Distribution of glucocorticoid receptors and 11beta-hydroxysteroid dehydrogenase isoforms in the rat inner ear

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Abstract

11β-hydroxysteroid dehydrogenase (11β-HSD) is an enzyme complex responsible for the conversion of hormonally active cortisol to inactive cortisone, and two isoforms of the enzyme (11β-HSD1 and 11β-HSD2) have been cloned and characterized. An immunohistochemical study was performed to determine the precise distribution of glucocorticoid receptors (GRs) and the isoforms of 11β-HSD in the rat (postnatal day 1, 4, 10, and adult). Immunoreactivity of GRs was detected in the stria vascularis (SV), the outer hair cells (OHCs), the inner hair cells (IHCs), the spiral ligament (SLig), the spiral limbus (SLib), the spiral ganglion cells (SGCs), Reissner's membrane (RM), the cochlear nerve (CN), the vestibular hair cells (VHCs), the dark cells (DCs), and the vestibular nerve (VN) in the rats. Immunostaining of 11β-HSD1 was observed in almost all the tissues in the cochlea and the vestibule except SLig, SLib, SGCs, CN, VHCs, and VN during all developmental stages, whereas, immunoreactivity of 11β-HSD2 was not detected in any of the inner ear tissues. A polymerase chain reaction (PCR) study was also performed on GRs, 11β-HSD1, and 11β-HSD2 in the OC, SV and vestibule of the postnatal rats, and revealed that mRNAs were detected in all those and tissues in all the developmental days of postnatal days 1, 4, and 10. This data indicates that expression of GRs and 11β-HSD isoforms in the inner ear is tissue and age-specific,
and that different local steroid regulation by GRs and the isoforms of 11β-HSD is present in each part of the inner ear.

**Keywords**: glucocorticoid receptor, 11 beta-hydroxysteroid dehydrogenase, isoform, the cochlea, the vestibular organ, rat
1. Introduction

As glucocorticoids are widely used to treat various inner ear diseases, it is important to know their modes and/or sites of action in the inner ear. Glucocorticoids are thought to regulate sensory transduction and homeostasis in the inner ear. It has previously been demonstrated that glucocorticoid receptors (GRs) are present in the inner ear. Nevertheless, there are still inconclusive issues to be addressed. For instance, a study of GRs mRNA in adult rats showed it to be present in the spiral ligament (SLig) but not in the organ of Corti (OC) (Ten et al., 1993). Regarding immunostaining of GRs in the OC of BALB/c mice, a study clearly indicated the presence of GRs (Hargunani et al., 2006) while others did not (Shimazaki et al., 2002). As glucocorticoids are suggested to regulate potassium ion recycling (Embark et al., 2003; Lee and Marcus, 2002), GRs are assumed to play an essential role in the stria vascularis (SV). A previous study regarding mRNA has not provided any further evidence of GRs in adult rats (Ten et al., 1993), and only a slight to weak immunostaining of GRs was observed in both postnatal developmental days and in adults (Zuo et al., 1995).

GRs encoded by nuclear receptor subfamily 3, group C, member 1 (NR3C1) belong to the nuclear hormone receptor superfamily, and are expressed in the nucleus or
the cytoplasm of most cells (Niu et al., 2009). However, in the inner ear, precise subcellular localization and polarity of GRs have not been shown yet even in animal inner ears. Furthermore, little information on factors determining biological actions of glucocorticoids in animal inner ear is available.

The concentration of glucocorticoids in the target cells is known to be regulated by 11β-hydroxysteroid dehydrogenase (11β-HSD), an enzyme complex responsible for the conversion of hormonally active cortisol to inactive cortisone (Oppermann et al., 1997). Recently two isoforms of the enzyme (11β-HSD1 and 11β-HSD2) have been cloned and are known to be linked to human diseases such as insulin resistance, obesity, glaucoma, and hypertension (Oppermann et al., 1997; Seckl, 2004; Draper and Stewart, 2005). 11β-HSD1 is found in a wide range of tissues such as the liver, adipose tissues, the choroid plexus of the brain, and the eyes. In contrast, 11β-HSD2 is mainly present in mineralocorticoid-selective target tissues such as the kidney, colon, pancreas, and placenta (Oppermann et al., 1997; Seckl, 2004; Draper and Stewart, 2005). 11β-HSD is known to be partly present in the inner ear (Ten et al., 1994), whereas the precise distribution and developmental changes of the 11β-HSD isoforms have as yet not been fully understood, including animals. In the present study, the distribution of GRs and the identification of isoforms of 11β-HSD using immunostaining and polymerase chain
reaction (PCR) were investigated in adult and postnatal rat inner ear tissues. Since it is already known that GRs in the cytoplasm are translocated to the nucleus by the stimulation of glucocorticoids (Raddatz et al., 1996; Kawata, 2001) the present study investigates whether translocation of GRs by dexamethasone (DXM) occurs in the SV or not.
2. Materials and Methods

2.1. Experimental Animals

Wister adult rats (8-10 weeks) and postnatal rats (day 1, 4, 10) were used in the present study. Animal care and experimental procedures were performed in accordance with the Guidelines for Animal Experimentation of Nagasaki University with approval of the Institutional Animal Care and Use Committee. All work was performed with approval from the Nagasaki University Institutional Animal Care and Use Committee (approval number 0802130651).

2.2. Immunohistochemistry (paraffin section)

2.2.1. Tissue preparation of rats

Rats were deeply anesthetized by intraperitoneal injection of pentobarbital. In adult rats, perfusion with 30 ml of ice-cold 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS, pH7.4) via cardiac puncture was performed, and decapitation followed. However, cardiac perfusion was omitted in postnatal rats. The dissection was carried out in ice cold Hank’s balanced saline solution (HBSS)(138mM NaCl, 5mM KCl, 0.5mM MgCl₂, 1.3mM CaCl₂, 5mM glucose, pH 7.2) supplemented with 10mM HEPES buffer. The oval and round windows of the labyrinth were opened by
removal of the stapes footplate and the round window membrane, respectively. In addition, the bony cap covering the apical turn of the cochlea was removed. Samples were fixed with 4% PAF/PBS by gentle cochlear perfusion and by immersion of 4% PAF/PBS overnight at 4°C. Decalcification using 10% Ethylenediamine-N,N,N′,N′-tetraacetic acid, disodium salt, dehydrate (2Na(EDTA2Na) in PBS (pH 7.4) was performed for 10 days at 4°C. The samples were then dehydrated through graded series of alcohols, cleared in xylene, and embedded in paraffin.

2.2.2. Immunohistochemical labeling for GR, 11β-HSD1, and 11β-HSD2

Sections, 4μm in thickness, were deparaffinized with toluene and rehydrated by serially graded ethanol solutions. After being washed 3 times in PBS, the sections were autoclaved in 10mM citrate buffer for 10 min at 121°C. Following the inactivation of endogenous peroxidase activity with 0.3% H2O2 in methanol for 15 min at room temperature, the sections were preincubated with 500μg/ml normal goat IgG and 5% bovine serum albumin (BSA) in PBS for 1 hour to block nonspecific reaction with the first antibody. Polyclonal antibodies against GR (no.sc-1004, http://datasheets.scbt.com/sc-1004.pdf), 11β-HSD1 (no.sc-20175, http://datasheets.scbt.com/sc-20175.pdf) and 11β-HSD2 (no.sc-20176,
were prepared by immunization of rabbits against synthetic peptides (Santa Cruz Biotechnology Inc, California, USA). The antibodies showed a single band of the expected size in western blot analysis and the specificity was confirmed in previous other studies (Brewer JA, et al., 2002; Ge RS, et al., 2005; Honda, et al., 2008). The sections were incubated with the first antibody of anti-GR (1:200), anti-11β-HSD1 (1:100), and anti-11β-HSD2 (1:100) with 5% BSA in PBS for overnight, washed 4 times with 0.075% Brij35 (B4184, Sigma-Aldrich, St. Louis, USA) in PBS. Afterwards the sections were treated with Cy™3-conjugated AffiniPure Goat anti-Rabbit IgG (H+L) (Jackson immuno research, USA, dilution 1:200) for 1 hour. After the sections were washed with 0.075% Brij35 in PBS, covered with Vectashield mounting medium (Vector Laboratories, Inc.; California, USA) and observed in a confocal laser microscope (LSM 510 META, Zeiss, Germany). Images were digitally captured and then analyzed using imaging software (Zen, Zeiss, Germany) provided and downloaded from Zeiss. Control samples consisted of rat brain and kidney tissues known to contain GR, 11β-HSD1, or 11β-HSD2. Technique controls were performed for each specimen using the same method with normal rabbit IgG but omitting the use of the primary antibody. As positive controls for 11β-HSD2, the kidneys were stained using the same method with the primary antibody.
A double staining of GRs with 11β-HSD1 in the rat crista ampullaris was also performed to observe more precise distribution of GRs and 11β-HSD1. For the double staining, Zenon Rabbit IgG Labeling kits Z25360 (Molecular Probes, USA) was used.

2.3. reverse transcription – polymerase chain reaction (RT-PCR)

2.3.1. Tissue preparation for RT-PCR

RT-PCR was further performed to confirm the presence of GRs and 11β-HSD isoforms in postnatal rats. mRNA was isolated from 24 ears (4 rats at each postnatal stage of day 1, 4, and 10, respectively). Rats were deeply anesthetized by intraperitoneal injection of pentobarbital (0.4mg/g body mass, Tokyo Chemical Industry, Tokyo, Japan) and diethyl ether, exsanguinated via the left ventricle with 70% ethanol/RNase-free water, and then decapitated. The brain was removed from the skull, and the complete inner ear bony labyrinth capsules were dissected from the skull base. The dissection was carried out in ice cold 10mM HEPES-buffered saline with HBSS. The inner ear tissue of the SV, the vestibule, and OC were dissected under a stereomicroscope.
2.3.2. Condition of RT-PCR

RNA was extracted using a RNease Mini Kit following the manufacture’s protocol (No.74104, Qiagen, Inc.; California, USA), and total RNA quality was determined with a Nanodrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, Inc.; Massachusetts, USA). The extracted total RNA was further purified using DNase I Amplification Grade (no.18068-015, Invitrogen; California, US) in order to eliminate the DNA. Total RNA obtained was reverse transcribed into complementary DNA (cDNA) by a standardized technique. For reverse transcription, SuperScript™ III Reverse Transcriptase (no.18080-044, Invitrogen) was employed with random hexamer primers. The cDNAs were synthesized using TaKaRa Ex Taq Hot Start Version (no.RR006A, Takara; Shiga, Japan). The PCR reaction consisted of 4 min at 94°C, following by 35 cycles of 94°C (30 sec), 55°C (30 sec), 72°C (30 sec), ending with 10 min extension at 72°C. All PCR products were run on 2% agarose gels and detected by etidium bromide. cDNA fragments were then amplified using specific primers for rat GR, 11β-HSD1, and 11β-HSD2 (GeneBank accession No. NM_012576, NM_017080, NM_017081, respectively). Primer sequences were as follows: rat GR (forward 5’-AAAATGGGTCGGTGCTTCTA-3’, reverse 5’-TTACGCGGCTTGGTGCTATC-3’), rat 11β-HSD1 (forward
5'-AAATACCTCCTCCCCGTCCT-3’, reverse
5’-TCCTGCCTCAACAAACAATC-3’), rat 11β-HSD2 (forward
5’-CAAACCCTTCCCCCACAG-3’, reverse 5’-GCCACATCTCACGCTAAACTC-3’).

Regarding the primer design, a previous PCR study was referenced (Pondugula et al., 2006). The expected lengths of the PCR products were 319, 303, and 301 bp, respectively. As positive controls, brain was used for GRs and 11β-HSD1, and kidney was used for 11β-HSD2. Water was used as a negative control.

2.4. Translocation of GRs (Stria vascularis explants and cell counts)

2.4.1. Condition of culture

Twenty cochleae were dissected from the cochleae of 10 rats (P4) rats and immediately placed in cold (4°C) 10mM HEPES-buffered saline with HBSS. Twenty SV were removed from the cochleae and 10 explants put on a culture slide coated with 20μl Cell Tek (Becton Dickinson Labware, USA) diluted 1:5 and covered with a 300μl culture medium consisting of Dulbecco’s Minimum Essential Medium (D-MEM, No. 12320, GIBCO, USA) (Contains 1,000 mg/L D-glucose, L-glutamine, 25mM HEPES buffer, pyridoxine HCl, and 110 mg/L sodium pyruvate.) with 0.1mg/ml dexamethasone (DMX), and 100units/ml penicillin. The other 10 explants were put on a culture slid
with 300μl of the same culture medium without DMX. All explants were kept in a 5% CO₂ atmosphere at 37°C for up to 3 hours. Cells grown on the glass coverslips were fixed with 4%PFA/PBS for 20 min.

2.4.2. Immunohistochemical labeling for GRs

The cells were washed in PBS, permeabilized with 0.1% Triton X-100 in PBS for 10 min. Following the inactivation of endogenous peroxidase activity with 0.3% H₂O₂ in methanol for 15 min at room temperature, specimens were incubated with the same antibody to GR used in the sections at a dilution of 1:200 in 5%BSA/PBS at 4°C overnight in a humidified chamber. Specimens were then incubated with Cy³-conjugated Affinipure Goat anti-Rabbit IgG (H+L) (Jackson immuno research, USA, dilution 1:200) for 1 hr, washed, and mounted.

Then, immunostained specimens were analyzed with a confocal laser microscope (LSM 510 META, Zeiss,Germany). Images were digitally captured and then analyzed using imaging software (Zen) provided and downloaded from Zeiss (Germany). Technique controls were performed for each specimen using the same method but omitting the use of the primary antibody. Cells showing GRs in the nucleus were counted in an optic field of each explants and a ratio of the number of cells showing
GRs in the nucleus to cells showing GRs in the cytoplasm was calculated. The investigator performing the cell counts was blinded with regard to the identity of the specimens until all counts were completed. For SV cell counts, t-test was used for data analysis with P<0.05 considered significant.
3. Results

3.1. Cellular localization of GRs, 11β-HSD1, and 11β-HSD2 in the adult rat cochlea (Figures 1A, B, C, D, and Table 1)

3.1.1. GRs (Figures 1A)

In midmodiolar cochlear cross-sections of matured rats, intense immunofluorescence labeling of GRs was observed in all cell types of the SV, the epithelial and mesothelial cells of Reissner’s membrane (RM), the SLig, the spiral limbus (SLib), the inner hair cells (IHCs) and the outer hair cells (OHCs), the cochlear nerve (CN), and the spiral ganglion cells (SGCs) (Figure 1A). GRs were expressed mainly in the cytoplasm.

3.1.2. 11β-HSD1 and 11β-HSD2 (Figure 1B, C, D and E)

Intense immunofluorescence labeling of 11β-HSD1 was observed in the SV, the IHCs, and the OHCs. The labeling in the SV was particularly intense in the marginal and basal cells, while it was weak in the intermediate cells (Figure 1B). No immunofluorescence labeling of 11β-HSD2 was detected in any parts of the cochlea and in negative controls stained with normal rabbit IgG (Figure 1C and D). The kidney collecting duct showed a positive immunostaining of 11β-HSD2 (Figure 1E).
3.2. **Cellular localization of GRs, 11β-HSD-1, and 11β-HSD-2 in the adult rat crista ampullaris (Figures 2A to H, and Table 1)**

3.2.1. **GRs (Figures 2A, B)**

Intense immunofluorescence labeling of GRs was observed in the vestibular hair cells (VHCs), the vestibular nerve (VN) fibers, and the dark cells (DCs) of the crista ampullaris in adult rats. The immunofluorescence labeling of GRs was expressed in the cytoplasm.

3.2.2. **11β-HSD1 and 11β-HSD2 (Figures 2C to F)**

Immunostaining of 11β-HSD1 was present in the apical side area of the VHCs and weakly expressed also in the apical side of DCs in adult (Figure 2C and D). No immunofluorescence labeling was observed with the antibody of 11β-HSD2 (Figure 2E and F), and in negative controls stained with normal rabbit IgG (Figure 2G and H).

3.3. **Cellular localization and postnatal development of GRs, 11β-HSD1, and 11β-HSD2 in the rat cochlea (Figures 3A-F, and Table 1).**

3.3.1. **GR (Figures 3A to C)**

In the SV, intense immunofluorescence labeling of GRs was identified in all the
cells at all the postnatal days (P1, P4, and P10). In the cells of the Kölliker's organ, the IHCs, the OHCs, and the CN, it can be seen at P1 (Figure 3A). These findings did not change through all the postnatal developmental periods observed in the present study (Figure 3A, -B, and -C). In the supporting cells, it seemed to be positive at P1, but was not observed at P10.

3.3.2. $11\beta$-HSD1, and $11\beta$-HSD2 (Figures 3D to F)

At P1 (Figure 3D), moderate immunofluorescence labeling of $11\beta$-HSD1 was observed in the cells of Kölliker's organ, the IHCs, and the OHCs. In the SV, the labeling was weak at P1 and P4 (Figure 3D and E), but it was clearly identified in the marginal cells and the basal cells at P10 (Figure 3F). It seemed weak in the intermediate cells at P10. Immunoreactivity of $11\beta$-HSD2 was not detected in any area of the cochlea at any postnatal day (Data not shown).

3.4. Cellular localization and postnatal development of GRs, $11\beta$-HSD-1, and $11\beta$-HSD-2 in the crista ampullaris (Figures 4A, 4B, and Table 1)

3.4.1. GRs (Figures 4A-1 to A-6, and 4B1-2)

Intense immunofluorescence labeling of GRs was observed in the VHCs, the DCs,
and the VN of the crista ampullaris at all the postnatal days (P1, P4, and P10) (Figure 4A-1, -2, -3, -4, -5, and 6). It was clearly expressed in the cytoplasm of the type I and type II sensory cells of the crista ampullaris (Figure 4B-1, and 2).

3.4.2. 11β-HSD1 and 11β-HSD2 (Figures 4A-7 to A-12, and 4B1-2)

At all postnatal days, 11β-HSD1 was present in the apical side of the VHCs, while in the DCs, it was expressed very weakly. Immunofluorescence labeling of 11β-HSD2 was not observed in any area of the crista ampullaris (Data not shown). In a double staining with GRs and 11β-HSD1, GRs were clearly observed in the cytoplasm of the VHCs and 11β-HSD1 was observed in the cytoplasm in the apical area of the VHCs (Figure 4B-1, and 2).

3.5. RT-PCR analysis of GRs, 11β-HSD1, and 11β-HSD2 mRNAs (Figure 5)

RT-PCR products derived from mRNA of GRs, 11β-HSD1, and 11β-HSD2 were detected as single bands located in the expected size on agarose gel in the OC, the vestibular organ, and the SV. mRNAs of GRs, 11β-HSD1, and 11β-HSD2 were present already at P1, and this presence was observed in developmental days P1, P4, and P10. RT-PCR products derived from the mRNAs were detected in positive
control samples obtained from the brain and the kidney.

3.6. **Cellular localization of GRs in the stria vascularis in culture (Figures 6A, B, and Table 2)**

In the medium without DXM, GRs were identified in the cytoplasm of all cells of the SV (Figure 6A). In the medium containing of DXM, some cells showed GRs in the cytoplasm, however, the number of cells showing GRs in the nucleus was higher (Figure 6B). In the medium containing DXM, the number of cells showing GRs in the nucleus was 7.03±8.16 (mean±SD)%, and in the medium without DXM the number was 0% (Table 2). The number of cells showing GRs in the nucleus significantly was higher in the medium with DXM ($P<0.05$).
4. Discussion

In the present study, precise cellular localization of GRs and distribution of 11β-HSD isoforms were identified in the rat inner ear tissue, indicating that steroid regulation by GRs and 11β-HSD isoforms is present in the inner ear. This further indicates that a lot of site and structures may be targets for steroids, accounting for the clinical efficacy of steroids on various inner ear diseases. In particular, it should be noted that mRNA and intense immunofluorescent labeling of GRs were observed in the SV of the postnatal and adult rats; this had not been clearly indicated in previous studies (Ten et al., 1993; Zuo et al., 1995). Low expression of GRs in the SV in postnatal rats was explained as a coincidence with developmental changes of endocochlear potential (EP) (Zuo et al., 1995). The difference between our results and theirs is assumed to be due to differences in sensitivity in the respective experimental procedures.

In general, Na⁺,K⁺-ATPase is assumed to be regulated by the mineralcorticoid receptors (MR) and also in the inner ear, Na⁺,K⁺-ATPase is regulated by MR (Pitovski, et al., 1993). However, GRs also participate in the regulation of Na⁺,K⁺-ATPase (Hatou, et al., 2009; Derfoul. et al., 1998). Studies in rat have shown a positive correlation between serum levels of corticosteroid (glucocorticoid and mineralocorticoid) hormones and the amount of Na⁺,K⁺-ATPase in the inner ear (Curtis
et al., 1993; Rarey et al., 1989). Low levels of GRs in immunostaining and ELISA were observed in the previously studies in the SV (Rarey and Curtis, 1996; Zuo et al., 1995). In the rat SV, Na$^+$,K$^+$-ATPase is highly expressed, and that such low levels of GRs do not coincide with the high expression of Na$^+$,K$^+$-ATPase (Erichsen et al., 1998). Our results regarding GRs in the SV coincide with the high expression of Na$^+$,K$^+$-ATPase. Although it is known that the absence of adrenal hormones does not affect the gross cochlear potentials (endocochlear potential: EP) (Lohuis et al., 1990), depletion of endogenous glucocorticoids by bilateral adrenalectomy in rats was reported to cause a decrease in the volume of the marginal cells leading to shrinkage of the scala media (Lohuis et al., 2000). Thus, regulation of fluid homeostasis by the SV is assumed to be highly dependent on circulating glucocorticoids. Furthermore, the strong GRs expression seen in the SV at the early developmental stages in this study is assumed to be required for maintaining fluid homeostasis and further maturation as well as EP formation.

In general, activity of glucocorticoids is classically explained by genomic mechanisms involving binding to a cytosolic receptor and stimulation (trans-activation) or inhibition (trans-repression) of a target gene transcription. Currently, it is widely accepted that in absence of the ligand, the receptors are part of a large protein
heterocomplex containing a number of chaperone proteins stabilizing the complex in the cytoplasm (Sarabdjitsingha RA, 2009). GRs bound to glucocorticoids are present in the nucleus, while those not bound are present in the cytoplasm. In the previous immunohistochemical studies of GRs in the inner ear, immunolabeling of GRs was observed in the cytoplasm but not in the nucleus. Also in the present study, immunostaining of GRs was observed in the cytoplasm in nearly all sections. To investigate whether subcellular localization of GRs in the SV changes as a consequence of corticosteroid treatment, a short time culture of the SV with a high dose of DXM was performed. In the medium without DXM, almost all of GRs was located in the cytoplasm. However, in the medium containing DXM, GRs were translocated to the nucleus. This indicates that what we observed in the sections of the SV are GRs not bound to glucocorticoids in the cytoplasm and also that GRs present in the cytoplasm are translocated to the nucleus by stimulation of glucocorticoid. According to a classic model, the free molecules of glucocorticoids enter the target cell by passive diffusion through the plasma membrane and form a ligand–receptor complex. Subsequent activation or repression of the target gene reflects the selective effect of a given glucocorticoids. This genomic process is normally slow, i.e. it takes hours before the outcome of the hormonal signaling manifests itself. Recent studies suggest that
glucocorticoids use receptors on the plasma membranes and/or cytoplasm both to gain access to the intracellular compartment and to modulate cellular functions through non-genomic mechanisms (Moore et al., 1995; Falkenstein et al., 2000; Johnson et al., 2005; Hu et al., 2008). The presence of non-genomic regulation of potassium ion recycling is known to occur in the SV (Lee and Marcus, 2002), and therefore, a part of GRs present in the cytoplasm may be required for the non-genomic rapid action such as potassium ion recycling and GRs translocated may show the genomic action. GRs expression was observed also in the cochlear hair cells, the supporting cells, and RM. Glucocorticoids protect the cochlear hair cells against cytotoxicity (Tahera Y, et al., 2006; Haake SM, et al., 2009) or pathological states such as noise trauma (Tahera Y, et al., 2007; Meltser I, et al., 2009). The supporting cells are known to be related to hair cell regeneration (Löwenheim H, et al., 1999) and ion regulation (Mistrik and Ashmore, 2009). In the RM of mice, PCR and electrophysiological methods have indicated that Na\(^+\) absorption is mediated by apical epithelial sodium channel (ENaC) and/or other amiloride-sensitive channels, basolateral Na\(^+\)-K\(^+\) ATPase, and K\(^+\) -permeable channels under the control of glucocorticoids (Kim et al., 2009). GRs possibly participate in the regulation of cell cycle, gene, ion, and fluid transport in the rat cochlea.

There has also been some controversy regarding the presence of GRs in the
peripheral vestibular organs. In the BALB mice, a report suggested that the immunoreactivity of GRs was hardly distinguishable, and that the VHCs were not likely to be direct targets of glucocorticoid (Shimazaki et al., 2002). However, functional regulation by glucocorticoids in the peripheral vestibular system has already been proven. For instance, corticosteroids are known to have a protective effect also in the vestibular maculae (Pondugula et al., 2004) and to regulate ions such as ENaC in animal experiments of the semicircular canal duct epithelium (Pondugula et al., 2006; Taura et al., 2006). In the present study, immunoreactivity of GRs was observed in the cytoplasm of the VHCs, the DCs, and the VN. This result implies that these tissues are likely to be targets of glucocorticoids, and that their function may be under control of genomic and non-genomic effects of glucocorticoids.

Although SV and DCs were found to contain GRs in this study, in a previous study, 11β-HSD was found in SLig but not found in the SV and the DCs by immunoblotting and immunocytochemistry by use of an antibody generated against rat liver 11β-HSD (Ten et al., 1994). However, so far no study has dealt with the precise distribution of 11β-HSD isoforms in the cochlea and the vestibular organs. In the present study, 11β-HSD1 was found in a lot of sites in the cochlea and vestibule, as well as in the CN and the VN in the adult rat. In a study that dealt with postnatal rats (Ten et al., 1997),
a) 11β-HSD was not found in the cochlea of rats at birth, b) the expression appeared at low levels at the 12th postnatal day in the SLig, and c) adult-like higher levels of expression were observed from the 15th postnatal day on. These findings may account for the EP changes during development. In our study, although both expression of mRNA and immunoreactivity of 11β-HSD1 increased during development, the expression of 11β-HSD1 as well as GRs was found even at postnatal day 1. Although the discrepancy between our results and the previous study is assumed to be based on the difference of technical application, the expression of GRs and 11β-HSD isoforms at early developmental stages implies that the isoforms also participate in the maturation and maintenance of the homeostasis of the cochlea with GRs. Especially, 11β-HSD1 is suggested to start controlling the cellular concentration of glucocorticoids in the hair cells at the early developmental stages. Localization of 11β-HSD1 does not completely overlap with that of GRs in the inner ear, which is observed in other organs. 11β-HSD1 is not always expressed in the site where GRs are present in other organs, the concentration of glucocorticoids in the target cells is regulated by other enzyme as well as 11β-HSD1 (Oppermann et al., 1997; Seckl, 2004; Draper and Stewart, 2005).

11β-HSD2 was not observed in any site in the inner ear in the present study. In the previous PCR study (Pondugula et al., 2006), a quantitative PCR indicated that
11β-HSD1 was the predominant enzyme in the semicircular canal duct epithelium. In addition, it was also indicated that mRNA of 11β-HSD2 expression was comparatively small and was not affected by dexamethasone (Pondugula et al., 2006). In the present study, compared with 11β-HSD2, immunostaining of 11β-HSD1 in the crista ampullaris was predominant; this was compatible with the quantitative PCR study. Since in 11β-HSD2 the kidney used as the positive control was clearly stained, a failure of immunostaining of 11β-HSD2 in the inner ear tissues is not assumed to be due to a technical error. In the organ of Corti, expression of GRs mRNA or molecules related to stress is modulated by stress or sound condition (Terunuma, et al., 2001; Meltser, et al., 2009; Mazurek, et al., 2010). 11β-HSD1 is assumed to be the main isoform of 11β-HSD in the inner ear, however, 11β-HSD2 levels may have been too low as to be detected by immunostaining, or expressed under a special condition of intracellular glucocorticoid concentration. 11β-HSD1 is known to participate in the control of intraocular pressure and cerebrospinal fluid production, and therefore, 11β-HSD1 is likely to participate in controlling fluid homeostasis in the inner ear. In the present study, 11β-HSD1 is shown to be in the cytoplasm in the apical area of the cochlear and vestibular hair cells as well as in the SV. Since 11β-HSD1 converts inactive-form cortisone to active-form cortisol, it is suggested that 11β-HSD1 controls cellular
function of the inner ear at receptor and prereceptor levels, especially playing an important role in regulating glucocorticoids supplied from endolymph.
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Declaration of interest

The authors report no conflict of interest. The authors alone are responsible.
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Research Highlights

> We investigated the distribution of GRs and 11β-HSD isoforms in the rat inner ear. 
> GRs and 11β-HSD1 were present in most cells in the rat inner ear tissues. 
> Immunostaining of 11β-HSD2 was not observed in any of the inner ear tissues. > Local steroid regulation by GRs and 11β-HSD is present in the inner ear.
Table 1. Localization of GR and 11β-HSD1 in rat inner ear tissues.

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<td>Cochlea SLib</td>
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<tr>
<td>Cochlea SLig</td>
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<tr>
<td>Cochlea RM</td>
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<tr>
<td>Cochlea SV</td>
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<tr>
<td>Cochlea SGC</td>
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<tr>
<td>Vestibule CN</td>
<td>++++</td>
<td>++</td>
</tr>
<tr>
<td>Vestibule VHC</td>
<td>++++</td>
<td>++++</td>
</tr>
<tr>
<td>Vestibule DC</td>
<td>++++</td>
<td>++++</td>
</tr>
<tr>
<td>Vestibule VN</td>
<td>++++</td>
<td>++++</td>
</tr>
<tr>
<td><strong>11β-HSD1</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cochlea IHC</td>
<td>++++</td>
<td>++++</td>
</tr>
<tr>
<td>Cochlea OHC</td>
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<tr>
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<tr>
<td>Vestibule DC</td>
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</tr>
<tr>
<td>Vestibule VN</td>
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P1 = the postnatal day 1, P4 = the postnatal days 4, P10 = the postnatal days 10, GR = glucocorticoid receptors, 11β-HSD1 = 11β-hydroxysteroid dehydrogenase type 1, IHC = the inner hair cell, OHC = the outer hair cell, SLib = the spiral limbus, SLig = the spiral ligament, RM = Reissner’s membrane, SV = the stria vascularis, SGC = the spiral ganglion cell, CN = the cochlear nerve, VHC = the vestibular hair cell, DC = the dark cell, VN = the vestibular nerve, ++++ = intense staining, ++ = moderate staining, + = slight staining, – = negative
Table 2. Counts of cells showing translocation of GRs

<table>
<thead>
<tr>
<th>N/AC (%)</th>
<th>DXM (-)</th>
<th>DXM (+)</th>
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<tr>
<td>0</td>
<td>7.03 ± 8.16*</td>
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N/AC (%) = Ratio of the number of cells showing GRs in the nucleus to that of all cells in each observed area. DMX (-) = culture in the absence of dexamethasone, DXM (+) = culture in the presence of dexamethasone. Data are shown as mean±SD. * = p<0.05.
Figure 3

(A(P1))

RM

SV

IHC

OHCs

CN

50 μm

(D(P1))

RM

SV

IHC

OHCs

50 μm

(B(P4))

RM

SV

IHC

OHCs

CN

50 μm

(E(P4))

RM

SV

IHC

OHCs

50 μm

(C(P10))

RM

SV

IHC

OHCs

SLig

CN

50 μm

(F(P10))

RM

SV

IHC

OHCs

50 μm
Figure 5

<table>
<thead>
<tr>
<th></th>
<th>Positive control</th>
<th>Organ of Corti</th>
<th>Stria vascularis</th>
<th>Vestibular organ</th>
<th>Negative control</th>
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<td>M</td>
<td>-</td>
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<tr>
<td>GRs</td>
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