HEARING LOSS IN HDR SYNDROME,

a study of mice and men

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Contents

Chapter 1:

General introduction

“Begin at the beginning,” the King said gravely, “and go on till you come to the end: then stop.”

Uit ‘Alice’s Adventures in Wonderland’, Lewis Carroll.

Hereditary deafness and hearing loss

Hearing loss and deafness are major health issues; it has been estimated that over 70,000,000 people worldwide are unable to participate in normal verbal communication due to auditory handicaps (Tekin et al., 2001). The prevalence of severe hearing loss and deafness amongst 6-12 year olds in the Netherlands was 0.74 per 1000 in 1996, making the estimated total number of deaf and hard of hearing in the Dutch population 11,400 (de Graaf et al., 1998).

Considering either phenotypic manifestation or aetiology, there are many ways to classify hearing loss; for instance phenotypically, congenital early onset hearing loss can be discerned from congenital late onset, mild hearing loss from profound and conductive hearing loss from sensorineural (Tekin et al., 2001).

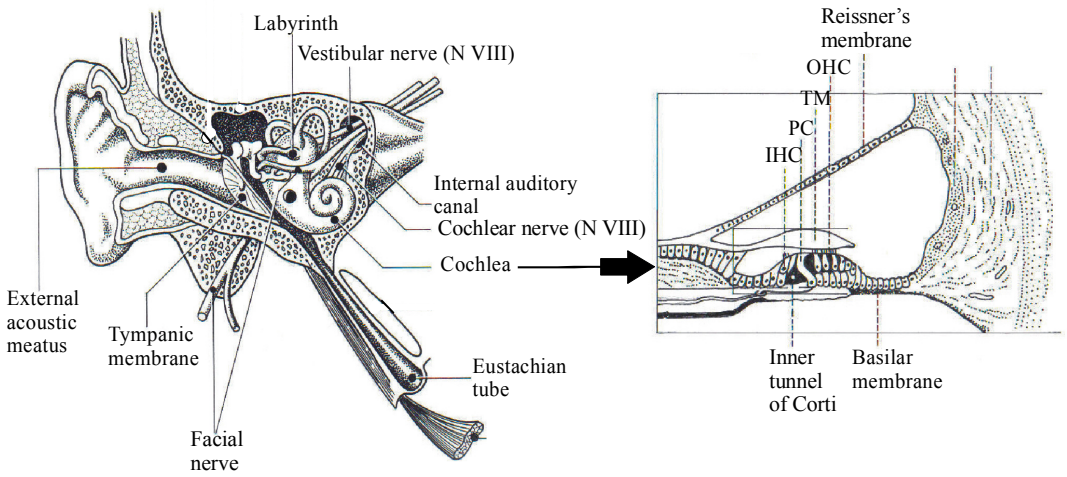

Figure 1.1: The peripheral auditory system is represented on the left, a cross section of the cochlear scala media containing the organ of Corti is represented on the right (adapted from van den Broek and Feenstra, 2007). Conductive hearing loss is caused by defects located distally from the cochlea, whereas sensorineural hearing loss is caused by defects of the cochlea or the central auditory pathways.
IHC = Inner Hair Cell, OHC = Outer Hair Cell, PC = Pillar Cell, TM = Tectorial Membrane

The etiological classification is based on a distinction made between environmental and genetic cause (or a combination of these) of hearing loss. Environmental and genetic factors have often been described to cause equal numbers of cases (Bitner-Glindzicz, 2002; Resendes et al., 2001). The proportion of environmental (or sporadic) as compared to genetic cases of hearing loss, however, in the Western world, appears to be decreasing over the last decades; this is possibly due to a better control of some environmental causes. For example, vaccination schemes and antibiotics reduce the incidence and severity of infectious diseases that may cause hearing loss.

Marazita et al. found, analysing a large group of deaf children that 37.2% were sporadic cases, the remaining 62.8% being 15.7% dominant and 47.1% recessive genetic cases (Marazita et al., 1993). A commonly used classification of genetic hearing loss is syndromic versus non-syndromic. Syndromic causes comprise 30% of genetic deafness and hearing loss, non-syndromic causes 70% (Resendes et al., 2001).

Rapid advancement in identification of genes involved in hearing loss and deafness is made, due to progress in the Human Genome Project, cochlea specific cDNA libraries and studies of murine models for hearing loss (Petit, 2006). Today, over 130 loci and 40 genes for non-syndromic hearing loss have been described, and new loci and genes are identified and characterized almost on a monthly basis (an up-to-date overview can be found on the hereditary hearing loss homepage: http://webh01.ua.ac.be/hhh/).

Non-syndromic hearing loss may be caused by a variety of pathophysiological mechanisms: for example by involvement of gene products in structural integrity, transportation of ions across membranes and regulation of transcription factors (Tekin et al., 2001). Denotation of non-syndromic hearing impairment is systematic: 'DFNA' indicating dominant loci, 'DNFB' recessive loci, 'DFN' X-linked loci and 'DFNM' modifying loci (Bitner-Glindzicz, 2002; Resendes et al., 2001).

Severity of hearing loss, caused by a single gene, may be very variable. Various factors influence the clinical outcome, such as the mutation itself, environmental factors and interacting genes. Mechanisms of gene interaction are; production of proteins interacting with normal gene products, genes providing alternative pathways and genes encoding transcription factors (Steel, 1999).

Some genes are involved in both syndromic and non-syndromic hearing loss, e.g. *PDS* (Pendred syndrome and non-syndromic deafness, respectively) and *MYO-7A* (Usher syndrome and non-syndromic deafness, respectively). One single gene can be involved in both dominant and recessive non-syndromic hearing loss (e.g. *MYO-7A*, *TECTA* and *GJB2*). Finally, phenotypic similarities are not necessarily caused by the same genetic condition (Resendes et al., 2001; Steel, 1999; Smith, 1995).

Today, several hundreds of syndromes comprising hearing loss have been described. The focus of this thesis will be on one of these syndromes: HDR syndrome. This clinical entity is, in its most classical form, characterized by hypoparathyroidism, deafness and renal dysplasia. Before addressing the syndrome in detail and defining the overall goals of this thesis, we will

review the more prevalent 'hearing loss – renal abnormalities association' and the rare 'hearing loss – hypoparathyroidism association'.

Hearing loss associated with renal abnormalities

Nowadays a wide range of syndromes is known in which the hearing loss and kidney anomalies co-occur. These syndromes are caused by an almost equally wide range of underlying patho-physiological mechanisms, several of which remain to be unravelled.[1]

Mutations in the *JAGGED1* gene cause Alagille syndrome, a complex malformative disorder that was initially described as the association of 5 major features: paucity of interlobular bile ducts, peripheral pulmonary artery stenosis, butterfly-like vertebral arch defect, posterior embryotoxon and a peculiar facies. Today, individuals with 3 out of the 5 main features are considered to have the syndrome (Crosnier et al., 2000). In addition to these main features, minor features such as skeletal defects, high-pitched voice, mental retardation, growth retardation, arterial stenosis, small bowel atresia and diabetes have been described (Crosnier et al., 2000). Patients often present with a variety of both developmental and functional renal abnormalities, ranging from renal cysts to hypoplastic kidneys (Crosnier et al., 2000). Another common feature is (mixed) hearing loss, that often is mild to severe in nature and predominantly affects the middle frequencies (Caignec et al., 2002). Pathophysiologically the disease is explained by the strong expression of *JAGGED1* during embryo-and fetogenesis in the vascular system, and other mesenchymal and epithelial tissues. The gene encodes a transmembrane protein, which is a ligand of the NOTCH receptors, which are implicated in the determination of cell fate (Crosnier et al., 2000; Kiernan et al., 2001; Li et al., 1997).

Alport syndrome is among the most prevalent auditory syndromes, its incidence being approximately 1:200,000 (Smith, 1995). Its main characteristics are: progressive nephropathy (hematuria progressing to renal failure), sensorineural hearing loss and ocular abnormalities (anterior lenticonus and perimacular flecks). Leiomyomatosis of the esophagus and the tracheobronchial tree, mental retardation and haematological abnormalities are symptoms sporadically associated with the disease. The phenotypic heterogeneity of Alport syndrome has long been recognized. However, in spite of this heterogeneity, all phenotypes are caused by mutations of type IV collagen α chains, which are expressed in basement membranes involved in the disease (glomerular basement membrane, anterior lens capsule, Descemet's membrane, Bruch's membrane, the cochlear basilar membrane, and the basement membranes

[1] A synopsis of characteristics of underlined syndromes described in this paragraph can be found in Table 1.1.

of the spiral prominence, the spiral limbus and the stria vascularis). The X-linked form of the syndrome affects approximately 80-85% of patients. This form of Alport syndrome is caused by mutations - several hundreds of which have been identified today - in the *COL4A5* gene (Xq22). Typically, males are more severely affected by renal disease than females. The remaining patients are for the greater part affected by autosomal recessive Alport syndrome, caused by mutations in *COL4A3* or *COL4A4* (2q35-37), and only a small minority is affected by autosomal dominant Alport disease, possibly arising from mutations in *COL4A3* or *COL4A4* (Hudson et al., 2003; Kashtan, 2000; Lemmink et al., 1997). Patients with early renal failure can be treated with angiotensin-converting-enzyme inhibitors and antihypertensive drugs, aiming to delay progression of the disease. The only available treatment for end-stage renal failure associated with the syndrome today is renal transplantation. Unfortunately, however, 1-5% of post-transplantation Alport patients develop specific anti-GBM nephritis, leading to renal graft loss (Hudson et al., 2003; Lemmink et al., 1997).

The autosomal recessive Alström syndrome is characterized by retinal pigmentary degeneration, early-onset truncal obesity, non-insulin-dependent diabetes mellitus, acanthosis nigricans, slowly progressive bilateral sensorineural hearing loss and chronic nephropathy. Renal failure usually commences between the second and fourth decades of life, the severity of the renal failure is an important determinant of prognosis regarding survival. Additional features are plenty and include cardiomyopathy, advanced bone age, developmental delay, asthma, hypothyroidism, hyperinsulinaemia, alopecia, hypertension, hypertriglyceridemia, elevation of hepatic enzymes, male hypogonadism, short stature, kyphosis, hyperostosis frontalis interna and scoliosis (Benso et al., 2002; Marshall et al., 1997). Mutations of the *ALMS1* gene on chromosome 2p13 bring about this rare disorder. In spite of structural similarities between *ALMS1* and mucin genes, the exact function of the former gene is as yet unknown (Hearn et al., 2002).

Defective salt transport by the thick ascending limb of Henle is the main feature of a group of autosomal recessive disorders known as Bartter syndrome. Five variants of the syndrome exist, sharing characteristic clinical features such as renal salt wasting with normal or lowered blood pressure, hypokalemic metabolic alkalosis, hypercalciuria and normotensive hyperreninemic hyperaldosteronism (e.g. Herbert, 2003; Shalev et al., 2003). In addition some patients show a typical facial appearance with a prominent forehead, triangular faces, drooping mouth and large eyes and pinnae (Vollmer et al, 2000). Sensorineural hearing loss is specific for Bartter's syndrome type IV. This variant is caused by mutations in the *BSND* or

Barttin gene on chromosome 1p (Birkenhäger et al., 2001;Vollmer et al., 2000). Barttin is essential for the proper functioning of chloride channels 'ClC-Kb' at the basolateral side of the thick ascending loop of Henle and 'ClC-Ka' at the basolateral side of the thin ascending loop of Henle. Interestingly, Barttin is also essential for chloride efflux at the basolateral side of the marginal cells of the stria vascularis, and defective chloride transport may influence the potassium secretion and endolymphatic potassium concentration -leading to impaired cochlear function and hearing loss-, and it may impede the generation of a high endocochlear potential – necessary for the sensory transduction of sounds by hair cells- (Naesens et al., 2004). Individuals with (antenatal) Bartter syndrome benefit from treatment with indomethacin (a cyclooxygenase inhibitor) (Herbert, 2003).

Branchio-Oculo-Facial (BOF) syndrome is a rare autosomal dominant disorder, bearing some similarities to the more prevalent BOR syndrome (see below). For this reason, although an exact gene locus has not been identified, it has been suggested that its locus may be expected to be on chromosome 8q. Phenotypically patients are affected by distinctive craniofacial, cervical, auricular, ophthalmologic and oral anomalies. Auricular abnormalities are common (low set, rotated ears, external auditory canal stenosis, preauricular pit, cochlear dysplasia) and 71 % of patients suffer from a conductive hearing loss. Sensorineural hearing loss is rare, but has been described in several patients (Raveh et al., 2000). Renal abnormalities are found in 37% of patients (Raveh et al., 2000).

Mutations in the eyeless (*EYA1*) transcription factor gene, as well as mutations in *SIX1* and *SIX5* (Hoskins et al., 2007; Ruf et al., 2004), lead to the most common (autosomal dominant) syndrome with an association of hearing impairment and renal abnormalities, namely Branchio-Oto-Renal (BOR) syndrome. Incidence is approximately 1:40.000 (Smith, 1995). Major phenotypic features of the syndrome are early-onset hearing loss, dysmorphology of the ears, branchial clefts and renal abnormalities (Bitner-Glindzicz, 2002; Steel, 1999; Tekin et al., 2001). Approximately 90% of patients are affected by hearing loss, which can be mixed, conductive or sensorineural in origin (Smith et al., 1998; Stinckens et al., 2001). Renal abnormalities range from vesico-ureteric reflux, ureteral pelvic junction obstruction, hydronephrosis, pelviectasis, calyectasis and bifid kidneys with double ureters to kidney agenesis, hypoplasia and dysplasia (Pierides et al., 2002; Smith et al., 1998).

No more than eight patients with Burn-McKeown syndrome have been described so far. Patients were characterized by bilateral choanal stenosis or atresia and a typical facial phenotype with hypertelorism, short palpebral fissures, lower eyelid coloboma, thin lips and prominent ears, as well as a prominent nasal bridge. Other features, such as cardiac defects,

hearing loss, cleft lip/palate (with oronasal fistula), micrognathia, short stature, pre-auricular tags, hypomimic face and (unilateral) kidney hypoplasia may be present. The facial phenotype seems to change significantly with age. Intrafamilial there may be a remarkable variation in phenotypic expression. It has been suggested that the syndrome may inherit in a X-linked recessive mode, however the causative gene(s) remain to be identified (Wieczorek et al., 2003).

CHARGE-syndrome (ocular Coloboma, Heart defects, Atretic choanae, Retarded growth or development, Genital hypoplasia and Ear anomalies or hearing loss) has an estimated birth incidence of 1:12,000 (Kallen et al., 1999). It is often caused by mutations in the *CHD7* gene (Vissers et al. 2004), but in a subset of patients mutations in *SEMA3E* may play a role in the pathogenesis of the disease (Lalani et al., 2004). The syndrome may reflect a polytropic developmental field defect involving neural crest cells or the neural tube. Sensorineural or mixed hearing loss affects the majority of patients, whereas renal anomalies occur in 15-25% of patients (Edwards et al., 2002; Tellier et al., 1998; Wang et al., 2001).

Based on a review of 140 cases, Nance et al. defined the requirements for the clinical diagnosis of classical Cockayne syndrome (CS I). According to these authors the diagnosis can be made in patients who are affected by 1) growth failure with (near) normal birth weight and 2) neurodevelopmental or neurological dysfunction, combined with at least three of the following five features 3) cutaneous photosensitivity, with or without thin or dry skin or hair, 4) progressive pigmentary retinopathy, and/or cataracts, optic disc atrophy, miotic pupils or decreased lacrimation, 5) sensorineural hearing loss, 6) dental caries and 7) cachectic dwarfism. In infants or toddlers the diagnosis may be more difficult; it based on the presence of the two obligatory features in combination with one of the five other criteria and additional biochemical evidence (i.e. fibroblast sensitivity to UV light) (Nance et al., 1992). Other symptoms compatible with the diagnosis are hypertension, renal dysfunction, hepatomegaly, serum transaminase elevation, undescended testes and anhidrosis. The mean age of death in reported cases was 12.3 years (Nance et al., 1992; Sato et al., 1988). Patients with CS II have a more severe and earlier onset of symptoms, they usually die by the age of 6 or 7 (Nance et al., 1992). The syndrome inherits in an autosomal recessive way. It is caused by the *CSA* and *CSB* genes, located on genes 5 and 10 respectively (Fryns et al., 1991; Henning et al., 1995).

The *ATP6(V1)B1* gene encodes the B1 subunit of H+-ATPase, one of the key membrane transporters for acid secretion in the α-intercalated cells of the medullary collecting duct in kidneys (Feldman et al., 2006; Hahn et al., 2003; Ruf et al., 2003). Mutations in *ATP6V1B1* cause Distal Renal Tubular Acidosis (DRTA) with sensorineural hearing loss; metabolic

acidosis of varying severity -accompanied by inappropriately alkaline urine- that if untreated may result in dissolution of bone, leading to osteomalacia and rickets. Other features of the disease include low serum potassium, elevated urinary calcium, growth failure, nephrocalcinosis and nephrolithiasis that may present as 'Steinstrasse' on abdominal X-ray (Peces, 2000). A final characteristic of the disease is early onset, sensorineural hearing loss, which is probably caused by defective endolymph pH homeostasis. The hearing loss is progressive and does not respond to the treatment of DTRA with alkali supplementation by sodium bicarbonate, Shoul solution, potassium citrate or other alkali preparations (Hahn et al., 2003). Interestingly, it has recently been demonstrated that mutations in another gene, *ATP6V0A4* cause DRTA associated with older onset, mild to moderate sensorineural hearing loss (Stover et al., 2002).

For a description of Duane- Radial Ray Syndrome see Okihiro syndrome.

Neuropathic pain, caused by nerve fiber degeneration due to lipid accumulation, is the clinical hallmark of Fabry disease (a.k.a. Anderson-Fabry disease), affecting as much as 77% of patients. The pain typically increases during exercise or overheating. Other common features are: angiokeratoma (71%), CVA, TIA, renal disease resulting in renal dialysis and renal transplant, left ventricular hypertrophy (88%), heart valve anomalies (29%), hypohidrosis (56%), gastrointestinal symptoms (abdominal pain, nausea and vomiting) and hearing loss (MacDermot et al., 2001). Approximately 50% of Fabry patients complain of hearing symptoms. Hearing loss may be uni- or bilateral, is moderate to severe and often has a sudden onset in childhood that may be preceded by tinnitus (Conti et al., 2003; Keilmann, 2003). Histological analysis of four temporal bones of Fabry patients demonstrated hyperplastic mucosa, strial and spiral ligament atrophy, basal hair cell loss and a reduced number of spiral ganglion cells, however glycosphingolipid accumulation (see below) could not be demonstrated (Schachern et al., 1989). Dysmorphic features, such as thick lips, bulbous nose and thickening of the nasolabial folds, are present in approximately 50% of patients. A birth frequency of 1: 100,000 makes Fabry disease the second most common glycospingolipid storage disorder. The disease is caused by a deficiency of X-linked lysosomal hydrolase, α-galactosidase, leading to the accumulation of uncleaved glycosphingolipids (especially Gb3) (MacDermot et al., 2001). Lipid deposits may be found in cortical and brain stem structures, in autonomic ganglia, in cardiac muscle and conducting fibres, in renal glomeruli and tubules, and in the endothelium and media of small vessels. Since Fabry disease is an X-linked disorder it predominantly affects males, although carrier females can, dependent on their X-chromosomal inactivation, also be affected to a mild or more severe degree (Jardim et al.,

2004). Current therapy involves symptomatic medical management, dialysis, enzyme replacement therapy, kidney transplantation and gene therapy (Inderbitzin et al., 2005). Agalsidase alfa therapy possibly even brings about a reversal of hearing deterioration in affected patients (Hajioff et al., 2003). The median cumulative survival of a cohort of UK patients was 50 years (MacDermot et al., 2001).

Fanconi anemia is a rare chromosome instability syndrome characterized by aplastic anemia, cancer and leukemia susceptibility and, at cellular level, a hypersensitivity to DNA cross-linking agents. Detection of increased chromosome breakage in cultured peripheral blood lymfocytes, following exposure to clastogenic agents, confirms the diagnosis (De Kerviler et al., 2000). The prevalence is 1-5/1,000,000, but as high as 1/22,000 in white South Africans. Patients typically develop bone marrow failure during the first decade of life, and by the age of 40 90% of patients are affected by it. By that age 33% of patients will have developed hematologic and 28% nonhematologic neoplasms (e.g. head and neck squamous carcinoma, gynecologic squamous cell carcinoma, esophageal carcinoma, liver tumors, brain tumors, skin tumors and renal tumors) (Taniguchi et al., 2006). In two thirds of cases the disease is associated with congenital malformations that may involve every organ system. Common features are skin pigmentation abnormalities, growth retardation, facial abnormalities, radial ray deformities, gastrointestinal deformities, microcephaly, eye abnormalities and congenital cardiopathies (De Kerviler et al., 2000). Hearing loss is present in 17.4-19% of patients, and is usually conductive in nature (Santos et al., 2002). Sensorineural hearing loss is rare, but has been described (Chevalier, 1983). External auditory canal stenosis (12%) and pinna deformations (6%), with low ear implant are not uncommon (Santos et al., 2002). The urinary system is affected in approximately 30% of patients, and hypo- or aplastic kidneys, ectopic or pelvic kidneys, horseshoe and sigmoid kidneys may be found (De Kerviler et al., 2000). Fanconi anemia comprises at least 12 complementation groups (A, B, C, D1, D2, E, F, G, I, J, L and M) and today 11 of the 12 responsible genes have been identified. Although progress has been made in the molecular understanding of the disease - FA proteins appear to cooperate with several other proteins in a pathway leading to the recognition and repair of damaged DNA- some pathophysiological aspects remain to be clarified (Taniguchi et al., 2006).

Fechtner syndrome is a macrothrombocytopenia associated with sensorineural hearing loss, cataracts, nephritis and characteristic leucocyte inclusion bodies (Moxey-Mims et al., 1999). The disease bears resemblance to, and may even be a variant of, Alport syndrome; ultrastructually, renal biopsy findings are alike. Fechtner syndrome is associated with a mild

bleeding tendency, and in case of surgery, it may be necessary to administer DDAVP preoperatively to affected patients (Matzdorff et al., 2001). The gene responsible for the disease was linked to 22q11-q13 (Cusano et al., 2000), and it has been proposed that mutations in the *MYH9* gene in this region may result in Fechtner syndrome, the May-Hegglin anomaly or Sebastian syndrome (Seri et al., 2000).

Isolated microtia/anotia, oculoauriculovertebral spectrum (OAVS) and Goldenhar syndrome are often considered to be appearances of increasing severity of the same anomaly. The latter two forms comprise a variety of abnormalities in structures derived from the first and second branchial arches. Cases are usually sporadic. Albeit that nomenclature is not completely decisive, the combination of epibulbair dermoids, preauricular tags/pits, microtia and facial asymmetry due to an unilateral small jaw is usually termed OAVS, whereas this combination is known as Goldenhar syndrome when accompanied by thoracic hemivertebrae and cleft lip and/or palate (Wang et al., 2001). Apart from the obvious conductive hearing loss, sensorineural hearing loss affects 11% of patients suffering from hemifacial microsomia (Carvalho et al., 1999). Prevalence of renal malformations has been reported to be as high as 11% in isolated anotia and or microtia, and 70% in Goldenhar syndrome (Harris et al., 1996; Ritchey et al., 1994).

It has been estimated that Klippel-Feil syndrome occurs in approximately 1:40,000-42,000 births. The syndrome is characterized by the presence of a congenital fusion of either some or all cervical vertebrae, depending on the subtype (1-3). This fusion may lead to a classic triad of symptoms; a short neck, a low posterior hairline and limited range of motion of the neck. An immense and variable spectrum of anomalies is known to be associated with the syndrome – e.g. congenital scoliosis (>50% of patients), rib abnormalities (33%), Sprengel's deformity (20-30%), synkinesia (15-20%), cervical ribs (12-15%), cardiovascular abnormalities (4-29%), craniofacial abnormalities, vocal impairment, psychomotor retardation, hypothyroidism and polydactyly (da Silva et al., 1993; Papagrigorakis et al., 2003; Tracy et al., 2004). Genitourinary abnormalities occur in 25-35% of patients (Tracy et al., 2004). Hearing loss is present in 30% of patients, and may be sensorineural, mixed or occasionally conductive (Papagrigorakis et al., 2003; Tracy et al., 2004). Inheritance is either autosomal recessive (types 1 and 3) or dominant (type 2). It has been suggested that the locus for type 2 Klippel-Feil syndrome lies on chromosome 2, 5, 8 or 15 (Papagrigorakis et al., 2003), but no Klippel-Feil genes have been identified so far.

The very rare Levy Hollister syndrome is characterized by dysplasia of different organ systems. Commonly found anomalies are radial ray defects of the upper limbs, which often

are associated with radioulnar synostosis and lacrimal malformations. Other features are xerostomia (due to aplasia of major salivary glands), dysplasia of the auricles (cup ears), dental anomalies, hearing loss (either sensorineural, conductive or mixed) and malformations of the kidneys (ranging from agenesis to sclerosis). Although it has been recognised that the disorder inherits in an autosomal dominant way, the exact underlying genetic cause remains to be elucidated (Fierik et al., 2003, Kreutz et al., 1988).

The combination of progressive sensorineural hearing loss, cognitive impairment, facial dysmorphism, late onset pancytopenia and variable renal and genitourinary abnormalities inheriting in an X-linked recessive way, is known as Martin-Probst syndrome. Additional features such as microcephaly, short stature, teleangiectasias and dermatoglyphics may be present. Although no exact underlying genetic cause has been identified today, skewed X-inactivation has been demonstrated in carrier females, and the disorder has been mapped to Xq1-21 (Martin et al., 2000; Probst et al., 2004).

Muckle-Wells Syndrome (MWS) is a dominantly inherited autoinflammatory disease, closely related to other associations such as Familial Cold autoinflammatory Syndrome (FCS) and Neonatal Onset Multisystem Inflammatory Disease (NOMID). MWS is characterized by rashes, fever, arthralgia, progressive sensorineural hearing loss and the frequent development of systemic AA amyloidosis (amyloidosis derived from the inflammatory serum amyloid-associated protein) (Hawkins et al., 2004). Occasionally ophthalmologic features may be present. Active inflammatory symptoms and visual loss respond well to subcutaneous injections of anakinra, a recombinant human IL-1 receptor antagonist (Alexander et al., 2005; Hawkins et al., 2004). The disease was linked to chromosome 1q44 and is probably caused by mutations in the *NALP3/CIAS1/PYPAF1* gene (Cuisset et al., 1999; Hawkins et al., 2004).

The exact pathophysiology underlying the very rare, Treacher Collins resembling, Nager syndrome (acrofacial dysostosis) remains unknown today. The paired related homeobox genes *Prrx1* and *Prrx2* (encoding DNA binding transcription factors essential for cellular differentiation and embryonic patterning), have been suggested to play a role in causing the syndrome. Although no mutations in the open reading frames of the genes have been found, mutations altering regulation of the genes have not yet been excluded (Norris et al., 2000). Patients affected by Nager syndrome have craniofacial features similar to those seen in Treacher Collins syndrome (e.g. downslanting palpebral fissures, hypoplasia of the maxilla, mandibular hypoplasia and external ear anomalies – causing conductive hearing loss), however in addition they suffer from defects of the upper, and sometimes the lower, limb (ranging from thumb hypoplasia to absence of the radial ray) (Friedman et al., 1996; Hunt et

al., 2002; Paladini et al., 2003). Wang et al. reviewed literature on Nager syndrome and found renal malformations in 9% of affected individuals (Wang et al., 2001).

The most characteristic features of Okihiro syndrome (or Duane- Radial Ray Syndrome) are radial ray defects and Duane anomaly (limitation of abduction of the eye and narrowing of the palpebral fissure with retraction of the globe on adduction). The phenotypic expression of Okihiro syndrome is variable. An extensive range of associated features has been reported, embracing anal stenosis, pigmentary anomalies, facial asymmetry, cardiac lesions (ASD, VSD), choanal atresia, renal malformations, external ear malformations and hearing loss (Kohlhase et al., 2003). The disease is inherited in an autosomal dominant way and results from mutations in the putative zinc finger transcription factor gene *SALL4* on chromosome 20 (Borozdin et al., 2004a; Kohlhase et al., 2003). Mutations in the same gene – that without exception produce preterminal stop codons - also have been shown to cause acro-renal-ocular syndrome, Holt-Oram syndrome and suspected thalidomide embryopathy. Although these disorders may all be part of a phenotypic spectrum caused by *SALL4* mutations, a clear genotype-phenotype correlation has not been established so far (Borozdin et al., 2004b; Kohlhase et al., 2005).

The autosomal recessively inherited Refsum's disease (Phytanic Acid Storage Disease, Heredopathia Atactica Polyneuritiformis) is characterised by the inability to metabolise dietary phytanic acid (by the primary α-oxidation pathway), resulting in excessive accumulation of phytanic acid in serum triglycerides and all fatty tissues (e.g. myelin sheaths, liver and kidneys). Patients are usually asymptomatic until the age of 20 to 30 years, when they are typically initially affected by night blindness. The 'full-blown' syndrome is characterised by the tetrad of retinitis pigmentosa, cerebellar ataxia, peripheral demyelinating neuropathy and high CSF protein. Other frequent findings are anosmia, vestibular impairment, pupillary abnormalities, shortened distal phalanges and broad short fingernails, lens opacities, ichtyosis, cardiac arrythmias and renal failure. A progressive sensorineural hearing loss affects up to 80 % of the patients. Usually this hearing loss commences asymmetrical in the second decade, whereas by the fourth decade its severity increases. The inner ear rather than the central nervous system is affected by the disease. Interestingly OHC function is normal and IHC function and/or auditory nerve function are impaired. The mainstay of management of Refsum's disease consists of dietary restriction of phytanic acid (dairy products, meats and ruminant fats) aiming to prevent acute attacks of the disease and progression of organ impairment. Therapeutic plasma exchange rapidly reduces plasma

phytanic levels during acute attacks. (Oysu et al., 2001; Weinstein, 1999). The Refsum disease gene was localized to chromosome 10p (Nadal et al., 1995).

The clinical presentation of Townes-Brocks syndrome is very variable, however characteristically anorectal abnormalities, abnormalities of hand and feet, deformities of the outer ear, preauricular tags and a sensorineural hearing loss occur. The hearing loss is slowly progressive, and apart from the predominant sensorineural component usually comprises a small conductive component. 27% of patients are affected by renal abnormalities (including renal hypoplasia, dysplasia, unilateral renal agenesis, horseshoe kidney, ureterovesical reflux, posterior urethral valves and meatal stenosis). The syndrome is caused by mutations in the *SALL1* gene encoding a zinc finger transcription factor, possibly involved in urological, renal, limb, ear, brain and liver development (Kohlhase, 2000; Powell et al., 1999; Rossmiller et al., 1994; Salerno et al., 2000).

Finally, Wolfram syndrome is a progressive, neurodegenerative condition, with an estimated prevalence of 1/770,000 in the UK. The syndrome is also known as DIDMOAD (Diabetes Insipidus, Diabetes Mellitus, Optic Atrophy, and Deafness). Diabetes mellitus usually is the first sign of the disease, it is followed in time by optic atrophy and diabetes insipidus (Minton et al., 2003; Piccoli et al., 2003). Urologic features, such as uretero-hydronephrosis and bladder dysfunction are present in the majority of patients. The features usually become manifest during late teenage-years (Ankotché et al., 1998; Cremers et al., 1977; Piccoli et al., 2003). A high frequency sensorineural hearing loss affects up to 62% of patients. A subgroup of Dutch female patients was recently identified, showing an exceptionally high degree of mid- and high frequency hearing impairment (Pennings et al., 2004). Other complications include psychiatric disorders (suicide), gastro-intestinal dysmotility, primary gonadal atrophy, olivo-ponto-cerebellar ataxia and anosmia (e.g Minton et al., 2003). The disease is usually caused by inactivating mutations in *WSF1*, whereas non-inactivating mutations in the same gene can be found in DFNA6/14 families (Cryns et al., 2003).

(A synopsis of the above syndromes is given in table 1.1)

Apart from the list of well-described syndromes above, that most likely will not be fully complete and will gradually expand, casuistic reports on the concurrence of renal problems and hearing loss are not uncommon. Sometimes the association could be incidental, for example in a boy affected by complete bilateral nerve deafness combined with familial iminoglycinuria (Rosenberg et al., 1968), in two brothers with sensorineural hearing loss, nephropathy, diabetes mellitus, premature atherosclerosis and progressive neurological

Table 1.1

Overview of syndromes with hearing loss – renal abnormalities association

Syndrome	Hearing loss	Renal abnormalities	Additional features	Cause
Alagille syndrome	- mixed - mild to severe - middle frequencies	- variable (horseshoe kidney, ureteral stenosis, renal cysts, hypoplastic kidneys, tubular acidosis, tubulopathies, glomerulopathies)	- paucity of interlobular bile ducts - pulmonary artery stenosis - vertebral arch defect - posterior embryotoxon - peculiar facies - skeletal defects - high-pitched voice - mental retardation - growth retardation - arterial stenosis - small bowel atresia - diabetes - congenital heart defects	*JAGGED1* (20p12)
Alport syndrome	- sensorineural - high frequency - M: 55%, F: 45%	- progressive nephropathy (hematuria, proteinuria, progressive renal failure)	- ocular abnormalities - leiomyomatosis - mental retardation - haematological abnormalities	XLAS: mutations of *COL4A5* gene (Xq22) ARAS: mutations of *COL4A3* or *COL4A4* (2q35-37) ADAS: mutations of *COL4A3* or *COL4A4*
Alström syndrome	- sensorineural - slowly progressive - bilateral	- chronic nephropathy (tubular atrophy, glomerular hyalinisation, moderate interstitial fibrosis) - start 2nd - 4th decade	- retinopathy - obesity - NIDDM - acanthosis nigricans - cardiomyopathy - hypertension - hypertriglyceridemia - elevation of hepatic enzymes - hypothyroidism - hyperinsulinaemia - developmental delay - male hypogonadism - skeletal abnormalities - asthma - alopecia	mutations of *ALMS1* (2p13)
Bartter syndrome	- sensorineural - early onset	- renal salt wasting, decrease in glomerular filtration rate,	- polyhydramnios - premature birth	Barttin gene or *BSND* gene on chromosome 1p.

		occasionally end-stage renal failure	- frequent neonatal infection - renal salt wasting - hypokalemic metabolic alkalosis - hypercalciuria - normotensive hyperreninemic hyperaldosteronism - facial dysmorphisms	
BOF syndrome	- conductive (71%) - sensorineural (rare)	- renal abnormalities (e.g.; multicystic kidney, uretero pelvic junction obstruction) - 37%	- branchial anomalies (supra auricular sinus, aplastic cervical skin lesions, ectopic thymus, malformed auricles, stenosis of external auditory canal) - ocular abnormalities (microphthalmia, lacrinal duct obstruction, coloboma, cataract, myopia, strabismus, telecanthus, upslanting palpebral fissures) - pseudocleft of upper lip - developmental delay, mental retardation (41%) - facial nerve paralysis (rare) - limb/ finger abnormality (32%)	gene unknown
BOR syndrome	- mixed, conductive or sensorineural - early onset - 90%	- vesico-ureteric reflux, ureteral pelvic junction obstruction, hydronephrosis, pelviectasis, calyectasis, bifid kidneys with double ureters, kidney agenesis, hypoplasia, dysplasia	- dysmorpholgy of the ears - branchial clefts	mutations in *EYA1* transcription factor (8q13.3), *SIX1*, *SIX5*
Burn-McKeown syndrome	- conductive	- kidney dysplasia/hypoplasia	- bilateral choanal stenosis or atresia - typical facial phenotype (see text) - cardiac defects - cleft lip/palate - micrognathia,, short stature - pre-auricular tags - hypomimic face.	autosomal recessive or X-chromosomal recessive
CHARGE-syndrome	- sensorineural or mixed	- 15-25%	- ocular coloboma - heart defects - atretic choanae - retarded growth or development - genital hypoplasia	*CDH7* (8q12.1) *SEMA3E* (7q21.11)

			-ear anomalies	
Cockayne syndrome	- sensorineural - mild to severe - bilateral - high frequency? - 60%	- renal dysfunction, ranging from decreased creatinine clearance to renal failure, global sclerosis of glomeruli - 10%	- growth failure - neurodevelopmental or neurological dysfunction - cutaneous photosensitivity - progressive pigmentary retinopathy, cataracts, optic disc atrophy, miotic pupils, decreased lacrimation - dental caries - cachectic dwarfism - hypertension - hepatomegaly - serum transaminase elevation - undescended testes - anhidrosis.	autosomal recessive *CSA* (5q12) *CSB* (10q11)
Distal Renal Tubular Acidosis	- sensorineural - moderate to severe vs. mild to moderate - high frequency - early onset vs. older onset - progressive - bilateral	- renal tubular transport disorder, elevated urinary calcium, nephrocalcinosis, nephrolithiasis	- osteomalacia - rickets - growth failure - low serum potassium	*ATP6V1B1* gene (2q13) *ATP6V0A4*
Duane-Radial Ray syndrome *see* Okihiro syndrome				
Fabry disease	- sensorineural - mild to severe - unilateral or bilateral - 2-3 kHz - tinnitus (38.3%, age of onset 12-15 years) - strial and spiral ligament atrophy, basal hair cell loss	- proteinuria, hypertension, glycolipid accumulation in glomerular, vascular and interstitial cells, deposition in distal tubules and loops of Henle	- neuropathic pain - cerebrovasculair complications - angiokeratoma - hypohidrosis - TIA/CVA - chest pain/ palpitation - left ventricular hypertrophy - heart valve anomalies - GI symptoms - fatique - facial dysmorphias	X-linked α-galactosidase gene
Fanconi anaemia	- conductive (rarely sensorineural) - average are bone gap 29.3 dB	- renal tumour, hypoplastic-, aplastic-, ectopic-, or pelvic kidneys, horseshoe, sigmoid kidney, double collecting	- skin (café au lait spots, hyper-, and hypopigmentation) - growth retardation, short stature (60%)	AR (all but FA-B), X-linked (FA-B) *FANCA* (16q24.3) *FANCB* (Xp22.31) *FANCC* (9q22.3)

	- 17.4-19%,	system, vesico-ureteric reflux, hypospadia, hypogenitalia.	- facial (elfin, broad nasal base, epicanthal folds, micrognathia) - radial ray defects (49-66%) (thumb malformations, absent or hypoplastic radius) - gastrointestinal (atresia, imperforate anus) - microcephaly (37%), hydrocephalus, ventriculomegaly - external ear malformations - eye (strabismus, microphthalmia, ptosis) - patent ductus arteriosus, VSD, ASD, tetralogy of Fallot, mitral valve prolaps - displaced radial artery	*FANCD1/BRCA2* (13q12-13) *FANCD2* (3p25.3) *FANCE* (6p21-22) *FANCF* (11p15) *FANCG/XRCC9* (9p13) *FANCJ/BACH1/BRIP1* (17q22-q24) *FANCL/PHF9/POG* (2p16.1) *FANCM/Hef* (14q21.3)
Fechtner syndrome	- sensorineural - high frequency	- microscopic haematuria – end-stage renal disease	- macrothrombo-cytopenia - cataract - leucocyte inclusions - mild bleeding tendency - liver function disorder? - hypercholesterolaemia?	*MYH9* (22q11-q13)
- isolated microtia/anotia - OAVS - Goldenhar syndrome	- conductive or sensorineural (11%)	- 11-70%	- epibulbair dermoids - preauricular tags/pits - microtia - facial asymmetry - thoracic hemivertebrae - cleft lip and/or palate	usually sporadic gene unknown
Klippel-Feil syndrome	- sensorineural, mixed or occasionally conductive - 30%	- genitourinary abnormalities - 25-35%	- congenital fusion of cervical vertebrae - congenital scoliosis - rib abnormalities - Sprengel's deformity - synkinesia - cervical ribs - cardiovascular abnormalities - craniofacial abnormalities - vocal impairment - psychomotor retardation - hypothyroidism - polydactyly	autosomal recessive (types 1 and 3) or dominant (type 2) gene unknown

Levy Hollister syndrome	- sensorineural, occasionally conductive or mixed - 50%	- agenesis - sclerosis	- radial ray defects - lacrimal malformations - xerostomia - dysplasia of the auricles - dental anomalies	autosomal dominant gene unknown
Martin – Probst deafness – mental retardation syndrome	- sensorineural - severe to profound - progressive - bilateral	- dysplasia, small kidneys	- facial dysmorphisms (telecanthus, hypertelorism, epicanthic folds, broad mouth, low set ears) - mild to severe cognitive impairment - teleangiectasias - widely spaced nipples - umbilical hernia - dermatoglyphics - microcephaly - short stature - pancytopenia - bifid scrotum, small undescended testicles and phallus, absence of vas deferens, chordee - mild myopia(?)	recessive X-linked, Xq1-21
Muckle-Wells syndrome	- progressive sensorineural (absence of organ of Corti, and vestibular epithelium, cochlear nerve atrophy and basilar membrane ossification)	- AA amyloidosis with renal involvement and end stage renal failure - 25%	- rashes (urticaria like) - fever - arthralgia - myalgia - conjunctivitis - visual loss	autosomal dominant *NALP3/CIAS1/PYPAF1* (1q44)
Nager syndrome (acrofacial dysostosis)	- conductive	- renal malformations - 9%	- craniofacial features (downslanting palpebral fissures, maxillar hypoplasia, mandibular hypoplasia, external ear anomalies) - defects of limbs	homeobox genes *Prrx1* (1q23) and *Prrx2* (9q34.1)?
Okihiro syndrome	- conductive or sensorineural - mild to severe - unilateral or bilateral	- renal agenesis, dystopic kidney, horseshoe kidney	- radial ray defects, radial aplasia, radial club hands, triphalangeal thumb, thumb reduplication, shortened upper limbs - Duane anomaly (70%) - anal stenosis - pigmentary disturbances	*SALL4* (20q13.13-13.2) zinc finger protein, acting as a transcription factor

			- facial asymmetry - cardiac lesions (ASD, VSD, foramen ovale) - external ear malformations, slit-like openings of auditory canal - choanal atresia	
Refsum's disease (Phytanic Acid Storage Disease, heredopathia atactica polyneuritiformis)	- sensorineural - progressive (start 2nd decade, increase 4th decade) - asymmetrical - OHC function normal - IHC and/or N. VIII function impaired - 80 %	- renal failure	- anosmia - vestibular impairment - pupillary abnormalities - shortened distal phalanges and broad short fingernails - lens opacities - ichtyosis - cardiac arrythmias	mutations in phytanoyl-CoA hydroxylase cDNA leading to inability to metabolise dietary phytanic acid (10p)
Townes-Brocks syndrome	- sensorineural with a small conductive component - slowly progressive	- renal hypoplasia, dysplasia, unilateral renal agenesis, horseshoe kidney, ureterovesical reflux, posterior urethral valves, meatal stenosis - 27%	- anorectal abnormalities - abnormalities of hand and feet - deformities of the outer ear - preauricular tags	*SALL1* (16q12.1) gene encoding a zinc finger transcription factor
Wolfram syndrome	- sensorineural - high frequency - 62%	- hydronephrosis, acute renal failure - 54-90%	- diabetes insipidus - diabetes mellitus - optic atrophy - psychiatric disorders - gastro-intestinal dysmotility - primary gonadal atrophy - olivo-ponto-cerebellar ataxia - anosmia	autosomal recessive *WFS1* (4p16), *WFS2* (4q22-q24)

Characteristic features of syndromes with a hearing loss – renal anomaly association.

In the second column the nature of hearing loss is described (when different types of hearing loss have been described, the most common form is mentioned first). Overall percentages of patients affected by hearing loss are represented where data were available, when between braces percentages indicate the proportion of patients affected by a specific subtype (either sensorineural, conductive or mixed). The nature of renal abnormalities and percentage of affected patients are represented in the third column. When known causative genes and between braces loci are indicated in the last column.

deterioration with photomyoclonic epilepsy (Feigenbaum et al., 1994) and in two sibs affected by Senior Loken syndrome (nephronophthisis and retinal dystrophy) and moderate sensorineural hearing loss (Clarke et al., 1992), but some of these associations may turn out to be new clinical (syndromic) entities in the future.

Crew et al. for example describe two sibs with a single nucleotide insertion in exon 5 of *CD151* (11p15.5) with end stage renal failure, sensorineural hearing loss, pretibial epidermolysis bullosa and β-thalassemia minor (Crew et al., 2004), whereas Hussain et al describe the combination of infantile hyperinsulinism, severe enteropathy, profound sensorineural deafness and renal tubulopathy, due to a 122 kb deletion of the short arm of chromosome 11, also deleting 26 exons of the *USH1C* gene (Hussain et al., 2004). The acronym BRESEK/BRESHECK was used by Reish et al. to describe a potential new X-linked syndrome affecting two maternally related half brothers. The clinical phenotype comprised brain anomalies, retardation, ectodermal dysplasia, skeletal deformities, Hirschsprung disease, eye anomalies, cleft palate, cryptorchidism, ear anomalies, mixed hearing loss and kidney dysplasia (Reish et al., 1997).

Bilateral macular colobomas combined with apical dystrophy of the hands and feet is known as Sorsby syndrome; another very rare, autosomal dominantly inherited condition. In one affected family sensorineural hearing loss was found in a young male patient with no kidney abnormalities and mixed hearing loss was found in his mother, who, like two other family-members had only a single kidney (Thompson et al., 1988).

The overlap in symptomatolgy between Melnick-Needles syndrome and several other syndromes has led to some controversy regarding nomenclature in recent decades. The existence of a fronto-otopalatodigital-osteodysplasty syndrome has been suggested, after clinical evidence indicated an overlap of frontometaphyseal dysplasia (FMD), oto-palato-digital (OPD) syndrome type I and II and Melnick-Needles syndrome (MNS). Common features in affected patients are (severe) skeletal dysplasia and characteristic facial features (FMD, MNS), but urogenital anomalies and hearing loss may also be part of the clinical spectrum (Kristiansen et al., 2002; Morova et al., 2003; Robertson et al., 1997). Serpentine fibula – polycystic kidney syndrome, or Hadju-Cheney syndrome bears a lot of similarities to Melnick-Needles syndrome, and multiple papers have addressed the question whether or not the syndromes are mere variants of one syndrome or whether they represent distinct clinical entities. An elongated curved, or serpentine, fibula present in the former seems to be the distinguishing feature (Exner, 1988; Majewski et al., 1993; Ramos et al., 1998).

Diagnosis of many of the above syndromes is often difficult for several reasons. First, all symptoms do not necessarily occur simultaneously. Secondly, often different symptoms are not equally apparent. And, finally, the rarity of many of these syndromes unavoidably leads to ignorance considering the syndromes in question, causing misdiagnosis or underdiagnosis.
Awareness amongst medical specialists may lead to a reduction in mis- and underdiagnosis as may targeted diagnostic tests. Malformations of the external ear (such as pre-auricular pits and tags, anotia or microtia, lop or cup ears) are associated with an increased incidence of renal anomalies of clinical significance – e.g. in CHARGE syndrome, Townes-Brocks syndrome, oculoauricolovertebral spectrum, BOR syndrome, BOF syndrome, Nager syndrome, Levy Hollister syndrome and Fanconi anemia (e.g. Wang et al., 2001) -, we therefore believe that these malformations when present, and especially when combined with hearing loss warrant renal ultrasound.

Hearing loss associated with hypoparathyroidism

The combination of hearing loss with hypoparathyroidism is much less common than that with renal abnormalities. Still some papers devoted attention to the subject. Some of these propose a causal connection, some a common (genetic) cause for the hypoparathyroidism-hearing loss association.
Garty et al. demonstrated sensorineural hearing loss in 3 out of 17 patients affected by idiopathic hypoparathyroidism (and conductive hearing loss in 2 out of 17) and hereafter proposed hearing impairment to be either a complication or an associated feature of hypoparathyroidism (Garty et al., 1994). Likewise, sensorineural hearing loss was found in 7 out of 21 Japanese patients with untreated hypoparathyroidism or chronic hypocalcaemia. Ikeda et al. suggested that a low calcium level in the inner ear fluid or the direct effect of vitamin D deficiency on the inner ear was responsible for the malfunctioning of these inner ears, and thus for the sensorineural hearing loss found in these patients (Ikeda et al., 1987). Unfortunately neither paper states explicitly if and how other causes of hearing loss were excluded. Therefore other possible causes of hearing loss might have been overlooked. Indeed for some of the cases described, a syndromic etiology might be considered.
As early as in 1983 a connection has been suggested between non-hypoparathyroidism induced vitamin D deficiency (and its resulting deficiency of calcium) and hearing loss. Brookes described 10 patients with progressive bilateral hearing loss and vitamin D deficiency. Different pathophysiological mechanisms for the coming about of the hearing loss

were proposed, e.g. disruption of the neurosensory epithelium or the supporting bone structure of the otic capsule and calcium dependent disturbances in membrane permeability and neuromuscular excitability (Brookes, 1983). Since -to our knowledge- until now the above hypotheses have not been tested systematically, a causative role for hypocalcaemia in hearing loss cannot be excluded in hypoparathyroidism-hearing loss associations.

Several distinct genetic (mitochondrial) defects have been described, causing a clinical phenotype in which hearing loss and hypoparathyroidism co-occur. The best known of these is probably Kearns-Sayre Syndrome, a neurological disorder with variable encephalomyopathic symptoms amongst which hearing loss. Less than 10% of Kearns-Sayre patients suffer from hypoparathyroidism, hyperaldosteronism, hypomagnesemia or thyroid dysfunction. However, hearing loss and hypoparathyroidism may be the initial major manifestations of the syndrome. If so, there is an association with pleioplasmic mitochondrial DNA rearrangements. It has been suggested that ATP deficiency is involved in the pathogenesis of both the hearing loss and PTH deficiency (Wilichowski et al., 1997). An A3243G mutation of mitochondrial DNA was found in a 54-year old woman with diabetes mellitus, hearing loss, muscle weakness and hypocalcaemia due to idiopathic hypoparathyroidism. Point mutations at the 3243 position are known to cause maternally inherited diabetes mellitus and deafness (MIDD), but hypoparathyroidism is a rare finding in this association. Once again, since the parathyroids require high-energy supply, it is not unthinkable that the hypoparathyroidism in this patient is caused by a disturbed mitochondrial oxidative phosphorylation (Tanaka et al., 2000). Finally, Hameed et al., describe a 6 year old boy with focal segmental glomerulosclerosis, hypoparathyroidism and sensorineural deafness, who after developing progressive neurological deterioration, was diagnosed with mitochondrial myopathy and neuropathy (see also next paragraph).

In some other patients affected by both hearing loss and hypoparathyroisim, although a genetic or syndromic cause was suspected, no distinct genetic or chromosomal defects could be demonstrated. Six members of one family were affected by autosomal dominant familial hypoparathyroidism and sensorineural deafness, without renal dysplasia. The proband had a normal 46, XY karyotype, without microdeletions in chromosome 22q11.2 as tested by FISH (Watanabe et al., 1998). Unfortunately, the authors do not describe the mitochondrial DNA, or chromosome 10p (see next paragraph). Finally, the existence of a familial idiopathic hypoparathyroidism – familial progressive sensorineural deafness – human leucocyte antigen syndrome was proposed by Yumita et al., after describing 3 cases from two families with these characteristics (Yumita et al., 1986).

Hypoparathyroidism, Deafness, Renal dysplasia (HDR) syndrome

In 1977 Bakarat et al. described two male siblings with hypoparathyroidism, 'nerve deafness' and nephrotic syndrome, both of whom died of renal failure during childhood. The authors suggested that this combination of symptoms might comprise a new association, possibly inheriting in an autosomal recessive way (Bakarat et al., 1977). However, it was not until several years later, in 1992 to be exact, when a larger family, affected by a distinct autosomal dominant syndrome of hypoparathyroidism, sensorineural hearing loss and renal dysplasia, was described (Bilous et al., 1992), that the triad of symptoms was recognized as a distinct clinical entity, that only since 1997 has been known by its acronym 'HDR syndrome' (Hasegawa et al., 1997).

The clinical presentation of HDR patients has been very diverse, their most prominent symptoms and signs ranging from seizures due to hypocalcaemia (this thesis; Beetz et al., 1997) and generalized psoriasis (Aksoylar et al., 2004), to recurrent cerebral infarctions (Fujimoto et al., 1999), and severe mental retardation, autism and calcifications of the basal ganglia (Verri et al., 2004). The variability in phenotypic expression may sometimes delay (Ishida et al., 2001) or even prevent diagnosis of HDR syndrome. The latter may hold true for a small number of patients, presented in medical literature over the last decades (Hameed et al., 2001; Shaw et al., 1991; Yumita et al., 1986) (see Table 1.2 for details, ** indicates patients that were never diagnosed with HDR syndrome).

Genetic cause of HDR syndrome

DiGeorge syndrome is a developmental field defect, characterized by facial dysmorphysms, congenital conotruncal heart defects, hypoparathyroidism and thymushypo- or aplasia (or T cell defects). Most patients are affected by a microdeletion in chromosome 22q11 (DGCRI). However in a small minority of patients the syndrome is caused by partial deletion of chromosome 10p (DGCRII). In the late 1990's several papers reported patients with Di-George or velo-cardio-facial like syndromes, lacking congenital heart disease but suffering from additional features such as renal malformations and sensorineural hearing loss. In some of these patients chromosomal aberrations telomeric from the DGCRII were detected (e.g. van Esch et al., 1999a). In a large retrospective review study, van Esch et al., compared the classical DiGeorge phenotype to the so-called 10p deletion syndrome, and found that in the latter in addition to the classical DiGeorge features severe mental retardation, sensorineural hearing loss, renal anomalies and severe growth retardation were commonly found (van Esch

Table 1.2

Overview of clinical and genetic features in (possible) HDR patients

Author / journal,	Clinical features	Diagnosis as made in paper	Genetic features
Barakat et al./ J Pediatr, 1977	***P***1- M (sibling *P*2) SNHL (90%), muscular weakness, carpopedal spasm, HPT renal biopsy: fetal like glomeruli † age 8 Path: small kidneys, cortical fetal lobulations, sclerosis of glomeruli, no parathyroids identified, normal thymus	?	- AR?
	P2 – M (sibling *P*1) bilateral SNHL, nephritic syndrome, cough, tachypnea, generalized seizure, carpopedal spasm † age 5 Path: kidneys cf *P*1, ectopic parathyroid glands, involuted thymus	?	- AR?
Beetz et al./ Monatsschr Kinderheilkd, 1997	***P***1 – M generalized seizures, SNHL, hypertelorism, epicanthic folds, psychomotor retardation, HPT, RD (small right kidney, bilateral vesicoureteral reflux grade 3)	?	?
	P2 – M (see also chapter 5 of this thesis) RD (right multicystic kidney, left dysplastc kidney), SNHL, HPT, intracerebral calcifications	?	?
Bilous et al./ N Engl J Med, 1992	***P***1 – M (sibling P2, father of P3-8) HPT (no clinical features), SNHL (since age 1, bilateral symmetric, all frequencies), RD	HDR	AD, normal karyotype
	P2 – M (sibling P1, uncle to P3-8) HPT (no clinical features), SNHL (since adulthood, bilateral symmetric, all frequencies), RD, diabetes	HDR	AD
	P3 – F (child of P1, niece of P2, sister of P4,7,8, half-sister of P5,6) HPT (no clinical features), SNHL (since infancy, bilateral symmetric, all frequencies), RD (small, irregular kidneys, with compressed collecting system)	HDR	AD
	P4 - F (child of P1, niece of P2, sister of P3,7,8, halfsister of P5,6) HPT (no clinical features), SNHL (since 5 years of age, bilateral symmetric, all frequencies), RD (small, irregular kidneys, with compressed colleting system) renal biopsy: dysplastic areas with compressed an partially sclerosed	HDR	AD

	glomeruli, atrophic tubules, fibrotic interstitium and focal lymphocytic infiltrate ***P***5 – M (child of P1, nephew of P2, brother of P6, half-brother of P3,4,7,8) severely handicapped after possible encephalitis at 3 months of age † age 8, no postmortem examination ***P***6 - M (child of P1, nephew of P2, brother of P5, half-brother of P3,4,7,8) † sudden death, infancy Path: normal kidneys ***P***7 - F (child of P1, niece of P2, sister of P3,4,8, half-sister of P5,6) RD, normal serum calcium concentrations, normal audiogram ***P***8 – M (child of P1, nephew of P2, brother of P3,4,7, half-brother of P5,6) RD † neonatal death Path: severe renal dysplasia, no normal renal tissue	Possible HDR Possible HDR Partial HDR Partial HDR	AD AD AD AD
Fujimoto et al./ Am J Med Genet, 1999	***P***1 – M recurrent left and right hemiplegia due to recurrent cerebral infarctions, mildly delayed psychomotor development, moderate mental retardation, HPT, SNHL (from age 7; bilateral, symmetric 40-50 dB), RD (pelvocalyceal deformity of kidneys)	HDR	46,XY,del(10)(p14P15.1) de novo
Hameed et al./ Postgrad Med, 2001	** ***P***1 – M cough, generalized edema, hypoalbunaemia, hypercholesterolaemia, HPT, RD (focal, segmental and glomerular sclerosis – kidney transplant), loss of motor functions, neuropathy, SNHL (from birth) Family history: profoundly deaf sister	Mitochondrial cytopathy	PCR of genome indicated a rearrangement no deletion of 10p13, no comments on possible mutations.
Hasegawa et al./ Am J Med Genet, 1997	***P***1 – F ventricular septal defect (spontaneous closure by age 2) , epilepsy, psychomotor retardation, hypertelorism, low set ears, low nasal bridge, micrognathia, bilateral clinodactyly of 4th toes, HPT, SNHL (90-100 dB), absent right kidney, pelvocalyceal deformity left kidney	HDR	46,XX, del(10)(p13)
Ishida et al./ Intern Med, 2001	***P***1 –F tonic-clonic seizure, hypocalcaemia, HPT, symmetrical calcifications in basal ganglia, SNHL (50 dB 1-2 kHz), RD (no right kidney on abdominal CT) Family history: Patients father HPT, SNHL (asymmetrical) ! Muroya paper: P1's daughter affected by hearing loss ! Ishida paper: P1's brother affected by hearing loss	familial idiopathic HPT SNHL and RD	no chromosome abnormalities (standard trypsin G-banding analysis) !see Muroya P7

Lichtner et al./ J Med Genet, 2000	***P***1 – F facial dysmorfism, psychomotor retardation, downslanting palpebral fissures, hypertelorism, blepharophimosis, ptosis, epicantic folds, curled eyelashes, stenosis of the lacrimal ducts, low nasal root, flat nose with anteverted nostrils, double left mandibular incisor, high arched palate, micrognathia, small round low set posteriorly rotated ears, preauricular sinus, short neck, widely spaced nipples, right sided accessory nipple, genital labial synechia, partial syndactyly of toes 2 and 3, clinodactyly of toe 4 and muscular hypotonia. SNHL (80 dB left and 50 dB right), tonic-clonic seizures from age 12, HPT, renal insufficiency ***P***2 – F ventricular septal defect, HPT, SNHL (60-80 dB), RD, delayed developmental milestones, hypertelorism, low set ears, low nasal bridge, bilateral clinodactyly of 4th toes, mild hypertrichosis	HDR HDR	46, XX, del (10)(p13) terminal deletion chromosome 10p, lack of paternal allele GATA-P19252, GATA-P34271
Mino et al./ Clin Exp Nephrol, 2005	***P***1 – F (mother of P2) renal dysfunction, hypertension, RD (absence of left kidney), HPT, SNHL, diffuse goiter ***P***2 – F (daughter of P1) hypocalcaemia, RD (vesicoureteral reflux), HPT, SNHL (left ear), goiter Family history: sister deceased of congenital heart disease, son aborted at 21 weeks multicystic kidney, rectal atresia, atrial septal defect, undescended testis, rocker-bottom feet	HDR HDR	heterozygous unusual mutation at exon 3 (709insC), resulting in premature stop at codon 302, loss of both ZnF domains “”
Muroya et al./ J Med Genet, 2001	***P***1 – F neonatal hypocalcaemia, SNHL (>105 dB), RD (bilateral pelvicalyceal deformity) ***P***2 – M hypocalcaemia, SNHL (>90 dB), RD (gross renal scar left –pyloric stenosis-, vesicoureteral reflux grade 4 right, grade 5 left) ***P***3 – F see P1 Hasegawa ***P***4 – M see P1Fujimoto ***P***5 – M (child of P6) HPT, SNHL (70 dB), RD (left renal hypoplasia, chronic renal failure, on dialysis)	HDR HDR see Hasegawa see Fujimoto HDR	nonmosaic chromosomal aberration; 46,XX,der(10)t(10;18)(p13; p11.22)pat nonmosaic chromosomal aberration; 46,XY,del(10)(p14)de novo see Hasegawa see Fujimoto heterozygous missense mutation ZF1 domain exon4 (T823A,W275R)

	P6 – F (mother of P5) hypocalcaemia, hearing aids, RD (left renal hypoplasia, chronic renal failure, on dialysis) † age 80 Family history: two deceased brothers (age 80 and 85) suffering from chronic renal failure, on dialysis	HDR	?
	P7 – F see P1 Ishida ! Muroya paper: P7's daughter affected by hearing loss ! Ishida paper: P7's brother affected by hearing loss	HDR	heterozygous unusual mutation at exon 4 (900insAA plus 901insCCT or C901AACCCT) resulting in premature stop-codon at 357 and loss of ZF2 domain
	P8 – F HPT, SNHL (hearing aids since childhood), proteinuria and haematuria Family history: one daughter normal ABR, but speech delay	HDR	heterozygous nonsense mutation at exon 6 (C1099T, R367X)
	P9 – M HPT, SNHL (bilateral, 30-40 dB), RD (right renal aplasia)	HDR	no mutations identified, only G/A polymorphism
	P10 – M HPT, SNHL (bilateral, > 105 dB), chronic renal failure, bilateral retinitis pigmentosa, short stature Family history: three aunts and one sister died in early neonatal period, one uncle affected by chronic renal failure, on dialysis	HDR	no mutations identified, only G/A polymorphism
Shaw et al./ Arch Dis Child, 1991	** ***P***1 – F (case 3 in paper) (cousin of P2) renal insufficiency (distal renal tubular acidosis), hypocalcaemia, hypotonia, HPT, possible infarcts left hemisphere, SNHL (right ear, absent ABR) † age 7 months Family history: consanguineous parents, neonatal death of brother	HPT with renal insufficiency and developmental delay	AR normal chromosomal pattern
	** ***P***2 – M (case 4 in paper) (cousin of P1) bilateral talipes equinovares, metabolic acidosis, hyperkalaemia, hypocalcaemia, renal insufficiency, HPT, developmental delay, SNHL (bilateral absent ABR), failure to thrive † age 14 months Path: absence of parathyroid glands, nephrocalcinosis, microcalcifications of the brain Family history: consanguineous parents	HPT with renal insufficiency and developmental delay	AR normal chromosomal pattern
Van Esch et al./ Clin Genet, 1999	***P***1 – M bilateral choanal atresia, developmental delay, SNHL (85 dB), multiple exostoses (knee and ankle joints), HPT, RD (solitary right kidney), retrognathia, small mouth, downslanting palpebral fissures,	Partial DiGeorge syndrome	46, XY ins (8;10) (8pter→8q13::10p14→10p13::8q24.1→8qter) ins(10;8)(10pter→10p14::8q24.1 → 8q13::10p13→ 10qter)

	hypertelorism ***P***2 – F convulsions, hypocalcaemia, RD (single dysplastic left kidney, vesico-ureteric reflux), SNHL (bilateral, 50 dB), uterus bicornus, mental retardation, hypertelorism, synophoris, small dysplastic ears with adherent lobuli, HPT, high arched palate	Partial DiGeorge syndrome	deletion distal part chromosome 10p
Van Esch et al./ Nature, 2000	***P***1 HPT, SNHL, renal agenesis ***P***2-5 (4 patients) HPT, SNHL, RD ***P***6 HPT, SNHL, RD ***P***7 HPT, SNHL, RD ***P***8,9 – M+F (mother and son) HPT, SNHL	HDR HDR HDR HDR HDR?	D10S1751-D10S1779, 990 kb deletion D10S1779, 250 kb deletion Frameshift from codon 156, exon 3 *GATA3* In-frame deletion codons 316-319, exon 5 *GATA3* 828C→T, Exon 4
Verri et al./ Ann Genet, 2004	***P***1 – M inguinal hernia, hypocalcaemic seizures, low set ears, short palpebral fissures, facial asymmetry, bristle hair, pectus excavatum, scoliosis, bilateral complete cataract, hypotonia, extrapyrimidal features, language impairment, ritualistic behavior, SNHL? (altered Auditory Evoked Potentials), basal ganglia calcifications normal renal function and ultrasounds	HDR?	haploinsufficiency of *GATA3* (FISH), partial deletion chromosome 10p
Yumita et al./ Tohoku J Exp Med, 1986	** ***P***1- M (case 3 in paper) tetany, SNHL (bilateral, 40-60 dB), bilateral cataract, calcification of basal ganglia, RD (aplasia or hypoplasia right kidney, HPT Family history: siblings, father and niece affected by SNHL	Familial idiopathic HPT and progressive SNHL	?
Zahirieh et al./ J Clin Endocrinol Metab, 2005	***P***1 – F SNHL (bilateral, high frequency), polycystic ovarian disease, hypertension, renal failure, HPT Family history: mother affected by SNHL (bilateral), mild mental retardation, sister affected by SNHL (bilateral, high frequency), hypocalcaemic seizures, HPT, mild mental retardation	HDR	heterozygous missense mutation, nonconservative change of a single amino acid (R276P) in ZnF1 domain

** = patients never diagnosed with HDR syndrome
† = deceased
AR = autosomal recessive inheritance
cf = in conformity with
F = female
HPT = hypoparathyroisdism
M = male
P = patient
Path = postmortem pathological examination
RD = renal dysplasia
SNHL = sensorineural hearing loss

et al., 1999b). Recently, it has been suggested that *BRUNOL3* may be responsible for heart and thymus defects associated with partial monosomy 10p (Lichtner et al., 2002) However, to remain within the scope of this thesis, from here on the focus will be mainly on HDR syndrome.

In 1997 Hasegawa et al. had already suggested that the putative gene(s) responsible for HDR syndrome should be located at a 10pter→p13 region, after demonstrating a terminal deletion of chromosome 10 (Del(10)(p13)) in one Japanese patient with HDR syndrome (Hasegawa et al., 1997). Three years later an HDR syndrome locus was officially mapped distal to the DiGeorge Critical Region on 10p13/14 (Lichtner et al., 2000), and shortly hereafter, van Esch et al. demonstrated that the syndrome is caused by haploinsufficiency of the *GATA3* gene (van Esch et al., 2000). Further evidence of this was provided by Muroya et al., demonstrating *GATA3* abnormalities in seven out of nine Japanese families with the HDR syndrome (Muroya et al., 2001).

An extensive analysis of different *GATA3* mutations in HDR syndrome, is given in a 2004 review paper (Nesbit et al., 2004). In this paper Nesbit et al. draw attention to the curious fact that a correlation between the *GATA3* mutations and the observed HDR phenotype has not been established so far; the same mutation in different families (or even within a single family) may lead to different phenotypes. Two new mutations have been identified since the appearance of Nesbit's review paper leading to a total of 21 mutations known today (Mino et al., 2005; Zahirieh et al., 2005). It is not unreasonable to expect that more mutations will be identified in the future: in fact we describe a new mutation identified in a Dutch HDR patient in Chapter 5 of this thesis.

Transcription factor GATA3

In mammals immature, multipotent precursor cells develop into single cell lineages. Lineage specific transcription factors play an important role in this cellular differentiation. A specific group of transcription factors consists of several zinc finger proteins, characterized by a GATA element (A/T GATA A/G), which together constitute the so-called "GATA family". As compared to other families of transcription factors, that may include dozens to hundreds of members, the GATA family is relatively small with three to four factors found in *Drosphila* and up to eleven in *Caenorhabditis elegans*. Six GATA factors have been identified in vertebrates, these are expressed in different tissues; GATA1 in haematopoietic progenitor cells, different blood cells and testes, GATA2 in haematopoietic progenitor cells, different

blood cells, endothelial cells and embryonic brain, GATA4 in heart, intestinal epithelium, primitive endoderm and gonads, GATA5 in heart, lung, gut and spleen and GATA6 in heart, lung, gut, liver and gonads. GATA3 is expressed in T-lymphocytes, endothelial cells, placenta, kidney, adrenal gland, embryonic brain, adult CNS and adult PNS (Patient et al., 2002; Simon, 1995).

The human *GATA3* gene was cloned in 1994 by Labastie et al., who demonstrated that the gene contains six exons distributed over 17 kb of DNA (Labastie et al., 1994). The two zinc fingers are encoded by two different exons. ZnF2 (or the C-terminal zinc finger) binds DNA, whereas this binding is stabilized by the N-terminal zinc finger (ZnF1). Besides, ZnF1 interacts with other multitype zinc finger proteins, e.g. the friends of GATA (FOG) (Nesbit et al., 2004).

Although the exact target genes of GATA3 remain unknown today, a recent extensive microarray study indicated no less than 261 possible target genes, grossly belonging to two groups; genes involved in transcription regulation and genes involved in cellular signaling (Airik et al., 2005).

The broad distribution of GATA3 and the large number of possible target genes, suggest the gene to be involved in a great variety of processes. Indeed over time it has been shown that GATA3 is –to name only a few examples- involved in the development of serotonergic neurons in the caudal raphe nuclei (van Doorninck et al., 1999), cellular proliferation in the thymus and development of T-cell progenitors (Hendriks et al., 1999). Mouse embryos, that are entirely deficient in Gata3 die at 11 days post conception, due to noradrenaline deficiency of the sympathetic nervous system (Lim et al., 2000), massive internal bleeding, marked growth retardation, severe deformities of the brain and spinal cord and gross aberrations in fetal liver haematopoiesis (Pandolfi et al., 1995). In contrast, mice heterozygous for the *Gata3* mutation survive, being even fertile (Pandolfi et al., 1995).

During early developmental stages Gata3 is expressed inside the otocyst and the surrounding periotic mesenchyme as well as in a population of neurons in rhombomere 4, where it is involved in morphogenesis of the ear and pathfinding of efferent neuron axons that navigate to the ear respectively. An elongated cyst is what remained of the entire ear in *Gata3* mutant embryo's (E13) (Karis et al., 2001). In 1998 Rivolta and Holley found downregulation of Gata3 in the mouse cochlea during hair cell differentiation, leading them to suggest that Gata3 could act as a repressor of genes involved in cell differentiation in the organ of Corti – enabling formation of the adult cellular pattern (Rivolta et al., 1998). In chapter 3 of this

thesis we contradict this suggestion by demonstrating Gata3 expression in adult murine cochleae.

With increasing knowledge about this transcription factor, *GATA3* seems to be an increasingly 'hot-topic'. It has been suggested that GATA3 variants may contribute to tumorigenesis in ESR1-positive breast tumors, through their involvement in growth control and maintenance of a differentiated state in epithelial cells (Usary et al., 2004). Moreover, *GATA3* has also been subject of recent otological publications. It is a well-known fact that in contrast to mammalian cochlear hair cells, avian cochlear hair cells have the capacity to regenerate after being damaged. Interestingly, it has been demonstrated that *GATA3* is amongst the up-regulated genes in regenerating avian cochleas (Hawkins et al., 2003).

Audiometry in mouse models of hearing loss

Great structural similarities exist between the ears of almost all (equatrian) mammals; this also applies to the murine and human ear. All structures found in the human ear are present in the murine ear as well. There are, however, some differences. One of those is the presence of a persistent stapedial artery in mice, a condition that is only very rarely found in man. In humans the inner ear lies within the bony trabeculations of the mastoid, whereas in mice the cochlea protrudes into the tympanic bulla, a 3.5 x 5.0 mm air-filled cavity. Another distinction is the length of the cochlear duct; 2.5 turns in man, as compared to, 1.5-2 turns in the mouse (Bohne et al., 1997; Jero et al., 2001b). The length of the mouse cochlea varies both within and across mouse strains (Ou et al., 2000).

Although previously the mouse was not considered an ideal animal model for studying hearing loss, due to disadvantages related to its small size, it is now rapidly becoming the animal of choice for several reasons. Great progress has been made in mouse genomics and several mouse models of hearing loss have been developed. As stated earlier, there is great similarity between murine and human ears. Finally, breeding characteristics of mice, where big litters are born after a relatively short gestation, make this animal an ideal model for studying genetic defects.

Preyer's reflex / Acoustic Startle Response

A method that has been extensively used (in the past) for the evaluation of hearing in laboratory mice is Preyer's reflex; the observation of a startle response elicited by a loud auditory stimulus. This method has certain advantages; it is easy and it can be rapidly

performed in large number of animals, and there's no need for any equipment to elicit the response. There are however some limitations; the test is based on a subjective observation of the animal by the investigator, and based on the test outcome no distinction between sensorineural and conductive hearing loss can be made. When categorizing mice as being genetically deaf or not, animals could easily be misclassified due to (possibly) self-resolving middle-ear problems. Furthermore the method is not very sensitive; Jero et al. found that animals with an ABR threshold equal to or under 76 dB SPL had positive reflexes. They suggested that the Preyer's reflex should only be used for rough screening of hearing in mice (Huang et al., 1995; Jero et al., 2001a).

Auditory Brainstem Response

Auditory Evoked Potentials (AEP's) are electrical potentials occurring in response to auditory stimulation, recorded at various levels of the auditory system. Already in 1974 Picton et al. categorized various types of Auditory Evoked Potentials as 'long latency responses'

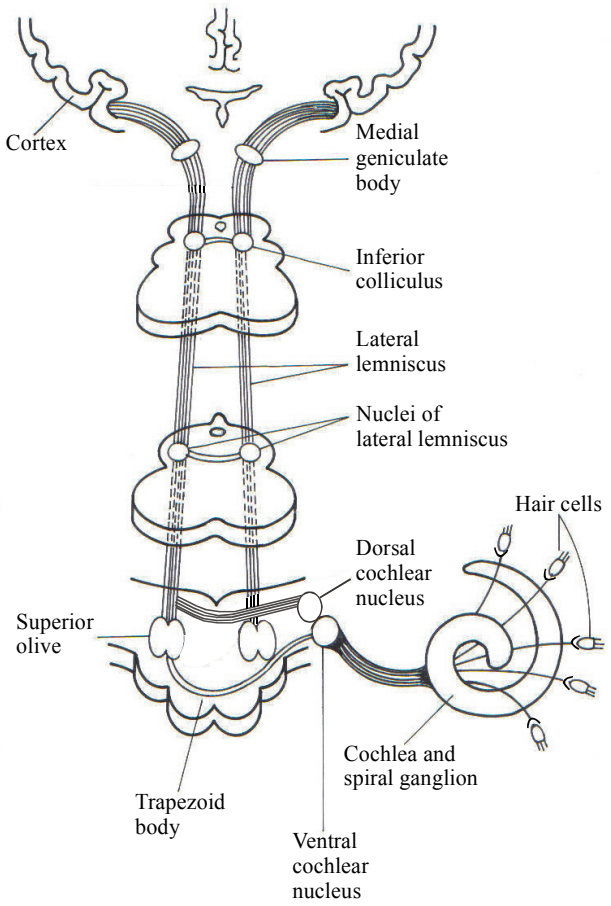

Figure 1.2: The ascending pathways of the auditory system in man (adapted from van den Broek and Feenstra, 2007).

- probably representing widespread activation of the frontal cortex-, 'middle latency responses' – representing the auditory thalamus and cortex - and 'early responses' – representing activation of the cochlea and the auditory nuclei of the brainstem – (Picton et al., 1974). Of these the early responses, and more specifically the Auditory Brainstem Response (ABR), have proved to be most useful, both in clinical practice and in experimental research.
In man Auditory Brainstem Responses (ABRs) are commonly used to screen for retrocochlear pathology (e.g. Godey et al, 1998; Rupa et al, 2003), to test hearing in newborns being at high risk for hearing impairment (e.g. Watson et al., 1996) and to objectively determine hearing thresholds, when subjective hearing evaluation is impossible.
The origins of the different ABR-peaks, in anaesthetised laboratory mice, were described by Henry in 1979. Based on lesion techniques and local recordings he concluded that PI corresponds with the cochlea and the action potential from the auditory nerve, and that PII-V originate from the ipsilateral cochlear nucleus, the contralateral olivary nuclei and the nucleus of the trapezoid body, a source between the cochlear nuclei and inferior colliculi and from the lateralmost portion of the contralateral inferior colliculus, respectively (Henry, 1979). Several years later an anatomical frequency-place map for the mouse organ of Corti was developed, by comparing noise-induced cochlear lesions with ABR-thresholds shifts in C57BL/CBA F1 mice (Ou et al., 2000). Nowadays, the recording of ABRs by subcutaneous needle electrodes, has become a routine method for hearing evaluation in laboratory mice.

Distortion Product Otoacoustic Emissions

In 1978 Kemp was the first to describe an acoustic phenomenon present in healthy ears, but absent in ears with cochlear hearing loss. He suggested that the non-linear acoustic responses to auditory stimulation he recorded in the external acoustic meatus originated in the cochlea, and depended on normal functioning of the cochlear transduction process (Kemp, 1978). Today these responses are known to be by-products of the active motility of outer hair cells, serving as amplifiers of the displacement of the cochlear partition. Various subgroups of these so-called otoacoustic emissions are recognised, occurring both spontaneous (SOAE's – Spontaneous Otoacoustic Emissions-) and after stimulation (Evoked Otoacoustic Emissions; TEOAE's – Transient Evoked Otoacoustic Emissions-, CEOAE's – Continuous Evoked Otoacoustic Emissions-, DPOAE's – Distortion Product Otoacoustic Emissions- and SFOAE's – Stimulus Frequency Otoacoustic Emissions-) (e.g. van Zanten et al., 2002).

Interestingly, nowadays (evoked) otoacoustic emissions (OAE's) represent the most accurate measure for testing OHC function (Schrott et al., 1991), with a broad range of applications in neonatal hearing screening, clinical practice and scientific research. In murine auditory research otoacoustic emissions have been used to study (amongst others) age related hearing loss (Jimenez et al., 1999; Parham, 1997; Parham et al., 1999), noise induced hearing loss – NIHL - (Jimenez et al., 2001; Vazquez et al., 2001), effects of gender on hearing (Guimaraes et al., 2004), effects of hypothyroidism on OHCs (Li et al., 1999) and the effects of genetic mutations on the auditory system (Huang et al., 1995; Huang et al., 1998; Konrad-Martin et al., 2001) – see also chapter 4 of this thesis.

Apart from the above-mentionted, commonly used techniques, other specific acoustic tests such as ECochG and patch clamp studies of central auditory nuclei may provide additional useful electrophysiological information on specific parts of the auditory pathway (in laboratory animals). However, discussion of these techniques is beyond the scope of this thesis.

Scope of this thesis

In recent years great progress has been made in the understanding of genetically induced hearing impairment. Papers of great interest on this subject are published frequently. In this thesis the nature of hearing loss in Hypoparathyroidism, Deafness and Renal dysplasia syndrome is studied with a variety of different techniques.

Hearing acuity in laboratory mice is often assessed by measuring Auditory Brainstem Responses (ABR) under general anesthesia. Some anesthetics are known to cause an increase in ABR-peak latencies in laboratory animals such as gerbils and rats. In **chapter 2** we evaluate the possibility of hearing assessment in alert mice. The possible effects of general anesthesia on peak and interpeak latencies and on ABR-thresholds are studied. In addition we look into the effects of varying post stimulus time frame duration, and stimulus repetition rate.

In **chapter 3** and **chapter 4** we assess whether the auditory system of heterozygous *Gata3* mice may serve as model for the auditory system of patients affected by the HDR syndrome. Hearing in the mouse model is evaluated by ABR-recordings and distortion product otoacoustic emissions (DPOAE)-measurements, whereas cochlear histology is analyzed by light microscopical examination of cochlear quarter turns and of semithin slices of the organ of Corti and by electron microscopical examination of cochlear hair cells. **Chapter 3** also

addresses the expression patterns of Gata3 in both the inner ear and the auditory parts of the central nervous system. Using the combined results of the abovementioned experiments we evaluate the nature of the hearing loss and postulate a hypothesis on the pathogenesis of the hearing loss in HDR syndrome.

A link between the basic scientific experiments, described in the previous chapters and clinical research is tentatively made in **chapter 5**. The hearing of two patients affected by HDR syndrome is described in detail. Human and murine audiometrical data are compared.

Experimental data described in chapters 2 to 5 are discussed in **chapter 6.** In this chapter some new developments in mouse auditory research are discussed, that may lead to directions for future research.

The main goal of this PhD study is to contribute new knowledge to the field of genetic hearing impairment, and more specifically to elucidate the underlying mechanisms causing hearing loss in HDR syndrome. In addition it is tried to bridge the gap between basic scientific and clinical research by comparing results of auditory tests performed in mice and men.

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Chapter 2:

Impact of conventional anesthesia on Auditory Brainstem Responses in mice

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“Don’t spend time beating on a wall, hoping to transform it into a door. ”

Coco Chanel.

Summary

Anesthesia is known to affect the Auditory Brainstem Response, ABR, in animals often used in hearing research. This study describes the differences in ABRs, between awake and anesthetized FVB/N mice. Intracranial electrodes connected to a head fixation pedestal were used for click-evoked ABR recordings. This pedestal served to immobilize mice, either awake or under anesthesia, in a 'free' sound field.

The presence of myogenic noise in the awake condition obviously increases recording time. However it is demonstrated that recording times can be significantly reduced by increasing the stimulus repetition rate from 23 up to 80 impulses per second. This causes only a small but significant increase in absolute peak latencies in the awake condition, but has no significant effect on the overall ABR-waveform, nor on the ABR-threshold, nor on the ABR interpeak latencies, nor on the absolute peak latencies in the anesthetized condition.

Anesthesia with ketamine/xylazine caused a significant prolongation of ABR-peak latencies and interpeak latencies as well as a significant upward shift (8.0 ± 1.8 dB) of ABR-thresholds as compared to the awake condition. Under anesthesia the measurement accuracy of peak latencies, interpeak latencies and thresholds decreases. In conclusion, the awake condition is preferable for more accurate measurements of ABR characteristics, in spite of the myogenic noise concomitant with this condition.

Keywords: ABR, anesthesia, awake, mouse

Introduction

The mouse is often used as a model to study effects of various genetic deficits on hearing. Anatomically the mouse cochlea is similar to that of the human (Steel and Bock, 1983) and, analogues between murine and human hereditary abnormalities in hearing are well established (Brown and Steel, 1994, Call and Morton, 2002; Steel, 1995; Tekin et al., 2001).

Assessment of hearing acuity in mice is done in many studies (e.g. Ou et al, 2000; Rusch et al, 2001; Trune et al., 1996; Zheng et al., 1999) by measuring the Auditory Brainstem Response (ABR) thresholds. Invariably those assessments are done in anesthetized animals. The combination of ketamine and xylazine is often used to effectuate narcosis in mice during ABR measurements (Henry, 2002; Huang et al., 1995; Jero et al., 2001; Miller et el., 1998; Ou et al., 2000). Several studies have demonstrated that ketamine increases peak latencies in gerbil and rat, while ABR thresholds remained unchanged (Church and Gritzke, 1987; Smith and Mills, 1989; Smith and Mills, 1991). Here we evaluate the possibility of hearing assessment by ABR in awake mice. ABR recording in awake animals is obviously more time consuming than in anesthetized mice because of myogenic noise, which triggers the artifact-rejection mechanism of the recording system. On the other hand, recording time can be reduced, possibly without significantly affecting the ABR, by increasing the stimulus repetition rate to values exceeding the often-used range of up to 20 per second. For eliciting the ABRs we simply used click stimulation as we do expect anesthesia to influence the various frequency regions in the cochlea alike.

The questions to be answered in this study are:

1) Is it possible to measure ABR thresholds in the awake mouse? ; 2) Does anesthesia with ketamine/xylazine affect the ABR waveform? ; 3) What is the accuracy of threshold measurements in both conditions? ; 4) Do peak latencies and thresholds of the responses significantly differ between awake and anesthetized animals? ; 5) Do these differences, if present, significantly depend on stimulus repetition rate?

Material and Methods

Mice

Twelve mice (7 females, 5 males) of the FVB/N strain were included in this study. At recording time their age range was 12-16 weeks.

Experiments were performed in accordance with the "Principles of laboratory animal care" (NIH publication No. 86-23, revised 1985) and the guidelines approved by the Erasmus University animal care committee (DEC; protocol No. 138-01-03). No (apparent) signs of distress (such as weight loss, stereotypical behavior, squeaking, scratching or eye-blinking) were observed in our mice during or after experiments.

Surgical technique

For the purpose of fixation of the animal in the experimental set-up and for connection of electrode leads, each mouse was operated upon approximately one week before ABR measurements. Animals were anesthetized with a mixture of O_2, N_2O and 2% halothane. Two platinum wires, serving as reference electrodes, were placed in hand-drilled holes in both left and right occipital bones at the level of Crus2 of the cerebellum. A stainless steel screw, which served as the active electrode, was fixed to the skull between the two parietal bones, as close as possible to the sagittal suture. Finally, another stainless steel screw, serving as common ground electrode, was drilled into either the left or the right frontal skull. All electrodes and wires as well as four other screws, placed solely for the purpose of fixation, were covered with dental cement, in which a four-poled connector was fixed. Total time of surgery was approximately 45 minutes. Morbidity and mortality were low.

ABR-recording

The active electrode was connected with the positive inputs of a 2-channel physiological amplifier and the reference electrodes to the negative inputs. ABR recording was carried out with help of custom-made equipment. We used the EUPHRA-I system (Erasmus University Physiological Response Averager) for stimulus control and response recording. This 2-channel response averaging system consists of a PC combined with components for stimulus presentation and response acquisition and averaging (DSP Motorola 56002, 32 MHz). The filter bandpass was 20-8000 Hz. Artifact rejection was used during averaging, the rejection criterion being ±40 μV. Stimulus clicks were generated by the DSP board (duration 100 μs; repetition rates 23 up to 80 per second). Five hundred accepted sweeps were averaged per response.

Clicks of alternating polarity were binaurally presented in a frontal free field condition with a one-tweeter loudspeaker (Radio Shack Super Tweeter 40-1310B) at a constant 40 mm distance between the cone tip of the tweeter and the external auditory canals of the animal. In subsequent peak latency determinations the 0.117 ms acoustic delay was not corrected for. A Bruel & Kjaer sound level meter (2218 with 1613 octave filter set and a ½ inch 4134 microphone) was used for acoustic calibration in dBpeSPL.

Response peaks were labeled with roman numerals I through V. As response waveforms appear to be strain dependent (see waveforms reported in (Le Calvez et al., 1998, Zheng et al., 1999)) we adopted a naming convention, for the anesthetized condition as much in correspondence with existing literature as possible, e.g. Figures 2.2 and 2.3.

Response thresholds were determined by decreasing the stimulus level with 20 dB steps from 80 or 90 dBpeSPL until no response peaks were detectable, and by subsequently increasing the level with 5 dB steps until the peaks reappeared (Huang et al., 1995). Reproduction of responses was done at and around the threshold level. The response threshold was defined as the lowest stimulation level at which the response for a specific stimulus waveform showed a reproducible peak, as judged subjectively by two experienced judges. Responses were stored in the PC for off-line statistical processing.

Experiments

Figure 2.1 shows a photograph a mouse in the experimental set-up. To enable positioning in the experimental set-up all animals were anesthetized shortly with a mixture of O_2, N_2O and 2% halothane. Before 'awake state' recordings were started, at least a 20-minute time span was observed, allowing the mouse to fully recover from this momentary anesthesia.

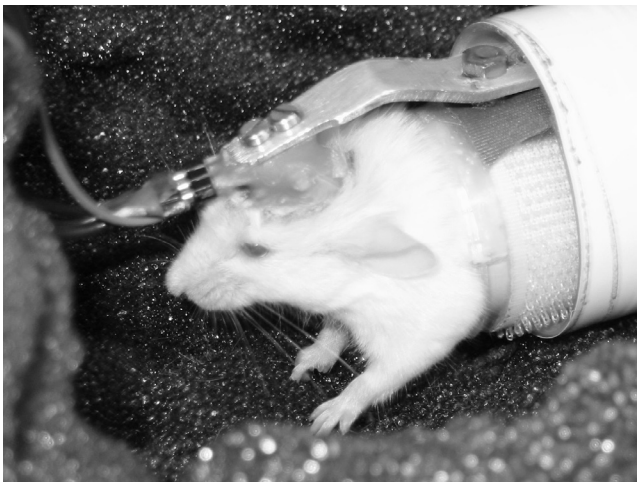

Figure 2.1: An awake mouse immobilized in the experimental set up.

During hearing assessment, mice were placed in a sound-attenuating box (50 cm x 50 cm x 50 cm) made out of plywood and with insides and all free surfaces covered with sound absorbing

foam rubber (see Figure 2.1). Tests were carried out in a quiet laboratory environment. During the recordings, the environmental sound level in the box complied with the NR10 standard in the frequency range 3 kHz and up.

In eight animals ABR waveforms were measured in both the awake as well as in the anesthetized condition in order to establish qualitative differences. When animals were assessed in both conditions on a particular day the awake condition was assessed first.

For hearing assessment in the anesthetized state, a conventional method of anesthesia was used (120 mg ketamine + 7.5 mg xylazine per kg body weight I.P.). This sufficed for a minimum of 45 minutes duration of anesthesia allowing completion of the hearing assessment. The individual durations varied from 45 minutes to 90 minutes.

During measurements body temperature was monitored via a copper-constantan thermocouple and a DC-Voltmeter, a set-up with a 0.5 degree resolution and calibrated against a conventional mercury thermometer between 32 and 44 °C. Body temperature was maintained constant with help of a heating bulb switching on when body temperature dropped by 0.5 degree below 37 °C.

In the same eight animals as mentioned before the response peak latencies and response thresholds were measured in both conditions as a function of stimulus repetition rate. In four additional animals, only the awake condition was assessed.

The following outcome variables were recorded: Response waveforms in the awake and anesthetized condition, response waveforms as a function of duration of the post-stimulus time frame (being varied between 5 and 20 ms), response thresholds (dBpeSPL) (stimulus level was varied between 0 and 90 dBpeSPL), peak latencies (ms), and finally interpeak latencies (ms). Furthermore, for a frame duration of 5 ms, the repetition rate of stimulation was varied between 23 and 80 per second.

Data processing

Animal identification data were stored in a spreadsheet file (Excel). Data values of the experimental and outcome variables were stored in a database (Access), containing each measured response with its annotations, e.g. the peaks' latencies. Statistical processing of the imported data was done with help of SPSS for Windows, version 11.0. Mean and median values, ranges, and standard deviations were calculated and tests of statistical significance of differences between various experimental conditions were done. Differences were considered to be statistically significant when p-values were below 0.02.

Results

ABR waveforms of six mice for both the awake and anesthetized condition are shown in Figure 2.2. The upper trace in each panel was recorded in the awake condition and the lower trace under anesthesia. It appears from this figure that peak latencies increase and amplitudes decrease when animals are anesthetized.

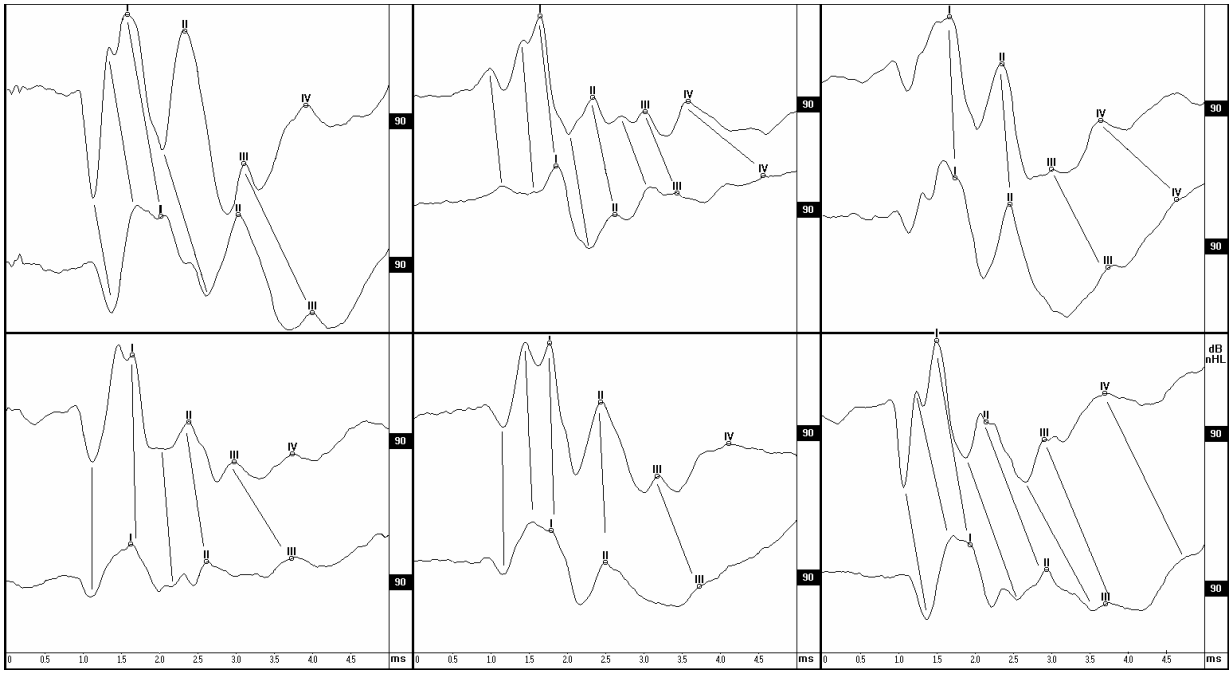

Figure 2.2: Examples of ABRs recorded at 90 dB nHL (normal Hearing Level) in six different mice. In each panel the ABR recorded in the awake state is the upper trace, and in the anesthetized state the lower one. In both states identical positive and negative peaks are identifiable, as indicated with connecting lines. In the anesthetized state amplitudes of response peaks are smaller and peaks are delayed as compared to the awake situation. The delay increases with peak number. The anesthesia affects the ABR of different mice differently.

Post stimulus time frame duration and repetition rate

Response waveforms to 80 dBpeSPL click stimuli are shown in Figure 2.3, for a short and for long post stimulus time frame duration. Both responses were acquired in the anesthetized condition. Peak V is the final peak present for both conditions and the response is flat after approximately 10 ms. For the awake condition the responses are similar in shape except for the differences already shown in Figure 2.2.

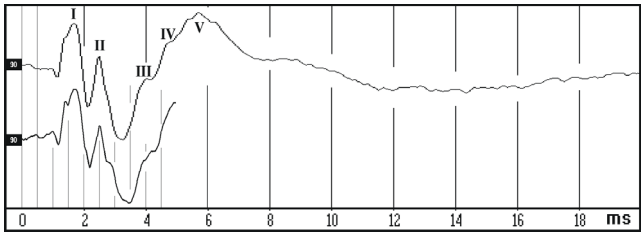

Figure 2.3: ABR recorded at 80 dBpeSPL in mouse 2 for two time-frame durations for post stimulus recording. No peaks are present at latencies above 10 ms. A repetition frequency up to 100 impulses per second can safely be used, without the risk of waveform distortion due to aliasing.

Effects on (inter)peak latencies

The overall response waveform did not change with stimulus repetition frequency in either condition. Table 2.1 shows the means and standard deviations of the absolute latencies of peak I through III and the interpeak latencies for different stimulus repetition frequencies, for both conditions. It is clear that in the anesthetized condition, latencies are longer and increasingly so with higher peak numbers. The differences between the mean values for the awake and anesthetized condition are all significant (Mann-Whitney U test, $p<0.02$).

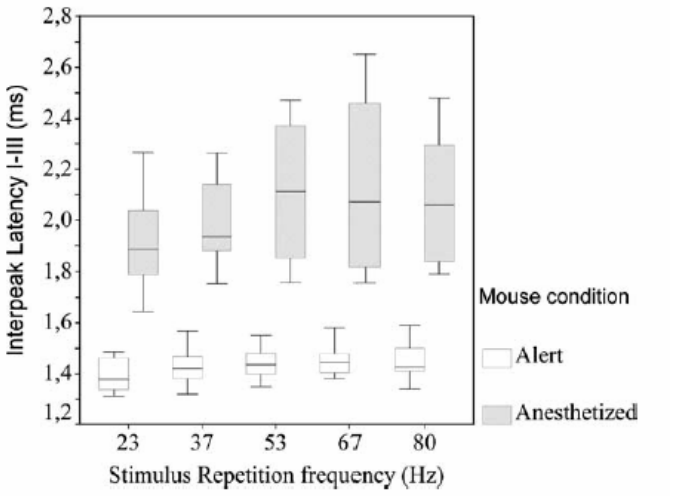

Figure 2.4: Boxplot showing, for a stimulus level of 80 dBpeSPL, vertically the ranges (whiskers), the interquartile ranges (boxes), and median values (horizontal bars in boxes) of the interpeak latency I to III at each stimulus repetition rate (horizontal), for the awake (open boxes) and the anesthetized condition (shaded boxes).

Figure 2.4 shows a boxplot of median values, ranges, and interquartile ranges for the interpeak latency of peaks I and III for both conditions, as a function of stimulus repetition rate. In the awake condition, the absolute peak latencies increase significantly with stimulus repetition rate (linear regression analysis, $p<0.02$). However, the absolute increase is about the

Stimulus repetition rate (per second)		Condition			
		Awake		Anesthetized	
	Peak	Mean	Standard Deviation	Mean	Standard Deviation
23	I	1.68	.05	1.87	.06
	II	2.36	.05	2.72	.12
	III	3.07	.07	3.80	.18
	IPL_I-II	.68	.05	.85	.11
	IPL_I-III	1.39	.07	1.92	.21
37	I	1.68	.07	1.86	.06
	II	2.37	.05	2.76	.15
	III	3.11	.06	3.88	.21
	IPL_I-II	.69	.04	.89	.15
	IPL_I-III	1.43	.08	1.99	.21
53	I	1.71	.07	1.86	.05
	II	2.37	.04	2.72	.10
	III	3.15	.07	3.95	.21
	IPL_I-II	.66	.04	.86	.11
	IPL_I-III	1.44	.06	2.11	.32
67	I	1.74	.06	1.86	.05
	II	2.39	.04	2.72	.12
	III	3.17	.07	3.99	.34
	IPL_I-II	.65	.05	.86	.11
	IPL_I-III	1.45	.07	2.14	.40
80	I	1.75	.08	1.87	.05
	II	2.42	.04	2.72	.11
	III	3.20	.11	3.95	.21
	IPL_I-II	.67	.06	.85	.11
	IPL_I-III	1.45	.08	2.09	.29

Table 2.1: Per repetition rate of the 80 dBpeSPL click-stimulus, means and standard deviations are given for the latencies of peak I, II, and III, as well as for the interpeak latency between peaks II / III and I, for both the awake and anesthetized condition. The differences between the mean values for the awake and anesthetized condition are all significant (Mann-Whitney U test, $p<0.02$).

same for all 3 peaks considered. A linear regression analysis between interpeak latencies and repetition rate revealed no statistically significant correlation of interpeak latency with increasing stimulus repetition rate. In the anesthetized condition, neither peak latencies nor interpeak latencies increased significantly with stimulus repetition rate.

Effects on ABR thresholds

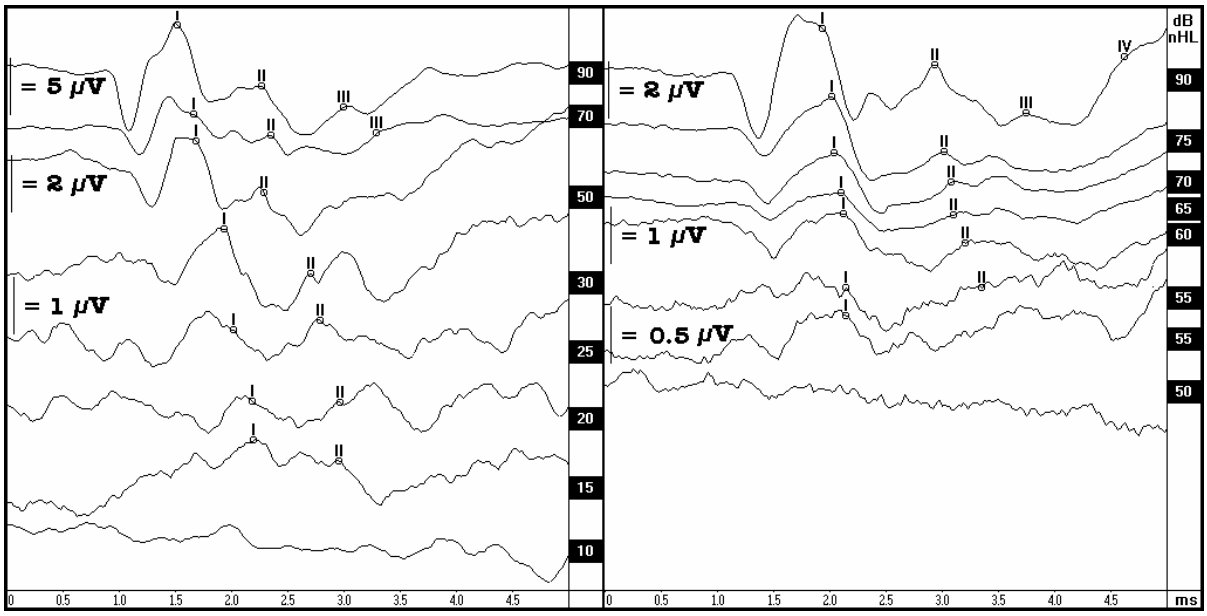

Figure 2.5: Examples of ABR patterns a mouse in the awake state, left panel, and in the anesthetized state, right panel. In the latter state the response amplitude is lower, the peak latencies are higher and the response threshold is also higher than in the awake state (55 and 15 dB nHL (normal Hearing Level) respectively).

Response patterns for one animal in both the awake –left panel– and anesthetized –right panel– conditions are shown in Figure 2.5. The response thresholds are 15 and 55 dBpeSPL, respectively. The figure shows the largest difference found between an awake and an anesthetized condition in any animal. Between one and four response patterns were acquired for each mouse in each condition. Per mouse and per condition the mean, the lowest and the highest thresholds were calculated. Overall, the mean ABR threshold for awake mice was 25.1 dB (s.d. 4.3 dB, s.e. 0.8 dB) whereas under anesthesia it was 33.1 dB (s.d. 8.6 dB, s.e. 1.6 dB), i.e. an increase of 8.0 ± 1.8 dB. This increase is statistically significant (T-test, $p<0.0005$; Mann-Whitney, $p<0.0005$). It is clear that under anesthesia, thresholds are higher and more variable. Figure 2.6 shows a box plot of median values, ranges and interquartile ranges of the mean response thresholds as a function of stimulus repetition rate for both

conditions. Linear regression analysis failed to show a significant effect of the stimulus repetition rate on the response thresholds for both conditions.

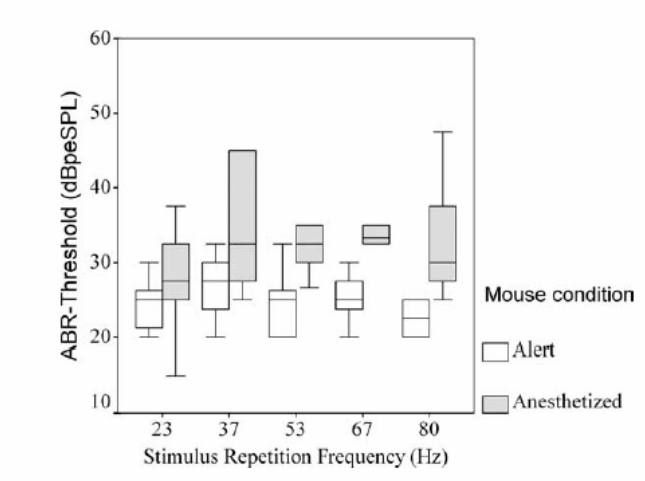

Figure 2.6: Ranges (whiskers), interquartile ranges (boxes), and median values (horizontal bars in the boxes) of the response threshold for both conditions and for increasing repetition rate of the stimulus. Linear regression analysis failed to show a significant relation between ABR-thresholds and repetition rate.

Discussion

In this study we evaluate the effects of ketamine/xylazine anesthesia on the outcomes of hearing assessment by ABR in mice, since this is the animal of choice for studying genetically induced hearing defects. Several past studies reported that ketamine induced increases in peak latencies in gerbil and rat without altering ABR thresholds (Church and Gritzke, 1987; Smith and Mills, 1989; Smith and Mills, 1991).

We demonstrate that it is possible to record ABR waveforms and ABR thresholds in the awake mouse. The pedestal for immobilizing the animal in a sound field used in this study stays in place for at least 3-6 weeks, and in some animals considerably longer (up to 5 months in this series), and loosening of the pedestal occurred without apparent signs of distress and infection. Infections were very rare and could successfully be treated with locally applied antibiotic eye drops.

Although we were successful in immobilizing the animals' heads, both ears were free to move. Therefore in the awake condition sound perception may have been influenced by pinna orientation. This confound might theoretically be avoided with a closed sound stimulation system. We feel that in the awake animal such a system would cause ethically unacceptable discomfort and stress.

We compared the waveforms for the anesthetized condition with waveforms reported in the literature for other mouse strains (CD1: Le Calvez et al, 1998; CBA/CaJ: Zheng et al 1999). From this comparison, we adopted a convention for peak labeling as much in line with existing literature as possible. For the FVB/N mouse strain that we used examples of waveform patterns are shown in Figures 2.2, 2.3, and 2.5. We inferred the peak identification for the awake condition directly from the anesthetized condition (see Figure 2.2).

Subjective judgment of the ABR threshold, as used in this study, is conventional in clinical settings (e.g. Sininger, 1993; Watson et al., 1996). This method is also used in animal studies, although sometimes not specifically reported (e.g. Huang et al., 1995, Zheng et al., 1999). An objective method of determining peak presence and latency, by applying an absolute amplitude criterion, as used for example in Ou et al. (2000), is very suitable for responses recorded in the anesthetized condition, because the residual noise in the averaged response is lower than in the awake condition. In the latter condition the background noise in the averaged responses is due to myogenic potentials. We estimated an objective method less suitable for this study than the subjective method, because the former will probably identify spurious peaks as response peaks.

The threshold we found for the anesthetized condition is in line with results from the literature. Using click stimulation at a rate of 19.1 per second, Zheng et al. (1999) reported the ABR thresholds for the FVB/N strain to be 33 and 38 dB SPL, at 9 and 28 weeks of age, respectively. These findings correspond very well with the threshold we determined under anesthesia at ages 12-16 weeks, which was 33.1 dB.

As could be expected in general, the effect of increasing the duration of the post-stimulus recording time frame is merely a lengthening of the response waveform (see Figure 2.3). For the FVB/N mouse strain used in this study, it is also clear that the response is flat after about 10 ms. We concluded that the duration of the ABR-assessment could be reduced by a factor four to five by increasing the repetition rate of the stimulus up to 100 per second without risking significant waveform changes due to response aliasing effects. This is not necessarily true for other stimulation conditions than the clicks, e.g. for tone pips. In this study a maximum repetition rate of 80 impulses per second was used.

For the awake condition, increasing the stimulus repetition rate causes a small but significant increase of absolute peak latencies, but not of interpeak latencies (see Table 2.1). The increase in absolute latencies is due to adaptation (Lasky 1997; Paludetti et al, 1983; Soucek and Mason, 1992), an effect that is thought to be caused by neural fatigue (Terkildsen et al, 1975).

For the anesthetized condition, this effect is not significant. The lack of statistical significance in the anesthetized condition is probably due to the higher dispersion of data in this condition (see Figure 2.4 and Table 2.1). There was a small effect of repetition rate on latency but it was largely overwhelmed by the variance due to anesthesia.
In this series, the ABR threshold does not depend significantly on the stimulus repetition rate in the range of 23 to 80 per second. We are of the opinion that it is unlikely that the threshold will be lower at slower repetition rates.

Importantly, however, response thresholds are significantly lower in the awake than in the anesthetized condition (difference of 8.0 ± 1.8 dB). Although ketamine has been demonstrated to affect ABR peak latencies and amplitudes in laboratory animals such as gerbils (e.g. Smith and Mills, 1989; Smith and Mills, 1991), to our knowledge, no effects on ABR thresholds have been described. One possible explanation for this could be that, whereas in the past in studies on the effect of anesthesia on ABR thresholds, occasionally 10 dB steps were used to obtain these thresholds (Smith and Mills, 1989), we used 5 dB steps. So, we conclude that the 8 dB threshold increase is due to the anesthesia. We can however not exclude that this increase is partially due to pinna orientation by the awake animal. The use of anesthesia also significantly increases absolute latencies of peaks I through III, and interpeak latencies I-II and I-III. The increase in absolute latency of peak I is in contrast with the results reported by Smith and Mills (1989). The prolongation of peak I (originating from the cochlea and VIIIth nerve (Henry, 1979)) might be caused by ketamine induced alterations in conductance of n. VIII. Ketamine decreases resting membrane potentials, leading to a profound effect on electrical activity of nerve fibers and nerve endings in the brain (Shrivastav, 1977).
Another important effect of the anesthesia on the ABR-threshold is increased variance of the data (see Figure 6). So, for accurate studies of effects on the response thresholds, the increased variance in the anesthetized condition necessitates either a larger number of examined animals, a larger number of trials, or a better control of the depth of anesthesia.

Conclusions

With the fixation method used in this study, it is possible to measure ABRs in the awake mouse in free field stimulation. For the FVB/N strain used during this study we can furthermore conclude that:

1. ABR-peak latencies, interpeak latencies and ABR-thresholds are significantly increased in the anesthetized condition compared to the awake condition.
2. The variance of peak latencies, interpeak latencies and thresholds is increased in the anesthetized condition.
3. Increasing the stimulus repetition rate from 23 up to 80 per second causes a small but significant increase in absolute peak latencies in the awake condition only. However, in either condition the repetition rate has no significant effect on the overall ABR-waveform, the ABR-threshold, or the ABR interpeak latencies.

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Chapter 3:

Hearing loss following GATA3 haploinsufficiency is caused by cochlear disorder

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“There’s not much point in finding a magic ring that lets you into other worlds, if you’re afraid to look at them when you’ve got there.”

Uit ‘The magician’s nephew’, C.S. Lewis

Summary

Patients with HDR syndrome suffer from hypoparathyroidism, deafness and renal dysplasia due to a heterozygous deletion of the transcription factor GATA3. Since GATA3 is prominently expressed in both the inner ear and different parts of the auditory nervous system, it is not clear whether the deafness in HDR patients is caused by peripheral and/or central deficits. Therefore, we have created and examined heterozygous *Gata3* knockout mice. Auditory brainstem response thresholds of alert heterozygous *Gata3* mice, analyzed from 1 to 19 months of age, showed a hearing loss of 30 dB compared to wild type littermates. Neither physiological nor morphological abnormalities were found in the brainstem, cerebral cortex, the outer or the middle ear. In contrast, cochleae of heterozygous *Gata3* mice showed significant progressive morphological degeneration starting with the outer hair cells at the apex and ultimately affecting all hair cells and supporting cells in the entire cochlea. Together these findings indicate that hearing loss following *Gata3* haploinsufficiency is peripheral in origin and that this defect is detectable from early postnatal development on and maintains through adulthood.

Keywords: *GATA3*, HDR, cochlea, deafness, degeneration, otic, mouse, hair cell, ABR, brain stem maturation

Introduction

The zinc finger transcription factor GATA3 is essential for proper development of several tissues and organs during embryogenesis (Pandolfi et al., 1995). *Gata3* knockout mice show aberrations of the nervous system, cardiovascular and lymphatic system, as well as of the liver and renal system (Lim et al., 2000; Pandolfi et al., 1995; Pata et al., 1999; Ting et al., 1996; van Doorninck et al.; 1999 Zheng and Flavell, 1997). Unless treated with catechol intermediates (Lim et al., 2000), homozygous *Gata3* knockouts die between embryonic day (E) 10.5-11.5 (Pandolfi et al., 1995).

The prominent role of GATA3 during development is also exemplified by the fact that haploinsufficiency of *GATA3* in humans leads to HDR syndrome, which is characterized by hypoparathyroidism, deafness, and renal defects (Bilous et al., 1992; Van Esch et al., 1999; 2000; Lichtner et al., 2000; Muroya et al., 2001). While the hypoparathyroidism and renal defects appear to be local defects (e.g. Lim et al., 2000), it remains to be demonstrated to what extent the deafness is caused by deficits in the peripheral and/or central auditory system. Both types of deficits may contribute to hearing loss, because GATA3 is prominently expressed during development in the ear as well as in the central nervous system (CNS). In the inner ear GATA3 is expressed in virtually all cell types that occur during otogenesis including inner hair cells (IHCs), outer hair cells (OHCs), and various supporting cells (Rivolta and Holley, 1998; Debacker et al., 1999; Karis et al., 2001; Lawoko-Kerali et al., 2002). In the central auditory nervous system GATA3 is expressed in progenitor neurons in rhombomere 4 of the hindbrain that will form the cochlear and vestibular efferents (Pata et al., 1999; Karis et al., 2001) and in higher order afferent neurons such as those that will form the inferior colliculus (van Doorninck et al., 1999; Karis et al., 2001). To date, it is not clear whether these cell types in the ear and the brain that abundantly express GATA3 during development continue to do so throughout adulthood.

Here, we address the question of whether the deafness in HDR syndrome is caused by deficits in the peripheral and/or central auditory system by investigating heterozygous *Gata3* mice as a model system. We investigated 1) whether the auditory brainstem responses (ABRs) of these mice show signs of peripheral and/or central deficits; 2) what cells in the ear and central auditory system express GATA3 through adulthood; and 3) whether these cells and their connections show morphological aberrations at the adult stage.

Materials and methods

Mouse lines

Heterozygous *Gata3* knockout mice have one of their *Gata3* alleles replaced either by an nlsLacZ reporter gene, which localizes β-galactosidase to the cell nucleus, or by a tauLacZ reporter gene, which allows β-galactosidase transport into axons (for construct details see (van Doorninck et al., 1999). The original F1 of 129/SvEvBrd x C57BL/6 Gata3-nlsLacZ mice were bred at least four times into FVB/N background, which is reported as a normal hearing mouse strain (Zheng et al., 1999) before being used in these experiments. Heterozygous mice were compared to wild type mice born from the same or a similar litter. Animal experiments were performed in accordance with the "Principles of laboratory animal care" (NIH publication No. 86-23, revised 1985) and the guidelines approved by the Erasmus Medical Center animal care committee (DEC; protocol No. 138-01-03). A few days before surgery and throughout experiments mice were kept in an inverted day/night cycle of 12 hours.

Surgery

To test their hearing capacity, heterozygous *Gata3* and wild type mice were prepared for longitudinal ABR measurements in the alert state. For surgery, animals were anaesthetized with a mixture of O_2, N_2O and 2% halothane and received an acrylic head fixation pedestal equipped with electrodes. Two platinum reference electrodes were placed in hand-drilled holes in both left and right occipital bones at the level of Crus2 of the cerebellum. A third stainless steel screw, which served as the active electrode, was fixed to the skull between the two parietal bones. Finally, a common ground electrode was drilled into either the left or the right frontal skull. All transcranial screws were covered with dental cement, in which a three-poled connector was fixed. The two reference electrodes and the active electrode were soldered to this connector, while the common ground electrode was wired to a separate connector.

ABR recordings

ABR recordings were performed in 31 heterozygous *Gata3* knockout mice and 25 wild type littermates at 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 14, 15, 16, 17 and 19 months of age. The ABRs were performed in alert mice, since anesthesia significantly prolongs ABR latencies and causes an upward shift of thresholds (our unpublished observations). Mice were immobilized by placing them in a plastic holder and fixing their pedestals under short-term anesthesia. A 10-minute interval was always taken between short-term anesthesia and ABR recordings. Each mouse

was tested alert in both binaural and monaural stimulation conditions. For monaural recordings, the left and right ears were reversibly plugged with silicon elastomer. As reported (Henry, 1979), plugging resulted in a 30-35 dB threshold elevation in the plugged ear. Sound stimuli were presented in a sound attenuated box in frontal free field condition with a one-tweeter loudspeaker (Radio Shack Super Tweeter 40-1310B) at a constant 40 mm distance between the cone tip of the tweeter and the external auditory canals of the animal. We used the EUPHRA-I system (Erasmus University Physiological Response Averager) for stimulus control and response recording. This 2-channel response averaging system consists of a PC combined with components for stimulus presentation and response acquisition and averaging (DSP Motorola 56002, 32 MHz). Stimulus clicks were generated by the DSP board (duration 50 μs; repetition rates 23 or 80 Hz), while stimulus tone bursts were generated by a Hewlett Packard 33120A signal synthesizer (carrier frequencies of 4, 8, 16 and 32 kHz; repetition rate of 80 Hz). The tone bursts had a rectangular envelope with 1 ms duration and either zero or π radians starting phase of the carrier (i.e. alternating polarity of the carrier). Five hundred accepted post-stimulus time frames were averaged. Artifact rejection level was set at 40 μV.

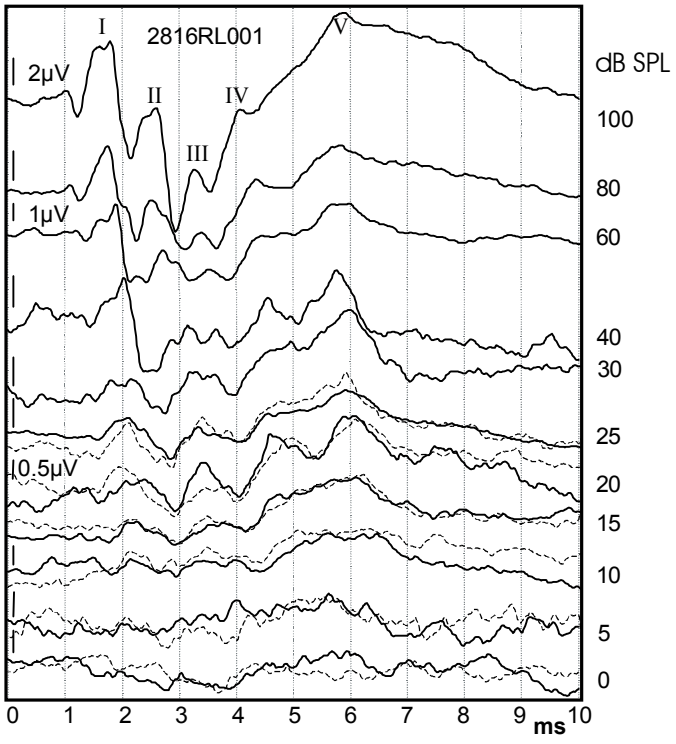

Figure 3.1: Representative example of an ABR test-retest pattern of a 7-month-old male wild type mouse. Five distinct peaks are generally observed. For reasons of clarity only the uppermost trace was labeled. Scale bars represent 2 μV at 100 and 80 dB SPL, 1 μV at 60-25 dB SPL and 0.5 μV for lower sound pressure levels, respectively. The ABR threshold for this mouse was estimated to be 10 dB SPL.

A Bruel & Kjaer sound level meter (2218 with 1613 octave filter set and a ½ inch 4134 microphone) was used for acoustic calibration. Peaks were identified and manually labeled by one and reviewed by a second observer. Response thresholds were determined by lowering the stimulus level with 20 dB steps from 110 dB sound pressure level (SPL) until no response peaks were detectable, and by subsequently increasing it with 5 dB steps until the peaks reappeared (Huang et al., 1995). The ABR threshold was defined as the lowest stimulus level at which a response peak was reproducibly present (for example see Figure 3.1). Statistical description of the data and tests were done with SPSS for windows version 10.0. An effect was considered significant when $P < 0.02$.

Histology

For histological examination at the light microscopic level we processed and analyzed 25 ears and 7 brains of 11 heterozygous *Gata3* and 10 wild type mice. Animals were between 1 and 15 months of age. The animals were deeply anaesthetized with Nembutal and perfused transcardially with either 1% paraformaldehyde, 0.5% glutaraldehyde in 0.05M phosphate buffer or with 4% paraformaldehyde in 0.1M phosphate buffer. The first fixation protocol was used to optimize visualization of β-galactosidase activity in both the ear and brain (van Doorninck et al., 1999). The second protocol was used to process the brains for immunohistochemistry against acetyl choline transferase, serotonin and glutamic acid decarboxylase (for details see De Zeeuw et al., 1996; Jaarsma et al., 1997; van Doorninck et al., 1999). Brains were embedded in gelatin, cryosectioned, processed, mounted and cover slipped in a standard fashion (see Jaarsma et al., 1995). Counting of cell nuclei in Nissl stained sections was done on comparable brain sections of three mice for each genotype. Cochleae were postfixed in 1% osmium tetroxide for 1 hr, embedded in plastic (Durcupan from Fluka, Buchs, Switzerland), and dissected into 10 'quarter' turns (Bohne and Harding, 1997). In the material of each consecutive ‘quarter’ both the length of the organ of Corti and the numbers of present OHCs, IHCs, and pillar cells were determined. In addition, the percentage of missing nerve fibers was estimated by extrapolation of the diameter of the bundles. N=4 cochleae for each genotype at 2 and 15 months of age, n=2 for each genotype at 9 months. For developmental analysis, β-galactosidase activity was visualized in ten fetal cochleae, after which they were decalcified, embedded in paraffin, sectioned and counterstained with neutral red (Kharkovets et al., 2000).

In situ hybridization

Non-radioactive *in situ* hybridization with digoxigenin-labeled antisense *Gata3* or *LacZ* probes (all around 800 nucleotides in length) was done according to Schaeren-Wiemers

(Schaeren-Wiemers and Gerfin-Moser, 1993) on inner ears of 1-, 5-, 7-, and 26-week-old wild type and heterozygous *Gata3* mice. Nine-micrometer-thick cryosections of paraformaldehyde-fixed decalcified cochleae (Whitlon et al., 2001) were hybridized at 65ºC with either an exon 2-3 probe comprising the 5' part of the *Gata3* cDNA (EcoRI-XhoI) or an exon 6 probe containing the 3' part of the *Gata3* cDNA (HindIII-EcoRI) or an EcoRV-ClaI *LacZ* fragment containing the 5' part of this gene as a negative control (when hybridized to wild type cochlear sections). Cochlea were counterstained with methyl green.

Results

Heterozygous Gata3 knockout mice show hearing loss

To investigate whether heterozygous *Gata3* knockout mice have a hearing defect, longitudinal ABR thresholds were determined for 31 heterozygous *Gata3* knockout mice and 25 wild type littermates. Measurements were done with clicks and tone bursts of respectively 4, 8, 16, and 32 kHz at ages ranging from 1–19 months under alert conditions in a total of 75 recording sessions. Figure 3.2 shows that both types of mice show the lowest ABR thresholds at 16 kHz, and that thresholds increase with age in both groups. The average monthly increase was 2.2 dB. As compared to wild type littermates, heterozygous *Gata3* mice showed a significant threshold elevation of approximately 30 dB at virtually all frequencies and all ages ($P < 0.01$, Mann-Whitney). When all ABR thresholds at 32 kHz were analyzed an apparent convergence of regression lines was detected, this however was an artifact, due to a ceiling effect (the level of the loudest sound stimulus was 110 dB SPL): when analyzing only thresholds of mice under 10 months of age the constant 30 dB threshold difference between the two groups was observed as well. We did not observe any difference in thresholds due to different repetition rates of the stimulus (23/s and 80/s, top panels in Figure 3.2), due to gender differences, or between left and right ears (data not shown). The mean difference between binaural and monaural thresholds was 5.95 dB (0.02 dB, SEM), which is comparable to findings in man (Conijn et al., 1990). Thus the persistent hearing deficits found in the *Gata3* mice appear to be reliable and specifically caused by the genetic mutation, and they indicate that the heterozygous *Gata3* mice can serve well as a model for studying the cause of deafness in HDR syndrome.

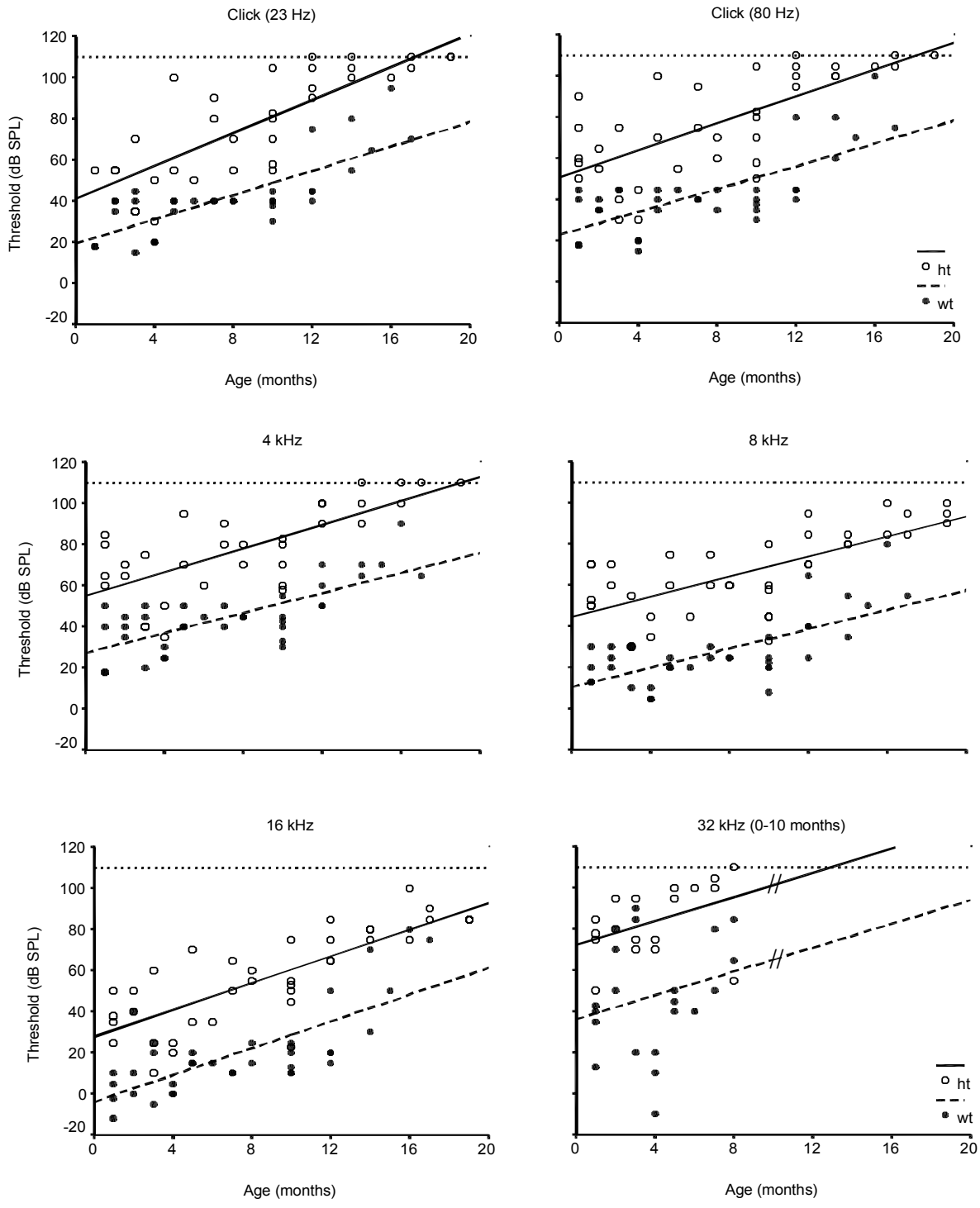

Figure 3.2: Binaural thresholds (dB SPL) as a function of age (months) for click stimuli and 4, 8, 16 and 32 kHz pure tone conditions. Open circles indicate heterozygous *Gata3* knockout mice and filled circles represent wild type controls. Note that due to overlapping data points the number of represented measurements may appear smaller than the actual number of measurements performed. Regression lines through wild type and heterozygous data points are shown. A reference line at 110 dB SPL indicates the maximum stimulus level. All panels demonstrate an approximate 30 dB threshold elevation in heterozygous *Gata3* knockout mice as compared to wild type controls.

Expression of GATA3 in the inner ear and auditory parts of the CNS

To learn whether and where Gata3 is expressed in the inner ear and/or auditory parts of the CNS during development and adulthood, we investigated β-galactosidase activity in mouse lines in which either an *nlsLacZ* (for labeling of cell nuclei) or a *tauLacZ* (for labeling of axonal connections) allele is inserted at the transcription start site of the *Gata3* gene. Inner ear expression of Gata3 starts at E9 in the otic placode (Lawoko-Kerali et al., 2002) and by E18 Gata3 driven β-galactosidase activity (GATA3-LacZ) was observed in both sensory and non-sensory cell types including cells in the developing cochlear duct and spiral ganglion (SG) (Figure 3.3A). At postnatal day 1 GATA3-LacZ was abundantly expressed in nerve fibers, hair cells, pillar cells and supporting cells throughout the entire cochlea (Figure 3.3B). Although it was reported that Gata3 expression could not be detected by immunofluorescence in the adult sensory epithelium of the ear (Rivolta and Holley, 1998), we can easily detect GATA3-LacZ throughout adulthood. In adult cochleae GATA3-LacZ was found in neurons of the SG, outer and inner hair cells, and various supporting cells including Claudius' cells, pillar cells, inner and outer sulcus cells, interdental cells and cells in the spiral prominence and ligament (Figure 3.3C). These expression profiles in the cochlea remained throughout adult life without any sign of reduced intensity at older ages. To verify the distribution patterns of GATA3-LacZ in the ear during young-adulthood and adult life we investigated its expression also with the use of *in situ* hybridization. We used two *Gata3* probes recognizing different parts of the gene that gave identical results. In heterozygous mice, a *LacZ* probe that hybridizes to the *LacZ* sequence inserted in the *Gata3* gene, gave results indistinguishable from both *Gata3* probes (data not shown). We used this *LacZ* probe as a negative control in wild type cochlear sections (see Figures 3.3D,G). In 5-7-week-old animals, Gata3 mRNA was detected in the organ of Corti and surrounding cells (Figure 3.3E, H) and in the SG (Figure 3.3E,I). The *in situ* hybridization experiments showed that Gata3 mRNA was strongly expressed in the cochlea from early life (P8) until half-a-year-of-age (the oldest age examined for *in situ* hybridization), without reduced expression levels at older ages. GATA3-LacZ staining therefore fully reproduces Gata3 *in situ* hybridization; both are detected in neurons of the spiral ganglion, outer and inner hair cells, Claudius' cells, pillar cells, inner and outer sulcus cells, interdental cells and cells in the spiral prominence and ligament.

No expression of GATA3-LacZ is found in the ossicles of the middle ear (data not shown). In the auditory parts of the CNS expression of Gata3 starts around E9 (Pata et al., 1999) and expression continues until adulthood and is observed in cell bodies and fibers in the superior paraolivary nucleus, nucleus of the lateral lemniscus, and inferior colliculus as well as in

efferent fibers in the cochlear nucleus (Figure 3.4). Similar to the expression in the inner ear, GATA3-LacZ expression in these neurons and axonal connections remained at high densities beyond the age of 1 year.

In conjunction our data show that Gata3 is continuously expressed from early development on through adulthood in various parts of both the inner ear and central auditory nervous system. Thus, these temporal-spatial expression patterns leave open all possibilities to explain whether the hearing loss in heterozygous *Gata3* mutants is caused by a peripheral or a central defect.

Peak and interpeak latencies do not differ

To find out whether auditory parts of the CNS of heterozygous *Gata3* knockout mice were affected, we compared the latencies of their ABR peaks to latencies of ABR peaks of wild type littermates. Stimulus levels of 80 dB SPL or higher that were at least 30 dB above threshold levels were used for these comparisons. In the ABRs of both wild types and heterozygous *Gata3* mutants usually 5 peaks (I to V) were readily discriminated (see also Figure 3.1). We aligned average peak I latencies obtained with frequency-specific stimulation (4, 8, 16 and 32 kHz) with those obtained with click stimulation to avoid frequency-dependent bias effects due to the tonotopy in the cochlea. The latencies of subsequent peaks were corrected with the same amount, leaving interpeak latencies unchanged. None of the latencies of the pooled data of the *Gata3* mutants were significantly different from those obtained in wild types (for corrected average latencies see Table 3.1). In addition, interpeak latencies do not differ between heterozygous *Gata3* and wild type control mice (e.g. $P > 0.8$ for peak I-III interpeak latencies, Student's t-test).

Interestingly, the interpeak latencies developed in both the wild types and mutants at a similar pace (Figure 3.5). In both cases these latencies were shortened by approximately 0.3 ms during the first 100 days. A similar maturation pattern can be observed in humans (Salamy, 1984) and is likely due to increases of the diameter of the myelin sheaths during maturation of the nervous system, which results in faster axonal conductance and thus in a decrease of peak and interpeak latencies. Nonlinear regression analysis shows that interpeak latency I-III is significantly age-dependent ($P < 0.0001$, F-test) with an R-square of 0.21. The age-effect therefore explains 21% of the variance in the interpeak latency I-III data. Together these latency data show that the auditory parts of the CNS in *Gata3* mutants mature similar to wild

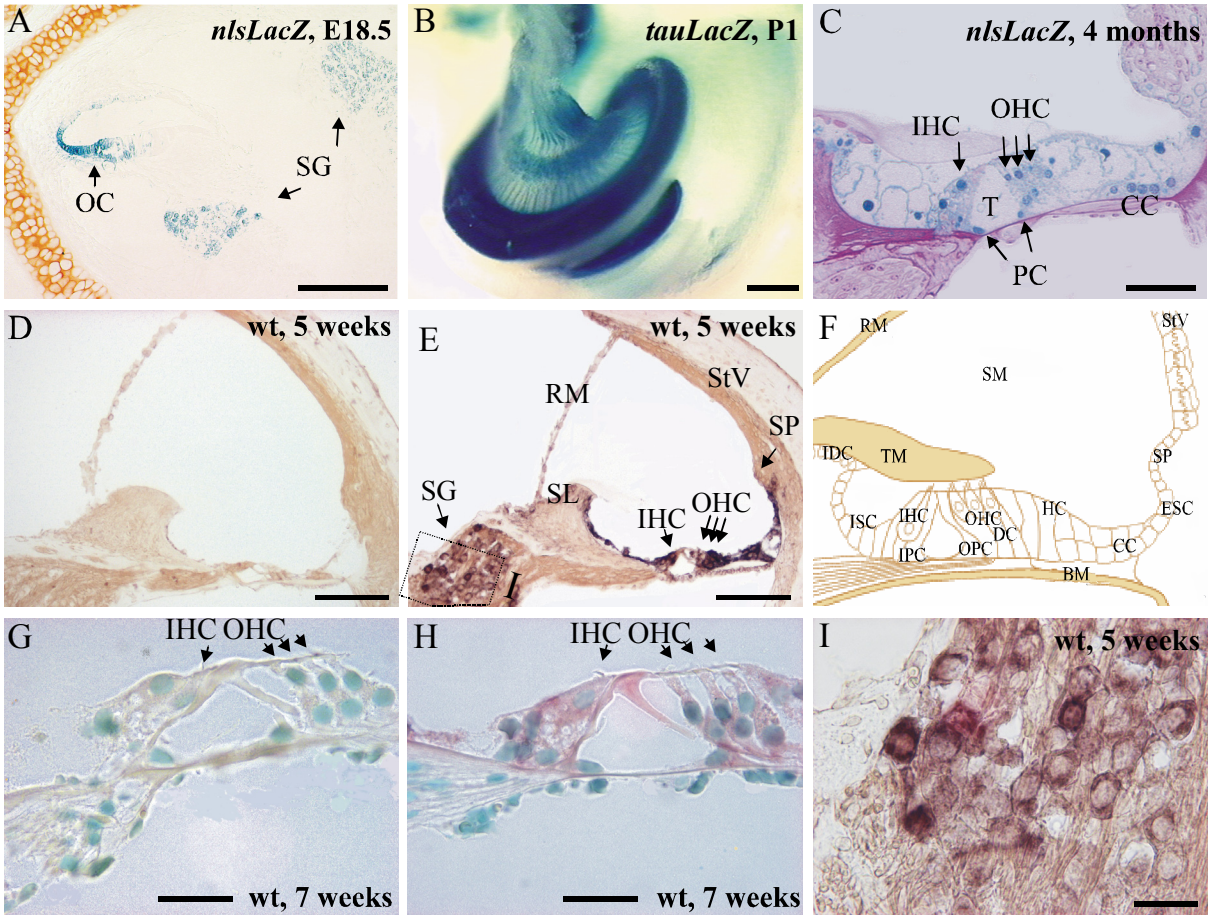

Figure 3.3: (A-C) Expression of Gata3 visualized by β-galactosidase activity (blue staining) and (D-I*) in situ* hybridization (purple) in mouse cochleae. (A) Section through the cochlea of an E18.5 heterozygous GATA3-nlsLacZ fetus. GATA3-LacZ expression is visible in and adjacent to the developing organ of Corti (OC) and in spiral ganglion (SG) cells. (B) Overview of a cochlea of a heterozygous *Gata3*-tauLacZ mouse at postnatal day 1 (P1). (C) Cross section of a cochlea of a 4-month-old heterozygous GATA3-nlsLacZ mouse. β-Galactosidase is predominantly present in the cell nuclei. Tunnel of Corti (T), inner hair cell (IHC), outer hair cells (OHC), pillar cells (PC), and Claudius' cells (CC) are indicated. (D, G) *In situ* hybridization on wild type cochlea hybridized with a LacZ probe (negative control). (E, H) Cochlea hybridized with a *Gata3* probe shows Gata3 mRNA expression in IHCs, OHCs, various supporting cells, inner and external sulcus cells, spiral ganglion (SG) cells identical to LacZ staining. Note that G and H have a nuclear methyl green counter staining. (F) Schematic drawing of an adult cochlea to indicate the different cell types (Van Camp and Smith, 2004).(I) Detail of E, showing the presence of Gata3 mRNA in the cytoplasm of spiral ganglion cells. Scale bars: A, 250 µm; B, 400 µm; C, 40 µm; D, E, 100 µm; G,H, I, 20 µm. BM Basilar membrane, DC Deiters' cells, ESC External sulcus cells, HC Hensen's cells, IDC Interdental cells, IPC Inner pillar cell, ISC Inner sulcus cells, SL Spiral limbus, OPC Outer pillar cell, RM Reissner's membrane, SP Spiral prominence, StV Stria vascularis, TM Tectorial membrane.

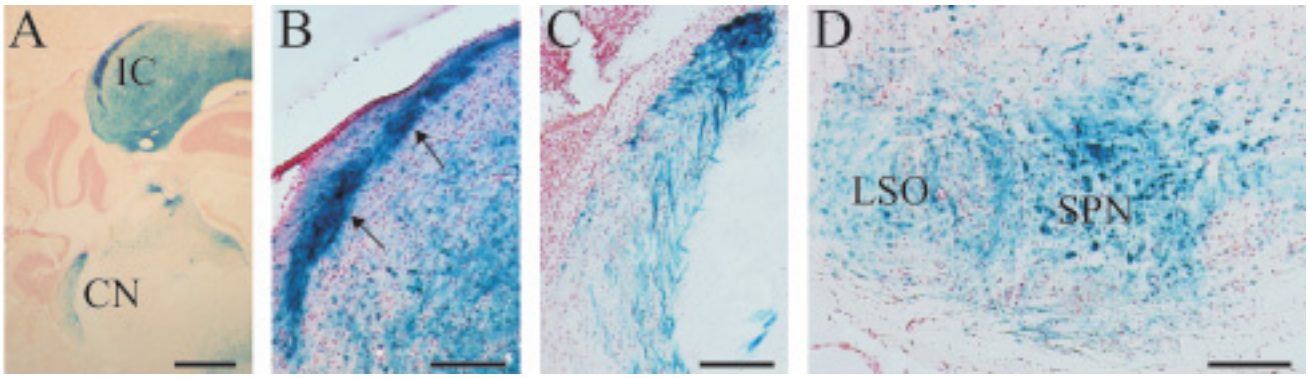

Figure 3.4: Distribution of Gata3 positive, β-galactosidase labeled fibers and neurons in various parts of the auditory system in the CNS of a 9-month-old heterozygous *Gata3-tauLacZ* mouse. (A) Overview of transverse section of the brainstem with labeled fibers in the cochlear nucleus (CN) and with both labeled cell bodies and fibers in the inferior colliculus (IC). (B) Magnification of IC labeling in (A). Note the patches of dense labeling in the external cortex of the IC (arrows). (C) Magnification of CN labeling in (A). The labeled fibers run through the ventral CN. (D) Both the lateral superior olive (LSO) and superior paraolivary nucleus (SPN) contains many labeled fibers and cell bodies. Scale bar: A, 500 µm; B-D, 100 µm.

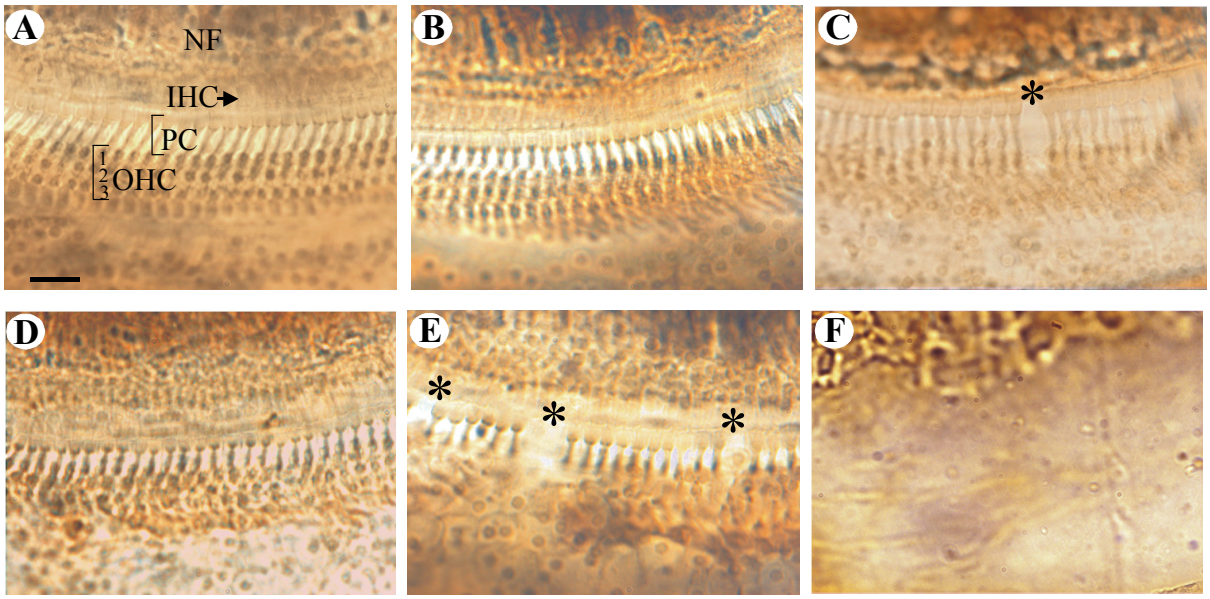

Figure 3.6: Light microscopic examinations of cochleae of wild type (A,B,C) and heterozygous mice (D,E,F) mice of 2 (A,D), 9 (B,E) and 15 (C,F) months of age. Of the 10 quarter pieces cut from the whole cochlea, the number 4 pieces are shown in this figure (apex is one, base is ten). Three rows of outer hair cells (OHC), a row of outer pillar cells with visible protruding necks and heads that are flanking the lighter stained heads of the inner pillar cell row (PC) and one row of inner hair cells (IHC) are indicated in (A). Whereas in (A) three distinct rows of OHCs can be seen, in (D) the pattern is irregular, this is due to missing OHCs and the appearance of underlying Deiters' cells (lighter circles). A complete loss of all cell types is seen in 15-month-old heterozygous mice (F), whereas in wild type mice (C), NF, PCs and IHCs are missing sporadically and only OHCs are almost completely lost. Asterisks indicate missing pillar cells. Scale bar in A (for A-F) 20 µm.

type mice during postnatal development and that they reach the same functional level that is maintained until at least 1 year of age.

Genotype		Peak I	Peak II	Peak III	Peak IV	Peak V
Wild type	mean	1.72	2.52	3.32	4.25	5.75
	s.d.	.09	.14	.14	.24	.25
Heterozygous	mean	1.70	2.47	3.30	4.14	5.68
	s.d.	.11	.21	.20	.23	.24

Table 3.1. Latencies of ABR peaks I to V of wild type and heterozygous *Gata3* mice.
ABR-peak I-V latencies, corrected for tonotopic effects (see Materials and methods), do not differ significantly between heterozygous *Gata3* and wild type control mice. Differences in peak latencies would indicate dissimilarities in conduction velocity in the (auditory) brainstem between groups. Stimulus level was 80 dB SPL, and data were pooled over all frequencies, stimulus modes and age groups.

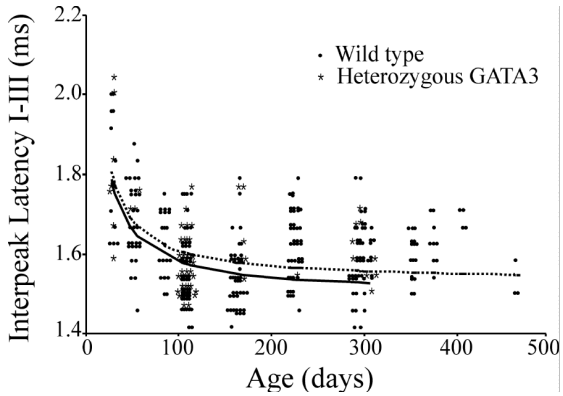

Figure 3.5: Maturation of the I-III interpeak latencies as a function of age. Mean interpeak latencies were determined for ABR peaks elicited by stimulus levels of 80 dB SPL or higher that were at least 30 dB above threshold levels. The lines are the least squares fits with an inverse function through the data. Dotted line represents fit for wild type mice data and solid line indicates fit for heterozygous mice. No data points were available for heterozygous mice older than 300 days because the thresholds were too high.

The inner ear, but not the brain, shows morphological abnormalities

The threshold elevations and normal latencies of the ABRs described above show that the cause of hearing loss in *Gata3* mutants is not central but peripheral, and that hearing loss

starts during early postnatal development and maintains through adulthood. To find out whether these physiological data are corroborated by morphological pathological phenomena, we investigated whether, when and to what extent the central auditory nervous system and ear show morphological aberrations.

Nissl stained sections of the brains of heterozygous *Gata3* mice of 1, 3 and 9 months old did not show any abnormality in cyto-architecture or cell counts when compared to wild types. Sections of the cochlear nucleus, superior olive, nucleus of the lateral lemniscus and inferior colliculus that were stained with antisera against a neurotransmitter (serotonin) and two neurotransmitter producing enzymes (acetyl choline transferase and glutamic acid decarboxylase) did not reveal any abnormal distribution or density of terminals (data not shown). Both macroscopic and light microscopic examination of the external and middle ear did not show any abnormality during development or adulthood (data not shown).

In contrast, the inner ears of heterozygous mice showed clear abnormalities. In Figure 3.6 mid-apical quarter pieces of cochleae of wild type and heterozygous *Gata3* mice isolated at different ages are depicted. Cochleae of 2-month-old wild type mice showed an organized pattern of one row of IHCs and 3 orderly rows of OHCs (Figure 3.6A). The heterozygous mice at 2 months of age however, showed an irregular pattern with remnants of the 3 OHC rows while the nuclei of underlying Deiters' cells became visible (Figure 3.6D). The stria vascularis and Reissner's membrane appeared normal in heterozygous mice. At 1 month of age, OHCs in the mutant mouse and to a lesser extent their auditory nerve fibers were partially missing at the apex and this became more prominent with age (Figure 3.6). Quantitative analyses of the OHCs, IHCs, pillar cells and nerve fibers revealed a progressive loss of cells and fibers that significantly preceded and exceeded the loss in wild types (Figure 3.7). During the same period, some degeneration occurred at the basal turn of the cochlea, but the level of this process was comparable to that in wild type mice (Figure 3.7). In 9-month-old mutants, all OHCs had disappeared, while in the wild types OHCs were only affected at the base of the cochlea (Figures 3.6B,E and 3.7B,E). Concomitantly, the IHCs, pillar cells and nerve fibers of the mutants substantially decreased in number at both the apical and basal turns. Finally, the organ of Corti of 15-month-old heterozygous *Gata3* mice was almost completely degenerated including supporting cells, whereas wild type littermates still had normal numbers of IHCs, pillar cells and nerve fibers in the upper half of the organ of Corti (Figures 3.6C,F and 3.7C,F). Intermediate ages showed intermediate patterns of degeneration

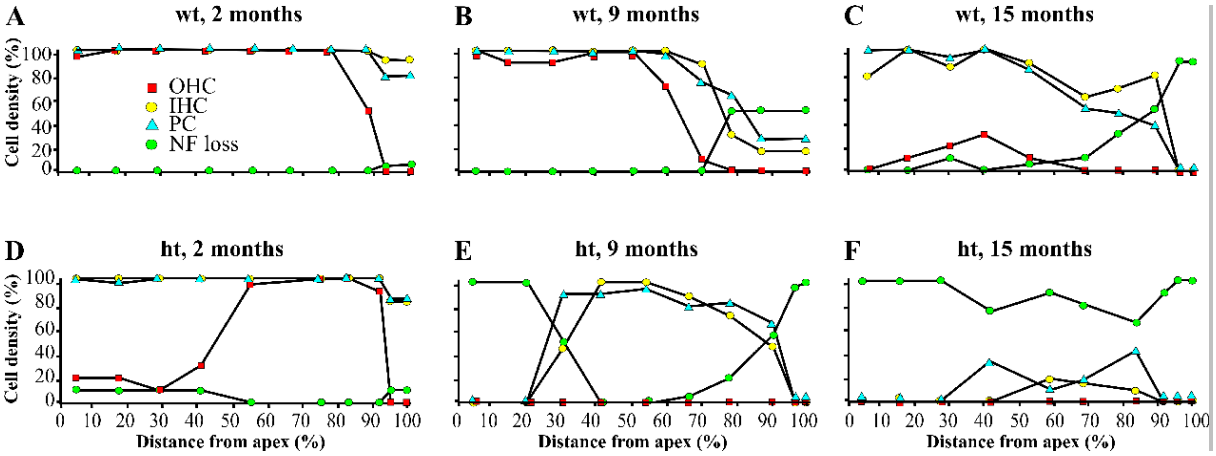

Figure 3.7: Presence of outer hair cells (OHC, red squares), inner hair cells (IHC, yellow circles), pillar cells (PC, blue triangles), and absence of nerve fibers (NF loss, green circles), in cochleae of wild type (A,B,C) and heterozygous *Gata3* (D,E,F) mice of 2 (A,D), 9 (B,E) and 15 (C,F) months of age (these data are from the same mice as shown in Figure 3.6). Markers indicate the percentage of cells present or the percentage of nerve fibers absent in the 10 different cochlear pieces, plotted against the distance to the apex of the analyzed cochlear part. Hair cells responsive to 4 kHz, 8 kHz, 16 kHz and 32 kHz tones are found at approximately 25%, 40%, 60% and 75% distance from the apex respectively (Ou et al., 2000).

(data not shown). Thus, *Gata3* haploinsufficiency-induced degeneration started at the apex and ultimately progressed into sensory epithelia in the middle turns of the cochlea.

In sum, our morphological analyses showed no abnormalities in the central auditory regions, while clear degeneration was observed peripherally in the cochlea and hence confirm the physiological data that showed involvement of the peripheral but not the central auditory nervous system in *Gata3* related hearing loss.

Discussion

Gata3-related hearing loss is cochlear in origin

Mice with a mutation in one allele of the *Gata3* gene may serve as a mouse model to study the involvement of GATA3 in different HDR disease phenotypes (hypoparathyroidism, deafness and renal dysplasia). Here we have addressed the question as to whether heterozygous *Gata3* mice indeed suffer from deafness, and if so, whether this disorder is caused by deficits in the peripheral and/or central auditory system. This question turns out to be particularly pertinent as we showed with the use of *in situ* hybridization and a *Gata3* regulated *LacZ* reporter gene that Gata3 is expressed in several auditory components of both

the central and peripheral nervous system from embryogenesis until adulthood. We used a combination of physiological and histological techniques to analyze different auditory parameters in brain and ear. Our ABR measurements showed that *Gata3* mutant mice suffer from sensorineural hearing loss similar to HDR patients (Bilous et al., 1992). The average threshold elevation in mutant mice was 30 dB at all ages ranging from 1 to 19 months.

In both the mutants and wild type littermates we observed a mild form of age-related hearing loss, which is characterized by functional and morphological deficits that start at the base of the cochlea (high frequencies) and progress towards the apex (low frequencies) during life. This was unexpected since the genetic background of these mice was 94 % FVB/N and FVB/N has been reported to be a good hearing mouse strain (Zheng et al., 1999). However, we are confident that this form of hearing loss did not confound the deficit and phenotype that is caused by *Gata3* haploinsufficiency, because the age-related hearing loss was equal in mutants and wild types, and because the dominant hair cell loss in the *Gata3* mutants started at the apex of the cochlea instead of the base.

To distinguish the impacts of a loss of GATA3 at the different neuro-anatomical levels we analyzed ABR peak latencies, because they are a measure for the conductance efficiency of the different auditory connections. ABR peaks I to V originate in the afferent auditory pathway between the cochlea and VIIIth nerve (peak I) and the inferior colliculus (peak V) (Henry, 1979). Comparisons of the peak and interpeak latencies of heterozygous *Gata3* and wild type mice showed that the velocities of the different nerve conductances of the mutant mice were indistinguishable from those of wild type animals. The absence of a prolonged latency of peak I together with the absence of morphological defects in the middle ear bones exclude a middle or outer ear origin (van der Drift et al., 1988). The normal interpeak latencies indicated that the origin of deafness was not located in the central nervous system, which was confirmed by the absence of detectable abnormalities in the brain. In contrast, histological analysis of the cochleae in heterozygous *Gata3* mice showed a gradual degradation of all cell types of the organ of Corti starting with the OHCs at the apex. At 15 months of age the organ of Corti was completely degenerated. Together, these results indicate that the observed hearing defects in heterozygous *Gata3* mice have a cochlear origin.

Primary cause of deafness

Our data clearly indicated that the hearing loss observed in *Gata3* mutant mice is most likely caused by a cochlear deficit, however, the exact cellular cause of this deafness remains to be elucidated. Several observations suggest that a defect of the OHCs may be the primary cause

of deafness: First, the OHCs were the first type of cells in the *Gata3* mutant mice that showed signs of degeneration. Second, the level of hearing loss corresponds well to that observed in other disorders with a degeneration of the OHCs. For example, the loss of OHCs in chinchillas induced by noise also causes a 30 dB permanent threshold shift (Hamernik et al., 1989). Third, a loss of OHCs in other mutants such as the *Prestin* and *Barhl1* knockouts and the $Kit^{(W\text{-}v)}$ and Jackson Shaker mouse mutants also causes comparable hearing deficiencies (Schrott et al., 1990a, 1990b; Kitamura et al., 1991, 1992; Dallos, 1996; Li et al., 2002; Liberman et al., 2002). Still, it should be noted that IHCs and pillar cells also express Gata3 and that functional loss of these cells may also contribute to the hearing loss.

We have shown abundant expression of Gata3 mRNA in the adult ear, either by the detection of Gata3 induced β-galactosidase or by direct *in situ* hybridization (Figure 3.3). However, GATA3 protein expression has been shown in mouse cochleae up till P0 by Holley and co-workers but was no longer visible at P14 (Rivolta and Holley, 1998). As Gata3 mRNA is readily detectable but apparently subject to strong translational control, it remains a question whether GATA3 protein is too low to be detected in adult cochleae or no longer present. If GATA3 protein is not present in adult life, the hearing deficiency and progressive degeneration that we observed in heterozygous *Gata3* mice are a result of developmental GATA3 shortage. An inducible *Gata3* knockout mouse, that deletes GATA3 after birth, will be tested to distinguish between a developmental and maintenance problem.

Does GATA3 haploinsufficiency cause cell-autonomous degeneration in the cochlea?

Different cell types in the cochlea express GATA3 but it is not known whether the observed cellular degeneration is due to cell-autonomous loss of GATA3. The first cells in the *Gata3* mutant mouse to degenerate are OHCs followed by IHCs and pillar cells. This is reminiscent of the noise-induced hearing loss model in which outer hair cells are lost first and surrounding supporting and sensory cells degenerate as a secondary event (Bohne and Harding, 2000), i.e. non cell-autonomous. However, the noise-induced cell loss is rapid (days, Bohne and Harding, 2000) whereas the IHC loss we observed in the *Gata3* mice lagged behind OHC loss by several months. Loss of OHCs (or OHC function) does not in itself cause loss of IHCs and pillar cells as several mouse mutants exist that show independent loss of OHCs and IHCs: The *Barhl1* mouse mutant shows opposed gradients of hair cell loss with OHC degeneration from apex to base and IHC degradation from base to apex (Li et al., 2002). The Jackson Shaker mouse, which carries a mutation in the scaffold protein Sans (Kikkawa et al., 2003), shows degeneration of basal OHCs in the first weeks while basal IHCs degenerate only 3

months later (Kitamura et al., 1991; Kitamura et al., 1992). The $Kit^{W\text{-}v}$ mouse mutant lacks almost all OHCs from birth onwards, but does not show any IHC or supporting cell degeneration (Sterbing and Schrott-Fischer, 2002). For these reasons, we think it likely that OHC, IHC, and pillar cell losses in the heterozygous *Gata3* mice are cell-autonomous events. This would be in line with our expression data (Figure 3.3), which show that all three cell types express Gata3. However, cell non-autonomous events could play a role in the observed degeneration. To distinguish between these possibilities we will investigate (cochlear) cell type specific *Gata3* knockout mice that will help to elucidate the exact role of each cell type in the observed cochlear degeneration.

Cochlear cells are likely to be dependent on a number of downstream genes regulated by the transcription factor GATA3. They may include neurotrophin-like factor genes, similar to GATA3 regulated induction of interleukines in T-lymphocytes (Zhang et al., 1998) or neurotransmitter genes, similar to the defective serotonin and tyrosine hydroxylase expression in respectively Raphe nuclei and sympathetic ganglia in homozygous *Gata3* knockout cells (van Doorninck et al., 1999; Lim et al., 2000). A reduced amount of neurotrophin-like substances would affect the correct development of the organ of Corti or its resistance against normal injury occurring throughout life.

The present study analyzed the deafness phenotype in heterozygous *Gata3* mice as a model system for the deafness of HDR patients. Our data show that *Gata3* haploinsufficiency leads to a peripheral auditory defect. This finding opens the possibility for the development of local peripheral treatment for the hearing problems in HDR patients using for example virally mediated transfer of GATA3 or GATA3 target genes into the cochlea.

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Chapter 4:

GATA3 haploinsufficiency causes a rapid deterioration of distortion product otoacoustic emissions (DPOAEs) in mice

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…

Zo ging hij telkens een stap dichter naar de tor toe, tot hij voor zijn oor stond en tamelijk luid riep: “Tor.”

Maar de tor sliep door.

De mus wendde zich tot de eekhoorn en zei: “Hij slaapt onmeetbaar diep.”

“Wat is onmeetbaar?” vroeg de eekhoorn.

“Ja…” zei de mus, “dat is bijvoorbeeld hoe warm de tijd is of wie er het meest van de lucht houdt of het jarigst is...”

“O,” zei de eekhoorn.

….

Uit ‘Misschien wisten zij alles’, Toon Tellegen, Em. Querido’s Uitgeverij B.V., 2005.

Summary

Human HDR (hypoparathyroidism, deafness and renal dysplasia) syndrome is caused by haploinsufficiency of zinc-finger transcription factor GATA3. The hearing loss due to *GATA3* haploinsufficiency has been shown to be peripheral in origin, but it is unclear to what extent potential aberrations in the outer hair cells (OHCs) contribute to this disorder. To further elucidate the pathophysiological mechanism underlying the hearing defect in HDR syndrome, we investigated the OHCs in heterozygous *Gata3*-knockout mice at both the functional and morphological level. While the signal-to-noise ratios of distortion product otoacoustic emissions (DPOAE) in wild-type mice did not change significantly during the first half-year of live, those in the heterozygous *Gata3* mice decreased dramatically. In addition, both light microscopic and transmission electron microscopic analyses showed that the number of OHCs containing vacuoles was increased in the mutants. Together these findings indicate that outer hair cell malfunctioning plays a major role in the hearing loss in HDR syndrome.

Key words: DPOAE, OHC, vacuole, HDR syndrome, EM, hearing loss, GATA3

Introduction

In 1992, HDR syndrome was first described as a new clinical entity, comprising hypoparathyroidism, deafness and renal dysplasia (Bilous et al., 1992).

The sensorineural hearing loss in HDR patients can be both symmetric or asymmetric, and as tested by auditory brainstem response (ABR), conditioned orientation reflex tests or pure tone audiometry it ranges in level from 40 dB to 105 dB (Bilous et al., 1992; Fujimoto et al., 1999; Hasegawa et al., 1997; Lichtner et al., 2000; Muroya et al., 2001). Although it is clear that hearing loss in HDR is usually somewhat more severe at the higher end of the frequency spectrum (Lichtner et al., 2000; Muroya et al., 2001), no systematic audiometric evaluation has yet been made, and the exact pathophysiological mechanism causing the hearing defect remains largely unknown. Genetically HDR syndrome is caused by haploinsufficiency of zinc finger transcription factor GATA3 (Van Esch et al., 2001a; Van Esch et al., 2001b; Van Esch et al., 2000), which in mice is essential for development of several tissues and organs including the lymphatic system, the sympathetic nervous system, brain, kidney, jaw and inner ear (Lim et al., 2000; Pandolfi et al., 1995, Pata et al., 1999;, Ting et al., 1996; van Doorninck et al., 1999; Zheng and Flavell, 1997). During development human and murine GATA3 is prominently expressed in virtually all cell types of the inner ear including inner hair cells (IHCs), outer hair cells (OHCs) and supporting cells as well as in various cell types of the central auditory system including neurons in the superior olive and inferior colliculus (Debacker et al., 1999; Karis et al., 2001; Lawoko-Kerali et al., 2002; Rivolta and Holley, 1998; van der Wees et al., 2004;van Doorninck et al., 1999).

In line with HDR syndrome in humans, heterozygous *Gata3* mice show an elevation in their Auditory Brainstem Response-thresholds. Thresholds are elevated by approximately 30-dB, while no interpeak latency differences can be found. In addition *Gata3* heterozygous mice showed extensive apical cochlear cell loss from young ages onwards, without any morphological abnormalities in the central nervous system. This led us to conclude that hearing loss due to GATA3 haploinsufficiency is peripheral in origin (van der Wees et al., 2004). Building upon the above-mentioned findings, and considering the fact that at light microscopical level outer hair cells (OHCs) degenerated first before subsequent loss of inner hair cells (IHC), pillar cells (PC) and nerve fibers (NF), we hypothesized that OHC malfunctioning could be an important factor causing hearing loss in GATA3 haploinsufficiency.

The most accurate measure for testing OHC function are otoacoustic emissions (OAE's) (Schrott et al., 1991). Otoacoustic emissions have been used in mice auditory research for a variety of purposes, amongst others to study age related hearing loss – AHL - (Jimenez et al., 1999; Parham, 1997; Parham et al., 1999), noise induced hearing loss – NIHL - (Jimenez et al., 2001; Vazquez et al., 2001) and effects of hypothyroidism on OHCs (Li et al., 1999). Moreover, OAE's have also been used for auditory phenotyping and to study effects of genetic mutations on the auditory system (Huang et al., 1995; Huang et al., 1998; Konrad-Martin et al., 2001).

To shed light on the potential role of dysfunction of OHCs in the hearing loss found in HDR patients, we investigated the function of these cells in heterozygous *Gata3* mice. We investigated functioning of their OHCs by measuring the distortion product otoacoustic emissions (DPOAE) and by further analyzing the morphology of these cells at both the light microscopic and electron microscopic level at young ages (1 to 2 months).

Materials and Methods

Mouse lines/subjects

Heterozygous *Gata3* knockouts (129/C57BL/6 GATA3-nlsLacZ mice bred 6 to 8 times into FVB/N background) were compared to wild type mice from the same litter. Animal experiments were performed in accordance with the "Principles of laboratory animal care" (NIH publication No. 86-23, revised 1985) and the guidelines approved by the Erasmus University animal care committee (DEC; protocol No. 138-02-01).

Light microscopy and electron microscopy

Outer hair cell loss was analyzed at the light microscopic level in 24 cochleae of 7 heterozygous *Gata3* and 7 wild type mice, in the age range between 1 and 15 months. The animals were deeply anaesthetized with Nembutal and perfused transcardially with 4% paraformaldehyde in 0.1M phosphate buffer.

Cochleae were post fixed in 1% osmium tetroxide for 1 hr, embedded in plastic (Durcupan from Fluka, Buchs, Switzerland), and dissected into 10 'quarter' turns (Bohne and Harding, 1997). In the material of each consecutive 'quarter' both the length of the organ of Corti and the number of present OHCs was determined.

A total of 11 cochleae (left and right ears) obtained from 3 mice of each genotype (of which one was 1 month old and two were 2 months old) were used for analysis of differences between heterozygous and wild type mice at light microscopical level. Cochlear pieces

containing those sections of the organ of Corti that are responsive to approximately 4, 16 and 32 kHz were used for histological analysis (Ou et al., 2000). Semithin slices (1µm thick) were cut on a Leica Reicherts Ultratome and counterstained with a Toluidin blue-staining containing 1% Toluidin blue, 1% Methylene blue, 0,2% Sodiumhydroxide, 1% Borax 30% Ethanediol Ethyleneglycol and 70% MilliQ water. Slices were examined under a Leica DM-RB microscope. Per cochlear piece 50 to 163 slices were examined. The number of OHCs containing vacuoles was scored and entered in Excel data-files, which were subsequently imported into an SPSS-data file. Statistical analysis of histological data was done using SPSS for Windows version 11.0. An effect was considered significant when $p < 0.02$. Non-parametric (Mann-Whitney) tests were used to analyze the difference in numbers of outer hair cells containing vacuoles between genotypes. Apart from this, the effect of age and location of the OHC in the cochlea on this difference was analyzed.

In order to assess possible additional signs of (outer) hair cell degeneration, such as mitochondrial changes, changes in endoplasmatic reticulum and disappearance of ribosomes ultrathin slices were cut for electron microscopy, and contrasted with uranyl acetate and lead citrate (Oei et al., 2003) of inner ears from three 2 months old mice for each genotype. Slices were analyzed using a Philips CM100 electron microscope.

DPOAE-test procedure

Per age group, genotype and gender at least 5 mice were subjected to DPOAE measurements (i.e. at least 20 mice were examined per age group). Measurements were performed both longitudinally and transversally (32 mice were tested once, 25 mice twice, 9 mice trice and 7 mice four times). Animals were anesthetized with a mixture of 5% Fluothane, N2O and O2. After induction of anesthesia, mice were placed on a heating pad (Thermocomfort, Brisk BV, Venray, The Netherlands), to maintain constant body temperature. In each mouse the right and left ear were consecutively tested. Cerumen obstruction and infection were excluded prior to experiments by otoscopically inspecting ear canals and eardrums. Mice suffering from obvious outer- or middle ear problems were temporarily excluded from experiments. To equalize the anesthesia induced increase in middle ear pressure, the pars flaccida of the tympanum was perforated using a Microlance® needle. The success rate of this procedure was high. Occasionally however, the perforation was unsuccessful or closed during measurements, resulting in a decrease of signal to noise ratios of emissions. If so, a second tympanocentesis was performed. Repeated measurements (and ear drum perforations) in individual mice did not influence DPOAE levels; an illustrative example of this is given in Figure 4.1. Signal to noise ratios of two 5 months old mice are compared, one after a single,

and the other after four test sessions. Next, foam stripped Etymotic® ER10C-14C ear tips were fitted in the external ear canals to enable recording of DPOAE.

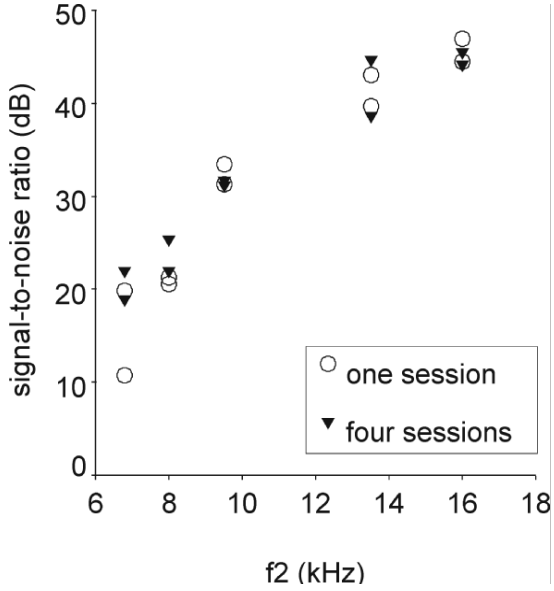

Figure 4.1: DPOAE signal to noise ratios (dB) of two 5 months old wild type mice, open circles indicate results after a single measurement, closed triangles represent results after four test sessions. Data for both ears are represented. Repeated measurements and paracentesis in individual mice did not influence DPOAE levels.

DPOAE measurements

2f1-f2 DPOAEs were measured using a commercially available recording system (DP 2000 – Starkey). After introduction of the probe and prior to actual recordings calibration of the stimulation conditions was done over a 0.1-20 kHz frequency range. The primary frequency ratio (f2/f1) was 1.2 (Le Calvez et al., 1998b). The targeted primary tone levels, L1/L2, were 65/55 dB SPL. Data were collected with f2 at 6.8, 8.0, 9.5, 11.3, 13.5 and 16.0 kHz. All experiments were performed in a quiet but not sound treated room. Recordings were repeated (at least 6 times).

DPOAE data processing and analyses

DPOAE data processing was done using SPSS for Windows version 10.0. Raw data, as stored in the database of the DPOAE recording system, were imported into SPSS-data files. Data were included for statistical analysis after a strict selection as to quality of calibration curves of both stimulus channels, on noise floor and stimulus condition quality during recordings. Symmetry criteria of the calibration curves of both loudspeakers in the probe were used for selection of good quality recordings. Furthermore, recordings with calibration curves showing a probable air leakage or occlusion of probe channels by the ear canal wall were excluded, as

were recordings with background noise levels over –2 dB SPL and recordings with stimulation levels differing too much from targeted levels (more than 15 dB). Finally only recordings with primary tone level differences ranging from –1 to 12 dB were included.

age (months)	f2 frequencies (kHz) and genotypes											
	6.8		8.0		9.5		11.3		13.5		16.0	
	wt	ht	wt	ht	wt	ht	wt	ht	wt	ht	wt	ht
1.0	8	9	9	11	9	11		1	6	9	9	11
2.0	7	10	7	11	7	11	2	2	6	10	6	10
3.0	9	12	10	12	10	12	3	2	9	12	10	12
4.0	9	8	10	8	10	8	5	3	6	8	9	8
5.0	11	10	11	11	11	11		5	11	10	11	11
6.0	11	10	11	10	11	10	3	4	11	10	11	10
7.0	5	8	7	9	7	9		3	5	9	7	9

Table 4.1: Number of mice per age group, genotype and frequency (f2) after selection of data on DPOAE recording-quality criteria.
For the f2 = 11.3 kHz only a limited number of data met the selection criteria, therefore this frequency was excluded from further statistical analysis. (wt; wild type, ht; heterozygous).

Table 4.1 displays the number of mice per age group, genotype and frequency (f2) after strict selection of data. For the f2= 11.3 kHz target levels of the primaries often failed to meet the selection criteria. Therefore this frequency was excluded from further statistical analysis. Subsequently, of every individual mouse the emission with the best signal to noise ratio per age, per frequency, and per ear was selected for analysis. Since each individual mouse was measured on both ears, we analyzed ear effects prior to further analyses. Within individual mice a significant correlation existed between right and left ears (Pearson's correlation coefficient = 0.81, significance < 0.0005). Therefore data were aggregated for each mouse (means of right and left ears), at each age and frequency, for further analysis of genotype and gender influence. Statistical analysis of DPOAE-data was done using SPSS for Windows version 10.0. An effect was considered significant when $p < 0.02$.

Effects of genotype on signal to noise ratio's were analyzed, as were age effects, and gender effects.

Results

Light microscopy

Quantitative analyses of the OHCs revealed a progressive loss of apical outer hair cells in heterozygous mice from one month onward, significantly preceding and exceeding loss in wild types. At the same time some degeneration occurred at the basal turn of the cochlea, but the level of this process was comparable to that in wild type mice. By the age of 9-months, in mutants, nearly all OHCs had disappeared, while in the wild types OHCs were still only affected at the base of the cochlea (Figures 4.2a and b). Thus, *Gata3* haploinsufficiency-induced degeneration started at the apex and ultimately progressed into the middle turns of the cochlea.

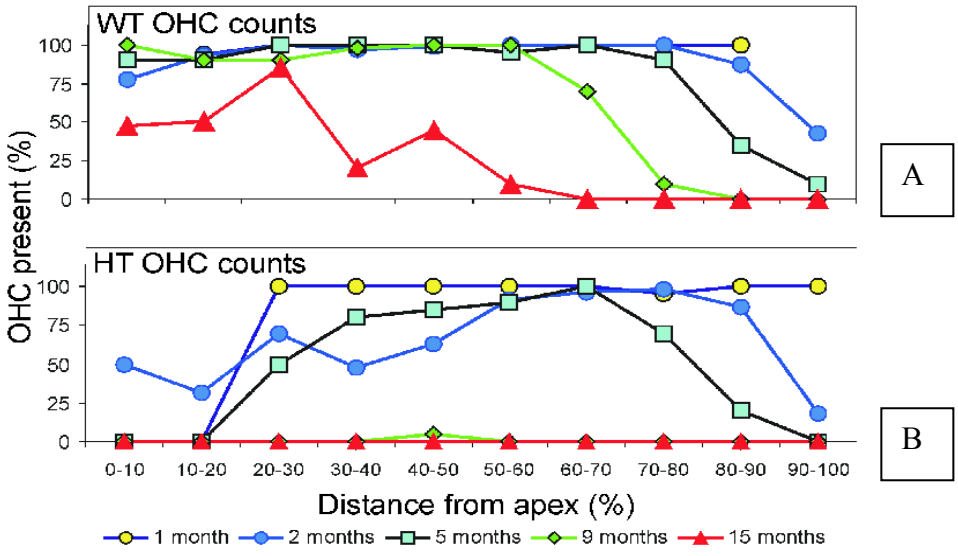

Figure 4.2: Presence of outer hair cells (OHC) in cochleae of wild type (A: upper panel) and heterozygous (B: lower panel) *Gata3* mice of 1, 2, 5, 9 and 15 months of age. Markers indicate the percentage of cells present in the 10 different cochlear pieces, plotted against the distance to the apex of the analyzed cochlear part. Hair cells responsive to 4 kHz, 8 kHz, 16 kHz and 32 kHz tones are found at approximately 25%, 40%, 60% and 75% distance from the apex respectively (Ou et al., 2000). OHCs in both the apical and basal cochlear regions degenerate in heterozygous mice, and progressively so with age. Wild type controls in contrast, are only affected by (normal) loss of basal outer hair cells. (Previously, data were presented partially in 'van der Wees et al., 2004')

To investigate effect of *Gata3* haploinsufficiency on the morphological structure of outer hair cells from heterozygous *Gata3* mice we analyzed semithin plastic sections and compared them to sections from wild types. We examined 1151 slices, representing 11 cochleae of 6 mice, at light microscopic level. The estimated width of OHCs in the cochlear regions examined in our study was approximately 5 µm, so that a maximum of 5 semithin slices per OHC may have been included in histological analysis. No compelling differences between heterozygous mice and wild type controls were found other than numbers of OHCs showing vacuoles. OHCs in heterozygous mice contained vacuoles more often than those in controls, an illustrative example of this can be seen in Figures 4.3a and b. In heterozygous mice vacuoles were found throughout the cell, whereas when present in wild type mice they were predominantly seen in the apical region.

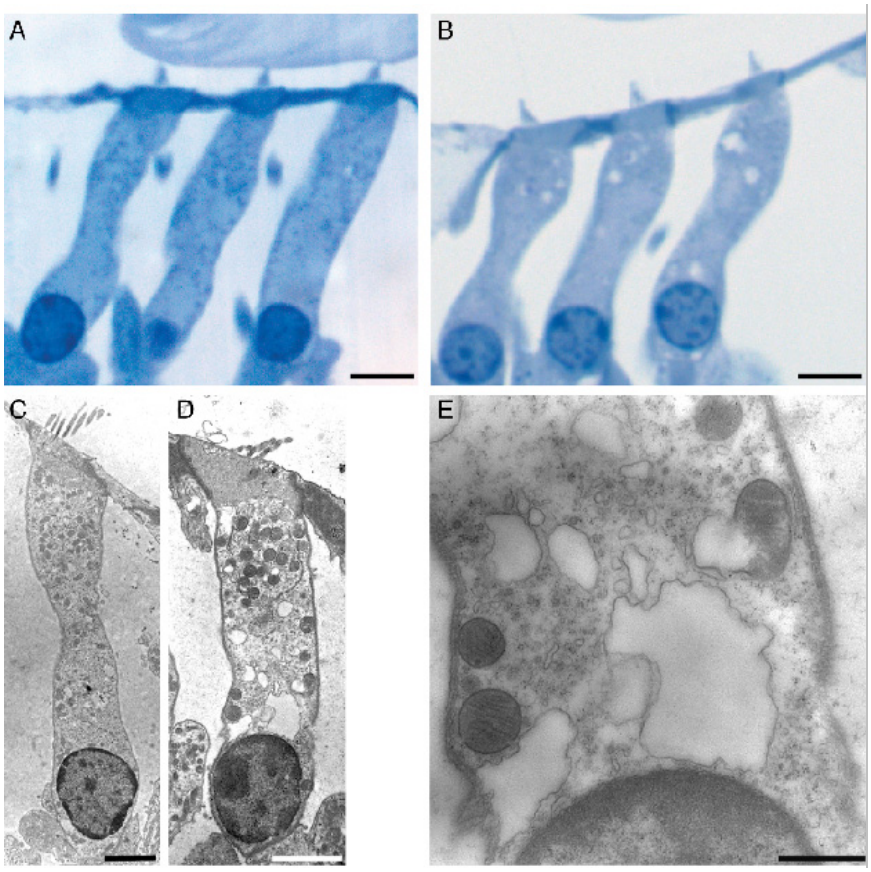

Figure 4.3: Vacuolization in *Gata3* heterozygous OHCs. Plastic sections of normal OHCs from a 2-months old wild type mouse (A) and of abnormal OHCs, with vacuoles in all three OHC rows, from a 2-months old heterozygous *Gata3* mouse (B). Electronmicroscopy of a normal wild type (C) and a *Gata3* +/- OHC (D) shows multiple vacuoles in the mutant OHC that otherwise looks normal. Panel E is a magnification of panel D and shows the irregular shape of the unfilled vacuoles that have a single layer membrane. Bars are 2 µm for panels A-D and 0.5 µm for panel E.

The difference in numbers of OHCs containing vacuoles was age-dependent: At one month of age this number significantly differed between genotypes for the first row of OHCs only (Mann Whitney, $P < 0{,}02$), whereas at two months of age the difference was significant (Mann Whitney, $P < 0{,}02$) for all three rows (see Figure 4.4a).

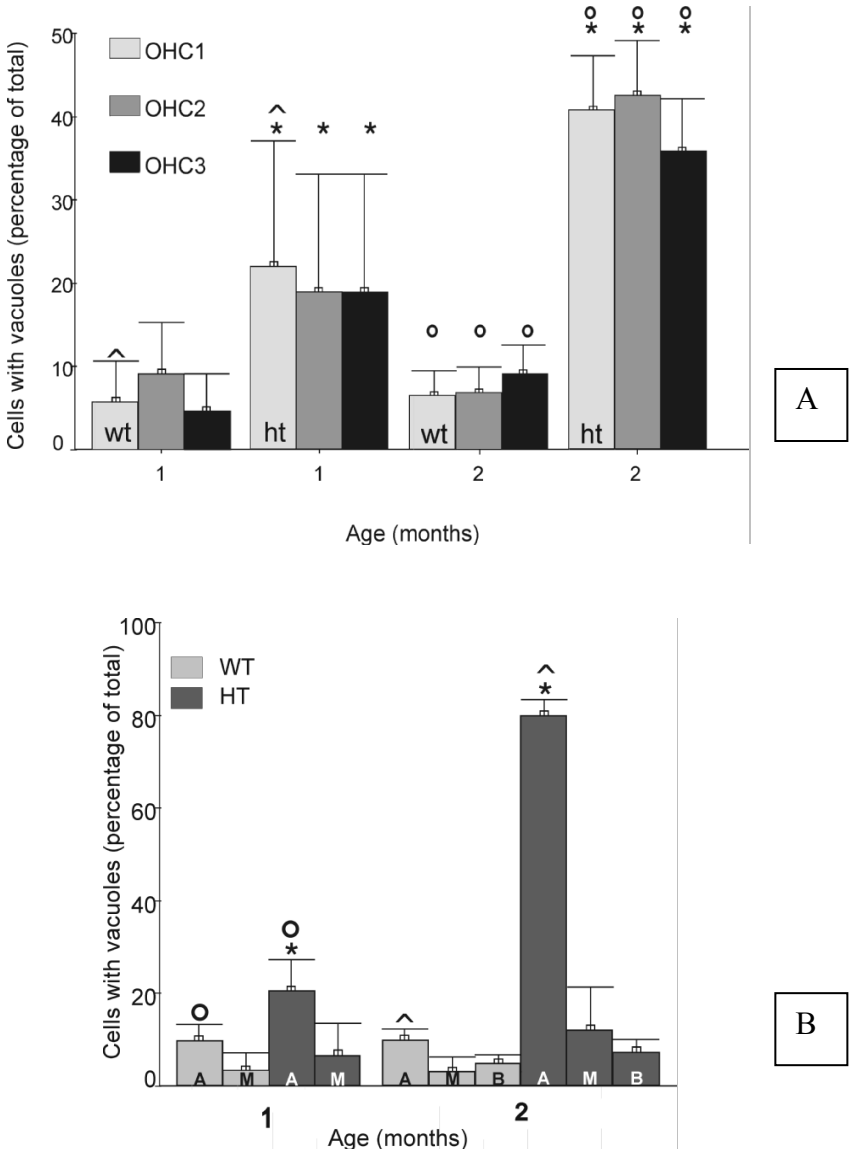

Figure 4.4: Panel A: Percentages of cells containing vacuoles versus age and genotype (wt; wild type, ht; heterozygous). Numbers of OHCs containing vacuoles significantly differed between genotypes for the first row of OHCs at one month of age (^), at two months of age this difference was significant for all three rows (**o**). Analysis within genotypes demonstrated an increase in the number of cells with vacuoles in the second as compared to the first month in heterozygous mice. This increase was significant for all three rows of OHCs at $P< 0.02$ (*). In wild types less than 10% of cells contained vacuoles. Error bars indicate 95% confidence intervals.

Panel B: Percentages of cells containing vacuoles versus age per genotype and cochlear localization. Analysis of influence of cochlear localization demonstrated the increase in the number of cells with vacuoles as described in heterozygous mice in Figure 4.4a, to be due to the highly significant increase in vacuoles in all rows of OHCs in the apical pieces in the second as compared to the first month of age (*****) (Mann-Whitney, P< 0.001). No significant differences could be demonstrated in the mid-cochlear or basal pieces. Numbers of cells containing vacuoles in the cochlear apex, however differed significantly between genotypes, both at one (**o**) and two months (^) of age (Mann-Whitney, P<0,02). Error bars indicate 95% confidence intervals. A = apical, M = mid-cochlear, B = basal.

Analysis within genotypes demonstrated an increase in the number of cells with vacuoles in the second as compared to the first month in heterozygous mice. This increase was significant for all three rows of OHCs at $P < 0.02$ (see Figure 4.4a). In wild types numbers of cells containing vacuoles were low constantly over the observed age-range.

The significant age-dependent increase in affected OHCs in heterozygous mice is mainly due to the highly significant increase in vacuoles in all rows of OHCs in the apical pieces (Mann-Whitney, $P < 0.001$). In the mid-cochlear pieces a similar trend was seen, but no significant differences could be demonstrated (see Figure 4.4b).

In conjunction, our data show that GATA3-haploinsufficiency induces OHC degeneration, especially in the apical regions of the cochlea, in mice as young as one month of age. These deleterious effects increase with increasing age.

Electron microscopy

To closer examine the nature of the observed vacuoles in the OHCs, we investigated them at the ultrastructural level. The vacuoles were irregularly shaped and surrounded by a single layer membrane (Figures 4.3 d, e). The OHCs did not show any sign of apoptosis or immediate cell death such as pyknotic nuclei or abnormal mitochondria. Thus, the first sign of GATA3-driven degeneration of OHCs seems to be the appearance of irregularly shaped vacuoles, which can be observed at both the light microscopic level and at electron microscopic level.

Otoacoustic emissions

Outer hair cell functionality was examined by measuring DPOAE levels in both *Gata3* heterozygous and wild type mice at various ages. DPOAE signal to noise ratios differed

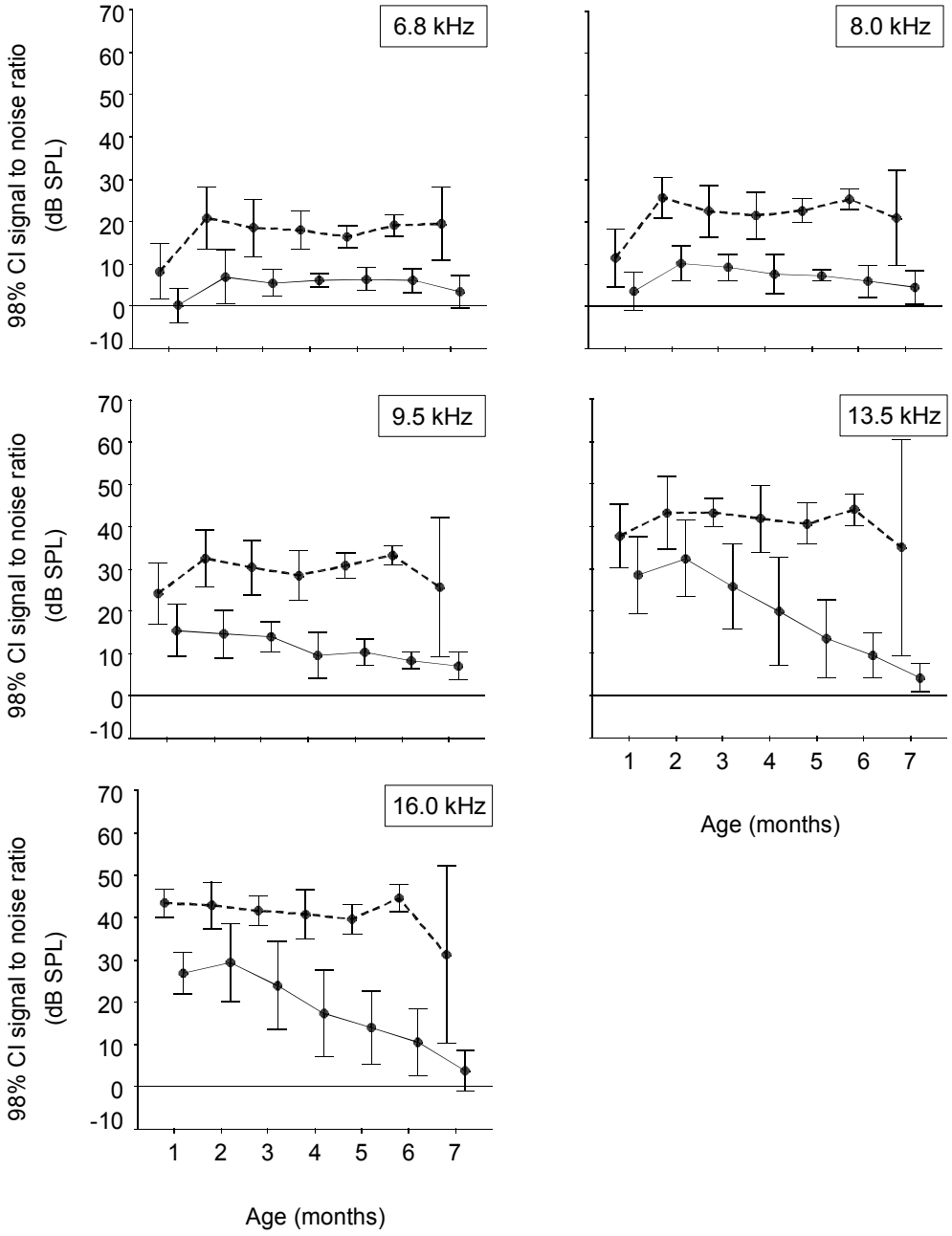

Figure 4.5: 98% confidence intervals of DPOAE signal to noise ratios (dB SPL) at f2's 6.8-16.0 kHz for wild type (discontinuous line) and heterozygous (continuous line) mice aged 1 to 7 months. At all ages and frequencies emissions in wild types were stronger than in heterozygous mice. At 7 months of age hardly any emissions could be generated in heterozygous knockouts, whereas signal to noise ratios in wild types remained fairly constant. Signal to noise ratios at 11.3 kHz are not represented graphically, since statistical analysis was unreliable due to small numbers of data (see Table 4.1).

significantly between *Gata3* heterozygous mice and wild types when data were pooled over all f2's and ages (Mann-Whitney Test, $P < 0.001$) (Figure 4.5). When separate ages and f2's were analyzed, this significance was consistent (at the $P < 0.02$ level) for groups larger than 6

(see Table 4.1), with the sole exception of 7 months old mice at 9.5 kHz (P = 0.023). Emission levels in the f2 frequency range from 6,8 kHz to 16,0 kHz remained fairly constant in wild type mice during the first seven months of life, whereas in the mutants signal to noise ratios rapidly decreased during this period (Figures 4.5 and 4.6). At 7 months of age signal to noise ratios of DPOAEs in heterozygous mice no longer differed from 0 dB. Signal to noise ratios did not differ significantly between genders for pooled data (T-test, P > 0.2), or within specific age groups (T-tests, P > 0.02).

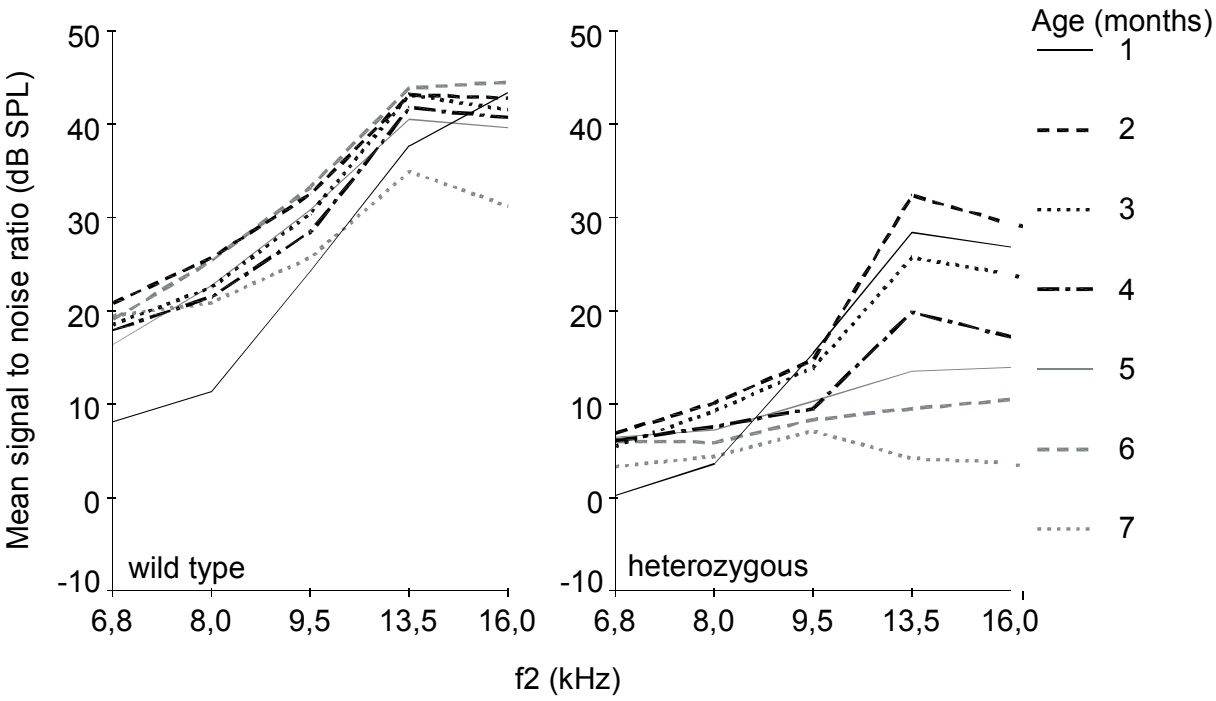

Figure 4.6: Signal to noise ratios (dB SPL) of 1-7 months old wild type and heterozygous mice in the f2 frequency range 6,8 to 16,0 kHz. During the first seven months of life, emission levels remain fairly constant in wild type mice, whereas in heterozygous mice signal to noise ratios rapidly decrease.

In sum, our acousto-physiological analyses demonstrated a rapid deterioration of OHC functionality in *Gata3* heterozygous mice, starting apically in the cochlea and progressing onto its base.

Discussion

Here, we show that young heterozygous *Gata3* mice generate abnormal Distortion Product Oto-Acoustic Emissions (DPOAEs) and that their outer hair cells (OHCs) are commonly affected by vacuoles. Together these data raise the possibility that dysfunctional OHCs form

one of the prime causes of hearing loss in these mutants. Since HDR patients, who suffer from the same haploinsufficiency as these mouse mutants, suffer from hearing loss as well, this pathological mechanism may also hold for HDR patients.

Outer hair cells are affected by both histological and physiological aberrations

Otoacoustic emissions (OAE's) are the best means for testing OHC function in vivo (Schrott et al., 1991). In our present study Distortion Product Otoacoustic Emissions were recorded in 1-7 months old *Gata3* heterozygous mice and in age- and sex matched controls. In both genotypes the loudest emissions were found in the higher end of the frequency range (6.8 kHz - 16 kHz). Emission levels decreased rapidly over time in heterozygous knockout mice, whereas in wild type controls they remained fairly constant during the first 7 months of life. Signal-to-noise emission levels in the FVB/N controls in this study are equal to, or even slightly better than emission levels in the CBA/J mouse model, which is considered a well hearing strain until relatively late in life (Parham et al., 1999). During DPOAE-recordings we used gas anesthesia, since this type of narcosis provides flexibility (e.g. short-term anesthesia), despite the known increase in middle ear pressure caused by N2O, necessitating paracentesis. Moreover, we demonstrated that repeated measurements in itself did not influence DPOAE levels (Figure 4.1). Thus, in contrast to the commonly used intraperitoneal or intramuscularly anesthesia (e.g. Sun et al., 1999, Jimenez et al. 2001, Konrad-Martin et al., 2001), gas anaesthesia may be quite feasible for (longitudinal) DPOAE-recordings.

Heterozygous mice also differed from controls in numbers of OHCs containing vacuoles, increasingly so with age. Curiously, no gross abnormalities were found in other cochlear cell types expressing GATA3. Further histological analysis of OHCs by electron microscopy excluded other changes at the cellular level such as apoptosis. Vacuoles are frequently seen as the first signs of (hair) cell death (Vetter et al., 1996). At two months of age the numbers of outer hair cells containing vacuoles in heterozygous mice were especially high in the apical region of the cochleae, which is responsive to frequencies of approximately 4 kHz (Ou et al., 2000), whereas in the middle (responsive to 16 kHz) and basal regions (responsive to 32 kHz) these numbers remained low. These findings are in agreement with the outcomes of the DPOAE measurements, which showed that the loudest emissions were found in the vacuole-free frequency regions. Thus, although the current data do not allow us to conclude that there is a direct causal relation between the deficits in DPOAEs and the occurrence of vacuoles in the OHCs, both findings suggest that functional deficits in OHCs may contribute to hearing loss in HDR syndrome.

Role of outer hair cells in hearing loss in HDR

Our findings that GATA3 haploinsufficiency induces an age-dependent loss of OHC-function as well as concomitant histological anomalies in OHCs raise the question as to whether the OHCs can be held solely responsible for the hearing loss in HDR syndrome. Although at present it is not possible to answer this question conclusively, several findings suggest that we cannot exclude this possibility either.

First, deficits in both DPOAEs and ABRs start to occur in the first few months of life (see also van der Wees et al., 2004).

Second, DPOAE signal to noise ratios in heterozygous *Gata3* mice as compared to controls are lower for all f2-frequencies in the 6,8–16,0 kHz range, agreeing with the fact that ABR-thresholds in these mice were elevated for all tested frequencies (4-32 kHz) in the age-range of 1-19 months as compared to controls. Moreover, ABR-thresholds are worse at 4 kHz, compared to 16 kHz for ages up to 19 months, this corresponds to lower DPOAE-signal to noise ratio's in the lower end of the frequency range described here.

Third, ABR-thresholds in heterozygous mice are on average elevated by approximately 30 dB (van der Wees et al., 2004), which agrees well with numerous other animal models in which there is a presumptive selective deficit of the OHCs. For example, Kanamycin sulfate treatment, causing complete loss of OHCs, with preservation of IHCs, results in threshold shifts of 40 dB and more in chinchillas (Woolf et al., 1981). In the same species, the first approximate 30 dB of noise-exposure induced permanent threshold shifts is induced by primarily outer hair cell losses (Hamernik et al., 1989). On the other hand, neomycin in combination with acoustic trauma causes decreases in cochlear sensitivity amounting up to 62 dB, averaged across all frequencies, in guinea pigs with losses of cochlear OHCs close to 100% (Brown at al., 1978). Unfortunately, the effect of the used treatment on other cochlear cell types was not described. Finally, homozygous *Kit W*-v mice (Wv/Wv), which have normal IHCs but lack 98% of cochlear OHCs, have an elevation of ABR- thresholds up to about 50 dB, as compared to control mice (Schrott et al., 1990).

It should be noted that the exact correlation between DPOAEs and ABR-thresholds remains to be established. DPOAEs were below the level of detection in laboratory animals such as mice and chinchilla's when ABR-threshold shifts exceeded approximately 40 dB (e.g. Kakigi et al., 1998, Le Calvez et al., 1998a), however when tentatively correlating DPOAE levels to ABR-thresholds variances were fairly large and no significant correlation was established so far. When considering our data we find that the best DPOAE signal to noise ratios in heterozygous *Gata3* mice were recorded in the upper half of the tested frequency spectrum

during the first months, when ABR-thresholds of corresponding frequencies (8 and 16 kHz) ranged approximately between 30 and 50 dB SPL. Moreover, in wild type controls DPOAE signal to noise ratios were high and remained fairly constant over the time span presented in this paper, where ABR-thresholds did on average not exceed 40 dB SPL during the first 7 months of life for the 4-16 kHz range.

Thus, taken together we can conclude that it appears likely that OHC dysfunction caused by GATA3 haploinsufficiency contributes to hearing loss in HDR syndrome, but it remains to be demonstrated whether it is the sole cause.

Conclusions

GATA3 haploinsufficiency is known to cause hearing loss in both humans affected by the HDR syndrome and mice. The goal of the present study was to elucidate the role of outer hair cells in this process. We demonstrated that GATA3 haploinsufficiency causes a rapid deterioration of OHC function in mice. Apical OHCs show early signs of cell degeneration in affected mice as young as one to two months in that they exhibit multiple vacuoles and produce abnormal emissions. We conclude that, outer hair cell malfunctioning can play a major role in the hearing loss in HDR syndrome.

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Chapter 5:

Characteristics of Hearing Loss in HDR (hypoparathyroidism, sensorineural deafness, renal dysplasia) syndrome

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“We still did not answer the questions that are important to us.”

Paulo Coelho

Summary

Haploinsufficiency of zinc-fingertranscriptionfactor GATA3 causes the triad of hypoparathyroidism, deafness and renal dysplasia, known by its acronym HDR syndrome. The purpose of the current study was to describe in detail the auditory phenotype in human HDR patients and compare these to audiometrical and histological data previously described in a mouse model of this disease.

Pure tone audiometry, speech audiometry, speech in noise, Auditory Brainstem Responses (ABR) and Transiently Evoked Otoacoustic Emissions (TEOAE) were measured in two patients affected by HDR syndrome. Both patients were affected by a moderate to severe sensorineural hearing loss. Speech reception thresholds were shifted and speech recognition in noise was disturbed. No otoacoustic emissions could be generated in either patient. Auditory brainstem response interpeak intervals were normal.

The human and murine audiological phenotypes seem to correspond well. Hearing loss in HDR syndrome is moderate to severe, seems to be slightly worse at the higher end of the frequency spectrum and may be progressive with age. The absence of otoacoustic emissions and loss of frequency selectivity suggest an important role for outer hair cells in causing the hearing loss.

Key words: HDR syndrome, GATA3, Audiometry, OHC

Introduction

Hearing loss in combination with hypoparathyroidism and renal dysplasia was first described in patients in 1977 (Barakat et al., 1977). However, it was not until several years later that it was recognised as a specific clinical entity (Bilous et al., 1992). Since then the triad of hypoparathyroidism, deafness and renal dysplasia has been known by its acronym HDR syndrome. This syndrome is inherited as an autosomal dominant trait. Molecular genetic and deletion-mapping studies mapped the HDR gene to 10p 13/14 (Hasegawa et al., 1997; Lichtner et al., 2000), and identified that it was caused by abnormalities of the *GATA3* gene (van Esch et al., 2000).

Since the first description of HDR syndrome several papers reporting on the clinical features of the syndrome in individual patients or (small) groups of patients have been published (e.g. Mino et al., 2005; Muroya et al., 2001; Zahirieh et al., 2005). These patients, as tested by ABR (auditory brainstem response), conditioned oriented reflex tests or pure tone audiometry, were affected by either a symmetric or an asymmetric sensorineural hearing loss (ranging from moderate (40 dB) to profound (105 dB)). Hearing loss was usually more severe at the higher end of the frequency spectrum (Bilous et al., 1992; Fujimoto et al., 1999; Hasegawa et al., 1997; Lichtner et al., 2000; Muroya et al., 2001; Yumita et al., 1986). Hitherto, however, hearing loss in HDR patients has not been systematically evaluated, and no attempts have been undertaken to identify the underlying cause of the hearing loss.

Previously we attempted to shed light on the pathophysiological mechanism causing the hearing defect in a mouse model of the syndrome. Our studies led us to conclude that hearing loss due to GATA3 haplo-insufficiency is peripheral in origin and that OHCs (outer hair cells) probably play a major role in the hearing loss in HDR syndrome (van Looij et al., 2005; van der Wees et al., 2004).

Here, we describe in detail the hearing loss in two patients, one who was newly diagnosed with the HDR syndrome and one in whom the phenotypic spectrum has previously been described, however without a detailed description of audiometry (Beetz et al., 1997; van Esch et al., 2000).

Case descriptions

Case 1

The clinical details of this 19-year old German boy have been previously reported (Beetz et al., 1997; van Esch et al., 2000). He is the first child to normally hearing parents. He has three

younger brothers with normal hearing. He had been affected by a multicystic right kidney and a dysplastic left kidney since infancy. When he first started attending kindergarten, his teacher suspected mental retardation. However, medical examination demonstrated his learning difficulties to be due to hearing loss, rather than retardation, and after being fitted with hearing aids at the age of 8 years, he performed well in school. At the age of eleven years he was affected by Lyme borreliosis, for which he was treated with intravenous antibiotics. Apart from this, he had no other risk factors for acquired hearing loss in his medical history. Throughout childhood he recurrently suffered from episodes of renal disease. A diagnosis of HDR syndrome was established after laboratory findings indicated hypoparathyroidism.
GATA3 mutation analysis demonstrated a 465-513 deletion, resulting in a frameshift from codon 156 (van Esch et al., 2000).
This case has been described previously by (Beetz et al., 1997; van Esch et al., 2000).

Pure tone audiometry
Pure tone audiometry was recorded, using a Madsen OB822 clinical audiometer and TDH-39 earphones. A symmetric sensorineural hearing loss was found, ranging from 40 dB in the lower end of the tested frequency range, to approximately 65 dB at 4 kHz (Figure 5.1).
Speech audiometry
The shift of the speech reception threshold (50% speech recognition level) relative to normal was approximately 55 dB in the left ear, and approximately 60 dB in the right ear (Figure 5.1).
Speech in noise
The speech in noise threshold (Freiburger Zahlentest) was 1.8 ± 0.6 dB for the left ear and 2.4 ± 0.6 dB for the right ear. This is about 6 dB worse than normal.
ABR (Auditory Brainstem Responses)
Auditory Brainstem Responses were recorded with custom-made equipment, the EUPHRA-1 system (Erasmus University Physiological Response Averager). When stimulating with 90 dB nHL clicks ABR peaks I (1.6 ms) through V (5.8 ms) were clearly present in both ears. Absolute latencies, as well as the I-III interpeak interval and the I-V interpeak interval were within the normal range (Figure 5.1).
TEOAE's (Transiently Evoked Otoacoustic Emissions)
TEOAE's were recorded using standard ILO88 equipment (Otodynamics). No transiently evoked otoacoustic emissions could be generated in either ear when stimulating with 81 dB peSPL (Figure 5.1).

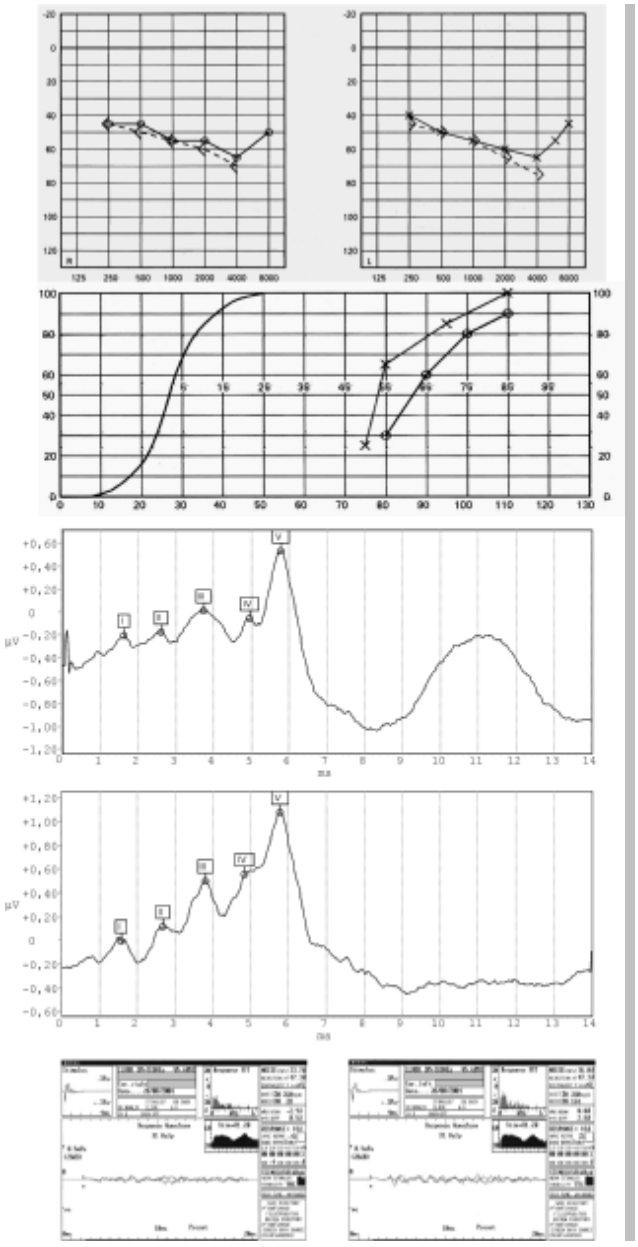

Figure 5.1: Case 1, the uppermost panel displays pure tone audiometry; the right ear is displayed on the left and the left ear on the right side.
The second panel represents speech audiometry. The third and fourth panel display ABR traces at 90 dB nHL for the right and left ear respectively. The fifth panel represents the TEOAE measurement for the right (left panel) and left (right panel) ear.
See text for details.

Case 2

A 33-year old Dutch female, had been affected by idiopathic hearing loss, for which hearing aids had been prescribed since the age of four. Apart from an isolated episode of otitis media in early childhood, her medical history did not reveal any risk factors for hearing loss, such as the use of ototoxic medication or a trauma of the head or ears. Both her parents and only sister had normal hearing. In her mid-twenties the patient started to suffer from cramp-like pains in her hands, and in her late twenties, she suffered from three generalised seizures due to hypocalcaemia that was secondary to hypoparathyroidism.

The patient requested for genetic counseling, in the context of reproductive decision-making. She was noted to have suffered from a single episode of pyelonephritis several years earlier, and renal ultrasonography demonstrated an asymptomatic cyst in the right kidney. This was confirmed by an abdominal CT-scan. On the basis of these findings a diagnosis of HDR syndrome was considered.

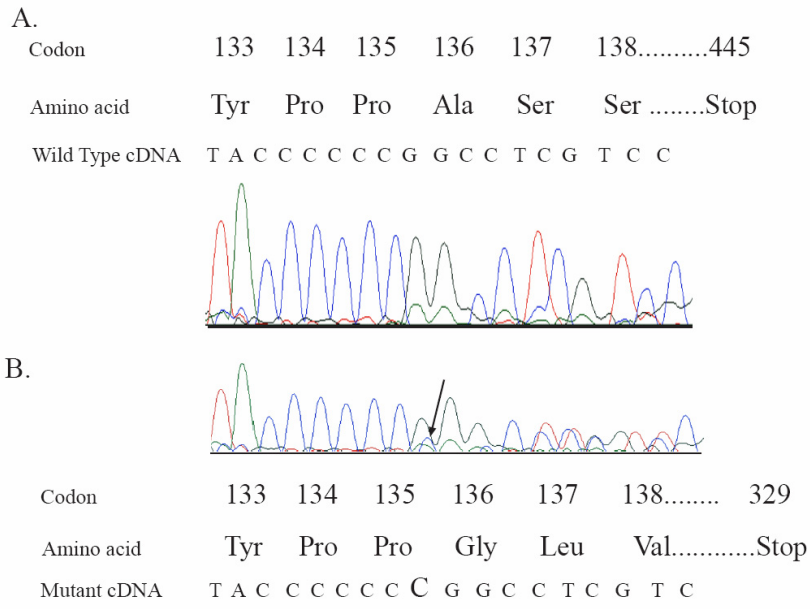

Figure 5.2: Detection of mutation in exon 3 of the Case 2 individual by DNA sequence analysis.

DNA sequence analysis of a 33 year old individual (Case 2) revealed the insertion of a C at codon 135 of exon 3 (indicated by arrow). This led to a frameshift that resulted in 193 missense amino acids from codon 136 to codon 328, followed by a premature termination signal (Stop) at codon 329. This change did not result in the alteration of a restriction enzyme site, hence was confirmed by repeat sequencing. This mutation is predicted to result in the loss of zinc-fingers 1 and 2 and haplo-insufficiency. Panel A shows a normal wild-type sequence of codons 133-138 and panel B shows the mutant sequence of the Case2 individual, with insertion of a C (indicated by arrow) and resulting frameshift.

Venous blood was obtained after informed consent, as approved by the local ethical committee, and used to extract leukocyte DNA (Nesbit et al., 2004). Nine pairs of GATA3 specific primers were used for PCR amplification of the six exons and ten intron-exon boundaries using 50 ng of genomic DNA as described (Nesbit et al., 2004). The DNA sequences of both strands were determined by Taq polymerase cycle sequencing and resolved on a semi-automated detection system (ABI 377XL sequencer, Applied Biosystems, Foster City, CA) (Nesbit et al., 2004). The DNA sequence abnormality, was confirmed by repeat sequence analysis, and was demonstrated to be absent in the DNA obtained from 55 unaffected unrelated individuals, using methods previously described (Nesbit et al., 2004).
Chromosomal analysis demonstrated a normal female karyotype (46, XX), but *GATA3* mutation analysis demonstrated an insertion of a nucleotide C of codon 135 in exon 3 that resulted in a frameshift (Figure 5.2). This is predicted to lead to a missense peptide from codon 136 with a premature termination at codon 329, such that there is a loss of zinc fingers 1 and 2. The mutation was not detected in either of her parents, and hence she represents a *de novo* mutation.

Pure tone audiometry

Pure tone audiometry was recorded, using a Madsen OB822 clinical audiometer and TDH-39 earphones. A moderate to severe sensorineural hearing loss was demonstrated, that was most pronounced in the highest frequencies. Losses ranged from 45 dB HL at 250 Hz to a maximum of 75 dB HL at 4 kHz in the right ear and from 45 dB HL at 250 Hz and 500 Hz to a maximum loss of 85 dB HL at 8 kHz in the left ear. A mixed hearing loss was found only at 250 Hz, with a conductive component of 30 and 20 dB for the right and left ear respectively (Figure 5.3).
Interestingly, we were able to compare these data to audiograms made during childhood. The first audiogram at the age of eight years, revealed that she was affected by a flat, predominantly sensorineural hearing loss, with a conductive component of 10-20 dB on average, with thresholds ranging from 40-60 dB HL (Figure 5.4), thus it appears that in this patient the hearing loss is progressive with age, especially so in the higher end of the tested frequency range.

Speech audiometry

Speech recognition level thresholds as compared to normal were shifted by 50 dB and 60 dB for the right and left ear respectively. Due to a 'plateau' in the curve, 100% speech

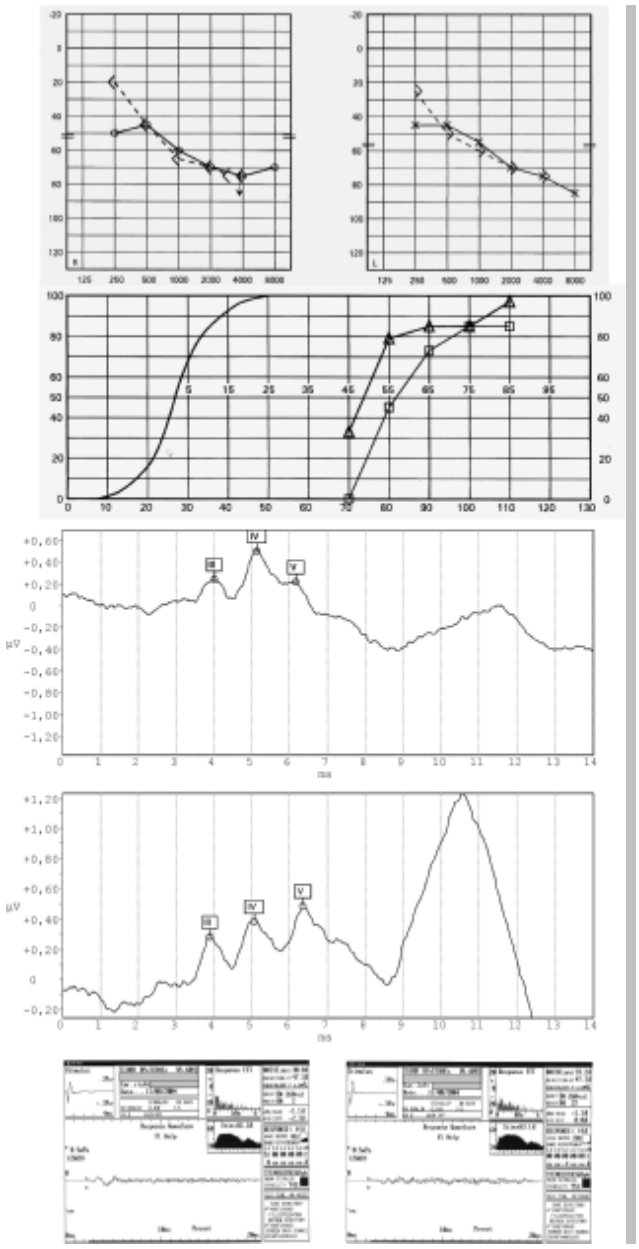

Figure 5.3: Case 2, the uppermost panel displays pure tone audiometry; the right ear is displayed on the left and the left ear on the right side.
The second panel represents speech audiometry. The third and fourth panel display ABR traces at 90 dB nHL for the right and left ear respectively. The fifth panel represents the TEOAE measurement for the right (left panel) and left (right panel) ear.
See text for details.

recognition was attained only at the 110 dB sound level in the right ear, whereas in the left ear maximum of 85% speech recognition was attained at the 100 dB sound level (Figure 5.3).

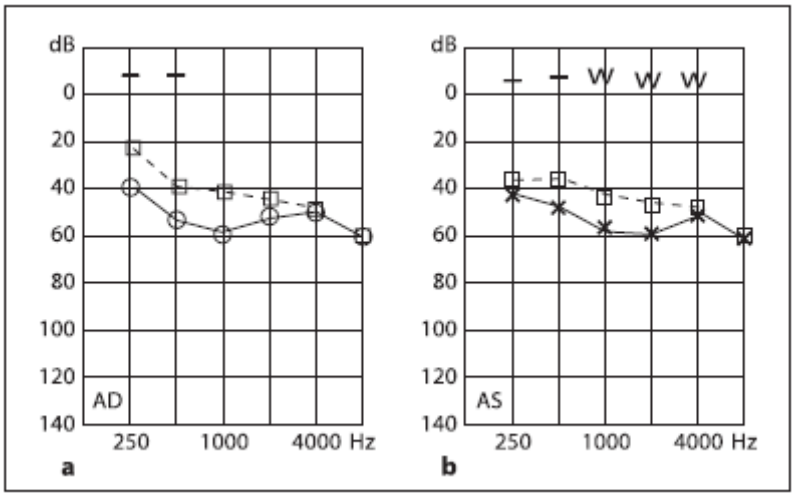

Figure 5.4: Pure tone audiometry of case two, at age eight. The left panel (a) represents the right ear; the right panel (b) represents the left ear.
See text for details.

Speech in noise

Free field testing by the Plomp and Mimpen test (Plomp et al., 1979) without hearing aids for the right ear yielded an average signal to noise ratio of 0.7 dB, whereas for the left ear this ratio was –2.0 dB. The average binaural signal to noise ratio was 0.4 dB.

ABR (Auditory Brainstem Responses)

Auditory Brainstem Responses were recorded with custom-made equipment, the EUPHRA-1 system (Erasmus University Physiological Response Averager). When stimulating with 90 dB nHL peaks III (3.9 ms and 4.0 ms, left and right ear respectively), IV and V (6.2 ms and 6.3 ms, right and left ear respectively) were clearly present. The III-V interpeak interval was not prolonged.

The absolute latency of peak V was prolonged for both ears. However, this was consistent with hearing thresholds as demonstrated by pure tone audiometry (Figure 5.3).

TEOAE's (Transiently Evoked Otoacoustic Emissions)

TEOAE's were recorded using standard ILO88 equipment (Otodynamics). No transiently evoked otoacoustic emissions could be generated in either ear when stimulating with 82-83 dB peSPL (Figure 5.3).

Discussion

The HDR syndrome is an autosomal dominant condition, defined by the triad hypoparathyroidism, deafness and hearing loss. Although phenotypic expression may be variable, it seems that hearing loss is a rather consistent feature, affecting the majority of patients (Muroya et al., 2001). So far, only few data have been published reporting the nature of the hearing loss in HDR patients.

Presently, we describe in detail hearing loss in two patients affected by HDR syndrome. Both patients suffered from a moderate to severe sensorineural hearing loss, which was worse in the higher end of the tested frequency range. No increases in interpeak latencies were demonstrated by ABRs, indicating a probably peripherally hearing loss. Speech audiometry corresponded well with pure tone audiometry, however both patients performed poor on speech in noise tests. The latter finding suggests a reduced frequency selectivity, which may be due to a loss of outer hair cells (Harrison et al, 1981-a; Harrison et al., 1981-b). Interestingly, in neither patient TEOAEs could be generated. The combination of the above findings – hearing loss with reduced speech in noise performance, absent otoacoustic emissions and normal ABR waves - substantiates the suggestion that outer hair cells might be lost, or that their functionality is reduced. Taking into consideration the severity of the hearing loss in our patients and patients previously described in literature, it is likely that inner hair cells also play a role in the coming about of the hearing loss.

When defining hearing loss due to a specific cause, it is important to exclude the influence of other possible risk factors for hearing loss. Our first case suffered from Lyme borreliosis at the age of eleven years, which may cause sudden hearing loss, although the causal relationship between the infection and sensorineural hearing loss is difficult to verify (Peltomaa et al., 2000). However, since the infection was adequately treated, and since hearing did not deteriorate in the peri-infectious period, it would seem that Borreliosis wasn't a causative factor for the hearing loss in this patient. The second patient did not yield any risk factors for hearing loss other than one single episode of otitis media in early childhood, which is unlikely to have caused the progressive loss of hearing.

In order to elucidate the underlying pathophysiology causing HDR syndrome we previously studied hearing loss in a mouse model of the disease. We demonstrated Auditory Brainstem Response-thresholds in heterozygous (+/-) *Gata3* mice to be elevated by approximately 30-dB as compared to those in controls, in absence of interpeak latency differences (van der Wees et al., 2004). Apart from this, distortion product otoacoustic emission levels (f2 frequency range

from 6,8 kHz to 16,0 kHz) rapidly decreased during the first seven months of life in the +/- mutants, whereas in the wild type (+/+) mice they remained fairly constant (van Looij et al., 2005). In addition, *Gata3* heterozygous mice, showed early signs of outer hair cell degeneration, especially in the cochlear apex, at both light microscopic level and electron microscopic level, without any morphological abnormalities in the central nervous system. Outer hair cell loss was followed by an extensive loss of both inner hair cells and supporting cells. This degenerative process progressed in an apico-basal direction from young ages onwards, and in combination with a normal age-related basal cell loss, ultimately affected all cochlear hair cells and supporting cells (van Looij et al., 2005; van der Wees et al., 2004). These findings led us to conclude that hearing loss due to GATA3 haplo-insufficiency is peripheral in origin and that OHCs probably play a major role in the hearing loss in HDR syndrome.

Mutant mouse models of hearing loss are very valuable in auditory research aimed at elucidating pathophysiological mechanisms underlying deafness and for gene identification by positional cloning, and often there is good agreement in pathology between mice and man (Steel, K.P., 1995). Occasionally however, differences in phenotypic expression occur between man and mice. A well-known example of this is the *MYO7A* gene, mutations of which cause Usher 1B syndrome in man, but hearing loss and vestibular dysfunction without blindness in shaker-1 mice. It has been hypothesized however, that the absence of visual problems may be due to the time span needed for retinal degeneration to develop (Keats and Savas, 1999; Steel, 1995). Another illustrative example of different phenotypic expression is seen in Pompe's disease; children affected by the classic infantile form of Pompe's disease are affected by a moderate to severe hearing loss, whereas no differences in auditory function - as measured by ABRs - could be demonstrated between a knockout mouse model of Pompe's disease and wild-type controls (Kamphoven et al., 2004).

These observations indicate that cautious interpretation is required when comparing murine and human data. However, our present study data from HDR patients indicated that the audiological phenotypes are similar between man and mouse, even though histological data from human studies are not available.

In conclusion, hearing loss in HDR syndrome is moderate to severe and it appears to be worse at the higher end of the frequency spectrum. Possibly, auditory functioning in HDR patients deteriorates with age. The hearing loss is brought about by disorders in the peripheral part of the auditory system and absence of otoacoustic emissions and loss of frequency selectivity

suggest a substantial causative role for outer hair cells, without however precluding a role for inner hair cells. Although no histological data of cochleae of patients affected by HDR syndrome are available to date, it appears that otological or at least audiological phenotypes between HDR patients and heterozygous *Gata3* knock-out mice correspond well.

Acknowledgements

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Chapter 6:

General discussion

…

“Morgen ga ik vliegen”, zei de slak. “Tenminste, ik ga eerst oefenen”.

De mier liep naar huis en sliep die nacht slecht.

De volgende dag betrapte hij zich erop dat hij telkens naar de lucht keek. Maar, dacht hij dan, vliegen kan hij nooit. Nooit! Dat bestaat niet!

Laat in de middag kwam de slak voorbij, traag en stuntelig fladderend. Hij slingerde, botste tegen de toppen van grassprieten aan, helde soms vervaarlijk opzij. Maar hij vloog.

Toen hij de mier zag wuifde hij en riep: “Kom jij ook vliegen? Het valt best mee. Je hoeft alleen maar op te stijgen.”

De mier keek naar de grond en trapte met volle kracht tegen een losliggende wortel.

Uit ‘Misschien wisten zij alles’ , Toon Tellegen, Em. Querido’s Uitgeverij B.V., 2005.

The last decennia great scientific progress has been made in the field of both syndromic and non-syndromic hereditary deafness. Studies of audiology, physiology, inner ear metabolism at molecular level and histology, as well as family, gene-linkage, and gene-identification studies have provided knowledge – and surely in the future will do even more so - about genotype-phenotype correlations and the natural course of hereditary deafness. This knowledge is very important in clinical otological practice. It will contribute to the development and selection of guiding patterns for patients with hereditary hearing loss, and in the future possibly even to the development of new treatments of sensorineural hearing loss.

The study described in this thesis was aimed at elucidating the nature of hearing loss in Hypoparathyroidism, Deafness and Renal dysplasia (HDR) syndrome. Although embryologically the kidneys develop from the intermediate mesoderm and the inner ears from the epidermal otic placode (Larsen, 1993) renal anomalies and hearing loss often co-occur. One possible pathophysiological mechanism causing both renal disease and hearing loss, may be the malfunctioning of transporters or channels - for e.g. chloride, bicarbonate, protons, sodium, potassium or water - that are found in both systems (as reviewed by Peters et al., 2004). In Table 1.1 of the introduction of this thesis an overview of syndromes in which hearing loss and renal anomalies co-occur is given. From this table, we can conclude that disturbed endolymph homeostasis is at least responsible for the clinical features of Bartter syndrome and Distal Renal Tubular Acidosis (DRTA) with sensorineural hearing loss. For some of the remaining syndromes other common pathophysiological mechanisms can be discerned; metabolic accumulation is the cause of Fabry disease and Refsum's disease, whereas BOR syndrome, Nager syndrome, Okihiro syndrome and Townes Brocks syndrome are caused by mutations involving transcription factors.
When studying syndromes involving both the kidneys and the inner ears, one needs to bear in mind that although microarray hybridization analysis of gene expression in these organs suggests a relationship between the tissues at genomic level, genes in either organ may be over- or underexpressed as compared to the other (Liu et al., 2004).

Experimental set-up: impact of anesthesia on audiometry

In this thesis, we used a variety of techniques to study the nature of hearing loss in HDR syndrome in knock-out mice as well as in patients. Chapter 2 reports on a pilot-study, conducted in healthy laboratory mice, aimed at developing an objective and accurate method

to assess hearing acuity in these animals. One commonly used method of testing the auditory function of laboratory animals is the recording of auditory brainstem response (ABR) thresholds. In addition to providing hearing thresholds, ABR's inform us on the functioning of the central auditory pathways up to the level of the inferior colliculus (Henry, 1979). In spite of previous publications, demonstrating an anesthesia (ketamine) induced increase in peak latencies in gerbil and rat (Church and Gritzke, 1987; Smith and Mills, 1989, 1991), possibly due to a decrease in resting membrane potentials of n. VIII (Shrivastav, 1997), ABR assessments in mice were invariably done in anesthetized animals (Henry, 2002; Huang et al., 1995; Jero et al., 2001; Miller et al., 1998; Ou et al., 2000).

We describe a surgical technique, allowing for ABR recordings in alert mice. With this technique longitudinal experiments can be performed without the need for repetitive prolonged general anesthesia, apart from the initial 45 minutes of anesthesia needed for surgery. We demonstrate that ABR thresholds are significantly elevated when recorded under general (ketamine/xylazine) anesthesia. We propose that this upward threshold shift is mainly due to a ketamine/xylazine effect, but we cannot exclude for sure the effects of pinna orientation. Furthermore, we show that general anesthesia causes a significant prolongation of ABR-peak latencies and I-III interpeak latencies as well as a decrease of the accuracy of peak- and interpeak latency, and threshold measurements. Therefore, our method as compared to conventional ABR recordings using subcutaneous needle electrodes, allows a more accurate evaluation of central auditory pathways up to the level of the inferior colliculus. This may be especially useful in (mouse models of) complex diseases, possibly affecting both the peripheral and central auditory system. Finally, it is demonstrated that the stimulus repetition rate can be as high as 80 per second without affecting ABR thresholds, interpeak latencies or waveforms.

In addition to influencing auditory brainstem responses, high-dose ketamine anesthesia also affects otoacoustic emission (OAE) recordings, probably by alteration of middle ear dynamics (Hatzopoulos et al., 2002). Moreover, ketamine is not the only anesthetic agent known to influence middle ear pressure. Nitrous oxide and pentobarbital induce negative middle ear pressure in children (Chinn et al., 1997) and gerbils respectively (Zheng et al., 1997). In order to avoid the considerable influences of general anesthesia on audiological outcomes we tried to develop a way to record otoacoustic emissions in alert mice. Mice were anesthetized. Using a ventral approach, an incision was made between the mandibular angle and the middle of the clavicle. The neurovascular bundle and digastric muscle were carefully retracted, allowing for access to the tympanic bulla. The bulla was opened and carefully avoiding the stapedial

artery, an electrode was placed in the cochlear round window. This electrode was diverted subcutaneously and connected to a head fixation pedestal (described in chapter 2). The round window electrode allowed us to record electrical variations of the intracochlear potentials. These variations result from outer hair cell activity, amongst other sources. In conventional electrocochleography one of the extracted components of these variations of potentials is called cochlear microphonics. The waveform of this component is almost an electrical analogue of the acoustic waveform of the stimulus used to generate it, hence it's name. Under stimulation conditions used for generating distortion product otoacoustic emissions, we expected that spectral analysis of the potential variations would reveal activity at the frequencies of the two stimulation tones ('the microphone signal'), but also at the frequencies of the distortion products generated in the cochlea. This approach would enable electrical recording of the distortion products, including the 2f1-f2 distortion product (usually recorded acoustically in the outer ear canal as the distortion product emission).

Apart from being rather time-consuming, unfortunately this method proved to be successful in only a small percentage of cases. This may have been due to difficulties we experienced in securing a stable electrical connection to the cochlea. Therefore, otoacoustic emissions in laboratory mice were eventually recorded using a mixture of 5% Fluothane, N_2O and O_2. Fluctuations in middle ear pressure – and concomitant fluctuations in signal strength of otoacoustic emissions – were prevented by perforating the pars flaccida of the tympanic membrane using a Microlance® needle (chapter 4).

Evaluation of hearing in a murine model of HDR syndrome

HDR syndrome is caused by haploinsufficiency of transcriptionfactor GATA3. The effects of *Gata3* haploinsufficiency on hearing in mice are described in chapters 3 and 4. Over a one-to-nineteen months age-range, heterozygous *Gata3* knock-out mice show a significant ABR-threshold elevation (approximately 30 dB), as compared to wild-type littermates. This threshold elevation is present at all tested frequencies. Both the heterozygous knock-outs and the wild type controls are affected by an age-related hearing loss, causing an average monthly increase in ABR-threshold of 2.2 dB.

In addition ABR- peak-and interpeak latencies were compared. Neither differed significantly between genotypes. The above findings led us to suggest that the hearing loss observed in the mutant mice is most likely caused by a peripheral – i.e. cochlear - deficit. This suggestion was substantiated by the fact that in the knock-out mice the outer hair cell (OHC) function, as

tested by distortion product otoacoustic emissions (DPOAEs), rapidly deteriorates during the first seven months of life.

Cochlear histology in a murine model of HDR syndrome

The auditory system of *Gata3*+/- mice and wild types was not only studied electrophysiologically, but also histopathologically. Heterozygous *Gata3* mice show a progressive morphological cochlear degeneration starting with apical OHCs from one month onwards that ultimately affects all hair cells and supporting cells in the entire cochlea, as well as the nerve fibers. This type of degeneration is rare, but has also been described in mice deficient for the *Barhl1* homeobox gene (Li et al, 2002.). In addition to the apical degeneration only found in the knock-out mice, some mild age-related morphological deficits starting at the cochlear base and progressing in a baso-apical direction, were demonstrated in both the affected mice and their control littermates (chapters 3 and 4).

The first sign of GATA3-driven degeneration is the appearance of irregularly shaped vacuoles in all three rows of outer hair cells. These vacuoles can be observed at both the light – and electron microscopic level (chapter 4).

Correlation between audiometric and histological data in a murine model of HDR syndrome

In chapters 3 and 4 of this thesis we have demonstrated a *Gata3* haploinsufficiency induced hearing loss in heterozygous knock-out mice. Since Gata3 is prominently expressed during different developmental stages in various parts of both the inner ear and the central auditory nervous system, one of the most apparent questions to be answered was whether *Gata3* haploinsufficiency induced hearing loss is peripheral or central in origin. ABR-recordings demonstrated a threshold shift in heterozygous knock-out mice, however without affecting interpeak latencies, indicating a peripheral deficit. The absence of morphologic abnormalities in the central auditory pathways supports this notion. GATA3 is not expressed in the middle ear ossicles, and no absolute ABR peak latency differences were demonstrated, suggesting that the hearing loss is caused by cochlear disorder. This was confirmed by our histological studies, demonstrating an extensive loss of OHCs – both sensors and mechanical feedback amplifiers – followed in time by a loss of inner hair cells (IHCs), pillar cells (PCs) and nerve fibers (NFs).

The lowest ABR-thresholds in both types of mice were found at 16 and 8 kHz, frequencies that are respectively perceived at 60 and 40 percent distance of the cochlear apex. This is very much in agreement with the histological findings in the knock-out mice, who show both an apical and basal age-related cochlear degeneration. The constant ABR-threshold difference between genotypes at 4 kHz, in this respect however, is unexpected and sofar unexplained.
When comparing ABR-thresholds to DPOAE signal to noise ratios, we find both the lowest ABR-thresholds and the best DPOAE signal to noise ratios at 16 kHz. Furthermore we find that disappearance of DPOAEs corresponds with an approximate ABR-threshold shift of 40-60 dB. This threshold shift is most probably for the greater part caused by OHC loss. However, the literature on the contribution of OHCs to hearing thresholds is not fully conclusive (as reviewed in the discussion section of chapter 4), and a contribution to this shift of e.g. IHC loss cannot be excluded.

Hearing loss in HDR syndrome, a comparison of mice and men

In Chapter 5 of this thesis, we describe the results of extensive audiometric testing of two human HDR patients. A moderate to severe, (probably) age-related hearing loss is demonstrated. Although this hearing loss affects all frequencies, it is slightly more pronounced in the higher end of the tested frequency spectrum. We compare the audiometric results obtained from the two patients to those from heterozygous *Gata3* knock-out mice (described in chapters 3 and 4). Based on the similarities in hearing loss between the two species, the loss of frequency selectivity in combination with normal auditory brainstem interpeak latencies in man and the histological data obtained from the mice, we conclude that the otological, or at least the audiological phenotypes between the two species seem to correspond well.
Mouse models of hearing loss for reasons discussed earlier in this thesis, today have become indispensable for basic auditory research. In some instances mouse mutations are identified even before the detection of the human disorder (e.g. Melchionda et al., 2001[2]), whereas in others the detection of a human 'deafness gene', induces the development of a murine knock-out model. Overviews of available mouse models have been published in several review papers (e.g. Ahituv et al., 2002; Morton 2002; Petit, 2006). However, the phenotypes, including the degrees of hearing loss, caused by a given mutation, may occasionally differ considerably between humans and mice. In Chapter 5 we describe two well-known examples

[2] E.g. *Myo6* and it's associated phenotype were described in Snell's Waltzer Mice before the identification of humans affected by DFNA22

of this phenomenon; Usher 1B syndrome and Pompe's disease. These differences might result amongst others from modifications in corresponding proteins, compensatory mechanisms, or different evolutionary developments (Petit, 2006). Finally, modifier genes, genes influencing the expression or function of other genes and thus accounting for phenotypic differences observed in a given disease, may be of influence in both inter- and intraspecies variability.

Suggestions for future research

In this thesis we have studied the nature of *GATA3* haploinsufficiency induced hearing loss by audiometric and histopathologic experiments. In order to fully understand the exact pathophysiological mechanisms leading to the cochlear degeneration and consequent hearing loss in HDR syndrome it would be beneficial to define all target genes of GATA3 and their functions and possible interactions with other genes. Recent studies have commenced in doing so. For example, a microarray analysis comparing gene expression levels of otic epithelia at an early vesicle stage between *Gata3 -/-* and wild type mice, identified 250 up-regulated and 63 down-regulated genes in the mutants. Amongst the up-regulated genes were gap junction membrane channel protein beta2 (*Gjb2*), encoding connexin 26, essential for the formation of intercellular membrane channels (gap junctions), and *Epha4* and *Ephb4*, encoding receptors that are involved in migration, adhesion and repulsion in multiple developmental events. In contrast secreted phosphoprotein 1 (Spp1), an extracellular matrix protein mediating cell-matrix interactions, cell-adhesion and cellular signaling, was down-regulated. Taking together these observations Lilleväli et al. suggested that cell motility in *Gata3 -/-* otic epithelium is changed (Lilleväli et al., 2006). The same group suggested a regulatory relationship between *Gata3* and fibroblast growth factor *Fgf10* (Lilleväli et al., 2006; Lilleväli et al., 2007). Furthermore, GATA3 was recently demonstrated to regulate the transcriptional activity of tyrosine hydroxylase through interaction with yet another transcriptionfactor; CREB, prompting Hong et al. to propose a role for GATA3 in the development of the sympathetic nervous system (Hong et al., 2006). Although the abovementioned studies, amongst several others, shed light on some of the functions and interactions of GATA3, a lot remains to be unraveled.

In chapter 4 we have described EM findings in OHCs of 2 months old heterozygous knock-out mice and healthy controls. It would be interesting to extend the examined age range, both to examine possible changes occurring at the ultrastructural before the occurrence of vacuoles

as well as to record the changes occurring hereafter. It would also be interesting to perform SEM, to closer examine the stereocilia.
In addition to the above, it would be (clinically) relevant to perform extensive audiometry in a larger group of patients, preferably of different age groups, allowing us to create age related typical audiograms (ARTA's).

General considerations regarding the future of basic auditory research

The discussion of this thesis would not be complete, without shortly addressing some exciting new developments in basic otologic research.
The primary cause of hearing loss is damage to or death of cochlear hair cells, as it also is in the syndrome described in this thesis. In contrast to birds and other non-mammalian vertebrates, mammals lack the capacity to regenerate sensory hair cells and consequently lose their cochlear hair cells through apoptosis when ageing or when exposed to severe damage such as noise exposure or exposure to ototoxic drugs. Although recently great advances have been made in the treatment of sensorineural hearing loss, in both the preventative (protein kinase inhibitors protecting against acoustic trauma and ototoxicity) and prosthetic (e.g. middle ear implantable hearing assistive devices, cochlear implants, hybrid devices and auditory brainstem implants) field (e.g. Holley, 2005; Murugasu, 2005), preliminary results of studies on regenerative therapies – aimed at repairing or replacing damaged hair cells – suggest that a cure for sensorineural hearing loss might no longer be an utopia in the future. Today, three different approaches aimed to develop regenerative therapies of hearing loss; stem cell transplantation, gene manipulation and gene therapy. In short, stem cells are cells with the capacity to develop into multiple cell types, the fate of the progeny of these cells is dependent on the microenvironment in which the cells reside. Current stem-cell related studies are for example aimed at finding 'niches' (groups of undifferentiated and multipotent stem cells) in the ear and at means to make stem cells differentiate into specific target cell types. Gene manipulation studies examine the possibility to interfere with cell cycle inhibitors, blockers that down-regulate mitosis in sensory epithelium and thus establish a mature, non-proliferating sensory epithelium. Finally, gene therapy is the delivery and (regulated) expression of a gene in a specific cell or tissue aimed producing a therapeutic effect in this cell or tissue. The cochlea seems quite well suited for this type of therapy, being isolated from the rest of the body by the blood-labyrinth barrier, and with endocochlear fluids permitting liquids to rapidly reach the entire cochlea. Genes may be delivered either by means

of viral vectors or in liposomes. Focus has mainly been on the Drosophila atonol *Atoh1* gene, and its murine and human homolog, *Math1* and *Hath1* respectively. The results obtained so far are promising: Adenoviral expression of *Hath1* in rats initiated robust regeneration of hair cells, whereas *Math1* gene transfer induced both structural and functional recovery of cochlear hair cells in vivo in guinea pigs (Atar et al., 2005; Holley, 2005; Izumikawa et al., 2005; Matsui et al, 2005; Van de Water et al, 1999)

It seems that some of the above studies make "the ability to cure sensory defects, a tangible goal that is now within our reach" as stated by Atar and Avraham in 2005.

Conclusions

One of the objectives of this thesis was to develop means to test hearing in alert laboratory animals and to evaluate the effects of anaesthesia on audiometry outcomes. In chapter 2 we describe a surgical technique, allowing us to measure ABRs longitudinally in alert mice. Moreover, we describe a significant anesthesia induced upward shift of ABR thresholds as well as a significant prolongation of ABR-peak latencies and I-III interpeak latencies.

The main goal of this study, however, was to assess whether the auditory system of heterozygous *Gata3* mice is a proper model for that of patients affected by the HDR syndrome. We have demonstrated that *Gata3* haploinsufficiency causes a peripheral, cochlear hearing loss in mice, and that malfunctioning and degeneration of OHCs plays a major role in the coming about of this hearing loss. In addition, we have demonstrated a moderate to severe cochlear hearing loss in human patients, with a proposed substantial causative role for OHCs.

We conclude that the otological or at least the audiological phenotypes between HDR patients and heterozygous *Gata3* knock-out mice correspond well, and that hearing loss in HDR syndrome is mainly caused by OHC malfunctioning and degeneration, however without precluding a role for inner hair cells.

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Summary

Hearing loss and deafness are major health issues. Estimations are that over 70,000,000 people worldwide are unable to participate in normal verbal communication due to auditory handicaps. In the Western world, the reduction of the proportion of exogenous or environmental cases of deafness - due to vaccination schemes, use of antibiotics, and knowledge about rhesus antagonism - has led to a relative increase in genetic cases; inherited causes are now believed to represent over 60% of deafness cases in children.
A commonly used classification of genetic hearing loss is that of syndromic versus non-syndromic or isolated. Several hundreds of syndromes with hearing loss have been described today. Hearing loss in one of these syndromes, HDR syndrome, is extensively analyzed in both affected mice and human patients in this thesis. HDR syndrome is an autosomal dominant disorder, characterized by hypoparathyroidism, sensorineural hearing loss and renal dysplasia, although the phenotypic expression may be quite diverse.

Chapter 2 reports on a pilot-study, conducted in healthy laboratory mice, aimed at developing an objective and accurate method to assess hearing acuity in these animals.
The auditory function of laboratory animals is commonly tested by the recording of auditory brainstem responses (ABRs). These responses provide hearing thresholds, as well as information on the functioning of the central auditory pathways up to the level of the inferior colliculus. In the past it was demonstrated that anesthesia induced an increase in ABR- peak latencies in gerbil and rat. In order to avoid anesthesia-effects we developed a surgical technique allowing longitudinal ABR recordings in alert mice. We demonstrated that ketamine/xylazine anesthesia induced a significant upward shift of ABR thresholds as well as a significant prolongation of ABR-peak latencies and I-III interpeak latencies. In addition, we showed that the accuracy of peak- and interpeak latency, and threshold measurements decreases under general anesthesia. Finally, we demonstrated that the stimulus repetition rate can be as high as 80 per second without affecting ABR thresholds, interpeak latencies or waveforms.

HDR syndrome is caused by haploinsufficiency of transcriptionfactor GATA3. The effects of *Gata3* haploinsufficiency on hearing in mice are described in chapters 3 and 4.
Over a one-to-nineteen months age-range and at all tested frequencies, heterozygous *Gata3* knock-out mice showed a significant ABR-threshold elevation, of approximately 30 dB, as compared to wild-type littermates. Both the heterozygous knock-outs and the wild type

controls were affected by an age-related hearing loss, causing an average monthly increase in ABR-threshold of 2.2 dB.
No significant differences in ABR- peak-and interpeak latencies between genotypes were found. The above findings suggest that the hearing loss observed in the mutant mice is probably caused by a peripheral – i.e. cochlear - deficit. This was substantiated by the fact that in the knock-out mice the outer hair cell (OHC) function, as tested by distortion product otoacoustic emissions (DPOAEs), rapidly deteriorates during the first seven months of life.

In addition to the electrophysiology, chapters 3 and 4 describe the histopathology of the auditory system of *Gata3* knockout mice and wild types.
From one month onwards, heterozygous *Gata3* mice showed a progressive morphological cochlear degeneration starting with apical OHCs and ultimately affecting all hair cells and supporting cells in the entire cochlea, as well as the nerve fibers. In addition some mild age-related morphological deficits starting at the cochlear base and progressing in a baso-apical direction, were demonstrated in both the affected mice and their control littermates.
The first sign of GATA3-driven degeneration is the appearance of irregularly shaped vacuoles in all three rows of outer hair cells that can be observed at both the light – and electron microscopic level.

In Chapter 5 of this thesis, the results of extensive audiometric testing - by means of pure tone audiometry, speech audiometry, speech in noise, ABR and Transiently Evoked Otoacoustic Emissions (TEOAE) - of two HDR patients are described. We demonstrated a moderate to severe, (probably) age-related hearing loss that affected all frequencies, but was slightly more pronounced in the higher end of the tested frequency spectrum. ABR interpeak latencies were normal, but both patients performed poor on speech in noise tests and in neither patient TEOAEs could be generated. These results suggest that hearing loss in HDR syndrome is probably due to loss of, or a reduced functionality of outer hair cells. A role a role for inner hair cells however, cannot be precluded.

We conclude that *Gata3* haploinsufficiency causes a peripheral, cochlear hearing loss in both mice and men and that hearing loss in HDR syndrome is mainly due to OHC malfunctioning and degeneration, however without precluding a role for inner hair cells.

Samenvatting

Geschat wordt dat wereldwijd meer dan 70.000.000 mensen door een auditieve handicap niet kunnen participeren in normale mondelinge communicatie. Dit maakt slechthorendheid en doofheid een gezondheids- en economisch probleem van aanzienlijk belang. In de westerse wereld is er, onder andere dankzij de invoering van vaccinatieschema's, het beschikbaar komen van (steeds betere) antibiotica en de toegenomen kennis over rhesusantagonisme, de laatste decennia een afname geweest van het percentage patiënten die doof of slechthorend zijn door een exogene oorzaak. Hierdoor is er een relatieve toename van het percentage ziektegevallen met een genetische oorzaak opgetreden; meer dan 60% van ernstige slechthorendheid en doofheid bij kinderen wordt tegenwoordig toegeschreven aan een erfelijke oorzaak.
Erfelijke slechthorendheid wordt vaak onderverdeeld in syndromale en niet syndromale, of geïsoleerde slechthorendheid. Er zijn inmiddels honderden syndromen beschreven, waarbij slechthorendheid onderdeel van het ziektebeeld is. In de experimenten beschreven in dit proefschrift wordt de slechthorendheid binnen een van deze syndromen, namelijk het HDR syndroom, uitgebreid geanalyseerd, zowel in een muismodel van de aandoening als in menselijke patiënten. Het HDR syndroom is een autosomaal dominant overervende aandoening, die zich wisselend kan presenteren, maar waarvan de belangrijkste kenmerken hypoparathyreoïdie, perceptieslechthorendheid en nierdysplasie zijn.

In hoofdstuk 2 wordt een pilot-study beschreven, die tot doel had een objectieve en accurate methode te ontwikkelen om het gehoor van laboratoriummuizen te onderzoeken. Het gehoor van proefdieren wordt veelal onderzocht met behulp van hersenstamaudiometrie (brainstem evoked response audiometry - BERA). Met deze methode wordt informatie verkregen over zowel gehoorsdrempels, als over het functioneren van de centrale gehoorbanen tot op het niveau van de colliculus inferior. In het verleden, werd aangetoond dat het gebruik van anesthesie in ratten en woestijnratten een toename gaf van BERA-latentietijden. Teneinde deze anesthesie-geïnduceerde effecten te vermijden ontwikkelden wij een techniek, die het mogelijk maakt longitudinaal BERA's te meten in wakkere muizen. We demonstreren dat narcose met de in proefdieren vaak gebruikte middelen ketamine en xylazine, een significante BERA-drempel vehoging teweeg brengt, evenals een significante toename van BERA-piek latentietijden en van het I-III interpiek interval. Daarnaast laten wij zien dat de nauwkeurigheid van piek-, interpiek- en drempelmetingen afneemt onder algehele anesthesie. Tenslotte tonen we aan dat de 'stimulus repetition rate' (de frequentie waarmee de stimulus die de BERA induceert wordt gegeven) kan worden opgehoogd tot 80 per seconde, zonder de

BERA-drempel, de interpiek latenties of het golfpatroon te veranderen - hiermee kan een aanzienlijke tijdswinst worden behaald tijdens het meten van BERA's in wakkere muizen.

Het HDR syndroom wordt veroorzaakt door haplo-insufficiëntie van de transcriptiefactor GATA3. Het effect van deze genetische afwijking op het gehoor van muizen beschrijven wij in hoofdstuk 3 en 4.
BERA-drempels van heterozygote *Gata3* knock-out muizen in de leeftijd van 1 tot en met 19 maanden zijn bij alle onderzochte frequenties significant (ongeveer 30 dB) hoger dan die van gezonde controle muizen. Daarnaast vertonen zowel de heterozygote knock-out muizen als de controle muizen een leeftijdsgerelateerd gehoorsverlies; maandelijks neemt de drempel met gemiddeld 2.2 dB toe. Tussen de genotypen bestaan geen significante verschillen in piek- en interpiek latentietijden.
De hierboven beschreven bevindingen tonen aan dat het gehoorsverlies in de mutante muizen wordt veroorzaakt door een perifere, en specifieker een cochleaire afwijking. De snelle achteruitgang de van buitenste haarcelfunctie in de knock-out muizen gedurende de eerste zeven levensmaanden, zoals wij aantoonden met behulp van otoacoustische emissies, levert hiervoor nog verder bewijs.

Naast electrofysiologische experimenten beschrijven wij in hoofdstuk 3 en 4 ook de histo(patho)logie van het auditieve system van heterozygote *Gata3* knock-out en controle muizen.
Heterozygote *Gata3* knock-out muizen vertonen een met de leeftijd progressief cochleair celverlies. Het eerste teken van de door GATA3 haplo-insufficiëntie veroorzaakte degeneratie zijn onregelmatig gevormde vacuoles in alle rijen buitenste haarcellen, die op zowel licht- als electronenmicroscopisch niveau te zien zijn. De degeneratie treedt al op vanaf de eerste levensmaand en betreft in eerste instantie alleen de apicale buitenste haarcellen, maar later alle haarcellen, steuncellen en zenuwvezels in de cochlea.
Daarnaast worden in zowel de aangedane als de controle muizen normale leeftijdsafhankelijke veranderingen gezien. Deze veranderingen treden aanvankelijk op aan de basale zijde van de cochlea, maar breiden zich met het toenemen van de leeftijd uit in apicale richting.

In hoofdstuk 5 van dit proefschrift beschrijven we het gehoor van twee menselijke HDR patiënten, dat wij onderzochten met behulp van toonaudiometrie, spraakaudiometrie, spraak

testen in ruis, BERA metingen en otoacoustische emissie metingen (Transiently Evoked Otoacoustic Emissions -TEOAE). Wij demonstreren in deze patiënten een matig tot ernstig, vermoedelijk leeftijdsafhankelijk gehoorsverlies. Alle onderzochte frequenties zijn aangedaan, maar het verlies is het meest uitgesproken in de hoge tonen.
De BERA interpiek latenties zijn niet afwijkend, maar otoacoutische emissies zijn niet opwekbaar en beide patiënten scoren slecht bij spraakverstaan in ruis. Alle resultaten in ogenschouw nemend, kunnen wij stellen dat gehoorsverlies binnen het HDR syndroom vermoedelijk wordt veroorzaakt door verlies of minder functioneren van buitenste haarcellen, een rol voor de binnenste haarcellen kunnen wij echter niet volledig uitsluiten.

Wij concluderen dat GATA3 haplo-insufficiëntie zowel in muizen, als in mensen een perifeer, cochleair gehoorsverlies veroorzaakt. Het gehoorsverlies bij HDR syndroom wordt vooral veroorzaakt door verminderd functioneren en degenereren van buitenste haarcellen, maar binnenste haarcellen spelen mogelijk ook een rol.

Dankwoord

"Everything that can be counted does not necessarily count, everything that counts cannot necessarily be counted." (A. Einstein).

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Curriculum vitae

"The only reason for time is so that everything doesn't happen at once." (A. Einstein).

Marjolein Annigje Johanna van Looij werd op 14 juni 1975 in Haarlem geboren. In 1993 behaalde zij het eindexamen gymnasium ß aan het Kennemer Lyceum te Overveen. In datzelfde jaar begon zij met de studie Biomedische Wetenschappen aan de Rijksuniversiteit Leiden, maar een jaar later maakte zij de overstap naar de studie Geneeskunde. Tijdens haar studie was zij als student-assistent verbonden aan de afdeling anatomie en werkte zij als gids voor het anatomisch museum. Haar afstudeeronderzoek "Chimaerism after Allogenic Bone Marrow Transplantation in Relation to Relapse – A study of mixed chimaerism in DNA isolated from stored blood smears" deed zij aan de afdelingen antropogenetica en paediatrische haemato-oncologie van het LUMC. Na het doorlopen van de reguliere co-schappen liep zij haar keuze-coschap in het Deborah Retief Memorial Hospital, Mochudi, Botswana.

Zij was, na het behalen van het artsexamen, korte tijd werkzaam als AGNIO heelkunde in het Diaconessenhuis in Leiden. Sinds mei 2001 is zij als "AGIKO" verbonden aan de afdeling KNO-heelkunde van het Erasmus MC. Onder begeleiding van dr. G.A. van Zanten, Prof. dr. L. Feenstra en Prof. dr. C.I. de Zeeuw werkte zij op de afdeling neurowetenschappen aan het onderzoek dat heeft geleid tot dit proefschrift. Op 1 juli 2008 hoopt zij haar opleiding tot KNO-arts te voltooien.

De auteur woont samen met André Vis.

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List of abbreviations

ABR	Auditory Brainstem Response
AEP	Auditory Evoked Potentials
AHL	Age-related Hearing Loss
ARTA	Age Related Typical Audiograms
ASD	Atrial Septal Defect
ATP	Adenosine Triphosphate
BOF	Branchio-Oculo-Facial (syndrome)
BOR	Branchio-Oto-Renal (syndrome)
BRESEK	Brain anomalies, Retardation , Ectodermal dysplasia, Skeletal malformations, ear/eye anomalies, Kidney dysplasia/hypoplasia and reflux
BRESHECK	Brain anomalies, Retardation , Ectodermal dysplasia, Skeletal malformations, Hirschsprung disease, ear/eye anomalies, cleft palate/cryptorchidism, Kidney dysplasia/hypoplasia and reflux
CEOAE's	Continuous Evoked Otoacoustic Emissions
CNS	Central Nervous System
CS I	Classical Cockayne Syndrome
CS II	Type II Cockayne Syndrome
CSF	Cerebro Spinal Fluid
CVA	Cerebro Vascular Accident
dB	Decibel
dBHL	Decibel Hearing Level (biological scale)
dB SPL	Decibel Sound Pressure Level (environmental scale)
DDAVP	Desamino-D-Arginine Vasopressin
DIDMOAD	Diabetes Insipidus, Diabetes Mellitus, Optic Atrophy and Deafness
DFN	X-linked non-syndromic deafness
DFNA	Dominant non-syndromic deafness
DFNB	Recessive non-syndromic deafness
DFNM	Modifying non-syndromic deafness
DGCRI	DiGeorge Critical Region I
DGCRII	DiGeorge Critical Region II
DNA	Deoxyribonucleic Acid
DPOAE	Distortion Product Otoacoustic Emission
DRTA	Distal Renal Tubular Acidosis
EM	Electron Microscopy
FA	Fanconi Anemia

FCS	Familial Cold autoinflammatory Syndrome
FISH	Fluorescence In Situ Hybridization
Fgf	Fibroblast growth factor
FMD	Fronto Metaphyseal Dysplasia
FOG	Friends Of GATA
GATA	A/T GATA A/G
GBM	Glomerular Basal Membrane
Gjb2	Gap junction membrane channel protein beta2
HDR	Hypoparathyroidism, Deafness, Renal dysplasia
Ht	Heterozygous
IHC	Inner Hair Cell
MIDD	Maternally Inherited Diabetes mellitus and Deafness
MNS	Melnick-Needles Syndrome
MWS	Muckle-Wells Syndrome
NIHL	Noise Induced Hearing Loss
NF	Nerve Fiber
NOMID	Neonatal Onset Multisystem Inflammatory Disease
OAE	Otoacoustic Emission
OAVS	Oculoauriculovertebral spectrum
OHC	Outer Hair Cell
OPD	Oto-Palato-Digital syndrome
PI	Peak I
PII	Peak II
PIII	Peak III
PIV	Peak IV
PV	Peak V
PC	Pillar Cell
PNS	Peripheral Nervous System
PTH	Parathyroid Hormone
SFOAE's	Stimulus Frequency Otoacoustic Emissions
SOAE's	Spontaneous Otoacoustic Emissions
TEOAE's	Transient Evoked Otoacoustic Emissions
TIA	Transient Ischemic Attack
TM	Tectorial Membrane
VSD	Ventricular Septal Defect.
Wt	Wild type