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Altered malate dehydrogenase activity in nucleus magnocellularis of the chicken following cochlea removal

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The metabolism of second order auditory neurons in nucleus magnocellularis (NM) in the chick brainstem can be profoundly altered when excitatory input from the cochlea is removed. Within hours of cochlea removal, NM neurons show an increase in histochemical staining for the Kreb's cycle enzyme succinate dehydrogenase (SDH), followed in several days by decreases in SDH staining. We examined the activity of another Kreb's cycle enzyme, malate dehydrogenase (MDH) using a histochemical stain and a sensitive quantitative biochemical assay for comparison. We found changes in MDH staining similar in time course and magnitude to those of SDH; within 4 h of cochlea removal, MDH activity increases in ipsilateral NM neurons. By 9 days after cochlea removal MDH activity decreases, although not to the same degree as seen with SDH. Biochemical measurements of MDH activity also showed an early increase in activity in ipsilateral NM, followed at 9 days survival by a decrease in activity. Biochemical measurements of the activity of other enzymes in NM may be useful in further defining the metabolic consequences of deafferentation.

Deafferentation; Oxidative metabolism; Auditory; Cochlear nucleus

Introduction

Alterations in afferent input have profound effects on neurons in the central nervous system. To understand the cellular mechanisms involved in the response of neurons to afferent manipulation, an analysis of cellular processes such as metabolism and protein synthesis are crucial. The central nervous system presents a special challenge for biochemical analysis. Unlike other organs made up of many similar repeating subunits, the brain is composed of discrete regions, each subserving a different specific function. The ultimate biochemical analysis would involve examination of compartments within individual neurons.

Histochemical stains have long been employed to examine biochemical properties of neurons on a cellular or even subcellular level (e.g., Pearse, 1980). Histochemical stains for cytochrome oxidase and succinate dehydrogenase activity have been used to delineate central nervous system biochemical organization and the response to functional changes (Kageyama and Wong-Riley, 1986; Land, 1987; Yip et al., 1987; Hyde and Durham, 1990). Such stains commonly involve deposition of a reaction product in tissue sections in a manner which is proportional to the amount of the substance of interest and in the location in which the substance occurs in the tissue. When the substance is the product of an enzymatic reaction occurring in the tissue, the deposition of reaction product in a given period of time can be used to estimate enzyme activity.

Histochemical stains provide good spatial resolution, but have some inherent problems for quantitative analysis as compared to direct biochemical assays. First, it is extremely difficult to create 'standards' to test the range or linearity of the reaction product produced with respect to tissue enzyme activity. Second, most histochemical reactions involve several additional steps between the enzymatic reaction in question and the deposition of reaction product. Alterations in any one of these reactions may be reflected in the observed deposition of reaction product. Finally, quantitative analysis of the concentration or density of reaction product in tissue sections is difficult, requiring sophisticated densitometry.

In vitro biochemical assays, in which the amount of a given substance or enzyme activity is determined in a non-homogeneous sample, offer solutions to some of the problems outlined above. Greater control over assay conditions and the ability to run multiple control reactions can produce quantitative results. A potential drawback of biochemical assays is their relatively low sensitivity, requiring larger samples which may involve overlapping functional regions of the nervous system. The inability to obtain more discrete samples from the nervous system can also hamper use of more sensitive

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direct assays. The microchemical analytical techniques of Lowry and colleagues (Lowry and Passonneau, 1972) offer solutions to some of these problems, particularly for measurement of enzyme capacities and metabolic intermediates. Sensitive assays coupled with exact tissue dissection methods can allow determination of the metabolic parameters of individual neurons (Kato and Lowry, 1973; Godfrey and Matschinsky, 1983).

Brainstem auditory nuclei in the chicken have been a useful system in which to examine the effects of afferent manipulation on CNS neurons. Second-order neurons in nucleus magnocellularis (NM) receive their only known excitatory input from the cochlea via the eighth nerve (Boord, 1969; Parks and Rubel, 1978). Cochlea removal in hatchling chickens results in rapid changes in a variety of cellular and subcellular parameters (Born and Rubel, 1985; Steward and Rubel, 1985; Rubel et al., 1991; Hyde and Durham, 1993b; Rubel et al., 1990), glucose metabolism (Lippe et al., 1980; Heil and Scheich, 1986; Born et al., 1991), blood flow (Richardson and Durham, 1990), and glial elements (Rubel and MacDonald, 1992). More recent studies suggest that changes in afferent activity underlie many of the postsynaptic changes which have been observed in NM (Robb et al., 1988; Born and Rubel, 1988; Canady and Rubel, 1992; Hyson and Rubel, 1989).

The purpose of the present study was to confirm previous results with a histochemical stain for enzyme activity, using a more quantitative direct biochemical assay for a similar enzyme in the chick auditory system. Using a histochemical stain, we have described previously a biphasic change in the activity of the Kreb's cycle enzyme succinate dehydrogenase in the chick brainstem auditory neurons following cochlea removal (Durham and Rubel, 1985). We wished to measure directly enzyme activity in the same system, and following the same manipulation, employing a quantitative microchemical method (Lowry and Passonneau, 1972) to measure enzyme activity quantitatively. Since no sufficiently sensitive quantitative assay is available for SDH, we measured the activity of another Kreb's cycle enzyme, malate dehydrogenase (MDH) using both staining and direct biochemical assays. Some of these data have been presented previously in preliminary form (Durham et al., 1985).

Materials and methods

Animals and surgery

A total of 28 hatchling chickens (H and N strain, Truslow Farms Hatchery) served as subjects for this study. Animals were raised from eggs in a forced draft incubator and housed in communal brooders with free access to food and water after hatching. At 9 to 12 days of age, animals were anesthetized with ketamine (Vetalar, 80 mg/kg, i.m.) and sodium pentobarbital (Nembutal, 18 mg/kg, i.p.) and one basilar papilla (cochlea) was removed through the oval window with forceps as described in detail elsewhere (Born and Rubel, 1985). This manipulation severs the peripheral processes of auditory ganglion cells but does not directly damage their central processes and somata. Animals survived for 4 h to 16 days and then were deeply anesthetized and decapitated. Brains were removed immediately, blocked, and frozen in heptane cooled to −65°C with dry ice or liquid nitrogen. Not more than 3 min elapsed between decapitation and freezing of the brain. Unoperated animals whose brains were removed and frozen in the same manner as those undergoing cochlea removal served as controls.

Slide histochemical assays

Brains from 12 animals were processed solely for histochemical staining. Serial 12 μm coronal sections through brainstem auditory nuclei were cut on a cryostat at −22°C. Two alternate series of sections were thaw-mounted on gelatin-chrome alum subbed slides and stored on a 37°C hotplate. One series was stained with thionin and the second series was stained to demonstrate MDH enzyme activity. The sections stained for MDH activity were processed within 4 h of cutting to minimize loss of enzyme activity. No appreciable loss was observed when staining was accomplished within 4 h. Sections were stained on a 37°C hotplate. An incubation solution consisting of 100 mM malate, 5.4 mM KCN, 1.2 mM β-nicotinamide adenine dinucleotide (NAD^+), and 0.55 mM nitro blue tetrazolium in 100 mM phosphate buffer, pH 7.6, was puddled onto the slides. After incubating the sections for 30 min the stain solution was poured off and the slides stored at least overnight in 100 mM phosphate-buffered 10% formalin (pH 7.6). Sections were dehydrated through graded alcohols, xylenes and coverslipped with DPX. Non-specific staining was assessed by incubating sections in staining solution without malate; no staining was observed. In some animals the effects of fixation on staining pattern and intensity were investigated. For these tests, alternate series of cryostat sections were prepared. One set was stained as described above. The other was stained after sections were postfixed for 12 min in 100 mM phosphate-buffered 10% formalin (pH 7.6) and washed three times in 100 mM phosphate-buffered saline (pH 7.6) prior to staining for MDH.

Test tube histochemical assays

For the 16 brains from which samples were to be taken for quantitative histochemical analysis, 20 μm

coronal sections were cut on a cryostat at −22°C. A one-in-five series of sections was thaw-mounted on subbed slides and stained for MDH as described above or for other enzymes as part of other studies. Remaining sections in the series were kept in order and remained in aluminum tissue racks in the cryostat. Tissue racks filled with frozen sections were transferred to vacuum tubes and then were dried under vacuum overnight at −40°C. Frozen-dried tissue sections were stored under vacuum at −20°C until used for analysis.

Samples to be analyzed biochemically for MDH activity were dissected free-hand from frozen dried tissue sections (Fig. 1). Dissections were carried out in

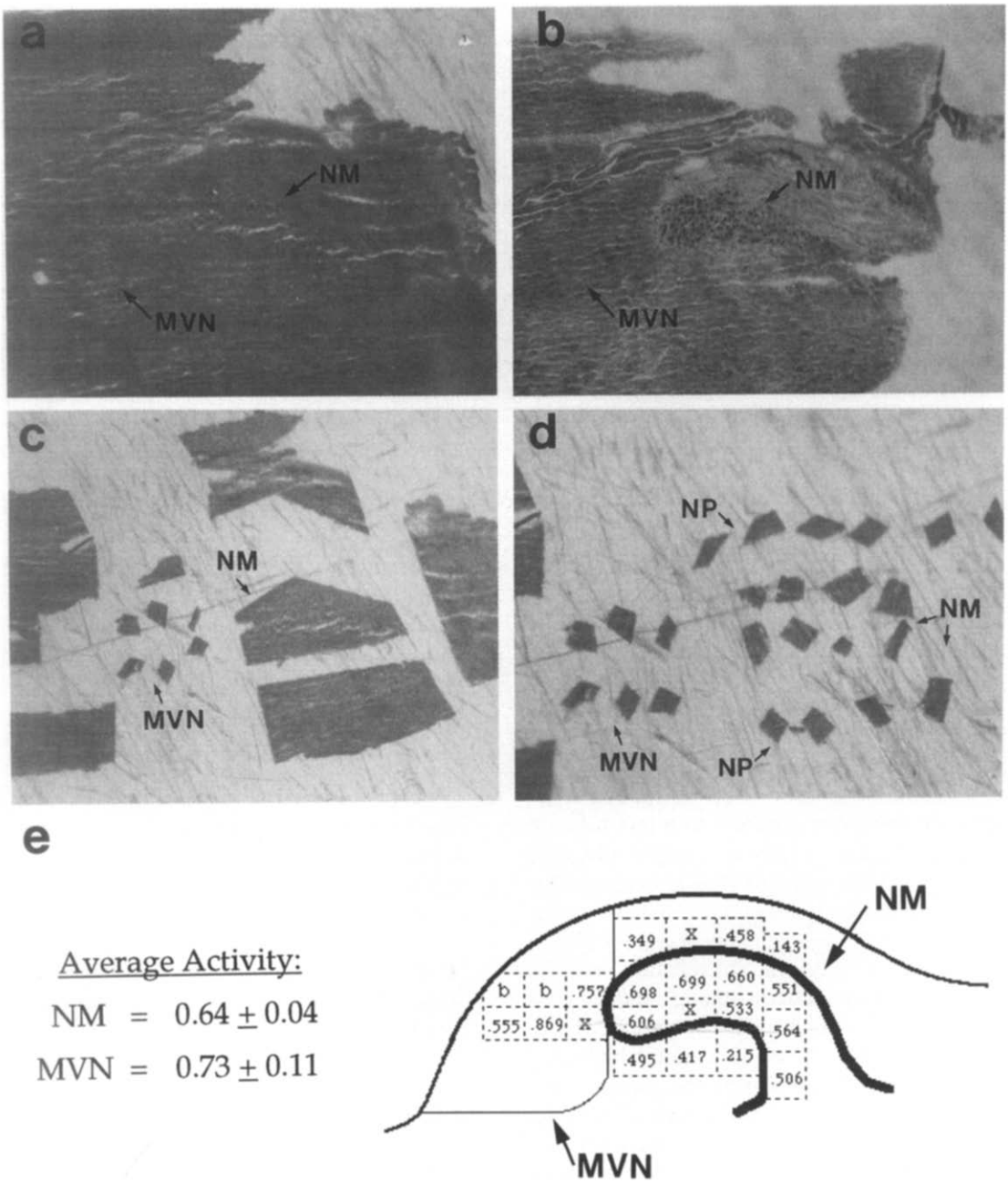

Fig. 1. Photomicrographs of coronal sections through the chick brainstem auditory nuclei to show stages in typical dissection for biochemical measurements. In unfixed, 20 μm frozen dried tissue section (a), borders of n. magnocellularis (NM) and medial vestibular nucleus (MVN) can be visualized by comparison to adjacent section stained for MDH (b). In (c), samples from MVN have been dissected and NM dissected as a whole from surrounding tissue. In (d), samples from NM and adjacent neuropil (NP) have been dissected. The location of all samples is recorded on a rough map of the section such as that shown in (e). The maps are drawn free-hand. Heavy line denotes border of NM, broken lines show borders of individual samples. Values for MDH activity (mol/kg dry weight/min) obtained in the biochemical assay are shown for each sample on the map in (e). Magnification (a) (b) ×25, (c) ×30, (d) ×35.

an environmentally-controlled room (20°C and 50% relative humidity) using hand-made dissection tools (Lowry and Passonneau, 1972). Sections were viewed with a Zeiss dissecting microscope at 50 × magnification for dissection. Using nearby stained sections from the same brain for orientation, the outlines of all relevant nuclei could be visualized clearly in the unstained frozen dried tissue sections under brightfield illumination. For each animal, samples from 2 or 3 sections located at approximately 60% of the anterior-to-posterior extent of NM were dissected for analysis. Each sample contained approximately 10 to 15 NM neurons and surrounding neuropil. Samples were dissected from NM and from surrounding tissue on both sides of the brain. Samples also were dissected from the nearby medial vestibular nucleus, which is unaffected by cochlea removal (see Results and Durham and Rubel, 1985) and served as a 'control' nucleus (Figs. 1c,d). The location of each sample was noted on a drawing of the section (Fig. 1e). Each sample was weighed on a quartz fiber balance (Lowry and Passonneau, 1972) and loaded into one well of an oil well rack (Matschinsky et al., 1968). The average weight of individual NM samples was 75 ng. Sections were stored in oil well racks overnight at −20°C under vacuum until assayed.

Sections were assayed for MDH activity using a modification of the protocol described by Hintz and colleagues (Hintz et al., 1980). Activity was measured in the direction of oxalacetate formation; end product inhibition was prevented by the addition of glutamate and glutamate oxalacetate transaminase (GOT) to the incubation solution. Samples in oil well racks were suspended in 5 μl of preincubation solution (50 mM 2-amino-2-methyl-1,3-propanediol-HCl [AMP], 0.6 M KCl, and 0.05% bovine serum albumin [BSA], pH 9.1) and quickly covered with oil. After 30 min, a 3 μl aliquot from each sample was added to a test tube on ice containing 100 μl of reaction reagent (50 mM AMP, pH 9.1, 10 mM malate, 10 mM glutamate, 300 μM NAD^+, 0.02% BSA, and 5.6 μg/ml GOT). Addition of all samples to the reagent was accomplished in less than 15 min. Because the rate of reaction on ice is very slow, errors in MDH activity among samples contributed by the order in which samples were added to the reagent are negligible. After all samples had been added, the test tube rack containing the samples was transferred to a 37°C water bath for exactly 45 min. At the end of the incubation period, the reaction was stopped by immersing the test tubes in a boiling water bath for 2 min. After the test tubes had cooled for 10 min on ice, 1 ml of 50 mM carbonate buffer (pH 10.0) was added to each tube and the fluorescence read in a Farrand ratio fluorometer. The fluorometer was calibrated with a standardized solution of NADH. For each sample, the enzyme activity was divided by the weight of the sample to express the enzyme activity in terms of mol/kg dry weight/min. Blanks consisting of tissue samples incubated in a reagent without malate were run with each assay. The assay was linear with respect to tissue weight and incubation time for the conditions used.

Data analysis

Slide assays

Sections stained to demonstrate MDH activity were examined under brightfield illumination for differences in the density of reaction product among NM neurons or medial vestibular nucleus. Differences in staining intensity were quantified using a variable aperture microdensitometer (Leitz MVP-DADS) as described previously (Durham and Rubel, 1985). Briefly, the optical density of reaction product in the cytoplasm of individual NM neurons on the two sides of a single tissue section was measured and values for NM neurons on each side were averaged. In addition, 3 measurements of optical density in the medial vestibular nucleus were made on each side of the brain and averaged. Within MVN, individual neurons are not distinguishable in stained sections, so measurements were made over areas of the neuropil. For NM measurements, the ratio of the average optical density on the ipsilateral, deafferented to that on the contralateral, control side of the brain was calculated. Similar ipsilateral to contralateral ratios were calculated for measurements in medial vestibular nucleus for each animal. Ratios were averaged among animals in a group. Average ratios for each group were compared using both parametric and non-parametric statistical analyses.

Test-tube assays

Values for enzyme activity in individual tissue samples from NM on each side of the brain in a given animal were averaged. A ratio of average MDH activity in NM ipsilateral to that in NM contralateral to cochlea removal was calculated for each animal. Average ratios were calculated for each survival group. Both absolute MDH activity and average ratios were compared statistically among animals using both parametric and non-parametric analyses.

Results

The two methods we employed to estimate MDH activity in brainstem auditory nuclei provide complementary information. The first, staining histochemistry, has good spatial resolution but can only provide information about relative amounts of activity between neurons on the two sides of the brain. Test-tube assays provide quantitative estimates of enzyme activity but

have less precise spatial resolution. We will present first the results of staining histochemistry followed by those for test tube assays. Both methods gave similar results regarding changes in MDH activity following cochlea removal.

Slide histochemistry

The pattern of staining for MDH is consistent with its location both in mitochondria and cytoplasm. As seen in Fig. 2, the cytoplasm of both neuronal and glial cell bodies in NM contain MDH reaction product. The pattern of staining appeared qualitatively similar in fixed and unfixed tissue, suggesting that no significant movement of reaction product is occurring in unfixed tissue. A biphasic change in staining intensity occurs in NM after cochlea removal. At early survival times (Figs. 2a,b) neurons on the side of the brain ipsilateral to cochlea removal appear more darkly stained than neurons on the contralateral side. With longer survival, NM neurons ipsilateral to cochlea removal are more lightly stained (Figs. 2c,d). Neuronal loss is also apparent in the longer-surviving animals, consistent with previously published data (Born and Rubel, 1985).

The time course of changes in relative density of MDH reaction product in NM is shown in Fig. 3. An increase in MDH staining is seen as early as 4 h after cochlea removal, while the long-term response is a decrease in density in ipsilateral neurons. Increases in MDH activity appear to occur more rapidly than for SDH (4 h survival), and MDH activity decreases more rapidly than that for SDH (2 days survival). In addition, MDH decreases at long survival times were not as great as those seen previously for SDH.

A between-group Kruskal Wallis analysis of average ipsilateral/contralateral optical density ratios shows that a reliable difference exists as a function of survival time ($H = 20.93$, $P < 0.001$). Posthoc pairwise comparisons (Mann-Whitney U tests) show that percent changes in optical density were significantly different from control animals for all survival times except 9 and 16 days after cochlea removal ($P < 0.05$). Although average percent changes in optical density were not significantly different from control at long survival

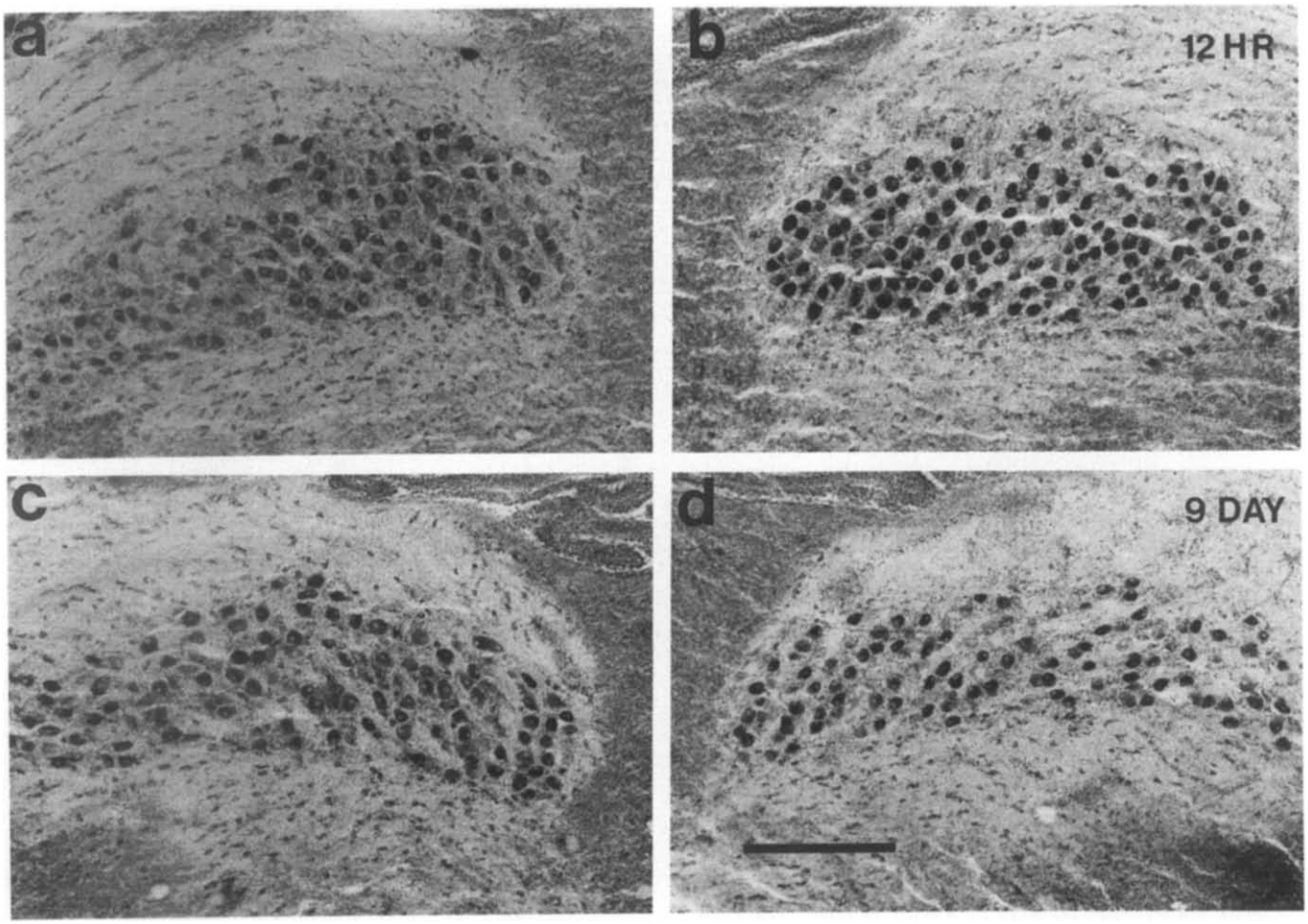

Fig. 2. Photomicrographs of MDH-stained sections of NM from animals sacrificed either 12 h (a,b) or 9 days (c,d) after unilateral cochlea removal. The nuclei shown are from two sides of the same tissue section. Scale bar in d is 200 μm and applies to all photographs.

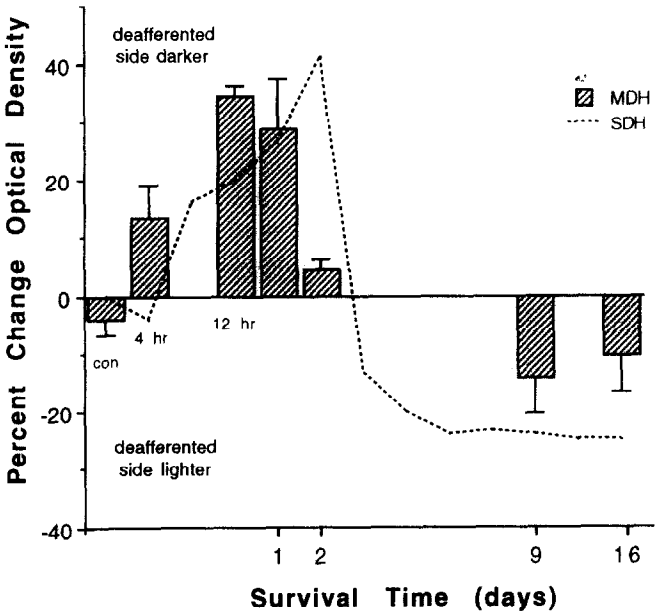

Fig. 3. Time course of change in histochemical staining for MDH after cochlea removal. The average percent change in optical density in NM neuronal cytoplasm, calculated from densitometry measurements, is plotted as a function of survival time. A relative increase in staining is measured at early survival times, followed by a decrease. The time course of previously obtained changes in SDH staining (Durham and Rubel, 1985) is shown also for comparison. Error bars indicate standard error of the mean. Survival times less than 24 h are shown below MDH bars.

times, t-tests in individual animals suggests that differences in MDH staining exist. In these groups, average optical density in ipsilateral NM neurons was significantly lower than that among contralateral NM neurons ($P < 0.05$ in all animals at 9 days survival, 2 of 3 animals at 16 days survival). Optical density ratios for medial vestibular nucleus were not reliably different from one another for any survival time (Kruskal Wallis and Mann-Whitney U tests).

Test tube histochemistry

Table I shows the results of our biochemical measurements of MDH activity. Average absolute values for MDH activity were somewhat variable among animals within a given group (e.g., unoperated NM in animals surviving 9 days after cochlea removal). In histochemically stained sections, only neuronal MDH activity is assessed by the optical density measurements. However, biochemical samples include both neuropil and glial cells within NM. Differences in MDH activity among animals may reflect different densities of neuronal vs non-neuronal elements in samples or actual differences among animals.

Changes in MDH activity following cochlea removal measured biochemically were qualitatively similar to those seen with histochemical stains (Fig. 4). Increases

TABLE I

Average MDH activity measured with a biochemical assay

Animal No.	Survival	Ipsilateral NM	Contralateral NM	Ratio
2701	control	0.606 ± 0.062 (8)	0.660 ± 0.025 (7)	0.92
2702	control	0.687 ± 0.041 (16)	0.775 ± 0.042 (19)	0.89
27P3	control	0.672 ± 0.063 (7)	0.696 ± 0.046 (14)	0.97
2703	4 h	0.627 ± 0.032 (17)	0.676 ± 0.050 (15)	0.93
2704	4 h	0.713 ± 0.055 (13)	0.706 ± 0.051 (13)	1.01
2729	4 h	0.748 ± 0.043 (13)	0.749 ± 0.042 (16)	1.00
2705	12 h	0.769 ± 0.047 (8)	0.660 ± 0.031 (11)	1.17
2706	12 h	0.685 ± 0.039 (14)	0.510 ± 0.017 (13)	1.34
2727	12 h	0.757 ± 0.039 (11)	0.600 ± 0.039 (9)	1.26
2709	9 days	0.370 ± 0.032 (8)	0.565 ± 0.039 (8)	0.66
2708	9 days	0.443 ± 0.029 (14)	0.563 ± 0.029 (19)	0.79
2725	9 days	0.512 ± 0.045 (13)	0.838 ± 0.057 (13)	0.62
2734	9 days	0.443 ± 0.046 (8)	0.673 ± 0.041 (8)	0.66

Averages for MDH activity (mol/kg dry weight/min) for all samples from individual animals are shown ± standard error of the mean. Number of samples averaged is shown in parentheses. Last value in each line is the ratio of MDH activity in NM neurons ipsilateral to cochlea removal to that in neurons contralateral to cochlea removal.

in activity in ipsilateral, deafferented NM were seen at early survival times (12 h) followed by decreases at 9 days. Absolute values for MDH activity were reliably different as a function of survival time in ipsilateral NM [$F(3,9) = 22.05$, $P < 0.0002$] but not in contralateral NM [$F(3,9) = 1.23$, $P > 0.35$] or medial vestibular nucleus [$F(3,9) = 0.07$, $P > 0.97$, ipsilateral side; $F(3,9) = 0.47$, $P > 0.71$, contralateral side]. Ratios of MDH activity (ipsilateral/contralateral NM) also were signif-

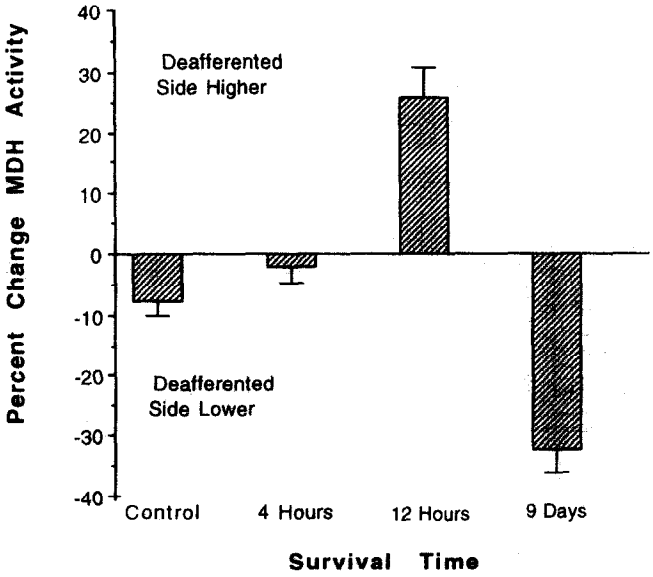

Fig. 4. Average percent change in MDH activity determined from quantitative assays. Activity increases in ipsilateral NM at early survival times and decreases at long survivals. Error bars indicate standard error of the mean.

icantly different as a function of survival time (Kruskal Wallis test, $H = 10.9$, $P < 0.01$). Posthoc pairwise comparisons of absolute MDH activity in operated NM showed only 9 day survival times to be reliably different from control animals (Fisher PLSD, $P < 0.05$). However, when ipsilateral/contralateral ratios of absolute MDH activity are compared, ratios for both 12 h and 9 day survival times are reliably different from control animals (Fisher PLSD, $P < 0.05$).

Discussion

The results of both a histochemical stain and a biochemical assay for MDH demonstrate a biphasic change in the activity of this enzyme in cochlear nucleus following cochlea removal. The time course of the MDH changes differs slightly from that previously described for SDH in that MDH increases more rapidly and returns to baseline levels sooner than SDH. In addition, the long-term decrease in MDH staining is less pronounced than that for SDH. We found quantitatively similar changes in MDH activity measured with a biochemical assay as seen with histochemical staining. We will first discuss several methodological issues relevant to our data, followed by a comparison of these results to other studies of deafferentation.

Methodological considerations

SDH vs MDH

Since both of these enzymes are involved in the Kreb's cycle, we expected to see similar changes in histochemical staining in NM following cochlea removal. Most of the differences between MDH and SDH staining patterns can probably be attributed to their different distributions within the cell. While SDH is found strictly in the mitochondria, MDH is located both in mitochondria and within the cytoplasm as part of the malate/aspartate shuttle. Since glial somata in NM contain relatively few mitochondria, the presence of MDH in both locations may explain why glial cell bodies are stained for MDH and not SDH. In addition, we observed a greater decrease in SDH activity in NM neurons at long survival times than that seen with MDH. In ultrastructural studies of NM neurons after cochlea removal, mitochondrial volume density decreases at long survival times (Hyde and Durham, 1993b), which would most likely result in decreases in staining for mitochondrial enzymes. However, NM neurons show a 25% decrease in cross sectional area at these times (Born and Rubel, 1985), which might be expected to result in an increase in staining density for cytoplasmic enzymes. Biochemical measurements of other enzymes that are strictly mitochondrial in location, such as citrate synthase, might be expected to parallel SDH histochemical staining changes more closely.

Slide vs test tube assays

In general, the results of our histochemical stain for MDH were similar to those of the biochemical assay for enzyme activity. We did observe more variability among animals in biochemical assays than with histochemical staining. As mentioned above, this may be due to the greater sensitivity of the biochemical method to detect differences among animals. Similar variability in absolute values among animals were observed for our measurements of MDH activity in MVN. Differences in the magnitude of the changes we observed with the two methods may also be a result of differences in sample composition. We observed a 35% increase in MDH activity as measured with histochemical stains at 12 h survival, while only a 25% change was measured biochemically. If greater changes in MDH activity were occurring in neuronal cell bodies than in other parts of NM (e.g., neuropil and glial cell bodies), then a greater change would be observed with the histochemical method, since only neurons are sampled. Based on staining intensity, NM neuropil and glia appear to have lower MDH activity than neuronal somata, although biochemical measurements in cat AVCN suggest that absolute levels may be similar (Godfrey and Matschinsky, 1983). Thus, biochemical samples might be expected to provide an underestimate of changes in neuronal enzyme activity. In addition, measurements of the density of neuronal somata within NM in similarly prepared tissue suggest that neurons comprise a smaller percentage of NM at 9 days survival (Durham, unpublished observations). Thus, greater decreases in MDH activity measured biochemically at long survival times may reflect the fact that biochemical samples contain fewer neurons per unit weight.

Comparisons to other studies

In most other systems, decreases in afferent input such as occurs in NM following cochlea removal results in decreases in metabolic enzymes or metabolites (Land and Akhtar, 1987; Yip et al., 1987; Dietrich et al., 1981; Mawe and Gershon, 1986; Hevner and Wong-Riley, 1990). However, these studies have usually examined changes in enzyme activity at relatively long survival times, ranging from 1 to 30 days. Thus, the results of the present study are consistent with what has been reported in other systems. Recently we have shown that NM neurons undergo a rapid proliferation of mitochondria following cochlea removal (Hyde and Durham, 1993b). The magnitude of this morphological increase in mitochondria can easily account for the increase in oxidative enzyme activity seen here and in

previous studies (Hyde and Durham, 1990; Durham and Rubel, 1985). Blockade of mitochondrial protein synthesis by systemic administration of chloramphenicol increases the amount of neuronal cell death observed in NM following cochlea removal (Hyde and Durham, 1993a). These results suggest that enhanced mitochondrial capacity may be involved in neuronal survival following deafferentation. Analysis of other metabolic enzymes and metabolites in NM and the manner in which they change after cochlea removal will be important in elucidating the role played by mitochondria in neuronal survival.

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