THE NOVEL PMCA2 PUMP MUTATION TOMMY IMPAIRS CYTOSOLIC CALCIUM CLEARANCE IN HAIR CELLS AND LINKS TO DEAFNESS IN MICE

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The mechanotransduction process in hair cells in the inner ear is associated with the influx of calcium from the endolymph. Calcium is exported back to the endolymph via the splice variant w/a of the PMCA2 of the stereocilia membrane. To investigate further the role of the pump, we have identified and characterised a novel ENU induced mouse mutation, Tommy, in the PMCA2 gene. The mutation causes a non-conservative Glu629Lys change in the second intracellular loop of the pump which harbors the active site. Tommy mice show profound hearing impairment from P18, with significant differences in hearing thresholds between wild type and heterozygotes. Expression of mutant PMCA2 in CHO cells shows calcium extrusion impairment: specifically, the long-term, non-stimulated calcium extrusion activity of the pump is inhibited. Calcium extrusion was investigated directly in neonatal organotypic cultures of the utricle sensory epithelium in Tommy mice. Confocal imaging combined with flash photolysis of caged calcium showed impairment of calcium export in both Tommy heterozygotes and homozygotes. Immunofluorescence studies of the organ of Corti in homozygous Tommy mice showed a progressive base to apex degeneration of hair cells after P40. Our results on the Tommy mutation, along with previously observed interactions between cadherin-23 and PMCA2 mutations in mouse and humans, underline the importance of maintaining the appropriate calcium concentrations in the endolymph to control the rigidity of cadherin, and ensure the function of interstereocilia links, including tip links, of the stereocilia bundle.

Deflection of the stereocilia bundle that protrudes from hair cells into the endolymph causes the opening of mechanotransduction (MET) channels and influx of K+ and Ca2+ into the cells, ensuing in graded changes of the resting membrane potential (i.e. in a receptor potential) (1). Outer hair cells (OHC) of the mammalian cochlea are mechanosensors that, acting as motor cells (2,3), produce forces driven by their receptor potential and amplify the mechanical vibrations of the basilar membrane in response to sound (4,5). The amplification action activates the MET process in inner hair cells (IHCs) and consequent release of neurotransmitter to afferent dendrites of spiral ganglion neurons whose action potentials relay acoustic information to the central nervous system (6).
In all types of hair cells, MET channels are gated by tension applied through extracellular tip links, which are oriented along the main symmetry axis of the hair bundle from the apex of one stereocilium to the lateral wall of its taller neighbour (7). Recent work (8) has confirmed that MET channels are located at the lower end of the tip link, in the two shorter rows of stereocilia (9). Earlier experiments performed under unphysiological conditions of Ca\(^{2+}\) concentrations (mM range) had shown that approximately 90% of the MET current was carried by K\(^{+}\), and 10% by Ca\(^{2+}\) (10). More recent work has indicated that the fraction of MET current carried by Ca\(^{2+}\) decreases directly in proportion to its external concentration, accounting for only ~0.2 per cent of the current in a saline comparable to endolymph (11), in which the Ca\(^{2+}\) concentration (~20 µM) is about 100 fold lower than in all other extracellular fluids (12). The entry of Ca\(^{2+}\) has been so far considered vital for adaptation of the mechanotransduction process (1) but these concepts may now have to be revised (11).

Ca\(^{2+}\) entering through MET channels is rapidly buffered by endogenous mobile chelators, e.g., parvalbumin (13). However, Ca\(^{2+}\) must be eventually exported back to the endolymph by the PMCA pump, which is highly concentrated in the stereocilia plasma membrane (11,14,15). The pump isoform of the stereocilia, PMCA2, that is coded by the ATP2b2 gene, is known to be essential for hearing and balance (15-18). PMCA2 has properties that set it apart from all other PMCAs (19): it has very high affinity for the activator calmodulin, yet, its activity is only modestly activated by it (19,20). Unlike the other 3 PMCA basic isoforms, PMCA2 has peculiarly high activity in the absence of the activator calmodulin, i.e., it pumps Ca\(^{2+}\) out of cells at a relatively high constant rate.

All PMCA transcripts undergo alternative splicing at site A, in the cytosolic loop connecting transmembrane domains 2 and 3, and at site C in the cytosolic tail of the pump. The splicing process is uniquely complex in PMCA2, as it involves the insertion of up to 3 exons at site A, and of 2 at site C. The site A inserts are in frame, and generate variant w when all 3 exons are inserted; those at site C create instead a novel stop codon, truncating the pump prematurely and generating variant a. Recent work has conclusively shown that splicing at site A dictates apical targeting in hair cells for PMCA2 and that the rodent hair-bundle isoform is variant PMCA2\(w/a\); the basolateral plasma membrane of the hair cell contains instead PMCA1 (21,22). Tests of the activity of the various PMCA2s expressed in model cells have revealed a decreased ability of the w/a variant to control incoming Ca\(^{2+}\) pulses in comparison to the non inserted z/b variant (23) which, as mentioned, responds with a lower activation to the arrival of Ca\(^{2+}\) pulses with respect to the ubiquitous PMCA1 or PMCA4 pumps (19). The stereocilia of vestibular hair cells and OHCs are the only plasma membrane in which the w/a variant of PMCA2 has so far been detected in mammals (21). The unusually low concentration of Ca\(^{2+}\) in the endolymph that bathes them (12) has evidently led to the evolutionary selection of a PMCA variant with the peculiar Ca\(^{2+}\) exporting properties of PMCA2\(w/a\).

It has been known for over a decade that ablation of the ATP2b2 gene produces hereditary deafness in mice (15). PMCA2 mutations were then discovered linked to recessively inherited deafness in mice (17,18,24-26) and humans (23,27). In the present contribution, we describe PMCA2 mutant Tommy mice that exhibit a profound deafness phenotype\(^1\). The mutation affects a highly conserved residue located next to the active site in the cytosolic loop that connects transmembrane domains 4 and 5 of the enzyme. This residue is classified as E584 in the z/b variant of the PMCA2, but corresponds to E629 in the PMCA2\(w/a\) variant due to the 45 amino acid site A insertion in the first intracellular loop. The analysis of the defect in model cells overexpressing the mutated pump and in hair cells of organotypic utricular cultures has revealed a marked depression of the unstimulated, long term ability of the PMCA2\(w/a\) variant to export Ca\(^{2+}\) from the stereociliar cytosol.

\(^1\) A preliminary report of the Tommy mutant has been published in abstract form: Nicholas J. Parkinson, Francesca Mackenzie, Debra Brooker, Martin Fray, Pete Glenister, Steve D.M. Brown (2003), “The Tommy mouse mutant is a new allele of dfw that displays semi-dominant age- related hearing loss”. Association for Research in Otolaryngology, Abstract ref. ID 9065.
EXPERIMENTAL PROCEDURES

Mice. All animals used for Tommy mutant line derivation were housed and maintained in the Mary Lyon Centre at MRC Harwell, under Specific Pathogen Free (SPF) conditions in individually ventilated cages, with environmental conditions outlined in the Home Office Code of Practice. Animal procedures were carried out in line with Home Office regulations, and mice were euthanised by Home Office Schedule 1 methods. The Tommy mutant line was derived from a dominant ENU mutagenesis screen at the MRC MGU Harwell (28) in which BALB/c mice were treated with ENU, mated to C3H/HeH and F1 progeny subject to a variety of screening procedures.

All animals used for ABR recordings and fluorescence microscopy experiments were housed and maintained in the Venetian Institute of Molecular Medicine (VIMM), Padua, Italy. The care and use of the animals were approved by the Animal Care and Use Committee of the University of Padua.

Clickbox screening. Mice were screened according to the SHIRPA protocol (28) and placed in the palm of the hand and tested with a click box (Institute of Hearing Research, Nottingham, UK) which produces a brief audio stimulus ~20 kHz tone at 90 dB when held 30 cm away from the subject. Mice with unaffected hearing, elicit the ‘preyer’ response, a backwards flick of the ear pinnae. In most cases the preyer reflex is followed by startle response that can range from a contracting of the neck muscles to a rapid jump backwards. A lack of either of the responses was recorded as no response to the clickbox (29).

Genome scan. We used a pooling strategy employing DNA from affected mice and genome-wide fluorescent simple sequence length polymorphism-based screening (28) to provide an initial map position for the Tommy locus, Tmy, to a 20cM region of Chromosome 6 flanked by markers D6Mit67 and D6Mit295. Additional polymorphic markers within the critical region were identified from public databases [microsatellite markers: Mouse Genome Informatics (MGI), Broad Institute Genetic Map of the Mouse Genome (Whitehead/MIT) and Center for Inherited Disease Research (CIDR); single nucleotide polymorphism (SNP) markers: Ensembl, Genomics Institute of the Novartis Research Foundation website (GNF)] and were tested for polymorphism in the parental strains BALB/cAnN and C3H/HeH. For high-resolution mapping, polymorphic microsatellite and SNP amplicons were analysed on 6% acrylamide gels using the single-stranded conformational polymorphism (SSCP) method allowing the Tmy critical region to be reduced to a 6cM interval flanked by polymorphic microsatellite markers D6Mit287 and D6Mit150 and encompassing the dfw critical region.

In vivo recordings of Auditory Brainstem Response (ABR). Mice aged between P18 and P90 were anesthetized with an intraperitoneal injection of Zolazapam (25 mg/Kg) and Xylazine (10 mg/Kg). Supplemental doses were then administered as needed. Body temperature was kept at 38°C by a feedback-controlled heating pad. Each recording procedure lasted up to 70 min. Acoustic stimuli were produced in the free field within a foam-padded, shielded acoustic chamber by a System 3 Real-time Signal Processing System (Tucker-Davis Technologies, Alachua, FL, USA) combined with an ES1 electrostatic speaker mounted 4 cm in front of the mouse’s ear. Stimuli consisted in tone bursts (1 ms rise/decay; 3 ms plateau) at 8, 14, 20, 26, and 32 kHz, and clicks (0.1 ms) delivered at a repetition rate of 13 Hz. A maximum of 100 dB SPL was employed for all stimuli. To monitor electrical signals, a subdermal (active) needle electrode was inserted at the vertex, whereas ground and reference electrodes were inserted subdermally in the loose skin beneath the pinnae of opposite ears. Bio-electrical potentials were differentially amplified (50,000 times), filtered (5–8,000 Hz) and digitized (25 µs) for averaging (AT MIO 16XE-10, Labview 7.0, National Instruments Corp., Austin, TX, USA). ABR threshold was defined as the lowest step in a 10 dB decremented scale at which the IV wave could be recognized by a trained Audiometry specialist. Judgment of threshold was confirmed by a second specialist. In case the IV wave of the ABR could not be discerned in response to the maximum 100 dB SPL stimulus, a nominal threshold of 110 dB SPL was assigned.

Cloning and mutagenesis of pmRFP-PMCA2w/a. mRFP was amplified from pCDNA3.1/zeo-mRFP (donated by Dr. M. Zaccolo, Padua, Italy) using the following primers,
forward: 5’-GGCTAGCATGGCCTCCTCCGA
GGACGTCA-3’ and reverse: 5’-GCAGATCTAGGCGCCGGTGGAGTGGCGG-3’, bearing restriction sites for NheI and BglII, respectively (in bold). The PCR product was than digested with NheI and BglII and inserted in pEGFP-c1 (donated by Dr. E. Strehler, Rochester, MN) was excised by independent digestion with Sall-EcoRI and EcoRI-KpnI and inserted into Xhol-KpnI sites of pmRFP-c1 in a three-part ligation reaction resulting in pmRFP-PMCA2 w/a. The construct was controlled by sequencing. Site-directed mutagenesis was carried out to obtain the mutant cloned in the appropriate vector. pmRFP-PMCA2 w/a was used as target and experiments were performed according to the manufacturer’s standard protocol (Quick-change, Stratagene, Cedar Creek, TX). The following primers were used: Tommy 5’ AGCAAAGGTGCTTCGAAGAGTTGTGCTCAAAA 3’(forward) and 5’ TTTTGAAGACAATCTTGCGAAGCACCTTTGCT 3’ (reverse). The nucleotide which determines the mutation is underscored.

**Immunolocalization of overexpressed pumps and membrane fluorescence computation.** CHO cells were grown in Ham’s F12 medium, supplemented with 10% fetal calf serum (FCS). Before transfection, they were seeded onto 13 mm glass coverslips and allowed to grow to 50% confluence. Transfection with 3 µg of plasmid DNA (or 1.5 :1.5 µg in the case of co-transfection) was carried out with a Ca-phosphate procedure (30). Immunocytochemistry quantified the expressed pump proteins in the plasma membrane of transfected cells. CHO cells expressing the PMCA2 variants were stained with polyclonal isoform-specific PMCA antibody 2N (Affinity Bioreagent, Inc.,Golden, CO) or a monoclonal antibody recognizing all pump isoforms (5F10, Affinity Bioreagent, Inc.,Golden, CO), at a 1:100 dilution in PBS. Staining was carried out with Alexa 488 labelled anti-rabbit or anti-mouse secondary antibodies (Molecular Probes, Invitrogen Corp., Carlsbad, CA) at a 1:50 dilution in PBS, and total fluorescence intensity in membrane was quantified using software developed in the Laboratory, as previously described (23,25). For each construct, fluorescence was averaged over a total of 50 cells in 3 different slides.

**Preparation of membranes from CHO cells, SDS-PAGE and Western blot analysis.** 36 h after transfection CHO cells were washed twice with cold PBS buffer and scraped in lysis buffer [Tris-HCl (PH 8.0) 10 mM, ethylenediaminetetraacetic acid (EDTA) 1 mM, phenylmethylsulfonyl fluoride (PMSF) 2 mM and Dithiothreitol (DTT) 1 mM]. After centrifugation at 1000 g for 5 min, the cells were resuspended in 80 µl of lysis buffer and subjected to three cycles of freeze and thaw. The proteins of the lysates were quantified using the Bradford Reagent (Sigma-Aldrich, Milan, Italy) 20 µg of proteins were loaded on 7.5 % polyacrylamide gel and transferred to nitrocellulose membranes which were incubated with polyclonal PMCA2 antibody 2N (Sigma-Aldrich, diluted 1:1000) and monoclonal β-actin antibody (Sigma-Aldrich). After incubation with anti-rabbit horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), the blots were developed with ECL reagents (Amersham Life Science, Arlington Heights, IL, USA). The quantitative analysis was carried out by densitometric analysis using the Kodak 1D Image Analysis program (Kodak Scientific Imaging System, New Haven, CT). Antibodies against β-actin were used to normalized the data obtained from the densitometric analyses.

**Ca^{2+} measurements with recombinant aequorin.** Recombinant cytosolic aequorin, as produced inactive by CHO cell transfection, was reconstituted in functional chemiluminescent protein by incubating cells for 1–3 h with 5 µM of the prosthetic group coelenterazine in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 1% FCS, at 37 °C in a 5% CO2 atmosphere. Additions to the KRB medium (1 mM CaCl2, 100 µM ATP) were made as specified in the Figure legends. The experiments and luminescence calibration into [Ca^{2+}] values were carried out according to (30). Data are reported as mean ± SD. Statistical differences were evaluated by Student’s 2-tailed t-test for unpaired samples. A p value <0.01 was considered statistically significant. We have previously shown that the contribution of the native endoplasmic reticulum Ca^{2+} pump (SERCA) to the reduction of Ca^{2+} level in the
cytoplasm is marginal in cells which overexpress PMCA variants (23). SERCA pump inhibitors were thus not used in these experiments. Furthermore by comparison with the results of control experiments performed in naive cells (not transfected with PMCA constructs) we determined that the recovery phase is dominated by the overexpressed PMCA2 pump. Similarly, the contribution of plasma membrane Ca\(^{2+}\) influx channels, opened by the emptying of intracellular stores, to the lowering of cytosolic Ca\(^{2+}\) was disregarded, assuming it would have been the same in all conditions tested.

Preparation of organotypic cultures from utricle sensory epithelia. Utricles were excised from wild type, heterozygous or homozygous Tommy mice between P6 and P7. Postnatal mice were decapitated for inner ear extraction, the otic capsule was opened medially in a dissection solution and the utricle was extracted by micro forceps. The dissection saline was composed of Hank’s balanced salt solution (HBSS, catalog no. H6648; Sigma–Aldrich) with 10 mM Hepes, 10,000 units/liter penicillin and 25 µg/liter fungizone. The endolymphatic compartment was cut open and the otolithic membrane was removed by the flux of a syringe filled with saline. The utricle epithelium was fixed by Cell-Tak (BD Biosciences, Bedford, MA) mixed with 90% NaHCO\(_3\), to the lateral side of a glass capillary (1.5 mm diameter, 5 mm length) that had been previously glued to a microscope slide by a small drop of Sylgard Silicon Elastomer (Dow Corning, Wiesbaden, Germany). The organs were preserved in culture for 1 day at 37°C in a complete medium of 95% DMEM/Ham’s F-12 (1:1) (1, liquid, with L-glutamine, without Hapes; GIBCO, Carlsbad, CA) and 5% FBS.

Confocal Ca\(^{2+}\) imaging combined with flash photolysis of caged Ca\(^{2+}\) in hair cells. At recording time, i.e. one day after dissection, utricle cultures were loaded with 10 µM Fluo-4 AM (Invitrogen) and 10 µM o-nitrophenyl-EGTA AM (Invitrogen) for 50 minutes at 37 °C in DMEM (GIBCO) supplemented with 25 µM sulfipyrazone and Pluronic F-127 (0.1%). For deesterification, cultures were superfused for 10 min with HBSS (GIBCO) supplemented with 4.5 g/liter glucose and 2 mM anhydrous CaCl\(_2\) (pH 7.4, Osm 330) and transferred to a chamber mounted on the stage of an upright confocal system (Bio-Rad Radiance 2100) incorporating an Eclipse E600FN optical microscope (Nikon Instruments Europe B.V., 1183AS Amstelveen, The Netherlands) equipped with a water immersion objective (Fluor 60×, 1.0 N.A., Nikon).

Intracellular Fluo-4 fluorescence was excited by the 488 nm line of an argon-ion laser and selected around 528 nm by a HQ528/50m narrow-band interference filter. A rapid increase in cytosolic Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_i\)) was achieved by the photorelease of Ca\(^{2+}\) from the Ca\(^{2+}\)-bound state of \(\alpha\)-nitrophenyl-EGTA. The ultraviolet (UV) illumination used for Ca\(^{2+}\) uncaging covered an area of about 10,000 µm\(^2\), comprising a few hair cells, and was generated by a 375-nm solid state laser connected to the microscope through a 600-nm-diameter optical fiber. The recollimated fiber output was reflected off a dichromatic beam splitter (400 DCLP, Chroma) positioned at 45° just above the microscope objective lens. UV light was delivered for 60 ms during the 40th frame (frame rate 7 Hz), under control of a transistor–transistor logic (TTL) signal generated by a PC that monitored the frame trigger output of the confocal microscope. After obtaining readout for the Fluo-4 baseline fluorescence, \(f_0\), in the hair cell, exposure to UV light generated a uniform Ca\(^{2+}\) transient distributed over the whole cell. Ca\(^{2+}\) concentration change was probed by the \(\Delta f/f_0\) signal, defined as the percentage change of Fluo-4 fluorescence \(f\) relative to \(f_0\) (31). Image sequences were stored on disk and processed off-line as previously described (23,25) using the Matlab 7.0 software package (The MathWorks, Inc., Natick, MA, USA). Fluorescence traces were obtained by spatially averaging pixel signals within the stereociliary compartment of wild type, heterozygous and homozygous Tommy mice. The parameter used to quantify the stereocilia ability to extrude Ca\(^{2+}\) was the decay time constant (\(\tau\)) of the single-exponential curve fitting the first part of the \(\Delta f/f_0\) trace after the UV flash.

Transversal and horizontal cochlear section preparation. Cochleae were dissected from P40 and P60 Tommy mice, fixed in 4% PFA overnight and decalcified for the next three days in Dulbecco’s phosphate buffered saline (DPBS, GIBCO) with 0.3 M EDTA. After 3 washes in DPBS, cochleae were included in 3% agarose.
(dissolved in DPBS) and cut in 100 µm thickness slices, either parallel or perpendicular to the modiolar axis (for transversal and horizontal sections, respectively). Sample orientation was controlled by a crossed pair of goniometers (Thorlabs GmbH, Dachau/Munich, Germany) coupled to a vibratome (VT 1000S, Leica Microsystems, Wetzlar, Germany). Section were cut with Platinum double−edge razor blades (Gillette) at speed 6 and frequency 6, both on a scale of 10 on the vibratome range.

**Immunohistochemistry of PMCA2 in hair cells.** Utricle cultures (UC) and cochlear slices (CS) were rinsed in DPBS containing 1% BSA (rinse solution). After permeabilization at room temperature with 0.1% Triton X-100 dissolved in 2% BSA solution (1 h for UC, 3 h for CS), PMCA2 was immunolabelled by overnight incubation at 4°C with polyclonal isoform-specific PMCA antibody 2N (Thermo Scientific ABR, PA1-915) diluted in rinse solution (1:400). After washing three times in PBS (5 minutes each time for UC, 1 h for CS), the secondary antibody (Alexa Fluor 488 goat anti−rabbit IgG, Invitrogen) was applied at 5 µg/ml for 1 h at room temperature. Nuclei were stained by incubation (1 h for UC and over night for CS) in 4´,6-diamidino-2-phenylindole dihydrochloride (DAPI, Invitrogen) diluted (5 µM) in rinse solution. The same procedure was used to stain F–Actin by Texas Red-X phalloidin (Invitrogen) in CS. Membranes in UC were stained by 20 minutes incubation in FM4-64FX (Invitrogen) diluted at 15 µM in DPBS. After washing for three times in DPBS, CS were mounted onto glass slides with a mounting medium (FluorSave™ Reagent, Merck KgaA, Darmstadt, Germany) and imaged with an inverted confocal microscope (TCS SP5, Leica Mikrosysteme Vertrieb GmbH, Wetzlar, Germany) equipped with oil immersion objectives (Leica 40X,1.25 N.A., or Leica 63×, 1.4 N.A.). UC were imaged by an upright confocal system (Bio-Rad Radiance 2100 incorporating a Nikon Eclipse E600FN microscope) equipped with a water immersion objective (Nikon Fluar 60X, 1.0 N.A.). For CS, Alexa Fluor 488 was excited by the 488 nm line of an air-cooled argon-ion laser and its fluorescence emission was collected in a spectral window between 495 nm and 540 nm. Texas Red was excited by the 561 nm line of a diode-pumped solid-state laser and its emission was collected between 600 nm and 690 nm. DAPI was excited by the 405 nm line of a diode laser module (emission light was collected between 410 nm and 440 nm) or excited by a pulsed Tsunami laser (Spectra-Physics, Santa Clara, CA 95054, USA) at 830 nm (emission light was collected with a HQ450/80 filter). For UC, Alexa Fluor 488 was excited by the 488 nm argon-ion laser of the Bio-Rad system and its fluorescence emission was collected by using a HQ515/30 narrow-band interference filter. FM4-64 was excited by the same argon laser line and observed by a long pass interference filter E570LP.

**RESULTS**

**Isolation and initial characterization of Tommy mutants.** The Tommy (Tmy) founder mouse was isolated from an aged cohort of BALB/cENU/C3H F1 mice produced during a dominant genome wide mutagenesis screen carried out by our facility (28). Mice from within this cohort were SHIRPA tested at four weeks post partum, aged, then repeat SHIRPA tested at 26 weeks. At four weeks of age the founder male exhibited a wild-type ear flick ‘Preyer’ reflex response to a transient 20kHz pure tone burst stimulus of ≈90dB SPL emitted by a calibrated click-box. At age 26 weeks no such reflex was observed with identical stimuli indicating a profound deafness. No other phenotype was observed with SHIRPA at either age. The founder BALB/cENUC3HF1-Tmy/+ animal was backcrossed to C3H/HeH then affected animals successively backcrossed to C3H/HeH to maintain the colony. Following confirmation of inheritance progeny were repeatedly tested for presence of a wild type hearing response using the click-box assay. Profound deafness in Tmy/+ animals was first observed at approximately 12 weeks of age and is nearly fully penetrant after 30 weeks. Middle and inner ear dissections of affected animals found no gross morphological defects of the cochlea, vestibular labyrinth or ossicles. In addition no evidence was found of middle ear infection or disease (data not shown).

Affected N4 backcross progeny were intercrossed to investigate potential homozygous phenotypes. Resultant litters produced animals with more severe phenotypes at frequencies consistent with them being Tmy/Tmy homozygotes (22% displayed an enhanced phenotype, total
progeny N=65). Such animals were visibly smaller than their littermates and failed to exhibit any response to click-box analysis from birth indicating a profound congenital deafness. In addition these animals were severely ataxic displaying a hesitant wobbly gait with frequent hyperextension of the rear limbs.

**Genetic mapping and candidate screening.** Using semi-automated fluorescent genome wide linkage analysis, we initially positioned the Tmy locus within a ~30cM region of mouse chromosome 6 flanked by polymorphic microsatellite markers D6Mit287 and D6Mit295. An additional 78 affected heterozygous animals were used to refine the initial region of linkage to a 1.5cM critical region between markers D6Mit287 and D6Mit366. This 3.1Mb chromosomal segment is predicted by ENSEMBL to contain 36 known genes including the Atp2b2 locus. Given the substantial overlap displayed between the phenotypes of Tmy/Tmy and Atp2b2dfw/dfw animals the Atp2b2 locus became the focus of a mutation screen. Direct sequence analysis of the Atp2b2 locus revealed a single nucleotide change in exon 7 corresponding to a non-conservative G1750A transition in the full length coding sequence. This mutation was not seen in either BALB/c or C3H/HeN homozygote control DNAs and is predicted to result in a Glu584Lys (with numeration referring to PMCA2 pump, and corresponding to position 629 in the w/a variant, which is the variant present in the hair cells, as mentioned in the Introduction section).

**Hearing impairment in Tommy mutant mice.** Hearing in Tommy mice was quantified by monitoring auditory brainstem responses (ABRs, see Experimental Procedures), which are electrical signals evoked from the brainstem following the presentation of sound stimuli (Figure 1). We measured the IV wave ABR thresholds for click and tone burst (8-14-20-26-32 kHz ) stimuli in wild type, heterozygous and homozygous Tommy mutant mice aged between P18 and P45 (Figure 2). Profound hearing impairment was found in the homozygote from P18. Measurements at younger stages were not performed due to limitations intrinsic in the ABR technique. A significant difference (p < 0.001, n=5) between wild type and Tommy heterozygote thresholds was observed with clicks as well as tone bursts at frequencies of 14 kHz and above.

**PMCA2w/a expression and function in model cells.** We investigated the effect of the replacement of a glutamic acid with a lysine at position 584 in the PMCA2 z/b sequence (corresponding to position 629 in the w/a variant) using an expression system in model cells. As documented in Figure 3 the corresponding E584 residue of PMCA2z/b is well conserved among the PMCA isoforms in different species (Panel A) and different Ca^{2+} pumps (Panel B). The wild type mammalian PMCA2w/a protein or the E629K mutant were overexpressed in CHO cells. We verified by Western Blotting analysis that transfected cells expressed equal levels of wild type and mutant pumps (Figure 4A and B). We also verified by immunocytochemistry that the overexpressed pumps were correctly delivered to the plasma membrane in equivalent amounts (Figure 4C and D). To monitor Ca^{2+} transients, CHO cells co-transfected with the cDNA encoding the cytosolic Ca^{2+} sensitive photoprotein aequorin (cytAEQ) were stimulated with the purinergic agonist ATP to evoke InsP_3-mediated cytosolic Ca^{2+} increases. As shown in Figure 5A and B, essentially no differences were detected between the two pump variants in the immediate response to the sudden increase of Ca^{2+} induced by ATP stimulation. Thus, the Ca^{2+} peak was 3.16±0.25 µM (n=15) in cells overexpressing wild type PMCA2 w/a, 3.01±0.27 µM (n=15) in those overexpressing the Tommy mutation (E629K), and 3.06±0.17 µM (n=6) in control cells (Figure 5B).

However, the mutation affected significantly (p<0.001), the declining phase of the Ca^{2+} transient that represented the recovery to basal Ca^{2+} levels (Figure 5C). Namely, the half time of the declining phase was 61.8±7.85s (n=6) in control cells, 6.77±0.6 s (n=13) in those overexpressing the wild type pump and 39.38±6.71 s (n=14), in those overexpressing the mutant pump.

**PMCA2 expression and function in vestibular hair cells of P6-P7 Tommy mice.** The PMCA2 ability to extrude Ca^{2+} was then investigated in situ using neonatal (P6-P7) organotypic cultures of utricle sensory epithelium. The choice of utricles was dictated, on the one hand, by the larger dimension of the sensory hair bundle in vestibular hair cells compared to cochlear hair cells and, on the other hand, by the greater ease with which the utricular preparation can be fixed to the coverslip
to acquire confocal images with the cell main axis resting in the focal plane (Figure 6A-C). Hair cells in this preparation were stimulated by the rapid photorelease of Ca\textsuperscript{2+} from a cytosolic caged precursor (o-nitrophenyl-EGTA, Figure 6D). The hair cell dissipate this sudden increase of [Ca\textsuperscript{2+}]i, by the action of endogenous buffers, uptake by mitochondria (11) and transport by Ca\textsuperscript{2+}-ATPases (15). The PMCA2w/a isoform is present at very high concentration in the hair cell stereocilia (11,22,32). To quantify the recovery of [Ca\textsuperscript{2+}]i after the UV flash, we used the time constant \( \tau \) of a single-exponential fit (Figure 6D, red dashed line) to the \( \Delta f/f_0 \) trace obtained by spatially averaging the Ca\textsuperscript{2+} transient within the stereocilia compartment. The ability of PMCA2 to extrude Ca\textsuperscript{2+} depends, amongst other things, also on the [Ca\textsuperscript{2+}]i levels, thus we decided to select only experiments with similar \( \Delta f/f_0 \) transients (Figure 6E, left panel). In wild type mice, the recovery to baseline [Ca\textsuperscript{2+}], was faster (\( \tau = 2.78 \pm 0.27 \) s, \( n=11 \) cells from \( m=3 \) mice) than in heterozygous (\( \tau = 3.80 \pm 0.80 \) s, \( n=11, m = 3; *p=0.21, \) one-way ANOVA Test) and, especially, homozygous Tommy mice (\( \tau = 4.47 \pm 0.87 \) s, \( n=18, m=4; **p=0.12, \) one-way ANOVA Test) (Figure 6E, right panel).

PMCA2 expression in situ and organ of Corti degeneration. We performed confocal imaging immunoassays in the neonatal utricle preparation using antibodies selective for the PMCA2 (Figure 7). P7 utricle cultures from wild type, heterozygous and homozygous Tommy mice presented with selective localization of the PMCA2 in the plasma membrane of the stereocilia, with comparable levels of fluorescence. Although not quantitative, these results suggest that the differences in the recovery following Ca\textsuperscript{2+} photoliberation highlighted above reflected a dysfunction of the pump more than problems with its expression or targeting.

Immunofluorescence was also used to monitor the progressive degeneration of the organ of Corti, which correlates with the lack of auditory function in the homozygote (Figure 2). By immunolabeling transversal and orthogonal sections of the cochlea, we observed a progressive base to apex loss of PMCA2 in the hair cells of homozygous Tommy mice after P40 (Figures 8 and 9). At P60, PMCA2 immunofluorescence signal was virtually absent in the basal part of the cochlea and signs of hair cell degeneration were evident by the lack of several nuclei stained with DAPI.

**DISCUSSION**

The Tommy mouse mutation described here was identified as a new PMCA2 pump mutant with progressive deafness from an ENU mutagenesis screen. It is the 4th PMCA2 point mutation so far found to be linked to hereditary hearing loss in mice. Other deafness-related mutations in the mouse *Atp2b2* gene have been described that led in some cases to the truncation of the molecule, and to its eventual disappearance from the stereocilia of hair cells (15,18,26). Three of the described mutations were instead point mutations that did not compromise the reading frame of the gene, and were thus compatible with the expression of the full-length PMCA2w/a variant of the pump: they all affected residues that are highly conserved in all PMCA isoforms across species and in other P-type pumps. The original deafwaddler mutation was a G-S replacement at position 283 in the first cytosolic loop of the pump (17). A second unnamed mutation was a T-K substitution at position 692 (737 in the w/a variant) in the C-terminal half of the large cytosolic loop that connects transmembrane domains 4 and 5 of the molecule (24). In the Oblivion mutation a S was replaced by a F at position 877 (position 922 in the w/a variant) in the 6th transmembrane domain of the pump (25).

Recent work has indicated that the amount of Ca\textsuperscript{2+} entering through the MET channels corresponds to only \( \sim 0.2 \% \) of the total inward current (11). Thus, with K\textsuperscript{+} as the monovalent cation driven by the endolymphatic potential and with an estimated \( \sim 40\% \) MET channels open at rest, the Ca\textsuperscript{2+} fraction of the total MET current in vivo should correspond to 3 pA in apical OHCs. The maximum Ca\textsuperscript{2+} load when all MET channels are gated open is estimated to correspond to a current of 7 pA. Both figures are well within the maximal clearing rate estimated for a density of \( \sim 6000/\mu m^2 \) PMCA2 pumps which, assuming a conservative turnover rate of 100 ions/s as measured in erythrocytes (33), could safely clear a sustained influx corresponding to a current of 20 pA (11). However, OHCs in the basal (high
frequency) region of the cochlea may have to cope with five-fold larger currents through MET channels (34,35). Other things being equal, the larger Ca\(^{2+}\) load could exceed the extrusion capacity of the PMCA2 in these cells. Therefore, mutations that decrease this capacity might completely disrupt Ca\(^{2+}\) homeostasis, with the basal turn of the cells more severely affected than the apex, and with OHCs more affected than IHCs, as found in both the Oblivion and Tommy mice. A diminished Ca\(^{2+}\) removal from the cells is also expected to affect the MET currents. Indeed, the pharmacological blockade of the PMCA2 pump shifted the current-displacement (I-X) curve in the positive direction and reduced its slope considerably (11). Similar effects have been reported by mutation or knock out of the PMCA2 isoform of the plasma membrane Ca\(^{2+}\) pump (23).

The only cochlear PMCA2 exposed to endolymph is that of the stereocilia (36,37). Thus, if less Ca\(^{2+}\) is exported from the stereocilia, as occurs in the PMCA2 mutants discussed here, its concentration in the endolymph is expected to fall (37). This may provide a clue as to why, in some cases, mutations in the gene of the PMCA2 pump potentiated the deafness phenotype induced by coexisting mutations of cadherin-23 (USH1D), a single pass membrane Ca\(^{2+}\) binding protein that is abundantly expressed in the stereocilia. Cadherin-23 has a prolonged extracellular portion exposed to the endolymph that contains 27 cadherin (Ca\(^{2+}\) binding) domains (38). Its homodimers interact in trans with homodimers of protocadherin-15 (USH1F), another cadherin that has 15 external cadherin domains (39). Both cadherins are defective in Usher syndrome type I (sensorineural deafness and blindness due to retinitis pigmentosa). The two cadherins interact together and form transient lateral links interconnecting the stereocilia and anchor to the stereocilia actin filaments (40), which provide the initial cohesion of the developing hair bundle. After completion of hair bundle morphogenesis, cadherin-23 and protocadherin-15 form the upper and lower parts of the tip link, respectively, i.e., the molecular machinery of the MET complex is anchored to the cytoplasmic region of protocadherin-15 (39,41).

Recent work (38) has solved the 3D structure of the first two repeats of cadherin 23, which show typical cadherin folds with an elongated N-terminus which contributes to forming the N-terminal Ca\(^{2+}\) binding site. The interaction of Ca\(^{2+}\) with binding sites in cadherin-23 is critical to the function of the tip links. A reduced concentration of endolymphatic Ca\(^{2+}\) has been found to affect the degree of their stiffness, which is required for the opening of the MET channels, even possibly leading in extreme cases to their disappearance (42). The Ca\(^{2+}\) affinity of the cadherin-23 fragment, estimated from models with three or four Ca\(^{2+}\) binding sites, corresponded to K\(_{D}\)S increasing from 1.9 to 71.4 µM, i.e., in the range of the Ca\(^{2+}\) concentration in the endolymph. Molecular dynamics simulations suggested the repeats to be stiff: removal of Ca\(^{2+}\), which is known to abolish the MET currents (42,43), reduced the rigidity, which was also decreased by reducing the affinity of the binding sites for Ca\(^{2+}\). This occurred in the deafness-inducing cadherin-23 mutant D101G, which was modeled using three Ca\(^{2+}\) binding sites, and in which the K\(_{D}\)S for Ca\(^{2+}\) increased from 3.9 µM to more than 100 µM. The degree of rigidity and the unfolding strength of cadherin 23 were thus critically sensitive to the concentration of Ca\(^{2+}\) in the environment i.e. in the endolymph, whose value in the bulk is poised at ~20 µM by the Ca\(^{2+}\) export function of the PMCA2 pump (higher values, up to about 50 µM, might however be present in the immediate proximity of the MET channels) (44).

The cooperation of the cadherin-23 and the PMCA 2 pump is evidently critical in the molecular events that ultimately permit the neural encoding of the acoustic signal. It is thus easy to appreciate the importance of their mutations in the generation of the hearing loss phenotype: indeed, a G753A polymorphism of cadherin-23 was detected in the original deafwaddler mice (45). Furthermore, in one human family a homozygous mutation in cadherin-23 (F1888S) caused the hearing loss in five siblings whereas a co-existing heterozygous PMCA2 pump mutation (V586M in the z/b nomenclature) was associated with increased loss in the three most severely affected siblings (27). In another human family in which both parents had normal hearing, a T→S substitution at position 1999 was detected in the cadherin-23 of the father but not in that of the mother, who presented instead with the G293S mutation of the PMCA2 pump. It is noteworthy that the G293S mutation of PMCA2 involved the same couple of residues of the original
deafwaddler mutation, and occurred ten residues downstream of the mouse residue. One son, affected by severe bilateral hearing impairment without vestibular involvement, inherited both mutations, whereas a brother with normal hearing was negative for the G293S mutation like the father (23). The link between cadherin 23 and the PMCA2 pump, therefore, is important. However, it is not obligatory, as shown by the results of the Tommy mutant described here, and those on the Oblivion mutant (25). Evidently, the biochemical defect of the homeostasis of Ca$^{2+}$ in the stereocilia of OHCs has different degrees of severity. Homozygous cadherin-23 mutations that impair the ability of the protein to bind Ca$^{2+}$, as detected in one of the human families described (27), may be sufficient to disrupt the opening properties of the MET channels, generating the hearing loss phenotype, which is then only exacerbated by the concomitant PMCA2 pump mutation. On the other hand, homozygous PMCA2 pump mutations, as was the case for the Oblivion mutant, and is the case for the Tommy mutant, may per se be sufficient to decrease the Ca$^{2+}$ concentration in the endolymph to a degree that would affect the function of the otherwise normal cadherin-23 in the tip links, and to generate the hearing loss phenotype, without the contribution of cadherin-23 mutations.

The Tommy mutation (E584K in the z/b nomenclature) affects a highly conserved residue located in the second intracellular loop of the pump only two residues upstream the site of the conservative mutation reported in (23). Based on the nearly identical position in the sequence (both residues are located in the domain that binds ATP), it could have been expected that the mutations would have the same effect on the hearing phenotype. However, the character of the replacement in the Tommy mutation (E/K) is much more dramatic than the human V586M conservative substitution. In principle, thus, it could be expected that the defect of the pump in the Tommy mutant would be more severe. The effect of the human V586M mutation on the PMCA2 pump has only been defined as a decrease of the pumping activity, although molecular modeling of the V586M replacement on a SERCA pump template had suggested structural disturbances of the ATP-binding site by the presence of the sterically larger methionine side chain. It would thus be useful to analyze the V586M in the same molecular detail as the Tommy mutant.

The results presented here, showing a reduced Ca$^{2+}$-pumping ability of the Tommy mutant PMCA2, and the discussion of the PMCA2 and cadherin-23 mutants, underline the importance of the concerted functioning of the two molecules for the neural encoding of acoustic stimuli. They show that the special properties of the variant of the PMCA2 pump of the stereocilia are a critical factor: the ability of the pump to export Ca$^{2+}$ at a constant level essentially irrespective of the presence of activators, e.g., calmodulin, which is present in the stereocilia (46) at a concentration of ~70 µM (47), ensures that the endolymph will be continuously replenished with Ca$^{2+}$ to compensate for the (minor) amount that enters into the stereocilia. This in turn ensures the correct functioning of the tip-links-MET channel machinery. The discussion has concentrated on the interplay between the PMCA2 pump and cadherin 23: possibly, however, PMCA2 pump defects could have much wider importance: i.e., they could act as modifiers of other hearing loss phenotypes, genetic and/or environmental, characterized by pathological processes affecting hair cells (27).

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**FIGURE LEGENDS**

**Figure 1.** Hearing thresholds measured by the ABR technique. Representative ABR recordings in response to click stimuli from wild type (postnatal day 31, black traces), heterozygous (postnatal day 27, blue traces) and homozygous (postnatal day 24, red traces) *Tommy* mice.

**Figure 2.** ABR audiograms for click and tone bursts at 8-14-20-26-32 kHz obtained from wild type (black line), heterozygous (blue line) and homozygous (red line) *Tommy* mice, aged between P18 and P45. Bars represent standard deviation. Note that click responses are plotted at an arbitrary point on the frequency axis (the position does not reflect the frequency content of click stimuli).

**Figure 3.** Sequence alignment of several PMCA family members showing E584 is highly conserved. Similarity analysis was performed using the ClustalW program. Human PMCA2 sequence is listed (GenBank accession number NP_001674) with other PMCAs sequences from other species (A) and with those of other P-type human ATPases (B) GenBank accession numbers are listed: AY928176 (Rhesus Macaque, PMCA4), AAV75643 (Mus musculus, PMCA2), BC109173 (Mus musculus, PMCA4); NP_036640 (Rattus norvegicus, PMCA2), XP_509257 (Pan troglodytes, PMCA1), NP_777121 (Bos Taurus, PMCA1), NP_999517 (Sus scrofa, PMCA1), Q00804 (Oryctolagus cuniculus, PMCA1), AAK11272 (Rana catesbeiana, PMCA1), AAH77905 (Xenopus laevis, PMCA3), EU559285 (Dario rerio, PMCA4); AAR28532 (Procambarus clarkia, PMCA3), AA66551 (Caenorhabditis elegans, PMCA3); XP_653525 (Entamoeba histolytica), EAL62716 (Dictyostelium discoideum), NP_001001323 (PMCA1), NP_068768 (PMCA3), NP_001675 (PMCA4), NP_004311 (SERCA1), NP_737765 (SERCA2), NP_77715 (SERCA3), AAP35375 (SPCA1), NP_000693 (Na+/K+ ATPase) and AAH31609 (H+/K+ ATPase).

**Figure 4.** Expression and immunolocalization of recombinant PMCA2w/a pumps in CHO cells. (A) Western blotting analysis; The band of ~130 kDa corresponds to PMCA2 and the band of ~42 kDa to β-
actin and (B) densitometric analysis of relative expression of wild type PMCA2w/a and PMCA2w/a harboring the Tommy mutation. The amounts of pump protein was normalized for β-actin. (C) Immunolocalization of wild type (left) and mutant (right) PMCA2w/a in transiently transfected CHO cells. The interaction with the 2N antibody was probed by the AlexaFluor488-conjugated secondary antibody. (D) Fluorescence level in the plasma membrane quantified as described in Experimental Procedures. Bars represent standard deviation.

Figure 5. Activity of recombinant PMCA2 pumps in CHO cells. (A) Cells were transiently either co-transfected with the PMCA2 variants and cytAEQ, or with cytAEQ alone (control). They were then perfused with KRB supplemented with 1 mM CaCl2. 100 μM ATP was used to produce a transient cytosolic Ca2+ increase. (B-C) Histograms show the means of Ca2+ peaks and the half peak decay times, respectively (n indicates the number of experiments considered for the statistical analysis) * p<0.01 calculated with respect to the wild type pump. Bars represent standard deviation.

Figure 6. Activity of native PMCA2w/a pumps in vestibular hair cells. (A) Utricle fixation to the lateral side of a glass capillary permits to observe hair cells along their main axis, thus to better estimate [Ca2+]i, changes both in the hair cell stereocilia and body compartments. (B) UV laser light (375 nm), controlled by a transistor–transistor logic (TTL) signal generated by a computer connected to the Bio-Rad 2100 scanning system, was delivered for 60 ms to elicit a homogeneous [Ca2+]i increase in an area covering a few hair cells. (C) Fluo-4 absolute fluorescence f, peaked at 515 nm, is shown green color-coded (scale bar 5 μm). The averaged f in the stereociliary compartment (bounded by the red line) was used to compute the [Ca2+]i change parameter Δf/f0. (D) A representative Δf/f0 stereociliary time course (UV flash delivered at time indicated by the violet arrow) is shown for a wild type Tommy mouse. The decay time constant τ of the single-exponential curve (red dashed line) fitting the Δf/f0 trace after the UV flash was used to quantify Ca2+ clearance rate from the stereocilia. (E) The experiments were selected in order to have similar Δf/f0 transients (left panel). In wild type (WT) mice, the recovery to baseline Ca2+ was faster (right panel, τ = 2.78 ± 0.27 s, mean ± standard error of the mean, n=11 from 3 mice) than in the heterozygous (τ = 3.80 ± 0.80 s, n=11 from 3 mice; *p=0.21 in the ANOVA test) and in the homozygous Tommy mice (τ = 4.47 ± 0.87 s, n=18 from 4 mice; **p=0.12 in the ANOVA test).

Figure 7. PMCA2 immunolabeling in neonatal utricle. Confocal imaging immunoassays of PMCA2 (stained by Alexa 488 conjugated, green; scale bar 10 μm) in P7 utricle cultures from wild type, heterozygous and homozygous Tommy mice (for details see Experimental Procedures). Membranes (red) are stained by FM4-64FX while nuclei (blue) by DAPI. Scale bar 15 μm.

Figure 8. PMCA2 immunolabeling in P60 cochlea whole mounts. Horizontal sections (orthogonal to the modiolus) of cochlea whole mounts from P60 wild type (left column) and homozygous (right column) Tommy mice. Images from apical, medial and basal regions are obtained by maximal back-projection of 20 confocal optical sections from a 2 μm step z–stack of wild type and homozygous Tommy mice. PMCA2 expression was probed by a PMCA2 selective antibody (Alexa 488 conjugated, green) and nuclei were stained with DAPI (blue). Scale bar: 15 μm.

Figure 9. PMCA2 immunolabeling in basal cochlear transversal sections (parallel to the modiolus) of P60 Tommy mice. Images were obtained by maximal back-projection of 2 to 6 confocal optical sections from a 1 μm step z–stack of wild type (top panel) and homozygous (bottom panel) Tommy mice. Actin filaments were stained with phalloidin (Texas Red conjugated, red) and nuclei with DAPI (blue). Scale bar 15 μm.
Fig. 6

A. Top view
- Utricle
- Glass capillary
- Coverslip

B. Scan system (Bio-Rad 2100)
- Frame start signal
- Fiber optics
- UV beam
- 488 nm
- 528 nm
- 400 DCLP dichroic
- 60× WI objective
- UV spot

C. Side view
- UT B
- WI objective
- UV Laser 375 nm
- UV spot

D. Single exponential fit
- \( y = a \exp\left(-\frac{t}{\tau}\right) \)

E. Graphs
- \( \Delta f/f_0 \) peak
  - WT
  - Hetero
  - Homo
- \( \tau \) (s)
  - WT
  - Hetero
  - Homo

Note: The graphs show data for different conditions, with error bars indicating variability.
Fig. 7

PMCA2

Membranes

Merge +
nuclei

WT

Hetero

Homo
WT
Nuclei/PMCA2/Actin

Homo
Nuclei/PMCA2/Actin

Fig. 9
The novel PMCA2 pump mutation Tommy impairs cytosolic calcium clearance in hair cells and links to deafness in mice
Mario Bortolozzi, Marisa Brini, Nick Parkinson, Giulia Crispino, Pietro Scimemi, Romolo Daniele De Siati, Francesca Di Leva, Andrew Parker, Sai da Ortolano, Edoardo Arslan, Steve D. Brown, Ernesto Carafoli and Fabio Mammano

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