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In vivo application of mitochondrial pore inhibitors blocks the induction of apoptosis in axotomized neonatal facial motoneurons

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Abstract

Axotomy induces apoptosis in motoneurons of neonatal rodents. To identify the key players in motoneuron apoptosis, we assessed the progression of apoptosis at 4 h intervals following facial motoneuron axotomy. The mitochondrial release of cytochrome *c*, caspase-3 activation and nuclear condensation were first observed in the motoneuron cell bodies 16 h postaxotomy. *In vivo* application of inhibitors of the mitochondrial permeability transition pore, Bongkrekic acid and cyclosporin A prevented cytochrome *c* release as well as caspase-3 activation and attenuated motoneuron apoptosis. Similarly, *in vivo* application of RU360, an inhibitor of the mitochondrial calcium uniporter, also protected axotomized motoneurons from apoptosis. Taken together, our results show that cytochrome *c* release and subsequent caspase-3 activation are critical events that precipitate the apoptotic death of axotomized neonatal motoneurons *in vivo*. In addition, these results provide evidence that application of mitochondrial pore inhibitors *in vivo* can block the induction of apoptosis following motoneuron axotomy.

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Abbreviations: MPTP, mitochondrial permeability transition pore; ANT, adenine nucleotide translocator; BKA, Bongkrekic acid; CsA, cyclosporin A

Introduction

Axotomy results in the apoptotic death of motoneurons in neonatal rodents but not of motoneurons in adult rodents, which typically survive axotomy.[1–3] The induction of apoptosis has been attributed to a loss of trophic factor support as neonatal motoneurons are dependent on target contact for a supply of trophic factors.[4–6] Loss of trophic support, however, may not be the sole determining factor responsible for neonatal motoneuron apoptosis. Axonal injury results in a massive influx of calcium into the injured axon with concomitant depolarization.[7,8] Applications of MK-801, an NMDA receptor antagonist, or calcium channel blockers such as flunarizine and cinnarizine rescues motoneurons from axotomy-induced apoptosis suggesting an excitotoxic/Ca^{2+} component to motoneuron apoptosis.[9–11]

The activation of apoptosis and the apoptotic pathway responsible for motoneuron death *in vivo* have yet to be elucidated. Bax, a proapoptotic gene, is required for motoneuron apoptosis as motoneurons in Bax knockout mice survive axotomy.[12] The proapoptotic actions of Bax are associated with its translocation to the mitochondria followed by the release of death-inducing factors such as cytochrome *c*, caspase-9, AIF and SMAC/Diablo into the cytosol, ultimately resulting in caspase activation.[13–19] We have previously shown that caspase-3 is activated following axotomy of neonatal motoneurons and is essential for nuclear condensation.[20]

In vitro studies have shown that, at least in some cases, the release of death-promoting factors from the mitochondria is a crucial step in committing a cell to an apoptotic death.[21] The mechanism by which proapoptotic factors are released from the mitochondria is presently unclear. Studies have demonstrated the formation of a mega-ion pore, the mitochondrial permeability transition pore (MPTP).[22,23] Upon opening, the MPTP behaves as a nonselective ion channel allowing a massive influx of Ca^{2+} and K^+ into the mitochondrial matrix resulting in loss of mitochondrial membrane potential, swelling due to osmotic changes and eventual rupture of the outer mitochondrial membrane.[23] Bax has been shown to activate MPTP by interacting with the inner mitochondrial membrane adenine nucleotide translocator (ANT).[15,24] Excitotoxic insults have also been shown to activate MPTP via an influx of calcium into the mitochondria.[25–28] Since both excitotoxicity and Bax-mediated apoptotic pathways involve the mitochondria, we hypothesized that MPTP-related changes may play a pivotal role in the induction of apoptosis in axotomized neonatal motoneurons.

Results

In vitro studies have shown that the release of death-promoting factors from the mitochondria into the cytosol is a

crucial step in committing a cell to an apoptotic death.[21] To determine whether mitochondria release proapoptotic factors following motoneuron axotomy *in vivo*, sections of the axotomized facial nucleus were examined for the translocation of cytochrome *c* from mitochondria to cytosol as demonstrated by a change from punctate to diffuse immunostaining. In uninjured P0 facial motoneurons, the punctate immunoreactivity of cytochrome *c* colocalized with the punctate immunostaining of COX IV (Figure 1a,c). At 24 h following facial motoneuron axotomy in neonatal rats, the immunostaining pattern of cytochrome *c* became diffuse (Figure 1d) or disappeared completely. In contrast, COX IV immunoreactivity retained its punctate pattern (Figure 1b) consistent with its role as an integral protein of the mitochondrial inner membrane that is not released from the mitochondria during apoptosis.

Mitochondrial release of cytochrome c precedes caspase-3 activation in axotomized motoneurons

In neonatal rodents, the majority of motoneurons are dead within 3–5 days after axotomy.[1] Previous studies by Rossiter *et al.*[3] and de Bilbao and Dubois-Dauphin,[2] who each characterized DNA fragmentation and the rate of cell death over 5 days postaxotomy in rats and mice respectively, found that motoneuron cell death within the first 24 h was 10–20%. In neonatal rats the facial nucleus consists of ~4500 motoneurons at birth; therefore a loss of 10% would equal ~450 motoneurons. Both studies also found that DNA fragmentation appeared to peak at 20 or 28 h postaxotomy in rats and

mice, respectively. This was associated with a precipitous loss of 40–50% of axotomized motoneurons by 48 h.[3,18] Therefore, to assess the initiation of apoptosis in axotomized neonatal facial motoneurons, we focused on the first 24 h postaxotomy.

Motoneuron cell bodies examined at 4, 8 and 12 h postaxotomy were similar to their uninjured counterparts. Cytochrome *c* immunostaining was punctate, the cell's nucleus was noncondensed and intact, and caspase-3 activation was not observed (Figure 2a,d,g). Following axotomy of P0 motoneurons, cytochrome *c* immunoreactivity changed from a punctate to a diffuse staining pattern, caspase-3 was activated and nuclei became condensed as shown at 24 h postaxotomy in Figure 2b,e,h. Increases in the numbers of axotomized P0 motoneurons positive for diffuse cytochrome *c* immunoreactivity, active caspase-3 and condensed apoptotic nuclei were observed at 16 and 24 h postaxotomy (Figure 3).

In contrast, at 24 h postaxotomy in adult rats, no motoneurons were found positive for cytochrome *c* release, active caspase-3 or condensed apoptotic nuclei in either the axotomized or contralateral facial nuclei of the six adult rats examined (Figures 2c,f,i, 3a–d).
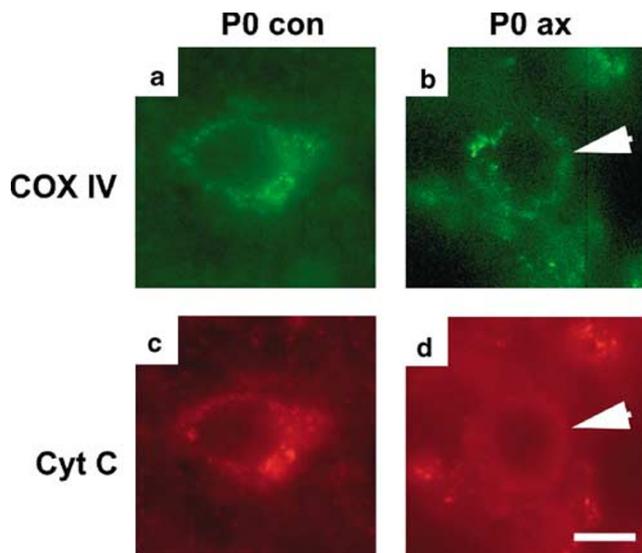
In the uninjured contralateral P0 facial nucleus, a small number of motoneurons were positive for cytochrome *c* release and active caspase-3 and had condensed nuclei indicative of developmental cell death as previously reported.[20]

Quantification of cells positive for cytochrome *c* release, active caspase-3, double-labeling and apoptotic nuclei at 4 h intervals following P0 motoneuron axotomy suggests that these changes take place in the cell soma 16–24 h postaxotomy (Figure 3a–d). Cytochrome *c* release, caspase-3 activation and nuclear condensation all occur rapidly within a short time. Examining the staining of motoneurons for diffuse cytochrome *c*, active caspase-3 and apoptotic nuclei suggested a specific sequence of events.

Active caspase-3 immunostaining was only observed with either diffuse cytochrome *c* or a complete loss of cytochrome *c* immunoreactivity. Since no cell positive for active caspase-3 had punctate (i.e. mitochondrial) cytochrome *c* immunoreactivity, these results indicate that cytochrome *c* release may be a prerequisite for caspase-3 activation following motoneuron axotomy.

Similarly, apoptotic, condensed nuclei were only observed in cells immunoreactive for diffuse cytochrome *c* and active caspase-3 or in cells with a complete loss of cytochrome *c* and active caspase-3 immunoreactivity, but not in cells with punctate cytochrome *c*. These results suggest that the sequence of events following motoneuron axotomy proceeds with the release of cytochrome *c* from the mitochondria, followed by caspase-3 activation and then nuclear condensation.

Numerous cells were observed lacking immunoreactivity for cytochrome *c* and active caspase-3 but with condensed, apoptotic nuclei. The complete lack of immunoreactivity for cytochrome *c* and active caspase-3 in cells with apoptotic nuclei suggests that the duration a cell is immunoreactive for diffuse cytochrome *c* and active caspase-3 is shorter than the duration a cell is positive for apoptotic, condensed nuclei. Our

Figure 1 Double immunohistochemistry for cytochrome *c* and cytochrome oxidase IV (COX IV) of representative motoneurons 24 h postaxotomy in the axotomized and contralateral nonaxotomized P0 facial nuclei. (**a,c**) Cytochrome *c* immunostaining (red) appears punctate and colocalizes with the mitochondrial marker, COX IV (green) in nonaxotomized P0 facial motoneurons. (**b,d**) Following axotomy, cytochrome *c* immunoreactivity changes from a punctate to a diffuse staining pattern in axotomized P0 motoneurons. The diffuse cytochrome *c* immunoreactivity no longer colocalizes with COX IV immunoreactivity demonstrating mitochondrial release of cytochrome *c* into the cytoplasm. Scale bar=10 μm
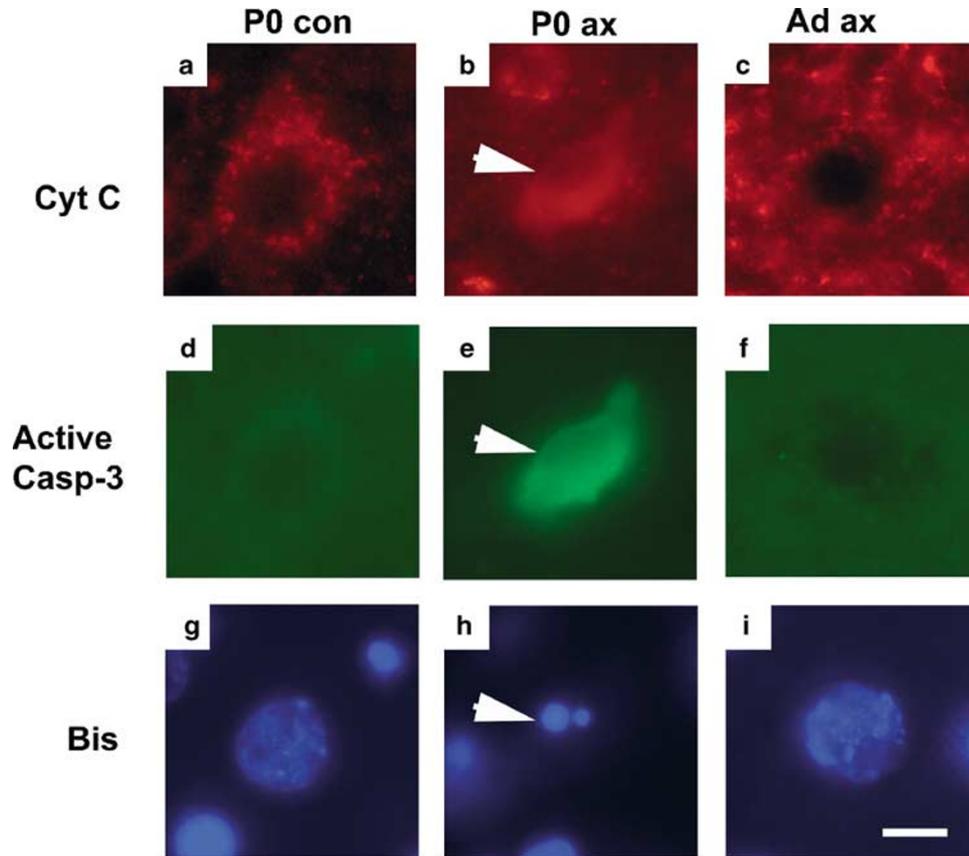
Figure 2 Cytochrome *c* release and caspase-3 activation occur following neonatal but not adult facial motoneuron axotomy. Representative facial motoneurons immunostained for cytochrome *c* (red) and active caspase-3 (green) followed by the nuclear stain, bisbenzimide. (**a,d,g**) Control, uninjured P0 rat facial motoneuron; (**b,e,h**) a P0 rat facial motoneuron 24 h following axotomy; (**c,f,i**) an adult rat facial motoneuron 24 h following axotomy. Note that 24 h postaxotomy, cytochrome *c* immunoreactivity becomes diffuse, caspase-3 is activated and nuclei are condensed in neonatal motoneurons, whereas axotomized adult motoneurons retain punctate cytochrome *c* immunoreactivity, do not activate caspase-3 and nuclei remain intact and noncondensed. Scale bar=10 μm

quantitative data in Figure 3 demonstrate that the number of apoptotic nuclei was significantly ($P<0.05$) greater than in uninjured nuclei at 16 h postaxotomy, whereas the number of cells positive for diffuse cytochrome *c*, or active caspase-3, or both reached significance at 24 h postaxotomy, when a larger number of cells were undergoing apoptosis. In other words, a longer lasting event results in a larger number of cells that are positive at a given time of observation.

Mitochondrial pore inhibitors block cytochrome c release and axotomy-induced apoptosis

We have previously shown that caspase-3 is activated during neonatal facial motoneuron apoptosis and is important for the rapid breakdown of the cell and nuclear condensation. Axotomized motoneurons in caspase-3 null mice, however, are not rescued from cell death; rather motoneuron death is delayed.[20] Since the present data show that diffuse cytochrome *c* staining occurred prior to caspase-3 activation, we examined whether blocking the release of cytochrome *c* could prevent activation of caspase-3 and motoneuron apoptosis.

The release of cytochrome *c* and other proapoptotic proteins from the intermembrane space of mitochondria is believed to occur through a mega-ion pore, the MPTP. The composition of the MPTP is unclear; however, the ANT is believed to be one of the components.[23,29] *In vitro* application of inhibitors of the ANT, specifically Bongkrekic acid (BKA) and cyclosporin A (CsA), have previously been shown to block the mitochondrial release of cytochrome *c*.[30–32] To determine whether the release of cytochrome *c* is necessary for activation of apoptosis *in vivo,* we applied these inhibitors to the axotomized facial nerve. Since few studies have used BKA and CsA *in vivo,* several different concentrations of each inhibitor were used to determine an effective dosage (data not shown). BKA was found to be toxic at high concentrations (1 mM), had the most significant effect at a dosage of 100 μM and was less effective at 10 μM. CsA was most effective at a concentration of 40 mM. Applications of either CsA or BKA on the axotomized nerve stump significantly inhibited the release of cytochrome *c* in the cell soma in comparison with vehicle control (Figure 4a). Blocking the mitochondrial release of cytochrome *c* also significantly reduced the number of cells positive for active caspase-3 (Figure 4b), double-labeled cells (Figure 4c) and the number of cells with condensed nuclei (Figure 4d).

Axonal injury results in a massive influx of calcium into the injured axon with concomitant depolarization.[7,8,33] Previous
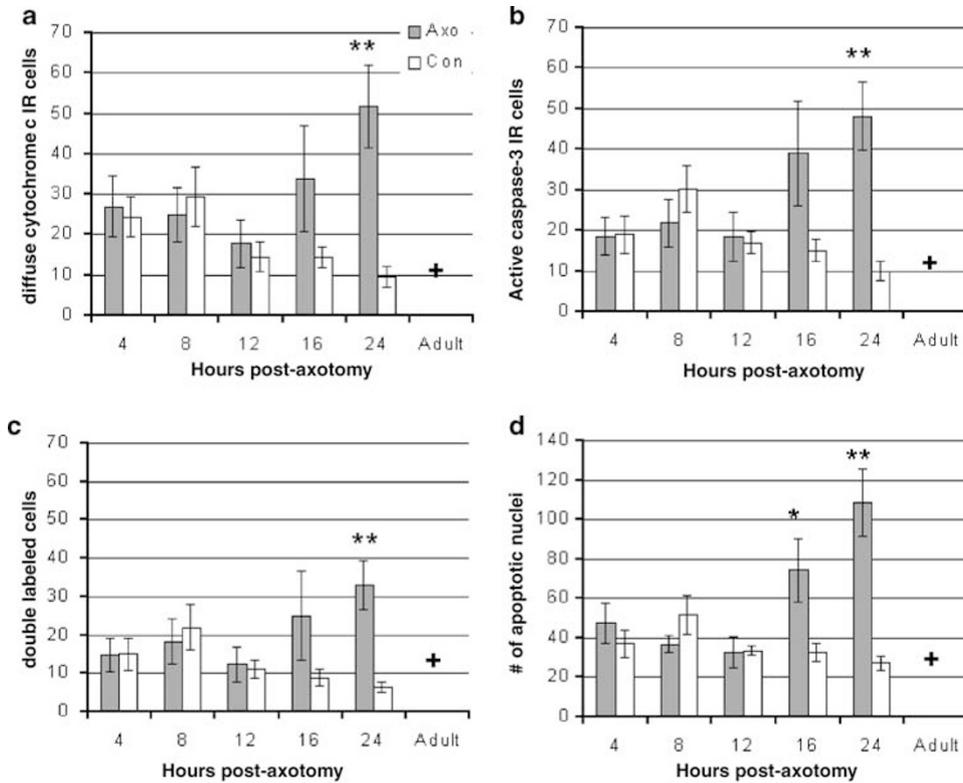
Figure 3 Time course for the release of cytochrome *c*, caspase-3 activation and nuclear condensation following facial motoneuron axotomy. Quantification of the number of motoneurons per facial nucleus that are positive for (**a**) diffuse cytochrome *c*; (**b**) active caspase-3; (**c**) double-labeled (diffuse cytochrome *c* and active caspase-3 immunoreactivity); and (**d**) condensed, apoptotic nuclei at 4, 8, 12, 16 and 24 h following neonatal motoneuron axotomy ($n=4$ or 5 per time point) and at 24 h following adult motoneuron axotomy ($n=6$). $*P<0.05$ and $**P<0.005$ indicate significance between axotomized and contralateral, nonaxotomized neonatal facial nuclei. $+P<0.001$, significance between axotomized neonatal and axotomized adult facial nuclei at 24 h postaxotomy
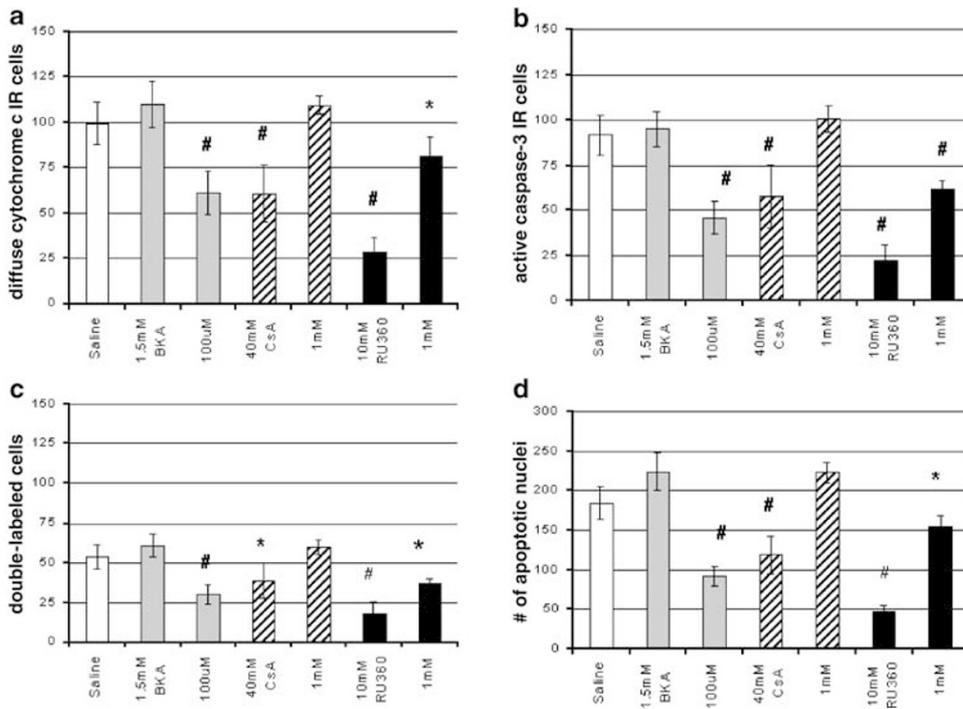
Figure 4 Effect of mitochondrial inhibitors on cytochrome *c* release, caspase-3 activation and apoptotic nuclei formation 24 h following facial motoneuron axotomy. Graphs show the number of cells positive for (**a**) diffuse cytochrome *c*; (**b**) active caspase-3; (**c**) double-labeled (diffuse cytochrome *c* and active caspase-3 immunoreactivity); and (**d**) condensed apoptotic nuclei per neonatal facial nucleus following treatments of vehicle, CsA, BKA and RU360. $*P<0.05$ and $P<0.01$ indicate significance between vehicle-treated and inhibitor treated

studies have rescued motoneurons with applications of cation pore inhibitors and the NMDA inhibitor, MK-801, suggesting a role for calcium in motoneuron apoptosis.[9–11,34] An increase in intramitochondrial calcium has been shown to induce formation of the mitochondrial permeability transition pore.[35] To further explore whether mitochondrial involvement in motoneuron apoptosis could be attributed to an influx of calcium, we applied RU360, an inhibitor of the mitochondrial calcium channel uniporter, to the injured axons. RU360 significantly inhibited cytochrome *c* release at both 1 and 10 mM concentrations. It is interesting to note that for each of the pore inhibitors, the dose that significantly blocked the release of cytochrome *c* also significantly inhibited caspase-3 activation, nuclear condensation and apoptosis (Figure 4a–d).

Discussion

Using the translocation of cytochrome *c* from mitochondria to cytosol as a marker of mitochondrial involvement, our results show that cytochrome *c* release occurs within 24 h following axotomy of motoneurons in neonatal but not adult rats. Consistent with the sequential relationship of cytochrome *c* release and caspase-3 activation from *in vitro* models of apoptosis, caspase-3 activation occurred rapidly following cytochrome *c* release and was closely followed by nuclear condensation. Applications of mitochondrial pore inhibitors rescued neonatal motoneurons from axotomy-induced death by blocking the induction of apoptosis.

Time course of apoptosis following motoneuron axotomy

Immunohistochemical analysis of motoneurons at 4, 8, 12, 16 and 24 h postaxotomy was performed to study the progression of apoptosis. Axotomy-induced changes in the cell soma were first observed at 16 h postaxotomy with the mitochondrial release of cytochrome *c* and caspase-3 activation. These results are consistent with our previous results examining caspase-3 activation.[20] Bisbenzimide staining revealed an increase in the number of neurons positive for nuclear condensation by 16 and 24 h postaxotomy, consistent with the findings of previous studies using terminal deoxynucleotide nick-end labeling (TUNEL) or *in situ* end-labeling (ISEL) to detect DNA fragmentation.[2,3] Rossiter *et al.*[3] demonstrated that an axotomized motoneuron stains positive for DNA fragmentation (ISEL) for ∼2 h. Our results indicate that the number of motoneurons positive for diffuse cytochrome *c*, active caspase-3 or both reached significance at 24 h postaxotomy. This suggests that the duration a cell is positive for diffuse cytochrome *c*, active caspase-3 or both is shorter than the duration a cell is positive for DNA fragmentation and therefore less than 2 h. Hence, a much greater percent of cells must be undergoing apoptosis before one observes a significant increase in the number of cells immunoreactive for diffuse cytochrome *c* or active caspase-3 at a given time point.

Motoneuron axotomy in adult rodents does not result in the induction of apoptosis within 24 h, in contrast with motoneurons from neonates (for review see Lowrie and Vrbova[1]). Our

results support these findings, such that cytochrome *c* immunoreactivity remained punctate and colocalized to the mitochondria, caspase-3 was not activated and nuclear condensation did not occur.

Cytochrome c release occurs prior to caspase-3 activation in axotomized motoneurons

Double immunohistochemistry for cytochrome *c* and active caspase-3 suggested that the release of cytochrome *c* preceded caspase-3 activation. Motoneurons immunoreactive for active caspase-3 had either diffuse cytochrome *c* staining or had lost their cytochrome *c* immunoreactivity all together, while punctate cytochrome *c* was not observed with activated caspase-3 in the same cells. In addition, our results using mitochondrial pore inhibitors BKA, CsA, and RU360 not only blocked cytochrome *c* release but also prevented caspase-3 activation and nuclear apoptosis indicating the caspase-3 activation requires mitochondrial involvement. Collectively, these results demonstrate two distinct points, firstly that mitochondria are involved in axotomy-induced neonatal motoneuron apoptosis and secondly that neonatal motoneuron apoptosis progresses with the mitochondrial release of cytochrome *c* – followed by caspase-3 activation – and lastly nuclear condensation.

Mitochondria act at a pivotal point in axotomy-induced motoneuron apoptosis

Several studies have suggested that the release of proapoptotic factors from the mitochondria during apoptosis occurs through the formation of a mega-ion pore, the MPTP, of which the ANT is believed to be a component.[23,29,35] In this study, we applied BKA and CsA, pharmacological inhibitors of the ANT, *in vivo* to determine whether the MPTP was responsible for the release of cytochrome *c* in motoneuron apoptosis. Application of either BKA or CsA significantly blocked cytochrome *c* release and subsequent apoptosis. BKA inhibits MPTP by binding to a competitive atractyloside site on the ANT and preventing the transport of ADP into the mitochondria and its subsequent oxidative phosphorylation.[36,37] CsA competes for a cyclophilin D binding site on the ANT.[23] In response to oxidative stress, cyclophilin D, a mitochondrial specific cyclophilin, binds to the ANT causing a conformational change in the ANT, thereby making it more sensitive to changes in matrix Ca^{2+} and activating MPTP.[35,38,39] The binding of CsA to the closed ANT prevents cyclophilin D from interacting with the ANT and activating MPTP. The ANT is required for the generation of ATP, by transporting ADP into the mitochondrial matrix in exchange for ATP; hence, long-term inhibition of the ANT results in a depletion of ATP and in turn ultimately cell death.[36,40]

Short-term *in vitro* applications of either BKA or CsA have been shown to block mitochondrial-mediated apoptosis, by preventing the loss of mitochondrial membrane potential, cytochrome *c* release and nuclear condensation.[30–32] However, Budd *et al.*[41] showed that although BKA inhibited mitochondrial membrane depolarization and caspase-3 activation, BKA did not prevent cytochrome *c* release in

NMDA-induced cortical neuron death. In addition, *in vitro* studies suggest that cytochrome *c* release may in some instances be reversible.[42] Therefore, it remains a possibility that cells employ different methods of cytochrome *c* release depending on the cell type and apoptotic stimuli. In this study, cytochrome *c* release was rapidly followed by caspase-3 activation, and blocking cytochrome *c* release with mitochondrial pore inhibitors also prevented caspase-3 activation and apoptosis.

MPTP is also activated by increases in mitochondrial matrix $[Ca^{2+}]$ and subsequently results in the release of proapoptotic factors from the mitochondria and caspase-3 activation.[35,43] An influx of Ca^{2+} into the motoneuron soma occurs following motoneuron axotomy through both the lesioned axon and NMDA receptors.[9–11,34] Application of MK-801, an NMDA receptor antagonist that blocks Ca^{2+} entry into the cell, rescued $\sim 60\%$ of axotomized neonatal facial motoneurons[9] as well as axotomized neonatal sciatic motoneurons[10,34,44,45] and axotomized adult retinal ganglion cells from apoptosis.[46] Collectively these results suggest that Ca^{2+} excitotoxicity plays a role in axotomy-induced apoptosis of neurons.

In the present study, application of RU360 significantly blocked the release of cytochrome *c*, caspase-3 activation and motoneuron apoptosis. RU360, a ruthenium red derivative, is a polycationic dye that acts as an inhibitor of the mitochondrial calcium uniporter.[47,48] Long-term application of RU360 is toxic because it disrupts oxidative phosphorylation and causes an increase in intracellular Ca^{2+} levels.[49] Our results indicate that short-term application of RU360 *in vivo* blocks the induction of apoptosis following motoneuron axotomy. Recent *in vitro* studies have demonstrated that overexpression of Bax in cancer cell lines causes an accumulation of calcium in the endoplasmic reticulum and mitochondria, and subsequently results in the release of proapoptotic factors including cytochrome *c* from the mitochondria.[50,51] Application of RU360 effectively blocked the Bax-mediated influx of calcium into the mitochondria, cytochrome *c* release, caspase activation and apoptosis.[50,51]

Applications of mitochondrial pore inhibitors BKA and CsA also rescue neurons from Ca^{2+}/excitotoxicity-mediated apoptosis. CsA application prevented the glutamate-mediated increase in mitochondrial Ca^{2+} and mitochondrial membrane depolarization in neurons *in vitro*.[52] Application of CsA *in vivo* rescued neurons from excitotoxicity-induced death following traumatic brain injury.[53–56] Similarly, application of BKA *in vivo* rescued neurons from apoptosis following ischemia by blocking cytochrome *c* release and the downstream activation of caspases 9 and 3.[57]

Our results support a model whereby the Bax-mediated apoptotic pathway converges with a calcium-mediated apoptotic pathway at the mitochondria to induce MPTP and the release of proapoptotic factors from the mitochondria and subsequent caspase activation. Our results demonstrate that in the presence of Bax expression, the induction of motoneuron apoptosis can be blocked with the application of mitochondrial pore inhibitors. BKA and CsA, inhibitors of the ANT, likely function to directly prevent MPTP activation and the subsequent release of proapoptotic factors from the mitochondria. In contrast, RU360, an inhibitor of the mitochondrial calcium uniporter, appears to function upstream of

MPTP activation by blocking the influx of calcium into the mitochondria. Whether these inhibitors function to directly prevent Bax from interacting with the MPTP/mitochondria or subsequent mitochondrial depolarization requires further inquiry.

In summary, our results show that mitochondria act at a pivotal point in the cell death pathway of axotomized motoneurons. Mitochondrial release of cytochrome *c* occurs prior to caspase-3 activation and blocking its release from mitochondria also prevents caspase-3 activation and apoptosis demonstrating that mitochondrial involvement is required for the induction of motoneuron apoptosis. In addition, the rescue of axotomized motoneurons with RU360 supports previous findings demonstrating an excitotoxic component in axotomy-induced apoptosis. These results suggest that the excitotoxic cell death pathway converges with the Bax-mediated death pathway at the mitochondria.

Methods and Materials

Animals

Male and female Sprague–Dawley rats (University of British Columbia's Animal Care Centre, Vancouver, Canada) were maintained in an environment-controlled room with a 12 h light/dark cycle, and provided with standard rodent chow and water *ad libitum*. Two litters of rats were used for the developmental study and five litters of rats were used for the mitochondrial inhibitor study. All experiments were approved by the University of British Columbia's Animal Care ethics committee adhering to the Guidelines of the Canadian Council on Animal Care.

Facial motoneuron axotomy

Axotomy of the left facial nerve was performed on neonatal (P0) rats, hours after birth and on adult male rats at 8 weeks of age.[20] Neonatal rats were euthanized at 4, 8, 12, 16 and 24 h ($n=5$ per time point) and adults rats ($n=6$) at 24 h postaxotomy with a lethal intraperitoneal injection of chloral hydrate (900 mg/kg) and transcardially perfused with phosphate-buffered saline (PBS, pH 7.4) followed by cold, freshly hydrolyzed 4% paraformaldehyde in PBS. Brainstems were dissected, cryoprotected in 22% sucrose in PBS and frozen on dry ice. Frozen brainstems were sectioned at 14 μm on a Zeiss cryostat from the rostral to caudal extent of the facial nucleus.

Mitochondrial pore inhibitor application

Mitochondrial inhibitors were applied by gelfoam (Johnson & Johnson, Co) to the axotomized facial nerve of neonatal rats. Gelfoam was cut into 5 mm^3 pieces and soaked in 10 μl of one of the following solutions: CsA (1, 10 and 40 mM, Novartis Pharma Canada Inc., Canada), BKA (10 μM, 100 μM, and 1 mM, BIOMOL, Plymouth Meeting, PA, USA) and RU360 (1 and 10 mM, Calbiochem-Novabiochem Corp., La Jolla, CA, USA) or vehicle (20 mM PBS containing 2% rat serum albumin and 0.2% dimethyl sulfoxide) and placed at the site of lesion. The skin was sutured over the gelfoam to keep it in place. The gelfoam remained in the lesion site until the neonates were euthanized at 24 h postaxotomy.

Immunohistochemistry

Double immunohistochemical staining was performed with antibodies to cytochrome oxidase subunit IV (1 : 500, cat# A6431, Molecular Probes) and cytochrome *c* (1 : 1000, cat# 665971A, Pharmingen) or cytochrome *c* and active caspase-3 (1 : 500, cat# 559565, Pharmingen, ON, Canada) and incubated overnight at 4°C. Secondary antibodies, Alexa-conjugated goat anti-mouse IgG (1 : 200, Molecular Probes) or Rhodol Green-conjugated goat anti-rabbit IgG (1 : 200, Molecular Probes, Eugene, OR, USA) and indocarbocyanine (Cy3)-conjugated donkey anti-mouse IgG (Molecular Probes) were applied for 1 h at room temperature. Sections were stained with bisbenzimide (Hoechst 33258, Sigma Aldrich Chemical Co., Oakville, ON, Canada) and mounted with Sigma™ mounting medium (Sigma Aldrich Chemical Co.). Immunofluorescent photomicrographs were taken with a SPOT digital colour camera (Diagnostic Instruments Inc., Sterling Heights, MI, USA) mounted on a Zeiss Axioskop compound microscope (Carl Zeiss Inc., Germany).

Cell counts

A total of 10 sections per neonatal facial nucleus and 12 sections per adult facial nucleus (due to developmental enlargement of nucleus) were collected for counting from the rostral to caudal extent of the nucleus (every fourth section), to avoid counting the same cell twice. Sections from 4–5 different time points and/or treatment groups were mounted on each microscope slide to minimize differences due to treatments of the slides. Sections were immunostained for cytochrome *c*, active caspase-3 and then stained with bisbenzimide (Sigma Aldrich Chemical Co.). An individual, blind to the experimental treatments, counted the number of motoneurons immunoreactive for (i) diffuse cytochrome *c*, (ii) active caspase-3, (iii) double-labeled (diffuse cytochrome *c* and active caspase-3) and (iv) apoptotic nuclei per section of the facial nucleus from neonatal and adult rats ($n=4$–5 rats/treatment). The criterion for a 'cell positive for cytochrome *c* release' was assessed as a change in the morphology of the immunostaining pattern from a punctate (cytochrome *c* within mitochondria) to a diffuse (cytochrome *c* released into the cytoplasm) staining pattern (see Figures 1d, 2b,e). Cells negative for cytochrome *c* immunoreactivity were not included in the cytochrome c release counts and probably represent a late stage of the apoptotic process. The criterion for an 'apoptotic nucleus' was complete condensation (i.e. solid circular structures of bisbenzimide staining as shown in Figure 2h).

Statistics

To determine the total number of cells per facial nucleus that was positive for each (i) diffuse cytochrome *c*, (ii) active caspase-3, (iii) double-labeled (diffuse cytochrome *c* and active caspase-3) and (iv) apoptotic nuclei, the counts were multiplied by 4 (every fourth section counted) and then multiplied by the Abercrombie correction factor. The Abercrombie correction factor was calculated as follows: $C = T/(T+h)$, where $T=14\ \mu m$ for the section thickness and $h=4\ \mu m$ for the height of the nucleus (~ 3–$5\ \mu m$). The mean number of cells per facial nucleus was calculated from $n=4$ or 5 rats/time point or treatment and plotted *versus* time postaxotomy (Figure 3) or plotted *versus* treatment at 24 h postaxotomy (Figure 4). Student's *t*-tests were performed to compare the mean number of immunopositive cells in the axotomized *versus* contralateral, uninjured nucleus at each time point, and significant differences were assessed at $\alpha=0.05$. In addition, *t*-tests were used to compare the number of immunopositive cells at 24 h postaxotomy between adult and neonatal facial nuclei, and significant differences were assessed at values of $\alpha=0.05$. To examine the effect of mitochondria inhibitors, a one-way analysis of variance was performed on the means for each of the treatment groups and a Fisher's LSM *post hoc* was performed on the individual means, with $P < 0.05$ as a significant difference.

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