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Brn3a is a transcriptional regulator of soma size, target field innervation and axon pathfinding of inner ear sensory neurons

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SUMMARY

The POU domain transcription factors Brn3a, Brn3b and Brn3c are required for the proper development of sensory ganglia, retinal ganglion cells, and inner ear hair cells, respectively. We have investigated the roles of Brn3a in neuronal differentiation and target innervation in the facial-stato-acoustic ganglion. We show that absence of Brn3a results in a substantial reduction in neuronal size, abnormal neuronal migration and downregulation of gene expression, including that of the neurotrophin receptor TrkC, parvalbumin and Brn3b. Selective loss of TrkC neurons in the spiral ganglion of *Brn3a*$^{-/-}$ cochlea leads to an innervation defect similar to that of *TrkC*$^{-/-}$ mice. Most remarkably, our results uncover a novel role for Brn3a in regulating axon pathfinding and target field innervation by spiral and vestibular ganglion neurons. Loss of Brn3a results in severe retardation in development of the axon projections to the cochlea and the posterior vertical canal as early as E13.5. In addition, efferent axons that use the afferent fibers as a scaffold during pathfinding also show severe misrouting. Interestingly, despite the well-established roles of ephrins and EphB receptors in axon pathfinding, expression of these molecules does not appear to be affected in *Brn3a*$^{-/-}$ mice. Thus, Brn3a must control additional downstream genes that are required for axon pathfinding.

Key words: Brn3a, POU domain, Transcription factor, Spiral ganglion, Vestibular ganglion, Innervation, Axon pathfinding, Mouse

INTRODUCTION

The vertebrate sensory neurons convey different modalities of sensory information to the central nervous system. In the inner ear, sensory neurons in the spiral ganglion innervate cochlear hair cells that are auditory sensory receptors; those in the vestibular ganglion innervate vestibular hair cells that are sensory receptors for head position and movement. Similarly, neurons within the geniculate ganglion innervate taste buds in the tongue that transmit gustatory information. During mouse embryogenesis, neurons in the spiral and vestibular ganglia are derived from the otic placode, while those in the geniculate ganglion arise from the first epibranchial placode. The neuronal progenitors derived from these two placodes migrate and coalesce around embryonic day 9 (E9) into a single sensory ganglion primordium – the facial-stato-acoustic ganglion. By E12.5, the primordium differentiates into the geniculate ganglion and vestibulocochlear ganglion, which remain physically connected (Sher, 1971; Fig. 1K). By E13.5-E14.5, the vestibulocochlear ganglion segregates into the vestibular and spiral ganglion primordia. By E17.5, the spiral and vestibular ganglia are physically separated inside and outside the otic capsule, respectively. The vestibulocochlear ganglion neurons start to innervate their peripheral targets as soon as the sensory epithelia are formed in the otocyst at E10.5 (Sher, 1971; Morsli et al., 1998).

Although the ontogenies of sensory neurons within the inner ear and geniculate ganglia have been well described, much less is known about the molecular mechanisms controlling this process. Several lines of evidence have indicated that transcription factors are critically involved in the development of these ganglia. For example, the bHLH factors neurogenin 1 (Ngn1; Neurod3 – Mouse Genome Informatics) and neurogenin 2 (Ngn2; Atoh4 – Mouse Genome Informatics) are expressed in the cranial ganglion placodes as early as E8.5. Targeted disruption of *Ngn1* or *Ngn2* in mice, respectively, abolishes the development of the proximal cranial ganglia, which include the vestibular and cochlear ganglia, or the distal cranial ganglia, which include the geniculate ganglion (Fode et al., 1998; Ma et al., 1998). Consistent with the role of their *Drosophila* paralog *atonal* (Jarman et al., 1993), *Ngn1* and *Ngn2* have been shown to be essential for cell fate commitment

(Fode et al., 1998; Ma et al., 1998; Ma et al., 1999; Ma et al., 2000a). In addition, the homeodomain-containing transcription factor Phox2a (Arix – Mouse Genome Informatics) has been shown to be required for differentiation and maintenance of geniculate ganglion neurons (Morin et al., 1997). Gene targeting analyses have also demonstrated essential roles for neurotrophins and their Trk receptors in supporting the survival of sensory neurons (reviewed in Farinas and Reichardt, 1997). In the inner ear sensory ganglia, brain-derived neurotrophic factor (BDNF), neurotrophin (NT) 3 (Ntf3 – Mouse Genome Informatics), and their receptors TrkB and TrkC exert complementary roles in promoting neuronal survival (Ernfors et al., 1995; Minichiello et al., 1995; Schimmang et al., 1995; Bianchi et al., 1996; Fritzsch et al., 1997a). For example, deletion of the genes for BDNF or NT3 in mice results in loss of more than 80% of neurons within the vestibular or spiral ganglion, respectively. In a compound mutant lacking genes for both BDNF and NT3, there is complete loss of vestibular or spiral ganglion neurons. Similar analyses have shown that BDNF, NT4, NT5 and NT3 are survival factors for geniculate ganglion neurons (Farinas et al., 1994; Conover et al., 1995; Liu et al., 1995; Fritzsch et al., 1997b; Liebl et al., 1997).

The POU domain transcription factors Brn3a and Brn3b, also known as Brn3.0 and Brn3.2, respectively (Pou4f1 and Pou4f2 – Mouse Genome Informatics), have been shown to be expressed in the trigeminal, dorsal root and inner ear sensory ganglia during mouse embryogenesis (Xiang et al., 1993; Xiang et al., 1995; Gerrero et al., 1993; Turner et al., 1994; Fedtsova and Turner, 1995; Ryan, 1997). Deletion of *Brn3a* results in a significant loss of spiral ganglion neurons and defects in their migration (McEvilly et al., 1996). In addition, consistent with the multiple sites of Brn3a expression, *Brn3a*$^{-/-}$ neonates show neuronal losses in the somatosensory ganglia and in several brainstem nuclei, which eventually lead to perinatal lethality (Xiang et al., 1996; McEvilly et al., 1996). For example, in the mutant trigeminal ganglion, approximately 70% of the neurons are lost by postnatal day 0 (P0) as a result of apoptotic cell death caused by a dramatic and specific downregulation of neurotrophin receptors in the absence of Brn3a (Huang et al., 1999b). In contrast, absence of Brn3b does not have overt effects on the development of inner ear sensory ganglia. Instead, its absence results in improper differentiation and loss of a large set of retinal ganglion cells (Gan et al., 1996; Gan et al., 1999; Erkman et al., 1996; Xiang, 1998). Interestingly, expression of Brn3b precedes that of Brn3a in the retina (Xiang, 1998). It remains unknown whether expression of Brn3a precedes that of Brn3b in the spiral ganglion, where Brn3a appears to have a leading role.

Despite significant advances in our understanding of the molecular mechanisms governing the specification and maintenance of auditory and gustatory sensory neurons, relatively little is known about their differentiation. In this report, we show that Brn3a is expressed in the facial-stato-acoustic ganglion prior to sensory neuron differentiation and innervation of the otocyst. Loss of Brn3a leads to downregulation of *TrkC*, *Brn3b* and the gene for parvalbumin in the spiral ganglion, indicating that these are downstream targets of Brn3a. More importantly, we provide evidence that Brn3a is required for proper growth and migration of inner ear and gustatory sensory neurons, and is critically involved in target innervation and axon guidance by spiral and vestibular ganglion neurons. Our data indicate that Brn3a controls survival and differentiation of sensory neurons by regulation of different downstream genes.

MATERIALS AND METHODS

Experimental animals

Brn3a$^{+/-}$, *Brn3b*$^{+/-}$ and *Brn3b*$^{-/-}$ mice were derived by targeted gene disruption as previously described (Gan et al., 1996; Xiang et al., 1996).

Immunohistochemistry

For section immunohistochemistry using anti-Brn3a, anti-Brn3b, anti-NF 150, anti-p75^{NTR}, and anti-parvalbumin, staged embryos and neonates were fixed in 4% paraformaldehyde, cryo-protected in 30% sucrose, embedded in OCT compound and sectioned at 10-14 μm using a cryostat. Immunohistochemistry for TrkB and TrkC antibodies has been described previously (Huang et al., 1999a). The color reaction was developed using the NovaRed substrate kit when Hematoxylin counterstaining was applied. Antibodies were obtained from the following sources: anti-Brn3a and anti-Brn3b (Xiang et al., 1993; Xiang et al., 1995); anti-TrkB and anti-TrkC (Huang et al., 1999a; Huang et al., 1999b); anti-NF 150 and anti-p75^{NTR} (Chemicon); anti-parvalbumin (SWant); and anti-EphB antibodies (Santa Cruz Biotechnology).

Histochemistry and quantitation

The silver impregnation technique was performed as described by Ungewitter (Ungewitter, 1951). Cresyl Violet labeling was performed as described by LaBossiere and Glickstein (LaBossiere and Glickstein, 1976). To determine the total number of neurons in each sensory ganglion, serial sections were stained with Cresyl Violet and scored every other section by light microscopy. All neuronal profiles larger than glial cells were counted and counts were not corrected for split neurons. All data were tested for significance using two sample Student's *t*-test with unequal variances. To compare neuronal size in wild-type and *Brn3a*$^{-/-}$ or *Brn3b*$^{-/-}$ sensory ganglia, cross-sectional areas of neuronal profiles were measured in a given region of the ganglion using the NIH Image program.

Analyses of afferent and efferent innervation patterns

Analyses of afferent innervations to the inner ear were performed as previously described (Fritzsch and Nichols, 1993). Briefly, lipophilic dye DiI (1,1′-diotadecyl-3,3,3′,3′-tetramethylindocarbocyanine percholate; Molecular Probes) -soaked filter strips were applied to the alar plate rostral to the statoacoustic (octaval) nerve root in E13.5 and P0 *Brn3a*$^{-/-}$ mice and their wild-type littermates (n=4 for each genotype). After a diffusion time of 3-8 days at 37ºC, the ears with otic ganglia and facial nerve attached, were dissected from adjacent mesenchyme and photographed as whole mounts with an Olympus compound microscope. After analyzing the DiI-labeling results, the ears were subsequently processed for immunohistochemical staining using α-acetylated tubulin antibodies (Sigma, T9026). Briefly, cochleae were defatted, incubated with primary antibody for 2 days, washed and incubated with a secondary antibody conjugated to horseradish peroxidase.

RESULTS

Sequential expression of Brn3a and Brn3b during development of the facial-stato-acoustic ganglion

To better understand the roles of Brn3a and Brn3b in development of the inner ear sensory and geniculate ganglia,

we first examined the expression patterns of these two transcription factors in the developing mouse facial-stato-acoustic ganglion, the precursor to these ganglia. A Brn3a antibody strongly labeled the facial-stato-acoustic ganglion primordium located medioventrally to the otic vesicle at E9.5 (Fig. 1A) and E10.5 (data not shown); by contrast, anti-Brn3b did not label this ganglion at these early developmental stages (Fig. 1B). At E12.5, following partial separation of the geniculate ganglion from the vestibulocochlear ganglion, Brn3a continues to be expressed strongly in both ganglia, while Brn3b is now weakly expressed in a small fraction of cells in both ganglia (Fig. 1C,D). Thus, Brn3a is expressed in the facial-stato-acoustic ganglion at least 3 days before the onset of Brn3b expression. At E16.5, however, both Brn3a and Brn3b are prominently expressed in perhaps all of the neurons in the spiral, vestibular and geniculate ganglia (Fig. 1E,F). Expression of both transcription factors continues to be strong in all neurons at E17.5 (Fig. 1G-J). As elucidated in Fig. 1K, Brn3a is strongly expressed in the facial-stato-acoustic ganglion at E9 and continues to be strongly expressed in derivatives of this ganglion throughout embryogenesis. In contrast, weak expression of Brn3b is found in a few cells only at E12.5 and expression of this transcription factor does not become widespread until E16.5.

As inner ear sensory neurons become postmitotic between E11.5 and E15.5 (Ruben, 1967), the onset of Brn3a expression at E9 in the primordium of these ganglia suggests that Brn3a is expressed in dividing neuronal progenitors before neuronal differentiation. Previous work has indeed detected Brn3a expression in proliferative cells of the vestibulocochlear ganglion (Fedtsova and Turner, 1995). Moreover, Brn3a expression significantly precedes peripheral target innervation by vestibulocochlear neurons and migration of cochlear neurons, both of which begin at E10.5 (Sher, 1971). In contrast, Brn3b is present prevalently in the facial-stato-acoustic ganglion only after initiation of these events.

Differential loss of neurons in *Brn3a*$^{-/-}$, but not *Brn3b*$^{-/-}$, spiral, vestibular and geniculate ganglia

Given that expression of Brn3a is initiated in the facial-stato-acoustic ganglion at E9 and persists in all of its derivative ganglia throughout embryogenesis (Fig. 1), we investigated whether the absence of this transcription factor affects neuronal survival. We determined the number of neurons within the spiral, vestibular and geniculate ganglia in control and in *Brn3a*$^{-/-}$ mice by Cresyl Violet staining. In the geniculate ganglion at P0, there are significantly fewer neurons in *Brn3a*$^{-/-}$ mice than in the wild type (Fig. 2A,B). In sections of the *Brn3a*$^{-/-}$ vestibular ganglia at P0, however, we were unable to visualize any obvious decrease in neuronal number (Fig. 2C,D). We next determined the total number of neurons within each sensory ganglion by scoring neurons on serial sections

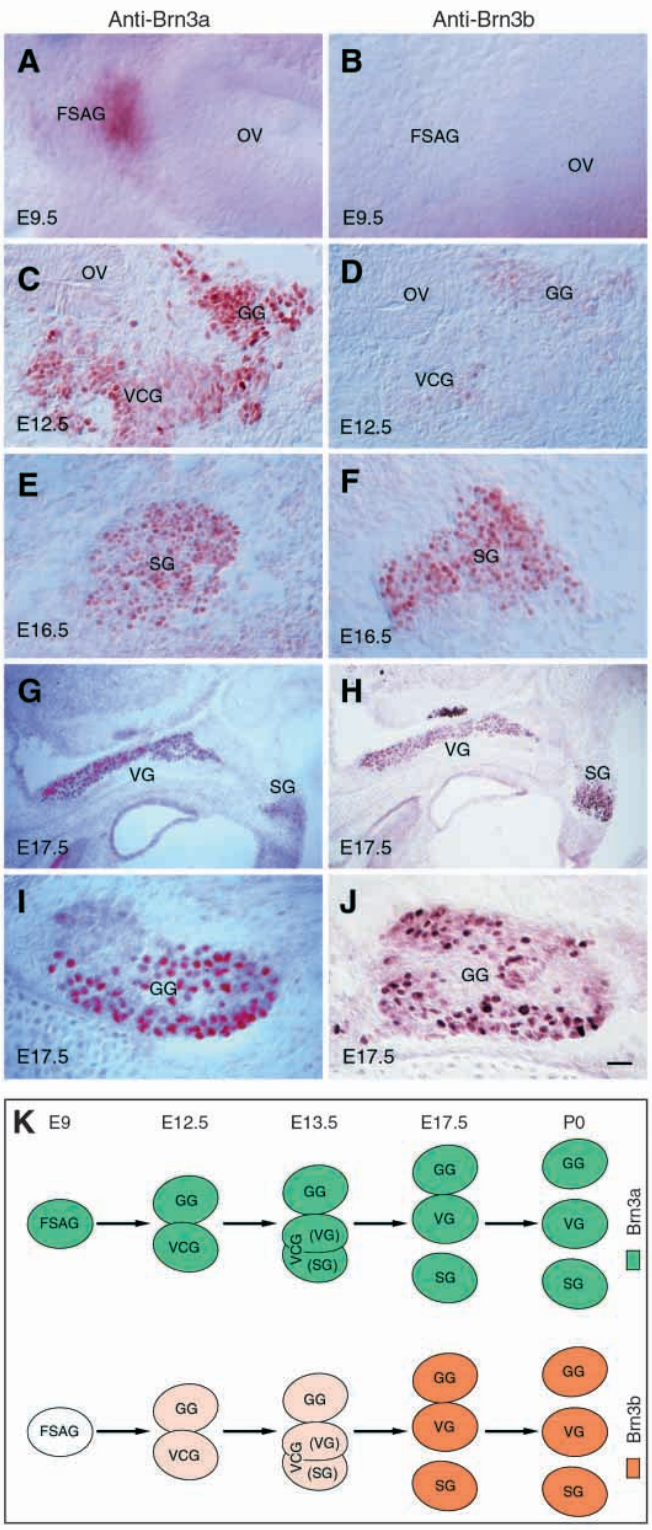

Fig. 1. Expression of Brn3a and Brn3b during development of the mouse facial-stato-acoustic ganglion. (A-J) Whole-mount (A,B) and inner ear sections (C-J) from embryos at the indicated stages were immunostained with anti-Brn3a (A,C,E,G,I) and anti-Brn3b (B,D,F,H,J) antibodies. Prominent Brn3a expression is initially found in the facial-stato-acoustic ganglion (FSAG) by E9-E9.5, and persists at later embryonic stages in all the ganglia derived from it, including the vestibulocochlear ganglion (VCG), spiral ganglion (SG), vestibular ganglion (VG) and geniculate ganglion (GG). Brn3b exhibits a much more delayed temporal expression pattern than Brn3a with no expression in the FSAG at E9-E10.5, weak expression in some cells of the VCG and GG by E12.5-E14.5, and prominent expression in the SG, VG and GG starting only at E16.5. The location of the otic vesicle (OV) is indicated in A-D. (K) Schematic illustrating the derivation of inner ear and gustatory sensory ganglia during embryogenesis, and temporal expression patterns of Brn3a and Brn3b in these ganglia and their primordia. Scale bar: 25 μm in A-F,I,J; 100 μm in G,H.

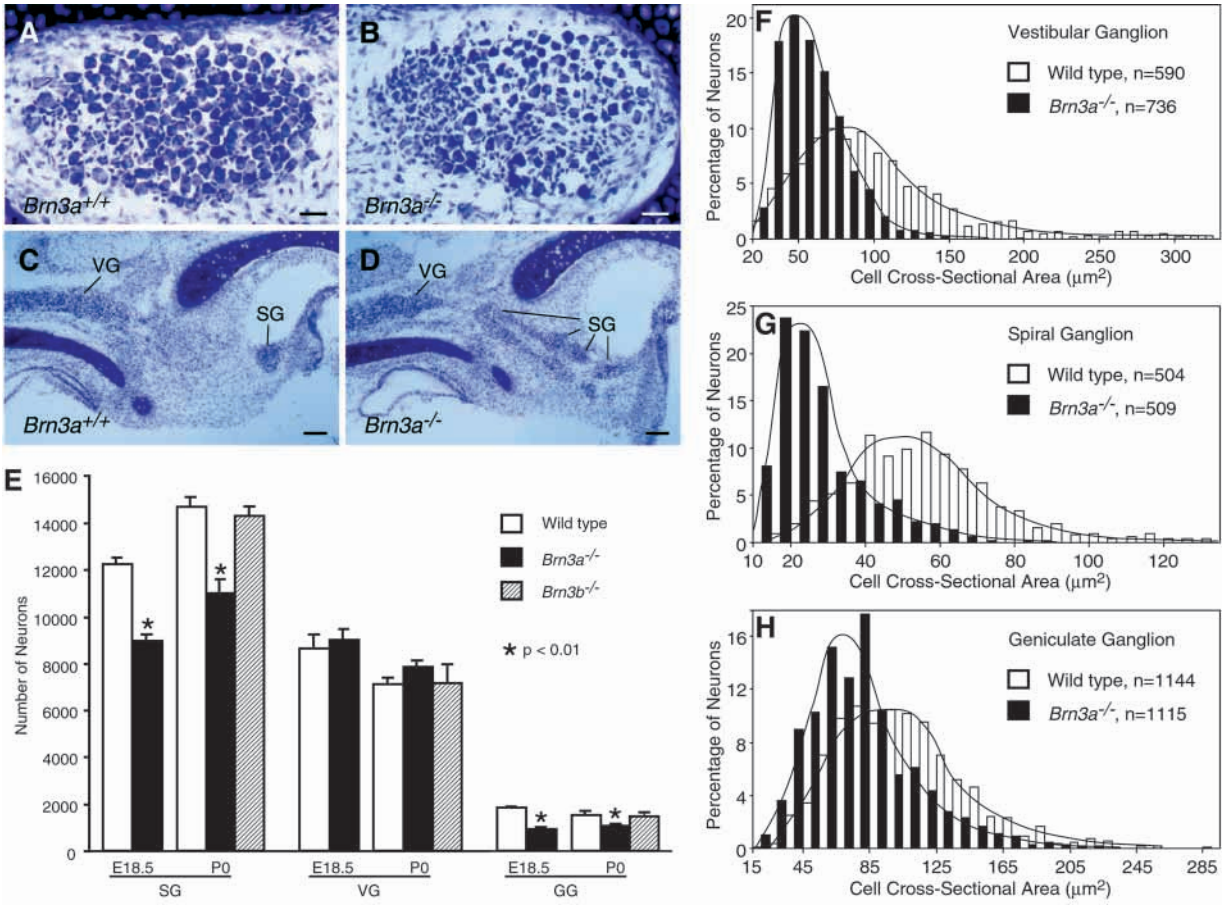

Fig. 2. Neuronal loss, neuron size reduction and migration defects in the spiral, vestibular and geniculate ganglia of *Brn3a*$^{-/-}$ mice. (A,B) Geniculate ganglion sections from P0 *Brn3a*$^{+/+}$ (A) and *Brn3a*$^{-/-}$ (B) mice were stained with Cresyl Violet. Compared with the wild type, fewer neurons are present in the mutant geniculate ganglion and they appear to be reduced in size. (C,D) Inner ear sections from P0 *Brn3a*$^{+/+}$ (C) and *Brn3a*$^{-/-}$ (D) neonates were labeled with Cresyl Violet. Spiral ganglion (SG) neurons are clustered in the modiolus of the wild-type cochlea (C), whereas they are widely scattered in the mutant (D). (E) Quantitation of neuron number in developing spiral (SG), vestibular (VG) and geniculate (GG) ganglia of wild-type, *Brn3a*$^{-/-}$ and *Brn3b*$^{-/-}$ mice. Each histogram represents the mean±s.d. for three to four ganglia. Wild type includes both *Brn3a*$^{+/+}$ and *Brn3a*$^{+/-}$ ganglia, which are indistinguishable in neuron number. (F-H) Distributions of cross-sectional areas of neuronal profiles in Cresyl Violet-labeled vestibular ganglion (F), spiral ganglion (G), and geniculate ganglion (H) from P0 wild type and *Brn3a*$^{-/-}$ mice. Cross-sectional areas were measured for neuron profiles using NIH Image and the data were plotted using Microsoft Excel. For all three ganglia, the mutant shows a much narrower distribution with the peak substantially shifted to the left compared to the wild type. Scale bar: 25 μm in A,B; 100 μm in C,D.

labeled with Cresyl Violet. As shown in Fig. 2E, at E18.5 and P0, there is a 30-50% reduction of neurons in the geniculate ganglion, and an approximately 30% decrease in the spiral ganglion in *Brn3a*$^{-/-}$ mice compared with controls. By contrast, there is no significant reduction in the number of neurons present in the vestibular ganglion at E18.5 or P0. Therefore, absence of Brn3a affects the survival of sensory neurons in the three ganglia to different extents. A similar quantitative analysis detected no significant differences in the number of neurons within the spiral, vestibular and geniculate ganglia between controls and mutant *Brn3b* mice at P0 (Fig. 2E). Thus, the presence of Brn3b is not essential for the maintenance of these sensory neurons.

Diminution of neuronal size in *Brn3a*$^{-/-}$ spiral, vestibular and geniculate ganglia

To investigate possible effects of *Brn3a* absence on differentiation of inner ear and geniculate sensory neurons, we compared these neurons using Cresyl Violet-labeled sections of wild-type and mutant ganglia. Two notable phenotypes were detected in the mutant ganglia. First, in contrast to the wild-type spiral ganglion (in which neurons are tightly packed) the spiral ganglion neurons in *Brn3a*$^{-/-}$ mice are scattered in the entire cochlear modiolus with many barely separated from the vestibular ganglion (Fig. 2C,D). Second, although the vestibular ganglion is well-formed and vestibular neurons appear to survive up to P0 in *Brn3a*$^{-/-}$ mice (Fig. 2E), we noticed a dramatic decrease in the volume of the vestibular ganglion and in the size of its neurons (Fig. 2F and data not shown). To quantify changes in neuronal size, the areas of neuronal cell profiles were measured in vestibular, spiral and geniculate ganglia using sections from both wild-type and mutant mice. In wild-type P0 neonates, the large majority of neurons in the vestibular ganglion have areas between 30-200 μm^2, with a peak at ~90 μm^2. In addition, a significant number of these neurons are more than 200 μm^2 with a few as large as

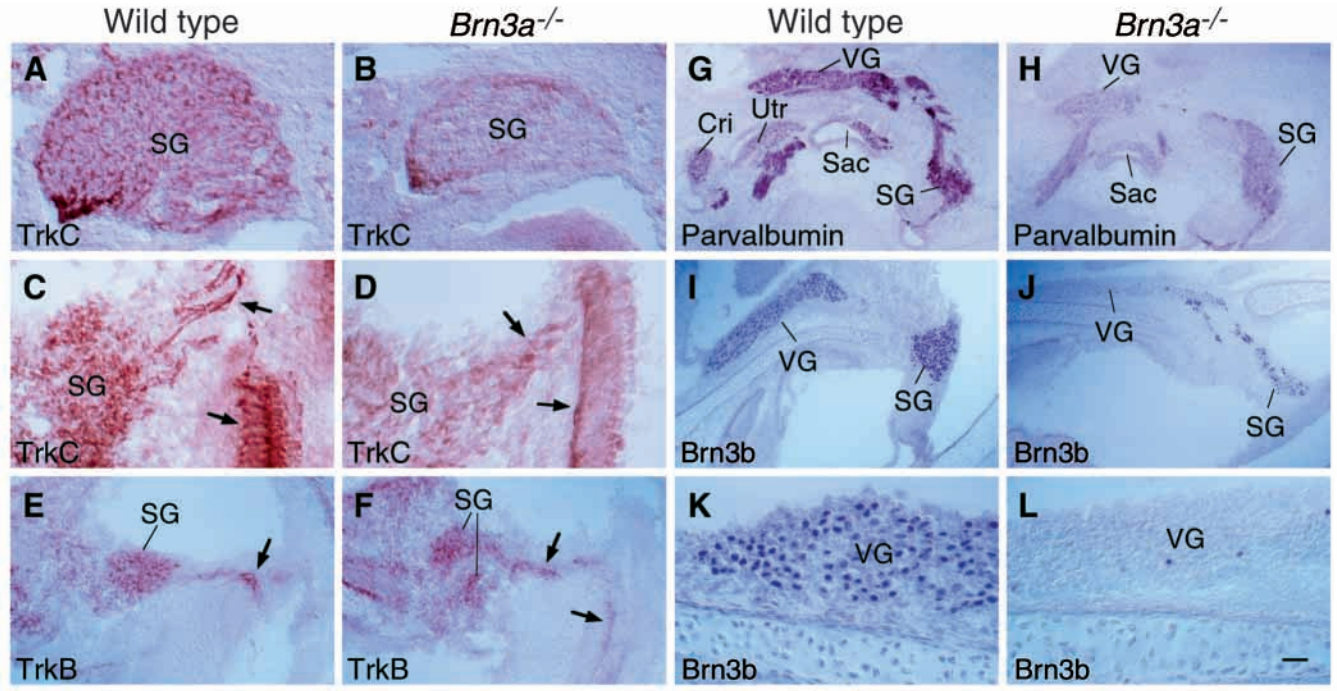

Fig. 3. Reduction of TrkC, parvalbumin and Brn3b expression in the *Brn3a*$^{-/-}$ spiral ganglion. (A-F) Compared with the wild-type ganglia (A,C), TrkC immunoreactivity is greatly reduced in the E15.5 (B) and E18.5 (D) mutant spiral ganglia. By contrast, a similar level of TrkB immunoreactivity is present in E18.5 spiral ganglion between wild-type (E) and *Brn3a* mutant (F). Arrows point to radial fibers. (G,H) Parvalbumin immunoreactivity shows substantial decrease in the mutant spiral and vestibular ganglia and their nerve fibers. (I-L) In contrast to the robust expression of Brn3b in wild-type ganglia, the intensity of Brn3b expression and the number of neurons positive for Brn3b is dramatically diminished in E18.5 mutant embryos. Cri, crista; Sac, saccule; SG, spiral ganglion; Utr, utricule; VG, vestibular ganglion. Scale bar: 25 μm in A-D,K,L; 50 μm in E,F; 100 μm in G-J.

390 μm^2 in area (Fig. 2F). In *Brn3a*$^{-/-}$ vestibular ganglia at P0, however, the areas of most of the neuronal profiles are distributed between 20-110 μm^2, with a peak at only ~50 μm^2 (Fig. 2F). Similar decreases in the areas of neuronal profiles were seen at E16.5 and E18.5 in mutant vestibular ganglia (data not shown). The areas of neuronal profiles in control and mutant spiral and geniculate ganglia were also measured. In the wild-type spiral ganglion at P0, the areas of most neurons range between 25 and 85 μm^2, with a peak at ~55 μm^2 (Fig. 2G). In the mutant at P0, the range is 10-50 μm^2, with a peak at ~25 μm^2 (Fig. 2G). In the wild-type geniculate ganglion at P0, most neurons have profiles between 35 and 175 μm^2, with a peak at ~105 μm^2 (Fig. 2H). In the mutant, profile areas range from 25 to 135 μm^2, with a peak at ~65 μm^2 (Fig. 2H). Therefore, in the absence of Brn3a, neuronal size is diminished in all three ganglia, including the vestibular ganglion (in which normal numbers of neurons survive). In contrast, examination of *Brn3b* mutants revealed neither loss of neurons in these ganglia nor any reduction in neuronal size in these ganglia (Fig. 2E and data not shown), indicating that Brn3b plays little or no role in regulating the size of these neurons.

Altered gene expression in *Brn3a*$^{-/-}$ spiral, vestibular and geniculate ganglia

As members in the POU domain factor family are required for cellular differentiation during development, it is possible that Brn3a may control downstream genes that are required for the survival and differentiation of sensory neurons. In the trigeminal ganglion, for example, Brn3a has been shown to play a major role in the survival of sensory neurons by regulating the expression of neurotrophin receptors TrkA, TrkB and TrkC (Ntrk1, Ntrk2 and Ntrk3 – Mouse Genome Informatics; McEvilly et al., 1996; Huang et al., 1999b). To determine whether Brn3a similarly regulates neurotrophin receptor expression in the spiral, vestibular and geniculate ganglia, we examined by immunostaining the expression levels of TrkB, TrkC and p75^{NTR} in these ganglia in E13.5-E18.5 *Brn3a*$^{-/-}$ embryos. As early work has shown that TrkB and TrkC are the major neurotrophin receptors controlling neuronal survival in these ganglia, we wished to determine the effect of Brn3a on the expression of TrkB and TrkC (Pirvola et al., 1994; Minichiello et al., 1995; Schimmang et al., 1995; Fritzsch et al., 1997b). In wild-type mice, both TrkB and TrkC immunoreactivities were detected in spiral ganglia and radial fibers of the cochlea (Fig. 3A,C,E). While the TrkB immunoreactivity in the *Brn3a*$^{-/-}$ was comparable with that in the wild-type control (Fig. 3F), TrkC immunoreactivity was markedly reduced by E15.5 and E18.5 (Fig. 3B,D). At E13.5, however, similar levels of TrkC immunoreactivity were observed in the vestibulocochlear ganglia and their projecting fibers between the wild-type and mutant (data not shown). Thus, the neuronal loss in *Brn3a*$^{-/-}$ cochleae is likely to result from a failure to maintain proper TrkC expression in the spiral ganglion neurons. In *Brn3a*$^{-/-}$ vestibular and geniculate ganglia, TrkB and TrkC were expressed at a level similar to that of the control (data not shown). Similar to the trigeminal ganglion (Huang et al., 1999b), we found that p75^{NTR} was expressed at comparable levels in wild type and mutant in the

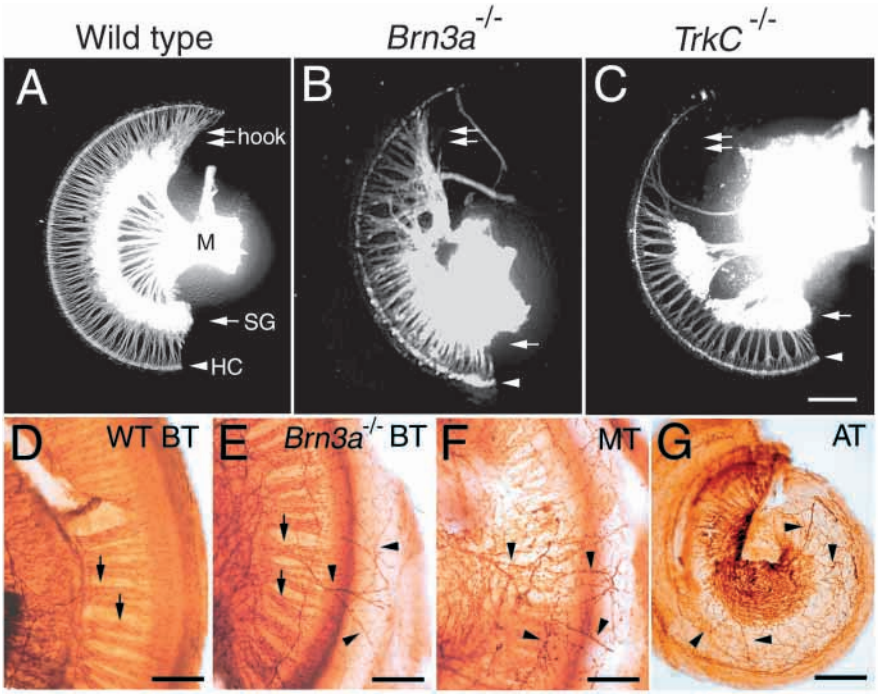

Fig. 4. Afferent and efferent projections to the cochlea in wild type, *Brn3a*$^{-/-}$ and *TrkC*$^{-/-}$ mice. (A-C) Afferent projection to the basal turn of cochlea at P0 is demonstrated by labeling the fibers with DiI. This labeling highlights the modiolus (M), spiral ganglion (SG) and inner ear hair cells (HC). In contrast to the densely organized afferent fibers in the wild-type cochlea (A), there is an overall reduction in fiber density in the basal turn of cochlea in *Brn3a*$^{-/-}$ mice, including an almost complete absence of the hook region of the ganglion in the basal turn (double arrowheads) and gapping between fiber bundles (B). These abnormalities in the *Brn3a*$^{-/-}$ cochlea are similar to those present in *TrkC*$^{-/-}$ mice (C). (D-G) The acetylated tubulin antibody labels both afferent (arrows) and efferent (arrowheads) fibers of the cochlea. In contrast to the orderly arrangement of fibers in wild-type mice (D), there is disordered outgrowth of efferent fibers in the basal turn (BT), middle turn (MT) and apical turn (AT) of the *Brn3a*$^{-/-}$ cochlea (E-G). Scale bar: 200 μm in A-C; 100 μm in D-G.

fibers and cell bodies of spiral, vestibular and geniculate sensory neurons (data not shown). Thus, the expression of TrkC in the spiral ganglion requires Brn3a, whereas the expression of TrkB and p75^{NTR} is independent of Brn3a.

In addition to the Trk receptors, we also examined the expression of parvalbumin, Brn3b and neurofilament 150 (NF 150) in the spiral, vestibular and geniculate ganglia of E16.5 and E18.5 *Brn3a*$^{-/-}$ embryos. In the control, the Ca^{2+}-binding protein parvalbumin was prominently localized in the cell bodies, as well as processes of spiral and vestibular ganglion neurons, but this immunoreactivity was greatly diminished in the mutant (Fig. 3G,H). Similarly, the number of neurons expressing Brn3b in the spiral ganglion was dramatically reduced in the mutant (Fig. 3I,J). This decrease was even more pronounced in the mutant vestibular ganglion, where neurons positive for Brn3b could barely be seen (Fig. 3I-L). In the mutant geniculate ganglion, Brn3b expression was also greatly reduced (data not shown). However, no significant difference in the level of NF 150 expression was observed in the neurons of spiral, vestibular and geniculate ganglia between the *Brn3a* wild type and mutant (data not shown). Thus, Brn3a may differentially control differentiation of sensory neurons by regulating the expression of genes common to all sensory ganglia as well as genes specific to different sensory ganglia.

Defects in target field innervation and axon pathfinding in vestibulocochlear ganglion of *Brn3a*$^{-/-}$ mice

As expression of Brn3a is initiated in vestibulocochlear neurons before the beginning of peripheral target innervation, it is possible that Brn3a helps regulate this important event. To test this possibility, we examined the afferent and efferent innervation patterns of wild-type and mutant inner ears at various developmental stages by DiI tracing and immunohistochemistry.

In the P0 wild-type cochlea, DiI labeling reveals that the axons of spiral ganglion neurons in the entire basal turn fasciculate into closely spaced radial fibers to centrifugally innervate hair cells in the organ of Corti (Fig. 4A). In contrast, the afferent innervation by these sensory neurons in the *Brn3a*$^{-/-}$ cochlea was sparse in the basal turn with an increased distance between fiber bundles and a paucity of radial fibers at the hook region (Fig. 4B). In addition, some radial fibers close to the base have shifted trajectories and innervate hair cells in the hook region. Consistent with the quantitation of neuronal numbers (Fig. 2E), spiral ganglion neurons are depleted in the base and reduced in the basal turn of the mutant cochlea (Fig. 4B). Interestingly, the topographical losses of sensory neurons and deficiencies in afferent innervation present in the *Brn3a*$^{-/-}$ cochlea resemble those found in *TrkC*$^{-/-}$ mutants (Fritzsch et al., 1998; Fig. 4C), consistent with the observation of a nearly complete absence of TrkC neurons in the *Brn3a*$^{-/-}$ spiral ganglion by P0 (Fig. 3A-D). In order to determine the timing of this deficit and its relation to the downregulation of TrkC expression, we performed similar experiments in early embryonic stages before loss of TrkC expression occurs. At E13.5, while the cochlear sensory epithelium of wild-type animals is densely innervated by afferent fibers (Fig. 5A), only a small number of afferent fibers are labeled in the Brn3a mutant cochlea (Fig. 5B). As the afferent innervation was eventually established in the P0 cochlea, the data from the embryonic stage indicate that afferent innervation to the cochlea is initially delayed in the absence of *Brn3a*.

The overall patterns of cochlear innervation in *Brn3a*$^{-/-}$ mice were further investigated by immunohistochemistry, using an anti-acetylated tubulin antibody that labels both afferents and efferents. In the P0 *Brn3a*$^{-/-}$ cochlea, tubulin-immunoreactive fibers display an innervation pattern defect similar to afferent fibers as revealed by DiI labeling. More notably, there is exuberant outgrowth of misrouted fibers throughout the cochlea. Many of these fibers project beyond the boundary of hair cells, some reaching as far as the

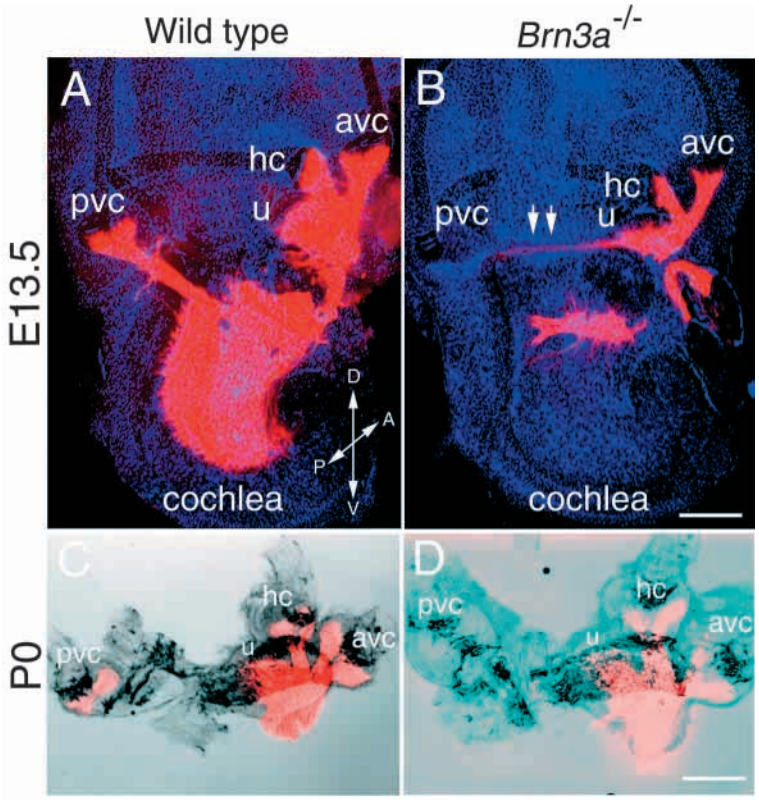

Fig. 5. Abnormal afferent fiber projections in the semicircular canals of *Brn3a*$^{-/-}$ mice. (A,B) DiI-labeled flat mount of E13.5 inner ears shows a dramatic reduction in fiber projection to the posterior vertical canal (pvc) and the cochlea in *Brn3a*$^{-/-}$ embryos. Although slightly reduced, the innervation to the anterior vertical canal (avc), horizontal canal (hc) and utricle (u) in *Brn3a*$^{-/-}$ mice is comparable with that in the wild type. (C,D) In contrast to the dense innervation in the wild type, the posterior vertical canal of *Brn3a*$^{-/-}$ mice shows essentially no innervation at P0. Scale bars: 200 μm.

mesenchyme surrounding the organ of Corti (Fig. 4E-G). None of these aberrant fibers are present in the control wild-type cochlea (Fig. 4D). As these abnormalities are not present in the DiI-labeled afferent pathways (Fig. 4B), it is most likely that these misrouted fibers represent cochlear efferent fibers in the mutant.

In the P0 *Brn3a*$^{-/-}$ vestibular system, despite the marked reduction of vestibular ganglion neuronal size, DiI labeling shows grossly normal afferent innervation of the maculae of saccule and utricle, and the cristae of anterior vertical and horizontal canals. However, there is a complete absence of afferent fiber projection to the posterior vertical canal (Fig. 5C,D). This innervation defect exists as early as E13.5, when the afferent fibers innervating the posterior vertical canal in the mutant show a severe reduction in the fiber bundle size. More importantly, the few fibers that do innervate the posterior vertical canal appear to arise dorsally from the utricle, rather than by the more ventral and separate route (Fig. 5A,B). As there are no fibers innervating the posterior vertical canal in the mutant by P0, this suggests that the originally misrouted fibers have failed to maintain their projection.

We examined the overall patterns of vestibular innervation in *Brn3a*$^{-/-}$ mice by immunostaining with antibodies against NF 150 and p75^{NTR}, and by silver impregnation, all of which stain both afferent and efferent fibers (Fig. 6). In the E16.5 and E18.5 vestibular system, several innervation anomalies were observed in the mutant. For example, in at least half of mutant embryos, a large fiber bundle splits from the bundle that innervates the crista of the anterior vertical canal, penetrates the bony labyrinth, and projects into the middle ear with or without further branching (Fig. 6B-D,F). In addition, the fiber bundle that innervates the saccular macula often abnormally branches away to project into the cochlea of the mutant (Fig. 6E). As these aberrant fibers are not found in DiI-labeled afferent projections (Fig. 5), they probably represent vestibular efferent fibers similar to those present in the mutant cochlea (Fig. 4E-G). Therefore, these data together indicate that Brn3a is required for proper innervation and axon pathfinding of both afferent and efferent fibers in the cochlear and vestibular systems.

Loss of Brn3a does not affect expression of members in the Eph family

The deficits in axon pathfinding in the inner ear of *Brn3a*$^{-/-}$ mutants are reminiscent of the phenotypes in mice with targeted deletion in *EphB2* and raise the possibility that *EphB2* could be a target gene whose expression is regulated by Brn3a. Because members in the ephrin and Eph family have been implicated in axon pathfinding and cell migration, it is possible that loss of Brn3a may affect their expression. To test this hypothesis, we examined the expression of EphB1, EphB2 and EphB6 in sensory ganglia and brainstem nuclei of *Brn3a*$^{-/-}$ mutants. Our data indicate that by immunohistochemistry there is no detectable difference in the amount of EphB1 protein in the spiral ganglion neurons, trigeminal neurons and neurons in the inferior olivary nucleus between *Brn3a*$^{-/-}$ mutants and their wild-type littermates (Fig. 7). Immunostaining of EphB2 and EphB6 shows less intense expression of both proteins in the spiral and vestibular neurons and there is no detectable difference between the wild type and *Brn3a*$^{-/-}$ at E12.5 and E16.5 (data not shown).

DISCUSSION

Our analyses of the development of facial-stato-acoustic ganglion in the *Brn3a*$^{-/-}$ mice provide evidence to indicate novel functions of Brn3a in regulating neuronal survival, differentiation, migration and axon pathfinding of these ganglia (Fig. 8). We show that loss of *Brn3a* leads to downregulation of gene expression, most notably that of *TrkC*, *Brn3b* and parvalbumin (Fig. 8B). In addition, our results uncover previously unidentified roles of Brn3a in regulating axon pathfinding and target field innervation in spiral and vestibular ganglion neurons (Fig. 8A). The innervation defects appear earlier than the downregulation of TrkC expression, suggesting that these two mutant phenotypes probably are independent of each other. This is further supported by the almost complete absence of innervation to the posterior vertical canal, a phenotype not identified in *TrkC*$^{-/-}$ mice. Interestingly,

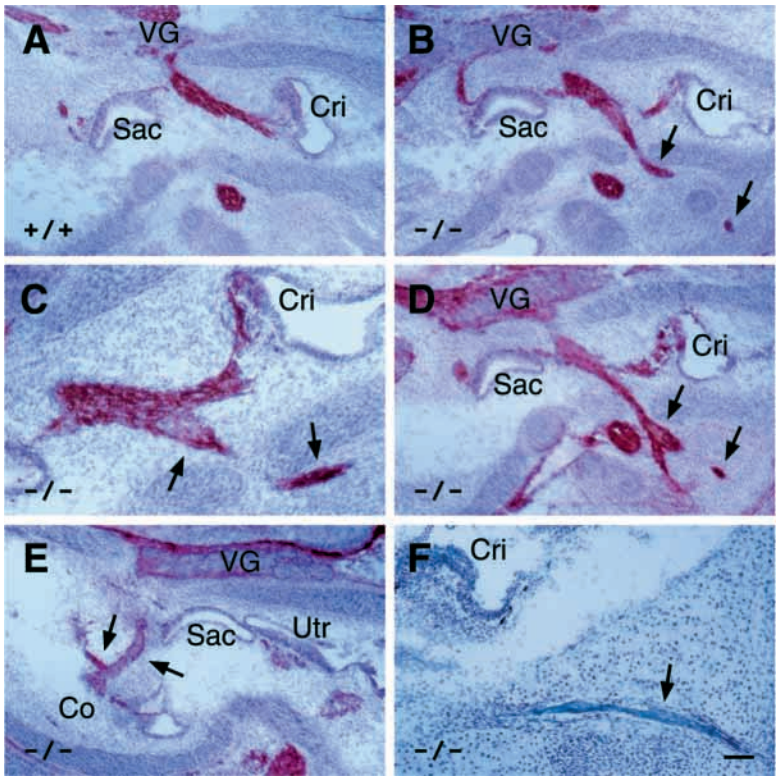

Fig. 6. Axon misrouting in the *Brn3a*$^{-/-}$ vestibular system. Inner ear sections from E16.5 (A-E) and E18.5 (F) *Brn3a*$^{+/+}$ (A) and *Brn3a*$^{-/-}$ (B-F) embryos were immunostained with anti-NF 150 (A-C) and anti-p75^{NTR} (D,E) antibodies with counterstaining by Hematoxylin, or labeled by silver impregnation (F). Although in the wild type (A), vestibular ganglion (VG)-derived nerve fibers immunoreactive for NF 150 or p75^{NTR} invariantly follow pathways leading to innervation of only sensory epithelia of the saccule (Sac), utricule (Utr) and crista (Cri), they often form aberrant branches in the mutant that project into the outside of the inner ear (B-D), or into the cochlea (Co) (E). Tracing nerve fiber trajectories by silver impregnation reveals similar anomalous fiber branches that penetrate the temporal bone and project away from the inner ear (F). Arrows indicate abnormal nerve fiber branches. Scale bar: 50 μm in C,F; 100 μm in A,B,D,E.

although the afferent axons eventually reach their targets, the selective loss of TrkC neurons in the spiral ganglion of *Brn3a*$^{-/-}$ cochlea leads to an innervation defect similar to that of *TrkC*$^{-/-}$ mice. In addition, efferent axons that use the afferent fibers as scaffold during pathfinding also show severe misrouting to the mutant inner ear. Despite similar axon pathfinding defect in *Brn3a*$^{-/-}$ mutants and *EphB2* mutants, loss of *Brn3a* apparently does not result in dramatic loss of *EphB1*, *EphB2 or EphB6*. Thus, these data indicate that Brn3a controls additional factors that are required for axon pathfinding in the developing inner ear.

Sequential expression and crossregulation of Brn3a and Brn3b during sensorineural development

During genesis of the inner ear and geniculate ganglia, the onset of Brn3a expression is observed in neuronal progenitors in the facial-stato-acoustic ganglion prior to fate commitment and differentiation (Fig. 1). Thereafter, Brn3a continues to show robust expression in differentiated neurons in these ganglia. Strong Brn3b expression, however, is not seen until E16.5 when soma growth, axon targeting and migration of sensory neurons have been established (Fig. 1; Sher, 1971). Interestingly, in contrast to the severe deficits in the trigeminal and facial-stato-acoustic ganglia of *Brn3a*$^{-/-}$ mice, no obvious neuronal defects are present in *Brn3b*$^{-/-}$ mice (Fig. 2E; Gan et al., 1996; Xiang et al., 1996; McEvilly et al., 1996). In contrast, we have previously shown that Brn3b is turned on in retinal ganglion cells two days before Brn3a during retinogenesis (Xiang, 1998). Targeted deletion in *Brn3b* results in loss of ~70% of retinal ganglion cells whereas *Brn3a* deletion causes no overt

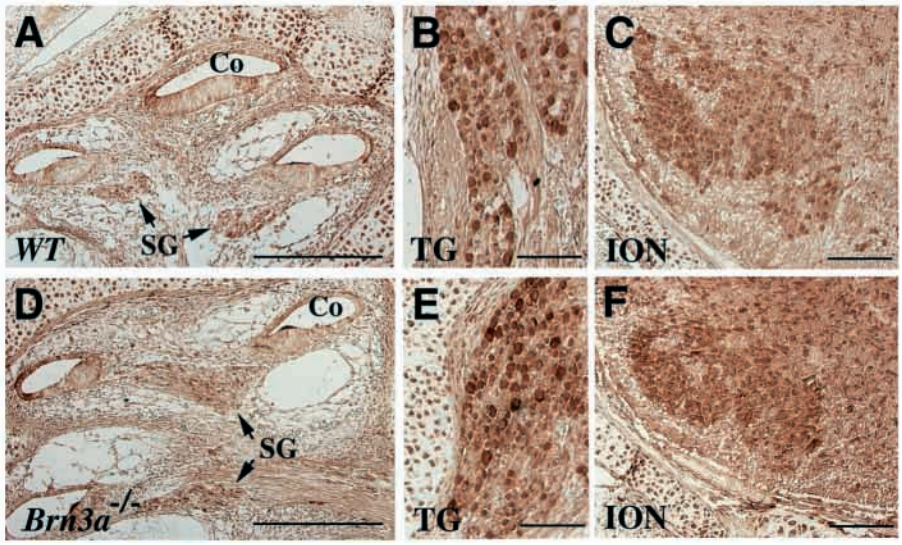

Fig. 7. Expression of EphB1 in the nervous system of *Brn3a* mutant mice. Sections of E16.5 wild-type (A-C) and *Brn3a*$^{-/-}$ (D-F) embryos show no difference in the expression of EphB1. Neurons in the spiral ganglion, trigeminal ganglion and inferior olivary nucleus, which are affected by *Brn3a* mutation, express similar level of EphB1 protein in both wild type and *Brn3a* mutants. Co, cochlea; ION, inferior olivary nucleus; SG, spiral ganglion; TG, trigeminal ganglion. Scale bar: 100 μm in A,D; 50μm in B,C,E,F.

defects in the retina (Gan et al., 1996; Erkman et al., 1996; Xiang et al., 1996).

Thus, these results indicate that the differential effects of *Brn3a* and *Brn3b* deletions on development of the sensory ganglia and retina correlate with the temporal expression orders of Brn3a and Brn3b in these sensorineural structures. Although members in the Brn3 family show significant overlap in their expression patterns, the ones that demonstrate earlier and broader expression always have more dominant roles in regulating cell differentiation in specific sensory ganglia (Fig. 8B). Indeed, Brn3a and Brn3b have been shown to share the same DNA-binding sites and both can activate gene expression (Turner et al., 1994; Gruber et al., 1997; Trieu et al., 1999; Liu et al., 2000a; Liu et al., 2000b), indicating that each may be able to compensate for the other when co-expressed in the same cell. Such a model predicts that forced expression of members of Brn3 family will compensate for the functions of the others. Consistent with this prediction, our recent data in the developing chick retinal ganglion cells show that all Brn3 factors are capable of promoting the differentiation of these neurons (Liu et al., 2000a).

The regulation by Brn3a of expression of Brn3b has added a layer of interesting complexity to the biological functions of these factors (Fig. 8B). In the sensory ganglia, for example, the absence of Brn3a causes an almost complete loss of Brn3b expression (Xiang et al., 1996; McEvilly et al., 1996; Fig. 3I-L), whereas elimination of Brn3b results in a dramatic reduction of Brn3a expression in the retina (Gan et al., 1996; Xiang, 1998). Thus, elimination of Brn3a in the sensory ganglia and Brn3b in the retina effectively leads to a functional double knockout. Although the exact mechanism for the crossregulation between Brn3a and Brn3b is not clear, one likely explanation is that there may be a direct transcriptional cross-activation of Brn3b by Brn3a and vice versa. Consistent with this model, Brn3a and Brn3b have been shown to be capable of activating each other's promoter in vitro (Trieu et al., 1999; Liu et al., 2001).

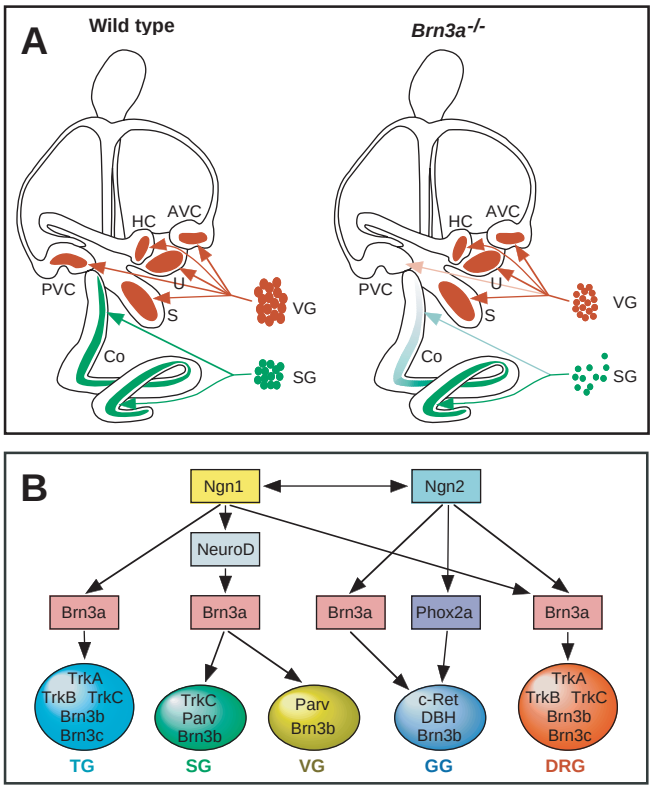

Fig. 8. Schematics illustrating inner ear defects and genetic interactions revealed by analysis of *Brn3a* mutant mice. (A) Inner ear defects in *Brn3a*$^{-/-}$ mice. In the mutant, the spiral ganglion (SG) loses ~30% of neurons by P0, whereas the vestibular ganglion (VG) contains normal number of neurons. Substantial reduction in soma size of neurons is seen in both *Brn3a*$^{-/-}$ SG and VG. In addition, the mutant SG neurons are defective in migration and thus do not become clustered. In the mutant cochlea (Co), there is overall decrease in afferent fiber density in the basal turn with a nearly complete loss of innervation at the base (broken green arrow). Moreover, efferent innervation displays profound pathfinding defects throughout the entire cochlea. In the mutant vestibular system, the saccule (S), utricle (U), and anterior vertical (AVC) and horizontal (HC) canals are all well innervated by afferent fibers. However, the posterior vertical canal (PVC) lacks afferent innervation (broken pink arrow). (B) Genetic interactions between Brn3a and other transcriptional regulators during sensory gangliogenesis. Brn3a is required for differentiation and survival of sensory neurons in the trigeminal (TG), spiral, vestibular, geniculate (GG) and dorsal root (DRG) ganglia. For fate commitment of neuron progenitors, the TG, SG and VG require Ngn1; the GG requires Ngn2; and the DRG requires both Ngn1 and Ngn2. Therefore, Ngn1 and Ngn2 genetically act upstream of Brn3a. Brn3a regulates expression of TrkA, TrkB, TrkC, Brn3b and Brn3c in TG and DRG, and that of parvalbumin (Parv) and Brn3b in SG and VG. In addition, it controls Brn3b expression in GG, and TrkC expression in SG. During GG development, Phox2a also genetically acts downstream of Ngn2 as it is only required for differentiation and survival of GG neurons. Phox2a has been shown to control expression of c-Ret and dopamine-β-hydroxylase (DBH) in GG.

Role of Brn3a in the control of neuronal growth, axon pathfinding and innervation during development of the facial-stato-acoustic ganglion

In the nervous system, specific neuron types have a characteristic soma size. As neurons primarily grow to a particular size after exit from cell cycle, neuronal growth constitutes an important part of neuronal differentiation which must be subject to tight developmental regulation. However, there is little knowledge at present about what controls the size of neurons and neural tissues (Conlon and Raff, 1999). Sensory neurons usually have a relatively large cell body, perhaps manifesting the requirement for a high biosynthetic capacity imposed by their long axons. The substantial reduction in soma size of neurons within *Brn3a*$^{-/-}$ spiral, vestibular and geniculate ganglia indicates a pivotal role for Brn3a in neuronal growth in these sensory ganglia. The insulin signaling pathway has recently been shown to be conserved from *Drosophila* to vertebrates in the regulation of cell size and number (Bohni et al., 1999; Montagne et al., 1999). It is therefore possible that Brn3a may control neuronal growth by regulating expression of components of the insulin signaling pathway or other growth regulators.

Axon pathfinding and innervation are other important aspects of neuron differentiation program that occur after terminal mitosis. During neurogenesis, the axons of inner ear sensory neurons must navigate long distances to sort out and innervate their appropriate peripheral and central targets for

proper hearing and balance. It is generally believed that this complicated feat is achieved in part by a chemotactic mechanism – the axonal growth cone integrates and responds to a large number of guidance cues including both attractive and repulsive factors (Dodd and Jessell, 1988; Tessier-Lavigne and Goodman, 1996). Recent genetic analyses have provided new evidence that guidance of axon pathfinding can be controlled by transcription factors. For example, the homeodomain transcription factor Hb9 and LIM homeodomain transcription factors, Lim1 and Lmx1b, are required for specifying motoneuron identity and establishing fidelity of trajectory for motoneuron axons (Arber et al., 1999; Thaler et al., 1999; Kania et al., 2000). Members of the class IV POU domain transcription factors have been shown to be important regulators in axon pathfinding and synaptic targeting. Deletion of *Brn3a* or *Brn3b* in mice results in delay and misrouting in axon projection in the vestibulocochlear neurons and retinal ganglion cells, respectively (this study and Erkman et al., 2000). Most intriguingly, other class IV POU domain factors Unc-86 and Acj6 have also been shown to be important in regulating motoneuron migration and axon projection in *Caenorhabditis elegans* and central neuron synaptic targeting in *Drosophila* (Sze et al., 1997; Certel et al., 2000). Thus, the functions of class IV POU domain transcription factors appear to be conserved in evolution.

The innervation to the hair cells of cochlea and vestibular system provides an ideal system for investigating the molecular mechanisms that control this process. In the mouse otocyst, the earliest axons are detected by late E10.5 (Sher, 1971). The onset of Brn3a expression in the facial-stato-acoustic ganglion at E9 suggests a potential role for Brn3a in axon guidance and innervation (Fig. 1). Indeed, multiple defects in axon pathfinding and innervation are observed in inner ears of mice that lack *Brn3a*, including afferent innervation defects in the cochlea and posterior vertical canal and axon misrouting of cochlear and vestibular efferents (Figs 4-6, 8A). These axon innervation defects are present as early as E13.5 in the developing inner ear (Figs 4, 5), consistent with an early expression pattern of Brn3a in the vestibulocochlear ganglia. Interestingly, although the final establishment of innervation by the afferent fibers is achieved at P0, there is a complete loss of innervation to the basal turn of the cochlea and the posterior vertical canal. Furthermore, significant gapping of afferent fibers is present in the middle and apical turns of the cochlea (Fig. 4). Thus, our data strongly suggest that Brn3a controls the initiation of axon pathfinding in the cochlea and posterior vertical canal. These data also suggest that additional factors may be capable of compensating for the loss of Brn3a to establish the final innervation to middle and apical turns of the cochlea and to other vertical canals.

The afferent innervation defects and neuron loss in *Brn3a*$^{-/-}$ cochleae are similar to those found in *TrkC*$^{-/-}$ and *Nft3*$^{-/-}$ (NT3) mutants (Fritzsch et al., 1997a; Fritzsch et al., 1998). While our data indicate that this may be a result of severe down-regulation of TrkC in *Brn3a*$^{-/-}$ spiral ganglia by midgestation stage, it is possible that other mechanisms also contribute to these defects. In the inner ear, *TrkC* appears to be just one of the effector genes of *Brn3a* that are required to explain the multiple defects in *Brn3a*$^{-/-}$ mutants. Several innervation and pathfinding defects found in the *Brn3a*$^{-/-}$ inner ear are not observed in *TrkC*, *TrkB*, *Ntf3* or *Bdnf* mutants (reviewed by Fritzsch et al., 1999). These include the cochlear and vestibular afferent and/or efferent guidance defects, lack of innervation of the posterior vertical canal, and the developmental delay in cochlear afferent projection. Furthermore, the innervation defects occur as early as E13.5, before the downregulation of TrkC, it is hence conceivable that Brn3a may also regulate expression of other guidance molecules and signaling proteins required for proper afferent and efferent pathfinding and innervation in the inner ear. The absence of EphB2 ephrin receptor has recently been reported to cause growth delay and abnormal navigation of inner ear efferents (Cowan et al., 2000). Although its expression does not appear to be affected by Brn3a, it would be interesting to examine whether other ephrin receptors or ligands expressed in the ear (see Cowan et al., 2000 for a review) are altered in *Brn3a*-null mutants.

A cascade of transcriptional regulation in neuronal survival and differentiation in sensory ganglia

The development of sensory neurons in different ganglia appears to be controlled by a cascade of transcriptional regulation (see Anderson, 1999 for a review). Our data and earlier work have demonstrated an essential role for Brn3a in the differentiation and survival, but not fate commitment of neurons in the spiral, vestibular, geniculate, trigeminal, and dorsal root ganglia (Xiang et al., 1996; McEvilly et al., 1996; Huang et al., 1999b). In contrast, expression of the basic helix-loop-helix genes *Ngn1* and *Ngn2* precedes that of *Brn3a* in these sensory ganglia and they are required singly or in combination for generation of these neurons, indicating they act upstream of *Brn3a* to determine sensory neuron progenitors (Fig. 8B; Fode et al., 1998; Ma et al., 1998; Ma et al., 1999). Indeed, ectopic expression of Ngn1 in non-neural tissue in chick induces expression of Brn3a and TrkC (Anderson, 1999; Perez et al., 1999). Interestingly, recent analyses on the inner ear sensory neurons of *Ngn1* mutant mice showed a more severe phenotype with a complete absence of both afferent and efferent fiber projections (Ma et al., 2000a). In contrast, compared with *Brn3a* mutant, mice with targeted deletion in *NeuroD* (*Neurod1* – Mouse Genome Informatics) a downstream target of *Ngn1*, showed similar, albeit slightly more severe, defects in the axon projections to the cochlea and to the posterior vertical canal (Liu et al., 2000b; Kim et al., 2001). Together these observations strongly suggest that a cascade of transcription factors are required for survival and differentiation of inner ear sensory neurons (Fig. 8B).

Sensory neurons have distinct morphology, innervation targets, survival requirements and physiological properties. This diversity must be generated by differential patterns of gene expression during development. Brn3a appears to regulate different sets of downstream genes in different sensory ganglia (Fig. 8B). In the *Brn3a* mutant, the expression levels of TrkA, TrkB and TrkC are markedly reduced in the trigeminal and dorsal root ganglia (McEvilly et al., 1996; Huang et al., 1999b); whereas in the vestibular and geniculate ganglia, the expression of TrkB and TrkC is essentially normal (Fig. 3 and data not shown). In the *Brn3a*$^{-/-}$ spiral ganglion, TrkB expression is similarly not affected, but expression of TrkC is found at a greatly reduced level (Fig. 3A-D). The differential effects of Brn3a in controlling Trk receptor expression in different sensory ganglia may in part explain why neuronal

survival is affected to different extents in these ganglia of *Brn3a*$^{-/-}$ animals. Although the exact mechanisms for this disparity remain unclear, recent characterization of the *TrkA* enhancer has revealed multiple binding sites for a wide variety of transcription factors (Ma et al., 2000b). Thus, it is possible that different combinatorial arrays of transcription factors may be required for proper Trk receptor expression in different sensory ganglion. Despite this downstream gene specificity, however, Brn3a appears to control expression of a common set of genes involved in differentiation and survival of all the affected ganglia. For examples, Brn3b expression is dramatically reduced in all the ganglia affected in *Brn3a*$^{-/-}$ mice, including the trigeminal, inner ear, geniculate and dorsal root ganglia (Figs 3I-L, 8B; Xiang et al., 1996; McEvilly et al., 1996).

Brn3a may also function in combination with other transcriptional regulators to generate diversity and specificity of sensory neurons, much as unique combinations of LIM homeodomain proteins underlie the diversification of motor neuron subclasses (Tsuchida et al., 1994). In this regard, the homeodomain factor Phox2a begins to express in the geniculate ganglion at the onset of Brn3a expression and genetically acts downstream of Ngn2 (Morin et al., 1997; Fode et al., 1998; Fig. 8B). The absence of Phox2a causes significant neuronal loss in the geniculate ganglion and prevents expression of dopamine-β-hydroxylase and c-Ret (Morin et al., 1997). Conceivably, Brn3a may act in concert with as yet unidentified transcription factors in other sensory ganglia to control differentiation programs characteristic of each particular sensory ganglion.

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