THE JOURNAL OF COMPARATIVE NEUROLOGY 426:561–571 (2000)

Patterns of Cell Death in Mouse Anteroventral Cochlear Nucleus Neurons After Unilateral Cochlea Removal

SAM P. MOSTAFAPOUR, SARAH L. COCHRAN, N. MAE DEL PUERTO, AND EDWIN W. RUBEL*
Virginia Merrill Bloedel Hearing Research Center and Department of Otolaryngology-Head and Neck Surgery, University of Washington, Seattle, Washington 98195

ABSTRACT

Developmental changes that influence the results of removal of afferent input on the survival of neurons of the anteroventral cochlear nucleus (AVCN) of mice were examined with the hope of providing a suitable model for understanding the cellular and molecular basis for these developmental changes in susceptibility. We performed unilateral cochlear ablation on wild-type mice at a variety of ages around the time of hearing onset to determine developmental changes in the sensitivity of AVCN neurons to afferent deprivation. In postnatal day 5 (P5) mice, cochlea removal resulted in 61% neuronal loss in the AVCN. By age P14, fewer than 1% of AVCN neurons were lost after this manipulation. This reveals a rather abrupt change in the sensitivity to disruption of afferent input, a critical period. We next investigated the temporal events associated with neuron loss after cochlea removal in susceptible animals. We demonstrate that significant cell loss occurs within 48 hours of cochlea removal in P7 animals. Furthermore, evidence of apoptosis was observed within 12 hours of cochlea removal, suggesting that the molecular events leading to cell loss after afferent deprivation begin to occur within hours of cochlea removal. Finally, we began to examine the role of the bcl-2 gene family in regulating afferent deprivation-induced cell death in the mouse AVCN. AVCN neurons in mature bcl-2 knockout mice demonstrate susceptibility to removal of afferent input comparable to neonatal sensitivity of wild-type controls. These data suggest that bcl-2 is one effector of cell survival as these cells switch from afferent-dependent to -independent survival mechanisms. J. Comp. Neurol. 426:561–571, 2000.

Indexing terms: deafferentation; bcl-2; apoptosis; auditory neuron; cochlear nucleus; critical period

In several species, and in a variety of sensory systems, it has been shown that there is a "critical period" during development when afferent removal or changes in afferent activity have dramatic effects on mature neuronal structure and function (Hubel and Wiesel, 1970; Van der Loos and Woolsey, 1973; Brunjes, 1994). However, little is known about the biological basis of these critical periods. One model that has proved useful for understanding afferent regulation of neuronal survival is the brainstem auditory system of birds and mammals. In this system, the sensitivity of the cochlear nucleus neurons to removal of afferent input decreases with time during the critical period. This change from afferent-dependent to -independent survival was first described in the avian cochlear nucleus by Born and Rubel (1985) and was not temporally related to hearing onset. In contrast, studies on mammals have indicated that the change in sensitivity to afferent deprivation in the mammalian ventral cochlear nucleus (VCN) is temporally related to hearing onset. Several studies point to the presence of an age-dependent differential in sensitivity to deprivation-induced cellular changes in the brainstem auditory system (Trune, 1982; Nordeen et al., 1983; Webster, 1983, 1988; Hashisaki and Rubel, 1989; Kitzes et al., 1995). For example, Hashisaki and Rubel (1989) demonstrated that gerbils un-

Grant sponsor: NIH; Grant number: training grant DC-00018; Grant sponsor: NRSA; Grant numbers: DC-00299, DC-03829.

*Correspondence to: Edwin W. Rubel, Virginia Merrill Bloedel Hearing Research Center and Department of Otolaryngology-Head and Neck Surgery, University of Washington, Box 357923 CHDD CD176, Seattle, WA 98195. E-mail: rubel@u.washington.edu

Received 12 April 2000; Revised 12 July 2000; Accepted 12 July 2000

dergoing unilateral cochlear removal at postnatal day 7 (P7) were considerably more sensitive than those at age 20 weeks. Specifically, there was a 58% reduction in anteroventral cochlear nucleus (AVCN) neuron number in animals 1 week old at the time of surgery, whereas there was no significant cell loss in AVCN in gerbils 20 weeks old at the time of surgery.

More recently, Tierney et al. (1997) examined this phenomenon in more detail. They found a remarkably narrow window during which the sensitivity of gerbil cochlear nucleus (CN) neurons to deafferentation-induced cell death abruptly decreases. Cochlear removal at or before P7 results in 50–80% cell loss in the CN. The same manipulation performed at P9 or later results in no significant cell loss. The molecular basis for this dramatic change from afferent-dependent to afferent-independent survival of CN neurons remains unknown.

The morphologic and physiologic changes associated with removal of input to CN neurons in embryonic or neonatal chickens provide some clues. For example, dramatic changes in the integrity and morphology of CN neurons occur rapidly after removal of afferent input either by cochlear removal or pharmacological blockade of eighth nerve activity in the chicken (Born and Rubel, 1985; Rubel et al., 1990; Garden et al., 1994; Hyde and Durham, 1994; Zirpel et al., 1995; Hartlage-Rübsamen and Rubel, 1996; Lachica et al., 1996). Similar changes have been observed in the ventral CN of the gerbil (Hashisaki and Rubel, 1989; Pasic and Rubel, 1989, 1991; Sie and Rubel, 1992) and mouse (Trune, 1982). The similarity between some of the morphologic changes observed in CN neurons after removal of afferent input and apoptosis in other systems suggests that genes involved in programmed cell death may regulate susceptibility to neuronal loss after afferent deprivation.

The protooncogene bcl-2 represents the first described member of a family of genes that have been found to be important in controlling programmed cell death (Hockenbery et al., 1990; Adams and Cory, 1998) and has been shown to be important for neuronal survival during development (Allsopp et al., 1995; Michaelidis et al., 1996; Pinon et al., 1997). Expression of the caspase cpp32 is highly regulated in the developing mouse nervous system (de Bilbao et al., 1999a). The possible role of cell death regulators in the switch from afferent-dependent to -independent survival of sensory system neurons has yet to be investigated.

In the present study we sought to establish the presence, timing, and extent of the critical period for afferent deprivation-induced cell death in anteroventral CN neurons in mice. In this effort we have established a model for future investigation of the molecular mechanism by which neurons switch from afferent-dependent to -independent survival. As a first step, we use this model to test the hypothesis that inactivation of the antiapoptotic gene bcl-2 results in increased sensitivity to afferent deprivation in AVCN neurons in mature animals.

MATERIALS AND METHODS

Animals

C57Bl/6 mice (B and K Universal, Inc., Kent, WA) were used for examination of the temporal pattern of cell death after deafferentation. In these studies, pups arrived (with parent) at the animal care facility at age P3 and were operated on at the ages indicated. For study of the critical period for cell death after deafferentation as well as comparison of cell death in wild-type and bcl-2 null mice, breeding pairs of B6/129 mixed background mice (Jackson Laboratories, Bar Harbor, ME) were used to establish a breeding colony. The B6/129 strain, which provides a genetic background comparable to that of the transgenic mice, was used as a wild-type control. Breeder pairs of mice heterozygous for the bcl-2 null mutation (Jackson Laboratories) were also used to establish a colony (Veis et al., 1993). Homozygous bcl-2 null mice (bcl-2 −/−) were identified by tail clip DNA analysis using polymerase chain reaction. All mouse cages were checked daily for new litters; new pups were considered 0 days of age on the day of birth (P0). All animals were weaned at about P21. The numbers of animals for each analysis are given in Results. All procedures were approved by the University of Washington Animal Use Committee.

Surgical procedures

C57Bl/6 mice underwent unilateral ablation of the cochlea at P7. Wild-type (B6/129) animals underwent unilateral ablation of the cochlea at ages P5, 7, 11, 14, 21, 40 and 180 (6 months). Homozygous bcl-2 −/− mice were examined at ages over P21. Mice of all ages were anesthetized using inhaled methoxyflurane until areflexic; this level of anesthesia was maintained throughout the surgical procedure. In animals P14 and younger, an incision was made inferior to the pinna, and the tympanic membrane was identified. Middle ear mesenchyme (when present) was aspirated, ossicles were removed, and the basal turn of the cochlea was visualized. Using a 30-gauge needle, the bony wall was penetrated and the contents were aspirated using a fine glass pipette. The modiolus was visualized and destroyed. The skin incision was closed using cyanoacrylic glue. Litters were returned to parents within 2 hours. In animals P21 and older, a transtympanic approach was used. A small superior and posteriorly based flap of skin was raised in the external acoustic canal (this was later used for closure). The tympanic membrane and ossicles were visualized and removed. A 23-gauge needle was used to penetrate the bony cochlea and its contents were aspirated. A pick was used to destroy the modiolus. The skin incision was closed with cyanoacrylic glue.

Histology

C57Bl/6 mice were sacrificed at 3, 6, 12, 24, 48, 96, or 336 hours (2 weeks) after unilateral ablation of the cochlea. Animals of all other genetic backgrounds were sacrificed after a uniform 2 week postsurgical survival period. Animals were sacrificed with CO_2 intoxication and transcardially perfused with phosphate-buffered 4% paraformaldehyde, pH 7.4. After perfusion, the brain was immediately dissected from the skull and placed in fresh fixative for 24–48 hours. Brains were blocked in a coronal plane, and a notch was made in the right ventral surface with a razor blade. A sample of temporal bones was carefully harvested, and soft tissue was removed and decalcified in 15% EDTA, pH 7.0, for 21 days to verify ablation of the cochlea. Both brains and temporal bones were serially dehydrated in graded alcohols, embedded in paraffin, and serially sectioned at 10 μm. A one-in-five series of sections was mounted on gelatin-coated slides, dewaxed in xylene,

thionin-stained, and coverslipped with DPX mounting medium. Temporal bones were sectioned approximately parallel to the midmodiolar plane and mounted similarly. These were stained with hematoxylin and eosin and coverslipped as described above. Photomicrocraphs were prepared using Adobe Photoshop 5.5 software (Adobe Systems, Inc., Seattle, WA).

Analysis

Only cases in which every section through the AVCN on both sides of the brain was intact were included in this study. Every mounted section through the entire anteroposterior extent of the AVCN was examined using standard light microscopy. The posterior boundary of the AVCN was defined by the appearance of the dorsal CN. Neuron counts were performed on a Leitz Aristoplan microscope with a 40× objective and a 10 × 10 reticule. All AVCN neurons in a given section were counted. The criteria for a neuron to be counted were a well-defined cytoplasm and nuclear outline and a clearly visible nucleolus. Neuron counts were obtained from the AVCN on both sides of the brainstem. The AVCN on the contralateral side provided a within-animal control. Total AVCN neuron number was defined as total number of neurons = No. counted × 5. Percentage of AVCN neuron loss on the lesioned side was calculated as: 100 × [1 − (No. neurons on ablated side/No. neurons on control side)]. Corrections for "double counting" were not used because the nucleolus is small compared to section thickness. Stereological procedures were not employed because our primary goal was the comparison of the two sides of the brainstem rather than the absolute numbers of neurons. Statistical comparisons were made using either a paired Student's t-test for comparison of neuron loss within a single age group or an unpaired Student's t-test for comparison amongst animals at various ages and genotypes.

TUNEL labeling of AVCN neurons

To derive an estimate of the timing and numbers of neurons undergoing apoptotic cell death, a one-in-five series of sections from C57Bl/6 animals was processed with the terminal deoxytransferase-mediated deoxyuridine triphosphate nick end-labeling (TUNEL) method using the TdT-FragEL Kit from Oncogene Research Products (Calbiochem, Cambridge, MA). TUNEL-labeled cells were judged to be neurons by size (in comparison to glial cells). Positive and negative controls were generated using slides provided in the kit. In addition, we routinely processed sections of colon from the experimental animals as positive and negative controls. Negative controls on colon and brain tissue were generated by omitting the TdT enzyme from the TdT Labeling Reaction Mix. After processing, slides were counterstained with methyl green, dehydrated, and coverslipped with DPX.

Confirmation of cochlear ablation

The temporal bones of animals were examined by light microscopy under low-power magnification (10×). The organ of Corti appeared intact and normal on the side contralateral to lesioning; most or all recognizable elements of the organ of Corti were absent on the lesioned side. No direct damage to the CN was observed.

RESULTS

Effect of cochlear ablation in wild-type (B6/129) animals

General observations. Figures 1 and 2 show representative sections through the AVCN of wild-type, background-matched B6/129 mice operated on at ages P5 and P21, respectively, and allowed to survive for 2 weeks. At P5, unilateral cochlear ablation results in a gross reduction in the size of the CN ipsilateral to the lesion, as demonstrated in Figure 1A,B. Higher power views of the same section (Fig. 1C,D) show that there is a prominent gliosis in the affected nucleus at this age. In contrast, at P21 unilateral cochlear ablation appears to have little effect on the cells in the CN. As shown in Figure 2A,B, the AVCN is grossly symmetric in the P21 animal. Higher magnification reveals an increased density of glia on the side ipsilateral to lesioning but no obvious difference in neuron density compared to the contralateral side (Fig. 2C,D).

Neuron number. To quantify the effects of deafferentation on AVCN neurons, neuron counts in the AVCN were performed on animals having undergone unilateral cochlea removal at ages P5, 7, 11, 14, and 21 and 6 months. In this experiment, all animals survived for 2 weeks. As is shown in Table 1, cochlea removal resulted in a large amount of neuronal cell loss at age P5 (mean change = 4,505, $P < 0.01$ between sides of the brainstem; n = 5) and P7 (mean change = 2,407, $P < 0.01$ between sides of the brainstem; n = 7). The extent of cell loss in AVCN decreased by P11 (mean change = 1,509, $P = 0.21$ between sides of the brainstem; n = 5) and was minimal by age P14 (mean change = 37, n = 5). In addition, ablation of the cochlea resulted in no loss of AVCN neurons in animals as old as 6 months of age (Table 1).

These data may also be represented as percentage neuron loss, as illustrated in Figure 3. In P5 mice, cochlea removal resulted in loss of 61% of AVCN neurons. By P14, AVCN neuronal loss was less than 1%. No deprivation-induced neuronal loss was observed in mice 6 months old. Thus, a decrease in the amount of afferent deprivation-induced neuronal cell death in the AVCN occurs between postnatal days 5 and 14, with no appreciable differences thereafter.

Effect of cochlear ablation in transgenic bcl-2 knockout (bcl-2 −/−) animals

General observations. Having established the presence and timing of a critical period for deafferentation-induced cell death in the wild-type mouse, we began to examine the molecular mechanisms underlying this switch from afferent input dependence to independence in AVCN neurons. We chose to begin with the antiapoptotic gene bcl-2 as a likely candidate for involvement in this switch. To test whether homozygous deletion of bcl-2 increases afferent deprivation-induced cell death in mature animals, bcl-2 null mutant (bcl-2 −/−) mice were obtained. Three mice, ages P24, P27, and P70, were examined. As shown above, these are ages at which essentially no afferent deprivation-induced cell death occurs in the AVCN of wild-type mice. Each bcl-2 −/− mouse underwent unilateral cochlear ablation exactly as described for their wild-type counterparts. It should be noted that, given the underlying comorbid diseases induced by deletion of bcl-2 in these mice (Veis et al., 1993), we encoun-

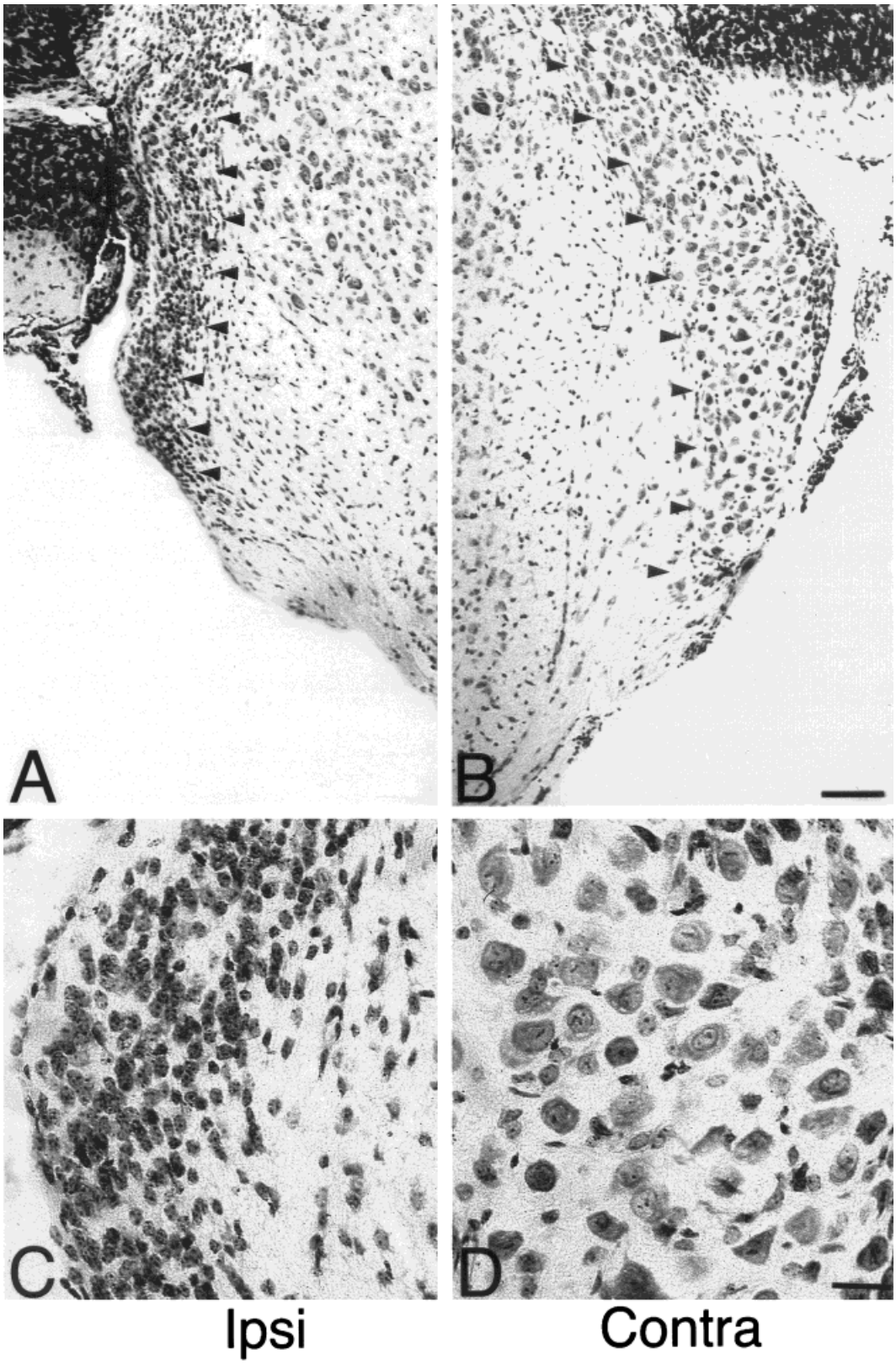

Fig. 1. Representative photomicrographs of coronal sections through AVCN of a P5 wild-type (B6/129) mouse after unilateral cochlear ablation and 2 week survival. Comparison of sections on sides ipsilateral (**A**) and contralateral (**B**) to the ablation shows gross reduction in the size of the nucleus. Comparison of higher power views of the same specimens reveals a marked gliosis and reduction in neuron density in the nucleus ipsilateral to the lesion (**C**) compared to the contralateral side (**D**). Ipsi, ipsilateral, or Contra, contralateral to cochlear ablation. Scale bars = 100 μm for A,B; 30 μm for C,D.

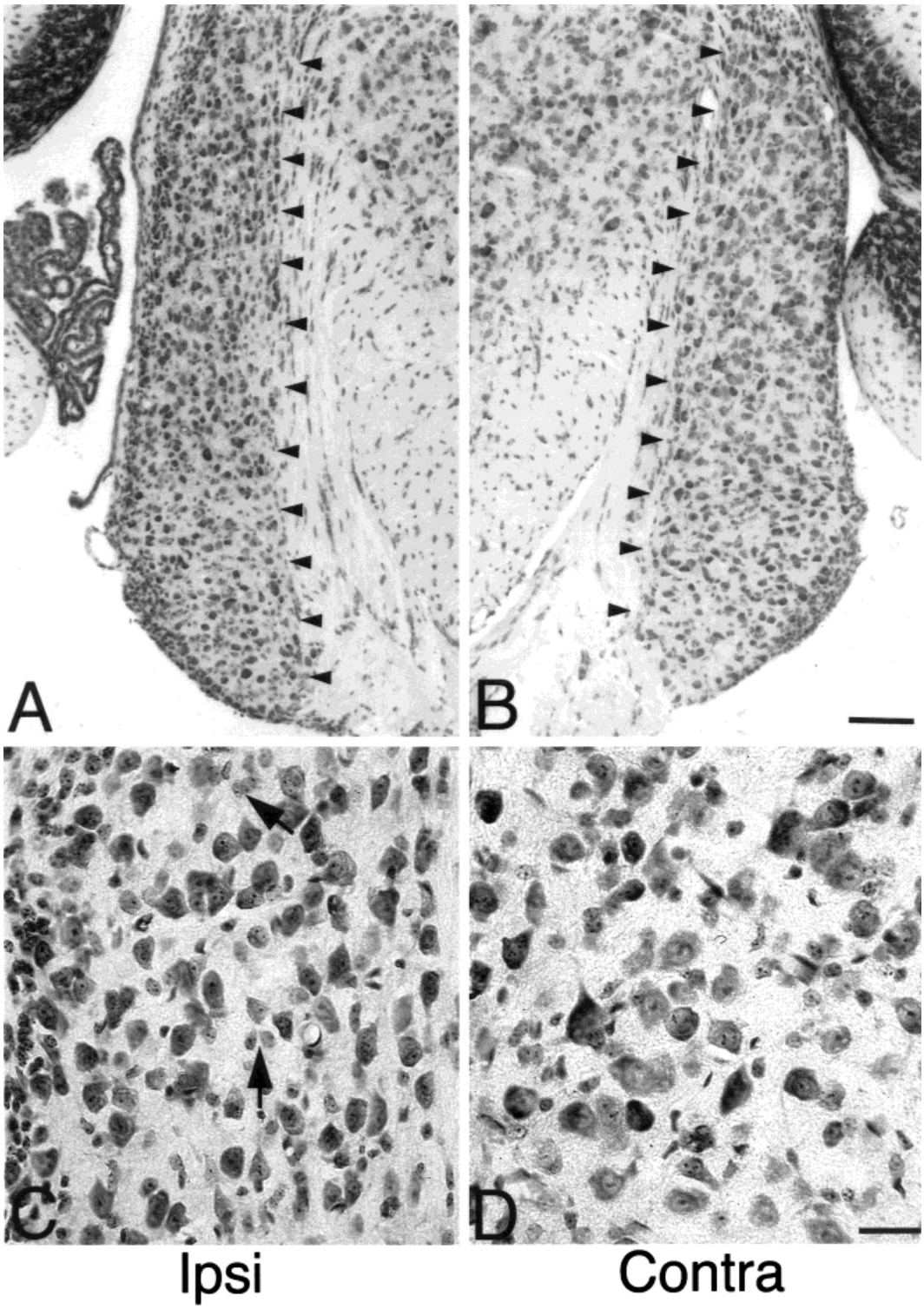

Fig. 2. Representative photomicrographs of coronal sections through AVCN of a P21 wild-type (B6/129) mouse after unilateral cochlear ablation. Comparison of sections through the AVCN on sides ipsilateral (**A**) and contralateral (**B**) to the ablation shows minimal, if any, reduction in the size of the nucleus. Comparison of higher power views of the same specimens reveals a small increase in the density of glial cells but no reduction in neuron density in the nucleus ipsilateral to the lesion (**C**) compared to the contralateral side (**D**). Ipsi, ipsilateral, or Contra, contralateral to cochlear ablation. Scale bars = 100 μm for A,B; 30 μm for C,D.

TABLE 1. Comparison of AVCN Neuron Number Ipsilateral and Contralateral to Cochlea Removal in B6/129 Mice at Various Ages

Age at surgery (postnatal days)	n	Neuron number (mean ± SEM)	
		Ipsilateral	Contralateral
5	5	2,618 ± 98	7,123 ± 756
7	7	4,949 ± 340	7,356 ± 378
11	5	7,130 ± 872	8,639 ± 704
14	5	8,089 ± 679	8,126 ± 190
21	7	7,444 ± 677	7,025 ± 728
40	3	9,113 ± 723	9,256 ± 1,024
180	4	7,146 ± 1,388	6,918 ± 1,308

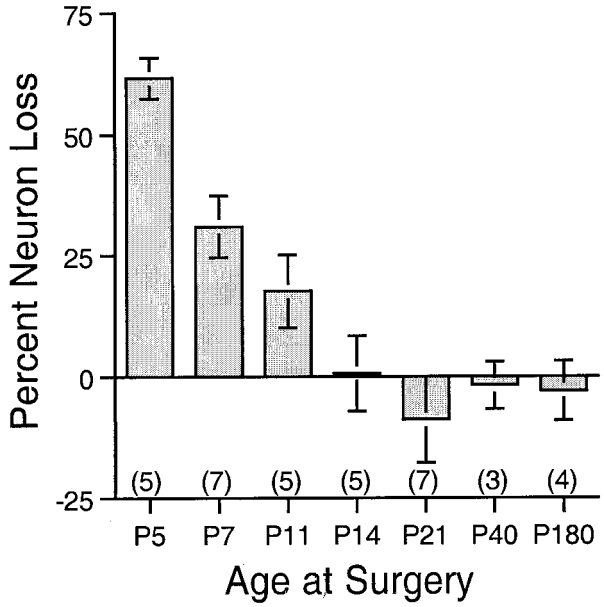

Fig. 3. Percentage neuronal cell loss in AVCN after cochlea removal at ages P5 through 6 months. All animals survived 2 weeks after the ablation. Percentage AVCN neuron loss on the lesioned side was calculated as: 100 × [1 − (No. neurons on ablated side/No. neurons on control side)]. Numbers shown represent mean ± SEM. Number of animals is indicated in parentheses.

tered difficulty in achieving surgical and postsurgical 2 week survival.

AVCN neurons in the bcl-2 −/− mice demonstrated increased susceptibility to cell death after removal of afferent input compared to wild-type animals of similar age. Figure 4A,B shows representative sections through the AVCN, demonstrating the grossly decreased size of the nucleus on the side ipsilateral to the lesion. At higher magnification (Fig. 4C,D), decreased neuronal density is readily apparent. Interestingly, less gliosis was present in the ipsilateral AVCN of bcl-2 knockout animals in comparison to wild-type (Figs. 1C, 4C).

Neuron number. To confirm that neuronal number in the bcl-2 −/− mice was unaffected prior to lesioning, we compared contralateral AVCN neuron counts in age-matched wild-type B6/129 mice and bcl-2 −/− mice. As is shown in Table 2, bcl-2 −/− mice demonstrated slightly fewer AVCN neurons than wild-type animals on the side contralateral to the lesion, although the difference did not approach statistical significance ($P = 0.26$). Similarly, there was no statistical difference in neuron number between the ipsilateral and the contralateral AVCN in wild-type animals older than age P21. However, bcl-2 −/− mice demonstrated a large decrease in AVCN neuron number on the side ipsilateral to lesioning (mean change = 2,048; n = 3), which was statistically different from that observed in wild-type, age-matched mice ($P < 0.01$).

When expressed as percentage neuron loss, we see that AVCN neurons of bcl-2 −/− mice demonstrate significantly more cell death than that observed in wild-type controls of similar age (38% ± 17% and –7 ± 6%, respectively; $P < 0.01$; Table 2). Furthermore, this degree of cell death appears to be comparable to that of wild-type mice at ages within the sensitive period.

Timing of cell death after deafferentation in immature C57Bl/6 mice

Neuronal loss in AVCN after cochlea removal in P7 and P21 mice. Given the evidence for the importance of the antiapoptotic gene bcl-2 in this model, we sought to define better the timing of cell death during the critical period. This would provide a temporal framework upon which to base future studies of cell death regulator expression during afferent deprivation-induced cell death. Postnatal day 7 C57Bl/6 mice underwent unilateral ablation of the cochlea as described in Materials and Methods. Animals survived for 3, 6, 12, 24, 48, 96, or 336 hours (2 weeks). Mice were then killed and their brainstems were processed for neuronal counts. Percentage loss of AVCN neurons in these animals over time is shown in Figure 5. Up to 24 hours after removal of eighth nerve input, less than 10% neuronal loss occurs. However, after this time, rapid loss of neurons occurs such that, by 96 hours, over 50% of AVCN neurons have degenerated. Neuron number remains stable from this time on, as demonstrated by similar neuronal loss measured in animals surviving for 2 weeks.

In that the experiments described above (Figs. 3, 4) were carried out on B6/129 mice, we thought it important to determine whether animals of the C57/B6 strain also demonstrate loss of susceptibility to afferent deprivation as they mature. Therefore, P21 animals of this strain also underwent unilateral cochlear ablation and survived for 96 hours. As is shown also in Figure 5, these mice demonstrated no neuronal loss 96 hours after cochlea removal, by which time P7 mice had undergone maximal AVCN neuronal loss.

TUNEL labeling of AVCN neurons after cochlea removal. The first step in determining the cellular cascades that precede neuron loss in AVCN is to develop an accurate model for the timing of cell death and neuron loss in the nucleus. Given that most cell loss occurs by 96 hours after deafferentation, we sought to examine afferent-deprived neurons in the nucleus in the hours leading up to this time. TUNEL labeling of neurons provides one method for examining DNA fragmentation and cell death in situ (Gavrieli et al., 1992). Postnatal day 7 C57Bl/6 mice underwent unilateral ablation of the cochlea as described in Materials and Methods. Animals survived for 3, 6, 12, 24, 48, 96, or 336 hours. We examined AVCN neurons for TUNEL labeling. As is shown in Figure 6A,B few TUNEL-positive neurons are present in the AVCN on either side of the brainstem 6 hours after unilateral cochlea ablation. However, by 12 hours after cochlea removal, TUNEL-positive cells begin to appear in the ipsilateral, but not contralateral, AVCN (Fig. 6C,D). By 24 hours, the number

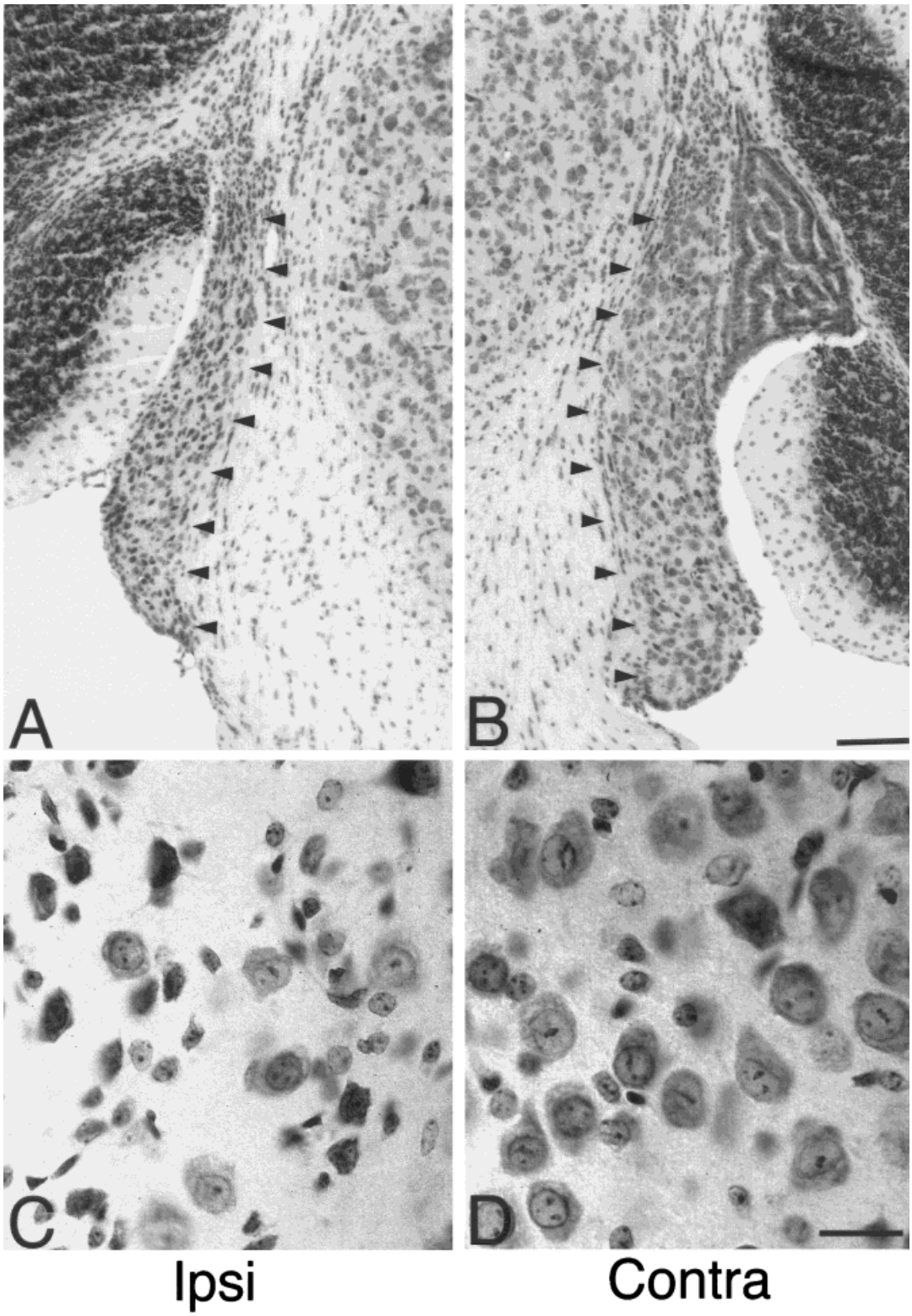

Fig. 4. Representative photomicrographs of coronal sections through AVCN of a P27 bcl-2 −/− mouse after unilateral cochlear ablation. Comparison of sections through the AVCN on sides ipsilateral (**A**) and contralateral (**B**) to the ablation shows a gross reduction in the size of the nucleus. Comparison of higher power views of the same specimens reveals a reduction in neuron density in the nucleus ipsilateral to the lesion (**C**) compared to the contralateral side (**D**). Ipsi, ipsilateral, or Contra, contralateral to cochlear ablation. Scale bars = 100 μm for A,B; 30 μm for C,D.

TABLE 2. Comparison of AVCN Neuron Number Ipsilateral and Contralateral to Cochlea Removal in Adult B6/129 and bcl-2 Knockout Mice

Genotype	Neuron number (mean ± SEM)		Percentage neuron loss
	Ipsilateral	Contralateral	
Wild-type (B6/129)	7,716 ± 541	7,472 ± 580	−7 ± 6
bcl-2 Knockout	3,903 ± 1,274	5,951 ± 629	38 ± 17

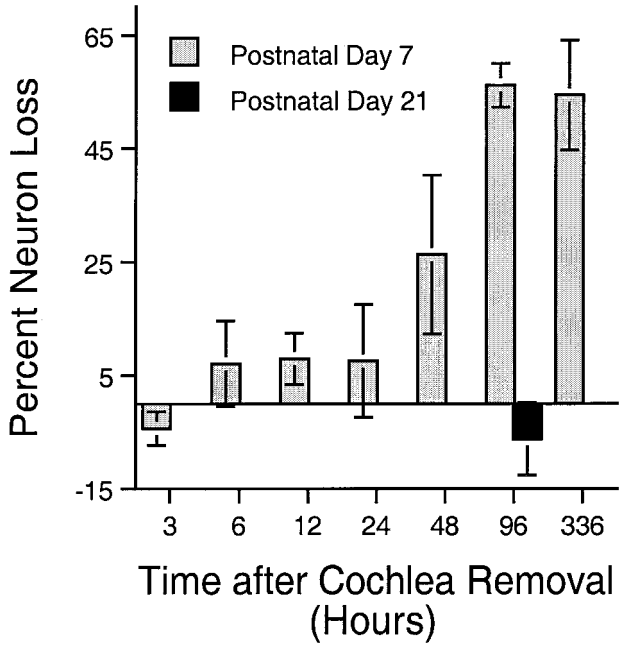

Fig. 5. Percentage neuronal cell loss in AVCN after cochlea removal as a function of survival time. C57Bl/6 mice underwent cochlea removal at age P7 (shaded bars) or P21 (solid bar) and were allowed to survive for the time indicated on the abscissa prior to sacrifice and brainstem processing. Percentage AVCN neuron loss on the lesioned side was calculated as: 100 × [1 − (No. neurons on ablated side/No. neurons on control side)]. Numbers shown represent mean ± SEM. All sections through the AVCN of three to five animals were counted at each time point.

of TUNEL-positive cells has continued to increase (Fig. 6E,F).

TUNEL-positive neurons were counted in each section through AVCN at each survival time. The ratio of the number of positive cells on the side ipsilateral: contralateral to lesioning are shown in Figure 7. At 48 hours after removal of the cochlea, the ipsilateral AVCN demonstrates over 10 times as many positive cells as the opposite, control side. Interestingly, this peak precedes the maximal cell loss as determined by cell counting, which occurs at 96 hours after cochlea removal. By 336 hours (2 weeks), no difference in numbers of TUNEL-positive neurons was observed between AVCN ipsilateral and contralateral to cochlea ablation. In addition, as is shown by the solid bar in Figure 7, no difference in TUNEL labeling between sides was observed in AVCN of P21 animals at 96 hours after cochlea removal (one-way ANOVA).

DISCUSSION

The present work provides a model for examining afferent deprivation-induced cell death in the mammalian central auditory system. We demonstrate 1) the age dependence (or critical period) of afferent deprivation-induced cell death in the mouse AVCN, 2) the timing of both neuronal loss and TUNEL labeling during the critical period, and 3) the importance of the cell death regulator bcl-2 in this process. These data provide an important framework for future studies of the molecular mechanisms of afferent-regulated neuronal death and survival in the mammalian CNS.

Age dependence of afferent deprivation

The sensitivity of AVCN neurons to removal of afferent input (removal of the cochlea) is age-dependent. We demonstrate a narrow window during which the sensitivity of AVCN neurons to afferent deprivation-induced cell death decreases dramatically. The age dependence data were acquired using B6/129 mixed background mice to facilitate comparison to bcl-2 knockout mice (discussed below). The amount of neuronal loss observed is maximal at the youngest age examined (P5), after which it decreases steadily. By age P14, AVCN neurons no longer degenerate following removal of afferent input from the eighth nerve. The lack of sensitivity to removal of afferent input is stable over time, to at least 6 months of age. Interestingly, AVCN neurons in P7 C57/Bl6 mice showed increased sensitivity to deafferentation in comparison to P7 B6/129 mice. Notably, the C57/Bl6 mice were from a different vendor, and mice at age P7 appeared qualitatively underdeveloped in comparison to P7 B6/129 mice. All mice, however, showed decreased sensitivity to deafferentation by age P21.

The presence of a narrow temporal window was demonstrated in the CN of the gerbil by Tierney et al. (1997) and suggested in the rat by Moore et al. (1998). Similarly to these findings, we find that the loss of sensitivity to deafferentation approximately coincides with the timing of hearing onset (Ehret, 1976). These results are also in agreement with those of Willott et al. (1994), who found that premature hearing loss in young adult mice did not result in cell loss in the CN. Although the steepness of the drop in sensitivity appears slightly greater in the gerbil, the age-related drop in sensitivity is considerably more abrupt in the mouse than that observed in the chicken by Born and Rubel (1985). The molecular basis for this age-related change in sensitivity remains unknown. An intriguing possibility is that the rapid change in sensitivity during this critical period is due to altered expression of genes involved in the apoptotic pathways.

Kinetics of cell death and neuron loss

The rapidity of the change in sensitivity to removal of afferent input implies similarly rapid changes in gene expression. To study this further, we have begun to delineate the kinetics of both cellular changes and cell loss in AVCN after deafferentation in animals during the critical period. Evidence of an apoptotic pathway, as determined by TUNEL labeling of AVCN neurons, occurs within 12 hours of removal of input from the cochlea in P7 animals and peaks at approximately 48 hours. These data suggest that a cell death pathway is activated in AVCN neurons within a few hours following the removal of eighth nerve

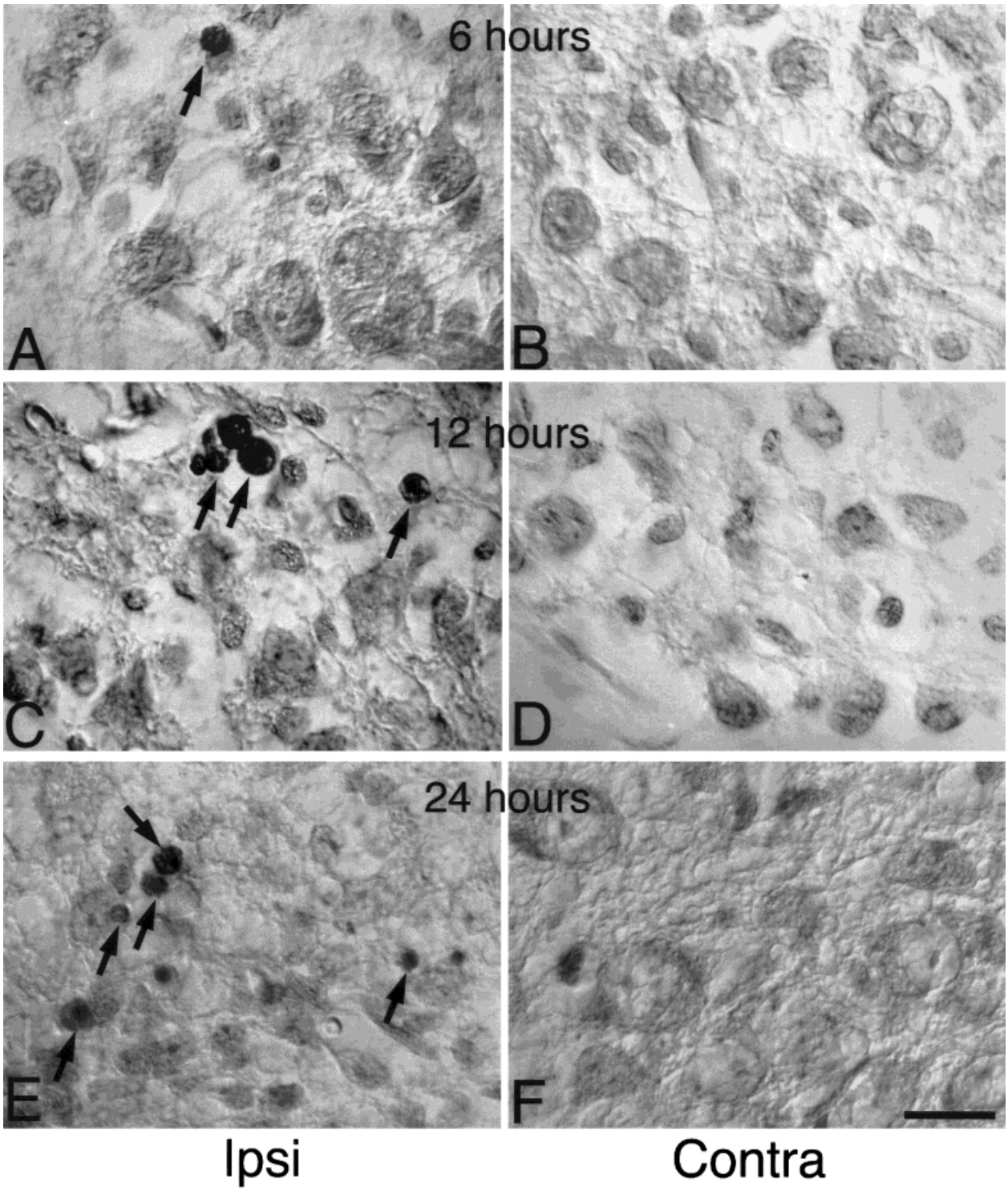

Fig. 6. Representative photomicrographs through AVCN of P7 C57Bl/6 mice after unilateral cochlear ablation and TUNEL labeling. C57Bl/6 mice underwent cochlea removal and were allowed to survive for the time indicated prior to sacrifice and brainstem processing and TUNEL labeling (as described in Materials and Methods). Shown are representative sections through the AVCN on sides ipsilateral (**A,C,E**) and contralateral (**B,D,F**) to the ablation. Arrows indicate TUNEL-positive neurons. Survival times after cochlea removal are indicated on each panel. Ipsi, ipsilateral, or Contra, contralateral to cochlear ablation. Scale bar = 15 μm.

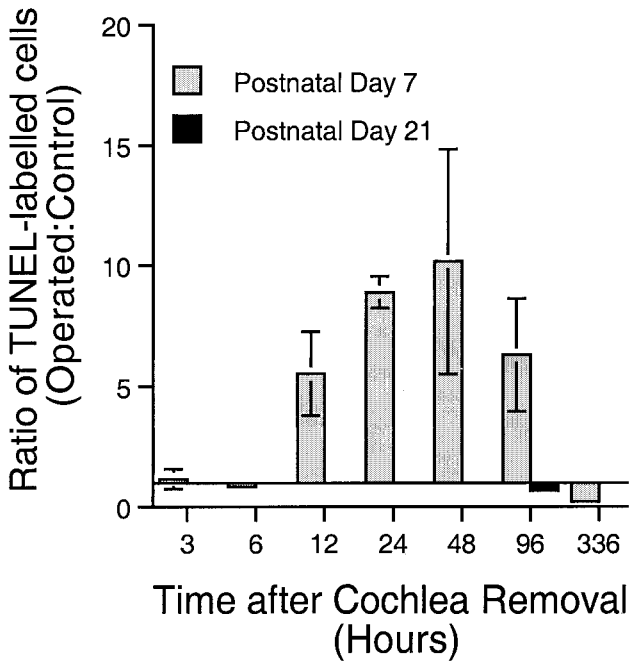

Fig. 7. TUNEL-positive neurons in AVCN after cochlea removal as a function of survival time. Brainstem sections were prepared and TUNEL labeled as described in Materials and Methods. The ratio of TUNEL-positive neurons on the two sides of the brain was calculated as No. of labeled AVCN neurons on side ipsilateral:No. of labeled AVCN neurons contralateral to cochlea removal. At least six sections of brainstem from two to four animals were examined at each time point. Numbers shown represent mean ± SEM.

input. Neuron loss after deafferentation, as determined by counting of neurons in Nissl-stained brainstem sections, follows the appearance of apoptosis within the AVCN. In this case, neuron loss became evident at 48 hours and was maximal by 96 hours after deafferentation. No TUNEL labeling was observed in P21 animals, consistent with the absence of significant cell death.

The timing of cell death in the brainstem after peripheral axotomy has been examined in the facial nerve nucleus. Facial nerve transection in P2 mice results in the appearance of apoptotic neurons (as detected by TUNEL labeling) as early as 16 hours after lesioning (de Bilbao and Dubois-Dauphin, 1996). Overexpression of bcl-2 blocked apoptosis after axotomy in these mice (de Bilbao and Dubois-Dauphin, 1996). The caspase cpp32, an important effector of the apoptotic cascade (Chinnaiyan et al., 1996), is up-regulated in this model within 12 hours of lesioning (de Bilbao et al., 1999b). These studies are interesting in that they provide information regarding the genes that may be involved in apoptosis in target-dependent control of neuronal survival. The importance of these genes/gene products in afferent regulation of neuronal survival remains to be investigated.

Some recent work indicates that at least some of the same genes may be involved in the afferent regulation of neuronal survival. The caspase cpp32 has been recently demonstrated to be widely expressed in the CN of P7 C57Bl/6 mice, whereas the adult brainstem was nearly devoid of cpp32 expression (de Bilbao et al., 1999a). The expression of this cell death regulator overlaps the critical period that we demonstrate is important for determining afferent input-dependent AVCN neuron survival.

Adult bcl-2 knockout mice demonstrate a prolonged critical period

We chose to investigate further the age dependence in sensitivity to deafferentation by examining the effect of deafferentation in a transgenic mouse in which the anti-apoptotic gene bcl-2 has been deleted. Expression of bcl-2 is widespread in the developing murine nervous system, including expression in the subventricular zone though P10, although no studies have examined the CN specifically (Merry et al., 1994). Our data show that deletion of this gene results in sensitivity to removal of afferent input in AVCN neurons in adolescent and adult animals, ages at which such sensitivity has ceased in wild-type mice. The data was statistically significant, although a relatively low number of experimental knockout animals were used in the comparison. However, these data suggest that bcl-2 acts as one necessary effector of cell survival in these neurons as they switch from afferent-dependent to afferent-independent survival. This does not necessarily mean that bcl-2 operates exclusively of other mechanisms. For example, complete cell loss is not observed in these animals. This may mean that not all AVCN neurons are sensitive to deafferentation, regardless of the presence or absence of bcl-2 in the genome. An alternative explanation, though not exclusive of the former, is that other mechanisms of cell protection are operable despite the inactivation of bcl-2. In this case, the absence of bcl-2 may be compensated for by modified expression of related molecules of the apoptotic pathway or by extracellular events activating a "survival pathway" (Garden et al., 1994). Interestingly, mice overexpressing bcl-2 showed no significant change in cpp32 expression, also supporting the notion that non-bcl-2 dependent pathways are at work to determine neuronal survival (de Bilbao et al., 1999a).

Several studies have indicated that regulation of apoptotic pathways is one mechanism by which neurotrophins mediate cell survival in target neurons. For example, it has been demonstrated that bcl-2 plays a role in the brain-derived neurotrophic factor (BDNF) survival response of chick ciliary neurons (Allsopp et al., 1995). Caspase activation occurs in mouse dorsal root ganglion neurons after nerve growth factor (NGF) deprivation (Mukasa et al., 1997). Several studies have examined the role of bcl-2 in embryonic and early postnatal development and survival of neurons in mice. For example, bcl-2 −/− mice were found to have continued neuronal cell death in sympathetic, motor, and sensory nuclei after the period of normally occurring cell death (Michaelidis et al., 1996). Because neuronal number in these nuclei did not diverge from that of wild-type mice during the period of normally occurring cell death, this was interpreted as an indication that bcl-2 does not play a role in this process. However, cell death continued in these nuclei into the postnatal period in bcl-2 −/− mice. Thus, one may view the continued cell death in bcl-2 −/− nuclei as an indication that its presence is necessary for survival of these neurons as they compete for neurotrophins or for some other survival signal. In another study, it was demonstrated that bcl-2 was required for the survival response of embryonic trigeminal ganglion neurons to neurotrophins during the period of naturally occurring cell death and that its absence led to decreased survival of these neurons in vivo (Pinon et al.,

1997). Taken together, these studies point toward the important role that bcl-2 plays in neural development.

In our model for afferent input-dependent neuronal survival, a change in cell survival from afferent-dependent to -independent mechanisms occurs at about the time of hearing onset. During the sensitive period, removal of afferent input sets in motion cellular events that lead to neuronal cell death within 48 hours. The cell death or survival mechanisms that mediate this process on the molecular level remain to be investigated. The finding that bcl-2 −/− mice demonstrate continued afferent dependence indicates that the presence of bcl-2 is important in effecting the "survival switch." Because most neurons survived afferent deprivation in this model, a role of other pro- or antiapoptotic molecules in this process is likely, and this remains to be investigated.

ACKNOWLEDGMENTS

The authors thank Drs. JiaLin Shang and David Hockenbery for technical assistance with this study and Dr. M. K. Mostafapour for critical reading of the manuscript.

LITERATURE CITED

Adams JM, Cory S. 1998. The Bcl-2 protein family: arbiters of cell survival. Science 281:1322–1326.

Allsopp TE, Kiselev S, Wyatt S, Davies AM. 1995. Role of Bcl-2 in the brain-derived neurotrophic factor survival response. Eur J Neurosci 7:1266–1272.

Born DE, Rubel EW. 1985. Afferent influences on brain stem auditory nuclei of the chicken: neuron number and size following cochlea removal. J Comp Neurol 231:435–445.

Brunjes PC. 1994. Unilateral naris closure and olfactory system development. Brain Res Brain Res Rev 19:146–160.

Chinnaiyan AM, Orth K, O'Rourke K, Duan H, Poirier GG, Dixit VM. 1996. Molecular ordering of the cell death pathway. Bcl-2 and Bcl-xL function upstream of the CED-3-like apoptotic proteases. J Biol Chem 271: 4573–4576.

de Bilbao F, Dubois-Dauphin M. 1996. Time course of axotomy-induced apoptotic cell death in facial motoneurons of neonatal wild type and bcl-2 transgenic mice. Neuroscience 71:1111–1119.

de Bilbao F, Guarin E, Nef P, Vallet P, Giannakopoulos P, Dubois-Dauphin M. 1999a. Postnatal distribution of cpp32/caspase 3 mRNA in the mouse central nervous system: an in situ hybridization study. J Comp Neurol 409:339–357.

de Bilbao F, Guarin E, Nef P, Dubois-Dauphin M. 1999b. The mouse cpp32 mRNA transcript is early up-regulated in axotomized motoneurons following facial nerve transection. Neurosci Lett 266:65–68.

Ehret G. 1976. Development of absolute auditory thresholds in the house mouse (*Mus musculus*). J Am Audiol Soc 1:179–184.

Garden GA, Canady KS, Lurie DI, Bothwell M, Rubel EW. 1994. A biphasic change in ribosomal conformation during transneuronal degeneration is altered by inhibition of mitochondrial, but not cytoplasmic protein synthesis. J Neurosci 14:1994–2008.

Gavrieli Y, Sherman Y, Ben-Sasson SA. 1992. Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. J Cell Biol 119:493–501.

Hartlage-Rübsamen M, Rubel EW. 1996. Influence of mitochondrial protein synthesis inhibition on deafferentation-induced ultrastructural changes in the nucleus magnocellularis of developing chicks. J Comp Neurol 371:448–460.

Hashisaki GT, Rubel EW. 1989. Effects of unilateral cochlea removal on anteroventral cochlear nucleus neurons in developing gerbils. J Comp Neurol 283:5–73.

Hockenbery D, Nunez G, Milliman C, Schreiber RD, Korsmeyer SJ. 1990. Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death. Nature 348:334–336.

Hubel DH, Wiesel TN. 1970. The period of susceptibility to the physiological effects of unilateral eye closure in kittens. J Physiol (Lond) 206: 419–436.

Hyde GE, Durham D. 1994. Rapid increase in mitochondrial volume in nucleus magnocellularis neurons following cochlea removal. J Comp Neurol 339:27–48.

Kitzes LM, Kageyama GH, Semple MN, Kil J. 1995. Development of ectopic projections from the ventral cochlear nucleus to the superior olivary complex induced by neonatal ablation of the contralateral cochlea. J Comp Neurol 353:341–363.

Lachica EL, Zirpel L, Rubel EW. 1996. Intracellular mechanisms involved in the afferent regulation of neurons in the avian cochlear nucleus. In: Auditory system plasticity and regeneration. New York: Thieme. p 333–353.

Merry DE, Veis DJ, Hickey WF, Korsmeyer SJ. 1994. bcl-2 Expression is widespread in the developing nervous system and retained in the adult PNS. Development 120:301–311.

Michaelidis TM, Sendtner M, Cooper JD, Airaksinen MS, Holtmann B, Meyer M, Thoenen H. 1996. Inactivation of bcl-2 results in progressive degeneration of motoneurons, sympathetic and sensory neurons during early postnatal development. Neuron 17:75–89.

Moore DR, Rogers NJ, O'Leary SJ. 1998. Loss of cochlear nucleus neurons following aminoglycoside antibiotics or cochlear removal. Ann Otol Rhinol Laryngol 107:337–343.

Mukasa T, Urase K, Momoi MY, Kimura I, Momoi T. 1997. Specific expression of CPP32 in sensory neurons of mouse embryos and activation of CPP32 in the apoptosis induced by a withdrawal of NGF. Biochem Biophys Res Commun 231:770–774.

Nordeen KW, Killackey HP, Kitzes LM. 1983. Ascending projections to the inferior colliculus following unilateral cochlear ablation in the neonatal gerbil, *Meriones unguiculatus*. J Comp Neurol 214:144–253.

Pasic TR, Rubel EW. 1989. Rapid changes in cochlear nucleus cell size following blockade of auditory nerve electrical activity in gerbils. J Comp Neurol 283:474–480.

Pasic TR, Rubel EW. 1991. Cochlear nucleus cell size is regulated by auditory nerve electrical activity. Otolaryngol Head Neck Surg 104:6–13.

Pinon LG, Middleton G, Davies AM. 1997. Bcl-2 is required for cranial sensory neuron survival at defined stages of embryonic development. Development 124:4173–4178.

Rubel EW, Hyson RL, Durham D. 1990. Afferent regulation of neurons in the brain stem auditory system. J Neurobiol 21:169–196.

Sie KC, Rubel EW. 1992. Rapid changes in protein synthesis and cell size in the cochlear nucleus following eighth nerve activity blockade or cochlea ablation. J Comp Neurol 320:501–508.

Tierney TS, Russell FA, Moore DR. 1997. Susceptibility of developing cochlear nucleus neurons to deafferentation-induced death abruptly ends just before the onset of hearing. J Comp Neurol 378:295–306.

Trune DR. 1982. Influence of neonatal cochlear removal on the development of mouse cochlear nucleus: I. Number, size, and density of its neurons. J Comp Neurol 209:409–424.

Van der Loos H, Woolsey TA. 1973. Somatosensory cortex: structural alterations following early injury to sense organs. Science 179:395–398.

Veis DJ, Sorenson CM, Shutter JR, Korsmeyer SJ. 1993. Bcl-2-deficient mice demonstrate fulminant lymphoid apoptosis, polycystic kidneys, and hypopigmented hair. Cell 75:229–240.

Webster DB. 1983. A critical period during postnatal auditory development of mice. Int J Pediatr Otorhinolaryngol 6:107–118.

Webster DB. 1988. Conductive hearing loss affects the growth of the cochlear nuclei over an extended period of time. Hearing Res 32:185–192.

Willott JF, Bross LS, McFadden SL. 1994. Morphology of the cochlear nucleus in CBA/J mice with chronic, severe sensorineural cochlear pathology induced during adulthood. Hearing Res 74:1–21.

Zirpel L, Lachica EA, Lippe WR. 1995. Deafferentation increases the intracellular calcium of cochlear nucleus neurons in the embryonic chick. J Neurophysiol 74:1355–1357.