Effects of Unilateral Cochlea Removal on Anteroventral Cochlear Nucleus Neurons in Developing Gerbils

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ABSTRACT

Afferent regulation of neurons in the cochlear nucleus as a function of age was investigated at the light microscope level. Unilateral cochlea removal was performed on Mongolian gerbils of three age groups: 1, 8, and 20 weeks postnatal. Animals survived for either 2 days or 2 weeks. An additional group of neonatally operated animals had a prolonged survival of 9 weeks. The number of neurons in anteroventral cochlear nucleus (AVCN) was counted, and cross-sectional area measurements of large spherical cells in AVCN were made.

In animals 1 week old at the time of surgery, there was a 35% reduction in neuron number in AVCN after 2 days, a 58% reduction after 2 weeks, and a 59% reduction 9 weeks after inner ear destruction. However, in animals 20 weeks old at the time of surgery, there was no cell loss in AVCN either 2 days or 2 weeks after surgery. Animals in each age group showed a reduction in cross-sectional area of large spherical cells in AVCN after cochlea ablation. The gerbils that underwent cochlea removal at 8 and 20 weeks showed an average decrease of 14–18%. This effect was seen as early as 2 days after cochlea removal. Animals that underwent cochlea removal at 1 week exhibited the greatest change; a 25% decrease at 2 days progressed to 38% at 2 weeks following cochlea removal. No appreciable further changes were seen at 9 weeks after neonatal cochlea removal.

The results indicate greater susceptibility of 1-week-old gerbil cochlear nucleus neurons to peripheral loss than found in older animals. In all groups the changes in cell size and neuron number occurred considerably sooner after cochlea ablation than previously has been reported. Interestingly, these changes were seen before the onset of hearing and before the onset of measurable cochlear microphonic responses to sound.

Key words: deafferentation, cell size, cell number, afferent influences

Transneuronal regulation of neuronal and glial elements has been of interest for a variety of reasons. Many clinical situations arise in which there is interruption of the connections between peripheral and central elements or compromise of central structures. In addition, there are numerous examples indicating that normal ontogeny and maintenance of neural structure and function depend heavily on the tissue interactions between afferents and target tissues (Cowan, '70; Globus, '75; Jacobson, '78; Purves and Lichtman, '85). Finally, as an adjunct to understanding the cellular principles underlying experiential regulation of neural function, the elimination of afferents provides an extreme manipulation which can be profitably used to suggest the cellular processes, signals, or developmental restrictions underlying more subtle forms of neural plasticity.

Changes in the structure, metabolism, or development of neurons or neural networks following destruction of peripheral end organs have been investigated in most sensory systems in both vertebrate and invertebrate preparations (e.g., Levi-Montalcini, '49; Powell and Erulkar, '62; Matthews and Powell, '62; Guillery, '73; Van der Loos and Woolsey '73; Murphey et al., '75; Kalil, '80; Trune, '82; Nordeen et al., '83). The major conclusions of Levi-Montalcini ('49) and Cowan ('70) still appear valid. First, the severity of trans-

neuronal changes is proportional to the percent of afferents destroyed. Second, the severity of change is inversely related to the age of the animal (after the time of normal synapse formation).

In marked contrast to progress toward understanding the cellular events underlying nerve-muscle "trophic" interactions (Purves and Lichtman, '85), relatively little recent progress has been forthcoming regarding either the cellular events or the signals underlying afferent regulation of postsynaptic neurons. Recent experiments using the brainstem auditory system of the chick have provided several interesting results. First, the transneuronal effects of cochlea removal are remarkably rapid; changes in protein synthesis can be observed within the first hour (Steward and Rubel, '85; Born and Rubel, '88), and changes in cell number and cell size are virtually complete within 2 days (Born and Rubel, '85). Second, the "critical period" when the nervous system is particularly sensitive to afferent manipulations extends well past the time of synaptogenesis and the time during which functional maturity is attained. Finally, in vivo experiments using tetrodotoxin to block eighth nerve activity and in vitro experiments using direct electrical stimulation of the eighth nerve suggest that the afferent signal involves neural activity and/or an activity-dependent release of some molecule (Born and Rubel, '85; Hyson and Rubel, '86).

The purpose of this paper and the one that follows is to test the generality of these findings to a mammalian species. Here, we examine variations in the amount of transneuronal cell loss and neuronal atrophy in the gerbil anteroventral cochlear nucleus (AVCN) following unilateral cochlea destruction as a function of both survival time and age of the animal at the time of the surgery. In the succeeding paper (Pasic and Rubel, '89), we examine rapid changes in cell area following reversible blockade of eighth nerve activity with tetrodotoxin and compare these changes to those following complete destruction of the cochlea.

MATERIALS AND METHODS
Subjects

Adult Mongolian gerbils (*Meriones unguiculatus*) were obtained from Tumblebrook Farms or our own breeding colony derived from this stock. All pups were from our colony. Each litter was culled to a maximum of six pups. The pups were considered 1 day old on the day after birth. All animals had free access to food and water.

Three age groups were used: 1, 8, and 20 weeks of age at the time of initial surgery. At 1 week of age, gerbil pups have an immature auditory system; no measurable cochlear microphonic or sound-evoked auditory brainstem responses have been recorded at this age (Woolf and Ryan, '84; Harris and Dallos, '84; Sanes and Rubel, '87; Smith and Kraus, '87). The 8-week-old animals were prepubertal but otherwise fully developed. By auditory brainstem response (ABR) or single-unit response criteria, their hearing levels are at adult values. By 20 weeks of age, gerbils are sexually mature adults.

Surgery

In 1-week-old animals unilateral cochlea removal was performed under hypothermic anesthesia. A small incision was made caudal to the right ear. Through this opening the tympanic membrane was identified and perforated. The middle ear mesenchyme was partially aspirated until the bony

cochlear wall could be identified. The cochlear wall was penetrated by a needle aspirator, and the cochlear contents were removed by aspiration. The modiolus was then transected and aspirated. The skin incision was reapproximated with cyanoacrylate glue and the animal was returned to its parents and littermates after regaining normal temperature. This operation took approximately 15 minutes.

Eight- and 20-week-old gerbils were anesthetized with ketamine (40 mg/kg; IM) followed by pentobarbital sodium (40 mg/kg; IP). The middle ear bulla was identified and opened. A hand drill was used to enlarge the round window antrum, exposing the round window. A needle aspirator was used to perforate the round window, and the cochlear contents were aspirated. The modiolus was then transected. The stapedial artery, persistent after birth in the gerbil, was left undisturbed. The skin incision was reapproximated with cyanoacrylate glue.

Histology

After a survival period of either 2 days or 2 weeks, five animals from each age group were given an overdose of pentobarbital sodium (120 mg/kg; IP) and transcardially perfused with 10% phosphate-buffered formalin or Heidenhain's solution. An additional group of five animals undergoing cochlea removal at 1 week of age were overdosed and perfused after a 9-week survival period.

Brains perfused with Heidenhain's solution were postfixed for 12 hours in Heidenhain's solution and then transferred to 10% buffered formalin. Brains were removed from the skull after 7 days in fixative, dehydrated in a series of graded ethanols, embedded in paraffin, and sectioned in the coronal plane at 10 μm. A one-in-four series of sections was mounted on glass slides, stained with thionin, dehydrated, and coverslipped with DPX.

Quantitative analyses

Cell counts. Neurons in AVCN were counted in animals operated on at either 1 week or 20 weeks of age. One-week-old animals were allowed to survive 2 days (n = 3), 2 weeks (n = 3), or 9 weeks (n = 3); 20-week-old animals were allowed to survive either 2 days (n = 3) or 2 weeks (n = 3). Every other section (i.e., a one-in-eight series of sections) through the entire anterior-to-posterior extent of AVCN was examined and the total number of neurons in AVCN on each side of the brain was counted. The contralateral AVCN served as an intraanimal control. The criteria for a neuron to be counted were well-defined cytoplasm and nuclear outlines and a clearly visible nucleolus. The AVCN neurons extended through 24–30 counted sections (0.96–1.2 mm) on each side of the brainstem. Four predominant cell types were identified in AVCN and we attempted to count them separately. These were spherical, globular, small spherical, and multipolar cells. Unfortunately, it was not possible to unequivocally separate the cell types, especially on the side ipsilateral to the cochlea removal. Therefore, counts of these cell types were summed and then multiplied by a factor of eight to obtain an estimate of the total number of neurons in AVCN on one side of the brainstem. Neuron counts were also collected from the right and left AVCN of a single nonoperated control animal at each age. No correction factor was used for cell counts since the size of the nucleolus is small compared to the section thickness. In addition, comparisons were made within animals, and there were no indications of a significant change in nucleolar area in the experimental animals.
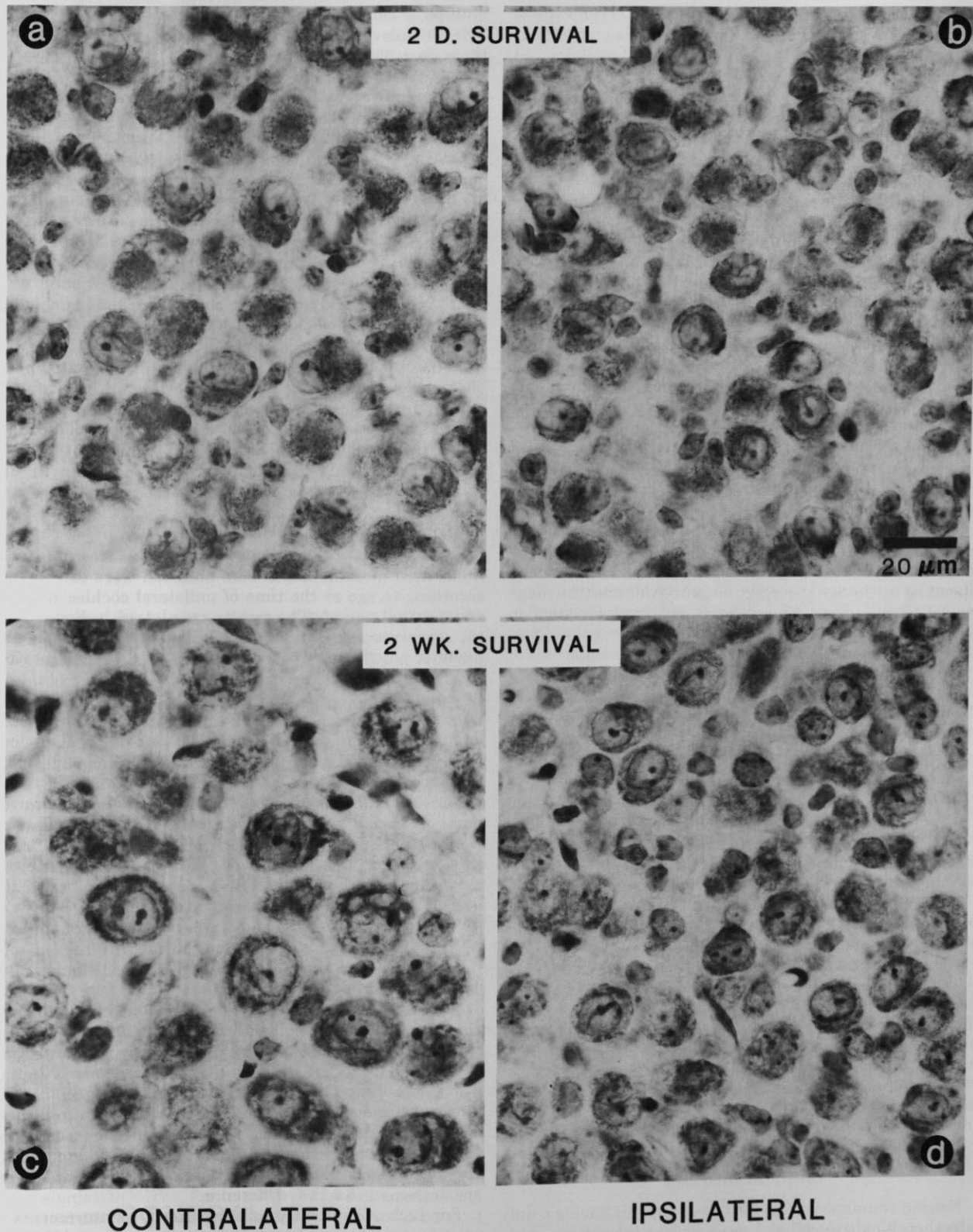
Fig. 1.   Representative photomicrographs of large spherical cell region of AVCN from two subjects oper-
ated on at 1 week of age. **a** and **b**: Contralateral and ipsilateral side of the brain, respectively, 2 days after
unilateral cochlea removal. **c** and **d**: Contralateral and ipsilateral sides of the brain, respectively, 2 weeks
following unilateral cochlea removal.

Cell size. Measurements of neuron soma cross-sectional area were obtained from animals of all seven experimental groups: animals with a unilateral cochlea removal at 1 week of age followed by a 2-day (n = 5) or 2-week (n = 5) survival; animals with unilateral cochlea removal at age 8 weeks with 2 days (n = 5) or 2 weeks (n = 5) survival; animals with cochlea removal at 20 weeks with 2 days (n = 5) or 2 weeks (n = 5) survival; and a long-term survival group consisting of animals with unilateral cochlea removal at 1 week of age and surviving for 9 weeks after surgery (n = 5). In addition, two unoperated animals at each age were examined.

Large spherical cells represent a uniform population of easily identified cell types in rostral AVCN. They correspond to bushy cells seen in Golgi-impregnated tissue (Brawer et al., '74). They are also the predominant cell type in the rostral region of AVCN and receive excitatory afferent input from the cochlea via large end bulbs of Held. For these reasons, they were selected for neuron soma area measurements. For consistency, all soma area measurements were taken at the same relative location in the nucleus. A tissue section was selected at 25% of the anterior-to-posterior extent of AVCN and *every* large spherical cell with an identifiable nucleolus was measured at its largest diameter. One hundred to 130 neurons per side of the brainstem were measured in each animal except in those animals which had extensive neuron loss following cochlea removal. For these animals, all large spherical cells in the section were measured, but the total number of neurons varied widely, depending on the age at surgery and survival time (the range was 36–130). A Zeiss Videoplan morphometry system in conjunction with a ×100 planapochromatic objective (N.A. 1.3) was used for cross-sectional area measurements.

RESULTS

Figures 1–3 show representative photomicrographs of large spherical cells from AVCN of gerbils operated on at 1 week, 8 weeks, and 20 weeks of age, respectively. In all cases the two sides of the same tissue section are shown. At the top of each figure are examples from the control (contralateral) and experimental (ipsilateral) side of an animal surviving 2 days; at the bottom are similar examples from an animal surviving 2 weeks.

When the cochlea was destroyed at 1 week of age and animals survived 2 days there was an apparent reduction in the cross-sectional area of large spherical cells on the ipsilateral side. After 2 weeks the size difference was more striking, and neurons on the experimental side also appeared more densely packed, similar to the density at the time of the operation (Fig. 1c vs. 1d). In an animal operated on at 8 weeks of age (Fig. 2) a qualitatively similar difference in soma area was apparent but no consistent differences in packing density were seen. Finally, when the operation was performed on adult animals the difference between the two sides of the brain at either 2 days or 2 weeks after the surgery was only marginally apparent (Fig. 3).

Neuron number

Neuron counts were collected from animals having a unilateral cochlea removal at 1 week or 20 weeks of age. Animals survived either 2 days or 2 weeks (and for the young age group an extended period of 9 weeks) following surgery. The results are presented in Figure 4. Total cell count refers to the sum of large spherical, globular, small spherical, and multipolar cells.

In animals operated at 1 week of age and surviving 2 days, there were 35% fewer neurons in the ipsilateral AVCN vs. the contralateral AVCN. For animals having a unilateral cochlea removal at the same age, but surviving for 2 weeks, the transneuronal degeneration in the ipsilateral AVCN had progressed to a mean of 58%. With a survival period of 9 weeks there was no further reliable increase in cell loss as compared to that seen at 2 weeks; an average neuronal loss of 59% was observed. While there were only three animals in each experimental group, the cell counts were quite consistent, as shown by the standard errors of the mean plotted in Figure 4. The apparent increase in cell number between 1 week and 3 weeks of age in normal animals is probably artifactual. Histological processing of the youngest animals did not reveal cytological details as well, and thus fewer neurons met our counting criteria.

Unilateral cochlea removal in adult gerbils resulted in no observable change in neuron number in AVCN. Animals operated on at 20 weeks of age and surviving either 2 days or 2 weeks showed no reliable differences between cell counts in the ipsilateral and contralateral AVCN. In addition, control animals at each age showed less than 6% difference in cell number between the two sides of the brain.

Cross-sectional area

The animals from which AVCN neuron soma cross-sectional area measurements were obtained were categorized according to age at the time of unilateral cochlea removal and survival period. The results are plotted in Figure 5 as the mean percent difference between the two sides of the brain. A positive number indicates that the spherical cells were smaller on the experimental than on the control side of the brain. The average difference in cell size was computed by determining the percent difference in cell size for each animal ([mean contralateral cell size − mean ipsilateral cell size/mean contralateral cell size] × 100) and then averaging these scores across the 5 animals in each group.

For neonatal gerbils operated on at 1 week, prior to the onset of hearing, the results are as follows. With a 2-day survival period, there was an average 25% decrease in cross-sectional area of surviving spherical cells on the ipsilateral side of the brain. For the animals surviving 2 weeks, the average difference in cross-sectional area progressed to 39%. This difference remained relatively constant thereafter; animals surviving 9 weeks had a mean difference of 43% between the size of AVCN large spherical cells on the two sides of the brain.

Gerbils operated on at 8 weeks of age continued to show a pattern of statistically significant and rapid change in neuron size. At both 2 days and 2 weeks following a unilateral cochlea removal, an average 17% difference was found. Finally, the adult gerbils, having a unilateral cochlea removal at the age of 20 weeks, showed results similar to those of the 8-week-old animals. Two days following a unilateral cochlea removal there was a reliable 18% reduction in large spherical cell cross-sectional area, and at 2 weeks, this average decreased to a 14% difference.

For each group of animals examined, measurements of cross-sectional area of large spherical cells from AVCN on the contralateral (control) side were not significantly different from measurements obtained from AVCN of non-operated, age-matched control animals.
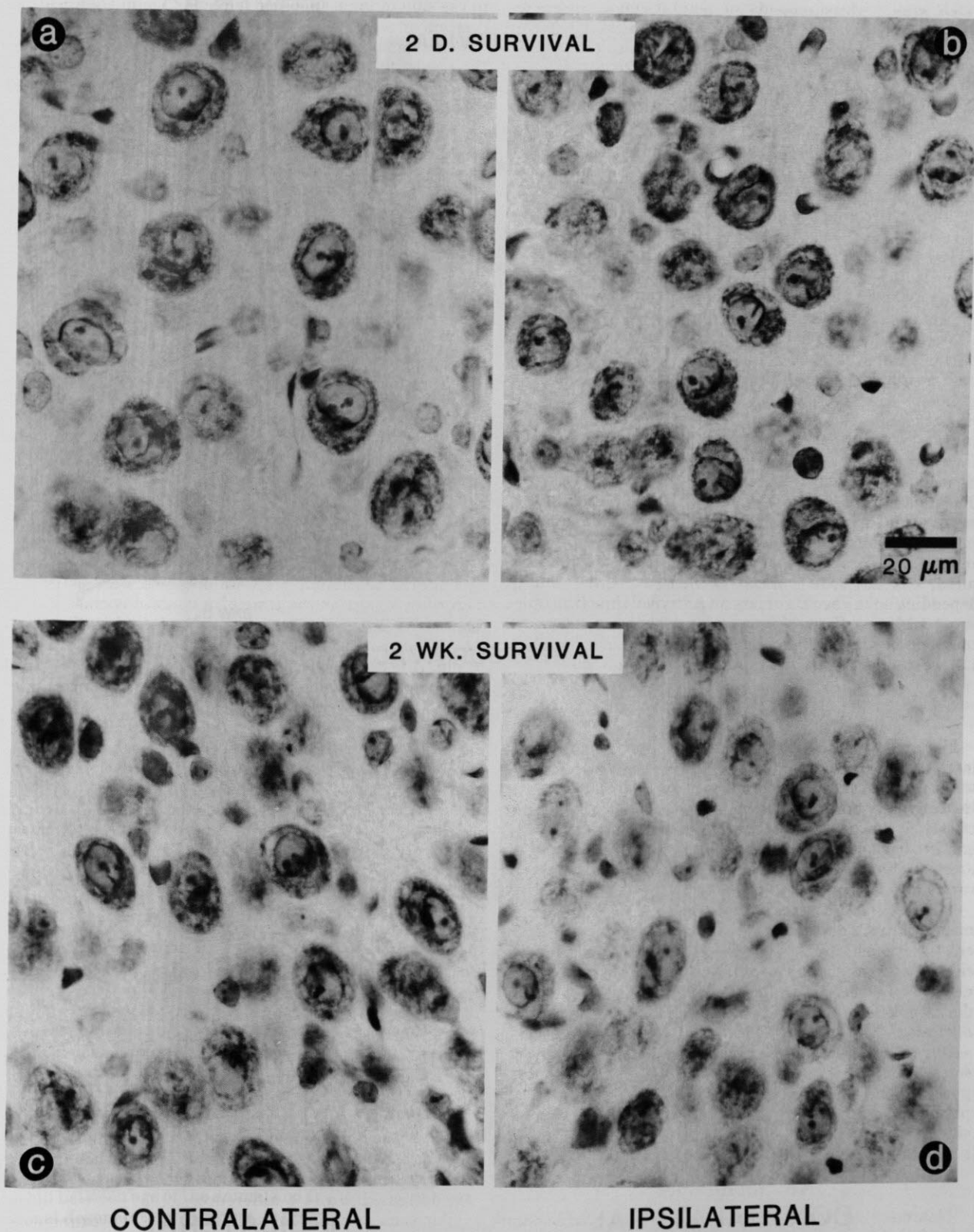
Fig. 2. Representative photomicrographs of large spherical cell region of AVCN from two subjects operated on at 8 weeks of age. **a** and **b**: Contralateral and ipsilateral side of the brain, respectively, 2 days after unilateral cochlea removal. **c** and **d**: Contralateral and ipsilateral sides of the brain, respectively, 2 weeks following unilateral cochlea removal.
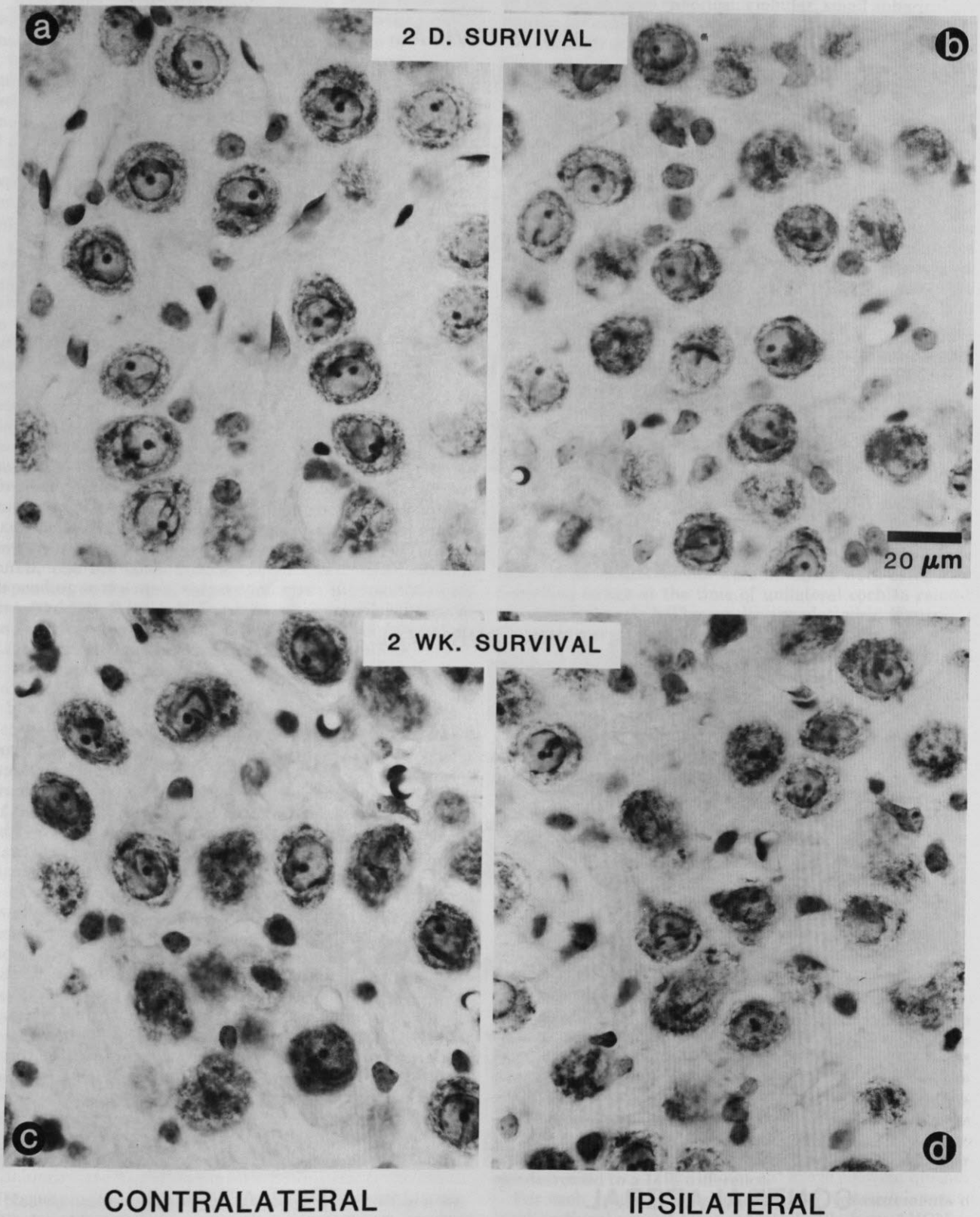
Fig. 3.   Representative photomicrographs of large spherical cell region of AVCN from two subjects operated on at 20 weeks of age. **a** and **b**: Contralateral and ipsilateral side of the brain, respectively, 2 days after unilateral cochlea removal. **c** and **d**: Contralateral and ipsilateral sides of the brain, respectively, 2 weeks following unilateral cochlea removal.
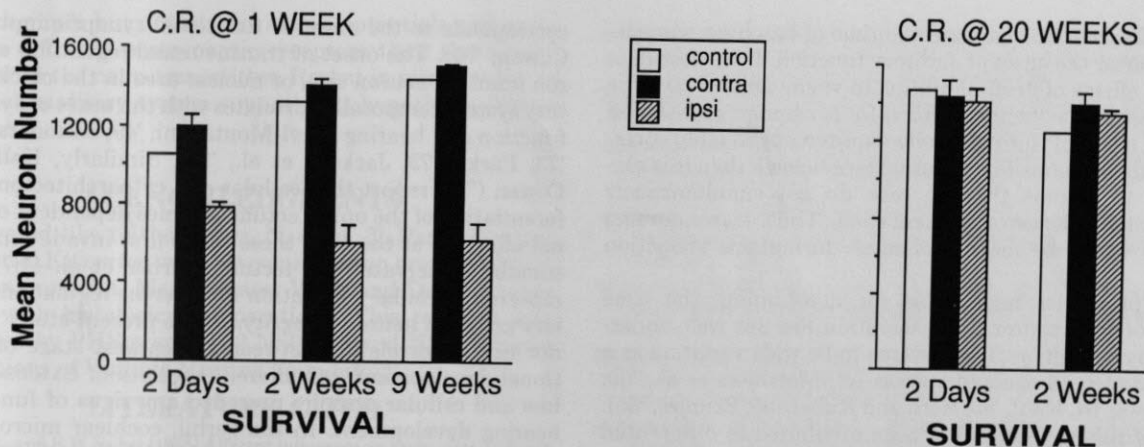
Fig. 4. Average neuron counts in AVCN (±1SEM) for each group of animals. **Left graph:** Results for animals operated on at 1 week of age and surviving 2 days, 2 weeks, or 9 weeks. **Right graph:** Counts for animals operated on at 20 weeks of age and surviving either 2 days or 2 weeks.
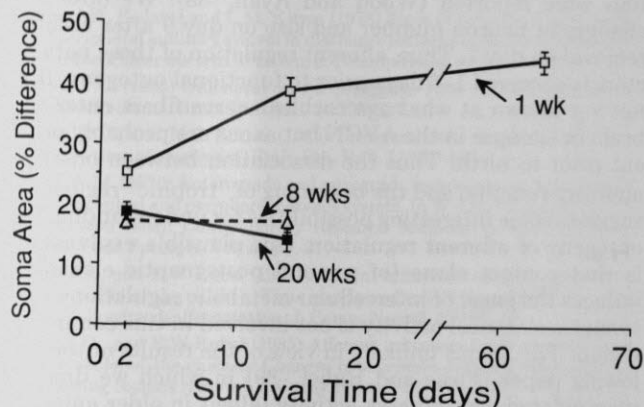

Fig. 5. Mean % decrease (±1SEM) in soma cross-sectional area as a function of survival time for subjects operated on at 1 week of age, 8 weeks of age, or 20 weeks of age.

DISCUSSION

Changes in the number and size of neurons in the gerbil AVCN following cochlea removal appear to be rapid and profound, particularly in young animals. These findings are not surprising given the wealth of data indicating that destruction of sensory receptors results in a cascade of transneuronal changes in the developing nervous system (e.g., Cowan, '70; Guillery, '74; Globus, '75; Woolsey and Wann, '76; Kalil, '80; Rubel et al., '84). The presynaptic signals which may trigger these changes are the subject of the following paper and will be discussed therein. The major goals of our experiment were to assess the time course of transneuronal changes in the cochlear nucleus and the relationship between age of the animal and the severity of transneuronal degeneration.

Time course of transneuronal degeneration

The majority of studies examining orthograde transneuronal cell death and atrophy in the central nervous system have assessed the long-term consequences of such manipulations. In order to discover the cellular mechanisms underlying transneuronal regulation of neuron number and neuronal metabolism, however, it is of considerable importance to identify the time course of postsynaptic changes. Recent studies of the avian brainstem auditory system have revealed that changes in some size, dendritic size, and neuron number are extremely rapid (Deitch and Rubel, '84; Born and Rubel, '85). Neuronal loss and atrophy in the avian homolog of AVCN, nucleus magnocellularis (NM), are essentially complete within 2 days after receptor removal. Metabolic changes, not surprisingly, are revealed much earlier (Durham and Rubel, '85; Steward and Rubel, '85). Other sensory systems, when examined at short intervals and in young animals, also reveal rapid structural changes in neurons (see Kalil, '80). In the present study changes in both cell number and soma size were apparent within 2 days and, with the exception of our youngest group, did not change significantly at a survival time of 2 weeks. We assume that these properties will remain stable after this time, but that assumption remains to be proven. In animals 7 days of age at the time of cochlea ablation, however, neither soma atrophy nor cell death was complete by 2 days following the surgery. For this reason an additional group of animals was allowed to survive for 9 weeks (until 70 days of age). Comparison of these two dependent variables between 2 weeks and 9 weeks survival revealed no further changes in the degree of transneuronal change. We again assume, but have not proven, that an asymptotic level of transneuronal change is seen by 2 weeks following cochlea destruction.

Several correlates can be identified which may be related to prolongation of the atrophic changes in the 1-week-old animals. Most obvious is the fact that the cochlear nucleus is quite immature at this time. The cells are undergoing a period of rapid soma growth, presumably accompanied by process maturation and synapse formation. Physiological function, as measured by responses to acoustic stimulation, has not yet begun. Although none of these events suggests a cellular mechanism for differences between this age group and the older animals, they are consistent with temporal relationships found in other species. For example, both Levi-Montalcini ('49) and Parks ('79) found that atrophic changes following otocyst removal in chicks did not reach

their full extent until around the time of hatching, some 9–10 days after the onset of auditory function. If we postulate that the effects of deafferentation in young animals are due to withholding a "factor" needed for *continued* growth and that the *onset* of this metabolic requirement roughly correlates with the time of innervation (see below), then it is reasonable to assume that all cells do not simultaneously acquire dependence on afferentation. Thus, transneuronal changes would be more prolonged during this transition period.

The properties responsible for determining the time course of transneuronal degeneration are not well understood in any system. There seems to be wide variation as a function of both age and species (cf. Matthews et al., '60; Matthews, '64; Kalil, '80; Born and Rubel, '85; Brunjes, '85). Some of this variability has been attributed to differential presence of other afferents but in most cases a causal relationship has not been established. More thorough understanding of the variables which determine the precise timing of transneuronal atrophy and cell death may place logical constraints on the cellular mechanisms involved.

Effects of age on transneuronal degeneration

In the present study, we observed changes in the size of large spherical cells in all three age groups: neonates, before the onset of auditory function; prepubertal juveniles; and adult gerbils. These changes were greatest in young animals and were equivalent in the two oldest groups. Cell loss, on the other hand, was confined to the neonates (although it was not measured in the 8-week-old animals). While these results are consistent with other studies of cochlear nucleus changes following peripheral destruction (Powell and Erulkar, '62; Trune, '82; Nordeen et al., '83; Born and Rubel, '85), several patterns can be observed across sensory systems in various species. For example, neurons in n. magnocellularis of immature chickens (hatchlings through at least 6 weeks of age) show equivalent changes in both transneuronal cell death and cell atrophy following cochlea removal. However, adult animals (>1 year old) do not show either effect (Born and Rubel, '85). In the cat visual system a similar pattern is seen with respect to cell loss. Eye removal in neonates or at 1 month of age results in essentially equivalent cell loss in the lateral geniculate nucleus (LGN), but there is little or no cell loss in adult cat LGN following enucleation (Guillery, '73; Kalil, '80). Cell atrophy in cat LGN, however, shows a different pattern. Guillery ('73) reports considerable atrophy in mature or adolescent cats at long survival times, which is similar to that observed following enucleation at 1 month of age and *more* severe than enucleation at 1 week of age (Kalil, '80). Yet another pattern appears to be seen in monkey LGN. In adults as well as neonates considerable cell loss and cell atrophy is seen in LGN following enucleation (Matthews et al., '60; Matthews, '64; Haseltine et al., '79). In other species and other sensory systems the age of central nervous system sensitivity to peripheral manipulations appears much more restricted to early development (e.g., Kelly and Cowan, '72; Brunjes and Borror, '83). In summary, there does not appear to be a consistent pattern in the relationship between maturation and resistance to either cell loss or neuronal atrophy following afferent destruction. Certainly, we cannot conclude that functional (i.e., synaptic) maturation marks the termination of a "sensitive" or "critical" period.

What about the onset of afferent regulation of postsynaptic neuron integrity? Most previous studies suggest that this corresponds to the onset of functional synaptic input (see Cowan, '70). The onset of transneuronal regulation of neuron number, neuron size, or nuclear area in the chick auditory system temporally correlates with the onset of synaptic function and hearing (Levi-Montalcini, '49; Saunders et al., '73; Parks, '79; Jackson et al., '82). Similarly, Kelly and Cowan ('72) report that cellular and cytoarchitectonic differentiation of the optic tectum becomes dependent on retinal afferents at the time these fibers first invade (and presumably innervate) the tectum. Furber et al. ('87) have reported a similar correlation for afferent regulation of ciliary ganglion neuron integrity. In the present study we did *not* find a correlation between the earliest stage of functional development and afferent regulation. Extensive cell loss and cellular atrophy preceded any signs of functional hearing development. In the gerbil, cochlear microphonic (CM) responses to airborne acoustic stimuli are first seen at postnatal day 12 (Woolf and Ryan, '84) and the first neural responses are seen a day or 2 later (Ryan et al., '82; Woolf and Ryan, '85; Sanes and Rubel, '88). Recently, use of a mechanical stimulator to directly drive the ossicles has allowed a CM to be detected at day 10 but no neural potentials were reported (Woolf and Ryan, '88). We observed changes in neuron number and size on day 9 after cochlea removal on day 7. Thus, afferent regulation of these parameters is observed 1–3 days prior to functional ontogeny. It is not yet known at what age cochlear nerve fibers enter the brain or synapse in the AVCN but axons are probably present prior to birth. Thus the dissociation between onset of auditory function and the beginning of "trophic" regulation suggests some interesting possibilities for understanding the ontogeny of afferent regulation. One plausible explanation is that contact alone (of pre-and postsynaptic elements) induces the onset of intercellular metabolic regulations, and synaptic electrical activity is not involved in this communication. This seems unlikely in view of the results of the following paper (Pasic and Rubel, '89) in which we directly manipulated eighth nerve activity (albeit in older animals) and observed similar effects on cell size.

Another possibility is that synaptic activity precedes the onset of auditory function by several days in this system, and "spontaneous" activity of the auditory nerve is the critical signal early in development. This hypothesis receives support from reports of intrinsic rhythmic activity in the immature auditory system (Sanes and Constantine-Patton, '85; Lippe, '86). The fact that hair cell-mediated "spontaneous" activity is sufficient to maintain cellular integrity in the avian homolog of the AVCN (Tucci and Rubel, '85; Tucci et al., '87) also lends support for this hypothesis. In addition, Wu and Oertel ('87) have recently elicited postsynaptic potentials in the mouse cochlear nucleus by electrical stimulation of the eighth nerve by 4–5 days after birth. Since the mouse shows a time course of hearing development similar to the gerbil it is likely that synaptic function is established prior to 7 days of age. On the other hand, most investigators have noted a marked paucity of spontaneous electrical activity while recording extracellular potentials from immature sensory systems of anesthetized animals (e.g., Rubel, '70; Brugge et al., '78; Sanes and Constantine-Patton, '85; Woolf and Ryan, '85). Finally, a third possibility is that contact-induced intercellular metabolic regulation involves the continual release of a "trophic" factor (such as nerve growth factor) which is coupled to, but not dependent on, electrical potentials in the presynaptic element. This hypothetical molecule could be released via calcium-acti-

vated channels but not require action potentials and could bind to different postsynaptic receptors than those associated with the neurotransmitter. Recent in vitro studies on the chick auditory system support this hypothesis (Hyson and Rubel, '89).

ACKNOWLEDGMENTS

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