The Potential Role of Mitochondria in Pediatric Traumatic Brain Injury

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Abstract

Mitochondria play a central role in cerebral energy metabolism, intracellular calcium homeostasis and reactive oxygen species generation and detoxification. Following traumatic brain injury (TBI), the degree of mitochondrial injury or dysfunction can be an important determinant of cell survival or death. Literature would suggest that brain mitochondria from the developing brain are very different from those from mature animals. Therefore, aspects of developmental differences in the mitochondrial response to TBI can make the immature brain more vulnerable to traumatic injury. This review will focus on four main areas of secondary injury after pediatric TBI, including excitotoxicity, oxidative stress, alterations in energy metabolism and cell death pathways. Specifically, we will describe what is known about developmental differences in mitochondrial function in these areas, in both the normal, physiologic state and the pathologic state after pediatric TBI. The ability to identify and target aspects of mitochondrial dysfunction could lead to novel neuroprotective therapies for infants and children after severe TBI.

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Introduction

Growing numbers of preclinical and clinical studies have defined a key role for brain mitochondria in the pathologic cascades that occur after traumatic brain injury (TBI) [1, 2] (fig. 1). In addition, many effective neuroprotective strategies directly target mitochondria or have indirect effects on mitochondrial function. Developmental differences in brain mitochondria of normal brain have been well described over the last 50 years [3–8], and studies are beginning to address how these baseline differences could contribute to the response of the young brain to injury. Limitations in the current knowledge of the role the mitochondria play in pediatric TBI center around four main groups of questions:

(1) Excitotoxicity

(a) What are the developmental differences in mitochondrial regulation of intracellular calcium homeostasis?

(b) During periods of excitotoxicity, is the maximal calcium uptake capacity of immature mitochondria similar to that of mature mitochondria? If not, is this detrimental or protective for the brain?

(c) What role do developmental differences in cell surface N-methyl-D-aspartate (NMDA) receptors play in the calcium load experienced by mitochondria after TBI? Also, how do developmental differences in other cell surface receptors (GABA$_A$, AMPA) influence this?

(d) What role, if any, does the mitochondrial permeability transition pore (mPTP) play in the response of the developing brain to TBI?

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Fig. 1. Mitochondria play a central role in many physiologic and pathologic pathways after pediatric TBI, including intracellular calcium homeostasis, ROS generation and detoxification, and cerebral energy metabolism. In addition, important apoptogenic proteins such as cytochrome c and AIF reside in the mitochondrial intermembrane space.
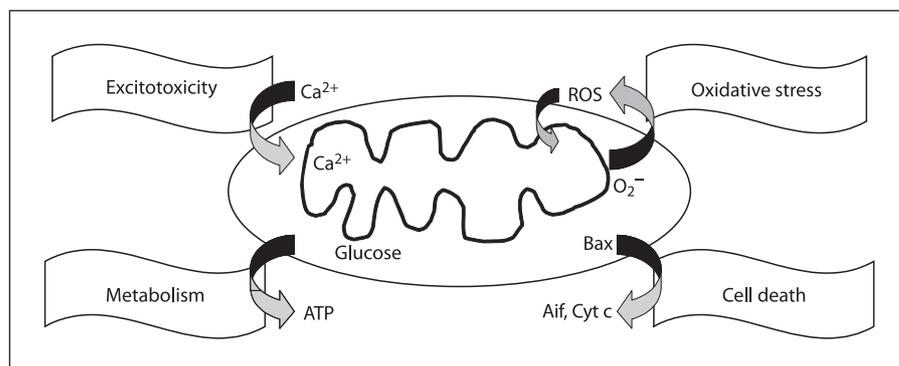
(2) Oxidative stress

(a) What are the age-dependent differences in baseline mitochondrial reactive oxygen species (ROS) production? Similarly, what are the age-dependent differences in mitochondrial ROS detoxification?

(b) What are the functional differences in immature brain mitochondrial detoxification of ROS in the posttrauma setting? Specifically, how effectively do immature mitochondria upregulate important detoxification enzymes, such as manganese superoxide dismutase (MnSOD) and glutathione peroxidase (GPX)?

(c) What role does mitochondrial nitric oxide synthase (mtNOS) play in the response of the developing brain to TBI?

(3) Energy Metabolism

(a) What are the unique developmental aspects of cerebral energy metabolism related to substrate utilization and transport?

(b) What are the time course and pattern of the posttraumatic metabolic derangements in the immature brain? Are enzymes of glucose metabolism, the TCA cycle and the electron transport chain equally affected?

(c) Following TBI, is the immature brain uniquely able to utilize fuels, such as ketone bodies or lactate, as alternatives to glucose?

(4) Apoptosis

(a) What pro- and antiapoptotic proteins are normally located at the mitochondrial level during brain development? Is there a greater tendency for certain proteins to translocate to mitochondria in immature brains?

(b) With a different profile of pro- and antiapoptotic proteins at the mitochondrial level, how do immature brain mitochondria respond differently to proapoptotic stimuli after TBI?

(c) Are the apoptotic profiles seen in developing animals similar in human infants and children?

This review will highlight what is known about developmental differences in brain mitochondria in relationship to these questions. We will also summarize the more limited body of knowledge specific to mitochondrial dysfunction after pediatric TBI.

Excitotoxicity and Calcium Uptake

Many forms of acute brain injury, including traumatic injury, result in high levels of cellular Ca^{2+}. The ability of mitochondria to effectively buffer this Ca^{2+} can be a determinant of cell survival or cell death [9]. Importantly, excessive mitochondrial Ca^{2+} sequestration can lead to induction of the mPTP. Formation of the mPTP is thought to involve the voltage-dependent anion channel from the outer mitochondrial membrane, the adenine nucleotide translocator from the inner mitochondrial membrane and cyclophilin D, a prolyl isomerase, from the mitochondrial matrix. Induction of the pore can lead to mitochondrial swelling, rupture of the outer mitochondrial membrane and nonspecific release of important mitochondrial proteins. Although cytochrome c can be released in this process, it is clear from recent studies with cyclophilin-D-deficient mice that, while the mPTP plays a critical role in necrotic cell death secondary to calcium overload and oxidative stress [10], it plays a very limited role, if any, in apoptotic cell death after acute brain injury [10–13].

Unique Features of the Developing Brain
NMDA-type glutamate receptors are responsible for the majority of the pathologic influx of Ca^{2+} into cells after injury. The regional expression of NMDA receptors is developmentally regulated [14], which can lead to a profound developmental effect on the Ca^{2+} load experi-

enced by brain mitochondria. Multiple subunits of the receptor (NR1, NR2A-D, NR3) all play varying roles in glutamate recognition and binding, ultimately influencing channel open time and conductance [for a review, see ref. 15, 16]. As a result of developmental shifts in relative subunit expression and developmental changes in NMDA receptor expression and sensitivity, the young brain is generally felt to have increased sensitivity to excitotoxic insults [17–19]. However, other studies found greater resistance to NMDA toxicity at younger ages [20], and others have shown that NMDA inhibition in immature TBI reduced early, necrotic damage but promoted greater secondary, apoptotic damage [21]. Important insight into the vulnerability, or lack of vulnerability, of the immature brain to pathologic insults can be gained in studies of epilepsy. In vitro work with immature neurons and in vivo work with young animals demonstrate increased vulnerability to seizure onset and generalization, but less vulnerability to cell death and permanent behavioral deficits [for a review, see ref. 22].

Calcium influx through other ion channels (AMPA, kainate) also changes with development, with the greatest density in early postnatal development [23, 24]. More recently, in addition to glutamate receptors, investigators have discovered a role for $GABA_A$ receptors in excitatory transmission during early development [for a review, see ref. 25]. In immature neurons, $GABA_A$ receptor activation causes depolarization, which can activate voltage-dependent Ca^{2+} channels and increase intracellular Ca^{2+}. In hippocampal cells and slices, it appears that GABAergic interneuronal circuits develop earlier than glutamatergic synapses. Therefore, $GABA_A$ receptors are initially responsible for the majority of excitatory activity in the first 2–3 postnatal days (PNDs). Subsequently, by the end of the first postnatal week, $GABA_A$ receptors transition to their classic inhibitory profile. Taken together, all studies would suggest that excitotoxicity in the developing brain is very different from that of the mature brain. As a result, any approach at reducing mitochondrial Ca^{2+} load by blocking intracellular Ca^{2+} influx must take into account the maturational profile of ionotropic cell surface receptor expression and interaction.

Once Ca^{2+} has gained entry into the cell, mitochondria play a key role in regulating intracellular Ca^{2+} homeostasis. Mitochondria from the immature brain may have differing capacity for maximal Ca^{2+} uptake than adult brain mitochondria. Using isolated brain mitochondria, we demonstrated that brain mitochondria isolated from 16- to 18-day-old rats had a lower maximal, respiration-dependent Ca^{2+} uptake capacity than those isolated from adult rats in the presence of ATP at pH 7.0 and 6.5 [26]. However, in conditions similar to those in the posttraumatic environment (absence of ATP), immature brain mitochondria exhibited greater maximal Ca^{2+} uptake at both pH 7.0 and 6.5. Other groups have demonstrated similar age-dependent changes in the basal Ca^{2+} uptake of isolated mitochondria [27]. These investigators showed that mitochondrial Ca^{2+} uptake increased in selected brain regions (cerebellum, cortex, hippocampus) with increasing age (PND 7, PND 21 and adult rats). Differences in Ca^{2+} uptake specific to brain region were only seen in PND 21 rats, with rates in the cerebellum much higher than in the cortex or hippocampus. Additional information on the mitochondrial response to intracellular Ca^{2+} loads has been gained from in vitro studies using hippocampal neurons isolated from rats of different ages (PND 1–25) [28–30]). Original observations in dissociated neurons revealed that neurons from older rats (PND 21–25) had much greater increases in intracellular Ca^{2+} in response to glutamate stimulus than neurons from younger rats (PND 1–3 and PND 6–8), which was associated with increasing morphologic changes in the older neurons [28]. Using dissociated neurons maintained 5 days in vitro, investigators found a significant loss in intracellular Ca^{2+} homeostasis in those neurons from older rats (PND 20–32) compared with those from younger rats (PND 0–9) [29]. Furthermore, they found that NMDA markedly dissipated mitochondrial membrane potential ($\Delta\Psi$) in older neurons, with very little change in $\Delta\Psi$ in younger neurons with NMDA stimulus. They concluded that the mitochondrion was responsible for the age-related vulnerability to excitotoxicity seen in this hippocampal neuronal population. Recent studies have revealed that mtNOS was responsible, in part, for the decreased NMDA vulnerability of younger neurons (PND 5) by mild dissipation of $\Delta\Psi$ and for decreasing the mitochondrial Ca^{2+} uptake [30]. In contrast, cytosolic NOS appears to be the main contributor to NMDA toxicity in older neurons (PND 19).

Direct evidence for the presence of the mPTP in the normal, developing brain is very limited. In mitochondria isolated from uninjured, immature rat brain (PND 16–18), the maximal Ca^{2+} uptake capacity and Ca^{2+}-induced cytochrome c release in vitro was not altered in the presence of the immunosuppressant, cyclosporin A (CsA) [26]. CsA acts by binding to cyclophilin D, thereby inhibiting the opening of the mPTP. Studies in animal models of prenatal and perinatal brain injury have supported a role for the mPTP, but CsA has not been universally neu-

roprotective. Puka-Sundvall et al. [31] demonstrated a significant increase in entrapment of ^{14}C-2-deoxyglucose in mitochondria both early (<1 h) and later (6.5–8 h) during reperfusion, documenting occurrence of mPTP. Interestingly, CsA did not show significant neuroprotective effects in this model. Studies in utero have included models of intrauterine ischemia [32], in utero ethanol exposure [33] and prenatal glucocorticoid exposure [34]. These studies utilized in vitro measurements to demonstrate evidence for mPTP in isolated mitochondria or permeabilized cells in injured brain tissue compared with control tissue. In the study by Nakai et al. [32], additional evidence for mPTP was provided by evaluation of the neuroprotective properties of CsA administered into the maternal tail vein 1 h after uterine artery occlusion. Treatment with CsA preserved mitochondrial respiration and tissue ATP levels and reduced mitochondrial swelling in the fetal brains. Additional insight into the role of the mPTP in the developing brain can be gained from studies comparing CsA with another immunosuppressant, FK-506. Both FK-506 and CsA accomplish immunosuppression through inhibition of the protein phosphatase, calcineurin. However, FK-506 does not bind to cyclophilin D, and therefore, does not have direct effects on inhibiting the mPTP. One study of seizure susceptibility showed that FK-506 was able to ameliorate the course of epilepsy following acute brain injury at PND 6 or PND 30 [35]. However, treatment with CsA increased mortality rates in PND 6 injured rats and lengthened seizure duration in PND 30 injured rats. Therefore, the neuroprotective properties of CsA may be very dependent on the age at injury and the model of injury being studied.

Excitotoxicity and Mitochondria after Pediatric TBI
The most detailed evidence for Ca^{2+} influx in pediatric TBI is found in a study of lateral fluid percussion in developing (PND 17, PND 28) and adult rats [36]. These investigators used $^{45}Ca^{2+}$ autoradiography to demonstrate an acute and sustained accumulation of Ca^{2+} after TBI at all ages, with both the time course and location of Ca^{2+} accumulation being age dependent. The youngest rats (PND 17) had a shortened course of Ca^{2+} accumulation and did not show the significant, delayed accumulation in the ipsilateral thalamus that was seen in PND 28 and adult rats. However, this study did not directly evaluate intracellular compartmentation of Ca^{2+}, so it is unclear what the specific mitochondrial Ca^{2+} burden was in relationship to age. In addition, all studies directly comparing immature with adult TBI are limited by the ability to control for differing injury severity between ages.

Distinct differences in body size, brain size, brain water content and the biomechanical properties of the brain and supporting structures make direct comparisons challenging in both small animal [37], large animal [38] and human [39] studies.

In children after severe TBI, elevated concentrations of glutamate and glycine were seen in the cerebrospinal fluid of injured patients versus controls, which correlated with poor outcome [40]. The greatest increases were seen in the youngest children (<4 years old) and in victims of child abuse. Studies using proton magnetic resonance spectroscopy (MRS) also demonstrated increases in occipital glutamate/glutamine content in injured pediatric patients [41].

To date, to our knowledge, no study has directly evaluated the response of mitochondria in the developing brain to the massive intracellular increases in Ca^{2+} that occur in the posttraumatic setting. Supportive evidence comes from adult TBI studies [42–45], neonatal hypoxia-ischemia (HI) models [46–48] and one study of seizures in immature and adult rats [49]. In adult TBI, mitochondria isolated from the injured hemisphere showed significant perturbations in Ca^{2+} homeostasis and excessive Ca^{2+} absorption with inhibition of mitochondrial respiration [42, 45]. Administration of CsA preserved mitochondrial morphology and function [43, 50] and reduced the Ca^{2+}-related induction of the mPTP in vitro [43]. Furthermore, CsA reduced overall tissue damage in models of both focal [51, 52] and diffuse [50, 53] TBI in adult rats. In neonatal HI models, similar to adult TBI, mitochondria showed significant Ca^{2+} accumulation and associated mitochondrial swelling at 3 and 24 h of reperfusion [48]. Treatment with the NMDA receptor antagonist MK-801 immediately after HI improved mitochondrial respiratory capacity and tissue ATP and phosphocreatine levels [46]. However, as described above, CsA was not neuroprotective in this model [31]. One study of kainate-induced seizures in immature (PND 10–11) and adult rats isolated mitochondria and demonstrated developmental differences in mitochondrial respiratory patterns and ROS production after injury [49]. These investigators hypothesized that age-related differences in vulnerability to excitotoxic injury with seizures was due to high levels of mitochondrial uncoupling proteins in the younger rats, as a result of the high-fat diet from suckling. When young rats were placed on an isocaloric low-fat diet after injury, the mitochondrial function in vitro resembled that of adult brain mitochondria. Taken together, these studies suggest a potentially important effect of high intracellular Ca^{2+} on mitochondrial function after pediatric TBI.

They also suggest that mitochondria from the developing brain could have unique aspects of both vulnerability and resistance to excitotoxic insult. Future studies should be aimed at evaluating the relationship between mitochondrial injury and long-term neurologic outcome after pediatric TBI. In addition, studies should evaluate the efficacy of certain neuroprotective strategies, such as antiexcitotoxic agents or mPTP inhibitors (CsA), in the specific setting of traumatic injury to the developing brain. The study by Pohl et al. [21] highlights the importance of studying these neuroprotective agents in immature animal models, as NMDA antagonists significantly increased apoptotic cell death in very young rats (PND 7) after TBI.

Mitochondrial Role in Oxidative Stress

Mitochondria are one of the most significant generators of ROS in the injured brain. In addition, many mitochondrial components are targets of prooxidants, including important metabolic enzymes, such as the pyruvate dehydrogenase complex and several complexes of the electron transport chain. Therefore, the relative balance of ROS production and detoxification within brain mitochondria is a major determinant of mitochondrial oxidative injury [for a review, see ref. 54].

Unique Features of the Developing Brain
Mitochondria are the main source of superoxide in physiologic conditions. As a result, the detoxification of superoxide by mitochondrial Mn-SOD plays an important role in neuroprotection and ROS homeostasis. Superoxide is converted to hydrogen peroxide (H_2O_2), which is further detoxified to water and oxygen by the enzyme GPX. This appears to be a very efficient system, as normal, uninjured mitochondria have the capacity for net absorption and detoxification of exogenous H_2O_2 [55]. Additional studies have demonstrated that intact mitochondria have an overall capacity for detoxification that far exceeds the highest rate of endogenous ROS production [54]. Another important component of oxidative injury involves nitric oxide, which, in combination with superoxide, can form peroxynitrite. Peroxynitrite diffuses freely throughout the cell causing oxidative damage to various cellular constituents. Nitric oxide is produced by the enzyme NOS, and recent studies have provided evidence for the presence of NOS in brain mitochondria [56–60]. Clearly, the intrinsic mitochondrial ROS balance is complex and involves many endogenous antioxi-

dant enzymes and cofactors. The interplay between the mitochondrial and cytoplasmic enzyme defense systems is important in determining the ultimate level of oxidative stress in the immature brain, both during normal development and in pathologic states.

A global discussion of the developmental aspects of all intracellular ROS production and removal is too extensive for this review. Therefore, we will specifically focus on aspects of ROS production and detoxification that occur in brain mitochondria. Studies have shown pathologic increases in mitochondrial ROS production with advanced aging [61, 62], in neurodegenerative disease models [63, 64] and in the trisomy 16 rat model of Down's syndrome [65]. However, to our knowledge, no studies have directly investigated age-dependent differences in brain mitochondrial ROS production in young, immature animals. In contrast, more extensive investigation has documented developmental differences in cellular antioxidant defense systems, with some studies specifically focused on those antioxidant systems found within mitochondria. Mavelli et al. [66] prepared subcellular fractions (cytoplasmic and mitochondrial) of brains from rats from birth to 2 months of age and measured the activities of Cu,Zn- and Mn-SOD, glutathione peroxidase and catalase. These authors found increases in mitochondrial Mn-SOD over the first 3–4 weeks of life. This did not reflect a nonspecific increase in mitochondrial protein, as the same developmental pattern was seen when expressed relative to cytochrome oxidase activity. There was no significant change with age of Cu,Zn-SOD or GPX activity in the mitochondrial fraction, and catalase activities generally increased with age in the mitochondrial fraction but were difficult to interpret in total brain homogenate because of the low content of the enzyme in the brain. A similar evaluation was completed in mouse brain, looking at both protein levels and enzyme activity of antioxidant enzymes [67]. Interestingly, this study showed a surge of Mn-SOD activity around birth (E18 to PND 1), with a steady decline between PND 1 and PND 21 in the mouse. This pattern correlated with greater Mn-SOD protein levels in the brain at younger ages (E18 to PND 7) compared with PND 14 and PND 21. These developmental changes in Mn-SOD are different from those found with the cytoplasmic antioxidant Cu,Zn-SOD, which showed highest activity at E18 and generally unchanged activity from PND 1 to PND 21. Similar to Mn-SOD, the enzyme GPX showed a surge in activity between E18 and PND 1 and a decline between PND 1 and PND 21, while the protein level steadily increased. The discrepancy between GPX enzyme activity and protein levels is

thought to relate to the relative contribution of the non-selenium-dependent fraction of the enzyme. This nonselenium-dependent fraction is usually minimal in most tissues, but represented >50% of total GPX activity in this study, suggesting an important role for this fraction in protecting the developing brain from oxidative stress. Overall, these two studies describe a unique profile for antioxidant enzyme content and activity during pre- and postnatal development. As explained by the authors, the newborn brain experiences a surge in oxygen tension as the fetus moves from relative hypoxia in utero to a relatively hyperoxic environment. This would necessitate compensatory increases in endogenous antioxidant enzymes, as seen in these studies, to protect the newborn brain from oxidative stress.

The ultimate fate of superoxide generated by mitochondria depends on the relative concentration of superoxide, the presence of nitric oxide and the activity of Mn-SOD. A study by Riobó et al. [68] evaluated the developmental profile of mtNOS activity in rat brain and examined this enzyme in comparison with Mn-SOD. Using immunological characterization, the authors describe a 144-kDa mtNOS isoform, which was unique from the 157-kDa neuronal NOS (nNOS) isoform found in cytosolic fractions. They found that mtNOS expression and activity was greatest in late embryonic and early postnatal (E19 to PND 8) life and then markedly decreased. This was distinctly different from cytosolic nNOS expression and activity, which steadily increased after birth to peak in the 2nd to 3rd postnatal week. Using isolated mitochondria from PND 2–4, PND 15 and adult rats, these investigators also demonstrated functional differences in mitochondrial H_2O_2 production. Nitric oxide binds with high affinity to and inhibits cytochrome oxidase (complex IV) of the electron transport chain, causing inhibition of mitochondrial respiration. This increases mitochondrial superoxide production, and in the presence of SOD, leads to increases in mitochondrial H_2O_2. Neonatal mitochondria showed a biphasic response to H_2O_2 production in response to nitric oxide, which was similar to adult mitochondria, with a maximal production at 0.2 μM nitric oxide in neonates compared with 0.1 μM nitric oxide in adults. However, only mitochondria from the neonatal rats showed a significant increase in H_2O_2 production in response to the NOS substrate, L-arginine. Investigators attributed this to the relatively limited expression of mtNOS at older ages (PND 15 and adult), making mitochondria less sensitive to L-arginine activation. Interestingly, they found that the developmental profile of Mn-SOD activity mirrored that of mtNOS.

There was also a linear relationship between the activities of Mn-SOD and Cu,Zn-SOD with their respective mtNOS and nNOS activities.

Human brain developmental studies in the area of oxidative stress are very limited. One group evaluated the prenatal pattern of antioxidant enzymes in the telencephalic white matter of the human fetus and found a developmental lag in SOD-1 and SOD-2 when compared with GPX and catalase levels [69]. Taken together, preclinical and clinical studies suggest that brain antioxidant enzymes have an age-dependent profile of activity and expression and that each enzyme may have a unique pattern of development that may be region and species specific. Furthermore, the pattern within mitochondria may be unique from the pattern found in other subcellular compartments.

Oxidative Stress and Mitochondria after Pediatric TBI
Mitochondrial ROS production and detoxification in the brain after pediatric TBI has not been directly evaluated. Insight into the relative ROS balance in mitochondria after injury can be gained from adult TBI studies and studies of hypoxic-ischemic brain injury in neonatal rats. Oxidative stress has been shown to be a significant contributor to neurologic injury in a number of preclinical studies in adult animals after TBI [70–75]. However, the contribution of mitochondrial ROS generation and detoxification cannot be separately evaluated in these studies. Some of the best evidence in vivo comes from studies using mice with genetic alterations in Mn-SOD. Adult mice deficient in Mn-SOD had greater mitochondrial release of cytochrome c after TBI and greater overall cell death [76], while transgenic mice overexpressing Mn-SOD were less sensitive to traumatic injury [77]. Xiong et al. [78] directly evaluated functional differences in mitochondria isolated from Cu,Zn-SOD and Mn-SOD-altered adult mice after TBI. They found that TBI induced a significant decrease in mitochondrial respiratory capacity, which was not seen in transgenic mice overexpressing either Cu,Zn-SOD or Mn-SOD. Furthermore, a milder degree of injury only compromised mitochondrial function in mice deficient in either enzyme, but not in wildtype mice. Given the relatively greater levels of mitochondrial SOD seen in younger rats [67, 68], this would suggest potentially greater reserve against mitochondrial superoxide generation and detoxification in the immature brain after injury. However, in a transgenic mouse model of neonatal HI, 7-day-old mice with overexpression of Cu,Zn-SOD had greater cortical infarct [79]. The reason for this was evaluated in a subsequent study, where

immature mice were found to have significant reduction in GPX activity 24 h after HI. Because SOD dismutates superoxide to H_2O_2, the transgenic mice had a resultant greater accumulation of H_2O_2 [80]. In contrast, immature transgenic mice overexpressing GPX had reduced histologic injury scores and increased GPX activity at 24h after injury [81]. Although these studies did not directly examine the mitochondrial role, other studies would suggest that deficiency of GPX can lead to increased vulnerability to mitochondrial toxins [82] and that altered levels of mitochondrial glutathione induce mitochondrial damage [83].

The literature in pediatric TBI relevant to mitochondrial oxidative stress is limited. Clinical studies of cerebrospinal fluid (CSF) from infants and children after severe TBI have documented evidence for significant lipid peroxidation [84, 85], reduced antioxidant reserve and reduction in total levels of ascorbate and glutathione [85]. In a study of neonatal asphyxia, infants with the greatest degree of hypoxic-ischemic encephalopathy had the greatest activities of SOD, GPX and catalase in CSF [86]. One study in this issue of *Developmental Neuroscience* (see Kochanek et al.) describes reductions in cytoplasmic Cu,Zn-SOD and mitochondrial Mn-SOD in the ipsilateral hippocampus after TBI in the immature rat. These investigators used proteomic analysis to measure changes 2 weeks after CCI. Another study has investigated the unique, functional aspects of oxidative stress after pediatric TBI. In this study, adult mice had increased GPX activity within 24 h in response to injury [87]. However, the immature mouse did not show this compensatory increase. Baseline levels of GPX in the uninjured immature mouse were the same as in the adult mouse. The reason for a developmental difference in upregulation of GPX expression was not investigated in this study, but could relate to differences in transcriptional activation of antioxidant genes. One of the major transcription factors responsible for upregulating the antioxidant response is nuclear factor E2-related factor 2, which is especially enriched in glia. Therefore, developmental differences in the relative ratio of neurons to astrocytes could contribute to differences in antioxidant gene response.

These limited studies highlight the prominent role of oxidative stress after pediatric TBI. Since mitochondria are prominent generators and targets of prooxidants after TBI, a more detailed analysis of mitochondrial ROS production and oxidative injury should be performed in the developing brain. This is especially important when the unique developmental profile of antioxidant enzymes is considered. This evaluation could lead to a greater under-

standing of the degree, time course, localization and endogenous response of immature brain mitochondria to the prooxidant state following TBI, which could ultimately guide the development of neuroprotective antioxidant strategies.

Mitochondria and Energy Metabolism

Most of the enzymes responsible for energy metabolism are located in the mitochondrial matrix and inner membrane, and many of these enzymes have a developmental profile that is different from the mature brain. Following TBI, a period of metabolic dysfunction occurs that can last for hours to days. Mitochondrial components of the metabolic machinery of the cell may be especially vulnerable to injury in this environment.

Unique Features of the Developing Brain

Although glucose is an obligate fuel for both the adult and immature brain, many aspects of cerebral energy metabolism show marked developmental changes. Important differences relate to substrate utilization and transport [for a review, see ref. 88]. In general, glucose utilization is low in the postnatal period and steadily increases throughout maturation. Mitochondrial enzymes important for the oxidative metabolism of glucose, such as pyruvate dehydrogenase, have relatively lower activities than those from the adult brain [89–91]. This coincides with the relatively higher activities of enzymes for ketone bodies utilization [92]. In addition, the developing brain may have a greater ability to utilize lactate as a fuel, especially in the immediate postnatal period [93]. As would be expected, the ability to utilize alternative substrates is mirrored by developmental changes in nutrient transporters for glucose and monocarboxylic acids [94]. In addition, these developmental increases in glucose transport and oxidative glucose metabolism are accompanied by increases in the activity of the complexes of the mitochondrial electron transport chain [95, 96]. Specifically, using isolated nonsynaptosomal mitochondria from rat brain, investigators have demonstrated that complexes II, III and IV increase over the first 2 months of life, while complexes I and V mature more rapidly, reaching adult levels in the first 2–3 weeks of life [95]. Overall, the pattern is more rapid in synaptosomal mitochondria, with most complexes reaching adult levels by 10–21 days, with very little maturation beyond 21 days in any complex [96]. The distinction between nonsynaptosomal and synaptosomal mitochondria is an important

one, as those from nonsynaptosomal samples contain a mixture of mitochondria from both astrocytes and neurons, while synaptosomal samples are predominantly neuronal. Separate evaluation can help with the interpretation of developmental differences, as the ratio of astrocytes to neurons changes with age. However, newer, improved techniques will be necessary to adequately analyze developmental differences in astrocytic and neuronal mitochondria directly. Importantly, the developmental increases in the activity of important mitochondrial enzyme complexes are matched by developmental increases in overall mitochondrial respiratory rates and ATP production [3, 5, 6].

Specific data on direct measurement of the TCA cycle and respiratory chain enzyme activity are not available in humans. However, developmental changes in indirect markers, such as oxygen consumption and glucose utilization, have been measured using noninvasive techniques, showing steady increases throughout early development. Kinnala et al. [97] measured the local cerebral metabolic rate for glucose (LCMRGlc) in 20 neonates using positron emission tomography. They found a positive correlation between postconceptional age and LCMRGlc, with regional trends in LCMRGlc reflecting maturation of these areas. Similarly, a study in premature infants in the neonatal intensive care unit used near infrared spectroscopy to show a steady increase in cerebral oxygen consumption with advancing gestational age [98]. In later development, children continue to show a steady rise in glucose utilization, with a peak at 4–10 years of age and a steady decline to adult values at 16–18 years [for a review, see ref. 99]. The general trends in cerebral oxygen consumption and cerebral blood flow match those of glucose utilization, with a peak in early childhood and adult levels by the teenage years [100, 101]. From these indirect measurements, it is unclear whether the developmental trends in glucose utilization and oxygen consumption are driven by maturation of metabolic enzymes or whether the metabolic machinery is simply responding to increasing energy demands during periods of brain growth and maturation.

Cerebral Metabolism and Mitochondria after Pediatric TBI

Direct evidence for alterations in mitochondrial energy metabolism caused by TBI in the developing brain is very limited. Preclinical [42, 102] and clinical [103] studies in adults have documented profound reduction in mitochondrial respiratory capacity after TBI. Alterations in cerebral glucose metabolism are seen in immature rats, with early, immediate hyperglycolysis and secondary metabolic depression over 1–3 days after TBI [104]. This pattern was unique to the developing brain, as studies in the same laboratory in adult rats demonstrated that the period of metabolic depression was much longer, extending for up to 2 weeks after the injury [105]. Furthermore, the duration of metabolic depression after TBI correlated with the duration of neurologic impairment seen on Morris water maze testing, with significant age-related differences [106]. During this period of metabolic dysfunction after TBI, the use of alternative fuel substrates, such as ketone bodies, may also be developmentally regulated. This was studied in the controlled cortical impact injury model of TBI, where the ketogenic diet reduced cortical contusion volume in PND 35 and PND 45 rats, but did not protect the youngest (PND 17) or oldest (PND 65) rats studied [107]. Additional studies are needed to evaluate the developmental differences in utilization of ketone bodies, specifically the effects on cellular energy metabolism that may be responsible for neuroprotection. Importantly, one study in this special issue used gel-based proteomics to study alterations in protein expression 2 weeks after CCI in PND 17 rats (see Kochanek et al. in this issue of *Developmental Neuroscience*). These investigators found significant reduction (approximately 30–35%) in the pyruvate dehydrogenase E1 β- and α-subunits in injured rats compared with sham. This has clear implications in energy metabolism after pediatric TBI, as pyruvate dehydrogenase is the critical enzymatic link between glycolysis and the TCA cycle. Furthermore, reductions in the enzyme may necessitate the use of alternative fuels to sustain aerobic energy metabolism in the injured brain.

Advances in imaging modalities available for clinical use after TBI have provided additional description of the global metabolic alterations after pediatric TBI. A series of studies by Ashwal et al. [41] have used proton MRS to study infants and children in the first 2 weeks after severe closed head injury [108, 109]. They observed marked elevations in brain lactate, a marker of anaerobic energy metabolism, and reductions in N-acetyl aspartate (NAA), a marker of neuronal integrity [109]. Importantly, these metabolic derangements correlated with long-term neurologic outcome, and the combination of clinical markers with spectroscopic data has predictive value in neuropsychological testing performed up to many years after injury [110]. As would be expected, limitations of clinical MRS studies include the inability to control for exact time after injury when obtaining the scan. In addition, most MRS studies in pediatric TBI have been obtained 3 days

Fig. 2. ^{1}H nuclear magnetic resonance of 17-day-old rat brain 24 h after TBI. Rats (PND 17) underwent controlled cortical impact injury to the left temporal cortex. Twenty-four hours after injury, brains were rapidly removed, separated into left (injured) and right (control) hemispheres and frozen immediately in liquid nitrogen. Hemispheres were extracted with perchloric acid, neutralized, lyophilized and resuspended in D_2O. ^{1}H spectra were obtained using the Varian Inova 500-MHz spectrometer. ^{1}H spectra from the injured hemisphere (**a**) shows an increased lactate/NAA ratio compared with the uninjured hemisphere (**b**), predominantly due to reductions in NAA signal. Lac = Lactate.
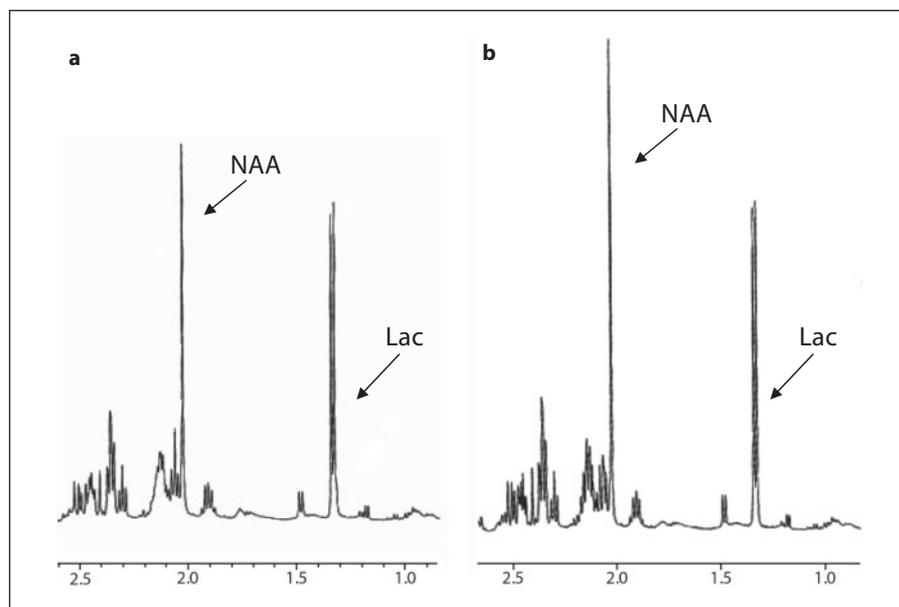
or later after TBI, due to the general instability of the patients early (first 24 h) after injury. Therefore, valuable information about very early metabolic alterations is missing.

Studies using ex vivo nuclear magnetic resonance spectroscopy after TBI in adult animals have begun to evaluate these potential early changes in cerebral metabolism in the first hours or day after injury. In the fluid percussion model of TBI in adult rats, reduction in both NAA and glutamate was seen 1 h after injury in both ipsilateral cortex and hippocampus [111]. However, lactate levels were not significantly different in injured rats compared with sham or control rats. The metabolic variations were more pronounced in a study of controlled cortical impact, with very significant elevations in lactate levels in the pericontusion cortex compared with the uninjured contralateral cortex [112]. The additional use of injection of stable isotopes such as ^{13}C-labeled glucose can provide valuable information about oxidative metabolism and alternative pathways of metabolism after brain injury [for a review, see ref. 113]. For instance, adult rats after TBI have increased glucose metabolism via the pentose phosphate pathway [114], an important alternative pathway responsible for the production of NADPH. The pentose phosphate pathway can be activated by oxidative stress. This may be especially important after acute brain injury, as NADPH is necessary for maintenance of reduced glutathione, thereby contributing to effective antioxidant response.

To date, to our knowledge, no studies have reported the time course of early cerebral metabolic alterations following pediatric TBI in immature animals. Preliminary work in our laboratory using the controlled cortical impact model of TBI in 17-day-old rats has shown an increase in the lactate to NAA ratio occurring early (4 h) and sustained for the first 24h after injury, predominantly influenced by reductions in NAA (fig. 2). Future studies should be directed at utilization of ex vivo and in vivo proton and ^{13}C MRS to define the specific time course and severity of metabolic alterations after TBI that may be unique to the developing brain. Additionally, these advanced metabolic studies could provide important endpoints for evaluating metabolic neuroprotective strategies.

Mitochondria and Molecular Cell Death Pathways

It is important to highlight that the understanding and nomenclature of cell death pathways has continued to evolve rapidly in the last few years (for an in-depth review, see *Cell Death and Differentiation,* Supplement: Morphology, November 2005). This has led investigators to understand that 'classic' apoptotic profiles, as seen in vitro with agents such as staurosporine, are rarely seen in vivo. Instead, the cell death process is often a complex mix of both apoptotic and necrotic features, with mitochondrial ATP levels strongly influencing this balance. This section of the review will focus on the molecular aspects of cell
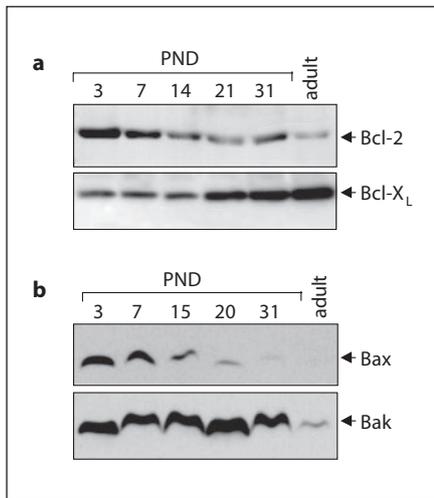
Fig. 3. Expression of Bcl-2 family proteins in brain mitochondria during postnatal development. **a** The relative levels of antiapoptotic Bcl-2 family proteins in brain mitochondria were examined by immunoblotting using equal amounts of mitochondria (50 µg/lane) isolated from rat brains at various postnatal ages (PND 3 to PND 31) and from adult rat brains. A differential pattern of regulation is observed for the antiapoptotic proteins, with a shift from a predominant Bcl-2 expression in the immature brain mitochondria to strong expression of Bcl-X$_L$ in the adult brain mitochondria. **b** Expression of the multidomain proapoptotic Bax and Bak was examined similarly as in **a**. Mitochondrial Bax is detected at high levels at early postnatal ages (PND 3 to PND 7), but declines rapidly after PND 14. Although the expression of Bak is high in postnatal mitochondria up to PND 31, both proteins are almost undetectable in the adult brain mitochondria.

death pathways that relate directly to mitochondrial function and mitochondrial membrane interaction.

The intermembrane space in mitochondria is the storage site for many proapoptotic proteins, including cytochrome c and apoptosis-inducing factor (AIF). Many of the pathologic cascades previously described, such as elevated intracellular Ca^{2+} and ROS, can promote the release of cytochrome c into the cytosol. Although this can occur through generalized mitochondrial swelling and nonspecific release, emerging evidence supports a more detailed process of cytochrome c release. Many proapoptotic Bcl-2 family proteins (e.g., Bax, Bid) promote cytochrome c release by translocating to and binding the outer mitochondrial membrane. This interaction then induces a selective permeability of the outer mitochondrial membrane, releasing cytochrome c and other proteins involved in apoptosis, such as AIF, endonuclease G, Smac/Diablo and others. The antiapoptotic Bcl-2 family proteins oppose these activities by binding to and sequester-

ing proapoptotic proteins. Thus, brain mitochondria play a central role in the process of molecular cell death pathways, both during normal brain development and in the pathologic, posttrauma environment.

Unique Features of the Developing Brain

Although necrotic cell death is always the result of brain injury, apoptotic cell death is an important part of normal brain development. Throughout the pre- and postnatal period, the brain normally undergoes selective, programmed cell death, with more than 50% of some neuronal populations dying during early development [115]. A broad overview would suggest that, in general, most apoptosis-related proteins are expressed at higher levels in the immature brain leading to a greater propensity for apoptotic cell death, but this explanation is likely to be too simplistic. Some important components of the apoptotic cascade, such as AIF and Bcl-X$_L$, show steady levels in the brain throughout development [116–118]. Therefore, it is important to also consider the level of these proteins that are normally present at the mitochondrial level and/or readily translocate from the cytosol to the mitochondrial membrane surface in response to stimuli. The release of cytochrome c and other caspase-independent proteins from the mitochondrial intermembrane space is strongly influenced by the interaction of these pro- and antiapoptotic proteins.

Previous work in our laboratory demonstrated that brain mitochondria isolated from 8- and 17-day-old rats had readily detectable Bax, with barely detectable levels in adult brain mitochondria [119]. Furthermore, when immature mitochondria were exposed in vitro to a synthesized peptide containing the cell death domain of proapoptotic proteins, there was a significant release of cytochrome c, which was not seen in adult brain mitochondria. Thus, both the relative levels of mitochondrial Bax and the functional response to proapoptotic stimuli were unique in the immature rat. We have recently undertaken additional experiments to delineate the pattern of other Bcl-2 family proteins in mitochondria during postnatal development. Studies have shown a differential pattern of expression of pro- and antiapoptotic Bcl-2 family proteins (fig. 3). In general, the proapoptotic proteins were downregulated during development, with Bak expression persisting longer than Bax. The antiapoptotic protein Bcl-2 declined steadily but remained detectable in adult mitochondria. In contrast, expression of the antiapoptotic protein Bcl-X$_L$ increased with development, with the greatest expression in mature brain mitochondria (fig. 3). These trends are similar to work of other in-

vestigators in whole brain evaluation of Bcl-2 family proteins in rats and mice [120–122].

Mitochondria and Cell Death after Pediatric TBI

Extensive literature confirms the susceptibility of the immature brain to apoptotic cell death following hypoxic-ischemic insult [123–126], which is much greater than similar levels of injury in the adult rat [116, 127, 128]. One study demonstrated that mitochondrial release of cytochrome c and AIF, as well as nuclear translocation of AIF, was more pronounced in the immature than in the adult brain following a similar level of hypoxic-ischemic injury [116]. This supports the concept that developmental differences in the upstream regulators of the intrinsic (mitochondrial) cell death pathway could be responsible, in part, for the increased sensitivity of the immature brain to initiate apoptosis during acute brain injury. In contrast to HI, direct evidence for apoptotic pathways in the immature brain after TBI is very limited. One study used electron microscopy to show morphologic evidence for apoptosis in the immature brain after TBI [129]. Trauma induced both excitotoxic (nonapoptotic) and apoptotic morphology, both of which were different from the pattern of physiologic cell death. Using the weight drop method of TBI in rats, this group found that trauma-induced apoptosis was greatest in the youngest rats (PND 3 and PND 7), with a rapid decline between PND 7 and PND 30 [130]. The younger rats (PND 7) had early (4 h) excitotoxic cell death limited to the pericontusion region, but were found to have widespread, significant apoptotic neurodegeneration over the first 24 h after TBI [21]. Antiexcitotoxic intervention (NMDA antagonism) reduced the early cell death, but dramatically worsened the secondary apoptotic damage, highlighting important developmental differences in response to neuroprotective strategies. It should be noted that the description of 'apoptotic' cell death in these studies was based primarily on ultrastructural changes seen using electron microscopy. A more complete understanding of the cell death pathways in studies of immature TBI would include evaluation and quantification with additional cell death assays and support the morphologic findings with additional biochemical measurements, such as caspase activation [131]. Future studies utilizing transgenic mice with overexpression or deletion of key pro- and antiapoptotic proteins, and/or utilization of other methods of genetic suppression (i.e. 'knock-down' approaches with short interfering RNA), may help delineate the time course and degree of molecular cell death pathways after pediatric TBI and the role of mitochondria.

Clinical studies in pediatric victims of severe TBI have also shown evidence for apoptosis. CSF concentrations of Bcl-2 protein were increased in children after TBI compared with control subjects, and elevated Bcl-2 was associated with survival [132]. Additional analysis of resected cortical tissue from 2 patients in this study (ages 3 and 11 years) demonstrated DNA fragmentation by TUNEL staining and detectable Bcl-2 by Western immunoblot. A postmortem analysis of head-injured patients also documented TUNEL-positive cells, consistent with apoptotic profiles, predominantly in the white matter of pediatric victims (ages 4–12 years) [133]. Recently, a pediatric study described increases in CSF cytochrome c after TBI [134]. This increase was independently associated with inflicted TBI and female gender. There were also increases in other proteins involved in apoptosis in the CSF after TBI, including Fas and caspase 1; however, these were not directly associated with inflicted injury.

Conclusions

In the United States alone, it is estimated that approximately 9,000 children per year die secondary to TBI, and that >50,000 children per year suffer severe TBI requiring hospitalization (National Institute of Child and Human Development). The lifetime emotional and societal burden of a severely injured child can be even more profound than an equivalent injury in an adult, yet no effective neuroprotective intervention exists. Furthermore, treatment strategies that have been effective in adult models of TBI may be detrimental in the developing brain [21]. Investigation of age-dependent aspects of the subcellular response to injury would help promote knowledge in this field and could lead to targeted, mitochondrial neuroprotective strategies for pediatric victims of TBI. Such strategies include agents that promote energy metabolism, e.g., ketone bodies [107] and acetyl-L-carnitine [135], and compounds such as CsA that inhibit the opening of the mPTP [44]. Others include mitochondrially targeted antioxidants [136, 137] and small molecules that suppress apoptosis by interfering with the ability of Bax to form pores in the outer membrane [138].

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