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Neuronal Death and Oxidative Stress in the Developing Brain

Chrysanthy Ikonomidou[1] and Angela M. Kaindl[2]

Abstract

The developing brain is particularly vulnerable to reactive oxygen and reactive nitrogen species–mediated damage because of its high concentrations of unsaturated fatty acids, high rate of oxygen consumption, low concentrations of antioxidants, high content of metals catalyzing free radical formation, and large proportion of sensitive immature cells. In this review, we outline the dynamic changes of energy resources, metabolic requirements, and endogenous free radical scavenging systems during physiologic brain development. We further discuss the involvement of oxidative stress in the pathogenesis of neuronal death after exposure of the infant brain to hyperoxia, hypoxia/ischemia, sedative drugs, ethanol, and mechanical trauma. Several approaches have been developed to combat oxidative stress, but neuroprotective treatment strategies are limited in the clinical setting. *Antioxid. Redox Signal.* 14, 1535–1550.

Introduction

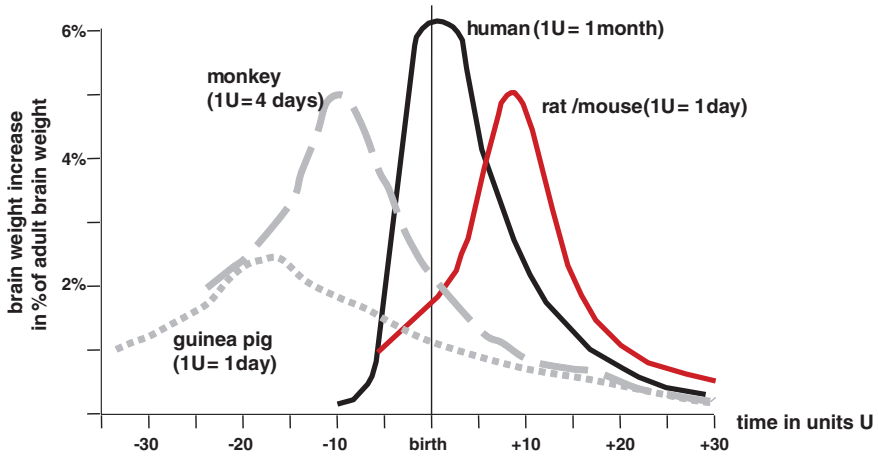
Growth and maturation of the mammalian brain can be divided into six phases: (a) neural cell genesis followed by (b) neuronal migration, (c) glial cell proliferation, (d) axonal and dendritic proliferation, (e) synaptogenesis and appearance of electrical activity, and (f) axonal myelination (46, 79, 113, 140). These developmental steps can occur at different times in various brain regions, often after a caudal-to-rostral progression of development in brain ontogeny. Anatomic, biochemical, and functional changes occur first in phylogenetically older brain regions. Also, maturation is an "inside-out'' phenomenon in the cerebral cortex (*i.e.*, the deeper layers develop morphologically and functionally before the relatively younger outer layers) (80, 114, 115, 141). Globally, the brain does not grow at a uniform pace throughout development but rather undergoes a period when it increases its weight most rapidly, the so-called ''brain growth spurt'' (39–41). Weight increase during this developmental period is largely due to glial cell multiplication and myelination (neuronal proliferation being already almost complete). The timing of the brain growth-spurt period varies among mammalian species and can occur prenatally, such as in humans, guinea pigs, sheep, and monkeys, or postnatally (*e.g.*, in rats and rabbits) (see Fig. 1) (34, 37–40).

More than half of the initially formed neurons are deleted during normal development through programmed cell death (PCD), a phenomenon by which unsuccessfully connected neurons are deleted by apoptosis (cell suicide). The term apoptosis is used to describe specific morphologic manifestations of PCD and is characterized by a sequence of very distinctive morphologic changes in the dying neuron (35, 86). Many of the effectors of this developmental cell-death program are highly expressed in the developing brain, making it more susceptible to accidental activation of the death machinery. When cells are exposed to various stressors, triggering and potential overshooting of cell-death pathways can occur. Oxidative stress is one of the pathomechanisms that can trigger cell death in the developing brain.

Energy Resources During Development

The growing mammalian brain is a very dynamic system in terms of energy metabolism. In general, an increase in the activities of energy-producing pathways occurs throughout development, and this occurs in response to an increasing demand by ATP-consuming reactions that aim mainly at maintaining ionic equilibrium (46). Both in the immature and the adult mammalian brain, a close positive correlation exists between neural electrical activity and mitochondrial density (188), cytochrome oxidase activity, cerebral metabolic rate for glucose, cerebral blood flow, and blood vessel density (19). Erecinska and colleagues (46) nicely reviewed studies on brain morphogenesis and energy-producing pathways during development of mammalian brains. In their review, they concluded that energy-producing pathways develop to support the key brain ATP-consuming reaction, the Na^+/K^+ pump, which maintains and restores ionic gradients and generates electrical potentials (46). It has been proposed that

[1]Department of Neurology, University of Wisconsin, and Waisman Center, Developmental Brain Injury Laboratory, Madison, Wisconsin.
[2]Department of Pediatric Neurology, and Waisman Institute of Cell Biology and Neurobiology, Center for Anatomy, Charité-Universitätsmedizin Berlin, Berlin, Germany.



HUMANS (WEEKS)	3.5-4.0	4.1-5.2	5.3-5.7	5.8-6.6	6.7-7.0	7.1-7.4	7.5-7.9	8.0-9.9	10.0-11.9	12.0-14.9	15.0-18.9	19.0-23.9	24.0-27.9	28.0-31.9	32.0-35.9	36.0-40.0
RATS (DAYS)	GD11	GD12	GD13	GD14	GD15	GD16	GD17	GD18	GD19	GD20	GD21-22	PND 0-3	PND 4-7	PND 8-11	PND 12-15	PND 16-19
Spinal cord																
Medulla																
Pons																
Cerebellum																
Mesencephalic tegmentum																
Mesencephalic tectum																
Thalamus																
Hypothalamus																
Pallidum																
Striatum																
Amygdala																
Neocortex and limbic cortex																
Entorhinal cortex																
Subiculum																
Hippocampus CA1-3																
Hippocampus dentate granule cells																

FIG. 1. Brain growth-spurt phase in various mammalian species. The developing brain experiences a period of rapid growth during which various otherwise innocuous environmental factors cause widespread apoptotic neuronal death. The brain growth spurt starts at about midpregnancy in humans and extends well into the third postnatal year. In mice and rats, this developmental period occurs within the first 3 postnatal weeks. In contrast, monkeys and guinea pigs are predominantly prenatal brain developers. Modified figure from Dobbing and Sands, 1973 (39). The table shows estimated timeline of neurogenesis in rats and humans. GD, gestational day; PND, postnatal day. (Adapted from reference 85.) (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

energy expenditure for the delivery of energy to the brain is minimized and the risk of a compromise for energy-consuming neuronal activity is reduced through the close spatial organization of neurons and blood vessels (189).

Mitochondria and Development

Changes in number, size, and morphology of brain cells occurring during development are accompanied by structural alterations within mitochondria. An electron-microscopic study of rat inferior colliculus has shown that in the neuropil, the mitochondrial volume fraction and the number of mitochondria are low during the first week of postnatal life, increase rapidly by about 6 times between postnatal day (P) 7 and 25 and thereafter increase gradually until adulthood (148). The mean size of mitochondria remains relatively stable, but the number of cristae almost doubles. This combined effect of increases in the mitochondrial volume and the content of cristae results in an overall 11-fold enhancement in mitochondrial capacity throughout development. Moreover, the number of mitochondria per cell also increases (65, 152) as does the amount of mitochondrial protein per gram of tissue until around P21 in the rat (30, 102). The developmental profile of mitochondria coincides with developmental processes such as neuronal differentiation and synaptogenesis. Augmentations in mitochondrial number, volume, and cristae are related to increasing levels of synaptic activity (30, 46, 102).

Age Dependence of Metabolic Requirements

Metabolic substrate preferences of the mammalian brain change during development. Shortly after birth, the brain switches its metabolism briefly to lactate. At birth, plasma glucose concentrations are 50% the adult levels and achieve adult levels at P10 in rats. During the suckling period, the brain metabolizes glucose and ketone bodies. The capacity of the immature brain to take up and metabolize ß-hydroxybutyrate (ßOHB) can be up to 6 times greater than that of the adult rat brain (29, 72, 130, 173). Suckling animals display higher systemic concentrations of ketones, greater

numbers of blood–brain-barrier transporters, and greater enzymatic activities of ketone-metabolizing enzymes (18, 181). On weaning, arterial ketone concentrations decrease, cerebral uptake decreases, and the monocarboxylate transporters are downregulated. After weaning, mammals switch to glucose as the main energy substrate. An increase in systemic glucose availability precedes increase in the expression of cerebral glucose transporters Glut1 and Glut3 (44, 181) and activity of glycolytic enzymes (103–105). These parameters reach maturation around P30 in the rat (130).

Maturation of Endogenous Free Radical–Scavenging Systems

Free radical production is a normal part of cellular physiology. Oxidative stress results from an imbalance between overproduction of reactive oxygen species (ROS) and reactive nitrogen species (RNS) and a deficiency of antioxidants, resulting in (per)-oxidations of lipids, proteins, and DNAs (175). ROS include superoxide ($O_2^{\cdot}S$), hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\cdot}OH$), and peroxyl radical ($ROO^{\cdot}$), whereas RNS include nitric oxide ($NO^{\cdot}$) and the highly toxic peroxynitrite (ONOOS). The brain is particularly vulnerable to oxidative damage because of its high oxygen utilization, its high content of oxidizable polyunsaturated fatty acids, and the presence of redox-active metals (Cu and Fe). The defense systems against oxidative and nitrosative stress consist of enzymes such as superoxide dismutase (SOD), glutathione peroxidases (GPx), catalase, and of nonenzymatic antioxidants, which include glutathione (GSH), ascorbic acid (vitamin C), α-tocopherol (vitamin E), carotenoids, and flavonoids (175).

The expression of antioxidant systems changes with brain maturation (5, 131). During early development, cytoplasmic antioxidant activities are generally higher, whereas the primary mitochondrial antioxidant activity is low. During this developmental period, age-related differences in endogenous reactive oxygen species (ROS) levels are found. Rat striatal synaptosomes from P7, P12, and P21 have higher ROS levels than do adult synaptosomes (43). After methylmercury application to induce elevated ROS levels, synaptosomes from the younger brains show greater ROS production than do those from adults. Infant animals, serving as models for premature babies, have been found to be highly susceptible to oxidative stress for several reasons.

1. They have decreased levels of antioxidant enzymes/scavengers in their tissue and serum (51,106,108,120, 161,162,165). Serum vitamin E levels, for example, have been found to be 80% lower in premature infants than in adults and to reach adult levels only after about 8 weeks of age, even with supplementation (95, 106, 153). Antioxidant defenses in preterm infants are also compromised by relative deficiencies in selenium and taurine (2, 27, 78, 109, 172).
2. They fail sufficiently to synthesize/upregulate antioxidant enzymes such as glutathione peroxidase and glutathione (55, 68). As a result, the free radical–trapping capacity in plasma of premature newborns is diminished and continues to decline during the initial postnatal period (176, 187). These findings are consistent with the idea that immature mitochondrial free radical scavenging systems render the younger brain more vulnerable to oxidative stress.

Oxidative Stress and Brain Damage in the Preterm and Term Infant

Oxidative stress is involved in the pathogenesis of early developmental brain injuries due to hyperoxia, hypoxia/ischemia, drugs, and mechanical trauma.

Oxygen toxicity

Oxygen is essential for life, but can also cause damage when administered in relative excess or to patients with impaired antioxidant defenses or both. Principles on the use of oxygen in neonatal medicine have changed dramatically in the last decades, based on compelling evidence that high oxygen concentrations during the neonatal period are associated with oxidative stress–induced damage to various organs, including the eyes (*e.g.*, retinopathy of prematurity), the lung (*e.g.*, bronchopulmonary dysplasia), and the brain (*e.g.*, cerebral palsy). Oxygen was first given to babies as a respiratory gas in the late 19th century, and its continuous administration was still recommended in the early 20th century (160). It was only in 2006, when the International Liaison Committee on Resuscitation (ILCOR) officially warned against these adverse effects and stated that room air is as effective as 100% oxygen for the resuscitation of most infants at birth (168).

Premature infants are particularly sensitive to the deleterious effects of oxygen, and this has been attributed in part to their immature defense mechanisms against oxidative stress, to oxygen-induced vasoconstriction leading to reduced brain perfusion, and to the susceptibility of the rapidly developing brain itself to environmental changes (23, 151). Conditions after premature birth are always hyperoxic relative to conditions *in utero*, where the average arterial oxygen tension (Pao_2) is extremely low, with about 32 mm Hg in the umbilical vein and 22 mm Hg in the descending aorta. Fetal hemoglobin exhibits a shifted hemoglobin dissociation curve and is, under physiologic conditions, saturated to 90% with oxygen at a Pao_2 of about 40 mm Hg (45). Under relatively hypoxic conditions, this enables an efficient maternal–fetal oxygen transfer at the placental interface and thus adequate oxygenation of the fetus. Immediately after birth, the Pao_2 increases to adult levels of about 100 mm Hg (157), and mechanical ventilation or oxygen supplementation or both used to ensure adequate tissue oxygenation and pulmonary vasodilation may lead to "relative hyperoxia" (*i.e.,* unphysiologically high oxygen levels). This increase in Pao_2 may be even more dramatic in asphyxiated infants with abnormally low oxygen tension, and the consequences may be most dramatic in preterm infants who are physiologically unprepared and exhibit developmental immaturity of their free radical defenses (24). These immature newborns are also more likely to encounter further situations of increased oxidative stress, such as oxygen supplementation, to aid impaired respiration, or systemic infections.

Although many studies have described the role of oxygen in the emergence of retinopathy of prematurity and bronchopulmonary dysplasia (54), only a few research teams have focused on the effect of hyperoxia on the immature brain. In 2004, we demonstrated that an increased oxygen tension of 80% leading to an Sao_2 of 100% and a Pao_2 of about 180 mm Hg causes oxidative stress in the brain and is a powerful trigger for widespread apoptotic cell death in the grey and white matter of immature brains from 7-day-old rats (Fig. 2) (48). Hyperoxia-induced cell death is age dependent: On P0,

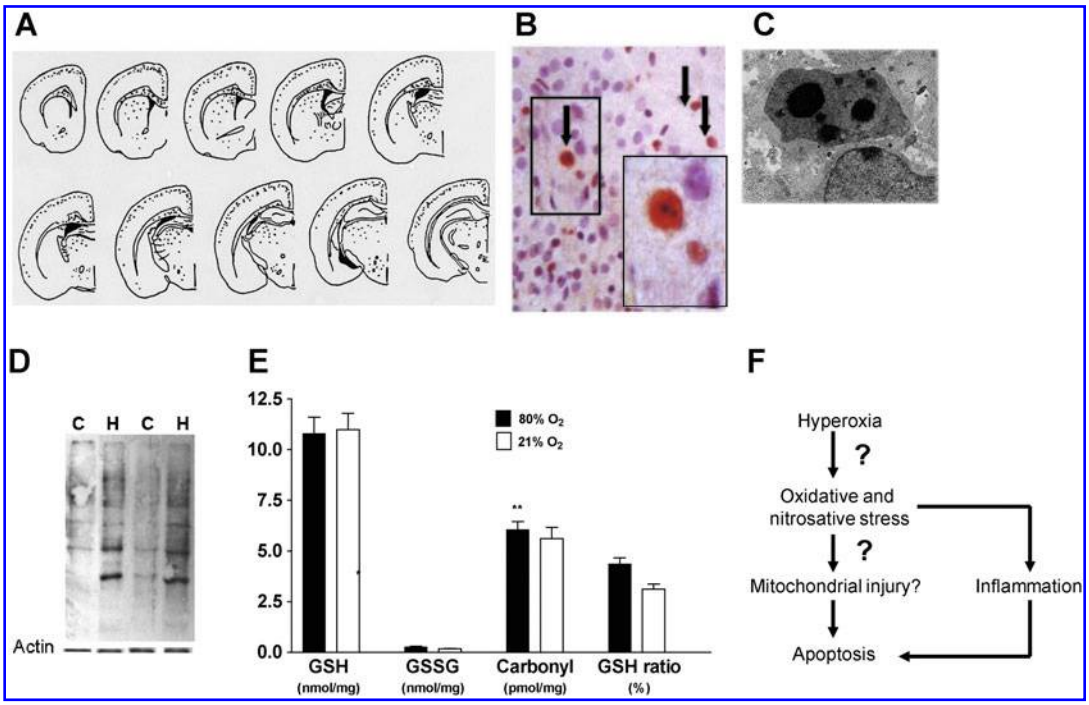


FIG. 2. Hyperoxia causes cell death and oxidative stress in the developing rodent brain. (A) Distribution pattern of hyperoxia-induced apoptosis in infant rats. **(B)** Immunohistochemical staining for activated caspase 3 is shown in the laterodorsal thalamus of a rat subjected to hyperoxia. Many activated caspase 3–positive cells are found (*arrows*). The magnified view of the framed area illustrates that immunopositive cells for activated caspase 3 show pycnotic changes in the nuclei that are indicative of apoptosis. **(C)** An electron micrograph from a cortical neuron in the brain of an infant rat subjected to hyperoxia at the end of a 12-h exposure. The neuron is in a middle stage of apoptosis and demonstrates formation of spherical chromatin masses, intermixing of nucleoplasmic and cytoplasmic contents and condensation. **(D)** Analysis of brain lysates from hyperoxia-treated (H) and control mice (C) after SDS-PAGE reveals increased levels of protein carbonyls after hyperoxia. Incubation of the same blots with anti-actin antibody confirms equivalent loading of proteins in each lane. **(E)** Levels of reduced and oxidized glutathione (GSH and GSSG), protein carbonyls, and the lipid peroxidation product malondialdehyde (MDA) in hyperoxic rat brains. A significant increase of GSSG and the GSSG/GSH ratio was detected in the brains of rats exposed to hyperoxia compared with those exposed to room air. Protein carbonyls showed a trend toward increase in the brains of rats exposed to hyperoxia. **(F)** Diagram showing what is known about the effects of hyperoxia on neuronal death in the developing brain. The involved pathomechanisms are largely unknown. Figures modified from reference 48. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

mainly thalamic nuclei, caudate nucleus, putamen, hypothalamus, and white matter tracts were affected. The most severe involvement of cortical areas was seen at the age of 7 days, and the highest overall vulnerability at the ages of 3 and 7 days. In 14-day- old rat pups, some degenerating cells were detected within the dentate gyrus after exposure to 80% oxygen over a 24-h period (48). When hyperoxia is applied to rat pups from birth to postnatal day 5, they develop a significant reduction of their brain weight (194). Moreover, hyperoxia is associated with an increased production of reactive oxygen intermediates (48, 90, 198), upregulation of proinflammatory cytokines, including interleukin (IL) 1β and IL18, decreased expression of neurotrophins, and decreased activation of neurotrophin-regulated pathways (48, 51). Mice deficient in IL-1 receptor–associated kinase 4 (IRAK4), which is pivotal for both IL-1β and IL-18 signal transduction, were protected from hyperoxia-mediated neurotoxicity (51). Moreover, co-treatment with erythropoietin proved to be neuroprotective (156, 195).

Hyperoxia-induced brain damage also affects the white matter of 7-day-old rats, leading to apoptotic cell death in pre-oligodendrocytes and immature oligodendrocytes but not in mature oligodendrocytes *in vivo* and *in vitro* and to a reduction of the myelin basic protein in P3, P6, but not P10 pups (58). This mechanism of white-matter damage may be relevant to the white-matter injury observed in infants born preterm and exposed to absolute or relative hyperoxia, because extensive oligodendrocyte migration and maturation occurs in the postconception weeks 23 to 32, and preoligodendrocytes and immature oligodendrocytes predominate during this time period (58). Cell death could be strongly reduced by (a) blockage of the caspase-dependent apoptotic pathway through the pancaspase inhibitor zVAD-fmk; (b) overexpression of BCL2 (*Homo sapiens* B-cell chronic lymphocytic leukemia/lymphoma 2); (c) application of the lipoxygenase inhibitors 2,3,5-trimethyl-6-(12-hydroxy-5-10-dodecadiynyl-1,4-benzoquinone and *N*-benzyl-*N*-hydroxy-5-phenyl-pentamide; and (d) co-treatment with erythropoietin or 17β-estradiol (59, 60, 156). Conversely, overexpression of superoxide dismutase SOD1 dramatically increased injury to pre-oligodendrocytes (OLs) but not to mature OLs (59). The superoxide dismutases convert superoxide into H_2O_2, which is further converted to water and oxygen by catalase or glutathione peroxidase.

To gain further insight into developmental events influenced by a premature exposure to high oxygen levels and to identify proteins engaged in neurodegenerative and reparative processes, we analyzed brain proteome changes 1 day, 1 week, and 1 month after 12 h of hyperoxia with 80% oxygen at P6. Protein changes were consistent with results of histologic and biochemical evaluation of the brains that revealed widespread apoptotic neuronal death and increased levels of protein carbonyls. Furthermore, we detected changes in proteins involved in synaptic function, cell proliferation, and formation of neuronal connections, suggesting interference of oxidative stress with these developmental events. These protein changes were age dependent, as they did not occur in mice subjected to hyperoxia in adolescence (90) and could be strongly inhibited by co-administration of erythropoietin (89).

Perinatal asphyxia

Whereas hyperoxia has only recently been acknowledged as a risk factor for perinatal brain injury, the central role of perinatal cerebral hypoxia/ischemia (HI) has long been known as a major cause of chronic disability. Approximately one to six of every 1,000 newborns will experience an HI insult, with a mortality rate of 15% to 20% (69, 178). Experimental models of HI have been studied in immature rats, rabbits, guinea pigs, and monkeys (81, 82, 150, 177, 179, 180). Studies examining the influence of age on histologic outcomes after HI injury revealed P2–P3 rat pups to be resistant to HI injury, whereas P7–P10 rats showed increasing cerebral lesions with age and with hypoxic duration (82, 171). Age-dependent and selective vulnerability of brain regions to HI injury are determined by susceptibility of the maturing neurons to excitotoxicity, vascular maturation, and metabolic demands. The metabolic preference for ketones appears to shape the vulnerability of the immature brain to HI insults. Hyperglycemia induced during HI worsens the histologic outcome in adults (129, 144) but is neuroprotective in P7 rats (180). Insulin-induced hypoglycemia before HI exacerbated injury in P7 rats (128, 191) but fasting-induced hypoglycemia or ß-hydroxybutyrate administration were both histologically neuroprotective in the immature rat (191). These findings suggest a relation between cerebral maturation and cerebral substrate utilization during injury.

Clinical observations from HI newborns and infants have shown that HI causes decreases in ATP and increases in

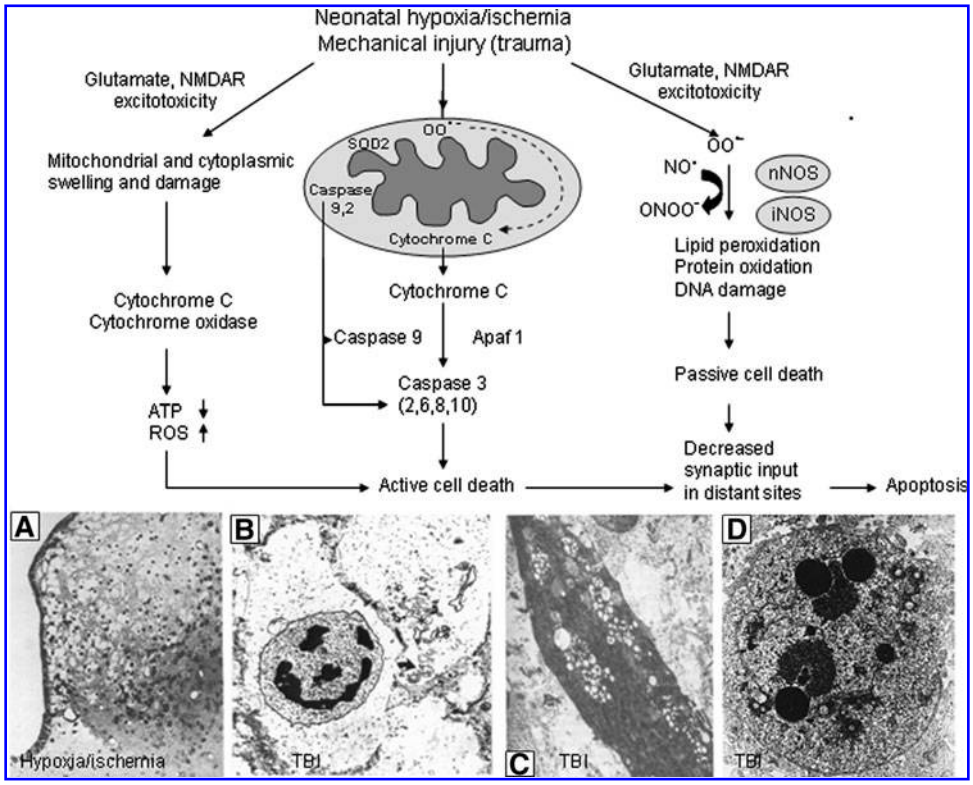


FIG. 3. Oxidative stress in hypoxic/ischemic and traumatic injury to the developing brain. Oxidative stress signaling after hypoxia/ischemia or traumatic brain injury. Injury generates reactive oxygen species (ROS) within the mitochondria, which then signal the release of cytochrome *c*. Cytochrome *c* binds to Apaf-1, followed by caspase-9, to form a complex that activates caspase-3 and other caspases. The activation of the *N*-methyl-D-aspartate (NMDA) receptor and formation of O_2^- and nitric oxide (NO) by neuronal nitric oxide synthase (nNOS) may lead to release of cytochrome *c* from the mitochondria or formation of peroxynitrite ($ONOO^-$). Resulting hydroxyl radicals can damage lipids, proteins, and DNA and lead to passive cell death. In the developing brain, delayed apoptotic neuronal death is observed in distant sites after hypoxia/ischemia or trauma and is thought to result partly from deafferentation of neuronal targets and the resulting lack of trophic support. During a period of ongoing programmed cell elimination, such events trigger neuronal suicide. (Modified from reference 176) **(A–D)** Examples of types of neuronal degeneration seen in the immature brain after hypoxia/ischemia or traumatic brain injury (TBI). **(A, B)** Neurons undergoing excitotoxic death in the P7 rat brain at 4 h after hypoxia/ischemia or TBI. Both cells display disrupted nuclear and cytoplasmic membranes, swollen mitochondria, and cytoplasmic debris. **(C)** A neuron 4 h after TBI in P7 rat brain displaying dark cytoplasm filled with vacuoles. This neuron is degenerating in a more-delayed fashion than that shown in **A** and **B**. **(D)** Electron micrograph depicting a neuron in an early stage of apoptosis within the cingulated cortex of a 7-day-old rat subjected to head trauma 16 h before it was killed. The nuclear chromatin has formed several electron-dense masses but is still confined to the nuclear compartment. The nuclear membrane has already begun to break down. (Electron micrographs adapted from references 35, 83, and 84.)

lactate levels due to anaerobic glucose metabolism with resulting ionic changes, membrane depolarization, and release of glutamate (67, 143). Cell damage and energetic breakdown induce a surge of glutamate that accumulates in the synaptic gap when such neurotransmitter levels reach a level that astrocytes cannot recycle at an adequate pace (Fig. 3) (87, 101, 145). In asphyxiated infants, CSF glutamate concentrations were significantly elevated at 16 h after birth (66, 142). The toxic effects of such high glutamate levels on brain tissue are referred to as excitotoxicity and are particularly deleterious in the developing brain (30). Pathologically high intrasynaptic glutamate levels activate in an excessive manner the postsynaptic glutamate receptors of the *N*-methyl-D-aspartate (NMDA), α-3-amino-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA), kainate (KA), and metabotropic (mGlu) type, and cause a massive influx of calcium. The latter can cause secondary damage, including cell death. This can activate several pathways leading to oxidative stress and ultimately neuronal cell death, for example, through high intracellular calcium level–induced mitochondrial dysfunction and increased production of free radicals (87, 100, 145). Plasma concentrations of the lipid peroxidation product malondialdehyde was increased fourfold within 12 to 24 h after birth in asphyxiated neonates (100). The increased fatty acid content, the immature antioxidant system, and the higher concentrations of free iron make the newborn brain particularly vulnerable to free radicals (122).

Mitochondria have been identified to be a major target of ROS attack and also a major site of ROS production through electron leakage from the electron-transport chain after ischemia (16, 17, 136, 173). These organelles are sensitive to ROS and peroxynitrite *in vitro*, and most data suggest that oxidative stress contributes to the postischemic impairment of mitochondrial respiration (53). When ROS levels exceed the capacity of a cell and its mitochondria to scavenge and render them harmless, the resulting oxidative stress may initiate mitochondrial permeability transition (mtPT; *i.e.*, an increase in mitochondrial membrane permeability through opening of mitochondrial permeability transition pores) (182). Proapoptotic factors are too large to pass directly through mtPT pores, but opening of mtPTs triggers the release of cytochrome *c* and other intermembrane proteins into the cytoplasm. In addition, the release of proapoptotic intermembrane proteins may also occur in an mtPT-independent fashion in the context of HI (98). Thus, oxidative stress can, directly or indirectly, influence the release of proapoptotic proteins (56, 125, 165). Drugs that block mtPT pore formation, like cyclosporin A, provide neuroprotection in adult models of ischemia and prevent the release of proapoptotic proteins (117, 197). In the developing brain, mtPT has been demonstrated to

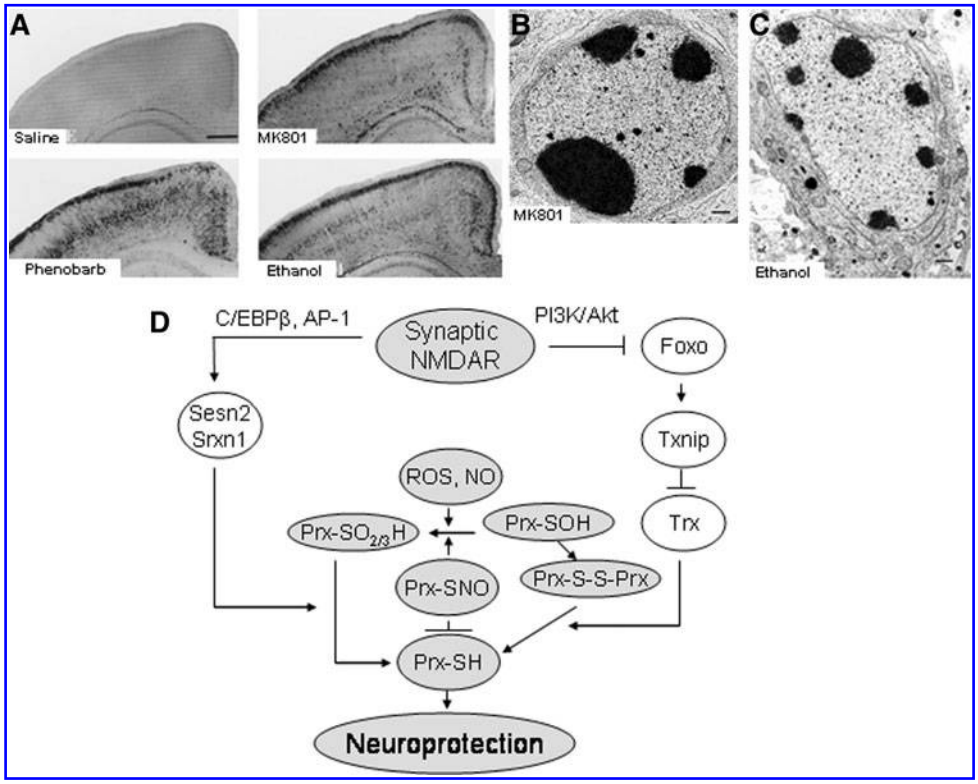


FIG. 4. Neurodegeneration induced by drugs in the developing brain. (A) Low-magnification light-microscopic overviews of silver-stained transverse sections from the parietal and cingulated cortex of 8-day-old rats treated 24 h previously with saline, the NMDAR antagonist MK801, the $GABA_AR$ agonist phenobarbital, or ethanol. Degenerating neurons (*small dark dots*) are present in several brain regions after MK801, phenobarbital, or ethanol, but are only sparsely present after saline treatment. **(B, C)** Electron micrographs depicting the early apoptotic changes induced in neurons by MK801 or ethanol. The nuclear membranes are intact, and the nucleus contains one or more large chromatin balls. Cytoplasmic organelles are relatively normal. **(D)** Stimulation of synaptic NMDARs strengthens antioxidant defenses in neurons. Stimulation of synaptic NMDARs activates the phosphatidylinositol 3 kinase (PI3K)–Akt signaling pathway and phosphorylates the transcription factor FOXO. Phosphorylated FOXO undergoes nuclear export and cannot activate transcription of Trx inhibitor (Txnip). Trx reduces oxidized Prxs (Prx-SOH or disulfide Prx-S-S-Prx) back to Prx so that they can inactivate ROS. Synaptic NMDAR channels allow Ca^{2+} influx, activation of the transcription factors C/EBPβ and AP-1, transcriptional activation of sulfiredoxin (Srxn1) and Sestrin2 (Sesn2), and reduction of overoxidized Prx-SO_2/3H back to Prx. Prx becomes available to detoxify additional ROS and protect from oxidative insults. Nitric oxide (NO) reacts with the same cysteine thiol groups on Prx as ROS to form Prx-SNO. RSH, protein thiol; Sesn2, Sestrin2; Srxn1, sulfiredoxin; -SH, sulfhydryl or thiol group; -SNO, *S*-nitrosylated derivative; -SOH, sulfenic acid derivative; and -SO_2/3H, sulfinic/sulfonic acid derivative (Modified from references 35, 80, 107).

occur after HI, as indicated by entrapment of deoxyglucose in mitochondria. However, in contrast to adult models of HI, cyclosporin A did not provide neuroprotection after HI in infant models (144).

The involvement of two of the three major superoxide dismutases, SOD1 (Cu,Zn-SOD), SOD2 (Mn-SOD), and SOD3 (EC-SOD), in HI has been studied. SOD1 is found mainly in the cytosolic and lysosomal fractions, but also in the mitochondrial intermembrane space, whereas SOD2 is located in the mitochondrial matrix. The neurologic outcome and infarctions were aggravated in *SOD2*-deficient mice after both transient (97) and permanent (126) focal ischemia in adult mice. Overexpression of *SOD2* prevented apoptosis and reduced tissue damage after focal ischemia (94, 164). Similarly, *SOD1* overexpression reduced the injury after transient focal ischemia in adult mouse brains (191), but in the immature brain, it aggravated the tissue damage after HI (36). Similarly, the SOD mimetic M40403 could inhibit phencyclidine-induced cortical apoptosis and deficits in rodent pups (184). One explanation for these latter surprising findings could be that the immature brain has a limited capacity to convert the accumulated H_2O_2 into water and oxygen, due to lower levels of catalase and glutathione peroxidase (57).

Developmental Neurotoxicity of Sedative and Antiepileptic Drugs and Ethanol

Compounds that are used as sedatives, anesthetics, or anticonvulsants in medicine have been identified as potent triggers for widespread apoptotic neurodegeneration throughout the developing brain when administered to immature rodents (14, 80, 81, 87). Such substances include NMDA-receptor antagonists (*e.g.*, ketamine, nitrous oxide), γ-amino-butyric acid subtype A ($GABA_A$)-receptor agonists (*e.g.*, barbiturates, benzodiazepines, propofol), combined NMDAR antagonist and $GABA_AR$ agonists (*e.g.*, alcohol) and/or sodium channel blockers (*e.g.*, phenytoin, valproate). We have found that rodents are particularly vulnerable to these drugs during the first 2 postnatal weeks of life, a period that coincides with the brain growth spurt and is comparable to the last trimester of human pregnancy up to several years after birth (Fig. 4) (41, 80, 81).

Glutamatergic synaptic transmission undergoes substantial changes during development and may be important for the selective susceptibility of the developing brain to drugs that influence neurotransmission: whereas α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) and kainic acid receptors dominate in the mature brain, NMDARs are more active in the immature brain because of the spatiotemporal expression of different NMDAR subunits (subunit switch) (8, 31, 70, 76, 96). The expression of NR2 subunit mRNAs, for example, is differentially regulated during development. In the embryonic rodent brain, only the NR2B and the NR2D-subunit mRNAs are expressed (8, 96, 118, 166). During the first 2 postnatal weeks, the NR2A-subunit mRNA appears in the entire brain, the NR2C subunit mRNA can be detected in the cerebellum, NR2B-subunit expression becomes restricted to the forebrain, and the NR2D-subunit mRNA is greatly decreased (166). The NR1-subunit mRNA is ubiquitously expressed in the brain throughout development (166). NR2-subunit compositions result in altered electrophysiologic NMDA-receptor properties: more immature NR1/NR2B receptors allow enhanced activation of the channel (through weak magnesium blockade), in contrast to the adult NR1/NR2A form, and thereby increase its capability to strengthen synapses and to learn (166). During critical periods of development and synaptogenesis, NMDARs play an essential role in activity-dependent plasticity and synaptic refinement (119, 147, 155, 158). Most NMDARs are located on postsynaptic dendrites and dendritic spines in postsynaptic densities (154); they may also be found on cortical astrocytes and presynaptically (26). NMDARs are distributed ubiquitously throughout the CNS (70) but predominantly are located in cortical structures, basal ganglia, and sensory-associated systems in the rat brain (28). In most regions, AMPA receptors are colocalized with NMDA sites, whereas kainate receptors have a different distribution (29). Developmental changes have been reported for non-NMDARs. Early in development, during a phase of intense synaptogenesis, increased expression of non-NMDARs is followed by a decline in expression (118). Furthermore, the distribution pattern of non-NMDAR subunits is also developmentally regulated (118). Developmental changes in the expression of mGluR (10, 11, 97, 148) and the GABA receptors (7) also have been described.

Several mechanisms have been implicated in the proapoptotic action of NMDAR antagonists and $GABA_AR$ agonists in the developing brain. One mechanism relates to changes in synthesis of neurotrophins (*i.e.,* brain-derived neurotrophic factor, neurotrophins 3 and 4, as well as reduced levels of the active phosphorylated forms of extracellular signal-regulated kinase (ERK1/2) and protein kinase B (AKT). ERK1/2 and AKT act within major survival-promoting pathways and can be activated by binding of growth factors to tyrosine kinase receptors. SynRas transgenic mice, which postnatally overexpress activated Ras in neurons and display higher levels of phosphorylated ERK1/2 in the cortex, were less susceptible to the proapoptotic effect of the NMDAR antagonist MK801 (71). Interestingly, 17β-estradiol counteracted inactivation of the ERK1/2 and AKT pathways and protected against the neurotoxicity of some antiepileptic drugs (4). A further mechanism can be the interaction of the neurotransmitter system with intrinsic antioxidant defenses that are essential for neuronal survival. In a recent study, Papadia, Hardingham, and colleagues (135) demonstrated that synaptic NMDAR-mediated activity boosts antioxidant defenses through changes to the thioredoxin-peroxiredoxin system. Synaptic activity enhances thioredoxin activity, facilitates the reduction of overoxidized peroxiredoxins, and promotes resistance to oxidative stress. Resistance is mediated by coordinated transcriptional changes (Fig. 4). Thus, interference with synaptic NMDAR activity may influence the progression of pathologic processes associated with oxidative damage (135). Whether sedatives and antiepileptic drugs acting via activation of the $GABA_A$ receptor also use this mechanism to produce apoptosis in the developing mammalian brain must be addressed in future studies. Slikker and co-workers (200) demonstrated that prolonged NMDAR antagonism by ketamine results in accelerated neurodegeneration and upregulation of the NR1 subunit of the NMDAR in rodent and monkey primary neuronal cell cultures. Later, they were able to demonstrate that such ketamine-induced neurotoxic effects were effectively inhibited by co-application of the selective neuronal nitric oxide (NO) synthase blocker 7-nitroindazole (185). They

thus speculated that NR1 upregulation facilitates a pathologic calcium entry into the neuron, leading to a release of reactive nitrogen species and excitotoxicity. This supports the relevance of oxidative stress in developmental drug neurotoxicity.

Evidence that ethanol, the most widely abused drug in the world, has NMDA antagonist- and $GABA_A$-agonist properties prompted evaluation of its ability to mimic the proapoptotic effects of other NMDAR antagonists and $GABA_AR$ agonists. Transplacental ethanol exposure of the human fetus *in utero* can cause craniofacial anomalies, microcephaly, mental retardation, and neurobehavioral disturbances ranging from hyperactivity/attention deficit and learning disabilities to depression and psychosis. The fetotoxic effects of ethanol can also manifest as a syndrome consisting of neurobehavioral disturbances without craniofacial malformations, referred to as fetal alcohol effects or alcohol-related neurodevelopmental disorder. A new term that represents all clinicopathologic manifestations of the fetotoxic effects of alcohol is fetal alcohol–spectrum disorder (6).

A series of studies from our group led to the observation that during the developmental period of synaptogenesis, brief exposure to ethanol can trigger widespread apoptotic neurodegeneration in the *in vivo* mammalian brain (80, 81). The window of vulnerability to ethanol-induced apoptosis is the same as that of NMDA antagonists and $GABA_A$ agonists (80, 81, 132). *In utero* ethanol exposure has been shown to elicit oxidative stress in the rat fetus by several research groups.

1. Increased levels of oxidative-stress markers were detected in fetal brains and in the placental villi after a short period of ethanol exposure during gestation (75, 92).
2. Alcohol administration during pregnancy results in differential gene expression in the stress-signal pathway, particularly in c-fos, in the embryos of pregnant mice (138).
3. The activity of glycogen synthase kinase 3beta (GSK3beta), a multifunctional serine/threonine kinase that responds to various cellular stresses, was affected by ethanol (6). GSK3beta inhibition provided protection against ethanol neurotoxicity, whereas high GSK3beta activity/expression sensitized neuronal cells to ethanol-induced damage *in vitro*.
4. NADPH oxidase (NOX) has been identified as an important source of ROS in ethanol-exposed embryos, and expression of NOX1 and NOX3 together with that of p53 was increased in cerebellar granule cells of P1 rats after daily ethanol exposure of their dams (75).
5. Such a treatment similarly resulted in decreased mRNA levels of mitochondrial genes encoding complexes IIA, IV, and V and increased immunoreactivity for 4-hydroxy-2,3-nonenal and 8-OjdG, suggesting that mitochondrial dysfunction, oxidative stress, and DNA damage represent toxic effects of ethanol in fetal alcohol syndrome (25).
6. Glutathione content has been found to be an important predictor of neuronal sensitivity to ethanol-mediated oxidative stress and subsequent cell death in cultured fetal cortical neurons (*i.e.*, apoptotic indices are preferentially increased after ethanol exposure in cells with low glutathione content (92).

Additional mechanisms beyond oxidative stress also contribute to brain damage after ethanol exposure during critical developmental periods. Pretreatment with antioxidants did not ameliorate alcohol-induced Purkinje cell loss in the developing rat cerebellum (66). However, vitamin E was reported to protect against alcohol-induced cell loss and oxidative stress in hippocampal cultures (124) and in the hippocampus in neonatal rats that received intragastric intubation on P7 to P9 and were coadministered 2 g/kg vitamin E. Vitamin E treatment alleviated the increase in protein carbonyls and the reduction in CA1 pyramidal cells seen in the ethanol-exposed group. However, the treatment did not improve spatial learning in the ethanol-exposed animals. These results suggest that whereas oxidative stress–related neurodegeneration may be a contributing factor in fetal alcohol syndrome, the antioxidant protection against alcohol-induced oxidative stress and neuronal cell loss in the rat hippocampus does not appear to be sufficient to prevent the behavioral impairments associated with fetal alcohol syndrome. These findings suggest that additional mechanisms beyond oxidative damage of hippocampal neurons also contribute to the disorder (74, 115). Dong *et al.* (42) studied the role of nuclear factor erythroid 2-related factor 2 (Nrf2) signaling in transcriptional activation of detoxifying and antioxidant genes in an *in vivo* fetal alcohol mouse model. Pretreatment with the Nrf2 inducer D3T resulted in a significant decrease in ethanol-induced reactive oxygen species generation and apoptosis in mouse embryos. Future studies must further explore this point.

Mechanical Trauma to the Brain

Traumatic brain injury (TBI) is a major cause of long-term morbidity and mortality in the industrialized world, with an incidence of about 1.5 million per year in the United States, leading to long-term disability in 6% of the patients (22, 45, 63, 141, 163, 169, 170). Children under the age of 6 years not only sustain TBI more frequently than do any other age group (1), but those younger than 4 years also show the worst neurologic outcomes (1, 100, 111, 167). Even mild injuries may result in long-term morbidity in preschool children (116, 121). Although perinatal mechanical brain injuries, often associated with asphyxia, and child abuse are frequent causes of TBI in newborns and infants, accidents (especially traffic accidents) are the number one cause of TBI in toddlers and young schoolchildren (33, 47, 73, 93, 183).

In TBI, the primary damage may result in diffuse axonal injury, intraparenchymal contusions, intracranial hematomas, or a combination of these (141). These events are often followed by a secondary cascade of biochemical, cellular, and molecular derangements as well as extracerebral complications that generate further damage (Fig. 3) (139, 141). Although similarities exist in the pathomechanisms triggered by TBI to pediatric and adult brains, an injury to a developing brain poses a unique challenge because of the often diffuse pattern of injury, the increased vulnerability of the brain, and ongoing developmental processes. TBI can trigger two types of neurodegeneration in the developing brain, passive and active cell death. Within the area of impact, excitotoxic cell death occurs and expands rapidly within approximately 4 h in rodent brains (11, 84, 139, 141). Approximately 6 h after TBI, this local excitotoxic response is followed by a delayed, but much more extensive disseminated apoptosis in many brain

regions ipsi- and contralateral to the trauma site hours after the excitotoxic degeneration has run its course (11, 15, 50, 84, 139, 141). Trauma-induced cell death is associated with oxidative stress, an activation of the intrinsic and the extrinsic apoptotic pathways, as well as an increase in the transcription of neurotrophins (11–13, 49, 50, 99, 139). The severity of apoptotic neurodegeneration after TBI is age dependent; in rats, the magnitude of apoptotic response was highest in 3- and 7-day-old animals and subsequently was followed by a rapid decline. The timing of greatest vulnerability to TBI coincides with the peak of the brain growth spurt (11–13, 15, 49, 50, 84, 139).

Cell damage and energetic breakdown induces a surge of glutamate that accumulates in the synaptic gap when such neurotransmitters reach a level that astrocytes cannot recycle any more at an adequate pace. The deleterious effects of such high glutamate levels on brain tissue can activate pathways leading to oxidative stress and cause secondary damage, including passive and active cell death (Fig. 3) (88, 146). For example, influx of calcium into intracellular compartments can induce the xanthine pathway, the synthesis of NO, and the peroxidation of membrane fatty acids. All these pathways lead to the production of free radicals, including ROS and RNS (Fig. 3).

Studies in pediatric TBI models have demonstrated mitochondrial dysfunction and altered cerebral metabolism (13, 91, 99). Early hyperglycolysis occurs, followed by a 1- to 3-day period of metabolic depression in TBI in immature rats (17 days old) (52). The ability of alternative substrates, such as ketone bodies, to rescue brain metabolism is developmentally regulated (112). Robertson and colleagues (192) studied isolated brain mitochondria early (1–4 h) after cortical impact TBI in immature rats (17 days old). Mitochondria had significant alterations in respiratory capacity, with increases in state 4 respiration (1 h) and decreases in state 3 respiration (4 h). Mitochondria also had reduced cytochrome *c* content and decreased activity of pyruvate dehydrogenase. Others used gel-based proteomics to show significant reductions in pyruvate dehydrogenase subunit expression that extended to 2 weeks after TBI in the developing brain (189). Loss of pyruvate dehydrogenase activity in pediatric TBI may be especially important, as it is the critical enzymatic link between glycolysis and the TCA cycle. Metabolic derangements in clinical studies of pediatric TBI have been reported based on imaging investigations (123). Magnetic resonance spectroscopy demonstrated marked elevations in brain lactate and reductions in *N*-acetyl aspartate, a marker of neuronal or mitochondrial integrity (or both), in children after TBI, and these metabolic alterations correlate with long-term neurologic outcome (123). Casey and colleagues (133) used proton (1H) spectroscopy after controlled cortical impact in immature rats (16–17 days old) to evaluate the time course of metabolic alterations. They showed that metabolic derangements begin early (by 4 h) and are sustained for at least 7 days after TBI in the developing brain. Another study used ^{13}C-nuclear magnetic resonance spectroscopy to evaluate glucose metabolism 5–6 h after cortical contusion injury in immature rats (21–22 days old) (167). This study showed that neuronal oxidative metabolism of glucose is delayed in both the ipsilateral and contralateral hemispheres, compared with uninjured sham controls. Findings were consistent with impairment at the level of pyruvate dehydrogenase and possibly α-ketoglutarate dehydrogenase, as well as impairment of the malate–aspartate shuttle.

In parallel, after a hemorrhage, the reduction of iron within heme molecules further promotes the production of free radicals. Physiologically, scavengers such as SODs and catalase recycle these free radicals. As mentioned earlier, this capacity of recycling free radicals is limited during the fetal and neonatal phase, which renders the developing brain particularly vulnerable to oxidative stress. We recently reported changes of infant rodent brain proteome after TBI and thereby identified a subacute increase of the two redox-regulating proteins peroxiredoxin 1 and 6 (Prdx1, Prdx6) and of DJ-1 protein (Dj1), as well as of stress-induced phosphoprotein 1 (Sti1) and heat-shock protein (Hsp) 90 family member Hsp84 (91). These protein changes likely reflect a physiologic activation of endogenous antioxidant defense mechanisms to prevent oxidative damage. For example, a neuroprotective role of peroxiredoxins against oxidative damage and an association with cell proliferation has been demonstrated in the neonate in a model of NMDA receptor–mediated brain lesions and in oxygen-mediated injury of the lung in neonatal baboons (32, 135). Several studies have shown that sequelae such as oxidative stress after brain injury is not restricted to the acute phase but is ongoing as late as 1 month in rodents after TBI (77, 159, 168, 185). Acute antioxidant treatment strategies, such as those with erythropoietin, *N*-acetylcysteine, and melatonin, have demonstrated efficacy in infant and adult rodent models of TBI (52, 112, 123, 133, 141, 190, 193).

Is Oxidative Stress a Clinically Relevant Therapeutic Target in the Developing Brain?

The clinical approach to the fetus and newborn at risk for cerebral damage is clearly a high priority, and an understanding of the specific pathologic processes preceding the onset of irreversible cerebral injury is essential to the design of effective interventions. Oxidative stress has been identified in several animal models and also in patients to hold a central role in the pathomechanism of perinatal brain damage.

Two main approaches have been developed to combat oxidative stress. The first consists in a reduction of free radical production through xanthine oxidase inhibition, of lipid peroxidation, and of iNOS. The second approach consists of increasing antioxidant defense mechanisms through an increase of ROS and RNS scavengers. Although many antioxidant drugs have been used in clinical and experimental approaches to reduce oxidative stress in neonatal brain diseases, the results are still uncertain:

1. Allopurinol reduces oxidative stress as an inhibitor of the xanthine oxidase and additional effects such as directly scavenging free radicals (61, 128). However, despite promising results in therapeutic essays in infants with hypoxic-ischemic (HI) encephalopathy (reduction of serum NO after HI in newborns HI and amelioration of their neurologic outcome) (149, 198), a meta-analysis of various therapeutic essays for this indication failed to show a beneficial effect of allopurinol on mortality or on progression of neonatal convulsions (110).
2. Vitamin E has an antioxidant effect through its capacity to scavenge ROS and RNS, but can induce neuronal apoptosis *in vitro* when high doses are used, and its supplementation favors the progression of sepsis in

newborns, as reported in a recent Cochrane review (21, 169).

3. Vitamin C, an antioxidant by being available for energetically favorable oxidation, showed no significant benefits or harmful effects when given to very preterm infants (32).
4. *N*-Acetylcysteine clears free radicals by the induction of glutathione and has been shown to be neuroprotective in several animal models of perinatal brain damage (134, 186). No adverse effects have been reported when it was given through the parenteral route in the premature infant (159).
5. Melatonin, a chief hormone of the pineal gland that participates physiologically in sleep regulation, has been shown to be neuroprotective in several animal models through its antioxidant potential (direct scavenger of OH^-, O_2^-, and NO) and other effects (20, 61, 62, 64, 77, 137, 174, 196).
6. Moreover, the ketogenic diet has been shown to have an antioxidant effect (redox status in human blood improved) and a neuroprotective effect in animal models of perinatal brain damage (3, 110, 128, 142, 149, 199). Although long used as an adjunctive therapy for intractable childhood epilepsy, the neuroprotective benefits of alternative substrate metabolism have not been investigated in detail in acquired pediatric brain injuries.
7. Similarly, L-carnitine has been shown to have a neuroprotective effect in animal models of perinatal brain damage (201) and is also administered to children with inborn errors of metabolism (127) but is not an established therapy for children with acquired brain injuries.

In conclusion, oxidative stress plays a central role in developmental brain injuries, and various processes during development make the immature brain particularly vulnerable to this type of stress. Neuroprotective treatment strategies are limited because of difficulties of performing clinical studies in infants with drugs that have not, for the most part, been FDA approved for that age in their primary indication, because of the heterogeneity of the population, the prolonged time needed for many free radical scavengers and inhibitors to penetrate the blood–brain barrier, the narrow therapeutic range, and the mostly moderate results in animal models.

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Address correspondence to:
Chrysanthy Ikonomidou
Department of Neurology
University of Wisconsin
600 Highland Avenue, CSC H6/574
Madison, WI 53792

E-mail: ikonomidou@neurology.wisc.edu

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Abbreviations Used

AKT = protein kinase B
AMPA = α-3-amino-hydroxy-5-methyl-4-isoxazole propionic acid
ßOHB = β-hydroxybutyrate
BCL2 = *Homo sapiens* B-cell chronic lymphocytic leukemia/lymphoma 2
CNS = central nervous system
CSF = cerebrospinal fluid
ERK = extracellular signal regulated kinase
$GABA_A$ = γ-amino-butyric acid subtype A
GD = gestational day
Glut1 = glucose transporter 1
Glut3 = glucose transporter 3
GPx = glutathione peroxidase
GSH = glutathione
GSK3beta = glycogen synthase kinase 3beta
GSSG = oxidized glutathione
HI = hypoxia–ischemia
H_2O_2 = hydrogen peroxide
Hsp = heat-shock protein
IL = interleukin
ILCOR = International Liaison Committee on Resuscitation
IRAK4 = IL-1 receptor–associated kinase 4
KA = kainate
MDA = malondialdehyde
mGlu = metabotropic glutamate receptor
MK801 = dizocilpine
mtPT = mitochondrial permeability transition
NMDA = *N*-methyl-D-aspartate
nNOS = neuronal nitric oxide synthase
NO^- = nitric oxide
NOX = NADPH oxidase
NR = *N*-methyl-D-aspartate receptor subunit
Nrf2 = erythroid 2-related factor 2
^-OH = hydroxyl radical
OL = oligodendrocyte
ONOOS = peroxynitrite
O_2^-S = superoxide
P = postnatal day
Pao_2 = arterial oxygen tension
PCD = programmed cell death
PND = postnatal day
Prdx = peroxiredoxin
RNS = reactive nitrogen species
ROO^- = peroxyl radical
ROS = reactive oxygen species
RSH = protein thiol
Sesn2 = sestrin2
-SH = sulfhydryl or thiol group
-SNO = *S*-nitrosylated derivative
$-SO_2/3H$ = sulfinic/sulfonic acid derivative
SOD = superoxide dismutase
-SOH = sulfenic acid derivative
Srxn = sulfiredoxin
Sti1 = stress-induced phosphoprotein 1
TBI = traumatic brain injury

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