Biochemical and Biophysical Research Communications **284,** 142–149 (2001)
doi:10.1006/bbrc.2001.4939, available online at http://www.idealibrary.com on IDEAL®

Deafness Due to Degeneration of Cochlear Neurons in Caspase-3-Deficient Mice

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Received April 25, 2001

Mice that lack caspase-3, which functions in apoptosis, were generated by gene targeting and shown to undergo hearing loss. The ABR threshold of the caspase-$3^{-/-}$ mice was significantly elevated compared to that of caspase-$3^{+/+}$ mice at 15 days of age and was progressively elevated further by 30 days. Distortion product otoacoustic emissions were not detectable in caspase-$3^{-/-}$ mice at 15 days of age. Caspase-$3^{-/-}$ mice exhibited marked degeneration of spiral ganglion neurons and a loss of inner and outer hair cells in the cochlea at 30 days of age, although no such changes were apparent at 15 days. The degenerating neurons manifested features, including cytoplasmic vacuolization, distinct from those characteristic of apoptosis. Spiral ganglion neurons and cochlear hair cells thus appear to require caspase-3 for survival but not for initial development. The mapping of both the human caspase-3 gene and the locus responsible for an autosomal dominant, nonsyndromic form of hearing loss (DFNA24) to chromosome 4q35 suggests that the caspase-$3^{-/-}$ mice may represent a model of this human condition.

Key Words: **apoptosis; caspase-3; gene targeting; spiral ganglion; hair cell; ABR; DPOAE; vacuolization; hearing loss; DFNA24.**

The vertebrate inner ear is a complex organ that comprises the vestibular structures responsible for maintenance of balance and the cochlear structures required for hearing. The cochlea is a spiral duct that contains the organ of Corti, a specialized epithelium important in the reception of auditory stimuli that consists of two types of sensory receptor cells, or hair cells, arranged along the entire length of the cochlea (1, 2). The inner hair cells (IHCs) form a single row, whereas the outer hair cells (OHCs) are arranged in three parallel rows. The organ of Corti receives afferent innervation from sensory neurons of the cochlear (spiral) ganglion, which convey auditory information from the hair cells to auditory (cochlear) nuclei in the brainstem.

The development of the inner ear from a simple otocyst to the intricate mature sensory structure is accompanied by the proliferation, differentiation, and programmed death (apoptosis) of various cell types (3–9). Studies with genetically engineered mice have suggested that the auditory and vestibular neurons of the statoacoustic ganglion require one or more of the neurotrophins, including brain-derived neurotrophic factor and neurotrophin-3, for survival but not for their initial development (10–13). These neurotrophins may thus surppress activation of the apoptosis machinery in the inner ear.

Caspases are cysteine proteases that play an important role in the effector phase of apoptosis. Of the large number of caspases that have been identified, caspase-3 appears to be a prominent mediator of neuronal apoptosis (8, 14, 15). Activation of caspase-3 during apoptosis is achieved by the proteolytic processing of procaspase-3 into the mature form of the enzyme

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0006-291X/01 $35.00

(16, 17). Once activated, caspase-3 in turn directly activates specific DNases and cleaves other cellular proteins, resulting in DNA fragmentation, condensation of cellular organelles, and other morphological hallmarks of apoptosis (18–21). Activated caspase-3 is abundant both in the peripheral nervous system, including dorsal root ganglia, and in the cortical plate of the central nervous system during the later stages of mammalian development (22, 23). Brain development is markedly abnormal in caspase-3-deficient mice, being characterized by a variety of hyperplasias and disorganized cell deployment as a result of a reduced extent of apoptosis (24). Caspase-3 thus plays an important role in neuronal death during development.

Although the morphological sequence of events, including apoptosis, that underlies the development of the inner ear is relatively well characterized, the molecular mechanisms responsible for the development and maintenance of the complex structure of this organ remain unclear. We have now shown that activated caspase-3 is expressed in the statoacoustic ganglion complex, the cells of which develop into spiral ganglion neurons, during early mouse embryonic development. Furthermore, we generated caspase-3 knockout mice and showed that these animals develop deafness with accompanying degeneration of spiral ganglion neurons and hair cells in the inner ear, suggesting that caspase-3 is required for the survival of ganglion cells and hair cells. Given that the human caspase-3 gene has been mapped to a chromosomal position close to that implicated in a form of nonsyndromic hearing loss (25–27), the caspase-3-deficient mice may represent a model for this congenital form of human deafness.

MATERIALS AND METHODS

Generation of caspase-3 knockout mice. Cloned genomic DNA corresponding to the caspase-3 locus was isolated from a 129/Sv mouse genomic library (Stratagene). The targeting vector, pCasp3.KO, was constructed by replacing a 9.8-kb *Hinc*II–*Hin*dIII genomic fragment containing the nucleotide sequence for the QACRG pentapeptide motif of the catalytic site of caspase-3 with a PGK-neo-poly (A) cassette (28). The vector thus contained 0.9-kb (*Spe*I–*Hinc*II) and 6.7-kb (*Hin*dIII–*Hin*dIII) regions of homology 5′ and 3′ of the neomycin resistance marker, respectively. The PGK-tk-poly (A) cassette was ligated at the 3′ end of the insert. The maintenance, transfection, and selection of embryonic stem (ES) cells were performed as described (29). The mutant ES cells were microinjected into C57BL/6 blastocysts, and the resulting male chimeras were mated with female C57BL/6 mice. Heterozygous offspring were intercrossed to produce homozygous mutant animals. Homozygous caspase-3 null mice were backcrossed to C57BL/6 mice for more than 3 years. All mice were maintained in a specific pathogen-free animal facility at the Medical Institute of Bioregulation, Kyushu University.

Hearing tests. Caspase-$3^{+/+}$, caspase-$3^{+/-}$, and caspase-$3^{-/-}$ mice, ranging in age from 15 days to 12 months, were studied. The hearing of the mice was tested by measurement of the auditory brainstem response (ABR) and distortion product otoacoustic emissions (DPOAEs). The animals were tested every 3 or 4 days from postnatal day (P) 15 to P30, after which they were tested once a month. The mice were anesthesized by intraperitoneal injection of pentobarbital (60 mg per kilogram of body mass). All tests were performed in a soundproof room. The ABR was recorded with a SYNAX ER1100 system (NEC, Tokyo, Japan). The subcutaneous needle electrodes were placed at the vertex, in the right retroauricular region, and in the presacral region of the mice. For each recording, responses to 1000 clicks (100 μs duration) were averaged. The stimuli were delivered through a probe designed to fit the external ear canal of the mice. The thresholds were determined visually in 5-dB steps.

DPOAEs were analyzed with an ILO292 system (Otodynamics, Herts, UK). The acoustic probe was lengthened with a tapered plastic tube to ensure a tight fit in the external ear canal and the formation of a closed acoustic system. The f_2/f_1 frequency ratio was maintained at 1.22, and the value of f_2 was varied from 1 to 6 kHz (eight points). The intensities of the sounds at each frequency (f_1 and f_2) were ~60 dB. The signal-to-noise ratios (SNRs) of DPOAE ($2f_1-f_2$) data at the various f_2 values were obtained. A DPOAE was defined as present when the SNR exceeded 3 dB.

Immunohistochemistry. Inner ears were fixed with 4% paraformaldehyde in phosphate-buffered saline (pH 7.5) (PBS) and processed for immunohistochemical analysis. For detection of myosin VIIa and F-actin, dissected tissue was permeabilized with 0.2% Triton X-100 in PBS and then incubated overnight at 4°C with rabbit polyclonal antibodies to myosin VIIa (kindly provided by C. Petit); immune complexes were detected with fluorescein isothiocyanate-conjugated goat antibodies to rabbit immunoglobulin G (Jackson ImmunoResearch). The tissue was subsequently incubated with rhodamine-conjugated phalloidin (Molecular Probes) to reveal the F-actin-rich stereocilia of hair cells. Micrographs were compiled from *z* series scans with a Zeiss LSM 410 inverted laser-scanning microscope. For detection of activated caspase-3, dissected tissue was treated with 0.3% H_2O_2 for 15 min at room temperature, after which nonspecific sites were blocked by incubation with normal goat serum for 60 min. Tissue was then incubated overnight at 4°C with rabbit polyclonal antibodies to activated caspase-3 (kindly provided by T. Momoi). After three 5-min washes with PBS, sections were exposed to biotinylated secondary antibodies and immune complexes were detected with a streptavidin-biotin-peroxidase detection kit (Vectastain Elite; Vector, Burlingame, CA) and diaminobenzidine (Wako, Osaka, Japan).

Transmission electron microscopy (TEM). Cochleae from P30 and P60 mice were perfused through the round window with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) and then subjected to immersion fixation for 2 h at 4°C. After incubation for 1 h at room temperature with 1% osmium tetroxide in the same buffer, they were rinsed with distilled water, dehydrated in a graded series of ethanol solutions, and embedded in Epon 812. Thin sections (1 μm) were prepared with a Sorvall MT2-B ultramicrotome, stained with uranyl acetate and lead citrate, and examined with a JEM-1200EX electron microscope operated at 100 kV.

RESULTS

Expression of activated caspase-3 in the developing inner ear of wild-type mice. With the use of gene targeting in ES cells, we have generated mice that lack caspase-3. Unlike caspase-$3^{-/-}$ mice previously described (20, 24), our caspase-3 knockout mice exhibit neither premature death nor morphological abnormalities in the brain; this difference in phenotype is likely due to a difference in genetic background resulting from the extensive backcrossing of our caspase-$3^{-/-}$ mice with C57BL/6 mice. The absence of caspase-3 expression in the homozygous mutant animals was confirmed by reverse transcription and polymerase chain reaction analysis (data not shown) and by immu-

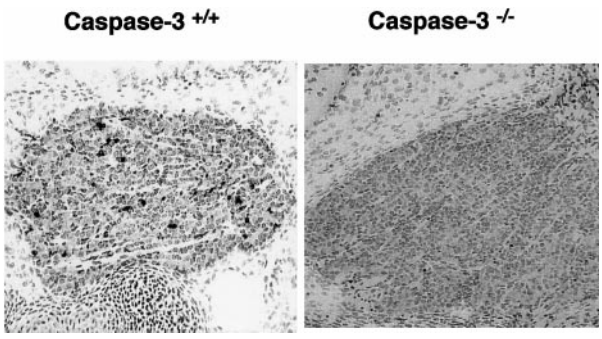

FIG. 1. Expression of activated caspase-3 in the developing statoacoustic ganglion of wild-type mice. Sections of the statoacoustic ganglion from caspase-$3^{+/+}$ (left panel) and caspase-$3^{-/-}$ (right panel) mice at embryonic day 14.5 were subjected to immunohistochemical analysis with antibodies to activated caspase-3. Scattered positive signals are apparent in the wild-type mice, but not in the caspase-$3^{-/-}$ mice. Original magnification, ×200.

nohistochemistry (Fig. 1). The active form of caspase-3 was detected in the statoacoustic ganglion, the primordial spiral ganglion, early during inner ear development (embryonic day 14.5) in wild-type mice but not in caspase-$3^{-/-}$ mice (Fig. 1). The expression of activated caspase-3 in the developing inner ear of wild-type mice suggests that certain statoacoustic ganglion neurons undergo apoptosis during this period, probably as a result of insufficiency of neurotrophins.

Increased threshold of the ABR in caspase-$3^{-/-}$ mice. Given the expression of activated caspase-3 in the developing inner ear of wild-type mice, we tested caspase-$3^{+/+}$, caspase-$3^{+/-}$, and caspase-$3^{-/-}$ mice for the Preyer reflex. Most of the caspase-$3^{-/-}$ mice showed a negative response (data not shown). We then subjected caspase-$3^{+/+}$ ($n = 22$), caspase-$3^{+/-}$ ($n = 17$), and caspase-$3^{-/-}$ ($n = 26$) mice, ranging in age from 15 days to 12 months, to click-evoked ABR testing. The ABR threshold was higher in 15-day-old caspase-$3^{-/-}$ mice than in caspase-$3^{+/+}$ littermate controls of the same age (Fig. 2A). Indeed, the threshold already exceeded 85 dB in some 15-day-old caspase-3 knockout mice (data not shown). The ABR threshold progressively decreased for both wild-type (Figs. 2A and 2B) and heterozygous (data not shown) mice between 15 and 30 days after birth. In contrast, the ABR threshold of caspase-$3^{-/-}$ mice progressively increased from 15 to 30 days after birth (Figs. 2A and 2B).

C57BL/6 mice undergo age-related hearing loss after 5 to 8 months of age (30). Nevertheless, the difference in ABR threshold between caspase-$3^{+/+}$ or caspase-$3^{+/-}$ mice and caspase-$3^{-/-}$ mice was still apparent in older mice (>4 months) (Fig. 2C). Whereas the ABR threshold of some caspase-3 knockout mice was >85 dB (the maximum sound pressure level tested), no heterozygous or wild-type animals exhibited such a high threshold at any age.

Absence of DPOAEs in caspase-$3^{-/-}$ mice. Cochlear function is critical for the generation of otoacoustic emissions (31–33). We therefore further evaluated cochlear function in caspase-$3^{-/-}$ mice by measurement of DPOAE ($2f_1-f_2$) at various times after birth. The SNRs of DPOAE values at f_2 frequencies of 1 to 6 kHz were obtained, with a DPOAE being defined as present when the SNR is >3 dB. We compared the results obtained from mice of different caspase-3 genotypes under the condition ($f_1 = 5200$ Hz, $f_2 = 6348$ Hz) that yielded the largest DPOAE. The earliest age at which a positive DPOAE was obtained from all wild-type mice was 15 days. At this age, all of the heterozygous mice tested also showed positive DPOAEs but none of the caspase-$3^{-/-}$ mice did (Fig. 3, Table 1). Identical results were obtained at 30 days (Table 1) and at all ages tested up to 3 months (data not shown). Together with the results of ABR testing, these data indicate that the lack of caspase-3 results in a profound hearing loss.

Loss of hair cells in the inner ear of caspase-$3^{-/-}$ mice. Cochleae dissected from caspase-3-deficient mice and wild-type littermates at 15 and 30 days of age were processed for whole-mount immunostaining with antibodies to myosin VIIa (Fig. 4) and staining of F-actin with rhodamine-phalloidin (data not shown). IHCs and OHCs were recognized on the basis of positive staining for myosin VIIa and the presence of stereocilia containing F-actin. At 15 days of age, the morphology of IHCs and OHCs throughout the entire cochlea of caspase-$3^{-/-}$ mice appeared identical to that of hair cells in wild-type mice. However, 30-day-old caspase-$3^{-/-}$ mice exhibited a loss of IHCs and OHCs from various regions of the organ of Corti, disrupting the continuity of hair cell distribution. The distribution of IHCs and OHCs in the organ of Corti was normal, with no degeneration apparent, in heterozygous mice at both 15 and 30 days of age (data not shown).

Progressive degeneration of the spiral ganglion in caspase-$3^{-/-}$ mice. We next examined the development and survival of the spiral ganglion in caspase-$3^{+/+}$ and caspase-$3^{-/-}$ mice by histological analysis at 15, 30, and 60 days of age. No marked change was apparent in the spiral ganglion of knockout mice at 15 days of age, although the number of cells appeared slightly increased (Fig. 5). However, a pronounced degeneration of the spiral ganglion, with a marked reduction in cell number, was observed in 30-day-old caspase-$3^{-/-}$ mice. This phenotype was further exaggerated in 60-day-old caspase-$3^{-/-}$ mice; most neurons had been lost from the ganglion and the remaining cells appeared to consist predominantly of microglia.

The death of the ganglion neurons in caspase-$3^{-/-}$ mice did not exhibit characteristics of apoptosis; neither chromatin condensation nor nuclear fragmentation was evident. Rather, cytoplasmic damage, with extensive swelling of organelles and vacuolization, was

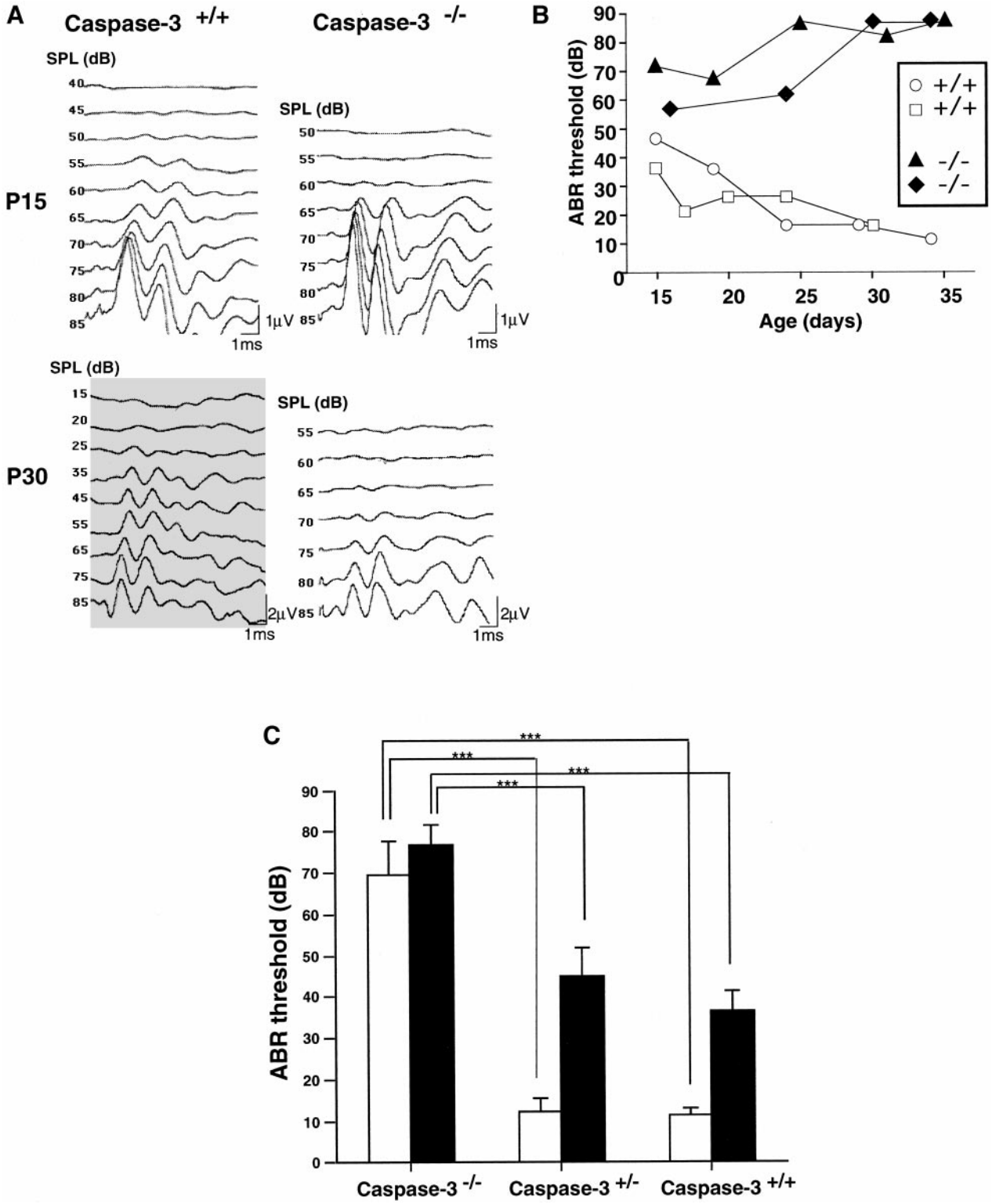

FIG. 2. Increased threshold of the ABR in caspase-$3^{-/-}$ mice. (A) ABR recordings from caspase-$3^{+/+}$ and caspase-$3^{-/-}$ mice at 15 (P15) and 30 (P30) days of age. SPL, sound pressure level. (B) Changes in the ABR threshold of two representative caspase-$3^{+/+}$ and caspase-$3^{-/-}$ mice with age. (C) ABR thresholds of caspase-$3^{+/+}$, caspase-$3^{+/-}$, and caspase-$3^{-/-}$ mice at 1 to 3 months (open columns) and 4 to 12 months (closed columns) of age. Data are means ± SEM of values from 17 to 26 mice. ***$P < 0.001$ (Student's t test).

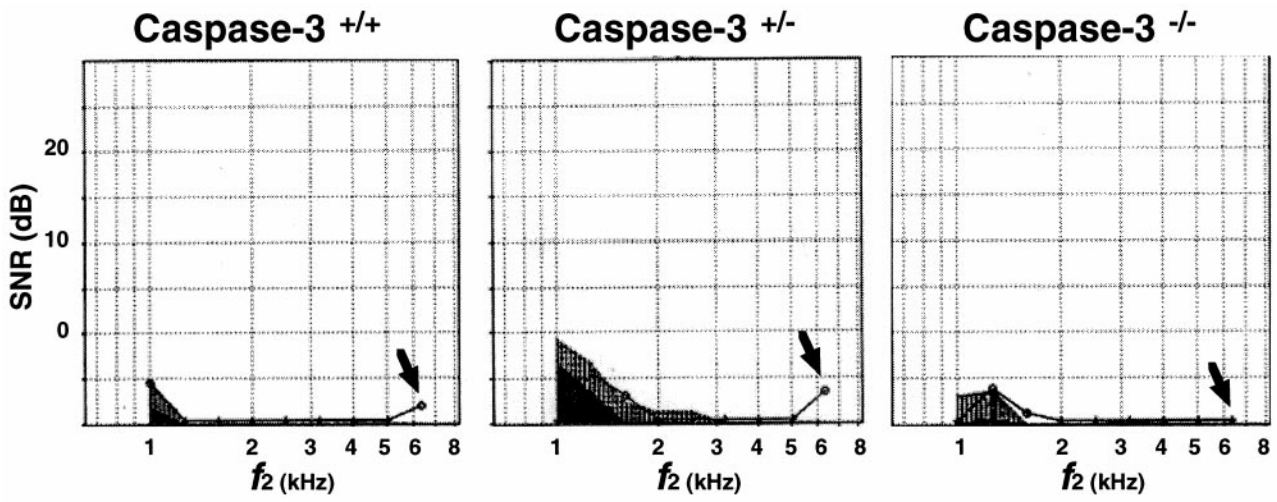

FIG. 3. Absence of DPOAEs in caspase-3$^{-/-}$ mice. DPOAEs were recorded from caspase-3$^{+/+}$, caspase-3$^{+/-}$, and caspase-3$^{-/-}$ mice at 15 days of age. Representative recordings are shown, and the results obtained from all animals tested are summarized in Table 1. SNRs of >3 dB for the f_2 value of 6348 Hz (arrows) were defined as positive.

the most prominent characteristic revealed (Fig. 6A). A similar type of neuronal cell death was recently described (34, 35). Microglial cells in the degenerated spiral ganglia of caspase-3$^{-/-}$ mice both extended processes toward the dead neurons and contained myelin (Figs. 6B and 6C), probably as a result of phagocytosis.

DISCUSSION

Caspase-3 is thought to be a central player in apoptosis. Previous studies showed that brain development is markedly impaired in caspase-3$^{-/-}$ mice, being characterized by various hyperplasias and disorganized cell differentiation, and that these animals die at 1 to 3 weeks of age (20, 24). The caspase-3$^{-/-}$ mice described in the present study, however, exhibit neither such histopathologic abnormalities, other than that of the inner ear, nor premature death. This difference in the phenotypes of these two lines of caspase-3 knockout mice is likely due to a difference in genetic background, which in our mice is virtually identical to that of the C57BL/6 strain. The dependence of brain development and survival on caspase-3 may thus be lower in the C57BL/6 strain than in the 129 strain background of the previously generated caspase-3$^{-/-}$ mice. Nevertheless, the inner ear appears to require caspase-3 for maintenance of its highly elaborate structure in our caspase-3 knockout mice.

TABLE 1

The Numbers of Caspase-3$^{+/+}$, Caspase-3$^{+/-}$, and Caspase-3$^{-/-}$ Mice Showing Positive DPOAEs

Age (days)	Caspase-3$^{+/+}$	Caspase-3$^{+/-}$	Caspase-3$^{-/-}$
15	9 (9)	3 (3)	0 (5)
30	13 (13)	4 (4)	0 (4)

Note. The total numbers of mice tested are shown in parentheses.

With the use of functional tests, including measurement of the ABR and DPOAEs, we have shown that caspase-3 deficiency results in hearing loss. The ABR threshold of the caspase-3$^{-/-}$ mice was significantly elevated at 15 days of age, and thereafter progressively elevated until 30 days after birth. DPOAEs were not detected in caspase-3$^{-/-}$ mice at 15 days of age or older. Histopathologic analyses further revealed that this sensory deficiency is accompanied by a loss of hair cells in the organ of Corti as well as by degeneration of spiral ganglion neurons. Although microscopic analysis detected no prominent abnormalities in the cochlea of 15-day-old caspase-3 knockout mice, some of the IHCs and OHCs of these animals were lost and spiral ganglion neurons had degenerated by 30 days of age. By 60 days after birth, most spiral ganglion neurons of the mutant mice had been lost. TEM analysis of caspase-3$^{-/-}$ mice at this age revealed that microglial cells in the spiral ganglion contained myelin, likely derived from engulfment and phagocytosis of neurons.

Degenerating spiral ganglion neurons exhibit the distinct morphological features of apoptosis. Light and electron microscopic analysis of caspase-3$^{-/-}$ mice revealed that the degenerating spiral ganglion neurons exhibited morphological features of necrosis, which is characterized, in part, by the onset of cytoplasmic vacuolization before changes in the nucleus are apparent. The degenerating cells thus did not manifest an early coalescence of nuclear chromatin into multiple nuclear bodies, compaction of the cytoplasm, cell shrinkage, or the budding-off of "apoptotic bodies," all of which are features of apoptosis. Recent studies have also de-

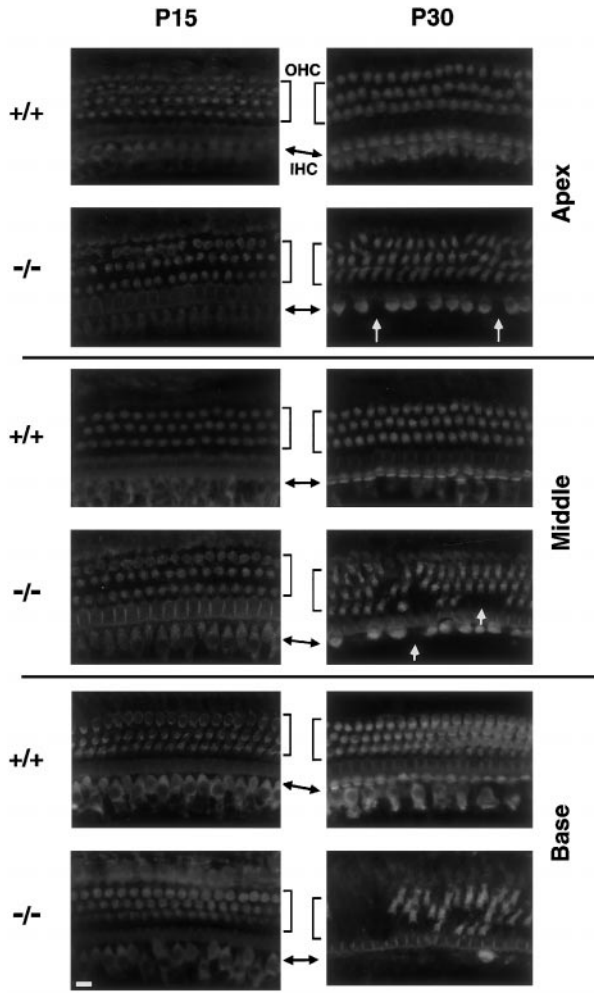

FIG. 4. Loss of hair cells in caspase-3$^{-/-}$ mice. Cochleae dissected from wild-type (+/+) and caspase-3 knockout (−/−) mice at 15 days (P15) or 30 days (P30) of age were processed for whole-mount immunostaining with antibodies to myosin VIIa. The positions of IHCs and OHCs, recognized by positive staining for myosin VIIa, are indicated by the double-headed arrows and brackets, respectively, for the apex, middle portion, and base of the cochlea. At 15 days of age, the morphology of IHCs and OHCs throughout the cochlea appeared identical in wild-type and caspase-3$^{-/-}$ mice. The 30-day-old homozygous mutants, however, exhibited a loss of IHCs and OHCs (white arrows) in various regions of the organ of Corti. Scale bar, 10 μm.

scribed a type of neuronal death, characterized by extensive vacuolization, similar to that apparent in the spiral ganglion neurons (34, 35). We therefore suggest that this type of cell death may not require caspase-3.

Although our caspase-3$^{-/-}$ mice did not exhibit a loss of hair cells or degeneration of spiral ganglion neurons until 30 days of age, a functional impairment of the inner ear was detected by ABR and DPOAE testing as early as 15 days after birth. We therefore cannot exclude the possibility that the observed structural changes are not the cause of the functional defect. A more detailed morphological analysis of the cochlea of caspase-3$^{-/-}$ mice at the ultrastructural level throughout the time course of hearing loss will be required to provide greater insight into the relation between the structural and functional abnormalities.

Although the molecular mechanism responsible for the cell death in the inner ear of caspase-3$^{-/-}$ mice remains unclear, we propose two possibilities. Firstly, substrates normally cleaved by caspase-3 may accumulate in the mutant animals. Such substates might be expressed specifically in the inner ear, or they might be expressed ubiquitously but specifically damage sensory neurons in the inner ear. Alternatively, cells that are destined to die during normal development might remain in the caspase-3$^{-/-}$ mice, resulting in an insufficiency of neurotrophic factors for neurons in the organ of Corti or in the spiral ganglion. Neurotrophin-3 is expressed in the developing spiral ganglion (36–38), and most spiral ganglion neurons undergo degeneration in mice deficient in this protein (11–13), suggesting the importance of neurotrophic factors for the survival of spiral ganglion neurons.

The human caspase-3 gene has been mapped to chromosome 4q35 (25–27), a region that also contains the locus for DFNA24, an autosomal dominant, nonsyndromic form of hearing loss (27). Nonsyndromic hearing loss is one of the most genetically heterogeneous

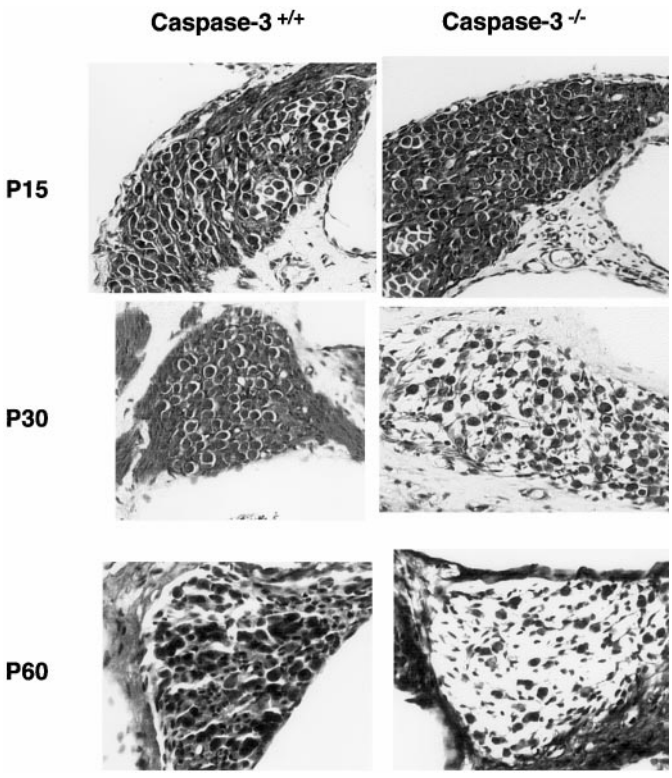

FIG. 5. Progressive degeneration of spiral ganglion neurons in caspase-3$^{-/-}$ mice. Spiral ganglia from caspase-3$^{+/+}$ and caspase-3$^{-/-}$ mice at 15 days (P15), 30 days (P30), and 60 days (P60) of age were subjected to histological analysis (hematoxylin-eosin staining). Original magnification, ×400.

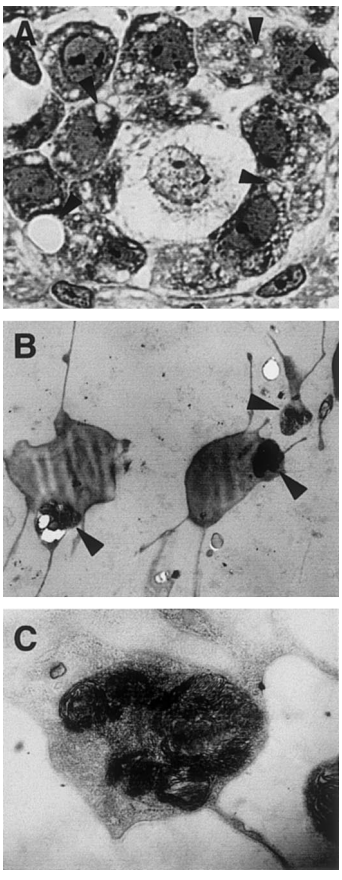

FIG. 6. Ultrastructural analysis of dying neurons and of microglia in the spiral ganglion of caspase-$3^{-/-}$ mice. (A) Extensive vacuolization (arrowheads) in the spiral ganglion neurons of a caspase-$3^{-/-}$ mouse at 30 days of age. Original magnification, ×1000. (B) Microglial cells extending processes toward and engulfing dead neurons (arrowheads) in the spiral ganglion of a 60-day-old caspase-$3^{-/-}$ mouse. Original magnification, ×6000. (C) Microglia in the spiral ganglion of a 60-day-old caspase-$3^{-/-}$ mouse exhibiting the laminar structure of myelin derived from engulfed neurons. Original magnification, ×30,000.

inherited traits known (39), with more than 60 loci having been mapped. Although the hearing loss apparent in caspase-$3^{-/-}$ mice is recessive (no hearing impairment was detected in heterozygotes), it remains possible that individuals with DFNA24 may contain a dominant negative mutation in the caspase-3 gene.

During preparation of this manuscript, Takahashi *et al.* also described degeneration of acoustic sensory neurons in caspase-3-deficient mice (40). Only young animals (<5 weeks of age) were analyzed in this previous study, which focused on the abnormal morphology of hair cells and supporting cells. In contrast, our study focuses on the degeneration of spiral ganglion neurons and presents data obtained with animals up to 1 year of age. Together, both studies indicate that the lack of caspase-3 causes severe hearing loss as a result of the degeneration of sensory neurons in the inner ear.

ACKNOWLEDGMENTS

We thank C. Petit for antibodies to myosin VIIa; T. Momoi for antibodies to activated caspase-3; Y. Yamada and K. Shimoharada for technical assistance; and M. Kimura for help with preparation of the manuscript. This study was supported in part by a grant from the Ministry of Education, Science, Sports, and Culture of Japan.

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