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Perinatal brain injury

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1 Introduction

Year after year, around a thousand children in Germany alone incur brain damage as a result of a perinatal hypoxic-ischemic insult [152, Perinatal statistics for the Federal Republic of Germany]. Depending on the extent and location of the insult these children can develop spastic paresis, choreo-athetosis, ataxia and disorders of sensomotor coordination (figure 1). Nor is it uncommon for damage to the auditory and visual systems and impairment of intellectual ability to develop later [197]. The resulting impact on the children affected and their families is considerable and their subsequent care demands a high level of commitment and co-operation between pediatricians, child neurologists, physio-, speech- and psychotherapists and other specialists. Conservative estimates of the costs to society for treatment and care of such cases per birth year lie around 1 billion German marks. However, despite the severe clinical and socio-economic significance, no effective therapeutic strategies have yet been developed to counteract this condition; one possible explanation being that perinatal management up to now has focused on preventing hypoxic-ischemic brain damage altogether [197]. The pathophysiology of ischemic brain lesions has not been investigated in depth until recently. One of the most urgent tasks for obstetricians and neonatologists will now be to develop therapeutic strategies from these pathophysiological models and to test them in prospective clinical studies.

Figure 1. Spastic diplegia in a child with cerebral palsy [117 a].

This review article presents our current understanding of the pathophysiology of hypoxic-ischemic brain damage in mature neonates. The situation in premature neonates is discussed separately wherever necessary. We first deal with the causes of ischemic brain lesion, especially intrauterine asphyxia of the fetus, and their effects on the cardiovascular system and cerebral perfusion. Next the typical neuropathological findings arising from reduced perfusion of the fetal brain are described. Also of key importance are the cellular mechanisms that are triggered by an ischemic insult. These will be discussed in detail, with particular emphasis on alterations of energy metabolism, intracellular calcium accumulation, the release of excitatory amino acids and protein biosynthesis. A considerable portion of neuronal cell damage first occurs during the reperfusion phase following an ischemic insult. The formation of oxygen radicals, induction of the nitric oxide system, inflammatory reactions and apoptosis will therefore be discussed in depth in this context.

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Finally, therapeutic concepts will be presented that have been developed out of our understanding of these pathophysiological processes and have been tested in animal experiments. Of these, intravenous administration of magnesium and induction of cerebral hypothermia appear to be of the greatest clinical relevance. This article is a short summary of a previously published paper [18].

2 Causes of hypoxic-ischemic brain lesions in neonates

With a few exceptions, acute hypoxic-ischemic brain lesions in neonates are caused by severe intrauterine asphyxia [197]. This is usually brought about by an acute reduction in the uterine or umbilical circulation [103], which in turn can be caused by abruptio placentae, contracture of the uterus, vena cava occlusion syndrome, compression of the umbilical cord etc.

3 Circulatory centralization and cerebral perfusion

The fetus reacts to an oxygen deficit of this severity by activating the sympathetic-adrenergic system and redistributing the cardiac output in favor of the central organs (brain, heart and adrenals) [103]. The lowered oxygen and raised carbon dioxide partial pressures lead to vasodilatation of the cerebral vascular bed causing cerebral hyperperfusion. This affects the brainstem in particular, while the bood flow to the white matter of the brain is hardly increased at all [7, 120, 104]. Depending on the extent of the oxygen deficit and the maturity of the fetus, this cerebral hyperperfusion can reach 2−3 times the original rate of blood flow. If the oxygen deficit persists the anaerobic energy reserves of the heart become exhausted. The cardiac output and the mean arterial blood pressure fall. At mean arterial blood pressures of below 25−30 mmHg there is an increasing loss of cerebral autoregulation, and a consequent reduction of the cerebral blood flow [119]. This affects the parasagittal region of the cerebrum and the white matter most of all. Immature fetuses seem to be particularly endangered by their limited ability to increase the cerebral circulation through vasodilatation.

If the supply of oxygen to the fetus can be improved, cerebral hyperperfusion is brought about by the progressive postasphyxial increase in cardiac output [103]. This hyperperfusion can be demonstrated in experiments using animal models of isolated cerebral ischemia (figure 2) [26]. Vasodilatation induced by acidosis in cerebral tissues and a reduction of blood viscosity at higher rates of blood flow have been put forward as possible causes of such hyperperfusion. The initial hyperperfusion of the brain is followed directly by a phase of hypoperfusion (figure 2) [26, 175]. Postischemic hypoperfusion may be caused by oxygen radicals formed during the reperfusion phase after ischemia. Rosenberg and co-workers demonstrated that this phenomenon can be prevented by inhibiting the synthesis of oxygen radicals after ischemia [175]. In addition, a so-called no-reflow phenomenon can be observed after severe cerebral ischemia. This failure of reperfusion in various brain areas is a consequence of the greater viscosity of stagnant blood, compression of the smallest blood vessels through swelling of the perivascular glial cells, formation of endothelial microvilli, increased intracerebral pressure, postischemic arterial hypotension and increased

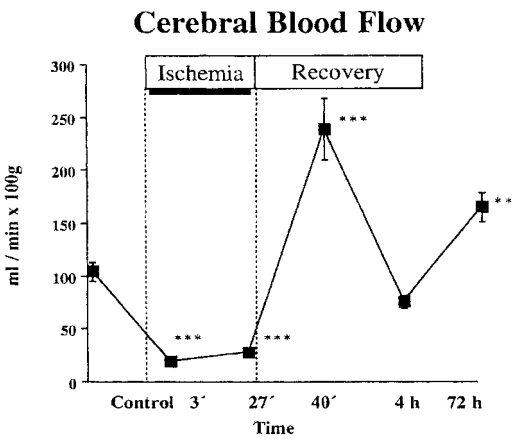

Figure 2. Blood flow to the cerebrum (ml/min × 100 g) in fetal sheep near term before, during and after global cerebral ischemia of 30 min duration. Cerebral ischemia was inducted by occluding both carotid arteries. Results are given as mean ± SD. The data were analyzed for intragroup differences by multivariate analysis of variance for repeated measures. Games-Howell-test was used as post-hoc testing procedure (** $P < 0.01$, *** $P < 0.001$ (ischemia/recovery vs. control)) [26, 32].

intravascular coagulation. The extent of the no-reflow phenomenon depends on the duration and type of cerebral ischemia. It is most pronounced when the vessels are engorged with blood after venous congestion [99]. Directly after postischemic hypoperfusion the cerebral blood flow recovers or overshoots into a second phase of hyperperfusion (figure 2) [26, 169]. Since this hyperperfusion is often accompanied by an isoelectric encephalogram, it is regarded as an extremely unfavourable prognostic factor [169].

4 Neuropathology of hypoxic-ischemic brain lesions

There are essentially six forms of hypoxic-ischemic brain lesion: selective neuronal cell damage, status marmoratus, parasagittal brain damage, periventricular leucomalacia, intraventricular or periventricular hemorrhage and focal or multifocal ischemic brain lesions (table I) [197].

In mature fetuses, selective neuronal cell damage is found most frequently in the cerebral cortex, hippocampus, cerebellum and the anterior horn cells of the spinal cord [66, 111, 148, 197]. As shown in animal experiments, the damage occurs after ischemia of only 10 min. [204]. Within the cortex, the border zones between the major cerebral arteries are the worst affected. The cell damage is mostly parasagittal and more marked in the sulci than in the gyri, i. e. the pattern of distribution is strongly dependent on perfusion. The neurones show the most damage while the oligodendrocytes, astroglia and microglia remain largely unscathed [197].

Table I. Hypoxic-ischemic brain damage in the fetus and neonate

Neurologic lesion	Topographic localization
Selective neuronal necrosis	cortex cerebri cerebellum hippocampus anterior horn cells of the spinal cord
Status marmoratus	basal ganglia thalamus
Parasagittal cerebral injury	cortex cerebri and subcortical substantia alba
Periventricular leucomalacia	substantia alba
Intra-, periventricular hemorrhage	germinal matrix substantia alba ventricles
Focal/multifocal ischemic brain damage	cortex cerebri and subcortical substantia alba

Status marmoratus, which is observed in only 5 % of children with hypoxic-ischemic brain lesions, chiefly affects the basal ganglia and the thalamus. The complete picture of the disease does not emerge until 8 months after birth although the insult begins to take effect during the perinatal period. Status marmoratus is characterized by loss of neurones, gliosis and hypermyelination. The increased number of myelinated astrocytic cell processes and their abnormal distribution give the structures affected, especially the putamen, a marbled appearance [66, 173].

Parasagittal brain damage caused by cerebral ischemia is mostly reported in mature neonates [66, 111, 148, 197] and affects the parietal and occipital regions in particular. The damage usually arises through insufficient perfusion of the border zones between the main cerebral arteries during cerebral ischemia. This form of damage has been reproduced in animal models (figure 3). The extent of the brain lesions was found to be closely dependent on the duration and severity of the cerebral ischemia [26, 204].

Periventricular leucomalacia is characterized by damage to the white matter dorsal and lateral to the lateral ventricle [111, 148]. It occurs most frequently in immature fetuses and chiefly affects the radiatio occipitalis at the trigonum of the lateral ventricle and the white matter around the foramen of Monroe. Six to twelve hours after an ischemic insult necrotic foci can be observed in these areas [10]. As the disease progresses small cysts develop out of the necrotic foci that can be identified by ultrasonography [56, 162]. As gliosis progresses the cysts begin to constrict. The lack of myelinization owing to the destruction of the oligodendrocytes and an enlargement of the lateral ventricle then become the most prominent features of the disease [53, 173, 186]. Periventricular leucomalacia around the Radiatio occipitalis

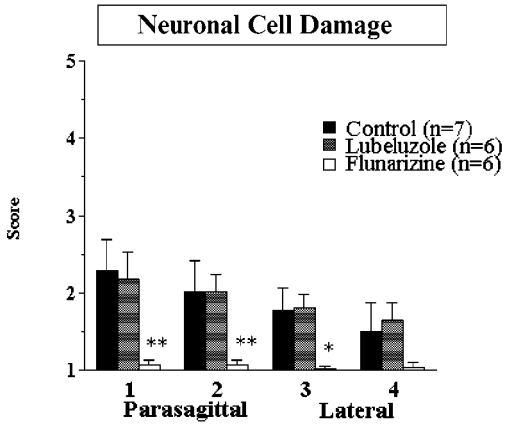

Figure 3. Neuronal cell damage in the cerebrum in fetal sheep near term 72 h after induction of global cerebral ischemia of 30 min duration. Cerebral ischemia was induced by occluding both carotid arteries. Neuronal cell damage was quantified as follows: 0−5 % damage (score 1), 5−50 % damage (score 2), 50−95 % damage (score 3), 95−99 % damage (score 4), and 100 % damage (score 5). Neuronal cell damage was most pronounced in the parasigittal regions, whereas in the more lateral part of the cortex only minor neuronal damage occurred. There was a tremendous reduction in neuronal cell damage after pretreatment with the calcium antagonist flunarizine (1 mg/kg estimated fetal body weight), whereas glutamate antagonist lubeluzole failed to protect the fetal brain. Values are given as mean ± SD. The data were analyzed within and between groups using a two-way ANOVA followed by Games-Howell post test (* $P < 0.05$, ** $P < 0.01$ (treated vs. untreated)) [32, 70].

at the trigonum of the lateral ventricle and in the white matter around the foramen of Monroe arises through vascular problems. The ability to increase blood flow by vasodilatation during and after a period of arterial hypotension appears to be extremely limited in these brain areas. After the 32 nd week of pregnancy the vascularization of these vulnerable areas is considerably increased and the incidence of periventricular leucomalacia thereby reduced.

Intra- or periventricular hemorrhage is another typical lesion of the immature neonate brain [197]. It originates in the vascular bed of the germinal matrix, a brain region that gradually shrinks until it has almost completely disappeared in the mature fetus [92, 140, 144]. Blood vessels in this brain region burst very easily. Sub- and post-partum fluctuations in cerebral blood flow can therefore lead to rupture of these vessels causing intra- or periventricular hemorrhage [27, 67, 74, 104, 134]. Possible consequences of a brain hemorrhage are destruction of the germinal matrix, a periventricular hemorrhagic infarction in the cerebral white matter or hydrocephalus [197].

Focal or multifocal brain damage usually occurs within areas supplied by one or more of the main cerebral arteries. This form of insult is not normally observed before the 28th week of pregnancy. The incidence then rises with increasing maturity of the fetus [12]. Focal or multifocal brain lesions are often the result of infections, trauma or twin births, especially monochoriotic ones [15, 166, 178]. It is thought that thromboplastic material or emboli from a miscarried co-twin sometimes occludes the cerebrovascular circulation of the living twin. Brain damage may also be caused by anemia or polycythemia and subsequent cardiac insufficiency and cerebral hypoperfusion arising from a feto-fetal transfusion. Alternatively, focal or multifocal brain damage can arise from systemic arterial hypotension, so that there is little distinction between this and other forms of brain damage such as selective neuronal cell damage, status marmoratus, parasagittal brain damage or periventricular leucomalacia [197].

5 Energy metabolism and calcium homeostasis

The normal function of the brain is essentially dependent on an adequate oxygen supply to maintain energy metabolism. Whereas, during moderate hypoxemia, the fetus is able to maintain adequate levels of ATP by speeding up the rate of anaerobic glycolysis [22, 23, 28], an acute reduction of the fetal oxygen supply will lead to a breakdown of energy metabolism in the cerebral cortex within a few minutes (table II) [20, 21]. The ionic gradients for Na^+, K^+ and Ca^{2+} across the cell membranes can no longer be regulated since the Na^+/K^+-pump stops working through lack of energy. The membrane potential approaches 0 mV [93]. The energy depleted cell takes up Na^+, and the subsequent fall in membrane potential induces an influx of Cl^- ions.

Table II. Concentrations of high-energy phosphates in the cerebral cortex of fetal guinea pigs near term during acute asphyxia caused by arrest of uterine blood flow [27, 28]

	Brain metabolite [μmol/g]		
	Control	Asphyxia 2 min	Asphyxia 4 min
Adenosine triphosphate	2.59 ± 0.15	2.03 ± 0.21**	1.35 ± 0.32**
Adenosine diphosphate	0.37 ± 0.07	0.76 ± 0.13**	1.05 ± 0.15**
Adenosine monophosphate	0.04 ± 0.02	0.17 ± 0.09**	0.52 ± 0.21**

Values are given as mean ± SD. ** $P < 0.01$ (asphyxia vs. control)

This intracellular accumulation of Na^+ and Cl^- ions leads to swelling of the cells as water flows in through osmosis. Cell edema is therefore an inevitable consequence of cellular energy deficiency [183].

In addition, loss of membrane potential leads to a massive influx of calcium down the extreme extra-/intracellular concentration gradient. It is currently thought that the excessive increase in intracellular calcium levels, the so-called calcium-overload, leads to cell damage by activating proteases, lipases and endonucleases [183]. Some of the cellular mechanisms that are activated by the calcium influx occurring during ischemia are shown in figure 4: alteration of the arachidonic acid cycle affecting prostaglandin synthesis, disturbances of gene expression and protein synthesis and increased production of free radicals and obstruction of the axonal transport system through disaggregation of microtubuli.

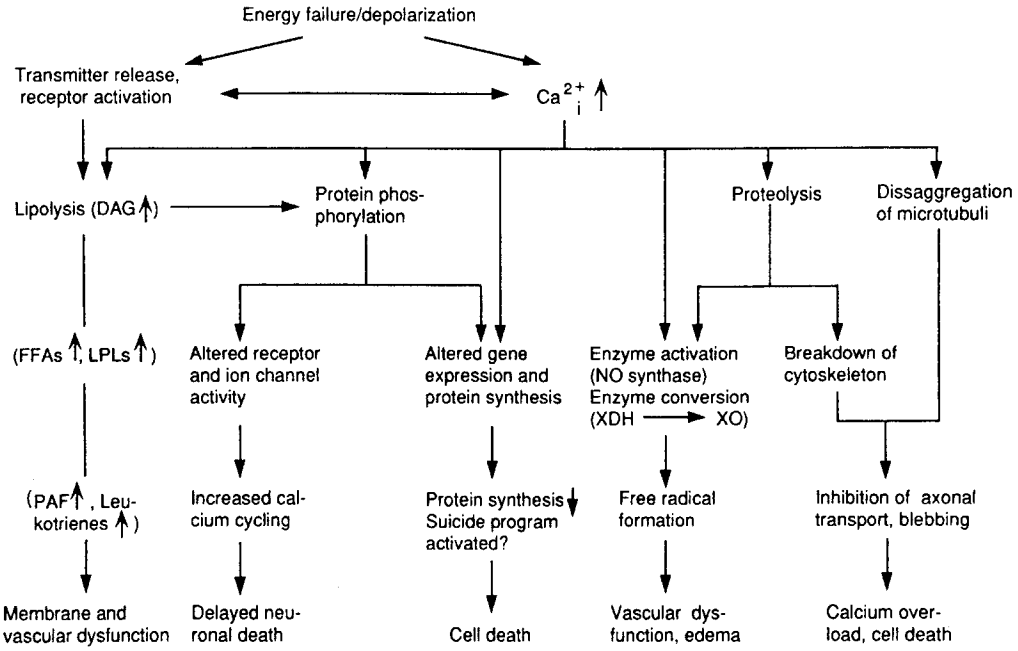

Figure 4. Primary secondary effects of the increased intracellular calcium concentration during and after cerebral ischemia [183]. XDH, xanthine dehydrogenase; XO, xanthone oxidase; PAF, platelet aggregating factor; FFAs, free fatty acids; DAG, diacylglyceride; LPL, lysophospholipids.

6 Excitatory neurotransmitters

As early as 1969 Olney succeeded in demonstrating that neuronal cell death could be induced by the exogenous application of glutamate, an excitatory neurotransmitter [155]. In subsequent years, this observation was confirmed in both immature and adult animals of various species including primates [156]. In 1984, Rothman showed that glutamate antagonists could prevent anoxic cell death in hippocampal tissue cultures [176]. That same year, Benveniste and co-workers reported an excessive release of glutamate into the extracellular space during cerebral ischemia in vivo [16], from which they concluded that glutamate might play an important role in neuronal cell death following ischemia [157, 176].

Glutamate activates postsynaptic receptors that form ionic channels permeable to cations (figure 5) [180]. The NMDA-receptor regulates a calcium channel, the metabotropic receptors induce an emptying of intracellular calcium stores while the AMPA/KA receptors open a voltage-dependent calcium channel by membrane depolarization. The increase in free calcium within the cell activates proteases, lipases and endonucleases that then initiate processes leading to cell death [46, 182].

There is no longer any doubt that glutamate release plays a critical role in neuronal cell death after focal cerebral ischemia such as that caused by an arterial embolus. Glutamate antagonists have been shown to exert a strong neuroprotective effect against hypoxic-ischemic brain damage in adult [109, 163, 195] and even in neonatal animals [6, 63, 73, 95, 130, 149]. In neonatal rats it was shown that glutamate release during and after an hypoxic-ischemic insult could evoke epileptogenic activity and that this effect was dependent on the maturity of the brain. In rats, the most marked effect was observed 10 to 12 days after birth [105]. The reason for this seems to be a developmental change in the composition of the glutamate receptor, which increases the neurone's permeability to calcium [106, 107]. Furthermore, the levels of GABA, one of the most important inhibitory neurotransmitters, in neuronal tissue are very low at this stage of development [50].

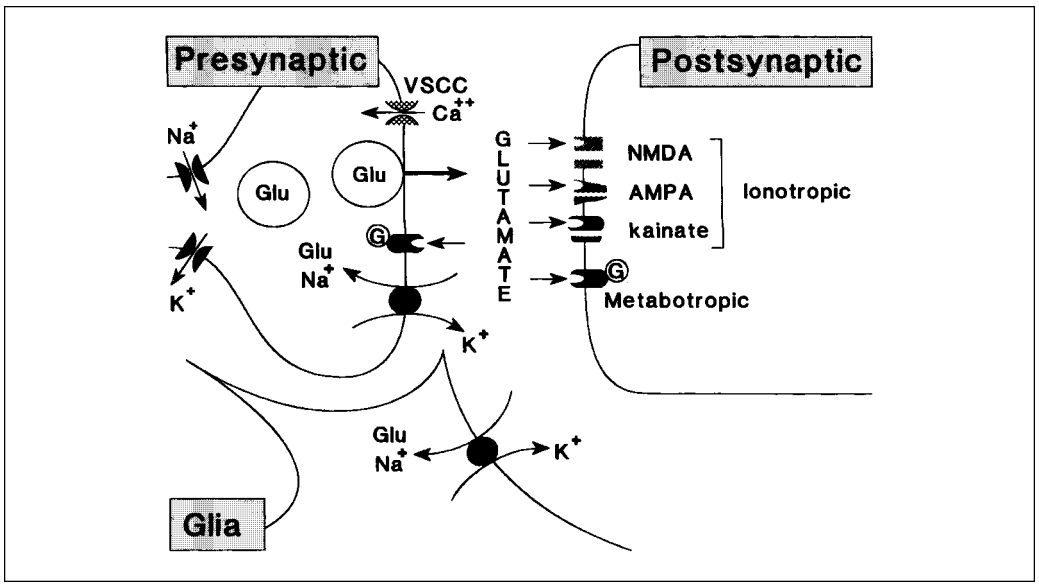

Figure 5. Regulation of glutamate-mediated synaptic transmission. After depolarization of the presynaptic neuron vesicular glutamate is released by exocytosis into into the synaptic cleft. Released glutamate activates postsynaptic ionotropic (NMDA, AMPA, Kainate) receptors and pre- or postsynaptic metabotropic (G-protein coupled) receptors. Glutamate action is terminated by Na+ dependent uptake in the presynaptic neuron as well as glial cells [151].

As shown in adult animals epileptogenic impulses in the vicinity of a brain infarct cause a considerable rise in metabolic activity. In an inadequately perfused section of brain tissue such as the penumbra surrounding an infarct, this can rapidly lead to an imbalance between cell metabolism and blood circulation, resulting in brain damage. In addition, the formation of LTP's (long term potentials), that play an important role in synaptic plasticity and hence in learning processes, may be disturbed by the induced epileptogenic activity [34]. Long-term neurological damage is the inevitable consequence in the children affected.

In global ischemia, such as that caused by cardiac insufficiency, the situation is quite different to that in focal ischemia. As shown in adult animals it is far less clear whether glutamate is directly involved in neuronal cell death [2, 3]. As Hossmannn points out in his 1994 review article, a number of observations argue against any major involvement of glutamate in processes leading to neuronal cell death after global ischemia [100]:

(1) Neither the pattern of glutamate release during ischemia nor the cerebral distribution of glutamate receptors matches the regional manifestation of brain damage after global ischemia.
(2) Glutamate toxicity in cell cultures from vulnerable brain areas was found to be no higher than in cultures from non-vulnerable regions.
(3) In contrast to the effects of in-vitro ischemia, application of glutamate to cell cultures or hippocampal tissue slices caused no prolonged inhibition of protein synthesis.

Since then, the possibility of glutamate's playing a key role in the induction of brain damage either during or directly after global ischemia, even in the immature brain, has been effectively excluded by the following observations: Application of glutamate or glutamate antagonists to hippocampal slices from guinea pig fetuses did not affect postischemic protein biosynthesis, a parameter used as an early marker of neuronal cell death (figure 6) [29]. Furthermore, the glutamate antagonist lubeluzole was found to have no neuroprotective effect on a model of cerebral ischemia in mature sheep fetuses (figure 3 [70]). However, it is possible that later, during the reperfusion phase after cerebral ischemia, glutamate-induced epileptogenic activity does cause brain damage. This possibility will be discussed further on.

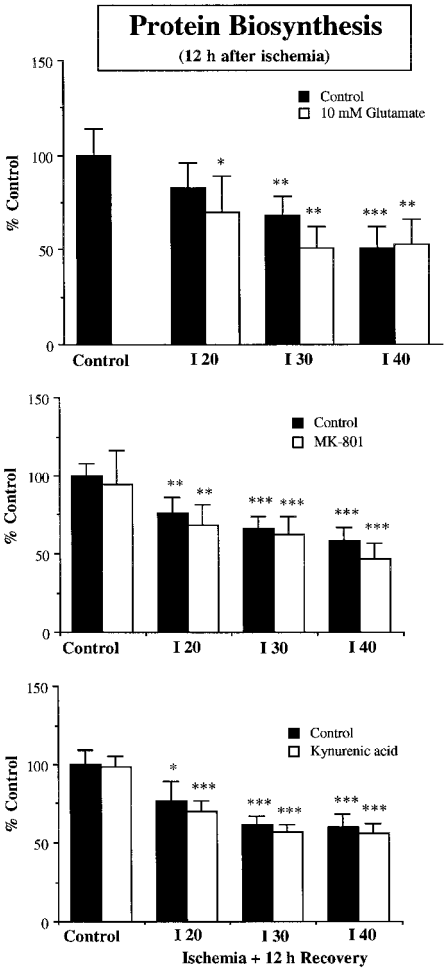

Figure 6. Protein synthesis rate in hippocampal slices from mature fetal guinea pigs 12 h after in vitro ischemia. The ischemic period lasted between 20 and 40 min (I20, I30, I40). Protein synthesis rate was not affected neither by application of glutamate nor by glutamate antagonists (MK-801, Kynurenic acid). Values are given as mean ± SD. Statistical analysis was performed by ANOVA followed by Scheffé's F-test (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (ischemia vs. control)) [29].

7 Protein biosynthesis

As animal experiments show, inhibition of protein synthesis plays a key role in the postischemic processes leading to neuronal cell damage [98]. Protein synthesis is reduced both during ischemia and in the early postischemic phase in vulnerable and non-vulnerable brain areas [108]. At the end of the ischemic period, protein synthesis in non-vulnerable regions recovers to pre-ischemic levels, while in vulnerable regions it remains inhibited [37, 98]. Thus the inhibition of protein synthesis appears to be an early indicator of subsequent neuronal cell death [98]. This observation ties in with the results of experiments demonstrating the neuroprotective effect of hypothermia or barbiturates after cerebral ischemia [201, 205]: Shortly after cerebral ischemia, the usual inhibition of protein synthesis set in, however, the recovery phase in the normally vulnerable areas was now much shorter (figure 7), and was accompanied by far less pronounced neuronal cell damage. Similar findings were reported in connection with developmental variations in the response of the brain to ischemic insults: Protein synthesis in the fetal brain was found to recover much faster from ischemic insults than that in adult brains [24]. The prolonged inhibition of protein synthesis is therefore an early indicator and possibly also one of the causes of neuronal cell damage arising after ischemia [98].

8 Secondary cell damage during reperfusion

In cerebral tissue capable of regeneration after an ischemic insult, energy metabolism can be seen to recover rapidly [24, 98]. A few hours later, however, the energy status is diminished once again in the affected tissue [35, 167]. Simultaneously, a secondary cell edema develops, followed a little later by epileptogenic activity that can be monitored on EEG. These events are quite

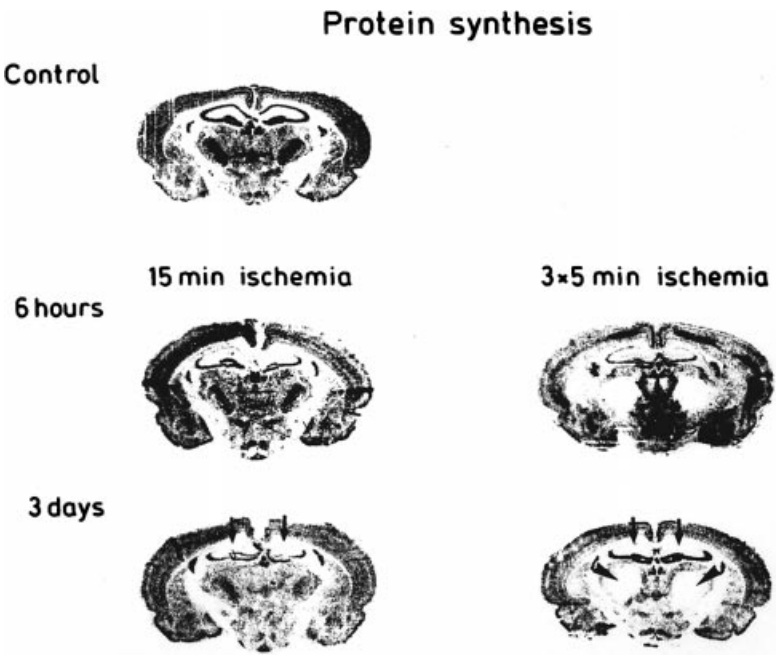

Figure 7. Autoradiographic evaluation of protein synthesis before (control) and at two recirculation times (2 hours and 2 days) after 5 min bilateral carotid artery occlusion in gerbil. Left: untreated animals. Right: treated animals (50 mg/kg pentobarbital intraperitoneal, shortly after ischemia). Note similar reduction of protein synthesis after 2 h of recirculation but recovery in all regions including CA1 sector in the barbiturate-treated animals after 2 days recovery (arrows) [98].

probably brought about or modulated by oxygen radicals, nitric oxide, inflammatory reactions and excitatory amino acids, particularly glutamate.

8.1 Oxygen radicals

During cerebral ischemia, the cut back in oxidative phosphorylation rapidly diminishes reserves of high-energy phosphates. Within a few minutes considerable amounts of adenosine and hypoxanthine accumulate. During reperfusion these metabolic products are metabolised further by xanthine oxidase to produce xanthine and uric acid [129]. Especially, the breakdown of hypoxanthine by xanthine oxidase in the presence of oxygen, produces a flood of superoxide radicals. These are then converted by superoxide dismutase to hydrogen peroxide [64, 65]. By the Haber-Weiss reaction shown below, hydrogen peroxide and tissue iron can then combine to form hydroxyl radicals.

Numerous studies have shown that oxygen radicals play an important role in processes leading to neuronal cell damage [190, review 89]. In adult animals various degrees of neuroprotection against ischemic insults can be achieved through the inhibition of xanthine oxidase or by application of oxygen radical scavengers and iron chelators [13, 33, 44, 88, 117, 127, 136, 165]. Oxygen radicals also appear to be involved in mechanisms underlying neuronal cell death in immature animals. The rate of lipid peroxidation was found to be considerably increased after hypoxia in fetal guinea pigs and newborn lambs [1, 75, 137]. The longer the gestational age, the greater this increase was [137]. Furthermore, marked production of oxygen radicals was observed after hypoxia both in vitro, in cultures of fetal neurones, and in vivo, in neonatal mice [94, 153]. There is also evidence that the infarct volume can be reduced in a model of focal ischemia in neonatal rats by application of allopurinol, an inhibitor of xanthine oxidase and oxygen radical scavengers [157].

8.2 Nitric oxide

During cerebral ischemia, a massive influx of intracellular calcium takes place through various channels, regulated, among other things, by the neurotransmitter glutamate [47, 182]. The rise in intracellular calcium activates NO-synthase [59, 71], which produces NO, citrulline and water from arginine, NADPH and oxygen.

$$\begin{array}{l} \text{Arginine} \\ + \text{NADPH} \\ + H^+ \\ + O_2 \end{array} \xrightarrow{\text{NO-Synthase}} \begin{array}{l} \text{NO} \\ + \text{Citrulline} \\ + NADP^+ \\ + H_2O \end{array}$$

There is also an accumulation of cGMP [14]. Since there is no oxygen available during ischemia, NO cannot be synthesized until the reperfusion phase [14]. Likewise, large numbers of superoxide radicals are produced by xanthine oxidase and via other pathways in the mitochondria during and, to an even greater extent, after ischemia [122]. During reperfusion, NO and superoxide radicals combine to produce peroxynitrite, leading to the formation of more potent radicals. Destruction of the tissue is the inevitable result [14].

Investigations of the action of inhibitors of NO-synthase in models of cerebral ischemia in adult animals have yielded highly variable results [43, 52, 55, 91, 110, 143, 147, 207]. This can be explained by the fact that the neuroprotective effect of NO-synthase blockers after ischemia, that is brought about by a lowering of NO production and consequent reduction of the build-up of potent radicals, is counteracted by a marked vasoconstriction induced by the fall in NO concentration in endothelial cells [53]. Thus Moskowitz and co-workers found markedly smaller infarct loci after occlusion of the A. cerebri media in mice whose expression of the neuronal form of NO-synthase had been blocked than in the wild type of the animal [102]. The same group was also able to protect the brain from ischemic insults by application of selective blockers of neuronal NO-synthase [53].

To date hardly any studies have investigated the importance of nitric oxide in neuronal cell death in neonates or fetuses. After a hypoxic-ischemic insult in neonatal rats, a greater number of neurones was found to contain NO-synthase [96]. The activity of this NO-synthase, however, appeared to be diminished [107]. Furthermore, two peaks of NO production were detected in this animal model: one during hypoxia and the other one during the reoxygenation period. The neuronal and

the inducible form of NO-synthase seems to be differently involved in this process [97]. Some authors succeeded in preventing ischemic lesions in the brains of immature animals through application of NO-blockers [8, 90, 191], while other research teams were unable to achieve this effect or observed, instead, a worsening of the damage [125, 185]. As already mentioned, this discrepancy may have arisen from the different effects of NO-blockers on vascular endothelia and neurones. In our investigations of the effect of blocking NO-synthase we therefore by-passed the cardiovascular system, by carrying out experiments on hippocampal slices [31]. Although postischemic NO-production could be completely blocked with NO-inhibitors, this intervention had no influence on the postischemic inhibition of protein biosynthesis, a parameter used as an early indicator of neuronal cell death (figure 8). Whether or not NO is directly involved in the pathogenesis of neuronal cell death following ischemia in fetuses therefore remains an open question.

8.3 Inflammatory reactions

As various studies have shown, ischemia and subsequent reperfusion can set off an inflammatory reaction in the brain (figure 9) [61, 177]. Expression of a wide variety of cytokines, e. g. IL-1, IL-6, transforming growth factor-β, and fibroblast growth factor, was observed. In rats, mRNA of IL-1 was expressed within 15 min of global cerebral ischemia [135]. Cytokines appear to be formed in activated microglia [72, 132, 141]. They are thought to mediate the migration of inflammatory cells within the reperfused tissue.

Through increased expression of the adhesion molecules P- and E-selectin and ICAM-1 on the endothelial cells and of integrins on leukocytes, granulocytes become attached to the endothelium, migrate through the vessel wall and accumulate in the interstitium [60, 76, 93, 128, 154, 160]. There, after further activation by cytokines, they synthesize oxygen radicals, especially superoxide radicals that proceed to damage neuronal tissue. The role of inflammatory cells in the pathogenesis of secondary cell damage was further elucidated in reperfusion experiments using blood lacking granulocytes, or antibodies to adhesion molecules and trials on transgenic mice [172, 196].

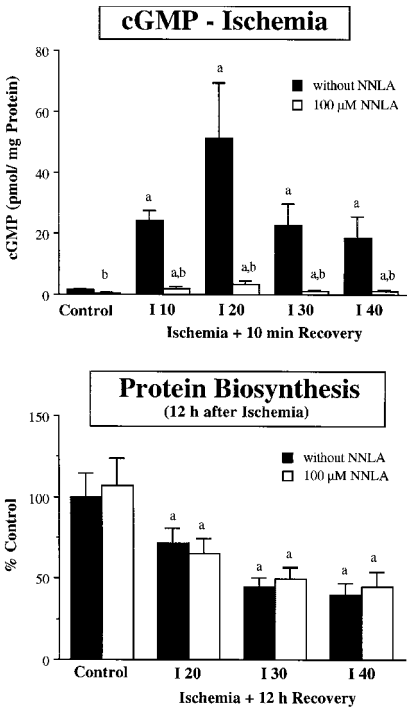

Figure 8 a. cGMP concentrations in hippocampal slices from mature fetal guinea pigs after different durations of in vitro ischemia (10−40 min). A portion of the tissue slices was incubated for 30 min, before, during and 10 min after ischemia, in 100 µM N-nitro-L-arginine (NNLA). After 10 min recovery from 10 to 40 min of ischemia, a marked rise in cGMP levels was observed in tissue slices that had not been incubated in NNLA. Note that application of NNLA blocked the ischemia-induced elevation of cGMP almost completely. **8 b.** Protein synthesis rate in hippocampal slices from mature fetal guinea pigs after different durations of in vitro ischemia (20−40 min) and a recovery period of 12 h. A portion of the tissue slices was incubated in 100 µM N-nitro-L-arginine (NNLA) for 30 min before, during and 12 hours after ischemia. Protein synthesis rate was reducted to 50 % of initial levels after 40 min ischemia. Note that blocking of NO-synthase with NNLA did not improve the postischemic recovery of protein synthesis. The statistical significance of differences between groups was assessed by ANOVA and the Scheffé post-hoc test (a: $P < 0.05$ (ischemia vs. control), b: $P < 0.05$ (NNLA vs. without NNLA)) [31].

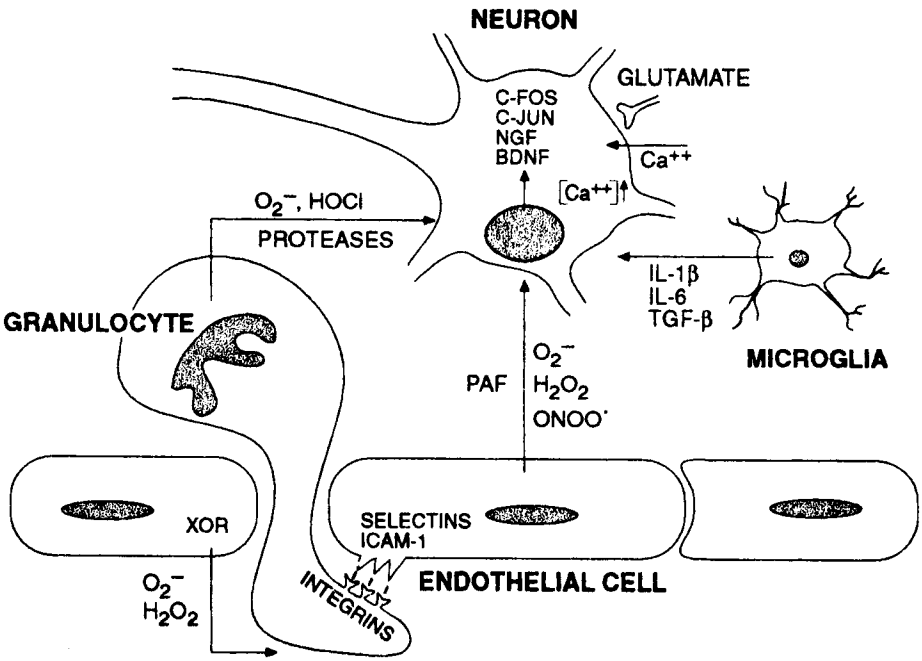

Figure 9. Mechanisms of recirculatory induced brain damage. Ischemia and recirculation are possible inductors of gene expression and formation of oxygen radicals. Endothelium derived oxygen radicals induce expression of adhesion molecules to allow granulocytes crossing the blood brain barrier. The formation of oxygen radicals, glutamate-induced excitotoxicity, and cytokines produced by activated microglia are damaging neuronal cells. NGF, nerve growth factor; BDNF, brain-derived neurotrophic factor; TGF, transforming growth factor; PAF, platelet-aggregating factor; ICAM-1, intercellular adhesion molecule 1; IL, interleukin; $ONOO^-$, Peroxynitrite [61].

Interestingly, there is increasing evidence from recent clinical studies that perinatal brain damage is closely associated with ascending intrauterine infection before or during birth [27, 55, 104, 206,]. However, it remains unclear whether fetal brain damage due to endotoxemia is the result of cerebral hypoperfusion caused by circulatory decentralization or is caused by a direct effect of endotoxins on cerebral tissue. To clarify this point we performed two sets of in-vitro experiments as well as in-vivo experiments.

First, we studied the influence of lipopolysaccharides (LPS) on nitric oxide (NO) production, energy metabolism and protein synthesis after oxygen-glucose deprivation (OGD) in hippocampal slices from fetal guinea pigs. Incubating slices in LPS (4 mg/L) for as long as 12 h did not modulate NO production significantly. Nor did addition of LPS to the incubation medium alter protein synthesis or energy metabolism measured 12 h after OGD [19]. In a second set of experiments we elucidated the effects of LPS on circulatory responses in immature fetal sheep before, during, and after 2 min of intrauterine asphyxia. Within 1 h after i.v. injection of LPS (53 ± μg per kg fetal weight) there was a steep fall in arterial oxygen saturation and pH. Whereas blood flow to the placenta severely decreased, that to the carcass rose (figure 10). Shortly after asphyxia there was an arrest of oxygen delivery to the cerebrum.

LPS-induced effects on fetal circulation, therefore, seem to play a central role in the development of fetal brain damage due to intrauterine infection. A direct toxic effect of LPS on immature brain tissue may not be very likely. However, at present delayed activation of LPS-sensitive pathways that are involved in apoptotic-like cell death, or damage limited to a small subgroup of cells such as oligodendrocyte progenitors cannot be fully excluded.

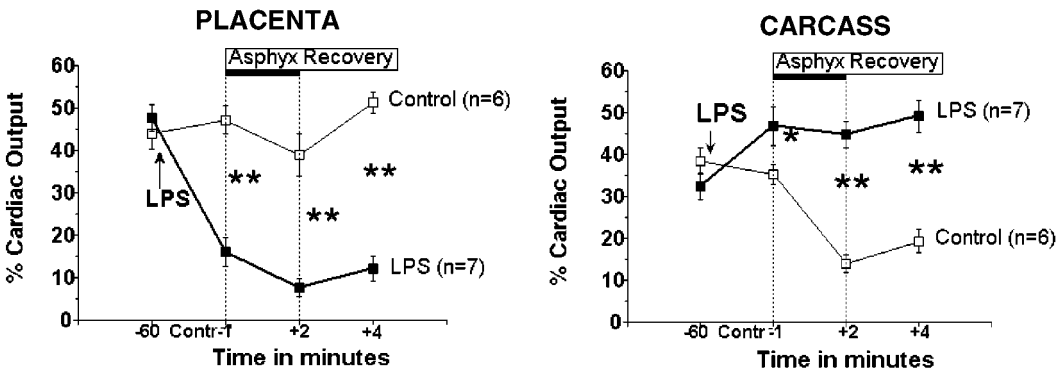

Figure 10. Combined ventricular output directed to the placenta and carcass in control (n = 6) and LPS treated (n = 7) immature fetal sheep before, during and after arrest of uterine blood flow for 2 min. Unlike in control fetuses, there was a significant fall in LPS treated fetuses in the percentage of combined ventricular output directed to the placanta while that directed to the carcass significantly increased. During arrest of uterine blood flow the portion distributed to the carcass remained elevated in fetuses of the study group ($P < 0.001$) [68]. Values are given as means ± SEM. The data were analyzed within and between groups using a two-way ANOVA followed by Games-Howell post-hoc test (* $P < 0.05$, ** $P < 0.01$).

8.4 Glutamate

Williams and co-workers observed epileptiform activity in mature sheep fetuses about 8 hours after 30 min of global cerebral ischemia that reached a peak 10 hours after the ischemic period [203]. They were able to completely inhibit this epileptiform activity by application of the glutamate antagonist MK-801, and show that the resulting brain damage was markedly reduced in the treated animals [187]. This suggests that a secondary wave of glutamate release or an imbalance between excitatory and inhibitory neurotransmitters during reperfusion may induce epileptiform bursts of neuronal activity that can lead to an uncoupling of cell metabolism and blood flow. This would automatically impair pathways of energy metabolism and cause a secondary wave of cell damage [100].

9 Apoptosis and postischemic genome expression

It is still unclear whether secondary cell death after ischemia is necrotic or apoptotic. The latter condition is characterized by a shrinking of the cell, blessing of the cell membrane, condenzation of chromatin and DNA fragmentation induced by a calcium-dependent endonuclease (figure 11) [40]. In DNA electrophoresis this fragmentation can be recognised by a typical DNA ladder [131]. In neuronal cell cultures, apoptosis can be prevented by postischemic inhibition of protein synthesis using cycloheximide, or inhibition of RNA synthesis with actinomycin or through inhibition of endonuclease with aurin tricarboxylic acid. In addition, the amount of apoptotic cell death was reduced by inhibition of caspases in neonatal rats after a hypoxic-ischemic insult [45]. These findings all point towards the existence of a built-in cellular suicide programme [170, 174]. It is also possible that the form of secondary cell death following ischemia is determined by the severity of the primary insult. Thus Dragunow and co-worker were able to demonstrate that delayed cell death in immature rat brains subjected to a 15-min period of hypoxic-ischemia was of an apoptotic nature, while after a 60-min insult the neuronal damage was predominantly necrotic [57]. Other investigators have also reported correlations between the severity of the insult and the extent of apoptotic cell death [116, 133].

As has since been shown in numerous studies, including some on immature animals, cerebral ischemia can induce the expression of a whole series of proto-oncogenes [36, 62, 142]. Proto-oncogenes themselves code for proteins that act as transscription factors and regulate the expres-

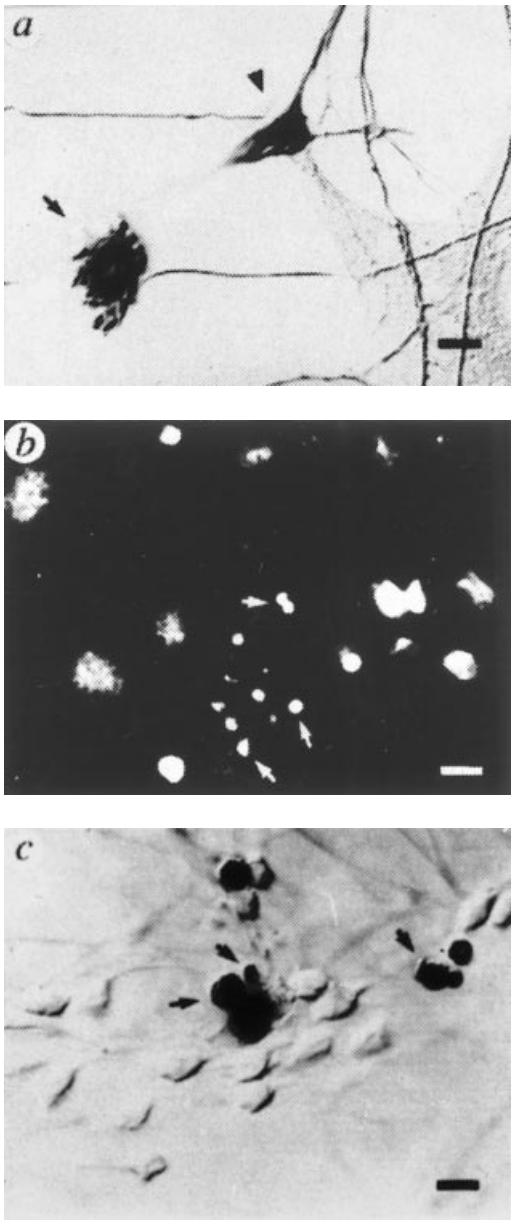

Figure 11. Apoptosis in neuronal cell culture. **a.** Illustration of an intact neuron (arrowhead) and an apoptotic neuron with typical intracytoplasmic vesicles (arrow). **b.** Fluorescence staining of 10 day old apoptotic neurons which shows fragmentation of nuclei and condenzation of chromatin (arrows). **c.** DNA-fragmentation in neurons illustrated by the TUNEL-method [40].

sion of genes modulating cell growth and differentiation. They are also termed 'immediate early genes' since they are expressed within a few minutes of an insult. These include c-fos, c-jun, jun-B, jun-D. The transscriptional activity of proteins of the fos-family is caused by a heterodimer formation with proteins of the jun-family [118]. Fos- and jun-proteins can also form dimers with proteins of the ATF- and CREB families and thereby increase their promotor affinity [85].

As already mentioned, transscription factors control the expression of genes participating in cell growth and differentiation. Depending on the severity of the insult, these factors are therefore capable of initiating processes leading to apoptotic cell death or triggering a recovery programme. Recent research findings have indicated that the proto-oncogenes and cell cycle-dependent proteins such as cyclin D1 [189, 202], and tumor suppressor genes such as p53 are critically involved in this control function.

Depending upon the developmental stage of the injured brain and the extent of cell damage on the one hand, and upon damage-induced p53 expression on the other, neurons may attempt cell cycle entry, a process that will involve a certain amount of DNA repair, or may only attempt transscription-coupled DNA repair. The cell death decision may result from the impossibility to proceed with both processes. Indeed, it has recently been shown in vitro that the p53 transscription factor, besides its role in halting replication while favoring repair, attenuates Bcl-2 expression, and is a direct transscriptional activator of the Bax gene, whose product is shown to induce apoptosis [5, 138, 139, 171].

10 Therapeutic strategies

Despite the critical clinical and socio-economic consequences of perinatal brain damage, no effective therapeutic strategies have yet been developed to prevent its causes. However, as already mentioned, some promising possibilities have been revealed through animal experiments that could be developed and tested in clinical studies. Since a significant proportion of neuronal cell damage is brought about by pathophysiological

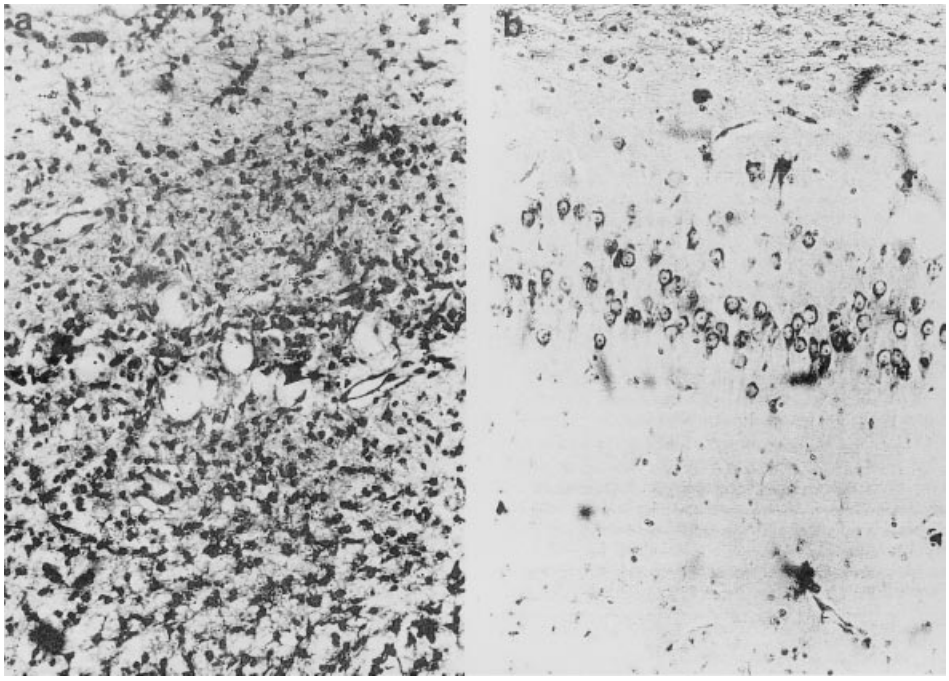

Figure 12 a, b. Section of the parasagittal cortex in (370 fold magnification) in term fetal sheep 5 days after 30 min of cerebral ischemia followed by normothermia (a) or mild hypothermia (b). **a.** Complete neuronal necrosis (normothermic group). **b.** Minor degree of neuronal cell damage (hypothermic group) [80].

processes that first begin several hours or even days after an ischemic insult (see secondary cell damage and apoptosis), the setting up of a therapeutic window would be feasible. In the following passages, current therapeutic concepts will be described by which neuroprotection has been achieved in animal models.

10.1 Hypothermia

The induction of mild hypothermia has raised interesting possibilities for neuroprotection from cerebral ischemia [124]. Various publications dating back to the 1950s, have described the therapeutic benefits of hypothermia in brains subjected to a wide variety of insults including brain trauma [164, 181], cerebral hemorrhage [101], cardiac arrest [17], carbon monoxide poisoning [51], neonatal asphyxia [200] and seizures [39]. Based on these findings, routine induction of hypothermia was introduced early on in heart and brain surgery to protect the brain in the event of iatrogenic intraoperative cardiac arrest [38, 58, 115, 121, 145]. Over the last few years, induction of mild hypothermia has been examined once again as a means of protecting the brain from ischemically induced damage. Experimental studies on adult animals have shown that lowering of the brain temperature by 3−4 °C during global cerebral ischemia reduces neuronal cell damage dramatically [41, 49, 77, 199, 201]. Furthermore, the treated animals were found to perform better than controls in subsequent learning and behavioral tests [77].

The author's research team were also able to demonstrate a neuroprotective effect of mild hypothermia in fetal brain tissue subjected to ischemic insults. They found that the postischemic recovery of protein synthesis and energy metabolism in hippocampal slices from mature guinea pig fetuses was considerably improved, in comparison to controls, by induction of mild hypo-

thermia [25, 30, 69 a]. In a recently published study, Gunn and co-workers described the effects of moderate hypothermia in sheep fetuses subjected to severe global cerebral ischemia in utero [80]. Hypothermia was initiated during the reperfusion phase, 90 min after induction of 30 mins of ischemia, in a 4-vessel occlusion model, and maintained for 72 hours. By this method it was possible to reduce the extent of neuronal cell damage in areas of the cortex cerebri by up to 60 % (figure 12) [80]. Even if hypothermia was started not before several hours after ischemia, neuroprotection could be observed in various animal models [69 a, 82]. Based on these results, many authors now consider the induction of hypothermia during and particularly after a hypoxic-ischemic insult to be an effective therapeutic strategy [42, 80]. In fact, Gunn and co-workers demonstrated in a recent clinical study that selective head cooling in newborn infants after perinatal asphyxia is a safe and convenient method of quickly reducing brain temperature [81].

10.2 Pharmacological intervention

Now that the pathophysiological mechanisms underlying neuronal cell damage are better understood, diverse possibilities present themselves for pharmacological intervention. Interest is currently focused on the administration of oxygen radical scavengers, NO inhibitors, glutamate antagonists, calcium antagonists, growth factors and anticytokines. Table III presents all the potential neuroprotective substances currently under investigation [modified according to 194].

10.3 Magnesium

The last interesting therapeutic approach to be discussed emerged from a retrospective analysis carried out by Nelson and Grether. Recently, in a population of 155,636 infants, these authors showed that ante-partum application of magnesium considerably lowered the incidence of cerebral palsy in newborns weighing less than 1500 g [146]. The incidence of moderate to severe cerebral palsy was 4.8 % in this group. 75 matched pairs were compared with the 42 children with cerebral palsy. In the control group 36 % of the children had been treated with magnesium, whereas, in the group with cerebral palsy only 7 % had been treated. This difference was statistically highly significant. Almost identical results were recently obtained in a retrospective study carried out by Schendel and co-workers [179].

The neuroprotective effect of magnesium has been attributed to a variety of effects on pathophysiological mechanisms during and after cerebral ischemia, i. e. vasodilation, inhibition of the NMDA-receptor, anti-convulsive properties. Furthermore, magnesium also seems to block the activation of NO-synthase after cerebral ischemia [69]. On the strength of these results several clinical studies have been conducted to test the effect of magnesium on the incidence of cerebral palsy in preterm fetuses.

11 Conclusion

Perinatal brain damage in the mature fetus is usually brought about by severe intrauterine asphyxia following an acute reduction of the uterine or umbilical circulation. Owing to the acute reduction in oxygen supply, oxidative phosphorylation in the brain comes to a standstill. The Na^+/K^+ pump at the cell membrane has no more energy to maintain the ionic gradients. In the absence of a membrane potential, large amounts of calcium ions flow through the voltage-dependent ion channel, down an extreme extra-/intracellular concentration gradient, into the cell. Additionally to the influx of calcium ions into the cells via voltage-dependent calcium channels, calcium also enters the cells through glutamate-regulated ion channels. Current research suggests that the excessive increase in levels of intracellular calcium, so-called calcium overload, leads to cell damage through the activation of proteases, lipases and endonucleases. A second wave of neuronal cell damage occurs during the reperfusion phase. This cell damage is thought to be caused by the postischemic inhibition of protein synthesis, release of oxygen radicals, synthesis of nitric oxide, inflammatory reactions and an imbalance between the excitatory and inhibitory neurotransmitter systems. Part of the secondary neuronal cell damage may be caused by induction of a kind of cellular suicide

Table III. Pharmacological intervention on hypoxic/ischemic brain damage in various models of hypoxia/ischemia [335]

Treatment class	Treatment details	Age/species	Hypoxic/ ischemic insult	Time of treatment with respect to insult	Neuro-protection/ pathology	Refer-ence
VSCC's anatgonists	Flunarizine (30 mg/kg)	7 days/rat	UCO + 2 h 8 %O2	pre	partial	[184]
	Flunarizine (30 mg/kg)	7 days/rat	UCO + 3 h 8 %O2	pre	partial	[48]
	Flunarizine (30 mg/kg)	21 days/rat	UCO + 2 h 8 %O2	pre	partial	[78]
	Flunarizine (9 mg/kg)	fetal sheep	30 min BCO (+VOAO)	pre	partial	[79]
	Flunarizine (1 mg/kg)	fetal sheep	30 min BCO (+VOAO)	pre	partial	[32]
	Nimodipine (70 µg/kg or 0.5 mg/kg)	7 days/rat	UCO + 3 h 8 %O2	pre	no effect	[48]
	Nimodipine (0.5 mg/kg)	0−3 days/pig	30 min BCO + hypotonia & 15 min 6 % O2	post	no effect	[114]
NMDA anatgonist	MK-801 (10 mg/kg)	7 days/rat	BCO + 1 h 8 %O2	pre	total	[95]
	MK-801 (10 mg/kg)	7 days/rat	BCO + 1 h 8 %O2	post	partial	[95]
	MK-801 (1 mg/kg)	7 days/rat	UCO + 3 h 8 %O2	pre, intra	partial	[123]
	MK-801 (10 mg/kg)	7 days/rat	UCO + 2 h 8 %O2	pre, intra	partial	[63]
	MK-801 (0.3 bzw. 0.5 mg/kg)	7 days/rat	UCO + 1.5 h 7.6 %O2	post (0 h)	partial	[84]
	MK-801 (0.75 mg/kg)	7 days/rat	UCO + 1.5 h 7.6 %O2	post (0 h)	no effect	[84]
	MK-801 (3 mg/kg)	0−3 days/pig	30 min BCO + hypotonia & 15 min 6 % O2	post (0 h)	no effect	[113]
	MK-801 (0.3 mg/kg +	Schaffet	30 min global ischemia	post (6−36 h)	partial	[187]
	Felbamate (300 mg/kg)	7 days/rat	BCO + 1 h 6.5 %O2	post	partial	[198]
AMPA antagonist	NBQX (20+20 mg/kg)	7 days/rat	UCO + 1.5 h 7.6 % O2	post (0 + 1 h)	partial	[84]
Glutamate release inhibitor	BW1003C87 (10 mg/kg)	7 days/rat	UCO + 1.5 h 7.7 % O2	pre	partial	[73]
Nonspecific glutamate antagonist	Kynurenic acid (300 mg/kg)	7 days/rat	UCO + 2 h 7.7 % O2	post	partial	[4]
	Kynurenic acid (200−300 mg/kg)	7 days/rat	UCo + 1.5 h 8 % O2	pre (1 h)	partial	[149]
Antioxidant enzymes	PEG-SOD + PEG-Catalase (10.000 U/kg)	0−3 days/pig	30 min BCO + hypotonia u. 15 min 6 % O2	post	no effect	[114]
Iron chelator	Deferoxamine (100 mg/kg)	7 days/rat	UCO + 2.25 h 8 % O2	post (5 min)	partial	[159]
Free radical scavengers	Allopurinol (135 mg/kg)	7 days/rat	UCO + 3 h 8 % O2	pre or post (15 min)	partial	[157,158]
	U74006F (7.5 mg/kg)	7 days/rat	UCO + 2 h 7.7 % O2	post or pre & post pre & post	partial	[9]
	U74689F (10 mg/kg)	7 days/rat	UCO + 3 h 8 % O2	pre & post	no effect	[48]
NO synthase inhibitors	Nitro-L-Arginine	7 days/rat	UCO + 2.5 h 8 % O2	pre & post	partial/ no effect	[90]
	Nitro-L-Arginine (50−100 mg/kg)	7 days/rat	UCO + 8 % O2	pre	partial	[191]
Glucocorticoids	Dexamethasone (0.01−0.5 mg/kg/Tag)	7 days/rat	UCO + 3 h 8 % O2	pre	total	[11]

Glucocorticoids	Dexamethasone (0.1 mg/kg)	7 days/rat	UCO + 3 h 8 % O2	pre	total/no effect	[11]
					partial	[48]
	Methylprednisolone (0.7 mg/kg)	7 days/rat	UCO + 3 h 8 % O2	pre (24 h)	partial	[193]
	Corticosterone (40 mg/kg)	7 days/rat	UCO + 2 h 8 % O2	pre (24 + 5 h)	partial	[192]
	Dexamethasone (0.1 mg/kg)	14 days/rat	UCO + 1 h 8 % O2	pre (24 + 5 h)	partial	[193]
	Dexamethasone (0.1 mg/kg)	1 month/rat	UCO + 30 min 8 % O2	pre (24 + 5 h)	no effect	[193]
Antiinflammatory	Interleukin-1-receptor-antagonist (100 mg/kg)	7 days/rat	UCO + 2 h 7.5 % O2	pre & post	partial	[126]
	Osteogenic protein-1 (50 µg)	12 days/rat	BCO + 20 min 8 % O2	pre	partial	[168]
	Antineutrophil serum	7 days/rat	UCO + 2.25 h 8 % O2	pre	partial	[161]
Growth factor	bFGF (100 µg/kg)	7 days/rat	UCO + 1.5 h 8 % O2	pre (30 min)	partial	[150]
Gangliosides	GM1 (50 mg/kg/Tag)	7 days/rat	2 h 7 % O2	pre & post	partial	[83]
	GM1 (30 mg/kg/Tag)	fetal sheep	30 min ischemia	pre	partial	[188]
Anticonvulsants	Zonisamide (75 mg/kg)	7 days/rat	UCO + 2.5 h 8 % O2	pre	partial	[86]
	Phenytoin (50 mg/kg)	7 days/rat	UCO + 2.5 h 8 % O2	pre	partial	[87]
Inhibition of caspases	boc-aspartyl-fluoromethyl-ketone	7 days/rat	UCO + 2 h 7.5 % O2	post	partial	[45]

UCO: unilateral occlusion of carotid arteries, BCO: bilateral occlusion of carotid arteries, VOAO: occlusion of the vertebro-occipital anastomoses, BP: arterial blood pressure, bFGF: basic fibroblast growth factor

programme known as apoptosis. Interestingly, there is increasing evidence from recent clinical studies that perinatal brain damage is closely associated with ascending intrauterine infection before or during birth. However, a major part of this damage is likely to be of hypoxic-ischemic nature due to LPS-induced effects on fetal cerebral circulation. Knowledge of these pathophysiological mechanisms has enabled scientists to develop new therapeutic strategies with successful results in animal experiments. Among these intravenous administration of magnesium and postischemic induction of cerebral hypothermia may be of clinical relevance during the next years.

Abstract

Perinatal brain damage in the mature fetus is usually brought about by severe intrauterine asphyxia following an acute reduction of the uterine or umbilical circulation. The areas most heavily affected are the parasagittal region of the cerebral cortex and the basal ganglia. The fetus reacts to a severe lack of oxygen with activation of the sympathetic-adrenergic nervous system and a redistribution of cardiac output in favor of the central organs (brain, heart and adrenals). If the asphyxic insult persists, the fetus is unable to maintain circulatory centralization, and the cardiac output and extent of cerebral perfusion fall. Owing to the acute reduction in oxygen supply, oxidative phosphorylation in the brain comes to a standstill. The Na^+/K^+ pump at the cell membrane has no more energy to maintain the ionic gradients. In the absence of a membrane potential, large amounts of calcium ions flow through the voltage-dependent ion channels, down an extreme extra-/intracellular concentration gradient, into the cell. Current research suggests that the excessive increase in levels of intracellular calcium, so-called calcium overload, leads to cell damage through the activation of proteases, lipases and endonucleases. During ischemia, besides the influx of calcium ions into the cells via voltage-dependent calcium channels, more calcium enters the cells through glutamate-regulated ion channels. Glutamate, an excitatory neurotransmitter, is released from presynaptic vesicles during ischemia following anoxic cell depolarization. The acute lack of cellular energy arising during ischemia induces almost complete inhibition of cerebral protein biosynthesis. Once the ischemic period is over, protein biosynthesis returns to preischemic levels in non-vulnerable regions of the brain, while in more vulnerable areas it remains inhibited. The inhibition of protein synthesis, therefore, appears to be an early indicator of subsequent neuronal cell death. A second wave of

neuronal cell damage occurs during the reperfusion phase. This cell damage is thought to be caused by the postischemic release of oxygen radicals, synthesis of nitric oxide (NO), inflammatory reactions and an imbalance between the excitatory and inhibitory neurotransmitter systems. Part of the secondary neuronal cell damage may be caused by induction of a kind of cellular suicide programme known as apoptosis. Interestingly, there is increasing evidence from recent clinical studies that perinatal brain damage is closely associated with ascending intrauterine infection before or during birth. However, a major part of this damage is likely to be of hypoxic-ischemic nature due to LPS-induced effects on fetal cerebral circulation. Knowledge of these pathophysiological mechanisms has enabled scientists to develop new therapeutic strategies with successful results in animal experiments. The potential of such therapies is discussed here, particularly the promising effects of intravenous administration of magnesium or postischemic induction of cerebral hypothermia.

Keywords: Fetal brain damage, asphyxia, hypoxia-ischemia, glutamate, endotoxin.

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