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RESEARCH ARTICLE

Altered expression of securin (Pttg1) and serpina3n in the auditory system of hearing-impaired *Tff3*-deficient mice

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Abstract
Introduction *Tff3* peptide exerts important functions in cytoprotection and restitution of the gastrointestinal (GI) tract epithelia. Moreover, its presence in the rodent inner ear and involvement in the hearing process was demonstrated recently. However, its role in the auditory system still remains elusive. Our previous results showed a deterioration of hearing with age in *Tff3*-deficient animals.
Results Present detailed analysis of auditory brain stem response (ABR) measurements and immunohistochemical study of selected functional proteins indicated a normal function and phenotype of the cochlea in *Tff3* mutants. However, a microarray-based screening of tissue derived from the auditory central nervous system revealed an alteration of *securin* (*Pttg1*) and *serpina3n* expression between wild-type and *Tff3* knock-out animals. This was confirmed by qRT-PCR, immunostaining and western blots.
Conclusions We found highly down-regulated *Pttg1* and up-regulated *serpina3n* expression as a consequence of genetically deleting *Tff3* in mice, indicating a potential role of these factors during the development of presbyacusis.

Keywords Trefoil peptides · Cochlea · Inferior colliculus · Presbyacusis · Expression patterns

Abbreviations

ABR	Auditory brain stem responses
GI	Gastrointestinal
huACT	Human antychymotrypsin
muACT	Murine antychymotrypsin
IC	Inferior colliculus
IBD	Inflammatory bowel disease
IHC	Inner hair cell
OHC	Outer hair cell
PI3K	Phosphoinositide 3-kinases
PI-6	Protein inhibitor 6
SPIs	Serin peptidase inhibitors
SV	Stria vascularis
TFF	Trefoil factor family

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Introduction

The trefoil factor family (TFF) in mammals comprises three peptides: TFF1, 2 and 3, predominantly expressed in the gastrointestinal (GI) mucosa but also in several other tissues [1–3]. TFFs play an important physiological role and are upregulated in many pathological conditions of mucosal injury such as inflammatory bowel disease (IBD), gastric and duodenal ulcers [4]. TFF3 maintains the GI integrity [5], promotes intestinal epithelial wound healing

[6] and modulates adhesion, migration and survival of epithelial cells [7]. Moreover, *Tff3* synthesis was shown in the rat and human hypothalamus [8]. The presence of *Tff3* exclusively in oxytocinergic neurons but not in vasopressinergic neurons suggested its role as a neuropeptide acting together with oxytocin along the hypothalamo-pituitary axis [9].

Previously, we demonstrated a new expression pattern of *Tff3* in the rodent cochlea and the peptide's involvement in the hearing process [18]. We showed that *Tff3* knock-out mice display a deterioration of hearing with age, a feature characteristic for presbyacusis (age-related hearing loss) [11, 12]. However, in *Tff3*-deficient animals we did not observe any degeneration of cochlear hair cells and spiral ganglion neurons or atrophy of the stria vascularis (SV) [10].

Presently, we focused our analysis on ABR responses and spatial and temporal expression of particular marker proteins involved in normal hearing comparing *Tff3* knock-out to wild-type animals. By studying ABR responses we investigated if the auditory processing pathway is obstructed in *Tff3* knock-out animals, whereas examination of different cochlear protein markers may reveal if lack of *Tff3* affects expression of particular genes in the cochlea. Lack of striking differences in the cochlea using these assays together with interesting data about functional changes underlying age-related hearing loss in the inferior colliculus (IC) [13, 14] turned our attention to the auditory central nervous system (CNS). We thus performed a microarray-based expression screening of genes differentially expressed in the IC followed by confirmation using quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR). Two conspicuous candidate sequences showed significantly altered expression levels. Neither the presence of *Pttg1* and serpina3n in the auditory system nor their interplay with trefoil peptides has been noted so far. Therefore, the differential expression pattern of these genes caused by genetic deletion of *Tff3* suggests their potential involvement during presbyacusis, which requires additional elucidation of the mechanisms involved.

Materials and methods

Animals

Tff3-deficient animals [5] were obtained from Prof. D.K. Podolsky (Harvard Medical School). These animals were backcrossed to C57BL/6 and to 129/Sv mice, and maintained on a mixed background. *Tff3* homozygous sister lines were established representing a *Tff3*-deficient ($Tff3^{-/-}$) and a wild-type genotype ($Tff3^{+/+}$), respectively. Animal experiments were approved and complied with all requirements at the University of Tübingen and the German law for use and welfare of laboratory animals (Tierschutzgesetz). Animal studies were also in line with the 'Ethical principles and guidelines for scientific experiments on animals' of the Swiss Academy of Medical Sciences. All efforts were made to minimize the number of animals used and their suffering. Animals were killed by decapitation following anesthesia with carbon dioxide, and cochleae were removed according to national ethical guidelines.

ABR responses

A minimum of three wild-type and $Tff3^{-/-}$ mutants were used for each measurement. All measurements were made under anesthesia. A dose of 50 mg/kg ketamin hydrochloride (Ketamin 50 Curamed, CuraMED Pharma, Germany), 8 mg/kg xylazin hydrochloride (Rompun 290, Bayer Leverkusen, Germany) and 0.25 mg/kg atropin sulfate (Atropinsulfat, Braun, Germany) was used. The mixture was applied intraperitoneally. Depth of anesthesia was checked every 30 min by testing the pedal withdrawal reflex. If necessary, additional injections of about 50% of the initial dose were given. Body temperature was maintained at 37°C using a heating pad and thermo resistor placed under the animals' body.

ABR recordings were performed in a soundproof chamber (IAC, Niederkrüchten, Germany). For stimulus generation and recording of responses, a multi-function IO-Card (PCI-6259, National Instruments, Austin, TX) was used, housed in an IBM-compatible computer. Sound pressure level was controlled with an attenuator (custom-made) and amplifier. Stimuli were delivered to the ear in a calibrated open system by a loudspeaker (DT911, Beyerdynamics, Heilbronn, Germany) placed 3 cm lateral to the animals' pinna. Sound pressure was calibrated on-line prior to each measurement with a microphone probe system (Brüel & Kjaer 4191, Bremen, Germany) placed near the animals' ear. Recorded signals were amplified (100 dB total amplification) and bandpass filtered (0.2–5 kHz). Clicks of 100-µs duration or tone pipes of 3-ms duration (including 1-ms rise and fall time) were presented. To record bioelectrical responses, subdermal silver-wire electrodes were inserted at the vertex (active) and ventro–lateral (reference) to the measured ear. Electrical signals were averaged over 64 repetitions of stimulus pairs with alternating phase. ABRs were measured for clicks or stimulus frequencies between 2.0 and 45.2 kHz and sound pressure levels from 20 to 110 dB SPL in 5-dB steps. To construct the ABR input-output functions, the peak-to-peak amplitudes of the ABR waveforms at the different sound pressure levels were determined. Thresholds were defined

as the sound pressure level where a stimulus-correlated response was clearly identified by visual inspection of the averaged signal.

Tissue preparation

Wild-type and *Tff3* knock-out mice were decapitated following anesthesia with carbon dioxide. The bullae were removed, and the cochlear spiral was dissected in Hanks' Balanced Salt Solution (HBSS: KCl 5.36, $MgSO_4 \cdot 7H_2O$ 0.405, $MgCl_2 \cdot 5H_2O$ 0.491, NaCl 141, HEPES 9.98, L-glutamin 3.42, $CaCl_2 \cdot 2H_2O$ 1.56, D-glucose 6.30; concentration of all components in mMol/l) with a pH of 7.4. Cochleae were fixed by immersion in 2% paraformaldehyde (SIGMA, Munich, Germany; all chemicals from SIGMA, unless indicated otherwise), 125 mM sucrose in 100 mM phosphate-buffered saline (PBS), pH 7.4, for 2 h. Cochleae of animals older than postnatal day 10 were decalcified after fixation for 15 min to 2 h in rapid bone decalcifier (Eurobio, Fischer-Scientific, 61130 Nidderau, Germany). After overnight incubation in 25% sucrose in PBS, pH 7.4, cochleae were embedded in OCT compound (Miles Laboratories, Elkhart, IN), cryosectioned at 10 µm, mounted on SuperFrost*/plus microscope slides, dried for 1 h and stored at −20°C before use. For the detection of securin protein in brain sections, brains were removed and fixed for 48 h in 4% paraformaldehyde, embedded in 4% agarose and stored in PBS + 0.4% paraformaldehyde at 4°C. The tissue was sectioned at 60 µm with the VT 1000S vibrating microtome (Leica, Wetzlar, Germany). Slices were kept in PBS in a 24-well plate.

Fluorescence immunohistochemistry of different markers

For immunohistochemistry, mouse cochlea sections were stained and imaged as described [15, 16]. The following antibodies were used: goat polyclonal anti-KCNQ4 (Santa Cruz Biotechnology, Santa Cruz, CA; sc-9385, lot B161), goat polyclonal anti-megalin (Santa Cruz Biotechnology, lot D0914), rabbit polyclonal anti-KCNQ1 (Santa Cruz Biotechnology, sc-20816, lot B2103), sheep polyclonal anti-neurofilament 200 (NF200, The Binding Site, Heidelberg, Germany, PH510), rabbit polyclonal anti $K_{ir}4.1$ (Alomone Labs, Jerusalem, Israel, APC-035), rabbit polyclonal anti-otoferlin [17], goat polyclonal anti-synaptophysin (Santa Cruz Biotechnology, sc-7568, lot E2308), rabbit polyclonal anti-securin (Abcam, ab26273), goat polyclonal anti-serpina3n (R&D, AF4709, Lot CBKW01) and rabbit polyclonal anti-*Tff3* [18]. To detect prestin, a polyclonal rabbit antibody directed against the C-terminal epitope of rat prestin was used [19]. Primary antisera were detected with fluorescently labeled secondary IgG antibodies (Cy3-conjugated antibodies, Jackson ImmunoResearch Laboratories, West Grove, PA; or Alexa Fluor-488 conjugated antibodies, Molecular Probes, Eugene, OR). Sections were embedded with Vectashield mounting medium with 4′,6-diamidino-2-phenylindole (DAPI), staining cell nuclei in blue (Vector Laboratories, Burlingame, CA). Specimens were imaged using an Olympus AX-70 microscope equipped with epifluorescence illumination and 40× (numerical aperture 1.0) or 100× oil immersion objectives (numerical aperture 1.35). Images were acquired using a CCD color view 12 camera and imaging system analysis (SIS, Münster, Germany) and additionally processed with Adobe Photoshop 6.0. Immunohistochemical comparison between control and *Tff3* knock-out mice were all performed using the same antibody titre for wild-type and mutant mice, and identical exposure times were used when comparative photographs were taken. Immunohistological analyses were performed from cochlea samples taken from three to five *Tff3* knock-out and wild-type mice. Representative images were chosen for presentation.

For localization of securin protein in the IC, brain sections of wild-type mice were prepared as described above. For protein detection on these slices, sections were washed briefly in PBS containing 0.05% Tween 20, and endogenous peroxidases were blocked in 3% H_2O_2. Blocking of streptavidin-biotin was carried out using the streptavidin-biotin blocking kit (Vector Laboratories) according to the manufacturer's instructions. Sections were incubated over night at 4°C with the primary securin antibody (Abcam, ab26273). Following incubation with the secondary antibody (biotinylated goat anti-rabbit, Vector Labs, Burlingame, CA), the sections were washed in PBS, and the chromogenic detection was carried out (3-amino-9-ethylcarbazole, AEC). Nuclei were counterstained with methyl-green (Vector Laboratories) and viewed using an Olympus AX70 microscope.

Western blot analysis

For Western blot analysis, cochlear tissues of control and $Tff3^{-/-}$ mice were homogenized and lysed in CelLytic MT Tissue Lysis/Extraction Reagent (Sigma-Aldrich C3228) supplemented with a protease inhibitor cocktail (1:50, Sigma–Aldrich P8340). Nuclei and cell debris were pelleted by low-speed centrifugation at 280*g* for 5 min at 4°C, and the supernatant was used for Western blotting. Protein contents were determined using the Bradford method. SDS-PAGE and Western blotting were carried out using the XCell II *SureLock*™ Mini-Cell and XCell II Blot Module from Invitrogen; 35 µg protein lysate per lane of each cochlear or IC sample was loaded on a 4–12% Tris-glycine gel (Invitrogen), resolved and transferred onto PVDF membrane (Invitrogen) according to the

manufacturer's instructions. Blotted proteins were incubated with rabbit polyclonal anti-securin antibody (1:750, Abcam ab26273) together with mouse monoclonal anti-ezrin antibody (0.5 μg/ml, Dianova DLN-10378) as a housekeeping protein, to ensure loading of equal amounts of proteins, followed by ECL™ peroxidase-labeled anti-rabbit or anti-mouse antibodies, respectively (1:2,000, GE Healthcare). Labeled proteins were detected by chemiluminescence using ECL Plus Western blotting detection reagents (GE Healthcare).

Chip hybridization

RNA was isolated from IC of 12-month-old mice using the RNA NOW kit (Biogentex, Seabrook, TX). Two independent RNA samples were obtained from wild-type and *Tff3* knock-out mice. RNA preparation, hybridization to oligonucleotide arrays (Set 430A and 430B) and scanning of the arrays were performed as described in the Affymetrix GeneChip expression analysis manual (http://www.affymetrix.com). Scanned files were analyzed with GeneChip (Affymetrix, Santa Clara, CA), and the expression data were saved as an Excel file containing expression values (average difference).

Quantitative RT-PCR

For qRT-PCR, 3–4 independent RNA samples were extracted from the adult IC and the cochlea at postnatal day 10 (P10), and at 4 and 14 weeks. The IC was identified in accordance with the mouse atlas of Franklin and Paxinos [21]. For RNA preparation, tissue was dissected with small forceps and immediately frozen in liquid nitrogen and stored at −70°C before use; 2 μg of RNA was reverse transcribed with the Transcriptor First Strand cDNA Synthesis Kit (Roche, Basel, Switzerland). Quantitative gene expression analysis was performed on a Lightcycler 480 II (Roche) using SYBR Green. The following primers were used (all sequences from *Mus musculus*): *Gapdh* (Genebank accession Nr. NM_008084): forward 5′-TCCTGCACCACCAACTGCTT-3′, reverse 5′-GTGGCAGTGATGGCATGGAC-3′, *Rpgrip1* (NM_023879): fw 5′-TGTCACATGCAGAGACCACA-3′, rev 5′-ATGCGGCTGTTCTTGAAGTC-3′, *Pttg1* (NM_013917): fw 5′-GGCATCTAAGGATGGGTTGA-3′, rev 5′-CATAGGCTTTTCGGCAACTC-3′, *Prl* (NM_011164): fw 5′-CCACTTCTTCCCTGGCTACA-3′, rev 5′-GATGTATTCGGGGGCTTCTT, *Qk* (NM_021881): fw 5′-GCAGCTGATGAACGACAAGA-3′, rev. 5′-CGTCAGGCAATTCTGCACTT-3′, *Serpina3n* (NM_009252): fw 5′-AGGACATTGATGGTGCTGGT-3′, rev 5′-TAGGGTGTGGTCAGGTCCTC-3′, *Kcnj9* (NM_008429): fw 5′-CCTCGAGAGGGACGACTTC-5′, rev 5′-CTCAAAGGTTTCGTGGAAGC-3′, *Cap1* (NM_007598): fw 5′-CTGGAAGGCAAGAAATGGAG-3′, rev 5′-ACCAGGCCAAGCTTCTTACA-3′, *Tnfrsf12a* (NM_013749): fw 5′-TTGGCGCTGGTTTCTAGTTT-3′ and rev 5′-GAATGAATGGACGACGAGTG-3′. The cycling condition comprised 10 min of preincubation at 95°C followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. All primers were validated for their amplification efficiency. Each sample was analyzed in triplicate, and data were analyzed with the $2^{-\Delta\Delta Ct}$ method.

Results

Hearing measurement

To investigate if auditory processing in *Tff3* knock-out animals is obstructed, a detailed study of ABR thresholds and amplitudes was performed by analyzing the animals' responses to clicks and pure tone bursts. Figure 1 presents averaged ABR-threshold losses of wild-type and *Tff3* knock-out mice at different ages; as a reference the average audiogram of P23 wild-type mice was used. In wild-type mice threshold loss was observed at all ages beyond P23, increasing from low to high frequencies at any age. This corresponds to the normal threshold loss pattern seen in mice showing accelerated presbyacusis [22]. In knockout-mice a conspicuous threshold loss in the low-frequency range was observed even in the youngest animals measured. With increasing age the threshold loss increased to values of up to 45–50 dB loss in the frequency range above 20 kHz. Compared to the wild-type mice, at 11 and 15 months the increased pan-cochlear threshold loss of approximately 20 dB in the knockout mice was statistically significant ($p < 0.05$; *t* test) at any frequency. The analysis of the ABR waveforms, corrected for elevated thresholds in the knockout mice, showed no alterations in latency or amplitudes (data not shown).

Immunohistochemical analysis of the cochlea of *Tff3* mutants

Tff3 knock-out mice developed hearing loss progressing with age. Expression of *Tff3* in the organ of Corti, including in hair cells and supporting cells, has been described previously [10, 18]. However, our previous preliminary study revealed no gross morphological abnormalities in the cochlea of *Tff3* mutant animals [10]. Therefore, we now performed a detailed study in adult wild-type and *Tff3*-deficient mice and surveyed the distribution of a series of key proteins related to auditory function.

The Ca^{2+}-binding protein otoferlin, which resides in synaptic vesicle membranes, showed a normal expression pattern in *Tff3*-deficient animals as well as the neurofilament

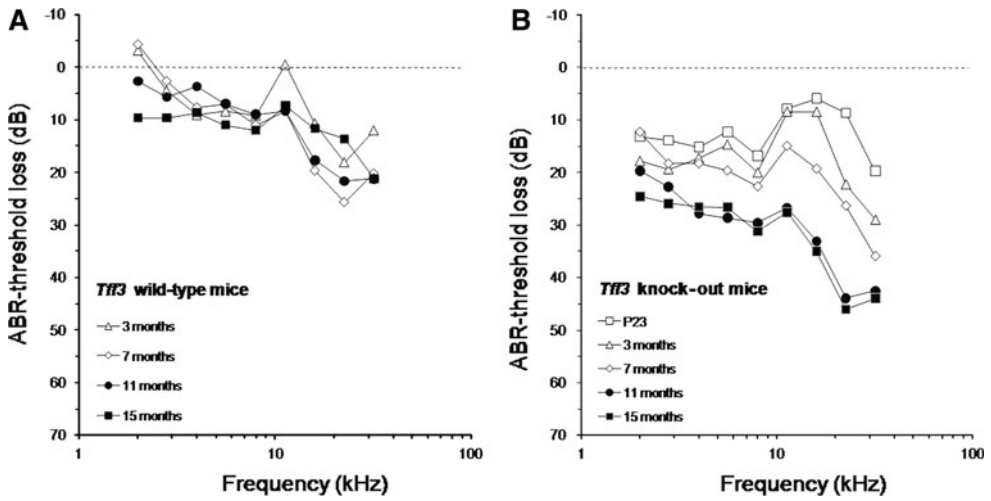

Fig. 1 Average mouse audiograms at different ages for **a** wild-type and **b** *Tff3* knockout animals. ABR-threshold curves were measured at the ages indicated in the legend. Threshold loss was calculated relative to the average threshold of the wild-type mice at postnatal day 23. In wild-type mice a threshold loss was observed, increasing from low to high frequencies at any age. In knockout-mice a conspicuous threshold loss in the low-frequency range was observed even in the youngest animals measured. With increasing age the high-frequency threshold loss increased. At 11 and 15 months the difference between knockout and wild-type animals was significant at any frequency

subunit 200 (NF200), which is a marker for afferent fibers (Fig. 2A). Moreover, outer hair cell-specific markers, such as the voltage-gated potassium channel KCNQ4, was detected at the basal pole of OHCs (Fig. 2b), and the OHC motor protein prestin was present in low-frequency (apical/medial) and high-frequency (midbasal/basal) cochlear turns (Fig. 2c). Expression of KCNJ10, the inwardly rectifying potassium channel, was not changed in the organ of Corti of *Tff3* knock-out mice (Fig. 2d). Likewise, a marker for sites of synaptic transmission, synaptophysin, was present throughout efferent boutons in OHCs (Fig. 2e).

Next to the already documented expression of *Tff3* in the organ of Corti and spiral ganglion [10, 18], we also observed expression in the SV and its absence in *Tff3* mouse mutants (Fig. 3a). To examine potential defects caused by loss of *Tff3* in the SV, we examined several marker proteins expressed in this tissue. Expression of KCNJ10 was localized in the intermediate cells (Fig. 3b). Strong expression of the voltage-activated potassium channel KCNQ1 and megalin, a protein that is essential for endocytosis of lipoproteins and low molecular weight proteins in absorptive epithelia, was observed in the apical membrane of marginal cells of the SV (Fig. 3c, d respectively). Thus, in summary we found no evidence that loss of *Tff3* affects gene expression in the cochlea.

A microarray-based screen for *Tff3*-regulated genes during presbyacusis

Since we observed no evidence for molecular changes in *Tff3* mutants in the cochlea we next concentrated on the analysis of the central auditory system. Within the central auditory system the IC, the principal midbrain nucleus of the auditory pathway, has been described to undergo morphological and functional changes underlying age-related hearing loss [13, 14]. To identify potential target genes for *Tff3* in the IC we used a microarray-based screen. We employed gene chips covering the expression of 34,000 known mouse genes and expressed sequence tags. In our analysis, we compared the expression profile of the IC from aged *Tff3* knock-out mutants to wild-type controls. As a result, a list of genes showing altered expression levels in the absence of *Tff3* was generated. We focused our study on a set of selected genes that were highly down- or upregulated as presented in Table 1. These genes corresponded to: *retinitis pigmentosa GTPase regulator interacting protein 1* (*Rpgrip1*; Mouse genome informatics (MGI) reference 1932134), *pituitary-transforming gene 1* (*Pttg1*; (*securine*), MGI 1353578), *prolactin* (*Prl*; MGI 97762), *quaking* (*Qk*; MGI 97837), *serine* (*or cysteine*) *peptidase inhibitor*, *clade A*, *member 3N* (*Serpina3n*; MGI 105045), *potassium inwardly rectifying channel*, *subfamily J*, *member 9* (*Kcnj9*; MGI 108007), *adenylate cyclase-associated protein 1* (*Cap1*; MGI 88262) and *tumor necrosis factor receptor superfamily*, *member 12a* (*Tnfrsf12a*; MGI 1351484).

Confirmation of microarray data by qRT-PCR

We next employed qRT-PCR to confirm the differential expression of the selected genes independently. Total RNA from the inferior colliculi of adult mice was extracted and served as a template for qRT-PCR with gene-specific

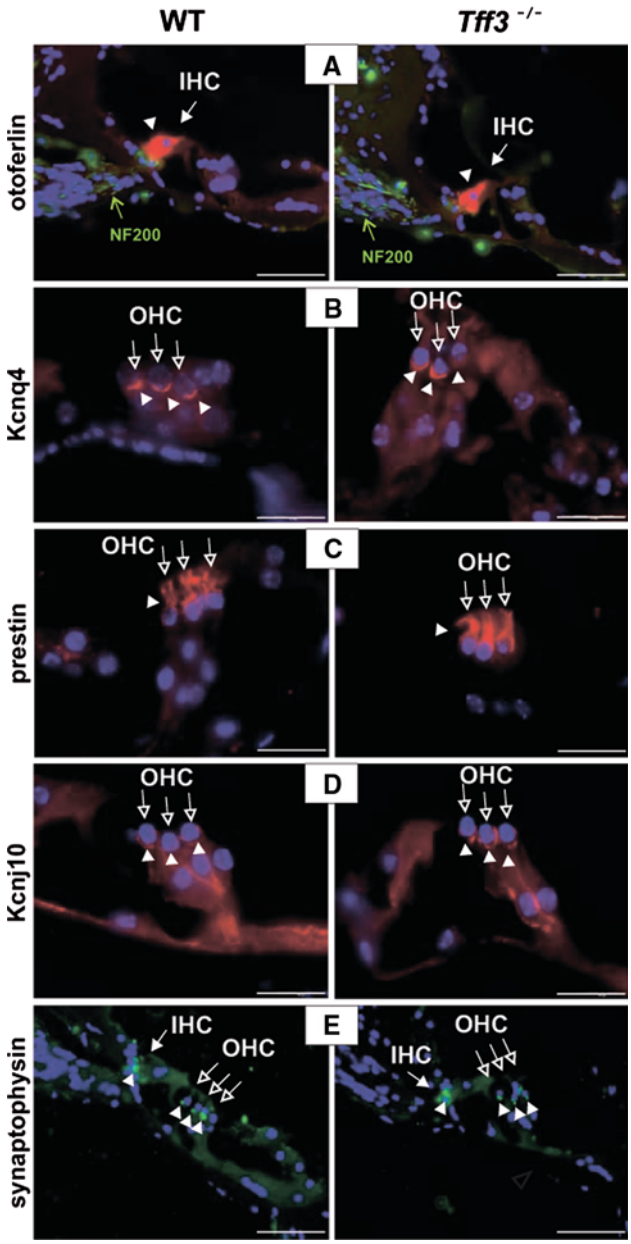

Fig. 2 Immunohistochemistry showing expression of otoferlin (*red*) and neurofilament subunit 200 (NF200, green) **a**, potassium channel KCNQ4 (*red*) **b**, prestin **c**, potassium channel KCNJ10 (*red*) **d**, and **e** synaptophysin (*green*) in 12-month-old wild-type and *Tff3* knock-out mice. Expression of all proteins was normal in *Tff3* knock-out animals in comparison to wild-type mice. Nuclei are stained in *blue* with DAPI. *Open arrows* indicate outer hair cells (*OHC*), *filled arrows* indicate inner hair cells (*IHC*), *filled arrowheads* point out staining with the respective antibodies. *Scale bars* **a**, **b** 50 µm, **c**–**e** 20 µm

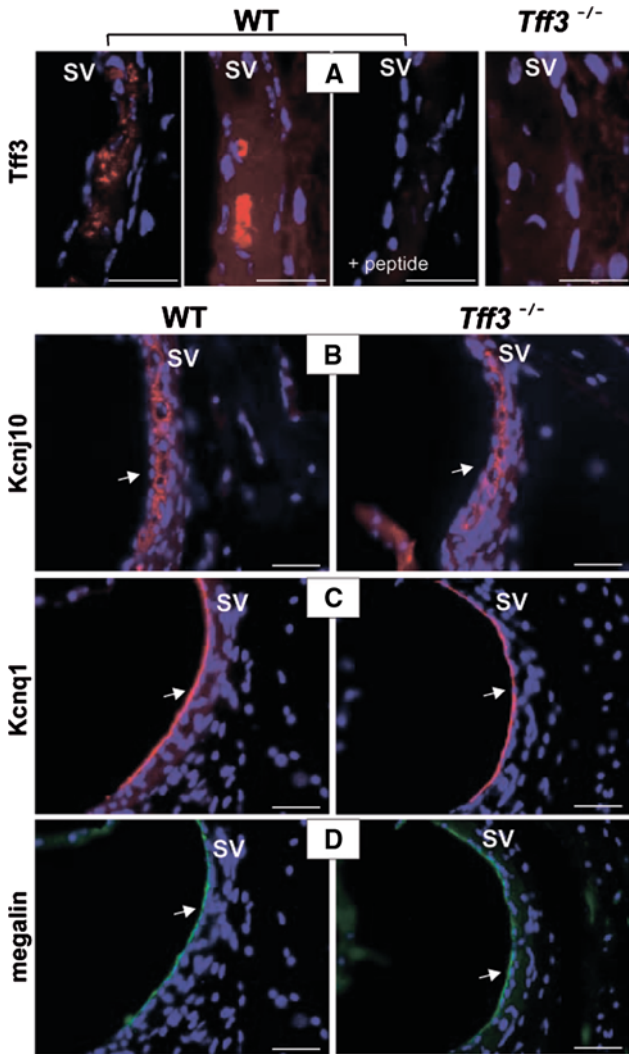

Fig. 3 Immunohistochemistry showing expression of **a** *Tff3* (*red*), **b** potassium channel KCNJ10 (*red*), **c** potassium channel KCNQ1 (*red*), and **d** megalin (*green*) in the stria vascularis (SV) of 12-month-old wild-type and *Tff3* knock-out mice. In *Tff3* mutants and in wild-type mice in the presence of a *Tff3* peptide no *Tff3* expression was observed (**a**). Expression of KCNJ10, KCNQ1 and megalin was normal in *Tff3* knock-out animals in comparison to wild-type mice. Nuclei are stained in blue with DAPI. *Arrows* indicate staining in the SV. *Scale bars* left panels in **a** and **b**–**d** 50 µm, right panels in **a** 20 µm

primers (see methods). As a control, amplification of the housekeeping gene *glyceraldehyde 3-phosphate dehydrogenase* (*Gapdh*) was performed. A strong downregulation of *Pttg1* (*securin*) in the IC of *Tff3* knock-out mice (Fig. 4a) was noted, whereas *serpina3n* was highly upregulated compared to wild-type animals (Fig. 4c), which was consistent with the results from the expression chips (Table 1). In contrast, *Prl*, *Qk*, *Cap1*, *Kcnj9* and *Tnfrsf12a* failed to show a significant difference in mRNA levels between wild-type and *Tff3* knock-out animals, and expression of *Rpgrip1* was not detected (data now shown).

Due to a strong regulation of expression observed for *Pttg1* and *serpina3n* in the IC, the question arose if these genes were also present and differentially expressed in the peripheral auditory system. Expression of both genes was detected in the cochlea of *Tff3* wild-type mice at postnatal

Table 1 Genes decreased or increased in the inferior colliculus of aged *Tff3* knockout mutants versus wild-type animals

Gene	Average fold change	Mouse genome informatics (MGI)
Retinitis pigmentosa GTPase regulator interacting protein 1 (*Rpgrip1*)	−13.9	1932134
Pituitary-transforming gene 1 (*Pttg1*, *Securin*)	−10.5	1353578
Prolactin (*Prl*)	−7.8	97762
Quaking (*Qk*)	−5.7	97837
Serine (*or cysteine*) *peptidase inhibitor*, *clade A*, *member 3n* (*Serpine 3n*)	+42.8	105045
Potassium inwardly-rectifying channel, *subfamily J*, *member 9* (*Kcnj9*)	+6.6	108007
Adenylate cyclase-associated protein (*Cap1*)	+6.2	88262
Tumor necrosis factor receptor superfamily, *member 12a* (*Tnfrsf12a*)	+3.5	1351484

day 10 (P10), and at the age of 4 and 14 weeks by qRT-PCR (data not shown). Moreover, in the cochlea of adult *Tff3* knock-outs as compared to wild-type animals, *Pttg1* and *serpina3n* were down- or upregulated, respectively, at a similar level as seen in the IC (Fig. 4b, d, respectively).

Expression and downregulation of securin in *Tff3* mutants

We next were interested to confirm expression of *Pttg1* (securin) and serpina3n at the protein level. Using commercially available antibodies raised against serpina3n, we were unable to observe a signal using immunohistochemistry or Western blotting (data not shown). In contrast polyclonal antibodies raised against securin allowed us to localize immunoreactivity in IC and cochlea. In the adult cochlea securin was strongly expressed in Deiter's cells, mesenchymal tissue underlying the organ of Corti and spiral ganglion neurons (Fig. 5a). In age-matched *Tff3* mutants we observed no immunoreactivity for securin (Fig. 5b). To further confirm this point, we performed Western blots of cochlear protein lysates at P22 and 12 months (Fig. 5c). At P22 *Tff3* expression appeared only weakly reduced in cochlear lysates of *Tff3* mutants compared to wild-type animals. At 12 months securin expression was found to be downregulated in comparison to P22 in wild-type animals. In cochlear lysates of *Tff3* mutants at 12 months of age, no securin expression was observed. Finally, on sections of the IC of adult wild-type mice immunoreactivity for securin was detected at 9 months of age but was strongly downregulated in age-matched *Tff3* mutants (Fig. 5d, e). Taken together these data confirm the downregulation of securin mRNA in the cochlea and IC also at the protein level.

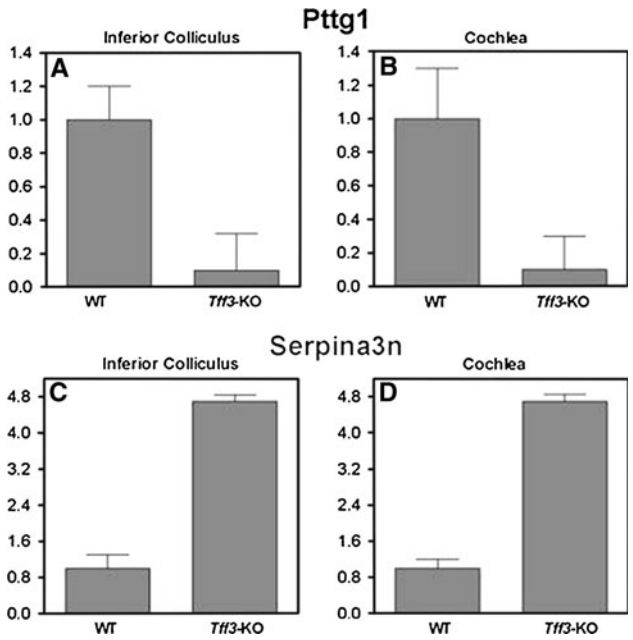

Fig. 4 Differential expression of *securin* (*Pttg1*) (**a**, **b**) and *serpina3n* (**c**, **d**) in adult *Tff3* knock-out versus wild-type animals. Results of quantitative RT-PCR (qRT-PCR) experiments performed with RNA samples isolated from the inferior colliculus (**a**, **c**) and cochlea (**b**, **d**) of wild-type (WT) and *Tff3* knock-out (KO) animals. The level of expression in wild-type animals was arbitrarily set to 1. Expression of *Gapdh* was used as control reference. Differences were tested by a two-tailed Student's *t* test, $p < 0.01$

Discussion

Earlier studies demonstrated expression of *Tff3* in the spiral ganglion and vestibular ganglion neurons. Moreover, in *Tff3* knock-out mice a pronounced deterioration of hearing with progressing age was observed. Analysis of middle and inner ear morphology, however, excluded existence of any gross abnormalities, which may affect the hearing process [10].

In the present study, we further analyzed the inner ear phenotype of *Tff3* mutants by examining the expression of eight specific marker proteins that are important for normal hearing. When comparing wild-type to *Tff3* knock-out animals, no difference in expression patterns of these

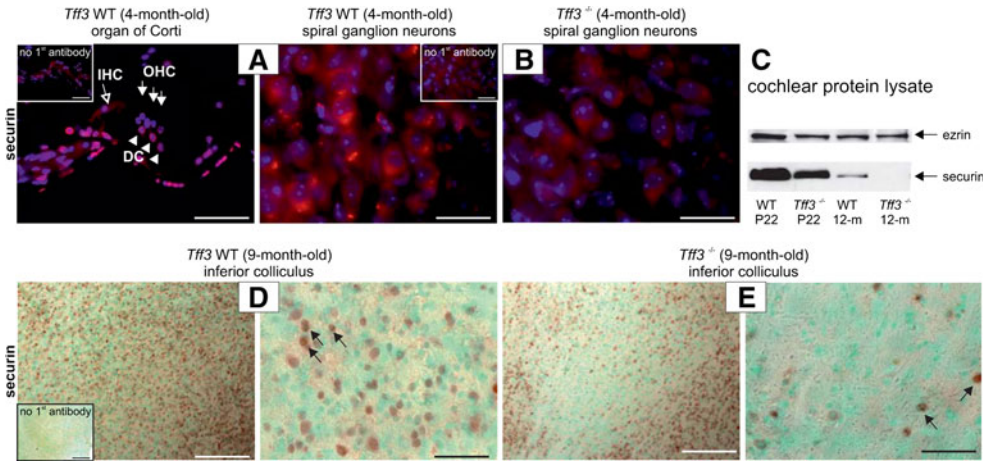

Fig. 5 **a** Securin expression in the organ of Corti (left panel) and in spiral ganglion neurons (right panel) shown for a 4-month-old *Tff3* wild-type mouse. *Filled arrowheads* point at Deiter's cells. *Filled arrows* indicate outer hair cells (*OHC*); *open arrows* indicate inner hair cells (*IHC*). Insets show images where the primary antibody was omitted. **b** No securin expression could be detected in the spiral ganglion neurons of an age-matched $Tff3^{-/-}$ mouse. *Scale bars* left panel in **a** 50 μm, right panel in **a** 20 μm, **b** 20 μm. **c** Semiquantification of the securin protein level in cochlear tissue of *Tff3* wild-type and $Tff3^{-/-}$ mice at indicated ages was performed using Western blots. Ezrin was co-detected to confirm the loading of approximately equivalent amounts of protein. Securin protein is highly expressed in young animals (P22), both in wild-type as well as in knockout mice, although expression of securin protein in P22 $Tff3^{-/-}$ mice appears slightly reduced. In adult wild-type mice the level of securin protein in cochlear tissue is downregulated compared to P22. In 12-month-old $Tff3^{-/-}$ mice, securin protein could not be detected at all. **d**, **e** Securin expression in the inferior colliculus shown for a 9-month-old *Tff3* wild-type (**d**) and an age-matched $Tff3^{-/-}$ mouse (**e**). Left panels show overview images, and the right panels show a higher magnification. Securin protein is stained in brown (*arrows*). Inset shows an image where the primary antibody was omitted. Note that securin expression in the inferior colliculus of an age-matched $Tff3^{-/-}$ mouse is strongly reduced compared to *Tff3* wild-type. *Scale bars* left panel in **d**, **e** 200 μm, right panel in **d**, **e** 50 μm

proteins was noted, excluding their possible contribution to the hearing defect in *Tff3* knock-out mice. Furthermore, quantification of spiral ganglion neurons of wild-type and *Tff3* knock-out mice at different ages (postnatal day 20 (P20), 4- and 15-month) did not reveal any gross difference in their number or morphology (data not shown). Finally, analysis of data from ABR measurements indicated that the processing of auditory information is not hampered in the *Tff3* knock-out mice. All these facts implied that the cause for the auditory defect may be localized at a different level.

In the peripheral auditory system both mice and humans undergo similar changes during hearing loss [23]. A common type of presbyacusis occurs at high frequencies and is caused by hair cell degeneration at the basal end of the cochlea [24]. This peripheral hearing loss eliminates evoked activity in high frequency auditory nerve fibers and translates into loss of sensitivity to high frequencies in central auditory nuclei [25]. Loss of high frequency sensitivity at the periphery eliminates neural responses to high frequency simple sounds in the central auditory system. However, it is unclear how age-related hearing loss affects more complex neural responses such as spectral integration, which is important for the analysis of complex sounds [26]. Multiple tuned and combination-sensitive neurons are found in the IC of both mustached bats [26] and mice [27]. Felix and Portfors (2007) found that the majority of combination-sensitive and multiple tuned neurons are sensitive to high frequencies, which led them to suggest that response properties of these types of neurons will be affected by age-related high-frequency hearing loss. They further hypothesized that high frequency hearing loss reduces spectral integration properties in the IC. They based their study on the examination of neural responses in the IC of CB57Bl/6 mice to single and a combination of tones to analyze the extent of spectral integration in the IC after age-related high-frequency hearing loss.

When ABR thresholds in wild-type and *Tff3* knock-out mice were assessed, we observed a deterioration of hearing in both groups over age, most pronounced in the high-frequency range [10], a feature quite characteristic for presbyacusis [11]. However, high-frequency hearing loss was more pronounced in the *Tff3* knock-out than in the wild-type mice. The *Tff3* knockout mouse thus shows the characteristic hallmark of presybacusis and therefore may be a useful model to identify genes involved during this process. Since no degeneration of hair cells, SV or spiral ganglion neurons in the cochlea of *Tff3*-deficient mice was noted [10] and no changes of molecular markers were observed in the cochlea (present study), we extended our investigation to the auditory CNS and particularly to the IC. To uncover genes related to the effect of presbyacusis, expression microarrays were applied.

A highly significant change in expression of two genes in the IC and cochlea of *Tff3* knock-out mice was observed. One of these genes, downregulated in *Tff3*-deficient animals, is named *securin* (*Pttg1*). Pituitary tumor-transforming gene-1 (*Pttg1*) was isolated from rat pituitary tumor cells in 1997 [28] and subsequently identified as a vertebrate securin, which regulates sister-chromatid separation [29]. *Pttg1* is transcriptionally regulated by various growth factors, is highly expressed in most of the tumors and tumor cell lines analyzed to date, and is thus defined as an oncogene (for review see [30]). Securin directly binds to several gene promoters and regulates transcriptional processes by interacting with other proteins such as p53, Sp1, c-Myc, FGF2, cyclin D3, p21 prolactin and MMP2 (for review see [31]). Upregulation of *Pttg1* increases cell proliferation, induces cellular transformation and promotes tumor development in nude mice. Conversely, downregulation of *Pttg1* in cancer results in suppression of tumor growth and angiogenesis, suggesting that *Pttg1* may serve as an important target gene for the treatment of cancer. The molecular mechanism by which *Pttg1* mediates its tumorigenic function is still unclear [30]. *Pttg1* functions in cell replication [29], cell cycle control [32], DNA damage/repair [33], organ development, metabolism and cell transformation processes [34]. It was shown to be required for human fetal brain development [35] and telencephalic neurogenesis [36].

The second gene, *serpina3n*, was strongly upregulated in *Tff3*-deficient animals. Serpins (serin peptidase inhibitors or SPIs) are the largest family of protease inhibitors and extend to all branches of life [37, 38]. Members of this class of protein perform roles in diverse physiological processes such as the blood clotting cascade, apoptosis and chromatin condensation [39]. Gene expression studies suggest that serpina3n (also known as EB22.4 and referred to as muACT-n, murine anti-chymotrypsin) is the closest murine orthologue of huACT (human anti-chymotrypsin). In particular, it is the only member of the serpina3 cluster that is expressed in the murine brain under resting conditions [40], consistent with evidence that huACT plays a role in the inflammatory response in that organ. As with huACT, muACT-n shows a wide tissue distribution: it is found in the liver, brain, testis, lung, thymus and spleen, and to a lesser extent bone marrow, skeletal muscle and kidney [40]. In the rat liver, three members of the SPI family have been cloned and characterized [41]. SPI-1 and SPI-2 genes are expressed in normal rat liver, but SPI-3 is virtually silent in normal rats and only becomes transiently active during acute inflammation in the rat liver [42] and after transient ischemia in the rat brain [43]. Endogenous expression of SPI-3 in response to inflammatory stimulation was also demonstrated in specific cells of ocular tissues (epithelial cells of the iris and ciliary body, and astrocytes in the retina) [44]. Since SPI-3 is expressed only under inflammatory conditions, previous studies speculated that it might have protective effects against inflammatory damage [43, 45]. Many SPIs have been involved in potent protective activities such as wound-healing [46]. SPI-3 may have similar anti-inflammatory properties under local inflammatory conditions. It has been shown that *SPI-3* gene is strongly induced during the acute-phase reaction, and it is regulated at the transcriptional level by interleukin 6 (IL-6) [42] and also by interferon gamma (IFNgamma) [47]. IL-6 and IFNgamma are cytokines with pleiotropic activities, each playing an important role in the host defense system. It was demonstrated that transcription factors such as STAT3 and STAT5B are involved in the induction of the *SPI-3* promoter, whereas C/EBPbeta reduced the transcriptional activity of the promoter [48]. Intestinal restitution induced by TFF3 is also associated with IL-6/Gp130/STAT signaling [49]. TFF3 activates STAT3 [50], which exerts anti-apoptotic and mitogenic effects [51]. Additionally, unpublished data suggest that optic nerve injury does not induce expression of *SPI-3*, whereas rat motor nerve transection dramatically induced *SPI-3* expression in the injured motor neurons [44]. A human homolog of *SPI-3*, protein inhibitor 6 (PI-6), was shown to inhibit cathepsin G, which activates a proapoptotic protease, caspase-7 [52, 53]. This may suggest that SPI-3 may prevents caspase-7-mediated apoptosis, which is caused by damage such as nerve injury or inflammatory stimulation. *Tff3*-deficient mice showed increased sensitivity to intestinal damage with augmented apoptosis, suggesting a protective role of *Tff3*. *Tff3* prevents apoptosis after injuries in a range of cell lines, an effect that requires activation of both the epidermal growth factor (EGF) and phosphoinositide 3-kinase (PI3 K) receptors [49]. Together these data suggest that upregulation of *serpina3n* plays an important compensatory role when *Tff3* protein is missing and may not act as a protector and anti-apoptotic factor. Further studies involving analysis of neurons and synapses of the IC of *Tff3* knock-out mice may confirm this postulate.

Presently there are no known patients with hearing impairment linked to both new candidate genes. *SERPINA3* is localized on 14q32.1, and there is no known gene related to hearing nearby. *PTTG1* is on 5q35.1. Somewhat in the vicinity, in 5q31, there are two genes, *DIAPH1* and *POU4F3*, and unknown genes for DFNA42 and DFNA54, but on the molecular genetic level this distance excludes *SERPINA3* and *PTTG1* as being involved at the present time. Therefore, the expression changes observed in both proteins may be unrelated to hearing loss. However, loss of *Pttg1* has been related to senescence in the pancreas and pituitary, suggesting a general defect in various organs during aging [54, 55]. In the case of *serpina3n* it is worthwhile mentioning that a member of the serpin gene

family, *SERPINB6*, has recently been associated with hearing loss in human patients [56]. Therefore, further studies directed at the function of *Pttg1* and *serpina3n* in the *Tff3* knockout mouse model may provide important insights on the roles of these genes during the development of age-related hearing loss.

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