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Gangliosides and hearing

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ABSTRACT

Severe auditory impairment observed in GM3 synthase-deficient mice and humans indicates that glycosphingolipids, especially sialic-acid containing gangliosides, are indispensable for hearing. Gangliosides associate with glycoproteins to form membrane microdomains, the composition of which plays a special role in maintaining the structural and functional integrity of hair cells. These microdomains, also called lipid rafts, connect with intracellular signaling and cytoskeletal systems to link cellular responses to environmental cues. During development, ganglioside species are expressed in distinctive spatial and temporal patterns throughout the cochlea. In both mice and humans, blocking particular steps of ganglioside metabolism produces distinctive neurological and auditory phenotypes. Thus each ganglioside species may have specific, non-overlapping functions within the cochlea, central auditory network, and brain.

1. Background

Deafness is the most common hereditary disability in humans, affecting 1 per 1000 newborns, and more than 40% of people older than 65 years suffer from age-related hearing loss. Cell-surface glycoconjugates—proteoglycans, glycoproteins, and glycosphingolipids (GSLs)—have specific spatial and temporal patterns of expression in the developing cochlea and appear to subserve important roles in hearing [1]. Gangliosides (sialic acid-containing GSLs) are abundant in mammalian brains, where they typically reside in the outer leaflet of cell membranes and cluster in microdomains (lipid rafts) specialized for adhesion and signaling [3,4]. They comprise 0.5% of mature human brain (~1.8% of non-water brain weight) [5], have 3-fold greater abundance in gray relative to white matter, and are requisite for the normal growth, differentiation, and maintenance of neural tissues [2].

GM3 synthase (a.k.a lactosylceramide sialyltransferase; SIAT9, ST3GAL5) is encoded by *ST3GAL5* and mediates the sialylation of lactosylceramide (LacCer) to form GM3, the root structure for all downstream a- and b-series gangliosides (Fig. 1A) [6–8]. B4GALNT1 subsequently transfers *N*-acetylgalactosamine to LacCer, GM3, or GD3 to form GA2, GM2 or GD2, respectively. Based on this enzymology, various patterns of systemic ganglioside deficiency have been modeled in transgenic mice [9].

St8sia1 −/− (GD3 synthase null) mice lack b-series gangliosides (GD3 and its downstream derivatives) and suffer from thermal hyperalgesia and mechanical allodynia [10]. *B4galnt1* −/− mice express only two ganglioside species (GM3 and GD3) and manifest delayed nerve conduction, axonal degeneration, and ataxia [11,12]. *B4galnt1* −/−: *St8sia1* −/− double knockout animals express only GM3 and have fatal audiogenic seizures and peripheral neuropathy [7,13]. Knocking out both *St3gal5* and *B4galnt1* produces mice deficient of GM3 as well as all downstream a-, b-, and o-series gangliosides; these animals have lethal neurodegenerative disease characterized by micrencephaly, aberrant axon-glial interactions, and axonal degeneration [14].

We generated *St3gal5* −/− mice lacking GM3 as well as a- and b-series gangliosides [9]. These animals have no overt neurodegenerative phenotype, perhaps owing to compensatory B4galnt1-mediated production of o-series ganglisosides [14]. *St3gal5* −/− mice do not startle in response to sound but startle normally to puffs of air, which suggests hearing impairment [15]. This is corroborated by electrophysiological and

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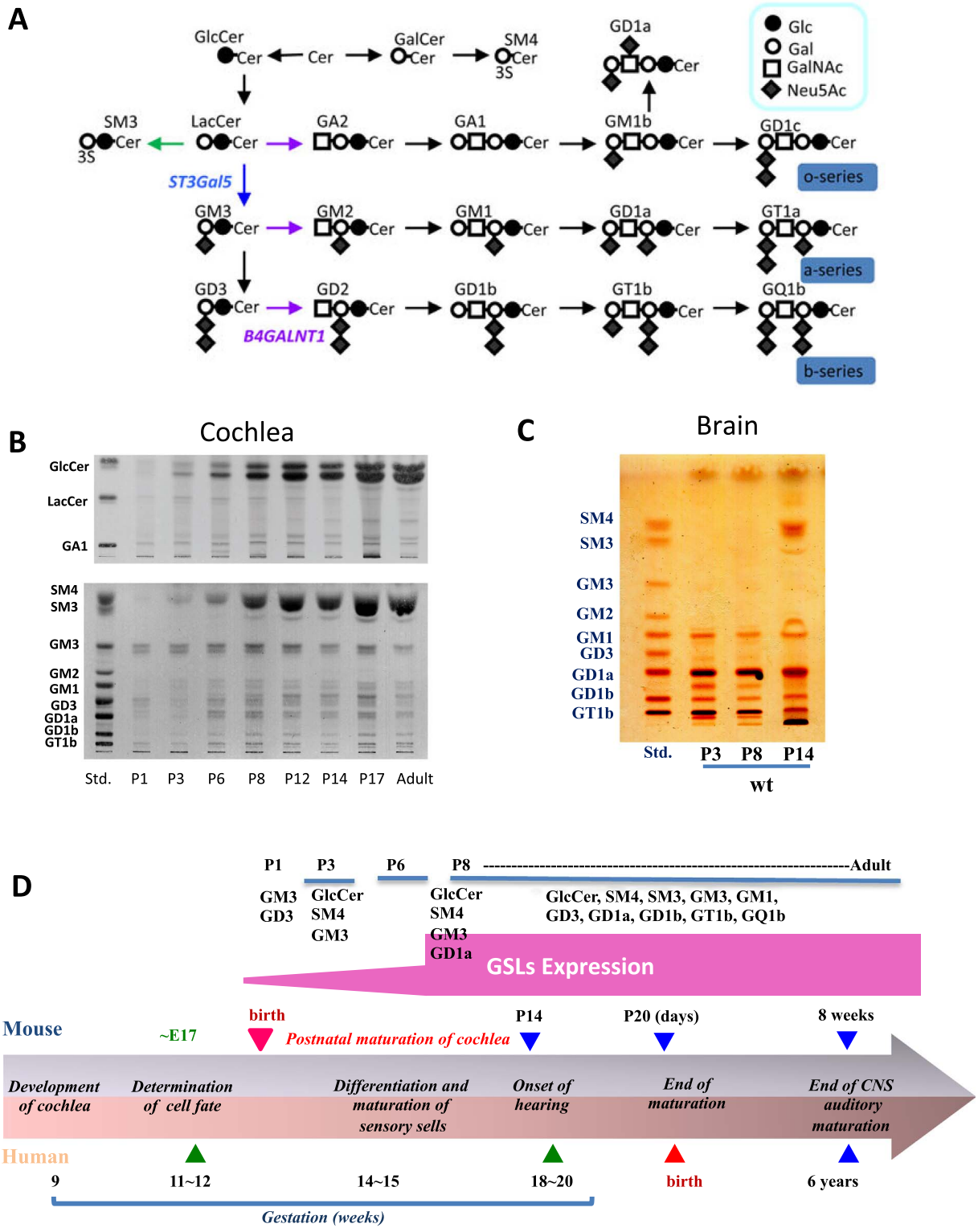


Fig. 1. Marked increase of GSLs in cochlea during postnatal maturation period. (A) Schematic presentation of GSL biosynthesis. (B) High performance-thin-layer chromatograms (HPTLC analysis of gangliosides in cochlea of wild-type mice during the postnatal maturation period. Upper panel is neutral glycolipids and lower panel is acidic glycolipids. Cochlea were isolated and subjected for the purification of GSL analysis as described in ref. [16]. Each purified fraction was spotted as 2 mg protein per lane. (C) HPTLC analysis of gangliosides in whole brain of wild-type mice during the postnatal maturation period (D) Summary of stage-specific expression of GSLs during postnatal maturation of cochlea based on the information of Fig. 1B and LC-MS/MS analysis of ganglioside species reported in ref. [16]. Comparative development of the cochlea of both humans and mice are also shown.

histological analyses of the auditory system, which reveal selective degeneration of the organ of Corti [15,16]. Interestingly, auditory impairment is the herald sign of disease in newborns with ST3GAL5 deficiency [16], indicating that GM3 may be indispensible for murine and human hearing.

2. Scope of review

We provide an overview of glycoconjugate expression in the murine auditory system and consider the association between ganglioside deficiency and hearing, including recent auditory findings in ganglioside-

Table 1
Phenotypes of hearing loss in mice depleted enzymes related to glycoconjugate metabolism.

Mouse	Type of hearing loss	Symptom, histology	Ref.
Neu1 knockout (Lysosomal sialidase)	Conductive	Inflammation in middle ear Thickened mucosa	[51]
	Sensorineural	Reduce of endolymphatic potential	
Fbs2 knockout (Ubiquitin ligase of glycoproteins)	Sensorineural (progressive)	Degradation of supporting cells	[52]
Cmah knockout (Sialic acid metabolism)	Sensorineural(reducing hearing ability with age)	Degradation of outer hair cells	[53]
Mucopolysaccharidosis type VII model (Lysosomal glucuronidase)	Hearing loss with age	No obvious cell loss	[54]
ST3GAL5 knockout (GM3 synthase)	Sensorineural	Degeneration and loss of outer and inner hair cells	[15,16]
Prosaposin knockout (Glycosphingolipid catabolism)	Sensorineural (progressive)	Hypertrophy of hair cells and dieters cells Loss of outer hair cells	[55]
SaposinB knockout (Glycosphingolipid catabolism)	Sensorineural	Degeneration of spiral ganglions	[56]

deficient mice and humans.

3. Major conclusions

3.1. Hearing loss in human and animals with disorders of glycoconjugate metabolism

Abundant glycans (carbohydrate portions of glycoconjugates) in cochlear tissue have been reported from 1980s using various stainings such as reagents, lectins and antibodies against specific glycan [15–18,36–50]. Further insights about the relationship between glyconjugates and hearing emerged from studies of transgenic mice. Deletion of enzymes that synthesize (Cmah and St3Gal5) and catabolize (Neu1, Fbx2, glucuronidase, and Saposins) glycoconjugates cause hearing loss in mice, and each enzymopathy entrains a particular pattern and mechanism of hearing loss (Table 1) [15,16,51–56].

Mixed type (conductive and sensorineural) hearing loss is observed in humans with lysosomal storage diseases (LSDs) and congenital disorders of glycosylation (CDGs), which result from mutations that interfere with the catabolism and assembly of glycoconjugates, respectively. Nerve defects and middle ear disease are observed in these patients, but the molecular mechanisms of hearing loss may be more specifically related to alterations of glycoconjugate expression within the cochlea (Table 2, [57–66]).

The study of autosomal recessive ST3GAL5 (GM3 synthase) deficiency provides the strongest link between ganglioside metabolism and auditory function in humans. Biallelic damaging *ST3GAL5* variants cause infantile-onset epileptic encephalopathy, slow brain growth, stagnant psychomotor development, growth failure, blindness, dyspigmentation, and deafness [19–23]. We examined eight children (ages 4.1 ± 2.3 years) from the Old Order Amish community who were homozygous for pathogenic *ST3GAL5* c.694C > T variants [16].

Table 2
Hearing loss in human LSDs and CDGs.

Human diseases	Causative gene	Ref.
Lysosomal storage disorders		
· Gaucher disease	Glucocerebrosidase	[57]
· Fabry disease	Alpha-galactosidase	[58]
· Mucopolysaccharidosis	hydrolases of glycosaminoglycans	[59]
· Pompe disease	Alpha-glucosidase	[60]
· Sialidosis	Neuraminidase	[61]
· GM2 gangliosidosis	Beta-hexosaminidase	[62]
· Mannosidosis	Mannosidases	[63]
Congenital disorder of glycosylation		
· Type Ig	ALG12	[64]
· Type II	SLC35A2 (UDP-Gal transporter)	[65]
	SLC39A8 (Manganese transporter)	[66]

Plasma of affected children had undetectable GM3 and downstream a- and b-series derivatives accompanied by elevations of LacCer, globoside, and paragloboside species. All *ST3GAL5* c.694C > T homozygotes had the characteristic phenotype of slow postnatal head growth, intractable epileptic encephalopathy, severe psychomotor delay, visual impairment, and hearing loss; the latter manifesting as a failed newborn hearing screen.

Auditory brainstem responses (ABRs) had abnormal thresholds in all ears tested and cochlear microphonics (CM) were consistently absent (Table 3). One child had no reproducible ABR waves. Wave I was observed in one subject, questionable in another, and absent in remaining patients. In six children, only Waves III and V could be clearly defined. Wave latencies were normal (80 dB) in the majority of patients. In most ears, comparison of condensation and rarefaction responses revealed waveform phase reversals (Fig. 2). Although cortical auditory-evoked potentials (CAEPs) were detectable in all 8 *ST3GAL5* c.694C > T homozygotes (Table 3), their morphology was abnormal in 7 (88%) and P2/N2 latency was delayed in 6 (75%).

3.2. Gangliosides and mammalian auditory development

3.2.1. Significant increase of glycosphingolipids during postnatal maturation of murine cochlea

We investigated the expression of GSLs in wild-type murine cochlea during the postnatal period of auditory system maturation. Healthy mice begin to recognize sound by postnatal day 12 (P12), designated the 'onset of hearing'. As shown in Fig. 1B, GM3 is the dominant cochlear GSL at P1. After P3, there is marked increase of cochlear GM3 as well as other GSLs, including glucosylceramide, complex gangliosides (GM1, GD1a, GD3, GD1b, GT1b), and sulfatides (SM3 and SM4) (Fig. 1B). In contrast, expression of cerebral gangliosides reaches a stable, mature pattern as early as P3 (Fig. 1C).

Significant alterations of ganglioside expression in murine cochlea after P3 reveal the vital role these molecules play in hearing onset [16]. After auditory maturation, GM3 and GM1 are discretely distributed among inner hair (IHC), outer hair (OHC), Dieters, and pillar cells of the organ of Corti (Table 4). Both IHCs and OHCs express GM3, which is preferentially distributed to the apical surface, cuticular plate, and stereocilia. GM1 is the primary IHC ganglioside and predominates on the apical surface of supporting cells, but is largely absent from OHCs (Fig. 3) [16].

3.2.2. Glycocalyx integrity and membrane cycling in GM3 synthase-deficient mice

Apical membranes of stereocilia are covered with a glycocalyx composed of sialic acid-containing glycoproteins and glycolipids (including gangliosides) [24] to create a dense negative charge field that normally prevents fusion of adjacent stereocilia. In experimental animals, aminoglycoside administration reduces expression of sialo-

Table 3
Auditory brainstem responses and CAEPs in children homozygous for ST3GAL5 c.694C > T.

Age	Auditory brainstem responses												CAEPs			
(years)	CM		Morphology		Waves (80 dB)		Thresholds		Latency (80 dB)		Phase reversal		Morphology		Latency	
	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right
1.6	Abs	Abs	–	–	Abs	Abs	–	–	–	–	–	–	Abn	Abn	D	D
2.0	Abs	Abs	Abs	Abs	I, III, V	I, III, V	50	50	N	N	No	Yes	Abn	Abn	D	D
2.8	Abs	Abs	Abs	Abs	III, V	III, V	70	70	D	D	Yes	Yes	Abn	Abn	D	D
3.1	Abs	Abs	Abs	Abs	III, V	III, V	80	80	N	N	No	Yes	Abn	Abn	D	D
3.6	Abs	Abs	Abs	Abs	III, V	III, V	80	80	N	N	Yes	Yes	Abn	Abn	D	D
4.5	Abs	Abs	Abs	Abs	I[a], III, V	I[a], III, V	DNT	DNT	N	N	Yes	Yes	Abn	Abn	N	N
7.2	Abs	Abs	Abs	Abs	III, V	III, V	60	50	N	N	No	Yes	N	N	N	N
7.9	Abs	Abs	Abs	Abs	III[a], V[a]	III[a], V[a]	80	80	N	D	No	a	Abn	Abn	D	D

Abn, abnormal; Abs, absent; CM, cochlear microphonic; D, delayed; N, normal; DNT, did not test.
[a] Equivocal result.

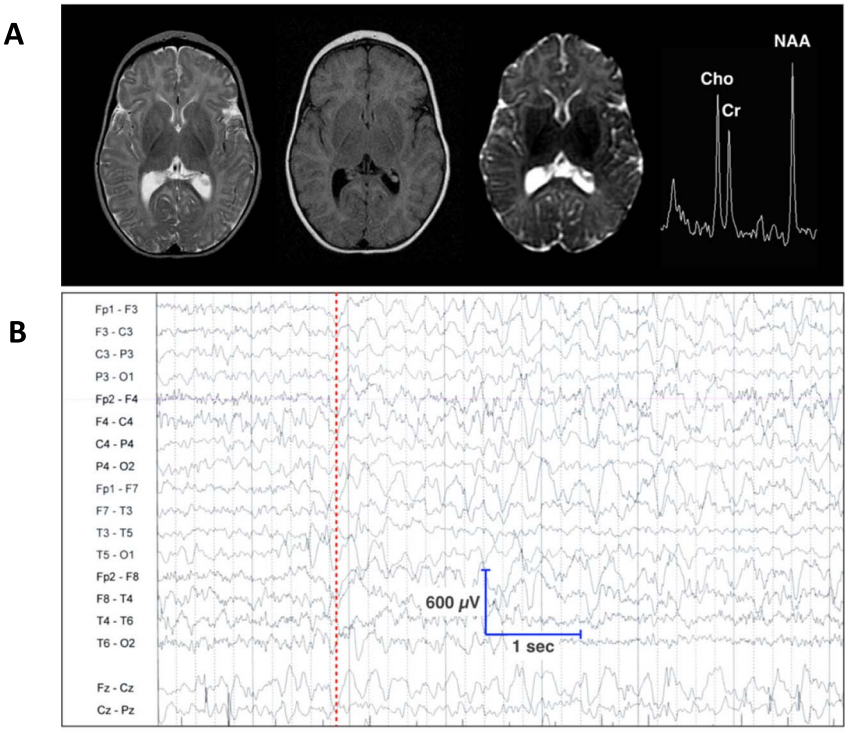


Fig. 2. Neurological and auditory phenotype of ST3GAL5 (GM3 synthase) deficiency in humans. (A) From left to right, T2, fluid-attenuated inversion recovery, diffusion-weighted, and 1H-MR spectroscopy at 1.5 T show normal cortical and subcortical structure, but severe hypomyelination and increased water diffusion throughout the corona radiata, as well as elevated choline (Cho) relative to creatine–phosphocreatine (Cr) and N-acetylaspartate (NAA) 1H signals (single voxel PRESS, TE 144/TR 1500). (B) Electroencephalogram of a 2 year-old child homozygous for ST3GAL5 c.694C > T shows a slow, chaotic background, absent posterior rhythm and no discernable change through a sleep–wake cycle. Beginning at the dashed red line, there is a burst of generalized, frontal predominant, 3 Hz spike-slow wave discharges in excess of 450 µV. (C) In the majority of affected children, auditory brainstem responses show waveform reversals [16].

Table 4
Comparison of GM3 and GM1 expression of specific regions in the organ of Corti at P14.

	OHC			IHC			DC		PC
	Stereocilia	Cuticular plate	Cell body	Stereocilia	Cuticular plate	Cell body	Cell surface	Cell body	Cell body
GM3	++	+	–	++	+	–	–	+	–
GM1	–	–	–	++	+	+	++	+	+

OHC; outer hair cell, IHC; inner hair cell, DC; Deiters cell, PC; pillar cell.

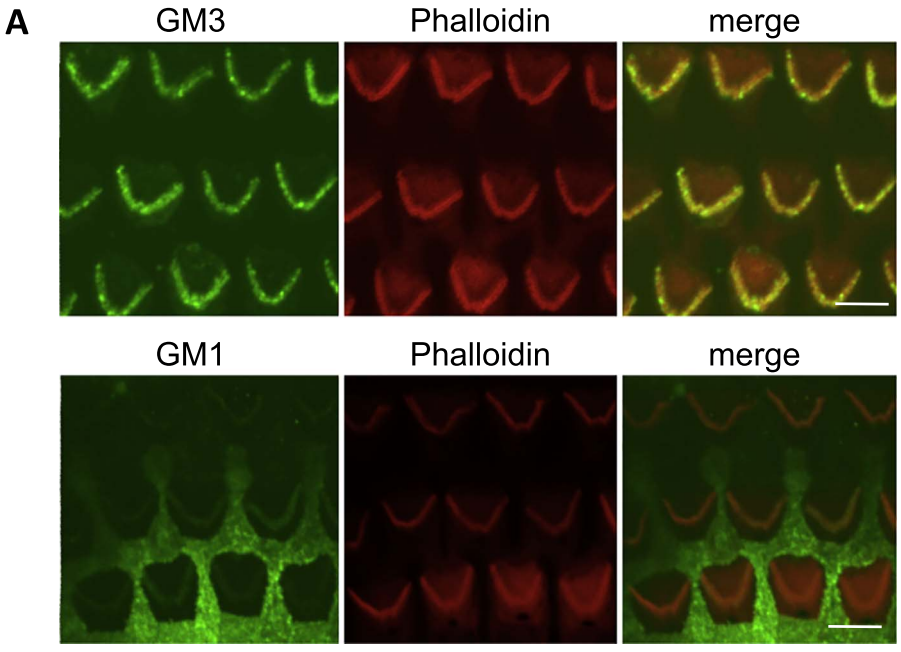
glycoconjugates in the OHC glycocalyx and precipitates fusion of stereocilia [25,26].

To better define the role of various ganglioside species in hearing, we compared auditory function between *St3gal5* −/− and *B4galnt1* −/− mice, which have distinctive patterns of GSL expression within the organ of Corti (Fig. 4A). Murine *B4galnt1* −/− cochlea has rich GM3 content, intact hair bundles, and normal auditory function whereas *St3gal5* −/− cochlea, devoid of GM3, are associated with deafness (Fig. 4B). Using confocal laser microscopy with phalloidin staining, we observe normal IHC and OHC morphology in 4 week-old *B4galnt1* −/− mice (Fig. 4C and D). In contrast, *St3gal5* −/− OHCs have blebs and intracellular vesicles, the latter composed of membranous structures (Fig. 4E), indicative of unbalanced endocytosis and exocytosis [16]. As noted above, GM3 is expressed in both IHCs and OHCs of wild-type mice, whereas only IHCs express GM1 (Table 4). This may explain in part why OHCs degenerate in advance of IHCs in *St3gal5* −/− mice.

3.2.3. GM3-enriched membrane organization, PTPRQ-myosin VI complex localization and hair cell morphology

Hair cells are specialized to mediate auditory and vestibular transduction. Projecting from their apical surface are filopodial processes called stereocilia, each filled with hundreds of cross-linked actin filaments. Certain proteins—e.g. unconventional myosins, Usher proteins, and some deafness-related gene products (e.g. PTPRQ, cadherin23, protocadherin15, Usherin, and VLGR1)–are expressed predominantly or exclusively in stereocilia to form a structured, interactive network that supports mechanoelectrical transduction (Fig. 5) [27].

In the normal organ of Corti, functional complexes of PTPRQ and myosin VI appear critical to maintaining the integrity of this network [28]. PTPRQ, a shaft connector at the tapered base of stereocilia, is composed of an extracellular domain containing 18 fibronectin III (FNIII) repeats, a membrane spanning domain, and a cytoplasmic domain with both phosphatidylinositol and tyrosine phosphatase activities [29,30]. Myosin VI is an actin-based motor protein that associates



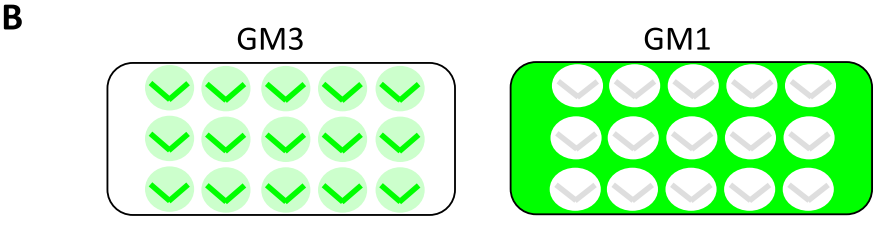


Fig. 3. Comparison of gangliosides expression in hair cells of mice aged 4 weeks. (A) Whole-mount immunostaining was performed. GM3 (upper panel, green) and GM1 (lower panel, green) of OHC stereocilia. Only GM3 was highly enriched in OHC stereocilia without staining of GM1. On the other hand, the expression levels of GM1 but not GM3 was especially high on the surface of supporting cells. (B) Schematic images of distinct expression of GM3 and GM1 in OHC.

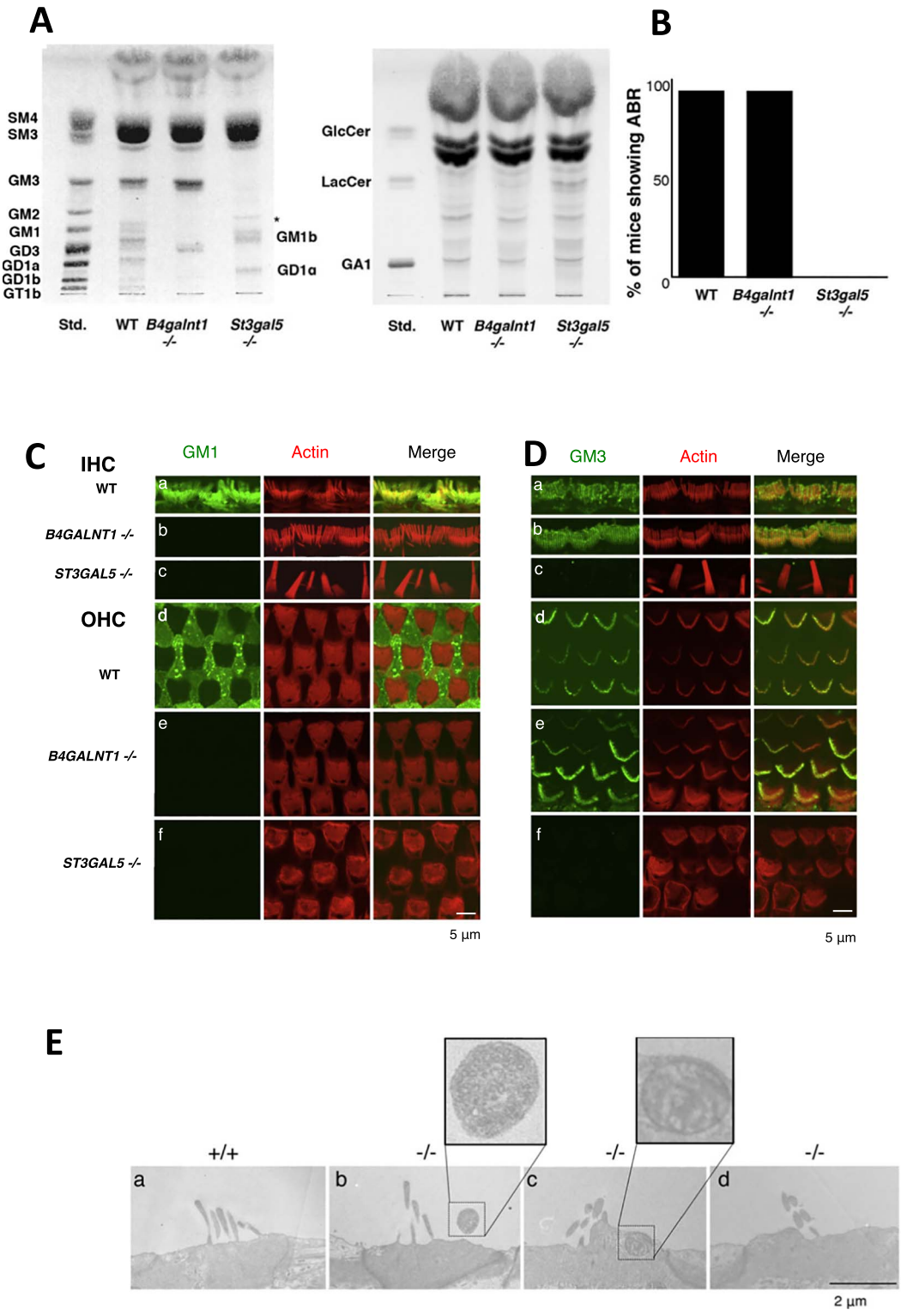


Fig. 4. Identification of GM3 as an essential molecule for hair cell function. (A) HP-TLC of acidic (left) and neutral (right) GSLs from the cochlea of 4-week-old wild-type (WT), B4galnt1 −/− and St3gal5 −/− mice. The cochlea of B4galnt1 −/− mice were mainly expressed GM3 and GD3. Each purified fraction was spotted 2 mg protein per lane. (B) Auditory-evoked brainstem response (ABR) of wild-type, B4galnt1 −/− and St3gal5 −/− mice at 4 weeks old. (C and D) Comparison of gangliosides expression in hair cells among wild-type, B4galnt1 −/− and St3gal5 −/− mice aged 4 weeks. Whole-mount immunostaining was performed. GM1 (C, green) and GM3 (D, green) of IHC (a–c) and OHC (d–f) stereocilia in wild-type (a and d), B4galnt1 −/− (b and e) and St3gal5 −/− (c and f) mice. In IHC, both GM1 and GM3 were expressed along the stereocilium from the taper region to top (Ca and Da). On the other hand, only GM3 was highly enriched in OHC stereocilia (Dd) without staining of GM1 (Cd). The stereocilia of IHC and OHC in B4galnt1 −/− mice were expressed GM3 but not GM1 (Cb and Db). In St3gal5 −/− mice, fused stereocilia was observed in IHC (Cc and Dc) and irregular phalloidin staining was observed in OHC (Cf and Df). Notably, the expression levels of GM1 but not GM3 was especially high on the surface of supporting cells (Deiters cell) (Cd and Dd). (E) TEM images of OHC in St3gal5 +/+ and −/− mice at P17 [16].

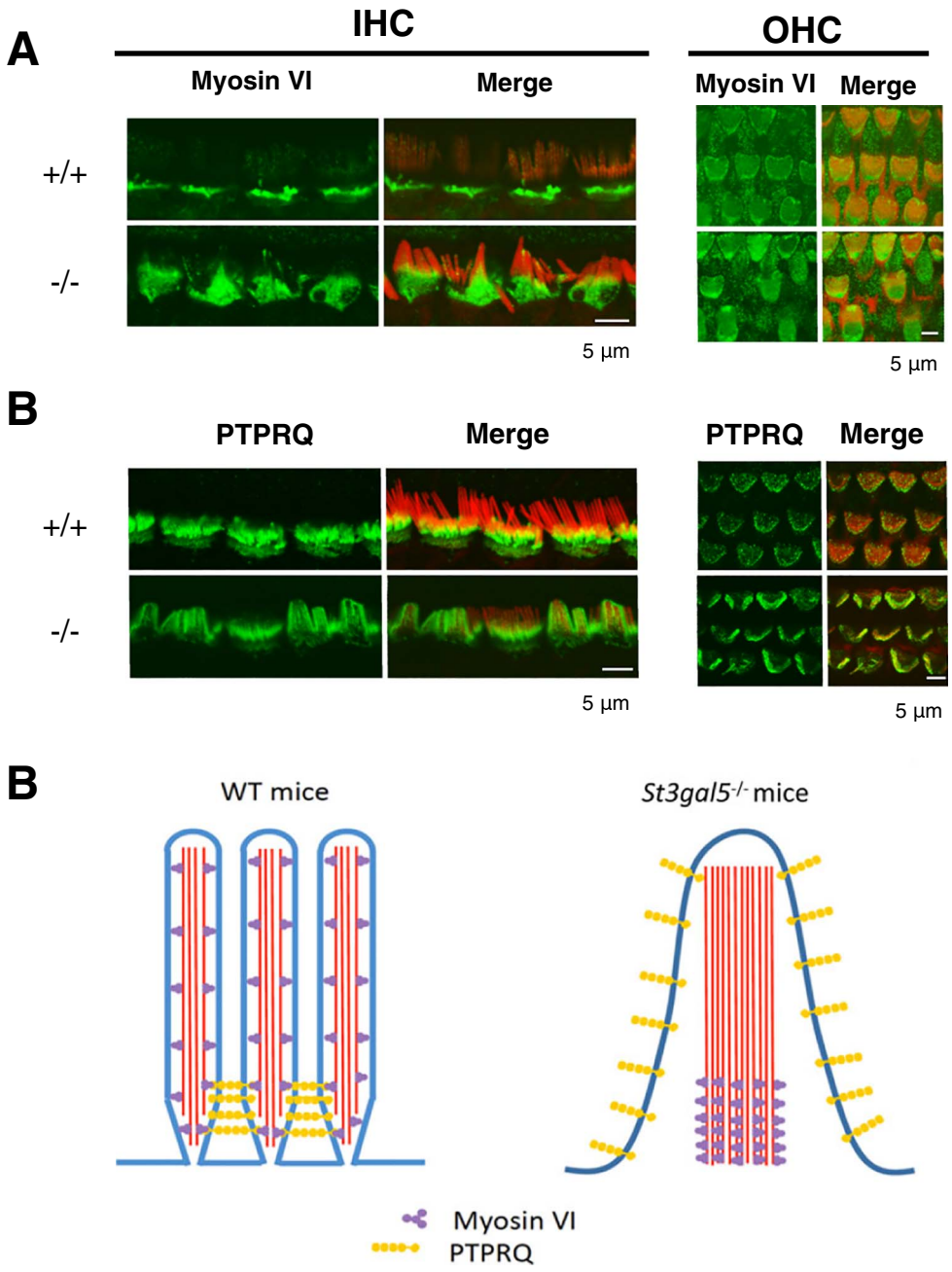


Fig. 5. Dislocalization of PTPRQ and myosinVI in the stereocilia of St3gal5 null mice. Confocal images showing stereocilia of IHCs and OHCs of St3gal5 +/+ and −/− mice stained for myosinVI (A) PTPRQ (B) (green) and F-actin (phalloidin, magenta). (C) Schematic images for dislocalization of PTPRQ and myosin VI in the IHC stereocilia of St3gal5 −/− mouse [16].

with PTPRQ to form a complex mediating interaction between the plasma membrane and cytoskeleton [31]. Mice lacking PTPRQ are deaf and lack tapering at the base of stereocilia, which become fused [28]. Myosin VI-deficient mice are also deaf and have comparable structural changes of hair cells accompanied by maldistribution of PTPRQ along the length of stereocilia [28,32,33].

In bull frogs, PTPRQ colocalizes with ganglioside-rich membrane domains in basal stereocilia [34]. We used immunostaining to further investigate the relationship between gangliosides and the basal PTPRQ-myosin VI complex (Fig. 5). In wild-type murine IHCs, PTPRQ localizes exclusively to the stereocilia base, whereas PTPRQ is maldistributed along shafts of fused *St3gal5* −/− stereocilia (Fig. 5B) and myosin VI is present from base to midshaft but absent from distal regions (Fig. 5A). Such structural disturbances likely reflect loss of normal ciliary motor action. In *St3gal5* −/− OHCs, myosin VI expression is concentrated at the surface of the cuticular plate, close to vestigial kinocilia (Fig. 5A).

Together, these observations suggest that membrane microdomains enriched with GM3 are crucial to the formation and proper localization of PTPRQ-myosin VI complexes in hair cells. In the absence of GM3, aberrant expression of PTPRQ-myosin VI complexes has important structural consequences for stereocilia and likely impairs their ability to transduce auditory signals (Fig. 5C).

4. Conclusions

Our results implicate a specific and indispensable role for GM3 in the development, function, and viability of cochlear hair cells. Although hearing is already impaired at birth in humans with GM3 synthase deficiency, the murine *St3gal5* −/− auditory phenotype indicates that ganglioside repletion might partially rescue auditory function during a critical postnatal window. These findings inform our understanding of hearing loss in humans with ST3GAL5 deficiency, but do not explain the broader neurological phenotype associated with this condition (Fig. 2). Thus future studies should more carefully delineate the role of lipid

rafts generally, and GM3 specifically, in the development and physiology of the central nervous system.

New concepts and techniques will allow us to determine novel therapies for hearing impairment. In a recent report, functional hair cell-like cells were generated from embryonic and induced pluripotent stem cells [35]. This and other strategies will give rise to new frontiers of hearing research, further advancing studies of glycoconjugate-rich microdomains and their auditory actions. We hope such research will engender new and effective therapies to restore hearing in humans.

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