain histamine system is involved in many functions, such as arousal and sleep, activation of the sympathetic nervous system, anxiety, stress-related release of hormones from the pituitary and of central aminergic neurotransmitters, water retention, and suppression of eating [25].

Histamine release in the CNS is enhanced under different pathologic conditions (dehydration, hypoglycaemia) or by a variety of stressors [25]. Perinatal ischaemia-reperfusion induced differences in the concentrations of this endogenous amine in various body and brain compartments have not been known previously. Histamine acts on different types of histamine receptors (mainly H_1-, H_2- and H_3-receptors) which have a widespread and specific distribution in the CNS [25] (Table 3).

Table 3 **Synaptic and cerebral localization of different histamine receptors**

Receptor subtype	Synaptic localisation (predominantly)	Brain localisation (areas with high densities)
H_1	postsynaptically	hypothalamus, other limbic regions
H_2	postsynaptically	hippocampus, amygdala, basal ganglia
H_3	presynaptically	basal ganglia

A number of electrophysiological mechanisms are involved in mediating the activation of histamine receptors depending on the type of the receptor [25]:

- H_1-receptors coupled positively to phospholipase C and activation of these receptors causes large depolarisations *via* blockade of a leak potassium (K^+) conductance, activation of a non-specific cation channel, or activation of a Na^+-Ca^{2+} exchanger.
- H_2-receptors are coupled positively to adenylate cyclase. Activation of H_2-receptors leads to mainly excitatory effects through blockade of Ca^{2+}-dependent K^+-channels and modulation of the hyperpolarisation-activated cation channel.
- H_3-receptors are coupled negatively to adenylate cyclase; the activation of this receptor subtype could reduce the release of histamine itself and other neurotransmitters, most likely *via* inhibition of presynaptic Ca^{2+}-channels.

Intracerebral histamine is also a known regulator of cerebral blood flow (CBF) [45]. It is released under pathological conditions [6,107,120] and plays an important role in brain oedema formation [45,75]. There are three distinct histamine pools within the brain, such as neuronal elements, perivascular mast cells, and cerebral endothelial cells. Brain capillaries have low activity of the histamine synthesizing (L-histidine decarboxylase) and metabolising (histamine-*N*-methyltransferase) enzymes [80]. Histamine receptors (mainly H_2-type), however, were supposed to be present on both sides of the BBB [75]. Indeed, an *in vitro* study revealed that cultured primary cerebral endothelial cells have the ability to take up histamine from both luminal and abluminal sides, but they released it mainly luminally [70]. This finding suggested that the brain microvessels may use the endothelial polarized transport-system in pathological conditions providing an important protective mechanism against the increase of cerebral histamine concentration.

1.3.3. Hormones

Experimental and clinical data revealed asphyxia-induced various hormonal changes in neonates (see hormones and references in Table 4). These results show that different type of hormones are involved in the pathogenetic process of neonatal brain injury, *e.g.*: stress hormones (ACTH, β-endorphin, cortisol, vasopressin, prolactin, TSH, epinephrine, norepinephrine); regulators of the carbohydrate metabolism (epinephrine, norepinephrine, insulin, C-peptide, growth factors) and of the water balance (aldosterone, ANP and related peptides, vasopressin). The majority of these hormones plays a role in deterioration of neonatal brain function during asphyxia, whereas adrenocorticotropin (ACTH) and related peptides provide protection against ischemia-reperfusion injury of brain stem [69], kidney [29], and heart [58,59, unpublished observation by Vecsernyés *et al.*]. Cardioprotective effect of melanocortins was ten times more efficient administering the exogenous hormone intracerebroventricularly (i.c.v.) [59]. In a rat model of myocardial ischaemia-reperfusion induced arrhythmias the heart protection was mediated *via* MC_3 receptors [59].

Table 4 **Perinatal asphyxia-induced hormonal changes**

hormones	body fluid	species (age)	references
ACTH ↑	blood	sheep (foetal D118)	[53]
aldosterone ↑	blood (cord)	human (0-18-24 h)	[111, 128]
ANP ↑	blood	human (0 h-newborn)	[28, 71, 111]
BNP ↑	blood (umbilical vein)	human (at birth)	[71]
catecholamines ↑	blood (umbilical)	human (at birth)	[92]
CCK ~	blood	human (day 1)	[168]
cortisol ↑	blood	human (0-12-72 h) sheep (foetal D118)	[86, 129, 130] [53]
C-peptide ↑	blood	human (newborn)	[33]
DHEA-S ↑	blood (cord)	human (12-18 h)	[130]
β-endorphin↑	blood	human (1-3 days)	[129]
epinephrine ↑	blood	human (newborn) rat (7 days) sheep (foetal D135)	[55] [73] [48]
epinephrine ↑	CSF	human (mature)	[19]
epinephrine ↓	CSF	human (premature)	[19]
erythropoietin~	blood (cord arterial)	human (at birth)	[138, 139]
growth hormone ↑	blood	human (2-4 h)	[171]
glucagon ~	blood	human (newborn)	[33]
IGF-1 ↓	blood	sheep (foetal D90-93)	[17]
IGF-2 ~	blood	sheep (foetal D90-93)	[17]
IGFBP-1 ↑	blood	sheep (foetal D90-93)	[17]
IGFBP-2 ~	blood	sheep (foetal D90-93)	[17]
insulin ↑	blood	human (newborn) sheep (foetal D90-93) pig (4-12 h)	[31, 33, 145] [17] [4]
insulin ↑	CSF	pig (4-12 h)	[4]
α-MSH ↑	blood	human (newborn) pig (4-12 h)	[100] [88]
α-MSH ↓	CSF	pig (4-12 h)	[88]
norepinephrine ↑	blood	human (newborn) rat (7 days)	[55] [73]
prolactin↑	blood	human (0-2-4 h)	[137, 171]
somatostatin ↑	blood	human (day 1)	[168]
T3, total ~ T3, free ~/↓	blood (cord)	human (at birth)	[160] [20, 160]
T4, total ~/↓ T4, free ~/↓	blood (cord)	human (at birth)	[51, 72, 160] [20,51,160]
TBG ~/↓	blood	human (at birth)	[72, 160]
TSH ~/↑ TSH ↓	blood (cord) blood (cord)	human (at birth) human (at birth)	[137, 160] [20]
vasopressin (AVP)↑ vasopressin (AVP) ↑/~ vasopressin (LVP)↑	blood (cord arterial)	human (0-12 h) sheep (foetal D123-144) pig (4 days)	[14, 111, 138, 139,152] [48, 132] [94]
vasopressin (AVP)↑	CSF	human (12 h)	[14]

Abbreviations used: ↑, increased concentration; ~, no significant change; ↓, decreased concentration; ANP; atrial natriuretic peptide; AVP, arginine-vasopressin; BNP; brain natriuretic peptide; CCK, cholecystokinin; CSF, cerebrospinal fluid; D, day; DHEA-S, dehydro-epiandrosterone sulphate; HIE, hypoxic-ischaemic encephalopathy; IGFBP-1, insulin-like growth factor-1 binding protein; IGBP-2, insulin-like growth factor-1 binding protein IGF-2, insulin-like growth factor-1; IGF-2, insulin-like growth factor-2; IRDS, infant respiratory distress syndrome; LVP, lysine-vasopressin; α-MSH, α-melanocyte stimulating hormone; T3, triiodothyronine; T4, thyroxin; TBG, thyroxin-binding globulin; TSH, thyrotropin.

α-melanocyte-stimulating hormone (α-MSH), a tridecapeptide derivative of proopiomelanocortin (POMC), is processed in the pituitary gland and released from the intermediate lobe after various stimuli. The release of α-MSH is regulated by tuberohypophysial dopaminergic neurons, but β-adrenergic system, peripheral circulating epinephrine, and hypothalamic histaminergic neurons might also mediate this process during stress [39,83]. Neonatal asphyxia resulted in elevated histamine release [89], which might also influence endogenous α-MSH levels. Perinatal stress increases α-MSH secretion of human pituitary intermediate lobe, but this activity declines shortly after birth [100].

Similarly to other ACTH-related melanocortins, α-MSH can cross the BBB [11], thereby the blood-borne hormone may affect brain functions [8]. On the other hand, receptors for α-MSH, with an apparent molecular weight of 45 kDa, are expressed by cerebral microvascular endothelial cells [34], and melanocortins may influence the BBB permeability for other solutes [11]. Intraperitoneal (i.p.) administration of α-MSH induces hypothermia, increases learning capacity, and elevates the pain threshold level in newborn rats [182]. The potential pharmacological application of exogenous α-MSH during resuscitation [58] and in the prevention of postischemic organ injuries [29,69] raised the question how endogenous α-MSH levels in body fluids would change during pathological conditions.

1.4. Porcine models of neonatal asphyxia and hypoxic-ischaemic brain damage

Perinatal asphyxia models are necessary to obtain knowledge of the pathophysiology of hypoxia-ischaemia and to test potential neuroprotective strategies [121]. Animal experiments are widely used for investigating asphyxia induced brain damages. Different species (mouse, rat, gerbil, guinea-pig, sheep, *etc.*) and various experimental models and settings (trauma, cold injury, hypobaric or hypoxic conditions, cardiac arrest, hypoperfusion, different types of shock, *etc.*) are utilised for getting deeper inside to the pathomechanism of organ injuries provoked by hypoxia-ischaemia. Newborn pig is considered to be one of the most appropriate animals for studying the physiologic and pathologic changes during the neonatal period, as the size and structure of its brain has similarities to the human CNS [164]. Several models on piglets (hypothermia, carotid artery occlusion, decreased fraction of inspired oxygen, interrupting ventilation, *etc.*) were developed to observe the effect of asphyxia and global or focal ischaemia-reperfusion on neonatal brain [12]. These models proved that asphyxiated newborn pig resembles many changes

in cerebral anatomy and function similar to human alterations observed in clinical praxis. These made this species generally accepted for studying neonatal hypoxic-ischaemic brain damages.
In the neonatal asphyxia and reperfusion model [163] used in our study, a deterioration of cerebral circulation [3], brain oedema formation [2,162,163,165], early neurological symptoms and cerebral histopathological damages [164] were previously observed. As our basic purposes were to further investigate the pathology and mediators of asphyxia-induced neonatal brain oedema, we used mainly this piglet model in our experiments.

2. AIMS OF THE STUDY

I. To check the changes of histamine concentrations simultaneously in different compartments (plasma from the internal jugular vein; cerebrospinal fluid, and isolated cerebral microvessels) during asphyxia-reperfusion evoked by experimental PTX in newborn pigs.

II. To measure the effect of asphyxia and reperfusion on the plasma and cerebrospinal fluid (CSF) concentration of α-MSH in newborn pigs.

III. To determine the rate of cytotoxic and vasogenic type brain oedema formation and to reveal the possible regional differences during neonatal asphyxia and postasphyxial reperfusion in piglets.

IV. To investigate the effect of intracarotid histamine administration on the permeability of the BBB in healthy newborn pigs.

V. To reveal the effects of H_1 or H_2 histamine receptor antagonists on postasphyxial brain oedema formation in piglets underwent bilateral PTX.

VI. To evaluate the influence of treatment with two *N*MDA-type glutamate receptor antagonists (magnesium sulphate and D-2-amino-5-phospho-valeric acid, APV) on asphyxia and reperfusion induced brain oedema in newborn pigs.

3. MATERIALS AND METHODS

3.1. Animal models

3.1.1. Experimental settings in different studies

Newborn piglets of either sex (birth weight: 1000-1610 g) aged between 3 and 8 h of life were included in all experiments. To reach the special aims of the thesis the following experimental settings were used:

I., II, III: Bilateral PTX was induced according to the model originally described by Temesvári *et al.* [163] under general anaesthesia with ketamine hydrochloride (Ketanest, Parke-Davis, 10 mg/kg b.w., i.m.) maintained with α-chloralose in newborn pigs fixed on their backs. Under local anaesthesia with lidocaine hydrochloride (Egis; 1 ml, 1% solution, s.c.) the animals were tracheotomized, intubated (2.5-3.0 tubes; Portex Ltd, Hythe, Kent, U.K.), paralysed with pipecuronium bromatum (Arduan, Richter), and ventilated with a constant-volume, pressure-limited infant respirator (MTA-KUTESZ, Budapest, Hungary). Initial ventilator parameters were: tidal volume, 10-16 ml; frequency, 40/min; peek inspiratory pressure, 1.18-1.48 kPa; end-expiratory pressure, 0 kPa; FiO_2:0.21. One of the umbilical arteries was cannulated for continuous blood pressure monitoring and intermittent blood sampling. Drugs and infusions were administered mainly through a drain into the umbilical vein. After reaching a stable cardiorespiratoric state ("basal stage" = B), as it was described in details previously [3,163] drains (Intermedicut chest tubes, 14-gauge) were inserted at the forth intercostal space in the mammary line 0.5 cm deep into the pleural cavity. Then the animals were divided into two groups: in asphyxia group PTX was induced through indwelling drains by increasing the intrapleural pressure, while sham-operated piglets served as control group. At the critical stage (C) of the disease severe bradycardia, arterial hypotension, hypoxaemia and combined acidosis developed. Then, extra-alveolar air was drained/removed from the pleural cavity and after the resuscitation (R) a 3-h recovery (R180) period was allowed to the piglets.

V: In the study revealing the effects of antihistamines on PTX-induced brain oedema formation the animals were not intubated and ventilated during the otherwise similar

experimental procedure. After 4 h survival the animals were killed with 2 mmol/kg KCl, and pieces of the parietal cortex (n=6 per animal) were taken for the measurements of water, electrolytes and permeability tracer (Evan's blue-labelled albumin).

VI: While investigating the role of *N*MDA-antagonists on PTX-induced vasogenic brain oedema formation pentobarbital (Nembutal, 30 mg/kg) was used instead of ketamine for general anaesthesia in the same experimental setting.

IV: In histamine-induced brain oedema study, after pentobarbital (30 mg/kg) anaesthesia, one of the umbilical arteries was catheterised, cardiovascular, blood gas and acid-base parameters were monitored [4]. The left internal carotid artery of the animals was catheterised through the external branch, and exogenous histamine diluted in 0.5 ml isotonic saline was given in slow intra-arterial injection. The vasoactive mediator was administered in the following doses: 0 mol, 10^{-6} mol, $5x10^{-6}$ mol, 10^{-5} mol, $5x10^{-5}$ mol, 10^{-4} mol (n=12 in each group). Animals receiving isotonic saline served as control. Then the catheter was removed and the external carotid artery was ligated. Newborn pigs were sacrificed 1 h after the challenge. BBB permeability changes were determined in the frontal, parietal, and occipital cortex, hippocampus, and periventricular white matter of 6 animals in each group. Cortical microvessels were also isolated from the brain tissue of the latter animals [167].

3.1.2. Clinical parameters measured

The body temperature was monitored and regulated between 38 and 39°C by using a heating pad. Heart rate (HR), mean arterial blood pressure (MABP), and central venous pressure (CVP) in superior vena cava was continuously monitored by Statham P230 transducer. Lung-thoracic compliance (LTC) was calculated by dividing tidal volume with peak inspiratory pressure and body weight [4]. Arterial blood gases and acid-base balance were analysed with an automatic analyser (Radiometer, Copenhagen), using the standard Astrup method.

3.2. Measurement of histamine

At the basal stage (B), at the critical state (C) and 15 and 180 minutes thereafter (R15 and R180) samples were taken from the right internal jugular vein (plasma), cisterna magna (CSF), and cerebral microvessels were isolated from cortex. The capillary-rich fraction was prepared by gradient ultracentrifugation according to the method of Joó and Karnushina [74]. The purity of fraction was tested under the light microscope after staining with Toluidine Blue [80]. The concentration of histamine in different brain compartments was measured using the slightly modified radioenzymatic method described by Beaven and Horakova [15]. Briefly, 25 µl of sample was added to a reaction mixture of 25 µl histamine-*N*-methyltransferase and 25 µl of 0.1 M phosphate buffer (pH 7.9) which contains 1 µCi of S-adenosyl[^{3}H-methyl]methionine (Amersham, 60-80 Ci x mmol^{-1}). Histamine-*N*-methyltransferase, the enzyme that converts histamine into a stable metabolite, methylhistamine, in the presence of [^{3}H]*S*-adenosyl methionine was prepared from rat kidney by a modified method of Verburg *et al.* [176]. The samples were incubated at 37°C for 90 min, and the reaction was stopped by adding 10 µl of 2.4 N perchloric acid. The labelled histamine was extracted with 10 µl of 10 N NaOH and 800 µl chloroform. The radioactivity was determined in a Beckman LS 100C liquid scintillation counter. Within and between the assays, variation coefficients of the method were 10.1 and 13.8% (at 5-25 nmol x l^{-1} range), respectively.

3.3. Measurement of α-melanocyte stimulating hormone

The α-MSH radioimmunoassay (RIA) procedure was performed as previously described in detail [141,172,173]. Briefly, the antiserum produced in rabbit gave a cross-reaction with des-acetyl α-MSH (68%), and ACTH 1-24 (0.2%). Less than 0.01% cross-reaction was detected with various ACTH fragments (1-39, 1-32, 1-16, 1-10, 1-8, 4-10, 11-24, 12-24), and the POMC-related peptides β-endorphin, β-lipotropin, and N-terminal fragment of POMC. The intra-assay coefficients of variation at 62.5, 125, 250, and 500 pg /tube were 8.8, 5.0, 9.7, and 9.2%, while inter-assay coefficients were 11.7, 13.4, 14.9, and 17.9%, respectively.

Before assay procedure, α-MSH was extracted from plasma with ethanol, as it was described previously [172,173]. The concentration of α-MSH in CSF was determined as follows: CSF samples were lyophilised, the residues were re-dissolved in RIA buffer, and subjected to RIA

procedure according to the method described by Vecsernyés *et al.* [172,173]. Identity of α-MSH originated from biological samples was verified by HPLC technique. All samples were measured in duplicate, and in the same assay from a particular experiment.

3.4. Determination of vasogenic and cytotoxic brain oedema formation

Three groups were formed (n=16 in each): (i) sham-operated control pigs not receiving intrapleural air; (ii) asphyxiated pigs in cardiovascular and metabolic shock; and (iii) pigs after a 3-h postasphyxial reperfusion period.

Development of cytotoxic brain oedema was measured in each group (n=6), as it was previously described [163]. Anaesthetised pigs were given intravenous injection of KCl (10%, 2.0 ml), then their brain was removed and samples from 10 regions (frontal, parietal and occipital cortex, hippocampus, striatum, thalamus, internal capsule, cerebellum, pons, medulla) were quickly removed. Wet weight of the samples was measured immediately, and percentage water content was determined after drying the tissue at 110°C for 40 h. To determine electrolyte contents, dry brain tissues were ashed at 550°C for 20 h, then dissolved in 5 ml 3 M nitric acid (Suprapur, Merck), and diluted 10-fold with deionised water. Na^+ was measured at the wavelength of 330.3 nm, K^+ at 404.4 nm and Ca^{2+} at 422.7 nm in an air-acetylene flame by a Perkins-Elmer 306 atomic absorption spectrophotometer, slit width was 0.7 mm in all measurements. Values determined were expressed as mmol x kg^{-1} dry weight.

Vasogenic brain oedema formation was determined by the extravasation of sodium fluorescein (SF, mw: 376) and Evan's blue-labelled albumin (EBA, mw: 67,000), as it was described [1]. Pigs (n=8 in each group) were given a solution of both tracers in isotonic saline (2%, 5 ml x kg^{-1}) in an intravenous injection 30 min before the end of the experiments. Intravascular dyes were removed by a perfusion with 200 ml x kg^{-1} isotonic saline. In the quantitative study, plasma and tissue samples from 10 regions of perfused brain were homogenized in 3.0 ml of cold 7.5 % trichloroacetic acid, and centrifuged with 10,000 *g* for 10 min. The concentration of tracers was measured in supernatants by a Hitachi F2000 fluorimeter (Tokyo, Japan), the absorbency of Evan's blue at 620 nm, whereas the emission of SF at 525 nm after excitation at 440 nm. Extravasation was expressed as brain tissue concentration divided by plasma concentration, such as μg dye x mg^{-1} brain tissue x (μg dye x μl^{-1} plasma)$^{-1}$ for both dyes.

3.5. Therapeutic studies to prevent neonatal brain oedema formation

3.5.1. Histamine receptor antagonists

For testing the effect of histamine receptor blockers, H_1-receptor antagonist mepyramine maleate (Specia), as well as H_2-receptor antagonists cimetidine, metiamide, and ranitidine (GlaxoSmithKline) were used. These substances solved in Krebs-Ringer buffer (pH 7.4) were injected i.p. 4, 2 and 0 hour before the onset of PTX, in a dose of 5 mg/kg b.w. (n=5 in each group). In 15 animals the receptor blockers were injected intrathecally (i.th.). In this group 0.3 ml CSF was drawn off from the animals through lumbar puncture and drugs dissolved in artificial CSF were injected back in 5 mg/kg concentration in the same volume. Since metiamide could not be dissolved in the above volume and concentration, only the mepyramine, cimetidine, and ranitidine were administered in this way. Artificial CSF alone was used as control for the i.th. treatment.

3.5.2. Magnesium sulphate treatment

Effect of postasphyxial magnesium sulphate ($MgSO_4$) infusion (in 5 ml/kg/h isotonic saline infusion for 30 min given immediately after the critical phase of experimental bilateral PTX) was tested on the BBB permeability for SF and EBA in 10 brain regions (frontal, parietal, and occipital cortex; hippocampus; striatum; thalamus; internal capsule; cerebellum; pons; and medulla) of newborn pigs. Treatment groups (n=8 in each) included: sham-operated control pigs; and asphyxiated groups treated either with vehicle or with 100, 200, or 400 mg/kg of $MgSO_4$.

3.5.3. *N*-methyl-D-aspartate receptor antagonist therapy

The possible protective effect of postasphyxial D-2-amino-5-phosphovaleric acid (APV) i.v. bolus administration on the BBB permeability for SF and EBA was investigated in the frontal and parietal cortex, hippocampus, striatum, cerebellum, and pons/medulla of newborn pigs (n=6 in each group). The following experimental groups were involved: sham-operated control pigs, as well as asphyxiated animals treated either with vehicle or 0.1 and 1.0 mg/kg APV i.v. immediately after resuscitating the animals from the cardiovascular collapse induced by bilateral PTX.

3.6. Statistical analysis

Data presented are means ± S.D. or S.E.M. The values were compared between groups using the analysis of variance (ANOVA), Kruskal-Wallis or Friedman's repeated measure ANOVA on ranks, by the SPSS 9.0 statistical software (Chicago, IL, U.S.A.). *Post hoc* tests used include Student-Newman-Keuls test, Scheffe's test, and Dunnett T3-test. The differences between treatment groups were considered significant at $P<0.05$, although in case of α-MSH measurements trends ($P<0.10$) were also indicated.

4. RESULTS

4.1. Clinical parameters of neonatal asphyxia and reperfusion in newborn pigs

The clinical symptoms of PTX-induced severe hypoxaemia were bradycardia, arterial hypotension and combined acidosis in the critical phase. During the recovery stage, moderate tachycardia, arterial hypotension and mainly metabolic acidosis were observed (Table 5). The measured changes in cardiovascular, blood-gas and acid base parameters in asphyxiated animals are similar to changes observed in human newborns during and after asphyxia-reperfusion [157].

4.2. Histamine release during neonatal asphyxia and reperfusion in newborn pigs (Fig. 1)

Plasma histamine levels in the venous blood (Fig. 1A) raised significantly ($P<0.05$) in animals during hypoxic cardiovascular and metabolic failure compared to the values measured in the control group (13.5±1.9 *vs.* 2.2±0.5 nM x l^{-1}, respectively), preceding any detectable change of histamine concentration in CSF or in cerebral microvessels. Blood histamine levels in the jugular vein samples remained high during the early (R15: 16.2 ± 4.3 nM x l^{-1}) and late (R180: 15.3 ± 2.9 nM x l^{-1}) recirculation periods, too (Fig. 1A).

Table 5 **Clinical data of newborn piglets**

Time (min)	Basal	Critical	R + 15	R + 180
Heart rate (min^{-1})				
Control	164 ± 3	166 ± 3	167 ± 3	167 ± 3
PTX	163 ± 3	52 ± 2[a,b]	190 ± 4[a,b]	189 ± 3[a,b]
Mean arterial blood pressure (kPa)				
Control	8.08 ± 0.29	8.77 ± 0.41	8.45 ± 0.25	8.49 ± 0.29
PTX	7.53 ± 0.27	2.24 ± 0.07[a,b]	9.57 ± 1.03	7.03 ± 0.81[a]
Mean right atrial pressure (kPa)				
Control	1.00 ± 0.08	0.96 ± 0.05	0.96 ± 0.07	1.00 ± 0.05
PTX	0.89 ± 0.08	1.91 ± 0.05[a,b]	1.14 ± 0.07[a]	0.89 ± 0.07
Lung-thorax compliance (l kPa^{-1} kg^{-1})				
Control	15.5 ± 1.0	14.6 ± 0.7	13.9 ± 0.6	13.6 ± 0.5
PTX	16.0 ± 0.8	–	9.9 ± 0.6[a,b]	9.2 ± 0.6[a,b]
Arterial pH				
Control	7.41 ± 0.01	7.39 ± 0.02	7.36 ± 0.02	7.34 ± 0.03
PTX	7.41 ± 0.02	6.89 ± 0.04[a,b]	7.08 ± 0.05[a,b]	7.22 ± 0.03[a,b]
Arterial HCO_3^{-1} (mM l^{-1})				
Control	24.8 ± 0.4	23.8 ± 0.5	22.9 ± 0.8	23.4 ± 1.2
PTX	22.8 ± 0.7	10.6 ± 1.2[a,b]	12.4 ± 0.8[a,b]	16.0 ± 2.0[a,b]
Arterial pCO_2 (kPa)				
Control	5.11 ± 0.15	5.39 ± 0.28	5.52 ± 0.16	5.80 ± 0.21
PTX	4.48 ± 0.25	11.57 ± 1.05[a,b]	7.55 ± 0.08[a,b]	5.03 ± 0.37
Arterial pO_2 (kPa)				
Control	8.16 ± 0.26	7.19 ± 0.35	7.60 ± 0.32	7.23 ± 0.23
PTX	7.83 ± 0.31	3.43 ± 0.34[a,b]	5.34 ± 0.52[a,b]	6.64 ± 0.41[a]

All values are means ± SEM., $n = 12$ in both groups. Values measured in PTX group differed significantly $^{a}P < 0.05$ from data in control group and $^{b}P < 0.05$ from basal values of pneumothorax group.

By contrast, histamine concentration was increased considerably in CSF samples (Fig. 1B) obtained from the early reoxygenation period only, and not in CSF samples 180 min after resuscitation (12.8 ± 6.5 nM x l^{-1} *vs.* 5.2 ± 1.9 nM x l^{-1}, respectively).

The presence of histamine was detected in the cerebral microvessels prepared from experimental and control animal groups, alike (Fig. 1C). However, significant elevation of histamine level was

seen in microvessels of piglets with PTX only in the late reoxygenation period (R180: 18.6 ± 5.3 *vs.* Basal: 7.2 ± 1.6 pM x (mg protein)$^{-1}$) There was no significant change of histamine levels in the cerebral microvessels prepared 3 hours after anaesthesia from the control group (Fig. 1C).

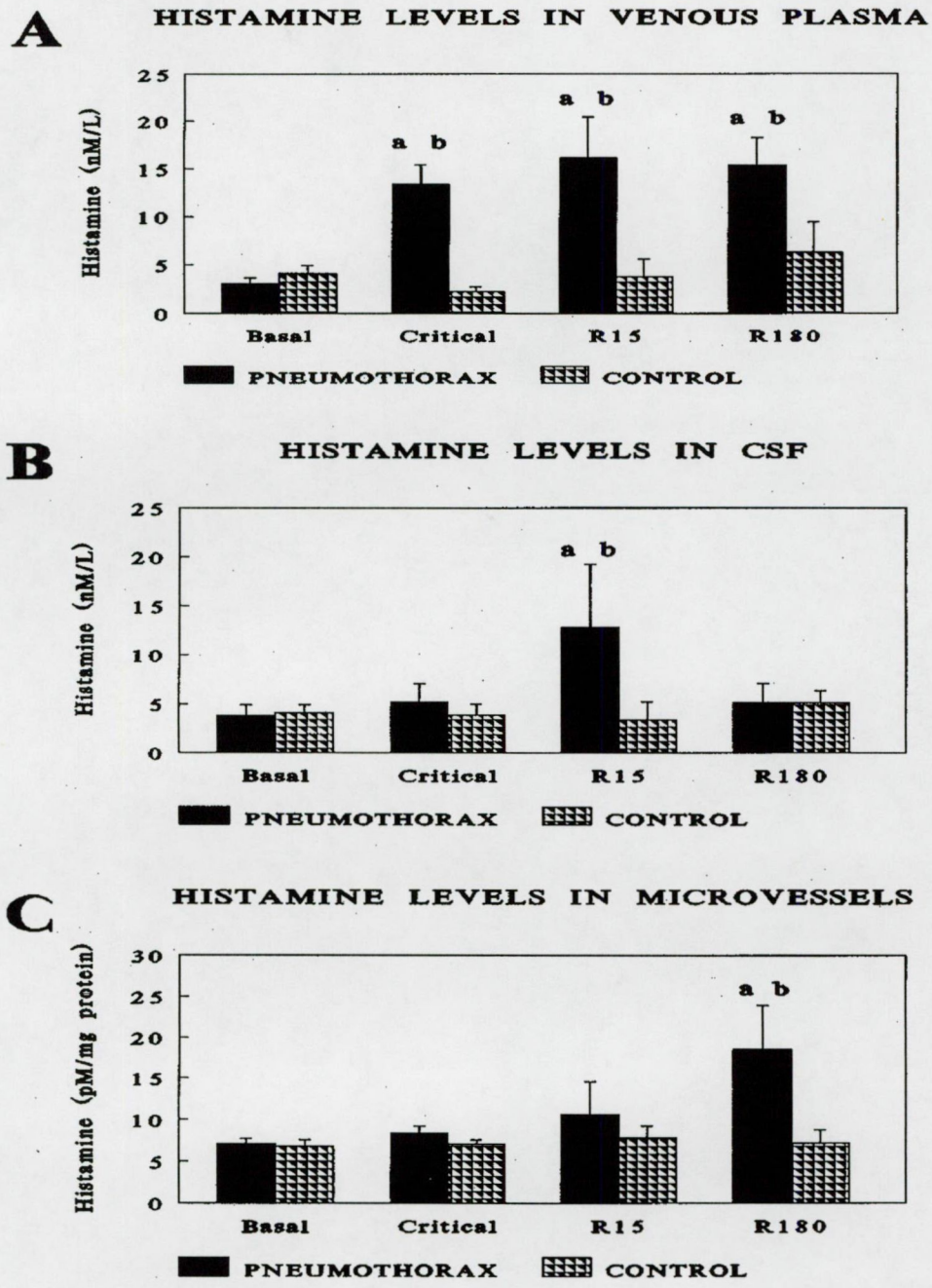
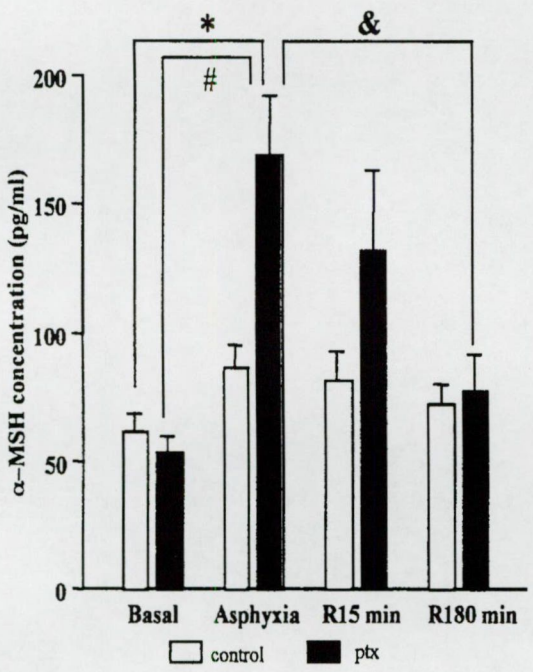


Fig. 1 Changes in histamine concentrations in venous plasma (Fig. 1A), in cerebrospinal fluid (Fig. 1B) and in isolated cerebral microvessels (Fig. 1C) before and during asphyxia and recovery in newborn pigs with experimental PTX. All values are means ± S.E.M., n=5 in each group. Values measured in PTX group differed significantly $^{a}P<0.05$ from data in control group and $^{b}P<0.05$ from basal values of PTX group.

4.3. α-MSH release during neonatal asphyxia and reperfusion of newborn pigs (Fig. 2)

Asphyxia resulted in significant increase in plasma concentration (Fig. 2A.). Plasma α-MSH values measured after asphyxia in PTX group significantly differed (ANOVA followed by Dunnett T3 *post hoc* test for multiple comparisons) from basal values in control (P=0.022) and PTX (P=0.011) groups, as well as data measured in the end of experiment in control group (P=0.045) (Fig. 2A). A trend for difference (P=0.100) was also seen between asphyxia and R180 values in PTX group.

Asphyxia-reperfusion decreased α-MSH concentration in CSF (Fig. 2B). This value was significantly lower in PTX group after 15 min of reperfusion, than basal levels in control (P=0.036) and PTX (P=0.027) groups (Fig. 2B). Trends for difference were seen between R180 value in PTX group and basal values in control (P=0.053) and PTX (P=0.058) groups (Fig. 2B).



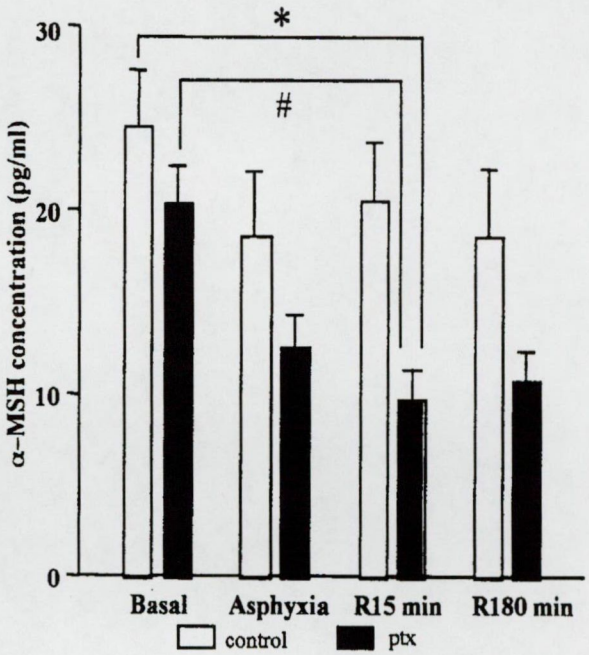


Fig. 2A Asphyxia-induced changes in the plasma α-MSH concentrations in newborn pigs suffering from pneumothorax (ptx; filled bars). Basal hormone levels, and concentrations measured in asphyxia and 15 min (R15) and 180 min (R180) of reperfusion were compared to data obtained on appropriate control pigs (open bars). Statistical analysis was done by ANOVA followed by Dunnett T3 *post hoc* test. *P<0.05 significantly differs from the basal values measured in control group; while in ptx group: #P<0.05 significantly differs from the basal value, and &P<0.05 from concentration measured in asphyxia

Fig. 2B Asphyxia-induced changes in the CSF α-MSH concentrations in newborn pigs suffering from pneumothorax (ptx; filled bars). Basal hormone levels, and concentrations measured in asphyxia and 15 min (R15) and 180 min (R180) of reperfusion were compared to data obtained on appropriate control animals (open bars). Statistical analysis was done by ANOVA followed by Dunnett T3 post hoc test. *P<0.05, and #P<0.05 significantly differ from the basal values measured in control and ptx groups, respectively

4.4. Cytotoxic and vasogenic brain oedema in newborn pigs

4.4.1. Neonatal asphyxia-induced cytotoxic brain oedema formation (Fig. 3)

Though significant ($P<0.05$) regional differences were seen in the distribution of water in the brain of control animals (water content was higher in all cortical regions and hippocampus compared to that in other regions), we could not find similar changes in the distribution of electrolytes (Fig. 3). Brain water content was increased in all regions during PTX. Significant ($P<0.05$) fluid accumulation was detected in parietal cortex and hippocampus in asphyxia group, and in the 3 cortical regions, in hippocampus and striatum during postasphyxial recovery (Fig. 3A). Asphyxia and reperfusion also resulted in increased Na^+- (Fig. 3B) and decreased K^+-concentrations (Fig. 3C) in several brain regions investigated. Brain tissue Ca^{2+}-content (Fig. 3D) was significantly ($P<0.05$) elevated in frontal cortex, hippocampus, striatum and thalamus in both asphyxiated and reperfusion groups, and also in parietal cortex and internal capsule in the reperfusion group.

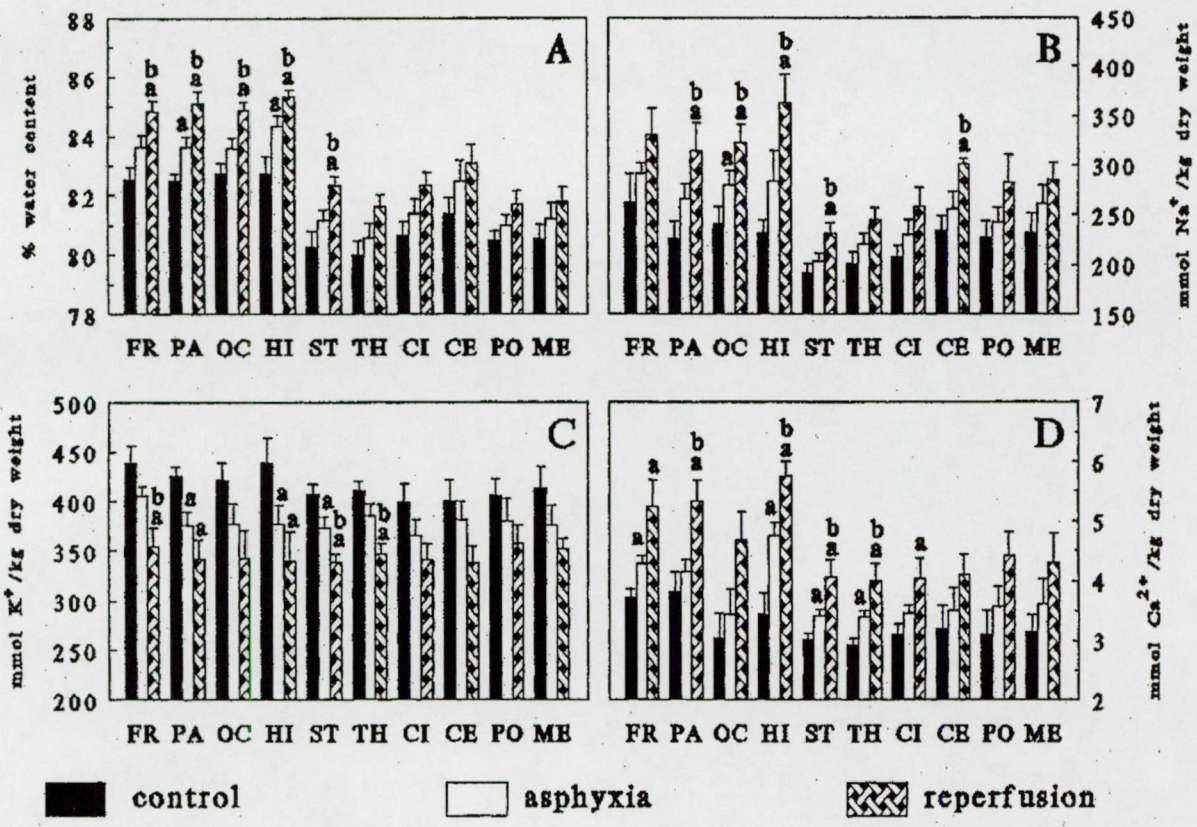


Fig. 3 Development of cytotoxic brain oedema in newborn pigs during asphyxia and reperfusion. Changes in percentage water content (Fig. 3A) and the concentrations (mmol x kg^{-1} dry weight) of sodium (Fig. 3B), potassium (Fig. 3C) and calcium (Fig. 3D) are seen in 10 brain regions (FR, frontal cortex; PA, parietal cortex; OC, occipital cortex; HI, hippocampus; ST, striatum; TH, thalamus; CI, internal capsule; CE, cerebellum; PO, pons; ME, medulla). All values are means ± S.E.M., n=6 in each group. Values differed significantly ($P<0.05$) from data measured in a: control group and b: in asphyxia group.

4.4.2. Vasogenic brain oedema induced by asphyxia-reperfusion (Fig. 4)

There were regional differences in the permeability of BBB for SF but not for EBA in control animals: the extravasation of SF was significantly ($P<0.05$) higher both in pons and medulla than that in other brain regions (Fig. 4). Asphyxia resulted in significantly ($P<0.05$) increased BBB transport of SF in all regions, except for the internal capsule and medulla (Fig. 4A). In reperfusion group, the SF permeability further increased and became significant in each region. The BBB permeability for EBA in asphyxia group did not differ significantly from data measured in control group, but it became significantly ($P<0.05$) increased in all regions examined in reperfusion group (Fig. 4B). After a 3-h postasphyxial reperfusion period, the highest relative elevations (8- to 9-fold) in albumin flux were seen in thalamus, striatum, and parietal cortex (Fig. 4B).

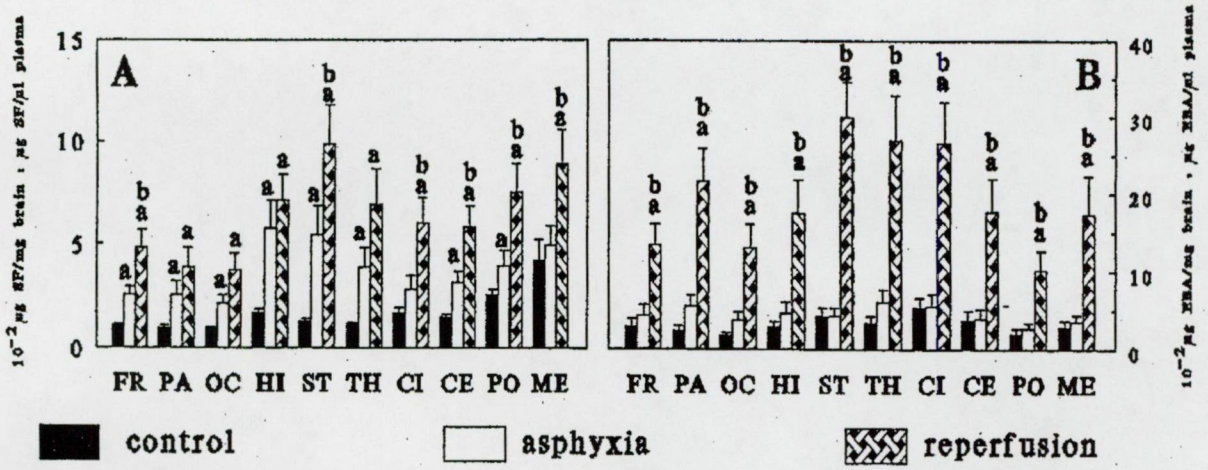


Fig. 4 Development of vasogenic brain oedema in newborn pigs during asphyxia and recovery. Changes in blood-brain barrier permeability (Fig. 4A) for sodium fluorescein (SF), and (Fig. 4B) for Evans blue-albumin (EBA) are seen in 10 brain regions (FR, frontal cortex; PA, parietal cortex; OC, occipital cortex; HI, hippocampus; ST, striatum; TH, thalamus; CI, internal capsule; CE, cerebellum; PO, pons; ME, medulla). All values are means ± S.E.M., n=8 in each group. Values differed significantly ($P<0.05$) from data measured in a: control group and b: in asphyxia group.

4.4.3. Vasogenic brain oedema induced by intracarotid histamine administration (Fig. 5)

Histamine administration did not change significantly the blood gas and acid parameters of the animals during 1 hour of experiment (data not shown). Although a temporary cardiac arrhythmia was detected within 5 min in 2 of 12 newborn pigs receiving 10^{-4} mol histamine, the rest of animals were unaffected, and no significant change in the MABP and HR compared to the values measured in control pigs was recorded (data not shown).

Intracarotid histamine administration tended to increase the BBB permeability in each brain region (Fig. 5), but only higher doses resulted in significant ($P<0.05$) changes: 10^{-4} mol for SF (Fig. 5A) , and $5x10^{-5}$ and 10^{-4} mol for EBA transport (Fig. 5B). The highest dose of histamine caused a 3- to 6-fold increase in the permeability of tracers in each brain region compared to that in control group.

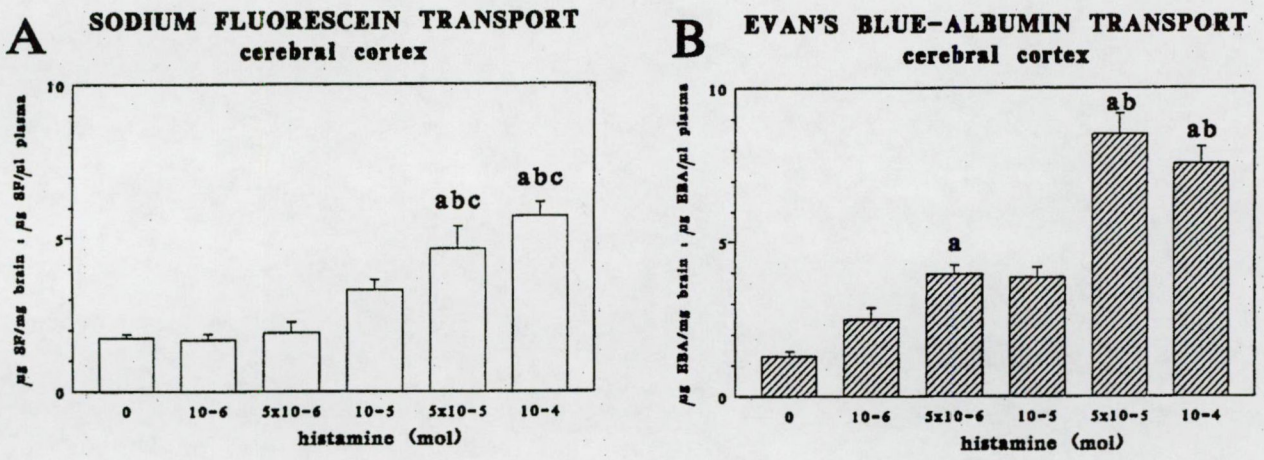


Fig. 5 Development of vasogenic brain oedema of newborn pigs 1 h after intracarotid histamine challenge. BBB permeability markers were SF (Fig. 5A) and EBA (Fig. 5B). Symbols indicate significant differences ($P<0.05$) compared to the following treatments: a: 0 mol; b: 10^{-6} mol; c: $5x10^{-6}$ mol histamine.

4.5. Prevention of neonatal brain oedema formation in newborn pigs

4.5.1. The effect of antihistamines on asphyxia-induced brain oedema

In asphyxiated pigs the effects of histamine receptor blockers on the brain water, sodium and potassium is demonstrated in Table 6. Both i.p. and i.th. administrations of H_1 receptor antagonist mepyramine could significantly reduce the water content of the brain tissue in a dose of 5 mg/kg, however only i.th. injection could decrease PTX-induced increase in brain sodium concentration. Of the H_2-receptor antagonists used, only ranitidine was an effective inhibitor of the developing brain oedema. Ranitidine was able to reduce the water accumulation in all areas investigated by both routes of administration, and it could also reduce the accumulation of sodium after i.th. injection. No remarkable change was seen in brain potassium levels in the experimental groups (Table 6).

Table 6 **The effect of histamine receptor antagonists on the brain water, sodium and potassium content in asphyxiated piglets 4h after the critical phase.**

	Water (%)	Na^+ (mmol/kg dry weight)	K^+ (mmol/kg dry weight)
Control ($n = 4$)	83.84 ± 0.88	271.4 ± 22.4	467.2 ± 27.1
Pneumothorax only ($n = 4$)	86.64 ± 0.77	329.6 ± 24.0	482.8 ± 32.0
Mepyramine			
i.p. ($n = 4$)	84.40 ± 1.04*	323.0 ± 16.2	472.1 ± 35.6
i.th. ($n = 4$)	85.69 ± 0.75*	294.9 ± 35.3*	464.6 ± 44.6
Ranitidine			
i.p. ($n = 4$)	85.31 ± 0.51*	316.1 ± 12.2	496.7 ± 39.3
i.th. ($n = 4$)	84.95 ± 0.49*	296.0 ± 29.6*	474.0 ± 40.0
Metiamide			
i.p. ($n = 4$)	87.48 ± 1.92	307.5 ± 38.2	508.9 ± 41.3
Cimetidine			
i.p. ($n = 4$)	86.32 ± 1.09	312.0 ± 40.1	456.8 ± 39.9
i.th. ($n = 4$)	86.20 ± 0.75	329.5 ± 16.7	486.5 ± 37.5

Values are mean ± S.D.
*$P < 0.01$ compared to the group "pneumothorax only".

Similarly to the results obtained by determining water and electrolytes, mepyramine and ranitidine could also prevent the leakage of serum albumin from the blood into the brain (Table 7).

		Mean ± S.D.
Control	($n = 3$)	0.10 ± 0.10
Pneumothorax only	($n = 3$)	0.48 ± 0.11
Mepyramine		
i.p.	($n = 3$)	0.14 ± 0.08*
i.th.	($n = 3$)	0.11 ± 0.07*
Ranitidine		
i.p.	($n = 3$)	0.15 ± 0.03*
i.th.	($n = 3$)	0.10 ± 0.09*

*$P < 0.01$ compared to the group "pneumothorax only".
(Evans Blue concentration, μg/g wet weight.)

Table 7 **The effect of histamine receptor antagonists mepyramine (H_1) and ranitidine (H_2) on the extravasation of Evans blue-albumin into the porcine brain 4 h after the critical phase of pneumothorax**

4.5.2. The effect of magnesium sulphate on asphyxia-induced brain oedema (Fig. 6)

Postasphyxial administration of $MgSO_4$ infusion in the doses applied did not result in significant changes in clinical data (MABP, HR, CVP, LTC) compared to those measured in PTX group treated with vehicle (data not shown). $MgSO_4$ administration could dose-dependently decrease the BBB permeability for EBA, but not for SF (Fig. 6). Both 100 and 200 mg/kg $MgSO_4$ could protect against the albumin extravasation in each brain region tested (Fig. 6A-J), whereas 400 mg/kg was effective only in parietal cortex (Fig. 6B), striatum (Fig. 6E), and medulla (Fig. 6J). However, $MgSO_4$ infusion failed to prevent the asphyxia-induced increase in SF permeability (Fig. 6A-J), and a dose of 400 mg/kg did even elevate the extravasation of this tracer in cerebellum (Fig. 6H).

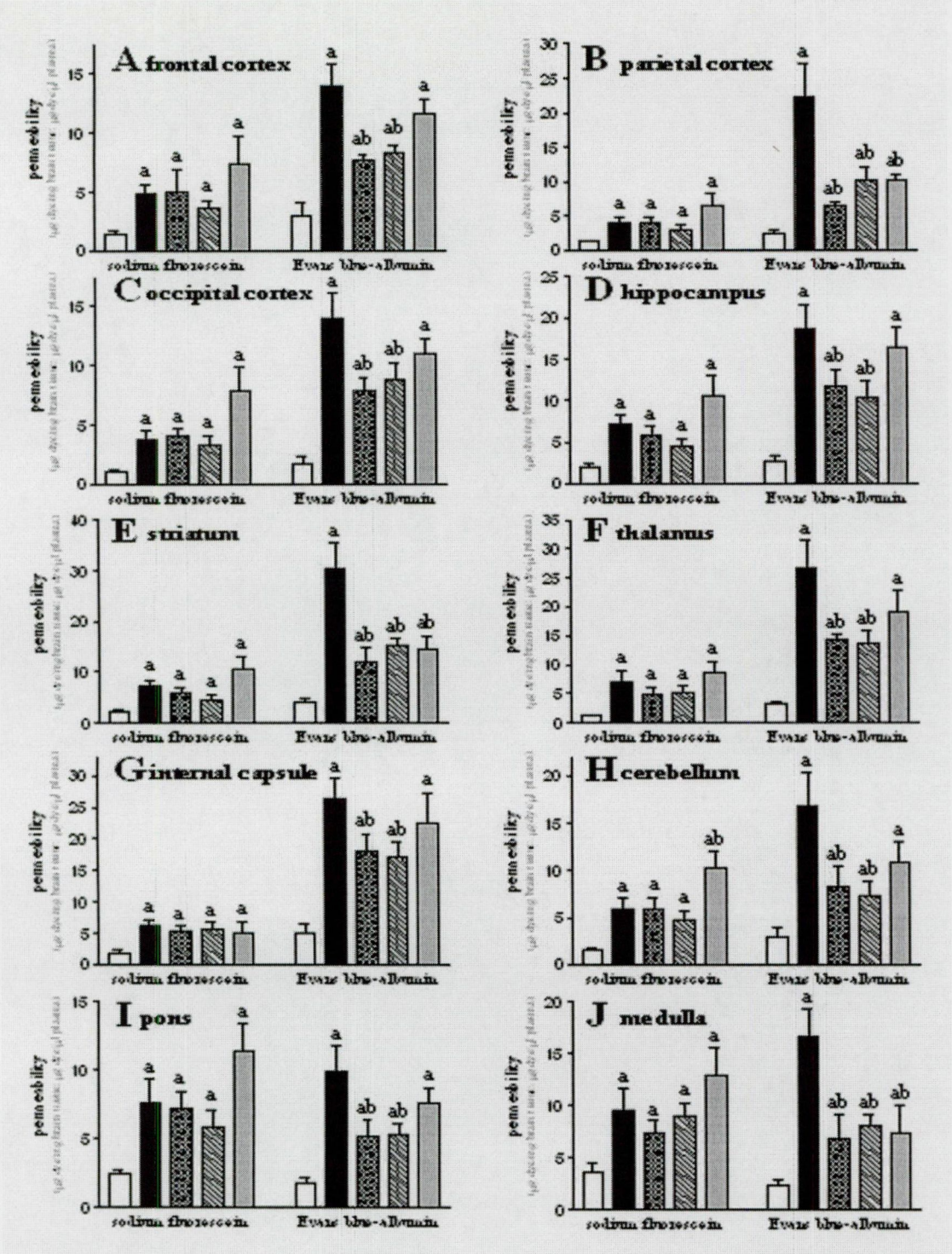


Fig. 6 Effect of postasphyxial magnesium sulphate ($MgSO_4$) infusion on the blood-brain barrier permeability for sodium fluorescein (SF) and Evans blue-labelled albumin (EBA) in newborn pigs. Treatment groups included: (i) sham-operated control pigs (open bars); (ii) asphyxiated animals treated with vehicle (filled bars); (iii) asphyxiated animals treated with 100 mg/kg $MgSO_4$ (cross-hatched bars); (iv) asphyxiated animals treated with 200 mg/kg $MgSO_4$ (hatched bars); and (v) asphyxiated animals treated with 400 mg/kg $MgSO_4$ (grey bars). Brain regions tested were as follows: A, frontal cortex; B, parietal cortex; C, occipital cortex; D, hippocampus; E, striatum; F, thalamus; G, internal capsule; H, cerebellum; I, pons; J, medulla. All values are means ± S.E.M., n=8 in each group. Values differed significantly ($P<0.05$) from data measured in a: control group and b: in asphyxia group.

4.5.3. The effect of D-2-amino-5-phosphovaleric acid on asphyxia-induced brain oedema (Fig. 7)

Treatment with APV, a competitive antagonist of *N*MDA receptor, could dose-dependently decrease the vasogenic brain oedema formation in newborn pigs (Fig. 7). A dose of 1.0 mg/kg did not only prevent the asphyxia-induced albumin extravasation in each brain region tested (Fig. 7A-F), but it could also significantly reduce the BBB permeability for SF in hippocampus (Fig. 7C), striatum (Fig. 7D), and cerebellum (Fig. 7F). The smaller dose (0.1 mg/kg) could decrease the EBA permeability only in hippocampus (Fig. 7C) and striatum (Fig. 7D).

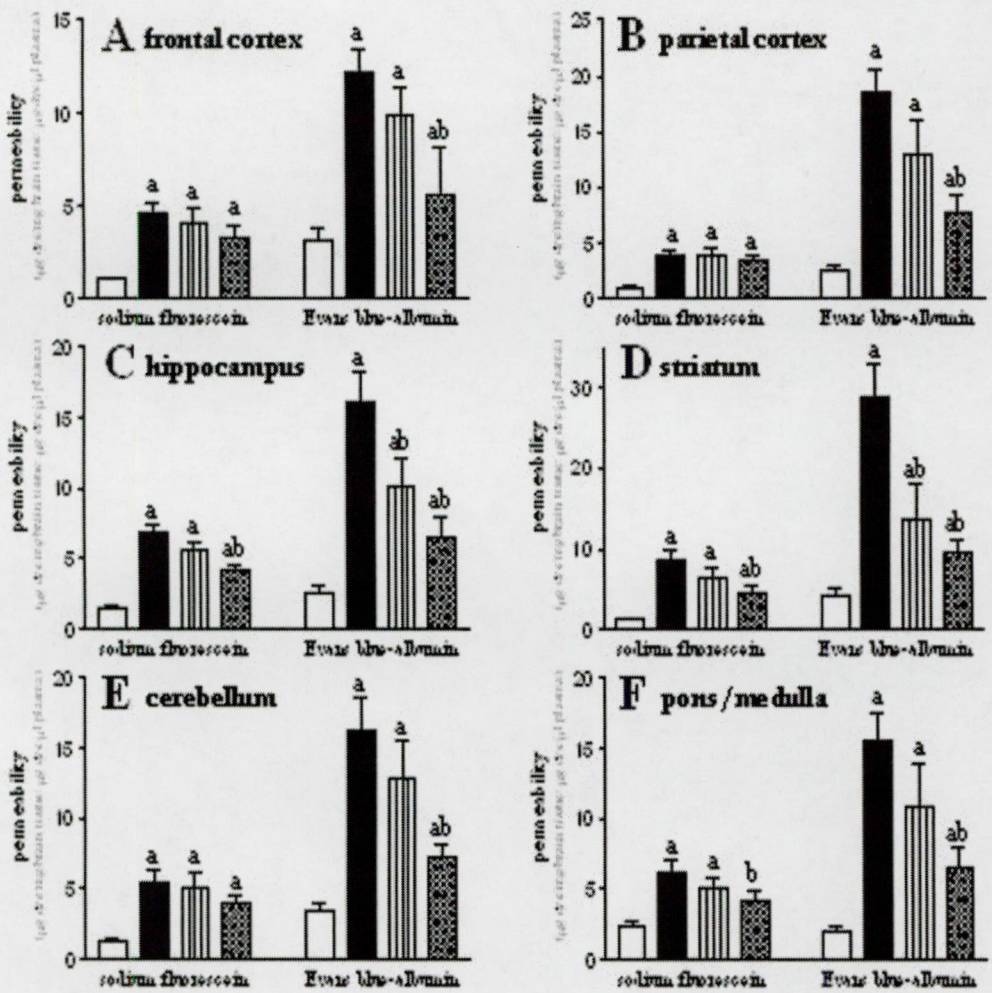


Fig. 7 Effect of postasphyxial D-2-amino-5-phosphovaleric acid (APV) i.v. bolus administration on the blood-brain barrier permeability for sodium fluorescein (SF) and Evans blue-labelled albumin (EBA) in newborn pigs. Treatment groups included: (i) sham-operated control pigs (open bars); (ii) asphyxiated animals treated with vehicle (filled bars); (iii) asphyxiated animals treated with 0.1 mg/kg APV (cross-hatched bars); (iv) asphyxiated animals treated with 1.0 mg/kg APV (vertical line bars). Brain regions tested were as follows: A, frontal cortex; B, parietal cortex; C, hippocampus; D, striatum; E, cerebellum; F, pons/medulla. All values are means ± S.E.M., n=6 in each group. Values differed significantly ($P<0.05$) from data measured in a: control group and b: in asphyxia group.

5. DISCUSSION

5.1. Pathogenesis of asphyxia-induced brain oedema in newborn pigs

Oedematous swelling, *i.e.* abnormal accumulation of fluid and electrolytes within the brain parenchyma intra- or extracellularly, or both, frequently accompanies cerebral diseases with different origin, such as tumours, trauma, infections, hypoxic, toxic and metabolic disorders. Cerebral oedema is a frequent consequence of severe neonatal hypoxia-ischaemia. A wide variety of inter-related factors, such as ATP depletion, ionic imbalances, oxidative stress, up- and down-regulation of specific genes, release of excitatory neurotransmitters and other mediators, neuronal apoptosis and necrosis, can contribute to the development of postasphyxial brain damages in neonates [50,89,159,161]. There is a growing evidence that cyclic nucleotides, free radicals, excitotoxins, lipid mediators including prostanoids, vasoactive amines, cytokines, and other factors are implicated in the pathogenesis of brain oedema formation in asphyxiated newborn pigs [1,7,82,85,87,89,91,104-106,115,123,143,144,153,162,163,183].

According to the classical neuropathological views [see for review: 75,179] brain oedema may have cytotoxic and vasogenic components. Cellular energy failure, resulted in water and electrolyte disturbances, is the essential event in the development of cytotoxic oedema. In the pathogenesis of vasogenic oedema three mechanisms play a role, namely (i) extravasation of serum constituents through the BBB; (ii) enhancement of driving forces including a bulk flow into the interstitial space of the CNS; and (iii) retention of fluid [179]. These changes can also be regarded as an "open-barrier oedema" [18] because the permeability of the BBB is increased and brain oedema results from the oncotic forces generated by an influx of serum proteins into brain.

In our animal model, bilateral PTX-induced neonatal asphyxia resulted in abnormal cerebral accumulation of fluid and electrolytes and an increased BBB transport for sodium fluorescein tracer, but not for albumin, in a region-dependent manner in newborn pigs. A 3-h reperfusion period further aggravated these changes, and also caused a significant albumin extravasation into the brain tissue. We proved that asphyxia and reperfusion could induce both cytotoxic and vasogenic type of brain oedema in newborn pigs. The cytotoxic form was already present at the critical phase [163], while the appearance of vasogenic component was delayed, so permeability for high molecular weight BBB tracers was increased only in the recovery period [7,44,163].

5.1.1. Cytotoxic brain oedema

Our data demonstrate a tendency for elevation of brain tissue water content during PTX in all porcine brain regions observed. Asphyxia and reperfusion also resulted in increased Na^+- and decreased K^+- concentrations in several brain regions investigated. Cerebral Ca^{2+}-content was significantly elevated in frontal cortex, hippocampus, striatum and thalamus in both asphyxiated groups, and also in parietal cortex and internal capsule in the reperfusion group. The elevated Ca^{2+}-concentration may be a consequence of a net cellular uptake of calcium, whereas the opposite changes in total Na^+- and K^+-concentrations in brain tissue samples may suggest a disturbance in the activity of Na^+/K^+-ATPase pump, an enzyme responsible for the maintenance of neuronal excitability and brain cell volume [161]. In contrast to this speculation, Bari *et al.* [12] could not measure significant decrease in the Na^+/K^+-ATPase activity in brain tissue homogenates of newborn pigs during reperfusion after (i) global cerebral ischemia, (ii) asphyxia, or (iii) incomplete forebrain ischemia.

Our data suggest that loss of cellular osmoregulation leading to cytotoxic oedema formation may occur soon after the "primary" energy failure in some particular brain regions of the newborn pig. In another porcine model of transient cerebral hypoxia-ischemia, however, no significant decrease in the apparent diffusion coefficient of brain (a change reflecting the redistribution of water in favour of intracellular compartment, i.e. cytotoxic oedema) was found in "medial" and "lateral" gray matter at 2-4 h after resuscitation [161,166]. In that study, the abnormal diffusion became statistically significant 21 h after the hypoxia-ischemia, and a strong linear correlation was seen between this parameter and diminished phosphocreatine/inorganic phosphate ratio during the "secondary" failure of energy metabolism [166].

5.1.2. Vasogenic brain oedema

Bilateral PTX induced asphyxia in newborn pigs caused a prolonged increase in permeability for SF, a small molecular weight tracer (mw: 376) thought to be a paracellular permeability marker, whereas extravasation of EBA (mw: 67,000), a transendothelial tracer, increased only in reperfusion. Our results on asphyxia-induced BBB opening confirm the validity of previous intravital observations of Temesvári *et al.* [162,165] on pial microvessels using the open cranial window technique in the same model. In these experiments a biphasic increase in the BBB permeability was found, an early opening for SF in the critical phase of PTX, and later

extravasation of fluorescein-labelled dextran (mw: 40,000) during the recovery period [162,165]. The BBB passage of a molecule after a noxious stimulus not necessarily correlates with the molecular weight of the tracer, because lipid solubility or presence of a specific transport process may also influence it. In our model, insulin concentration was elevated in the CSF during postasphyxial reperfusion [4], which can be mediated by either an increase in receptor-mediated transcytosis of insulin (mw: 5,778) through the BBB, or an increased cerebral synthesis. On the other hand, blood to brain transport of [^{14}C]sucrose (mw: 342) was unchanged after 20 min of cerebral ischemia followed by 30 min of reperfusion and significant increase was only seen after 2 h of reperfusion in newborn pigs [48]. In contrast to these observations, a resistance to the development of brain oedema formation in neonates was suggested by Stonestreet *et al.* [155], based on a study in which BBB integrity was maintained for sodium (^{22}Na or ^{24}Na) and [^{14}C]mannitol (mw: 182) in the brain of the newborn pig exposed to severe systemic hypoxia/hypercapnia and phlebotomy-induced hypotension. However, the blood to brain transfer of these tracers was measured in whole brain 1 and 24 h after the noxa and ^{125}I-serum albumin was used to determine the brain vasculature space [155]. It is assumed that regional differences in BBB permeability or albumin extravasation from cerebral vessels during recovery might modify the accuracy of these measurements [155]. Another important difference is that in our model of asphyxia, similarly to the clinical situation, hypertensive periods developed during induction of PTX and early recovery. Hypertension results in irreversible cerebral vasodilatation in newborn pigs and may be a predisposing factor to the subsequent BBB damage [3]. Interestingly, a dramatic increase in permeability for EBA, but no significant change for SF, was found in the 4-vessel occlusion model of cerebral ischemia-reperfusion in adult rats [114]. Species-, age-, and stimulus-specific differences are thought to be responsible for the controversial findings about the BBB permeability obtained on different animal models.

Our quantitative data obtained on 10 brain regions demonstrate regional changes in cytotoxic and vasogenic brain oedema formation in a clinically relevant model of neonatal asphyxia-reperfusion. It also provides information unavailable in human studies about the BBB permeability during asphyxia and first hours of recovery. Recent prospective clinical investigations using magnetic resonance imaging [10,13] revealed specific patterns of brain injury in asphyxiated full-term newborns in the first 10 days of life. These findings include: (i) gray matter damage (oedema in thalami, globi pallidi, posterior putamina, caudate nuclei); (ii) predominant damage of cerebral

cortex and subcortical white matter; (iii) periventricular white matter injury; and (iv) mixed injury pattern. These specific regional patterns of cerebral damage in human neonates correspond to postasphyxial oedema formation, especially the increased albumin permeability, in porcine brain. Thus, porcine PTX-model offers the possibility to scan drugs for their potential to prevent asphyxia-induced brain damages.

5.2. A role for histamine during neonatal brain oedema formation

5.2.1. Endogenous histamine (released from brain compartments)

The following conclusions can be drawn from our comparative histamine measurements carried out simultaneously on venous blood, CSF and isolated microvessel samples of asphyxiated newborn pigs [89]:

- The rise in venous histamine content may originate from blood corpuscles and endothelium.
- The increase in histamine content of CSF in the early (15-min) reoxygenation phase may be interpreted as increased histamine release from intracerebral sources, *i.e.* from neuronal elements and perivascular mast cells. Severely disturbed brain purine metabolism, *i.e.* release of reactive oxygen species during posthypoxic reoxygenation, may contribute to the histamine release [162].
- The histamine content of brain microvessels increased significantly at the end of reoxygenation phase (180-min). This fact may be connected with triggering of selective albumin transport through the brain microvessels [7,35,163]. The microvascular histamine could be derived mainly from the brain intercellular space.

We showed also that cerebral microvessels can take up histamine from the cerebral extracellular space during recovery from asphyxia in newborn pigs. Taken together our present *in vivo* data with results of previous *in vitro* observations [70] it may be concluded that, in cases of ischaemic challenges, the cerebral endothelial cells can not only accumulate but also release histamine towards the blood circulation and thereby take part actively in the elimination of histamine from the extracellular space. Similar detoxifying mechanisms for removal the excess amount of mediator have already been described, *e.g.* Na^+-dependent glutamate transporters (EAAT-1,-2,-3) are present on the abluminal membrane of the BBB [119]. Further studies are warranted to

elucidate the nature and characteristics of supposed histamine transporters operating in the cerebral endothelial cells.

In addition to the contribution to physiological mechanisms, histamine has also been implicated to human pathological changes in brain or gastrointestinal system [21-23,25,45,146,157,170]. Increased blood histamine levels could be measured in neonates suffering from infant respiratory distress syndrome [42], or in newborns undergoing the arterial switch operation for simple transposition of the great arteries [148]. Paediatric systemic diseases may also result in the release of inflammatory mediators into the bloodstream, and excess amount of histamine can provoke BBB changes and CNS symptoms. Sunder *et al.* [156] described children suffering from angioedema with high blood histamine level, who developed neurological manifestations (seizures, headaches, focal and generalized deficits). Severe malaria could also induce an about 5-fold increase in serum histamine level of affected children [47]. Histamine-associated pathological increases in brain, lungs, kidney, or intestine are well-known predictive factors in severe or cerebral malaria [47]. Moreover, because of increased plasma concentration and brain uptake of histidine, children with malaria would also elicit enhanced histamine synthesis in the brain [47,146]. Impaired inactivation of excess cerebral histamine by antimalarial drugs may increase brain levels of imidazole-4-acetic acid, an inducer of sleep-like state associated with seizures, and may contribute to the malaria-related encephalopathy [47]. We suppose that histamine-induced brain oedema with increased BBB permeability contributed to the appearance of these cerebral symptoms.

Histamine has long been known to increase the BBB permeability mainly by H_2-receptor-dependent ways, most probably by the activation of adenylate cyclase enzyme [45,75-78,169]. Histamine, similarly to cyclic adenosine 3'5'-monophosphate (cAMP), increased the formation of pinocytotic vesicles in cerebral endothelium *in vivo* [75]. However, in an *in vitro* reconstituted model of the BBB, cAMP treatment resulted in a rapid decrease in paracellular permeability, while histamine administration increased permeability for albumin, but not for tight junction markers [35]. It was proved that histamine might affect the BBB permeability by a H_1-receptor dependent, phosphoinositol-mediated mechanism, too [45,75,78,101].

On the other hand, histamine could also increase the BBB permeability through consequent phospholipase C activation, release of Ca^{2+} from intracellular stores, induction of nitric oxide synthase, stimulation of guanylate cyclase, and the formation of cyclic guanosine 3'5'-

monophosphate (cGMP) [101]. In rat cerebral endothelial cells, histamine elevated the intracellular [Ca^{2+}] concentrations in vitro, while cGMP increased the rate of pinocytosis *in vivo* [78,101].

Though the emerging role of histamine, as an intracellular second messenger [78], has been established in the regulation of cellular processes in a wide variety of cell types [23], no information was published about the role of Hic receptors in the regulation of the BBB properties until recently. Karlstedt *et al.* [79] found lack of histamine synthesis in immortalized brain endothelial cells, but provided evidence that the internalised histamine was distributed in the cytoplasm and nucleus of the cells. It is known, however, that polyamines synthesised by ornithine decarboxylase can act as intracellular messengers and mediate the BBB breakdown [84]. In a recent study, Hic antagonist DPPE induced an increased BBB permeability for intravascular tracers, which supports a role for the intracellular histamine in the maintenance of the barrier properties [36].

5.2.2. Exogenous histamine induced brain oedema

In newborn pigs, intracarotid administration of exogenous histamine resulted in a dose-dependent increase in the BBB permeability for intravascular tracers in each brain region examined 1h after drug injection [116]. Exogenous histamine caused marked deterioration of BBB function characterised by significant rise in permeability for both SF and EBA at concentrations similar to those measured during asphyxia [116]. In an *in vitro* study histamine enhanced the transcellular passage of albumin through the BBB, but not the permeability for paracellular markers (sucrose, inulin) [35]. In adult rats increased pinocytotic activity was also seen after intracarotid histamine administration [45,56,57,76-78]. EBA, similar to other molecules with large molecular weight, is supposed to pass the BBB by pinocytosis, while SF is thought to extravasate both para- and transcellularly.

5.2.3. Effects of antihistamines on brain oedema formation

We have found in our model of neonatal asphyxia [3,162,163], that antihistamines could prevent hypoxic brain oedema in newborn pigs [44]. Among the investigated drugs, mepyramine and ranitidine were found to decrease the accumulation of water, sodium and albumin in the porcine cerebral cortex. Protective effect of antihistamines suggests that histamine plays a pathogenetic role in cerebral oedema formation in the neonatal period [44,76,78].

Histamine receptor antagonists have a protective effect on increased BBB permeability and brain oedema formation in other animal models [27,43,75-78,142,169]. Cerebral microvessels contain primarily histamine receptors of H_2-type that are linked to the endothelial adenylate cyclase [7,78,80], but we could demonstrate the involvement of H_1-receptors in the development of perinatal brain oedema [44]. The applied doses of antihistamines were in good agreement with the effective doses described in other *in vivo* animal experiments [56,57].

The H_1-blocker mepyramine, being a lipid-soluble molecule, easily penetrates the BBB, and may act also on H_1-receptors being present in mast cells or neurons on the "brain side" of the barrier. Although ranitidine can not cross the intact BBB, the drug was found in CSF of patients with neurological illnesses which suggests that ranitidine can pass the impaired BBB [99]. In our experiment i.p. administered ranitidine could probably penetrate the disturbed BBB and was also effective in reducing the permeability changes during asphyxia-reperfusion. Butt and Jones [27] showed that histamine resulted in a 75% decrease in transendothelial electrical resistance in brain-surface microvessels of rats and caused an increase in BBB permeability which was mediated *via* endothelial H_2-receptors.

5.3. Possible involvement of α-melanocyte-stimulating hormone in asphyxia

Asphyxia resulting in hypoxaemic cardiovascular and metabolic failure induced an increase in plasma α-MSH level in newborn pigs in our experiments. It is known that perinatal stress also causes an acute release of ACTH-related peptides including α-MSH in human neonates [100, 129]. We described a decrease in CSF concentration of α-MSH during postasphyxial reperfusion in newborn pigs. The dissimilar changes found in the hormone levels in plasma and CSF may be explained by different mechanisms responsible for the regulation of α-MSH levels in plasma, CSF, and brain [38,39,108,141,147]. Plasma α-MSH is derived from the pituitary, whereas CSF α-MSH from the CNS, which may suggest a differential secretion of α-MSH [39]. Similarly, drug-induced changes [141] or circadian variations [108,147] in the release of α-MSH from the pituitary and various brain areas are also not synchronous. On the other hand, α-MSH found in CSF might derive from blood to brain transport, too [11,38]. α-MSH crosses the BBB by a nonsaturable process [11], thereby a hypoxic energetic failure seems unlikely to block the transport. However, CSF α-MSH has a short half-time disappearance [38], so increased plasma supply or even elevated

brain interstitial concentration does not necessarily result in rapid changes in α-MSH level in CSF from cisterna magna.

Porcine model of PTX-induced cardiovascular failure [2,3,4,88,89,163] is suitable for studying neuroendocrinological changes during perinatal stress [4,88,89]. In the same model, our data indicated an asphyxia-induced release of histamine into blood and CSF, and it was suggested that cerebral microvessels might be involved in the elimination of histamine from brain interstitial fluid [89]. Neonatal pneumothorax also increased cerebrovascular permeability for sodium fluorescein (mw: 376 Da), and Evan's blue-albumin (mw: 67 kDa) [2]. Brain endothelial cells express melanocortin receptors [34], and α-MSH might alter BBB transport of different substances [11]. A co-incidence was seen between the increases in the BBB permeability [2], and α-MSH release into the circulation during asphyxia and reperfusion [88]. We assume that local delivery of histamine by hypothalamic histaminergic neurons is, at least partly, responsible for the α-MSH release in asphyxiated newborn pigs, though significant increase in histamine concentration of CSF originated from cisterna magna was only seen after 15 min of postasphyxial reperfusion [89].

The biological significance of the changes in α-MSH concentrations during neonatal asphyxia-reperfusion remains to be elucidated. α-MSH is a member of the main endogenous anti-analgesic system, and it also plays a role in neuroimmunomodulatory host reactions including fever, inflammation and shock [58,96]. Anti-inflammatory effects of α-MSH thought to be beneficial in myocardial infarction, rheumatic disorders, endotoxaemia, and human immunodeficiency virus-1 (HIV-1) infection [96]. It was suggested that administration of pharmacological doses (160 μg x kg^{-1}) of exogenous α-MSH into blood might be a simple, non-toxic first-aid treatment during resuscitation of prolonged respiratory arrest or haemorrhagic shock [58]. In animal studies α-MSH also protected against ischaemia-reperfusion injury of brain stem [69], kidney [29] and heart [unpublished observation by Vecsernyés *et al.*]. The neurotrophic effects of α-MSH are mediated by various neuronal melanocortin receptors (MC_{1-5}) coupled to G proteins and cyclic AMP signal transduction pathway [8]. On the other hand, α-MSH exerts some anti-cytokine peptide-like effects on peripheral macrophages and neutrophil leukocytes [96]. It is known that neonatal hypoxia-ischemia stimulates the expression of cytokines including tumor necrosis factor-α (TNF-α) [159], which mediator induces cerebrovascular changes and increases the BBB permeability in newborn pigs [1,104,105]. Because α-MSH can directly inhibit the production of TNF-α [133], an asphyxia-induced decrease in α-MSH concentration in the brain interstitial fluid may further aggravate the

adverse effect of this cytokine. In a recent study performed on adult rats, systemic α-MSH administration could reduce CNS leukocyte accumulation, but did not attenuate pneumococcal meningitis-associated intracranial complications including BBB disruption or brain cytokine levels [81]. However, the possible therapeutic effect of exogenous α-MSH on organ injuries evoked by neonatal hypoxia-ischaemia, remains to be elucidated.

5.4. Therapeutic effects of glutamate receptor antagonists in neonatal brain oedema

Both *N*MDA receptor antagonists tested provided therapeutic benefit in postasphyxial brain oedema formation: $MgSO_4$ and APV dose-dependently attenuated the increase in the BBB permeability. Although *N*MDA receptors are widely distributed in the neurons of the developing brain [37], the site of action for the prevention of vasogenic brain oedema formation is suggested to be the cerebral endothelial cells, the morphological basis of the BBB. Koenig *et al.* [48] have described that *N*MDA receptors may regulate BBB function in isolated cerebral capillaries, whereas Krizbai *et al.* [90] provided evidence that rat primary cerebral endothelial cells express functional glutamate receptors and *N*MDA receptor antagonist MK-801 (dizocilpine) could prevent glutamate-induced increase in intracellular Ca^{2+} accumulation. Since then molecular biological and functional studies confirmed the presence of glutamate receptors in primary human [30] and porcine [122] brain endothelial cells, even if a study, performed on cerebral endothelial of allegedly human origin with high passage number, failed to demonstrate the receptors [109]. Recent study indicated that *N*MDA receptor antagonist memantine could attenuate the loss of BBB function and recruitment of inflammatory cells in experimental allergic encephalomyelitis [125]. The beneficial effect of $MgSO_4$ treatment has been demonstrated in various models of neonatal brain injuries [26,59,102,103,144]. Experimental and clinical data show that $MgSO_4$ infusion can be safely administered both to newborn animals, and human neonates [5,95], and older patients [49], although it caused systemic vasodilation and hypotension in a study on immature neonatal rabbits [24]. No significant changes in the clinical parameters tested was induced by $MgSO_4$ infusion in our study. Although Gee *et al.* could not detect increase in brain intracellular magnesium concentration by magnetic resonance spectroscopy in newborn minipigs [54], we could measure dose-dependent increases in plasma and CSF magnesium concentrations (data not shown), in accordance with data of Rivera *et al.* [136].

$MgSO_4$ infusion protected against the hypoxia-induced modification of *N*MDA receptor and increased the activity of Na^+/K^+-ATPase, whereas it did not alter the cortical levels of ATP and phosphocreatine [67,68]. In newborn rats, i.p. magnesium administration prevented *N*MDA-mediated neurotoxicity [103]. Neonatal hypoxia-ischaemia results in a loss of *N*MDA receptor-mediated vasodilatation associated with neuronal nitric oxide production in newborn pigs [41,174,175]. Extracellular magnesium concentration may modify the basal formation and release of nitric oxide and thus alter arterial smooth muscle tone in cerebral circulation [158].

Experimental studies indicate therapeutic benefit after the use of *N*MDA receptor blockers. In a model of rat neonatal hypoxia-ischaemia *N*MDA receptor antagonist MK-801 in i.p. doses of 0.3 and 0.5 mg/kg could significantly reduce the brain damage, whereas a higher dose provided no benefit [59]. MK-801 attenuated the neonatal brain injury from hypoxic-ischaemic damage [102], and it could protect against caspase-3 activation and DNA fragmentation after hypoxia-ischaemia in 7-day-old rats [131]. Memantine, an *N*MDA receptor-associated channel blocker provided protection in a photothrombotic model of cerebral focal ischaemia in neonatal rat [154]. Ketamine could partially protect against the hypoxic-ischaemic injury induced changes in hippocampus, such as decrease in levels of high energy phosphates, and neuropathological damage [144]. *N*MDA receptor blockade with dextromethorphan (DM), a non-competitive channel blocker, prevented decrease in cortical thickness, and seizures in neonatal rats subjected to hypoxia [93]. APV attenuated the calcium redistribution and electroencephalographic changes induced by 15-min cerebral ischaemia in adult rabbit [134]. Selective *N*MDA antagonist APV reduced the brain ischaemia-induced accumulation of purine metabolites hypoxanthine, xanthine, inosine and adenosine in rat striatum [61].

6. CONCLUSIONS

I. It has been established that neonatal asphyxia-reperfusion induces time-dependent increases in the concentration of histamine in serum from the jugular vein, in CSF, and in isolated cerebral microvessels. The investigations have revealed the role of cerebral microvessels in the elimination of excess histamine released from brain compartments during postasphyxial reperfusion in newborn piglets.

II. During asphyxia-reperfusion an increase in serum α-MSH concentration and a decrease in cerebrospinal fluid hormone level were observed in newborn pigs. It is known that the release of α-MSH from the intermediate lobe of the pituitary gland is regulated, among others, by hypothalamic histaminergic neurons in perinatal stress.

III. It has been proved that temporal and regional distribution of asphyxia-induced cytotoxic and vasogenic oedema in newborn pigs resembles to the characteristics of brain damages in human neonates. This observation further supports that porcine bilateral experimental PTX model is appropriate for studying the pathomechanism and therapy of brain damages evoked by neonatal asphyxia.

IV. Intracarotid administration of exogenous histamine could induce vasogenic brain oedema formation in healthy newborn pigs. These data may serve as an indirect proof for the involvement of excess endogenous histamine in the BBB permeability changes.

V. Moreover, the contribution of histamine to the development of cerebral injury is further supported by the efficacy of H_1 and H_2 histamine receptor antagonists in reduction of brain oedema formation in porcine model of neonatal asphyxia.

VI. It has been also demonstrated that development of brain oedema in asphyxiated newborn pigs could be attenuated by postasphyxial treatment with magnesium sulphate or D-2-amino-5-phospho-valeric acid, two *N*MDA type glutamate receptor antagonists. The crucial role for glutamic acid in the pathogenesis of neonatal encephalopathies has been previously established in series of experimental and clinical studies.

7. THE POSSIBLE CLINICAL RELEVANCE OF THIS STUDY

7.1. Antihistamines

The major achievement of this study is supposed to be the experimental evidence provided about the involvement of histamine in the development of postasphyxial brain oedema in newborns. The pathogenetic role of histamine was supported by three-fold proof, namely (i) neonatal asphyxia-reperfusion induces histamine release to plasma and CSF [89]; (ii) infusion of exogenous histamine to common carotid artery provokes vasogenic brain oedema in newborn pigs [116]; and (iii) pre-treatment with histamine H_1 and H_2 receptor antagonists could attenuate neonatal asphyxia-induced brain oedema [44]. Pharmacological use of histamine receptor antagonists can also be considered in other paediatric diseases, because histamine, among other substances, certainly takes part in the development of the pathological alterations including infant respiratory distress syndrome [42], transposition of the great arteries [148], angioedema associated with neurological symptoms [156], and cerebral malaria [47] in newborns, infants, and children.

Histamine is also suggested to be involved in the pathogenesis of cerebral palsy. Elevated perinatal circulating cytokine levels, including TNF-α, and several interleukins (IL-1β, IL-6, IL-8, IL-9), were found to correlate with the occurrence of cerebral palsy in human neonates [112,113]. In a mouse model, administration of interleukin-9 could potentiate the excitotoxicity of glutamate analogue ibotenate, and periventricular leukomalacia by activating various immune cells including mast cells and increasing the production of toxic factors, such as histamine [124]. Administration of cromoglycate, an inhibitor of mast cell degranulation, or combination of histamine H_1 (dexchlorpheniramine) and H_2 (ranitidine) receptor antagonists could significantly protect against neurotoxicity in this model [124].

7.2. α-melanocyte stimulating hormone

α-MSH, similarly to other POMC-derivates (ACTH and its fragments lacking the C-terminal sequence) have a life-saving effect in animals and humans in hypoxaemic conditions induced by different types of shock [58], or by prolonged respiratory arrest [59]. It is suggested that this beneficial effect of melanocortins is due to their capacity to inhibit the overproduction of oxygen-free radicals and to their peculiar anti-inflammatory activity [58,96]. Selective melanocortin receptor agonists and antagonists may have favourable effects on increasing survival rate of

animals with shock. Besides the prevention of severe cardiovascular problems (ventricular arrhythmias, fall in systemic arterial pressure) α-MSH or analogues could reduce BBB permeability and subsequent brain injuries, since MR_3-subtype of melanocortin receptor is expressed also in different brain areas and in cerebral microvasculature [58]. Recently other hormones were also suggested to be neuroprotective, growth hormone [60] and basic fibroblast growth factor [118] were beneficial in a rat model of neonatal hypoxia-ischaemia, whereas melatonin attenuated free radical injury in asphyxiated human neonates [52]. Further studies are expected to characterise these processes, and to reveal the advantages of such potential drugs.

7.3. *N*-methyl-D-aspartate type glutamate receptor antagonists

The potential use of various *N*MDA receptor antagonists in the prevention of neonatal and adult brain injuries has long been investigated, and a thousands of papers were published [reviewed in 32,40,97]. The excitement following early studies, however, was soon dissipated when protective effects were associated severe side-effects including temperature reduction [97]. Although *N*MDA receptor antagonists may protect against neonatal asphyxia or hypoxia-ischaemia related brain injuries in several models [59,93,102,131,144,154], to our best knowledge there is no proprietary synthetic molecule for neonatal brain injuries in an advanced phase of drug development. On the other hand, $MgSO_4$, a generic drug widely used by generations of medical doctors, has limited therapeutic margin. Considering the important role of *N*MDA receptors in the physiology and pathology in developing brain, it is difficult to predict the time until when concentrated research and development activity can produce an effective and safe *N*MDA receptor antagonist for the prevention of neonatal brain injuries.

The present investigations on the pathophysiology and therapy of neonatal brain oedema aimed to contribute to the better understanding of hypoxic-ischaemic neonatal brain damages, to the treatment of acute life-threatening complications, and to the prevention of neurological sequelae. If the professional experience gained during the years of research could improve life-expectancy of a single patient, my efforts were useful.

8. REFERENCES

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