Supporting Information

Gómez-Casati et al. 10.1073/pnas.1008938107

SI Materials and Methods

In Situ Hybridization. Mice were perfused with 4% paraformaldehyde (PFA) and temporal bones were dissected out and fixed overnight at 4 °C. Then, temporal bones were washed two times for 5 min in PBS solution with 0.1% Tween-20 (PBT) and cleared with 6% hydrogen peroxide for 1 h at room temperature. After 20-min treatment with protease K (0.2 U/mL) at 37 °C, temporal bones were washed at room temperature for 10 min with 2 mg/mL of glycine in PBT and postfixed in PFA for 20 min followed by two washes for 5 min with PBT. Then, temporal bones were treated with proteinase K (0.2 U/mL) at 37 °C for 30 min with a 2 mg/mL of sodium hypochlorite (NaCl) solution. After washing three times for 5 min with 2X sodium phosphate buffered saline solution and then washed three times for 5 min with Tris-buffered saline solution and Tween-20 (TBST; 0.1 M NaCl; 2.7 mM KCl; 10 mM Tris, pH 7.5; 0.1% Tween-20). Subsequently, temporal bones were blocked for 2.5 h with 10% sheep serum in TBST and then incubated overnight at 4 °C in sheep anti–DIG-Fab fragments conjugated to alkaline phosphatase (0.15 U/mL; Boehringer). After washing, temporal bones were incubated with three times with TBST through the round window to get rid of most of the remaining antibody solution and left overnight in TBST at 4 °C. The next day, they were washed three times for 10 min in NTMT (100 mM Tris, pH 9.5; 50 mM MgCl2; 100 mM NaCl; 0.1% Tween-20) and developed in the dark with NBT/BCIP in NTMT (Roche). The development was stopped by washing, temporal bones were treated with proteinase K (0.2 U/mL) at 37 °C for 20 min followed by two washes for 5 min in PBS solution with 0.1% Tween-20. Subsequently, temporal bones were embedded in araldite and were sectioned at 200 nm on a Reichert Ultracut S ultramicrotome and conventionally dehydrated in graded ethanol and propylene oxide, samples were embedded in Araldite:DDSA resin. Ultrathin sections were cut on a Reichert Ultracut S ultramicrotome and contrasted with uranyl acetate followed by lead citrate.

VsEP Recordings. VsEPs were recorded from mice anesthetized with ketamine (100 mg/kg, i.p.) and xylazine (20 mg/kg, i.p.). Stainless steel electrodes were placed s.c. at the midline close to the neural crest, behind the left pinna, and at the back (ground). Mice were positioned on their backs, with the head coupled securely to a shaker platform. Stimuli were linear acceleration ramps, 2 ms in duration, applied in the earth-vertical axis at 16/s with alternating stimulus polarity (2). An accelerometer, mounted near the head, was used to calibrate the resultant jerk, which is expressed in dB re: 1.0 g/ms. Electrophysiological activity was amplified (10,000×), filtered (300–3,000 Hz), and digitized (125,000 Hz), and 512 responses were averaged at each stimulus level. We collected an intensity series in 5-dB steps encompassing stimulus levels above and below threshold.

Immunostaining. The freshly dissected utricular maculae were fixed by methanol at 4 °C for 20 min as described in ref. 3 for BDNF immunostaining; or ethanol for 20 min as described in ref. 4 for RIBEYE, GluR2/3, and NFH immunostaining. Thereafter, the preparations were washed three times in phosphate buffer (for BDNF) or PBS solution (for RIBEYE, GluR2/3, and NFH) and blocked for 2 h in 10% normal goat serum, 0.1% Triton X-100, 0.2% Tween (BDNF), or 16% normal goat serum, 450 mM NaCl, 0.3% Triton X-100, and 20 mM phosphate buffer, pH 7.4. The following antibodies were used: chicken anti–BDNF (1:50; Promega), mouse anti–CitP2 (also recognizing the ribonucleoprotein RIBEYE; 1:150; BD Biosciences), rabbit anti–GluR2/3 (1:1,000; Chemicon), chicken anti–NFH (1:1,000; Chemicon), and secondary Alexa Fluor-488, 568, and 647–labeled antibodies (1:300; Invitrogen).

Real-Time Quantitative RT-PCR. Real-time quantitative RT-PCR was performed as in ref. 5. Both inner ears from a given animal were processed as one sample. Only RNA samples that fulfilled the criteria that were established previously (5) were used for cDNA synthesis using iScript cDNA synthesis kit (Bio-Rad). Samples without reverse transcriptase were processed in parallel and served as negative controls. Measurements were made using a thermal cycler (Bio-Rad) and IQ SYBR Green Supermix kit (Bio-Rad). The following forward (F) and reverse (R) primers were used. For 18S rRNA (accession no. 006068): F, GCCGCTCACACATCACA-GGAA; R, GCTGGAATTACCGCGGCTT which generate a 187-bp amplicon. For BDNF (accession no. NM_005235): F, GCC TGC GAA TTAGGAGGATG; R, GATACCGGCGATCTTCT TCTAGGAC, which generate a 101-bp amplicon. For NT-3 (accession no. 53257): F, GCC CCC TTC CCT ATA CCT AAT G; R, CAT AGC GTT TCC TCC GTG GT, which generate a 83-bp amplicon. For GDNF (accession no. U36499): F, GTGCTGTG- TGACAGTATAGGAGTGG; R, GATACCGGCGATCTTCTCTTAGGAC, which generate a 101-bp amplicon. For NT-3 (accession no. 53257): F, GCC CCC TTC CCT ATA CCT AAT G; R, CAT AGC GTT TCC TCC GTG GT, which generate a 83-bp amplicon. For GDNF (accession no. U36499): F, GCC ACC ATT AAA AGA CTG AAA AGG; R, GCC TGC CGA TTC TCT TCT CT, which generate a 77-bp amplicon.

**Fig. S1.** DN-erbB4 is expressed in SC of GFAP-DN-erbB4 mice. In situ hybridization with DN-erbB4 probe produces no signal in the utricular maculae of P21 WT (A) but labels SCs (white arrowhead) in the maculae of GFAP-DN-erbB4 mice (B). No DN-erbB4 signal is observed in hair cells (black arrowhead) (B) or vestibular ganglia neurons (C). (Scale bar: 20 μm.)

**Fig. S2.** Vestibular maculae of GFAP-DN-erbB4 mice have normal overall structure and hair cell mechanotransduction. (A) Representative images by standard light microscopy from WT and GFAP-DN-erbB4 utricular maculae at P21 illustrate that there is no change in the gross appearance of the sensory epithelium. [Scale bars: 100 μm, 20 μm (inset.)] White arrows in inserts point to afferent axons. (B) Quantification of saccular and utricular maculae surface area at P6 and P21 demonstrates no significant changes in GFAP-DN-erbB4 mice (P = 0.6348 and P = 0.4793 for saccules at P6 and P21, respectively; P = 0.7556 and P = 0.2136 for utricles at P6 and P21, respectively; n = 3 for WT and GFAP-DN-erbB4 mice at both ages). Error bars represent SEM. (C) Quantification of utricular hair cells, afferent fibers, and neuronal cell bodies in the Scarpa ganglia at P21 demonstrates no significant changes in GFAP-DN-erbB4 mice (P = 0.9660 for utricular hair cells/100 μm; P = 0.3074 for afferent fibers/100 μm, and P = 0.7312 for neuronal cell bodies/mm²; n = 3 for both WT and GFAP-DN-erbB4 mice). Error bars represent SEM. (D and E, Left) Top view of utricles stained for neurofilament shows that calyceal terminals on hair cells are normal in the mutants at P21. Arrowheads point to nerve terminals. (Insets) Side views of the same tissue stained for neurofilament (green) and nuclei (blue). Arrowheads point to afferent axons. (D and E, Right) EM images of hair cells and their calyceal terminals in WT and Tg utricles. (Scale bars: 10 μm in D–G.) (F) Top view of utricular maculae stained with phalloidin shows no changes in hair bundle density and orientation in the Tgs. (Scale bar: 50 μm.) (G and H) Analysis of mechanotransduction by FM1-43 uptake. (G and H, Left) Top view of utricles incubated with FM1-43 dye (green) shows that dye uptake is similar in hair cells from WT and Tg. Red shows hair bundles stained with phalloidin. (G and H, Right, Lower) Side view of cells shown in G and H (Left). (G and H, Right, Upper) FM1-43 uptake is blocked in both genotypes by La³⁺, indicating that the FM1-43 signal reflects mechanotransduction channel activity.

**Fig. S3.** Knockdown of BDNF expression in SCs does not result in neuronal loss. Neurofilament immunostaining of P26 utricular maculae from tamoxifen treated control (BDNF⁺⁺) and BDNF conditional KO (BDNF⁺⁺::PLP/CreER¹) mice, and BDNF⁺⁺::GATA-1/Cre mice shows that reduction in BDNF expression in SCs late in embryonic development has no impact on the calyceal terminals on macular hair cells, whereas, when BDNF is eliminated globally and early in embryogenesis, in the case of BDNF⁺⁺::GATA-1/Cre mice, terminals are lost like in BDNF-null mice (6). Arrowheads point to calyx nerve terminals. (Scale bar: 10 μm.)

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Fig. S4. Peak 1 amplitude of the VsEP correlates with synaptic density in the utricle. VsEP peak 1 amplitude (in μV at 5 dB) for BDNF$^{ff}$ and BDNF$^{ff}$::PLP/CreER$^T$ mice injected with tamoxifen versus the corresponding synaptic puncta [defined by colocalization of presynaptic (RIBEYE) and postsynaptic (GluR2/3) markers] in the utricle.

Movie S1. GFAP-DN-erbB4 mice are ataxic. Video recordings of WT and GFAP-DN-erbB4 mice show that the Tg mice have severe ataxia, including head shaking, circling behavior, difficulty walking on a balance beam, and abnormal swimming.