Histochemical and Fluorescent Analyses of Mitochondrial Integrity in Chick Auditory Neurons following Deafferentation

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Abstract

Background: Neurons rely exclusively on mitochondrial oxidative phosphorylation to meet cellular energy demands, and disruption of mitochondrial function often precipitates neuronal cell death. Auditory neurons in the chick brain stem (n. magnocellularis [NM]) receive glutamatergic innervation exclusively from ipsilateral eighth nerve afferents. Cochlea removal permanently disrupts afferent support and ultimately triggers apoptotic cell death in 30–50% of ipsilateral, deafferented neurons. Here, we evaluated whether disruption of mitochondrial function occurs during deafferentation-induced neuronal cell death.

Purpose: To determine whether mitochondrial dysfunction occurs preferentially within dying NM neurons.

Research Design: An experimental study. All birds underwent unilateral cochlea removal. Normally innervated neurons contralateral to surgery served as within-animal controls.

Study Sample: Hatchling broiler chickens between 8 and 12 days of age served as subjects. A total of 62 birds were included in the study.

Intervention: Cochlea removal was performed to deafferent ipsilateral NM neurons and trigger neuronal cell death.

Data Collection and Analysis: Following unilateral cochlea removal, birds were sacrificed 12, 24, 48, or 168 hours later, and brain tissue was harvested. Brainstems were sectioned through NM and evaluated histochemically for oxidative enzyme reaction product accumulation or reacted for Mitotracker Red, an indicator of mitochondrial membrane potential (m) and cytoplasmic TdT-mediated dUTP Nick-End Labeling (TUNEL), an indicator of cell death. Histochemical staining intensities for three mitochondrial enzymes, succinate dehydrogenase (SDH), cytochrome c oxidase (CO), and ATP synthase (ATPase) were measured in individual neurons and compared in ipsilateral and contralateral NM. Comparisons were made using unpaired t-tests (CO) or Kruskal Wallis one way ANOVA followed by Dunn’s post hoc pairwise comparisons (ATPase, SDH). Mitotracker Red tissue was examined qualitatively for the presence of and extent of colocalization between Mitotracker Red and TUNEL label in NM.

Results: Results showed global upregulation of all three oxidative enzymes within deafferented NM neurons compared to contralateral, unperturbed NM neurons. In addition, differential SDH and ATPase staining intensities were detected across neurons within the ipsilateral nucleus, suggesting functional differences in mitochondrial metabolism across deafferented NM. Quantitative analyses revealed that deafferented neurons with preferentially elevated SDH and ATPase activities represent the subpopulation destined to die following cochlea removal. In addition, Mitotracker Red accumulated intensely within...
the subset of deafferented NM neurons that also exhibited cytoplasmic TdT-mediated dUTP Nick-End Labeling (TUNEL) and subsequently died.

Conclusions: Taken together, our results demonstrate that a subset of deafferented NM neurons, presumably those that die, preferentially upregulates SDH, perhaps via the tricarboxylic acid (TCA) cycle. These same neurons undergo ATPase uncoupling and an eventual loss of Δψm.

Key Words: ATPase, auditory system, cytochrome c oxidase, deafferentation, Mitotracker Red, succinate dehydrogenase, TUNEL

Abbreviations: Δψm = mitochondrial membrane potential; AMPA = α-amino-3-hydroxy-5-methyl-4-isoxazolpropionic acid; ATPase = adenosine triphosphate synthase; CMXRos = chloromethyl-X-rosamine; CO = cytochrome c oxidase; DPX = dibutyl phthalate xylene; ETC = electron transport chain; NM = nucleus magnocellularis; NO = nitric oxide; OD = optical density; PBS = phosphate buffered saline; ROS = reactive oxygen species; SDH = succinate dehydrogenase; TCA = tricarboxylic acid; TUNEL = terminal deoxynucleotidyl transferase–mediated dUTP Nick-End Labeling

In the nervous system, neuron number is rigorously regulated to ensure proper development and maintenance of neurological function (Oppenheim, 1991). In response to traumatic or injurious stimuli, neurons, like most cells in the body, either rapidly adapt or undergo a program of self-destruction, the latter often producing devastating consequences (Honig and Rosenberg, 2000; Mattson, 2000; Martin, 2001; Friedlander, 2003; Vila and Przedborski, 2003). Elucidating the molecular events that modulate programmed neuronal death will provide valuable insight toward reversing or preventing the loss that accompanies neurological trauma or disease (Forman et al, 2004).

Second-order neurons in the chick auditory nucleus, nucleus magnocellularis (NM), are exceptional models in which to study the cell death process (Rubel and Parks, 1988; Rubel et al, 1990; Rubel and Fritzsch, 2002). NM neurons represent a homogeneous population of sensory neurons (Rubel and Parks, 1975) whose sole source of excitatory input derives from VIIIth nerve afferents from the ipsilateral cochlea (avian basilar papilla [Parks and Rubel, 1978]). Cochlea removal permanently disrupts the afferent signal to NM (Rubel et al, 1990; Born et al, 1991) and triggers a series of cellular perturbations across the entire population of deafferented neurons. Immediately following cochlea removal, electrical activity within NM ceases (Born et al, 1991). Within an hour, neuronal glucose utilization dramatically declines (Heil and Scheich, 1986; Born et al, 1991), and intracellular calcium levels rise (Zirpel et al, 1995; Zirpel and Rubel, 1996). By 6 hr, mitochondria proliferate across the deafferented nucleus (Hyde and Durham, 1994), and a subset of neurons develops signs of ultrastructural compromise, including vacuolization of mitochondria (Hyde and Durham, 1994), degradation of polyribosomes (Born and Rubel, 1985; Rubel et al, 1991), and loss of protein synthetic machinery (Steward and Rubel, 1985; Kelley et al, 1997; Lu et al, 2004). Two to five days later, the morphologically compromised neurons (approximately 30–50% of the deafferented population) begin to degrade (Born and Rubel, 1985; Edmonds et al, 1999; Smittkamp et al, 2005; Karnes et al, 2009).

Although the entire population of NM neurons relies on excitatory, glutamatergic innervation from VIIIth nerve afferents, only a subset succumbs to death following afferent withdrawal. Cochlea removal activates the mitochondrially mediated intrinsic apoptotic cascade: cytochrome c, active caspase-9 (Wilkinson et al, 2003), and active caspase-3 (Karnes et al, 2009) have been detected in deafferented NM neurons. Interestingly, however, these apoptotic indicators are not specific to dying cells, a finding that suggests cell fate may ultimately be influenced by alternative pathways.

Recent work in our laboratory revealed the presence of fragmented DNA, via terminal deoxynucleotidyl transferase–mediated dUTP Nick-End Labeling (TUNEL), in deafferented NM neurons targeted to die (Karnes et al, 2009). Surprisingly, TUNEL labeling accumulated in neuronal cytoplasm, implicating fragmentation of mitochondrial DNA in the deafferentation-induced death process. Mitochondria are intimately involved in the neuronal response to deafferentation in NM (Durham and Rubel, 1985; Hyde and Durham, 1990; Garden et al, 1994; Hyde and Durham, 1994; Hartlage-Rubsamen and Rubel, 1996).

It was our goal to further characterize how mitochondria influence neuronal fate in deafferented NM. Here, we evaluated histochemical reaction products of three mitochondrial enzymes across deafferented NM: cytochrome c oxidase (CO), succinate dehydrogenase (SDH), and adenosine triphosphate synthase (ATPase). In addition, we utilized a marker of mitochondrial inner membrane potential, Δψm, which is directly linked to mitochondrial adenosine triphosphate production (Mitchell, 1961; Saraste, 1999; Scheffler, 2001), to predict cell fate. We describe
altered SDH and ATPase activity, as well as the loss of ΔΨm, within a subset of deafferented NM neurons undergoing mitochondrial DNA fragmentation and, subsequently, death.

**EXPERIMENTAL PROCEDURES**

**Animal Preparation and Surgery**

Cornish Rock broiler chickens (Ross 308 × Ross 308) were obtained as day-old hatchlings from a commercial supplier (Welp Hatchery, Bancroft, Iowa) and were housed in communal brooders at the University of Kansas Medical Center Laboratory Animal Resources center with ad libitum access to food and water. Eight- (P8) to 12-day (P12) posthatch chicks were anesthetized with intramuscular injections of ketamine HCl (35 mg/kg; Phoenix Pharmaceutical, Inc., St. Joseph, Missouri) and xylazine (2 mg/kg; Lloyd Laboratories, Shenandoah, Iowa) and subjected to unilateral cochlea (basilar papilla) removal, as described previously (Born and Rubel, 1985; Hyde and Durham, 1990). Briefly, the left tympanic membrane was punctured, and the columella was extracted. The cochlea was removed from the oval window aperture using fine forceps and was examined to ensure complete removal of the organ. Cochlea removal severs the peripheral processes of cochlear ganglion neurons but leaves cell bodies intact (Born et al, 1991). The cochlear duct was aspirated following surgery, and animals were allowed to recover. Contralateral cochleae remained intact, and contralateral NM neurons served as within-animal controls.

To assess mitochondrial membrane potential (ΔΨm), unilaterally operated birds were anesthetized as before 12 hr (n = 6), 24 hr (n = 9), 48 hr (n = 6), or 168 hr (n = 4) following cochlea removal; treated topically with lidocaine HCl (10 mg/ml; Hospira, Inc., Lake Forest, Illinois) just above the odontoid process of the axis; and subjected to intrathecal injections of 0.5 mM Mitotracker Red chloromethyl-X-rosamine (CMXRos; Molecular Probes, Eugene) in dimethyl sulfoxide and 0.1 M phosphate buffered saline (PBS; pH 7.4) containing 2.5% glycerol as described previously (Sekido and Lovell-Badge, 2007). Mitotracker Red is a positively charged, fixable, fluorescent probe containing CMXRos that is utilized to detect changes in ΔΨm (Macho et al, 1996). Following intrathecal injection, animals were maintained under anesthesia for 3 hr prior to sacrifice. All experimental protocols were approved by the University of Kansas Medical Center Institutional Animal Care and Use Committee and carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23). Every effort was made to minimize the number and suffering of all animal subjects.

**Tissue Preparation**

Animals were sacrificed by intraperitoneal injections of Beuthanasia-D (Schering-Plough, Union, N.J.) and decapitated 12 hr (n = 8), 24 hr (n = 7), 48 hr (n = 10), and 168 hr (n = 12) following cochlea removal for histochemical analyses or 3 hr following intrathecal injections for Mitotracker Red analyses. For histochemical evaluation of mitochondrial enzyme reaction product accumulation, brains were extracted and rapidly frozen in heptane cooled by dry ice. Brain stems were sectioned coronally through nucleus magnocellularis, and 20 μm sections were mounted serially onto four series of slides, which were subsequently reacted for Thionin, SDH, CO, or ATPase, respectively. Slides were stored at –20°C or stained immediately. For Mitotracker Red analyses, animals were perfused intracardially with Avian Ringer’s solution (2M NaCl, 2M KCl, 2M MgCl2, and 100 mM ethylenediaminetetra-acetic acid) in PBS; and brains were removed, postfixed for 6 hr in modified Carnoy’s fixative (containing ethanol, chloroform, Avian Ringer, and glacial acetic acid), stored overnight in 70% ethanol, and embedded in paraffin as previously described (Lurie and Durham, 2000).

**Histologic Examination of Mitochondrial Enzymes**

**Thionin Staining**

Frozen slide-mounted sections were defatted in xylenes, rehydrated through graded ethanolns, stained with Thionin, dehydrated, and cover-slipped with dibutyl phthalate xylene (DPX) mounting medium (VWR International Ltd., Poole, U.K.).

**SDH Staining**

Sections were reacted for SDH activity as described previously (Nachlas et al, 1957; Durham and Rubel, 1985). Briefly, sections were stained in a solution containing (per 25 ml) 0.05 M phosphate buffer (pH 7.4), 0.338 g succinic acid, and 10.2 mg nitro blue tetrazolium (Sigma, St. Louis, Missouri) for 10 min at 37°C. Sections were then immersed in 10% buffered formalin for at least 1 hr and dehydrated through graded ethanolns and xylenes prior to cover-slipping with DPX.

**CO Staining**

Sections were reacted for CO activity as described previously (Wong-Riley, 1979; Hyde and Durham, 1990). In brief, slide-mounted sections were immersed in a solution containing (per 100 ml) 50 mg 3,3′-diaminobenzidine tetrahydrochloride (Sigma, St. Louis, Missouri), 20 mg cytochrome c, and 4 g sucrose in 0.1 M...
PBS (pH 7.4) for 40 min at 37°C. Sections were then washed three times in PBS, dehydrated through graded ethanol and xylenes, and cover-slipped with DPX.

**ATPase Staining**

Sections were reacted for ATPase enzyme activity as described previously (Meijer and Vloedman, 1980). In brief, sections were immersed in cold (4°C) acetone for 5 min and then stained with freshly prepared ATPase-staining solution, containing (per 100 ml) 50 mg adenosine triphosphate (Sigma, St. Louis, Missouri), 40 ml 0.2M Tris maleate buffer (Tris maleate, Tris base, and 10N NaOH in distilled water; pH 7.4), 35.2 mg dinitrophenol (Sigma, St. Louis, Missouri), 6 ml 0.06M lead nitrate, and 10 ml 0.05M magnesium chloride in distilled water for 10 min at 37°C. Sections were rinsed with Tris maleate buffer and immediately washed for 1 min in 1% ammonium sulfide. Sections were then dehydrated and cover-slipped with DPX.

**TUNEL Labeling of Fragmented DNA**

To directly evaluate the relationship between $\Delta \psi_m$ and cell death in deafferented NM, brain stem sections from animals injected with Mitotracker Red were colabeled with terminal deoxynucleotidyl transferase–mediated dUTP Nick-End Labeling. Coronal 10 μm paraffin-embedded sections at the 50th percentile along the anterior-to-posterior extent of NM were reacted for TUNEL using Promega’s DeadEnd Fluorometric TUNEL System (Promega Corporation, Madison, WI) as described previously (Karnes et al, 2009) according to the manufacturer’s instructions. Briefly, sections were deparaffinized through xylenes and rehydrated through graded ethanol. Sections were washed in 0.85% NaCl followed by PBS and fixed in 4% paraformaldehyde. Neurons were permeabilized with 20 μg/ml proteinase K, washed in PBS, and equilibrated in the kit's Equilibration Buffer. Sections were incubated with rTdT enzyme, nucleotide mix, and Equilibration Buffer in a humidity chamber in the dark for 1 hr. Following incubation, sections were immersed in 1× SSC (150mM NaCl and 15mM sodium citrate) buffer and washed in PBS and distilled water prior to cover-slipping with Vectashield antifade mounting medium (Vector Laboratories, Burlingame, California).

**Data Analysis**

**Analysis of Histologic Enzyme Reaction Product**

For each NM montage, OD measurements of ATPase, CO, and SDH reaction products were acquired in individual neurons (in a medial to lateral progression) until at least 50 neurons per nucleus were measured. The outline of each neuronal cytoplasm (excluding the non-staining nucleus) was traced, and a mean grayscale value was calculated for each neuron. Mean grayscale values were converted to OD measurements using a standard curve generated from measurements of a calibrated OD step tablet (Tiffen Company, Hauppauge, NY) photographed using Photoshop CS3 Extended (Adobe, San Jose, California), which is equipped with scientific and analytic image analysis software.

For analysis of TUNEL and Mitotracker Red staining, NM sections were visualized using a Nikon Digital Eclipse C1Si confocal microscope system under 40× oil magnification. Photomicrographs were acquired using a Nikon Digital Sight DS-Fi1 high-definition color camera and NIS-Elements F-3.0 software.

**Image Acquisition**

Optical densities (ODs) of SDH, CO, and ATPase reaction products were measured in individual NM neurons and compared across survival times using methods similar to those described elsewhere (Hyde and Durham, 1990; Smittkamp et al, 2003). Briefly, neurons within a single section at the 50th percentile along the anterior-to-posterior frequency axis of both ipsilateral and contralateral NM were visualized under bright-field illumination at 40× magnification and photographed using a Nikon Digital Sight DS-Fi1 high-definition color camera and NIS-Elements F-3.0 software.

For analysis of TUNEL and Mitotracker Red staining, NM sections were visualized using a Nikon Digital Eclipse C1Si confocal microscope system under 40× oil magnification. Photomicrographs were acquired using a Nikon Digital Sight DS-Fi1 high-definition color camera and NIS-Elements F-3.0 software.

$Z = (\text{OD} - \text{mean contralateral OD})/\text{(standard deviation of contralateral OD)}$

$Z$-scores were generated for all ipsilateral and contralateral OD values from NM neurons. $Z$-scores for all animals at a given survival time were plotted as histograms using DeltaGraph 5 (Red Rock Software, Inc., Salt Lake City, UT), and the shapes of histograms were evaluated for differences in enzymatic activity as a function of survival time.
Neuronal Counts

To estimate the extent of neuronal death in NM, neuron number in photomontages at the 50th percentile from animals surviving seven days after cochlea removal was evaluated. Neuronal profiles with visible and intact nuclei and with enzymatic reaction products present in the cytoplasm were quantified in ipsilateral and contralateral NM. Percentages of cell loss were derived using the following formula: ([# neurons in contralateral NM – # neurons in ipsilateral NM] / # neurons in contralateral NM) × 100%. The mean percentage of cell loss at seven days was calculated for each enzymatic stain using SigmaStat 3.5 (SYSTAT Software, Inc., San Jose, California).

Statistical Analyses

To compare differences in CO reaction product intensity between ipsilateral and contralateral brain stem nuclei, the mean OD value (from each CO-stained nucleus) at every survival time was calculated using Microsoft Excel. Unpaired t-tests were performed using SigmaStat 3.5 (SYSTAT Software, Inc., San Jose, California) to compare mean OD values for CO activity per animal across survival times. Differences were deemed significant if p < .05.

To compare differences in SDH and ATPase reaction product intensities in ipsilateral versus contralateral NM neurons, ipsilateral neurons were first categorized based on their Z-score OD value. For SDH and ATPase stains, ipsilateral neurons with a Z-score ≥2 were assigned to one group, while neurons with a Z-score <2 were assigned to a second group. This arbitrary partition was determined by visually examining the distributions of OD histograms. OD values from each ipsilateral group, as well as OD values from contralateral NM, for both stains (SDH and ATPase) were compared using a Kruskal Wallis one-way analysis of variance (ANOVA) on ranks, followed by a series of pairwise, post hoc comparisons using Dunn’s Method.

RESULTS

Deafferentation-Induced Changes in Histochemical Enzyme Activities

Thionin staining of ribosomal Nissl substance has been used to distinguish dying from surviving neurons in deafferented NM (Born et al., 1991; Rubel et al., 1991). Neurons that stain poorly with Thionin, so-called ghost neurons, represent the subpopulation of deafferented neurons that will die following cochlea removal (Karnes et al., 2009). Figure 1 reveals the characteristic Thionin staining pattern in NM from an animal sacrificed 24 hr after cochlea removal (Fig. 1A–B). Nissl staining is normal in unperturbed, contralateral NM neurons (Fig. 1A). In ipsilateral NM, Thionin-positive neurons are present, as are an abundant number of Thionin-negative “ghost” neurons (Fig. 1B, inset). To assess differences in mitochondrial function between deafferented, surviving NM neurons and deafferented, dying NM neurons, we stained adjacent sections for succinate dehydrogenase (Fig. 1C–D), cytochrome c oxidase (Fig. 1E–F), and ATPase (Fig. 1G–H) and compared staining intensities of enzyme reaction products in individual neurons. Previous reports on NM have shown global oxidative upregulation early following cochlea removal, including widespread upregulation of CO and SDH across the deafferented nucleus (Durham and Rubel, 1985; Hyde and Durham, 1990), as well as a marked expansion of mitochondria (Hyde and Durham, 1994). Here CO staining appears uniformly increased in ipsilateral NM (Fig. 1F), while the intensity of SDH (Fig. 1D) and ATPase (Fig. 1H) reaction products appear more variable across the nucleus. A subset of neurons showing a marked increase in staining appears similar in number to that of “ghost” neurons. The differential staining intensities for SDH and ATPase across the deafferented nucleus suggest that mitochondrial functional differences may exist between dying, “ghost” neurons and surviving, Thionin-positive neurons.

Quantitative Evaluation of Histochemical Staining

SDH Histochemistry

SDH is a multisubunit enzyme involved in both the electron transport chain (ETC) and the tricarboxylic acid (TCA) cycle. Comparison of median OD values ipsilateral and contralateral to cochlea removal using the Kruskal-Wallis one-way ANOVA on ranks across all time points suggests significant deafferentation-induced differences in SDH activity (H = 1053, 11 degrees of freedom, p ≤ .001). Interestingly, prior reports of SDH activity in NM, where OD measurements were made in a small region within the cytoplasm of each neuron, showed relatively uniform enzymatic levels across all deafferented neurons (Durham and Rubel, 1985). However, our results from measurements of the entire cytoplasm suggest otherwise. Figure 2 illustrates a broad distribution of SDH reaction product intensity in NM neurons ipsilateral to cochlea removal, with a subset of neurons demonstrating intense staining. Twelve to 24 hr after deafferentation, ipsilateral NM neurons (blue bars) separate into two categories based on their staining intensity for SDH: (1) those with median SDH OD values comparable (12 hr = 0.313, 24 hr = 0.463) to those of contralateral neurons (12 hr =
0.00677, 24 hr = 0.00916) and (2) those with significantly (p < .05) elevated median SDH OD values (12 hr = 2.89, 24 hr = 2.779) compared to contralateral neurons (Fig. 1D and Fig. 2A–B). By 48 hr, when 30–50% of deafferented NM neurons begin to degrade, SDH staining intensity declines in ipsilateral NM and shifts toward a unimodal distribution of enzyme activity (Fig. 2C). By seven days, SDH levels in ipsilateral neurons have declined, although a broad distribution of SDH staining intensity persists (Fig. 2D). Previous reports, using less precise measurements of SDH reaction product, demonstrated a reduction in SDH activity 3–35 days following co-chlea removal (Durham and Rubel, 1985). Our results suggest that the population of ipsilateral neurons that survives deafferentation is transitioning to a reduced metabolic state, although at the last time point examined,
Deafferented neurons exhibit a wide range of OD values compared to contralateral neurons (Fig. 2D).

To assess percent neuronal loss, the number of neurons reacting positively for SDH was compared in ipsilateral and contralateral NM at seven days. On average, $31.0 \pm 12.9\%$ of deafferented neurons underwent cell death. To determine whether NM neurons with increased SDH staining at 12 and 24 hr represented the population eventually targeted to die, we calculated the average number of ipsilateral neurons with OD Z-scores $\geq 2$ at 12 and 24 hr survival times to be 29.3% and 29.4%, respectively. Our results suggest that neurons destined to die experience early upregulation of SDH levels compared to adjacent ipsilateral NM neurons that survive deafferentation.

**CO Histochemistry**

CO is a large, multisubunit protein that comprises complex IV of the mitochondrial ETC (Saraste, 1999). As has been described previously, CO reaction product in deafferented NM (Fig. 3, blue bars) increases significantly compared to contralateral neurons (Fig. 3, orange bars; $t$-test, $p = .009$) as early as 12 hr following cochlea removal (Fig. 3A). By 48 hr, the average OD of CO reaction product is not significantly different than in contralateral neurons ($t$-test, $p = .222$; Fig. 3C). By seven days, there is a reduction in the number of neurons ipsilateral to cochlea removal (42.0 ± 3.9%), consistent with the cell death that is known to occur by this time, but no appreciable difference in CO activity exists between ipsilateral and contralateral brain stem nuclei. Both ipsilateral and contralateral neurons exhibit relatively normal, unimodal OD distributions of CO reaction product accumulation (Fig. 3), indicating that all deafferented neurons within NM exhibit similar demands for CO, albeit at higher levels than in contralateral neurons.

**ATPase Histochemistry**

ATPase utilizes the electrochemical gradient of $H^+$ generated by the ETC to synthesize adenosine triphosphate, thus coupling the electrochemical gradient to energy generation. The histochemical staining solution used to detect the coupling state of mitochondrial ATPase includes the known uncoupling agent...
dinitrophenol. OD measurements of cytoplasmic ATPase reaction product in contralateral NM show a relatively normal, unimodal distribution of staining intensities (Fig. 4, orange bars). In deafferented NM neurons ipsilateral to cochlea removal, average ATPase levels are increased compared to contralateral neurons (Kruskal-Wallis ANOVA on ranks: \( H = 661, 11 \) degrees of freedom, \( p \leq .001 \); Fig. 1H and Fig. 4, blue bars), indicative of mitochondrial distress throughout the nucleus. As early as 24 hr, two neuronal populations with different levels of ATPase staining emerge in deafferented NM: one with a median OD value (0.224) similar to that of contralateral neurons (0.0405) and one with a significantly \(( p < .05)\) greater median OD value (2.722) compared to contralateral neurons (Fig. 1H, Fig. 4B). By 48 hr, as the number of ipsilateral neurons undergoing mitochondrial uncoupling increases, pronounced differences in staining intensity are evident in the deafferented nucleus: ipsilateral NM neurons with less intense ATPase reaction product (median OD value = 0.649) are significantly \(( p < .05)\) different from ipsilateral, “uncoupled” NM neurons (median OD value = 3.134; Fig. 4C). The number of ipsilateral neurons with \( Z \)-scores \( \geq 2 \) at 48 hr (38.6\%) approximates the number of neurons that die by seven days (39.8 \( \pm \) 14.0\%), thereby suggesting that deafferented neurons that undergo ATPase uncoupling represent the subset that dies following deafferentation.

**Mitotracker Red**

We used Mitotracker Red, a fluorescent, cationic, lipophilic dye, to evaluate qualitative fluctuations in mitochondrial membrane potential (\( \Delta \psi_m \)) in our fixed tissue (Macho et al, 1996). Like other indicators of \( \Delta \psi_m \) (e.g., rhodamine 123, cationic cyanine dyes, and safranine 0), Mitotracker Red passively diffuses across the plasma membrane and subsequently accumulates within the negatively charged inner membrane of intact mitochondria, where it is partially quenched (Johnson et al, 1980; Mostafapour et al, 1997; Nicholls and Budd, 2000; Buckman et al, 2001). Following mitochondrial depolarization, or loss of \( \Delta \psi_m \), the dye redistributes from the mitochondrial matrix to the cytoplasm, where it is dequenched and visualized by an increase in fluorescence intensity (Johnson et al, 1980; Johnson et al, 1981; Mostafapour et al, 1997; Nicholls and Budd, 2000).

Although our methods do not allow visualization of \( \Delta \psi_m \) reversal in situ, our results demonstrate that Mitotracker Red staining intensity corresponds well with cell death. Intact mitochondria, with negatively maintained membrane potentials, accumulate Mitotracker Red from the cytoplasm and do not fluoresce; accordingly, intact NM neurons in contralateral (Fig. 5A) and in some regions of ipsilateral NM (Fig. 5B) exhibit no evidence of Mitotracker Red fluorescence. In

![Figure 3. Histograms showing cytochrome c oxidase (CO) histochemical reaction product measured as optical density and plotted as Z-scores from animals sacrificed 12 hr (A), 24 hr (B), 48 hr (C), or seven days (D) following cochlea removal. Across all time points examined, nucleus magnocellularis (NM) neurons contralateral (orange bars) and ipsilateral (blue bars) to cochlea removal exhibit unimodal distributions of CO activity. At 12 hr, CO reaction product in ipsilateral neurons is significantly higher (\( t \)-test, \( p = .009 \)) than in contralateral neurons (A). Thereafter, no significant differences between sides exist. By seven days, approximately 42.0 \( \pm \) 3.9\% of ipsilateral neurons have died (D).](image-url)
contrast, deafferented NM neurons destined to die contain dysfunctional mitochondria, which have already undergone $\Delta \psi_m$ reversal. These depolarized mitochondria cannot accumulate Mitotracker Red and, consequently, exhibit intense cytoplasmic fluorescence as early as 24 hr after cochlea removal (Fig. 5B). This increase in Mitotracker Red intensity is observed in the subset of deafferented neurons also undergoing mitochondrial DNA fragmentation, as evidenced by TUNEL labeling (Fig. 5B and 5D). This correlative pattern persists 48 hr following deafferentation (data not shown) but is gone at seven days when cell death is complete (Fig. 5F and 5H). These results demonstrate that $\Delta \psi_m$ reversal precedes cell death.

In summary, we have shown that mitochondrial function is impaired in the subset of deafferented NM neurons destined to die. SDH, CO, and ATPase activities are globally elevated in ipsilateral NM neurons compared to contralateral control neurons (Figs. 1, 2, 3, and 4). Furthermore, differences in SDH and ATPase activities emerge within deafferented NM as early as 12 hr and 24 hr, respectively, following cochlea removal (Fig. 2 and Fig. 3). This demonstrates that early alterations in mitochondrial function may selectively identify neurons susceptible to deafferentation-induced cell death. In addition, we have shown that NM neurons destined to die undergo mitochondrial uncoupling and loss of $\Delta \psi_m$ (Fig. 1H, Fig. 4, Fig. 5B).

**DISCUSSION**

In nucleus magnocellularis auditory neurons, mitochondria are clearly involved in the cellular response to deafferentation. Previous studies revealed significant morphological changes in neuronal mitochondria, including rapid proliferation across the entire deafferented nucleus (Hyde and Durham, 1994), ultrastructural vacuolization within the subset undergoing ribosomal degradation and endoplasmic reticulum (ER) fragmentation (Born and Rubel, 1985; Rubel et al, 1991; Hyde and Durham, 1994; Hartlage-Rubsamen and Rubel, 1996), and loss of mitochondrial DNA integrity in the subset destined to die (Karnes et al, 2009). Here, we demonstrate disturbances in mitochondrial functional status in deafferented neurons as a corollary to the previously described alterations of mitochondrial structure. Although increased CO activity is observed transiently among all deafferented NM neurons, increased staining intensities for SDH and ATPase reaction products occur in a subset of neurons, suggesting differential enzyme activity in dying versus eventually undergo cell death (C, D). By seven days, approximately $39.8 \pm 14.0\%$ of ipsilateral neurons have died (D). Arrows in B and C indicate mean OD for all ipsilateral neurons, with OD $\leq 2$ (left arrow) or $> 2$ (right arrow).
surviving neurons (Figs. 2 and 4). We assessed mitochondrial membrane potential status ($\Delta \psi_m$) in fixed NM tissue sections and showed that a loss of $\Delta \psi_m$ occurs in neurons undergoing mitochondrial DNA degradation prior to dying (Fig. 5). In the paragraphs that follow, we describe the significance of our results in the context of our model system and propose a mechanism underlying cell fate determination in deafferented NM neurons.

Cochlea removal permanently disrupts delivery of glutamate to NM (Nemeth et al, 1983; Zhou and Parks, 1992a, 1992b), abruptly reducing the activity of metabotropic glutamate receptors (Lachica et al, 1995; Zirpel and Rubel, 1996; Hyson, 1998; Nicholas and Hyson, 2004) and leading to a sizable increase in intracellular calcium (Lachica et al, 1995; Zirpel and Rubel, 1996; Zirpel et al, 1998; Kato and Rubel, 1999). The source of this calcium influx remains undetermined but has been speculated to derive from $\alpha$-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor activation by residual glutamate (Zirpel et al, 2000b), N-type voltage-gated calcium channels at the plasma membrane (Lu and Rubel, 2005), or release from internal stores (Lachica et al, 1995).

Intracellular calcium accumulation, beyond a certain threshold, is detrimental to neurons (Choi, 1988; Zirpel et al, 2000b; Pfeiffer et al, 2001; Nicholls, 2008). A rise in intracellular calcium triggers an abundant mitochondrial expansion (Gunter et al, 1994; Hyde and Durham, 1994; Zirpel et al, 1998; Pinton et al, 2008) as well as a global upregulation in oxidative enzyme activity within neurons (Duchen, 2004; Gunter et al, 2004; Nicholls, 2008). In NM, this is evidenced by the elevated SDH, CO, and ATPase staining intensities observed throughout the ipsilateral nucleus as early as 12 hr following cochlea removal. Mitochondria scavenge calcium as part of a protective buffering mechanism (Gunter and Gunter, 1994; Hansford, 1994; Budd and Nicholls, 1996; Scheffler, 2001; Nicholls, 2008).

**Figure 5.** Photomicrographs of ipsilateral (B, D, F, H) and contralateral (A, C, E, G) sections through nucleus magnocellularis (NM) from an animal subjected to intrathecal injection of Mitotracker Red 24 hr (A, B) or seven days (E, F) following cochlea removal. Following sacrifice, NM sections were reacted for terminal deoxyribonuclease–mediated dUTP Nick-End Labeling (TUNEL; C, D, G, H). A subset of ipsilateral neurons at 24 hr exhibit diffuse cytoplasmic TUNEL labeling, indicative of mitochondrial DNA fragmentation (D). Mitotracker Red accumulates with greater staining intensity within the same subset of neurons (B). By seven days, when cell death is complete, no evidence of TUNEL labeling (H) or Mitotracker Red accumulation (F) exists in deafferented NM neurons. Scale bar $= 50 \mu m$. 

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In deafferented NM, however, a subset of neurons contains morphologically impaired mitochondria (Hyde and Durham, 1994), which presumably compromises organelle function as well as calcium uptake mechanisms. These same neurons undergo ER fragmentation (Born and Rubel, 1985; Rubel et al, 1991), loss of protein synthetic capacity (Steward and Rubel, 1985; Kelley et al, 1997; Lu et al, 2004), and cell death. We speculate that loss of ER and mitochondrial integrities, together, perpetuate the death that occurs in some deafferented NM neurons and that the mechanism of death involves a calcium-mediated attack on mitochondrial function. Mitochondria and ER are intimately linked both in structure and in function (Hajnoczky et al, 1995; Rizzuto et al, 1998; Pinton et al, 2008), and physical apposition facilitates direct transfer of substances across ER–mitochondrial junctions (Vance, 1990; Pinton et al, 2008). These ER–mitochondrial junctions are enriched sites of lipid transfer (Piccini et al, 1998; Stone and Vance, 2000) and calcium exchange (Berridge,}

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**Figure 6.** A schematic illustrating the proposed mechanism of deafferentation-induced cell death in nucleus magnocellularis (NM) neurons. In deafferented NM neurons destined to die, calcium accumulates beyond sustainable levels. Calcium most likely enters deafferented NM neurons via three routes: (1) plasma membrane α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPA R), (2) N-type voltage-sensitive calcium channels (NCC), and/or (3) release from internal organelle stores. Compromised mitochondria and degenerating endoplasmic reticulum perpetuate the release of lipids and calcium into the neuronal cytoplasm. Calcium-mediated lipid peroxidation makes available fatty acid substrates for tricarboxylic acid (TCA) cycle energy production, preferentially upregulating succinate dehydrogenase (SDH) and other TCA cycle dehydrogenases. Calcium, as well as reactive oxygen species from lipid peroxidation, attack adenosine triphosphate synthase (ATPase) and facilitate formation of the mitochondrial transition pore, which metabolically uncouples energy production, perpetuates the intrinsic mitochondrialy mediated apoptosis cascade, and activates endonucleases that degrade mitochondrial DNA. ROS = reactive oxygen species; CO = cytochrome c oxidase; MTP = mitochondrial transition pore; mtDNA = mitochondrial DNA.
2002; Pinton et al, 2008; Rimessi et al, 2008), and their disruption has been increasingly linked to apoptotic death in many systems (Demaurex and Distelhorst, 2003; Orrenius et al, 2003; Rizzuto et al, 2003; Scorrano et al, 2003).

Increased intracellular calcium can activate several parallel pathways that target mitochondria for destruction and may explain the deafferentation-induced oxidative changes we have observed. First, calcium triggers nitric oxide synthase generation (Alderton et al, 2001) and nitric oxide (NO [Knott et al, 2008; Nicholls, 2008]) production. NO, along with calcium directly, attacks constituents of the mitochondrial inner membrane, disrupting ATPase activity (Cleeter et al, 1994) and perpetuating production of reactive oxygen species (ROS [Inoue et al, 1999; Stewart and Heales, 2003; Turrens, 2003; Balaban et al, 2005; Pope et al, 2008]). Lipid peroxidation as a consequence of ROS generation has been described in deafferented NM neurons 6 hr after cochlea removal (Nicholas and Hyson, 2006) and probably contributes to our observed increases in SDH activity within some neurons. As a TCA cycle enzyme, SDH utilizes a number of substrates, including oxidized fatty acids for energy production (Duchen, 2004). A switch in metabolic substrate may account for the increased SDH activity observed in a subset of deafferented NM neurons (Fig. 1D, Fig. 2).

Second, intracellular calcium directly attacks the ATPase proton pump (Shiva and Darley-Usmar, 2003); a phenomenon that disrupts electron transport chain efficiency, inhibits mitochondrial capacity to maintain the energy needs of the cell, and produces cell death from adenosine triphosphate uncoupling (Pfeiffer et al, 2001; Nicholls, 2008). The differential staining intensities for ATPase across deafferented NM (Fig. 4) suggest that mitochondrial uncoupling occurs in the subset of NM neurons targeted to die.

Third, calcium overload, beyond the buffering capacity of mitochondria, also triggers the formation of a permeability transition pore in the mitochondrial outer membrane (Nicholls, 2008; Pinton et al, 2008), which results in loss of $\Delta \psi_{m}$ as well as collapse of the cellular energetic apparatus (Gunter et al, 2004). Membrane potential reversal correlates strongly with cytochrome $c$ release and initiation of intrinsic mitochondrial-mediated apoptosis (Foster et al, 2006). Mitotracker Red fluoresces intensely in the cytoplasm of deafferented NM neurons known to undergo cell death. This suggests that deafferentation-induced death in NM neurons is accompanied by loss of $\Delta \psi_{m}$.

Although abundant evidence supports the use of fluorescent, cationic, lipophilic dyes to accurately detect $\Delta \psi_{m}$ (Johnson et al, 1980; Johnson et al, 1981; Macho et al, 1996; Mostafapour et al, 1997), some controversy surrounds this premise (Poot et al, 1996; Nicholls and Budd, 2000; Buckman et al, 2001; Foster et al, 2006). Mitotracker Green, in particular, is not equipped with chloromethyl moieties and consequently is insensitive to fluctuations in $\Delta \psi_{m}$. This dye has been used as a general index of mitochondrial mass (Poot et al, 1996; de la Monte et al, 2000; Buckman et al, 2001). Mitotracker Red and Orange, on the other hand, are equipped with chloromethyl-X-rosamine groups, uptake of which has been shown to be $\Delta \psi_{m}$ dependent (Macho et al, 1996). Mitotracker Red fluorescence is also influenced by ROS generation (Buckman et al, 2001; Kim et al, 2002). ROS generation is known to occur in deafferented NM (Nicholas and Hyson, 2006); whether Mitotracker Red measures ROS accumulation or $\Delta \psi_{m}$ reversal must be determined in future experiments.

CONCLUSION

In summary, the results presented here suggest that mitochondrial functional deficits differ across the deafferented nucleus, most likely differentiating neurons that will die from neurons that will survive deafferentation. Figure 6 shows a model for intracellular events culminating in neuronal cell death in NM.

Cochlea removal abruptly terminates activation of neuronal metabotropic glutamate receptors, which are the primary regulators of intracellular calcium in NM (Zirpel and Rubel, 1996; Zirpel et al, 1998; Kato and Rubel, 1999; Zirpel et al, 2000a). Calcium dysregulation leads to calcium influx most probably via activation of AMPA receptors by residual glutamate (Zirpel et al, 2000b), entry through N-type calcium channels (Lu and Rubel, 2005), and/or potential release from internal stores (Misset, 1991). Calcium influx triggers rapid proliferation of mitochondria within NM neurons (Hyde and Durham, 1994). Mitochondria unable to buffer the rise in calcium undergo vacuolization (Hyde and Durham, 1994; Hartlage-Rubsamen and Rubel, 1996), whether in response to persistent elevated calcium levels (Brookes et al, 2004) or from the loss of ER–mitochondrial junctional integrity (Pinton et al, 2008). ER–mitochondrial junctional impairment releases lipids and calcium into the cellular cytoplasm, which perpetuates ROS generation (Nicholas and Hyson, 2006; Nicholls, 2008), shifts metabolism toward the TCA cycle (Duchen, 2004; Gunter et al, 2004), and increases SDH levels. Calcium directly impairs ATPase activity (Cleeter et al, 1994), leading to metabolic uncoupling; activates Bcl2 gene expression (Wilkinson et al, 2002; Scorrano et al, 2003), which, in ribosomal-deficient, dying neurons, cannot be translated to protein; activates mitochondrial permeability transition, which elaborates intrinsic apoptosis (Gunter et al, 2004); and directly activates endonucleases, which degrade mitochondrial DNA. Future investigations of
the molecular genetic contributions to cell-specific death in deafferented NM should further elucidate this mechanism.

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