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A corticotropin-releasing factor system expressed in the cochlea modulates hearing sensitivity and protects against noise-induced hearing loss

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ABSTRACT

Noise-induced hearing loss is a highly prevalent occupational injury, yet little is known concerning the signals controlling normal cochlear sensitivity and susceptibility to noise-induced trauma. While the corticotropin-releasing factor (CRF) system is involved in activation of the classic hypothalamic–pituitary–adrenal axis, it is also involved in local physiological responses to stress in many tissues, and is expressed in the inner ear. We demonstrate that mice lacking the CRF receptor CRFR2 exhibit a significantly lower auditory threshold than wild type mice, but this gain of function comes at the price of increased susceptibility to acoustic trauma. We further demonstrate that glutamatergic transmission, purinergic signaling, and activation of Akt (PKB) pathways within the cochlea are misregulated, which may underlie the enhanced sensitivity and trauma susceptibility observed in CRFR2$^{-/-}$ mice. Our data suggest that CRFR2 constitutively modulates hearing sensitivity under normal conditions, and thereby provides protection against noise-induced hearing loss.

Introduction

Corticotropin-releasing factor (CRF) is a 41 amino acid peptide critically important to hypothalamic–pituitary–adrenal (HPA) axis function (Vale et al., 1981). While three receptors have been cloned (Grammatopoulos and Chrousos, 2002; Bale and Vale, 2004), only CRFR1 and CRFR2 are expressed in mammals. In addition to its role in HPA axis physiology, CRF and its receptors are expressed in the central nervous system (Sawchenko et al., 1993; Van Pett et al., 2000), suggesting functions for CRF beyond its classic hormonal role. CRF receptors are involved in sensitivity to stress and anxiety (Smith et al., 1998; Bale et al., 2000, 2002; Kishimoto et al., 2000; Vetter et al., 2002), cellular stress responses of the skin (Slominski et al., 1998; Slominski et al., 1999, 2000, 2001), mood disorders (Nemeroff, 1988, 1992; Nemeroff et al., 1988; Bale and Vale, 2003), energy balance and metabolism (Pelleymounter et al., 2000), hemodynamics (Brown et al., 1986), vascularization (Bale et al., 2003) and differentiation of neuronal dendrites within the hippocampus (Chen et al., 2004).

Within the cochlea, hair cells are responsible for encoding auditory stimuli, while various support cells are important for homeostatic regulation of the endolymph, a specialized fluid of the scala media bathing the hair cell apices. Endolymph is an unusual extracellular fluid by virtue of its high potassium content, and relatively low calcium level. The exact ionic composition of the endolymph can be altered by acoustic overexposure (Marcus et al., 1998; Jentsch, 2000; Housley et al., 2006) and by other endogenous signals, that alter cochlear sensitivity, thereby serving a protective role against release of potentially excitotoxic levels of glutamate from the hair cells.

Because cochlear hair cells are spontaneously active, and are constantly stimulated by the environment, the cochlea is under constant physical and metabolic stress. Damage and subsequent loss of cochlear hair cells results in permanent hearing loss in mammals. Although noise-induced hearing loss is the most prevalent occupational injury reported in the United States, knowledge concerning mechanisms underlying susceptibility to noise-induced hearing loss, and general protein expression changes that take place within the cochlea in response to noise exposure, is incomplete. We have previously demonstrated the existence of urocortin, a CRF-related peptide, and CRFR1 and CRFR2 in the murine cochlea (Vetter et al., 2002) in regions involved with homeostatic regulatory functions of the inner ear, as well as neural processing of hair cell responses. Given that other systems such as the skin use local CRF signaling to maintain homeostasis and protect against physical damage, we hypothesized that the CRF system may play a similar role in the inner ear, and may serve to protect against pathologies such as noise-induced hearing loss. We therefore investigated whether CRF is expressed in the cochlea, determined the role of CRFR2 activity in cochlear function, and attempted to define some of the possible mechanisms by which CRFR2 may exert its protective effects using a CRFR2 null mouse line.

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Materials and methods

Animals, housing, and noise assessment

CRFR2$^{-/-}$ mice have been described previously (Bale et al., 2000). Mice were raised under standard vivarium conditions (12 h light/dark cycles) in ventilated Thoren cage racks. Alternatively, some mice were raised in an IAC acoustic chamber on static shelving. One octave filter measurements of sound intensities over a spectrum of frequencies from 63 to 16,000 Hz were measured in the standard vivarium to assess ambient sound levels. Two measures were taken; one on the actual shelf the cages are suspended from, and one from the room as an open field measure approximately four feet from the floor. A Bruel and Kjaer SLM model 2231 audiometer was calibrated on site just prior to measurements using a Bruel and Kjaer pistonphone. The audiometer was equipped with a Filter set type 1625 with a ½ in. microphone, model 4125. Linear unfiltered measures were also obtained (see Supplemental data). As expected, the most intense sound was at the lowest frequency (63 Hz, 74 dB SPL). Intensities fell linearly with increasing frequency, and stabilized between 58 dB (at 500 Hz) and 50 dB (at 16,000 Hz). Examination of the mouse ABRs reveals that mice generally have very poor hearing at frequencies below 8–10 kHz, and therefore the most intense sounds do not reach threshold and are not of concern for data interpretation. However, at 16 kHz, mice exhibit very sensitive hearing, and thus one may assume that the mice held in the standard vivarium are sensitive to the 50 dB constant noise from the ventilated housing racks.

Immunolabeling

Cochleae were fixed in 4% paraformaldehyde for 1 h at room temperature and decalcified overnight at 4 °C in solution containing 8% EDTA buffered in 1× (final) PBS. Cochleae were then handled either as whole mount dissections, or embedded for cryosectioning. For whole mount processing, decalcified cochleae were stripped of the surrounding bone, and the lateral wall was trimmed down to the basilar membrane level using microdissection scissors. The turns were then separated into an apical and a basal turn (hook region was generally not recovered) and put into PBS in an eppendorf tube, in which the remaining immunolabeling and wash steps were carried out. For cryosectioning, decalcified cochleae were cryoprotected with 15% sucrose in 1× PBS for 2–4 h at 4 °C, and then transferred to a 30% sucrose solution made in 1× PBS overnight on a rotator. Cochleae were then moved to OCT embedding medium and slowly injected with OCT via round and oval windows using a 10 cc syringe and an 18 gauge needle. Cochleae were embedded in fresh OCT in peel away paraffin embedding molds and frozen in isopentane previously cooled with, and maintained on, dry ice. Cryostat sections were cut 10 μm thick and only mid-modiolar sections were used for examination. All tissue was incubated in blocking solution (5% normal goat serum and 0.5% Triton X-100 in 1× PBS) for 1 h at room temperature and incubated in primary antibody solution (1% normal goat serum, 0.1% Triton X-100) overnight at room temperature. Primary antibodies included: polyclonal rabbit anti-CRFR2 (1:200, Chemicon/Millipore, Billerica, MA), polyclonal rabbit and monoclonal mouse anti-calbindin (1:1000, Swant, Bellinzona, Switzerland), mouse anti-CtBP2 (1:200, Millipore), polyclonal rabbit anti-CRF (1:200, Chemicon/Millipore), monoclonal mouse anti-TuJ1 (1:1000, Neuromics, Edina, MN), rabbit monoclonal anti-total Akt1 (1:100), anti-Akt1 pThr 308 (1:50), and anti-Akt1 pSer473 (1:50) (all Akt antibodies from Cell Signaling Technology (Danvers, MA). Following primary incubation, tissue was washed three times in 1× PBS and then incubated in secondary antibody for 1 h at room temperature. Secondary antibodies used for fluorescent immunolabeling were either goat anti-mouse Alexa488, goat anti-rabbit Alexa594, or goat anti-rabbit Oregon Green (1:200, Invitrogen, Eugene, OR). For DAB immunostaining (calbindin), biotinylated goat anti-rabbit secondary (1:100 Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) was used, followed by incubation in standard ABC (Vector Labs, Burlingame, CA). For CRF immunolabeling, 15% (v/v) saturated picric acid was added to the 4% paraformaldehyde fixative. Images were gathered using a Leica TCS SP2 AOBS confocal microscope. Controls for each primary antibody consisted of a no primary step in which primary antibody was replaced with PBS. All other steps were as described above.

Auditory physiology

Briefly, mice were anesthetized with xylazine (20 mg kg^{-1} intraperitoneally, i.p.) and ketamine (100 mg kg^{-1} i.p.). For auditory brainstem responses, needle electrodes were inserted at vertex and pinna, with a ground near the tail. Stimuli were 5-ms tone pips delivered at 35 s^{-1}. At each test frequency, the sound-pressure level was varied in 5 dB steps. DPOAEs were measured with an ER-10C system. Two primary tones ($f_2/f_1 = 1.2$) were presented with f_2 level 10 dB < f_1. We computed a fast Fourier transform and extracted sound pressures at f_1, f_2 and $2f_1$-f_2 after spectral averaging from five serial waveform traces. We interpolated the iso-response contours for DPOAEs from the amplitude-versus-level functions: the criterion response was a $2f_1$-f_2 DPOAE of 0 dB SPL.

Assessment of susceptibility to noise-induced auditory threshold shifts

Awake and unrestrained mice were exposed to sounds free-field in a small reverberant chamber. Acoustic trauma consisted of a 2-h exposure to an 8–16 kHz octave band noise presented at 100 dB SPL. The exposure stimulus was generated by a custom white-noise source, filtered (Brickwall Filter with a 60 dB/octave slope), amplified (Crown power amplifier), and delivered (JBL compression driver) through an exponential horn fitted securely to a hole in the top of a reverberant box. Sound pressure levels previously measured at four positions within the holding cage (using a 0.25 in. Bruel and Kjaer condenser microphone) was found to vary by less than 0.5 dB across all positions.

Western blot

Whole cochlear lysates were prepared from at least four mice (eight cochleae). Mice were raised either in continuous noise (standard vivarium conditions, referred to as moderate noise environment), or in an acoustic chamber (referred to as quiet condition). Homogenates were prepared in buffer containing either T-Per plus protease inhibitor cocktail (Pierce, Rockford, IL) on ice or 2% SDS at 95–100 °C. Protein concentration was quantified using a Micro BCA kit (Pierce) and either 75 μg (or 150 μg for GluR4 analysis) of total protein (100 μg for phosphorylation assays) was loaded onto an 8% polyacrylamide gel (15% for connexin detection). Proteins were resolved using SDS-Page and transferred to a PVDF membrane. The membrane was blocked with 10% dry nonfat milk in TBST (50 mM Tris, 150 mM NaCl, pH 7.6, .05% Tween-20) for 1 h at room temperature. Primary antibodies were diluted into solution containing 1% non-fat milk in TBST and incubated with the membrane overnight at 4 °C. Primary antibodies included: polyclonal rabbit anti-GluR4 (1:500, Millipore), polyclonal rabbit anti-P2X2 (1:2000, Abcam, Cambridge, MA), polyclonal rabbit anti-P2Y4 (1:1000, Sigma, St. Louis, MO), monoclonal mouse anti-Connexin 26 or 30 (1:500, Zymed, Carlsbad, CA). Following primary antibody incubation, membranes were washed 3× for 5 min with TBST and incubated with HRP-labeled goat anti-rabbit or goat anti-mouse secondary (1:2000, Jackson ImmunoResearch Laboratories, Inc.) for 1 h at room temperature. Secondary antibodies were diluted into solution containing 1% non-fat milk powder in 0.5% TBST. Following secondary incubation, blots were washed 2× for 10 min in distilled water and then 1× for 5 min in TBST.