

Research paper

Mineralocorticoid receptor mediates glucocorticoid treatment effects in the autoimmune mouse ear

Dennis R. Trune *, J. Beth Kempton, Neil D. Gross

Oregon Hearing Research Center, Department of Otolaryngology – Head and Neck Surgery; Oregon Health and Science University, Mail Code NRC04, 3181 SW Sam Jackson Park Road, Portland, OR 97239-3098, USA

Received 27 May 2005; accepted 7 October 2005
Available online 22 November 2005

Abstract

The standard treatment for many hearing disorders is glucocorticoid therapy, although the cochlear mechanisms involved in steroid-responsive hearing loss are poorly understood. Cochlear dysfunction in autoimmune mice has recently been shown to be controlled with the mineralocorticoid aldosterone as effectively as with the glucocorticoid prednisolone. Because aldosterone regulates sodium, potassium, and other electrolyte homeostasis, this implied the restoration of hearing with the mineralocorticoid was due to its impact on cochlear ion transport, particularly in the stria vascularis. This also suggested glucocorticoids may be controlling hearing recovery in part through their binding to the mineralocorticoid receptor in addition to their glucocorticoid receptor-mediated anti-inflammatory and immunosuppressive functions. Therefore, the aim of the present study was to better delineate the role of the mineralocorticoid receptor in steroid control of hearing in the autoimmune mouse. Spironolactone, a mineralocorticoid receptor antagonist, was administered to MRL/MpJ-*Fas*^{lpr} autoimmune mice in combination with either aldosterone or prednisolone to compare their hearing and systemic disease with mice that received either steroid alone. ABR thresholds showed either aldosterone or prednisolone alone preserved hearing in the mice, but spironolactone prevented both steroids from maintaining normal cochlear function. This suggested both steroids are preserving hearing through the mineralocorticoid receptor within the ear to regulate endolymph homeostasis. The spironolactone treatment did not block normal glucocorticoid receptor-mediated immune-suppression functions because mice receiving prednisolone, either with or without spironolactone, maintained normal body weights, hematocrits, and serum immune complexes. Thus, reducing systemic autoimmune disease was not sufficient to control hearing if mineralocorticoid receptor-mediated functions were blocked. It was concluded the inner ear mineralocorticoid receptor is a significant target of glucocorticoids and a factor that should be considered in therapeutic treatments for steroid-responsive hearing loss.

© 2005 Elsevier B.V. All rights reserved.

Keywords: Autoimmune hearing loss; Spironolactone; Prednisolone; Aldosterone; Mineralocorticoid; MRL/MpJ-*Fas*^{lpr} autoimmune mice

1. Introduction

Glucocorticoids (prednisone, prednisolone, dexamethasone) are commonly used to treat sudden and idiopathic hearing loss, as well as hearing loss associated with sys-

temic autoimmune diseases, such as rheumatoid arthritis, Cogan's syndrome, and systemic lupus erythematosus (Moskowitz et al., 1984; Nadel, 1996; Wilson et al., 1980; Grandis et al., 1993; Alexiou et al., 2001). Despite their clinical efficacy, very little is known about the action of steroids in the ear. Glucocorticoid functions include immune suppression, anti-inflammation, and sodium reabsorption (Schimmer and Parker, 1996; Munck et al., 1990). The immune-suppressive and anti-inflammatory functions are mediated through the glucocorticoid receptor, whereas sodium reabsorption is controlled by glucocorticoid activation of the mineralocorticoid receptor (Barnes and Adcock,

Abbreviations: ABR, auditory brainstem response; dB, decibel; kHz, kilohertz; Na⁺, K⁺ – ATPase, sodium, potassium – adenosine triphosphatase; *p*, probability; spir, spironolactone; SPL, sound pressure level; χ^2 , Chi-square statistic

* Corresponding author. Tel.: +1 503 494 2931; fax: +1 503 494 5656.

E-mail address: truned@ohsu.edu (D.R. Trune).

1993; Smith et al., 2001; Funder, 1997; Arriza et al., 1987). Steroid-mediated hearing improvement has traditionally been attributed to the anti-inflammatory and immunosuppressive activities of glucocorticoids, but they also are effective in cases of sudden and rapidly progressing hearing loss with no demonstrable immunologic problem. Glucocorticoids actually have a high binding affinity to the mineralocorticoid receptor (Claire et al., 1993; Rupprecht et al., 1993; Munck et al., 1990; Arriza et al., 1987) and this potential impact on ion homeostasis in the ear has largely been ignored in explaining steroid-responsive ear disease. If hearing loss is due to disruption of stria vascularis functions, the efficacy of glucocorticoids may be due to their restoration of normal endolymph ion balances.

The MRL/MpJ-*Fas*^{lpr} autoimmune disease mouse is an established model of spontaneous autoimmune sensorineural hearing loss (Ruckenstein et al., 1993; Trune et al., 1997; Trune and Kempton, 2001). These mice carry a *Fas* gene defect that prevents apoptosis of self-recognizing T lymphocytes, causing T cell proliferation and polyclonal B cell activation (Watanabe-Fukunaga et al., 1992). This leads to the systemic autoimmune disease condition of elevated serum immune complexes and anti-DNA antibodies, lowered hematocrit, splenomegaly, lymphadenopathy, and increased body mass. The resultant inner ear disease is pathology of the stria vascularis (Trune, 1997), disruption of the blood-labyrinth barrier (Lin and Trune, 1997), depression of the endocochlear resting potential (Ruckenstein et al., 1999), and elevated auditory thresholds (Ruckenstein et al., 1993; Trune et al., 1997; Trune and Kempton, 2001). These various sequelae of stria vascularis dysfunction suggests endolymph homeostasis is compromised in autoimmune mouse hearing loss.

Hearing loss in the autoimmune mice is prevented or reversed with glucocorticoids (Trune et al., 1999a,b; Trune and Kempton, 2001), providing significant parallels with human steroid-responsive hearing loss. However, this laboratory also has recently shown the mineralocorticoid aldosterone is equivalent to the glucocorticoid prednisolone in preventing hearing loss in this autoimmune mouse model (Trune and Kempton, 2001). Because of the significant role of mineralocorticoids in control of sodium and potassium balances, it is possible that both steroids are improving hearing through their activation of the mineralocorticoid receptor. Both mineralocorticoid and glucocorticoid receptors occur in the ear (Rarey et al., 1991; Pitovski et al., 1993a) and it is well established that glucocorticoids have

an affinity for the mineralocorticoid receptor that is equal to or higher than affinity for their own receptor (Claire et al., 1993; Munck et al., 1990; Arriza et al., 1987; Rupprecht et al., 1993). It is therefore conceivable that glucocorticoid-responsive hearing recovery in the mice, as well as patients, is partially mediated via the mineralocorticoid receptor function of restoring ionic homeostasis in the stria.

The aim of this study was to better delineate the role of the mineralocorticoid receptor in glucocorticoid-mediated hearing preservation. Preliminary studies suggested steroid-mediated hearing recovery is prevented with spironolactone (Gross et al., 2002), a competitive antagonist for the mineralocorticoid receptor. Therefore, cochlear function and systemic autoimmune disease were assessed in mice treated with a glucocorticoid or mineralocorticoid in conjunction with spironolactone. Spironolactone will block the mineralocorticoid receptor-mediated actions of the two steroids and differentiate their respective mechanisms of action in control of auditory function. Such pharmacologic information could be helpful in developing better targeted drug therapies for patients with idiopathic and autoimmune hearing loss.

2. Materials and methods

2.1. Animal model

MRL/MpJ-*Fas*^{lpr} autoimmune mice ($n = 118$) were purchased from Jackson Laboratories (Bar Harbor, ME) at 2 months of age. The onset of systemic autoimmune disease occurs at 3–4 months of age and cochlear thresholds rise shortly thereafter. Therefore, the mice were tested with auditory brainstem response (ABR) audiometry at 2–3 months of age to establish pretreatment baseline auditory thresholds. The mice were then assigned to one of several treatment groups and ABR audiometry was repeated after 2, 3, and 4 months of treatment. At each ABR test, body weights were taken and 100 μ l of sera were collected retro-orbitally to assess the impact of treatment on systemic autoimmune disease progression.

2.2. Steroid treatment

Mice were randomly assigned to one of several drug treatment groups (Table 1) to establish the impact of the mineralocorticoid receptor blocker spironolactone. All drugs were given in approximately therapeutic doses. The treatment groups were: aldosterone (mineralocorticoid), prednisolone (glucocorticoid), spironolactone (mineralocorticoid receptor antagonist), and the combinations of spironolactone + aldosterone and spironolactone + prednisolone. The normal progression of systemic disease and related hearing loss was assessed in untreated water controls. The treatment drugs were given in the drinking water, shown previously to be effective (Trune et al., 1999a,b; Trune and Kempton, 2001). Mice drink 3–5 ml

Table 1
Drug treatment groups

Treatment group	Initial # mice	Dose (/kg/day)	Amount per 500 ml bottle	Effective dose (/day)	% (#) Survival six months
Water control	24				58 (14)
Prednisolone	10	5 mg	15 mg	0.15 mg	60 (6)
Aldosterone	10	15 μ g	45 μ g	0.45 μ g	50 (5)
Spironolactone	10	5 mg	15 mg	0.15 mg	40 (4)
+Prednisolone	28	5 mg	15 mg	0.15 mg	50 (14)
+Aldosterone	26	15 μ g	45 μ g	0.45 μ g	77 (20)
ETOH control	10		750 μ l	7.50 μ l	80 (8)

of water daily, so the final effective dose of each drug was estimated from this assumed consumption. The advantage of this method is that it provides a constant source of steroid, parallels oral administration in patients, and avoids the trauma of daily injections. Some drugs needed to be dissolved in a small volume of ethyl alcohol (ETOH) before dilution in the drinking water bottle. Therefore, an alcohol control group of mice was given an equivalent amount of ETOH in their drinking water to determine if this had any impact on hearing thresholds or systemic autoimmune disease.

2.3. Treatment groups

2.3.1. Prednisolone ($n = 10$)

The glucocorticoid group was given daily oral doses (5 mg/kg/day) of prednisolone sodium phosphate (Spectrum Quality Products, Inc., Gardena, CA). The steroid was provided orally by dissolving it in the standard 500 ml drinking water bottle, which has been shown to maintain normal systemic levels (Zhou et al., 1994; Hunneyball et al., 1986; Van der Kraan et al., 1993). The effective dose was approximately 0.15 mg/day (Table 1). This dose was shown previously to prevent cochlear threshold elevations and systemic autoimmune disease symptoms (Trune and Kempton, 2001).

2.3.2. Aldosterone ($n = 10$)

The mineralocorticoid group was given a daily oral dose of 15 μ g/kg/day of aldosterone (d-aldosterone, Sigma, St. Louis, MO). Aldosterone drinking water was prepared by dissolving 45 μ g of steroid in 50 μ l of 100% ETOH, then diluting it in the 500 ml water bottle (Hausler et al., 1992) to reach the final effective dose of 0.45 μ g. The final ETOH concentration in the water bottle was 0.009% and was considered negligible. This aldosterone dose was shown previously to prevent the cochlear threshold elevations but not systemic autoimmune disease symptoms because it has no immune suppression activity (Trune and Kempton, 2001).

2.3.3. Spironolactone ($n = 10$)

Spironolactone is a competitive antagonist to aldosterone for binding with the mineralocorticoid receptor (Claire et al., 1993; Luzzani and Glaser, 1984; McInnes et al., 1982; Wambach and Casals-Stenzel, 1983). Therefore, it was given to block access to this receptor by the steroids. This treatment group was given daily oral doses (5 mg/kg/day) of spironolactone (Sigma, St. Louis, MO) in the drinking water. Spironolactone drinking water was prepared by dissolving 15 mg in 0.75 ml of ETOH and diluting it into a 500 ml water bottle. The final effective dose was approximately 0.15 mg/day and the final ETOH concentration was 0.15%.

2.3.4. Spironolactone + aldosterone ($n = 26$)

This treatment group was given 15 μ g/kg/day of aldosterone in addition to the 5 mg/kg/day of spironolactone. The drugs were prepared as above and delivered in the same water bottle. The combined ETOH concentration from both compounds was 0.16% (0.15% + 0.009%).

2.3.5. Spironolactone + prednisolone ($n = 28$)

This treatment group was given daily oral doses (5 mg/kg/day) of prednisolone sodium phosphate in addition to the 5 mg/kg/day of spironolactone. This combination was delivered by dissolving 15 mg of prednisolone in the same 500 ml drinking-water bottle as the spironolactone. The effective dose of each drug was 0.15 mg/day.

2.3.6. Water controls ($n = 24$)

Untreated control mice were given regular tap water in their bottles to assess the normal progression of auditory dysfunction and autoimmune disease.

2.3.7. Alcohol controls ($n = 10$)

The greatest alcohol concentration given above was 0.16%. Although this amount was considered negligible, it still was necessary to determine if this amount of alcohol solvent had any impact on hearing and autoim-

mune disease. Therefore, a control group of mice was given 750 μ l of ETOH in the 500 ml drinking bottle for a final concentration of 0.15% and a final effective dose of 7.5 μ l/day.

2.4. Cochlear function

Auditory brainstem response (ABR) audiometry to pure tones was used to evaluate cochlear function and followed our standard protocol (Mitchell et al., 1999). Prior to treatment, mice were anesthetized and their individual ears were stimulated with a closed-tube sound delivery system sealed into the ear canal. The ABR to tone-burst stimuli at 4, 8, 16, and 32 kHz were recorded and analyzed independently and thresholds at each frequency were determined for each ear. Following their respective treatments, ABR thresholds were again determined and the threshold shift at each of the four frequencies was determined for each ear.

Considerable variability in ABR thresholds occurs in autoimmune mice, which makes statistical comparisons difficult due to large within group variances. Because the critical determination in this study is whether an individual ear responded to treatment, its pre- and post-treatment thresholds were compared. The total shift across the four frequencies was calculated by arithmetically summing the four frequency threshold shifts (Trune et al., 1999a,b; Trune and Kempton, 2001). An ear was judged as improved if this post-treatment threshold summation was lower by 20 dB or more, which would represent an average improvement of at least 5 dB per frequency. Conversely, an ear was classified as worse if this combined threshold shift was higher by 20 dB or more (average threshold of 5 dB or more per frequency higher than baseline). Any combined threshold shift of 19 dB or less (+ or -) was considered unchanged. The number of ears in each outcome category was statistically compared to water controls with the χ^2 nonparametric test.

2.5. Serum and body weight analyses

The severity of systemic autoimmune disease was evaluated according to previous protocols (Trune et al., 1989). Serum immune complexes increase with autoantibody production and hematocrits are reduced due to anti-erythrocyte autoantibodies. Both of these autoimmune disease effects are reversed with prednisolone (glucocorticoid) treatment (Trune and Kempton, 2001). Therefore, hematocrits and immune complexes were measured to determine if blocking the mineralocorticoid receptor prevents glucocorticoid control of these symptoms. Body weight also increases with systemic autoimmune disease, primarily due to hemopoietic proliferation that increases the size of the spleen (7–10 \times), lymph nodes (50–100 \times), and thymus (2 \times) (Murphy, 1981). Therefore, to assess this impact of disease and treatment on body mass, animal weights were recorded at each ABR test. Each animal's baseline and post-treatment values for these various measures were compared with a paired *t*-test.

The use of the animals reported in this study was approved by the Oregon Health & Science University Institutional Animal Care and Use Committee to ensure compliance with federal animal welfare guidelines.

3. Results

3.1. Steroid treatment

The mice tolerated all of the drug treatments very well and did not show any adverse side effects. None appeared dehydrated, which would have been expected if they avoided the drinking water. Because these various drugs were given in therapeutic doses, it is expected that they

altered the taste of the water little if at all. The treatment groups showed similar attrition rates due to systemic autoimmune disease (Table 1), which normally is 50% by five months of age.

3.2. Cochlear function

Treatment effects on cochlear thresholds were analyzed for the mice at six months of age, or approximately three months of drug treatment. This time period was chosen because it was long enough for disease related hearing loss to develop, but short enough that a sufficient number of mice still remained alive for reliable conclusions to be drawn. Post-treatment ABR analyses were limited to the number of mice surviving to six months for each treatment (Table 1), which are generally the least diseased mice. The progression of hearing loss in the autoimmune mice is demonstrated in the untreated control group (Fig. 1). Analysis of variance revealed a significant progression of rising thresholds ($F = 22.98$, $p < 0.0001$) over the three month treatment period. Threshold elevation occurred in most of the frequencies, which is typical for the autoimmune mice as disease progresses.

There was considerable threshold variation among mice within the various treatment groups. Some mice develop a greater threshold elevation with disease progression than others, while some respond more to treatments. The sickest animals die (Table 1), eliminating those with the potential for the greatest hearing loss and impact of treatment. Thus, a survival (statistical) bias is introduced if attempts are made to compare all mice alive at each treatment time. Because the key issue is the effect each treatment had on the hearing of individual animals, the threshold shift (or

its lack) within individual ears was analyzed to more accurately assess the impact of the treatments. Therefore, for each ear, the baseline and post-treatment ABR thresholds at the four frequencies were compared to establish change attributable to treatment. The shifts for each frequency within an ear were added to derive a total shift in threshold per ear. The ear was considered to be improved if the combined threshold shifts were lower by 20 dB or more (average 5 dB per frequency). The ear was considered unchanged if the combined threshold shifts were ± 19 dB and rated as worse if the combined thresholds shifts were higher by 20 dB or more. The χ^2 analyses compared the number of ears within each outcome category for each steroid relative to controls receiving water.

The mice receiving either aldosterone or prednisolone alone showed significantly better cochlear function than the untreated water controls run in parallel with them (Fig. 2). Nearly all (91.7%) ears of the untreated water controls at six months of age were worse by 20 dB or more than they were at the three month baseline measurement. This reflects the typical progression of hearing loss that accompanies development of systemic autoimmune disease. However, both the aldosterone and prednisolone groups showed thresholds in approximately 60% of the ears were unchanged at six months. This resulted in significant differences by χ^2 analysis between the steroid treatments and water controls (Fig. 2). No ears in any of these three groups were improved, presumably because the steroid treatments were started before hearing loss onset.

The various spironolactone treatment groups all had ABR thresholds that were similar to untreated (water control) mice (Fig. 3). The mice receiving spironolactone alone were similar to the water controls, as were mice receiving the alcohol alone ($p > 0.05$). Spironolactone also blocked the hearing protective effects of aldosterone and prednisolone (Fig. 3). When spironolactone was given in combination with either of these steroids, the mice did not demonstrate any slowing or prevention of the elevated thresholds. This suggested the spironolactone interfered with the receptor that was mediating the effects of each steroid. Because of the implication the glucocorticoid prednisolone is mediating its effects through the mineralocorticoid receptor, a further analysis was conducted of the difference in cochlear function between prednisolone (Fig. 2) and spironolactone + prednisolone (Fig. 3) treated mice. A χ^2 analysis showed the treatment effect was significant ($\chi^2 = 9.15$; $p = 0.010$) as the prednisolone mice had better thresholds than the spironolactone + prednisolone mice.

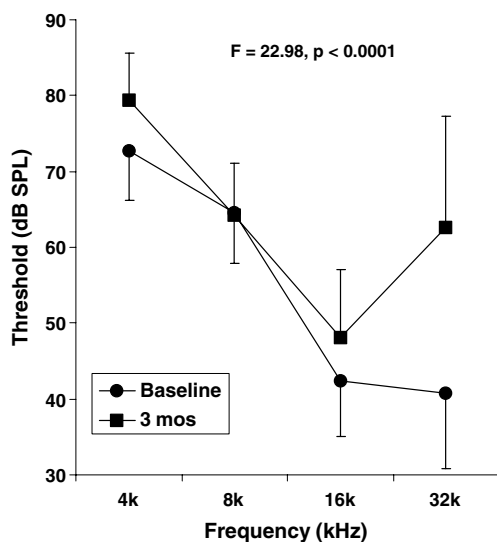


Fig. 1. Average auditory brainstem response thresholds for untreated autoimmune mice alive after three months of treatment ($N = 14$, 28 ears) compared to their baseline values. Analysis of variance showed a significant time \times frequency interaction ($F = 22.98$, $p < 0.0001$). Vertical bars represent 1 SD.

3.3. Body weight and serum factors

3.3.1. Body weight

A normal impact of systemic autoimmune disease is an increase in body weight, due mainly to disease related symptoms of lymphadenopathy, splenomegaly, etc. Glucocorticoid treatment generally prevents this increase in body weight because of its immune suppression effects. The

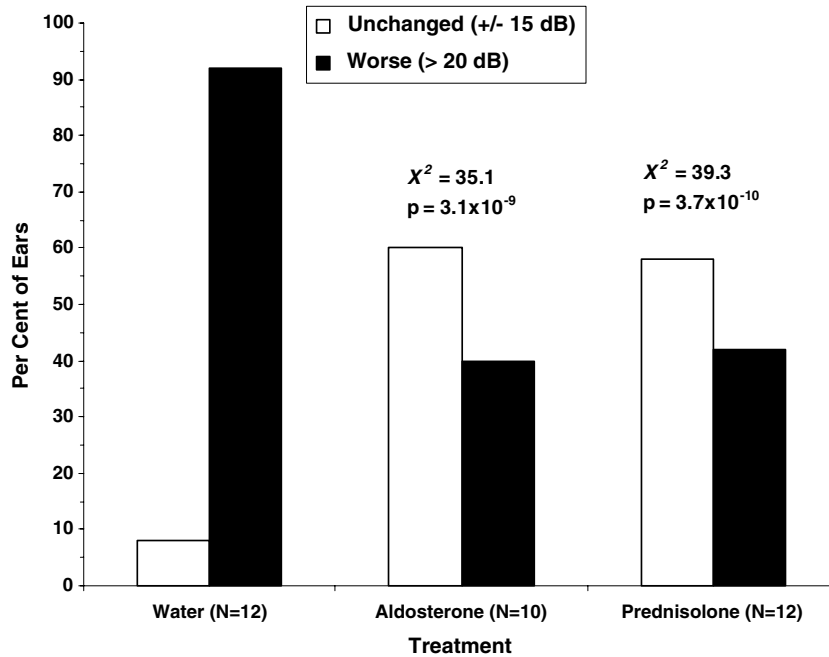


Fig. 2. Autoimmune mice treated for three months with either aldosterone or prednisolone had significantly better hearing thresholds compared to untreated water controls. Nearly all ears (91%) in control (water) mice had combined threshold shifts that were worse by 20 dB or more at the end of the treatment period. However, mice treated with either aldosterone or prednisolone had threshold shifts worse by 20 dB or more in only 40% of their ears, the remaining 60% were unchanged (± 15 dB). χ^2 analyses showed both steroid treatment groups were statistically different from untreated water controls. N , number of ears tested in surviving mice in parentheses.

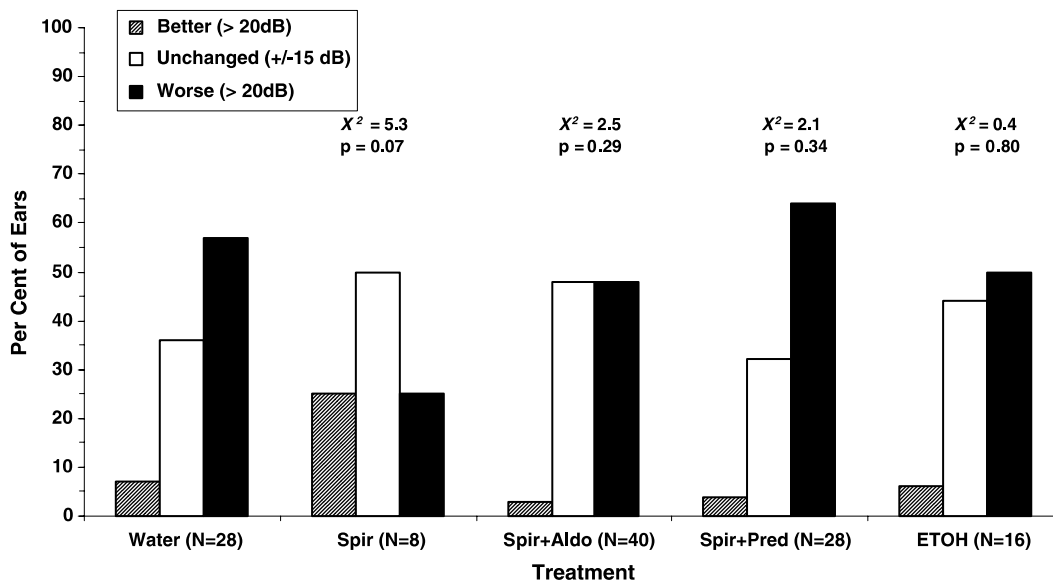


Fig. 3. Spironolactone treatments prevented steroids from preserving hearing in autoimmune mice. Autoimmune mice had hearing thresholds similar to untreated water controls (χ^2 , $p > 0.05$) when spironolactone was combined with either aldosterone (Spir + Aldo) or prednisolone (Spir + Pred). Mice treated with either spironolactone (Spir) or alcohol (ETOH) alone also were not different from untreated control mice. N , number of ears tested in surviving mice in parentheses.

untreated mice showed this typical increase in body weight, going from a mean baseline weight of 31.8 g to a six month average weight in the survivors of 37.6 g (Fig. 4). A paired t -test of baseline and six month weights in the surviving mice showed this degree of increase to be statistically significant ($t = 2.29$; $p = 0.04$). Similarly, all treatment groups

had significant increases in body weights except those receiving the glucocorticoid prednisolone, either alone or with spironolactone (Fig. 2). These latter mice that received prednisolone actually declined in body weight due to the steroid treatment. The spironolactone + prednisolone mice had a statistically significant decline in weight ($p = 0.03$).

The mice receiving prednisolone alone had a decline from 36.2 to 31.3 g, although this did not reach statistical significance. Thus, the increase in body weight typically seen with autoimmune disease did not occur in mice receiving the immune suppressive prednisolone and blocking the mineralocorticoid receptor with spironolactone did not interfere with this.

3.3.2. Immune complexes

Serum immune complexes (antigen–antibody complexes) increase with systemic disease due to production of autoantibodies. The immune suppressive effect of the glucocorticoid prednisolone is to reduce these immune complex levels, or keep them from developing if treatment is started prior to disease onset. The normal immune complex development in autoimmune disease is best demonstrated in the untreated mice (Fig. 5). Mice receiving water only showed a progressive increase in serum immune complex levels from a baseline of 1475–6133 $\mu\text{g}/\text{ml}$ after two months ($t = 3.53$; $p = 0.003$). The slight decline in levels between two and four months of treatment reflect the survivor effect as the sickest mice die and the group mean improves. Paired t -tests of values at baseline and two months of treatment showed a similar pattern in the mice treated with aldosterone, alcohol, spironolactone, or spironolactone + aldosterone (Fig. 5). All of these groups showed statistically significant elevations in serum immune complexes after two months of treatment. Conversely, mice receiving prednisolone, either alone or combined with spironolactone, did not develop elevated levels of immune complexes (Fig. 5; $p > 0.05$). Like the body weight data, the immune suppressive effects of the glucocorticoid prednisolone were not impaired by blocking the mineralocorticoid receptor with spironolactone.

3.3.3. Hematocrit

The hematocrit, or cellular portion of blood, declines in autoimmune disease due to anti-erythrocyte autoantibodies. Normal mouse hematocrit is 44–47%. The baseline (2–3 months of age) hematocrit for the various autoimmune mouse groups was between 44% and 49% (Fig. 5). Once autoimmune disease began, hematocrits in untreated (water) mice dropped from 47% to less than 43% after two months. This typical pattern of hematocrit decline was observed in all mouse groups except those receiving the glucocorticoid prednisolone, either alone or with spironolactone (Fig. 5). Paired t -test comparisons of hematocrits at baseline and two months of treatment showed that all treatment groups not receiving prednisolone had significantly lower hematocrits. The surviving aldosterone mice had a hematocrit of 41% at two months of treatment ($p = 0.06$), but by four months of treatment this had declined to 31%. On the other hand, mice receiving prednisolone or spironolactone + prednisolone had hematocrits that were not significantly different from baseline ($p > 0.05$). In fact, hematocrits for both of these groups actually showed a trend of increasing due to the immune suppression effects of the steroid. This indicated that the usual protective effect of the glucocorticoid prednisolone on hematocrit was not prevented by spironolactone blockage of the mineralocorticoid receptor.

3.4. Summary of treatment effects

Overall, spironolactone blockage of the mineralocorticoid receptor appeared to interfere with steroid effects on the ear, but did not impair steroid effects on systemic immune disease. Mice receiving spironolactone in combination with either prednisolone or aldosterone did not

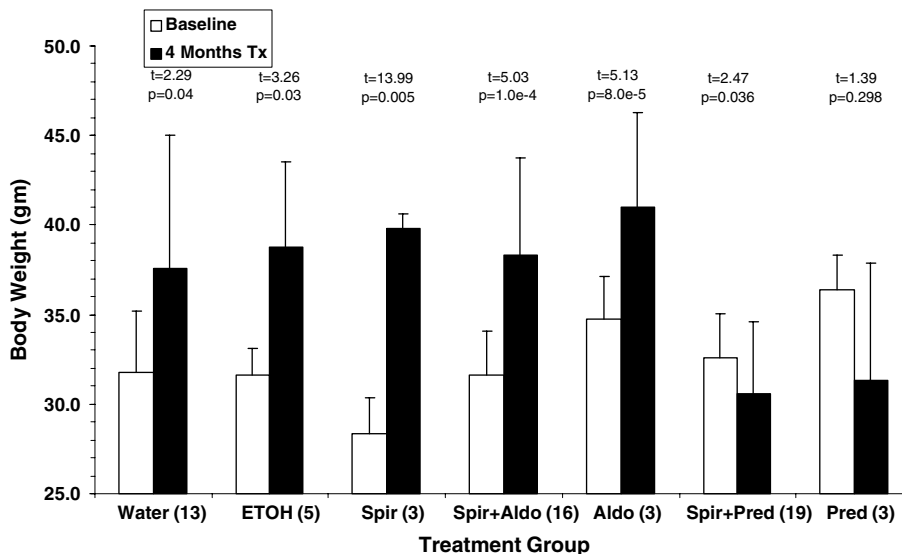


Fig. 4. Body weights for autoimmune mice at baseline and after four months of treatment. Paired t -test values and probabilities of baseline versus post-treatment weights are over each plot. All groups showed a significant increase in body weight except the mice receiving prednisolone or spironolactone + prednisolone, both of which trended lower.

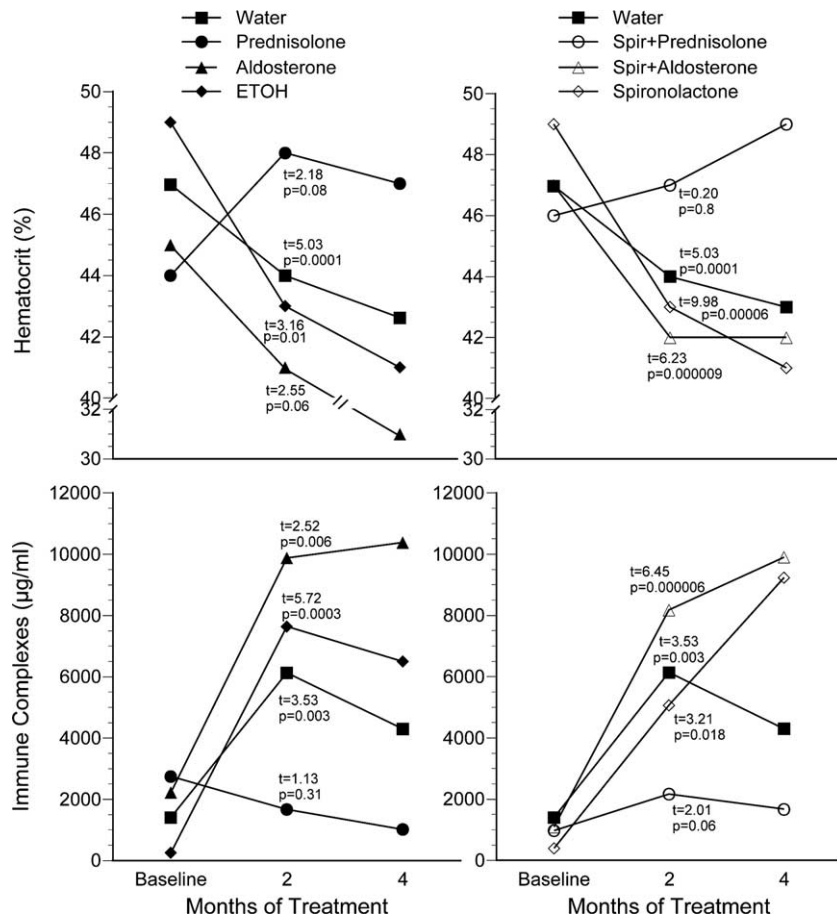


Fig. 5. Hematocrits and serum immune complex levels for autoimmune mice in the various treatment groups. Paired *t*-tests of baseline and two months of treatment showed all groups had significantly decreased hematocrits except those mice receiving prednisolone or spironolactone + prednisolone ($p > 0.05$). The probability for the aldosterone group at 2 months was at the cutoff level of significance ($p = 0.06$), but hematocrits in this group declined further to 31% by four months. The various treatment groups also showed similar patterns of increased levels of serum immune complexes except those mice receiving prednisolone or spironolactone + prednisolone ($p > 0.05$). The water control group is shown in each plot for reference. Sample sizes are the same as Fig. 4.

show the hearing preservation that these two steroids by themselves normally cause. Thus, blocking the mineralocorticoid receptor interfered with the cochlear effects of both steroids. However, the normal immune suppression effects of the glucocorticoid prednisolone, which are mediated through the glucocorticoid receptor, were not impaired by blocking the mineralocorticoid receptor. Mice receiving prednisolone, either alone or combined with spironolactone, maintained baseline body weights, immune complexes, and hematocrits. These observations suggest that the cochlear effects of both steroids are mediated through the mineralocorticoid receptor, whereas the systemic effects of glucocorticoids on the immune system are mediated through the glucocorticoid receptor.

4. Discussion

The present results for MRL/MpJ-*Fas*^{lpr} autoimmune mice parallel earlier studies that showed rising cochlear thresholds due to systemic disease were reversed or prevented with either prednisolone or aldosterone treatment (Trune et al., 1999a,b; Trune and Kempton, 2001). Because

glucocorticoids bind to both receptors with equal affinity (Arriza et al., 1987; Munck et al., 1990; Rupprecht et al., 1993), it was not determinable in previous studies which steroid receptor mediated hearing preservation with the glucocorticoid prednisolone. Blocking the mineralocorticoid receptor with spironolactone in the present study helped differentiate the role of each steroid receptor in steroid-responsive mechanisms of the ear.

Mice treated only with spironolactone were not different from the untreated controls. Spironolactone is a competitive antagonist for the mineralocorticoid receptor, although its affinity for binding is only about 5–15% of the binding affinity of aldosterone (Claire et al., 1993; Luzzani and Glasser, 1984; Alnemri et al., 1991). However, because the natural aldosterone produced by untreated autoimmune mice is not sufficient to prevent hearing loss, it would not be expected to preserve hearing in mice simultaneously receiving the antagonist spironolactone. Furthermore, the additional aldosterone in mice receiving spironolactone + aldosterone still was not sufficient to maintain normal hearing. Therefore, because aldosterone only treatment in this and previous studies (Trune and

Kempton, 2001) has consistently preserved hearing, and this was prevented by the receptor antagonist spironolactone, one can conclude that the ear is responsive to aldosterone through the mineralocorticoid receptor.

Mice receiving only the glucocorticoid prednisolone showed hearing preservation consistent with previous studies from this laboratory (Trune et al., 1999a,b; Trune and Kempton, 2001). In contrast, mice receiving spironolactone + prednisolone showed hearing decline similar to untreated mice, suggesting blockage of the mineralocorticoid receptor interfered with prednisolone effects in the ear. This is compelling evidence that the glucocorticoid restored hearing through mineralocorticoid receptor-mediated processes.

This is interpreted that the stria vascularis pathology (Lin and Trune, 1997; Ruckenstein et al., 1999) caused by systemic autoimmune disease is restored by reestablishment of normal endolymph ion balances mediated by mineralocorticoid receptor functions. On the other hand, prednisolone functions of immune suppression (immune complexes, hematocrits, and body weights), which are regulated through the glucocorticoid receptor, were not affected by the mineralocorticoid receptor block. Thus, glucocorticoid receptor-mediated functions suppress the underlying systemic autoimmune disease, while mineralocorticoid receptor-mediated functions reverse one of the resulting symptoms, i.e., disruption of stria homeostasis. These findings emphasize the differential roles these receptors play in inner ear responses to glucocorticoids and challenge current thinking that it is only the anti-inflammatory actions of glucocorticoids that restore hearing in steroid-responsive hearing loss.

It is interesting to note that circulating immune complex levels were reduced similarly in both prednisolone groups, but this was not sufficient to restore hearing in the spironolactone + prednisolone mice. This lack of a relationship between hearing recovery and immune complex levels parallels observations of the present and earlier studies (Trune and Kempton, 2001) that aldosterone preserved hearing in the autoimmune mice while not reducing the elevated serum immune complex levels. This also is evidence the two steroid receptors play different roles in steroid-responsive hearing loss. The observation that prednisolone + spironolactone will reduce immune complexes, but not improve hearing, suggests the underlying pathology in the ear is not reversed with glucocorticoid-receptor mediated processes (immune suppression and anti-inflammation). Furthermore, the observation that aldosterone will improve hearing without reducing immune complexes indicates that mineralocorticoid-related processes (ion homeostasis) are involved in some cases of steroid-responsive hearing loss. Awareness of these differences will be critical in development of therapies for hearing loss.

Although our conclusion is that spironolactone impaired prednisolone's impact on hearing control by blocking the mineralocorticoid receptor, it is possible that spironolactone interfered directly with the glucocorticoid

receptor. However, this mechanism seems unlikely in view of the fact that both aldosterone and spironolactone have very little binding affinity for the glucocorticoid receptor. Competitive binding studies have shown that spironolactone has only 0.1% of the binding affinity of glucocorticoids for the glucocorticoid receptor (Claire et al., 1993). To effectively block this receptor, spironolactone would have to be 100–1000 times the concentration of the glucocorticoids, well beyond the physiologic levels used in this study (Campen and Fanestil, 1982; Couette et al., 1992; Arriza et al., 1987; Clore et al., 1988). Furthermore, the normal glucocorticoid receptor-mediated effect of immune suppression occurred in the mice whether they received spironolactone or not, which also suggests that there was no interference with glucocorticoid receptor function. Therefore, direct spironolactone interference with the glucocorticoid receptor seems an unlikely explanation for the present results.

Aldosterone activation of the cytoplasmic mineralocorticoid receptor (Schimmer and Parker, 1996; Benos et al., 1995; Garty and Palmer, 1997) results in the expression of multiple gene products called aldosterone-induced proteins (AIPs). These proteins are responsible for increased sodium and potassium transport across the cell by activation of existing sodium channels, synthesizing new ones, and increasing cellular Na^+ , K^+ -ATPase to drive the process (Mujais et al., 1985; Kleyman et al., 1989; Horisberger and Rossier, 1992). Aldosterone also increases expression of calcium channels and the Na^+/Cl^- -cotransporter (Falkenstein et al., 2000; Lesouhaitier et al., 2001; Abdallah et al., 2001). Thus, the mineralocorticoid receptor has significant control over local ion transport, which is blocked by spironolactone (Claire et al., 1993; Luzzani and Glasser, 1984; McInnes et al., 1982; Wambach and Casals-Stenzel, 1983). The mineralocorticoid receptor in the inner ear (Pitovski et al., 1993b; ten Cate et al., 1993, 1994; Furuta et al., 1994; Yao and Rarey, 1996) controls endolymph homeostasis through these same ion channels and transporters on cells lining the cochlear duct, including the stria vascularis and spiral ligament (Wangemann, 2002; Weber et al., 2001; Sage and Marcus, 2001; Takeuchi et al., 2001).

It is assumed that the major impact of these steroid treatments is on the ion homeostatic functions of the stria vascularis and spiral ligament. Studies from numerous laboratories have shown that this region is primarily affected in autoimmune mice, as determined by both histology and loss of endocochlear potentials (reviewed in Trune, 2001). Furthermore, steroid treatment leads to recovery of function, as well as the qualitative (Trune and Kempton, 2001) and quantitative (Kaylie and Trune, 2002) improvement in stria morphology. While there may be additional cochlear compartments that are functionally affected by autoimmune disease, thus far these are unidentified. Therefore, findings from the present study are interpreted that it is this stria vascularis pathology that is prevented by either glucocorticoid or mineralocorticoid activation of the mineralocorticoid receptor, while both are ineffective if the antagonist

spironolactone is present. Certainly future studies will be necessary to fully characterize the steroid-mediated gene expression in the ear that underlies hearing recovery.

Membrane receptors also have been identified that increase sodium channels and transporters via rapid nongenomic mechanisms (Falkenstein et al., 2000; Zhou and Bubien, 2001). However, these membrane aldosterone receptors, in contrast to the classic cytoplasmic receptors above, are not blocked by spironolactone. Therefore, their function in the present study probably would not be affected. It also is possible that the mineralocorticoid effects on the ear are secondary to steroid-responsive mechanisms in other organs (e.g., kidney) that affect systemic electrolyte homeostasis. While this cannot be completely ruled out, the extensive distribution of steroid receptors in the ear suggest direct cochlear effects of circulating hormones are more likely.

These findings offer new insights into the mechanism by which prednisolone and other glucocorticoid treatments for hearing loss operate in the ear by activating mineralocorticoid receptors. This relationship of the two groups of steroids to their receptors has been established for other organ systems and is proposed here for cochlear steroid-responsive function, as well (Fig. 6). Glucocorticoids given for autoimmune hearing loss can enhance inner ear ion transport functions through the mineralocorticoid receptor and suppress inflammation or immune disease through the glucocorticoid receptor. The immune suppression function also could secondarily affect hearing (long-term) by reducing the elevated serum immune complexes that are causing homeostasis dysfunction in the first place. This proposed mechanism also could explain why prednisone (glucocorticoid) treatment is effective for sudden and idiopathic hearing loss (Nadel, 1996; Alexiou et al., 2001; Wilson et al., 1980). Prednisone-responsive hearing loss, in the absence of systemic immune disease or inflammatory problems, cannot be explained by the glucocorticoid's role of suppressing the immune system. The glucocorticoid function of regulating sodium and potassium ion transport is an alternative explanation for the recovery of hearing in these clinical disorders with no demonstrable immunologic basis. This also suggests that the common interchangeable use of the clinical terms "autoimmune hearing loss" and "steroid-responsive hearing loss" may need to be revised. If disorders of cochlear ion homeostasis recover with glucocorticoid treatment, then steroid-responsive hearing loss will include more auditory problems that just those with an immune-mediated pathology.

Although additional studies will be necessary to better understand steroid-responsive mechanisms in the ear, the present results offer provocative new insight into their respective receptors' roles. Future confirmation of the present observations may lead to alternative steroid therapies for sudden and idiopathic hearing loss. Effective treatment of these disorders with steroids that selectively target the mineralocorticoid receptor may reestablish normal stria vascularis and/or cochlear function without the systemic side effects seen with glucocorticoids like prednisone. On

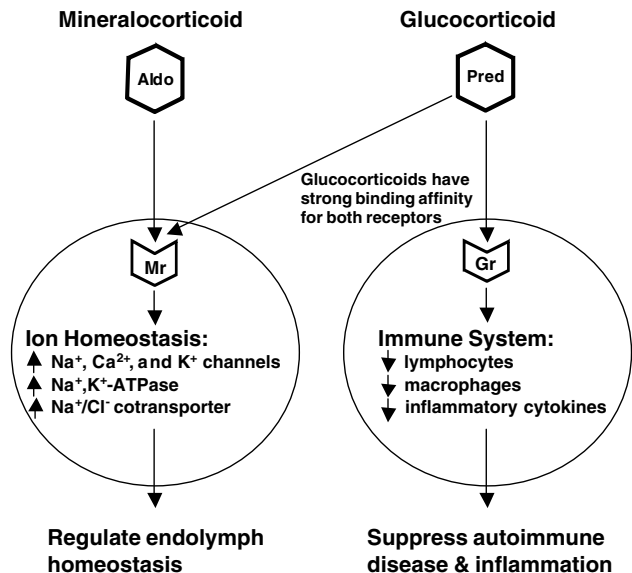


Fig. 6. Functional relationship of the steroids to the mineralocorticoid receptor (Mr) and glucocorticoid receptor (Gr) within the inner ear. The mineralocorticoid aldosterone (Aldo) and the glucocorticoid prednisolone (Pred) each bind to their respective cytoplasmic receptors, while prednisolone (and most other glucocorticoids) also has high binding affinity for the mineralocorticoid receptor. Mineralocorticoid receptor-mediated functions include increasing the number and activity of ion channels and transporters involved in endolymph homeostasis. Glucocorticoid receptor-mediated functions include the suppression of inflammation and immune disease by decreasing the production and function of lymphocytes, macrophages, and pro-inflammatory cytokines.

the other hand, if treatment of autoimmune inner ear disease requires systemic immune suppression in addition to cochlear homeostasis restoration, then combined mineralocorticoid and glucocorticoid therapy may be more advantageous. Combination treatment may allow each to be given in lower doses to reduce detrimental side effects. The findings of the present study suggest there is significant clinical value in further research into the differential role of these receptors in the ear.

Acknowledgements

Research supported by NIH-NIDCD R01 DC05593, NIDCD R01 DC03573, and VA RR&D National Center for Rehabilitative Auditory Research RCTR 597-0160, Portland VAMC.

References

- Abdallah, J.G., Schrier, R.W., Edelstein, C., Jennings, S.D., Wyse, B., Ellison, D.H., 2001. Loop diuretic infusion increases thiazide-sensitive Na^+/Cl^- -cotransporter abundance: role of aldosterone. *J. Am. Soc. Nephrol.* 12, 1335–1341.
- Alexiou, C., Arnold, W., Fauser, C., Schratzenstaller, B., Gloddek, B., Fuhrmann, S., Lamm, K., 2001. Sudden sensorineural hearing loss: does application of glucocorticoids make sense? *Arch. Otolaryngol. Head Neck Surg.* 127, 253–258.
- Alnemri, E.S., Maksymowych, A.B., Robertson, N.M., Litwack, G., 1991. Overexpression and characterization of the human mineralocorticoid receptor. *J. Biol. Chem.* 266, 18072–18081.

- Arriza, J.L., Weinberger, C., Cerelli, G., Glaser, T.M., Handelin, B.L., Housman, D.E., Evans, R.M., 1987. Cloning of human mineralocorticoid receptor complementary DNA: structural and functional kinship with the glucocorticoid receptor. *Science* 237, 268–275.
- Barnes, P.J., Adcock, I., 1993. Anti-inflammatory actions of steroids: molecular mechanisms. *Trends Pharm. Sci.* 14, 436–441.
- Benos, D.J., Awaysda, M.S., Ismailov, L.I., Johnson, J.P., 1995. Structure and function of amiloride-sensitive Na⁺ channels. *J. Membrane Biol.* 143, 1–18.
- Campen, T.J., Fanestil, D.D., 1982. Spironolactone: a glucocorticoid agonist or antagonist? *Clin. Exper. Hyperten. Theory Pract.* A4, 1627–1636.
- Claire, M., Faraj, H., Grassy, G., Aumelas, A., Rondot, A., Auzou, G., 1993. Synthesis of new 11 β -substituted spironolactone derivatives. Relationship with affinity for mineralocorticoid and glucocorticoid receptors. *J. Med. Chem.* 36, 2404–2407.
- Clore, J.N., Estep, H., Ross-Clunis, H., Watlington, C.O., 1988. Adrenocorticotropin and cortisol-induced changes in urinary sodium and potassium excretion in man: effects of spironolactone and RU486. *J. Clin. Endocrinol. Metab.* 67, 824–831.
- Couette, B., Marsaud, V., Baulieu, E.-E., Richard-Foy, H., Rafestin-Oblin, M.-E., 1992. Spironolactone, an aldosterone antagonist, acts as an antiglucocorticoid on the mouse mammary tumor virus promoter. *Endocrinology* 130, 430–436.
- Falkenstein, E., Christ, M., Fearing, M., Wehling, M., 2000. Specific nongenomic actions of aldosterone. *Kid. Int.* 57, 1390–1394.
- Funder, J.W., 1997. Glucocorticoid and mineralocorticoid receptors: biology and clinical relevance. *Ann. Rev. Med.* 48, 231–240.
- Furuta, H., Mori, N., Sato, C., Hoshikawa, H., Sakai, S., Iwakura, S., Doi, K., 1994. Mineralocorticoid type I receptor in the rat cochlea: mRNA identification by polymerase chain reaction (PCR) and in situ hybridization. *Hear. Res.* 78, 175–180.
- Garty, H., Palmer, L.G., 1997. Epithelial sodium channels: function, structure, and regulation. *Physiol. Rev.* 77, 359–396.
- Grandis, J.R., Hirsch, B.E., Wagener, M.M., 1993. Treatment of idiopathic sudden sensorineural hearing loss. *Am. J. Otol.* 14, 183–185.
- Gross, N.D., Kempton, J.B., Trune, D.R., 2002. Spironolactone blocks glucocorticoid mediated hearing preservation in autoimmune mice. *Laryngoscope* 112, 298–303.
- Hausler, A., Persoz, C., Buser, R., Mondadori, C., Bhatnagar, A., 1992. Adrenalectomy, corticosteroid replacement and their importance for drug-induced memory-enhancement in mice. *J. Steroid Biochem. Mol. Biol.* 41, 785–789.
- Horisberger, J.D., Rossier, B.C., 1992. Aldosterone regulation of gene transcription leading to control of ion transport. *Hypertension* 19, 221–227.
- Hunneyball, I.M., Crossley, M.J., Spowage, M., 1986. Pharmacological studies of antigen-induced arthritis in BALB/c mice. I. Characterization of the arthritis and the effect of steroidal and non-steroidal anti-inflammatory agents. *Agents Actions* 18, 384–393.
- Kaylie, D.M., Trune, D.R., 2002. Steroid treatments restore stria vascularis capillary size in MRL/MpJ-Fas^{lpr} autoimmune mice. Presented at the Assoc Res Otolaryngol Midwinter Mtg, St. Petersburg Beach, Jan.
- Kleyman, T.R., Cragoe Jr., E.J., Kraehenbuhl, J.P., 1989. The cellular pool of Na⁺ channels in the amphibian cell line A6 is not altered by mineralocorticoids. Analysis using a new photoactive amiloride analog in combination with anti-amiloride antibodies. *J. Biol. Chem.* 264, 11995–12000.
- Lesouhaitier, O., Chiappe, A., Rossier, M.F., 2001. Aldosterone increases T-type calcium currents in human adrenocarcinoma (H295R) cells by inducing gene expression. *Endocrinology* 142, 4320–4330.
- Lin, D.W., Trune, D.R., 1997. Breakdown of stria vascularis blood-labyrinth barrier in C3H/lpr autoimmune disease mice. *Otolaryngol. Head Neck Surg.* 117, 530–534.
- Luzzani, F., Glasser, A., 1984. Characterization of spironolactone binding sites distinct from aldosterone receptors in rat kidney homogenates. *Biochem. Pharm.* 33, 2277–2281.
- McInnes, G.T., Shelton, J.R., Ramsay, L.E., Harrison, I.R., Asbury, M.J., Clarke, J.M., Perkins, R.M., Venning, G.R., 1982. Relative potency and structure activity relationships of aldosterone antagonists in healthy man: correlation with animal experience. *Brit. J. Clin. Pharm.* 13, 331–339.
- Mitchell, C.R., Kempton, J.B., Creedon, T.A., Trune, D.R., 1999. The use of a 56-stimulus train for the rapid acquisition of auditory brainstem responses with multiple frequency and intensity tone-bursts. *Audiol. Neurootol.* 4, 80–87.
- Moskowitz, D., Lee, K.J., Smith, H.W., 1984. Steroid use in idiopathic sudden sensorineural hearing loss. *Laryngoscope* 94, 664–666.
- Mujais, S.K., Chekal, M.A., Jones, W.J., Hayslett, J.P., Katz, A.I., 1985. Modulation of renal sodium-potassium-adenosine triphosphatase by aldosterone. Effect of high physiologic levels on enzyme activity in isolated rat and rabbit tubules. *J. Clin. Invest.* 76, 170–176.
- Munck, A., Mendel, D.B., Smith, L.I., Orti, E., 1990. Glucocorticoid receptors and actions. *Am. Rev. Respir. Dis.* 141, S2–S10.
- Murphy, D.D., 1981. Lymphoproliferation (*lpr*) and other single-locus models for murine lupus. In: Gershwin, M.E., Merchant, B. (Eds.), *Immunologic Defects in Laboratory Animals*. vol. 2. Plenum Press, New York, pp. 143–173.
- Nadel, D.M., 1996. The use of systemic steroids in otolaryngology. *ENT-Ear Nose Throat J.* 75, 502–516.
- Pitovski, D.Z., Drescher, M.J., Kerr, T.P., Drescher, D.G., 1993a. Aldosterone mediates an increase in [³H]ouabain binding at Na⁺, K⁺-ATPase sites in the mammalian inner ear. *Brain Res.* 601, 273–278.
- Pitovski, D.Z., Drescher, M.J., Drescher, D.G., 1993b. High affinity aldosterone binding sites (Type I receptors) in the mammalian inner ear. *Hear. Res.* 69, 10–14.
- Rarey, K.E., Lohuis, P.J.F.M., ten Cate, W.-J.F., 1991. Response of the stria vascularis to corticosteroids. *Laryngoscope* 101, 1081–1084.
- Ruckenstein, M.J., Mount, R.J., Harrison, R.V., 1993. The MRL-*lpr/lpr* mouse: a potential model of autoimmune inner ear disease. *Acta Otolaryngol.* 113, 160–165.
- Ruckenstein, M.J., Milburn, M., Hu, L., 1999. Strial dysfunction in the MRL-Fas^{lpr} autoimmune mouse. *Otolaryngol. Head Neck Surg.* 121, 452–456.
- Rupperecht, R., Reul, J.M.H.M., van Steensel, B., Spengler, D., Soder, M., Berning, B., Holsboer, F., Damm, K., 1993. Pharmacological and functional characterization of human mineralocorticoid and glucocorticoid receptor ligands. *Eur. J. Pharm. Mol. Pharm.* 247, 145–154.
- Sage, C.L., Marcus, D.C., 2001. Immunolocalization of CIC-K chloride channel in stria marginal cells and vestibular dark cells. *Hear. Res.* 160, 1–9.
- Schimmer, B.P., Parker, K.L., 1996. Adrenocorticotrophic hormone; adrenocortical steroids and their synthetic analogs; inhibitors of the synthesis and actions of adrenocortical hormones. In: Hardman, J.G., Limbird, L.E., Molinoff, P.B., Ruddon, R.W. (Eds.), *Goodman and Gilman's The Pharmacological Basis of Therapeutics*, ninth ed. McGraw-Hill Companies, New York, pp. 1459–1485.
- Smith, P.J., Cousins, D.J., Jee, Y.-K., Staynov, D.Z., Lee, T.H., Lavender, P., 2001. Suppression of granulocyte-macrophage colony-stimulating factor expression by glucocorticoids involves inhibition of enhancer function by the glucocorticoid receptor binding to composite NF-AT/activator protein-1 elements. *J. Immunol.* 167, 2502–2510.
- Takeuchi, S., Ando, M., Sata, T., Kakigi, A., 2001. Three-dimensional and ultrastructural relationships between intermediate cells and capillaries in the gerbil stria vascularis. *Hear. Res.* 155, 103–112.
- ten Cate, W.J.F., Curtis, L.M., Small, G.M., Rarey, K.E., 1993. Localization of glucocorticoid receptors and glucocorticoid receptor mRNAs in the rat cochlea. *Laryngoscope* 103, 865–871.
- ten Cate, W.J.F., Monder, C., Marandici, A., Rarey, K.E., 1994. 11 β -hydroxysteroid dehydrogenase in the rat inner ear. *Am. J. Physiol.* 266, E269–E273.
- Trune, D.R., 1997. Cochlear immunoglobulin in the C3H/lpr mouse model for autoimmune hearing loss. *Otolaryngol. Head Neck Surg.* 117, 504–508.

- Trune, D.R., 2001. Mouse models for immunologic diseases of the auditory system. In: Willott, J.F. (Ed.), *Handbook of Mouse Auditory Research: From Behavior to Molecular Biology*. CRC Press, Boca Raton, pp. 505–531.
- Trune, D.R., Kempton, J.B., 2001. Aldosterone and prednisolone control of cochlear function in MRL/MpJ-*Fas^{lpr}* autoimmune mice. *Hear. Res.* 155, 9–20.
- Trune, D.R., Craven, J.P., Morton, J.I., Mitchell, C., 1989. Autoimmune disease and cochlear pathology in C3H/*lpr* strain mouse. *Hear. Res.* 38, 57–66.
- Trune, D.R., Kempton, J.B., Hefeneider, S.H., Bennett, R.M., 1997. Inner ear DNA receptors in MRL/*lpr* autoimmune mice: potential 30 and 70 kilodalton link between autoimmune disease and hearing loss. *Hear. Res.* 105, 57–64.
- Trune, D.R., Wobig, R.J., Kempton, J.B., Hefeneider, S.H., 1999a. Steroid therapy improves cochlear function in the MRL.MpJ-*Fas^{lpr}* autoimmune mouse. *Hear. Res.* 137, 160–166.
- Trune, D.R., Wobig, R.J., Kempton, J.B., Hefeneider, S.H., 1999b. Steroid treatment in young MRL.MpJ-*Fas^{lpr}* autoimmune mice prevents cochlear dysfunction. *Hear. Res.* 137, 167–173.
- Van der Kraan, P.M., Vitters, E.L., Postma, N.S., Verbunt, J., van den Berg, W.B., 1993. Maintenance of the synthesis of large proteoglycans in anatomically intact murine articular cartilage by steroids and insulin-like growth factor I. *Ann. Rheum. Dis.* 52, 734–741.
- Wambach, G., Casals-Stenzel, J., 1983. Structure–activity relationship of new steroidal aldosterone antagonists. Comparison of the affinity for mineralocorticoid receptors in vitro and the antialdosterone activity in vivo. *Biochem. Pharm.* 32, 1479–1485.
- Wangemann, P., 2002. K^+ cycling and the endocochlear potential. *Hear. Res.* 165, 1–9.
- Watanabe-Fukunaga, R., Brannan, C.I., Copeland, N.G., Jenkins, N.A., Nagata, S., 1992. Lymphoproliferation disorder in mice explained by defects in Fas antigen that mediates apoptosis. *Nature* 356, 314–317.
- Weber, P.C., Cunningham III, C.D., Schulte, B.A., 2001. Potassium recycling pathways in the human cochlea. *Laryngoscope* 111, 1156–1165.
- Wilson, W.R., Byl, F.M., Laird, N., 1980. The efficacy of steroids in the treatment of idiopathic sudden hearing loss. A double-blind clinical study. *Arch. Otolaryngol.* 106, 772–776.
- Yao, X., Rarey, K.E., 1996. Localization of the mineralocorticoid receptor in rat cochlear tissue. *Acta Otolaryngol.* 116, 493–496.
- Zhou, Z.-H., Bubien, J.K., 2001. Nongenomic regulation of ENaC by aldosterone. *Am. J. Physiol. Cell Physiol.* 281, C1118–C1130.
- Zhou, N.N., Nikai, S., Kawakita, T., Oka, M., Nagasawa, H., Himeo, K., Nomoto, K., 1994. Combined treatment of autoimmune MRL/MP-*lpr/lpr* mice with a herbal medicine, Ren-shen-yang-rong-tang (Japanese name: Ninin-Youyi-to) plus suboptimal dosage of prednisolone. *Int. J. Immunopharm.* 16, 845–854.