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RESEARCH ARTICLES

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Co-induction of growth-associated protein GAP-43 and neuronal nitric oxide synthase in the cochlear nucleus following cochleotomy

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Abstract In adult animals, cochlear lesioning leads to a reactive synaptogenesis with a reemergence of growth-associated protein, GAP-43, in the auditory brainstem nuclei. In addition, nitric oxide (NO) is also implicated in synaptogenesis. Three isoforms of nitric oxide synthase (NOS) responsible for generating NO have been identified and, in neurons, the predominant isoform is neuronal NOS (nNOS). Studies in visual or olfactory systems have found that the NOS expression often correlates with periods of axonal outgrowth and synapse formation; whether NO plays a similar role in the auditory brainstem needs to be examined. In the present study, a unilateral cochleotomy was performed in adult mice to examine the relationship between the reemergence of GAP-43 and the expression pattern of nNOS. Following surgery, GAP-43 re-emerged in the ipsilateral anterior ventral cochlear nucleus (AVCN) and the immunoreactivity reached a climax around postoperative day (POD) 8; the same expression pattern as that reported in the previous literature is the indicator of synaptogenesis. As for the nNOS immunoreactivity, a dramatic redistribution from a mostly cytoplasmal to a predominantly membranous localization in the ipsilateral AVCN was found especially at POD 4. A similar redistribution pattern in the ipsilateral AVCN for the N-methyl-D-aspartate (NMDA) receptor was also observed at POD 4, corresponding to the fact that the activation of nNOS is coupled to calcium influx via the NMDA-receptor. Furthermore, the expression of cyclic guanosine monophosphate (cGMP) is an indicator for activity of soluble guanylyl cyclase (sGC), the substrate of NO, which reveals the target area of NO. Therefore, cGMP immunoreactivity was also examined and an obvious increase of cytoplasmal cGMP expression was observed around POD 4. Accordingly, it is suggested that nNOS activity correlates closely with the reactive synaptogenesis following a cochleotomy. Further evidence is shown by the results of fluorescent double staining; nNOS-positive cells were surrounded by GAP-43 labeled regions that appeared to be presynaptic boutons, and the vast majority of nNOS-positive cells also expressed cGMP. The former result indicates that, after surgery, there should be new terminal endings projecting onto the nNOS-positive cells in the AVCN. Furthermore, the latter result suggests a possible role of an autocrine mediator for nNOS in the AVCN.

Keywords Auditory brainstem · Mice · Plasticity · Synaptogenesis

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Introduction

Mammalian brains retain the ability to respond to a changed environment with modifications of their structure and function throughout maturity, commonly known as a kind of "neuronal plasticity." One important component of the molecular machinery underlying neuronal plasticity is the neuron-specific growth-associated protein GAP-43 (Strittmatter et al. 1992). During neurite outgrowth and early stages of synaptogenesis, high levels of GAP-43 expression are shown in every nerve cell (Mahalik et al. 1992). With maturation, its expression is down-regulated by most neurons (Benowitz and Routtenberg 1997). However, synaptic remodeling in adult animals again raises the level of GAP-43 expression (Gispen et al. 1991; Strittmatter et al. 1992; Benowitz and Routtenberg 1997). For example, in the adult animal, an axotomy may reinvoke the expression of GAP-43 in several types of neurons (Liabotis and Schreyer 1995; Elliott et al. 1997). Sensory deprivation may also alter the pattern of GAP-43 expression in distinct brain regions (Baekelandt et al. 1994; Ohno et al. 1994). Thus, GAP-43 has been

suggested as a useful marker for events related to axonal growth, synaptogenesis, and synaptic modification (Gispen et al. 1991; Lin et al. 1992; Benowitz and Routtenberg 1997).

Following synthesis in the cell body, GAP-43 is rapidly transported to distal axonal processes (Benowitz and Routtenberg 1997; Oestreicher et al. 1997), in which GAP-43 regulates the cytoskeletal organization in nerve endings, thus causing it to serve as an intrinsic determinant of neuronal development and neuronal plasticity (Benowitz and Routtenberg 1997). In addition, the gas nitric oxide (NO) is also reported to play a role in several models of neuronal plasticity, including long-term potentiation in the hippocampus (Haley et al. 1992; Hawkins et al. 1998), cerebellar long-term depression (Crepel 1998), and activity-dependent refinement of visual connections during development (Cramer et al. 1996; Cogen and Cohen-Cory 2000). Previous studies have shown that postsynaptic activity in target structures significantly influences the structure and contacts of presynaptic axons, suggesting the presence of a retrograde messenger (Esguerra et al. 1992; Simon et al. 1992; Ramoa and McCormick 1994). Recently, investigators found that NO may act as a diffusible retrograde signal that can mediate multiple aspects of neuronal plasticity (Hawkins et al. 1998; Cogen and Cohen-Cory 2000).

The gas NO is generated from the oxidation of L-arginine, catalyzed by nitric oxide synthase (NOS) (Bredt and Snyder 1992). Three main isoforms of NOS, neuronal (nNOS), inducible (iNOS), and endothelial (eNOS), have been identified. Among these, NO formed by nNOS has major signaling functions in the central nervous system (Garthwaite and Boulton 1995). nNOS is a calcium-dependent enzyme, releasing NO as a consequence of Ca^{2+} influx resulting from N-methyl-D-aspartate (NMDA) receptor activation (Garthwaite 1991; Bredt and Snyder 1994). Once the gas NO is formed, it can stimulate a cytosolic protein, soluble guanylyl cyclase (sGC), to produce cyclic guanosine monophosphate (cGMP) (Bredt and Snyder 1994). Cumulative evidence has shown that the NO/cGMP pathway modulates diverse functions, including the neuronal plasticity, in the central and peripheral nervous system (Boulton et al. 1995; Jaffrey and Snyder 1995).

The role of NO in central auditory processing remains unclear; however, nNOS and mRNA coding for sGC have been reported in the auditory brainstem (Fessenden et al. 1999; Burette et al. 2001). In addition, both the central auditory system and the NO/cGMP pathway are activated by the neurotransmitter L-glutamate (Helfert et al. 1991; Bilak et al. 1996). The NMDA-type glutamate receptors are found in the spiral ganglion, the cochlear nucleus (CN), and the superior olivary complex (SOC) (Niedzielski and Wenthold 1995; Bilak et al. 1996; Sato et al. 1998, 2000). Since the nNOS is physically coupled to the NMDA-receptors, glutamate also preferentially activates the NO/cGMP pathway in the auditory brainstem (Brenman et al. 1996; Kiss and Vizi 2001). According to these results, it has been suggested that the NO/cGMP pathway is involved in both the ascending and descending pathways of the auditory brainstem (Fessenden et al. 1999; Reuss and Riemann 2000).

Numerous experimental studies have investigated the structural, metabolic and functional effects of sound deprivation upon the auditory pathways. In particular, after a unilateral cochlear lesion in a neonatal animal, the connectivity of the brainstem is altered with aberrant synapses in both the CN and inferior colliculus (IC) (Nordeen et al. 1983; Trune 1983; Moore and Kitzes 1985; Moore and Kowalchuk 1988), which indicates that the neonatal auditory brainstem was subjected to reactive synaptogenesis. On the other hand, the existence of GAP-43 in the mature nervous system, including the auditory system, indicates a potential of plastic remodeling in the mature auditory pathways (Benowitz et al. 1988; Benowitz and Routtenberg 1997; Illing 2001; Plunet et al. 2002). According to the studies of Illing and his colleagues, after a unilateral cochlear lesion in an adult animal, a substantial increase in the expression of GAP-43 was observed in the neuropil (fibers and boutons) of all subnuclei of the ipsilateral CN and in neuronal cell bodies of the lateral superior olive (LSO). They concluded that the sudden loss of spiral ganglion cells leads to the reemergence of GAP-43, indicating that reactive synaptogenesis had occurred in the auditory brainstem nuclei (Illing and Horvath 1995; Illing et al. 1997).

In the present study, a unilateral cochleotomy was performed on adult animals to evaluate the involvement of GAP-43 and NO in this kind of synaptogenesis. As previously mentioned, studies investigating the development of visual or olfactory systems have demonstrated that an NOS expression often correlates with periods of axonal outgrowth and synapse formation, and that NO may act as a retrograde messenger to coordinate axonal targeting and synaptogenesis (Roskams et al. 1994; Williams et al. 1994; Tenorio et al. 1995). However, evidence that NO performs a similar property in the auditory brainstem is lacking. Therefore, in this study, the spatial and temporal relationships that exist between the expressions of GAP-43, nNOS, NMDA-receptor, and cGMP were examined via immunohistochemistry (single labeling) and fluorescent double staining (double labeling). In the ascending auditory pathway, the CN is the first relay station and is particularly accessible to experimental study, leading it to be of particular interest. Among its three major subdivisions, the anterior ventral CN (AVCN) was the target for observation and analysis, since its properties are well known (Romand and Avan 1997). In addition, the animal's auditory processing ability was evaluated by recording brainstem auditory evoked potentials (BAEPs) before and after the cochleotomy, as well as before sacrifice in order to confirm the success of the unilateral cochlear lesion.

Materials and methods

Animals

A total of 54 C57BL10/ScSn (B-10) mice were assessed in this study and were divided into six groups for different postoperative survival days. All animals were healthy looking and aged 10 weeks (25–30 g) at the beginning of this study. They were housed in an air-conditioned room (25±1°C). The light/dark cycle was 12/12h with the lights on from 6:00 to 18:00. Free access to food and water was given to all animals throughout the experiment, except during the cochleotomy. The experiments were conducted according to the "Principles of laboratory animal care" (NIH publication No.86-23, revised 1985) and approved by the Animal Care and Use Committee of the Kaohsiung Medical University.

BAEPs recording

BAEPs were recorded from the scalp during the first 10 ms after acoustic stimulation and reflect neural transmission in the auditory brainstem. Five positive waves designated as waves I to V have been identified and correlated with activity levels of the cochlear nerve, cochlear nucleus (CN), SOC, lateral lemniscus and inferior colliculus, respectively (Fig. 1a). The method for recording BAEPs in rodents has been established previously (Chen and Chen 1990, 1991; Chen et al. 2002). The animals were deeply anesthetized with an intraperitoneal injection of 50 mg/kg sodium pentobarbital. BAEPs were picked up by needle electrodes located subcutaneously over the vertex and in the retroauricular region ipsilateral to monaural sound stimuli. An additional electrode attached to the retroauricular region on the other side served as the ground. All experimental animals used in this study were examined initially by recording their BAEPs, and only those presenting parallel BAEPs, despite the side of sound stimuli, were included.

A signal averager (Neuropack 2, Nihon Kohden Co., Tokyo, Japan) was used for recording the BAEPs. The animal was placed in a sound-attenuating chamber with a background noise level below 30 dBA (Leq) (monitored by using a sound level meter, Model NL-01A, Rion Co., Tokyo, Japan). Monaural sound stimuli were provided through a speaker placed 2 cm away from the animal's ear, and the contralateral ear was masked. Clicks of 80 dB (re 20 μPa) delivered at a rate of 17 Hz were supplied, at which the BAEP waveform was the largest with very little artifacts. The evoked potentials were amplified and filtered with a bandpass of 50–3,000 Hz. Each BAEP recording had an average of 1,000 click-evoked responses in the first 10 ms after the stimulation. Routine recordings of BAEPs were performed before and after the cochleotomy, as well as before the animal's sacrifice, to document the success and permanent lesion effect of the cochleotomy.

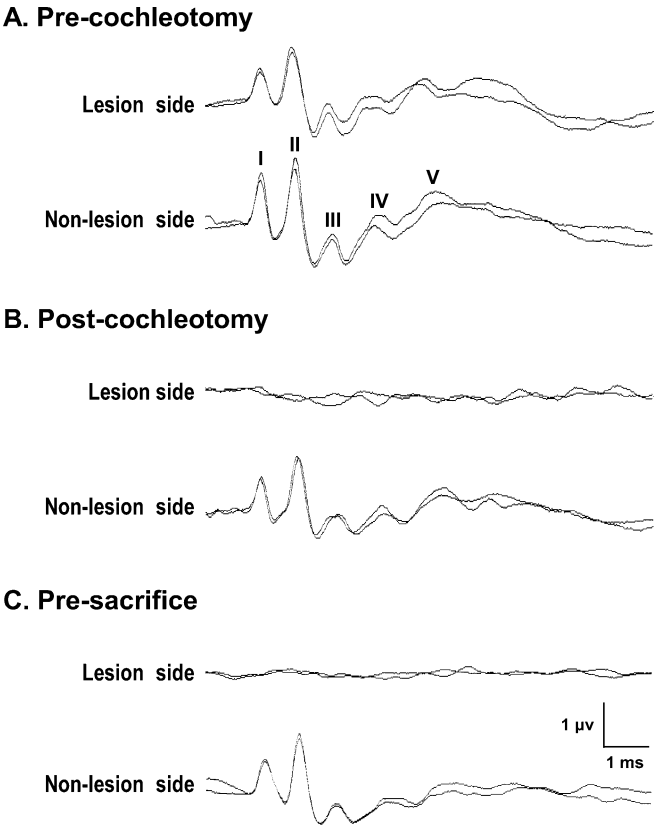

Fig. 1a–c Alterations of the BAEP waveforms after unilateral cochleotomy in one representative mouse. **a** Normal BAEP waveforms were shown on both sides before surgery. Five positive peaks could be discerned clearly. **b** Immediately after the cochleotomy, flat BAEP waveforms were observed on the lesion side, indicating successful surgery, while the non-lesion side retained a normal BAEP waveform. **c** Before sacrifice, no recovery of the BAEP waveform could be found on the lesion side, indicating a permanent cochlea lesion

Cochleotomy

Following pre-surgery BAEPs recordings, a supplementary injection of sodium pentobarbital was given if needed. In addition, an injection of 400 μg/kg atropine sulphate was given to the animal intraperitoneally 30 min before the surgery to keep the rodent free from airway obstruction. After shaving the hair around the animal's right ear, a retroauricular incision was made to approach the bulla tympani while the facial nerve was cut upon its exit from the skull. The bulla was opened, starting from the outer ear canal and working

Table 1 Experimental parameters

Survival days after unilateral cochleotomy	Total animal number	Immunohistochemistry (single labeling)				Fluorescent double staining (double labeling)	
		GAP-43	nNOS	NMDA-receptor	cGMP	GAP-43/nNOS	nNOS/cGMP
2	*n* =9	*n* =3		*n* =3		*n* =3	
4	*n* =9	*n* =3		*n* =3		*n* =3	
8	*n* =9	*n* =3		*n* =3		*n* =3	
14	*n* =9	*n* =3		*n* =3		*n* =3	
30	*n* =9	*n* =3		*n* =3		*n* =3	
60	*n* =9	*n* =3		*n* =3		*n* =3	

n number,*GAP-43* growth-associated protein 43, *nNOS* neuronal nitric oxide synthase, *NMDA-receptor* N-methyl-D-aspartate receptor, *cGMP* cyclic guanosine monophosphate

caudally until the bulging cochlea was visible. The bony wall of the cochlea was perforated with a spherical drill head and the interior of the cochlea, including the spiral ganglion, was cleared. Subsequently, the opened bulla was irrigated with a phosphate buffered solution and then filled with wax. The wound was finally closed with surgical clamps.

Tissue preparation

Six specified survival times (2, 4, 8, 14, 30, and 60 days) were chosen for observing the alterations of temporal and spatial expression patterns among GAP-43, nNOS, NMDA-receptor, and cGMP. Nine B-10 mice were included for the study at each survival time, respectively, with six mice for single labeling and three mice for double labeling (Table 1).

After specified survival times ranging from 2–60 days, animals had their BAEPs measured in order to determine whether a permanent cochlear lesion existed. Then the animal was given a lethal dose of sodium pentobarbital. Transcardial perfusion was performed subsequently with a perfusion buffer containing 8 g/l NaCl, 0.25 g/l KCl, and 0.5 g/l $NaHCO_3$ in 0.2 M phosphate buffer (pH 7.4), followed by a fixative containing 3% paraformaldehyde, 0.1% glutaraldehyde, and 15% picric acid in a 0.2 M perfusion buffer (pH 7.4). The brain was quickly removed and immersed in a 30% sucrose solution for 2 days at 4°C. Following freezing in liquid-nitrogen iced isopentane and embedding in an OCT compound (Sakura Finetek USA, Inc., Torrance, CA, USA), the brain was cut on a cryostat into 20-μm (for single labeling) or 10-μm (for double labeling) coronal sections. These sections were mounted immediately onto slides precoated with poly-L-lysine and then stored at −70°C until the procedures for single labeling or double labeling were performed. In order to obtain consistent results of staining, series of brain sections obtained from animals survived for various days (2–60 days) after surgery were collected and were stained at the same time. Three animals were assigned for each specified immunoreactive staining (GAP-43 etc.), and at least three repetitions of staining were made for each animal. Thus a total of nine or more stains were obtained for each immunoreactivity.

Immunohistochemistry (single labeling)

Excluding the different primary antibody added, the immunohistochemical procedures for observing the expressions of GAP-43, nNOS, NMDA-receptor, and cGMP in the auditory brainstem were identical. Each animal was assigned for two stains, thus alternate sections were used for each respective stain. Brain sections were treated with 1% H_2O_2 in a phosphate buffered saline (PBS) for 5 min, and then preincubated in a blocking buffer containing 10% normal goat serum and 0.2% Triton X-100 in a PBS for 30 min, both at room temperature. Subsequently, sections were incubated overnight at 4°C with a primary antibody (mouse anti-GAP-43 monoclonal antibody in 1:1000 dilution, Sigma, St. Louis, MO, USA; rabbit anti-nNOS polyclonal antibody at 1:100 dilution, Santa Cruz Biotechnology, Santa Cruz, CA, USA; mouse anti-NMDAR-1 monoclonal antibody in 1:1000 dilution, Upstate Biotechnology, Inc., Lake Placid, NY, USA; rabbit anti-cGMP polyclonal antibody in 1:200 dilution, Sigma, St. Louis, MO, USA). Sections were then incubated for 60 min at room temperature in a biotinylated goat anti-mouse or anti-rabbit secondary antibody (1:300 dilution; DAKO Co., Carpinteria, CA, USA) and for 30 min in 1% ABC reagent (streptavidin-biotinylated horseradish peroxidase, DAKO Co., Carpinteria, CA, USA); peroxidase was visualized histochemically with diaminobenzidine (DAB) (DAKO Co., Carpinteria, CA, USA). Sections were rinsed with PBS and air dried before being coverslipped with a gelatin mounting medium (DAKO Co., Carpinteria, CA, USA).

Fluorescent double staining (double labeling)

Fluorescent double staining was performed for examining the temporal and spatial expression patterns of GAP-43/nNOS and nNOS/cGMP. Alternate sections of each animal were also used for these two kinds of double labeling. Brain sections were first pretreated in 1% sodium borohydride for 15 min to quench free aldehyde groups and then preincubated in a blocking buffer containing 10% normal goat serum for 30 min. After an overnight incubation at 4°C with two kinds of primary antibodies, mouse anti-GAP-43 monoclonal antibody in 1:1000 dilution and rabbit anti-nNOS polyclonal antibody in 1:100 dilution, or mouse anti-nNOS monoclonal antibody in 1:200 dilution (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and rabbit anti-cGMP polyclonal antibody in 1:600 dilution, sections were reacted for 1 h at room temperature with two kinds of secondary antibodies simultaneously: rhodamine-conjugated goat anti-mouse IgG and fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (Both in 1:800 dilution, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) for double labeling of GAP-43 and nNOS, or rhodamine-conjugated goat anti-rabbit IgG and FITC-conjugated goat anti-mouse IgG for double labeling of nNOS and cGMP. Subsequently, sections were rinsed with PBS and air dried before being coverslipped with a gelatin mounting medium (DAKO Co., Carpinteria, CA, USA).

Controls

Two additional groups of age-matched control mice, three non-treated mice and three sham-treated mice, were included for the single labeling study. All experimental procedures were the same as those mentioned previously except that no cochleotomy was given to these mice. Comparisons were made for the staining patterns shown in non-treated or sham-treated mice with those shown in post-cochleotomy mice. After various survival days (2–60 days) following cochleotomy, the target brain area contralateral to lesion showed no difference in the staining patterns as compared with the corresponding area in control mice. For simplicity, the effect of unilateral cochleotomy on the target brain area was compared between ipsilateral and contralateral sides.

In a control study for immunohistochemistry, sections were processed through the same immunohistological sequence, except that primary antibody was omitted. In the double-immunofluorescence staining, in order to control for possible cross reaction between the first primary antibody and the second secondary antibody, the sections were processed as described above; however, the second primary antibody was omitted. In all cases, the omission of primary antibody resulted in the lack of specific labeling, confirming the specificity of immunohistochemical methods.

Image acquisition and analysis

All brain sections containing the AVCN were examined carefully with a Nikon light microscope (Tokyo, Japan) and photographed with a Nikon film camera (Tokyo, Japan). In order to show the detailed staining patterns of four kinds of immunoreactivities (GAP-43, nNOS, NMDA-receptor, and cGMP) and to compare staining intensities more closely, sections were examined with the light microscope at high power (200×). To provide comparability between lesion and non-lesion side, fields of evaluation were chosen at matching position as specified in Fig. 2. All photographs were scanned into the computer for further image processing. For comparisons of staining patterns, the background staining levels were adjusted to almost the same intensity. Although the absolute staining levels were difficult to measure, the constancy of staining in this study made it feasible and reasonable to identify the peak of immunoreactivity.

The results of double labeling were evaluated with a Zeiss fluorescent microscope (Axioskop 2 plus, Germany). Images viewed

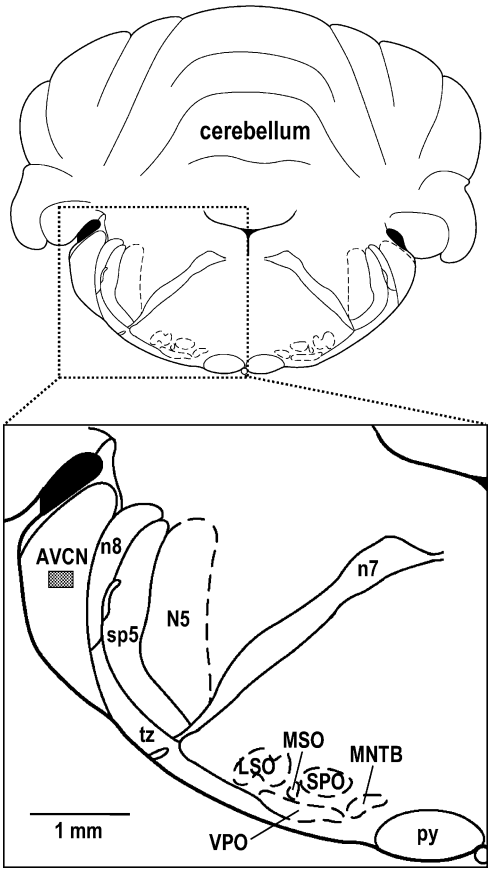

Fig. 2 The brain section at 1.04 mm posterior to the interaural line. The position of the field chosen for close up analysis of the staining pattern is shown in the *gray rectangle* in the anteroventral cochlear nucleus (*AVCN*). *LSO* lateral superior olive, *MNTB* medial nucleus of the trapezoid body, *MSO* medial superior olive, *n7* facial nerve, *n8* vestibular branch of the eighth cranial nerve, *N5* trigeminal nucleus, *py* pyramidal tract, *sp5* spinal trigeminal tract, *SPO* superior periolivary nucleus, *tz* trapezoid body, *VPO* ventral periolivary nucleus

at a magnification of 400× were acquired with a CoolSNAP cf digital camera (RS Photometrics, Downingtown, PA, USA) coupled to a personal computer with an Intel Pentium III. To sharpen images, RS Image Software (ver. 1.07, RS Photometrics, Downingtown, PA, USA) was used to adjust the brightness and contrast level. Eventually, double immunofluorescent images were displayed as double-color merged images by using Color Stacker software (ver. 1.0, Polaroid Co., Cambridge, MA, USA).

Results

Evaluating the unilateral cochlear lesioning by BAEPs

Due to the initial screening of normal animals at the beginning of this study, all animals showed normal BAEP responses (Fig. 1a). However, following unilateral cochleotomy, obvious changes were observed in the BAEP responses recorded from the lesion side. A marked decline of BAEP response or flat waveform was the evidence for a successful cochleotomy. Normal BAEP responses were retained on the contralateral side (Fig. 1b). No recovery was found before sacrifice, indicating the irreversibility of the cochlea lesion regardless of survival time (Fig. 1c). All animals showing this BAEP alteration were included in the study.

Choosing of evaluation field in the AVCN

For comparing the effects of a unilateral cochleotomy on the immunoreactivity of GAP-43, nNOS, NMDA-receptor, and cGMP between the lesion and the non-lesion side AVCN, a fixed field (shown in Fig. 2) was assayed. This area was selected because it demonstrated strong immunoreactivity. Figure 3 illustrates the expression patterns of GAP-43 (Fig. 3a) and nNOS (Fig. 3b) immunoreactivity on the lesion side at postoperative day (POD) 4 following unilateral cochleotomy. Heavy GAP-43- or nNOS-labeled cells were observed in AVCN; however, in the most ventral region of the AVCN, the staining was too dense to identify individual cells (Fig. 3). The staining for NMDA-receptor and cGMP immunoreactivity in the ventral AVCN region was less dense but still difficult to observe. Therefore, the most ventral region of the AVCN was not suitable for evaluation and the central region, showing the clearest expression pattern, was selected for this study.

Alteration of GAP-43 expression in the AVCN following cochleotomy

Little staining of GAP-43 was found in the adult AVCN. As a consequence of removing the inner ear, GAP-43 immunoreactivity in the AVCN rose dramatically on the side of lesion (Fig. 4).

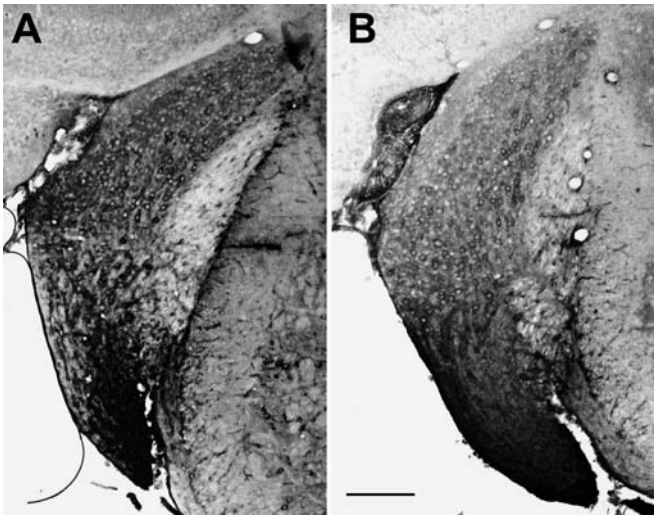

Fig. 3a, b Frontal sections at the AVCN level in two representative animals. These sections were obtained from sacrificed animals at POD 4, following unilateral cochleotomy, and only the lesion/right side of the AVCN was shown. **a** Sections stained for GAP-43 immunoreactivity. **b** Sections stained for nNOS immunoreactivity. For both GAP-43 and nNOS immunoreactivities, most animals showed heavy labeling around cells in almost the entire AVCN, with dense staining in the most ventral region. Scale bar = 200 μm

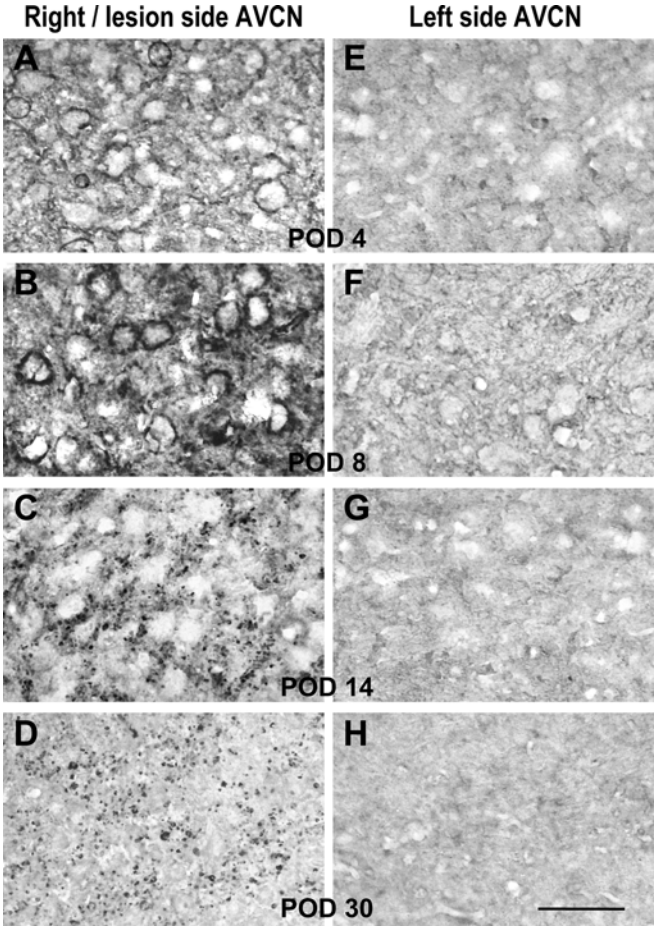

Fig. 4a–h The pattern and time course of GAP-43 immunoreactivity in the AVCN following unilateral cochleotomy. Images for each survival time were taken from the same animal. After removing the inner ear, GAP-43 immunoreactivity in the AVCN rose dramatically on the lesion side. A peak of GAP-43 immunoreactivity was reached at POD 8 (**b**). *AVCN* anterior ventral cochlear nucleus, *POD* postoperative day. Scale bar = 50 μm

It took a few days to reveal the lesion-induced re-expression of GAP-43. By POD 2, GAP-43 immunoreactivity had not yet changed as compared with the unaffected contralateral side. By POD 4, the first unequivocal increase of GAP-43 immunoreactivity was observed in the AVCN. Dense GAP-43 staining appeared in the neuropil. This staining was mostly accumulated in axonal swellings, while immunoreactive cell bodies were not observed (Fig. 4a).

The response took 8 days for a full change. That is, the intensity of GAP-43 immunoreactivity reached a peak around POD 8. Heavily labeled axonal swellings were seen around cell bodies and in the neuropil (Fig. 4b). By POD 14, GAP-43 immunoreactivity appeared to be reduced again and was clearly weaker than a week earlier. The intensity of the staining was comparable to POD 4, but the texture of the staining had changed clearly, as immunoreactive structures were no longer concentrated around cell bodies. Instead, stained dots that appeared to be presynaptic boutons were rather diffusely distributed in the neuropil (Fig. 4c). After 1 month, the pattern of staining had changed again. While still fewer immunoreactive dots were visible, some of these were particularly large (Fig. 4d). By 60 days postoperatively, GAP-43 immunoreactivity steadily decreased until it had decreased almost to low adult levels.

Alteration of nNOS expression in the AVCN following cochleotomy

To identify potential sources of NO in the AVCN, immunostaining for nNOS was performed. Neurons with somatic staining for nNOS were observed in the AVCN of the normal animal. The staining was confined mainly to somatic cytoplasm and the most proximal portions of dendrites.

Following unilateral cochleotomy, a dramatic redistribution of nNOS, from a mostly cytoplasmal to a predominantly membranous localization, and an increase in nNOS immunoreactive neurons were observed in the AVCN on the lesion side. In contrast, the staining for nNOS retained cytoplasmal expression patterns on the contralateral non-lesion side throughout the whole period of observation (Fig. 5).

By POD 2, few neurons showed membranous staining for nNOS immunoreactivity in the lesion side AVCN. By POD 4, obvious nNOS immunostaining lining the cytoplasmal membrane was observed, and the largest number of nNOS immunoreactive neurons was found (Fig. 5a). The obvious membranous nNOS expression decayed gradually. By POD 8, both cytoplasmal and membranous nNOS immunoreactivities were observed. In addition, nNOS expression could also be observed in the neuropil, indicating that nNOS might distribute to dendrites at that time (Fig. 5b). Two weeks after the cochleotomy, most neurons expressed cytoplasmal nNOS immunoreactivity, while only a few still possessed membranous nNOS (Fig. 5c). After 1 month, the staining for nNOS had achieved a normal pattern (Fig. 5d). By POD 60, no obvious difference could be determined in the expression pattern and the number of nNOS immunoreactive neurons between the lesion and non-lesion side.

Alteration of the NMDA-receptor expression in the AVCN following cochleotomy

In the AVCN of the normal mouse, staining for the NMDA-receptor was prominent in the somatic cytoplasm, excluding the nucleus. Occasionally, the staining was confined not only to the somata but also to the most proximal portions of the dendrites.

Similar changes to those of the nNOS expression were observed following the cochleotomy. In the AVCN on the side of lesion, a dramatic redistribution of the NMDA-receptor from a mostly cytoplasmal to predominantly membranous and dendritic localizations was found. In contrast, the staining for the NMDA-receptor showed the

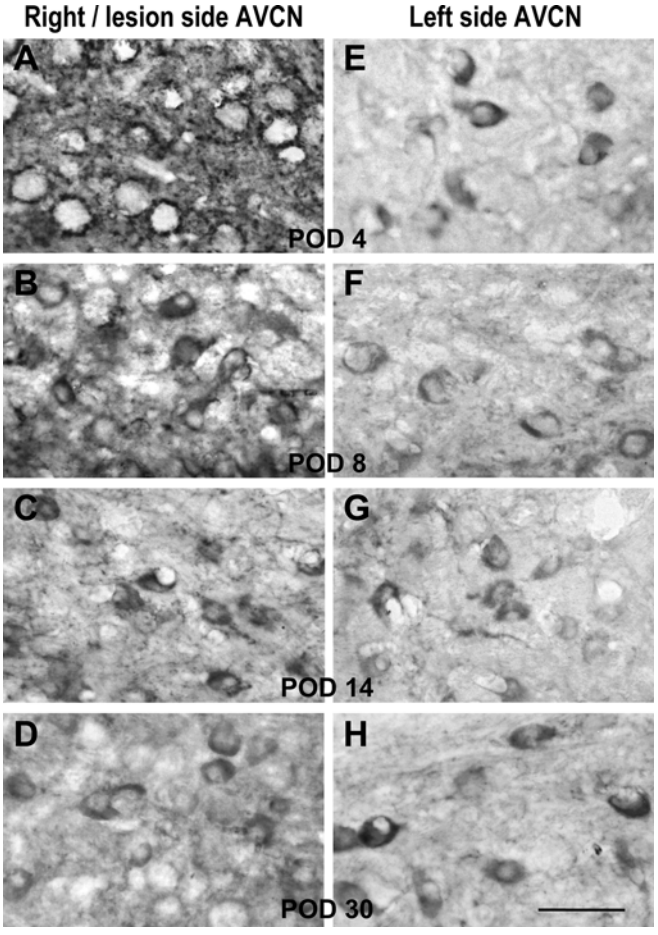

Fig. 5a–h The pattern and time course of nNOS immunoreactivity in the AVCN following unilateral cochleotomy. Images for each survival time were taken from the same animal. After removing the inner ear, a dramatic redistribution of nNOS from a mostly cytoplasmal to a predominantly membranous localization was first observed in the AVCN on the lesion side at POD 4 (**a**). *AVCN* anterior ventral cochlear nucleus, *POD* postoperative day. Scale bar = 50 μm

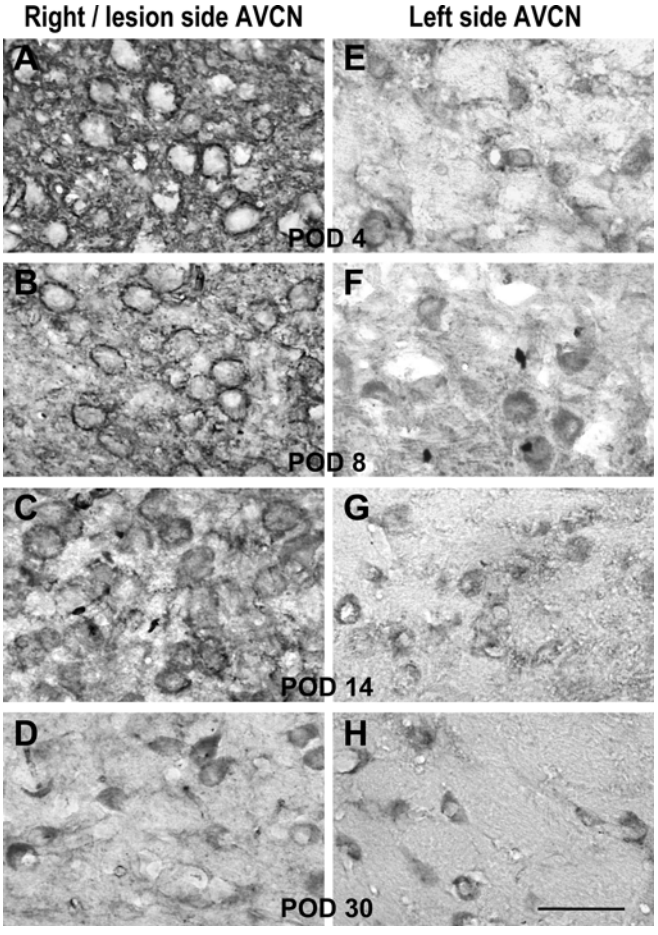

Fig. 6a–h The pattern and time course of the NMDA-receptor immunoreactivity in the AVCN following unilateral cochleotomy. Images for each survival time were taken from the same animal. After removing the inner ear, redistribution changes similar to those of the nNOS expression were observed. The membranous NMDA-receptor expression was first observed at POD 2 and reached the strongest at POD 4. *AVCN* anterior ventral cochlear nucleus, *POD* postoperative day. Scale bar = 50 μm

normal pattern on the contralateral non-lesion side throughout the whole period of observation (Fig. 6).

By POD 2, a few neurons showed membranous staining for NMDA-receptor immunoreactivity in the lesion side. By POD 4, the membranous NMDA-receptor expression was at its strongest. In addition, NMDA-receptor expression was also observed in the neuropil (Fig. 6a). By POD 8, most neurons showed a membranous location of the NMDA-receptor. The staining for the NMDA-receptor in the neuropil declined (Fig. 6b). After 1 month, the staining for NMDA-receptor had approached a normal pattern (Fig. 6d). By POD 60, no obvious difference could be determined in the expression pattern and the number of NMDA-receptor immunoreactive neurons between the lesion and non-lesion side.

Alteration of cGMP expression in the AVCN following cochleotomy

sGC is a receptor for NO. Anti-cGMP immunostaining was performed to assess the catalytic activity of sGC. Perikaryal staining for cGMP was observed in the AVCN of the normal animal.

Following unilateral cochleotomy, cGMP immunoreactive neurons were observed on the lesion side with a dense staining intensity. In contrast, very slight changes in the cGMP expression patterns were found on the contralateral non-lesion side (Fig. 7). By POD 4, deeply stained neurons were shown in the ipsilateral AVCN. Dense particles within the cytoplasm were noted in these neurons. The staining was most intense at POD 4 (Fig. 7a). By POD 8, expression pattern was similar to that of POD 4 but with a slightly reduced immunoreactivity (Fig. 7b). By POD 14, cGMP immunoreactivity reduced (Fig. 7c). Two months after the cochleotomy, the staining pattern for cGMP on the lesion side showed no

differences as compared to the contralateral side and with normal patterns.

Colocalization of GAP-43 and nNOS

Double-label experiments were carried out to investigate the relationship of GAP-43 and nNOS in the reactive synaptogenesis, which occurred in the AVCN following the cochleotomy.

Double labeling of GAP-43 and nNOS was examined at POD 4, 8, 14, and 30. Following the unilateral cochleotomy, little GAP-43 expression could be found in the contralateral AVCN, regardless of survival days after surgery, while the nNOS expression retained considerable levels throughout the experiment. Colocalization of GAP-43 and nNOS was prominent in the ipsilateral AVCN at POD 4. The vast majority of the nNOS-positive cells were circumscribed by the GAP-43 labeled regions that appeared to be presynaptic boutons (Fig. 8). Similar results were also found on POD 8. By POD 14, the GAP-43 staining declined markedly, and thus colocalization was scarce. After POD 14, no GAP-43 fluorescent staining could be examined in the ipsilateral AVCN.

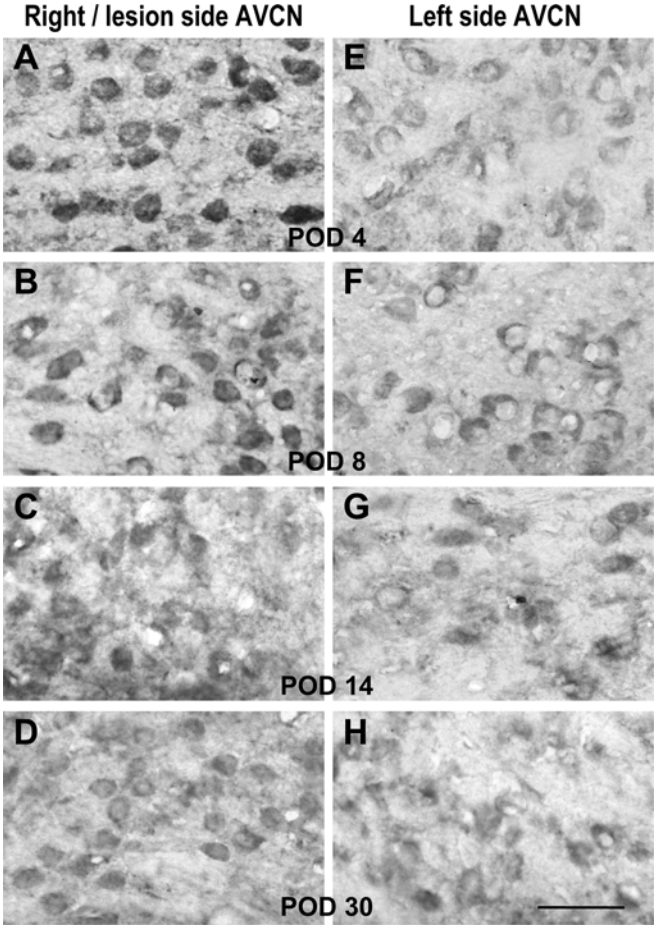

Fig. 7a–h The pattern and time course of cGMP immunoreactivity in the AVCN following unilateral cochleotomy. Images for each survival time were taken from the same animal. After removing the inner ear, a deeper staining intensity was observed on the lesion side, with a peak of staining reached at POD 4. *AVCN* anterior ventral cochlear nucleus, *POD* postoperative day. Scale bar = 50 μm

Colocalization of the nNOS and cGMP

The expression of cGMP is an indicator for sGC activity and reveals the target area of NO. Therefore, double-label experiments were performed to investigate the relationship of NO-producing cells and cGMP-positive areas.

Double labeling of nNOS and cGMP was also examined at POD 4, 8, 14, and 30. The characteristic of nNOS expression was similar to that shown in the previous experiment for colocalization of the GAP-43 and nNOS. Prominent colocalization of the nNOS and cGMP was observed in the ipsilateral AVCN at POD 4 and 8 (Fig. 8). The vast majority of nNOS-positive cells also expressed cGMP. At POD 4, a specific expression pattern could be found with nNOS lined somata and cGMP in the cytoplasm. At POD 8, both the nNOS and cGMP were expressed mainly in the cytoplasm, confirming the previous finding that the membranous nNOS began reappearing in the cytoplasm by POD 8.

Discussion

In the study made by Illing et al. (1997), stains for glutamate and calretinin were performed at varying postoperative times to confirm the success of cochleotomy. Based on the knowledge that fibers of the cochlear nerve contain glutamate (Altschuler et al. 1989) as well as calretinin (Winsky and Jacobowitz 1995), degeneration of the nerve fibers on the lesion side was easily detectable in both stains from POD 4 to POD 56, indicating that the auditory brainstem was indeed deprived from the cochlear input (Illing et al. 1997). However, the success of the lesion could not be confirmed via these stains until the animals were sacrificed. In our study, a non-invasive BAEP was recorded to evaluate the success of a unilateral cochleotomy. This convenient electrophysiological recording affords an opportunity to confirm the success of a unilateral cochlear lesion immediately after surgery and to assure the permanence of the lesion.

The expression patterns of GAP-43, nNOS, NMDA-receptor, and cGMP following unilateral cochleotomy were studied in the present study. GAP-43 immunoreactivity re-emerged in fibers and boutons-like regions, but not in the cell bodies of the ipsilateral AVCN following unilateral cochleotomy. A peak of staining was reached around POD 8, and then faded gradually. nNOS immunoreactivity showed a dramatic redistribution from a mostly cytoplasmal to a predominantly membranous localization in the AVCN ipsilateral to inner ear destruction. Most neurons characterized by membranous nNOS were observed at POD 4. As for the NMDA-receptor immunoreactivity, similar changes to those of the nNOS redistribution were found after the unilateral cochlear lesion. Most neurons at POD 4 were characterized by

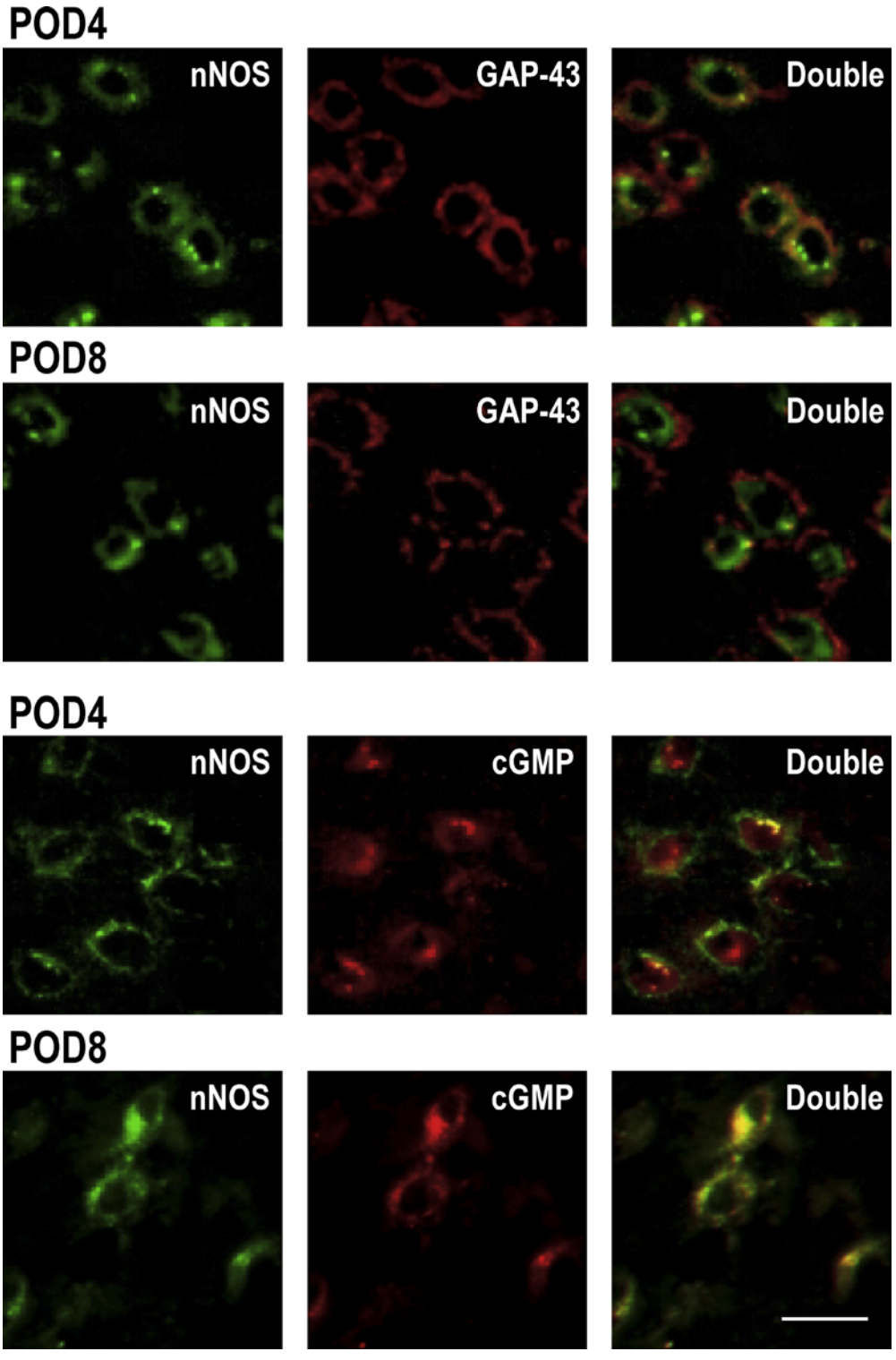

Fig. 8 Double immunofluorescent labeling revealing the spatial and temporal relationships of GAP-43/nNOS and nNOS/cGMP. Following unilateral cochleotomy, no obvious changes could be found in the non-lesion side AVCN; thus, only the stains in the lesion side AVCN at POD 4 and 8 which showing prominent changes were presented. Double merged images showed that membranous nNOS-labeled cells (especially at POD 4) were circumscribed by the GAP-43 labeled regions that appeared to be presynaptic boutons. As for the double staining for nNOS and cGMP, double merged images showed that the vast majority of nNOS-positive cells also expressed cGMP. At POD 4, the nNOS lined somata while the cGMP resided in cytoplasm. However, at POD 8, both nNOS and cGMP were expressed mainly in the cytoplasm. Scale bar = 20 μm

membranous NMDA-receptor. Perikaryal staining of cGMP immunoreactivity was increased in the ipsilateral AVCN following unilateral cochleotomy. Changes for the cGMP expression pattern were milder than those for the GAP-43 or nNOS; however, the most prominent expression was shown around POD 4. All stainings in the AVCN contralateral to the lesion showed no obvious changes.

Illing et al. (1997) showed that the most striking effect of a cochlear lesion was the rise of GAP-43 immunoreactivity in fibers and boutons, but not in the cell bodies of the CN. Concomitant rises in GAP-43 mRNA and its protein expression were also observed in the cell bodies of the LSO (Illing et al. 1997, 1999). Therefore, they suggest that the immunoreactive boutons belong to neurons that do not reside in the CN itself, but in other structures of the

auditory brainstem, possibly in the LSO. These LSO neurons could project descending fibers to the cochlea and synthesize GAP-43 in response to the cochlear lesion and export the protein to the CN (Illing et al. 1997). Although the origin of the re-expressed GAP-43 in the CN is not clarified definitively, this reactive synaptogenesis following alterations of the auditory input is still evidence for plasticity in the adult auditory brainstem (Illing et al. 1997). Searching for the origin of GAP-43 expressed in the CN was not the aim of this study; however, investigating whether NO is implicated in the reactive synaptogenesis of the CN following cochleotomy was of particular interest.

Changes of the GAP-43 expression pattern in the AVCN ipsilateral to the cochleotomy were the same as those found by Illing et al. (1997). Dramatic changes in the nNOS subcellular distribution and the nNOS-positive cells surrounded by GAP-43 labeled regions that appeared to be presynaptic boutons lend support to the idea that NO correlates with synaptogenesis in the AVCN. GAP-43 expression is enhanced during reactive synaptogenesis following a nerve injury and may serve as an indicator of synaptic remodeling (Aarts et al. 1998). While the nNOS expression often correlates with axonal outgrowth and synapse formation, it also reveals the role of NO as a mediator of synaptic plasticity (Roskams et al. 1994; Williams et al. 1994; Tenorio et al. 1995; Rentería and Constantine-Paton 1999). The primary effects appeared around POD 4–14 for the re-expression of the GAP-43 and re-distribution of the nNOS in the AVCN following the cochleotomy. However, the most prominent changes of the nNOS expression pattern (POD 4) were earlier than those of the GAP-43 (POD 8), which indicated that the NO signaling might be upstream of the synaptogenesis in the AVCN following cochleotomy.

NO has been suggested as an important candidate for the elusive retrograde messenger in synaptic plasticity, thus attempting to account for presynaptic alterations after the postsynaptic NMDA receptor activation, in both mature and developing brains (Gally et al. 1990; Edelman and Gally 1992). Therefore, after demonstrating that nNOS expression patterns changed reversibly within several days following the cochleotomy, an effort was made to examine the effects of lesions on the expression of the NMDA-receptor. Similar to the nNOS redistribution, cytosolic NMDA-receptors redistributed to cytoplasmal membrane at POD 4. This indicates that the nNOS activity correlated closely with the NMDA-receptor activation.

Previous studies indicate that a major portion of the nNOS is associated with membranes (Hecker et al. 1994). Membrane association of the nNOS in neurons appears to be mediated by the PDZ domain, and interactions with proteins such as PSD-95 and 93 (Brenman et al. 1996). In this study, however, nNOS immunoreactivity was normally confined to somata and the most proximal processes, which correlated with findings reported by Burette et al. (2001). There have been more than ten differently spliced nNOS transcripts reported (Brenman et al. 1996; Eliasson et al. 1997). The principal form is nNOSα, accounting for the great majority of catalytic activities in the brain (Huang et al. 1993). PSD-95 appeared to anchor nNOSα to neuronal membranes in the vicinity of the NMDA-receptor (Aoki et al. 1993; Kornau et al. 1995), thus coupling NOS activation to calcium influx, via the NMDA-receptor. In contrast, nNOSβ is the dominant splice variant in the VCN (Eliasson et al. 1997), and it lacks the PDZ domain (Brenman et al. 1996). Therefore, nNOSβ is localized to the cytosolic fraction (Brenman et al. 1996), which may account for the findings in this study. Since cytosolic nNOSβ might not be anticipated to respond to an NMDA-receptor stimulation, their ability for in vivo activation is unclear. However, the evidence that nNOSβ possesses activity comparable to nNOSα (Brenman et al. 1996) and that nNOSβ is catalytically active in vivo (Eliasson et al. 1997) indicates that membrane association is not required for catalytic activity. In the present study, cGMP immunoreactivity increased for several days following cochleotomy, indicating that the NO/cGMP pathway was activated via nNOS activity. In addition, following the cochlea lesion, both cytosolic nNOS and cytosolic NMDA-receptor redistributed to the cytoplasmal membrane. Thus, the possibility of nNOSβ activation via NMDA receptor stimulation should not be eliminated.

It has been suggested that the calcium concentration required to activate nNOS is sufficiently high for inhibiting sGC activity and, in turn, cGMP accumulation (Vincent 1994). Thus, the cells that accumulate cGMP in response to NO are typically distinct from (although close to) the neurons that synthesize NO. This organization suggests that NO normally acts in a paracrine manner, serving as an intercellular messenger. However, in the present study, fluorescent double staining showed the overwhelming colocalization of the nNOS and cGMP in the same cells, suggesting a possible autocrine or intracellular function for NO in the AVCN. This suggestion has been discussed completely by Burette et al. (2001).

Although NO is likely to be produced in the postsynaptic cell after activation of the NMDA-receptors, it is available simultaneously to both presynaptic axons and postsynaptic cells. Thus, in addition to the autocrine effect, the paracrine, or retrograde, effect of NO should not be overlooked. Many studies concerning long-term potentiation and visual system development provide strong evidence that NO, which is produced downstream of the NMDA-receptor activation, acts as a retrograde messenger molecule to induce changes in pre-synaptic structures or functions (Haley et al. 1992; Malgaroli et al. 1995; Cramer et al. 1996, 1998; Cogen and Cohen-Cory 2000). Possible substrates for NO in the presynaptic terminal during long-term potentiation include guanylate cyclase (GC) (East and Garthwaite 1991), adenosine diphosphate ribosyl transferase (ADPRT) (Schuman et al. 1994), or a combination of both (Abe et al. 1994). Since the results shown in this study revealed that cGMP localized in the cell bodies (represented nNOS) but not presynaptic boutons (represented GAP-43) in the AVCN, the retrograde action of NO on the presynaptic terminal may not

via sGC activation. In addition, an intriguing finding is that NO acts on GAP-43, via a possible mode of action with respect to ADPRT (Coggins et al. 1993). Therefore, NO may influence GAPs, via non-cGMP pathways, mediating the effects of NO on elongating neurites. Since the process of synaptogenesis involves the progressive elaboration of connections in appropriate regions, as well as the retraction and loss of connections from inappropriate regions, NO may act as a retrograde stop signal for the modulation of neuronal connections.

Taken together, our results revealed an apparent redistribution of nNOS from cytoplasm to membrane and new terminal endings surrounding nNOS-positive cells, demonstrating that NO may play a role in reactive synaptogenesis following cochleotomy. Both autocrine and paracrine (retrograde) effects of NO are possible mechanisms for coordinating axonal targeting and synaptogenesis in the AVCN.

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