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Math5 expression and function in the central auditory system

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Abstract

The basic helix-loop-helix (bHLH) transcription factor *Math5* (*Atoh7*) is required for retinal ganglion cell (RGC) and optic nerve development. Using *Math5-lacZ* knockout mice, we have identified an additional expression domain for *Math5* outside the eye, in functionally connected structures of the central auditory system. In the adult hindbrain, the cytoplasmic *Math5-lacZ* reporter is expressed within the ventral cochlear nucleus (VCN), in a subpopulation of neurons that project to medial nucleus of the trapezoid body (MNTB), lateral superior olive (LSO), and lateral lemniscus (LL). These cells were identified as globular and small spherical bushy cells based on their morphology, abundance, distribution within the cochlear nucleus (CN), co-expression of Kv1.1, Kv3.1b and Kcnq4 potassium channels, and projection patterns within the auditory brainstem. *Math5-lacZ* is also expressed by cochlear root neurons in the auditory nerve. During embryonic development, *Math5-lacZ* was detected in precursor cells emerging from the caudal rhombic lip from embryonic day (E) 12 onwards, consistent with the time course of CN neurogenesis. These cells co-express *MafB*, *Math1* and *Math5* and are post-mitotic. *Math5* expression in the CN was verified by mRNA *in situ* hybridization, and the identity of positive neurons was confirmed morphologically using a *Math5-Cre* BAC transgene with an alkaline phosphatase reporter.

The hindbrains of *Math5* mutants appear grossly normal, with the exception of the CN. Although overall CN dimensions are unchanged, the *lacZ* positive cells are significantly smaller in *Math5* $-/-$ mice compared to *Math5* $+/-$ mice, suggesting these neurons may function abnormally. The Auditory Brainstem Response (ABR) of *Math5* mutants was evaluated in a BALB/cJ congenic background. ABR thresholds of *Math5* $-/-$ mice were similar to those of wild-type and heterozygous mice, but the interpeak latencies for Peaks II-IV were significantly altered. These temporal changes are consistent with a higher level auditory processing disorder involving the CN, potentially affecting the integration of binaural sensory information.

Keywords

Math5; cochlear nucleus; AVCN; PVCN; mouse; atonal; Math1; bHLH; globular bushy cells; spherical bushy cells; cochlear root neurons; auditory processing; ABR latency; binaural integration;

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interaural intensity difference; interaural time difference; phase locking; Calyx of Held; Kv1.1; Kv3.1; Kcnq4; mafB; Cre recombinase; fluorogold; tract-tracing; inferior colliculus; multipolar cells; rhombic lip

Introduction

In *Drosophila melanogaster*, the *atonal* basic helix-loop-helix (bHLH) transcription factor is required for development of R8 photoreceptors and the chordotonal and antennal mechanosensory organs (Eberl, 1999; Jarman et al., 1995). There are two *atonal* semi-orthologs in mammals, *Math1* and *Math5* (also known as *Atoh1* and *Atoh7*, Mouse Genome Informatics). *Math1* is expressed during development in the cerebellar external granule layer (EGL); in progenitors that develop into ventral cochlear nucleus, pontine nucleus and dorsal spinal tract neurons; and in peripheral mechanosensory structures, including cochlear hair cells and cutaneous touch receptors (Ben-Arie et al., 1997; Bermingham et al., 1999; Wang et al., 2005). *Math1* homozygous mutant mice lack these tissues and die shortly after birth (Ben-Arie et al., 2000). *Math5* is transiently expressed in the developing retina, where it controls early steps in cell fate determination (Brzezinski and Glaser, 2007). Homozygous *Math5* knockout mice are viable but lack retinal ganglion cells (RGCs) and optic nerves, and are insensitive to circadian photoentrainment (Brown et al., 2001; Brzezinski et al., 2005; Wang et al., 2001).

These data suggest the proprioceptive and visual functions of invertebrate *atonal* have been discretely partitioned in mammals between *Math1* and *Math5*, respectively (Hassan and Bellen, 2000), most likely through gene duplication and divergence of modular enhancers during evolution (Force et al., 1999; Hufnagel et al., 2007). Relatively few examples of complete functional complementarity between paralogous genes are known. In most cases, paralogs exhibit some degree of functional redundancy, where the activity or expression domains overlap, spatially and temporally.

To test partitioning of sensory functions between mammalian *atonal* homologs, we have used mice with a cytoplasmic *lacZ* (β -galactosidase) reporter cassette targeted to the *Math5* locus (Brown et al., 2001). Endogenous *Math5* regulatory elements drive *lacZ* expression, which can be easily visualized. In this report, we demonstrate *Math5-lacZ* expression in the ventral cochlear nucleus of adults and embryos, and co-expression of *Math5* and *Math1* during development.

The magnocellular region of the rodent VCN contains six major neuron classes (small and large spherical bushy cells, globular bushy cells, octopus cells, and type I and II multipolar [stellate] cells) with distinct functions, response patterns and central projections. Although specific molecular markers are lacking, these cell types can be distinguished by their size, shape, distribution, connectivity, and firing properties (Cant and Benson, 2003; Martin, 1981; Rhode and Smith, 1986; Rouiller, 1997; Webster and Trune, 1982). Using some of these criteria, we have identified *Math5*-positive cells as globular and small spherical bushy cells, which are known to have roles in central auditory processing of binaural information. Auditory brainstem responses (ABRs) recorded from *Math5* mutant mice revealed changes consistent with dysfunction of these cell types.

Materials and Methods

Mice

Math5-lacZ mice, with a targeted deletion of *Math5* and in-frame insertion of the *E. coli* γ -galactosidase gene (*Atoh7^{tm1Gla}*), were generated previously (Brown et al., 2001). Congenic BALB/cJ mice (>12 generations) were intercrossed to obtain F2 mice for auditory testing.

Some anatomical analyses were performed on outbred CD-1 *Math5-lacZ* mice. All experimental procedures were approved by the University Committee on Use and Care of Animals (UCUCA) at the University of Michigan.

β-Galactosidase Histochemistry

Adult tissues and embryos were fixed in 4% paraformaldehyde (PFA), 0.1 M Na PO₄ (pH 7.4) by transcardial perfusion or immersion, respectively. For sectioning, tissues were cryopreserved in graded 10% to 30% sucrose, and frozen in OCT (Tissue Tek, Torrance, CA) with 2 mM MgCl₂. Tissue sections and wholemount embryos were washed twice in phosphate-buffered saline (PBS pH 7.4) with 2 mM MgCl₂, and equilibrated in 0.1 M Na PO₄ (pH 7.4), 2 mM MgCl₂, 0.01% Na deoxycholate, 0.02% NP-40. Staining was performed at 37°C in the same buffer (Brown et al., 2001), with 5 mM K₃Fe(CN)₆, 5 mM K₄Fe(CN)₆, and 1 mg/mL 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal). Sections were counterstained with neutral red, dehydrated, preserved in Vectamount (Vector Laboratories, Burlingame, CA), and photographed using a Nikon Eclipse E800 microscope with SPOT camera.

mRNA Analysis

Hindbrain and eye tissues from wild-type CD-1 embryos and adult mice were harvested and homogenized in TRIzol reagent (Invitrogen, Carlsbad, CA) for RNA isolation. The hindbrain included all neural tissue from the spinal cord to the inferior colliculus except the cerebellum. For RT-PCR, total RNA (2 μg) was treated with RNase-free DNase I (Roche, Indianapolis, IN) and reverse-transcribed using the SuperScript II First Strand Synthesis kit (Invitrogen, Carlsbad, CA). Aliquots of cDNA were used as templates for parallel quantitative PCR reactions containing *Math5* or *Hprt* primers. *Math5* primers are: 5'-CCTCCCTATCTCCACTTCTCTGTG-3' (M5 3'UTR F) and 5'-TTTCTCCACCTCCTGAATGACG-3' (M5 3'UTR R). *Hprt* primers are: 5'-CAAACCTTGCTTTCCCTGGT-3' (Hprt F) and 5'-CAAGGGCATATCCAACAACA-3' (Hprt R). Quantitative PCRs were performed using an iCycler instrument (Bio-Rad, Hercules, CA) with 60 sec annealing steps at 55°C. The product was monitored using SYBR green fluorescence at the end of each 72°C elongation cycle. Melting curves showed a single peak at the temperature corresponding to the PCR product. Six replicate reactions were performed for each sample. Relative expression levels were determined from cycle thresholds (C_T) and normalized using the 2^{-ΔΔC_T} method (Livak and Schmittgen, 2001). *In situ* hybridization was performed on cryosectioned tissue using previously described *Math5* cDNA probes and conditions (Brown et al., 1998).

Morphometric Analysis in the Cochlear Nucleus

Math5 +/- and -/- mice were perfused transcardially with 4% PFA, 0.1 M Na PO₄ (pH 7.4). After dissection, the brains were post-fixed in 4% PFA for 3 hours at 4°C, washed in PBS, equilibrated in 30% sucrose, and embedded in OCT media. Coronal sections were prepared from 6 animals of each genotype through the cochlear nucleus (10 μm, 2 sections per slide) using a Leica-Jung CM1800 cryostat. To map anatomical borders within the CN, every 15th slide was evaluated by Nissl staining. We analyzed *Math5-lacZ* cells within three regions: [1] the anteroventral cochlear nucleus (AVCN); [2] a transition zone (TZ) that overlaps the posteroventral CN (PVCN) and dorsal CN (DCN); and [3] the DCN. Within each region, we immunostained the central and third flanking slides. The slides were blocked with 4% nonfat dry milk in Tris-buffered saline with 0.1% Tween-20 (TST) as described (Brown et al., 2001), and incubated overnight at 4 °C with rat polyclonal β-galactosidase (β-gal) antisera (1:500). Slides were then washed in 1% milk TST and incubated with biotinylated donkey anti-rat IgG (Jackson ImmunoResearch, West Grove, PA) for 2 hours. After additional washes in 1% milk TST, β-gal antigen was visualized by applying avidin-horseradish peroxidase for 1

hour (ABC Elite, Vector), and staining in 0.5 mg/ml diaminobenzidine (DAB), 0.06% H₂O₂ for 40 min at room temperature. Sections were photographed using a Zeiss Axioscope 20 microscope and SPOT camera. The outlines of immunopositive cells were traced digitally, and their lengths, widths and areas were determined using MetaMorph software (Molecular Devices, Downingtown, PA). Sections were analyzed in random order with respect to genotype, with minimal variation between replicate measures of the same cell. Mutant and heterozygous cell dimensions were compared using two-sided *t*-tests of statistical significance (for *n* = 200–400 cells from six mice of each genotype). For the CN cell distribution analysis, two additional flanking slides were counted from each region. The density of cells (per million μm^3) was determined by counting all *lacZ*⁺ soma within a circumscribed region, and dividing this value by the area and section thickness (10 μm).

5-Bromo-2-deoxyuridine (BrdU) labeling

Cycling cells in the cochlear nucleus were identified by short-term labeling with the thymidine analog BrdU. Pregnant dams were given a single intraperitoneal injection of BrdU (0.1 mg/g body mass) on day E12.5, E14.5, E16.5, or E18.5, one hour prior to harvesting embryos.

Fluoro-gold (FG) Tract-tracing

Math5-lacZ heterozygous mice were anesthetized with ketamine (90 mg/kg) and xylazine (5 mg/kg), and placed in a stereotaxic holding device (model 900 with 921-E adaptor, David Kopf Instruments, Tujunga, CA). The retrograde tracer Fluoro-gold (FG, Fluorochrome LLC, Denver, CO) (Schmued and Fallon, 1986) was injected into one inferior colliculus (IC) using stereotaxic coordinates (1 mm lateral to the midline, 1.5 mm caudal to lambda, and ≤ 0.2 mm ventral to the dural surface). A small volume of FG (1 μl 2 % solution in PBS pH 7.4) was delivered using a 20 μl Hamilton syringe equipped with a glass micropipette (20–30 μm tip) as described (Frisina et al., 1998; Zhou and Shore, 2006). Six days after the FG injection, animals were euthanized with pentobarbital (150 mg/kg) and perfused transcardially with 100 ml 10 U/ml heparin, 0.5% NaNO₂ in 0.05 M PBS (pH 7.4), followed by 200 ml 4% PFA in 0.1 M PBS. Following perfusion, the brains were dissected, immersed in 4% PFA, 0.1 M NaPO₄ for 2 hr at 4°C, equilibrated stepwise in 10% to 30% sucrose 0.1 M NaPO₄ at 4°C, and embedded in OCT media (Tissue-Tek, Torrance, CA). Cryosections (20 μm) were prepared from brainstems in the coronal plane, mounted on glass slides, and air-dried. The highest concentration of FG labeling was observed in neurons at the IC injection site, with significant labeling of cells in the ipsi- and contralateral CN and the ipsilateral LL and superior olivary complex (SOC). To identify *Math5-lacZ* neurons, sections were immunostained with polyclonal rat anti- βgal sera (1:500), biotinylated donkey anti-rat IgG, and dichloro-triazinyl-amino-fluorescein (DTAF)-coupled streptavidin. Slides were mounted in Fluoromount (Calbiochem, San Diego, CA) and photographed using Olympus BX51 epifluorescence and FV-500 UV laser confocal microscopes. The wavelengths for confocal imaging of FG and DTAF were 364 nm and 488 nm (excitation), with ≥ 475 nm LP and 505–530 nm BP (emission), respectively.

Histochemical Analysis of *Math5-Cre*; Z/AP Mice

Math5-Cre lines 360 and 872 have been described in detail (Brzezinski and Glaser, 2007). These mice carry 205 kb C57BL/6J genomic BAC (bacterial artificial chromosome) transgenes that encompass the *Math5* transcription unit. The single-exon *Math5* coding region in the BAC was precisely replaced by a *Cre* recombinase cassette, and is flanked by >100 kb genomic DNA, including *cis* regulatory elements that drive endogenous *Math5* expression in the retina and hindbrain. The *Math5-Cre* transgene accurately recapitulates the *Math5-lacZ* expression pattern during embryogenesis. To demonstrate *Math5*⁺ neurons in the CN, *Math5-Cre* mice were crossed to mice carrying the Z/AP reporter transgene (Lobe et al., 1999), obtained from

The Jackson Laboratory. In double transgenic offspring, *Cre* irreversibly activates Z/AP by precisely excising a *loxP*-flanked stop segment, leading to expression of heat-stable human placental alkaline phosphatase (hPLAP) in *Math5*⁺ cells and their descendants. As a glycosylphosphatidylinositol (GPI)-tethered enzyme, hPLAP associates with the plasma membrane, revealing the detailed cellular morphology of labeled neurons (Badea et al., 2003). Brains from double transgenic adult mice were fixed by overnight immersion in 4% PFA at 4°C, equilibrated in 30% sucrose and frozen in OCT. Slides containing air-dried cryosections (10 µm) were incubated in Coplin jars in 70°C PBS for 45 min to inactivate endogenous AP and stained for hPLAP at room temperature using 5-Bromo-4-chloro-3-indolyl phosphate (BCIP) and nitroblue tetrazolium (NBT) as described (Lobe et al., 1999). Staining was terminated by washing in PBS with 20 mM EDTA and post-fixation in 4% PFA. Slides were counterstained with 0.05% neutral red, dehydrated, and coverslipped in Permount. Vibratome sections (250 µm) were prepared from adult brains that had been immersion-fixed in 4% PFA, 2 mM MgCl₂ overnight at 4°C, heat-treated for 45 min in PBS, 2 mM MgCl₂ at 70°C, and embedded in 3% agarose (SeaPlaque, FMC BioProducts, Hercules, CA). These were stained for hPLAP activity as floating sections in 24-well plates, washed extensively in PBS, dehydrated through graded ethanol, cleared with BABB (1:2 benzyl alcohol : benzyl benzoate), and transferred to glass slides, which were coverslipped with Permount and viewed using DIC (Nomarski) optics.

Immunohistochemistry

Cryosections (10 µm) of PFA-fixed tissues were blocked with 10% normal donkey serum (NDS) in 0.1 M NaPO₄ (pH 7.4), 0.25% Triton X-100, 1% bovine serum albumin (BSA), and incubated at 4°C overnight with primary antibodies diluted with 3% NDS in PBS. Primary mouse antibodies were: anti-calbindin 28-kDa (CB-955 monoclonal, 1:500, Sigma) and anti-parvalbumin (PARV-19 monoclonal, 1:500, Sigma). Primary rabbit antibodies were: anti-Kcnq4 (gift of Thomas Jentsch, University of Hamburg, Germany, 1:400), anti-Kv1.1 (Alomone Labs, Jerusalem, Israel, 1:300), anti-Kv3.1b (Alomone Labs, 1:750), anti-Math1 (gift of Jane Johnson, University of Texas Southwestern, Dallas, TX, 1:250) and anti-β-galactosidase (Cappell, 1:5000). Primary rat antibodies were: anti-BrdU (BU1/75 monoclonal, 1:100, Harlan Seralab, Indianapolis, IN) and anti-β-galactosidase (1:500). The polyclonal rat β-gal antiserum was generated by immunizing Wistar rats with *E. coli* β-galactosidase (G5635, Sigma grade VIII). Secondary antibodies were FITC-conjugated donkey anti-rabbit or anti-mouse IgG, Texas Red-conjugated donkey anti-rat IgG (Jackson ImmunoResearch), or Alexafluor 488-conjugated chicken anti-rat IgG (Molecular Probes, Eugene, OR). MafB was detected after antigen retrieval for 5 min in 0.1 M Tris pH 9.5 at 95°C, with polyclonal goat anti-MafB peptide antisera (sc-10022, 1:2000, Santa Cruz Biotechnology, Santa Cruz, CA), biotinylated donkey anti-goat IgG (Jackson), and AlexaFluor 594-conjugated streptavidin (Molecular Probes). Slides were photographed using a Nikon Eclipse E800 or Olympus BX51 fluorescence microscope with SPOT camera.

Cochlear Histology

Vestibular and cochlear structures were dissected from adult temporal bones, fixed in 2% PFA with 3% EGTA and 2 mM MgCl₂ for 2.5 hours at 4 °C, and decalcified in 10% EGTA with 2 mM MgCl₂ at 4°C until pliable. Tissues were then equilibrated in 30% sucrose, 0.1M NaPO₄ (pH 7.4), embedded in OCT, cryosectioned, and processed for immunostaining or X-gal histochemistry as described above.

Auditory Brainstem Response (ABR) Analysis

ABRs of BALB/cJ congenic N₁₂F₂ *Math5* ^{+/+}, ^{+/-} and ^{-/-} mice were compared at 8 weeks of age. Animals were anesthetized with an intraperitoneal injection of ketamine (65 mg/kg),

xylazine (3.5 mg/kg) and acepromazine (2 mg/kg). Body temperature was maintained with heating pads and heat lamps. ABRs were recorded in an electrically and acoustically shielded chamber (Acoustic Systems, Glendale Heights, IL). Subdermal needle electrodes were placed at the skull vertex (active) and pinnae of the test (reference) and contralateral (ground) ears. Tucker-Davis Technologies (Alachua, FL) System II hardware and SigGen/Biosig software were used to present stimuli and record responses. Tones were generated by a Beyer driver (Beyerdynamic, Mt. Prospect, IL) with an aluminum-shielded enclosure made in-house, and were delivered by a speculum placed just inside the tragus. Stimuli were presented as 15 msec tone bursts, with 1 msec rise/fall times, and were repeated 10 times per second. Up to 1024 responses were averaged for each stimulus intensity and frequency. Responses were collected at 4, 12 and 20 kHz for a range of stimulus levels, separated by 10 dB steps at higher intensities and 5 dB steps near threshold. Thresholds were interpolated between the lowest stimulus intensity producing a response, and the highest stimulus where no response was observed (5 dB increment). Interpeak latencies were defined as the time intervals between positive peaks.

Results

***Math5-lacZ* expression in the adult auditory hindbrain and cerebellum**

To investigate *Math5* expression in the central nervous system, we tested adult brains from *Math5-lacZ* mice using a sensitive β -galactosidase histochemical stain. In previous studies, we showed that the *Math5-lacZ* allele is expressed transiently by post-mitotic cells in the embryonic retina (Brown et al., 2001; Brzezinski and Glaser, 2007) and identified *Math5* mRNA in the developing brainstem (Brown et al., 1998). Wholemount brains from *Math5* +/– and *Math5* –/– adult mice showed *lacZ* expression in the cerebellum (Fig. 1A-B) and trapezoid body (TB), a major transverse fiber tract in the auditory pathway also known as the ventral acoustic stria (Fig. 1C-D). Coronal sections through the brainstem (Fig. 1F-G) showed *Math5-lacZ* expression in additional structures of the auditory hindbrain, including the cochlear nucleus (CN) (Fig. 1J), lateral superior olive (LSO) (Fig. 1I), and medial nucleus of the trapezoid body (MNTB). In more rostral sections, we saw *Math5-lacZ* expression in the ventral nucleus of the lateral lemniscus (LL) (Fig. 1H). These structures are functional components of the central auditory pathway (Willott, 1983). However, the CN is the only location in the hindbrain where *Math5-lacZ* was observed in cell somata (Fig. 1J). This complex expression pattern can be explained by diffusion of *lacZ* polypeptide through the cytoplasm and axons of CN neurons, which have extensive projections to other hindbrain structures. The *lacZ*⁺ cells are interspersed across the CN in this plane of section, with greatest concentration in the central and dorsal regions.

The morphology of *Math5* –/– and +/– hindbrains is grossly similar. *Math5-lacZ* is not expressed in the adult cortex, and no *lacZ* activity was detected in medial superior olive (MSO) or higher-level auditory processing structures such as the inferior colliculus or auditory cortex (data not shown). Within the cerebellum, *Math5-lacZ* is expressed by a dispersed subpopulation of granule cells (data not shown).

***Math5-lacZ* expression in the developing auditory hindbrain**

To determine the origin of *Math5* expressing cells, we followed *lacZ* activity in the hindbrain from E12.5 to E18.5 (Fig. 2). Wholemount E12.5 *Math5* +/– embryos showed robust *lacZ* staining in the retina (Fig. 2A, arrowhead) and faint expression within or close to the caudal rhombic lip (cRL), a major germinal zone for hindbrain neurons (Wingate, 2001) (Fig. 2A, bottom arrow). *Math5-lacZ* was also weakly expressed near the rostral rhombic lip (rRL), which gives rise to cerebellar granule cells and a subset of neurons comprising the cerebellar nuclei (Fig. 2A, upper arrow). Neurons of the ventral cochlear nucleus (VCN) are generated between E11 and E15 (Martin and Ricketts, 1981; Taber Pierce, 1967). The expression of

Math5-lacZ near the cRL corresponds spatially and temporally with the migration of cells from this region into the developing CN via the cochlear extramural stream (CES) (Wang et al., 2005).

To further evaluate *Math5-lacZ* cells in the developing CN, we performed pulse-labeling experiments on embryos from E14.5 to E18.5 (Fig. 2B-D). The *lacZ*⁺ cells did not incorporate BrdU and are thus post-mitotic, in contrast to adjacent cells within the CES. The *Math5-lacZ* CN cells were also immunopositive for *Math1* (Fig. 2E-G), which is expressed by newly formed VCN neurons during migration and development (Wang et al., 2005). Finally, the *Math5-lacZ* CN cells were immunoreactive for MafB (Fig. 2H-I, supplementary Fig. 1), a bZIP transcription factor that initially patterns rhombomeres 5 and 6, but is subsequently expressed in specific hindbrain nuclei, including the preBötzing complex, trigeminal spinal nucleus, vestibular nuclei, SOC and the ventral (but not dorsal) CN (Blanchi et al., 2003; Cordes and Barsh, 1994; Eichmann et al., 1997; Farago et al., 2006; Howell et al., 2007; Moens et al., 1998). Most or all VCN neurons express MafB (Howell et al., 2007). A subset of these MafB⁺ cells were *lacZ*-positive at E16.5 and P8 (Fig. 2I, supplementary Fig. 1).

As CES neurons delaminate, they extend axons that will later interconnect adult auditory structures (Rubel et al., 1998). To demonstrate these events in *Math5*⁺ cells, we followed the progression of *lacZ* activity from E14.5 to E18.5 in a series of horizontal (Fig. 2K-M) and coronal (Fig. 2N-V) sections from *Math5*^{+/-} embryos. At E14.5, *Math5-lacZ* expression was mainly localized in the presumptive VCN (Fig. 2K,N). The first axons were observed emerging from the CN, and extending via the TB (Fig. 2O) toward the ipsilateral LSO (Fig. 2P). By E16.5, the extent of *Math5-lacZ* expression had expanded in the VCN (Fig. 2L,Q), TB and LSO (Fig. 2R,S). At E18.5, *Math5-lacZ* expression was particularly intense in the VCN (Fig. 2M,T), and axons from these cells (Fig. 2U) had formed extensive collateral branches in the LSO (Fig. 2V). *Math5-lacZ* expression was also detected in TB fibers crossing the midline at E18.5 (data not shown), similar to the pattern observed in the adult hindbrain (compare Figs. 2U-W and 1F-G). This sequence is consistent with the successive birth of neurons comprising the CN, MNTB and LSO (Altman and Bayer, 1980) and the stepwise formation of afferent connections from the CN (Kandler and Friauf, 1993).

Hindbrain *Math5-lacZ* expression, assayed by β -galactosidase histochemical and antibody staining, appeared more robust in embryos than adult mice (compare Figs. 1J and 2Q). This may reflect decreased expression in individual neurons, or interspersed non-expressing cells (e.g. glia) in the CN and surrounding structures as development proceeds and the hindbrain expands. To verify expression independently of the *lacZ* reporter, and estimate relative *Math5* mRNA levels in the developing hindbrain, we used quantitative reverse transcriptase PCR (qRT-PCR) and *in situ* hybridization. These data show highest expression at E16.5, with a 12-fold lower level of *Math5* mRNA in the adult hindbrain (Fig. 3A,B). At the time of peak expression, *Math5* transcripts are 28-fold less abundant in the hindbrain than the eye (Fig. 3C). *Math5* mRNA was clearly localized to the CN by *in situ* hybridization in the E16.5 hindbrain (Fig. 3D). Persistent expression of *Math5* mRNA in the adult cerebellum, but not in the adult eye, was also demonstrated by Northern blot analysis (supplementary Fig. 2).

***Math5-lacZ* expression in the auditory nerve and peripheral vestibulocochlear structures**

Since *Math5-lacZ* is expressed in multiple central auditory structures, and *Math1* is required to form inner ear hair cells (Bermingham et al., 1999), we evaluated *Math5-lacZ* expression in the peripheral auditory system (Fig. 4A-D). In mid-modiolar sections through the cochlea, we observed a small group of *Math5-lacZ* neurons within the acoustic nerve (Fig. 4B, arrowheads). There was no obvious difference in staining between *Math5*^{+/-} and *-/-* sections (Fig. 4C-D). The *Math5-lacZ* positive cells in the modiolar nerve root co-expressed calbindin and parvalbumin (Fig. 4E-F). These cells may represent a satellite population of vestibular

ganglion neurons in close proximity to the acoustic nerve (Dechesne and Thomasset, 1988; Dememes et al., 1993), but are most likely cochlear root neurons (CRNs), a group of extranuclear globular cells that are embedded in the vestibulocochlear nerve in rodents, before it bifurcates in the CN. These neurons receive input from primary afferents through numerous axosomatic synapses, respond to short latency stimuli, and mediate the acoustic startle reflex (Lee et al., 1996; Lopez et al., 1999; Merchan et al., 1988). CRNs express calbindin, and extend axons that pass through the CN and cross the midline via the TB (Lopez et al., 1999). To evaluate *Math5-lacZ* expression in CRNs directly, we immunostained coronal sections of the CN and attached nerve root with anti- β gal sera (Fig. 4G,H). The distribution and density of β gal+ neurons in the cochlear nerve strongly resembles the original descriptions of CRNs in rats and mice (Harrison et al., 1962; Merchan et al., 1988; Webster and Trune, 1982).

We also detected weak expression of *Math5-lacZ* in sensory epithelia of the saccule and utricle (supplementary Fig. 3A-C), in a punctate pattern consistent with processes of afferent vestibular ganglion neurons, which transmit signals from otolithic sensory hair cells to the hindbrain. There was no *Math5-lacZ* expression in the cristae ampullaris or vestibular nuclei. *Math5-lacZ* was not detected in inner or outer auditory hair cells in the adult cochlea or in spiral ganglion neurons. However, we did observe transient activity in the cochlear duct epithelium between E14.5 and E17.5, with peak expression at E15.5 (supplementary Fig 3D-I).

Distribution and size of *Math5-lacZ* positive cells in the VCN

Although the hindbrain morphology of *Math5* +/- and -/- mice is similar (Fig. 1), we noted differences in the appearance of *lacZ*+ cells in the mutant VCN (Fig. 5A,B). In these cells, the X-gal staining was diffuse, with a lacy, reticular pattern compared to wild-type. This morphological difference may reflect an alteration in the intracellular structure or environment that favors a punctate versus uniform distribution of the β -galactosidase product (Friedrich et al., 1993). To evaluate these cells quantitatively, we used *lacZ* immunohistochemistry, which stains the somata uniformly (Fig. 5F,G), to measure the distribution and size of *lacZ*-positive cells in coronal sections across the CN (Fig. 5C-E). No *lacZ*+ cells were detected in the dorsal CN (DCN). The density of *lacZ*+ cells was determined at five points across the anteroventral CN (AVCN) and posteroventral (PVCN) (Fig. 5J). These cells were distributed similarly in *Math5* +/- and -/- mice, with highest density in the rostral and central AVCN (27–42 cells/million μm^3) and lowest in the caudal PVCN (3–6 cells/million μm^3). This pattern conforms well with the established distribution of bushy cells in the mouse VCN (Martin, 1981; Webster and Trune, 1982). Spherical bushy cells (SBCs) are the major cell type in the rostral AVCN, but are also present in the caudal AVCN. Globular bushy cells (GBCs) are concentrated in the central AVCN, near the cochlear nerve root, and extend into the PVCN. Multipolar and octopus cells are located primarily in the PVCN, where *lacZ* staining was minimal.

Cell dimensions were determined from the outlines of *lacZ*+ somata by metamorphic analysis (Fig. 5H,I,J,K). Immunopositive cells in the AVCN of *Math5* +/- mice have a mean cross-sectional area ($\pm$ SEM) of $109.1 \pm 3.4 \mu\text{m}^2$ compared to $97.3 \pm 2.4 \mu\text{m}^2$ in the *Math5* -/- mice. This 11% decrease in area ($t = 2.80$; $df = 9$; $P = 0.020$) corresponds to a 15–20% decrease in cell volume. In the PVCN, mean cell area was also reduced, but the difference between *Math5* +/- ($109.4 \pm 5.6 \mu\text{m}^2$) and *Math5* -/- ($105.1 \pm 4.5 \mu\text{m}^2$) mice was not statistically significant ($t = 0.60$; $df = 10$; $P = 0.563$). Likewise, the length ($14.3 \pm 0.2 \mu\text{m}$) and width ($9.7 \pm 0.2 \mu\text{m}$) of *lacZ*+ cells in *Math5* -/- AVCN were significantly smaller than the length ($15.2 \pm 0.2 \mu\text{m}$) and width ($10.2 \pm 0.2 \mu\text{m}$) of cells in the *Math5* +/- AVCN ($t_{\text{length}} = 3.99$; $df = 10$; $P = 0.003$; $t_{\text{width}} = 2.27$; $df = 10$; $P = 0.047$). These values did not differ significantly in the PVCN ($t_{\text{length}} = 0.40$; $df = 10$; $P = 0.695$; $t_{\text{width}} = 0.72$; $df = 10$; $P = 0.492$). The dimensions of the *Math5-lacZ* cells are typical of medium-sized VCN neurons, including spherical and

globular bushy cells and multipolar cells, which have diameters between 10–18 μm in mice (Martin, 1981). They are larger than granule neurons (4–6 μm), and smaller than octopus cells (15–25 μm).

***Math5-lacZ* cells co-express bushy cell neuronal markers**

To better characterize *Math5-lacZ* positive cells, we performed double immunolabeling experiments with rat anti- β -galactosidase and antibodies to markers associated with CN neurons. Bushy cells in the VCN are known to express a number of voltage-gated potassium channels with rapid kinetic properties, including Kv1.1 (*Kcna1*) and Kv3.1b (*Kcnc1*) (Pal et al., 2005; Perney and Kaczmarek, 1997; Song et al., 2005). Kcqn4 is expressed in the CN, in other auditory structures, and in CRNs; however, the cell-type specificity of this potassium channel within the CN is not known (Kharkovets et al., 2000). *Math5-lacZ* positive cells were found to co-express all three channels (arrowheads in Fig 6A-F). Additional cells were immunoreactive for Kv1.1, Kv3.1b and/or Kcqn4 but did not express *Math5-lacZ* (arrows in Fig 6 A-F). The *Math5-lacZ* positive cells could be subdivided into calbindin-positive (arrowheads in Fig. 6G-H) and negative (closed arrows) populations, which were interspersed. Other cells expressed calbindin without *Math5-lacZ* (open arrows). This heterogeneity may reflect functional differences among *Math5*⁺ bushy cells. There was no obvious difference between *Math5* mutant and heterozygous mice in the staining pattern with these markers (Fig. 6B,D,F,H).

***Math5-lacZ* neurons are not multipolar or octopus cells: retrograde tracing and cytomorphology**

To further evaluate *Math5-lacZ* cells as CN bushy neurons, we performed three additional experiments. First, to test whether any *Math5-lacZ* neurons are multipolar cells, we injected the retrograde tracer fluoro-gold (FG) into one inferior colliculus (IC) of adult *Math5*^{+/−} mice, and compared the pattern of FG and β gal staining in the hindbrain after six days (Fig. 7A-F). This tract-tracing experiment was designed to specifically mark individual type I multipolar (T-stellate) cells within the CN, since these are the most abundant class of multipolar neurons, and most or all of these cells project to the ipsi- or contralateral IC, whereas bushy cells do not (Josephson and Morest, 1998; Oertel, 1985; Schofield and Cant, 1996; Smith and Rhode, 1989). We observed numerous FG⁺ cells in both CN, intermingled with *Math5-lacZ* cells, but no β gal⁺ neurons were co-labeled with FG (< 1.8%), consistent with the absence of X-gal staining in the IC (Fig. 1). We conclude that few if any type I multipolar neurons express *Math5*.

Second, to test whether any *Math5-lacZ* neurons are octopus cells, we co-stained the PVCN with Kv1.1 and β gal antibodies (Fig. 7G-H). Octopus cells express Kv1.1 strongly (Grigg et al., 2000; Jung do et al., 2005) and are concentrated in the central PVCN. They have distinct morphology with large soma and thick, tapered dendrites oriented in the same direction (Brawer et al., 1974; Oertel et al., 1990; Osen, 1969; Rhode et al., 1983). We observed numerous Kv1.1⁺ neurons with typical octopus cell morphology (Fig. 7H), but none of these cells was β gal⁺.

Third, to demonstrate the detailed morphology of *Math5*⁺ cells, we used *Math5-Cre* BAC (Brzezinski and Glaser, 2007) and Z/AP reporter (Lobe et al., 1999) transgenes to label these cells in the adult CN with human placental alkaline phosphatase (hPLAP). This membrane-associated enzyme can be used histochemically to reveal the fine structure of neuronal processes (Badea et al., 2003). Although somewhat less efficient than the *Math5-lacZ* allele, we observed abundant hPLAP⁺ neurons in the anterior VCN of adult double transgenic mice (Fig. 8A), consistent with the anterior/posterior distribution of *lacZ*⁺ neurons (Fig. 5J). Most of these cells have unipolar morphology, with 1–2 primary dendrites that arborize extensively (Fig. 8B-D). These features are typical of bushy neurons, but not multipolar cells, which have

multiple primary dendrites and stellate morphology (Brawer et al., 1974; Disterhoft et al., 1980; Martin, 1981; Oertel et al., 1990; Osen, 1969). We also observed hPLAP staining in decussating fibers of the trapezoid body and in large axon terminals within the MNTB (Fig. 8E-F), which we interpret to be calyces of Held, specialized nerve endings of globular bushy cells (Smith et al., 1991; Spirou et al., 1990). Apart from histology, this experiment also demonstrates *Math5* expression in CN bushy cells independently of the *lacZ* allele.

The anatomical and molecular data presented thus far suggest that the *Math5-lacZ* cells are spherical and globular bushy neurons. Together, these cells represent 92% of neurons in the mouse VCN (Trune, 1982). Since the mouse AVCN contains 80 neurons per million μm^3 (Willott et al., 1994) and the density of *lacZ*+ cells in the central AVCN is 27–42 cells/million μm^3 , we estimate that *Math5-lacZ* cells comprise 40–60% of all bushy neurons, or approximately 5,000–10,000 cells in the VCN.

***Math5* $-/-$ mice exhibit ABR latency shifts**

The *Math5* $-/-$ mice exhibit a Preyer reflex, consistent with grossly normal hearing. To evaluate the auditory phenotype in more detail, we recorded auditory brainstem responses (ABRs) of *Math5* $+/+$, $+/-$ and $-/-$ mice on a congenic BALB/cJ background at 8 weeks of age. This time point was chosen to avoid age-related hearing loss, which occurs around 10 months in BALB/cJ mice (Willott et al., 1998). ABR thresholds at 4, 12, and 20 kHz did not differ between genotypes (Fig. 9A); neither did ABR peak amplitudes. However, there were consistent temporal differences in the ABR profiles, which we analyzed as interpeak latencies. The characteristic peaks of the ABR waveform reflect field potentials generated by different nuclei along the auditory processing pathway (Melcher and Kiang, 1996). Peaks I and II of the mouse ABR have been attributed to synchronous firing of the auditory nerve and neurons of the CN, respectively (Henry, 1979; Martin, 1981). Peaks III–V are complex cross-field potentials generated by the CN, MNTB, SOC, LL and IC. They represent multiple pathways with different conduction speeds, polarity and spatial orientations (Galbraith et al., 2006). The latency period between peaks can be viewed roughly as the time required for sound information to travel from one processing center to the next.

Fig. 9B shows an overlay of representative waveforms from a *Math5* $-/-$ mouse (red) and a *Math5* $+/+$ mouse (black) at 12 kHz. Similar results were obtained at 20 kHz and for other stimulus levels (supplementary Fig. 4). The interval between Peaks II and III was significantly shorter in the *Math5* $-/-$ ABR, by 0.10 to 0.41 msec, compared to the wild-type ABR, over a range of stimulus intensities from 40 to 80 dB SPL (sound pressure level), at frequencies of 12 and 20 kHz (Fig. 9C,D). Conversely, the latency between Peaks III and IV was significantly increased by 0.10 to 0.35 msec for *Math5* $-/-$ mice compared to wild-type mice over the same intensity and frequency range (Fig. 9C,D).

In *Math5* $-/-$ mice, peak III occurs earlier than in wild-type mice. Although relatively small, this shift is greater than the interaural time difference (ITD) for mice (0.1 msec) and the synaptic transmission delay for GBC terminals in the MNTB (Taschenberger and von Gersdorff, 2000; von Gersdorff and Borst, 2002). The latency differences between *Math5* mutant and wild-type mice are consistent with a disruption of brainstem auditory signaling and binaural processing.

Discussion

We have demonstrated *Math5* expression in the auditory hindbrain and identified *Math5*+ cells as a subset of VCN globular and small spherical bushy cells. These neurons are significantly smaller in *Math5* $-/-$ mice, and the ABR interpeak latencies are altered. Based on our findings,

we propose that *Math5* may be necessary for normal central auditory processing of binaural information.

***Math5* is expressed in globular and small spherical bushy cells of the VCN**

The *Math5*⁺ cells are most likely globular and small spherical bushy cells for five reasons. First, *Math5-lacZ* cells, and those marked with *Math5-Cre*, are located throughout the AVCN and rostral PVCN, coextensive with the known distribution of GBCs and small SBCs in rodents (Martin, 1981; Webster and Trune, 1982). Neither cell type alone can account for this pattern. Moreover, the cell diameter, shape and density data support this conclusion.

Second, the axonal projection pattern of *Math5-lacZ* cells in the hindbrain is completely overlapping with that of globular and small spherical bushy cells. Although *Math5* encodes a nuclear protein, the cytoplasmic reporter reveals somata and axonal projections extending via the trapezoid body to the LSO, MNTB, and VNLL. Small spherical bushy cells project to the ipsilateral LSO, whereas globular bushy cells extend axons to the contralateral MNTB and VNLL, with major collateral branches to the ipsilateral LSO (Cant and Benson, 2003; Friauf and Ostwald, 1988; Kuwabara et al., 1991). Principal cells in the MNTB receive GBC input through large synaptic endings (calyces of Held) and generate an inhibitory signal that is relayed to the LSO (von Gersdorff and Borst, 2002). Large spherical bushy cells can be effectively excluded, as a group, since these cells project to the medial superior olive (MSO), where no *lacZ* staining was observed (Cant and Casseday, 1986; Smith et al., 1993). However, the MSO is relatively small in mice compared to other mammals that are sensitive to lower frequencies and that rely primarily on interaural time differences for sound localization (Irving and Harrison, 1967; Tollin, 2003). Likewise, type I multipolar cells can be excluded because nearly all of these neurons project to the IC (Josephson and Morest, 1998) where no *Math5-lacZ* expression was observed. Indeed, our retrograde tract-tracing experiment directly shows that *lacZ*⁺ neurons in the CN do not project to the IC. Finally, type II multipolar cells and octopus cells can be excluded since their projections exit the VCN via the intermediate acoustic striae (Cant and Benson, 2003) and not the TB, and octopus cells were further excluded by Kv1.1 double-labeling analysis. While we cannot completely rule out the possibility that *Math5* is expressed in rare neurons of the excluded cell types, these are unlikely to contribute significantly to the *Math5-lacZ* population.

Third, *Math5-lacZ* is expressed by post-mitotic cells emerging from the cRL beginning at E12. This corresponds to the onset of birthdates for spherical and globular bushy cells and multipolar cells (Martin and Rickets, 1981; Taber Pierce, 1967). It follows the birthdates of large octopus cells and precedes the birthdates of small neurons and granule cells within the VCN.

Fourth, *Math5-lacZ* cells in the VCN express a combination of molecular markers that are characteristic of bushy cells. Every *lacZ*⁺ cell in the VCN expresses the potassium channels Kv1.1, Kv3.1b and Kcnq4, which have rapid activation kinetics and are associated with fast-spiking neurons (Kopp-Scheinpflug et al., 2003; Song et al., 2005). Octopus cells also express Kv1.1 and Kv3.1b (Bal and Oertel, 2001; Ferragamo and Oertel, 2002; Perney and Kaczmarek, 1997) but as stated previously, their large size, unique morphology, projection patterns, and restricted localization in the dorsomedial PVCN distinguish them from *Math5-lacZ* cells.

Fifth, the specific ABR latency shifts in *Math5* mutant mice are consistent with changes in small spherical and globular bushy neurons. ABR testing of *Math5*^{-/-} mice revealed a decrease in the II-III interpeak latency and an increase in the III-IV interpeak latency. Although ABRs are complex field potentials reflecting the activity of multiple generators, kainic acid lesioning studies in the cat CN have shown peaks II and III are generated by the spherical and globular bushy cells and their targets (Fullerton and Kiang, 1990; Melcher et al., 1996; Melcher and Kiang, 1996). Likewise, kainic acid lesioning of guinea pig MNTB neurons (which receive

input mainly from globular bushy cells) reduced the amplitude of peak III by up to 60% (Gardi and Bledsoe, 1981). In principle, a reduction in the magnitude of inhibitory input from MNTB cells to LSO neurons via the ascending pathway, or from MNTB neurons to globular bushy cells via descending projections (Schofield, 1994) could advance the timing of ABR peak III. This effect could also be produced if peak III had two underlying components, and the amplitude of the slower component was diminished. This phenomenon has been reported in cats, where wave III is often double-peaked after a midline lesion through the trapezoid body (Fullerton and Kiang, 1990). In *Math5*^{-/-} mice, this type of effect could occur if globular bushy cells in the contralateral CN fail to adequately stimulate MNTB principal cells that inhibit LSO firing. The lengthening of the III-IV interval is most likely due, indirectly, to the shift of peak III.

***Math5* auditory hindbrain expression**

Transcription factors of the bHLH family have major roles as proneural genes that control cell fate specification during neural development and terminal differentiation (Bertrand et al., 2002). One defining characteristic of proneural genes is that loss of function results in the absence of cell lineage(s) specified by that gene (Hassan and Bellen, 2000). In *ato* flies, and *Math5* mutant retinas, for example, the R8 photoreceptors and RGC neurons fail to develop, respectively. In contrast, there is no VCN cell loss in *Math5* null mice, which suggests that *Math5* is not needed for the genesis or survival of these brainstem cell types. Moreover, although bushy cells are moderately smaller, *Math5-lacZ* expression is retained in the LSO, LL, MNTB and TB of adult mutant mice, suggesting that these CN cells have grossly normal projection patterns.

There are relatively few proneural bHLH transcription factors with major roles in the adult brain (Elliott et al., 2001; Lee et al., 2000). Although β -galactosidase is known to perdure in tissues after termination of gene expression, the residual activity typically lasts on the order of days (Hall et al., 1983; Wang et al., 1999), and is undetectable after three days in progenitors that give rise to CN neurons (Farago et al., 2006). *Math5* mutant mice over 18 months of age showed identical *lacZ* expression patterns in the hindbrain as younger mice (data not shown) but no *lacZ* activity in the retina. Moreover, the staining pattern was confirmed by *Math5* mRNA analysis (Fig. 3). The persistence of *Math5* expression in adults and cytological changes in mutant mice suggest an ongoing, cell-autonomous need for *Math5* in the maintenance or function of a subset of VCN cells. The decrease in VCN cell size in the *Math5* mutants is similar in magnitude to changes observed in young gerbils and guinea pigs following cochlear ablation, associated with loss of early trophic signals (Lesperance et al., 1995; Pasic and Rubel, 1989). In this respect, *Math5* is also reminiscent of *ato*, which regulates neurite arborization but does not act as a proneural gene in the *Drosophila* brain (Hassan et al., 2000).

All or most VCN neurons derive from the anteriormost territory of the caudal rhombic lip (rhombomeres 2–4) and subjacent progenitor zone (Farago et al., 2006). These cells delaminate and migrate tangentially in the CES to form CN neurons, with regional identities that correspond to their rostrocaudal origins in the cRL. *Math5* expression coincides temporally and spatially with *Math1* at the cRL and within the VCN, although the domain of *Math1* expression extends significantly beyond these structures (Machold and Fishell, 2005; Wang et al., 2005). *Math1* may partially complement *Math5* function during VCN histogenesis. However, endogenous *Math5* activity is not sufficient to allow VCN neuron development in *Math1*^{-/-} mice, which lack all CES derivatives (Wang et al., 2005). *Math5* and *Math1* proteins have limited sequence similarity outside the bHLH domain (Brown et al., 2002) and are likely to have different molecular properties. Indeed, *Math1* can almost completely replace *Drosophila ato* function *in vivo* (Ben-Arie et al., 2000), whereas *Math5* rescues photoreceptor development in *ato* mutants weakly (Sun et al., 2003). These observations suggest that

functional partitioning between *Math1* and *Math5* in the auditory hindbrain is incomplete. While *Math1* acts at multiple levels in the development of auditory and proprioceptive structures, ranging from mechanosensory receptors to brainstem nuclei, *Math5* acts at the same level in both auditory and visual pathways – in the formation of second-order neurons, CN bushy cells and RGCs, which receive input from bipolar-type primary neurons in the spiral ganglion and inner nuclear layer, respectively (Rouiller, 1997; Sharpe and Stockman, 1999). GBCs and RGCs have parallel roles in the contralateral representation of auditory and visual space in the cortex, via decussating fibers in the TB and optic chiasm, respectively (Jenkins and Masterton, 1982). These findings also provide direct evidence that SBCs projecting to the LSO and MSO differ at the molecular level.

Role of *Math5*-positive cells in auditory processing

Sound localization depends on the ability of auditory brainstem nuclei to accurately preserve and transmit timing information from acoustic stimuli. Phase-locked activation of SOC neurons by CN bushy cells is important for the detection of interaural intensity differences and sound localization (Cant, 1991; Grothe, 2003; Helfert, 1991; von Gersdorff and Borst, 2002). The unique molecular characteristics of bushy cells, including the expression of specific voltage-gated potassium channels, facilitate rapid responses to sound stimuli, contribute to the preservation of timing, and enhance neural synchronization (Joris et al., 1994). Kv1.1 and Kv3.1b are both outward rectifiers. Kv1.1 is a low-threshold channel with rapid activation kinetics; these properties minimize temporal summation of inputs and preserve timing information across synapses (Brew and Forsythe, 1995; Kopp-Scheinpflug et al., 2003). Kv3.1b is a high-threshold channel that repolarizes neurons rapidly, creating short-duration action potentials (Trussell, 1997, 1999; Wang et al., 1998). The *Math5*-expressing cells contain both of these channels, whose complementary properties ensure precise timing of responses. Kcnq4 likewise favors a short membrane time constant (Kharkovets et al., 2000). Co-expression of Kv1.1 and Kv3.1b has not been previously demonstrated in CN cells.

In rodents, where sensitivity to low-frequency sound is poor, globular and small spherical bushy cells, and their respective target neurons in the MNTB and LSO, detect interaural intensity (level) differences, which are the primary cues used to determine the spatial origin of high-frequency sound (Tollin, 2003; von Gersdorff and Borst, 2002). This circuit compares inhibitory and excitatory signals that converge upon the LSO, respectively, from globular bushy cells via MNTB principal neurons, and small spherical bushy cells (Pfeiffer, 1966; Smith et al., 1991). In this way, LSO neurons integrate information from both ears (Oertel, 1999). Globular bushy cells that synapse upon inhibitory MNTB and LNTB neurons can similarly adjust the sensitivity of MSO cells to detect interaural time differences (Grothe, 2003). Dysfunction of *Math5*⁺ neurons in the VCN would be expected to disrupt both mechanisms. These cells may also mediate the crossed middle ear muscle (MEM) reflex, which protects the inner ear from intense acoustic stimuli (Lee et al., 2006).

Preservation of high-frequency timing information is particularly important for decoding complex sounds, including speech recognition in humans (Oertel, 1999). Circumscribed lesions in the trapezoid body have been associated with loss of speech discrimination, loss of crossed acoustic reflexes, and masking level difference abnormalities, with preservation of pure-tone thresholds and ipsilateral reflexes (Cohen et al., 1996; Egan et al., 1996; Griffiths et al., 1998). These clinical findings are consistent with binaural interaction defects. Likewise, demyelinating brainstem lesions in multiple sclerosis patients have been associated with loss of interaural time discrimination (Furst et al., 1995; Levine et al., 1994; van der Poel et al., 1988).

Over one-hundred mouse models of sensorineural hearing loss have been described involving the peripheral auditory system (Avraham, 2003). In contrast, few mouse or human mutations

have been shown to cause retrocochlear hearing defects (Parkinson et al., 2001; Starr et al., 2004) and the neuropathologic basis for auditory processing disorders is poorly understood (Liu, 2006; Wright et al., 1997). *Math5* is a specific molecular marker of CN bushy cells, and the knockout mice represent a unique model for studying high-frequency binaural pathways.

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Supplementary Material

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Figure 1.

Math5-lacZ expression in the adult auditory hindbrain (X-gal histochemical stain). A-D. Wholemount brains from heterozygous (*M5* ^{+/+}) and mutant (*M5* ^{-/-}) mice. Dorsal (A, B) and ventral (C, D) views showing *lacZ* activity in the cerebellum and trapezoid body. The optic chiasm and tracts (arrows in C,D) are absent in *M5* ^{-/-} mice (Brown et al., 2001). E. Diagram showing the pattern of *Math5-lacZ* expression (blue) and plane of section in panels F, G, I and J. F-G. Coronal sections of *M5* ^{+/+} and *M5* ^{-/-} hindbrains, counterstained with neutral red, showing *Math5-lacZ* expression in the cochlear nuclei, lateral superior olives, trapezoid body and medial nuclei of the trapezoid body. *M5* ^{+/+} and *M5* ^{-/-} brains have grossly similar morphology. H. More rostral section showing *Math5-lacZ* expression in the lateral lemnisci.

I, J. Higher magnification views of the LSO and CN, respectively, from panel G. Abbreviations: CE, cerebellum; CN, cochlear nucleus; LL, lateral lemniscus; LSO, lateral superior olive; MNTB, medial nucleus of the trapezoid body; TB, trapezoid body. Scale bars 500 μm in F, H;. 100 μm in I, J.

Figure 2.

Math5-lacZ expression during auditory hindbrain development. A. M5 +/- E12.5 wholemount embryo stained with X-gal showing strong *lacZ* activity in the retina (arrowhead), and weaker expression rostral and caudal rhombic lips (arrows), which form cerebellar granule cells and hindbrain structures including the ventral cochlear nuclei, respectively. B-D. Coronal sections of M5 +/- embryos (E14.5-E18.5) after a 1-hr BrdU pulse, showing the presumptive cochlear nucleus (CN) and migrating cRL cells immunostained for β -galactosidase (red) and BrdU (green). The *lacZ*-positive cells are post-mitotic. E-G. Coronal section of an E15.5 M5 +/- hindbrain showing co-expression of *Math1* (red) and *Math5-lacZ* (green) in the CN. H,I. Coronal sections of an E15.5 M5 +/- hindbrain showing β gal (green) in cytoplasmic processes

of VCN neurons, which express the nuclear transcription factor MafB (red). J. Diagram showing the orientation of sections in panels B-V. K-M. Horizontal sections, stained with X-gal and neutral red, showing the progression of *Math5-lacZ* activity in M5 +/- hindbrains between E14.5 and E18.5. There is no obvious difference in the expression pattern between M5 +/- and M5 -/- embryos. N-V. Coronal hindbrain series showing *Math5-lacZ* expression in the developing left CN of M5 +/- embryos between E14.5 and E18.5. At E14.5, *lacZ* activity is mainly restricted to the CN (panel N) but has begun to extend ventromedially toward the trapezoid body (TB) and lateral superior olive (LSO) (panels O,P). After E16.5 *Math5-lacZ* expression continues in the CN (panels Q,T) and extends via the TB (panels R,U) to the LSO (panels S,V). Abbreviations: CN, cochlear nucleus; LSO, lateral superior olive; TB, trapezoid body. Scale bars 100 μ m.

Figure 3.

Math5 mRNA analysis. A. RT-PCR analysis showing *Math5* expression in the developing and adult hindbrain. B. Quantitative RT-PCR showing *Math5* mRNA levels during hindbrain development relative to *Hprt* ($\pm$ SD). *Math5* is expressed at relatively low levels in the adult hindbrain (normalized to 1.0). C. Comparison of *Math5* expression in the dissected hindbrain and eye at E16.5. The level of *Math5* mRNA in the eye is 28 times higher than in the hindbrain. D,E. *In situ* hybridization with antisense and sense probes showing *Math5* mRNA localized in the developing VCN. Abbreviations: vcn, ventral cochlear nucleus; hb, hindbrain; cb, cerebellum; ctx, cortex. Scale bar 100 μ m.

Figure 4.

Math5-lacZ expression within the cochlear nerve. A,B. Mid-modiolar section of an adult *M5*^{-/-} cochlea stained with X-gal, hematoxylin and eosin, showing *lacZ* expression in a small group of cells within the vestibulocochlear nerve (arrowheads). C,D. Sections through *M5*^{+/-} and *M5*^{-/-} cochlear nerves, stained with X-gal and eosin. The *lacZ*-positive cells appear similar in *Math5* mutant and heterozygous mice, are likely to be cochlear root neurons (CRNs), and. E,F. Adjacent sections showing co-localization of *Math5-lacZ* (red) with calbindin (green) and parvalbumin (green) in neurons within the cochlear nerve (arrowheads). G,H. Coronal sections through the AVCN and PVCN regions of an adult *M5*^{+/-} mouse showing *lacZ*-positive cells embedded within the cochlear nerve root (arrowheads). The boundaries of the

AVCN, DCN and PVCN are indicated in black and red. Abbreviations: SG, spiral ganglion; SM, scala media; ST, scala tympani; SV, scala vestibuli; Cb, cerebellum; ChP, choroid plexus. Scale bars 500 μm in A; 100 μm in D, F, G.

Figure 5.

Math5-lacZ positive cells are enriched in the AVCN, and are significantly smaller in *Math5* mutant mice. A,B. Coronal cryosections through the CN of *M5*^{+/-} and *-/-* mice, respectively, stained with X-gal and neutral red. The size and shape of *lacZ*-expressing cells differs between these genotypes. C-E. β-galactosidase immunohistochemistry showing the graded distribution of *Math5-lacZ* positive cells across the CN. *Math5-lacZ* cells are concentrated in the AVCN (panel C) and less abundant in the PVCN (panel D), but are absent in the DCN (panels D and E). F,G. Coronal cryosection of the CN immunostained with β-galactosidase sera and DAB. H,I. High-power comparison of *Math5*^{+/-} and *-/-* CN neurons, respectively, showing cell size metrics. J. Density of *lacZ*-positive cells across the CN of heterozygous and mutant mice. The apparent increase in cell density in the midventral AVCN of *M5*^{-/-} mice is most likely due to higher *lacZ* dosage and antibody sensitivity. K,L. Morphometric comparison of *lacZ*-positive cells in the AVCN and PVCN of *M5*^{+/-} and *M5*^{-/-} mice. Dimensions are given ± SEM. K. The cross-sectional cell area is significantly smaller in the AVCN of mutant mice. L. The length and width of AVCN cells are reduced proportionately in *M5*^{-/-} mice.

Abbreviations: AVCN, anteroventral cochlear nucleus; DCN, dorsal cochlear nucleus; PVCN, posteroventral cochlear nucleus. * $P < 0.05$; ** $P < 0.01$. Scale bars 100 μm .

Figure 6.

Math5-lacZ is coexpressed with bushy cell markers in the ventral cochlear nucleus A-H. Cryosections through the VCN of M5 +/- mice (panels A, C, E, G) and M5 -/- mice (panels B, D, F, H) were immunostained for β -galactosidase (red) neuronal antigens (green). A-D. The Kv1.1 and Kv3.1b potassium channels are expressed by all *Math5-lacZ* cells (arrowheads) and a roughly equal number of *lacZ*-negative cells (arrows). The co-labeled cells represent a subset of globular and spherical bushy cells within the VCN. E, F. The Kcnq4 channel is similarly expressed by all *Math5-lacZ* positive cells (arrowhead) and a larger number of *lacZ*-negative cells (arrow). G, H. Calbindin is expressed by a subset of *Math5-lacZ* positive cells in the VCN (arrowhead). Additional cells are positive for calbindin only (open arrow) or β -gal only (closed

arrow). This indicates that the *Math5*-expressing cell population is heterogeneous. Abbreviations: VCN, ventral cochlear nucleus. Scale bar, 100 μ m.

Figure 7.

Math5-lacZ is not expressed by type I multipolar or octopus neurons. A-F. Mutually exclusive labeling of VCN neurons with β gal (green) and a fluorogold (FG) retrograde tracer (magenta), which was injected into the ipsilateral inferior colliculus (IC) of an adult *Math5* +/- mouse. Coronal sections through the AVCN were imaged using epifluorescence (A-C) and confocal (D-F) microscopy, and are oriented with dorsal toward the upper right. Type I multipolar cells project to the IC and were labeled with FG, but do not express *Math5-lacZ*. The two populations are intermingled, consistent with the distribution of multipolar and bushy cell neurons. G,H. Octopus neurons do not express *Math5-lacZ*. Coronal sections through the anterior (G) and posterior (H) VCN of an adult *Math5* +/- mouse immunostained for β gal (red) and Kv1.1 (green). The octopus cells (arrowheads) can be identified easily by their large size, distinct morphology and location in the central PVCN. They strongly express Kv1.1 and clearly differ from the smaller *lacZ*+ cells (arrows). Abbreviations: dcn, dorsal cochlear nucleus; cb, cerebellum. Scale bars 100 μ m.

Figure 8.

Cytomorphology of *Math5*+ bushy neurons in *Math5-Cre* BAC; Z/AP mice. Vibratome sections (250 μ m; panels A, E, F) and cryosections (20 μ m; panels B-D) of double transgenic hindbrains were stained for hPLAP activity using BCIP-NBT histochemistry to reveal the distribution and fine structure of *Math5*+ neurons. A. Horizontal section through the VCN showing hPLAP+ cells with bushy cell morphology clustered toward the anterior pole. B-D. High-power views of representative cells with bushy cell morphology, including 1–2 dendritic trunks (arrowheads), counterstained with nuclear red. E. Coronal view of the trapezoid body showing hPLAP+ fibers crossing the midline (arrowhead) and a particulate pattern of staining in the MNTB. F. high-power view of the MNTB showing calyces of Held (arrowheads), large

tulip-shaped nerve endings originating from globular bushy neurons in the contralateral CN. Abbreviations: cp, cerebellar peduncle; mntb, medial nucleus of the trapezoid body. Scale bars: 100 μm in A, E; 25 μm in B-D, F.

Figure 9.

Altered auditory brain stem responses (ABR) in M5 $-/-$ mice. **A**. ABR thresholds for 4, 12, and 20 kHz pure-tone stimuli. There are no threshold differences between M5 $+/+$, $+/-$ and $-/-$ mice. **B**. ABR waveforms from representative M5 $+/+$ (black) and M5 $-/-$ (red) mice at 12 kHz and 80 dB SPL were overlaid for comparison. The time intervals (latency) between Peaks II and III, and Peaks III and IV are indicated with brackets. The peak amplitudes were heavily dependent on electrode placement and did not vary consistently between genotypes. **C**, **D**. Graphs comparing interpeak latencies ($\pm$ SEM) for 40–80 dB SPL stimuli at 12 and 20 kHz. The genotype symbols are defined in panel A. M5 $-/-$ mice have a decreased latency between Peaks II and III, and an increased latency between Peaks III and IV. The differences between

Math5 +/- and ++ mice were not statistically significant. $n = 10$ M5 +/+, 10 M5 +/- and 11 M5 -/- mice. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ for comparisons between M5 -/- mice and M5 ++ (black) or M5 +/- (gray) mice, using an unpaired t -test with unequal variances.