Loss of αCGRP Reduces Sound-Evoked Activity in the Cochlear Nerve

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Running head: Auditory Phenotype of αCGRP knockout mice

Abstract: 206 words
Introduction: 1036 words
Discussion: 1976 words
Text pages: 21
Figures: 5
Tables: 0

Key Words: olivocochlear, noise-induced hearing loss, hair cell.

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Abstract

α-Calcitonin Gene-Related Peptide (αCGRP) is one of several neurotransmitters immunolocalized in the unmyelinated component of the cochlear efferent innervation, the lateral olivocochlear (OC) system, which makes axo-dendritic synapses with cochlear sensory neurons. In rodents, CGRP is also immunocolocalized in the myelinated medial OC system, which contacts cochlear outer hair cells (OHCs). To understand the role(s) of this neuropeptide in the OC system, we characterized the auditory phenotype of αCGRP-null mice. Cochlear threshold sensitivity was normal in mutant mice, both via a neural metric, the auditory brainstem response (ABR), and an OHC based metric, distortion product otoacoustic emissions (DPOAEs). Medial OC function and resistance to acoustic injury were also unaffected by αCGRP deletion: the former assessed by measuring cochlear response suppression with electrical stimulation of the OC bundle, the latter by measuring temporary threshold shifts after exposure to high level sound. However, significant abnormality in αCGRP-null mice was seen in the growth of cochlear neural responses with increasing stimulus level. This observation, contrasted with normal amplitude-vs.-level functions for DPOAEs, is consistent with a selective, post-synaptic effect on cochlear neurons via αCGRP release from lateral OC terminals. This constitutes the most direct evidence to date for a functional role of the lateral OC system in the auditory periphery.
Introduction

CGRP is a 37 amino-acid neuropeptide (Fig. 1A), found throughout the central and peripheral nervous system in mammals. This peptide has been implicated in a range of biological effects including peripheral vasodilation, gastric acid secretion, the protective effects of preconditioning with mild ischemia, and control of acetylcholine receptor synthesis and function (Li and Peng, 2002; Mulle et al., 1988; Miles et al., 1989). There are two isoforms of the CGRP peptide. αCGRP is produced by the tissue-specific alternative splicing of the primary transcript of the calcitonin/αCGRP gene (Rosenfeld et al. 1983). A second gene, βCGRP, bears high sequence homologies with the α form (Amara et al. 1985). As shown in Figure 1A, the rat peptides are of identical length and differ at only two amino acids; the two isoforms in mouse differ at only three sites. These closely related peptides have overlapping, but not identical, expression patterns and share many of the same biological effects.

CGRP actions are mediated via a complex of proteins forming CGRP receptors (Luebke et al. 1996; McLatchie et al. 1998; Evans et al. 2000). Activation of this complex results in increased levels of intracellular cAMP, suggesting a G-protein-coupled receptor. The CGRP receptor is composed of a ligand binding protein – Calcitonin Receptor-Like Receptor (CRLR) – that has the stereotyped structure of a seven transmembrane receptor, but that is inactive when expressed alone (Fluhmann et al. 1997; Chang et al. 2001). CRLR requires two accessory proteins for function: 1) the Receptor Activity Modifying Protein 1 (RAMP1) that acts as a molecular chaperone and that is required for routing CRLR to the cell surface (McLatchie et al. 1998), and 2) the CGRP Receptor Component protein (RC) that is required for coupling the receptor to the cellular signal transduction pathway (Luebke et al. 1996; Evans et al. 2000; Prado et al. 2001).

CGRP is almost universally found in the efferent innervation of hair cell systems, including the lateral organs in fish and amphibian, as well as cochlea and vestibular organs in mammals, where it is typically co-localized with cholinergic markers (Fex and Altschuler 1986; Eybalin 1993; Maison et al. 2003). In the lateral line of the frog, bath application of either αCGRP or βCGRP increases spontaneous discharge in primary afferent neurons as it decreases neural response to mechanical stimulation of the lateral-line hair cells they innervate (Bailey and Sewell 2000a). Pharmacological studies suggest that both α and β effects in the lateral line are mediated via the CGRP1 receptor subtype (Bailey and Sewell 2000b).

CGRP function in the inner ear is unknown. In the mammalian cochlea, α/βCGRP immunoreactivity is present in two different fiber systems: 1) autonomic pathways innervating the cochlear vasculature (e.g. Vass et al. 1998); and 2) the unmyelinated component of the
cochlear efferent innervation, the lateral olivocochlear (LOC) system (Vetter et al. 1991; Eybalin 1993; Maison et al. 2003). The peripheral targets of these LOC fibers, which co-localize cholinergic markers, are the dendrites of cochlear afferent neurons, in the region near the afferent synapses with inner hair cells (Liberman 1980). There is cytochemical evidence that the LOC neurons are activated by sound (Adams 1995). However, the effects of the LOC system on afferent function, and therefore the effects of the CGRP it contains, are currently unknown, because it has proven difficult to electrically activate this unmyelinated fiber tract (Gifford and Guinan 1987).

In rodent cochleae, CGRP immunoreactivity is also prominent in the peripheral terminals of the myelinated medial olivocochlear (MOC) efferent system, which targets the OHCs and also co-expresses cholinergic markers (Fex and Altschuler 1986; Vetter et al. 1991; Eybalin 1993; Maison, 2003). OHCs display electromotility, in vitro, which, in vivo, amplifies cochlear vibration and increases cochlear sensitivity 100-fold (Holley 1996; Liberman et al. 2002). Activation of the MOC system suppresses cochlear responses by decreasing this OHC contribution to cochlear amplification (Guinan 1996). This classic OC-induced cochlear suppression, in turn, is mediated via the members of a novel class of nicotinic cholinergic receptors on OHCs, the α9/α10 receptor complex (Vetter et al. 1999). The role of the CGRP co-expressed in these MOC terminals is unknown.

To study the role of CGRP in the cochlear efferent innervation, both LOC and MOC subsystems, we have examined a transgenic mouse line with targeted deletion of the αCGRP gene. Previous study of this mouse line has failed to detect abnormalities in systemic cardiovascular function or in neuromuscular development (Lu et al. 1999). Using immunohistochemistry, in the present study we show that both isoforms of CGRP are absent in the cochlea of the knockouts. To differentially diagnose CGRP-related alterations in cochlear afferent neural function from alterations in OHC function, we measured both the auditory brainstem response (an electrical potential including the summed sound-evoked activity of cochlear afferent fibers) and the distortion product otoacoustic emissions (sounds generated and amplified by OHCs and transmitted back to the ear canal where they can be recorded). To assess a possible CGRP role in modulating cholinergic OC effects on OHCs, we measured the magnitude and time course of OC-mediated suppression of cochlear responses evoked by electrical activation of the OC bundle. Finally, given reports of a role for CGRP in cardiac protection from ischemia (Li and Peng 2002), and the large literature suggesting a protective role of the olivocochlear system in acoustic injury (Rajan 1988; Kujawa and Liberman 1997; Maison and Liberman 2000; Maison et al., 2002), we also assessed the differences in acoustic...
vulnerability of the knockout vs. wildtype animals by comparing threshold shifts following a brief, intense acoustic overexposure.

Materials and Methods

Experimental procedures: Transgenic lines were created, maintained as heterozygotes in the original C57BL/6 strain background, and genotyped in the Emeson laboratory (a description of the methods is available in Lu et al. 1999). Transgenic mice and their wildtype littermates were shipped to Boston where investigators were blinded to the genotype until all data acquisition was complete. For all experiments, including baseline testing of auditory sensitivity, magnitude of OC suppression as well as in acoustic overexposure experiments, mice were 16 wks old. All electrophysiological experiments were conducted in a temperature-controlled soundproof chamber maintained at ~90 °F. Although the C57BL/6 strain displays age-related cochlear degeneration, the functional changes and histopathological correlates are minimal at this age (Hequembourg and Liberman 2001). The care and use of the animals reported in this study were approved by the IACUC of the Massachusetts Eye & Ear Infirmary.

Cochlear Immunostaining: Following intracardial perfusion with 10% formalin, cochleae were decalcified in EDTA, and half-turns were dissected with fine forceps and immunostained as whole mounts, with a rabbit anti-rat CGRP (Peninsula, San Carlos, CA) as the primary. Tissue was incubated in primary antisera overnight, followed by biotinylated secondary antibodies, avidin-biotin-HPR complex (ABC kit, Vector), and DAB/H2O2, embedded in plastic and mounted on glass slides.

Auditory Brainstem Responses: ABRs were measured in each animal before and after acoustic overexposure. For the measurement, mice were anesthetized with xylazine (20 mg/kg i.p.) and ketamine (100 mg/kg i.p.). Needle electrodes were inserted at vertex and pinna, with a ground near the tail. ABR potentials were evoked with 5-ms tone pips (0.5-ms rise-fall with a \(\cos^2\) onset envelope, delivered at 35/sec). The response was amplified (10,000 X), filtered (100 Hz - 3 kHz), and averaged with an A-D board in a LabVIEW-driven data-acquisition system. Sound level was raised in 5 dB steps from 10 dB below threshold up to 80 dB SPL. At each sound level, 1024 responses were averaged (with stimulus polarity alternated), using an ‘artifact reject’ whereby response waveforms were discarded when peak-to-peak amplitude exceeded 15 \(\mu\)V. Upon visual inspection of stacked waveforms, ‘threshold’ was defined as the lowest SPL level at which any wave could be detected, usually corresponding to the level step just below that
at which the peak-peak response amplitude rose significantly above the noise floor (~0.25 µV). For amplitude vs. level functions, the wave I peak was identified by visual inspection at each sound level and the peak-to-peak amplitude computed.

**DPOAE Responses:** Animals were anesthetized as for ABR testing. DPOAEs at 2f₁-f₂ were recorded with a custom acoustic assembly consisting of two 1/4” condenser microphones to generate primary tones (f₁ and f₂ with f₂/f₁ = 1.2 and f₂ level 10 dB < f₁ level) and a Knowles miniature microphone (EK3103) to record ear-canal sound pressure. Stimuli were generated digitally (National Instruments, AO-6). Ear-canal sound pressure was amplified and digitally sampled at 20 µs (National Instruments, A-2000). FFTs were computed and averaged over 5 consecutive waveform traces, and 2f₁-f₂ DPOAE amplitude and surrounding noise floor were extracted, a procedure requiring ~4 seconds of data acquisition and processing time.

**Medial Olivocochlear Assay:** Animals were anesthetized as for ABR testing, and a posterior craniotomy and partial cerebellar aspiration were performed to expose the floor of the IVth ventricle. To stimulate the OC bundle, shocks (monophasic pulses of 150 µs duration presented at 200/sec) were applied through fine silver wires (0.4 mm spacing) placed along the midline, spanning the OC decussation. Shock threshold for facial twitches was determined, muscle paralysis induced with α-d-tubocurarine (1.25 mg/kg i.p.), and the animal connected to a respirator via a tracheal cannula. Shock levels were raised to 6 dB above twitch threshold. During the OC suppression assay, f₂ level was set to produce a DPOAE ~10-15 dB > noise floor. To measure OC effects, repeated measures of baseline DPOAE amplitude were first obtained (n=12), followed by a series of 12 interleaved periods in which DPOAE amplitudes were measured with (n=6) and without (n=6) simultaneous shocks to the OC.

**Acoustic overexposure:** Animals were exposed, awake and unrestrained, within cages suspended inside a small reverberant sound–exposure box (Liberman and Gao 1995). The exposure stimulus was generated by a custom -made white-noise source, filtered (Brickwall Filter with a 60 dB/octave slope), amplified (Crown power amp), and delivered (JBL compression driver) through an exponential horn fitted securely to a hole in the top of a reverberant box. Sound exposure levels were measured at four positions within each cage using a 1/4-in Bruel and Kjaer condenser microphone: sound pressure was found to vary by < 0.5 dB across these measurement positions. Sound pressure was calibrated daily by positioning the microphone at the approximate position of the animal’s head.
Results

As previously reported (Lu et al. 1999), mice homozygous for the αCGRP null mutation showed no obvious difference from wildtype littermates in growth rate, fertility or open-field behavior. In addition, their systemic cardiovascular function was indistinguishable from wildtype, as were a number of measures of acetylcholine receptor expression and distribution at the neuromuscular junction (Lu et al. 1999).

Cochlear immunostaining

Cochlear whole-mounts of both mutant and wildtype mice were immunostained with rabbit anti-rat CGRP antibodies. In the inner spiral bundle (ISB) of wildtype mice, beneath the inner hair cells, light-microscopy showed large numbers of CGRP immunoreactive boutons, usually in the form of varicosities of fine (<1 µm) immunostained axons (Fig. 1B). As previously described in mice, the average density of CGRP-immunopositive terminals (~4-5 µm²/µm of cochlear length) showed little variation across cochlear location (Maison et al. 2003). In the OHC area, immunopositive terminals were larger (~5 µm² on average) and commonly located near the base of the OHC, where they often appeared in clusters. Terminal distributions showed no radial gradient across the three OHC rows.

In mutant mice, no immunoreactivity was found within the inner hair cell region, the tunnel of Corti, or the OHC region (Fig. 1C). Given that only 3 amino acids differ between α and βCGRP in the mouse (Fig. 1A), the antibodies are unlikely to distinguish between them. Thus, the absence of immunostaining in the mutants suggests that 1) neither αCGRP nor βCGRP is present in the organ of Corti and 2) CGRP expression in wildtype cochlear terminals arises from the αCGRP gene.

Cochlear function

Assessment of baseline cochlear function was performed both by measurement of auditory brainstem responses (ABRs) and distortion product otoacoustic emissions (DPOAEs). The ABR measure represents synchronous neural activity generated at several levels of the ascending auditory pathways, including the auditory nerve. The DPOAEs are sounds created within the cochlea, amplified by the action of OHCs, propagated back through the middle ear, and finally to the ear canal where they can be measured with a microphone (Kemp 1986). Although the ear creates DPOAEs at a number of frequencies, the largest is that at the frequency 2f₁-f₂, where f₁ and f₂ are the values of the two primary frequencies presented to the ear (f₂>f₁).
For ABRs and DPOAEs, data were gathered in such a way as to allow both 1) a measure of the “threshold” of response (Figs. 2A and 3B), i.e. the lowest stimulus level required to produce a criterion response chosen to be just above the measurement noise floor, and 2) the growth of response magnitude with sound pressure level (Figs. 2B,C and 3C,D). For growth functions, data are shown at two test frequencies, however results were similar for all test frequencies measured (all frequencies tested can be read from Fig. 2A).

Wildtype and αCGRP-null mice exhibited similar ABR and DPOAE thresholds across the range of test frequencies. The small differences in the mean values seen in Figures 2A and 3B were not statistically significant. Similarly, DPOAE growth functions were unaffected by the lack of αCGRP (Fig. 2B-C). On the other hand, ABR suprathreshold responses of mutant mice were systematically lower in amplitude (Fig. 3C) from 5.66 to 32 kHz (Two-way ANOVA - 5.66 kHz: F(1,16)=8.7, p=0.01; 8 kHz: F(1,16)=15.4, p=0.01; 11.33 kHz: F(1,16)=43.7, p<0.001; 16 kHz F(1,16)=9.3, p=0.008; 22.65 kHz: F(1,16)=5.3, p=0.036; 32 kHz F(1,16)=18.6, p=0.001). These data reflect the peak-to-peak amplitude of wave 1 of the ABR response, which represents the summed sound-evoked activity of the cochlear afferent fibers (Møller and Jannetta 1985). Comparison of full ABR waveforms revealed amplitude reductions in all waves, without striking latency shifts (e.g. Figure 3A), consistent with the view that the effects of the CGRP deletion are produced in the cochlea and then relayed to higher auditory centers.

When the differences in mean amplitude between the groups are expressed as a percentage change in response (Δ amplitude), that percentage is roughly constant at 20 - 25% across sound level: data from two of the test frequencies are shown in Figure 3D. The same pattern was obtained at the other test frequencies, and the average magnitude of the amplitude reduction was also roughly constant across test frequency (Figure 3E).

The normality of DPOAE threshold and suprathreshold measures in the mutant animals indicates that cochlear mechanics and OHC function are not significantly altered by the absence of αCGRP. That observation, coupled with the systematic lowering of suprathreshold wave 1 amplitudes in the mutant mice, suggests that loss of αCGRP is altering cochlear processes involved in synaptic transmission between the inner hair cell and the primary cochlear afferents.

Medial olivocochlear function

Given the close relation between OHC function and DPOAE amplitude, the degree of DPOAE suppression evoked by electrical stimulation of the medial OC system can be a sensitive measure of the cholinergic actions of this efferent pathway in the inner ear. Because of potent effects of CGRP on cholinergic responses in other systems (Mulle et al. 1988; Miles et al. 1989)
and given the co-localization of the CGRP and cholinergic markers in medial OC terminals in the OHC area of the mouse (Maison et al., 2003), we investigated whether the lack of $\alpha$CGRP affects the magnitude or time course of OC-mediated cochlear suppression.

DPOAEs were measured while electrically stimulating the OC bundle at the floor of the IVth ventricle, where the OC axons run close to the surface of the brainstem (Warr et al. 1986). As illustrated in Figures 4A, our assay of OC-mediated cochlear suppression involved repeated measures of DPOAE magnitude without or with simultaneous electrical activation of the OC bundle with 200/sec shock trains. DPOAE magnitude is clearly reduced during the periods when shocks were presented. The “MOC effect” was defined (Panel A) as the difference (in dB) between 1) the average DPOAE amplitude for the 12 trials before the first shock epoch and 2) the average DPOAE amplitude for the 6 with-shocks trials (triangles in Figure 4 A,C). The MOC effect recorded from mutant animals was not statistically different from wildtypes (Figure 4B).

For both genotypes, the suppression is greatest for acoustic stimuli near 16 kHz and declines for cochlear frequencies apical and basal to that point. This pattern is seen in all mouse strains investigated (Vetter et al. 1999; Maison et al. 2002) and presumably reflects the analogous longitudinal gradient in the density of MOC terminals on OHCs along the cochlear spiral (Maison et al. 2003).

The magnitude of the MOC effect tends to decay with repeated epochs of electrical stimulation (Figure 4A). Similar types of MOC effect decay have been observed in other in vivo systems (Wiederhold and Kiang, 1970; Sridhar et al., 1995, 1997), and some portion of this phenomenon may be due to the receptor desensitization, as has been studied in vitro for cholinergic receptors, in general, and the $\alpha_9/\alpha_{10}$ cholinergic complexes expressed by OHCs, in particular (Elgoyhen et al. 2001). Given the role of CGRP in modulating cholinergic receptor desensitization in other systems, we looked for differences in this effect decay across genotypes.

As shown by the data in Figure 4C, the magnitude of the MOC effect decay seen in 3 animals of each genotype is fundamentally similar, although small differences could have been hidden by the scatter in the data and the relatively small sample size.

*Acoustic overexposure*

To evaluate whether the absence of $\alpha$CGRP affects vulnerability to acoustic injury, groups of transgenic and wildtype animals were exposed for 2 hrs to a noise band (8-16 kHz) at 88 dB SPL. After a 12-hrs recovery period, the degree of temporary noise-induced threshold shift was measured via recording of ABRs (Fig. 5A) and DPOAEs instead (Fig. 5D).

When measured 12h after exposure, temporary threshold shifts (TTSs) were maximal near 16 kHz for both genotypes (Fig. 5A,D); 1 wk later, mean thresholds had returned to normal (data not shown), demonstrating the reversibility of the damage from this exposure. Mean values of
TTS were not significantly different between mutant and wildtype mice over the whole range of test frequencies (Two-way ANOVA - ABR: F(1,16)=0.055, p=0.818; DPOAE F(1,16)=0.167, p=0.688). When viewed as amplitude-vs.-level functions, DPOAE responses were not different between genotypes (Fig. 5E and F) and ABR suprathreshold responses (Fig. 5B and C) were, in general, no more significantly shifted in mutant mice than what was observed prior to acoustic overexposure (Fig. 3). At one test frequency (16 kHz, Figure 5C), ABR amplitudes at near-threshold sound pressures are more suppressed in mutants re. wildtype than before exposure (Figure 3C). This was not the case at other test frequencies, as reflected in the fact that only at 16 kHz does the mean TTS in the CGRP knockouts appear larger than in the wildtypes. Two-way ANOVAs were used to compare post-exposure ABR amplitude-vs.-level functions: differences between the genotypes remained significant from 8 to 22 kHz (8 kHz: F(1,16)=4.759, p=0.044; 11.33 kHz: F(1,16)=5.958, p=0.027; 16 kHz F(1,16)=5.418, p=0.033; 22.65 kHz: F(1,16)=4.496, p=0.05). Two octave-spaced test frequencies are illustrated for both ABRs and DPOAEs; however results were similar for the other test frequencies measured.

Discussion

In our experiments, mutant mice lacking αCGRP (Fig. 1) displayed an auditory phenotype consisting of a selective reduction of sound-evoked activity in primary afferent fibers (Fig. 3), without changes in cochlear mechanics or OHC contributions to cochlear amplification (Fig. 2). Furthermore, no changes in the electrically evoked (Fig. 4) or sound-evoked (Fig. 5) effects of the cholinergic innervation of OHCs was observed.

There are several CGRPergic neuronal systems in the cochlea: the olivocochlear efferents targeting hair cells and neurons within the sensory epithelium, as well as two autonomic pathways innervating the cochlear vasculature. Theoretically, the loss of CGRP from any of these systems could contribute to an auditory phenotype. In the following sections, we review these systems and conclude that the lack of CGRP in the LOC innervation of cochlear afferent fibers is the most likely origin of the effects reported here.

CGRP and the innervation of the cochlear vasculature

There is immunohistochemical evidence for CGRP in two neuronal systems projecting to the cochlear vasculature: 1) the adrenergic innervation of the cochlea, originating in the superior cervical ganglion (Qiu et al. 2001) and 2) a second autonomic pathway originating in the trigeminal ganglion and co-localizing a number of vasoactive peptides including substance P (Vass et al. 1998). Both systems innervate the spiral modiolar artery of the cochlea (O’Connor
and Kooy 1988; Miller and Dengerink 1988; Carlisle et al. 1990) and arterioles in the modiolus and osseous spiral lamina, where axons of cochlear afferents are found (Spoendlin and Leichtensteiger 1966; Densert 1974; Spoendlin 1981; Vass et al. 1998). CGRPergic fibers are absent from the stria vascularis, the highly vascularized part of the cochlear epithelium, which is responsible for maintaining the high-K⁺ nature of endolymph and the endolymphatic potential which drives sensory transduction.

Infusion of CGRP into the cochlear arterial network induces a dose-dependent increase in cochlear circulation (Quirk et al. 1994), and enhances cochlear neural responses. Thus, the depression of cochlear neural output associated with CGRP deletion (Fig. 3) represents a change in the appropriate direction. However, alterations in cochlear blood flow due to loss of CGRP would be expected to affect both the neurally based cochlear responses, such as wave 1 of the ABR, as well as the OHC-based cochlear responses, such as the DPOAEs, or the cochlear microphonic (CM), the summed receptor potentials generated by OHCs. Indeed, such parallel alterations with blood flow manipulations have been consistently observed. For example, in the CGRP-infusion study cited above, both the cochlear compound action potential and the cochlear microphonic were enhanced (Laurikainen et al. 1997). Similarly, betahistine hydrochloride, which increases cochlear blood flow, also increases both cochlear neural and hair-cell based potentials (Laurikainen et al. 1993). Finally, in a study of pressure-induced reductions in cochlear blood flow, both DPOAEs and ABRs were reduced in parallel, and the DPOAEs were a more sensitive index of reductions in cochlear blood flow than wave 1 of the ABR (Mom et al. 1999).

Thus, the selective reductions in sound-evoked activity of the cochlear nerve, as seen in the ABR, associated with a lack of changes in DPOAEs, the mechanical non-linearity which is arguably the most sensitive index of OHC function, is hard to reconcile with a change in cochlear blood flow associated with the targeted deletion of αCGRP. Furthermore, although changes in sympathetic nervous activity have been detected in different αCGRP-null mice (Gangula et al. 2000; Oh-Hashi et al. 2001), the loss of αCGRP in these mutants was not associated with detectable abnormalities in heart rate or blood pressure, under basal or exercise-induced conditions (Lu et al. 1999).

**CGRP and the Olivocochlear Efferent System**

The cytochemistry of the OC system is complex: there are 1) two main subsystems (lateral vs. medial), 2) a variety of possible functional/chemical subgroupings within each subsystem, and 3) apparent species differences. Nevertheless, efferent innervation of the cochlear sensory epithelia in most vertebrates includes at least one population of cholinergic neurons which co-localize CGRP (Fex and Altschuler, 1986; Vetter et al., 1991; Eybalin, 1993; Maison et al.,
Co-localization of CGRP and acetylcholine (ACh) is seen in a variety of other cholinergic systems, including the neuromuscular junction and the adrenal medulla, where effects of CGRP on cholinergic transmission via muscle and neuronal nicotinic acetylcholine receptors (nAChRs), respectively, have been documented (Miles et al. 1989; Fernandez et al. 1999; Delarue et al. 2001; Nistri and di Angelantonio 2002). In these cholinergic systems, CGRP has been implicated in the modulation of nAChR desensitization (Nistri and di Angelantonio 2002) and expression of nAChRs and acetylcholinesterase at the synapse (Fernandez et al. 1999). These effects are induced via second-messengers, occurring downstream to CGRP action on its G-protein-coupled receptor complex (Juaneda et al. 2000). CGRP has also been implicated in suppression of ACh effects via direct interactions with the nAChRs themselves (Giniatullin et al. 1999).

In the rodent, there is a cholinergic / CGRPergic innervation to both inner and OHC areas (Vetter et al. 1991; Eybalin 1993). In the mouse, virtually all OC terminals in both areas immunostain for CGRP and a cholinergic marker (VAT) (Maison et al. 2003); the OHC innervation arises via the myelinated medial (M)OC system, whereas the cochlear afferent innervation in the inner hair cell area arises from the unmyelinated, LOC group (Maison et al. 2003).

**MOC system:** There is immunohistochemical evidence for expression of several types of cholinergic receptors by hair cells and afferent neurons, including members of muscarinic and nicotinic classes (Guth and Norris 1996; Elgoyhen et al. 1994, 2001; Ito and Dulon 2002; Khan et al. 2002). However, the only cochlear cholinergic receptors with well-delineated function, *in vivo*, are the α9/α10 complex expressed by hair cells. Targeted deletion of the α9 gene eliminates suppressive effects of activating the MOC system (Vetter et al. 1999), normally mediated by a reduction in the OHC contribution to cochlear mechanical amplification (see Guinan 1996). In the present study, the magnitude of this cholinergic effect of MOC activation was unchanged by CGRP deletion (Fig. 4B). This lack of phenotype suggests that αCGRP does not play an important role in the development or maintenance of nAChRs within OHCs. The DPOAE-based assay we used can detect changes in receptor expression of ~ 1.6 fold: transgenically driven α9 overexpression enhanced the DPOAE suppression evoked by MOC activation, using the same assay as reported here (Maison et al. 2002).

In the α9 overexpressor mouse, the enhanced MOC effects elicited via electric activation were coupled with increased resistance to acoustic injury, suggesting that it is cholinergic effects on OHCs mediated by the α9 receptor that underlie the well-studied protective effects of the OC system (Maison et al., 2002). Results from the present study are consistent with this view: despite the loss of CGRP, the magnitude of electrically evoked MOC effects on OHCs is
unchanged (Figure 4) and, correspondingly, the degree of resistance to acoustic injury is also unaffected by the loss of CGRP (Figure 5).

Desensitization of these α9/α10 receptor complexes has been described in vitro (Elgoyhen et al. 2001), and the exponential decay in MOC effect we observed in vivo (Fig. 4A,C) may include a component due to receptor desensitization (as well as components due to action-potential failure at MOC branch points and/or depletion of synaptic vesicles at the MOC terminal). Thus, present results also suggest that αCGRP also does not play a major role in regulating the rate of desensitization of these receptor complexes.

**LOC system:** The decrease in sound-evoked cochlear neural responses observed in this αCGRP knockout (Fig. 3) is best explained via the loss of CGRP from LOC terminals. The neural responses we measured (wave 1 of the ABR) are dominated by radial afferent fiber contacting inner hair cells; the OHC afferents represent only 5% of the auditory neurons and may not respond to moderate-level sound (Liberman and Brown 1986; Brown 1989). The peripheral actions of the LOC system in mammals are unknown, because the unmyelinated axons of this pathway are difficult to electrically activate. However, the location of their synaptic terminals, i.e. axo-dendritic to radial afferents in the inner hair cell area, is consistent with the notion that LOC transmitter release (e.g. CGRP) should affect neural output from the inner hair cells without changing OHC function or DPOAEs.

The most direct evidence for LOC peripheral effects comes from experiments in guinea pigs with selective lesion of the LOC system, by stereotaxic injection of neurotoxins into the superior olivary complex (LePrell et al. 2003). Although the number of successful lesions was small, some animals showed a similar phenotype to that reported here: i.e. reduction in suprathreshold amplitudes of the cochlear compound action potential. The present results suggest that this effect of LOC lesion may be due to the loss of CGRP.

Consistent with these gross-potential measures of cochlear ensemble neural activity, chronic cochlear de-efferentation (which removes both MOC and LOC components) caused a reduction in spontaneous discharge rates in single cochlear afferents in cat (Liberman 1990). Although maximum sound-evoked rates in single fibers were unchanged, relative to normal fibers of similar spontaneous rate, the population shift towards lower spontaneous rates should cause a decrease in ensemble sound-evoked activity (i.e. wave 1 of the ABR), since sound-evoked rates are significantly lower in low-spontaneous than high-spontaneous-rate fibers (Winter et al. 1990).

**Mechanisms of Action:** The effect of CGRP on hair cell afferents has been extensively studied in the lateral line of Xenopus laevis (Sewell, 1990; Sewell and Starr, 1991; Bailey and
Sewell 2000a,b), where the cholinergic / CGRPergic efferent fibers project directly to the hair cells. In this system, CGRP increases spontaneous discharge rates in afferent fibers, as it increases the frequency, but not the amplitude, of EPSPs. These effects are blocked by cobalt, suggesting a pre-synaptic effect of CGRP on the transmitter release rate at the hair cell afferent synapse (Sewell and Starr, 1990). This CGRP-induced increase in spontaneous rate is associated with a decrease in stimulus-evoked rate (Bailey and Sewell, 2000a), which is reminiscent of the mammalian phenomenon of excitatory masking, whereby increasing steady evoked neural activity (e.g. by addition of background noise) decreases sound-evoked discharge rates due presumably to vesicle depletion at the afferent synapse.

The mechanisms of CGRP action in the mammalian cochlea are likely to be different from those in the lateral line, as the primary mammalian targets of the CGRPergic LOC neurons are post-synaptic to the hair cell, rather than on the hair cell, as seen in the lateral line. Correspondingly, if loss of CGRP decreased spontaneous activity by pre-synaptic mechanisms similar to those postulated for the lateral line, one might expect an enhancement of sound-evoked discharge rates, by reversal of the arguments re. vesicle depletion described above. Furthermore, the changes in afferent spontaneous rate in the mammalian ear associated with removal of the OC system require > 12 hours to appear; i.e., they are not observed after acute section of the OC bundle (Liberman, unpublished). This suggests a mechanism involving changes in gene expression, for example, in the nature or number of the glutamate receptors mediating afferent transmission at the inner hair cell. Although there is no evidence that effects of cochlear de-efferentation are due exclusively to the loss of CGRP, the present results are well explained by hypothesizing a reduction of glutamate receptor expression in the afferent neurons of the αCGRP knockout mice, as such an effect would likely result in a reduction in afferent discharge of a constant percentage across input sound pressure levels, as was observed in the present study.

**Acknowledgements**

We thank Dr. W.F. Sewell for critically reading the manuscript. This work was supported by NIH grant RO1 DC-0188 and the Charles A. King Trust – Fleet National Bank, Edward Dane and Richard Lovell, Co-Trustees.
References


Figure 1: Amino acid sequences for α and β isoforms of CGRP in rat and mouse (A), and CGRP immunostaining in cochlear whole-mounts (B,C). Panel A: Alignments of αCGRP and βCGRP amino acid sequences of rat and mouse. Panel B: Light micrograph of the organ of Corti in a wildtype mouse immunostained for CGRP showing 1) the plexus of immunopositive terminals beneath inner hair cells in the inner spiral bundle (ISB; unfilled arrow), 2) immunostained terminals beneath almost every OHC (e.g. at white arrow) and 3) fascicles of efferent fibers crossing the tunnel of Corti (e.g. at black arrow). OHC rows are numbered. Panel C: Light micrograph of an identically processed cochlear whole mount from αCGRP -/- animal. The only labeled structures are red blood cells (due to reaction of endogenous peroxidases with the chromogen). Scale bar in Panel B applies also to Panel C.
Figure 2: αCGRP deletion does not affect distortion product otoacoustic emissions (DPOAEs). **Panel A:** Mean DPOAE “thresholds” (+/- SEM) for groups of 16-wk mutants and wildtype littermates. Numbers of animals in each group are shown in the key. "Threshold" functions are derived as iso-response contours, i.e. the primary-tone levels, at each primary-tone frequency, necessary to produce a 2f₁-f₂ DPOAE of 5 dB SPL. **Panel B and C:** Mean amplitude-vs.-level functions (+/- SEM) for the DPOAEs are shown for 2 primary frequencies (f₂ is indicated in each panel). Data are from the same animal groups illustrated in Panel A.
Figure 3: αCGRP deletion reduces auditory brainstem responses (ABRs) by a constant percentage at all suprathreshold sound levels. Data are from the same animals analyzed in Figure 2. **Panel A:** Comparison of mean ABR waveforms (16 kHz - 80 dB SPL) reveals amplitude reductions in all waves without striking latency shifts. **Panel B:** ABR “thresholds” are determined by visual inspection of waveforms obtained at 5 dB increments in sound pressure level. Group means (± SEM) are plotted, and numbers of animals in each group are indicated in the key. **Panel C:** Mean ABR amplitude-vs.-level functions for mutants and wildtypes are shown for two test frequencies (16 and 32 kHz), as indicated. Wave 1 of the ABR is defined as the earliest negative-positive deflection in the response waveform and corresponds to the summed activity of cochlear afferents. **Panel D:** Data from panel B are replotted to show the percent change in response amplitude, at each sound pressure level, due to the CGRP deletion. Only suprathreshold values are shown. **Panel E:** Percentage decrease in response amplitude due to CGRP deletion is constant across ABR test frequency. Each point in this panel represents the mean value of a Δ amplitude-vs.-level function such as shown in panel C.
Figure 4: αCGRP deletion does not affect MOC-evoked cochlear suppression. **Panel A:** Measurement of MOC effect magnitude for one primary frequency ($f_2 = 22.62$ kHz) in a wildtype mouse. To quantify MOC-evoked effects, repeated measures of baseline DPOAE amplitudes are obtained ($n=12$), followed by a series of 12 interleaved periods in which DPOAE amplitudes are measured with ($n=6$) or without ($n=6$) simultaneous shocks to the OC bundle. MOC effect magnitude is the difference (in dB) between the mean DPOAE for the 6 with-shocks trials and the mean for the 12 baseline measures. Noise floor measures the average spectral level for the frequency points surrounding $2f_1-f_2$. **Panel B:** Mean MOC effect magnitudes (+/- SEM) are compared between wildtype and αCGRP -/- animals for each of 7 $f_2$ frequencies tested. Technique for deriving MOC effect are illustrated in Panel A. **Panel C:** To compare the MOC effect decay between wildtype and knockout mice, DPOAE amplitudes for each individual were normalized and averaged within groups. Numbers of animals in each group are indicated in the key.
Figure 5: αCGRP deletion does not affect vulnerability to acoustic injury. **Panel A & D:** Mean temporary threshold shifts (+/- SEM) for wildtype vs. CGRP -/- animals. Animals in both groups were exposed for 2 hrs to an 8-16 kHz noise band (gray box) at 88 dB SPL, and ABRs (Panel A) and DPOAEs (Panel D) were measured 12 hrs later. **Panels B and C:** Mean post-exposure amplitude-vs.-level functions for ABR are shown for 2 test frequencies, as indicated. **Panels E and F:** Mean post-exposure amplitude-vs.-level functions for DPOAEs are shown for 2 f2 frequencies, as indicated.