Protecting Sub-Types of Primary Auditory Neurons from Acoustic Trauma

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Abstract

Disabling hearing loss represents a global public health concern. At least 33% of adults aged 65 and above have age-related hearing deficits. In recent years, there is also an increasing prevalence for acoustic trauma in young adults; 19% of adults aged 20 to 29 have evidence of early noise-induced damage. Unfortunately, age-related (AHL) and noise-induced (NIHL) hearing loss can result in permanent synaptopathy and axonopathy in spiral ganglion neurons (SGNs), the primary auditory neurons of the inner ear. Loss of these synapses can lead to social isolation, tinnitus, and a drop in a patient’s quality of life.

Prior work has shown that AHL and NIHL target a subpopulation of differentially vulnerable SGN synapses. However, the molecular basis for this heterogeneity remains unknown. To address this challenge, I leveraged our group’s single-cell RNAseq database of adult Type I SGNs and showed that Lypd1, a nicotinic acetylcholine receptor modulator, and Calb2, an intracellular calcium buffer, define differentially vulnerable populations of SGNs. In particular, Lypd1+ SGNs share neuroanatomical features with low spontaneous firing rate (SR) SGNs, a functional SGN subtype previously shown to be susceptible to AHL and NIHL. SmFISH analysis of aged mice cochlea also showed that AHL preferentially targets Lypd1+ SGNs. At 108 weeks, the density of surviving Lypd1+ SGNs is severely attenuated, while the density of Calb2+ SGNs remains largely unchanged.

Traditional therapies for hearing loss fail to fully compensate for SGN synapse loss and lack the ability to protect against future damage. To protect Lypd1+ SGNs from AHL and NIHL,
I investigated the therapeutic potential of Bclw, an anti-apoptotic protein previously shown to be critical for the long term survival of sensory neuron axons. I injected adeno-associated viruses packaged with Bclw into the inner ears of early postnatal and adult mice and showed that overexpression of Bclw protected the SGNs from noise-induced synaptopathy. I also found that the viral treatment led to functional auditory protection, suggesting that Bclw overexpression can act as a prophylactic therapy against future acoustic trauma. As a result, we propose that Bclw is an attractive drug target for the prevention of AHL and NIHL.
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— CHAPTER ONE —

Introduction
"My defective hearing appeared everywhere before me like a ghost; I fled from the presence of men, was obliged to appear to be a misanthrope although I am so little such."

— Ludwig van Beethoven

For most of human history, the only therapeutic recourse for those with hearing loss was to cup their hand behind their ear. This hurdle would persist until the development of rudimentary hearing devices and sign language in the early 17th century (Alexander, 1998). The earliest practical treatment for encroaching deafness was the ear trumpet. Initially made from hollowed out animal horns, these funnels slowly evolved to become specialized metal trumpets that could collect sounds and focus them into the ear. Critically, these trumpets were unable to amplify sounds, limiting their effectiveness to those with mild hearing loss. The first assistive listening devices truly capable of amplifying sounds would have to wait until the 19th century with the introduction of the electric hearing aid (Mills, 2011). In the intervening centuries, these hearing aids would become more discrete to wear, more sensitive to external acoustic stimulation, and more customizable to the individual patient. Today, those who are hard of hearing have access to advanced digital hearing aids and cochlear implants. Modern hearing aids allow those with even the most severe hearing loss to detect rudimentary sounds from the outside world.

However, despite these advances in medical technology, the burden of disabling hearing loss as a global public health concern continues to grow. In a 2012 report, the World Health Organization (WHO) estimated that 33% of adults aged 65 and above had disabling hearing loss. As the world becomes more and more industrialized, the young are starting to become affected as well. In the United States alone, 12.5% of children aged 6-19 years of age have noise-induced
auditory threshold shifts (Niskar et al., 2001). Without intervention, continued exposure to excessively loud sounds can lead to progressive hearing loss, reduced productivity, and social isolation. The prevalence of hearing impairment is expected to grow over the next 10 years as economic development and population growth increases the number of people exposed to hazardous levels of noise (NIDCD). Tens of millions of people across the world need treatment for hearing loss or face a substantial drop in their quality of life (WHO). This chapter provides an overview of the structure of the inner ear, the pathology of acoustic trauma, and the need to develop new therapies to combat this growing public health concern.

**Organization of the Inner Ear**

The perception of sound is initiated when sound waves from the outside world bounce into the outer ear, travel through the ear canal, and strike the eardrum. The resulting vibrations are conducted through the middle ear via three small bones: the malleus, the incus, and the stapes (Zwislocki, 2005). These three bones oscillate and transmit mechanical vibrations from the eardrum to the cochlea, a snail-shaped structure within the inner ear that houses all the primary auditory sensory receptors and neurons (Figure 1.1A). This process also converts mechanical acoustic energy into traveling waves that ripple through the fluid in the cochlear spiral (Gold, 2006; Reichenbach and Hudspeth, 2014). As the traveling wave moves along the cochlear spiral, different regions of the cochlea flex, causing the primary sensory epithelium to move up and down (Dallos, 1992). This oscillation of the epithelium stimulates hair cells sitting on top of the membrane by deflecting their stereocilia; each hair cell detects a narrow range of sound frequencies, dictated by its location along the length of the sensory epithelium (LeMasurier and Gillespie, 2005). Hair cells located near the base of the cochlear spiral respond best to high
frequency noise, while hair cells located near the apex respond best to low frequency noise (Russell and Sellick, 1978). A cross-section through the epithelium reveals two types of hair cells: inner hair cells (IHCs) and outer hair cells (OHCs). IHCs form a single row of primary sensory receptors that spiral along the cochlea from base to apex. IHCs convert mechanical vibrations in the cochlea to neuronal electro-chemical signals (Hudspeth and Jacobs, 2006). Directly adjacent to the IHCs are three rows of OHCs which serve to mechanically amplify noise-induced vibrations in the inner ear (Figure 1.1B) (Ashmore, 2008; Zheng et al., 2000).

IHCs capture the intensity, frequency, and timing of all incoming sounds. This information is then transmitted to spiral ganglion neurons (SGNs). SGNs serve as the only afferent connection between the cochlea and higher-level auditory processing centers in the central nervous system (CNS), making them critically important for auditory perception. SGNs are bipolar neurons; they extend short peripheral processes to the basolateral surface of hair cells and long central processes to the cochlear nucleus complex in the auditory brain stem (Spoendlin, 1969). In the mammalian auditory system, there are two broad populations of SGNs, Type I and Type II, which are distinguished by their structural morphology and the target of their peripheral projections (Liberman et al., 1990). Type I SGNs innervate IHCs and typically make up 90-95% of the total afferent nerve population. Each Type I SGN will receive input from only one IHC (Liberman et al., 1990); however, each IHC may establish synaptic coupling with 4-28 separate Type I SGNs (Liberman, 2017), depending on the species and location of the IHC along the cochlea spiral (Liberman, 1980a; Merchán-Perez and Liberman, 1996; Stamataki et al., 2006). Type I SGNs are also myelinated except at the distal end of the peripheral axon near the IHC/SGN synaptic complex. In contrast, Type II SGNs are unmyelinated and target OHCs, forming en passant synapses with multiple OHCs (Brown, 1987).
Figure 1.1: Schematic of the inner ear. (A) Sound waves hit the eardrum causing it to vibrate. These vibrations are transmitted through the middle ear via three small bones (orange): the malleus, incus, and stapes. These small bones convert the mechanical motions of the eardrum into traveling waves that propagate through the cochlea (brown) from base to apex. As the traveling wave ripples through the cochlea, the sensory epithelium deflects, stimulating the hair cells to send electro-chemical signals to the primary auditory neurons (SGNs). (B) A cross-section through the sensory epithelium reveals one row of inner hair cells (IHCs) and three rows of outer hair cells (OHCs).
These SGNS represent 5-10% of the total SGN population (Simmons and Liberman, 1988). While it is clear that Type II SGNs integrate electro-chemical signals across multiple OHCs, the role Type II SGNs play in the inner ear remains poorly understood. There is, however, gathering evidence to support their role in nociception within the inner ear (Flores et al., 2015).

**Heterogeneity within Type I SGNs**

Functionally, the Type I SGN population can be further divided by analyzing their heterogeneous electro-physiological characteristics. In cats, Type I SGNs exhibit a range of spontaneous firing rates (SR), with high-SR (≥20 spikes per second) neurons making up 60% of neurons in the inner ear (Liberman and Kiang, 1978). These neurons are thought to operate in environments with low ambient sounds (Winter et al., 1990). The remaining 40% of neurons are characterized with low-SR (Liberman, 1978; Tsuji and Liberman, 1997), offering their firing patterns a greater dynamic range across a wider spectrum of sound pressure levels (Costalupes et al., 1984; Schalk and Sachs, 1980). These low-SR SGNs are believed to be utilized in environments with high levels of complex ambient sounds. Neuro-anatomical studies with HRP-labeling have revealed other differences between low-SR and high-SR SGNs. Low-SR SGNs preferentially project towards the modiolar side of the IHC, while high-SR SGNs extend peripheral projects to the opposite pillar side of the IHC (Kawase and Liberman, 1992; Liberman, 1980a, 1982) (Figure 1.2). Differential vulnerability to damage has also been associated with functional subtypes of Type I SGNs (Furman et al., 2013). Morphological studies in aging mice and gerbils reported a biased loss of small afferent terminals on IHCs, which correspond to fibers with low-SR (Furman et al., 2013; Schmiedt et al., 1996). Ouabain-induced neuropathy also preferentially targets low-SR
Figure 1.2: Functional subtypes of Type 1 SGNs innervate IHCs. The inner hair cell is innervated by two functional subtypes of Type 1 SGNs. Each can be distinguished by their spontaneous firing rate and neuro-anatomy. Low-SR SGNs preferentially form synapses along the modiolar side of the IHC, while high-SR SGNs project towards the pillar side of the IHC.
SGNs in a dose dependent manner (Bourien et al., 2014). It has been proposed that low-SR SGNs are critically involved in extracting acoustic information from backgrounds with high levels of continual noise. The preferential loss of low-SR fibers may explain why aging listeners appear to distinguish sound in quiet environments perfectly well, but rapidly decline in performance when moved to louder areas (Hickox and Liberman, 2014; Kujawa and Liberman, 2009). Critically, despite the well characterized physiological differences within the Type I SGN population, the molecular basis for this heterogeneity remained unknown.

**Inner Ear Synapse**

While IHCs are tasked with capturing and converting all sound waves into electro-chemical signals, they lack an axon to directly transmit information to the auditory brainstem. As a result, highly efficient synaptic signaling between IHCs and SGNs is needed to capture all the important features of the incoming acoustic stimulus. The crucial component of the IHC/SGN synaptic complex is the synaptic ribbon, an electron-dense, pre-synaptic structure that tethers up to 20 synaptic vesicles to the hair cell active zone for efficient release and refilling (Buran et al., 2010; Frank et al., 2010; Khimich et al., 2005). This unique feature allows a single IHC/SGN synaptic complex to maintain high rates of synaptic signaling and to drive all the activity of an individual cochlear afferent fiber. Opposing the synaptic ribbon is an unusually large postsynaptic density (PSD) (Sobkowicz et al., 2018). The SGN PSD contains large amounts of AMPA-type receptors, including GluR2, GluR3, and GluR4, as well as NMDA-receptors (Matsubara et al., 1996). The density of AMPA-type receptors within the PSD protects against glutamate saturation and further facilitates high-frequency synaptic transmission between IHCs and SGNs (Glowatzki and Fuchs, 2002).
Decades of prior work has shown that accurate perception of sound depends on the integrity of the inner ear. However, most of the focus has been on hair cell degeneration and the development of therapeutics to restore hair cell function following acoustic damage. This hair cell-centric view developed from early animal work studying damage to the inner ear following exposure to acute, loud sounds or ototoxic drugs. In these experiments, hair cells disintegrated within hours (Bohne and Harding, 2000; Lawner et al., 1997; Suzuki et al., 2008; Webster and Webster, 1978), leaving behind a seemingly intact SGN peripheral process. Retraction and loss of the peripheral processes would only be observable weeks to months after the initial insult, suggesting that the hair cells were the most vulnerable elements of the inner ear (Miller et al., 1997; Sugawara et al., 2005; Webster and Webster, 1978). As a result, the underlying pathology of SGN degeneration following inner ear trauma has been underappreciated and little studied for a long time.

**Permanent vs. Temporary Acoustic Trauma**

Overexposure to extreme sounds can lead to temporary or permanent hearing loss. Such scenarios are typically referred to as noise-induced hearing loss (NIHL). Historically, the prevailing view of NIHL focused on the transformation of intense acoustic energy into permanent, mechanical destruction of IHCs and OHCs (Liberman and Kiang, 1978; Remenschneider et al., 2014; Rybalko et al., 2015). Early histological studies following permanent NIHL showed extensive hair cell death or damage to their stereocilia bundles within minutes after acoustic trauma (Liberman and Dodds, 1984). In these early studies, it appeared as though the peripheral processes of SGNs continued to project towards the auditory sensory epithelium, despite the clear loss of their IHC targets (Le Prell et al., 2007; Sugawara et al.,
These observations led to the idea that SGN loss was secondary to the rapid loss of hair cells and supporting cells. Numerous studies with ototoxic antibiotics also supported the view that degeneration of sensory neurons almost always occurred following loss of their hair cell targets (Bohne and Harding, 2000; Lawner et al., 1997; Wang et al., 2002). Hearing tests after NIHL suggested severe functional impairment between IHCs and SGNs (Dallos and Harris, 1978). Traditionally, cochlear function and auditory impairment can be measure using two techniques: auditory brainstem response (ABR) and distortion product otoacoustic emissions (DPOAE). ABRs measure the sound-evoked electrical potentials generated by auditory synaptic junctions following exposure to a tone stimulus at a given sound pressure level (SPL). This measure provides insight on the health of the auditory neural pathway, as well as the integrity of the IHC synaptic complex. Disruption of HC function, loss of SGN synapses, and attenuation of synchronous SGN firing can all lead to decreases in measured electrical potentials. In contrast, DPOAEs measure the strength of OHC function and the ability of the inner ear to amplify incoming sounds. Immediately following acute acoustic trauma, maximum ABR and DPOAE threshold shifts are observed (Kujawa and Liberman, 2009). Depending on the intensity of the acoustic trauma, limited recovery can be expected. Weeks after permanent NIHL, ABR and DPOAE thresholds shifts can slowly decrease, but ultimately stabilize at an elevated value (Cho et al., 2013; Remenschneider et al., 2014).

However, recent work in mice, humans, and guinea pigs has revealed a new form of pathogenesis following temporary NIHL (Kujawa and Liberman, 2009; Liberman and Kujawa, 2017; Liberman and Liberman, 2015). In temporary NIHL, hair cells and supporting cells are exposed to attenuated SPLs that cause only transient ABR/DPOAE threshold shifts. The full recovery of ABR/DPOAE threshold shifts suggests no permanent damage to the hair cells and
supporting cells of the inner ear. Strikingly, despite full functional recovery of the hair cells, there is a rapid and acute loss of IHC/SGN synapses and the retraction of Type I SGN unmyelinated neurites away from the IHCs (Kujawa and Liberman, 2009). Immunostaining against the pre-synaptic zone and post-synaptic density revealed a loss of 40-50% of all IHC/SGN synapses following temporary NIHL (Kujawa and Liberman, 2009). The immediate synaptopathy following temporary NIHL is also irreversible; time course studies show minimal synaptogenesis months after the initial trauma (Kujawa and Liberman, 2009; Liberman and Liberman, 2015). Prior electron microscopic studies have shown that each auditory nerve fiber contacts a single IHC via a single terminal bouton and is paired with a single pre-synaptic ribbon (Liberman, 1980a; Stamataki et al., 2006). Thus, synaptic counts via immunostaining and confocal imaging provide an accurate estimate of the maximum number of SGNs that are available to carry information from the cochlea to the brain. Following the initial synapse loss and retraction of the unmyelinated neurite, the SGN peripheral axon undergoes gradual degeneration, slowly pulling back to the cell body (Kujawa and Liberman, 2009). While the subsequent SGN axon degeneration progresses over the course of months, there is a close agreement between the initial amount of synapse/terminal loss and the eventual loss of SGN cell bodies. This suggests that the loss of SGN peripheral synapses is a key marker for eventual SGN degeneration and that the long-term fates of SGNs are sealed before any observable hair cell degeneration.

Interestingly, a similar phenomenon was also observed following age-related hearing loss (AHL). Work with aged mice raised in sound-controlled rooms showed that SGN synaptopathy preceded degeneration of all other cell types in the inner ear (Ruan et al., 2014). Synaptic counts decreased steadily from 4 weeks to 144 weeks of age, while IHC counts remained largely stable.
until the very end of the time course (Sergeyenko et al., 2013). SGN peripheral axon loss was also highly correlated with the loss of IHC/SGN synapses during AHL and occurred before the loss of hair cell targets (Fernandez et al., 2015; Liberman and Liberman, 2015). As with prior work on temporary NIHL, IHC/SGN synapses in the aging cochlea appeared to be the most vulnerable element—not hair cells as previously believed. In humans, there is also increasing evidence to suggest that primary synaptopathy precedes age-induced IHC death (Makary et al., 2011). There have been reports of elderly subjects with 80% loss of SGN innervation, despite a complete complement of IHCs (Viana et al., 2015). Thus, in contrast to prior beliefs, primary neural degeneration may also be a key component underlying AHL.

Pathology Underlying Acoustic Trauma

Little is known about the cellular mechanisms and molecular effectors that induce synaptopathy and neuronal death in the cochlea following acoustic trauma. A common observation following both permanent and temporary NIHL is disruption of the SGN dendrites via excitotoxic damage (Pujol et al., 1985, 1993). The unmyelinated distal tips of SGN peripheral processes rapidly swell following exposure to acoustic trauma (Puel et al., 1998; Robertson, 1983; Spoendlin, 1971). Large concentrations of glutamate are released in response to noxious noise; toxic concentration of this amino acid can lead to excessive amounts of sodium and potassium ion influx across the post-synaptic membrane (Le Prell et al., 2007). This osmotic imbalance results in extracellular fluid entry and the characteristic swelling of SGN peripheral dendrites. It is believed that this continuous osmotic imbalance eventually ruptures the dendritic cell membrane, leading to the retraction of the unmyelinated portion of the SGN peripheral terminal away from hair cells and surrounding supporting cells (Fritzsch et al., 2004; Le Prell et
Prior work has shown that both hair cells and supporting cells secrete neurotrophins (NTs) (Stankovic et al., 2004; Wheeler et al., 1994; Ylikoski et al., 1993) which may serve to sustain the SGN peripheral axon. As a result, trauma-induced retraction of the SGN peripheral process away from the neighboring IHCs and supporting cells may suppress neurotrophin-mediated survival (Fritzsch et al., 2004; Ramekers et al., 2012; Stankovic et al., 2004).

Continuous neurotrophin (NT) support from neighboring IHCs and supporting cells is necessary for the long term survival of SGNs (Zilberstein et al., 2012). Acoustic trauma or ototoxic antibiotics can damage or destroy hair cells and supporting cells (Dodson and Mohuiddin, 2000; McFadden et al., 2004; Takeno et al., 1998), disrupting neurotrophic support onto the SGN peripheral terminal. Brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT3) appear to be necessary for the survival of SGNs during development (Fritzsch et al., 2004). Expression of their receptors, tyrosine kinase B (TrkB) and tyrosine kinase C (TrkC), is also necessary for intact SGN development (Fritzsch et al., 1997; Green et al., 2012; Postigo et al., 2002). Loss of NT receptors or ligands during mouse development results in complete loss of sensory neurons in the vestibular and auditory systems (Wise et al., 2005). BDNF and NT3 continue to be expressed in the inner ear after birth. In the postnatal inner ear, BDNF is expressed by IHCs, OHCs, and supporting cells; however, the expression pattern becomes restricted to supporting cells after P10 (Ernfors et al., 1995). NT3 is also broadly enriched in the early postnatal sensory epithelium, but becomes restricted to IHCs, supporting cells, and SGNs by adulthood (Hansen et al., 2001b, 2001a; Sugawara et al., 2007). When culturing SGNs in vitro, addition of BDNF and NT3 greatly enhances the survival rate of these neurons (Mou et al., 1997; Wang and Green, 2011). In addition, following acoustic trauma, direct infusions of NT3 into the cochlea in vivo appears to promote SGN regeneration and protection (Budenz et al.,
In vitro, NT3 helps promote synaptogenesis following excitotoxic damage (Wang and Green, 2011). However, in all of these studies, the entire inner ear was exposed to elevated levels of NTs, making it difficult to assess the efficacy of local NT stimulation on peripheral terminal survival following noise-induced synaptopathy and axon degeneration.

Recent work has shown that supporting cell-derived NT3 regulates synapse density, while supporting cell-derived BDNF has a negligible role (Wan et al., 2014). Overexpression of NT3 by adult supporting cells also promoted the regeneration of IHC/SGN synapses and unmyelinated terminals following acoustic trauma. In contrast, knockout of NT3 from adult supporting cells led to a decrease in the synaptic density between IHCs and SGNs (Wan et al., 2014). Morphologically, supporting cells closely ensheath the unmyelinated terminal of the SGN peripheral axon (Sugawara et al., 2007; Zilberstein et al., 2012). Proximity of the SGN peripheral terminal to neighboring supporting cells may be essential for the maintenance of NT support. Retraction of the unmyelinated terminal away from supporting cells may be sufficient to attenuate downstream effectors that normally protect SGN synapses and the integrity of the peripheral axon. Nevertheless, little is known about how NTs promote the survival of SGN synapses and peripheral axons; it is still unclear what the downstream effectors of NT support are and how these effectors mediate their protective function.

**Bclw May Protect SGNs**

*Bclw*, a death protecting member of the *Bcl2* family, is an attractive therapeutic target to combat AHL and NIHL. Broadly speaking, members of the *Bcl2* family play an important role in regulating cell survival (Cusack et al., 2013; Nikolaev et al., 2009). Some members of this
family, such as Bcl2, Bclx, and Bclw, actively inhibit apoptosis, while others, such as Bax and Bak, promote cell death (Cory et al., 2003; Ross et al., 2001). Different members of the Bcl2 family tend to operate by antagonizing each other’s function through the formation of heterodimers or indirect interactions (Ross et al., 1998; Yan et al., 2003). Early work in the mouse testis revealed that Bclw promotes the survival of Sertoli cells by inhibiting Bax-induced apoptosis (Ross et al., 1998, 2001). Further molecular studies confirmed that Bclw co-immunoprecipitates with Bax and Bak, suggesting that Bclw normally inhibits apoptosis by directly binding to pro-apoptotic members of the Bcl2 family (Holmgreen et al., 1999).

However, Bclw is unique from the rest of its anti-apoptotic counterparts because of its sub-cellular localization and regulation via a retrograde neurotrophin signaling cascade (Cosker et al., 2013; Courchesne et al., 2011; Pazyra-Murphy et al., 2009). Only NT stimulation of the DRG distal axon leads to an increase in Bclw mRNA in both the cell body and axon; in contrast, NT stimulation of the DRG cell body did not change Bclw mRNA levels in either location (Cosker et al., 2013). In-situ hybridization wholemounts reveal that Bclw mRNA is localized to both the DRG cell body and the peripheral axon, but not the central axon (Courchesne et al., 2011). Subsequent data also suggests that NTs regulate the transport of newly made Bclw mRNA transcript to the distal axon and induces its local translation (Cosker et al., 2013). The preferential induction of Bclw by NT stimulation on distal axons demonstrates that Bclw may be involved in promoting the survival of neurons that have successfully extended axons out to their NT-secreting targets. This distinct regulatory pattern in DRGs may fit with the current model of SGN survival. Here, we have a system with localized NT stimulation at the SGN peripheral axon terminal via IHCs and neighboring supporting cells. Disruption of NT support onto the SGN peripheral axon, through retraction of the unmyelinated terminal, may attenuate Bclw protein
expression in the peripheral axon and increase susceptibility to primary SGN synaptopathy and axonopathy.

In fact, recent work has shown that \textit{Bclw} protein is critical to the survival of the DRG peripheral axon. Young \textit{Bclw-/-} mice have normal DRG innervation density and comparable responses to noxious thermal stimulation as controls. Aging \textit{Bclw-/-} show progressively worse reflexive response to noxious stimuli and undergo progressive loss of peripheral epidermal innervation (Courchesne et al., 2011). Interestingly, the neuropathy found in \textit{Bclw-/-} animals involves the slow retraction of peripheral axons rather than the loss of DRG cell bodies, further supporting the hypothesis that \textit{Bclw} predominately functions in the long axons of sensory neurons. It has also been shown that overexpression of \textit{Bclw} protein into axons protects against axonal degeneration caused by NT deprivation (Cosker et al., 2013). Both NIHL and AHL are characterized by increased synaptopathy and slow retraction of the SGN peripheral axon; loss of \textit{Bclw} may play a role in this observed pathology. Investigating the possibility that \textit{Bclw} is involved in sustained SGN survival may open alternative therapeutic treatments to combat hearing loss.

Finally, \textit{Bclw} is associated with intracellular membranes, particularly the mitochondrial membrane (O’Reilly et al., 2001). Damage to the mitochondria can lead to apoptosis via release of cytochrome c and subsequent activation of pro-apoptotic molecules, such as the caspase family. Pro-apoptotic members of the \textit{Bcl2} family are also known to localize around the mitochondria (Cory et al., 2003; Cosker et al., 2013; Pease-Raissi et al., 2017). In \textit{Bclw-/-} mice, there is elevated caspase6 activation in the peripheral axons of DRGs (Cosker et al., 2013). Activated levels of caspase6 could be attenuated with localized overexpression of recombinant \textit{Bclw} protein into DRG axons. It has been previously shown that \textit{Bclw} binds to Bax and Bak to
prevent caspase activation; inhibition of Bclw’s ability to bind to Bax and Bak resulted in DRG axon degeneration and caspase6 activation following NT withdrawal (Cosker et al., 2013; Pazyra-Murphy et al., 2009). There is gathering evidence to suggest that Bak is also involved in the initiation of SGN and hair cell degeneration (Someya et al., 2009; Yamasoba et al., 2007). In particular, Bak -/- mice have delayed onset of AHL. In SGNs, Bclw may normally suppress and sequester Bak away from axonal mitochondria. As a result, investigating Bclw in the context of NIHL and AHL may elucidate the cellular and molecular mechanism that underlies synaptopathy and neuronal death in the cochlea.

**Significance**

Currently, treatments for disabling hearing loss are largely restricted to hearing aids and cochlear implants (NHS). However, both hearing aids and cochlear implants can have limited efficacy, as they depend on the continued survival of SGNs following acoustic trauma for their therapeutic effects (Pfingst et al., 2015). In addition, those with cochlear nerve deficiencies cannot benefit from such treatments. These traditional therapies also struggle in environments with loud ambient noise and fail to protect against future acoustic damage. As a result, developing new therapies that help SGNs survive against a lifetime of acoustic stress and trauma is critical for efforts to treat hearing disorders. My thesis identifies the molecular basis for Type I SGN heterogeneity and seeks to develop Bclw as a new therapeutic tool to protect against AHL and NIHL.
— CHAPTER TWO —

Sensory Neuron Diversity in the Inner Ear Is Shaped by Activity

Brikha R. Shrestha, Chester Chia, Lorna Wu, Sharon G. Kujawa, M. Charles Liberman, and Lisa V. Goodrich
C.C. validated the single-cell RNAseq database via smFISH (Figure 2.3). C.C. also performed and analyzed all experiments illustrating the neuro-anatomy of SGN-types and differential vulnerability to AHL (Figure 2.4 and 2.5). B.S. generated the single-cell RNAseq database and defined the activity-dependent maturation of SGN identity. L.W. performed smFISH showing the development of SGN identity during early neonatal time points. S.K. and C.L. provided the aged cochleae for the AHL experiment. This chapter was reproduced with permission from *Cell* 174 (2018), pp. 1229-46, doi: 10.1016/j.cell.2018.07.007

**Introduction**

Heterogeneity is a hallmark of the nervous system, with distinct types of neurons forming networks that subserve specific functions. Defining their cellular components is a necessary step toward understanding such networks. Across circuits with clearly different functional outputs, assignment of cell identity is relatively straightforward. For instance, in the somatosensory system, neurons that encode pain and light touch are readily distinguished by the identities of their peripheral end organs and morphologies of their endings (Abraira and Ginty, 2013). However, classification of neurons that share the same position in a circuit, perform similar function, and underlie a single modality can be challenging. For instance, the primary sensory neurons of the auditory system, the Type I spiral ganglion neurons (SGNs), are all bipolar and make connections with the same sensory cell type, consistent with their shared ability to encode sound. Their key differences are physiological, which could be imposed by other cells in the circuit. Whether Type I SGNs also exhibit endogenous differences that might contribute to auditory function has remained elusive.
Type I SGNs differ in sensitivity to sound and spontaneous firing rate (SR), as revealed over 50 years ago via single-fiber recordings in the cat auditory nerve (Kiang et al., 1965). Based on the relation between threshold and SR, they were classified into 3 groups: low-SR, medium-SR and high-SR (Liberman, 1978). These three subtypes are present in all regions of the cochlea and in many species (Barbary, 1991; Borg et al., 1988; Schmiedt, 1989; Taberner and Liberman, 2005; Winter et al., 1990). SGNs with different SRs form synapses at different positions along the basolateral surface of inner hair cells (IHCs) (Liberman, 1982) and project to different cell types in the brainstem. Such diversity enables the wide dynamic range of sound intensities encoded in the cochlea and helps maintain hearing in noisy environments (Costalupes et al., 1984; Liberman, 2017; Winter et al., 1990). In addition, immature SGNs display different firing properties in the base versus apex of the cochlea, which encode high and low sound frequencies, respectively (Mann and Kelley, 2011). Whether mature SGNs retain these differences and how they vary among SR subtypes are not known.

Because Type I SGNs have been classified physiologically, the nature and origin of their underlying heterogeneity are unclear. At one extreme, there may be one basic type of SGN that exhibits different firing properties because of the input received from its pre-synaptic partners. Both heterogeneity in presynaptic vesicle release probability in IHCs (Frank et al., 2009) and regulation by olivocochlear efferents (Guinan, 2017; Liberman, 1980b) have been suggested to contribute to SGN physiological diversity. Alternatively, SGNs may express different collections of receptors and ion channel regulators that shape their sensitivity and SR. Indeed, hints of molecular heterogeneity have been noted (Adamson et al., 2002; Chen et al., 2011; Liu and Davis, 2014; Liu et al., 2014), although it was not possible to link any one marker with known
electrophysiological signatures. Without a comprehensive understanding of SGN molecular profiles, the ways SGNs actively influence auditory coding remain to be defined.

The lack of knowledge of subtype-specific molecular signatures represents a major barrier in understanding SGN biology and function, with direct implications for human health. Low-SR SGNs are lost after noise damage (Furman et al., 2013) and as a consequence of aging (Schmiedt et al., 1996), which may explain the difficulties understanding speech amid background noise in older individuals. However, in the absence of subtype-specific molecular tools, efforts to assign functions to individual subtypes and to understand the mechanisms underlying selective vulnerability have been limited. Similarly, since SGN subtypes do not exhibit mature physiological properties until the end of the first month in mice (Liberman and Liberman, 2016), the factors that give rise to their diverse properties during development remain elusive, despite the potential for SGN pathology in congenital deafness.

Single cell transcriptome-based approaches offer a powerful way to investigate heterogeneity in neuronal populations. To characterize neuronal diversity in the cochlea, we generated high-resolution transcriptomic profiles of individual SGNs. We found three molecularly distinct subtypes (Ia, Ib, Ic) that match the anatomical features of high-, medium- and low-SR SGNs, with additional variation along the tonotopic axis. SGN identities emerge gradually in development and this diversification requires IHC-driven activity. This comprehensive view of SGN heterogeneity uncovers endogenous differences that could directly affect auditory coding and highlights the importance of activity for establishing these differences during development, with important implications for the effects of age-related and congenital hearing loss on SGN composition.
Experimental Procedures

Single cell RNA-sequencing

High throughput scRNA-seq approaches such as Dropseq and Indrop offer ease of profiling and classifying cells but at the cost of low information content per cell. Since our goal was to both classify neurons and gain a deep understanding of the transcriptomic basis of neuronal identities, we chose to combine manual neuron collection with the Smart-seq2 approach that offers a high rate of transcript sampling per cell and sequencing of full-length transcripts. Our workflow is described in detail below.

Collection of single neurons. Cochleae of P25 - P27 mice (Genotypes: Bhlhb5\textsuperscript{Cre/+}; Ai14/+ and Bhlhb5\textsuperscript{Cre/+}; Ai14/+; Vglut3\textsuperscript{-/-}) were dissected out of the inner ear. Each cochlea was further dissected to isolate either the most apical, middle, or basal turn and subsequent processing of each turn was done separately to permit tracking of each neuron’s origin along the tonotopic axis. Pieces of the cochlea were then digested first with collagenase (25 min at 37 °C) then 40 U/ml papain (25 min at 37 °C) (Worthington, LK003150) before passing through a discontinuous density gradient of ovomucoid protease inhibitor (Worthington, LK003182). The crude dissociation extract was passed through a 40 μm cell strainer (Corning, Inc., 352340) and placed in a petri dish with a glass bottom microwell (Cellvis, D60-14-1N) for manual collection. Neurons expressing tdTomato were identified under a dissecting microscope (Olympus, MVX10) and a small number of them (typically <100) were gently transferred to a clean droplet of transfer buffer (10% ovomucoid protease inhibitor in 1x EBSS (Invitrogen, 14155-063)) using a glass micropipette attached to suction tubing. To get rid of debris and non-neuronal cells that accompany tdTomato\textsuperscript{+} neurons during the transfer, cells were rinsed five times by aspirating and
expelling them sequentially into a series of clean transfer buffer droplets on the same petri dish, taking care to aspirate only tdTomato\(^+\) cells each time. After the final rinse, a neuron-enriched pool with no visible tdTomato-negative cells or floating debris was obtained. Neurons were then placed one-by-one at the bottom of individual 0.2 mL tubes (Axygen, 321-10-061) using a fine glass micropipette (typically 40 µm internal diameter at the tip) attached to suction tubing. To avoid contamination resulting from cells that lyse during transfer, glass micropipettes and tubes were never reused after failed transfers. A typical transfer resulted in 1-2 µl of transfer buffer in the tube, most of which was aspirated out of the tube, leaving ~0.3 µL behind. To ensure collection of a healthy single neuron per tube, maintenance of strong red fluorescence, lack of cell shrinkage, and absence of other neurons in the same tube were visually verified for every sample under high magnification. Any sample that did not meet all three of these criteria were discarded. Cells were immediately frozen after transfer and stored at -80 °C for further processing. All cells were collected between 1 PM and 8 PM. Collection of single cells started ~75 minutes after sacrificing the animal and ended after 2 hrs. Time elapsed between euthanization of the animal and freezing of cells varied between 90 and 195 minutes. Both ears of every mouse and mice of both sexes were used.

**cDNA and library preparation.** cDNA libraries were made by the Smart-seq2 method, essentially as described previously (PMID 24385147) but with the following modifications: 1) ERCC spike-in controls (1 µl at 1:50000 dilution, Ambion, 4456740) were added at the cell lysis step; 2) first-strand synthesis was done using Primescript Reverse Transcriptase (Takara, 2680A); 3) 19 cycles of PCR amplification was done; 4) the following primers were used:

Oligo-dT\(_{30}\)VN: 5’-AATGATACGCG ACCACCGATCGT\(_{30}\) VN-3’
Template-switching oligo: 5’-AGACGTGTGCTCTCCGATCTNNNNNrGrGrG-3’

ISPCR: 5’-AGACGTGTGCTCTCCGATCT-3’

Sample quality and yield were analyzed using the high-sensitivity DNA kit in a bioanalyzer (Agilent).

**qPCR screening.** SGN cell bodies in mice are naturally ensheathed by satellite glia after neonatal stages. We found that a small proportion of SGNs dissociated using the protocol described above lost their associated ensheathing glia, but most retained it. This posed a challenge for obtaining neuron-specific transcriptome due to possible contamination from transcripts derived from satellite glia. Given the low proportion of cells that visibly lacked ensheathing glia under high magnification, we chose to pick tdTomato-positive SGNs without regard to their ensheathment status, and instead enriched for neuron-specific libraries by screening out those containing glia-derived transcripts by qPCR. Specifically, presence of Mpz (F: 5’-GTCAAGTCCCCCAGTAGAA-3’, R: 5’-AGGAGCAAGAGGAAAGCAC-3’) and Plp (F: 5’-AGCAAAAGTCAGCCGCAAAC-3’, R: 5’-CCAGGGAAGCAAGGGGG-3’) were probed and only libraries that were negative for both transcripts were included in the library enrichment step of the Smart-seq2 protocol.

**Tissue processing for mRNA/protein detection**

Cochlear whole mounts and sections were processed as described previously. The following primary antibodies were used: anti-Calb2 (1:600), anti-Pou4f1 (anti-Brn3a) (1:300), anti-Parvalbumin (1:500), anti-GluA2 (1:500), anti-CtBP2 (1:500), anti-Myo7a (1:1000), anti-dsRed (1:1000), anti-NF-H (1:1000). For immunostaining of Pou4f1, antigen retrieval in 10 mM citrate
buffer (pH 6.0) was done for 20 minutes before commencing with the staining protocol. For mRNA detection by RNAscope (Advanced Cell Diagnostics), the manufacturer’s protocol was used with the exception that at the end of the protocol, tissues were stained overnight with anti-Parvalbumin, followed by incubation in the appropriate secondary for 1 hr the next day. The following probes were used: Mm-Calb2 (Cat#313641-C3), Mm-Pou4f1 (Cat#414671-C2), Mm-Th (Cat#317621), Mm-Tsc22d3 (Cat#448341), Mm-Epha4 (Cat#419081), Mm-Lypd1 (Cat#318361), Mm-Hcrtr2 (Cat#460881), Mm-Kcn3 (Cat#467371).

QUANTIFICATION AND STATISTICAL ANALYSIS

Sequencing and bioinformatic analysis

Sequencing was done in a NextSeq platform (Illumina) over 2 runs, with similar numbers of samples corresponding to different genetic backgrounds, ages, and cochlear origin mixed across the runs. De-multiplexed raw reads were processed using custom-written UNIX scripts in a shared high-performance compute cluster running CentOS Linux. The following steps and settings were used: 1) low-quality and short reads were trimmed using Trimmomatic (0.33) (PE -phred33 SLIDINGWINDOW:4:20 MINLEN:22 PE -phred33 SLIDINGWINDOW:4:20 MINLEN:22); 2) reads were aligned using STAR to the mouse genome (mm10) to which sequences corresponding to ERCC spike-ins and tdTomato had been appended (--sjdbOverhang 37 \ --outFilterScoreMinOverLread 0.4 \ --outFilterMatchNminOverLread 0.4); 3) reads mapped to the mouse transcriptome were counted using the featureCounts program of the Subread package (v1.4.6). Transcriptome-wide counts for all samples were merged into a table, which was then imported into R (v3.3.2). Analyses and visualization of results within the R environment were done using a custom-written script that utilized Seurat (v 1.4.0.12) for
dimension reduction, tSNE, unsupervised clustering and differential expression analyses, edgeR for normalization, and randomForest for supervised classification.

Data normalization. Type II SGNs have smaller cell bodies than Type I neurons (Brown, 1987). In addition, heterogeneities in cell size both locally and across the tonotopic axis have been noted (personal observations and (Echteler and Nofsinger, 2000)). To account for differences in RNA content arising from cell size heterogeneity, we performed trimmed mean of M-values (TMM) normalization on raw read counts using the edgeR package before importing the expression matrix into Seurat. Cell classification was also conducted without TMM normalization with nearly identical results. No other normalization or transformation was done beside log normalization of counts performed within Seurat. All log-normalized maximum count values for individual genes reported in tSNE plots were normalized further to account for gene length differences. However, these values were used strictly for visual reporting in such plots and not for any other aspect of our analyses.

Cell filter. Only libraries meeting the following criteria were analyzed: 1) free of glial-markers (i.e., Mpz, Gjc3, Mbp all ≤ 1); 2) total reads >750000; 3) proportion of ERCC spike-in transcripts < 20%; 4) proportion of transcripts from mitochondrial genome < 10%; 4) genes detected ≥ 4000.

Dimension reduction. Principal component analysis (PCA) was done using variable genes defined as described in the Seurat manual (Satija, 2018). Genes expressed (>1 count) in fewer cells than a set cutoff (i.e., cubed root of no. of cells in the quality-filtered dataset) were deemed
to be ultra-sparse and hence omitted from the variable gene list used for PCA. In addition, all exogenous transcripts (ERCC spike-in and tdTomato) were removed. Between 8 and 20 top PCs were then selected to generate 2-dimensional embeddings for data visualization using tSNE for various subsets. The set of PCs used always included those contained within the first elbow in a scree plot as well as those deemed statistically significant in Jack Straw plots and explained 60-70% variance in the dataset. Because tSNE outcome can vary slightly from run to run even under the same settings, tSNE coordinates generated in initial runs were saved and re-used in subsequent runs for consistency in visualization.

Unsupervised clustering. scRNA-seq profiles were classified via an unsupervised graph-based clustering algorithm implemented in Seurat (v1.4.0.12) that embeds cells in a K-nearest neighbor (KNN) graph and determines clusters by iteratively grouping cells while optimizing the standard modularity function (Butler et al., 2018; Satija et al., 2015). In this approach, granularity of the clustering is dependent on a user-specified ‘resolution’ parameter within the FindCluster function in Seurat. To ensure unbiased selection of the appropriate number of clusters for classifying Type I SGNs, clustering was conducted using 46 different values of this parameter, ranging from 0.4 to 1.3 with increments of 0.02. Predictions of 3 clusters were obtained across 42 out of those 46 runs (91%) (Supplemental Figure 1B), with 97% of cells being assigned the same identity across such runs (Supplemental Figure 1C). This led to the conclusion that the major structure in the single-cell transcriptomic data was captured by classification into 3 clusters, and by extension, reflected the existence of 3 distinct neuronal subtypes. Additionally, hierarchical clustering based on Ward’s method and Euclidean distances was conducted using
the hclust package in R. In both approaches, only highly variable genes (as described above) were used.

*Training an ensemble learning-based classifier.* Consistent with previous reports, we observed that deriving consistent cell type classification became progressively harder with decreasing cell number. To rule out that identifying subtypes among neurons from Vglut3<sup>−/−</sup> mice was not affected by sample size, we implemented an ensemble learning method to derive an independent classification. The same 3 subtypes identified across all Type I SGNs (n = 179) in our data could be identified even when we analyzed SGNs from the middle turn alone (n = 88). Therefore, we employed ensemble learning to generate a classifier trained on just the middle turn neurons using the *randomForest* package in R. The training set consisted of 44 randomly selected neurons (15, 18, 11 of A, B, C subtypes, respectively). We reasoned that differentially expressed genes must be detectable broadly within a cluster for it to be informative for classifying unknown cells, so only genes expressed in >50% of cells in the cluster they are markers for and <50% in the rest were selected. This resulted in a 44 x 233 cell-by-gene matrix, which was used as the training data set. To assess the classifier’s performance, we used it to predict classes for all wildtype cells not part of the training set, which comprised the test set and included 44, 50, and 41 cells from the middle, apex, and base, respectively. The results were compared against classification produced by graph-based clustering implemented in Seurat. Training parameters were tuned until the median out-of-bag error rate reached zero and performance in the test set peaked (99%) across >10000 combinations of the mtry, nodesize and ntree parameters. The following settings were picked: mtry = 3, nodesize = 1, ntree = 1000.
Image acquisition and analysis

All RNAscope images were acquired at Nyquist settings using a point-scanning confocal microscope (Leica SP8, 63x oil-immersion objective) equipped with hybrid detectors. Image stacks were then analyzed semi-automatically using Imaris (Bitplane, UK). Briefly, cell volumes were segmented using either anti-Parvalbumin stain or anti-dsRed stain (in animals expressing the tdTomato transgene in SGNs). Any cell clipped along the XYZ axes was removed before automatic detection of RNAscope puncta. Parameters for both cell segmentation and puncta detection were set after visually guided search for optimal parameters. To account for variability in signal:noise ratio across experimental conditions, puncta counts for cells from each image were rescaled to fit max and min values across all cells within each biological group. Cells with puncta count above 10% of the maximum value were considered positive for the marker.

Confocal images of cochlear wholemounts were acquired using the same hardware as above. Images were taken as a 1024 x 1024 pixel raster at 63x (2x digital zoom) and a Z-sampling rate of 0.308 µm. Image stacks were analyzed using Amira (FEI, USA). Volumes of individual pre-synaptic ribbons were computed using Amira’s 3D rendering and masking functions. Briefly, each pre-synaptic ribbon was reconstructed by a 3D ‘isosurface’ which surrounded all the pixels that defined the ribbon. The volume of the isosurface defined the volume of the pre-synaptic ribbon. Differences in sample preparations were normalized by dividing each isosurface by the median isosurface size in a given Z-stack. The spatial segregation of individually labelled Type I SGN fibers along the basolateral surface of the inner hair cell were analyzed by generating an isosurface around both the afferent fiber and the target pre-synaptic puncta. The YZ coordinates of the pre-synaptic ribbon and adjacent afferent terminal were remapped onto an XY plane and
assessed. A line bisecting the inner hair cell nucleus and cuticular/basolateral axis defined the 0 position of the pre-synaptic ribbons along the modiolar/pillar axis.

Optical sections of the OSL bundle were taken 50 µm away from the base of the IHCs in Amira (FEI, USA) and all subsequent analyses involving fluorescence intensity measurements was done using Image J (1.49V). To analyze CALB2 levels in SGN fibers in the OSL, regions of interest (ROI) were drawn around SGN fibers positive for NF-H in optical cross-sections of confocal Z-stacks. Mean fluorescence intensity values for CALB2 were then calculated for all ROIs and normalized to the highest value within an image. Somatic CALB2 fluorescence intensity was quantified in Mafb<sup>CreERT2;Ai9</sup> animals, which exhibit sparse labeling of SGN cell bodies with the tdTomato reporter. ROIs were drawn around tdTomato<sup>+</sup> SGN cell bodies and fluorescence intensity values for CALB2 across all ROIs within an image were normalized to the highest mean fluorescence value in the same image.

**Clustering of RNAscope-based expression data.** An unsupervised machine learning algorithm (K-means clustering) was utilized to group the CALB2 OSL fluorescence intensity data (Supplemental Figure 5C, D) into a specified number of clusters. The number of clusters (k) was determined based on an elbow plot (sum of squared errors, SSE vs. k clusters) and chosen at the point where the slope approached zero. A total of 3 clusters were found to be appropriate for the CALB2 OSL dataset. Cluster boundaries defined by K-means clustering was used to set the fluorescence intensity cut-offs for ‘low’, ‘medium’, and ‘high’ CALB2 groups (Supplemental Figure 5D). K-means clustering was also performed on the RNAscope-based *Lypd1/Calb2* expression data presented in Figure 2.6. Elbow plots utilized for each dataset are shown in Supplemental Figure 6D, E. All statistical analyses of *in situ* expression data and
immunostaining data in Figs. 4 and 5 were done in GraphPad Prism 6.0. Analyses of such data in all other figures were done in R.
Results

Type I and Type II SGNs exhibit clear molecular differences

To survey SGN heterogeneity in an unbiased manner, we manually collected individual SGNs from the basal, middle and apical turns of cochlea of young adult mice (P25-P27, 11 animals) and analyzed them by single-cell RNA sequencing (scRNA-seq) (Figure 2.1A; see Methods). A total of 186 wildtype SGN single-cell libraries passed all quality control criteria (Supplemental Figure 1A). In a two-dimensional representation generated using t-stochastic neighbor embedding (tSNE), SGN libraries segregated into several distinct groups (Figure 2.1B). Type I and II SGNs, which exhibit well-characterized differences in anatomy, glutamate-responsiveness, myelination, and synapse number (Berglund and Ryugo, 1987; Glowatzki and Fuchs, 2002; Spoendlin, 1979; Weisz et al., 2009), fell into distinct groups enriched for known markers such as Eph4 and Th, respectively. Presumed Type I and Type II SGNs also express different levels of Gata3 ($p = 5.7\times10^{-13}$), Mafb ($p = 1\times10^{-4}$) and Prox1 ($p = 2.3\times10^{-5}$), as previously reported (Figure 2.1C) (Nishimura et al., 2017). Housekeeping genes such as Gapdh ($p = 0.37$) (Figure 2.1C) and Eif2a ($p = 0.43$, not shown) are expressed at comparable levels in all SGNs. Overall, Type I and II SGNs comprised 96% and 4% of the samples, respectively, which is close to histological estimates of Type I/II proportions ( Spoendlin, 1969).

Type I and II SGNs exhibit profound differences in connectivity and function that predict similar distinctions at the molecular level. The myelinated Type I SGNs encode all the signals we think of as "hearing" (Kiang et al., 1967; Sachs and Abbas, 1974), whereas unmyelinated Type II SGNs may mediate auditory pain and nociception (Flores et al., 2015). Like other neurons representing different modalities, Type I and Type II SGNs exhibit broad differences at the molecular level, with >1700 differentially expressed genes ($p<0.01$ or lower).
Figure 2.1: Type I and II SGNs can be detected as molecularly distinct cell populations using scRNA-seq. (A) Workflow for single cell RNA-seq (scRNA-seq) of spiral ganglion neurons (SGNs). Numbers indicate time elapsed (in minutes) since animal euthanization. (B) t-stochastic neighbor embedding (tSNE) of neuronal profiles (n = 186, 11 P25-P27 animals) revealed several clusters. Clusters of Type I (blue) and II (orange) SGNs were identified by expression of *Epha4* and *Th*, respectively (insets). In this and all subsequent plots, numbers in the upper right corner indicate highest expression (Max) observed for each gene. (C) Violin plots comparing gene expression among Type I and II SGNs illustrate increased expression of *Gata3* and *Mafb* in Type II SGNs ($p = 5.7 \times 10^{-13}$ and $1 \times 10^{-4}$, respectively), and of *Prox1* in Type I SGNs ($p = 2.3 \times 10^{-5}$), with no difference in expression of the housekeeping gene *Gapdh* ($p = 0.37$). White dots and error bars indicate mean and standard deviation, respectively. (D) Heat map showing genes expressed differentially between Type I and II SGNs, with examples of Type I-enriched (top panel) and Type II-enriched (bottom panel) genes listed on the right. Superscripted numbers indicate gene functional groups annotated manually. (E-F) Several genes exhibit binary ON/OFF expression between the two subtypes (E), with clear correspondence between scRNA-seq (F) and RNAscope (F’) quantification in P25-P27 tissue sections for *Epha4* and *Th*, as well as the novel Type I marker *Tsc22d3*. In images showing RNAscope puncta (F’), SGN cell bodies are outlined in yellow as visualized by immunostaining for parvalbumin (not shown). In scatterplots (F, F’), the two dots in each column indicate counts for two different genes in the same neuron, and neurons are sorted along the X-axis by the level of the gene in magenta. $\rho$ represents Pearson’s correlation coefficient. See also Supplemental Figure 1.
Figure 2.1 (Continued)

A. cochlear turns dissected → cells dissociated → neurons placed into tubes → cDNA libraries made → Bioinformatics → Validation

B. tsNE 2

C. Gata3 $\rho = 5.7 \times 10^{-13}$, Prox1 $\rho = 2.3 \times 10^{-5}$

D. Differentially expressed genes

E. Tsc22d3: Type I 5.9, Type II 4.8

F. scRNA-seq, in situ mRNA detection by RNAseq

G. $\rho = -0.87$, $\rho = -0.52$, $\rho = -0.82$

1. Ion channel-related
2. ATP response
3. Postsynaptic function
4. Neurite guidance
5. Presynaptic function
Of these, 335 genes show a binary ON/OFF expression between the two cell classes (defined operationally as expressed in >70% and <20% of cells for ON and OFF, respectively) (examples in Figure 2.1E). Gene ontology (GO) analysis of differential expression revealed overrepresentation of molecules involved in neuronal function and innervation (Supplemental Figure 1H). Additional manual categorization of the top 50 genes highlighted a stark difference in the presynaptic vesicle-associated protein profiles between Type I and II SGNs, with highly specific expression of \( Sv2b, Sv2c, Syt2, Syt12 \) in Type I SGNs and of \( Snca, Vamp8 \) in Type II SGNs (Figure 2.1D). In addition, whereas both subtypes co-express several glutamate receptor subunits (e.g., \( Grina, Gria2, Gria4 \), data not shown), Type II SGNs show unique expression of \( Grin2c \) and \( Grik3 \) (Figure 2.1D). Type I and Type II SGNs also express different sets of potassium channel subunits, with \( Kcns1 \) and \( Kcnip1 \) in Type I and \( Kcnc4 \) in Type II SGNs (Figure 2.1D). These results suggest distinctions in glutamate responsiveness, presynaptic release properties, and neuronal excitability that are consistent with previous reports of different electrophysiological signatures between these SGN subtypes (Glowatzki and Fuchs, 2002; Weisz et al., 2014).

Validation using RNAscope, an \textit{in situ} approach for semi-quantitative RNA detection (ACD Bio), confirmed that the variation detected by scRNA-seq represents \textit{bona fide} differences. In tissue sections of P25-P27 mouse cochleae, levels of \( Th \), which mark the small Type II subpopulation in our scRNA-seq data (Figure 2.1B, inset), were highest in cells with the lowest levels of \( Epha4 \), a Type I marker (Figure 2.1F’). \( Tsc22d3 \) transcripts were detected in neurons negative for the Type II marker \( Th \) (Figure 2.1F’), and overlapped with expression of the Type I marker \( Epha4 \) (data not shown), indicating that it may be a reliable Type I marker as predicted by scRNA-seq (Figure 2.1F). Thus, our approach allowed sampling and detection of
the Type II subtype comprising only a small minority of SGNs (Spoendlin, 1969). Despite the small size and number of Type II SGNs, an average of 6320 genes/cell and >11000 genes across all cells could be detected, including cell type-specific molecules that influence neuron physiology and connectivity. These findings confirm that transcriptomic SGN profiles can be used to establish a molecular framework for subtype classification.

Three molecularly-defined subtypes of Type I SGNs

We next focused our analysis on Type I SGNs, which were identified as $Epha4^+ Th^-$ cells in the scRNA-seq libraries (Figure 2.1B, insets). In a tSNE plot, Type I SGNs further segregated into three distinct groups (Figure 2.2A). Independent classification using an unsupervised machine-learning strategy (i.e., graph-based clustering, see Methods) categorized Type I SGNs into three distinct subtypes (Ia, Ib, Ic) that constitute 35%, 40%, and 25%, respectively, of the total population (Figure 2.2B). Both projection of SGN transcriptomes onto principal component analysis (PCA) space (Supplemental Figure 1D) and hierarchical clustering by Ward’s method (Supplemental Figure 1E) revealed three broad subtypes and close correspondence with the cell identities that were independently predicted by the graph-based clustering approach (Figure 2.2A). Thus, multiple statistical methods indicate that there are three molecularly distinct Type I SGN subtypes.

This classification scheme was not influenced by experimental and biological variables. First, the clusters do not correspond to where the neurons originated in the cochlea (Chi-squared test, $p = 0.37$) (Figure 2.2C). Second, clustering is not driven by the acoustic exposure history of the animal, as activity-induced genes such as $Fos$ and $Nrn1$ were expressed in comparable proportions of cells (Figure 2.2D). Furthermore, no significant differences were
Figure 2.2: Three molecular subtypes of Type I SGNs exist in the mouse inner ear. (A-B) tSNE embedding of Type I SGN transcriptomes (A) depicting three clusters — A, B, C — predicted by graph-based clustering, which are indicated by dot color. Overall proportions are illustrated in B. (C-D) SGN subtypes are present in all regions of the cochlea (C) and show expression of the activity-induced genes *Fos* and *Nrn1* in all clusters (D). (E-G) The clusters exhibit broad differences in their transcriptomes, illustrated in a heat map for the top 100 differentially expressed genes (E) and in tSNE plots for individual genes that show subtype-specific expression patterns (F). Numbers in the upper right corner indicate highest expression (Max) observed for each gene. (G) Genes enriched across the three subtypes encode proteins associated with many aspects of neuronal differentiation and function. Superscripted numbers indicate gene functional groups annotated manually. (H) Examples of differentially expressed genes that encode proteins localized to different neuronal compartments, indicating that input and output properties vary among SGNs. For each gene, expression level among SGN subtypes is indicated by the size of each colored dot. (I) Expression of select genes relevant to neuronal physiology is illustrated in dot matrix plots of individual libraries, which are grouped by subtype. Some genes are expressed uniformly across all libraries (top row for each group), whereas others vary across subtypes (all other rows). Numbers on the right indicate the highest expression (Max) observed for each gene. (J-K) Differentially expressed genes identified by scRNA-seq (J) showed the same patterns of expression in individual SGNs analyzed by RNAscope of P25-P27 tissue sections (J’). SGN cell bodies are outlined in yellow as visualized by immunostaining for parvalbumin (not shown). Similarly, immunostaining (K) for CALB2 (calretinin) (green),
Figure 2.2 (Continued)

POU4F1 (magenta) and tdTomato (yellow) in tissue sections of P25-P27 bhlhb5\textsuperscript{Cre/+}: Ai14/+ mice revealed inverse gradients of CALB2 and POU4F1 expression, quantified below. In scatterplots (J, J’, K), the two dots in each column indicate expression levels of two different markers in the same neuron, and neurons are sorted along the X-axis by the level of the gene in magenta. Scale bars: 10 µm (K). See also Supplemental Figure 1.
Figure 2.2 (Continued)
found across the clusters in experimental factors such as animal identity, cDNA processing batch, sequencing batch, genes detected, or proportions of spike-in and mitochondrial transcripts (Supplemental Figure 1F,G). Lastly, none of the single-cell libraries were positive for glial markers (Supplemental Figure 1H).

Differential expression analysis among Ia, Ib, and Ic SGNs revealed 425 genes (all at \( p < 0.01 \)) with subtype-enriched expression (Supplemental Figure 1B). The segregation into three subtypes is associated with striking differences in expression of multiple genes, including Calb2, Pou4f1, Ntng1, and Lypd1. Of these, Calb2 and Pou4f1 are expressed in complementary gradients: Calb2 is highest in Ia and lowest in Ic, with Pou4f1 highest in Ic and lowest in Ia (\( \rho = -0.73 \), Figure 2.2F). Ib and Ic SGNs both express high levels of Ntng1, but can be further distinguished by expression of Lypd1, which overlaps partially with Pou4f1 and Ntng1 (\( \rho = 0.71 \), 0.49, respectively), and is expressed at higher levels in Ic SGNs than in Ib SGNs (Figure 2.2F). Pcdh20, Cacna1b, Lrrc52, Grm8 were also expressed in subtype-specific patterns (Figure 2.2F). In contrast, pan-neuronal genes, such as Tubb3, Gria2, and Atp1b1, were expressed broadly (100%, 99%, 99% of cells, respectively) (Supplemental Figure 1I).

Genes that are expressed differentially across subtypes are mostly involved in regulating neuronal differentiation, connectivity and physiology. In a GO analysis, ‘potassium channel activity’, ‘calcium ion binding’, ‘signal transducer activity’, ‘neuron differentiation’, ‘regulation of ion transport’, and ‘axon development’ were among the 54 biological processes enriched significantly (\( p < 0.01 \)) (Supplemental Figure 1K). Genes encoding proteins that localize to ‘synapse’, ‘cation channel complex’, and ‘plasma membrane’ were also overrepresented. Manual annotation revealed combinatorial expression of several transcription factors, further indicating that SGN subtypes express distinct cohorts of genes that might influence their differentiation:
Rxrg is expressed mostly in Ia, Runx1 is primarily in Ib and Ic, and Pou4f1 is in a decreasing gradient from Ic to Ia. Similarly, the subtypes express unique combinations of genes encoding Ca\(^{2+}\)-binding proteins, with Calb2, Calb1 and Ncald enriched in Ia, Ib and Ic SGNs, respectively. Ib and Ic SGNs also show enriched expression of molecules related to neurite guidance (e.g., Ntn1, Sema3a, Dcc, Slit2) and presynaptic release (e.g., Slc17a6, Cplx2) (Figure 2.2G).

The molecular signatures of SGN subtypes suggest meaningful physiological differences. Several proteins related to synaptic transmission or neuronal physiology were among those differentially expressed, and a comprehensive assessment of their gene families revealed both broad and subtype-specific expression (Figure 2.2I). Among glutamate receptors, several AMPA-type subunits (e.g., Gria2, Gria3, Gria4) were uniformly expressed, but Grik4 was expressed in an increasing gradient from Ia to Ic while the metabotropic receptor subunit Grm8 was expressed specifically in Ic SGNs (Figure 2.2I). Whereas the cholinergic receptor subunits Chrna1 and Chrna4 were enriched in Ia SGNs, Ic SGNs showed enriched expression of Drd1, which encodes a dopamine receptor subunit (Figure 2.2I). Among genes encoding K\(^+\) channel subunits, Kcnq4 and Kcnd2 were detected mostly in Ia and Ib, respectively, with enriched expression of Kcnip2 and Kcnf9 in Ib and Ic SGNs (Figure 2.2I). The voltage-gated Ca\(^{2+}\) channels Cacna1b, Cacna1h and Cacna2d1 were all enriched in Ia SGNs (Figure 2.2I). Expression of the leak Na\(^+\) channel Nalcn was restricted to Ib and Ic neurons, whereas the voltage-gated Na\(^+\) channel subunit Scn2b was expressed in a decreasing gradient from Ic to Ia (Figure 2.2I). Taken together, these findings suggest that the three molecularly defined Type I SGN subtypes constitute physiologically distinct subpopulations.

RNAscope validation confirmed that the same genes that drive SGN segregation in the scRNA-seq data can be used to identify Ia, Ib, and Ic subtypes in cochlear sections of P25-P27.
mice. As seen by scRNA-seq (Figure 2.2J), Calb2 and Pou4f1 are expressed in opposing gradients among SGNs ($\rho = -0.78$) (Figure 2.2J'). Pcdh20 levels are highest in cells negative for Pou4f1 transcripts and lower in all other cells, indicating a weak negative correlation ($\rho = -0.38$). Lypd1 expression is restricted to cells largely off or low for Calb2 ($\rho = -0.79$). Thus, these results validated both individual gene-level variation as well as gene-gene relationships across SGN populations. Furthermore, a negative correlation was observed between CALB2 and POU4F1 protein levels in SGNs ($\rho = -0.36$) (Figure 2.2K), suggesting that the observed transcript-level differences may be functionally significant.

**Tonotopic differences**

To gain further insights into Type I SGN heterogeneity, we examined other sources of variation in the data revealed by PCA. We found that although differences corresponding to the Ia, Ib, and Ic subtypes can be accounted for largely by PC2, additional heterogeneity represented by PC5 diversified SGNs within each subtype (Figure 2.3A). This orthogonal axis of heterogeneity corresponded to the tonotopic origin of the SGNs, which was recorded for every cell during dissociation and manual collection. This result motivated further analysis of differences related to tonotopy both at the single-cell and tissue levels.

We grouped the single-cell transcriptomic profiles based on cochleotopic position instead of molecular identity and examined gene expression differences among such groups. As predicted by PCA results (Figure 2.3A), this analysis revealed an additional but subtler variation, with 114 genes expressed differentially among neurons from the three cochlear regions,
Figure 2.3: Type I SGNs exhibit both broad and subtype-specific tonotopic differences. (A) Molecular heterogeneity exists along the tonotopic axis of the cochlea. Projection of single cell transcriptomes onto principal component analysis (PCA) space shows that PC2 reflects differences among the A, B, C subtypes while PC5 captures heterogeneity that corresponds to tonotopic origin. (B) Violin plots illustrate examples of genes that show either differential or uniform expression across the three tonotopic regions. Dunn’s test was used to assess significance for each possible comparison, as indicated by colored dots next to p values. White dots and error bars in B represent mean and standard deviation, respectively. See Keys. (C-D) Regional differences in expression of Kcns3 and Hcrtr2 were confirmed by RNAscope of P25 tissue sections (C), quantified in D. White dots and error bars in D represent mean and standard deviation, respectively. (E) Further analyses of scRNA-seq data revealed that some genes exhibit regional variation in a subtype-specific manner. Trends for all SGNs are shown in grey solid lines and for Ia (green), Ib (purple) and Ic (blue) SGNs in dashed lines. Dots and error bars represent mean and standard error of the mean. Pairs of dots indicate p values for comparisons across tonotopic regions by Tukey’s HSD test if the data were normally distributed and Dunn’s test otherwise. P values are reported only for statistically significant differences. (F) SGN subtypes are present in all regions of the cochlea, as revealed by tSNE plots showing the anatomic origin of cells (apex, middle, base) in each cluster. However, the proportions differ in the basal turn of the cochlea compared to the apex and the middle (G). Scale bars: 10 μm (C). See also Supplemental Figure 4.
Figure 2.3 (Continued)
compared to 425 genes that differ across subtypes \((p < 0.01)\) (Supplemental Figure 4A). Unlike the genes that drive the Ia, Ib, and Ic classification, region-enriched genes were expressed mostly in gradients (Figure 2.3B). For example, genes for two \(K^+\) channel subunits \((Kcns3, Kcnip4)\) and several guidance-related factors \((NtnG2, Wnt3)\) were enriched in the apex (Supplemental Figure 4A). RNAscope of P25-P27 mouse cochlea validated the tonotopic variation in expression observed by scRNA-seq. Levels of \(Kcns3\), which encodes the \(Kv9.3\) \(K^+\) channel subunit, were highest in the apex, lower in the middle and even lower in the base of the cochlea (median number of puncta per cell: 11.5, 9, 6, respectively, Figure 2.3C,D). Similarly, expression of \(Hcrtr2\), which encodes the hypocretin receptor type 2, was highest in the middle of the cochlea and lower in both the apex and the base (median number of puncta per cell: 44, 34, 33, respectively, Figure 2.3C,D), consistent with scRNA-seq results (Figure 2.3B). By contrast, expression of the pan-neuronal marker \(Tubb3\) showed minimal variation (median number of puncta per cell: 86, 85, 89 in apex, middle, base, respectively, Supplemental Figure 4B), indicating that the observed variability in \(Kcns3\) and \(Hcrtr2\) transcripts is unlikely to be due to technical artifacts.

Closer analysis revealed that some genes vary along the tonotopic axis in a subtype-specific manner. For example, \(Efna1\), which encodes the axon guidance molecule Ephrin A1, is expressed at higher levels in the middle and base relative to the apex among Ib SGNs, but only enriched in the base among Ia and Ic SGNs (Figure 2.3E). Both \(Kcnip2\) and \(Cpne6\) are expressed in an apical-basal ascending gradient among Ib and Ic SGNs, but selectively enriched in the base among Ia SGNs (Figure 2.3E). \(Cacng5\), which encodes a voltage-gated \(Ca^{2+}\) channel subunit, shows a particularly complex pattern, with an apical-basal descending gradient among Ic SGNs, an apical-middle descending gradient among Ia SGNs, and relatively uniform expression among
Ib SGNs. *Pcdh9* is expressed in apical-basal gradient but only among Ic SGNs. We did not observe subtype-specific variation in expression for known pan-neuronal genes, such as *Tubb3* and *Atp1b1*, or other weakly-expressed genes that exhibit tonotopic variation, such as *Ntng2* and *Wnt3* (Supplemental Figure 4C), suggesting that such differences are not artifactual. Overall, analysis across the tonotopic axis for each individual class of SGNs revealed 184, 157 and 183 genes with regional differences in expression among Ia, Ib, and Ic SGNs, respectively. Of the 524 total genes, 494 were unique, which is 3 times higher than the genes identified in subtype-blind comparisons. Only 77 of the 524 genes overlapped with the gene list derived from subtype-blind comparisons. Thus, a subtype-aware analysis of scRNA-seq data unmasked a new layer of tonotopic variation among SGNs.

Even some of the genes that vary among SGN subtypes exhibit additional variation along the cochlear axis. The most robust subtype markers, such as *Calb2*, *Cacna1b*, *Ntng1*, and *Lypd1*, were expressed at similar levels in the apex, middle and base (Supplemental Figure 4D). However, some functionally relevant genes (e.g., *Lrrc52*, *Kcnip4*, *Anxa5*, *Ryr3*) showed additional regional differences (Supplemental Figure 4E). For instance, the potassium channel subunit *Kcnip4* was enriched in Ia SGNs in the apex and middle, but in Ic SGNs in the base. This indicated that, although the molecular identities of Ia, Ib, and Ic SGNs across the cochlea are largely similar, subtle variations exist in some regions, particularly in the base.

To further investigate how SGNs vary across the three major turns of the cochlea (apex, middle, base), we annotated the anatomic origin of every neuron in tSNE plots that depict SGN subtypes. We observed different proportions of SGN subtypes in each turn (Figure 2.3F). In the middle turn, there were 33% Ia, 45% Ib, and 22% Ic SGNs. The proportions were similar in the apex (31% Ia, 43% Ib, and 26% Ic) (Figure 2.3G). However, the base contained a significantly
higher proportion of Ia SGNs (46%), largely at the expense of Ib SGNs (24%). Thus, tissue-level variation in SGN subtype proportions is a tonotopic feature that is superimposed on gene expression gradients across the mouse cochlea.

Type Ia, Ib, and Ic SGNs exhibit stereotyped anatomical and morphological differences

Previous studies showed synapse size differences and spatial segregation of the peripheral projections of the three SR groups (Kawase and Liberman, 1992; Liberman, 1982; Liberman et al., 2011). To map our molecular classification onto the known anatomy, we took advantage of sparse labeling of SGNs in the Maft<sup>CreERT2;Ai9</sup> transgenic line to trace neuronal projections from the cell body to the peripheral synapse. Consistent with scRNA-seq and RNAscope results, levels of the calcium-binding protein CALB2 (calretinin) varied among SGN fibers (Figure 2.4B-B”). Double staining for CALB2 revealed that levels in peripheral processes correlated strongly (\(\rho = 0.86\)) with those in the cell body (Supplemental Figure 5A, B). Thus, CALB2 levels in individual fibers can be used to identify SGN molecular subtypes.

We observed a striking organization of Ia, Ib, and Ic peripheral processes coursing through the osseous spiral lamina (OSL), which is flanked by *scala vestibuli* (SV) and *scala tympani* (ST) (Figure 2.4A). In optical cross sections of image stacks capturing CALB2- and neurofilament (NF)-labeled afferent processes in the OSL in the P25-P27 cochlea (Figure 2.4C-C”), fibers with the highest levels of CALB2 (CALB2<sup>high NF</sup>) were closer to ST, while those with the lowest level of CALB2 (CALB2<sup>lo NF</sup>) were closer to SV. The mid-OSL region was occupied by fibers with intermediate CALB2 levels (CALB2<sup>mid NF</sup>) (Figure 2.4D).
Figure 2.4: Type I SGN peripheral processes and synapses are anatomically segregated by subtype. (A) Schematic depicting a cross-section of the cochlea (left) with a magnified view of the boxed area on the right. The three perspectives corresponding to the cochlear wholemount images in B-H are indicated (right). Blue rectangle represents the plane of section through confocal image stacks of afferent fibers (red) extending through the osseous spiral lamina (OSL) to terminate along the basolateral surface of the hair cell (HC) (green). (B-C) Side (B) and cross-sectional (C) views of a wholemount cochlea stained for CALB2 (green, B,C) and NF-H (neurofilament heavy chain) (red, B’,C’), with merged images (B”,C”). CALB2+ fibers preferentially project towards the pillar side of the inner hair cell (IHC) compared to the total population of all NF-H+ SGN processes and are segregated along the scala vestibuli (SV)-scala tympani (ST) axis in the OSL (C-C”). CALB2 antibody also labels IHCs. (D) Afferent fiber distribution in the OSL depicted as box and whisker plots. CALB2 fluorescent intensity levels were measured for all NF-H+ fibers in the OSL cross-section (n = 5 animals). Fibers were split into three groups based on CALB2 levels: ‘low CALB2’ (n = 165 fibers), ‘medium CALB2’ (n = 82 fibers), and ‘high CALB2’ (n = 174 fibers). Distance from the median center of each nerve bundle was calculated for individual fibers from each cluster. Error bars represent minimum and maximum values. P values indicate results of Tukey’s HSD test following one-way ANOVA. (E-H) Individual tdTomato-labeled fibers (red) (E, E”) were traced in cochlear wholemounts from Mafb^CreERT2;Ai9 animals that were also stained for CALB2 (green, E’, E”) to assign subtype identity. Presynaptic ribbons were defined by co-staining for CtBP2 (white, F-H). In this example, three individual tdTomato-labeled SGN fibers (arrows) express ‘high’ (2), ‘medium’ (3), and ‘low’ (1) levels of CALB2 as they project through the OSL (E, E”). The same three fibers segregate along the modiolar-pillar axis of the IHC, shown in side view in F. Each
**Figure 2.4 (Continued)**

tdTomato-labeled fiber terminates opposite a single presynaptic ribbon, shown in high resolution reconstructions (H). (I-J) Quantification of all analyzed fibers (n = 61, 5 animals) revealed that both fiber position (I; \( \rho = 0.72 \)) and ribbon size (J; \( \rho = -0.70 \)) correlate with CALB2 intensity. (K) Type Ia (green), Ib (purple), and Ic (blue) SGNs extend peripheral processes that are segregated in the OSL and along the modiolar-pillar axis of the IHC where they are apposed by presynaptic ribbons that decrease in size along the same axis. These features match those described for high, medium, and low-SR SGNs. Scale bars: 10 \( \mu \)m (B, C, E, F); 5 \( \mu \)m (G). See also Supplemental Figure 5.
Figure 2.4 (Continued)
This organization matches the projections of low-, medium-, and high-SR SGNs along the SV-ST axis (Kawase and Liberman, 1992). Examination of tdTomato-labeled olivocochlear efferent axons in ChatCre;Ai14 mice indicated comparatively even distribution across the OSL (Supplemental Figure 5E-G) in contrast with tdTomato NF+ SGN processes. Thus, the presence of CALB2lo NF+ fibers closer to SV is not conflated by olivocochlear axon organization. The position and morphology of synapses also matched known differences among SR subtypes. By following sparsely labeled SGN processes from the OSL to their IHC terminals (n = 61 SGNs, 5 P25-P27 MafbCreERT2;Ai9 animals; Figure 2.4E-H), we found that peripheral axons with low CALB2 expression (Ic subtype) ran closer to SV and could be traced to the modiolar aspect of IHCs (Figure 2.4I), where they formed synapses with large presynaptic ribbons (Figure 4J). Conversely, fibers with high CALB2 levels (Ia subtype) were closer to ST, projected to the pillar face (Figure 2.4I), and formed synapses with small ribbons (Figure 2.4J). Processes from neurons with medium CALB2 levels (Ib subtype) were flanked by high and low CALB2 processes (Ia and Ic, respectively) along the modiolar-pillar axis and were apposed to medium-sized synaptic ribbons. Overall, there was a strong positive correlation between CALB2 fiber intensity and synapse position (\(\rho = 0.72\)) (Figure 2.4I), and a strong negative correlation (\(\rho = -0.70\)) between CALB2 fiber intensity and the size of the associated ribbon (Figure 2.4J). Thus, Ia, Ib, and Ic fibers are topographically organized in the OSL and this order is preserved along the modiolar-pillar axis of the IHC, accompanied by a gradient of presynaptic ribbon size in IHCs (Figure 2.4K). Overall, based on fiber position, synapse location, and synapse morphology, the Ia, Ib, and Ic subtypes match the features of high-, medium- and low-SR subgroups, respectively.
Type Ic SGNs are selectively vulnerable to age-related loss

In aging animals, SGNs are progressively lost, and low-SR fibers are the most affected (Schmiedt et al., 1996; Sergeyenko et al., 2013). Thus, we assessed Type Ia, Ib, and Ic SGNs in 32, 64, and 108 week old mice, as determined by expression of Calb2 and Lypd1 (Figure 2.5). SGN loss in aging animals was characterized by a gradual loss of cells expressing low levels of Calb2 and high levels of Lypd1 (Figure 2.5A), as evident in population histograms (Figure 2.5B,C). To assess the composition of SGNs at each stage, we defined Calb2hi Lypd1off, Calb2mid Lypd1off, Calb2lo Lypd1hi neurons as Ia, Ib, Ic subtypes, respectively. As expected, overall SGN density declined with age (Figure 2.5D). Only Type Ic SGNs showed a similar decrease, dropping from 30% at 32 weeks (wks) to 20% at 64 wks and still further to 11% at 108 wks (Figure 2.5E). The proportion of Ia and Ib SGNs increased. Thus, Ic SGNs are selectively vulnerable to age-related hearing loss.

Signatures of SGN subtypes are present at birth and refined over the first postnatal week

The discovery of molecularly distinct SGN subtypes raises the important question of how this heterogeneity is established in development. In many systems, identities are apparent as soon as neurons become post-mitotic, with additional activity-dependent modification as the neurons are integrated into functional circuits. In the mouse cochlea, Type I and Type II SGNs exhibit distinct anatomical features by E16.5 (Koundakjian et al., 2007). By contrast, variation among Type I SGNs has not been detected until postnatal stages, after the peripheral processes have reached their IHC targets, and IHC-SGN synapse morphology is not fully mature until the 4th postnatal week (Liberman and Liberman, 2016). Since the nature of the synapses linking IHCs to Type I SGNs likely influences neuronal thresholds (Liberman et al., 2011), this relatively slow
Figure 2.5: Type I SGN subtypes show differential vulnerability to age-related hearing loss.

(A) SGN subtype identity was assessed using RNAscope to quantify levels of Calb2 and Lypd1 transcripts at 32, 64 and 108 weeks, shown in representative tissue sections. (B-C) Histograms show the frequency distribution of Calb2 (B) and Lypd1 (C) mRNA levels for all analyzed SGNs at each age (n = 212 cells, 5 animals at 32 weeks (top); 175 cells, 5 animals at 64 weeks (middle); 155 cells, 5 animals at 108 weeks (bottom)). The Type Ic population, defined by low levels of Calb2 and high levels of Lypd1, is shaded in yellow. (D-E) SGN density (% relative to the 32 wk time point) decreases over time (D) and this loss is matched by a decrease in the proportion of Ic SGNs (E). Type Ia and Ib SGNs increase in proportion over the same time frame, indicating that loss of Ic SGNs likely accounts for the overall decrease in density. Filled dots and error bars represent mean and standard deviation, respectively. P values indicate results of Dunn’s test following one-way ANOVA. Scale bar: 10 µm (A). See also Supplemental Figure 6.
Figure 2.5 (Continued)

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<th>64 Wks</th>
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*Calb2* *Lypd1*

B

**Calb2**

Frequency (%)

C

**Lypd1**

Frequency (%)

D

$p = 0.003$

E

La

$p = 0.003$

Lb

$p = 0.004$

Lc

$p = 0.001$
and late emergence of functional heterogeneity suggests that SGN subtype identity may not be fully established until the end of the first month of life. However, in the absence of a molecular toolkit, analysis of SGN development has been limited to young adult and adult stages, obscuring early developmental events that shape SGN identities.

Using subtype-specific markers, we found that SGNs begin to diversify perinatally, followed by a period of refinement over the first postnatal week (Figure 2.6). The Ia marker \textit{Calb2} and Ic marker \textit{Lypd1} are already expressed in SGNs by late embryonic stages (E18.5) and at P0.5, but differ sharply in breadth of expression: whereas \textit{Calb2} is expressed in almost all neurons (>90%), \textit{Lypd1} expression is more restricted (60-70%) at both time points (Figure 2.6A, Supplemental Figure 7A-C). \textit{Calb2} levels are relatively uniform at these early stages, unlike the strong variability observed in the mature cochlea (Figure 2.2J). Given the extensive co-expression and lack of a strong \textit{Calb2} gradient, SGN identities cannot be assigned confidently.

By P3, \textit{Calb2} levels are more heterogeneous, and a small fraction of cells begin to show mutually exclusive expression of \textit{Calb2} and \textit{Lypd1}, with significantly more \textit{Lypd1}$^{+}$\textit{Calb2}$^{-}$ neurons compared to P0.5 (Figure 2.6B, magenta dots). The gradient of \textit{Calb2} expression is even sharper at P7, and more low-\textit{Calb2} or \textit{Calb2}$^{-}$ neurons express high levels of \textit{Lypd1}. At P26, \textit{Calb2} and \textit{Lypd1} show mutually exclusive expression in ~90% of SGNs (Figure 2.6D). These observations suggest that identities take shape through the gradual downregulation of \textit{Calb2}, followed by maintenance of \textit{Lypd1} in select SGNs. Consistently, most SGNs initially express \textit{Calb2}, but this proportion drops significantly after P3 (\(p = 0.03\), Figure 2.6C inset, green line). Expression of \textit{Lypd1}, in comparison, first ramps up, possibly reflecting a slight delay in the onset of \textit{Lypd1} relative to \textit{Calb2}, and then decreases over time (\(p = 5 \times 10^{-5}\), Figure 2.6C inset, magenta line).
Figure 2.6: Type I SGN subtypes emerge gradually over the first postnatal week. (A-B) Representative images of *Calb2* (green) and *Lypd1* (magenta) mRNA detected using RNAscope in tissue sections of cochlea at various developmental stages (A). Scatterplots (B) show expression levels of *Calb2* alone (green), *Lypd1* alone (magenta) or both markers (orange) in individual SGNs (n = 100 randomly selected cells at each time point). (C-D) Over time, the proportion of cells expressing both *Calb2* and *Lypd1* decreases (orange, B, C), shown also for expression of each gene individually (magenta and green, insets). In parallel, there is an increase in the proportion that express a single subtype marker (D), shown for the whole population (black), as well as separately for *Lypd1*+ only SGNs (magenta, inset) and *Calb2*+ only SGNs (green, inset). Means are shown in solid dots, with raw data from each individual animal in open circles. *P* values indicate results of Tukey’s HSD test (left) and one-way ANOVA (right, inset). Scale bars: 10 μm (A).
Figure 2.6 (Continued)

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B

![Graph showing the relationship between Calb2 and Lypd1](image)

C

Coexpressing subtype markers

![Graph showing the proportion of coexpressing markers over age](image)

D

Expressing a single subtype marker

![Graph showing the proportion of single marker expression over age](image)
These trends coincide with a sharp decline in the proportion of cells co-expressing subtype-specific markers both within the first postnatal week (P3 to P7: $p = 0.03$) and over the first 3 weeks of postnatal development (P3 to P26, $p = 3 \times 10^{-5}$) (Figure 2.6C, yellow line). At the same time, the proportion of cells expressing only one of the two markers increases (P3 to P26: $p = 2 \times 10^{-4}$; P3 to P7: $p = 0.07$) (Figure 2.6D). Thus, SGN molecular identities begin to take shape around birth, with broad and overlapping expression of markers that becomes progressively restricted and signs of adult-like mutually exclusive expression appearing by the end of the first postnatal week.

**Synaptic transmission from IHCs is necessary for SGN diversification**

Mice begin to respond to airborne sound around P12 and cochlear function continues to mature into the 3rd postnatal week (Shnerson and Pujol, 1981). However, our data indicate that molecularly distinct SGNs emerge just after peripheral synapses begin to form at birth. To investigate whether synaptic communication influences SGN diversification, we performed scRNA-seq in P25-P27 $Vglut3^{-/-}$ mice, in which glutamatergic transmission from IHCs to SGNs is abolished. Transcriptional profiles of 40 SGNs from $Vglut3^{-/-}$ animals and 88 origin-matched wildtype SGNs were analyzed as described above. In a tSNE plot, wildtype (WT) middle-turn SGNs again segregated into three distinct groups (Figure 2.7A). SGNs from $Vglut3^{-/-}$ animals, on the other hand, split into only two highly asymmetric groups. Unsupervised graph-based clustering sorted WT SGNs into 3 clusters (M1, M2, M3), containing 33%, 43% and 24% of the cells, respectively. In contrast, 80% of $Vglut3^{-/-}$ SGNs formed a single cluster (M4), with the remaining 20% mostly in a small fifth cluster (M5). Based on expression of subtype-specific markers, WT cells in the M1, M2, M3 clusters were identified as Ia, Ib, and Ic SGNs,
Figure 2.7: SGN heterogeneity is altered in a mouse model of congenital deafness. (A) tSNE embedding of single cell transcriptomic profiles from wildtype (WT, circles) and Vglut3<sup>−/−</sup> (triangles) animals, with 5 distinct clusters (M1-M5) predicted by graph-based unsupervised clustering indicated by color. M1, M2 and M3 correspond to WT Ia, Ib, and Ic SGNs, respectively, whereas M4 and M5 consist of SGNs from Vglut3<sup>−/−</sup> animals. (B) Subtype identities of neurons from Vglut3<sup>−/−</sup> animals were assigned using supervised clustering by the Random Forest method. WT cells are shown in grey, and cells from Vglut3<sup>−/−</sup> animals are shown in colors corresponding to their predicted subtype identities. Cluster M4 (brown, A) consists of Type Ia-like SGNs (green, B), whereas the remaining neurons in cluster M5 (red, A) are either more like Type Ib (purple, B) or Type Ic (blue, C) SGNs. (C) Subtype proportions are significantly altered in Vglut3<sup>−/−</sup> animals compared to WT, with a dramatic loss of Ic SGNs. P values indicate results of Test of Equal Proportions between Ia, Ib, and Ic subtypes in the two genetic backgrounds. (D-E) SGNs from WT and Vglut3<sup>−/−</sup> animals show broad differences in gene expression reflective of a shift from Ic to Ia identity, shown both in a heatmap (D) and in scatterplots (E) of the level of expression of Ia (Calb2, Rxrg, Pcdh20) and Ib/Ic (Lypd1, Pou4f1, Ntng1, Runx1) subtype markers (WT, top; Vglut3<sup>−/−</sup>, bottom). Yellow shading marks cells belonging to B/C or C clusters. (F-H) RNAscope of tissue sections from P3 (F), P8 (G), and P27 (H) WT (left) and Vglut3<sup>−/−</sup> (right) animals shows that expression of Calb2 (green) and Lypd1 (magenta) initiates normally at P3, quantified in F’. However, the proportion of Lypd1<sup>+</sup> SGNs is decreased at P8 (G’), and there are almost no Lypd1<sup>+</sup> SGNs remaining at P27 (H’). Scatterplots (F’-H’) show individual cells expressing Calb2 alone (green), Lypd1 alone (magenta) or co-expressing both markers (orange). (I-I”) Proportions of SGNs in control (blue circles) and Vglut3<sup>−/−</sup> (red triangles) animals that express any Lypd1 (I), that express only Lypd1 (I’), or that express only Calb2 (I”). Means
Figure 2.7 (Continued)

shown in solid symbols, with raw data from individual animals in open symbols. *P* values refer to results of independent samples t-test between the two genetic backgrounds at each time point.

(J) Schematic showing developmental emergence of mutually exclusive expression between *Calb2* and *Lypd1*. In *Vglut3*<sup>-/-</sup> animals, in which glutamate release from IHCs is abolished, *Lypd1*<sup>+</sup> SGNs are severely underrepresented compared to control animals, beginning after P3, resulting in overabundance of Ia SGNs by the fourth postnatal week. See also Supplemental Figure 7. Scale bars: 10 µm (F-H).
Figure 2.7 (Continued)
respectively. The mutant cells in cluster M4 expressed Ia markers (such as Calb2) (Supplemental Figure 7D), while those in M5 expressed mostly Ib markers (Ning1, Pou4f1) and very low or zero levels of Ic markers (Lypd1, Grm8) (Supplemental Figure 7D). Thus, the vast majority of SGNs (80%) from Vglut3−/− animals are Ia-like and the rest are Ib/Ic-like (20%). To corroborate these results, we utilized Random Forest, an ensemble machine learning approach, for supervised classification of Vglut3−/− cells based on subtype definitions derived from WT cells (Figure 2.7B). Importantly, this approach is independent of sample size. After verifying that the classifier performed robustly (Supplemental Figure 7E-G, see Methods), predictions for SGNs from Vglut3−/− animals were generated (colored triangles in Figure 2.7B). We found that 73% of the mutant cells were transcriptionally similar to Ia SGNs, 25% were Ib-like, and 3% were Ic-like (Figure 2.7C). Consistent with the results of unsupervised classification, this indicated an overabundance of Ia-like neurons at the expense of Ib and Ic subtypes. Indeed, pooled comparison of scRNA-seq profiles of mutant and WT SGNs revealed a broad transcriptional shift towards the Ia phenotype, with downregulation of multiple Ib and Ic markers, such as Ttn and Lypd1, respectively (Figure 2.7D).

Variability in Calb2 expression further supported this interpretation: Calb2 levels ranged from moderate to high (standard deviation, s = 0.07) across all Vglut3−/− cells, as expected for a population comprised mostly of Ia SGNs. This contrasts with the steep gradient from zero to high expression observed among WT cells (standard deviation, s = 0.78) (Figure 2.7E). In addition, Lypd1 expression was limited to ~18% of cells, all of which lie near the WT Ib/Ic clusters in the tSNE plot (Supplemental Figure 7D). Changes in proportions of neurons expressing other Ib/Ic-specific or Ia-enriched transcription factors and axon guidance molecules
(Figure 2.7E) are consistent with the interpretation that SGN subtypes are significantly altered in \textit{Vglut3}^{-/} animals.

**Defective consolidation of Ic identity in \textit{Vglut3}^{-/} animals**

Depolarization of IHCs triggers action potential in SGNs, both in pre-hearing animals (<P12), when waves of spontaneous activity propagate from IHCs to SGNs and up the auditory axis, and in post-hearing animals, when IHC-SGN transmission is essential for mEPSCs and sound-evoked action potentials. The change in SGN proportions seen in \textit{Vglut3}^{-/} animals could arise from disruption of activity at either stage. To determine when SGN diversification goes awry, we examined expression of subtype-specific markers at earlier developmental stages. At P3, both \textit{Calb2} and \textit{Lypd1} are expressed in the expected proportion of SGNs in \textit{Vglut3}^{-/} animals compared to WT (all \textit{Lypd1}^{+} SGNs: 0.63 and 0.72, \(p = 0.13\); only \textit{Lypd1}^{+} SGNs: 0.07 and 0.13, \(p = 0.38\); only \textit{Calb2}^{+} SGNs: 0.33 and 0.26, \(p = 0.26\) in control and \textit{Vglut3}^{-/}, respectively) (Figure 2.7F-F’, I-I”). Thus, the onset of subtype specification does not require IHC-driven activity. However, by P8, the proportion of cells expressing the Ic marker \textit{Lypd1} is decreased significantly in \textit{Vglut3}^{-/} animals (All \textit{Lypd1}^{+} SGNs: 0.55 and 0.22, \(p = 7.7x10^{-3}\); only \textit{Lypd1}^{+} SGNs: 0.13 and 0.01, \(p = 0.049\) in control and \textit{Vglut3}^{-/}, respectively) (Figure 2.7G-G’, I-I”). This is accompanied by an increased proportion of \textit{Calb2}^{+} SGNs (only \textit{Calb2}^{+} SGNs: 0.40 and 0.68, \(p = 7.5x10^{-3}\) in control and \textit{Vglut3}^{-/}, respectively) (Fig 2.7I”). These changes persist into the fourth postnatal week (Figure 2.7H-H’), with significantly lower \textit{Lypd1}^{+} SGN proportion at P27 in \textit{Vglut3}^{-/} animals (All \textit{Lypd1}^{+} SGNs: 0.30 and 0.03, \(p = 4.2x10^{-4}\); only \textit{Lypd1}^{+} SGNs: 0.22 and 0.007, \(p = 4x10^{-3}\) in control and \textit{Vglut3}^{-/}, respectively) (Figure 2.7I-I”), consistent with scRNA-seq results (Figure 2.7A-E).
Comparison of marker expression over time suggests that $Vglut3^{−/−}$ animals fail to produce Ib/c subtypes from among a pool of SGNs with mixed identity, which instead assume Ia identities. In control animals, the proportion of $Lypd1^+$ SGNs (-13%) decreases modestly between P3 and P8 (Figure 2.7I, blue line), consistent with the expected gradual refinement of SGN identities (Figure 2.6). However, in $Vglut3^{−/−}$ animals, the proportion of $Lypd1^+$ SGNs drops drastically (-70%) (Figure 2.7I, red line). This is unlikely to reflect accelerated maturation because the proportions continue to decrease to near-absence of $Lypd1^+$ SGNs by P27 (-96% vs. P3, in contrast to -52% in control). At the same time, the proportion of SGNs that express only $Lypd1^+$ (and not $Calb2$) does not increase over time, in contrast to controls (Figure 2.7I’).

Instead, the proportion of $Calb2^+$-only SGNs increases between P3 and P8 to a much greater extent in $Vglut3^{−/−}$ animals (+161%) than in controls (+21%) (Figure 2.7I”). Thus, in $Vglut3^{−/−}$ animals, the $Calb2$ gradient does not sharpen and there is a dramatic loss in $Lypd1$ expression, as if most remaining SGNs retained an Ia identity rather than taking on a Ib/c identity in the first postnatal week and beyond (Figure 2.7J).

Thus, our data indicate that IHC-driven activity is required for the developmental refinement of SGN identities from an early phase of broad and overlapping marker expression to subtype-restricted expression, with impairment of this process causing a drastic shift in SGN subtype proportions within the first postnatal week, which persists in the mature cochlea (Figure 2.7J).
Discussion

SGNs enable the wide dynamic range needed to sense sounds from the rustle of a leaf to the roar of a jet engine. Although SGNs show well-characterized differences in morphological and functional properties that subserve dynamic range expansion (Kawase and Liberman, 1992; Liberman, 1982, 1991; Liberman et al., 2011; Taberner and Liberman, 2005), the molecular correlates of such heterogeneity have been a mystery. Using single-cell transcriptomics in mice, we define three subtypes of Type I SGNs that match the anatomical features of those originally defined by physiology, including the subtype that is preferentially affected by age-related hearing loss. These SGN subtypes express distinct cohorts of ion channels, receptors, synaptic proteins, and adhesion molecules that vary both locally and along the tonotopic axis, indicating that a complex reservoir of SGNs is available for coding auditory information. Unexpectedly, SGNs with distinct identities emerge during the first postnatal week in an activity-dependent manner. Together, these results establish a new molecular logic for cochlear circuitry, reveal the potential for many endogenous differences among SGNs, and suggest a new model for when and how SGN diversification is achieved, with important implications for the treatment of congenital deafness.

It was not a given that SGNs could be parsed molecularly, since the response properties that differentiate them could be imposed entirely by other cells in the circuit, e.g. functional presynaptic differences within IHCs (Wichmann and Moser, 2015) or differential innervation by olivocochlear efferents (Yin et al., 2014). To the contrary, we find that individual SGNs are intrinsically different in ways that could fundamentally alter their input and output properties. For instance, each subtype expresses unique cohorts of K⁺ channel subunits, Na⁺ channel subunits, and Ca²⁺-binding proteins that could yield distinct responses to input from IHCs and
efferent neurons, such that both endogenous and circuit-level differences underlie SR-group
heterogeneity. Notably, one of the most robust markers for Ic SGNs is Lypd1, which encodes a
transmembrane receptor implicated in cholinergic signaling (Tekinay et al., 2009). Thus, Type Ic
SGNs may exhibit unique responses to acetylcholine released by olivocochlear efferents.
However, Lypd1 is only one of many class-specific genes that could create other, as yet
uncharacterized, diversity in SGN electrical properties beyond SR and threshold. For instance,
Type Ib and Ic SGNs are similar at the molecular level, suggesting they might be difficult to
distinguish physiologically and highlighting the need for other means of classification. Similarly,
whole-cell recordings from immature Type I somata reveal a range of electrical properties
(Crozier and Davis, 2014; Liu et al., 2014), some of which depend on voltage-gated K+ channels
(Liu et al., 2014) or correlate with levels of Ca2+-binding proteins (Liu and Davis, 2014). Given
this broad range of possible physiological differences, SR differences are unlikely to be
explained by single genes, and other variable features may also contribute to auditory coding in
ways we do not yet understand.

SGNs may also differ in how they transmit signals to their brainstem targets in the
cochlear nucleus complex (CNC). Type Ia, Ib, and Ic SGNs express distinct complements of
axonal molecules, including proteins associated with presynaptic terminals such as the
metabotropic glutamate receptor Grm8 and the exocytosis regulator Cplx2. Such molecular
differences could generate distinct modes of plasticity and presynaptic release centrally,
consistent with suggestions that presynaptic release is not uniform in the CNC (Cao and Oertel,
2010). Given the differences in expression of presynaptic molecules, as well as cell-adhesion
molecules such as the Netrin family gene Ntng1, SGN subtypes may stimulate separate networks
of CNC targets by virtue of their unique synaptic properties and connectivity. Indeed, previous
studies have revealed both mixed and selective innervation of central targets by different SR groups. Multipolar cells in one part of the anteroventral cochlear nucleus (AVCN) receive inputs from all SR groups, whereas multipolar cells in another part of the AVCN, and in the small-cell cap region, are almost exclusively innervated by low- and medium-SR fibers (Liberman, 1991; Ryugo, 2008).

With the discovery of molecular differences among Type I SGNs comes the opportunity to not only test long-standing hypotheses but also pursue fresh questions regarding their functional properties and significance. Much has been learned about the physiology of Type I SGNs in adult ears via single-fiber recordings from the VIII\textsuperscript{th} nerve, work that showed correlations between SR and numerous other physiological features, as well as vulnerability to aging and noise damage. Our results make it possible to further elucidate the features and functions of individual subtypes, for instance by assessing the circuit-level contributions of each subtype to auditory behavior by using genetic approaches to perform targeted ablations. Some caution is warranted before embarking on such studies, however, due to the broad distribution of subtype-specific markers early in development. Careful consideration of expression dynamics will be critical for obtaining interpretable results. In this regard, combining subtype-specific markers with approaches that permit precise spatiotemporal control of gene expression, for instance via intersectional targeting strategies, may prove useful. Our data also stimulate new hypotheses regarding the mechanistic basis of SGN physiology, including but not limited to SR. Due to the technical difficulties of recording from individual adult SGNs both \textit{in vivo} and \textit{in vitro}, it will take time to work out how individual molecules contribute to SGN subtype function and ultimately hearing. Indeed, the breadth of molecular differences uncovered here identifies
new questions beyond the origins of SR heterogeneity and opens many doors that will fuel the field for years to come.

Multiple layers of tonotopic variation among Type I SGNs

Tonotopy is a fundamental organizing principle in the auditory system that originates with the orderly gradient of frequency tuning along the cochlear spiral. Although emphasis is placed on this frequency axis and the gradient in hair cell mechanics that underlies it, tonotopic differences in SGN physiology have also been described, such as differences in action potential kinetics at neonatal stages (Adamson et al., 2002). However, with only a few genes identified (Adamson et al., 2002; Flores-Otero and Davis, 2011), neither the origin nor the extent of such variation is clear. Our results reveal differences in molecules spanning diverse gene families across the tonotopic axis. Many are involved in neuronal connectivity, such as the Ephrin gene EFNA1, and might therefore help maintain topographic organization within the VIIIth nerve and in the CNC. Others could influence SGN physiology, such as the K+ channel subunit KCNS3, as well as CACNG5, CPNE6, and VAMP2. Thus, rather than serving as identical conduits within a topographically ordered circuit, SGNs may exhibit endogenous differences in their response properties that affect encoding of low versus high sound frequencies. Intriguingly, some tonotopic variation was limited to individual SGN subtypes, such that there could be three overlapping maps along the cochlear spiral. Nonetheless, the difference across subtypes, rather than tonotopic variation, was the dominant form of molecular heterogeneity in the adult cochlea. This suggests that the ear's dynamic range is extended by the development of molecularly distinct cohorts of SGNs that are further specialized based on their tonotopic position.
Detection of complex sounds can depend not only on the properties and connectivity of SGN subtypes, but also on the proportions of these subtypes along the tonotopic axis. Interestingly, the distribution of SR rates is uniform in some species (Liberman, 1978; Tsuji and Liberman, 1997), whereas others show striking differences across the tonotopic axis (Schmiedt, 1989). The situation in mice is ambiguous, since it is harder to record from large populations of SGNs in such small animals (Taberner and Liberman, 2005). Here, we find a larger proportion of Ia neurons and smaller proportion of Ib neurons in the base compared to the rest of the cochlea. Such regional variation could enable heightened responses to certain, ethologically relevant sound frequencies, much as the presence of a high cone:rod photoreceptor ratio in the fovea of the retina enables strong color perception (Curcio and Hendrickson, 1991). Thus, SGNs could contribute to stimulus coding at multiple levels, from cell-based differences within and across SGN classes to tissue-level differences in their pattern of distribution along the cochlear spiral.

SGN subtypes emerge early via an activity-dependent process

The presence of molecular SGN subtypes presents a challenge for wiring the developing cochlea, where one IHC connects to 10-30 SGNs that share the same basic patterns of connectivity, but also exhibit diverse anatomical and functional phenotypes (Liberman, 1982; Liberman et al., 2011). Heterogeneity in the size of postsynaptic glutamate receptor patches is detectable by P14 in mice, but other features such as synapse number, efferent innervation density, ribbon size gradient, and ion channel localization reach maturity only between the third and fourth postnatal weeks (Kim and Rutherford, 2016; Liberman and Liberman, 2016). It was therefore surprising that Ib/Ic markers appear in subsets of SGNs shortly after their processes reach hair cells around E15.5 (Koundakjian et al., 2007), and that well-defined subtypes emerge
coincident with a period of synaptogenesis and pruning during the first postnatal week (Huang et al., 2012; Meyer et al., 2009). Thus, SGN diversity is seeded soon after they form connections, pointing to a major role for signaling between IHCs and SGNs in the acquisition of mature identities.

Although mature Type I SGNs express class-specific genes, these differences emerge after several days of co-expression (Figure 2.6). Transition from such a mixed state is defined by a singular rule: those that maintain Ib/Ic-specific genes lose Ia identity and those that do not continue as Ia neurons. Although transient periods of co-expression are a recurrent theme in the developing nervous system, conflicting identities are typically resolved intrinsically, for instance by mutual repression of class-specific transcription factors within individual progenitors (Jukam and Desplan, 2010). SGN identity, on the other hand, appears to be shaped by neuronal activity. When IHCs can no longer release glutamate to activate SGNs, as occurs in Vglut3−/− animals (Seal et al., 2008), the proportion of Ib/Ic SGNs is drastically decreased. Since markers for Ib/Ic SGNs appear normally at P3 in these animals, activity seems to bias how the mixed state is ultimately resolved, rather than directly specifying Ib/Ic identity. Furthermore, additional unbiased transcriptional profiling in Vglut3−/− animals confirmed that most SGNs assume a nearly normal Ia identity. Thus, IHC-driven activity appears to select a subset of SGNs to take on Ib and Ic fates from a pool of SGNs that would otherwise become Ia.

Although we have focused on IHC-driven events, any change in cochlear activity may also contribute to the final mix of SGNs. Indeed, SGN activity in the neonatal cochlea depends not only on IHCs but also on input from the olivocochlear efferent system. For example, medial efferents form transient cholinergic synapses with IHCs during the first postnatal week (Glowatzki and Fuchs, 2000) and are thus poised to influence IHC-evoked SGN activity during
this period of SGN diversification. Lateral efferents could also influence SGN identity, perhaps in a maintenance role as suggested previously (Yin et al., 2014). In this issue of Cell, (Sun et al., 2018) also report that Type I SGNs can be classified into three broad molecular subtypes that match those we describe here. Consistent with our findings, they demonstrate that subtype-specific marker expression changes upon disruption of mechanotransduction (Tmie<sup>-/-</sup>) or glutamate release from IHCs (Vglut3<sup>-/-</sup>), both of which significantly alter SGN spontaneous activity patterns. Regardless of which factors pattern SGN activity during this critical developmental window, their effects may materialize as changes in expression or function of subtype-specific transcription factors, such as Pou4f1 and Runx1. In fact, both Pou4f1 and Runx1 contribute to diversification of dorsal root ganglion neurons (Chen et al., 2006; Qi et al., 2017; Yoshikawa et al., 2007; Zou et al., 2012) and could play similar roles in SGNs, acting downstream of a generic differentiation program headed by the master regulator Gata3 (Appler et al., 2013; Yu et al., 2013). Interestingly, a similar activity-dependent mechanism diversifies cortical interneurons with distinct firing properties, in this case via the transcription factor Er81 (Dehorter et al., 2015). Such a mechanism of neuronal diversification may be particularly effective in the cochlea, where patterns of IHC-driven activity during an early plastic phase, achieved for instance via efferent modulation, could yield mature subtype proportions across the cochlea from a finite pool of generic postmitotic neurons. Under this model, SGN heterogeneity is established by both intrinsic and extrinsic mechanisms: presynaptically patterned activity informs identity during development, yet the SGNs also express transcription factors that induce and maintain subtype-specific cohorts of genes to further shape each neuron’s mature functional properties. An important question for the future is whether SGN identities remain sensitive to activity into adulthood or whether these early developmental events lock them in place.
Implication for disease and therapy

Loss of $Vglut3^{-/-}$ causes total deafness in mice (Seal et al., 2008), while mutation in $Vglut3$ underlies the deafness DFNA25 in humans (Ruel et al., 2008). Therefore, a major implication of the influence of IHC-driven activity on SGN identity is that some forms of congenital deafness—specifically those that disrupt SGN activity before the onset of hearing—may have an associated SGN pathology. This underscores the need to look beyond the primary molecular lesion in assessments of cellular phenotypes associated with deafness-causing mutations. An outstanding question is whether restoring expression of a defective gene in IHCs is sufficient to correct the sequelae of SGN phenotypes associated with a congenital lesion. The answer lies in part in whether plasticity of SGN identity, particularly its susceptibility to extrinsic influence, persists into adulthood. Should it be necessary to directly target SGNs to re-establish correct proportions, valuable insights may be gained from studies focusing on molecular genetic regulation of SGN subtype identity during development and beyond.
— CHAPTER THREE —

Bclw Prevents Noise-Induced Synapse Loss in the Inner Ear

Chester Chia, Cyrille Sage, Evan Hale, and Lisa V. Goodrich
C.C. performed and analyzed the postnatal and adult Bclw overexpression experiments. C.C. also performed adult injections in the aging mouse subjects. Early preliminary work on Bclw overexpression was done by C.S., who also generated the AAV2/9-\textit{HA-Bclw} virus used in this project. E.H. helped perform adult injections in the aging mouse subjects and will be doing the analysis for that experiment.

**Introduction**

Sensorineural hearing loss is a major public health concern. As communities around the world become increasingly industrialized, people are constantly subjected to a dizzying array of loud sounds. In the United States alone, it is estimated that 24% of adults aged 20-69 suffer from noise-induced hearing loss (NIHL) (CDC). Age-related hearing loss (AHL) is also a common condition, with 33% of adults over the age of 65 reporting significant hearing impairment (WHO). Historically, the therapeutic target for hearing loss has been the hair cells (HCs). Inner hair cells (IHCs) serve as the primary auditory receptors within the inner ear, while outer hair cells (OHCs) operate as a mechanical amplifier (Ashmore, 2008; Hudspeth and Jacobs, 2006; Zheng et al., 2000). Through IHCs, all acoustic information from the outside world is captured and transmitted to spiral ganglion neurons (SGNs), the primary auditory neurons of the inner ear. For decades, loss of HCs was considered to be the primary pathology behind AHL and NIHL; loss of SGNs was thought to be merely a secondary effect (Le Prell et al., 2007; Sugawara et al., 2005; Webster and Webster, 1978).

However, recent work has revealed the vulnerability of IHC/SGN synapses to moderate sound intensities (Hickox and Liberman, 2014; Kujawa and Liberman, 2009; Viana et al., 2015). In both AHL and NIHL, permanent synaptopathy can occur long before IHCs are damaged,
suggesting that SGNs synapses are particularly sensitive to acoustic overexposure and may be the primary target for acoustic trauma. Further work has shown that loss of SGN synapses can result in peripheral axonopathy, and if left untreated, the eventual cell death of the SGN (Kujawa and Liberman, 2009). The synaptic silencing of whole populations of SGNs can go unnoticed to traditional tests of hearing acuity (Kujawa and Liberman, 2009; Schaette and McAlpine, 2011), leading to the term ‘hidden hearing loss’. Ongoing work suggests that patients with hidden hearing loss can struggle to operate in complex acoustic environments, develop tinnitus, and experience a drop in their quality of life (Liberman, 2017). Developing a new generation of therapies to restore and protect SGN synapses from acoustic trauma could provide relief to tens of millions of patients around the world.

Crucially, the axonopathy and cell death of SGNs following primary synaptopathy is a process that takes months to years, during which time the neurons remain physiologically active (Kujawa and Liberman, 2009, 2015). This provides patients with a large temporal window in which regenerative therapies can be introduced into the inner ear. Work with Neurotrophin-3 (NT3) overexpression in the inner ear has shown that protection and restoration of the IHC/SGN synapse is possible (Wan et al., 2014; Wang and Green, 2011). However, the use of neurotrophins to restore function has certain limitations. For one, it is unclear how long the prophylactic effect of NT3 overexpression lasts. The nature of overexpressing a diffusible trophic factor also results in variable branching and regeneration of Type I SGN peripheral processes and synapses. Leveraging effector proteins downstream for NT3-mediated survival in SGNs may result in a more controlled and precise therapy.

In this study, we sought to leverage an endogenous survival system to protect SGNs from AHL and NIHL. Previous studies in dorsal root ganglion (DRG) have identified Bclw, an anti-
apoptotic member of the \textit{Bcl2} family, as a promising therapeutic candidate to protect against axonopathy (Cosker et al., 2013; Courchesne et al., 2011; Pazyra-Murphy et al., 2009; Pease-Raissi et al., 2017). \textit{In vitro} and \textit{in vivo} work has shown that \textit{Bclw} is required for the long-term maintenance of peripheral projections in sensory neurons. Loss of \textit{Bclw} disrupts neurotrophin-mediated survival of axons and overexpression of \textit{Bclw} protein can protect against neurotrophin deprivation (Cosker et al., 2013; Courchesne et al., 2011). Here, we show that overexpression of \textit{Bclw} via adeno-associated viral (AAV) injections into the inner ear can protect SGNs from noise-induced synaptopathy. We also show that treatment leads to functional protection of the inner ear, suggesting that \textit{Bclw} overexpression can act as a prophylactic therapy against future acoustic trauma. Our results suggest that \textit{Bclw} is an attractive drug target for the prevention of hidden hearing loss.
Experimental Procedures

Animal Models

CBA/CaJ male mice were obtained from Jackson Laboratories at 10 weeks of age and bred to age mice to experimentally relevant time points. Bclw -/- mice (Courchesne et al., 2011) were obtained from Rosalind Segal at the Dana Farber Cancer Institute. All experimental procedures were approved by the Institutional Animal Care and Use Committee of the Harvard Medical School and conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Noise Exposure and Cochlear Function Testing

Awake mice were exposed to an octave-band noise (8–16 kHz) for 2 hrs at 100 dB sound pressure level (SPL) in a custom sound-exposure box. The sound was digitally generated with an amplified signal processing filter. The octave-band noise was then funneled through the roof of the exposure box via a loudspeaker and exponential horn. Unrestrained mice were suspended in a wire basket directly under the horn.

Auditory brainstem responses (ABRs) and distortion product otoacoustic emissions (DPOAEs) were recorded in mice anesthetized with an intraperitoneal injection of ketamine (100 mg/kg) and xylazine (15 mg/kg). All recordings were performed in sound-proof and electrically-shielded chamber. During testing, mice were kept on a heating pad held at 37°C. A custom probe tube coupled with two miniature earphones was inserted into the ear canal, allowing for direct delivery of acoustic stimuli into the middle ear. ABR responses were collected for stimulus levels in 5 dB steps from 20 dB to 80 dB SPL. Waveforms were subsequently collected and analyzed in Massachusetts Eye and Ear’s ABR Peak Analysis
Software (Version 1.0.0.6). ABR thresholds were defined as the lowest dB SPL needed to produce a structured and reproducible waveform.

**Cochlear Processing and Immunohistochemistry**

After perfusion, cochleae were dissected from the temporal bones and post-fixed overnight in 4% PFA at 4°C. Cochleae were then rinsed in PBS and decalcified in 120mM EDTA at room temperature for 3 days. Following complete decalcification, cochleae were micro-dissected into 3 cochlear turns (base, middle, and apex) for further processing. For immunostaining, cochlear turns were blocked with 3% normal donkey serum and 1% Triton X-100. Primary antibodies were incubated overnight at 37°C with vigorous agitation. The following primary antibodies were used: mouse (IgG1) anti-CtBP2 at 1:500 (BD Transduction Labs) for pre-synaptic ribbons; mouse (IgG2a) anti-GluR2 at 1:1000 (Millipore) for post-synaptic densities; Goat anti-Calb2 (Swant) at 1:500 for wholemount IHCs and SGNs, rabbit anti-HA (EMD) at 1:500 to stain virally expressed HA-Bclw, and rabbit anti-Myosin 7a at 1:500 (Proteus Biosciences) for OHCs and IHCs. Cochlear turns were washed with 1% Triton X-100 and then incubated for 2-3 hrs at 37°C in species-appropriate secondary antibodies. Cochlear turns were mounted using Fluoromount-G (Thermo Fisher).

**Wholemount Single-Molecule Fluorescent In-Situ Hybridization**

For wholemount mRNA detection by RNAscope (Advanced Cell Diagnostics), the manufacturer’s protocol was used; however, cochleae were also immunostained with anti-CALB2 (Swant), followed by incubation in the appropriate secondary for 1 hr the next day. The following probes were used: Mm-Bclw (424921-C1) and Mm-Tubb3 (423391-C3).
Adult Vestibular Canal Injection

Adult CBA/J mice were anesthetized with an intraperitoneal injection of ketamine (100 mg/kg) and xylazine (15 mg/kg) and placed on a 37°C heating pad. A small incision was made behind the external pinna with spring scissors. The soft tissue was pushed apart using #5 forceps to expose the surface of the posterior semicircular canal. A small hole was hand drilled into the canal using a sharpened microprobe. A pre-made 35G micro-tube was inserted through the lateral hole and glued in place with tissue glue. Once the glue hardened, a Nanoliter 2010 Injector (World Precision Instruments) was used to inject 1.2 uL of virus (10^{10} GCs of AAV2/9-HA-Bclw) directly into the semicircular canal. After the injection, the surgical incision was closed with 7-0 Vycril surgical sutures.

Neonatal Round Window Injection

P0/P1 pups were placed in ice and anesthetized by hypothermia. A small incision was made directly behind the external pinna. The incision was held open with cotton balls while the round window was being located. Once located, a pulled glass micro-tube was inserted into the round window. A Nanoliter 2010 Injector (World Precision Instruments) was used to inject 0.7 uL of virus (10^{10} GCs of AAV2/9-HA-Bclw) directly into the inner ear space. The surgical incision was closed with 7-0 Vycril surgical sutures. Pups were allowed to warm up before being reintroduced to their litter.

Image Acquisition and Analysis

All images were collected on a Leica SP8 Confocal microscope with an oil-immersion 63X objective (1.3 N.A.) and 2X digital zoom. For all images, the Z-dimension was sampled at
0.308 μm. Each Z-stack included 10-12 adjacent inner hair cells for future analysis. Image analysis was performed in proprietary image processing software (Amira, Visage Imaging). Pre- and post-synaptic ribbons in each Z-stack were isolated as Amira iso-surfaces and counted using the “connected components” function in Amira. Inner hair cells in each stack were counted using the hair cell staining from the anti-Myosin VIIa. The number of individual synapses was then assigned to specific inner hair cells to obtain ‘synaptic density per IHC’. For RNAscope samples, images were acquired using Nyquist settings. The Z-dimension was set to capture all the SGN cell bodies within a given optical field.

**Statistical Analysis**

All statistical comparisons were done using Prism software (GraphPad, La Jolla, CA) and presented as mean ± S.D. Sample size for all experiments was determined via power analysis. Specific details for each experiment are included in the text (n values and their meaning) or in the Figure legends (statistical tests used).
Results

Bclw mRNA is enriched in the inner ear

Bclw has unique protective effects against sensory neuron axonopathy (Pease-Raissi et al., 2017). Prior studies have shown that neurotrophins prevent DRG axonopathy by increasing levels of Bclw in both DRG cell bodies and peripheral axons (Cosker et al., 2013). In the cochlea, SGNs receive trophic support along their peripheral processes from neighboring IHCs and supporting cells (Sugawara et al., 2007; Wan et al., 2014). To assess whether Bclw may play a similar role in the inner ear, we sought to first characterize Bclw’s mRNA expression pattern within the inner ear. We performed single-molecule fluorescent in-situ hybridization (RNAscope) in P27 cochlear wholemounts (n = 2 cochlea, 2 CBA/J mice) (Figure 3.1). Consistent with prior scRNA-seq results (Shrestha et al., 2018), Tubb3 mRNA was seen in all labelled SGNs and was restricted to the SGN cell bodies (Figure 3.1A and 1B). We observed expression of Bclw mRNA puncta in both the cell bodies and peripheral processes of SGNs (Figure 3.1A and 1B). Bclw mRNA was also expressed in the IHCs (Figure 3.1C).

Bclw is required for maintenance of inner ear function

The connectivity between IHCs and SGNs is very stereotyped. In mice, each IHC receives innervation from 10-20 SGNs, with each individual SGN forming one synaptic junction along the basolateral surface of the IHC (Liberman, 1982, 1993; Merchan-Perez and Liberman, 1996). Through these synapses, all auditory information is transmitted from the inner ear to the brainstem for further information processing. As a result, the health and connectivity of an inner ear can be readily assessed by counting the number of pre- and post-synaptic puncta present.
Figure 3.1: Bclw mRNA is expressed in both SGNs and IHCs. (A) Representative P27 cochlear wholemounts immunostained for CALB2 and co-stained for Tubb3 (green) and Bclw (red) mRNA via RNAscope. Both Tubb3 and Bclw mRNA are expressed in SGN cell bodies. (B) Representative P27 confocal images of SGN peripheral processes in cochlear wholemounts. Tubb3 (green) mRNA expression is restricted to the SGN cell body, while Bclw (red) mRNA can be seen along the peripheral process. White arrows mark Bclw mRNA in the peripheral axons. (C) Confocal maximum projections of P27 SGN peripheral distal tips and IHCs. Bclw (red) mRNA expression can be seen in both SGN distal tips and IHCs, suggesting multiple functions within the inner ear. White arrows mark Bclw mRNA in the peripheral axons.
Figure 3.1 (Continued)

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SGN Cell Body

B

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SGN Peripheral Process

C

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IHC/SGN Distal Tip
within individual IHCs. It is also now known that synapse loss precedes IHC loss in AHL (Kujawa and Liberman, 2015). To assess Bclw’s involvement in the maintenance of inner ear function, we analyzed 9-month old cochleae from Bclw -/- and quantified synapse loss across tonotopic regions. We observed that Bclw -/- had pre- and post-synaptic density loss in high frequency regions of the inner ear (Figure 3.2A). At low and middle frequency regions, there was no detectable difference in synaptic density between control (n = 8 cochlea, 4 mixed mice) and Bclw -/- (n = 8 cochlea, 4 mixed mice) (Figure 3.2B and 3.2C), suggesting that continued Bclw expression plays a role in maintaining the integrity of synapse health in high frequency regions of the inner ear. High frequency cochlear regions are also known to be more susceptible to damage following AHL and NIHL (Cruickshanks et al., 1998; Kujawa, 2006; Kujawa and Liberman, 2009).

Since we also saw Bclw mRNA expression in the IHCs, we tested whether Bclw -/- mice had any changes in hearing acuity. To test hearing acuity, we recorded auditory brainstem responses (ABRs) and distortion product otoacoustic emissions (DPOAEs). ABRs and DPOAEs serve as functional tests that probe integrity of the IHC/SGN synapse and outer hair cell (OHC) function, respectively. We observed that Bclw -/- mice were functionally deaf at 2-months of age, well before normal onset of AHL. Both ABRs (Figure 3.2D) and DPOAEs (Figure 3.2E) response curves were flat with minimal electrical signal detected. However, wholemount immunostaining in 2-month old control and Bclw -/- mice revealed no changes in synaptic or IHC density (Figure 3.2F and 3.2G). This suggests that Bclw may have multiple functions in the inner ear.
Figure 3.2: Bclw +/- are deaf at 2-months and have reduced synaptic density in high frequency regions of the cochlea at 9-months. (A) Representative 9-month old cochlear wholemounts at the 64 kHz region of the cochlea. Pre- and post-synaptic puncta were stained with anti-Ctbp2 (green) and anti-GluR2 (magenta), respectively. Anti-Myosin VIIa (blue) stained the IHCs. Bclw +/- mice have reduced synaptic density in high frequency regions of the inner ear. (B-C) Quantification of synaptic counts per IHC in 9-month old cochleae. Synaptic density was assessed across various tonotopic areas. At low (8 kHz) and middle (16 kHz) frequencies, Bclw +/- mice (n = 4) did not have reduced pre- and post-synaptic counts compared to control (n = 4). However, from 32 kHz and above, there was a steep decline in synaptic density, suggesting Bclw is needed for long term maintenance of IHC/SGN synapses. (D-E) Hearing acuity was assessed in 2-month old Bclw +/- mice. Both ABR and DPOAE tests suggest that 2-month old Bclw +/- are functionally deaf, well before onset of AHL. Bclw is necessary for the proper function of the inner ear. (F-G) Interestingly, the synaptic counts in 2-month old Bclw +/- was not significantly different from control, suggesting that the early observed deafness may be an IHC phenotype. The statistical significance between Bclw +/- vs. Bclw +/- is shown by asterisks (B-E). *p < 0.05 by Student’s t-test. Error bars indicate s.d.
Figure 3.2 (Continued)

A

B

C

D

E

F

G

9-mth Bclw +/- 64 kHz

Myosin Vila Ctbp2 GluR2

9-mth Bclw +/- 64 kHz

# of CBP2 Puncta per IHC

# of GluR2 Puncta per IHC

# of CBP2 Puncta per IHC

# of GluR2 Puncta per IHC

ABR Threshold (dB SPL)

DPOAE Threshold (dB SPL)

Frequency (kHz)

Frequency (kHz)

10 μm
Neonatal overexpression of Bclw

Prior studies have reported adeno-associated viruses (AAV) to be effective tools for delivering gene therapy into mouse retina and inner ears. In particular, AAV2/9 was shown to preferentially target SGNs for viral transduction (Landegger et al., 2017; Shibata et al., 2017). As a result, we packaged Synapsin-driven, HA-tagged Bclw into an AAV2/9 capsid for injection (Askew et al., 2015). We first assessed transgene delivery efficiency by injecting AAV2/9-HA-Bclw into P0/P1 pups via round window micro-injections (RWI) (Figure 3.3A). P0/P1 injections also offered the maximum amount of time for transgenic HA-Bclw to be overexpressed in both the SGN cell body and peripheral process. After 3-months, we harvested the cochleae and stained for HA-tag and Calb2, a marker for IHCs and most Type I SGNs. AAV2/9-HA-Bclw has high transfection efficiency in SGN cell bodies (Figure 3.3B). Promisingly, we also observed transgenic HA-Bclw making its way up the SGN peripheral processes and towards the basolateral surface of the IHCs (Figure 3.3C).

To determine whether neonatal overexpression of Bclw could protect against future acoustic trauma, we injected AAV2/9-HA-Bclw into P0/P1 pups as described above. The pups were kept until 3-months of age before being exposed to acoustic trauma (8-16 kHz broadband noise @ 100 dBs for 2 hours). Two weeks later, ABRs and DPOAEs were performed and the inner ears were harvested. In mice that received AAV2/9-HA-Bclw, we observed a striking reduction in noise-induced synaptopathy at high-frequency regions (32 and 64 kHz) (Figure 3.3D). Virally treated mice (n = 7, CBA/J) experienced 26% ± 5.34% loss in pre-synaptic counts and 23% ± 6.85% loss in post-synaptic counts. In contrast, mice that received only sham surgery (n = 7, CBA/J) experienced 57% ± 8.11% loss in pre-synaptic counts and 51% ± 8.86% loss in
Figure 3.3: P0/P1 overexpression of BCLW protects against noise-induced trauma (A)

Schematic timeline of the experimental protocol. Neonatal pups received a single RWI of AAV2/9-HA-Bclw and were aged to 3-months before being exposed to acoustic trauma (8-16 kHz band noise @ 100 dBs for 2 hrs). (B-B”) Representative 3-month old cochlear wholemounts stained for CALB2 (green) and HA-tag (red). AAV2/9-HA-Bclw induced Bclw overexpression across SGN cell bodies. (C-C”) Representative 3-month old cochlear wholemounts stained for CALB2 (green) and HA-tag (red); images were taken directly under the basolateral surface of the IHC. HA-tagged BCLW overexpression was also seen along the distal tips of transfected cochleae. Dotted yellow outline corresponds to an individual IHC. (D-D”) Confocal maximum projections of IHCs and synapses from the 32 kHz regions of a cochlear wholemount. Pre- and post-synaptic puncta were stained with anti-Ctbp2 (green) and anti-GluR2 (magenta), respectively. Anti-Myosin VIIa (blue) stained the IHCs. In control subjects, a full complement of synaptic densities could be observed on a per IHC basis (D). 2-wks post-trauma, there is permanent primary synaptopathy (D’); however, overexpression of BCLW in P0/P1 pups protects the IHC/SGN synapse from acoustic trauma (D”). (E-F) Quantification of synaptic counts per IHC following acoustic trauma and viral treatment. Animals that received AAV2/9-HA-Bclw (n=7) had more pre- and post-synaptic densities than sham-treated animals (n=7) post-trauma. (G) ABR P1 amplitudes also increased in BCLW overexpressing mice, suggesting functional preservation of the IHC/SGN synaptic junction. P1 amplitudes were collected following exposure to an 80 dB SPL stimulus. (H) ABR threshold response curve shows that our acoustic trauma protocol did not cause permanent ABR threshold shifts 2-wks post-trauma. Loss of synapses and attenuation of ABR P1 amplitude can be attributed to primary synaptopathy.

88
Figure 3.3 (Continued)

(I-J) Quantifications of synapse number between ‘No trauma’ controls (n=5) and ‘No trauma + HA-Bclw’ (n=5). Early overexpression of BCLW did not result in increased numbers of synaptic densities in the mature inner ear. This suggests that there was no developmental compensation behind our observed protective effects above. The statistical significance of ‘Trauma’ vs. ‘Trauma + HA-Bclw’ is shown by asterisks (E-G). *p < 0.05 by Tukey’s multiple comparisons (2-way ANOVA). Error bars indicate s.d. See also Supplemental Figure 8.
Figure 3.3 (Continued)

A

<table>
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B Composite

B' Calb2

B'' HA-Bclw

C Composite

C' Calb2

C'' HA-Bclw

D No Trauma

D' Trauma

D'' Trauma + BCLW

E

F

G

H

I

J

<Graphs and data plots related to cochlear regions and auditory brainstem responses>
post-synaptic counts (Figure 3.3E and 3.3F). This suggested that Bclw could indeed protect against noise-induced synaptopathy if overexpressed early enough in the maturing mouse.

In order to test if these surviving synapses were still functional, we also assessed ABRs and DPOAEs. The magnitude of the Wave 1 amplitude corresponds to the number of IHC/SGN synapses firing synchronously in response to an acoustic stimulus. Here we noticed that the Wave 1 amplitude of mice that received AAV2/9-\textit{HA-Bclw} (n = 7, CBA/J) was not significantly different from that of control mice (n = 5, CBA/J) that received no acoustic trauma at all, suggesting that Bclw overexpression could preserve the function of surviving synapses following noise-induced damage (Figure 3.3G). Control mice that received only a sham surgery had greater synaptic loss and reduced Wave 1 amplitudes. No permanent ABR threshold shifts were observed, suggesting that our noise trauma protocol did not lead to permanent NIHL (Figure 3.3H). We next tested to see if early overexpression of an anti-apoptotic protein would cause changes in IHC/SGN synaptic density. Changes in baseline postnatal synaptic density would impact our assessment of Bclw’s protective effects against primary synaptopathy by offering the possibility of developmental compensation. To assess this possibility, we injected another cohort of mice with AAV2/9-\textit{HA-Bclw} at P0/P1 and collected their cochleae at 3-months for synapse staining. We observed that across the tonotopic axis, there were no changes in synapse counts between mice that received AAV2/9-\textit{HA-Bclw} (n = 7, CBA/J) or sham surgeries (n = 5, CBA/J) (Figure 3.3I and 3.3J). Overexpression of Bclw had no impact on postnatal synaptic density. Taken together, this set of experiments suggested that early Bclw overexpression could act as a promising prophylactic treatment against NIHL.
**Adult overexpression of Bclw**

Bclw’s ability to protect against noise-induced synaptopathy after early overexpression suggests that it may be a useful therapeutic candidate. However, the most at-risk population for hidden hearing loss are those patients who have already been exposed to decades of loud sounds (Fernandez et al., 2015; Sergeyenko et al., 2013). As a result, we wanted to test whether Bclw overexpression in adult CBA/J mice could also lend protection against NIHL. We injected AAV2/9-HA-Bclw (n = 7, CBA/J) into the posterior semicircular canal of 2-month old CBA/J mice; the perilymph in the semicircular canal is contiguous with the cochlea. This allowed us to bypass the fully calcified cochlea and round window and overexpress HA-Bclw in adults with minimal surgical trauma. 1-month later, we exposed the mice to acoustic trauma, as described above, and analyzed ABRs, DPOAEs, and synaptic counts (Figure 3.4A). Following adult injections, we also observed high levels of SGN transfection efficiency (Figure 3.4B and 3.4C). Further analysis of synaptic counts also revealed that adult Bclw overexpression could protect the inner ear from noise-induced primary synaptopathy (Figure 3.4D). However, the protective effect was more modest. Virally treated mice (n = 7, CBA/J mice) experienced 49% ± 6.37% loss in pre-synaptic counts and 36% ± 5.92% loss in post-synaptic counts. In contrast, mice that received only sham surgery (n = 5, CBA/J) experienced 64% ± 8.90% loss in pre-synaptic counts and 62% ± 9.23% loss in post-synaptic counts (Figure 3.4E and 3.4F). The Wave 1 amplitude for mice that received AAV2/9-HA-Bclw at 2-months also showed improvement when compared to sham controls (Figure 3.4G). However, like the synaptic counts, the effect of the protective effect was attenuated compared to the Wave 1 amplitudes in early Bclw overexposed mice. Taken together, this suggests that while Bclw can have an effect in mature
Figure 3.4: Adult overexpression of Bclw protects against noise-induced trauma. (A)

Schematic timeline of the experimental protocol. 2-month old CBA/J adults received a single injection of AAV2/9-HA-Bclw into their posterior canal and were aged to 3-months before being exposed to acoustic trauma (8-16 kHz band noise @ 100 dBs for 2 hrs). (B-B”) Representative 3-month old cochlear wholemounts stained for CALB2 (green) and HA-tag (red). AAV2/9-HA-Bclw induced Bclw overexpression across SGN cell bodies. (C-C”) Representative 3-month old cochlear wholemounts stained for CALB2 (green) and HA-tag (red); images were taken directly under the basolateral surface of the IHC. HA-tagged BCLW overexpression was also seen along the distal tips of transfected cochleae. Dotted yellow outline corresponds to an individual IHC. (D-D”) Confocal maximum projections of IHCs and synapses from the 32 kHz regions of a cochlear wholemount. Pre- and post-synaptic puncta were stained with anti-Ctbp2 (green) and anti-GluR2 (magenta), respectively. Anti-Myosin VIIa (blue) stained the IHCs. In control subjects, a full complement of synaptic densities could be observed on a per IHC basis (D). 2-wks post-trauma, there is permanent primary synaptopathy (D’); however, overexpression of Bclw in adults protects the IHC/SGN synapse from acoustic trauma (D”). (E-F) Quantification of synaptic counts per IHC following acoustic trauma and viral treatment. Animals that received AAV2/9-HA-Bclw (n=7) had more pre- and post-synaptic densities than sham-treated animals (n=7) post-trauma. However, the effect was more modest than early neonatal injections. (G) ABR P1 amplitudes also saw modest protection in Bclw overexpressing mice. P1 amplitudes were collected following exposure to an 80 dB SPL stimulus. The statistical significance of ‘Trauma’ vs. ‘Trauma + HA-Bclw’ is shown by asterisks (E-G). *p < 0.05 by Tukey’s multiple comparisons (2-way ANOVA). Error bars indicate s.d. See also Supplemental Figure 9.
Figure 3.4 (Continued)
ears, it either needs more time to elicit its protective effect or simply needs to be present early in development to protect the inner ear from NIHL.

**Overexpression of Bclw post-trauma**

In order to fully characterize Bclw’s protective abilities in noise-induced primary synaptopathy, we also assessed Bclw overexpression post-trauma. We exposed 3-month old CBA/J mice to acoustic trauma as described above. 24-hours later, we injected AAV2/9-*HA-Bclw* (n = 5, CBA/J) into the posterior semicircular canal and waited 4-weeks post-trauma before assessing inner ear health with ABRs, DPOAEs, and synaptic counts (Figure 3.5A). While we observed transfection in the ganglia and in the peripheral SGN processes (Figure 3.5B and 3.5C), we saw no improvement in synapse counts or Wave 1 amplitudes in mice that received AAV2/9-*HA-Bclw* post-trauma (Figure 3.5D-G). This suggests that once the damage is done, Bclw is unable to help recover function.
Figure 3.5: Post-trauma overexpression of Bclw does not prevent decrease in ABR P1 amplitudes. (A) Schematic timeline of the experimental protocol. 3-month old CBA/J adults were exposed to acoustic trauma (8-16 kHz band noise @ 100 dBs for 2 hrs). 24-hours later, exposed mice received a single injection of AAV2/9-HA-Bclw into their posterior canal and were allowed to recover for 1-month before undergoing hearing acuity tests and synapse quantification. (B-B”) Representative 4-month old cochlear wholemounts stained for CALB2 (green) and HA-tag (red). AAV2/9-HA-Bclw induced Bclw overexpression across SGN cell bodies. (C-C”) Representative 4-month old cochlear wholemounts stained for CALB2 (green) and HA-tag (red); images were taken directly under the basolateral surface of the IHC. HA-tagged BCLW overexpression was also seen along the distal tips of transfected cochleae. Dotted yellow outline corresponds to an individual IHC. (D-D”) Confocal maximum projections of IHCs and synapses from the 32 kHz regions of a cochlear wholemount. Pre- and post-synaptic puncta were stained with anti-Ctbp2 (green) and anti-GluR2 (magenta), respectively. Anti-Myosin VIIa (blue) stained the IHCs. In control subjects, a full complement of synaptic densities could be observed on a per IHC basis (D). 4-wks post-trauma, there is permanent primary synaptopathy (D’). Overexpression of Belw in adults post-trauma did not protect the IHC/SGN synapse from acoustic trauma (D”). (E-F) There was no observed synapse protection in mice that received AAV2/9-HA-Bclw 24 hrs post-trauma. (G) P1 amplitudes collected following exposure to an 80 dB SPL stimulus. Animals that received AAV2/9-HA-Bclw post-trauma (n=5) still had significantly lower ABR P1 amplitudes than no-trauma controls (n=5). This suggests that Bclw’s protective function is limited to prophylactic therapies. The statistical significance of ‘Trauma’ vs. ‘Trauma + HA-Bclw’ is shown by asterisks (E-G). *p < 0.05 by Tukey’s multiple comparisons (2-way ANOVA). Error bars indicate s.d. See also Supplemental Figure 10.
Figure 3.5 (Continued)
Discussion

Primary synaptopathy underlies both AHL and NIHL (Kujawa and Liberman, 2009; Liberman and Liberman, 2015; Sergeyenko et al., 2013). Loss of synaptic coupling is also an early indicator of which SGN will undergo peripheral axonopathy following noise trauma. Left untreated, this form of sensorineural hearing loss can result in hidden hearing loss, a progressive disease that results in poor speech discrimination, difficulty hearing in environments with complex ambient noise, and depression (Kujawa and Liberman, 2009; Liberman, 2015; Liberman et al., 2016). As a result, new therapies that seek to protect the synapses will need to be developed.

Overexpression of NT3 has proven to be effective in protecting the inner ear from acoustic trauma. Previous studies show that NT3 overexpression promotes recovery from noise-induced synaptic degeneration and the associated decrements in auditory evoked potentials (Budenz et al., 2012; Stankovic et al., 2004; Wan et al., 2014). However, the long-term efficacy of such treatments remains unknown. In rodents, excess neurotrophin exposure can lead to branching and sprouting of Type I SGNs, potentially affecting the processing of information that is transmitted from the IHCs to higher cortical processing regions.

Prior work has highlighted Bclw as a unique member of the Bcl2 super-family. Bclw mRNA is trafficked directly to the peripheral axon of DRGs; while there, evidence suggests that Bclw mediates a local anti-apoptotic function by inhibiting caspase6-mediated axonal apoptosis (Cosker et al., 2013; Courchesne et al., 2011; Pazyra-Murphy et al., 2009). Bclw has also been shown to regulate neurotrophin-dependent axon survival. All of these factors combine make Bclw a compelling therapeutic candidate to tackle our pathology of interest.
In this study, we found that Bclw overexpression protects against noise-induced primary synaptopathy. In both neonatal and adult BCLW overexpression, there were significant increases in the number of surviving IHC/SGN synapses following acoustic trauma. However, we note that the protective effect following adult injections was more modest than earlier Bclw overexpression. The cause of this discrepancy remains unclear. It is possible that following the 2-month adult injections, 1-month of exposure to AAV2/9-HA-Bclw was not enough time to produce a sufficient quantity of Bclw in the peripheral processes of transfected SGNs. Alternatively, early Bclw overexpression may change the way pre- and post-synaptic densities mature during neonatal development. While our data suggests that same number of synapses survive post-pruning, early Bclw over-expression may stabilize the synapses in such a way as to make them more resistant to acoustic damage.

The DRGs in Bclw -/- mice experience peripheral axonopathy with age (Courchesne et al., 2011). We also observe that loss of Bclw results in loss of synapses in high frequency regions of the inner ear. We hypothesize that sufficient levels of Bclw expression is required for the long-term maintenance of inner ear synapses. To assess this hypothesis, we have injected AAV2/9-HA-Bclw into CBA/J mice at P0, 16-weeks, 32-weeks, and 48-weeks of age. Previous studies have shown that by 64-weeks of age, mice lose ~20% of their SGN cell bodies. The loss of IHC/SGN synapses at 64-weeks is even greater, as synaptopathy precedes SGN degeneration by months. As a result, we can assess whether Bclw overexpression at various time points slow age-induced synapse loss. We are also planning on supplementing this experiment with smFISH to assess Bclw mRNA levels during aging. Loss of Bclw expression may precede eventual SGN synaptopathy.
This work offers a new strategy for tackling hidden hearing loss. Our findings indicate that specific anti-apoptotic molecules can blunt the full impact of acoustic trauma. Further work will need to be done to fully characterize the therapeutic window of Bclw overexpression and to assess if Bclw mRNA expression can serve as an early signal for SGN synaptopathy and axonopathy.
— CHAPTER FOUR —

Concluding remarks and future directions
The goals of my thesis work are to better understand the heterogeneity of primary auditory neurons within the inner ear and to develop a new approach to prevent cochlear synaptopathy in differentially vulnerable populations of Type I SGN subtypes. In this body of work, we identify novel molecular markers for subtypes of inner ear neurons and characterize their diversification over postnatal development. Our data reveals molecular markers that correspond to functionally distinct populations of Type I SGNs. Lypd1 and Calb2 are some of the first genes identified to distinguish low-SR and high-SR SGNs. We show that Lypd1+ SGNs are more susceptible to age-related SGN degeneration. We also show that neonatal synaptic transmission between IHCs and SGNs is critical for the development of SGN identity. Synaptic silencing results in a shift in SGN heterogeneity; in the absence of synaptic transmission, fewer Lypd1+ SGNs can be seen in the adult inner ear. Further work will have to be done to understand the mechanisms underlying differential SGN vulnerability and activity-dependent identity formation.

By characterizing Bclw’s protective role in the inner ear, our work opens up the possibility of using endogenous survival mechanisms to protect against noise- and age-induced cochlear synaptopathy. Bclw mRNA is enriched in the inner ear; loss of Bclw results in high-frequency cochlear synaptopathy in 9-month old mice cochlea. We show that neonatal and adult overexpression of Bclw via AAV2/9 is sufficient to protect SGNs from noise-induced synaptopathy. We are now also actively pursuing other lines of research to assess Bclw’s protection function and expression pattern during AHL. Future studies will seek to leverage Bclw’s unique protective effects for the development of new drug targets against NIHL and AHL. Dissecting Bclw’s mechanism within SGNs will also be necessary to better understand the pathology of SGNs following noise trauma. Taken together, the work presented in this
dissertation provides new insight on the complexity of SGN identities and the pathology of inner ear neuropathy.

**Molecular mechanisms underlying differential SGN vulnerability**

Using single cell transcriptomics, we have been able to molecularly define three subtypes of Type I SGNs and match them to previously characterized functional subtypes. While the subtypes we have characterized express unique ion channels, proteins, and receptors, it is still unclear what drives the differential vulnerability observed in Type I SGNs. We now know that *Lypd1*+ SGNs share anatomical features with previously described low-SR SGNs. Our work also showed that *Calb2*+ SGNs appear to be anatomically similar to the high-SR SGNs. Prior studies have shown that low-SR SGNs are more vulnerable to acoustic damage (Furman et al., 2013). As Calb2 is a calcium binding protein, it is possible that differences in calcium buffering drives differences in vulnerability to sound. Electron microscopy studies on low-SR and high-SR SGNs have revealed differences in mitochondria density in each subtype (Liberman, 1980a). Low-SR fibers tend to have low mitochondria content and larger pre-synaptic ribbons. In contrast, high-SR fibers are characterized with high mitochondria content and smaller pre-synaptic ribbons (Liberman, 1980a). Mitochondria supply ATP for calcium ion pumps that are critical for the proper regulation of intracellular Ca$^{2+}$ (Szydlowska and Tymianski, 2010). Excessive influx of calcium is currently thought to be a primary initiator of glutamate excitotoxicity and downstream caspase activation (Puel, 1999; Puel et al., 1995; Pujol and Puel, 1999). It is possible that the greater density of mitochondria, combined with increased levels of Calb2, offers high-SR SGNs a greater buffer against calcium-induced apoptosis (Figure 4.1).
In addition to its intrinsic calcium buffering capabilities, Calb2 has also been shown to activate other biological processes. *In-vitro* overexpression of Calb2 in neuronal and non-neuronal cell lines resulted in increased activation of the Akt/PKB survival pathway (Dong et al., 2012). The Akt/PKB pathway promotes downstream cell survival by blocking pro-apoptotic members of the Bcl2 superfamily, such as BAD (Datta et al., 1997; Del Peso et al., 1997) and BIM (Dijkers et al., 2002). Activation of the Akt/PKB pathway also blocks the cascade of caspase activation following cell stress (Cardone et al., 1998). Lower levels of Akt/PKB activity within low-SR SGNs may make them more vulnerable to age-related and noise-induced acoustic stress.

Finally, differential glutamate clearance efficiency along the IHC synaptic cleft runs parallel to the Calb2 expression gradient. Rapid removal of glutamate away from terminal synapses is critical to protect neurons from excitotoxic damage (Puel et al., 1994; Pujol and Puel, 1999). Immunostaining for GLAST, a glutamate-aspartate transporter expressed within inner ear supporting cells, shows lower concentration along the modiolar side of the IHC (Furness and Lawton, 2003). Loss of GLAST also results in exacerbated excitotoxicity within SGN afferent terminals following noise exposure (Hakuba et al., 2000). This suggests that glutamate clearance is less efficient along the low-SR side of the IHC and may lead to prolonged Ca$^{2+}$ influx into the SGN peripheral process.
Figure 4.1: Model for Bclw and Calb2-mediated protection in SGNs. Calb2 buffers excess Ca\(^{2+}\) influx induced by acoustic trauma, thereby reducing intracellular Ca\(^{2+}\) levels and downstream activation of IP\(_3\)R1 and Bak. Bclw interacts with both IP\(_3\)R1 and Bak to prevent release of Ca\(^{2+}\) from ER stores and the formation of Bak channels along the mitochondrial outer membrane. Overall, this leads to reduced activation of caspase-pathways and a protective effect against primary noise-induced synaptopathy and axonopathy.
In our prior work (Shrestha et al., 2018), we show that MafbCreERT2; Ai9 sparsely labels Type I SGNs in adult mice cochlea. Further analysis revealed that labelled SGNs preferentially projected to the modiolar face of the IHC and formed synapses with large pre-synaptic ribbons (Figure 2.4). This suggested that MafbCreERT2; Ai9 sparsely labels the low-SR population of Type I SGNs in the inner ear. We can leverage this tool to further explore the relationship between Calb2 expression and SGN survival. In cultures of rodent cortical neurons, differential vulnerability along a Calb2 expression gradient has been previously characterized (Lukas and Jones, 1994). Cortical neurons with high levels of Calb2 expression appear to be significantly more resistant to glutamate-mediated excitotoxicity. In contrast, low Calb2 expression cortical neurons undergo cell death within hours of excitotoxic damage (Lukas and Jones, 1994). One idea is to inject a virus packaged with CAG-LSL-Calb2-eGFP into neonatal and adult MafbCreERT2 cochleae. This will allow for the selective overexpression of Calb2 in a subset of low-SR SGNs. We can then take transfected MafbCreERT2 mice and induce temporary NIHL. It would be interesting to see if eGFP positive fibers are more resistant to noise-induced synaptopathy. The sparse labelling of MafbCreERT2 offers a system where we have clear internal controls; we can easily compare the impact that Calb2 overexpression has on individual low-SR Type I SGNs. In a complementary experiment, we can leverage existing Calb2 +/- mice or deplete Calb2 expression in high-SR SGNs and assess whether there is increased synaptopathy along the pillar axis following NIHL. Calb2 is not the only calcium-binding protein in the inner ear (Liu and Davis, 2014). If Calb2 does mediate differential vulnerability in Type I SGNs, it would be interesting to evaluate whether other calcium-binding proteins have similar effects or if Calb2 mediates a unique protective pathway. This question can be tackled by overexpressing other calcium-binding proteins, such as Calb1, in Calb2 +/- mice and seeing if
that can protect against primary synaptopathy. Future experiments designed to overexpress Calb2 or other related calcium-binding proteins in low-SR SGNs can help provide insight on the molecular basis underlying differences in SGN vulnerability.

**Bclw and Ca\(^{2+}\)-dependent degeneration**

Intense acoustic trauma can lead to excessive release of glutamate from IHCs into the synaptic cleft (Kujawa and Liberman, 2009; Puel et al., 1994; Pujol et al., 1993). This wave of glutamate excitotoxicity results in rapid SGN dendritic swelling, over-activation of glutamate receptors, and leakage of extracellular Ca\(^{2+}\) into the SGN peripheral process. The influx of extracellular Ca\(^{2+}\) following noise trauma can damage organelles, such as mitochondria and endoplasmic reticulum, resulting in the release of more Ca\(^{2+}\) from intracellular stores into the cytoplasm (Pease-Raissi et al., 2017). Prior work has shown that the rapid rise of toxic levels of cytoplasmic Ca\(^{2+}\) can trigger downstream degenerative cascades, such as the activation of caspases, calpains, and other Ca\(^{2+}\)-dependent proteases (Pease-Raissi et al., 2017; Szydlowska and Tymianski, 2010; Zündorf and Reiser, 2011).

Bclw appears to be well placed to mitigate the downstream effects of excitotoxicity and Ca\(^{2+}\) overload following acoustic trauma (Cosker et al., 2013; Courchesne et al., 2011). Extensive work has shown that Bclw is capable of protecting DRGs from traumatic axon injury and downstream calcium dysregulation within the DRG mitochondria and endoplasmic reticulum (Pease-Raissi et al., 2017; Pease and Segal, 2014). Through interactions with axonal IP\(_3\)R1, Bclw is able to inhibit paclitaxel-induced calcium release from intracellular stores and the initiation of the calpain degeneration pathway (Pease-Raissi et al., 2017). Bclw also inhibits pro-apoptotic proteins, Bax and Bak, along the mitochondrial membrane, preventing the formation of non-
specific pores and the release of cytochrome c and mitochondrial Ca$^{2+}$ (Holmgreen et al., 1999; Ross et al., 1998, 2001). Loss of Bclw results in reduced mitochondrial membrane potential, increased calcium dysregulation, and increased caspase6 activation (Cosker et al., 2013; Courchesne et al., 2011). Ultimately, Bclw may have its protective effects by limiting the ability of extracellular Ca$^{2+}$ influx to compound its effects through downstream release of Ca$^{2+}$ from intracellular stores.

**Bclw’s mechanism in SGNs**

Recent work has shown that Bclw in DRGs interacts with axonal IP$_3$R1 (Pease-Raissi et al., 2017), endoplasmic reticulum (ER) calcium channels, to prevent paclitaxel-induced axonopathy. This interaction is unique to Bclw; other anti-apoptotic members of the Bcl2 family do not bind to axonal IP$_3$R1. Further studies have also shown that Bclw’s BH4 domain is sufficient to protect against paclitaxel-induced axonopathy, while loss of Bclw exacerbates the neuropathy (Pease-Raissi et al., 2017). Loss of axonal IP$_3$R1 fully blocks paclitaxel-induced axonopathy; this protective effect is independent of Bclw expression. As a result, Bclw appears to function as a unique upstream regulator of axonal IP$_3$R1 activity (Pease-Raissi et al., 2017). Disruption of axonal IP$_3$R1 activity via mechanical or toxic injury can lead to increased release of calcium ions from intracellular ER stores (Twiss et al., 2014). This results in increased cytoplasmic calcium concentration. Ongoing work suggests that excessive calcium release from the ER can lead to the opening of mitochondrial permeability transition pores (MPTPs), a non-specific channel that lines the inner mitochondrial membrane (Court et al., 2011; Twiss et al., 2014). Opening of these pores leads to noxious oxidative stress, reduction in ATP production, and eventual axon degeneration (Bernardi et al., 2006; Court and Coleman, 2012). Inhibiting ER
calcium release channels or MPTPs protects DRGs from induced oxidative stress (Twiss et al., 2014). Taken together, it appears neural survival depends on tightly controlled internal calcium concentration. We have already described differences in SGN calcium buffering (Calb2 expression), mitochondrial density, and vulnerability to excitotoxicity; all of these factors may influence the Bclw- IP3R1-survival cascade. Ultimately, Bclw and axonal IP3R1 may play a role in the regulation of SGN survival.

Our work with Bclw provides a possible mechanism following noise-induced trauma. We show that Bclw overexpression in both neonatal and adult mice protects the IHC/SGN synaptic complex from noise-induced excitotoxicity. Maintenance of these synapses is required for the long-term survival of the SGN peripheral process and cell body (Kujawa and Liberman, 2009; Sergeyenko et al., 2013). Overexpressing Bclw in SGNs may increase the ‘brake’ on IP3R1’s activity and pro-degenerative effects. This reduction in receptor activity could protect SGN peripheral processes by restricting the downstream release of intracellular calcium stores, inhibiting formation of MPTPs, and preventing calcium-mediated synaptopathy (Figure 4.1). We can test this model by first assessing the vulnerability of IP3R1-/- mice to temporary NIHL. We would predict that loss of IP3R1 will lend a protective effect against acoustic trauma. Bclw binding affinity to IP3R1 can be tested using cochlear lysate pulldowns; this will allow us to assess whether observed molecular interactions in DRGs are also conserved in the SGNs. Our model proposes upstream Bclw inhibition of IP3R1 in the peripheral processes of Type 1 SGNs. In order to assess this molecular pathway, we can overexpress Bclw in mice with IP3R1 knockdown and expose these mice to temporary NIHL. If Bclw mediates its unique protective effects in SGNs through a separate molecular pathway, we may observe improved synapse protection as multiple survival pathways are being recruited. In a separate experiment, we could
knockdown IP₃R1 in Bclw -/- mice and quantify synapses along the tonotopic axis following AHL. If we observe normal synapse density in the high frequency region of aged Bclw -/- cochleae, that could also suggest a functional relationship between these two proteins. Future work will assess Bclw and IP₃R1’s role in NIHL, AHL, and drug-induced synaptopathy.

**Conclusion**

In conclusion, the work in this dissertation provides a new level of insight into how SGN heterogeneity is formed, the pathology underlying NIHL, and offers a new potential candidate for therapies against sensorineural hearing loss.
— APPENDIX —

Supplemental figures
Supplemental Figure 1. Quality Control Metrics and Gene Ontology Results. (A) Scatterplots showing technical and biological criteria used to filter cells before analysis. Red dotted lines indicate cutoff value and red arrow indicates direction of exclusion. (B) Setting the resolution parameter to values between 0.40 and 1.30 for graph-based clustering in Seurat yielded 2 to 5 clusters, with 42 out of 46 values (91%) predicting 3 clusters. (C) Agreement with regard to cell identity among all variations of the resolution parameter that yielded 3 clusters. For each cell, a consensus score was calculated as the percentage of times the same identity was assigned to it across clustering runs that varied only in terms of resolution. Consensus was 100% for 174 out of 179 cells (97%). (D) Projection of SGN transcriptomes onto PCA space (PC2 versus PC4) reveals groups that correspond closely with those identified by graph-based clustering. Each group in the PCA plot contains SGNs from all tonotopic regions. (E) Results of hierarchical clustering by Ward’s method depicted as a dendrogram. The three major branches correspond well with SGN subtype identities predicted by graph-based clustering but not with their tonotopic position. (F) Segregation of SGNs is not driven by technical variables or experimental factors such as sequencing batch. P values refer to Chi-squared test of independence. (G) There were no significant subtype-specific differences in the detection of spike-in transcripts, mitochondrial transcripts, or number of genes detected. Box plots depict median (horizontal line), 25%-75% quartiles (shaded rectangle), smallest observation ≥ 25% quartile-1.5*interquartile range (lower error bar), largest observation ≤ 25% quartile + 1.5* interquartile range (upper error bar). P values are indicated next to pairs of dots that represent subtypes compared by Tukey’s HSD test if the data was normally distributed data and Dunn’s test otherwise. (H,I) Absence of glial gene expression (H) and uniform expression of known pan-neuronal genes (I) across the three Type I SGN clusters. Numbers in upper right corner indicate
Supplemental Figure 1 (Continued)

highest expression (Max) observed for each gene. (J, K) Graphical representation of results of Gene Ontology analysis of genes expressed differentially between Type I and II SGNs (J) or among Type I SGNs (K). A few examples of statistically significantly enriched categories are named.
Supplemental Figure 1 (Continued)
Supplemental Figure 2. SGN Subtypes Express Unique Cohorts of Genes that Influence Neuronal Physiology. Dot-matrix plots showing expression of gene families relevant to neuronal physiology. Each dot is a neuron profiled by scRNA-seq and neurons are grouped based on subtype identity assigned by unsupervised clustering. Numbers on the right indicate the highest expression (Max) observed for each gene.
Supplemental Figure 3. SGN Subtypes Express Unique Cohorts of Genes that Influence Neurotransmission and Neurite Guidance. Dot-matrix plots showing expression of gene families relevant to neurotransmission and neurite guidance. Each dot is a neuron profiled by scRNA-seq and neurons are grouped based on subtype identity assigned by unsupervised clustering. Numbers on the right indicate the highest expression (Max) observed for each gene.
### Supplemental Figure 3 (Continued)

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**Dopaminergic receptors**

| Drd1                  | 3.8                 |
| Drd2                  | 0                   |
| Drd3                  | 0                   |
| Drd4                  | 1.0                 |
| Drd5                  | 3.2                 |

0.1 Max 0
Supplemental Figure 4. Tonotopic Differences in Gene Expression across all SGNs and within SGN Subtypes. (A) Heat map showing the top 100 most differentially expressed genes across the tonotopic axis. Each column represents a single cell and genes are in rows. Cells were grouped based on tonotopic origin. Panels on the right show examples of genes enriched in the apex (top), middle (middle) and base (bottom). (B) RNAscope of tissue sections (P25-P27) confirms even expression of the pan-neuronal gene *Tubb3* in the apex, middle, and base of the cochlea, quantified below. (C) Plots showing expression (mean ± SEM) of genes that were invariant across the tonotopic axis for all pooled SGNs (dashed lines) and for each subtype (Ia, green; Ib, purple; Ic, blue). (D) Examples of genes that are robust markers for Ia, Ib, and Ic subtypes across all tonotopic regions. Mean expression levels (± SEM) are shown for Ia (A), Ib (B), and Ic (C) SGNs taken from the apex (gray), middle (red), or base (blue) of the cochlea. (E) Examples of genes whose cross-subtype variation in expression (± SEM) differs among SGNs from different cochlear regions (apex, gray; middle, red; base, blue). For example, there is significantly lower expression of *Anxa5* in Ia SGNs from the base relative to those from the middle and the apex, but little variation among Ib or Ic SGNs. Pairs of dots indicate *p* values for comparisons across tonotopic regions by Tukey’s HSD test if the data was normally distributed data and Dunn’s test otherwise. *P* values are reported only for statistically significant differences. Scale bars: 10 µm (B).
**Supplemental Figure 4 (Continued)**

### A

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**Example Table:**

- **Tmbb3**: Apex, Middle, Base
- **Alp1b1**: Apex, Middle, Base
- **Nmc2**: Apex, Middle, Base
- **Wnt3**: Apex, Middle, Base

### B

- **Tmbb3**
- **Alp1b1**
- **Nmc2**
- **Wnt3**

### C

- **Keys for C,D,E**
  - **A**: Apex
  - **B**: Middle
  - **C**: Base
  - **- - -**: Apex vs. Base
  - **- - -**: Middle vs. Base
  - **- - -**: Base vs. Base

### D

- **Caib2**
- **Cacna1b**
- **Nmc2**
- **Lypd1**

### E

- **Lrc52**
- **Kcnip4**
- **Anxa5**
- **Ryr3**
Supplemental Figure 5. Operational Definition of SGN Subtypes According to CALB2

Intensity. (A-B) Wholemount cochlea from \textit{Mafb}^{CreERT2}; \textit{Ai9} animals were double-stained for CALB2 (green) and tdTomato (red). Individual tdTomato labeled SGNs could be traced from their cell bodies to the organ of Corti, shown in a top down (XY) view (top). Position in the OSL was assessed in cross-sectional (XZ) views through confocal image stacks (below). CALB2 intensities in cell bodies and processes were correlated for each neuron. Examples of one CALB2$^+$ SGN (yellow arrow) and one CALB2$^{low}$ SGN (white arrow) are shown, with quantification for all analyzed SGNs in B. (C-D) SGN subtypes were operationally defined based on CALB2 levels. K-means clustering of all measured CALB2 fluorescence intensities in fibers revealed that the expression values are best split into three clusters (red dotted line, C). Fibers were divided into low (n=113), medium (n=78), and high (n=116) CALB2$^+$ groups as defined by the K-means cluster boundaries, indicated by colored shading in a histogram of all analyzed fibers (n=214, 5 animals). (E) Cross-sectional views (XZ) through the OSL of \textit{Chat}^{Cre};\textit{Ai14} cochlear wholemount preparations immunostained for CALB2 (green) and tdTomato (red). \textit{Chat}^{Cre};\textit{Ai14} labels olivocochlear efferents. Dotted lines indicate the upper limit (towards SV), mid-point and lower limit (towards ST) of the afferent fiber bundle. (F-G) Histograms show even distribution of efferent fibers (red) but SV-biased distribution of low CALB2 (blue) fibers in the OSL (F). The average number of fibers per region is quantified in G. Error bars in G represent standard deviation. \textit{P} values indicate results of Kolmogorov–Smirnov’s test (F) and results of Tukey’s HSD test following one-way ANOVA (G). Scale bars: 10 µm.
Supplemental Figure 5 (Continued)

A

B

C

D

E

F

G

KS: tdTomato vs. Low CALB2: $p = 0.001$
Supplemental Figure 6. Analysis of Lypd1 and Calb2 mRNA Levels in the Aging Mouse Cochlea. (A-B) In sections of the cochlea at 108 wks, autofluorescent aggregates (arrows) appear in all fluorescent channels (anti-parvalbumin (PARV), A; Calb2 RNAscope, A’; Lypd1 RNAscope, A”). In order to count mRNA puncta reliably, this signal was removed prior to cell segmentation and quantification by applying a Gaussian filter across the fluorescent channels (B-B”). (C) Scatterplots of Lypd1 and Calb2 levels in all SGNs analyzed at 32, 64, and 108 wks (n=5 animals, 212 cells at 32 weeks, n=5 animals, 175 cells at 64 weeks, n=5 animals, 151 cells at 108 weeks, respectively). (D-E) K-means clustering analysis showed that SGN populations could be sorted into three groups based on Calb2 levels at 32 wks (top) and 64 wks (middle), but only into 2 groups at 108 wks (bottom), consistent with a loss of Type Ic SGNs. Likewise, whereas two clusters of Lypd1+ SGNs can be defined at 32 and 64 wks, only one cluster remains at 108 wks (E). Scale bar: 10 µm (A,B).
Supplemental Figure 6 (Continued)
**Supplemental Figure 7. Marker Expression in Embryonic and Vglut3 -/- Animals, and Comparison of Supervised and Unsupervised Clustering Approaches.** (A-C) Detection of *Lypd1* (magenta) and *Calb2* (green) transcripts by RNAscope at E18.5 reveals early co-expression in individual SGNs, with *Calb2* expressed broadly but *Lypd1* expressed in a subset, quantified in B. Whereas the vast majority of SGNs express *Calb2*, a smaller proportion express *Lypd1* (C). Box plots in C depict median (horizontal line), 25%-75% quartiles (shaded rectangle), smallest observation ≥ 25% quartile-1.5* interquartile range (lower error bar), largest observation ≤ 25% quartile + 1.5* interquartile range (upper error bar). (D) The level of expression of select subtype-specific markers is overlaid in a tSNE plot containing both WT (M1-M3 clusters) and *Vglut3*-/- cells (M4-M5 clusters). Cluster identities in the key to the right are based on Figure 7A. Numbers on the upper left indicate the highest expression (Max) observed for each gene. (E) tSNE plots with dot colors indicating subtype identity assigned by unsupervised graph-based clustering (left) and by Random Forest (right), a supervised clustering approach. (F) Scatterplot showing subtype identity of every cell predicted by unsupervised (X-axis, “Graph-based”) and supervised clustering (Y-axis, “Random Forest”) methods. Only cells that had not been used for training the Random Forest classifier are shown. Cells that fall on the diagonal dotted line are the ones predicted correctly by the supervised method. (G) Subtype proportions among SGNs from three tonotopic regions predicted by each clustering method. Scale bar: 10 µm (A).
Supplemental Figure 7 (Continued)

A

E18.5

Lypd1

Calb2

Merged

B

C

D

Calb2

Pou4f1

Lypd1

Ntng1

Grm8

9.8

5.6

6.4

4.5

4.4

0.1

Max

0

E

Graph-based clustering

Random Forest predictions

F

G

predicted by Random Forest classifier

Apex

Middle untrained

Base

n=51

n=86

n=41

Graph-based

Random Forest

A

B

C

Apex

Middle

Base
Supplemental Figure 8. I/O Graphs for ABR P1 Amplitudes Following P0 Viral Injection. (A) Mean amplitude vs. SPL for ABR P1 at 8 kHz. (B) Mean amplitude vs. SPL for ABR P1 at 16 kHz. (C) Mean amplitude vs. SPL for ABR P1 at 32 kHz. (D) Mean amplitude vs. SPL for ABR P1 at 64 kHz. The statistical significance of ‘Trauma’ vs. ‘Trauma + HA-Bclw’ is shown by asterisks. *p < 0.05 by Tukey’s multiple comparisons (2-way ANOVA). Error bars indicate s.d.
Supplemental Figure 9. I/O Graphs for ABR P1 Amplitudes Following Adult Viral Injection. (A) Mean amplitude vs. SPL for ABR P1 at 8 kHz. (B) Mean amplitude vs. SPL for ABR P1 at 16 kHz. (C) Mean amplitude vs. SPL for ABR P1 at 32 kHz. (D) Mean amplitude vs. SPL for ABR P1 at 64 kHz. The statistical significance of ‘Trauma’ vs. ‘Trauma + HA-Bclw’ is shown by asterisks. *p < 0.05 by Tukey’s multiple comparisons (2-way ANOVA). Error bars indicate s.d.
Supplemental Figure 9 (Continued)

A 8 kHz

B 16 kHz

C 32 kHz

D 64 kHz

\[ \text{ABR P1 Amplitude (uV)} \]

Sound Pressure Level (dB SPL)

\[ \text{No Trauma} \]

\[ \text{Trauma} \]

\[ \text{Trauma w/ HA-Bclw} \]
Supplemental Figure 10. I/O Graphs for ABR P1 Amplitudes Following Post-Trauma Viral Injection. (A) Mean amplitude vs. SPL for ABR P1 at 8 kHz. (B) Mean amplitude vs. SPL for ABR P1 at 16 kHz. (C) Mean amplitude vs. SPL for ABR P1 at 32 kHz. (D) Mean amplitude vs. SPL for ABR P1 at 64 kHz. The statistical significance of ‘Trauma’ vs. ‘Trauma + HA-Bclw’ is shown by asterisks. *p < 0.05 by Tukey’s multiple comparisons (2-way ANOVA). Error bars indicate s.d.
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