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Research Report

Auditory nerve input is not an absolute requirement for the expression, distribution and calcium permeability of AMPA receptors in the adult rat ventral cochlear nucleus

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ABSTRACT

In order to understand whether glutamatergic excitatory presynaptic input is an absolute requirement for the adult regulation of postsynaptic glutamate receptors we analyzed if a period of 11 days of excitatory deprivation affects the expression, distribution and Ca^{2+} permeability of AMPA receptor subunits in the ventral cochlear nucleus of the rat. Bilateral cochlear ablations were performed in 30-day-old rats. After 11 days of survival, immunohistochemistry for GluR1, GluR2/3 and GluR4 AMPA receptor subunits showed no changes in the normal pattern of distribution, with GluR2/3 and GluR4 immunoreactivity predominating, and little GluR1. No changes in the amount of these AMPA receptor subunits were found between normal and cochleotomized rats in Western blots. AMPA receptors lacking the GluR2 subunit are Ca^{2+} permeable. Kainate-induced Co^{2+} uptake histochemistry, which labels AMPA Ca^{2+} permeable receptors, demonstrated no changes in somatic labeling intensity for Co^{2+}, 11 days after cochleotomy. Therefore, our data indicate that excitatory input is not an absolute requirement to maintain AMPA receptor subunit expression, distribution and functional properties such as Ca^{2+} permeability in VCN neurons. Nevertheless, subtle changes in AMPA receptors through regulatory post-transductional mechanisms cannot be ruled out.

1. Introduction

Excitatory activity influences the survival and functional maintenance of central neurons. Neurons in the cochlear nuclei (CN), the first central relay station of the auditory pathway in the brainstem (Morest, 1993), receive primary excitatory glutamatergic input from auditory nerve axons originating from spiral ganglion neurons in the cochlea. Many CN neurons die when connections from the auditory nerve are interrupted during a critical developmental period extending to the time of hearing onset. When this critical period is over, interruption of afferent cochlear input to CN neurons no longer leads to substantial neuronal death (Born and Rubel, 1985; Lachica et al., 1996; Mostafapour et al., 2000; Rubel and Fritzsch, 2002; Suneja et al., 2000; Tierney et al., 1997). However, adult CN neurons deprived of excitatory input, in particular spherical cells in its ventral division (ventral cochlear nucleus, VCN), show atrophy as seen by

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decreased neuronal size and altered shapes (Lesperance et al., 1995; Moore, 1990; Tierney et al., 1997). Atrophy is a generic cellular reaction to injury, which usually reflects impaired protein synthesis and turnover. Lowered rates of protein synthesis have been found in CN neurons after cessation of auditory activity (Steward and Rubel, 1985). Along with impaired protein synthesis, diminished oxidative metabolism (Durham and Rubel, 1985) and altered electrophysiological properties (Francis and Manis, 2000) have been also reported in CN neurons after auditory deprivation.

Such cellular reactions are relevant to establish the general framework of the neuronal response to the loss of excitatory input. However, the specific impact of excitatory deprivation will be largely a function of the involvement of excitatory synaptic activity in the regulation of specialized signaling molecules in specific neuronal populations. It is therefore important to address whether the expression, distribution and functions of neurotransmitter receptors involved in signal processing in adult CN neurons depend on the integrity of auditory nerve endings. In this regard, postsynaptic ionotropic glutamate receptors are of particular interest. They mirror auditory nerve endings, thus being under the most direct influence of excitatory activity from the auditory nerve.

Ionotropic glutamate receptors of the AMPA class predominate in adult CN neurons (Hunter et al., 1993; Petralia et al., 2000; Wickesberg and Oertel, 1989). The AMPA receptor is a tetrameric complex that forms an ion channel primarily permeable to Na^+ and K^+ which is assembled by combinations of four different polypeptide subunits, GluR1 to GluR4 (Mano and Teichberg, 1998; Rosenmund et al., 1998). It has been shown by immunohistochemistry that AMPA receptors in the VCN of the adult rat are assembled mainly from GluR3 and GluR4 subunits (Caicedo and Eybalin, 1999; Wang et al., 1998). Electrophysiological studies have also revealed that most cell types receiving auditory nerve input in the VCN have AMPA receptors that share subunit composition and properties; they are assembled by GluR3 and GluR4 subunit variants with exceptionally fast kinetic properties (Gardner et al., 2001; Lawrence and Trussell, 2000). Moreover, absence of the GlurR2 subunit from AMPA receptors in CN neurons provides these receptors with important Ca^{2+} permeability properties (Jonas et al., 1994; Otis et al., 1995).

In this work, we have studied how the lack of auditory nerve activity after bilateral cochleotomy in young adult rats (Caminos et al., 2005) affects the expression, distribution and Ca^{2+} permeability properties of AMPA receptors in the VCN. To achieve this goal the expression and distribution of AMPA receptor subunits was analyzed with a combination of immunohistochemistry and Western blot. To test Ca^{2+} permeability of AMPA receptors a Co^{2+} uptake histochemistry method was used. Our results show that neither AMPA subunit presence, localization or Ca^{2+} permeability properties of AMPA receptors are altered in the VCN after 11 days of acoustic deprivation, an arbitrarily defined time period when auditory nerve terminal and fiber degeneration are well established (Benson et al., 1997; Caminos et al., 2005; Illing et al., 1997).

2. Results

2.1. *Assessment of nerve fiber degeneration in cochleotomized rats*

To assess auditory nerve fiber loss in the VCN after cochlear ablation, calretinin immunostaining was compared in normal and cochleotomized rats (Caminos et al., 2005; Illing et al., 1997). In Fig. 1A, abundant fiber immunostaining for calretinin can be seen in a control section of the VCN. However, most immunolabeled calretinin fibers disappeared from the neuropil in the VCN after 11 days of acoustic deprivation (Fig. 1B).

2.2. *Immunohistochemical distribution of AMPA subunits in the VCN from normal and cochleotomized rats*

The distribution of polypeptide subunits assembling AMPA type glutamate receptors was analyzed by immunohistochemistry in the VCN from normal and cochleotomized rats, 11 days after cochlear ablation.

In control animals, very light GluR1 labeling was seen in VCN cells. The cellular distribution of such faint labeling was difficult to appreciate, but it seemed to be preferentially located in somata (Fig. 2A). This labeling pattern was in deep contrast with the more intense labeling seen in the superficial layers of the dorsal cochlear nucleus (DCN), where neuropilar elements showed distinct GluR1 staining (not shown). Localization of GluR2 and/or GluR3 subunits was visualized with an antibody which binds an epitope shared by these two AMPA receptor subunits. GluR2/3 immunoreactivity was clearly visible in many cell bodies of the VCN, with very little neuropilar staining (Fig. 2D). As far as GluR4 is concerned, staining for this subunit was also present in the VCN. The pattern of staining was more diffuse than that of GluR2/3 (Fig. 2G). Both cell bodies and neuropilar structures showed GluR4

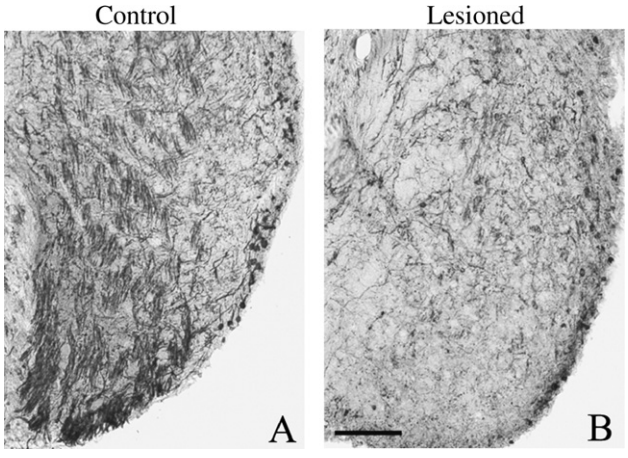

Fig. 1 – Low magnification photomicrographs showing massive nerve fiber loss in the VCN as seen by calretinin immunostaining. (A) Coronal section from a control animal. (B) Coronal section at the same level 11 days after cochlear ablation. Abundant calretinin immunoreactive fibers are observed in control animals in contrast to the dramatic decrease seen in the lesioned animals. Scale bar: 100 μm.

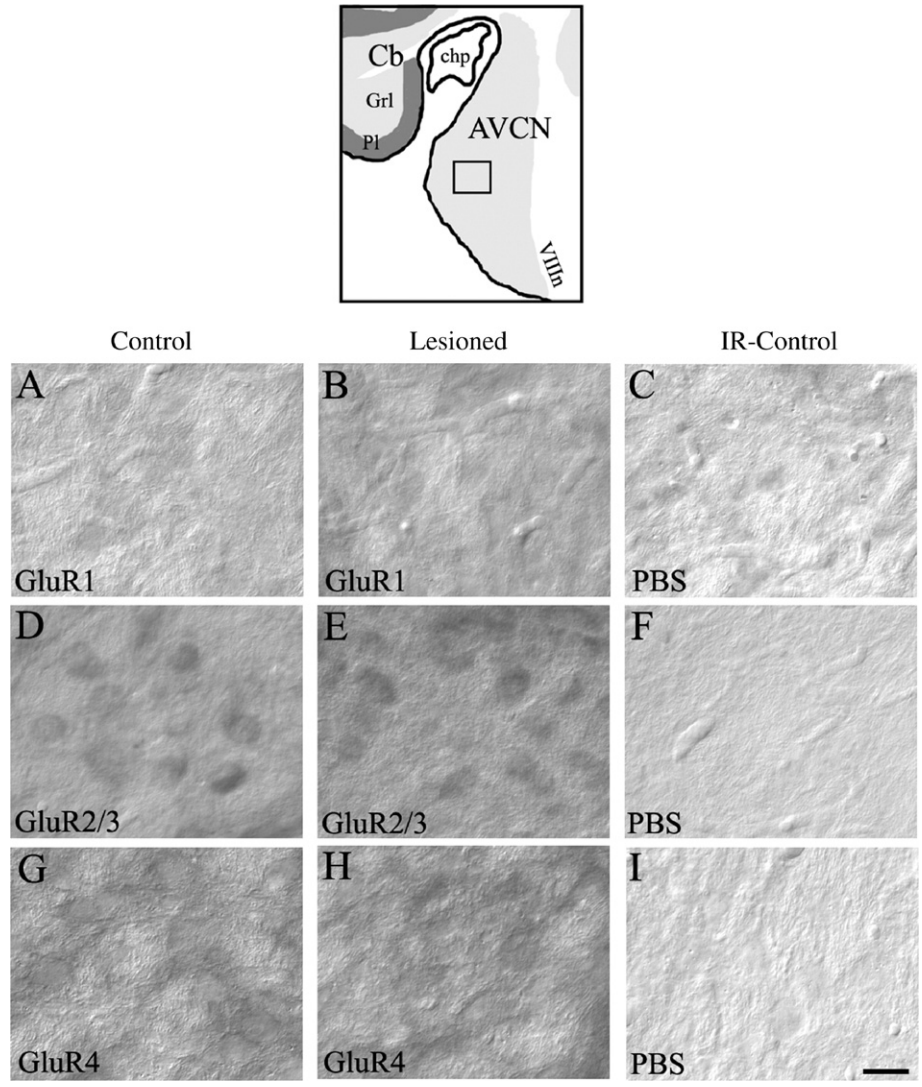

Fig. 2 – Immunohistochemical labeling of AMPA receptor subunits in the VCN from control and lesioned rats. The square in the schematic drawing shows the region of the VCN where the pictures were obtained. GluR1 immunoreactivity in control (A) and lesioned (B) animals. Labeling is scarce in both conditions. GluR2/3 immunoreactivity in control (D) and lesioned animals (E). GluR2/3 labeling is clearly visible, with no staining differences between the cell bodies of control and deafferented VCN neurons. GluR4 immunoreactivity in sections from control (G) and lesioned (H) animals. GluR4 staining is unchanged after the lesion. No specific immunostaining is detected in control sections (IR-control: immunoreactivity control) processed without primary antibody (C, F and I). AVCN: anteroventral cochlear nucleus; Cb: cerebellum; chp: choroid plexus; Grl: granule cell layer; Pl: Purkinje cell layer; VIIIn: vestibulocochlear nerve. Scale bar: 20 μm.

labeling in the VCN. It was not unusual to see dendritic trunks steming from parent cell bodies which were labeled for this AMPA receptor subunit.

In the VCN of cochleotomized animals, labeling for these AMPA receptor subunits was identical to that seen in control animals. Specific GluR1 immunoreactivity was faint and scarce (Fig. 2B). GluR2/3 immunoreactivity patterns were identical to those found in control rats with labeling concentrated in cell bodies (Fig. 2E). Similar to what was found in normal rats, GluR4 labeling in cochleotomized rats was distributed in cell bodies and neuropilar structures of the VCN (Fig. 2H). No immunoreactivity was found in control sections processed in the absence of primary antibody (Figs. 2C, F and I).

2.3. *Western blot analysis of prevalent AMPA receptor subunits in the CN of normal and cochleotomized rats*

Our immunohistochemical findings showed predominant labeling for GluR2/3 and GluR4 subunits. Western blot analysis was carried out to investigate the possibility that an interruption of auditory nerve activity regulates protein amount of these two prevalent AMPA receptor subunits in the adult VCN.

Western blot experiments in the cochlear nuclei of control animals showed that GluR2/3 and GluR4 antisera labeled one band of approximately 108 kDa, corresponding to the expected molecular weights of the corresponding GluR subunits (Wenthold et al., 1992). An identical distribution of bands was seen in animals in which both cochleae had been surgically removed. Optical density values of GluR2/3 and GluR4 bands from experimental animals were 102% and 99%, respectively, of those found in control animals (Fig. 3). No statistical differences for GluR2/3 ($p=0.88$) and GluR4 ($p=0.92$) between extracts from control and those from cochleotomized animals were found.

2.4. *Ca^{2+} permeability through AMPA channels in VCN neurons from normal and cochleotomized rats.*

AMPA receptors lacking the GlurR2 subunit, such as those which seem to be predominantly expressed in the VCN (Gardner et al., 2001, Wang et al., 1998) are permeable to Ca^{2+} (Jonas et al., 1994). They can be identified with Co^{2+} uptake histochemistry (Pruss et al., 1991). To study possible mid- to long range effects of excitatory deprivation on Ca^{2+} permeability of the AMPA receptor, kainate-induced Co^{2+} uptake was performed in hindbrain slices from normal and cochleotomized rats, 11 days after cochlear ablation. A representative low magnification image of the histochemical labeling for Co^{2+} uptake histochemistry in a brainstem coronal slice containing the VCN is shown in Fig. 4A. Intense Co^{2+} staining was visualized in cell bodies of VCN neurons, in both control and cochleotomized rats (Fig. 4B). Mean values (±SEM) of the relative gray value levels (RGV_{soma}) obtained from sections of control ($n=70$ cells) and lesioned animals ($n=101$ cells) were 53.75±2.74 and 57.86±2.27 respectively (Fig. 4C). No significant differences were detected ($p=0.25$), suggesting that no changes in Ca^{2+} permeability through AMPA receptors take place 11 days after excitatory deprivation of VCN neurons.

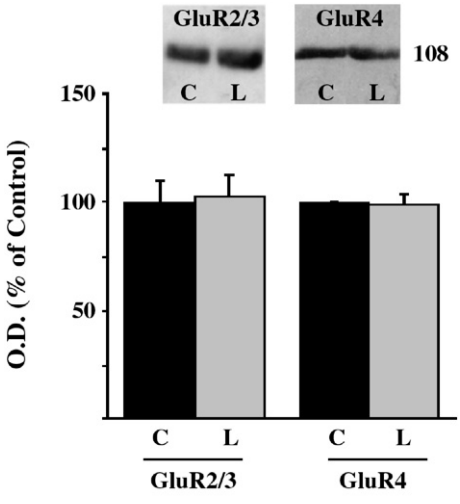

Fig. 3 – Afferent deprivation does not alter protein levels of AMPA receptor subunits in the VCN. Optical density mean values from Western blots of GluR2/3 and GluR4 proteins obtained from control (C lanes) and lesioned animals (L lanes). Differences between control and lesioned animals were not statistically significant (unpaired Student's *t* test).

3. Discussion

We have tested whether the interruption of excitatory activity produced after 11 days of cochlear ablation, alters the cellular integrity of polypeptide subunits assembling ionotropic glutamate AMPA receptors in second order neurons of the VCN. Regardless possible time-related changes, which are not analyzed in this work, 11 days after cochlea removal, a time period long enough to lead to auditory nerve degeneration, there are no changes either in the amount of major AMPA receptor subunits in the adult VCN as seen by Western blot, in their distribution, as seen by immunocytochemistry, or in Ca^{2+} permeability properties tested by Co^{2+} uptake histochemistry.

3.1. *Removal of auditory nerve input to VCN neurons*

Neurons in magnocellular regions of the VCN receive excitatory glutamatergic input from auditory nerve fibers and this is the major source of glutamatergic excitation that these neurons receive (Altschuler et al., 1981; Altschuler et al., 1986; Juiz et al., 1993, Rubio and Juiz, 1998; Wickesberg and Oertel, 1989). The interruption of auditory nerve axons shuts down excitatory activity on VCN neurons, providing a useful model to study the influence of excitatory glutamatergic activity on the expression and distribution of postsynaptic signaling molecules.

Eleven days after bilateral cochlea removal there was a dramatic decrease in calretinin immunoreactivity in nerve fibers of the VCN, an indication of massive fiber degeneration. Extensive decrease in calretinin immunostaining has been shown previously 11 days after cochleotomy in the rat CN (Caminos et al., 2005). Actually, such a decrease was detectable 4 days after cochlea removal (Illing et al., 1997). Also, abundant fiber degeneration was found 7 days after cochleotomy in the VCN of adult guinea pigs (Benson et al., 1997). Furthermore, auditory-evoked brainstem response (ABR) thresholds are virtually undetectable in both sides 11 days after bilateral cochleotomy (unpublished observations). Therefore our experimental approach of bilateral cochlear ablation leads to massive degeneration of auditory nerve axons and terminals and abolishes excitatory input to VCN neurons.

3.2. *Distribution of AMPA receptor subunits in the VCN by immunocytochemistry in normal and cochleotomized animals*

Using light microscopy immunohistochemistry, we tested whether excitatory deprivation alters the cellular or subcellular distribution of AMPA receptor subunits in adult VCN neurons. AMPA receptors are the most abundant and best characterized glutamate receptors in the main principal projecting neurons of the VCN: spherical, globular, stellate and octopuss cells (Isaacson and Walmsley, 1995; Wickesberg and Oertel, 1989). Furthermore, in this CN division, AMPA receptors exhibit very rapid deactivation and desensitization

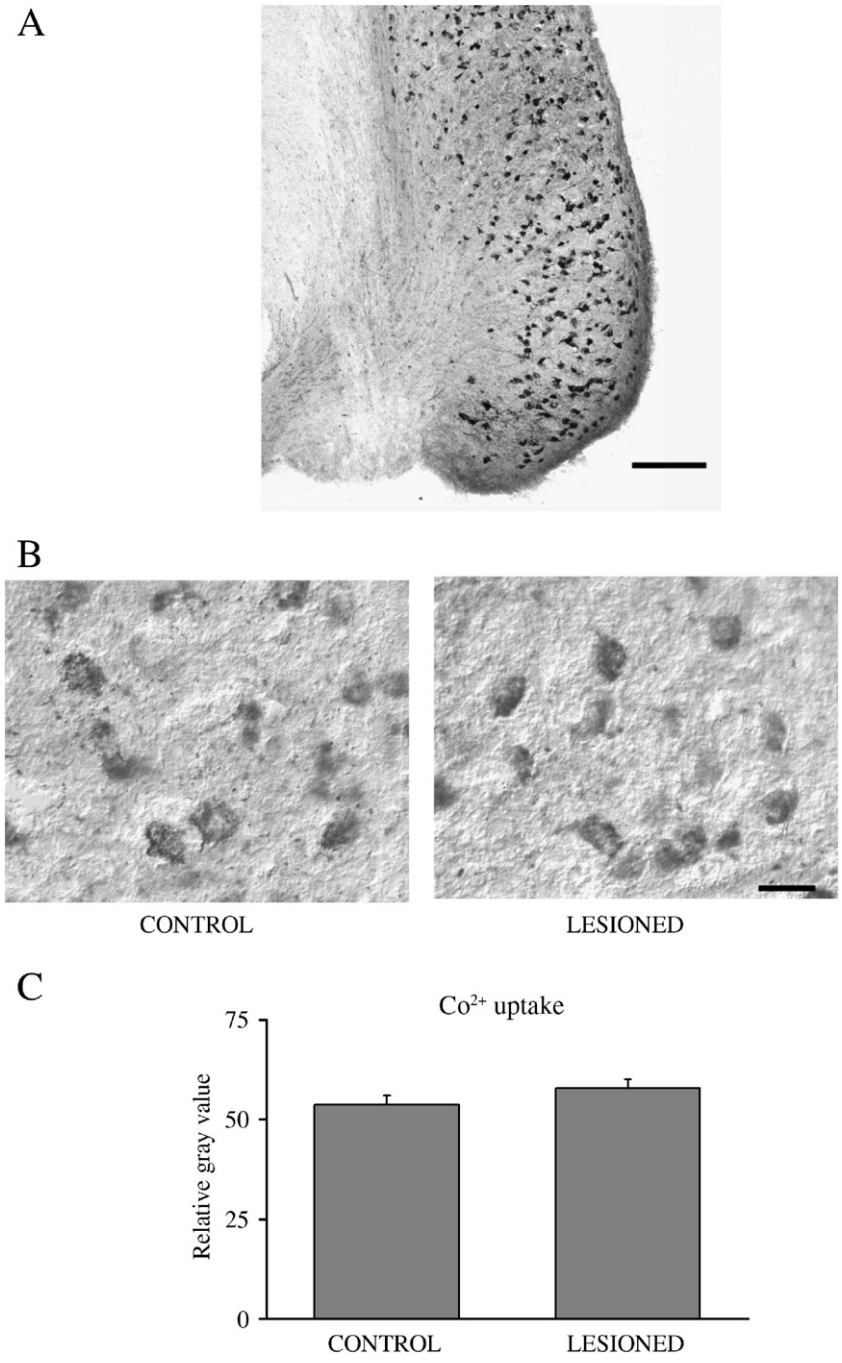

Fig. 4 – Ca^{2+} permeability of AMPA receptors in the VCN is unchanged after deafferentation. (A) Representative low magnification photomicrograph of Co^{2+} uptake histochemistry from a cryostat section obtained from a hindbrain slice containing the VCN. (B) High magnification photomicrographs of Co^{2+} uptake labeling in the VCN from control and lesioned animals show no staining differences. (C) Densitometry analysis of Co^{2+} uptake labeling in cell bodies of the VCN shows no significant differences between control and lesioned animals (unpaired Student's *t* test). Scale bars: (A) 200 μm, (B) 20 μm.

rates with high Ca^{2+} permeability (Gardner et al., 2001; Lawrence and Trussell, 2000; Raman et al., 1994). This points to an abundance of AMPA receptors assembled by combinations of GluR3 and GluR4 subunits, likely with "flop" splice variants, which are responsible for such rapid kinetic properties. Conversely, GluR1 and GluR2 subunits barely participate in the assembly of AMPA receptors in adult VCN neurons (Gardner et al., 2001). In agreement with these data, our results in control animals showed hardly detectable staining for GluR1. In contrast, there was somatic labeling for GluR2/3, and both somatic and dendritic labeling for GluR4. This pattern is coincident with the AMPA subunit distribution found in

previous immunohistochemical studies in the adult CN (Caicedo and Eybalin, 1999; Wang et al., 1998).

The GluR subunit labeling pattern seen in control animals was preserved 11 days after cochlear ablation, with labeling intensities and cellular and subcellular distributions identical to those found in control animals. These results support that afferent deprivation does not necessarily or permanently alters the distribution of AMPA receptor subunits in VCN neurons. Neither it changes the combination of AMPA subunits expressed in this CN division. However, the possibility that the amount rather than the cellular distribution of polypeptide subunits had been altered after cochlear ablation and cessation of auditory nerve activity needed to be further explored.

3.3. *Expression of AMPA receptor subunits in the VCN by Western blot in normal and cochleotomized animals*

Western blot of GlurR2/3 and GluR4 subunits were performed in control and cochleotomized rats. For the first time, Western blot was used to demonstrate presence of AMPA GluR subunits in the rat CN. In control animals, GluR2/3 and GluR4 labeling were found in bands corresponding to their expected molecular weights. No significant quantitative changes were found in the CN of cochleotomized animals, suggesting that the amount of AMPA GluR subunits expressed in CN neurons was not affected by the lack of glutamatergic excitatory presynaptic activity from the auditory nerve. The possibility of changes in the amount of AMPA receptor subunits in the CN after cochlear ablation was suggested by increases in [^{3}H]AMPA binding in the CN ipsilateral to the lesioned side at survival times from 13 to 145 days (Suneja et al., 2000). However, after correcting for postlesion shrinkage, increases in binding levels were not evident (Suneja et al., 2000). On the other hand, Marianowski et al. (2000) reported that as a consequence of bilateral deafening by aminoglycoside administration, the developmental switch between "flip" and "flop" forms of the subunits of the AMPA receptor is eliminated, with "flip" isoforms predominating. Therefore, the possibility of changes in the receptor structure at more subtle levels than protein amount remains open.

3.4. *Ca^{2+} permeability of AMPA receptors in VCN neurons from normal and cochleotomized rats*

A property common to AMPA receptors in VCN neurons is Ca^{2+} permeability. Kainate-induced Co^{2+} uptake histochemistry (Pruss et al., 1991) allows visualization of neurons with Ca^{2+} permeable AMPA receptors and is useful to estimate Ca^{2+} influx through AMPA receptors. Along with supplying information about pharmacological properties of AMPA channels in slices maintained "in vitro", this technique also allows to determine presence or absence of GluR2 subunits in the composition of AMPA receptors in specific cell types. Stimulation of normal control brainstem slices with kainate in the presence of $CoCl_2$ showed high calcium permeability through AMPA receptors in the VCN, as shown previously (Caicedo et al., 1998; Ravindranathan et al., 2000; Zhou et al., 1995). This supports that the GluR2 subunit does not participate substantially in the assembly of AMPA receptor in this CN division (Gardner et al., 2001). The finding that Ca^{2+} permeability, as determined by densitometry of somatic labeling, does not change after auditory deprivation in the VCN, suggests that the GluR2 subunit is not overexpressed after cessation of auditory nerve activity.

Therefore, AMPA receptor composition, distribution and Ca^{2+} permeability in CN neurons 11 days after interruption of excitatory activity from the auditory nerve is virtually identical to that seen in normal animals. Other glutamate ionotropic receptors, i.e. NMDA receptors, seem to have a similar behavior. Unpublished observations from our laboratory indicate that lack of excitatory input does not affect the amount of the NMDAR1 subunit in the CN. Interestingly, Sato et al. (2000) reported recovery of NMDAR1 mRNA levels to control levels 20 days after cochlear surgery. Also, no changes were found in NMDAR1, NMDAR2A and GluR2 expression in vestibular nuclei 2 weeks after unilateral labyrinthectomy (King et al., 2002).

Although we did not find changes in AMPA receptor composition by Western blot, in their distribution, by immunocytochemistry or in their function, by histochemical measures of Ca^{2+} permeability after a period of excitatory deprivation, regulation by excitatory signals could still be taking place at the post-transductional level in CN neurons, for instance with changes in AMPA receptor trafficking (Sheng and Lee, 2001), which may lead to redistribution of AMPA receptor subunits as shown recently by Rubio (2006). Also changes in the phosphorylation state of the receptor cannot be ruled out (Wang et al., 2005). These possibilities, as well as different mechanisms of regulation by excitatory activity taking place at different levels of the auditory pathway (Holt et al., 2005; Kotak et al., 2005) need to be further explored.

4. Experimental procedures

4.1. *Distribution of experimental animals*

Twenty-seven 30-day-old Wistar rats (Charles River, Barcelona, Spain and Animal House of the Universidad de Castilla-La Mancha, Albacete, Spain) were used. The care and handling of the animals were supervised by the Animal House Facility of the Universidad de Castilla-La Mancha, and were approved by institutional committees. Experimental procedures were in accordance with the EU and with national regulations for the use and care of animals in biomedical research.

Bilateral cochlear ablations were performed on fifteen rats, as described below. Twelve rats were used as matched controls. The number and distribution of animals according to the methods utilized in this work are summarized in Table 1.

4.2. *Surgical procedure for bilateral cochlear ablation*

Animals were deeply anaesthetized with ketamine (100 mg/kg, Parke-Davis, Alcobendas, Spain) and xylazine (10 mg/kg, Dibapa, Barcelona, Spain). Following a retroauricular incision, the bulla was opened until the bulging cochlea was visible. The bony wall of the cochlea was perforated and the interior, including the modiolus with the spiral ganglion, was cleared.

Table 1 – Summary of the distribution of experimental animals

P41 (11 days after bilateral cochleotomy)	P41 (controls)	Methods
3	3	Immunohistochemistry
6	6	Western blot
6	3	Co^{2+}-uptake histochemistry

Total number of animals = 27.

After surgery, animals were allowed to recover on a hot blanket until they regained consciousness, and were housed under the same standard conditions as the control animals. Microscopic inspection of the dissected cochlea was performed in order to evaluate the complete extent of cochlear ablation.

Eleven days after surgery (at P41), experimental animals and matched controls were anaesthetized as indicated above and sacrificed. The brains were dissected out and processed for immunocytochemistry, Western blot analysis or Co^{2+} uptake histochemistry, by following the protocols described below.

Random sets of sections from the same animals processed for AMPA receptor subunit immunocytochemistry were used to test the extension of auditory nerve fiber loss in the CN after cochlear ablation by running immunocytochemical labeling with a polyclonal antibody against calretinin (Swant, Bellinzona, Switzerland) at a dilution of 1:500, following the method described previously (Caminos et al., 2005) (Fig. 1).

4.3. *Immunocytochemistry*

Eleven days after surgical ablation of the cochlea, lesioned animals and their corresponding controls (Table 1) were deeply anaesthetized with ketamine and xylazine as described above. They were perfused through the left ventricle with 4% paraformaldehyde in 0.1M phosphate buffer (PB), pH 7.4. Their brains were dissected out and postfixed overnight in fresh fixative at 4 °C. Fifty μm thick coronal sections of the hindbrain containing the cochlear nuclei were cut using a tissue slicer (VT1000S; Leica, Nussloch, Germany). Free floating sections were incubated in phosphate buffered saline (PBS) containing 3% hydrogen peroxide (H_2O_2) for 30 min at room temperature, to block endogenous peroxidase activity. After washing three times in PBS for 5 min, sections were incubated in normal horse serum for 30 min followed by incubation overnight at room temperature in the following polyclonal antibodies: anti-GluR1, anti-GluR2/3 or anti-GluR4 (from Chemicon, Temecula, CA, USA) at 1 μg/ml. After three washes of 5 min each in PBS, sections were incubated in a biotinylated goat anti-rabbit antibody (Vector Laboratories, Burlingame, CA, USA) at 1:100 for 60 min at room temperature. This was followed by extensive washes in PBS, after which sections were incubated in an avidin-biotinylated peroxidase complex (Vectastain ABC-kit, Vector Laboratories, Burlingame, CA, USA) in PBS for 60 min. After washing in PBS, bound peroxidase was reacted with 3,3′-diaminobenzidine as chromogen, diluted at 0.05% in PBS containing 0.005% H_2O_2. After rinsing in PBS the sections were mounted onto gelatin-coated slides and allowed to air-dry, dehydrated and coverslipped. Immunohistochemical labeling was visualized by microscopic inspection with a Leica DMRX-A microscope equipped with differential interference contrast optics. To test the specificity of the immunocytochemical detection system, in all experiments sets of control sections were processed in the absence of primary antibodies, substituting this step by vehicle (PBS) incubation and keeping the remaining steps of the method identical. No staining was found in such control preparations (Fig. 2).

4.4. *Western blots*

Animals in which both cochleae had been surgically ablated, and their corresponding controls (Table 1), were anaesthetized with carbon dioxide prior to decapitation, after which both CNs were removed and homogenized in 2–4 ml of homogenization buffer (250 mM sucrose, 10 mM Tris, 10 mM HEPES, and 10 mM EDTA, pH 7.2) containing protease inhibitors (Sigma, St. Louis, MO, USA). The homogenate was centrifuged at 10,000 rpm for 10 min at 4 °C (Heraeus Biofuge, Charlotte, NC, USA), and the resulting supernatant was centrifuged again at 20,000 rpm for 1 h at 4 °C (Optima XL-100K, Beckman Instruments, Fullerton, CA, USA). The resulting pellet, enriched in membrane proteins, was resuspended in 200–400 μl of homogenization buffer with protease inhibitors, and was stored at −20 °C. Protein concentration was determined using a Micro-BCA protein kit (Pierce, Rockford, IL, USA). Twenty μg of protein were resolved by a SDS-PAGE using an 8% Laemmli system. Gels were electrophoretically transferred to nitrocellulose membranes (Hybond, Amersham Bioscience, Uppsala, Sweden) for 2 h at 1 mA/cm^2 membrane surface using a semidry blotter (Bio-Rad Labs, Hercules, CA, USA). Nitrocellulose-bound proteins were visualized by being stained with Poinceau S, and were then blocked in TBST-milk (50 mM Tris, pH 7.5, 200 mM NaCl, 0.1% Tween 20 and 5% non-fat dry milk) for 1 h. They were further incubated in the same buffer overnight at 4 °C with the following polyclonal antibodies (1 μg/ml): anti-GluR2/3 and anti-GluR4 (from Chemicon, Temecula, CA, USA). After 3 washes in TBST, the secondary antibody (horseradish peroxidase-conjugated goat anti-rabbit IgG, Vector Labs, 1: 1000) was applied for 1 h. After three washes in TBST, bound antibody was detected using an enhanced chemiluminescence assay (Amersham Biosciences). The intensity of the Western blot bands from the CN of cochleotomized vs. control animals was measured with a fixed-size sampling area using the image analysis software Quantity One (Bio-Rad Laboratories, USA). Gray level values/mm^2 of band were obtained from a total of five Western blots for GluR2/3 and three Western blots for GluR4. Results were expressed as average gray level values. Differences were statistically compared by means of an unpaired Student's t test.

4.5. *Co^{2+} uptake and histochemical detection*

Animals were deeply anaesthetized with ketamine and xylazine as described above. Their brains were rapidly removed and placed in ice-cold artificial cerebrospinal fluid (ACSF) containing 124 mM NaCl, 5 mM KCl, 1.2 mM KH_2PO_4,

26 mM $NaHCO_3$, 1 mM $MgCl_2$, 2 mM $CaCl_2$ and 10 mM glucose, continuously bubbled with 95% O_2 and 5% CO_2 to reach a pH of 7.3. Six hundred μm thick hindbrain coronal slices containing CN from lesioned animals and their corresponding controls (Table 1) were cut with a tissue slicer (VT1000S; Leica, Nussloch, Germany). One or two slices were obtained from each animal. Slices were pre-incubated in ACSF for 1 h. Experiments were performed at 22 °C.

Co^{2+} uptake labeling was carried out as described by Pruss et al. (1991) with minor modifications. Slices from control and lesioned animals were transferred to a dish containing oxygenated uptake buffer with the following composition: 140 mM NaCl, 4 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES and 10 mM glucose, and washed 2x10 min. Slices were incubated for 20 min in uptake buffer containing 2.5 mM $CoCl_2$, 250 μM kainate and 100 μM AP-5 (to block unusual Co^{2+} permeation through NMDA receptors). Slices were then washed for 10 min in uptake buffer plus 2 mM EDTA to remove extracellular Co^{2+}, followed by 10 min in uptake buffer. Intracellular Co^{2+} was precipitated by incubating the slices for 5 min in uptake buffer containing 1.2% $(NH_4)_2S$. Following washes in uptake buffer, slices were transferred to 0.1 M PB and fixed overnight in 4% paraformaldehyde. Slices were further cryoprotected by immersion in 30% sucrose for 2 days and resectioned in the coronal plane at 40 μm with a cryostat (CM3050S; Leica, Nussloch, Germany). Sections were then washed in PB and Co^{2+} precipitates were finally made visible by silver precipitation with a commercially available kit (IntenSEM; Amersham, Buckinghamshire, UK). Incubation time in the kit solution mixture was 36 min. Finally, sections were washed in PB, allowed to air-dry, dehydrated and coverslipped.

In order to estimate nonspecific labeling under our experimental conditions, we "loaded" slices by exposure to 2.5 mM $CoCl_2$ either alone or with the specific AMPA/kainate receptor antagonist CNQX (100 μM), or with kainate plus CNQX. As the Co^{2+} uptake technique has been extensively applied in the hippocampus (Pruss et al., 1991; Toomim and Millington, 1998; Yin et al., 1999), we used hippocampal slices for these test experiments. Specific staining was significantly decreased in all conditions and entirely abolished in the slices incubated in $CoCl_2$ plus CNQX as previously described (Pruss et al., 1991; Toomim and Millington, 1998; Yin et al., 1999).

DL-2-amino-5-phosphopentanoic acid (AP-5), 6-cyano-7-nitroquinoxaline-2, 3-dione (CNQX) and kainate were purchased from Tocris (Essex, UK). Drugs were prepared as stock solutions in distilled water, stored at 4 °C and prepared to working concentrations in uptake buffer immediately prior to use.

Histochemical labeling was analyzed by microscopic inspection with a Leica DMRX-A microscope equipped with differential interference contrast optics. Differences in staining intensities between experimental and control sections were assessed by densitometry. Gray values were measured using the Cell^R imaging software from Olympus Biosystems GmbH (Planegg, Germany). Digitized images with an 8-bit resolution were used to calculate absolute gray values of somata (AGV_{soma}). In order to compensate for noise, background labeling was determined for each section by measuring absolute gray values ($AGV_{background}$) in an outlined area where labeled somata were absent. Relative gray value from somata (RGV_{soma}) was calculated through the following equation:

$$RGV_{soma} = 100 - (AGV_{soma}/AGV_{background} \times 100).$$

A random sample of 101 labeled cell bodies picked from 7 cryostat sections from 7 slices (from 6 lesioned animals) and 70 cells from 5 cryostat sections from 5 slices (from 3 control animals) were used to measure RGV_{soma}. The statistical significance of differences between mean values in control and lesioned animals was assessed using an unpaired Student's *t* test.

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