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Enhancement of the medial olivocochlear system prevents hidden hearing loss

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Title Page

Title: Enhancement of the medial olivocochlear system prevents hidden hearing loss

Abbreviated Title: Medial olivocochlear system and cochlear synaptopathy

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Abstract

Cochlear synaptopathy produced by exposure to noise levels which only cause transient auditory threshold elevations is a condition that affects many people and is believed to contribute to poor speech discrimination in noisy environments. These functional deficits in hearing, without changes in sensitivity, have been called hidden hearing loss (HHL). It has been proposed that activity of the medial olivocochlear (MOC) system can ameliorate acoustic trauma effects. Here we explore the role of the MOC system in HHL by comparing the performance of two different mouse models: an α9 nicotinic receptor subunit *knock-out* (*Chrna9* KO) which lacks cholinergic transmission between efferent neurons and hair cells, and a gain of function *knock-in* (*Chrna9L9'T* KI) carrying an α9 point mutation that leads to enhanced cholinergic activity. Animals of either sex were exposed to sound pressure levels that in wild-type (WT) produced transient cochlear threshold shifts and a decrease in neural response amplitudes, together with the loss of ribbon synapses, indicative of cochlear synaptopathy. Moreover, a reduction in the number of efferent contacts to OHCs was observed. In *Chrna9* KO ears, noise exposure produced permanent auditory threshold elevations together with cochlear synaptopathy. In contrast, the *Chrna9L9'T* KI was completely resistant to the same acoustic exposure protocol. These results show a positive correlation between the degree of HHL prevention and the level of cholinergic activity. Notably, enhancement of the MOC feedback promoted new afferent synapse formation, suggesting that it can trigger cellular and molecular mechanisms to protect and/or repair the inner ear sensory epithelium.

Significance Statement

Noise overexposure is a major cause of a variety of perceptual disabilities, including speech-in-noise difficulties, tinnitus and hyperacusis. Here we show that exposure to noise levels that do not cause permanent threshold elevations nor hair cell death, can produce a loss of cochlear nerve synapses to inner hair cells (IHCs) as well as degeneration of MOC terminals contacting the outer hair cells (OHCs). Enhancement of the MOC reflex can prevent both types of neuropathy, highlighting the potential use of drugs that increase $\alpha9\alpha10$ nicotinic cholinergic receptor activity as a pharmacotherapeutic strategy to avoid HHL.

Introduction

Noise-induced hearing loss (NIHL) is growing as one of the most prevalent types of non-congenital hearing loss. It has recently been shown that exposure to loud sounds, causing only transient cochlear threshold elevations, can produce a loss of synapses between IHCs and auditory nerve fibers (Kujawa and Liberman, 2009). This cochlear synaptopathy, also known as HHL (Schaette and McAlpine, 2011), occurs within hours post acoustic exposure and it is proposed to be a type of glutamate excitotoxicity (Liberman and Mulroy, 1982; Pujol et al., 1993; Pujol and Puel, 1999). Recent studies have demonstrated that auditory nerve fibers with high-threshold and low spontaneous rates (SR) are most vulnerable to noise damage compared to low-threshold high-SR fibers (Furman et al., 2013; Liberman and Liberman, 2015). These observations provide an explanation to the absence of auditory threshold elevations in cochlear synaptopathy models. Due to the wider dynamic range and threshold distribution, it was suggested that these low-SR fibers are critical for signal coding in noisy backgrounds (Costalupes, 1985; Young and Barta, 1986).

MOC efferent neurons form a negative feedback gain-control system that inhibits amplification of sounds by OHCs (Galambos, 1955; Wiederhold and Kiang, 1970; Guinan, 2011). Activation of the MOC pathway reduces cochlear sensitivity through the action of acetylcholine on $\alpha9\alpha10$ cholinergic nicotinic receptors (nAChRs) at the base of OHCs (Elgoyhen et al., 1994, 2001; Ballestero et al., 2011; Guinan, 2011). Several lines of evidence have demonstrated that the MOC system has an important role in the protection from NIHL: 1) stimulation of MOC fibers during sound overexposure produces a reduction of sensitivity loss (Reiter and Liberman, 1995); 2) chronic sectioning of the olivocochlear bundle renders the ear more vulnerable to permanent acoustic injury (Handrock and Zeisberg, 1982; Kujawa and Liberman, 1997; Maison et al., 2013) 3) the strength of the olivocochlear reflex is inversely correlated with the degree of NIHL

(Maison and Liberman, 2000) and 4) genetically modified mice where the magnitude and duration of efferent cholinergic effects are increased, exhibit higher tolerance to noise-induced trauma (Taranda et al., 2009). However, the precise role of the MOC reflex on HHL remains mostly undefined. Recent studies show enhanced cochlear synaptopathy after olivocochlear bundle lesion, which removes much of the MOC innervation to OHCs with almost no alteration in the lateral olivocochlear (LOC) neurons (Maison et al., 2013). A variety of data indicate that MOC neurons release not only acetylcholine but also gamma-aminobutyric acid and calcitonin-gene related peptide (Maison et al., 2003; Wedemeyer et al., 2013). Thus, cochlear de-efferentation produced by surgical lesion to the olivocochlear pathway removes all the different neurotransmitter/neuromodulator components and possibly some LOC neurons. Here we explored the effect of the strength of the cholinergic component of the MOC reflex on the cochlear synaptopathy that occurs in HHL by using genetically modified mice with different levels of $\alpha9\alpha10$ nAChR activity. In addition, we analyzed for the first time the effect of acoustic trauma in ears exposed at sound levels well below those that cause hair cell damage and permanent threshold shifts on efferent synaptic terminals contacting the OHCs. We used mice at the early onset of puberty, i.e. at three weeks of age, a period of enhanced sensitivity to NIHL (Henry, 1984; Ohlemiller et al., 2000; Kujawa and Liberman, 2006). Our results show that the degree of cochlear synaptopathy is negatively correlated to the level of $\alpha9\alpha10$ nAChR activity. Notably, enhancement of cholinergic activity not only prevented cochlear synaptopathy but also promoted new afferent synapse formation after acoustic trauma. Moreover, exposure to loud noise produced plastic changes leading to degeneration of MOC terminals synapsing on OHCs. We propose that both cochlear synaptopathy and the loss of MOC terminals contribute to HHL, compromising the performance of complex listening tasks such as understanding speech in a noisy environment. Therefore, our findings unequivocally show that strengthening of the MOC feedback by enhancement of $\alpha9\alpha10$ nAChRs activity can prevent the development of HHL

symptoms after noise exposure.

Materials and Methods

Animals. *Chrna9* KO and *Chrna9L9´T* KI mice have been previously described (Vetter et al., 1999; Taranda et al., 2009) and were backcrossed with congenic FVB.129P2-*Pde6bþ Tyrc-ch/AntJ* strain (https://www.jax.org/strain/004828, RRID:IMSR_JAX:004828) for seventeen generations (i.e., N-17). We used a similar male/female ratio in all the experimental groups in the different genotypes. All experimental protocols were performed in accordance with the American Veterinary Medical Association (AVMA) Guidelines for the Euthanasia of Animals (June 2013) as well as Instituto de Investigaciones en Ingeniería Genética y Biología Molecular (INGEBI) Institutional Animal Care and Use Committee (IACUC) guidelines, and best practice procedures.

Cochlear function tests. Inner ear physiology, including auditory brainstem responses (ABRs) and distortion-product otoacoustic emissions (DPOAEs), was performed in mice of either sex anesthetized with xylazine (10 mg/kg, i.p.) and ketamine (100 mg/kg, i.p.) and placed in a soundproof chamber maintained at 30°C. The first recording was performed at postnatal day 21 (P21), followed by noise exposure and the additional measurements 1, 7, 8 and 14 days post-exposure. Sound stimuli were delivered through a custom acoustic system with two dynamic earphones used as sound sources (CDMG15008–03A; CUI) and an electret condenser microphone (FG- 23329-PO7; Knowles) coupled to a probe tube to measure sound pressure near the eardrum (for details, see https://www.masseyeandear.org/research/otolaryngology/investigators/laboratories/eaton-peabody-laboratories/epl-engineering-resources/epl-acoustic-system). Digital stimulus generation and response processing were handled by digital I-O boards from National Instruments driven by custom software written in LabVIEW (generously given by Dr. M. Charles Liberman, Eaton-Peabody Laboratories, Massachusetts Eye & Ear Infirmary, Boston, MA.). For

ABRs, needle electrodes were placed into the skin at the dorsal midline close to the neural crest and pinna with a ground electrode near the tail. ABR potentials were evoked with 5 ms tone pips (0.5 ms rise-fall, with a $\cos^2$ envelope, at 40/s) delivered to the eardrum at log-spaced frequencies from 5.6 to 45.25 kHz. The response was amplified 10,000X with a 0.3–3 kHz passband. Sound level was raised in 5 dB steps from 20 to 80 dB sound pressure level (SPL). At each level, 1024 responses were averaged with stimulus polarity alternated. Threshold for ABR was defined as the lowest stimulus level at which a repeatable peak 1 could be identified in the response waveform. The ABR peak 1 amplitude was computed by off-line analysis of the peak to baseline amplitude of stored waveforms. The DPOAEs in response to two primary tones of frequency f1 and f2 were recorded at 2f1-f2, with f2/f1=1.2, and the f2 level 10 dB lower than the f1 level. Ear-canal sound pressure was amplified and digitally sampled at 4 μs intervals. DPOAE threshold was defined as the lowest f2 level in which the signal to noise floor ratio is >1.

Noise exposure. Animals were exposed under anesthesia to a 1-16 kHz noise at 100 dB SPL for 1 h in the same acoustic chamber used for cochlear function tests. Noise calibration to target SPL was performed immediately before each acoustic overexposure.

Cochlear processing and immunostaining. For the immunolabeling and quantification we divided animals for each genotype in two groups: 'Control' and 'seven days after acoustic trauma (AT + 7d)'. First, we tested the auditory function at P21 in both 'Control' and 'AT + 7d' groups. Immediately after, the control group was introduced in the acoustic chamber under anesthesia for 1 hour for a 'sham' condition while the 'AT + 7d' group was exposed to 100 dB SPL for 1 hour in the same chamber. Acoustic thresholds in both groups were measured again at one and seven days post-acoustic trauma and after the final auditory test (postnatal day 28), tissues were collected for immunostaining. Cochleae were perfused intralabyrinthly with 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS), post-fixed with 4% PFA overnight,

and decalcified in 0.12 M EDTA. Cochlear tissues were then microdissected and permeabilized by freeze/thawing in 30% sucrose (for CtBP2/GluA2 immunostaining) or directly blocked (for synaptophysin immunostaining). The microdissected pieces were blocked in 5% normal goat serum with 1% Triton X-100 in PBS for 1 h, followed by incubation in primary antibodies (diluted in blocking buffer) at 37°C for 16 h (for CtBP2/GluA2 immunostaining) or 4°C for 16 h (for synaptophysin immunostaining). The primary antibodies used in this study were: 1) mouse anti-synaptophysin antibody (Millipore, Billerica, MA; MAB5258, RRID:AB_2313839, 1:1000) to reveal the efferent terminals 2) anti-C-terminal binding protein 2 (mouse anti-CtBP2 IgG1; BD Biosciences, San Jose, CA; #612044, RRID:AB_399431, 1:200) to label the presynaptic ribbon and 3) anti-glutamate receptor 2 (mouse anti-GluA2 IgG2a; Millipore, Billerica, MA; MAB397, RRID:AB_11212990, 1:2000) to label the post-synaptic receptor plaques. Tissues were then incubated with the appropriate Alexa Fluor-conjugated fluorescent secondary antibodies (Invitrogen, Carlsbad, CA; 1:1000 in blocking buffer) for 2 h at room temperature (for CtBP2/GluA2 immunostaining) or processed with biotinylated secondary, ABC reagent (Vector Labs, Burlingame, CA, RRID:AB_2336827), and diaminobenzidine for standard light microscopy (for synaptophysin immunostaining). Finally, tissues were mounted on microscope slides in FluorSave mounting media (Millipore, Billerica, MA). For IHCs synaptic counts, confocal *z*-stacks (0.1 µm step size) of the apical, medial and basal regions from each cochlea were taken using a Leica TCS SPE microscope equipped with 63X (1.5X digital zoom) oil-immersion lens. Image stacks were imported to Fiji software (RRID:SCR_002285) (Schindelin et al., 2012), where IHCs were identified based on their CtBP2-stained nuclei. Each image usually contained 10 to 20 IHCs. For each stack, a custom Fiji plugin was developed to automatize the quantifications of synaptic ribbons, glutamate receptor patches and co-localized synaptic puncta. Briefly, each channel was analyzed separately and maximum projections were generated to quantify the number of CtBP2 or GluA2 puncta. Additionally, a composite between the two channels was produced to draw the different ROIs that correspond to each IHC taking the

CtBP2 stained nuclei as a reference. The maximum projections from the single channels were multiplied to generate a merged 32-bit image of the two channels. Then, they were converted to binary images after a custom thresholding procedure. Automatic counting of the number of particles on each ROI was performed.

Statistical analysis

Data were analyzed with R Statistical Software (RRID:SCR_001905). Shapiro-Wilks test was used for testing normal distribution of the residuals. We used non-parametric tests because our data were non-normally distributed. For one-group comparisons that involved repeated measures (ABRs thresholds, peak 1 amplitudes and latencies and DPOAEs thresholds) Friedman tests were used followed by a post-hoc test (Pohlert, 2014). For group comparisons that do not involve repeated measures (ABR comparisons of unexposed animals, IHCs synaptic counts), Kruskal-Wallis non-parametric ANOVA followed by a Dunn´s post-tests were used to determine statistical significance. For independent, two-group comparisons (MOC terminals per OHC), two-sample Kolmogorov-Smirnov test was applied. Statistical significance was set to $p<0.05$.

Results

Auditory function in mice with different degrees of efferent inhibition after acoustic trauma

In order to evaluate cochlear function we recorded ABR, the sound-evoked potentials generated by neuronal circuits in the ascending auditory pathways. We used genetically modified mice with different levels of $\alpha9\alpha10$ nAChR activity: *Chrna9* KO mice which lacks cholinergic transmission between MOC neurons and hair cells (Vetter et al., 1999), and a gain of function *Chrna9L9´T* KI mice carrying an $\alpha9$ point mutation that leads to enhanced responses to MOC activity (Taranda et al., 2009). We examined ABR responses at P21 in WT, *Chrna9* KO and *Chrna9L9´T* KI unexposed mice and found no difference in the median ABR thresholds between WT and *Chrna9* KO mice except at the highest frequency tested (Kruskal-Wallis test, df=2, p=0.04 at 45.25 kHz). However, median ABR thresholds were slightly elevated by 5 to 10 dB in *Chrna9L9´T* KI mice at all the frequencies, except for high frequency stimuli (Kruskal-Wallis test, df=2, $p<0.05$ at 5.6, 8, 11.33 and 16 kHz) (Figure 1a). ABRs peak 1 amplitudes at 80 dB, the summed activity of the cochlear nerve, were not modified in the three genotypes (Kruskal-Wallis test, df=2, $p>0.05$ at all the frequencies tested) (Figure 1b).

We then exposed WT, *Chrna9* KO and *Chrna9L9´T* KI mice to a 1-16 kHz noise at 100 dB SPL for 1 h and tested the animals one and seven days post acoustic trauma (Figure 2). As shown in Figure 2b, large auditory threshold shifts from 10 to 35 dB SPL were found one day after exposure in WT and *Chrna9* KO mice (Friedman test, df=2 $p<0.05$ at all the frequencies tested for both genotypes). One week later, auditory thresholds returned to pre-exposure values in WT (Friedman test, df=2, $p>0.05$ at all the frequencies tested), a result known to occur in mice with clear signs of HHL (Kujawa and Liberman, 2009). However, in *Chrna9* KO ears, which lack a functional MOC system feedback, auditory thresholds did not recover (Friedman test, df=2, $p<0.05$ at all the frequencies tested), indicating a persistence of damage (Figure 2b, middle

239 panel). In contrast, the *Chrna9L9'T* KI gain-of-function mouse model with an enhanced $\alpha9\alpha10$
240 nAChRs activity was completely resistant to the same acoustic exposure protocol (Friedman
241 test, df=2, p>0.05 at all the frequencies tested) (Figure 2b, right panel).

242 One signature of cochlear synaptopathy observed in HHL in mice is a reduction of ABR peak 1
243 amplitudes without permanent changes in ABR thresholds (Kujawa and Liberman, 2009). This
244 peak represents the summed sound-evoked spike activity at the first synapse between IHCs
245 and afferent nerve fibers (Buchwald and Huang, 1975; Antoli-Candela and Kiang, 1978). As
246 shown in Figure 2a and c, a large reduction in amplitudes were observed at 11.33, 16 and 22.65
247 kHz in WT mice one day after acoustic trauma (Friedman test, df=2, p=0.048 at 11.33 kHz;
248 p=0.026 at 16 kHz and p=0.018 at 22.65 kHz), which did not completely recover after seven
249 days (Friedman test, df=2, p=0.048 at 16 kHz and p=0.003 at 22.65 kHz). Similarly, in *Chrna9*
250 KO mice, ABR peak 1 amplitudes were reduced one day after trauma at 5.6, 16, 22.65 and 32
251 kHz (Friedman test, df=2, p=0.048 at 5.6 kHz; p=0.007 at 16 kHz; p=0.01 at 22.65 kHz and
252 p=0.026 at 32 kHz). Seven days after acoustic overexposure ABR peak 1 amplitudes did not
253 completely recover, however, it was not significantly different (Friedman test, df=2, p>0.05 at all
254 the frequencies tested) (Figure 2a and c). Interestingly, ABR peak 1 amplitudes were not
255 modified in the *Chrna9L9'T* KI mice after acoustic trauma (Friedman test, df=2, p>0.05 at all the
256 frequencies tested), suggesting that enhanced $\alpha9\alpha10$ nAChRs activity might prevent the loss of
257 afferent synapses. The ABR peak 1 latencies were not altered after acoustic trauma in any of
258 the evaluated genotypes (Friedman test, df=2 p<0.05 at all the frequencies tested for all the
259 genotypes) (Figure 2d), indicating that variation in the MOC strength does not affect the
260 conduction velocity of cochlear nerve fibers.
261
262 **Noise-induced cochlear synaptopathy in mice with different levels of MOC feedback**

263 Cochleae were harvested and fixed for histological analysis at P28 in all the different groups.
264 Hair cell count showed no loss of IHCs or OHCs in the different genotypes in controls and
265 exposed groups (data not shown). To analyze the IHCs-auditory nerve fibers synapses, organ
266 of Corti whole-mounts were immunostained with antibodies against CtBP2-Ribeye, a critical
267 protein present at the presynaptic ribbon (Khimich et al., 2005) and GluA2 AMPA-type
268 glutamate receptors, which are expressed at the postsynaptic afferent terminal (Matsubara et al.,
269 1996; Liberman et al., 2011; Maison et al., 2013). IHC-afferent synapses were identified by
270 colocalization of a pair of CtBP2/GluA2 puncta at the base of the IHC (Liberman et al., 2011). In
271 each whole mount organ of Corti, counting was done at three different cochlear locations: apical,
272 medial and basal. The number of pre, post or colocalized synaptic markers was averaged from
273 10 to 20 IHCs per each imaged cochlear section (4 to 14 animals per genotype) to generate the
274 synaptic density per IHC.

275 First, we compared the number of pre, post and colocalized synaptic markers per IHC in
276 unexposed WT, *Chrna9* KO and *Chrna9L9´T* KI mice (Figure 3). Synaptic counts were lower in
277 *Chrna9* KO compared to WT mice in the apical cochlear end (Kruskal-Wallis test, apical:
278 $\chi2=17.4$, df=1, $p<0.001$) (Figure 3, right panel). However, there was a slight, but significant
279 increase in the number of ribbon synapses in the medial cochlear region (Kruskal-Wallis test;
280 medial: $\chi2=6.1$, df=1, $p=0.01$) (Figure 3, right panel). It is important to note that synaptic counts
281 showed the same spatial distribution in *Chrna9* KO compared to WT mice: the number of ribbon
282 synapses peaks in the medial region where the cochlea is most sensitive to sound (Figure 3).
283 Nevertheless, in *Chrna9L9´T* KI mice synaptic counts were comparable to WT mice in low and
284 high frequency areas, but in the middle cochlear region a large reduction in synaptic counts of
285 around 15% was observed (Kruskal-Wallis test, $\chi2=19.3$, df=1, $p<0.0001$) (Figure 3, right panel).
286 Thus, the spatial distribution from base to apex seen in WT and *Chrna9* KO ears seems to be
287 lost in the *Chrna9L9´T* KI mice.

The impact of noise exposure on the number of auditory nerve synapses is summarized in Figure 4. Ribbon (CtBP2) counts in WT exposed ears were significantly reduced in the three cochlear regions (Figure 4a). Reduction was more pronounced in the low (apical-end) and high (basal-end) frequency regions with a 10% reduction compared to controls (Kruskal-Wallis test; apical: χ^2=23.6, df=1, p<0.0001 and basal: χ^2=8.9, df=1, p=0.002). Similarly, GluA2 postsynaptic receptors were diminished in the whole cochlea of traumatized WT mice with a bigger reduction at the apical and medial turns (Kruskal-Wallis test; apical: χ^2=42.2, df=1, p<0.0001 and medial: χ^2= 5.8, df=1, p=0.01). Putative ribbon synapse counts, defined as juxtaposed CtBP2- and GluA2-positive puncta showed a 10% reduction only in the apical turn after acoustic trauma in WT mice (Kruskal-Wallis test, χ^2=26.4, df=1, p< 0.0001) (Figure 4a, right panel).

In *Chrna9* KO mice there was also a reduction in the number of pre, post and colocalized puncta, depending on cochlear frequency/location after exposure to noise (Figure 4b). Ribbon puncta in *Chrna9* KO exposed ears were reduced up to a 20% of the control at the basal cochlear end after acoustic trauma (Kruskal-Wallis test, χ^2=53.8, df=1, p<0.0001). GluA2 postsynaptic receptors were reduced by 13% in both the apical and basal turns of traumatized mice (Kruskal-Wallis test; apical: χ^2=25.4, df=1, p<0.0001 and basal: χ^2=24.4, df=1, p<0.0001). Synaptic puncta showed a 20% reduction only in the basal turn after acoustic trauma (Kruskal-Wallis test, χ^2=41.7, df=1, p<0.0001) (Figure 4b, right panel).

Surprisingly, after seven days of acoustic exposure, higher density (approximately 10-15% increase) of pre-synaptic ribbons, post-synaptic GluA2 receptor patches, and putative synapses at all cochlear regions were observed in *Chrna9L9'T* KI ears (Figure 4c) (synaptic counts, Kruskal-Wallis test; apical: χ^2=14.9, df=1, p=0.0001, medial: χ^2=64.8, df=1, p<0.0001 and basal: χ^2=6.7, df=1, p=0.009) (Figure 4c, right panel). These results suggest that potentiation of $\alpha9\alpha10$

311 nAChRs responses can not only prevent the noise-induced functional signs of HHL and afferent
312 synaptic loss but also might promote synapse formation after acoustic trauma.
313
314 **OHC function and efferent innervation pattern after noise exposure**

315 OHC function was assessed through DPOAEs, which can be measured from the external
316 auditory canal (Shera and Guinan, 1999). When two tones are presented, electrical distortions
317 are created, amplified and conducted back into mechanical motion of the sensory epithelium at
318 the distortion frequencies by normally functioning OHCs and can be detected by a microphone
319 in close proximity to the tympanic membrane. As shown in Figure 1c, DPOAEs responses at
320 P21 in unexposed WT and *Chrna9* KO mice were similar except at 45.25 kHz (Kruskal-Wallis
321 test, df=2, p=0.004 at 45.25 kHz). However, there was a small elevation of around 5 dB of mean
322 DPOAEs thresholds in unexposed *Chrna9L9´T* KI mice at some frequencies (Kruskal-Wallis test,
323 χ^2=7.3, df=2, p=0.020 at 11.33 kHz; χ^2=5.7, df=2, p=0.004 at 16 kHz and χ^2=10.8, df=2, p=0.030
324 at 22.65 kHz) (Figure 1c). Threshold elevations in *Chrna9L9´T* KI mice were similar in
325 magnitude whether measured by ABRs (Figure 1a) or DPOAEs (Figure 1c), as already reported
326 by Taranda et al. 2009. Thus, compared to *Chrna9* KO mice, in which baseline cochlear
327 thresholds were normal, elevated thresholds in unexposed *Chrna9L9´T* KI mice suggest that
328 they may arise from the enhancement of cholinergic activity on OHCs, revealing cholinergic
329 MOC effects under resting conditions. However, we cannot disregard that unexposed
330 *Chrna9L9'T* KI mice have fewer afferent synapses per IHC at the medial cochlear region (Figure
331 3) and this may also contribute to generating higher auditory thresholds in these mice.

332 In WT ears, with normal MOC feedback, exposure to loud sounds produced DPOAEs threshold
333 shifts from 5 to 20 dB one day after acoustic trauma at some frequencies (Friedman test, df=2,
334 p=0.019 at 11.33 kHz; p=0.012 at 16 kHz; p=0.001 at 22.65 kHz and p=0.012 at 32 kHz) that
335 returned to almost normal at day 7 (Friedman test, df=2, p>0.05 at all the frequencies tested)

(Figure 5, left panel). In contrast, in *Chrna9* KO mice, which are functionally de-efferented, noise exposure produced DPOAEs threshold shifts from 5 to 35 dB one day after trauma at some frequencies (Friedman test, df=2, p= 0.022 at 11.33 kHz; p=0.003 at 16 kHz; p= 0.017 at 22.65 kHz; p=0.014 at 32 kHz and p=0.014 at 45.25 kHz). DPOAEs thresholds remained elevated for at least seven days after exposure (Friedman test, df=2, p=0.009 at 11.33 kHz; p=0.009 at 16 kHz; p=0.030 at 22.65 kHz; p=0.014 at 32 kHz and p=0.043 at 45.25 kHz) (Figure 5, middle panel). Remarkably, there were no changes in DPOAEs thresholds after acoustic trauma in *Chrna9L9'T* KI mice with enhanced MOC feedback (Friedman test, df=2, p>0.05 at all the frequencies tested) (Figure 5, right panel). These results show that the degree of functional OHC damage depends on the level of $\alpha 9\alpha 10$ nAChRs activity.

In order to evaluate if exposure to loud sounds can alter the distribution of MOC terminals in OHCs, a quantitative analysis of whole mount organ of Corti immunostained for synaptophysin, an integral protein of the synaptic vesicle membrane, was performed (Figure 6). In WT mice, MOC terminals typically occur in clusters under OHCs, along the entire cochlea from base to apex (Vetter et al., 1999). As seen in Figure 6b, left panel, in the middle cochlear turn, the number of terminals per OHC in WT ranged from 1 to 5, with most of the OHCs contacted by 2 to 3 synaptic terminals (39.8 and 41.7 %, respectively). However, seven days after acoustic trauma there was a reduction in the number of MOC terminals per OHC with almost 40% of terminals occurring as singlets rather than as clusters. The mean number of terminals under OHCs before and 7 days after acoustic overexposure was reduced from 2.60 $\pm$ 0.02 to 1.80 $\pm$ 0.01, respectively. Thus, the distribution of MOC contacts under OHC was significantly changed after noise exposure (two-sample Kolmogorov-Smirnov test, D=0.3, p<0.0001).

As previously reported (Vetter et al., 1999), quantification of MOC endings in cochlear sections of *Chrna9* KO mice revealed abnormalities in the number of terminals. Most OHCs were contacted by 1 to 2 terminals (39.3 and 52.4 %, respectively) and very few by more than 2. After

361 acoustic trauma, there were no changes in the pattern of labeled terminals in *Chrna9* KO mice
362 (Figure 6a and b, middle panels). In contrast, the *Chrna9L9'T* KI gain-of-function mouse model
363 already showed a basal increase in the mean number of efferent terminals per OHC, with some
364 cells contacted by as many as 7 terminals (Figure 6a, right panel). Previous studies in
365 *Chrna9L9´T* KI mice have shown the same increase in the mean number of efferent terminals
366 per OHC (Murthy et al., 2009). Notably, no changes were observed in the number and
367 distribution of efferent terminals under OHCs in the *Chrna9L9'T* KI mice after exposure to loud
368 sounds (Figure 6a and b, right panels). Altogether, these observations suggest that $\alpha 9\alpha 10$
369 nAChR activity influences olivocochlear pre-synaptic terminal differentiation required for proper
370 synapse assembly.

371 Although healthy adult IHCs lack MOC innervation, it has been recently suggested that MOC
372 contacts return to IHCs in the damaged and aged cochlea (Ruel et al., 2007; Lauer et al., 2012;
373 Zachary and Fuchs, 2015). To determine if the present noise exposure protocol can produce
374 changes in MOC synapses on IHCs we analyzed synaptophysin-stained cochlea in the IHCs´
375 area. Figure 6c shows light microscopy micrographs of the inner spiral bundle that reveal the
376 olivocochlear innervation. Synaptophysin labels both MOC and LOC fibers, which cannot be
377 differentiated. As previously demonstrated by Vetter et al., 1999, in unexposed WT mice the
378 spiraling plexus of efferent terminals consist of a dense matrix at levels well below the IHC base,
379 on the modiolar side, where terminals contact dendrites of auditory nerve fibers. Moreover, a
380 sporadic plexus of terminals positioned around the IHC soma on the pillar side was observed
381 (Figure 6c, top left panel, arrowheads). Seven days after noise exposure there was no apparent
382 change in the efferent terminals of the IHCs area (Figure 6c, bottom left panel). Thus, similar to
383 unexposed ears, the dense matrix of efferent terminals at the base of the IHCs showed no
384 alteration and a much sparser innervation in the IHC somata region was observed. Interestingly,
385 as previously shown (Vetter et al., 1999), under control conditions the population of efferent

terminals on the pillar side of IHCs appears to be absent in *Chrna9* KO mice, whereas the terminals on the modiolar side was not modified (Figure 6c, upper middle panel). After acoustic trauma, there was no overt change in the pattern of labeled terminals in *Chrna9* KO mice (Figure 6C, lower middle panel). Finally, in the organ of Corti of unexposed *Chrna9L9´T* KI mice, synaptophysin immunostaining revealed an intense cholinergic innervation (Figure 6c, upper right panel) with an apparent increase in the number of terminals in the pillar side. Furthermore, efferent terminals appeared disorganized compared to WT mice. Seven days after acoustic overexposure, there was no overt change in the pattern of efferent innervation in *Chrna9L9´T* KI mice (Figure 6c, lower right panel).

The reduction in the number of efferent contacts in WT OHCs after noise exposure (Figure 6a and b, left panels) led us to analyze if further de-efferentation would occur when applying a second noise challenge of 1h at 100 dB SPL, that could render the organ of Corti totally devoid of efferent feedback. At P28 (i.e., seven days after the first noise exposure), we exposed WT again and tested their cochlear function the next day and seven days after. This second exposure caused the same ABR threshold elevations one day post acoustic trauma (Friedman test, df=4, p=0.0375 at 11.33 kHz; p=0.016 at 16 kHz; p=0.049 at 22.65 kHz; p=0.009 at 32 kHz and p=0.022 at 45.25 kHz), which completely recovered to pre-exposure values by seven days after the second noise challenge (Friedman test, df=4, p>0.05 at all the frequencies tested) (Figure 7a). The distribution pattern of MOC terminals in the OHC area, analyzed by synaptophysin immunostaining, showed no further de-efferentation (Figure 7b). The mean number of MOC terminals under OHCs after the first and second acoustic overexposure was 1.80 $\pm$ 0.01 and 2.09 $\pm$ 0.01, respectively. These results suggest that with a consecutive noise exposure there is no additional degeneration of MOC terminals and that their cochlear sensitivity was not altered.

411 **Discussion**

412 HHL was recently described as an auditory neuropathy believed to contribute to perceptual
413 abnormalities including tinnitus, hyperacusis and speech discrimination in noisy environments
414 (Kujawa and Liberman, 2009; Schaette and McAlpine, 2011). The causative contribution of
415 cochlear synaptopathy to HHL has become the most suitable explanation since in the noise-
416 exposed and/or aging ear significant IHC de-afferentation takes place well before elevation of
417 auditory thresholds (Kujawa and Liberman, 2009). Here we show an inverse correlation
418 between activity of the $\alpha9\alpha10$ nAChRs and the HHL phenotype, which is suggestive of the
419 critical role of the MOC system in cochlear synaptopathy.
420 Until now, delivery of neurotrophins appeared as the only way of triggering the repair of noise-
421 induced damage to cochlear synapses (Wan et al., 2014; Suzuki et al., 2016). Our study shows
422 that enhancement of the MOC feedback can counteract noise-induced cochlear synaptopathy
423 and the loss of MOC terminals. Thus, potentiation of α9α10 nAChR-mediated responses by a
424 pharmacological positive modulator could have a potential therapeutic use in the prevention or
425 treatment of HHL.
426
427 **Noise-induced cochlear synaptopathy and MOC feedback**
428 Noise-induced cochlear synaptopathy was initially described in adult mice with an up to 40% of
429 synaptic loss (Kujawa and Liberman, 2009) and afterwards validated in guinea pigs with a
430 smaller reduction of 30% (Furman et al., 2013). A more recent study in mice revealed a similar
431 degree of cochlear synaptopathy at 6 weeks of age (Jensen et al., 2015). Similarly, in aging
432 mice, there is a 25-30% loss of cochlear nerve synapses, well before there is any loss of hair
433 cells or significant threshold elevation (Sergeyenko et al., 2013). Our work shows noise-induced
434 cochlear synaptopathy at P21, i.e. just at the early onset of puberty and also an early stage of
435 the development of the hearing system. The smaller reduction in our ribbon synapses counts
436 (up to 20%) compared to that described by Kujawa and Liberman (2009) can derive from

differences in mice strains and/or from a reduced time of the noise exposure protocol. Although most studies have been performed in rodents, a recent work has described noise-induced loss of ribbon synapses in non-human primates (Valero et al., 2017). Several lines of evidence indicate that humans are less vulnerable to noise injury (Dobie and Humes, 2017). Nonetheless, emerging information in humans show that auditory nerve fibers are more susceptible than hair cells, as in rodents (Viana et al., 2015). Taken together, these results suggest that cochlear synaptopathy is a general phenomenon that occurs in different species and at different stages of development and might be a more important factor in noise-induced and age-related hearing loss than previously valued.

It has been proposed that the MOC reflex in adults controls the dynamic range of hearing (Guinan, 1996), improves signal detection in background noise (Kawase et al., 1993), is involved in selective attention (Delano et al., 2007), and protects from acoustic injury (Liberman, 1991). Before hearing onset, MOC neurons establish transient synapses on IHCs somata mediated by $\alpha9\alpha10$ nAChRs (Glowatzki and Fuchs, 2000; Katz et al., 2004; Gómez-Casati et al., 2005). This transient MOC activity modulates the temporal fine structure of spontaneous activity and plays a role in the maturation of the IHC synaptic machinery and central synapse formation (Glowatzki and Fuchs, 2002; Johnson et al., 2013; Clause et al., 2014). However, the importance of the MOC system on the maintenance of cochlear nerve synapses after damage has not been deeply studied. Recent work in mice with cochlear de-efferentation induced by surgical lesion, showed a dramatic loss of ribbon synapses after exposure to noise (Maison et al., 2013). Although the surgery spares most of the LOC neurons, some of them can be affected. The present work provides evidence that MOC-mediated protection of cochlear synaptopathy is via the $\alpha9\alpha10$ nAChRs complexes on OHCs. However, a developmental effect of an altered α9α10 nAChRs activity on the susceptibility to damage in IHCs and auditory nerve fibers cannot be precluded. The magnitude of cochlear synaptopathy is inversely correlated with the activity of

462 α9α10 nAChRs: high in *Chrna9* KO and undetectable in *Chrna9L9'T* KI mice. Previous work
463 with *Chrna9L9´T* KI mice showed that increasing the magnitude of MOC effects rendered mice
464 more resistant to acoustic trauma (Taranda et al., 2009). This is consistent with studies showing
465 that overexpressing the α9 channels also increased the resistance of the ear to noise injury
466 (Maison et al., 2002). We now show that the enhanced α9α10 nAChRs activity also prevents
467 cochlear synaptopathy and points to the importance of the integrity of MOC synapses as a
468 feedback pathway to protect the inner ear from everyday acoustic environments.
469 The reduction in synaptic counts can account for the decrease in neural response amplitudes
470 after acoustic trauma in WT and *Chrna9* KO mice. A decrease in the number of ribbon synapses
471 could reduce the amount of synchronous excitatory postsynaptic potentials in response to
472 sounds altering the amplitude of ABR peak 1 (Kiang et al., 1976; Kujawa and Liberman, 2009).
473 It will be interesting to investigate if the remaining synapses are equally capable of releasing
474 glutamate as before trauma. Similarly, the lack of reduction in ABR amplitudes in *Chrna9L9´T* KI
475 mice correlates with the absence of synaptic loss after acoustic overexposure, indicating that
476 enhancement of the MOC reflex prevents the neuronal loss.

477 The observation in *Chrna9L9´T* KI mice of an increase in synaptic counts after noise exposure,
478 is intriguing. In unexposed WT ears, synaptic counts show a spatial distribution from base to
479 apex: the number of ribbon synapses per IHC peaks at the medial region, where the cochlea is
480 most sensitive to sound (Meyer et al., 2009). However, in *Chrna9L9´T* KI this distribution is lost:
481 synaptic counts were comparable to WT mice at low and high frequency regions, except in the
482 middle turn where a significant reduction was observed. One possibility to explain the abnormal
483 cochlear distribution of ribbon synapses in the *Chrna9L9´T* KI mouse model is that before the
484 onset of hearing, the enhanced transient MOC activity could modify the pattern of spontaneous
485 IHC firing, leading to an alteration in the definitive number of auditory nerve synapses.
486 Nevertheless, after acoustic trauma there was an increase in synaptic puncta across the

cochlea suggesting that enhancement of MOC activity together with sound overexposure prompted new afferent synapse formation. An alternative explanation may be that the cochlea already contains synaptic contacts, but without pre- and post-synaptic specializations necessary for neurotransmission, which then gain function following noise trauma. The functionality of these new synapses is still an open question, since there were no changes in ABR peak 1 amplitudes in *Chrna9L9´T* KI mice after acoustic trauma. Currently, we can speculate that acoustic overexposure in this gain of function mouse model could lead to longer lasting increases in intracellular calcium concentration (Wedemeyer et al., 2018) serving as a second messenger to increase the expression of, for example, neurotrophic factors which might finally allow the formation of new synapses. Taken together, the present results show for the first time that potentiation of the MOC system not only strengthens cochlear suppression *in vivo,* but also triggers cellular and molecular pathways that protect and/or repair the inner ear sensory epithelium after injury.

OHCs function after noise exposure and MOC feedback

DPOAEs threshold measurements show the same inverse correlation between the strength of MOC activity and the degree of acoustic damage. WT ears presented a transient elevation after acoustic trauma indicating a full recovery of OHCs function throughout the ear's dynamic range. In contrast, *Chrna9* KO mice thresholds remain elevated, suggesting OHCs dysfunction after noise overexposure. It is interesting to note that after acoustic trauma we did not observe any loss of OHCs in the *Chrna9* KO, suggesting that elevation of DPOAEs thresholds is not caused by OHCs death. Instead, it could indicate that acoustic overexposure without a functional inhibitory MOC reflex can alter the OHC electromotility by changing the mechanotransduction and/or prestin properties. It remains to be shown whether OHCs are more prone to degeneration in *Chrna9* KO mice after more than a week post-exposure. In agreement with our observations, it has been shown that cochlear de-efferentation produces permanent DPOAEs

513 threshold elevations without or with minimal OHC loss in aged and noise-exposed mice,
514 respectively (Maison et al., 2013; Liberman et al., 2014). Conversely, DPOAEs thresholds in
515 *Chrna9L9´T* KI mice were not modified with the same noise protocol, showing that enhanced
516 MOC activity can protect OHCs damage after acoustic overexposure.
517
518 **Noise-induced degeneration of MOC terminals**
519 The present work shows for the first time that noise exposure leading to temporary threshold
520 shifts and cochlear synaptopathy also causes a reduction of MOC terminals to OHCs. There is a
521 sparse literature dealing with degeneration of MOC terminals after exposure to noise. Only few
522 studies have shown acute damage to efferent nerve endings immediately following noise
523 exposure, but they used an acoustic trauma protocol that causes permanent auditory threshold
524 elevations concomitantly with OHCs loss in some cochlear regions (Omata et al., 1992; Canlon
525 et al., 1999). It has been suggested that degeneration of MOC neurons could be a contributing
526 factor to age-related hearing loss (Liberman et al., 2014; Chumak et al., 2016). However, recent
527 work by Lauer (2017) with the *Chrna9* KO mice did not find accelerated onset of hearing loss up
528 to 15 months of age. Considering that MOC neurons regulate several aspects of auditory
529 processing like the dynamic range of hearing and detection of relevant auditory signals in
530 background noise (Maison et al., 2001; Guinan, 2011), we propose that the interruption in
531 synaptic communication between MOC terminals and OHCs after acoustic trauma contributes,
532 together with cochlear synaptopathy, to the HHL reported symptoms.
533
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719 **Figure Legends**

720 **Figure 1: Auditory function in unexposed WT, *Chrna9* KO and *Chrna9L9´T* KI mice.**

721 (**a**) ABRs thresholds for control WT (n=12), *Chrna9* KO (n=14) and *Chrna9L9´T* KI (n=15) mice
722 at P21. (**b**) ABR peak 1 amplitudes at 80 dB SPL. (**c**) DPOAEs thresholds in the same
723 unexposed mice in the different genotypes. Cochlear thresholds are elevated in *Chrna9L9´T* KI
724 ears, as measured by either ABRs (**a**) or DPOAEs (**c**). In all cases median and interquartile
725 ranges are shown and the comparisons were made by a Kruskal-Wallis non-parametric ANOVA
726 followed by a Dunn´s post-test. Dark grey asterisks represent the statistical significance
727 between *Chrna9* KO and WT and light gray asterisks between *Chrna9L9´T* KI and WT mice
728 (*=p<0.05 and **=p<0.01).

729

730 **Figure 2. ABR measurements before and after acoustic trauma (AT).**

731 (**a**) Representative ABRs traces from WT, *Chrna9* KO and *Chrna9L9´T* KI mice at P21 before
732 trauma (Pre-AT, black trace), 1 day after AT (AT+1d, dark gray trace) and 7 days after AT
733 (AT+7d, light gray trace). Arrow indicates peak 1 amplitude. Scale bars: vertical=0.4 μV,
734 horizontal=1 ms (**b**) ABR thresholds in WT (n=12), *Chrna9* KO (n=14) and *Chrna9L9´T* KI (n=15)
735 mice at P21 before trauma, 1 day after AT and 7 days after AT. WT and *Chrna9* KO mice
736 showed a significant increase in ABR thresholds 1 day after AT. A recovery of ABR thresholds
737 was observed a week after exposure only in WT mice. *Chrna9L9´T* KI mice did not present any
738 changes in ABR thresholds at any time after AT. (**c**) ABR peak 1 amplitudes at 80 dB SPL in
739 WT, *Chrna9* KO and *Chrna9L9´T* KI mice at the same time points shown in (**b**). WT and *Chrna9*
740 KO mice displayed a reduction in amplitudes 1 day after AT and only a partial recovery a week
741 after exposure. Peak 1 amplitudes in *Chrna9L9´T* KI mice were unaffected by noise exposure.
742 (**d**) ABR peak 1 latencies in WT, *Chrna9* KO and *Chrna9L9´T* KI mice at the same time points
743 shown in (**b**). Latencies were not affected in any of the genotypes after AT. Median and
744 interquartile ranges are shown and the comparisons were made by Friedman tests followed by

a post-hoc test. Dark grey asterisks represent the statistical significance of AT+1d values compared to Pre-AT and light gray asterisks represent AT+7d values compared to Pre-AT controls. (∗=p<0.05; ∗∗=p<0.01; ∗∗∗=p<0.001).

Figure 3. Analysis of IHCs ribbon synapses in unexposed WT, *Chrna9* KO and *Chrna9L9´T* KI mice.

Quantitative data obtained from unexposed WT, *Chrna9* KO and *Chrna9L9´T* KI mice at P28. For each IHC we analyzed the number of CtBP2 puncta, post-synaptic GluA2 receptor patches, and putative ribbon synapses, defined as juxtaposed CtBP2 and GluA2 positive puncta. Horizontal lines inside the boxplots represent the median and whiskers correspond to percentiles 10 to 90. Comparisons were made by a Kruskal-Wallis non-parametric ANOVA followed by a Dunn´s post-test. Dark grey asterisks represent the statistical significance between *Chrna9* KO and WT and light gray asterisks between *Chrna9L9´T* KI and WT mice (*=p<0.05; **=p<0.01; ***=p<0.001).

Figure 4. Analysis of the degree of IHC synaptopathy a week after AT.

Left panel: representative confocal images of IHCs synapses from cochleae immunolabeled for pre-synaptic ribbons (CtBP2-red) and post-synaptic receptor patches (GluA2-green) (scale bar=10 μm). The dashed lines show the approximate outline of one IHC. CtBP2 antibody also weakly stains IHC nuclei. **Right panel:** Quantitative data obtained from WT, *Chrna9* KO and *Chrna9L9´T* KI mice at P28. For each IHC we analyzed the number of CtBP2 puncta, post-synaptic GluA2 receptor patches, and putative ribbon synapses. (**a**) In traumatized WT mice there was a reduction in the number of CtBP2 puncta, GluA2 receptor patches and putative synapse, depending on cochlear frequency/location ($nWT_{control}$ =360 IHCs at the apical, 350 IHCs at the medial and 185 IHCs at the basal region from 8-10 animals; nWT_{AT+7d} =394 IHCs at

the apical, 492 IHCs at the medial and 166 IHCs at the basal region from 6-11 animals). (**b**) In traumatized *Chrna9* KO mice there was a reduction in the number of pre, post and colocalized puncta that was more pronounced at the basal turn ($nKO_{control}$ =234 IHCs at the apical, 200 IHCs at the medial and 218 IHCs at the basal region from 6-8 animals; nKO_{AT+7d} =291 IHCs at the apical, 271 IHCs at the medial and 130 IHCs at the basal region from 4-7 animals). (**c**) In traumatized *Chrna9L9´T* KI mice we found a significant increase in the number of pre-synaptic ribbons, postsynaptic AMPA receptors and colocalization puncta for the three regions of the cochlea ($nKI_{control}$ =262 IHCs at the apical, 309 IHCs at the medial and 135 IHCs at the basal region from 7-12 animals; nKI_{AT+7d}=179 IHCs at the apical, 406 IHCs at the medial and 119 IHCs at the basal region from 5-14 animals). Horizontal lines inside the boxplots represent the median and whiskers correspond to percentiles 10 to 90. Comparisons were made by a Kruskal-Wallis non-parametric ANOVA followed by a Dunn´s post-test (*=p<0.05; **=p<0.01; ***=p<0.001).

Figure 5. Evaluation of OHCs functional integrity before and after AT.

DPOAEs thresholds for WT (n=12), *Chrna9* KO (n=13) and *Chrna9L9´T* KI (n=11) mice at the same time points as in Figure 2. DPOAEs thresholds showed a significant increase 1 day after AT in WT and *Chrna9* KO, but not in *Chrna9L9´T* KI mice. 7 days after AT, DPOAEs thresholds recovered only in WT whereas DPOAEs thresholds remain elevated in *Chrna9* KO mice. Median and interquartile ranges are shown and the comparisons were made by Friedman tests followed by a post-hoc test. Dark grey asterisks represent the statistical significance of AT+1d values compared to Pre-AT and light gray asterisks represent AT+7d values compared to Pre-AT controls. (*=p<0.05; **=p<0.01; ***=p<0.001).

Figure 6. Olivocochlear synaptic boutons a week after AT.

(**a**) Synaptophysin immunostaining of the OHC region in control and traumatized WT, *Chrna9* KO and *Chrna9L9´T* KI mice at P28 reveals MOC terminals under OHCs (scale bar=10 μm). In WT ears, MOC terminals regularly occurred as clusters, such as the quadruplet indicated by four arrows. After AT, there was a reduction in the number of MOC terminals per OHC. In *Chrna9* KO, most terminals occurred as singlet or doublet indicated by one or two arrows, respectively. After AT, there was no alteration in the number of efferent contacts. In *Chrna9L9´T* KI mice, each OHC is contacted by a larger than normal contingent of MOC terminals (arrows) with no modification after AT. (**b**) Cumulative frequency histograms of the efferent innervation pattern to OHCs in control and 7 days after AT in the different genotypes ($nWT_{control}$=1418 OHCs from 8 animals, nWT_{AT+7d}=2226 OHCs from 6 animals, $nKO_{control}$=2017 OHCs from 8 animals, nKO_{AT+7d}=1319 from 7 animals, $nKI_{control}$=2860 OHCs from 10 animals and nKI_{AT+7d}=1795 OHCs from 8 animals). **(c)** Synaptophysin immunostaining of the inner spiral bundle (ISB) region in control and traumatized WT, *Chrna9* KO and *Chrna9L9´T* KI mice (scale bar=10 μm). The dashed lines show the approximate outline of one IHC. WT mice exhibit a regular progression of efferent terminals along the modiolar side of the IHCs (ISB; arrows). These boutons are larger than those on the pillar side of the IHC (arrowheads). After AT, there was no alteration in the efferent innervation pattern. In *Chrna9* KO, there was a reduction in efferent synapses on the pillar side that was not modified after AT. In *Chrna9L9´T KI* mice, there was an increase of efferent terminals on the pillar side with no modification after AT.

Figure 7. Auditory function and OHC connectivity after a second noise exposure in WT mice.

7 days after AT, WT mice were traumatized again with the same acoustic protocol (n=6 animals). (**a**) ABR thresholds of WT control, 1 day after AT (AT+1d), 7 days after AT (AT+7d), 1 day after a second noise exposure (2AT+1d) and 7 days after the second trauma (2AT+7d). There were large threshold elevations both the day after the first and the second AT with a complete

recovery 7 days after either acoustic overexposure. Median and interquartile ranges are shown and the comparisons were made by Friedman tests followed by a post-hoc test. Grey asterisks represent the statistical significance of AT+7d values compared to 2AT+1d (∗=p<0.05; ∗∗=p<0.01). (**b**) Cumulative frequency histograms of MOC innervation pattern after the first and the second noise exposure. Data corresponding to P21 control mice (Control P21) and 7 days after AT (AT+7d) are the same as shown in Figure 6. After the second noise exposure, there was not any further reduction in the number of MOC synapses to OHC. We also showed the quantification of MOC terminals in unexposed P35 control mice and found no difference compared to unexposed P28 mice.