The Role of Saposins in Auditory and Vestibular Systems

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Abstract

Prosaposin, and its cleaved byproducts saposin A-D, are crucial proteins necessary for glycoshpingolipid degradation and metabolism. Mutations in these proteins can lead to devastating lysosomal storage diseases, including Gaucher's disease, symptoms of which often include hearing loss. Until recently, little was known about the role that prosaposin and saposins A-D played in auditory and vestibular system function. Early studies in knockout mouse models showed prosaposin to be important for normal cochlear innervation and maintenance of normal hearing (Akil et al., 2006). Severe vestibular dysfunction paired with inclusion body accumulation in the vestibular end-organs of prosaposin KO mice suggests its role in the maintenance of normal hearing and the maturation of normal vestibular system function (Akil et al., 2012). To determine if these phenotypes were attributable to the loss of prosaposin as a whole or one of its constituent proteins, saposin A-D, KO mice of individual saposins were subsequently studied. Given the nature of the hearing loss, as well as efferent and afferent neuronal sprouting in the prosaposin KO mouse, it was hypothesized that saposin C, a protein known for its neurotigenic properties, was responsible for these changes. However, a null-mutant mouse lacking both saposin C and D showed no effect on hearing (Lustig et al., 2015). In contrast, a loss of functional saposin B led to a progressive sulfatide accumulation in satellite cells around cochlear spiral ganglion (SG) neurons resulting in satellite cell degeneration, SG degeneration, and ultimately, loss of hearing (Akil et al., 2015). While saposin B KO mice did not show any vestibular dysfunction phenotype, vestibular evoked potentials demonstrated profound vestibular dysfunction likely attributable to the large-scale neuronal degeneration (Akil et al., 2015). Furthermore, the data suggests that saposin B appears to have a much greater role in auditory neuronal maintenance and balance than saposin C and/or D.

Key Words: prosaposin, saposin A, B, C, D, glycosphingolipid hydrolases, cochlea, hearing, balance, vestibular, auditory

1. Introduction

Prosaposin and its cleaved byproducts saposin A-D are crucial proteins necessary for glycoshpingolipid (GSL) degradation, mutations of which can lead to devastating lysosomal storage diseases such as Gaucher's disease. These proteins are important targets of study as they offer potential treatment for such disorders as well as offer insight into neuronal pathfinding. Until recently, little was known about prosaposin or saposins' role in the ear but many of these lysosomal storage diseases include symptoms of progressive hearing loss in patients, and thus draw interest to the ear. Here we review what is known about prosaposin and each saposin (A-D) constituent as they relate to auditory and vestibular function.

1.1 Sphingolipid Overview

Sphingolipids, also known as glycosylceramides, are a class of lipids, made up of different combinations of an amide-linked fatty acid and a long-chain (sphingoid) base, that were discovered in 1870 by Johann Thudicum when examining brain extracts. Thudicum deemed the enigmatic chemical structure "sphinx-like" and so named the molecules 'sphingosin' (reviewed by Hakomori, 1983). The biochemistry of these and related compounds are based on a ceramide backbone. Ceramides are sphingolipids with an R group consisting solely of a hydrogen atom. Sphingomyelin is similar to a ceramide but the R group is phosphocholine. If the R group is a sugar monomer, the compound is a cerebroside, and if it is a sugar polymer it is termed a globoside; together these two compounds are known as glycosphingolipds (GSLs).

As a group, the sphingolipids are critical for neuronal signal transmission and cell recognition. Initially they were believed to only serve as structural components of cell membranes, but it is now recognized that they are involved in signaling cell fate as well as neuronal signaling (reviewed by Colombaioni et al., 2004). Sphingolipd metabolites have a clear role as signal transducers involved in cell differentiation, migration, growth, apoptosis, proliferation, and survival (Colombaioni et al., 2004). Clinically they are important - genetic and metabolic abnormalities of these compounds result in a number of diseases with a variety of neuronal deficits, including Fabry disease, Krabbe disease, metachromatic leukodystrophy, Niemann-Pick disease, and Tay-Sachs disease (Sandhoff et al., 1970).

Sphingolipid activator proteins, termed saposins, are four small glycoproteins all generated in lysosomes from a single precursor protein, prosaposin (Kishimoto et al., 1992). Each of the four saposins A-D are necessary for activity of specific lysosomal glycosphingolipid hydrolases (GSLs) involved in the metabolism of various sphingolipids (Sun et al., 2002). These proteins are similarly important as individual saposin mutations are associated with lysosomal storage diseases, some associated with progressive hearing loss such as Gaucher's disease (Holtschmidet et al., 1991; Paton et al., 1992; Bamiou et al., 2001; Campbell et al., 2004).

1.2 Prosaposin Overview

Prosaposin is a multifunctional protein (70kDa) encoded by a single gene on chromosome 10 with extra and intra cellular functions (Inui et al., 1985; O'Brien et al., 1988; Fujita et al., 1996). Intracellularly, prosaposin is cleaved into constituent lysosomal glycoproteins saposin A-D that degrade specific glycosphingolipids through activation of GSL hydrolases.

Prosaposin moves from the endoplasmic reticulum to the Golgi and then the endolysosomal compartment where it is proteolytically processed into four homologous saposins of roughly 80 amino acids each (Sandhoff et al., 1998). Extracellularly, prosaposin is involved in lipid transfer and nerve outgrowth properties (O'Brien et al., 1995).

Originally named SGP-1, prosaposin was first isolated in rat then detected in various human tissues, primarily the brain and testes followed by kidney, spleen, and liver (O-Brien et al., 1988). Hineno *et al.* detected uncleaved prosaposin, in various human body fluids, such as cerebrospinal fluid, seminal plasma, milk, pancreatic juice, blood plasma, and bile, suggesting several functions of prosaposin (Hineno et al., 1991). Prosaposin and its constituent saposins are widely expressed in many tissues and can be considered "housekeeping proteins" necessary for lysosomal hydrolysis (Radin et al., 1984). As such, most studies on prosaposin expression have been done in the brain, kidney, and liver with little or no focus in the ear. A null mutation of the prosaposin gene in mice leads to a complex neurodegenerative phenotype as well as severe leukodystrophy and abnormally increased levels of brain gangliosides (Fujita et al., 1996).

1.3 Saposin A-D Overview

Saposins A-D are vital for sphingolipid and membrane degradation at inner lysosomal membranes. They bind lipids and transfer them as water-soluble complexes from vesicular membranes to catabolizing enzymes or acceptor membranes. Each saposin has six conserved cysteine residues forming disulfide bonds, two prolines, as well as an N-glycosylation site and so are incredibly heat stable and resist most proteases (O'Brien et al, 1991). The four saposins are structurally similar to each other, yet the specificity and mode of activation of sphingolipid hydrolases differs.

The first saposin SAP-1, later termed saposin B (SapB), was discovered by Jatzkewitz *et al* in 1964. Research analyzing enzymatic sphingolipid degradation showed water-soluble lysosomal hydrolases cleave their sphingolipid substrates effectively only in the presence of the right detergents (Sandhoff K, 1970; Scholte et al., 1991). Jatzkewitz *et al.* were searching for *in vivo* factors that substitute for detergents when they purified arylsulfatase A and discovered SapB. SapB is a small, unspecific glycoprotein shown to activate hydrolysis of cerebroside sulfate by arysulfatase A and activate hydrolysis of ganglioside G_1 (GM1) by acid β-galactosidease (Sandhoff et al., 2012). As such, SapB is a physiological detergent that facilitates the partial extraction of GSLs from membranes and presents them to the enzymes arylsulfatase A and α-galactosidase A (Sandhoff, 1992). In humans, SapB inherited deficiency causes the progression of metachromatic leukodystropy-like disease caused by arylsulfatase A deficiency (Zhang, 1990).

The second saposin SAP-2, now called saposin C (SapC), was discovered by Ho and O'Brien in 1971 and found to increase activity of the lysosomal enzyme β-glucosidase. Its deficiency leads to a Gaucher-like disease with neurological manifestations due to decreased glucosylceramide metabolism (Zhang, 1990).

As more saposins were identified, the nomenclature changed based on the placement of the four saposin domains in prosaposin, reading from amino- to

carboxyl-terminal, saposins A, B, C, and D (Morimoto et al., 1988). This change cleared up confusion surrounding the naming of SAPs across species and emphasized their common genetic origin.

Saposins A and D were discovered in 1989 and 1988 from cloning and sequencing prosaposin cDNA (Morimoto et al., 1988; 1989). Less is known about these proteins. Fabbro and Grabowsky found saposin A (SapA) to stimulate glucocerebrosidase, and thus its function appears similar to SapC (Fabbro et al., 1991; reviewed by Kishimoto et al., 1992). While no patients with SapA deficiency have been identified, SapA knockout (KO) mouse models exhibit similar pathology to that seen in chronic form of globoid cell leukodystrophy and lysosomal storage disorder Krabbe disease (Matsuda, 2004).

Saposin D (SapD) is the least understood of the saposins. Located near the C-terminus of prosaposin, SapD stimulates sphingomyelinase activity. SapD deficiency, in both mice and humans, has been tied to cerebellar Purkinje cell degeneration.

Mutations in SapD have also been tied to urinary system defects manifested as polyuria and polydipsia (Azuma, 1994; Matsuda, 2004). SapD null mutation led to urinary and cerebellar deficits, yet a combined saposin CD KO mouse led to changes in prosaposin trafficking and ceramide accumulation (Matsuda, 2004).

Saposin research was prioritized when it was observed that patients with mutations in the prosaposin gene developed lysosomal storage diseases accompanied by an accumulation of sphingolipidosis in many tissues (Kishimoto et al., 1992). The liver and brain of patients with Niemann-Pick, Tay-Sachs, Sandhoff, and Gaucher diseases all show considerable excess of saposins in tissues where sphingolipids accumulate. Much work has been done to explore saposins in these organs but it was not until hearing loss phenotypes associated with these diseases were observed that research was done into saposins' role in auditory function. In one study of patients with Gaucher's disease, related to a saposin C-like deficiency, hearing loss was felt to have been attributable to an efferent auditory defect; two-thirds of the patients in the study had abnormal contralateral suppression of otoacoustic emissions (Bamiou et al., 2001). Despite these associations, not much is known about saposins' role in auditory and vestibular systems. In this review we describe the current understanding of the precursor glycolipid, prosaposin, and its four constituent's role in the auditory and vestibular systems.

2. Prosaposin

2.1 Prosaposin in Auditory Function

Initial work describing prosaposin in the auditory system identified the protein in the ear via a yeast-two-hybrid screen, traced its expression in the ear over time, and phenotypically characterized prosaposin KO mice (Akil et al., 2006). This work not only connected prosaposin to the auditory system but also identified prosaposin's role in maintaining normal innervation patterns to the organ of Corti.

Akil *et al.* were screening for protein-protein interactions involved in the rodent efferent auditory system when they identified prosaposin. The authors found diffuse expression of prosaposin in the organ of Corti at birth, gradually localizing to supporting cells, Deiters' cells, inner hair cells (IHCs), inner pillar cells, and the synaptic region of outer hair cells (OHCs) by post-natal day 21 (P21). Additionally,

quantitative polymerase chain reaction (q-PCR) and immunohistology experiments on microdissected OHCs and Deiters' cells localized prosaposin mRNA to both populations, and found that protein was predominantly localized to the apex of the Deiters' cells.

Further work in prosaposin KO mice demonstrated a slight reduction in hearing in mice up to P19 and deafness by P25 accompanied by reduced distortion product otoacoustic emissions from P15 onward (Akil et al 2006) Simultaneously, beginning at P12, the prosaposin KO mice showed histologic organ of Corti changes including cellular hypertrophy in the region of the IHCs and greater epithelial ridge, a loss of OHCs from cochlear apex, and vacuolization of OHCs. Immunofluorescence revealed exuberant overgrowth of auditory afferent neurites in the region of the IHCs and proliferation of auditory efferent neurites in the region of the tunnel of Corti. IHC electrophysiologic recordings from these KO mice showed normal current-voltage curves and responses to applied acetylcholine, indicative of normal hair cell function. This work was supported by later work from Terashita *et al* (2007) who also demonstrated localization of prosaposin within the rat cochlea. Taken together, these results strongly suggest that prosaposin or one of its cleaved products is required for maintenance of adult cochlear innervation patterns in the organ of Corti, and as a result the maintenance of normal hearing (Akil et al., 2006). Clinically, these finding could potentially contribute to the effort to improve neuronal survival for profoundly deaf patients who may be cochlear implant candidates as improved neuronal survival may improve cochlear implant performance (Gao, 1998; Marzella and Clark, 1999; Shepherd and Hardie, 2001; Nakaizumi et al., 2004). Prosaposin and/or one of its derivatives was found to be critical for competent neuronal path finding and subsequent maintenance of normal hearing. Observations of prosaposin KO mice behavior during auditory work led Akil *et al*. to question if prosaposin had a similar role in the vestibular system.

2.2 Prosaposin in the Vestibular System

During these early studies on prosaposin in the cochlea, it was noted that prosaposin KO mice demonstrated behaviors consistent with vestibular dysfunction, including circling, an unsteady gait, and difficulties in maintaining balance. These observations suggest that in addition to its role in hearing, prosaposin also contribute in maintaining vestibular function. The vestibular system is made up of three end-organs: the semicircular canals that detect changes in angular acceleration; the utricle that detects changes in linear acceleration; and the saccule that detects changes in head position with respect to gravity (Walls, 1962; Land, 1999; Spoor et al., 2002). In normal wild-type (WT) mice, prosaposin localizes to the three vestibular end-organs and Scarpa's ganglion as shown by RT-PCR, Western blot analysis, and immunofluorescence (Akil et al., 2012).

Ablation of prosaposin function caused severe vestibular dysfunction on a series of behavioral tasks. Histologically, the KO mice demonstrated an exuberant cellular proliferation below the vestibular hair cells with disruption of the supporting cells. Electron microscopy further demonstrated inclusion bodies and cellular proliferation disturbing the normal neuroepithelial structure of the vestibular end-organs, reminiscent to what was seen in the organ of Corti (Akil et al., 2006). Immunofluorescence staining with anit-

neurofilament F200 and anti-synaptophysin antibodies in the vestibular tissues sections suggested that the cellular proliferation corresponds to afferent and efferent neuronal overgrowth, similar to what was seen in the organ of Corti (Akil et al., 2006). From these studies it was concluded that prosaposin played a role not only in the maintenance of normal hearing, but was also critical in the neuronal maturation processes of the vestibular sensory epithelium and the maintenance of normal vestibular system function.

However, previous work done in prosaposin KO mice, which demonstrated a number of abnormalities within the central nervous system, must be considered while interpreting these findings. These studies found similar pathologic changes as seen in the inner ear, including an inclusion body accumulation with leukodystrophy and gylcosphingolipid accumulation (Fujita et al., 1996; Sun et al., 2002). As a result, it is possible that a central pathology is a key contributor to the balance dysfunction observed in KO mice. Perhaps it is a general central nervous system phenotype observed in prosaposin KO mice as opposed to vestibular dysfunction, or a combination of both.

These initial studies raised the question whether the severe changes seen in organ of Corti and vestibular end-organs were due to the loss of prosaposin itself or one of its cleaved end products, Saposin A-D. This question motivated additional studies exploring the roles of individual saposins in maintaining auditory and vestibular function.

3. Saposin B

Saposin B (SapB) is a non-enzymatic GSL activator protein that has lipid transfer properties and stimulates the degradation of sulfatide, a major lipid component of myelin, as well as other glycolipids (Li et al., 1988; Ciaffoni et al., 2006). SapB facilitates partial extraction of target GSLs from membranes, including sulfatide and globotriaosylceramide, forming soluble protein-lipid complexes and presenting them for cleavage to arylsulfatase A (ASA) and α-galactosidase A, respectively (Sandhoff et al., 1992; Furst et al., 1992). In humans, Sap B deficiency leads to elevated sulfatide levels in the kidneys and brain, variable neurological phenotypes and a metachromatic leukodystrophy-like disease similar to that observed with the deficiency of ASA (O'Brian et al., 1995; Gieselmann et al., 2010; de Hossen et al., 2011).

Metachromatic leukodystrophy (MLD) is an autosomal recessive neuro degener-ative lysosomal disease caused by defective ASA activity. MLD variants are also characterized by accumulation of sulfatides, progressive demyelination, and extensive white matter damage (Hess et al., 1996). Like the human disease, transgenic mice with diminished ASA enzymatic activity develop accumulation of sulfatide in white matter, and an early onset neurological phenotype without evidence of demyelination (Hess et al., 1996). These ASA-deficient mice were also noted to be deaf, lacking auditory brainstem response (ABR). Several cases of MLD-like diseases caused by Sap B deficiency have also been reported with lysosomal storage of cerebroside sulfate (Wenger et al., 1989; Scholte et al., 1991). The Sap B defects in these cases arise from various mutations that produce substitutions for critical cysteine residues, insertions, transversions or obliteration of a glycosylation site (Sandhoff et al., 1992; Deconinkik et al., 2008).

Recently, Sap B deficient mice (B-/-) were developed by introducing a point mutation

into its coding sequence within prosaposin, destroying one of its three essential disulfide bonds (Sun et al., 2008). This mutation leads to an unstable and undetectable Sap B protein, but preserved prosaposin and saposin A, C and D proteins with their related functions. These B-/- mice mimicked the biochemistry and phenotype of the human disease and developed neurological impairment, including ataxia, head tremor, and impaired neuromotor coordination (Sandhoff et al., 2001). At the microscopic level, neuronal storage of sulfatide in the auditory nerve was also identified (Sun et al., 2008).

3.1 Saposin B in Auditory Function

Based on prior work in ASA-deficient mice that exhibited hearing loss (Hess, 1996), additional studies were performed by Akil *et al.* to evaluate cochlear changes in SapB deficient mice to elucidate its role in hearing (Akil et al., 2015). Histological analysis of B-/- mice revealed normal organ of Corti morphology as compared to WT mice but a significant reduction of spiral ganglion (SG) and cochlear nerve fibers in Rosenthal's canals that progressed with age. As opposed to prosasposin KO mice, neuronal overgrowth was not observed. Further work showed that while gross morphology of the SG was identical to WT mice, there were abnormal inclusions in B-/- satellite cells highly correlated to loss of SG neurons that worsened with age. The inclusion bodies in B-/- mice satellite and SG cells were determined to be sulfatide accumulation (Akil et al., 2015).

These findings in the SapB KO mouse shed important light on the role of saposins in auditory function. The histological results from these data indicate a progressive degeneration of SG cells and nerve fibers in the B-/- cochlea. The osseous spiral lamina, Rosenthal canal, and modiolus, which normally harbor the dendritic and neurotic processes of the SG neurons, were almost completely devoid of SG neurons and myelinated nerve fibers. In contrast, the overall architecture of the organ of Corti and the stria vascularis were structurally unaltered in the B-/-mice. The pattern of SG alteration resembled the one found in ASA-deficient mice (Hess et al., 1996). Histological study of the SG neurons revealed a progressive accumulation of inclusion bodies in the satellite cells surrounding the SG cells starting from P1mo onwards. The degree of storage accumulation in the satellite cells increased in older mice, which may have been the cause of SG degeneration that took place between P4mo and P15mo in the B-/- mice. However, the most severe alterations of SG neurons occurred between 4 and 8 months of age in the ASA-deficient mice (D'Hooge et al., 1999), indicating that in the B-/- mice, there was a milder and slower neurological and neuropathological pheonotype to that observed in the ear of the ASA (-/-) mice (Coenen et al., 2001). Similar observations were also seen for other organs in the B-/- mice (Sun et al., 2008). The exact reason for this difference between the B-/- mice and the ASA-deficient mice is unknown. It is possible that a lack of alternative ASA-independent pathways for sulfatide metabolism is responsible for the difference (Sundaram et al., 1995). It is also possible that, despite undetectable levels of Sap B, the mouse model still has residual SapB function based on the nature of the mutation. Alternatively, SapB deficiency may lead to accumulation of fewer GSLs than in ASA-deficient mice, and hence a more mild phenotype (Akil et al., 2015).

In addition to characterizing the histological

changes in the organ of Corti in SapB KO mice, auditory physiology was also studied using auditory brainstem response (ABR) testing (Akil et al. 2015). No significant differences in hearing between the B-/- mice, heterozygote and WT mice were observed until P6mo. However beyond 6 months, a significant increase of ABR thresholds were observed for each of the pure tone-burst stimuli (8, 16 and 32 kHz), but not click stimuli, in the B-/- mice. Increases in ABR click stimuli thresholds in the B-/- mouse were not seen until P13mo. No defect in OHC function was observed in B-/- mice through P8mo despite 57% of SG being lost.

It is interesting to note that ABR click stimuli thresholds were normal until P13mo, despite the severe morphologic changes seen in the spiral ganglion neurons developing during this same time period. Similar results have been documented in mice models of hearing recovery after noise exposure, in which even ‘‘reversible’’ noise exposure with recovery of auditory thresholds leads to long-term afferent nerve terminal degeneration while retaining ‘‘normal’’ ABR thresholds (Kujawa et al., 2009; Lin et al., 2011).

These results imply a causative effect of Sap B deficiency on sphingolipid accumulation in inclusion bodies leading to neurological degradation and reduced hearing. The observed pattern of hearing loss in both ASA-deficient and B-/- mice reflects the morphologic changes observed (Akil et al., 2015). ASA-deficient mice showed greater ABR threshold shifts at an earlier age, correlating with deterioration of the SG neurons and nerve fibers (D’Hooge et al., 1999). The intact efferent response is suggestive of several possibilities. The efferent system may be more robust than the afferent neuronal system to loss of Sap B, and/or efferent neurons employ alternative metabolic pathways for GSL metabolism that allowed them to be spared the changes seen with the afferent system (Akil et al., 2015). This idea is supported by findings that the dorsal root ganglia in B-/- mice and other Saposin KO mice have inclusions, but motor neurons are generally not affected by these changes (Sun et al., 2007, 2008) these results suggest alternative manifestations of Sap function in afferent versus efferent neurons. It thus seems likely that OHC function was preserved because there is no accumulation of sulfatide in the type 2 SG neurons.

3.2 Saposin B in Vestibular Function

Sun *et al.* reported that the B-/- mice demonstrate head tremor that may have been caused by the vestibular dysfunction and an abundant storage material accumulation in the vestibular ganglion. However, Akil *et al*. did not find any clear evidence for peripheral vestibular dysfunction in B-/- mice. No head bobbing phenotype in the B-/- mice was observed and the mutants were indistinguishable from the WT siblings even at older ages. Despite the abundant storage material accumulation in the vestibular ganglion, the B-/-mice presented no visible signs of balance dysfunction. While B-/- mice did not show any vestibular dysfunction phenotype, vestibular evoked potentials (VsEPs) demonstrated profound vestibular dysfunction likely attributable to large-scale neuronal degeneration in myelinated SG neurons. Because type 2 SG neurons are spared degeneration, the efferent system was preserved. The normal observed balance, despite the neuronal loss, is reminiscent of the normal click ABR thresholds observed in the same mouse where only 15% of the cochlea nerve fibers

remain at P15mo. This implies that not all vestibular ganglion neurons are required for normal vestibular function or alternatively the vestibular system has more compensatory systems. Unlike hearing, balance requires the integration of several organ systems including vision and the musculoskeletal system, and it is possible that these other systems offset the loss of vestibular function, leading to a lack of an observable phenotypic change in balance.

4. Saposin C and Saposin CD

4.1 Saposin C and Saposin CD in the auditory system

It was unclear from early studies of prosaposin if the severe inner ear deficits were due to actions of prosaposin itself, known to have intrinsic nerve-growth-factor like qualities (Furst et al., 1992; Sun et al., 2002). In a further attempt to determine if the severe inner ear deficits observed in prosaposin KO mice were due to a lack of prosaposin or individual constituents of prosaposin, investigators turned to examining the role of saposin C (SapC) in the ear. As SapC is known for having neurotigenic properties, it was hypothesized that SapC may be responsible for the hearing loss and efferent and afferent neuronal sprouting seen in the prosaposin KO mice (Lustig et al., 2015). Given the neuritogenic and nerve outgrowth properties of the n-terminal region of SapC it seemed likely that SapC was behind the neuronal proliferation observed in the prosaposin KO mice. To explore this idea, two saposin transgenic mice were generated; one lacking SapC and the other combining the null mutation of saposin C and D to generate a saposin CD (SapCD) KO mouse (Bamiou et al., 2001; Matsuda et al., 2004; Akil et al., 2006). In both models, mutations were made in the coding region to eliminate one of the protein's three essential disulfide bridges, creating a deficiency selectively in only the targeted saposin (Akil et al., 2006).

When comparing mutant mice lacking either SapC or SapCD to WT littermates, investigators found minimal histologic changes in the organ of Corti and no effect on hearing. Morphology of inner and outer hair cells, supporting cells, stria vascularis, and spiral ganglion neurons were all normal through P30 in both the SapC and SapCD KO mice (Lustig et al., 2015). Additionally, surface preparations stained with phalloidin showed normal inner and outer hair cell counts throughout the cochlea. As compared to WT littermates, neither mutation resulted in notable changes in ABR thresholds, and both mutants exhibited normal outer hair cell function, as seen through DPOAE, demonstrating normal afferent and efferent auditory neuronal function in the KO mice through P55. These results suggest that the loss of SapC or SapCD has no observable effect on the organ of Corti through this age.

4.2 Saposin C and Saposin CD in the vestibular system

While auditory function appeared normal, SapCD KO mice exhibited difficulty walking and generally abnormal movement, bringing the vestibular system into question. The vestibular morphology of SapCD KO mice was similar but not as severe as it was for prosaposin KO mice. Lustig group found that inclusion bodies form in SapCD KO mice in all three end-organs and upon immunofluorescence staining for afferent and efferent auditory fibers; these cellular proliferations were found to be consistent with neuronal overgrowth. The abnormal movement

patterns and inclusion bodies were not seen in the SapC KO mice nor in WT littermates, are suggesting a lack of SapD responsible for these inclusions.

To explore if the inner ear deficits found in the prosaposin KO mouse were due to an absence of prosaposin itself or if one of the end-products, SapCD, KO mice of SapC and SapCD were studied. While SapC seemed like a likely contributor to neuronal overgrowth phenotype given the 'neurotigenic' portion of prosaposin is found in the n-terminal region of SapC (O'Brien et al., 1994), this hypothesis was not supported - SapC KO mice showed normal hearing and morphology with no obvious abnormalities in end-organs while SapCD KO mouse showed accumulation of inclusions in the vestibular system similar but not severe to those seen in prosaposin KO mouse.

While these vestibular findings, in isolation, seem to identify lack of sapD as a likely cause of gait abnormalities, when compared with systemic effects found in SapCD KO mice, the abnormalities in the ear seem mild and thus it could not be concluded that loss of SapD alone was responsible. Sun *et al.* found a severe neurological phenotype in SapCD KO mice including ataxia, kyphotic posturing, and hind limb paralysis. Further, storage bodies in the neurons of the spinal cord, brain, and dorsal root ganglion as well as accumulation of alpha-hydroxy ceramides and glucosylceramides in brain and kidney were also seen. These central changes were more severe than those seen in the vestibular system in SapCD KO mice and thus likely explain the gait abnormalities observed (Sun et al., 2007). In this model, beyond 6 weeks loss of Purkinje cells was noted, along with storage bodies in neurons of the spinal cord, brain and dorsal root ganglion. These mice similarly showed large accumulations of glucosylceramides and alpha-hydroxy ceramides in their brains and kidneys. Thus, while there are abnormalities noted within the vestibular system in the SapCD KO mice, they appear mild in comparison to the central changes seen by Sun *et al*.

5. Discussion: Saposins function in the ear

When one takes into account the cumulative work on prosaposin and studies on saposins B, C and D, a number of interesting generalizations emerge. Ablation of prosaposin, as the precursor of all the saposins, causes the most significant defect in the inner ear (Akil et al., 2006; Akil et al., 2012). This conforms to findings in other organs of mouse model of prosaposin ablation (Sun et al., 2002). Given the nature of the hearing loss, as well as efferent and afferent neuronal sprouting in the prosaposin KO mouse, it was hypothesized that SapC, a protein known for its neurotigenic properties, was responsible for these changes. However, the SapCD KO mouse has minimal histologic changes, and no effect on hearing. In contrast, though much milder than the full prosaposin null mutation, the Sap B KO mouse has a more severe deficit with hearing loss starting at ~6 months and progressing thereafter. Thus SapB appears to have a much greater role in auditory neuronal maintenance and balance than SapC and/or D.

However, as others have noted, one cannot simply sum up the constituent saposins A-D to determine the overall role of prosaposin. Deficiencies in single saposin or combined saposins have exhibited significantly different physiological effects than were expected by summing the individual

deficiencies (Sun et al., 2007). Prosaposin itself can act as neurite outgrowth or nerve regeneration factor or may be involved in preventing cell death (Hiraiwa et al., 1992; Kotani et al., 1996; Sun et al., 2002). It is thus possible that individual saposins may be acting synergistically with the progenitor prosaposin to function effectively, and no 'single' saposin is responsible for maintenance of the auditory system.

Investigating a synergistic function as opposed to individual roles is complicated by the fact that prosaposin is also detected by antibodies to individual saposins. This makes cellular localization of individual saposins not currently possible, complicating the question of which saposins are the most active in the inner ear. Additionally, assigning specific functions of individual saposins based on the findings in the prosaposin KO mice phenotype is also not straightforward. As has been pointed out by Sun *et al*, in mice, deficiencies of saposin A cause a Krabbe-like demyelinating disease while loss of SapD results in ceramide accumulation and bladder defects; yet neither of these abnormalities are seen in the prosaposin KO mouse. Analogous discrepancies have been seen in SapB and C KO mice as compared to prosaposin KO mice (Sun et al., 2002). Thus, deficiencies of saposin A or D might similarly yield auditory and vestibular phenotypes markedly different than that seen in the prosaposin KO mouse. Table 1 summarized the auditory and vestibular findings in KO models of prosaposin, SapA, SapB, SapC, SapD, SapC+D.

Table 1. Summary of auditory and vestibular findings in KO models of prosaposin, SapA, SapB, SapC, SapD, SapC+D.

Knockout Model	Auditory Findings	Vestibular Findings
Prosaposin	Important for normal cochlear innervation and maintenance of normal Hearing Expression of prosaposin observed in organ of Corti, supporting cells Deiters' cells, IHCs, inner pillar cells, synaptic region of OHCs Reduction in hearing in mice up to P19, deafness by P25, reduced DPOAE from P15 onward	Critical in neuronal maturation process of vestibular sensory epithelium and maintenance of normal vestibular function Prosaposin localized to three vestibular end-organs and Scarpa's ganglion Vestibular dysfunction on behavioral tasks Exuberant cellular proliferation below vestibular HCs with disruption of supporting cells

	Cellular hypertrophy in IHCs and greater epithelial ridge, loss of OHCs form apex, vacuolization of OHCs Overgrowth of auditory afferent neurites in region of IHCs and proliferation of auditory efferent neurites in region of tunnel of Corti Normal HC function	Inclusion bodies and cellular proliferation disturbing normal neuroepithelial structure of vestibular end-organs corresponding with afferent and efferent neuronal overgrowth
SapA	Little is known. Not studied yet in the ear	Little is known. Not studied yet in the vestibular
SapB	progressive sulfatide accumulation in satellite cells around spiral ganglion (SG) neurons resulting in satellite cell degeneration highly correlated to SG degeneration, and ultimately, loss of Hearing Normal organ of Corti morphology, significant reduction of SG and cochlear nerve fibers in Rosenthal's canals progress with age No neuronal overgrowth observed	Head tremor No clear evidence for peripheral vestibular dysfunction phenotype Storage material accumulation in vestibular ganglion VsEPs showed profound vestibular dysfunction likely from large-scale neuronal degeneration in myelinated SG neurons
SapC	No histologic changes in organ of Corti No effect on hearing	No effect on the vestibular function (No abnormal movement patterns, no inclusion bodies)
SapD	Least understood. Not studied yet in the Ear	Suggested that lack of SapD is responsible for vestibular abnormalities seen in SapCD KO mice
Sap C+D	No histologic changes in organ of Corti No effect on hearing	Difficulty walking, abnormal movement Inclusion bodies in SapCD KO mice in all three end-organs Cellular proliferations consistent with neuronal overgrowth

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