Requirement for Brn-3c in maturation and survival, but not in fate determination of inner ear hair cells

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SUMMARY

Mutations in the POU domain gene Brn-3c causes hearing impairment in both the human and mouse as a result of inner ear hair cell loss. We show here that during murine embryogenesis, Brn-3c is expressed in postmitotic cells committed to hair cell phenotype but not in mitotic progenitors in the inner ear sensory epithelium. In developing auditory and vestibular sensory epithelia of Brn-3c−/− mice, hair cells are found to be generated and undergo initial differentiation as indicated by their morphology, laminar position and expression of hair cell markers, including myosins VI and VIIa, calretinin and parvalbumin. However, a small number of hair cells are anomalously retained in the supporting cell layer in the vestibular sensory epithelia. Furthermore, the initially differentiated hair cells fail to form stereociliary bundles and degenerate by apoptosis in the Brn-3c−/− mice. These data indicate a crucial role for Brn-3c in maturation, survival and migration of hair cells, but not in proliferation or commitment of hair cell progenitors.

Key words: Brn-3c, POU domain, Inner ear, Sensory hair cell, Apoptosis, Mouse

INTRODUCTION

The mammalian inner ear contains two sensory organs, the cochlea and vestibule, which are responsible for the senses of hearing and balance, respectively. In each of the sensory organs, there are specialized sensory neuroepithelia that are composed of highly organized mechanosensory hair cells and supporting cells. During inner ear development, the sensory epithelia are derived from the otic vesicle, which originates from an ectodermal placode. In the mouse, previous birth-dating analyses using tritiated-thymidine labeling have shown that most of the cochlear hair cells undergo terminal mitosis at E13-E15, and most of the vestibular hair cells at E14-E18 (Ruben, 1967). The initial differentiation of hair cells appears to occur immediately after terminal mitosis, as judged by expression of an early hair cell marker, calretinin (Zheng and Gao, 1997). During late embryonic and early postnatal stages, hair cells start to mature by growing stereociliary bundles shortly after initial differentiation.

The early inner ear development involves several processes including acquisition of placodal competence, specification of the otic field, commitment of the otic fate, and regional and cell fate specification and differentiation (Fekete, 1996; Torres and Giraldez, 1998). All these processes depend on intricate cell-cell interaction, and inductive/inhibitory signals from surrounding mesodermal and neuroectodermal tissues. Recent molecular genetic studies have identified a number of transcription factors that play an important role in these developmental processes. These include the basic leucine zipper factor Kreisler, and the homeodomain factors Hoxa-1 and Pax-3, which are expressed only in neural tube and crest but affect inner ear development (Deol, 1964, 1966; Epstein et al., 1991; Chisaka et al., 1992; Cordes and Barsh, 1994), and homeodomain factors Pax-2 and Nkx5.1/Hmx3, which are required for regional specification of the otocyst (Torres et al., 1996; Favor et al., 1996; Hadrys et al., 1998; Wang et al., 1998).

Two POU domain transcription factors are also found to be critical for proper inner ear development. The POU domain transcription factors are a family of important developmental regulators. Many of them have distinct expression patterns in the nervous system, and have been shown by genetic studies in mice and humans to play critical roles in neural development (reviewed in Rosenfeld, 1991; Treacy and Rosenfeld, 1992; Wegner et al., 1993; Herr and Cleary, 1995; Ryan and Rosenfeld, 1997). The POU-domain functions as a bipartite DNA binding domain that contains a POU-specific domain of about 70 amino acids and a POU-homeodomain of about 60 amino acids, joined by a variable linker. It was first identified in the mammalian pituitary-specific factor Pit-1, the octamer binding proteins Oct-1 and Oct-2, and the C. elegans factor Unc-86 (Herr et al., 1988). Based on sequence homology in...
the POU domain, the POU domain protein family members are subdivided into several classes. The class III factor Brn-4/RHS2/POU3F4 is expressed in the otic vesicle in the developing rat (Le Moine and Young, 1992; Mathis et al., 1992), and mutations in the corresponding human gene result in progressive sensorineural deafness and stapes fixation (de Kok et al., 1995). In developing and adult mice, the class IV factor Brn-3c/Brn-3.1/POU4F3 displays strong expression in the sensory hair cells of both the cochlear and vestibular systems. Targeted null mutation of this gene leads to loss of all the cochlear and vestibular hair cells, resulting in complete deafness and profound deficit in the vestibular system of Brn-3c-/- mice (Gerrero et al., 1993; Ninkina et al., 1993; Erkman et al., 1996; Xiang et al., 1995, 1997a,b). In the human, a small deletion in the Brn-3c coding region has also been linked to autosomal dominant hearing loss (Vahava et al., 1998). Therefore, Brn-3c plays an essential role in the development of inner ear sensory hair cells.

The absence of hair cells within Brn-3c-/- sensory epithelia indicates a requirement for the Brn-3c function in one or multiple steps of hair cell development, which could include proliferation and fate specification of hair cell progenitors, and initial differentiation, maturation and maintenance of the hair cell type. To determine the step(s) in which the Brn-3c gene may act during murine hair cell development, we performed immunolabeling with a Brn-3c antibody at various developmental stages. We show in this report that Brn-3c initiates its expression in the sensory epithelia as early as E12.5, temporally concomitant with the initial generation of hair cells during inner ear development. We also provide evidence that Brn-3c initiates its expression exclusively in postmitotic cells in developing inner ear sensory epithelia, suggesting an unlikelyhood for Brn-3c to be involved in the division of hair cell precursors. Using various histochemical and immunohistochemical analyses, we demonstrate that generation and initial differentiation of hair cells occur in the Brn-3c-/- inner ear. However, these initially differentiated hair cells fail to mature and form stereociliary bundles. In Brn-3c-/- vestibular sensory epithelia, a small set of cells are labeled positive for early hair cell markers but fail to properly migrate into the lumenal layer. By TUNEL labeling, we show in Brn-3c-/- mice that the immature, differentiating hair cells progressively degenerate via apoptosis during development. This loss of hair cells also causes apoptotic degeneration of secondary neurons in the spiral and vestibular ganglia. Our data indicate that Brn-3c is required for maturation, survival and proper positioning of hair cells, whereas it plays little role in commitment and initial differentiation of hair cells.

**MATERIALS AND METHODS**

**Experimental animals**

Wild-type C57BL/6J mice were purchased from the Jackson Laboratory. The day on which the copulatory plug was observed was referred to as embryonic day 0.5 (E0.5), and the day of birth was considered postnatal day 0 (P0). Brn-3c-/- mice were derived by targeted gene disruption as described (Xiang et al., 1997a). For comparison, only animals within a litter derived from Brn-3c-/- x Brn-3c-/- matings were analyzed. Genotyping was done by PCR amplification of mouse tail genomic DNA.

**Immunohistochemistry and histochemistry**

To prepare cryosections from the inner ear, embryos and neonates were fixed in 4% paraformaldehyde, cryoprotected in 30% sucrose, and embedded in OCT compound as described previously (Xiang et al., 1993; 1995). Immunostaining was performed using the ABC system (Vector Laboratories) according to the manufacturer’s protocol, except that sections were incubated overnight at 4°C in primary antibodies. Double immunostaining with anti-Brn-3c and anti-BrdU was carried out as described in Xiang et al. (1993), but rhodamine-conjugated goat anti-rabbit IgG was used. For double immunolabeling with anti-Brn-3c and anti-myosin VI or anti-myosin VIIa, single immunostaining with anti-Brn-3c was done first using the ABC system. Following the color reaction, the sections were rinsed with PBS, blocked in 5% normal goat serum, and incubated overnight at 4°C with anti-myosin VI or anti-myosin VIIa. Following three 7-minute washes in PBS, the sections were incubated with rhodamine-conjugated goat anti-rabbit IgG. Antibodies were obtained from the following sources: anti-Brn-3c, anti-Brn-3b and anti-Brn-3a (Xiang et al., 1993, 1995), anti-myosin VI (Hasson and Mooseker, 1994), anti-myosin VIIa (Hasson et al., 1995), anti-calretinin and anti-BrdU (Sigma) and anti-parvalbumin (SWant). Cresyl Violet labeling was performed as described (LаБossiere and Glickstein, 1976).

**BrdU pulse-labeling in vivo**

To label dividing cells in the inner ear sensory epithelia, timed pregnant C57BL/6J mice at E13.5 and E14.5 were injected intraperitoneally twice at 2-hour intervals with 5-bromodeoxyuridine (Sigma) in PBS at a dose of 100 mg/kg body mass. The labeled embryos were collected 2 hours after the second injection and processed for immunostaining as described above.

**Terminal dUTP nick end labeling (TUNEL)**

Following inactivation of endogenous peroxidase activity in 3% H2O2 in methanol for 3 minutes, inner ear sections were rinsed in ddH2O for 5 minutes and permeated in 0.1% Triton X-100 in PBS for 2 minutes at room temperature. After three 3-minute rinses in ddH2O, sections were incubated for 1 hour at 37°C in terminal transferase buffer (25 mM Tris-HCl, pH 6.6, 200 mM sodium cacodylate, 2.5 mM CoCl2 and 0.25 mg/ml bovine serum albumin) with 4 μM biotin-16-dUTP and 250 units/ml terminal transferase. Following three 5-minute washes in PBS, sections were processed for ABC staining (Vector Laboratories).

**RESULTS**

**Correlation of the onset expression of Brn-3c with the initiation of sensory hair cell production during the mouse inner ear development**

The absence of sensory hair cells in postnatal and adult Brn-3c-/- mouse inner ears indicates an essential role for Brn-3c in hair cell development (Erkman et al., 1996; Xiang et al., 1997a). However, it is not known whether the targeted null mutation of Brn-3c affects proliferation of hair cell progenitors, or differentiation and survival of hair cells. To distinguish these possibilities, we investigated by immunolabeling when Brn-3c was initially expressed during genesis of the mouse inner ear (Fig. 1). Serial otic cryosections from E11.5- E14.5 embryos were examined by immunostaining with a specific anti-Brn-3c antibody (Xiang et al., 1995).

In the otocyst, while no Brn-3c protein expression is found at E11.5 (Fig. 1A), by E12.5, Brn-3c commences its expression in scattered cells of the presumptive sensory epithelium, a small thickening of the otocyst wall (Fig. 1B). At E13.5, Brn-
3c is found in more sensory epithelia of the otocyst (Fig. 1C). Therefore, Brn-3c expression is temporally concomitant with the morphological appearance of sensory end organs during embryogenesis, formation of three cristae ampullaris and macula utriculi by E12, and separation of cochlea and macula sacculi by E13 (Sher, 1971; Li et al., 1978; Morsli et al., 1998). By E14.5 and E15.5, when all the inner ear sensory epithelia are formed, Brn-3c is expressed in increasing numbers of hair cells within all the sensory organs in both the cochlea and vestibular system, including the organ of Corti, maculae and cristae. This spatial pattern of Brn-3c expression persists to postnatal stages and adulthood (Fig. 1E-J; Erkman et al., 1996; Xiang et al., 1997a,b).

During mouse inner ear development, the sensory hair cells undergo terminal mitosis in a period spanning from E11-P2 (Ruben, 1967). Thus, the onset expression of Brn-3c at E12.5 temporally correlates with the initial generation of hair cells in the inner ear. Interestingly, in the developing vestibular sensory epithelia, not only is Brn-3c expressed in hair cells of the lumenal layer, but within the supporting cell layer it is also localized in a small population of cells which appear to stream toward the lumenal layer (Figs 1B,D, 3A,C,E). This phenomenon is observed in all the early developmental stages examined ranging from E12.5 to P2, including stages E17.5 and E19.5 not shown in Fig. 1. Similarly, Brn-3b has been shown to be turned on initially in the presumptive migrating postmitotic retinal ganglion cell precursors in the ventricular zone during early retinogenesis (Gan et al., 1996; Xiang, 1998). Therefore, the minor number of cells expressing Brn-3c in the supporting cell layer may represent cells that have just undergone terminal mitosis but have not properly positioned in the lumenal layer.

**Restrictive expression of Brn-3c in postmitotic inner ear sensory hair cells but not in mitotic progenitors**

To determine whether mitotic hair cell progenitors express Brn-3c, S-phase cells in E13.5 and E14.5 otocysts were pulse-labeled by the thymidine analog 5-bromodeoxyuridine (BrdU), followed by double-immunostaining using anti-Brn-3c and anti-BrdU antibodies (Fig. 1K). Within the vestibular sensory epithelia, anti-Brn-3c stained hair cells of the lumenal layer, as well as a small number of cells (arrowheads) in the supporting cell layer, which appear migrating toward the lumenal layer but do not overlap the S-phase cells stained by anti-BrdU. Anti-BrdU labeled a number of dividing cells in both the hair and supporting cell layers. However, none of the cells positive for Brn-3c colocalized with the proliferative cells, indicating an exclusive expression of Brn-3c in postmitotic cells in the mouse inner ear sensory organs.

In the developing sensory epithelia, the cells that express Brn-3c in the supporting cell layer could be either freshly generated, undifferentiated postmitotic hair cells, or dislocated differentiated hair cells. To distinguish these two possibilities, we screened for early structural hair cell markers using antibodies against myosin VI, myosin VIIa, calretinin and

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**Fig. 1.** Expression of Brn-3c in the developing mouse inner ear. (A-J) Temporal and spatial patterns of Brn-3c expression in the inner ear sensory epithelia. Cortal inner ear sections at indicated developmental stages were immunostained with anti-Brn-3c antibody. Brn-3c expression is initiated in presumptive hair cells in the otocyst at E12.5 and persists in cochlear and vestibular hair cells to adulthood. Arrows point to labeled cells in the supporting cell layer, which appear streaming toward the lumenal layer. Co, cochlea; Cri, crista; IHC, inner hair cell; OHC, outer hair cell; Oto, otolith organ; OV, otic vesicle; Sac, sacculae; Utr, utricule. (K) Postmitotic expression of Brn-3c in the inner ear sensory epithelia. Inner ear sections from BrdU-labeled E14.5 embryos were double immunostained with anti-Brn-3c (red) and anti-BrdU (green) antibodies. Anti-Brn-3c labeled hair cells in the lumenal hair cell (HC) layer, as well as a small number of cells (arrowheads) in the supporting cell (SC) layer, which appear migrating toward the hair cell layer but do not overlap the S-phase cells stained by anti-BrdU. Bar, 25 μm (A,B,D,E,H-K), 50 μm (C,F), 100 μm (G).
The hair cell-like cells present in the Brn-3c−/− mutant (Fig. 4F,H,J, arrows) compared with controls (Fig. 4E,G,I). To analyze more quantitatively the hair cell-like cells in Brn-3c−/− vestibular sensory epithelia, we counted the number of cells within the luminal hair cell layer of E18.5 and P4 maculae and cristae on serial sections labeled by Cresyl Violet (Fig. 7A). This quantitation showed that on average there were approximately 27%, 28% and 26%, respectively, of hair cell-like cells in Brn-3c−/− maculae and cristae of saccules and utricles, and in cristae of semicircular canals at later developmental stages in the Brn-3c−/− mutant (Fig. 4F,H,J, arrows) compared with controls (Fig. 4E,G,I). To analyze more quantitatively the hair cell-like cells in Brn-3c−/− vestibular sensory epithelia, we counted the number of cells within the luminal hair cell layer of E18.5 and P4 maculae and cristae on serial sections labeled by Cresyl Violet (Fig. 7A). This quantitation showed that on average there were approximately 27%, 28% and 26%, respectively, of hair cell-like cells in the sensory epithelia of the saccule, utricule and crista in the E18.5 Brn-3c−/− embryo compared to the Brn-3c+/+ embryo. At P4, these numbers were reduced to about 23%, 15% and 12%, respectively. Therefore, the hair cell-like cells within the vestibular sensory epithelium appear to gradually degenerate during development in the Brn-3c−/− mutant.

The hair cell-like cells present in the Brn-3c−/− inner ear sensory epithelia could be hair cell precursors, partially differentiated hair cells, or fully differentiated mature hair cells. To distinguish these possibilities, we examined by immunolabeling the expression patterns of several hair cell markers.

**Generation and initial differentiation of hair cells occur in developing Brn-3c+/− inner ear sensory epithelia**

Although it has been shown that targeted deletion of Brn-3c in mice causes complete loss of cochlear and vestibular hair cells in the adult (Xiang et al., 1997a), it remains unclear whether hair cells are initially generated in developing sensory epithelia of Brn-3c−/− mice (Erkman et al., 1996; Xiang et al., 1997a). To resolve this issue, we first examined all sensory epithelia of Brn-3c−/− mice at stages E16.5, E18.5, P1 and P4 by immunostaining the expression patterns of several hair cell markers. To distinguish these possibilities, we examined by immunolabeling the expression patterns of several hair cell markers.
markers in the inner ear sensory epithelia from E15.5-P8 Brn-3c−/− and control Brn-3c+/+ and Brn-3c+/− animals (Fig. 5). These hair cell markers included myosins VI and VIIa, calretinin and parvalbumin. Here we describe several observations made in the Brn-3c−/− inner ear using these hair cell markers.

First, in Brn-3c−/− sensory epithelia of both the cochlear and vestibular systems from E15.5-P8 animals, anti-myosin VI, anti-myosin VIIa and anti-calretinin all immunostained a small population of cells (Fig. 5A-R). Similarly, anti-parvalbumin also labeled a small number of cells in late embryonic and early postnatal stages in the mutant (Fig. 5S,T). Therefore, the morphologically identified hair cell-like cells present in Brn-3c−/− inner ear sensory epithelia (Fig. 4) are indeed hair cells that have undergone initial differentiation. Furthermore, a large number of hair cells appear to be degenerated in Brn-3c−/− sensory epithelia by as early as E15.5 (Fig. 5A,B), a peak time point of hair cell production (Ruben, 1967). Second, anti-myosin VI and anti-myosin VIIa stained a minor fraction of cells within the supporting cell layer in developing Brn-3c−/− vestibular sensory epithelia (Fig. 5B, arrows), whereas in the control Brn-3c+/+ and Brn-3c+/− epithelia, the cells positive for myosins VI and VIIa are located only to the hair cell layer (Fig. 5A). Therefore, it appears likely that the small number of fresh postmitotic hair cells normally expressing Brn-3c in the supporting cell layer can initiate hair cell differentiation but have difficulty in migrating or positioning into the lumenal layer in developing Brn-3c−/− vestibular sensory epithelia. Third, myosin VIIa is shown to be localized in cell bodies as well as in stereociliary bundles of adult inner ear hair cells (Hasson et al., 1997). We show by P8 that anti-myosin VIIa can clearly identify hair bundles of the cochlear and vestibular hair cells in control Brn-3c+/+ and Brn-3c+/− mice (Fig. 5M, arrowheads). However, no stereociliary bundles were labeled by anti-myosin VIIa in Brn-3c−/− cochlear and vestibular labyrinth by P8 although there are a significant number of hair cell bodies labeled (Fig. 5N). Consistent with this result, early electron microscopic studies have also failed to detect any stereociliary bundles in Brn-3c−/− inner ear sensory epithelia from P0 to adult stages (Erkman et al., 1996; Xiang et al., 1997a). Together, these data show that in the absence of Brn-3c, inner ear hair cells cannot undergo further maturation to form stereociliary bundles even though they are produced and able to initiate early hair cell differentiation.

**Degeneration of neurons in developing Brn-3c−/− spiral and vestibular ganglia**

Previous histochemical characterization of Brn-3c−/− mice has shown that loss of hair cells in cochlear and vestibular sensory epithelia causes severe to complete degeneration of secondary neurons in spiral and vestibular ganglia by late postnatal and adult stages (Erkman et al., 1996; Xiang et al., 1997a). We examined the generation and degeneration of spiral and vestibular ganglion neurons in Brn-3c−/− E15.5-P5 animals by Cresyl Violet labeling, and by immunostaining with antibodies against ganglion neuron markers, which included Brn-3a, Brn-3b, parvalbumin and calretinin (Fig. 6 and data not shown). In the developing sensory ganglia of wild-type mice, Brn-3a, Brn-3b, calretinin and parvalbumin are each expressed in most of the neurons (data not shown).

In the cochlea, Cresyl Violet labeling showed nearly normal development of Brn-3c−/− spiral ganglia by E16.5 (Fig. 6A,B). At E18.5, quantitation of neurons on serial spiral ganglion sections revealed a loss of approximately 13% of neurons in the Brn-3c−/− spiral ganglion compared to the Brn-3c+/− one (Fig. 7B). Only by P4/P5 does the Brn-3c−/− spiral ganglion become overtly smaller than the control Brn-3c+/+ and Brn-3c+/- spiral ganglia (Fig. 6L,J). Antibodies against Brn-3a, Brn-3b, calretinin and parvalbumin also stained a reduced number of cells in Brn-3c−/− spiral ganglia by P4/P5, as exemplified in Fig. 6O,P. Quantitation of neurons on serial sections showed a loss of approximately 29% of neurons in the Brn-3c−/− spiral ganglion at P4 (Fig. 7B).

In the Brn-3c−/− vestibular system, Cresyl Violet labeling showed a much faster degeneration of vestibular ganglion neurons than of spiral ganglion neurons. At E16.5, the Brn-3c−/− vestibular ganglion was significantly diminished in size compared to the control (Fig. 6C,D). By P4, the large majority of neurons in the Brn-3c−/− vestibular ganglion were lost (Fig.

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**Fig. 3.** Expression of Brn-3c in presumptive, newly produced hair cells in developing vestibular sensory epithelia. Inner ear sections from E15.5 (A-D) and P0 (E and F) embryos and pups were double immunostained with anti-Brn-3c (A,C,E) and anti-myosin VI (B) or anti-myosin VIIa (D,F). In developing vestibular end organs, hair cell nuclei positive for Brn-3c within the lumenal layer are colocalized with cells that express myosins VI and VIIa, whereas in the supporting cell layer, a small number of cells labeled by anti-Brn-3c (indicated by arrows) do not express either myosin VI or VIIa. Cri, crista; Utr, utricule. Bar, 25 µm (A-F).
Based on quantitation of neurons on serial sections, we estimated a loss of approximately 34% and 77%, respectively, of vestibular ganglion neurons at E18.5 and P4 in Brn-3c−/− mice (Fig. 7B). Immunostaining analysis of Brn-3c−/− vestibular ganglia showed a similar rapid degeneration of neurons positive for Brn-3a, Brn-3b or parvalbumin during late embryonic and early postnatal stages (Fig. 6G,H). Interestingly, the large and small ganglion neurons were temporally differentially degenerated in Brn-3c−/− vestibular ganglia. The large neurons degenerated first during development, with most of the large neurons lost by E16.5 (Fig. 6E,F). The degeneration of large neurons was completed by P4 and at this stage the 23% of neurons left in the Brn-3c−/− vestibular ganglion were all small neurons (Fig. 6M,N).

**Apoptosis-mediated degeneration of sensory hair cells and ganglion neurons in the Brn-3c−/− inner ear**

To understand how the partially differentiated hair cells degenerate during inner ear development in Brn-3c−/− mice, we tested by TUNEL assay whether there was an increased apoptotic cell death within the sensory epithelia of E15.5-P0 Brn-3c−/− animals (Fig. 8). Serial sections of vestibular sensory epithelia from Brn-3c−/− and control Brn-3c+/+ litters were labeled by TUNEL, and the number of apoptotic cells were quantitated and compared. At E18.5 and P0, we detected a low level of apoptotic cells within the maculae of saccule and utricle, and the cristae of semicircular canals in the wild type (Fig. 8A,C; Table 1), consistent with an earlier observation in the rat (Zheng and Gao, 1997). In the vestibular sensory epithelia of Brn-3c−/− mice, however, a significantly higher number of apoptotic cells was observed (Fig. 8B,D). An increase ranging from 23% to 375% of TUNEL-labeled cells was consistently found in Brn-3c−/− vestibular sensory epithelia, depending on different experiments and end organs (Table 1). Therefore, the partially differentiated hair cells within Brn-3c−/− inner ear appear to degenerate by apoptosis, indicating a critical role for Brn-3c in the maintenance of sensory hair cells. At E15.5 and E16.5, no significant rate change of apoptotic cell death was seen in Brn-3c−/− sensory epithelia (data not shown). This was because a large number of cells were labeled by TUNEL in control Brn-3c+/+ maculae and cristae, rendering it difficult to detect any small change in the number of apoptotic cells within Brn-3c−/− sensory epithelia.

The survival of spiral and vestibular ganglion neurons is speculated to depend on interaction with and neurotrophic supply of hair cells (Ernfors et al., 1994, 1995; Fritzsch et al., 1997). We investigated by TUNEL assay whether loss of hair cells resulted in the observed degeneration of inner ear sensory ganglion neurons by apoptosis in Brn-3c−/− mice. In control Brn-3c+/+ spiral and vestibular ganglia, a significant number of neurons were found undergoing apoptosis at E18.5 and P0 (Fig. 8E,G,I). In Brn-3c−/− mice, we observed a marked increase in the number of TUNEL labeled cells (Fig. 8F,H,J). Quantitation of serial sections showed an increase of 83-140% and 44-68% of apoptotic cells in the Brn-3c−/− spiral ganglion and vestibular ganglion, respectively (Table 1). Thus, the spiral and vestibular ganglion neurons gradually degenerate via apoptosis subsequent to the loss of sensory hair cells in the Brn-3c−/− inner ear.

**DISCUSSION**

The time course of inner ear hair cell development includes fate commitment/determination, initial differentiation, maturation/acquisition of stereociliary bundles and maintenance (Kelley et al., 1993; Fekete, 1996; Torres and...
Giraldez, 1998). The experiments reported here identify steps of hair cell development in which Brn-3c plays an important role. Examining expression patterns of Brn-3c and myosins VI and VIIa in the embryonic mouse inner ear by immunolabeling showed onset of Brn-3c expression at E12.5, corresponding to a time point when hair cells begin to be produced and express myosins VI and VIIa. Double immunostaining of developing vestibular sensory epithelia with anti-Brn-3c and anti-myosin VI or anti-myosin VIIa revealed that, while the large majority of Brn-3c-positive cells expressed myosins VI and VIIa, a small set of Brn-3c-positive cells within the supporting cell layer did not. Pulse-labeling of dividing cells by BrdU and double-immunostaining demonstrated the expression of Brn-3c was restricted only to postmitotic hair cells. Therefore, these expression data were consistent with a role for Brn-3c in differentiation and/or maintenance of hair cells but not in proliferation of mitotic hair cell precursors. We then set out to test this presumption by examining developing Brn-3c−/− sensory epithelia using Cresyl Violet staining, and immunolabeling with several hair cell markers including myosins VI and VIIa, calretinin and parvalbumin. We found in the Brn-3c−/− inner ear that: (1) hair cells can be initially generated; (2) hair cells can undergo initial differentiation; (3) the initially differentiated hair cells cannot mature to grow stereociliary bundles; and (4) a small number of hair cells fail to properly migrate into the lumenal hair cell layer. These observations indicate a crucial role for Brn-3c in maturation and proper migration of hair cells. Furthermore, using a TUNEL-labeling assay, we found Brn-3c is also essential for survival of hair cells as the absence of...
Table 1. Number of TUNEL-labeled cells in vestibular end organs and spiral and vestibular ganglia of Brn-3c<sup>+/+</sup> and Brn-3c<sup>−/−</sup> mice

<table>
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<th>Experiment</th>
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<td>Brn-3c&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>33</td>
<td>22</td>
<td>6</td>
<td>221</td>
<td>489</td>
</tr>
<tr>
<td></td>
<td>Percentage increase</td>
<td>64</td>
<td>57</td>
<td>22</td>
<td>530</td>
<td>803</td>
</tr>
<tr>
<td></td>
<td>Percentage increase</td>
<td>94</td>
<td>159</td>
<td>267</td>
<td>140</td>
<td>64</td>
</tr>
</tbody>
</table>

To determine the total number of cells undergoing apoptosis in each structure, serial sections from a pair of Brn-3c<sup>+/+</sup> and Brn-3c<sup>−/−</sup> mice of the same litter at E18.5 or P0 were labeled in situ by TUNEL, and the number of labeled cells was counted in all sections. Structures in both right and left ears were analyzed in each experiment (except for the crista), so each value represents the mean for two structures. For crista, each value represents the mean for four structures (cristae in superior, posterior and lateral semicircular canals were not differentiated for this analysis). Cri, crista; Sac, macula sacculi; SG, spiral ganglion; Utr, macula utriculi; VG, vestibular ganglion.

Fig. 6. Neuronal loss in Brn-3c<sup>−/−</sup> developing spiral and vestibular ganglia. (A-F and I-N) Cresyl Violet labeling of Brn-3c<sup>+/+</sup> (A,C,E,I,K,M) and Brn-3c<sup>−/−</sup> (B,D,F,J,L,N) spiral ganglia (SG) (A,B,I,J) and vestibular ganglia (VG) (C-F and K-N) from E16.5 (A-F) and P4 (I-N) mice. In Brn-3c<sup>−/−</sup> mice, vestibular ganglion neurons degenerate much faster than those of the spiral ganglia (A-D and I-L). In addition, the large VG neurons degenerate first and by P4 they are completely lost (E,F,M,N; the arrows in M point to large neurons, and the arrowheads to small neurons). (G,H) P2 VG sections from Brn-3c<sup>+/+</sup> (G) and Brn-3c<sup>−/−</sup> (H) mice immunolabeled with anti-parvalbumin. (O,P) P5 VG sections from Brn-3c<sup>+/+</sup> (O) and Brn-3c<sup>−/−</sup> (P) mice immunolabeled with anti-Brn-3b. Staining with these antibodies reveal a marked neuronal loss in VG by P2 (G,H), but only a moderate neuronal loss in SG by P5 (O,P). Bar, 50 μm (A-D,G,H,I-L), 16.7 μm (E,F), 12.5 μm (M,N); 25 μm (O,P).
Brn-3c protein in Brn-3c−/− mice prompts apoptotic hair cell death.

**Onset of Brn-3c expression temporally correlates with the beginning of terminal mitoses of hair cell progenitors and initial expression of early hair cell markers**

The terminal mitoses of mouse inner ear hair cell progenitors occur between E11 and P2 (Ruben, 1967). Cells expressing Brn-3c first emerge in the sensory epithelia around E12.5 (Fig. 1), suggesting a good correlation between Brn-3c expression and hair cell genesis. Interestingly, myosins VI and VIIa are also found to be early hair cell markers as they commence their expression in hair cells at E13.5 (Fig. 2), 1 day after the onset of Brn-3c expression. Since myosins VI and VIIa are structural proteins, their expression may mark a commitment to hair cell fate and beginning of initial hair cell differentiation. Similarly, calretinin has been shown to initiate its expression in hair cells of the rat vestibular sensory epithelia at E15 (equivalent to E14 in mouse), a time point of initial vestibular hair cell differentiation (Zheng and Gao, 1997). Thus, Brn-3c appears to be expressed in freshly produced hair cells shortly before they initiate early differentiation. In support of this conclusion, it is found that in support cell layer of vestibular sensory organs, a small set of cells, presumably cells just coming out of cell cycle, express Brn-3c but do not express myosins VI and VIIa (Fig. 3). Demonstration of Brn-3c expression only in postmitotic cells is also consistent with the notion that Brn-3c initiates its expression in fresh hair cells that have just undergone the final cell division.
The spatiotemporal expression patterns of Brn-3c in the inner ear closely resemble those of its homologues Brn-3a and Brn-3b in the central nervous system. During retinogenesis, Brn-3b initiates its expression at E11.5 in presumptive migrating postmitotic ganglion cell precursors within the ventricular zone, temporarily and spatially correlating with the histogenesis of retinal ganglion cells (Xiang et al., 1993; Turner et al., 1994; Gan et al., 1996; Xiang, 1998). In the brainstem, Brn-3a is similarly found to be expressed only in postmitotic neurons (Gerrero et al., 1993; Xiang et al., 1995, 1996; Fedtsova and Turner, 1995).

**Brn-3c is not required for commitment of hair cells but is required for migration, maturation and survival of hair cells**

Several lines of evidence suggest that Brn-3c is required for proper migration, maturation and maintenance of hair cells, but not required for determination and initial differentiation of hair cells. First, the loss of hair cells in the adult Brn-3c-/- inner ear could be a result of developmental arrest in proliferation of mitotic hair cell progenitors. However, the exclusive expression of Brn-3c in postmitotic cells in developing sensory epithelia precludes this possibility. Second, in developing Brn-3c-/- organs of Corti and vestibular sensory epithelium, cells with a hair cell-like morphology can be unambiguously identified; moreover, they express early hair cell markers including myosins VI and VIIa, and calretinin. Thus, Brn-3c is unlikely to play a role in commitment and initial differentiation of hair cells. Third, by immunostaining stereociliary bundles of hair cells with the anti-myosin VIIa antibody, no hair bundles can be seen on the apical surface of sensory epithelia in the Brn-3c-/- inner ear. In agreement with this observation, previous light and electron microscopic analyses have also failed to find any stereociliary bundles on Brn-3c-/- sensory epithelia at postnatal and adult stages (Erkman et al., 1996; Xiang et al., 1997a). Therefore, Brn-3c must be critically involved in the maturation of hair cells. Fourth, Brn-3c appears also to be required for proper migration of newly produced hair cells, as indicated by a small number of hair cells anomalously retained in the supporting cell layer of embryonic Brn-3c-/- vestibular sensory epithelia. Presumably, these are derived from the small population of fresh hair cells that would normally express Brn-3c in the supporting cell layer (Figs 1, 3). Finally, TUNEL-labeling assay detects a significant increase of apoptotic cell death in developing Brn-3c-/- vestibular sensory epithelium, indicating a crucial role for Brn-3c in maintaining differentiated hair cells.

Based on these data and previous studies (Gan et al., 1996; Erkman et al., 1996; McEvilly et al., 1996; Xiang et al., 1996, 1997a; Xiang, 1998), it appears that the Brn-3 family members (Brn-3a, -3b and -3c) may function in a very similar mode during mammalian neural development; that is, they are all required for differentiation, maintenance, and/or migration of sensory neurons, but not involved in fate commitment. In Brn-3b-/- mice, a large set of postmitotic retinal ganglion cell precursors are produced but then fail to properly differentiate, and eventually degenerate by apoptosis during late embryonic and early postnatal stages. However, the migration of postmitotic retinal ganglion cell precursors from the ventricular zone to the ganglion cell layer does not seem to be affected (Gan et al., 1996; Erkman et al., 1996; Xiang, 1998). Brn-3a has also been shown to be required for differentiation of various types of neurons, and for maintenance of these neurons by regulating neurotrophin-mediated signaling pathways (Xiang et al., 1996; McEvilly et al., 1996). Interestingly, Brn-3a may or may not be involved in neuronal migration, depending on different neuronal cell types. In Brn-3a-/- mice, the red nucleus neurons can be generated and migrate to the correct location in early development. In contrast, the neurons of the compact formation in the nucleus ambiguous, and the spiral ganglion neurons in the cochlea, fail to migrate to their appropriate positions (McEvilly et al., 1996). As discussed above, Brn-3c is similarly required for proper migration of fresh hair cells into the lumenal hair cell layer in the vestibular sensory organs.

**Brn-3c-/- spiral and vestibular ganglion neurons display distinct kinetics of apoptotic degeneration**

In the Brn-3c-/- inner ear, spiral and vestibular ganglia show severe to complete loss of neurons by late postnatal and adult stages. Since Brn-3c is exclusively expressed in the hair cells, it is postulated that the loss of sensory ganglion neurons results from depletion of trophic supply from hair cells due to hair cell degeneration in Brn-3c-/- mice (Erkman et al., 1996; Xiang et al., 1997a). In support of this hypothesis, we demonstrate here that the spiral and vestibular ganglion neurons degenerate via a mechanism of apoptotic cell death. A careful examination of Brn-3c-/- spiral and vestibular ganglia at early stages reveals distinct rates of neuronal degeneration in the two ganglia (Figs 6, 7B). For instance, by P4, there is a loss of approx. 77% of total neurons in the vestibular ganglion in Brn-3c-/- mice, whereas the spiral ganglion shows a loss of only approx. 29% (Fig. 7B). Because both auditory and vestibular hair cells degenerate at a similar rate in developing Brn-3c-/- mice, the distinct degeneration kinetics of spiral and vestibular ganglia may manifest the differential requirement of these two types of ganglion neurons for brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) (Ernfors et al., 1994, 1995a,b; Fritzsch et al., 1997). In addition, the cause of hair cell degeneration also appears to influence the degeneration kinetics of spiral and vestibular ganglion neurons. For example, in the Snell’s waltzer mutants, the spiral ganglion neurons show a much faster degeneration than those of the vestibular ganglion following postnatal hair cell degeneration in the cochlear and vestibular systems (Deol and Green, 1966; Deol, 1983). Intrinsic properties of ganglion neurons may also contribute to different degeneration kinetics, as large neurons of the vestibular ganglion degenerate at a much faster rate than the small ones in Brn-3c-/- mice (Fig. 6). Similarly, it has been shown that in the cat retina, axotomy results in differential degeneration of different subtypes of ganglion cells (Silveira et al., 1994).

**Molecular implication in hair cell development and regeneration**

In this report, we have shown that Brn-3c is critically involved in the maturation and maintenance of inner ear hair cells, and in the proper positioning of newly produced hair cells. However, Brn-3c appears not to be required for commitment and initial differentiation of hair cells. Therefore, like other developmental processes, hair cell development must require...
combinatorial functions of a complicated network of regulatory molecules including Brn-3c. It will be interesting to know what factor(s) play a part in the specification of the hair cell fate versus the supporting cell identity, and in the initial differentiation of hair cells. Lateral inhibition mediated by Notch signaling has been proposed to control cell-fate choices in the sensory epithelia (Whittfield et al., 1997). Other signaling molecules such as BMP may also play a role (Oh et al., 1996; Morsli et al., 1998).

Recent studies have demonstrated regeneration of hair cells from supporting cells via proliferation-mediated and nonproliferation-mediated mechanisms following acoustic and ototoxic damages (reviewed in Cotanche and Lee, 1994; Corwin and Oberholtzer, 1997; Stone et al., 1998). Identification of the molecular determinant of hair cell fate may be key to understanding hair cell regeneration and for artificially triggering hair cell regeneration from supporting cells. Given its role in migration, maturation and maintenance of hair cells, Brn-3c is likely to be critical for later stages of hair cell regeneration, for the newly regenerated hair cells must migrate to the appropriate laminar layer, mature and survive for proper function. Brn-3c may also be essential for un lethally damaged hair cells to repair their stereociliary bundles in the mammalian inner ear sensory epithelia (Sobkowicz et al., 1992; Zheng et al., 1997). It will be interesting to know whether overexpression of Brn-3c in supporting cells would be sufficient to induce or trigger conversion of supporting cells into hair cells.

In the mouse inner ear, Brn-3c shows an onset expression in hair cells at E12.5 (Fig. 1), and myosins VI and VIIa commence their expression in hair cells at E13.5 (Fig. 2). Therefore, Brn-3c, myosins VI and VIIa join calretinin as early markers for hair cell differentiation and regeneration (Zheng and Gao, 1997). Moreover, Brn-3c, myosins VI and VIIa have the advantage of being specific only to hair cells; by contrast, calretinin is expressed in hair cells as well as in ganglion neurons (Deschesne et al., 1994; Zheng and Gao, 1997). Since in Brn-3c-/- mice, myosins VI and VIIa, calretinin, and parvalbumin are all found to be expressed in the inner ear sensory epithelia (Fig. 5), Brn-3c appears not to play a role in controlling their expression. Although the Brn-3c target genes are currently unknown, it is conceivable that Brn-3c may regulate genes that promote migration, maturation and survival of hair cells.

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REFERENCES


