Thrombospondins 1 and 2 are important for afferent synapse formation and function in the inner ear

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Abstract

Thrombospondins (TSPs) constitute a family of secreted extracellular matrix proteins that have been shown to be involved in the formation of synapses in the central nervous system. In this study, we show that TSP1 and TSP2 are expressed in the cochlea, and offer the first description of their putative roles in afferent synapse development and function in the inner ear. We examined mice with deletions of TSP1, TSP2 and both (TSP1/TSP2) for inner ear development and function. Immunostaining for synaptic markers indicated a significant decrease in the number of formed afferent synapses in the cochleae of TSP2 and TSP1/TSP2 knockout (KO) mice at postnatal day (P) 29. In functional studies, TSP2 and TSP1/TSP2 KO mice showed elevated auditory brainstem response (ABR) thresholds as compared with wild-type littermates, starting at P15, with the most severe phenotype being seen for TSP1/TSP2 KO mice. TSP1/TSP2 KO mice also showed reduced wave I amplitudes of ABRs and vestibular evoked potentials, suggesting synaptic dysfunction in both the auditory and vestibular systems. Whereas ABR thresholds in TSP1 KO mice were relatively unaffected at early ages, TSP1/TSP2 KO mice showed the most severe phenotype among all of the genotypes tested, suggesting functional redundancy between the two genes. On the basis of the above results, we propose that TSPs play an important role in afferent synapse development and function of the inner ear.

Introduction

The sensory epithelium in the cochlea of the inner ear, the organ of Corti, is a precisely patterned structure consisting of the inner hair cells (IHCs), outer hair cells (OHCs), and several types of supporting cell (SC). In rodents, cochlear spiral ganglion neurons (SGN) projection towards hair cells starts at embryonic day (E) 12.5. The cochlea then goes through a critical process of synapse formation and refinement during the first two postnatal weeks (Rubel, 1978; Knipper et al., 1995; Appler & Goodrich, 2011), with the onset of hearing occurring by postnatal day (P) 12–14. Any defect at this stage of maturation can result in a dysfunctional hearing organ, and is one of the causes of auditory neuropathy or synaptopathy (Starr et al., 2008; Santarelli, 2010).

Recent studies have revealed important roles for extracellular matrix molecules such as thrombospondins (TSPs) in promoting synapse formation during development in the central nervous system (CNS) and in repairing synaptic connections after stroke (Christopherson et al., 2005; Liauw et al., 2008; Xu et al., 2010). TSPs are multifunctional proteins that are being increasingly recognised as having a variety of important physiological roles in development and disease (Toone et al., 1998; Swinnen et al., 2009; Kim et al., 2012; Tran et al., 2012) in many systems, including the cardiovascular system and CNS. Their physiological actions are mediated by regulation of cell–cell and cell–matrix interactions through partnering with an array of membrane receptors, other extracellular matrix proteins, and cytokines. TSPs are secreted by astrocytes in the CNS (Bornstein, 2000; Bentley & Adams, 2010; Kishner & Erglu, 2012), and can be structurally divided into two groups: group A contains the highly homologous TSP1 and TSP2; and group B consists of TSP3, TSP4, and TSP5 (Adams & Lawler, 2004; Carlson et al., 2008). Mice deficient in TSP1 and TSP2 or both can develop abnormalities in lungs, muscles, bones, heart, and brain (O’Shea et al., 1990; Kyriakides et al., 1998; Lawler et al., 1998; Swinnen et al., 2009). In a purified retinal ganglion cell system, TSP1 and TSP2 signaling from astrocytes was shown to be necessary and sufficient to stimulate excitatory synaptogenesis (Christopherson et al., 2005). Intriguingly, TSP2 miRNA was upregulated in a thyroid-deficient mouse model with defective synaptic maturation, as compared with wild-type (WT) controls (Sendin et al., 2007) (our data, unpublished). On the basis of the above findings, we hypothesised that TSPs may be involved in synapse formation in the cochlea.

In this study, we examined whether TSPs play a role in cochlear and vestibular afferent synapse formation and function, by using mice with targeted deletions of either one or more than one TSP gene.
TSP3, TSP4 and TSP5 knockout (KO) mice did not show an auditory phenotype. TSP2 and TSP1/TSP2 KO mice had reduced numbers of afferent ribbon synapses and varying degrees of impaired auditory and vestibular function as compared with WT littermates, with TSP1/TSP2 KO mice showing the most severe phenotype. In summary, these findings indicate that TSP1 and TSP2 act redundantly to ensure normal synapse formation and function of the inner ear.

Materials and methods

Animals

Mice of either sex lacking TSP1 (Lawler et al., 1998), TSP2 (Kyriakides et al., 1998) or both (Agah et al., 2002) and their littermate WT controls were used for experiments. These mice are on an FVB/NJ genetic background. At least three mice per genotype per experiment were used, and experiments were repeated two to three times for validation. All experiments were performed according to national animal care guidelines, and were approved by the Stanford University Administrative Panel on Laboratory Animal Care.

RNA extraction and quantitative polymerase chain reaction (qPCR)

RNA was extracted from whole cochleae dissected from WT FVB mice at P0 (n = 4), P6 (n = 3), P10 (n = 4), P15 (n = 5) and P29 (n = 5) for TSP2, and additionally at P60 (n = 3), P180 (n = 5) for TSP1, or from the whole vestibular organ dissected from WT FVB mice at P6 (n = 6), P10 (n = 4), P16 (n = 7) and P30 (n = 4) for all genes tested, with an RNAqueous-Micro Kit from Ambion (Grand Island, NY, USA), according to the protocol of the manufacturer. Extracted RNA was converted to cDNA with a High Capacity RNA to cDNA kit (Applied Biosystems, Grand Island, NY, USA). This cDNA was used in polymerase chain reaction assays with Taqman (Applied Biosystems, Grand Island, NY, USA) proprietary probes and primers for TSP1 (assay ID Mm01335418_m1) and TSP2 (assay ID Mm01279240_m1). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (assay ID Mm0332249_g1) was used as the internal standard. All Taqman qPCR assays were performed on a Bio-Rad (Hercules, CA, USA) CFX96 Real-Time System with the internal standard. All Taqman qPCR assays were performed on a Bio-Rad (Hercules, CA, USA) CFX96 Real-Time System with a Bio-Rad (Hercules, CA, USA) CFX96 Real-Time System with the internal standard. GAPDH. Error bars represent standard errors of the mean (SEMs).

In situ hybridisation

TSP-specific in situ probes were generated from the 5′-part of the full-length cDNA. Fragments were subcloned from IMAGE clones (Openbiosystem ID 64050917 and Openbiosystem ID 30062369, respectively) into the pGEM-T vector (Promega, Madison, WI, USA), with the following primers: TSP1, ATGGAGCCTCCTCCGGG GACTA and ATCAGGAACTGTGGCCTGGACGA (1098 bp); and TSP2, ATGCTCTGGGACACTGGCCCTGCT and TTCACATAACAGTGGTGGCCCACA (1101 bp). In situ hybridisation was carried out as previously described (Schwander et al., 2007).

Immunohistochemistry

Inner ears were dissected into cold phosphate-buffered saline (PBS), and, after opening of oval, round windows and the bone on the cochlear apex, cochleae were perfused with 4% paraformaldehyde in PBS and left in fixative solution for an additional 10 min. Samples were washed in PBS for 10 min, and then blocked in PBS containing 0.5% Triton X-100 plus 5% bovine serum albumin for 30 min at room temperature. The same blocking buffer was used for diluting antibodies. Primary antibodies were incubated at 4 °C for 36 h, and this was followed by three washes in 0.1% PBS–TWEEN. Secondary antibodies were added for 1 h at room temperature, and this was followed by three washes in 0.1% PBS–TWEEN. The following primary antibodies were used: goat anti-CtBP2 (1 : 200; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for RIBEYE, rabbit anti-glutamate receptor 2 and 3 (1 : 100; Millipore Biosciences Research Reagents, Billerica, MA, USA), rabbit anti-HOMER1 (1 : 200; Synaptic System, Goettingen, Germany), and rabbit anti-SHANK1 (1 : 200; Neuromics, Minneapolis, MN, USA). Secondary antibodies used were Alexa Fluor 488-conjugated anti-goat and Alexa Fluor 546-conjugated anti-rabbit (1 : 500; Invitrogen, Grand Island, NY, USA). After immunostaining, cochleae were decalciﬁed in 10% EDTA for 1 h at room temperature, and further ﬁxed by immersion in 4% paraformaldehyde in PBS for 15 min. Cochleae or vestibular utricles were washed in PBS and mounted on slides in ProLong (Invitrogen) anti-fading medium.

Confocal analysis of synapse number

Images of the immunostained organ of Corti or vestibular utricle were collected on a Carl Zeiss AG (Oberkochen, Germany) AxioVert confocal inverted microscope. Optical sections were line-averaged and frame-averaged, and collected at 0.3-μm intervals with a 1024 × 1024 raster. Detector gain and excitation thresholds were adjusted per tissue to cover the same range of pixel values, and the pinhole was calibrated for the WT animal tissues and kept constant for all samples. A cochlear frequency map (Muller et al., 2005) was estimated for every sample to localise hair cells from different frequency regions. Cochlear z-stacks from a selected frequency region were taken for each sample. Each stack contained the entire synaptic pole of 15–20 IHCs. Synapses were automatically counted through the series of optical sections by use of VICTOL3D IMAGE ANALYSIS SOFTWARE. To conﬁrm the accuracy of the counts, z-stacks were manually quantiﬁed for synapse number by projecting a series of optical sections and counting synaptic puncta in each projection volume. On average, one stack per cochlear/vestibular region was obtained from six different mice. For tissues double-labeled with presynaptic and postsynaptic markers, a total of at least four scans per genotype at P29 were analysed by use of the automatic counts in VICTOL3D IMAGE ANALYSIS SOFTWARE, and also conﬁrmed manually. Statistical analyses were performed with an SPSS (STATISTICS PREMIUM GRAD PACK, Version 20.0 for Mac OS; IBM Corporation, Armonk, NY, USA) software package. One-way analysis of variance (ANOVA) of synaptic marker count as dependent variable and genotype (WT, TSP1, TSP2 and TSP1/TSP2 KO) as factor, followed by Scheffe’s post hoc test, was performed for RIBEYE, SHANK1, and colocalised (synaptic) puncta. P < 0.05 was considered to be statistically signiﬁcant.

Auditory brainstem responses (ABRs)

ABR recordings were conducted in a sound-attenuating room at the Auditory Core for the Department of Otolaryngology, Stanford University. Mice were weighed and anaesthetised with ketamine (100 mg/kg) and xylazine (10 mg/kg), injected intraperitoneally. Core body temperature was kept at 37.0 °C for all recordings with a homeothermic heating pad (FHC, Bowdoin, ME, USA). Stimulus
presentation, ABR acquisition, equipment control and data management were coordinated by use of the computerised intelligent hearing system (Intelligent Hearing Systems, Miami, FL, USA). A high-frequency transducer was coupled with the intelligent hearing system to generate specific acoustic stimuli. Acoustic stimuli were delivered to the ear canals via plastic tubes channelled to the speaker at 8, 16, and 32 kHz. ABRs were recorded via subdermal needle electrodes inserted at the vertex (active electrode), behind the left pinna (reference electrode), and in the left leg (ground electrode). Electrophysiological activity was amplified, filtered, and averaged. Sound levels were increased in 5-dB steps from 10 to 20 dB below threshold to 80 dB (for 8 and 16 kHz) or 100 dB (for 32 kHz). The threshold for an ABR was defined as the lowest stimulus level at which replicable waves I and V could be identified in the response waveform. The amplitude analysis was performed by peak-to-peak measurement of the ABR waveform, and latency was calculated as time from stimulus onset until the respective peak. ABR waveform amplitude and latency analysis was performed for 16 kHz at 65 dB. Statistical analyses were performed with an SPSS software package. One-way ANOVA of either amplitude or latency as dependent variable and genotype (WT, TSP1 KO, TSP2 KO, and TSP1/TSP2 KO) as factor, followed by Scheffe’s post hoc test, was performed at specific ages (1 month or 3 months). Two-way ANOVA of the ABR data was performed with ABR threshold as dependent variable and genotype (WT, TSP1 KO, TSP2 KO, and TSP1/TSP2 KO) and age as independent factors. On the basis of significant main effects and interactions from this statistical test, planned comparisons were performed by use of a one-way ANOVA with ABR threshold as dependent variable, followed by Scheffe’s post hoc test to identify differences between genotypes, or across the ages tested, at a given frequency (8, 16, or 32 kHz). In all statistical tests, P < 0.05 was considered to be statistically significant.

**Vestibular evoked potentials (VsEPs)**

Gravity receptor neural function was assessed by the use of linear VsEPs. VsEPs were recorded from mice of either sex at 1.5 months of age from four genotypes. The recording method was similar to that of Mock et al. (2011). In short, mice were weighed and anesthetised with ketamine and xylazine, similarly as for ABR recordings. Core body temperature was maintained at 37.0 °C. The active electrode was placed subcutaneously at the midline just posterior to the lambdoidal suture; the reference electrode was placed behind the right pinna, and the ground at the right leg. Mice were positioned supine; a non-invasive head clip was used to secure the skull behind the right pinna (reference electrode), and the ground at the right leg. Mice were delivered to the ear canals via plastic tubes channeled to the middle ear. ABRs were recorded via subdermal needle electrodes inserted at the vertex (active electrode), behind the left pinna (reference electrode), and in the left leg (ground electrode). Electrophysiological activity was amplified, filtered, and averaged. Sound levels were increased in 5-dB steps from 10 to 20 dB below threshold to 80 dB (for 8 and 16 kHz) or 100 dB (for 32 kHz). The threshold for an ABR was defined as the lowest stimulus level at which replicable waves I and V could be identified in the response waveform. The amplitude analysis was performed by peak-to-peak measurement of the ABR waveform, and latency was calculated as time from stimulus onset until the respective peak. ABR waveform amplitude and latency analysis was performed for 16 kHz at 65 dB. Statistical analyses were performed with an SPSS software package. One-way ANOVA of either amplitude or latency as dependent variable and genotype (WT, TSP1 KO, TSP2 KO, and TSP1/TSP2 KO) as factor, followed by Scheffe’s post hoc test, was performed at specific ages (1 month or 3 months). Two-way ANOVA of the ABR data was performed with ABR threshold as dependent variable and genotype (WT, TSP1 KO, TSP2 KO, and TSP1/TSP2 KO) and age as independent factors. On the basis of significant main effects and interactions from this statistical test, planned comparisons were performed by use of a one-way ANOVA with ABR threshold as dependent variable, followed by Scheffe’s post hoc test to identify differences between genotypes, or across the ages tested, at a given frequency (8, 16, or 32 kHz). In all statistical tests, P < 0.05 was considered to be statistically significant.

**Results**

**Expression of TSP1 and TSP2**

To determine the relative expression levels of TSP1 and TSP2 transcripts in the mouse cochlea, we performed qPCR with specific, validated primers sets on RNA extracted from cochleae of WT FVB/NJ mice at different developmental ages. We found that TSP1 mRNA levels were substantially higher between P0 and P29 than between P48 and P60 (Fig. 1A). At P180, TSP1 expression levels remained low. TSP2 was highly expressed at birth, and its expression decreased thereafter (Fig. 1B). By P29, TSP2 expression was almost undetectable in the cochlea. Elevated levels of mRNAs for TSP3, TSP4 and TSP5 were also seen between P0 and P29 (data not shown).

In order to more precisely localise TSP expression in the cochlea, we performed *in situ* hybridisation at late embryonic and early postnatal periods, when afferent synaptogenesis is ongoing in the cochlea (Sobkowicz et al., 1982; Appler & Goodrich, 2011). At E17 and P1, TSP1 expression was observed in the SCs of the organ of Corti, surrounding the IHCs and OHCs, including the inner sulcus, phalangeal, pillar and Dieter’s cells (Fig. 1C, E, and E′). At P5, TSP1 expression was restricted to pillar cells and to inner sulcus cells (Fig. 1E″) of the sensory epithelium. TSP1 expression was also widespread in the cochlear bony tissue. No signal was observed with control sense probe (Fig. 1D and F–F″). Expression of otoferlin (OTOF) is shown as a positive control that marks the IHCs and OHCs (Fig. 1G–G″). We speculated that TSP2 would also be expressed by the SCs of the cochlea, but *in situ* hybridisation with multiple TSP2 probes did not yield convincing results for cochlear tissue at the same ages as described for TSP1. The above results show that TSP1 and TSP2 are both expressed in the cochlea and are developmentally regulated. We next examined whether these molecules are involved in cochlear synaptogenesis.

**Cochlear synaptogenesis in TSP mutants vs. control WT mice**

Initial examination of gross cochlear morphology with plastic-embedded sections did not show significant differences between the WT strain and the different mutant strains (data not shown). However, to determine whether TSP1 and TSP2 may cause more subtle changes, as would be expected with a synaptogenic role, we looked at the number of synapses in the mature organ of Corti at P29 in WT and TSP1, TSP2 and TSP1/TSP2 KO mice. We examined presynaptic and postsynaptic afferent markers in the IHCs by immunostaining. We focused on the 16-kHz region, which has the highest synaptic density and hearing sensitivity in mice (Liu & Davis, 2007; Meyer et al., 2009). Confocal images of whole mount-prepared cochleae were reconstructed in z-stacks with VOLocity 3D IMAGE ANALYSIS SOFTWARE to count numbers of synaptic puncta. First, we looked at the presynaptic marker RIBEYE, which is a main protein of synaptic ribbons (Schmitz et al., 2000). A one-way ANOVA of RIBEYE marker count as dependent variable and genotype as independent factor was significant (*F*3,40 = 8.001, *P* = 0.000). Scheffe’s post hoc test showed that there was a significant reduction in the number of RIBEYE-positive puncta in TSP2 KO mice (*P* = 0.002) and TSP1/TSP2 KO mice (*P* = 0.007) as compared with WT mice (Fig. 2A and B). To examine the postsynaptic side, we labeled the AMPA receptor glutamate receptor 2/3 (Tanabe et al., 1992; Nakanishi, 1994; Puel, 1995) and the scaffolding proteins of postsynaptic density HOMER1 (Xiao et al., 1998, 2000) and SHANK1 (Naisbitt et al., 1999; Tu et al., 1999) (data shown for SHANK1)
only). With the same confocal approach, counts in the 16-kHz region indicated no significant differences (one-way ANOVA, $F_{3,25} = 1.662$, $P = 0.201$) in SHANK1 staining between WT mice and TSP single and double KO mice (Fig. 2C and D).

To verify that stained puncta correspond to synapses (as defined by juxtaposition of presynaptic and postsynaptic terminals, and henceforth referred to as ‘synapses’), we double-labeled cochlear whole mount preparations from WT and TSP KO mice with antibodies against RIBEYE and SHANK1 (Fig. 2E and F). There was a 16% decrease in synapses in TSP1 KO mice (8.0 per IHC as compared with 8.8 in WT mice), a 25% decrease in TSP2 KO mice (6.4 per IHC as compared with 8.8 in WT mice), and a 43% reduction
in TSP1/TSP2 KO mice (3.7 per IHC as compared with 8.8 in WT mice) (Fig. 2E and F). There was more orphan RIBEYE staining in TSP1/TSP2 KO mice but not as much orphan SHANK1 staining. As the above results showed that IHCs of TSP1, TSP2 and TSP1/TSP2 KO mice have reduced numbers of synapses, we next examined the effect of these synaptic deficits on auditory function.

**Assessment of ABR thresholds in TSP1 and TSP2 KO mice**

To determine the functional consequences of loss of TSP1 and/or TSP2 for hearing function, we performed neurophysiological ABR tests in all three types of TSP KO mice at different ages, starting from P15 and ending by 18 months of age. WT littermates on the
same FVB/NJ background were used as controls. To determine auditory thresholds, ABR measurements were obtained for three frequencies representing each turn of the cochlea: 8 kHz for the apical turn, 16 kHz for the middle turn, and 32 kHz to assess function at the base of the cochlea. Mice are born deaf, with the onset of hearing occurring at around P12–14. Therefore, we started testing for ABR thresholds at P15 to assess cochlear function as soon as possible after the onset of hearing. We measured hearing again at 1, 3, 6 months (data not shown), 12 and 18 months of age. For statistical analysis, a two-way ANOVA of the ABR data was performed, with ABR threshold as dependent variable and genotype (WT, TSP1 KO, TSP2 KO, and TSP1/TSP2 KO) and age (P15, P1, P3, P6, P12, and 18 months) as independent factors. This analysis showed a significant main effect of genotype × age interaction ($F_{15,193} = 8.668, P = 0.000$). Planned comparisons were performed with a one-way ANOVA, with ABR threshold as dependent variable and either age or genotype as independent factor. This analysis was followed by Scheffe’s post hoc test to identify differences between genotypes, or across the ages tested, at a given frequency (8, 16, or 32 kHz). TSP1/TSP2 KO mice showed elevated thresholds at all frequencies tested, starting as early as P15 (Fig. 3). At P15, TSP2 KO mice showed elevated thresholds in the 8- and 32-kHz regions (Fig. 3A and C). TSP1 KO mice had normal hearing thresholds at all frequencies tested (Fig. 3) at P15. There were no further changes in ABR thresholds for the different KO mice at 1 month, 3 months (Fig. 3) and 6 months (not shown) of age. However, at 12 months of age, there were greater threshold shifts at all frequencies tested in TSP2 and TSP1/TSP2 KO mice (Fig. 3). At 12 months of age, TSP1 KO mice showed a significant ($P = 0.000$) threshold elevation only at 32 kHz. Beyond 12 months, all genotypes show profound threshold elevation at 32 kHz owing to aging, and therefore no comparisons were made beyond 12 months for this frequency. The stronger threshold shifts in TSP1/TSP2 KO mice than in single KO mice suggests functional compensation between TSP1 and TSP2. ABRs of 1-month-old TSP3, TSP4 and TSP5 KO mice were normal as compared with their WT controls (data not shown). However we cannot rule out whether these genes would have effect on hearing when in combination of double or triple KO. The above results show that TSP2 KO mice had a more severe phenotype than TSP1 KO mice, and that TSP1/TSP2 KO mice had the strongest hearing defect among the genotypes tested. To assess the relative contributions of TSP1 and TSP2 to cochlear neuronal activity, we next examined the ABR waves in these mutants.

**Analysis of the first ABR wave amplitude and latency**

It has been shown that the ABR threshold does not always reflect subtle phenotypes in the number of neurons that are firing (Liberman, 1982; Kujawa & Liberman, 2009). Therefore, we further analysed the ABR data to identify possible changes in peak amplitude or latency. We analysed ABR responses at 65 dB SPL, which is at least 30 dB above the hearing threshold in mice, and provides a robust measure of sound-evoked neuronal activity (Fig. 4A and B). Furthermore, 16 kHz represents the most sensitive hearing region in mice. A one-way ANOVA with amplitude as dependent variable and genotype as independent factor showed significant differences between groups ($F_{3,33} = 4.789, P = 0.007$). Scheffe’s post hoc test showed a significant ($P = 0.026$) reduction in the wave I response amplitude (shown as P1 – N1 values, where P1 is the first robust positive peak in the ABR trace, and N1 is the negative component following P1) of TSP1/TSP2 KO mice as compared with WT mice, starting from 1 month of age (Fig. 4A and C). TSP2 KO mice showed no significant wave 1...
amplitude changes at 1 month or at 3 months (Fig. 4C and D). Likewise, TSP1 KO mice did not show significant changes in wave I amplitudes at 1 month or 3 months (Fig. 4C and D). The peak latency of the wave I ABR was significantly prolonged in TSP1/TSP2 KO mice at 1 month \((P = 0.031)\) and 3 months \((P = 0.001)\) of age as compared with WT mice. Latencies for TSP1 and TSP2 KO mice were similar to those of WT mice at both tested ages (Fig. 4E and F). Analysis of the wave I ABR peak at 8 kHz also showed significant differences in amplitude and latency in TSP1/TSP2 KO mice vs. WT mice at 1 month of age (data not shown). Analysis of the wave II ABR peak performed for 16 kHz at 65 dB SPL at this age showed results similar to those for the wave I ABR peak (data not shown). Amplitudes of the wave I ABR peaks in TSP3, TSP4 and TSP5 KO mice at 16 kHz and 65 dB SPL were similar to those of their WT controls (data not shown). The above results show that the amplitude and latencies of TSP1/TSP2 KO mice were the most affected, starting at 1 month of age when those of the other KO mice were similar to those of WT mice.

**Characterisation of vestibular function in TSP KO mice**

As many genes that have been shown to be important for cochlear function have also been reported to be involved in vestibular function (Moller, 2002; Nandi & Luxon, 2008), we investigated whether TSP1 and TSP2 are expressed in the vestibular system, and whether they play a role in the function of the inner ear gravity receptor organs (Eatock & Songer, 2011). qPCR analysis of vestibular tissue showed expression profiles for TSP1 and TSP2 that were similar to those in cochlear tissue (Fig. 5A and B). The highest expression levels for both genes were seen at P6, the earliest age tested. TSP1 expression remained at the same level from P6 to P16, and then declined, but TSP2 expression declined soon after P6. To more precisely localise

![Fig. 4](image-url)

**Fig. 4.** Analysis of auditory nerve firing in TSP KO mice at 1 month and 3 months of age. ABR wave I response amplitude and latency were analysed at 65 dB for 16 kHz. Statistical analyses were performed with one-way ANOVA followed by Scheffe’s post hoc test. (A) Representative waveforms of WT mice (black line) and TSP1/TSP2 KO mice (gray line) at 1 month of age. The difference between P1 and N1 values indicates the amplitude of response. Distance from time when stimulus was given (0 on the x-axis) and time of response (P1 on wave I) is the latency of the response. (B) ABR thresholds in WT and TSP KO mice at 16 kHz indicate no significant threshold shift in TSP1 or TSP2 KO mice as compared with WT mice. (C) ABR wave I amplitude was significantly reduced in TSP1/TSP2 KO mice as compared with WT mice at 1 month \((P = 0.026)\). (B and D) TSP1/TSP2 KO mice had prolonged latency as compared with WT mice at 1 month \((P = 0.031)\) (B) and 3 months \((P = 0.001)\) (D) of age. (C) At 3 months of age, the amplitude of wave I in TSP1/TSP2 KO mice was significantly reduced \((P = 0.038)\). The amplitude shift in TSP1 KO mice was not significant. TSP2 KO mice had an insignificant shift in amplitude at this age. The n-value near the indicated genotype gives the number of mice that were used for the experiment. Quantification data are presented as mean ± SEM. Significant differences: \(*P < 0.05\), \(**P < 0.01\), and \(***P < 0.001\).
TSP expression in the vestibular system, we performed in situ hybridisation on the neonatal utricle at P1 (Fig. 5C). Strong TSP1 mRNA expression was seen at the apical side of the utricle sensory epithelium (Fig. 5C and C'). We were again unable to specifically localise TSP2 mRNA with this technique. No signal was observed with the control sense probe (Fig. 5D and D'). Expression of OTOF is shown as a positive control that marks the type I and type II hair cells (Fig. 5E and E'). Immunostaining for the presynaptic marker RIBEYE and the postsynaptic marker SHANK1 on the sensory epithelium of the P29 mouse utricle showed reduced (P = 0.001) synaptic ribbon staining in TSP1/TSP2 KO mice as detected with a RIBEYE antibody (Fig. 6A, D, and G), whereas SHANK1 staining appeared to be similar between WT mice and TSP1/TSP2 KO mice (Fig. 6B, E, and H). The number of colocalised synaptic puncta was also significantly (P = 0.000) reduced in TSP1/TSP2 KO mice as compared with WT controls (Fig. 6I). We also examined cryosections of the vestibular utricle for these markers, and obtained similar results (data not shown).

To assess gravity receptor function, we measured VsEP responses in 1.5-month-old WT and KO mice. One-way ANOVA of VsEP data showed significant differences among genotypes for VsEP thresholds (P = 0.020) and wave I (P1 N1) amplitudes (P = 0.040). Post hoc comparisons revealed no differences in VsEP thresholds between TSP1 KO mice and WT mice at this age. However, TSP2 KO mice (P = 0.020) and TSP1/TSP2 KO mice (P = 0.010) had significantly elevated thresholds as compared with WT mice (Fig. 7B). Amplitudes at +6 dB for the wave I response peak were also significantly reduced in TSP1/TSP2 KO mice as compared with WT controls (P = 0.011) (Fig. 7A and C). Analysis of the wave I peak latencies at +6 dB did not show significant differences among genotypes (Fig. 7D). Vestibular behavioral dysfunction, as measured with a swimming test, did not reveal obvious differences between the various KO and WT strains (data not shown). Overall, these results show that, whereas the vestibular phenotype is rather subtle, TSP2 and TSP1/TSP2 KO mice have reduced sensitivity of the peripheral gravity receptor organs.

Discussion

The data presented in this study show, for the first time, that TSP1 and TSP2 play important synaptogenic roles in the cochlear and vestibular organs. Furthermore, the physiology experiments demonstrate that synaptic defects in mice deficient in TSP1, TSP2 or both could at least partially account for the hearing loss seen in these mice. The more severe phenotype of the TSP1/TSP2 KO mice than of TSP1 or TSP2 KO mice points to a redundancy in this system. TSP2 appears to compensate somewhat for the absence of TSP1, whereas the reverse effect of TSP1 compensating for the absence of TSP2 does not appear to be robust. These studies are novel in their scope, and provide the first demonstration to date of the effect of synaptogenic molecules of the extracellular matrix in the development and function of cochlear and vestibular systems.

**TSP expression in the inner ear**

The in situ hybridisation analysis for TSP1 (Fig. 1) showed early expression in the SCs, such as the inner phalangeal, pillar and Dietz’s cells, up to P5. This result suggests that SCs of the inner ear...
may play a similar role to glial cells in the brain in providing critical factors for synapse formation and maintenance (Pirvola et al., 1994; Ullian et al., 2001; Dityatev et al., 2006; Sugawara et al., 2007; Gomez-Casati et al., 2010; Tritzsch & Bergles, 2010; Zuccotti et al., 2012; Clarke & Barres, 2013). Restriction of TSP1 expression to a small subset of SCs at P5, from initial widespread expression in the cochlea, also points to diversity in the functions of the various types of SC in this tissue (Río et al., 2002; Monzack & Cunningham, 2013; Wan et al., 2013). Our qPCR studies showed high expression of TSP1 and TSP2 during the first postnatal week in the mouse cochlea. TSP2 expression decreased by P10, paralleling a reported decrease in synaptogenesis in the cochlea (Huang et al., 2007, 2012). TSP1 expression subsequently decreased at P60, by which time the cochlea is fully mature. Interestingly, whereas expression of TSP2 was practically undetectable by P29, expression of TSP1 did not entirely disappear at later ages, suggesting a more sustained role for these genes in the cochlea. These spatial and temporal gene expression data strengthen the notion that SCs are involved in providing critical factors, such as TSPs, that are required for afferent synapse formation (Christopherson et al., 2005).

Involvement of TSPs in cochlear synaptogenesis

We saw a significant reduction in staining with the presynaptic ribbon marker RIBEYE but not with the postsynaptic marker SHANK1 in the cochlea of TSP KO mice by P29. This observation is in agreement with previous data showing that addition of TSP1 or TSP2 alone to the cell culture medium mainly affected the presynaptic side, leading to the formation of silent synapses, and did not appear to be involved in the maturation of postsynaptic densities (Christopherson et al., 2005; Allen et al., 2012). TSP2 and TSP1/TSP2 KO mice showed significant reductions in colocalised synaptic puncta, supporting the important role of TSPs in cochlear synaptogenesis. Reductions in presynaptic ribbon numbers in these mutants would result in decreased numbers of ribbons being available to form functioning synapses with postsynaptic structures. In turn, this decrease in the number of functioning synapses would result in fewer synapses firing in response to a given stimulus than in WT controls, as reflected by the ABR data.

As TSP1 and TSP2 are highly homologous genes, one could speculate on a certain degree of redundancy in this system, as is indeed suggested by the severe phenotype in TSP1/TSP2 KO mice as compared with TSP2 KO mice. The greater loss of synaptic numbers in TSP2 KO mice than in TSP1 KO mice also indicates that TSP2 is able to partially compensate for TSP1, and that the role of TSP2 is possibly more crucial in establishing the neuronal network in the inner ear.

Contribution of TSPs to cochlear physiology

A single SGN forms a synapse with a single IHC (Spoendlin, 1969; Liberman, 1980; Liberman et al., 1990; Raphael & Altschuler, 2003), and SGNs that contact a single IHC can be divided into at

Fig. 6. Sensory epithelium of utricle stained with synaptic markers in WT and TSP1/TSP2 KO mice. Counts were statistically analysed with one-way ANOVA followed by Scheffe’s post hoc test. (A–C) WT utricle stained with RIBEYE (A) and SHANK1 (B) at P29. An overlay view is shown in C. (D–F) TSP1/TSP2 KO utricle stained with RIBEYE (D) and SHANK1 (E). Colocalisation of both markers is shown in F. (G) Synaptic ribbon counts per 40 μm² of the utricle in TSP1/TSP2 KO and WT mice. Ribbon numbers in TSP1/TSP2 KO mice were lower than in WT mice (P = 0.001). (H) Quantification of SHANK1. No significant difference was observed between WT and TSP1/TSP2 KO mice. (I) Average number of synapses determined by RIBEYE that colocalised with SHANK1 per 40 μm² in WT and TSP1/TSP2 KO mice. The number of synapses was reduced in TSP1/TSP2 KO mice as compared with WT mice (P = 0.000). The number of animals tested per genotype and type of staining are indicated on the graphs. The scale bar in A is 10 μm, and applies to B–F. Results are expressed as mean ± SEM. Significant difference: ***P < 0.001.
least two groups on the basis of the rate of firing: high spontaneous rate fibers and low spontaneous rate fibers (Liberman, 1982; Taberner & Liberman, 2005; Liberman et al., 2011; Furman et al., 2013). Measurements of ABR thresholds in TSP KO mice and comparison with WT controls allowed us to study the general function of the cochlea. ABR threshold responses compared across the different mutants and throughout the hearing ages of a mouse (P15 – 540 or 2 weeks and 18 months of age) showed varying degrees of hearing loss in the KO mice as compared with WT controls. The stronger phenotype in TSP1/TSP2 KO mice suggests that TSP2 is able to compensate for the loss of TSP1 at younger ages. However, for the TSP1 KO mice, elevated ABR thresholds were seen at all frequencies by 12 months of age as compared with WT controls, suggesting that lack of TSP1 may affect the long-term maintenance of hearing. This effect on long-term maintenance is likely to be more attributable to the initial effects of TSP1 during development than to the effects at later ages, as we saw very low levels of TSP1 mRNA beyond P29.

Therefore, TSP1 may be important in establishing those elements of a stable neuronal network in the developing cochlea that are needed to sustain auditory function over the course of a rodent’s hearing lifetime. The observation of an early threshold shift at P15 at the 32-kHz frequency in TSP2 KO mice suggests that TSP2 may play an important role in the onset of hearing in the high-frequency range. It is not surprising to see mutations in a synapse-related protein causing progressive high-frequency hearing impairment, as this was previously described in patients and mice with vesicular glutamate transporter 3 mutations (Ruel et al., 2008). In addition, gross morphology and staining for prestin and myosin 7 in the mutant OHCs appeared to be normal as compared with WT controls (data not shown).

The results from synapse counting and ABR threshold measurements allow us to draw a connection from the establishment of functional synapses early in development by a network of genes (such as those encoding TSPs) to the onset and maintenance of hearing function. A lack or defect in one of these genes may not manifest in hearing impairment (such as in the TSP1 KO mice), as synaptic defects can be very subtle, and also because of inherent redundancy in the system, whereby another molecule with a similar structure can substitute for the missing factor. However, when more than one such gene is affected (as in the TSP1/TSP2 KO mice), a more obvious hearing phenotype becomes apparent. Furthermore, the lack of normal synapse patterning during development may impact on the ability of an affected individual to retain normal hearing over a lifetime that may include challenges in the form of environmental insults, aging, or disease.

Fig. 7. Contribution of TSP1 and TSP2 to vestibular function. (A) Representative VsEP waveforms collected at +6 dB (relative to 1.0 g/ms) for the four genotypes tested. P1 – N1 represents the first response peak measured for latency and amplitude. One-way ANOVA followed by a post hoc test was used for statistical analysis. (B) On average, VsEP thresholds were significantly elevated in TSP1/TSP2 KO mice (downward triangle) as compared with WT mice (square) (P = 0.010) and TSP1 KO mice (circle) (P = 0.023). TSP2 KO mice (upward triangle) also had VsEP thresholds significantly higher than those of WT mice (P = 0.020). (C) VsEP wave I amplitudes were significantly reduced for TSP1/TSP2 KO mice as compared with WT mice (P = 0.011) and TSP1 KO mice (P = 0.014). (D) VsEP wave I latencies were not significantly different across genotypes. Significant difference: *P < 0.05.
To better estimate the number of neurons firing in these animals, we measured the amplitude and latency of ABR wave I, and picked a frequency (16 kHz) where TSP1 and TSP2 KO mice showed no elevation in threshold. We hypothesised that amplitude loss in TSP KO mice would correlate with loss of synaptic function. Analysis of the first peak of ABRs in TSP KO mice was in agreement with histological data showing reductions in juxtaposed presynaptic and postsynaptic markers. Physiologically, this result correlated with the most significant reduction in first peak amplitude seen in TSP1/ TSP2 KO mice, followed by TSP2 KO mice. These data suggest that TSP2 may functionally compensate for the absence of TSP1, at least initially.

Contribution of TSPs to vestibular physiology

We saw a reduced density of synaptic ribbons in the vestibular organs of TSP1/TSP2 KO mice, further supporting the idea that lack of TSPs results in loss of synapses. As a lack of genes affecting synapse function may not necessarily cause a visible behavioral defect, such as circling, we used VsEPs as a measure of vestibular neural activity (Jones et al., 2002). VsEP thresholds were elevated in both TSP2 and TSP1/TSP2 KO mice as compared with controls, similarly to the auditory phenotypes in these animals. The onset of the compound action potential was not altered in TSP KO mice, although such a functional defect was observed in the cochlea through the prolonged latencies in TSP1/TSP2 KO mice.

In summary, this study shows, for the first time, that TSPs expressed in the inner ear during the critical window of postnatal synaptogenesis play an important role in establishing connectivity between IHCs and SGNs. The data presented above show that a lack of TSPs leads to defective synapse formation in the inner ear. This synaptic defect, in turn, leads to fewer functional synapses responding to a given stimulus, and impaired auditory and vestibular function, especially in mice that are deficient in both TSP1 and TSP2. These results also indicate that, although TSP2 plays a more important role in the development of auditory function and is able to mostly compensate for the lack of TSP1, there could be some redundancy in the system. This study shows that the components of the extracellular matrix, such as TSPs, play an important role in afferent synapse development and functional maturation in the inner ear, and are candidates for screening in patients with various forms of inner ear dysfunction.

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Abbreviations

ABR, auditory brainstem response; CNS, central nervous system; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; E, embryonic day; IHC, inner hair cell; KO, knockout; OTOF, otosteril; P, postnatal day; PBS, phosphate-buffered saline; qPCR, quantitative real-time polymerase chain reaction; SC, supporting cell; SEM, standard error of the mean; SGN, spiral ganglion neuron; TSP, thrombospondin; VsEP, vestibular evoked potential; WT, wild type.

Synaptogenic role of TSPs in the inner ear

References


