MODULATION OF THE HPA AXIS ALTERS THE SENSITIVITY OF THE COCHLEA TO ACOUSTIC TRAUMA

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In memory of my beloved Dada
Modulation of the HPA axis alters the sensitivity of the cochlea to acoustic trauma
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ABSTRACT

The hypothalamic pituitary adrenal axis (HPA axis) regulates nearly all neuroendocrine responses of the body. An active HPA axis is crucial to maintaining homeostasis. An altered reactivity of the HPA axis can affect several physiological systems including the auditory system. The mechanism underlying the interaction between the HPA axis and the auditory system is not known. The overall objective of this thesis was to define the functional relationship between the HPA axis and the auditory system and to evaluate the underlying mechanisms.

The results presented in this thesis demonstrate how the cochlea is protected against acoustic trauma with previous activation of HPA axis. Activation of the HPA axis with either restraint stress or sound conditioning prior to acoustic trauma significantly elevates plasma corticosterone, activates GRs in the paraventricular nucleus (PVN), spiral ganglion neurons (SGN) and significantly lowers ABR threshold shifts. This protective effect was block by either adrenalectomy or combine use of glucocorticoid synthesis inhibitor (metyrapone) or receptor antagonist (RU486). Moreover, the combined use of metyrapone and RU486 prior to acoustic trauma leads to the exacerbation of hearing loss indicating that an optimal HPA axis response is crucial for the recovery from trauma.

Nuclear factor kappa B (NFκB), a GR regulated transcription factor, was also involved in this corticosterone mediated protective mechanism. Enhancement of nuclear translocation of NFκB in the spiral ganglion neurons coincides with lower ABR threshold shifts indicating an interaction between GR and NFκB in the SGNs. This can be the main factor, which contributes to the protection of cochlea against acoustic trauma. A selective NFκB inhibitor PDTC was use to confirm the role of NFκB in the cochlea. Pretreatment of PDTC caused an elevation of auditory thresholds and lowered nuclear translocation of NFκB in the SGNs. Steroid receptor co activator 1 (SRC-1), a GR co- regulatory protein also contributes to this GR mediated protective mechanism of hearing. The expression of SRC-1 in the SGN was increase after sound conditioning and acoustic trauma, which could be the probable mechanism of sound conditioning triggered protection of hearing.

In conclusion, the findings from this study imply that the HPA axis modulates the sensitivity of the cochlea against acoustic trauma by the release of glucocorticoid. Glucocorticoid regulates the expression and activation of GR locally in the cochlea. Active GR interacts with transcription factor NFκB and this interaction ultimately determines the susceptibility of cochlea against acoustic trauma.

Key words: HPA axis, corticosterone, glucocorticoid receptor, NFκB, spiral ganglion neurons, cochlea, acoustic trauma, paraventricular nucleus
LIST OF PUBLICATIONS

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


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1 BACKGROUND

1.1 THE COCHLEA

The cochlea resides deep in the temporal bone and is responsible for the perception of sound. Throughout its length, the basilar membrane and Reissner’s membrane divide the cochlea into three fluid filled compartments, the scala tympani, scala vestibule and scala media. The two outer compartments contain perilymph and the inner compartment contains endolymph. The hearing organ, the organ of Corti is located in the scala media. Within the organ of Corti, there are two types of sensory hair cells, the inner hair cells (IHCs) and the outer hair cells (OHCs), epithelial cells and supporting cells. The inner and outer hair cells are arranged in rows from base to apex through the basilar membrane. There is one row of IHCs and three rows of OHCs in mammalian cochlea. The stria vascularis and spiral ligament lie close to the bone along the lateral wall of cochlea. The stria vascularis is made up of three layers of cells, marginal cells, intermediate cells and basal cells arranged from medial to lateral. One of the major roles of the stria vascularis is to pump $\text{Na}^+$ away from endolymph and maintenance of the ionic composition of endolymph. Presence of Na-K ATPase system in the marginal cell suggests that stria vascularis plays an important role in generation of the endocochlear potential (EP). The intermediate cells, derived from neural crest generally termed as melanocytes also play an important role in generation of EP (Hilding et al., 1977; Steel and Barkway, 1989). The spiral ligament is located between the stria vascularis and the otic capsule. It is composed connective tissue elements and cells from mesenchymal origin (Morera et al., 1980). Spiral ligament contains the capillary bed for supply and drainage of the cochlea. It provides mechanical support to the stria vascularis and anchors the lateral aspects of basilar membrane (Fig 1). In addition, spiral ligament plays a crucial role in ionic balance of the cochlea. Spiral ligament with the help of Na-K ATPase system pump $\text{K}^+$ from the perilymph and transport it to the endolymph and thus maintaining the high concentration of $\text{K}^+$ in the endolymph. Spiral ligament contains five types of fibrocytes of which type II, IV and V are functioning in ion transport (Spicer and Schulte, 1991; Spicer and Schulte, 1996).

Figure 1 Principal structures of the cochlea (Ades et al., 1974)
1.2 THE SPIRAL GANGLION NEURONS (SGN)

The spiral ganglion neurons are the cell bodies of afferent neurons located in the central part of bony cochlea in a special channel called Rosenthal’s canal. These neurons are bipolar, extending one process to hair cells and other to auditory nuclei in the brain stem (Kiang et al., 1982; Berglund and Ryugo, 1987). There are two types of SGNs, type I and type II. The type I, SGNs synapses only to IHCs and are bipolar, large in size, myelinated and comprise 90-95% of the total fibers (Spoendlin, 1971; Bernard and Spoendlin, 1973). The ratio of type I neurons to hair cell innervations is 20:1. On the other hand, the type II fibers are unmyelinated, pseudo bipolar, smaller and synapse to OHCs. They branch repeatedly and synapse to several OHCs, approximately 15-20 OHCs make synapse with each neuron (Liberman, 1980) and constitutes only 5-10% of total SGNs. Degeneration of SGNs is one of the major cause of sensorineural hearing loss. Cochlea infections such as bacterial and viral labyrinthitis, ototoxic drugs, and acoustic trauma are the common causes of degeneration of SGNs (Kerr and Schuknecht, 1968; Otte et al., 1978; Nadol et al., 1989). The survival of SGNs depends on different factors, of which the neurotrophic factors are crucial (Gillespie and Shepherd, 2005; Roehm and Hansen, 2005). Several neurotrophic factors such as neurotrophic factor 3 (NT-3), nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), and glial derived neurotrophic factor (GDNF) support the survival of SGNs. In addition to neurotrophins, other trophic factors such as TGF (beta) superfamily, FGF, ciliary neurotrophic factor (CNTF), and cytokines participate in the survival of SGNs (Lefebvre et al., 1991; Staeccker et al., 1995). Recently, the role of nuclear factor kappa B (NFκB), a transcription factor that regulates many inflammatory and apoptotic genes has been demonstrated in the survival of SGNs (Lang et al., 2006). The expression of NFκB in the SGNs and its activation after acoustic trauma (Masuda et al., 2006; Tahera et al., 2006) also implies NFκB as a prominent target for SGNs function.

1.3 HEARING DISORDERS

Hearing loss is a major health problem affecting millions of people worldwide. According to the report of World Health Organization in 2002, approximately 250 million people suffering from hearing loss. In addition, hearing loss is one of the common disorders among the adult population and its prevalence is continuously increasing. Hearing disorder affects about 10% of the total population. Hearing loss arising in the auditory periphery is divided into two different type’s namely conductive and sensorineural loss. The conductive type of hearing loss is due to disturbances in the external ear canal and middle ear, most commonly due to disrupted impedance transformation in the middle ear. Hearing loss arising from cochlea or auditory nerve damage is known as sensorineural hearing loss. Acoustic trauma is one of the major causes of hearing disorders. One striking feature to acoustic trauma-induced hearing loss is the large variability among individuals (Davis et al., 2003). One possible cause for this large inter-individual variation may be due to the variations in the set point of the hypothalamic-pituitary-adrenal (HPA) axis and the subsequent influences on neuroendocrine responses.

1.4 THE HYPOTHALAMIC PITUITARY ADRENAL (HPA) AXIS

The hypothalamic pituitary adrenal (HPA) axis regulates nearly all neuroendocrine responses at both physiological and pathological condition. The appropriate activation of HPA axis is important in protecting the organism against adverse stimuli.
Corticotrophin releasing hormone (CRH), a 41 amino acid peptide is the principal regulator of the HPA axis. In non-stressful condition, CRH secretes in the portal system in a circadian fashion (Chrousos, 1992). The amplitude of high CRH in the early morning, eventually results elevated ACTH and corticosterone. In response to stress, the amplitude of release of (CRH) increases, which stimulates the anterior pituitary to secret adrenocorticotropin (ACTH) hormone, which in turn stimulates the adrenal gland to secret glucocorticoid (Chrousos, 1992). A negative feedback mechanism maintains the optimum activation of HPA axis by inhibiting over production of CRH (Fig.2). Though the optimum activation of the HPA axis is crucial for maintaining homeostasis, a hypo or hyperactive HPA axis can lead to adverse effect on organism. However, the degree of HPA axis activation depends on the intensity and duration of the exposure to stressors.

### 1.4.1 Paraventricular nucleus (PVN)

It is well documented that the paraventricular nucleus of the hypothalamus is the main regulator of complex stress responses. The paraventricular nucleus of the rat consists of approximately 20,000 neurons (Kiss et al., 1991), and contains 20 neuropeptides and several other neurotransmitters (Kiss et al., 1988; Meister et al., 1990). This nucleus is located in a triangular area in the middle of the hypothalamus between the third ventricle and the fornix. There are three subdivision of the area namely magnocellular, mediocellular and parvocellular nuclei. Among the different neuropeptides and neurotransmitters present in the PVN the corticotrophin-releasing hormone (CRH) a 41-amino acid peptide, vasopressin and oxytocin play the important role in response of various stressors. CRH neurons projects from parvocellular region to the median eminence and stimulate anterior pituitary to release ACTH. Subsequently, ACTH stimulates adrenal glands to secret glucocorticoid, which inhibit further release of CRH thus maintaining the negative feed back mechanism. The actions of glucocorticoid in the brain are exerted by glucocorticoid receptors and mineralocorticoid receptors (McEwen et al., 1986). GRs are present everywhere in the brain but most abundant in the CRH expressing neurons of PVN. Similar to the CRH neurons, parvocellular subdivisions of PVN expresses most of the glucocorticoid receptors. These receptors are actively involved in GC negative feed back mechanism leading to reduce the HPA axis activity (De Kloet and Reul, 1987).

### 1.4.2 Stress induced activation of the HPA axis

Stress refers as a response to a physical or psychological stimulus that disrupts ongoing homeostasis. Any sorts of physical or psychological stress activate the HPA axis results in a series of neuroendocrine cascade known as stress response. The stress response allows the body to make necessary physiological and metabolic changes to cope with the adverse stimuli and balance homeostasis. When a stressor is of short duration, it called acute stress. Acute stress results in an activation of HPA axis for shorter period. The time limited nature of acute stress renders its most of the effect as beneficial rather than damaging. On the other hand, when a stressor is of longer
period or chronically exposed it called chronic stress. Chronic stress leads to constant hyperactivity of the HPA axis resulting chronically elevated level of GC, which leads to several maladaptive and pathological conditions. Moreover, chronic stress can also results in a hypoactive HPA axis. Both hypo and hyperactive HPA axis contributes to different neuro physiological disorders (Miller and O’Callaghan 2002).

1.4.3 Models of activating the HPA axis

Restraint stress
Restraint stress is a widely used animal model in the area of stress research. In restraint stress, the animals are physically restrained and kept in an isolated area without any food and water. Over the past few years, the models of restraint stress are using to examine the interaction between the HPA axis and other physiological system. Restraint stress activates the HPA axis and elevates endogenous glucocorticoid that exerts protective effect against a variety of disorders. Endogenous glucocorticoids control the cerebral innate immunity and protects the brain against neurotoxic effect of lipopolysaccharide (LPS) (Nadeau and Rivest, 2003). The protective effect of restraint stress has been shown in digestive system against acute pancreatitis (Abe et al., 2004), against the oxidative stress by induction of metallothionein (Ghoshal et al., 1998) against the seizure induction in both male and female rats (Chadda and Devaud, 2005). The protective effect of restraint stress has been demonstrated also in cochlea (Wang and Liberman, 2002; Tahera et al., 2006). It has been shown that restraint stress alters the GRs level tissue specifically in the cochlea, the level increase in the spiral ligament tissue whereas decrease in organ of corti (Curtis and Rarey, 1995).

Sound conditioning
Sound conditioning is a well-studied paradigm where prior exposure to low levels of acoustic stimuli reduces the subsequent traumatic damage in the cochlea (Canlon et al., 1988). Sound conditioning mediated protection has been found in different species as guinea pig, rabbit, chinchilla, gerbils, rat and mice (Canlon et al., 1988; Campo et al., 1991; Ryan et al., 1994; Pukkila et al., 1997; Yoshida and Liberman, 2000). The clinical significance of the sound conditioning paradigm has been established in young adults (Miyakita et al., 1992). In spite of its well-documented protective effect on hearing, the underlying mechanism of sound conditioning mediated protection was not well characterized. Several mechanisms have been suggested to play a role in auditory protection by sound conditioning including the activation of antioxidant enzymes (Jacono et al., 1998; Harris et al., 2006), inhibition of apoptosis (Niu et al., 2003), increased tyrosine hydroxylase activity in the lateral efferent system (Niu and Canlon, 2002), and glucocorticoid activation (Yoshida and Liberman, 2000). Some studies showed that the protective effect contributed primarily by cochlea. Conditioning mediated protection was not abolished by cutting middle ear muscle or olivocochlear efferent (Ryan et al., 1994; Kujawa and Liberman, 1997) and failure to get protection in both ears by unilateral conditioning (Yamasoba et al., 1999) support this notion. Another study showed that short-term sound conditioning significantly elevates plasma glucocorticoid and protects hearing against subsequent acoustic trauma. The protection disappeared when treatment trauma interval is more than 48 hours (Yoshida and Liberman, 2000), which suggests that the protective effect is a contribution of endogenous glucocorticoid secreted by the stress response exerted by sound conditioning. Protection of hearing from sham surgery irrespective of conditioning also suggests that conditioning mediated
protection could arise from generalized stress response (Kujawa and Liberman, 1997).

1.5 THE HPA AXIS AND THE AUDITORY SYSTEM

Depending on the strength of its activation, the reactivity of the HPA axis response affects nearly all systems in the body including the auditory system. The different stressors that affect auditory function include restraint stress, heat shock, and acoustic trauma. Restraint stress and acoustic trauma has been shown to activate the HPA axis as revealed by elevation of plasma corticosterone (Wang et al., 2002) (Michaud et al., 2003). Moreover, acoustic trauma induces c-fos expression in the PVN and central auditory areas (Campeau and Watson, 1997). However, a direct anatomical connection between the auditory system and the hypothalamus has not been demonstrated yet but indirect interconnections via the bed of the stria terminalis to the auditory brainstem have been shown using dextran amine tracing (Campeau and Watson, 2000). Restraint stress elevates plasma glucocorticoid that protects cochlea from subsequent acoustic trauma (Wang and Liberman, 2002; Tahera et al., 2006). Up regulation of HSP by heat stress also protect cochlea from acoustic trauma (Yoshida et al., 1999; Mikuriya et al., 2005), as well as from endotoxin-induced inflammation (Sone et al., 2005). Elevation of plasma glucocorticoid after heat stress coincides with protection of cochlea that suggests glucocorticoid as a potential contributor of heat shock mediated protective mechanism of cochlea (Paz et al., 2004). The ultimate effect of these stressors on the cochlea is exerted primarily by glucocorticoid the major stress hormone. Naturally, glucocorticoid (GC) helps to regulate most of the physiological function by restoration of energy and glucose uptake in post stress condition.

1.6 SYNTHESIS OF GLUCOCORTICOID

Glucocorticoid is the major stress hormone secreted from the adrenal glands in response of various physiological and pathological conditions. A hormone is defined as a biomolecule that is secreted by the ductless glands directly into the blood and instantly transported to the target tissues where they exert their physiological and cellular effects. Structurally, all the hormones are divided in to diverse groups depending on their chemical properties and physiological functions as proteins, polypeptides, aminoacids and their derivatives and steroids. GCs are the member of classical steroid hormone family in which mineralocorticoid; androgen, estrogen and progesterone are other subclasses. GCs synthesized in the adrenal cortex within the zona glomerulosa and zona reticularis from circulating cholesterol by a complex series of enzymatic reaction (Fig 3). In human, glucocorticoid is termed as cortisol whereas in rodents it called corticosterone. Glucocorticoids were first discovered in 1930s, they named glucocorticoid due to their capability to stimulate glucose synthesis from the liver and the kidney. However, glucocorticoids have additional effects on both protein and fat metabolism. The most important metabolic effect of glucocorticoids is their ability to stimulate gluconeogenesis and decreased glucose utilization that finally elevates blood glucose concentration. Other important metabolic effects include reduction of cellular protein, increased blood amino acids and mobilization of fatty acids. By these metabolic effects...
effects, glucocorticoids play a crucial role in regulation of basal and stress related homeostasis. In spite of regulating homeostasis, glucocorticoids have their potential anti-inflammatory effects. Regardless of these, glucocorticoids have their physiological effect on several other physiological systems including the auditory system. Both endogenous and exogenous GC affects the auditory system. Elevated amount of endogenous GC by restraint stress or sound conditioning protect hearing against acoustic trauma (Yoshida and Liberman, 2000; Wang et al., 2002). Pretreatment of synthetic GC as dexamethasone also showed protection against acoustic trauma (Takahashi et al., 1996; Takemura et al., 2004). More over, in clinical practice GC are extensively used to treat different inner ear disorders such as sudden hearing loss, tinnitus, Menière’s disease, and auto-immune diseases (McCabe, 1979; Moskowitz et al., 1984; Alexiou et al., 2001; Dodson and Sismanis, 2004). The ability of GC to act on target organs and elicit their effects depends on the presence and availability of their receptor, the glucocorticoid receptor (GR).

1.7 THE GLUCOCORTICOID RECEPTOR

An intracellular protein named glucocorticoid receptor (GR), which is ~ 94-kDa in size, mediates the effects of GC. GR is the member of nuclear hormone receptor superfamily in which mineralocorticoid; androgen, progestin, estrogen, thyroid and a number of orphan receptors are the other members. GR is also known as ligand dependent transcription factor as they are capable of modulate the activity of several target gene promoters in its hormone bound state (Evans and Hollenberg, 1988; Mangelsdorf et al., 1995). Being a member of the nuclear receptor super family, GR is composed of three domain structures (Fig. 4). The N-terminal domain is responsible for activation of target genes and interacts with other transcription factor (Giguere et al., 1986; Dahlman-Wright et al., 1994). In steroid receptors, the N-terminal domain is most variable in terms of length and variability. The N-terminal domain contains many antigenic sites that have been used for developing antibodies against GR (Wikstrom et al., 1986). The N-terminal domain is the major target for ligand dependent phosphorylation at multiple serine residues (Bodwell et al., 1991). The DNA binding domain consists of two highly conserved zinc finger motifs in the central part of receptor which is essential for DNA binding at special promoter sites (Zilliacus et al., 1995). This domain is also important for receptor dimerization, nuclear translocation and transactivation (Picard and Yamamoto, 1987; Tsai et al., 1988; Lefstin et al., 1994). The C-terminal or ligand binding domain specifically binds with hormonal ligand (Giguere et al., 1986; Warriar et al., 1994). Besides ligand binding LBD has several function as it contains an important sequence for binding of heat shock protein (HSP) (Dalman et al., 1991; Hutchison et al., 1993), as well as for nuclear translocation (Picard and Yamamoto, 1987), dimerization (Dahlman-Wright et al., 1994) and transactivation (Godowski et al., 1987; Hollenberg et al., 1987). The constitutive activation of receptor after removal of LBD suggests that LBD can prevent the activation of receptor (Godowski et al., 1987). The LBD of GR showed a very flexible structure that helps the receptor to binds with variety of ligands (Bledsoe et al., 2004).

**Figure 4.** Functional domain of glucocorticoid receptor. The black box represents DNA binding domain, LBD indicates ligand-binding domain.
1.7.1 GR expression in the cochlea

Although GR is ubiquitously expressed in almost all tissues, its concentration varies (Bamberger et al., 1996). In the auditory system, GR is expressed in both neuronal and non-neuronal tissues of different species (ten Cate et al., 1992; Rarey et al., 1993; Rarey and Curtis, 1996; Shimazaki et al., 2002). Among different parts of the cochlea, the spiral ganglion neurons, spiral ligament show the highest expression and the hair cells, stria vascularis has lower GR expression. The strong expression of GR was observed in type III and type II fibrocytes of spiral ligament (Shimazaki et al., 2002). Spiral ligament fibrocytes play a crucial role in regulating inner ear homeostasis. Presence of Na-K-ATPase (Spicer and Schulte, 1991) in the spiral ligament fibrocytes further indicates that spiral ligament has some role in regulating the inner ear homeostasis. The GR expression and expression of Na-K-ATPase show similar pattern in developing cochlea (Erichsen et al., 1996) which suggests that GR probably regulating the expression of Na-K-ATPase expression. Moreover, the presence of GR in spiral ligament fibrocytes indicates that glucocorticoid could be an effective treatment of certain inner ear disorder as endolymphatic hydrops, labyrinthitis or otitis media, all of which showed reduced Cx 26 immunoreactivity in spiral ligament fibrocytes (Ichimiya et al., 1994; Ichimiya et al., 1998). It has been found that glucocorticoid receptors in the cochlea are sensitive to different stressors as restraint stress and acoustic trauma (Curtis and Rarey, 1995; Terunuma et al., 2001; Tahera et al., 2006). Acoustic trauma down regulates the expression of both GR mRNA (Terunuma et al., 2003) and protein expression (Rarey et al., 1995). GR protein expression was significantly down regulated in organ of corti after noise exposure converse to a non significant decrease in spiral ligament tissue (Rarey et al., 1995). On the other hand, restraint stress elevates the expression of GR protein in the cochlea (Curtis and Rarey, 1995). Expression of GR in the cochlea and its responsiveness to different stressor indicates that cochlea is a stress responsive organ and the sensitivity of cochlea is varied depending upon the intensity of stressors and its duration of exposure.

1.8 MOLECULAR MECHANISM OF GLUCOCORTICOID RECEPTOR

The unliganded GR resides in the cytoplasm as a multiprotein complex comprises of receptor itself, two molecules of hsp90, one molecules hsp70 and hsp56 and an immunophillin called FK506 (Hutchison et al., 1993). The non liganded GR multiprotein complex mainly resides in to the cytoplasm of the cells (Fig 5), although a small part of GR in association with HSP may resides in the nucleus (Wikstrom et al., 1987). The association of HSP to the LBD of GR keeps GR in an optimum conformation for binding of LBD as well as prevents GR activation (Bresnick et al., 1989). However, in absence of ligand, this complex can also undergoes a continuous process of dissociation and reassociation which is an ATP and hsp70 dependent process (Hutchison et al., 1993; Hu et al., 1994). First step of GR activation starts with binding of glucocorticoid with receptor. As a lipophilic substance, glucocorticoid readily crosses the cell membrane and binds with the receptor in the cytoplasm. The hormone bound glucocorticoid complex dissociates from HSP complex and enters in to the nucleus (Hutchison et al., 1993; Truss and Beato, 1993; Tsai and O'Malley, 1994). Within the nucleus, ligand bound GR can act by two ways, either by interacting with specific DNA sequences or by direct protein-protein interaction with other transcription factors without specific DNA binding. GC exerts its most of the endocrine and metabolic effects by binding with GRE; whereas the anti-inflammatory effects are mediated by interacting with other transcription factors.
Figure 5. Mechanism of GR action. In non-active state, GR resides in the cytoplasm bound with a multiprotein complex of hsp protein90 and hsp70. Upon ligand binding GR dissociates from HSP complex and ligand, bound GR enters in to the nucleus where it transactivates several genes either by DNA binding with specific GRE sites or by interacting with other transcription factors.

1.9 NUCLEAR FACTOR KAPPA B (NFκB)

The ultimate effect of GR depends on its interaction with other transcription factors as AP-1 and NFκB as well as with several other co-activators or co-repressors (De Kloet, 2004). Of several transcription factor NFκB is one of the crucial factor that is directly interacts with GR. NFκB is composed of homo or heterodimers of Rel proteins as RelA (p65), RelB, c-Rel, NFκB1 (p105/p50) and NFκB2 (p100/p52) (Zandi and Karin, 1999). The most studied dimer of NFκB is the p65/p50 heterodimers of which p65 is the transcriptionally active subunit (De Bosscher et al., 2003). A wide variety of stimulation including inflammation, oxidative stress, and different bacterial and viral protein activates NFκB. Recently acoustic trauma has been shown to activate NFκB in the cochlea (Masuda et al., 2006). In inactivate state NFκB resides in the cytoplasm binding with an inhibitory protein IκB (Baeuerle and Baltimore, 1988). IκB masks the nuclear localization signal sequence of p65 and inhibits NFκB activation. Phosphorylation and degradation of IκB by IκB kinase complex (IKK) leads to activation of NFκB, results its nuclear translocation (Fig 6). IκB family consists of several members of which IκB α, IκB β and IκB ε are the most important. In nucleus NFκB activates several target genes by binding with high affinity kappa B elements in their promoters (Baldwin, 1996). NFκB is involved in several gene regulations that are responsible for inflammation, apoptosis, differentiation and cell survival (Pahl, 1999). NFκB has a dual effect depending upon the cell type being investigated. In immune system, activation of NFκB upregulates proinflammatory cytokines and enhances inflammation on the other hand in nervous system, its activation is neuroprotective.
Figure 6. Mechanism of NFκB nuclear translocation. Inactivate NFκB resides in the cytoplasm binding with IκB. Upon stimulation phosphorylation and degradation of IκB leads NFκB to translocate into nucleus where it activates several responsive genes.

1.9.1 GR and NFκB interaction

The cross talk between GR and NFκB has been discussed over the years. GR and NFκB interact physically and they function as a mutual transcriptional antagonist. The mutual antagonism between GR and NFκB is more prominent in immune system. Several proinflammatory cytokines that are positively regulated by NFκB, is suppressed by glucocorticoids. GR represses NFκB transcriptional activity by physically interacting with the p65 subunit of NFκB. On the other hand, NFκB has also been shown to negatively regulate GR transcription (McKay and Cidlowski, 1998; Tao et al., 2001). The exact mechanism how GR repressing NFκB is still controversial. Several in vitro studies demonstrated that GR associate with p65 subunit of NFκB. And this interaction occurs in the nucleus of cell and is known to involve the p65 subunit of NFκB and DNA binding domain of GR (Caldenhoven et al., 1995). Nevertheless, the interaction of GR and NFκB has been demonstrated in the cytosol of rat liver (Widen et al., 2003). However, the proposed mechanism of GR mediated NFκB repression is that the interaction of GR and NFκB sequesters NFκB, inhibiting its binding to DNA (McKay and Cidlowski, 1998). Another suggested probable mechanism is that both GR and NFκB compete for mutual cofactors, such as cAMP response element binding protein (CREB) binding protein (CBP) and steroid receptor co-activator -1 (SRC-1). This mechanism was supported by the findings that both GR and NFκB required CBP and SRC-1 for their transcriptional activity (Gerritsen et al., 1997; McKay and Cidlowski, 2000). This finding was further established when over expression of SRC-1 and CBP abrogated GR mediated repression of NFκB (Sheppard et al., 1998). Contrary to this, it has also been suggested that CBP facilitates the interaction of GR and NFκB (McKay and Cidlowski 2000). In addition to these mechanisms, GR may repress NFκB gene activation by histone modification or chromatin remodeling. GR may also disrupt the transcriptional complex by interfering with basal transcriptional machinery (Smoak
and Cidlowski, 2004). Finally, GC may also repress NFκB activity through induction of NFκB inhibitor IκB. GR can increase IκB expression, which prevents nuclear translocation of NFκB (De Bosscher et al., 2003). However, a positive correlation between nuclear translocation of both GR and NFκB has also been demonstrated (Erlandsson et al., 2002; Nakamori et al., 2005). Simultaneous elevation of both GR and NFκB nuclear translocation was observed in herpes simplex virus infection (Erlandsson et al., 2002). Levels of both intranuclear GR and NFκB were elevated in activated polymorphonuclear leukocytes of systemic inflammatory response syndrome patients (Nakamori et al., 2005). All these findings suggest that GR and NFκB interaction probably involved multiple pathways and it is very much tissue or cell type specific.

1.9.2 Role of NFκB in the cochlea

Several studies have suggested that NFκB is expressed in the cochlea (Ramkumar et al., 2004; Jiang et al., 2005; Masuda et al., 2006; Tahera et al., 2006). It activates in response of acoustic trauma. Nagashima et al., 2005 showed that NFκB DNA binding and nuclear translocation of its p65 subunit significantly increased after acoustic trauma (Nagashima et al., 2005). Enhanced activation of NFκB as revealed by increased nuclear translocation was also observed in the lateral wall of mice cochlea after acoustic trauma (Masuda et al., 2005). On the other hand, a decrease of nuclear translocation of NFκB was found in the SGN of cochlea immediately after acoustic trauma (Tahera et al., 2006). However, four hours after acoustic trauma of same intensity showed an elevation of the nuclear translocation of NFκB in the SGN (Tahera et al., 2006). This findings suggest that acoustic trauma induced activation of NFκB is time dependent and this activation is directly related with protection of hearing (Tahera et al., 2006; Tahera et al., 2006). NFκB mediated protection of cochlea has been demonstrated by several other studies, Jiang et al., 2005 showed that NFκB pathways play a crucial role in hair cell survival against aminoglycoside ototoxicity (Jiang et al., 2005). Mice lacking with p50 subunit of NFκB showed auditory nerve degeneration and enhanced acoustic trauma induced hearing loss (Lang et al., 2006). The protective role of NFκB has also been demonstrated in the developing cochlea. Inhibition of NFκB caused apoptosis of immature hair cell in vitro (Nagy et al., 2005). Contrary to these reports, Masuda et al, 2005 suggests that activation of NFκB contributes to acoustic trauma induced hearing loss (Masuda et al., 2006). NFκB mediated hair cell death in the cochlea by expression of iNOS in the stria vascularis and spiral ligament has also been shown (Watanabe et al., 2002).
2 AIMS

The overall purpose of the study is to define a relationship between the HPA axis and the auditory system and to determine how the HPA axis modulates the sensitivity of cochlea against acoustic trauma.

The specific aims are:

- To determine the role that glucocorticoid and the glucocorticoid receptor has on the cochlea after acoustic trauma and to elucidate the down stream transcription factors of glucocorticoid receptor.

- To determine if restraint stress modulates glucocorticoid receptors in the inner ear.

- To determine the underlying mechanism responsible for restraint stress mediated protection of hearing against acoustic trauma.

- To determine if sound conditioning activates the HPA axis and protects hearing against acoustic trauma.
3 MATERIALS AND METHODS
(For detailed description, please see the individual paper)

3.1 ANIMALS
All animals used in this thesis were CBA male mice, aged 10 - 12 weeks (25 - 29 g) without any evidence of middle ear pathology. The animals were housed in groups of five animals per cage on an artificial light/dark cycle (12/12 h, lights on at 0700h), with free access to food and water. The Ethical Committee at the Karolinska Institute approved the care and use of animals in this experiment.

3.2 EXPERIMENTAL PARADIGM
The experimental paradigm in each study was different in the treatment and time point of tissue collection. Acoustic trauma used in the paper I and III was different from paper IV.
In paper I, animals were injected with either vehicle or metyrapone (corticosterone synthesis inhibitor) and RU486 (GR antagonist) 1.5 hours prior to acoustic trauma. Cochlea was collected in different time point as immediately after (to evaluate GR mRNA), 4 hours after (to evaluate NFκB nuclear translocation) and 24 hours after (to evaluate GR nuclear translocation) acoustic trauma.
In paper II, animals were injected with either vehicle or metyrapone and RU486, 1.5 hours prior to exposure them in restraint stress. After 4 hours of restraint stress, cochlea was collected immediately. In another group, cochlea was collected 24 h after restraint stress.
In Paper III, animals were injected with either vehicle or metyrapone and RU486, 1.5 hours prior to expose them in restraint stress for 4 hours. Immediately after restraint stress animals were exposed to acoustic trauma of 6-12 kHz, 100 dB, for 45 min. Cochlea was collected immediately after acoustic trauma. In another group, cochlea was collected 24 h after acoustic trauma.
In paper IV, either animals were adrenalectomized or sham operated 1 week prior to sound conditioning. Another group of mice was injected with either vehicle or metyrapone and RU486, 1.5 hours prior to expose them in to sound conditioning (8-16 kHz, 89 dB, 15 min). Twenty-four hours after sound conditioning animals were exposed to acoustic trauma of 8-16 kHz, 100 dB, for 2 hours. Cochlea and brain was collected immediately.

3.3 AUDITORY BRAINSTEM RESPONSE
Animals were anesthetized with an intra-peritoneal injection of ketamine (20 mg/kg) and xylazine (50 mg/kg). Supplementary injection of ketamine was given when needed. Auditory sensitivity was assessed with ABR thresholds for the frequencies of 8, 12.5, 16 and 20 kHz. ABR thresholds were recorded with subcutaneously stainless-steel electrodes as the potential difference between an electrode on the vertex and an electrode on the mastoid, while the lower back served as ground. The body temperature of the animals was maintained at $38.0$ °C using heating pad and cotton coverings. The stimulus signals were generated through Tucker-Davis Technologies (Gainesville, FL, USA) equipment controlled by computer and delivered by an earphone (Telephonix TDH 39, Farmingdale, NY, USA). The acoustic signal was delivered through the system inserted into the external auditory meatus. The evoked responses were amplified 100 000 times and averaged. The stimuli were presented at
a repetition rate of 20/second. At first, the stimuli were presented at intensity well above threshold and then decreased in 10 dB steps until threshold was found, and then in 5 dB steps until the ABR wave 1 disappeared. Threshold was defined as the lowest intensity at which a visible ABR wave was seen in two averaged runs.

### 3.4 MODULATION OF HPA AXIS

**Acoustic trauma**

Un anaesthetized animals were individually placed in to small wire mesh cages without any food and water in a sound proof box, free field broad band noise was then generated with a noise generator. In Paper I and III, animals were exposed to acoustic trauma at frequency 6-12 kHz, at an intensity of 100 dB for 45 minutes. In paper IV, animals were exposed to acoustic trauma at frequency 8-16 kHz, 100 dB, for 2 hours.

**Sound Conditioning**

Sound conditioning is a low level of acoustic stimuli, where un anaesthetized animals was individually placed in to small wire mesh cages without any food and water in a soundproof box. Sound stimulus (8-16 kHz, 89 dB, 15 minutes) was then applied with a noise generator (*paper IV*).

**Restraint Stress**

Animals were placed into 50 ml conical tubes with ventilation holes made throughout the tube. The mice were placed into the tube and were not able to turn in any direction. The mice were maintained in these tubes for 4 hours in a soundproof booth without any access to food or water (*paper II and III*).

**Adrenalectomy**

Bilateral adrenalectomy was done under ketamine (20 mg/kg) and xylazine (50 mg/kg) anesthesia with dorsal approach. Drinking water was replaced with normal saline. Control animals were sham operated (*paper IV*).

**Pharmacological manipulation**

Glucocorticoid synthesis inhibitor was dissolved in distilled water and given intraperitoneally at a dose of 200 mg/kg of body weight. GR antagonist was dissolved in vegetable oil and was given subcutaneously at a dose of 100 mg/kg of body weight (*paper I, II, III, IV*). Dexamethasone dissolved in distilled water and given I/P at a dose of 0.5 mg/kg (*paper I*). NFκB inhibitor pyrrolidine dithiocarbamate ammonium salt (PDTC) was dissolved in saline and given at a dose of 100 mg/kg body weight (*paper I*).

### 3.5 ELISA

The animals were sacrificed by cervical dislocation and trunk blood was collected in heparinized tubes. Blood was taken at the same time of day to minimize the circadian fluctuation (between the hours of 10:00 and 14:00). Plasma was separated by spinning at 6,000 rpm at room temperature and immediately stored at –20°C until assay. The plasma ACTH (*paper IV*) and Corticosterone (*paper I, II, III, IV*) level was determined by commercially available ELISA kit (MD biosciences, St. Paul, USA; Assay design, MI, USA)
3.6 RT-PCR

RT-PCR was performed according to standard practice. RNA was isolated from whole cochlea using TRIZOL LS reagent (Life technologies, Invitrogen, MD). The yielded RNA was DNase treated (RNase-Free DNase Set and cleaned with RNeasy Mini Kit (QIAGEN, Valencia, CA) and was stored in -80°C until further assay. RT-PCR was done using specific primers for GR cDNA (GI 51057): forward 5’ aagcttcggtatgccattatgg 3’, reverse 5’atataacacctcaggctcgat 3’. GAPDH was used as an internal control (GI 193423); forward 5’agctgaacgggaagctcact 3’, reverse 5’ cctgttgctgtagccgtatt 3’ (paper I).

3.7 IN-SITU HYBRIDIZATION

In situ hybridization was performed to examine the GR mRNA expression changes in spiral ganglion locally. To avoid any RNA degradation cochlea was rapidly removed and was frozen without being decalcified. Temporal bones were isolated and immediately dissected in ice-cold phosphate-buffered saline (PBS). The bony wall surrounding the cochlea was removed and the remaining soft tissue was attached to a piece of cork with Cryomount and frozen in liquid isopentone (- 40°C). Cochlea was then sectioned at (14 µm) at – 30°C, mounted on slides and stored at – 80°C until fixation. In-situ hybridization was then done by using specific GR riboprobes (Promega, Madison, WI), both sense and antisense, which were generated from 673 bp EcoRI- PstI rat GR cDNA fragment (position: 1691 – 2364 bp, corresponding to the 3’portion of the coding region), sub cloned into the vector pSP64 (paper I and III).

3.8 IMMUNOHISTOCHEMISTRY

Immunohistochemistry were done on cochlea and PVN sections using specific antibody. Following primary antibodies were used:

Glucocorticoid Receptor- Polyclonal rabbit anti glucocortcoid receptor antibody (catalogue no PA1-511A, Affinity Bioreagents, Golden, CO, USA) at a concentration of 5 µg/ml (paper I, II, III, IV)
NFkB- NF-kappa B (Catalogue no-SC 7151, Santa Cruz Biotechnologies, Inc., CA, USA) at a concentration of 1 µg/ml (paper I, II, III).
SRC-1- SRC-1 (Catalogue no-SC 7151, Santa Cruz Biotechnologies, Inc., CA, USA) at a concentration of 2 µg/ml (paper IV).
NeuroN antibodies- (cat. MAB377, Chemicon International, CA, USA) at a dilution of 1:500 (paper IV).

The Vectastain kit and DAB nickel (Vector lab, Burlingame, CA) was used to obtain visible reaction product. Cochlear sections were then analyzed in a Zeiss Axiovert microscope at 40x magnification. The PVN sections were analyzed with stereology.

3.9 WESTERN BLOT ANALYSIS

Mice were sacrificed by transcardiac perfusion with ice-cold PBS with heparin, cochleae were immediately removed. Protein was isolated and preserved in to -80.0°C until further assay. Proteins from individual sample (8-10 µg) were separated by SDS-PAGE. Proteins were then transferred to a polyvinylidene difluoride (PVDF) membrane (Amersham Pharmacia Biotech, Little Chalfont, UK) blocked for 1 h and incubated with primary antibodies followed by incubation with appropriate secondary antibodies. The membrane was then developed with an enhanced chemiluminescence
Western blot detection kit (Pierce Super Signal® West Dura, Rockford, IL, US). The bands of protein were quantified by densitometry.

Antibodies used for western blot:

*Anti-NF-κB p65*, 1/1000 (catalogue no 3034, Cell Signalling Inc., Beverly, MA, US),
*Anti-IκBα* 1/1000 (catalogue no 9242, Cell Signalling Inc., Beverly, MA, US) and anti-*GAPDH* 1/10000 (catalogue no ab9484, Abcam Ltd., Cambridge, UK).

#### 3.10 STEREOLOGICAL ANALYSIS

The stereological analysis was performed using an Olympus video stereological system. The software program CAST GRID 1.2 (Olympus, Denmark) was used to generate the counting frame to delineate the area of interest. The systematically random sampled sections were first viewed on the screen with low magnification (5× objective). The entire PVN was delineated on the screen. Subsequently, an automated systematic sampling procedure within the area of interest was performed using a 100× oil immersion objective with a high numerical aperture (1.3). The stereological probe was superimposed on the microscopic image, and immunoreactive neurons were sampled if their nucleoli were inside the sampling frame and did not touch either one of the two (left and bottom) forbidden lines. The neuron was classified as immunopositive when a black deposit from the DAB staining was darker than background staining. The optical fractionator (Gundersen et al., 1988) was used to obtain unbiased estimates of total numbers of neurons in PVN. The coefficient of error was calculated according to West et al., (West et al., 1991) and was accepted when the CE was <10%.

#### 3.11 STATISTICAL ANALYSIS

Data were presented as a mean values ± SEM. Statistical analysis used in this thesis includes Student’s t test and one-way ANOVA followed by a post hoc Tukey test using sigma stat program (Jandel, version 2.03). Difference was considered statistically significant when p value was <0.05.
4 RESULTS

4.1 INHIBITION OF GC SYNTHESIS AND GR EXACERBATES HEARING LOSS AFTER ACOUSTIC TRAUMA (PAPER I)

In paper I, we have shown how the sensitivity of hearing is differentially regulated in a normally activated HPA axis or with an altered HPA axis response. When an acoustic trauma of a moderate level (6-12 kHz, 100 dB, 45 min) was applied to the male CBA mice, they showed hearing threshold shifts of 10-30 dB immediately after trauma. On the other hand, treatment of GC synthesis inhibitor (metyrapone) and GR antagonist (RU486) prior to acoustic trauma exaggerated the threshold shift to 25-60 dB across the frequencies. Twenty-four hours after acoustic trauma, the vehicle treated group showed nearly complete recovery of hearing while the metyrapone and RU486 treated group continued to show elevated hearing thresholds. Pretreatment with dexamethasone, a GC agonist, significantly lowered hearing threshold both immediately after and 24 hours after acoustic trauma. Plasma corticosterone demonstrated a normal HPA axis response in the vehicle treated group as revealed by significant elevation of plasma corticosterone (152.6 ± 40 ng/ml) immediately after acoustic trauma compared to metyrapone + RU486 treated group (29 ± 10 ng/ml). By two hours post trauma, plasma corticosterone went back to baseline in vehicle treated group while the metyrapone and RU486 treated group showed a tendency of elevation. RT-PCR analysis of GR mRNA expression in the cochlea demonstrated a significant elevation in metyrapone and RU486 treated group compared to vehicle treated group when evaluated two hours after acoustic trauma. In situ hybridization confirmed the RT-PCR results as shown by an elevation of GR mRNA in the spiral ganglion neurons of metyrapone and RU486 treated group compared to vehicle treated group. GR protein expression was significantly down regulated in the spiral ganglion neurons of vehicle treated group compared to metyrapone and RU486 treated group when evaluated 24 hours post trauma. Vehicle treated group showed 12 % of SGNs with nuclear translocated GR while metyrapone and RU486 group showed 26.6 % of neurons with GR nuclear translocation. Nuclear factor kappa B (NFκB), a GR regulated transcription factor, was also evaluated to determine the underlying mechanism of GC action in the inner ear. NFκB nuclear transport was significantly decreased in metyrapone and RU486 treated animal compared to vehicle treated group when evaluated four hours after acoustic trauma. This decrease of NFκB nuclear translocation correlates with an elevated hearing thresholds in that group. The role of NFκB activation in hearing protection was further confirmed by using a selective NFκB inhibitor pyrrolidine dithiocarbamate ammonium salt (PDTC). The group treated with PDTC also showed an elevation of hearing thresholds as high as metyrapone and RU486 treated group, which is significant in all frequencies, compared to vehicle treated group. Nuclear translocation of NFκB in the SGNs was significantly reduced in the PDTC group compared to vehicle treated group.
4.2 RESTRAINT STRESS ELEVATES PLASMA CORTICOSTERONE AND ACTIVATES GLUCOCORTICOID RECEPTORS AND NFκB IN THE COCHLEA (PAPER II)

In paper II, we have shown that restraint stress elevates plasma corticosterone that results in activation of GR in the spiral ganglion neurons of cochlea. In vehicle treated group corticosterone level was 27.8 ± 4.9 ng/ml when measured 5.5 hours after injection. Restraint stress significantly elevates plasma corticosterone (175 ± 20.8 ng/ml) compared to the vehicle group. Immediately after restraint stress, no changes were observed in GR protein expression in the modiolus. However, GR nuclear translocation in the SGNs of cochlea was significantly differed among groups when evaluated immediately after restraint stress. GR immunoreactivity revealed that 34% of SGNs showed nuclear translocated GR in the vehicle group, which was significantly increased (55%) after restraint stress. Pretreatment with RU486 + Metyrapone significantly decreased GR nuclear translocation in the SGN (22%). Western blot analysis demonstrated a significant decrease of GR protein expression 24 hours after restraint stress in the vehicle group compared to the RU486+Metyrapone group. The expression of NFκB, a GR regulated down stream transcription factor, was also evaluated in the SGNs of cochlea. The vehicle group showed 56% of SGNs with nuclear translocated NFκB, which was not altered by restraint stress (52%). However, treatment of RU486 + MET before restraint stress significantly decreased nuclear translocation of NFκB in the SGNs (22%). The protein expression of NFκB was significantly decreased after restraint stress. However, the expression of NFκB was decreased more in presence RU486 + Metyrapone with or without restraint stress. IκBα protein was significantly decreased in presence of RU486 + Metyrapone compared to vehicle treatment. Restraint stress led a slight decrease in IκBα expression but the magnitude of decrease was significantly lower in presence of RU486 + Metyrapone.

4.3 RESTRAINT STRESS PROTECTS HEARING AGAINST ACOUSTIC TRAUMA BY AN INTERACTION OF GLUCOCORTICOID RECEPTORS AND NFκB IN THE COCHLEA (PAPER III)

Restraint stress (RS), when applied prior to acoustic trauma significantly reduced auditory threshold shifts. When measured 24 hours post acoustic trauma, it was found that restraint stress significantly protects hearing against acoustic trauma. However, this protective effect was abolished by treatment of RU486 + Metyrapone prior to restraint stress. Acoustic trauma demonstrated threshold shifts of 21-30 dB, while RS prior to trauma significantly reduced the threshold shifts to 8-19 dB across the frequencies. Treatment with RU486 + Metyrapone before restraint stress abolished the protective effect of RS as revealed by threshold shifts which was between 13-33 dB across the frequencies. Immediately after acoustic trauma, plasma corticosterone (CORT) was significantly elevated in the trauma group (96.1 ± 21.4 ng/ml) compared to the vehicle treated control (28 ± 5 ng/ml). When RS applied before acoustic trauma the plasma CORT rises to 242 ± 37 ng/ml, which was significantly higher than trauma only group. RU486+Metyrapone showed also an elevation of CORT concentration (135 ± 20 ng/ml). RU486 + Metyrapone followed by RS and trauma also showed higher CORT (275 ± 35 ng/ml), but the magnitude of elevation was twice higher in RS group while compared to time matched control. When the CORT
induced GR nuclear translocation was evaluated in SGNs, it was found that restraint-pretreated group demonstrates highest number of GR nuclear translocation. Trauma group showed 29% of neurons with nuclear translocated GR. A significant elevation of GR nuclear translocation was observed in RS + Trauma group (45%). RU486 + Metyrapone treatment followed by RS and trauma showed only 20% of SGNs with GR positive nuclei, which was a significant reduction, compared to RS + Trauma group. To evaluate how GR transcription is modulated by RS, the GR mRNA expression was evaluated in SGNs by ISH. RS itself caused a slight decrease of GR mRNA compared to control. RU486 + Metyrapone treatment followed by RS and trauma showed a significant elevation of GR mRNA when compared to RS + Trauma group. The GR protein expression in the modiolus demonstrated similar pattern of changes when measured 24 hours post acoustic trauma. The RU486 + Metyrapone group showed a significant elevation of GR protein expression compared to RS + Trauma group. No changes in protein expression were observed at immediate post trauma period. To define the interaction between GR and NFκB in the SGNs, the nuclear translocation of NFκB was evaluated immediately after acoustic trauma. The control group showed 56% of neurons with NFκB nuclear translocation. Acoustic trauma caused a significant decrease in the NFκB nuclear translocation (31%). RS prior to acoustic trauma prevents this down regulation of NFκB nuclear translocation (50%). However, RU486 + Metyrapone group abolished this effect. Western blot analysis revealed a significant elevation of NFκB protein expression in RU486 + Metyrapone + RS + trauma group compared to all other group. Trauma or RS + Trauma group did not show any difference in protein expression of NFκB. However, the IκB α protein expression was significantly increased in trauma group, which corresponds with lower nuclear translocation of NFκB in this group. RS + Trauma group did not show any changes in IκB α expression but RU486 + Metyrapone + RS + Trauma group showed significant lower expression of IκB α compared to RS + Trauma group.

4.4 SOUND CONDITIONING PROTECTS HEARING AGAINST ACOUSTIC TRAUMA BY ACTIVATING THE HPA AXIS (PAPER IV)

In paper IV, we have demonstrated that 15 minutes of sound conditioning (8-16 kHz, 89 dB) activates the HPA axis as revealed by elevated plasma ACTH and corticosterone and glucocorticoid receptor expression in paraventricular nucleus and cochlea, which ultimately protect hearing against subsequent acoustic trauma. The trauma group demonstrated threshold shifts of 40-50 dB across the frequencies when measured immediately after. While the sound conditioning group showed 15-20 dB, lower threshold shifts an effect that was blocked by both surgical and pharmacological adrenalectomy (GC synthesis inhibitor and GR antagonist treatment) prior to sound conditioning. Sound conditioning activates the HPA axis as revealed by elevation of plasma CORT and ACTH. Plasma CORT in control animals were 40 ± 8 ng/ml. Acoustic trauma cause an elevation of CORT (77 ± 8 ng/ml) compared to control while sound conditioning alone caused significant elevation of CORT concentration (98 ± 20 ng/ml). Pretreatment with sound conditioning before acoustic trauma caused a significant two-fold elevation of CORT (144 ± 17 ng/ml) compared to trauma only group. However, RU486 + Metyrapone before sound conditioning prevent this sound conditioning induced CORT elevation (60 ± 13 ng/ml). Adrenalectomy animals showed the CORT concentration of only (8 ± 3 ng/ml) which implies the effectiveness of adrenalectomy. Sham operated animals showed plasma
corticosterone concentration of 135 ± 40 ng/ml. Plasma ACTH was significantly elevated by sound conditioning (345 ± 26 pg/ml) compared to control animal (16 ± 6 pg/ml). Sound conditioning combining with acoustic trauma also resulted in significant elevation of ACTH (347 ± 12 pg/ml) compared to trauma only group (83 ± 19 pg/ml). RU486 + Metyrapone treatment prior to sound conditioning prevent this elevation (167 ± 37 pg/ml) of ACTH by sound conditioning. Adrenalectomy group showed highest elevation of ACTH (445 ± 54 pg/ml) which confirmed the effectiveness of surgery. Activation of the HPA axis was further evaluated by analyzing GR expression in the PVN. In control group, 17 % of neurons were GR immunopositive. Sound conditioning alone group showed 21% of GR immunopositive neurons. However, acoustic trauma significantly decreased the number of GR immunopositive neurons (10%) in PVN. Sound conditioning prior to acoustic trauma prevent this trauma induced down regulation (20%) of GR expression. Whereas, the group treated with RU486 + Metyrapone prior to sound conditioning showed only 8% of GR immunopositive neurons in the PVN. To determine the sound conditioning induced activation of GR in the cochlea, we evaluated nuclear translocation of GR in SGNs of cochlea. In control animals 27% of neurons showed GR nuclear translocation. Sound conditioning alone (26%) or acoustic trauma (22%) did not show any changes compared to control. Sound conditioning prior to acoustic trauma significantly increased in GR nuclear translocation compared to all other groups (43%) which was blocked by pretreatment of RU486 + Metyrapone before sound conditioning. Adrenalectomy group also demonstrated lower percent of GR nuclear translocated neurons (18%) which were significant compared to sham operated group (39%).Western blot analysis revealed that GR protein expression in whole cochlea was significantly decreased after acoustic trauma compared to control. Sound conditioning alone also caused a decrease in GR protein expression but the magnitude of decrease was much higher in acoustic trauma group. However, when sound conditioning was applied prior to acoustic trauma, GR protein expression was significantly elevated than acoustic trauma group. RU486 + Metyrapone treatment prior to sound conditioning significantly down regulates GR protein expression compared to sound conditioning + trauma group. The expression of GR regulated co-activator SRC-1 was also evaluated in the SGNs of cochlea. Immunohistochemical analysis revealed, no effect of sound conditioning only or acoustic trauma on the expression of SRC-1 compared to control. However, sound conditioning + trauma group showed highest expression of SRC-1 in the SGN. Treatment with RU486 and metyrapone prior to sound conditioning significantly reduced the expression of SRC-1.
5 DISCUSSION

The optimum activation of the HPA axis is an important factor for the maintenance of essential physiological homeostasis in both basal and stressful condition. The individual response to a stressful stimulus varies between individuals (McEwen, 2002). Variability in the HPA axis response is the main determinant of this interindividual variation. The variation among individuals in the HPA axis activity is thought to be a combination of different factors including individual’s feedback sensitivity, the secretion and circadian rhythm of glucocorticoid and the peripheral sensitivity of glucocorticoid (Meikle et al., 1988). Thus, glucocorticoid the final product of the HPA axis, determines the ultimate effect of HPA axis activation on different physiological system. Like all other systems in the body, the auditory system is also regulated by the neuroendocrine responses of the HPA axis. We have demonstrated that, the auditory system responds differentially depending upon the magnitude of HPA axis activation. Since the degree of activation of the HPA axis depends on the nature of exposed stressor, the duration and intensity of the stressor is crucial in this regard. Different stressors that have been shown to affect the auditory system include acoustic trauma (Terunuma et al., 2003; Tahera et al., 2006), restraint stress (Wang and Liberman, 2002; Tahera et al., 2006), heat stress (Yoshida et al., 1999) and sound conditioning (Yoshida and Liberman, 2000). It is well documented that the effect of stress on target organ is bi-directional. The acute stress induced short-term activation of the HPA axis leads to stimulate the classical stress response pathway and demonstrates protection of several physiological systems against subsequent adverse stimuli. On the other hand chronic stress demonstrates a detrimental effect on several physiological system and contributes to different pathological condition (McEwen, 2002).

Acoustic trauma of moderate intensity activates the HPA axis as revealed by elevation of plasma corticosterone, which helps to recovery of cochlea and minimize hearing loss. When HPA axis response was altered with pharmacological manipulation (metyrapone and RU486), the hearing loss was exacerbated (Paper I). In accordance with this finding when a prior activation of the HPA axis was implied before acoustic trauma either by restraint stress or by sound conditioning, the hearing was protected against subsequent acoustic trauma. This protective effect was abolish by adrenalectomy or combined use of metyrapone and RU486 before restraint stress or sound conditioning (Paper III and IV). The common factors which playing the key role in this protective mechanism are corticosterone and their receptor glucocorticoid receptors.

The cascade of this protective mechanism initiates by the elevation of endogenous plasma corticosterone (CORT). Sound conditioned and restraint induced animal showed higher corticosterone after acoustic trauma compared to only trauma group. We found that this elevated CORT contributes to protect hearing by activating GRs in the cochlea.

We demonstrated that, in the normal functioning HPA axis, the initial elevation of CORT returns to base line within two hours. On the other hand, metyrapone and RU486 treatment showed an altered response as revealed by initial inhibition of CORT rise and a subsequent elevation (paper I, II, III). The dynamics of plasma CORT in these groups follow the pharmacokinetics of used drugs. The effect of metyrapone persists only up to 4 hours in contrast of long lasting effect of RU486, which is about 90 hours (Foldesi et al., 1996). However, we cannot exclude the rebound effect of the HPA axis response. Adrenalectomy completely inhibits sound
conditioning induced elevation of plasma CORT as revealed by very low CORT in this group. The effectiveness of adrenalectomy was further evident by highest rise of plasma ACTH in this group (paper IV).

Changes in the corticosterone level by the modulation of the HPA axis also reflects in the central markers of HPA axis activity. We demonstrated that the GR expression in the PVN was differentially regulated depending upon the intensity of stressor. When an acoustic trauma of relatively higher intensity was applied for longer duration (2 hours), the expression of GR in the PVN was down regulated. However, a low level of acoustic stimuli (sound conditioning) for shorter period (15 minutes) did not show this effect. Moreover, priming of the HPA axis with sound conditioning prior to acoustic trauma prevents this trauma induced down regulation of GR, an effect that was block by either adrenalectomy or combined use of metyrapone and RU486 prior to sound conditioning (paper IV).

Corticosterone is the main regulator of the GR expression, not only in the central HPA axis as PVN but also in all other peripheral GR expressing tissues. GR expressed in both neuronal and non-neuronal tissues of inner ear (ten Cate et al., 1992; Rarey and Curtis, 1996; Shimazaki et al., 2002; Tahera et al., 2006). In addition, these receptors are activated by both endogenous and exogenous corticosterone. Upon hormone binding activated GR translocated into nucleus where it exerts its transcriptional activity (Beato, 1989). When restraint stress or sound conditioning was applied, a significant increase in the GR nuclear translocation was observed in the SGN of cochlea immediately after acoustic trauma. The elevation of nuclear translocation of GR in the SGNs was block by adrenalectomy or combine use of metyrapone and RU486 pretreatment (paper III, IV). On the other hand, 24 hours after acoustic trauma GR nuclear translocation was down regulated in the SGNs compared to metyrapone and RU486 group (paper I). The lower expression of GR in the acoustic trauma group probably reveals GRs auto regulation to protect itself by over exposure of ligand (Dong et al., 1988). Moreover, increased nuclear translocation of GR in presence of RU486 can still occur as it has been shown previously (Pariante et al., 2001). Above all, these findings imply the complex dynamics of GR regulation over time in the SGNs. As demonstrated by western blot analysis, GR protein expression was not altered immediately after restraint stress (paper II, III) but was significantly lowered after 24 hours as it has been demonstrated previously (Okret et al., 1991). The increased nuclear translocation and lower protein expression after 24 hours implies the relatively higher activity of GR in this group (paper II, III). Contrary to this result, GR protein expression was significantly down regulated immediately after acoustic trauma, and was prevented by prior sound conditioning (paper IV). Since the time lag of GR protein expression is about 18-24 hours after ligand treatment (Gustafsson et al., 1987; Okret et al., 1991) the relative elevation of GR protein expression after sound conditioning followed by acoustic trauma is probably due to the inhibition of GR degradation not new synthesis (Paper IV). GR mRNA expression was also down regulated immediately after restraint stress and acoustic trauma, but significantly elevated after metyrapone and RU486 treatment which implies the inactivation of GR (paper I, III). Summarizing the above findings, it should be concluded that an over riding mechanism of this restraint stress and sound conditioning mediated protection of cochlea depends on the maintenance and availability of GR protein expression and its activation. The transactivation of several genes by active GR determines the ultimate effect of CORT on the target tissue. When active GR stimulates different intra and extra cellular protective system, the target organ shows protection against adverse stimuli. Several protective systems are stimulated by active GR as heat shock proteins, antioxidants, and neutrophins all of which protect cochlea against acoustic trauma (Agerman et al., 1999; Minami et
al., 2004). In spite of transactivation of several protective genes, GR can interact with other transcription factors by direct protein interaction (Umland et al., 2002). NFκB, a GR regulated transcription factor has been shown to protect cochlea against amino glycoside ototoxicity (Jiang et al., 2005). In our study, we have also demonstrated a protective function of NFκB in the cochlea against acoustic trauma (paper I, III).

The event of NFκB mediated gene regulation starts with its nuclear translocation. We demonstrated that immediately after acoustic trauma the nuclear translocation of NFκB in the SGNs were decreased; however, restraint stress prevents this trauma induced down regulation of NFκB nuclear transport. In presence of metyrapone and RU486 restraint stress did not prevents this down regulation (paper III). On the other hand, in paper I, we have demonstrated an elevation of NFκB nuclear transport in the SGN, 4 hours after acoustic trauma. The experimental paradigms in these two studies though differ from treatment and time point of tissue collection but it also indicates that nuclear translocation of NFκB in the SGNs is sensitive to time point when it is analyzed. Moreover, these results imply that NFκB nuclear translocation is a crucial factor to protect cochlea against acoustic trauma as hearing loss was less in those groups where NFκB nuclear translocation was higher. The further support of NFκB mediated protection of cochlea was established by using selective NFκB inhibitor PDTC. Pretreatment of PDTC prevents nuclear transport of NFκB in the SGNs and elevates auditory thresholds. One possible mechanism of PDTC mediated inhibition of NFκB nuclear translocation could be the suppression of release of IKK and thereby degradation of IκB (Yamamoto and Gaynor, 2004). IκB the main regulator of NFκB activity (Yamamoto and Gaynor, 2004) was involved in regulation of NFκB activity in the SGNs after acoustic trauma with or without restraint stress. Western blot analysis demonstrated an upregualtion of IκB expression after acoustic trauma, which coincided with lower nuclear translocation of NFκB in this group (paper III). This result indicates that IκB dependent NFκB activation occur in the SGNs after acoustic trauma. However, considering the unaltered nuclear translocation of GR in this group (acoustic trauma), it should be concluded that NFκB activity is not under direct control of GR at least at this time point. In presence of restraint stress, the trauma group did not show any changes in IκB expression or NFκB nuclear translocation when GR was activated. Thus restraint induced GR activation prevents NFκB down regulation immediately after acoustic trauma, which implies a positive correlation between GR, and NFκB nuclear translocation (paper III) which has been demonstrated previously (Nakamori et al., 2005). Since restraint stress prior to acoustic trauma group only showed this interaction of GR and NFκB, we suggest that this interaction can be the basis of restraint-mediated protection of hearing against acoustic trauma (paper III). In addition, this interaction of GR and NFκB in the SGNs was altered by metyrapone and RU486 treatment. A previous study showed the impairment of this interaction by RU486 (Garside et al., 2004). A simultaneous down regulation of both IκB expression and NFκB nuclear translocation in presence of metyrapone and RU486 suggest that the classical IκB regulated NFκB activation was disrupted in this group (paper III). However, we cannot exclude the direct inhibitory effect of RU486 on NFκB as it has been shown previously (Han and Sidell, 2003). On the other hand, the decreased IκB expression in presence of GR antagonist in SGNs could be the consequence of positive regulation of IκB synthesis by GR, as it has been already shown in some tissues (Scheinman et al., 1995). The findings from paper III, suggests that GR regulates the activity of NFκB in the SGNs in a complex and time dependent manner.
The underlying mechanism how GR and NFκB interaction protects cochlea against acoustic trauma was not explored in this study. However, we speculate that several factors could be involved in this mechanism. The key finding that co-relate with protection of cochlea against acoustic trauma is nuclear translocation of NFκB in the SGNs of cochlea. The role of NFκB varied depending upon the cell type being investigated (Marchetti et al., 2004). Even in the same type of cell NFκB exhibits dual function, it can stimulates both pro and anti inflammatory cytokines, pro and anti-apoptotic genes, can enhance both tumor promoting and tumor suppressing effect simultaneously (Perkins and Gilmore, 2006). In general, in immune and inflammatory system, NFκB regulates immune response, stimulate proinflammatory cytokines and enhance inflammation (Li and Verma, 2002). On the other hand, in central nervous system its activation protects against oxidative stress (Kratsovik et al., 2005), long term depression (Albensi and Mattson, 2000), and NMDA excitotoxicity (Yu et al., 1999; Lipsky et al., 2001). In auditory systems, most studies demonstrated the protective effect of NFκB activation (Jiang et al., 2005; Nagy et al., 2005; Lang et al., 2006). The activation of NFκB could upregulates some neurotrophic factors, which contributes this protective mechanism. Up regulation of neurotrophic factors as NT-3 and BDNF has been shown to protect hearing (Agerman et al., 1999; Duan et al., 2000). Moreover, NFκB activation can reduce the acoustic trauma induced excitotoxicity in neurons (Lang et al., 2006). Nevertheless, the activation of NFκB probably upregulates some anti-inflammatory cytokines that minimize the acoustic trauma induced inflammation of cochlea and thereby reduce the hearing loss. NFκB activation has been found in the resolution phase of inflammation, which coincides, with upregulation of anti-inflammatory cytokines. Inhibition of NFκB increased the inflammatory cells after 72 hours of inflammation suggests that NFκB activation has an anti-inflammatory role (Lawrence et al., 2001). An inhibitory role of NFκB was also observed with LPS induced shock in mice (Gadjeva et al., 2004). However, further studies are needed to make a conclusion of this protective mechanism.

Another probable candidate in the GR mediated protective effect in cochlea could be the involvement of its co activator steroid receptor co activator-1 (SRC-1) (Paper IV). The SRC-1 expression was not altered by either acoustic trauma or sound conditioning but was elevated in sound conditioning + acoustic trauma group. We speculate that this elevated SRC-1 expression provides relatively high GR activity in the cochlea that helps to maintain the protective effect of sound conditioning. However, further studies regarding the interaction of SRC-1 with other co-regulatory proteins and NFκB is needed to elucidate the exact mechanism of this pathway.
6 SUMMARY

Prior activation of the HPA axis protects the hearing against acoustic trauma. This finding defines new information regarding the interaction between the HPA axis and the auditory system. Several basic mechanisms have been characterized to determine how the HPA axis modulates the sensitivity of hearing. The degree of HPA axis activation depends on the intensity and duration of exposed stressors. Depending on the magnitude of HPA axis activation, the degree of hearing loss can be increased or decreased. When the HPA axis is activated by either acute restraint stress or short-term sound conditioning the hearing was protected against subsequent acoustic trauma. Activation of HPA axis results in an elevation of endogenous corticosterone, which in turn activates the GRs locally in the cochlea. Glucocorticoid receptors interact with its down stream transcription factor NFκB and provide protection against acoustic trauma. The effect of HPA axis activation on auditory function was further established when surgical or pharmacological adrenalectomy prior to sound conditioning or restraint stress abolished this protective effect. Our findings established that this endogenous protective mechanism of cochlea is triggered by generalized stress response. Glucocorticoid receptor and NFκB are the possible mediators of this corticosterone mediated protective mechanism of cochlea. This finding will help us to develop new pharmacological strategies to protect hearing against acoustic trauma.
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