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AFFERENT REGULATION OF CYTOCHROME-C AND ACTIVE CASPASE-9 IN THE AVIAN COCHLEAR NUCLEUS

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Abstract

During development, a subpopulation (approximately 30%) of neurons in the avian cochlear nucleus, nucleus magnocellularis (NM), dies following removal of the cochlea. It is clear that neuronal activity coming from the auditory nerve provides trophic support critical for cell survival in the NM. Several aspects of the intracellular signaling cascades that regulate apoptosis have been defined for naturally occurring, or programmed cell death, in neurons. These intracellular cascades involve the extrusion of cytochrome-c from the mitochondria into the cytosol and the subsequent activation of proteolytic caspase cascades, which ultimately act on substrates that lead to the death of the cell. In contrast, the intracellular signaling cascades responsible for deafferentation-induced cell death are not fully understood. In the present series of experiments, the potential extrusion of cytochrome-c from the mitochondria into the cytosol, and the activation of caspases were examined in the NM following deafferentation. Cytochrome-c immunoreactivity increased within 6 h following deafferentation and persisted for at least 3–5 days following surgery. However, cytochrome-c was not detectable within immunoprecipitates obtained from cytosolic fractions of deafferented NM neurons. This suggests that the increased immunoreactivity of cytochrome-c is related to mitochondrial proliferation. As a positive control, cytochrome-c was detected in cytosolic fractions of deafferented NM neurons treated with kainic acid, a substance known to cause cytochrome-c release into the cytosol. In addition, immunoreactivity for downstream active caspase-9 did increase following cochlea ablation. This increase was observed within 3 h following cochlea removal, but was not observed 4 days following surgery, a time point after the dying population of NM neurons have already degenerated. Together, these findings suggest that deafferentation of NM neurons results in caspase activation, but this activation may be cytochrome-c independent.

Keywords

deafferentation; apoptosis; auditory system; n. magnocellularis

Programmed cell death (or apoptosis) occurs widely throughout the developing nervous system (Oppenheim, 1991; Jacobson et al., 1997). Several of the molecular events that underlie programmed cell death (PCD) have been elucidated. The molecules, implicated as regulators of cell death cascades in neurons, include but are not limited to the bcl-2 gene family, apoptosis activating factor-1 (Apaf-1), cytochrome-c, and the caspases (Yaun and Yankner, 2000; Denecker et al., 2001). While “programmed” neuronal cell death does occur naturally during the course of development, additional cell death can be induced in developing organisms by eliminating sources of trophic support. It appears that for many sensory neurons, some form of activity-dependent trophic support is provided by their afferents. Consistent with the results

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obtained from systems used to study PCD, cell death initiated by the elimination of synaptic input is more highly pronounced in the developing organism (Peusner and Morest, 1977; Rubel et al., 1990; Galli-Resta et al., 1993; Brunjes, 1994; Linden, 1994; Johnson and Leon, 1999). However, it is not known if the intracellular signaling cascades commonly activated during PCD are also responsible for deafferentation-induced cell death.

The mitochondrion has been recognized as a pivotal regulator of cellular life and death (Petit et al., 1996; Gross et al., 1999; Duchen, 2000; Desagher and Martinou, 2000). In several systems, mitochondria have been implicated in the regulation of PCD/apoptosis by controlling the release of apoptogenic factors into the cytosol. One of these apoptogenic factors is cytochrome-c, a molecule that is normally located in the space between the inner and outer mitochondrial membranes. Following an apoptotic stimulus, bioenergetically challenged mitochondria can become permeabilized and potentially extrude cytochrome-c into the cytosol where it interacts to form a complex of molecules known as the 'apoptosome' (Li et al., 1997; Green and Reed, 1998). An apoptosome is an oligomerization of cytochrome-c, Apaf-1, and procaspase-9 in the presence of dATP (Li et al., 1997). An autocatalytic reaction causes the activation of caspase-9, which subsequently cleaves and activates caspase-3. The activation of caspase-3 results in the proteolytic cleavage of a range of cellular targets that ultimately leads to the demise of the cell (Bratton et al. 2000; Jiang and Wang, 2000; Yuan and Yankner, 2000). However, it should be noted that PCD can occur in the absence of these molecules (Cregan et al., 2002; Oppenheim et al., 2001; Yaginuma et al., 2001).

Although the intracellular cascades leading to PCD are largely defined, it remains unclear whether all neurons vulnerable to cell death during developmentally defined critical periods die by means of the aforementioned cascades. The brainstem auditory system of the chick has been widely studied as a model of activity-dependent cell regulation and survival (Rubel et al., 1976, 1990). In young birds, cessation of auditory nerve (nVIII) activity by cochlea ablation results in the death and atrophy of postsynaptic target neurons in the ipsilateral avian cochlear nucleus, the nucleus magnocellularis (NM). Approximately 30% of NM neurons in the ipsilateral NM die and the remaining 70% are markedly smaller within 2 days following cochlea removal (Born and Rubel, 1985). It appears that the neuro-transmitter glutamate provides some form of activity-dependent trophic support to NM neurons through activation of metabotropic glutamate receptors (Zirpel and Rubel, 1996; Hyson, 1998).

Rapid changes in both the ultrastructure and the metabolic activity of NM neurons are observed following removal of excitatory afferent activity. A rapid decrease in protein synthesis and an increase in intracellular calcium concentration are observed across the entire population of deafferented neurons within 1–3 h after elimination of afferent activity (Steward and Rubel, 1985; Hyson and Rubel, 1989; Zirpel et al., 1995). By 6 h after cochlea removal, the majority of cells recover protein synthesis capacity and survive; however, approximately 30% of NM neurons never regain protein synthesis capabilities and it is believed that these are the neurons that are destined to die. The dying population of NM neurons is also delineated by a dramatic reduction of overall RNA synthesis and a degradation of polyribosomes (Rubel et al., 1991; Garden et al., 1995). Meanwhile, surviving NM neurons retain normal ribosomal ultrastructure as well as the ability to synthesize new RNA and protein, albeit, at reduced levels (Rubel et al., 1990; Garden et al., 1994, 1995). Although some cellular events delineating dying from surviving populations of NM neurons have been identified, it remains unclear whether the molecular events that commonly occur during PCD are initiated in this system.

The biochemical variables that determine individual cell fate are unknown; however, earlier studies support a determinate role for mitochondria. An increase in mitochondrial-related function provides a notable exception to the dramatic decreases in macromolecular synthesis described above. Within 6 h of cochlea removal, a robust increase in both mitochondrial density

and oxidative enzyme capacity is evident (Durham and Rubel, 1985; Hyde and Durham, 1990, 1994a). Furthermore, blockade of mitochondrial proliferation results in increased neuronal death following deafferentation (Hyde and Durham, 1994b). Thus, proliferation of mitochondria following afferent deprivation may be a regulated process that is pivotal in the determination of cell survival.

Apoptotic cascades have been reported to be involved in other preparations that investigate the effect of deafferentation on target neurons. For example, *bcl-2*, a molecule that has been shown to regulate cell death cascades in model systems of PCD, also appears to regulate deafferentation-induced cell death. Olfactory epithelial neurons overexpressing *bcl-2* are protected from cell death following olfactory bulb ablation (Jourdan et al., 1998). *Bcl-2* overexpression also eliminates deafferentation-induced cell death in the anteroventral cochlear nucleus, and these neurons show no increase in caspase-3 activation (Mostafapour et al., 2002). In NM, we have shown that *bcl-2* mRNA expression is transiently up-regulated following cochlea removal (Wilkinson et al., 2002).

Since the response of NM neurons to cochlea removal appears to include components of apoptotic signaling cascades, we sought to identify whether additional downstream apoptotic molecules were involved in deafferentation-induced NM cell death. In this report, we examine the potential release of cytochrome-c into the cytosol and the potential activation of caspase-9 following deafferentation.

EXPERIMENTAL PROCEDURES

Subjects and surgical procedures

All subjects were posthatch chicks (Ross×Ross) hatched and reared at The Florida State University. The surgical procedures used in these experiments were approved by the Animal Care and Use Committee at The Florida State University and conform to the guidelines set forth by the National Institutes of Health. All efforts were made to minimize the number of animals used and their suffering. Cochlear ablations were performed as previously described (Born and Rubel, 1985). Subjects were anesthetized with Halothane. The ear canal was widened with a small incision, and the tympanic membrane was punctured. The columella (middle ear ossicle) was removed followed by the extraction of the basilar papilla through the oval window. The middle ear cavity was packed with Gelfoam and the external incision sealed with surgical adhesive. Extracted tissue was examined under a dissection microscope to assure complete removal of the cochlea. Each NM receives excitatory input from only the ipsilateral nVIII; therefore, unilateral cochlea removal eliminates all afferent drive to neurons in the ipsilateral NM, while innervation to the contralateral NM remains intact (Born et al., 1991). This procedure allows for a within subject (within tissue-section) control for comparisons between NM on the deafferented side and NM on the intact side of the brain.

Immunocytochemistry

Standard immunocytochemical (ICC) procedures were utilized for these experiments. Subjects were perfused with 0.9% saline followed by ice cold 4% paraformaldehyde. Brainstems were blocked and post-fixed in 4% paraformaldehyde for 1–2 h followed by overnight cryoprotection in a 20% sucrose solution at 4 °C. For cryosectioning, brainstems were rapidly frozen in 2-methylbutane and embedded in Tissue-Tek OCT compound. Twenty micrometer sections were free-floated and processed for either anti-cytochrome-c (33–8200, Zymed, South San Francisco, CA, USA) or anti-cleaved caspase-9 D315, Cell Signaling, Beverly, MA, USA) ICC. Sections processed with anti-cytochrome-c antibody were obtained from subjects that survived 3 h ($n = 4$), 6 h ($n = 4$), 12–24 h ($n = 6$), or 3–5 days ($n = 4$) following unilateral cochlea removal. Free-floating sections were preincubated in 4% normal horse serum and then

incubated overnight with anti-cytochrome-c antibody diluted 1:500 in 0.3% Triton X-100. Sections were then washed with phosphate buffer saline, and incubated in biotinylated anti-mouse secondary (1:200) for 1 h, and detected through the avidin–biotin–peroxidase complex (ABC kit; Vector Laboratories, Burlingame, CA, USA). Peroxidase activity was visualized with diaminobenzidine (Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA). Free-floating sections were mounted on gelatin-subbed slides, dehydrated, and coverslipped. Controls with no primary antibody were run to assure specificity of the reaction, and no immunolabeling for cytochrome-c was observed in these control sections (data not shown). Anti-cleaved caspase-9 processed sections were obtained from subjects that survived 1 h ($n = 4$), 3 h ($n = 4$), 6 h ($n = 4$), 12 h ($n = 3$), 24 h ($n = 4$), or 4 days ($n = 3$) after cochlea removal. Tissue from a control group ($n = 4$) that did not receive surgery was also processed for active caspase-9. ICC procedures were identical to those described above with the following exceptions: preincubation in normal goat serum, washes in Tris-buffered saline (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, and 0.1% Triton X-100), and incubation in biotinylated anti-rabbit secondary antibody (1:200; Vector Laboratories).

Objective analyses of anatomical tissue

All ICC studies compared opposite sides of the same tissue section in order to control for variability in absolute labeling densities owing to tissue processing variables. Measurements of labeling were accomplished using video image analysis and NIH Image software. For the objective analysis of relative differences in immunolabeling, the mean gray-scale density over NM neurons on each side of the brain was measured in several sections from each subject's brain (three to four sections/brain). Approximately 40 cells within each NM in a given tissue section were analyzed, and measurements were taken systematically with analyses beginning at the medial edge of the nucleus and proceeding laterally. Differences in the density of labeling on the manipulated and normal sides of the section were analyzed using an analysis of variance (ANOVA).

Tissue preparation, immunoprecipitation, and Western analysis

Brain tissue was obtained from subjects that survived for 12 h ($n = 10$) after bilateral cochlea removal surgery and a control group ($n = 10$) that did not have surgery. The auditory nuclei were micro-dissected from brainstem slices into harvesting buffer (0.32 M sucrose, 10 mM Tris base, 50 mM KCl, and 1 mM EDTA) in deionized H₂O plus a mixture of protease inhibitors (1:100, pH 7.8; Sigma, St. Louis, MO, USA). Extracted tissue was homogenized on ice with a Teflon pestle (40 strokes). Homogenates were centrifuged at 1000×g for 10 min at 4 °C to remove the nuclei. The resulting supernatant was then centrifuged at 8000×g for 30 min at 4 °C to remove the crude mitochondrial fraction. The remaining supernatants were further centrifuged at 100,000×g for 1 h at 4 °C, and the resulting supernatant was used as the cytosolic fraction.

Immunoprecipitation procedures were modified from Fadool et al. (2000). Cytosolic fractions were lysed with protease and phosphatase inhibitor (PPI) solution (25 mM Tris, 250 mM NaCl, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 1% Triton X-100, 1 µg/ml each leupeptin and pepstatin, and 2 µg/ml aprotinin; pH 7.5) for 40 min on an orbital rotary at 4 °C. Cell lysates were then clarified by centrifugation at 14,000×g for 10 min at 4 °C and incubated for 1 h with 3 mg/ml protein-A sepharose (Amersham-Pharmacia, Piscataway, NJ, USA), and then re-centrifuged to remove the protein-A sepharose. Cytochrome-c was immunoprecipitated from the clarified lysate by overnight incubation with 0.5 µg/ml mouse anti-cytochrome-c (Zymed 33–8500) at 4 °C. Samples were then incubated with protein-A sepharose for 2 h and centrifuged as before. Immunoprecipitates were washed four times with ice-cold PPI solution (modified to 0.1% Triton X-100). Crude mitochondrial fractions and immunoprecipitates from the cytosolic fractions were diluted in sodium dodecyl sulfate (SDS)

gel-loading buffer containing Na_3VO_4 (Sambrook and Russell, 2000) and stored at -20°C until used. Proteins were separated on 12% acrylamide SDS gels and electrotransferred to nitrocellulose for Western analysis. For the crude mitochondrial fraction, protein concentration was measured by the bicinchoninic acid method (Biorad, Hercules, CA, USA) and 10 μg of protein/lane was loaded on the gel. Membranes were blocked with 4% non-fat dry milk at room temperature for 30 min and then incubated overnight at 4°C in primary antibody against cytochrome-c followed by horseradish peroxidase-conjugated mouse IgG (Sigma) secondary antibody for 90 min at room temperature. Labeled proteins were visualized through enhanced chemiluminescence (Amersham-Pharmacia) exposure on X-ray film (Kodak, Rochester, NY, USA). Equal loading of lanes was assured by Bradford assay. Films were scanned on a Hewlett-Packard Photosmart Scanner (model 106–816) and analyzed using line scan densitometry with Quantiscan software (Biosoft, Cambridge, UK).

Kainic acid exposure

A kainic acid exposure paradigm adapted from Liu et al. (2001) was utilized as a positive control for cytochrome-c release and detection in brainstem slice preparations. Brainstem slices containing the auditory nuclei were obtained from fully innervated posthatch chicks ($n = 10$; Hyson and Rubel, 1989). Slices (300–400 μm thick) were prepared in oxygenated (95:5 O_2/CO_2) artificial cerebrospinal fluid (ACSF; 130 mM NaCl, 3 mM KCl, 2 mM CaCl_2 , 2 mM MgCl_2 , 26 mM NaHCO_3 , 1.25 mM NaH_2PO_4 , 10 mM D-glucose). Slices were then incubated in 50 μM kainic acid (Tocris, Ellisville, MO, USA) in ACSF for 3 h at 34°C . The preparation was bubbled with a 95:5 O_2/CO_2 mixture in order to ensure the integrity of the tissue. Following the exposure to kainic acid, brainstem slices were washed three times in drug-free ACSF and then processed as described above.

RESULTS

Changes in cytochrome-c following deafferentation

An increase in cytochrome-c immunoreactivity is evident across the entire population of ipsilateral NM by 6 h following unilateral cochlea removal, and immunoreactivity remains elevated for at least 5 days following cochlea removal. Fig. 1 shows a representative example of increased cytochrome-c immunolabeling at 24 h after cochlea removal. Labeling density of individual neurons was objectively analyzed by densitometry and the percent differences between labeling density of NM neurons on the two sides of the brain are displayed in Fig. 2. Statistically reliable differences in immunolabeling emerged by 6 h following cochlea removal and immunoreactivity remained elevated in the deafferented NM for the duration of these experiments. A Side $\times$ Survival Time mixed analysis of variance of the average density measurements from each side of the brain produced a reliable main effect of Side ($F_{1,14} = 55.2$, $P < 0.05$). Post hoc comparisons (Student-Newman-Keuls, $P < 0.05$) revealed that immunolabeling in the deafferented (ipsilateral) NM was reliably greater than labeling in the intact (contralateral) NM in all groups, except for the 3 h survival time. The specificity of the reaction was assured by incubating control sections in the absence of primary antibody; these sections did not produce cytochrome-c immunolabeling (data not shown).

This ICC study provided valuable temporal information regarding cytochrome-c expression following deafferentation; however, this technique is not optimized for determining whether the cytochrome-c is located in the cytosol or if it is located within intact mitochondria. Consequently, the localization of cytochrome-c was assessed by immunoprecipitation followed by Western analysis of cytochrome-c in mitochondrial and cytosolic fractions of deafferented neurons. A 12 h time point was chosen for the Western analysis based on the temporal data obtained from the ICC experiments. Cytochrome-c immunoreactivity is reliably elevated following cochlea removal at this time. A single time point was also chosen because

of the large numbers of subjects needed to generate adequate levels of protein to perform immunoblot procedures. The quality of the antibody was first examined using Western analyses of whole brain extracts. A protein band at the appropriate molecular weight, approximately 15 kD, was detected in extracted neuronal tissue from seven to 10 posthatch chickens. Membranes incubated without primary antibody did show some non-specific labeling from the secondary antibody; however, the non-specific labeling did not occur below 20 kD, nor did it appear in the immunoprecipitates. Thus, we are confident that the anti-cytochrome-c antibody used in these experiments detects chicken cytochrome-c and is specific for this antigen.

Immunoprecipitates from the cytosolic fractions of NM obtained from control subjects and from NM obtained from subjects that survived 12 h following deafferentation were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and Western analysis for cytochrome-c. Cytochrome-c was not detected in the cytosolic fractions of either group (Fig. 3). This was confirmed by densitometric measurements using Quantiscan software, which showed that the pixel density in the 15 kD region of the lanes containing immunoprecipitates from the cytosolic fractions of NM did not rise above background. Mitochondrial fractions from both the group receiving cochlea removal and the control group, processed on the same gel and measured over the same region, showed substantial levels of cytochrome-c: 12 h (pixel density = 284.95) and control (pixel density = 218.76).

The potential for NM mitochondria to release cytochrome-c into the cytosol was analyzed by exposing extracted NM tissue to cytotoxic insult. Tissue slices containing the brainstem auditory nuclei were treated with kainate, a substance known to induce cytochrome-c release from mitochondria (Liu et al., 2001). Lysates from kainate-exposed NM were then processed via immunoprecipitation followed by Western analysis. Cytochrome-c was detected in both the cytosolic and the crude mitochondrial fractions from NM neurons treated with kainate (Fig. 4). Protein bands analyzed for immunodensitometry revealed that cytochrome-c was contained in both the cytosolic fraction (density net area = 134.38) and the crude mitochondrial fraction (density net area = 1425.87). These results show that under cytotoxic conditions NM neurons can be induced to release cytochrome-c from their mitochondria, and that the methods used in this study are sensitive enough to detect this release.

Caspase-9 activation in NM following deafferentation

In order to characterize whether apoptotic events downstream of cytochrome-c release are activated in NM neurons following activity deprivation, the presence of cleaved caspase-9 was investigated using an antibody that specifically recognizes the cleaved (active) form of caspase-9. Within hours following cochlea removal active caspase-9 immunoreactivity was elevated in ipsilateral NM neurons when compared with NM neurons on the contralateral (intact) side of the same tissue section (Fig. 5). By 4 days following cochlea removal, there is no longer a significant difference in immunolabeling between the ipsilateral and contralateral NM. Although only a subpopulation of deprived neurons ultimately die following cochlea ablation, the density of labeling for active caspase-9 showed a normal distribution at all time points and labeling was not restricted to a subpopulation of deafferented NM neurons. Cellular labeling above background was not observed in any other areas of the brain stem, with the possible exception of the cochlear nucleus, nucleus angularis, where labeled neurons were frequently observed on the operated side of the brain. Control sections, omitting primary antibody incubation, did not yield significant immunolabeling of either NM or the surrounding tissue.

Fig. 6 illustrates the percentage difference in the mean density of labeling between ipsilateral and contralateral NM neurons. A Side×Survival Time mixed analysis of variance on the density measurements revealed a reliable main effect of Side ($F_{1,20} = 26.3$, $P < 0.05$). The main effect of Survival Time and the Side×Survival Time interaction were also statistically significant

($F_{6,20} = 6.7$, and $F_{6,20} = 46.9$, respectively; $P < 0.05$). Post hoc comparisons (Student-Newman-Keuls, $P < 0.05$) revealed that differences between sides of the brain were reliable for the 3, 6, 12, and 24 h groups, but not for the control, 1 h and 4 day groups (Fig. 6). These analyses confirm that activation of caspase-9 begins approximately 3 h following cochlea removal and is no longer activated 4 days following the cessation of afferent activity.

DISCUSSION

PCD (apoptosis) is preceded by a cascade of well-characterized intracellular events that culminate in cell death. The goal of this research was to identify whether the intracellular events that precede cell death in the avian cochlear nucleus (NM) following deafferentation includes molecules known to be involved in forms of PCD. Our findings indicate that cytochrome-c immunoreactivity is up-regulated in deafferented NM neurons, but cytochrome-c is not detected in cytosolic fractions obtained from deafferented NM. Rather, it appears that cytochrome-c is retained within the mitochondria of deafferented NM neurons and the increase in immunoreactivity is likely attributable to the proliferation of mitochondria (Hyde and Durham, 1994a). An unexpected and intriguing finding was the transient presence of active caspase-9 following deafferentation; caspase-9 expression was detectable by 3 h following deafferentation but was no longer evident by 4 days following surgery. Together, these results suggest that deafferentation leads to caspase activation and a type of cell death in NM neurons that occurs in the absence of cytochrome-c release from mitochondria.

The lack of evidence for the release of cytochrome-c into the cytosol could be attributable to methodological limitations, or it could be that mitochondria within deafferented NM maintain their integrity and do not extrude this apoptogenic factor into the cytosol following deafferentation. If cytochrome-c is not released, then the question remains as to how caspase-9 becomes activated in these neurons. The remainder of this discussion will address each of these issues in turn.

A methodological consideration that must be addressed is whether the procedures used are sensitive enough to detect cytochrome-c released into the cytosol of deafferented NM neurons. The cytochrome-c antigen could simply be in such low abundance, compared with the overall volume of sample used in these experiments, that it is not detectable. We used immunoprecipitation procedures in an effort to further concentrate the sample used for analysis. In addition, experiments were performed that dialyzed and then lyophilized sample homogenates from both deafferented and control subjects; these experiments also failed to show cytochrome-c in the cytosol of deafferented NM (data not shown). As a positive control, we detected cytochrome-c release in NM following treatment with kainate. It is possible that release was detected following kainate exposure and not following cochlea removal because a greater percentage of NM neurons released cytochrome-c following kainate, making cytochrome-c within the cytosolic fraction more readily detectable. Nevertheless, there was clear detection of signal following kainate, whereas there was absolutely no indication of cytochrome-c present in the cytosolic fraction of NM following cochlea removal. While it is impossible to prove the absence of cytochrome-c in the cytosol (i.e. prove the null effect), the fact that kainate exposure did lead to detectable cytochrome-c release from NM provides some confidence that had cytochrome-c been released following deafferentation, the experimental procedures used would have detected its presence.

It is possible that the lack of cytochrome-c accumulation in the cytosol of deafferented NM neurons simply reflects that the mitochondria in these neurons are not compromised following deafferentation. Previous findings in activity-deprived NM neurons suggest that mitochondria function to ameliorate cell death in NM neurons (Durham and Rubel, 1985; Hyde and Durham, 1990; Durham et al., 1993). An increased oxidative capacity is accompanied by a proliferation

of mitochondria following deafferentation (Hyde and Durham, 1994a), and blockade of mitochondrial proliferation in NM results in increased deafferentation-induced cell death (Hyde and Durham, 1994b). It is likely that the increased cytochrome-c immunoreactivity observed in NM neurons reflects mitochondrial proliferation after cochlea removal. An increase in mitochondria containing cytochrome-c would likely manifest as more intensely immunolabeled NM neurons. Immunodensity measurements from the mitochondrial fractions seem to support this hypothesis in that there is more intense cytochrome-c labeling in the mitochondrial fractions of deafferented subjects than in the mitochondrial fractions from control subjects. An alternative approach might be to examine cytochrome-c localization with confocal microscopy using double-labeled immunofluorescence for cytochrome-c and a mitochondrial marker; however, the dense proliferation of mitochondria following cochlea removal (Hyde and Durham, 1994a) could easily lead one to erroneously conclude that cytochrome-c is located in the cytoplasm.

The apparent absence of cytochrome-c in the cytosol of deafferented NM neurons leads to some speculation on how caspase-9 may get activated following deafferentation. During classic apoptosis, the activation of caspase-9 is believed to be intimately related to cytochrome-c release from mitochondria and the subsequent formation of the apoptosome. While it remains possible that small, undetectable amounts of cytochrome-c are released in NM neurons leading to caspase activation, the time course of changes in cytochrome-c and active caspase-9 immunoreactivity also suggest that activation may be cytochrome-c independent. Increased immunolabeling for cytochrome-c was not reliably observed until 6 h following cochlea ablation, whereas active caspase-9 immunoreactivity showed reliable increases by 3 h following surgery. Caution is necessary for both lines of evidence, but together they suggest that caspase-9 may be activated in NM by a cytochrome-c independent mechanism.

There is evidence for cytochrome-c-independent activation of caspase-9 in other systems. For example, *in vitro* experiments have shown that both caspase-9 and caspase-3 are cleaved without the release of cytochrome-c into the cytosol in multiple myeloma cells following dexamethasone treatment (Chauhan et al., 1997, 2001). Other mitochondrial proteins may also activate caspases. The release of second mitochondrial-derived activator of caspase (Smac)/direct inhibitor of apoptosis (IAP)-binding protein with low pI (DIABLO) acts to eliminate the inhibitory effect of IAPs, which suppress apoptosis by binding to and inactivating caspases (Du et al., 2000; Verhagen et al., 2000). The result is a more efficient activation of the apoptosome-dependent activation of caspase-3 (Denecker et al., 2001). Smac/DIABLO can also potentiate caspase-9 activation using the dexamethasone procedure, which does not require cytochrome-c release to initiate apoptosis, and blockade of dexamethasone-induced apoptosis prevents the release of Smac/DIABLO (Chauhan et al., 2001). It is possible that deafferentation-induced cell death in NM does not follow the apoptosome-dependent apoptotic pathway, but rather, a Smac/DIABLO or an as yet unknown, pathway of intracellular events that leads to caspase activation. Future experiments will have to examine whether Smac/DIABLO is present and regulated by afferent activity in NM neurons.

Although it appears that cytochrome-c is either subtly involved or not released at all in NM neurons following deafferentation, we have provided evidence that caspase-9 is activated following deafferentation. While caspase activation may not always be related to cell death (Yaginuma et al., 2001), it is notable that caspase activation appeared to be restricted to the cochlear nuclei after ablation, suggesting that it may contribute to death in this system. The expression of active caspase-9, however, does not appear to be restricted to a subpopulation of deafferented NM neurons as one would expect if it were initiating proteolytic caspase cascades in the 30% of NM neurons that ultimately die following cochlea ablation. One explanation of the global increase in active caspase-9 is that some NM neurons, presumably the living population, express proteins that inhibit the activity of caspase-9. Another molecule

that is believed to play a role in regulating cell death, bcl-2, is transiently up-regulated in a subpopulation of NM neurons with a similar time course following cochlea removal (Wilkinson et al., 2002). Bcl-2 is believed to have a pro-survival influence in other systems, but surprisingly, its expression was restricted to 30%, presumably the dying subpopulation, of deafferented NM neurons. Consequently, it seems unlikely that bcl-2 is playing a role in protecting cells from the effects of caspase-9 activation. Alternatively, a family of proteins referred to as IAPs are capable of binding and inhibiting the action of caspase-9 (Deveraux et al., 1997). Additional studies will have to confirm whether IAPs are differentially regulated in NM following deafferentation, and if so, whether they directly contribute to cell survival in NM neurons. The widespread increase in cleaved caspase-9 suggests that competing cell death and cell survival mechanisms must work downstream of caspase-9 activation to regulate cell death in NM neurons.

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References

- Born DE, Rubel EW. Afferent influences on brain stem auditory nuclei of the chicken: neuron number and size following cochlea removal. *J Comp Neurol* 1985;231:435–445. [PubMed: 3968247]
- Born DE, Durham D, Rubel EW. Afferent influences on brainstem auditory nuclei of the chick: nucleus magnocellularis neuronal activity following cochlea removal. *Brain Res* 1991;557:37–47. [PubMed: 1747768]
- Bratton SB, MacFarlane M, Cain K, Cohen GM. Protein complexes activate distinct caspase cascades in death receptor and stress-induced apoptosis. *Exp Cell Res* 2000;256:27–33. [PubMed: 10739648]
- Brunjes PC. Unilateral naris closure and olfactory system development. *Brain Res Brain Res Rev* 1994;19:146–160. [PubMed: 8167658]
- Chauhan D, Pandey P, Ogata A, Teoh G, Krett N, Halgren R, Rosen S, Kufe D, Kharbanda S, Anderson K. Cytochrome c-dependent and -independent induction of apoptosis in multiple myeloma cells. *J Biol Chem* 1997;272:29995–29997. [PubMed: 9374472]
- Chauhan D, Hideshima T, Rosen S, Reed JC, Kharbanda S, Anderson KC. Apaf-1/cytochrome c-independent and Smac-dependent induction of apoptosis in multiple myeloma (MM) cells. *J Biol Chem* 2001;276:24453–24456. [PubMed: 11356822]
- Cregan SP, Fortin A, MacLaurin JG, Callaghan SM, Cecconi F, Yu SW, Dawson TM, Dawson VL, Park DS, Kroemer G, Slack RS. Apoptosis-inducing factor is involved in the regulation of caspase-independent neuronal cell death. *J Cell Biol* 2002;158:507–517. [PubMed: 12147675]
- Deveraux QL, Takahashi R, Salvesen GS, Reed JC. X-linked IAP is a direct inhibitor of cell-death proteases. *Nature* 1997;388:300–304. [PubMed: 9230442]
- Denecker G, Vercammen D, Declercq W, Vandenabeele P. Apoptotic and necrotic cell death induced by death domain receptors. *Cell Mol Life Sci* 2001;58:356–370. [PubMed: 11315185]
- Desagher S, Martinou JC. Mitochondria as the central control point of apoptosis. *Trends Cell Biol* 2000;10:369–377. [PubMed: 10932094]
- Du C, Fang M, Li Y, Li L, Wang X. Smac, a mitochondrial protein that promotes cytochrome c-dependent caspase activation by eliminating IAP inhibition. *Cell* 2000;102:33–42. [PubMed: 10929711]
- Duchen MR. Mitochondria and calcium: from cell signaling to cell death. *J Physiol* 2000;529:57–68. [PubMed: 11080251]
- Durham D, Rubel EW. Afferent influences on brain stem auditory nuclei of the chicken: changes in succinate dehydrogenase activity following cochlea removal. *J Comp Neurol* 1985;231:446–456. [PubMed: 3968248]
- Durham D, Matschinsky FM, Rubel EW. Altered malate dehydrogenase activity in nucleus magnocellularis of the chicken following cochlea removal. *Hear Res* 1993;70:151–159. [PubMed: 8294259]

- Fadool DA, Tucker K, Phillips JJ, Simmen JA. Brain insulin receptor causes activity-dependent current suppression in the olfactory bulb through multiple phosphorylation of Kv1.3. *J Neurophysiol* 2000;83:2332–2348. [PubMed: 10758137]
- Galli-Resta L, Ensini M, Fusco E, Gravina A, Margheritti B. Afferent spontaneous electrical activity promotes the survival of target cells in the developing retinotectal system of the rat. *J Neurosci* 1993;13:243–250. [PubMed: 8423471]
- Garden GA, Canady KS, Lurie DI, Bothwell M, Rubel EW. A biphasic change in ribosomal conformation during transneuronal degeneration is altered by inhibition of mitochondrial, but not cytoplasmic protein synthesis. *J Neurosci* 1994;14:1994–2008. [PubMed: 8158254]
- Garden GA, Redeker-DeWulf V, Rubel EW. Afferent influences on brainstem auditory nuclei of the chicken: regulation of transcriptional activity following cochlea removal. *J Comp Neurol* 1995;359:412–423. [PubMed: 7499538]
- Green DR, Reed JC. Mitochondria and apoptosis. *Science* 1998;281:1309–1312. [PubMed: 9721092]
- Gross A, McDonnell JM, Korsmeyer SJ. BCL-2 family members and the mitochondria in apoptosis. *Genes Dev* 1999;13:1899–1911. [PubMed: 10444588]
- Hyde GE, Durham D. Cytochrome oxidase response to cochlea removal in chicken auditory brainstem neurons. *J Comp Neurol* 1990;297:329–339. [PubMed: 2168902]
- Hyde GE, Durham D. Rapid increase in mitochondrial volume in nucleus magnocellularis neurons following cochlea removal. *J Comp Neurol* 1994a;339:27–48. [PubMed: 8106659]
- Hyde GE, Durham D. Increased deafferentation-induced cell death in chick brainstem auditory neurons following blockade of mitochondrial protein synthesis with chloramphenicol. *J Neurosci* 1994b; 14:291–300. [PubMed: 8283237]
- Hyson RL, Rubel EW. Transneuronal regulation of protein synthesis in the brain stem auditory system of the chick requires synaptic activation. *J Neurosci* 1989;9:2835–2845. [PubMed: 2769367]
- Hyson RL. Activation of metabotropic glutamate receptors is necessary for transneuronal regulation of ribosomes in chick auditory neurons. *Brain Res* 1998;809:214–220. [PubMed: 9853113]
- Jacobson MD, Weil M, Raff MC. Programmed cell death in animal development. *Cell* 1997;88:347–534. [PubMed: 9039261]
- Jiang X, Wang X. Cytochrome c promotes caspase-9 activation by inducing nucleotide binding to apaf-1. *J Biol Chem* 2000;275:31199–31203. [PubMed: 10940292]
- Johnson, B.; Leon, M. The role of early experience in olfactory bulb cell survival. In: Hyson, RL.; Johnson, F., editors. *The biology of early influences*. New York: Kluwer Academic/Plenum Publishers; 1999. p. 73–85.
- Jourdan F, Moyse E, De Bilbao F, Dubois-Dauphin M. Olfactory neurons are protected from apoptosis in adult transgenic mice over-expressing the bcl-2 gene. *Neuroreport* 1998;9:921–926. [PubMed: 9579691]
- Li P, Nijhawan D, Budihardjo I, Srinivasula SM, Ahmad M, Alnemri ES, Wang X. Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell* 1997;91:479–489. [PubMed: 9390557]
- Linden R. The survival of developing neurons: a review of afferent control. *Neuroscience* 1994;58:671–682. [PubMed: 8190251]
- Liu W, Liu R, Chun JT, Bi R, Hoe W, Schreiber SS, Baudry M. Kainate excitotoxicity in organotypic hippocampal slice cultures: evidence for multiple apoptotic pathways. *Brain Res* 2001;916:239–248. [PubMed: 11597611]
- Mostafapour SP, Del Puerto NM, Rubel EW. Bcl-2 overexpression eliminates deprivation-induced cell death of brainstem auditory neurons. *J Neurosci* 2002;22:4670–4674. [PubMed: 12040073]
- Oppenheim RW. Cell death during development of the nervous system. *Annu Rev Neurosci* 1991;14:453–501. [PubMed: 2031577]
- Oppenheim RW, Flavell RA, Vinsant S, Prevette D, Kuan CY, Rakic P. Programmed cell death of developing mammalian neurons after genetic deletion of caspases. *J Neurosci* 2001;21:4752–4760. [PubMed: 11425902]
- Petit PX, Susin SA, Zamzami N, Mignotte B, Kroemer G. Mitochondria and programmed cell death: back to the future. *FEBS Lett* 1996;396:7–13. [PubMed: 8906857]

- Peusner KD, Morest DK. Neurogenesis in the nucleus vestibularis tangentialis of the chick embryo in the absence of the primary afferent fibers. *Neuroscience* 1977;2:253–270. [PubMed: 904776]
- Rubel EW, Smith DJ, Miller LC. Organization and development of brain stem auditory nuclei of the chicken: ontogeny of n. magnocellularis and n. laminaris. *J Comp Neurol* 1976;166:469–489. [PubMed: 1270618]
- Rubel EW, Hyson RL, Durham D. Afferent regulation of neurons in the brain stem auditory system. *J Neurobiol* 1990;21:169–196. [PubMed: 2181062]
- Rubel EW, Falk PM, Canady KS, Steward O. A cellular mechanism underlying activity-dependent transneuronal degeneration: rapid but reversible destruction of neuronal ribosomes. *Brain Dysfunct* 1991;4:55–74.
- Sambrook, J.; Russell, D. *Molecular cloning: a laboratory manual*. 3. Woodbury, New York: Cold Spring Harbor Laboratory Press; 2000.
- Steward O, Rubel EW. Afferent influences on brain stem auditory nuclei of the chicken: cessation of amino acid incorporation as an antecedent to age-dependent transneuronal degeneration. *J Comp Neurol* 1985;231:385–395. [PubMed: 3968244]
- Verhagen AM, Ekert PG, Pakusch M, Silke J, Connolly LM, Reid GE, Moritz RL, Simpson RJ, Vaux DL. Identification of DIABLO, a mammalian protein that promotes apoptosis by binding to and antagonizing IAP proteins. *Cell* 2000;102:43–53. [PubMed: 10929712]
- Wilkinson BL, Sadler KA, Hyson RL. Rapid deafferentation-induced upregulation of bcl-2 mRNA in the chick cochlear nucleus. *Brain Res Mol Brain Res* 2002;99:67–74. [PubMed: 11869810]
- Yaginuma H, Sato N, Homma S, Oppenheim RW. Roles of caspases in the programmed cell death of motoneurons in vivo. *Arch Histol Cytol* 2001;64:461–474. [PubMed: 11838706]
- Yuan J, Yankner BA. Apoptosis in the nervous system. *Nature* 2000;407:802–809. [PubMed: 11048732]
- Zirpel L, Lachica EA, Lippe WR. Deafferentation increases the intracellular calcium of cochlear nucleus neurons in the embryonic chick. *J Neurophysiol* 1995;74:1355–1357. [PubMed: 7500157]
- Zirpel L, Rubel EW. Eighth nerve activity regulates intracellular calcium concentration of avian cochlear nucleus neurons via a metabotropic glutamate receptor. *J Neurophysiol* 1996;76:4127–4139. [PubMed: 8985906]

Abbreviations

ACSF	artificial cerebrospinal fluid
ANOVA	analysis of variance
Apaf-1	apoptosis activating factor-1
EDTA	ethylenedi-aminetetraacidic acid
IAP	inhibitor of apoptosis
ICC	immunocytochemical
nVIII	auditory nerve, eighth nerve
NM	nucleus magnocellularis
PAGE	

polyacrylamide gel electrophoresis

PCD

programmed cell death

PPI

phosphatase inhibitor

SDS

sodium dodecyl sulfate

Smac/DIABLO

second mitochondrial-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low pI

Fig. 1. Representative photomicrograph showing anti-cytochrome-c peroxidase-labeled coronal tissue section at 24 h following cochlea removal. There is a significant difference in immunolabeling between the deafferented and intact sides of the same tissue section. This increased immunoreactivity is evident across the entire extent of the deafferented NM.

Fig. 2.

Deafferentation-induced changes in cytochrome-c immunoreactivity in NM can be observed as soon as 6 h following deafferentation. Immunolabeling was compared on opposite sides of the same tissue sections. Within subject comparisons (ANOVA followed by Student-Newman-Keuls pairwise comparisons, $P < 0.05$) revealed a significant difference in labeling density between the deafferented and intact sides of the same tissue section at 6 h, 12 h, and 3+ days following unilateral cochlear ablation; these differences were not statistically reliable at 3 h. Asterisk (*) indicates a statistically reliable difference between the deafferented and intact sides of the brain. Bars=S.E.M.

Fig. 3.

Immunoprecipitation followed by Western analysis failed to detect cytochrome-c within the cytoplasmic fractions from either subjects that survived 12 h following bilateral cochlea removals or control subjects that did not undergo any surgical procedure. Proteins were immunoprecipitated with 5 $\mu\text{g}/\mu\text{l}$ of cytochrome-c antibody, separated by 12% SDS-PAGE, and then electrotransferred to nitrocellulose. Nitrocellulose was probed with 1:1000 anti-cytochrome-c. Quantitative densitometry did not yield any difference between the 12 h survival group, the control group, and background labeling in the cytosolic fractions. Mitochondrial fractions, where cytochrome-c is normally found, did show a strong presence of cytochrome-c under both conditions. Arrows denote the location of bands from molecular weight markers on the same membrane.

Fig. 4.

Cytochrome-c can be detected in cytosolic fractions from tissue treated with kainate. Cytochrome-c was immunoprecipitated and separated by SDS-PAGE as in Fig. 3. When brain slices were exposed to 50 μ M kainic acid for a period of 3 h, the mitochondria in NM neurons did extrude cytochrome-c into the cytosol. Arrows denote the location of bands from molecular weight markers on the same membrane.

Fig. 5. Active caspase-9 is up-regulated in NM following deafferentation. Photomicrographs from a subject that survived 12 h following cochlea removal. The increase in active caspase-9 immunoreactivity was observed across the entire extent of the deafferented NM.

Fig. 6.

Within 3 h following cochlea removal, active caspase-9 immunoreactivity is elevated in NM neurons. Immunolabeling was compared on opposite sides of the same tissue sections. Within subject comparisons (ANOVA followed by Student-Newman-Keuls pairwise comparisons, $P < 0.05$) revealed a significant increase in active caspase-9 immunolabeling 3 h following deafferentation. This difference is no longer apparent 4 days following surgery. Asterisk (*) indicates a statistically reliable difference between the deafferented and intact sides of the brain. Bars=S.E.M.