Review Article

Water Waves to Sound Waves: Using Zebrafish to Explore Hair Cell Biology

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ABSTRACT

Although perhaps best known for their use in developmental studies, over the last couple of decades, zebrafish have become increasingly popular model organisms for investigating auditory system function and disease. Like mammals, zebrafish possess inner ear mechanosensory hair cells required for hearing, as well as superficial hair cells of the lateral line sensory system, which mediate detection of directional water flow. Complementing mammalian studies, zebrafish have been used to gain significant insights into many facets of hair cell biology, including mechanotransduction and synaptic physiology as well as mechanisms of both hereditary and acquired hair cell dysfunction. Here, we provide an overview of this literature, highlighting some of the particular advantages of using zebrafish to investigate hearing and hearing loss.

Keywords: lateral line system, auditory system, mechanosensation, hearing loss

INTRODUCTION

Hearing loss is extremely common in the United States, affecting nearly 1 in 4 individuals over the age of 12

(Goman and Lin 2016). Not only is it pervasive, hearing loss often affects high-frequency hearing first, a pattern that is particularly problematic for communication. Since consonant speech sounds (e.g., “s”) have higher frequencies, with just mild or moderate hearing loss, our ability to perceive speech without amplification is dramatically reduced (Yoshinaga-Itano et al. 2017). The organ devoted to hearing is housed within a bony inner ear structure known as the cochlea. Despite being protected by this structure, cochlear cells are highly susceptible to genetic disease and environmental toxicity. Understanding the biology of auditory cells and the mechanisms of cell damage is critically important for considering new preventative and restorative treatments for hearing impairment.

Hair cells, the sensory cells that mediate hearing and balance, are mechanosensory receptors that transduce mechanical stimulation into electrical responses (Eatock et al. 2006). Hair cells synapse with afferent neurons that convey auditory information to the brain. Complementing mammalian studies, zebrafish have become an increasingly attractive and popular model system for investigating hair cell and neuronal function. The zebrafish inner ear is well developed during larval stages as measured by behavioral measurements of auditory and vestibular behaviors (Lu and DeSmidt 2013; Bhandiwad et al. 2013; Bagnall and Schoppik 2018).

Although fish possess inner ear sensory epithelia, hair cells mediate sensation in the lateral line sensory system as well. The lateral line allows fish and other aquatic organisms to detect directional water flow.

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The zebrafish model system offers many advantages for auditory and vestibular research, including (1) zebrafish produce offspring in large numbers, providing the opportunity for quantitative analysis across many animals; (2) embryos and larvae are optically clear and lateral line hair cells are superficially located, making them amenable for *in vivo* imaging; (3) their small size allows them to be arrayed in multiwell plates for drug screening; and (4) genetic expression constructs can be easily introduced into animals, and protocols are well established for both forward and reverse genetic manipulation, including CRISPR. Although not within the scope of this review, zebrafish have also been quite powerful in elucidating mechanisms of sensory development and hair cell regeneration (for recent reviews, see Thomas et al. 2015 and Kniss et al. 2016). Here, we address different aspects of hair cell function and the ways in which zebrafish studies—with a focus on the lateral line system—have contributed to our understanding of auditory neuroscience.

The Zebrafish Lateral Line System

Zebrafish are found throughout Southeast Asia in flowing streams and slow flowing or stagnant pools (reviewed in Parichy 2015). Many of the predatory organisms that forage for zebrafish rely on suction feeding; thus, at relatively early developmental stages, zebrafish must be capable of detecting and responding to high velocity water flow (Engeszer et al. 2007; Arunachalam et al. 2013). The lateral line sensory system allows fish and other aquatic vertebrates to do just that. This mechanoreceptive sense, described as “touch at a distance,” allows fish to orient in water currents and detect both predators and prey (Dijkgraaf 1963; Webb 2013). The lateral line organs, called neuromasts, contain clusters of mechanosensory hair cells and non-sensory support cells and are found on the surface of the zebrafish in a stereotyped arrangement (see Fig. 1) (Metcalf et al. 1985; Raible and Kruse 2000). Neuromasts found on the head comprise the anterior lateral line system (aLL), whereas those along the trunk form the posterior lateral line system (pLL). Lateral line hair

cells are innervated by bipolar sensory neurons with central axonal projections terminating in the hind-brain and peripheral axons at the neuromasts. The afferents are organized into two ganglia near the ear based on the body positioning of the neuromasts they innervate. The axons of the aLL and pLL ganglia terminate in distinct locations within the hindbrain, leading to somatotopic mapping of lateral line sensory input (Alexandre and Ghysen 1999; Pujol-Marti and Lopez-Schier 2013). Zebrafish lateral line efferent neurons provide both excitatory and inhibitory input to hair cells and are also functionally segregated based on whether they innervate neuromasts of the aLL or pLL (Bricaud et al. 2001; Toro et al. 2015; Haehnel-Taguchi et al. 2018). Dopaminergic efferent innervation enhances the responses of lateral line hair cells, likely by enhancing the activity of presynaptic Cav1.3 channels (Toro et al. 2015). Dopaminergic neurons also innervate the sensory epithelia of the zebrafish inner ear (Haehnel-Taguchi et al. 2018), although the functional significance has not been determined. Studies from *Xenopus* and other fish taxa suggest that cholinergic signaling likely decreases hair cell activity and may act as an adaptive filter during swimming activity (Flock and Russell 1973; Montgomery and Bodznick 1994; Dawkins et al. 2005).

Studies examining the behavioral relevance of the lateral line in zebrafish larvae have focused on the characteristic C-start escape response, in which the body curls and then quickly accelerates away from an impending threat while straightening (McHenry et al. 2009; Stewart and McHenry 2010; Stewart et al. 2013, 2014). In one of the earlier studies, zebrafish larvae were exposed to accelerated water flow, mimicking a predator strike, and the probability and latency of the response were measured. Upon ablation of lateral line hair cells, the response probability was significantly reduced. Behavioral responses recovered following hair cell regeneration, thus demonstrating a role for lateral line hair cell input (McHenry et al. 2009). Hair cell stimulation may also be sufficient to drive this behavior or at least particular facets of it. Later work revealed that escape responses could be elicited through optogenetic stimulation of hair cell activity; however, this stimulation paradigm activated hair cells

FIG. 1. Anatomy of the zebrafish lateral line. **a** Schematized visualization of the anterior (aLL) and posterior (pLL) lateral line of a larval zebrafish. Neuromasts are represented by the green dots and innervation is represented by the magenta lines. **b** Schematized cross section of a lateral line neuromast, including structures and cell types

of both the lateral line and inner ear (Monesson-Olson et al. 2014). In goldfish, lateral line input specifically has been shown to contribute to the latency and directionality of the c-start response (Mirjany et al. 2011). Another behavior mediated by lateral line input (as well as visual input) is rheotaxis, or orientation to constant water flow (Suli et al. 2012). Similar to escape responses, chemically ablating hair cells or disrupting of hair cell transduction both significantly reduced the number of animals performing rheotaxis. Allowing for hair cell recovery (both in number and function) restored the behavior.

The functional contributions of different neuromasts to behavioral responses may vary with position along the body. In an examination of lateral line function during swimming bursts relative to a suction source, ablation of just the most caudal neuromasts had a disproportionate behavioral effect that was not significantly different from depleting all pLL hair cells (Olszewski et al. 2012). Similar results were obtained in a study that combined behavioral experiments with mathematical modeling to study rheotaxis (Oteiza et al. 2017). As in previous work, significant reduction in rheotaxis was observed when the lateral line was ablated; however, while the pLL neuromasts were required for the behavior, the aLL neuromasts were not, suggesting that the lateral line system has topographic subdivisions that mediate distinct behaviors. In addition to position along the length of the fish, larval fish seem to require the integration of bilateral hair cell input for rheotaxis, since laser ablation of hair cells on a single side led to a similar change in behavior compared to bilateral ablation. What about the contribution of a single neuromast? Although stimulation of a single neuromast was initially found to be insufficient to elicit motor behavior (based on electrophysiological recordings in the brainstem ventral motor root), a later study found that single neuromast stimulation was in fact sufficient to drive fictive swimming and motor responses in anesthetized larvae (Liao 2010; Haehnel-Taguchi et al. 2014). The ability of a single neuromast to elicit behavior may stem from the fact that a given neuromast can be stably innervated by multiple neurons (Liao 2010; Haehnel-Taguchi et al. 2014; Pujol-Martí et al. 2014). Moreover, developmental differences in afferent innervation and electrophysiological properties could also contribute to behavioral output (Liao and Haehnel 2012).

Zebrafish Genetic Models of Hearing Loss

The relationship between mechanosensory sensation and behavioral output is a clear benefit of using this system to study sensory and cell function. Many genes required for hair cell function were initially found

through a large mutagenesis screen for animals with behavioral movement and balance deficits (Nicolson et al. 1998). The identified mutants, so-called circler mutants, all responded to touch, but swam in a circular motion due to significant inner ear and lateral line hair cell dysfunction. As the individual genes underlying these phenotypes were identified, each shared homology with mammalian genes, some of which are also known human hereditary deafness genes.

Although acquired human hearing loss is more common, congenital deafness is also quite prevalent, affecting 2–3 out of every 1000 newborn children (Morton and Nance 2006). Roughly 80 % of congenital hearing loss is genetic, the forms of which can be categorized as (1) non-syndromic, occurring in isolation; or (2) syndromic, occurring as part of a complex genetic disorder (Shearer et al. 2017). Although zebrafish cannot be used to study sensory deficits associated with dysfunction of cochlea-specific structures (e.g., the stria vascularis), they have been valuable models for uncovering aspects of both syndromic and non-syndromic deafness conditions (Whitfield 2002; Nicolson 2005, 2017). The many zebrafish models of human hereditary deafness genes (DFNs) are listed in Table 1.

While most zebrafish mutations were identified by behavioral deficits associated with inner ear dysfunction such as circling or loss of acoustic startle, those involving genes that regulate hair cell function affect both the inner ear and the lateral line. Others affect structural elements such as otoliths/otoconia (reviewed in Lundberg et al. 2015), with no obvious effects on lateral line function. While study of hair cells in the lateral line has the advantage of direct access for manipulation, the larger size of inner ear stereocilia is advantageous for studies of protein localization and trafficking. Studies therefore often use both inner ear and lateral line hair cells to assess structure and function. Broadly, lateral line hair cells have physiological properties similar to those reported for mammalian vestibular organs and auditory organs of other vertebrates such as goldfish, but different than mouse cochlear hair cells (Olt et al. 2016). It is likely, however, that there are specific characteristics of lateral line (and inner ear) hair cells that are not conserved across organs and species.

One of the first zebrafish models of human genetic deafness disorders came from the identification and characterization of mutations in the unconventional myosin gene, *myosin7a* (*myo7a*). Myo7a is motor molecule that serves as a scaffolding protein required for bundle integrity (Ernest et al. 2000; Blanco-Sánchez et al. 2014). The *myo7aa* zebrafish mutants, also known as *mariner*, were characterized by their lack of acoustic or vibrational sensitivity, absent microphonic potentials, and splayed bundle phenotype

TABLE 1

Zebrafish models of human hereditary hearing loss

DFNA/B	Human Gene	Zebrafish Gene	Zebrafish Reference
DFNA2A	KCNQ4	<i>kcnq4</i>	(Wu et al. 2014) ¹
DFNA3A,B DFNB1A,B	GJB2, GJB6	<i>cx30.3*</i>	(Chang-Chien et al. 2014) ²
DFNA5	GSDME	<i>gsdmeb</i>	(Busch-Nentwich et al. 2004) ²
DFNA8,12 DFNB21	TECTA	<i>tecta</i>	(Stooke-Vaughan et al. 2015) ²
DFNA10	EYA4	<i>eya4</i>	(Wang et al. 2008) ^{2,3}
DFNA11 DFNB2	MYO7A	<i>myo7aa</i>	(Nicolson et al. 1998; Ernest et al. 2000) ^{2,3}
DFNA22 DFNB37	MYO6	<i>myo6b</i>	(Seiler et al. 2004; Kappler et al. 2004) ^{2,3}
DFNA23	SIX1	<i>six1a</i>	(Bricaud and Collazo 2006, 2011) ²
DFNA25	SLC17A8	<i>slc17a8</i>	(Obholzer et al. 2008) ³
DFNA28	GRHL2B	<i>grhl2b</i>	(Han et al. 2011) ^{2,3}
DFNA36 DFNB7,11	TMC1	<i>tmc2*</i>	(Chou et al. 2017) ³
DFNA50	MIRN96	<i>mir96</i>	(Li et al. 2010) ²
DFNA68	HOMER2	<i>homer2</i>	(Azaiez et al. 2015) ²
DFNA71	DMXL2	<i>rbc3a</i>	(Einhorn et al. 2012) ³
DFNB4	SLC26A4	<i>slc26a3*</i>	(Bayaa et al. 2009) ¹
DFNB6	TMIE	<i>tmie</i>	(Shen et al. 2008; Gleason et al. 2009) ^{2,3}
DFNB9	OTOF	<i>otofa/b</i>	(Chatterjee et al. 2015) ³
DFNB12	CDH23	<i>cdh23</i>	(Nicolson et al. 1998; Söllner et al. 2004; Blanco-Sánchez et al. 2014) ^{2,3}
DFNB18	USH1C	<i>ush1c</i>	(Phillips et al. 2011; Blanco-Sánchez et al. 2014) ^{2,3}
DFNB18B	OTOG	<i>otog</i>	(Stooke-Vaughan et al. 2015) ²
DFNB23	PCDH15	<i>pcdh15a</i>	(Seiler et al. 2005; Maeda et al. 2014, 2017) ^{2,3}
DFNB24	RDX	<i>msna</i>	(Pataky et al. 2004) ¹
DFNB25	GRXCR1	<i>grxcr1</i>	(Blanco-Sánchez et al., 2018)

DFN genes were identified using the Hereditary Hearing Loss website (<http://hereditaryhearingloss.org/>) and cross referenced using the Zebrafish Information Network (ZFIN) online database (<https://zfin.org/>)

¹Denotes studies with expression data only

²Denotes studies in which a morphological and/or developmental mutant phenotype was observed

³Denotes studies in which a physiological and/or behavioral mutant phenotype was observed

⁴Note: Phenotypic inconsistency observed between CRISPR knockout (Imtiaz et al. 2018) and morpholino knock-down (Delmaghani et al. 2016) methodologies

*Homologous gene

§Characterization of protein function

(Nicolson et al. 1998; Ernest et al. 2000). Paralleling mammalian discoveries, *Myo7A* was also one of the first genes identified as part of the mammalian cochlear hair cell mechanotransduction apparatus through characterization of the *shaker-1* mouse mutant (Gibson et al. 1995; Self et al. 1998). Mutations in human *MYO7A* are known to cause Usher syndrome (clinical subtype 1) (*USH1*), a disorder characterized by progressive retinal degeneration and profound hearing and balance deficits (Reiners et al. 2006).

In addition to identifying mutations in genes required for hair cell function, understanding the trafficking, targeting, and assembly of these proteins has become a more active area of investigation. These studies provide additional insight into the mechanisms underlying human hereditary deafness (Blanco-Sánchez et al. 2014; Maeda et al. 2014; Erickson et al. 2017). For example, following the identification of *myo7aa* mutations, additional zebrafish mutants were identified in genes homologous to the human *USH1* genes, including *cdh23*, *pcdh15*, *ush1c* (*harmonin*), and *clarin1* (Söllner et al. 2004; Seiler et al. 2005; Phillips et al. 2011; Gopal et al. 2015). Subsequently, investigation of these zebrafish models provided unique insight into the causes of hair cell death associated with *USH*. Three of the *USH1* proteins—*Myo7aa*, *Cdh23*, and *Ush1c* (and the intraflagellar transport protein, *Ift88*)—form complexes in the endoplasmic reticulum (ER) that are required for protein trafficking. Mutations in *cadherin23* or *ush1c* lead to defects in protein targeting, as well as induction of ER stress and apoptotic cell death. This may be linked to activation of the unfolded protein response in the ER, as components of the complex are missing and the complex is misassembled. Suppressing ER stress has been shown to reduce hair cell death in both zebrafish and mouse models of *USH1* (Blanco-Sánchez et al. 2014; Hu et al. 2016).

Mutations in *grxcr1*, encoding zebrafish orthologues of glutaredoxin domain-containing cysteine-rich 1 (*Grxcr1*) protein, also result in changes in *USH* protein distribution (Blanco-Sánchez et al. 2018). *Grxcr1* is an enzyme that post-translationally modifies proteins by adding glutathione; glutathiolation of *Ush1c* protein promotes its interactions with *Ush1g* (*Sans*). Loss of activity results in thinning of inner ear stereocilia bundles, similar to previous reports of *Grxcr1* mutation in the *pirouette* (*pi*) mouse (Odeh et al. 2010). No loss of mechanotransduction-dependent behaviors was found in zebrafish mutants (Blanco-Sánchez et al. 2018), suggesting potential compensation by other *GRXCR* genes.

Protein trafficking is also highlighted in the characterization of the zebrafish *transmembrane O-methyltransferase* (*tomt*) mutant, *mercury*, a model of non-syndromic deafness *DFNB63*. Although it was initially thought that

hearing loss associated with *DFNB63* was related to deficient catecholamine metabolism, Erickson et al. (2017) revealed that *mercury* mutants exhibit abolished mechanotransduction due to defects in *Tmc1/2* protein targeting, specifically (Erickson et al. 2017). Unlike other proteins required for mechanotransduction, *Tomt* is not expressed in the hair cell bundle, but rather in the Golgi apparatus. It remains unclear exactly how *Tomt* regulates *Tmc* trafficking in the hair cells; however, its role is conserved in mammalian hair cells (Cunningham et al. 2017). Trafficking defects also underlie loss of function in zebrafish *ap1b1* mutants, with deficits in mechanotransduction (Clemens Grisham et al. 2013). This gene encodes for part of the adaptor complex involved in clathrin-mediated transport. Mutations in the related *AP1S1* gene in humans underlie *MEDNIK* syndrome that includes hearing loss (Montpetit et al. 2008).

Mechanotransduction Activity

Mechanotransduction is mediated by a specialized apical structure, the hair cell bundle, which is characterized by several stereocilia and an eccentric kinocilium. Notably, some of the first zebrafish models of human deafness had deficits in mechanotransduction. The mechanism and components of hair cell mechanotransduction have been recently reviewed in Nicolson (2017), but will be described in brief.

The stereocilia of the hair cell bundle are composed primarily of actin filaments and have a staircase-like arrangement, such that they become progressively taller closer to the kinocilium, a true cilium. Stereocilia are connected by tip links, which gate mechanosensitive, non-selective cation channels at the tips of the stereocilia (Hudspeth and Corey 1977; Hudspeth and Jacobs 1979; Corey and Hudspeth 1979). Deflection of the stereocilia toward the kinocilium results in mechanotransduction channel opening and hair cell depolarization, while deflection away from the kinocilium leads to channel closing. Extracellular microphonic recordings were used to determine that stereocilia deflection promotes channel opening (Corey and Hudspeth 1980; Hudspeth 1982).

Although directional selectivity is a key element of hair cell mechanotransduction, this feature of mechanosensitivity develops with bundle maturation. Immature hair cells in both the cochlea and lateral line can be stimulated in the direction opposite their morphological polarity to elicit mechanotransduction currents (Waguespack et al. 2007; Kindt et al. 2012). While the immature cochlear hair cells lack directional sensitivity, immature lateral line hair cells in fact exhibit reverse directional sensitivity. This is followed by an intermediate stage of bi-directional sensitivity and finally mature directional sensitivity (which coincides with

stereocilia tip link formation). The reversed mechanosensitivity of immature lateral line hair cells is mediated by the kinocilia and kinocilial links, as mutants lacking these structures (*ift88* mutants) do not exhibit early mechanosensory responsiveness or a reversal of functional polarity (Kindt et al. 2012). Although mammalian auditory hair cells lose their kinocilia following the onset of mechanotransduction, perhaps there is a role for the kinocilia in the early maturation of these cells as well (Lim and Anniko 1985).

The structure and components of the apical bundle are conserved between mammals and zebrafish. One of the proteins comprising the stereocilia tip links, for example, was identified in tandem in both zebrafish and mouse models. Both *waltzer* mice and *sputnik* circler mutants were found to have mutations in the gene encoding the calcium-dependent adhesion molecule cadherin23 (*Cdh23*) (Siemens et al. 2004; Söllner et al. 2004). *Cdh23* interacts with Protocadherin15 (*Pcdh15*) to form tip link filaments (Ahmed et al. 2006; Kazmierczak et al. 2007). The splayed bundle phenotype of inner ear hair cells of the *waltzer* and *sputnik* mutants results in loss of mechanotransduction and corresponding auditory and balance defects, well recognized as circular ambulatory or swimming movement. Mutations in human *CDH23* and *PCDH15* also lead to auditory and vestibular dysfunction, as well as visual loss, associated with the genetic disease Usher syndrome (Reiners et al. 2006).

In addition to structural components of the hair cell bundle, proteins of the mechanotransduction channel complex are also conserved across taxa, including *Tmc* (Shen et al. 2008; Gleason et al. 2009; Zhao et al. 2014), *Lhfpl5* (Longo-Guess et al. 2005; Maeda et al. 2017), the transmembrane channel-like proteins (*Tmc1* and *2*) (Pan et al. 2013; Maeda et al. 2014; Kurima et al. 2015; Erickson et al. 2017; Chou et al. 2017; Pan et al. 2018), and *Tomt*, a protein necessary for *Tmc* localization (Cunningham et al. 2017; Erickson et al. 2017). The exact pore-forming protein(s) of the mechanotransduction channel still remain unknown. Mirroring mammalian studies, zebrafish *Trp* channels were initially proposed, but with conflicting results. An early study of the unconventional *Trp* channel *NompC* (*Trpn1*) demonstrated a potential role in mechanotransduction, while mutation of *trp1a* had no effect (Sidi et al. 2004; Prober et al. 2008). Primarily through study of mammalian hair cells, more recent evidence has established *TMC1/2* as the best candidates (Pan et al. 2013; Kurima et al. 2015; Pan et al. 2018). Support for this in the zebrafish literature stems from the interaction of *Pcdh15* and *Tmc2*, as well as mechanotransduction deficits that result from improper *Tmc1/2* trafficking (Maeda et al. 2014; Erickson et al. 2017).

Development of Cell Polarity

Given the importance of the hair bundle orientation for cell function and directional sensitivity, establishment and maintenance of hair cell planar cell polarity (PCP) is crucial. During development and regeneration in the lateral line system, sibling hair cells form apical bundles exhibiting mirror symmetrical orientation (López-Schier et al. 2004; López-Schier and Hudspeth 2006). Sibling pairs are born from a common progenitor that divides in a plane perpendicular to the axis of polarity of the neuromast. The developing hair cells then undergo rearrangements ultimately leading to cells with oppositely oriented bundles (Wibowo et al. 2011). In this way, lateral line hair cells display PCP relative to each other as well as to the body axis.

The *vangl2* gene is known to be required for proper PCP in both mammalian inner ear and lateral line hair cells (Montcouquiol et al. 2003; Wang et al. 2005, 2006; López-Schier and Hudspeth 2006; Mirkovic et al. 2012). Like mammals, the zebrafish *Vangl2* protein is asymmetrically localized within lateral line hair cells, with protein accumulating in the apical side of the cells. *Vangl2*, or *trilobite*, mutants display misalignments in progenitor cell division and disrupted hair cell orientation, where hair cells are oriented randomly within the neuromast rather than in 180 degree-opposing directions (López-Schier and Hudspeth 2006; Mirkovic et al. 2012). *Vangl2* overexpression also disrupts orientation, as hair cells are observed with randomized orientation or uniform orientation bias within a neuromast.

Whereas *Vangl2* is expressed, albeit asymmetrically, in all hair cells, another important regulator of PCP, the transcription factor *Emx2*, is only expressed in half of the hair cells within the neuromast (Jiang et al. 2017). *Emx2* CRISPR knockout leads to loss of mirror symmetry, as all hair cells become uniformly polarized in one direction (Jiang et al. 2017). Conversely, upon *emx2* overexpression, hair cells become uniformly polarized in the opposite orientation. These polarity changes have functional consequences. Calcium imaging experiments demonstrated that after ectopic expression of *emx2*, all hair cells maximally responded to stimulation in one orientation. Asymmetric *Emx2* expression was also observed in mouse and chick utricles along the line of polarity reversal, a delineation of two regions of the organ with opposing hair cell polarities. As in the zebrafish experiments, *Emx2* knockout and overexpression in the mouse utricle also disrupted mirror symmetry (Holley et al. 2010; Jiang et al. 2017), together demonstrating a conserved role for *Emx2* in PCP across vertebrates. In addition to regulating hair cell polarity, *Emx2* has also been shown to regulate afferent neuron directional selectivity of hair cell synaptic targets (Ji et al. 2018).

In the lateral line, hair cell polarity is also important for innervation, as afferents demonstrate strict synaptic selectivity for hair cells of the same polarity (Nagiel et al. 2008, 2009; Faucherre et al. 2009; Dow et al. 2015; Dow et al. 2018). Illustrating this specificity, directional selectivity persists when contacts are formed with regenerating hair cells post-ablation, as well as during axonal regeneration (Nagiel et al. 2008; Faucherre et al. 2009). Curiously, although lack of mechanotransduction activity seems to alter the complexity of afferent peripheral arbors (Faucherre et al. 2010; Pujol-Martí et al. 2014), central and peripheral afferent innervation occurs normally in the absence of hair cell mechanotransduction activity or synaptic vesicle release (Nagiel et al. 2008, 2009; Faucherre et al. 2010; Pujol-Martí et al. 2012, 2014). Moreover, afferent innervation retains preference for one of two sibling hair cells in *vangl2* mutants, even while innervating hair cells of random polarity, consistent with the idea that coordinated activity is unnecessary for innervation specificity (Dow et al. 2018). By contrast, selectivity for individual hair cells from sibling pairs is lost after activation of Notch signaling by overexpression of the Notch intracellular domain, where all hair cells become polarized in a single direction simultaneously (Dow et al. 2018).

In the mammalian cochlea, hair cell activity is certainly important for hair cell maturation and cochlear wiring, as spontaneous action potentials occur during development prior onset of auditory-induced stimulation (Johnson et al. 2011, 2013; Wang and Bergles 2015). However, like zebrafish, hair cells can be innervated even when neurotransmitter release is reduced or absent. Such innervation was observed in rodent *Vglut3* knockouts and *Cav1.3* knockouts (although these synapses do degenerate over time) (Glueckert et al. 2003; Nemzou et al. 2006; Seal et al. 2008; Ruel et al. 2008). The role of these conserved genes in synaptic activity is discussed below.

Synaptic Activity

Hair cells transduce mechanical stimulation into electrical signals through graded receptor potentials and neurotransmitter release to post-synaptic lateral line afferents. Patch clamp electrophysiology experiments reveal that lateral line hair cells exhibit K^+ and Ca^{2+} currents similar to those of other fish, birds, and mammalian vestibular hair cells (Ricci et al. 2013; Olt et al. 2014, 2016). These include A-type and delayed-rectifier type K^+ currents. While the details of current profiles are distinct from those of the mammalian cochlea, hair cell conductances vary across the cochlea itself (Johnson 2015; Olt et al. 2016). Nevertheless, it is likely that the lateral line system will be

most useful for studying general principles of hair cell physiology in an intact system.

Similar to the identification of proteins involved in mechanotransduction, circler mutants were critical in early studies of the lateral line hair cells and their afferent synapses. Although mutations were isolated on the basis of inner ear dysfunction, physiological studies have largely been performed in the lateral line system, as afferent neurons and hair cells can be more easily accessed. Identification of *slc17a8*, which encodes for the glutamate transporter Vglut3, as the gene underlying the *asteroid* mutation showed that lateral line hair cells—like cochlear hair cells—rely on glutamatergic synaptic transmission (Obholzer et al. 2008). Neurotransmitter release depends on calcium influx through L-type voltage-gated calcium channels, Cav1.3, localized to the basal membrane (Moser and Beutner 2000; Sidi et al. 2004; Sheets et al. 2012). As in *asteroid* mutants, lateral line afferents in *gemini* mutants (caused by a mutation in the *cacna1d* gene encoding for Cav1.3) do not respond to hair cell stimulation or exhibit any spontaneous activity due to abolished neurotransmitter release (Obholzer et al. 2008; Trapani and Nicolson 2011). The function of these genes is conserved in mammalian hair cells, as transmission is significantly impaired in both *Cav1.3* and *Vglut3* mouse mutants (Glueckert et al. 2003; Nemzou et al. 2006; Seal et al. 2008; Ruel et al. 2008).

Precise timing of hair cell neurotransmitter release allows afferent neurons to phase-lock their activity to hair cell stimulation, maintaining signal fidelity. The importance of proper vesicle release is illustrated by two mutants: the *comet* mutant and the *pinball wizard* mutant. The gene underlying the *comet* mutation is *synptojanin1* (*synj1*), which is required for synaptic vesicle recycling. Although less severe than the other transmission mutants, *comet* mutants display delayed hair cell output relative to mechanical stimulation and disrupted afferent phase-locking (Trapani et al. 2009). In mice, synptojanin dysfunction leads to more drastic phenotypes. *Synj1* mutants do not survive long after birth, although examination of cultured cortical neurons from mutant animals revealed reduced vesicle pools and reduced vesicle recycling with prolonged periods of stimulation (as assayed in cortical neurons) (Kim et al. 2002). *Synj2* mutants, also known as *Mozart*, however, exhibit an auditory system-specific phenotype wherein cochlear hair cells degenerate (Manji et al. 2011). Timing of neurotransmitter release is also implicated in *pinball wizard* mutants, affecting the *wrb* gene that encodes a small transmembrane protein required for membrane insertion of tail-anchored proteins. Mutants show reduced synaptic vesicle reserve pool and a reduction in proteins associated with synaptic vesicles, suggesting a lack of ability to replenish the vesicle pool. These animals display visual deficits in

addition to balance deficits and diminishing auditory startle (Lin et al. 2016). Mutations in the mouse *Wrb* gene also cause reduced hearing as a result of synaptic disruption (Vogl et al. 2016). The WRB protein is targeted to the endoplasmic reticulum, where it regulates the membrane insertion of the hair cell synaptic regulator Otoferlin.

Perhaps the most important structure allowing hair cells to transmit both the timing and intensity of mechanical stimulation is the synaptic ribbon. Synaptic ribbons are presynaptic electron densities that tether glutamate-filled vesicles near the active zone, thus permitting rapid and sustained neurotransmitter release (Matthews and Fuchs 2010; Nicolson 2015). Hair cell synaptic ribbons are largely composed of the protein Ribeye, which is encoded by two paralogous genes in zebrafish: *ribeye a* and *b*, also known as *ctbp2a* and *ctbp2l* (Wan et al. 2005; Sheets et al. 2011). With the ability to easily induce genetic mutation and transgenesis, zebrafish have been useful models for investigating ribeye function in intact organisms.

Ribeye plays a critical role in directing ribbon organization and synaptic organization, particularly as related to appropriate localization and clustering of Cav1.3 channels (Sheets et al. 2011, 2017; Lv et al. 2016). In *ribeye* CRISPR knockouts, Lv et al. observed “ghost ribbons,” or ribbons that lacked synaptic densities and did not appose any efferent or afferent neuronal connections (in addition to aberrant Cav1.3 channel clustering). Curiously, this did not correspond with any obvious deficits in post-synaptic activity. Overexpression of *ribeye* can lead to enlargement of hair cell ribbons; however, this did not increase Cav1.3 channel localization at the synapse (Sheets et al. 2017). Additionally, while these hair cells had more associated synaptic vesicles relative to wildtype and exhibited increased calcium signaling, ribbon enlargement corresponded with reduced afferent neuron spontaneous activity and increased response latency to the onset of hair cell stimulation. Investigating Ribeye activity has been complicated by the difficulty of completely disrupting gene and protein function. For example, in the CRISPR knockout study, Ribeye expression was dramatically reduced in double knockouts, but still detectable, albeit at low levels, at the presynapse by immunohistochemistry (Lv et al. 2016). In mouse, *Ctbp2* knockout is embryonic lethal (Hildebrand and Soriano 2002); however, Ribeye disruption has been achieved through the generation of a Cre knockout mouse (Maxeiner et al. 2016). Cre-mediated excision removed the Ribeye A domain, but left the B domain, which is essentially identical to *Ctbp2*. Truncation of the protein was found to interfere with ribbon formation, however presynaptic active zones still formed, perhaps explaining the relatively small effect

on synaptic transmission and mild hearing deficit observed (Becker et al. 2018; Jean et al. 2018). These results suggest that compensatory mechanisms may be masking the importance of RIBEYE function in both mammalian and zebrafish models.

While Ribeye is important for calcium channel localization and synaptic organization, ribbon size can in turn be influenced by calcium (Sheets et al. 2012). Additionally, although a post-synaptic target is not required for ribbon formation, the presence of a post-synapse does influence ribbon maintenance (Suli et al. 2016). Lack of innervation, as in *neurogenin1* mutants or with lateral line nerve transection, corresponds with smaller ribbon size and improper ribbon localization. During hair cell regeneration, Ribeye clustering at the basal cell membrane corresponds with the timing of hair cell innervation (Suli et al. 2016). There also appears to be a dynamic aspect to the structure, as Ribeye protein is mobile within the ribbons and exchanged at a low rate, suggesting some level of turnover and renewal (Chen et al. 2017; Graydon et al. 2017). In addition to the importance of Ribeye, these studies highlight mechanisms that influence ribbon formation and maintenance.

Although the formation of synaptic structures is relatively uniform across neuromast hair cells, some hair cells within individual neuromasts are synaptically silent upon mechanical stimulation despite robust depolarization (Zhang et al. 2018). Synaptically inactive hair cells can become active within minutes after ablation of a neighboring active hair cell, suggesting the process is dynamically regulated. This regulation of presynaptic activity is independent of innervation and appears to depend on intact K⁺ handling by surrounding supporting cells. It will be interesting to see whether this tight regulation of synaptic activity extends to hair cells in other systems.

This model has also been used to identify mechanisms of damage that are quite difficult to address in the mammalian auditory system due to its inaccessible anatomical location. For example, neuronal damage following noise exposure was recently modeled in a study of glutamatergic transmission and excitotoxicity affecting lateral line afferents (Sebe et al. 2017). Hair cell stimulation leads to robust calcium influx into post-synaptic afferent terminals through calcium-permeable AMPA receptors. Prolonged receptor activation with glutamate receptor agonists led to calcium accumulation, causing swelling and decreased afferent responsiveness, while blocking the calcium-permeable AMPA receptors prevented the excitotoxic effects. These results parallel mammalian studies of excitotoxicity in the cochlea following noise exposure (Puel et al. 1998). Calcium influx is followed by swelling of the spiral ganglia neuron terminals contacting inner hair cells, which can be caused or

mitigated by activating or inhibiting AMPA receptors, respectively (Puel et al. 1998). As evidence from Sebe et al. (2017) supports functional conservation of calcium-permeable AMPA receptors across vertebrates, these channels are likely crucial players in the synaptic damage that occurs following noise overexposure leading to hearing loss (Sebe et al. 2017). Beyond damage to terminals, glutamate excitotoxicity can lead to apoptotic lateral line hair cell death, even in the absence of afferent or efferent innervation (Sheets 2017). These findings suggest that glutamate accumulation following noise exposure may have a direct pathological effect on the hair cells as well as the afferents.

Hair Cell Death

Hearing loss can occur due to aging, loud noise exposure, and ototoxic compound exposure. Of these, damage or death of the sensory hair cells is a common feature. In addition to their use as genetic models of hearing loss, zebrafish have been extraordinary models for understanding mechanisms of hair cell death, particularly as a result of ototoxic drug exposure. In addition to their sensitivity to heavy metals (e.g., copper), lateral line hair cells are susceptible to the otherwise therapeutic aminoglycoside antibiotics (e.g., neomycin and gentamicin) and chemotherapeutic agents such as cisplatin (Harris et al. 2003; Ton and Parng 2005; Linbo et al. 2006; Hernández et al. 2006; Ou et al. 2007; Olivari et al. 2008; Van Trump et al. 2010). Despite their importance for treating disease, the unwanted side effect of these compounds has led to an effort to understand their specific off-target toxicity as well as the development of means to protect hair cells from the ensuing damage. Larval zebrafish are particularly well suited to this line of inquiry. Ototoxic compounds can be added to the media of free-swimming larvae and hair cell loss can be easily monitored via vital dye staining or transgenic animals with fluorescently labeled hair cells. Moreover, the accessibility of lateral line hair cells allows for live-imaging studies of drug entry and the post-exposure events that lead to cell death. Importantly, these compounds also kill hair cells in a reliably dose-dependent fashion (Harris et al. 2003; Ou et al. 2007). In combination with the ability to obtain many zebrafish larvae at once, the ability to generate a reliable dose-response curve has provided a valuable tool for evaluating means of hair cell protection and sensitization in both genetic and small molecule screens (Fig. 2).

Mitochondrial Dysfunction Leading to Cell Death. Zebrafish have been quite useful for understanding subcellular events leading to hair cell death, with a particular focus on mitochondria. Mitochondrial

dysfunction is a universal feature of multiple modes of hair cell damage and death, including noise damage, age-related hearing loss, and aminoglycoside exposure (Kopke et al. 1999; Pickles 2004; Böttger and Schacht 2013). Following aminoglycoside antibiotic treatment, dying hair cells across vertebrate taxa exhibit a suite of cellular and morphological changes, including dilated and swollen mitochondria (Duvall and Wersäll 1964; Bagger-Sjöback and Wersäll 1978; Lang and Liu 1997; Hirose et al. 2004; Owens et al. 2007). Additional evidence of mitochondrial distress stems from the production of reactive oxygen species (ROS) and the modest protective effects of treatment with antioxidants or expression of ROS-reducing enzymes (Clerici et al. 1996; Sha and Schacht 2000; Sha et al. 2001a, b; McFadden et al. 2003; Kawamoto et al. 2004; Jiang et al. 2005; Ton and Parng 2005; Choung et al. 2009; Someya et al. 2009, 2010; Jensen-Smith et al. 2012; Chen et al. 2013; Quan et al. 2015; Takumida et al. 2016). Incidentally, ROS production is not limited to aminoglycoside treatment, as similar results have been observed with copper treatment in larval zebrafish (Olivari et al. 2008).

Mitochondrial ROS production in response to treatment with neomycin occurs due to toxic calcium elevation and transfer between intracellular compartments. Through live-imaging studies during neomycin exposure, Esterberg et al. (2013, 2014) determined that calcium flows from the ER to the mitochondria, causing depolarization of the organelles (Fig. 3). As mitochondrial membrane potential is lost, calcium is then released into the cytoplasm, and cells ultimately lose membrane integrity. Manipulation of calcium flow can either protect or sensitize cells to neomycin treatment. Calcium flow into the mitochondria results in an increase in ROS production, yet specifically reducing mitochondrial ROS production using a targeted ROS sink confers hair cell protection (Esterberg et al. 2016).

Given the importance of mitochondria for both hair cell function and death, research devoted to understanding the role of mitochondrial networks, biogenesis, and dynamics will likely be an important area of exploration. In support of this avenue, a recent study found that the cumulative history of mitochondrial activity correlated with susceptibility to death from neomycin exposure (Pickett et al. 2018). Application of mdivi-1—a mitochondrial fission inhibitor—protected hair cells from cisplatin-induced hair cell toxicity (Vargo et al. 2017), suggesting that mitochondrial function may broadly influence susceptibility of hair cells to damage. This suggests that mitochondrial dynamics may play a role in hair cell viability. Beyond understanding ototoxic-induced hair cell dysfunction, mitochondria-focused studies would provide additional insight into the hearing impairment caused by mutations in mitochondrial genes

FIG. 2. Using a dose-response curve to assay hair cell death modulators. **a** Lateral line hair cells labeled with parvalbumin in control or antibiotic-treated conditions. Scale bar=10 μ m. **b** Hair cells can be counted following treatment with different concentrations of toxic aminoglycoside antibiotics. As antibiotic concentrations increase, the number of hair cells remaining decreases. The resulting dose-response curve can be reliably reproduced across

experiments. As a result, mutations or compounds can easily be assayed for effects on hair cell death. Hair cell protection or sensitization would be indicated by shifts in the curve (pink and blue lines, respectively). Data points represent the mean percentage of hair cells remaining. Error bars = SD. Figure adapted from Stawicki et al. (2014). Copyright © 2014. Published by The Company of Biologists Ltd.

that lead to syndromic and non-syndromic hearing loss (Kokotas et al. 2007; Luo et al. 2013; Ding et al. 2013). While mutations in these mitochondrial deafness genes are known to affect several aspects of mitochondrial function—including mitochondrial protein synthesis and DNA replication, mitochondrial metabolism, and mitochondrial fission and fusion—their cellular location

of action remains unknown, particularly with regard to impacts on hair cells versus other cell types within the sensory system. In addition, investigation of potential interactions between mitochondrial mutations and aminoglycoside susceptibility may be important. Precedent for such interactions is highlighted by the A1555G mutation in the MTRNR1 gene encoding 12S rRNA, which has been shown to dramatically increase susceptibility to damage in humans (Prezant et al. 1993).

Genetic Screening. As noted above, an advantage of the zebrafish system is the ability to perform genetic screens. Genes revealed by screening might include potential targets for therapies to block ototoxicity, but also might reveal underlying vulnerabilities of hair cells that are important for otherwise normal function. A major facet of drug-induced toxicity is entry into hair cells (Olivari et al. 2008; Coffin et al. 2009; Thomas et al. 2013; Hailey et al. 2017). Across taxa, active mechanotransduction is required for drug entry, presumably through the large mechanotransduction channels (Gale et al. 2001; Marcotti et al. 2005; Alharazneh et al. 2011). As a result, blocking mechanotransduction, and thus drug uptake, remains one of the most potent means of protecting hair cells. This is evidenced in part by the fact that the MET-incapable mutants in both mouse and zebrafish are resistant to neomycin (Richardson et al. 1997; Seiler and Nicolson 1999; Vu et al. 2013; Thomas et al. 2013). In order to investigate other genetic modulators of hair cell death, Owens and colleagues conducted a forward genetic screen for hair cell resistant phenotypes (Owens et al. 2008). Although the genes identified had

Fig. 3. Relative timing of subcellular events during aminoglycoside-induced hair cell death, as measured by mitochondrial dyes and targeted, genetically encoded Ca²⁺ indicators. Box-and-whisker plots indicate the time at which the half-maximal change in fluorescence of each indicator is reached relative to mitochondrial depolarization (dotted line). Bars indicate minimal and maximal values. (Modified from Esterberg et al. 2014)

quite disparate functions, many of them influence hair cell mechanotransduction to some extent (Owens et al. 2008; Hailey et al. 2012; Stawicki et al. 2014, 2016).

Two of the mutants, *persephone* and *merovingian*, were found to have mutations in genes that relate to ionic balance homeostasis. *Persephone* possesses a mutation in the *slc4a1b* gene encoding a chloride/bicarbonate exchanger, which fails to properly target to the cell membrane. *Persephone* mutants are resistant to multiple aminoglycosides and partially resistant to cisplatin. Recapitulating the mutant phenotype, pharmacological blockade of the exchanger and elevated extracellular bicarbonate concentration can be used to protect wildtype hair cells (Hailey et al. 2012). A mutation in the *gcm2* gene was found to underlie the *merovingian* mutant. Gcm2 is a transcription factor involved in the production of a particular type of ionocyte (H⁺-ATPase-rich), which maintains and regulates whole body pH in fish. Like *persephone*, *merovingian* mutants displayed strong neomycin resistance and moderate resistance to cisplatin, in addition to an acidified extracellular environment surrounding lateral line hair cells (Stawicki et al. 2014). Both *persephone* and *merovingian* mutants exhibit decreased mechanotransduction and drug uptake, highlighting the importance of ionic balance for both proper hair cell function and protection. This is further bolstered by the finding that elevated extracellular calcium or magnesium concentration have a similar effect (Coffin et al. 2009).

Identification of another mutant from the screen led to the investigation of a completely different group of genes: cilia genes related to intraflagellar transport (IFT) and the ciliary transition zone (Stawicki et al. 2016). Mutations affecting genes implicated in intraflagellar transport include *dync2h1*, *wdr35*, *ift88*, and *traf3ip*, and those affecting the ciliary transition zone include *cc2d2a*, *mks1*, and *cep290*. Mutations in cilia-related genes typically lead to a variety of diseases known as ciliopathies. For lateral line hair cells, mutations in the IFT genes have different effects compared to the transition zone mutants. Unlike the transition zone mutants, IFT mutants show defects in kinocilia formation in addition to protection. For both groups, the stereocilia of the hair cells appear normal. IFT mutations caused decreases in FM1-43 uptake reflecting decreased mechanotransduction, and reduced uptake of aminoglycoside antibiotics, accounting for their protective effects. How transition zone genes alter aminoglycoside susceptibility remains unknown.

Small Molecule Screening. In addition to genetic screening, zebrafish larvae are ideal model organisms for small molecule screening, due in part to their small size and the ability to obtain many hundreds of animals for a given experiment. Moreover, the surface location of lateral line hair cells means that hair cell survival or

death can be determined based on relative fluorescence with animals in a 96-well plate. A number of small molecule screens have been conducted to identify ototoxins as well as compounds that confer hair cell protection (Ton and Parg 2005; Owens et al. 2008; Ou et al. 2009; Coffin et al. 2010, 2013b; Kruger et al. 2016).

The zebrafish model has the potential to identify new ototoxic compounds and interrogate compounds with suspected ototoxic properties, such as those previously identified by anecdotal patient reports. Currently there is no systematic screening of drugs for ototoxicity as part of approval for use. However, using the lateral line, screening of FDA-approved drugs alone or in combinations has uncovered ototoxic drugs that should be studied further in mammalian systems (Chiu et al. 2008; Hirose et al. 2011). For example, a recent study extended screening to herbal supplements and found that some popular flavonoids from Ginkgo biloba may be moderately ototoxic (Neveux et al. 2017). These screens of FDA-approved compounds revealed drugs that were known to be ototoxic or were suspected in isolated case reports. In addition, several drugs previously unrecognized as ototoxic were identified, including the antiprotozoal pentamidine and anticholinergic propantheline that were subsequently shown to be toxic to mammalian hair cells (Chiu et al. 2008). The chemotherapeutic sunitinib was not previously identified as ototoxic until screening in zebrafish (Hirose et al. 2011), but has been subsequently been implicated in patients (Dekeister et al. 2016). In addition, chemotherapeutic drugs with previous known ototoxicity were shown to have synergistic effects (Hirose et al. 2011), suggesting that hearing should be closely monitored with combination therapies.

To prevent drug-induced ototoxicity, particularly for therapeutic compounds, small molecule screens have also been conducted with the goal of hair cell protection. Depending on the design, one downside of the broad screens is that the mechanistic action of the compounds is unknown. In a screen conducted by Coffin et al. (2013b), a more focused approach was taken in selecting compounds with known intracellular targets that might confer protection against neomycin, gentamicin, or cisplatin exposure. Some compounds were protective against multiple insults; however, most of the hits were protective against only one or two of the toxins. For example, autophagy inhibitor 3-MA protected hair cells from both aminoglycosides and cisplatin, while an antioxidant, D-methionine, protected hair cells from gentamicin and cisplatin. Interestingly, caspase inhibition was not found to protect zebrafish hair cells, contrasting studies of inner ear hair cells in rodent and chick

(Cheng et al. 2005), suggesting that some mechanisms of cell death may not be conserved across taxa.

Both the genetic and small molecule screens highlight the multitude of mechanisms that contribute to hair cell susceptibility or protection, likely reflecting the multiple cell death pathways activated during drug-induced hair cell death. Recent exploration of aminoglycoside drug entry and trafficking has provided some additional insight into how different cell death mechanisms may be activated. Hailey et al. (2017) observed that entry was mediated both by mechanotransduction and endocytosis, consistent with many studies finding that toxicity is mechanotransduction dependent (as discussed above), and with a prior documentation of apical vesicle pools in hair cells that could mediate entry (Seiler and Nicolson 1999). Once entering hair cells, aminoglycosides accumulate diffusely in the cytoplasm and in discrete lysosomes, the latter of which is mediated by endocytosis. Interestingly, intracellular accumulation differs depending on the aminoglycoside used. While neomycin remains largely diffuse and more slowly accumulates into lysosomes, the opposite is true for gentamicin. Inhibition of vesicle budding (and thus endocytosis) reduces lysosomal accumulation for both compounds and increased gentamicin-induced cell death, but not neomycin-induced cell death. Alternatively, increasing lysosomal accumulation relative to the cytoplasmic accumulation reduced cell death. Together, these data suggest that lysosomal compartmentalization has a protective effect. Overall, they also support the idea that different aminoglycosides activate different cell death pathways (Owens et al. 2009; Coffin et al. 2013a).

The ultimate goal of studying the mechanisms of hair cell death and protection is to develop therapeutic strategies for preventing human hearing loss. In screening protective compounds, ideal candidate molecules must show protection (1) at low doses; (2) without interfering with the therapeutic function of the drugs; and (3) without causing hearing loss themselves. In progressing toward this goal, recent small molecule screens with zebrafish have been used to identify protective compounds that were also validated in mammalian systems. Starting with a zebrafish screen, Kenyon et al. (2017) identified 13 compounds that also protected hair cells in mammalian cochlear cultures from the aminoglycoside antibiotic gentamicin. This study focused on compounds that block mechanotransduction, preventing aminoglycoside entry. Another recent screen built on previous identification of the protective compound PROTO-1, this time conducting a new search with analogs synthesized to develop a structure-activity relationship (Owens et al. 2008; Chowdhury et al. 2018). Use of a new compound, ORC-13661, was validated in rats in vivo during co-administration with

the aminoglycoside antibiotic amikacin. With oral administration, ORC-13661 treatment provided significant hearing protection compared to rats treated with amikacin only, as determined via ABR testing. While taking different approaches, both studies highlight using zebrafish in progressing the development of otoprotectants that will alleviate hearing loss associated with therapeutic drug treatment. ORC-13661 has been approved for use in humans and is currently in clinical trials.

CONCLUSION

The zebrafish is a unique and convenient model for investigating the biology of genes, cells, and structures that share homology with the mammalian auditory system. Among other advantages, the surface location of the lateral line as well as the ease of genetic manipulation in zebrafish have facilitated important studies contributing to our understanding of human hereditary deafness as well as acquired hearing loss due to hair cell or neuronal toxicity. Moreover, the use of zebrafish in small molecule screens constitutes the first steps in developing therapeutic compounds to protect against cochlear damage. With the emergence of CRISPR and other technologies, zebrafish studies have the potential to significantly advance understanding of subcellular structures and events that contribute to hair cell function and disease.

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