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Cochlear nucleus neurons redistribute synaptic AMPA and glycine receptors in response to monaural conductive hearing loss

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Abstract

Neurons restore their function in response to external or internal perturbations and maintain neuronal or network stability through a homeostatic scaling mechanism. Homeostatic responses at synapses along the auditory system would be important for adaptation to normal and abnormal fluctuations in the sensory environment. We investigated at the electron microscopic level and after postembedding immunogold labeling whether projection neurons in the cochlear nucleus responded to modifications of auditory nerve activity. After unilaterally reducing the level of auditory inputs by ~ 20 dB by monaural earplugging, auditory nerve synapses on bushy cells somata and basal dendrites of fusiform cells of the ventral and dorsal cochlear nucleus, respectively, upregulated GluR3 AMPA receptor subunit, while inhibitory synapses decreased the expression of GlyR α 1 subunit. These changes in expression levels were fully reversible once the earplug was removed, indicating that activity affects the trafficking of receptors at synapses. Excitatory synapses on apical dendrites of fusiform cells (parallel fibers) with different synaptic AMPA receptor subunit composition, were not affected by sound attenuation, as the expression levels of AMPA receptor subunits were the same as in normal hearing littermates. GlyR α 1 subunit expression at inhibitory synapses on apical dendrites of fusiform cells was also found unaffected. Furthermore, fusiform and bushy cells of the contralateral side to the earplugging upregulated the GluR3 subunit at auditory nerve synapses. These results show that cochlear nucleus neurons innervated by the auditory nerve, are able to respond to small changes in sound levels by redistributing specific AMPA and glycine receptor subunits.

Keywords

earplugging; glutamate receptors; glycine α 1 subunit; postembedding immunogold labeling; ultrastructure

Introduction

Perturbation of auditory processing after hearing loss might be the consequence of dysfunctional central synaptic processing including alterations of synaptic strength, neuronal excitability and kinetic properties (Syka, 2002; Kotak et al., 2005; Xu et al., 2007). Given the well-known CNS changes induced by deafness, there may be accompanying changes in

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excitatory and inhibitory receptor expression. In the brain, neurons can restore their function at a set-point level when challenged by external or internal perturbations and thus maintain neuronal or network stability through homeostatic scaling mechanisms (Turrigiano and Nelson, 2004; Davis, 2006). In response to decreased neuronal activity homeostatic scaling leads to compensatory changes in the surface expression of excitatory (up) and inhibitory (down) neurotransmitter receptors (Kilman et al., 2002; Wierenga et al., 2005). These changes affect synaptic strength by changing quantal amplitude (Turrigiano and Nelson, 2004; Davis, 2006). Moreover, receptor movements in and out of synapses could constitute the molecular basis of the adaptive regulation of receptor number at synapses (Lévi et al., 2008). This is important considering that alterations of the properties and number of neurotransmitter receptors can change the efficiency of synaptic transmission, and therefore alter the connectivity between neurons (Bear and Malenka, 1994; Malinow and Malenka, 2002).

Auditory neurons' responses to acoustic stimulation must maintain rapid transmission and maximize temporal fidelity through their synaptic networks (Trussell, 1999; Davis and Young, 2000). It has been shown that AMPA glutamate receptor subunits, which mediate fast synaptic transmission in the auditory pathway, are selectively targeted to postsynaptic sites (Rubio and Wenthold, 1997; 1999; Wang et al., 1998; Gardner et al., 1999, 2001). However, we know very little how auditory nerve synapses adapt to changes in hearing sensitivity. In addition, neuronal networks have to maintain a balance between excitation and inhibition for normal brain function. In cochlear nucleus, glycine mediates the action of the auditory nerve through the activation of glycine receptors (Hirsch and Oertel, 1988; Mildbrandt and Caspary, 1995; Golding and Oertel, 1996; Potashner et al., 2000). How glycinergic synapses respond to hearing sensitivity has been underexplored. We aimed to investigate these questions *in vivo* by using the synaptic circuitry of the cochlear nucleus in young adult rodents (Fig. 1), and to modify sound by a conductive hearing loss paradigm. The ultrastructure of the circuitry of rodent cochlear nucleus is known and key synaptic excitatory (AMPA) and inhibitory (glycine) receptors have been identified by postembedding immunocytochemistry, especially for the main projection neurons: bushy cells (BC) and fusiform cells (FC) (Rubio and Wenthold, 1997; Wang et al., 1998; Rubio and Juiz, 2004; Rubio, 2006). In this study, we manipulated hearing *in vivo* and examined the expression of synaptic AMPA and glycine receptor subunits after postembedding immunogold labeling. We provide evidence that only cochlear nuclei neurons innervated by the auditory nerve responded to sound reduction by upregulating specific AMPA and downregulating glycine receptor subunits.

Material and Methods

Animals

A total of seven postnatal day 30 Sprague-Dawley rats were used in these experiments. Six animals were used for acoustic brain response (ABR) hearing tests, four of which were used for structural analysis. The animals that followed structural analysis were separated into one control (n= 3) and one experimental group (n= 4). The handling of the animals prior to and during the experimental procedures was approved and supervised by the University of Connecticut IACUC and followed NIH guidelines. Rats were anesthetized with isoflurane for ABRs or with a mixture of ketamine 60 mg/kg and xylazine 6.5 mg/kg for insertion and/or removal of the earplug and for the intracardiac perfusion, or with CO₂ for the biochemical procedures.

Monaural Earplugging and Acoustic brainstem responses (ABRs)

After anesthesia, animals were put on a warm blanket under a stereomicroscope. The skin was disinfected and foam earplugs (Ear Classic, Aearo Company, Indianapolis, IN) were cut to appropriate size and introduced into the right external canal. ABR testing was performed

immediately before ear plugging. Twenty-four hours later the animals were retested before, and then after, removal of the earplug. Three subcutaneous pin electrodes were placed on the midline of the scalp located at the vertex (+), posterior midline (−) and the anterior-most region of the neck (ground). Evoked potentials were amplified (AM System Inc. Model 1700 Differential AC Amplifier, 1000x Gain, pass band 1Hz to 10 kHz), digitized (12-bit A/D conversion at 50 kHz sampling rate) and averaged. Stimuli consisted of 200–500 clicks (1 msec duration) presented separately to each ear canal through a calibrated, custom-built earphone. During stimulation, stimulus intensity was monitored with a Knowles FG-23629-P16 2.565 mm diameter microphone. The threshold of averaged ABR responses were determined off-line by determining the stimulus intensity at which the short latency component first became evident (Fig. 2A). The thresholds were confirmed by a second, independent observer. The attenuation provided by each ear plug was determined by comparing the ABR threshold before and after removal of the plug. The attenuation measured in each animal was averaged to determine the average ear plug attenuation. Average ABR thresholds were compared using the paired student t-test.

Structural analysis

Seven animals (3 controls and 4 experimental) were used for structural analysis. In the group of experimental animals, two followed intracardiac perfusion after 1-day of being earplugged. In the other two, the foam earplugs were removed after 1-day of earplugging and the animals were returned to their cage for another day. At the indicated survival time animals were anesthetized and after checking anesthetic depth, were perfused through the heart with 4% paraformaldehyde and 0.5% glutaraldehyde in 0.12 M phosphate buffer (pH 7.2). Low glutaraldehyde fixation was followed by freeze-substitution or conventional electron microscopy as previously described (Rubio and Wenthold, 1997; Rubio, 2006; Tzounopoulos et al., 2007; Rubio et al., 2008).

Freeze-substitution and postembedding immunogold labeling

For the detection of GluR2, GluR2/3 and GluR4 AMPA receptor subunits, and GlyR α 1 subunit with immunogold labeling after freeze-substitution in the cochlear nucleus, a protocol similar to that described in detail was used (Rubio and Wenthold, 1997; Rubio, 2006; Rubio et al., 2008). The anterior ventral and the dorsal cochlear nucleus were dissected and processed for freeze-substitution and low-temperature embedding. For postembedding immunocytochemistry, ultrathin sections (80 nm in thickness) on nickel grids were incubated in sodium borohydride and glycine in Tris-buffered saline solution with Triton X-100. After being pre-blocked with serum, the sections were incubated with affinity purified polyclonal primary antibodies for GluR2/3 (1.5 μ g) or GluR4 (1.5 μ g) (gift from Dr. Robert Wenthold), or the monoclonal antibodies against GluR2 N-terminus (1.5 μ g; Chemicon, Temecula, CA; Rubio 2006; Rubio et al., 2008), or GlyR α 1 (1:250; Alexis Biochemicals, San Diego, CA). Primary antibodies were detected with secondary antibodies conjugated 5 nm gold particles in diameter (1:20; Amersham GE Healthcare, Buckinghamshire, UK). To check the specificity of primary and secondary antibodies, sections were prepared either in the absence of the primary antibody during the incubation step or by preadsorption of GluR2/3, GluR2, and GluR4 antibodies with the corresponding peptides (Rubio and Wenthold, 1997; 1999; Matsui et al., 2005; Rubio, 2006). No gold particles were observed on the ultrathin sections after any of the control procedures. Ultrathin sections were analyzed with a TECNAI G2 Spirit Biotwin TEM (FEI, Hillsboro, OR, USA). The images were captured with an AMT XR40 4 megapixel side mounted CCD camera (Denvers, MA, USA) at 49,000x or 68,000x magnification. Image processing was performed with Adobe Photoshop using only the brightness and contrast commands to enhance gold particles.

Postembedding immunogold labeling of the ipsilateral-earplugged, contralateral sides to the earplug and normal-hearing littermates was performed simultaneously. Only well-identified synapses were included in the analysis.

Identification of excitatory and inhibitory endings on fusiform cells (FC), and bushy cells (BC)

The identification at the electron microscope of auditory nerve synapses on fusiform and bushy cells and of parallel fibers synapses on fusiform and cartwheel cells was done as previously described (Ryugo and May, 1993; Ryugo et al., 1997; Rubio and Wenthold, 1997; Rubio and Juiz, 2004; Rubio, 2006; Tzounopoulos et al., 2007; Wang et al., 1998). The ultrastructural identification of inhibitory synapses on basal and apical dendrites of FCs was performed following criteria by Rubio and Juiz (2004). To analyze inhibitory synapses on BC somata we took into account the same ultrastructural parameters as the ones on FC or elsewhere (Altschuler et al., 1986; Rubio and Juiz, 2004). In this study we concentrated on the synaptic endings making symmetric synaptic contacts (Gray type II), and containing flattened synaptic vesicles, which are characterized for being glycinergic (Altschuler et al., 1986; Rubio and Juiz, 2004). The two major sources for glycinergic inhibition within the cochlear nucleus modulating auditory nerve activity are from tuberculoventral cells in the dorsal cochlear nucleus and stellate cells (type-D) within the ventral cochlear nucleus (Young and Voigt, 1982; Wickesberg and Oertel, 1988, 1990; Oertel and Wickesberg, 1993; Nelken and Young, 1994). Both cell types innervate basal dendrites of FC and the cell body of BC. The main modulators of activity on apical dendrites of fusiform cells are from cartwheel and stellate cells in the molecular layer of the DCN (Wouterlood and Mugnaini 1984; Mugnaini, 1985; Golding and Oertel, 1996). The inhibitory endings were clearly distinguishable from those of the auditory nerve and parallel fibers.

Quantitative analysis—The distribution and relative density of GluR2/3, GluR2 and GluR4 AMPA receptor subunits and GlyR α 1 immunolabeling in the ventral and dorsal cochlear nucleus was performed in a total of 1,436 postsynaptic densities (PSD; Table I). The analysis in the auditory nerve and inhibitory synapses on the somata of bushy cells and basal dendrites of fusiform cells was determined for 1,174 PSDs (n= 689 (BC), n = 485 (FC) and 25–40 PSDs per antibody). For the quantitative analysis of parallel fibers on fusiform and cartwheel cells 222 PSDs were analyzed (approximately 120 per cell type and 30 per antibody). Forty PSDs were analyzed for the glycine receptor subunit expression on apical dendrites of FC. Data were collected from cases only where the PSD was well defined; the length of the PSDs was measured with ImageJ (<http://rsb.info.nih.gov/ij>), and the numbers of gold particles was counted. The density of gold particles per PSD was calculated by dividing the number of gold particles per the length of the corresponding PSD as previously described (Rubio and Wenthold, 1997; Rubio and Juiz, 2004; Rubio, 2006). We also calculated the average number of gold particles per PSD, by dividing the total number of gold particles by the total number of PSDs (Wang et al., 1998). Only gold particles clearly seen at the PSD and within the synaptic cleft were counted. The maximum distance allowed between the PSD and a gold particle was approximately 15 nm, based on the spatial resolution of the immunogold technique (Merighi and Polak, 1993). The same imaging software was used to calculate the thickness of the PSD as described elsewhere (Dosemici et al., 2001; Rubio, 2006). Briefly, the cytoplasmic outline of a PSD, including the associated dense material, was traced, and this area was then enclosed by tracing the postsynaptic membrane (length of PSD). The area was then divided by the length of the postsynaptic membrane to derive an average thickness for the PSD. The results are presented $\pm$ SEM. Two-tailed tests (assuming unequal variance) were used for statistical comparison. Control and experimental animals, as well as each side of the brainstems were blindly assigned with a random number.

Results

ABR Thresholds were used to confirm effectiveness of earplugging and to assess auditory sensitivity

All animals used in this study exhibited an acoustic startle reflex suggesting that their auditory systems were functioning normally. Auditory sensitivity was tested further in seven animals by measuring ABR thresholds of each ear separately before and after monaural earplugging. In addition, ABR thresholds were measured for the plugged ear to determine the effectiveness of the plug. Electrodes were positioned on the midline of the head. Although ABRs recorded from the midline electrodes may differ slightly from other electrode orientations (Galbraith et al., 2006) the midline geometry was selected for the convenience it offered in allowing us to use the single set of electrodes to record ABRs in response to monaural stimulation of each ear (Fig. 2A). Prior to earplugging the average ABR threshold monaural click-stimulus thresholds were 33.4 ± 4.4 dB SPL on the control side and 33.0 ± 3.5 dB SPL on the treatment side (Fig. 2B). Earplugs were inserted monaurally, and the animals were returned to their cages. Upon insertion, earplugs increased ABR thresholds by approximately 20 dB (data not shown). The effectiveness of the earplugs was confirmed after the 24 hour treatment by comparing ABR thresholds measured immediately before, and after, removing the plugs. After the 24 hour treatment period the plugged ears were shown to have an average ABR threshold of 53.4 dB SPL (Fig. 2B₁). Paired comparison of thresholds before and after removing earplugs revealed that the earplugs attenuated sound by at least 15 dB in each of the 6 animals tested (mean = 19.2 ± 2.6 dB attenuation, range 15–22.5 dB, $n=6$). ABR thresholds measured in the untreated (Fig. 2B₂) and treated (Fig. 2B₁) ears after removing the ear plug showed that there was no significant permanent change in monaural ABR thresholds in either ear as a result of the earplugging.

Response of auditory nerve synapses to unilateral conductive hearing loss

Studies done in the spinal cord, cortical and hippocampal neurons in culture have shown that excitatory synapses upregulate AMPA receptor (AMPA) subunits in response to reduction or block of synaptic activity (O'Brien et al., 1998; Harms et al., 2005; Turrigiano and Nelson, 2004; Hou et al., 2008; Ibata et al., 2008). Previous data from our laboratory have shown that just after 4 hours of unilateral deafferentation AMPAR subunits redistributed at the synapse of the auditory nerve on fusiform cells of the dorsal division of the cochlear nucleus (Rubio, 2006). In this study we asked whether two different projection neurons directly innervated by the auditory nerve reapportioned AMPARs in response to reduction of sound and if they did, whether it happened in the same manner. As a model system we chose projection neurons in the ventral (bushy cells: BC) and in the dorsal (fusiform cells: FC) cochlear nucleus that receive glutamatergic endings of the auditory nerve. These synapses are made up mainly of GluR3 and GluR4 with little or no GluR1 and little GluR2 (Rubio and Wenthold, 1997; Wang et al., 1998; Gardner et al., 1999; 2001).

We performed unilateral earplugging to reduce sound by ~20 dB for 1-day as described above (Fig. 2) and analyzed quantitatively after postembedding immunogold labeling the expression of GluR2, GluR3 and GluR4 AMPAR subunits at the synapse of the auditory nerve on the cell body of BCs and on basal dendrites of FCs (Fig. 1, 3 and 4). There were not any ultrastructural changes at the postsynaptic neuron or the presynaptic terminal that could indicate any type of axonal degeneration after earplugging. The length of the PSD was the same as normal hearing littermates (BC: 0.27 ± 0.03 ; FC: 0.25 ± 0.01 ; $p > 0.05$), and although the PSD was slightly thicker in earplugged animals, the difference was not statistically significant (normal hearing: ~25nm; $p > 0.05$). No significant difference in the density of gold particles at the PSD was measured among the animals comprising the control group (ANOVA $p > 0.05$). There was also no significant difference in the densities measured among the animals in the experimental group

(ANOVA $p > 0.05$). Therefore, we were able to use pooled results to compare the control group to the experimental group. The density of gold particles for GluR2/3, GluR2 and GluR4 at the PSD of excitatory synapses on BC and FC revealed that GluR2/3 was upregulated (earplugged: BC: 34.8 ± 2.7 , FC: 21.6 ± 2.1 ; normal hearing: BC: 15.4 ± 1.3 , FC: 12.8 ± 1.6 ; $p < 0.001$) while the values obtained for the GluR2 were the same as normal hearing littermates (BC: 15.4 ± 1.3 , FC: 12.8 ± 1.6 ; $p > 0.05$) (Fig. 3 and 4). The measurements indicate that it is the GluR3 subunit that increases at the auditory nerve synapse in response to reduction of sound independently of the neuronal target. Interestingly, we observed a downregulation of the GluR4 subunit but only at the synapse formed by the auditory nerve on FCs (6.7 ± 2.2 ; normal hearing 15.4 ± 2.5 ; $p < 0.001$). A downregulation of the GluR4 also occurred at the auditory nerve-FC synapse after unilateral deafferentation (Rubio, 2006). The analysis of the average number of gold particles per PSD showed similar results (Table 2). This suggests that although BCs and FCs responded to sound reduction, the underlying cellular mechanism to produce such response, depends on the postsynaptic target.

Excitatory synapses on apical dendrites of FC remain unchanged to 1-day monaural earplugging

We addressed whether other excitatory synapses in the circuitry that are not directly affected by the modifications of auditory nerve activity also responded to sound reduction by upregulating the synaptic expression of AMPAR subunits. We studied the glutamatergic synapse of the granule cells (parallel fibers) on apical dendrites of FCs in the dorsal cochlear nucleus after 1-day unilateral earplugging (Fig. 1 and 5). This synapse has been shown to contain GluR2 and GluR3 AMPAR subunits (Rubio and Wenthold, 1997; Gardner et al., 1999; Rubio, 2006). Our data showed that the density of gold particles for GluR2/3 (14.3 ± 2.0) and GluR2 (9.1 ± 2.1) in the monaural ear plugged animals was the same ($p > 0.05$) as in normal hearing littermates (12.2 ± 4.3 and 12.0 ± 3.3 , respectively). Parallel fibers also synapse on cartwheel cells, which are the main inhibitory interneuron in the superficial layers of the nucleus to innervate FCs (Fig. 1). We also observed no change ($p > 0.05$) at the PF-cartwheel cell synapse in the expression for GluR2/3 or GluR2 between experimental (14.3 ± 2.5 ; 8.6 ± 1.2 , respectively) and control animals (GluR2/3: 14.8 ± 3.9 ; GluR2: 8.1 ± 0.4) (Fig. 5). The average number of gold particles per PSD, showed similar results (Table 2).

Glycinergic synapses on bushy and fusiform cells respond to monaural earplugging

Decreased neuronal activity leads to compensatory changes in the surface expression of excitatory and inhibitory neurotransmitter receptors, increasing and decreasing, respectively (Turrigiano and Nelson, 2004; Davis, 2006). Thus, we anticipated that if after earplugging AMPAR expression increased at the synapses of the auditory nerve on BCs and FCs, these neurons would need to compensate for the imbalance of activity by downregulating inhibitory receptors. Glycine is a major inhibitory neurotransmitter in the cochlear nucleus through the activation of glycine receptors (GlyR) (Hirsch and Oertel, 1988; Mildbrandt and Caspary, 1995; Golding and Oertel, 1996; Potashner et al., 2000). The $\alpha 1$ is the ligand-binding subunit of the mature brain (Malosio et al., 1991; Sato et al., 1991), and has high strychnine binding affinity (Kuhse et al., 1995). In the adult cochlear nucleus the presence of GlyR $\alpha 1$ subunit has been well documented by a variety of procedures including postembedding immunogold labeling (Altschuler et al., 1986; Wenthold et al., 1988; Friauf et al., 1997; Sato et al., 2000; Piechotta et al., 2001; Rubio and Juiz, 2004). For these reasons, we concentrated on the analysis of this subunit and investigated by postembedding immunogold labeling, whether its expression decreased at inhibitory synapses on basal dendrites FC and on BC somata (Fig. 1 and 6). The sample only included synaptic endings containing flattened synaptic vesicles and making symmetric synaptic contacts immunolabeled for GlyR $\alpha 1$. Tuberculoventral cells in the dorsal cochlear nucleus and stellate cells (type-D) within the ventral cochlear nucleus are the two major sources for glycinergic inhibition on BCs and basal dendrites FCs (Young and Voigt,

1982; Wickesberg and Oertel, 1988, 1990; Oertel and Wickesberg, 1993; Nelken and Young, 1994).

After one day of unilateral earplugging our results showed a significant decrease of GlyR α 1 at inhibitory synapses on the cell body of BCs (5.3 ± 0.9 ; $p < 0.001$) and on basal dendrites of FCs (8.3 ± 1.1 ; $p < 0.01$) when compared to normal hearing littermates (BC: 10.5 ± 1.1 ; FC: 21.4 ± 1.5) (Fig. 6). The number of gold particles per PSD also decreased for the earplugged animals (Table 2). We also investigated the expression levels of the GlyR α 1 subunit on apical dendrites of FC receiving the glycinergic input of cartwheel cells. Data showed no change in the expression levels for GlyR α 1 in response to earplugging (normal hearing: 30.00 ± 3.2 ; experimental: 28.3 ± 4.2). These results suggest that cochlear nucleus neurons compensate for the imbalance of excitation and inhibition caused by mild modifications of auditory nerve activity by decreasing their glycinergic synaptic strength.

Reversible expression of synaptic AMPA and GlyR α 1 receptor subunits after earplug removal

We have shown signs of synaptic scaling at excitatory and inhibitory synapses in response to ~20 dB monaural sound reduction. If those changes in receptor expression were directly related to modifications of synaptic activity due to the earplugging, we could expect that the expression levels for those AMPA and glycine receptor subunits that responded to the hearing loss (GluR3, GluR4 and GlyR α 1) will go back to normal levels when the ear plug is removed. To directly address this issue, we designed an experiment in which we removed the earplug after 1-day of ear plugging and left the animal alive for another day. ABRs on these animals were the same as normal hearing animals (data not shown) thus we anticipated that the results in the expression of AMPA and glycine receptor subunits in the earplugged animals would be as in normal hearing littermates. In fact, that is what we found (Fig. 7), after earplug removal the synaptic levels of expression at the auditory nerve or inhibitory synapses for GluR2/3 (20.8 ± 2.2) and GlyR α 1 (15.0 ± 1.2) on BC somata, and for GluR2/3 (15.7 ± 1.7), GluR4 (13.7 ± 1.5) and GlyR α 1 (19.4 ± 2.0) on cell bodies and/or basal dendrites of FCs were similar to normal hearing littermates ($p > 0.05$).

BC and FC of the contralateral side to the earplugging respond to unilateral conductive hearing loss

Contralateral excitatory inputs to the cochlear nucleus seem to compensate rapidly for large changes in afferent input (Sumner et al., 2005). The imbalance of activity in CN neurons of the contralateral side may cause a change in the expression of synaptic AMPARs. Accordingly, a recent study from our laboratory showed that after two days of unilateral deafferentation, the synapses of the auditory nerve on FCs of the contralateral cochlear nucleus increased the amount of AMPA receptors subunits by upregulating the synaptic expression of the GluR3 subunit (Rubio, 2006). In this study, we hypothesized that the auditory nerve synapses contralateral to the earplugging would also sense the sound reduction and compensate for changes of activity by reapportioning up or down synaptic AMPA and glycine receptors on both cell types. Data showed (Figs. 3 and 4) that the synapse of the auditory nerve on BCs and on FCs significantly increased the expression for GluR2/3 (BC: 24.5 ± 2.9 , $p < 0.01$; FC: 15.0 ± 3.1 , $p < 0.05$), when compared to normal hearing littermates (BC: 11.4 ± 1.3 , FC: 12.8 ± 1.6 SEM). The expression for GluR2 and GluR4 at both synapses was found similar to control animals ($p > 0.05$). The density values in the experimental animals for GluR2 were 4.9 ± 0.7 (BC) and 8.3 ± 2.1 (FC), compared to 6.7 ± 1.5 (BC) and 9.0 ± 1.0 (FC) in normal hearing animals. The values found for GluR4 were 12.7 ± 1.4 (BC) and 9.5 ± 1.2 (FC) for experimental, and 9.1 ± 1.6 (BC) and 15.4 ± 2.5 (FC) for control animals. When we analyzed the data for the GlyR α 1 subunit (Fig. 6), we found that it only decreased at inhibitory synapses on basal

dendrites of FC (15.0 ± 1.9 ; $p < 0.05$) but not on the cell body of BC (8.8 ± 2.6 ; $p > 0.05$) when compared to normal hearing animals (FC: 21.4 ± 1.5 ; BC: 8.8 ± 1.2).

Discussion

In the auditory system, plasticity at synapses is important for adaptation to fluctuations in the sensory environment (Molitor and Manis, 1997; Turecek and Trussell, 2000; Fujino and Oertel 2002; Kaltenbach et al., 2005; Illing and Reisch, 2006). In this *in vivo* study, we have shown that in response to monaurally attenuating auditory input by ~20 dB for 1-day, projection neurons of the cochlear nucleus scaled excitatory (up) and inhibitory (down) synapses in a fully reversible fashion. In contrast, parallel fibers and inhibitory synapses on apical dendrites of FC do not show a scaling response. We also provide evidence that the same neurons of the contralateral side to the earplug redistributed AMPAR subunits.

Synaptic scaling of cochlear nucleus neurons

Auditory nerve synapses respond to sound attenuation similarly to the visual cortex after monocular deprivation, or in hippocampal, spinal cord and cortical cultured cells after a decrease or block of synaptic activity. This response is characterized by upregulating synaptic AMPARs (O'Brien et al., 1998; Harms et al., 2005; Sutton et al., 2006; Hou et al., 2008; Ibata et al., 2008). After 1-day of monaural earplugging the auditory nerve synapses on BCs and FCs, increased the expression of AMPAR GluR3. Unlike the results of studies in cultured cells from other brain regions (O'Brien et al., 1998; Wierenga et al., 2005) we did not detect changes for GluR2. The upregulation for this subunit might depend on the experimental procedure (Sutton et al., 2006; Hou et al., 2008; Rabinowitch and Segev, 2008). The synapses of the auditory nerve on BCs and FCs are mainly characterized by AMPAR complexes formed by GluR2, GluR3 and GluR4. Both synapses, however, differ in their permeability to Ca^{++} due to lower presence of GluR2 at the auditory nerve-BC synapse (Wang et al., 1998; Gardner et al., 2001). Therefore, the selective targeting and incorporation of GluR3 subunits at these two excitatory synapses can result in the formation of more Ca^{++} -permeable AMPA channels. This is consistent with recent reports suggesting that reduced synaptic activity causes the formation of GluR2-lacking AMPARs (Ju et al., 2004; Sutton et al., 2006; Thiagarajan et al., 2005; Hou et al., 2008). Although the mechanism is still unknown, it is suggested that the expression of homeostatic regulation might involve AMPAR-gated calcium signaling and/or glia released TGF- α (tumor necrosis factor- α) that has been shown to increase surface expression of AMPARs in response to a decrease in neuronal activity (Stellwagen and Malenka, 2006). Supporting the role of glial cells in this process we found signs of astrocytic activation in response to sound attenuation (E.C., M.E.R., unpublished observations). Consequently, more calcium-permeable AMPARs might enhance the plasticity of auditory nerve synapses to compensate for the loss of hearing by earplugging. Interestingly, we showed that bushy and fusiform cells responded differentially to conductive hearing loss, since the auditory nerve-FC synapse presented also a decrease in the expression for GluR4. This was an unexpected result that might not fit under the definition of synaptic scaling, which is defined as an accumulation of synaptic AMPARs in response to decrease synaptic activity (Turrigiano and Nelson, 2004; Davis, 2006). A downregulation for GluR4 was found at this synapse few hours after peripheral deafferentation (Rubio, 2006). Studies in other systems did not investigate how synapses scale the GluR4 subunit in response to modifications of synaptic activity. Thus, we are unable to compare our results to published literature. Nevertheless, our data suggest that this downregulation is related to modifications of auditory nerve activity, since it reversed to control levels after earplug removal. All together, our results suggest that the synaptic/neuronal response to activity is individually regulated and that it probably depends on the utilization of distinct molecular pathways by the postsynaptic neuron.

Neuronal networks have to maintain a balance between excitation and inhibition for normal brain function. Thus, as with excitatory synapses, inhibitory synaptic strengths are scaled up or down by changes in activity (Nusser, 1998; Kilman et al., 2002). Synaptic scaling of inhibitory synapses is achieved by changing the number of GABA_ARs or GlyRs clustered at synaptic sites (Nusser et al., 1998; Kilman et al., 2002; Lévi et al., 2008). In cochlear nucleus, glycine mediates the balance of auditory nerve excitation through the activation of GlyRs (Hirsch and Oertel, 1988; Mildbrandt and Caspary, 1995; Golding and Oertel, 1996; Potashner et al., 2000). Excitation increases GlyR levels at synapses of spinal cord cultures (Lévi et al., 2008). Similarly, inhibition of activity may decrease GlyR levels at synapses. After 1-day of monaural conductive hearing loss, inhibitory synapses on bushy and fusiform cells presented a fully reversible response by decreasing GlyR α 1. The same neurons had shown reappointment of specific AMPAR subunits at the auditory nerve synapse. Thus, by scaling up or down excitatory and inhibitory synapses, cochlear nucleus neurons can integrate and transmit precisely, normal and abnormal fluctuations of auditory nerve activity to upper auditory pathways. ABR thresholds indicated that earplugging effectively attenuated sound by an average of 19 dB. Furthermore, after removal of the earplug the ABR thresholds of the plugged and unplugged ears were indistinguishable from their pretreatment level. Thus, although we observed changes in AMPA and GlyR subunit expression and distribution we did not detect any significant changes in the monaural sensitivity of the peripheral auditory system to broadband click stimuli. This does not preclude the possibility that differences in auditory sensitivity might be revealed through more refined electrophysiological measures of auditory function.

Parallel fibers on FCs do not show scaling in response to 1-day monaural conductive hearing loss

Neurons in general, can globally adjust synaptic strength in response to changes in their own firing rates (Ibata et al., 2008), meaning that neurons proportionally scale all the synapses up or down (Turrigiano and Nelson, 2004; Burrone and Murthy, 2003). Alternatively, there could be a local induced synaptic scaling response, which would cause the synapse to be locally scaled in strength and this could contribute to the stabilization of neuronal activity (Rabinowitch and Segev, 2008). These two ideas are currently under debate. In this study we addressed *in vivo* and ultrastructurally whether changes of activity of inputs to the basal dendrites of FCs affects synapses in a separate group of dendrites, the apical dendrites receiving the parallel fibers. As discussed above, the auditory nerve synapse responded to 1-day earplugging by redistributing specific AMPAR subunits. Our data, however, showed no change in the expression for the two main AMPAR subunits (GluR2 and GluR3) at parallel fibers synapses. Earplugs would be expected to have very little effect on the activity of parallel fibers because few granule cells are driven by acoustic input (Brown et al. 1988; Benson and Brown, 2004). These neurons preferentially receive somatosensory, vestibular and descending inputs (Itoh et al. 1987; Weinberg and Rustioni 1987; Caicedo and Herbert 1993; Weedman and Ryugo 1996; Wright and Ryugo 1996; Shore et al. 2000; Haenggeli et al. 2005; Zhou and Shore 2004; Schofield and Coomes 2005) that would not be expected to be altered by attenuating the acoustic input to one ear. Alternatively, our inability to detect changes in expression might be due to a slower response compared to the other synapses. Although, this study did not address this issue, we recently reported changes in AMPARs at parallel fiber-FC synapses after 2-days of peripheral denervation (Rubio, 2006). This would suggest that other excitatory synapses on the same neuron could show scaling, but at a longer time-scale.

The contralateral side to the earplugging responds to ~20 dB sound reduction

The first site for convergence of binaural information occurs at the level of the cochlear nucleus (see for review Cant and Benson, 2003). Cochlear nuclei interact with each other through commissural projections (Alibardi, 2003; Shore et al., 2003) or via descending inputs from

upper auditory nuclei (Sprangler et al., 1987; Shore et al., 1991). Evidence of this communication includes studies showing that contralateral sound stimulation suppresses auditory nerve activity through the olivocochlear bundle (Liberman and Brown, 1986; Warren and Liberman, 1989a,b; Darrow et al., 2006). In addition, another set of experiments indicate that contralateral inputs to cochlear nucleus compensate rapidly for large changes in afferent input (Sumner et al., 2005), probably by redistributing AMPA receptors at auditory nerve synapses as occurs after unilateral peripheral denervation (Rubio, 2006). In this study, we investigated whether bushy and fusiform cells on the side contralateral to earplugging present a homeostatic response to compensate the unilateral sound reduction. As occurs after deafferentation (Rubio, 2006), the synapses of the auditory nerve of the contralateral side upregulated AMPAR GluR3, but not GluR2. As discussed above, this may result in the formation of more Ca^{++} -permeable AMPA channels, thus strengthening the existence of calcium mediated postsynaptic signaling mechanisms, triggered by modifications of presynaptic activity. In addition, the study by Darrow and colleagues (2006) showed that lateral olivocochlear efferents balance the strength of inputs to the two ears. It seems then possible that the reduction of sound level to one ear affects signaling by the lateral olivocochlear efferents that then alter the firing rate of auditory nerve fibers contralateral to the plugged ear. Therefore, the observed changes in AMPA receptors could be the response to altered firing rates of afferents.

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Figure 1. Schematic of the synaptic circuitry of the cochlear nucleus

AN: auditory nerve on basal dendrites of fusiform cells (FC) and on the cell body of bushy cells (BC). The AN on the right represents the side of the earplug; PF: parallel fibers of the granule cells (GC) synapsing on apical dendrites of FC. GC can receive excitation from the AN type-II fibers, and from somatosensory inputs (SS). *Intrinsic inhibitory interneurons:* CwC: cartwheel cells on apical dendrites of FC; VC: vertical cells on basal dendrites of FC and BC somata. The auditory nerve also synapses on VC (not shown). Other inhibitory inputs on basal dendrites of FC and BC are represented as small endings. These endings may belong to T-stellate cells in the ventral cochlear nucleus or from upper auditory nuclei. Dash line represents the midline and the arrow indicates the existence of commissural fibers communicating the cochlear nucleus of both sides.

Figure 2. ABR thresholds

A) ABR response to 1msec click stimulus delivered 20dB above threshold. Scale bar: 1 msec, 0.5 μ V. The first short latency component is clearly visible (arrow).

B) Average ABR threshold for treated and untreated side ($n = 7$ animals). **B₁**. Average ABR threshold prior to ear plugging was 33.4 ± 4.4 dB SPL. With the earplug in place the average ABR threshold increased to 53.4 ± 5.7 dB SPL (** $p < 0.01$). When the earplug was removed after being in place for 24 hours the ABR threshold for the treated side (32.6 ± 4.3 dB SPL) was not significantly different from the pretreatment threshold ($p > 0.05$). **B₂**. There was no significant difference in the ABR threshold of the untreated side before (33.0 ± 3.5 dB SPL) and after (34.3 ± 3.4 dB SPL) treatment of the contralateral ear ($p > 0.05$).

Figure 3. Unilateral earplug affects the synaptic expression of GluR2/3 but not GluR2 or GluR4 at the auditory nerve-bushy cell synapse

A) Electron micrographs show postembedding immunogold labeling (5 nm gold) for GluR2/3, GluR2 and GluR4 at the PSD of the auditory nerve (AN) on bushy cells (BC) somata of normal hearing (A1, A4, A7) and earplugged animals (A2–3, A5–6, A8–9). Scale bar: 0.25 μm for low and 0.1 μm for magnified images.

B) Histograms show the density of gold particles/ μm length of PSD $\pm$ SEM for GluR2/3, GluR2 and GluR4 at the AN on BC of normal hearing animals (control $n=3$) and after 1-day of unilateral earplugging ($n=2$) of the plugged side and contralateral-unplugged side to the earplug (** $p<0.01$; *** $p<0.001$).

Figure 4. Unilateral earplug affects the synaptic expression of GluR2/3 and GluR4 but not GluR2 at the auditory nerve-fusiform cell synapse

A) Electron micrographs show postembedding immunogold labeling (5 nm gold) for GluR2/3, GluR2 and GluR4 at the PSD of the auditory nerve (AN) on basal dendrites of fusiform cells (FC) of normal hearing (A1, A4, A7) and ear plugged animals (A2–3, A5–6, A8–9). Scale bar: 0.25 um for low and 0.1um for magnification images.

B) Histograms show the density of gold particles/um length of PSD $\pm$ SEM for GluR2/3, GluR2 and GluR4 at the AN on FC of normal hearing animals (control $n=3$) and after 1-day of unilateral earplugging ($n=2$) of the plugged side and contralateral-unplugged side to the earplug (* $p < 0.05$; *** $p < 0.001$).

Figure 5. Unilateral earplug does not affect the synaptic expression of GluR2/3, GluR2 and GluR4 at the synapse of the parallel fibers on fusiform and cartwheel cells

A) Electron micrographs show postembedding immunogold labeling (5 nm gold) for GluR2/3 and GluR2 at the PSD of the parallel fibers on apical dendrites of fusiform cells, as well on cartwheel cells. Scale bar: 0.25 μ m for low and 0.1 μ m for magnification images.

B) Histograms show the density of gold particles/ μ m length of PSD $\pm$ SEM for GluR2/3, and GluR2 and GluR4 at the parallel fibers on fusiform and cartwheel cells of normal hearing animals and after 1-day of unilateral earplugging of the plugged side to the earplug ($p > 0.05$).

Figure 6. Decrease in the expression of GlyRa1 at inhibitory synapses on bushy and fusiform cells
A) Electron micrographs show postembedding immunogold labeling (5 nm gold) for GlyRa1 at the PSD of inhibitory synapses on BC somata and on basal dendrites of FC of normal hearing (A1 and A4), and earplugged animals (A2–3). Scale bar: 0.25 μm for low and 0.1 μm for high magnification images.

B) Histograms show the density of gold particles/ μm length of PSD $\pm$ SEM for GlyRa1 at the inhibitory synapses on BC somata and on basal dendrites of FC of normal hearing animals (control $n=3$) and after 1-day of unilateral earplugging ($n=2$) of the plugged side and contralateral unplugged side to the earplug (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

Figure 7. Synaptic changes in expression for AMPA and GlyR subunits are reversible after earplug removal

Histograms show the density of gold particles/ μm length of PSD $\pm$ SEM for GluR2/3, GluR4 at the AN on BC and on FC, and GlyR α 1 at inhibitory synapses on BC somata and basal dendrites of FC of normal hearing animals (control $n=2$) and after 1-day of earplug removal ($n=2$).

Table 1

Number of postsynaptic densities analyzed quantitatively after postembedding immunogold labeling in normal and earplugged (EP) animals.

	AMPA			Glycine R
	GluR2	GluR2/3	GluR4	$\alpha 1$
Normal hearing (n=3)				
AN/BC	40	81	58	---
Inhibitory synapses on BC	---	---	---	50
AN/FC	40	30	30	---
Inhibitory synapses on FC (basal dendrites)	---	---	---	30
Inhibitory synapses on FC (apical dendrites)	---	---	---	20
PF/FC	30	30	---	---
PF/CwC	30	30	---	---
1 day EP (n=2)				
AN/BC				
Ipsi.	40	58	52	---
Contra.	54	61	43	---
Inhibitory synapses on BC				
Ipsi.	---	---	---	40
Contra.	---	---	---	35
AN/FC				
Ipsi.	37	43	40	---
Contra.	25	30	30	---
Inhibitory synapses on FC (basal dendrites)				
Ipsi.	---	---	---	38
Contra.	---	---	---	27
Inhibitory synapses on FC (apical dendrites)				
Ipsi.	---	---	---	20
PF/FC-Ipsi.	30	30	---	---
PF/CwC-Ipsi.	30	32	---	---
1 day EP + 1 day after removal (n=2)				
AN/BC-Ipsi.	---	35	---	---
Inhibitory synapses on BC-Ipsi	---	---	---	32
AN/FC-Ipsi.	---	30	25	---
Inhibitory synapses on FC-Ipsi	---	---	---	30

Table 2

Range and number of gold particles per PSD

	Normal Hearing	EP-Plugged side	EP-Unplugged side
AVCN			
AN-BC			
GluR2/3			
Range	0 – 8	2 – 22	1 – 25
Gold part./PSD	1.8	7.4	7.3
GluR2			
Range	0 – 8	0 – 6	0 – 8
Gold part./PSD	2	1.8	1.5
GluR4			
Range	0 – 6	0 – 8	0 – 9
Gold part./PSD	2.3	2.3	3.0
GlyRa1			
Range	1 – 11	0 – 5	1 – 9
Gold part./PSD	2.5	1.6	2.8
DCN			
AN-FC			
GluR2/3			
Range	0 – 8	1 – 11	1 – 8
Gold part./PSD	2.0	4.8	3.3
GluR2			
Range	0 – 8	0 – 7	1 – 8
Gold part./PSD	2.0	1.5	3.3
GluR4			
Range	0 – 7	0 – 5	1 – 5
Gold part./PSD	3.7	1.5	2.7
Inhibitory synapses on FC (basal dendrites)			
GlyRa1			
Range	1 – 13	0 – 8	0 – 11
Gold part./PSD	5.3	2.0	4.1
PF-FC			
GluR2/3			
Range	0 – 9	0 – 10	
Gold part./PSD	3.9	4.1	
GluR2			
Range	0 – 8	0 – 7	
Gold part./PSD	2.6	2.4	
PF-CwC			
GluR2/3			
Range	1 – 11	0 – 13	

	Normal Hearing	EP-Plugged side	EP-Unplugged side
Gold part./PSD	4.1	4.0	
GluR2			
Range	0 – 7	0 – 7	
Gold part./PSD	3.0	2.8	
Inhibitory synapses on FC (apical dendrites)			
GlyRa1			
Range	1 – 11	1 – 12	
Gold part./PSD	6.5	7.0	