Genetic Inactivation of Trpml3 Does Not Lead to Hearing and Vestibular Impairment in Mice

Simone Jörs*, Christian Grimm*, Lars Becker, Stefan Heller*

Departments of Otolaryngology – Head and Neck Surgery and Molecular and Cellular Physiology, Stanford University School of Medicine, Stanford, California, United States of America

Abstract

TRPML3, a member of the transient receptor potential (TRP) family, is an inwardly rectifying, non-selective Ca\(^{2+}\)-permeable cation channel that is regulated by extracytosolic Na\(^{+}\) and H\(^{+}\) and can be activated by a variety of small molecules. The severe auditory and vestibular phenotype of the TRPML3(A419P) varitint-waddler mutation made this protein particularly interesting for inner ear biology. To elucidate the physiological role of murine TRPML3, we conditionally inactivated Trpml3 in mice. Surprisingly, lack of functional TRPML3 did not lead to circling behavior, balance impairment or hearing loss.

Introduction

The mammalian TRPML gene family consists of TRPML1, TRPML2, and TRPML3. Mutations in human TRPML1 cause the lysosomal storage disease Mucolipidosis Type IV [1–3]. The murine Trpml2 and Trpml3 genes were identified in a positional cloning approach to find the gene responsible for the varitint-waddler (Va) phenotype [4]. Heterozygote Va mice have pigmentation defects, hearing loss, and circling behavior, whereas homozygotes display perinatal lethality [4–6]. The Va phenotype is caused by a single mutation (A419P) in the predicted fifth transmembrane-spanning domain (TM5) of TRPML3, which leads to a constitutively open channel, resulting in elevated [Ca\(^{2+}\)]

Results and Discussion

Generation of a floxed Trpml3 allele (Trpml3\(^{lox}\))

TRP channel knockouts have successfully been generated through targeted deletion of a genomic region encoding the presumptive pore-loop domain of the ion channel [18–20]. Therefore, we decided to target exon 11 of Trpml3, which encodes the pore-loop and the pore lining TM6 (Fig. 1A). We generated a targeting vector that, after homologous recombination, resulted in a modified Trpml3 allele carrying two loxP sites flanking exon 11 (Fig. 1B). A neo\(^{R}\) cassette, which was used for G418/geneticin selection, was removed with Flp recombinase before the ES cells were injected into host blastocysts to generate chimeric mice (Fig. 1C). After germline transmission and continued breeding, PCR with genomic DNA from progeny of wild-type, heterozygous and homozygous animals showed proper recombination and inheritance of the Trpml3\(^{lox}\) allele (Fig. 2A,B). Exon 11 of the Trpml3\(^{lox}\) allele and the genomic sequences surrounding the recombination site were verified by sequencing.

Excision of Trpml3 exon 11

To investigate whether TRPML3 plays a role in development, we used the X-linked Hprt\(^{lox}\)/Hprt\(^{lox}\) driver, which mediates ubiquitous and highly efficient excision that is complete at the first stages of development [21]. Hprt\(^{lox}\)/Trpml3\(^{lox}\)/Hprt\(^{lox}\) females were mated with Trpml3\(^{lox}\)/Hprt\(^{lox}\) males. The floxed exon 11 was always excised...
regardless of \textit{Hprt}\textsuperscript{Cre} inheritance. Oocytes of \textit{Hprt}\textsuperscript{Cre} females have sufficiently stored Cre recombinase to excise floxed DNA segments already at the zygote or early stages [21], which most likely resulted in \textit{Trpml3} inactivation already in the fertilized egg. The resulting \textit{Trpml3}\textsuperscript{D} mutation was stably inherited (Fig 2C–E).

To examine whether the \textit{Trpml3}\textsuperscript{D} allele is being transcribed, RT-PCRs were conducted (Fig. 2D,E). mRNA from kidney and inner ear was purified from dissected organs of mice of each genotype. RT-PCR amplification of specific cDNA sequences before and after the site of deletion indicated that wild-type and \textit{Trpml3}\textsuperscript{D} mRNA was expressed in kidney and the inner ear of all animals (Fig. 2D,E; RT-2 and 3). Amplification of cDNA encoding exon 11 (RT-1), however, was only possible from mRNA of wild-type (\textit{Trpml3}\textsuperscript{+/+}) and heterozygous animals (\textit{Trpml3}\textsuperscript{A/+}), but not from mRNA of \textit{Trpml3}\textsuperscript{A/A} mice.

An additional oligonucleotide pair was used (RT-4) to amplify the coding region comprising exons 10, 11, and 12. Sequence analysis revealed that in mutated animals, splicing occurred between exon 10 and exon 12, resulting in a 207 bp shorter message encoding a protein lacking 69 amino acids of the pore loop and TM6, but without frame shift. To demonstrate that a pore-less TRPML3 protein does not function as an ion channel, we generated cDNA encoding a fusion protein of TRPML3(\textit{D} exon11) with yellow fluorescent protein and expressed the mutant channel protein in HEK293 cells. The subcellular distribution of the mutant channel TRPML3(\textit{D} exon11), analyzed by confocal

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{targeting_strategy.png}
\caption{Targeting strategy for disruption of the \textit{Trpml3} gene. A, Transmembrane-spanning domains are depicted as grey bars and numbered from 1–6. The orange frame indicates the part of the TRPML3 protein that is encoded by exon 11, which will be deleted (pore loop and TM6). Exons are shown as black and orange bars on the schematic genomic map below. B, Shown are the targeting vector and the targeted allele after homologous recombination. The blue bar represents the PGK promoter-driven neo\textsuperscript{R} expression cassette, which was used for positive selection. The DTA cassette, used for negative selection, is shown in green. The black and red arrowheads symbolize position and orientation of FRT and loxP sites. C, Targeted allele after Flp site-specific recombination in ES cells, resulting in excision of neo\textsuperscript{R} cassette, and leaving one FRT site behind. D, Excision of exon 11 using Cre site-specific recombinase, resulting in disruption of \textit{Trpml3} gene.
\label{targeting_strategy}
\end{figure}
**Figure 2. Genotyping analysis.** A, Schematic drawing of the Trpml3 targeted allele before (Trpml3^lox^) and after Cre recombination (Trpml3^Δ^). The positions of sense (sn) and antisense (asn) oligonucleotides are indicated with arrows. The names of PCR products and the corresponding lengths are parenthesized. The names of PCR products and the corresponding lengths are parenthesized. B, PCR amplification products of Trpml3 locus of homozygous (lox/lox), heterozygous (lox/+), and wild-type (+/+ ) Trpml3^lox^ mice are shown: lox: 309 bp and/or 471 bp, neo: 374 bp, and left arm (LA): 3176 bp and right arm (RA): 3298 bp. C, Genotyping after Cre recombination of representative Hprt^Cre/;Trpml3^Δ^ mice. All mice are heterozygous for cre (210 bp). The same sets of oligonucleotides were used as in (B), however lox-PCR only displayed the wild-type 309 bp PCR fragment, since the lox asn-oligonucleotides cannot hybridize after the targeted exon 11 was excised; and RA: 2340 bp, since exon 11 was excised by Cre. The last panel on the right shows the shortened fragment (indicated with a red arrow): 1952 bp.
mice, and HprtCre/;Trpml3 Δ/Δ (Fig. 3A,B). Interverwave latencies between wave I and wave III at 70 dB, which are indicative of the afferent auditory nerve conductance were 1.9±0.026 msec in HprtCre/+; Trpml3 Δ/+ animals (n = 6), compared to 1.87±0.026 msec in HprtCre/+; Trpml3 Δ/+ (n = 6), and 1.86±0.019 msec in HprtCre/+; Trpml3 Δ/Δ littermates (n = 6). These results suggested that TRPML3 is not essential for hair cell function, synaptic signal transmission, performance of spiral ganglion neurons, and auditory nerve function. Nevertheless, because the inactivation of Trpml3 using HprtCre−mediated recombination was introduced very early in development, compensation effects cannot be excluded with this approach.

With the goal to circumvent potential compensatory mechanisms, we used tamoxifen-inducible Math1-CreER™ mice [24] to inactivate TRPML3 in cochlear hair cells between P0–P3. ABR measurements of these mice at three weeks of age revealed no differences (Fig. 3C), suggesting that compensatory mechanisms most likely do not explain the lack of a TRPML3 hearing phenotype. Mice were also analyzed at 3 months of age to determine possible enhancement or early onset of age-related hearing loss. But reviewing audiograms revealed no ABR threshold difference between mutant mice and control mice (data not shown). These experiments have two important limitations. First, the tamoxifen-inducible recombination in cochlear hair cells is not complete, as revealed by analysis of crosses of Math1-CreER™ mice with Rosa26-lacZ reporter mice [24]. Therefore, a substantial number of cochlear hair cells might have been unaffected by Cre-mediated recombination. The fact that mutant TRPML3 protein is being expressed (Fig. 3A) complicates the analysis of Cre-mediated recombination because the mutant pore-less TRPML3 protein is likely to be detectable by immunohistochemistry. Second, three weeks of loss of TRPML3 function might be enough for potential compensatory mechanisms to become effective. Nevertheless, as long as there are no clear candidates or...
mechanisms known that could provide compensation for inactivation of Trpml3, it is difficult to speculate about the timing of compensatory mechanisms. In summary, the use of Math1-CreER<sup>TM</sup> mice did not reveal potential compensatory mechanisms of Trpml3 inactivation, but because of the limitations of this experiment, we cannot exclude that loss of TRPML3 function is being compensated by an unknown mechanism.

To investigate whether acoustic challenge of the auditory system would reveal a more subtle role of TRPML3, we exposed Hprt<sup>Cre/+; Trpml3<sup>+/-</sup></sup> and Hprt<sup>Cre/+; Trpml3<sup>Δ/Δ</sup></sup> mice for 4 hr to 4 kHz pure tone at 125 dB SPL [25,26]. We compared ABR thresholds of littermates of both genotypes before and one week after the noise exposure, and we detected no difference in noise susceptibility between the two groups (Fig. 4D). Unlike knockout of TRPV4, an ion channel expressed by cochlear hair cells, spiral ganglion neurons, and stria vascularis marginal cells [27], which displays increased susceptibility to acoustic injury [25], mice carrying two inactive Trpml3 alleles did not show increased acoustic vulnerability.

Besides evaluating the auditory system, we also assessed vestibular function in Hprt<sup>Cre/+; Trpml3<sup>+/-</sup></sup> and Hprt<sup>Cre/+; Trpml3<sup>Δ/Δ</sup></sup> mice.

**Figure 4. ABR measurements.** A, Graph shows representative ABR waveforms of 3-week-old Hprt<sup>Cre/+; Trpml3<sup>+/-</sup></sup> and Hprt<sup>Cre/+; Trpml3<sup>Δ/Δ</sup></sup> mice in response to a click stimulus. ABRs were recorded at sound stimulation intensities of 25–70 dB. ABR waves I–V are indicated above the peaks. Red arrow highlights the hearing threshold, which is at 35 dB in this representative example pair. B, Shown are ABR thresholds (mean values ± SEM) to click, 8-, 16-, and 32 kHz stimuli of Hprt<sup>Cre/+; Trpml3<sup>+/-</sup></sup> (n = 6), Hprt<sup>Cre/+; Trpml3<sup>Δ/Δ</sup></sup> (n = 6), and Hprt<sup>Cre/+; Trpml3<sup>Δ/Δ</sup></sup> (n = 6) and C, of Math1-CreER<sup>Cre/+; Trpml3<sup>+/-</sup></sup> (n = 3), Math1-CreER<sup>Cre/+; Trpml3<sup>Δ/Δ</sup></sup> (n = 3), and Math1-CreER<sup>Cre/+; Trpml3<sup>Δ/Δ</sup></sup> (n = 3), respectively. Statistical comparisons of means of different genotypes were made using one-way ANOVA followed by Tukey’s post test; p < 0.05. D, ABR threshold shifts of 3-month-old Hprt<sup>Cre/+; Trpml3<sup>+/-</sup></sup> (n = 7) and Hprt<sup>Cre/+; Trpml3<sup>Δ/Δ</sup></sup> (n = 7) 1 week after the acoustic overexposure of 125 dB SPL at 4 kHz for 4 hr. Shown are mean values ± SEM. No significant differences were observed between genotypes (p > 0.05, one-way ANOVA, followed by Tukey’s post test).

doi:10.1371/journal.pone.0014317.g004
mice. We did not notice circling behavior, head-bobbing, waddling, or imbalance when walking or when walking along the top of the 3 mm thin cage wall, suggesting normal vestibular function (data not shown). To obtain more objective and quantitative data on potential balance deficits, we performed Rotarod tests [28]. We did not measure a significant difference between HprtCre/+;Trpml3+/+ and HprtCre/+;Trpml3A/Δ mice for the 6-day-period tested (Fig. 5), indicating that inactivation of Trpml3 does not lead to balance defects in this test.

Despite the strong Va phenotype, which is caused by constitutively active TRPML3, no overt inner ear phenotype was detectable in mice with ubiquitous Trpml3 inactivation or when the gene was inactivated from P0–P3 onwards. This finding supports the previous conclusion that the Trpml3A/+ mutation of Va mice is a gain-of-function mutation, which has been hypothesized to cause Ca2+-loading of cells and subsequently apoptotic cell death [7–11]. Loss of functional Trpml3, on the other hand, is not causing a detectable phenotype in the inner ear. Moreover, HprtCre/+;Trpml3A/Δ mice were indistinguishable from their wild-type littermates in size and weight. Trpml3A/Δ mice inherited the mutation stably and homozygous mutant animals displayed normal breeding performance.

We suggest several possible explanations for the lack of a phenotype in Trpml3A/Δ mice. The first possibility is that Trpml3 is not essential for inner ear function and that in Va mice, the channel protein is turned into a constitutively active and cytotoxic channel. This would mean, however, that the native Trpml3 channel does not fulfill essential detectable functions in the inner ear in vivo. Another possibility is that our tests were not sufficient to reveal more subtle roles of Trpml3 such as potential modulatory roles in hair cells or other parts of the auditory and vestibular system.

Finally, it is a distinct possibility that native TRPML3 proteins are dispensable subunits of heteromeric channels. A different TRP channel, such as the related TRPML2 might compensate for loss of TRPML3. Heteromerisation of TRPML channels has been shown previously [12,15]. This hypothesis is supported by the observation that a dominant negative isoform of TRPML3, when transfected into epidermal melanocytes, is able to inhibit activation of a presumptively heteromeric channel consisting of TRPML3 and other unknown subunits [13]. Such a mechanism is potentially testable by generation of mice with inactivating mutations in Trpml3 and one or more additional genes that encode potential heteromeric subunits. This strategy might ultimately reveal the physiological function of TRPML3-containing channels in sensory hair cells and other cell types of the body.

Materials and Methods

Targeting construct

Genomic 129sv/svJ mouse DNA was used for PCR-amplification of DNA sequences flanking exon 11 of Trpml3 (Fig. 1). pBluescript II SK(+) vector (Stratagene); Left arm (SfiI), FRT-flanked neomycin resistance marker (neoR) (HindIII), loxP-flanked Intron-Exon11-Intron (EcoRI), right arm (SmaI), and Diphtheria toxin A (DTA) cassette (Sphl and Sadl). The sequence-verified and linearized targeting vector DNA was electroporated into 129sv/svJ ES cells (Stanford transgenic research center (http://med.stanford.edu/transgenic)) and two independent clones with proper homologous recombination were selected by PCR amplification and sequencing of the integration sites. The neoR-cassette was excised with Fpl recombinase and the ES cells carrying the targeted Trpml3 allele were injected into blastocysts to generate chimeras. Mating of male chimeras with C57BL/6J females produced heterozygous transgenic founders that were crossed and maintained in FVB/NJ background, which was selected for analysis because this strain does not exhibit early onset age-related hearing loss [29].

Mice and Genotyping

FVB/NJ mice (Jackson Laboratory stock number 001800), Math1-CreERT2TM mice (provided by Dr. S.J. Baker, St. Jude, Memphis, TN), and HprtCreERTTM mice (provided by Dr. M. Krasnow, Stanford University) were used. Both driver strains were crossed for at least ten generations into FVB/NJ background before crossing them with mice carrying the Trpml3lox allele. Trpml3lox mice had been crossed into FVB/NJ background for at least three generations. Genomic DNA was isolated from mouse tails (DNEasy, Qiagen, Valencia, CA). Animal studies were conducted in accordance with protocols approved by the Administrative Panel on Laboratory Animal Care at Stanford University. The protocol number is #11961.

Induction of Cre activity

Tamoxifen (Sigma) was dissolved in prewarmed sterile corn oil (Sigma) at a concentration of 3 mg/ml. A 26-gauge needle insulin syringe was used for intraperitoneal injections between P0 and P3 at 3–4 mg/40 g body weight. Three injections were separated by 24 hours.

Heterologous expression of TRPML3 isoforms and calcium imaging

HEK293 cells were grown and maintained in a standard humidified 37°C incubator, with 95% air and 5% CO2. The cells were maintained in DMEM (Cellgro), supplemented with 10% fetal bovine serum (Omega scientific), and 100 μg/ml penicillin and streptomycin (Cellgro). For calcium imaging experiments and localization studies all plasmid constructs were transiently expressed in HEK293 cells with the use of Genejammer (Stratagene), and analyzed 24 hr after transfection. Expression vectors were based on

Figure 5. Rotarod experiments. The average latencies to fall ± SEM (in sec) are shown for 3-month-old HprtCre/+;Trpml3+/+ and HprtCre/+;Trpml3A/Δ (n = 8) mice. The experiment was executed over a time range of 6 days. The day 6 experiment was performed in the dark to exclude compensation via visual cues. The difference between genotypes was not statistically significant at any given time point (p>0.05, one-way ANOVA, followed by Tukey’s post test).

doi:10.1371/journal.pone.0014317.g005
pcDNA3.1 (Invitrogen). For calcium imaging experiments, the cells were loaded with 4 μM fura-2-AM (Invitrogen) in a solution containing 130 mM NaCl, 6 mM KCl, 2 mM MgCl₂, 2 mM CaCl₂, 10 mM HEPES, and 5.5 mM D-glucose (300 mOsmol/kg and adjusted to pH 7.4 with NaOH). Measurements of [Ca²⁺], with this fluorescent indicator were performed using a monochromatic-based imaging system (iMIC platform and Polychrome V monochromator, TILL Photonics). TRPML3 activator SN-2 (5-mesityl-3-oxa-4-azatricyclo[5.2.1.0²,6]dec-4-ene) was used at a final concentration of 10 μM [13].

Auditory brainstem response (ABR) measurements and noise exposure

The ABR procedure was done as previously described [11]. The absolute threshold was obtained for each animal by reducing the stimulus intensity in 5 dB steps to identify the lowest intensity at which the first ABR wave was detectable. The latency (time delay from when the stimulus is presented (0 msec) until its peak occurs) was determined after detecting all peaks in click ABR waveforms from when the stimulus is presented (0 msec) until its peak occurs) was determined after detecting all peaks in click ABR waveforms I-V at 70 dB. Interwave latencies between the peaks of ABR waves I and III were calculated. Three months old Hprt+/−, Ttpml3+/− and Hprt+/−, Trpml3 Δ/Δ mice were exposed 4 hr to 100 dB SPL (sound pressure level) in a truncated pyramid-shaped exposure box [26] to examine the effects of TRPML3 inactivation on acoustic injury of the cochlea. Shifts in the ABR thresholds were determined after 1 week.

Motor coordination tests

A four track Rotarod (Columbus Instruments) was used to test for a balance or motoric impairment in 3 months old Hprt+/− mice.

References

1. Bargal R, Avidan N, Ben-Asher E, Orleneder Z, Zeigler M, et al. (2000) Identification of the gene causing mucolipidosis type IV. Nat Genet 26: 118-123.
2. Sun M, Goldin E, Stahl S, Falardeau JL, Kennedy JC, et al. (2000) Identification of the gene causing mucolipidosis type IV. Nat Genet 26: 118-123.
3. Di Palma F, Belyantseva IA, Kim HJ, Vogt TF, Kachar B, et al. (2002) Mutations in Mcoln3 associated with deafness and pigmentation defects in varitint-waddler (Va) mice. Proc Natl Acad Sci U S A 99: 14994-14999.
4. Cable J, Steel KP (1998) Combined cochleo-saccular and neuroepithelial abnormalities in the Varitint-waddler-J (VaJ) mouse. Hear Res 123: 125-136.
5. Kim HJ, Jackson T, Nohen-Traut K (2003) Genetic analyses of mouse deafness mutation Varitint-waddler (Va) and Jerker (Espl). J Assoc Res Otolaryngol 4: 83-90.
6. Grimm C, Cuajungco MP, van Aken AF, Schnee M, Jors S, et al. (2007) A helix-breaking mutation in TRPML3 leads to constitutive activity underlying deafness in varitint-waddler mice. Proc Natl Acad Sci U S A 104: 19583-19588.
7. Kwan KY, Allchorne AJ, van MA, Christensen AP, Zhang D, et al. (2006) TRPA1 contributes to cold, mechanical and chemical nociception but is not essential for hair-cell transduction. Neurosci 59: 277-289.
8. Bautista DM, Jorde SE, Nikai T, Tsuruda PR, Read Aj, et al. (2006) TRPA1 mediates the inflammatory action of environmental irritants and pruridogenic agents. Cell 124: 1269-1282.
9. Moriquich A, Wook Hwang S, Earley TJ, Petrus MJ, Murray AN, et al. (2005) Impaired thermosensation in mice lacking TRPV3, a heat and camphor sensor in the skin. Science 307: 1468-1472.
10. Tang SHE, Silva FJ, Tsark WMK, Mann JR (2002) A Cre/Iso-Pedeleter transgenic line in mouse strain 129S1/SvImJ. Genesis 32: 199-202.
11. Cuajungco MP, Grimm C, Heller S (2007) TRP channels as candidates for hearing and balance abnormalities in vertebrates. Biochimica et Biophysica Acta 1772: 1022-1027.
12. Jero J, Coling DE, Lalwani AK (2001) The use of Preyer's reflex in evaluation of hearing in mice. Acta Oto-Laryngologica 121: 585-589.
13. Chow LML, Tian Y, Weber T, Corbett M, Zuo J, et al. (2006) Inducible Cre recombinase activity in mouse cerebellar granule cell precursors and inner ear hair cells. Dev Dyn 235: 2991-2998.
14. Tabuchi K, Suzuki M, Muzuno A, Hara A (2005) Hearing impairment in TRPV4 knockout mice. Neurosci Lett 382: 304-308.
15. Liberman MC, Gao WY (1995) Chronic cholecalciferol deficiency and possibility to persistent acoustic hearing. Audiol Res 90: 158-166.
16. Liethke W, Choi Y, Martin-Reemdonn MA, Bell AM, Dennis CS, et al. (2000) Vanilloid receptor related osmotically activated channel (VR-OAC), a candidate vertebrate osmoreceptor. Cell 101: 525-535.
17. Jones BJ, Roberts DJ (1968) The quantitative measurement of motor inco-ordination in naive mice using an accelerating rotarod. J Pharm Pharmacol 20: 302-304.
18. Zhong YQ, Johnson KR, Erway LC (1999) Assessment of hearing in 80 inbred strains of mice by ABR threshold analyses. Hear Res 130: 94-107.