Increased Deafferentation-induced Cell Death in Chick Brainstem Auditory Neurons following Blockade of Mitochondrial Protein Synthesis with Chloramphenicol

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Second-order auditory neurons in nucleus magnocellularis (NM) of the chick brainstem undergo a series of rapid metabolic changes following unilateral cochlea removal, culminating in the death of 25% of NM neurons. Within hours of cochlea removal, ipsilateral NM neurons show marked increases in histochemical staining for the mitochondrial enzymes succinate dehydrogenase and cytochrome oxidase (CO). We have shown previously in an ultrastructural study that these increases in oxidative capacity are mediated in part by a rapid increase in mitochondrial volume within deafferented neurons. In neurons that are destined to die as a result of deafferentation, mitochondria are smaller, stain poorly for CO, and often contain vacuoles associated with oxidative dysfunction.

Our present set of experiments is designed to test the hypothesis that increases in oxidative metabolism are necessary for NM neuronal survival following removal of afferent input. We used chloramphenicol (CAP), a mitochondrial protein synthesis inhibitor, to block the characteristic increase in CO activity and mitochondrial proliferation following cochlea removal. We then studied the effects of CAP on NM neuronal survival following deafferentation.

When CAP was administered continuously at 600 mg/kg/d for 5 d following cochlea removal, deafferentation-induced neuronal death in NM was significantly increased from 22% to 36%. Higher-dose (1200 mg/kg/d) pulses of CAP were administered for the first 6, 12, or 24 hr following cochlea removal. After 5 d survival, greater increases in neuronal cell death were found in animals treated for the first 12 or 24 hr (65% neuronal death). CAP administration for the first 6 hr had no significant effect on neuronal survival.

The effects of CAP on neuronal cell death in NM are not likely to be due to systemic effects of the drug, but instead to a specific change in mitochondrial function. Our results

suggest that enhanced oxidative enzyme function plays an important role in the survival of NM neurons during the first 24 hr after deafferentation. The nature of the signal(s) eliciting mitochondrial enhancement and the means by which it influences NM survival are not known.

[Key words: nucleus magnocellularis, deafferentation, mitochondria, mitochondrial biogenesis, neuronal cell death, avian]

Removal of afferent input can have profound effects on neurons in the CNS, eventually culminating in death of the postsynaptic cell in some systems (e.g., Kalil, 1980; Trune, 1982; Moore, 1990). The cellular mechanisms involved in neuronal cell death are not known in detail but are the subject of intense interest. Ultrastructural features of dying cells have been examined in an effort to elucidate the cellular mechanisms at work (Trump et al., 1984; Walker et al., 1988; Clarke, 1990). Recent work examining cell death in a number of situations has implicated changes in intracellular calcium levels as being a potential final common mediator of cell death (Schanne et al., 1981; Choi, 1988; Siesjo, 1988; Trump and Berezsky, 1992).

The effects of deafferentation have been particularly well studied in second-order auditory neurons in the chick brainstem (Rubel et al., 1990). Neurons in nucleus magnocellularis (NM) receive their only known excitatory input from the cochlea via the ipsilateral eighth nerve (Boord, 1969; Parks and Rubel, 1978). By performing unilateral cochlea removal or blocking eighth nerve activity, all excitatory input to NM neurons can be removed. Studies using this system have shown rapid changes in protein synthesis (Steward and Rubel, 1985; Rubel et al., 1991), glucose uptake (Lippe et al., 1980; Heil and Scheich, 1986; Born et al., 1991), oxidative enzyme activity (Durham and Rubel, 1985; Hyde and Durham, 1990; Durham et al., 1993), and glial processes (Canady and Rubel, 1992; Rubel and MacDonald, 1992). Two to five days after deafferentation, approximately 25% of NM neurons die, and surviving neurons show decreases in soma size (Born and Rubel, 1985).

Previous studies in our laboratory have shown that NM neurons display a transient increase in oxidative enzyme capacity within hours of cochlea removal. Deafferented NM neurons show increases in histochemical staining for succinate dehydrogenase (SDH) (Durham and Rubel, 1985), cytochrome oxidase (CO) (Hyde and Durham, 1990), and malate dehydrogenase (MDH) (Durham et al., 1993) within hours of cochlea removal. After neuronal death has occurred, surviving NM neurons show reduced levels of these enzymes. Recently we have shown with

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transmission electron microscopy that these initial increases in oxidative capacity are mediated in large part by an increase in mitochondrial volume in NM neurons (Hyde and Durham, 1993).

As described above, only 25% of NM neurons will eventually die as a result of cochlea removal. Neurons destined to die can be identified within hours of cochlea removal by the ultrastructural appearance of their ribosomes (Rubel et al., 1991). In our light microscopic analysis of changes in oxidative enzymes, all deafferented NM neurons showed equivalent increases in SDH or CO activity, suggesting that oxidative enzyme capacity could not be used to differentiate surviving versus dying NM neurons at the light microscopic level. However, in our ultrastructural study, we did observe differences between NM neurons destined to survive or die that suggested they differ in oxidative capacity. All deafferented NM neurons show increases in mitochondrial volume after cochlea removal. However, in neurons showing ribosomal dissociation indicative of cell death, mitochondria are small in size, more lightly stained for CO, and often display swelling and vacuolization associated with mitochondrial dysfunction. These results suggested that dying NM neurons differ from surviving neurons in oxidative functional capacity.

Most mitochondrial proteins are synthesized in the cytoplasm and imported into the mitochondria (Tzagoloff and Myers, 1986; Attardi and Schatz, 1988; Nicholson and Neupert, 1988; Hartl and Neupert, 1990). However, key subunits of several oxidative enzyme complexes, including CO, are coded by mitochondrial DNA and are manufactured *in situ* by mitochondrial protein synthetic machinery (Hoppeler et al., 1981; Nelson, 1987; Ozawa et al., 1987). The activity of these oxidative enzymes can be impaired if synthesis of these subunits is blocked. Chloramphenicol (CAP) specifically inhibits the synthesis of proteins manufactured in mitochondria by binding to the 70S subunit of mitochondrial ribosomes (Lamb et al., 1968; Tzagoloff, 1982; Sande and Mandell, 1990). Although there are several antibiotics that will inhibit mitochondrial protein synthesis, only CAP freely crosses the blood–brain barrier (Sande and Mandell, 1990). Based on our previous ultrastructural study (Hyde and Durham, 1993), we observed that dying NM neurons contain mitochondria that appear to be functionally deficient. These observations suggested that additional inhibition of mitochondrial function might increase the amount of neuronal cell death seen in NM after cochlea removal. To test this hypothesis, we examined animals in which we blocked mitochondrial protein synthesis with CAP and determined the extent of neuronal cell death in NM after cochlea removal. Our results suggest that blockade with CAP does increase cell death in a dose-dependent manner.

Materials and Methods

Animal preparation

Inbred White Leghorn chickens (H&N International, Redmond, WA) were the subjects of these experiments. Ten- to eighteen-day-old chicks were obtained by incubating and hatching eggs in a forced-draft incubator. Prior to and after surgery, the birds were housed in a communal brooder with *ad libitum* water and food.

Thirty minutes prior to cochlea removal, the birds were anesthetized with ketamine (Vetalar, 80 mg/kg, i.m.) and sodium pentobarbital (Nembutal, 18 mg/kg, i.p.). A loading dose of CAP (500–1000 mg/kg) or vehicle (equal volume of sterile water) was given intramuscularly. CAP was subsequently administered either by hourly intramuscular injection or by an Alzet osmotic pump (Alza Corp., Palo Alto, CA). Pumps filled with CAP dissolved in sterile water were implanted subcutaneously on the animal's back. Pumps containing sterile water only were used in vehicle-control animals. Pumps were primed in normal

saline at 37°C for 4 hr prior to implantation. With either method, the dose of CAP varied from 600 to 1200 mg/kg/d.

At the time of pump implantation, the cochlea (basilar papilla) was removed as described previously (Born and Rubel, 1985). Briefly, the right tympanic membrane and columella were removed and the cochlea pulled out through the oval window with jeweler's forceps. Cochlea removal severs the peripheral processes of eighth nerve ganglion cells, but leaves their cell bodies intact. The ear cavity was packed with Gelfoam and the wound closed with cyanoacrylate glue. Animals were killed at various times after cochlea removal.

Effects of CAP on NM neurons

Cytochrome oxidase measurements. We initially used a histochemical stain for CO to measure the degree of mitochondrial protein synthesis inhibition by CAP. Three animals underwent unilateral cochlea removal and received hourly injections of CAP at a dose of 600 mg/kg/d. Six hours after cochlea removal, birds were deeply anesthetized with Nembutal and perfused transcardially with a solution containing 4% paraformaldehyde, 1% glutaraldehyde, and 4% sucrose in 0.1 M phosphate buffer (PB) (pH 7.4) for 10 min. Brainstems were processed for CO histochemistry as described previously (Hyde and Durham, 1990). Briefly, 25 μ m coronal sections were cut on a cryostat and collected in PB for CO histochemical staining. Sections were incubated at 37°C in a CO staining solution that consisted of (per 100 ml) 50 mg diaminobenzidine; 20 mg cytochrome C, type III (Sigma Chemical, St. Louis, MO); and 4 gm sucrose in 0.1 M PB at pH 7.4. After a 60–90 min incubation, the sections were washed in four changes of PB to stop the reaction and then were mounted on chrome-alum–subbed slides. The slides were then dried on a 37° hot plate for at least 30 min before being dehydrated in graded alcohols and coverslipped with DPX.

Changes in the optical density of CO in individual neurons were quantified as previously described (Hyde and Durham, 1990). Briefly, a computer-assisted video image analysis system (Bioquant System IV, R&M Biometrics) was used to measure the optical density of CO reaction product in individual NM neurons. Fifty NM neurons were sampled medially to laterally on each side of the brain in a single tissue section located at the midpoint of the rostral to caudal extent of NM. Only those neurons in which a nucleus was visible were selected for measurement. The boundary of each NM neuron was outlined under Nomarski optics and the optical density of the bright-field image averaged over this area. The area of the nonstaining nucleus in each NM neuron was excluded from the optical density calculation. The average optical density of NM neurons ipsilateral to cochlea removal was compared to the average on the contralateral side of the brain for each animal using an unpaired *t* test.

Electron microscopic analysis. In addition to CO histochemical measurements, we also examined the effects of CAP treatment on mitochondrial ultrastructure and number in NM neurons using transmission electron microscopy. Under pentobarbital/ketamine anesthesia, two birds received a loading dose of CAP (1000 mg/kg), and a pump that delivered 1200 mg/kg/d of CAP was implanted. One cochlea then was removed and the animals were allowed to survive for 6 hr. At the time of death, birds were deeply anesthetized with Nembutal and perfused transcardially with a solution containing 4% paraformaldehyde, 1% glutaraldehyde, and 3% sucrose in 0.1 M phosphate buffer (pH 7.4) for 10 min. The birds were then decapitated and the posterior cranium was removed, exposing the cerebellum and brainstem. The heads were immersed in room temperature fixative (25°C) for 1 hr, after which the brains were removed, blocked, and washed in three changes of phosphate buffer for at least 15 min per change.

Brainstems were sectioned in the coronal plane at a thickness of 80 μ m using a vibratome. Sections at approximately 30% and 50% of the caudal to rostral extent of NM were osmicated and stained en bloc with saturated aqueous uranyl acetate before embedding in PolyBed 812 resin using standard protocols. Plastic-embedded tissue was sectioned on an ultramicrotome using a diamond knife. Thin sections (70–90 nm) were taken, floated on distilled water, and picked up on Formvar-coated slot copper grids in random orientation. Grids were stained with 0.3% lead citrate in 0.1% NaOH prior to viewing on a JEOL transmission electron microscope at 60 kV.

Thin sections were systematically photographed at 3600 $\times$ magnification by beginning at the medial edge of NM and photographing every recognizable neuronal somata profile, progressing laterally until a minimum of 30 neurons were photographed for each NM nucleus. From 8 $\times$ 10 photographic prints of these electron micrographs, at a final

magnification of 8800 $\times$, qualitative observations were made on mitochondria in NM neurons.

Neuronal survival following CAP treatment

Two groups of animals were prepared to examine the effects of CAP treatment on neuronal survival in NM following cochlea removal. In the first group, animals received a loading dose of 500 mg/kg of CAP, followed by implantation of a pump designed to deliver 500–600 mg/kg/d of CAP for 5 d ($n = 6$). Animals receiving an injection of sterile water and pumps containing water served as vehicle controls ($n = 3$). All birds underwent unilateral cochlea removal and were killed 5 d later. In the second group, the effects of varying lengths of mitochondrial protein synthesis inhibition were studied. Birds were treated with CAP (1200 mg/kg/d) for 6, 12, or 24 hr following cochlea removal ($n = 3$ per group). Animals treated with sterile water for similar periods served as controls ($n = 3$). Following the treatment period, the osmotic pumps were removed under halothane anesthesia and the animals were killed 5 d following cochlea removal.

At the time of death, all birds were deeply anesthetized with Nembutal and perfused transcardially with 10% buffered formalin for 10 min. The birds then were decapitated and the posterior cranium removed, exposing the cerebellum and brainstem. The heads were placed in chilled (4°C) perfusion solution overnight. The brains then were removed and the brainstems blocked. The brainstems were washed with running tap water for at least 8 hr and stored overnight in 70% ethanol.

The brainstems were embedded in paraffin and sectioned at 10 μ m using a paraffin microtome. A one-in-four series of sections through NM was mounted on chrome-alum-subbed slides. Sections were deparaffinized in xylenes, rehydrated through serial alcohols, stained with thionin, dehydrated in serial alcohols and xylene, and coverslipped with DPX.

An estimate of the total number of NM neurons on each side of the brainstem was made using previously described methods (Born and Rubel, 1985). Briefly, NM neurons in which a nucleolus was visible were counted in the thionin-stained sections. The number of neurons counted on each side was then multiplied by 4 to estimate the total number of NM neurons in each nucleus. No correction factor was used as the size of the nucleolus is small relative to the section thickness (Konigsmark, 1970). The estimated totals for the ipsilateral and contralateral nuclei of each treatment group were averaged and compared using a one-way ANOVA. Significant differences between groups were detected by post hoc pairwise comparison with the Fisher protected least significant difference (PLSD) test.

Results

Short-term effects of CAP

CAP was administered to the animals in this study to inhibit mitochondrial protein synthesis in NM neurons. However, since the drug was given systemically, we examined our animals for signs of more global effects of CAP administration. Outwardly, animals receiving CAP showed no obvious ill effects. They ate, drank, and gained weight exactly like vehicle-control animals and showed no other signs of ill health. As will be described below, the ultrastructure of neurons in NM contralateral to cochlea removal appeared normal, and no cell death was observed in contralateral NM.

Initially we examined CO activity in individual NM neurons to determine how the doses of CAP we administered affected mitochondrial protein synthesis in NM. CO is located exclusively in mitochondria and one of its subunits is synthesized by the mitochondrial protein synthetic machinery. Since the activity of CO is dependent on the presence of all subunits, CO activity as estimated by histochemical staining should accurately reflect the effects of CAP on mitochondrial protein synthesis.

In animals undergoing cochlea removal alone, histochemical staining for CO increases in NM neurons ipsilateral to cochlea removal within hours of the surgery (Hyde and Durham, 1990). By 6 hr after cochlea removal, a 22% increase is seen. In animals

Figure 1. CO staining in animals receiving low doses of CAP. Optical density (OD) of CO reaction product was measured in individual NM neurons in animals undergoing cochlea removal alone (*CR Only*) and in animals undergoing cochlea removal and receiving 600 mg/kg/d of CAP (*CR + CAP*). *CR Only* animals were prepared as part of another study (Hyde and Durham, 1990). Both groups were killed 6 hr after cochlea removal. Percentage change in CO optical density was obtained by $[(\text{mean OD ipsilateral NM neurons} / \text{mean OD contralateral neurons}) - 1] \times 100$. Error bars are SEM. Without CAP, NM neurons ipsilateral to cochlea removal show a 22% increase in CO activity. Treatment with CAP abolishes the increase.

receiving 600 mg/kg/d of CAP and killed 6 hr after cochlea removal, this increase in CO is abolished. Figure 1 shows the results of optical density measurements from CAP-treated animals and those undergoing cochlea removal alone. A Mann-Whitney *U* test comparing changes in optical density in the two groups showed them to be reliably different ($p < 0.05$). Thus, relatively low doses of CAP prevent the increase in mitochondrial functional capacity seen after cochlea removal, as measured by the activity of an oxidative enzyme.

In addition to mitochondrial function, we also examined mitochondrial morphology in NM neurons from CAP-treated animals. We have shown previously that at 6 hr after cochlea removal, mitochondria proliferate in ipsilateral NM neurons (Hyde and Durham, 1993). Figure 2 shows representative electron micrographs from an animal killed 6 hr after cochlea removal but with no CAP treatment, prepared as part of another study (Hyde and Durham, 1993). The NM neuron from the side of the brain ipsilateral to cochlea removal (Fig. 2*b*) shows a clear increase in the number of mitochondrial profiles compared to the contralateral, control neuron (Fig. 2*a*). Stereological estimates of mitochondrial volume show a 53% increase in ipsilateral NM compared to contralateral NM at 6 hr after cochlea removal (Hyde and Durham, 1993). In addition to greater numbers, mitochondria are also more darkly stained for CO in ipsilateral NM neurons.

CAP treatment greatly reduces the amount of mitochondrial proliferation seen after cochlea removal. Figure 3 shows two neurons from an animal receiving 1200 mg/kg/d CAP and killed 6 hr after cochlea removal. Deafferented NM neurons in CAP-treated animals (Fig. 3*b*) show only a slight increase in the number of mitochondrial profiles when compared to contralateral NM neurons with intact afferent input (Fig. 3*a*). Mitochondria in deafferented neurons are often smaller than normal and tend to be clustered around the nucleus (Fig. 3*b*). Contralateral, control neurons in CAP-treated animals appear normal

Figure 2. Ultrastructural changes in NM neurons after cochlea removal without CAP treatment: transmission electron micrographs from an animal killed 6 hr after unilateral cochlea removal. Deafferented neurons ipsilateral to cochlea removal (*b*) show a striking increase in the number of mitochondria compared to contralateral, control neurons (*a*). Magnification, 4800 $\times$.

Figure 3. Ultrastructural changes in NM neurons after cochlea removal in CAP-treated animal: transmission electron micrographs of NM neurons in an animal receiving 1200 mg/kg/d of CAP and undergoing unilateral cochlea removal. Animal was killed 6 hr after cochlea removal. NM neuron from contralateral side of the brain (*a*) looks normal. Ipsilateral NM neuron (*b*) shows some mitochondrial proliferation, but not as much as that seen without CAP treatment (Fig. 2). Magnification, 6130 $\times$.

Figure 4. Neuronal cell loss with low doses of CAP. NM neuronal number ($\pm$ SEM) is plotted for animals undergoing cochlea removal and receiving 600 mg/kg/d of CAP for 5 d (CR + CAP) and for animals undergoing cochlea removal but receiving only sterile water (CR + Veh). Note 22% neuronal cell loss in vehicle animals and 36% loss in animals receiving CAP. The number of contralateral neurons is equal in the two groups.

in morphology, suggesting that brief CAP treatment does not have any major deleterious effect on cellular function.

The results of our CO and ultrastructural examination of CAP-treated animals suggest that CAP treatment blocks the increase in mitochondrial functional capacity seen in NM after cochlea removal. The entire increase in CO activity normally seen after cochlea removal is blocked by relatively low doses of CAP (600 mg/kg/d). Although some mitochondrial proliferation is seen with twice the dose of CAP, our histochemical results would suggest that mitochondria, although present, may be functionally impaired. Substantially higher doses of CAP (1800 mg/kg/d) are required to block mitochondrial proliferation completely (data not shown).

CAP blockade and neuronal cell death in NM

The main purpose of this study was to test the hypothesis that the increase in mitochondrial capacity seen in NM neurons after cochlea removal is necessary for neuronal survival. If this hypothesis is correct, we would predict that blockade of mitochondrial protein synthesis will result in greater neuronal death after cochlea removal. We first tested this hypothesis in animals receiving a relatively low dose of CAP (average 590 mg/kg/d). CAP was administered continuously from the time of cochlea removal until the animals were killed 5 d later. At this dose, the increase in CO activity ordinarily seen at 6 hr after cochlea removal is abolished (Fig. 1).

The results of NM neuronal cell counts from these animals are shown in Figure 4. The absolute number of NM neurons on both sides of the brain is plotted for vehicle-control animals, which undergo cochlea removal but receive water instead of CAP, and for animals treated with CAP for 5 d beginning at the time of cochlea removal. The number of contralateral, control NM neurons is the same in the two groups, which suggests that CAP is not having a systemic toxic effect. As hypothesized, greater neuronal cell loss is seen in ipsilateral NM in CAP-

treated animals compared to vehicle-control animals (36% vs 22% cell loss). A one-way ANOVA showed that CAP-treated animals have reliably fewer neurons in ipsilateral NM compared to vehicle-control animals [$F(1, 7) = 12.86$; $p < 0.01$]. The number of neurons in NM contralateral to cochlea removal does not differ in the two groups [$F(1, 7) = 0.176$; $p > 0.68$]. Thus, blockade of mitochondrial protein synthesis with CAP results in a modest increase in the amount of deafferentation-induced neuronal cell death in NM, and is apparently not toxic to NM neurons receiving normal afferent input.

The increase in mitochondrial capacity of NM neurons ipsilateral to cochlea removal, characterized by increases in CO and SDH activity and in the number of mitochondrial profiles, occurs within hours of cochlea removal. This increase is transient, and within several days of cochlea removal the activity of these enzymes decreases. We wished to determine whether brief blockade of mitochondrial protein synthesis, just during these early survival times, might be effective in increasing deafferentation-induced neuronal cell death in NM. For these experiments we administered CAP for either the first 6, 12, or 24 hr after cochlea removal. CAP administration then ceased and the animals were killed 5 d later. Because we administered CAP for shorter periods, and thus were at less risk of toxic systemic effects, we increased the dose to 1200 mg/kg/d. Our electron microscopic data show that at this dose, mitochondrial proliferation in deafferented NM neurons is reduced 6 hr after cochlea removal (Fig. 3).

When CAP is administered for at least the first 12 hr after cochlea removal, we observed greatly increased neuronal cell death in NM. Figure 5 shows photomicrographs of NM neurons in all higher-dose treatment groups. Contralateral, control NM neurons (seen in the right column of Fig. 5) appear normal in all groups. In vehicle-treated animals or those receiving CAP for just the first 6 hr after cochlea removal, some neuronal cell death is evident in ipsilateral NM. However, when CAP is administered for the first 12 or 24 hr after cochlea removal, greatly increased neuronal cell death is evident in ipsilateral NM. Most of the neurons that remain in NM in these animals are in the medial portion of the nucleus.

Neuronal cell counts for animals receiving brief higher doses of CAP are shown in Figure 6. As with lower doses of CAP, neuron number in contralateral, control NM is not reliably different among any treatment group, suggesting that higher doses of CAP have no deleterious effect on neuronal survival in neurons with intact afferent input. A one-way ANOVA showed no reliable difference in neuron number in contralateral NM as a function of treatment group [$F(3, 8) = 0.78$; $p > 0.54$]. In ipsilateral NM, however, much greater neuronal cell death is seen when CAP is administered for the first 12 or 24 hr after cochlea removal. A one-way ANOVA showed a reliable effect of treatment group in ipsilateral NM [$F(3, 8) = 5.59$; $p < 0.05$]. Post hoc pairwise comparisons showed that neuron number on the deafferented side in animals receiving CAP for the first 6 hr did not differ from vehicle-control animals (Fisher PLSD, $p > 0.05$). However, animals receiving CAP for either 12 or 24 hr after cochlea removal contain reliably fewer neurons in ipsilateral

Figure 5. NM neurons in animals receiving higher doses of CAP for short periods after unilateral cochlea removal. Control NM neurons contralateral to cochlea removal are shown on the right (B, D, F, H), ipsilateral NM neurons on the left (A, C, E, G). All animals underwent unilateral cochlea removal. Animals received sterile water (A, B) or CAP for the first 6 hr (C, D), 12 hr (E, F), or 24 hr (G, H) after cochlea removal. All animals were killed 5 d after cochlea removal. Note greater loss in ipsilateral NM in animals receiving CAP for 12 or 24 hr. Scale bar in G, 250 μ m.

Figure 6. Neuronal cell counts from animals treated with CAP for short periods after cochlea removal. The number of NM neurons contralateral to cochlea removal is equal in all groups, indicating no toxic effect of CAP on neurons with normal afferent input. With CAP treatment for 12 or 24 hr, greater neuronal loss is seen in ipsilateral NM. Asterisk indicates reliable differences in ipsilateral NM neuronal number compared to vehicle-control animals (Fisher PLSD, $p < 0.05$).

NM than in vehicle-control animals (Fisher PLSD, $p < 0.05$). The average amount of neuronal cell death is not reliably different between the 12 or 24 hr treatment groups (Fisher PLSD, $p > 0.05$). Thus, blockade of mitochondrial protein synthesis for just the first 12 or 24 hr after cochlea removal is sufficient to increase greatly the amount of neuronal cell death seen 5 d later.

Discussion

We have provided evidence that blockade of mitochondrial protein synthesis with CAP increases the amount of neuronal cell death seen in chick cochlear nucleus after cochlea removal. Relatively low doses of CAP (600 mg/kg/d) block the rapid increase in CO activity ordinarily seen in NM within hours of cochlea removal. In animals receiving this dose of CAP for 5 d, 36% of NM neurons ipsilateral to cochlea removal will die, compared to 22% with cochlea removal alone. When higher doses of CAP are given, greater neuronal cell death is seen. Relatively brief exposure to 1200 mg/kg/d of CAP during the first 24 hr after cochlea removal causes approximately 65% of deafferented NM neurons to die 5 d later. CAP must be administered for at least the first 12 hr after cochlea removal for increases in neuronal cell death to occur. In the remainder of this report we will first discuss whether the increases in neuronal cell death we observe are due to a specific effect of CAP on NM mitochondrial protein synthesis. Then we will examine the relationship between mitochondrial protein synthesis and neuronal cell death in NM.

Specificity of CAP effects

The purpose of CAP administration in our study was to inhibit mitochondrial protein synthesis in NM neurons and to assess the effects of that inhibition on neuronal cell death due to deafferentation. However, since CAP was administered systemically, the possibility exists that the increased neuronal cell death we observed is due to some nonspecific effects of CAP. Possibilities include a generalized deterioration in the animal's health, a direct neurotoxic effect of CAP, and the occurrence of known side effects of CAP administration.

The general health of an animal can effect neuronal survival following an insult. For example, the renal effects of systemic

gentamicin or the hypothermia that accompanies administration of MK-801 can confound the interpretation of CNS responses to the administration of these agents. Our CAP-treated birds showed no obvious behavioral, feeding, or growth differences from the vehicle-control animals. If some nonspecific toxic effect of CAP were responsible for the increased NM neuronal death we observed, then animals made ill by some other drug might also be expected to show more cell death. In experiments designed to test whether inhibition of cytoplasmic protein synthesis affects deafferentation-induced neuronal cell death, birds were administered cyclohexamide for brief periods after cochlea removal (Garden et al., 1991). Birds appeared ill when given doses required to inhibit cytoplasmic protein synthesis in NM but did not show any significant increase in NM neuronal death following cochlea removal. Further, in our experiments the animals showing the greatest increase in cell death received CAP for only the first 12 or 24 hr after cochlea removal. In contrast, the normal rate of turnover of mitochondrial proteins is 5–6 d (Luzikov, 1985). Animals receiving lower doses of CAP for 5 d, in which a greater degree of nonspecific interactions might be expected to occur, showed less neuronal cell death than those receiving CAP for brief periods.

To exclude a direct neurotoxic effect of CAP on NM neurons, we compared the total numbers of contralateral NM neurons in CAP-treated animals to those in vehicle-control birds and to previously published results of the number of NM neurons following cochlea removal alone (Born and Rubel, 1985). There were no significant differences in the number of contralateral NM neurons in CAP-treated animals compared to vehicle-control animals (Fig. 6). The average number of contralateral NM neurons in CAP- and vehicle-treated animals was less than 1 SD from the average number of contralateral NM neurons found by Born and Rubel (1985) following cochlea removal alone. In addition, electron microscopic examination of contralateral NM neurons in animals receiving high doses of CAP (1800 mg/kg/d) shows no obvious signs of altered morphology. Thus, CAP does not appear to have a direct neurotoxic effect on NM neurons.

Finally, the most common known side effect of CAP administration is bone marrow suppression (Scott et al., 1965; Ward, 1966), particularly in animals with liver or renal abnormalities. Significant decreases in blood cell counts are usually not found until after 5–7 d of treatment. Since our animals were killed prior to this period, it is doubtful that bone marrow suppression played a role in our results.

In summary, since we have no evidence of any deleterious systemic or direct neurotoxic effects of CAP administration, we attribute the increased neuronal cell death in NM to a specific effect of CAP on inhibition of mitochondrial protein synthesis after deafferentation.

Relationship between mitochondrial proliferation and neuronal cell death

Our principal finding is that blockade of mitochondrial protein synthesis during at least the first 12 hr after deafferentation greatly increases neuronal cell death in auditory neurons. This result and the fact that deafferented NM neurons undergo a burst of mitochondrial proliferation in response to cochlea removal suggest that ordinarily mitochondrial protein synthesis is involved in the survival of NM neurons after deafferentation. One possible mechanism by which this could occur is by export into the cytoplasm of a protein synthesized in mitochondria that in

some way enhances neuronal survival after deafferentation. Blockade of mitochondrial protein synthesis would block production of this protein and thereby enhance neuronal cell death. However, proteins manufactured in mitochondria are thought to be exported only under rare circumstances (Nelson, 1987; Attardi and Schatz, 1988; Nicholson and Neupert, 1988; Hartl and Neupert, 1990).

The more likely explanation for our results is that CAP administration inhibits some increase in mitochondrial oxidative function that is necessary for neuronal survival after deafferentation. As discussed in our report describing increases in mitochondrial volume in NM after deafferentation (Hyde and Durham, 1993), we know of no other example of deafferentation-induced proliferation of mitochondria. The specific cellular signals that elicit production of mitochondria are not known, but are commonly assumed to be in response to an increased energy requirement (Salmons, 1980; Hoppeler et al., 1981; Salmons and Henriksson, 1981; Eisenberg, 1983; Saltin and Gollnick, 1983). Restoration of some ionic imbalance resulting from deafferentation might require additional energy and stimulate production of mitochondria. A large body of evidence suggests, for example, that increases in intracellular calcium concentrations are a common feature in cell death resulting from a variety of causes (Wroegemann and Pena, 1976; Trump et al., 1980; Farber et al., 1981; Jennings and Reimer, 1981; Schanne et al., 1981; Cheung et al., 1986; Bondy and Komulainen, 1988; Choi, 1988; Siesjo, 1988; Siesjo and Bengtsson, 1989; Pazdernik et al., 1992). In addition, it has been suggested that mitochondria might play a more direct role in neuronal calcium homeostasis by taking up calcium (e.g., Miller, 1991), although most likely only under pathological conditions (McCormack et al., 1990). Alternatively, in the programmed cell death observed in NGF-dependent neurons, a sustained increase in levels of intracellular calcium suppresses cell death (Franklin and Johnson, 1992; Johnson et al., 1992). The increase in oxidative function in NM at early survival times after cochlea removal is also puzzling in light of decreases in glucose use (Lippe et al., 1980; Heil and Scheich, 1986; Born et al., 1991) and blood flow (Richardson and Durham, 1990) at similar survival times. Further analysis of the response of other enzymes or metabolites involved in energy metabolism at early times after cochlea removal will help to determine the role of increases in oxidative metabolism in NM.

Mitochondria proliferate very rapidly in NM after deafferentation, much more quickly than proliferation in muscle due to nerve stimulation or administration of thyroid hormone (Barker and Klitgaard, 1952; Winder et al., 1974; Jakovic et al., 1978; Salmons et al., 1978; Eisenberg and Salmons, 1981; Saltin and Gollnick, 1983). The rapidity with which mitochondrial changes occur and the observation that blockade of mitochondrial protein synthesis for just the first 12 hr after deafferentation enhances neuronal death suggest that mitochondrial changes are one of the primary events occurring during deafferentation-induced neuronal death. However, it is equally likely that some cellular signal not directly involved in mitochondrial proliferation occurs first and that it acts to enhance mitochondrial capacity in NM neurons. For example, Bcl-2, a protein shown to prevent programmed cell death in some tissues (Nunez et al., 1990; Vaux et al., 1992), is located in the mitochondrial inner membrane and is thought to exert its effects by altering mitochondrial function (Hockenbery et al., 1990). Similarly, the primary cellular target of thyroid hormone is the nucleus, and

effects on mitochondrial capacity are secondary (Nelson, 1987, 1990; Oppenheimer et al., 1987).

One final aspect of our data deserves comment. In animals receiving short-term CAP treatment, blockade of mitochondrial protein synthesis for at least the first 12 hr was necessary to enhance neuronal death. These results imply that some specific event may occur between 6 and 24 hr that requires mitochondrial protein synthesis to enhance neuronal survival. Alternatively, these data could be interpreted to suggest that blockade of a certain percentage of the mitochondrial proliferation that ordinarily occurs is necessary to increase neuronal death. In either event, further analysis of the effects of changing the timing of mitochondrial protein synthesis blockade will help to resolve this issue.

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