# Biochemical Basis of Hypoxic-Ischemic Encephalopathy

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**Abstract**

Hypoxic-ischemic encephalopathy (HIE), the most common cause of neurologic disease during the perinatal period, is associated with a high mortality and morbidity rate and also has long-term consequences like cerebral palsy, mental retardation, and seizures. Perinatal HIE is caused by processes that alter the cerebral blood flow (CBF) in the fetus and newborn compromising the supply of oxygen to the brain. They may develop antepartum (20%), intrapartum (30%), antepartum and intrapartum (35%), or postpartum (10%). Acute or long-term consequences of HIE are related either to necrosis or to apoptosis of neuronal cells. Cell necrosis will lead to generalized disruption of internal homeostasis and eventually to the lysis of the cells, which give rise to an inflammatory response with the release of oxygen free radicals and activation of the microglial cells. Apoptosis is programmed cell death, not associated with the lysis of the plasma membrane and inflammation, which can be triggered by hypoxia. It is crucial to restore any failures in the respiratory and circulatory systems, in order to prevent neuronal cell death. However, neonatologists should also be aware of the hazards of medically induced hyperoxia (high $FIO_2$) because this condition may increase the production of oxygen free radicals thus worsening the neuronal insult. Elucidating basic cellular mechanisms in response to hypoxia of the developing brain will enable the development of novel strategies for preventing or attenuating the deleterious effects of hypoxia in the human newborn.

## 125.1 Salient Points

- Acute- or long-term consequences of hypoxic-ischemic encephalopathy are related to necrosis or apoptosis of neuronal cells.
- During hypoxia, the fall of ATP levels includes cell membrane depolarization and disruption of voltage-dependent ion channels allowing excessive amounts of $Ca^{++}$ to enter the cytosol and initiating the release of glutamate with consequent activation of N-methyl-D-aspartate (NMDA).
- Excessive activation of NMDA and non-NMDA receptors may initiate a number of biochemical events that lead to free radical generation and cell death. In addition to $Ca^{++}$ mediation, there are other potential mechanisms for free radical generation during hypoxia.
- Hypoxic brain injury is associated with the formation of nitric oxide, which has been reported to cause neuronal damage though various mechanisms.
- The ratio of proapoptotic protein Bax to antiapoptotic protein Bcl-2 increases in all compartments of the cell during hypoxia. This may lead to activation of a hypoxia induced cascade resulting in neuronal death.
- Neuroprotective treatments that may be effective in humans include the administration of magnesium sulfate, allopurinol, opioids, and hypothermia.

## 125.2 Introduction

Perinatal hypoxia-ischemia is the most common cause of neurologic disease during the neonatal period. Hypoxic-ischemic encephalopathy (HIE) is associated with a high mortality and morbidity rate, also cerebral palsy, mental retardation, and seizures (Volpe 2001). The incidence of perinatal asphyxia is about 1.0–1.5% in most centers and is usually related to gestational age and birth weight. It occurs in 9.0% of infants less than 36 weeks of gestation and in 0.5% of infants more than 36 weeks of gestation (Legido 1994; Hill and Volpe 1999). The etiology of perinatal HIE includes those circumstances that can affect the cerebral blood flow in the fetus and newborn compromising the supply of oxygen to the brain. They may develop antepartum (20%), intrapartum (30%), antepartum and intrapartum (35%), or postpartum (10%) (Raichle 1983).

HIE develops in the setting of perinatal asphyxia, which is a multiorgan system disease (Legido 1994). Assessment and management of these complications is an integral part of the treatment of perinatal asphyxia/HIE (Legido 1994). The present chapter primarily focuses on cellular and molecular mechanisms of the hypoxic neuronal injury in the newborn brain.

A large amount of information has been collected on the fetal cardiovascular and respiratory response to oxygen limitations, giving rise to a better understanding and management of neonatal deterioration induced by hypoxia. Besides these physiologic studies, cellular and biochemical mechanisms that result in brain cell death are being increasingly explored, particularly in the adult with respect to focal (stroke) and global (cardiac arrest) hypoxia-ischemia (Raichle 1983). These studies have shown that complex and interrelated biochemical alterations are triggered during hypoxia-ischemia in mature subjects that ultimately result in neuronal death. Studies are in progress to investigate mechanisms of hypoxic brain injury in the fetus and newborn brain (Legido et al. 2001). To focus on cellular and molecular mechanisms of hypoxic injury in the developing brain, it is important to recognize the factors that may determine the susceptibility of the developing brain to neonatal and perinatal hypoxia.

The determinants of the susceptibility of the developing brain to hypoxia include the lipid composition of the brain cell membrane, the rate of lipid peroxidation, the presence of antioxidant defenses, the development and modulation of the excitatory neurotransmitter receptors such as the *N*-methyl-D-aspartate (NMDA) receptor, and the intracellular $Ca^{++}$ influx mechanisms. In addition to the developmental status of these cellular components, the response of these potential mechanisms to hypoxia determines the fate of the hypoxic brain cell in the developing brain in the fetus and the newborn. Elucidating basic cellular mechanisms in response to hypoxia of the developing brain will enable the development of novel strategies for preventing or attenuating the deleterious effects of hypoxia in the human newborn. Several excellent reviews on different aspects of hypoxic-ischemic cell injury in the developing brain have been published recently (Delivoria-Papadopoulos and Mishra 1998; Fritz and Delivoria-Papadopoulos 2006; Davidson et al. 2015).

## 125.3 Steps of Posthypoxic Brain Injury

Acute or long-term consequences of hypoxic-ischemic encephalopathy are related either to necrosis or to apoptosis of neuronal cells. Necrosis is characterized by passive cell swelling; rapid energy loss; generalized disruption of internal homeostasis leading to eventual lysis of the nucleus, organelles, and plasma cell membranes; and the release of intracellular components that induce a local inflammatory response, resulting in edema and injury to the neighboring cells. This inflammatory response results in the expression of the cytokines interleukin-1-beta (IL-beta) and tumor necrosis factor-alpha (TNF-alpha) stimulating oxygen free radical release from neutrophils, activating microglial cells.

Necrosis is only one of the mechanisms of cell death following hypoxia or ischemia to the brain. Programmed cell death, or apoptosis, also appears to contribute to cell death following hypoxia-

ischemia, especially cell death that occurs days to weeks following the insult (Linnik et al. 1993; Ferrer et al. 1994). Apoptosis is an active process that requires the activation of a genetic program and specific endonucleolytic digestion of nuclear DNA. In contrast to necrosis, programmed cell death is characterized by cell shrinkage, coarse chromatin aggregation with extensive nuclear DNA fragmentation, nuclear pyknosis, and extrusion of membrane-bound cytoplasmic fragments or apoptotic bodies, but is not associated with the lysis of the plasma membrane (Wylie et al. 1980; Columbano 1995).

Studies in cell culture models have demonstrated that hypoxia can trigger programmed cell death (Rosenbaum et al. 1994). Programmed cell death as assessed by the cleavage of genomic DNA has also been shown to occur in the brain following focal (Linnik et al. 1993; Dragunow et al. 1994; Gillardon et al. 1996) and global ischemia (Ferrer et al. 1994; Kitada et al. 1996).

The mechanism by which hypoxia causes DNA fragmentation has been extensively studied but is not well understood.

### 125.3.1 Energy Breakdown

Under normal conditions, the oxidative phosphorylation and the synthesis of high-energy phosphates like adenosine 5-triphosphate or ATP need adequate oxygen supply. Under anaerobic conditions, the metabolic cost of ATP production is critically increased leading to a breakdown of energy balance depleting the brain cells of high-energy compounds, necessary for energy-dependent metabolic processes in neurons and glial cells.

### 125.3.2 Excitotoxic Mechanisms

The fall of ATP levels includes a cell membrane depolarization and a disruption of voltage-dependent ion channels allowing excessive amounts of $Ca^{++}$ to enter the cytosol, initiating the release of glutamate consequently activating *N*-methyl-D-aspartate (NMDA) receptors. The increased expression/activation of NMDA receptors further enhances cellular calcium influx. This whole mechanism is further accelerated by the dysfunction of the energy-dependent reuptake of glutamate both in neurons and in astrocytes in a vicious circle manner.

## 125.4 NMDA Receptors

Glutamate is the major excitatory amino acid neurotransmitter that contributes to a number of developmental processes such as synaptogenesis, synaptic plasticity, long-term potentiation (LTP), learning and memory, as well as neurodegeneration and hypoxia-induced injury (Choi 1990; Rothman and Olney 1986). The physiological and pathological effects of glutamate in the central nervous system (CNS) are mediated through its interaction with specific cell membrane receptors, of which the *N*-methyl-D-aspartate (NMDA), kainate, and AMPA subtypes are the best characterized (Monaghan et al. 1989). The use of specific antagonists (Monaghan et al. 1989; Bashir et al. 1991; Tacconi et al. 1993; Hoffman et al. 1994a) of the NMDA receptor supports the role of receptor activation in long-term potentiation and hypoxic-ischemic cerebral injury. The NMDA-type glutamate receptor is a predominant mediator of excitotoxicity in the immature as compared to the adult brain due to overexpression of the receptor in the developing immature brain (Johnston 1995). Within the developmental period, however, the extent of NMDA receptor-mediated processes such as LTP and hypoxia-induced excitotoxicity may depend on the ontogeny of the NMDA receptor sites and subunits leading to altered function of the ion-channel complex. In addition, the function of the receptor may be modified by intracellular mechanisms such as phosphorylation/dephosphorylation, nitration, and pathways of free radical generation.

### 125.4.1 Structure and Function of the NMDA Receptor

The activity of the NMDA receptor ion-channel complex is regulated by a number of pharmacologically distinct binding sites. The NMDA

receptor possesses a neurotransmitter binding site, or recognition site, which binds glutamate or NMDA, a coactivator site which binds glycine, a channel site that binds MK-801 in its open state, a voltage-dependent $Mg^{++}$ binding site, a polyamine site that binds spermine and spermidine, an ifenprodil site, and an inhibitory divalent cation site that binds $Zn^{++}$ (Monaghan et al. 1989). The activity of the receptor ion channel can also be modified by redox agents (Aizenman et al. 1989; Lipton 1999). Ligand binding studies indicate that there are two distinct binding sites or states associated with the glutamate recognition site, one that preferentially binds agonists and one that preferentially binds antagonists (Monaghan et al. 1988).

The NMDA receptor is associated with a cation-selective ion channel that gates $Na^{+}$, $K^{+}$, and $Ca^{++}$ ions, and, in the resting state, when blocked by Mg++ in a voltage-dependent manner (Nowak et al. 1984; Mayer et al. 1984), the blockade of the ion-channel complex by glutamate or NMDA is allowed, and the agonist-dependent $Ca^{++}$ influx occurs. The influx of $Ca^{++}$ ions is thought to initiate biochemical processes responsible for both NMDA receptor-induced plasticity in the developing brain and NMDA receptor-mediated excitotoxic cell death (Rothman and Olney 1986; Collingridge 1987; Tang et al. 1999).

Each of the regulatory sites of the NMDA receptor ion-channel complex is modified during brain development and may alter the site-dependent influx of NMDA receptor-mediated $Ca^{++}$ and site-specific responses of the receptor during development and during hypoxia.

### 125.4.2 Mechanism of NMDA Receptor Modification During Hypoxia

Brain tissue hypoxia modifies the NMDA receptor recognition, coactivator, and the ion-channel sites. A decrease in the apparent number of NMDA receptors and an increase in receptor affinity for MK-801 were observed in hypoxic fetal guinea pig and newborn piglet brain (Mishra and Delivoria-Papadopoulos 1992; Hoffman et al. 1994b). In these same studies, glutamate and glycine-dependent activation of the NMDA receptor was decreased, and spermine-dependent and basal-state receptor activation was increased during hypoxia. In brains of hypoxic newborn piglets, it was noted that hypoxia modified the recognition, coactivator, and modulatory sites of the NMDA receptor ion-channel complex, probably through NO-mediated nitration (Hoffman et al. 1994b; Fritz et al. 1996). Several lines of evidence support this conclusion.

First, in neurons of the central nervous system, neuronal nitric oxide synthase (nNOS) is colocalized with the NMDA receptor (Bhat et al. 1997; Aoki et al. 1997), thereby favoring nitration of the receptor. Second, nNOS activity is decreased by phosphorylation and increased by dephosphorylation (Bredt et al. 1992; Dawson et al. 1993), a condition that may be achieved during hypoxia.

Furthermore, dephosphorylation of the receptor will make tyrosine sites available for nitration by peroxynitrite, which is produced by NO and superoxide radicals, both of which are produced during hypoxia. Peroxynitrite-dependent nitration inhibits phosphorylation of proteins (Gow et al. 1996), indicating possible steric hindrance between the phospho- and nitro groups on the same tyrosine residue. Thus, dephosphorylation during hypoxia may facilitate peroxynitrite-mediated nitration on the 3-position of tyrosine. In view of these considerations, a critical role of the nitric oxide synthase (NOS) pathway in NO-mediated mechanism of hypoxia-induced modification of the NMDA receptor in newborn brain is strongly suggested.

In neurons of the central nervous system, nNOS is colocalized with NMDA receptors (Bhat et al. 1997; Aoki et al. 1997). In addition, neuronal NOS is activated by $Ca^{++}$ influx through the NMDA receptor ion channel; however, nNOS is not efficiently stimulated by activation of non-NMDA receptors that also induce $Ca^{++}$ influx (Kiedrowski et al. 1992). In synaptic plasma membranes, the nNOS immunoreactivity is associated with the NMDA receptor (Aoki et al. 1993). The synaptic localization of nNOS in the brain may be mediated by the postsynaptic density protein, PSD-95. Recently, it was demonstrated that

nNOS, PSD-95, and NMDA receptor subunit NR2B from the brain coimmunoprecipitate and that the PSD-95 is sufficient to assemble a tight ternary complex with nNOS and the NR2B subunit of the NMDA receptor (Christopherson et al. 1999).

In summary, results of these studies indicate that NO production in the brain is preferentially activated by $Ca^{++}$ influx through the NMDA receptor ion channel and that there is a specific structural and functional link between the NMDA receptor and nNOS.

## 125.5 Free Radicals

Free radicals are molecular species with unpaired electrons in the outer orbit with a strong tendency to initiate chain reactions that result in membrane peroxidation, protein oxidation, nucleic acid oxidation, and cell damage. Normally, more than 80% of the oxygen consumed by the cell is completely reduced by cytochrome oxidase to water without production of oxygen free radicals. The remaining 10–20% undergoes other oxidation reduction reactions in the cytoplasm and mitochondria that produce a superoxide anion radical.

### 125.5.1 Free Radical Generation in Cerebral Cortex of Newborn Piglets

The production of free radicals during hypoxia was documented by measuring the signal of spin adducts with electron spin resonance spectroscopy. Newborn piglets of 3–5 days were assigned to either normoxia ($PaO_2$ – 120 mmHg) or hypoxia ($PaO_2$ $<$20 mmHg) for 1 h. Cortical samples were obtained by biopsy from anesthetized, ventilated piglets.

The data provided direct evidence of increased free radical generation during hypoxia in the newborn model. On the basis of the characteristics of the spin adduct signal, the free radical species present in the hypoxic tissue was identified to be an alkoxyl radical.

These studies demonstrate increased free radical generation during hypoxia in the cerebral cortex of the fetus and the newborn, and intervening with the inhibitors of pathways of free radical generation reduced the hypoxia-induced production of free radical species. Alkoxyl radical appears to be the predominant free radical species identified during hypoxia, indicating that free radical-mediated lipid peroxidation is an ongoing event during cerebral hypoxia, a mechanism of hypoxic neuronal injury.

### 125.5.2 Mechanisms of Free Radical Generation During Hypoxia

There are a number of potential mechanisms of free radical generation under hypoxic conditions. During hypoxia, the increased accumulation intracellular $Ca^{++}$ due to excessive activation of NMDA (Zanelli 1999) and non-NMDA receptors is crucial in hypoxia-induced excitotoxicity. Increased intracellular $Ca^{++}$ can initiate a number of biochemical events that could lead to free radical generation and cell death such as (1) activation of phospholipase A2 leading to increased generation of oxygen free radicals from cyclooxygenase and lipoxygenase pathways; (2) activation of NOS, leading to peroxynitrite formation and generation of free radicals; (3) activation of proteases, leading to conversion of xanthine dehydrogenase to xanthine oxidase and resulting in increased free radical generation; (4) activation of phospholipase $C_1$ leading to IP3 formation and resulting in the release of $Ca^{++}$ from intracellular stores; and (5) free radical generation further triggering the release of additional excitatory amino acid neurotransmitters as well as influencing the activation of the NMDA receptor ion-channel activity through the redox site.

In addition to $Ca^{++}$ mediation, there are other potential mechanisms of free radical generation during hypoxia such as (1) reduction of electron transport chain components including ubiquinone (a component that undergoes autooxidation to produce free radicals), (2) increased release of ferritin under the conditions of decreased cellular high-energy compounds, and (3) increased degradation of ATP during hypoxia, increasing the substrate for the xanthine oxidase reaction and leading to increased free radical generation.

### 125.5.3 Nitric Oxide Free Radicals and Neuronal Injury

The role of nitric oxide (NO) in neuronal injury, both in vitro and in vivo, has been controversial (Dawson 1994a, b). This controversy may be due to the use of nonspecific NOS inhibitors. Three major isoforms of NOS have been identified: constitutive neuronal, constitutive endothelial, and inducible macrophage isoforms. Following ischemia, NO produced from neuronal NOS has toxic effects, but NO produced from endothelial NOS has protective effects in the brain (Huang 1994).

Hypoxic brain injury is associated with the formation of NO, a gaseous free radical (Beckman 1991; Cazevielle 1993). Although under normal conditions NO physiologically mediates cerebral vasodilatation (Faraci 1991), recent studies suggest that NO may react with superoxide anion to form peroxynitrite and cause neurotoxicity (Beckman 1990; Dawson 1991; Delivoria-Papadopoulos and Mishra 1998; Hamada 1994). Furthermore, *Nw*-nitro-L-arginine (NNLA), an NOS inhibitor, administration in a middle cerebral artery occlusion model reduced the volume of cortical infarct in the mouse, indicating the role of NO in neurotoxicity (Nowicki 1991).

Nitric oxide is reported to cause neuronal damage through various mechanisms. We tested the hypothesis that NO synthase inhibition by NNLA will result in decreased oxygen-derived free radical production, leading to the preservation of cell membrane structure and function during cerebral hypoxia (Numagami 1997). Results demonstrated that free radicals, corresponding to alkoxyl radicals, were induced by hypoxia but were inhibited by pretreatment with NNLA before inducing hypoxia. NNLA also inhibited hypoxia-induced generation of conjugated dienes, products of lipid peroxidation. $Na^+$-/$K^+$-ATPase activity, an index of cellular membrane function, decreased following hypoxia but was preserved by pretreatment with NNLA. This data demonstrated that during hypoxia NOS generates free radicals via peroxynitrite production, presumably causing lipid peroxidation and membrane dysfunction.

The appearance of primary free radicals, such as superoxide anion or hydroxyl radical, may not indicate oxidative injury. The reactivity of superoxide radicals is limited (Baum 1984; Sawyer 1981), but hydroxyl radicals are highly reactive to almost all molecules (Mishra and Delivoria-Papadopoulos 1999) so that they can target even noncritical molecules. Therefore, their concentration does not necessarily correlate with the degree of oxidative damage, particularly when assessing lipid peroxidation. Furthermore, these radicals damage cells in cooperation with other radical species or oxidants (Beckman 1990, 1991; Cazevielle 1993; Faraci 1991). In contrast, the production of secondarily formed lipid free radicals provides strong evidence of peroxidative injury. This is particularly true for alkoxyl radicals, which are generated from lipid peroxide by either iron or copper ions and can abstract hydrogen atoms from polyunsaturated fatty acids, leading to further lipid peroxidation (Mishra and Delivoria-Papadopoulos 1999). Our results suggested that NO has an in vivo role in the generation of alkoxyl radicals, leading to free radical-mediated lipid peroxidation.

The exact molecular mechanism of hypoxic membrane damage is not clear. An appealing hypothesis is that when peroxynitrite (formed by the reaction between superoxide anions and NO) is protonated, it decomposes rapidly to form nitrogen dioxide and hydroxyl radicals, both of which are strong oxidants and can initiate oxidative reactions (Beckman 1990, 1991; Radi 1991). It has been shown that peroxynitrite can cause lipid peroxidation in vitro (Radi 1991). Therefore, high concentration of NO during may result in an increased production of peroxynitrite, causing lipid peroxidation.

### 125.5.4 Inhaled Nitric Oxide and Neuroprotection

Nitric oxide is known to be involved in several critical processes in the developing brain. Recent studies have shown that exposure to inhaled nitric oxide (iNO) during the first week of postnatal life improves myelination in the developing brain and significantly reduced the size of excitotoxic lesion in the neonatal rat brain (Delivoria-Papadopoulos et al. 2011a). iNO also significantly improves

cerebral blood flow during ischemia and reduced the infarct volume (Delivoria-Papadopoulos et al. 2011b). In P7 rat model, exposure to 20 ppm iNO was associated with progressive and significant increase in cortical nitric oxide concentration of up to 140% of the basal nitric oxide concentration. Infarct volumes were significantly decreased in the 20 ppm iNO. Nitric oxide is known to react with oxygen free radicals to generate peroxynitrite that leads to nitration of cellular proteins, an index of cell damage. Exposure to 20 ppm iNO during ischemia decreased the density of nitrotyrosine-positive cells by 43% in the cortex. In neonatal rats subjected to postnatal hyperoxia following gestational hypoxia, iNO exposure during the first postnatal week significantly attenuated the cerebral white matter damage in neonatal rats (Lawn et al. 2005). iNO was associated with decreased astrogliosis, microglial activation, and apoptotic cell death through both caspase-dependent and caspase-independent pathways.

iNO has been demonstrated to be effective in various animal models (Kurinczuk et al. 2010). The iNO was protective in a well-characterized model of murine perinatal hypoxia-ischemia. Tissue loss and pathological scores were significantly attenuated by 50 ppm iNO 3 days after the insult. iNO, in an ovine model of ischemic stroke, selectively increased CBF in the ischemic penumbra as compared to a control animal not receiving iNO. These findings substantiate the view that inhalation of nitric oxide causes selective vasodilation of vessels in lowly perfused brain tissue. Recent studies have investigated whether the acute beneficial effects of iNO also translate into long-term neurological improvement; functional outcome was assessed over a period of 7 days after MCA occlusion. Mice treated with iNO (50 ppm) for 24 h after onset of ischemia performed significantly in neurological tests analyzing motor function, agility, and coordination. Furthermore, iNO-treated mice recovered significantly better from postischemic weight loss and showed an improved survival. These studies suggest the need for further preclinical and clinical investigations into the beneficial effects of iNO in neonates at high risk of brain damage.

## 125.6 Neuronal Nuclear $Ca^{++}$ Influx

A number of critical nuclear functions including regulation of transcription factors, cell cycle regulation transcription, DNA replication, and nuclear envelope breakdown are controlled by intracellular $Ca^{++}$ (Mishra and Delivoria-Papadopoulos 1999; Delivoria-Papadopoulos et al. 2003). Furthermore, nuclear $Ca^{++}$ signals potentially control a number of events leading to hypoxia-induced programmed cell death. Nuclear and cytosolic $Ca^{++}$ signals are differently regulated, and the extranuclear $Ca^{++}$ concentration determines the mode of $Ca^{++}$ entry into the nucleus.

The increased intracellular $Ca^{++}$ is a primary mediator of activity-dependent gene transcription under a number of experimental conditions (Ghosh and Greenberg 1995; Hardingham and Bading 1998; Chawla and Bading 2001). The patterns of neuronal impulse and the specific properties of the stimulus-induced calcium transients determine the nature and amplitude of the genomic response (Hardingham and Bading 1998; Fields et al. 1997). Several factors including the site of calcium entry and the amplitude and the spatial properties of the calcium signals determine the calcium-regulated gene expression (Lerea and McNamara 1993; Hardingham et al. 1999; Dolmetsch et al. 2001). Furthermore, the duration of calcium signal also contributes to the specificity of the transcription induction. In cells of the immune system, only a continuous rise in intracellular $Ca^{++}$ concentration, but not a brief spike, induced translocation of transcription factors, NF-ATc (Dolmetsch et al. 1997). Gene expression in neurons is also determined by the duration of calcium transients and the activity-dependent transcription is regulated by the duration of calcium transients (Chawla and Bading 2001).

In previous studies, we have shown that cerebral hypoxia results in increased nuclear $Ca^{++}$ influx in neuronal nuclei of the cerebral cortex of newborn piglets (Delivoria-Papadopoulos et al. 2003; Mishra and Delivoria-Papadopoulos 2000). The nuclear $Ca^{++}$ influx increased as a function of increase in cerebral tissue hypoxia, as measured by decrease in high-energy phosphates, ATP, and phosphocreatine (PCr). Cerebral hypoxia results in

increased $Ca^{++}$/calmodulin kinase (CaM kinase) IV activity and CREB protein phosphorylation in neuronal nuclei of newborn piglets (Vannucci 1990). NO donors increased neuronal nuclear $Ca^{++}$ influx (Mishra and Delivoria-Papadopoulos 2002) and hypoxia resulted in generation of NO free radicals and increased high-affinity $Ca^{++}$-ATPase activity in neuronal nuclei. The high-affinity $Ca^{++}$-ATPase activity increased as a function of increase in cerebral tissue hypoxia (Mishra and Delivoria-Papadopoulos 2001). In addition, IP3-dependent $Ca^{++}$ influx is increased in neuronal nuclei of hypoxic animals as compared to normoxic ones, and this increase was a function of cerebral tissue hypoxia.

During hypoxia, NO-mediated modification of the nuclear membrane high-affinity $Ca^{++}$-ATPase and IP3 receptor is a potential mechanism of increased intranuclear $Ca^{++}$ that leads to activation of $Ca^{++}$-dependent nuclear mechanisms and activates cascades of hypoxic programmed cell death.

## 125.7 Expression and Posttranslational Modification of Apoptotic Proteins

Bcl-2 family of proteins (including Bcl-2 and Bax) control cell proliferation, differentiation, and programmed cell death during normal brain development (Oltvai et al. 1993). Bax and Bcl-2 are inducible genes found in the developing and adult central and peripheral nervous systems (Chen et al. 1996; Reed 1996). Bcl-2 prevents apoptosis by forming a heterodimer with the proapoptotic protein Bax and protects cells from programmed cell death following hypoxia (Oltvai et al. 1993).

Prolonged cerebral hypoxia may lead to a state of primary energy failure, depletion of cellular reserves of high-energy compounds (ATP and phosphocreatine (PCr)), and mitochondrial dysfunction. Failure of the ability of the neuronal membrane to maintain its electrochemical homeostasis results in lipid peroxidation and subsequent modification of the NMDA receptor, influx of calcium into the cytosol, and free radical formation (Oltvai et al. 1993; Reed 1996). Following the calcium influx and the deactivation of the phosphatases located in the cytosol, there is activation of a protein complex that extends from the membrane to the nucleus and the mitochondria, the system of focal adhesions (FAs). The FAs include a number of proteins maintaining a dynamic state while activating each other and changing morphology thus contributing to the signal transduction the nucleus. The proteins of FAs play a crucial role to the cell migration and motility and are very important regulatory factors of the apoptotic cascade as they enter in the nucleus mediating phosphorylation of calmodulin, activation of CaM kinase IV, and CREB protein-mediated transcription of Bax. Subsequently, there is increased expression of caspases leading to DNA fragmentation and cell death (Kratimenos et al. 2018; Oltvai et al. 1993).

In the mitochondrial level, we know that hypoxia leads to mitochondrial outer membrane permeabilization (MOMP) and opening of the mitochondrial permeability transition pores (mPTP) leading to the leakage of the proapoptotic proteins into the cytosol. We also know that during hypoxia, two of the major mitochondrial proteins, second mitochondria-derived activator of caspases (Smac, direct inhibitor of apoptosis-binding protein with a low isoelectric point) and cytochrome c, are translocated into the cytosol. Smac/DIABLO, discovered in 2000, is a small-sized protein (27 kDa) initially formed in a premature form localized in mitochondria and is subsequently released into the cytosol as a bigger mature protein along with cytochrome c, maintaining a dynamic interaction. Cytochrome c protein (12 kDa) is a crucial element of the electron transport chain in mitochondria, and it is also involved in the initiation of apoptosis. It is released to the cytoplasm and binds apoptotic protease activating factor-1 (Apaf-1). Smac is released concurrently with cytochrome c from mitochondria into the cytosol during apoptosis and deactivates the inhibitor of apoptosis protein (IAP)-mediated inhibition leading to the activation of the caspases and cell death (Kratimenos et al. 2017; Oltvai et al. 1993).

Concentrating on focal adhesions, our previous data in guinea pigs show that hypoxia leads to decreased concentration of Smac and cytochrome c in mitochondria, while their expression increases in the cytosol. These findings indicate that there is probably translocation of Smac and cytochrome c from the mitochondria to the cytosol during hypoxia.

We have also shown that hypoxia increases the activation of several proteins of the focal adhesions, including the Src kinase. Since the mitochondria are located in the area of FAs, one of the generated questions is whether the proteins of the FAs contribute to the trafficking of the mitochondrial proteins in the cytosol.

Cerebral hypoxia results in increased expression of Bax protein in neuronal nuclei of the cerebral cortex of newborn piglets. The Bax protein increased as a function of increase in degree of cerebral tissue hypoxia as measured by decrease in high-energy phosphates, ATP, and phosphocreatine (Ravishankar et al. 2001). The expression of Bax protein increases in the mitochondrial, cytosolic, as well as neuronal nuclear fractions indicating increased expression of the protein rather than its translocation, e.g., from mitochondria to cytosol. The expression of anti-apoptotic protein Bax to anti-apoptotic protein Bcl-2 did not increase during hypoxia. Therefore, the ratio of proapoptotic protein Bax to anti-apoptotic protein Bcl-2 increases in all compartments of the cell during hypoxia that may lead to activation of hypoxia-induced cascade of neuronal death.

Administration of NOS inhibitor prevented the hypoxia-induced increased expression of proapoptotic protein Bax indicating that the hypoxia-induced increased expression of Bax is NO-mediated (Zanelli et al. 2002).

### 125.7.1 Posthypoxic Expression and Activation of Caspase-3, Caspase-8, and Caspase-9 in the Newborn Brain

Caspases are a unique family of proteases that play an important role in the initiation and execution of apoptosis (Delivoria-Papadopoulos et al. 2008). All caspases contain a cysteine residue at their active site and specifically cleave substrate proteins at an aspartic acid residue. These cysteine proteases reside predominantly in the cytosolic compartment of animal cells as inactive zymogens and become activated by proteolytic cleavage at internal aspartate residues on apoptotic stimulation (Mishra et al. 2001). These are divided into two main classes: those with long prodomain are the class I caspases and those with short prodomain are the class II caspases.

Class I caspases such as 8, 9, and 10 can autocatalyze their own activation and are activated in the early phase of apoptosis. These are called the initiator caspases. Class II caspases, such as 3, 6, and 7, require cleavage by another protease and are responsible for the breakdown of cells (Delivoria-Papadopoulos et al. 2008). These are called the effector or the executioner caspases. The upstream caspases activated during apoptosis lead to the activation of downstream caspases in a self-amplifying cascade (Delivoria-Papadopoulos et al. 2008).

Two well-studied pathways of caspase activation are the cell surface death receptor-mediated pathway and the mitochondria-initiated pathways. The recruitment and the cleavage of procaspase-8 to produce the active form of caspase-8 is a critical biochemical event in the death receptor-mediated apoptosis (Delivoria-Papadopoulos et al. 2008). Following its activation, caspase-8 can activate downstream caspase by direct cleavage or indirectly by cleaving the proapoptotic protein and inducing cytochrome c (cyt c) release from the mitochondria (Delivoria-Papadopoulos et al. 2008). In the proposed mitochondria-initiated pathway, caspase activation is triggered by formation of an oligomeric apoptotic protease activation factor (Apaf-1)/cyt c complex, which leads to recruiting and activating procaspase-9, an upstream caspase in this pathway (Mishra and Delivoria-Papadopoulos 2010). The complex formed by the combination of Apaf-1, cyt c, Bax/Bcl-2, and procaspase-9 is referred to as the apoptosome (Mishra and Delivoria-Papadopoulos 2010; Ashraf et al. 2007).

Two other less defined pathways of apoptotic caspase activation are the ceramide pathway that may act predominantly through the initiator caspase-8 or caspase-9, depending on the stimulus that induces ceramide synthesis, and the granzyme B-perforin pathway, which may be directly initiated by the effector caspase-3 and caspase-7. All these pathways, with the exception of the granzyme pathway, are known to be active in neurons.

Studies were conducted to investigate the role of caspase cascade-mediated programmed cell death. Using our 3- to 5-day-old newborn piglet model, we investigated the hypoxia-induced alterations in the activity and expression of caspase-3, caspase-8, and caspase-9. Results of these studies after 1 h of severe hypoxia in newborn piglets demonstrated that following hypoxia, there is an increase in the activity of the initiator caspase-8 and caspase-9 in the cytosolic fraction of the cerebral cortex. The activity of caspase-3 also increased in the cytosolic fraction of the cerebral cortex. In addition, the expression of caspase-8, caspase-9, and caspase-3 protein increased following hypoxia (Delivoria-Papadopoulos et al. 2008; Mishra and Delivoria-Papadopoulos 2010; Ashraf et al. 2007).

## 125.7.2 Mechanisms of Caspase Activation During Hypoxia in the Newborn Brain

To investigate the mechanism of caspase activation, we used selective inhibitors such as clonidine, an inhibitor of high-affinity $Ca^{++}$-ATPase; 7-nitroindazole sodium salt (7-NINA), an inhibitor of neuronal NO synthase; and z-Leu-Glu(OMe)-His-Asp(OMe)-fluoromethylketone (z-LEHD-FMK), a selective caspase inhibitor.

### 125.7.2.1 The Role of Nuclear $Ca^{++}$ Influx in Caspase-9 and Caspase-3 Activation

Studies performed in newborn piglets were specifically designed to investigate the role of nuclear Ca++ influx in caspase activation during hypoxia by administration of a $Ca^{++}$-ATPase inhibitor (clonidine) to block the nuclear $Ca^{++}$ influx. It was shown that the increased activity of caspase-9 and caspase-3 during hypoxia is mediated by nuclear $Ca^{++}$ influx. The levels of tissue high-energy phosphates (ATP and PCr) in the cerebral cortex of normoxic, hypoxic, and hypoxic pretreated with clonidine piglets were comparably decreased. The determination of caspase-9 activity in the normoxic, hypoxic, and hypoxic pretreated with clonidine groups of piglets showed that cerebral tissue hypoxia results in increased caspase-9 activity and that the pretreatment with high-affinity $Ca^{++}$-ATPase inhibitor prevents this hypoxia-induced increase in caspase-9 activity.

Assessment of the activity of caspase-3 in the normoxic, hypoxic, and hypoxic pretreated with clonidine groups of piglets demonstrated that cerebral tissue hypoxia resulted in increased caspase-3 activity, a consequence of caspase-9 activation, and that the pretreatment with clonidine prevented this increase in caspase-3 activity. These results demonstrate that hypoxia-induced increase in caspase-3 activity is mediated by nuclear $Ca^{++}$ influx.

### 125.7.2.2 The Role of NO in Caspase-9 and Caspase-3 Activation

These studies in newborn piglets were specifically designed to investigate the role of NO derived from nNOS in caspase activation during hypoxia by administration of a relatively selective inhibitor of nNOS, 7-NINA (Mishra and Delivoria-Papadopoulos 2006). After having confirmed that cerebral tissue hypoxia achieved in the hypoxic and hypoxic pretreated with 7-NINA groups was comparable, the activity of caspase-9 in the normoxic, hypoxic, and hypoxic pretreated with 7-NINA groups of piglets was determined, and the results demonstrated that cerebral tissue hypoxia results in increased caspase-9 activity and that the pretreatment with the nNOS inhibitor prevents the hypoxia-induced increase in caspase-9 activity.

Cerebral tissue hypoxia resulted in increased caspase-3 activity, a consequence of caspase-9 activation, and the pretreatment with the nNOS inhibitor prevented this hypoxia-induced increase in caspase-3 activity. It demonstrates that the

hypoxia-induced increase in caspase-9 is mediated by nNOS-derived NO.

#### 125.7.2.3 The Effect of Caspase-9 Inhibition During Hypoxia on Prevention of Downstream Events Including Caspase-3 Activation

To demonstrate that caspase-3 activation is a downstream event of caspase-9 activation during hypoxia, we used its selective inhibitor z-LEHD-FMK (Chiang et al. 2007, 2008).

The activity of caspase-9 was determined in the cytosolic fraction of the cerebral cortex of newborn piglets. Cerebral tissue hypoxia resulted in increased caspase-9 activity and the pretreatment with the caspase-9 inhibitor prevented the hypoxia-induced increase in caspase-9 activity.

The activity of caspase-3 in the cytosolic fraction of the cerebral cortex increased as a consequence of caspase-9 activation, and the pretreatment with a caspase-9 inhibitor prevented this hypoxia-induced increase. The same study showed that pretreatment with caspase-9 inhibitor prevents the hypoxia-induced increase in the expression of active caspase-9 and active caspase-3 (protein levels).

These studies demonstrate that caspase-9 can be activated during hypoxia by multiple mechanisms that are dependent on generation of nNOS-derived NO and neuronal nuclear $Ca^{++}$ influx. The increase in nuclear $Ca^{++}$ influx leading to $Ca^{++}$-dependent activation of Ca/calmodulin-dependent protein kinase IV may result in increased phosphorylation or cyclic AMP response element binding protein at serine 133 and transcription of caspases as well as proapoptotic proteins. NO-mediated phosphorylation of anti-apoptotic proteins may alter their anti-apoptotic potential due to a defect in dimerization and increased caspase-9 activation.

We have shown that NO increases $Ca^{++}$ influx in synaptosomes as well as neuronal nuclei. By increasing nuclear $Ca^{++}$ influx, NO can increase expression of caspase-9 as well as proapoptotic proteins. NO-mediated modification of caspase protein may alter its activation.

Thus, caspase activation during hypoxia in the newborn brain is mediated by transcription-dependent and transcription-independent mechanisms.

Studies using z-LEHD-FMK, a selective inhibitor of caspase-9, indicate that the role of caspase-9, the inhibitor caspase, is highly significant in the hypoxia-induced programmed cell death in the newborn brain.

## 125.8 DNA Fragmentation

It has been proposed that the cleavage of DNA at its intranucleosomal linkage region is produced by specific endonucleases that are $Ca^{++}$ dependent (Ishida et al. 1974).

Caspase-3 acting as cysteine protease cleaves and inactivates a chain reaction by nuclear enzymes like PARP, a DNA repair enzyme, and ICAD, the inhibitor of caspase-activated DNase. Then, the caspase-activated DNase enters the nucleus and cleaves genomic chromosomal DNA (Hameed et al. 1989; Tominaga et al. 1993). This nuclear genomic DNA fragmentation correlates exponentially with the degree of cerebral tissue hypoxia in newborn piglets (Waseem et al. 2001) and is characteristic of cellular apoptosis. However, in our study, there was no significant increase in fragmentation until the ATP and phosphocreatine levels decreased by more than 50%, compared with baseline levels.

## 125.9 Temporal Biochemical Changes

The steps of posthypoxic neuronal injury are evolving within hours in the necrotic process and within days in the apoptotic process. Temporal biochemical changes and associated nuclear fragmentation have been assessed in the cerebral cortex of newborn guinea pigs following hypoxia. Initial cellular injury may be followed by a failure of cellular repair mechanisms leading to further delayed brain injury. Neuronal nuclear $Ca^{++}$ influx increases immediately following hypoxia and remains elevated through 7 days of age. Similar nuclear Bax protein expression increases

immediately following hypoxia and remains elevated through 7 days where Bcl-2 protein remains similar to control during hypoxia.

All these temporal (biphasic) changes may reflect not only the primary hypoxic insult but also a secondary cellular damage due to a recurrent (continuing) free radical release during the reperfusion-reoxygenation phase.

## 125.10 Clinical Implications

The understanding of the very complex and interrelated mechanisms of cell death after a hypoxic-ischemic result may serve as background in critical care of the newborn.

Hypoxia at the cellular level is the consequence of failure in oxygen transport from the lung alveolar space to the mitochondria. In order to prevent neuronal cell death, to restore any insufficiency or failure in the respiratory and circulatory systems is an emergency in terms of minutes.

Among the hazards in restoring oxygen supply, medically induced hyperoxia (high FIO2) may worsen the neuronal insult by the production of additional oxygen free radicals.

The temporal evolution of the posthypoxic biochemical disturbances may alter the opportunity of pharmacologic interventions at key steps of the biochemical events, like magnesium sulfate, a NMDA receptor antagonist, or allopurinol, an inhibitor of the enzyme xanthine oxidase.

## 125.11 Neuroprotective Treatments with Suggested Efficacy in Humans

### 125.11.1 Magnesium Sulfate

In the clinical setting, $MgSO_4$ has been widely used in obstetrics practice for more than 60 years. Its indications include suppression of preterm labor and management of pregnancy-induced hypertension (Levene et al. 1999). A retrospective epidemiologic study by Nelson and Grether (1995) suggested that premature fetuses whose mothers received $MgSO_4$ for the treatment of preeclampsia or as a tocolytic agent are less likely to develop cerebral palsy compared to a gestational age-matched group of fetuses not exposed to the drug. The Collaborative Eclampsia Trial (1995) reported that babies of women who had been given $MgSO_4$ before delivery were significantly less likely to be intubated at the place of delivery or to be admitted to a special care nursery than the babies of mothers who had been given phenytoin. These studies suggested that $MgSO_4$ might provide a protective effect against brain damage in immature fetuses and newborn infants. Randomized, controlled, double-blind trials were established to examine this hypothesis. One was discontinued after interim analysis showed that administration of $MgSO_4$ to mothers in preterm labor before 34 weeks of gestation was associated with significant increase in infants' mortality (Mittendorf et al. 1997). However, other trials have not shown any difference in the mortality rates between the placebo and treatment groups (Benichou et al. 1997).

A multicenter randomized, controlled trial of $MgSO_4$ vs. placebo, for the prevention of cerebral palsy, in 2,241 women at risk of imminent premature delivery at 24–31 weeks of gestation carried out in the USA was published in 2008 (Rouse et al. 2008). The primary outcome was the composite of stillbirth or infant death by 1 year of corrected age or moderate or severe cerebral palsy at or beyond 2 years of corrected age. The primary outcome was not significantly different in the $MgSO_4$ group and the placebo group. However, in a prespecified secondary analysis, moderate or severe cerebral palsy occurred significantly less frequently in the $MgSO_4$ group (1.9% vs. 3.5%). A similar study in France followed up 606 infants of less than 33 weeks of gestation, whose mothers were treated with $MgSO_4$. Compared to placebo, treated infants showed a decrease of all primary end points (total mortality, severe white matter injury, and their combined outcome) and of all secondary end points (motor dysfunction, cerebral palsy, cognitive dysfunction, and their combined outcomes at 2 years of age). The decrease was nearly significant or significant for gross motor dysfunction and combined

criteria: death and cerebral palsy, death and gross motor dysfunction, and death, cerebral palsy, and cognitive dysfunction (Moriette et al. 2008). Doyle et al. (2009) reviewed the evidence of the neuroprotective effects of $MgSO_4$ given to women considered at risk of preterm birth. The authors concluded that the neuroprotective role for antenatal $MgSO_4$ therapy given to mothers at such risk is now established. The number of women needed to treat to benefit one baby by avoiding cerebral palsy is 63 (95% confidence interval 43–87). Given the beneficial effects of $MgSO_4$ on substantial gross motor function in early childhood, outcomes later in childhood should be evaluated to determine the presence or absence of later potentially important neurologic effects, particularly on motor or cognitive function.

### 125.11.2 Allopurinol

In experimental animal models, administration of allopurinol to immature rats 30 min before inducing focal hypoxia-ischemia reduced the severity of the secondary edema and the extent of the neuropathologic lesions in the treated compared with a control group (Palmer et al. 1990). Similarly, pretreatment with allopurinol preserved cerebral energy metabolism of the 7-day postnatal rat during hypoxia-ischemia (Williams et al. 1992). The same group of researchers found that oxypurinol, the active metabolite of allopurinol, administered at the same dose and at the same time as allopurinol after hypoxia-ischemia reduced brain injury in the immature rat (Palmer and Roberts 1991). Administration of allopurinol in newborn piglets prevented the hypoxia-induced modification of NMDA receptor as well as cell membrane preoxidation and neuronal dysfunction (Marro et al. 1994; Maro et al. 1998).

In the clinical setting, a 7-day course of enteral allopurinol (20 mg per kg) given after birth to 400 infants between 24 and 32 weeks of gestation did not change the incidence of periventricular leukomalacia (Russell and Cooke 1995). In a study of 22 asphyxiated newborn infants, intravenous allopurinol in a dose of 40 mg per kg given 4 h after birth resulted in a decreased mortality (2/11 vs. 6/11 in the control group) and in a beneficial effect on free radical formation, cerebral blood flow, and electrical brain activity, without toxic side effects (Van Bel et al. 1998). Clancy et al. (2001) conducted a clinical trial to test the hypothesis that allopurinol could reduce death, seizures, coma, and cardiac events in infants who underwent heart surgery using deep hypothermic circulatory arrest. They studied a total of 318 infants, 131 hypoplastic left heart syndrome (HLHS) and 187 non-HLHS. In HLHS surgical survivors, 40 of 47 (85%) allopurinol-treated infants did not experience any end point event, compared to 27 of 49 (55%) controls ($p = 0.002$). There were fewer seizure-only ($p = 0.05$) and cardiac-only ($p = 0.03$) events in the allopurinol versus placebo groups. Allopurinol did not reduce efficacy end point events in non-HLHS infants. Treated and control infants did not differ in adverse events. Recently, Bender et al. (2006) investigated whether postnatal allopurinol would reduce free radical-induced reperfusion/reoxygenation injury of the brain in severely asphyxiated neonates. In an interim analysis of a randomized, double-blind, placebo-controlled study, 32 severely asphyxiated infants were given allopurinol or a vehicle within 4 h of birth. The analysis showed an unaltered (high) mortality and morbidity in infants treated with allopurinol. The authors concluded that allopurinol treatment started postnatally was too late to reduce the early reperfusion-induced free radical surge. Allopurinol administration to the fetus with (imminent) hypoxia via the mother during labor may be more effective in reducing free radical-induced post-asphyxial brain damage.

Chaudhari and McGuire (2008) performed a meta-analysis to evaluate the evidence of the effect of allopurinol on mortality or morbidity in newborn infants with suspected hypoxic-ischemic encephalopathy. The authors concluded that the available data are not sufficient to determine whether allopurinol has clinically important benefits for newborn infants with hypoxic-ischemic encephalopathy, and, therefore, larger trials are needed. Such trials could assess allopurinol as an adjunct to therapeutic hypothermia in infants with moderate and severe encephalopathy and should be designed to exclude clinically important effects

on mortality and adverse long-term neurodevelopmental outcomes.

### 125.11.3 Opioids

The antinociceptive effects of opioids are mediated through a combination of pre- and postsynaptic hyperpolarization, which produces a decrease in the release of and the sensitivity to endogenous mediators like glutamate (Lee et al. 2004; Yamakura et al. 1999). This suggests that they may have a neuroprotective effect. Indeed, studies in cell cultures have demonstrated that endogenous and exogenous opioids may protect cortical neurons from hypoxia-induced cell death (Zhang et al. 2000, 2002). Similarly, opioids may induce ischemic tolerance in cerebellar Purkinje cells subject to ischemia-reperfusion (Lim et al. 2004). Antagonists of opioid receptors increase the survival time during severe hypoxia in intact animals (Mayfield and D'Alecy 1992, 1994) and enhance tissue preservation and survival time of organs used for transplants (Chein et al. 1994).

In 2005, Angeles et al. (2005) published the results of a retrospective study of 52 term newborns with perinatal asphyxia, in which they analyzed the relationship between treatment with opioid analgesics (morphine or fentanyl) and neurological damage. A total of 33% of them received opioids; in spite of having a more severe degree of asphyxia (higher levels of lactate, lower 5 min Apgar scores), this group of patients had less severe signs of brain damage on the MRI performed after 7 days of life. Moreover, their neurologic outcome at a mean follow-up of 13 months was better than the group of newborns who did not receive opioids. The same group of researchers also performed a follow-up study with magnetic resonance (MR) spectroscopy of 28 term newborns treated with opioids and 20 controls (Angeles et al. 2007). The results showed that occipital gray matter NAA/Cr was significantly decreased and lactate was present in a significantly higher amount in non-opioid-treated neonates compared with opioid-treated neonates. Also, compared with controls, untreated neonates showed large changes in more metabolites in the basal ganglia, thalami, and occipital gray matter with greater significance than treated neonates. The authors concluded that the use of opioids during the first week following perinatal asphyxia has no long-term adverse effects and may increase brain resistance to hypoxia-ischemia. The authors speculated that the neuroprotective effect of opioids may be mediated by increasing the levels of adenosine, and endogenous nucleoside with neuroprotective activity, or by inducing neuronal hyperpolarization, which results in diminishing intracellular penetration of calcium.

Despite the potential benefit of opioids on asphyxiated term neonates as indicated in these studies, caution must be exercised in the use of this class of medications. Available literature suggests that the routine use of opioid analgesics can be complicated by problems such as tolerance, withdrawal symptoms, and ventilator dependence. Very few studies have examined the long-term effects of exposure to opioids in the neonatal period. In addition, previous reports indicate that endogenous opioids can suppress DNA synthesis in vivo in mature cerebellar and glial cells (opioid receptors are widely distributed in the CNS with functions that include pain modulation, cardiorespiratory regulation), whereas exogenous opioids can exacerbate neurotoxicity in animal models of cerebral ischemia. Future prospective randomized trials are warranted to determine whether there is truly an immediate neuroprotective effect on hypoxic-ischemic brain injury and whether these agents can play a role in improving long-term outcome.

### 125.11.4 Hypothermia

Hypothermia has developed during the past few years as an alternative for treating perinatal asphyxia/HIE (Gunn and Gunn 1998; Wagner et al. 1999). Hypothermia during experimental cerebral ischemia is associated with potent dose-related, long-lasting neuroprotection. Conversely, hyperthermia of only 1–2 °C extends and markedly worsens damage and in particular tends to promote pannecrosis (Gunn and Gunn 1998).

Although the majority of such studies involved global ischemia in adult rodents (Coimbria and Wielock 1994), similar results were reported from studies on hypoxia-ischemia in 7-day-old rats (Trescher et al. 1997) and newborn piglets (Thorensen et al. 1995), kittens, rabbits, and puppies (Miller 1971).

The study of the mechanisms of action of hypothermic neuroprotection suggests that cooling affects many or all of the pathways leading to delayed cell death (Gunn and Gunn 1998). Hypothermia reduces the rate of oxygen-requiring enzymatic reactions and cerebral oxygen consumption, slows the fall of phosphocreatine/inorganic phosphate (PCr/Pi), and confers a protective effect of the brain after ATP exhaustion. In addition, hypothermia decreases oxygen consumption of the brain by 6–7% and cerebral energy utilization rate by 5.3% per degree. Additional experimental evidence suggests that hypothermia suppresses cytotoxic excitatory amino acid accumulation, inhibits nitric oxide synthase activity, decreases interleukin-1 levels, decreases the release of other cytotoxic cytokines by microglial cells, and suppresses free radical activity and delayed cell death by apoptosis. Hypothermia also decreases blood-brain barrier permeability and intracranial pressure and facilitates recovery of electrophysiologic function after cerebral ischemia.

The efficacy of hypothermia is dependent on a number of factors, such as the timing of initiation of cooling, its duration, and the depth of cooling attained. Mild hypothermia is defined as a reduction in core temperature of 1–3 °C, moderate as 4–6 °C, severe as 8–10 °C, and profound as 15–20 °C. Brief (0.5–3 h); mild-to-moderate hypothermia immediately after hypoxia-ischemic injury may be most effective after relatively mild insults. Protection appears to be lost if brief hypothermia is delayed by as little as 15–45 min after the primary insult. A more recent approach has been to try to suppress the secondary encephalopathic processes by maintaining hypothermia throughout the course of the secondary phase. An extended period of cooling (between 5 and 72 h) appears to be more consistently effective and remains effective after significant delays (possibly up to 6 h) between the primary insult and the start of cooling; however, the degree of neuroprotection progressively declines if cooling is initiated more than a few hours post-insult (Gunn and Gunn 1998). In addition, cerebral hypothermia seems to be less effective when started after postischemic seizures occur (Gunn et al. 1999).

Potential adverse effects of induced hypothermia (the risk increasing with depth of hypothermia) include increased blood viscosity, mild metabolic acidosis, decreased oxygen availability, intracellular shift of potassium, cardiac arrhythmias, coagulation abnormalities and platelet dysfunction, and choreic syndrome (Wagner et al. 1999).

### 125.11.5 Selective Head Cooling

The first study on neuroprotection of perinatal HIE with selective head cooling was published in 1998 by (Gunn et al. 1998), who basically proved the safety of this procedure. Later on, the same group of researchers published the results of other studies in a small number of patients, which confirmed the lack of side effects and a tendency to a better neurologic prognosis in those newborns with moderate or severe HIE treated with this hypothermia technique (Battin et al. 2001, 2003).

In 2005 Gluckman et al. (2005) published the results of the most important study that has produced reliable and significant data about the neuroprotective effect of selective head cooling hypothermia. It was a multicenter investigation that included 234 newborns with HIE and expected gestational age (EGA) above 36 weeks. Patients were randomized before 5.5 h of life into two groups: body normothermia or hypothermia of 34.5 °C, induced through selective head cooling during 72 h. Patients treated with hypothermia had a significantly higher incidence of arrhythmia (mostly sinus bradycardia). A total of 218 infants were followed up until 18 months of age. The presence of death or neurological disability was found in 66% of patients in the control group and 55% in the hypothermia group ($p < 0.1$). However, when newborns with severe

neurological depression or those who had seizures on the aEEG were excluded, 66% of infants in the control group and 48% in the hypothermia group died or had neurological disability ($p < 0.2$). Moreover, the presence of severe neurological disability was 28% and 12% in each group, respectively. The authors concluded that, except in newborns with the most severe forms of HIE, selective head cooling applied immediately following delivery may be a feasible therapeutic technique to decrease neurological sequelae of perinatal HIE.

### 125.11.6 Generalized Body Hypothermia

The first study with generalized body hypothermia in perinatal HIE was published in 2000 by Azzopardi et al. (2000), who found that prolonged hypothermia of 33–34 °C was associated with minimal physiological changes (e.g., decreased heart rate, increased blood pressure), but was well tolerated. During the next 3 years, other research protocols in a limited number of patients corroborated that generalized body hypothermia was a feasible and clinically safe technique (Shankaran et al. 2002; Debillon et al. 2003).

In 2005, Eicher et al. (2005) published the results of a pilot multicenter study about the safety and efficacy of generalized body hypothermia in the treatment of 32 newborns with perinatal HIE. Adverse effects included bradycardia, hypotension, decreased platelets, increased prothrombin time, and higher incidence of seizures, but none of them was severe, and they all responded to treatment (Eicher et al. 2005). The efficacy results showed a higher incidence of death or severe neurological motor involvement in the control group (82%) compared to the group of hypothermia-treated newborns (52%) ($p = 0.019$). A severe psychomotor developmental delay (less than 70%) was seen in 64% of infants in the control group and in 24% of those subjected to hypothermia ($p = 0.053$).

Also in 2005, Shankaran et al. (2005) published a large multicenter study on the use of body hypothermia to treat perinatal HIE. A total of 208 newborns with HIE and EGA more than 36 weeks were included and randomized before 6 h of life into two groups: body normothermia or hypothermia of 33.5 °C, induced by body cooling, during 72 h. Patients were followed up until 18–22 months. The incidence of mild complications was similar in both groups. The incidence of death or moderate or severe neurological disability was 62% in the control group and 44% in the hypothermia-treated group ($p = 0.01$). The incidence of cerebral palsy was 30% in the control newborns and 19% in those treated with hypothermia ($p = 0.20$). The authors concluded that generalized body hypothermia reduces the risk of death and neurological disability in newborns with moderate or severe HIE.

A magnetic resonance imaging (MRI) follow-up study of infants enrolled in the abovementioned trial (Shankaran et al. 2005) aimed to measure relative volumes of subcortical white matter. They were significantly large in hypothermia-treated than in control infants. Furthermore, relative total brain volumes correlated significantly with death or neurosensory impairments. Relative volumes of the cortical gray and subcortical white matter also correlated significantly with Bayley Scales psychomotor development index (Parikh et al. 2009). There is growing body of evidence supporting that total body hypothermia improves significantly survival and disability in full-term neonates with HIE with better neurodevelopmental outcomes in infancy and early childhood (Jacobs et al. 2013; Azzopardi et al. 2014; Shankaran et al. 2012).

Hypothermia has already translated from the clinical research experience to direct clinical application and has already become the standard of care to treat newborns with perinatal asphyxia (Zanelli et al. 2008; Kapetanakis et al. 2008; Tan and Parks 1999; Gunn et al. 2005; Sahni and Sanocka 2008; Wagner et al. 2002; Hoeger et al. 2006; Talati et al. 2005; Van Bel and Groenendaal 2008; Higgins et al. 2006; Jacobs et al. 2013; Azzopardi et al. 2014; Shankaran et al. 2012). However, the clinical trials with hypothermia in premature newborns and/or in combination with neuroprotective agents such as xenon, erythropoietin, melatonin,

and allopurinol have also addressed many questions, which need to be answered before they are implemented in routine clinical practice.

## References


Aizenman E, Lipton SA, Loring RH (1989) Selective modulation of NMDA responses by reduction and oxidation. Neuron 2:1257–1263

Angeles DM, Wycliffe N, Michelson D et al (2005) Use of opioids in asphyxiated term neonates: effects of neuroimaging and clinical outcome. Pediatr Res 57:873–878

Angeles DM, Ashwal S, Wycliffe ND et al (2007) Relationship between opioid therapy, tissue damaging procedures, and brain metabolites as measured by proton MRS in asphyxiated term neonates. Pediatr Res 60:614–621

Aoki C, Fenstemaker S, Lubin M et al (1993) Nitric oxide synthase in the visual cortex of monocular monkeys as revealed by light and electron microscopic immunocytochemistry. Brain Res 620:97–113

Aoki C, Rhee J, Lubin M et al (1997) NMDA-R1 subunit of the cerebral cortex co-localizes with neuronal nitric oxide synthase at pre and postsynaptic sites and in spines. Brain Res 750:25–140

Ashraf QM, Mishra OP, Delivoria-Papadopoulos M (2007) Mechanisms of expression of apoptotic protease activating factor-1 (Apaf-1) in nuclear, mitochondrial and cytosolic fractions of the cerebral cortex of newborn piglets. Neurosci Lett 415:253–258

Azzopardi D, Robertson NJ, Cowan FM et al (2000) Pilot study of treatment with whole body hypothermia for neonatal encephalopathy. Pediatrics 106:684–694

Azzopardi D, Strohm B, Marlow N, Brocklehurst P, Deierl A, Eddama O et al (2014) Effects of hypothermia for perinatal asphyxia on childhood outcomes. N Engl J Med 371:140–149. https://doi.org/10.1056/NEJMoa1315788

Bashir ZI, Alford S, Davies SN et al (1991) Long-term potentiation of NMDA receptor-mediated synaptic transmission in the hippocampus. Nature 349:156–158

Battin MR, Dezoete JA, Gunn TR et al (2001) Neurodevelopmental outcome of infants treated with head cooling and mild hypothermia after perinatal asphyxia. Pediatrics 107:480–484

Battin MR, Penrice J, Gunn TR, Gunn AJ (2003) Treatment of term infants with head cooling and systematic hypothermia (35.0 degrees and 34.5 degrees C) after perinatal asphyxia. Pediatrics 111:244–251

Baum RM (1984) Superoxide theory of oxygen toxicity is center of heated debate. Chem Eng News 9:20–28

Beckman JS (1990) Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. Proc Natl Acad Sci U S A 87:1620–1624

Beckman JS (1991) The double-edged role of nitric oxide in brain function and superoxide-mediated injury. J Dev Physiol 15:53–59

Bender MJ, Bos AF, Rademaker CM et al (2006) Early postnatal allopurinol does not improve short term outcome after severe birth asphyxia. Arch Dis Child Fetal Neonatal Ed 91:F163–F165

Benichou J, Zupan V, Fernandez H et al (1997) Tocolytic magnesium sulphate and pediatric mortality. Lancet 351:290–291

Bhat GK, Mahesh VB, Lamar CA et al (1997) Histochemical localization of nitric oxide neurons in the hypothalamus: association with gonadotropin-releasing hormone neurons and co-localization with N-methyl-D-aspartate receptors. Neuroendocrinol Lett 62:187–197

Bredt DS, Ferris CD, Snyder SH (1992) Nitric oxide synthase regulatory sites. Phosphorylation by cyclic AMP-dependent protein kinase, protein kinase C, and calcium/calmodulin protein kinase, identification of flavin and calmodulin sites. J Biol Chem 267:10976–10981

Cazevielle C (1993) Superoxide and nitric oxide cooperation in hypoxia/reoxygenation-induced neuron injury. Free Radic Biol Med 14:359–395

Chaudhari T, McGuire W (2008) Allopurinol for preventing mortality and morbidity in newborn infants with suspected hypoxic-ischemic encephalopathy. Cochrane Database Syst Rev 2:CD006817

Chawla S, Bading H (2001) CREB/CBP and SRE-interacting transcriptional regulators are fast on-off switches: duration of calcium transients specifies the magnitude of transcriptional responses. J Neurochem 79:849–858

Chein S, Oeltgen PR, Diana JN et al (1994) Extension of tissue survival time in multiorgan block preparation with a delta DADLE (D-Ala2, D-leu5)-enkephalin). J Thorac Cardiovasc Surg 107:964–967

Chen J, Zhu RL, Nakayama M et al (1996) Expression of the apoptosis- effector gene, Bax, is up-regulated in vulnerable hippocampal CA1 neurons following global ischemia. J Neurochem 67:64–71

Chiang MC, Ashraf QM, Ara J et al (2007) Mechanism of caspase-3 activation during hypoxia in the cerebral cortex of newborn piglets. Neurosci Lett 421:67–71

Chiang MC, Ashraf QM, Mishra OP, Delivoria-Papadopoulos M (2008) Mechanism of DNA fragmentation during hypoxia in the cerebral cortex of newborn piglets. Neurochem Res 33:1232–1237

Choi DW (1990) Cerebral hypoxia: some new approaches and unanswered questions. J Neurosci 10:2493–2501

Christopherson KS, Hillier BJ, Lim WAS et al (1999) PSD-95 assembles a ternary complex with the N-Methyl-D-Aspartic acid receptor and bivalent neuronal NO synthase PDX domain. J Boi Chem 274:27467–27473

Clancy RR, McGaurn SA, Goin JE et al (2001) Allopurinol neurocardiac protection trial in infants undergoing

heart surgery using deep hypothermic circulatory arrest. Pediatrics 108:61–70
Coimbria C, Wielock T (1994) Moderate hypothermia mitigates neuronal damage in the rat brain when initiated several hours following transient cerebral ischemia. Acta Neuropathol (Berlin) 87:325–331
Collingridge G (1987) Synaptic plasticity. The role of NMDA receptors in learning and memory. Nature 330:604–605
Columbano A (1995) Cell death: current difficulties in discriminating apoptosis and necrosis in the context of pathological processes in vivo. J Cell Biochem 58:181–190
Davidson JO, Wassink G, van den Heuij LG et al (2015) Therapeutic hypothermia for neonatal hypoxic-ischemic encephalopathy – where to from here? Front Neurol 14:6–198
Dawson VL (1991) Nitric oxide mediates glutamate neurotoxicity in primary cortical cultures. Proc Natl Acad Sci U S A 88:6368–6371
Dawson DA (1994a) Nitric oxide and focal cerebral ischemia: multiplicity of actions and diverse outcome. Cerebrovasc Brain Metab 64:299–324
Dawson TM (1994b) Gases as biological messengers: nitric oxide and carbon monoxide in the brain. J Neurosci 14:5147–5159
Dawson TM, Steiner JP, Dawson VL et al (1993) Immunosuppressant FK506 enhances phosphorylation of nitric oxide synthase and protects against glutamate neurotoxicity. Proc Natl Acad Sci U S A 90:9808–9812
Debillon T, Daoud P, Durand P et al (2003) Whole-body cooling after perinatal asphyxia: a study in term neonates. Dev Med Child Neurol 45:17–23
Delivoria-Papadopoulos M, Mishra OP (1998) Mechanisms of cerebral injury in perinatal asphyxia and strategies for prevention. J Pediatr 132:S30–S34
Delivoria-Papadopoulos M, Akhter W, Mishra OP (2003) Hypoxia-induced Ca2+ -influx in cerebral cortical neuronal nuclei of newborn piglets. Neurosci Lett 342:119–123
Delivoria-Papadopoulos M, Ashraf QM, Ara J, Mishra OP (2008) Nuclear mechanisms of hypoxic cerebral injury in the newborn: the role of caspases. Semin Perinatol 32:334–343
Delivoria-Papadopoulos M, Ashraf QM, Mishra OP (2001a) Brain tissue energy dependence of CaM kinase IV cascade activation during hypoxia in the cerebral cortex of newborn piglets. Neurosci Lett 491(2):113–117
Delivoria-Papadopoulos M, Ashraf QM, Mishra OP (2001b) Mechanism of CaM kinase IV activation during hypoxia in neuronal nuclei of the cerebral cortex of newborn piglets: the role of Src kinase. Neurochem Res 36(8):1512–1519
Dolmetsch RE, Lewis RS, Goodnow CC (1997) Differential activation of transcription factors induced by Ca2+ response amplitude and duration. Nature 386:855–858
Dolmetsch RE, Pajvani U, Fife K et al (2001) Signaling to the nucleus by an L-type calcium channel-calmodulin complex through the MAP kinase pathway. Science 294:333–339
Doyle LW, Crowther CA, Middleton P et al (2009) Magnesium bias sulphate for women at risk of preterm birth for neuroprotection of the fetus. Cochrane Database Syst Rev 1:CD004661
Dragunow M, Beiharz E, Sirimanne E et al (1994) Immediately early gene protein expression in neurons undergoing delayed death, but not necrosis following hypoxic-ischemic injury to the young rat brain. Brain Res Mol Brain Res 25:1933
Eicher DJ, Wagner CL, Katikaneni LP et al (2005) Moderate hypothermia in neonatal encephalopathy: safety outcomes. Pediatr Neurol 32:18–24
Faraci FM (1991) Role of endothelium-derived relaxing factor in cerebral circulation: large arteries vs. microcirculation. Am J Physiol 261:H1038–H1042
Ferrer I, Tortosa A, Macaya A et al (1994) Evidence of nuclear DNA fragmentation following hypoxia-ischemia in the infant rat brain, and transient forebrain ischemia in the adult gerbil. Brain Pathol 4:115–122
Fields RD, Esthete F, Stevens B et al (1997) Action potential-dependent regulation of gene expression: temporal specificity in Ca2+, cAMP-responsive element binding proteins, and mitogen-activated protein kinase signaling. J Neurosci 17:7252–7266
Fritz K, Delivoria-Papadopoulos M (2006) Mechanisms of injury to the newborn brain. Clin Perinatol 33:573–591
Fritz KI, Groenenedaal F, McGowan JE et al (1996) Effects of 3- (2-carboxy-piperzine-4-yl) propyl-1-phosphonic acid (CPP) on NMDA receptor binding characteristics and brain cell membrane function during cerebral hypoxia in newborn piglets. Brain Res 729:66–74
Ghosh A, Greenberg ME (1995) Calcium signaling in neurons: molecular mechanisms and cellular consequences. Science 268:239–247
Gillardon F, Lenz C, Waschle KF (1996) Altered expression of Bcl- 2, Bcl-X, Bax and c-Fos colocalizes with DNA fragmentation and ischemic cell damage following middle cerebral artery occlusion in rats. Brain Res Mol Brain Res 40:254–260
Gluckman PD, Wyatt JS, Azzopardi D et al (2005) Selective head cooling with mild systemic hypothermia after neonatal encephalopathy: multicenter randomized trial. Lancet 365:663–670
Gow AJ, Duran D, Malcom S et al (1996) Effect of peroxynitrite-induced protein modification on tyrosine phosphorylation and degradation. FEBS Lett 385:63–66
Gunn AJ, Gunn TR (1998) The 'pharmacology' of neuronal rescue with cerebral hypothermia. Early Hum Dev 53:19–35
Gunn AJ, Gluckman PD, Gunn TR (1998) Selective head cooling in newborn infants after perinatal asphyxia: a safety study. Pediatrics 102:885–892
Gunn AJ, Bennet L, Gunning MI et al (1999) Cerebral hypothermia is not neuroprotective when started after postischemic seizures in fetal sheep. Pediatr Res 46:274–280

Gunn AJ, Battin M, Gluckman PD et al (2005) Therapeutic hypothermia: from lab to NICU. J Perinat Med 33:340–346

Hamada Y (1994) Inhibitors of nitric oxide synthesis reduce hypoxic-ischemic brain damage in the neonatal rat. Pediatr Res 35:10–14

Hameed A, Olsen KJ, Lee MK et al (1989) Cytolysis by Ca-permeable transmembrane channels: pore formation causes extensive DNA degradation and cell lysis. J Exp Med 169:765–777

Hardingham GE, Bading H (1998) Nuclear calcium: a key regulator of gene expression. Biometals 11:345–358

Hardingham GE, Chawla S, Cruzalegui FH, Bading H (1999) Control of recruitment and transcription-activating function of CBP determines gene regulation by NMDA receptors and L-type calcium channels. Neuron 22:789–798

Higgins RD, Rahu TN, Perlman J et al (2006) Hypothermia and perinatal asphyxia: executive summary of the national institute of child health and human development workshop. J Pediatr 148:170–175

Hill A, Volpe J (1999) Hypoxic-ischemic cerebral injury in the newborn. In: Swaiman KF, Ashwal S (eds) Pediatric neurology, principles and practice. Mosby, St. Louis, pp 191–204

Hoeger H, Engidawork E, Stolzlechner D et al (2006) Long-term effect of moderate and profound hypothermia on morphology, neurological, cognitive and behavioural functions in a rat model of perinatal asphyxia. Amino Acids 31:385–396

Hoffman DJ, Marro PJ, McGowan JE et al (1994a) Protective effect of MgSO4 infusion on NMDA receptor binding characteristics during cerebral cortical hypoxia in newborn piglets. Brain Res 644:144–149

Hoffman DJ, McGowan JE, Marro PJ et al (1994b) Hypoxia-induced modification of the N-methyl-D-aspartate (NMDA) receptor in the brain of newborn piglets. Neurosci Lett 167:156–160

Huang Z (1994) Effects of cerebral ischemia in mice deficient neuronal nitric oxide. Science 265:1883–1885

Ishida R, Akiyoshi H, Takahashi T (1974) Isolation and purification of calcium and magnesium dependent endonuclease from rat liver nuclei. Biochem Biophys Res Commun 56:703–710

Jacobs SE, Berg M, Hunt R, Tarnow-Mordi WO, Inder TE, Davis PG (2013) Cooling for newborns with hypoxic ischaemic encephalopathy. Cochrane Database Syst Rev 1:CD003311.10.1002/14651858.CD003311.pub3

Johnston MV (1995) Neurotransmitters and vulnerability of the developing brain. Brain Dev 17:301–306

Kapetanakis A, Azzopardi D, Wyatt J et al (2008) Therapeutic hypothermia for neonatal encephalopathy: a UK survey of opinion, practice and neuron-investigation at the end of 2007. Acta Paediatr 98:631–635

Kiedrowski I, Costa E, Wroblewski JT (1992) Glutamate receptor agonist stimulate nitric oxide synthase in primary cultures of cerebellar granule cells. J Neuroch 58:335–341

Kitada S, Krajewski S, Miyashita T (1996) Gamma-radiation induces upregulation of Bax protein and apoptosis in radiosensitive cells in vivo. Oncogene 12:187–192

Kratimenos P, Koutroulis I, Marconi D et al (2014) Multi-targeted molecular therapeutic approach in aggressive neuroblastoma: the effect of Focal Adhesion Kinase–Src–Paxillin system. Expert Opin Ther Targets 18(12):1395–1406

Kratimenos P, Koutroulis I, Agarwal B, Theocharis S, Delivoria-Papadopoulos M (2017) Effect of concurrent Src kinase inhibition with short-duration hypothermia on Ca2+/calmodulin kinase IV activity and neuropathology after hypoxia-ischemia in the newborn swine brain. Sci Rep 7(1):16664. https://doi.org/10.1038/s41598-017-16983-1

Kratimenos P, Koutroulis I, Jain A, Malaeb S, Delivoria-Papadopoulos M (2018) Effect of Src kinase inhibition on cytochrome c, Smac/DIABLO and apoptosis inducing factor (AIF) following cerebral hypoxia-ischemia in newborn piglets. Neonatology 113(1):37–43. https://doi.org/10.1159/000480067

Kurinczuk JJ, White-Koning M, Badawi N (2010) Epidemiology of neonatal encephalopathy and hypoxic-ischaemic encephalopathy. Early Hum Dev 86(6):329–338

Lawn JE, Cousens S, Zupan J (2005) 4 million neonatal deaths: when? Where? Why? Lancet 365(9462):891–900

Lee J, Kim MS, Park C et al (2004) Morphine prevents glutamate-induced death of primary rat neonatal astrocytes through modulation of intracellular redox. Immunopharmacol Immunotoxicol 26:17–28

Legido A (1994) Perinatal hypoxic-ischemic encephalopathy: current advances in diagnosis and treatment. Int Pediatr 9:114–136

Legido A, Katsetos CD, Mishra OP et al (2001) Perinatal hypoxia-ischemia encephalopathy: current and future treatments. Int Pediatr 15:143–151

Lerea L, McNamara JO (1993) Ionotropic glutamate receptor subtypes activate c-fos transcription by distinct calcium-requiring intracellular signaling pathways. Neuron 10:31–41

Levene MI, Evans DJ, Mason S et al (1999) An international network for evaluation neuroprotective therapy after severe birth asphyxia. Sem Perinatol 23:226–233

Lim YJ, Zheng S, Zuo Z (2004) Morphine preconditions Purkinje cells against cell death under in vitro simulated ischemia- reperfusion conditions. Anesthesiology 100:562–568

Linnik MD, Zobirst RH, Hatfield MD (1993) Evidence supporting a role for programmed cell death in focal cerebral ischemia in rats. Strokes 24:2002–2008

Lipton S (1999) Redox sensitivity of NMDA receptor. Meth Mol Biol 128:121–130

Maro PJ, Hoffman D, Schneiderman R et al (1998) Effect of allopurinol on NMDA receptor modification following recurrent asphyxia in newborn piglets. Brain Res 787:71–77

Marro PJ, McGowan JE, Razdan B et al (1994) Effect of allopurinol on uric acid levels and brain cell membrane Na+, K+-ATPase activity during hypoxia in newborn piglets. Brain Res 650:9–15

Mayer ML, Westbrook GL, Guthrie PB (1984) Voltage-dependent block by Mg++ of NMDA responses in spinal cord neurons. Nature 309:261–263

Mayfield KP, D'Alecy LG (1992) Role of endogenous opioid peptides in the acute adaptation to hypoxia. Brain Res 582:226–231

Mayfield KP, D'Alecy LG (1994) Delta-1 opioid agonist acutely increases hypoxic tolerance. J Pharmacol Exp Ther 268:683–688

Miller JA (1971) New approaches to preventing brain damage during asphyxia. Am J Obstet Gynecol 110:125–132

Mishra OP, Delivoria-Papadopoulos M (1992) NMDA receptor modification of the fetal guinea pig brain during hypoxia. Neurochem Res 17:1211–1216

Mishra OP, Delivoria-Papadopoulos M (1999) Cellular mechanisms of hypoxic injury in the developing brain. Brain Res Bull 48:233–238

Mishra OP, Delivoria-Papadopoulos M (2000) Hypoxia-induced generation of nitric oxide free radicals in cerebral cortex of newborn guinea pigs. Neurochem Res 25:1559–1565

Mishra OP, Delivoria-Papadopoulos M (2001) Effect of graded hypoxia on high-affinity Ca2+-ATPase activity in cortical neuronal nuclei of newborn piglets. Neurochem Res 26:1335–1341

Mishra OP, Delivoria-Papadopoulos M (2002) Nitric oxide-mediated Ca++-influx in neuronal nuclei and cortical synaptosomes of normoxic and hypoxic newborn piglets. Neurosci Lett 318:93–97

Mishra OP, Delivoria-Papadopoulos M (2006) Effect of neuronal nitric oxide synthase inhibition on caspase-9 activity during hypoxia in the cerebral cortex of newborn piglets. Neurosci Lett 401:81–85

Mishra OP, Delivoria-Papadopoulos M (2010) Mechanism of tyrosine phosphorylation of procaspase-9 and Apaf-1 in cytosolic fractions of the cerebral cortex of newborn piglets during hypoxia. Neurosci Lett 480:35–39

Mishra OP, Fritz KI, Delivoria-Papadopoulos M (2001) NMDA receptor and neonatal hypoxic brain injury. Ment Retard Dev Disabil Res Rev 7:249–253

Mittendorf R, Covert R, Boman J et al (1997) Is tocolytic magnesium sulphate associated with increased total pediatric mortality? Lancet 350:1517–1519

Monaghan DT, Olvenman HJ, Nguyen L et al (1988) Two classes of N-methyl-D-aspartate recognition sites: differential distribution and differential regulation by glycine. Proc Natl Acad Sci U S A 85:9836–9840

Monaghan DT, Bridges RJ, Cotman CW (1989) The excitatory amino acid receptors: their classes, pharmacology, and distinct properties in the function of the central nervous system. Annu Rev Pharmacol Toxicol 29:365–402

Moriette G, Barrat J, Truffert P et al (2008) Effect of magnesium sulphate on mortality and neurologic morbidity of the very preterm newborn (of less than 33 weeks) with two-year neurological outcome: results of the prospective PREMAG trial. Gynecol Obstet Fertil 36:278–288

Nelson KB, Grether JK (1995) Can magnesium sulphate reduce the risk of cerebral palsy in very low birth weight infants? Pediatrics 95:263–269

Nowak L, Bregetovski P, Ascher P et al (1984) Magnesium gates glutamate-activated channels in mouse central neurons. Nature 307:462–465

Nowicki JP (1991) Nitric oxide mediates neuronal death after focal cerebral ischemia in the mouse. Eur J Pharmacol 204:339–340

Numagami Y (1997) Lipid free radical generation and brain cell membrane alteration following nitric oxide synthase inhibition during cerebral hypoxia in the newborn piglet. J Neurochem 69:1542–1547

Oltvai ZN, Milliman CL, Korsmeyer SJ (1993) Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death. Cell 74:609–619

Palmer C, Roberts RL (1991) Reduction of perinatal brain damage with oxypurinol treatment after hypoxic-ischemic injury. Pediatr Res 29:362–368

Palmer C, Vanucci RC, Towfighi J (1990) Reduction of perinatal hypoxic-ischemic brain damage with allopurinol. Res Pediatr 27:332–336

Parikh NA, Lasky RE, Garza CN et al (2009) Volumetric and anatomical MRI hypoxic-ischemic encephalopathy: relationship to hypothermia therapy and neurosensory impairments. J Perinatol 29:143–149

Radi R (1991) Peroxynitrite-induced membrane lipid peroxidation: the cytotoxic potential of superoxide and nitric oxide. Arch Biochem Biophys 288:481–487

Raichle ME (1983) The pathophysiology of brain ischemia. Ann Neurol 13:2–10

Ravishankar S, Ashraf QM, Mishra OP et al (2001) Expression of Bax and Bcl-2 proteins during hypoxia in cerebral cortical neuronal nuclei of newborn piglets: effect of administration of magnesium sulfate. Brain Res 901:23–29

Reed JC (1996) Mechanisms of Bcl-2 family protein function and dysfunction in health and disease. Behring Inst Mitt 97:72–100

Rosenbaum DM, Michaelson M, Batter DK et al (1994) Evidence for hypoxia induced programmed cell death of cultured neurons. Ann Neurol 25:19–33

Rothman SM, Olney JW (1986) Glutamate and the pathophysiology of hypoxic-ischemic brain damage. Ann Neurol 19:105–111

Rouse D, Hirtz DG, Thom E et al (2008) A randomized controlled trial of magnesium sulfate for the prevention of cerebral palsy. N Engl J Med 359:895–905

Russell GA, Cooke RW (1995) Randomized controlled trial of allopurinol prophylaxis in very preterm infants. Arch Dis Child Fetal Neonatal Ed 73:F27–F31

Sahni R, Sanocka UM (2008) Hypothermia for hypoxic-ischemic encephalopathy. Clin Perinatol 35:717–734

Sawyer DT (1981) How super is superoxide? Acc Chem Res 14:393–400

Shankaran S, Laptook A, Wright LL et al (2002) Whole-body hypothermia for neonatal encephalopathy: animal observations as a basis for randomized, controlled pilot study in term infants. Pediatrics 110:377–385

Shankaran S, Laptook AR, Ehrenkranz RA et al (2005) Whole-body hypothermia for neonates with hypoxic-ischemic encephalopathy. N Engl J Med 353:1574–1584

Shankaran S, Pappas A, McDonald SA, Vohr BR, Hintz SR, Yolton K et al (2012) Childhood outcomes after hypothermia for neonatal encephalopathy. N Engl J Med 366:2085–2092. https://doi.org/10.1056/NEJMoa1112066

Tacconi S, Ratti E, Marien MR et al (1993) Inhibition of (3H)- (+)-MK-801 binding to rat brain sections by CPP and 7-chlorokynurenic acid: an autoradiographic analysis. Br J Pharmacol 108:668–674

Talati AJ, Yang W, Yolton K et al (2005) Combination of early perinatal factors to identify near-term and term neonates for neuroprotection. J Perinatol 25: 245–250

Tan S, Parks DA (1999) Preserving brain function during neonatal asphyxia. Clin Perinatol 26:733–747

Tang YP, Shimizu E, Dube GR et al (1999) Genetic enhancement of learning and memory in mice. Nature 401:63–69

The Eclampsia Trial Collaborative Group (1995) Which anticonvulsant for eclampsia? Evidence from the Collaborative Eclampsia Trial. Lancet 345: 1455–1463

Thorensen M, Penrice J, Lorek A (1995) Mild hypothermia after severe transient hypoxia-ischemia ameliorates delayed cerebral energy failure in the newborn piglet. Pediatr Res 37:667–670

Tominaga T, Kagure S, Narisawa K et al (1993) Endonuclease activation following focal ischemic injury in the rat brain. Brain Res 608:21–26

Trescher WH, Ishiwa S, Johnston MV (1997) Brief post-HI hypothermia markedly delays neonatal brain injury. Brain Dev 19:326–328

Van Bel F, Groenendaal F (2008) Long-term pharmalogic neuroprotection after birth asphyxia: where do we stand? Neonatology 94:203–210

Van Bel F, Shadid M, Moison RM et al (1998) Effect of allopurinol on postasphyxial free radical formation, cerebral hemodynamics, and electrical brain activity. Pediatrics 101:185–193

Vannucci RC (1990) Experimental biology of cerebral hypoxia-ischemia: relation to perinatal brain damage. Pediatr Res 27:317–326

Volpe J (2001) Neurology of the newborn, 3rd edn. WB Saunders, Philadelphia

Wagner CL, Eicher DJ, Katikkaneni LD et al (1999) The use of hypothermia: a role in the treatment of neonatal asphyxia? Pediatr Neurol 21:429–443

Wagner BP, Nedelcu J, Martin E (2002) Delayed postischemic hypothermia improves long-term behavioral outcome after cerebral hypoxia-ischemia in neonatal rats. Pediatr Res 51:182–193

Waseem W, Ashraf QM, Zanelli SA et al (2001) Effect of graded hypoxia on cerebral cortical genomic DNA fragmentation in newborn piglet. Biol Neonate 79:187–193

Williams GD, Palmer C, Heitjan DF et al (1992) Allopurinol preserves cerebral energy metabolism during perinatal hypoxic-ischemia: a 31P NMR study in anaesthetized immature rats. Neurosci Lett 144:104–106

Wylie AH, Kerr JFR, Currie AR (1980) Cell Death, the significance of apoptosis. Int Rev Cytol 68:251–306

Yamakura T, Sakimura K, Shimoji K (1999) Direct inhibition of the N-methyl-D-aspartate receptor channel by high concentration of opioids. Anesthesiology 91:1053–1063

Zanelli SA (1999) NMDA receptor-mediated calcium influx in cerebral cortical synaptosomes of the hypoxic guinea pig fetus. Neurochem Res 24:434–446

Zanelli SA, Ashraf QM, Mishra OP (2002) Nitration is a mechanism of regulation of the NMDA receptor function during hypoxia. Neuroscience 112:869–877

Zanelli SA, Naylor M, Dobbins N et al (2008) Implementation of a "hypothermia for HIE" program: 2-year experience in a single NICU. J Perinatol 28:171–175

Zhang J, Haddad GG, Xia Y (2000) Delta-, but not mu- and kappa, opioid receptor activation protects neocortical neurons from glutamate- induced excitotoxic injury. Brain Res 885:143–153

Zhang J, Gibney GT, Zhao P (2002) Neuroprotective role of delta opioid receptors in cortical neurons. Am J Physiol 282:C1225–C1234