From DEPARTMENT OF PHYSIOLOGY AND PHARMACOLOGY
Karolinska Institutet, Stockholm, Sweden

THE ROLE OF GLUCOCORTICOID RECEPTORS AND MITOGEN-REGULATED PROTEIN KINASES IN THE COCHLEA

Inna Meltser

Karolinska Institutet

Stockholm 2009

Published by Karolinska Institutet. Printed by Universitetsservice US-AB.

ISBN 978-91-7409-560-9

ABSTRACT

Hearing loss induced by acoustic trauma is a common handicap for the human population. Acoustic trauma triggers a cascade of changes in the cochlea such as a direct mechanical damage of sensory hair cells, biochemical disturbances including reactive oxygen and nitrogen species generation, release of pro-inflammatory cytokines and exitotoxicity. Acoustic trauma can result in transient or permanent hearing loss depending on the physical and temporal characteristics of the acoustic stimulation as well as individual susceptibility. Interestingly, acoustic trauma triggers not only damaging, but also protective mechanisms in the cochlea such as the up-regulation of antioxidants, glucocorticoids and neurotrophins. The overall goal of the present study was to characterize the molecular mechanisms underlying the protective effects of glucocorticoid receptors (GR) and their interactions with the family of mitogen-activated protein kinases (MAPKs). The results demonstrate that GR plays an unequivocal role in modulating auditory sensitivity. In a GR-dependent manner, the p38 pathway is activated after restraint stress, and ERKs are down-regulated after restraint stress followed by acoustic trauma. ERKs and p38, as well as c-jun-N-terminal kinases (JNKs) regulate cell recovery and cell death. Cell recovery after acoustic trauma correlates with a down-regulation of p38 and an up-regulation of ERKs and JNKs 24 h post trauma. In contrast, hair cell loss is accompanied by immediate post-traumatic up-regulation of all three MAPKs. A critical factor for protecting against cochlear trauma includes GR and ERK interactions and the down-stream activation of the otoprotective neurotrophin, brain-derived neurotrophic factor (BDNF). BDNF is up-regulated in the cochlea after acoustic trauma, and the duration of its elevation correlate with the pattern of ERKs activation and the severity of cochlea damage. BDNF acts through receptor tyrosine kinase TrkB followed by the downstream activation of ERKs and p38 cascades. Severe acoustic trauma leads to a down-regulation of the truncated form of TrkB thus probably providing a more robust activation of the full-length TrkB, the main mediator of BDNF-induced protective effects. The knowledge of the glucocorticoids and MAPKs cellular mechanisms are of a great importance for clinical audiology since it opens new avenues for the prevention and treatment of hearing loss. These data will help to understand the nature of individual sensitivity to acoustic trauma since hypothalamic-pituitary adrenal (HPA) axis status is now demonstrated to be a critical factor for determining the overall sensitivity to acoustic trauma.

LIST OF PUBLICATIONS

This thesis is based on the following papers, which will be referred to the text by their Roman numerals.

I Tahera Y, Meltser I, Johansson P, Bian Z, Stierna P, Hansson AC, Canlon B (2006) NF-kappaB mediated glucocorticoid response in the inner ear after acoustic trauma. J Neurosci Res 83:1066-1076.

II Tahera Y, Meltser I, Johansson P, Hansson AC, Canlon B (2006) Glucocorticoid receptor and nuclear factor-kappa B interactions in restraint stress-mediated protection against acoustic trauma. Endocrinology 147:4430-4437.

III. Meltser I, Tahera Y, Canlon B (2009) Glucocorticoid receptor and mitogen-activated protein kinases activity after restraint stress and acoustic trauma. J Neurotrauma. Epub ahead of print.

IV. Meltser I, Tahera Y, Canlon B (2009) Differential activation of mitogen-activated protein kinases and brain-derived neurotrophic factor after temporary or permanent damage to a sensory system (Manuscript).

CONTENTS

LIST OF ABBREVIATIONS

ABR	auditory brainstem response
ACTH	adrenocorticotriopic hormone
BDNF	brain-derived neurotrophic factor
CRH	corticotrophin-releasing hormone
dB	decibel
ERK	extracellular signal-regulated kinases
GR	glucocorticoid receptor
HPA	hypothalamic-pituitary adrenal axis
IHC	inner hair cells
JNK	c-jun-N-terminal kinases
MAPK	mitogen activated protein kinases
NFkB	nuclear factor kappa B
OC	organ of Corti
OHC	outer hair cells
PTS	permanent threshold shift
RS	restraint stress
RT-PCR	reverse transcriptase polymerase chain reaction
SGN	spiral ganglion neurons
SPL	sound pressure level
TTS	temporary threshold shift

1 BACKGROUND

1.1 THE EFFECT OF THE ACOUSTIC TRAUMA ON THE AUDITORY SYSTEM

Hearing loss is a common disorder that affects the human population. In 2002 the World Health Organization reported about approximately 250 million people suffering from hearing loss. Hearing loss induced by acoustic trauma is related to occupational noise (Ishii et al., 1992; Ishii and Talbott, 1998) or to environmental noise, including leisure time noise. The problem of hearing impairment due to exposure to loud music and use of portable music devices is becoming more common for young adults. Acoustic trauma is a disorder caused by a high-intensity sound which can be hazardous for the auditory system by causing hearing loss. Acoustic trauma can result in temporary or permanent hearing loss depending on the duration and intensity of the stimulus and from the individuals´ susceptibility to it (Davis et al., 2003). If the hearing impairment is reversible, it is characterized by a temporary threshold shift (TTS) whereas irreversible damage is accompanied by a permanent threshold shift (PTS) of auditory sensitivity.

Cochlear damage induced by acoustic trauma primarily involves two types of sensory cells, the inner and outer hair cells and the surrounding supporting cells. Inner hair cells (IHC) are responsible for transferring electric impulses to the primary auditory neurons (spiral ganglion neurons, or SGN). The outer hair cells (OHC) serves as a signal amplifier and tuner, and they are particularly vulnerable to acoustic damage. Both types of hair cells have stereocilia at the apical pole of the cell which responds to stimulation by deflection that results in the opening of ion channels at their tips (named tip-links).

Acoustic trauma of a moderate intensity results in temporary functional and morphological damage to the cochlea. The morphological signs of temporary damage are characterized by disorganization of the stereocilia of the inner and outer hair cells and breakage of the tip links as a result of the mechanical damage, buckling of pillar cells, reduction in OHC stiffness and cell length, and detachment of OHC stereocilia from the tectorial membrane (Boettcher et al., 1992; Fridberger et al., 1998; Nordmann et al., 2000). The overstimulation of IHC results in the activation of reactive oxygen species (ROS) and nitric oxide (NO) generation, an increase in the intracellular Ca^{2+} and an excessive release of glutamate. These events lead to the loss of contact between IHC and SGN dendrites (swollen dendrites) within the first several hours post trauma (Stopp, 1983; Pujol et al., 1993). No hair cell loss or auditory neuron degeneration can be detected after temporary acoustic trauma (Nordmann et al., 2000), and the stereocilia can recover within hours to days.

The early morphological changes of the cochlea to permanent damage are similar to that of TTS, but also include the loss of hair cells (especially OHC) as well as the collapse, fusion or fracture of the stereocilia of IHC and OHC (Nordmann et al., 2000). Permanent damage also results in the degeneration of nerve fibers and auditory neurons (Pourbakht and Yamasoba, 2003). With intensive acoustic trauma (more than 120 dB SPL) a mechanical damage to the hair cells and detachment of the whole organ of Corti from the basilar membrane can occur (Nordmann et al., 2000) which causes hair cell death due to necrotic processes. Apoptotic processes are activated by ROS and NO

generation, excessive release of excitatory neurotransmitters, and Ca^{2+} overload. As a result of the damaging factors a degeneration of hair cells, supporting cells and auditory neurons occur (Gale et al., 2004). The decrease of intracochlear blood flow and the activation of pro-inflammatory cytokines (TNF, IL-6) are also contributing factors to noise-induced cochlear pathology (Zou et al., 2001; Satoh et al., 2002; Maeda et al., 2005; Fujioka et al., 2006).

1.2 THE HYPOTHALAMIC-PITUITARY ADRENAL AXIS

The hypothalamic-pituitary adrenal axis (HPA) is a neuroendocrine system which mediates a response to different stressor events. HPA includes the paraventricular nucleus of the hypothalamus which responds to stressful signals from the limbic system, brain stem and other brain regions by activating different genes including corticotrophin-releasing hormone (CRH) and arginin vasopressin (AVP). These peptides trigger the release of adrenocorticotropic hormone (ACTH) from anterior pituitary which, in turn, activates the synthesis and secretion of glucocorticoids from the adrenal glands (fig. 1).

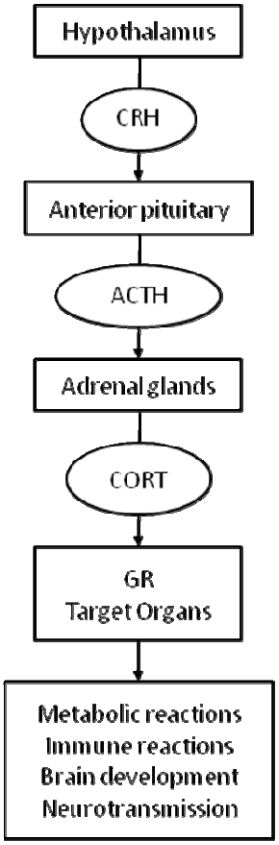

Figure 1. Hypothalamo-pituitary adrenal axis (HPA) is activated by stimulation of the hypothalamus. Neuroendocrine neurons of the hypothalamus secrete corticotrophin-releasing hormone (CRH) which stimulates the secretion of adreno-corticotriopic hormone (ACTH) from the anterior pituitary. ACTH activates the synthesis and release of glucocorticoids from the adrenal glands which acts through gluco-corticoid receptors (GR) in target organs to regulate gene expression.

Cortisol is the predominant glucocorticoid in humans whereas corticosterone dominates in rodents. Glucocorticoids are synthesized by cells of the zona glomerulosa and zona reticularis in the adrenal cortex having cholesterol as a substrate (fig. 2). ACTH increases the cholesterol concentration in mitochondria where synthesis takes place. The final steps of cortisol and corticosterone synthesis is mediated by steroid 11β-hydroxylase which can be pharmacologically inhibited by metyrapone. The concentration of cortisol and corticosterone in the blood has a specific pattern depending of the species, and diurnal activity. In rodents the peak of the corticosterone concentration is at night

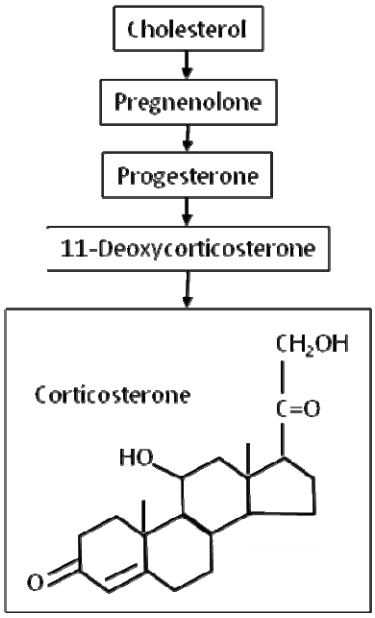

Figure 2. Synthesis of cortico-sterone from cholesterol. The conversion of 11-deoxycortico-sterone to corticosterone is mediated by 11β-hydroxylase. The glucocorticoid antagonist, metyrapone, blocks the synthesis of corticosterone.

time while for humans it is the first half of the day. The synthesis and secretion of the glucocorticoids is tightly controlled by negative feedback mechanisms. Inactivation of glucocorticoids occurs by the enzyme 11β-hydroxysteroid dehydrogenase (11β-HSD) which is found in peripheral tissues including brain. 11β-HSD is one of the regulators of glucocorticoids accessibility to GR, and its mRNA synthesis in the cochlea is up-regulated by dexamethasone (Kim et al., 2009).

There is a broad spectrum of pharmacological treatments that use glucocorticoid therapy. These include autoimmune and allergic diseases, other types of inflammatory conditions, tumors, and as a substitutive therapy in Cushing's syndrome and some types of adrenal gland insufficiency. For inner ear disorders, glucocorticoid therapy is used for the treatment of Meniere's disease, tinnitus, acoustic trauma, sudden hearing loss and autoimmune inner ear disorders (McCabe, 1979; Moskowitz et al., 1984; Alexiou et al., 2001; Dodson and Sismanis, 2004; Dodson et al., 2004). Unfortunately, the beneficial effect of glucocorticoid therapy on hearing lasts only during treatment and disappears upon tapering the therapy (Harris et al., 2003; Doyle et al., 2004). Therefore additional knowledge regarding glucocorticoid action in the auditory system is of a vital clinical importance.

1.3 GLUCOCORTICOID RECEPTORS

Glucocorticoid receptors (GRs) are ligand-activated transcription factors which belong to the nuclear receptor super family. They share common structural and functional features with other receptors of this family such as having a conserved DNA binding domain (DBD) and carboxyl terminal ligand binding domain. The amino terminal domain varies in different receptors and its functional activity depends on a ligand binding. Ligand free GR resides in the cytoplasm as a complex with chaperons such as heat shock protein 90 (hsp90) dimer, tetratricopeptide repeat (TPR) and p23 (Hutchison et al., 1993). The main function of this protein complex formation is a

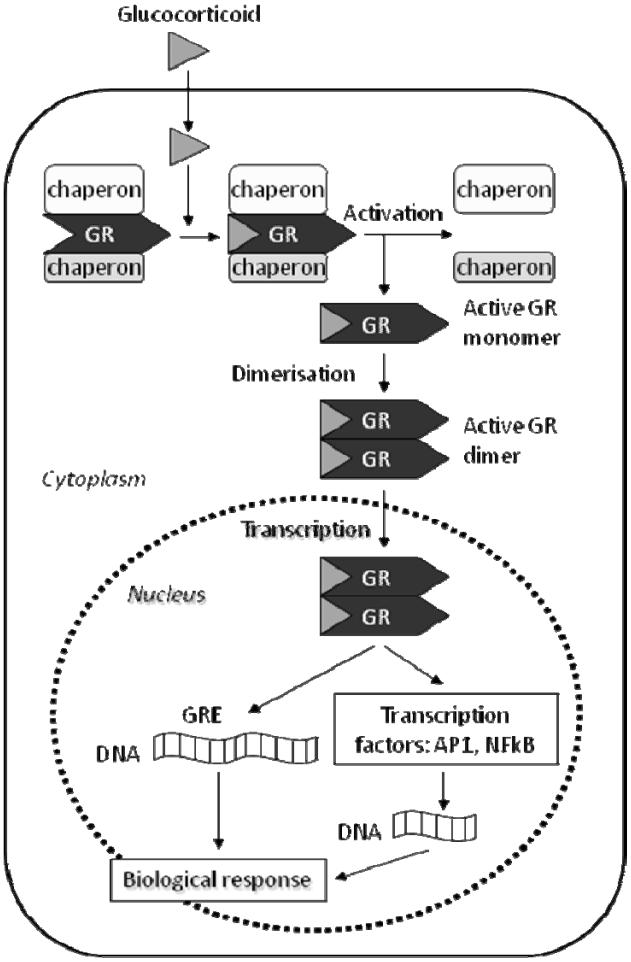

Figure 3. Molecular mechanisms of GR activation by glucocorticoids. Glucocorticoids are hydrophobic substances that can easily penetrate cellular membranes and bind glucocorticoid receptors (GRs). Inactive GRs reside mainly in the cytoplasm as a complex with chaperone proteins which dissociate upon ligand binding. GRs bound to glucocorticoids form homodimers and are translocated to the nucleus where they exert their transcriptional regulation through glucocorticoid response elements (GRE) or though the interaction with other transcriptional factors such as activation protein 1 (AP-1) or NF-kB. The resulting changes of a number of proteins determine the cell response to glucocorticoids.

prevention of DNA binding in absence of the ligand (Dalman et al., 1989). Corticosteroids trigger the cascade of events including dissociation of GR-hsp90 complex, GR homodimerization, translocation of the GR dimers to the nuclei (Tsai et al., 1988a; Tsai et al., 1988b; Hutchison et al., 1993; Basu et al., 2003) where they exert their transcriptional activity either through the direct binding to glucocorticoid response elements (GRE) (Freedman et al., 1988; Hard et al., 1990), or through the interaction with other transcriptional factors such as activator protein 1 (AP-1) and NF kappa B (De Bosscher et al., 2000; Widen et al., 2003; Wikstrom, 2003; Martens et al., 2005) (fig. 3). The genes controlled by GR include the key enzymes of the gluconeogenesis (phosphoenolpyruvate carboxykinase), amino acids (tyrosine aminotransferase) and fatty acids metabolism (Munck et al., 1984). GRs have a critical role in the immune response controlling many immune reactions and exerting anti-inflammatory and immunosuppressive functions (Smoak and Cidlowski, 2004) and apoptosis (Rocha-Viegas et al., 2006). They are also involved into blood glucose regulation (Saad et al., 1993; Ruzzin et al., 2005; Jin and Jusko, 2009), growth and development (Tronche et al., 1998; Wang et al., 2006). The role of GR in psychological disorders was recently revealed (Webster et al., 2002; Mackin et al., 2007; Gallagher et al., 2008; Gourley et al., 2009). Thus, GRs regulate a broad panel of functions and can modulate metabolic, immune and psychological response to stress. The level of expression GR varies in different tissues (Bamberger et al., 1996). Spiral ganglion neurons and spiral ligament of a cochlea has the highest level of GR expression whereas stria vascularis and organ of Corti have less (Rarey et al., 1993; ten Cate et al., 1993; Tahera et al., 2006b; Meltser et al., 2009). In the organ of Corti, GR was found in the hair cells and in the supporting cells (Shimazaki et al., 2002) (fig. 4). The functional role of GRs was shown for the Reisner's membrane epithelium were they regulate sodium transport through the activation of transcription of several factors involved in sodium transport regulation (Kim et al., 2009). It has to be noted that the large ionic difference between perylimph and endolymph is critical for the transduction of a sound signal into nerve impulse in the cochlea, and Reisner's membrane epithelium is partly responsible for the sustained ionic gradient. Particularly, this epithelium provides sodium absorption together with outer sulcus cells.

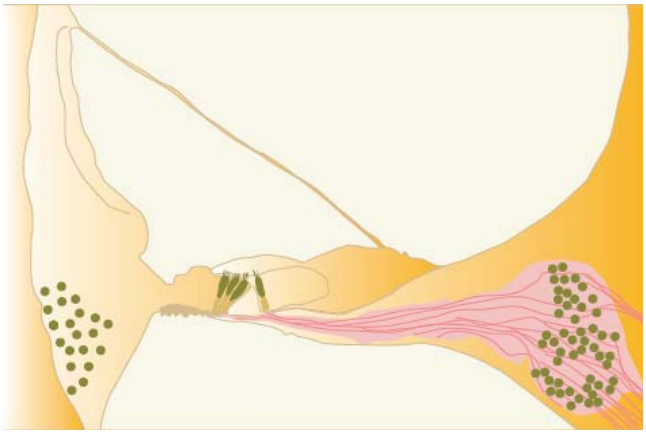

Figure 4. Glucocorticoid receptor (GR) expression in the cochlea. GR were detected in spiral ganglion neurons, inner and outer hair cells as well as in the fibrocytes of the spiral ligament.

In neuronal and sensory cells GR may control the level of neurotrophins and neurotrophin receptors (Schaaf et al., 1999; Yang et al., 2005). Neurotrophins belong to neurotrophic growth factors, and include nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT3) and 4 (NT4), and exert their biological functions through activation of tropomyosin receptor kinase from a family of tyrosine kinase receptors (Trk). The expression of neurotrophins and their receptors is critical for

auditory system development and function (Ernfors et al., 1996; Staecker et al., 1996; Duan et al., 2000; Agerman et al., 2003). Morover, the application of BDNF to mature cochlear provide otoprotection against different injuries and rescue hair cells and auditory neurons from apoptotic death and degeneration (Hartnick et al., 1996; Gabaizadeh et al., 1997b; Gabaizadeh et al., 1997a; Song et al., 2008). The neuroprotective effects of BDNF are mediated by TrkB receptors which are expressed in the cochlea (Schimmang et al., 2003; Tan and Shepherd, 2006). Glucocorticoids have been shown to activate Trk receptors through neurotrophin-independent mechanisms and thus provide neuroprotection mediated by GR transcriptional mechanisms (Jeanneteau et al., 2008). In addition, GR-TrkB interaction in cortical neurons is involved in BDNF-regulated glutamatergic signaling (Numakawa et al., 2009). Also GRs increase BDNF expression in hippocampus (Schulte-Herbruggen et al., 2006). Thus, factors controlling the sodium balance and BNDF – TrkB system are possible targets for GRs in the cochlea.

The turnover of GRs is relatively fast and depends on their activation (Rosewicz et al., 1988). The proteasomal degradation of the activated GR in the nucleus occurs within 2 h after binding to corticosterone and translocation into the nucleus (Conway-Campbell et al., 2007). The down-regulation of GR transcription after their activation was described for different tissues (Rosewicz et al., 1988; Wang and DeFranco, 2005) including the cochlea, and can be observed within 12-24 h after GR binding to corticosterone (Rarey et al., 1995; Terunuma et al., 2001). This rapid reaction of GR expression on the elevated glucocorticoids level provides the fine tuning of the glucocorticoids targeted control of different cellular functions. Moreover, the release of glucocorticoids has a pulsatile manner *in vivo*, and a rapid regulation of tissues sensitivity to them is important for balanced cell response to HPA axis modulation (Windle et al., 1998; Lightman, 2008)

1.4 MITOGEN ACTIVATED PROTEIN KINASES

Mitogen activated protein kinases (MAPKs) is a family of signal transducing enzymes that are involved in a variety of cellular functions. MAPKs include extracellular signal-regulated kinases (ERK) 1/2, c-jun-amino-terminal kinases (JNK1/2/3), p38 proteins (α/β/γ/δ), and ERK 5. MAPKs are connected to different types of the membrane receptors but they also can be activated by physical or chemical cellular stresses in a receptor-independent manner (Kyriakis and Avruch, 2001). The activation of different MAPKs is triggered by phosphorylation of a concerved serine/threonine and tyrosine residues catalized by specific MAPK kinases (MAPKK, or MKK): MEK1/2 for ERK1/2, MKK4/7 for JNK1/2/, and MKK3/6 for p38 (Lin et al., 1995; Wu et al., 1997; Pleschka et al., 2001). In turn, these kinases are activated upon dual phosphorylation mediated by MAPKK kinases (MAPKKK, or MAP3K) which are probably signal-specific. The subsequent activation of MAP3K-MKK-MAPK requires minimal three-enzyme module formation in response to a variety of signals. These interactions can be facilitated by scaffold proteins such as JIP1 for JNK1/2, or MP for ERK1 (Schaeffer et al., 1998; Whitmarsh et al., 1998). The formation of MAPK-activating module can occur in a cytoplasm or in a nucleus. The activation of MAPKs mainly effects the expression of different genes through the interaction with different transcription factors and translation regulators. In addition, apoptosis modulators are also typical targets of MAPKs activity.

Some of the MAPK pathways converge on certain substrates providing the multilevel control of cellular reaction to stress.

It is commonly accepted that ERK activation is related to cell survival, whereas JNK and p38 pathways are linked to apoptosis. However, it has been shown that the ultimate effect of each MAPK cascade depends on many factors including cell type, cell context and the signal duration. The ERK pathway is activated mainly by hormones such as insulin, growth factors (for example, neurotrophins including nerve growth factor (NGF) and BDNF), and neuromediators including glutamate and gamma-aminobutiric acid (GABA). The membrane receptors for insulin, neurotrophins or NMDA and GABAA interact with Ras, a small membrane GTPase which triggers MAP3K-MEK-ERK cascade. The most important targets of this cascade are proteins which belong to the family of 90-kDa ribosomal S6 kinases (RSK). Both RSK and ERK translocate into the nucleus upon activation where they interact with a variety of transcription factors whish including CREB, histone H3 acetyl transferase, c-fos and elk-1. The ERK pathway can be triggered by damaging factors such as hypoxia and oxidative stress (Hess et al., 2002; Chu et al., 2004) and are involved in the activation of apoptosis (Vanhoutte et al., 1999; Lubin et al., 2005). In addition, the ERK cascade can converge with the pro-apoptotic p38 pathway on down-stream kinase MSK1 which regulates the activity of the histone H3 acetylation and, in turn, the expression of AP-1 proteins c-fos and c-jun in response to NMDA receptor activation (Brami-Cherrier et al., 2007). JNK and p38 mainly respond to cellular stresses and therefore there are also referred as to stress activated protein kinases (SAPK). JNK plays a crucial role in regulating the activity of numerous transcription factors such as AP-1, activating transcription factor 2, Elk-1, and GR (Yang et al., 2003).

The half life of active MAPKs is controlled by their de-phosphorylation by MAPK phosphatases (MKP). The MKP family includes MKP1/2 which undergoes unselective activation upon binding with all three MAPK (Slack et al., 2001). In addition, active MAPKs can up-regulate the transcription of MKP1/2 (Sgambato et al., 1998; Sun et al., 2008) thus providing autoregulative feedback control.

The pattern of activation of different MAPKs in the cochlea depends on the degree of damage. For example, JNK activation was found in hair cells after acoustic trauma and impulse noise exposure (Wang et al., 2007; Murai et al., 2008), and inhibition of JNK cascade prevented noise-induced hair cell loss (Pirvola et al., 2000; Wang et al., 2003; Eshraghi et al., 2007). JNK activation correlated with aminoglycoside-induced spiral ganglion neurons and hair cell degeneration (Pirvola et al., 2000; Tan and Shepherd, 2006). ERK cascade in the cochlea can be triggered by damaging insults such as hypoxia and mechanical damage (Hess et al., 2002; Lahne and Gale, 2008). On the other hand, ERKs are critical for cochlea development (Urness et al., 2008). Moreover, it mediates the protective effect of the brain-derived neurotrophic factor (BDNF) and substance P in spiral ganglion neurons resulting in cell survival and axonal regeneration (Xia et al., 1995; Hetman et al., 1999; Aletsee et al., 2002; Lallemend et al., 2003; Arthur et al., 2006).

1.5 INTERACTION BETWEEN GLUCOCORTICOID RECEPTORS AND MAPK

GR provides the possibility for the interaction of the systemic (HPA axis – glucocorticoids) and cellular (MAPKs) response to different stressors in target organs which helps to regulate the overall reaction to stress (Fig. 5). These mechanisms can be divided in direct MAPKs – GR pathway interactions (or non-genomic GR effects), the regulation of the transcription of different proteins involved in GR or MAPKs turnover and activity, and the functional interaction of different intracellular pathways modulated by GR and MAPKs.

GR can bind to MAPK kinase-2 (MAPKK), or MEK-2 complex thus providing the control over ERK cascade (Hedman et al., 2006). The activation of p38 and JNK has been shown to activate GR phosphorylation resulting in inhibition of GR transcriptional activity (Irusen et al., 2002; Itoh et al., 2009). GRs can induce ERK-pathway activation through Ras-Raf proteins in hippocampus after stress (Revest et al., 2005). On the contrary, the genomic effects of GR activation on MAPKs are inhibitory. GR positively regulates the transcription of the dual specificity MAPK phosphatase-1 (DUSP-1), the MAPK-inactivating enzyme (Johansson-Haque et al., 2008). GR – ERK and GR – p38 pathways interplays are involved in the modulation of the inflammatory response and long-term potentiation (Ahmed et al., 2006; Bladh et al., 2009).

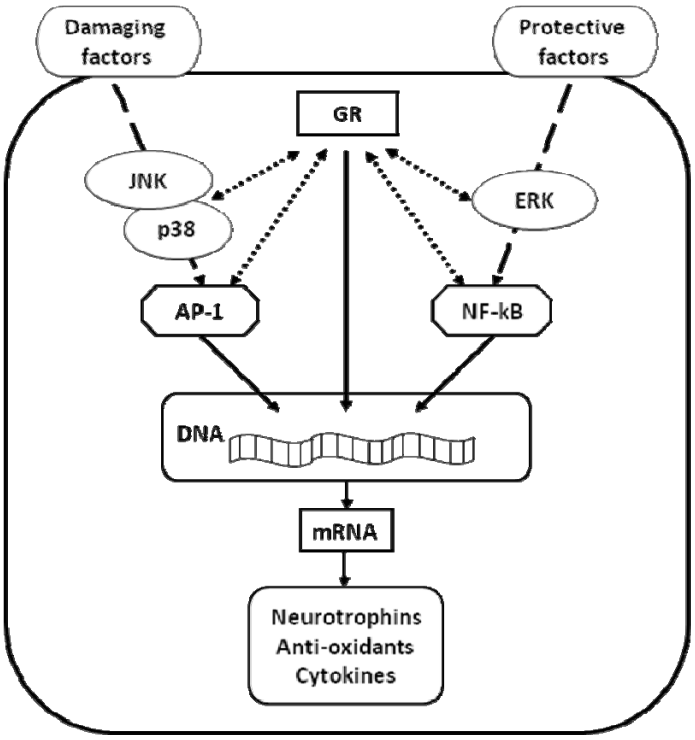

Figure 5. Hypothetical scheme of possible interactions of glucocorticoid receptors (GRs) and mitogen-activated protein kinases (MAPK) on the cellular level. Stress factors (reactive oxygen species, pro-inflammatory cytokines or the excess of cytotoxic neuromediators) activate JNKs and p38 and the transcriptional factor AP-1. Protective factors can act through ERKs which regulate the transcriptional activity of several transcriptional factors including NF-kB. GRs activated by corticosterone effect transcription directly or through the interaction with AP-1, NF-kB and other transcription factors. The integrated effects of GR and MAPKs determine the intensity and duration of transcription and gene expression.

2 AIMS

The overall aim of this study is to determine the mechanisms underlying hearing loss induced by acoustic trauma. The main focus has been on stress pathways involving the hypothalamic-pituitary adrenal axis and mitogen-activated protein kinases.

Specific aims:

- To evaluate the physiological and molecular role of glucocorticoids and glucocorticoid receptors after acoustic trauma

- To determine how restraint stress protects the auditory system against acoustic trauma by investigating mechanisms involving glucocorticoid receptors.

- To determine the correlation between glucocorticoid receptors and mitogen-activated protein kinases in the cochlea after restraint stress and acoustic trauma

- To characterize the expression of mitogen-activated protein kinases, brain-derived neurotrophic factor (BDNF) and TrkB in the cochlea after a temporary or a permanent hearing loss induced by acoustic trauma.

3 MATERIALS AND METHODS

3.1 ANIMALS

CBA/Ca/Sca male mice aged 10-12 weeks (25-29 g) without evidence of ear pathology were used for this study. The animals were housed in groups of five animals per cage on an artificial light/dark cycle (12/12 h, lights on at 07:00), with free access to food and water. The Northern Stockholm Ethical Committee approved the care and use of animals in this experiment.

3.2 EXPERIMENTAL DESIGN

All of the experimental procedures for this study were performed at the same day time in order to avoid variations of HPA status. All of the treatments were done at 7.30 – 12.00 am. ABR thresholds were determined in all animals 2-5 days prior to the acoustic trauma.

Paper I. Animals were injected with metyrapone and RU486 or with solvents (vehicle groups) 1.5 h prior to acoustic trauma to generate a temporary threshold shift (TTS) (free field broadband noise at 6 - 12 kHz was used for 45 min at intensity of 100 dB SPL). The controls for trauma were placed in acoustic chambers for 45 min, but no noise was generated. The collection of the material was performed at different time points post trauma: immediately, 4 h and 24 h after trauma cessation.

Papers II – III. Animals were injected with metyrapone and RU486 or with solvents (vehicle groups) 1.5 h prior to the restraint or sham restraint. After restraint or sham restraint for 4 h there were subjected to acoustic trauma to generate TTS (as described for Papers I and II). The controls for trauma were placed in acoustic chambers for 45 min, but no noise was generated. ABR thresholds were evaluated immediately, 24 h and 48 h after trauma. Biological material was collected at different time points (immediately, 2 h, 4 h, 24 h after trauma).

Paper IV. Animals were subjected to a free field broadband noise (frequency of 6 - 12 kHz) of 100 dB SPL for 45 min for generation of TTS, or 110 dB SPL for 2 h for permanent threshold shift (PTS). The controls for TTS and PTS were placed to the acoustic chamber in mesh cages for 45 min and 2 h respectively, but no acoustic stimulation was generated. ABR measurements were performed 30 min, 24 h, 48h, 72h, 1 week and 2 weeks after trauma. Biological material was harvested at 30 min, 2 h, 4 h, 24 h after trauma. For the PTS groups, the material was collected at 72 h and 2 weeks post trauma.

3.3 PHARMACOLOGICAL TREATMENTS

The steroid 11 β–hydrozylase inhibitor metyrapone [2-methyl-1, 2-di-3-pyridyl-1-propanone] 200 mg/kg, in water solution intraperitoneally (ip) and RU486 100 mg/kg, in vegetable oil subcutaneously(sc) were used in order to disrupt HPA axis response to stress. Solvents (deionized water ip and vegetable oil sc) were used as vehicle.

3.4 RESTRAINT STRESS

Animals were kept in 50 ml conical Falcon tubes with ventilation holes placed in a soundproof booth for 4 hours. They did not have access to food and water during restraint stress.

3.5 AUDITORY BRAINSTEM RESPONSE (ABR)

Animals were anesthetized with ketamine (20 mg/kg) and xylazine (50 mg/kg) ip. ABR thresholds were recorded with subcutaneously stainless-steel electrodes as described previously and assessed at frequencies 8, 12.5,16 and 20 kHz . The stimuli were generated through Tucker-Davis Technologies (Gainesville, FL) equipment controlled by a computer and delivered by EC1 speakers for closed-field stimulation (Tucker-Davis Technologies). Thresholds were defined as the lowest intensities at which visible ABR waves were detected in two averaged runs.

3.6 ELISA

Blood was collected in heparinized tubes after cervical dislocation, plasma separated by spinning (6 000 rpm, room temperature) and stored at – 20 °C. Corticosterone concentration was measured using ELISA kit (MD Biosciences, St. Paul, USA, Assay Design, MI, USA).

3.7 IMMUNOCYTOCHEMISTRY

Cochleae were fixed in 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) followed by 0.5 % PFA at +4°C, decalcified in EDTA, cryoprotected in sucrose and quickly deep frozen and sectioned (14 μm). The Vectastain® Elite ABC standard kit (Vector laboratories, Inc, Burlingame, CA) was used for rabbit primary antibody as described previously (Tahera et al., 2006c), and Vector® M.O.M Immunodetection Kit (Vector laboratories, Inc, Burlingame, CA) was used for mouse primary antibody as described in manufacturer's instruction. The last step was performed with DAB nickel. Counterstaining with cresyl violet was performed for staining with anti-pJNK and anti-synaptophysin antibodies.

3.8 RT-PCR

Animals were killed by cranial dislocation, cochlea were quickly removed and placed in TRIZOL LS reagent (Life Technologies, Invitrogen, MD, USA). First-strand cDNA was obtained with SuperScript III (First Strand cDNA synthesis kit, Life Technologies, Invitrogen, MD, USA). PCR was performed with Taq DNA polymerase /Roch Molecular Biochemicals, Mannheim, Germany) with specific primers for GR (GI 51057) and GAPDH (GI 193423). PCR products were resolved in 1.5% agarose gel, visualized with ethidium bromide, and quantified with Quantity One (BioRad, Hercules, USA).

3.9 HYBRIDIZATION IN SITU

Cochleae were quickly removed, frozen and dissected from the bony shell in ice-cold PBS. The soft cochlear tissues containing spiral ganglion was frozen for further dissection. Sections (14 μm) were used for hybridization with specific GR riboprobes as described (Hansson et al., 2003). Sections were counterstained with cresyl violet.

3.10 WESTERN BLOT

Animals were sacrificed with ice-cold PBS perfusion under ketamine-xylazine anesthesia. Cochleae were dissected in PBS supplemented with proteinase inhibitor cocktail (Roche Diagnostics, Mannheim, Germany), DTT and Na_3VO_4. Cochleae of 3-4 animals were pulled and frozen. Proteins were isolated on ice, resolved separated in SDS-PAGE gel with 4 % spacer and 12 % (w/v) separating phases, and transferred to PVDF membranes (Amersham Pharmacia Biotech, Little Chalfont, UK) by semi-dry electroblotting. The PVDF membranes were developed with an enhanced chemiluminescence Western blot detection kit (Pierce SuperSignal® West Dura, Rockford, IL) and exposed to Lumi-Film chemiluminescent detection films (Roche Diagnostics, Mannheim, Germany). The results were quantified using Tina software (Raytest, Isotopen Messergäte GmbH, Munich, Germany), GenTools (version 3.08, SynGene, Cambridge, England) or Quantity One (Version 4.6.6., Bio-Rad).

3.11 STATISTICAL ANALYSIS

The comparisons of means were performed with 1-way (Paper IV) or 2-way (Papers I, III and IV) ANOVA with Tukey correction. For the comparison of two groups the Students t-test was explored. A value of $P < 0.05$ was considered as statistically significant. The software SigmaStat version 2.03 (Systat Inc., Richmond, CA, USA) was used for statistical analysis.

4 RESULTS

4.1 PROTECTION AGAINST ACOUSTIC TRAUMA INVOLVES CORTICOSTERONE RELEASE AND GR NUCLEAR TRANSLOCATION IN THE SPIRAL GANGLION NEURONS

The protective role of glucocorticoids for hearing function was reported previously, but the mechanism underlying this protection has not been fully characterized. We hypothesized that acoustic trauma can trigger HPA activation, corticosterone release, and the activation of GR in the cochlea. Our main focus was on the spiral ganglion neurons because of their rich abundance of glucocorticoid receptors, their pivotal role in signaling auditory information to the central auditory nervous system.

In order to test our working hypothesis, a temporary hearing loss was generated in by acoustic trauma followed by ABR measurements. In the vehicle treated group, acoustic trauma resulted in ABR threshold shifts of 10 to 30 dB immediately post trauma in the frequencies between 8 and 20 kHz. The threshold shifts were reduced to 6 -18 dB 24 h post with a complete recovery to pre-trauma values by 48 h post. In the Met + RU 486 treated groups, ABR threshold shifts were higher immediately and 24 h post trauma with a statistical significant difference from the vehicle-treated animals at 8, 12,5 and 16 kHz. In contrast, pre-treatment with dexamethasone resulted in a decrease of ABR threshold shifts immediately (at 12.5 kHz) and 24 h (all measured frequencies) post trauma compared to vehicle + trauma group. Thus, the disruption of the HPA axis and GR activation by Met + RU 486 exacerbates the post-traumatic elevation of ABR threshold shifts while the GR agonist, dexamethasone, protects hearing and facilitates the post-traumatic recovery. The antagonist of MR spironolactone did not change post-traumatic ABR threshold shifts compared to the vehicle suggesting that MR are not involved into the acute response to acoustic trauma.

The plasma corticosterone was measured after different manipulations at different time points of the experiment. Blood was taken between 10 am and 2 pm to avoid the interference of the circadian fluctuation of corticosterone release. The corticosterone level was significantly elevated (90 ± 13 ng/ml) by the injection compared to the basal level for this day time (9 ± 1,5 ng/ml) when measured 1,5 h after injection of vehicle while the injection of Met + RU 486 resulted in a slightly elevated corticosterone 1,5 h after injection which was significantly less then after vehicle injection. After vehicle injection, acoustic trauma elevated plasma corticosterone levels and these values were significantly higher compared to pre-trauma vehicle levels and post-trauma Met + RU 486 values. There was no elevation of the corticosterone immediately post trauma in Met + RU 486 group compared to pre-trauma values. The evaluation of corticosterone concentrations 2 h post trauma showed a significant decrease in vehicle-treated group (30 ng/ml). On the contrary, the Met + RU 486-treated group showed an elevation of the corticosterone by 2 h post trauma which we attributed to the pharmacokinetics of the inhibitor of the corticosterone synthesis inhibitor metyrapone.

The expression of GR in spiral ganglion neurons was evaluated at different time-points post trauma. RT-PCR showed that GR mRNA expression in the cochlea is down-regulated 2 h post trauma, and Met + RU 486 significantly reversed this reaction

compared to the vehicle. *In situ* hybridization for GR mRNA of the spiral ganglion sections confirmed this finding. A similar trend was detected for GR protein expression 24 h post trauma. Specifically, the expression of GR protein in spiral ganglion neurons was down-regulated in the vehicle-treated group 24 h post trauma while in the Met + RU 486-treated group it was significantly elevated compared to the vehicle group. Vehicle alone and Met + RU 486 alone did not cause any of a change in GR mRNA or protein expression. These findings clearly demonstrated that spiral ganglion neurons have a negative feedback mechanism for GR synthesis, and that corticosterone effects on the cochlea is mediated by GR after the mild acoustic trauma.

4.2 RESTRAINT STRESS PROTECTS COCHLEA AGAINST HEARING LOSS INDUCED BY ACOUSTIC TRAUMA THROUGH THE ACTIVATION OF CORTICOSTERONE RELEASE AND GR NUCLEAR TRANSLOCATION IN THE COCHLEA

We used restraint stress as a model for activating the HPA axis followed by an acoustic trauma that resulted in a temporary hearing loss (TTS). In order to specify the role of corticosterone and GR several groups were injected with Met + RU468 prior to stress or acoustic trauma. We found that restraint stress reduced ABR threshold shifts 24 h post trauma compared to the trauma group. When the glucocorticoid pathway was disrupted by treatment with Met + RU 486 prior to restraint stress and trauma, no reduction of ABR threshold shifts was found suggesting that corticosterone and GR as key factors of a hearing protection induced by restraint stress. Plasma corticosterone was higher after restraint stress + trauma compared to post trauma only. The concentration of corticosterone in Met + RU486 + restraint stress + trauma was also elevated indicating that disruption of the negative feedback of glucocorticoid regulation is mostly due to the effect of RU486. By 2 h post trauma the corticosterone levels returned to control values. Immunocytochemical analysis demonstrated that the expression of GR in spiral ganglion neurons nuclei was not changed immediately post trauma alone, but was significantly elevated after restraint stress + trauma suggesting an activation of GR in spiral ganglion neurons. Met + RU 486 treatment prior to restraint and trauma abolished this elevation indicating even higher levels of plasma corticosterone were not able to activate GR in the cochlea in the presence of the GR antagonist. The level of GR is the main factor which determines the sensitivity of tissues to glucocorticoids. Particularly, the down-regulation of GR by glucocorticoids as a mechanism of a negative feedback has been demonstrated for different types of cells (Freeman et al, 2004, Conway-Campbell et al, 2007, Wang and DeFranco, 2005). We found that the expression of the GR mRNA was down-regulated in spiral ganglion neurons immediately post restraint stress, while the treatment with Met + RU 486 significantly up-regulated GR mRNA in the restraint stress + trauma group compared to vehicle-treated restraint stress + trauma group. The GR protein level in the cochlea was not changed by any of the treatments immediately post trauma, but a statistical significant increase was found in Met + RU 486-treated group compared to the vehicle treated group 24 h post restraint stress + trauma demonstrating that RU 486 interfered in the turnover process of the GR.

4.3 THE PROTECTIVE EFFECT OF RESTRAINT STRESS ON THE AUDITORY SYSTEM INVOLVES GR-DEPENDENT ERK CASCADE IN THE COCHLEA

In the previous papers we showed that acoustic trauma of 100 dB resulted in a temporary hearing loss (TTS) and triggers the activation of both general and local cochlear stress activated systems such as corticosterone and GRs, and that the local activation of the GR in a cochlea mediates the restraint stress induced protection of hearing. Here we analyzed the functional interplay between GRs and mitogen-activated protein kinases (MAPKs) in the cochlea after restraint stress and acoustic trauma. In addition, we characterized the expression pattern of GR and the phosphorylated forms of ERK, p38 and JNK in the cochlea.

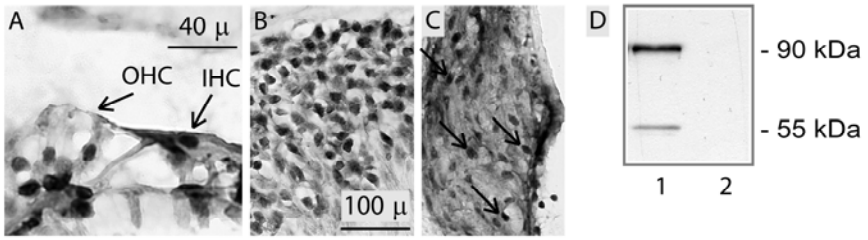

Figure 6. Immunocytochemical localization of the glucocorticoid receptor (GR) in the mouse cochlea. (A) Positive staining in the nuclei of the inner and outer hair cells (IHC and OHC). (B) GR-positive spiral ganglion neurons (SGN). (C) Positively stained fibroblasts of the spiral ligament (arrows). (D) The expression of GR in nuclear fraction (upper panel) was up-regulated in vehicle + trauma (lane 3) and vehicle + RS (lane 4) groups while in cytosolic extract (lower panel) from the same groups it was down-regulated. The Met + RU 486 pre-treatment (lane 2) combined with either trauma (Met + RU486 + trauma, lane 5) or RS (Met + RU 486 + RS, lane 6) did not change the nuclear or cytosolic expression of GR compared to vehicle (lane 1). The protein loading was verified by MemCode staining. (E) Representative blots of the expression of TBP and GAPDH in nuclear and cytosolic extracts in the vehicle group. TBP is abundant in nuclear extract while it is almost undetectable in cytosolic fraction. The expression of GAPDH is detectable in both fractions.

First, we found that GRs are abundantly expressed not only in the spiral ganglion neurons, but also in outer and inner hair cells, while their expression in fibrocytes of the spiral ligament was low-to-moderate (fig. 6). Moreover, we confirmed the nuclear translocation of GR in the cochlea after restraint stress and acoustic trauma using Western blot of cytosolic and nuclear protein extracts. We also found that Met + RU 486 treatment abolished GR nuclear translocation after restraint or acoustic trauma. The immunocytochemical mapping of pERK 1/2, phospho-p38 and pJNK 1/2 in the cochlea showed that all these MAPKs are expressed after vehicle treatment. Specifically, a moderate expression of pERK 1/2 in the nuclei of the inner and outer hair cells, the supporting (Deiters') cells, and spiral ganglion neurons was found, while the spiral ligament had only few positively-stained fibrocytes. The expression of pp38 was localized to the organ of Corti and spiral ganglion neurons while spiral ligament was negative. pJNK 1/2 were found only in the organ of Corti presumably in the cytoplasm of the outer hair cells and in the nerve endings of the spiral ganglion neurons below hair cells. Thus, the expression pattern of GR and different MAPK is overlapping in the cochlea making possible the direct interaction of them within these cells.

Proteins of the peripheral part of the auditory nerve and the cochlear tissues (lateral wall, and organ of Corti) immediately post acoustic trauma were analyzed using Western blot.

We found that neither restraint stress nor acoustic trauma changed the expression of pERK 1/2 in both fractions of the cochlea extracted immediately post trauma. Restraint stress + acoustic trauma decreased the expression of pERK 1/2 in both fractions. The administration of Met + RU 486 reversed the reaction of pERK 1/2 to the restraint stress alone and restraint stress + trauma, but not to the trauma alone. Specifically, Met + RU 486 alone down-regulated pERK 1/2 expression compared to the vehicle alone. pERK 1/2 was down-regulated in Met + RU 486 + restraint stress group compared to vehicle + restraint stress group. Restraint stress did not change the expression of pERK 1/2 after Met + RU 486 compared to Met + RU 486 alone. In contrast, restraint stress + acoustic trauma significantly up-regulated pERK 1/2 when applied after Met + RU 486. These findings suggest that the suppression of GR activity results in a suppression of pERK in the absence of other local disturbances (such as acoustic trauma). When acoustic trauma is applied in the presence of GR-antagonism there is an elevation of pERK. The expression of pp38 was elevated by restraint stress in the nerve fraction and in the cochlea tissues compared to the vehicle alone, and this elevation was abolished by Met + RU 486. The combined treatment with restraint stress + trauma resulted in a decrease of pp38 in the nerve fraction, but not in the cochlear tissues. This decrease was reversed by Met + RU 486 treatment. These data suggest that restraint stress, but not acoustic trauma increased pp38 expression in the auditory nerve in a GR-dependent manner. The relative activation of phospho-p38 after restraint stress + trauma in Met + RU 486 treated group was specific for the neuronal tissue of the cochlea. No changes of the expression of pJNK 1/2 were detected after any of the treatment in both fractions obtained from the cochlea immediately post trauma.

These findings demonstrate a GR-dependent ERK-mediated pathway that modulates auditory function in the cochlea. They also show that restraint stress activates cochlear p38 through GR, which plays a protective role for the cochlea.

4.4 TEMPORARY AND PERMANENT NOISE INDUCED HEARING LOSS RESULTS IN THE DIFFERENTIAL ACTIVATION OF MAPK, BDNF AND TRKB IN THE COCHLEA

In the previous papers we showed that mild acoustic trauma activates the HPA axis with a subsequent release of glucocorticoids, which in turn activates glucocorticoid receptors in the inner ear and facilitates recovery from a temporary hearing loss. This finding suggests that glucocorticoids regulate metabolic pathways which determine the fate of cells damaged by acoustic trauma. One of the most important intracellular stress-activated systems is a group of MAPKs including extracellular-signal regulated kinases 1/2 (ERK 1/2), c-jun-N-terminal-activated kinases 1/2 (JNK 1/2) and p38. Differential modulation of the activity of JNK by acoustic traumas of a different sound intensities has been previously reported (Selivanova et al., 2007; Murai et al., 2008). In order to characterize the changes in the activity of MAPK after acoustic trauma, we analyzed the expression of the phosphorylated forms of JNK1/2, ERK 1/2, and p38 within 24 h - 72 h

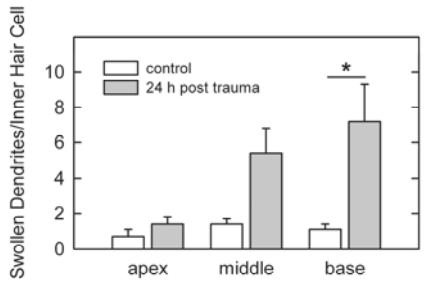

Figure 7. Quantification of the amount and size of swollen dendrites per inner hair cells. Statistically significan differences were found at the basal turn immediately post trauma compared to the control.
* $p<0.05$, t-test.

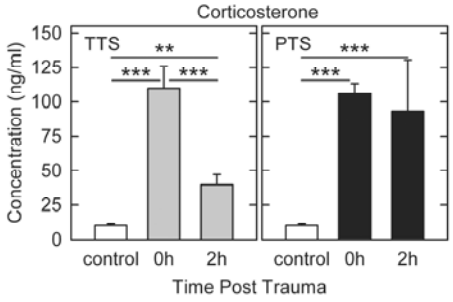

Figure 7. The values of blood corticosterone. Left panel. The dynamics of the corticosterone shows an elevation immediate post TTS with a significant decrease by 2 h. Right panel. The elevation of corticosterone immediate post PTS is up to the same level as post TTS. The elevation of corticosterone persists 2 h post PTS. ** $p<0.01$, *** $p<0.001$, t-test.

post trauma in the cochlea. The expression of BDNF and its receptor TrkB were assessed as possible pro-regenerative factors, and the expression of c-fos was evaluated as a marker of the cellular stress.

Animals were subjected to acoustic traumas at two different intensities: 100 dB SPL for 45 min or 110 dB SPL for 2 h. The physiological and morphological consequences of these two traumatic paradigms were evaluated by measurement of ABR thresholds, defining the amount and size of the auditory neuron dendrite endings and the analysis of hair cell loss. The activation of the stress-responsive systems was determined by the analysis of plasma corticosterone concentration and the expression of the phosphorylated ERK, JNK and p38 in total cochleae tissues at different time-point after traumas.

We found that an acoustic trauma of 100 dB resulted in a temporary ABR threshold shift (or TTS) and recovered by 48 h post trauma. The transitory de-afferentation of the inner hair cells (or swollen dendrites) was found immediately post trauma (Fig. 7), but no hair cell loss was detected after 2 weeks. The trauma of 110 dB resulted in ABR threshold shift which did not recover within 2 weeks post trauma. Hair cell loss in the basal turn of the cochlea was also found indicating that the damage caused by this type of trauma is permanent. Thus, acoustic trauma of 100 dB for 45 min results in TTS, and trauma of 110 dB for 2 h results in PTS. The corticosterone concentration was elevated up to approximately the same level after TTS and PTS, but after PTS this elevation was of a longer duration suggesting that the acoustic trauma of higher intensity had a longer and stronger effect on the HPA axis (Fig. 8).

The expression of the active (phosphorylated) forms of p38, pJNKs and pERKs was up-regulated 30 min post severe trauma (permanent hearing loss). A decrease (p38) or no change (ERKs and JNKs) of the expression of the active forms were detected after mild trauma. At 24 h post-trauma phospho-p38 was decreased in both transient and permanent hearing loss while pERKs and pJNKs were up-regulated only by transient hearing loss. The expression of pERKs and pJNKs were back to the control levels in permanent hearing loss at 24 h post trauma, and the expression of phospho-p38 and pJNKs was down-regulated at 72 h post trauma. The expression of BDNF was up-regulated in temporary and permanent hearing loss, but only after permanent hearing loss the elevation was detected at 24 and 72 h whereas in temporary hearing loss it returned to control values by 24 h. In the permanent hearing loss group the precursor of BDNF (pre-

BDNF) was elevated. The elevation of BDNF and pro-BDNF in the PTS group coincided with the down-regulation of the truncated form of the TrkB T1 receptor. The up-regulation of c-fos was detected at 30 min post TTS and PTS, and the immunocytochemical analysis showed an increase of the nuclear expression of c-fos in supporting and hair cells in organ of Corti, stria vascularis and spiral ganglion neurons.

5 DISCUSSION

Acoustic trauma activates the hypothalamic-pituitary-adrenal (HPA) axis and affects the cochlea through glucocorticoid receptors. Our results show that molecular cochlea changes in the cochlea induced by acoustic trauma are regulated by a complex interplay of glucocorticoid receptors and local cochlea stress-activated pathways such as mitogen-activated protein kinases (MAPKs), and neurotrophins. Particularly, the modulation of the sensitivity of the cochlea to acoustic trauma is regulated by glucocorticoids and glucocorticoid receptors (Papers I, III, IV). The level of the endogenous glucocorticoids and the sensitivity of glucocorticoid receptors depend on the previous stress experience and the intensity and duration of stress (Conway-Campbell et al., 2007; de Kloet et al., 2009). The stress experience modulates the response to acoustic trauma through the HPA axis as it has been demonstrated for restraint stress (Wang and Liberman, 2002; Tahera et al., 2006a) and sound conditioning (Tahera et al., 2007). The first response to stress is a release of glucocorticoids by adrenal glands, and the amount and duration of this release is critical for the ultimate effect of stress on the peripheral organs. Acoustic trauma also triggers the release of glucocorticoids (Muchnik et al., 1992; Paz et al., 2004), and we showed that the level of this release depends on the pre-traumatic stress experience. Glucocorticoids exert their effects through glucocorticoid and mineralocorticoid receptors (de Kloet et al., 1990; Reul et al., 1990). Mineralo- and glucocorticoid receptors are expressed in all tissues while their expression level and signaling pathways vary for specific tissues. Both types of receptors can bind glucocorticoids and regulate the transcription upon ligand binding, but the dynamics of their activity is different. The balance between MR and GR allows for the fine tuning of glucocorticoid signalling in different brain regions (Hansson et al., 2000), while acute elevation of glucocorticoids is most probably mediated by glucocorticoid receptors due to their rapid transcriptional (and possibly non-transcriptional) effects and their relatively fast turnover (Conway-Campbell et al., 2007). Both types of receptors are expressed in the inner ear (ten Cate et al., 1992; Rarey et al., 1993; Yao and Rarey, 1996; Erichsen et al., 2001). Nevertheless, only glucocorticoid receptors seems to be involved in response to acoustic trauma since the mineralocorticoid receptor antagonist did not change ABR threshold shifts (Paper I) although the effect of mineralocorticoids on ABR thresholds has been previously documented (Trune and Kempton, 2001). In contrast, the antagonist of the glucocorticoid receptor RU486 with corticosterone synthesis inhibitor metyrapone elevated ABR thresholds post trauma (Paper I) compared to vehicle + trauma suggesting the crucial role of the glucocorticoid receptors in response to trauma-induced stress.

Glucocorticoid receptors are expressed in most of the inner ear tissues and in the central auditory system (ten Cate et al., 1992; Rarey and Curtis, 1996; Shimazaki et al., 2002). We showed their expression in the inner and outer hair cells of organ of Corti, in fibroblasts of the spiral ligament (Paper III) and in spiral ganglion neurons (Papers I, III, IV). The activation of the glucocorticoid receptors leads to their homodimerization and translocation in the nuclei of target tissues where they exert their transcriptional activity (Freedman et al., 1988; Tsai et al., 1988b; Hutchison et al., 1993). Acoustic trauma and restraint stress resulted in the up-regulation of the glucocorticoid receptors in the nuclear

extracts of the cochlea relative to the GR cytosolic expression (Paper III) indicating that abovementioned stressors triggered an activation of GR-signaling in the cochlea. The localization of this activation occurred in spiral ganglion neurons where an increase of a nuclear staining for GR was detected (Papers I, III). Moreover, Met+RU486 abolished this nuclear up-regulation after restraint stress and acoustic trauma in the cochlea tissues (Paper III) and particularly in spiral ganglion neurons (Papers I, III) which coincided with deteriorative effect of these GR blockage to post-traumatic ABR threshold shifts (Papers I, III). Taken together, these data indicate that acoustic trauma activates the HPA axis with a subsequent release of glucocorticoids, which in turn activates glucocorticoid receptors in the inner ear and accelerates recovery from TTS (Paper I). These findings suggest that glucocorticoids regulate metabolic pathways which are involved in reaction to acoustic trauma and that the corticosterone-induced protection is mediated by glucocorticoid receptors in the cochlea tissues and in spiral ganglion neurons. Moreover, this corticosterone–glucocorticoid receptor interaction in the inner ear is crucial for the restraint-induced protection of the auditory system against acoustic trauma.

The mechanisms of this protection depend on further pathways of GR signaling, as well as on the GR interaction with other intracellular pathways. One GR–mediated signaling pathways involves an autoregulation through negative feedback(Dong et al., 1988; Rosewicz et al., 1988). Indeed, the sensitivity of a target cell to glucocorticoids depends on the amount and the activity of GRs, and the autoregulative GR mechanisms can vary in different cell types. Nevertheless, the most common cascade of GR autoregulation is a suppression of GR mRNA synthesis upon GR activation through specific DNA sequences – glucocorticoid response elements (GRE) (Freedman et al., 1988; Hard et al., 1990). We found that this negative feedback loop can be activated in spiral ganglion neurons of the cochlea in response to 9 – 15–fold elevation of the plasma corticosterone (Papers I and III) (Tahera et al., 2006a) and include a fast transcriptional response (down-regulation of the GR mRNA at 2 h after trauma or restraint stress + trauma) followed by GR protein decrease detected at 24 h post these challenges. The dynamics of GR can play an important role in cellular recovery after trauma, as it was confirmed by the experiment with RU486 + Metyrapone where this negative feedback loop was disrupted resulting in exacerbation of trauma-induced hearing loss (Papers I and III) (Tahera et al., 2006a). Moreover, this disruption can interfere in cellular recovery on the level of GR-interacting cascades involved in cellular reparation and neuroregeneration. These cellular cascades triggered by acoustic trauma include intracellular pathways regulated by mitogen activated protein kinases (MAPKs) (Selivanova et al., 2007; Wang et al., 2007; Murai et al., 2008).

MAPKs is a group of enzymes activated in response to extracellular stimuli including membrane receptor (cytokines, growth factors etc) and receptor-independent mechanisms such as direct cellular stress (physical and chemical) (Kyriakis and Avruch, 2001). MAPKs can interact with GR functionally or directly. Acoustic trauma activated MAPKs in the cochlea, and the pattern of their activation depended on the intensity of the acoustic stimulation (Paper IV). Particularly, mild acoustic trauma resulting in temporary threshold shifts (TTS) did not cause an immediate change in the phosphorylated forms of the extracellular signal-regulated kinases 1/2 (pERK 1/2) or c-jun-N-terminal kinases 1 and 2 (JNK 1/2), while severe trauma resulted in permanent ABR threshold shifts (PTS)

triggered an up-regulation of all these MAPKs at the same time point after trauma. After a recovery period of a 24 h, the activation of JNK1/2 and ERK1/2 were detected in TTS group in contrast with their expression 24 h after PTS where their expression returned to the control values.

MAPK activation showed temporal differences after TTS and PTS reflecting distinct damaging mechanisms. The patterns of MAPKs activation correlated with the cochlear cell fate indicating a dual role of their activation and the importance of understanding temporal aspects of MAPK signaling (Murphy and Blenis, 2006). Particularly, the activation of ERKs, JNKs and p38 immediately after severe trauma (PTS) was most probably due to glutamate-induced exitotoxicity, pro-inflammatory cytokines release and overloading with reactive oxygen and nitrogen species (Goldman et al., 2004; Ito et al., 2006; Nam, 2006; Moult et al., 2008; Ramiro-Cortes and Moran, 2009), while the increase of pJNKs and pERKs at 24 h post mild trauma (TTS) is possibly caused by an accumulation of reactive oxygen species and neurotrophins (Hess et al., 2002; Lallemend et al., 2003; Lallemend et al., 2005). Indeed, we found an up-regulation of brain-derived neurotrophic factor (BDNF), in a cochlea after acoustic trauma (Paper IV). BDNF is a short peptide form the neurotrophin family which activates neuroregeneration and prevents apoptosis upon binding with neurotrophin receptor TrkB on the target cell (Kim et al., 2004; Liu et al., 2007b). The application of BDNF protects cochlear cells from degeneration and death (Gillespie et al., 2004). The immature form of BDNF has opposite effects and induces cellular apoptosis through binding to p75 NTR, the receptor from the tumor necrosis factor receptor super family (Teng et al., 2005). The mature (regenerative) form of BDNF was up-regulated after severe and mild trauma while the pro-BDNF was up-regulated only after severe trauma (Paper IV). The up-regulation of BDNF immediately after mild trauma followed by ERKs activation after 24 h suggests the relationship between BDNF and ERK cascade. After severe trauma, the expression of mature form of BDNF was up-regulated immediate post trauma and lasted up to 72 h post, while the expression of pERK1/2 was up-regulated only immediately post trauma suggesting that triggers of the ERK cascade other than BDNF are involved. These triggers can include glutamate and Ca^{2+} overload (Vanhoutte et al., 1999; Lubin et al., 2005; Lahne and Gale, 2008), and this ERK activation can exacerbate cellular damage and lead to cell death (Satoh et al., 2000; Ho et al., 2008). In addition, pro-BDNF can induce apoptosis through the activation of p38 and JNK (Costantini et al., 2005), and an increase in pro-BDNF and phosphorylated p38 and JNK correlated with the hair cell loss after severe trauma.

A negative role of ERK cascade after acoustic trauma was demonstrated when GR pathway was disrupted by Met+RU486 (Fig. 9). In contrast, restraint stress applied before acoustic trauma correlated with post-traumatic down-regulation of pERK1/2 and improved ABR thresholds recovery (Paper III). The down-regulation of ERK by Met+RU486 in the cochlea suggests that the interaction between GR and ERK cascade occurs even at basal levels of corticosterone. The inverted reaction from pERKs on the restraint stress + trauma treatment in presence of the Met+RU486 in cochlear tissues (organ of Corti), but not in the auditory nerve demonstrated the cell-specific interaction of GR and ERK under the stress conditions.

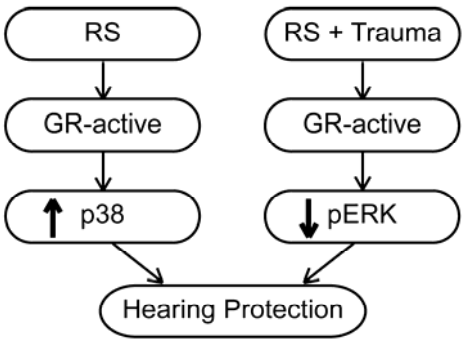

Figure 8. Diagram representing the summary of the results and a hypothetical pathway of the crosstalk between the glucocorticoid receptors (GRs) and MAP kinases. Restraint stress (RS) alone (left) activates GRs leading to p38 phosphorylation (activation) and protection against acoustic trauma. When GRs are blocked by Met + RU 486 there is no activation of GR or p38 and exacerbated hearing loss occurs. When RS is followed by acoustic trauma GRs are activated resulting in a decrease of pERK and protection against acoustic trauma. When GRs are inhibited there is an increase of pERK and exacerbated hearing loss occurs.

In contrast to ERK, p38 is mostly related to cell degeneration and is mediated by caspases and p53 (Sanchez-Prieto et al., 2000; Lee et al., 2008) although it can also mediate cellular protection induced by hypoxia-preconditioning in myocytes and neurons (Nishimura et al., 2003; Nagy et al., 2007). GR – p38 interaction was demonstrated for in vascular smooth muscle cells, myocytes, neutrophils and neurons (Qi et al., 2005; Kewalramani et al., 2008; Molnar et al., 2008; Saffar et al., 2008). The GR-dependent activation of p38 was found in the auditory nerve and in the cochlear tissues after restraint stress suggesting the protective role of p38 cascade (Fig. 9). These protective mechanisms probably include glutamate-induced transcription and an activation of antioxidant systems (Yin et al., 2000; Zepeda et al., 2008) (Yin et al., 2000).

The mechanisms underlying these GR effects on MAPKs may include direct action of GRs on ERKs as well as an indirect interplay of different cell types in the cochlea which differentially respond to GR signaling. For example, the down-regulation of MAPKs by GR activation through the up-regulation of the transcription of MAPK phosphatase-1 has been demonstrated in human epithelial carcinoma cells (Johansson-Haque et al., 2008) and can be attributed as a direct GR effects on MAPKs activity. The indirect effects of GR probably include the regulation of the synaptic transmission and the cytokines and neurotrophins which in turn regulates MAPKs cascades. The GR effect on synapses is specific for different brain regions and include the activation of NMDA upon GR binding to corticosterone if GR are localized in synapses (Liu et al., 2007a), as well as the transcriptional inhibition of NMDA receptor synthesis (Gourley et al., 2009). NMDA receptors activated by glutamate release after acoustic trauma (Pujol et al., 1993) triggered p38 and ERK-regulated cascades in hyppocampal neurons (Moult et al., 2008; Luo et al., 2009) providing with another possibility for GR – MAPKs interaction.

6 SUMMARY

The magnitude of hearing loss induced by acoustic trauma can be modified by modulating the level of corticosterone and the availability of glucocorticoid receptors (GR). Mineralocorticoid receptors (MR) are not involved in modulating auditory sensitivity to acoustic trauma. The local expression of GR in the cochlea is shown to be a critical factor in determining the overall sensitivity of the auditory system to acoustic trauma. The reaction to acoustic trauma involves the differential activation of mitogen activated protein kinases (MAPKs) in the cochlea which coordinate cellular stress responses by regulating gene expression. The activity of mitogen activated protein kinases (MAPKs) in the cochlea has different expression and temporal patterns after temporary (cell recovery) and permanent (cell death) insults. The expression of brain-derived neurotrophic factor (BDNF) was elevated in both TTS and PTS, but was elevated for a longer duration after PTS. The difference between permanent and temporary hearing loss also included the elevation of the immature form of BDNF (pro-BDNF) and the down-regulation of the truncated form of the neurotrophin receptor TrkB immediate post trauma. The interaction between central (corticosterone – GR) and cellular (MAPKs) stress response is involved in restraint stress induced protection from acoustic injury. Particularly, the correlation cross talk between GR and extracellular signal-regulated kinases 1/2 (ERK1/2) was involved in this protection.

The knowledge of the glucocorticoids and MAPKs cellular mechanisms are of a great importance for clinical audiology since it opens new avenues for therapeutic intervention for the prevention and treatment of hearing loss. These data can also help our understanding of the nature of individual sensitivity to acoustic trauma which may vary in accordance to the individuals HPA axis. The individual stress experience and HPA axis status may be critical for this sensitivity, and GR autoregulation and interaction with other stress-induced intracellular pathways will help to develop new treatments for hearing disorders.

7 ACKNOWLEDGEMENTS

I would like to express my sincere gratitude and appreciation to all who helped me to work on this thesis, and especially:

Professor Barbara Canlon, my supervisor, for her creative scientific ideas and her bright personality, for giving me the freedom to develop my research in the direction that was most interesting to me, for her positive criticism of my work and for inspiring me to have no fear to jump higher in my scientific ambitions. Special thanks for the creation of a nice friendly working atmosphere at her lab, I really enjoyed doing research there.

Agneta Viberg, for her inestimable help in preparation of our manuscripts and my thesis, for cochlea dissections, and for her valuable advices about the lab work. I would also like to thank her for our nice chats and her guidance during our travels to ARO meetings.

Zhao Bian, my co-supervisor during the first three years at KI, for giving me the first lessons in DNA and RNA technique, for his brave and original scientific ideas.

Stefan Ericsson and Bertil Fredholm, present and former head of the Department of Physiology and Pharmacology, for giving me the opportunity to work here and for their support

Yeasmin Tahera and Peter Johansson, my co-authors and friends, who were always ready to help me with my experiments and manuscript preparations. Our scientific and technical discussions were invaluable for me, and their good sense of humor made my research even more enjoyable and interesting.

Anita Hansson, my co-author, for her *in situ* lessons, expert help in molecular biology and sharing her immense knowledge in stress and brain research.

Ezma Idrizbegovic, Xianzhi Niu, Amzad Hossein, Saida Hadjab, Tina Charitidi, Dan Hasson, Martin Benka Wallen, Zlatan Alagic, Shaikh Nurunnabi, the former and current members of our group, for their help and support, and for bringing more warmth to the atmosphere of our lab.

Karima Chergui, Lubna Al-Khalili, Alex Chibalin, Abe Katz, for interesting chats and coffee-breaks.

Lars Gustaffson, Håkan Westerblad, Mats Rundgren, Bo Rydqvist for their help to resolve technical questions and for the lunch discussions at the kitchen.

Lilian Sundberg, Annika Olsson, Monica Tunberg, for their excellent technical assistance, for their advices about research, Sweden and family, and for their concern.

Ulla Lindgren, Louise Bovine, Camilla Fors-Holmberg, for their administrative help, for their understanding and advices, and also for our nice charts. Monica Pace-Sjöberg, Lena Öman and Dana Galuska for the administrative help.

Animal Department: Ann Hagström, Benny Gustafsson, Micke Elm, Melinda Verriere, for their help to order and take care of my mice.

Peter Wolf, Bo Tedner, Renee Andersson, for their help with cards and computers.

Many thanks to the former PhD students and postdocs of the Department of Physiology and Pharmacology, espessially Sietske Scotanus (and her cat), Ann-Louise Hemdahl, Olga Karovic, Lydia Bennedich Kahn and Holger Ulbrich.

Boris Kershengolz, Olga Kolosova, Alla Juravskaja, my first scientific advisors in Russia, for introducing me to the wonderful world of biological science, for teaching me all the basic lab techniques I know, and for our fruitful discussions. Vladimir Sharyko, my former colleague and my teacher in biochemistry, for all his help, support and understanding, and for being a devoted scientist.

Marpha Alekseeva, my clinical advisor, for introducing me in field of viral hepatitis and for all that support I received from her.

My ”Swedish” family, for their concern and constant support, and my Russian family for their understanding, warmth and having confidence in me. My father Mickail, who showed me what the science is at the very first years of my life, and for teaching me maths and logic. My mother Larisa, for her encouragement and especially for her help with David.

My small family, Igor, who was always skeptic to research but supportive to one researcher (me), and David, my little sunshine and the main person in my life.

This study was supported by Karolinska Institutet, the Swedish Medical Research Council, and the Stiftelsen Tysta Skolan.

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