ANNI HERRANEN

TRAUMA-INDUCED CELLULAR STRESS SIGNALLING AND
HAIR CELL DEATH IN THE COCHLEA

MOLECULAR AND INTEGRATIVE BIO SCIENCES PROGRAMME
FACULTY OF BIOLOGICAL AND ENVIRONMENTAL SCIENCES
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UNIVERSITY OF HELSINKI
TRAUMA-INDUCED CELLULAR STRESS SIGNALLING AND HAIR CELL DEATH IN THE COCHLEA

Anni Herranen

Molecular and Integrative Biosciences Research Programme
Faculty of Biological and Environmental Sciences
University of Helsinki

Doctoral Program Brain and Mind
Doctoral School of Health Sciences

DOCTORAL DISSERTATION

To be presented for public examination with the permission of the Faculty of Biological and Environmental Sciences of the University of Helsinki, in Auditorium 4 at Metsätalo, on the 5th of June, 2020 at 10 o’clock.

Helsinki 2020
Cover image: Whole mount preparation of a murine (CBA/Ca mouse) noise-exposed cochlea. Noise exposure was given for 2 h at 105 dB sound pressure level and the sample was collected 6 days after the exposure. Myosin7a staining shows the hair cells of the cochlea. Some outer hair cells have been lost in the basal, high frequency region of the cochlea.
La fixité du milieu intérieur est la condition de la vie libre.

To be or not to be, that is the question.
W. Shakespeare, “Hamlet”, 1604
## List of Original Publications

VII

## Abbreviations

VIII

## Abstract

IX

## Tiivistelmä

XI

## 1 Introduction

1

## 2 Review of the Literature

4

### 2.1 Compartments of the inner ear and their function

4

#### 2.1.1 Organ of Corti

5

#### 2.1.2 IHC versus OHC

8

#### 2.1.3 Stria vascularis and cochlear fluids

10

#### 2.1.4 Spiral ligament fibrocytes, root cells, and the gap junctional system

12

#### 2.1.5 Blood circulation, blood-labyrinth barrier, and immune cells in the cochlea

13

#### 2.1.6 Spiral ligament

14

#### 2.1.7 Spiral ganglion

14

#### 2.1.8 Vestibular labyrinth

16

### 2.2 Stress signalling networks and cell death in the hearing organ

16

#### 2.2.1 Various cellular functions of JNK/c-Jun and ERK1/2 stress signalling pathways

17

#### 2.2.2 Multifunctional NF-κB orchestrating cellular events

19

#### 2.2.3 Cell death

20

#### 2.2.4 ER stress and UPR

22

#### 2.2.5 ROS in physiology and pathophysiology

24

#### 2.2.6 Inflammation

26

#### 2.2.7 Systemic glucocorticoids in local stress responses

27

#### 2.2.8 Circadian rhythms

29

### 2.3 Studying hearing loss with mouse models

30

#### 2.3.1 Mouse as a man

30

#### 2.3.2 Mouse strain differences in hearing research

31

#### 2.3.3 Age-related hearing loss

32

#### 2.3.4 Noise vulnerability

33

#### 2.3.5 Ototoxic drug-induced lesion

35

### 2.4 Summary of the literature review

35
3 AIMS OF THE STUDY ........................................................................................................ 37

4 MATERIALS AND METHODS .................................................................................. 38

4.1 List of used methods ................................................................................................. 38

4.2 Mouse lines ........................................................................................................... 38
  4.2.1 Genotyping ......................................................................................................... 39

4.3 Noise exposure and ABR measurements ............................................................... 40

4.4 Kanamycin-furosemide and LPS applications ....................................................... 40

4.5 Immunohistochemistry ......................................................................................... 41

5 RESULTS AND DISCUSSION .................................................................................... 44

5.1 Trauma-induced stress responses in the organ of Corti (I,II) .............................. 44
  5.1.1 c-Jun phosphorylation in the traumatized organ of Corti ................................. 44
  5.1.2 ERK1/2 phosphorylation in the traumatized organ of Corti ......................... 47
  5.1.3 Transcriptional NF-kB activity is absent from the noise-exposed organ of Corti .... 48
  5.1.4 Systemic inflammation does not raise stress responses in the organ of Corti ........ 48

5.2 Cochlear stress outside of the organ of Corti (I,II,III) ........................................ 49
  5.2.1 Trauma-induced ERK1/2 and c-Jun phosphorylation in the spiral ligament ....... 49
  5.2.2 NF-kB, FOXO3a, and macrophages in the traumatized spiral ligament .......... 53
  5.2.3 Summary of spiral ligament stress responses .................................................... 54
  5.2.4 Stress responses in the stria vascularis ............................................................ 55
  5.2.5 Spiral limbus stress responses ........................................................................... 56
  5.2.6 Stress signalling in the spiral ganglion neurons .............................................. 57
  5.2.7 The other hair cells of the inner ear ................................................................. 57

5.3 ER stress and OHC loss in the Manf inactivated cochlea (III) .............................. 58

5.4 Mouse strain differences (II, III) ........................................................................... 61
  5.4.1 Mouse strain differences in noise vulnerability and noise-induced stress responses 61
  5.4.2 Mouse strain background influences the phenotype of Manf inactivation ............ 65

5.5 Summary of the cochlear stress responses in different trauma paradigms ............ 68
  5.5.1 The role of trauma-induced stress responses in the cochlea ......................... 69
  5.5.2 Supporting cells as harbingers of OHC death? ............................................. 71
  5.5.3 Therapeutical future perspectives ................................................................. 72

6 CONCLUDING REMARKS ......................................................................................... 74

ACKNOWLEDGEMENTS ............................................................................................. 75
LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following articles, which are referred in the text by their Roman numbers.


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The thesis also contains unpublished results.

Author contribution:

In article I, AH participated in planning and executing the experiments, analysing the results and writing the manuscript together with TA and UP. The star (*) refers to equal contribution.

In article II, AH did all the experiments and analysis, and designed and wrote the article with UP.

In article III, AH did all the experiments and analysis, and designed and wrote the article with UP.
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ABC</td>
<td>avidin-biotin complex</td>
</tr>
<tr>
<td>ABR</td>
<td>auditory brainstem response</td>
</tr>
<tr>
<td>AHL</td>
<td>age-related hearing loss</td>
</tr>
<tr>
<td>AP-1</td>
<td>activator protein 1</td>
</tr>
<tr>
<td>ATF6</td>
<td>activating transcription factor 6</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BL6</td>
<td>C57BL/6J mouse strain</td>
</tr>
<tr>
<td>BLB</td>
<td>blood-labyrinth barrier</td>
</tr>
<tr>
<td>CBA</td>
<td>CBA/Ca mouse strain</td>
</tr>
<tr>
<td>CDH23</td>
<td>cadherin 23</td>
</tr>
<tr>
<td>CHOP</td>
<td>C/EBP homologous protein</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EP</td>
<td>endolymphatic potential</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>ERAD</td>
<td>endoplasmic reticulum (ER)-associated degradation</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>FOXO3a</td>
<td>forkhead box class O 3a</td>
</tr>
<tr>
<td>GFI1</td>
<td>growth factor independent 1 transcriptional repressor</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>GRP78</td>
<td>78 kDa glucose-regulated protein (aka. binding immunoglobulin protein BiP)</td>
</tr>
<tr>
<td>HPA</td>
<td>hypothalamus-pituitary-adrenal gland</td>
</tr>
<tr>
<td>HSP</td>
<td>heat shock protein</td>
</tr>
<tr>
<td>IBA1</td>
<td>ionized calcium binding adaptor molecule 1</td>
</tr>
<tr>
<td>IHC</td>
<td>inner hair cell</td>
</tr>
<tr>
<td>IL</td>
<td>interleukine</td>
</tr>
<tr>
<td>IRE-1α</td>
<td>inositol-requiring protein 1α</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
</tr>
<tr>
<td>KO</td>
<td>knock-out</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>MAM</td>
<td>mitochondria-associated membrane</td>
</tr>
<tr>
<td>MANF</td>
<td>mesencephalic astrocyte-derived neurotrophic factor</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen activated protein kinase</td>
</tr>
<tr>
<td>MYO7a</td>
<td>myosin 7a</td>
</tr>
<tr>
<td>NF-κB</td>
<td>nuclear factor κB</td>
</tr>
<tr>
<td>OHC</td>
<td>outer hair cell</td>
</tr>
<tr>
<td>PDI</td>
<td>protein disulfide-isomerase</td>
</tr>
<tr>
<td>PERK</td>
<td>protein kinase RNA-like ER kinase</td>
</tr>
<tr>
<td>PVM</td>
<td>perivascular macrophage-like cell</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>S</td>
<td>serine</td>
</tr>
<tr>
<td>SG</td>
<td>spiral ganglion</td>
</tr>
<tr>
<td>SPL</td>
<td>sound pressure level</td>
</tr>
<tr>
<td>T</td>
<td>threonine</td>
</tr>
<tr>
<td>TNFα</td>
<td>tumor necrosis factor α</td>
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<tr>
<td>UPR</td>
<td>unfolded protein response</td>
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ABSTRACT

Cochlea is the peripheral receptive component of the auditory system. It contains two types of hair cells, inner and outer hair cells (IHCs and OHCs respectively). IHCs are the major receptive cells that convey auditory signals through spiral ganglion neurons to higher auditory pathways. OHCs amplify the signals. OHCs are also the most vulnerable cell type of the cochlea. OHC death leads to irreversible hearing impairment, because these cells are not regenerated in the mammalian cochlea. Thereby, prevention of OHC death would protect hearing. However, there are multiple cell death modes and if one mode is antagonized, another one might be activated. Therefore, intracellular events, namely stress responses, taking place prior to cell death are potential targets for preventing cell death. In this thesis the studied trauma paradigms are noise exposure, ototoxic drugs, ageing and systemic inflammation. The aim is to understand mechanisms leading to OHC death by using different mouse models.

We have studied stress-induced mitogen activated protein kinase signalling, c-Jun and extracellular signal-regulated kinase (ERK) 1/2 activation in the cochlea. In addition, we have studied nuclear factor κB (NF-κB) in different trauma paradigms as well as cochlear macrophages in noise exposure. In these studies, trauma paradigms led to the death of OHCs, but the stress responses - the activation of stress signalling networks - took place in the non-sensory, supporting cells and IHCs in the cochlea. As these cells did not die in the used trauma paradigms, we studied if their stress responses influence OHC survival. Though the full mechanism is still not known, we were able to show that noise-induced c-Jun phosphorylation promotes OHC death in a paracrine manner in the cochlea.

We have also studied the role of mesencephalic astrocyte-derived neurotrophic factor (Manf) inactivation in the cochlea. First, we showed that Manf is expressed in the cochlea in neurons, hair cells and certain non-sensory supporting cells. MANF is an unconventional neurotrophic factor that has a role in regulating endoplasmic reticulum (ER) homeostasis. Inactivation of Manf led to progressive OHC loss and hearing loss after the onset of hearing in mice. We linked this OHC death to ER stress activated pro-apoptotic unfolded protein response.

Furthermore, we have studied the effects of genetic background on noise-induced stress signalling and on the phenotype of Manf inactivation. In these studies, we used the inbred C57BL/6J (BL6) and CBA/Ca (CBA) mice as well as the outbred CD-1 (ICR) mouse line. Noise exposure at young age causes fourfold greater OHC loss in CBA compared with BL6 mice. We noticed that also the noise-induced spiral ligament stress response, namely c-Jun phosphorylation in fibrocytes, was only activated in CBA mice. Spiral ligament fibrocytes
Abstract

have important roles in ion transfer in the cochlea. They are likely to contribute to the maintenance of the high endolymphatic potential needed for hearing function. However, the spiral ligament stress response alone does not lead to OHC death. Older, noise-induced hearing loss resistant CBA mice did not display OHC loss, but the spiral ligament stress response was comparable to the younger, noise-vulnerable mice.

We observed a genetic background dependence on the phenotype of Manf inactivation as well. Manf inactivation in hybrid CD-1 and CBA mouse background resulted in two distinct groups: one with robust OHC loss and hearing impairment and another that was comparable to wildtypes in both OHC survival and hearing. In BL6 background, Manf inactivation in the cochlea always led to significant OHC loss and hearing loss. We concluded that Manf inactivation causes OHC loss only if combined to other genetic defects, most likely causing ER dysfunction since MANF has a role in the maintenance of ER homeostasis. These genetic modifications are present in BL6 and CD-1, but not in CBA mice.

To conclude, in this thesis, the role of trauma-induced stress responses in OHC survival is discussed. We have studied stress signalling networks in different cochlear compartments with histological methods. We found that stress signalling pathways have physiological roles in normal cellular metabolism. They are also upregulated upon traumas and can promote either cell death or survival, depending on the context, in cell intrinsic or extrinsic manners.


1 INTRODUCTION

For over 100 years, it has been known that loud sounds cause the death of cochlear hair cells (reviewed by Thurston, 2013), but still today the mechanisms and ways to prevent the death elude us. Thereby studying the cellular mechanisms in the traumatized cochlea is important. It is vital to understand the complexity of the events since a trauma induces a whole array of signalling cascades which affect each other, and the synergy of these pathways can result in a unique outcome.

Coordinated cell death is needed in adult tissues. For example, the regeneration of the cells in the lining of the gut or blood cells, which are renewed continuously, requires the death of the old cells. However, cell death can also be detrimental and measures to prevent it are needed. This is the case with the mammalian auditory sensory cells that are unable to regenerate after trauma. Loss of auditory sensory cells leads to hearing loss for which no efficient cure is currently available (reviewed by Roccio et al., 2019). Various cell death pathways are known, and therefore multiple potential targets for inhibiting cell death pathways exist. However, as new cell death mechanisms continue to be recognized, the complete picture of cell death is not yet understood. Moreover, preventing a controlled cell death pathway from proceeding can lead to a worse outcome: an uncontrolled cell death where the death of one cell harms the other cells of the tissue. Therefore, it is desirable to prevent the cell from activating the cell death pathways before the cell has advanced beyond the point of no return to survival and normal function (reviewed by Green and Llambi, 2015). The trauma-induced cellular events taking place prior to the activation of cell death pathways include the activation of stress signalling pathways. On the other hand, stress signalling is also fundamental to life, because it ensures the survival of cellular function in a changing environment. Also, many stress signalling pathways have other physiological roles in a cell as well, such as regulating growth and development (Klemke et al., 1997; reviewed by Cuenda and Rousseau, 2007; Waetzig et al., 2017). More information on the mechanisms of stress signalling is needed to prevent cell death by antagonizing stress signalling pathways. In addition, the thresholds between physiological and pathophysiological signalling should be defined.

In a multicellular organism cells form complex tissues and organs. For an organism to function as an entity, communication between cells is essential. This communication can be seen in the signalling networks of individual organs, such as the hearing organ, the cochlea. The cochlea has a fine cellular architecture, comprising multiple cell types, and functions as the peripheral unit to convey auditory cues to the central nervous system for hearing sensation. Even though the sensory receptor cells, the hair cells, are central to hearing, they could not
fulfill their function without various non-sensory epithelial and mesenchymal supporting cell types and neurons. Furthermore, cochlea is not isolated from the rest of the body. Systemic effectors, such as hormonal control, reach the cochlea through blood circulation. Cochlea is sensitive to many stressors such as noise, ototoxic lesion, and ageing. These traumas lead to stress responses in the form of activated stress signalling pathways in many of the different non-sensory cell types of the cochlea (reviewed by Monzack and Cunningham, 2013 and Francis and Cunningham, 2017). However, the most vulnerable cells are the hair cells. Little is known about the relationship between hair cell death and the stress responses of the cochlear non-sensory cells.

Traumas can activate multiple stress signalling pathways. Thereby a network of stress signalling decides the fate of an individual cell. It is noteworthy that the same response can promote either survival or death, depending on the situation. As an example, reactive oxygen species (ROS) signalling on the one hand has a physiological role and on the other a cell death promoting role. Low levels of ROS are needed for normal homeostasis. However, for example in inflammation, too low levels of ROS cause immunosuppression whereas too high levels lead to autoimmunity. Thereby, if ROS levels are lowered with drugs from the homeostatic level, immunosuppression is promoted (reviewed in Scieber and Chandel 2014). Together, the outcome of stress signalling in traumas is context-dependent.

The house mouse (Mus musculus) is used as a common model animal for cell death and stress signalling research. Mice have gained the leading role also in hearing research thanks to the genetic tools that allow the generation of mutant mouse models. The use of inbred mouse strains has diminished the genetic variability of mice between laboratories and hence reduced the variability of results. However, the phenotypes of inbred mouse strains can differ significantly. For example, age-related hearing loss begins at very different time points in different strains (reviewed by Ohlemiller et al., 2016). Because mouse strains differ significantly, they also have great impact on the results of the studies, and the discoveries might be mouse strain-specific (Johnson et al., 2006; Ohlemiller and Gagnon 2007; Ohlemiller et al., 2018). Together, genetic background dictates the mechanisms available for survival from trauma-induced stress.

This thesis aims to unravel physiological and pathophysiological roles of stress signalling in the traumatized cochlea. Three different acute traumas were used to study stress responses: noise exposure, ototoxic lesion with kanamycin-furosemide application, and systemic inflammation caused by bacterial cell wall component lipopolysaccharide (LPS). Chronic stress was assessed by ageing and genetic mutation models. Even though these traumas mainly cause the death of auditory hair cells, stress responses have been studied in the whole cochlea comprising many different cell types. A trauma-induced stress response involves a whole
network of signalling pathways of which mitogen activated protein kinase (MAPK) pathways c-Jun N-terminal kinase (JNK)/c-Jun and extracellular signal-regulated kinase (ERK1/2), nuclear factor κB (NF-κB), and endoplasmic reticulum (ER) stress were studied here. Many of these pathways have physiological functions in the cochlea and are not only activated upon traumas. In addition, the influence of the genetic background, both in trauma-induced stress signalling and in mutant mouse models, was studied. The underlying theme is that stress signalling is more than just a sentence to death since, in addition to pathophysiology, stress signalling also has important physiological functions (Fig 1). Figure 1 summarizes how a trauma-induced stress response, the activation of intracellular stress signalling, can lead to survival or cell death. Many factors, such as genetic background or the synergy of all the stress signalling pathways activated, influence the outcome.

Figure 1. A schematic picture representing the outcome, survival or death, of trauma-induced stress responses and some factors influencing the outcome.
2 REVIEW OF THE LITERATURE

Cochlea functions as the model tissue throughout this thesis. In order to understand stress responses in the cochlea, the different compartments of the inner ear and their function and vulnerability in traumas are described. Then, some intracellular stress signalling pathways and their role in physiology and hearing loss are addressed in detail. Systemic stressors, such as inflammation and glucocorticoids, influence the outcome of stress responses in the cochlea. Lastly, some aspects of the use of mice as an animal model for hearing loss caused by noise, ageing or ototoxic drugs focusing especially on inter-mouse strain differences are considered.

2.1 COMPARTMENTS OF THE INNER EAR AND THEIR FUNCTION

The inner ear consists of the hearing organ, the cochlea, and the balance organs, the vestibular labyrinth (Fig 2). It is surrounded by the temporal bone. The inner ear is connected to the central nervous system via the 8th cranial nerve. The middle ear ossicles – malleus, incus and stapes – connect the ear drum, the tympanic membrane, to the inner ear and transmit sound waves travelling through the air into the mechanical movement of the cochlear fluids. The movement of the fluid will frequency-specifically activate the mechanosensory auditory hair cells, which will transform the mechanical movement of their stereocilia into electrical signals through mechanotransduction. This electrical signal travels through different nuclei in the brainstem, where the auditory information is processed, and ends up in the auditory cortex where hearing sensation is in the end acknowledged.


2.1.1 ORGAN OF CORTI
The organ of Corti is the sensory epithelium of the mammalian cochlea. It is responsible for receiving the auditory input and transmitting this signal in an electrical form to the central auditory pathways. It consists of several different cell types which lie on top of the basilar membrane (Fig 3). The apical parts of the cells in the organ of Corti face the high potassium ($K^+$) concentration of the endolymph in the scala media while the rest of the cells are bathed by the perilymph which has a more conventional ionic composition of an extra-cellular fluid (reviewed by Wangemann, 2006). There are two types of sensory receptor cells, one row of inner hair cells (IHCs) and three rows of outer hair cells (OHCs). The hair cells are named after the stereociliary bundle at their apical end. The stereocilia do not contain microtubules but an actin cytoskeleton. An auditory signal stimulates the basilar membrane to move towards the tectorial membrane, making the stereocilia bend (reviewed by Fettiplace, 2017).

The stereocilia of a hair cell are interconnected via tip links. Tip links open the mechanotransduction channels in the stereocilia due to bending-induced tension when the hair cell is activated. The mechanotransduction channels are large ion channels permeable to cations. Tip links consist of cadherin 23 (CDH23) and protocadherin 15 (Kazmierczak et al., 2007). The $K^+$-rich endolymph flows inside the hair cells through the mechanotransduction
channels in the stereocilia depolarizing the hair cell. Depolarization opens voltage-gated calcium (Ca\(^{2+}\)) channels and the ensuing Ca\(^{2+}\) current causes the cell to release neurotransmitter glutamate that activates the interconnected neurons. K\(^{+}\) ions leave the hair cell at the basolateral end of the cells following their concentration gradient into the K\(^{+}\) poor perilymph. Thus, no energy expenditure is needed in the hair cells. Instead, stria vascularis, the cochlear component maintaining the ionic composition of endolymph, uses adenosine triphosphate (ATP) to pump K\(^{+}\) into the endolymph against its concentration gradient (reviewed by Fettiplace, 2017).

The organ of Corti is tonotopically arranged so that the sensory cells, the hair cells and the neurons, responsible for low frequency sounds are found in the apex of the spiraling cochlear duct and the cells receiving high frequency information reside in the basal part of the cochlea. IHCs are responsible for transducing the auditory stimuli while OHCs amplify the signals. Hair cells are surrounded by different types of supporting cells that keep these cells in the right orientation for auditory function (Taylor et al., 2012). The supporting cells serve also other supportive roles such as the clearance of extracellular glutamate from the synaptic cleft (Furness and Lawton, 2003). Inner and outer pillar cells form the tunnel of the organ of Corti that separates IHCs and OHCs. Deiters’ cells form a cup structure beneath each OHC and extend to the apical surface of the organ of Corti. Deiters’ cells participate in the formation of the reticular lamina that prevents endolymph leakage into the sensory epithelium (Taylor et al., 2012). In addition, a thick glyocalyx protects hair cells and supporting cells from the damaging effects of high K\(^{+}\) by forming a filamentous layer that is six times thicker on the endolymphatic side compared with the perilymphatic side (Prieto and Merchan 1986). Deiters’ cells have also important roles in OHC death, when they rapidly close the surface breach formed as an OHC dies. An actin-rich scar develops at the place where the OHC died. Deiters’ cells also phagocytose the remains of the dying OHCs (Anttonen et al., 2014; Hirose et al., 2017). However, an intense noise exposure can cause irreversible damage in the organ of Corti by making holes in the reticular lamina after noise exposure (Bohne and Rabbitt, 1983; Ohlemiller et al., 2018).

Lateral to each side of the organ of Corti are supporting cells that based on their protein composition have important roles in ion movement. Towards the modiolus, the central part of the cochlea where spiral limbus and spiral ganglion (SG) neurons are located, starting from the IHCs, there are inner sulcus cells whereas towards the lateral wall from the OHCs there are several supporting cell types: Hensen’s, Boettcher, Claudius, and outer sulcus cells. Hensen’s cells display dynamic movement towards the center of the cochlea after intense noise exposure, studied in guinea pigs (Flock et al., 1999). These cells are found next to the third row of OHCs. Boettcher cells are found above the basilar membrane, beneath the
Claudius cells. Boettcher cells are only present in the basal part of the cochlear spiral responsible for high frequency sounds (Kanazawa et al., 2004).

The supporting cells in the organ of Corti are interconnected by a gap junctional system while hair cells are excluded from this network (Jagger and Forge, 2015). This connectivity extends to the interdental cells of the spiral limbus and root cells of the lateral wall. The gap junctional syncytium provides a large volume for buffering intracellular changes, as well as necessary nutrients and waste removal for the metabolically active organ of Corti (reviewed by Kikuchi et al., 2000 and Wangemann, 2006). This is important, because only a small capillary remains in the organ of Corti after development. Most of the blood circulation of the cochlea occurs in the lateral wall.

Cochlea can be damaged by many different traumas, for example noise exposure, ototoxic drugs, and ageing. Following these stressors, the most sensitive cell type in the organ of Corti is the OHCs, especially the OHCs residing in the basal part of the cochlea. After OHC death, a flat epithelium, which consists of less-differentiated columnar cells, replaces the complex architecture of the organ of Corti (Taylor et al., 2012). Stereocilia are sensitive to noise exposure as well, and even though the treadmilling of stereocilia components is a controversial issue, at least parts of stereocilia are regenerated in mammalian cochlear hair cells (Schneider et al., 2002; Rzadzinska et al., 2004; Zhang et al., 2012a). Another sensitive part of the organ of Corti is the ribbon synapses between the IHCs and SG neurons. These have been shown to be the most vulnerable element of the cochlea in response to loud sounds (Kujawa and Liberman, 2009). Hair cells are not regenerated in the mammalian cochlea after trauma while the stereocilia damage can be repaired up to a certain point (Roberson and Rubel, 1994; Zhao et al., 1996). It is believed that ribbon synapse loss, auditory synaptopathy, is irreversible, but now evidence exists that ribbon synapses can be remade after trauma in certain cases (Kaur et al., 2019).
2.1.2 IHC VERSUS OHC

The sensory receptor cells of the organ of Corti, IHCs and OHCs, are distinctly different from each other both in their surroundings and function. The IHCs contain long stereocilia and are surrounded by inner border, inner phalangeal, and inner pillar cells. The fundamental function of IHCs is to transduce the mechanical auditory signal into electric signals which are
transmitted through the SG neurons into the central auditory pathways. On the other side of the tunnel of Corti, OHCs function as the amplifier for these signals, and each OHC has only one supporting cell beneath it, the Deiters’ cell that forms the cup structure where the OHC lies. Each IHC contains several synaptic ribbons, 10-18, depending on the tonotopical location of the IHC (Kujawa and Liberman, 2009). Each of these glutamatergic ribbon synapses connects to one SG neuron. OHCs also contain ribbon synapses, but only 2-4 per cell. A synaptic ribbon is an electron-dense structure in the basal part of the hair cell. This structure tethers several neurotransmitter glutamate-containing vesicles. During mechanotransduction, Ca$^{2+}$ flowing into the cells through voltage-gated channels causes the release of the glutamate-filled vesicles. The structure of synaptic ribbons allows the continuous and faithful activation of the synapse needed for constant hearing function (Jean et al., 2018). Ribbon synapses are the most sensitive structure of the cochlea, for example in noise exposure where the number of ribbon synapses is reduced before hair cell death occurs (Kujawa and Liberman 2009).

The three rows of OHCs reside on the lateral side of the tunnel of Corti. OHCs are directly under efferent neuronal control, whereas in IHCs efferent neurons contact the postsynaptic densities of the afferent dendrites of the SG neurons (reviewed by Vetter, 2015). In contrast to the IHCs, OHCs contain a motor protein prestin in their basolateral cell membrane. This makes the OHCs capable of movement needed for the amplification of auditory signals (Dallos et al., 2008; Liberman et al., 2004). The intracellular structure of OHCs also differs from IHCs. In OHCs, the ER and mitochondria are concentrated in the “neck” -region of the cell, above the nucleus and below the cuticular plate (Mammano et al., 1999; reviewed by Fettiplace and Nam, 2019). In IHCs, these cellular organelles are distributed more evenly around the cell (Bullen et al., 2015). The tips of the highest row of OHC stereocilia are embedded in the tectorial membrane while the IHC stereocilia are not firmly attached to the tectorial membrane (Andrade et al., 2016).

OHCs are also more vulnerable in many different traumas than IHCs, such as noise exposure, exposure to ototoxic drugs, or ageing. Further, it is the high frequency OHCs in the basal part of the cochlear duct that are the most vulnerable and typically die first. Intriguingly, the high frequency OHCs are the ones to die even upon low frequency noise exposures (reviewed by Fettiplace and Nam, 2019). Still, it is good to keep in mind that even though the IHCs do not die easily in traumas, they are prone to synaptopathy, the loss of ribbon synapses, which affects hearing function (Kujawa and Liberman, 2009). It has been suggested that intracellular Ca$^{2+}$ dyshomeostasis in OHCs may contribute to the vulnerability of the high frequency OHCs. Ca$^{2+}$ current through mechanotransduction channels is approximately three-times higher in the high frequency OHCs than low frequency OHCs. In addition, about half of the channels are constantly open in OHCs irrespective of tonotopical location. OHCs in in
the basal part of the cochlear duct are shorter than in the apical part, hence the cytosolic calcium buffering volume is smaller in the base. There is no tonotopical variation in the $\text{Ca}^{2+}$ currents in IHCs and the resting open probability is lower than in OHCs. Important players in the intracellular $\text{Ca}^{2+}$ homeostasis in hair cells are mitochondria, ER, and calcium buffer proteins, and differences in the function or concentration of these factors could explain differences in the vulnerability of hair cells (Fridberger et al., 1998; reviewed by Fettiplace and Nam, 2019). To conclude, IHCs and OHCs differ in many aspects, which is not surprising considering that their roles in hearing are also distinctly different. However, some of these differences could provide an explanation as to why OHCs are more vulnerable to traumas.

2.1.3 STRIA VASCULARIS AND COCHLEAR FLUIDS

The stria vascularis is located in the lateral wall of the cochlea (Fig 3). It consists of basal, intermediate, perivascular macrophage like melanin-containing cells and marginal cells and hosts a dense network of capillaries (Fig 4A,B). Stria vascularis is the powerhouse of the cochlea, because it maintains the high voltage and $\text{K}^+$ rich content of the endolymph. Endolymphatic potential (EP) is the driving force of mechanotransduction in hair cells. In a normal cochlea EP is approximately 100 mV. The endolymph is a very unusual extracellular fluid as its composition is closer to an intracellular fluid. It has high $\text{K}^+$ and bicarbonate ($\text{HCO}_3^-$) concentration, whereas the concentration of $\text{Ca}^{2+}$, sodium ($\text{Na}^+$), and proteins is low. Endolymph is found in the scala media while perilymph bathes the scala tympani and vestibuli (reviewed by Wangemann, 2006). The high $\text{K}^+$ concentration of the endolymph is toxic to the basolateral side of the OHCs (Zenner et al., 1994; Ahmad et al., 2003). Thereby it is important that the various cell types lining the scala media keep the epithelial barrier impermeable to endolymph.

The mammalian endolymphatic sac is a non-sensory structure of the inner ear that participates in the regulation of endolymph volume and pressure, the immune defense of the inner ear and removal of cellular waste from the endolymph. Instead of EP of 100 mV in the cochlear endolymph, the EP is 8-15 mV in the endolymphatic sac (reviewed by Thalmann and Thalmann, 1999 and Köppl et al., 2018). The high $\text{K}^+$ concentration in the cochlear endolymph is produced locally since even though the same the fluid space is shared with the vestibular labyrinth, the endovestibular potential is only 8 mV as in the endolymphatic sac (Marcus et al., 1994).

Marginal cells and vestibular dark cells secrete $\text{K}^+$ into the endolymph. The generation of EP was long thought to be generated by marginal cells. Now it is known that KCNJ10 (potassium inwardly rectifying channel subfamily J member 10, also known as Kir4.1) $\text{K}^+$
channel in the intermediate cells is the main factor generating EP. Marginal cells have an indirect role in EP generation, as they maintain the low K⁺ concentration in the intrastrial space. EP is generated across the basal cell barrier, not marginal cells. Intermediate cells are interconnected to basal cells, and the spiral ligament fibrocytes, through gap junctions whereas marginal cells are not part of the syncytium (Marcus et al., 2002).

A transient drop in EP is observed following noise exposure. It coincides with a temporary threshold shift caused by the noise exposure (Hirose and Liberman, 2003). EP drop can lead to OHC death that proceeds from base to apex along the cochlear duct. Notably, IHC survival is not sensible to EP drop (Liu et al., 2016). Noise exposure can also cause morphological damage to the stria vascularis. Transiently swollen stria vascularis has been observed following exposure to damaging noise levels (Hirose and Liberman, 2003). Thinning of the stria vascularis has also been associated to age-related hearing loss (Hequembourg and Liberman, 2001). Together, a functional stria vascularis is essential for normal hearing function and for OHC survival.

![Figure 4. Cochlear lateral wall vasculature and immune cells. Schematic pictures show in red the cochlear regions of stria vascularis and spiral ligament where the whole mount preparations have been taken from. Lateral wall blood vessels stained with PECAM-antibody in the stria vascularis (A) and spiral ligament respectively (C). The perivascular macrophage-like cells in the stria vascularis in an unstained light-microscopy image (B). These cells contain melanin, which is seen in brown (arrowhead). Erythrocytes can be observed in the capillaries. Tissue-resident macrophages (arrowhead) in the spiral ligament (D) are stained with ionized calcium binding adaptor molecule 1 (Iba1)-antibody. Scale bar in A is 50 µm for A; 22 µm for B; 90 µm for C; 60 µm for D.](image-url)
2.1.4 SPIRAL LIGAMENT FIBROCYTES, ROOT CELLS, AND THE GAP JUNCTIONAL SYSTEM

In addition to the stria vascularis, the cochlear lateral wall is comprised of fibrocyte types I-V and root cells (Fig 3). Each of these cell types has their distinct roles in the cochlea and are specialized in ion trafficking based on their molecular content. Root cells combine the organ of Corti to the lateral wall reaching from Claudius cells to type II fibrocytes (Jagger and Forge, 2013). The fibrocyte types differ in the region where they reside, in their morphology, and in their enzyme contents. Type I fibrocytes reside in the spiral ligament lateral to the stria vascularis while type II fibrocytes are found from the lateral wall towards the scala tympani side below type I cells. Type III fibrocytes line the most lateral side of the spiral ligament next to the temporal bone. Type I and III fibrocytes contain carbonic anhydrase and not Na,K-ATPase, while type II fibrocytes have the opposite composition. Both carbonic anhydrase and Na,K-ATPase contribute to ion movements (Spicer and Schulte, 1991). Type IV fibrocytes reside in the region next to the basilar membrane. Type V fibrocytes, which are found next to the conjunction of the Reissner’s membrane and the lateral wall, have been associated to the regulation of cochlear blood flow having pericyte-like abilities. They reside in the area where arterioles branch into precapillaries and into the two capillary networks of the lateral wall: stria vascularis and spiral ligament (Dai and Shi, 2011).

The lateral wall fibrocytes are interconnected with a gap junctional system. The gap junctional system has been associated with ion recycling from perilymph to endolymph (reviewed by Kikuchi et al., 2000). Participation of the gap junctional system to the dispersal of second messengers, such as ATP or Ca$^{2+}$, in the cochlea has also been suggested (reviewed by Evans et al., 2006 and Jagger and Forge, 2015). Gap junctions are formed between adjacent cells through connexins. In the cochlea, the most prominent connexins are connexin 26 and 30 (Cx26, Cx30). The importance of this gap junctional network to hearing function is emphasized by the fact that a large percentage of human inherited deafness is caused by mutations in these two connexins. Decreased levels of Cx26 and Cx30 in the lateral wall have also been observed seven days after exposure to damaging noise-levels (Yamaguchi et al., 2014).

Type IV fibrocytes are especially vulnerable to noise exposure. Noise exposure already at 94 dB sound pressure level (SPL) causes fibrocyte IV loss, studied two weeks after exposure (Wang et al., 2002; Hirose and Liberman, 2003). Also, in age-related hearing loss the loss of type IV fibrocytes has been reported. Type IV fibrocytes are more vulnerable to ageing than type I or II fibrocytes in the lateral wall. The loss of type IV fibrocytes precedes even age-related hair cell loss (Hequembourg and Liberman, 2001). Type II fibrocytes have also been observed to die after noise exposure, but some evidence exists that these fibrocytes could be
able to regenerate after the insult (Hirose and Liberman, 2003). The contribution of lateral wall fibrocytes to the hearing function is still not well-known.

2.1.5 BLOOD CIRCULATION, BLOOD-LABYRINTH BARRIER, AND IMMUNE CELLS IN THE COCHLEA

Blood vessels enter the cochlea from its apical part and divide into capillaries in the lateral wall, where they further divide into branches towards the stria vascularis and the spiral ligament, as well as to the modioli. The modiolar side capillaries serve the SG neurons and the organ of Corti. It has been demonstrated that physically impeding the blood flow of the spiral ligament and stria vascularis destroys cells in the lateral wall but leaves the organ of Corti intact. Impeding the blood flow in the modiolar blood vessels leads to localized death of OHCs at the site of injury. This implies that the nutrient supply and the waste removal of the organ of Corti is conveyed through the vas spirale, the blood vessel beneath the basilar membrane (Lawrence, 1966).

Systemic effects, like hormones or drugs, reach the cochlea through the blood stream. Additionally, perturbances in the cochlear blood flow have been associated to many traumas from age-related hearing loss to noise-induced trauma (reviewed by Shi, 2011). While blood vessel diameters in noise exposure decline only slightly, the blood flow decreases significantly in noise-exposed lateral wall (Arpornchayanon et al., 2011). An increase in blood flow follows later after a noise exposure (Attanasio et al., 2001).

The cochlea was long regarded as an immune-privileged organ thanks to the blood-labyrinth barrier (BLB). Despite the functional significance of the intrastrial BLB, the exact structure and component cells of the barrier remain unclear. The barrier includes the basement membrane and endothelial cell tight junctions. Inflammatory stimuli can destroy these tight junctions, for example through matrix metalloproteinase 9, which is primarily derived from macrophages. Pericytes surround capillaries as well and regulate the blood flow in cochlear lateral wall with their contractility. Blood is propelled forward with the aid of pericytes, which cover the lateral wall capillaries in approximately 1:2 ratio with endothelial cells (reviewed by Shi, 2011). Perivascular macrophage-like cells are also in contact with the capillaries in the stria vascularis and participate in the maintenance of the BLB integrity (reviewed by Shi, 2011; Zhang et al., 2012b; Fujioka et al., 2014). Lipopolysacharide (LPS), a component of gram-negative bacterial cell wall that causes an innate immune response in the host, can disrupt cochlear homeostasis and lead to hearing loss. LPS causes perivascular macrophage-like cells to become smaller and have thinner dendritic processes and less contact with capillaries. This leads to loose or broken tight junctions, less dense basement membrane, and
the migration of pericytes away from capillaries, leading to leakage of the BLB (Zhang et al., 2015).

The cochlea is not known to contain lymphocytes, but it does house a pool of tissue resident macrophages as well as the perivascular macrophage-like cells in the stria vascularis (Fig 4B,D). In traumas, such as noise exposure, macrophages are also recruited to the cochlea from the circulation (Hirose et al., 2005). The turnover of cochlear macrophages is longer than 6 months (Okano et al., 2008). The role of macrophages in the cochlear lateral wall is not known but has been suggested to involve phagocytosis of dying cells. Macrophages in different parts of the lateral wall might also serve different purposes. For example, macrophages in the area of type V fibrocytes have been shown to be coupled to microvessels and contributing to the fluid flow by inducing vasospasms in response to noise exposure (Dai and Shi, 2011). The perivascular macrophage-like cells in the stria vascularis participate in the regulation of blood flow, immunologic defense, BLB integrity and tissue-repair (Hess et al., 2004; Shi, 2010; Zhang et al., 2012b).

2.1.6 SPIRAL LIMBUS

Spiral limbus is the cochlear structure in the middle of the cochlea, in the modiolus, from which the tectorial membrane protrudes (Fig 3). It also contains a population of fibrocytes and, on the side lining the endolymph, specialized interdental cells that play a role in maintaining the ionic composition of the endolymph together with the stria vascularis (Spicer and Schulte, 1991; Eckhard et al., 2012). Inner sulcus cells leading from the organ of Corti to the modiolus contain a gap junctional system that extends to the interdental cells and has been associated to K⁺ ion recycling from the IHCs back to the endolymph (reviewed by Kikuchi et al., 2000). During development, a transient structure, called the greater epithelial ridge forms between the organ of Corti and the spiral ligament, but it disappears during post-natal development and is absent in adult tissue (Hou et al., 2019). It has been shown that in an intense noise exposure the fibrocytes of the spiral limbus die whereas the interdental cells survive (Wang et al., 2002).

2.1.7 SPIRAL GANGLION

Spiral ganglion (SG) in the modiolus of the cochlea contains two types of SG neurons and satellite cells, the supporting cells of the neurons. 95% of the SG neurons are type I, which connect to IHCs and convey the auditory stimuli to the brainstem. A minority, 5%, of the SG neurons are type II neurons that connect to OHCs. Several type I neurons contact a single IHC, whereas one type II SG neuron contacts several OHCs. Type I cell somas are full of
mitochondria, unlike type II cells, indicating the high metabolic status of these cells. The IHCs are not inactive in silence. Instead, they perform spontaneous firing. This spontaneous firing can be measured from the type I afferent neurons. The neurons can be classified into different groups based on their electrical activity. The low spontaneous rate, high threshold neurons, approximately 15% of the total neuronal population, contact IHCs on the modiolar side. The high spontaneous rate, low threshold neurons, 60%, contact the IHCs on the pillar side (Taberner and Liberman, 2005; Liberman et al., 2011). Type I SG neurons differ also in their molecular contents, hence the differences in the firing rates are not solely resulted from the IHC activity. Still, IHC function is required for the differentiation of the neuronal subtypes. Three subtypes, Ia, Ib and Ic, have been identified and their ratios in the SG are 35%, 40% and 25%, respectively. The electrical properties of the subtypes have not yet been determined (Shrestha et al., 2018; Sun et al., 2018).

SG neurons are lost in age-related hearing loss and following noise exposure (Hequembourg and Liberman, 2001; Kujawa and Liberman, 2009). In many trauma models, SG neuron loss is shown to slowly follow the initial trauma-induced hair cell loss. However, in a mouse model where IHCs are lost in adulthood, SG neurons were shown to survive for months. Thereby it appears that IHCs are not necessary for neuronal survival in mature tissue. In fact, the trauma-induced slowly progressing neuronal death could be due to the original damage of the trauma to the sensory neurons or supporting cells that produce SG neuron survival factors (Zilberstein et al., 2012).

Figure 5 summarizes cochlear pathology after noise trauma with different noise levels. Spiral ligament type IV fibrocytes seem to be the most vulnerable part of the noise-exposed cochlea followed by synaptopathy and OHC loss at 98 dB SPL. However, the time point when the pathology is observed may differ. OHC loss and synaptopathy take place during noise exposure whereas fibrocyte IV death has been observed only two weeks after trauma (Wang et al., 2002; Hirose and Liberman, 2003; Kujawa and Liberman, 2009; Liberman et al., 2015).

Figure 5. A summary of cochlear damage loci after exposure to 8-16 kHz noise for 2 hours shown in 8-weeks-old CBA/Ca mice after exposure to different noise levels. Datapoints have been collected from the following articles: Wang et al., 2002; Hirose and Liberman, 2003; Kujawa and Liberman, 2009; Liberman et al., 2015. Abbreviations IHC: inner hair cell, OHC: outer hair cell, SG: spiral ganglion, EP: endolymphatic potential.
2.1.8 VESTIBULAR LABYRINTH
In addition to the auditory system, the inner ear contains another sensory system: the vestibular organs and the semicircular canals responsible for balance and sensing acceleration. The vestibular labyrinth consists of two otolith organs, the utricular macula and the sacculus, and three ampullaris cristae located in the semi-circular canals. These organs contain two types of hair cells. These hair cells differ from the ones found in the organ of Corti. Type I hair cells are flask-shaped and innervated by a single calyceal afferent terminal that surrounds their basolateral wall. Type II hair cells are cylindrical and innervated by multiple bouton terminals (Wan et al., 2019). The vestibular side shares endolymph with the cochlear scala media though the composition of the fluid is different in these two compartments (reviewed by Hibino and Kurachi, 2006). As for the cochlear hair cells, K$^+$ influx governs the excitability of vestibular hair cells. Dark cells, located in the non-sensory epithelium in utriculus and the cristae, but not in sacculus, recycle the K$^+$ from perilymph to endolymph (reviewed by Wangemann, 2002 and Hibino and Kurachi, 2006).

As with progressing hearing loss in aging, also vestibular defects, such as dizziness, vertigo and imbalance are common in elderly people. Interestingly, age-related vestibular dysfunction seems to differ from age-related hearing loss mechanisms. Although age-related vestibular hair cell loss has been observed in the cristae in the semi-circular canals, the cell loss is not as obvious in the otolith organs. In the otolith organs age-related vestibular dysfunction has been recently associated to synaptopathy which precedes cell death. The calyceal synapses of type I vestibular hair cells seem to be especially vulnerable and disappear first (Wan et al., 2019).

2.2 STRESS SIGNALLING NETWORKS AND CELL DEATH IN THE HEARING ORGAN
A cell thrives to keep its internal state stable, to maintain its homeostasis, in the changing environment. A cell can modify its activity in response to changes by activating cellular stress signalling. This is what I have defined as a stress response. Stress helps overcome stressful situations. However, stress can also lead to cell death if the cause of the stress cannot be addressed by the stress responses. All in all, a sufficient level of stress responses is needed for survival while overactivation could lead to cell death. Stress can be caused by extracellular or intracellular cues. Stress can be viewed at three different levels: system, organ, and cellular level. At systemic level stress activates the hypothalamus-pituitary-adrenal gland (HPA) axis and results in increased glucocorticoid levels in the blood stream. Systemic effectors can influence local stress signalling. Stress can be acute or chronic and the stress responses need
to be adjusted accordingly. A stress response initially targeted for survival can become harmful if it lasts for extended periods of time.

Stress needs to be sensed by a cellular receptor for a stress response to be initiated. These receptors can be located on the cell membrane or in the cytoplasm. Activation of the receptor initiates a cellular stress response by activating signalling pathways. These pathways lead to changes in gene transcription, or other changes in cellular function, that affect cell survival or death. Stress responses are complex and their outcome varies depending on the context. For example, systemic effectors such as inflammation or circadian rhythms can regulate the outcome of stress. In addition, the outcome of stress responses can differ depending on the cell type activating them.

2.2.1 VARIOUS CELLULAR FUNCTIONS OF JNK/C-JUN AND ERK1/2 STRESS SIGNALLING PATHWAYS

Mitogen activated protein kinases (MAPKs) function as signalling cascades in many cellular events from cell growth to cell death. The signalling cascades: c-Jun N-terminal kinase (JNK)/c-Jun, extra-cellular signal regulated kinase (ERK) 1/2 (and ERK5), and p38 comprise parallel MAPK pathways (reviewed by Plotnikov et al., 2011). p38 pathway is commonly related to cell death, but the duration of its activity affects the outcome. This pathway can also regulate cytoskeletal proteins and transcription factors (reviewed by Cuenda and Rousseau, 2007). Application of a p38 inhibitor prior to noise exposure has been shown to confer protection from hearing loss and OHC loss (Tabuchi et al., 2010). ERK1/2 pathway is involved in the recovery after stress. It is activated, for example by brain-derived neurotrophic factor (BDNF) in cell survival. However, in the cochlea ERK1/2 activation has also been associated to cell death after mechanical trauma (Lahne and Gale, 2008; Meltser et al., 2010). The JNK branch of the MAPK signalling has several roles ranging from development, cytokine release, to stress-induced apoptosis. In the hearing organ, JNK activity has been mainly linked to hair cell death (Pirvola et al., 2000; Wang et al., 2007). Figure 6 gives an overview of MAPK activity in stressed adult tissue.
ERK1/2 activation was shown to exaggerate hair cell death in neonatal cochlear explants exposed to mechanical trauma or ototoxins. ERK1/2 activation was observed in supporting cells under OHCs and IHCs, and not in hair cells. Preventing ERK1/2 activation produced partial protection from hair cell death (Lahne and Gale, 2008). However, ERK2 has been shown to promote hair cell survival in traumatized cochlea as ERK2 knockout (KO) mice were more vulnerable to noise (Kurioka et al., 2015). In other models, ERK1/2 has roles both in the cytosol and in the nucleus where it activates transcription factors (Hodge et al., 1998). In the cytosol, ERK1/2 targets molecules involved in migration or cytoskeletal remodelling or can inhibit gap junction function (Klemke et al., 1997; Brandes et al., 2002).

There are multiple isoforms of JNKs, kinases that phosphorylate c-Jun. JNKs differ in their function and tissue location (reviewed by Davis, 2000). JNK1 has been shown to have a constitutive role in the cerebellar granule neurons, while JNK2/3 levels were very low and increased greatly upon withdrawal of trophic support (Coffey et al., 2002). JNKs target phosphorylation sites in c-Jun at serines (S) 63/73 and threonines (T) 91/93. JNKs can have c-Jun-independent roles as well (Behrens et al., 1999; Morton et al., 2003). JNKs can for example move into mitochondria where they facilitate the phosphorylation of pro-apoptotic factors (Putcha et al., 2003). However, JNK activity may also be fundamental to neuronal survival as was shown in PC12 neuronal cell model by Waetzig and colleagues (2017). They showed that too low a level of JNK decreased neuronal cell viability.
c-Jun is an immediate early gene and its expression levels increase within an hour of exposure to extracellular stress stimuli (Angel et al., 1988). The same stimuli can activate both the expression and phosphorylation of c-Jun (Smeal et al., 1991). c-Jun is a component of the transcription factor activator protein-1 (AP-1) complex. The Jun proteins, c-Jun, JunB, and JunD, can either homodimerize or heterodimerize with other Jun family members or with other proteins, such as Fos and activator transcription factors (ATF). Different dimers have different functions in the cell (reviewed by Karin et al., 1997 and Chinenov and Kerppola, 2001).

c-Jun phosphorylation by JNKs has been associated with cell death (reviewed by Dhanasekaran and Reddy, 2017) and neurodegenerative diseases, such as Alzheimers’s and Parkinson’s diseases (Silva et al., 2005; Pearson et al., 2006). On the other hand, c-Jun has been shown to be essential for axonal regeneration (Raivich et al., 2004; Nix et al., 2011). In cerebellar granule cells, apoptosis caused by trophic/potassium deprivation works through c-Jun phosphorylation. In addition, preventing c-Jun phosphorylation at S63/S73 by replacing these sites with alanines (JunAA/AA) prevents kainate-induced excitotoxic neuronal cell death (Behrens et al., 1999). Still, c-Jun phosphorylation at different phosphorylation sites could have different outcomes especially in neurons. Phosphorylation at S63 and at T91/T93 have been independently associated to nerve cell death whereas phosphorylation at S73 has been linked to survival (Raivich et al., 2004; Reddy et al., 2013). The C-terminal region of c-Jun has two important phosphorylation sites T239 and S243 which serve as a docking sites for a ubiquitin ligase. Ubiquitination of a protein targets it for degradation and hence the phosphorylation of T239 and S243 lead to the degradation of c-Jun (Wei et al., 2005). The N-terminal phosphorylation of c-Jun is needed for the C-terminal degradation pathway to be activated (Nateri et al., 2004). This could be a pathway to control JNK-mediated apoptosis.

MAPKs have various, even opposing roles in cells. It seems that MAPKs have physiological roles in the maintenance of cellular survival as well as trauma-induced functions. Many of the MAPK studies have been done with cell cultures taken from neonatal mice, and it is possible that MAPK signalling functions in an alternative manner in adult tissue. This could, in part, explain the variation of the outcome of MAPK activation.

2.2.2 MULTIFUNCTIONAL NF-κB ORCHESTRATING CELLULAR EVENTS

NF-κB is a transcription factor involved in various cellular processes from cell survival to cytokine transcription. The activation of NF-κB is cell-specific and, thus, not all activating stimuli will activate NF-κB in every cell type. NF-κB is necessary for the efficient transcription of many genes, but additional transcription factors may be required as well. The
need for several transcription factors increases the specificity of cellular processes (reviewed by Pahl, 1999). ROS activates NF-κB and has been suggested to be a common mediator of the activation of NF-κB through various pathways (Schreck et al., 1991).

NF-κB is a family of proteins that functions as either homo- or heterodimers. The members of the family include RelA, RelB, NF-κB1, NF-κB2, and c-Rel. A common component of each of these molecules is the DNA binding domain and nuclear translocation signal called the Rel-homology domain. Different dimers can have different functions (reviewed by May and Ghosh, 1998). RelA, RelB and c-Rel are transcribed as active proteins whereas NF-κB1 and NF-κB2 are produced as precursor molecules that are further processed into smaller, active components. NF-κB molecules reside in the cytosol bound to inhibitory molecules called IκBs. NF-κB is activated by phosphorylation and ubiquitination of the inhibitory IκB. Thereby, no new protein synthesis is needed for NF-κB activity (reviewed by Sun and Andersson, 2002). Due to the complexity of NF-κB activity, different methods to study its activity have been made. For example, the mouse model created by Bhakar and colleagues (2002) demonstrates the transcriptional activity of NF-κB instead of the expression of its subunits. A LacZ-construct is preceded by a general promoter and NF-κB binding sites and hence LacZ is transcribed only in cells in which NF-κB is promoting transcription.

Trauma-induced NF-κB activation in the cochlea has been shown in the fibrocyte types I and II of the cochlear lateral wall following noise exposure and LPS-induced systemic inflammatory response, respectively. No activation upon stressors was detected in the cochlear hair cells, supporting cells, or SG neurons. During systemic inflammation, no toll-like receptor 4 (TLR4), the innate immune system activating receptor which recognizes LPS, was found in the activated cochlear cell types. Thus, the cells that activate NF-κB in response to systemic LPS application, do not recognize LPS itself. On the other hand, dexamethasone, an anti-inflammatory corticosteroid drug, abolished the NF-κB activity, suggesting that systemic inflammatory cytokines are the likely cause of NF-κB activation in the cochlear lateral wall in LPS-induced systemic inflammation (Adams et al., 2009). Thereby, NF-κB activity has been associated to cochlear trauma-induced stress responses.

2.2.3 CELL DEATH

Cell death can be initiated by cell-extrinsic or -intrinsic mechanisms and it can proceed via numerous modes. The same stimulus does not necessarily kill all the cells receiving it (reviewed by Elmore, 2007 and Green and Llambi, 2015). Research is currently shedding light into the various modes of cell death. New cell death modes are still being discovered (reviewed by Nirmala and Lopus, 2019). In the cochlea, apoptosis and necrosis have been accepted as
the main modes for OHCs death (Anttonen et al., 2014; reviewed by Furness, 2015).

Cell death follows a certain pattern and many of these patterns are known in detail. Apoptosis is known as a programmed cell death pathway, where the cell death is controlled and the dying cell blebs off membrane-enclosed vesicles that the surrounding cells phagocyte. In contrast, necrosis is an uncontrolled cell death mode where characteristically the cell membrane is broken and the interior of the dying cell bursts out causing inflammation. Unlike apoptosis where the dying cell itself has an active role in the cell death, requiring energy-expenditure, necrosis is a passive process of cell destruction (reviewed by Zeiss, 2003 and Elmore, 2007). Apoptosis follows a certain pattern dictated by the cascading activation of caspase proteases that can be initiated by cell-intrinsic mechanisms through the opening of mitochondrial permeability transition pores or extrinsic death-receptor mediated mechanisms. Mitochondrial apoptosis is linked to cytochrome c release from the mitochondria through the mitochondrial permeability pore. Cytochrome c and apoptotic protease-activating factor-1 (Apaf 1) form an apoptosome that induces the caspase cascade leading to cell death. The mitochondrial phase of apoptosis is regulated by B-cell lymphoma 2 (Bcl-2) family of proteins. These include the pro-apoptotic proteins Bax and Bak, which create pores to the mitochondrial membrane promoting cytochrome c release. Another pro-apoptotic factor, Bim, maintains anti-apoptotic Bcl-2 inactive (reviewed by Nirmala and Lopus, 2019). The executioner caspases are the same for the intrinsic and extrinsic pathways, but these pathways are initiated by different caspases, caspase 9 and 8 respectively for intrinsic and extrinsic pathways (reviewed by Cohen, 1997 and Elmore, 2007). When the caspases are activated the fate of the cell seems to be sealed (reviewed by Elmore, 2007). In necrosis, cell swelling, mitochondrial damage and ruptured cell organelle membranes are the distinct morphological changes prior disruption of the cell membrane (Denecker et al., 2001; reviewed by Elmore, 2007).

OHCs are the most vulnerable cell type in the cochlea. They die rapidly in response to many stressors such as noise overexposure and ototoxic drugs or more gradually during ageing, mainly through necrosis or apoptosis, depending on the strength of the stressor (reviewed by Fulda et al., 2010 and Furness, 2015). What are the molecular mechanisms causing the OHC death? Intracellular Ca\(^{2+}\) cytotoxicity has been proposed to cause the OHC death in traumas and it would explain the extreme vulnerability of the high frequency OHCs (reviewed by Fettiplace, 2017). Evidence from zebrafish suggests that calcium flow from the ER to mitochondria explains in part the vulnerability of hair cells to ototoxins. Physiologically, an increase in Ca\(^{2+}\) uptake supports metabolically active cells to increase their ATP production, but prolonged Ca\(^{2+}\) uptake can be toxic (reviewed by Giorgi et al., 2012). Ca\(^{2+}\) overload promotes the opening of the mitochondrial permeability pores, which leads to
cell death (Esterberg et al., 2014). Accumulation of reactive oxygen species (ROS) is linked to cell death in many models (reviewed by Furness, 2015 and Nirmala and Lopus, 2019). Increased ROS levels likely follow the Ca\(^{2+}\) accumulation initiated by mitochondrial membrane depolarization (reviewed by Nicholls, 2005). To prevent the toxic endolymph from entering the organ of Corti during hair cell death, the epithelium lining the scala media needs to be rapidly sealed. The sealing of the epithelial surface and phagocytosis of the OHC debris is done by supporting cells (Abrashkin et al., 2006; Anttonen et al., 2014).

Preventing a programmed cell death pathway from proceeding can lead to an uncontrolled cell death, which would cause degradation of the surrounding tissue. A decrease in the availability of caspases or ATP shortage can change apoptotic cell death to necrosis (Leist et al., 1997; Denecker et al., 2001; Zeiss, 2003; reviewed by Elmore, 2007). Thereby, the events preceding the activation of cell death pathways, namely stress signalling pathways, could provide target molecules for the prevention of cell death. An important point in the process of cell death is to know when the fate of the cell is sealed, and nothing can be done to reverse the cell death. The threshold for this most likely varies depending on the situation.

### 2.2.4 ER STRESS AND UPR

Accumulation of unfolded or misfolded proteins in the endoplasmic reticulum (ER) causes ER stress. Misfolding of proteins can be caused by several factors such as the high secretory activity of the cell or changes in Ca\(^{2+}\) concentration inside the ER (reviewed by Carreras-Sureda et al., 2018). ER stress is linked to the activation of unfolded protein response (UPR) pathways and this system aims to restore proteostasis. UPR signalling cascades have also other physiological roles in the cell than relieving protein-folding stress, such as the regulation of innate immunity, metabolism, synaptic function, and cell differentiation (Cho et al., 2009; reviewed by Hetz, 2012 and Martínez et al., 2018; Mogilenko et al., 2019). Recently, a UPR-independent ER stress relieving pathway has been identified. It functions through a cell-surface hyaluronidase, transmembrane protein 2 (TMEM2), and MAPKs ERK and p38 (Schinzel et al., 2019).

Three different ER membrane localized receptors: inositol-requiring protein 1α (IRE1α), protein kinase RNA-like ER kinase (PERK), and activating transcription factor 6 (ATF6) initiate signalling cascades that aim to restore ER homeostasis by enhancing ER associated protein degradation (ERAD) and protein folding by upregulating ER chaperone expression as well as downregulating other protein translation. Unfolded proteins are sensed in the ER when the unfolded proteins bind to the ER resident chaperone 78 kDa glucose-regulated protein/immunoglobulin heavy chain binding protein (GRP78), also known as BIP or HSPA5.
GRP78 is also bound to all three ER membrane resident UPR-initiation receptors but following the accumulation of misfolded proteins in the ER, GRP78 detaches from these receptors allowing their activation (Carrara et al., 2015). Mesencephalic astrocyte-derived neurotrophic factor (MANF) is another protein that has been associated to the maintenance of ER homeostasis (Mizobuchi et al., 2007). Even though the initial role of UPR is to promote cell survival, prolonged UPR, when the ER stress cannot be addressed, leads to cell death (Rao et al., 2001; reviewed by Walter and Ron, 2011 and Hetz, 2012). An overview of the adaptive and pro-apoptotic UPR mechanisms is shown in Figure 7. In the adaptive UPR IRE1, PERK, and ATF6 receptors within the ER are activated. This activation leads to transcriptional changes that aim to alleviate the misfolded protein load within the ER. For example, protein folding is promoted, general protein synthesis is decreased, and ERAD-pathway upregulated. Pro-apoptotic UPR activity includes upregulation of C/EBP homologous protein (CHOP) and BH3-only proteins that lead to cytochrome c release from the mitochondria. In addition, IRE1 pathway activates caspase 2 and JNKs promoting apoptosis.

![Figure 7. A schematic of adaptive and cell death promoting ER stress activated UPR pathways. The figure is based on a review by Koopman et al., 2019. Although MANF has been shown to physically interact with GRP78, its role in ER stress is not understood as well that of GRP78. Abbreviations UPR: unfolded protein response, GRP78: 78 kDa glucose-regulated protein, MANF: mesencephalic astrocyte-derived neurotrophic factor, IRE1: inositol-requiring protein 1a, ATF6(c): activating transcription factor 6 (cleaved), PERK: protein kinase RNA-like ER kinase, XBP-1s: spliced x-box binding protein 1, eIF2α: eukaryotic initiation factor 2α, eIF2α: eukaryotic initiation factor 2α, ER: endoplasmic reticulum, ERAD: ER associated degradation, JNK: c-Jun N-terminal kinase, ATF4: activating transcription factor 4, CHOP: C/EBP homologous protein, ROS: reactive oxygen species.](image-url)
The storage of Ca$^{2+}$ into the ER requires energy and takes place through the activity of sarco/endoplasmic-reticulum Ca$^{2+}$ ATPase (SERCA) pumps. Ca$^{2+}$ is released from the ER continuously through several Ca$^{2+}$ -channels, and if the SERCA pumps are inhibited with thapsigargin, the entire calcium pool of ER is depleted in minutes, followed by ER stress and cell death (reviewed by Carreras-Sureda et al., 2018). Calcium is transferred from the ER to mitochondria through mitochondria-associated membranes (MAMs). Inositol 1,4,5-triphosphate receptors (IP3R) and ryanodine receptors in MAMs regulate in part Ca$^{2+}$ signalling in the cells (Csordás et al., 2010; reviewed by Carreras-Sureda et al., 2018). The tight association between ER and mitochondria is related to energy metabolism, cellular Ca$^{2+}$ homeostasis and signalling as well as to cell death. An increase in Ca$^{2+}$ concentration increases ATP production in mitochondria, but an overload of Ca$^{2+}$ can support the opening of a mitochondrial transition pore that leads to the release of pro-apoptotic factors from the mitochondria (reviewed by Decuyper et al., 2011, Bravo et al., 2012 and Carreras-Sureda et al., 2018).

ER stress mediated cell death has been studied in the cochlea with local application of tunicamycin, an ER stress activator that blocks N-acetylglucosamine transferases. Tunicamycin caused progressive hearing loss, OHC loss and degeneration of IHCs and SG neuron nerve endings (Fujinami et al., 2012). Increased ER stress has also been associated to noise exposure and the ensuing OHC loss to the activation of CHOP. CHOP is a pro-apoptotic factor of UPR. Application of integrative stress-response inhibitor (ISRIB), which alleviates ER stress, reduced noise-induced OHC loss. ISRIB leads to the inhibition of UPR pathway acting through ATF4 including inhibition of CHOP (Li et al., 2018). Hence, ER stress has a role in trauma-induced cellular stress responses in the cochlea.

### 2.2.5 ROS IN PHYSIOLOGY AND PATHOPHYSIOLOGY

Reactive oxygen species (ROS) have various physiological and pathophysiological roles in the cell. ROS regulate, for example, cell death, ER stress and ageing (Liu et al., 2008; Esterberg et al., 2016; Benkafadar et al., 2019). ROS are oxygen-based molecules that either act as free radicals or are readily capable of producing them. Free radicals have an unpaired electron and they eagerly interact with other molecules to receive an electron. This leads to destabilization of molecules such as lipids, proteins or DNA causing tissue damage. Superoxide anion (O$^{2-}$) and hydrogen peroxide H$_2$O$_2$ are examples of ROS. Inside cells, superoxides are rapidly converted to H$_2$O$_2$ by superoxide dismutases. A major source of ROS inside a cell is the electron transport chain in the mitochondria and high energy expenditure is likely to increase ROS production (reviewed by Fetoni et al., 2019). Increased levels of ROS
have been shown to increase cochlear vulnerability to high-level noise measured with ABRs and OHC loss (Morioka et al., 2018).

ROS are generally associated to detrimental effects, but they have physiological metabolic roles as well. When referring to these physiological effects of ROS, the term redox biology is used (reviewed by Schieber and Chandel, 2014). The term ‘redox’ comes from reduction and oxidation, which are chemical reactions where the oxidation states of atoms are changed. Usually in a redox reaction one molecule gets oxidated and another undergoes reduction. Such reactions are common in a cell. For example, the formation of disulphide bonds in protein folding functions through redox reactions. Protein disulfide isomerases (PDIs) are ER chaperones responsible for correct protein folding. They utilize redox reactions for disulphide bond formation in proteins (reviewed by Okumura et al., 2015). Cysteines in aminoacid chains generally exist as thiolate anions (Cys-S-) in an intracellular environment. During redox signalling \( \text{H}_2\text{O}_2 \) oxidizes the thiolate anion to the sulfenic form (Cys-SOH). This change causes allosteric changes and can lead to an altered function of the protein. Disulfide reductases return the sulfenic form to thiolate anion. However, high levels of \( \text{H}_2\text{O}_2 \) can oxidize the thiolate anions up to sulfinic (SO\(_2\)H) or sulfonic (SO\(_3\)H) species and these changes can be irreversible resulting in permanent protein damage (oxidative stress) (reviewed by Schieber and Chandel, 2014).

ROS have been shown to be essential for growth-factor-induced receptor tyrosine phosphorylation (Sundaresan et al., 1995). In immune responses, ROS have important roles as second messengers, but they can also lead to hyperactivation of inflammatory responses causing tissue damage (reviewed by Mittal et al., 2014). It could be that small elevation of ROS levels enhances immune system function and high levels promote the pathological outcomes (reviewed by Schieber and Chandel, 2014). In ageing, a revolutionary idea is that increasing ROS can activate cellular stress pathways to dampen tissue degeneration and promote healthy ageing (reviewed by Ristow and Schmeisser, 2011 and Schieber and Chandel, 2014). Physical exercise increases ROS production and results in activation of beneficial pathways (Ristow et al., 2009). Beneficial effects of exercise have been demonstrated for hearing function as well though the beneficial effects have not been directly associated to elevated ROS levels (Han et al., 2016).

In the cochlea, ROS has been observed to play a role in many traumas and antioxidant treatment has been shown to confer beneficial effects in many cases. For example, ROS has been associated to age-related hearing loss pathology (Benkafadar et al., 2019). Genetic over-production of ROS in the cochlea has been shown to heighten the vulnerability to noise exposure (Morioka et al., 2018). In addition, hair cell death initiated with ototoxic aminoglycoside antibodies has been linked to ROS (Esterberg et al., 2016).
To conclude, the dual role of ROS in signalling and trauma-inducing stress response could explain, in part, the controversy of antioxidant therapies in hearing loss. Low levels of ROS are needed for cellular physiology, elevated levels help to overcome stressful situations and high levels promote cell death (reviewed by Schieber and Chandel, 2014 and Ftoni et al., 2019).

### 2.2.6 INFLAMMATION

Inflammation is one of the body’s defense mechanism in immune responses. A stressor, a pathogen or cell debris can initiate an inflammatory response by activating immune cells which produce inflammatory cytokines. These cytokines activate processes that aim to exterminate the cause of the inflammation, for example by recruiting macrophages, dilating blood vessels and disturbing the integrity of the blood vessel walls. Prolonged inflammation can lead to tissue damage (reviewed by Mittal et al., 2014). Bone marrow-derived macrophages are recruited into the cochlea after traumas, such as noise, exposure to ototoxic drugs, and ageing (reviewed by Hirose et al., 2017).

The pro-inflammatory LPS has been shown to cause leakage of cochlear BLB (Hirose et al., 2014). Locally applied LPS disturbs the intra-strial fluid-blood barrier permeability, downregulating expression of the tight junction-associated proteins zonula occludens-1 (ZO-1), occludin, and vascular endothelial cadherin, and promoting transport activity (Zhang et al., 2015). Local stress to the cell populations of stria vascularis results from the leakage of the blood vessels since these cells are sensitive to changes in ion homeostasis. The stressed cells of the stria vascularis cannot maintain the concentration of the endolymph, thereby causing detrimental effects on the organ of Corti. In the traumatized cochlea, macrophages are recruited to the spiral ligament and limbus, but they do not readily enter the stria vascularis (reviewed by Hirose et al., 2017).

The proinflammatory cytokines, tumor necrosis factor α (TNF-α), and interleukines IL-1β and IL-6, are produced by the cochlear lateral wall fibrocytes after noise-induced damage (Fujioka et al., 2006). The cytokines participate in recruiting macrophages to the cochlea (Wakabayashi et al., 2010). The precise role for cochlear macrophages and inflammatory responses is not known, but it is currently an active research topic in hearing research.

OHCs are sensitive to disruptions of the lateral wall function (Liu et al., 2016). The effects of LPS might also be partly conveyed to the organ of Corti through the blood vessel beneath the basilar membrane. One putative role for the trauma-induced macrophages in the cochlea is phagocytosis of cell debris from the dying hair cells (reviewed by Hirose et al., 2017). In addition, activated macrophages have been associated with IHC synapse regeneration (Kaur
et al., 2019). A systemic inflammatory response can alter local stress signalling pathways and thereby affect, for example the cochlear trauma-induced recovery processes.

### 2.2.7 SYSTEMIC GLUCOCORTICOIDS IN LOCAL STRESS RESPONSES

At the systemic level, hormonal and neuronal systems control stress responses through HPA axis. The classical stress hormones, glucocorticoids, namely cortisol in humans and corticosterone in mice, are produced in the adrenal glands in response to stress response activation in the brain. These hormones influence the activity of almost all cells of the body and glucocorticoid receptors are expressed throughout the body, also in the cochlea (Shimazaki et al., 2002; reviewed by Buckingham, 2006). Glucocorticoids have complicated modes of action and their purpose in stress is not completely understood. They could 1) induce a stress response, 2) facilitate the emerging stress responses, or 3) act to suppress the stress response from becoming pathologically active (reviewed by Munck et al., 1984 and Sapolsky et al., 2000).

Hypothalamus secretes corticotropin-releasing hormone that acts on the pituitary gland where corticotropes are released into the blood stream and finally reach the target tissues in the adrenal gland where stress hormone glucocorticoids, are released. Glucocorticoids will prime the body for fight-or-flight situations. Glucocorticoids aim to promote survival, but they can cause harmful effects if the stress becomes chronic (reviewed by Buckingham, 2006). It is good to keep in mind that in an acute stress situation most of the actions of glucocorticoids take place after one hour from the initiation of the stress. This delay in response to stressors is explained by the transcriptional function of glucocorticoids. Transcriptional changes take approximately one hour to be executed (reviewed by Sapolsky et al., 2000).

There are two principal types of glucocorticoid receptors: mineralocorticoid, type I, and glucocorticoid, type II, receptors. Glucocorticoids are steroid hormones and travel in the blood stream throughout circulation. They may freely cross cell membranes and bind to their receptors in the cytosol. The glucocorticoid-receptor complex moves to the nucleus and acts as a transcription factor binding to specific glucocorticoid response elements in deoxyribonucleic acid (DNA) (Bamberger et al., 1996). However, glucocorticoid receptors do not necessarily have to bind to DNA to express their function. Instead, they can use so-called transcriptional cross-talk by tethering to other transcription factors such as AP-1. Heterodimers with c-Jun and c-Fos are repressed while c-Jun and c-Jun homodimers are enhanced by such cross-talk (Miner et al., 1991; Miner and Yamamoto, 1992; Bamberger et al., 1996; reviewed by Göttlicher et al., 1998 and Sapolsky et al., 2000). In addition, NF-κB
can interact with glucocorticoid receptors in a similar manner (reviewed by Göttlicher et al., 1998). The above-mentioned factors could influence the varied outcomes of glucocorticoids in stress. Also, duration and timing of the stress responses can affect the outcome of the stress response (reviewed by Munck and Náray-Fejes-Tóth, 1994, and Sapolsky et al., 2000).

Exposure to noise causes an increase in systemic stress hormone levels (reviewed by Babisch, 2003; Münzel et al., 2017). On the other hand, systemic glucocorticoids appear to regulate stress responses in the cochlea. This regulation has been demonstrated in the pre-conditioning experiments. The concept of hormesis means that a minor exposure to a damaging stress will confer protection from the following greater trauma (reviewed by Schieber and Chandel, 2014). Pre-conditioning with low-level stress that protects from a following damaging stress appears to work through systemic effectors in the cochlea as well. The low-level stress can be introduced in many forms ranging from mild noise exposure or heat stress to restrained stress (Canlon et al., 1988; Yoshida et al., 1999; Wang and Liberman, 2002). Hearing loss can be diminished if the damaging noise exposure is shortly preceded by restraint stress. In restraint stress, the mouse is prevented from moving freely for a certain amount of time. This causes the corticosteroid levels to rise and confers protection from permanent hearing loss caused by exposure to loud noise (Wang and Liberman, 2002). If the plasma levels of corticosteroids are lowered by drugs inhibiting glucocorticoid synthesis, no protection from hearing loss is produced (Tahera et al., 2006).

Glucocorticoid receptors have been found in many cochlear compartments, especially from type I-III fibrocytes in the lateral wall and SG neurons, and they are present also in the organ of Corti in supporting cells, but not in hair cells (Shimazaki et al., 2002). Therefore, it appears that glucocorticoids do not directly act on hair cells. It seems that in the cochlea, glucocorticoids have a protective role as seen in the pre-conditioning paradigm, and this protection is conferred via non-sensory or neuronal cells. Some locally induced protective stress responses might still be involved. In an experiment where one ear was blocked from the preceding minor noise exposure, this ear was not protected from the following damaging noise exposure. Purely systemic protective effectors would have reached the blocked ear as well (Yamasoba et al., 1999).

Steroids remain currently a frequently used drug treatment for sudden sensorineural hearing loss, though recent research does not support the therapeutic value of this treatment (Crane et al., 2015). It could be that the effective therapeutic window of corticosteroid treatment for treating hearing loss is very narrow as suggested by pre-conditioning experiments (Yoshida et al., 1999; Wang and Liberman, 2002). The function of glucocorticoids in pathological situations is not yet fully understood in the cochlea.

Noise-induced stress in humans has been associated to annoyance, stress, sleep
disturbance and impaired cognitive performance. These effects are likely to increase stress hormone levels and vascular oxidative stress (reviewed by Münzel et al., 2018). In a mouse study modelling aircraft noise exposure, noise-induced stress was associated with vascular dysfunction. Intriguingly, a comparable noise exposure with white noise did not cause similar changes as seen with the aircraft noise paradigm (Münzel et al., 2017). Stress can affect hearing through changes in blood circulation. Exercise has been shown to diminish age-related hearing loss. The capillaries in the stria vascularis stayed in better shape and the hearing thresholds of the old mice in the exercise group were lower than in the non-exercising controls (Han et al., 2016). Hence the noise-induced glucocorticoid release seems to be a two-edged sword. On one hand conferring protection from further noise-damage and on the other hand causing hearing loss due to prolonged vascular dysfunction.

2.2.8 CIRCADIAN RHYTHMS

A circadian rhythm refers to an approximately 24-hour cycle in the physiology of living organisms. This rhythm is endogenously generated, but external cues, such as feeding times and the light-dark cycle, regulate it. Many physiological processes in the body, for example hormone production and immune responses, vary at different times of the day. The rhythm is kept with an internal clock mechanism that is centrally controlled by the suprachiasmatic nucleus in the hypothalamus. The clock genes include Period, Timeless and Clock which have been extensively studied in the fruit fly Drosophila melanogaster. Clock and Cycle (BMAL in mammals) transcription factors activate the transcription of Period and Timeless. Period and Timeless proteins interact in the cytoplasm and when this complex is transferred into the nucleus it inhibits the activity of Clock and Cycle proteins. Old Period and Cycle complexes are degraded relieving the inhibition of Clock and Cycle, and a new cycle may begin. The amounts of Period and Timeless are regulated by degradation through Double-time (a casein kinase 1) and Chryptochrome, respectively. Chryptochrome is a blue light photoreceptor. It allows Timeless to be accumulated in the cells only at night-time, thereby linking the circadian clock to the environmental cycle. In mammals, the idea of the circadian clock is similar to the one in Drosophila, but there are more copies of the genes involved. Also, apparently Chryptochrome is not a photoreceptor in mammals but acts as a transcription factor that replaces Timeless in complexes with Period (reviewed by Basinou et al., 2017 and Honma, 2018).

Circadian rhythms can affect pathophysiology. The auditory sensitivity has been shown to vary depending on the time of the day. In humans, variation in OHC function according to the time of the day has been demonstrated (Haggerty et al., 1993). Studies done in mice,
nocturnal animals, showed that the same noise exposure caused a temporary threshold shift when given during the day, but at night resulted in a permanent threshold shift (Meltser et al., 2010). Later, it was shown that this differential noise-induced hearing loss is regulated by glucocorticoids (Cederroth et al., 2019a). Noise exposure was shown to decrease the expression of circadian clock genes in the cochlea. However, the role of the cochlear clock machinery to hearing sensitivity remains to be unravelled (Meltser et al., 2014). Still, noise is one potential external factor regulating the circadian rhythms so its influence on the clock machinery could be expected.

2.3 STUDYING HEARING LOSS WITH MOUSE MODELS

Currently, with the number of genetic models available, mice are widely used in hearing studies. In older studies though, other species, such as rat, guinea pig, cat, and chinchilla, have dominated as model animals in the auditory field. Animal models are often used to model human auditory function and hearing loss. Thereby, it is good to consider how well the model animal corresponds to human physiology. The following section is dedicated to a few remarks about using mice to model humans as well as considering the influence of mouse strain differences in hearing research.

2.3.1 MOUSE AS A MAN

Mice differ from humans in size, lifespan (up to 2-3 years) and metabolic rate. They are nocturnal prey animals (reviewed by Ohlemiller et al., 2016). Mouse hearing range is more extensive than that of humans. The frequency region of hearing in mice extends to ultrahigh frequencies, 100 kHz, whereas in humans the hearing range is 20 Hz-20 kHz in young people. High frequency hearing in humans decreases rapidly after childhood and typically adults hear only up to 16 kHz. Even though mice and humans share a large part of their genes, there might be more variance in the regulatory sequences of the genes causing major differences in gene expression (reviewed by Johnson et al., 2006). Thereby it is important to understand the mechanisms behind hearing loss when modelling genetic hearing loss in mice.

Mice serve as a good model for the human peripheral auditory system as well as offer suitable models for hereditary hearing loss (reviewed by Kikkawa et al., 2012). However, some inter-species differences exist in the hearing organ. For example, the cochlear capsule, the stapes and the bone covering the cochlear nerve are particularly thin in mice. Outer sulcus cells, which have fundamental roles in ion circulation in the cochlea, differ in their anion exchangers between mice and a common marmoset, Callithrix jacchus, which is used as a
primate model for human cochleas (Hosoya et al., 2016). Also, there are fewer root cells in human cochlear lateral wall than in mice (Santi et al., 2016). Naturally, the amount of hair cells differs in mice and men as well. Murine cochleas contain approximately 800 IHCs and 2600 OHCs (counted from a cochlear whole mount specimen) whereas the corresponding numbers in humans are 3500 and 12’000 (reviewed by Nadol, 1988).

2.3.2 MOUSE STRAIN DIFFERENCES IN HEARING RESEARCH
Multiple mouse strains have been developed for research purposes. Many of them are inbred strains. Inbred mouse strains are developed through 20 or more generations of sister-brother breeding. As a result, any two individuals of the same gender in an inbred strain are practically identical twins (reviewed by Ohlemiller et al., 2016). Inbreeding leads to the pairing of dominant with dominant alleles and recessive with recessive alleles. However, in addition to the gene expression level, the regulation of genetic machinery can take place at messenger ribonucleic acid (RNA) or protein level (reviewed by Davis et al., 2003). Genetic variance is characteristic of both mice and men, and the extensive use of inbred strains fails to cover this diversity (reviewed by Ohlemiller et al., 2016).

Many inbred mouse strains have been maintained for over 60 years and genetic selection in the individual animal facilities has created selective pressure to different traits. It is also known that mitochondrial DNA affects metabolism and has a high mutation rate, which could further add to variability between substrains. C57BL/6J (BL6) is the model mouse strain for many studies and it was the reference genome for the mouse genome sequence project. Many different substrains of BL6 mice exist nowadays (Enriquez, 2019; reviewed by Lesus et al., 2019). BL6 mice carry a mutation in Ahi gene (age-related hearing loss) that codes for otocadherin CDH23 (Johnson et al., 1997). CDH23 together with protocadherin 15 are the components of the tip links in the hair cell stereocilia (Kazmierczak et al., 2007). The defect in the Cdh23 gene is a transition of G to A in aminoacid position 753. This transition causes a frame shift in exon 7 and ultimately reduced message stability (Kane et al., 2012). The hearing of BL6 mice is normal at first but begins to worsen already at 3-months of age (Hequembourg and Liberman, 2001). Different BL6 substrains display identical hearing loss patterns (Kane et al., 2012).

The CBA strain was originally created in 1920. CBA/CaJ and CBA/J mouse strains arrived at the Jackson Laboratory for distribution at different times. CBA strains have been traditionally used as good hearing controls for BL6 mice since they have low hearing thresholds until old age (reviewed by Ohlemiller et al., 2016). CBA/J and CBA/CaJ seem to have a similar response and vulnerability to noise exposure (Ohlemiller and Gagnon, 2007).
However, during the early-vulnerability period of CBA mice, when noise exposure causes great damage in the cochlea, CBA/J strain is not as vulnerable to noise exposure as CBA/CaJ strain (Ohlemiller et al., 2011).

Some outbred mouse lines are also in common use, such as CD-1, also known as ICR, mice. Even though the mice of an outbred line are not clones of each other, each outbred mouse line is commonly derived from a few founders and hence the outbred mouse lines differ from each other (reviewed by Ohlemiller et al., 2016). CD-1 mice show early-onset, rapid progressive hearing loss, but the genetic basis of the hearing loss is not well defined (Shone et al. 1991; Le Calvez et al. 1998; Wu and Marcus, 2003).

2.3.3 AGE-RELATED HEARING LOSS

Ageing leads to the dysfunction of many organs. Age-related hearing loss, presbycusis, has traditionally been classified into three different classes: strial, neuronal and hair cell based. Nowadays the classification is less strict and age-related hearing loss can be a mixture of different pathologies (reviewed by Keithley, 2019). Many mouse lines, such as BL6 and CD-1, display early-onset age-related hearing loss and they serve as models for presbycusis research (reviewed by Ohlemiller et al., 2016).

Progressive hair cell loss from base-to-apex along the cochlear duct is typical for age-related hearing loss. OHCs are more vulnerable and die first and IHC death follows. In addition, lateral wall fibrocyte and SG neuron loss in the basal part of the cochlea are observed in the ageing BL6 and CD-1 mice. These pathologies have been observed also in some human specimens with presbycusis (Schuknecht, 1955; Hequembourg and Liberman, 2001; Wu and Marcus, 2003). Different from humans, EP does not change due to ageing in many age-related hearing loss mouse models, such as BL6 and CD-1 (Wu and Marcus, 2003; Ohlemiller et al., 2018; reviewed by Keithley, 2019). Despite the unchanged EP between young and old CD-1 mice, a significant reduction in K+ concentration in old CD-1 mice has been observed, which could partly explain the hearing loss (Wu and Marcus, 2003).

BL6 mice show early-onset age-related hearing loss. Even though this hearing loss is traditionally associated to the Cdh23 mutation, it is not likely that it is solely responsible for hearing loss. Cdh23 mutation transferred to the CBA mouse strain does not cause the hearing loss phenotype (Kane et al., 2012). However, correcting the Cdh23 mutation with a wildtype allele is enough to prevent the hearing loss in BL6 mice. BL6.CAS.T/EiJ mice harbour a wildtype Cdh23 allele from Mus musculus castaneus at the Ahl locus (Harding et al., 2005). Thereby it seems that Cdh23 mutation together with some other characteristic of BL6 mice cause the hearing loss phenotype. The age-related cell death in BL6 mice has been associated
with the mitochondrial intracellular apoptotic pathway. Inhibition of the pro-apoptotic protein Bak prevents hearing loss and OHC death (Someya et al., 2009).

Is age-related hearing loss associated to greater vulnerability to loud sounds in aged cochleas? Apparently not, since studies with chinchillas did not find heightened noise-vulnerability in older animals (Sun et al., 1994) and in mice older individual are generally more resilient to the detrimental effects of noise than young mice (Henry, 1982). For example, EP reduction after noise exposure takes place after higher noise levels (110 dB SPL) in older CBA/J mice (4-16 months old) compared with a lower noise level (101 dB SPL) in younger animals (6-7 weeks old). CBA mice are particularly vulnerable to noise exposure at young age, and display holes and tears of reticular lamina after exposure to 104 dB SPL for 2 hours. In older mice and in BL6 strain the reticular lamina stays intact after noise exposure though exposure to 119 dB SPL for 2 hours causes reticular lamina disruption irrespective of strain or age. However, OHC death in old BL6 mice does not cause a drop in EP (Ohlemiller et al., 2018).

2.3.4 NOISE VULNERABILITY
The duration of the noise exposure together with the SPL dictate the outcome of noise exposure in the cochlea. Though the type of noise matters as well. The higher the noise level is, the shorter exposure time is needed to cause damage (Ohlemiller et al., 2000). When noise exposure levels rise above 110 dB SPL in mouse studies, it is likely that part of the damage in the cochlea is caused by mechanical trauma to the tissue (Bohne and Rabbit, 1983; Ahmad et al., 2003; Ohlemiller et al., 2018).

Noise vulnerability often refers to elevated hearing thresholds after exposure to loud sounds. ABR measurements are commonly used as a functional measure of hearing. A typical ABR waveform contains five primary waves. The first wave corresponds to the summated potential of SG neurons. The following four peaks represent electrical activity measured at different brainstem nuclei. Thus, ABR does not measure electric activity from the auditory cortex. Hearing threshold for each tested frequency is determined as the lowest sound level that gives a detectable waveform. Elevated hearing thresholds mark hearing loss (Henry, 1978).

Since the first ABR wave represents the electrical input from the cochlea, cochlear damage can be monitored in the ABR measurements. OHCs lacking their characteristic electromotility in prestin KOs displayed approximately 40 dB higher hearing thresholds than controls (Liberman et al., 2004). Thereby, loss of OHCs, the cochlear amplifier, explains, in part, high ABR thresholds in the areas where OHC loss is detected. Problems in the function of stria
vascularis, cochlear gap junctions, or damage to the lateral wall fibrocytes can cause an EP drop. An EP drop interferes with the hair cell mechanotransduction and causes hearing loss (Hequembourg and Liberman, 2001; Mei et al., 2017). Still, not all morphological damage in the cochlea is reflected in the ABR thresholds. It is estimated that more than 50% of SG neurons need to be lost before a change can be detected in ABR thresholds, provided that OHCs are intact (Shucknect, 1955; Kujawa and Liberman, 2009). Synaptopathy is not readily detected in ABR thresholds. The most vulnerable ribbon synapses are the low-spontaneous rate, high-threshold ones, while the low-threshold ribbons are not as vulnerable (Kujawa and Liberman, 2009). The low-spontaneous rate neurons make up a minority, 15%, of the total neuronal population (Taberner and Liberman, 2005; Liberman et al., 2011).

Elevation of hearing thresholds after trauma can be temporary or permanent. The morphological markers of a temporary threshold shift after noise exposure include metabolic changes in neuronal metabolism in auditory brainstem, breaches in the reticular lamina, stereocilia damage, and detachment of the OHC stereocilia from the tectorial membrane. Common to all these changes is the ability to recover over time. Generally, the hearing thresholds regain their pre-exposure levels by one week after noise exposure (reviewed by Ryan et al., 2016). Noise-induced permanent threshold shift displays hair cell, lateral wall fibrocyte, and SG neuron loss (Hirose and Liberman, 2003). As hair cells and SG neurons are not regenerated in the mammalian cochlea, the hearing loss is not reversible.

Vulnerability to noise differs between individuals. In humans, workers exposed to the same noise levels develop different levels of noise-induced hearing loss (reviewed by Davis et al., 2003). Also, mouse strains show different responses to noise exposure and are thereby a good model to study the background of differences in noise vulnerability. BL6 mice are thought to be more vulnerable to noise than CBA mice (Davis et al., 2001). For example, BL6 hearing thresholds did not recover from noise exposure when compared with CBA mice which were shown to recover their hearing thresholds 3-7 days after noise exposure (reviewed by Davis et al., 2003). The mutation in Cdh23 gene has been thoroughly studied and no changes in the noise-induced permanent threshold shift were detected between BL6 mice and BL6.CAST mice that have a normal allele of the Cdh23 gene (Harding et al., 2005). Thereby, the Ahl gene responsible for the early-onset age related hearing loss in the BL6 mice does not augment vulnerability to noise exposure. In fact, a study comparing the cochlear pathology in noise exposure at a morphological level between CBA and BL6 mice, revealed more damage in CBA cochleas (Ohlemiller and Gagnon, 2007). The noise-induced drop in EP is also dependent on the mouse strain. While the CBA/J mice show EP reduction to 20 mV starting from exposure to 101 dB SPL, BL6 mice only show reduction in EP when noise level rises up to 110 dB SPL (Ohlemiller et al., 2018).
To conclude, it seems that BL6 mice are more likely to suffer hearing threshold changes after noise exposure while cochlear pathology at the cellular level takes place more readily in the CBA mice during the early-vulnerability period of this mouse strain. Other inbred mouse strains also display a period of higher vulnerability to noise at the age of 1-2 months, where the threshold shifts are higher and OHC loss more promoted than in the adult, 5-7 months old, mice after a similar noise exposure (Ohlemiller et al., 2000). Hearing measurements do not correspond one to one to the cochlear pathology observed at the light microscopy level. Thereby, it is important to know how the noise vulnerability has been assessed for a given mouse strain.

### 2.3.5 OTOTOXIC DRUG-INDUCED LESION

Ototoxic compounds, such as the aminoglycoside antibiotics, cause hair cell death and hearing loss. Aminoglycosides, like kanamycin, have direct damaging effect on hair cells though the exact mechanism is not known. OHCs have been shown to accumulate aminoglycosides (Imamura and Adams, 2003; reviewed by Jiang et al., 2017). To cause a cochlear lesion, long periods of drug application are needed. However, if aminoglycosides are given with loop-diuretics, they result in a synergistic effect that triggers rapid and widespread OHC loss. Loop-diuretics, such as furosemide, disrupt the $K^+$ secretion of the stria vascularis (Taylor et al., 2008).

The principles of hormesis have been demonstrated with aminoglycosides in the cochlea. CBA/J mice show protection from noise-induced hearing loss with a preceding low dose kanamycin application. Both hearing thresholds and OHCs were protected from noise-induced damage. This protection was not seen in BL6 mice, which suggests that a genetic component influences the internal response to kanamycin (Ohlemiller et al., 2011).

### 2.4 SUMMARY OF THE LITERATURE REVIEW

As we have seen in the literature review, many cochlear compartments are damaged in traumas and all these compartments are needed for hearing function. Figure 8 gives an overview of stress responses involved in OHC death in different lesions based on previous literature. Circadian rhythms and the genetic background lay the setting for local stress signalling and the outcome of trauma. Level 2 in the figure represents different stress signalling pathways. Many of these pathways get activated by stressors from level 3 simultaneously. ROS are placed separately at level 1, because they have such widely spread functions in stress signalling and are thus likely to be involved in all cases described at the levels above it. Noise
exposure increases systemic glucocorticoid levels. Glucocorticoids are a general stress response and they can function either as transcription factors through binding to their receptors or by binding to NF-κB or AP1 molecules. Even though elevated glucocorticoid levels can protect hearing thresholds in noise exposure, it is not known if glucocorticoids affect OHC survival.

Figure 8. A summary of various factors affecting OHC survival in different traumas based on previous studies. Abbreviations JNK: c-Jun N-terminal kinase, ERK: extracellular signal-regulated kinase, NF-κB: nuclear factor κB, UPR: unfolded protein response, AP1: activator protein 1, ROS: reactive oxygen species, OHC: outer hair cell.
3 AIMS OF THE STUDY

Many stress signalling pathways are induced by traumas in the cochlea. However, the mechanisms how these pathways influence hearing loss are often not known. Understanding the spatio-temporal changes of trauma-induced stress signalling in different cochlear cell types brings us closer to deciphering how trauma-induced cochlear damage and hearing loss develop.

The principal aims of this thesis are listed below.

1. To study the role of c-Jun phosphorylation in the cochlear traumas: ototoxic drug-induced lesion, noise exposure, and systemic inflammation.
2. To study how other cochlear cells, namely the supporting cells and the cells of the lateral wall, contribute to the death of the OHCs.
3. To characterize Manf expression and the consequences of Manf inactivation in the inner ear using a genetic mouse model.
4 MATERIALS AND METHODS

4.1 LIST OF USED METHODS

This thesis is based on experiments done with methods listed in table 1. The article or articles where the method has been utilized is marked in the table. Detailed descriptions of the methods can be found in articles I-III.

Table 1. A list of used methods. Abbreviations X-gal: X-galactose, ABR: auditory brainstem response, DIC: differential interference contrast microscopy.

<table>
<thead>
<tr>
<th>Method</th>
<th>Used in publication</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genotyping</td>
<td>I, II, III</td>
</tr>
<tr>
<td>Immunohistochemistry and histology for paraffin sections</td>
<td>I, II, III</td>
</tr>
<tr>
<td>Immunohistochemistry for whole mount preparations</td>
<td>I, II, III</td>
</tr>
<tr>
<td>X-gal histochemistry</td>
<td>II, III</td>
</tr>
<tr>
<td>ApopTag staining kit</td>
<td>I</td>
</tr>
<tr>
<td>ABR measurements</td>
<td>III</td>
</tr>
<tr>
<td>Noise exposure</td>
<td>I, II</td>
</tr>
<tr>
<td>Lipopolysacharide (LPS) application</td>
<td>II</td>
</tr>
<tr>
<td>Kanamycin-furosemide application</td>
<td>I</td>
</tr>
<tr>
<td>Microscopy (transmitted light, DIC, fluorescence)</td>
<td>I, II, III</td>
</tr>
<tr>
<td>Fiji (Image J) and Photoshop for image processing</td>
<td>I, II, III</td>
</tr>
<tr>
<td>Excel and Origin for statistical analysis</td>
<td>I, II, III</td>
</tr>
</tbody>
</table>

4.2 MOUSE LINES

Different mouse lines from various inbred strains to reporter mouse lines and KO or knock-in models have a central role in the studies included in this thesis. The used mouse lines are listed in table 2. In our experiments, CBA (CBA/Ca) strain, which preserves low hearing thresholds in old age, was used and compared with BL6 (C57BL/6J) strain that is known for early-onset age-related hearing loss (Johnsson et al., 1997; Wang et al., 2002). In addition, the outbred mouse line CD-1, which is also characterized by early-onset hearing loss was utilized (Shone et al., 1991; Ohlemiller et al., 2016). These mouse lines were used as the background for different genetic modifications, such as NF-κB reporter or Manf KO. The noise vulnerability phenotype, namely the acute EP drop and the lateral wall pathology, of CBA mice is inherited in a dominant manner to the F1 hybrids of CBA and BL6 strains (Ohlemiller and Gagnon 2007).
Materials and methods

Gfi1-Gfp knock-in mice, which lose the majority of hair cells by 4 weeks of age, were used to study if functional OHCs are needed for activating the c-Jun phosphorylation after noise trauma. In these mutants, a green fluorescent protein (GFP)-producing gene knocks out the growth factor independent 1 transcriptional repressor (Gfi1) gene. JunAA/AA mice were utilized to find out if impeding the c-Jun phosphorylation in S73 and S63, by replacing them with alanines, decreases OHC loss after noise trauma. NF-κB reporter mice were used to study the transcriptional activity of NF-κB in different trauma paradigms. Finally, Manf KO mice were utilized to study the expression of Manf and its role in the cochlea. Conditional Manf KO, Pax2-Cre Manf\textsuperscript{flox-flox}, was used to verify the local function of MANF in the hair cell phenotype as the conventional Manf KO mice have hyperglycemia that could affect hearing (Lindahl et al., 2014). However, it is to be noted that Pax2 is expressed also in the female germline, so Cre-positive females used in breeding for the cKO mice, produce offspring with conventionally knocked of allele of Manf. The other Manf allele received from the male is conditionally knocked off (Cederroth et al., 2019a). We used principally Cre-positive males instead of females in the breeding of the cKO mice.

Table 2: A list of mouse lines, their origin and in which publications they were used. Abbreviations Gfi1: growth factor independent 1 transcriptional repressor, Gfp: green fluorescent protein, NF-κB: nuclear factor κB, Manf: mesencephalic astrocyte-derived neurotrophic factor, cKO: conditional knockout.

<table>
<thead>
<tr>
<th>Mouse line</th>
<th>Origin</th>
<th>Used in publication</th>
</tr>
</thead>
<tbody>
<tr>
<td>CBA/Ca (CBA)</td>
<td>Envigo</td>
<td>I, II</td>
</tr>
<tr>
<td>C57BL/6J (BL6)</td>
<td>Charles River Laboratories</td>
<td>I, II</td>
</tr>
<tr>
<td>CD-1 aka ICR</td>
<td>Envigo</td>
<td>III</td>
</tr>
<tr>
<td>Gfi1-Gfp knock-in</td>
<td>Yücel et al., 2004</td>
<td>I</td>
</tr>
<tr>
<td>JUNAA/AA in mixed background</td>
<td>Behrens et al., 1999</td>
<td>I</td>
</tr>
<tr>
<td>NF-κB reporter in BL6 or CBA background</td>
<td>Bhakar et al., 2002</td>
<td>II</td>
</tr>
<tr>
<td>Manf KO in CD-1 and CBA-CD-1-hybrid background</td>
<td>Lindahl et al., 2014; article III</td>
<td>III</td>
</tr>
<tr>
<td>Pax2-Cre Manf\textsuperscript{flox-flox} cKO in BL6 background</td>
<td>Ohyama and Groves, 2004; Lindahl et al., 2014</td>
<td>III</td>
</tr>
</tbody>
</table>

4.2.1 GENOTYPING

Mutant mouse genotypes were identified by genotyping ear mark samples taken at postnatal day (P) 21. In younger mice, tail samples taken at the time of cochlea preparation were used as DNA samples. Primers used for each polymerase chain reaction (PCR)-reaction are listed in table 3. Each PCR protocol was optimized by our laboratory technician Sanna Sihvo.
Materials and methods

Table 3. List of used primers for genotyping the mouse lines. Abbreviations Gfi1: growth factor independent 1 transcriptional repressor, Gfp: green fluorescent protein, NF-κB: nuclear factor κB, Manf: mesencephalic astrocyte-derived neurotrophic factor, KO: knockout.

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primers</th>
<th>Used in publication</th>
</tr>
</thead>
<tbody>
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<td>JunAA/AA</td>
<td>5'-CTC ATA CCA GGT CGC ACA GGC GGC-3'</td>
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</tr>
<tr>
<td></td>
<td>5'-CCG CTA GCA CTC AGG TTG GTA GGC-3'</td>
<td></td>
</tr>
<tr>
<td>Gfi1Gfp/Gfp</td>
<td>5'-CCC TTC TCT CAG AAC TCA GAG-3'</td>
<td>I</td>
</tr>
<tr>
<td></td>
<td>5'-GGA AAC GAG GTG GCT TGG AG-3'</td>
<td></td>
</tr>
<tr>
<td></td>
<td>EGFP: 5'-GTC TTG TAG TTG CCG TCG TC-3'</td>
<td></td>
</tr>
<tr>
<td>NF-κB reporter</td>
<td>5'-CTG CAG ATA ACT GCC GTC ACT CC-3'</td>
<td>II</td>
</tr>
<tr>
<td></td>
<td>5'-CTT AAT CGC CCT GCA GCA CAT-3'</td>
<td></td>
</tr>
<tr>
<td>ManfKO</td>
<td>F: 5'-TGG AGT GAG CAC AAC TCA GG-3'</td>
<td>III</td>
</tr>
<tr>
<td></td>
<td>WT: 5'-GGC TTC GAC ACC TCA TTG AT-3'</td>
<td></td>
</tr>
<tr>
<td></td>
<td>KO: 5'-CCA CAA CGG GTT CTT CTC GT-3'</td>
<td></td>
</tr>
<tr>
<td>Manflox/lox</td>
<td>5'-TGG AGT GAG CAC AAC TCA GG-3'</td>
<td>III</td>
</tr>
<tr>
<td>Cre</td>
<td>5'-CTG TTT CTG AGC ATA CCT GGA-3'</td>
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</tr>
<tr>
<td></td>
<td>5'-AAT CTC CCA CCG TCA GTC AG-3'</td>
<td></td>
</tr>
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</table>

4.3 NOISE EXPOSURE AND ABR MEASUREMENTS

Noise at 8-16 kHz for various exposure times (1, 2 or 4 hours) and at different noise levels (85, 91, 105, 110, and 116 dB SPL) were used in our studies. Noise exposure was used to study stress signalling and OHC loss in the traumatized cochlea. Noise exposure at 105 dB SPL for 2 hours is well characterized in CBA mice. It is known to cause OHC loss and synaptopathy in the basal part of the cochlea, while no OHC loss takes place at the frequency region of the noise-band (Hirose and Liberman, 2003; Ohlemiller and Gagnon, 2007). In addition, a noise pre-conditioning experiment with 1 hour of 91 dB SPL noise followed 12 hours after by 1 or 4 hours of 105 dB SPL noise, was used to study if stress signalling is altered by pre-conditioning. Hearing measurements were made by recording auditory brainstem responses (ABR). ABRs were measured with clicks and tone pip stimuli at several frequencies (4, 8, 16, 23, 32, 40, and 45 kHz) starting from 90 dB SPL at 5 dB steps. For a detailed explanation of noise exposure see article I and ABR measurements see article III.

4.4 KANAMYCIN-FUROSEMIDE AND LPS APPLICATIONS

Systemic kanamycin-furosemide (see article I) injections and application of LPS (see article
II) were used to study the effects of systemic inflammation and ototoxic lesion in the cochlea respectively. To induce an ototoxic drug-induced lesion, kanamycin was given as a single subcutaneous injection (1 mg/g) followed 30 minutes later by a single intraperitoneal injection of furosemide (0.4 mg/g). LPS was given as a single intraperitoneal injection (1 mg/kg). Mice were sacrificed 3 hours after LPS injection or the injection was followed by noise exposure 2 hours after injection, and the mice were sacrificed immediately after noise exposure.

4.5 IMMUNOHISTOCHEMISTRY

Immunohistochemistry has a substantial role in the experiments of this thesis. Stainings were done with both peroxidase-based avidin-biotin complex (ABC)-staining and fluorescence methods on whole mount preparations and paraffin sections of the cochlea. The primary antibodies used in this thesis are listed in table 4. In whole mount preparations, DAPI for nuclei, and phalloidin for filamentous actin, were used as markers of basic cochlear morphology. Hematoxylin-eosin staining was used as a basic histological staining method to study the morphology of the cochlea in paraffin sections. Methyl green and Nuclear fast red were utilized as counter stains in immunohistochemistry. ApopTag kit was utilized to identify apoptotic fragments in paraffin sections of the cochlea. X-gal immunohistochemistry was used to identify the cells expressing LacZ-construct in NF-κB reporter mice and heterozygous Manf KO mice.
Spacial data of molecular changes in the tissue can be obtained with immunohistochemistry. Importantly, with immunohistochemistry cell type specific information on the activation of stress signalling can be achieved. The cochlea contains few proper sensory cells. In the murine cochlea there are approximately 800 IHCs and 2400 OHCs, and various other cell populations. Thereby, using biochemical assays on whole cochleas gives little information on the processes taking place in the sensory cells. In addition, it is hard

<table>
<thead>
<tr>
<th>Antibody against</th>
<th>Host</th>
<th>Cat #</th>
<th>Manufacturer</th>
<th>Used in publication</th>
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<tr>
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<td>5335</td>
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<td>Calreticulin</td>
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<td>Casp3</td>
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<td>9579</td>
<td>Cell Signaling Technology</td>
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<tr>
<td>CHOP</td>
<td>mouse</td>
<td>NB600-1335SS, clone 9C3</td>
<td>Novus Biologicals</td>
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<td>c-Jun</td>
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<td>WAKO</td>
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<td>310-100</td>
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<td>AB5539</td>
<td>Millipore</td>
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<td>p-c-JunS63</td>
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<td>p-ERK1/2</td>
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<td>SOX2</td>
<td>goat</td>
<td>5603</td>
<td>Santa Cruz Biotechnology</td>
<td>I</td>
</tr>
</tbody>
</table>
to separate specific cell populations from the adult organ of Corti, because the structure is very rigid in comparison with developing tissue (Anttonen et al., 2017).

Specificity of antibodies is important in immunohistochemistry. In this thesis, the specificity of the antibodies for c-Jun S73 and S63 and MANF were tested in appropriate KO-tissue, JunAA/AA and Manf KO, respectively. For GRP78 antibody, a preadsorption assay with the manufacturer’s immunizing peptide was utilized to confirm the lack of unspecific binding of GRP78 antibody. It is important to note that the preadsorption assay does not give information on the specificity of the antibody since even though specific binding to the antigen in question is shown, it does not reveal if the antibody would also bind to unspecific targets. The best way to prove the specificity of an antibody is to use KO tissue. Next best option would be to use KO cells. When the antigen of interest has been knocked out, no staining should be detected.

Enzymatic ABC-detection is a sensitive method for immunohistochemistry. It uses a biotinylated secondary antibody and ABC with horseradish peroxidase to amplify the signal. Typically, an antibody used both in fluorescence- and ABC-technique requires a more concentrated dilution of the primary antibody to work in fluorescence staining. For example, GRP78 antibody was used with 1:1000 dilution for fluorescent staining in whole mount preparations and 1:10’000 dilution for ABC-staining in paraffin sections in the experiments of this thesis. Four parallel stainings in one sample with fluorescence staining were done in our experiments. In paraffin sections, parallel sections were used for multiple stainings.

Some antibodies do not work in tissues after the heavy processing needed for paraffin embedding: removal of paraffin, rehydration, antigen retrieval, and dehydration, and also H₂O₂ treatment to abolish unspecific peroxidase activity in the tissue if ABC-detection is used. These antibodies might work better in whole mount preparations. For example, MANF antibody worked only in whole mount preparations for us. Cochlea is also a bony structure and adult murine cochleas need a decalcification step with ethylenediaminetetraacetic acid (EDTA) before whole mount dissection or sectioning. EDTA treatment can interfere with binding of certain antibodies. It is likely to interfere with caspase 3 staining that for us worked in developing cochleas with soft bone, but not in adult tissue where EDTA was utilized.

Binding of the antibodies in the tissue can be influenced with changes in pH. In this thesis, a buffer with pH 6 was used in the antigen retrieval step. For antigen retrieval, heating in a microwave worked better than a pressure kettle for the phosphorylated antigens in our experiments. Staining of phosphorylated-antigens, c-Jun S73, c-Jun S63 and ERK1/2, were done with tris-buffered saline (TBS) to avoid phosphates in the phosphate-buffered saline (PBS). For MANF and CHOP staining, a shorter fixation time, 2 hours at room temperature, was used instead of overnight at 4°C that was used for other antibodies.
5 RESULTS AND DISCUSSION

The results of this thesis are divided in four parts. First, trauma-induced stress responses are studied in the organ of Corti where the vulnerable OHCs reside. Many cell types in the organ of Corti activate stress signalling pathways upon trauma, but only OHCs die. Second, the role of stress responses in the lateral wall, spiral limbus, and SG neurons are addressed. Are these cochlear compartments related to OHC death? Third, the outcome of MANF-depletion in the inner ear by studying Manf KO mice is explained. Fourth, the influence of genotype on trauma-induced stress signalling and the phenotype of Manf inactivation is considered. In the Results-section, stress responses were studied in CBA mouse strain if not otherwise indicated.

5.1 TRAUMA-INDUCED STRESS RESPONSES IN THE ORGAN OF CORTI (I,II)

We used multiple approaches to study stress in the cochlea ranging from ototoxic compounds, inflammation causing LPS, genetic models to noise exposure. The trauma paradigms can be divided into two categories: acute and chronic. Genetic modifications causing OHC death, and ageing can be considered as chronic stressors while noise, ototoxic compounds and inflammation are acute stressors. A common feature for all these trauma paradigms was that OHCs in the basal part of the cochlea were the most vulnerable cells. However, they were not the only cell type to raise stress responses. Therefore, the question was whether stress responses in other than the vulnerable cell type promote cell death in a paracrine manner or if the activation of stress signalling pathways could have intrinsic survival promoting effects. Since the vulnerable OHCs reside in the organ of Corti, trauma-induced stress responses activated in the organ of Corti are considered first.

5.1.1 C-JUN PHOSPHORYLATION IN THE TRAUMATIZED ORGAN OF CORTI

Antagonizing JNK signalling in the cochlea is known to attenuate hearing loss and OHC death caused by ototoxic antibiotics and noise (Pirvola et al., 2000; Ylikoski et al., 2002; Wang et al., 2007). The protection in those studies was induced with small molecules inhibiting JNK signalling. We provided molecular details of this protection with a genetic mutation model, JunAA/AA mice (I: Fig 10). We showed that the protection is mediated, at least in part, by JNK/c-Jun interaction. This demonstration is important, because JNKs could also have a c-Jun-
independent role in the cochlea, as shown in other tissues (reviewed by Coffey, 2014). We showed that OHC loss after noise exposure is attenuated by preventing c-Jun phosphorylation. We used a genetic mouse model, JunAA/AA mice, where the serines 73 and 63 are replaced by alanines, thereby preventing c-Jun phosphorylation by JNKs. It is to be noted that the protection against noise exposure was not complete; JunAA/AA mice displayed approximately 50% reduction in noise-induced OHC loss compared with littermate controls (I: Fig 10).

To further explore the mechanism of c-Jun phosphorylation-mediated OHC death, we studied the spatial and temporal pattern of c-Jun phosphorylation with immunohistochemistry in the cochlea. This was important, because the protection studies mentioned above did not address the cellular mechanisms. We found c-Jun phosphorylation in the organ of Corti acutely after noise exposure (I: Fig 3,4,7; II: Fig 3A,E) and after kanamycin-furosemide application (I: Fig 5,9). c-Jun is an immediate early gene and, thus, it is not surprising that it responds rapidly to traumas (Angel et al., 1988). c-Jun phosphorylation was also rapidly downregulated after its initial activation. Following kanamycin-furosemide application, c-Jun phosphorylation was downregulated by 60 hours after injections (I: Fig 9). In response to noise exposure, the phosphorylation was downregulated already 12 hours after noise exposure and it showed pre-noise levels by 24 hours post-exposure (I: Fig 3). We did not observe c-Jun phosphorylation in the organ of Cort in response to inflammation, in ageing or in response to Manf inactivation (unpublished results). However, since ageing and Manf inactivation cause OHC loss in a chronic manner, it is possible that we have missed the time of the dynamic stress-induced c-Jun phosphorylation in the cochlea.

Kanamycin-furosemide application caused the death of the majority of OHCs by 60 hours post-injections (I: Fig 9C). Noise exposure at 8-16 kHz, for 2 hours at 105 dB SPL, caused a severe OHC death, progressing from the most basal part of the cochlea up to the 32 kHz region. Less than half of the total amount of cochlear OHCs died in our noise exposure paradigm. Intriguingly, both after the ototoxic lesion and noise trauma, OHCs were practically the only cell type of the organ of Corti that lacked c-Jun phosphorylation. c-Jun phosphorylation was observed in IHCs, pillar cells, Deiters’ cells, and Hensen’s cells along the whole cochlear duct after kanamycin-furosemide application. The same cell types displayed c-Jun phosphorylation following noise exposure, but this phosphorylation was seen only in the basal part of the cochlea where OHC loss also took place. Therefore, we concluded that c-Jun phosphorylation in the supporting cells of the organ of Corti promotes OHC death in a paracrine manner.

In addition to this suggested role of JNK/c-Jun signalling promoting OHC death, the activation of this signalling might have other roles. A dynamic c-Jun phosphorylation pattern was evident in the cells of the organ of Corti following traumas. In the IHCs and supporting cells adjacent to IHCs (inner border cells and inner phalangeal cells), phosphorylation
Results and discussion

coincided temporally and spatially with synaptopathy. We did not ourselves quantify the synapse loss between IHCs and SG neurons, but we used the same noise paradigm and mouse strain as have been used in the detailed synaptopathy studies by the Liberman group (Kujawa and Liberman, 2009). We found phosphorylation in the supporting cells adjacent to IHCs already one hour after noise exposure. It is known that a large part of synaptopathy has already taken place at this acute post-exposure time point (I: Fig 3; Kujawa and Liberman, 2009). One reason behind synaptopathy has been suggested to be excitotoxicity, the inability of the supporting cells around IHCs to handle the large amount of glutamate neurotransmitter that is exocytosed from IHCs into the synaptic cleft upon sound stimulation (Puel et al., 1998). It is possible that this increased glutamate uptake causes activation of JNK/c-Jun stress signalling in the supporting cells. There are only a few afferent synapses between OHCs and SG neurons and thus excitotoxicity is not a major problem in this location. Consequently, the trigger for JNK/c-Jun activation in Deiters’ cells is likely not to be the excess of glutamate. Also, excessive glutamate release from IHCs would presumably activate the auditory pathway, which should not take place in the ototoxic drug paradigm nor in the basal, high frequency region of the cochlea in the noise exposure paradigm. Since we did not study synaptopathy in the JunAA/AA mice, we cannot say if c-Jun phosphorylation promotes synaptopathy.

Hormesis means that a small amount of a detrimental factor can protect from the following stronger stressor. This has been shown in the cochlea in experiments where a low-level noise exposure preceding a more intense noise exposure protects hearing (Canlon et al., 1988). We studied c-Jun activation (phosphorylation) in the cochlea in relation to sound pre-conditioning. Our pre-conditioning noise paradigm, 91 dB SPL for 1 hour, does not cause OHC loss. However, it raised a robust c-Jun phosphorylation response in the supporting cells of the organ of Corti. We then studied the outcome of this pre-conditioning noise followed 12 hours afterwards with a damaging noise exposure (105 dB SPL for 1 or 4 hours). Interestingly, this paradigm decreased c-Jun phosphorylation in supporting cells. Even more interestingly, the paradigm raised c-Jun phosphorylation in OHCs, but only in the medial part of the cochlea where OHCs do not normally die following noise (I: Fig 6, 7F-G). These results show that cell-intrinsic c-Jun phosphorylation in OHCs does not cause cell death. Therefore, it appears that in addition to mediating cell death, JNK/c-Jun signalling has additional roles in the cochlea. We have speculated that, according to the hormesis concept, JNK/c-Jun stress signalling becomes detrimental (leading to cell death) when its levels exceed a threshold level and this level varies depending on many factors.

The only case when we witnessed c-Jun phosphorylation in degenerating OHCs was the Gfi1-mutant model where differentiating OHCs die soon after birth (I: Fig 2). We believe that this c-Jun activation in Gfi1-depleted OHCs is linked to the developmental status of these
Results and discussion

cells, as their differentiation path is perturbed. As said, c-Jun was not phosphorylated in the adult, degenerating OHCs following ototoxic or noise traumas. We confirmed this by studying c-Jun phosphorylation immediately after 15 minutes of exposure to 105 dB SPL noise. We found c-Jun activation in other, non-vulnerable cells after this paradigm, but not in OHCs. By decreasing noise levels, we found that exposure to 85 dB SPL (1 hour) was a sound level that no more triggered c-Jun activation (I: Fig 7E,F).

Also, when comparing the CBA and BL6 mice at the age of 2 months (II: Fig 1), we observed c-Jun phosphorylation in the supporting cells of both strains after noise exposure (II: Fig 3A,E). At this young adult age, there was a four-fold greater OHC loss in CBA compared with BL6 mice after noise exposure (II: Fig 1). The CBA strain becomes more resistant to noise at older age, shown in our unpublished experiments where 8-to-10-months-old mice were exposed to noise (2 hours at 105 dB SPL). There was no OHC loss (23±24, total OHC loss±SD, n=3) at this age. Despite the lack of OHC loss, the noise-induced c-Jun phosphorylation pattern in the older organ of Corti was comparable to that of the younger organ (Fig 9C’’,F’’; I: Fig 1, 2). Together, these observations point to the conclusion that c-Jun phosphorylation in the organ of Corti has roles additional to the role in mediating OHC death.

5.1.2 ERK1/2 PHOSPHORYLATION IN THE TRAUMATIZED ORGAN OF CORTI

Another stress-related MAPK that is upregulated in the organ of Corti upon noise exposure and kanamycin-furosemide application is ERK1/2. Phosphorylated ERK1/2 was found in the synaptic region of both IHCs and OHCs in the control, non-traumatized mice (II: Fig 5A-D). It likely has a physiological role in the hair cell synaptic region. ERK2 KO mice have been shown to be more vulnerable to noise than littermate controls, suggesting that ERK2 has a protective role in noise exposure (Kurioka et al., 2015). We observed that exposure to noise upregulates ERK1/2 phosphorylation in Deiters’ and pillar cells in the basal part of the cochlea (II: Fig 5). After the ototoxic drug lesion, ERK1/2 phosphorylation was similarly detected from the Deiters’ and pillar cells, but unlike after noise exposure, these cells expressed ERK1/2 phosphorylation along the whole cochlear duct. Though the antibody stained most likely unspecifically the microtubule-rich upper part of pillar cells, it was detected from the pillar cell nuclei after noise exposure, which is likely to be specific staining. The rich cytoskeleton of pillar cells has been shown to non-specifically bind to other polyclonal antibodies as well (Laos et al., 2017).

ERK1/2 phosphorylation had the same temporal pattern as c-Jun phosphorylation after
Results and discussion

trauma, it was rapidly upregulated upon noise exposure and was then dynamically downregulated. ERK1/2 phosphorylation in Deiters’ cells after trauma has been shown to cause OHC death in neonatal cochlear explant cultures (Lahne and Gale, 2008). In accordance with this finding, we observed ERK1/2 phosphorylation in a more restricted region in the basal high frequency area of cochlear duct than c-Jun phosphorylation after noise exposure. The OHC death was also centered at this region.

MAPKs JNK, ERK1/2 and p38 have all been shown to phosphorylate pro-apoptotic molecules (Lei and Davis, 2002). It could be that both JNK/c-Jun and ERK1/2 activity in the supporting cells of the organ of Corti are needed for a death-promoting activity of MAPK signalling to take place. Still, in the older CBA mice, both signalling pathways were active in the organ of Corti after noise exposure and no OHC loss followed (Fig 9C’’,F’’,I’’). It could be that the activity of MAPKs promotes cell death in a context-dependent manner since MAPK activity alone does not cause OHC loss, and the cells activating these pathways do not die themselves in our trauma paradigms.

5.1.3 TRANSCRIPTIONAL NF-KB ACTIVITY IS ABSENT FROM THE NOISE-EXPOSED ORGAN OF CORTI

We studied NF-κB activation with a genetic reporter model where LacZ-construct is placed after NF-κB binding sites so that β-galactosidase, is produced only when NF-κB is promoting transcription in the cell (Bhakar et al., 2002). With this method we cannot study the increases or decreases of NF-κB expression. We did not detect NF-κB transcriptional activity in the organ of Corti in any trauma paradigm we studied. NF-κB has many roles in cell survival and death but in the time points we studied it after noise exposure (acutely and 12 hours after exposure) or in the ageing BL6 cochlea, we did not detect its activity in the organ of Corti (II: Fig 6,8). Our results after noise exposure support the results of Adams and colleagues (2000) who did not observe transcriptional NF-κB activity in the organ of Corti after noise exposure.

5.1.4 SYSTEMIC INFLAMMATION DOES NOT RAISE STRESS RESPONSES IN THE ORGAN OF CORTI

Systemic LPS injection did not activate c-Jun or ERK1/2 phosphorylation in the organ of Corti. LPS activated c-Jun phosphorylation in the lateral wall but this effect did not cause cell death in the organ of Corti with the LPS concentration (1 mg/kg) used in our studies. Application of LPS prior to noise exposure did not alter the c-Jun phosphorylation taking place in the organ of Corti. In previous studies LPS has been shown a detrimental effect on hearing function (Zhang et al., 2015). Our studies did not reveal stress responses in the cells of the
organ of Corti, but it is possible that the detrimental effect of LPS affects hearing by disturbing the cells of stria vascularis and their function to maintain the ionic composition of the endolymph (see section 5.2.4).

5.2 COCHLEAR STRESS OUTSIDE OF THE ORGAN OF CORTI (I,II,III)

In the previous section it was noted that the stress signalling pathways do not need to be activated in the vulnerable cells themselves in order to influence the outcome of cell survival. In this section, the focus is on the non-sensory mesenchymal cells of the cochlea residing either in the lateral wall or in the spiral limbus, and in the cochlear neurons and the vestibular hair cells. Many types of fibrocytes with distinct roles in hearing function are found in the lateral wall. Stria vascularis is a fundamental structure for the auditory function and responsible for maintaining the unusual ion concentration of the endolymph required for the mechanotransduction of hair cells. Interdental cells in the spiral limbus also have a role in the maintenance of the endolymph.

We have studied trauma-induced stress responses in different cochlear compartments. OHC survival can be compromised through damage in these compartments. For example, a genetic mouse model lacking intermediate cells and hence possessing a dysfunctional stria vascularis has shown that OHC survival depends on a functional stria vascularis (Liu et al., 2016). Furthermore, in the cochlea, blood circulation is mainly located in the stria vascularis, the spiral ligament and the modiolus, hence systemic effectors reach the cochlea through these sites.

5.2.1 TRAUMA-INDUCED ERK1/2 AND C-JUN PHOSPHORYLATION IN THE SPIRAL LIGAMENT

We observed a progressive c-Jun phosphorylation in the lateral wall spiral ligament upon exposure to noise. Phosphorylation was first noted in the root cells in the basal part of the cochlea after 1 hour of noise exposure. By 4 hours, c-Jun phosphorylation was no longer detected in the root cells. Instead, it had reached fibrocyte types I and II (I: Fig 3; II: Fig 3AD). Another MAPK, ERK1/2, was phosphorylated after noise exposure in the lateral wall fibrocytes in the same manner as c-Jun. By 12 hours after noise exposure, only type III fibrocytes still showed ERK1/2 phosphorylation (II: Fig 5E-G). By 24 hours after noise exposure, all MAPK activation was back to pre-noise levels (I: Fig 3G). With Gfi1-mutant mice lacking hair cells at 4-weeks of age, we studied whether the MAPK activation in the noise-exposed lateral wall requires functional hair cells (I: Fig 3I-J). No c-Jun phosphorylation
Results and discussion

was detected acutely after noise exposure in either the supporting cells of the organ of Corti or in the root cells in the lateral wall. Therefore, we concluded that OHCs are needed for the lateral wall stress response to take place in noise exposure.

Since the lateral wall stress response, c-Jun and ERK1/2 phosphorylation, took place in the basal, high frequency part of the cochlea, where OHC loss is centered, we wondered if it is linked to OHC death. Older CBA mice, 8-to-10-months-old, are more resistant to OHC loss than at the age of 2-months. However, a robust lateral wall stress response was seen also in this older age group (Fig 9A-C’,D-F’,G-I’). Therefore, we concluded that c-Jun and ERK1/2 phosphorylation in the lateral wall do not explicitly promote OHC death. However, it is still possible that the death-promoting effect is context-dependent, similarly as concluded with the phosphorylation in the organ of Corti. We have not been able to antagonize c-Jun phosphorylation selectively in the organ of Corti or in the lateral wall, hence we cannot state which part is responsible for the partial protection from OHC loss observed in noise-exposed Jun^{AA/AA} mice (I: Fig 10).
## Results and discussion

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### J

**ABR before and 6 d after 2 h 105 dB SPL**

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</table>

- 2 mo control (n=6)
- 2 mo noise (n=5)
- 8-10 mo control (n=5)
- 8-10 mo noise (n=3)

### K

**Ratio of round/total lateral wall macrophages 12 h after 2h 105 dB SPL**

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### L

**Iba1**

- Control
- Noise

![Image](image14.png)
Results and discussion

Figure 9. Comparing noise-induced stress responses and hearing loss in young, 2-months-old, and older, 8-to-10-months-old, CBA/Ca mice. Non-noise-exposed control mice are stained with c-Jun (A), p-c-JunS73 (D) and p-ERK1/2 (G) antibodies respectively, 2-months-old mice acutely (0-2 hours) after exposure to 2 hours 105 dB SPL are stained for c-Jun (B), p-c-JunS73 (E) an p-ERK1/2 (H). 8-10 months-old mice exposed to 105 dB SPL for 2 hours and studied acutely (0-2 hours) after exposure are stained for c-Jun (C-C''), p-c-JunS73 (F-F'') an p-ERK1/2 (I-I''). The images were taken from paraffin sections of the basal, high frequency region of the cochlea. A diagram represents ABR measurements of 2- and 8-to-10-months-old mice without noise exposure and 6 d after exposure to 2 hours 105 dB SPL (J). The ratio of round macrophages versus total macrophage counts from the lateral wall spiral ligament of the basal part of the cochlear whole mount preparations stained with Iba1 were compared between young and older mice (K). Representative regions of the lateral wall macrophages are stained with Iba1 in the whole mount preparations of control (L) and noise-exposed (M) 8-to-10-months-old mice. Arrow in M points to a macrophage with round morphology. Scale bar in A is 50 µm for A-C,D-F,G-I; 30 µm for C',C'',F',F'',I',I'',L,M. Abbreviations ERK1/2: extracellular signal-regulated kinase 1/2, ABR: auditory brainstem response, SPL: sound pressure level, Iba1: ionized calcium binding adaptor molecule 1. Unpublished data.

Interestingly, c-Jun and ERK1/2 phosphorylation in the lateral wall, 2 hours after exposure to noise, coincide temporally with the reduction of blood flow through the stria vascularis, which has been shown to be reduced 2 hours after exposure to 106 dB SPL noise for 30 minutes (Arponchayanon et al., 2011). Reduced blood flow could cause the stress response in the lateral wall, since the energy demand of the cochlea is likely to be augmented during noise exposure. Noise exposure interrupts oxygen and nutrient supply in the cochlea and disturbs the elimination of waste products (Dai and Shi, 2011). Lateral wall cells could activate intracellular stress responses in response to these changes.

As for systemically applied stressors, we noticed that kanamycin-furosemide application caused a robust c-Jun phosphorylation response in the lateral wall. By 2 hours after the injections, c-Jun phosphorylation was detected from root cells and fibrocyte types I-III (I: Fig 2,9). Also, ERK1/2 phosphorylation was detected from the lateral wall fibrocytes (unpublished results). Unlike c-Jun phosphorylation that was observed throughout the whole cochlear duct, ERK1/2 phosphorylation was restricted to basal part of the cochlea. By 20 hours after the application, c-Jun phosphorylation was not anymore seen in root cells and by 60 hours after the injections, c-Jun phosphorylation in the lateral wall fibrocytes had returned to pre-trauma levels.

Another systemically applied trauma paradigm was inflammation caused by an intraperitoneal injection of LPS. LPS alone activated c-Jun phosphorylation in the endothelial cells of the capillaries in the whole cochlea, including the lateral wall (II: Fig 3I-K). LPS application followed by noise exposure potentiated the noise-induced c-Jun phosphorylation response in the lateral wall as c-Jun phosphorylation was seen simultaneously in root cells and lateral wall fibrocytes (II: Fig 3L-O). c-Jun phosphorylation in the lateral wall fibrocytes was detected only in the basal part of the cochlea as with the noise exposure paradigm.

MAPK activity after noise exposure and ototoxic drug-induced lesion seems to follow the gap junctional system in the lateral wall (I: Fig 3H). The gap junctional syncytium is a way for signalling molecules to spread (reviewed by Jagger and Forge, 2015). The stress signal
could be, for example, changes in K⁺ recycling (reviewed by Jagger and Forge, 2015), depletion of ATP (Nagashima et al., 2011) or cytokines (Fujioka et al., 2006). Extracellular ATP in the endolympathic compartment decreases EP through the action of the ATP-gated ion channels (P2X₂ receptors). Activation of these receptors leads to increased K⁺ influx into the cells and reduces EP. ATP is released to the endolymph in noise exposure, and hence causes the noise-related decrease in EP. In the organ of Corti, a large amount of P2X₂ receptors is found in the stereocilia, hence also the cells in the organ of Corti could sense changes in extracellular ATP levels. K⁺ influx into the cells lining the endolymph could cause the MAPK stress response that we have observed after noise exposure. In addition, ATP acts on P2Y receptors on marginal cells in the stria vascularis regulating the K⁺ secretion into the endolymph (reviewed by Housley et al., 2002). Decreased EP causes hearing loss, which may be transient, but it can also lead to OHC loss (Liu et al., 2016).

5.2.2 NF-KB, FOXO3A, AND MACROPHAGES IN THE TRAUMATIZED SPIRAL LIGAMENT

We detected NF-κB transcriptional activity in the lateral wall fibrocyte types I-III by 12 hours after noise exposure (II: Fig 6E-I). At this time point, the MAPK signalling was downregulated in the lateral wall fibrocytes. There was no NF-κB activity in the control, non-noise-exposed, lateral walls. Adams and colleagues (2000) found NF-κB transcriptional activity in the lateral wall fibrocytes type I 24 hours after noise exposure for 2 hours at 100 dB SPL. Therefore, NF-κB transcriptional activity may continue for a long time after noise exposure though we have not studied other time points. All activation of stress signalling in the lateral wall was detected in the basal, high frequency part of the cochlear duct.

Even though NF-κB has a central role in immune responses, we did not detect transcriptional NF-κB activity in the cochlear lateral wall after application of LPS (n=3, 12 hours after trauma, unpublished results). Adams and colleagues (2000) saw NF-κB activity with systemic LPS application in the type II fibrocytes. Since Adams and colleagues used a larger dose of LPS, 50 ng-200 µg per mouse, than us, approximately 20-to-30 ng per mouse, it could be that only a more robust immune response activates NF-κB transcriptional activity in the cochlear lateral wall.

Age-related spiral ligament stress responses were studied in BL6 mice that have early-onset age-related hearing loss. Fibrocytes behind stria vascularis showed transcriptional NF-κB activity (II: Fig 8H). Upregulation of NF-κB transcriptional activity in ageing has been shown in other tissues as well and this upregulation has been suggested to drive cell senescence (reviewed by Tilstra et al., 2011). In younger mice, we detected NF-κB activity in
the spiral ligament only after noise exposure and this activity was minor in BL6 mice compared to CBA mice (II: Fig 6I, 8H).

We detected forkhead box class O 3a (FoxO3a) expression in root cells. FOXO3a is known to antagonize oxidative stress in other tissues (reviewed by Storz, 2011). Furthermore, oxidative stress has been observed in the spiral ligament after noise exposure and kanamycin-furosemide application (Yamaguchi et al., 2014). However, our results showed that the FOXO3a expression was present also prior to noise and did not change after noise exposure (II: Fig. 4). This shows that FOXO3a is used in root cells for normal cochlear physiology, but it does not exclude the possibility that oxidative stress could be initiating other stress responses in the cochlear lateral wall.

Another stress response that we detected in the lateral wall 12 hours after noise exposure, was a morphological change in the lateral wall macrophages in the basal part of the cochlea. At this time point, the total amount of macrophages was not increased so we were studying the tissue resident macrophages of the lateral wall. We observed that one fifth of the basal lateral wall macrophages had changed their morphology from a ramified to a round phenotype (Fig 9K-M; I: Fig 7). Cytokine production has been shown to take place in the lateral wall fibrocytes after noise exposure. TNF-α, IL-1β and IL-6 upregulation in the cochlea have been observed acutely, 3-6 hours after noise exposure, in the fibrocytes type III and IV, not in the tissue resident macrophages (Fujioka et al., 2006). Therefore, it could be that the stress responses in the lateral wall fibrocytes (c-Jun and ERK1/2 phosphorylation) trigger cytokine release and cause the morphological change of the tissue resident macrophages.

5.2.3 SUMMARY OF SPIRAL LIGAMENT STRESS RESPONSES
Do the spiral ligament stress responses lead to OHC death? Minowa and colleagues (1999) have shown that in Brn-4 KO mice fibrocyte type I-III damage and severely augmented hearing thresholds take place in the absence of hair cell loss. They concluded that the fibrocyte damage disrupts K⁺ transport resulting in decreased EP, 38-39 mV, and elevated hearing thresholds in 11-weeks-old KOs. Notably, the fibrocytes did not die but displayed an altered phenotype (Minowa et al., 1999). We did not observe morphological changes or cell death in spiral ligament fibrocytes in our trauma paradigms, but our observations were only at the light microscopical level and thus we might have missed some subcellular changes.

The temporal sequence of the stress signalling in the noise-exposed spiral ligament studied in this thesis is 1) c-Jun and ERK1/2 phosphorylation, 2) NF-κB transcriptional activity and morphological change of macrophages. It is possible that, as with glucocorticoids (reviewed by Sapolsky et al., 2000), some of the many stress signalling pathways activated upon trauma
are preordained for enduring the original stress responses initiated by the other signalling pathways. For example, ERK1/2 has been suggested to inhibit gap junctional signalling (Brandes et al., 2002). In the cochlea, the inhibition of the gap junctional system would interfere the spread of stress signals in the lateral wall and could in this way mitigate the stress responses both in the organ of Corti and in the lateral wall. Additionally, inflammatory responses are well known to cause tissue damage if sustained for long periods, so maintaining their levels promotes homeostasis (reviewed by Mittal et al., 2014). The stress responses of the spiral ligament and their relation to hearing loss have just begun to be understood.

### 5.2.4 STRESS RESPONSES IN THE STRIA VASCULARIS

We detected noise-induced c-Jun phosphorylation in the stria vascularis in the basal cells and in a part of the intermediate cells, 2 hours after noise exposure, in the basal part of the cochlea. After 4 hours of 105 dB SPL noise exposure also marginal cells showed c-Jun phosphorylation (I: Fig 3; II: Fig 3D). We discovered that with kanamycin-furosemide application all cells in the stria vascularis displayed c-Jun phosphorylation (I: Fig 5). LPS caused c-Jun phosphorylation in all cells in the stria vascularis, including the endothelial cells of the capillaries that are also phosphorylated throughout the cochlea (II: Fig 3I-N). We detected the stress response along the whole cochlear duct with both systemically applied traumas; ototoxic drug lesion and LPS. Noise exposure following LPS injection in the LPS-noise paradigm did not change the c-Jun phosphorylation in the stria vascularis.

We did not detect NF-κB transcriptional activity in the stria vascularis in control mice. However, in 10-months-old BL6 mice, the stria vascularis is degenerated and the marginal cells show NF-κB activity (II: Fig 8). In addition, some of the melanin-containing, perivascular macrophage-like cells in the stria vascularis displayed transcriptional NF-κB activity. In the aged stria vascularis these macrophage-like cells contained a greater amount of melanin pigment compared with these cells in a 2-months-old BL6 (II: Fig 8A-G). We did not detect c-Jun or ERK1/2 phosphorylation in the aged cochleas (unpublished results).

The stress responses activated in the cells of the stria vascularis did not seem to lead to cell death, at least cell-intrinsically, in our trauma paradigms. Manf inactivation also caused accumulation of ER stress markers in the marginal cells but no cell death was observed in the stria vascularis (III: Fig 4M-S). However, since the stria vascularis has a fundamental role in maintaining the EP and hence hearing function, stress in these metabolically highly active cells could impede their function. Stria vascularis contains a vast microvasculature (Fig 4A), so systemic effectors circulating in the blood stream can affect this cochlear structure directly, provided they pass the BLB. Kanamycin-furosemide, LPS, and ageing could affect stria
vascularis directly through the blood circulation.

How is noise sensed in the stria vascularis? Hearing requires more extensive work from the strial cells, which could cause intracellular stress. In addition, noise-induced disturbances in blood flow in the stria vascularis coincide temporally with the c-Jun phosphorylation we observed in the basal-most part of the cochlea (I: Fig 3E-F). After exposure to 106 dB SPL noise for 30 minutes, reduced red blood cell velocity in the stria vascularis 2 hours after noise exposure has been detected in guinea pigs and it was further reduced up to 3.5 hours after the exposure (Arpornchayanon et al., 2011). Finally, disturbed recycling of K⁺ through the spiral ligament or other signalling molecules coming from the spiral ligament (reviewed by Jagger and Forge, 2015) could cause the stress responses we have observed in the stria vascularis.

5.2.5 SPIRAL LIMBUS STRESS RESPONSES

The interdental cells and fibrocytes in the modiolus have received, in general, less interest than the lateral wall fibrocytes and marginal cells. Still, as interdental cells serve similar functions as marginal cells in the cochlea, we studied stress responses in the spiral limbus upon different trauma paradigms as well. We detected an acute c-Jun phosphorylation in the interdental cells and ERK1/2 phosphorylation in the spiral limbus fibrocytes upon noise exposure (unpublished results). Interestingly, both MAPK phosphorylation events took place in the medial part of the cochlea, unlike MAPK activation in the organ of Corti or in the lateral wall, which were focused in the basal part of the cochlea where OHC loss took place. It has been shown that spiral limbus fibrocyte death after intense noise exposure takes place two weeks after trauma in the medial part of the cochlea (Wang et al., 2002). This trauma region corresponds to the frequency of the applied noise. We did not observe the death of modiolar fibrocytes or interdental cells in our trauma paradigms. However, the tonotopical location of MAPK phosphorylation observed in the spiral limbus after noise exposure suggests that the stress signalling could be initiated by mechanical movement of cochlear structures caused by the incoming sound stimulus. After all, the tectorial membrane is attached to the spiral limbus. Another possibility is that IHCs at the frequencies the noise targets, get activated and release some stress signalling molecules that the supporting cells under IHCs take up and further transport via the gap junctional system to the spiral limbus. The same stress response is not seen in more basal regions, because they are not targeted by the noise centered at 8-16 kHz.

Similarly, as in root cells, we found FOXO3a expression in the interdental cells in non-noise-exposed cochleas and this expression was not altered in noise-exposed mice (II: Fig 4G,H). Also, we found NF-κB transcriptional activity in interdental cells in non-noise-exposed controls. There was no change in the NF-κB activity after noise exposure (II: Fig 6A-
Even in the degenerated older BL6 cochleas some NF-κB transcriptional activity in the interdental cells was still present (II: Fig 8A,I,L,M). Therefore, it is likely that instead of stress signalling, FOXO3a and NF-κB have other physiological roles in these cochlear cells.

5.2.6 STRESS SIGNALLING IN THE SPIRAL GANGLION NEURONS
We found c-Jun phosphorylation (Fig 10, unpublished results), NF-κB transcriptional activity (II: Fig 6A-D, 8A,I-K), Manf expression (III: Fig 1A,B,L-N), as well as PDI and GRP78 expression (III: Fig 4F) in the control, non-traumatized SG neurons. NF-κB activity was seen only in a subset of SG neurons. The other molecules were detected in the whole population of SG neurons. We did not detect changes in these signalling molecules in our trauma paradigms, except in the ER stress markers PDI and GRP78 in Manf inactivated mice. It seems that, similarly as the modiolar interdental cells, the stress signalling pathways we have studied are not stress-inducible in the SG neurons. Rather, they are active as a part of normal physiological function of these cells.

5.2.7 THE OTHER HAIR CELLS OF THE INNER EAR
In addition to the cochlea, the inner ear consists also of vestibular organs that have sensory hair cells. Cochlear and vestibular hair cells have distinct functions in hearing and balance respectively. Some traumas, such as certain ototoxic drugs, cause degradation in both cochlea and the vestibular labyrinth (Ylikoski et al., 2002; reviewed by Jiang et al., 2017) while for example noise only targets the hearing organ. We saw c-Jun phosphorylation in vestibular hair cells in the sacculus after kanamycin-furosemide application, but not in other vestibular compartments (unpublished results). Robust expression of Manf and ER stress markers was seen in the vestibular hair cells of both control and, for the ER stress markers, Manf inactivated
mice. However, no apparent vestibular cellular damage, hair cell loss or behavioural changes, such as circling behaviour or head bobbing were observed in the Manf inactivated mice (III: Supplemental material Fig 1). Instead, the activation of stress signalling pathways in the vestibular hair cells did not lead to the death of the cells in our trauma paradigms. Activation of stress signalling pathways could help the cells to adjust to the trauma-induced stress.

5.3 ER STRESS AND OHC LOSS IN THE MANF INACTIVATED COCHLEA (III)

We studied ER stress with a genetic Manf inactivation model. MANF is an unconventional trophic factor that resides in the ER and regulates ER stress (Hellman et al., 2011; reviewed by Walter and Ron, 2011; Yan et al, 2019). First, we demonstrated the expression of MANF in the cochlea with immunohistochemistry in wildtype mice and with X-gal staining in heterozygous Manf KO mice, at P2, P12, 4 weeks, and 8 weeks of age. A LacZ-gene has been inserted in the middle of Manf gene knocking out the Manf allele (Lindahl et al., 2014). MANF was detected in IHCs, pillar cells, Deiters’ cells, SG neurons and OHCs (II: Fig 1). In addition, the vestibular hair cells showed MANF expression (III: Supplemental Fig 1). The expression was most robust in the IHCs and SG neurons.

To find out the function of MANF in the cochlea, we studied Manf gene KO mice. However, since the Manf KO mice suffer from hyperglycemia (Lindahl et al., 2014) which might affect hearing function, we generated a conditional Manf KO (cKO) mouse line by crossing Pax2-Cre and Manf flox-flox mouse lines. Pax2 is expressed in various tissues, such as the otic placode and the kidney (Ohyama and Groves, 2004). MANF ablation is more restricted in the cKO than in the conventional KO and, importantly, cKO mice did not display elevated blood glucose levels (III: Fig 3L,M). MANF ablation caused hearing loss measured by ABR (III: Fig 2D, 3D). At the cellular level, the primary target of the cell death was OHCs (III: Fig 2C, 3B,C). Even though IHCs and SG neurons displayed robust Manf expression, no IHC or SG neuron death was observed in 8-weeks-old Manf KOs (III: Fig 2, 3). Moreover, we did not detect any obvious damage to neurites in the organ of Corti area (III: Fig 3J,K). Despite the IHCs surviving much longer than OHCs, synaptic ribbons in the IHCs were lost from the high frequency region of the cochlea (III: Fig 3E). In the basal, high frequency part of the most damaged cochleas, the death of a few pillar cells and IHCs could be seen. OHC loss leads to the degradation of the whole organ of Corti, which could explain the destruction of IHCs and pillar cells following OHC death (Taylor et al., 2012). Therefore, we suggested that the primary cells targeted by Manf inactivation in the cochlea are the OHCs.

To find out the mechanism behind the OHC loss in Manf KO and cKO mice, we studied
ER stress in the cochlea knowing that MANF has a role as an ER homeostasis regulator. ER stress markers PDI and GRP78 were ubiquitously expressed in the organ of Corti, but they were upregulated in Manf KO and cKO mice (III: Fig 4A-G). This upregulation was seen both in the hair cells and supporting cells. However, the pro-apoptotic UPR-component CHOP was only upregulated in the OHCs of the basal part of the cochlea. Thereby the OHC death in the Manf KO and cKO mice seems to proceed through the activation of death promoting UPR-pathway after prolonged ER stress. Yet, this does not explain why OHCs are the only cochlear cell type to die while many cell types in the organ of Corti show ER stress in the Manf KOs and cKOs. ER stress-related cell death and survival have been associated to MAPKs (reviewed by Hetz, 2012; Schinzel et al., 2019). However, we did not detect c-Jun nor ERK1/2 phosphorylation in Manf inactivated mice at 4 to 5 weeks of age (n=6, unpublished data). In our other genetic hair cell stress model, the Gfi1 mutant model where OHCs die during postnatal development, c-Jun phosphorylation was detected in the dying OHCs (I: Fig 2). However, in Manf inactivated mice, OHC loss begins only after the onset of hearing function since the OHC population is intact at P12 (III: Fig 2A). JNK/c-Jun signalling appears to function differently in the regulation of cell death in developing and adult tissue, because c-Jun phosphorylation is detected in the dying cells during development but not in the adult tissue (I: Fig 4).

Insufficient maintenance of the Ca^{2+} homeostasis has been associated to the vulnerability of OHCs (reviewed by Fettiplace and Nam, 2018). Calcium is also a major component of ER homeostasis (Csordás et al., 2010). Problems concerning Ca^{2+} overload in OHCs could explain why they are so sensitive to ER stress. Dysfunctioning Ca^{2+} buffering in OHCs could lead to some extent of ER stress and any additional ER stress, such as stress caused by MANF depletion, could hence be fatal for the cells.

We detected stereocilia damage in the basal, high frequency region of the Manf KO and cKO cochleas. The stereocilia damage did not precede the OHC loss area so it could be nothing more than a sign of the demise of the dying cells. However, stereocilia are an important part of the mechanotransduction in the hair cells and CDH23, a tip link component, has been associated to ER stress in a zebrafish Usher syndrome model (Blanco-Sánchez et al., 2014). Usher syndrome is a condition characterized by hearing and vision loss (reviewed by Bonnet and El-Amraoui, 2012). Blanco-Sanchez and colleagues (2014) suggested that the flow of membrane from the ER through the ER-Golgi intermediate compartment is compromised in the zebrafish with Usher mutation, which could contribute to the reduced numbers of stereocilia they observed in mutant hair cells. Problems in maintaining the stereocilia bundle in the Manf inactivated mice could relate to accumulating ER stress inside the OHCs due to lack of regulation of ER homeostasis by MANF.
Results and discussion

OHC loss and the stereocilia damage of the OHCs took place in the basal part of the cochlea of Manf inactivated mice. Both progressed to more apical regions, lower frequencies, as the mice aged (II: Fig 2C, 3B,C). However, whereas the OHC loss doubled from 4 to 5 to 8 to 10 weeks of age, ABR thresholds showed comparable hearing loss at the high-frequency region in both age groups (II: Fig 2D, 3D). In the full-KO, ABR thresholds were elevated also in the low frequencies where no OHC loss was detected. We saw a complete OHC population at P12 in the Manf inactivated mice (II: Fig 2A), but the hearing measurements have not been done at this age, so we cannot say if the hearing function matures to littermate control levels at any point. To verify that the OHC amplification machinery is normally present in the Manf inactivated mice, we studied the motor-protein prestin that is required for the OHC electromotility (Liberman et al., 2002). No differences in prestin expression between control and Manf inactivated OHCs were observed (II: Fig 3F,G).

Other cochlear structures can affect OHC survival as well. We observed Manf expression in the marginal cells of stria vascularis (II: Fig 1O, 4S). However, we did not see cell death in stria vascularis, even though ER stress marker GRP78 was upregulated in the marginal cells in Manf inactivated mice (II: Fig 4M-R). Thus, ER stress in the cells secreting ions to maintain EP could disturb the composition of endolymph and the ensuing EP drop could promote OHC death. We have not measured EP in the Manf inactivated mice.

PDI and GRP78 were upregulated in the SG neurons of Manf inactivated mice (Fig 12G-L; II: Fig 4F,G). We did not detect the death of ER stressed SG neurons. However, it is possible that their function is disturbed by the intracellular stress and this might explain in part the augmented hearing thresholds of the Manf inactivated mice.

What other evidence is there for cell death due to ER stress in the cochlea? ER stress has been linked to noise trauma as well as to hair cell loss due to mutations in the Cdh23 gene (Blanco-Sánchez et al., 2014; Hu et al., 2016; Lin et al., 2019). ER stress caused by a local application of a chemical impeding protein glycosylation in the ER, tunicamycin, has been shown to cause damage in OHCs and SG neurons (Fujinami et al., 2012). In addition, markers of ER stress have been shown to be upregulated acutely after noise exposure (Li et al., 2018). Furthermore, upregulation of ER stress-related pro-apoptotic UPR-component CHOP has been observed in the old BL6 mice (Wang et al., 2015). ER stress has been associated to synaptic dysfunction in neurodegenerative diseases (reviewed by Freeman and Mallucci, 2016), so ER stress could lead to the loss of presynaptic ribbons as well. Together, it seems that ER stress is involved in many degrading events in the cochlea and hence its regulation provides potential targets for therapies for hearing loss. We have introduced MANF as a novel factor involved in maintaining ER homeostasis in the cochlea.
5.4 MOUSE STRAIN DIFFERENCES (II, III)

CBA mouse strain is widely used in hearing loss studies as a good-hearing mouse strain (reviewed by Ohlemiller et al., 2016) and was used as such also in this thesis. However, this inbred mouse strain is also very vulnerable to cell death after damaging noise exposure at young age. This early-vulnerability is a passing period and older CBA mice are not as sensitive to noise exposure (Ohlemiller et al., 2000). Another inbred mouse strain that was used in this thesis is BL6. This mouse strain is known for its early onset of age-related hearing loss (Johnsson et al., 1997). In many papers, BL6 has also been described as highly vulnerable to noise exposure (Davis et al., 2001). Many of these papers however, have only studied ABR measurements as a readout. A detailed comparative study of pathological morphological changes in the cochleas of CBA and BL6 mice in noise exposure showed greater damage in the lateral wall as well as an EP drop in CBA mice (Ohlemiller and Gagnon 2007).

One difference between BL6 and CBA mice at the young age, 4 weeks, is that the amplitude of the wave I in ABR is higher in CBA mice with sound levels higher than 70 dB SPL. Otherwise the ABR thresholds and latencies have been shown to be comparable between the mouse strains. (Liu et al., 2019). Differences in the formation of a flat epithelium after hair cell loss caused by ototoxic kanamycin-bumetanide application between BL6 and CBA mice has also been demonstrated. BL6 mice developed a flat epithelium faster than CBA mice after the ototoxic insult even though both mouse strains lost OHCs 48 hours after the application. In BL6 mice, flat epithelium started to appear few weeks after the application whereas in CBA mice it did not appear even after several months (Taylor et al., 2012). We found differential stress responses in CBA and BL6 mouse strains in different trauma paradigms as well as different phenotypes in a genetic mutant mouse model in these two genetic backgrounds.

5.4.1 MOUSE STRAIN DIFFERENCES IN NOISE VULNERABILITY AND NOISE-INDUCED STRESS RESPONSES

We examined noise vulnerability at the cellular level in the cochlea, instead of using only hearing measurements. We studied MAPK stress signalling, c-Jun and ERK1/2 phosphorylation, acutely after noise exposure in BL6 and CBA mice at the age of 2 months. At this age, OHC loss in CBA mice was four-fold greater in comparison with BL6 mice six days after noise exposure to 105 dB SPL for 2 hours (II: Fig 1). In addition, c-Jun and ERK1/2 phosphorylation in the lateral wall differed between the strains. Both CBA and BL6 mice showed an acute phosphorylation response in the supporting cells and IHCs in the organ of Corti in the basal part of the noise-exposed cochleas (II: Fig 3A-H; Fig 5). However, only in the noise-vulnerable CBA mice the stress response was detected also in the lateral wall acutely
Results and discussion

After noise exposure, BL6 mice did not lack the ability to raise MAPK stress signalling in the lateral wall, because when noise exposure was preceded by systemic application of LPS, also the lateral wall of BL6 mice showed phosphorylated c-Jun in a comparable manner to CBA mice (II: Fig 3I-O). NF-κB transcriptional activity studied 12 hours after noise exposure also showed a weaker activity in the BL6 than in CBA background in the lateral wall (II: Fig 6). In the constitutive activity of NF-κB in the interdental cells and SG neurons no difference was detected between the mouse strains. Despite the great difference in the amount of noise-induced OHC loss between the mouse strains, their ABR thresholds after noise exposure did not differ significantly (Fig 11, unpublished data). Noise-induced OHC loss in CBA mice extended to approximately 32 kHz region, where no OHC loss was detected in BL6 mice. Therefore, some other factor than OHC loss is behind the elevation of ABR thresholds. Notably, hearing loss measured less than a week from noise exposure could still represent a temporary threshold shift that may recover in time (Kujawa and Liberman, 2009).

Figure 11. ABR measurements from CBA/Ca (CBA) and C57BL/6J (BL6) mice before and 6 days after exposure for 2 hours at 105 dB SPL. Data presented in mean±SD. Abbreviations ABR: auditory brainstem responses, SPL: sound pressure level. Unpublished data.

BL6 and CBA mice are known to differ in the outcome of different cochlear trauma paradigms. Preconditioning with hypoxia or kanamycin did not confer protection from hearing loss in BL6 mice compared with CBA/J mice (Gagnon et al., 2007; Ohlemiller et al., 2011), which suggests that local stress responses, that are activated in CBA but not in BL6 mice, could participate in the protection. Gratton and colleagues (2012) observed differential heat-shock protein (HSP) activation in the lateral walls of different mouse strains with different
vulnerabilities to noise-induced hearing loss measured with ABR recordings. HSPs are a conserved cellular response to stress and serve as chaperones intracellularly though they have been shown to be secreted as well. One of the mouse strains used was BL6, and after noise exposure it did not have HSP activation in the lateral wall. They also found BL6 to have high hearing thresholds after noise (Gratton et al., 2012). HSPs are linked to cell survival in many tissues. HSPs could promote the survival of the stressed cells in the lateral wall, and this activity might not be needed in the BL6 mice, which do not activate stress responses in the lateral wall upon noise exposure.

Since the noise-vulnerability of the CBA mice changes during aging, we wanted to find out if the stress response to noise exposure changes as well. After all, BL6 mice that are not vulnerable to noise exposure at the age of 2 months do not show stress responses in the lateral wall after noise exposure. However, this was not the case for CBA mice. We studied 8-to-10-months-old CBA mice and saw that there was no OHC loss and that the hearing thresholds were not elevated after noise trauma. Despite the change in the OHC loss compared with 2-months-old CBA mice, the c-Jun phosphorylation in the lateral wall acutely after noise exposure in older mice was comparable to the younger mice (Fig 9A-I, unpublished data).

CBA/J mice show reduced EP after noise exposure at young age (3 months), whereas BL6 mice do not. Also, older CBA/J mice show decreased EP acutely after noise exposure (Ohlemiller et al., 2007). Thereby, an EP drop could cause the stress responses we observed in noise exposure in the lateral wall or the stress response could lead to the EP drop. The stress response observed in the organ of Corti is unlikely to be related to the EP drop since BL6 mice show MAPK phosphorylation in that cochlear compartment as well while they do not show an EP drop. EP drop is a dynamic event. Telang and colleagues (2010) did not see EP change after noise exposure in their experiments, when they studied young mice at the age of 4 months and used 100 dB SPL noise exposure for 48 hours in comparison with 110 dB SPL for 2 hours used by Ohlemiller and Gagnon (2007) who detected an EP drop. The ATP triggered K+ influx after noise exposure into the cells lining the endolymph through the P2X2 receptors in CBA mice could cause a stress response in these cells. The stress response could be freely distributed further through the gap junctional system in the lateral wall. Telang and colleagues (2010) showed that noise exposure increased the expression of ATP-gated ion channels, P2X2 receptors, more robustly in the spiral limbus and the organ of Corti in young (3-6 months) than in older (12-15 months) CBA/CaJ mice. This could be one factor explaining the difference in age-related vulnerability to noise in CBA mice.

Macrophages in the lateral wall of the BL6 mice did not show altered morphology, from ramified to round shape, after noise exposure (II: Fig 7). Interestingly, there were less tissue-resident macrophages in total in the lateral wall of the CBA mice compared with BL6 mice.
The temporal and spatial expression of the lateral wall c-Jun phosphorylation response suggests that it could lead to the morphological change of the lateral wall macrophages. c-Jun phosphorylation is downregulated in the lateral wall fibrocytes by 12 hours after noise exposure when the macrophage morphological change has taken place in the tissue resident macrophages. NF-κB transcriptional activity, studied 12 hours after noise exposure, did not colocalize with the Iba1-stained macrophages in the lateral wall (II: Fig 6F). In addition, when studying the older CBA mice, which have no OHC loss but still display a robust lateral wall stress response, we observed the same morphological change from ramified to round phenotype in the lateral wall macrophages as at the age of 2 months (Fig 9K-M). Perhaps the tissue resident macrophages release cytokines that prevent the lateral wall stress responses in BL6 mice, which have more lateral wall macrophages compared with CBA mice.

_Nnt_ gene encodes for a mitochondrial enzyme nicotinamide nucleotide known for its antioxidative capacity when it regenerates nicotinamide adenine dinucleotide phosphate (NADPH) from nicotinamide adenine dinucleotide (NADH). However, a pathological metabolic demand may reverse the function of _Nnt_ and cause ROS overload. BL6 mice lack _Nnt_ and are thus protected from oxidative stress and heart failure (Nickel et al., 2015). The cochlear lateral wall and endocochlear potential of BL6 mice resist noise-induced injury (Ohlemiller and Gagnon 2007; Wang et al., 2002). BL/6N mouse strain has functional _Nnt_ alleles but they still display a similar age-related hearing loss phenotype to BL6 mice (Ohemiller et al., 2016). Perhaps prolonged exposure to pathological noise levels causes energy depletion that activates the reverse function of _Nnt_ leading to oxidative stress. This would cause the activation of stress signalling pathways in the lateral wall in CBA mice while BL6 mice are protected from this effect due to the lack of _Nnt_. This energy-depletion theory for cells having major K⁺ pumping functions in the lateral wall has been suggested to explain the differences in noise-induced lateral wall pathology discovered between different mouse strains by Ohlemiller and Gagnon (2007).

To conclude, genetic background sets the stage for trauma-induced stress responses and should be considered when studying stress in the cochlea. Differences observed in the inbred mouse strains may explain inter-individual variation in trauma-induced hearing loss in mice of mixed genetic backgrounds. It is known that responses to traumas causing hearing loss vary in humans as well and these differences could have genetic causes (reviewed by Davis et al., 2003).
5.4.2 MOUSE STRAIN BACKGROUND INFLUENCES THE PHENOTYPE OF MANF INACTIVATION

When studying Manf inactivation under different mouse strain backgrounds, we came across an interesting phenotype. Initially, we received the conventional Manf KO line in CD-1 background (Lindahl et al., 2014). However, since CD-1 mice show signs of hearing and OHC loss early in life, we transferred the mutant line into the good-hearing CBA background (reviewed by Ohlemiller et al., 2016). Wildtype CBA-CD-1 hybrid offspring in F2 did not have OHC loss at the age of 8 weeks unlike their CD-1 grandparents. Thus, we used these mice for analysis. Interestingly, two separate groups in the Manf KO mice appeared: one, termed l-group, with robust OHC loss and hearing loss, and another, termed s-group, that was comparable to the wildtypes in both OHC survival and low hearing thresholds at all measured frequencies (III: Fig 2C,D). Both Manf KO groups had smaller body size and hyperglycemia compared with wildtype littersmates resembling the original CD-1 Manf KO line (III: Fig 2F,G; Lindahl et al., 2014). Also, both s- and l-group Manf KOs showed upregulated ER stress markers in the organ of Corti and in SG neurons (Fig 12).
Results and discussion

Figure 12. ER stress markers PDI and GRP78 are upregulated in Manf KO s- and l-group (no OHC loss and robust OHC loss respectively) compared to wildtype controls. PDI (A-C) and GRP78 (D-F) expression is shown in the organ of Corti respectively. PDI (G-I) and GRP78 (J-K) expression is displayed in the SG neurons, respectively. The images were taken from paraffin sections at the basal-medial part of the cochlea from 4-weeks-old mice. Scale bar in A is 20 µm. Abbreviations ctrl: wildtype control, KO: knockout, OC: organ of Corti, SG: spiral ganglion, GRP78: 78 kDa glucose-regulated protein, PDI: protein disulfide-isomerase.

We also generated a conditional Manf KO mouse line under Pax2-Cre in BL6 background and did not see two separate phenotypes in the cKOs (Fig 13). All cKOs showed OHC loss and elevated hearing thresholds (III: Fig 3). From these results, we concluded that MANF-depletion alone does not lead to OHC loss, but considering the ER stress regulator role of MANF (Hellman et al., 2011; reviewed by Walter and Ron, 2011; Yan et al, 2019), MANF-depletion together with a genetic phenotype predisposing to ER dyshomeostasis causes the damage observed in the l- group of full-KOs in a hybrid background and cKO cochleas in BL6 background.
Results and discussion

Figure 13. Comparison of the total OHC loss in Manf full knockouts (KO) in CBA/Ca-CD-1 hybrid background and conditional KO (cKO) in C57BL/6J background. Manf full KOs and wildtypes (WT) were studied at the ages of 4- and 8-weeks whereas cKOs and controls were studied at the ages of 5- and 8-to-10-weeks. In Manf KOs two groups: s- and l-group (red and blue respectively) without and with OHC loss could be detected. Means are marked with horizontal lines. Error bars represent SD.

It is known that the Cdh23 mutation is not by itself enough to cause the progressive early-onset age-related hearing loss characteristic of BL6 mice since the mutation in CBA/CaJ background had little effect on the onset of hearing loss (Kane et al., 2012). Therefore, it is likely that BL6 contain other mutations that combined with the Cdh23 mutation lead to the early-onset age-related hearing loss. Cdh23 is also a gene related to Usher syndrome. In a zebrafish model, problems of protein aggregation of Cdh23 along with other Usher syndrome-related proteins was associated to ER stress and hair cell pathology (Blanco-Sanchez et al., 2014). Thereby it is possible that in addition to the mutation in Cdh23 BL6 mice harbour a defect in ER homeostasis machinery making them prone to OHC death due to stress initiated by normal hearing function that begins at P12. The Cdh23 mutation might be enough to predispose MANF-deficient mice to OHC loss, if the mutant Cdh23 gene causes ER stress and an ER stress regulator, MANF, is absent. The genetic background of the early onset of age-related hearing loss in CD-1 mice is not known (Shone et al., 1991). The genetics of CD-1 mice is less studied probably because they are an outbred mouse line. However, similarities in the age-related pathology between BL6 and CD-1 mice suggest a similar predisposing
genotype as in BL6 mice (Shone et al., 1991). Another Cdh23 mutation, Erlong, has been shown to cause hearing loss that is associated to OHC death caused by ER stress (Hu et al., 2016).

It has been long known that the genetic background of mutant mouse models affects the phenotype (reviewed by Johnson et al., 2006 and Ohlemiller et al., 2016). By applying mutations to different mouse strains, we can avoid making misguided conclusions about the phenotype of the mutation. Since inbred mouse strains basically represent one individual, differences in the phenotypes in different genetic backgrounds could reflect the heterogeneity of an outbred population. As an example, we have shown that Manf inactivation requires additional cellular dysfunction, most likely in the ER homeostasis, to cause OHC loss.

5.5 SUMMARY OF THE COCHLEAR STRESS RESPONSES IN DIFFERENT TRAUMA PARADIGMS

The cochlear stress responses studied in this thesis include MAPKs JNK/c-Jun and ERK1/2, NF-κB, FOXO3a, ER stress markers GRP78, PDI and MANF, as well as the lateral wall macrophages. These factors were studied in different trauma paradigms: noise, ototoxic drug-induced lesion with kanamycin-furosemide application, inflammation with systemic LPS application, ageing in BL6 mice, which display early-onset age-related hearing loss, and Manf inactivation. Not all the stress responses were studied in all the trauma paradigms. For example, MANF, GRP78, and PDI were not studied in other trauma paradigms than MANF-depletion. Stress responses took place in different cell types at different cochlear compartments. Table 5 summarizes roughly the results. The final row demonstrates the percentage of the cochlear duct where the stress responses were detected. All trauma paradigms caused stress responses in the basal high frequency region of the cochlea, so the percentage is given from base-to-apex in the table. c-Jun and ERK1/2 represent their phosphorylation, FOXO3a, MANF, GRP78 and PDI for their expression, NF-κB for its transcriptional activity, and macrophages for their morphological change from ramified into round shape.
Results and discussion

5.5.1 THE ROLE OF TRAUMA-INDUCED STRESS RESPONSES IN THE COCHLEA

Despite robust activity or activation of stress signalling, many cell types did not die. For example, IHCs or SG neurons did not readily die in the trauma paradigms: noise exposure, ototoxic drugs or Manf inactivation even though they contained several active stress signalling pathways, such as c-Jun phosphorylation and ER stress markers.

Any trauma paradigm induces a whole network of stress signalling pathways in a cell and the outcome of this activation can depend on the synergistic effects of these pathways. Thereby studying individual stress signalling pathways might not be enough to understand the outcome of trauma-induced stress responses. For example, it has been shown that in CBA/CaJ mice immediately after exposure to 110 dB SPL broadband noise for 1 hour, 155 and 221 genes were upregulated and downregulated respectively in the cochlea. Out of all these gene expression changes, one major pathway activated in the damaging noise exposure was MAPK signalling (Alagramam et al., 2014).

Temporal control of the stress signalling networks is also crucial. Proof of the importance gives the development of a colorectal cancer drug oxaliplatin, which was originally abandoned.
Results and discussion

because of toxicity in phase I clinical trial, but when chronobiology was considered, the adverse effects of the drug were minimized and oxaliplatin has become one of the main drugs against colorectal cancer (reviewed by Graham et al., 2004 and Cederroth et al., 2019). It is possible that also in the cochlea, systemic effectors, such as cytokines, circadian rhythms, and glucocorticoids, influence the outcome of trauma-induced stress responses.

Perhaps the cochlear stress responses are a way to adjust to noise exposure. They could be a part of an endogenous mechanism of the cochlea to adjust its sensitivity. Other systems for this adjustment have been suggested to be the olivocochlear system, local release of ATP, and glucocorticoids (reviewed by Graham et al., 2011). ATP and glucocorticoids could activate cochlear stress responses studied in this thesis. On the other hand, the olivocochlear system is the efferent neural network extending to the IHCs and OHCs from the lateral and medial olivocochlear nucleus respectively. The efferent system contacts the afferent dendrites under IHCs and synapse with OHCs directly. The medial olivocochlear system fibers contain gamma-butyric acid (GABA) and acetylcholine, and it has been suggested to confer protection from loud noise exposure though some controversy exists in the matter (Kirk and Smith, 2003; Maison et al., 2013). It could be that both local stress responses and the olivocochlear system are used to control cochlear sensitivity to noise.

Together, as has been shown with ROS and glucocorticoids (reviewed by Sapolsky et al., 2000 and Schieber and Chandel, 2014), stress responses in the cochlea could have multiple roles. Activity of the stress signalling pathways is a part of the normal cellular metabolism in certain cell types, as we have seen with SG neurons in the cochlea. Also, if stress responses are not upregulated upon traumas, the cell could die in an uncontrolled manner (Fig 14). Appropriate amount of trauma-induced stress signalling maintains cellular homeostasis. However, if the stress signalling overcomes a threshold up to which it is supported, it becomes detrimental and leads to upregulation of programmed cell death pathways. These thresholds are not stationary but can change. Genetic background and systemic effectors are likely to influence the thresholds.

Figure 14. A model of physiological and pathophysiological roles of stress signalling. The idea of the figure is adapted from the review by Schieber and Chandel, 2014.
5.5.2 SUPPORTING CELLS AS HARBINGERS OF OHC DEATH?
Supporting cells in the organ of Corti have important roles in hearing function. Especially important is to maintain the function and survival of the auditory sensory receptor cells, IHCs and OHCs (reviewed by Monzack and Cunningham, 2013). However, supporting cells could also promote OHC death in certain situations. JNK/c-Jun communication between hair cells and supporting cells in the organ of Corti appears to be such case. c-Jun phosphorylation is not observed in the vulnerable OHCs but preventing the phosphorylation confers partial protection from noise-induced OHC loss. As seen with the Gfi1-mutant that lacks the majority of OHCs at 4 weeks of age, OHCs appear to be needed for c-Jun phosphorylation in the supporting cells of the organ of Corti after noise exposure (I: Fig 3I,J).

How do OHCs and Deiters’ cells communicate and what signal do the Deiters’ cells send to OHCs to promote OHC death? The third row of Deiters’ cells and Hensen’s cells attached to them has been shown to move towards the organ of Corti in noise exposure in guinea pigs. The movement is reversible and coincides with a temporary threshold shift after noise exposure (Flock et al., 1999). This mechanical movement could act as a signal for the activation of stress responses in the supporting cells but it seems unlikely to serve as death promoting signal for the OHCs.

In all our trauma paradigms, OHCs die in the high frequency (above 32 kHz) basal part of the cochlear duct. Thereby, OHC loss is not likely to be associated to hair cell mechanotransduction, since for example the noise exposure we utilize is targeted to 8-16 kHz region and the hair cells at this tonotopical region of the cochlea do not die. It is known that the high frequency OHCs are the most vulnerable to traumas, and differences in Ca$^{2+}$ homeostasis has been suggested to be the reason behind the differential vulnerability of OHCs (reviewed by Fettiplace and Nam, 2019). Also, EP drop is known to cause OHC loss. The mechanism of EP drop promoted OHC loss functions through the reduction of K$^+$ influx into OHCs. The membrane potential in hair cells is maintained by three factors: 1) K$^+$ concentration is higher in hair cells than in the surrounding tissue, 2) there is a K$^+$ leak through the mechanotransduction channels at rest, and 3) there is a Na$^+/K^+$ pump at the basolateral membrane of hair cells. A reduced K$^+$ concentration gradient across the basolateral membrane due to EP drop causes the resting membrane potential to shift towards zero. This causes Ca$^{2+}$ influx which further depolarizes the hair cell and leads to the accumulation of intracellular Ca$^{2+}$. High Ca$^{2+}$ concentration can lead to apoptosis if it cannot be controlled (Liu et al., 2016). The stress responses of the supporting cells could diminish their capacity to support the OHCs concerned with changes in K$^+$ concentration gradient and thereby promote the death of OHCs.

ATP and Ca$^{2+}$ released from the supporting cells, have been shown to promote OHC death in the case of ERK1/2 phosphorylation in the supporting cells (Lahne and Gale, 2008).
Another possibility is that heat shock proteins (HSPs), which can be released from cells to extracellular space, are produced by the supporting cells (reviewed by Lyon et al., 2019). Extracellular HSPs secreted by non-neuronal cells have been shown to promote the survival of neurons. A similar HSP-response could exist in the cochlea between the sensory hair cells and glial-like supporting cells. HSP secretion in astrocytes has been shown to be promoted by ERK1/2 activity and tempered by activation of JNK activity (Taylor et al., 2007). Therefore, JNK/c-Jun activity in the supporting cells after trauma could downregulate the HSP release aimed to support OHCs and increase the risk of OHC death. In the cochlea, HSPs have been shown to be upregulated in traumas and overexpression of HSPs has been shown to confer protection from hearing and OHC loss (Fairfield et al., 2005; Taleb et al., 2009). On the other hand, since Hsp72 has been shown to inhibit JNK activity (Park et al., 2001), HSP activation could function to downregulate JNK/c-Jun activity in the traumatized cochlea.

Also, MANF is expressed in the Deiters’cells under OHCs (III: Fig 1G,G’). MANF has been associated with a secretory function during UPR activation (Petrova et al., 2003). It could be that lack of secreted MANF from supporting cells promotes the damage of OHCs. Protective effects of secreted MANF have been demonstrated in many studies (Apostolou et al., 2008; Tadimalla et al., 2008; Airavaara et al., 2009; Glembotski et al., 2012). MANF could be a trophic factor for hair cell survival, but no evidence of MANF secretion in the cochlea exists at the present.

To conclude, supporting cells in the organ of Corti influence the cellular fate of OHCs. Yet the majority of the mechanisms remain unidentified. More studies are required for deciphering the mechanism of OHC loss in traumas.

5.5.3 THERAPEUTICAL FUTURE PERSPECTIVES
This thesis addresses the cellular biology of trauma-induced stress responses in the cochlea. To extrapolate the results to hearing loss mechanisms, it is important to note that in addition to the cochlea, hearing requires also several brainstem nuclei and the auditory cortex, which are not covered in the scope of this thesis. However, we should develop new methods to prevent hearing loss since wearing hearing protection is not always an efficient way to prevent hearing loss (Groenewold et al., 2014). Targeting cellular stress signalling leading to cell death would be a potential alternative for hearing protection. Preventive therapies could be targeted to people in high risk of developing hearing loss due to either genetic or environmental causes.

There are countless articles introducing potential therapeutic targets that provide protection from hearing loss. Just by looking at the titles of articles published in the hearing research field, one would assume that several treatments for the prevention of hearing loss
already exist. However, this is not the case. Most of the studies have been made in mice and the inter-species leap to treating humans could cause problems. There might also be some oversimplification, when one research paper focuses on one target molecule and declares that it is the reason behind hearing loss. After reading more of these articles, it is clear that a broader view on the matter is needed. Furthermore, one of the major challenges in creating a therapy for hearing loss is that when a potential candidate molecule for drug therapy has been found, we lack the mechanism how the protection is generated. These mechanisms might also differ depending on the context, such as other activated molecules or systemic effects. Moreover, the obvious conclusion is not always the right one. For example, testosterone was long thought to cause aggression. When testosterone levels were raised aggression increased and when the testosterone levels were decreased the aggression decreased as well. Still, in the end, testosterone does not cause aggression. It facilitates neural signalling and thereby aggravates the already existing behavioral patterns (reviewed by Simpson, 2001).

Many therapies for hearing loss are being developed. One therapy will not cure all since there are many factors causing hearing loss. Inter-individual variation between humans is also great and personalized medicine should be considered in hearing loss therapies (reviewed by Schilder et al., 2019). For example, D-JNK-1 inhibitor, AM111, has been in clinical trials for treating hearing loss by Auris Medical Inc. AM111 is a small, cell-permeable peptide and it was applied as one local application of the drug through the tympanic membrane to patients with sudden sensorineural hearing loss. Phase three trials were aimed at severe to profound hearing loss patients with hearing loss onset up to 72 hours. Even though amelioration of hearing thresholds were detected in the profound hearing loss group, the phase 3 clinical trial was terminated, since it failed to meet the goals set for hearing recovery (Staecker et al., 2019).

Our studies revealing differences in stress responses between inbred mouse strains as well as how the genetic background can affect gene mutation phenotype, offer explanations for the inter-individual variation in hearing loss. Based on the results in this thesis, we should consider more carefully when and to which cell types hearing loss therapies are applied to, especially if they are targeted to stress signalling networks. Many stress signalling networks are not only involved in pathophysiology but also have physiological roles in the cochlea. In addition, trauma-induced signalling can be rapid and dynamic narrowing the time window of the treatment. Circadian biology is becoming an important factor to consider in order to improve drug efficiency and diminish drug toxicity (reviewed by Cederroth et al., 2019b). The inner ear is also under circadian control. Perhaps we could get less variation in hearing loss therapy studies if circadian regulation would be accounted for.
6 CONCLUDING REMARKS

We studied cellular stress responses in the cochlea with different trauma paradigms. The aim was to unravel the influence of cochlear stress signalling on OHC survival. The principal conclusions of this thesis are:

1) Our studies revealed that c-Jun phosphorylation takes place in the cochlea in many traumas including ototoxic drug-induced lesion, noise exposure, and systemic inflammation. However, the vulnerable OHCs did not show c-Jun expression or phosphorylation in any of these situations.

2) We showed that upon traumas c-Jun phosphorylation is present in the supporting cells of the organ of Corti and in the cells of the lateral wall. The stress response in the lateral wall was dependent on the genetic background. Genetic ablation of c-Jun phosphorylation conferred partial protection from noise-induced OHC death. Thereby, c-Jun phosphorylation in other cell types than the dying OHCs affects OHC survival in a paracrine manner. The exact mechanism how the intercellular communication takes place remains to be shown.

3) We characterized the expression of an unconventional neurotrophic factor MANF in the inner ear. By using a genetic mouse model, we demonstrated that Manf inactivation in the inner ear causes OHC death and hearing loss. The hearing loss phenotype depended on the genetic background and the OHC death took place through a pro-apoptotic UPR pathway.

To conclude, we can think about the physiology and pathophysiology of stress responses as sailing a boat. You need water, wind and some sun to create the wind for sailing, meaning that some stress responses are needed in normal physiology. However, if there is too much wind or rain you risk capsizing the boat. Also, too much sun will evaporate all the water and the sailing will stop. The outcome of stress responses may lead to cell death. Or, sailing can continue as soon as the boat is back in water, meaning that normal homeostasis is regained after a stress response. BL6 mice have a sturdier boat than CBA mice in our noise exposure paradigm, hence the CBA mice capsize (activate a lateral wall stress response) more readily. The outcome of trauma also depends on where you are sailing at the moment of stress. In shallow waters running aground is more likely. For example, systemic stress preceding noise exposure overcame the threshold of noise-induced stress responses in the cochlear lateral wall of BL6 mice. In order to build sturdier boats that continue sailing regardless of the storms, we need to investigate these phenomena further.
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FACULTY OF BIOLOGICAL AND ENVIRONMENTAL SCIENCES
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