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Old Mice Lacking High-Affinity Nicotine Receptors Resist Acoustic Trauma

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Abstract

There is presently no clearly effective preventative medication against noise-induced hearing loss (NIHL). However, negative feedback systems that presumably evolved to modulate the sensitivity of the organ of Corti may incidentally confer protection. One feedback system implicated in protection from NIHL involves synaptic connections between the lateral olivocochlear efferent terminals and the afferent fibers of spiral ganglion neurons (SGNs). These connections operate via high affinity nicotinic acetylcholine receptors containing the $\beta 2$ subunit. We unexpectedly observed protection from NIHL in 9 month old knockout mice lacking the $\beta 2$ subunit ($\beta 2^{-/-}$); however, the same protection was not observed in 2-month old $\beta 2^{-/-}$ mice. This enigmatic observation led to the discovery that protection from acoustic trauma in older $\beta 2^{-/-}$ mice is mainly mediated by an age-related increase of corticosterone, not disruption of efferent cholinergic transmission. Significant protection of inner hair cells after acoustic trauma in $\beta 2^{-/-}$ mice was linked to the activation of glucocorticoid signaling pathways. However, significant loss of SGNs was observed in animals with chronically high systemic levels of corticosterone. These results suggested a “double-edge sword” nature of glucocorticoid signaling in neuronal protection, and a need for caution regarding when to apply synthetic glucocorticoid drugs to treat neural injury such as accompanies acoustic trauma.

Keywords

Noise-induced hearing loss; spiral ganglion neurons; hair cell; glucocorticoid; olivocochlear efferent synapse

1. Introduction

At least 30 million people in the USA encounter hazardous levels of noise that can cause acoustic trauma (DHHS, 2009). There are two injury phases after noise exposure, a

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temporary or compound threshold shift (TTS or CTS) that is most prominent in the first 24 hours, and a residual permanent threshold shift (PTS) that still remains 2-3 weeks later (Nordmann et al., 2000; Quaranta et al., 1998). Although certain molecular pathways such as glucocorticoid signaling exert protective effects against noise-induced hearing loss (NIHL) (reviews: Canlon et al., 2007; Le Prell et al., 2007; Ohlemiller, 2008), and promising therapeutic agents have been identified (Kopke et al., 2005; Lefebvre et al., 2002; Le Prell et al., 2007; Lynch and Kil, 2005; Niu and Canlon, 2002), no drugs are currently in clinical use against NIHL.

One way to protect against NIHL might be to potentiate negative feedback systems already present in the auditory system. Olivocochlear efferent feedback systems presumably evolved to enhance detection of signals in noise (Elgoyhen et al., 2009). Nevertheless, the efferent system has also been implicated in protection of the organ of Corti against noise injury (Maison et al., 2007). Electrical stimulation of the olivocochlear efferent pathway reduces hearing loss (Rajan, 1988; Reiter and Liberman, 1995). In addition, animals are more vulnerable to NIHL after surgical disruption of the olivocochlear efferent pathway (Kujawa and Liberman, 1997). The olivocochlear efferent system consists of two major divisions: the medial olivocochlear (MOC) pathway and the lateral olivocochlear (LOC) pathway. MOC efferents originate near medial nuclei of the superior olivary complex and make synaptic contact with outer hair cells (OHCs). LOC efferents originate near the lateral nuclei of the superior olive, and contact primary afferent dendrites of spiral ganglion neurons (SGNs) near their synapses with inner hair cells (IHCs). The mechanism by which the MOC system confers protection may involve modulation of OHC active mechanics. Yet how the LOC pathway may protect remains unclear, partly due to the anatomical and neurochemical complexity of this system. A recent study demonstrated increased vulnerability to NIHL after destruction of LOC neurons (Darrow et al., 2007). The LOC pathway makes cholinergic, dopaminergic, GABAergic, and peptidergic synaptic transmissions on SGN terminals (Eybalin, 1993; Puel, 1995). It is unknown which neurotransmitter or neuromodulator mediated this protection.

The cholinergic component of the LOC pathway may operate in part by activating a combination of nicotinic acetylcholine receptors (nAChRs), including high-affinity $\alpha 4\beta 2$ complex in SGNs. Cochlear SGNs express $\alpha 2$, $\alpha 4-7$, and $\beta 2-3$ nAChR subunits (Bao et al., 2005; Drescher et al., 1995; Housley et al., 1994; Morley et al., 1998). We therefore reasoned that transgenic mice lacking the $\beta 2$ nAChR subunit might be an ideal tool to isolate cholinergic protective mechanisms engaged by the LOC pathway. Accordingly, we hypothesized that *nAChR* $\beta 2^{-/-}$ mice would show increased vulnerability to NIHL due to the disruption of the LOC cholinergic pathway. Instead our findings indicate that transgenic $\beta 2^{-/-}$ mice at nine months old *resist* acoustic trauma mainly due to chronic systemic up-regulation of glucocorticoids.

2. Materials and Methods

2.1. Animal and Drug Treatments

A total of 90 mice B6.CAST-*Cdh23*^{CAST} congenic mice to C57BL/6J (henceforth B6.CAST) were used, including 24 mice at 2 months of age and 66 mice at 9 months. A total of 42 mice *nAChR* $\beta 2^{-/-}$ mice were used, including 16 at 2 months of age and 26 at 9 months. B6.CAST and *nAChR* $\beta 2^{-/-}$ mice on the same background were housed five per cage with food and water freely available. Our rationale for using the congenic mice was to eliminate the effects on age-associated and noise-induced hearing loss in C57BL/6J (B6) inbred mice of the *ahl* allele of cadherin 23 (*Cdh23*^{ahl}). The $\beta 2^{-/-}$ mice were originally established on the B6 background and the knockout allele was moved to the B6.CAST line.

Mice were maintained in a noise-controlled environment on a 12 hr light/dark cycle with light onset at 6:00 a.m.

In some experiments, the cholinergic agonist nicotine was applied systemically by addition to drinking water. In accord with previous studies (Kirch et al., 1992; Gaddnas et al., 2000), nicotine (Sigma, St. Louis, Missouri, USA) at 200 µg/ml in 2% sucrose was added to drinking water two weeks before noise exposure. Control mice were given water containing 2% sucrose only. The drug solution was maintained in colored bottles, and changed once every three days. The body weight and general health of all mice were monitored daily. To block nAChR receptors in some animals, mecamylamine (Sigma) was applied by i.p. injection at 1 or 2 mg/kg body weight, following protocols from previous studies (Rabenstein et al., 2006; Philips et al., 2007). To test for interactions between endogenous cholinergic- and steroid-based feedback systems, glucocorticoid agonists and antagonists were applied in some mice. Based on previous studies of glucocorticoid effects on NIHL (Tahera et al., 2006a; 2006b; Canlon et al., 2007), glucocorticoid synthesis inhibitor metyrapone (2-Methyl-1,2-di-3-pyridyl-1-propanone, 50 µg/ul; Sigma) was dissolved in distilled water. Glucocorticoid agonist methylprednisolone (300 mg/ml; Sigma) and glucocorticoid receptor antagonist RU486 (25 µg/ul; Sigma) were dissolved in vegetable oil. These drugs were injected i.p. six hours before noise exposure. The dosage for metyrapone was 5 mg/mouse, RU486 2.5 mg/mouse, and methylprednisolone 60 mg/kg. Control mice were injected with 200 µl vegetable oil six hours before noise exposure. All procedures were approved by the Animal Studies Committee at Washington University in St. Louis.

2.2. Noise exposure and auditory brainstem response (ABR) tests

Noise exposures were performed in a foam-lined, double-walled soundproof room (Industrial Acoustics). The noise exposure apparatus consisted of a 21×21×11 cm wire cage mounted on a pedestal inserted into turntable. The cage was rotated at 1 revolution/80 s. A Motorola KSN1020A piezo ceramic speaker (four total) was attached to each side of a metal frame surrounding the cage. Opposing speakers were driven by independent channels of a Crown D150A power amplifier. Noise was generated by two General Radio 1310 generators and filtered to 4.0-45.0 kHz by Krohn-Hite 3550 filters. The overall noise level was measured at the center of the cage using a B&K 4135 ¼ inch microphone in a combination with a B&K 2231 sound level meter set to broadband (0.2 Hz-70 kHz). Mice were exposed in pairs at 110 dB SPL for 30 min. Exposures were timed to begin at approximately 10 a.m.

ABR testing was performed prior to treatment, then 24 hours and 2-3 weeks after the noise exposure to estimate CTS and PTS, respectively. Thresholds were obtained as described previously (Ohlemiller et al., 2000; Bao et al., 2005). Prior to testing, mice were anesthetized using a combination of 80 mg/kg ketamine and 15 mg/kg xylazine (i.p.). Otoscopic examination was performed to ensure that tympanic membranes were normal. Core temperature was maintained at 37.5±1.0 °C using a thermostatically-controlled heating pad in conjunction with a rectal probe (Yellow Springs Instruments Model 73A). Platinum needle electrodes (Grass) were inserted subcutaneously just behind the right ear, at the vertex, and in the back (ground). Electrodes were led to a Grass P15 differential amplifier (100-10,000Hz, ×100), then to a custom amplifier providing another ×1,000 gain, and digitized at 30 kHz using a Cambridge Electronic Design Micro1401 in conjunction with SIGNAL™ and custom signal averaging software operating on a 120 MHz Pentium PC. Sinewave stimuli generated by a HP 3445 oscillator were shaped by a custom electronic switch to 5 ms total duration, including 1 ms rise/fall times. The stimulus was amplified by a Crown D150A amplifier and output to a KSN1020A piezo ceramic speaker. Toneburst stimuli at each frequency and level were presented 1,000 times at 20/sec. The minimum sound pressure level required for visual detection of wave I was determined at each

frequency using a 5 dB minimum step size. To calibrate sound stimuli, a B&K 4135 ¼ inch microphone was placed where the ear would normally be located.

2.3. Measurement of Fecal Corticosterone Level

To track systemic changes in endogenous steroid hormones, corticosterone was measured in feces of some animals. Each mouse was placed in a cage covered with filter paper to absorb the urine at 9 am, and the amount of feces recovered during one hour period was weighed. Each fecal sample was homogenized with 1 ml of 90% methanol for 30 min on a vortex. After centrifugation at 13K rpm for 10 min, each sample was divided into an aliquot of 200 µl, dried at 45 °C in a vacuum, and stored at -80 °C. The amount of fecal corticosterone was determined using the Correlate-EIA corticosterone enzyme immunoassay kit (#900-097, Assay Designs, Inc.; Ann Arbor, Michigan, USA).

2.4. Quantitative RT-PCR

Some cochleas were analyzed for changes in the level of mRNAs encoding glucocorticoid and mineralocorticoid receptors. Total RNA from two cochleae of each animal was extracted using RNAqueous (Ambion). To avoid any DNA contamination during RNA extraction, at the final step of extraction, one microliter of DNase I was added to 49 µl elution buffer to extract total RNA from an RNA extraction column. The solution was incubated at 37 °C for 15 minutes and heated to 100 °C for five minutes to kill the DNase I. Then the RNA was quantified with RiboGreen RNA quantization reagent (Molecular Probes, Eugene, OR). Prior to reverse-transcription to generate cDNA, the quality of RNA was determined by checking ribosomal RNA integrity on a 3% denatured agarose gel. Ten microliters of total 50 µl RNA were reverse-transcribed in total 20 µl using random hexamers and Superscript II reverse transcriptase (Invitrogen, CA). Standard curves for GAPDH, glucocorticoid receptor (GR), and mineralocorticoid receptor (MR) were made using their respective cDNA plasmids at five dilutions. Primers used for real-time RT-PCR of these mouse genes were:

GAPDH: 5-CCTGGCCAAGGTCATCCATGACAAC-3 and 5-TGTCATACCAGGAAATGAGCTTGAC-3;

GR: 5-GGGGACCTGGACGCACGCGAGT-3 and 5-CTGCTGGGGACTTCGTCTCT-3;

MR: 5-AGAGCCGTGGAAGGACAACA-3 and 5-CTCAAGGCAAACGATGATAGAC-3;

PCR protocols for the LightCycler System were optimized with the LightCycler FastStart DNA Master^{PLUS} SYBR Green I kit (Roche). Two microliters of each 20 µl cDNA were added in a 20 µl PCR reaction mixture containing 1 × PCR buffer, 0.5 µM each primer, and Master Mix from the kit. The number of PCR cycles when the fluorescence intensity exceeds a predetermined threshold was measured automatically during PCR. Quantification of the initial amount of template molecules was achieved by calculating this number of PCR cycles with the number from the standard samples. The difference in the initial amount of total RNA among the samples was normalized in every assay using the house keeping gene, GAPDH. Besides using melting curve analysis in the program of the LightCycler System to ensure the right PCR product, we also examined each PCR product on a 3% agarose gel after each experiment.

2.5. Quantification of SGNs and Hair Cells

Mice were overdosed with ketamine (100 mg/ml, 0.1 ml/mg body weight) and perfused transcardially with cold 2% paraformaldehyde and 2% glutaraldehyde in PBS. Each cochlea was rapidly isolated, immersed in the same fixative, and the stapes immediately removed.

Complete infiltration of the cochlea by fixative was ensured by making a small hole at the apex of the cochlear capsule and gently circulating the fixative over the cochlea using a transfer pipet. After decalcification overnight in sodium EDTA, cochleae were post-fixed in buffered 1% osmium tetroxide, dehydrated in an ascending acetone series, and embedded in Epon. Cochleae were sectioned perpendicular to the modiolar axis at a thickness of 50 μm . A Nikon microscope was equipped with a motorized stage controlled by the Stereo Investigator software for precise, well-defined movements along the x, y, and z axes. High-resolution images and a thin focal plane were obtained using a 60 $\times$ oil immersion objective lens from Nikon with a numerical aperture of 1.40. The optical fractionator method was used to sample the SGN number in a defined fraction of the spiral ganglion by the optical dissector. A clear nucleolus, a large nucleus, and a clearly defined, oval body were used to distinguish SGNs from Schwann cells. The specific feature chosen for counting was the nucleolus of each SGN.

For evaluation of IHC and OHC loss the entire length of the basilar membrane was assessed using Stereo Investigator software. Hair cell survival throughout the cochlea was examined using a 60 $\times$ oil immersion objective lens, setting the plane of focus at the level of the pillar heads. The criteria for a hair cell to be considered missing were the absence of a hair bundle and replacement of the cuticular plate by a phalangeal scar. The percent loss of IHCs and OHCs was then calculated for each of five segments representing 20% increments of organ of Corti length.

2.6. Statistical analysis

ABR and cell count data were subjected to one- or two-way ANOVA with post hoc Bonferroni multiple comparisons testing. No interactions between frequency or cochlear location and group were observed in any of the ANOVAs, thus only effects by group or cochlear place were reported. A t-test assuming unequal variances was used to analyze changes in corticosterone levels under various conditions. Significant differences were assumed to exist when the probability of a type I error was ≤ 0.05 .

3. Results

3.1. Protection against NIHL in 9 month old but not 2 month old $\beta 2^{-/-}$ mice

ABR recording showed no difference in hearing thresholds between control and $\beta 2^{-/-}$ mice at 9 months [Fig. 1A; $F(1,10) = 2.88$, $p = 0.12$]. Surprisingly, protection against NIHL was observed in the $\beta 2^{-/-}$ mice compared to controls, both 24 hours after the noise exposure [Fig. 1B; $F(1, 10) = 43.04$, $p < 0.001$], and three weeks after the noise exposure [Fig. 1C; $F(1, 10) = 35.84$, $p < 0.001$]. However, when the same noise exposure was repeated in 2 month old animals, PTS was similar in control and $\beta 2^{-/-}$ mice [Fig. 2; $F(1, 18) = 0.73$, $p = 0.404$]. The protection found in older animals appeared counter to any protective nature of the LOC cholinergic pathway acting via the $\beta 2$ receptor, although it was unclear why this should depend on age.

3.2. Testing the involvement of LOC cholinergic signaling

Based on the above observations, we tested whether pharmacologic activation of cholinergic synaptic transmission could increase NIHL, and whether pharmacologic blockade of cholinergic synaptic transmission could protect against NIHL in 9 month old B6.CAST wild type mice. First, the cholinergic agonist nicotine was administered via drinking water before noise exposure. Three weeks after exposure no significant differences in PTS were found between control and nicotine-treated mice [Fig. 3A; $F(1, 9) = 3.10$, $p = 0.112$]. Treatment with nAChR antagonist mecamylamine at 1 mg/kg also yielded no significant effect on ABR threshold shifts three weeks after [Fig. 3B; $F(1, 5) = 0.82$, $p = 0.407$]. However, when we

repeated the same test with mecamylamine at 2 mg/kg, significant protection against NIHL was observed [Fig. 3C; $F(1, 16) = 9.16$, $p = 0.008$]. Thus, pharmacologic modulations of nAChR receptors failed to explain the protection against NIHL in old $\beta 2^{-/-}$ mice.

3.3. Possible role of glucocorticoid signaling

It has been shown that both elevated endogenous glucocorticoids and exogenously applied glucocorticoid agonists can protect against NIHL (Canlon et al., 2007; Takahashi et al., 1996; Tahera et al., 2006a, 2006b; Wang and Liberman, 2002). Disruption of a single gene throughout the body could exert its effects at the cellular, tissue, or systemic level. It is possible, for example, that protection against NIHL in older $\beta 2^{-/-}$ mice reflects age-specific systemic effects due to the lack of nAChR $\beta 2$ subunits in the brain. Noting a previous discovery of age-related increases of corticosterone in the $\beta 2^{-/-}$ mice (see Figure 6 in Zoli et al., 1999), we measured fecal corticosterone levels 24 hours before, then 2 hours and 24 hours after noise exposure among 9 month old control mice, mice receiving 2 mg/kg of mecamylamine, and untreated $\beta 2^{-/-}$ mice (Fig. 4). Prior to noise exposure corticosterone levels were significantly higher in the knock-out mice than in controls (t-test, $df = 15$, $p < 0.05$). Two hours after noise exposure, corticosterone levels appeared similarly increased in all three groups. One day after noise, corticosterone levels similarly decreased to near baseline levels in all three groups. We subsequently measured GR and MR expression levels in the cochleae by a standard quantitative RT-PCR. No significant difference was observed for these two receptors among the same three groups (Fig. 5).

3.4. Protection against NIHL by high corticosterone levels

Our corticosterone measurements raised the possibility that age-related increases in corticosterone contribute to protection from NIHL in older $\beta 2^{-/-}$ mice. We first determined whether glucocorticoid protective cascades are preserved in the $\beta 2^{-/-}$ mice by applying the glucocorticoid agonist methylprednisolone in wild type control and $\beta 2^{-/-}$ mice at two months of age. Similar to previous reports (Canlon et al., 2007; Tahera et al., 2006a; 2006b), injection of methylprednisolone prior to noise exposure conferred protection in B6.CAST controls [Fig. 6A; $F(1, 10) = 17.69$, $p < 0.002$] and in $\beta 2^{-/-}$ mice [Fig. 6B; $F(1, 6) = 6.65$, $p = 0.042$]. We further reasoned that if the protection against NIHL in older $\beta 2^{-/-}$ mice were due to high levels of corticosterone, then inhibition of glucocorticoid signaling pathways should diminish this protection. Accordingly, 9 month old $\beta 2^{-/-}$ mice were injected with the glucocorticoid synthesis inhibitor metyrapone and glucocorticoid receptor antagonist RU486 six hours before noise exposure. Three weeks post-exposure, there were significant differences among B6.CAST wild type mice, untreated $\beta 2^{-/-}$ mice, and $\beta 2^{-/-}$ mice receiving the drug treatment [Fig. 6C; $F(2, 15) = 19.53$, $p < 0.001$]. PTS in $\beta 2^{-/-}$ mice receiving metyrapone and RU486 was significantly increased compared to untreated $\beta 2^{-/-}$ mice, PTS ($p = 0.015$), yet remained significantly reduced compared to wild types ($p = 0.004$). As expected there remained a significant difference between wild type mice and untreated $\beta 2^{-/-}$ mice ($p < 0.001$). Thus, protection against NIHL in older $\beta 2^{-/-}$ mice appeared consistent with a protective effect of chronically elevated corticosterone.

The primary targets of noise are hair cells and their afferent neurons (Ohlemiller, 2008). To determine the anatomic correlates of protection against NIHL in old $\beta 2^{-/-}$ mice, we therefore quantified hair cell and neuronal loss among the same three groups described in the preceding paragraph (Fig. 7). A significant difference by group was detected for noise-related IHC loss [Fig. 7A; $F(2, 10) = 35.67$, $p < 0.001$], such that loss of IHCs was less dramatic in older $\beta 2^{-/-}$ mice than in wild type mice at the same age ($p < .001$). Blocking glucocorticoid pathways before noise exposure largely removed this protection ($p < .001$). Regarding OHCs, old $\beta 2^{-/-}$ mice again showed significant preservation versus wild types [Fig. 7B; $F(2, 10) = 5.20$, $p = .028$] while blocking glucocorticoid pathways showed little

effect. Surprisingly, SGN counts revealed the opposite trend. By 9 months of age, $\beta 2^{-/-}$ mice with or without drug treatment retained significantly fewer neurons compared to wild [Fig. 7C; overall $F(2, 21) = 6.94$, $p = 0.005$; $p < 0.04$ between groups). Taken together, the data implicate a high level of corticosterone in older $\beta 2^{-/-}$ mice in protection against NIHL. The primary anatomic correlate of this protection appeared to be hair cell preservation. This preservation coincided with a seemingly anomalous finding of accelerated SGN loss in these mice.

4. Discussion

We show here that chronic impairment of $\beta 2$ -related cholinergic signaling confer protection against cochlear noise injury. This protection shows an age-dependence we cannot fully explain, but can be accounted for in part by assuming that removal of $\beta 2$ promotes chronic systemic elevation of glucocorticoids. Noise-exposed $\beta 2^{-/-}$ mice showed significantly less hair cell loss than did wild type mice, such that hair cell preservation could account threshold differences. By contrast, $\beta 2^{-/-}$ mice also showed significantly greater neuronal loss, the origins and effects of which we consider below.

4.1 Potential role of glucocorticoid signaling

Our data involving blockers for the glucocorticoid pathway (metyrapone and RU486) are consistent with a role of glucocorticoid pathways in the protection against NIHL. Glucocorticoid mimics such as methylprednisolone are used for clinical therapy of neural trauma such as spinal cord injury. They are also used to treat hearing loss in a variety of cochlear disorders such as autoimmune inner ear disease (Hughes and Burrows, 1993; McCabe, 1979; Nadol et al., 1996). Glucocorticoids are not presently clinically indicated in cases of acoustic trauma. However, protection against NIHL by restraint or elevation of body temperature (Paz et al., 2004; Wang and Libermann, 2002; Yoshida et al., 1999), is posited to involve glucocorticoid signaling (Canlon et al., 2007; Hirose et al., 2007; Takahashi et al., 1996; Takemura et al., 2004).

Linkage of noise resistance in old $\beta 2^{-/-}$ mice to elevation of glucocorticoids is consistent with a role for glucocorticoids in promoting resistance to acute stress (McEwen, 2008). However, this acute protection may be superimposed on a trend of progressive cellular injury inflicted by chronically elevated levels of corticosterone. (Landfield et al., 1978; Sapolsky, 1986). The latter phenomenon may explain present and previous observations of accelerated neuronal loss in the same $\beta 2^{-/-}$ mice (Bao et al., 2005; Zoli et al., 1999). Although the twin findings of hair cell preservation and accelerated neuronal loss in $\beta 2^{-/-}$ mice may seem incompatible, this need not be so. First, there is no contradiction between the hair cell and SGN results with respect to hearing thresholds. PTS is much more steeply dependent upon hair cell loss (particularly OHC loss) than on neuronal loss (Kujawa and Liberman, 2009). Thus, based on the data of Figure 7 we would expect $\beta 2^{-/-}$ mice to have better hearing than wild types. Second, we cannot say from Figure 7 that all the hair cell loss in $\beta 2^{-/-}$ mice is noise-related. Just as for neurons, part or all of this loss may have been caused by chronic glucocorticoid toxicity. A final anomaly of the data shown in Figure 7 is that the extent of IHC loss in 9 month old B6.CAST mice exceeds that in most descriptions of noise exposed C57BL/6J mice (e.g., Shen et al., 2007). The causes of this difference are unknown.

Glucocorticoids exert their effects via two receptors, the high-affinity mineralocorticoid receptor and the low-affinity glucocorticoid receptor (Reul and de Kloet, 1985). In the cochlea both receptors are expressed in SGNs, the spiral ligament, and the stria vascularis. The mineralocorticoid receptor is expressed in hair cells, while the glucocorticoid receptor is either weakly expressed or absent from the hair cells (Erichsen et al., 2001; Jin et al., 2009;

Shimazaki et al., 2002). Therefore, the glucocorticoid receptor could be involved in age-related SGN loss in $\beta 2^{-/-}$ mice. The protection of IHCs and OHCs in the same mice after noise exposure could be due to the expression of mineralocorticoid receptors, or to weak expression of the glucocorticoid receptor. Although downstream steps in the protective cascade are not well characterized, nuclear factor- κ B (NF κ B) has been implicated as a molecular target (Canlon et al., 2007). Further dissection of this pathway may provide new pharmacological agents that protect against acoustic trauma without detrimental side-effects.

Taken together, the present and previous results present a dilemma regarding the use of glucocorticoids to prevent sensory cell loss following noise and other stressors. Exposure duration appears pivotal for the outcome of glucocorticoids in neurons. An inverted-U shape function between glucocorticoid levels and neuronal function is well established in the hippocampus. Acute exposures to glucocorticoids improve hippocampal function, while chronic exposure leads to a decline of function. The hearing loss phenotype in $\beta 2^{-/-}$ mice shows similar features: elevated corticosterone protects against NIHL, while chronic exposure causes accelerated age-related SGN loss and potentially hair cell loss. Based on these observations, clinical application of glucocorticoids to treat sensory cell injury should focus on acute, not chronic, protocols.

4.2 Evidence against the involvement of LOC efferents

Both MOC and LOC pathways have been implicated in protection of the organ of Corti from noise injury (Darrow et al., 2007; Elgoyhen et al., 2009). MOC efferent fibers, which contain both GABAergic and cholinergic neurotransmitters, form synaptic terminals at the base of OHCs expressing the nAChR $\alpha 9/\alpha 10$ subunit (Eybalin, 1993). Data from transgenic mice that over-express the nAChR $\alpha 9$ subunit provide direct evidence that protection from acoustic injury by the MOC system is mediated by cholinergic synaptic transmission via the nAChR $\alpha 9$ complex on OHCs (Elgoyhen, 2009). To our knowledge, no direct evidence exists for a protective role of the LOC cholinergic synaptic transmission. Selective stereotaxic removal of LOC pathway reportedly increases vulnerability to acute acoustic injury (Darrow et al., 2007). The LOC pathway may reduce glutamate excitotoxicity in SGN terminals in the acute stage of acoustic injury (d'Aldin et al., 1995; Puel et al., 1998; Robertson, 1983), potentially via dopaminergic synaptic transmission (d'Aldin et al., 1995; Ruel et al., 2001). The nAChR $\beta 2^{-/-}$ mouse seems an appropriate animal model to test whether LOC cholinergic transmission via $\beta 2$ receptors can protect against NIHL. Our data from $\beta 2^{-/-}$ mice show that nAChR complexes containing the $\beta 2$ subunit are unlikely to contribute to any LOC-mediated protective mechanism. Because of the presence of other nAChR subunits in SGNs (Bao et al., 2005), we cannot completely exclude any contribution of cholinergic transmission to protection against NIHL by LOC neurons.

4.3 Conclusions

Our data argue that NIHL resistance seen in older $\beta 2^{-/-}$ mice is best explained by elevated systemic corticosterone levels. This discovery shows the importance of studying biological effects of a single gene at the molecular, cellular and systemic levels. In combination with previous findings (Bao et al., 2005; Zoli et al., 1999), our results emphasize the “double-edged sword” nature of glucocorticoids: acute neuronal protection and chronic neuronal loss. Because glucocorticoids have been widely used clinically for the treatment of neural trauma, autoimmune diseases, and inner ear diseases, it is critical for future studies to identify glucocorticoid signaling pathways that determine whether the dominant effect is neural protection or neural death. The $\beta 2^{-/-}$ mouse model can be an important tool for such studies.

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Fig. 1. Protection against NIHL in 9-month old $\beta 2^{-/-}$ mice. (A) ABR thresholds (Mean $\pm$ S.D.) for 9-month old B6.CAST (control) and $\beta 2^{-/-}$ mice. Each group included six animals with three males and three females. (B) and (C) ABR threshold shifts at 24 hours (CTS), and three weeks (PTS) after noise exposure from the same groups of animals. Significant protection against NIHL was found in the $\beta 2^{-/-}$ mice for both CTS and PTS.

Fig. 2.

No protection against NIHL in 2-month old $\beta 2^{-/-}$ mice on the B6.CAST background. ABR threshold shifts (Mean $\pm$ S.D.) for B6.CAST (n=12; control) and the $\beta 2^{-/-}$ mice (n=8) two weeks after noise exposure. Each group included an equal number of male and female mice.

Fig. 3. Effects on NIHL by pharmacological modulation of cholinergic transmission. (A) ABR threshold shifts (Mean $\pm$ S.D.) for 9-month old B6.CAST mice (females; n=7 without nicotine; n=4 with nicotine). (B) ABR threshold shifts for 9-month old B6.CAST mice (females; n=3 without mecamylamine, n=4 with mecamylamine at 1 mg/kg). (C) ABR threshold shifts for 9-month old B6.CAST mice without or with mecamylamine at 2 mg/kg (n=9/group with six males and three females in each group). Significant effects by treatment were found only for 2 mg/kg mecamylamine (C).

Fig. 4.

Comparison of fecal corticosterone levels before and after noise exposure among wild type control mice, controls treated with mecamylamine, and untreated $\beta 2^{-/-}$ mice. All animals were 9 months old. Control group and the drug treated wild types included six males and three females. $\beta 2^{-/-}$ mice included two males and six females. The corticosterone level (Mean $\pm$ S.D.) was determined 24 hours before the noise exposure, and 2 or 24 hours after the noise exposure.

Fig. 5. Comparison of GR and MR levels in the cochlea. The cochleae were isolated from the same three groups examined for Figure 4, and mRNAs from both cochleae of each animal were isolated and quantified as one data point. The ratio between the receptor and GAPDH (Mean $\pm$ S.D.) was used to eliminate possible difference in the amount of total mRNAs between animals.

Fig. 6.

Protection against NIHL by glucocorticoids. ABR threshold shifts (Mean $\pm$ S.D.) two weeks after the noise exposure for the B6.CAST mice with or without the treatment of methylprednisolone (n=6/group) at 2-month old (A), and for $\beta 2^{-/-}$ mice with or without the treatment by methylprednisolone (n=4/group) at 2 months of age (B). (C) ABR threshold shifts (Mean $\pm$ S.D.) for B6.CAST wild types and $\beta 2^{-/-}$ mice with or without treatment by metyrapone and RU486 at 9-month old (n=6/group) three weeks after noise exposure. Significant differences by group were found in all cases.

Fig. 7. Quantitative comparison of OHCs, IHCs, and SGNs by cochlear location among noise-exposed B6.CAST mice (n=6/group), $\beta 2^{-/-}$ mice (n=6/group), and $\beta 2^{-/-}$ mice (n=6/group) receiving methylprednisolone and RU486, as determined three weeks after exposure: (A) Percent missing IHCs. (B) Percent missing OHCs. (C) Total SGN number.