Deafness and Stria Vascularis Defects in S1P₂ Receptor-null Mice*

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The S1P₂ receptor is a member of a family of G protein-coupled receptors that bind the extracellular sphingolipid metabolite sphingosine 1-phosphate with high affinity. The receptor is widely expressed and linked to multiple G protein signaling pathways, but its physiological function has remained elusive. Here we have demonstrated that S1P₂ receptor expression is essential for proper functioning of the auditory and vestibular systems. Auditory brainstem response analysis revealed that S1P₂ receptor-null mice were deaf by one month of age. These null mice exhibited multiple inner ear pathologies. However, some of the earliest cellular lesions in the cochlea were found within the stria vascularis, a barrier epithelium containing the primary vasculature of the inner ear. Between 2 and 4 weeks after birth, the basal and marginal epithelial cell barriers and the capillary bed within the stria vascularis of the S1P₂ receptor-null mice showed markedly disturbed structures. JTE013, an S1P₂ receptor-specific antagonist, blocked the S1P-induced vasoconstriction of the spiral modiolar artery, which supplies blood directly to the stria vascularis and protects its capillary bed from high perfusion pressure. Vascular disturbance within the stria vascularis is a potential mechanism that leads to deafness in the S1P₂ receptor-null mice.

Sphingosine 1-phosphate (S1P)³ is a sphingolipid metabolite that functions as a signaling ligand through interactions with G protein-coupled S1P receptors. Five high affinity receptors (S1P₁–S1P₅) have been described that trigger distinctive intracellular signaling pathways (1, 2) following binding of the S1P ligand. Three of these receptors, S1P₁, S1P₂, and S1P₅, are widely expressed on cells and tissues, whereas expression of the S1P₄ and S1P₅ receptors are largely confined to cells of the immune and nervous systems. The ligand S1P is produced through the phosphorylation of sphingosine by sphingosine kinases 1 and 2 and can be degraded by S1P-specific enzymes that include phosphatases and a lyase (3). Micromolar levels of the S1P ligand, bound primarily to high density lipoproteins, are found in plasma and may provide a source for tonic signaling. Acute S1P signaling may result from enhanced secretion of S1P from cells, such as platelets and mast cells, upon activation.

Genetic deletion of receptors within mice has been an important means of identifying the physiologic roles of S1P receptor signaling. These studies have demonstrated that the signaling pathways are biologically significant and potentially clinically relevant within the vascular (4–6), immune (7, 8), pulmonary (9), and nervous systems (10).

Here we report that S1P₂ receptor expression is essential for proper functioning of the auditory and vestibular systems. S1P₂ receptor-null mice exhibit profound deafness early in life with severe associated pathologic changes within the cochlea. Early cellular defects were found to be in the stria vascularis, a compartment that harbors the main vasculature of the inner ear, helps maintain homeostasis of the inner ear fluids, and whose proper function is critical for hearing.

EXPERIMENTAL PROCEDURES

Materials—S1P was purchased from Biomol. The S1P₂ receptor blocker JTE013 (11–13) was purchased from Tocris Bioscience (Ellisville, MI).

S1P₂-null Mice—S1P₂ receptor-null mice were generated and genotyped as described previously (6). The mice used in this study were on a 129/SvTac, C57BL/6 mixed background.

Auditory and Vestibular Testing—Auditory function was evaluated by auditory brainstem response (ABR) analysis as described previously (14). Distortion product otoacoustic emissions (DPOAEs) were recorded with an acoustic probe (ER-10C, Etymotic Research, Elk Grove Village, IL) using the DP2000 DPOAE measurement system, version 3.0 (Starkey Bioscience, Ellisville, MI).

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§ The abbreviations used are: S1P, sphingosine 1-phosphate; ABR, auditory brainstem response; DAPI, 4',6-diamidino-2-phenylindole; db, decibel; DPOAE, distortion product otoacoustic emission; SPL, sound pressure level; PBS, phosphate buffered saline; PFA, paraformaldehyde; MOPS, 4-morpholinepropanesulfonic acid.

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Laboratory, Eden Prairie, MN). Two primary tones with a frequency ratio $f_2/f_1 = 1.2$ were presented at $L_1 = 65$ db SPL and $L_2 = 55$ db SPL. $f_2$ was varied in one-sixth octave steps from $\sim 4$ to $16$ kHz. Distortion product (DP)-grams comprised $2f_1 - f_2$ DPOAE amplitudes as a function of $f_2$. Possible vestibular function defects were evaluated by standard rotarod, balance beam walking, swimming, and contact righting reflex tests (15).

Histological Analysis of Inner Ear—Mice were perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4; PFA). The skulls were removed, immersed in 4% PFA for 4 h at room temperature, decalcified in Decalcifier II (Surgepath Medical Industries Inc., Richmond, IL) overnight at $4^\circ$C and embedded in paraffin blocks. The 5-$\mu$m serial sections were obtained from paraffin blocks and stained with hematoxylin and eosin.

Fluorescence Confocal Microscopy—For whole-mount staining, the stria vascularis was dissected out and fixed in 4% PFA for 30 min on ice. The tissue was treated in blocking buffer (1% bovine serum albumin in phosphate-buffered saline (PBS) with 0.1% Triton X-100) for 30 min at room temperature. The tissue was stained overnight at $4^\circ$C to visualize the vasculature with Alexa Fluor 594-conjugated isoelectin GS-IB4 (Molecular Probes) and DAPI to visualize nuclei. Tissue was washed in PBS, and the stria vascularis with the spiral ligament was dissected out. The vestibular epithelia were transferred to fresh PBS and imaged using a Stemi-11 stereomicroscope (Carl Zeiss MicroImaging, Inc.) equipped with a ZVS-3C75DE digital camera and Digital Acquire application software (Carl Zeiss MicroImaging, Inc.). The stria vascularis with the underlying spiral ligament were mounted using immu-mount solution (ThermoShandon, Pittsburgh, PA) and examined with a Nikon eclipse 80i upright microscope (Nikon Instruments, Inc.) using differential interference contrast.

Real-time PCR—Tissue RNA was isolated with TRIzol® reagent (Invitrogen). Stria vascularis RNA was pooled from nine wild-type mice. For other tissues, RNA was prepared from separate samples from three wild-type mice. cDNA was synthesized from 100 ng of total RNA. Real-time PCR was performed using an ABI PRISM 7700 sequence detection system (Applied Biosystems). Assay-on-Demand™ gene expression products for $S1P_1$, (catalog number Mm00514644_m1), $S1P_2$ (Mm01177794_m1), $S1P_3$ (Mm00515669_m1), Spk1 (Mm00448841_g1), Spk2 (Mm00445021_m1), Sgpl1 (Mm00486079_m1), and TaqMan Universal PCR Master Mix (Applied Biosystems) were used for real-time PCR under conditions recommended by the manufacturer. For quantitation, $Gapdh$ was amplified simultaneously, and the gene expression of $S1P_1$, $S1P_2$, $S1P_3$, $Spk1$, $Spk2$, $Sgpl1$, and $Sgpp1$ was normalized to the $Gapdh$ expression.

Preparation of the Spiral Modiolar Artery—The isolation of the spiral modiolar artery has been previously described in detail (16). The artery segments (~1.5 mm in length) were canulated and pressurized hydrostatically (30 mm Hg). The functional experiments were performed at 37 $^\circ$C in MOPS-buffered salt solution (3 mM MOPS, 145 mM NaCl, 4.7 mM KCl, 3 mM CaCl$_2$, 1.17 mM, MgSO$_4$.$7$H$_2$O, 1.2 mM NaH$_2$PO$_4$.$2$H$_2$O, 2 mM pyruvate, 0.02 mM EDTA, and 5.0 mM glucose). Changes in the outer diameter were detected using videomicroscopy (Crescent Electronics, Windsor, Ontario, Canada). Artery viability was confirmed by testing its vasomotor response to 0–10 mM Ca$^{2+}$ under depolarizing conditions (125 mM KCl).

RESULTS

We have previously reported that ~50% of $S1P_2$,$S1P_3$ receptor double null mice die in utero with angiogenic defects (6). We noted that ~20% of the surviving $S1P_2$,$S1P_3$ receptor double null mice developed a pronounced head tilt as adults, suggesting a possible inner ear abnormality (Fig. 1A). Histological evaluation of the inner ears of six-month-old double null mice demonstrated a striking bilateral absence of the spiral ganglion neurons (Fig. 1B) but not in the $S1P_1$, single null mouse. Scale bar in $B$ is 100 $\mu$m and applies also to $C$ and $D$.

![FIGURE 1. Phenotype of S1P2; S1P3 receptor double null mice. A, characteristic head tilt of a 3-month-old S1P2;S1P3 double null mouse. B–D, histological sections of inner ears from 6-month-old mice demonstrating the absence of spiral ganglion neurons in double null and S1P2, single null mice (arrows) but not in the S1P1, single null mouse. Scale bar in B is 100 $\mu$m and applies also to C and D.](image-url)
S1P₂ and Deafness

using one- and three-month-old S1P₂ null and S1P₂ heterozygous mice from the same litters (Fig. 2, A and B). At one month of age, heterozygous mice already had normal adult-like ABR waveforms and thresholds. In contrast, age-matched S1P₂ receptor-null mice showed no characteristic ABR waveforms at intensities of 100 db of sound pressure level (SPL) to all four stimuli tested (click, 8, 16, and 32 kHz). These results demonstrated that the S1P₂ receptor-null mice were profoundly deaf shortly after hearing function usually becomes fully established in mice (∼3 weeks) (17, 18). We next measured distortion product otoacoustic emission (DPOAE), an indicator of the mechano-sensory function of outer hair cells within the organ of Corti. We found that S1P₂ receptor-null mice at one and three months of age had no measurable DPOAE (Fig. 2C), which suggested that the function of the outer hair cells was disrupted either because of abnormalities of these and other cells of the organ of Corti or because of disturbed homeostasis of the inner ear fluids that are critical for mechanotransduction in the organ of Corti.

We noted during the S1P₂ receptor-null inner ear dissections that otoconia of the utricular epithelium of both ears were largely absent and that the few remaining were enlarged, although otoconia of the saccular epithelium were indistinguishable from wild-type controls (Fig. 3, A and B). Although the otoconia defect could underlie manifestations of vestibular dysfunction, such as the head tilt originally displayed by the double null mice, the S1P₂ receptor-null mice exhibited balance and motor function indistinguishable from wild-type littermates when tested on a rotarod by balance beam walking or in a swimming test (data not shown).

To understand the mechanism of hearing loss in S1P₂-null mice, we examined the inner ear histology of S1P₂ receptor-null and normal hearing wild-type littermates at various ages. The cochlea duct histology of null and wild-type mice was similar at postnatal day 7 (data not shown). However, at postnatal day 14, the stria vascularis, which produces the endocochlear potential within the inner ear endolymphatic space, was nearly double the thickness in S1P₂ receptor-null mice compared with wild-type littermates (Fig. 3, C and F). Within the stria vascularis of the S1P₂ receptor-null mice, blood vessels appeared dilated. At postnatal day 31, the stria vascularis of the S1P₂ receptor-null mice showed abnormally increased pigmentation, an indicator of damage and altered function of intermediate cells (19) (Fig. 4, A–C). The sensory hair cells in the organ of Corti in S1P₂ receptor-null mice appeared relatively normal at postnatal day 14 (Fig. 3, C and F) and were only mildly affected by postnatal days 17–21 (Fig. 3, D and G). By postnatal day 31 there was base-to-apex degeneration, which was more severe in the basal turn of the organ of Corti and milder in the middle and apical turns, with the degeneration progressively increasing at later times (Fig. 3, E and H; Fig. 4, D–G). The spiral ganglion neurons of S1P₂ receptor-null mice were present up to postnatal day 21 but had largely disappeared by 4 months (Fig. 3, E and H). Because the stria

FIGURE 2. Analysis of S1P₂ receptor-null mice auditory function. A, shown is a sample ABR analysis for a click stimulus of a 4-week-old S1P₂ receptor-null (−/−) and heterozygous (+/−) littermate. The horizontal axis is time in milliseconds; the vertical axis is sound intensity in db SPL. Observed thresholds in this sample were 40 db SPL for the S1P₂−/− mouse and 100 db SPL for the S1P₂+/− mouse. B, mean ABR thresholds (db SPL) of S1P₂−/− and S1P₂+/− mice at 1 and 3 months old. C, distortion product otoacoustic emissions (DPOAEs) in S1P₂ receptor-null mice and their heterozygous littermates. Relationships between DPOAE and stimulating frequency (DP-grams) are shown for S1P₂ receptor-null mice at 1 and 3 months of age. Heterozygous littermates had normal DPOAEs at both of these ages and, therefore, the data were combined. Values are presented as mean ± S.E. Gray area indicates noise floor.
vessel was severely affected at an early time in the temporal sequence of pathologic changes within S1P2 receptor-null cochlea, we analyzed this structure in more detail.

Confocal microscopic image analysis of the strial vascularis, after staining of cortical actin structures by phalloidin, showed a striking disorganization of marginal and basal cell barriers in S1P2 receptor-null mice as early as about 2 weeks of age (Fig. 5A, B, D, and E). Double staining with phalloidin and DAPI revealed both multinuclear and anuclear marginal cells of heterogeneous sizes in S1P2 receptor-null stria vascularis (Fig. 5, A and D). The capillary network of the stria vascularis was imaged by isolectin staining and confocal microscopy. The blood vessels of the stria vascularis from S1P2 receptor-null mice, at one month of age, were remarkably dilated and tortuous compared with vessels from the controls (Fig. 5, C and F).

The spiral modiolar artery, which directly supplies blood to the vessels of the stria vascularis, expresses the S1P2 receptor and responds to the S1P ligand by exhibiting Rho GTPase-dependent vasoconstriction (20). We therefore investigated whether S1P-stimulated vasoconstriction in spiral modiolar arteries isolated from the gerbil cochlea could be inhibited with an antagonist of the S1P2 receptor. Gerbil spiral modiolar arteries were used because the mouse cochlea anatomy does not permit reliable spiral modiolar artery isolation. Canulated spiral modiolar arteries were stimulated with increasing concentrations of S1P under control conditions and in the presence of the specific S1P2 receptor blocker, JTE013 (11–13). Fig. 6A shows a recording of dose-dependent vasoconstrictions induced by S1P in a spiral modiolar artery segment. In these typically biphasic responses to a given concentration of S1P, strong initial vasoconstrictions were followed by a weaker more sustained phase. Comparison of both peak and sustained phases of the response to S1P (Fig. 6, B (peak) and C (sustained)) revealed significant inhibition of both phases by JTE013.

The expression of S1P2 mRNA within the stria vascularis in wild-type mice was determined by real-time PCR and compared with S1P2 expression in the organ of Corti, brain, and liver. S1P2 RNA expression was highest in the stria vascularis followed by the organ of Corti and was substantially higher in these inner ear structures than in brain or liver (Fig. 7). S1P3 RNA expression was also higher in stria vascularis and organ of Corti compared with brain and liver. In contrast, S1P1 RNA expression was higher in brain relative to stria vascularis and organ of Corti. Expression of genes that regulate S1P levels, such as the sphingosine kinases (Sphk1, Sphk2), S1P lyase (Sgly1), and S1P phosphatase (Sgpp1), were also measured by real-time PCR in wild-type mice and were expressed at levels either comparable or higher relative to the levels found in the brain, lung, and liver.

**DISCUSSION**

We have shown here that S1P2 receptor-null mice are profoundly deaf. This striking phenotype is not shared by S1P3 receptor-null mice. A substantial incidence of head tilt in double null S1P2; S1P3 receptor mice first prompted us to examine inner ear structures. The head tilt, a possible vestibular manifestation, was observed much less frequently in the S1P2 receptor-null mice. However, the bilateral absence of normal with the presence of few enlarged utricular otoco-
nia in all of the S1P2 receptor-null inner ears indicate the perturbed ionic composition of vestibular labyrinth fluids and suggest that more subtle vestibular dysfunction may indeed exist. Other prominent pathologic features of the S1P2 receptor-null inner ear included cellular abnormalities within the stria vascularis by postnatal day 14 and subsequent base-to-apex degeneration of sensory hair cells within the organ of Corti, which were later accompanied by the loss of spiral ganglion neurons.

How does the loss of S1P2 receptor function cause deafness and the associated inner ear pathology? A potential mechanism may be gleaned from the early cellular changes that occur within the stria vascularis. The stria vascularis is a highly vascularized, multilayer structure with (i) marginal cells facing the endolympathic space, (ii) layers of flat interleaved basal cells facing the perilymphatic space, and (iii) capillaries and intermediate cells in the center. A major function of this structure is to establish the endocochlear potential by secreting K+ ions against an electrical and chemical gradient into the endolympathic space, which is essential for mechanosensitive hair cell signal transduction (21).

Our imaging results demonstrate that the cellular organization of the S1P2 receptor-null stria vascularis is dramatically disturbed around the time when the endocochlear potential is normally established. Within the stria vascularis, the marginal and basal epithelial barrier layers showed irregular cortical actin patterns, and the vasculature exhibited a highly dilated and abnormally twisted morphology. Importantly, the spiral modiolar artery, which directly supplies blood to the vessels of the stria vascularis and may protect the stria vascularis against high blood pressure, also expresses the S1P2 receptor and responds to the S1P ligand by exhibiting Rho GTPase-dependent vasoconstriction (20). As shown here, the specific S1P2 receptor antagonist JTE013 inhibited S1P-mediated vasoconstriction of the artery, implicating the S1P2 receptor in this process. Because the regulation of vasoconstriction may be an important mechanism to protect adjacent capillary beds from high pressure, the stria vascularis vessels in the S1P2 receptor-null mouse could experience an unusually high pressure load as they develop, resulting in the observed morphologic abnormalities of capillary dilation and distortion. Significantly, an in vivo role for the S1P2 receptor in determining vascular tone has recently been demonstrated (22).

In S1P2 receptor-null mice, structural changes within the

**FIGURE 4. Increased pigmentation in stria vascularis and degeneration of the organ of Corti in S1P2 receptor-null mice.** A–C show light microscopy images of the whole-mount preparation of the stria vascularis and underlying spiral ligament. Intermediate cells of the stria vascularis are pigmented cells, and the pigmentation is seen as brown spots all over the stria vascularis. Note the abnormally increased pigment accumulation in the stria vascularis of one- and six-month-old S1P2 receptor-null mice. Images were obtained by using a 10× objective and differential interference contrast. Scale bar in A is 100 μm and applies to B and C. E–G show degeneration of the organ of Corti in the S1P receptor-null mouse at postnatal day 31. D, middle turn of the organ of Corti of a wild-type control littermate. The rhodamine-phalloidin staining of filamentous actin (red) highlights one row of inner hair cells (IHCs, arrow) and three rows of outer hair cells (OHCs, arrowheads). Arrowheads point to cuticular plates of OHCs with a characteristic V-shape of stereocilia bundles. Cuticular plate of an IHC is indicated by the arrow. The stereocilia of IHCs are present but are not visible, because they are out of the focal plane of this optical section. F, basal turn of the organ of Corti of postnatal day 31 S1P2 receptor-null mouse. Arrow points to IHC stereocilia. Arrowheads point to remnants of severely degenerated OHCs in all three rows. Also shown are middle (F) and apical (G) turns of the organ of Corti of the postnatal day 31 S1P2 receptor-null mouse. Arrows point to IHC stereocilia. Arrowheads point to the degenerating OHCs. Scale bars in D and G are 10 μm. Scale bar in G applies to E and F.

**FIGURE 5. Organization of marginal and basal cell barriers and capillaries in the stria vascularis.** The marginal (A and D) and basal (B and E) cell barriers were visualized by whole-mount phalloidin staining of stria vascularis (green). A and B illustrate the normal morphology of the marginal and basal cell barriers of 3-week-old wild-type mice. D and E illustrate the disorganization of the marginal and basal cell barriers in 3-week-old S1P2 receptor-null mice. The cell nuclei were visualized by DAPI (blue) in A and D. The stria vascularis capillaries of 1-month-old wild-type mice were visualized by isoelectin in C (wild-type) and F (S1P2 receptor-null). Scale bars in A and C are 20 μm. Scale bar in A also applies to B, D, and E. Scale bar in C applies to F.
vascular beds of the stria vascularis, where the S1P2 receptor RNA is relatively highly expressed, may further increase vulnerability to perfusion pressure. The endothelial S1P1 receptor is required to regulate the interaction of endothelial cells and associated smooth muscle cells for the formation of stable, intact blood vessels during embryonic development (4, 5). Likewise, the S1P2 and S1P3 receptors, functioning in concert with the S1P1 receptor, participate in the formation of an intact embryonic vasculature, as shown by the resulting hemorrhage after their simultaneous genetic deletion (6).

As has been described previously (19, 23–28), a defect in the stria vascularis can result in the inability to produce an endocochlear potential resulting in deafness and the subsequent degeneration of hair cells and the spiral ganglion neurons as seen in the S1P2 receptor-null mice. The vestibular otoconia abnormality in the S1P2 receptor-null mice is also consistent with the altered homeostasis of inner ear fluids (29). Thus, the early failure in structure and function of the stria vascularis is sufficient to explain subsequent inner ear findings in the S1P2 receptor-null mice.

The essential involvement of the S1P2 receptor in auditory function suggests the possibility that defects in S1P signaling may underlie some inherited forms of deafness in humans. Furthermore, pharmacologic enhancement of the signaling pathway may be explored as a possible therapeutic approach in vascular-related inner ear pathologies.

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Addendum—After this work was largely completed, a paper appeared in press describing that the S1P2 receptor is essential for auditory and vestibular function in a independently derived line of S1P2 receptor-null mice (30).

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