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Research report

# Mitochondrial response to calcium in the developing brain

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**Abstract**

Developmental differences in mitochondrial content and metabolic enzyme activities have been defined, but less is understood about the responses of brain mitochondria to stressful stimuli during development. Cerebral mitochondrial response to high $Ca^{2+}$ loads after brain injury is a critical determinant of neuronal outcome. Brain mitochondria isolated from 16–18-day-old rats had lower maximal, respiration-dependent $Ca^{2+}$ uptake capacity than brain mitochondria isolated from adult rats in the presence of ATP at both a pH of 7.0 and 6.5. However, in the absence of ATP, immature brain mitochondria exhibited greater $Ca^{2+}$ uptake capacity at pH 7.0 and 6.5, indicating a greater resistance of immature brain mitochondria to $Ca^{2+}$-induced dysfunction under conditions relevant to those that exist during acute ischemic and traumatic brain injury. Acidosis reduced the maximal $Ca^{2+}$ uptake capacity in both immature and adult brain mitochondria. Cytochrome *c* was released from both immature and adult brain mitochondria in response to $Ca^{2+}$ exposure, but was not affected by cyclosporin A, an inhibitor of the mitochondrial membrane permeability transition. Developmental changes in mitochondrial response to $Ca^{2+}$ loads may have important implications in the pathobiology of brain injury to the developing brain.
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*Theme:* Development and regeneration
*Topic:* Neuronal death

*Keywords:* Cytochrome *c*; Brain mitochondria; Development; Acidosis

## 1. Introduction

Mitochondrial function is essential for normal cerebral energy metabolism. Following injury to the brain, mitochondria have an important role in both neuronal recovery and neuronal death. One well-described role of cerebral mitochondria is the buffering of elevations in neuronal cytoplasmic $Ca^{2+}$ [6,7,47,49]. High levels of cellular $Ca^{2+}$ can occur after many forms of brain injury, such as cerebral ischemia and traumatic brain injury. The ability to effectively buffer high levels of intracellular $Ca^{2+}$ is one determinant of ultimate cell survival. However, excessive $Ca^{2+}$ sequestration by mitochondria can produce injury, leading to respiratory inhibition, uncoupling of oxidative phosphorylation, and ultimately to the release of cytochrome *c* from the mitochondrial membrane into the cytosol. Cytochrome *c* then activates the family of caspase enzymes, leading to apoptotic cell death. Many studies have proposed mechanisms for cytochrome *c* release, most of which involve the formation of a nonspecific pore in the mitochondrial inner membrane called the permeability transition pore (PTP, reviewed in Ref. [29]). The PTP allows solutes to transverse the membrane with a loss of mitochondrial membrane potential and mitochondrial swelling, which can lead to lysis of the outer mitochondrial membrane and the release of cytochrome *c*. Alternatively, other studies have described the release of cytochrome *c* in the absence of PTP formation [2,10]. Regardless of the specific mechanisms, it is clear that the mitochondrial response to high $Ca^{2+}$ loads is an important determinant of cell survival or cell death.

During normal brain development, many cellular and subcellular changes occur. Developmental differences in

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brain mitochondria have been well documented and include changes in mitochondrial content and mitochondrial metabolic enzyme activities [9,17,18,25,41]. A series of studies by the laboratory of Holtzman and others have detailed developmental differences in brain mitochondrial activity in immature (< 4 weeks old) and mature (adult) rats. These studies demonstrated differences in respiration at different ages, with a general trend of lower rates of respiration in the youngest animals (< 2 weeks old), which reached adult levels by the 4th week of life [17,18]. These developmental differences may contribute to both normal neuronal survival after birth and to mitochondrial susceptibility following brain injury. Recent studies have explored mitochondrial dysfunction following hypoxic–ischemic brain injury in immature rats (7–21 days old), documenting alterations in mitochondrial respiration [12,21,36], energy metabolism [12,50] and redox state [46,50,51]. In addition, studies have demonstrated prolonged, apoptotic neuronal death in the developing brain after hypoxic–ischemic injury [3,31], with evidence of an important role for caspase-3 in neuronal death in immature rats [19,31,36], which was not found in mature rats [19].

Given the significant role of mitochondrial $Ca^{2+}$ sequestration in the response to brain injury and the potentially significant developmental differences, we evaluated these responses and compared brain mitochondria isolated from immature rats to those from adult rats. We hypothesized that mitochondria from the brains of immature rats (16–18 days old) would respond differently to in vitro exposure to conditions that occur after brain injury, such as high calcium, low pH and ATP depletion.

## 2. Methods

### 2.1. Mitochondrial isolation

Forebrains were removed from immature rats (16–18 days old) or adult rats and were immediately placed in ice-cold isolation buffer and minced. For immature rats, two brains per mitochondrial preparation were used. Mitochondria (synaptosomal plus non-synaptosomal) were isolated according to the method of Rosenthal et al. [38] in which digitonin is employed to disrupt synaptosomal membranes, with a minor modification for immature animals (1/2 the volume of digitonin added per isolation tube in the adult rat brain mitochondrial preparation). Isolated mitochondria were kept on ice for the duration of the experimental protocols.

### 2.2. Measurement of mitochondrial respiration

Mitochondrial oxygen consumption was determined using a Clark-type oxygen electrode. All assays were conducted in a medium (KCl medium) containing 125 mM KCl, 2 mM $KH_2PO_4$ and 20 mM HEPES–KOH at a pH of 7.0. For assays examining the effects of $Ca^{2+}$ uptake, mitochondria (0.25 mg/ml) were added to KCl medium at 37 °C, supplemented with 5 mM glutamate, 5 mM malate and 1 mM $MgCl_2$ in a total volume of 0.5 ml. State 3 respiration was initiated by the addition of 0.4 mM ADP. In the presence of $Mg^{2+}$, significant ATP hydrolysis can occur in suspensions of isolated mitochondria due to even minor contamination with synaptosomal membranes containing $Mg^{2+}$-dependent ATPases. To obtain the minimum rate of non-phosphorylating, State 4 respiration, the mitochondrial ATP synthetase inhibitor oligomycin (2.2 μg/ml) was added 3 min after the addition of ADP. Mitochondrial respiratory energy coupling was evaluated by determining the Acceptor Control Ratio (ACR), calculated as the ratio of the rate of ADP-stimulated State 3 respiration to the State 4 rate in the presence of oligomycin.

### 2.3. Measurement of mitochondrial calcium uptake

Samples of mitochondria (0.25 mg/ml) were placed in KCl medium in 1 ml volumes in a clear, quartz cuvette. KCl medium was supplemented with 5 mM malate and 5 mM glutamate Fluorescence measurements were made using the Perkin-Elmer LS-3 Fluorescence spectrometer at a controlled temperature of 37 °C. $Ca^{2+}$ uptake was measured by fluorescent detection of the decrease in $Ca^{2+}$ added to the media using the dye Calcium Green 5N (0.1 μM) as previously described [28]. Measurements were made with an excitation wavelength of 506 nm and an emission wavelength of 532 nm and recorded on either a strip-chart recorder or using data collection software. Maximal $Ca^{2+}$ uptake capacity was determined both in the presence and absence of ATP (3 mM) and at a pH of 7.0 and 6.5. $MgCl_2$ was added in amounts based on the presence or absence of ATP (4 and 1 mM, respectively), maintaining the medium free $Mg^{2+}$ concentration at approximately 1 mM. In experimental conditions without ATP, maximal $Ca^{2+}$ uptake was also measured in the presence and absence of 1 μM cyclosporin A, a known inhibitor of the mitochondrial permeability transition pore.

All experiments were conducted with both immature (16–18 days old) and adult rats (3–4 months old) for comparison. $Ca^{2+}$ calibration curves were constructed in all experimental conditions using the standard KCl media with the same substrates but without mitochondria. For the calibration curves, $Ca^{2+}$ was added in incrementally increasing amounts of a known $Ca^{2+}$ concentration from 0 to 320 μM. The Calcium Green 5N fluorescence response was logarithmically related to added $Ca^{2+}$ at both pH 7.0 and 6.5. For each mitochondrial measurement, the amount of $Ca^{2+}$ added to the suspension was adjusted so that the total accumulated was greater than 75% but less than 100% of that added. The final fluorescence level was in a sensitive range of the calibration curve (Fig. 1). The difference between the $Ca^{2+}$ remaining in the medium and the concentration present prior to the addition of $Ca^{2+}$ was then

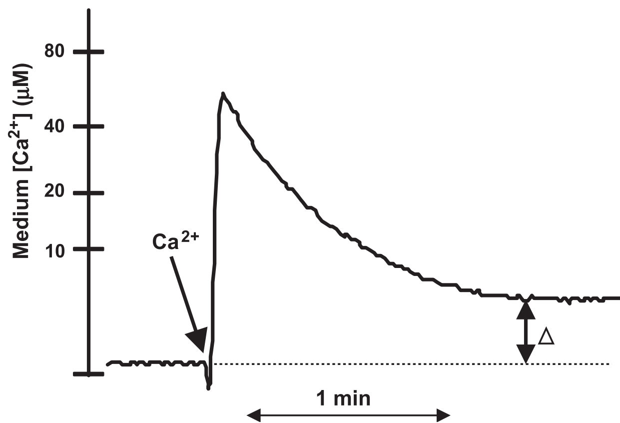


Fig. 1. Calcium Green 5N measurement of $Ca^{2+}$ uptake by brain mitochondria isolated from an immature, 17-day-old rat. Isolated forebrain mitochondria were suspended at a concentration of 0.25 mg/ml in medium maintained at 37 °C containing 125 mM KCl, 5 mM glutamate, 5 mM malate, 2 mM $KH_2PO_4$, 1 mM $MgCl_2$, 0.1 μM Calcium Green 5N and 20 mM HEPES–KOH (pH 6.5). Two minutes after the addition of mitochondria, 100 μM $CaCl_2$ was added. Fluorescent recordings of the medium [$Ca^{2+}$] were continued until the minimum [$Ca^{2+}$] was attained. The difference between the minimum [$Ca^{2+}$] attained following mitochondrial $Ca^{2+}$ uptake and the basal [$Ca^{2+}$] present prior to the addition of $Ca^{2+}$ (Δ), was subtracted from the added $Ca^{2+}$ and defined as the mitochondrial uptake capacity. The Calcium Green fluorescence was calibrated against total added $Ca^{2+}$ for different experimental conditions and on each day experiments were performed.

subtracted from the total added $Ca^{2+}$ to give the maximum uptake capacity, expressed as nmol/mg protein.

### 2.4. Determination of cytochrome c release

Mitochondrial suspensions were exposed to $Ca^{2+}$, removed 8 min later, and centrifuged at 12,000 × *g* for 5 min at 4 °C. The supernatant (200 μl) was removed and supplemented with 30 μl of Protease Inhibitor Cocktail (Sigma). Supernatant and pellet were frozen and stored, and were later assayed for the presence of cytochrome *c* by immunoblot as described [24]. Briefly, aliquots (13 μl) are run on to 4–12% Bis–Tris gradient gels. Proteins are electrotransferred to PVDF membranes that are then rinsed with Tris-buffered saline with 0.05% Tween 20 buffer (Kirkegaard and Perry Labs, Maryland) and blocked for 1 h in buffer supplemented with 1.5% BSA and 1.5% dry milk. Cytochrome *c* is immunostained with primary 7H8 mouse anti-cytochrome *c* antibody (PharMingen, San Diego, CA) plus secondary anti-mouse IgG bound to horseradish peroxidase (Amersham, Arlington Heights, IL). Peroxidase activity is detected using the Enhanced ChemiLuminescence detection kit (Amersham) and X-ray film.

### 2.5. Statistical analysis

Data are expressed as mean and standard error of the mean, where applicable. A Student's *t* test was used to assess statistical differences in calcium uptake between immature and adult mitochondria at each condition. A $p$ value $<0.05$ was considered significant.

## 3. Results

### 3.1. Respiration

Brain mitochondria from immature rats exhibited a lower ACR, with a mean ACR of 6.8 ± 0.4 versus a mean ACR of 8.6 ± 0.6 in adult rats ($p<0.05$) at 37 °C. The State 3 respiratory rate was significantly slower in immature rats (157.6 ± 14.8 vs. 296.4 ± 20.3 nmol $O_2$/min/mg mitochondrial protein, $p<0.01$). The oligomycin-induced State 4 rate was also slower in immature rats, but the difference from adult rats was not statistically significant (24.4 ± 3.1 vs. 35.1 ± 4.7 nmol $O_2$/min/mg, $p=0.06$).

### 3.2. Calcium uptake

A typical Calcium Green 5N fluorescent tracing of medium [$Ca^{2+}$] following the addition of $Ca^{2+}$ and subsequent uptake by isolated forebrain mitochondria from 17-day-old rats is shown in Fig. 1. The goal of these measurements was to determine the maximum amount of $Ca^{2+}$ that can be actively accumulated by brain mitochondria of immature and mature rats under conditions in vitro that simulate at least some of those present within normal cells and those that can exist following acute brain injury. In the experiment shown in Fig. 1, the suspending medium contained the respiratory substrates glutamate plus malate and the pH was adjusted to 6.5, representing a level of acidosis that can exist in the brain following acute brain injury due to ischemia or trauma. Upon addition of 100 μM $CaCl_2$, equivalent to 400 nmol/mg protein, the Calcium Green fluorescence increased rapidly, followed by a decrease due to respiration-dependent mitochondrial $Ca^{2+}$ uptake. In the absence of respiratory substrates or in the presence or respiratory inhibitors, no reduction in the $Ca^{2+}$-induced rise in fluorescence was observed (data not shown). When respiration was active, the added $Ca^{2+}$ was accumulated to varying extents, depending on the amount of $Ca^{2+}$ to which the mitochondria were exposed. In this experiment, net $Ca^{2+}$ uptake ceased at a medium [$Ca^{2+}$] of 5 μM. Therefore the accumulated $Ca^{2+}$ was $100-5=95$ μM. As the mitochondrial protein concentration was 0.25 mg/ml, this represents 380 nmol $Ca^{2+}$ accumulated per mg protein. In order to optimize the accuracy of these net uptake measurements, the amount of added $Ca^{2+}$ was adjusted for each experiment to provide a final medium [$Ca^{2+}$] in the 5–20 μM range where the Calcium Green fluorescence is most sensitive.

The average maximal $Ca^{2+}$ uptake values for immature ($n$=6–8/condition) and mature ($n$=4/condition) brain mitochondria obtained under different conditions in vitro and quantified as described in Fig. 1 are presented in Figs. 2–4.

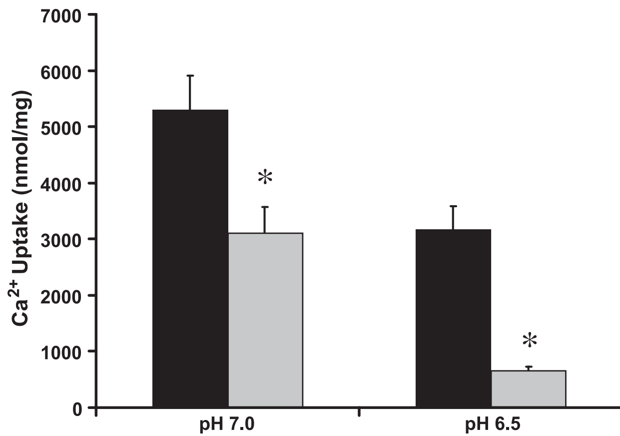


Fig. 2. Calcium uptake capacity of mature and immature brain mitochondria in the presence of ATP. Maximal $Ca^{2+}$ uptake is lower in immature (gray bars) versus adult (black bars) rat brain mitochondria at both a pH of 7.0 ($5305 \pm 598$ vs. $3176 \pm 466$ nmol $Ca^{2+}$/mg mitochondrial protein, *$p < 0.05$) and a pH of 6.5 ($3110 \pm 409$ vs. $645 \pm 66$ nmol $Ca^{2+}$/mg mitochondrial protein, *$p < 0.05$) in the presence of 3 mM ATP. Other conditions were as described in the Methods.

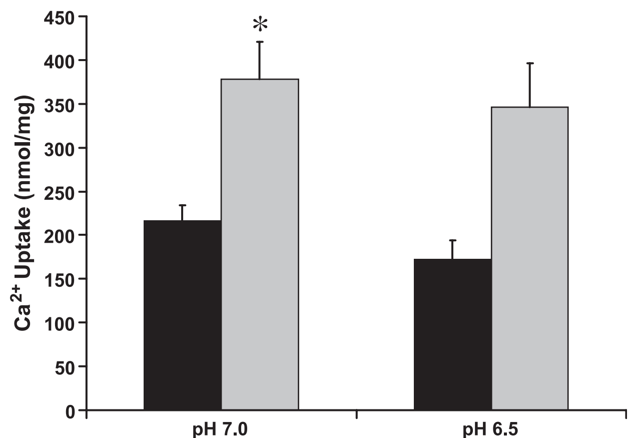


Fig. 3. Calcium uptake capacity of mature and immature brain mitochondria in the absence of ATP. Maximal $Ca^{2+}$ uptake is increased in immature (gray bars) versus adult (black bars) rat brain mitochondria at a pH of 7.0 ($378 \pm 43$ vs. $217 \pm 17$ nmol $Ca^{2+}$/mg mitochondrial protein, *$p < 0.05$) with a trend toward increased calcium uptake at a pH of 6.5 ($346 \pm 50$ vs. $173 \pm 21$ nmol calcium/mg mitochondrial protein, $p = 0.06$) in the absence of ATP. Other conditions were as described in the Methods.

In the presence of ATP at a pH of 7.0, brain mitochondria isolated from immature rats exhibited a significantly lower (approximately 40%) $Ca^{2+}$ uptake capacity than mitochondria from mature animals (Fig. 2). This difference was even more pronounced at a pH of 6.5, with the $Ca^{2+}$ uptake capacity of immature rat brain mitochondria being approximately 80% less than that of adult rats. Thus, acidic pH reduced the mitochondrial $Ca^{2+}$ uptake capacity for both ages but the extent of reduction was much greater for immature brain mitochondria (80%) than for mature brain mitochondria (40%).

Mitochondrial $Ca^{2+}$ uptake was also measured in the absence of ATP as this condition is particularly relevant to the early, post-ischemic scenario where mitochondria are exposed to extremely high levels of $Ca^{2+}$ present in the cytosol. As expected from previous studies with adult brain mitochondria [23], $Ca^{2+}$ uptake capacity was much lower (>90%) in the absence of ATP than in its presence for both adult and immature brain mitochondria (Fig. 3). At a pH of 7.0, immature rat brain mitochondria accumulated an average of approximately 380 nmol $Ca^{2+}$/mg mitochondrial protein, which was significantly greater than the uptake capacity of approximately 220 nmol $Ca^{2+}$/mg mitochondrial protein for adult brain mitochondria. Immature rat brain mitochondria also had a greater calcium uptake capacity at a pH of 6.5, but this did not reach statistical significance. In contrast to the profound effect of acidic pH observed in the presence of ATP, no effect was observed in the absence of ATP for immature or mature brain mitochondria.

The capacity of mitochondria to actively transport and retain exogenous $Ca^{2+}$ is limited by the deleterious effects of accumulated $Ca^{2+}$. High mitochondrial $Ca^{2+}$ can reduce the ability of mitochondria to generate and maintain a high membrane potential, the force that drives electrophoretic $Ca^{2+}$ uptake. One mechanism by which intramitochondrial $Ca^{2+}$ disrupts the membrane potential is through activation of the inner membrane permeability transition pore. To probe for the involvement of this pore in our experiments, $Ca^{2+}$ uptake capacity was measured in the absence and presence of cyclosporin A, a well-established inhibitor of the membrane permeability transition. Cyclosporin A had no effect on the $Ca^{2+}$ uptake capacity of either immature or mature brain mitochondria in the absence of ATP at pH 7.0 (Fig. 4). The lack of effect of cyclosporin A persisted at pH 6.5 (data not shown). Other experiments also demonstrated a lack of effect of cyclosporin A in the mitochondrial $Ca^{2+}$ uptake capacity in the presence of ATP (data not shown).

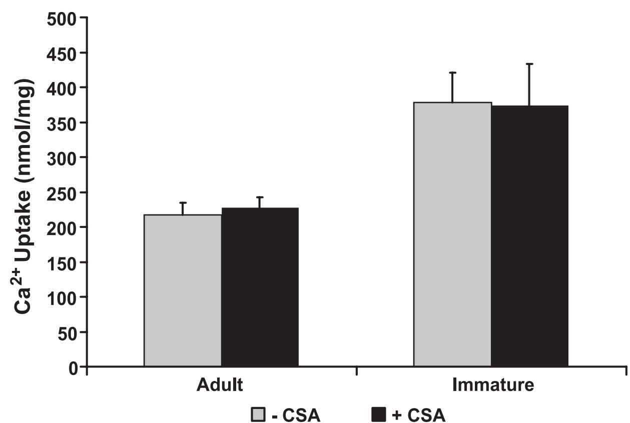


Fig. 4. Effect of cyclosporin A on the calcium uptake capacity of brain mitochondria measured at pH 7.0. In the absence of ATP, maximal $Ca^{2+}$ uptake is unchanged in the absence (gray bars) and presence (black bars) of 1 μM cyclosporin A in both adult ($217 \pm 17$ vs. $228 \pm 15$ nmol $Ca^{2+}$/mg mitochondrial protein, $p$ = NS) and immature ($378 \pm 43$ vs. $374 \pm 59$ nmol $Ca^{2+}$/mg mitochondrial protein, $p$ = NS) rat brain mitochondria. Other conditions were as described in the Methods.

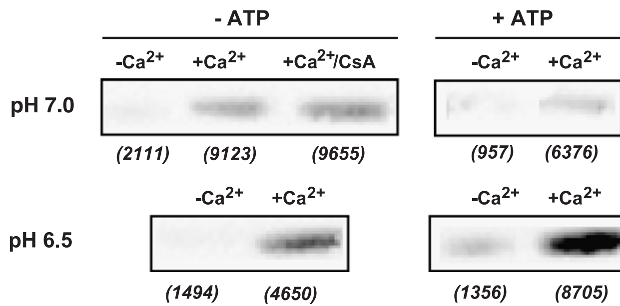


Fig. 5. Immunoblots of cytochrome *c* released from immature brain mitochondria in response to $Ca^{2+}$. Brain mitochondria isolated from 16–18-day-old rats were suspended under the same conditions described in Figs. 2–4 and exposed to either 300 nmol $Ca^{2+}$/mg protein (− ATP) or 2000 nmol $Ca^{2+}$/mg protein (+ ATP). Following 8 min of exposure, the suspensions were centrifuged and the supernatant used for immunoblot determinations of cytochrome *c*, as described in the Methods. Numbers in parentheses refer to relative band densities. These blots are representative of two to four experiments for each condition.

### *3.3. Cytochrome c release*

A potential mechanism by which $Ca^{2+}$ uptake causes mitochondrial dysfunction and also triggers the apoptotic pathway of cell death is the induction of the release of cytochrome *c* from the mitochondrial intermembrane space into the surrounding milieu. Immunoblot analysis of cytochrome *c* present in the supernatant following centrifugation of the suspension of immature brain mitochondria after exposure to $Ca^{2+}$ revealed an approximately fivefold increase in extramitochondrial cytochrome *c* (Fig. 5), similar to what we reported earlier for adult rat brain mitochondria [2]. $Ca^{2+}$-induced cytochrome *c* release was not inhibited by the permeability transition inhibitor cyclosporin A either in the absence of ATP (Fig. 5) or in its presence (data not shown), again as shown previously for mature brain mitochondria [2]. Densitometric analysis revealed a similar, fivefold increase in extramitochondrial cytochrome *c* following exposure to $Ca^{2+}$ in the absence of ATP at either pH 7.0 or 6.5 or in the presence of ATP at 6.5. In the presence of ATP at 7.0, the extent of cytochrome *c* release was similar to that observed under other conditions but the increase compared to the timed control was greater.

## 4. Discussion

In this initial evaluation comparing the response of immature and adult rat brain mitochondria to supranormal levels of $Ca^{2+}$, several differences were established. As mitochondria appear to be the primary target of toxic, elevated intracellular $Ca^{2+}$ during brain hypoxia, ischemia and, possibly, trauma (reviewed in Ref. [11]), these differences could be important in characterizing the contribution of different subcellular mechanisms of neural cell death in pediatric and adult populations.

The effect of acidic pH on mitochondrial $Ca^{2+}$ uptake capacity is one relationship that we compared between immature and adult rats. Cerebral acidosis has been reported in both animal and human studies following ischemic and traumatic brain injury, with reduction in brain tissue pH to as low as 6.0–6.5 during periods of maximal ischemia [20,26,27,30,39,45]. Acidosis may produce mitochondrial dysfunction via effects on respiration, reactive oxygen species (ROS) generation and $Ca^{2+}$ sequestration. The influence of acidosis on mitochondrial activities has been demonstrated in studies using animals [16,37] and with in vitro evaluation of pH on isolated mitochondria [1,15]. However, none of these investigations have analyzed mitochondria from immature animals. Studies using immature animals and pediatric patients suggest unique differences in post-traumatic cerebral metabolism that may make the developing brain more vulnerable to acidosis. For example, following traumatic brain injury, young rats have a shortened period of cerebral metabolic depression in response to injury compared to adult rats while both exhibit substantial post-traumatic hyperglycolysis [44]. In clinical studies, pediatric victims of head injury show significant elevations in tissue lactate in regions of contused brain [43]. We found that acidosis decreased maximal $Ca^{2+}$ uptake capacity in brain mitochondria isolated from both adult and immature rats and measured in the presence of ATP (Fig. 2). However, immature rat brain mitochondria exhibited a much greater decrease in maximal calcium uptake with an 80% reduction versus a 40% reduction in adult rats, when comparing a pH of 7.0 to a pH of 6.5. The reduced capacity of mitochondria from immature animals to uptake $Ca^{2+}$ in an acidotic environment could lead to higher cytosolic $Ca^{2+}$. Moreover, the inhibition of acidic pH on mitochondrial $Ca^{2+}$ uptake capacity represents an increase in the sensitivity of brain mitochondria to bioenergetic failure, which, in turn, limits the ability of mitochondria to accumulate and retain $Ca^{2+}$.

The profound effect of acidosis on mitochondrial $Ca^{2+}$ uptake capacity was only observed in the presence of ATP, as acidic pH only mildly depressed the uptake capacity of mature or immature brain mitochondria in the absence of ATP (Fig. 3). Mitochondrial $Ca^{2+}$ uptake is driven by the negative-inside membrane potential established by either respiration- or ATP hydrolysis-dependent $H^+$ efflux. The finding that acidic pH had little effect on uptake capacity in the absence of ATP suggests that the well-characterized inhibitory effect of acidosis on mitochondrial respiration is not responsible for the inhibition of $Ca^{2+}$ accumulation observed in the presence of ATP.

The $Ca^{2+}$ uptake capacity was more than 10-fold greater in the presence of ATP for both immature and mature brain mitochondria. The mechanism by which acidosis inhibits net $Ca^{2+}$ uptake in the presence of ATP is therefore likely related to the potentiation of massive mitochondrial $Ca^{2+}$ accumulation by adenine nucleotides. This phenomenon has been ascribed to two different mechanisms. Adenine nucleotides and ATP in particular promote the precipitation

of $Ca^{2+}$ and phosphate within the mitochondrial matrix, thus maintaining low osmotically active $Ca^{2+}$ (micromolar) in the face of exceedingly high (millimolar to molar) concentrations of total $Ca^{2+}$ [13,48]. As calcium phosphate precipitation is inhibited by acidic pH, this could explain the inhibition of massive mitochondrial $Ca^{2+}$ uptake by acidosis observed in the presence of ATP. Adenine nucleotides are also known to inhibit the $Ca^{2+}$-induced mitochondrial membrane permeability transition, which destroys the driving force behind active mitochondrial $Ca^{2+}$ transport [14,40]. Although acidic pH can inhibit the MPT under some conditions [4], recent evidence indicates that acidosis actually promotes the MPT under conditions where mitochondria are energized, such as those used in our study [22]. Promotion of the MPT by acidic pH is unlikely to be responsible for pH effects on mitochondrial $Ca^{2+}$ uptake capacity as the MPT inhibitor cyclosporin A had no effect on $Ca^{2+}$ accumulation by either adult or immature brain mitochondria at pH 7.0 or 6.5 (Fig. 4 and data not shown). It is, however, possible that an acid pH-sensitive and cyclosporin A-insensitive MPT limits the $Ca^{2+}$ uptake capacity in the presence of ATP as we and others have reported a cyclosporine-A insensitive MPT with brain mitochondria that is particularly apparent in the presence of ATP [5,8].

In addition to impairing the ability of mitochondria to accumulate and retain $Ca^{2+}$, acidosis promoted the cyclosporin A-insensitive release of cytochrome *c* caused by $Ca^{2+}$ uptake (Fig. 5). This effect was particularly evident in the presence of ATP, suggesting a link to the pronounced effect of acid pH on $Ca^{2+}$ uptake under these conditions. The release of cytochrome *c* results in both respiratory inhibition and stimulation of mitochondrial ROS generation [34,42]. As the effect of pH on cytochrome *c* release was measured only with immature brain mitochondria, future experiments will be directed at comparing the influence of acidosis on the release of cytochrome *c*, respiration and ROS production by both immature and mature brain mitochondria. In addition to possibly providing further insight into the mechanisms of action of acidosis on mitochondrial dysfunction, these experiments may contribute to the knowledge of age-selective mechanisms of neural cell injury caused by factors relevant to acute brain ischemia and trauma.

Quantitative differences between the $Ca^{2+}$ uptake capacity of immature and mature brain mitochondria were detected in addition their relative sensitivity to acidic pH. While in the presence of ATP, the $Ca^{2+}$ uptake capacity of immature mitochondria was less than that of the adult's (Fig. 2), the uptake capacity was significantly greater than the adult mitochondria in the absence of ATP (Fig. 3). It is therefore possible that the relative resistance of immature brain mitochondria to dysfunction caused by $Ca^{2+}$ in the absence of ATP could confer the neural cells of immature animals with decreased vulnerability to acute cell death caused by excitotoxic levels of intracellular $Ca^{2+}$ at early periods following ischemia or trauma when cellular ATP levels are very low. On the contrary, the lower $Ca^{2+}$ uptake capacity of immature brain mitochondria in the presence of ATP could potentially increase the sensitivity of cells within the immature brain to excitotoxic $Ca^{2+}$ caused by, e.g., post-traumatic seizure activity, when cellular ATP has recovered.

The molecular explanation for the differences in the response of immature and mature brain mitochondria to high levels of $Ca^{2+}$ in the presence of various conditions in vitro is at this juncture unknown. Previous comparisons of non-synaptosomal brain mitochondria during postnatal development have focused on rates of respiration and enzyme activities of respiratory enzymes, both being generally lower in immature animals [9,17,18]. We also observed lower rates of State 3 respiration and lower Acceptor Control Ratios for immature synaptosomal plus non-synaptosomal brain mitochondria. These differences are unlikely to be responsible for the age-related differences in $Ca^{2+}$ uptake capacity as the maximum uptake by immature brain mitochondria is greater in the absence ATP, where $Ca^{2+}$ uptake can only be driven by respiration, and is lower in the presence of ATP, where both ATP hydrolysis and respiration can generate the membrane potential that drives $Ca^{2+}$ uptake. In addition to exploring the molecular mechanism responsible for changes in $Ca^{2+}$ uptake capacity of brain mitochondria during postnatal development, it will also be important to determine the relative changes that occur in synaptosomal compared to non-synaptosomal mitochondria, as these fractions are enriched with mitochondria from neurons and astrocytes, respectively. Finally, this study only evaluated one postnatal time point (16–18 days old), and there are likely to be distinct mitochondrial differences at other ages, which warrant further study. Understanding these differences in the normal (uninjured) "neonatal" (3–10 days old) and "pediatric" rat (10–20 days old) will be important in order to make comparisons with commonly used animal models of brain injury, such as the neonatal hypoxia–ischemia and pediatric traumatic brain injury models.

Multiple studies of immature rats using models of hypoxia–ischemia have demonstrated changes in the respiratory chain and other mitochondrial enzyme activity after hypoxia–ischemia [12,21,50], and many of these changes were different from those seen in adult rats [21]. Other studies documenting the relative importance of apoptosis in hypoxic–ischemic and traumatic brain injury in immature animals suggest an important role for mitochondrial abnormalities other than metabolic dysfunction [3,32,33]. We found that endogenous levels of the pro-apoptotic protein Bax are higher in immature compared to mature brain mitochondria, allowing for increased release of cytochrome *c* in response to exposure to other pro-apoptotic proteins, e.g., tBid [35]. As this finding has implications regarding the relatively greater contribution of apoptosis in immature brain injury, it will be equally important to identify the

molecular basis of the age-dependent response of brain mitochondria to $Ca^{2+}$, considering its relevance to excitotoxic cell death.

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