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THE EFFECT OF CALORIC RESTRICTION ON AGE-RELATED HEARING LOSS AND THE IMPACT OF REPEATED SOUND EXPOSURES

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To my beloved family

ABSTRACT

The auditory system is the sensory system responsible for hearing and crucial for communication between individuals. While aging, many organs of the body start losing their function, including the hearing organ. The severity of age-related hearing loss depends on numerous factors. Intense noise and different ototoxic drugs are well-known damaging external factors, which can be avoided if precaution is taken. However, the moderate sound levels that surround the environment in every-day life are difficult to avoid, and the knowledge about their impact on the age-related hearing loss is limited. There are however ways of delaying age-related hearing loss. Caloric restriction is a proven method of prolonging life expectancy and to delay the age-related diseases, such as the age-related hearing loss.

The structural effects of caloric restriction on inner ear tissues of aging Sprague-Dawley rats were investigated. The age-related degenerative shrinkage of the metabolic important structure stria vascularis was delayed by a life-long, 70% dietary restriction. Moreover, the restricted feeding regime preserved hearing function at median life-expectancy age, compared to age-matched littermates.

The impact of repeated moderate sound exposures on hearing was investigated in the female Sprague-Dawley rat. The aim was to develop an animal model, to mimic human life-time noise exposure. Different exposure intensities were compared to determine an appropriate level, which could be repeated several times without causing a permanent hearing loss. At the intensity level of 104 dB SPL, the exposures were repeated six times, six weeks apart, with hearing thresholds returning to normal levels. Interestingly, exposures at this level made the animals more resistant to later overstimulation with intense noise.

The elderly population increases every year in the developed countries. Thus, there is significant need, not only to investigate the causes of age-related hearing loss, but also to explore ways to delay or prevent the disability. The life-time noise exposure model will thus be a realistic tool for this purpose. Caloric restriction and other possible intervention therapies can further be investigated, and its impact on age-related hearing loss can be studied.

LIST OF PUBLICATIONS

I. Mannstrom P., B. Ulfhake, M. Kirkegaard, M. Ulfendahl. 2013. Dietary restriction reduces age-related degeneration of stria vascularis in the inner ear of the rat. Experimental gerontology 48(11): 1173-1179.

II. Mannstrom P., M. Kirkegaard, M. Ulfendahl. Repeated moderate noise exposure in the rat – a life-time noise exposure model (manuscript in prep.).

CONTENTS

LIST OF ABBREVIATIONS

ABR	Auditory brain stem response
AL	Ad libitum
ANOVA	Analysis of variance between groups
Ca^{2+}	Calcium ion
COX	Cytochrome c oxidase
dB	Decibel
DNA	Deoxyribonucleic acid
DR	Dietary restriction
ihc	Inner hair cell
K^{+}	Potassium ion
kHz	Kilohertz
LX- PCR	Long-extension polymerase chain reaction
ms	Millisecond
N.VIII	Vestibulocochlear nerve
Na^{+}	Sodium ion
Na, K-ATPase	Sodium-potassium adenosine triphosphatase
ohc	Outer hair cell
Pa	Pascal
PTS	Permanent threshold shift
ROS	Reactive oxygen species
SGN	Spiral ganglion neuron
SPL	Sound pressure level
SV	Stria vascularis
TTS	Temporary threshold shift

1 INTRODUCTION

Aging is a process in the body when cells and organs deteriorate during the passage of time. Aging is often accompanied by age-related diseases and the age-related hearing loss is one of them. Mild hearing loss develops already during the middle age and progresses over time. As the elderly population is steadily growing, there is emergent need for better understanding of the mechanisms behind the deterioration. The present thesis addresses the effect of aging and a life-time noise exposure on the peripheral hearing organ.

The hearing organ is a complex system. It efficiently converts sound waves into electrical nerve impulses, which are encoded in the auditory- and language-centers of the brain. Many factors can disrupt this delicate system e.g. exposure to loud noise [1], ototoxic drugs [2], or solvent chemicals [3]. The genetic background is also an important component [4, 5]. The onset and severity of age-related hearing loss is indeed dependent on many of the above mentioned risk factors. Today, there is no true treatment for patients suffering from age-related hearing loss, instead residual hearing is amplified by hearing aids or when severe, by cochlear implants. Extensive efforts are made to discover possible pharmacological treatments that could slow down age-related hearing loss and when effective, to explore the mechanisms that are triggered by the treatment.

This thesis consists of two studies, both addressing the effects of aging. The first study is focused on the characterization of age-related degenerative changes in the peripheral hearing organ at the cellular level and what structures are preserved by a restrictive diet. The second study relates to what extent repeated moderate, and non-damaging, sound exposures affect hearing. Both of these two studies are carried out in animal models with an aim to develop a test model for future studies involving repeated moderate noise exposures and the morphological effects on age-related hearing loss. This model can further be used to study various preventive treatments aiming at delaying the onset and severity of age-related hearing loss for the elderly population.

1.1 ANATOMY AND PHYSIOLOGY OF SOUND TRANSDUCTION

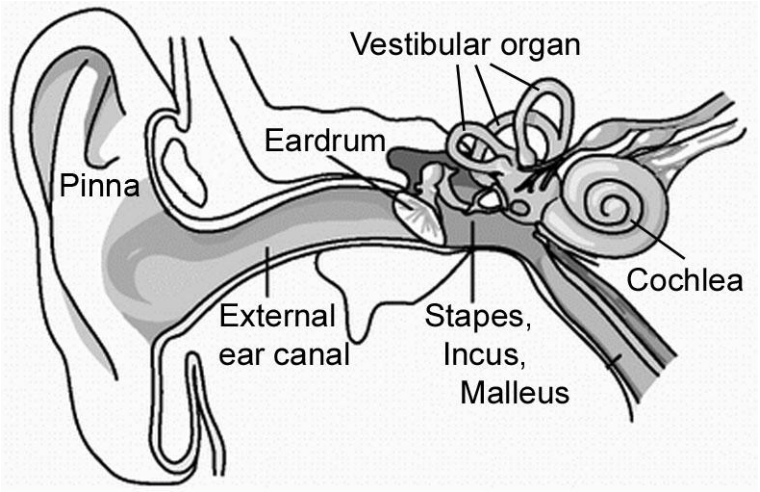
1.1.1 The auditory organ

The mammalian peripheral auditory organ is divided into the outer, middle and inner ear. The outer ear consists of the pinna and the external ear canal (fig. 1). The pinna captures and directs sound waves towards the external ear canal. The eardrum (tympanic membrane) is a thin membrane that separates the outer ear from the three connecting ossicles (malleus, incus and stapes) in the air-filled cavity of the middle ear.

A sound wave sets the ear drum into vibration with a subsequent mechanical movement of the ossicles, which serves as a lever and amplifies the vibrations. The footplate of the stapes pushes against the oval window membrane, which separates the middle ear from the inner ear.

Figure 1

Schematic drawing illustrating the human ear.
Modified from: www.medicalook.com



The inner ear consists of the vestibular organ with its semi-circular canals and the spiral-shaped hearing organ, the cochlea. Three fluid-filled separated tubes: scala tympani, scala media and scala vestibuli run in parallel from the base to the apex of the cochlea (fig. 2). The fluids inside these tubes differ from each other by their ionic concentrations; scala media contains the K^+ rich endolymph and the other scalae contain Na^+ rich perilymph. The sensory epithelium rests on the basilar membrane between the scala tympani and scala media.

During sound stimulation, the oval window membrane moves inwards and outwards of the cochlea, eliciting a vibratory motion of the basilar membrane that spreads from the base to the apex. This is usually referred to as the traveling wave. The vibration has its maximum amplitude at a specific location along the basilar membrane depending on the frequency of the sound wave. A high frequency sound has its maximum close to the base of the cochlea, whereas a low frequency sound creates the highest amplitude close to the apex. This is the fundamental principle for how different frequencies of the sound are discriminated.

1.1.2 Sensory hair cells

When the travelling wave reaches its maximum, the sensory hairs, the stereocilia of the hair cells (fig. 2), deflect with a subsequent opening of ion channels. The stereocilia are located on top of the hair cells towards the potassium ion-rich scala media. Potassium ions from the scala media enter the hair cells, which depolarize and open Ca^{2+} channels, thus eliciting the release of neurotransmitters to the adjacent afferent nerve fibers.

The hair cells are arranged in rows along the spiral of the cochlea. There are about 15 000 hair cells in the human cochlea, whereas the cat has about 12 500, the guinea pig has 9 500 and the rat has been reported to host 4 700 hair cells [6, 7]. One row of inner hair cells (ihc) transmits the information via afferent nerves to the brain. Three rows of outer hair cells (ohc) receive information from the brain via efferent nerves and act as a pre-amplifier by influencing the mechanical behavior of the cochlea.

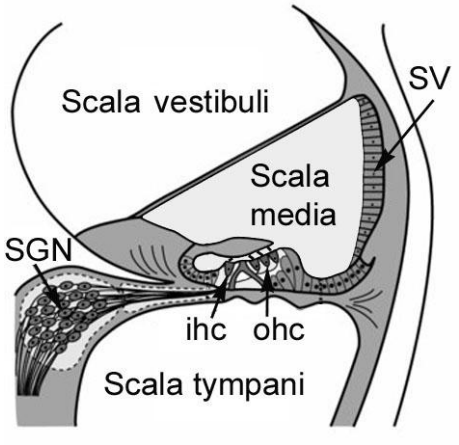


Figure 2
Schematic drawing, showing a cross-sectional view of the cochlea. Modified from Wise and Gillespie, 2012

1.1.3 Spiral ganglion neurons

Rosenthal's canal is a centrally placed canal that follows the spiral of the cochlea and contains the spiral ganglion neurons (SGNs, fig. 2). The total number and cell soma area of SGNs varies among the mammalian species [7]: humans are believed to have about 30 000 with a volume between 1 000 to 7 000 μm^3 [8], while the cat is believed to have 50 000 SGN and the rat 15 800 [9].

There are two types of neurons; the type I SGNs, which are more numerous (approximately 95%) and bipolar. They have a large cell soma (cell body), which in some species (guinea pig, mouse, rat and cat but not in humans) are covered with a myelin sheath. The type I SGNs receive sensory information from one single ihc. During sound stimulation, the release of the neurotransmitter glutamate initiates action potentials in the auditory nerve which in turn transmits the information further to the central auditory pathways. The type II SGNs are less numerous, pseudomonopolar, with a small unmyelinated cell soma. They receive information from the central auditory pathway and innervate several of the outer hair cells.

1.1.4 Stria vascularis

The lateral wall refers to the inside of the bony shell of the cochlea and consists of the stria vascularis (SV, fig. 3), which is lining scala media, and the adjacent spiral ligament. The SV is rich in blood vessels and has a high metabolic and secretory activity. It is composed of three cell layers; 1) *the marginal cells* with microvilli, facing the endolymph in scala media. On the basolateral side, they contain numerous mitochondria-rich fine processes, that interdigit with the underlying cell layers. The cells are linked to each other by electron-dense tight junctions, which allow ions to pass through from the intrastrial space to the endolymph. 2) *The intermediate cells*, which in

pigmented animals contain pigmented melanin granules, are discontinuously spread throughout the SV. This cell layer connects to the underlying basal cell layer via gap junctions. Blood vessels are located between the marginal cells and intermediate cells. 3) *The basal cells* are flat and create a border against the adjacent spiral ligament by tight junctions.

Inside the spiral ligament are collagen fibers located in between fibrocytes and blood vessels. There are five types of fibrocytes that are recognized both by their location within the spiral ligament and their histological appearance.

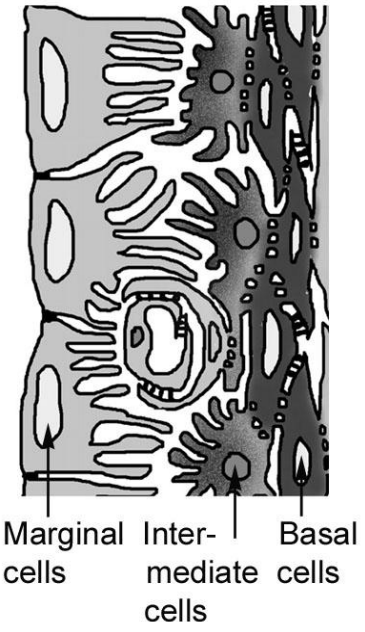


Figure 3
Cell types within the SV. Modified from Wangemann, 2006

One of the main purposes of the SV is to maintain a high K^+ concentration of the scala media. Potassium ions are thus actively transported from the marginal cells in SV into the endolymph in scala media and thereby maintaining a +80 mV potential of the endolymph with respect to the perilymph. This potential difference is called the endocochlear potential and is a vital component of hearing. During sound transmission, the sensory hair cells become depolarized by the K^+ ion influx. The K^+ ions are then actively transported back with help from the enzyme Na, K-ATPase through the fibrocytes in the spiral ligament, to the cell layers of the SV [10]. The delicate system of interdigitating processes of the marginal cells in the strial tissue is important for a functioning ion-circulation and imbalance of this complex recycling system affects the endocochlear potential. This is proposed to be the cause of strial presbyacusis [11-13].

1.2 FACTORS INFLUENCING HEARING

1.2.1 Age-related hearing loss

Age-related hearing loss, presbycusis, is associated with cochlear impairment due to the natural aging process. The hearing loss starts slowly with mild symptoms and progresses with age both in terms of frequency range and amplitude. Today, about 40% of the population at the age of 60 suffers from age-related hearing loss in the developed countries [14-17]. With an increasing aging population it becomes a growing problem for the individual as well as for the society [18]. Hearing aids compensate for hearing loss to some extent. However there are many situations when the amplified sound rather is tiring for the users, for example during multidirectional communication (when many people are talking at the same time) or in noisy environments. Hearing loss impairs everyday life for people in both communicative and psychological ways, and subsequently reduces the quality of life. With an increasing elderly population, this is a

growing problem. Societal socio-economic aspects are also significant, for example increasing cost for medical care and sick leave.

Schuknecht [19] divided age-related hearing loss into three major subtypes according to their origin: 1) *Sensory presbycusis*, which refers to degeneration of the sensory cells and reflects hearing loss of the higher frequencies observed by the pure tone audiometry test. 2) *Neural presbycusis* demonstrates a similar hearing loss across the measured frequency range, with a concomitant loss of word discrimination. Degeneration of SGNs and/or central auditory pathways of the brain are the cause of the hearing loss. 3) Strial or metabolic presbycusis is caused by degeneration of the SV and the adjacent fibrocytes in the spiral ligament of the cochlea. This type of degeneration reflects similar flat audiometric pattern as for the neural presbycusis, but in this case, subjects perform better in word discrimination tests. Over the years, this classification system has been debated since combinations of the different types have been identified. The underlying mechanisms may have multiple causes, both from the genetic background and previous history of various environmental factors such as exposure to noise, ototoxic drugs or infection which affect the total degeneration of the hearing organ.

The use of animal models in research of age-related hearing loss is widely spread, as similar condition as described for humans can also be shown in other mammals. There are, however, both species- and strain-specific differences regarding the onset and severity of age-related hearing loss. The degenerative changes also vary, since some strains demonstrate an early loss of the sensory cells, while the degeneration in other strains starts in the SGNs and/or in the SV [9, 12, 13, 20-23]. Additionally, the external factors as for example exposure to noise can more easily be controlled in a laboratory environment in order to minimize variations due to extrinsic factors.

1.2.2 Dietary restriction

A reduced dietary intake can extend life expectancy in a multiple of species, from single-cell organisms to humans [24-26]. In addition to the increased longevity, diet restriction also slows down the incidence of age-related diseases such as cancer, neurodegenerative disorders, cardiovascular diseases, autoimmune diseases and type II diabetes mellitus. Other behavioral explorative tasks have been described to be beneficial from caloric or dietary restriction in the literature. Better outcome in both sensorimotor dependent activities and physical activity tests (locomotion and rearing) has been reported together with a preserved muscle mass [27, 28].

Age-related hearing loss in the mammalian hearing organ can also be postponed by a restrictive diet [29]. Better maintained hearing thresholds as well as preservation of the sensory hair cells and SGNs have been reported as beneficial effects from caloric restriction [30, 31].

The mechanisms behind the beneficial effects from a restricted diet are still unclear, however speculations aiming at several different pathways that might be involved regarding the cells metabolism and energy production. This would result in reduced oxidative stress and enhanced autophagy, which in turn is health beneficial for the organism [26].

1.2.3 Noise exposure

One of the most common causes to hearing loss is noise exposure [1]. The degenerative effect on the hearing organ depends both on the intensity and the duration of the exposure, and is calculated as the total content of energy. For example, a gunshot has a very high intensity but the duration is within milliseconds. On the other hand, noise from loud machines in industry might not have the same high intensity, but the duration of the exposure is much longer and can go on for eight hours five days a week. The consequences for the hearing organ from the industrial noise exposure can thus be as devastating as exposure from a gunshot, as the energy content will be equally strong.

At the cellular level, the effect of different exposures may be quite different [32]. Very intense noise usually creates mechanical damage to the hearing organ with rupture of the tympanic membrane or death of the sensory hair cells with permanent hearing loss, so called permanent threshold shift (PTS) as consequence [33]. Milder noise primarily creates metabolic change of the cells, which may change the important ion balance in the fluids of the cochlea, and cause degeneration of the cells in the lateral wall [32]. This type of exposure usually leads to a temporary threshold shift (TTS), and hearing levels normalizes after a couple of weeks. However, studies have shown that although the hearing returns to normal levels, other permanent changes still occur as reduction in the number of synaptic vesicles of the ihc [34]. The vesicles are important for the transmission of nerve impulses, and the loss might have an impact on the onset and severity of the age-related hearing loss.

After noise exposure at intensities creating metabolic damage, formation of reactive oxygen species (ROS, free radicals) immediately starts and proceeds for 7-10 days [35]. The formation of ROS activates the apoptotic pathways and can lead to cell death. An increased production the free Ca^{2+} in the ohc is another consequence of overstimulation, which also triggers the apoptotic and necrotic pathways [36]. Overproduction of Ca^{2+} leads in turn to excessive release of neurotransmitter glutamate, from the ihc synapses, which in higher concentration is excitotoxic for the SGN [34]. During noise exposure, the cochlear blood flow decreases and can cause ischemia in the hair cells. The exact mechanisms for this is not fully explored, but it is well known that blood flow is important for establishing and maintaining the endocochlear potential [37, 38].

Today, in developed countries, people use hearing protection more frequently and are not so often exposed to loud noise levels. Instead, it is the everyday life noise, the so called leisure noise, which people are more frequently exposed to when listening to loud music in tight earphones, going to rock concerts and cinemas with loud music etc. This is a type of moderate exposure, which is repeated several times during a lifetime and little is still known how it will affect the hearing with increasing age [39, 40].

1.3 TOOLS TO ASSESS ANATOMY AND PHYSIOLOGY OF HEARING

1.3.1 Morphology assessment

Different techniques are used for visualizing anatomy of tissues. Choice of method depends on the current issue of the study. When the aim is to quantify cell populations in tissue sections, the stereology method is a good choice. If intracellular structures are to be studied, there is a need for a microscope which can produce images of higher magnifications. This can be implemented by using an electron microscope.

1.3.1.1 Stereology

Stereology is an unbiased statistically proven method to perform three-dimensional estimates regarding cell number and volumes from two-dimensional histological sections [41]. The method can be applied to all sorts of tissues and has earlier been used in hearing research to estimate total numbers of SGN in the cochlea [42]. However, the technique can also be applied to other cell types and structures of the inner ear. The technique requires a well-defined randomized sampling-design to get a reliable estimate of the complete population. Depending on the actual issue, different probes can be used, all geometrically well-defined. For example, point probes are used for estimation of volumes, lines for estimation of surfaces and three-dimensional probes (disectors) for number estimations.

When the sampling design is set and the appropriate probe is chosen, the actual counting on the sections can begin, starting from a randomized chosen start section. After the counting, the acquired data are calculated for final result. The precision of the result is dependent partly on the biological variability, which always exists when working with biological samples, and partly on the methodological error, which is dependent on the stereological design [43]. These two variables provide information about the total variation, and if the variation is too great, either the number of individuals or the amount of sampling fractions has to be increased. Therefore, a pilot study in one or two individuals is necessary, to control the methodological design.

1.3.1.2 Electron microscopy

The standard method to study morphology at the cellular level is by using electron microscopy. The transmission electron microscope is used for studying intracellular structures, while the scanning electron microscopy is used for scanning of tissue surfaces. The tissues that will be examined with a transmission electron microscopy

needs to be sectioned in ultrathin slices and stained with heavy metals. Electron beams inside the electron microscope will illuminate the tissue, become absorbed by the metals and the un-scattered electrons will produce a negative image of the tissue on a fluorescent viewing screen. A high-resolution image can be magnified up to 10,000,000X, unlike a light microscope, were the limit of magnification is 100X.

1.3.2 Hearing assessment

Different methods are used to evaluate hearing status in humans. The choice of method partly depends on the subject´s ability to participate and partly on the question at hand. Pure tone audiometry is the most common test for evaluating hearing thresholds for specific frequencies. It requires the subject´s participation, and the hearing threshold is determined as the weakest sound detectable by the subject for each frequency.

For individuals who themselves cannot cooperate (newborns or young children for example), screening for otoacoustic emissions or recording auditory brain stem responses (ABR) are used. The otoacoustic emission screening is a fast and non-invasive method that reflects the patient´s hearing status and can detect hearing loss caused by damage to the outer hair cells. The emissions are sounds produced by the sensory cells themselves as a response to vibrations of the sound stimulus. The emissions are detected by a probe inserted in the ear canal. ABR is widely used to monitor hearing status by recording the electric responses from the auditory nerve during sound stimulation. It is thus a method to investigate neural and/or central damages. In the auditory animal research, hearing assessment by ABR is a commonly used method to determine hearing loss, whereas the Preyer reflex (see below) is frequently used as a screening method for normal hearing.

1.3.2.1 Preyer reflex test

Already in 1882, Preyer described a method to assess hearing in guinea pigs [44]. Since then, the technique has been widely used in various other animal models to screen for normal hearing, as the method is easy to perform and reproducible. It is also a fast method without any need for expensive and complicated equipment. However, the method is not as sensitive as the ABR, and when using this method, conductive hearing loss cannot be distinguished from sensorineural hearing loss. Moreover, it is not possible to differentiate unilateral hearing loss from normal hearing.

A sound is presented close to the tested subject’s ears. If the auditory system is functioning, it will respond with a positive reflex, shown as either a contraction of the ears (as in the guinea pig and rat) or by a sudden movement of their bodies [45, 46]. The presented sound can either be a handclap, or more precise by a recorded sound from specific or multiple frequencies.

1.3.2.2 Auditory brainstem response (ABR)

ABR is a more precise measure of the hearing status, where the electrophysiological response from the vestibulocochlear nerve (N.VIII) and the auditory brainstem structures are tested [47]. The stimulus presented in the ear canal is either a frequency-specific sound or a click-stimulus, which is a sound burst that includes multiple frequencies. The response requires functionally intact middle and inner ear as well as an intact auditory nerve. In studies where the hair cells are damaged and the status of the auditory nerve is of interest to study, the electrically-evoked ABR method is used. The auditory nerve is then directly electrically stimulated by an electrode implanted close to the nerve. Thus, the damaged hair cells are bypassed and the electric response from the auditory nerve can be recorded using the ABR method.

The response from the auditory nerve is recorded via electrodes and displayed as waveforms, containing several peaks that occur within 10 milliseconds (ms) after the presented stimulus. Each of the peaks is believed to be generated from different locations along the auditory pathway. Peak I is generated by the peripheral portion of N.VIII, peak II from the central portion of N.VIII, peak III from the cochlear nucleus, peak IV from the superior olivary complex and peak V from the lateral lemniscus.

The hearing threshold is determined as the lowest presented sound stimulus in dB SPL that results in a reproducible ABR waveform in an average amount of responses. In addition to hearing thresholds, more information can be extracted from the ABR, for example, the amplitude of the first peak, which gives information of the total activity and the synchrony in firing rate from the cochlear nerve. The latency of the same peak gives information of the transmission rate, the time it takes for the neurons to fire after the onset of the sound stimuli. Interpeak latencies between first and fifth peak can also be extracted, which give information about the brainstem transmission time, the time for the neural impulse to be conducted from the auditory nerve to the lateral lemniscus in the midbrain. Differences between latencies of left and right ears can be studied by comparing the latency of the fifth peak. The latency is commonly used as a clinical diagnostic tool and abnormalities in the latency can be implications of a lesion along the auditory pathway.

1.4 AIMS OF THE STUDY

This thesis consists of two studies. The aim of the first, (study I) was to investigate the morphological changes in the aging female Sprague-Dawley rat cochlea and the effects from a life-long restricted diet regime. The specific objectives for this study were:

- To establish stereological methods to characterize and quantitatively estimate total volume of stria vascularis (SV), total amount of sensory hair cells, total amount and soma size volume of spiral ganglion neurons (SGNs) in the rat cochlea from histological sections.
- To compare patterns of age-related histological degenerations in aged animals on different feeding regime; either with free access to food (*ad libitum*, AL) or with a 70% dietary restriction (DR) on the above mentioned parameters. Young adult animals serve as controls.
- On the cellular level, by use of transmission electron microscopy, to study the degree of degeneration in the SV of young and old rats with the different feeding regimes.

The second study (study II) aimed at exploring a new model for repeated moderate noise exposures in the female Sprague-Dawley rat, which would mimic a life-time exposure in humans. The objectives of the second study were:

- To determine an optimal intensity level of moderate noise exposures that can be repeated at least six times every six weeks without creating permanent threshold shift (PTS).
- To assess hearing with auditory brainstem responses (ABR), regarding threshold shifts, amplitudes and latencies of the response before and after each noise exposure.
- To study noise susceptibility changes after different intensities of non-damaging repeated moderate noise exposures by acoustic overstimulation with intense noise.

These studies aim to gain a better understanding of hearing; how different structures of the inner ear are affected by various factors such as age, dietary restriction and noise. The significance of the two studies will provide knowledge and appropriate tools to continue further investigations of life-time exposures and to study the morphological effects on age-related hearing loss with possible intervention therapies. The long-term intention is to find treatments that prevent age-related hearing loss and thus will facilitate communication for elderly people.

2 MATERIAL AND METHODS

2.1 SUBJECTS

Female albino Sprague-Dawley rats were used in all experiments. In study I, a total number of 66 animals were delivered from Scanbur BK (Sollentuna, Sweden) and kept on a 12/12 hour day and night cycle at the animal facility at the Department of Neuroscience, Karolinska Institutet. Post-weaning, 50 of them were kept on a life-long diet of standard pellet (Lactamin, R70, Lantmännen, Sweden), either with free access or on a 70% restriction. The remaining rats served as young controls.

In study II, 48 rats from the same strain as in study I were delivered from Harlan laboratories (the Netherlands) and kept on a 12/12 hour day and night cycle with free access to food and water and kept at the animal facility, at the Department of Comparative Medicine located at Karolinska University Hospital.

All animal experiments and procedures followed the local ethical guidelines from Karolinska Institutet, and were in accordance with national regulations (approvals N122/06 and N394/09 for study I, and N33/07, N12/10 and N300/11 for study II).

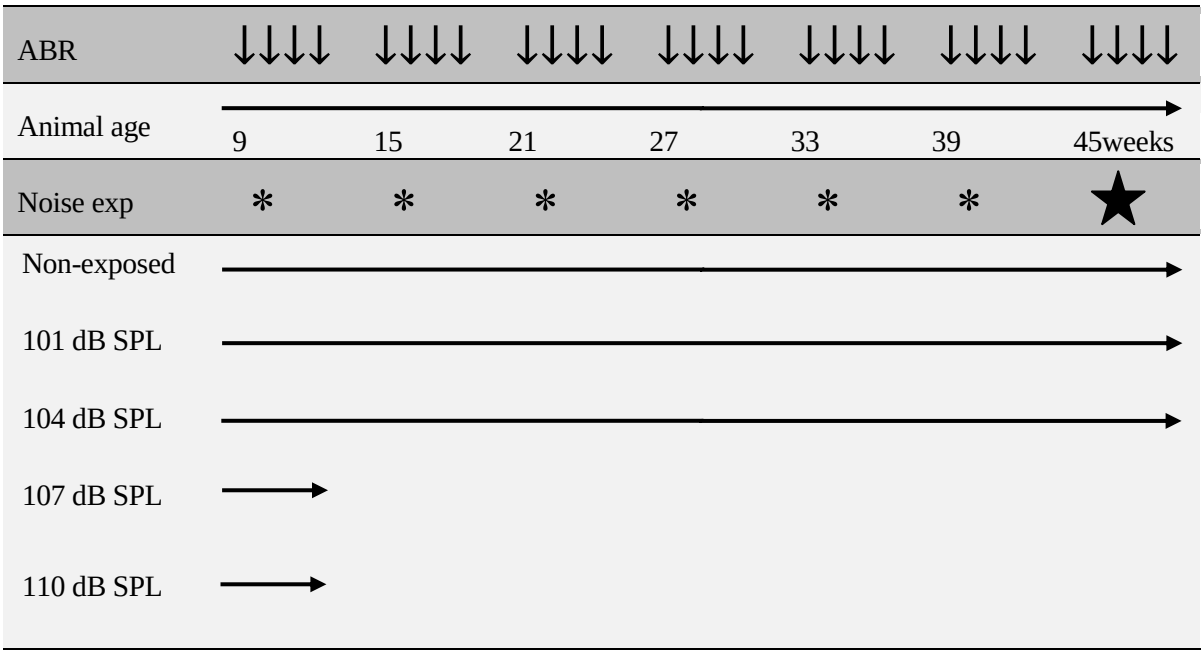
2.2 EXPERIMENTAL DESIGN

In study I, one group of animals (n=30) were post-weaning maintained with free access to food (*ad libitum,* AL) while another, age-matched group (n=20) was maintained on a life-long diet with 70% dietary restriction (DR). Animals from both the two aging groups were sacrifized at their median survival age, at 30 months. Animals from a young adult control group (n=10) were sacrifized at the age of 9 weeks. Hearing was assessed by testing the Preyer´s reflexes of the animals before they were sacrifized. Animals were then given a lethal dose of pentobarbital sodium, and their cochleae were dissected out for histological processing to estimate cell number, volumes and fine structures of the inner ear tissues.

In study II, the animals were divided in groups for moderate noise exposures with different intensity levels: 101 dB SPL (n=8), 104 dB SPL (n=8), 107 dB SPL (n=7), 110 dB SPL (n=7) together with a non-exposed control group (n=10), (table 1). Eight animals that died from anesthesia were excluded from the study. Moderate noise exposures were repeated every six weeks to explore which intensity level could be repeated several times without creating a permanent hearing loss. ABR was used for hearing assessment 1 week before first exposure and 24 hours, 1 week, 2 weeks and 5 weeks after each exposure, to determine the hearing thresholds as well as the amplitudes and latencies of the responses. The exposures were repeated either until the subjects in each intensity group displayed a permanent hearing loss, or for a maximum of six repetitions. The groups that did not show any PTSs after six repetitions (i.e. 101

dB SPL and 104 dB SPL groups) were then together with the previously non-exposed control group, exposed to acoustic overstimulation using intense noise. The intention was to explore possible changes in susceptibility to intense noise after non-damaging repeated moderate noise exposures.

Table 1
Experimental design:



*Downward arrows indicate timepoints for ABR measurements. Timepoints for the repeated moderate noise exposure are indicated with * and the acoustic overstimulation with intense noise are indicated with a star. Horisontal arrows indicate time spent in experiment for each animal group at the specific intensity level. Modified from study II.*

2.3 HEARING ASSESSMENT

2.3.1 Preyer reflex test

In study I, hearing was assessed with the Preyer´s reflex test in 10-13 animals per group. The test was performed inside the animal facility before the rats were sacrifized. A recorded sound burst, at multiple frequencies and intensity at 60 dB SPL was presented above the cage, while carefully observing the animals. Animals that either contracted their ears or suddenly moved their bodies when the sound was presented were considered to have positive reflexes and normal hearing.

2.3.2 Auditory brainstem response

Frequency-specific ABR was used for hearing assessment in study II. Detailed description is presented in the manuscript. In short, tone bursts at specific frequencies (3.5, 7, 14 and 28 kHz) were generated by a TDT system II (BioSig 32, Ver 3.12, Tucker Davis Technologies, FL, USA). The tone bursts were presented through an electrostatic speaker (EC1), with an earphone placed in the left or right ear canal of the anaesthetized animal. The same ear was used for all ABR recordings throughout the study. Sub-dermal needle electrodes were placed at the vertex (active), behind the recorded ear (reference), and in the hind leg (ground). Electrical responses to the stimuli were collected with the electrodes and displayed as waveforms in the software program (fig. 4). The second peak was used as the reference peak, since it is the largest ABR peak in rat and easy to identify, also at lower stimulus intensities. The intensity of the tone burst was decreased until the waveform disappeared and the threshold was determined as the lowest presented stimulus that elicited a reproducible response after 2000 averages. The minimum step length of the stimulus was 5 dB SPL.

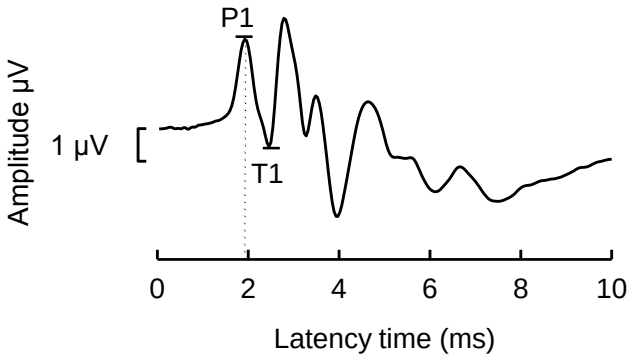


Figure 4

ABR waveforms from a young rat. Amplitude measured as the difference between the peak (P1) and the trough (T1) of waveform I. The dotted line indicates the latency. Modified from study II.

The amplitude (fig. 4) was measured as peak to peak $_{(p\text{-}p)}$ amplitude difference of wave form I; from peak 1 (P1) to its trough (T1) [48]. The latency was defined as the time from the onset of the stimulus to the peak of waveform I.

ABRs were compared over time for the separate groups to investigate the impact of the repeated moderate noise exposures and to explore eventual aging effects. The different groups were compared after the repeated moderate noise exposures and after the acoustic overstimulation.

2.4 NOISE EXPOSURES

Noise exposures were performed in study II. Freely moving animals were placed two at a time in individual cages, centrally placed inside a soundproof box with a loudspeaker (horn TD 360, Spain) attached to the ceiling. The sound was generated from a Brüel & Kjær 3560-C PULSE hardware and a LAB 300 amplifier with PULSE LabShop

Version 13.1.0.246 software (Brüel & Kjær, Denmark). A calibration microphone (Brüel & Kjær, Denmark) was centrally placed at the same level as the animal cages and used for adjusting the intensity level of the noise.

2.4.1 Repeated moderate noise exposures

Every six weeks, the animals were exposed during 90 minutes to broadband noise (2-20 kHz, centered at 11 kHz) at a 50% duty cycle (burst time on, during 500 ms every second). The intension was to find an appropriate intensity level that could be repeated several times without causing PTS. The tested intensity levels were: 101, 104, 107 and 110 dB SPL. The exposures were repeated until the group displayed a PTS or for a total of six repetitions.

2.4.2 Acoustic overstimulation

The animal groups that still had normal hearing after six repeated exposures, and the animals in the previously non-exposed control group were then exposed to acoustic overstimulation using 110 dB SPL narrow band noise (3.2 kHz, centered at 4 kHz) for 4 hours. ABR were compared between the groups to determine if the previously non-damaging repeated moderate exposures had any impact on the animals´ noise susceptibility.

2.5 HISTOLOGICAL EVALUATION

In study I, histological evaluation regarding cell volume of SV, total amount of hair cells and SGN as well as their soma volume were estimated with the stereological method. Transmission electron microscopy was used to study the fine structures of SV. No histological examination was performed in study II.

2.5.1 Stereology

Cochleae from the different groups (young n=8, AL n=9, DR n=9) in study I were dissected out, fixed and decalcified before embedded in a 2-hydroxyethyl metacrylate-based resin (Technovit 7100, Heraeus, Germany). Serial sections, 24 µm thick, were cut and stained with regular nuclear and cytoplasmic stain (haematoxylin and eosin). A bright field microscope (Axioplan, Zeiss, Germany) was equipped with a motorized stage (Prior ProScan II), which could be moved in x, y and z – axes, controlled by the newCAST software program (Visiopharm, Denmark).

The Cavalieri principle was used for estimating the tissue volume of SV in a predetermined amount of sections, at 20X magnification, with a random starting number [43]. A counting grid with test points was digitally placed on the section and all test points that covered the SV were counted with the newCAST software (fig. 5A). The total volume was then calculated by multiplying the counted test points with the area per test point of the counting grid and the average distance between the counted sections.

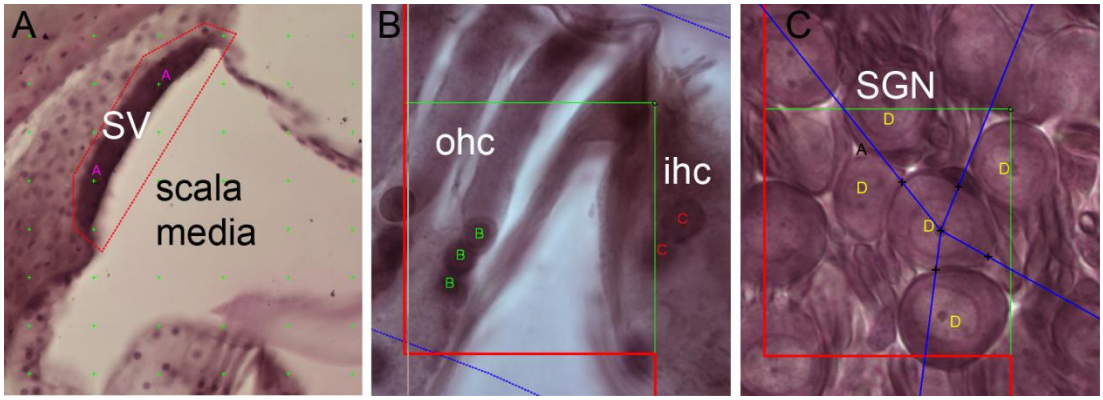


Figure 5

Stereological estimations. A) SV volume; all test points that covered the SV were counted and marked "A". B) All ohc and ihc inside the counting frame (or touching the green line) were counted, marked "B" or "C". C) All SGN inside the counting frame (or touching the green line) were counted, marked D. The length of four randomly placed test lines was used for estimation of cell soma volumes of the SGNs.

The total amount of hair cells (ihc and ohc) as well as the SGNs was estimated using the optical fractionator technique [41, 42, 49], from inner ear tissues divided into sampling fractions. The fractions were determined in three levels; 1) *the section sampling fraction* (the ratio of counted sections per cochlea), 2) the *area sampling fraction* (percentage size of counting frame out of the area of interest) and 3) *the height sampling fraction*, defined by dividing the height of the counting frame with the average section thickness. A pilot experiment was conducted to determine the fraction sizes, were approximately 200 cells in at least seven sections should be counted in each cochlea. A 100X magnification oil immersion objective (NA=1.3) was used for counting. Counting frames with an area of 1600 µm^2 and height of 12 µm were digitally placed over the sections from the same (randomly determined) start section as for the SV estimations. Cells that met the criteria for counting rules (were within the counting frame or touched the green inclusion line) were counted in all predetermined sections (fig. 5B and C). The total amount of cells was estimated by multiplying the counted number with the inverted number of each and one of the fractions. Both ihc and ohc were counted as well as both types of SGN (type I and II).

The average volume of the type I SGN somas were estimated with the nucleator technique [49-51]. The average length of four randomly placed test lines, from the most centrally located nucleolus to the cell surface, was calculated in between 100 and 150 evenly distributed neurons (fig. 5C). The location (apex, mid or base) of neurons with extremely small (less than 1500 µm^3) or large (greater than 5000 µm^3) volumes were noted.

2.5.2 Transmission electron microscopy

Three cochleae from each group in study I were further studied on cellular level using transmission electron microscopy. After dissection and fixation, the cochleae were post-fixed in 1% osmium tetroxide, decalcified and embedded in Agar 100 Resin Kit (Agar Scientific limited, England). The basal and apical parts were dissected out and remounted on a blank resin block. Sections at 1 µm thickness of were stained with toluidine blue and inspected in light microscope to control the angle of the specimen. Ultra-thin sections were cut, put on grids coated with formvar and stained with heavy metals (uranyl acetate and lead citrate) before examination with transmission electron microscope (JEOL 1230, JEOL GmbH, Germany). Digital images were captured to compare degenerative changes in the SV and the adjacent spiral ligament, between young and aging animal groups from the different feeding regimes.

2.6 STATISTICS

All statistical calculations were performed using SigmaPlot for Windows, version 11.0. Values from the different groups from the stereological estimations in study I, and ABRs from the different groups in study II were statistically compared using one-way ANOVA. Repeated measurements one-way ANOVA were used for ABR comparison over time in study II. When a statistically significant difference was present, post-hoc analysis using the Holm-Sidak multiple comparison test was used. If the normality test failed, Kruskal-Wallis One way ANOVA on ranks, were used together with Dunn´s method for all pair wise multiple comparison procedure. The significance level was set to $P < 0.05$ for all comparisons.

The total variation for each group of the stereological estimates were also calculated to control the stereological sampling design, were the level for variance of error of the method was set to be below 0.5.

3 RESULTS AND DISCUSSION

3.1 STUDY I – DIETARY RESTRICTION REDUCES AGE-RELATED DEGENERATION OF STRIA VASCULARIS IN THE INNER EAR OF THE RAT

3.1.1 Age-related degeneration of the rat cochlea

The main age-related degenerative findings were a 25% decrease in total number of sensory hair cells and a reduced volume of the SV epithelium by 27%, as estimated with the stereological method. The majority (85%) of the aging AL fed animals were lacking positive Preyer reflexes, indicating a decline in hearing. Since some animals still displayed positive reflexes, it indicates that individual differences exist in this animal strain. The Sprague-Dawley rat is an out-bred strain with more variability between individuals than in an in-bred strain. The strain was therefore chosen considering the similarities to humans, in respect to individual differences. Interestingly, the total number of SGN was only slightly reduced in the aging cochleae (10%), and the average cell soma volume was unaltered compared to young animals´. The cell soma volume of individual SGN neurons, within the same cochlea, varied between 250 and 8700 μm^3, also for the young animals. The smallest cell somas were found in the apex of the cochlea whereas the largest were found close to the base. A frequency distribution analysis with a grouping interval of 500 μm^3 revealed that the relative amount of neurons with larger cell somas was more frequent for the aging groups compared to the young group.

The reduced SV volume reflected a variety of degenerative changes observed by electron microscopy; from retraction of the marginal cell borders to the more advanced changes, with reduction of SV to a single cell layer. Degeneration of the fine processes of the marginal cells, lobular inclusions, cell debris and thickening of basement membrane surrounding the blood were other changes observed in the aging animals. Degenerative changes were also observed in the adjacent spiral ligament, which contained many empty intercellular spaces between the fibrocytes.

Similar age-related loss of sensory cells has previously been described in the literature both in the Sprague-Dawley rat [6], in other rat strains and in other rodents [52, 53]. Age-related degenerative changes of processes, cell loss and inclusion of lobules in the SV and spiral ligament degeneration have previously been studied in senescent gerbils with strial presbycusis [12, 13, 54]. Other researchers have reported a more prominent age-related loss of SGN in the Sprague Dawley rat [9] compared to our study, but sub-strain differences, gender and choice of method could explain the different results. The broad variation of SGN volumes that was observed in our study has also been described in human SGNs [8]. Even though no prominent age-related SGN loss was detected in

our aging rats, the increased amount of cells with larger cell somas could be a predictor of cell death preceded by swelling of the neurons.

3.1.2 Preservation by dietary restriction

A life-long restricted diet led to a preserved volume of the metabolic important structure SV. The age-related reduction was only 12% in the DR animals compared to 27% in the AL fed animals. The auditory status was also significantly better in the DR animals, where as much as 73% of the animals displayed positive Preyer reflexes compared to only 15% in the AL group. About 20% higher survival rates and lower body weight were other beneficial outcomes from the restricted regime.

No age-related protective effect from sensory cell loss was obtained from the dietary restriction. This in contradiction to what Seidman and coworkers found after dietary restriction in the Fisher rat [30]. However, in this study, only a small sample size (3-5 individuals/group) was used and neither exact number for the hair cell loss, nor significance values were presented. The different result could also depend on differences in strain and gender (not specified in their study). Interestingly, 73% of animals in our study displayed positive Preyer reflexes, despite a 25% sensory hair cell loss, suggesting a functional hearing, even with a reduced amount of sensory hair cells. The Preyer reflexes are a measure of normal auditory status, reflecting aspects both from the peripheral and central pathways of the auditory organ. The dietary restriction may also have beneficial effects on the central auditory pathways, which not been investigated in this study.

The SV is a structure with high metabolic and secretory activity, which responsible for the ionic circulation in the cochlea, and accordingly an important structure for hearing function. A high metabolic rate in cells, leads to high production of ROS, a by-product of energy production in the mitochondria, which after increasing age accumulates, leading to oxidative stress and eventually cell death. Dietary restriction is believed to slow down the metabolic rate in cells and it is not surprising that the SV, which contain cell types with high metabolism, is the structure that will benefit most from the dietary regime.

3.1.3 Choice of methods

In this study, the Preyer reflex test was chosen as the method to assess hearing status. The test is a crude, but fast method that can be performed in a silent room, without requirements of a sound proof box or complex instrumentation. The ABR method is a more sophisticated and precise measure. Due to a period of infectious disease at an adjacent section of the animal department, we were not allowed to move the animals to the laboratory for ABR-measurements as was the plan. Instead, the animals had to be in quarantine until the time for them to be sacrificed. The animals in this study showed no signs of infectious disease and were determined healthy by a veterinarian. To still gain

some functional information, we tested the Preyer reflexes (which could be performed on site). The Preyer reflex test is however, a proven, reliable test of hearing function, well documented in the literature [45, 46]. Lack of response can be caused by for example: conductive hearing loss, sensorineural hearing loss with degeneration of the sensory or neuronal cells or deterioration of the central auditory pathways. Thus, the reflex test reveals a combined response, which reflects the status not only from the peripheral organ but also from the central pathways.

Traditionally, investigators calculate hair cell and SGN loss in cochleae, using two separate methods for each cell type. If both cell types are to be studied, the cochlea from one ear is used for surface preparation and sensory hair cells loss is calculated using a cytocochleogram with a frequency positioning map, which can determine the locations of the cell loss along the cochlea. The cochlea from the other ear is prepared for histological mid-modiolar sectioning and the density of the SGN is determined for each of the mid-modiolar turns of the cochlea. In our study, we used the same cochlea for stereological estimations of the total amount of hair cells and SGN, as well as the volume of structures such as SV, and cells like SGNs. Also other cell types and structures could be estimated from the same specimen. Density measurements, which traditionally are used to estimate SGN status, are not fully comparable with total number estimations. Swelling or shrinkage of the Rosenthal´s canal, which hosts the SGN, can alter the density estimation without a necessary change of the cell number. The stereology method is thus a better tool to determine an actual cell population.
By using the stereology method, all cells within the complete cochlea have the same chance of being counted, not only the ones in the mid-modiolar region. The need for a precise sectioning angle is not necessary, as for the mid-modiolar sectioning method. This unbiased method is considered the gold standard for cell estimations and since the error of the method can be calculated from the total variation of the sample, an appropriate design can be set to optimize the method. As in most methods there are drawbacks, the relatively labor-intensive work is one. Neither can the actual location of specific cell loss be specified with this method, which is possible when using the surface preparation method. With the stereology method, only visual inspection could monitor the specific location for the cell loss and smaller local differences are thus difficult to identify. The operator must have the skills to differentiate the specific cell types to be counted, even in sections that not are mid-modiolar, which is another drawback. This could however be overcome by using immunohistochemistry labeling of specific cell types.

Spontaneous mitochondrial mutations and dysfunctions are believed to accumulate over years in aging cells and also in the cochlea. The mitochondrial deletions are considered to produce excess of ROS [55, 56]. With this fact in mind, we wanted to measure the rate of mitochondrial DNA deletions in cochlear tissues, to investigate if the DR animals obtained fewer mutations than the age matched AL animals. Cochleae from the

young animals served as controls. DNA was extracted from the cochleae and amplified by long-extension DNA polymerase chain reaction (LX- PCR). Unfortunately, a high rate of deletions were detected even in tissues from the young animals, probably due to degradation of the tissues during handling, thus we were unable to detect any differences. Another way of detecting mitochondrial deficiency is by an enzymatic staining of the mitochondrial enzyme cytochrome c oxidase (COX), which is a crucial enzyme-complex in the mitochondrial respiratory chain. The enzyme activity has been shown to decline by aging [56]. However also here, the methodology failed, probably due to degradation during decalcification of the bony shell surrounding the cochlea. The tissue needs to be soft before sectioning of the cochlea into slices can be performed. No enzymatic reaction from the decalcified tissues was detected.

3.1.4 Conclusion

With advancing age, the sensory hair cells and the metabolic structure SV were degenerated in the female Sprague-Dawley rat leading to a decline in the hearing function. SV was however preserved by a 70% restricted dietary regime which also maintained the hearing function. The preservation was statistically proven by estimating the complete volume of the coil-shaped structure with the stereological method. The structure was then studied in detail on cellular level by electron microscopy, and the degenerative changes were found to be less advanced in the DR animals. The SV is an important structure for maintaining the endocochlear potential of the inner ear fluids, and important for hearing function. Degeneration of SV is believed to be the cause of metabolic presbycusis. In this study, animals on dietary restriction demonstrated preserved strial tissue and a delayed progression of the age-related hearing loss, suggesting a maintained function of the endocochlear potential. To prove this hypothesis, the endocochlear potential in aging animals on different feeding regime, would be of interest to study.

The stereology method is for the first time in this study used and proven to function as a good method to simultaneously estimate total numbers and volumes of different cell types and structures within the cochlea. In the future, other cell types could be interesting to study, for example the different fibrocytes in the spiral ligament, which also are known to be degenerated by age, possibly before the strial degeneration [12]. To be able to identify the specific fibrocytes, an antibody labeling against cell specific proteins could be used in combination with the stereology method.

3.2 STUDY II - REPEATED MODERATE NOISE EXPOSURE IN THE RAT – A LIFE-TIME NOISE EXPOSURE MODEL

3.2.1 Repeated moderate noise exposures

Exposures at moderate intensity levels of 101 and 104 dB SPL could be repeated up to six times without any permanent hearing was detected. This was in stark contrast to the animal groups exposed to intensities levels of 107 and 110 dB SPL. These subjects

obtained a permanent hearing loss already after the first exposure. When examining the wave $1_{p\text{-}p}$ amplitudes, measured 5 weeks after each repeated exposure, significant reductions were found already after the first noise exposure for the group exposed to 101 dB SPL. Reductions of the amplitudes in the group exposed to 104 dB SPL on the other hand were not significant until after the 4th exposure. However, even in the non-exposed control group amplitudes were significantly reduced at the time point when ABR was measured after the 3rd exposure. Reduced amplitudes are an indicator of decline in neural activity, and as the control group also displayed reduced amplitudes, we concluded that the decline depended on age rather than noise exposure. The latencies of the ABR wave I were only prolonged in some of the later time-point for some frequencies in all intensity groups.

Interestingly, the small change of 3 dB in the intensity level made a dramatic difference in hearing thresholds; from a TTS following the 104 dB SPL exposures, to a PTS already after a single exposure at 107 dB SPL. However, as the dB scale is logarithmic, the actual sound pressure will increase by approximately 40% (from 3.17 to 4.48 Pa) after an increase of 3 dB SPL and would thereby partly explain the dramatic physiological change. Another possible explanation of the dramatic effect caused by an increase of a few dB (from 104 to 107 dB SPL) could be that other mechanisms are triggered by a louder noise. The TTS observed in the groups exposed to the milder noise (101 and 104 dB SPL) will mostly create a metabolic change for the cells, while the higher intensity levels (107 and 110 dB SPL) also causes mechanical damage. Further studies at the structural level would be beneficial for the understanding of the differences following repeated moderate noise exposures.

Other researchers have also studied the effects of repeated moderate noise exposures, with reduced ABR amplitudes even for animals with normal thresholds. In a study by Wang and Ren [57], CBA / CaJ mice were exposed three times to broadband noise at 100 dB SPL during 2 hours, two weeks apart. Already after the first exposure, which only gave a temporary hearing loss, the amplitudes were reduced. Only after the third exposure, the ABR threshold shifts became permanent. The reduced amplitudes detected after the first exposure, were explained as an early sign of degeneration of the cochlear nerve after a moderate noise exposure. This finding is in agreement with our study, were the amplitudes were significantly reduced already after a single exposure at 101 dB SPL, in animals displaying normal thresholds.

Reduced amplitudes have in the literature been associated with a structural loss of synaptic ribbons of the ihc. The synaptic ribbons are the connection site between the ihc and the peripheral axon of the SGN. During sound transmission, they transmit the release of the neurotransmitter glutamate to the afferent nerve. In another study, animals with normal hearing thresholds and intact amount of hair cells and SGN, a loss of synaptic ribbons was reported [58]. The loss was believed to reduce the connection

between the ihc and the cochlear nerve, leading to a decline in the nerve fibers, especially those that have a high threshold and a low spontaneous rate. The low spontaneous rate nerve fibers are believed to be an important structure for the possibility of hearing in noisy environments and the authors claim that the loss off synaptic ribbons can be an explanation for the poor frequency resolution in hearing among elderly people.

3.2.2 Noise susceptibility after repeated moderate noise exposures

The hypothesis that repeated moderate noise exposure would change the susceptibility for later acoustic overstimulation with a more intense noise was tested in the animals that maintained normal hearing thresholds after six moderate exposures. The animals previously exposed six times at either 101 or 104 dB SPL and the non-exposed groups were acoustically overstimulated during four hours, at 110 dB SPL using a narrowband noise. All animals received PTSs, but the thresholds for the animals previously exposed at 104 dB SPL were somewhat lower at all measured time points, compared to the animals in the other groups, and significantly lower after 2 weeks at 28 kHz. We could then conclude that a certain level of repeated noise exposures does lead to improved noise resistance. Repeated exposures at 104 dB SPL would thus be the ideal exposure level, to achieve the highest level of noise resistance for this strain of animals, when using this exposure set up.

There are two types of “training” models well documented in the literature named sound conditioning and toughening. By sound conditioning, animals are typically exposed to sound levels at 55-95 dB SPL for a longer period of time (days or weeks) which makes the animals more resistant to a later more intense noise exposure. By toughening, animals are exposed to noise giving TTSs in an interrupted time schedule over days or weeks. The thresholds, recorded directly after the exposures, are decreasing over time as a consequence of the repeated exposures. However neither of these methods fully resembles our model, as they either use lower intensities for the training noise, longer exposure durations or different intervals than in our study. Still, one can speculate, that the same mechanisms involved in sound conditioning or toughening are involved in our study.

It is however promising to find that repeated moderate exposures are not damaging for the hearing if the intensity level not is too high. The exposures can actually also improve the resistance to louder noise. Most likely, the sound exposures trigger an up-regulation of endogenous feed-back system that makes the ear more resistant to acoustic overstimulation. Systems that produce endogenous antioxidants or neurotrophic factors are indeed candidates that would be of interest to study in future experiments. Those are factors that are beneficial for the rescue of hearing.

3.2.3 Conclusion

In the present model, 104 dB SPL was the highest intensity level that could be used for repeated noise exposures without producing a PTS. This level can be used to mimic a human life-time exposure. At this level, the animals´ amplitudes also remained normal even after several exposures and their hearing were more resistant to noise after an acoustic overstimulation, both compared to lower level of previous exposures or without prior exposures.

Repeated moderate noise exposures are most likely not damaging for the hearing. Advantageous if the exposures are at the level and repetition rate where only metabolic changes occur, which are likely to trigger the endogenous rescue systems in the hearing organ. However, how the effect on age-related hearing loss will be is still unclear. Eventually, even moderate exposures will probably reduce the activity of the cochlear nerve, as indicated by the reduced amplitudes from the ABR recordings over time. Subjects will then experience age-related hearing difficulties as a result from the reduced neural activity.

4 SUMMARY AND FUTURE PERSPECTIVES

The major age-related histological degenerations found in the female Sprague-Dawley were a reduction of the total volume of SV together with sensory hair cell loss. When the end-point effect of a 70% dietary restriction was evaluated, a more preserved volume of the SV with a lower level of cellular degeneration was observed, as compared to age-matched animals fed *ad libitum*. The dietary restriction thus appears to slow down the cellular metabolism over life which is beneficial for the inner ear as well as for many organs of the body. The SV is the metabolic site of the inner ear, rich in blood vessels and essential for the ionic balance of the different fluid compartments of the inner ear. The slow-down in metabolic rate, that the dietary restriction entails, will preserve the strial cellular structures and delay the age-related hearing loss.

Dietary restriction is one way of challenging the onset, severity and progression of age-related hearing loss, other aspects as noise exposures earlier in life, genetic background etc. also influence the disability. Here we developed a model to mimic life time noise exposure in a rat model. The animals could repeatedly be exposed, with a repetition rate of six weeks interval, at the optimal intensity level of 104 dB SPL. Interestingly, repeated moderate exposure at this non-damaging level produced a protective effect that made the animals more resistant to overstimulation of a more intense noise.

To gain a better understanding of the aging processes in the hearing organ we have now the tools to evaluate the outcome of different therapies for age-related hearing loss in an animal model. In the future we will be able to study long-terms effect of life-time exposure on age-related hearing loss under more realistic conditions with moderate exposures over a life-time. With this model, we can continue studying intervention therapies. Dietary restriction is probably the only proven approach to prolong life and lower the incidence of age-related diseases such as age-related hearing loss. Anyhow, most people are not willing to reduce their food intake by 30%, therefore other intervention therapies will be interesting to study, which could have the same effect on hearing and/or trigger the same protective mechanisms as with the dietary restriction. The antioxidants; vitamin A, C and E, together with the antioxidant and vasodilator Magnesium, are currently used in our laboratory, given as a food supplement to rats that will be exposed to life-time exposures and grow old. This combination of antioxidants and vasodilator has in previous studies been shown to lower the hearing threshold shifts after damaging noise. Now, the antioxidant impact on age-related hearing loss after a life-time exposure will further be investigated with the aim to find a therapy that delays the age-related hearing loss and prevents poor frequency resolution in the elderly population.

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