Congenital Deafness and Recent Advances Towards Restoring Hearing Loss

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Congenital hearing loss is the most common birth defect, estimated to affect 2-3 in every 1000 births. Currently there is no cure for hearing loss. Treatment options are limited to hearing aids for mild and moderate cases, and cochlear implants for severe and profound hearing loss. Here we provide a literature overview of the environmental and genetic causes of congenital hearing loss, common animal models and methods used for hearing research, as well as recent advances towards developing therapies to treat congenital deafness.

Keywords: animal models • congenital deafness • gene therapy • hearing loss

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INTRODUCTION

The most common birth defect in the U.S. is congenital deafness, which ranges from 2 to 3 cases of clinically deaf infants for every 1000 births (CDC prevention 1999-2007). Congenital deafness can range from mild (26-40dB), to moderate (41-55dB), moderate-severe (56-71dB), severe (71-90dB), and profound (>91dB) (American speech-language-hearing association- website: *https://www.asha.org/public/hearing/Degree-of-Hearing-Loss/*). It can affect one or both ears. A recent study separating unilateral and bilateral hearing loss in children showed that the former accounts for 29% of the cases and the latter for the remaining 71% (van Beeck Calkoen et al., 2019). Studies estimated that the congenital permanent bilateral hearing loss is much rarer, with a prevalence of 1.2 per 1000 births (Zahnert, 2011) Surprisingly, 90% of deaf children have hearing parents (Mitchell & Karchmer, 2004). Congenital hearing loss affects children's speech and language acquisition as well as social and cognitive development. There are multiple causes for congenital hearing loss, and currently there are no cures. Cochlear implants and hearing aids together with speech therapy can help improve the quality of life and communication skills of hearing-impaired children. However, these are palliative approaches and do not restore hearing. In the best of cases, cochlear implants produce a sensation of hearing that can only mimic a fraction of the sounds that a healthy ear can perceive.

It has been estimated that 50% of congenital hearing loss is due to genetic causes and 50% to environmental factors (CDC prevention 1999-2007).

The environmental causes can be divided into different categories depending on their origins, which can be intrauterine infections,

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drugs, or trauma. Several pathogens have vertical transmission capacity, meaning that the maternal-fetal barrier does not protect the fetus from infection. The most common infections that can be transmitted to the fetus with a teratogenic effect are regrouped under the acronym TORCH (Toxoplasmosis–Others–Rubella–Cytomegalovirus–Herpes simplex). Among them, cytomegalovirus, rubella, herpes simplex, lymphocytic choriomeningitis, and Zika virus infection can lead to congenital hearing loss (Macedo-da-Silva, Marinho, Palmisano, & Rosa-Fernandes, 2020).

The genetic causes of congenital deafness can be divided in two subgroups: syndromic and non-syndromic. The former, which is associated with other symptoms, accounts for 30% of the cases of genetic origin, while in the latter, where deafness is the only symptom, accounts for 70% of cases (Lammens, Verhaert, Devriendt, Debruyne, & Desloovere, 2013; Smith, Bale, & White, 2005).

Here, we will review environmental and genetic causes of congenital hearing loss and the recent advances towards developing treatments, from basic science to the clinic. We will start with a brief overview of the animal models and methods used in studying congenital deafness.

ENVIRONMENTAL CAUSES OF CONGENITAL HEARING LOSS

Environmental causes of congenital hearing loss, more common in low-income and developing countries, are diverse and include viral, parasitic or bacterial infections, as well as drug and alcohol use during pregnancy.

Viral infections

The most common non-genetic cause of congenital deafness is an *in utero* infection with cytomegalovirus (CMV), which affects 0.6% to 0.7% of newborns in industrialized countries (Dollard, Grosse, & Ross, 2007).

Cytomegalovirus

In the U.S., it is estimated that congenital CMV infections affect roughly 26,500 newborns per year, with 10% to 15% of them presenting with symptoms at birth. Hearing loss related to congenital CMV infection can manifest at birth or develop over the 3 first years of life (Cohen, Durstenfeld, & Roehm, 2014). The earlier the infection occurs in fetal development, the higher the chances are to present with a symptomatic infection at birth as well as sensorineural hearing loss. Several studies report that the risk of *in utero* transmission is higher during the third trimester, even if this infection will lead to fewer consequences for the fetus (Enders, Daiminger, Bader, Exler, & Enders, 2011). Fetal CMV infection can be detected by ultrasound, although the gold standard to confirm it is an amniocentesis (Yinon et al., 2010). Despite its teratogen risk and its prevalence in developed as well as developing countries, there are no effective vaccines to prevent congenital CMV. Hopefully, there will be a CMV vaccine in the near future, as several vaccines are currently in clinical trials (Schleiss & Diamond, 2020).

The pathogenic mechanism of congenital CMV infection is not fully understood, but several human analyses and mouse studies suggest the involvement of the spiral ganglion neurons and the stria vascularis in SNHL (sensorineural hearing loss) (Fig. 1). In 2011, Lokensgard and his team described a neonatal mouse model of CMV infection that lead to SNHL with labyrinthitis in 100% of the mice. In this study, the virus was found in the perilymph (*scala tympani* and *scala vestibuli*) and the spiral ganglion neurons 7 days after infection. The SNHL was explained by the absence of sensory cells around 21 days post infection. The author concluded that the loss of hair cells was not due to the direct infection by the virus but by a secondary effect due to the inflammation of the inner ear (Schachtele, Mutnal, Schleiss, & Lokensgard, 2011). The same year, another team developed a model in which the mice were infected earlier (E12.5) during active neurogenesis (developing central nervous system). The DNA of the virus was detected again in the spiral ganglion neurons as shown in the other study. However, they also found the virus in the endolymph structures such as the stria vascularis and the spiral ligament (Juanjuan et al., 2011). More recently, several postnatal infection studies showed the same virus localization in the inner ear and demonstrated the degeneration of the vascularization of the stria vascularis and spiral ligament (Bradford et al., 2015; Carraro et al., 2016). These studies are more consistent with some temporal bone analyses of congenitally CMV-infected human samples in which the viral inclusion bodies were predominantly in the stria vascularis (Teissier, Bernard, Quesnel, & Van Den Abbeele, 2016). Another human analysis showed the absence of the virus in the organ of Corti but its presence near the Reissner's membrane and the dark cells of the semi-circular canal and utricle, which correspond to the vestibular analog of the stria vascularis. They also found

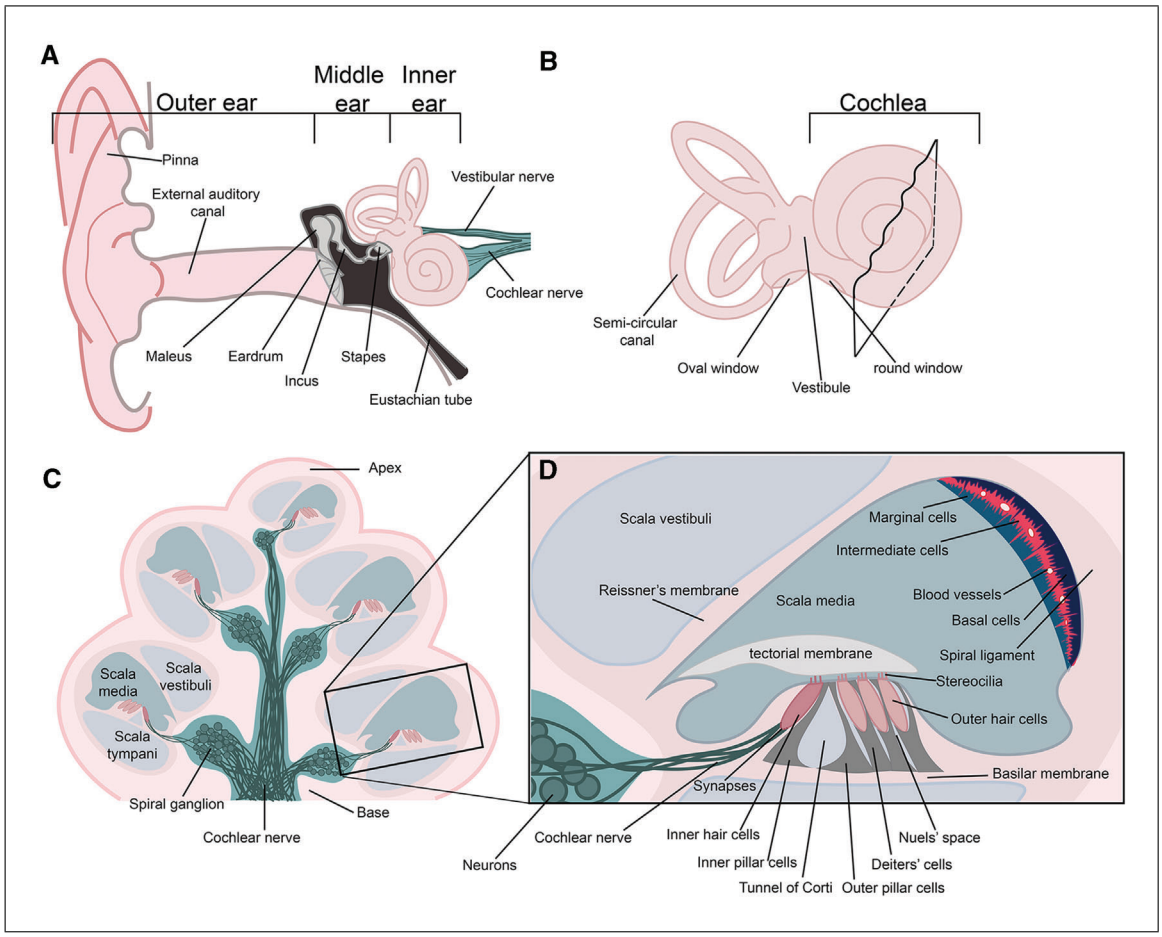

Figure 1 Anatomy of the ear. (**A**) The outer ear transmits sound vibrations to the middle ear through the external auditory canal. The bones in the middle ear transmit the pressure of the sound vibrations into the perilymph-filled *scala vestibuli* of the inner ear through the oval window. (**B**) The inner ear houses the sensory organs of the vestibular system responsible for detecting motion and acceleration, and the cochlea, which houses the auditory organ of Corti. (**C**) Section through the turns of the cochlea. (**D**) Magnification of one turn of the cochlea showing supporting cells, inner and outer hair cells of the organ of Corti, synapses of inner hair cells with the neurons of the VIIIth nerve (cochleovestibular ganglion), and marginal, intermediate, and basal cells of the Stria Vascularis.

that the stria vascularis seems hypervascularized, with enlarged blood vessels, in contrast to the mouse study, which demonstrated devascularization (Tsuprun, Keskin, Schleiss, Schachern, & Cureoglu, 2019). This year, a postnatal mouse study showed the rapid loss of pre-synaptic ribbons, which suggests a neurodegeneration as a mechanism of SNHL in congenital CMV infection (Almishaal et al., 2020). A defect in the stria vascularis could also explain the secondary loss of hair cells and the progressive SNHL in some infants.

CMV is a host-specific virus which does not replicate in non-human tissues. This species-specific virus needs cells of human origin to replicate, with the exception of the closest phylogenetic relative of humans, the chimpanzee, in which the skin fibroblasts are also permissive for human CMV (Perot, Walker, & Spaete, 1992). To overcome this problem, researchers have several options: use an *in vitro* human model, a CMV corresponding to the animal model, or a humanized mouse model in which human cells or tissue are engrafted. This last one is particularly important for the discovery of drugs (Bidanset, Rybak, Hartline, & Kern, 2004; Kern, 2006). To study the mechanism of pathogenesis of congenital CMV infection, researchers used guinea pig models and mouse models. The guinea pig model was particularly used for the vertical transmission rate of CMV, which is similar to the congenital infection rate of humans (Griffith & Hsiung, 1980). Contrary to the guinea pig, mouse CMV does not pass the mouse placental barrier to infect the fetus. To overcome this problem, several groups developed different approaches: an *in vitro* culture of the inner ear at E15.5 in a medium containing the virus (Melnick & Jaskoll, 2013),

or a placental injection in order to infect the fetuses of the mouse during pregnancy (Juanjuan et al., 2011). Finally, several studies showed that a brain injection of CMV in neonatal pups creates SNHL as well (Bradford et al., 2015; Carraro et al., 2016; Ikuta et al., 2015; Schachtele et al., 2011). This difference between the postnatally infected mouse that developed SNHL in contrast to human can be due to the fact that in the mouse, pups are born before developing hearing, as the inner ear is still in development, which is not the case in humans (Hastings & Brigande, 2020; Vollmer et al., 2004)

Rubella

Rubella is the leading vaccine-preventable cause of birth defects (WHO). In 2010, the number of children born worldwide with congenital rubella virus was estimated at more than 100,000 per year (Vynnycky et al., 2016). With the creation of vaccines, this risk is very low in vaccinated countries but congenital rubella is still considered a public health problem in other nations such as India (Harley et al., 2020). The infection of a pregnant mother does not always lead to congenital rubella syndrome. A fetus has 90% risk of presenting symptoms if the infection arises between 2 and 10 weeks of development. This risk drops drastically to 0% after 19 weeks (Lee & Bowden, 2000; Thompson, Simons, Badizadegan, Reef, & Cooper, 2016). Sensorineural hearing loss is among the most common sequelae of congenital rubella syndrome, varying between 38% and 100% depending on the study (Givens, Lee, Jones, & Ilstrup, 1993; Harley et al., 2020; Herini et al., 2017). Hearing loss can be present at birth, but most often is detected between 6 and 12 months of life (Cohen et al., 2014). Previous research showed that the proportion of bilateral hearing loss was seven times higher than unilateral hearing loss (Simons, Reef, Cooper, Zimmerman, & Thompson, 2016).

The pathophysiology of rubella virus is not well understood. The virus can infect any organ and create a non-lytic infection in fetuses. Several human studies showed change in the stria vascularis, such as dilation of the capillaries and atrophy of the stria vascularis with presence of lymphocytes and mononuclear cells in the intermediate cell layer. Most deaf newborns examined had a collapse of the cochlear duct and saccule (Brookhouser & Bordley, 1973; Gussen, 1981).

In terms of animal models, few options were studied in the past including mice, rats, rabbits, ferrets, and monkeys. Some researchers showed the development of cataracts, heart, and inner ear defects with a low incidence, while others did not show these defects. This low incidence of defects and their inconsistency between studies has limited the usefulness of these models (Webster, 1998). Today, with relative control of epidemics and an effective immunization, research on the mechanism of pathogenicity of the rubella virus has drastically decreased (Lee & Bowden, 2000).

Lymphocytic choriomeningitis virus (LCMV)

The incidence of LCMV on congenital birth defects is difficult to assess, mainly due to its resemblance to other congenital birth defects and the absence of prenatal diagnostic guidelines (Jamieson, Kourtis, Bell, & Rasmussen, 2006; Panchaud, Stojanov, Ammerdorffer, Vouga, & Baud, 2016). This rodent-borne zoonotic infection is mainly carried by the common mouse, and no horizontal transmission from human to human has been reported except via organ transplantation (Barton & Hyndman, 2000; Barton, Peters, & Ksiazek, 1995; Wright et al., 1997). Congenital infection with LCMV leads to up to 35% of mortality, and survivors present with defects in the development of the central nervous system if infected early in the developmental process (Wright et al., 1997). Several studies have reported that SNHL is not a common symptom of congenital LCMV infection, as its prevalence varies between 7% and 20% depending on the study (Anderson et al., 2014; Barton et al., 1995; Barton, Mets, & Beauchamp, 2002; Kinori, Schwartzstein, Zeid, Kurup, & Mets, 2018).

The pathological mechanism of LCMV has been studied mainly in the central nervous system (Bonthius, 2009; Bonthius & Perlman, 2007). Recent research with *in vitro* models revealed the absence of virus replication in term placental explants as opposed to first-trimester explants (Enninga & Theiler, 2019).

The rat has been shown as the animal model of choice for congenital LCMV study as it can reproduce the diverse neurological problems observed in humans (Bonthius & Perlman, 2007). These studies demonstrated the critical role of the age of the fetus at the time of infection for the outcome of the defects, with a tropism for mitotically active neuronal precursors (Bonthius, Nichols, Harb, Mahoney, & Karacay, 2007).

Herpes simplex

Genital herpes caused by HSV serotype 1 or 2 is one of the most prevalent sexually transmitted diseases worldwide (Cherpes, Matthews, & Maryak, 2012). The prevalence of HSV-1, most commonly causing labial herpes, was estimated to affect roughly 48% of the American population between 14 and 49 years old in 2015, while HSV-2, which commonly affects the genitals, was estimated to affect 12% of the population (Looker, Garnett, & Schmid, 2008; McQuillan, Kruszon-Moran, Flagg, & Paulose-Ram, 2018). Despite this virus being widely spread, the percentage of seroconversion during pregnancy is estimated at less than 2%, while in this group, less than 3% of the newborns will be contaminated by the virus (Brown et al., 1997). Several studies showed that either first infection or reactivation of the virus in pregnant women led to an extremely low risk of *in utero* transmission (Brown et al., 1997; Hutto et al., 1987). The majority of neonatal herpes cases are perinatally acquired (Cherpes et al., 2012). Regardless of the fact that *in utero* infection is extremely low, fetal infection leads to a poor outcome, with high morbidity and mortality (Hutto et al., 1987; Pichler et al., 2015). Most of the morbidity associated with fetal herpes infection comes from small case reports. Among the congenital symptoms, several researchers noticed the presence of hearing loss in 10% to 33% of the newborns (Dahle & McCollister, 1988; Hutto et al., 1987).

The physiopathology of congenital hearing loss due to herpes simplex infection is not known, but several studies in adult guinea pigs showed the deterioration of the stria vascularis and the organ of Corti. Histological analyses revealed a swollen stria vascularis and the disappearance of the sensory cells at the basal turn of the cochlea (Esaki et al., 2011; Stokroos, Albers, & Schirm, 1998, 1999; Stokroos, Albers, & Tenvergert, 1998).

Zika virus

A recent teratogenic infection, the Zika virus, was shown as a public health problem when the epidemic spread through South America in 2013. This mosquito-borne pathogen is able to pass through the placental barrier to infect the fetus and create developmental problems (Almeida et al., 2020; Cugola et al., 2016). The incidence of birth defects is difficult to assess with lack of access to the RT-PCR equipment necessary to rule out the infection in affected areas (Barbosa et al., 2020; Fandino-Cardenas, Idrovo, Velandia, Molina-Franky, & Alvarado-Socarras, 2019; Leal, Muniz, Caldas Neto, van der Linden, & Ramos, 2020). This problem could explain the wide range of hearing deficits assessed in different studies, which range from 0% to 68% of infected fetuses (Barbosa et al., 2020; Fandino-Cardenas et al., 2019; Satterfield-Nash et al., 2017). Another explanation could be the presence of different strains of ZIKV with different levels of aggressiveness (Russo, Jungmann, & Beltrao-Braga, 2017). Several vaccines and antiviral peptide therapies have been developed, and some vaccine candidates are currently in phase I of clinical trials to protect the population against the Zika virus (Camargos et al., 2019; De Lorenzo et al., 2020; Trovato, Sartorius, D'Apice, Manco, & De Berardinis, 2020).

The pathogenesis of ZIKV was investigated in different *in vitro* and animal models. A bioinformatics and *in vitro* study showed the activation of a proliferative and pro-apoptotic P-53 related response in human neural progenitor cells in response to ZIKV (Ghouzzi et al., 2016). Infection in adult mice revealed the presence of vacuolization in the stria vascularis, the dark cells of the vestibule, and the spiral ganglion (Yee, Neupane, Bai, & Vetter, 2020). *In utero* infection of mice led to hearing deficits in 25% to 66% of the offspring, with a greater deficit at higher frequencies (Julander et al., 2018). The study also suggests that the hearing loss did not involve damaged hair cells (Julander et al., 2018). Chicken embryo research where the virus was injected *in ovo* suggested that all regions of the inner ear epithelium are susceptible to ZIKV when the infection is contracted early enough (Thawani et al., 2020).

In terms of animal models, several types of immunocompetent or immunocompromised mice were analyzed. Neonatal infection or intrauterine injection of the virus can be performed in wild-type (WT) mice, as the virus will be able to replicate (Shi et al., 2018). But in case of adult mice, immunocompromised models are used, as WT mice are resistant to ZIKV infection. Some studies also showed that the virus cannot pass the placental barrier in C57BL/6 mice, which is not the case for SJL strain (Cugola et al., 2016). The AG129 mouse strain was widely used, as it lacks both alpha/beta and gamma interferon receptors which removes a key component of the antiviral immunity (Julander et al., 2018; Lazear et al., 2016; McDonald, Anderson, Wilusz, Ebel, & Brault, 2020). For a review of the

different mouse and non-human primate models developed for the study of Zika virus, please refer to Morrison and Diamond minireview (Morrison & Diamond, 2017).

Parasitic infections

Toxoplasmosis

Toxoplasma gondii is an intracellular protozoan parasite that can infect any warm-blooded animal. This common parasitic zoonoses uses the cat as a host. The seroprevalence, reflecting the exposure of a population, varies greatly by country and increases with age (Wilking, Thamm, Stark, Aebischer, & Seeber, 2016). This prevalence is relatively low in countries such as Norway, the U.K., and the U.S.A. (7%-10%), and high in countries like France, Germany, and Brazil (40%-50%) (Pappas, Roussos, & Falagas, 2009; Pleyer, Gross, Schluter, Wilking, & Seeber, 2019). A German study estimated the risk of seroconversion, which corresponds to the first infection, to be 1% during pregnancy (Wilking et al., 2016). An infection by T. *gondii* often goes unnoticed, as only 5% of infected pregnant women present with symptoms (Dunn et al., 1999). The risk of vertical transmission is evolving from 6% at the beginning of pregnancy to up to 72% at 36 weeks (Dunn et al., 1999). Between 2003 and 2004, the rate of positive newborn infection was evaluated at 0.6 per 1000 living births (de Andrade et al., 2008). Depending on the study, the risk of deafness varies between 0% and 27% (de Andrade et al., 2008; Fontes et al., 2019). The low rate of SNHL in certain studies is attributed to successful treatment started less than 2.5 months after birth (Brown, Chau, Atashband, Westerberg, & Kozak, 2009; McGee et al., 1992; Vos, Senterre, Lagasse, SurdiScreen, & Leveque, 2015).

The mechanism of pathogenesis of SNHL by *T. gondii* is not known, and the lower rate of SNHL in treated children decreases the need for research. In 2013, a temporal bone study performed on deceased newborns showed Toxoplasmosis cysts of the parasite in the stria vascularis, spiral ligament, and saccular macula. The researchers concluded that the cysts may be a dormant cyst form as the presence of the cyst was not associated with tissue necrosis or inflammation (Salviz, Montoya, Nadol, & Santos, 2013).

Although the parasite is able to infect mice, no research regarding the physiopathology of toxoplasmosis infection on hearing has been done so far.

Bacterial infections

Treponema pallidum

While congenital syphilis is often noted as a risk factor for congenital hearing loss, recent analyses of congenital syphilis showed no cases of congenital hearing loss associated with *in utero* infection (Chau, Atashband, Chang, Westerberg, & Kozak, 2009; Gleich, Urbina, & Pincus, 1994; Vos et al., 2015). Some previous studies pointed to congenital syphilis as a cause of congenital deafness, but the physiopathology is not clear (Kerr, Smyth, & Cinnamond, 1973; Yune, Miyamoto, & Yune, 1991). A major factor for this modification is the introduction of penicillin as a treatment that alleviated the morbidity associated with the fetal infection (Chau et al., 2009).

Alcohol and opioids

While alcohol consumption during pregnancy is the most preventable cause of birth defect, its prevalence reaches 7 per 1000 births in the United State (Gentile, Cowan, & Dixon, 2019; Lange et al., 2017). The most drastic consequences of alcohol consumption, called fetal alcohol syndrome, was estimated at 1 birth in every 500 to 1000 births in Australia (Elliott, Payne, Morris, Haan, & Bower, 2008). While some studies showed only a trend toward association between prenatal alcohol exposure and hearing defects (Yoshida, Wilunda, Kimura, Takeuchi, & Kawakami, 2018), a meta-analysis showed a prevalence of 56% for conductive hearing loss and 57% for SNHL in children suffering from fetal alcohol syndrome versus the general population in the U.S.A. (Popova et al., 2016). Although the pathophysiology associated with hearing loss is not known, a study showed an incidence of 19% in rats suffering from fetal alcohol syndrome, confirming the association between alcohol and hearing defects (Church, 1987). Alcohol toxicity in the formation of the first and second branchial arch during embryogenesis has been suggested as an explanation of the SNHL and conductive hearing loss (Church & Gerkin, 1988).

Studies analyzing the consequences of cocaine or opioid consumption during pregnancy showed no significant increase in SNHL in children from cocaine or opioid-abusing mothers (Carzoli, Murphy, Hammer-Knisely, & Houy, 1991; Grimmer, Buhrer, Aust, & Obladen, 1999).

Neonatal complications

Jaundice is a common perinatal problem created when the bilirubin contained in red blood cells is present in excess amounts in the blood due to a temporary lack of clearance by the liver. This hyperbilirubinemia that occurs during the first week after birth is present in 60% to 84% of newborns and usually clears on its own (Bhutani et al., 2013; Okhravi, Tarvij Eslami, Hushyar Ahmadi, Nassirian, & Najibpour, 2015). However, 7% of newborns require a phototherapy treatment or in severe cases an exchange transfusion to avoid neurological injury. Unfortunately, a sensorineural hearing loss is seen in 12% of those newborns (Nunez-Batalla, Jaudenes-Casaubon, Sequi-Canet, Vivanco-Allende, & Zubicaray-Ugarteche, 2019; Xu et al., 2019).

The pathophysiology of hyperbilirubinemia includes an auditory pathway problem with normal peripheral function of the ear such as non-affected hair cells (Belal, 1975; Shaia, Shapiro, & Spencer, 2005). Studies have showed increase in ABR (Auditory Brainstem Response) latencies and interpeak intervals in hyperbilirubinemia animal models (Shapiro & Hecox, 1989; Teixeira, Borges, Riesgo, & Sleifer, 2020). This phenomenon can be explained by the degenerative appearance of the spiral ganglion, where the neurons demonstrate decreased size and a lack of myelinated axons (Shaia et al., 2005). Toxicity occurs when the increased concentration of bilirubin in the blood is superior to the albumin binding capacity. This unconjugated bilirubin diffuses across the blood-brain barrier and the cell membrane to create neuroinflammation (Okhravi et al., 2015; Olds & Oghalai, 2015; Zucker, Goessling, & Hoppin, 1999). Previous studies highlight the production of oxidative stress and endoplasmic-reticulum stress (ER-stress) as a cause of the neuronal toxicity (Schiavon, Smalley, Newton, Greig, & Forsythe, 2018; Vodret et al., 2018).

Different animal models have been used to understand the mechanisms by which hyperbilirubinemia induces neuronal toxicity and hearing loss. Among them, the Gunn rat model has the advantage of a missing uridine diphosphate-glucuronosyltransferase enzyme, and so presents an elevated concentration of circulating bilirubin through the rat's lifespan (Belal, 1975; Schutta & Johnson, 1967; Shaia et al., 2005). Different teams also studied acute hyperbilirubinemia by administration of sulfadimethoxine to reduce serum binding (Waddell, He, Tang, Rizzuto, & Bearer, 2020). A mouse model of inactive glucuronosyltransferase 1a1 enzyme ($Ugt1^{-/-}$) was also developed. In this mouse model, the phenotype is more drastic; a jaundice develops soon after birth and newborns die a couple days later due to the bilirubin neurotoxicity (Bortolussi et al., 2012). Intraperitoneal injection of bilirubin and sulfadimethoxine was also used in wild-type mice to induce hyperbilirubinemia (Schiavon et al., 2018). Those studies not only allowed the comprehension of the pathogenicity of hyperbilirubinemia, but also the development of potential treatment to reduce the risk of bilirubin-induced neurological damage such as hearing loss. So far, several neuroprotective and anti-inflammatory compounds have been tested on those animal models to find adjuvant therapy to improve the protocol already in place (Vodret et al., 2018).

Another neonatal complication that can lead to hearing loss is intraventricular hemorrhage. This pathology usually develops during the first week of life of extremely preterm newborns. The prevalence is calculated at 16% of newborns under 28 weeks, showing an increased risk with decrease in gestational age and weight (Robinson, 2012). While the precise cause is not yet perfectly understood, the immaturity of the central nervous system and the alteration of cerebral blood flow in preterm babies can lead to intraventricular hemorrhage (Tan, Svrckova, Cowan, Chong, & Mankad, 2018). In 9% of the cases, the newborn will develop hearing loss as a long-term consequence of the intraventricular hemorrhage (Patra, Wilson-Costello, Taylor, Mercuri-Minich, & Hack, 2006).

While environmental causes account for 50% of congenital deafness cases, most of them can be prevented by vaccination (rubella), awareness of the adverse effects (alcohol), or early treatment (*T. gondii*, hyperbilirubinemia). Those interventions drastically decrease the prevalence of congenital deafness. While research has led to the development of vaccines and treatments to avoid the consequences of fetal infections, new viruses can emerge at any time, as was shown in 2013 in South America during the Zika virus outbreak or in 2020 worldwide with SARS-CoV-2 (coronavirus, COVID-19). Because research on COVID-19 is too recent and still ongoing, there is insufficient information to know the risk of vertical transmission and congenital hearing loss in infected pregnant women. Laboratory tests and newborn hearing screenings should be administered at birth as a precaution and to ensure early intervention if needed (Juan et al., 2020).

GENETIC CAUSES OF CONGENITAL HEARING LOSS

Over the past century, more than 180 deafness-associated genes have been identified and studied (Hereditary Hearing Loss Homepage, October 2020; see Internet Resources). Genetic deafness can be classified according to the pathological mechanism leading to hearing loss. The genetic causes of deafness can be a ciliopathy, a neuropathy, a synaptopathy, a neurocristopathy, or a homeostasis disorder. Ciliopathy is a category of diseases caused by disruption of the physiological functions of cilia. Cochlear ciliopathies are a group of diseases that affect the development or maintenance of the mechanosensory stereocilia bundles present at the apical surface of the sensory hair cells essential for hearing. While stereocilia are not cilia per se, these actin-based structures require the hair cell primary cilium—the kinocilium—for correct development and orientation (Pollock & McDermott, 2015). Auditory neuropathies refer to a problem in the transmission of sound from the cochlea to the brain. More specifically, this problem arises downstream of the hair cells and is caused by disruption of the nerve impulses traveling from the spiral ganglion neurons to the brain. Auditory synaptopathies, a pathologies just upstream of the auditory neuropathy, come from an impairment in the inner hair cell ribbon synapses (Moser & Starr, 2016). Problems in inner ear homeostasis are also well known to cause hearing loss. Cellular junctions, ion channels, and their regulators play an essential role in the specific composition of the endolymph required for hearing. Finally, a neurocristopathy is characterized by a problem in the development of the neural crest cells. For hearing, the neural crest cells contribute to a significant amount of ear structure such as the cartilage of the pinna, the ossicles, and the glial and intermediate cells of the cochlea (Ritter & Martin, 2019). All of those pathologies can be inherited recessively or dominantly to create congenital or progressive hearing loss.

Syndromic deafness

Syndromic deafness is characterized by being associated with other symptoms. Those symptoms can affect a wide variety of organs such as eyes, skin, kidneys, and heart. Here we will discuss the five most common causes of syndromic congenital hearing loss. Please refer to Table 1 for a detailed list of the mutations that cause these types of syndromic deafness.

Usher syndrome

Usher syndrome is an autosomal recessive ciliopathy divided in three categories based on the severity of the symptoms as well as the ages of onset. Usher type 1 is characterized by a congenital profound deafness, a vestibular dysfunction, and a progressive vision loss starting during the first decade of life. Usher syndrome type 2 is less severe, with a mild to moderate congenital deafness and the onset of blindness after puberty. Usher syndrome type 3 is rare and features post-lingual progressive hearing loss and blindness starting during adulthood. In 2010, the prevalence of Usher syndrome was estimated at up to 9% of congenital deafness (Kimberling et al., 2010). For Usher type1, *MYO7A* (Myosin 7a) was determined as the most frequent gene implicated (53%), for which half of the mutations were predicted to lead to truncated proteins (Le Quesne Stabej et al., 2012). The other genes implicated in Usher type 1 are *USH1C*, *CDH23*, *PCDH15*, *USH1G*, and *CIB2*. For the second type of Usher which lead to congenital hearing loss as well, *USH2A* was determined as the gene responsible in 79.3% of the cases (Le Quesne Stabej et al., 2012). The other genes implicated in Usher syndrome type 2 are *VLGR1* (also called GPR98) and *WHRN*. A recent meta-analysis calculated the frequency of those genes in all Usher patients. The study showed that *USH2A* coding for Usherin contributes to 50% of all Usher cases and *MYO7A* for 21% (French, Mellough, Chen, & Carvalho, 2020; Jouret et al., 2019).

As presented above, Usher syndrome is a heterogeneous genetic disorder for which 10 genes have already been discovered so far (French et al., 2020). Each gene encodes a protein expressed in the hair cells of the organ of Corti. While their roles are different, each of the Usher proteins participate in the formation and maintenance of the hair bundle or the synapse ribbon, two specialized structures of the mechanosensory hair cells. Mouse models were used to better understand the role played by each Usher protein; some of them listed are in Table 1. Those models allowed researchers to notice a common phenotype of abnormal hair bundle shape characteristic of sensory cells (Lefevre et al., 2008; Mburu et al., 2003; McGee et al., 2006; Michel et al., 2017). Extensive reviews regarding the genetics and mechanisms of Usher syndrome are available (El-Amraoui & Petit, 2014; French et al., 2020; Geleoc & El-Amraoui, 2020; Toms, Pagarkar, & Moosajee, 2020).

Table 1 Syndromic Congenital Hearing Loss with Mouse Models

Pendred	Pendrin	SLC26A4 KO	Pds$^{-/-}$ or Slc26a4$^{-/-}$	Everett et al. (2001)
	Pendrin	SLC26A4c.919-2A.G	Slc26a4$^{tm1Dontuh/tm1Dontuh}$	Y. C. Lu (2011)
	Pendrin	SLC26A4 p.H723R	Slc26a4$^{tm1Dontuh/tm2Dontuh}$	Y. C. Lu (2014)
	Pendrin	SLC26A4 hH723R	mPDS$^{-/-}$	Choi et al. (2020)
	Pendrin	SLC26A4 L236P	Slc26a4^{L236P}	Wen et al. (2019)
	Pendrin	SLC26A4 p.S408F	Slc26a4 $^{loop/loop}$	Dror & Avraham (2010)
	Pendrin	SLC26A4 conditional KO	Slc26a4$^{\Delta/\Delta}$	Choi et al. (2011)
Usher Syndrome				
type 1	Myosin 7a	MYO7A	*Shacker-1* or Myo7a^{Sh}	Gibson et al. (1995)
type 1	Harmonin	USH1C	*Deaf circler* or *dfcr* or Ush1c^{dfcr}	Johnson et al. (2003)
type 1	Cadherin 23	CDH23	*Waltzer* or Cdh23^{v}	Di Palma et al. (2001); Wilson et al. (2001)
type 1	Protocadherin 15	PCDH 15	*Ames waltzer* or Pcdh15$^{av\text{-}TgN2742Rpw}$	Alagramam et al. (1999)
type 1	Sans	USH1G	*Jackson shaker* or Ush1g$^{Js/Js}$	Kikkawa et al. (2003)
type 2	Usherin	USH2A	Ush2a$^{-/-}$	Liu et al. (2007)
type 2	Vlgr1	GPR98	VLGR1^{delTM}	McMillan & White (2004)
type 2	Whirlin	WHRN	Whrn$^{wi/wi}$	Mburu et al. (2003)
type 2	Cib2	CIB2	CIB2$^{-/-}$	Michel et al. (2017)
Jervell & Lange-Nielsen				
		KCNE1	Isk$^{-/-}$	Vetter et al. (1996)
			Kcne1^{pkr}	Letts et al. (2000)
		KCNQ1	Kcnq1$^{-/-}$	Casimiro et al. (2001)
Waardenburg		EDNRB exon 2-3 deletion	WS4	
		EDNRB exon 3 deletion	Ednrb$^{-/-}$	
		EDNRB KO	*Pieldbal-lethal* or Ednrb$^{s\text{-}l}$	Hosoda et al. (1994)
		EDN3	*Lethal spotting* Edn3^{ls}	Baynash et al. (1994)
		MITF Dominant mutation	Mi^{Wh}	Steingrímsson et al. (1994)
		MITF Recessif form	Mi^{Vi}	Steingrímsson et al. (1994)
		SNAI2	*Snai2^{del1}*	Jiang et al. (1998)
		PAX3	Pax3$^{Cre/Cre}$	Kim et al. (2014)
B-O-R				
		EYA1	Eya1$^{-/-}$	Xu et al. (1999)
		EYA1	Eya1 ^{Bor}	Johnson et al. (1999)
		SIX1	Six1 $^{cwe/cwe}$ (catweasel)	Bosman et al (2009)

Pendred syndrome

Pendred syndrome is characterized by hearing loss, a dilated vestibular aqueduct, and the formation of a goiter (enlargement of the thyroid gland) that usually develops during puberty (Cremers et al., 1998). The symptoms can vary between patients from congenital to late onset, hearing loss, and from a simple vestibular aqueduct enlargement to a cochlea malformation called a Mondini deformity, which adds an incomplete partitioning of the apical portion of the cochlea to the enlargement. In addition, the thyroid is also often affected with hypothyroidism, even if some rare Pendred patients already present hyperthyroidism (Kusano, 2020). This syndrome is created by a mutation in a protein called pendrin, encoded by the *SLC26A4* gene (Everett et al., 1997). More than 200 different mutations of the *SLC26A4* gene have been found in humans so far (*https://databases.lovd.nl/shared/genes/SLC26A4*). The prevalence of Pendred syndrome varies from country to country, while the predominant mutations causing the disease also show ethnic and geographical differences. As an example, Pendred syndrome accounts for 5% of all prelingual hearing loss in East Asia (Park et al., 2003). While most patients with Pendred syndrome present a mutation in either or both alleles of *SLC26A4* gene, a few do not. Mutations in the cis-regulatory elements critical for *SLC26A4* expression as well as mutations in the transcription factors necessary for its activation can also lead to Pendred syndrome (Yang et al., 2007).

Extensive studies have tried to understand the physiopathology of Pendred syndrome in the ear. In adult mice, pendrin is located in the apical membrane of the endolymphatic sac and duct, the external sulcus associated with long projections into the spiral ligament, as well as the vestibular transitional cells in the utricle and saccule, but not in the dark cells (Royaux et al., 2003; Yoshino et al., 2004). A complete knockout of pendrin in mice revealed a degenerated organ of Corti, irregular shape of the marginal cells of the stria vascularis, and a decrease in the expression of the potassium channel *KCNJ10*. The endolymphatic dilatation is visible by E15.5, but the authors conclude that the malformation is probably a consequence of a progressive deterioration and swelling of the near-mature endolymph compartment rather than an arrest in the development of the cochlea (Everett et al., 2001). Electrophysiological studies of the cochlea showed a normal potassium concentration, but the abolishment of the endocochlear potential (Royaux et al., 2003). The acidification of the endolymph and an increased concentration in calcium were also noted (Wangemann et al., 2007). Researchers hypothesized that the absence of pendrin, which might secrete HCO_3^- in the endolymph, leads to an alkalinization of the intrastrial space, which would enhance free radical and oxidative stress. The presence of free radicals and the acidification of the endolymph would result in a reduction of *KCNJ10* expression, essential for the generation of endocochlear potential and therefore normal hearing (Wangemann et al., 2004; Yang et al., 2009). For a comprehensive review about the role of pendrin in inner ear development as well as on mouse models, see Wangemann (2013). While these studies showed the physiopathology of the absence of pendrin, most human mutations lead to amino acid substitutions or frame shift mutations. To understand the molecular mechanism and cellular defects created by these mutations, several teams studied them *in vitro*. Most of the prevalent mutations in pendrin create a retention of the protein in the intracellular region instead of the protein reaching the plasma membrane. This retention happens mainly in the endoplasmic reticulum, which recently became the target for developing a therapy for this syndrome (Rotman-Pikielny et al., 2002; Yoon et al., 2015).

In terms of mouse models for pendrin, several have been developed over time. The first presents a total absence of pendrin, which leads to profound hearing loss and vestibular problems, but does not reflect the phenotype in some of the less severely affected patients. For this reason, other researchers developed point mutations corresponding to mutations found in Pendred patients such as *H723R*, the most common among Asian populations (Park et al., 2003). Surprisingly, this mutation in the mouse did not lead to hearing loss (Choi et al., 2020). In consequence, the team decided to study this point mutation in the human sequence of pendrin, which leads to a less severe hearing loss than the complete knockout, more closely reflecting the human disease (Adam et al., 1998).

Waardenburg syndrome

Waardenburg syndrome was previously estimated to account for between 2% and 5.38% of all congenital hearing loss, but a recent study stated that the prevalence is much higher than previously reported and can reach up

to 9%, affecting 1 in every 10,000 newborns (Nayak & Isaacson, 2003; Tamayo et al., 2008; Zaman, Capper, & Baddoo, 2015). Waardenburg syndrome is a neurocristopathy, and can be divided into four categories based on clinical symptoms. Waardenburg syndrome type 2 is characterized by hearing loss and pigmentation abnormalities. Waardenburg syndrome type 1 adds dystopia canthorum to the two previous symptoms, while type 3 adds musculoskeletal abnormalities of the upper limbs to the first three symptoms. Waardenburg syndrome type 4, also called Shah-Waardenburg, is characterized by hearing loss and pigmentation abnormalities associated with Hirschsprung's disease, which correspond to the absence of innervation of the intestine leading to an aganglionic megacolon (Pingault et al., 2010). A meta-analysis performed in 2015 showed that Waardenburg type 1 accounts for almost 50% of the Waardenburg population, type 2 accounts for 32.6%, type 3 for only 1.7%, and type 4 for 18.9%. The study also showed that hearing loss is present in 71% of the patients (Song et al., 2015). This syndrome is genetically heterogeneous, where six pathogenic genes are known, *PAX3* (type 1 and 3), *SOX10* (type 2 and 4), *EDNRB* (type 2 and 4), *ET3* (type 2 and 4), *MITF* (type 2), and SNAI2 (type 2) (Attie et al., 1995; Edery et al., 1996; Pingault et al., 1998; Sanchez-Martin et al., 2002; Tassabehji et al., 1992; Tassabehji, Newton, & Read, 1994). The penetrance and phenotypes can be widely variable between patients even in the same family (Somashekar et al., 2019). An extensive review of mutations causing Waardenburg syndrome was made 10 years ago (Pingault et al., 2010). Other neurocristopathies are known to affect hearing, and are presented in a recent review by Donna Martin's lab (Ritter & Martin, 2019).

The association of hearing loss and hypopigmentation that defines Waardenburg syndrome is the consequence of a defect in the development of neural crest cells of melanocytic fates. The genes involved in Waardenburg syndrome are all implicated in the development of neural crest cells and their derivatives. Several studies about lineage tracing and gene deletion have allowed researchers to link the syndrome with the disappearance of the melanocytic cells in the skin and the stria vascularis of the cochlea (Matsushima et al., 2002; Motohashi, Hozawa, Oshima, Takeuchi, & Takasaka, 1994). The intermediate cells, originating from the neural crest cells that migrate in the stria vascularis, are essential for the formation of the endocochlear potential, a key component of hearing (Steel & Barkway, 1989).

Several mouse models have helped the comprehension of Waardenburg syndrome, and each gene implicated was analyzed in mice, showing the participation of recessive and dominant mutations (Table 1). Several mutations in one gene have been shown to lead to different phenotypes, as shown for MITF (Steingrímsson et al., 1994). The specific deletion of *EDNRB* expression in neural crest cells confirmed that Waardenburg phenotype arises from developmental neural crest cell defects (Druckenbrod, Powers, Bartley, Walker, & Epstein, 2008).

Branchio-oto-renal (BOR) syndrome

As is the case for several rare syndromes, the precise prevalence of BOR syndrome is still unknown. Two estimates made by Fraser in 1976 and 1980 suggest that BOR accounts for 0.15% or 2% of all congenital deafness. The real number is probably in this range of prevalence (Coppage & Smith, 1995). Another obstacle to determining the real prevalence is the overlap of symptoms with several other syndromes such as otofacio-cervical syndrome and Townes-Brocks syndrome (Engels, Kohlhase, & McGaughran, 2000; Morisada, Nozu, & Iijima, 2014). The most common phenotypes of BOR syndrome are hearing loss (92%-98%), second branchial arch anomalies (such as branchial cleft cyst palpable in the neck, 50%-68%), preauricular pits (53%-83%), and renal anomalies such as renal hypoplasia (38%-40%) (Chang et al., 2004; Morisada et al., 2014). The deafness can be conductive (20%), SNHL (30%), or mixed (40%), and some studies have also found progressive hearing loss in non-congenitally deaf patients (Morisada et al., 2014; Song et al., 2013). The most common gene affected in BOR patients is *EYA1* (Abdelhak et al., 1997). Two other genes from the SIX family were discovered later to be causes of BOR syndrome (Hoskins et al., 2007; Ruf et al., 2004). Most of those mutations are unique to individuals' families (Song et al., 2013). The effects of *EYA1* and *SIX1* mutations were studied in different mouse models. The *EYA1* and *SIX1* genes are expressed early in development, before the formation of the ear. At E9, *EYA1* and *SIX1* are expressed in the ventral region of the otic vesicle. Later, *SIX1* is expressed in the region that will give rise to the stria vascularis and the future prosensory domain until the cell exits the cell cycle. In the fully differentiated

organ of Corti, *SIX1* is also expressed in the hair cells, which could explain the progressive hearing loss observed in some patients (Zheng et al., 2003). In a non-functional Eya1 or Six1 protein mouse model, the heterozygous mice present abnormal sound conduction due to morphological abnormalities in the ossicles of the middle ear (Xu et al., 1999; Zheng et al., 2003). The homozygous mutant for Eya1 dies at birth but shows open eyelids, severe skeletal and craniofacial defects, no kidneys, preauricular pits, malformed ossicles, and no inner ear structure or acoustic ganglion (Xu et al., 1999). Another study with an hypomorphic mutation of Eya1 manifests a less severe phenotype. Histological analyses revealed a reduced cochlea, a quarter of the normal size, the presence of a spiral ligament but without an associated stria vascularis, and a completely absent organ of Corti (Johnson et al., 1999). A mouse model with a hypomorphic mutation for Six1, named Catweasel, was used to the study of the implication of Six1 in ear development. The heterozygous mutant presents a less severe phenotype, with the absence of the eminentium cruciation running between the middle of the posterior crista sensory patch and additional inner hair cells along the cochlea. The homozygous mutant has a truncated cochlea with no sensory cells, similar to the Eya1$^{-/-}$ mutant. The ossicles are normal except for the incus, which does not present the long process. The lateral semi-circular canal and the oval and round windows are absent (Bosman, Quint, Fuchs, Hrabe de Angelis, & Steel, 2009). The otic vesicle formed but failed to develop further, creating a severely reduced otic vesicle (Laclef, Souil, Demignon, & Maire, 2003; Zheng et al., 2003). Other research highlights the increase of cell death present in both homozygous mice otic vesicles as the reason for the small inner ear structure (Xu et al., 1999; Zheng et al., 2003).

Jervell and Lange Nielsen syndrome

Jervell and Lange-Nielsen syndrome is a rare autosomal recessive syndrome characterized by sensorineural deafness and a prolonged QT interval on the electrocardiogram, leading to recurrent syncope and tachyarrhythmia (Schulze-Bahr et al., 1997). Its prevalence is estimated at 1 in every 200,000 children under 10 years old in Sweden (Winbo et al., 2012). This prevalence might be underestimated because of the high mortality rate due to cardiac arrest from ventricular tachyarrhythmias of untreated patients (Winbo et al., 2012). This syndrome originates from mutations in two specific genes: *KCNQ1* and *KCNE1* (previously named KvLQT1, KV7.1 or KCNA8 and LQTS, Mink or Isk, respectively). While mutations in *KCNE1* are associated with a lower risk of cardiac events compared to *KCNQ1*, *KCNQ1* accounts for 90% of the patients and *KCNE1* accounts for the remaining 10%. (Schwartz et al., 2006).

In terms of physiopathology, *KCNQ1* encodes a potassium channel that can form heteromultimers with Kcne1 to create a delayed-rectifier potassium channel where Kcne1 is a regulatory subunit (Strutz-Seebohm et al., 2011). This heteromultimeric KCNE1/KNCQ1 channel plays an essential role in the endolymph production, as seen in the mouse model *KCNE1* knockout (KO), where the endolymph compartment is entirely collapsed due to the collapse of the Reissner membrane at P3 (before the onset of hearing in mouse). The hair cells and the spiral ganglion degenerate later (Vetter et al., 1996). KCNE1 is localized at the apex of the marginal cells of the stria vascularis and the dark cells of the vestibule (Nicolas, Dememes, Martin, Kupershmidt, & Barhanin, 2001; Sakagami et al., 1991). KCNQ1 is also localized at the apex of the marginal cells (Chang, Wang et al., 2015). A study of *KNCQ1* KO also showed the collapse of the Reissner's membrane, but the volume shrinkage of the endolymphatic space was less important than in the *KCNE1* mutant (Casimiro et al., 2001). Interestingly, a patient with an intronic mutation in a splice regulatory site of *KCNQ1* which led to only 10% of functional *KCNQ1* transcript showed no hearing defect. This homozygous patient indicates that 10% of the functional KCNQ1 channel was able to maintain normal hearing, but was insufficient for the heart where a long QT was still observed (Bhuiyan et al., 2008).

Non-syndromic

Non-syndromic hearing loss patients present no symptoms other than deafness. For the most part, autosomal dominant non-syndromic hearing loss starts after speech acquisition in the first to fourth decade of life (Van Laer, McGuirt, Yang, Smith, & Van Camp, 1999). In contrast, autosomal recessive non-syndromic hearing loss is congenital or pre-lingual, and profound with few exceptions (Sundstrom, Van Laer, Van Camp, & Smith, 1999). The non-syndromic deafness pathologies can be classified by their locus names, which correspond to the abbreviation DFNA for autosomal dominant and DFNB for autosomal recessive, followed by a number that

corresponds to their discovery order (Table 2). As some genes can possess a recessive and a dominant variant, some DFNA correspond to the same position on the chromosome as DNFB; this is the case for DFNB1 and DFNA3, as presented later. To date, more than 180 loci associated with hearing loss have been mapped, but only 121 deafness-associated genes are known so far (Hereditary Hearing Loss Homepage, October 2020; see Internet Resources). In this section, the most common non-syndromic congenital hearing loss will be discussed.

GJB2 or Connexin 26

The most common non-syndromic hearing loss is caused by mutations in the protein named connexin 26 (Kelley et al., 1999). Mutations in the *GJB2* gene can cause recessive or dominant hearing loss depending on the mutation present in the locus DFNB1A or DFNA3, respectively, as they both map to the same region of chromosome 13 (Kelsell et al., 1997). A meta-analysis performed in 2013 assessed a worldwide prevalence of autosomal recessive congenital hearing loss due to *GJB2* mutations at 16.9% (Chan & Chang, 2014). The prevalence was the highest in Europe with 27.1% and the lowest in sub-Saharan Africa (5.6%). Different pathogenic variants were overrepresented in different populations, such as 35delG in Europe, 235delC in East Asia, or W24X in India, showing possible founder mutations (Chan & Chang, 2014). A recent study in Argentina showed that 38% of non-syndromic hearing loss patients present a mutation in *GJB2* or *GJB6* (Buonfiglio et al., 2020). Many genetic alterations of *GJB2* that lead to deafness have been described so far; a recent review reports the differences between these variants (Del Castillo & Del Castillo, 2017).

Connexin 26 can assemble with itself or other connexins to create a channel called a connexon. Two homo- or heteromeric connexons on adjacent cells can create a gap-junction that allows intercellular communication between the two cells, where ions and small molecules can pass. A rat study showed that the connexin 26 protein is expressed in the supporting cells of the organ of Corti, as well as in the inner sulcus and border cells, while on the lateral wall of the cochlea the protein is expressed in the fibroblasts, intermediate cells, and basal cells of the stria vascularis. This expression in the cochlea creates two gap junction systems, namely the epithelial and connective tissue gap junction systems (Kikuchi, Kimura, Paul, & Adams, 1995). A human temporal bone study showed a similar localization of the protein (Kelsell et al., 1997). The deletion of connexin 26 in the cochlea allows further analyses of the mechanism of pathogenesis associated with *GJB2* mutations. In this study, researchers showed the absence of hearing in the adult mice, a lower EP (endocochlear potential), and potassium concentration in the endolymph, as well as the death of hair cells in the organ of Corti (Cohen-Salmon et al., 2002). They hypothesized that the mechanism of hearing loss came from a local accumulation of potassium under the sensory cells that could lead to their death by oxidative stress (Cohen-Salmon et al., 2002). The important role of gap junctions in potassium recycling was already established, and this theory became largely accepted (Kikuchi, Adams, Miyabe, So, & Kobayashi, 2000; Spicer & Schulte, 1996). While the underlying mechanism of deafness is still unknown today, several results are in contradiction with this previously accepted hypothesis. One of the first conflicting pieces of evidence was the presence of no significant cell loss in the organ of Corti or the spiral ganglion after the onset of hearing, even if the mice presented profound congenital deafness (Liang, Zhu, Zong, Lu, & Zhao, 2012). Later, another study showed that the reduction of EP observed in the absence of connexin 26 was not significant enough to account for the deafness. Furthermore, the deletion of connexin 26 before P4 led to a collapsed tunnel of Corti, while deletion later than P6 did not lead to deafness after the onset of hearing (Chen, Chen, Zhu, Liang, & Zhao, 2014). The early absence of connexin 26 also leads to immature synapses at the hair cells. This finding was not sufficient to explain the deafness, but confirmed a developmental problem in the cochlea in the absence of connexin 26 (Chang, Tang, Kim, & Lin, 2015). Finally, the absence of developmental defects in a mouse model without connexin 30 raises the possibility that connexin 26 is necessary for intercellular genetic communications through miRNA for proper development of the organ of Corti (Zhu, Zong, Mei, & Zhao, 2015). Recently, a study showed a relation between malformed pillar cells and hearing loss in connexin 26−deficient mice (Xie et al., 2019). More recently, an *in vitro* study showed that autosomal mutations in *GJB2* lead to a loss of function, and that connexin 26 is not colocalized with connexin 30. In contrast, autosomal dominant mutations of connexin 26 interact with the other connexins and could

Table 2 Non-Syndromic Congenital Hearing Loss with Their Locus Classified by Cause of Deafness

	LOCUS	GENE	Protein	Mouse model	References
Homeostasis					
	DNFB1	GJB2	Connexin 26	Cx26 $^{flox/flox}$	Cohen-Salmon et al. (2002)
	DFNA3	GJB2 or GJB6	Connexin 26 and 30	$Cx26^{R75W}$	Inoshita et al. (2008)
	DNFB4	SCL26A4	Prendrin	$mPDS^{-/-}$	Choi et al. (2020)
	DFNB39	HGF	Hgf	*Hgf* $^{Fl/Fl}$	Schultz et al. (2009)
				Hgf ^{del10Neo} and Hgf ^{del10}	Morell et al. (2020)
Synaptopathies					
	DFNB9	OTOF	Otoferlin	$Otof^{-/-}$	Roux et al. (2006)
				Otof $^{I515T/I515T}$	Strenzke et al. (2016)
				Otof $^{C2C/C2C}$	Michalski et al. (2017)
Ciliopathies					
	DNFB2	MYO7A	Myosin 7a	Shaker-1	Gibson et al. (1995)
	DNFB3	MYO15	Myosin 15	Shaker-2	Probst et al. (1998)
	DNFB6	TMIE	Tmie	$Tmie^{sr/sr}$	Mitchem et al. (2002)
	DFNB7/11	TMC1	Tmc1	*deafness* or Tmc1 $^{dn/dn}$	Kurima et al. (2002)
	DFNB12	CDH 23	Cadherin 23	*Waltzer*	Di Palma et al. (2001); Wilson et al. (2001)
	DFNB18	USH1C	Harmonin	*Deaf circler* or $Ush1c^{dfcr}$	Johnson et al. (2003)
	DFNB23	PCDH15	protocadherin 15	*Ames Waltzer* or $Pcdh15^{av\text{-}TgN2742Rpw}$	Alagramam et al. (1999)
	DFNB28	TRIOBP	Triobp	$Triobp^{\Delta ex8/\Delta ex8}$	Kitajiri et al. (2010)
	DFNB29	CLDN14	Claudin 14	$Cldn14^{-/-}$	Ben-Yousef et al. (2003)
	DFNB30	MYO3A	Myosin 3A	$Myo3a^{KI/KI}$	Walsh et al. (2011)
	DFNB31	WHRL	Whirlin	$Whrn^{wi/wi}$	Mburu et al. (2003)
	DFNB36	ESPN	Espin	*Jerker or* $Espn^{Je}$	Sekerková, Richter, & Bartles (2011)
	DFNB37	MYO6	Myosin 6	Snell's waltzer or $Myo6^{sv}$	Avraham et al. (1995)
Others					
	DNFB21	TECTA	alpha-tectorin	*Tecta-Cre*	Babola, Kersbergen, Wang, & Bergles (2020)
	DFNA8/12	TECTA	alpha-tectorin	$Tecta^{C1509G/+}$	Xia et al. (2010)

explain transdominant properties (Beach et al., 2020).

SLC26A4 or pendrin

A study performed in France showed that in the Caucasian population, mutation in *SLC26A4* can account for up to 4% of non-syndromic deafness in children, making this gene the second most frequent cause of non-syndromic deafness (Albert et al., 2006). The physiopathology of *SLC26A4* mutation was already explained in the discussion of Pendred syndrome earlier in this review, as this gene can lead to Pendred syndrome as well as *DFNB4*.

OTOF or Otoferlin

Another important contributor to recessive congenital hearing loss is the gene *OTOF* (Locus DFNB9). Different studies showed that the prevalence of *OTOF* pathogenic mutations account for 2.3% to 7.3% of recessive pre-lingual hearing loss, depending on the region of the world studied (Choi et al., 2009; Duman, Sirmaci, Cengiz, Ozdag, & Tekin, 2011; Iwasa et al., 2019; Migliosi et al., 2002). Surprisingly, in Saudi Arabia, this gene is accountable for 33% of sensorineural hearing loss (Almontashiri et al., 2018). Most patients affected present pre-lingual severe to profound hearing loss (Iwasa et al., 2013). The gene *OTOF* contains 28 coding exons and encodes multiple long and short isoforms (Yasunaga et al., 1999). A study of an affected family showed that the long isoforms are required for hearing function (Yasunaga et al., 2000). The pathogenic mutations are widely dispersed among the gene, and the majority of them are unique to one family. Some recurrent variants have been discovered in different ethnic populations. Among the most recurrent mutations, we can find Q829X in Spanish populations and p.R1939Q in Japanese populations (Iwasa et al., 2013; Migliosi et al., 2002).

Several studies unraveled the role of otoferlin in the hearing process. Immunolabeling on mouse tissue showed the expression of otoferlin in the sensory hair cells of the organ of Corti as early as E16. The spiral ganglion is also reactive for otoferlin antibody for a short window of time between P2 and P7. While the labeling persists at adult stages in the inner hair cells, the outer hair cells stop expressing otoferlin after P6, which corresponds to the disappearance of the afferent synaptic contacts present on the outer hair cells (Roux et al., 2006). In inner hair cells, the protein is located at the basolateral region of the hair cells, associated with the synaptic vesicles. The study of a mouse model deficient for otoferlin highlights the absence of hearing of those mice at every frequency, with the proper function of the outer hair cells shown by *DPOAE*. Due to the defects in the auditory pathway, but not in outer hair cells function, this auditory synaptopathy was classified as an auditory neuropathy (Starr, Picton, Sininger, Hood, & Berlin, 1996). Histological studies demonstrated the normal morphology of the hair cells and their ribbon synapses, even if their numbers were lower in the homozygous knockout. The research team concluded that otoferlin is necessary for the synaptic exocytosis of the neurotransmitter between the hair cells and the neurons of the spiral ganglion (Roux et al., 2006). For proper sound coding, the inner hair cells afferent synapses are able to transmit the sound into nerve impulse at a high rate and with a high degree of precision. For this specific purpose, the basolateral region of the cell contains several tethered vesicles facing a single ribbon synapse (Nouvian, Beutner, Parsons, & Moser, 2006). Recent research focused on the specific role of otoferlin in those ribbon synapses. They showed that the protein functions as a calcium sensor, triggering the synaptic vesicles' fusion with the presynaptic membrane as well as vesicle pool replenishment at the inner hair cells' ribbon synapse (Hams, Padmanarayana, Qiu, & Johnson, 2017; Michalski et al., 2017; Takago, Oshima-Takago, & Moser, 2018). More recently, sheep were used to create a large-mammal animal model of otoferlin deficiency-related hearing loss, to allow the study of possible therapies (Menchaca et al., 2020).

Congenital deafness, whether syndromic or not, should be determined as early as possible to ensure the best possible outcome. In cases of syndromic hearing loss, other organs could be affected, and an appropriate treatment could be lifesaving, such as for Jervell-Lange-Nielsen syndrome where the patient is at high risk of cardiac arrest (Winbo et al., 2012). In addition, it has been well established that delays in treatment can lead to delays in speech as well as cognition. Another example of the importance of early diagnosis is the possibility of progressive worsening of hearing or the appearance of other symptoms such as blindness in Usher syndrome. Early diagnosis allows for comprehensive counseling with the parents and the ability to address vision and balance issues early in the patient's life. Finally, with the recent advances in development

of the hereditary hearing loss panel (HHLP) assay, which analyzes 166 genes related to both non-syndromic and syndromic hearing loss, the possibility of early identification of the gene affected could become an accessible reality (Butz et al., 2020).

ANIMAL MODELS

In choosing an animal model to study congenital deafness, several parameters have to be considered such as the developmental timing, host-specific diseases, or possible vertical transmission during fetal development. These parameters will influence the experimental design depending on the outcome desired, such as the comprehension of the mechanism of progression or the discovery of a drug that targets the disease studied. In this perspective, it is important to note that there are dramatic developmental differences between maturation of the human auditory system and that of other mammals. For this reason, it is often necessary to extrapolate the developmental stage to study congenital disease and treatment in an animal model. For a review about the developmental differences between mouse and human ear development as well as the translation of mouse to human therapies, please refer to this recent review (Hastings & Brigande, 2020).

Zebrafish

The zebrafish, *Danio rerio*, has become a popular alternative animal model for hearing research in the last two decades (Yang, Sun, Chen, Li, & Chen, 2017). To study the sensory cells, the zebrafish provides several advantages as an animal model. First, it possesses hair cells in the inner ear and the superficial lateral line, which allows easy physical access to the hair cells. Second, it develops rapidly within a few days after external fertilization, and the hair cells mature in a day (Kindt, Finch, & Nicolson, 2012; Yang et al., 2017). This allows for rapid performance of experiments and quick data generation. Third, many transgenic fish lines are already available, and the genetic expression of fluorescent constructs can be easily attained. Moreover, the transparent embryos and larvae make it an excellent model for *in vivo* imaging (Kindt & Sheets, 2018; Pickett & Raible, 2019). Finally, the small size and sociability of this small fish makes it easy to maintain in a small facility, which is an advantage for high-throughput drug screening and mutagenesis (Vona, Doll, Hofrichter, Haaf, & Varshney, 2020). But this model also contains some drawbacks, such as an increased evolutionary distance to humans in comparison to other mammals such as rodents (Yang et al., 2017). The lateral line's sensory cells possess physiological properties similar to immature mammalian vestibular organs, but are different from mature mouse cochlear hair cells (Olt, Johnson, & Marcotti, 2014). And finally, while this fish is a powerful model for drug screening, the fact that it lives underwater makes the testing of chemicals with low solubility in water problematic (Cassar et al., 2020). In conclusion, the zebrafish is a great animal model for soluble drug compound screening for ototoxicity or *in vivo* imaging for hair cell mechanotransduction and synaptopathy.

Chick

The chick model organism is the most attractive animal model to construct gene regulatory networks. Its embryology is very well described and similar to human development (Streit et al., 2013). The chick embryo has a larger size and is easily accessible, making this model an attractive organism for experimental gene manipulation and *in vivo* imaging to study dynamic biological processes and developmental processes such as cell fate decisions. The otocyst (inner ear precursor) is fairly easily accessible for surgical manipulation, and leads to a good embryo survival rate (Seal, Lilian, Popratiloff, Hirsch, & Peusner, 2019). Eggs can be produced in large numbers, with the production of one egg per day per chicken, allowing large-scale experiments. Another advantage of the chick model is that, unlike mammals, hair cells can regenerate, which has allowed for the identification of pathways for regeneration (Saunders, 2010). However, the chick brings some concerns such as the morphological differences between the basilar papilla and the organ of Corti, and the long generation time for sexual maturity (6 months). Recently, researchers highlight the Japanese quail as an attractive alternative to the chicken model. The quail present several advantages over the chick such as a smaller size and an accelerated sexual maturity of 6 weeks, which is shorter than the mouse (8 weeks) and the zebrafish (12 weeks) presented in this section (Serralbo et al., 2020).

Rodents

Rodents have become the most widely used animal model for hearing research over the last decade (Ohlemiller, Jones, & Johnson, 2016), as the anatomy and physiology of the inner ear and central auditory system are similar between rodents and humans. Small rodents

allow a relatively short gestation period—as short as 3 weeks—and maturity in 3 months. Moreover, they can generate several litters per year, making this a cost-efficient animal model (Bowl & Dawson, 2015; Escabi, Frye, Trevino, & Lobarinas, 2019). In comparison to the zebrafish and the chick, rodents share more similarities with humans, as about 99% of mouse genes have human orthologs (Brown, Hardisty-Hughes, & Mburu, 2008). Different rodents such as the mouse, the rat, and the guinea pig have different advantages. The mouse model offers a large variety of genetic mutants, which can save time and money. Genetically identical inbred strains are also a valuable advantage to identify gene function (Bryda, 2013). However, like other model organisms, mice have several drawbacks, such as their low sociability in comparison to the rat and guinea pig and the existence of several mouse strains that show progressive hearing loss as early as 2 months (Holt, Kuhl, Braun, & Altschuler, 2019; Turner, Parrish, Hughes, Toth, & Caspary, 2005). The early onset of hearing loss in these models creates a very narrow window in which to investigate therapeutics. The rat is a good compromise between the high sociability of the guinea pig and the large genetic mutation repertoire already available in the mouse (Escabi et al., 2019). Their hearing frequency, ranging from 520 Hz to 70 KHz, is also closer to the human range in the low frequencies, as mouse hearing ranges from 2 KHz to 85 KHz, while the human is between 2 Hz and 20 KHz (Holt et al., 2019). On the other hand, the rat is particularly susceptible to otitis media, which impacts hearing analysis (Albuquerque, Rossato, Oliveira, & Hyppolito, 2009). Finally, the guinea pig is appreciated for its ease of handling, learning attributes, and larger size, which makes surgery easier (Albuquerque et al., 2009; Escabi et al., 2019). The guinea pig also allows for vertical transmission of maternal infections such as CMV, unlike the mouse model.

METHODS USED TO STUDY HEARING LOSS

To understand the hearing process and the physiopathology present in deafness models, several histological and electrophysiological methods have been developed. While histological studies cannot detect whether hearing is functional, these methods have been used routinely to understand the physiopathology underlying deafness. Surface electron microscopy and immunohistochemistry are common methods used to study the proper development of hair cells and their stereocilia. To study the electrophysiology of hearing, several methods have been developed. Here we will discuss the more widely used ones.

ASR: Acoustic startle reflex

This method, based on the motor response after an animal hears a brief loud sound, is effective in identifying profound sensorineural hearing loss (also see Current Protocols article: Falls, 2002). This non-invasive method is simple and can be used in different species. In the rodent, this reflex can be analyzed by a full body movement or by a small ear movement, called a Preyer reflex (Jero, Tseng, Mhatre, & Lalwani, 2001). In the zebrafish, the reflex is assessed by a curvature of the body followed by a quick acceleration of the small fish (Henry et al., 2009). The disadvantage of this test is that it is subjective, reflects only the better ear, and is insensitive to less severe hearing loss (Jero et al., 2001). Recently, to overcome the subjectivity of this test, a team developed an automated classification of acoustic startle reflex waveforms using a machine learning method (Fawcett, Cooper, Longenecker, & Walton, 2020).

ABR: Auditory brainstem response

ABR is one of the most commonly used objective electrophysiological measures of hearing (also see Current Protocols article: Willott, 2006). It is a non-invasive method to assess the health of the auditory pathway from the cochlear nerves to the brainstem (Jewett & Williston, 1971). This method is used in laboratory rodents, and it is also used in the clinic to screen newborns, infants, and adults for hearing loss. Electrodes are placed on the scalp of the subject to record electrical activity from the cochleovestibular nerve to the brainstem after an acoustic stimulus. The reading produces a 7-wave graph (5 waves in rodents), where each wave represents the function and latency of a different portion of the auditory pathway (Young, Cornejo, & Spinner, 2020). Each ear can be tested independently. In rodents, the measurement requires anesthesia, but it is often not required in human tests. In addition to help diagnose hearing loss, ABR are also used to detect schwannomas and brainstem lesions (Biacabe, Chevallier, Avan, & Bonfils, 2001).

EP: Endocochlear potential

A +80 mV endocochlear potential in the *scala media* is essential for cochlear

amplification. This provides part of the voltage source needed to drive the outer hair cells (OHC) transducer current, which leads to OHC electromechanical force (Wang, Fallah, & Olson, 2019). Determination of the EP is an invasive terminal procedure in laboratory rodents. The surgery required is difficult to establish in a lab, as it requires specific materials and a very delicate and precise procedure. It involves carefully placing reading electrodes in the *scala media* of the inner ear to record voltage. Because the electrode has to puncture the lateral wall of the cochlea, there is a risk of endolymph leaking giving inaccurate readings (Bosher & Warren, 1971). EP measurements are not a readout of hearing, but rather a readout of endolymph properties, which are necessary for the function of the hair cells. Reduced or absent EP due to defects in the potassium transport or in the stria vascularis can lead to severe hearing loss and/or congenital deafness.

DPOAE: Distortion product otoacoustic emission

This measurement is based on the fact that the cochlear amplifier (outer hair cells) responds to sound by creating a vibration along the basilar membrane. To assess the DPOAE, two pure tones of close frequencies (f1 and f2) are generated simultaneously. The stimulation of the cochlea at two positions close to each other leads to the production of a third tone at a lower frequency known (2f1-f2) that can be recorded (Avan, Buki, & Petit, 2013). When DPOAEs are present and normal in amplitude and configuration, this indicates that the cochlear amplifier (i.e., OHC motility) is normally functional. In the absence of neurological or isolated inner hair cell dysfunction, this result is consistent with normal hearing. When DPOAEs are absent, this indicates that there is some dysfunction in the cochlea, though the level of dysfunction and, thus, degree of the hearing loss is not clear (Abdala, 2001). DPOAE measurement is now part of standard hearing loss screening in newborns, and for infants and adults it does not require sedation. In the lab, DPOAE is measured in rodents under anesthesia.

RECENT DEVELOPMENTS TOWARD CONGENITAL HEARING LOSS TREATMENTS

Currently, there are no cures for hearing loss whether it is congenital or acquired. The available options for people suffering hearing loss consist of hearing aids for mild cases and cochlear implants for more profound to severe cases, provided that the patient is a good candidate to receive the implant. Although the technology for hearing devices has advanced rapidly in the recent past, neither the sound amplification from hearing aids nor the stimulation of the auditory nerve by electrodes of the cochlear implant can compare to natural hearing. In the best-case scenario, they provide a sensation of hearing that allows patients to develop almost normal speech patterns when implemented prior to language acquisition age in cases of congenital deafness.

Over the last decade, next generation sequencing (NGS) has allowed the identification of multiple deafness related genes and pathways. Together with recently developed molecular biology tools, this has opened the field to the development of hearing loss therapies that include gene delivery and editing, stem cell transplantation, and targeted drug delivery systems. These tools and therapies are summarized in Table 3.

Gene therapy

Gene therapy, or the use of genetic material to treat diseases, has gained a lot of traction in hearing loss research due to recent successes in rescuing deafness caused by different gene defects in animal models. The key factors for success are a delivery route that will be minimally invasive and relatively specific, and the optimization of vectors that carry the genetic material.

Some of the early studies done in this area delivered either adeno-associated viruses (AAV) or lentiviruses (LV) carrying GFP (green fluorescent protein) into the otocyst of mouse embryos *in utero*. These experiments targeted hair cells and supporting cell progenitors in the prosensory region of the ear. While GFP expression from AAV labeled inner hair cells, in outer hair cells and supporting cells GFP expression was relatively low. In comparison, GFP from lentiviruses labeled a higher number of outer hair cells, but the animals showed a significant decrease in hearing thresholds, raising concerns about ototoxicity (Bedrosian et al., 2006). Since then, optimization of AAV vectors has allowed for a much higher efficiency using this delivery method, both in the cochlea and in the vestibular sensory organ (Hu et al., 2020).

Another commonly used delivery route is injection through the round window membrane of the inner ear that connects to the perilymph in the *scala tympani* and *scala vestibuli*.

Table 3 Recent Developments Towards Congenital Hearing Loss Treatment

Intratympanic		Oishi et al. (2013)
Round window		Pietola et al. (2008)
Post semi-circular canal		Suzuki et al. (2017)
Cochleostomy		Akil et al. (2012); Chang et al. (2015); Wang, Kempton, & Brigande (2018); Wang et al. (2013)
Intra-amniotic		Depreux et al. (2016); Wang et al. (2020)
Gene therapy	Adeno-associated viruses	Al-Moyed et al. (2019); Askew et al. (2015); Chang et al. (2015); Gyorgy et al. (2019); Landegger et al. (2017); Nist-Lund et al. (2019); Tan et al. (2019)
	Lentivirus	Bedrosian et al. (2006)
Gene editing	Cas9-sgRNA	Gao et al. (2018)
	Antisense oligonucleotides	Depreux et al. (2016)
	Short interfering RNA	Oishi et al. (2013)
Drug treatments	BF844	Alagramam et al. (2016)
	Salicylate	Ishihara et al. (2010)
	Rapamycin	Fujioka et al. (2020)
	Cell therapy	Nabeyama et al. (2017)
Cell therapy	Human stem cell	M. Y. Lee et al. (2017); Chen et al. (2018)

Although this is probably the least invasive of the delivery routes, early studies of GFP delivery via this method, using either LV or AAV, resulted mainly in expression in marginal cells of the stria vascularis and cells lining the perilymph ducts, but not in the organ of Corti or the spiral ganglion (Pietola et al., 2008). Recently developed AAV vectors with modifications that allow the virus to cross the mesothelial membranes that separate the perilymph from the organ of Corti have resulted in more efficient gene transfer in supporting cells, inner hair cells, and outer hair cells of the cochlea (Landegger et al., 2017; Tan et al., 2019).

A third delivery method consists of a cochleostomy, a small incision in the lateral wall of the cochlea to inject the viral vector directly into the *scala media*. This method has been used extensively with relative success because the viral particles can come in direct contact with hair cells and supporting cells in the *scala media* (Akil et al., 2012; Chang, Wang et al., 2015; Wang et al., 2013; Wang, Kempton, & Brigande, 2018). However, the procedure is invasive, and the surgery itself can cause hearing loss if the tissues are damaged.

The first report of a successful use of gene therapy to restore hearing came from a study using a mouse model of hereditary deafness. Autosomal dominant DFNA25 is caused by a missense mutation in the gene that encodes VGLUT3, a vesicular glutamate transporter that is expressed by inner hair cells. VGLUT is an essential component for synaptic transmission between inner hair cells and the afferent nerve synapse. VGLUT3 KO mice are deaf and show a reduction of about 20% in spiral ganglion neurons. By injecting an AAV1 carrying a functional copy of the *VGLUT* gene, the authors were able to confirm expression of the gene in inner hair cells, which restored hearing to normal levels in these mice within 7-14 days after injection. Both the cochleostomy and the round window membrane routes were tested, with a more efficient gene transfer achieved with the latter. Although hearing thresholds were restored, as measured by ABR (auditory brainstem response), there was no increase in the number of spiral ganglion neurons (Akil et al., 2012).

A similar approach was taken to rescue mouse models of DFNB7/11 and DFNA36, which have autosomal recessive and dominant deafness, respectively. These congenital deafnesses are produced by mutations in the Tmc (transmembrane channel-like) proteins present in the tips of stereocilia and necessary for mechanotransduction of both inner and outer hair cells. Mice lacking functional Tmc proteins lack sensory transduction, are deaf, and suffer balance dysfunctions although their hair cells appear normal. After injection of an AAV2/-Cba-Tmc construct, the authors of the study reported modest gains in hearing thresholds, but the virus failed to transfect outer hair cells (Askew et al., 2015). A few years later, the same group experimented with a modified synthetic adenovirus (sAAV-Tmc1) which not only transfected inner but also outer hair cells. In addition to restoring hearing to almost normal levels in $Tmc^{-/-}$ mice, the virus injection also rescued balance dysfunctions caused by lack of Tmc in hair cells of the vestibular organs (Nist-Lund et al., 2019).

One of the main challenges in the use of AAVs for gene therapy is their limited capacity in terms of the DNA that can be packed in the virus, about 5 KB in size. The otoferlin gene, whose mutation is responsible for DFNB9 deafness, is about 6 KB, and therefore too large to transfect with adenoviruses or to efficiently transfect hair cells with lentiviruses or via electroporation. Over one thousand pathogenic mutations have been identified within this gene, affecting between 2% and 10% of patients suffering from hereditary non-syndromic hearing loss. The product of this gene, otoferlin, is expressed in inner hair cells, and is required postnatally for exocytosis, vesicle replenishment, vesicle reformation, and endocytosis. Inner hair cells in $Otof^{-/-}$ mice have a 40% reduction in the number of synaptic ribbons. To circumvent the limited capacity of adenoviruses, Al-Moyed et al. (2019) designed two sets of dual AAV half-vectors each containing one half of the otoferlin coding sequence and splicing donor and acceptor sequences, respectively. When co-injected through the round window, these vectors were able to efficiently transfect inner hair cells, and to a lesser extent outer hair cells. The two halves of the coding sequence formed a full-length transcript that was detected by PCR. In $Otof^{-/-}$ mice, injection at P6 produced normal otoferlin protein at about 30% of the level of wild-type mice. This was sufficient to partially rescue hearing, with ABR thresholds between 40 and 60 dB (normal wild-type thresholds are 30±5, and complete deafness is >80 dB). The injections, however, did not restore the number of synaptic ribbons (Al-Moyed et al., 2019).

Three separate studies have recently reported hearing rescue using gene therapy in mouse models of Usher syndrome. The first study used a mouse model of Usher syndrome type 1, in which a point mutation of the harmonin gene (*USH1c*) recapitulates the human syndrome causing progressive hearing loss and deafness. Injecting a synthetic adenovirus containing a functional copy of harmonin through the round window, the authors were able to efficiently transfect both inner and outer hair cells. This resulted in a functional recovery of sensory transduction and rescue of hearing loss as measured by ABRs (Pan et al., 2017). In the second study, the authors generated a mouse model of Usher syndrome III that recapitulates the progressive hearing loss seen in this syndrome, where expression of the gene clarin-1 is dramatically down-regulated postnatally, causing the mice to be profoundly deaf by P21-P25. By injecting AAV2 or AAV containing a full-length copy of the clarin-1 gene through the round window, the authors were able to preserve hearing in these mice through adulthood, in contrast to control mice in which hearing loss progressed after P2 (Geng et al., 2017). The third study used a $Clnr1^{-/-}$ mouse model, which do not recapitulate the progressive hearing loss observed in Usher syndrome III patients, but rather presents hair-cell defects and deafness from birth. In this case, the hearing rescue was achieved by P1 injection of an AAV9 containing a functional copy of the clarin-1 gene. This vector has the additional advantage of transfecting inner and outer hair cells not only in mice, but also in non-human primates, which makes it a promising vector for translation into clinical trials (Gyorgy et al., 2019).

In addition to the examples above where gene therapy was successfully used to rescue hearing defects caused by mutations in sensory hair cells, there has been some success in treating other types of syndromic deafness associated with non-sensory cell types in the cochlea. Mutations in the potassium channel subunit KCNQ1 in humans cause Jervell and Lange-Nielsen syndrome, which is characterized by severe congenital deafness. This channel is expressed in the marginal cells of the stria vascularis, and is necessary for the

generation of endolymph and endocochlear potential. In its absence, the *scala media* collapses, resulting in the subsequent death of cells in the auditory organ of Corti. Injection of a modified AAV containing a functional copy of the *Kcqn1* gene directly into the *scala media* of Kcqn1$^{-/-}$ mice resulted in efficient transfection of marginal cells and a complete rescue of hearing thresholds. Although the injection also resulted in ectopic expression of the protein in other cell types in the cochlea, this did not have a detrimental effect on the hearing rescue (Chang, Wang et al., 2015).

Gene editing

A dominant negative missense mutation in the TMC1 channel in hair cells causes progressive sensorineural hearing loss in humans. The Beethoven mutation in the mouse *Tmc1* gene (*Tmc1*$^{Bth/+}$) causes a similar phenotype, with elevated hearing thresholds and inner hair cell loss followed by outer hair cell death starting at 1 month of age. Gao et al. (2018) designed small guide RNAs (sgRNA) that target the missense mutation in the Bth allele of the *Tmc* gene. They first tested the efficiency of several sgRNAs by transfecting fibroblasts derived from *Tmc1*$^{Bth/Bth}$ mice *ex-vivo* using cationic lipids to deliver a Cas9-sgRNA complex into the cells. They identified a complex, Cas9-Tmc1-mut3, which yielded the best results. They then injected the lipid complex containing Cas9-Tmc1-mut3 directly into the *scala media* of *Tmc1*$^{Bth/+}$ mice at P1. The injection resulted in enhanced survival of both inner and outer hair cells compared to un-injected ears. Four weeks after injection, treated animals showed a significantly lower ABR threshold than controls, suggesting that the Cas9-Tmc1-mut3-lipd complexes were effective in reducing progressive hearing loss. This is the first example of *in vivo* gene editing to correct a missense mutation in the inner ear (Gao et al., 2018).

Antisense oligonucleotides (ASO) are short modified nucleic acids that can bind to specific RNA sequences. Because of their favorable pharmacokinetics and toxicological properties, they are ideal candidate gene-editing tools for drug therapeutics. Intra-amniotic injection of an ASO that was meant to target other organs resulted in a significant down-regulation of the targeted gene in the inner ear (Depreux et al., 2016). This result motivated researchers to use this method to deliver a specific ASO that had been shown in neonatal mice to correct a missense mutation that produces an ectopic splicing site in the harmonin gene in a mouse model of Usher syndrome (Lentz et al., 2020). The embryonic intra-amniotic injection resulted in a more efficient rescue of the hair cell phenotype than was observed in intraperitoneal neonatal injections of ASO; however, hearing and balance improvement were not significant. The same group later modified their injection, and instead of delivering ASO systemically through the amniotic cavity, they targeted the inner ear by injecting ASO directly into the inner ear cavity of E12.5 mouse embryos. Delivery of ASO into the inner ear improved the efficiency of the mutant gene editing in hair cells as well as hair cell morphology in both the cochlea and the vestibular organs, and rescued hearing and balance phenotypes into adulthood (Wang et al., 2020).

Drug treatments

The combination of advances in mouse and cell-based models, high-throughput screening, and identification of deafness-related genes has allowed the development of novel and known drugs to treat some types of congenital deafness. A high-throughput screening of over 50,000 molecules identified several compounds that were able to stabilize and rescue the proper localization of a mutant variant of the *CLARIN* gene in HEK293 cells. This mutation is one of the causes of Usher syndrome type III in humans. The candidate molecules were analyzed in a secondary screen for toxicity and pharmacokinetics, and this led to the identification of a compound that was later modified chemically to enhance its potency and physicochemical properties. In further cell studies, this compound, BF844, was found to act post-translationally together with chaperone proteins to stabilize the mutant protein and properly localize it to the plasma membrane. When tested in a mouse model of Usher syndrome III, BF844 was able to stop progressive hearing loss and lower hearing thresholds compared to control animals (Alagramam et al., 2016). A similar approach was taken to identify molecules that could correct the improper localization of several mutant forms of pendrin. Pendrin is the product of the *SLC26A4* gene, responsible for Pendred syndrome, the most common form of syndromic deafness. In a stable HEK293 cell line expressing the pendrin mutant P123S, researchers tested the efficiency of 20 salicylate derivatives chemically modified to direct the proper localization of the mutant protein. They had previously shown that salicylate can act as a molecular chaperone to rescue pendrin

folding mutants (Ishihara et al., 2010). These experiments led to the identification of a small molecule with low cytotoxicity that was able to rescue the misfolding and localization of pendrin to the plasma membrane (Nabeyama, Ishihara, Ban, Wada, & Nakamura, 2017). Another study focusing on Pendred syndrome generated cochlear epithelial cells from induced pluripotent stems cells derived from Pendred patients (Hosoya et al., 2017). This approach allowed the study of mutant forms of human *SLC26A4* and circumvented the fact that there are no good animal models for Pendred syndrome. The authors found aggregates of the mutant protein that failed to localize in the plasma membrane and instead associated with the ubiquitin proteasome system in the cytoplasm of the cells. The intracellular aggregation of the mutant protein led to cell death, and this could be ameliorated by activation of autophagy. Treatment of the cells with rapamycin, a well known activator of autophagy, enhanced cell survival and decreased the number of activated caspase 3 positive cells. Because rapamycin is a well-known drug currently used in other therapeutic applications, this study opened the door to a Phase I/II trial for the treatment of Pendred syndrome, which is currently ongoing (Fujioka et al., 2020).

CONCLUDING REMARKS

Congenital deafness is the most common birth defect. It affects 2-3 in every 1000 births. Currently there are no effective treatments for congenital or acquired hearing loss. Causes of congenital deafness are varied and can be environmental or genetic. Determination of the cause of congenital deafness in individual cases will guide the preferential treatment for the best outcome. Development of animal models for each particular cause of congenital deafness will allow researchers to improve treatment methods and, in the future, to personalize a treatment for each cause. Some strategies currently being developed are specific to certain causes of deafness and will not apply to others. For example, deafness due to the absence of a protein will not be treated the same way as a deafness caused by a dominant toxic protein that would have to be suppressed. Some specific cases such as deafness caused by neurocristopathies have an entire cell type missing, in which case the expression or suppression of a protein will not correct the problem, as the underlying issue arises earlier in the development of the fetus and cannot be easily corrected. In such cases, research is still ongoing to treat deafness with cell therapies in which a missing cell type could be re-introduced to correct the problem. In addition to congenital deafness, other cell, genetic, and drug-based therapies are being developed to treat acquired and age-related hearing loss. No therapy has been successfully used on humans thus far, but this could become a reality in the near future.

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The authors declare no conflict of interest.

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INTERNET RESOURCES

https://www.asha.org/public/hearing/Degree-of-Hearing-Loss/

American Speech-Language-Hearing Association (ASHA). https://hereditaryhearingloss.org

Hereditary hearing loss home page.