Essential role of BETA2/NeuroD1 in development of the vestibular and auditory systems

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BETA2/NeuroD1 is a bHLH transcription factor that is expressed during development in the mammalian pancreas and in many locations in the central and peripheral nervous systems. During inner ear ontogenesis, it is present in both sensory ganglion neurons and sensory epithelia. Although studies have shown that BETA2/NeuroD1 is important in the development of the hippocampal dentate gyrus and the cerebellum, its functions in the peripheral nervous system and in particular in the inner ear are unclear. Mice carrying a BETA2/NeuroD1 null mutation exhibit behavioral abnormalities suggestive of an inner ear defect, including lack of responsiveness to sound, hyperactivity, head tilting, and circling. Here we show that these defects can be explained by a severe reduction of sensory neurons in the cochlear-vestibular ganglion (CVG). A developmental study of CVG formation in the null demonstrates that BETA2/NeuroD1 does not play a primary role in the proliferation of neuroblast precursors or in their decision to become neuroblasts. Instead, the reduction in CVG neuron number is caused by a combination both of delayed or defective delamination of CVG neuroblast precursors from the otic vesicle epithelium and of enhanced apoptosis both in the otic epithelium and among those neurons that do delaminate to form the CVG. There are also defects in differentiation and patterning of the cochlear duct and sensory epithelium and loss of the dorsal cochlear nucleus. BETA2/NeuroD1 is, thus, the first gene to be shown to regulate neuronal and sensory cell development in both the cochlear and vestibular systems.

[Key Words: BETA2/NeuroD1; CVG; hair cells; inner ear; delamination]

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The mammalian inner ear is a complex and delicate sensory organ for hearing and balance. The cochlea is responsible for auditory sensation, the otolith organs (utricle and saccule) detect linear acceleration and head position with respect to gravity, and the semicircular canals detect angular head movements. Development of the inner ear begins as an ectodermal thickening, leading to a placode lateral to the rhombencephalon, which subsequently invaginates to form a rudimentary structure, the otocyst [otic vesicle; Sher 1971; Rubel 1978]. The cochlear and vestibular sensory epithelia and the respective ganglia are all derived from the otocyst [Noden and Van de Water 1992; Bissonnette and Fekete 1996]. Development of the otocyst requires intrinsic and extrinsic factors that regulate proliferation, differentiation, and apoptosis to form the mature three-dimensional inner ear structures [Van de Water and Represa 1991; Fekete 1996]. The otic epithelium becomes polarized and cell fate is determined at an early stage of development [Li et al. 1978; Anniko and Wikstrom 1984; Morsli et al. 1998]. The first lineage to differentiate is the sensory neuronal precursors that form the cochlear-vestibular ganglion [CVG, ganglion of cranial nerve VIII; Hemond and Morest 1991]. Specification of the neuroblast lineage occurs immediately after, if not simultaneously with, the irreversible determination of the otic placodal field [E8 in mice; Jacobson 1963; Swanson et al. 1990]. Formation of the CVG starts between embryonic day E9 and E9.5, when neuronal progenitors delaminate from the otic placode, migrate toward the ventral side of the otocyst, and aggregate to form the ganglion primordium.
normalities in the cochlear and/or vestibular systems [for reviews, see Torres and Giraldez 1998; Holme and Steel 1999]. These defects result from failure in various steps of inner ear development, such as (1) inductive signals from the neural tube [e.g., Hoxa-1, fgf-3; Chisaka et al. 1992; Vendrell et al. 2000], (2) field specification [e.g., ear vs. eye, dlx3 and six1], (3) regional and cell-fate determination [e.g., RARα/γ, Pax2, Brn3.1, Nkx5.1, Ngn1, Otx-1, and Otx-2 and Math1; Lohnes et al. 1994; Erkman et al. 1996; Torres et al. 1996; Hadrys et al. 1998; Ma et al. 1998; Wang et al. 1998; Morsli et al. 1999; Bermingham et al. 1999], (4) target-derived neurotrophic support for vestibular and cochlear sensory neurons [neurotrophins and their receptors; Minichiello et al. 1995; Bianchi et al. 1996; Farinas and Rechardt 1996], and (5) functionality [e.g., TRβ, KCNQ4; Rüschi et al. 1998a; Kubisch et al. 1999]. However, to date, no genes have been identified to regulate both sensory hair cell and neuronal development simultaneously in the vestibular and auditory systems.

**BETA2/NeuroD1** is a tissue-specific basic helix-loop-helix transcription factor originally cloned in our laboratory by its ability to up-regulate insulin gene expression [Naya et al. 1995]. It was also cloned [Lee et al. 1995] as a gene required for neuronal differentiation, named NeuroD1. We now refer to the gene as BETA2/NeuroD1. Like many bHLH family members that play important roles in regulating various developmental systems [Jan and Jan 1993], BETA2/NeuroD1 is essential for development of the pancreas and brain. Our laboratory has shown that the pancreatic islet cells in the BETA2/NeuroD1 null are not properly maintained and undergo apoptosis. Morphogenesis of the islet itself is also defective in the null [Naya et al. 1997]. In addition, both the secretin-and cholecystokinin-expressing enteroendocrine cells are missing in the null gut [Mutoh et al. 1998]. Ectopic overexpression of BETA2/NeuroD1 in Xenopus embryos promotes neurogenesis and induces premature differentiation of neuronal precursors [Lee et al. 1995]. Finally, the granule cells of the cerebellum and hippocampal dentate gyrus in the null fail to differentiate properly and are present in greatly reduced numbers [Miyata et al. 1999; Liu et al. 2000; Schwab et al. 2000]. Thus, both gain-of-function and loss-of-function experiments have implicated BETA2/NeuroD1 in promoting cell cycle withdrawal and cellular differentiation. However, recent results both from our laboratory [see below] and others [Miyata et al. 1999; Lee et al. 2000; Liu et al. 2000] have revealed that BETA2/NeuroD1 expression is not restricted to postmitotic neurons but is also detected in proliferating neural precursor cells in some tissues.

In this study, we have analyzed the auditory and vestibular defects in the BETA2/NeuroD1 null. Our results indicate that BETA2/NeuroD1 is required for neurogenesis of the cochlear-vestibular ganglion. During inner ear development, BETA2/NeuroD1 expression is first detected prominently within the otic vesicle wall. This expression is first seen in the sensory neuroblast precursors [E8.75] and later [E13.5 and E15.5] in sensory epithelia of both vestibular organs and the cochlea. Adult BETA2/NeuroD1 null animals displayed an 80% decrease in vestibular ganglion neurons as compared to the controls, while there was an almost total loss (>95%) of cochlear ganglion neurons. A detailed developmental study in the null demonstrates that BETA2/NeuroD1 does not act either by reducing the proliferation of neuroblast precursors or by changing their decision to become neuroblasts. Instead, the early CVG deficit is attributed to the failed delamination of CVG neuroblasts from the otic vesicle wall. At later stages, inadequate trophic support by the peripheral targets and excess apoptosis also contribute to the great reduction in CVG neurons. Examination of the sensory epithelia showed that there was misalignment, misplacement, and duplication of hair cells in the organ of Corti in the null. Phenotypically, the BETA2/NeuroD1 null is completely deaf and suffers from severe deficiencies in balance and coordination. Taken together, these data establish BETA2/NeuroD1 as a critical gene for development of the auditory and vestibular systems.

**Results**

**Defects in balance and hearing**

As reported previously [Liu et al. 2000], we have established a BETA2/NeuroD1 null mouse line that survives to adulthood and is fertile. However, the null mothers do not nurse their pups, possibly because of CNS and/or sensory defects. The null animals were generally underweight and more susceptible to parasitic infection. By 7–10 d of age, all surviving null animals show deficits in balance, manifested by head tilting, lack of coordination, and an inability to right themselves when laid on their sides or backs (but they all learned this skill by 1 mo of age). Between weeks two and three, they displayed abnormal hyperactivity and circling behavior. BETA2/NeuroD1 is expressed in sensory organs such as the eye [Morrow et al. 1999], olfactory bulb [Lee et al. 2000] and the inner ear [this study]. As circling behavior is a characteristic of mouse mutants with inner ear defects [Gibson et al. 1995; Bussoli et al. 1997; Rogers et al. 1999], we first examined the hearing abilities of the BETA2/NeuroD1 null by measuring the auditory brainstem responses (ABR) evoked by acoustic transients (clicks). The ABR consists of multiple waves: Wave 1 is believed to reflect activation of the primary afferent nerve terminals in brainstem nuclei, and wave 2 reflects activation of the cochlear nuclear complex [Mitchell and Clemis 1977]. The null showed no click-evoked ABR at any stimulus level, even up to a value that is 25 dB more intense than the mean threshold for wild-type littermates [Fig. 1]. This indicates complete deafness in the null. Interestingly, heterozygotes had a significantly higher threshold (15 dB higher) than the wild type. The fact that the null lacked any ABR indicates that the hearing deficit may occur in the auditory pathway as early as in the cochlear afferent nerve fibers and, possibly, in the cochlea.

In addition to the spontaneous seizures [Liu et al.
2000) and circling behavior, the null showed severe ataxia reflecting a cerebellar defect (Miyata et al. 1999; M. Liu and M.-J. Tsai, unpubl.). Therefore, some of the classical balance tests such as the rotarod test could not be performed. However, abnormal vestibular function in the null was clearly indicated by the circling behavior and lack of a righting reflex. Also, when suspended by their tails, the null animals displayed a hindlimb clutching response indicative of impaired motor coordination (Crawley and Paylor 1997). Therefore, the BETA2/NeuroD1 null has severe defects both in the cochlea and the vestibule.

Reduction of vestibular and spiral ganglion neurons

The behavioral and electrophysiological defects in the BETA2/NeuroD1 null are consistent with inner ear abnormalities. Histological examination revealed that the most dramatic sensory deficit was the severe loss of neurons in the cochlear and vestibular ganglia. The cell bodies of the primary auditory neurons [spiral ganglion] are located within the cochlear modiolus, while primary vestibular neurons [Scarpa’s ganglion] are located in the internal auditory meatus. During embryonic and early postnatal stages, these neurons undergo proliferation, axonal growth, and apoptosis, reaching maturity by 2 wk of age (Altman and Bayer 1982; Anniko 1983). At P8, the BETA2/NeuroD1 null already showed significant reductions in the number of vestibular ganglion neurons [VG; 85% reduction in Fig. 2a,b] and cochlear ganglion neurons [CG; 95% in Fig. 2c–f]. Also, axon fibers from these neurons were obviously reduced in number in the null [Fig. 2b,f]. Table 1 summarizes the reduction in neurons at different embryonic and neonatal stages. By E13, there was already a ~30% and ~40% loss of CG and VG neurons, respectively. We suspect that the decrease in ganglion neurons was not caused solely by degeneration, as the decrease was observed as early as E9.5, when development of the cochlear-vestibular ganglion complex (CVG) begins (see below). At the cellular level, the residual ganglion neurons appeared unhealthy and lacked nucleolar staining but did have inclusion bodies (see insert in Fig. 2b). Aside from the loss of afferent innervation (cf. Fig. 2e and f), the organ of Corti was grossly intact in the null (Fig. 2d,f; although scanning electron microscopy revealed some defects that will be discussed in a later section).

BETA2/NeuroD1 expression in the developing inner ear

Once the otic anlage is set up [E8.5 in mice], cells of the otic vesicle proliferate and undergo complex morphogenetic changes and programmed cell death. The outgrowth of the future cochlea is from the ventromedial part of the otocyst, while the dorsolateral wall of the otocyst later gives rise to the vestibular apparatus (Fekete 1996). To understand the defects in the null, we examined the expression pattern of BETA2/NeuroD1 in the developing inner ear. β-galactosidase (β-gal) expressed from the BETA2/NeuroD1 locus was used to monitor BETA2/NeuroD1 expression in heterozygotes (Naya et al. 1997). The expression in the ear anlage was detected as early as E8.75, the otic cup stage (data not shown). At E9, when cranial nerve VIII neurons begin to differentiate, prominent BETA2/NeuroD1 expression was observed in the ventral part of the otic vesicle.
3a,c), the location of the future CVG and cochlea. The CVG expression persisted throughout its development (Fig. 3b; see Fig. 4). In the vestibular sensory epithelia, \textit{BETA2/NeuroD1} expression began at E13-E14 during the onset of hair cell differentiation, and it persisted at least until the sensory structures were well defined. Expression was clearly evident throughout the sensory epithelia of the utricle, saccule (Fig. 3d), and crista ampullaris (Fig. 3e). The expression in the cochlear sensory epithelium (organ of Corti) began at E15.5 and was weak compared to other \textit{BETA2/NeuroD1}-expressing regions in the inner ear (Fig. 3f,g).

Mechanism of cochlear-vestibular ganglia defects
The CVG neurons are believed to be primarily of placodal otic origin with a minimal neural crest cell contribution [D’Amico-Martel and Noden 1983; Van De Water 1986]. CVG neuroblasts are born in the ventral otocyst wall around E9 and go through a period of intense cell proliferation between E9.5 and E13.5. Then they delaminate from the otic epithelium and differentiate into ganglion neurons [Ruben 1967]. The pattern of \textit{BETA2/NeuroD1} expression correlates well with CVG ontogenesis. Interspersed patches of \textit{BETA2/NeuroD1}-expressing cells were initially observed in the ventral side of the otocyst wall [Fig. 3c], where CVG neurons are born and from which cochlear components are derived. The CVG initially forms as one complex that subsequently separates into distinct vestibular and cochlear components. At the beginning of its formation, the CVG complex, as marked by \textit{BETA2/NeuroD1} expression, is contiguous at its dorsal edge with the geniculate ganglion (Fig. 4a). Starting from E10.5, the single cell mass of the VII-VIII ganglion complex splits ventrally into a medial part, the acoustic ganglion, and a lateral part, the geniculate ganglion (Fig. 4c). At E11.5, the VIIIth nerve ganglion begins to separate into cochlear and vestibular components (shown at E12.5 in Fig. 4g). The cells of the medial, cochlear portion has more densely packed nuclei than does the lateral, vestibular portion, which has cells with more densely stained cytoplasm. At E13.5, the medial half, the primordium of the cochlear ganglion, terminates in a loop between the ventral end of the saccule and the newly formed half-coil of the cochlear duct [Altman and Bayer 1982]. Thus, the spatial and temporal expression of \textit{BETA2/NeuroD1} clearly correlated with the generation, delamination, and differentiation of the CVG. Double-staining for β-gal and an antibody against a neuron-specific isofrom of β-tubulin, an early pan-neuronal marker, showed that the \textit{BETA2/NeuroD1}-positive cells within the ventral otocyst wall were also positive for β-tubulin, confirming their neuronal identity (data not shown).
In the \textit{BETA2/NeuroD1} null, we found clear defects even at the onset of CVG formation. First, there was a dramatic increase in the number of \textit{BETA2/NeuroD1}-positive cells within the null otic vesicle wall compared to heterozygous controls. As early as E9.5, there were more \(\beta\)-gal-positive cells within the null epithelium than within the heterozygous epithelium. Interestingly, in the heterozygous control, the CVG neuroblasts were more basal in the epithelium; while in the null, they were dispersed across the epithelium, from the apical (luminal) side to the base (Fig. 4, cf. a,c,e with b,d,f). By E11.5, the otocyst epithelium had few proliferating cells in the null (Naya et al. 1997); surviving islet cells fail to form a normal round-shaped mature islet but, instead, aggregate into small clusters. We speculate that a defect in cell–cell interactions is the cause of this morphogenetic defect, but the molecules responsible have not been identified.

In the mouse, VG neurons are born between E9.5 and E12.5, with a peak in proliferation at E11.5, while CG neurons are born between E10.5 and E13.5 with peak production at E12.5 (Ruben 1967). As \textit{BETA2/NeuroD1} is expressed in the otic vesicle during the active proliferation period of CVG formation, we used BrdU staining and BrdU and \(\beta\)-gal double staining to examine whether the decrease in CVG neurons was the result of defective proliferation. The number of BrdU-labeled cells in the ventral otic epithelium and the forming CVG was largely similar between the null and control [outlined in Fig. 5a,b]. This was confirmed by counting BrdU positive cells in double-stained [\(\beta\)-gal and BrdU] sections [Fig. 5e]. Thus, cell-fate determination and the proliferation steps during CVG development were largely unaffected in the null inner ear. Interestingly, \textit{BETA2/NeuroD1} was expressed in many dividing [BrdU-positive] cells during CVG formation [black arrows in Fig. 5c,d]. The number of double-stained cells within the otocyst wall was higher in the null [Fig. 5d] than in heterozygotes [Fig. 5c]. These data confirm that \textit{BETA2/NeuroD1} is expressed in some dividing cells as has been shown for the granule cells of the cerebellum and dentate gyrus [Miyata et al. 1999; Lee et al. 2000; Liu et al. 2000].

### Lack of trophic support and increased apoptosis contribute to the loss of CVG neurons

The loss of CVG neurons in the \textit{BETA2/NeuroD1} null occurred up to E18.5 [Table 1], after which most residual neurons appeared to survive to adulthood. As described above, we propose that delay or failure of neuroblast precursors to move from the otocyst causes the first wave of neuronal loss [E9.5–E12.5], but the magnitude and the timing of this defect can not completely account for the neuronal loss evident later on. Delamination of CVG neuroblasts was minimal in controls by E13.5, but CVG neuron loss continued, relative to the control, until E18.5 [Table 1]. We therefore looked for additional effects of \textit{BETA2/NeuroD1} on CVG neuron number.

During normal development, pioneering afferent nerve fibers from the CVG first penetrate the vestibular epithelium at E12 and cochlear epithelium at E13, with a significant increase in the number of nerve fibers by E16 [Galinovic-Schwartz et al. 1991]. Thus, the postdelamination reduction of CVG neurons in the null [E14-E18] occurs during target innervation. The survival and maintenance of CVG neurons depends on the neurotrophins BDNF and NT3 produced by the cochlear and vestibular epithelia. As null mutants for these proteins lose CG and VG neurons at late gestation [Ernfors et al. 1995], we therefore examined the effect of the \textit{BETA2/NeuroD1} null mutation on the expression of TrkB, the receptor for the neurotrophins BDNF and NT3 [Pirvola et al. 1994]. At E9, when the size of the null CVG was still comparable to the control, \(TrkB\) expression was already greatly reduced in the null [Fig. 6A, see a,b]. By E11.5, a large

### Table 1. Neuron counts from the vestibular and cochlear ganglia of \textit{BETA2/NeuroD1} mice

| Age and ganglion | Wild type\(^{a,b}\) | Null\(^{a,b}\) | % reduction\(^{c}\) |
|------------------|-----------------|------------|------------------|
| E13:             |                 |            |                  |
| Cochlear         | 3212 ± 262 [4]  | 2248 ± 118 [6] | 30*               |
| Vestibular       | 2860 ± 161 [4]  | 1716 ± 212 [6] | 40*               |
| E14.5:           |                 |            |                  |
| Cochlear         | 4211 ± 176 [6]  | 1275 ± 156 [6] | 70**             |
| Vestibular       | 3104 ± 224 [6]  | 1092 ± 76 [6]  | 65*               |
| E16.5:           |                 |            |                  |
| Cochlear         | 5562 ± 156 [6]  | 1206 ± 205 [6] | 90**             |
| Vestibular       | 4762 ± 168 [6]  | 570 ± 107 [6]  | 75**             |
| E18.5:           |                 |            |                  |
| Cochlear         | 7021 ± 212 [4]  | 812 ± 78 [4]  | 95*               |
| Vestibular       | 5072 ± 134 [4]  | 355 ± 112 [4]  | 84*               |
| P2:              |                 |            |                  |
| Cochlear         | 6850 ± 121 [4]  | 778 ± 88 [4]  | 95**             |
| Vestibular       | 4568 ± 90 [4]   | 345 ± 65 [4]  | 83**             |
| P7:              |                 |            |                  |
| Cochlear         | 6210 ± 140 [4]  | 652 ± 89 [4]  | 95*               |
| Vestibular       | 4375 ± 135 [4]  | 315 ± 116 [4] | 85*               |

\(^{a}\)Ganglionic neuron counts from 8-µm serial sections and mean number of neurons (±SEM) are shown. Number of samples is indicated in parentheses.

\(^{b}\)At each stage examined, number of neurons in the wild type was set to 100%.

\(^{c}\)Statistical significance performed by Students t-test.

\(P < 0.05.\)

\(** P < 0.002.\)
The population of differentiating and TrkB-expressing CVG neurons was missing in the null [Fig. 6A, see c,f]. According to the prevailing neurotrophic model, up to 25% of ganglion cells born will die during the competition for limited amounts of trophic factors (Ard et al. 1985). Therefore, we next sought to examine whether increased apoptosis contributes to the loss of CVG neurons in the BETA2/NeuroD1 null. Results obtained with the TUNEL assay at E12 [Fig. 6B, see a,b] indicated that cell death was indeed dramatically increased (sixfold, n = 4) in the delaminated and differentiating null CVG [arrowheads]. Apoptosis was also increased within the ventral otic epithelium of the null relative to the control [arrowheads, Fig. 6B, see d]. The elevated level of apoptosis in the null CVG was observed as early as E10 and persisted to E15 [data not shown].
Given the essential roles of neurotrophins and their receptors in the survival, growth, and differentiation of CVG neurons (Fritzsch et al. 1997), it is expected that a decreased level of TrkB would produce a smaller CVG. However, the decreased TrkB expression level and apoptosis in the BETA2/NeuroD1 null occurred earlier than the times at which deficits are first seen in mice that are null for TrkB or for BDNF and NT3 (E16; Schim-mang et al. 1995; Ernfors et al. 1995). Therefore, there may be other factor(s) downstream of BETA2/NeuroD1 that act earlier than TrkB (between E10 and E15) to regulate the initial survival and maintenance of CVG neurons.

Shortening of the cochlear duct

Paint-filled membranous labyrinths from the BETA2/NeuroD1 null revealed a significantly shortened cochlear duct but otherwise normal labyrinth morphology (Fig. 7). The cochlear duct develops as an extension of the ventral part of the otic vesicle, beginning around E12. The increase in length of the proximal portion (the hook) of the cochlear duct occurs concurrently with the coiling of the distal portion (the coil). At E13.5, the snail-shaped duct normally consists of approximately one turn. In the BETA2/NeuroD1 null, there was no difference in length compared to the control (compare red outlines of CVG in c–h). At E12.5, the residual CVG neurons formed scattered clusters [b] rather than the compact ganglion complex seen in the control [g]. Note that the geniculate ganglion (gg, arrowheads in a–f) near the head vein [n.VII, facial ganglion, spherical shape] is unaffected in the null [scale bar 100 µm].

Figure 4. Failure/delay in CVG neuroblast delamination in the null otic vesicle. Early development of CVG neurons (β-gal positive, arrow) in BETA2/NeuroD1 heterozygous [a,c,e,g] and homozygous null [b,d,f,h] from E9.5 to E12.5. As early as E9.5, there is an increase in CVG neuroblasts retained within the null otic epithelium compared to the controls [cf. a,b]. The retained CVG precursors in the nulls were located throughout the epithelium, from the apical (lumenal) side to the basal side. In contrast, in the heterozygous animals, the blue CVG precursors are located more toward the base of the epithelium, where they are readily engaged to delamine. This failure/delay in delamination became more severe during the subsequent peak period of CVG formation [E10.5–E12.5]. By E10.5 and E11.5, the forming CVG is obviously reduced in size in the null compared to the control (compare red outlines of CVG in c–h). At E12.5, the residual CVG neurons formed scattered clusters [b] rather than the compact ganglion complex seen in the control [g]. Note that the geniculate ganglion (gg, arrowheads in a–f) near the head vein [n.VII, facial ganglion, spherical shape] is unaffected in the null [scale bar 100 µm].

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Defects in the CVG targets

Loss of central nuclei and atrophy of eighth nerve fibers in the brainstem Cochlear and vestibular neurons are bipolar with a short peripheral process contacting the hair cell mechanoreceptors in their respective sensory epithelia and a long central process projecting to the cochlear and vestibular nuclei within the medulla. The axons of the CG leave the base of the cochlea, join with the vestibular fibers, and enter the posterior cranial fossa accompanied by the facial nerve. These primary sensory neurons terminate in the dorsal and ventral cochlear nuclei at the junction of the brainstem and medulla. The dorsal cochlear nucleus (DCN) forms a small bump, the acoustic tubercle, in the brainstem. The acoustic tubercle was missing in mutant mice [Fig. 8a,b]. It is possible that the loss of the majority of CG neurons resulted in a loss of innervation and lead to problems in the central targets of these neurons. In addition, as BETA2/NeuroD1 is expressed in the cochlear and vestibular nuclei during embryonic development [data not shown], the null mutation may directly affect the formation of these nuclei. Cresyl violet staining [Fig. 8c,d] confirms the lack of the DCN in the null. Moreover, the eighth nerve fiber bundle connecting the brainstem and inner ear, which includes the central processes of the CG and VG neurons and efferent nerve fibers, was also drastically reduced and/or missing in the null [Fig 8e,f].
Figure 6.  ∆ Reduced expression of neurotrophin receptor TrkB in the null. TrkB in situ hybridization on cross sections from wild type [a,c,e] and the null [b, d, f] at E9.0 [a,b], E10 [c,d], and E11.5 [e,f]. At all stages analyzed, the level of TrkB expression was reduced in the null. By E11.5, the TrkB-expressing CVG population is largely missing in the null (arrowheads in e,f). Note that TrkB expression is relatively spared in the geniculate ganglion, n.VII [gg] in the null [arrows, compare c,d] scale bar = 120 µm.  | B | Increased apoptosis in CVG neurons in the null. Inner ear cross sections from E12 wild-type [a,c] and null [b,d] embryos stained by TUNEL. [a,b] Note the significant increase in TUNEL-positive cells (dark brown, arrowheads) in the null CVG [scale bar = 200 µm]. Since CVG neuron depletion is already very dramatic at E12 in the null and the residual neurons occupy a more lateral-caudal position than in the control, the null sections were taken at a more caudal level than the control [a,c]. The sections shown are from planes corresponding to the maximum size of the CVG in both the control and the null. [c,d] Apoptosis was also increased within the ventrolateral side of the null otic epithelium wall, where the retained CVG neuroblasts [β-gal positive] were located. Arrowheads point to TUNEL-positive cells, many of which are also positive for β-gal [BETA2/NeuroD1 positive, scale bar = 150 µm, insert = 200 µm].
addition, ectopic IHCs were found scattered among the first row of OHCs. Even more striking was the presence of two rows of IHCs rather than the usual single row (white arrowhead in Fig. 9b). The hair bundles in the null OHCs and IHCs appear normal [see insert in Fig. 9b].

The vestibular epithelia contain two types of receptor cells: The flask-shaped type I hair cells surrounded by a cup-shaped nerve ending [calyx], and the more cylindrical type II hair cells that receive multiple bouton-type afferent endings. In the null epithelia at 3 mo of age (Fig. 9d,f), both hair cell types were present. This is consistent with results from mice null for neurotrophins and their receptors, which, despite the loss of afferent innervation, have epithelia that appear normal [Minichiello et al. 1995; Schimmang et al. 1995; Silos-Santiago et al. 1997]. In the vestibular epithelia of the BETA2/NeuroD1 null, most afferent fibers were missing, as expected from the drastically reduced VG. However, at least in the saccular macula [Fig. 9d], some afferent nerve fibers persisted, principally in a cytoarchitectonically and physiologically distinct region called the striola. Taken together, our data show that at 3 mo of age, some ganglion cells and their peripheral processes persist but that, at least in some cases, there are no central processes into the brainstem of the BETA2/NeuroD1 null.

Discussion

The mammalian inner ear contains very sophisticated mechanosensory elements and a highly specific set of neuronal connections for the transduction of mechanical energy to electrical impulses in the cochlear and vestibular nerve. Many factors are required for regulating its development. Our results show that BETA2/NeuroD1 is essential for the formation of cochlear and vestibular ganglion neurons. At an early stage, BETA2/NeuroD1 is already prominently expressed in the otic vesicle, the delaminating neuroblasts, and the forming ganglia. Importantly, its expression is found not only in the differentiated neurons but also in the dividing neurons. This is of special interest as, until recently, it had been generally believed that BETA2/NeuroD1 was only expressed in postmitotic/differentiating cells [Miyata et al. 1999; Lee et al. 2000; Liu et al. 2000]. Our data indicate that lack of BETA2/NeuroD1 caused retention of a large population of neuroblast precursors within the otocyst. One explanation for our observation is that BETA2/NeuroD1 is required in the neuroblast precursors in the last round of cell division to promote cell cycle exit and to initiate differentiation. Similarly, in the case of enteroendocrine cells, it has been suggested that BETA2/NeuroD1 acts together with the coactivator p300 to induce the expression of p21, a cyclin-dependent cell cycle inhibitor [Mutoh et al. 1998]. If this were the case in the otic epithelium, one might expect enhanced proliferation. We saw no difference in proliferation between null and wild-type epithelia with a single BrdU labeling period of 1.5 h; however, for this question to be examined fully, a pulse-chase BrdU study should be performed. An alternative
hypothesis is that lack of BETA2/NeuroD1 in the neuroblast precursors resulted in failure of these cells to initiate differentiation, thus retarding delamination. Thus, although the initial cell fate determination and proliferation of CVG neurons appeared to be largely unaffected in the null, BETA2/NeuroD1 is required for CVG delamination and differentiation.

The TrkB-labeling experiments revealed another form of altered differentiation. Down-regulation of neurotrophin receptors such as TrkB in the BETA2/NeuroD1 null is expected to compromise CVG maturation and survival. In mice lacking the neurotrophins and/or their receptors, CVG neurons are born, but in NT-3 and TrkC null mice, most cochlear neurons degenerate later in gestation (Fritzsch et al. 1997). In contrast, the CVG deficit in the BETA2/NeuroD1 null occurs earlier during ontogeny of these ganglia. This suggests that there must be other growth factors, downstream targets of BETA2/NeuroD1.

Figure 8. Absence of the dorsal cochlear nucleus (DCN) and eighth nerve in the null. (a,b) Ventral view of whole-mount β-gal stained heterozygous (a) and null (b) brains at 6 wk of age. Note that n.VIII (see bracket in a, arrowhead in b) and the small bump representing the whole population of the DCN are missing in the null (* in a,b; scale bar = 200 µm). This loss is confirmed by cresyl violet-stained horizontal sections (DCN in c,d) and H&E stained cross sections (8n in e, f; scale bar = 200 µm). VCP, ventral cochlear nucleus, posterior; vg: vestibular ganglion; 8vn, vestibular nerve root; sp5, spinal tract of Vth cranial nerve; Tn, nucleus of sp5; cb, cerebellum; cp, choroid plexus.
NeuroD1, that are required at the early stage of CVG development. This does not mean that TrkB does not have an effect at the earlier stages of CVG development but may play a role in combination with these other growth factors. In addition, the initial CVG afferent contacts with their targets, which are a prerequisite for neurotrophic action, may have been reduced during the first wave of ganglion neuron loss (E9.5–E12.5). However, whether the sensory epithelia are defective in providing neurotrophic support is one possibility that remains to be examined.

There are other gene-targeted mouse models in which CVG neurons are decreased or lost. Neurogenin 1 (ngn1), acting as a proneural cell fate determination gene, is a positive upstream regulator of BETA2/NeuroD1. Mouse embryos lacking ngn1 fail to generate the proximal subset of cranial sensory neurons, including those in the CG and VG, as a result of defective cell fate determination of CVG neuronal precursors (Ma et al. 1998). Neurogenin3 (ngn3) is also an upstream regulator of BETA2/NeuroD1 (Huang et al. 2000; Gradwohl et al. 2000). Thus, the question remains as to what extent the phenotypes iden-
tified in the neurogenin null mice result from the loss of BETA2/NeuroD1 function.

The BETA2/NeuroD1 null has an unusually short and widened cochlear duct. The oldest part of the organ of Corti appears at the apex, while the youngest is at the base (Ruben 1967). Whether this shortening of the null cochlear duct is a direct or indirect effect of the null mutation is unclear. One possibility is that growth factors that regulate cell proliferation in the inner ear are down-regulated in the BETA2/NeuroD1 null ear. In vitro explant studies have shown that IGF-1 is a potent growth-promoting and survival factor for otic vesicle development (Oesterle et al. 1997). Consistent with the down-regulation of TrkB, a maintenance and survival factor for neurons in the null, growth-promoting genes like IGF-1 may be candidate downstream targets of BETA2/NeuroD1 in the ear.

The analysis of the BETA2/NeuroD1 null ears revealed some defects in hair cell organization in the organ of Corti, including duplication of, and ectopic addition of, IHCs and misalignment of OHCs. Increased numbers of hair cells were also seen in mice with null mutations for Jagged 2, a Notch 1 ligand (Lanford et al. 1999), and p27Kip1, a cell cycle inhibitor (Chen and Segil 1999). In the Jagged 2 null, the mechanism is a cell fate switch from supporting cell to hair cell, as a result of decreased Notch-mediated lateral inhibition. In the p27Kip1 null, hyperplasia of both IHCs and OHCs results from the loss of control of cell cycle progression. Because BETA2/NeuroD1 is believed to promote exit from the cell cycle, its absence in the null may explain the presence of ectopic and duplicated IHCs. Excess numbers of IHCs could secondarily lead to misalignment of OHCs. Alternatively, the abnormal retention of CVG neuroblast precursors in the null otic epithelia may have affected the organization of the developing hair cells.

This study has found several important contributors to the hearing loss documented by ABR testing in the BETA2/NeuroD1 null: The severe depletion of CG neurons and at least one central target (the DCN), shortening of the cochlear duct, and abnormalities within the organ of Corti. It is interesting that heterozygous mice showed a higher ABR threshold when compared with the wild type. Recently, mutations of BETA2/NeuroD1 were implicated in the development of familial human type II diabetes in the heterozygous state (Malecki et al. 1999). The ABR test might be useful for screening diabetic patients for possible BETA2/NeuroD1 mutations, allowing a better correlation of hearing impairment and diabetes.

The BETA2/NeuroD1 null also showed defective balance behaviors. Balance depends on complex interactions of sensory and motor systems. The atrophy of the VG shown in this study contributes to the imbalance in the null, but any defects in the central vestibular system or the motor system could also be factors. BETA2/NeuroD1 is also expressed in spinal cord, vestibular nuclei, and cerebellar granule cells (Miyata et al. 1999; Lee et al. 2000; Liu et al. 2000). Central processes of VG neurons form the vestibular division of the eighth nerve, which terminates in the vestibular nuclear complex in the floor of the fourth ventricle. In addition, a small number of these axons terminate in the flocculonodular lobe of the cerebellum. The medial and inferior vestibular nuclei have reciprocal connections with the cerebellum (vestibulocerebellar tract) that allow the cerebellum to coordinate balance during movement. We [M. Liu and M.-J.Tsai, unpubl.] and others (Miyata et al. 1999) have found that the posterior part of the cerebellum, including the vestibulocerebellum (lobule X), is more severely affected (loss of granule cells) than the anterior cerebellum in the null brain. This might in part be secondary to the loss of the VG, or vice versa, or caused by a mutual loss. The degree and mechanism of involvement of the CNS component in the manifestation of the phenotype is unclear and under investigation.

In summary, lack of BETA2/NeuroD1 causes a severe loss of CVG neurons, loss of the DCN, and alterations in the mechanosensory hair cells and structural components of the inner ear. Thus, BETA2/NeuroD1 is the first gene in which mutations produce both neuronal and sensory defects in both vestibular and cochlear organs. Moreover, this gene acts centrally in both systems: Its loss eliminates the DCN and the granule cells of the posterior cerebellum. This is the first molecular evidence that a specific gene has evolved to regulate critical developmental events such as balance in functionally related parts of the mammalian peripheral and central nervous systems.

Materials and methods

Animals

BETA2/NeuroD1 null mice were generated in the 129/SvJ background as described (Liu et al. 2000).

Auditory Brainstem Response (ABR)

ABR was recorded at 10 wk of age to assess hearing abilities. Mice were anesthetized with 45 mg Ketamine/kg and 5.4 mg xylazine/kg. Electrodes were placed at the vertex (active) and behind the ear (reference). Tucker-Davis Technology software and hardware was used to generate the stimulus and collect ABR data. Pulses 5 msec long were presented at a rate of 20/sec to a speaker [Entymotic ER-2]. Intensity levels are given in dB re 0.001 volts [RMS] into the speaker, and were presented from 100 to 25 dB in 5-dB decrements. At each intensity, 500 responses were averaged. Threshold is defined as the level at which wave 2 disappeared.

Tissue processing and neuron counting

E9-E12 embryos or heads [E13-P8] were collected and fixed in 4% paraformaldehyde in PBS at 4°C for periods between 6 h and overnight. The older animals were anesthetized with avertin [150 mg/kg] and transcardially perfused. Inner ears were dissected from postnatal heads, fixed overnight, decalcified with 10% formic acid for 4 d at room temperature, and then processed for paraffin embedding. Neuron counts were done on serial 8-µm sections stained either with 0.5% cresyl violet or hematoxylin and eosin [H&E]. Neurons with a clear nucleus...
and nucleoli were counted in every fifth section. Counts were not corrected for double or split nucleoli.

**Paint-filling**

Inner ears were excised from staged wild-type and null embryos, fixed in Bodian’s fixative, dehydrated in ethanol, and cleared in methyl salicylate. The membranous labyrinths were visualized using latex paint injections as previously described [Morsli et al. 1999]. At least two pairs of inner ears were injected for each stage presented.

**Semithin sections and electron microscopy**

Semithin sectioning and staining was carried out as described previously (Lyshakowski and Goldberg 1997). Briefly, wild-type and null littermates at 2 wk and 3 mo of age were anesthetized with avertin [150 mg/kg body weight] and perfused transcardially with 30 mL of a warm 0.1 M sodium cacodylate 0.9% NaCl buffer (pH 7.4), followed by 100 mL of a warm trisaldehyde fixative consisting of 3% glutaraldehyde, 2% paraformaldehyde, 1% Acrolein, and 5% sucrose in 0.08 M cacodylate buffer (DeGroot et al. 1987). Temporal bones were dissected, postfixed for 1 h in either 1% OsO4 in 0.1 M cacodylate buffer, decalified in Cal-Ex for 1–2 h, dehydrated in graded ethanol and propylene oxide, and embedded in Araldite (Durcupan). Temporal bones were sectioned in the horizontal plane. Serial sections, 4 µm thick, were cut with a diamond knife through the whole temporal bone, mounted on glass slides, and stained with Richardson’s stain. Hair cells and supporting cells were classified using morphological criteria developed previously [Rüsch et al. 1998b]. Tissue preparation for SEM consisted of osmication (1% OsO4 in cacodylate buffer), dehydration, critical-point drying, and sputter-coating with gold. Specimens were examined in a JEOL 35S electron microscope.

**β-gal activity staining, in situ hybridization, and immunohistochemistry**

Embryos were isolated and prepared for double-labeling with X-Gal and BrdU antibody as previously described [Liu et al. 2000] on 8-µm serial cross sections. BrdU (Amersham Life Sciences) was injected intra-peritoneally. 1 h before sacrifice at 150 µg/g body weight. In situ hybridization was performed as described (Qiu et al. 1994) using a TrkB antisense RNA probe (Klein et al. 1990). At least three animals were analyzed at each stage. Animals were treated according to animal care guidelines at Baylor College of Medicine and the University of Illinois at Chicago.

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Essential role of BETA2/NeuroD1 in development of the vestibular and auditory systems

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