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Hereditary susceptibility to inner ear stress agents studied in heterozygotes of the German waltzing guinea pig

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In memory of my dear brother Kaj

1 ABSTRACT

The German waltzing guinea pig is a strain of animals expressing deafness and severe balance disorders already at birth. The mutation arose spontaneously in a breeding facility in Germany and as the affected animals show a characteristic waltzing behavior, the strain is named the German waltzing guinea pig. The strain is presently bred only at Karolinska Institutet.

The hereditary inner ear impairment has a recessive mode of inheritance and the strain thus produces not only affected homozygotes but also symptom-free heterozygotes and fully normal offspring. The outcome depends solely on the genotype of the parents. The heterozygotes, which have obtained the "waltzing" gene from one parent only, have normal hearing and no balance dysfunction. The heterozygous animals appear normal but will, in turn, carry the genetic defect to the next generation. The present thesis is focused on these animals.

Noise and ototoxic drugs are well known stress factors that interfere negatively with the hearing organ in both humans and animals, causing hearing impairment. However, the interindividual variability in susceptibility to auditory stress factors is surprisingly large, most likely due to different genetic predisposition. In this study heterozygous animals of the German waltzing guinea pig, animals carrying a genetic defect known to cause severe hearing impairment, were used to study how an unexplored gene for deafness interacts with the auditory stress agents; noise exposure and the ototoxic drugs gentamicin and cisplatin.

Animals were exposed to both narrowband as well as broadband noise at different ages and hearing threshold were conducted using ABR. Heterozygote animals of the German waltzing guinea pigs showed less threshold shifts compared to control strains. Old animals were less affected of the noise trauma than younger animals.

To explore the hypothesis that the efferent system contribute to the protection of the inner ear against noise trauma, measurements using the new methods of post onset adaptation of the DPOAE and maximum adaptation magnitude were used. The post onset adaptation of the DPOAE could detect a strain difference at the higher frequency region while in the maximum adaptation magnitude method showed no difference between the strains.

The heterozygous animals of the German waltzing guinea pig displayed a distinctly increased resistance to noise exposures, manifested as reduced threshold shifts and faster recovery following acoustic overstimulation. However, when exposed to ototoxic drugs, the heterozygous carriers suffered from a more pronounced hearing loss.

It is concluded that endogenous resistance to noise in the heterozygotes does not offer any protection against ototoxic drugs. The detailed mechanisms still need to be explored.

Key words: age-dependent hearing loss, cisplatin, DPOAE, efferent system, gender, gentamicin, noise trauma, ototoxic drugs, protection

LIST OF PUBLICATIONS

This thesis is based on the following papers. They will be referred to by their Roman numerals (I-IV).

I. Skjönsberg Å, Herrlin P, Duan M, Johnson AC, Ulfendahl M. (2005) A guinea pig strain with recessive heredity of deafness, producing normal-hearing heterozygotes with resistance to noise trauma. *Audiology & Neurootology* 10:323-330.

II. Halsey K, Skjönsberg Å, Ulfendahl M, Dolan DF. (2005) Efferent-mediated Adaptation of the DPOAE as a Predictor of aminoglycoside toxicity. *Hearing Research* 201:99-108.

III. Skjönsberg Å, Halsey K, Ulfendahl M, Dolan DF. (2006) Exploring efferent-mediated adaptation of the DPOAE in three different guinea pig strains. *Submitted to Hearing Research*.

IV. Skjönsberg Å, Bucinskaite V, Laurell G, Ulfendahl M. (2006) Augmented ototoxic effect of cisplatin in heterozygotes of the German waltzing guinea pig. *Submitted to Audiology & Neurootology*.

CONTENTS

LIST OF ABBREVIATIONS

+/+	Wild type animals
ABR	Auditory brainstem response
ANOVA	Analysis of variance between groups
CNS	Central nervous system
dB	Decibel
DPOAE	Distortion product otoacoustic emission
EP	Endocochlear potential
gw/+	Heterozygous animal of the German waltzing guinea pig
gw/gw	Homozygous animal of the German waltzing guinea pig
i.m.	Intramuscular (injection)
i.p..	Intraperitoneal (injection)
i.v.	Intravenous (injection)
IHC	Inner hair cell
K	Potassium
N.VIII	Eight cranial nerve
NIHL	Noise-induced hearing loss
OAE	Otoacoustic emission
OHC	Outer hair cell
PTS	Permanent threshold shift
ROS	Reactive oxygen species
s.c.	Subcutaneous (injection)
SD	Standard deviation
SM	Scala media
SOAE	Spontaneous otoacoustic emission
SPF	Specific pathogen free
SPL	Sound pressure level
ST	Scala tympani
St.v	Stria vascularis
SV	Scala vestibuli
TEOAE	Transient evoked otoacoustic emission
TTS	Temporary threshold shift

2 INTRODUCTION

The ability of hearing is very important to humans as well as animals, not only in communication between individuals but also for the possibility of detecting an imminent danger. The sense of hearing is also necessary when it comes to incorporating cultural expressions such as music and theatre. Two normal functioning ears make it possible to distinguish the direction of a sound source.

Hearing dysfunction can originate in the external ear or middle ear, in the cochlea or be of retro-cochlear origin. The site of the lesion also dictates the nature and to some extent to the degree of the hearing loss. The external ear (ear canal and pinna) can involve problems like external deformities and otitis externa but is rarely associated with hearing loss. If the problems are located in the middle ear hearing will be attenuated but the perception of sound quality will not be very much changed. Middle ear dysfunction, whether congenital or acquired, is also much more promising for surgical restoration, or will have a positive prognosis for hearing aid support. In the case of cochlear damage, sound perception will not only be attenuated but also distorted, often in combination with a diminished dynamic range between the hearing threshold and the uncomfortable sound pressure level, i.e. loudness recruitment.

With exception of cochlear implantation, surgical treatment of inner ear damage is still not possible. However, in the future, therapies involving stem cell technology as well as pharmacological treatment of the inner ear are expected to emerge. Until then, the only treatment is the use of hearing aids and other technical support to ease the patients' every-day life. Loss of hearing, totally or partly, is not of any life threatening danger but there is a large population of people that affected by hearing loss or deafness. In Sweden about 10 % of the population is hearing impaired (www.hrf.se) and 0.1% is deaf (www.sdrf.se).

2.1 ANATOMY AND PHYSIOLOGY

The ear is divided into three parts: outer, middle and inner ear. The outer ear consists of the pinna and the ear canal. The middle ear, an air filled cavity containing the ossicular chain, is functionally demarcated by the tympanic membrane towards the ear canal and the oval window towards the inner ear. The ossicular chain consists of the mallus, attached to the tympanic membrane, the incus and the stapes. The stapes footplate is placed in the oval window. The oval window separates the middle ear from the inner ear. The inner ear consists of two functional parts, the vestibular apparatus and cochlea (figure 1).

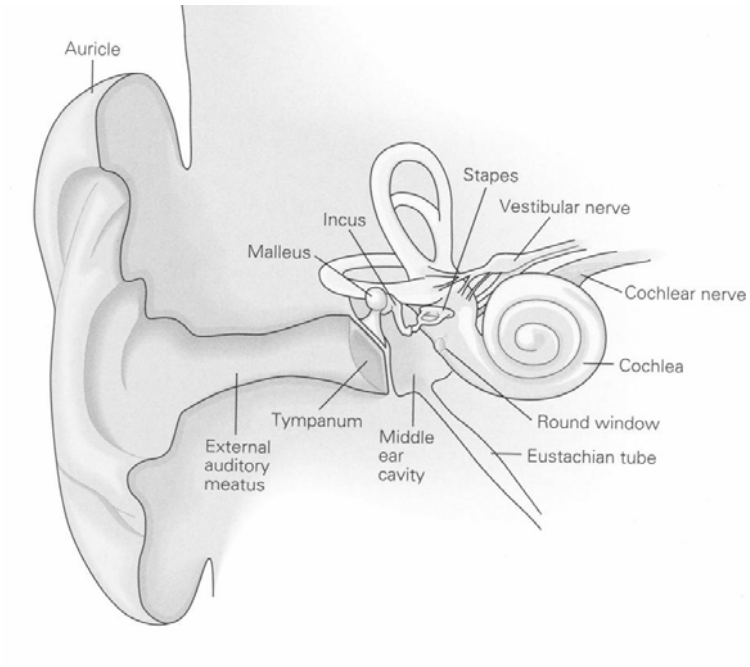


Figure 1 The different parts of the ear, the outer, middle and inner ear.

The cochlea is a snail-shaped structure ("kokhlias" is the Greek word for snail) containing the sensory organ of hearing. The cochlea contains three canals, the scala tympani (ST), scala vestibuli (SV) and scala media (SM). The hearing organ, the organ of Corti, is located in SM. The three scalae are separated by membranes: Reissner's membrane between SM and SV and the basilar membrane between SM and ST. The fluid in the SV and ST is called perilymph and contains a low K^+ (potassium) concentration, while the SM is filled with endolymph, which contains a high K^+ concentration (figure 2). The SV and ST are connected via a small opening at the apex of the cochlea, helicotrema. The stria vascularis (St.v) a highly vascularized and metabolically active structure located in the lateral wall in the SM. The stria vascularis consists of marginal cells, intermediate cells and basal cells, and is the source of the endocochlear potential (EP), maintaining the high K^+ concentration in the SM.

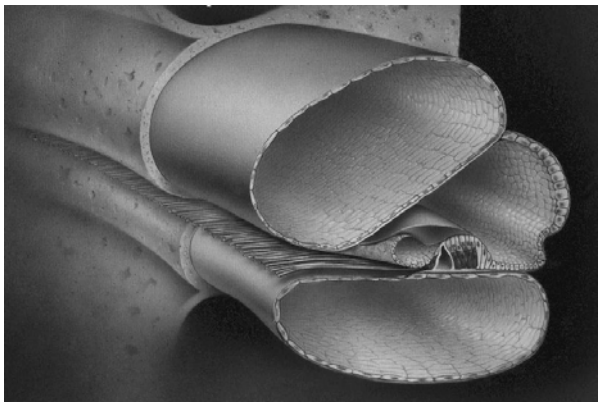


Figure 2 The three fluid filled canals in the cochlea, and the organ of Corti inside scala media (adapted from www.earaces.com)

The organ of Corti contains, in addition to different supporting cells, the sensory cells, the inner and outer hair cells. The hair cell is named after the mechanically sensitive hair bundle located at the apical pole of the cell body. The hair cells, one row of inner hair cells (IHC) and three rows of outer hair cells (OHC) respond to acoustic stimulation (figure 3). The inner hair cells act as receptors while the outer hair cells also function as effectors. The outer hair cells are also responsible for the frequency specific tuning (Ulfendahl et al., 1998). About 90% of the cochlear ganglion cells terminate on the IHCs, each axon innervates one single hair cell. About 10% of the cochlear ganglion cells innervate the OHC where each axon projects to several OHC.

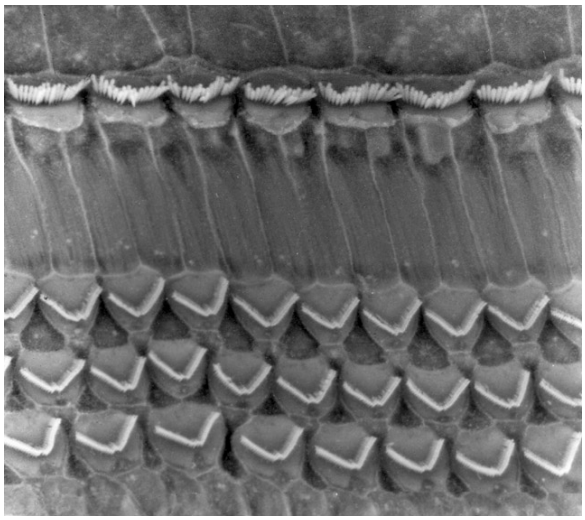


Figure 3 The surface of hearing organ showing the hair bundles of the three rows of outer hair cells and one row of inner hair cells (adapted from the Auditory Science Lab at the Hospital for Sick Children)

The vestibular part of the inner ear contains the semicircular canals and the utricle and the saccule. The semicircular canals respond to rotational acceleration while the saccule and utricle respond to linear acceleration.

The whole auditory system is tonotopically organized, from the hair cells through the acoustic nerve (N.VIII) to the auditory cortex. When the sound wave traveling through the ear canal hits the tympanic membrane, the energy will be transferred to the inner ear through the ossicular chain. The ossicular chain impedance adjusts the energy from the larger area of the tympanic membrane to the smaller area of the oval window (1:20). When the stapes (due to sound stimulation) presses on to the oval window, waves are produced in the fluid. This leads to a traveling wave along the basilar membrane. Due to the mechanical-structural properties of the basilar membrane, the traveling wave has a maximum at a specific point depending on the frequency of the sound stimulus. Movements of the basilar membrane and the hearing organ with respect to the overlying tectorial membrane cause deflections of the hair bundles of the sensory cells. This initiates the mechano-electrical transduction in the hair cells, which in turn generates electrical signals in the afferent nerve.

The auditory pathways have also an efferent direction, i.e. the central nervous system (CNS) transferring signals *from* the central regions *to* the cochlea. This efferent innervation reaches the cochlea via the medial part of the olivocochlear bundle, both the ipsilateral and contralateral cochleae, and synapse at the base of the outer hair cells. The efferent innervation controls the cochlear amplifier and thereby by also controls the dynamic range of hearing. The lateral

efferent innervation terminates on the dendrites of the auditory nerve radial afferent fibers below the IHC: Very little is known about the rule of the lateral efferent physiology.

2.2 AGE-RELATED HEARING LOSS

The biological aging process in humans (and in animals) is associated with hearing loss in the high frequency area (in humans, above 2000 Hz). Aging causes changes within the cochlea at several levels: degeneration of the sensory hair cells, degeneration of cochlear neurons, changes in the stria vascularis, as well as changes in the basilar membrane leading to mechanical disruption (Ohlemiller, 2004; Pickles, 2004; Schacht et al., 2005). However, the hearing loss in elderly people cannot be explained by aging itself but is rather a result of contributions from several factors that also affect hearing, including noise exposure, exposure to ototoxic drugs, exposure to solvents, genetic factors, gender and life style factors e.g. smoking, alcohol consumption, and hypertension(Christensen et al., 2001; Guimaraes et al., 2004; Johnson, 1993; Johnson et al., 1995; Li et al., 1994; Toppila et al., 2001). It is very common that elderly people are under permanent pharmacological treatment, daily taking in one or more drugs that can act ototoxically, either alone or synergistically with the aging ear. Hearing loss usually increases with age. In society, the elderly population is rapidly increasing, which means that age-related hearing loss will affect in increasing number of people.

2.3 NOISE INDUCED HEARING LOSS

Noise is defined as an undesired sound of any source that is experienced as unpleasant. It can be hazardous to the auditory system but not necessarily. However, hearing loss following exposure to noise is very common in both humans and in research animals. In humans, the noise trauma can also cause other hearing problems such as tinnitus and hyperacusis. Noise induced hearing loss (NIHL) can either be reversible, only resulting in a temporary threshold shift (TTS), or irreversible, resulting in a permanent threshold shift (PTS). Some degree of recovery occurs in most cases but the hearing threshold might not recover to pre-exposure levels. The time window for this event is not clearly defined. In research studies with a clinical TTS approach, it is common that a recovery time spanning from minutes, hours and as much as one week is used (Olszewski et al., 2005; Quaranta et al., 2004). In the basic research area a PTS is often confirmed in the time span of within 2-6 weeks (Duan et al., 1996; Perez et al., 2004; Skjönsberg et al., 2005). The differences between TTS and PTS have recently been studied at a molecular level, e.g. by detecting differences in gene regulation, and interestingly, an up-regulation of certain genes immediately after noise exposure has been found (Altschuler et al., 2002; Cho et al., 2004; Van Laer et al., 2006).The degree of hearing loss following noise trauma is to some extent dependent on the sound pressure level, frequency range and the duration of the noise, but there is also a large individual variability in noise susceptibility, both in humans and in animals. Some individuals will suffer from a pronounced hearing loss after the same exposure levels that will leave others totally unaffected. The underling factors of this phenomenon are not clear but are likely to be associated with gender, age, genetics and exposure to solvents or ototoxic drugs (Boettcher et al., 1987; Davis et al., 1999; Davis et al., 2001; Erway et al., 1996; Gratton et al., 1990; Hultcrantz et al., 2006; Johnson, 1993; Johnson et al., 1994; Johnson et al., 1995; Li et al., 1993; Skjönsberg et al., 2006; Skjönsberg et al., 2005)

Mechanisms: Reactive oxygen species (ROS) can lead to cell death but can also stimulate the formation of antioxidants, which in turn enhance cell survival. The mechanisms include

ischemia, swollen dendrites and Ca^{2+} influx. The ischemia causes NIHL through changes in the cochlear blood flow that leads to increased levels ROS and/or free radicals. The distortion of the cochlear blood supply results in temporary swelling of the stria vascularis followed by permanent loss of intermediate cells, which alter the size of the stria vascularis. The altered blood flow can be prevented by pharmacological factors that ease the blood flow in several ways. The swollen dendrites are caused by the influx of glutamate into the synapses, which overstimulates the receptors of the postsynaptic cell leading to the swelling of dendrites. Since this sometimes is a reversible condition it has been proposed to be linked to TTS (Robertson, 1983).

Protection: Studies have shown that by adding antioxidants directly to inner ear or systemically (Duan et al., 2004; Henderson et al., 1999) the noise-induced threshold shift can be diminished. Interestingly, toughening the inner ear with a noise of moderate level has been shown to protect the inner ear against subsequent noise exposure (Canlon et al., 1988; Harris et al., 2006; Henderson et al., 1999; Hu et al., 1997). It is thought that the conditioning sound stimuli are increasing the endogenous antioxidant enzymes (Henderson et al., 2006). Antioxidants are molecules that act as free radical scavengers and reduce the ototoxic effects of the ROS.

Another mechanism that has been discussed to serve as a protector is activation of the efferent system. The efferent innervation serves as a feedback system to control the cochlear amplifier (Guinan, 1996). Today the only prevention of NIHL is to avoid the hazardous exposures and if that is not possible, to use hearing protectors like ear muffs or ear plugs. Hereto it has not been possible to predict the individual susceptibility to noise. However, the strength of the medial olivocochlear reflex has been suggested to serve as a non- invasive measurement for predicting the individual resistance to noise: the stronger reflex, the lesser NIHL (Maison et al., 2000). Clinical treatment of NIHL is not yet available but in the future it will probably be possible to pharmacologically prevent hearing impairment and rescue the auditory function following noise trauma.

2.4 HEARING LOSS INDUCED BY OTOTOXIC DRUGS

There are several drugs that induce hearing loss, either permanent or temporary. Among the drugs that cause temporary hearing loss, salicylates and diuretics can be mentioned. Drugs that produce permanent threshold shifts include, for example, aminoglycosides like streptomycin, neomyin and gentamicin, kanamycin (Rybak et al., 2005). Aminoglycosides were developed in the 40s but were soon shown to produce hearing loss and vestibular damage (Schacht et al., 2006). Chemotherapeutic drugs like cisplatin are also known to causes hearing loss. In the tumor-killing situation the cisplatin binds to and interacts with the nuclear DNA of the tumor cell and triggers apoptosis (McAlpine et al., 1990; Thomas et al., 2006).

2.4.1 Gentamicin

The aminoglycoside gentamicin affects the protein synthesis of bacteria. It has a very broad antibacterial spectrum (Blanchet et al., 2000; Forge et al., 2000) and is successfully used to treat e.g. severe lung infections. Gentamicin is commonly used in developing countries due to its effectiveness and low costs but have negative side-effects like nephrotoxicity and ototoxicity, mainly affecting the vestibular part of the inner ear. In the western world it is used as treatment for cystic fibrosis and in renal dialysis(Chen et al., 2006). The cochlear pathology is associated with outer hair cell loss starting in the basal turn, i.e. causing high frequency hearing loss, but progressing to more apical regions (Forge et al., 2000). ROS formation is thought to generate the

cell death since the damage, to some extent, can be prevented by antioxidants (Chen et al., 2006; Duan et al., 2000; Sha et al., 2000; Sinswat et al., 2000) The vestibulotoxic nature of gentamicin renders it feasible for treatment of Ménières disease to ease vertigo.

2.4.2 Cisplatin

Cis-diaminodichloroplatinum, or cisplatin, is a widely used chemotherapeutic drug, however, with the undesirable side effect of ototoxicity. The dose-limiting factor is not only ototoxic: cisplatin has also other targets for toxicity such as nephrotoxicity and neurotoxicity. In patients, the nephrotoxicity can be prevented by hydration and pharmacological induced diuresis (Cvitkovic et al., 1977; Hayes et al., 1977) while the neurological damage is in worst cases irreversible (Quasthoff et al., 2002). The hearing loss and cochlear pathology is very similar to the gentamicin-induced ototoxicity but the vestibular part of the inner ear is not, or only occasionally, affected. The damage is located to the OHC and the stria vascularis (van Ruijven et al., 2005a). There is a large variability between individuals and in different animals species regarding the susceptibility to cisplatin (Ekborn et al., 2000; Hoeve et al., 1987). Like in noise-induced hearing loss this may be related to genetic factors and previous exposures to inner ear insults or other stress factors (Laurell, 1992; Miettinen et al., 1997). The hearing loss following administration of cisplatin is normally in the high frequency region, i.e. in the basal turn of the cochlea, and is dose dependent. (Rybak et al., 2005). The hearing impairment is often associated with tinnitus(Kaltenbach et al., 2002).Hearing impairment due to cisplatin treatment is permanent but the ototoxic effect can, however, be reduced by antioxidants and free radical scavengers (Campbell et al., 1996; Campbell et al., 1999; Cheng et al., 2005; Drottar et al., 2006; Korver et al., 2002; Lynch et al., 2005; Rybak et al., 2005; Rybak et al., 1999; Rybak et al., 2000). Cisplatin-induced hearing loss is thus thought to be caused by ROS and/or free radicals. Cisplatin also interacts with DNA to form cisplatin-DNA adducts, which can be detected in almost all cells in the organ of Corti as well as in the lateral wall, in guinea pigs following long-term, low dose treatment with cisplatin (van Ruijven et al., 2005b) as well as after a single-injection of high dose of cisplatin (Thomas et al., 2006).

2.5 AUDITORY BRAINSTEM RESPONSE

In the auditory research, measuring the auditory brainstem response (ABR) is a routine method. The ABR is obtained by measuring the electrical response in the auditory nerve (N.VIII) when stimulating the hearing organ, either using a sound or by electrically stimulation (eABR). When stimulated with sound various acoustic stimuli can be used, either frequency specific stimulation or a click stimulus (including multiple frequencies). The interpeak latency following click stimulation is a clinically important tool that brings insight to the localization of the lesion i.e. cochlear or retrocochlear damage, and is the most used diagnostic tool in the clinic. Frequency specific hearing thresholds can also be measured. In the pre-clinical situations, both click stimuli and frequency specific stimulation are used, depending on the aims of the study, equipment, species, and the available time etc. Another advantage that makes ABR an important clinical tool and research instrument is that the subject (patient or animal) does not need to participate during measurements. This allows hearing tests in neonatal babies, or otherwise difficult tested subjects like patients with dementia or other mental problems. ABR can be used as a diagnostic tool in patients with hearing disorders as well as neurological disordes such as multiple sclerosis, cranial tumors and also vascular diseases (Eggermont, 1985; Kaga et al., 2004; Kon et al., 2000; Middleton et al., 1997; Munoz et al., 1983; Stein et al., 1981; Zimmerman, 1994).The response is seen as wave forms including 4-5 peaks, (figure 4) depending on the species. Each peak is

representing a certain connection site along the auditory pathway, from the VIII cranial nerve to the brainstem. The first peak represents the peripheral part of N.VIII, the second peak the central part of the auditory nerve, the third peak the cochlear nuclei, the forth peak the superior olive complex, and finally the fifth peak originates from the lateral lemniscus. This waveform including the five peaks occurs within 10 milliseconds (ms).

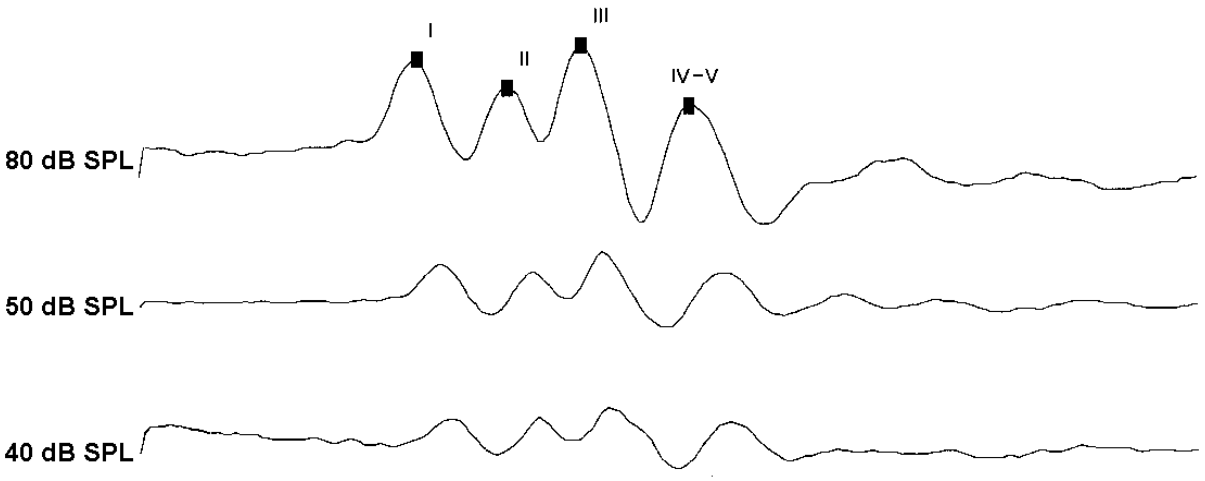


Figure 4 Auditory brainstem response from a guinea pig. In guinea pigs typically four peaks are present. Note that the latencies of the peaks decays as the sound pressure level decrease.

Additional to these responses there are potentials that are apparent at later time points, up to 300 ms (middle-, late, and long-latency auditory evoked potentials). The responses are picked up by electrodes or subdermal needle electrodes in animals, and standard electro-encephalogram (EEG) disc electrodes in humans. ABRs have been recorded from a wide range of species: mice, rats, guinea pigs, gerbils, cats, dogs, monkeys and even dolphins and horses (Addison et al., 2006; et al., 2005; Borg, 1982; Cooper et al., 1990; Famula et al., 2001; Harland et al., 2006; Hultcrantz et al., 2000; Johnson et al., 1994; Kretzmer et al., 2004; Mayhew et al., 1990; Pfingst et al., 1979; Ridgway et al., 1981; Snyder et al., 1990; Sockalingam et al., 2000; Williston et al., 1982; Wilson et al., 2005; Zhou et al., 2006). In the present study the ABR measurement method was used to establish hearing thresholds before and after exposure to different traumatic agents, and as well as for monitoring threshold changes in aging animals.

2.6 OTOACOUSTIC EMISSIONS

Otoacoustic emissions (OAE) are sounds that are measurable in the ear canal. The sound is created in the cochlea and then transmitted backwards, via the middle ear ossicles, to the tympanic membrane. The OAE are by-products of the activity in the outer hair cells (Kemp, 2002). Since these by-products are a result of the active mechanism, the cochlear amplifier, the OAE requires a healthy cochlea and middle ear to be present. There are two types of OAEs: evoked (EOAE) and spontaneous (SOAE). While the evoked emissions occur during or after acoustical stimulation, the spontaneous emissions occur in the absence of external stimulation. The evoked OAEs are differentiated by the way they are elicited. Measurements of OAEs require an earpiece consisting of a microphone for the recordings and speakers for stimulation. The earpiece needs to be tightly fixed into the ear canal to avoid the background noise to interfere with the results. Measurement results can also be affected by the subjects' movements,

vocalization and external sound sources that are present in the environment. The OAEs are mainly reflecting the state of the outer hair cells why the measurements are useful tool in monitoring early signs of drug-induced hearing loss as well as the decline of hearing in people working in a noisy environment. The measurements do not require any collaboration of the subject measured why it is a suitable method to use in patients that cannot corporate in a test situation as well as in animals.

SOAE: The spontaneous emissions are present in more than 50% of the human population but the prevalence varies in different studies, probably due to the quality of measurements, equipment, criteria and overall setup. SOAEs are more common and are more frequent in females with the largest difference between genders in the early period of life. SOAEs decrease by age but seem stable during the first year, and they are more common in the right side ear (Lamprecht-Dinnesen et al., 1998). The SOAEs are also present in animals (Manley, 2004; Ohyama et al., 1991; Taschenberger et al., 1997; Wit et al., 1989).

TEOAE: The transient evoked OAE (TEOAE) are elicited by a brief stimulus and are non-frequency specific responses. Clinically, TEOAEs give information about the cochlea's ability to produce a transient response or not, i.e. the status of the inner and middle ear. The measurements are fast and non-invasive and are therefore used as an important screening tool in neonatal babies.

DPOAE: Distortion Product Otoacoustic Emissions (DPOAE) are evoked by two simultaneous tones, f_1 and f_2, presented to the inner ear. The non-linearity of the cochlea admits the distortions to arise and several tones consisting of algebraic alterations of the stimuli primaries. Depending on what is referred to (frequency or SPL) the primaries are labeled as f_1 and f_2 (frequency) or L_1 and L_2 (SPL) The most prominent DP is the $2f_1$-f_2 (Kemp, 2002). Ratios of f1/f2 ≈1.2 are shown to generate a strong DP in both humans and animals. (Gaskill et al., 1990). The sound pressure levels (SPL) of the primaries can either be equal or unequal (preferably with a higher SPL of the L_1). DPOAE is a frequency-specific measurement, which allows investigations of different regions of the cochlea. The DPOAE can either be analyzed as an input/output function of the primaries being stimulated with at different SPLs or as the level the DP-response reaches with as certain stimuli.

2.6.1 Adaptation of DPOAE and the efferent system

In the present study variants of the DPOAE was used to trace the decrease (or increase) in DP level by time and the maximum adaptation magnitude i.e. the DPOAE as function of the L_1 and L_2. These measurements are not in clinical practice at present but pre-clinically investigations are performed to study the efferent activity in the cochlea. The auditory efferent system is not fully understood but is thought to be involved in improving the signal-to-noise ratio, protection from NIHL, to contribute to attention, and to control the mechanical state of the cochlea via outer hair cell motility (Guinan, 1996). In the hearing aid fitting situation it is very common that patients are having problems to adjust to the new and to some extent unnatural sound picture. Patients often experience that the sound is unpleasant, unnatural and even painful. This phenomenon is normally explained by the fact that the auditory cortex needs to re-code the auditory information but it is likely to speculate that the efferent output from the CNS is not coping with afferent input from the aided ear. Efferent-mediated fast adaptation is visible as a change in $2f_1$-f_2 intensity over approximately the first 200-500 ms of the DPOAE response.

2.7 THE GERMAN WALTZING GUINEA PIG

2.7.1 Homozygotes of German waltzing guinea pig

The German waltzing guinea pig arose spontaneously in a breeding facility in Bayreuth, Germany, in 1996, where two normal-behaving animals had a litter consisting of five animals, where two progenies (one male, one female) showed a characteristic circling behavior. The two waltzing animals were interbred, resulting in two litters of which both consisted of two waltzing animals. All six waltzing animals were sent to Karolinska Institutet, Stockholm, Sweden, where they have been bred ever since. Initially, the strain underwent systematically breeding showing the typical pattern of a monohybrid autosomal recessive Mendelian mode of inheritance (table 1). Animals from this strain show a normal variance in coat color for pigmented guinea pigs, from bright yellowish to dark brown, occasionally including white or black spots. The life span and fertility of these animals do not differ from other guinea pig strains (unpublished data). Affected animals from the German waltzing guinea pig strain are deaf and have dysfunctional vestibular system already at birth. The vestibular dysfunction is seen as circling, or waltzing, behavior and tilting head movements, which remain through out life. The Preyer reflex, a startle reflex trigged by a sudden sound stimulus, is absent already in newborn animals.

Table 1 The classical pattern of recessive inheritance. Systemic breeding of the German waltzing guinea pig has confirmed the expected percentages.

Parents genotype	Homozygotes gw/gw	Heterozygote gw/+	Normal +/+
(gw/gw) X (gw/gw)	100%		
(gw/gw) X (gw/+)	50%	50%	
(gw/gw) X (+/+)		100%	
(gw/+) X (gw/+)	25%	50%	25%
(gw/+) X (+/+)		50%	50%

The gene(s) underlying this genotype is still unknown but the morphological and histological findings in newborns and adult animals are further described in Jin et al. (2006) (Jin et al., 2006). In short, the scala media is absent in the gw/gw animals, following a collapse of Reissner's membrane. The stria vascularis is thinner and shorter, showing only one cell layer instead of the normal three cell layers. This single cell layer consists of degenerated marginal cells, intermediate cells and lack basal cells. In old gw/gw animals the number of spiral ganglion cells is also diminished. The vestibular part shows similar structural changes, especially a loss of the endolymphatic compartment. Serial sections reveal that there are no dark cells in the transition epithelia. In young animals a normal population of vestibular hair cells is seen, but they appear to degenerate by age (Ernstson et al., 1999).

2.7.2 Heterozygotes of the German waltzing guinea pig

The work in this study was mainly based on the heterozygotes (gw/+) of the German waltzing guinea pig strain. These animals have normal hearing and normal balance behavior and cannot by visual observation be distinguished from wild-type guinea pigs. The endocochlear potential (EP) has been shown to be normal (Ernstson et al., 2000). The heterozygous animals constitute an interesting population for studies of how an unexpressed hereditary deafness interacts together with auditory stress factors. In papers I and III noise was used, and in paper IV the

ototoxic chemotherapeutic drug cisplatin was administered. The carriers have also been exposed to the ototoxic antibiotic drug gentamicin (unpublished data). Results will be presented here. The German waltzing guinea pig strain is bred together with the Sahlin strain to produce heterozygous animals. We have also bred the heterozygotes (gw/+) together with the purpose to get litters consisting of animals of all three possible genotypes i.e. (gw/gw), (gw/+), and (+/+) and exposed these littermates to noise (unpublished data). Results from the littermate study will be presented here.

2.8 AIMS OF THE STUDY

This thesis consists of four studies of which the aims were

1. to characterize the German waltzing guinea pig with respect to its hereditary pattern
2. to investigate the hearing status at different ages in the heterozygous animals
3. to explore any gender differences in hearing status in heterozygous animals
4. to examine the susceptibility to noise, gentamicin and cisplatin heterozygous animals
5. to explore the correlation between the efferent-mediated adaptation of the DPOAE and the susceptibility to the ototoxic aminoglycoside gentamicin
6. to use the efferent-mediated adaptation of the DPOAE in three different guinea pig strains to explore genetic differences in the reflex strength

3 MATERIALS AND METHODS

3.1 ANIMALS

All animals included in this study were free from middle ear infections, as shown by otoscopic inspection prior to all experiments, and did not show any signs of other health problems. Animals were housed in an animal facility (12 hours daylight) with free access to food and water. Because the guinea pig species lack the ability to produce necessary C-vitamin, ascorbic acid was added to the water at all times. All animals were pigmented and overall healthy.

Guinea pigs from four different sources were used in this study. The German waltzing guinea pig is not commercially available but is bred at Karolinska Institutet. Here the heterozygote animals were used (papers I, II, IV). The Sahlin strain (papers I-IV) is a commercially available guinea pig strain (Bio Jet Service, Uppsala, Sweden). At the Center for Hearing and Communication Research this strain is used for two purposes, both as an experimental animal in a wide range of studies of studying the auditory system and for breeding. As it is presently impossible to distinguish the heterozygous animals from normal littermates in the German waltzing guinea pig, the Sahlin strain is used in the breeding with homozygous German waltzing guinea pigs to produce heterozygotes. The Lidköping strain, supplied by a commercial breeder (Lidköping Kaninfarm, Lidköping, Sweden), was used in paper III. This strain was added as an additional control strain in the study of noise and age effects, and in the study of maximum adaptation of the DPOAE (paper III). The aim was to minimize the risk of incorrect conclusion drawn from the results. Finally, one study (paper II) also includes specific pathogen free animals obtained from Elm Hill Breeding Laboratories (Chelmsford, MA, USA)
All experiments were approved by Swedish and American committees for use and care of animals, numbers 7/98, N10/01, N11/01, N13/01, N465/03, N423/04 and 8422.

3.2 AUDITORY BRAINSTEM RESPONSE

Hearing thresholds were established in one ear using frequency specific ABR. Different equipment, hardware and software were used in the studies, detailed information regarding setup are described in each paper (I- IV). Frequencies were selected with respect to the specific experimental design. Equipment from Tucker Davis Technologies was used (system II in paper I-III, system III in paper IV), and Biosig software. Subcutaneous (s.c.) needle electrodes were used in paper I, III, and I. In paper II permanent electrodes was implanted into the skull bone, at the time of pre-exposure recordings.

Animals were anesthetized during all measurements, for details see paper I- IV. In paper II animals were anesthetized at the pre-exposure and final measurements, but daily screening measurements were also performed in conscious animals. The body temperatures in the animals were not controlled but were maintained during measurements using heating pads.

The hearing threshold was defined as the lowest level where a reproducible response could be recorded. The third wave peak served as the reference for detection. In paper II the hearing threshold was interpolated between the lowest level of response and the sub threshold level 5 dB below.

In paper I ABR from the right ear were conducted at 4, 6.3, 8, and 12.5 kHz before noise exposure and at 24 hours, one week, and four weeks after exposure to a narrow-banded noise for six hours (110 dB SPL, paper I). In paper II the animals were stimulated by 2, 8, and 16 kHz in anesthetized animals prior to and after administration of gentamicin, as well as daily at 16 kHz in animals that were not anesthetized (paper II) In paper III the ABR were obtained at 4, 6.3, and 12.5 kHz in young and old animals from three animal strains (carriers of German waltzing guinea pig, Sahlin strain, and Lidköping strain). Animals from the same strains were also exposed to noise and measurements took place before and 24 hours, one week, and four weeks after exposure (paper III). In paper IV ABR was recorded in the right ear) at 3.5, 7, 14, and 28 kHz prior to and 96 hours following intra venous injections of cisplatin of two different dosages (5mg/kg, and 8mg/kg respectively).

3.3 POST-ONSET DISTORTION PRODUCT OTOACOUSTIC ADAPTATION

Ten heterozygotes of the German waltzing guinea pig strain (six males and four females) and four guinea pigs of the Sahlin strain (two males and two females) were used to investigate the correlation between the adaptation of the DPOAE-levels and susceptibility to noise, both regarding differences between strains and differences between individuals (Skjönsberg et al., 2003).

An earpiece consisting of two Beyer speakers for stimulation and one Etymotic (ER10B+) microphone for recording of the response was mounted on a holder and inserted into the ear canal. Stimulus presentations were coordinated and responses obtained using Tucker Davis Technologies (TDT) system II hardware and a custom MATLAB^{TM} script. Adaptation strength was defined as the DPOAE level at steady state subtracted from the DPOAE level at the onset of the primary tones. Two different f_1, first at 8 000 Hz, and then at 14548.23 Hz were used. These two f_1 frequencies were chosen based on previous experiments at the Dolan laboratory at Kresge Hearing Research Institute (University of Michigan), to bring out the strongest DPOAE.

Measurements from both ears were conducted. The two primaries were presented at an equal level, each 1 second of duration. The stimulus was given 25 times with a time gap of 2 seconds. The mean value of the 25 stimuli was calculated in discrete time points and plotted as a function of time vs. level of DPOAE-response. The efferent mediated reflex was measured with the primaries at three different levels at the two $2f_1$-f_2´s in order to create the most optimal DPOAE-response. In the group comparisons the mean value of the three L_1 were calculated and the means from both ears were added. In each animal the recording that produced the largest adaptation was selected independent on what level of the primaries.

3.4 EFFERENT-MEDIATED ADAPTATION

Animals from three guinea pig strains were used. Six carriers of German waltzing guinea pig of which one was a male and five were females. Seven animals from the Sahlin strain (four males and three females) and ten animals came from the Lidköping strain (two males and eight females).

Stimuli primaries were delivered through two Beyer speakers and the responses were recorded by an Etymotic (ER10B+) microphone, mounted on an earpiece inserted into the ear canal. Tucker Davis technologies system II hardware and a custom MATLAB^{TM} script were used. Efferent mediated $2f_1$-f_2 DPOAE adaptation magnitude was collected. f_2/f_1 ratio was 1.2. In this study f_1=8000 Hz was used. Primaries were presented in 1-second bursts, with 10 ms on and off ramps. Responses were collected at 25 ms intervals, starting 25 ms after the onset of the primary tones and ending at 975 ms after the onset. A fast Fourier transform (FFT) was performed on the response waveform, and the sound pressure level of the distortion product was obtained for each time point.

Typically, four one-second presentations and responses were recorded, with a two-second pause between each presentation. An average of the four responses was calculated and plotted in a 3D graph as a function of L_1 and L_2. For definition of the maximum adaptation magnitude the largest "negative adaptation" was subtracted from the largest "positive adaptation". The recordings consisted of two parts, coarse grid and fine grid as defined below.

Coarse grid: At starting point the L_1 and L_2 were of equal levels. Then L_1 was held fixed and L_2 was decreased in 1 dB steps for 12 different L_1; L_2 combinations. Six different L_1 were used. This method resulted in 72 different L_1; L_2 combinations.

Fine grid: After analyzing the results from the coarse grid, the starting levels of L_1 and L_2 were selected from were the maximum adaptation was seen in the coarse grid. Then, the L_1 was kept fixed and L_2 was decreased in 0.4 dB steps for 12 different L_1; L_2 combinations. Again, six different L_1 were used and 72 different L_1; L_2 combinations were run through. It was possible to add on more L_1; L_2 combinations in both the coarse grid and the fine grid if it was suspected that the whole positive and/or negative peak was not present. Finally, the coarse grid and the fine grid were added together and the maximum adaptation magnitude was defined by calculating the difference between the maximum negative value and the maximum positive value. The DPOAE as a function of all L_1; L_2 combinations were plotted in a 3D-graph (see figure 5).

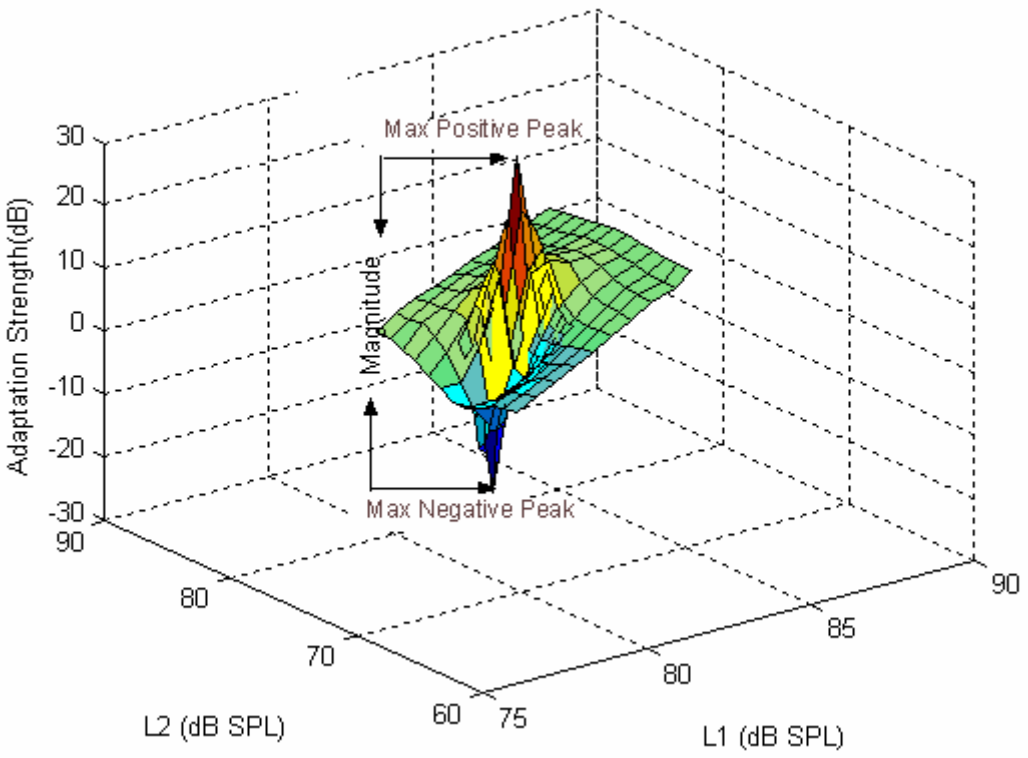


Figure 5 Example of the maximum adaptation magnitude, the difference between the maximum positive peak and the maximum negative peak as a function of L_1 and L_2.

3.5 NOISE EXPOSURE

In paper I heterozygotes of the German waltzing guinea pig strain and animals from the Sahlin strain were exposed to a free-field noise with a band-width of 800 Hz centered at 4 kHz. The exposure lasted for six hours and was presented at a level of 110 dB SPL (peak level measured at 4 kHz, see paper I for details).

During the exposure animals were kept in a wire-mesh cage divided into four isolated parts. Each part consisted of one animal at the time. The cage was positioned inside of a sound-isolated box (1x1x2 m) equipped with a speaker-horn mounted in the ceiling. During the noise exposure animals had free access to food and water. Before the exposure baseline ABR thresholds at 4, 6.3, 8, and 12.5 kHz were established and compared to the ABR hearing thresholds recorded twenty-four hours, one week and four weeks after the noise exposure.
In paper III animals from three different strains (heterozygotes of the German waltzing guinea pig strain, Sahlin strain and Lidköping strain) were exposed to the same noise as described above. In this experiment animals were kept in their ordinary cages placed inside the same sound-proof box as described earlier. The ABR hearing thresholds were recorded at 4, 6.3, and 12.5 kHz at the same time points as above.

We also exposed heterozygotes of German waltzing guinea pig strain to broadband noise. Un-anesthetized animals were placed in a wire cage on a turntable (approximately one revolution per minute) in an acoustically insulated chamber. A speaker was placed about 30 cm from the edge of the turntable at the same height as the guinea pig ears. The noise was a continuous 2-20 kHz broadband noise presented at 103 dB (A) for two hours. Sound levels were measured inside the exposure booth using a ½ inch condenser microphone and precision sound level meter (B&K Instruments, Inc.) (Skjönsberg et al., 2003).

One-year-old heterozygotes of the German waltzing guinea pig strain and animals from the Sahlin strain of were noise exposed using the same exposure set-up as in paper I. Hearing thresholds were established before and at 24 hours and 4 weeks after the noise exposure (paper III).

3.5.1 Littermate study

To further explore the reduced susceptibility to noise in the heterozygotes of the German waltzing guinea pigs strain a blind study was initiated using breading couples of only heterozygotes. This allows offspring of all three possible outcomes i.e. gw/gw (25 %), gw/+ (50 %), and +/+ (25 %).

The gw/gw animals are easy to identify by their waltzing behavior but it is not possible to distinguish between heterozygotes and normal animals. Littermates consisting of heterozygotes and normal animals were exposed to noise (the same protocol as in paper I) and confirmed hearing thresholds using ABR at frequencies 2, 4, 6.3, 8, and 12.5 kHz prior to and at 24 hours, 1 week, and 4 weeks after exposure.

At the time of exposure animals were of different age varying from approximately one month up to about six months. After completed experiments animals went back to the breeding facility to be "genotyped" by the characteristics of their offspring. Breeding a possible heterozygote ("non-genotyped"), a gw/+ animal, with a gw/gw animal will result in a litter consisting of both waltzing animals and heterozygotes whereas breeding a normal animal with gw/gw animal will give rise to only carriers and no waltzers. Thus, the occurrence of waltzing animals in the litter demonstrates that the "non-genotyped" parent must have been a heterozygote. If no waltzing animals appeared in the litter, the couple was allowed to rebreed to provide at least two more litters.

After confirmation of the animals' genotypes, the ABR data were grouped and compared at the different time points.

3.6 EXPOSURE TO OTOTOXIC DRUGS

3.6.1 Gentamicin

To explore the correlation between the susceptibility to the ototoxic aminoglycoside gentamicin and the efferent-mediated adaptation of the DPOAE animals were subcutaneously injected with gentamicin (American Pharmaceutical Partners, Inc, Schanberg, IL, USA) once a day for 14 consecutive days.

The Sahlin strain and heterozygotes of the German waltzing guinea pig strain were given the 120 mg/kg daily but there were no blood sample collected from the Sahlin strain and neither from the heterozygotes. The Specific pathogen free (SPF) animals were given 100, 130, 145, and 160 mg/kg. Animals were stored in the ordinary animal facility and had free access to food and water but additive nutritional supplements (Nutri-Cal EVSCO Pharmaceuticals) were given orally. Subcutaneous saline (Abbott Laboratories) injections were given to support the animal's state of health during the protocol.

Hearing thresholds were established (see above) and the efferent-mediated adaptation of the DPOA E (see above) was recorded prior to the injections. ABR were also performed at the end point of the experiment as well as a daily screening ABR at 16 kHz. The criteria of a remaining threshold shift of 20 dB compared to baseline levels were used in paper II when screening for the day of deafness onset. At day 10 of gentamicin dosing blood samples were collected from the SPF animals for analyzes of the general health.

3.6.2 Cisplatin

The heterozygotes of the German waltzing guinea pig strain was shown in paper I and III to be less susceptible to noise exposure and to explore whether this phenomenon reflects a general protection mechanism animals were exposed to the ototoxic chemotherapeutic drug cisplatin. Cisplatin was injected into the left jugular vein in anaesthetized heterozygotes of the German waltzing guinea pig strain and guinea pigs from the Sahlin strain. The animals received either 5 mg/kg, or 8 mg/kg cisplatin (Platinol® 1 mg/ml, Bristol-Myers Squibb AB, Bromma, Sweden). ABR hearing thresholds were measured before and 96 hours following the injections. Directly after surgery as well as daily during the experimental protocol the animals received subcutaneous saline injections to avoid dehydration. Analgesic and antibiotics injections were also given. Cardiatic blood samples were collected and analyzed for urea and albumin.

Quantification of hair cell loss: Hair cell loss was examined by creating cytocochleograms (paper IV). After decapitation, the left cochleae were removed and transferred to 4% parafomaldehyde in phosphate buffered saline (pH 7.4). The cochleae were carefully rinsed through the round window and a small hole in the apical part of the cochlea. The cochleae were left in the fixative for one hour and then kept in 0.5% paraformaldehyde at 4°C until the organ of Corti was dissected. The organ of Corti was stained with FITC-labeled phalloidin and cut into 3 mm pieces. The percentage of missing hair cells were quantified in each ¼ mm and plotted as a function of distance from the round window (in mm). The mean value from both strains and the two dosage groups were compared.

Cochlear platinum analysis: The right cochleae were collected as above. After the storage in 0.5% paraformaldehyde at 4°C the organ of Corti was dissected out from the bony part and weighed. The total-platinum analysis was performed at Analytica, Luleå, Sweden.

3.7 STATISTICAL ANALYSIS

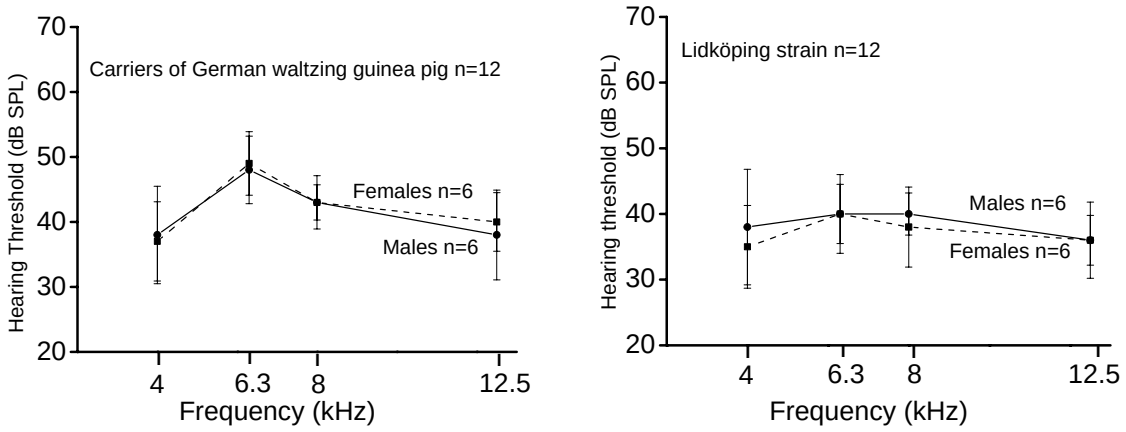
In paper I, an ANOVA model for repeated measurements was used. The statistical significance level was set to $p<0.01$. In paper II, unpaired Student's *t*-test (levels of significance at $p<0.05$, $p<0.01$, and $p<0.001$), linear regression, and exponential curve fitting were used. In paper III One-Way ANOVA was used for comparison of ABR hearing thresholds and threshold shifts and the level of significance was set at $p<0.01$ was used as indicator of significant difference. A Student's *t*-test was used for testing significant differences between groups regarding the maximum adaptation magnitude at a level of $p<0.01$. In paper IV the significant difference between groups were determined by Student's *t*-test; levels of $p<0.05$, $p<0.01$, $p<0.001$) was used to explore the statistical strength.

4 RESULTS

The focus of this thesis was to study heterozygous animals of the German waltzing guinea pig strain with regards to their susceptibility to different stress agents. The recessive autosomal Mendelian mode of heredity was confirmed by systematic breeding. The homozygous animals of this strain are deaf at birth, which was confirmed by the lack of Preyer reflexes and ABR responses. Compromised vestibular function was evident by a distinct waltzing behavior.

The initial hypothesis was that the hetreozygotes of the German waltzing guinea pig strain should progressively loose their hearing soon after birth but it has been shown that they keep their hearing throughout life. The hearing thresholds in young heterozygous animals do not remarkably differ from animals from other guinea pig strains. In one study it was possible to detect a difference at 12.5 kHz (paper III). In very young animals, approximately three weeks of age, there were no significant differences between heterozygotes of the German waltzing guinea pig strain and animals from the Sahlin strain as tested by ABR at 3.5, 7, 14, and 28 kHz (paper IV).

When comparing the ABR hearing thresholds obtained in males and females in heterozygotes of the German waltzing guinea pig strain and the Lidköping strain there was no significant gender difference (figure 6). In animals about one year of age (hetreozygotes of the German waltzing guinea pig strain, Sahlin, and Lidköping strains), it was possible to differentiate the Lidköping strain as a guinea pig strain with an early onset of age-dependent hearing loss as compared to the other two strains (paper III).



a) b)

Figure 6 Hearing thresholds in young unexposed males and females of carriers of the German waltzing guinea pig (a) and the Lidköping strain (b), measured with ABR. There was no significant difference between genders in either guinea pig strain.

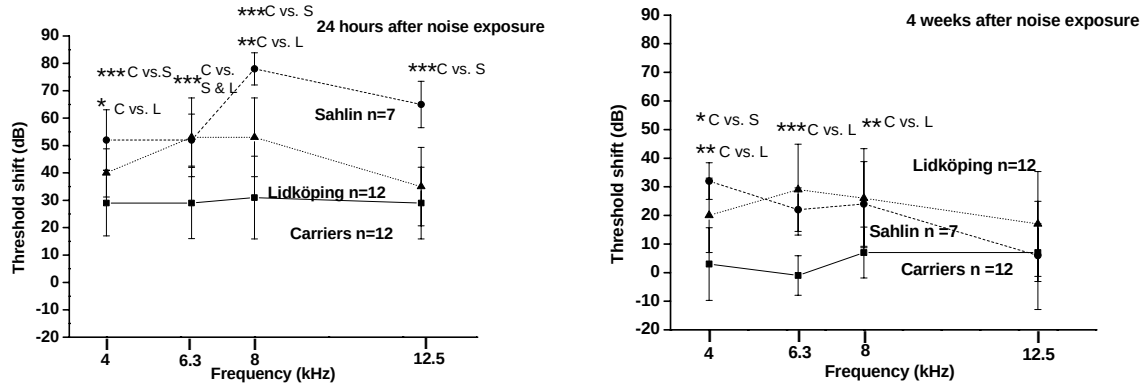
4.1 NOISE EXPOSURE

The hypothesis was that the heterozygotes of the German waltzing guinea pig strain would be more susceptible to acoustic overstimulation. To test this hypothesis, the heterozygotes of the German waltzing guinea pig were exposed to different types of noise and at different ages.

The results from a series of experiments showed that the heterozygotes were in fact less susceptible to noise exposure.

In paper I we used a narrowband noise resulting in a TTS in both heterozygotes of the German waltzing guinea pig and the control strain (Sahlin strain) 24 hours after the noise exposure. There was as a progressive recovery from hearing loss in both strains, evident already at one week of recovery. The heterozygotes of the German waltzing guinea pig had a less pronounced hearing loss at both 24 hours as well as 1 week after the exposure. When measuring the animals at 4 weeks after the exposure the heterozygotes had recovered to pre-exposure levels at the two lower frequencies (4, and 6.3 kHz) but a remaining threshold shift of about 7 dB still present at 8, and 12.5 kHz. The control animals (Sahlin strain) had significantly higher threshold shifts at 4, 6.3, and 8 kHz, but at 12.5 kHz there was no difference (paper I).

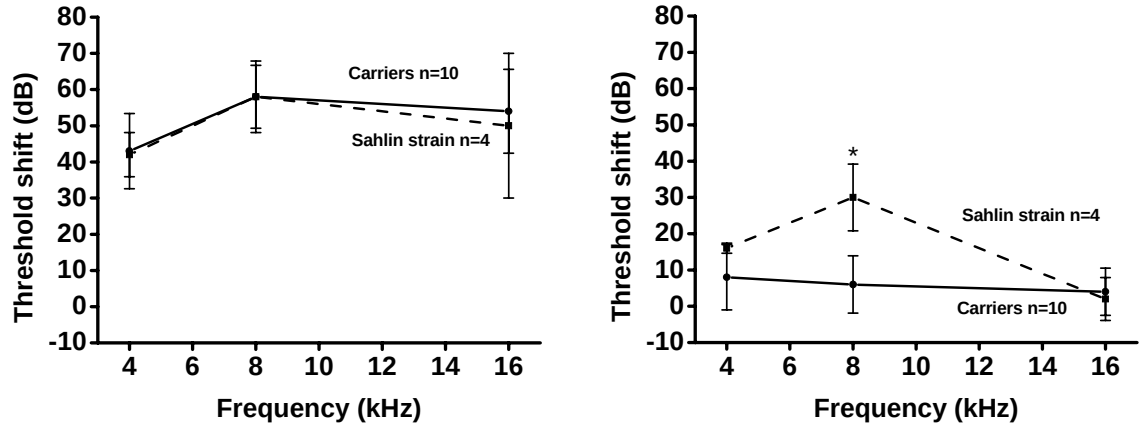
Nearly the same type of noise exposure as above (paper I) was used in paper III only differing in the cages used during exposure. Here, an additional control strain was added, the Lidköping strain. Also in this study the heterozygotes were less affected by the noise trauma as compared to control animals. Twenty-four hours after the noise exposure the carriers had significantly lesser threshold shifts compared to the Lidköping strain at 4 ($p<0.05$), 6.3 ($p<0.001$) and at 8 kHz ($p<0.01$). Compared to the Sahlin strain the difference was $p\leq0.001$ at all frequencies measured (figure 7 a). At 4 weeks after the exposure the carriers had significantly lower threshold shifts compared to the Lidköping strain at 4 ($p<0.01$), 6.3 ($p<0.001$) and at 8 kHz ($p<0.01$). The difference compared to the Sahlin strain was significant only at 4 kHz ($p<0.05$ (figure 7 b). The recovery was also more rapid in the carriers. This is most obvious at 6.3 kHz were the most prominent threshold shift was seen (paper III).



a) b)

Figure 7 ABR threshold shifts in carriers of the German waltzing guinea pig, Sahlin and Lidköping animals at 24 hours (a) and 4 weeks (b) after noise exposure.

Animals from both groups suffered from TTS one hour following exposure to a broadband noise. There were no significant differences between groups. However, the heterozygotes recovered from the TTS measured 1 hour post noise exposure to a less than 10 dB threshold shift two weeks after noise exposure. Control animals suffered from PTS at 8 kHz (Figure 8).

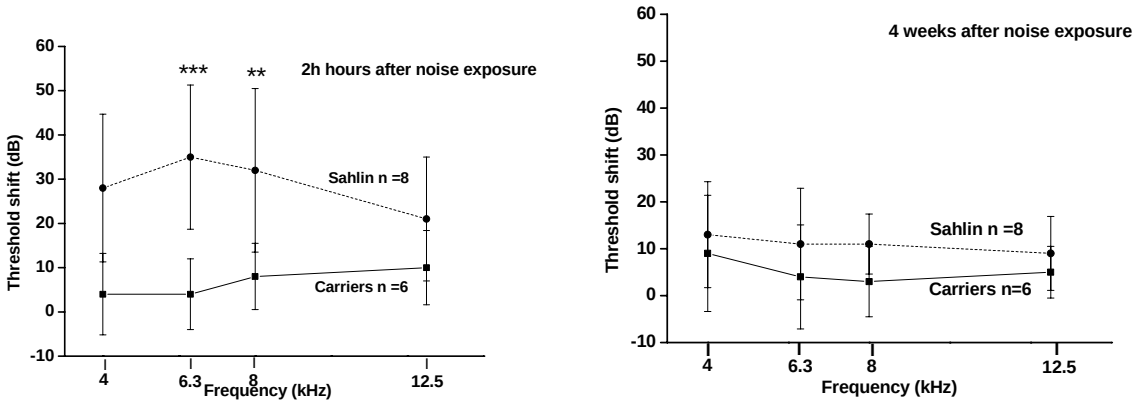


a) b)

Figure 8 Threshold shift (measured by ABR) 1 hour after a broadband noise exposure (a) and 2 weeks after noise exposure (b) (left ear). Carriers were less affected by the noise. The difference was significant (p=0.000291) at 8 kHz.

When one-year-old heterozygotes and animals from the Sahlin strain were exposed to noise, the hearing thresholds were not affected to the same extent as in younger animals. At 24 hours following the noise-exposure the carriers had significantly raised hearing thresholds only at 12.5 kHz ($p<0.05$) compared to pre-exposure levels. After four weeks there was no significant difference compared to pre-exposure thresholds. At 24 hours after exposure the animals from the Sahlin strain had significantly elevated hearing thresholds at all frequencies measured ($p<0.01$ at 4, 8, and 12.5 kHz; $p<0.001$ at 6.3 kHz). After 4 weeks of recovery, the ABR hearing thresholds of the Sahlin strain animals were still significantly different compared to pre-exposure levels at all frequencies measured ($p<0.01$ at 4, and 8 kHz; $p<0.05$ at 6.3 and 12.5 kHz) (data not shown). Between groups (carriers and Sahlin animals) there was a significant difference at 6.3 kHz ($p=0.001$) and at 8 kHz ($p=0.01$) 24 hour after the noise exposure (figure 9a). Four weeks after the noise exposure there were no significant differences between the two strains (figure 9b).

The carriers of German waltzing guinea pig have repeatedly shown that their auditory system is equipped with some mechanisms defending the hearing system from noise trauma. This can only be inherited from the German waltzing guinea pig since the breeding mate is used as control strain (the Sahlin strain). It was also shown in the littermate study.



a) b)

Figure 9 Threshold shifts in old animals from carriers and the Sahlin strain 24 hours and 4 weeks after noise exposure. Asterisks corresponds to ** p≤ 0.01, *** p≤ 0.001 differences between groups.

4.2 POST ONSET DPOAE ADAPTATION

The decay of DPOAE by time gave contradictory information. It was possible to detect a significant difference between groups at f_l=14548.23 Hz (p=0.0153). At the lower frequency (8000 Hz) there was no difference between groups. Individual animals from the heterozygotes of the German waltzing guinea pig strain showed extremely strong reflexes, none of the control animals showed exceptional strong reflexes.

However, there was no correlation between reflex strength and threshold shift following noise exposure. Animals expressing the strongest reflex were not the ones showing the smallest threshold shifts following noise exposure. From the example (figure 10) of the two animals showing the strongest reflexes the control animal showed a greater threshold shift at 8 kHz. It was also the frequency where groups were significantly divided. On the other hand, at 16 kHz the heterozygote animal suffered from a greater threshold shift than the control animal did. At 4 kHz the threshold shifts were similar for both the heterozygote animal and the control animal.

The heterozygote's reflex strength was also stronger than in the control group but the difference was not significant. The individual animal of the heterozygotes that showed extremely strong reflexes had changes in DPOAE amplitude as high as 8.89 dB at f_l=14548.23 Hz compared to 3.36 dB in the Sahlin strain animal with the strongest reflex (figure 10). In the Sahlin strain group it was no significant difference between males and females, neither at 8000 Hz nor at 14548.23 Hz. In the heterozygous animal group there was a significant difference (p≤0.05) between males (mean 0.64 dB) and females (mean 1.55 dB) at 8000 Hz but it was no significant difference between genders at 14548.23 Hz. The diverse results from the two frequency regions calls for further investigation of this method, preferably using a more standardized initial selection of the primaries.

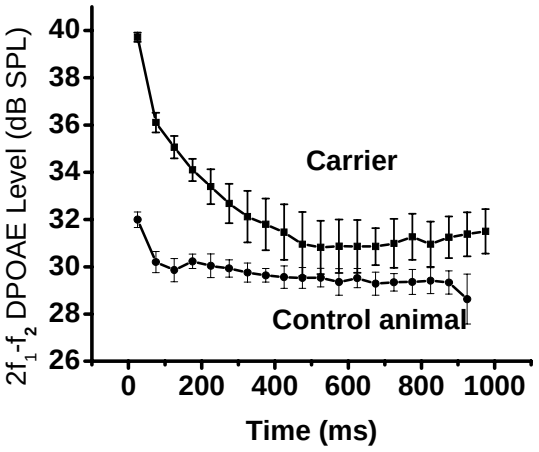


Figure 10 Post onset DPOAE adaptation from the animals that showed the greatest drop-off in each strain (heterozygotes of the German waltzing guinea pigs and Sahlin strain) Δ DPOAE in the heterozygote was 8.89 dB and 3.36 in the Sahlin strain animal.

4.3 MAXIMUM ADAPTATION MAGNITUDE

When plotting the adaptation of the DP (as a response of the L_1 and L_2 matrix, where the L is referring to the SPL of the two primaries) in the three-dimensional graph at least one negative and positive peak will occur. The maximum adaptation magnitude is the difference between the maximum negative, and the maximum positive peak. We could not find any difference between groups in either the adaptation magnitude or in the amplitude of the peaks. The adaptation and/or the positive and negative peak can thus not be used to predict any strain characteristics regarding the noise resistance or age-dependent hearing loss. Female animals of the Lidköping strain had significantly greater maximum adaptation magnitude compared to males ($p \leq 0.05$) but in the Sahlin strain animals no gender differences were found. Since the heterozygote animal group only consisted of one male animal no gender comparison were possible to make.

Table 2. Mean value and SD (in dB) for the maximum adaptation magnitude and the maximum positive and negative peaks in the carriers, Sahlin strain animals and Lidköping animals.

	Adaptation (mean and SD)	Max positive (mean and SD)	Max negative (mean and SD)
Heterozygotes	40.3 (9.5)	20.3 (3.7)	-20.7 (5.1)
Sahlin	39.5 (5.5)	18.7 (3.2)	-20.8 (3.3)
Lidköping	37.9 (4.9)	17.6 (3.4)	-20.3 (2.0)

4.4 GENTAMICIN INDUCED HEARING LOSS

A relatively large group of animals in different dosage groups did not survive throughout the entire experiment. Female animals were more susceptible to gentamicin and were more prone to die following the injections. Female animals had significantly higher levels of gentamicin in the blood as shown by the assays. The daily s.c. gentamicin injections resulted in a dose-dependent elevation of the hearing thresholds. In addition a gender-specific effect were detected as females suffered from a more pronounced hearing loss than males.

As expected, the thresholds at the high frequencies (8 and 16 kHz) were more affected than the thresholds at the low frequency (2 kHz). The heterozygotes of the German waltzing guinea pigs

strain were more negatively affected by the drug as compared to the control animals. The female heterozygotes were even more susceptible. The numbers of days before onset of deafness was animals receiving the higher doses negatively correlated to the efferent-mediated DPOAE adaptation magnitude; the larger magnitude, the earlier the onset of deafness following gentamicin.

Up to about 10 days after onset of gentamicin-administration, most animals passed the criteria of deafness. After day 10, all dosage groups started to loose hearing rapidly; the higher the dose, the earlier the onset of deafness. It was a significant difference regarding the onset of deafness between animals (females) receiving the lowest dose (100 mg/kg) compared to animals (males) receiving the two highest dosage groups (160 mg/kg, and 145 mg/kg) (figure 8, paper II).

4.5 CISPLATIN INDUCED HEARING LOSS

Systemic cisplatin administration notably affected the general condition of the animals. All cisplatin treated animals, of both strains, survived throughout the experimental protocol but signs of weakening health were observed in a dose dependent manner. The fur looked fuzzy and the animals appeared much less active than normal. Cisplatin affected also gastrointestinal function, as seen in by altered faeces.

Animals in the heterozygote group were more affected by the drug than the weight-matched controls, appearing more lethargic and apathetic compared to the control strain animals (Sahlin). Cisplatin had a negative impact on the body weight but the size of the dose was more important that the genotype of the animals. Animals in the high dose group significantly lost body weight starting from the first day following the cisplatin administration. In the low dosage group there was only a significant difference in body weight in the heterozygote group at day 3 post cisplatin administration compared to starting weight.

As expected, the hearing thresholds were negatively affected by the cisplatin injections and there was a dose-dependent effect but also an effect related to the genotype. The hearing thresholds in the heterozygotes of the German waltzing guinea pig strain were more elevated. Animals that received the higher dose (8 mg/kg) had significantly larger threshold shifts at the three lower frequencies compared to control animals ($p<0.05$ at 3.5 and 7 kHz, and $p<0.01$ at 14 kHz, see figure 4 in paper IV). There was no significant difference at 28 kHz, even if both groups had large threshold shifts compared to pre-exposure levels (mean value 39 dB in the Sahlin group and 47 dB in the heterozygote group) compared to pre-exposure levels. In animals receiving the lower dose (5 mg/kg) there was a significant difference between the groups at 7 kHz ($p<0.05$).

The pattern of threshold shift of the Sahlin animals strain injected with the higher dose was similar to that seen in the heterozygotes receiving the lower dose. This is even more obvious when averaging the threshold shifts over the whole frequency range measured (figure 11).

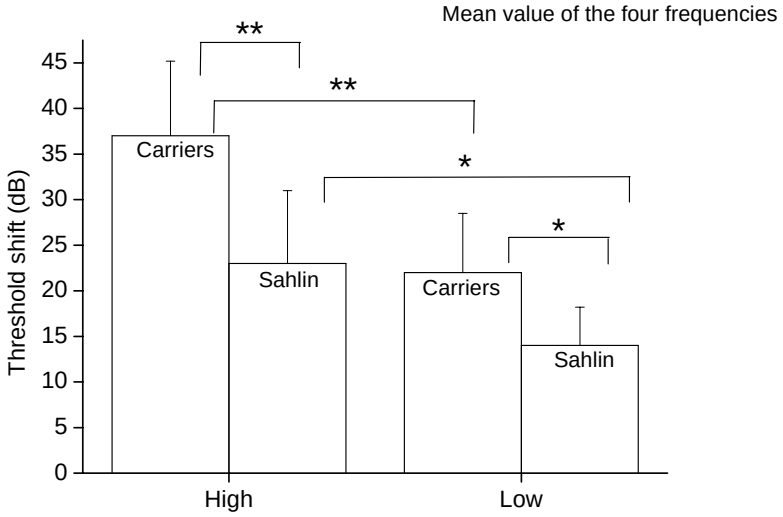


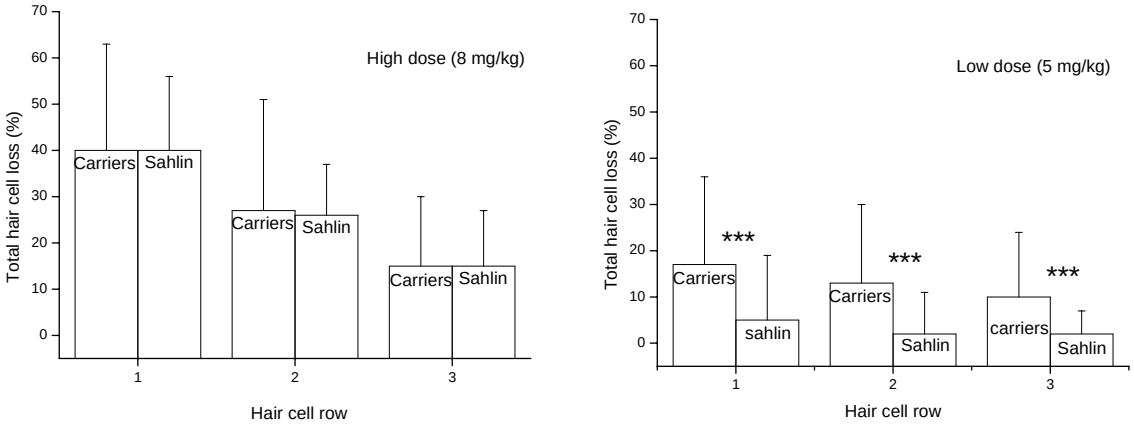
Figure 11 Mean value and SD of the threshold shifts at all frequencies measured (3.5, 7, 14, and 28 kHz). Carriers in the cisplatin high dose group had significantly higher mean threshold shifts ($p<0.01$) compared to Sahlin high dose animals as well as heterozygotes low dose animals. The Sahlin high dose animals had a significantly higher threshold

The inner hair cells were not affected by the cisplatin injections in either dosage group (data not shown). The first row cells of the OHC was more affected in both the heterozygotes and the Sahlin strain in both dose groups (figure 7 and figure 6, in paper IV). When the mean value of hair cell loss was plotted as a function of distance from the round window, the heterozygotes were shown to have a flatter pattern of hair cell loss compared to the Sahlin strain, especially in the first row of OHC. The carriers thus had significantly more hair cell loss (in percentage) at 15-17 mm from the round window compared to the Sahlin animals (table 2).

Table 3 The areas were there were significant differences in the outer hair cell loss in animals exposed to the higher dose of cisplatin.

	17 mm	**16 mm**	**15 mm**
OHC 1	P<0.05		
OHC 2	P<0.01	P<0.05	P<0.05
OHC 3	P=0.01		P<0.05

When averaging the hair cell loss in the whole cochlea the hair cell loss in the high dose groups (heterozygotes and Sahlin) were very similar (figure 12a). The first row was the most affected followed by the second and the third. The Sahlin animals in the low dose group suffered from a very small hair cell loss. The heterozygotes had a greater percentage of hair cell loss. A more flat pattern of the hair cell loss as was seen in the high dose animals (figure 6, paper IV). However, the difference was only significant ($p<0.05$) at 7 mm from the round window (data not shown). Interestingly, when averaging the hair cell loss in the whole cochlea the heterozygotes had significantly more hair cell loss (in percentage) at all three OHC rows ($p<0.001$, figure 12 b).



a) b)

Figure 12 Mean value and SD of the outer hair cell loss in each row (in percent) over the whole cochlear region.

Blood analysis revealed in a significant difference ($p<0.05$) in urea levels between the heterozygotes that received the high dose of cisplatin (74.6 µmol /l, SD29.6) compared to heterozygotes exposed to the lower dose (32.1 µmol /l, SD 21.4). The levels measured in the Sahlin strain animals showed no significant difference ($p<0.05$) between the high dose group (60.7 µmol /l, SD 20.7) and the low dose group (35.3 µmol /l, SD 18.7). The albumin levels did not differ between strains and dosage groups (data not shown). The heterozygotes that received the higher dose (8 mg/kg) had significantly higher levels of platinum in the cochleae compared to both control animals in both dosage groups as well as the heterozygotes receiving the lower dose (5 mg/kg) cisplatin (figure 13).

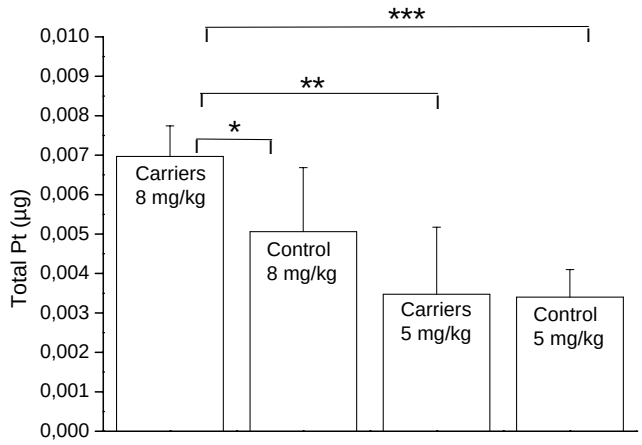


Figure 13 Levels of platinum in whole cochleae. Heterozygotes in the high dose group had significantly higher levels compared to control animals in the high dose group ($p\leq0.05$), control animals in the low dose group ($p\leq0.001$) and the carriers low dose group ($p\leq0.01$.

4.6 LITTERMATE STUDY

Forty-three animals were used in the littermate study. Nine of these were gw/gw animals, a genotype obvious already at birth. The other 34 animals were noise exposed and were allowed

to rebreed. At the time of this thesis 18 animals have been identified as gw/+ and four have been identified as +/+ animals. The additional 12 animals had either got one or two litters where "genotyping" was not possible or had no offspring. Twenty-one percent of the animals were shown to be waltzing animals, 42 % were heterozygotes, 9 % were normal animals and 28 % were not genotyped.

There was no significant difference between the hearing thresholds in gw/+ and +/+ animals before the noise exposure (figure 14 a). Twenty-four hours after the noise exposure the gw/+ animals had significantly lower threshold shifts compared to +/+ animals at 4 and 8 kHz ($p \leq 0.05$) (figure 14 b). One week after the noise trauma there was no significant difference between groups.

The heterozygotes, however, had about 10 dB lesser threshold shift over the whole frequency range compared to +/+ littermates (figure 14 c). Finally, after a 4-week recovery there was a significant difference between gw/+ and +/+ animals at 2 ($p \leq 0.01$), 4 and 12.5 kHz ($p \leq 0.05$) (figure 14 d).

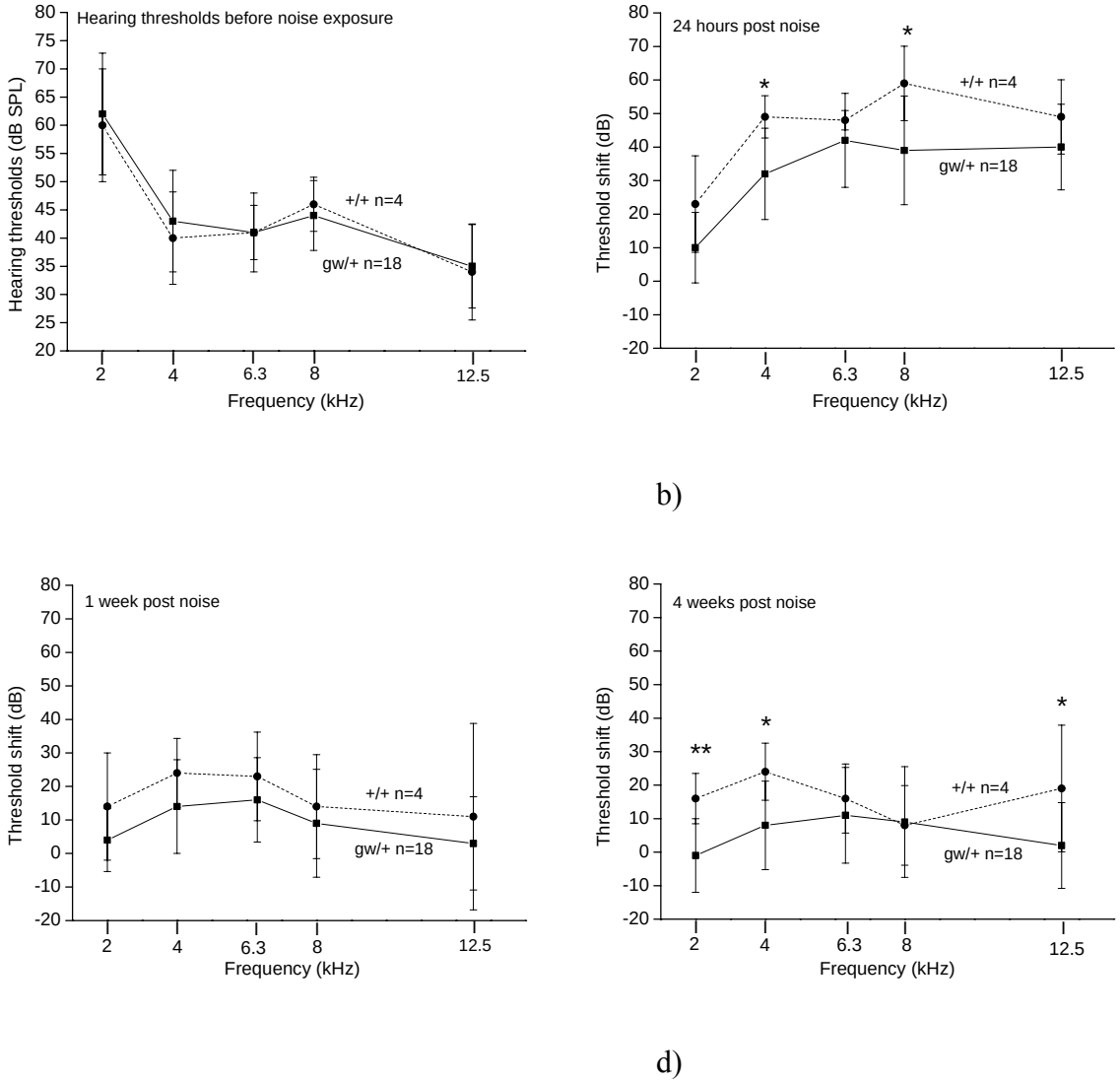


Figure 14 Hearing thresholds before noise exposure (a) and threshold shifts at 24 hours (b) 1 week (c) and 4 weeks (d) after exposure to noise.

5 DISCUSSION

The aims of this thesis were to functionally characterize the heterozygotes of the German waltzing guinea pig strain regarding their auditory system and their response to different stress agents; not only noise but also the ototoxic drugs gentamicin and cisplatin, which are known to cause inner ear damage just as specifically the acoustic overstimulation do. Methods used in this study were both routine measurements (ABR) and more advanced, and less explored, measurements like the variants of the DPOAE analyze. The seemingly unaffected heterozygous animals have given interesting results after been exposed to different auditory stress agents, presented in details below.

Effects of noise exposure: Young heterozygotes of the German waltzing guinea pig strain seem to be protected against PTS caused by noise exposure. The strain does suffer from TTS especially seen in the broadband noise study (figure 8); they also recover more rapidly (figure 2, in paper III) from the narrowband noise exposure. The narrowband noise did not produce as much shift as the broadband noise did. It should be pointed out that in the broadband noise study the TTS effect was measured as early as one hour after the exposure while in the narrowband studies the animals were allowed to recover for 24 hours. It is reasonable to speculate that the TTS would be more prominent one hour after the exposure compared to 24 hours after noise. However, the difference might also depend on the frequency range of the noise exposure used in respective study. When noise exposing older animals (approx. one year of age) to noise the effects of the noise trauma, shown as ABR threshold shift, were not as extensive as in younger animals no matter what strain used. These results are totally opposite to the results in the heterozygote animals of $Cdh23^{v}$ mice both regarding, the hearing status noise susceptibility and age-dependent hearing loss (Holme et al., 2004). Similar to the German waltzing guinea pig, the $Cdh23^{v}$ mice arose spontaneously and are deaf with a waltzing behavior. The heterozygous offspring have low- and high frequency hearing loss and are twice as susceptible to noise trauma compared to +/+ littermates. A study in rats showing that older rats are more susceptibility to noise than young adult rats (Fraenkel et al., 2003). The increased resistance to noise trauma in both young and old heterozygotes of the German waltzing guinea pig strain might be explained by an effective activity of endogenous antioxidant system at least in the auditory system. Based on the knowledge of oxidative stress in relation to noise induced hearing loss (Henderson et al., 2006) speculations at several levels can be made. Since the carriers seem to suffer from TTS and the synaptic damage are thought to be responsible for about 50% of the acute threshold shift (Puel et al., 1998) it would be interesting to compare the size and shape of the dendrites in the carriers of the German waltzing guinea pig and in control strains immediately after the noise exposure. If there are no differences found in dendrites size comparison the protection mechanisms might be explained at the outer hair cell level. This has, however, not yet been investigated. The disruption of cochlear blood flow leading to the increased levels of ROS could be absent or altered in the heterozygotes of German waltzing guinea pig strain meaning that the formation of ROS chain reactions might be blocked. The blood flow in relation to noise exposure has not been evaluated in the heterozygotes of the German waltzing guinea pig strain.

Effect of ototoxic drugs: The ototoxic drugs affected animals from all strains negatively. In the gentamicin study it was possible to show a significant difference gender-effect with females to be more susceptible to gentamicin. The question whether this is due to hormonal differences could be answered if comparisons were made between females and males before onset of fertility. Receptors for the female sex hormone estrogen have been shown to be present in the

inner ear at different regions e.g. inner and outer hair cells and in stria vascularis (Stenberg et al., 1999). The estrogen receptors are not explored in any of the strains included in this study. Due to the high incidence of death following the injections it was not possible to statistically prove the strain differences. The heterozygotes of the German waltzing guinea pig strain, especially females, showed poorer survival than the specific pathogen free animals and animals from the Sahlin strain. The reason for the higher mortality in the carrier animals can at this point only be speculated on, but an altered metabolic uptake of drugs could be one explanation. However, it has been no signs of elevated susceptibility to the anesthesia used.

In the cisplatin study the heterozygotes had greater threshold shifts and more pronounced hair cell loss as well as higher levels of platinum in the cochleae. Cisplatin affects the cochlea with the stria vascularis as the possible primary target (Tsukasaki et al., 2000; Wolters et al., 2004) but no morphological investigations of the stria vascularis or EP measurements were done in this study. However, since the stria vascularis is abnormal in the gw/gw animals but seems normal in the gw/+ animals post-exposure, investigations of the stria vascularis in the heterozygotes of the German waltzing guinea pig strain and/or EP measurements would be interesting. The antioxidant D-methionine are shown to protect the stria vascularis and the outer hair cell from damage (Campbell et al., 1999). The heterozygotes might serve as a suitable animal model using D-methionine or other antioxidative agents as protective component.

Efferent effect: In this study the hypothesis of the relationship between efferent reflex strength and strain differences was tested. The earlier findings of (Maison et al., 2000) could not be confirmed using our methods (as described in papers II and III). Prior to these studies a pilot study using a different setup was performed (unpublished data). Interestingly, in that study (results presented under Post-onset Distortion product otoacoustic adaptation) it was possible to correlate the post onset DPOAE adaptation with the reduced susceptibility to noise between strains at the higher f_1 frequency used (f_1=14548.23 Hz). At the lower frequency (f_1=8000) used no difference between strains could be detected. The hearing loss 1 hour after the noise exposure was equal between the strains and also rather flat (40-60 dB) over the frequency range. The PTS measurement (2 weeks after the exposure) showed recovery in the heterozygotes and a significant difference compared to the Sahlin strain at 8 kHz but not in the other two frequencies measured. The frequency range where there was a significant difference between groups in the reflex strength, there was no difference in threshold shifts. On the other hand, at the frequency range were it was no difference in reflex strength it was a significant difference in threshold shifts 2 weeks after the noise exposure. The methods in this set-up were not as standardized as in the second study were the maximum adaptation magnitude was measured. The initial level of the primary tones were selected by the criteria were the $2f_1$-f_2 distortion product was most prominent, meaning that the initial L_1 and L_2 were different in all cases. In the following adaptation studies the primaries had the same starting levels and were following the same scheme in the descending levels so that the DP level were recorded at the same144 L1;L2 combinations. Here gender differences were found in the study of post onset adaptation of the DPOAE. It has been shown that the SOAE differs by the monthly cycle in females why the eventual strain differences possibly were influenced by hormones levels (Bell, 1992; Haggerty et al., 1993).

In order to further develop the previous method (post onset adaptation) the maximum adaptation of the DPOAE were conducted. It was not possible correlate the maximum adaptation magnitude to the noise resistance or age-dependent hearing loss at the strain level. However, the noise resistance was not investigated in the individual animals used in later study (paper III) why the result might be misleading. On the other hand the heterozygotes of

the German waltzing guinea pigs is a strain that has repeatedly been shown to exhibit protection against noise trauma, which makes it possible to expect that the difference in maximum adaptation magnitude would be detected if there were any. In earlier studies, the correlation between the individual susceptibility to noise trauma were shown (Kim et al., 2001; Maison et al., 2000). In the case of the heterozygotes the situation is opposite, they are *less* susceptible to the noise and the method might not screen for that. Notably, the heterozygotes are also shown to suffer from a more pronounced hearing loss following cisplatin, which obviously cannot be predicted at the strain level using the maximum adaptation magnitude method. It was not possible to predict the strain differences in susceptibility to gentamicin at the strain level, but at the individual level it was (paper II). The method does not seem to contribute with new knowledge about the early onset of age-dependent hearing loss when using young animals from the Lidköping strain.

However, if the efferent system is contributing to the protection from acoustic trauma the protection mechanisms would need to respond very fast. From that aspect the post onset adaptation of the DPOAE measurements might bring more light to this theory than the maximum adaptation magnitude. However, authors Kirk and Smith suggest that the primary role of the efferent system is to improve signal-to-noise ratio (Kirk et al., 2003).

Effect of genetic component: The Guinea pig is widely used as an experimental animal in the field of hearing research. This is due to the easy access to the inner ear and that the guinea pig is having a normal-hearing frequency range that is similar to humans. The guinea pig inner ear is also fairly large, the length of the basilar membrane in guinea pig are approx. 19 mm compared to 12 mm in rats and 7 mm in mice which makes it practical in surgical procedures etc. Despite the many advantages very little is known regarding guinea pig strain characteristics. While the different strains of mice and rats are well characterized, both genetically and functionally, guinea pigs are often referred to simply as “guinea pigs”. In most cases, guinea pigs are only distinguished by fur color (albino or pigmented) or in some cases as waltzing animals (Canlon et al., 1993; Ernstson, 1970; Ibsen et al., 1929). In paper III we added an additional guinea pig strain, the Lidköping strain, to serve as reference to the Sahlin animals and provide information about the strength of the results shown in paper I. Since the carriers also are genetically related to the Sahlin strain it needed to be ruled out that the carriers’ reduced susceptibility to noise exposure was not related to inherited genes specific to the Sahlin strain genes. The results in the present study clarify that the reduced susceptibility to noise trauma is indeed an effect of genes coming from the German waltzing guinea pig. This study also showed that the age-dependent hearing loss in guinea pigs has different onset depending on strain. This emphasizes the importance of using well-defined guinea pig strains in the field of hearing research. The origin of the age-dependent hearing loss found in the animals from the Lidköping strain was not further explored in present study. It can be due to both middle ear and inner ear factors. Factors compromising the middle ear in aged individual are associated with progressive stiffness of the ossicular chain and tympanic membrane. In present study the middle ears was only examined by otoscope why it is not possible draw any conclusion regarding the status of middle ear beyond what can be detected visually. The role of estrogen in the auditory system is not clear but estrogen seems to be important in protecting the inner ear from for example presbyacusis (Guimaraes et al., 2004; Stenberg et al., 2001).

Quantification of hair cell loss: In this study (paper IV) the hair cell loss was quantified by using the standard technique of surface preparation. The way of plotting the cochleogram is however not standardized leading to difficulties when comparing different studies (Viberg et al., 2004). Normally the cochleograms are shown as data from individual animals but in the present study

group comparisons were performed by calculating the mean value of the hair cell loss (see figure 6, paper IV). In the plots presented in paper IV the standard deviations were not included in order to simplify the ability to compare between groups. The variation were however large and very few regions revealed significant differences between the strains. The mean value of the total outer hair cell loss in each row, including the SD, is seen in figure 8.

5.1 CONCLUSIONS

The German waltzing guinea pig strain shows a recessive autosomal Mendelian mode of heredity. The affected (gw/gw) animals are deaf and vestibular defected. The heterozygote animals (gw/+) on the other hand have normal hearing and balance which are maintained throughout life. There is no difference in hearing thresholds between females and males. The heterozygotes of German waltzing guinea pig strain have protection from PTS through an endogenous mechanism not yet discovered. The resistance to noise trauma is evident no matters what control strain is used. The protection is inherited from the German waltzing guinea pig genes, which is further shown in the noise exposure study using littermates. No strain differences regarding the maximum adaptation could be made but the adaptation of the DPOAE seems more prominent in the high frequency region. Some gender differences regarding both post onset adaptation and maximum adaptation magnitude is seen. The protection mechanism is, however, not efficient for animals being exposed to the ototoxic drugs gentamicin and cisplatin.

These interesting findings in the heterozygotes of the German waltzing guinea pig open for the opportunity to study inherited protection mechanisms in the inner ear.

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